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# Spread of *Aspergillus flavus* and aflatoxin accumulation in postharvested maize treated with biocontrol products

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ABSTRACT

Maize is a major staple crop and calorie source for many people living in Sub-Saharan Africa. In this region, *Aspergillus flavus* causes ear rot 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 reduce aflatoxin levels in maize grain 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 postharvest drying period, the time between harvest and the point when grain moisture is safe for storage. In Sub-Sahara Africa, this period often is extended by weather and the complexities of postharvest drying practices. Maize grain was collected from fields in Texas and North Carolina that were treated with commercial biocontrol products and untreated control fields. 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 °C for 6 days. Although the initial number of kernels infected by fungal species was high in most samples, less than 24% of kernels were infected with *Aspergillus flavus* and aflatoxin levels were low (<4 ppb). Both toxigenic and atoxigenic strains grew 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 may have migrated to untreated fields. These results also indicate that the population of toxigenic *A. flavus* in the harvested grain will increase and produce aflatoxin during the drying period when moisture is high. Therefore, we conclude that preharvest biocontrol applications will not replace the need for better postharvest practices that reduce the drying time between harvest and storage.



## 1. Introduction

Maize in Sub-Sahara Africa is often infected by the aflatoxin-producer *Aspergillus flavus* prior to harvest and during post-harvest handling of the product. 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 post-harvest fungi (Hell et al., 2010). However, achieving safe levels of moisture by the sun-drying methods used in some Sub-Sahara regions is difficult. Hence, post-harvest 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). A study in Benin by Hell et al. (2000) showed that post-harvest contamination of maize with aflatoxin increased when harvesting took more than five days. After harvest, maize is often stacked 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, a delay in the drying process results in increased contamination (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 and subsequent aflatoxin

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production in maize, the population in Sub-Saharan countries is chronically exposed to aflatoxin in their diet. Ingesting high levels of aflatoxin can result in 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). Several outbreaks of aflatoxin contamination have been reported, including in Kenya where human deaths have been reported 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., 2000, 2003).

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 (Ojiambo et al., 2018). This strategy seeks to competitively exclude aflatoxin-producing strains from crops by 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. An initial screen of 4200 *A. flavus* isolates in Nigeria lead to the identification of four strains that reduced aflatoxin levels in maize by up to 99%, which subsequently were used to the formulate the biocontrol product Aflasafe in Africa (Atehnkeng et al., 2008; Donner et al., 2010; Bandyopadhyay et al., 2016).

In the United States, two commercial biocontrol products are available, Afla-Guard and AF36. The Afla-Guard strain lacks the entire cluster of genes responsible for aflatoxin biosynthesis. 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 harvested maize inoculated with both AF36 and a toxigenic strain of *A. flavus* resulted in aflatoxin concentrations that were lower than inoculations with only the toxigenic strain. A study in Texas by Dorner (2009) on Afla-Guard 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 Senegal, with a 75–93% reduction of aflatoxin in fields treated with AflaSafe SN01 (Bandyopadhyay et al., 2016).

The study described here attempts to address the question of whether preharvest application of biocontrol strains can affect aflatoxin accumulation by toxigenic strains during the post-harvest drying-period. Atehnkeng et al. (2014) attempted to answer this question by rewetting stored maize that was previously harvested from Aflasafe-treated and non-treated fields. They observed that the level of aflatoxin contamination increased dramatically after rewetting. Although the final level of aflatoxin in the Aflasafe-treated maize was less than the control grain, no evidence was presented to indicate that the biocontrol strains directly affected the growth and aflatoxin production by toxigenic strains. Our study tested the hypothesis that treatment of maize fields with biocontrol strains during pre-harvest period will interfere with the growth of toxigenic *A. flavus* strains during the postharvest drying period. Here we examined postharvest changes in biocontrol-treated maize in studies conducted in Texas and North Carolina.

## 2. Materials and methods

### 2.1. Maize samples

In 2017, maize samples were collected from AF36-treated (TX) and untreated fields (Control TX) in Hill and Burleson Counties in Texas. Afla-Guard-treated (NC) and untreated samples (Control NC) were also collected from fields in Rocky Mount, 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 field sites where maize was treated at the V7 and V9 stages of development with AF36 and Afla-Guard. 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 Afla-Guard 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. All samples collected from North Carolina were from fields that were previously treated with Afla-Guard.

### 2.2. Storage experiments

All maize samples from 2017 to 2018 were shipped to Purdue University, where they were stored in sealed plastic bags until use. Grain moisture was adjusted to 20% (wet basis) by the method described by Williams et al., 2014 with some modifications. About 1.5 kg of the maize with the appropriate amount of water were placed in a rotary tumbler (C&M Topline Goleta. CA) for 2 h. Thereafter, the grain was incubated at 4 °C for 72 h 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 − 8 cm × 8 cm × 16 cm) with perforated lids (lid diameter - 8.5 cm). These jars were incubated for 6 days at 28 °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 after incubation. At the same time-points, a 50 g sample was collected and stored at −20 °C until analyzed for aflatoxin.

### 2.3. Isolation and enumeration of fungi in kernels

Kernels from each time-point 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 (8%) 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). The number of infected kernels also was enumerated and the proportion of infected kernels recorded. *A. flavus* from infected kernels was isolated into pure cultures and stored at −80 °C until further characterization.

### 2.4. Characterization of A. flavus isolates

*Aspergillus flavus* isolates were grown on Rose Bengal agar medium to characterize sclerotia production. Isolates were designated as either sclerotia producers or nonproducers. By microscopic measurements, sclerotia were characterized as L-type (>400 μm in diameter) or S-type (<400 μm in diameter) (Cotty, 1989).

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 h 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 and stored at −20 °C until aflatoxin analysis.

### 2.5. 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 the 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). Aflatoxin quantification was based on comparisons with aflatoxin standards that were included on each TLC plate.

### 2.6. 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 and Arnold, 1985). Purified DNA was used as the template in a PCR amplification of *aflC* with primers *aflC*-forward (5′-TTAGATCGGTCCCTTTACTTT-3′) and AFLC-reverse (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 Afla-Guard-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 at 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 (Ehrlich and Cotty, 2004).

## 3. Results

### 3.1. Effects of high moisture conditions

In 2017, we received maize harvested from fields in Texas and North Carolina that were treated with and without biocontrol strains of *A. flavus*. At the start of the experiment, the number of fungal-infected kernels was high (>80%) in the North Carolina samples and the control sample from Texas (Table 1). The proportion of kernels from the AF36-treated field that were infected was only 40%. The number of surface fungi followed the same trend as kernel infection, with over 1000 CFU/kernel in the samples for North Carolina and the Texas control (Table 1). Over the six-day incubation period, kernel infection levels and number of surface fungi increased (Table 1). Visible signs of fungi on the kernels were observed at days 4 and 6 after incubation.

In 2018, kernels samples from Texas experimental plots that were processed in this study were from field trials evaluating efficacy of several biocontrol strains. Samples from the Greenville site had a kernel infection level between 41% and 63%, with surface fungi exceeding $1 \times 10^4$ CFU/kernel (Table 1). The kernel samples from the Field 219 site were nearly all infected and the surface fungi were greater than $25 \times 10^4$ CFU/kernel. As observed with the 2017 samples, fungi infection and surface fungal counts increased during the six day period following incubation.

Fungi 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* was predominant in both the kernel

**Table 1**
Density of surface fungi and proportion of infected kernels at different time-points following incubation[a].

| Location | Treatment | Surface fungi (CFU × $10^4$/kernel)[b] | | | | Infected kernels (%)[c] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0[d] | 2 | 4 | 6 | 0 | 2 | 4 | 6 |
| 2017 | | | | | | | | | |
| North Carolina (NC) | Afla-Guard | 0.9 | 2.5 | 157 | 100 | 88 | 100 | 100 | 100 |
| | Control | 48 | 1.2 | 18 | 71 | 99 | 94 | 100 | 100 |
| Texas (TX) | AF36 | 0 | 0 | 0.3 | 1.8 | 40 | 53 | 66 | 64 |
| | Control | 0.9 | 1.8 | 5.3 | 33 | 90 | 94 | 100 | 99 |
| 2018 | | | | | | | | | |
| Greenville (GV) | Afla-Guard | 9.4 | -[e] | – | 323 | 62 | – | – | 100 |
| | AF36 | 1.9 | – | – | 41 | 41 | – | – | 100 |
| | Contro | 13 | – | – | 33 | 63 | – | – | 100 |
| Field 219 (219) | Afla-Guard | 34 | – | – | 307 | 96 | – | – | 99 |
| | Control | 25 | – | – | 221 | 100 | – | – | 100 |

[a] Maize moisture content was adjusted to 20% and incubated at 28 °C.
[b] Values are mean from 3 replicate jars (n = 360 kernels).
[c] Value are mean percent of infection from 3 replicate jars.
[d] Days of incubation.
[e] Sample not collected.

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 and Field 219 sites, respectively, produced small sclerotia.

### 3.2. Aspergillus flavus

Although the incidence of kernels infected by fungal species was high in the three of the 2017 samples, the proportion of kernels infected with *A. flavus* was 1–2% (Table 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 proportion of kernels initially infected with *A. flavus* was higher, and the fungus spread more rapidly during the incubation period, especially in the Greenville samples.

### 3.3. Aflatoxin accumulation in grain

At the start of the storage experiment, aflatoxin was not detected in any of the 2017 samples (Table 3). However, by the end of the six-day incubation period, high aflatoxin levels were found in the Texas samples and the Afla-Guard-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 levels of 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. Since aflatoxin levels were low, the storage period was extended to 10 days. Aflatoxin continued to accumulate in the samples and levels became detectable in all Field 219 samples. However, the Greenville AF36 sample remained free of aflatoxin contamination.

### 3.4. Characterization of A. flavus isolates

From the 2017 samples, we characterized 187 *A. flavus* isolates from North Carolina and 96 from Texas (Table 4). Only 17% and 9% of the isolates from the Afla-Guard-treated and control, respectively, produced aflatoxin in culture. Of the 84 isolates from the Afla-Guard-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 six 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 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 Afla-Guard-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**
Kernels infected with *Aspergillus flavus* before and after incubation of maize grain.

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

[a] Days of incubation.
[b] Samples were collected before incubation and after 6 days of incubation.
[c] Sample not collected.

**Table 3**
Production of $AFB_1$ in maize samples harvested from North Carolina and Texas at different time points following incubation.

| Location | Treatment | Aflatoxin level (ppb)[a] | | |
|---|---|---|---|---|
| | | Day 0 | Day 6 | Day 10 |
| 2017 | | | | |
| North Carolina (NC) | Afla-Guard | 0 | 11 (0–33) | – |
| | Untreated | 0 | 0 | – |
| Texas (TX) | AF36 | 0 | 211 (33–333) | – |
| | Untreated | 0 | 77 (33–200) | – |
| 2018 | | | | |
| Greenville (GV) | AflaGuard | 0 | 8 | 14 |
| | AF36 | 0 | 0 | 0 |
| | Untreated | 4 | 2 | 6 |
| Field 219 (219) | Afla-Guard | 0 | 0 | 1 |
| | Untreated | 0 | 1 | 1 |

[a] Values in parenthesis are the range (minimum and maximum) of aflatoxin levels in tested samples. Dashes indicate samples were not collected.

## 4. Discussion

At harvest, maize kernels, as well as other grains, are contamination with many fungal genera externally on the surface and internally within 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 pre-harvest management practices (Klich, 2007). Based on 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 contained *Aspergillus* species. Similarly, studies conducted in Brazil and Nigeria identified species of *Fusarium, Penicillium,* and *Aspergillus* in harvested maize (Atehnkeng et al., 2008; Orsi et al., 2000).

Maize harvested in the US and other developed countries is usually quickly shelled and dried to moisture levels that inhibit fungal growth. In many developing countries, including those in Sub-Saharan Africa where the majority of farmers lack modern

**Table 4**
Genotype determination of *Aspergillus flavus* isolates from infected kernels from treated and untreated fields in Texas and North Carolina in 2017[a].

| Location | Treatment | Days | Total *A. flavus* isolates | Toxigenic[b] | *aflC* + | *aflC* - | AF36-like[c] |
|---|---|---|---|---|---|---|---|
| North Carolina (NC) | Afla-Guard | 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 |
| | Untreated | 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 |
| | Untreated | 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 |

[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 and Cotty, 2004).

**Table 5**
Genotype determination of *A. flavus* isolates from infected kernels of treated and untreated fields from Texas in 2018.[a]

| Location | Treatment | Days | Total *A. flavus* isolates | Toxigenic[b] | *aflC* + | *aflC* - | AF36-like[c] |
|---|---|---|---|---|---|---|---|
| Greenville (GV) | Afla-Guard | 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 |
| | Untreated | 0 | 17 | 1 | 3 | 13 | 1 |
| | | 6 | 100 | 7 | 86 | 7 | 82 |
| Field 219 (219) | Afla-Guard | 0 | 18 | 8 | 10 | 0 | 8 |
| | | 6 | 104 | 23 | 19 | 62 | 3 |
| | Untreated | 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 and Cotty, 2004).

drying machinery, the postharvest period begins with maize plants cut and stacked in fields and may remain stacked for as long as 3 weeks, depending on the prevailing 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 moisture and temperature levels 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 conducted in Nigeria by Oyebanji and Efiuvwevwere (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 moisture content (17% and 20% MC) was more prone to deterioration during the storage period.

Due to the application strategy for biocontrol products, one would expect biocontrol-treated fields to have a high incidence of *A. flavus*-infected kernels. In 2011, Isakeit et al. (2011) found that Texas maize treated with Afla-Guard 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).

In this study, genotypic 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, and many isolates lacking the *aflC* possessed *aflQ.* These observations suggest 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.* However, we cannot rule out the possibility that some of these isolates are poor aflatoxin producers in artificial medium as described by Probst and Cotty (2012). Although the an analysis of the field population of *A. flavus* was not conducted prior to planting, 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 and Bhatnagar, 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.

While the biocontrol approach is highly effective in reducing pre-harvest aflatoxin contamination in maize, our results did not reveal evidence that the efficacy of pre-harvest biocontrol treatments extends through a prolonged post-harvest 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, who 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, tillage practices, weed control and insect control should also be integrated in these fields to reduce *A. flavus* infection and aflatoxin levels in maize during post-harvest periods (Lavkor and Var, 2017; Okoth et al., 2012).

## Declaration of competing interest

The Authors have no competing interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jspr.2019.101519.

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