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Degradation of Aflatoxins B_1 by Atoxigenic *Aspergillus flavus* Biocontrol Agents

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Abstract

Aflatoxins are potent *Aspergillus* mycotoxins that contaminate food and feed, thereby impacting health and trade. Biopesticides with atoxigenic *Aspergillus flavus* isolates as active ingredients are used to reduce aflatoxin contamination in crops. The mechanism of aflatoxin biocontrol is primarily attributed to competitive exclusion but, sometimes, aflatoxin is reduced by greater amounts than can be explained by displacement of aflatoxin-producing fungi on the crop. Objectives of this study were to (i) evaluate the ability of atoxigenic *A. flavus* genotypes to degrade aflatoxin B_1 (AFB1) and (ii) characterize impacts of temperature, time, and nutrient availability on AFB1 degradation by atoxigenic *A. flavus*. Aflatoxin-contaminated maize was inoculated with atoxigenic isolates in three separate experiments that included different atoxigenic genotypes, temperature, and time as variables. Atoxigenic genotypes varied in aflatoxin degradation but all degraded AFB1 >44% after 7 days at 30°C. The optimum temperature for AFB1 degradation was 25 to 30°C, which is similar to the optimum range for AFB1 production. In a time-course experiment, atoxigenics degraded 40% of AFB1 within 3 days, and 80% of aflatoxin was degraded by day 21. Atoxigenic isolates were able to degrade and utilize AFB1 as a sole carbon source in a chemically defined medium but quantities of AFB1 degraded declined as glucose concentrations increased. Degradation may be an additional mechanism through which atoxigenic *A. flavus* biocontrol products reduce aflatoxin contamination pre- or postharvest. Thus, selection of optimal atoxigenic active ingredients can include assessment of both competitive ability in agricultural fields and their ability to degrade aflatoxins.

Keywords: aflatoxin, *Aspergillus flavus*, atoxigenic biocontrol agent, degradation

Aflatoxins are naturally occurring carcinogenic secondary metabolites produced by several species in *Aspergillus* section *Flavi*, including *Aspergillus flavus* Link (Cotty et al. 1994). Aflatoxins contaminate crops such as maize, peanut, cassava, sorghum, cottonseed, rice, wheat, chilies, and tree nuts (Essono et al. 2009; Kachapulula et al. 2017; Picot et al. 2017; Probst et al. 2007; Singh and Cotty 2019). Aflatoxin B_1 (AFB1), the most common and potent aflatoxin, is classified as a group 1a human carcinogen by the International Agency for Research on Cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2002). Aflatoxin contamination impacts one-quarter of the world's population, causing acute and chronic health effects, including immune suppression, growth retardation, cancer, and death (Gong et al. 2002; Woo et al. 2011). The negative health impacts of aflatoxins are rare in countries where aflatoxin levels in the food and feed supply are strictly regulated. However, these regulations pose an economic burden, and trade markets are limited when crops become contaminated with high levels of aflatoxins (Wu 2015).

Agronomic practices, host genetics, and proper postharvest storage and handling of crops can be optimized to minimize aflatoxin contamination and accumulation (Hell et al. 2000); however, one of the most effective aflatoxin control strategies is the application of biocontrol products based on nonaflatoxigenic (atoxigenic) *A. flavus* genotypes. Atoxigenic *A. flavus*-based biocontrol products have been commercially available for over two decades (Adhikari et al. 2016; Atehnkeng et al. 2008; Cole and Cotty 1990; Dorner and Lamb 2006). Reduction in crop aflatoxin content by application of these atoxigenic *A. flavus* biocontrol products has been primarily attributed to competitive exclusion (Cotty and Bayman 1993, Mauro et al. 2018, Senghor et al. 2020). Atoxigenic strain applications change the structure of *Aspergillus* communities associated with crops so that aflatoxin producers are less common (Mauro et al. 2018). Community structure changes are driven by founder effects, competitive superiority of atoxigenic strains, advantages instilled by nutrients in the biopesticide formulation, and delivery of the product to the soil surface without incorporation into a trapping soil matrix (Bandyopadhyay et al. 2016; Cotty and Bayman 1993; Ortega-Beltran and Cotty 2018). However, in some cases, the reduction of aflatoxin observed in crops exceeds what would be expected from

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The authors receive no direct financial benefit from the marketing of the atoxigenic biocontrol products reported in this work. Initial patents for the use of atoxigenic strains to prevent aflatoxin contamination were filed in 1988 and awarded by the U.S. patent office to the United States Department of Agriculture in 1992 and 1994 with P. J. Cotty as the inventor. Patent protection has expired. The Aflasafe name is a Trademark of the International Institute of Tropical Agriculture (IITA). Manufacturing and distribution of the Aflasafe biocontrol products have transferred to the private companies. IITA charges a licensing fee to manufacturers for the use of the Aflasafe name and associated technology transfer. IITA employs R. Bandyopadhyay. The remaining authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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displacement of aflatoxigenic strains alone (Mehl and Cotty 2010). This reduction in aflatoxin has been attributed to additional mechanisms such as competition for nutrients and regulation of aflatoxin biosynthesis as a response of thigmo stimuli (Huang et al. 2011; Mehl and Cotty 2013). In addition to having reduced aflatoxins at harvest, crops treated with these atoxigenic biopesticides do not show the increases in aflatoxin content during postharvest handling and storage usually seen in contaminated crops (Brown et al. 1991; Dorner and Cole 2002). Postharvest benefits have been credited to lower levels of the aflatoxin producers present in the crop (Senghor et al. 2020) or the continued superior competitive ability of the atoxigenic biocontrol strains on the substrate (Mehl and Cotty 2010, 2013). However, another potential but unrecognized mechanism of aflatoxin biocontrol both pre- and postharvest may be degradation of aflatoxins by the atoxigenic *A. flavus* active ingredients in commercial biopesticide products.

Several studies have demonstrated that aflatoxin-producing *A. flavus* and *A. parasiticus* strains have the capability to degrade their own synthesized aflatoxin (Doyle and Marth 1978a; Hamid and Smith 1987; Huynh et al. 1984) but the exact adaptive functions for the production and degradation of aflatoxins are not well defined. In vitro, after aflatoxin biosynthesis stops, reductions in aflatoxin concentrations have been observed (Huynh and Lloyd 1984; Huynh et al. 1984). In one study, aflatoxin levels declined when mature cultures were aged in expended media, suggesting possible degradation and utilization of aflatoxin during the stationary phase (Doyle and Marth 1978a). The only *A. flavus* strain that failed to produce aflatoxin in these studies also failed to significantly degrade aflatoxin in media, and it was concluded that the ability of *A. flavus* isolates to degrade aflatoxins is correlated with aflatoxin-producing potential (Doyle and Marth 1978b). Based on these results, it was also suggested that genes in the aflatoxin biosynthesis cluster may play a role in the degradation process (Doyle and Marth 1978b; Hamid and Smith 1987). *A. flavus* isolates have different mutations conferring atoxigenicity ranging from point mutations (Donner et al. 2010; Ehrlich et al. 2007) to either partial (Donner et al. 2010) or full deletion of the aflatoxin biosynthesis gene cluster (Adhikari et al. 2016; Chang et al. 2005). There may be variation among atoxigenic *A. flavus* in aflatoxin-degrading ability based on the presence or absence of aflatoxin biosynthesis genes; however, no studies have examined this. Overall, very few studies have examined the ability of atoxigenic *A. flavus* to degrade aflatoxins, and those that did only included a single atoxigenic strain or were performed in liquid media (Cotty and Bayman 1993; Doyle and Marth 1978b; Raksha Rao et al. 2020; Xing et al. 2017). Furthermore, the ability of different atoxigenic *A. flavus* genotypes that are active ingredients in commercial biopesticide products to degrade aflatoxins during crop infection has not been explored.

The current study tests the hypothesis that, in addition to competitive exclusion, atoxigenic biocontrol strains of *A. flavus* have the potential to reduce both pre- and postharvest contamination via degradation of aflatoxins. Objectives of this study were to (i) evaluate the ability of atoxigenic *A. flavus* genotypes that are active ingredients in registered biocontrol products to degrade aflatoxin and (ii) characterize impacts of temperature, time, and nutrient availability on degradation of aflatoxins by atoxigenic *A. flavus*. Understanding degradation of aflatoxins can be an important factor in aflatoxin management.

Materials and Methods

Fungal isolates. Ten active ingredients from five commercially available biopesticides were evaluated for their ability to degrade aflatoxins (Table 1). Two isolates, NRRL18543 (Bock and Cotty 1999) and NRRL21882 (Dorner and Lamb 2006), were used in experiments aimed at evaluating the impacts of time, temperature, and nutrient availability on aflatoxin degradation by atoxigenic *A. flavus.* These isolates are the active ingredients in the two aflatoxin biopesticide formulations that are commercially available in the United States (AF36 Prevail and Afla-Guard). The aflatoxigenic *A. flavus* isolate AF13 (Bock and Cotty 1999) was used for AFB1 production. Isolates were obtained from the silica gel storage culture collection at the United States Department of Agriculture–Agricultural Research Service aflatoxin lab in Tucson, AZ. Fungal isolates were cultivated on 5/2 agar (5% V8 juice [Campbell Soup Company] and 2% agar [Difco Laboratories Inc.], pH 6.0] for 5 days at 31°C in the dark. After growth, six colonized 3-mm agar plugs were added to 2.5 ml of sterile distilled water (dH_2O) in vials and stored at 8°C. For each isolate, a 15-µl spore suspension from the vials was seeded in the center of 5/2 agar plates and incubated for 5 days at 31°C in the dark. Spores were harvested using sterile cotton swabs and transferred into glass vials containing 20 ml of sterile dH_2O with 0.01% Tween-80. The turbidity of each spore suspension was measured with a turbidity meter (Model 965-10; Orbeco-Hillige), and spore concentrations were calculated using a nephelometric turbidity unit (NTU) versus CFU curve: $Y = 49{,}937X$, where X = NTU and Y = spores per milliliter (Mehl and Cotty 2010). A spore suspension of each isolate was standardized to 10^6 spores/ml before inoculation.

Maize inoculation. To prepare aflatoxin-contaminated maize, 250 g of kernels of Pioneer hybrid N82VGT was placed in 1-liter Nalgene bottles and autoclaved at 121°C for 20 min to eliminate any contaminating microorganisms. Grain water content was measured with a moisture balance (HB43 Halogen Moisture Analyzer; Mettler-Toledo) and adjusted to 25% by adding sterile dH_2O. The kernels were then inoculated with 2 ml of AF13 at 1×10^6 spores/ml. The mouths of the bottles were sealed with Tyvek (Roll 1443R; DuPont Tyvek) to provide a sterile, gas-permeable barrier. Inoculated maize was incubated for 14 days at 31°C in the dark. Following incubation, the bottles were autoclaved at 121°C for 20 min to kill AF13. Kernels were then mixed and distributed into 50-ml Falcon tubes (5 g/tube). Four tubes were randomly selected for extraction and quantification of initial levels of AFB1 as described below. The remaining

Table 1. Atoxigenic *Aspergillus flavus* isolates used in the current study

Isolate	Culture accession, source[a]	Type[b]	Biocontrol product[c]	Citation
AF36	NRRL 18543, United States	S	AF36 Prevail	Adhikari et al. 2016; Bock and Cotty 1999; Donner et al. 2010; Ehrlich et al. 2007
Aflaguard	NRRL 21882, United States	C	Afla-Guard	Adhikari et al. 2016; Donner et al. 2010; Dorner and Lamb 2006
GP5G-8	IITA, Mozambique	S	Aflasafe MWMZ01/MZ02	Current study
MZM029-7	IITA, Mozambique	C	Aflasafe MWMZ01	Current study
MZM028-5	IITA, Mozambique	P	Aflasafe MZ02	Current study
MZM594-1	IITA, Mozambique	S	Aflasafe MWMZ01	Current study
Og0222	IITA, Nigeria	C	Aflasafe	Adhikari et al. 2016; Donner et al. 2010
Ka16127	IITA, Nigeria	S	Aflasafe	Adhikari et al. 2016; Donner et al. 2010
La3279	IITA, Nigeria	S	Aflasafe	Adhikari et al. 2016; Donner et al. 2010
La3304	IITA, Nigeria	S	Aflasafe	Adhikari et al. 2016; Donner et al. 2010
AF13	USDA-ARS, United States	N/A	N/A	Bock and Cotty 1999

[a] NRRL = Agricultural Research Service Culture Collection; IITA = The International Institute of Tropical Agriculture; and USDA-ARS = United States Department of Agriculture–Agricultural Research Service Aflatoxin Biocontrol Lab, Tucson, AZ.
[b] Type of atoxigenicity: C = complete deletion of the aflatoxin cluster, P = partial deletion, S = single nucleotide polymorphism in the aflatoxin biosynthesis cluster, and N/A = not applicable (aflatoxigenic, not a biocontrol active ingredient).
[c] Registered biocontrol product in which the indicated isolate is an active ingredient.

tubes were inoculated with 2 ml of atoxigenic *A. flavus* isolates at 1 × 10^6 spores/ml. Noninoculated control tubes consisted of autoclaved contaminated maize to which 2 ml of sterile dH_2O was added. The tubes were sealed with Tyvek and incubated in the dark. This protocol was used to conduct the three experiments described below, with a tube serving as an experimental unit.

Aflatoxin degradation by atoxigenic genotypes of *A. flavus*. To determine whether degradation of aflatoxins is associated with the type of mutation in the aflatoxin biosynthesis cluster, the aflatoxin-degrading abilities of atoxigenic isolates with different genotypes that resulted in atoxigenicity were compared. The mutations conferring atoxigenicity for the active ingredients in the biopesticides AF36 Prevail, Afla-Guard, and Aflasafe have been previously characterized (Table 1) (Adhikari et al. 2016; Chang et al. 2005; Donner et al. 2010; Ehrlich et al. 2007). The cluster amplification pattern method described previously (Callicott and Cotty 2015) was used to identify the type of mutation of genes within the aflatoxin biosynthesis cluster from the isolates of Aflasafe MWMZ01 and Aflasafe MZ02.

To test the ability of *A. flavus* genotypes with different mechanisms of atoxigenicity to degrade AFB1, aflatoxin-contaminated maize (AFB1 at mean = 11.7 ± 1.8 μg/g) was inoculated with 1 × 10^6 spores of the individual atoxigenic isolates. Maize inoculated with the aflatoxin-producing isolate AF13 and noninoculated maize were included as controls. The experiment followed a completely randomized design with 12 treatments and four replicates. The treatments were incubated for 7 days at 31°C in the dark. Aflatoxin was extracted and quantified as described below. To evaluate whether genes in the aflatoxin biosynthesis cluster affect the ability of atoxigenic isolates to degrade aflatoxin, quantities of aflatoxin degraded by three isolates with a complete deletion of the aflatoxin biosynthesis cluster (NRRL 21882, Og0222, and MZM029-7) were compared with quantities degraded by three isolates with single-nucleotide polymorphisms (SNPs) and a lack of large deletions in the gene cluster (NRRL 18543, Ka16127, and MZM594-1).

Influence of temperature on aflatoxin degradation. To evaluate the effect of temperature on both production and degradation of AFB1, aflatoxin-contaminated maize (AFB1 at mean = 19.3 ± 1.1 μg/g) was inoculated with aflatoxigenic isolate AF13 and two atoxigenic isolates (NRRL 18543, the active ingredient in AF36 Prevail, and NRRL 21882, the active ingredient in Afla-Guard). Inoculated maize was incubated at five different temperatures (10, 15, 25, 30, or 35°C) for 7 days in the dark. Treatments were arranged in a randomized factorial design with four replicates. At the end of the experiment, aflatoxin was extracted and quantified as described below.

Time course of aflatoxin degradation. To evaluate aflatoxin degradation over time, aflatoxin-contaminated maize (AFB1 at mean = 5.1 ± 0.7 μg/g) was inoculated with two atoxigenic isolates (NRRL 18543 and NRRL 21882) and incubated at 31°C in the dark for up to 21 days. A noninoculated control was also included to account for aflatoxin degradation in the absence of fungal growth. Treatments were arranged in a randomized factorial design with three inoculation treatments, six incubation times (3, 7, 10, 14, 17, and 21 days), and four replicates per treatment combination. At the end of the experiment, aflatoxin was extracted and quantified as described below. For each treatment, percent aflatoxin remaining at different time points was calculated by dividing the measured concentration AFB1 by the initial concentration of AFB1 (5.1 μg/g) and multiplying by 100%.

Impact of nutrient availability on aflatoxin degradation. To test whether aflatoxin can be used as a carbon source, an in vitro experiment was performed. First, aflatoxin produced on maize was extracted with acetone/dH_2O (85:15). Extracts were separated by thin-layer chromatography (TLC) (Silica gel G with Preadsorbent Zone; UNIPLATE, ANALTECH) with diethyl ether/methanol/water (96:3:1). All reagents were analytical grade and purchased from EMD Millipore. To isolate separated AFB1 from other compounds, the TLC plates were visualized under 365-nm UV light, and silica from the area with AFB1 as determined by comparison with aflatoxin standards (Aflatoxin Mix Kit-M; Supelco) was scraped off. The silica gel containing the separated AFB1 was concentrated as described previously (Cardwell and Cotty 2002). After drying, AFB1 was resuspended in a volume equivalent to 10% of the original acetone solution. The purified AFB1 was confirmed both by visualization of blue fluorescence under 365-nm UV light and by the presence of a single peak in the corresponding to the retention factor of AFB1 of the standard (Supplementary Fig. S1).

Czapek's (CZ) broth (Dox 1910) was modified by substituting glucose for sucrose as the sole carbon source. Glucose was added to the CZ broth at final concentrations of 0, 8, 16, 33, 66, and 166 mM. The above-described aflatoxin solution (3 ml) was added to 30 ml of the modified CZ broths in 250-ml Erlenmeyer flasks. Four flasks containing aflatoxin plus liquid media at the different glucose levels were randomly selected for extraction and quantification of the initial quantity of AFB1, which was 2.3 ± 0.24 μg/ml. The remaining flasks were individually inoculated with either 2 ml (1 × 10^6 spores/ml) of an atoxigenic isolate (NRRL 18543 or NRRL 21882) or 2 ml of sterile water (control). The experiment was a randomized factorial design with six glucose concentrations, three inoculation treatments, and four replicates of each treatment combination. The flasks were placed into two different VWR1575R refrigerated incubator shakers and incubated with shaking (150 rpm) at 31°C in the dark for 5 days. At the end of the experiment, mycelia were separated from the CZ broth using vacuum filtration onto Whatman No. 4 filter paper and dried in an oven at 60°C for 24 h. The filter paper plus mycelia was weighed, and the mycelial mass was calculated by subtracting the weight of the filter paper. Aflatoxin was extracted and quantified as described below. For each treatment, percent aflatoxin degraded was calculated by dividing the difference between the initial and final concentration of AFB1 by the initial concentration AFB1 (2.3 μg/ml) and multiplying by 100%. The quantity of aflatoxin degraded per gram of mycelia was calculated by dividing the total quantity of AFB1 degraded (difference between the initial and final concentration of AFB1 multiplied by the total volume) by the dry weight of the mycelia.

AFB1 extraction and quantification. For maize experiments, kernels were ground using a Geno/grinder 2010 (SPEX) with four sterile 6-mm stainless steel beads (2157; SPEX), and AFB1 was extracted from 5 g of ground maize with 35 ml of 85% acetone (Bertuzzi et al. 2012). For the in vitro experiment, AFB1 was extracted from 30 ml of CZ broth with 30 ml of 100% acetone. Extracts were separated on TLC plates (Silica gel 60; EMD) alongside aflatoxin standards (Aflatoxin Mix Kit-M; Supelco) with diethyl ether/methanol/water (96:3:1). All solvents were analytical grade and purchased from EMD Millipore. TLC plates were visualized under 365-nm UV light and AFB1 was quantified directly on plates using scanning fluorescence densitometry with a TLC Scanner 3 (Camag Scientific) (Probst and Cotty 2012). The limit of detection (LOD) of AFB1 was considered the lowest amount of AFB1 spotted on the TLC plate detectable by the scanner (Dolowy et al. 2015). The LOD of AFB1 determined by spiking concentrations of a serial dilution of the aflatoxin standard mix was 0.005 ng/μl (Supplementary Fig. S2).

Statistical analyses. AFB1 concentrations were $\log_{10}$-transformed and percent degradation data were arcsine transformed to meet assumptions of homogeneity of variance. Main treatment effects and their interactions were evaluated with an analysis of variance, and the means were compared using Tukey's honestly significant difference. A Student's *t* test was used to compare aflatoxin degradation by *A. flavus* genotypes with and without complete deletion of that aflatoxin biosynthesis gene cluster. Correlation analyses between AFB1 degraded per mass of mycelium, fungal growth, and glucose concentrations were performed. All statistics were conducted using JMP 11.1.1 (SAS Institute). The true means of the nontransformed data are reported.

Results

Aflatoxin degradation by atoxigenic genotypes of *A. flavus*. Ten atoxigenic genotypes of *A. flavus* that are active ingredients in five commercially available aflatoxin biocontrol products were evaluated for ability to degrade aflatoxins. The type of mutation, SNP, or deletion in the aflatoxin gene cluster conferring atoxigenicity varied among the isolates as determined previously or in the current study

(Table 1). When grown at 31°C for 7 days on aflatoxin-contaminated maize, all 10 atoxigenic genotypes degraded AFB1 as compared with the negative control, whereas the aflatoxin-producing strain AF13 increased AFB1 concentrations (Fig. 1A). Although all atoxigenic genotypes were able to degrade AFB1, there were differences among genotypes in the extent of degradation ($P < 0.0001$). Isolate NRRL 21882 degraded AFB1 the least (45%) while Og0222 reduced AFB1 concentrations the most (79%). Although there were differences in the amount of AFB1 degraded by the different genotypes, this variation was not associated with type of atoxigenicity (Fig. 1B). Atoxigenic biocontrol isolates that had a full deletion of the aflatoxin cluster degraded similar amounts of aflatoxin compared with isolates that only had point mutations in aflatoxin cluster genes ($P = 0.068$). The two isolates that are active ingredients in biocontrol products registered in the United States (NRRL 21882 and NRRL 18543) were similar in their ability to degrade aflatoxin and represent two different types of atoxigenicity; a full cluster deletion and an SNP, respectively. Thus, these two isolates were used for subsequent experiments aimed at characterizing factors that influence aflatoxin degradation.

Influence of temperature on AFB1 degradation. AFB1 in contaminated maize kernels was degraded by both atoxigenic isolates at all temperatures tested, resulting in significantly less AFB1 in the NRRL 18543 and NRRL 21882 treatments after 7 days as compared with the treatment with the aflatoxin-producing isolate AF13 ($P < 0.0001$) (Fig. 2). Overall, atoxigenic isolates did not differ in their ability to degrade aflatoxin ($P = 0.97$), and aflatoxin degradation varied by temperature ($P < 0.0001$). Even though optimal degradation was observed for both isolates at 25°C, NRRL 18543 degraded aflatoxin by 82% whereas degradation by NRRL 21882 was 62% ($P = 0.0036$). Aflatoxin degradation by the two atoxigenic isolates was similar at all other temperatures evaluated ($P > 0.05$). AFB1 production by the toxigenic isolate AF13 also reached its maximum at 25°C, and concentrations in maize more than doubled compared with the initial aflatoxin content (19.3 ± 1.1 versus 42.0 ± 1.0 µg/g) (Fig. 2). Interestingly, degradation of AFB1 was observed even at 10°C, a temperature that did not support significant AFB1 biosynthesis by AF13. Both aflatoxin degradation and biosynthesis increased between 10 and 25°C but, whereas aflatoxin production leveled off between 25 and 35°C, degradation decreased at temperatures greater than 25°C ($P < 0.0001$).

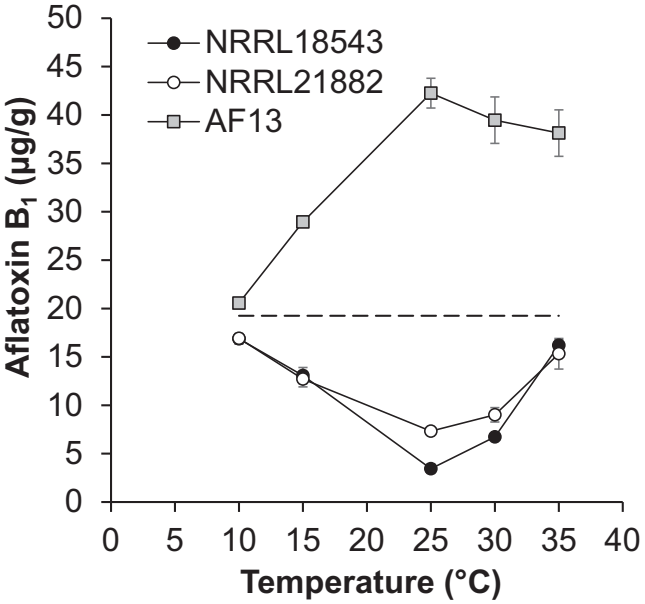

Fig. 2. Degradation of aflatoxin B_1 (AFB1) at different temperatures by active ingredients of commercial biocontrol products. Maize contaminated with AFB1 at 19.3 ± 1.1 µg/g was inoculated with NRRL 18543 (active ingredient in *Aspergillus flavus* AF36 Prevail), and NRRL 21882 (active ingredient in Afla-Guard). Treatments were incubated at five different temperatures and AFB1 remaining after 7 days was measured. A positive control of an aflatoxin-producing genotype (AF13) was also included. The dotted line indicates the initial concentration of AFB1. Data points are the average of four replicates. Error bars = standard error of the mean.

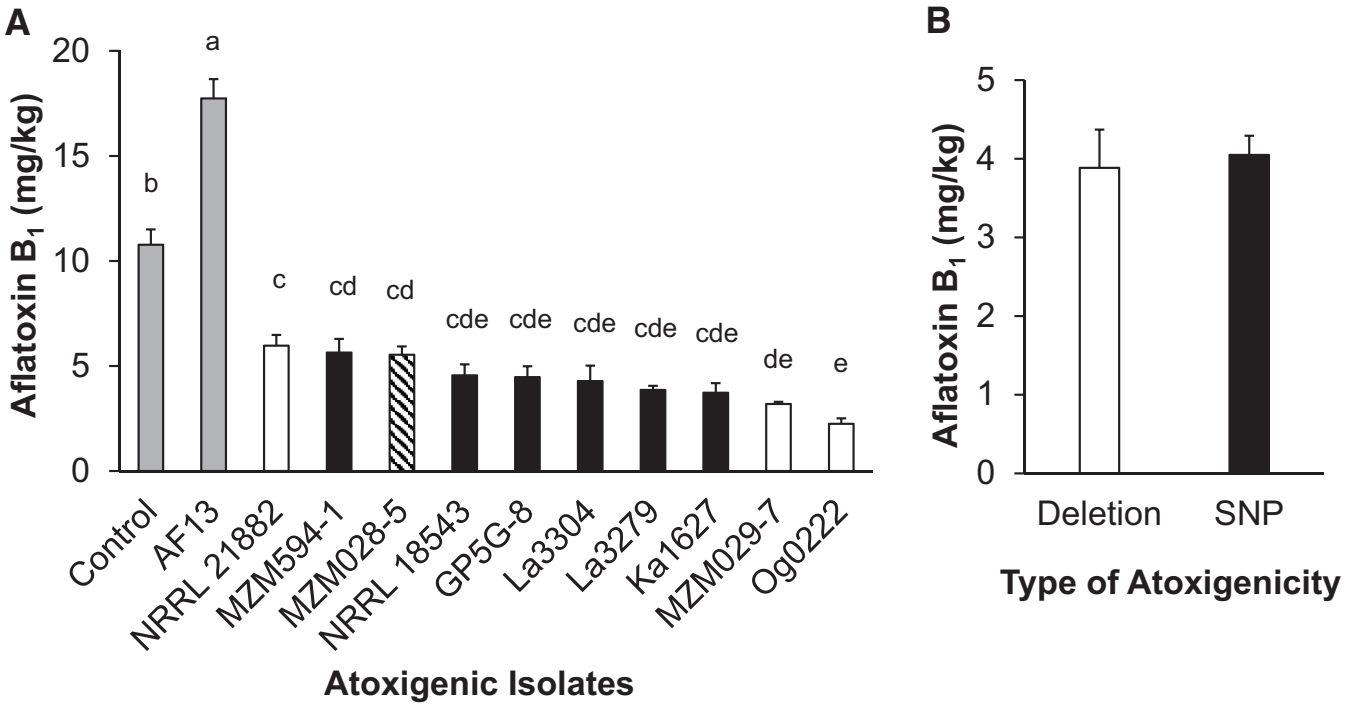

Fig. 1. Aflatoxin B_1 (AFB1) remaining after degradation by different atoxigenic genotypes of *Aspergillus flavus*. **A,** Maize contaminated with AFB1 at 11.7 ± 1.8 µg/g was inoculated with different atoxigenic *A. flavus* isolates and incubated (31°C, 7 days). Atoxigenic isolates had either single nucleotide polymorphisms (SNPs) in the aflatoxin biosynthesis cluster (black bars), partial deletions (striped bars), or complete deletion of the aflatoxin cluster (white bars). An aflatoxin-producing isolate (AF13) and noninoculated control were also included (gray bars). Means were compared using Tukey's honestly significant difference at the 95% confidence level. Means sharing the same letter do not differ significantly. Each value is the average of four replicates. **B,** The average amount of AFB1 remaining after degradation by isolates with complete deletions versus SNPs was similar ($P = 0.76$). Error bars = standard error of the mean.

Time course of aflatoxin degradation. AFB1 concentrations in maize kernels inoculated with atoxigenic isolates NRRL 18543 and NRRL 21882 decreased over time ($P < 0.00010$ (Fig. 3). Degradation by the two isolates was similar ($P = 0.52$) and there was not an isolate–time interaction ($P = 0.65$). Aflatoxin concentrations in the noninoculated control also decreased over time but AFB1 was degraded at a faster rate in treatments inoculated with atoxigenic isolates ($P < 0.001$). By day three, 27 and 35% of AFB1 was degraded by NRRL 18543 and NRRL 21882, respectively, and by day 21 both atoxigenic isolates reduced AFB1 by greater than 80% (Fig. 3). In contrast, 2 and 21% of AFB1 was degraded in the noninoculated control on days 3 and 21, respectively. The rate of aflatoxin degradation was greatest during the first 3 days following inoculation (10% of aflatoxin degraded per day, on average) and, after a week, average degradation per day was 4% or less (Fig. 3).

Impact of nutrient availability on aflatoxin degradation. CZ medium was supplemented with varying concentrations of glucose (0, 8, 16, 33, 66, and 166 mM) with and without AFB1 (mean = 2.3 ± 0.24 µg/ml) and, after 5 days of growth, the dry weight of *A. flavus* mycelia and concentrations of AFB1 were determined. Overall, mycelial growth was significantly greater with the addition of aflatoxin to the media (mean across all glucose concentrations: 0.30 versus 0.27 g of mycelia, $P = 0.0057$), and this response was similar for the two atoxigenic isolates (aflatoxin–isolate interaction: $P = 0.5058$). In addition, the effect was similar across all glucose concentrations (aflatoxin–glucose treatment interaction: $P = 0.7629$). A small quantity of mycelial growth occurred in media with aflatoxin as the only carbon source (mean = 0.04 g mycelia) (Fig. 4B); in contrast, to no mycelial growth in media lacking both aflatoxin and glucose was observed (Fig. 4A). This suggests that AFB1 was being utilized as a carbon source. Mycelial growth of both isolates increased as the glucose concentration increased ($P < 0.0001$), Though the two atoxigenic isolates differed slightly in their response to glucose at the 33- and 66-mM concentrations (glucose–isolate interaction, $P < 0.0001$) (Fig. 5A), the overall trend of increasing mycelial growth with increasing glucose was similar for both isolates.

Both atoxigenic isolates were able to degrade AFB1 in CZ media at all glucose concentrations, and percent degradation varied by glucose concentration ($P < 0.0001$). Though NRRL 18543 degraded a slightly greater percentage of AFB1 compared with NRRL 21882 at the 16-mM glucose concentration (isolate–glucose concentration interaction: $P = 0.0309$), percent degradation was similar at the other concentrations, and both isolates degraded the least aflatoxin at the greatest (166 mM) glucose concentration (Fig. 5B). When aflatoxin degradation was expressed as the quantity degraded per gram of mycelia, degradation was the greatest in the absence of glucose and decreased with increasing glucose concentration ($P < 0.0001$) (Fig. 5C). There was an isolate–glucose concentration interaction ($P = 0.0078$) due to NRRL 21882 degrading a greater quantity of AFB1 per gram of mycelia compared with NRRL 18543 in the absence of glucose. However, the two isolates degraded similar quantities of AFB1 per gram of mycelia when glucose was added to the media, and both isolates degraded the least amount in the treatment with the greatest glucose concentration ($P < 0.0001$). As expected, fungal growth was positively correlated with glucose concentration ($r = 0.98$; $P < 0.0001$). Absolute quantities of aflatoxin degraded were not significantly correlated with glucose concentration ($P = 0.0586$); however, there was a negative correlation between glucose concentration and aflatoxin degraded per mycelial mass ($r = -0.46$, $P = 0.0009$).

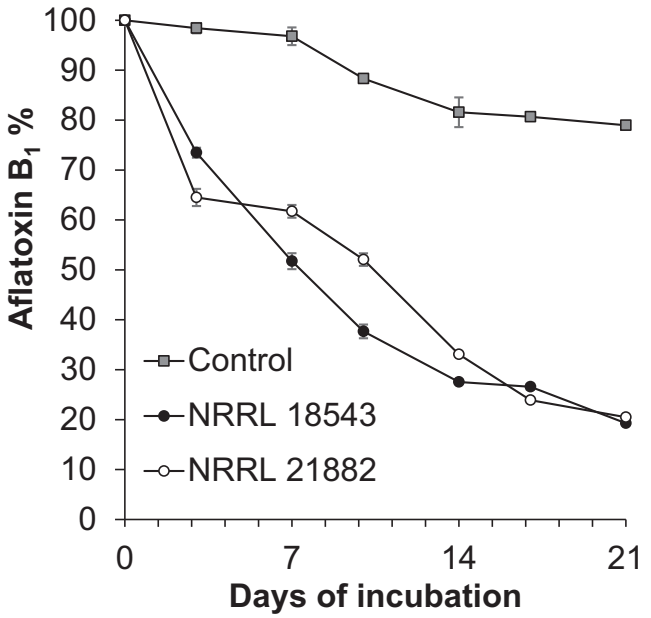

Fig. 3. Degradation of aflatoxin B_1 (AFB1) in maize by atoxigenic *Aspergillus flavus* over time. Maize kernels contaminated with AFB1 at 5.1 ± 0.7 µg/g were either noninoculated (control) or inoculated with atoxigenic isolates NRRL 21882 or NRRL 18543. Kernels were incubated at 31°C, and the percent AFB1 remaining relative to the initial aflatoxin concentration was calculated for each time point. Data points are the average of four replicates. Error bars = standard error of the mean.

Discussion

This is the first study to characterize aflatoxin degradation by atoxigenic *A. flavus* isolates that are active ingredients in commercial aflatoxin biocontrol products. Previous research has shown that some atoxigenic *A. flavus* biocontrol strains reduce aflatoxin contamination more than can be explained by competitive exclusion alone (Cotty and Bayman 1993; Mehl and Cotty 2010), thus suggesting that additional mechanisms of aflatoxin biocontrol may be in play. Experiments conducted in this study demonstrated that degradation is another mechanism through which atoxigenic biocontrol strains can reduce aflatoxin contamination in crops. Ten atoxigenic *A. flavus* isolates that are active ingredients in five different commercial biocontrol products were all able to degrade AFB1 in contaminated autoclaved maize grain by over 44% (Fig. 1A). There was some variability in aflatoxin degradation among atoxigenic isolates; however, this was independent of the type of mutation conferring atoxigenicity (Fig. 1B). Degradation occurred rapidly following inoculation of aflatoxin-contaminated maize with atoxigenic *A. flavus*, and the optimum temperature for aflatoxin degradation was 25°C. Furthermore, it was demonstrated that atoxigenic *A. flavus* can utilize AFB1 as a carbon source. The results of this study suggest that, under certain conditions pre- or postharvest, atoxigenic active ingredients in registered aflatoxin biocontrol products may degrade aflatoxins that are present in the crop. This is significant because aflatoxin-degrading ability of

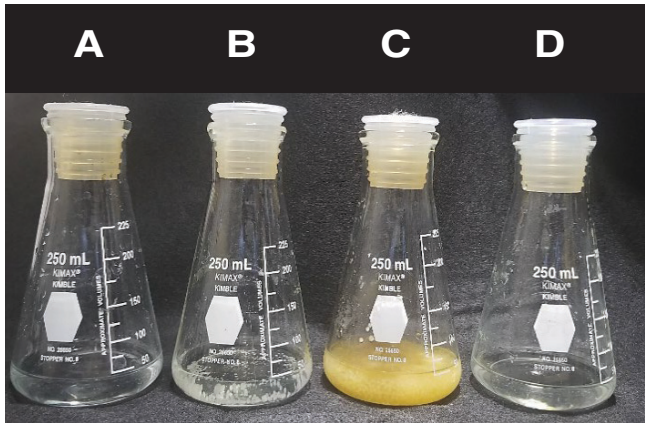

Fig. 4. Mycelial growth of atoxigenic *Aspergillus flavus* isolate NRRL 21882 in Czapek-Dox (CZ) broth **C,** with and **A** and **B,** without glucose and **B** and **C,** with and **A,** without aflatoxin B_1 (AFB1) at 2.3 ± 0.24 µg/ml. **D,** Noninoculated control. Flasks were incubated at 31°C for 5 days. There was a lack of *A. flavus* growth in the absence of glucose and AFB1 (A) but some mycelial growth was observed in the absence of glucose when aflatoxin was added to the CZ medium (B). As expected, CZ with glucose and AFB1 (C) supported more *A. flavus* growth that aflatoxin alone.

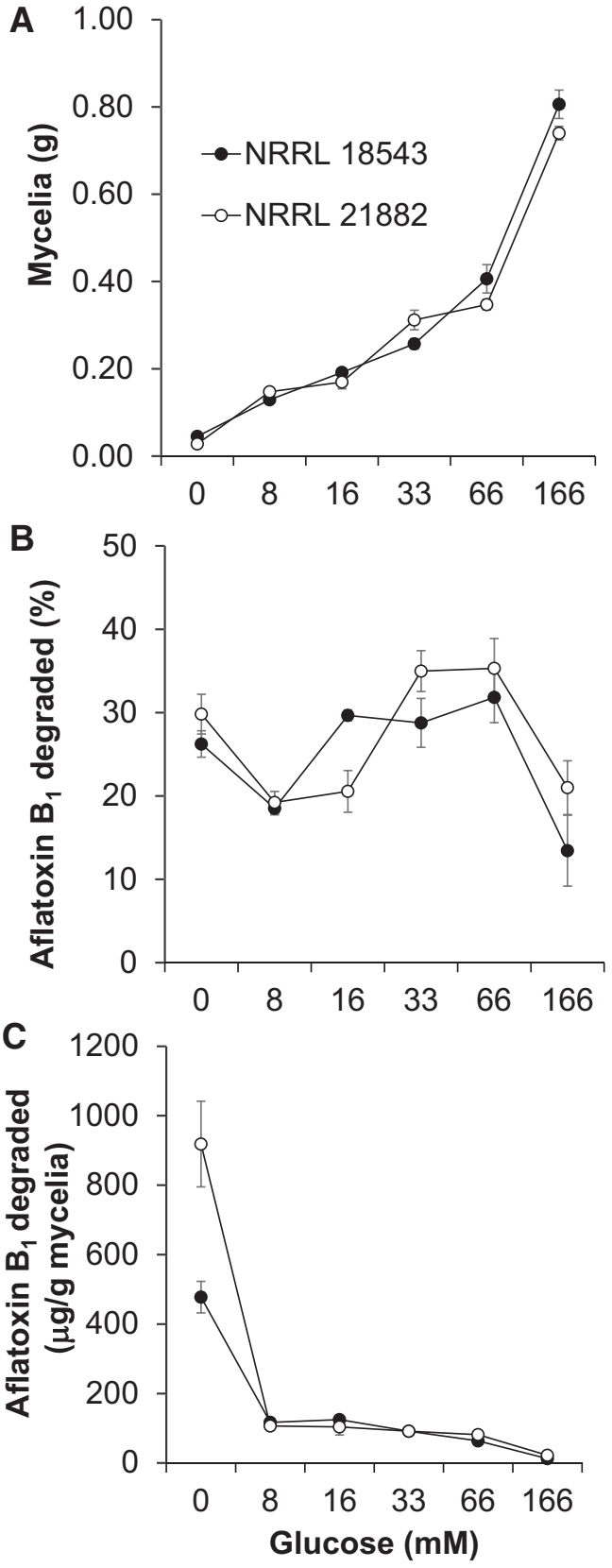

Fig. 5. Fungal growth and aflatoxin B_1 (AFB1) degradation by atoxigenic *Aspergillus flavus* isolates NRRL 18543 and NRRL 21882 in Czapek-Dox (CZ) broth medium with varying concentrations of glucose as the sole carbon source. Flasks containing CZ medium and AFB1 (2.3 ± 0.24 µg/ml) were inoculated and incubated at 31°C for 5 days. **A,** Dry weight of mycelia and **B,** percent AFB1 degradation relative to a noninoculated control were measured. **C,** Quantity of AFB1 (in micrograms) degraded per gram of mycelia at the different glucose concentrations. Data points are the average of four replicates. Error bars = standard error of the mean.

atoxigenic strains may contribute to the efficacy of currently registered biopesticides, and selection of new atoxigenic active ingredients with superior aflatoxin-degrading ability may allow for development of aflatoxin biocontrol products with improved efficacy.

Previous studies concluded that atoxigenic *A. flavus* isolates degrade little to no aflatoxin (Cotty and Bayman 1993; Doyle and Marth 1978b; Raksha Rao et al. 2020); however, the current study differs in that it is the first to test degradation on a crop substrate. The use of autoclaved maize kernels as a host substrate is supported by a previous study which showed that *A. flavus* growth and aflatoxin production was similar on autoclaved versus unautoclaved kernels (Probst and Cotty 2012). Furthermore, use of autoclaved maize kernels allows analysis solely of the impact of the inoculated atoxigenic *A. flavus* without interference from the toxigenic strain used to contaminate the maize or potential aflatoxin-degrading microorganisms that may be naturally associated with the maize. The results of the current study suggest that applied biocontrol strains have the potential to degrade aflatoxins. There was some variation in the extent to which atoxigenic isolates degraded aflatoxins but this is not unexpected due to the high levels of genotypic and phenotypic diversity within *A. flavus* that include differential production of cyclopiazonic acid, aflatoxins, sclerotia, and pectinase (Cleveland and Cotty 1991; Cotty et al. 1990). The differences in the amount of aflatoxin degraded among atoxigenic *A. flavus* isolates might suggest a differential ability to utilize aflatoxin as a nutrient source (Mehl and Cotty 2013).

To determine whether AFB1 could be degraded and used as a carbon source, atoxigenic *A. flavus* strains were grown in defined liquid media. Small amounts of fungal growth were observed when AFB1 was the sole carbon source added to the medium (Fig. 4); however, degradation declined when glucose was added (Fig. 5C). This suggests that, though AFB1 can be utilized for growth, it is not a preferred carbon source. In previous studies, the regulation of *A. flavus* growth, aflatoxin biosynthesis, and virulence by different carbon sources was attributed to carbon catabolite repression (Fasoyin et al. 2018; Fountain et al. 2016), and this may explain the suppression of AFB1 degradation in the presence of glucose. The presence of preferred carbon sources and subsequent suppression of aflatoxin degradation may also explain why little to no aflatoxin degradation by *A. flavus* was observed in previous studies conducted in defined liquid media (Cotty and Bayman 1993; Doyle and Marth 1978b; Raksha Rao et al. 2020). The dynamics of aflatoxin degradation over time in the current study also support the contention that aflatoxin degradation is suppressed in the presence of preferred carbon sources. The rate of aflatoxin degradation was greatest within the first few days following inoculation of maize with atoxigenic *A. flavus* (Fig. 3). Nutrient availability on crop surfaces is limited but, as the fungus invades internal host tissues, it has access to nutrient- and carbon-rich substrates (Dolezal et al. 2014; Lillehoj et al. 1976). In the presence of more nutrients, aflatoxin may be less preferred as a carbon source and, thus, the rate of degradation decreases.

Identifying mechanisms involved in aflatoxin degradation by atoxigenic *A. flavus* was beyond the scope of this work but the nature of potential mechanisms can be inferred based on the observations that (i) *A. flavus* is able to utilize aflatoxin as a carbon source and (ii) genes within the aflatoxin biosynthesis cluster do not need to be present for aflatoxin degradation to occur. Previous studies speculated that enzymes involved in aflatoxin biosynthesis may also be involved in degradation (Doyle and Marth 1978b; Hamid and Smith 1987) but the current study demonstrates that atoxigenic *A. flavus* with complete deletions of the aflatoxin biosynthesis cluster are able to degrade AFB1. Thus, it is not clear which enzymes may be involved in degradation of aflatoxin by atoxigenic *A. flavus* or what the specific degradation products might be. Most microorganisms that degrade aflatoxins do so through enzymatic pathway-dependent processes which involve complete catabolism of aflatoxins, or degradation to less toxic intermediates (Adebo et al. 2017; Alberts et al. 2009; Verheecke et al. 2016). However, specific pathways associated with aflatoxin degradation by both aflatoxigenic and atoxigenic *A. flavus* are not well understood. Some clues about degradation come from data suggesting that some *A. flavus* isolates convert AFB1 to

aflatoxicol-A and reconvert aflatoxicol-A to AFB1 (Bhatnagar et al. 1991; Karabulut et al. 2014; Nakazato et al. 1990). Aflatoxicol is less toxic compared with AFB1; however, it has similar potency to form an exo-epoxide which can bind to DNA and cause cancer (Karabulut et al. 2014). Also, a recent study described two metabolites of AFB1 as the potential degradation products of the furfuran and lactone ring of AFB1 (Xing et al. 2017). In the current study, there were unknown peaks present on TLC scans of atoxigenic biocontrol agents incubated with aflatoxin as compared with noninoculated control and those treatments without aflatoxin (Supplementary Fig. S1). These peaks may be degradation products or products of unrelated metabolism. Future work is necessary to identify the potential degradation products, or the potential incorporation of degraded aflatoxin compounds into fungal structures, as well as the degradation pathways used by atoxigenic isolates. Bacteria such as *Lactobacillus rhamnosus* (Hathout and Aly 2014) and yeasts such as *Saccharomyces cerevisiae* (Shetty et al. 2007; Singh et al. 2016) bind aflatoxin on their cell wall. In this study, if aflatoxin was bound to fungal cell walls, the acetone would likely extract it. However, future studies will be needed to test both whether cell walls of atoxigenic *A. flavus* isolates bind aflatoxin and the mechanism of aflatoxin degradation by atoxigenic *A. flavus*.

Competitive exclusion of aflatoxin-producing fungi by atoxigenic biocontrol strains reduces aflatoxin contamination of crops; however, based on the results of this study, we can speculate that some of the observed reductions in aflatoxin contamination both at harvest and during storage may be due to degradation of aflatoxin by atoxigenic *A. flavus*. Under storage conditions that favor *A. flavus* growth, aflatoxin can accumulate within a few days even if the crop was free from detectable concentrations of aflatoxin at harvest (Kachapulula et al. 2017). Parameters evaluated in the current study provide insight into the potential dynamics of aflatoxin degradation in storage. Degradation was rapid over the first 3 days postinoculation, followed by slower degradation over the next 18 days at 31°C, a temperature that is favorable for both production and degradation of aflatoxin. Several explanations for this observation are possible. For example, degradation may be concentration dependent, *A. flavus* may degrade aflatoxins more effectively during germination and early hyphal growth versus colonization of the host substrate, the fungus may quickly degrade the aflatoxin that is more accessible initially while degrading the less accessible aflatoxin at a slower rate, or access to preferred nutrients during ramification of host tissues may suppress aflatoxin degradation as discussed above. Further studies are needed to determine whether the initial rate differences are due to the location of aflatoxins in the grain, fungal growth, or the concentration of aflatoxins in the grain. Temperature also influenced the dynamics of aflatoxin degradation (Fig. 2), and atoxigenic *A. flavus* strains were able to degrade aflatoxins both under optimal conditions for aflatoxin biosynthesis (25 to 30°C) and under conditions outside those required for aflatoxin production (10°C). These data suggest that it may be possible to manipulate the storage environment to facilitate aflatoxin degradation while simultaneously inhibiting aflatoxin biosynthesis.

Over two dozen atoxigenic genotypes are in use as active ingredients in aflatoxin biocontrol products in various regions of the world, and these genotypes have been selected using various criteria that indicate that they will be successful competitors in target cropping systems (Bandyopadhyay et al. 2016; Dorner and Lamb 2006; Doster et al. 2014; Mauro et al. 2018; Pitt et al. 2015; Senghor et al. 2020). All active ingredients belong to the *A. flavus* L strain morphotype, which comprises many, diverse genotypes (Islam et al. 2018). These complex L strain populations contain significant proportions (10 to 40%) of atoxigenic fungi (Atehnkeng et al. 2008; Probst et al. 2011), providing resources for selection of superior genotypes for biocontrol. Such selection is a continuing objective of several research groups. Most active ingredients have been selected through a laborious process of population surveys to determine relative adaptation to the environment, laboratory competition studies with aflatoxin producers, field tests to determine both dispersal and overwintering during crop production, and efficacy in single-season aflatoxin management. Based on the results of the current study, it can be concluded that the extent to which atoxigenic genotypes are able to degrade aflatoxin will also contribute to their efficacy as biocontrol active ingredients. Additional studies are needed to understand the dynamics of aflatoxin degradation by atoxigenic fungi in the presence of aflatoxigenic strains under different environmental conditions and to identify potential mechanisms and products of aflatoxin degradation. However, the methods described here for quantifying aflatoxin degradation by atoxigenic genotypes in autoclaved maize provide a basis for rapid, quantitative, and reproducible assessment of the relative ability of atoxigenic *A. flavus* to degrade aflatoxin in crop substrates. Furthermore, these methods can be used to select atoxigenic *A. flavus* with superior ability to degrade aflatoxins that can be utilized as active ingredients in new aflatoxin biocontrol products.

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