RESEARCH EFFORTS, CHALLENGES AND OPPORTUNITIES IN MITIGATING AFLATOXINS IN FOOD AND AGRICULTURAL CROPS AND ITS GLOBAL HEALTH IMPACTS

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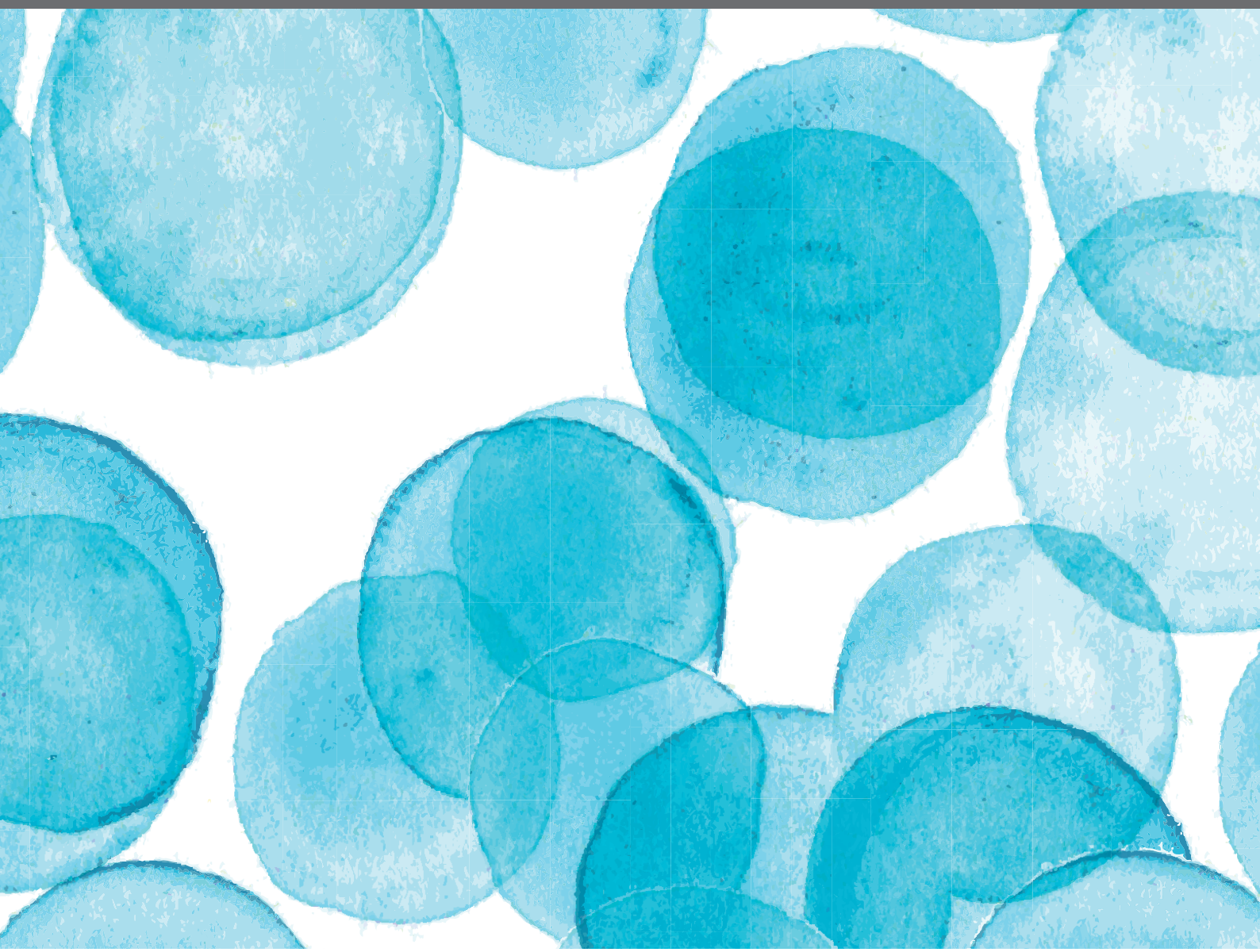

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RESEARCH EFFORTS, CHALLENGES AND OPPORTUNITIES IN MITIGATING AFLATOXINS IN FOOD AND AGRICULTURAL CROPS AND ITS GLOBAL HEALTH IMPACTS

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EDITORIAL
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Editorial: Research Efforts, Challenges, and Opportunities in Mitigating Aflatoxins in Food and Agricultural Crops and Its Global Health Impacts

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Editorial on the Research Topic

Research Efforts, Challenges, and Opportunities in Mitigating Aflatoxins in Food and Agricultural Crops and Its Global Health Impacts

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Aflatoxins are a group of polyketide mycotoxins that are produced during fungal development as secondary metabolites mainly by members of the *Aspergillus* section *Flavi* (Yu et al., 2004; Norlia et al., 2019; Uka et al., 2019). Contamination of food, feed and agricultural commodities by aflatoxins impose an enormous economic concern, as these chemicals are highly carcinogenic, they can directly influence the structure of DNA (Bbosa et al., 2013; Feng et al., 2016). They can lead to fetal maldevelopment and miscarriages, and are known to suppress immune systems (Ahmed Adam et al., 2017). In a global context, aflatoxin contamination is considered a perennial concern between the 35N and 35S latitude where developing countries are mainly situated. With the expansion of these boundaries, aflatoxins are increasingly becoming a problem in countries that previously did not have to worry about aflatoxin contamination. Given the continuing problems arising from aflatoxin contamination of food and agricultural commodities throughout the world, aflatoxins research is becoming one of the most exciting and rapidly developing areas of microbial toxins research. The applications include many disciplines, from medicine to agriculture. Nowadays, traditional research on aflatoxins has been expanded to modern technologies such as omics for understanding the regulation of aflatoxin biosynthetic pathway genes, the taxonomy, ecology, biochemistry, and evolution of aflatoxigenic fungi in addition to strategies to pre- and post-harvest management of aflatoxin contamination. This includes improving host resistance of susceptible crops such as cotton, maize, peanut, and tree nuts via genetic engineering.

The present Research Topic includes one review article, one mini-review and fifteen original research articles. Contributors highlighted challenges and opportunities in mitigating aflatoxins in food and agricultural crops and the current knowledge on the global health issues of aflatoxins and aflatoxigenic fungi. All aspects of aflatoxin contamination of food and agricultural crops from epidemiology to ecology, biochemistry, molecular biology, biocontrol strategies, natural inhibitors of fungal growth and aflatoxin production, transgenic hosts and pre- and post-harvest management strategies have been discussed.

Host resistance is an attractive area of aflatoxin research pertaining to various aspects of *A. flavus*-plant host interaction. In this context, the role of signaling pathway genes, gene silencing, and development of maize inbred in association with aflatoxin reduction *in vitro* and in field conditions have been extensively studied by different researchers. Parish et al. conducted a genome-wide survey of maize genes involved in maize-fungus interaction and signaling pathways by investigating the gene expression levels among the 12 maize QTL-NILs (quantitative trait loci-near isogenic lines) that carry maize resistance QTL regions. Seven calcium-dependent protein kinases and one respiratory burst oxidase displayed significant differential expression levels among the maize QTL-NILs. The authors concluded that the elucidation of differentially expressed signaling pathway genes involved in maize resistance to *A. flavus* can provide insights into maize disease resistance and enhance maize molecular breeding. In a promising study of managing aflatoxin contamination in maize through host-induced RNAi-based gene silencing strategy (HIGS), Raruang et al. selected *A. flavus* gene *aflM* encoding versicolorin dehydrogenase, a key enzyme involved in the aflatoxin biosynthetic pathway, as a target for suppression through HIGS. They reported up to 76.4% reduction in aflatoxin levels in the transgenic lines containing the HIGS construct targeting the *aflM* in comparison to the null controls under field inoculation conditions. Likewise, they further indicated that genetic transformation to suppress the fungal target genes through HIGS can also protect grains from post-harvest aflatoxin contamination since they reported a 95.3% reduction in aflatoxin levels of harvested transgenic maize kernels compared to the null kernels during a 7-day incubation under 100% humidity. This enhanced aflatoxin resistance was correlated to the presence of high levels of *aflM*-specific small RNAs. Transferring the resistant trait from these transgenic lines into elite maize background resulted in a 60–80% reduction in aflatoxin of the F1 crosses under field conditions. This study offered a more sustainable approach in managing aflatoxin contamination in maize and other susceptible crops. In an effort to reduce aflatoxin accumulation in maize through identifying associated quantitative trait loci (QTL), Womack et al. provided the map of QTL by a bi-parental population comprised of 241 F2:3 families derived from the cross of inbred lines Mp705 (susceptible) × Mp719 (resistant) in maize. The mapping population was characterized in replicated field trials in three environments for resistance to aflatoxin accumulation under artificial inoculation with an *A. flavus* spore suspension. The genetic linkage map was constructed with 1,276 single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) molecular markers covering a total genetic distance of 1,642 cM across all ten maize chromosomes. The authors concluded that the aflatoxin-reducing QTL in the chromosomal regions bin 1.06 and 3.09 are critically important for developing aflatoxin-resistant maize lines and hybrids and should be the primary targets to elite lines with marker-assisted breeding.

Biological control is another important approach to mitigate aflatoxin contamination of crops and agricultural commodities in pre- and post-harvest conditions. A promising approach is the use of competitive atoxigenic strains of *A. flavus* in the field. Over a 4-year period, Weaver and Abbas applied three biocontrol strains of *A. flavus* (NRRL 21882, 18543, and 30797) annually, to the 3.2-ha commercial corn field in the Mississippi Delta. They showed that after 4 years of biocontrol applications, the *A. flavus* population recovered from the grain was approximately 11% aflatoxigenic, regardless of the particular biocontrol treatment. As a conclusion, they indicated that biocontrol applications could be beneficial when the initial soil population has a high percentage of aflatoxigenic isolates. In a 10-year study as a field trial, Bandyopadhyay et al. applied Aflasafe, an advanced biocontrol-based product employing atoxigenic strains of *A. flavus* to control aflatoxin contamination of maize and groundnut in Nigeria. They showed that efficacy of the biocontrol product in limiting aflatoxin contamination was stable regardless of farming practices, crop varieties, or environmental challenges. Pertaining to this long-term efficient technology of aflatoxin management, the authors indicated that biocontrol could be considered as a preferred route for aflatoxin management. It also contributes to better health, increased income, and greater trading opportunities for groundnut and maize farmers throughout the world.

Climatic conditions of temperature and water activity together with drought stress are another important aspects of aflatoxin contamination of crops. In a study by Gasperini et al. post-harvest control of AFB_1 by non-toxigenic strains of *A. flavus* in non-GM and isogenic GM maize cultivars was evaluated with special focus on environmental conditions affecting fungal growth and aflatoxin production. The authors concluded that pre-harvest ripening stage of maize cobs and their inherent water availability, interacting variables such as type of cultivar, $T^{\circ}C$, CO_2 levels, and water availability conditions, resilencing of the non-toxigenic strains and finally formulation approaches of applied biocontrol agents affect pre- and post-harvest fungal growth and subsequent aflatoxin contamination of maize in practice. Hanano et al. studied the role of Caleosin/Peroxygenase system in elevating the virulence of *A. flavus* through increasing sporulation and aflatoxigenicity after exposure of the fungus to an environmental pollutant toxin named "2,3,7,8-tetrachlorinated dibenzo-*p*-dioxin" (TCDD). The authors highlighted the impact of climate changes on food safety as new challenges in a global context and the necessity of reinforce the global regulations of food and feed products.

Another important challenge of aflatoxin control is the use of natural inhibitors of growth and aflatoxin production by the fungus. In an effort to control aflatoxin production by *A. flavus* in laboratory conditions by plant-derived natural molecules, Nobili et al. used non-digestible hull of common buckwheat, *Fagopyrum esculentum* extracted by supercritical fluid extraction process using carbon dioxide (SFE-CO_2) to inhibit fungal growth and toxin production. They showed that SFE-CO2 extract of *F. esculentum* not only efficiently inhibited *A. flavus* growth and aflatoxin production *in vitro* probably due to containing higher amounts of polyphenols and lipophilic bioactive molecules but also it could be considered for open field applications considering its solvent-free nature which reduces the potential risk for plants and the environment. Gong et al. examined antifungal volatiles of a soil isolate of *Alcaligenes faecalis* to

control *A. flavus* growth and aflatoxin production in groundnut, maize, rice and soybean during storage. They identified more than 25 compounds in the volatiles of *A. faecalis,* of which two compounds i.e., disulfide dimethyl (DMDS) and methyl isovalerate (MI) were proven to be responsible for antifungal properties and thus, they may be considered as promising agents in biocontrol of *A. flavus* in practice. In a comprehensive review on targeting alternative oxidase (AOX), a crucial fungal enzyme that affects fungal pathogenesis, morphogenesis, stress signaling, drug resistance and even mycotoxin production in the case of sterigmatocystin as a precursor of aflatoxin biosynthesis, Tian et al. highlighted that AOX inhibitors alone or in combination with other known antifungals should be considered as promising antifungals against *A. flavus* growth and aflatoxin contamination of crops. They indicated that further understanding of fungal alternative respiration and fungal AOX structure, and screening of effective fungal-specific AOX inhibitors are needed for practical application of AOX in food industries.

Detoxification of aflatoxins and rapid screening techniques of elimination of contaminated grains are important aspects of mycotoxin research due to the unfavorable fungal growth and subsequent aflatoxin contamination of food and feed in many tropical countries and economic problems of contaminated food elimination in low-income countries. In a promising effort in develop a rapid screening method and detoxify contaminated grains by using a new strategy, Juodeikiene et al. used an acoustic strategy in combination with fermentation by modeling maize and nuts contaminated with different amounts of aflatoxins. They successfully used a portable acoustic spectrometer comparable with ELISA in cost and speed for high-throughput detection of aflatoxins in grains and nuts to eliminate contaminated ones from the production chain and also they achieved detoxifying aflatoxin-contaminated grains through ethanol fermentation as a novel approach. In a non-destructive rapid image-based screening, Hruska et al. showed the usefulness of fluorescence hyperspectral imaging to differentiate susceptible and resistant corn hybrids infected by a toxigenic and atoxigenic strains of *A. flavus*. They indicated a significant role for the intensity of fluorescence when using fluorescence hyperspectral imaging for early detection of maize kernels infected with toxigenic and atoxigenic *A. flavus* in resistant and susceptible corn varieties.

Host-fungus interaction is another challenging area of aflatoxin research due to its dynamic nature. Musungu et al. used systems biology approach involving transcriptomic dual RNA-seq to uncover interactions between *A. flavus* and *Zea mays*. They showed that the activation of *Z. mays* resistance genes effectively influenced the expression of specific *A. flavus* genes. As a result, transcripts and pathways of *A. flavus* contributed to endosomal transport, aflatoxin production, and carbohydrate metabolism were up-regulated. Sweany and Damann studied the dual challenging effects of volatiles produced by *A. flavus* on aflatoxin production. They showed that these volatiles either stimulate aflatoxin production or suppress toxin production by the fungus *in vitro*. They concluded that the contribution of fungal volatiles to quorum sensing and communication. Applying them in modified atmospheres during grain storage with the aim of minimizing aflatoxin contamination warrants further investigation.

In a well-designed review by Arenas-Huertero et al. on the role of aryl hydrocarbon receptor (AhR) pathway in aflatoxins activation, it has been shown that the mono-oxygenases, such as CYP1A1 which are expressed abundantly in the liver as aflatoxin activators, have elemental promoters which are used by the AhR activation. With evidences of *in vitro* induction of an aflatoxin B_1-induced increase in CYP1A activity and CYP1A transcription, in association with an enhanced AhR activity, the authors suggested that AhR pathway activation may be considered as a toxicity mechanism of AFB_1.

Human exposure to aflatoxins is another challenging but not well-studied area. In the concise overview of aflatoxin contamination of foods and related biomarker research, Yunus et al. described aflatoxin contamination of milk as a public health problem in Pakistan and how these problems have influenced the Pakistan population by highlighting the aflatoxin concentrations in basic food products and their comparison with established aflatoxin limits. They showed that around 80% of AFM_1 in milk was due to the use of contaminated cottonseed cake in dairy rations. The authors suggested a replacement of cottonseed cake with feedstuffs lower in aflatoxin contamination such as canola meal, and commercial concentrate feeds. They concluded that long-term mitigation strategies could be successfully used to reduce aflatoxin contamination in cottonseed cake and increase the safety of dairy animal feed. In a study by Ismail et al. on aflatoxin contamination of commercially available black tea in Pakistan, the authors estimated daily intake (EDI) of aflatoxins through branded and non-branded black tea consumption and the health risk assessment based on the margin of exposure (MOE) approach. As a result, they showed that aflatoxin contamination was common in both types of tea samples and the MOE values for aflatoxins after black tea consumption indicated a considerable public health risk problem. Results indicated the possible transfer of more than half of aflatoxins from tea leaves to the tea beverage and probable production of aflatoxins degradation products probably due to four to five times boiling of tea. The authors suggested that level of aflatoxins should be monitored constantly in tea and tea products by the health and regulatory agencies.

In conclusion, this Research Topic opens exciting perspectives on challenges and opportunities in mitigating aflatoxins in food and agricultural crops and their global health impacts with a special focus on the development of suitable strategies for preventing toxigenic fungal growth in the field and storage, thereby reducing or eliminating subsequent aflatoxin contamination of the food and feed supplies.

AUTHOR CONTRIBUTIONS

MR-A, Z-YC, MS-G, and MR designed the study. MR-A and MS-G prepared the draft. All authors read and approved final version of the manuscript. MR-A supervised the study. All authors contributed to the article and approved the submitted version.

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Inhibitory Effect of Volatiles Emitted From *Alcaligenes faecalis* N1-4 on *Aspergillus flavus* and Aflatoxins in Storage

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Controlling aflatoxigenic *Aspergillus flavus* and aflatoxins (AFs) in grains and food during storage is a great challenge to humans worldwide. *Alcaligenes faecalis* N1-4 isolated from tea rhizosphere soil can produce abundant antifungal volatiles, and greatly inhibited the growth of *A. flavus* in un-contacted face-to-face dual culture testing. Gas chromatography tandem mass spectrometry revealed that dimethyl disulfide (DMDS) and methyl isovalerate (MI) were two abundant compounds in the volatile profiles of N1-4. DMDS was found to have the highest relative abundance (69.90%, to the total peak area) in N1-4, which prevented the conidia germination and mycelial growth of *A. flavus* at 50 and 100 μL/L, respectively. The effective concentration for MI against *A. flavus* is 200 μL/L. Additionally, Real-time quantitative PCR analysis proved that the expression of 12 important genes in aflatoxin biosynthesis pathway was reduced by these volatiles, and eight genes were down regulated by 4.39 to 32.25-folds compared to control treatment with significant differences. And the *A. flavus* infection and AFs contamination in groundnut, maize, rice and soybean of high water activity were completely inhibited by volatiles from N1-4 in storage. Scanning electron microscope further proved that *A. flavus* conidia inoculated on peanuts surface were severely damaged by volatiles from N1-4. Furthermore, strain N1-4 showed broad and antifungal activity to other six important plant pathogens including *Fusarium graminearum*, *F. equiseti, Alternaria alternata*, *Botrytis cinerea*, *Aspergillus niger*, and *Colletotrichum graminicola*. Thus, *A. faecalis* N1-4 and volatile DMDS and MI may have potential to be used as biocontrol agents to control *A. flavus* and AFs during storage.

Keywords: *Aspergillus flavus*, aflatoxins, *Alcaligenes faecalis*, volatile, dimethyl disulfide, methyl isovalerate

INTRODUCTION

Aspergillus flavus is an important agricultural fungus which can infect many grain and oil crops in pre- and post-harvest such as groundnuts, maize, rice and cottonseed et al. (Yu et al., 2004). Moreover, some aflatoxigenic *A. flavus* can produce large amount of toxic aflatoxins (AFs) in crops and food, and seriously affect human and livestock health (Waliyar et al., 2015). Currently, 18 types of AFs produced by *Aspergillus* spp. have been identified. Among them, aflatoxin B_1 (AFB_1) was considered as the most dangerous mycotoxins to mammals due to its high toxicity and carcinogenicity (Asters et al., 2014; Adhikari et al., 2016). International Agency for Research on Cancer (IARC) classified AFB_1 as Group I carcinogen to humans (Robens and Cardwell, 2003; Zhuang et al., 2016).

Aspergillus flavus and AFs pose great risk of human health worldwide. Williams et al. (2004) estimated that more than 5 billion people are suffering from the chronic exposure to AFs. Vardon calculated that the annual economic losses caused by AFs was about $500 million in the United States (Wu, 2015; Outlaw et al., 2016), and the additional cost of disease management is more than $20–$50 million (Robens and Cardwell, 2003). In less developed countries, because of the poor income and scanty food supplication, AFs contamination in food is even more severe, resulting in serious harms to local people. In Burundi and Congo, the percentage of AFs contamination was 100% in 244 samples collected from local marker including crops, milk, and other products. In these samples, more than 50% crops were contaminated with large amount of AFs which were higher than the maximum limit set by the European Union (Udomkun et al., 2018). Azziz-Baumgartner et al. (2005) reported over 125 human deaths in Kenya due to the consumption of AFs contaminated foods during 2004–2005. More seriously, in hepatitis B/C virus endemic countries (such as China, India, Africa, etc.), AFs led to severe hazards to humans. Taking China as an example, under the dual pressure of hepatitis B/C virus and AFs, more than 3.7 million people died of liver cancer every year. This accounts for 50% of the world's liver cancer deaths (Chen et al., 2013).

Because of the great damage and serious risks to humans, many countries have legislatively defined the maximum levels of AFs intended for human and animal consumption. For example, in groundnut and their products, United States Food and Drug Administration permits the maximum AFs at 20 ppb (Torres et al., 2014). In European Union, maximum level is 4 ppb for AFs, and 2 ppb for AFB_1 (European Commission [EC], 2010). Upper limit of AFs is 10 and 15 ppb in Kenya (Wagacha et al., 2013) and Malaysia (Leong et al., 2010), respectively. In China, the maximum level for AFB_1 is 20 ppb, and total AFs has not yet regulated. No regulation for total AFs and AFB_1 in some developing counties (Kamika and Takoy, 2011).

Although legislations aimed to reduce the potential damage of AFs to humans is necessary, farmers in poor areas can not afford to monitor AFs (Probst et al., 2010). Thus, the effective strategies to eliminate AFs is controlling the initial infection of *A. flavus* in crops and grains (Abdel-Kareem et al., 2019). The *A. flavus* control in storage currently relies on physical drying, infected kernel sorting and chemical agents (Nurtjahtja, 2019). While the first two methods are safe and easy strategies and have been used for a long period (Bediako et al., 2018), the synthetic chemical agents may cause adverse effects on human health and environment due to long degradation period, toxic residues and potential undesirable biological effects (Prakash et al., 2015; Li et al., 2016). Bio-active compounds from organisms have been gradually applied in controlling *A. flavus* and AFs during storage (Kai et al., 2009; Passone and Etcheverry, 2014). Some essential oils extracted from plants have been widely used as promising agents in controlling *A. flavus* and AFs during storage, such as *Curcuma longa* (Hu et al., 2017), *Litsea cubeba* (Li et al., 2016), *Zanthoxylum molle* (Tian et al., 2014), *Cinnamomum jensenianum* (Tian et al., 2012), *Peumus boldus*, *Lippia turbinata* (Passone and Etcheverry, 2014), *Oxalis corniculata* (Rehman et al., 2015) and *Cuminum cyminum* (Kedia et al., 2014). Some compounds have been produced into commercial agents (DMC base Natural, owned by DOMCA S.A.), and successfully applied in disease control.

Despite the potential of plant extracts (i.e., bio-compounds and oils), plant growth requires a long period, dedicated maintenance and large cultivated areas. Moreover, extraction of bio-active compounds needs a large amount of plant sources which are not always available. Compared to plant materials, microbes are simple to obtain and culture, which are easier to obtain large amount. Till now, several microbes have been obtained and used in controlling *A. flavus* and AFs such as *Bacillus megaterium* (Chen et al., 2019), *B. pumilus* (Cho et al., 2009) and non-aflatoxigenic *A. flavus* strains (Ehrlich, 2013; Zanon et al., 2016). However, the biocontrol activity of these microbes mainly relies on antagonism (macromolecular metabolite) and competition, which always required direct contact with grains. Such contact may lead to secondary pollution to grains in storage. Compared to these contact phase, volatile phase can easily and uniformly distribute in the whole space of storage, and generated lower residual after volatilization. Recently, some evidences proved that the inhibitory effect of volatile phase on *A. flavus* in storage was consistently more effective than contact phase (Hu et al., 2017; Ma et al., 2017). To date, seldom microbe volatile was identified and used to control *A. flavus* and AFs during storage. Thus, the objectives of this study were to: (1) screening effective biocontrol microbes against *A. flavus* through the production of volatiles; (2) evaluating biocontrol efficacy of microbes against *A. flavus* and AFs in grain and food during storage; (3) identifying the primary antifungal bio-active volatiles; and (4) analyzing their antagonistic effect on cell ultra-structure of *A. flavus* and AFs biosynthesis.

MATERIALS AND METHODS

Microorganism and Plant Materials

Alcaligenes faecalis N1-4 was isolated from rhizosphere soil of tea plant in Cheyun mountain, Xinyang city, Henan, China

(Fang et al., 2014). The bacteria was suspended in 25% (v/v) glycerol and stored at −80°C for long-term use.

Regular-sized maize (cultivar Zhengdan 958), peanut (Silihong) and soybean (W82) were purchased from local markets. Rice (Huanghuazhan) samples were supported by Huazhong Agricultural University. These samples were divided into two parts, and adjusted water activity (a_w) to 0.8 and 0.9, respectively. Then, they were inoculated with *A. flavus* to detect the disease development and AFs contamination in storage.

Seven important phyto-pathogens were used in our test. These fungal strains included *A. flavus*, *Fusarium graminearum*, *F. equiseti, Alternaria alternata*, *Botrytis cinerea*, *Aspergillus niger*, and *Colletotrichum graminicola.* The pathogens were isolated from infected plants and stored in our lab for broad antifungal tests (Gong et al., 2014). *A. flavus* conidia was inoculated into PDB medium and cultured at 28°C for 48 h to produce mycelium coils.

Screening of Bacteria With Antifungal Volatile Production

The soil samples were collected from tea gardens containing large amounts of active humus. The soil was suspended in sterilized water and diluted to 10^{-5}. The suspension (100 μL) was spread on the surface of NA medium and cultured at 28 for 48 h. The obtained microbe clones were streaked on NA medium to produce pure culture. Bacteria bodies on NA surface were washed off and co-cultured with *A. flavus* in Face-to-face (FTF) cultural method to screen antagonistic bacteria.

In FTF tests, *A. flavus* mycelia coil was placed to the center of PDA medium bacteria bodies (OD600 = 1.8, 100 μL) were spread on NA medium. The two petri dishes were placed FTF with *A. flavus* above and bacterial plate at the bottom. PDA plate inoculated with *A. flavus* mycelia challenged with NA plate was used as control. All sets were cultured at darkness and 28°C for 3 days. The mycelium diameter in each group was recorded, and the inhibitory rate was calculated following the forma below.

$$\text{Inhibitory rate (\%)} = [(\text{the diameter of control} - \text{the diameter of antagonist treatment})/\text{the diameter of control}] \times 100.$$

Inhibitory effect of bacteria to *A. flavus* conidia germination was measured. Ten micro liter of *A. flavus* conidia (10^5 cfu/mL) were injected on a round filter paper (5 mm in diameter), and placed to the center of PDA plate. Isolated bacteria were spread on NA plate. The two petri dishes were located FTF with PDA above. PDA medium inoculated with *A. flavus* conidia was used as control. All sets were cultured at darkness and 28°C for 3 days. The diameter of conidia in each test was recorded, and the inhibition rate was calculated as before.

DNA Extraction and Phylogenetic Tree Analysis

Single clone of N1-4 was inoculated into NB medium and cultured at 200 rpm and 28°C for 48 h. The cultured broth was centrifuged at 12,000 rpm for 10 min, and the collected cell bodies were used for DNA extraction (Gong et al., 2014). The 16S rRNA sequences of strain N1-4 were amplified using two universal primers (27 f and 1541 r). Obtained 16S rRNA were sequenced in TIANYI HUIYUAN company, and blasted in GenBank database. The strains in GenBank database homologous to N1-4 were selected and used to construct the phylogenetic tree through MEGA program and neighbor-joining method (Ding et al., 2015; Chen et al., 2017).

Biochemical Analysis of Strain N1-4

Biochemical activity of N1-4 was conducted through BIOLOG MicroStation™ System (Biolog Inc., United States). Exactly, strain N1-4 was streaked on NA medium. Fresh single clone was transferred into IF-A GEN III Inoculating Fluid. Then the suspension was transferred into GEN III MicroPlate with 120 μL in each cell. The plate was cultured at 37°C in darkness for 12 h, and the optical density of each cell in MicroPlate was recorded in BIOLOG MicroStation™. All recorded data were aligned in bacterial database through BIOLOG MicroStation™ System (Banik et al., 2016).

Identification of Volatiles From N1-4

Volatiles from N1-4 were enriched with solid-phase micro-extraction (SPME) fiber (divinylbenzene/carboxen/polydimethlsiloxane), and identified through GC-MS/MS system (5975B-7890N, Agilent Technologies Inc.). N1-4 cell were spread on the surface of NA medium in a 150 mL flask. The flask containing NA medium without N1-4 bodies was used as control. Each flask was sealed with two layer of membrane, and placed at 37°C, and darkness for 24 h to produce volatiles. Volatiles in airspace of each flask were enriched with SPME system for 30 min, then analyzed with GC-MS/MS system equipped with an Agilent HP-5MS fused-C18 capillary column (length 30 m, internal diameter 0.25 mm, 0.25 μm thickness film). The samples were analyzed with splitless injection mode. The inlet temperature was 250°C, carrier gas was helium. The compounds were analyzed using the method as follows: initial temperature at 40°C for 3 min, following a linear heating rate of $3°C \cdot min^{-1}$ to 160°C, and contained for 2 min, $8°C \cdot min^{-1}$ to 220°C, and contained for 3 min. Each sample was conducted for two times. The compounds in N1-4 samples that were not present in the control samples were considered to be the final analyzes.

Minimal Inhibitory Concentration Analysis of Identified Volatiles

Identified compounds in N1-4 volatiles were selected for MIC analysis against *A. flavus* in FTF test (Gong et al., 2015). Ten micro liter fresh *A. flavus* conidia (5×10^5 cfu/mL) were inoculated to a round paper disk placed in the center of PDA plate. Another plate was inoculated with identified compound, respectively. Ethanol was used as control. All compounds was adjusted to the final concentration of 5, 10, 100 and 200 μg/L (compound weight to airspace volume), respectively. These two dishes were placed FTF and incubated at 28°C and darkness for 4 days. The diameter of *A. flavus* mycelium was calculated 4 dpi, and the inhibitory rate was calculated according to the following formula: Inhibitory rate (%) = [(the mycelia diameter of control – the diameter of N1-4 treatment)/the diameter of control] × 100.

Infection Control and AFs Prevention by N1-4 in Crops During Storage

Peanuts, maize, rice and soybean kernels were inoculated with *A. flavus* conidia, and challenged with N1-4 in sealed FTF culture test to detect the inhibitory effect of volatiles. In the tests, eight flasks was prepared, and two flasks as a group for one kind of kernel. 100 g of grains was added into each flask and autoclaved at 121°C and 1.01 MPa for 20 min, and cooled down to room temperature. 100 μL *A. flavus* conidia (at 1×10^5 cfu/mL) was added to each flask and mixed well. The two flasks in each group was inoculated with 10 mL (5 mL) of sterilized water, mixed well, and adjusted a_w to 0.9 (0.8) with Aqualab Series 3 model TE (Decagon Devices, Pullman, WA, United States), respectively.

Peanuts, maize, rice and soybean kernels in each flask were equally divided into two parts, and placed into two petri dishes, respectively. One petri dish was challenged FTF with strain N1-4 coated on NA medium. The other petri dish challenged with NA medium was used as control. All dishes were cultured at 28°C and darkness for 7 days. The disease incidence of each treatment was recorded. The grains were collected, dried at 60°C for 4 days, and milled well for AFs extraction.

Quantitative Analysis of AFs

Milled samples of each grain were used for AFs extraction and quantitative analysis. One gram of milled sample was re-suspended in 5 ml acetonitrile/water (84/16, v/v), and vortex for 5 min. The suspension was centrifuged at 12,000 rpm for 10 min. The supernatant was transferred into a new tube, and same volume of hexane was added into the tube, mixed well. The upper layer was obtained and used for AFs analysis. The quantitative analysis of AFs were conducted through LC-ESI-MS system containing Thermo Surveyor plus HPLC system coupled to a TSQ Quantum Ultra mass spectrometer (Thermo Scientific, CA, United States) (Warth et al., 2012). AFs (AFB_1, AFB_2, AFG_1, and AFG_2) purchased from Sigma (Sigma-Aldrich, St. Louis, MO, United States) were used as standards for quantitative analysis.

Ultra-Structural Analysis of *A. flavus* Cell on Peanuts Surface

Ultra-structural of *A. flavus* cell on peanuts coat of a_w 0.92 were analyzed through JSM-4800 scanning electron microscope (SEM, Hitachi, Tokyo, Japan). Peanuts in control and N1-4 treatments were fixed with 0.1% (v/v) osmic acid for 1 h. A piece (3∼5 mm × 3∼5 mm) of peanut coat was ripped down, and affixed to SEM stubs, respectively. Then, all samples were sprayed with gold and examined in SEM (Boukaew and Prasertsan, 2014).

Broad Spectrum Inhibitory Activity of Strain N1-4

Broad antifungal tests of N1-4 against other 6 fungal pathogens were conducted with FTF method as mentioned above. Fresh hyphae block (5 cm in diameter) was inoculated to the center of PDA medium, respectively. The plate was challenged with strain N1-4 on NA plate with FTF method, respectively. All tests were cultured at 28°C and darkness for 5 days. The inhibition rate was calculated according the methods above.

RNA Extraction and Quantitative Real-Time PCR Analysis

Fresh *A. flavus* conidia (100 μl, 10^6 cfu/mL) were spread on PDA medium in one petri dish. Another dish containing NA medium was spread with N1-4 cell bodies (10^8 cfu/mL, 100 μl). Then, the two dishes were challenged FTF with *A. flavus* on the top. *A. flavus* conidia on PDA plate challenged with NA medium was used as control. All treatments were cultured at 28°C and darkness for 48 h. The mycelia on PDA surface were collect and used for RNA extraction.

Total RNA in *A. flavus* mycelia was extracted by Trizol (Invitrogen) method (Li et al., 2017). The RNA was treated with DNase at a concentration of 1.5 unit/μg. cDNA was synthesized using a PrimeScript™ RT Reagent Kit (Takara). The cDNA products were diluted 20-fold with nuclease free deionized water. Reverse transcription quantitative PCR (RT-qPCR) was performed using a Bio-Rad iQ2 PCR system (Bio-Rad, United States). The PCR conditions were as follows: 95°C for 1 min; 40 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 30 s. Twelve important genes in AFs biosynthesis pathway were used in our tests including *aflR*, *AccC*, *aflCa*, *aflA*, *aflS*, *aflO*, *aflD*, *aflF*, *aflP*, *aflQ*, *aflX*, and *aflC* (Cleveland et al., 2009; Hua et al., 2014). β-*tubulin* was used as the endogenous control due to its relatively stable expression level. All primers used for RT-qPCR were shown in **Table 1**. The relative expression of genes in N1-4 compared to control was calculated by using the 2^-ΔΔCt method (Chen et al., 2017; Chai et al., 2018).

Data Analysis

All statistical analyses were performed by one-way analysis of variance (ANOVA) with SPSS 17.0 statistics software for windows (SPSS Inc., Chicago, IL, United States). Experiments were arranged in a completely randomized design with at least two replications. Mean comparisons were performed with Student's *t* test at $P < 0.05$.

RESULTS

Isolation of Active Bacteria With Antifungal Volatile Production

In screening tests, 693 bacteria were isolated from tea soil, and six bacteria showed great antifungal activity against *A. flavus* in FTF tests. Among these strains, N1-4 could completely inhibit the growth of *A. flavus* mycelium, as well as conidia germination *in vitro*. N1-4 was selected for further biocontrol analysis in the tests.

In control treatment, mycelia in PDA medium could grow quickly, and extended to 5.2 cm in diameter 5 days post inoculation (dpi). Conidia on PDA surface germinated to hyphae, and extended quickly. When challenged with bacteria, N1-4 in NA medium without contact to *A. flavus* cell, can greatly inhibited the conidia germination, as well as mycelia growth of *A. flavus* (**Figure 1**). Seldom fresh mycelia were formed in N1-4 treatment 5 dpi. Hence, the inhibition rate of N1-4 to mycelia

TABLE 1 | Primers used in RT-qPCR analysis.

Gene name	Gene functions	Primers used in the RT-qPCR
aflR	Pathway regulator	F: AGCACTACAAACACTGACCCAC R: CCAGCACCTTGAGAACGATAA
aflCa	Noranthrone monooxygenase, Norsolorinate-anthrone to norsolorinate	F: GCACCAATGGAGCCGTAT R: GCGGTGTTCGTAGCGTTC
aflA	FAS alpha subunit, Acetate to polyketide	F: CGTGAGGTCAAGGCATTCCT R: GACTTGGCCCCCCTTCTGT
aflS	Transcription enhancer, Pathway regulator	F: CCGAAGATTCCGCTTGGA R: TGAAGACATGCAGCAAAAGGA
aflO	O-methyltransferase B, dihydrodemethylsterigmatocystin to dihydrosterigmatocystin	F: TGCTGTGGCATCCATTCAAA R: GGACTGCGTCTTCCAAAAGG
aflD	NOR reductase, norsolorinic acid to averantin	ACTGCGACTCGGAAACTGATG TGCTCCTCCCGCAATGTC
aflP	O-methyltransferase A, sterigmatocystin to O-methylsterigmatocystin	F: TGTGTCGAGTGATGTGGGACTAG R: GCCACCCAGCTCAACCTACA
aflF	NOR dehydrogenase, norsolorinic acid to averantin	F: AAGATGCTGGGCACGTTTG R: CATGGGTGAGGACGAATTGG
aflQ	Oxydoreductase, O-methylsterigmatocystin to AFB_1 and AFG_1, dihydro-Omethylsterigmatocystin to AFB_2 and AFG_2	F: TTGCTGGGCTTGTGGATTC R: GAGGAGGACGCGTGTCTTTG
aflC	polyketide synthases, Acetate to polyketide	F: TCACAAGCGATGCACAGTTG R: AACTGACGAATGTGGGTCTTGTACT
aflX	Monooxygenase/oxidase, VA to DMST	F: ACCGCGTTGCACATCGT R: TGGGTGTCCACAACCTTCGT
AccC	Acetyl-CoA carbosylase, Acetyl-CoA to Malonlyl-CoA	F: ATGGTAAGACCTGCCTGCTA R: AGCGAGGATACCGAGGAT
β-*tubulin*	Endogenous control, Reference gene	F: AGCAGGCGAAGAAGGAGG R: ACGCCACGCATTTGATCTTC

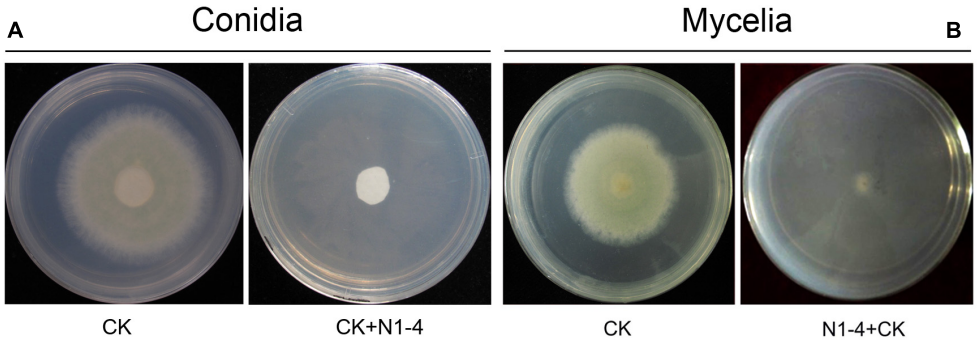

FIGURE 1 | Antifungal activity of strain N1-4 against the growth of *A. flavus* mycelia and conidia. Mycelia pellet and conidia of *A. flavus* was inoculated to the center of PDA plate was used as control (CK), respectively. *A. flavus* challenged with bacteria N1-4 (CK+N1-4) with FTF method was used to test the antifungal activity, respectively. **(A)** The inhibitory effect of N1-4 against conidia germination of *A. flavus*. **(B)** The inhibitory effect of N1-4 against mycelia growth of *A. flavus*.

growth and conidia germination was up to 100% compared to control (**Figure 1**).

When active charcoal added into the tests, the diameter of *A. flavus* mycelia is relative equal to control treatment without the presence of N1-4. It means that active charcoal showed no inhibitory effect to *A. flavus*. When N1-4 added into the reaction, the diameter of *A. flavus* (CK+C+N1-4) is shorter than charcoal treatment (CK+C), but longer than N1-4 (CK+N1-4) (**Figure 2**). Thus, we could deduce that strain N1-4 can produce abundant volatiles, spread in the whole cultural space, and eventually inhibited the growth of *A. flavus*.

Taxonomic Identification of Bio-Active Strain N1-4

The 16S rRNA sequence of strain N1-4 was sequenced and blasted in GenBank database. The results indicated that sequences of N1-4 showed great similarity to the species of *Alcaligenes faecalis*, *A. aquatilis* and *A. endophyticus*. The physiological tree was constructed based on 16S rRNA from N1-4 and other homologous strains (**Figure 3**). Strain N1-4 was classified into a sub-cluster with *A. faecalis*, and showed highly homologous and close genetic distance to *A. faecalis* cb-4 (FJ588233.1) and *A. faecalis* 47N3 (KX302626.1). Thus, we deduced that

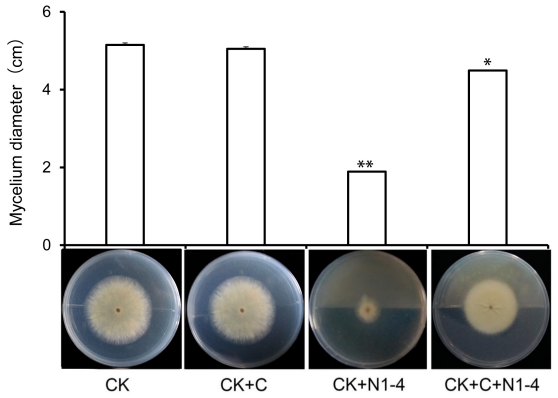

FIGURE 2 | Antifungal activity of volatiles from strain N1-4 against *A. flavus*. Mycelia pellet of *A. flavus* was inoculated to the center of PDA plate was used as control (CK). *A. flavus* challenged with active charcoal (CK+C), N1-4 (CK+N1-4), and both of them (CK+C+N1-4), respectively. * and ** mean significant difference at $p < 0.05$ and $p < 0.01$ leveles compared to CK treatment, respectively.

strain N1-4 was initially identified to be *A. faecalis* based on 16S rRNA sequences.

Biochemical analysis further proved that strain N1-4 exhibited great similarity to *A. faecalis* strain in BIOLOG MicroStation^{TM} System. Both of them can utilize more than 18 kinds of nutrients including methyl pyruvate, L-alanine, L-lactic acid and L-aspartic acid so on. They could grow at the presence of 1∼4% NaCl, and tolerant to 14 kinds of antibiotic such as troleandomycin, lincomycin, vancomycin, and rifamycin SV so on. But, bacteria N1-4 showed different activity to *A. faecalis* on the application of Tween 40 and D-Arabitol, as well as tolerance to 8% NaCl (**Table 2**).

Identification of Antifungal Volatiles From N1-4

More than 25 compounds were detected in the volatiles of N1-4 through SPME coupled with GC-MS/MS system. But, 23 fractions were also detected in control treatment. Only two compounds, including disulfide dimethyl (DMDS) and methyl isovalerate (MI) were proved exclusive in N1-4 volatiles. In the chromatogram, the retention time of DMDS and MI is between 3.04 and 4.34 min. And, both of them contain low molecular weight between 94 and 130 Dalton. DMDS showed the greatest relative abundance (compared to the total peak area) at 69.90%, which was higher than MI (3.38%) (**Figure 4**). Additionally, we purchased these two standards for further antagonistic effect analysis.

MIC Analysis of Identified Volatile Compounds

Two compounds, DMDS and MI, were serially diluted to 10, 50, 100, and 200 μL/L, and tested their inhibitory effect against *A. flavus* in FTF dual culture tests. In control treatment, *A. flavus* conidia germinated to hyphae, and mycelia grew quickly without the presence of volatiles. When DMDS or MI added into

TABLE 2 | Biochemical analysis of strain N1-4 through BIOLOG MicroStation^{TM} System.

Reaction	N1-4	*A. faecalis*	Reaction	N1-4	*A. faecalis*
Carbon utilization			**NaCl tolerance**		
p-HydroxyPhenylacetic Acid	++	++	1% NaCl	++	++
Tween 40	−	+	4% NaCl	+	++
Methyl Pyruvate	++	+	8% NaCl	−	+
D-Arabitol	+	−	**PH tolerance**		
L-Alanine	++	+	pH 6	++	++
L-Lactic Acid	++	+	**Compounds sensitivity**		
β-Hydroxy-D,L Butyric Acid	++	+	Troleandomycin	+	++
L-Aspartic Acid	++	+	Lincomycin	++	++
Citric Acid	++	++	Vancomycin	++	++
α-Ketobutyric Acid	+	+	Rifamycin SV	++	++
L-Glutamic Acid	++	++	Minocycline	+	++
α-Ketoglutaric Acid	++	+	1% Sodium Lactate	++	++
L-Histidine	++	++	Aztreonam	+	+
Propionic Acid	++	+	Fusidic Acid	++	++
L-Pyroglutamic Acid	++	+	Guanidine HCl	+	++
L-Malic Acid	++	++	Tetrazolium Viole	++	++
Acetic Acid	++	++	Lithium Chloride	++	++
D-Serine	+	+	D-Serine	++	++
Bromosuccinic Acid	++	+	Niaproof 4	++	++
Formic Acid	+	+	Tetrazolium Blue	++	++

Alcaligenes faecalis with the greatest similarity to N1-4 was selected as positive species from BIOLOG MicroStation^{TM} database. + means positive reaction, ++ means strong positive reaction, − means negative reactions.

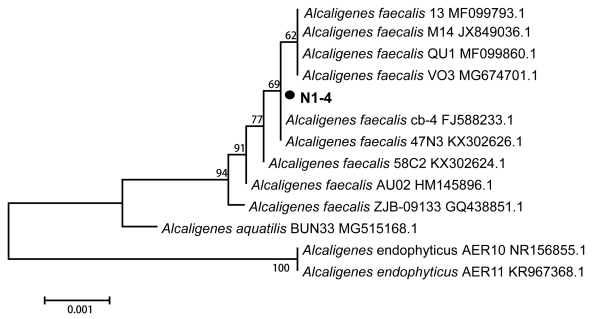

FIGURE 3 | Phylogenetic tree analysis of N1-4 and other homologous strains based on 16S rRNA sequences. The tree was constructed with neighbor-joining methods and the scale bar represents the number of substitutions per base position.

the treatment, the growth of *A. flavus* was greatly inhibited. Obviously, at 50 μL/L or higher concentration, DMDS can eventually prevent conidia germination of *A. flavus*. And at 100 μL/L or higher concentration, DMDS eventually prevented the growth of mycelia. Thus, the MIC of DMDS against conidia germination and mycelia growth of *A. flavus* was 50 and 100 μL/L, respectively. The MIC of MI against mycelia growth of *A. flavus* was 200 μL/L, but can not eventually prevent the germination of conidia (**Figure 5**). Thus, we can conclude that DMDS with the highest relative abundance (69.90%) and best antifungal activity is the main inhibitory factor in N1-4 volatiles.

Bio-Control Activity of N1-4 Against *A. flavus* and AFs in Grains During Storage

Volatiles from N1-4 can eventually control *A. flavus* infection in maize, peanut, soybean and rice grains during storage. In control treatment, the *A. flavus* conidia germinated to hyphae, and quickly infected these grains at high a_w. And the disease severity in peanuts was more serious than that in maize, soybean and rice samples. Additionally, in each grain, the disease severity of *A. flavus* at a_w 0.9 was more severe than that at a_w 0.8. Take peanuts as an example, the disease incidence in peanuts of a_w 0.9 is up to 100% (**Figure 6**). And a mass of fresh conidia were formed and covered the grain surface. But, in N1-4 added treatment, *A. flavus* conidia did not germinate and infect these grains, and no disease symptom was shown in each grains of two a_w. Hence, we could deduce that volatiles from N1-4 can greatly inhibit infection development in different grains during storage.

Aflatoxins concentration in four grains showed similar tendency to infected symptom of *A. flavus*. In control treatment of a_w 0.9, the total AFs in peanuts is 45.51 μg/g (AFB_1

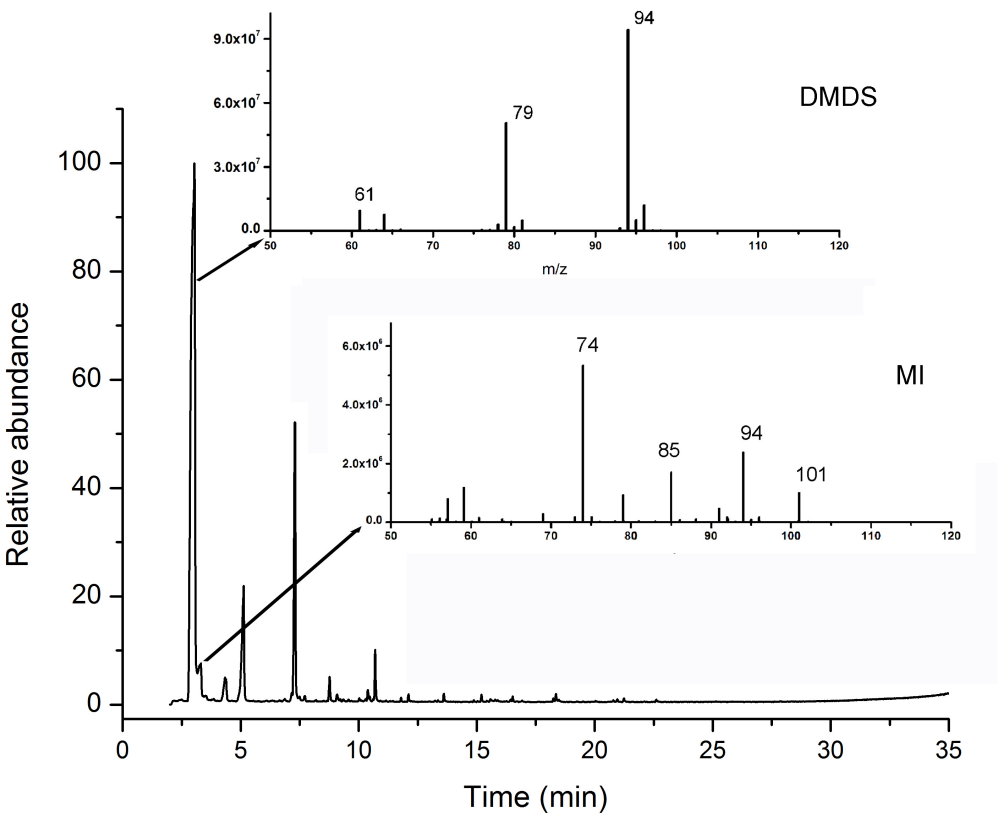

FIGURE 4 | Identification of volatile compounds from strain N1-4 through GC-MS/MS. Volatiles were enriched with SPME system, and identified through GC-MS/MS system. Two compounds, DMDS and MI, in N1-4 profiles and without presence in NA profiles were considered as authentic compounds.

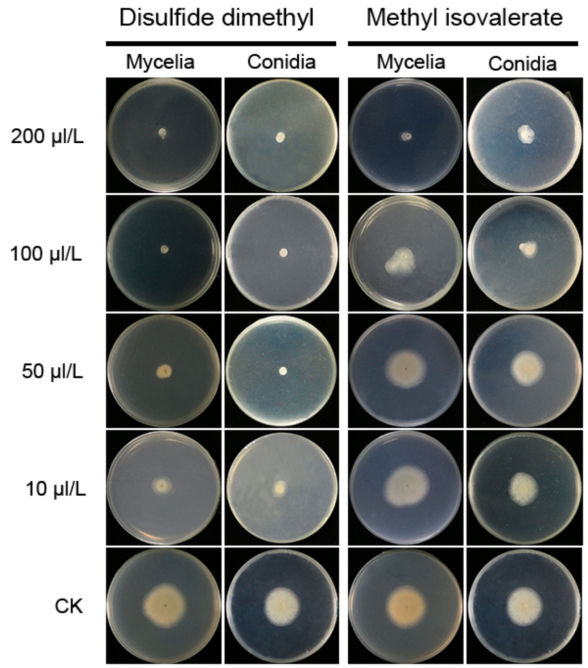

FIGURE 5 | Minimal inhibitory concentration analysis of identified volatiles against mycelia growth and conidia germination of *A. flavus*. Two individual compounds DMDS and MI were diluted to gradient concentrations, and co-cultured with *A. flavus* conidia and mycelia with FTF methods.

is 42.63 μg/g), the concentration is higher than that other grains including maize 37.54 μg/g (AFB_1 is 34.80 μg/g), rice 22.05 μg/g (AFB_1 is 20.86 μg/g) and soybean 2.13 μg/g (AFB_1 is 2.01 μg/g). In control of a_w 0.8, less AFs was detected in peanuts (0.37 μg/g), rice (0.01 μg/g) and soybean (0.01 μg/g), respectively. More importantly, no AFs was detected in each grain of 0.8 and a_w 0.9 with the presence of N1-4. These results indicate that volatiles from N1-4 not only inhibit the infection of *A. flavus*, it also eventually prevented the contamination of AFs in storage (**Table 3**).

TABLE 3 | Quantitative analysis of aflatoxins (AFs) in infected grains with or without the challenge of N1-4.

a_w	Treatment	Samples	AFB_1	AFB_2	Total AFs
0.8–0.85	CK	peanut	0.37 ± 0.00*	0.00	0.37
		maize	0.00	0.00	0.00
		rice	0.01 ± 0.00	0.00	0.01
		soybean	0.01 ± 0.00	0.00	0.01
	N1-4	peanut	0.00	0.00	0.00
		maize	0.00	0.00	0.00
		rice	0.00	0.00	0.00
		soybean	0.00	0.00	0.00
0.9–0.94	CK	peanut	42.63 ± 1.13*	2.88 ± 0.01*	45.51
		maize	34.80 ± 0.27*	2.74 ± 0.08*	37.54
		rice	20.86 ± 0.01*	1.19 ± 0.03*	22.05
		soybean	2.01 ± 0.04*	0.12 ± 0.01*	2.13
	N1-4	peanut	0.00	0.00	0.00
		maize	0.00	0.00	0.00
		rice	0.00	0.00	0.00
		soybean	0.00	0.00	0.00

Four grains including maize, peanut, soybean and rice were adjusted to proper a_w, inoculated with A. flavus conidia and co-cultured with N1-4 in sealed airspace. Asterisk indicated statistical significance at $P < 0.05$.

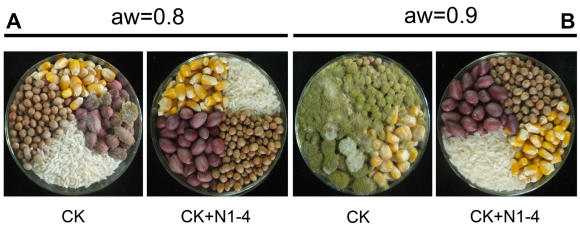

FIGURE 6 | Biocontrol efficacy of volatiles from N1-4 against *A. flavus* and AFs on four different grains of a_w 0.8 to 0.9. **(A)** Disease symptom of *A. flavus* inoculated grains with (N1-4+CK) or without (CK) the presence of strain N1-4 at 28°C for 7 days. **(B)** AFB_1 and AFB_2 in peanut samples were detected using LC-ESI-MS system.

Structural Analysis of *A. flavus* Inoculated on Peanut Surface

In scanning electron microscopy (SEM) analysis, *A. flavus* conidia germinated to hyphae, formed typical conidiophores, and produce abundant fresh conidia in control treatment (**Figure 7**). The hyphae and produced conidia covered the whole surface of inoculated peanut coat. But, the conidia in N1-4 added treatment, few conidia were found on peanut coat surface. These conidia were originated from the initial inoculation test. The germination of these conidia were completely inhibited by N1-4, they could not infect grains, produce conidiophore and fresh conidiospore, as well as induce secondary infection on grains. Additionally, these conidia appeared atypical structure with irregular surface and curving bodies. The regular ball around conidia surface were sunk and severely deformed. Finally, we deduce that strain N1-4 with effective antifungal volatiles production endowed valid functions in control *A. flavus* and AFs in grains during storage.

Broad Spectrum Antifungal Activity of Strain N1-4

Strain N1-4 was co-cultured with other six fungal pathogens by FTF methods to detect the broad antifungal activity. These fungi caused great damage to plant in pre- or post-harvest including *Fusarium graminearum*, *F. equiseti, Alternaria alternata*, *Botrytis cinerea*, *Aspergillus niger*, and *Colletotrichum graminicola.* The inhibitory rate was calculated 5 dpi. As shown in **Figure 8**, the mycelia in control grew quickly, and extended to 7 cm in length 5 dpi. And some pathogens including *F. graminearum, A. alternata* and *C. graminicola* produced different color of pigments in PDA medium, respectively. When N1-4 was added, the growth of all selected fungi was greatly inhibited by the produced volatiles. The inhibitory rate was ranged between 64.1 and 97.8%, respectively. These results proved that volatiles emitted from strain N1-4 exhibited a wide spectrum of antifungal activity against fungi from different genera.

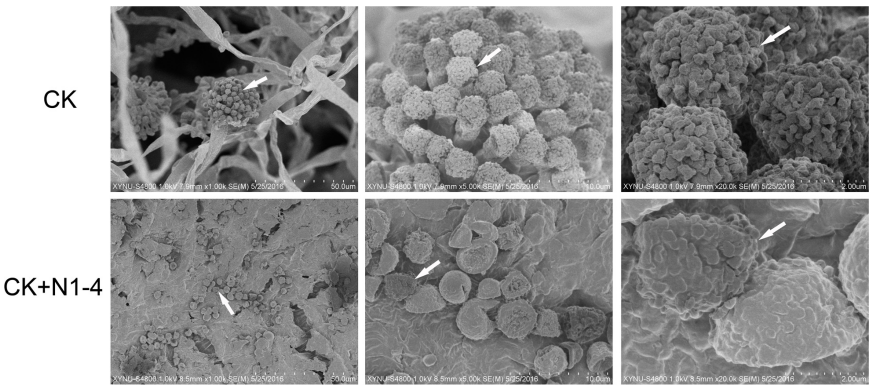

FIGURE 7 | Scanning electron microscope analysis of *A. flavus* cell structure on peanut surface. Peanuts of a_W 0.92 inoculated with *A. flavus* conidia was challenged with (N1-4+CK) or without (CK) the presence of strain N1-4 in FTF tests 7 dpi. Co, conidia; cp, conidiophore; my, mycelia; sg, sterigma.

Real-Time Quantitative PCR Analysis of Gene Expression in AFs Biosynthesis

In RT-qPCR analysis, the expression of 12 genes in N1-4 treatment was all reduced compared to control treatment. Eight genes were significantly down-regulated by 4.39 to 32.25-fold. The results further prove that volatiles from N1-4 greatly repress genes expression in *AFs* biosynthesis (**Figure 9**), and finally reduce AFs production.

DISCUSSION

Aspergillus flavus can infect many grain, food and feedstuff in storage, and produce toxic and carcinogenic AFs which causes great hazardous to humans and livestock. The control of *A. flavus* and AFs at storage is an urgent and crucial problem to scientists worldwide. However, except for the traditional physical and chemical methods, few effective and safe agents are applied in control *A. flavus* and AFs during storage recently. In our current work, we innovatively prove that *A. faecalis* N1-4 isolated from tea rhizosphere could produce two antifungal volatiles including DMDS and MI, significantly inhibit the mycelia growth and gene expression in aflatoxin biosynthesis, eventually inhibit

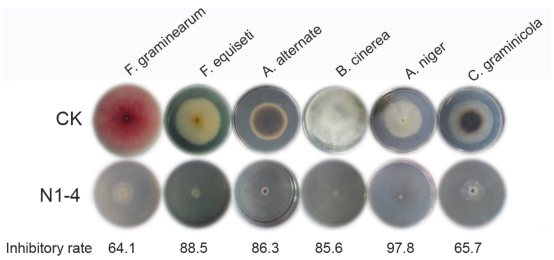

FIGURE 8 | Broad spectrum antifungal activity of strain N1-4 against six important fungal pathogens. Fresh fungal block were inoculated to the center of PDA plate and challenged with strain N1-4 in FTF test, respectively. The diameter of each fungus on PDA plate was measured to calculate the inhibition rate.

A. flavus infection and AFs production in four grains of high a_W, and severely damage *A. flavus* cell structure. Thus, we deduce that strain N1-4 and the produced volatiles provide novel and alternative strategies for control *A. flavus* and AFs during storage, and open new views for screening effective biocontrol agents.

Alcaligenes faecalis is a gram-negative bacterium, commonly existing in soil and water. These bacteria have been broadly applied in several fields including organic acid production (Yamamoto et al., 1991; He et al., 2009), plant growth promoting (Sayyed and Chincholkar, 2010; Sayyed et al., 2010), $NH4^+$-N removal in wastewater (Joo et al., 2005), biodegradation of phenol contamination (Jiang et al., 2007) and biocontrol activity (Shan et al., 2019). For example, Yokoyama et al. (2013) report that *A. faecalis* AD15 produce large amount of hydroxylamine (33.3 mg/L) in synthetic medium, and greatly inhibited the growth of two pathogens including *Pantoea agglomerans* and *Colletotrichum gloeosporioides*. Kakar et al. (2018) report that *A. faecalis* strains Bk1 and P1 greatly suppress the incidence of sheath blight diseases more than 70%. And both of them with phosphate solubilizing activity can produce indoleacetic acid, ammonia, siderophores, enrich the content of mineral nutrients in seedlings and improved plant growth. However, to our knowledge, the application of *A. faecalis* in preventing plant disease at storage has not been reported till now.

As we all know that *A. flavus* as an air-borne fungus produce abundant conidia during infection which spread in a long distance to infect grain and food. Hence, the control of *A. flavus* and AFs in storage is more difficult than other pathogens without conidia. Recently, Ma et al. (2017) report that the biocontrol activity of volatile phase on *A. flavus* is more effective than contact phase. Hence, screening antagonistic volatiles in control post-harvest *A. flavus* and AFs is urgent nowadays. So far, approximately 1,300 microbial volatiles have been obtained from microorganisms and registered in the volatile database[1] (Lemfack et al., 2014). However, less than 10 volatiles are proved useful in control *A. flavus* or AFs till now. In our current work,

[1]http://bioinformatics.charite.de/mvoc/

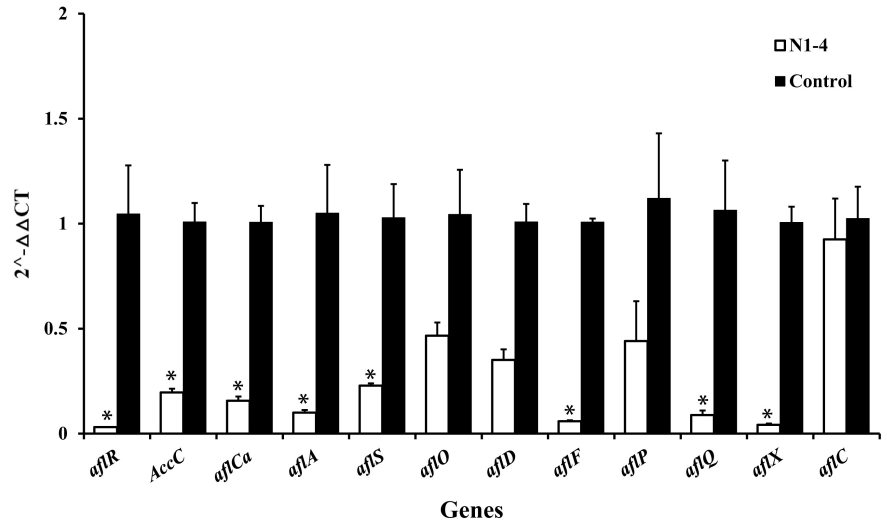

FIGURE 9 | Expression of genes (RT-qPCR) involved in aflatoxin biosynthesis pathway of *A. flavus* effected by volatiles from N1-4. The fold change of genes in N1-4 treatment was calculated with 2^-ΔΔct method. And ΔΔct = ΔCt in N1-4 treatment −ΔCt in control treatment. *means significant difference at $p < 0.05$ compared to CK treatment.

two novel antifungal volatiles, DMDS and MI, are identified, and show effective in control *A. flavus in vitro*. They both are first proved effective in control *A. flavus* in grains during storage. Especially for DMDS, it shows the highest relative abundance at 69.90% in N1-4 volatiles, and best antifungal activity to *A. flavus* with MIC at 50 μL/L. MI shows less inhibitory activity compared to DMDS, The MIC for MI is 200 μL/L. These results prove that DMDS with effective antifungal activity will provide more valid and alternative bio-active agents in control *A. flavus* and other fungal pathogens in storage.

Dimethyl disulfide (molecular weight 94 Dalton) is easily spread in storage condition. Some research reported that it is naturally existed in fresh *Allium porrum* and *Romanesco cauliflower* plants (Valette et al., 2003; Sébastien et al., 2004). It is always considered as common and safe compounds, and be used as spice additive in food. Some microbe can also produce DMDS such as *Bacillus cereus* (Huang et al., 2012), *pseudomonas aeruginosa* (Briard et al., 2016), *Serratia odorifera* (Kai and Piechulla, 2010). DMDS with broad resource has been applied in plant disease control field. For example, DMDS shows effective activity in control root knot nematodes and cyst nematodes (Curto et al., 2014; Fritsch et al., 2014; Sasanelli et al., 2014), Recently, Papazlatani et al. (2016) proves that DMDS at low dose (56.4 g m^{-2}) could drastically reduce the population of tomato soil borne pathogens *F. oxysporum* and *Rhizoctonia solani*. It also shows great activity to other pathogen including *Sclerotium rolfsii*, *Verticillium dahliae* and *R. solani* (Fritsch, 2004). Moreover, DMDS can induce systemic resistance in plant, and promote the growth of plant in field (Huang et al., 2012; Meldau et al., 2013; Piechulla et al., 2017). DMDS as novel bio-active compound with antagonistic activity in disease and pest control, plant growth promoting and ISR can be considered as potential biocontrol agent in field and storage.

Additionally, the volatiles produced by strain N1-4 also show broad antifungal activity against other 6 important fungal pathogens including *Fusarium graminearum*, *F. equiseti, Alternaria alternata*, *Botrytis cinerea*, *Aspergillus niger* and *Colletotrichum graminicola*. These fungi belong to different genera of two phylum including ascomycota, deuteromycotina. Each of them can cause great damage to crops, fruits and vegetables in field or storage. Our current work elucidates that volatiles from N1-4 can greatly prevent the growth of these different fungi *in vitro*. It may also inhibit the infection of these fungi in field or storage. Hence, we deduce that strain N1-4 and the produced volatiles may provide novel agents in controlling fungal pathogens and associated mycotoxins in practice.

DATA AVAILABILITY

GenBank accession numbers for our nucleotide sequences: N1-4: MK972333.

AUTHOR CONTRIBUTIONS

A-DG and Y-CL conceived and designed the experiments. A-DG, N-NW, X-WK, and F-YD performed the experiments. A-DG analyzed the data. J-HW, Z-ZY, S-JG, and Y-MZ contributed reagents, materials, and analysis tools. A-DG and M-JH wrote the manuscript.

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Field Displacement of Aflatoxigenic *Aspergillus flavus* Strains Through Repeated Biological Control Applications

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A popular pre-harvest strategy to mitigate aflatoxin contamination of corn involves field application of non-aflatoxigenic strains of *Aspergillus flavus*. The basis of this biological control may involve multiple factors, but competitive displacement of aflatoxigenic strains by the biocontrol strains is a likely mechanism. Three biocontrol strains (NRRL 21882, 18543, and 30797) were applied annually, over a 4-year period, to the same 3.2-ha commercial corn field in the Mississippi Delta, where we monitored their post-release establishment, spread, and persistence. Within 2 months of the first biocontrol application, the percentage of soil-inhabiting aflatoxigenic *A. flavus* strains in some plots was reduced from 48 to 9% of the population. The frequency of aflatoxigenic *A. flavus* strains was also significantly reduced in the rest of field. After 4 years, neighboring plots that had never received a biocontrol treatment, and distanced from our treatment plots by at least 20 meters, had less than 20% aflatoxigenic isolates. This significant halo effect might be attributed to movement of soil through tillage operations, but the aflatoxigenicity shift could be detected in the untreated plots within 2 months of the initial applications, at a time when there was no tillage. The *A. flavus* populations that colonized the grain were also monitored and found to be less than 15% toxigenic in the fourth year for all treatments. Over all treatments and years, less than 2 ppb of aflatoxin was detected, which could be a consequence of the field-wide shift of the inherent *A. flavus* population to predominately non-aflatoxigenic strains. This study supports the efficacy of using non-aflatoxigenic *A. flavus* strains as pre-harvest biocontrol, and shows that most of its effectiveness occurs with the first application.

Keywords: aflatoxin prevention, biopesticide, mycotoxin, biological control, atoxigenic

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INTRODUCTION

Aspergillus flavus is a common soil saprophyte and an opportunistic plant pathogen. Many agriculturally important crops can be infected by *A. flavus*. The resulting infections rarely cause appreciable yield loss, but can be important due to the resulting contamination by *A. flavus* mycotoxins. Several mycotoxins can be produced by *A. flavus* including aflatoxin (AF) and cyclopiazonic acid (CPA). Aflatoxin AF, a Group 1 carcinogen (IARC, 1993), is produced in several forms by various fungal species, and aflatoxin B_1 is considered an especially potent liver carcinogen. Aflatoxin contamination of food and feed is especially problematic in parts of the world where the lack of testing and proper storage conditions lead to periodic

aflatoxicosis, reduced growth in infants and children, and elevated risk of cancers (Wild et al., 2015). Europe and North America have enacted strict controls on the allowable levels of AF in foods and feeds, and the agricultural industry cooperates in monitoring AF throughout the food chain. Globally, the commodity that is most affected by AF is corn, resulting in tens to hundreds of millions of dollars in lost value annually. The level of economic loss varies greatly from year to year, but one model indicated a loss of up to US$ 1.06 billion in 2012, when weather conditions in the upper Midwest were particularly conducive to *A. flavus* infection and subsequent AF contamination, with corn prices at or near record highs (Mitchell et al., 2016). While there is comparatively less corn grown in the Southern U.S., it is contaminated by AF more frequently and at higher concentrations (Jones et al., 1981; Diener et al., 1987; Payne, 1992; Mitchell et al., 2016).

With these strong economic incentives, corn producers endeavor to reduce AF contamination. Because *A. flavus* is an opportunistic pathogen, efforts to promote host plant health and minimize plant stress may be helpful, but are often insufficient (Bruns, 2003; Wiatrak et al., 2005). AF contamination linked to insect activity, especially by the European Corn Borer (*Ostrinia nubilalis*) and Fall Armyworm (*Spodoptera frugiperda*), led to the expectation that transgenic insect control would reduce AF contamination (Dowd, 2001; Abbas et al., 2013). *Bt*-corn, developed to prevent host damage from insect predation, has been shown to be effective in the management of other mycotoxins, *e.g.,* fumonisin (Munkvold et al., 1999), but success with AF management by *Bt* insect control has been equivocal (Bowen et al., 2014; Weaver et al., 2017a,b). The most effective AF-reducing strategy is the pre-harvest application of *A. flavus* isolates that are naturally incapable of aflatoxin production. This strategy has been commercialized in the U.S. *via A. flavus* strain NRRL 21882 (AflaGuard GR, Syngenta Crop Protection, Greensboro, NC) and NRRL 18543 (AF36, Arizona Cotton Research and Protection Council), whose effectiveness has been repeatedly validated in U.S. field trials (Brown et al., 1991; Cotty, 1994; Bock et al., 2004; Dorner, 2010; Weaver et al., 2015), Europe (Mauro et al., 2018), and Africa (Atehnkeng et al., 2008; Bandyopadhyay et al., 2016).

While overall efficacy of this particular biocontrol approach for aflatoxin is well supported, questions remain regarding the multi-year dynamics of the indigenous *A. flavus* population in the context of biocontrol applications. We describe here an experiment involving 4 years of annual biocontrol applications, in a highly productive corn monoculture, to monitor *A. flavus* populations in the soil and in host kernel tissue. This experiment tested the hypotheses that applied biocontrol strains (1) displace the indigenous toxigenic strains, (2) persist beyond year of application; and (3) shift the indigenous *A. flavus* populations in a non-treated plot that is situated at a distance beyond the application site.

MATERIALS AND METHODS

Fungal Strains and Inoculum Preparation

Aspergillus flavus strains NRRL 21882, 18543 and 30797 are non-aflatoxigenic biocontrol strains that have been well characterized (Abbas et al., 2011) and sequenced (Weaver et al., 2017a,b). These strains were produced on wheat that had been moistened and autoclaved on two consecutive days, as described previously (Abbas et al., 2006). Briefly, bags containing 1,600 g of sterilized wheat were inoculated with each biocontrol strain and incubated, mixing twice per day, for 4 days before drying in a forced air drier set at 30°C. Autoclaved, but not inoculated, wheat was also dried for use as a control treatment in field applications.

Study Site

The field experiment was conducted from 2012 through 2015 in Washington County, Mississippi (Lat: 33.43°N; Lon: 90.94°W) on a 4-ha commercial corn field in continuous corn production. The soil type was a Bosket, very fine sandy loam without irrigation. The cultural and weather conditions are described in **Table 1**. Field preparation included fall deep tillage and raised beds to promote drainage, followed by tillage and reshaping of the beds in the spring.

Planting involved twin rows on raised beds with 97-cm spacing. Weed and insect pressures were closely managed to promote crop health. Plots were established by measuring 400-m^2 (20 rows × 20 m length) areas in April of 2012, geo-referencing the position of the plots and marking plot borders. The same plot borders were maintained throughout the 4-year study. Surface soil samples (0–5 cm, each a composite of five subsamples, approximately 400 g in total, near the center of every plot) were collected in May of each year from every plot, immediately before biocontrol applications, and once more in July, coinciding with kernel development and potential *A. flavus* infection. Biocontrol applications were made with hand-held fertilizer spreaders (Scotts 71,133) at a rate of 22 kg ha^{-1}, consistent with label guidelines for AflaGuard GR when corn

TABLE 1 | Growing conditions.

			Avg. Max/Min air temperature (°C)*		Precipitation (mm)*	
Year	Planting date	Hybrid planted	April–May	June–July	April–May	June–July
2012	3/20/2012	Rev 28R10	28/16	33/22	158	278
2013	4/10/2013	P16–15	24/13	31/20	312	141
2014	3/21/2014	DK66–97	25/14	31/21	407	268
2015	3/31/2015	DK62–05	27/16	34/23	317	136
Historical average			26/14	32/21	266	185

**Meteorological conditions recorded by Mississippi State University: http://deltaweather.extension.msstate.edu*

was at the five leaf-collar stage (V5). There were four replications of each treatment in a randomized complete block. To minimize interference between treatments, a 400-m^2 untreated buffer plot was included between all treatment plots. Treatments are described in **Table 2**, and the field layout is presented in **Figure 1A**. Grain was collected, by combine harvest, from the center two rows of each plot.

Sample Processing and Analysis

Soil samples were mixed with 0.1% Triton water and plated onto modified dichloran rose Bengal (MDRB) semi-selective medium to enumerate the *A. flavus* population (Horn and Dorner, 1998). Individual colonies were transferred to potato dextrose agar supplemented with β-cyclodextrin as a fluorescence enhancer and scored as presumptive aflatoxigenic or non-aflatoxigenic based upon production of a characteristic yellow pigment and a blue fluorescence. This methodology of Abbas et al. (2004) was used, except the scoring was done in 24-well culture dishes so that 22 isolates could be grown and scored alongside one known toxigenic strain and one known non-aflatoxigenic reference strain. This method was validated for 1,698 isolates by HPLC measurement of AF production, with the visual screen producing 89.93% accurate determination of toxigenicity.

A subsample of corn from each plot, weighing approximately 2 kg, was ground until at least 70% of the sample passed a 20-mesh screen, as per industry protocol (Texas State Chemist). A 50-g portion was extracted with 70% methanol for detection and quantification of AF, CPA, and fumonisin (Weaver et al., 2017a,b). An additional 20-g portion was mixed with 0.1% Triton water to isolate and characterize the *A. flavus* population using the same methods as the soil samples.

The colonization of the soil and grain by the non-aflatoxigenic strains was evaluated by analysis of variance (JMP 12.2, SAS Institute) at $\alpha = 0.05$. For each year, the percent of non-aflatoxigenic isolates in the soil was compared between the May and July sample dates to determine the effect of biocontrol product applications in that season. No significant differences between the three biocontrol strains (21,882, 18,543, and 30,797) were detected in any year (2012, $p = 0.08$; 2013, $p = 0.34$; 2014, $p = 0.34$; 2015, $p = 0.75$), so they were grouped together in the analysis. Similarly, there were no significant differences between the three biocontrol strains applied in alternating years, so these treatments were also considered together. Analysis of variance and Tukey's honestly significant difference test at $\alpha = 0.05$ were used to evaluate treatment effects on the *A. flavus* isolates that colonized grain, as measured at harvest.

TABLE 2 | Description of treatments.

	Applied inoculum			
Treatment	**2012**	**2013**	**2014**	**2015**
1. Control	Mock	Mock	Mock	Mock
2. Strain 21,182	21,182	21,182	21,182	21,182
3. Strain 30,797	30,797	30,797	30,797	30,797
4. Strain 18,543	18,543	18,543	18,543	18,543
5. Alternating 21,182	21,182	Mock	21,182	Mock
6. Alternating 21,182	Mock	21,182	Mock	21,182
7. Alternating 30,797	30,797	Mock	30,797	Mock
8. Alternating 30,797	Mock	30,797	Mock	30,797
9. Alternating 18,543	18,543	Mock	18,543	Mock
10. Alternating 18,543	Mock	18,543	Mock	18,543

RESULTS AND DISCUSSION

Mycotoxin Observations

Aflatoxin contamination is most common during periods of severe stress to the plant host, such as periods of high heat and drought, but is periodically a problem even in comparatively moist, highly productive sites (Abbas et al., 2012). This study took place during a period of historically high corn prices, leading to increased corn planting in the Mississippi Delta and supporting agronomic practices to maximize plant health. The local climatic conditions (**Table 1**) during our study also were favorable for corn production, as the state of Mississippi set record corn yields in 3 of the 4 years, and experienced an overall increase of 36% compared to the previous 10-year average (NASS). These conditions were not favorable for mycotoxin contamination and we did not detect any CPA or fumonisin during this experiment. Aflatoxin was only detected in 20 of the 88 kernel samples in 2012, and only three of the 88 kernel samples exhibited AF concentrations greater than 5 ppb. In 2013, there were 13 aflatoxin-positive samples, with nine having concentrations greater than 5 ppb. Six positive samples were found in 2014, five of which were over 5 ppb. In 2015, the final year of the study, no aflatoxin was found in any of the samples. There was no significant effect of treatment on the aflatoxin concentration in the harvested corn.

Colonization of Soil by *A. flavus*

The percentage of aflatoxigenic isolates collected from the soil is presented in **Figures 1B–F, 2**. At the initiation of the experiment (**Figure 1B**), and before any biocontrol treatments were applied, the soil *A. flavus* population was determined to be about 48% (standard deviation = 15%) aflatoxigenic. Approximately 2 months after the first phase of biocontrol products were distributed in their respective plots, each treatment area had a significantly lower frequency of aflatoxigenic isolates (**Figure 1C**). The plots that were to receive four annual biocontrol treatments went from harboring 49% aflatoxigenic *A. flavus* to 11% aflatoxigenic *A. flavus,* and the plots to receive biennial treatments (2012 and 2014) went from 53% aflatoxigenic *A. flavus* to just 8%. This reduction was to be expected, and is the purpose of the biocontrol application; however the co-occurrence of significant reductions in untreated plots that did not receive biocontrol treatments was not as obvious. Others have reported detection of biocontrol strains outside of the treatment area (Cotty, 1994; Bock et al., 2004). In the present study, we not only detected atoxigenic strains in untreated areas, but we observed a dramatic shift in the population in these untreated areas. Many of the "untreated" plots, scheduled to receive biocontrol treatment in subsequent years (2013 and 2015), shared a border with treated plots and may have benefited from a halo effect from an adjacent treatment.

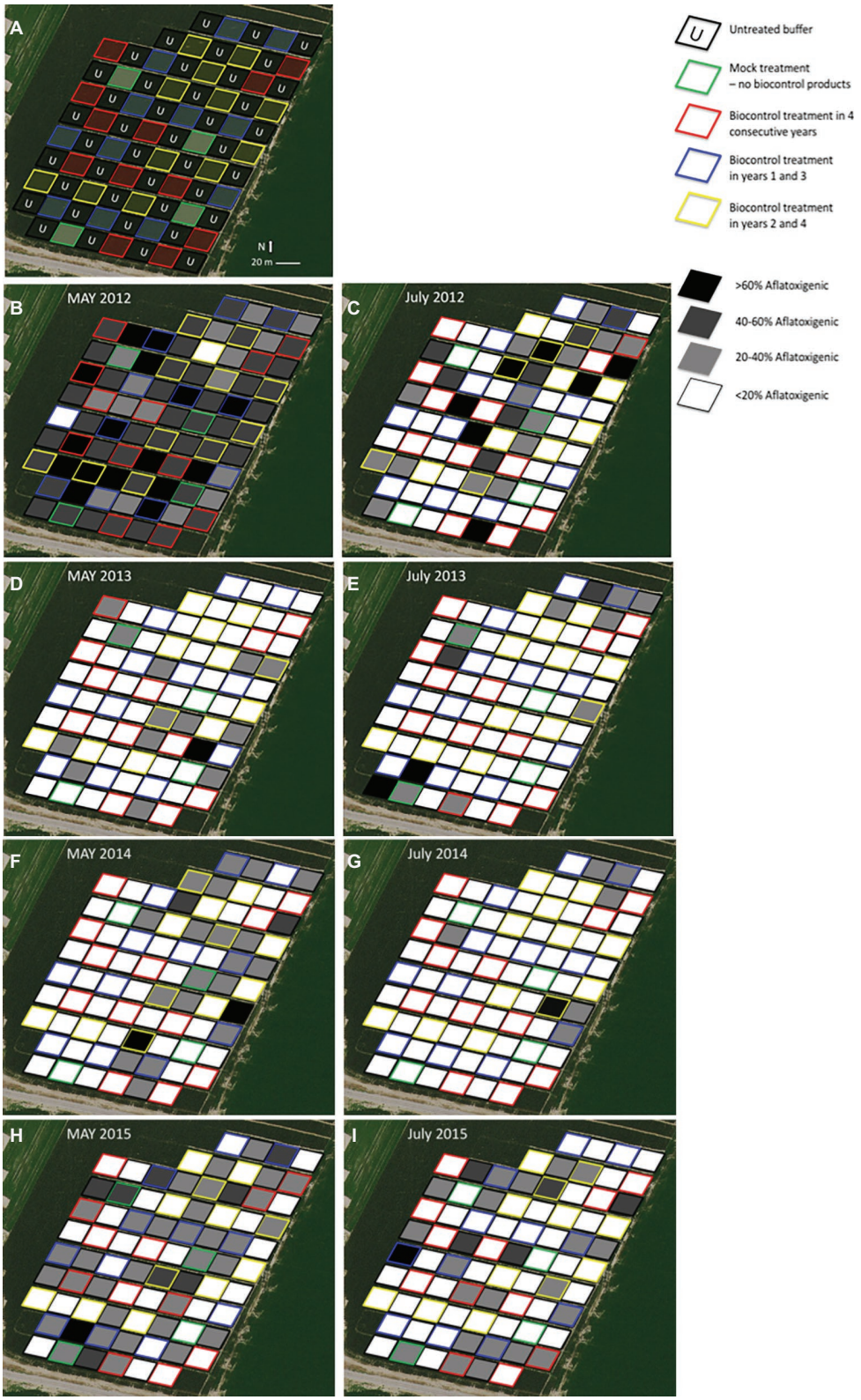

FIGURE 1 | Spatial arrangement of treatment plots within the commercial corn field and percentage of the *A. flavus* population that was aflatoxigenic within each plot at a particular time. The same plot borders were maintained throughout the 4 years of the experiment. Plot borders are color coded to indicate the treatments received and shaded to indicate the percent of *A. flavus* isolates from a plot that were aflatoxigenic. **(A)** clearly presents the treatments and experimental layout. **(B–I)** present the *A. flavus* population dynamics.

The mock inoculated treatment, which only received an application of sterilized wheat, also had a highly significant aflatoxigenicity shift: between May and July, the *A. flavus* population in plots that received no biocontrol treatment shifted from 48% aflatoxigenic to about 30% aflatoxigenic. By the following May, less than 20% of the strains isolated from these treatment areas were aflatoxigenic. Plots in these treatments were separated from plots with biocontrol treatments by a 20-m buffer, as indicated in **Figure 1A**. It is possible that the passive spread of these biocontrol strains, by normal agricultural field work, windborne spore dispersal, drift during occasional flooded conditions, or movement by insects or animals influenced the conditions of this experiment.

Plot-by-plot examination of the soil population over time reveals other patterns. In July 2012 (**Figure 1C**), nearly all of the plots with >20% aflatoxigenic isolates were either untreated buffers (plots with black outlines) or plots that would not receive their first treatments until the following year (plots with yellow outlines). In 2013 and 2014 (**Figures 1D–G**, respectively), many of the plots with >20% aflatoxigenic isolates were along the margins of the field; the North and South margins in 2013 and the South and East margins in 2014. If there was substantial movement of inoculum from the biocontrol treatments into nearby plots, it might be expected that plots on the edge would not shift as quickly as the central portion of the field. Results from 2015 are indicative of a shift in the population to a slightly more aflatoxigenic state, but still much less toxigenic than the initial, 2012 conditions.

The five treatments were compared for aflatoxigenicity shifts between May and July of each of the 4 years, for 20 comparisons in total (**Figure 2**). In the first year, as discussed above, all five treatments had a statistically significant shift toward a less toxigenic state. In the following 3 years, only five more occurrences of significant reductions in aflatoxigenic percentage were detected. Thus, of all the instances where the *A. flavus* population had a statistically significant shift, half of them were in the first year of treatments. The 10 occurrences of significant changes in July were also associated with comparatively highly aflatoxigenic states in May; i.e., measurable reductions in the aflatoxigenic percentage were more common when the starting population was relatively highly aflatoxigenic. Across all treatments, over the course of the entire experiment, the soil *A. flavus* population was 33% (S.E. = 5%) aflatoxigenic in May for the treatments that would have a significant reduction in the aflatoxigenic percentage by July, and just 15% (S.E. = 2%) in treatments that would not have a significant reduction. A reasonable inference is that the expense of a biocontrol product application is most likely to be warranted when the initial population is highly toxigenic, and that attempting to further shift an already less toxigenic population is not as likely to be effective. It is possible that it is simply difficult to detect a meaningful shift to a lower percentage when starting from a fairly low percentage. It is also reasonable for the biocontrol products to have limited efficacy when the background population has minimal aflatoxigenicity.

Colonization of Grain by *A. flavus*

In 3 of the 4 years encompassed in our study, it was not possible to discern a significant effect from the biocontrol treatments on the *A. flavus* populations that colonized the grain (**Figure 3**). Only in 2014 did one of the treatments, a biennial biocontrol application, have an *A. flavus* population with a significantly lower percentage of aflatoxigenic isolates. This "failure" of the biocontrol, however, must be seen in context. Even if the biocontrol applications did not significantly shift the population on the grain to a less aflatoxigenic state at the time of harvest, there was still nearly zero aflatoxin observed on the corn samples. Also, in two of the years without significant effect, the background *A. flavus* population (the untreated or mock inoculated plots) was only 10–20% aflatoxigenic, making it impossible for us to detect any further reduction with the resolution of our analytical methods. Furthermore, this experiment did not include any of the stresses commonly associated with high aflatoxin occurrence such as extremely high temperatures, drought stress, or abundant activity by ear-feeding insects, so the full value of the biocontrol

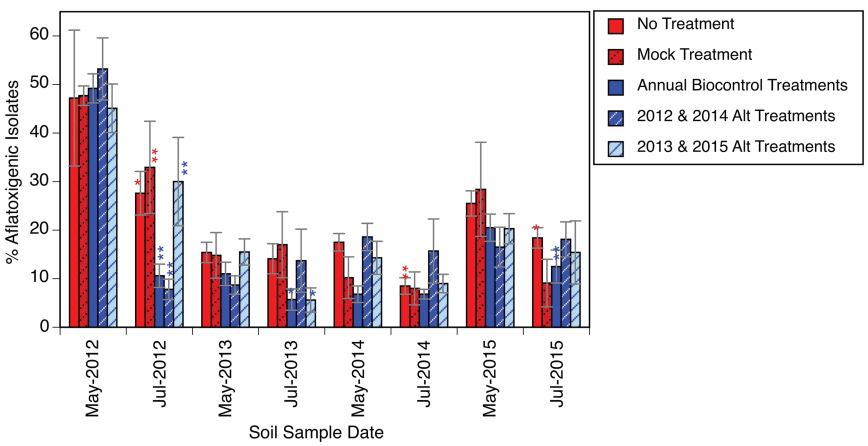

FIGURE 2 | Relative abundance of presumptive aflatoxigenic isolates in soil over time in response to biocontrol applications. Asterisk or asterisks used to indicate a significant ($\alpha = 0.05$) or highly significant ($\alpha < 0.001$) reduction, respectively in the percentage of presumptive aflatoxigenic isolates between a May and July sampling date within plots of a given treatment. Error bars indicate one standard error of the mean.

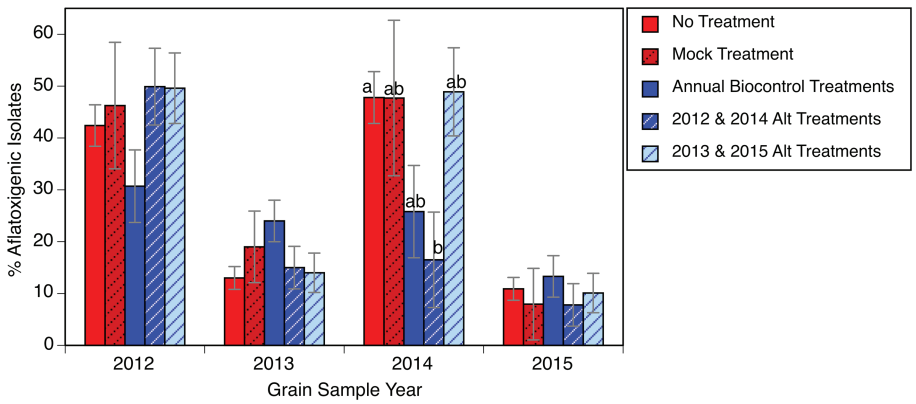

FIGURE 3 | Relative abundance of presumptive aflatoxigenic isolates in grain samples in response to biocontrol applications. Bars within the same year with different letters are significantly different by Tukey's honestly significant difference test. Error bars indicate one standard error of the mean.

applications may be somewhat masked. The reason for our observed overall increase in aflatoxigenic isolates from 2013 to 2014 is unknown and warrants further investigation. It has been suggested from vegetative compatibility group lineages that the *A. flavus* community has great variability from year to year, even within a given field (Bandyopadhyay et al., 2016). The frequency of aflatoxigenic isolates collected from all corn samples declined from a high of 43% (S.E. = 2.8%) in 2012 to 11% (S.E. = 1.5%) in 2015, consistent with an overall shift in the field population to a minimally aflatoxigenic level.

The experiment intended to address three hypotheses. Within the parameters of the current study, the *A. flavus* population in the soil shifted from 40 to 50% aflatoxigenic to consistently below 20% aflatoxigenic (hypothesis 1, **Figure 2**). In the final year of the experiment, there were several plots that appeared to revert from the "<20% aflatoxigenic" to the "20–40% aflatoxigenic" status (**Figure 1**). This could reflect weather conditions that year, migration of a different population, or allelic drift. Even with this change in the final year, the percentage of aflatoxigenc isolates was still less than half of the starting field average. The *A. flavus* population colonizing the grain from this field also declined, ending at approximately 10% aflatoxigenic (**Figure 3**). There was no evidence in the present experiment to support the expense of repeated, annual applications of biocontrol products. Similar reductions in toxigenic *A. flavus* were observed in plots that received biocontrol applications in alternating years (hypothesis 2). The shift to a lower frequency of aflatoxigenic isolates in untreated plots is consistent with the hypothesis that there is significant movement of inoculum (hypothesis 3). This observation could support alternative, more economical application methods.

CONCLUSION

While aflatoxin was nearly undetectable in the present experiment, it was possible to quantify the *A. flavus* population in soil and grain samples to monitor the decrease in the relative abundance of aflatoxigenic isolates of *A. flavus*. In the soil, significant aflatoxigenicity shifts were most common in the first year of biocontrol application. Biocontrol efficacy was positively associated with a relatively high frequency of aflatoxigenic isolates in May, immediately before application, but generally did not produce a significant effect if the background population was already <15% aflatoxigenic. Significant population changes in untreated plots were consistent with substantial passive movement of *A. flavus* inoculum beyond 20 meters. After 4 years of biocontrol applications, the *A. flavus* population recovered from the grain was approximately 11% aflatoxigenic, regardless of the particular biocontrol treatment. Biocontrol applications are most likely to be beneficial when the initial soil population has a high percentage of aflatoxigenic isolates. Future studies should be initiated when aflatoxin levels are at greater concentrations, and also should include a single biocontrol application treatment at year one, with no other applications in subsequent years, to determine if the number of aflatoxigenic strains increases to pre-application levels.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MW planned and executed the experiment, analyzed results, and wrote the manuscript. HA participated in planning the experiment, provided technical support to sample collection, and reviewed the manuscript.

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Buckwheat Hull Extracts Inhibit *Aspergillus flavus* Growth and AFB_1 Biosynthesis

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Fungal contamination poses at risk the whole food production chain - *from farm to fork* - with potential negative impact on human health. So far, the insurgence of pathogens has been restrained by the use of chemical compounds, whose residues have gradually accumulated determining toxic effects in the environment. Modern innovative techniques imply the use of natural and eco-sustainable bioactive plant molecules as pathogens and pests-control agents. These may be profitably recovered in large amounts at the end of industrial milling processes. This is the case of the non-digestible hull of common buckwheat (*Fagopyrum esculentum* Moench), a natural source of polyphenols, tocopherols, phytosterols and fatty acids. We extract these compounds from the hull of buckwheat; apply them to *Aspergillus flavus* - aflatoxin producer - under *in vitro* conditions, checking their ability to inhibit fungal growth and aflatoxin biosynthesis. Moreover, a solvent free method implying the adoption of supercritical CO_2 as solvent was set up to extract lipophilic molecules from the buckwheat' hulls. Positive results in controlling fungal growth and aflatoxin biosynthesis let infer that the extracts could be further tested also under *in vivo* conditions.

Keywords: *Aspergillus flavus*, biomolecules, *Fagopyrum esculentum*, industrial waste recovery, organic pest-control

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INTRODUCTION

At a time when the focus on the environmental issues is very high and the concepts of sustainability and circular economy are the key points of scientific community, researchers aim to find alternatives to synthetic pesticides whose use heavily affected the environment. The increasing yield losses associated to pests and diseases (up to 30% worldwide), and the expanding demand from the agro-food industry for a higher quality and availability of the products, led to a massive use of fertilizers, fungicides and synthetic pesticides (agropharmaceuticals) in the pre- and post-harvest (Janisiewicz and Korsten, 2002). Fungicides, and the effects determined by their use, normally receive minor attention compared to other types of agrochemicals such as insecticides and herbicides. It is a common belief that fungicides have a lower toxicity compared to other agrochemicals such as pesticides; however, recent studies demonstrated the opposite: i.e., fungicides revealed more toxic than herbicides and pesticides in a comparative assay (Mesnage et al., 2014).

Indeed, chemicals used to control fungal contamination along the agro-food chain, leave residues that tend accumulating causing a negative impact in the environment (Liu et al., 2008; Wightwick et al., 2010; Abdallah et al., 2018). The increasing pollution can be reduced through the application of a more restrictive legislation, appealing to clearer rules to regulate the approval process for plant protection products (Directive 2009/128/EC) and to take them to the market (Regulation (EC) No 1107/2009), showing a great attention to environmental sustainability and consumer health.

In this scenario, molecules extracted from plants tissues could represent an alternative more sustainable than synthetic agrochemical. Such compounds have been successfully tested for applications in various sectors such as food industry, cosmetics and agro-chemistry (Balandrin et al., 1985; Miyakado, 1986; Benner, 1993; Hedin and Hollingworth, 1997) and, more recently, they have been utilized within systems of integrated pest management (El-Habib, 2012; Omidpanah et al., 2015). These natural extracts can possibly be recovered from waste materials through technologies with low environmental impact; thus favoring sustainability by promoting a "circular economy." In the last decades emerged a growing interest for plant-derived bioactive substances possessing antioxidant, antibacterial and antifungal properties (Hussain et al., 2008; Venkateswarlu, 2013). In particular, the antimicrobial activity of polyphenols, such as flavonols, has been extensively investigated in various microorganisms, whilst, generally, antioxidant activities are associated to tocopherols, lipophilic compounds belonging to the vitamin E group (Daglia, 2012). Additionally, the antifungal effect of some lipid substances should not to be underestimated. Fatty acids and sterols can interact directly with the fungal cell membrane causing a generalized disorganization leading to changes in the growth pattern (Pohl et al., 2011).

The filamentous fungus *Aspergillus flavus* is widely spread; it affects a large number of plant species (e.g., maize, peanuts), with potential devastating consequences on yield and economic profits. Furthermore, *A. flavus* can produce aflatoxins, secondary metabolites that, in some instances, may result highly toxic (carcinogenic) for humans. Since now, the best strategy for controlling *A. flavus* resides on the field control measures provided by the use of antagonistic non-aflatoxigenic strains of the same species (Bhatnagar-Mathur et al., 2015; Mauro et al., 2018). It is possible to limit aflatoxin contamination at post-harvest level using some synthetic compounds (e.g., BHA) that nonetheless present their own toxicity (Nesci et al., 2003). In relation to this, it appears worth considering the possibility to control *A. flavus* and consequent aflatoxin production using plant-derived biomolecules. Similar compounds are present, in considerable amounts, in common buckwheat (*Fagopyrum esculentum* Moench) (Bonafaccia et al., 2003; Dziadek et al., 2016). The nutritional traits and the nutraceutical properties of buckwheat achenes have been studied and are well known (Soral-Smietana et al., 1984). Buckwheat grains are rich in phenolic compounds and tocopherols, fatty acids and phytosterols (Dorrell, 1971; Sedej et al., 2012). Among the various grain parts, the pericarp contains the highest amounts of these molecules (Dietrych-Szostak, 2004). Therefore, buckwheat hull, normally regarded in Europe as an industrial waste, may instead represent a low cost source of "beneficial" molecules, thus promoting the recycle of otherwise unusable exhausted raw materials.

To optimize for the sake of sustainability, the extraction of lipophilic biomolecules contained in buckwheat hull, an environmental-friendly extracting process, represented by the supercritical fluid extraction process using carbon dioxide (SFE-CO_2) was adopted. Due to its non-polar nature, SFE-CO_2 can easily solubilize lipophilic substances avoiding solvent contamination and chemical modification. Additionally, carbon dioxide has the advantage of being non-toxic, non-explosive, chemically inert, non-flammable, and inexpensive and, due to its volatility, at the end of the process, the extract can be considered as solvent-free (Raventós et al., 2002; Nautiyal, 2016).

In this study, we test - *in vitro* - lipophilic compounds - tocopherols, phytosterols and fatty acids - extracted using SFE-CO_2; and polyphenols, i.e., isoorientin, vitexin, rutin, isovitexin, hyperoside and quercetin obtained from buckwheat hull, to evaluate their efficacy to inhibit the growth of *A. flavus* and the biosynthesis of aflatoxin B_1 (AFB_1).

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich, United States.

Buckwheat Hull

A mix of hulls resulting from the milling of three *F. esculentum* varieties (Bamby, Špačinská e Lileja), was provided by "GARFAGNANA COOP," a small company located in Central Italy. For analytical purpose, buckwheat hull was grinded through a "CYCLOTEC 1093 Sample mill" (Tecator) and then sieved with $<$1000 μm mesh.

Polyphenols Analysis

Sieved hull was freeze-dried, powdered in liquid nitrogen, extracted with a 80:20 ethanol:water solution (Morishita et al., 2007) and filtrated with a 0.20 μm PTFE filter.

Total polyphenols were quantified as described by Emmons et al. (1999), with some modifications. A total of 250 μL of extract were added with 4 mL of water, 500 μL of Na_2CO_3 and 250 μL of Folin-Ciocâlteau (1:1 v/v in water), and shaken. After 25 min of incubation in the dark at 25°C, samples were centrifuged at 5000 rpm for 10 min. The absorbance was detected at 725 nm by a "Beckman DU530 UV/VIS" spectrophotometer, using a 80:20 ethanol:water mixture as blank. Polyphenols content was determined in comparison to a calibration curve of Gallic acid (3,4,5-Tri-hydroxybenzoic acid) in the range between 10 and 500 ppm. All samples, in triplicates, were measured three times and results expressed as ppm (mg/L) of Gallic acid and data are presented as the means (±SD) of 3 separate experiments (n = 9).

The antioxidant activity was evaluated, in triplicates, as described in Chitarrini et al. (2014) and data are presented as the means (±SD) of 3 separate experiments (n = 9).

The chromatographic separation of isorientin, vitexin, rutin, isovitexin, hyporoside, and quercetin was performed by HPLC (Agilent 1260, United States) equipped with a 1260 Quat pump

(Varian, United States), a 1260 DAD detector, an Infinity 1260 auto sampler (Agilent, United States), using a Supelco Ascentis C18 RP-Amide (25 cm × 4.6 mm, 5 μm) analytical column. The eluent mixture was composed of acetonitrile (solution A) and water (solution B) both acidified with 0.1% HCOOH. The flow rate was set to 1 mL/min, the column was thermostated at 30°C and the detection wavelength was set at 362 nm. The separations were performed using different solution A concentrations according to the following program: 20 min of linear gradient elution from 20 to 85%, 5 min of isocratic elution at 85%, 5 min of linear gradient elution from 85 to 20%, followed by 2 min of isocratic elution at 20%. The identification of individual compounds was performed based on their retention times and UV spectra. Libraries comprising retention times and UV-visible spectra were made by subjecting solutions of each standard. Using the Open LAB (Agilent, United States) software, a similarity index (SI) was calculated to evaluate how closer spectra of standard and corresponding phenolic compounds separated in the extracts resemble each other. According to the above software, SI closer to unity is indicative of higher similarity. In addition, the use of a purity index (PI), based on the comparison of all the spectra within a chromatographic peak to the spectrum at the peak apex, allowed one to exclude the presence of co-eluting substances in the peaks of the phenolic compounds separated from the extracts. Quantification of individual compounds was performed by calibration curves in the range of 50–1500 ng, using Kaempferol as internal standard (IS). Limit of detection (LOD) and limit of quantification (LOQ) were 5 and 11 ng, respectively. Recovery, performed adding spike compounds in amounts equal to 50, 100, and 150% of the measured analytes to lyophilized buckwheat hull, ranged between 81 and 97%, indicating a good accuracy of the method.

Lipophilic Compounds Extract Analysis

A rough characterization of the lipid fraction contained in buckwheat hull was carried out according to Christie and Han (2010) in order to confirm the presence of compounds under investigation. This preliminary assay was performed comparing the retention factor (Rf) of the spots of the lipid fraction with the Rf of the spots of standard compounds developed in the same conditions.

The ascertained presence of the species of interest in significant amounts allowed proceeding with the extraction of lipophilic compounds with a Dionex SFE-703 counter extractor, using carbon dioxide as sole extracting solvent, at 300 bar, 40°C for 180 min. Furthermore, the extraction can be performed avoiding thermal degradation and reducing energy consumes, thanks to the associated low critical values (Tc = 31.08°C; Pc = 73.8 bar) compared to those showed by similar gases. The most appropriate process conditions were suggested by the published literature (Wang et al., 2008; Tomita et al., 2014) and preferring a particle size of less than 1 millimeter to improve the surface/volume ratio to favor the diffusion process for extraction of the solute from inside the solid phase.

To verify the efficiency of SFE-CO_2 method, the pool of tocopherols, phytosterols and fatty acids was in parallel extracted with organic solvents, hereinafter reported as "conventional extraction."

Tocopherols and phytosterols conventional extraction was performed according to Slavin and Yu (2012), with minor modifications, and data are presented as the means (±SD) of three separate experiments (n = 9). Aliquots (1 g) were weighed and added with 250 μg of α-tocopherol acetate acting as internal standard. This mixture was added with 3 mL of an ethanolic solution containing 0.1% (w/v) of tert-Butyl hydroperoxide (TBH) and shaken for 10 s. The sample obtained was, in sequence, placed in a thermostatic bath at 85°C for 5 min, added with 190 μL of a 10 M potassium hydroxide solution (KOH), shaken for 10 s, incubated at 85°C for 10 min and finally cooled in ice for 10 min after the addition of 3 mL of NaCl 1M. Hexane extraction was carried out two consecutive times: samples were added with 3 mL of hexane, shaken for 10 s and centrifuged at 1000 rpm for 5 min at 4°C. The supernatant of the two extractions was combined in a new test tube, to be added with 5 mL of a 5% (w/v) Na_2CO_3 solution and subsequently centrifuged at 1000 rpm for 5 min at 4°C. The novel supernatant was washed with 5 mL of ultra-pure water and transferred to a clean test tube. The sample thus obtained was evaporated under nitrogen stream at room temperature. Separation, identification and quantification of α-, β-, γ-, δ-tocopherols, campesterol and β-sitosterol was performed by HPLC (Perkin Elmer Series 200) coupled to a mass spectrometer (AB Sciex QTrap 3200) equipped with Atmospheric Pressure Chemical Ionization source (APCI) in Multiple Reaction Monitoring (MRM). After appropriate dilution, the samples were separated by XBridge Phenyl column 150 × 2.1 mm, 3.5 μm (Waters), thermostated at 25°C with a flow of 0.25 mL/min. The mobile phases were as follows: Phase A containing H_2O acidified with 0.1% formic acid, Phase B containing acetonitrile acidified with 0.1% formic acid. Separation was performed by isocratic elution with phase B at 90%. The injection volume was 10 μL. The MS/MS acquisition was performed using an APCI source optimized with the following parameters: Curtain Gas (CUR) 40, Temperature Source (TEM) 400°C, Spray Gas (GS1) 30, Heater Gas (GS2) 30, Spray Current (NC) 5 mA, Entrance Potential (EP) 7 kV, dwell time 100 msec. Declustering Potential (DP) and Collision Energy (CE) were optimized for single transitions in MRM mode: α-tocopherol m/z 431/165, 431/137, DP 30, CE 20; β and γ-tocopherol, m/z 417/191, 417/151, DP 30, CE 25; campesterol, m/z 383/161, DP 30, CE 20; β-sitosterol, m/z 397/161, DP 30, CE 30, m/z 397/135, DP 30, CE 35; ergosterol, m/z 379/69, DP 30, CE 25; stigmasterol, m/z 395/297, DP 30, CE 15, m/z 395/83, DP 30, CE 35; δ-tocopherol, m/z 403/177, 403/137, DP 25, CE 25. α-tocopherol acetate was used as internal standard (IS) m/z 473/431, 473/207, DP 30, CE 20. Acquisition and processing of data was carried out using Analyst 1.5.1 software. The analytic identification was performed by comparing retention times and MRM transitions of a standard mix. The quantification of the analytes was performed with a calibration curve in the linear range of 0.1–10 ng. LOD and LOQ were 0.002 and 0.006 ng, respectively. Recovery results ranged between 85 and 97%, indicating a good accuracy of the method.

The extraction of fatty acids was carried out according to Phippen et al. (2006), with slight modifications, and data are presented as the means (±SD) of three separate experiments (n = 9). Grounded hulls (300 mg) were weighed in a 25 mL Pyrex flask with cap, added with 2 mL of 0.5 M KOH in methanol, stirred for 1 min and placed in a water bath at 60°C for 60 min. At this stage, the flask was further incubated at 60°C for 15 min following the addition of 2 mL of a 1 M solution of H_2SO_4 in methanol. Then the sample was added with 2 mL of ultrapure water, left cooling for 10 min and finally added with 2 mL of hexane containing non-methylated C17: 0 (margaric acid) as internal standard. Supernatant (1 mL) was collected and dried under nitrogen stream at room temperature. Fatty acids were analyzed by gas chromatography (GC) 7890 B (Agilent, United States) equipped with: an injector set at 250°C, 20.443 psi, flow rate 28.2 mL/min, purge flow 3 mL/min, split 1:2 with flow 24 mL/min; OmegawaxTM 250 silicon capillary column (30 m × 0.25 mm df 0.25 μm, Supelco Analytical) with flow 1.2 mL/min, 20.443 psi, speed 33.995 cm/s, hold time 1.4725 min, post run 0.78872 mL/min; oven at 170°C, rate 1°C/min and Tf 230°C in 16 min. The injected volume and run time were set at 1 μL and 16 min, respectively. Chromatographic peaks were identified comparing retention times of a standard mixture of 37 fatty acids including: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3). Once identified, the amounts of these five compounds were quantified using a second more specific standard mixture (F.A.M.E. RM-2) containing the above-mentioned standards and margaric acid as internal standard. The identification of individual compounds was performed based on their retention times and the application of the standard addition method (Wrona et al., 2013). Quantification of individual compounds was performed via the external standard method. Linearity was evaluated based on the calibration curves that were constructed by plotting the concentration of standards in μg/mL versus peak area. Linear least-squares regression analysis was employed to calculate slope, intercept, and correlation coefficient of the calibration curve. This last parameter resulted higher than 0.9998 for all analytes, indicating good linearity, verified in the range 7–70 ng for palmitic acid (C16:0), 5–50 ng for stearic acid (C18:0), 18–180 ng for oleic acid (C18:1), 35–358 ng for linoleic acid (C18:2) and 34–340 ng for linolenic acid (C18:3). Limits of detection (LOD) and limits of quantification (LOQ), were 0.8, 0.6, 1.9, 3.6, 3.5 and 2.1, 1.2, 3.8, 17.5, 17.1 ng respectively. Recoveries varied between 83 and 95%, thus proving an adequate degree of accuracy.

Evaluation *in vitro* of the Antifungal Activity of Extracts on *A. flavus* Growth

AFB_1-producing *A. flavus* strain - NRRL 3357 was maintained on Czapek Dox Agar (CDA), amended with $ZnSO_4$ (5 mg/L) and $NaMoO_4$ (1 mg/L), at 30°C. Extract fraction containing polyphenols (PE), and lipophilic compounds obtained with supercritical CO_2 (LE) were tested to verify the ability to modulate either mycelial growth or AFB_1 production at different time intervals: 4, 5, 6, and 7 days after inoculation (DAI). PE and LE concentrations showing higher inhibitory ability were determined after considering several combinations. In order to evaluate antifungal activity of buckwheat hull extracts on *A. flavus* growth, 5 Petri dishes (100 mm × 15 mm size) for each experiment, containing potato Dextrose Agar (PDA), amended with either PE (100 ng/mL), LE (10 μg/mL) or a mixture of both, were inoculated with a pure culture of the fungus. A positive control (ctr+), inoculated but not containing extracts and a non-inoculated negative control (ctr−) were also included. The incubation temperature was set up at 30°C for 7 days, until the appearance of mature spores according to Liu et al. (2016) and Alshannaq et al. (2018). At the end of the incubation period (7 DAI), mean radial mycelial growth was determined by measuring the size of the colony at two perpendicular directions and presented as the means (±SD), while for multiple comparison analysis (Tukey's test) on fungal growth inhibition (%), the mean growth values were recorded and compared to the control treatment to determine the mycelial growth inhibition (MGI) percentage through the formula,

$$MGI(\%) = ((d_c - d_t)/d_c) \times 100$$

where d_c and d_t represent mean mycelial growth size in control and treated Petri dishes, respectively.

Evaluation *in vitro* of the Antifungal Activity of Extracts on AFB_1 Production

Phenolic and lipophilic (LE) extracts' influence on aflatoxin B_1 production was evaluated on *A. flavus* grown on potato dextrose broth (PDB) over a period of 7 days and data are presented as the means (±SD) of five separate experiments. Beside different concentrations of PE (50, 100, 500 ng/mL) and LE (1, 10, 100 μg/mL) also the following combinations (PE 50 ng/mL and LE 1 μg/mL, PE 50 ng/mL and LE 10 μg/mL, PE 100 ng/mL and LE 10 μg/mL) were evaluated. Positive control (ctr+) adopted was extract free, whereas not inoculated growth media served as negative control (ctr−). AFB_1 determination was performed as previously reported (Fanelli et al., 2004), by extracting *A. flavus* culture in chloroform:methanol (2:1 v/v) three times. The extracts were collected after filtration on anhydrous Na_2SO_4 and concentrated under a N_2 stream. AFB_1 quantification was performed by HPLC (Agilent 1260, United States) equipped with a 1260 Quat pump (Varian, United States), a 1260 DAD detector, an Infinity 1260 auto sampler (Agilent, United States); using an analytical column GEMINI®C18 (LC Column, 150 mm × 4,6 mm, 5 μm, 110 Å, Phenomenex). Mobile phase was a mixture of water/acetonitrile (70:30 v/v). The flow rate was set to 1 mL/min, the column was thermostated at 40°C and the detection wavelength was 363 nm. Crystalline AFB_1 was used to prepare the standard solution. AFB_1 content was calculated on the basis of the calibration curve, 25–1000 ng of AFB_1 standard. Quantification of AFB_1 was performed by the external standard method. Linearity was evaluated on the basis of the calibration graphs that were constructed by plotting the concentration of standards 25–1000 ng of AFB_1 in μg/mL versus peak area. Linear least-squares regression analysis was employed to calculate slope, intercept, and correlation

coefficient of the calibration graph. The correlation coefficient of the calibration graph was higher than 0.9978, indicating good linearity. The linearity was verified in the range 15–500 ng. Limit of detection (LOD) and (LOQ) were 1.6 and 8.2 ng respectively. The accuracy of the method was evaluated by a recovery study, which was carried out according to the procedure afore mentioned for polyphenols. The recoveries were between 88.2 and 103.8%, indicating that the method has an adequate degree of accuracy. Data are presented as the means (±SD) of five separate experiments, while for multiple comparison analysis (Tukey's test) on inhibition of AFB_1 production; the mean values were normalized to the control treatment (ctr+).

Statistics

To verify if MGI and AFB_1 synthesis inhibition raw data means were significantly different from each other, a multiple comparison test was performed on the information returned by ANOVA test using Tukey's test honestly significant difference procedure. As it is known, two group means are significantly different if their comparison intervals are disjoint (we assumed a significance level of 0.05). In fact, values which present not overlapping errors bars are significantly different ($\rho < 0.05$). Correlations of variables, MGI and AFB_1 inhibition, were suggested by the scatter plot. All the statistical analyses were performed using MATLAB R2015b software.

RESULTS AND DISCUSSION

Buckwheat Hull Phenolic Content Analysis

The content in term of total polyphenols found in buckwheat hull is 4.89 mg/g Dry Weight-DW, confirming an antioxidant activity equal to 7.41 μmol Trolox EQ/g, in line with values previously associated with antifungal effect (Alvarez-Jubete et al., 2010; Żmijewski et al., 2015). Polyphenols extracted (PE) from buckwheat hull can alter the antioxidant system of the fungal cells, although temporarily, and are the main responsible for the inhibition of AFB_1 production as well as of fungal growth (Nesci et al., 2003).

Amongst the investigated polyphenols, vitexin and hyperoside are present in a significantly higher concentrations within the hulls (**Table 1** and **Supplementary Figure S1**), with average values of 426 and 440 μg/g, respectively. Concentration of rutin, the polyphenol, characterized by the highest antioxidant activity among those investigated (Dietrych-Szostak and Oleszek, 1999), was 206 μg/g DW, value similar to those observed in buckwheat grain (Brunori et al., 2010).

These evidences are in agreement with the current literature which reports that phenolic compounds are potent antioxidants showing often antifungal activity, e.g., against *Aspergillus* (Hua et al., 1999; Kim et al., 2005, 2006; Razzaghi-Abyaneh et al., 2008). Moreover, polyphenols may exert inhibitory activity on AFB_1 production modulating oxidative stress levels in the fungal cell (Bhatnagar et al., 2008; Kim et al., 2008; Brown et al., 2010). Reactive Oxygen Species (ROS) may regulate all the vitally important processes in fungi: phase development change, intercellular communications, and protection from interspecies competition proving to be a prerequisite and stimulatory factor for AFB_1 biosynthesis (Jayashree and Subramanyam, 2000; Zaccaria et al., 2015). At physiological concentrations, ROS play an important role in fungal developmental processes (Reverberi et al., 2008), but if their level exceeds the cell-scavenging capacity, cell membranes and cell metabolism may result damaged. Interestingly, the same effect can be obtained also with low ROS concentrations. Those seem likely to suppress the spore germination at a regulatory level (Gessler et al., 2007; Breitenbach et al., 2015).

TABLE 1 | Contents (μg/g) of polyphenols in buckwheat hull extract.

	μg/g
Isoorientin	247.3 ± 5.2
Vitexin	425.7 ± 9.1
Rutin	206.0 ± 4.8
Isovitexin	269.1 ± 5.0
Hyperoside	440.1 ± 9.3
Quercetin	39.1 ± 0.7

Values are reported as means ± SD.

Lipophilic Extract Characterization

In the present study, TLC analysis of the lipid fraction (LE) of buckwheat hull indicated high levels of polar lipids (PoL), phospholipids (PhL), sterols (St) and free fatty acids (FFA), and low amounts of triglycerides (TG) and sterol esters (ES) (**Figure 1**). Tocopherols, fatty acids and phytosterols extraction was performed with both conventional protocols and supercritical CO_2 fluid extraction (SFE-CO_2; **Supplementary Table S1**), which enables yield increase (up to three orders of magnitude). This extraction method proved to be more conservative favoring the preservation and augmenting the yield of lipophilic compounds (**Table 2**; Uquiche et al., 2015).

Compounds such as tocopherols, fatty acids and phytosterols gain relevance in fungal growth and mycotoxin synthesis inhibition processes (Gonelimali et al., 2018). Specifically, antifungal activities of non-polar compounds involve the alteration of the cell membrane; this, in fungi, may lead to a loss of membrane permeability and to the accumulation of toxic substances, which disrupt the cell metabolism and activate the cytolytic pathways (Pohl et al., 2011; Sedej et al., 2012).

In plant tissues, the main function of tocopherols - the most important and abundant lipid-soluble antioxidant - is to reduce the amount of Reactive Nitrogen Oxide Species (RNOS), capable in turn to induce oxidative stress (Falk and Munnè-Bosch, 2010). In plants, α-tocopherol deactivates photosynthesis-derived reactive oxygen species, and prevents the propagation of lipid peroxidation by scavenging lipid peroxyl radicals in thylakoid membranes (Munné-Bosch, 2005). Γ-tocopherol, an isomer of vitamin E, is regarded as the most potent free-radical remover, able to detoxify electrophiles, thanks to its ability to form a stable nitro adduct, 5-Nγ-tocopherol (Cooney et al., 1993; Jiang et al., 2001). Conversely, γ-tocopherol is very unstable and at risk of denaturation under "conventional extraction" conditions

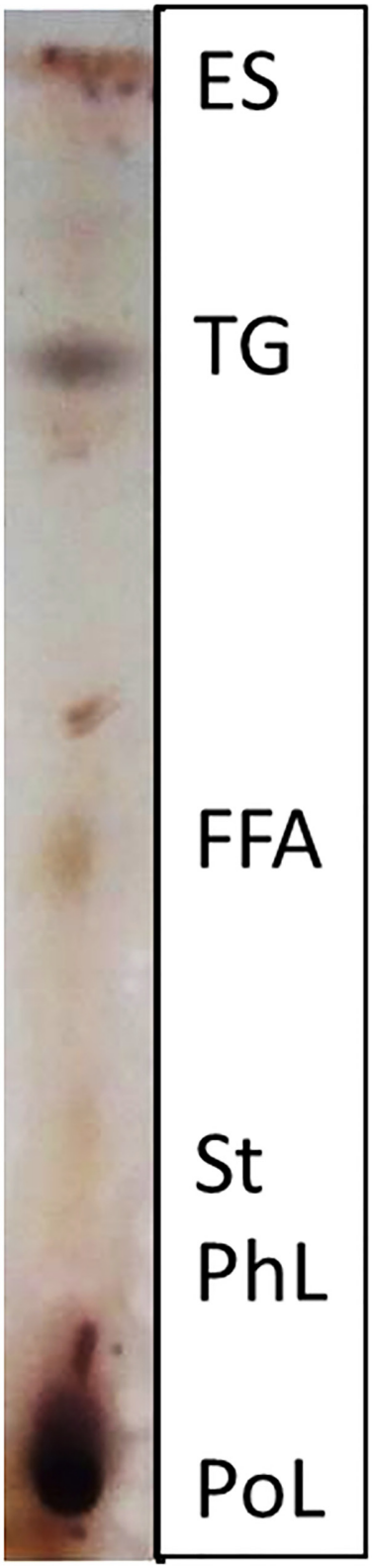

FIGURE 1 | Thin layer chromatography (TLC) analysis of lipid fraction. PoL, polar lipids; PhL, phospholipids; St, sterols; FFA, free fatty acids; TG, triglycerides; ES, esters.

TABLE 2 | Contents (μg/g) of tocopherols, sterols and fatty acids in hull extract obtained using conventional extractions or supercritical CO_2 as eluent.

	Conventional extractions	CO_2 extraction
α- tocopherol	144 ± 5	100 ± 4
δ-tocopherol	–	19.5 ± 0.6
γ-tocopherol	16.9 ± 0.5	602 ± 24
β-sitosterol	384 ± 12	9474 ± 452
campesterol	40.5 ± 0.4	1678 ± 51
stigmasterol	158 ± 5	5686 ± 194
C16:0	551.1 ± 9.1	$378.3 \times 10^3 \pm 4.7 \times 10^3$
C18:0	56.6 ± 0.9	$56.6 \times 10^3 \pm 0.8 \times 10^3$
C18:1	528.2 ± 9.2	$196.1 \times 10^3 \pm 5.1 \times 10^3$
C18:2	515.9 ± 9.2	$193.4 \times 10^3 \pm 5.3 \times 10^3$

Values are reported as means ± SD.

(Sabliov et al., 2009). This may be prevented when a more conservative method such as supercritical fluid extraction (SFE-CO_2) is adopted. Furthermore, concerning *A. flavus*, tocopherols have a high affinity to aflatoxin, reducing its bioavailability through the formation of stable associations (Daud et al., 2014).

Phytosterols, as well, express their toxicity against fungi via interference with cell membrane integrity (Augustin et al., 2011; Singh et al., 2016), playing an important role in fundamental biological processes like signal transduction, cellular sorting, cytoskeleton reorganization and infection process (Simons and Ikonen, 2000; Simons and Ehehalt, 2002). Regarding β-sitosterol, it has been reported that its exogenous application may favor an increased resistance to fungal diseases, presumably in connection with the loss of membrane integrity of the plant pathogens (Siebers et al., 2016).

Free fatty acids lipid fraction can cause an elevation in fluidity of cell membrane increasing the mobility of phospholipid acyl chains in the membrane bilayer in proportion with their degree of unsaturation (18:1 > 18:2 > 18:3) (De Kruyff et al., 1973; Avis and Bèlanger, 2001). Among other, oleic and linoleic acids, which resulted the most abundant in the lipophilic extract (LE), are known to express a strong antifungal activity either on the spore germination, on the mycelial growth or both depending on the fungus lifestyle (Altieri et al., 2007; Liu et al., 2008). Palmitic acid,

TABLE 3 | Fungal growth (cm) in cultural medium amended with phenolic extract (PE) and lipophilic extract (LE), monitored: 4, 5, 6 and 7 days after inoculation (DAI).

	4 DAI	5 DAI	6DAI	7 DAI
ctr+	1.06 ± 0.05^{A}	1.41 ± 0.07^{A}	5.06 ± 0.24^{A}	9.93 ± 0.49^{A}
PE 100 ng/mL	0.63 ± 0.03^{B}	1.08 ± 0.06^{B}	4.15 ± 0.21^{B}	6.21 ± 0.31^{B}
LE 10 μg/mL	0.51 ± 0.03^{B}	0.86 ± 0.04^{BC}	1.54 ± 0.08^{C}	2.58 ± 0.13^{C}
PE 100 ng/mL+ LE 10 μg/mL	0.35 ± 0.02^{B}	0.58 ± 0.03^{C}	0.86 ± 0.05^{D}	1.42 ± 0.07^{D}

Values are reported as means ± SD. Negative control (ctr–) and positive control (ctr+) corresponded, respectively, to non-inoculated growth medium amended with extracts, and infected growth medium without extracts. Small capital letter in the charts represent the significantly different groups ($p < 0.05$; Tukey test).

the other fatty acid present in high concentration, is characterized by an antifungal activity quite higher compared to unsaturated fatty acids (Liu et al., 2008).

Evaluation *in vitro* of Buckwheat Hull Extracts Influence on *A. flavus* Growth and AFB_1 Synthesis

To investigate the effectiveness of the buckwheat extracts against *A. flavus* mycelial growth under *in vitro* conditions, the lipid fraction (LE) obtained with SFE-CO_2 was added to the culture medium, at 10 μg/mL and 100 ng/ml, respectively, either alone (LE10; PE100) or in mixed combination (LE10PE100) with polyphenol extract (PE), (**Table 3** and **Supplementary Figure S2**). Raw data were normalized with the control obtaining a growth-inhibition trend (**Figure 2**). Despite the different timeline of the trends, the most evident inhibition was expressed by the combination of the two extracts (PE100LE10) in every time interval. By the end of the incubation period (7 DAI), *A. flavus* growth was reduced, respectively by 74 and 38% following LE10 and PE100 application, whereas the combination of the two fractions, determined a mycelial growth reduction of the 86%.

To outline the ability of PE and LE to inhibit AFB_1 biosynthesis in *A. flavus*, a wide range (**Table 4** and **Supplementary Figure S3**) of concentrations was tested as single as in combination. Notably, PE 500 ng/mL totally inhibited AFB_1 synthesis during the period of observation, while PE 50

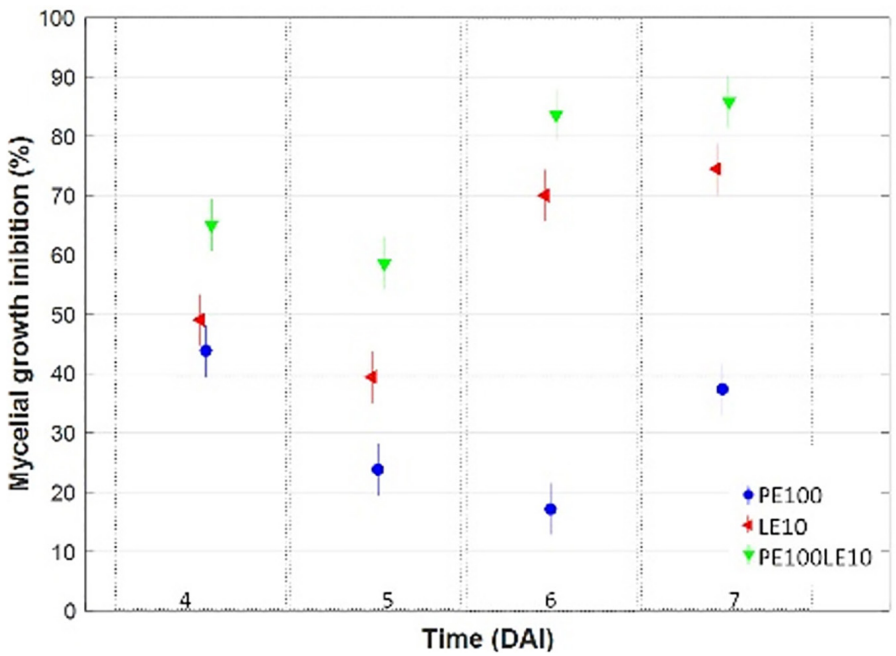

FIGURE 2 | Multiple comparison test (with Tukey's honestly significant difference procedure) on mycelial growth inhibition (%) trend occurred in growth media amended with PE (100 ng/mL), with LE (10 μg/mL) and a mix of both, at different days after inoculation (DAI).

TABLE 4 | Amount of aflatoxin B_1 (ng/mL) produced in growth medium amended with phenolic extract (PE) and lipophilic extract (LE) at different concentrations.

	4 DAI	5 DAI	6 DAI	7 DAI
ctr +	344.37 ± 17.28^{A}	951.94 ± 46.8^{A}	1479.47 ± 73.6^{A}	2207.29 ± 109.5^{A}
PE 500 ng/mL	0^{F}	0^{F}	0^{G}	0^{E}
PE 100 ng/mL	0^{F}	0^{F}	93.01 ± 4.65^{F}	157.01 ± 7.26^{D}
PE 50 ng/mL	0^{F}	77.33 ± 3.48^{E}	107.54 ± 5.34^{F}	170.60 ± 8.45^{D}
LE 100 μg/mL	84.50 ± 4.22^{E}	180.67 ± 9.15^{D}	364.23 ± 18.84^{C}	725.45 ± 35.72^{B}
LE 10 μg/mL	113.40 ± 5.62^{D}	207.44 ± 10.42^{CD}	209.41 ± 11.01^{E}	244.15 ± 12.94^{D}
LE 1 μg/mL	0^{F}	233.45 ± 11.75^{C}	274.90 ± 13.81^{D}	535.01 ± 26.88^{C}
PE 50 ng/mL + LE 1 μg/mL	172.28 ± 8.59^{C}	216.16 ± 10.79^{CD}	200.68 ± 10.82^{E}	619.10 ± 29.61^{C}
PE 50 ng/mL + LE 10 μg/mL	311.88 ± 14.7^{B}	356.79 ± 18.84^{B}	389.97 ± 19.59^{C}	742.48 ± 36.98^{B}
PE 100 ng/mL + LE 10 μg/mL	180.89 ± 9.12^{C}	192.07 ± 9.53^{D}	465.77 ± 23.56^{B}	726.72 ± 34.87^{B}

Values are reported as means ± SD. AFB_1 production was monitored: 4, 5, 6, and 7 DAI. Negative control (ctr−) and positive control (ctr+) corresponded, respectively, to non-inoculated growth medium amended with extracts, and infected growth medium without extracts. Small capital letter in the charts represent the significantly different groups ($p < 0.05$; Tukey test).

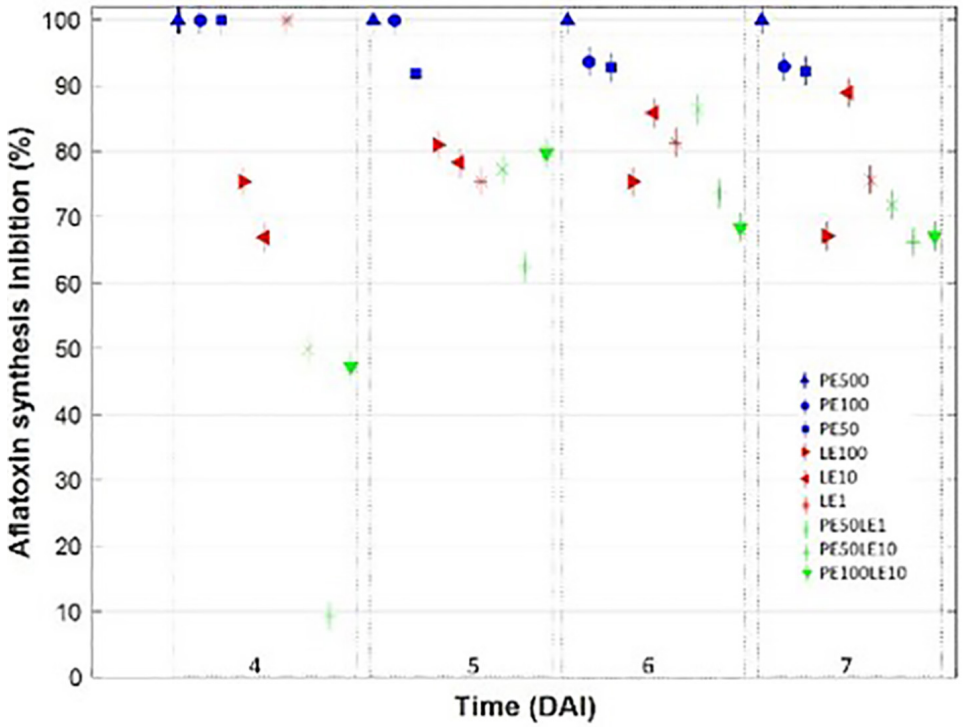

FIGURE 3 | Multiple comparison test (with Tukey's honestly significant difference procedure) on AFB_1 synthesis inhibition (%) trend occurred in growth media amended with PE, LE and a mix of both in different concentrations, at different days after inoculation (DAI).

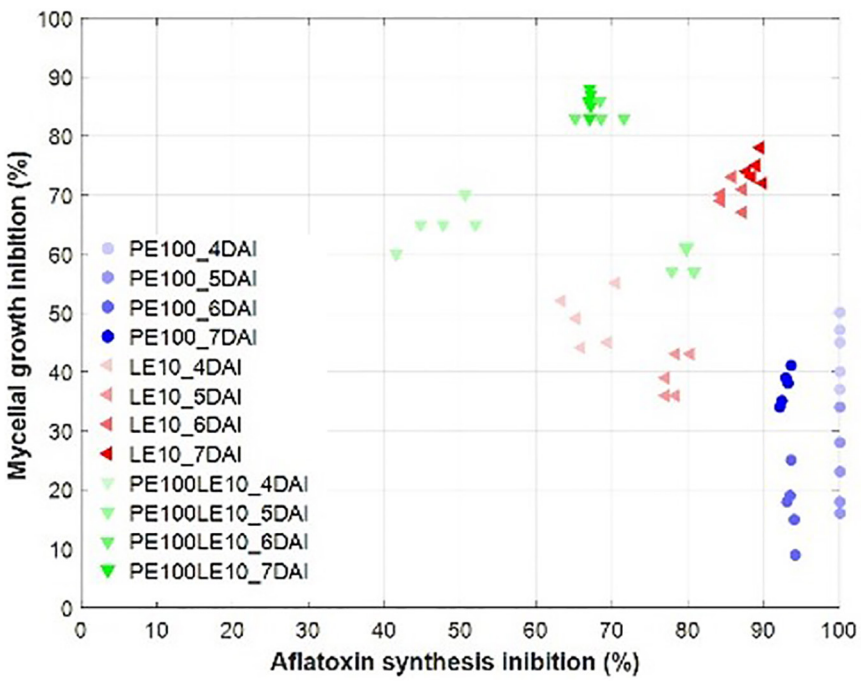

FIGURE 4 | Scatter plot between mycelial growth inhibition (%) and AFB_1 synthesis inhibition (%) for PE (100 ng/mL), LE (10 μg/mL) and their mix from 4 to 7 DAI.

and 100 ng/mL inhibition lasted up to 4 and 5 DAI, respectively (**Figure 3**). This evidence suggests that, at least for the PE fraction, the inhibition of AFB_1 production is dose-dependent: the higher the concentration the longer the inhibition.

Lipophilic application showed over time a lower and apparently not dose dependent inhibitory effect on AFB_1 synthesis. In general, no trend was evident except in the case of LE10, where the effect was continuosly increasingly with time. A peak value of 100% was observed for threatment of LE1 on 4 DAI followed by a rapid decrease, whereas the best performing threatment resulted LE10 with an average inhibition > 80% (**Table 4**). The behavior of the PE-LE mixtures appeared independent from the concentrations of the two fractions leading to a significant inhibition of AFB_1 biosynthesis attesting around 70%. Actually, at 7 days after inoculation, PE, regardless the concentration tested, showed an inhibitory effect on AFB_1 production significantly higher (>90%) compared to the other threatments. As regards LE, the highest inhibition, around 85%, was attained at 10 μg/mL, while any of the mixtures containing both lipofhilic fraction and poliphenols pooled around 70%. Nevertheless, none of the mixtures tested proved to inhibit AFB_1 production as much as the sole PE.

We draw a scatter plot for evaluating the combined ability of buckwheat hull extracts to limit the mycelium growth and the aflatoxin biosynthesis (**Figure 4**). The best synergic effect on both variables occurred in the presence of LE10 followed by PE100LE10 at 7 and 6 DAI, while PE alone acted almost exclusively on the aflatoxin synthesis reduction (>90%), inhibiting only up to 45% the fungal growth.

Results suggest the existence of important physiological mechanisms that induces PE and LE to counteract the mycotoxin production and to modulate the mycelial growth.

CONCLUSION

This study demonstrates that buckwheat hull extracts - rich in polyphenols and lipophilic molecules - can be used for limiting *A. flavus* growth and AFB_1 synthesis (Liu et al., 2008; Reverberi et al., 2012). It was observed, in fact, that the mixture of both extracts had the highest influence on fungal growth, while polyphenols exert their main effect on the production of AFB_1.

When "mild technologies" (extraction with supercritical CO_2) were applied, such compounds were extracted at a concentration significantly higher compared to conventional methods, with the further advantage that molecules of interest are solvent-free, suitable for open field applications with highly reduced potential risk for plants, operators and the environment.

The capacity demonstrated by active natural molecules extracted from a plant waste partly using an eco-friendly extraction technique could design a new strategy to counteract fungal contamination at field or storage level.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

CN and MR designed the study. CN, ADA, CB, GL, and SP performed the experiments. CN and DP analyzed the data. CN, AB, and MR wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01997/full#supplementary-material

TABLE S1 | Overview of analytical methods used to extract lipid fraction.

FIGURE S1 | HPLC-DAD (Agilent 1260, United States) chromatograms of polyphenols in PE extracts, identified by mean of their DAD profile and compared with standards as well as published spectra.

FIGURE S2 | Fungal growth (cm) in cultural medium amended with phenolic extract (PE) and lipophilic extract (LE), monitored at 4, 5, 6, and 7 days after inoculation (DAI). Positive control (ctr+) corresponded, respectively, to non-inoculated growth medium amended with extracts, and infected growth medium without extracts. Values represented the mean of $n = 5$ determinations ± SE.

FIGURE S3 | AFB1 biosynthesis in cultural medium amended with phenolic extract (PE) and lipophilic extract (LE), alone or in combinations of a range of concentrations (indications provided in the graph legend) monitored at 4, 5, 6, and 7 days after inoculation (DAI). Positive control (ctr+) corresponded, respectively, to non-inoculated growth medium amended with extracts, and infected growth medium without extracts. Values represented the mean of $n = 5$ determinations ± SE.

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REVIEW
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Involvement of Ahr Pathway in Toxicity of Aflatoxins and Other Mycotoxins

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The purpose of this review is to present information about the role of activation of aflatoxins and other mycotoxins, of the aryl hydrocarbon receptor (AhR) pathway. Aflatoxins and other mycotoxins are a diverse group of secondary metabolites that can be contaminants in a broad range of agricultural products and feeds. Some species of Aspergillus, Alternaria, Penicilium, and Fusarium are major producers of mycotoxins, some of which are toxic and carcinogenic. Several aflatoxins are planar molecules that can activate the AhR. AhR participates in the detoxification of several xenobiotic substances and activates phase I and phase II detoxification pathways. But it is important to recognize that AhR activation also affects differentiation, cell adhesion, proliferation, and immune response among others. Any examination of the effects of aflatoxins and other toxins that act as activators to AhR must consider the potential of the disruption of several cellular functions in order to extend the perception thus far about the toxic and carcinogenic effects of these toxins. There have been no Reviews of existing data between the relation of AhR and aflatoxins and this one attempts to give information precisely about this dichotomy.

Keywords: ***Aspergillus***, ***Alternaria***, **AFB1, alternariol (AOH), CYP1A1**

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INTRODUCTION

Aflatoxins and other mycotoxin are activators of the aryl hydrocarbon receptor (AhR), are a structurally diverse group of fungal secondary metabolites and a toxigenically and chemically heterogeneous assemblage. They are ubiquitous contaminants in a broad range of agricultural commodities and feed (**Table 1**) and can contaminate the food supply at any time during production, processing, transport or storage (Bräse et al., 2009). Currently, far more than 400 mycotoxins are produced by some 350 fungi species (Kuhn and Ghannoum, 2003), but scientific attention is given mainly to those that have proven to be carcinogenic and/or toxic (Zain, 2011). The aflatoxins and other toxins that can act as activators to AhR can be acutely or chronically toxic, depending on the kind of toxin, the dose, health, age and nutritional status of the exposed individual or animal, and the possible synergistic effects between mycotoxins (Bennett and Klich, 2003). Their ingestion, inhalation, or skin absorption can cause diseases in humans and animals and their greater impact on human health in industrial countries is due to the chronic exposure (Gil-Serna et al., 2014). In this case, aflatoxins are associated with different biological effects like carcinogenic, mutagenic,

TABLE 1 | Toxic effects of several mycotoxins in human.

Mycotoxin	General effects	Mycotoxin related symptoms/disease in humans	References
Aflatoxins	Hepatotoxic; immunotoxic, genotoxic, mutagenic, teratogenic carcinogenic	Acute/chronic liver disease, (hepatocellular carcinoma, hemorrhagic necrosis, fatty acid infiltration in liver cells), lung cancer, child growth impairment, immunosuppression, Kwashiorkor disease, change in protein metabolism and micronutrients (Zn, Fe, vitamin A), vomiting, abdominal pain, anorexia, diarrhea, depression, jaundice, photosensitivity, pulmonary or cerebral edema, encephalopathy, pulmonary interstitial fibrosis, reproductive system effects (particularly male)	Steyn, 1995; Bryden, 2007; Bräse et al., 2009; Marin et al., 2013; Gil-Serna et al., 2014; Wu et al., 2014
Alternaria toxins	Genotoxic, mutagenic, teratogenic carcinogenic	*Onyalai* disease, cardiovascular collapse, gastrointestinal hemorrhage, lung, and esophageal cancer	Ostry, 2008; Siegel et al., 2010; Marin et al., 2013; Gil-Serna et al., 2014; Lee et al., 2015; Fan et al., 2016
Fumonisin B_1	Neurotoxic, hepatotoxic, nephrotoxic, immunosuppressive, carcinogenic, teratogenic	Neural tube defects, esophageal and liver cancer, inhibit the uptake of folic acid via the folate receptor	Steyn, 1995; Ueno et al., 1997; Marasas, 2001; Missmer et al., 2006; Bryden, 2007; Voss et al., 2007; Bräse et al., 2009; Zain, 2011; Gil-Serna et al., 2014; Wu et al., 2014
Ochratoxin A	Nephrotoxic, hepatotoxic, immunotoxic, carcinogenic, genotoxic teratogenic	Renal diseases (Endemic nephropathy, urothelial, and kidney tumors)	Steyn, 1995; Schwartz, 2002; Bryden, 2007; Bräse et al., 2009; Gil-Serna et al., 2014; Wu et al., 2014

teratogenic, estrogenic, hemorrhagic, immunotoxic, nephrotoxic, hepatotoxic, dermotoxic and neurotoxic ones, including displays of both tumor and antitumor effect, cytotoxic, and antimicrobial properties (see **Table 1**). Their impact in human diseases has been the subject of intensive study since the early 1960s, since aflatoxicosis and other mycotoxicosis can provoke tumor formation or even rapid death (Bräse et al., 2009).

Some molds are capable of producing more than one toxin and some toxins are produced by more than one fungal species (Bräse et al., 2009; Zain, 2011). Aflatoxins are commonly found in many food products, such as cereals, fruits, nuts, and meat, among others.

INTERACTIONS OF AFLATOXINS AND OTHER FOODBORNE MYCOTOXINS WITH AHR

The major foodborne mycotoxins of public health interest are aflatoxins. However, there are several foodborne mycotoxins (*Alternaria* toxins, fumonisin B_1, ochratoxin A, and patulin) that should not be excluded due to their broad toxicity, as well as ability to activate AhR-pathway, the focus of this review. These mycotoxins account annually for millions of dollars losses worldwide in human health, animal health and discarded agricultural products (Zain, 2011).

AFLATOXINS

The name "aflatoxin" (*A. flavus* toxin) was assigned to a number of polyketide-derived furanocoumarins carcinogenic metabolites produced mainly by fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Kensler et al., 2011). The aflatoxins were structurally identified in the early 1960s and over the last 50 years have been extensively studied with respect to their mechanism of action, including their mutagenic and carcinogenic activity (Valencia-Quintana et al., 2014). Collectively, aflatoxins are a group of approximately 20 chemically related metabolites, although the most important and naturally occurring are aflatoxins B_1 (AFB_1), B_2, G_1, and G_2 (**Figure 1**). The AFB_1 is in this group the most potent hepatotoxin with highly toxic and carcinogenic characteristics for many animal species (Hendricks, 1994).

Aflatoxins are metabolically activated in the liver by major CYP isoenzymes as CYP1A1, CYP1A2, and CYP3A4, their biotransformation produces a highly reactive AFB_1-8,9-exo-epoxide and 8,9-endo-epoxide that can form adducts with DNA and RNA. In addition, CYP1A1 which also participates in AFB_1 transformation into epoxide, represents the highest fraction of extrahepatic CYP. After binding to DNA, the predominant 8,9-dihydro-8-(N7-guanyl)-9-hydroxy AFB_1 (AFB_1-N7-Gua) adduct is formed. AFB_1-N7-Gua may be converted to two secondary lesions, the one with apurinic site and the other with a more stable ring opened AFB_1-formamidopyrimidine (AFB_1-FAPY) adduct; the latter far more persistent *in vivo* than AFB_1-N7-Gua. Sufficient evidence from animal and epidemiological studies, such as the demonstration of a specific mutation in the *TP53* gene, led to the AFB_1 classification as a human carcinogen by the International Agency for Research on Cancer (IARC) (Hendricks, 1994; IARC, 2002; Krska et al., 2008; Valencia-Quintana et al., 2014). Among mycotoxicosis described in humans, aflatoxicosis is of the greatest concern; repetitive aflatoxin outbreaks were reported in recent years in Kenya, India, and Malaysia, often accompanied by fumonisins (Shephard, 2004; Lewis et al., 2005). In acute mycotoxication, it can cause impaired growth in children,

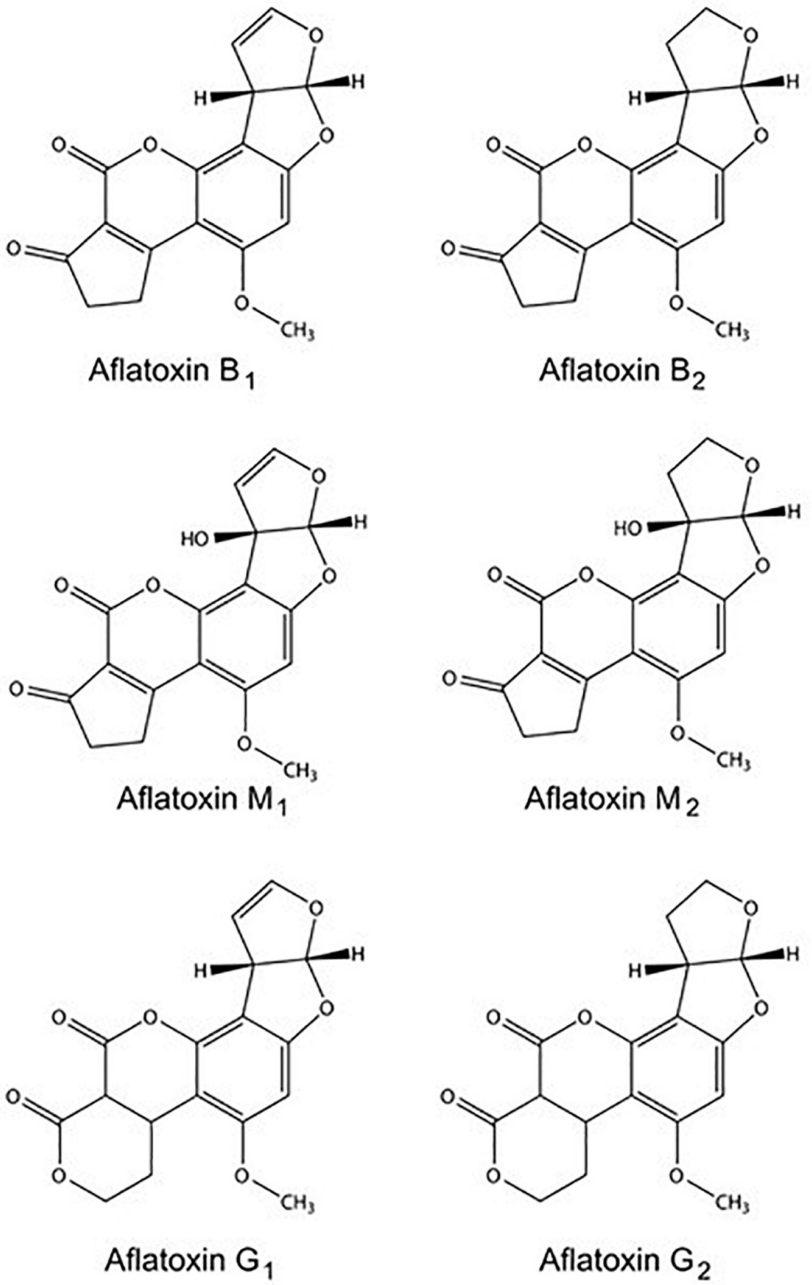

FIGURE 1 | Molecular structures of several aflatoxins.

vomiting, abdominal pain, pulmonary edema and liver necrosis (Valencia-Quintana et al., 2012).

Because of its planar structure, it could act as activators for the AhR, at the moment there is not evidence to prove that any mycotoxin is an agonist of AhR. That was first seen in H4IIE cells; AFB_1 induced an increase in CYP1A activity and CYP1A transcription, which was associated with an enhanced AhR activity, suggesting AhR pathway activation as a toxicity mechanism of AFB_1 (Mary et al., 2015). The major human cytochrome P450 (CYP) enzymes involved in aflatoxin metabolism are CYP1A1, CYP3A4, 3A5, 3A7, and 1A2 (Tan et al., 2004; Marin et al., 2013). The CYP enzymes activation is an indirect evidence of activating AhR pathway. It is known that AFB_1 causes carcinogenic, mutagenic and teratogenicity effects

(Krska et al., 2008; Zhang et al., 2015), as well as immunotoxicity, hepatotoxicity and even death in farm animals and humans (**Table 1**). The AFB_1 causes micronucleus (MN), sister chromatid exchanges (SCE), unscheduled DNA synthesis, and chromosomal strand breaks as well as adducts in rodent and human cells (Turkez and Geyikoglu, 2010). The mechanism of AFB_1 cellular damage has not been completely elucidated. Reactive oxygen species (ROS) and lipid peroxidation (LPO) have been considered to be an important mechanisms in the toxicity. The ROS and LPO are created via AhR activation and its target genes as several CYPs, already mentioned above. In the following sections, this review will describe how the AhR pathway participates via cytochrome activation and the ROS production.

Alternaria Toxins

The *Alternaria* genus is able to produce different mycotoxins, *A. alternata* is considered to be the main mycotoxin producer. *Alternaria* mycotoxins include a small percentage of more than 70 phytotoxins, and only few have been physicochemically characterized (Dall'Asta et al., 2014). They belong to three different structural groups: the dibenzopyrone derivatives, alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT); the perylene derivatives altertoxins (ATX-I, -II, -III); and the tetramic acid derivatives, tenuazonic acid (TeA) and iso-tenuazonic acid (iso-TeA) (Schrader et al., 2001; Ostry, 2008). The chemical structures of these mycotoxins are presented in **Figure 2** (Zain, 2011).

Alternariol monomethyl ether (AME) and alternariol (AOH) are not very toxic, but exert mutagenic, genotoxic and estrogenic activity (Schrader et al., 2001; Fehr et al., 2009; Dall'Asta et al., 2014), and also inhibit topoisomerase I and II (Fehr et al., 2009; Schreck et al., 2012; Dall'Asta et al., 2014). These toxins are planar benzopyrones that are quite similar to benzo(a)pyrene [B[*a*]P], a polycyclic aromatic hydrocarbon, and are metabolized by CYP1A1 and 1A2 cytochromes, which generate epoxides with marked activity on DNA and protein (Schreck et al., 2012). AOH and AME induce DNA strand breaks in cell lines (Pfeiffer et al., 2007), unscheduled DNA synthesis in cultured human amnion FL cells, chromosomal aberrations and sisters chromatid exchange in human peripheral blood lymphocytes, mutation in V79 cells and transformation of NIH 3T3 cells (Brugger et al., 2006; Schobert and Schlenk, 2008; Pahlke et al., 2016). These two toxins are AhR activators in mouse hepatoma cells (Schobert and Schlenk, 2008; Schreck et al., 2012; Pahlke et al., 2016). At very low concentrations altertoxin-II induces CYP1A1, which results in the production of ROS (Pahlke et al., 2016) that can cause DNA damage. However, DNA damage does occur, likely due to the generation of direct DNA adducts or the ROS induction, independently of cytochrome activity (Schreck et al., 2012; Pahlke et al., 2016). TeA is considered to be the most toxic among the *Alternaria* mycotoxins. It inhibits protein synthesis and is biologically active, exerting cytotoxic, phytotoxic, antitumor, antiviral and antibiotic effects (Schobert and Schlenk, 2008; Siegel et al., 2010), and has been deemed responsible for the outbreak of "onyalai" disease (Siegel et al., 2010). Also *Alternaria* toxins in grains might be responsible for esophageal cancer and other diseases (Dall'Asta et al., 2014; **Table 1**). Still, little is known about the mechanisms of action of *Alternaria* toxins.

FUMONISIN B_1

Since their discovery in 1988 (Gelderblom et al., 1988), fumonisins have been the subject of numerous toxicological investigations (Voss et al., 2007). Fumonisins are a group of mycotoxins with a strong structural similarity to sphinganine, the backbone precursor of sphingolipids, and are produced primarily by *Fusarium* species commonly associated mainly with cereal grains *Fusarium verticillioides*, *F. proliferatum* strains (García and Heredia, 2006; Marin et al., 2013). These toxins have been epidemiologically and experimentally associated with human esophageal cancer (Cortez-Rocha et al., 2003) and birth defects due to interference with cellular folate uptake (Stevens and Tang, 1997; Voss et al., 2007). FB_1 is a cancer promoter, but a poor cancer initiator; based on toxicological evidence, the IARC has classified FB_1 as a possible human carcinogen (group 2B) (IARC, 2002). FB_1 co-occurred with AFB_1 in a high-incidence area in human primary hepatocellular carcinoma in China, suggesting that the mixture may be involved in the development of the disease (Li et al., 2001). Toxic properties in general and the symptoms related to human exposure to FB_1 are listed in **Table 1**.

From a toxicological perspective, fumonisin B_1 (FB_1) is the most important fumonisin (**Figure 3**). The chemical name of this mycotoxin is 1,2,3-propanetricarboxylic acid, 1,10-(1-(12-amino-4,9,11-trihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediyl)ester (European Food Safety Authority [EFSA], 2005), and it elicits a spectrum of toxicities likely mediated through mechanisms involving disruption of sphingolipid metabolism and sphingolipid-mediated processes (Voss et al., 2007). This mycotoxin inhibits ceramide (CER) synthase that catalyzes the acylation of sphinganine (Sa) and recycling of sphingosine (So), increases intracellular Sa and other cytotoxic sphingoid compounds. This imbalance is mainly responsible for the toxicity (Voss et al., 2007). Thus, alterations of Sa to So ratio in tissues, urine and blood have been proposed as potential biomarkers of FB exposure, but studies have not allowed an accurate validation (Solfrizzo et al., 2011). Also, the AhR activation was demonstrated when FB_1 was evaluated in H4IIE hepatoma cells. As a result, this activation initiated the CYP1A1 and CYP1A2 activity (Mary et al., 2015).

Ochratoxin A

Ochratoxin A (OTA) is a secondary metabolite produced by *Penicillium verrucosum*, many species of the *Aspergillus* section *Circumdati* (*A. ochraceus*, *A. steynii,* and *A. westerdijkiae* are most important regarding OTA contamination in agricultural products) and a few *Aspergilli* section *Nigri* (Samson et al., 2007; Visagie et al., 2014). OTA (**Figure 4**) is recognized as a potential human health hazard (**Table 1**) associated with kidney disease in humans (García and Heredia, 2006) and has been classified as a possible human carcinogen (Tangni et al., 2002). Structurally, it is a phenylalanyl derivative of

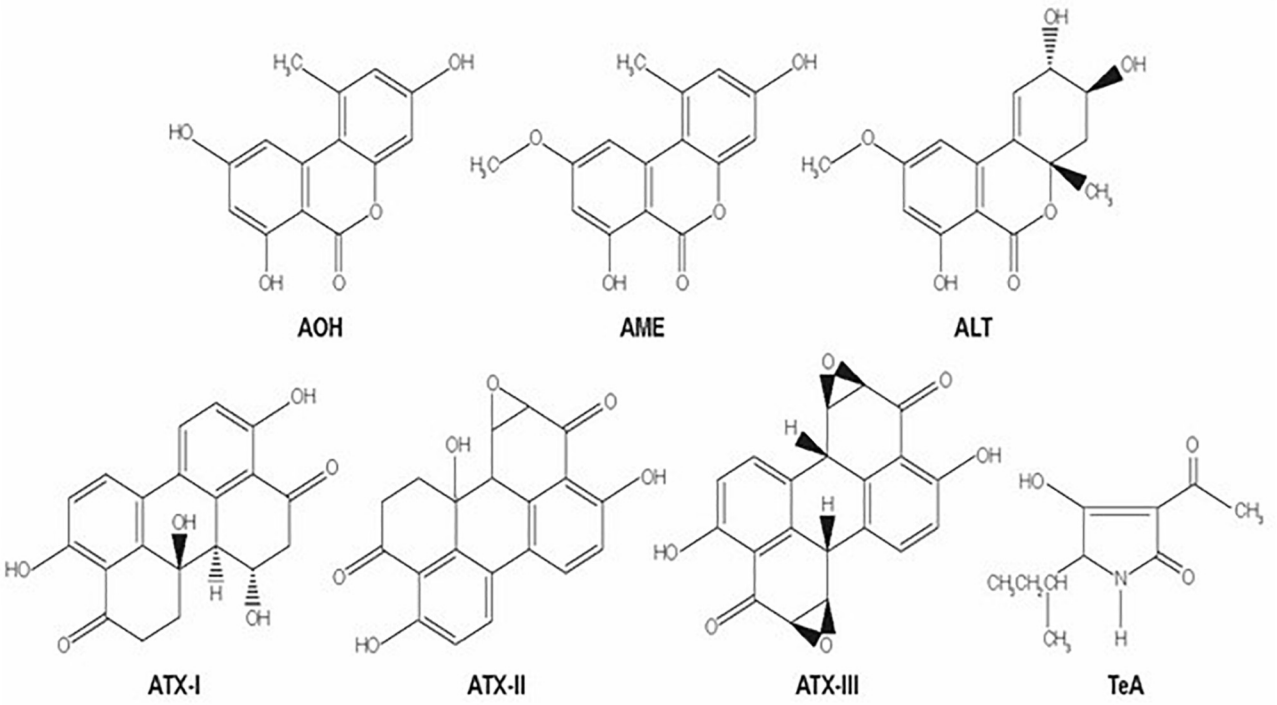

FIGURE 2 | Molecular structures of several *Alternaria* mycotoxins: alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT); the perylene derivatives altertoxins (ATX-I, -II, -III); and the tetramic acid derivatives, tenuazonic acid (TeA).

a substituted isocoumarin(R)-N-(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-y1)-carbonyl)-L-phenylalanine (Marin et al., 2013). Several mechanisms of OTA toxicity have been proposed including DNA damage coupled with forming direct DNA adducts via quinone formation and a network of interacting epigenetic mechanisms including inhibition of protein synthesis, oxidative stress, mitosis disruption and activation of specific signaling pathways (Mally, 2012; Pfohl-Leszkowicz and Manderville, 2012; Vettorazzi et al., 2013). In primary human hepatocytes cultures, it induced an increase in the transcription of the AhR gene and, thus, also CYP1A1 and 1A2 cytochromes gene transcription (Ayed-Boussema et al., 2012b).

Patulin

Patulin (PAT) (4-38 hidroxi-4H-furo(3,2-c)piran-2(6H)-ona) is mainly produced by *Penicillium expansum*, but other Penicillia can produce it including *P. crustosum, P. patulum* as well as *Aspergillus clavatus* (Samson et al., 2009; **Figure 5**). Apples, apple-based products and other fruits are major commodities contaminated with PAT and are responsible for PAT human exposure (Piqué et al., 2013). Animal model studies obtained convulsions, agitation, ulceration, edema, intestinal inflammation, vomiting and DNA damage in brain, liver and kidneys upon PAT exposure (McKinley and Carlton, 1980; de Melo et al., 2012). Chronic toxicity is related to neurotoxic, immunotoxic, genotoxic, and teratogenic effects in rodents (Boon et al., 2009). However, based on a lack of toxicological human data, the IARC has classified PAT in Group 3 as a non-human carcinogen (IARC, 2002).

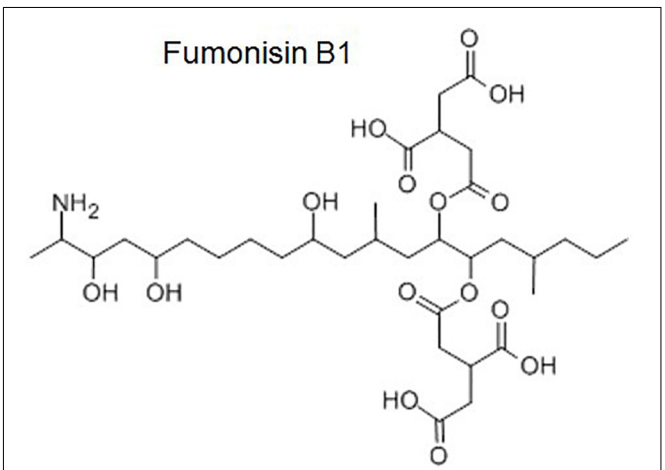

FIGURE 3 | Molecular structure of fumonisin B1.

PAT biotransformation has been poorly investigated; increase in CYP P450 content was reported in male mice and no increase was seen in the metabolic activity in human cells previously immortalized and transfected with human CYP 450 genes (Siraj et al., 1980; Lewis et al., 1999). The study that investigated involvement of AhR pathway in PAT toxicity is discussed

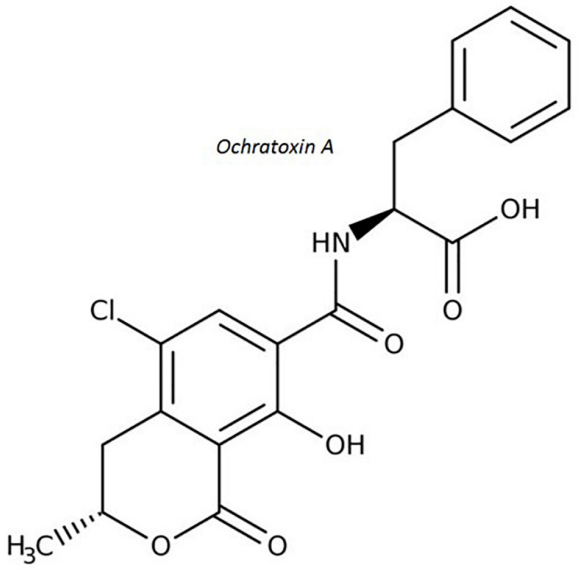

FIGURE 4 | Molecular structure of ochratoxin A.

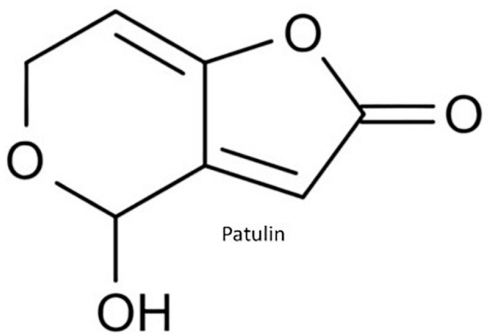

FIGURE 5 | Molecular structure of patulin.

later on in this review (section Activation of AhR Pathway by Other Mycotoxins).

THE ROLE OF THE ARYL HYDROCARBON RECEPTOR: HISTORICAL ASPECTS

The study of the AhR reveals a two-dimensional history, each with its own characteristics. One is a reality of our time; namely, exposure to synthetic organic compounds and its consequences. Back at the beginning, in the 1970s, many toxicologists and, later, biochemists and molecular biologists, centered their attention on the toxicological implications of exposure to the halogenated polycyclic hydrocarbon 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which was identified as a contaminant in the process of producing 2,4,5-trichlorophenol, a herbicide to which the workers who participated in its synthesis were exposed to (Schultz, 1957). After exposure, those workers suffered chloracne and *porphyria cutanea tarda* (Kimmig and Schulz, 1957). Later, studies proved that TCDD can elevate the expression of the δ- aminolevulinate synthase enzyme that participates in the heme biosynthesis (Poland and Glover, 1973). The second aspect is a rather serendipitous finding that appeared for reasons unknown at that time. In the early 1950s, studies observed that treating rats with the carcinogen 3-methylcholanthrene inhibited the hepatocarcinogenicity of 3′-methyl-4-dimethylamino azobenzene (Richardson et al., 1952). Afterward, it was proven that this carcinogenicity-inhibiting activity can be induced as well by other polycyclic aromatic hydrocarbons (PAH), such as B[*a*]P. Thus, it was demonstrated that these PAH can block the activity of an enzyme that modifies carcinogens, and it was suggested that, due to the constant use of B[*a*]P, it should be called B[a]P hydroxylase (Conney et al., 1957), which today corresponds to CYP1A1 hepatic cytochrome. Back then, in the 1960s, it was determined that the enzyme B[*a*]P hydroxylase, renamed at that moment as aryl hydrocarbon hydroxylase, or AHH, was inducible in some, but not all, syngeneic strains of mice (Nebert and Bausserman, 1970; Nebert et al., 1982). This response suggested the existence of a gene and AHH locus, denominated *Ah*, which is expressed, together with the enzyme, in the C57BL/6 strain, and corresponded to the Ah^b allele. Mice of the DBA/2 strain were not responsive, and that corresponded to the Ah^d allele (Nebert et al., 1972, 1982). Later, the role of the *Ah* locus was demonstrated in regulating the carcinogenicity, mutagenicity, teratogenicity and toxic responses to the PAH, based on inducing AHH activity (Nebert, 1986).

This provided the opportunity to test the inductive strength of AHH for TCDD and 3-methylcholanthrene, and subsequent research demonstrated that TCDD was 30,000 times more potent in AHH enzyme induction (Poland and Glover, 1974). As a result, this became the ideal test molecule for measuring all metabolisms of xenobiotic compounds in that period. Thanks to this, strains with phenotype Ah^d to 3-methylcholanthrene could be made responsive to TCDD (Poland and Glover, 1974). At that time, the study of steroid receptors was also in its apogee and researchers pondered the existence of a "receptor" with smaller affinity for 3-methylcholanthrene and a greater TCDD affinity, from the *Ah* locus that induces AHH (Yueh et al., 2003). Once the first radioactively-marked TCDD was synthetized, in 1976, the existence of a fraction bonded to the cytoplasm and another to the nucleus was successfully demonstrated by the differential fractioning method. Similarly to what occurred with the steroid receptors, once the TCDD bonded to its receptor, it was translocated to the nucleus with its *Ah* receptor (Poland et al., 1976). The weight of the receptor varied if it was isolated from the cytoplasm and, in fact, was heavier than that found in the nucleus (Gasiewicz and Bauman, 1987). This raised suspicions of the possible presence of other protein compounds. It was then demonstrated that induction of AHH activity requires the formation of the TCDD-AHR compound (Jones et al., 1984). In the 1980s, the nucleotide sequence to which the TCDD-AHR compound bonded on the AHH gene was identified, and it was confirmed that it corresponded to a gene of the group of enzymes denominated cytochromes, in

this case *Cyp1a1*. This sequence was named the dioxin response element (DRE) (Jones et al., 1985), which was shown to have the ability to activate expression of heterologous genes. At first, its existence was demonstrated in mice, but later in rats and humans as well. In another part of the world, in Japan, studies were being conducted, not regarding the response to TCDD, but to other xenobiotic compounds, and those reports found the same sequence where the TCDD-AHR compound should bond, though they called it the xenobiotic response element (XRE) (Fujisawa-Sehara et al., 1988), this sequence is: 5′-TNGCGTG-3′ (Pohjanvirta, 2011). Biochemical evidences continue to reveal differences between the molecular form of AhR found in the cytoplasm (i.e., heavier) and the one existing in the nucleus. This latter finding suggested the presence of another, very similar, weight unit. This led to the clonation and determination of the AhR nuclear receptor translocator (ARNT), a protein that forms the complex with the receptor and generates the bond with TCDD (Hoffman et al., 1991). First, the cloning of the AhR gene in humans in 1994 made it possible to prove that the initial enzyme, AHH, corresponded to the cytochrome CYP1A1. After that, researchers undertook explorations of its expression in a large number of tissues as well as in other organisms (Pohjanvirta, 2011). This information then made it possible to look for its expression in different pathologies, such as those characterized by high *Cyp1a1* activity, which suggested a "cross-talk" in different pathways that run from the one in charge of metabolizing and eliminating toxic xenobiotic compounds to the alteration of such processes as the response to steroid hormones in organs like the ovaries and mammary glands (Kociba et al., 1978). From that moment forward, expression of *Cyp1a1* and *Cyp1b1* in response to the application of natural compounds, drugs, other PAHs, and other compounds in general such as mycotoxins, could indirectly reflect the activation and/or participation of the AhR pathway, as shown below, in a wide variety of processes, including proliferation, death/apoptosis, differentiation, cellular adhesion, and drug-expulsing proteins (Pohjanvirta, 2011).

Finally, we now know that this molecule that emerged initially from the response to toxic compounds, providing on this way a linear explanation of toxicology, specific "only" to this response, is actually a protein that is a master regulator situated above all general cellular processes, including proliferation, differentiation, cellular adhesion, death and others that will surely be described in the years to come.

GENERAL CHARACTERISTICS OF THE AHR RECEPTOR

Organization of the Gene

In order to know and understand better the gene function, it is important to focus the analysis on the sequence organization, from the promoter zone to the codifying region. Before beginning to describe its important molecular and sequential characteristics, it is vital to mention that the AhR gene provides a clear example of the evolution of proteins in organisms. Genes homologous to this receptor exist, from *Caenorhabditis elegans*, called AHR-1, which also possesses a protein that forms a pair with AHR-1 and AHA-1, which is the homolog of ARNT. Also, other organisms, like *Drosophila melanogaster*, have a *Spineless* that is homologous to human AhR; and *Tango* which is homologous to human ARNT. Gene conservation during evolution in pluricellular organisms has an element in common related to the differentiation processes in the early stages of development (Pohjanvirta, 2011). Detailed information related to what occurs in humans is presented below.

Thanks to the cloning of the close promoter region (Eguchi et al., 1994), it is important to mention that one of the principle characteristics is that this promoter is without a TATA box, which characterizes the promoters of specific tissue genes, and has three *cis* GC sites that act as bonding sites to the factor *trans* Sp1; a feature of gene promoters that have a constitutive expression. It also possesses a *cis* bonding site to CRE factors, and another E-box type site for binding to Myc. This analysis is a product of the study of the sequence -468 to -911 close to the promoter. The human gene promoter is longer than the mouse one and therefore generates a longer complete gene (promoter plus codifying region) of 6.6 pb vs. 5.5 pb, respectively. Harper et al. (2006) analyzed the presence of these *cis* binding sites to other transcription factors, at -5000 pb over the promoter sequence. Their results showed multiple *cis* sites to the factor *trans* HNF (hepatic nuclear factor) that numbered approximately 23 (Kaestner et al., 1994). There were also 2 *cis* DLX3 regions (Distal-less 3), to which this home box factor binds and functions as a placenta-specific transcriptional regulator (Morasso et al., 1999). In addition, it contains 11 BRN3-like sites that serve to bind to transcription factors of the POU family specifically expressed in the nervous system of mammals in development and in adult state (Korkalainen et al., 2005). Moreover, this complex participates in the differentiation and survival of motor and sensory neurons (McEvilly et al., 1996). There are also four *cis* sites to STAT6 (a signal transducer and transcription 6 activator) which belong to the family of the transcription factors that respond to cytokines such as IL-4, and to growth factors like TGF-β1 (Tanaka et al., 2005). In addition, there are 7 *cis* regions that bind to factors such as Tcf/Lef, other transcription factors that realize their nuclear response to Wnt signaling by interacting with β-catenin (Eastman and Grosschedl, 1999). Finally, there are 11 *cis* binding sites to potential nuclear receptors of the progesterone-, androgen-, glucocorticoid-, proliferation-activated peroxisome-, the farsenoid X-, and vitamin D- receptors (Harper et al., 2006). This full information on the *cis* sites inside the complete promoter makes it possible to see and understand the constitutive expression and specific tissue of the receptor, while also allowing us to comprehend one additional characteristic: the fact that it is a constitutive gene with ubiquitous expression; due to the important biological functions it fulfills (Hahn, 2002). It is especially critical during embryogenesis and fetal development in the first post-natal stages, after which its levels decrease significantly. This suggests that its role is required less in the final phases of an organism's growth (Harper et al., 2006) and also allows for the explanation of the broad responses observed in several tissues and organs where xenobiotic compounds such as the aflatoxins, exert their

hepato-, nefro- and neurotoxic activity as well as mutagenic and carcinogenic properties.

The open reading frame has 12 exons, which are organized and formed in the mature messenger and the important domains of the protein, as it will be described below.

Important Domains of the Protein

The detailed information regarding the domains present in the protein was obtained principally by cloning mouse genes. The mouse's peptic sequence revealed the presence of 805 residues of amino acids organized in domains present in those bHLH-PAS transcription factors (Burbach et al., 1992). These factors contain a binding region to DNA basic Helix-Loop-Helix (bHLH) and a pair of domains that have been found in the Per proteins (a factor that participates in regulating the circadian cycle), in ARNT (i.e., the protein with which AhR realizes heterodimerization) and, finally, with a protein (that participates in neuronal development in *Drosophila*) called "single-minded," or Sim, which gives this domain its name: PAS (Pohjanvirta, 2011). Analyses based on the terminal amino domain show a region of basic amino acids residues, followed by the hHLH motif. The basic residues region is important for the AhR interaction with the response element *cis* sequence to the aryl hydrocarbons, ARE; while the hHLH motif is important for the realization of heterodimerization between AhR and Arnt. Ahead of this hHLH motif there are 2 repeated inversions regions of 51 amino acids residues that are also found in the PAS factors described above. These motifs perform multiple functions, such as directing the AhR union with 2 Hsp90 molecules and with the protein AIP (AhR interacting protein), or XAP2 (X-associated protein 2) in the cytoplasm (Ma and Whitlock, 1997), the bond to the ligand and the interaction of AhR and Arnt in the nucleus (**Figure 2**). The carboxy terminal contains three domains for the transcription activation (AT) that contain residues of amino acids that are acidic and rich in serine/threonine and glutamate. Between the PAS B and AT domains there is a small sequence of approximately 81 residues of amino acids that exerts an inhibiting activity on AT and so has been named the inhibiting domain (ID) (Ma et al., 1995).

Associated Proteins and Formation of the AhR Complex

One must recall that, historically, the study of the AhR was always conducted based on the results observed during exposure to, and interaction with, TCDD. The AhR in the cytosolic fraction was shown by ultracentrifugation to have a 9S sedimentation value. Upon adding the ligand, i.e., TCDD– this value decreases to 6S and, moreover, is found in the nuclear fraction (Pohjanvirta, 2011). These differences revealed the existence of two different forms in the two cellular compartments. In addition, electrophoretic studies demonstrated that this weight difference was due to the fact that the receptor protein in the cytoplasm is found in complex tetrameric compounds by that same receptor and three proteins with molecular weights of 96, 88, and 37 kDa, the first two corresponding to two isoforms of mouse HSP90 (Chen and Perdew, 1994). The protein XAP2 (X-associated protein 2) (Meyer et al., 1998), as well as AIP (AhR-interacting protein) (Ma and Whitlock, 1997) or ARA9 (AhR-associated protein 9) (Carver et al., 1998) is the one that corresponds to molecular weight 43–37 kDa (**Figure 6**). It has been shown that the interaction between Hsp90 and the AhR takes place in the PAS-B motif, and that this interaction is required to create the conformational change that allows binding with the ligand. The protein AIP has three repeated sequences of tetratricopeptides that allow protein-protein interaction (Schreiber, 1991). This explains why the receptor protein in the cytoplasm is more stable and has a half-life of approximately 28 h. This data corresponds to the earlier description which indicated that the onset of the induction of CYP1A1 does not require protein synthesis. After treatment with a ligand (TCDD or B[*a*]P), the receptor's half-life is reduced to just 3 h (Ma and Baldwin, 2000). Afterward, the AhR protein suffers degradation by the 26S proteasome (Pohjanvirta, 2011), which takes place in the nucleus (**Figure 6**), an important site for the degradation of other transcription factors in addition to AhR (Roberts and Whitelaw, 1999), such as TGF-β1 (Nebert, 1986; Lo and Massagué, 1999) and MyoD (Floyd et al., 2001).

THE CANONICAL PATHWAY OF AHR ACTIVATION THROUGH INTERACTION WITH ARNT

In order to explain the canonical pathway of AhR activation (**Figure 2**), the description must be based strongly on the activation of the genes studied that participate in the detoxification mechanism, or in phase I and phase II of the xenobiotic compounds metabolism such as the cytochromes

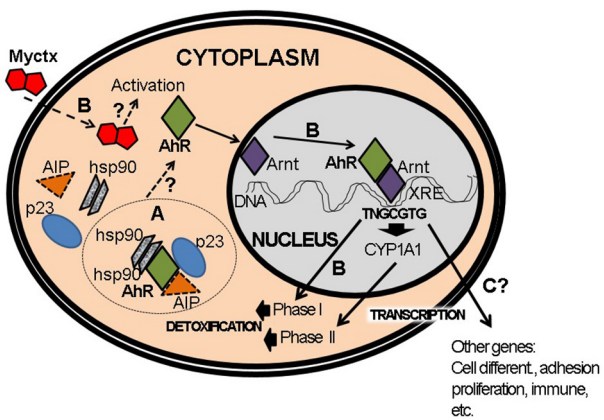

FIGURE 6 | Activation of AhR pathway by mycotoxins (Myctx). (A) The AhR is a protein that interacts with hsp90, AIP, and p23 proteins in cytoplasm. (B) Under activation by Myctx, the transcription of phase I and phase II genes is started in order to metabolize and detoxify. It is not proved if the Myctx are ligands/agonists of AhR, which is indicated by a question mark (?). (C) This activation can also start transcription of several genes such as those involved in cell differentiation, adhesion, proliferation and immune response, but in the case of Myctx these have to be proved if it is performed as ligands/agonists (?).

Cyp1a1 and *Cyp1a2*. This history starts with the binding of the ligand to the AhR, that binds and causes a conformational change in the PAS A domain which enables demonstration from the residues of amino acids 55–75 to a nuclear localization signal, allowing its translocation to the nucleus (**Figure 2**). As mentioned previously, it dissociates in the cytoplasm of the complex of 2 molecules of Hsp90, and translocates to the nucleus in a process fostered by importins (Ikuta et al., 1998). Once inside the nucleus, AhR is dimerized with another protein from the same family as factors bHLH-PAS, denominated Arnt. Arnt is only localized in the nucleus and this protein is also organized in domains similar to those that contain AhR, i.e., toward the amino terminal end we find the bHLH domain and the nuclear localization signal, followed by the PAS A and PAS B domains (**Figure 2**). Finally, the activation domain is contained toward the carboxyl terminal end (Pohjanvirta, 2011). The dimerization between AhR and Arnt is realized through the HLH regions (Reisz-Porszasz et al., 1994; Fukunaga et al., 1995) and is stabilized by a conformational change in the PAS A region (Soshilov and Denison, 2008). Observations have shown that modifications by phosphorylation occur for the binding function to the DNA via the protein kinase C (Mahon and Gasiewicz, 1995). Binding to the ARE *cis* site in the promoter sequences takes place with the consensus sequence 5′-TNGCGTG-3′, which is seen in many genes, such as cytochrome CYP1A1, which contains 8; CYP1A2, which contains 1, and CYP1B1, which contains 3 sequences (Pohjanvirta, 2011). For this reason, it is said that these genes are regulated by the canonical pathway and that CYP1A1 is the model gene for the study of the response to AhR. In the context of the binding, AhR binds to the middle of the 5′-T/NGC site, while Arnt binds by occupying the second half of 5′GTG (Bacsi et al., 1995). We also know that a series of nucleotides located near the ARE are important because its *cis* element contains the optimal sequence: GGGTGNAT(C/T)GCGTGACNNCC. This sequence establishes contact with the bHLH domain of the AHR/ARNT heterodimer (Chapman-Smith and Whitelaw, 2006). We also know that the promoter has a second sequence or response element to AHR II, whose sequence is CATG(N)C(T/A)TG, and which is conserved in humans, rats and mice, but does not seem to influence the canonical response to ARE's action on the *cis* site (Pohjanvirta, 2011). Thanks to the exhaustive studies that have been conducted on the regulation of the expression of the cytochromes during AhR activation, it was possible to identify various genes that have AREs (Gasiewicz et al., 2008), which can be grouped as follows:

1. Phase I genes of the metabolism of xenobiotics: CYP1A1, CYP1A2, CYP1B1, CYP2A5, CYP2S1, and CYP4B1.
2. Phase II genes of the metabolism of xenobiotics: aldehyde dehydrogenase 3A1, glutation-S-transferase, NAD(P)H- quinone oxidoreductase-1, UDP glucuronosyltransferase 1A1, and 1A6.
3. Cell cycle suppressors: p21, p27.
4. Cell cycle activators: c-jun, s-myc, junD, Insulin-like growth factor binding protein-1.
5. Others: Bax, cathepsin D, cyclooxygenase -2, epiregulin, filaggrin, Slug, etc.

There is also one exceptional case; that of poly/ADP-ribose polymerase, which contains 16 XRE *cis* sequences (Ma, 2002). One aspect of the consequences of cytochrome activation is the generation of modifications of the xenobiotic compounds that induce their activation and achieve control of the receptor activation while reducing ligand concentrations in the cell (Gu et al., 2000). All currently available evidence demonstrate that the AhR protein can function as an exogenous ligand sensor, and that these compounds appeared only recently in the human ecosystem such as polycyclic aromatic hydrocarbons (the technosphere). Thus, it can be said that the canonical pathway of AhR-Arnt may well be activated as a response to the presence of toxic compounds produced by humans, and that thanks to a process of conserved evolution, the AhR protein – belonging to a group of proteins known to be environmental sensors – is a response mechanism with the function to detoxify the organism from those "new" compounds that are foreign to nature. In this context, activation of the canonical pathway by AhR-Arnt is separated from its physiological role, in which surely many endogenous compounds perform functions less related to toxicity, and much more focused on the processes of development and differentiation of mammals, since this receptor and this group of PAS proteins also play important roles in development, a function in which AhR perhaps operates initially in the early stages of mammals, as mentioned above.

The Non-canonical AhR Pathway

In the past 10 years, the development of research analyzing the microarrays of genes in samples of the AhR from the organs of knockout mice, and cell lines principally from the liver, and their comparison with WT mice to AhR and in normal lines of gene expression, have revealed that in the absence of ligands exogenous to the receptor exists an expression of genes for which, upon analyzing the *cis* regions of some promoters using chromatin immunoprecipitation (or CHIP), it was possible to show that they present sequences distinct from those of ARE described in the canonical pathway, which have been denominated as non-consensus response elements to xenobiotics (NC-XRE) (Peters et al., 1999; Dere et al., 2011). As a result, the non-canonical AhR pathway refers to the expression of genes which promoters even in the presence of some ligand, such as TCDD, do not contain the characteristic *cis* element of the ARE. One of these genes is the plasminogen-1 activator inhibitor (PAI-1) (Tijet et al., 2006). Later studies corroborated that treatment with TCDD suppresses hepatic regeneration, and we now understand that this is due to PAI-1 inhibition of the urokinase-type plasminogen activator that is necessary for the activation of the hepatic growth factor (Mitchell et al., 2006). The characteristic of these promoters is that they contain a repeated tetranucleotide: 5′-GGGA-3′. In the case of the PAI-1 promoter, it is the second NC-XRE that is important for the binding of AhR. One important feature is the absence of interaction with Arnt in this gene activation (Huang and Elferink, 2012). It has also been proven that suppression of hepatic regeneration is realized by stopping cell proliferation by inducing a delay in phase G_1 by inhibiting the activity of kinase 2-dependent cyclin (CDK2) (Mitchell et al., 2006). This is dependent on the increase in the expression of kinase-dependent

cyclin inhibitors such as p21 and p27. These inhibitors negatively regulate the progression of the cell cycle by controlling CDK2, CDK4 and CDK6 (Harper et al., 1995). Later, through analyses of the p21 promoter sequence, it was discovered that they contain NC-XRE *cis* regions (Jackson et al., 2015). Also, an endogenous ligand must exist that allows, in the absence of a bond to some ligand, expression of AhR-dependent p21, as was proven in the hepatic regeneration model through partial hepatectomy (Jackson et al., 2014).

Recent evidence demonstrates that the Kruppel-like factor (KLF) forms a heterodimer with the AhR receptor (Wilson et al., 2013). Structurally, the carboxy terminal of AhR is where the bHLH and PAS A domains are found that must interact with the amino terminal end of KLF6 (Wilson et al., 2013). The KLF6 factor regulates numerous cellular processes that participate in differentiation, proliferation and apoptosis (Philipsen and Suske, 1999). Alterations to the KLF6 gene are associated with various types of cancer, including astrocytomas and gliomas (Jeng and Hsu, 2003). The KLF6 factor also activates expression of p21, forming the heterodimer, which is part of the activation of the non-canonical pathway of AhR activation (Andreoli et al., 2010). The KLF6 factor induces an increase in the expression of the E-cadherin genes, transforming the growth factor and the insulin-like growth factor 1 receptor (Rubinstein et al., 2004).

IMPLICATION OF THE AHR PATHWAY IN TOXICITY OF AFLATOXINS AND SOME OTHER MYCOTOXINS

It is important to understand, firstly, that the proposal for this review arose because so little has been written on this topic up to date. Secondly, we must be aware that the reports that do exist are mainly related to AFB_1 (Ayed-Boussema et al., 2011, 2012a; Merrick et al., 2013; Mary et al., 2015), and few to *Alternaria* toxins (Pahlke et al., 2016), ochratoxin A (Ayed-Boussema et al., 2012b), fumonisin and patulin (Ayed-Boussema et al., 2011, 2012a; Mary et al., 2015). All of these studies have demonstrated the activation of members of different families of CYP during transcription (Ayed-Boussema et al., 2012b; Mary et al., 2015; Pahlke et al., 2016), enzymes with UDP-glucuronidase activity (Fleck et al., 2012; Hanioka et al., 2012), and the role played by such receptors as pregnane X (PXP), and the constitutive androstane (CAR), retinoid X (RXR), glucocorticoids (RG), and Ah receptors (Fleck et al., 2012; Hanioka et al., 2012; Mary et al., 2015; Pahlke et al., 2016). Some of these studies also evaluated the cytotoxic and genotoxic potential of these toxins (Koliopanos et al., 2002; Ayed-Boussema et al., 2012a; Fleck et al., 2012). The following describe the key results that different working groups have obtained regarding aflatoxins and other mycotoxins and their relation to the AhR.

Activation of AhR Pathway by Aflatoxins

AFB_1 is a powerful hepatocarcinogen (Merrick et al., 2013). Due to its planar structure and activation of transcriptional expression of the CYP1A1 in H4IIE hepatoma cells, it was suggested to be agonist for the AhR, but at the moment the direct evidence is lacking (Mary et al., 2012). Increased expression has been observed in carcinomas of the pancreas and lungs (Koliopanos et al., 2002; Lin et al., 2003). This increase in its expression is also related to an increase in the invasion of gastric cancer cells. Consequences of the binding and activation of the AhR include the induction of free radicals and ROS (Mary et al., 2012). This seems to play an important role in carcinogenesis and the progression of carcinomas. In fact, their operation functions by reducing immunovigilance of the carcinogenic process because activation of the AhR affects the regulatory function of T CD4 + lymphocytes (Pot, 2012). The activation of AFB_1 to the AhR allows the activation of the rapid response of AhR and the activation of tyrosine kinases and the ERK and PKC1 signaling pathways (Tan et al., 2004; Schreck et al., 2012). In cultures of human hepatocytes, AFB_1 also increased the transcriptional expression of RXR due to the increased expression of the genes of CYP2B6 and CYP3A5 cytochromes, whose expression is regulated by this receptor (Ayed-Boussema et al., 2012b).

An analysis of genes differentially-expressed by RNAseq (transcriptome) from rat livers revealed an at least twofold increase, to a total of 1026 genes. These genes participate in the AhR, NF-E2-related factor 2 (Nrf2) and glutathione (GSH) pathways, the cell cycle of the metabolism of xenobiotic compounds, the extracellular matrix and cell differentiation (Merrick et al., 2013). Observations of the H4IIE hepatocellular carcinoma lines showed activation of the AhR and of the transcription of the CYP1A1 gene (Mary et al., 2015). Finally, in HepG2 hepatocellular carcinoma cells, a significant expression of UGTs of the families 1 and 2 was observed (Hanioka et al., 2012).

There are transcription factors (TF) associated to DNA damage response. After a combination of transcriptomic analyses and prediction of binding of TF, in HepG2 cells after exposure to 5μM of AFB_1, it was revealed that the canonical pathway is important in the response to modulate the DNA repair response by assessing the phosphorylation of H2AX histone (γH2AX), via ARNT. In the absence of ARNT, reduction in CYP1A1 expression and in γH2AX response demonstrated that canonical pathway of AhR is necessary for the DNA repair response after exposure to AFB_1 (Smit et al., 2017).

Finally, the efflux transporter ABCG2 plays an important role in the mammary excretion of toxins in humans. AFB_1 can interact with the ABCG2 transporter even at higher aflatoxin concentrations. This interaction produces an efflux effect of dioxin-like compounds such as PCB 126, but this function depends of AhR activation since it is inhibited by CH233191, an inhibitor of the AhR pathway. Therefore, this evidence opens new issues to research the role produced by the interaction between AFB_1 and the close action of the AhR pathway (Manzini et al., 2017).

Activation of AhR Pathway by *Alternaria* Toxins

Alternaria toxins, alternariol and its monomethyl ether are planar benzopyrones that are quite similar to B[*a*]P, compounds metabolized by CYP1A1 and 1A2 cytochromes, which generate epoxides with marked activity on DNA and

protein (Schreck et al., 2012). They have both mutagenic and genotoxic effects, induce micronuclei, and show estrogenic activity (**Table 1**; Fleck et al., 2012; Schreck et al., 2012; Pahlke et al., 2016). They are also related to the development of esophageal cancer and are inhibitors of DNA topoisomerases (Fehr et al., 2009). Only monomethyl ether stops the cells in G2/M. Apoptosis is induced at 48 h of exposure to both compounds (Schreck et al., 2012). These two toxins are AhR activators in mouse hepatoma cells (Burkhardt et al., 2012; Schreck et al., 2012; Pahlke et al., 2016). Also altertoxin-II, at very low concentrations, induces CYP1A1, resulting in the ROS production (Pahlke et al., 2016) that can cause DNA damage, independently of the AhR, since the siRNA of the receptor does not induce CYP1A1. However, DNA damage does occur, likely due to the generation of direct DNA adducts, or the ROS induction independently of cytochrome activity (Burkhardt et al., 2012). For this reason, it is important to evaluate, as well, whether they have the capacity to induce phase II enzymes of detoxification, such as glucuronidation, because the metabolites of these mycotoxins do not cause DNA damage. This could represent a strategy for reducing the toxic properties of these mycotoxins through conjugation with glucuronic acid.

Activation of AhR Pathway by Other Mycotoxins

So far, there have been only a few studies related to other mycotoxins, including OTA, FB_1 and patulin as activators of AhR. In primary cultured human hepatocytes treated with subcytotoxic OTA concentrations, significant upregulation of CYP3A4, CYP2B6, and, to a lesser extent, CYP3A5 and CYP2C9 was obtained. PXR mRNA expression increased in only 1 treated liver, whereas CAR mRNA expression was not affected. OTA induced an overexpression of CYP1A1 and CYP1A2 genes accompanied by an increase in AhR mRNA expression suggesting that OTA could activate both PXR and AhR in human hepatocytes (Ayed-Boussema et al., 2012b). Also, in human kidney cells HK-2 OTA activated both AhR and PXR by induction of the CYP1A1, CYP1A2, and CYP3A4 genes. The mRNA expression of phase II enzymes such as heme oxygenase-1, nicotinamide adenine dinucleotide phosphate-quinone oxidoreductase 1, and glutamate cysteine ligase catalytic subunit were upregulated by the activation of Nrf2. Nrf2 activation is directly induced by OTA-activated AhR and PXR, or indirectly by the adaptive response to ROS generated through metabolic processes between AhR, PXR-induced CYP enzymes, and OTA. Taken together, these studies indicate that OTA induces phase I and II enzymes through the activation of AhR, PXR, and Nrf2 signaling pathways, which may lead to cell injury (Lee et al., 2018).

Induction of CYP enzymes by FB_1 has been poorly investigated so far; but an increase of CYP1A activity in the liver of Wistar rats exposed to FB_1 has been confirmed (Martínez-Larrañaga et al., 1996). Recent study demonstrated that FB_1 applied alone caused a small increase in CYP1A activity and *cyp1A* gene transcription in H4IIE and rat spleen mononuclear cells, but to a lesser extent and duration than single AFB_1. However, *cyp1A* gene expression by FB_1 applied alone did not involve Ahr activation. On the other hand, a two-toxin mixture induced an overexpression of *cyp1A* and *ahr* genes in spleen cells indicating the interaction between both toxins in AhR pathway activation and subsequently in carcinogenesis (Mary et al., 2015).

In primary human hepatocytes, PAT induced an upregulation of PXR and CYP2B6, 3A5, 2C9, and 3A4 genes accompanied by AhR and CYP1A1 and 1A2 mRNA expression. Since this is the only study available in literature related to AhR activation by PAT, further studies should determine whether this mechanism could modulate PAT toxicity (Ayed-Boussema et al., 2012a).

CONCLUSION AND PERSPECTIVES

- Due to their toxic properties, aflatoxins and other mycotoxins are AhR activators and are also mutagenic and carcinogenic.
- As lipophilic compounds, they can easily cross cells biological membranes that are a part of the entrance pathway; for example, the intestinal wall. For this reason, they constitute as pro-carcinogenic molecules.
- Their activation as carcinogenic metabolites has centered on modification by mono-oxygenases, represented primarily by CYP1A1, that are expressed abundantly in the liver. Furthermore, the CYP1A1 promoter has 8 XRE-elements which are used by the AhR activation.
- The gene expression increase and the activity of such cytochromes such as CYP1A1 are important factors due to their activity, triggered by exposure to aflatoxins can go on for weeks or months, meaning that the carcinogenic process can be sustained due to the consequences of this activity.
- Aflatoxin metabolites are mutagenic and some produce adducts on the nitrogenous bases that can cause base changes and modifications in the methylation states of gene promoters and thereby establish a modified expression pattern. This area has not been yet fully explored.
- These metabolites also induce an increase in ROS that can cause damage to DNA, lipids or other proteins, and thereby initiate the process of carcinogenesis.
- Mutations can be produced in genes with important functions but they are also targets for mutagen modification such as p53 and K-Ras, among others.
- The increase in mRNA expression of the AhR is significant due to significant expression of this gene in liver and adipose tissue, with the latter potentially serving as a reservoir for lipophilic compounds like aflatoxins. It is therefore important to mention that in the AhR promoter there are several important *cis* elements which have an important function in interactions with *trans* factors. Some *cis* elements are: HNF (binding of the hepatic nuclear factor); DLX3 (regulatory placenta-specific); BRN2-like (binding of factors participating in neural development and adult stage in mammals, also performing differentiation of sensitive and motor neurons); STAT6 (responding to cytokines such as IL-4 and TGF-β1); Tcf/Lef (Wnt via/neurodevelopment). Binding sites to

potential nuclear receptors to the progesterone receptor, androgen receptors, glucocorticoid receptors, proliferation-activated peroxisome receptors, the farsenoid X receptor and the vitamin D receptor could explain the biological effects of these toxins. These *cis* elements partly enable the comprehension of the toxic effects caused by the activation of AhR, but this is a less studied area.

- Up to date, the AhR activation has been limited to groups of genes such as the cytochromes and UDP-glucuronidases. However, we know that AhR activation can participate in inflammatory responses, cell proliferation and differentiation, and the loss of cell adhesion. Thus, the implications of AhR activation by aflatoxins may be more serious than thought thus far.
- Certain polymorphisms can predispose tissues to cancer development, when they are exposed to aflatoxins. This field needs to be studied broadly since we know that at least some polymorphisms of enzymes like GCTP1, AKR, EPHX1, and EERC2 may participate in cancer development.
- The pathways that depend on the AhR activation have been explored, but there must be others, such as the non-canonical receptor pathway that participates in the toxic and carcinogenic effects by aflatoxins. At this point it is important to emphasize that AhR protein heterodimerizes with KLF6 and this complex can activate CYP1A1. Finally, KLF6 can regulate differentiation, proliferation and apoptosis.
- The study of the aflatoxins effects thus represents an area of opportunity in which diverse specialists, including researchers in omics, must work together in efforts to integrate the data that make their field of study so complex.

AUTHOR CONTRIBUTIONS

FA-H, RV-Q, and MS contributed mostly in final shaping and improving the manuscript, while the other authors also contributed in writing but also in designing the tables, figures, and correcting the manuscript with English and reference editing. All authors have contributed to the idea and form of the manuscript.

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Exposure of *Aspergillus flavus* NRRL 3357 to the Environmental Toxin, 2,3,7,8-Tetrachlorinated Dibenzo-*p*-Dioxin, Results in a Hyper Aflatoxicogenic Phenotype: A Possible Role for Caleosin/Peroxygenase (AfPXG)

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Aflatoxins (AFs) as potent food contaminants are highly detrimental to human and animal health. The production of such biological toxins is influenced by environmental factors including pollutants, such as dioxins. Here, we report the biological feedback of an active AF-producer strain of *A. flavus* upon *in vitro* exposure to the most toxic congener of dioxins, the 2,3,7,8-tetrachlorinated dibenzo-*p*-dioxin (TCDD). The phenotype of TCDD-exposed *A. flavus* was typified by a severe limitation in vegetative growth, activation of conidia formation and a significant boost in AF production. Furthermore, the level of reactive oxygen species (ROS) in fungal protoplast was increased (3.1- to 3.8-fold) in response to TCDD exposure at 10 and 50 ng mL^{-1}, respectively. In parallel, superoxide dismutase (SOD) and catalase (CAT) activities were, respectively, increased by a factor of 2 and 3. In contrast to controls, transcript, protein and enzymatic activity of caleosin/peroxygenase (AfPXG) was also significantly induced in TCDD-exposed fungi. Subsequently, fungal cells accumulated fivefold more lipid droplets (LDs) than controls. Moreover, the TCDD-exposed fungi exhibited twofold higher levels of AFB_1. Interestingly, TCDD-induced hyperaflatoxicogenicity was drastically abolished in the AfPXG-silencing strain of *A. flavus*, suggesting a role for AfPXG in fungal response to TCDD. Finally, TCDD-exposed fungi showed an increased *in vitro* virulence in terms of sporulation and AF production. The data highlight the possible effects of dioxin on aflatoxicogenicity of *A. flavus* and suggest therefore that attention should be paid in particular to the potential consequences of climate change on global food safety.

Keywords: dioxin, aflatoxicogenicity, aflatoxin, caleosin, peroxygenase

INTRODUCTION

Aflatoxins (AFs) are fungal lipid-derived toxins that provoke both acute and chronic toxicity in humans and animals. These toxins are produced by certain ascomycete fungi, most notably *Aspergillus flavus* and *Aspergillus parasiticus* (Yu et al., 2004; Shephard, 2008; Yu, 2012) and contaminate a range of fresh and stored food/feed products, therefore causing serious health,

economic and ecological troubles. Thus, AFs were evaluated by the International Agency for Research on Cancer (IARC) as Group-1 agents (IARC, 2002). Of these, aflatoxin B_1 (AFB_1) is considered as the most potent carcinogen contaminant identified to date, with hepatocellular carcinoma as a major risk factor (Yu et al., 2004; Yu, 2012).

From an ecological point of view, *A. flavus* is widely spread in different niches where environmental factors play crucial roles in the phenotyping of fungal aflatoxicogenicity. More particularly, fungal spores have a remarkable connection with soil where its physicochemical and biological properties are determinant modulators of conidia biogenesis. Interestingly, increasing attention has been recently paid to the possible adverse effects caused by climate change in connection with fungal aggressivity (Medina et al., 2014; Assuncao et al., 2018). As a possible consequence of climate change, persistent environmental pollutants could dramatically increase because of the increased incidence of large-scale forest fires that has occurred over the last decade. Such pollutants, namely polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), are considered the most toxic group of Persistent Organic Pollutants (POPs) (WHO, 2016). Due to their physicochemical properties, dioxins can persist in the environment and bioaccumulate in the organisms of a given ecosystem, including bacteria, fungi, plants, animals, and humans (Field and Sierra-Alvarez, 2008; Ishida et al., 2010; Anasonye et al., 2014; Hanano et al., 2014b).

The cytotoxicity of dioxins is expressed through their high lipophilicity which exercises a force driving on dioxins toward the cellular lipids, affecting therefore their metabolism and functions (Lawrence and Kerkvliet, 1998; Hanano et al., 2014a,b, 2018b; Cranmer-Byng et al., 2015). In connection with this, dioxin-induced alternations in lipid metabolism and oxidative status could have important feedback effects on the biology of AF-producing fungi in general and on their aflatoxicogenicity in particular. This is because aflatoxins are ultimately synthesized from acetyl-CoA via fatty acid polyketide under an excessive oxidative status (Reverberi et al., 2010; Fountain et al., 2018; Kenne et al., 2018). Beyond the modulatory role of reactive oxygen species (ROS), fungal lipids and their metabolites, more particularly a class of them know as oxylipins, can play as modulators of AF biosynthesis (Roze et al., 2007; Gao and Kolomiets, 2009; Fountain et al., 2014). The fungal oxylipin-biosynthesizing enzyme, the AfPXG, has been recently characterized as a caleosin with a peroxygenase activity and therefore referred to as Caleosin/Peroxygenase (Hanano et al., 2015). This AfPXG is necessary for fungal growth, development and AF production (Hanano et al., 2015). More recently, we presented detailed genetic, molecular and biochemical evidence on the direct implication of AfPXG in the biosynthesis of aflatoxins and their trafficking and extracellular secretion via lipid droplets (LDs) (Hanano et al., 2018a). A similar implication of fungal LDs in the sequestration and trafficking of lipid-soluble molecules has been also suggested in plants. The LDs isolated from oilseeds or date palm stones can sequester *in vitro* a variety of hydrophobic organic contaminants, with the greatest activity found for dioxins (Boucher et al., 2008; Hanano et al., 2016a). Likewise, the dioxin-sequestration activity of LDs was also demonstrated *in planta*, where the majority of experimentally administrated dioxins to Arabidopsis or date palm seedlings was found within LD fractions (Hanano et al., 2016b, 2018b). This high activity was based on the production of numerous small LDs, and this ensures the highest contact surface between the LDs and the dioxins (Hanano et al., 2016b, 2018b,c).

Based on the above, hydrophobic pollutants could have significant effects on the biology of AF-producing fungi and subsequently on their aflatoxicogenicity. To that end, we *in vitro* exposed the *A. flavus* NRRL 3357 to 2,3,7,8-tetrachloronated dibenzo-*p*-dioxin (TCDD), the most toxic congener of the dioxins group. Then, the toxicological effects of dioxins were characterized in terms of fungal growth, development and aflatoxin production. In a particular connection with the TCDD-induced accumulation of LDs, the transcripts and enzymatic activity of the caleosin/peroxygenase AfPXG were discussed using the wild-type (WT) and the AfPXG-silenced lines of *A. flavus*. Finally, the virulence of the TCDD-exposed *A. flavus* was assayed. This work highlights the biological effects on the aflatoxicogenicity of *A. flavus* following its exposure to the persistent environmental pollutant, the dioxin.

MATERIALS AND METHODS

Materials, Chemicals, Strains, Culture Conditions, and Treatments

Oligonucleotides were purchased from either Eurofins or Sigma-France. Aniline, cumene hydroperoxide, aflatoxin AFB_1 and all organic solvents were purchased from Sigma-Aldrich, Germany. The *A. flavus* strain NRRL3357 was supplied from the Faculty of Agricultural Sciences, Gembloux, Belgium. Stock cultures of *A. flavus* were maintained in slant tubes at 4°C on potato dextrose agar (PDA) (Difco Laboratories, United States). For solid or liquid cultures of *A. flavus*, stock cultures were transferred onto Petri dishes containing PDA or into a 500-mL Erlenmeyer flask containing 100 mL of PD broth and kept for 7 days at 28°C.

Biomass and Conidia Number Measurements

Fungal biomass, expressed as dry weight per plate, was measured as previously described (Hanano et al., 2015). In parallel, the total conidia fraction for each plate was harvested and taken up in 5 ml solution of 0.01% Tween 80, diluted to 1:10, and counted using a hemocytometer.

Preparation of *A. flavus* LDs Fraction and Peroxygenase Activities Assay

Isolation of fungal LD fractions was performed essentially as described by Ferreira de Oliveira and co-workers (Record et al., 1998; Ferreira De Oliveira et al., 2010) with brief modification as described previously (Hanano et al., 2015). In brief, 5 g of fungal mycelium was ground into a mortar in the presence of liquid nitrogen until a fine powder was obtained. The dried powder was immediately hydrated with 10 mL of buffer A (100 mM potassium

pyrophosphate, 0.1 M sucrose and pH 7.4). The mixture was then gently homogenized for 5 min using an ultra-dispenser (T25 digital ULTRA-TURRAX, IKA laboratory, Germany) and centrifuged for 10 min at 10,000 × *g*. The resulting supernatant was centrifuged at 100,000 × *g* for 1 h, and this enabled the obtainment of a floating white pad layer consisting of LDs. LDs were gently collected from the top of the tube using a Pasteur pipette, then carefully washed twice with 5 mL of buffer B (buffer A without sucrose). After a final centrifugation (100,000 × *g* for 1 h), the LD fraction was suspended in 1 mL of buffer B and stored at 4°C for further analysis. Peroxygenase activity was assayed by oxygenation of aniline as a substrate (Blee and Durst, 1987; Hanano et al., 2006).

Analysis of LDs

Microscopic imaging was performed at a magnification of 40 × under a LEICA MPS60 microscope using an Olympus FE-4000 camera. The purity of LD preparation, their native encapsulation and their number per mL were evaluated under a LEICA MPS60 light microscope, and the images were taken at a magnification of 40 × immediately after preparation.

SDS–PAGE and Western Blotting

Lipid droplet-associated proteins were isolated according to Katavic et al. (2006) and analyzed by SDS–PAGE using 12% polyacrylamide gels and electroblotted onto a PVDF membrane (Millipore) in a Semi-Dry Transfer Cell (Bio-Rad). Caleosins were immunodetected by incubating the membrane with a polyclonal antibody prepared from the complete sequence of the CLO1 caleosin isoform from *Arabidopsis thaliana*, as described previously (Hanano et al., 2018c).

Genes, Primers and Transcript Analysis

Nucleotide sequences of primers used in this section are listed in **Table 1**. For the gene expression studies, *A. flavus* was grown as described previously. Total fungal biomass was collected for total RNA isolation using an RNeasy kit according to the manufacturer's instructions (Qiagen, Germany). DNA traces were removed by 2 units of RNase-free RQI DNase (Promega, United States) for 1 h at 37°C. RNAs were diluted to 50 ng μL^{-1} using RNase-free water and stored at −80°C. cDNA synthesis was performed using M-MLV RT (Invitrogen) as described previously (Hanano et al., 2015). Real-time PCR was carried out in 96-well plates using an AriaMx Real-time PCR System from Agilent technologies, United States. The 25-µl reaction mixtures contained 0.5 mM of each target and reference gene primers, 12.5 µl of SYBR Green qRT-PCR mix (Bio-Rad, United States) and 2.5 µl of 10-fold diluted cDNA. qRT-PCR conditions were as previously described (Hanano et al., 2015). Each point was triplicated and the average of C_T was taken. The relative quantification RQ = $2^{(-\Delta\Delta CT)}$ of the target gene was calculated.

TABLE 1 | Primers used for the transcriptional analysis of AF-biosynthesis cluster genes.

Gene	Primers name	Nucleotide sequence (5′–3′)	Primer position	Amplicon (bp)
fas-a	*fasaF*	CAACGCCAACGCTATTCGAG	537–556	180
	fasaR	GTAATGCCACACGATTCGGC	697–716	
fas-b	*fasbF*	ATCCACTCGACATCATCGCC	2468–2487	115
	fasbR	TTGATGTCACGTCGGCTGAA	2563–2582	
pksA	*pksAF*	TAGTGTGCCTCTGCCAGTTG	254–273	107
	pksAR	GGAACCCATGCAGAATCCCA	341–360	
nor-1	*nor1F*	GCATCGGACGAGGTCTCATT	158–177	170
	nor1R	CTGGGCATCAGTTTCCGAGT	308–327	
norA	*norAF*	TTGGTACTGAGCGAGGAGGA	940–959	159
	norAR	TTCTAGCCGAGTGTTGCAGG	1079–1098	
avnA	*avnAF*	ATCGACGACTGTTGGCCTTT	386–405	188
	avnAR	CGAGTCTCCAAAAGCGAGGT	554–573	
adhA	*adhAF*	TCTAGAGACGGGGCAGAACA	147–166	172
	adhAR	TGCAAAGGAGACACCTGCAA	299–318	
avfA	*avfAF*	AGTACCGGCCTTCGTTCATC	411–430	177
	avfAR	AGTCTGTAGCCCGTTGGTTG	568–587	
estA	*estAF*	ACGCTACGAGATGATGCCAG	766–785	150
	estAR	TCCCCCGAAGAAAGTCCTCT	896–915	
vbs	*vbsF*	CCGCTCTGATGACTCCCTTC	1509–1528	158
	vbsR	GTCCGATGCAACAATCTCGC	1647–1666	
verb	*verbF*	GATGCTCAATAACGCTGCCG	963–982	186
	verbR	GTAAGGTACGGCAGATGCGA	1129–1148	
ver-1	*ver1F*	TGGTGAACTACGCCCATTCC	110–129	137
	ver1R	CACCGTCTCCGCCATTAACT	227–246	
verA	*verAF*	CCTCAGCAGCCACCCAAATA	290–309	128
	verAR	CCGCCACTTCTTCCAAGTCT	398–417	
omtB	*omtBF*	GCAAACGGCAAATTCAGGGT	71–90	173
	omtBR	CGCTAGAGTTATCGGCGTGT	224–243	
omtA	*omtAF*	ATGTGACGAAGTGATGCGGT	294–313	156
	omtAR	CTCGCATTTCAGCTGCGTTC	430–449	
ordA	*ordAF*	ATTTGTGTTCGGCTTTGGGC	1524–1543	107
	ordAR	TGGGCGAGATGAAGAAGCAG	1611–163	
apa-2	*apa2F*	CGCTATTGCTGCTTTTCGCT	691–710	158
	apa2R	GCATCGGTAGCCCTCTTGTT	829–848	
TE[a]	*TEF*	AGGCTTTCTTTGTGAGCCGT	357–376	132
	TER	ATAGCTGATGCTGACGGAGC	469–488	

[a] *TE – Transcriptional Enhancer encoding gene.*

Extraction, Clean-Up, TLC and HPLC Analysis of Aflatoxin

The extraction of AF was done according to Bertuzzi et al. (2011) using 50 mL of chloroform for 1 h on a rotary-shaker, and extracts were purified as described previously (Shannon et al., 1983). The extracted AF samples were analyzed by thin layer chromatography (TLC) using a C_{18} reversed-phase TLC plate (aluminum sheets measuring 20 × 20 cm with 200-µm layers, Merck, Germany) and the chromatogram was developed using a solvent system of chloroform/acetone (90:10, v/v). After development, the spot with the same *Rf*-value as the AFB_1 standard was scraped off, re-extracted with chloroform and evaporated to dryness under nitrogen. The extract was taken up in 100 µL acetonitrile in an amber-colored vial under refrigeration. AF was analyzed using a Jasco LC-2000 plus series HPLC system (Jasco, United States) with a fluorescence detector (RF-10Axl, Shimadzu) (λexc 247 nm; λem 480 nm) and a C18 column (Eclipse XDB-C18 150 × 4.6 mm, 5 µm; Agilent, United States, column temperature 35°C) as described previously (Hanano et al., 2015).

Detection and Quantification of ROS in Fungal Tissue

The accumulation of ROS was detected in fungal spores protoplast using a 2′,7′-dichlorofluorescin diacetate (DCFH-DA) staining protocol (Chang et al., 2011). The DCFH-DA, a cell-permeable non-fluorescent probe that turns to highly fluorescent 2′,7′-dichlorofluorescein in the presence of ROS (Sigma-Aldrich, United States) was dissolved into dimethyl sulfoxide (DMSO), and the stock solution was conserved at −20°C. Fungal protoplasts, previously washed with sterile deionized water, were incubated into 1 mL of freshly prepared solution of 10 μM DCFH-DA in phosphate-buffered saline (PBS) for 4 h at 28°C in the dark. Later, the protoplasts were collected by a brief centrifugation and washed twice with PBS and subsequently examined under a Nikon Eclipse Ti-U fluorescent microscope. Micrographs were taken at a magnification of 10 × using a Nikon Ti-U camera. The fluorescence intensity was analyzed using the software Image-Pro Plus 6.0.

SOD and CAT Enzymatic Activities

Preparation for enzyme activities was carried out as described previously (Zhang et al., 2012) with some modification. Briefly, 5 g of fungal tissues was homogenized with 5 mL potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol and 5% (w/v) insoluble PVPP on ice. Subsequently, the homogenate was centrifuged at 12.000 rpm for 5 min, and the supernatant was therefore used for analyzing the enzymatic activities. Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as described by Beauchamp and Fridovich (Beauchamp and Fridovich, 1971). Catalase (CAT) activity was measured by the method of Azevedo et al. (1998). Activity was determined by monitoring the decrease in absorbance due to H_2O_2 reduction at 240 nm for 2 min.

siRNA-Silencing of AfPXG Gene

Small interference RNAs (siRNA) design primers, preparation of fungal protoplast and siRNA delivery were performed according to Hanano et al. (2018a). In brief, delivery of siRNA to protoplasts was done in sterile 1.5 mL tubes. A total of 10 μL of siRNA primer (100 nM) was mixed with an equal volume of Lipofectin reagent (Invitrogen Life Technologies, United Kingdom) and kept for 15 min at 20°C. A volume of 50 μL of protoplasts was added, gently mixed and incubated at 20°C for 24 h to allow transfection to proceed. The transfected protoplasts were therefore inoculated in 10 mL of PD medium with 1.2 M of sorbitol for 7 days at 28°C in the dark. All experiments were performed using three biological replicates.

Inoculation of Maize Seeds With *A. flavus* and Biomass Estimation

A quantity of 100 g of maize (*Zea mays*) was sterilized by immersion into 70% ethanol for 1 min. After drying, grains were placed in a sterile petri plate and directly inoculated with 200 μl of liquid culture of *A. flavus* in PD broth. Inoculated grains were incubated at 28°C for 7 days. Fungal biomass was estimated on day 7 by careful washing of infected grains, filtration and then weighing the fungal growth expressed in grams of fresh weight per 100 g of grains.

Statistical Analysis

All data presented are expressed as means ± standard deviation (SD). Comparisons between control and treatments were evaluated by *t*-test. Difference from control was considered significant as $P < 0.05$, very significant as $P < 0.01$, and highly significant as $P < 0.001$.

RESULTS

Characterization of the TCDD-Exposed Phenotype of *A. flavus*

The photographs presented in **Figure 1A** show that the TCDD-exposed *A. flavus* were phenotyped by limitation in fungal growth and activation of conidia formation compared to control. Inversely to control, the mycelium dry weight of fungi per plate was significantly reduced when they were exposed to TCDD and formed only about half of control growth at the highest dose of TCDD (50 ng L^{-1}) on days 3, 5, and 7 after inoculation (**Figure 1B**). More surprisingly, the TCDD-exposed fungi tended to produce spores more actively compared to the control. For example, on day 7, while the fungus that was exposed to 50 ng L^{-1} produced more than 14×10^6 mL^{-1}, the control produced about 9.6×10^6 mL^{-1} (**Figure 1C**). Furthermore, the blue fluorescent spot of AFB_1 was increasingly detected on the TLC as a function of TCDD treatment (**Figure 1D**). Quantitatively speaking, the amount of AFB_1, measured by HPLC, was doubled in the TCDD-exposed fungi compared to control, where the highest concentration of AFB_1 (24.8 μg mL^{-1}) was detected when *A. flavus* was exposed to 50 ng L^{-1} on day 7 after inoculation (**Figure 1E**). Together, these data suggest that dioxin exposure causes certain limitation in fungal growth and boosts conidiation and AFB_1 production in *A. flavus*.

The TCDD-Exposed Phenotype of *A. flavus* Has an Enhanced Level of Oxidative Status

It is well know that AF biosynthesis is naturally stimulated under stress oxidative status caused by a high level of ROS (Reverberi et al., 2008). Thus, the high production of AF by the TCDD-exposed *A. flavus* raises the question on whether such exposure will boost the accumulation of ROS in fungal tissues. To evaluate that, the accumulation of ROS in fungal spore protoplasts was detected using the DCFH-DA, a cell-permeable non-fluorescent probe that turns to highly fluorescent 2′,7′-dichlorofluorescein in the presence of cellular ROS. As the photographs show, the intensity of green fluorescence that was proportionally related to the amount of ROS was remarkably augmented in a response to the exposure to TCDD at 10 and 50 ng mL^{-1} in fungal spore protoplasts compared to their respective controls (**Figure 2A**). When the fluorescence intensity was analyzed by the software

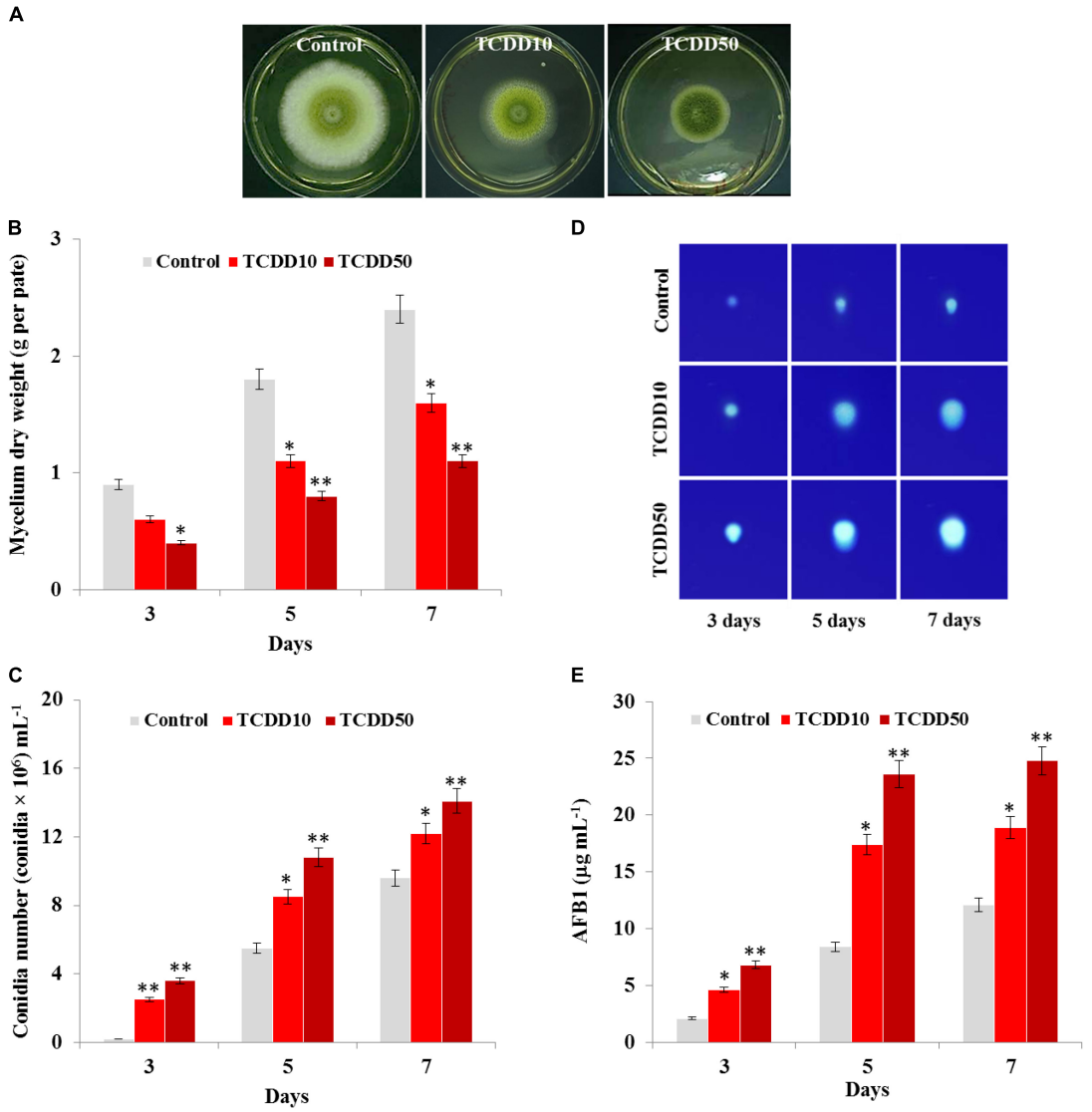

FIGURE 1 | Characteristic of *A. flavus* TCDD-exposed phenotype. **(A)** Photographs of 5-day-old cultures of *A. flavus* on PDA-plates upon the exposure to TCDD at 10 and 50 ng L^{-1} that referred as to TCDD10 and TCDD50, respectively, compared with non-exposed fungus control. **(B,C)** Variation in fungal mycelium dry weight and spore number, respectively, as a function of the exposure to TCDD at both doses. **(D)** Sections of Thin Layer Chromatography (TLC) plate showing the blue-fluorescent spots under the UV light that correspond to the AFB_1 secreted by the TCDD-exposed fungi compared to control. **(E)** Quantitative data of AFB_1 as estimated by UV-detector HPLC. All measurements were done in triplicate and the presented data are means ± *SD* (n = 3). Difference between treatments and control was significant (*$P < 0.05$) or very significant (**$P < 0.01$) when analyzed by *t*-test.

Image-Pro Plus 6.0, we stated that such intensity was higher – about 3.1- to 3.8-fold in protoplasts exposed to TCDD at 10 and 50 ng L^{-1}, respectively, compared to their representative controls (**Figure 2B**). In connection with ROS level, we have measured the cellular enzymatic activities of SOD and CAT; both enzymes are activated by the excess ROS and react to neutralize the $O^{2.-}$ and H_2O_2, respectively. SOD activity was stimulated by a factor of 2 and 3 in TCDD-exposed fungi to 10 and 50 ng mL^{-1}, respectively, compared to controls (**Figure 2C**). Also, CAT activity was induced 2.7- to 3.6-fold in such treatments compared to controls (**Figure 2C**). These data suggest that the exposure to dioxin led to enhanced ROS accumulation in fungal tissue, and subsequently the ROS-scavenging enzymatic activities were induced.

TCDD Induces the Expression of AfPXG, the Accumulation of LDs and Subsequently the Production of AFB_1 in *A. flavus*

We recently reported that the caleosin/peroxygenase AfPXG of *A. flavus* modulates the biosynthesis of AF and its trafficking via the LDs (Hanano et al., 2018a). Likewise, certain isoforms of plant caleosins/peroxygenase were strongly induced by

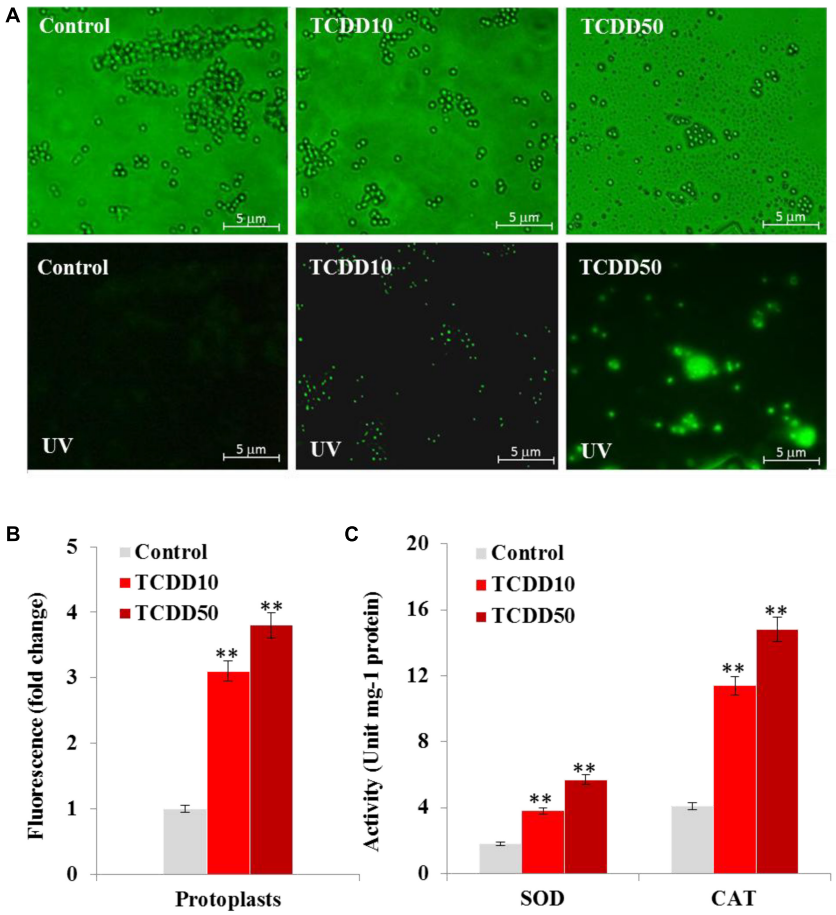

FIGURE 2 | The exposure to TCDD enhances the accumulation of ROS in fungal tissues. **(A)** Micrographs of fungal spore protoplasts stained with DCFH-DA, a cell-permeable non-fluorescent probe that turns to highly fluorescent 2′,7′-dichlorofluorescein in the presence of cellular ROS. Samples were examined under a Nikon Eclipse Ti-U fluorescent microscope and micrographs recorded at a magnification of 10 × a using a Nikon Ti-U camera. Bar represents 5 µm. **(B)** The ROS fluorescence intensity was analyzed by software Image-Pro Plus 6.0. The fold change in fluorescence intensity was estimated by comparing the TCDD-treated samples for spore protoplasts with their respective controls that were considered as 1. Data were shown as the mean ± *SD*. **(C)** Enzymatic activities of SOD and CAT in fungal tissues in response to TCDD exposure at indicated concentrations. All measurements were done in triplicate and the presented data are means ± *SD* ($n = 3$). Difference between treatments and control was significant (*$P < 0.05$) or very significant (**$P < 0.01$) when analyzed by *t*-test.

dioxin (Hanano et al., 2018b). In connection with this, we were interested in gaining more insight into the biological implication of AfPXG in fungal responses to dioxin exposure. At transcription level, the expression of AfPXG was significantly induced upon exposure to TCDD. The transcript level of AfPXG was augmented 6.5- and 9.2-fold as a function of exposure to TCDD at 10 and 50 ng mL^{-1}, respectively, compared with control (**Figure 3A**). This was synchronized with a similar increase in protein level of AfPXG as well as in its enzymatic activity, measured by hydroxylation of aniline, where this activity was increased from 0.3 in the control to 1.8 and 2.3 (µmol $min^{-1}mg^{-1}$) in the exposed fungi to 10 and 50 ng mL^{-1} of TCDD (**Figure 3B**). Subsequently, the TCDD-exposed fungi accumulated an increasing number of LDs as shown in the micrographs under light microscope (**Figure 3C**). Unlike the LDs isolated from control fungi, the number of LDs isolated from TCDD-exposed fungi was augmented by a factor of 5, and this number was stable 24 h after preparation compared with the critical stability of control LDs (**Figure 3D**). The increasing accumulation of LDs in the TCDD-exposed fungal tissues raises the question about their cellular activity in the trafficking and secretion of AF. Interestingly, despite the decreasing amount of AFB_1 that detected in the TCDD-exposed fungal tissues (**Figure 3E**), these fungi secreted more AFB_1 into the medium (**Figure 3F**) and this elevated their secretion ratio to about 0.9 versus 0.7 for the control (**Figure 3G**). The high production of AFB_1 upon exposure to TCDD was effectively related with important increases in the transcripts level of certain key genes, e.g., *alfD*, *alfG*, *alfI*, *alfL*, *alfM*, *alfN*, *alfQ*, *alfR*, and *alfS* of the AF biosynthesis pathway (**Figure 3H**). These data suggest that the exposure of *A. flavus* to dioxin induces the expression of AfPXG, increases the accumulation of stable LDs and therefore enhances the production of AF.

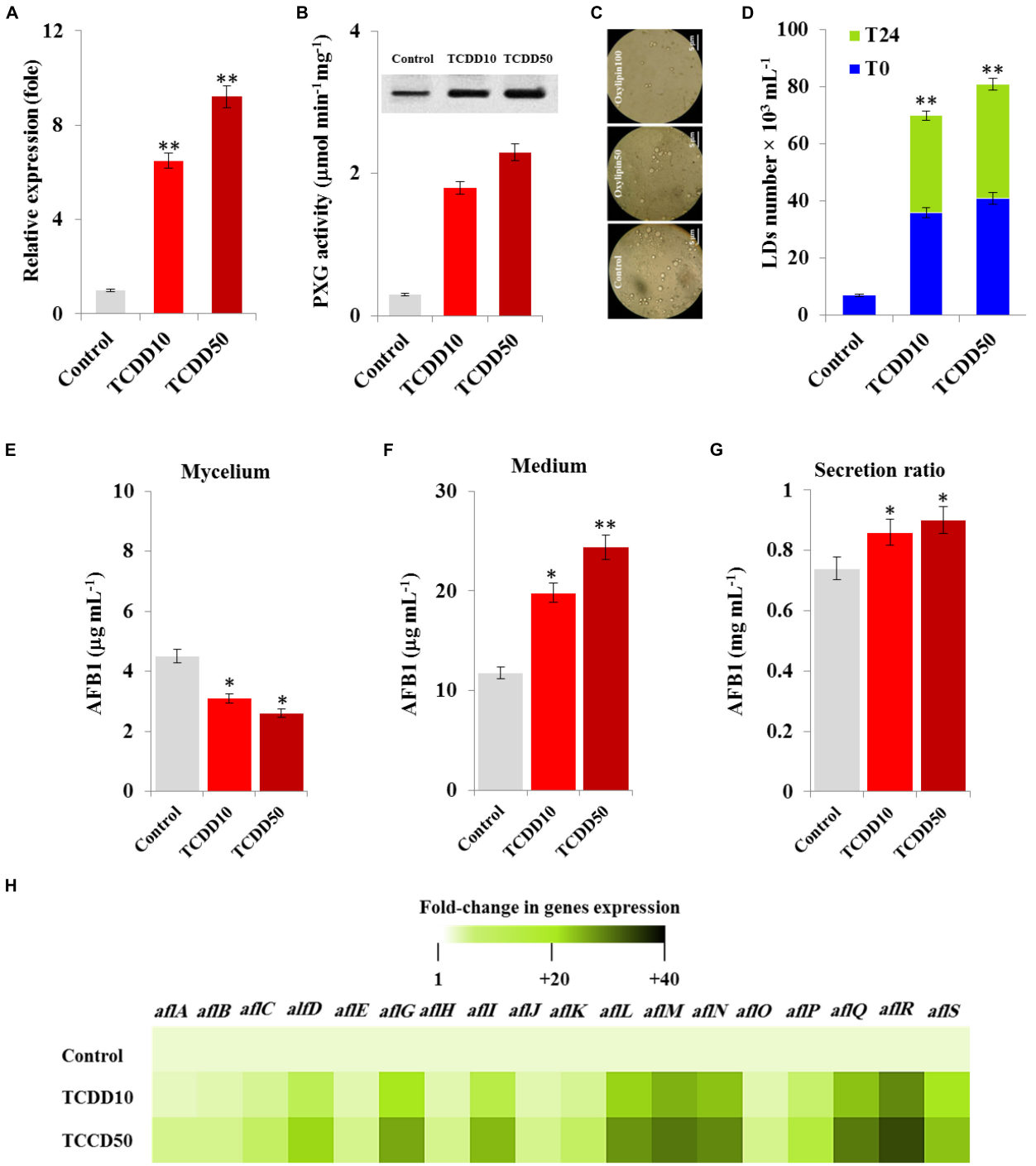

FIGURE 3 | The TCDD induces the functional expression of AfPXG, the accumulation of LDs and the production of AfB1. **(A)** Transcripts level of AfPXG gene, evaluated by RT-qPCR, in the fungal tissues exposed or non-exposed to TCDD. **(B)** The peroxygenase activity of AfPXG, estimated by the hydroxylation of aniline at 310 nm. **(C)** Micrographs of LDs isolated from fungal tissues exposed or not exposed to TCDD. Samples were examined under a LEICA MPS60 light microscope and the images were taken at a magnification of 40 × immediately after preparation. Bar represents 5 µm. **(D)** LD number was estimated using a hemocytometer. **(E,F)** Measurement of AFB1 in the fungal mycelium and in the culture medium of *A. flavus* exposed or not exposed to TCDD. **(G)** Secretion ratio of AFB1 by fungi exposed or not exposed to TCDD (calculated as the ratio of AFB1 in the medium to AFB1 in medium plus mycelia in a 100 mL culture). **(H)** Relative quantification $RQ = 2^{(-\Delta\Delta CT)}$ of AF-biosynthesis gene transcripts upon the exposure to TCDD compared to control. The color scale (white-green-black) indicates relative changes of transcripts of 1, +20, and +40-fold, respectively. For each gene, the expression level in the control was defined as 1, and the corresponding increase in gene transcript under treatments was calculated. All measurements were done in triplicate and the presented data are means ± *SD* ($n = 3$). The differences between control and treatments were significant when analyzed by *t*-test (*$P < 0.05$; **$P < 0.01$).

TCDD-Induced Hyperaflatoxicogenicity Is Reduced in AfPXG-Silencing Strain

To confirm the biological link between the TCDD-induced aflatoxicogenicity and the function of AfPXG, the TCDD was administrated to *A. flavus* WT as well as to AfPXG-silenced strain (referred as to siAfPXG) in which the AfPXG gene was silenced by about half using siRNAs (Hanano et al., 2015, 2018a). Our data indicated that the silencing of AfPXG was briefly restored upon treatment with TCDD, but its transcript level was still about 42- to 46-fold lower compared to the control (**Figure 4A**). In parallel, the silencing of AfPXG was also confirmed by the absence of peroxygenase activity in the control as well as in the TCDD-treated samples (**Figure 4B**). Furthermore, the treatment with TCDD, at both concentrations, did not lead to increased accumulation of LDs in the siAfPXG strain as it did in the WT (**Figure 4C**). Subsequently, the treatments with TCDD at both concentrations did not provoke an over-secretion of AFB_1 in the siAfPXG strain as it did in the WT. For example, while the production of AFB_1 in the WT treated with the highest dose of TCDD was highly stimulated to reach about 23.5 μg mL^{-1}, this treatment failed to provoke a similar effect in the siAfPXG strain where its AFB_1 production did not exceed 1.8 μg mL^{-1} (**Figure 4D**). Apparently, the failure of the TCDD-exposed siAfPXG strain in stimulating the AF production was related with a severe down-regulation of a set of key genes in AF-biosynthesis pathway. The heat map (**Figure 4E**) shows that upon its exposure to TCDD, the siAfPXG strain still had low levels of *alfB*, *alfC*, *alfD*, *alfG*, *alfI*, *alfL*, *alfM*, *alfN*, *alfQ*, *alfR*, and *alfS* transcripts compared with WT. Altogether, these results suggest that the TCDD-induced aflatoxicogenicity of *A. flavus* is likely mediated by AfPXG.

The TCDD-Exposed *A. flavus* Shows More Aggressivity to Infect Maize Grains

The induced aggressivity of *A. flavus* caused by the exposure to TCDD was assayed *in vitro* using grains of maize. For that, the spores of *A. flavus* WT strain grown in contaminated medium with TCDD were collected and used to infect the grains. Then, the fungal biomass and AF production were evaluated and compared with control. The photographs of infected grains (**Figure 5A**) show a brief limitation in fungal growth but a more pronounced activity in conidiation of TCDD-exposed fungi compared to the non-exposed fungus. This was confirmed by the decreasing amount of fungal biomass, expressed as (g dry weight per 10 g of grains) in the TCDD-exposed fungi, at both concentrations (2.3 and 3.5-fold) compared to control (**Figure 5B**). Inversely, the AF production was doubled and tripled in the infected grains with the exposed fungi to TCDD at 10 and 50 ng mL^{-1} compared to control (**Figure 5B**). These data indicate that the exposure of *A. flavus* to dioxin could likely boost its aflatoxicogenicity.

DISCUSSION

Aflatoxins are globally considered the most perilous food contaminants with proven toxicological, economic and social impacts. The fact that these toxins are mainly produced by certain fungal species that are known by their remarkable adaptive capabilities with various environmental niches, makes their management an effective challenge (Weckbach and Marth, 1977; Mehl et al., 2012). In this context, the focus on the biological impacts of environmental factors on the fungal growth, and development in general and its aflatoxicogenicity in particular, is a decisive rule for globally governing the contamination with aflatoxins in a sustainable minded manner. In line with this, many ecological studies have demonstrated the biological feedback of environments on the aggressivity/aflatoxicogenicity of AF-producing fungi (Gilbert et al., 2017; Medina et al., 2017). Unexpectedly, despite the ultimate connection of AF-producing fungi with soil, the biological impacts of soil contaminants, more particularly the POPs, are still obscure.

In light of this, the current study shows that the *in vitro* exposure of *A. flavus* to dioxin results in a phenotype typified by a peculiarly reducing in fungal vegetative growth and a noteworthy tendency to conidiation. First, in the absence of comparative data in fungi, the limitation in fungal mycelial growth caused by TCDD could be supported by a similar phenomenon in the experimental animals exposed to TCDD. A characteristic feature of acute exposure to dioxin is a dramatic decline in body weight, a phenomenon known as the wasting syndrome (Tuomisto et al., 1995; Tsujimoto et al., 2013; Hutin et al., 2018). With surprising similarity, a significant reduction in the fresh weight of leaves and seeds was also reported for plants that were experimentally exposed to TCDD (Zhang et al., 2012; Hanano et al., 2014a, 2018b). Beyond the wasting of weight, the dioxin-exposed organisms tended to implant a specific profile of cellular fatty acids, which leads to a puzzling and dramatic perturbation of the regulatory systems for energy balance (Hanano et al., 2018b,c). Moreover, the TCDD-induced phenotype of *A. flavus* was characterized by a remarkable ability to sporulate. Indeed, there are multiple lines of genetic, molecular and biochemical evidence that confirm the presence of a biological link between the fungal sporulation and the accumulation of secondary metabolites (SM) and more particularly the AF, where their production was also enhanced upon exposure to TCDD. In this context, earlier reports have shown that the environmental conditions required for sporulation and the accumulation of SM were often similar and were more strict than those for vegetative growth (Sekiguchi and Gaucher, 1977; Guzman-De-Pena and Ruiz-Herrera, 1997). This included, but was not limited to, temperature (Espeso et al., 1993), air-surface interface (Guzman-De-Pena and Ruiz-Herrera, 1997), medium pH (Buchanan and Ayres, 1975), nature of nutrients (Keller et al., 1997) and certain compounds present in seeds commonly infected by *Aspergillus* species (Goodrich-Tanrikulu et al., 1995; Burow et al., 1997; Calvo et al., 1999; Zeringue, 2000). In a more evolved manner, it was suggested that certain SMs act as sporogenic factors. For example, several lines of genetic evidence have shown that the accumulation of natural pigments in fungal tissues, such as melanin, is associated with fungal sporulation (Kawamura et al., 1999). Of particular interest, the relationship between fungal sporulation and mycotoxin biosynthesis has been corroborated for AF biosynthesis in *A. flavus* and *A. parasiticus* as well as for

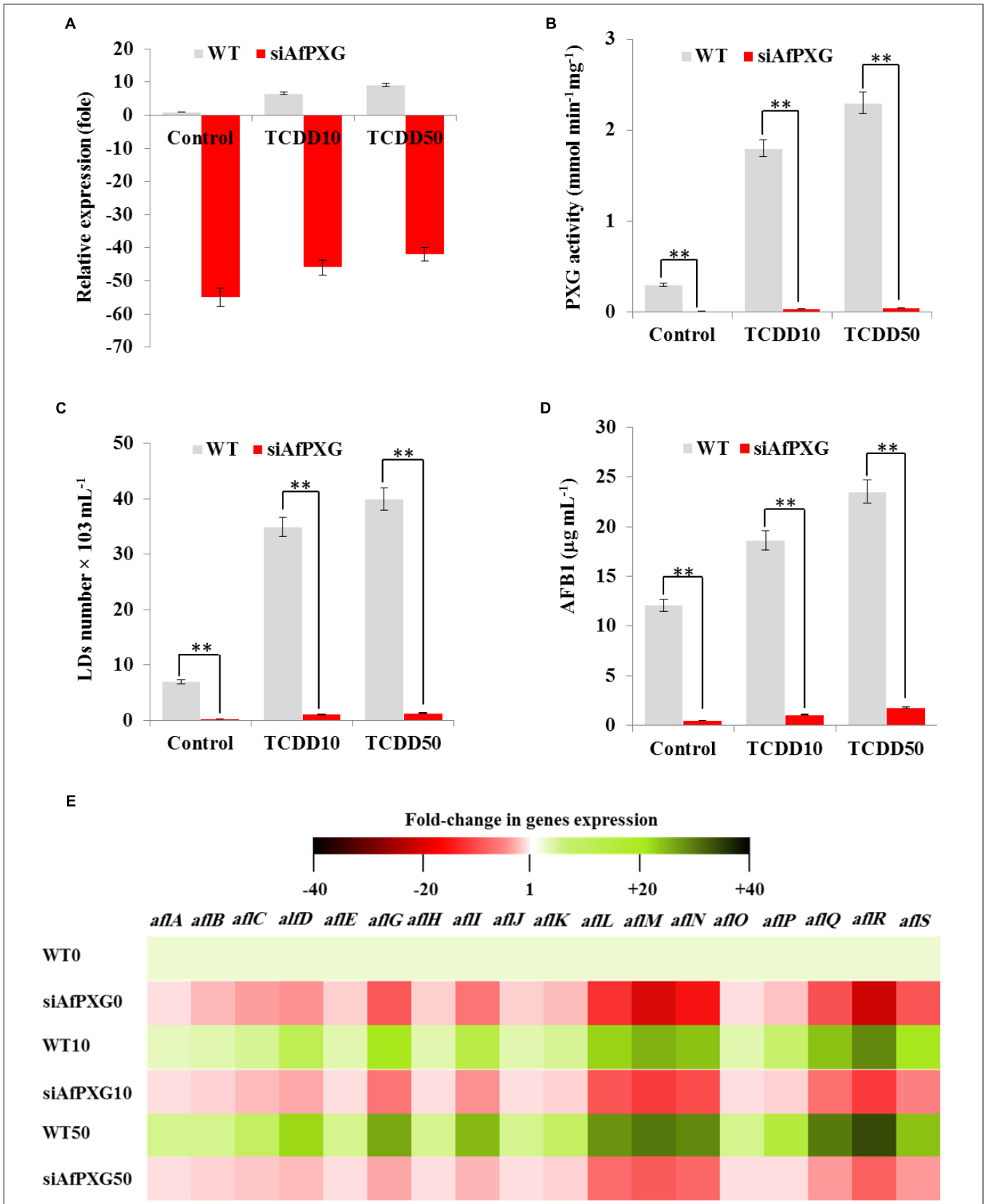

FIGURE 4 | Continued

FIGURE 4 | The TCDD-induced hyperaflatoxicogenic phenotype is abolished in the AfPXG-silencing strain. **(A)** Relative quantification of AfPXG transcripts in the AfPXG-silencing strain (siAfPXG) and the wild type (WT) upon their exposure to TCDD at the indicated concentrations. **(B)** Estimation of the peroxygenase activity in siAfPXG and WT after exposure to TCDD compared with their respective controls. The activity was estimated by the hydroxylation of aniline at 310 nm. **(C)** Number of LDs isolated from siAfPXG and WT after exposure to TCDD. **(D)** Concentration of AFB1 secreted by siAfPXG and WT upon their exposure to TCDD at both concentrations compared with their respective controls. **(E)** Transcripts levels of the AF-biosynthesis genes in fungal tissue of siAfPXG and WT after their exposure to TCDD at both concentrations. This was carried out using the RT-qPCR system. Three measurements were done in three cDNAs prepared from three individual fungal tissues for each line. The color scale (red-white-green) indicates positive changes of transcript abundance of - 40-, 1, and +40-fold, respectively. For each gene, the expression level in the control was defined as 1, and the corresponding abundance changes under treatments were calculated directly using the software installed in the Applied Biosystems qPCR system. The differences between treatments and control were significant when analyzed by *t*-test (*$P < 0.05$; **$P < 0.01$).

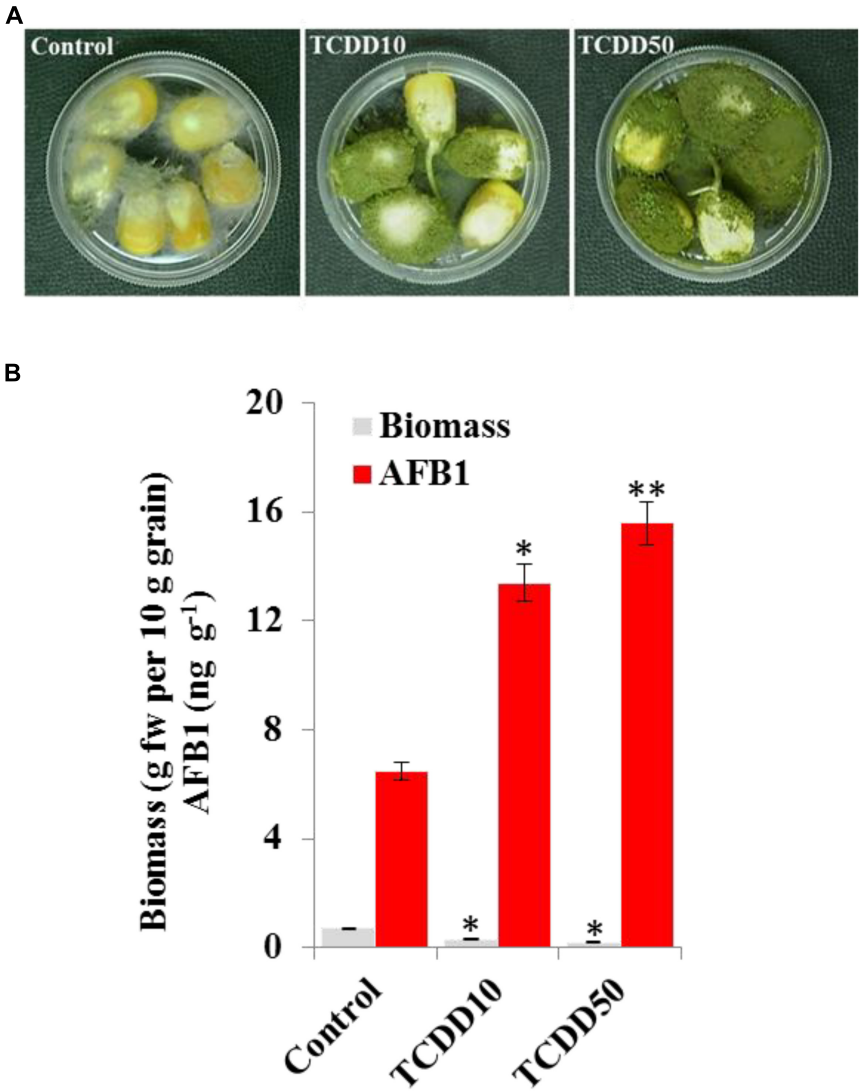

FIGURE 5 | The exposure to TCDD boosts the aggressivity of *A. flavus* against the maize grains. **(A)** Photographs of infected maize grains with *A. flavus* previously exposed to TCDD at 10 and 50 ng L^{-1} referred to as TCDD10 and TCDD50, respectively, compared with non-exposed fungus control. After sterilizing, grains were placed in a sterile petri plate and directly inoculated with 200 µl of liquid culture of *A. flavus* in PD broth contaminated or not contaminated with TCDD. The photographs were taken 8 days after inoculation. **(B)** Estimation of fungal biomass and AFB1 production in the three samples. Asterisks indicate significant differences between treatments and control (*$P < 0.05$; **$P < 0.01$).

sterigmatocystin (ST) biosynthesis in *Aspergillus nidulans*. The non-producing AF/ST mutants that had a deletion in *aflR*, a gene encoding a regulatory protein that modulates the expression of AF/ST-biosynthesis cluster genes, failed in the formation of spores in all three *Aspergillus* sp. (Trail et al., 1995). Inversely, the overexpression of *aflR* enhances the production of AF and the formation of spores (Yu et al., 1996), and this is in line with our data showing an increasing level in *aflR* transcripts in

fungal tissues after exposure to dioxin. Furthermore, the fluffy mutants of *A. nidulans* and *A. flavus* that have a defective G-protein signaling pathway resulted in an aconidial, aflatoxin-null phenotype (Calvo et al., 2002; Affeldt et al., 2012). More recently, the nucleoside diphosphate kinase (AfNDK), which was newly characterized in *A. flavus*, regulates spore and sclerotia development and is involved in plant infection (Wang et al., 2019). Additionally, the HosA, a new histone deacetylase recently identified in *A. flavus*, plays a determinant role in growth, development and AF biosynthesis (Lan et al., 2019).

Substantial dioxin-induced overproduction of AF can be an expected result of the increasing level of ROS in the fungal tissues upon their exposure to TCDD. In line with this, dioxins have a proven effect in inducing the accumulation of ROS in exposed animals (Bentli et al., 2016; Liu et al., 2016) and plants (Hanano et al., 2014a), suggesting a similar effect in fungi. Otherwise, the enhanced level of ROS was effectively accompanied with a significant stimulation of SOD and CAT activities, the two main scavengers of $O_2^{\bullet-}$ and H_2O_2, respectively. This could be viewed as a part of the complex anti-oxidative defense system used by the organism to minimize the oxidative damage initiated by the ROS access. The activities of both enzymes were stimulated in plant tissues upon exposure to dioxin, polyaromatic hydrocarbons and some pesticides (Liu et al., 2009; Zhang et al., 2012; Hanano et al., 2014a).

In respect to the induction of AfPXG by TCDD, the expression of this caleosin is modulated by various biotic and abiotic stresses in several fungal species e.g., *Aspergillus oryzae* (Machida et al., 2005; Akao et al., 2007), *Ustilago maydis* (Tollot et al., 2016), and *Erysiphe necator* (Wakefield et al., 2011). Furthermore, the TCDD-induced expression of AfPXG led to an increase in the accumulation and the stability of LDs in fungal tissues. This result can be supported by recent findings showing that the heterologous expression of AfPXG in yeast led to a massive accumulation of stable LDs (Hanano et al., 2015). Likewise, the AfPXG-overexpressed line of *A. flavus* accumulated more LDs than the wild type (Hanano et al., 2018a), and this was synchronized with an elevated level in AF secretion. It is likely that the expression of AfPXG could be coordinated by TCDD at two levels;

(i) At the level of AfPXG enzymatic activity, it is now well recognized that AfPXG experimentally catalyzes the reduction of fatty acid hydroperoxides (FA-OOH) into their corresponding alcohols (AF-OH) as the plant caleosins/peroxygenases typically do (Hanano et al., 2006, 2016b). The biological impacts of this activity, characterized as the newest branch of the oxylipins biosynthesis pathway, have been interestingly underlined in plants (Blee et al., 2014; Charuchinda et al., 2015) but less in fungi (Fan et al., 2015). For example, it was documented that the endogenous treatment of *Aspergillus* with plant-originated FA-OOH represses AF biosynthesis and lengthens the time during which the AF gene transcripts accumulate (Burow et al., 1997). More recently, the knockout of AfPXG in *A. flavus* resulted in a considerable accumulation of FA-OOH in fungal tissues that have been accompanied with developmental anomalies and lowering in AF production (Hanano et al., 2018a).

(ii) At the level of AfPXG's structural role in LDs assembly, the caleosins encoding genes are present in the vast majority of publicly available fungal genomic sequences including all *Aspergillus* spp., and they contain at least one copy of a highly conserved transmembrane domain enabling the caleosin proteins to be targeted in the monolayer membrane of LDs (Murphy, 2012; Hanano et al., 2015; Rahman et al., 2018a,b). The structural role of fungal caleosins in impacting the assembly and the stability of LDs is well demonstrated (Froissard et al., 2009; Jamme et al., 2013; Hanano et al., 2015), and this is in line with our results indicating that the TCDD-exposed fungi expressed more AfPXG and therefore accumulated more of LDs than the control. Otherwise, similar inductions of certain isoforms of caleosins were also reported in plants that have been experimentally exposed to TCDD (Hanano et al., 2016b, 2018b,c).

In connection with the TCDD-induced hyperaflatoxicogenicity, the implication of LDs in trafficking and exporting of AF has been recently reported (Hanano et al., 2018a), where the increasing number of LDs in the AfPXG-overexpressed line of *A. flavus* showed an increasing capability to sequestrate the AF and this was positively correlated with a high secretion ration of AF into the medium. In line with this, the role of LDs as transient repositories of lipophilic and hydrophilic compounds, from small molecules such as DNA, signal molecules, SM to large proteins, is well established (Chang et al., 2015; Gao and Goodman, 2015; Kory et al., 2016; Schuldiner and Bohnert, 2017). Taken together, our data suggest that the dioxin-induced hyperaflatoxicogenic phenotype of *A. flavus* is likely mediated by the caleosin/peroxygenase AfPXG. This could be particularly supported by using the *AfPXG*-silencing strain of *A. flavus,* where the typical TCDD-induced phenotype observed in the WT was completely abolished in the *AfPXG*-silencing strain. Finally, we found that the TCDD-exposed *A. flavus* had increased virulence against the grains of maize in terms of fungal invasion, sporulation and AF production. These observations suggest that contamination of soil with dioxin can influence the invasion capacity of soil-dwelling *A. flavus*, more particularly the fungal spores, empowering its aflatoxicogenicity. Similar worrying scenarios were suggested in response to possible adverse consequences of climate change (Medina et al., 2014; Assuncao et al., 2018).

CONCLUSION

Our study sheds light, for the first time, on the possible biological feedback of persistent environmental pollutants, notably the dioxins on the aggressivity of AF-producing fungi in terms of their ability to infect and contaminate the crops. The production of aflatoxins and their subsequent prevalence in agricultural commodities are affected by environmental changes including persistent pollutants such as dioxins, which are increasingly

recognized as direct contaminants resulting from the forests burning. Our data showed that the *in vitro* exposure of *A. flavus* to dioxin considerably increased its virulence in term of sporulation and aflatoxicogenicity. We suggest that this hyper-aflatoxicogenic phenotype is mediated by the caleosin/peroxygenase AfPXG that has proven functions in controlling the biosynthesis and trafficking of aflatoxins in *A. flavus*. This study highlights one unpredicted consequence of climate change that is relevant to the food safety. Our observations suggest that global food safety could be impacted by climate change, and this will possibly present new challenges in the near future to combat climate change in a global manner as well as to preserve, and even more to reinforce, the global regulations of food safety.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/supplementary files.

AUTHOR CONTRIBUTIONS

AH led the work, designed all experiments in biochemistry and molecular biology, and wrote the manuscript. MS and IA carried out all experimental work. All authors read and approved the final manuscript.

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"Ground-Truthing" Efficacy of Biological Control for Aflatoxin Mitigation in Farmers' Fields in Nigeria: From Field Trials to Commercial Usage, a 10-Year Study

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In sub-Saharan Africa (SSA), diverse fungi belonging to *Aspergillus* section *Flavi* frequently contaminate staple crops with aflatoxins. Aflatoxins negatively impact health, income, trade, food security, and development sectors. *Aspergillus flavus* is the most common causal agent of contamination. However, certain *A. flavus* genotypes do not produce aflatoxins (i.e., are atoxigenic). An aflatoxin biocontrol technology employing atoxigenic genotypes to limit crop contamination was developed in the United States. The technology was adapted and improved for use in maize and groundnut in SSA under the trademark Aflasafe. Nigeria was the first African nation for which an aflatoxin biocontrol product was developed. The current study includes tests to assess biocontrol performance across Nigeria over the past decade. The presented data on efficacy spans years in which a relatively small number of maize and groundnut fields (8–51 per year) were treated through use on circa 36,000 ha in commercially-produced maize in 2018. During the testing phase (2009–2012), fields treated during one year were not treated in the other years while during commercial usage (2013–2019), many fields were treated in multiple years. This is the first report of a large-scale, long-term efficacy study of any biocontrol product developed to date for a field crop. Most (>95%) of 213,406 tons of maize grains harvested from treated fields contained <20 ppb total aflatoxins, and a significant proportion (>90%) contained <4 ppb total aflatoxins. Grains from treated plots had preponderantly >80% less aflatoxin content than untreated crops. The frequency of the biocontrol active ingredient atoxigenic genotypes in grains from treated fields was significantly higher than in grains from control fields. A higher proportion of grains from treated fields met various aflatoxin standards compared to grains from untreated fields. Results indicate that efficacy of the biocontrol product in limiting aflatoxin contamination is stable regardless of environment and cropping system. In summary, the biocontrol technology allows farmers across Nigeria to produce safer crops for consumption and increases potential for access to premium markets that require aflatoxin-compliant crops.

Keywords: aflatoxin, biocontrol, efficacy trials, long-term efficacy, maize, groundnut, safe food

INTRODUCTION

Throughout sub-Saharan Africa (SSA), certain *Aspergillus* species frequently contaminate with aflatoxins several staple crops, including maize and groundnut (Shephard, 2008; Udomkun et al., 2017). In SSA, human and animal aflatoxin exposure is high (JECFA, 2018; Sirma et al., 2018; Blankson et al., 2019). Consumption of highly contaminated food can result in acute health effects such as liver diseases and death (Gieseker, 2004; Probst et al., 2010; Kamala et al., 2018). Chronic, sub-lethal exposure may cause child stunting, immunosuppression, impaired food conversion, and cancer (Coursaget et al., 1993; Gong et al., 2008; JECFA, 2018; Leroy et al., 2018; Voth-Gaeddert et al., 2018). Trade sectors also become affected. High aflatoxin content restricts farmers' access to local and international premium markets. This results in reduced income for farmers but also aggregators, processors, and exporters (Williams, 2008; Wu, 2015). Because of the challenges posed by aflatoxins, substantial efforts have been made to both understand the contamination process and design management programs to reduce food safety risks (James et al., 2007; Hell et al., 2008; Bandyopadhyay et al., 2016; Seetha et al., 2017).

Aflatoxin-producing *Aspergillus* species of agricultural importance belong to section *Flavi* (Frisvad et al., 2019). Some species produce only B aflatoxins while others produce both B and G aflatoxins. There are four major aflatoxins (B_1, B_2, G_1, G_2) with aflatoxin B_1 both the most toxic and prevalent and classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC, 2002; JECFA, 2018). The major causal agent of contamination is *A. flavus*, which produces only B aflatoxins (Amaike and Keller, 2011). *A. flavus* is composed of the L and S morphotypes, which differ in morphological, physiological, and genetic criteria (Cotty, 1989). Each morphotype can be further subdivided in numerous vegetative compatibility groups (VCGs) (Bayman and Cotty, 1991; Ortega-Beltran and Cotty, 2018). Genetic variation within members of a VCG is low because they descend from a single clonal lineage but members of different VCGs vary in several characters (Leslie, 1993; Grubisha and Cotty, 2010, 2015). Most L morphotype fungi produce aflatoxins (at varying levels) while others produce no aflatoxins (i.e., are atoxigenic) due to lesions in the aflatoxin biosynthesis gene cluster (Adhikari et al., 2016). Furthermore, there are L morphotype VCGs composed exclusively of atoxigenic members (Mehl et al., 2012; Grubisha and Cotty, 2015; Atehnkeng et al., 2016).

Species other than *A. flavus* may be important etiologic agents of aflatoxin contamination. In West Africa, fungi resembling the *A. flavus* S morphotype but that produce both B and G aflatoxins are associated with several crops, including maize and groundnut (Cotty and Cardwell, 1999; Atehnkeng et al., 2008a; Donner et al., 2009; Diedhiou et al., 2011; Agbetiameh et al., 2018; Ezekiel et al., 2019). These fungi were previously known as unnamed taxon S_{BG}. Fungi in this group may be any of recently described species that include *A. aflatoxiformans*, *A. austwickii*, *A. cerealis*, *A. minisclerotigenes*, and unnamed taxa (Probst et al., 2014; Frisvad et al., 2019; Singh and Cotty, 2019). As in a previous study (Ezekiel et al., 2019), in the current study we use the term S_{BG} strains for all fungi with S morphotype producing both B and G aflatoxins. Even though S_{BG} strains are found at low levels in some years/crops, the extremely high aflatoxin-producing potential of the group warrants special consideration as a major contributor to aflatoxin contamination events in West Africa.

Aflatoxin management strategies have been sought for more than 40 years. Most pre- and post-harvest strategies may reduce incidences and severities of aflatoxin contamination (Bruns, 2003; Cotty et al., 2008; Hell et al., 2008; Waliyar et al., 2015; Seetha et al., 2017; Mahuku et al., 2019). However, the use of a single management strategy in isolation may not prevent initiation of aflatoxin contamination and be insufficient to reduce aflatoxin contamination to acceptable levels [i.e., at least below 20 parts per billion (ppb)] (Bandyopadhyay et al., 2016). Therefore, aflatoxin management strategies must address the contamination process throughout crop production and until crops are consumed using holistic interventions (Ayalew et al., 2017; Logrieco et al., 2018).

The most promising strategy to control aflatoxins is the use of atoxigenic *A. flavus* strains to competitively displace aflatoxin producers (Amaike and Keller, 2011). This strategy favors the prevalence of atoxigenic strains in the treated fields and throughout the environment. When less aflatoxin producers are associated with a crop, less aflatoxins accumulate (Mehl et al., 2012). This aflatoxin management strategy protects crops from the field, throughout storage, and until consumption. The US Department of Agriculture – Agricultural Research Service (USDA-ARS) developed the first atoxigenic biocontrol product, *Aspergillus flavus* AF36, and initially registered it with the US Environmental Protection Agency (USEPA) for use in cotton (USEPA, 2003, 2004). Together with Afla-Guard®, a second atoxigenic biocontrol product, the biocontrol technology has been used commercially for >15 years in the US in several crops (Cotty et al., 2007; Dorner, 2009; Doster et al., 2014; Ortega-Beltran and Bandyopadhyay, 2019).

In 2003, the International Institute of Tropical Agriculture (IITA) started a collaboration with USDA-ARS to adapt and improve the biocontrol technology for use in Nigeria and subsequently in other SSA nations (Bandyopadhyay et al., 2016). Initial efforts in Nigeria included examining fungal communities associated with maize and field soils and assessing aflatoxin-producing potentials of the fungi (Atehnkeng et al., 2008a; Donner et al., 2009). Distribution and frequencies of atoxigenic genotypes across target environments in Nigeria were used as criteria to select those atoxigenic African *Aspergillus flavus* VCGs (AAVs) with superiority in adaptation, fitness, and competitiveness (Atehnkeng et al., 2016). In parallel, studies to determine genetic lesions causing atoxigenicity in atoxigenic AAVs were conducted (Donner et al., 2010). Moreover, representative isolates of selected atoxigenic AAVs were evaluated under laboratory conditions (Atehnkeng et al., 2008b), and in farmers' fields across Nigeria to further select competitive and widely adapted atoxigenic AAVs (Atehnkeng et al., 2014). Four superior isolates, each representing a unique AAV composed only of atoxigenic members, are the active ingredient fungi of the biocontrol product Aflasafe® (hitherto called 'biocontrol product'). In 2014, Nigeria's National Agency

for Food and Drug Administration and Control (NAFDAC) approved the full registration of the biocontrol product for unrestricted use in both maize and groundnut across Nigeria (Bandyopadhyay et al., 2016).

Several technologies aiming to improve crop productivity and/or quality—including technologies to reduce crop aflatoxin content—are often evaluated in experimental stations, or on farmer fields managed by researchers, and typically in a few fields (Alaniz Zanon et al., 2013; Weaver et al., 2015; Accinelli et al., 2018; Molo et al., 2019; Weaver and Abbas, 2019). Evaluating technologies under farmer field conditions, in multiple fields of multiple agro-ecological zones (AEZ), and in multiple years, under real-life situations that farmers face can aid in drawing unbiased conclusions about a technology's efficacy (Ortega-Beltran and Bandyopadhyay, 2019). Multi-year, multi-location evaluations are needed to determine if a technology is valuable to farmers. Indeed, this important biocontrol technology has been often questioned and one of the main concerns is that multi-year, multi-area studies are needed to clarify whether aflatoxin management using atoxigenic fungi is a valuable tool, particularly in African contexts (Ehrlich et al., 2015; Njoroge, 2018; Stepman, 2018; Pitt, 2019; Kagot et al., 2019).

The current study reports the longest-term efficacy study of any aflatoxin biocontrol product developed to date. We present results of biocontrol product efficacy across Nigeria at first in (i) field trials in maize and groundnut farmers' fields (2009–2012), and then in (ii) commercially-produced maize (2013–2018). Post-harvest benefits of using pre-harvest biocontrol are discussed as well. The results obtained during these 10 years demonstrate that biocontrol provides stable aflatoxin reductions and is a valuable aflatoxin mitigation tool used by maize and groundnut farmers across Nigeria regardless of their farming practices, crop varieties, or environmental challenges. Biocontrol allows farmers in Nigeria to produce maize and groundnut with aflatoxin concentrations safe for consumption and trade.

MATERIALS AND METHODS

The Active Ingredient Fungi of the Biocontrol Product

The biocontrol product contains four atoxigenic *A. flavus* L morphotype isolates (Ka16127, La3304, La3279, and Og0222) that belong to atoxigenic AAVs native and relatively common in the major maize and groundnut producing areas of Nigeria (Atehnkeng et al., 2014, 2016). Ka16127 was recovered from maize produced in Saminaka, Kaduna state; La3304 and La3279 were recovered from maize cropped in Lafia, Nasarawa state; Og0222 was recovered from maize cropped in Ogbomosho, Oyo state (Atehnkeng et al., 2016). The four strains are maintained in the fungal collection of both IITA and USDA-ARS as sporulating cultures in water vials and silica grain vials for short- and long-term storage, respectively.

The Treated Fields

The biocontrol product was applied in maize and groundnut farmer field trials (2009–2012) and in commercial maize (maize produced for commercial markets) fields (2013–2018) (**Table 1**). All fields belonged to smallholder farmers. Cropping systems, climatic conditions, maize and groundnut cultivars used by farmers, disease and pest pressure, among other factors were variable across the different areas of Nigeria where the biocontrol product was used. Results from field efficacy trials conducted in 2009 and 2010 were used to prepare a dossier for registration of the biocontrol product with NAFDAC (Bandyopadhyay et al., 2016). Trials conducted in 2011 and 2012 were used to demonstrate to regulators efficacy of the product under additional conditions and to create market-linkages with poultry and food industries seeking aflatoxin-compliant crops. Commercial maize fields treated with the biocontrol product during 2013–2018 were part of the AgResults Nigeria Aflasafe^{TM} Challenge Project, hitherto called as the 'AgResults Project' (Bandyopadhyay et al., 2016; AgResults, 2019; Schreurs et al., 2019).

Biocontrol Product Manufacturing

For the field trials (2009–2012), the biocontrol product was produced using a laboratory-scale method described earlier (Cotty et al., 2007; Atehnkeng et al., 2014). Briefly, spore suspensions of each of the four atoxigenic isolates were harvested from 5-day-old cultures on 5-2 agar [5% V8 juice (Campbell Soup Company, Camden, NJ, United States), 2% Bacto-agar (Difco Laboratories Inc., Detroit, MI, United States), pH 5.2 (Cotty, 1994)] using 0.1% TWEEN® 80. Suspensions were adjusted to a concentration of 10^6 spores ml^{-1} using a hemocytometer. White sorghum grains were soaked in water for 2 h, drained, and

TABLE 1 | Number of maize and groundnut samples from fields treated with an aflatoxin biocontrol product and accompanying untreated fields (field efficacy trials, 2009–2012) and samples taken from commercially treated and control maize (2013–2018).

Year	Crop	Purpose	Number of samples[z]	
			Biocontrol-treated	Control
2009	Maize	Farmer field efficacy trials	51	51
	Groundnut	Farmer field efficacy trials	8	8
2010	Maize	Farmer field efficacy trials	14	14
	Groundnut	Farmer field efficacy trials	16	16
2011	Maize	Development of market linkages	199	199
	Groundnut	Development of market linkages	82	82
2012	Maize	Development of market linkages	38	38
2013	Maize	Commercial use by farmers	660	0
2014	Maize	Commercial use by farmers	213	99
2015	Maize	Commercial use by farmers	292	109
2016	Maize	Commercial use by farmers	1,314	0
2017	Maize	Commercial use by farmers	2,451	257
2018	Maize	Commercial use by farmers	2,751	240

[z] *During 2009–2013, grain samples were collected from individual farmer's field. During 2014–2018, field officers of the AgResults Project collected a 5-kg sample from each 30-ton grain lots aggregated from multiple farmers by commercial enterprises. For example, in 2014, 213 maize samples represent a total of 6,390 tons of biocontrol-treated maize.*

autoclaved for 45 min in polyethylene bags (45 cm × 20 cm). Batches of 1-kg of grain were seeded independently with 100 ml of spore suspensions of each atoxigenic active ingredient isolate, mixed to spread the suspension uniformly, and incubated for 18 h at 31°C. Then, grains were dried at 55°C for 4 days to stop fungal growth. Several batches were prepared for each active ingredient isolate. The final product was formulated by combining batches of 2.5 kg of each isolate into a polyethylene bag and mixed by hand shaking. Product in the polyethylene bags were placed within 10-kg-capacity plastic containers, sealed, and stored at room temperature until use.

The biocontrol product applied in commercial maize fields (2013–2018) was produced in the Aflasafe Manufacturing Plant in IITA-Ibadan (Bandyopadhyay et al., 2016). Briefly, spores of the four atoxigenic isolates were obtained as mentioned above for field trials, and each isolate was multiplied separately in glass bottles containing sterilized sorghum grains that were pre-conditioned before in sterile 1-l plastic bottles. The pre-conditioning process raised the moisture content of sorghum grain to 30% by adding sterile distilled water to the bottles, which were subsequently rolled for 4 h on a 240 Vac Benchtop Roller (Wheaton, Millville, NJ, United States). Pre-conditioned grain (30 g) were added to 250-ml glass bottles along with two Teflon balls (1/2″ dia) and autoclaved (20 min, 121°C). Each cooled bottle containing sorghum was independently inoculated with 4 ml of spore suspension of each atoxigenic isolate. After incubation (7 days, 31°C), 125 ml sterile 0.1% TWEEN® 80 was added to each bottle to harvest spores. Bottles were placed on a Roto-Shake Genie reciprocal shaker (Scientific Industries, Bohemia, NY, United States) at 200 rpm for 20 min. The Teflon balls helped to dislodge spores from sorghum grains. For each atoxigenic isolate, a suspension was adjusted to 4×10^7 spores ml^{-1} using an Orbeco-Helling digital direct reading turbidimeter (Orbeco Analytical Systems Inc., Farmingdale, NY, United States) and a nephelometric turbidity unit (NTU) vs. CFU (colony-forming units) standard curve ($y = 49{,}937x$; x = NTU; y = spores ml^{-1}). To prepare 100 kg of the product, a spore suspension (1 l, 4×10^7 spores ml^{-1}) of the constituent atoxigenic isolates were individually combined with 150 ml of a polymer (Sentry™, Precision Laboratories, Waukegan, IL, United States) and 200 ml of a blue non-toxic dye (Prism™, Milliken and Company, Spartanburg, SC, United States), and coated on roasted, sterile sorghum grain with a seed treater (Bandyopadhyay et al., 2016).

Quality of the Biocontrol Product

Samples from biocontrol product batches produced either in the laboratory or the manufacturing plant were examined. For each sample, 100 sorghum grains were plated onto two plates each of 5-2 agar, Nutrient Agar (Lab M, United Kingdom; 28 g l^{-1}, 20 g l^{-1}glucose), and Violet Red Bile Agar (VRBA; Difco Laboratories, 41.5 g l^{-1}, pH 7.4). After incubation (7 days, 31°C) plates were examined to count numbers of grains colonized by *A. flavus* and to detect presence or absence of any other microorganism, including fecal coliforms on VRBA. Spore yield was evaluated by placing 24 grains from each batch in individual wells of 24-well cell culture plates, in triplicate, and incubated as above. After incubation, three replicates of two randomly selected grains were rinsed three times with 10 ml 100% ethanol. The resulting wash from each replicate was mixed with 10 ml distilled water and transferred into a turbidimeter vial. Spore yield was quantified by turbidity as above.

Frequencies of the atoxigenic AAVs to which the four active ingredient isolates belong to were determined in all biocontrol product batches. Microbial isolations were done following protocols previously described (Atehnkeng et al., 2008a; Agbetiameh et al., 2018). *Aspergillus* isolates were characterized and saved as previously described (Agbetiameh et al., 2018; Ezekiel et al., 2019). Mutants of *A. flavus* isolates (*nit*) were generated and saved as previously described (Atehnkeng et al., 2014, 2016). Assignment of isolates to one of the four atoxigenic AAV ingredients of the biocontrol product was conducted by performing vegetative compatibility analyses (VCA). In VCA, fungal suspensions of each atoxigenic AAV tester pair and a *nit* mutant being evaluated were individually seeded into wells, spaced by 1 cm in a triangular pattern, in starch media (36 g l^{-1} dextrose, 20 g l^{-1} soluble starch, 3 g l^{-1} $NaNO_3$, 20 g l^{-1} Bacto-agar, pH 6.0) (Cotty and Taylor, 2003). Complementary *nit* mutants produce regions of prototrophy indicating restoration of a functional nitrate reductase enzyme (Leslie, 1993). Complementation occurs only between *nit* mutants isogenic at loci governing vegetative incompatibility. *Nit* mutants complementing a tester pair were assigned to the atoxigenic AAV defined by that tester pair.

Farmers' Fields

Efficacy Trials

During 2009–2012, efficacy of the biocontrol product was tested by smallholder farmers who voluntarily consented to participate in the experiments in Enugu North, Isi-Uzo, Oji River, and Uzo-Uwani (Enugu state); Birnin-Gwari, Lere, Maigana, Giwa, Soba, and Ikara (Kaduna state); Gwarzo, Tudun Wada, Tsanyanwa, Danbata, Rinin Gado, Rano, Dawakin, Tofa, Albasu, Doguwa, and Warawa (Kano state); and Ogbomosho (Oyo state) (**Figure 1**). All participating farmers along with agricultural extension agents and trainers were made aware of aflatoxins and their impact (e.g., what aflatoxins are, occurrence in crops, impact on health and trade) and trained on use of biocontrol and other management practices. The on-farm efficacy of the biocontrol product was evaluated using paired plot experimental design (**Supplementary Table 1**). For each treated maize and groundnut field, there was an accompanying untreated control field of the same crop separated by at least 500 m to avoid interference from movement of biocontrol isolates from treated to control fields (Bock et al., 2004). To avoid carryover influence of inoculum from 1 year to the next, no treated field used in any year was used in subsequent years. Willingness of farmers to participate in the efficacy trials and the crops grown by them also determined the site of the treated and untreated fields. The size of fields ranged from 0.25 to 5.0 ha. Due to interplot interference from movement of inoculum from treated area to control area in small plot settings (Weaver and Abbas, 2019), it was not possible to replicate treatments several times within the small-sized farmer's fields. Instead, an entire farmer's field was considered as an

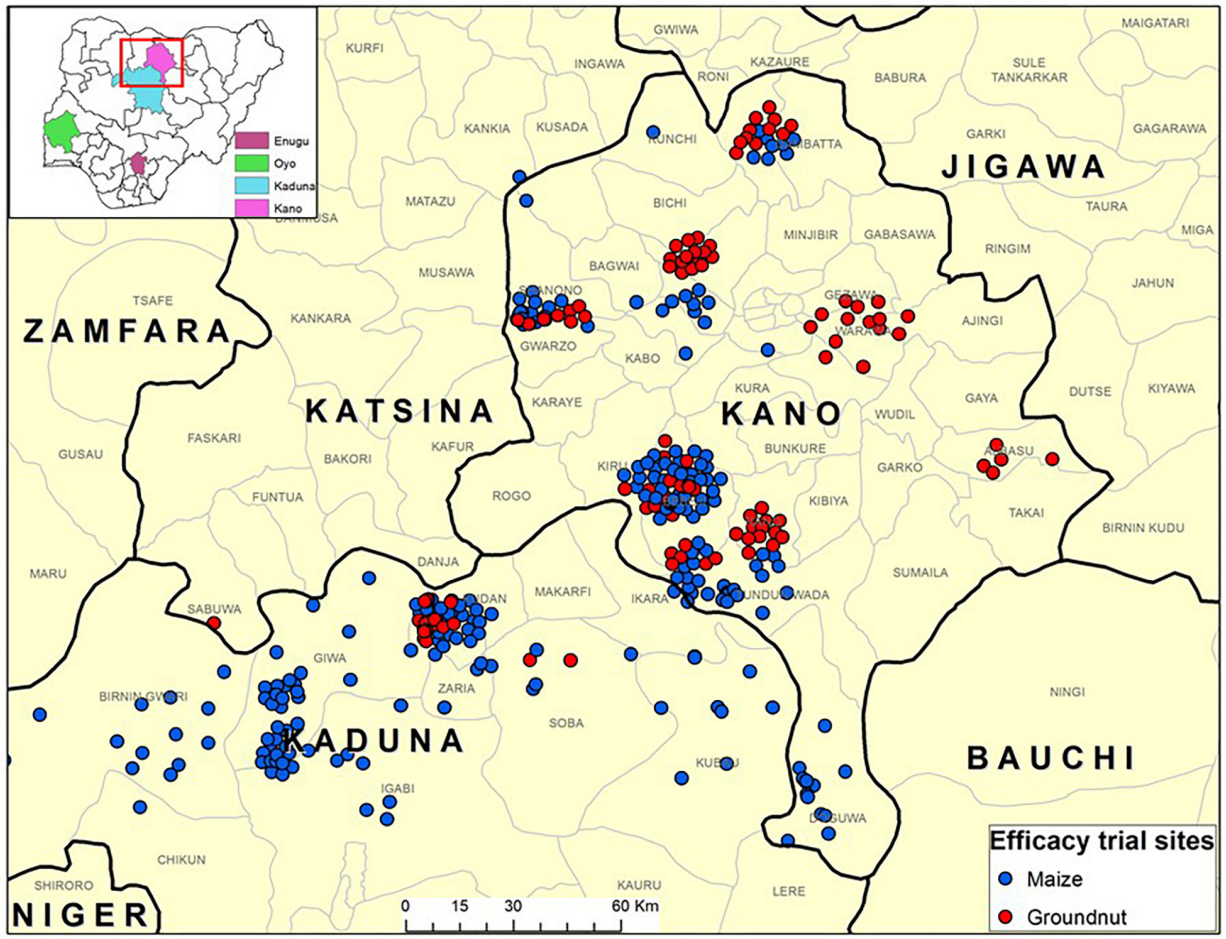

FIGURE 1 | Map of various states of Nigeria (inset) where efficacy trials of an atoxigenic biocontrol product were conducted by maize and groundnut farmers for aflatoxin mitigation during 2009 to 2012. Majority of the trials were conducted in Kano and Kaduna states while a few were conducted in Enugu and Oyo states (inset).

experimental unit (replicate) and was either treated or untreated. The number of replicates varied from year to year and are given in **Table 1**. Farmers grew their crops following their own agronomic practices. In general, farmers' fields were weeded, earthed-up (i.e., piling up soil around the base of the plants), and top-dressed with urea prior to application of the biocontrol product. Within 15 days post biocontrol product application, no other agronomic intervention was made to avoid burying the product. All fields were rain-fed. The number of treated and control fields for each year is given in **Table 1**. The biocontrol product was uniformly tossed across the field by hand, at a rate of 10 kg ha^{-1}. Prior to application, areas of fields were measured using Garmin eTrex GPS units (Garmin Ltd., Olathe, KS, United States) to determine the quantity of product required to treat individual fields. Extension agents, trainers, and/or IITA staff supervised biocontrol product application.

Commercial Use

During 2013–2018, biocontrol was used only in commercial maize fields by farmers and implementers of the AgResults Project (Bandyopadhyay et al., 2016; Schreurs et al., 2019). Briefly, implementers were private (mostly) or public sector enterprises that worked with groups of smallholder farmers and enabled them to produce biocontrol-treated maize. Implementers purchased and distributed the biocontrol product to farmers and had mutually agreed cost-recovery and profit-sharing arrangements. Implementers were selected by the AgResults Project based on several criteria: (i) ability to organize and coordinate smallholder farmers throughout the cropping season, (ii) ability to provide extension services and access to farm inputs, (iii) possess downstream market linkages to efficiently aggregate and sell quality crops at a premium, and (iv) committed to maximizing transparency, disclosing records, and document-sharing of premium payments or other benefits to their participating farmers, among others. Implementers were trained on improved maize production practices and both pre- and post-harvest aflatoxin mitigation strategies, including use of biocontrol. Once trained, the implementers passed the acquired knowledge to their farmers. During the project, in any given year, some implementers worked with less than 100 farmers while

others worked with over 9,000 farmers (**Figure 2**). The biocontrol product was applied in thousands of fields in the states of Benue, Edo, Ekiti, Enugu, Gombe, Kaduna, Kano, Kogi, Ogun, Osun, Oyo, Plateau, Taraba, Zamfara, and the Federal Capital Territory (FCT) (**Figure 3**). It is likely that several farmers applied the product in the same field for more than 1 year leading to an unknown extent of carryover effect of biocontrol inoculum from 1 year to the next. As much as possible, samples from non-treated maize were collected from fields about 500 m away from those of participating farmers to enable comparison of paired treated and non-treated samples. In contrast to the 2009–2012 period, the number of control samples was lower than the number of treated samples in 2014, 2015, 2017, and 2018 (**Table 1**). Control samples were not collected in 2013 and 2016.

Sampling and Sample Treatment

In both 2009 and 2010, soils of all treated and control fields were collected prior to biocontrol application. Soil from the top 2-cm layer were collected by sub-sampling (∼50 times) from three random locations within each field (Cotty, 1997). Composite samples were placed in cotton bags inside sealed plastic bags and transported to IITA-Ibadan Pathology laboratory. Samples were dried at 48°C for 48 h. Following elimination of soil clods in sterile mortars, samples were manually homogenized within the bags, and then stored at 4°C until subjected to further analyses.

During 2009–2012, 25 maize ears were randomly harvested by hand from treated and control fields. The ears were placed in paper bags and transported to the laboratory. Aflatoxins were quantified on two sets of grain: (i) grain immediately after harvest (15 ears) and (ii) grain subjected to simulated poor storage where 10 ears with husks were wetted for 4 h and allowed to dry slowly over a 10-day period, followed by drying at 48°C for 48 h. Ears from each set were husked and shelled for aflatoxin analysis (Atehnkeng et al., 2014). Sub-samples (500 g) were ground using a blender (Waring Commercial, Springfield, MO, United States) for 1 min in a 250 ml stainless steel blending jar (MC-2), which was decontaminated between samples with 80% ethanol to avoid

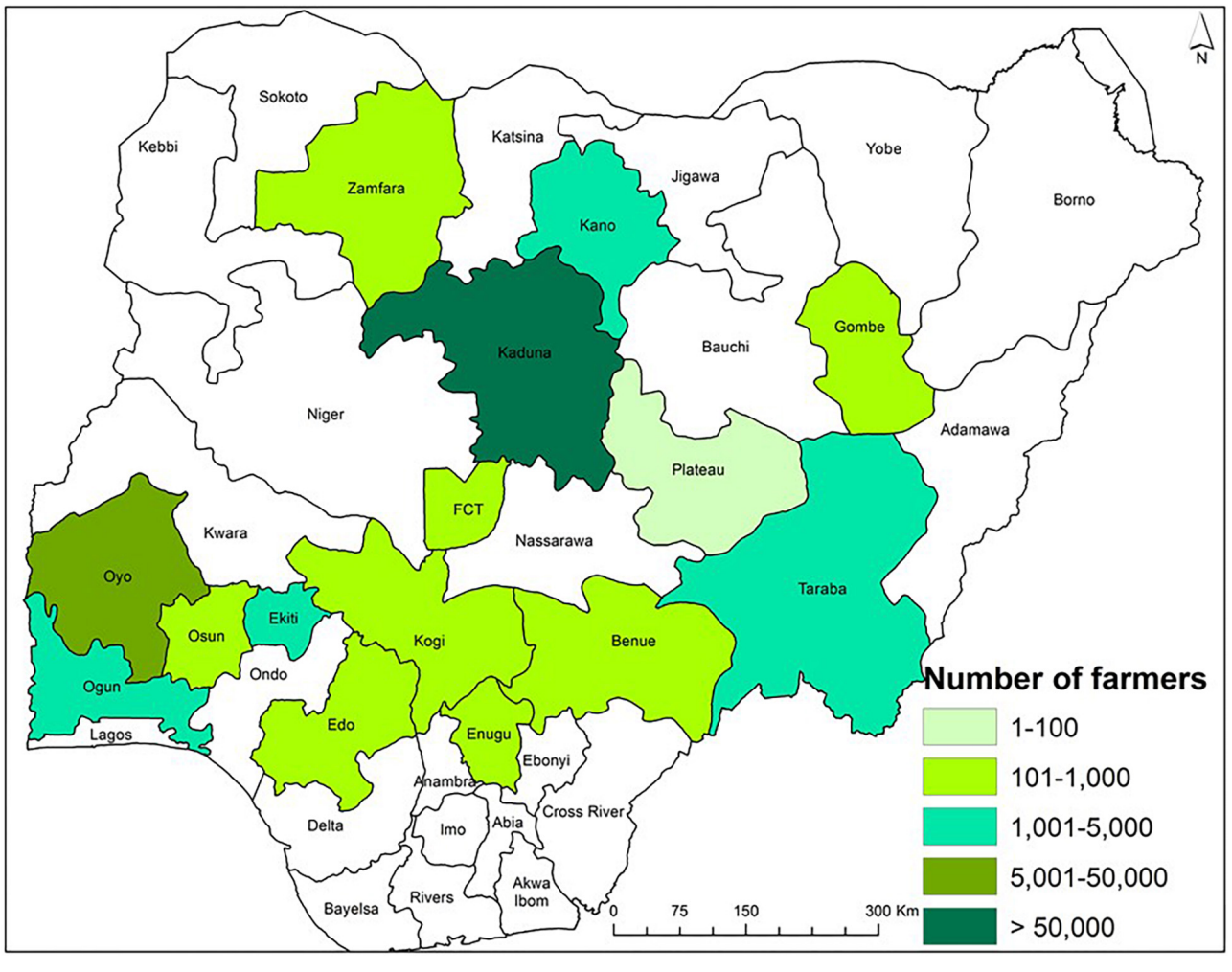

FIGURE 2 | Map of Nigeria showing the number of farmers that participated in the AgResults Project and applied an atoxigenic biocontrol product in maize in various states during 2013 to 2018.

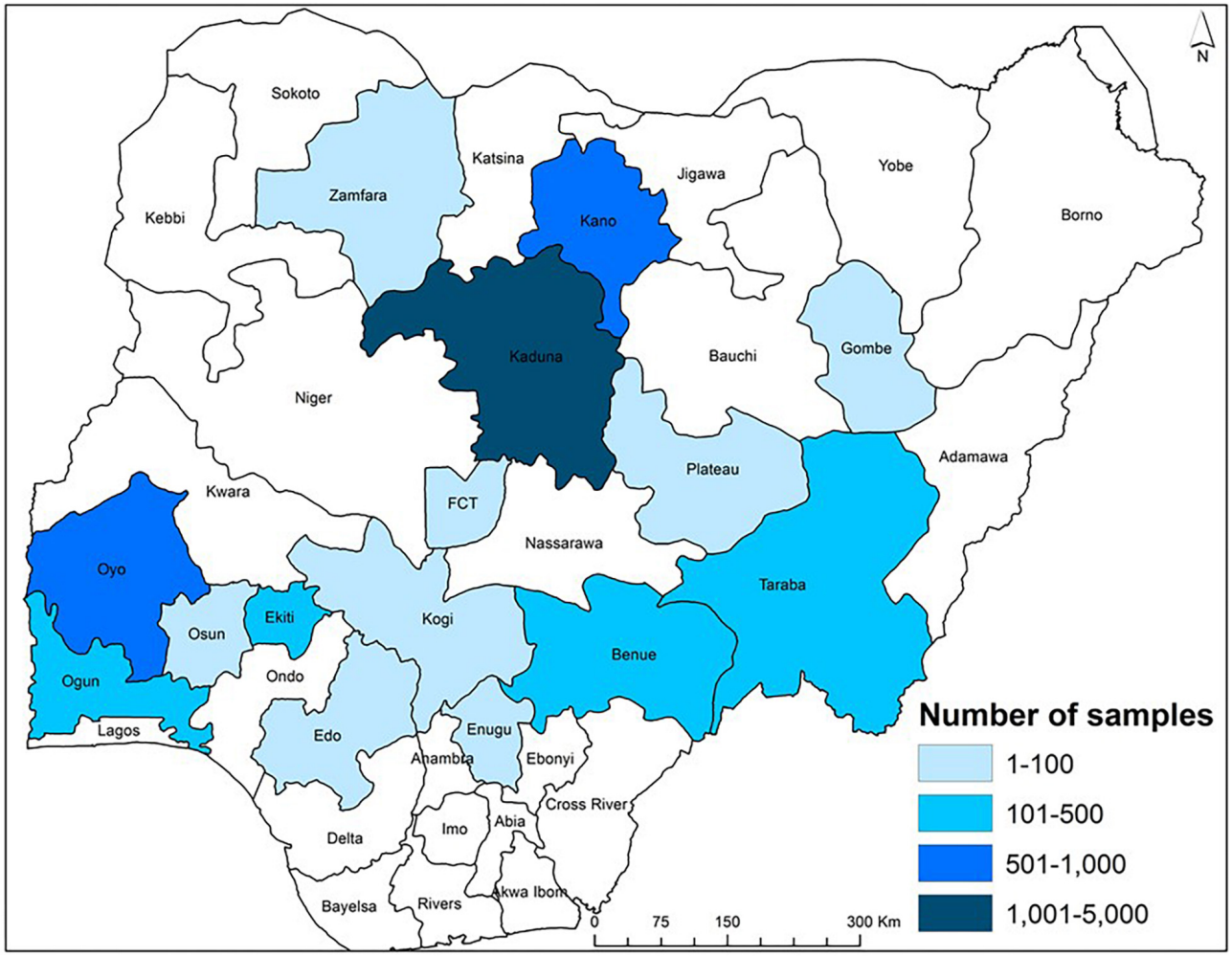

FIGURE 3 | Map of Nigeria showing the range in the number of maize samples collected from aggregation points in various states during 2013 to 2018. Each sample represents approximately a 30-ton grain lot aggregated from farmers who used an atoxigenic biocontrol product on a commercial basis.

cross-contamination. Blended and unblended grain samples were kept at 4°C until analyzed. During 2009–2011, groundnut was randomly harvested by hand from treated and control fields. Five kg of unshelled groundnut were placed in paper bags and transported to the laboratory. Aflatoxins were quantified on two sets of grains: (i) grain immediately after harvest (4 kg, 2009–2011) and (ii) grain (1 kg, 2010–2011) subjected to the simulated poor storage conditions, as described for maize. Pods from each set were manually shelled inside a biological safety cabinet and dried as above. Milling and storage of samples was done as above.

For maize from commercial fields (2013–2018), farmers harvested the ears and the implementers aggregated the maize in their stores. Some implementers assisted farmers with threshing, bagging, labeling, and transporting the maize to aggregation stores. At the aggregation point, maize bags (containing around 100 kg maize) were tagged with farmers' details and arranged according to the quantity supplied by each farmer. Since it was not possible to quantify aflatoxin concentration in individual fields of the >90,000 farmers, grain samples for each 30 tons of treated maize were examined, except for 2013 when samples were collected from individual farmers' fields. Field officers of the AgResults Project collected a 5-kg sample for each 30 tons of aggregated maize by randomly sampling 50 g of maize from each of 100 bags. The samples were transported to IITA-Ibadan, where they were homogenized, milled, and stored as above.

Densities of Aflatoxin-Producing Fungi in Soil Before Biocontrol Product Application and Grain at Harvest

These analyses were conducted for 2009 and 2010 biocontrol field efficacy trials. *Aspergillus* section Flavi fungi were obtained from soil and grain by dilution plate technique on Modified Rose Bengal Agar (MRBA) (Cotty, 1994). Briefly, 1 g sample was suspended in 10 ml sterile distilled water contained in 40 ml glass vials and vortexed for 1 min. Appropriate dilutions were plated on MRBA and plates were incubated for 3 days

(31°C, dark). Incidence of *Aspergillus* species was calculated as CFU g^{-1} of sample. Isolates from plates with less than 10 putative *Aspergillus* section Flavi colonies were transferred onto 5-2 agar and incubated (5 days, 31°C, dark). *Aspergillus* isolates were assigned to their corresponding species based on colony morphology, spore ornamentation, and aflatoxin production profile (Cotty, 1989; Cotty and Cardwell, 1999; Frisvad et al., 2019). Isolates were saved as agar plugs (3-mm dia) of sporulating cultures in 4 ml vials containing 2 ml sterile distilled water and maintained at room temperature until further characterization.

Vegetative Compatibility Analyses

Frequencies of the AAVs to which the four active ingredient isolates of the biocontrol product belong to were determined in both soils before application and grains at harvest during 2009 and 2010. Mutants of isolates (*nit*) were generated as previously described (Atehnkeng et al., 2014, 2016). Assignment of isolates to one of the four atoxigenic AAVs was conducted by performing VCA as above.

Quantifying Aflatoxin Concentration in Crops at Harvest and After Poor Storage

For crops examined during 2009–2012 (at harvest and after simulated poor storage), aflatoxins were extracted from sub-samples (20 g ground sample) by adding 100 ml 70% methanol (Atehnkeng et al., 2008a) for maize and 100 ml 80% methanol for groundnut (Cole and Dorner, 1993). Aflatoxin extraction and quantification was conducted as previously described (Atehnkeng et al., 2008a).

For maize from commercial fields (2013–2018), aflatoxins were quantified using GIPSA-approved Neogen Reveal® Q+ for Aflatoxin kit (Neogen Corp., Lansing, MI, United States). Briefly, a 20-g sub-sample was transferred into a 500 ml media bottle and 100 ml 65% ethanol was added. The mixture was shaken for 3 min using an orbital shaker at 200 rpm, allowed to settle for 3 min, and then filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, United Kingdom) into a Tri-Pour® beaker. Thereafter, 500 µl of sample diluent was transferred to a sample cup and 100 µl of sample filtrate was added. A 100-µl aliquot of diluted sample was transferred into a new cup and mixed thoroughly and mixed by aspiration. A new test lateral flow strip was placed in the sample cup for 6 min ensuring that the strip touched the mixture. Then the strip was removed and read either on a Neogene AccuScan® Pro or Gold Reader.

Data Analysis

Data on CFU g^{-1}, *Aspergillus* species distribution, incidence of atoxigenic AAVs, and aflatoxin concentration (response variable, x) were transformed using the equation $y = \log_{10}(1 + x)$ to stabilize the variance prior to analysis. Depending on the comparisons, means were separated using paired t-tests (PROC T TEST, $\alpha = 0.05$) or protected Fisher's least significant difference test (LSD, $\alpha = 0.05$) using SAS software (version 9.2, SAS Institute Inc., Cary, NC, United States). Untransformed data are presented in summary tables and graphs in this paper.

RESULTS

Quality of the Biocontrol Products

During the 10-year study, 382 batches of the biocontrol product were produced and examined for quality control parameters. Batches include the biocontrol formulation produced using both the laboratory- and industrial-scale processes. The quantity of the biocontrol product produced per year varied (range = 1.54 tons in 2009 to 779.42 tons in 2017). In all examined batches, 100% of carrier grains were colonized by *A. flavus*. Other microorganisms were not found in any of the examined carrier grains of any of the 382 batches. VCA revealed that the recovered fungi belonged solely to the four atoxigenic AAV active ingredients of the biocontrol product. In general, each of the four atoxigenic AAVs was found on 25 ± 8% of the carrier grains. On an average, there were 3,500 ± 500 CFU g^{-1} of product.

Fungal Densities in Field Efficacy Trials: 2009 and 2010

Across treated and control fields, *Aspergillus* section Flavi densities ranged from 304 CFU g^{-1} to 1,050 CFU g^{-1} of soil (**Table 2**). In all cases, population densities in soil prior to biocontrol application were similar ($P > 0.05$) in treated and control fields. At harvest, maize and groundnut grains from treated fields contained *Aspergillus* section Flavi densities ranging from 185 CFU g^{-1} to 4,117 CFU g^{-1} compared to 72 CFU g^{-1} to 4,839 CFU g^{-1} in grains from control fields (**Table 2**). There were no significant ($P > 0.05$) differences in CFU g^{-1} between grains from treated and control fields.

Distribution of *Aspergillus* Section Flavi in Field Efficacy Trials: 2009 and 2010

The native *Aspergillus* fungi found in soil were potentially high aflatoxin producers in all farmers' fields where efficacy trials were conducted. In 2009, groundnut fields had high levels of

TABLE 2 | Colony-forming units (CFU) g^{-1} of *Aspergillus* section *Flavi* fungi in soil before biocontrol application and in maize and groundnut grains harvested from farmers' fields that were either treated or not treated during 2009 and 2010.

Year	Crop	*n*	Treatment	CFU g^{-1} [z]	
				Soil before inoculation	Grain at harvest
2009	Maize	51	Biocontrol	957[a]	1,387[a]
		51	Control	850[a]	1,325[a]
	Groundnut	8	Biocontrol	375[a]	185[a]
		8	Control	1,050[a]	72[a]
2010	Maize	14	Biocontrol	304[a]	4,117[a]
		14	Control	491[a]	4,839[a]
	Groundnut	16	Biocontrol	1,012[a]	1,963[a]
		16	Control	748[a]	2,447[a]

[z] CFU g^{-1} were log transformed ($\log_{10}$[CFU g^{-1} + 1]) prior to analysis to stabilize the variance. The means of paired treated and control fields for a crop within each column with different letters are significantly different according to Student's t-test ($\alpha = 0.05$).

TABLE 3 | Frequencies of *Aspergillus* section *Flavi* fungi in soil before biocontrol application, and in maize and groundnut grains from biocontrol -treated and control fields during 2009 and 2010.

Year	Crop	*n*	Treatment	*Aspergillus* fungi distribution (%)[y]							
				Soil before inoculation[z]				Grain at harvest			
				L	S_{BG}	P	T	L	S_{BG}	P	T
2009	Maize	51	Biocontrol	85.9^{aA}	3.5^{aB}	1.0^{aB}	9.6^{aB}	99.7^{aA}	0.3^{aB}	0.0^{aB}	0.0^{aB}
		51	Control	87.4^{aA}	2.7^{aB}	1.7^{aB}	8.2^{aB}	93.3^{aA}	5.9^{aB}	0.5^{aB}	0.3^{aB}
	Groundnut	8	Biocontrol	99.2^{aA}	0.5^{bB}	0.3^{aB}	0.0^{aB}	100.0^{aA}	0.0^{aB}	0.0^{aB}	0.0^{aB}
		8	Control	82.4^{bA}	11.5^{aB}	0.3^{aB}	5.8^{aB}	95.6^{aA}	1.6^{aB}	2.8^{aB}	0.0^{aB}
2010	Maize	14	Biocontrol	89.9^{aA}	7.1^{aB}	3.0^{aB}	–	100.0^{aA}	0.0^{bB}	0.0^{aB}	–
		14	Control	77.9^{aA}	14.8^{aB}	7.3^{aB}	–	83.4^{bA}	16.3^{aB}	0.3^{aC}	–
	Groundnut	16	Biocontrol	97.8^{aA}	2.2^{aB}	0.0^{aB}	–	100.0^{aA}	0.0^{aB}	0.0^{aB}	–
		16	Control	96.3^{aA}	2.8^{aB}	0.9^{aB}	–	95.7^{aA}	3.5^{aB}	0.8^{aB}	–

[y] *The frequency (%) of fungi (x) were log transformed ($log_{10}[x + 1]$) prior to analysis to stabilize the variance. L, A. flavus L morphotype; S_{BG}, S_{BG} strains; P, A. parasiticus; T = A. tamarii (not found in 2010).* [z] *The means of paired treated and control fields for a crop within each column with different lowercase letters are significantly different (Student's t-test, α = 0.05). Means of fungi within a row with different uppercase letters are significantly different (Fisher's LSD test, α = 0.05).*

S_{BG} strains while in 2010, maize fields had high levels of both S_{BG} strains and *A. parasiticus* before treatment (**Table 3**). In treated and control maize and groundnut fields, the *A. flavus* L morphotype dominated both soils before inoculation and grains at harvest. In the corresponding treated and control fields, frequencies of each fungal type were similar ($P > 0.05$) except for both the L morphotype and S_{BG} strains in treated groundnut soil during 2009 (**Table 3**) and treated maize grain during 2010 (**Table 3**). When comparing fungal types within treated or control samples, in all cases, the L morphotype had significantly ($P > 0.05$) higher frequencies than the other types (**Table 3**). Members of the S_{BG} strain, which produce large concentrations of B and G aflatoxins, were rarely detected in treated maize and *A. parasiticus* was never detected in treated maize or groundnut fields (**Table 3**). These aflatoxin producers were almost completely replaced by *A. flavus* L morphotype fungi, to which the four atoxigenic active ingredient isolates of the biocontrol product belong (**Table 3**).

Vegetative Compatibility Analyses in Samples From Field Efficacy Trials: 2009 and 2010

In both 2009 and 2010, frequencies of the biocontrol active ingredient AAVs in soils before inoculation were not significantly ($P > 0.05$) different between fields to be treated (range = 1.1–6.5%) and their respective control fields (range = 0.7–7.8%) (**Table 4**). The observed frequencies prior to product application demonstrate that the biocontrol active ingredient AAVs are relatively common across the major maize and groundnut producing regions of Nigeria.

Frequencies of the biocontrol product active ingredient AAVs were always higher ($P < 0.05$) in treated crops (range = 68.8–83.8%) than in control crops (range = 2.5–11.6%). In treated fields, the frequencies of atoxigenic AAVs in soil before inoculation were always lower ($P < 0.05$) than in grains at harvest (**Table 4**). In contrast, in all control fields, frequencies of atoxigenic AAVs were similar ($P > 0.05$) in soils before inoculation and grains at harvest (**Table 4**).

Frequencies did not differ significantly among the biocontrol product AAVs in soils before treatment in control plots (**Table 5**). There were, however, some differences ($P < 0.05$) among atoxigenic AAV frequencies in maize soil to be treated during both 2009 and 2010 and groundnut soil to be treated during 2010 (**Table 5**). Ka16127 had higher frequencies than the other active ingredients in maize soil during 2009. Both La3279 and Ka16127 had higher frequencies than the other two atoxigenic AAVs in both maize and groundnut soil during 2010. In grains of treated fields, both La3279 and Ka16127 generally dominated communities of both crops during both years (**Table 5**). In grain

TABLE 4 | Incidence of atoxigenic African *Aspergillus flavus* vegetative compatibility groups (AAVs) active ingredients of a biocontrol product in soil before its application, and maize and groundnut grains from treated fields and control fields during 2009 and 2010.

Year	Crop	Treatment	Biocontrol AAVs (%)[y]	
			Soil before inoculation[z]	Grain at harvest
2009	Maize	Biocontrol	1.1^{aB}	75.9^{aA}
		Control	0.7^{aA}	11.6^{bA}
	Groundnut	Biocontrol	6.5^{aB}	71.9^{aA}
		Control	7.8^{aA}	4.7^{bA}
2010	Maize	Biocontrol	3.9^{aB}	68.8^{aA}
		Control	3.5^{aA}	2.5^{bA}
	Groundnut	Biocontrol	4.8^{aB}	83.8^{aA}
		Control	2.3^{aA}	3.7^{bA}

[y] *Percentage of the four atoxigenic active ingredient AAVs = [(y × 100)/n] where y, number of isolates belonging to any of the four AAVs, and n, total number of A. flavus L morphotype isolates. Isolates were assigned to AAVs using vegetative compatibility analysis.* [z] *The means of paired treated and control fields for a crop within each column with different lowercase letters are significantly different (Student's t-test, α = 0.05). Means of the AAVs within the row with different uppercase letters are significantly different (Student's t-test, α = 0.05).*

TABLE 5 | Incidence (%) of the four atoxigenic African *Aspergillus flavus* vegetative compatibility groups (AAVs) constituting an aflatoxin biocontrol product in soil before its application and in maize and groundnut grains from treated and control fields during 2009 and 2010.

Year	Crop	AAVs	Incidence (%) of AAV[y]			
			Soil before treatment[z]		Grain at harvest	
			Treated	Control	Treated	Control
2009	Maize	La3279	0.4[b]	0.0[a]	31.9[a]	7.0[a]
		Ka16127	1.1[a]	0.4[a]	20.3[b]	1.7[b]
		La3304	0.2[b]	0.4[a]	12.5[bc]	1.8[b]
		Og0222	0.1[b]	0.1[a]	9.1[c]	1.2[b]
	Groundnut	La3279	3.9[a]	3.1[a]	29.7[a]	1.6[a]
		Ka16127	0.8[a]	3.1[a]	19.0[ab]	0.8[a]
		La3304	0.1[a]	0.8[a]	7.0[b]	1.6[a]
		Og0222	0.8[a]	0.8[a]	15.6[ab]	0.8[a]
2010	Maize	La3279	1.8[a]	0.9[a]	29.0[a]	0.9[a]
		Ka16127	1.3[ab]	0.4[a]	21.4[ab]	1.3[a]
		La3304	0.9[b]	0.0[a]	16.1[ab]	0.9[a]
		Og0222	0.0[b]	0.0[a]	7.6[b]	0.4[a]
	Groundnut	La3279	1.6[ab]	0.4[a]	35.2[a]	4.3[a]
		Ka16127	2.7[a]	0.8[a]	29.7[a]	2.3[ab]
		La3304	0.4[b]	0.0[a]	5.1[b]	0.4[b]
		Og0222	0.0[b]	0.0[a]	3.5[b]	0.4[b]

[y]Percentage of AAVs = [(y × 100)/n] where y, number of isolates belonging to the specific AAVs, and n, total number of A. flavus L morphotype isolates. Isolates were assigned to AAVs using vegetative compatibility analysis. [z]The means of paired treated and control fields for a crop within each column with different lowercase letters are significantly different (Fisher's LSD test, α = 0.05).

from control fields, La3279 had higher frequencies in maize (2009) and groundnut (2010); Ka16127 and La3279 had similar frequencies in groundnut (2010) (**Table 5**).

Aflatoxin Concentrations in Grain at Harvest and After Poor Storage

At harvest, treated crops from field efficacy trials (2009–2012) generally contained low aflatoxin content and significantly ($P < 0.05$) less aflatoxins (82–95% less) than untreated crops (**Table 6**). The only case in which the average of treated crops contained >20 ppb total aflatoxin was maize in 2010 (21 ppb). However, during that year, average aflatoxin content in maize from the 14 examined control fields was 372.4 ppb (**Table 6**), a high and unsafe level.

When crops were subjected to simulated poor storage conditions, the protection provided by biocontrol continued, with treated crops accumulating 80% to 100% less aflatoxins than untreated crops (**Table 6**). Aflatoxin content in untreated crops increased dramatically due to simulated poor storage conditions. For example, the total aflatoxin content in maize at harvest from 2009 was 14.8 ppb and after simulated poor storage it increased to 245.1 ppb. Large increases in aflatoxin content occurred in the other poorly stored control grains, with up to 40-fold aflatoxin increase (maize 2012; **Table 6**).

In commercial maize grain samples, there were 72–94% less aflatoxin in treated maize compared to controls during 2015, 2017, and 2018 (**Table 7**). During years when aflatoxin levels were <10 ppb in untreated grains (2014 and 2015), aflatoxin reductions provided by the biocontrol were less pronounced (72–76%) compared to years when untreated maize contained high aflatoxin levels (88–94%; **Table 7**). A few samples with high aflatoxin level were encountered among treated samples in 2016 and 2017 (**Table 7**). Variance of aflatoxin concentration in grain samples from treated fields was 53% (in 2014) to 99% (in 2018) lower than samples from control fields (**Table 7**). Although aflatoxin concentration was measured in grains from treated fields in 2013 and 2016, aflatoxin reduction could not be quantified due to lack of samples from control fields.

TABLE 6 | Total aflatoxin concentration in freshly harvested and poorly stored maize and groundnut grains from biocontrol-treated and control fields in Nigeria during 2009 to 2012.

Crop and its stage	Treatment	Aflatoxin (ppb)							
		2009		2010		2011		2012	
		Mean[x]	Red (%)[y]	Mean	Red (%)	Mean	Red (%)	Mean	Red (%)
At harvest									
Maize	Biocontrol	2.7[b]	82	21.0[b]	94	3.7[b]	83	1.8[b]	86
	Control	14.8[a]		372.4[a]		22.3[a]		12.9[a]	
Groundnut	Biocontrol	0.0	–	2.7[b]	95	3.1[b]	85	–	–
	Control	0.0		54.6[a]		20.3[a]		–	
After simulated poor storage									
Maize	Biocontrol	18.4[b]	82	26.2[b]	93	25.8[b]	89	50.3[b]	90
	Control	245.1[a]		399.0[a]		238.2[a]		527.4[a]	
Groundnut	Biocontrol	–[z]	–	9.5[b]	80	29.9[b]	80	–	–
	Control	–		47.5[a]		152.4[a]		–	

[x]The means of paired treated and control fields for a crop within each column with different letters are significantly different according to the Student's t-test (α = 0.05). Mean aflatoxin concentration in grains from maize fields (51 in 2009, 14 in 2010, 199 in 2011, and 38 in 2012) and groundnut fields (8 in 2009, 16 in 2010, and 82 in 2011). [y]Red denotes aflatoxin reduction (%) = [1 – (aflatoxin in treated sample)/(aflatoxin in control sample)] × 100. [z]Not tested.

TABLE 7 | Aflatoxin concentration in samples from commercial maize grain aggregated from biocontrol-treated and control fields by commercial enterprises in Nigeria during 2013–2018.

Year	Treatment	Min (ppb)	Max (ppb)	Variance	Mean (ppb)[x]	Reduction (%)[y]
2013	Treated	0.0	70	11	0.5	–
	Control	–[z]	–	–	–	
2014	Treated	0.0	141	108	1.7[b]	72
	Control	0.0	103	233	6.1[a]	
2015	Treated	0.0	134	106	2.4[b]	76
	Control	0.1	147	711	9.7[a]	
2016	Treated	0.0	1,094	6,347	16.9	–
	Control	–	–	–	–	
2017	Treated	0.0	870	473	3.6[b]	88
	Control	0.0	1,971	17,995	29.6[a]	
2018	Treated	0.0	174	144	3.3[b]	94
	Control	0.0	738	11,348	55.3[a]	

[x]The mean of aflatoxin concentration in sampled grains from treated (660 in 2013; 213 in 2014; 292 in 2015; 1,314 in 2016; 2,451 in 2017; and 2,751 in 2018); and untreated maize samples (99 in 2014, 109 in 2015, 257 in 2017, and 240 in 2018). The means of treated and control pairs for any year within each column with different letters are significantly different according to the Student's t-test (α = 0.05). [y]Aflatoxin reduction (%) = [1 – (aflatoxin in treated sample)/(aflatoxin in control sample)] × 100. [z]Not tested.

Samples Meeting Standards

Treated crops in efficacy trials had higher proportions of samples below the European Union 4 ppb maximum allowable level for total aflatoxins both at harvest (2009–2012) and after simulated poor storage (2009 and 2010) compared to controls (**Table 8**). In 2009, 2010, and 2012, none of the treated crops exceeded the US action level of 20 ppb total aflatoxins at harvest. In 2011, only 6.5% of the treated maize and 9.8% of the treated groundnut had >20 ppb total aflatoxins at harvest (**Table 8**), even though during that year high total aflatoxin content occurred in both control maize (avg. = 372.4 ppb) and control groundnut (avg. = 54.6 ppb) (**Table 6**). Also see **Supplementary Table 2**.

After simulated poor storage, frequencies of biocontrol-treated crops containing <4 ppb total aflatoxins ranged from 7.9 to 31.4% while for untreated crops the range was 0–5.5% (**Table 8**). The proportion of crops containing >20 ppb total aflatoxins ranged from 28.6 to 73.7% for treated crops and from 71.4 to 100% for control crops (**Table 8**).

For commercially produced maize treated with the biocontrol product during the 2013 to 2018 seasons, the proportion of samples containing <4 ppb total aflatoxins ranged from 65.8 to 98.5% (**Table 9**) with only 0.6–9.7% having >20 ppb total aflatoxins. The proportion of crops from control fields examined in 2014, 2015, 2017, and 2018 containing <4 ppb total aflatoxins were 81, 84, 43, and 25%, respectively. The proportion of control samples containing >20 ppb total aflatoxins in the same years were 9.1, 11.9, 23.0, and 37.1%, respectively (**Table 9**). Also see **Supplementary Table 3**.

DISCUSSION

The current study provides a decade-long summary of efficacy of the aflatoxin biocontrol product Aflasafe® in maize and groundnut cropped in Nigeria. Aflasafe® is the first aflatoxin biocontrol product registered for use in any African nation and the third worldwide. The 10-year record provides substantial evidence of the stability of the biocontrol product efficacy in limiting aflatoxin content in farmers' fields across the diverse cropping systems of Nigeria within fluctuating and sometimes challenging environmental conditions. Biocontrol-treated crops became contaminated with significantly less aflatoxins than untreated crops. Most of the treated crops met the stringent tolerance thresholds of local and international food and feed premium markets. A significant proportion (94%) of the 7,681 biocontrol-treated commercial crop samples that were examined had less than the World Food Program maximum limit of 10 ppb total aflatoxins. Initially (2009–2012), biocontrol efficacy was evaluated in a relatively modest number of maize and groundnut fields (14–199 for maize; 8–82 for groundnut; total of 408 treated and 408 control fields). However, the number of fields and the size of each field (∼1 ha) during the 2009–2012 efficacy evaluations were considerably larger than other reported biocontrol efficacy studies that used fewer than five replicates per year with some plots less than 0.01 ha (Alaniz Zanon et al., 2013, 2016; Pitt et al., 2015; Weaver et al., 2015; Molo et al., 2019; Weaver and Abbas, 2019). After the testing and refinement phase, aflatoxin biocontrol usage in Nigeria grew exponentially through the AgResults Project (2013–2018) in which thousands of farmers used biocontrol and other good agricultural practices. Over 210,000 tons of maize with acceptable aflatoxin concentrations (<10 ppb) were produced through the AgResults Project. A large portion of the commercially produced biocontrol-treated maize entered premium markets in Nigeria while the other portion was either consumed by the farmers and their families or sold in local, informal markets (Schreurs et al., 2019). Reduced aflatoxin exposure brought health benefits for maize famers and consumers, and economic benefits for farmers and associated industries (Narayan et al., 2019).

Several crops grown in Nigeria frequently become contaminated with unsafe aflatoxin concentrations (Bandyopadhyay et al., 2007; Singh and Cotty, 2017; Ezekiel et al., 2018, 2019; JECFA, 2018) leading to unacceptable aflatoxin exposure in humans (Ezekiel et al., 2014). This negatively impacts health, income, and trade. One example is lack of access of Nigerian groundnuts to European markets due to high aflatoxin levels. Agricultural, climatic, cultural, infrastructural, and institutional practices and/or conditions all contribute to aflatoxin contamination (Bandyopadhyay et al., 2016). Therefore, aflatoxin management is best with a holistic approach (Ayalew et al., 2017; Logrieco et al., 2018). Intervention during the initial stages of crop infection by aflatoxin producers is a desired component of such strategies and use of atoxigenic *A. flavus* as biocontrol agents during crop development is effective (Cotty and Mellon, 2006).

TABLE 8 | Proportion of farmers meeting various total aflatoxin standards in freshly harvested and poorly stored maize and groundnut grains from farmers' fields that were either treated or not treated (control) with a biocontrol product in Nigeria during 2009 to 2012[u].

Crop and its stage[v]	Aflatoxin content (ppb)[w]	Proportion of farmers' fields (%)[x]							
		2009		2010		2011		2012	
		Biocontrol	Control	Biocontrol	Control	Biocontrol	Control	Biocontrol	Control
At harvest									
Maize	<4	76.5[a]	29.4[b]	50.0[a]	0.0[b]	50.8[a]	12.6[b]	78.9[a]	52.6[b]
	<10[y]	96.1[a]	54.9[a]	64.3[a]	21.4[b]	71.9[a]	30.7[b]	94.7[a]	71.1[b]
	<20[y]	100.0[a]	72.5[a]	100.0[a]	35.7[b]	93.5[a]	70.9[b]	100.0[a]	76.9[b]
	>20	0.0[b]	27.5[a]	0.0[b]	64.3[a]	6.5[b]	29.1[a]	0.0[b]	23.1[a]
Groundnut	<4	100.0[a]	100.0[a]	62.5[a]	68.8[a]	50.0[a]	22.0[b]	–	–
	<10	0.0	0.0	81.3[a]	87.5[a]	75.6[a]	41.5[b]	–	–
	<20	0.0	0.0	100.0[a]	93.8[a]	90.2[a]	58.5[b]	–	–
	>20	0.0	0.0	0.0[a]	6.2[a]	9.8[b]	41.5[a]	–	–
After simulated poor storage									
Maize	<4	31.4[a]	0.0[b]	21.4[a]	0.0[b]	31.3[a]	5.5[b]	7.9[a]	0.0[b]
	<10	56.9[a]	0.0[b]	57.1[a]	9.7[b]	44.5[a]	10.9[b]	10.5[a]	0.0[b]
	<20	70.6[a]	3.9[b]	71.4[a]	28.6[b]	63.3[a]	18.0[b]	26.3[a]	0.0[b]
	>20	29.4[b]	96.1[a]	28.6[b]	71.4[a]	36.7[b]	82.0[a]	73.7[b]	100.0[a]
Groundnut	<4	–[z]	–	73.3[a]	40.0[b]	75.8[a]	28.8[b]	–	–
	<10	–	–	86.7[a]	40.0[b]	83.3[a]	37.9[b]	–	–
	<20	–	–	93.3[a]	73.3[b]	87.9[a]	43.9[b]	–	–
	>20	–	–	6.7[b]	26.7[a]	12.1[b]	56.1[a]	–	–

[u]For maize, the number of pairs of treated and control fields were 51 in 2009, 14 in 2010, 199 in 2011, and 38 in 2012. The respective numbers for groundnut were 8 in 2009, 16 in 2010, and 82 in 2011. [v]Maize grain and groundnut kernel samples for aflatoxin analysis were either processed immediately after harvest or subjected to simulated poor storage conditions. [w]<4 ppb is the European Union maximum total aflatoxin limit for human consumption; <10 ppb is the World Food Program maximum total aflatoxin limit; <20 ppb is the United States Food and Drug Administration action level for total aflatoxins in food; and >20 ppb is universally considered unacceptable for human consumption. [x]The means of paired treated and control fields of a crop for a year within each row with different letters are significantly different according to the Student's t-test (α = 0.05). [y]The number of samples within a category was divided by total number of samples and then the quotient was divided by 100 to calculate the percentages. Note that the number of samples having <4 ppb aflatoxins were added to the number of samples having 4 to <10 ppb aflatoxins to derive the number of samples with <10 ppb aflatoxins. Similarly, the counts for <20 ppb also contained counts for samples with <10 ppb, and thus, also counts for samples with <4 ppb. [z]Groundnut not examined during the year/stage.

TABLE 9 | Proportion of samples meeting various total aflatoxin standards in freshly harvested maize grain from farmers' fields that were treated commercially with a biocontrol product in Nigeria during 2013–2018 and from control fields in nearby locations that did not apply the product.

Aflatoxin content (ppb)[x]	Proportion of samples (%)[w]											
	2013		2014		2015		2016		2017		2018	
	Biocontrol	Control	Biocontrol	Control	Biocontrol	Control	Biocontrol	Control	Biocontrol	Control	Biocontrol	Control
<4[y]	98.5	–[z]	93.9	80.8	94.9	84.4	65.8	–	89.7	43.2	87.9	24.6
<10	99.1	–	96.2	82.8	96.9	84.4	85.6	–	93.7	49.8	94.0	50.0
<20	99.4	–	98.6	90.9	98.6	88.1	90.3	–	96.5	77.0	97.3	62.9
>20	0.6	–	1.4	9.1	1.4	11.9	9.7	–	3.5	23.0	2.7	37.1

[w]The number of examined samples were 660 in 2013; 213 in 2014; 292 in 2015; 1,314 in 2016; 2,451 in 2017; and 2,751 in 2018. The respective numbers for untreated samples were 99 in 2014, 109 in 2015, 257 in 2017, and 240 in 2018. In 2013, 660 samples were collected from stores of individual farmers' fields soon after harvest. Each sample during 2014–2018 represent a grain lot of approximately 30 tons aggregated by commercial enterprises from farmers. [x]<4 ppb is the European Union maximum total aflatoxin limit for human consumption; <10 ppb is the World Food Program maximum total aflatoxin limit; <20 ppb is the United States Food and Drug Administration action level for total aflatoxins in food; and >20 ppb is universally considered unacceptable for human consumption. Maize grain samples for aflatoxin analysis were processed shortly post-harvest after aggregation. [y]The number of samples within a category was divided by total number of samples and then the quotient was divided by 100 to calculate the percentages. Note that the number of samples having < 4 ppb aflatoxins were added to the number of samples having 4 to <10 ppb aflatoxins to derive the number of samples with <10 ppb aflatoxins. Similarly, the counts for <20 ppb also contained counts for samples with <10 ppb, and thus, also counts for samples with <4 ppb. [z]Samples from control fields not collected in 2013 and 2016.

Improvement in Biocontrol

The atoxigenic biocontrol technology developed by USDA-ARS uses a single atoxigenic genotype as active ingredient fungus (Cotty et al., 2007). This strategy protects crops from field to plate because after sporulating in the field and becoming associated with the treated crop, the beneficial fungi move with

the crops to storage and continue to prevent aflatoxin production should conditions for production again become favorable (Mehl et al., 2012; Atehnkeng et al., 2014; Bandyopadhyay et al., 2016; Senghor et al., 2019). The adaptation and improvement of the technology for use in SSA included use of four atoxigenic AAVs as active ingredient fungi rather than a single isolate (Bandyopadhyay et al., 2016). Use of multiple atoxigenic isolates belonging to diverse AAVs may provide longer-term protection compared to using single genotypes (Probst et al., 2011; Mehl et al., 2012). In the current study, four atoxigenic AAVs native to Nigeria were used in a biocontrol formulation to limit maize and groundnut aflatoxin content.

Testing Under Natural Setting and Product Registration

When IITA and USDA-ARS initiated talks with NAFDAC, the biopesticide regulator in Nigeria, to develop an aflatoxin biocontrol program for Nigeria, it was decided that the work would require applications in multiple fields covering several agro-ecologies over multiple years. Testing the biocontrol product under natural settings without researcher interventions (i.e., farmers used their traditional practices) other than farmer training in application method and provision of the biocontrol product for a single application allowed testing under real-life situations that smallholder Nigerian farmers face. A natural-setting strategy, although in large-scale commercial agriculture, was conducted during efficacy trials to register the biocontrol products AF36 (Cotty, 2006) and Afla-Guard® (Dorner, 2004) in the United States (USEPA, 2003, 2004). The results from 2009 and 2010, used for preparing a dossier for registration, demonstrated that, compared to control, biocontrol application (i) did not increase fungal densities in treated crops (**Table 2**), (ii) significantly lowered frequencies of aflatoxin producers in treated crops (**Table 3**), (iii) promoted a higher incidence of atoxigenic AAVs in treated crops (**Tables 4**, **5**), and (iv) lowered aflatoxin content in treated crops both at harvest and after simulated poor storage (**Tables 6**, **7**). Because of these results, in 2014 NAFDAC approved the unrestricted use of Aflasafe® for aflatoxin mitigation in maize and groundnut throughout Nigeria. The registration allowed thousands of smallholder farmers in Nigeria to use biocontrol commercially for producing aflatoxin standard-compliant maize. In addition, the biocontrol product has also been proven to effectively limit aflatoxin contamination of chili peppers although its registration with NAFDAC for unrestricted use in chili peppers is still pending (Ezekiel et al., 2019).

Aspergillus Community Modulation by Biocontrol

In treated crops, *A. flavus* L morphotype predominated, and most of the L morphotype isolates belonged to the atoxigenic active ingredient AAVs of the biocontrol product (**Table 4**). The high frequency of atoxigenic AAVs in grains from treated crops illustrate excellent competitiveness for displacing aflatoxin producers. Across substrates and years, AAVs La3279 and Ka16127 were consistently the most commonly recovered genotypes in treated fields (**Table 5**). La3279 and Ka16127 were also the most commonly recovered genotypes from treated fields of the preliminary experiments (Atehnkeng et al., 2014). Both genotypes appear to be more competitive than the two other atoxigenic AAVs composing the biocontrol product. Some of the factors that affect the competitiveness of biocontrol isolates are differential ability to infect and colonize hosts/debris, produce spores, disperse from soil to crop, cause secondary infection, among others (Mehl et al., 2012). The observed low aflatoxin levels in the treated crops are a direct consequence of the almost complete replacement of high aflatoxin producers by the atoxigenic AAVs composing the biocontrol product. Even when S_{BG} strains and *A. parasiticus* occur at a relatively low frequency, as in the control crops (range = 0.3–16.3%; **Table 3**), aflatoxin levels can reach to over 300 ppb (maize in 2010; **Table 6**). Therefore, the biocontrol application promoted communities with less aflatoxin-producing potential and consequently lower aflatoxin levels were detected in treated crops both at harvest and after the simulated poor storage.

One of the advantages of changing the *Aspergillus* community structure in favor of the applied atoxigenic AAVs is that the safer *Aspergillus* community structure translates to not only nearly uniform low aflatoxin levels but also reduced variance in aflatoxin concentration in grains. Relatively recently, it was reported that Aflasafe SN01® usage in Senegal resulted in reduced variance in aflatoxin content of treated crops in efficacy trials in farmers' fields in groundnut and maize (Senghor et al., 2019). Our studies on maize in commercial fields in Nigeria confirmed the previous observations in Senegal. Low variance in aflatoxin content indicates that sample to sample variations in aflatoxin concentration are low. This reduces the risk that lots measured as low in aflatoxin would actually have unacceptable aflatoxin content and be rejected after receipt in a location with high value markets. Thus, biocontrol treatments might be expected to reduce both aflatoxin-concentration heterogeneity and costs associated with shipment of crops that ultimately become rejected and destroyed or turned away at the destination. High variance in control grain lots suggests significant risk of undetected aflatoxin contamination in the absence of biocontrol treatment. Maximum aflatoxin level in a sample from treated maize reached 1,094 ppb in a grain lot of 2016. Thus, although the sample was considered as treated, the high aflatoxin content suggests that some or few of the farmers that contributed to that 30-ton lot did not apply the biocontrol product at the indicated rate and/or at the mandatory maize growth stage (2-to-3 weeks before flowering).

Aflatoxin Reduction by Biocontrol

Even though use of biocontrol results in excellent aflatoxin reductions at harvest and after storage (**Tables 6–9**), there were cases in which the protection provided by biocontrol was insufficient to limit aflatoxin content to below tolerance thresholds. For example, treated maize in 2010 had an average total aflatoxin content of 21 ppb (**Table 6**) and, in 2016, 10% of treated samples contained >20 ppb total aflatoxins (**Table 9**). In addition, if treated crops were stored poorly, aflatoxin content, although dramatically lower than poorly stored control crops, may increase above tolerance thresholds (**Table 6**). Therefore, it is critical to use biocontrol along with all other available, practical

management practices. Relatively recently, use of biocontrol without other intervention was considered a pitfall (Njoroge, 2018). One of the objectives of the preliminary experiments (Atehnkeng et al., 2008b, 2014) and of the field efficacy trials reported in the current study was to demonstrate the value of biocontrol with no other intervention. This allowed determining the extent of protection by biocontrol alone. Aflatoxin reduction by biocontrol was prominent when aflatoxin pressure was high. In years when biocontrol efficacy resulted in less than 80% reduction, the aflatoxin content in untreated crops was low (<10 ppb total aflatoxin). Therefore, the efficacy of biocontrol in those years was not as obvious as in years where higher aflatoxin concentrations occurred in control crops (**Table 6**). Our investigations during 2009 to 2012 revealed that the atoxigenic AAVs of the biocontrol product are effective at limiting contamination well over 70% and up to 100% even in the absence of improved agronomic and storage practices. Certainly, if other management strategies were used during both the initial studies (Atehnkeng et al., 2008b, 2014) and the farmer-field efficacy trials (2009–2012) reported in the current study, the aflatoxin concentrations both at harvest and after storage would have been even lower, especially after storage since at harvest most treated crops contained low total aflatoxin levels.

Protection provided by atoxigenic genotypes during post-harvest conditions has been questioned (Villers, 2014; Ehrlich et al., 2015; Gressel and Polturak, 2018). However, treated crops become associated with high frequencies of the applied fungi before harvest (**Tables 4**, **5**). If conditions for fungal infection and aflatoxin formation occur during storage, most *Aspergillus* fungi growing in stored treated crops would be the applied atoxigenic isolates. Less aflatoxin content occurred in treated crops compared to untreated crops (**Table 6**) and this is attributed to high frequencies of atoxigenic AAVs composing the biocontrol product in the treated crops (**Tables 4**, **5**). Thus, treating crops with the biocontrol product during crop development provided protection during deliberately poor post-harvest storage (**Table 6**). Post-harvest benefits when treating groundnut with Aflasafe SN01® in Senegal (Senghor et al., 2019) and maize with an experimental biocontrol formulation in Nigeria (Atehnkeng et al., 2014) have been reported.

Paradigm Shifts in Perception of Biocontrol in Africa

There is the notion that in SSA, and consequently in Nigeria, biocontrol usage has not gone beyond the research stage because smallholder farmers cannot afford biocontrol and there is no incentive to use the technology since aflatoxin contamination is not a factor that determines price competitiveness of grains in market (Njoroge, 2018; Stepman, 2018; Pitt, 2019). In general, these are indeed challenges for scaling up any aflatoxin mitigation technology, including biocontrol, to reach thousands of farmers. Nevertheless, through diverse innovative mechanisms and partnership arrangements with various stakeholders, large-scale use of biocontrol and other aflatoxin management interventions has reached thousands of farmers in Nigeria by implementing strategies for sustainable biocontrol use through the AgResults Project and other initiatives (Bandyopadhyay et al., 2016; AgResults, 2019; Schreurs et al., 2019). The strategies include awareness and sensitization campaigns, use of improved agronomic practices, improved pre-harvest practices, use of the aflatoxin biocontrol product, improved harvest and post-harvest practices, sorting of moldy/diseased grains, proper storage and use of hermetic bags, testing, market development, policies, and any other novel, practical, and available management tool for farmers. These holistic interventions have helped to create markets willing to pay for aflatoxin standard-compliant maize (Johnson et al., 2018, 2019) resulting in farmers' willingness to pay $11-19/ha for biocontrol (Ayedun et al., 2017). Farm-based agricultural enterprises have enabled thousands of farmers to adopt aflatoxin management strategies, centered in biocontrol, to produce and commercialize more than 200,000 tons of aflatoxin-compliant maize demonstrating that sustainability and scaling of the technology is possible. It is necessary to increase awareness about aflatoxin and create new market opportunities for aflatoxin-compliant crops for farmers to further enhance adoption of aflatoxin-mitigation practices, including biocontrol.

Biocontrol applications of atoxigenic strains have long-term carryover effects and as a result additive benefits accrue as the product is used over multiple years (Cotty et al., 2007). However, because aflatoxins influence human health managing the toxins to the lowest possible level is desired, and it is recommended that the products be applied each season maize or groundnut are produced. Costs associated with applying biocontrol products every crop cycle have been viewed as a negative aspect of the biocontrol technology (Ehrlich et al., 2015; Njoroge, 2018; Molo et al., 2019). However, optimal crop production requires application of inputs (e.g., certified seeds, fertilizers, insecticides) on a yearly basis. All of these critical inputs must be applied each cropping season and it should not be expected that atoxigenic genotypes must replace toxigenic strains with a single application. Farmers producing commercial crops in Nigeria and other SSA nations utilize inputs to increase crop production and many are now using biocontrol as a necessary input to protect crops and access premium markets. This is similar to what has occurred in high-risk portions of Texas where use of aflatoxin biocontrol products is considered a necessary cost of maize production.

Long-term studies on the efficacy and stability of aflatoxin biocontrol agents have been recommended to determine the true value of the technology in SSA (Kagot et al., 2019). It takes over a decade to develop aflatoxin biocontrol programs because it is necessary to sensitize farmers and several key stakeholders, screen for appropriate biocontrol agents, test their efficacy in multiple years, multiple areas, in real-farming conditions, develop delivery methods, register the biocontrol product with national authorities, develop commercialization strategies, establish market entry strategies, create sustainable models for biocontrol adoption in a nation-wide manner, develop infrastructure to manufacture and distribute the biocontrol product en masse, among other actions (Mehl et al., 2012; Bandyopadhyay et al., 2016; Ortega-Beltran and Bandyopadhyay, 2019; Schreurs et al., 2019). We are hopeful that the substantial evidence of the benefits of integrated management strategies centered on biocontrol products presented in this long-term

study may change certain ambiguous and sometimes negative perceptions about the effectiveness of biocontrol in African contexts, its adoption, and its sustainability.

CONCLUSION

Our results demonstrate that an aflatoxin biocontrol product registered for use in maize and groundnut in Nigeria, is a practical, cost-effective, and environmentally safe aflatoxin mitigation tool that enables farmers in Nigeria to produce both crops with little to no aflatoxin content. This work is the most extensive published study of the long-term efficacy of any aflatoxin biocontrol product. The results indicate that biocontrol is a stable, effective aflatoxin management tool regardless of environment or cropping system and is highly effective on crops grown by smallholder farmers. In addition, evidence of large-scale adoption of the biocontrol product in Nigeria is provided. The results suggest that biocontrol is a preferred component of holistic aflatoxin management strategies tackling agricultural, behavioral, institutional, and policy challenges. A holistic maize and groundnut aflatoxin management strategy with biocontrol as the centerpiece is contributing to better health, increased income, and greater trading opportunities. Farmers and consumers of other susceptible crops (e.g., chili peppers, sesame, sorghum) in Nigeria and elsewhere in SSA would benefit if the technology is adapted and registered for its use in those crops.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

RB and PC designed the overall projects from which data are derived. JA, AA, AO-B, PC, and RB contributed to the conception and design of the experiments. JA, AA, AO-B, and TF conducted the experiments and field studies, and collected and analyzed the data. AA, PC, and RB provided guidance. AO-B drafted the original manuscript. JA, TF, PC, and RB edited the manuscript. All authors read, reviewed, and approved the final manuscript. RB secured funds for the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02528/full#supplementary-material

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Resilience of Biocontrol for Aflatoxin Minimization Strategies: Climate Change Abiotic Factors May Affect Control in Non-GM and GM-Maize Cultivars

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There has been significant interest in the development of formulations of non-toxigenic strains of *Aspergillus flavus* for control of toxigenic strains to reduce the aflatoxin B_1 (AFB_1) contamination of maize. In the future, climate change (CC) abiotic conditions of temperature (+2–4°C), CO_2 (existing levels of 400 vs. 800–1,200 ppb), and drought stress will impact on the agronomy and control of pests and diseases. This study has examined (1) the effect of two-way interacting factors of water activity × temperature on colonization and AFB_1 contamination of maize cobs of different ripening ages; (2) the effect of non-toxigenic strains of *A. flavus* (50:50 inoculum ratio) on relative control of toxigenic *A. flavus* and AFB_1 contamination of ripening cobs; (3) post-harvest control of AFB_1 by non-toxigenic strains of *A. flavus* in non-GM and isogenic GM maize cultivars using the same inoculum ratio; and (4) the impact of three-way interacting CC factors on relative control of AFB_1 in maize cobs pre-harvest and in stored non-GM/GM cultivars. Pre-harvest colonization and AFB_1 production by a toxigenic *A. flavus* strain was conserved at 37°C when compared with 30°C, at the three ripening stages of cob development examined: milk ripe (R3), dough (R4), and dent (R5). However, pre-harvest biocontrol with a non-toxigenic strain was only effective at the R3 and R4 stages and not at the R5 stage. This was supported by relative expression of the *aflR* regulatory biosynthetic gene in the different treatments. When exposed to three-way interacting CC factors for control of AFB_1 pre-harvest, the non-toxigenic *A. flavus* strain was effective at R3 and £4 stages but not at the R5 stage. Post-harvest storage of non-GM and GM cultivars showed that control was achievable at 30°C, with slightly better control in GM-cultivars in terms of the overall inhibition of AFB_1 production. However, in stored maize, the non-toxigenic strains of *A. flavus* had conserved biocontrol of AFB_1 contamination, especially in the GM-maize cultivars under three-way interacting CC conditions (37°C × 1,000 ppm CO_2 and drought stress). This was supported by the relative expression of the *aflR* gene in these treatments. This study suggests that the choice of the biocontrol strains, for pre- or post-harvest control, needs to take into account their resilience in CC-related abiotic conditions to ensure that control of AFB_1 contamination can be conserved.

Keywords: resilience, biocontrol, aflatoxins, climate change, non-toxigenic *Aspergillus flavus*, non-GM maize, GM maize

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INTRODUCTION

There has been significant interest in developing biocontrol agents for aflatoxin B_1 (AFB_1) control in staple commodities, especially maize. Indeed, there are some commercial products based on individual non-aflatoxigenic *A. flavus* strains or mixtures of such strains for reducing AFB_1 contamination of maize and groundnuts in West and East Africa and in the USA for control of toxin contamination in cotton and groundnuts (Lyn et al., 2009; Abbas et al., 2011; Bandyopadhyay et al., 2016; Mauro et al., 2018; Kagot et al., 2019).

Abiotic factors, especially drought stress, can have a major impact on maize growth especially during the critical silking period, which can allow both pest damage and increase in the colonization by *A. flavus* resulting in AFB_1 contamination. In some cases, pest damage has been reduced by the use of GM cultivars, which have resistant genes for pesticides and/or herbicides. This can reduce the entry points for *A. flavus* and reduce contamination. However, under expected climate change (CC) conditions, which involves interactions between key abiotic factors such as the predicted increase in environmental CO_2 (400 vs. 1,000–1,200 ppm), elevated temperature (+2–4°C), and extreme changes in drought/wet regimes, there have been few studies to examine the resilience of non-toxigenic *A. flavus* biocontrol strains used for toxin reduction in maize and groundnuts. Many maize growing regions are considered hot spots for an impact of these environmental pressures. Indeed, work on *A. flavus* colonization of maize grain has shown that interacting conditions of +2–5°C, elevated CO_2 (650–1,000 ppm), and drought stress can result in an increase in both regulatory (*aflR*) and structural genes (*aflD*) involved in AFB_1 biosynthesis as well as other secondary metabolite genes in maize grain and lead to a significant stimulation in AFB_1 contamination (Battilani et al., 2016; Medina et al., 2017a; Gilbert et al., 2018). Bearing this in mind, it is thus surprising that while biocontrol of toxigenic *A. flavus* using microbial antagonists and non-toxigenic strains of *A. flavus* has been examined for many years, their resilience has never been examined under expected CC regimes (Cotty, 1994; Abbas et al., 2011; Bandyopadhyay et al., 2016; Weaver et al., 2016; Kagot et al., 2019). Some studies have examined the impact of non-toxigenic *A. flavus* strains and other microbial biocontrol agents on temporal control of AFB_1 in maize stored under different temperature and water availabilities (Mohale et al., 2013; Al-Saad et al., 2016; Medina et al., 2017a). However, the relative resilience of the biocontrol strains for control of mycotoxin production under CC scenarios has not previously received any attention (Medina et al., 2017b). It is very important to understand how the potential biocontrol strains targeting AFB_1 control in maize may behave under interacting CC abiotic factors and whether they have the necessary resilience to reduce biosynthesis of AFB_1 *in situ*.

The objectives of this study were to examine (1) the effect of ripening stage of maize cobs on rates of colonization and AFB_1 production by *A. flavus* in relation to interactions between two-way abiotic factors of temperature × water availability (water activity, a_w); (2) the effect of these two-way interacting factors on the control of AFB_1 contamination using 50:50 ratios of non-toxigenic and toxigenic strains in maize cobs of different ripening stages and in stored non-GM and isogenic GM maize cultivars; and (3) the effect of three-way interacting CC abiotic factors on resilience of non-toxigenic *A. flavus* strains in terms of reducing the expression of key biosynthetic genes involved in aflatoxin synthesis, and on phenotypic AFB_1 contamination, in these two types of maize cultivars.

MATERIALS AND METHODS

Fungal Strains

Two non-toxigenic strains of *A. flavus* isolated from Mexican and Brazilian maize were used in these experiments (AFL^-Mex02; $AFL4^-$). Both strains were examined molecularly to confirm that key genes in the biosynthetic cluster for aflatoxins were deleted using the multiplex PCR method developed by Callicott and Cotty (2015). Different toxigenic strains including one Mexican strain (ALF^+-Mex01, isolated from Mexican maize), a type strain NRRL3375 ($AFLe^+$), and one Brazilian toxigenic strain ($AFLb^+$, isolated from Brazilian maize), all known AFB_1 producers were used. **Table 1** summarizes the strains used in this study. The type strain was kindly provided by Prof. D. Bhatnagar, Southern Regional Research Centre, New Orleans, LA, USA.

Pre-harvest Studies With Maize Cobs of Different Ripening Ages for Resilience of Biocontrol of AFB_1

Maize cobs of different ripening stages (R3: Milk; R4: Dough; R5: Dent) were obtained from the NIAB farm (National Institute of Agriculture and Botany; Cambridge, UK). The type of maize was ES Regain (Euralis Semences; forage maize). Harvested maize cobs of different ripening ages were brought to the laboratory where the water activity (a_w) of sub-samples of detached kernels from the entire cob (5–10 maize kernels from the apical, middle, and distal parts of the cobs) was measured (AQUALAB® Series TE4; Decagon Devices Inc., Pullman, WA, USA). Maize cobs were then divided into batches and snap frozen in liquid nitrogen and stored at −20°C for later use in the experiments. The a_w of the R3 (milk ripe), R4 (dough), and R5 (dent) stages was found to be 0.985, 0.976, and 0.958, respectively.

TABLE 1 | List of toxigenic and non-toxigenic strains used in this study.

Strain code	Source	Aflatoxin B_1 producer +/−	Deleted genes
$AFL\text{-}MEX01^-$	White maize/Mexico	Naturally very low producer*	None
AFL-MEX02	White maize/Mexico	Producer	
$AFL4^-$	GM maize P30F53 H®, Brazil	Non-producer	*aflN, aflE, aflS. aflB. aflA*
$AFLB^+$	Landrace maize red grain, Brazil	Producer	
NRRL3357	Peanuts (type strain, ARS, USDA)	Producer	

Less than the limit of quantification.

The fungal strains were all point inoculated on 3% Maize Meal Agar (MMA, 0.98 a_w) and incubated at 25°C for 7 days, and the conidial spore suspensions made by using a loop and decanting spores into 9 ml of sterile water +0.5% Tween 8-0 solution in 25 ml Universal bottle. The conidial spore concentrations were measured with a hemocytometer, and then diluted with sterile water as required, to obtain a final concentration of 1×10^4 spores/ml.

The flash-frozen cobs were thawed at 4°C for 24 h. The maize cobs were divided into three segments, and these were point inoculated. Each treatment consisted of 3–4 replicate maize cob segments taken at random from the batch of each ripening age. These were point inoculated by damaging a single kernel with a surface sterilized needle and then decanting a 10 µl droplet containing 10^4 conidia/ml of the *A. flavus* type strain. These were incubation in separate environmental chambers at 30 and 37°C. The equilibrium relative humidity (ERH) of the atmosphere was maintained at the actual a_w levels of the cobs by using glycerol/water solutions of the same a_w as the maize cob ripening stages. The colonization rate was measured over a 10-day period, and the AFB_1 contamination quantified at the end of the experimental period only.

Subsequent studies examined the biocontrol of the toxigenic *A. flavus* strain and AFB_1 control in each of the ripening stages as described previously by Samsudin et al. (2017). The different ripening stages of the cobs were incubated in separate environmental chambers to stabilize for 3 hours at 25°C until inoculation. The control treatment used was inoculated with the toxigenic *A. flavus* ($MEX01^+$) or the type NRRL3375 strain alone. The biological control agent (BCA) treatments consisted of a 50:50 conidial inoculum ratio of pathogen:antagonist. This was used based on previous studies where different inoculum ratios were used of toxigenic:non-toxigenic strains of *A. flavus*, which showed that 50:50 or 25:75 ratios, respectively, gave similar levels of AFB_1 control as described previously (Medina et al., 2017a). The cob sections were point inoculated with 100 µl of the treatments after damage to a single kernel using a surface sterilized needle and incubated at 30°C for 10 days (Samsudin et al., 2017). In all cases, 3–4 replicates per treatment were used. Each environmental chamber included 250 ml of a sterile solution of glycerol/water to maintain the ERH at the same level as the cob ripening stage a_w. The colony diameter was measured at the end of the incubation period. For *aflD* and *aflR* gene expression, contaminated kernels from the colonized area were carefully removed at random with a pair of forceps and immediately frozen in liquid N_2 and kept at −80°C for subsequent RNA extraction and RT-qPCR as detailed previously (Al-Saad et al., 2016). The rest of the colonized cob was kept at −20°C for AFB_1 extraction; clean-up was done using an immune-affinity column (IAC) and quantified by HPLC-FLD. The limit of detection was <1.0 ng/g.

Post-harvest Studies With Non-GM and GM-Maize Cultivars

Two cultivars of non-GM maize and its isogenic GM lines were selected for the post-harvest biocontrol studies (**Table 2**). For these studies, batches of the maize grain were gamma-irradiated (12–15 kGy) in order to eliminate the natural contaminants but retain germinability of the kernels. The a_w of the maize treatments was modified to 0.98 and 0.95 with the addition of sterile water based on the moisture absorption curve for each cultivar. These were mixed thoroughly to ensure that the spore inoculum was well distributed throughout the maize grain. The number of spores added to each cultivar was calculated as 10 spores per gram of maize from a solution of 10^3 spores/ml. After the addition of the water, the maize kernels were kept at 4°C for 24 h for full absorption and equilibration. The water availability was checked by measuring the a_w using the AquaLab® 4TE (Decagon, USA).

TABLE 2 | Maize cultivars (non-GM and GM) selected for biocontrol *in situ* as substrate for *A. flavus* development.

Conventional cultivar (non-GM)	Isogenic line (GM)	Event name[1]	Inserted gene	Traits tolerance
M20-A78 CON	M20-A78 PW®	MON89034 + NK603 + TC1507	CP4 EPSPS	HT-Glyphosate
			PAT	HT-Glufosinate ammonium
			Cry2Ab2	
			Cry1F	IR-Lepidopteran
			Cry1A.105	
P30F53 CON	P30F53 H®	DAS1507 + T25	PAT	HT-Glufosinate ammonium
			BLA	Antibiotic resistance
			Cry1F	IR-Lepidopteran

IR, insect resistance; HT, herbicide tolerance; PW®, PowerCore; H®, Pioneer Hi-Breed.
[1]Event name refers to the unique code to access the information about the trait at http://www.isaaa.org/gmapprovaldatabase/eventslist/default.asp

The non-toxigenic and toxigenic strains of *A. flavus* were incubated on MMA for 7 days at 30°C and then used to prepare the conidial spore inoculum. A final concentration of 10^3 spores/ml was obtained. A ratio of 50:50 (toxigenic:non-toxigenic strains) was used. Previous showed that this as an effective inoculum load for biocontrol of AFB_1 production (Mohale et al., 2013; Samsudin and Magan, 2016). The spore suspensions were mixed in a 50:50 ratio prior to inoculation of the maize kernels. The controls consisted of 100:0 and 0:100 ratios for the two control treatments.

A 10 g sample of maize grain were placed aseptically into glass culture vessels (Magenta™, Sigma, USA) with vented lids (10 mm with a polypropylene membrane 0.22 µm pore size) to allow gase exchange but keeping the environment inside the vessel sterile. These were inoculated with a 100 µl conidial mixture and shaken to distribute the conidia. The jars were placed in closed plastic environmental chambers that also contained a glycerol/water solution of the same a_w as the maize kernels to keep the ERH the same as the target treatments. The glycerol/water solution was renewed every 3 days. The environmental chambers were incubated at 30°C for 20 days. Samples were destructively sampled after 10 and 20 days storage for quantification of AFB_1.

The samples for AFB_1 quantification were oven dried at 65°C for 48 h to remove the water and stop fungal growth. These were then ground using a laboratory blender with a

stainless steel blade (Waring, Stamford, USA). The effects on *aflD* and *aflR* gene expression were only examined after 10 days in all the treatments and replicates. The samples for gene expression were snap frozen in liquid nitrogen and stored at −80°C for subsequent RNA extraction. All studies were carried out with two non-GM and their isogenic GM cultivars. All experiments were carried out with at least three replicates per treatments and repeated once.

Studies of Resilience of Non-toxigenic Strains of *A. flavus* When Controlling Aflatoxin B_1 Contamination of Maize Cultivars Under Climate Change Conditions

Fungal Strains

Studies were carried out with two non-aflatoxigenic strains of *A. flavus* (AFL$^-$:Mex02; AFL4$^-$) isolated from Mexican and Brazilian maize, respectively, based on their ability to reduce AFB_1 in previous *in vitro* and *in situ* studies (Rodríguez-Sixtos, 2017; Marcon Gasperini, 2019). The native toxigenic strains (AFL$^+$MEX01; AFLb$^+$; and the AFLe type strain) with known AFB_1 production capacity were used as the toxigenic pathogen in these studies.

Pre-harvest studies involved the use of maize cobs at the R4 (dough) and R5 (dent) stages only. Post-harvest studies used the two non-GM and isogenic GM maize cultivars. The same modification procedures were used as detailed previously. A ratio of 50:50 conidia (toxigenic *A. flavus*: non-toxigenic *A. flavus*) were used. The controls consisted again of only the toxigenic or non-toxigenic conidial concentrations as detailed previously.

The post-harvest maize grain treatments were modified to 0.98 and 0.95 a_w with the addition of sterile water using the moisture adsorption curve of each cultivar to obtain the target treatment regimes. The maize treatments and replicates were inoculated with a conidial mixture of non-toxigenic and toxigenic strains as described previously (10^3 conidia/ml and addition of the mixture resulting in approx. 10 conidia/g maize). The only exception was that for the cultivars AS 1555 CON and PRO®, and P2530 CON and Hx® were only conducted at 0.98 a_w because of a limited amount of maize grain of these cultivars available for the experiment.

For the CC study, the separate treatments were placed in plastic environmental chambers (Lock & Lock HPL890 16 L) containing a glycerol/water solution of the same a_w as the treatments as described previously. The environmental conditions were set to flush the CO_2 treatments of 400 ppm (atmospheric CO_2) and 1,000 ppm. The elevated CO_2 content was achieved by using a gas cylinder containing a certified concentration of 1,000 ppm CO_2/synthetic air at 200 bar prepared by the British Oxygen Company (Guildford, UK). The chambers were vented, and for the 1,000 ppm treatment flushed with CO_2, every 12 h for the 20-day experimental period. The concentration of CO_2 was regulated to 3 L/min (LPM) with a gas flow meter (Alicat Scientific, Arizona, USA) and flushed for 10–12 min, which corresponded to 2× the volume of each chamber. After flushing, the inlet and outlet valves of the chambers were immediately closed, and they were incubated at 30 or 35°C. The control chambers were flushed with air (400 ppm) and similarly incubated. At the end of the experiment, samples were destructively sampled for gene expression studies (10 days) and for AFB_1 quantification (10, 20 days). The samples for toxin quantification were oven dried at 65°C for 48 h to remove the water and stop any fungal growth. The samples were ground using the laboratory blender as described previously.

Gene Expression Studies

The gene expression studies were performed using the samples from the different maize ripening stages (pre-harvest studies) and those from the stored maize grain experiments after 10 days incubation. This time frame was chosen based on previous studies with both *A. flavus* and *A. parasiticus* that suggested gene expression of several of the toxin biosynthetic genes had optimal peaks of expression after 8–10 days growth (Schmidt-Heydt et al., 2008). The gene expression of the chosen genes was only performed for the interaction between toxigenic/non-toxigenic strains MEX01$^+$:MEX02$^-$ and AFLb$^+$:AFL4$^-$, respectively. The type strain AFLe$^+$ (NRRL3357) treatments were not included because the AFB_1 production was lower than the native Mexican or Brazilian toxigenic strains.

The treatments/replicates were stored at −80°C and transferred to reinforced 7 ml tubes designed for use in the Precellys 24® (Bertin, FR) homogenizer with three glass beads (6.5 mm). The tubes were kept in liquid N_2 until use. The kernels were homogenized into a fine powder using a 6,500 rpm cycle for 30 s (2 × 15 s) and then immersing them in liquid N_2 for 5 min and the cycle repeated. Approximately 50 mg of the powder was transferred to a 2 ml Eppendorf RNase/DNAse free to proceed with the extraction of the total RNA.

RNA Extraction

Total RNA isolation was carried out using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich Co, USA) according to the manufacturer instructions. The observations for samples with high amounts of starch were taken into account. For this reason, samples were incubated at room temperature, and 1 ml of lysis buffer added to the 50 mg of powder. RNA samples were treated with RNase-Free DNAse set (Qiagen, Hilden, Germany). The purity and concentrations of RNA were examined by measuring the absorbance of 2 µl of sample Genova-Nano spectrophotometer (JenWay, Staffordshire, UK). Samples were considered to be of good purity when the ratio A_{260}/A_{280} was ≥2.0. The RNA integrity was verified using Experion™ RNA StdSens in an Experion™ automated electrophoresis system (Bio-Rad, California, USA) or by gel electrophoresis, and an RQI >6 was used as a threshold for integrity. The samples were kept at −80°C until use.

Relative Gene Expression Using Quantitative Polymerase Chain Reactions

Real-time quantitative PCR (RT-qPCR) assays were used to amplify the structural *alfD* and the regulatory gene *afl*R of

the aflatoxin biosynthetic pathway as target genes (**Table 3**). The β-tubulin gene was used as the control gene. The *afl*D qPCR was previously optimized by Abdel-Hadi et al. (2010), whereas *afl*R was optimized by Medina et al. (2015) following the same method as that for the *afl*D gene.

Two RT-qPCR assays were carried out, one optimized to amplify the target *afl*D and the housekeeping β-tubulin genes, and the other one to quantify the *afl*R gene expression and for the β-tubulin gene. The qPCR reactions were prepared in triplicate for each biological replicate (n = 9). The TaqMan system with different primers and probes was used in all cases. Both reaction mixtures consisted of 6.25 µlm Premix Ex Taq™ (Takara Bio Inc., Otsu, Japan), 830 nM of each primer, 330 nM of each probe, and 1.5 µl of cDNA template in a final volume of 12.5 µl. The optimal thermal cycling conditions included an initial step of 10 min at 95°C and all 50 cycles at 95°C for 15 s, 55°C for 20 s, and 72°C for 30 s. The assays were carried out using a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, CA, USA).

Relative Quantification of the Expression

Relative quantification of *afl*D and *afl*R genes was performed using the housekeeping gene β-tubulin (ben) as an endogenous control to normalize the quantification of the target in the relative quantification assays and used for all treatments. Quantification cycle (Cq) determinations were automatically performed by the instrument using the default parameters, and the expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method as proposed by Livak and Schmittgen (2001). The control of each condition corresponded to $AFLb^{+}$ without the presence of the BCA.

Mycotoxin Quantification

For the extraction of AFB_1, a 2 g sample was ground and transferred to a glass vial and mixed with 8 ml of extraction solution (methanol:water; 80:20 v/v). The samples were agitated with a magnetic stirrer for 1 h at room temperature and then centrifuged to allow phase separation. The liquid phase was transferred to a new 15 ml polypropylene tube and 100 µl of the extract mixed with 900 µl of mobile phase (methanol: acetonitrile:water 30:15:60 v/v/v) in a 2 ml Eppendorf tube and vortexed and filtered using a nylon filter (13 mm × 0.22 µm) directly into an amber silanized vial. The samples were injected into the HPLC system, and samples below the limit of detection (<1.0 ng/g) were cleaned-up and concentrated using an IAC as described previously and then reinjected.

IAC Analysis

The extract of the samples was diluted 2:20 in 1× PBS (phosphate buffered saline, Fisher Scientific, USA). The pH of the extract was checked to ensure that this was not lower than 7.0 to ensure good performance of the IACs (AflaStar™ R, RomerLabs, Austria). The IACs were brought to room temperature prior to use and were attached to a SPE vacuum manifold (Phenomenex, CA, USA). Above each IAC, a 25 ml reservoir was used to hold the sample extract. Following the manufacturers' instructions, the buffer in the IAC was removed, and the sample extract passed through the column at a speed of 1 to 3 ml/min. This was followed by 20 ml of 1× PBS for the clean-up (10 ml was added in the reservoir and 10 ml added directly into the IAC). The last step was the elution with 1.5 ml of methanol. For best recovery, the elution was performed by adding 3 × 500 µl of methanol. The eluted samples were evaporated to dryness using a vacuum evaporator (miVac Quattro Concentrator – Genevac, Leicestershire, UK) at 45°C for 3 h. The dried extract was re-suspended in 500 µl mobile phase, transferred to an amber silanized vial, and injected into the HPLC for AFB_1 quantification.

HPLC Analysis

The quantification of AFB_1 in the maize grain was done by reverse-phase HPLC. The HPLC system used was an Agilent 1,200 series (Agilent, Santa Clara, USA) with a fluorescence detector (λ_{exc} 360 nm; λ_{em} 440 nm) and post-column derivatization with a UVE photochemical reactor with UV-Light (LCTech GmbH, Germany). A C_{18} column (Agilent Zorbax® Eclipse Plus, 2.1 × 100 mm, 3.5 µm particle size) preceded by a Phenomenex® Gemini C18 guard column cartridge 3 mm × 3 µm (Phenomenex, CA, USA) was used for separation. Followed by isocratic elution with methanol:water:acetonitrile (30:60:15, v/v/v) and a mobile phase flow rate of 1.0 ml/min. The injection volume was 5–50 µl according to each set of samples. A set

TABLE 3 | Nucleotide sequences of primers for RT-qPCR assays designed on the basis of the *aflD*, *aflR,* and β-tubulin genes.

Primer pairs	Gene	Nucleotide sequences (5′–3″)	Position
*nor*Taq – 1		GTCCAAGCAACAGGCCAAGT	516[a]
*nor*Taq – 2	*aflD*	TCGTGCATGTTGGTGATGGT	562[a]
nor-Probe		[FAM]TGTCTTGATCGCGCCCG[BHQ2]	537[a]
*afl*RTaq – 1		TCGTCCTTATCGTTCTCAAGG	1,646[b]
*afl*RTaq – 2	*aflR*	ACTGTTGCTACAGCTGCCACT	1,735[b]
*afl*R-Probe		[FAM]AGCAGGCACCCAGTGTACCTCAAC[BHQ2]	1,689[b]
benTaq – 1		CTTGTTGACCAGGTTGTCGAT	65[c]
benTaq – 2	β-tubulin	GTCGCAGCCCTCAGCCT	99[c]
ben-Probe		[CY5]CGATGTTGTCCGTCGCGAGGCT[BHQ2]	82[c]

[a]Positions are in accordance with the published sequence of the aflD gene of Aspergillus flavus (GenBank accession no. XM_002379908.1); [b]Positions are in accordance with the published sequences of aflR gene of Aspergillus flavus (GenBank accession no. AF441435.2); [c]Positions are in accordance with the published sequences of β-tubulin (benA56) gene of Aspergillus flavus (GenBank accession no. AF036803.1).

of standards was injected (0.05–4 ng of a mixture of aflatoxins per injection), and standard curves were generated by plotting the peak areas against the amounts of each aflatoxin. The recovery of the extraction method for AFB_1 in maize was 80%.

Statistical Analyses

The data from pre-harvest cobs of different ripening stages and the *in situ* post-harvest experiments (gene expression data, AFB_1) were analyzed using the Shapiro-Wilk tests to determine normality and Levene's test to assess variance homogeneity. However, the data violated the two assumptions for ANOVA even after transformations and therefore non-parametric tests (Kruskal-Wallis/Wilcoxon; $p = 0.05$) were used for analyses (Chan and Walmsley, 1997). Where there was significance after the Kruskal-Wallis test, median comparisons for each pair were made using the Wilcoxon-Each Pair test ($p = 0.05$). The correlation of relative gene expression × AFB_1 production was checked using non-parametric Spearman's (ρ) rank correlation coefficient for each a_w level. The statistical package JMP®14 (SAS Institute Inc., 2018, Cary, NC, USA) was used to perform the analyses.

The data sets for the effect of CC scenarios on AFB_1 production satisfied the requirements for ANOVA after transformation to the cube root. Tests were thus performed comparing the interactions of temperature × CO_2 × a_w for each cultivar of maize. The relative gene expression for this study violated the two assumptions for ANOVA, and the differences were compared using non-parametric tests (Kruskal-Wallis/Wilcoxon; $p = 0.05$). The calibrant (control sample) for the biocontrol experiment was the toxigenic pathogen strain ($AFLb^+$) in the same conditions as the non-toxigenic strain was applied. For the effects of CC, the control sample refers to normal environmental conditions (30°C, 400 ppm CO_2) for each cultivar. The statistical package JMP®14 (SAS Institute Inc., 2018, Cary, NC, USA) was used to perform the analyses.

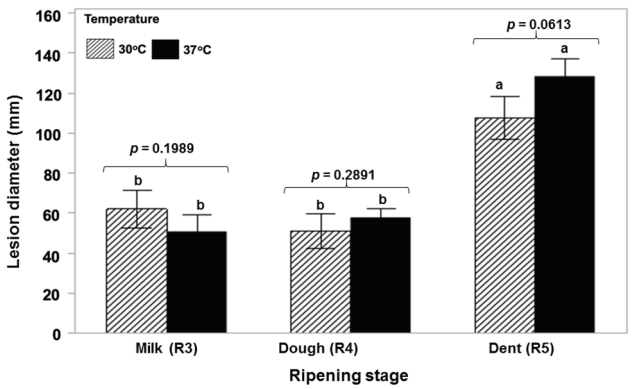

FIGURE 1 | Relative colonization rates of maize cobs of different ripening ages (R3, R4, R5) by a toxigenic strains of *A. flavus* (NRRL 3375) when point inoculated and incubated at the water activity levels of the different growth stages at 30 and 37°C. Statistical test performed after lesion diameter (mm) data was transformed to Log(x + 1) to achieve normality fit (Shapiro Wilk $p \geq 0.05$). The values of p indicate no difference between temperatures. Treatments with the same letters indicate no differences in the ripening stages based on the Tukey's HSD test ($p \geq 0.05$).

RESULTS

Effect of Maize Cob Ripening Stage and Temperature on Colonization and Aflatoxin B_1 Contamination

Figure 1 shows that a toxigenic strain of *A. flavus* is able to colonize ripening maize cobs of different ages at both

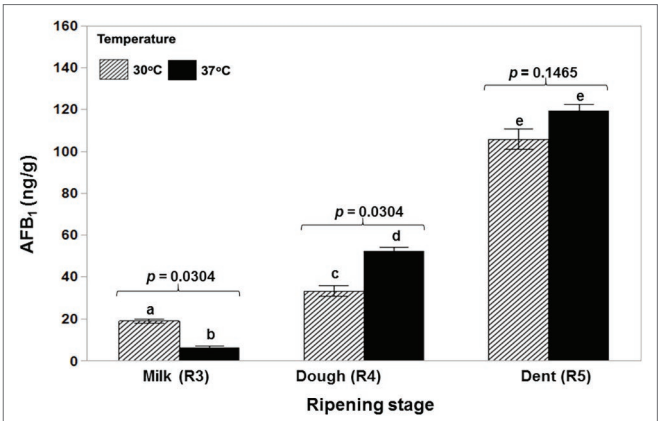

FIGURE 2 | Relative contamination of maize cobs of different ripening ages (R3, R4, R5) with aflatoxin B_1 when incubated at the actual water activity levels of the maize kernels at the different growth stages at 30 and 37°C. The toxigenic strain NRRL 3375 was used. The values of p indicate no evidence of difference between temperatures. Ripening stage treatments with the same letters were not significantly different using the Wilcoxon for each pair test ($p \geq 0.05$). Overall analyses was done using the Non-Parametric data – Wilcoxon Test. Ripening stages: R3 × R4 × R5, $p = 0.0009$; Temperatures: 30 × 37°C, $p = 0.7728$.

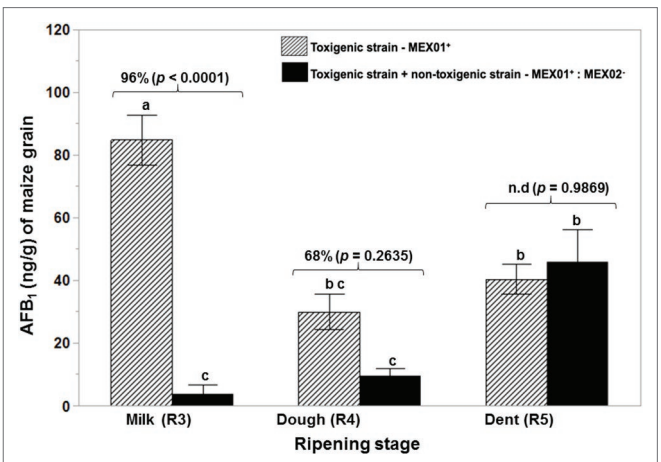

FIGURE 3 | Effect of a non-toxigenic strain (AFL-MEX02) of *A. flavus* when co-inoculated with a wild type toxigenic strain (AFL + MEX01) on aflatoxin B_1 production when applied as a mixed inoculum (50:50) to maize cobs of different ripening ages (R3, R4, R5) after 10 days at 30°C. The percentage values above the bars show relative reduction of AFB_1, and the values of p (<0.05) indicate evidence of difference from the control (toxigenic strain). Treatments with the same letters show no differences in the ripening stages using the Tukey's HSD test at 5% significance. n.d. – AFB_1 reduction not detected.

30 and 37°C in a relatively similar manner with no significant difference between cob ripening stage and temperature. AFB_1 production was significantly lower initially at the R3 (milk) stage and 37°C and then significantly higher at the R4 (dough) stage and the same temperature. There was no difference at the R5 (dent) stage (**Figure 2**). Statistically, there was a significant overall effect of ripening stage but not of temperature.

Relative Control of AFB_1 Contamination in Ripening Ages of Maize Cobs Using a Non-toxigenic *A. flavus* Strain

Figure 3 shows the effect of the non-toxigenic strain (AFL^-:MEX02) on control of AFB_1 production by the toxigenic strain (AFL^+MEX01) on maize cobs of different ripening stages. This shows that the level of control of AFB_1 was maximum at the R3 (milk) and R4 (dough) stages ($p = 0.05$). At the R5 (dent) stage, there was no control of toxin production. **Plate 1** shows an example of the colonization of maize cobs segments at the R5 stage. This was confirmed by measurement of the relative expression of the regulatory *aflR* gene, which was downregulated at the R3 and R4 stages in the maize cobs, while in the R5 stage, there was no difference from the toxigenic control gene expression levels (**Figure 4**).

Post-harvest Control of Aflatoxin Under Different Water Availability Conditions in Non-GM and GM Maize Grain

Figure 5 summarizes the effect of a non-toxigenic strain ($AFL4^-$) on AFB_1 control in maize grain contaminated with two different toxigenic *A. flavus* ($AFLb^+$; $AFLe^+$) strains on AFB_1 control in GM and non-GM cultivars (mean of two cvs of each). This showed that the toxigenic $AFLb^+$ strain produced significantly more AFB_1 than the $AFLe^+$ (NRRL3375) strain. Overall, there was better control of AFB_1 in the GM-maize cultivars than the non-GM ones. Although in both GM and non-GM cultivars, >90% control of AFB_1 contamination of the maize grain was achieved using the initial inoculum ratio of 50:50 of non-toxigenic:toxigenic conidial inoculum. These studies were carried out at 30°C and showed that efficacy was consistent across non-GM and GM cultivars. **Figure 6** compares the relative expression of both the *aflD* and *aflR* genes in one of the non-GM and GM cultivars at 0.98 and 0.95 a_w. The efficacy of the non-toxigenic strain on the toxigenic strains was supported by the effects on the structural and regulatory genes examined. These were significantly downregulated in both non-GM and GM cultivars, although this was more pronounced in the latter one.

Resilience of Non-toxigenic Strains of *A. flavus* for Control of Aflatoxin B_1 Under Three-Way Interacting Climate Change Abiotic Conditions in Non-GM and GM Strains

Two sets of studies were carried out. Pre-harvest studies with a 50:50 mixed conidial inoculum of the non-toxigenic:toxigenic strains were used in maize cobs at the R4 and R5 stages and incubated at 30 or 35°C and exposed to elevated CO_2 for 10 days. This showed that there were no statistically significant differences in AFB_1 contamination between the control and the elevated CO_2 treatments ($p = 0.05$; means of R4: 545 vs. 390; R5 = 1,125 vs. 780 ng/g AFB_1 in maize, respectively). The relative expression of the *aflD* and *aflR* genes showed that there was an inhibition of the former structural gene expression but not of the latter regulatory gene (data not shown).

Exposure to interacting climate change abiotic factors in post-harvest storage studies was carried out with the two non-GM and isogenic GM cultivars. **Figure 7** compares the relative effect of the biocontrol strain $AFL4^-$ when co-inoculated with the toxigenic strain $AFLb^+$ in one of the non-GM and GM cultivars when carried out under existing CO_2 conditions (400 ppm) at two a_w stress levels and comparisons with exposure

PLATE 1 | Example of colonization of maize cob sections by mixtures of 50:50 inoculum of the toxigenic and non-toxigenic strains (AFL + MEX01:AFL-MEX02), respectively, at the R4 stage.

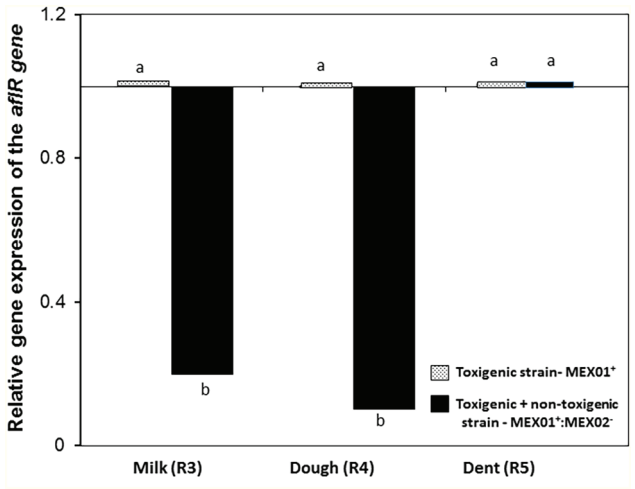

FIGURE 4 | Relative gene expression of the regulatory *aflR* gene in maize cobs of different ripening stages when co-inoculated with the non-toxigenic and toxigenic strains of *A. flavus* (AFL-MEX02+ AFL + MEX01) after 10 days incubation at 30°C. Treatments with different letters are significantly different ($p = 0.05$).

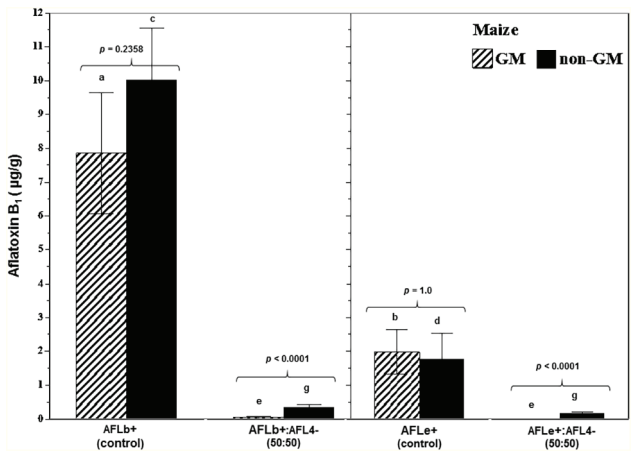

FIGURE 5 | Overall aflatoxin B_1 contamination of post-harvest stored maize grain treated with a mixture of a non-toxigenic and a toxigenic strain of *A. flavus* in non-GM and GM maize cultivars taking into account the parameters of water availability (0.98, 0.95), time of incubation (10, 20 days), and type of maize cultivar (non-GM vs. GM). The grain was inoculated with two different Brazilian or NRRL type toxigenic strains (AFL^+ or $AFLe^+$) and the non-toxigenic Brazilian strain $AFL4^-$ in a 50:50 inoculum ratio. Different letters indicate a significant difference ($p < 0.05$) between the strains of the same maize type. The values of p show differences in overall AFB_1 content comparing GM and non-GM maize at 5% significance. Bars represent mean ± SE.

to 1,000 ppm CO_2. There was higher AFB_1 production at 0.98 a_w than at 0.95 a_w. In addition, in the non-GM cultivar, more AFB_1 was produced by the toxigenic strain. Overall AFB_1 control was more effective at 0.98 a_w than at 0.95 a_w in both existing and future CO_2 scenarios. For both GM and non-GM maize cultivars, the control of AFB_1 was similar, suggesting relative resilience of this non-toxigenic strain.

Figure 8 compares the relative expression of the regulatory *aflR* gene at 35°C and 0.98 a_w in the different CO_2 treatments (400 and 1,000 ppm CO_2) with the control at 30°C/400 ppm CO_2 in both non-GM and GM cultivars. This shows that the *aflR* gene expression was significantly affected at 35°C when comparisons were made between existing (400 ppm) and future (1,000 ppm) CO_2 treatments. The relative expression values were relative to those at 400 ppm CO_2 and 30°C (existing conditions).

DISCUSSION

This study has examined the pre-harvest and post-harvest resilience of non-toxigenic strains of *A. flavus* for control of AFB_1 contamination of maize cultivars, including non-GM and isogenic non-GM with herbicide/pesticide resistant traits for the first time. The ability of the toxigenic strains of *A. flavus* to colonize ripening maize cobs at both 30 and 37°C suggests that it is important to screen non-toxigenic biocontrol strains for resilience to fluxes in temperature and the ability to tolerate a range of water availability conditions to ensure that competitiveness both pre- and post-harvest can be maintained. The fact that AFB_1 control was more effective at the milky ripe (R3) and dough stages (R4) of maize cobs pre-harvest suggests that the window for control based on the a_w range at these stages may be an important consideration for application of the biocontrol strain. Recently, Giorni et al. (2019) showed that relative infection of ripening maize cobs by *A. flavus*, *Fusarium verticillioides* and *F. graminearum* influenced the colonization and the mixture of mycotoxins contaminating the maize at harvest. Thus, the presence of *A. flavus* impacted on the contamination of the maize grain with other mycotoxins such as fumonisin B_1 (FB_1) and deoxynivalenol, perhaps because of the its wider range of temperature and water availability during silking.

The biocontrol of AFB_1 contamination at different ripening stages is important. They represent different a_w levels as well as different nutritional compositions during the silking process. However, since ripening stage did not affect the ability of the toxigenic strain to colonize the cobs, this suggests that toxigenic *A. flavus* strains are able to colonize maize cobs rapidly during silking, if entry points are available for infection. Thus, the resilience of the non-toxigenic biocontrol strains is critical to facilitate niche exclusion or effectively outcompete the toxigenic strains either in soil or in the later silking process (Medina et al., 2017b). Previously, it has been suggested that *A. flavus* is adapted to the ripening stages of maize, expressing specific genes to utilize the available carbon sources (CS; Reese et al., 2011; Dolezal et al., 2014). The non-toxigenic strain was effective in controlling AFB_1 production at the milky ripe and dough stages. However, at the dent stage, it was less effective. This was supported by the effects on the gene expression of one of the key regulatory genes (*aflR*) involved in secondary metabolite production. Previously, Verheecke et al. (2015) studied the efficacy of *Streptomyces* strains against toxigenic *A. flavus* strains on synthetic media. They examined five different

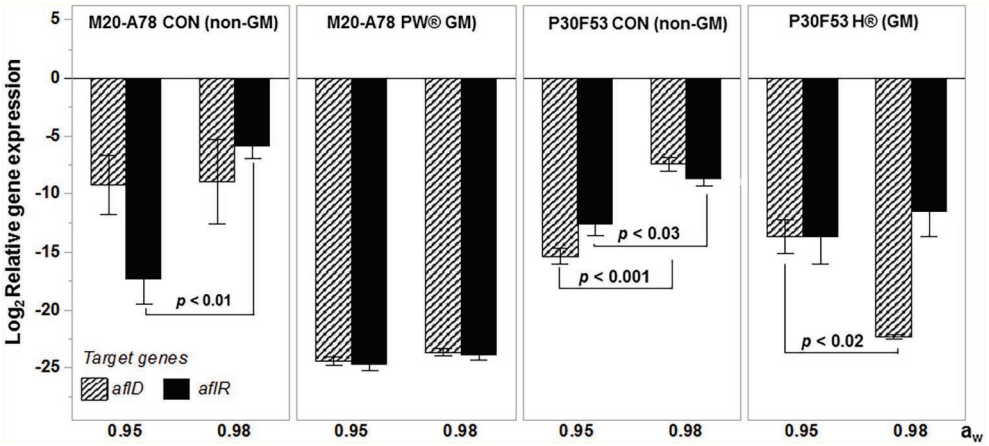

FIGURE 6 | Effect of biocontrol using the Brazilian AFL4^{-}:AFLb^{+} mixed non-toxigenic and toxigenic strain 50:50 ratio inoculum on the relative gene expression of the structural *aflD* and regulatory *aflR* genes on one of the non-GM and isogenic-GM cultivars stored post-harvest at both 0.98 and 0.95 water activity. The values of *p* show differences in overall AFB_1 content comparing GM and non-GM maize at 5% significance. Bars represent mean ± SE.

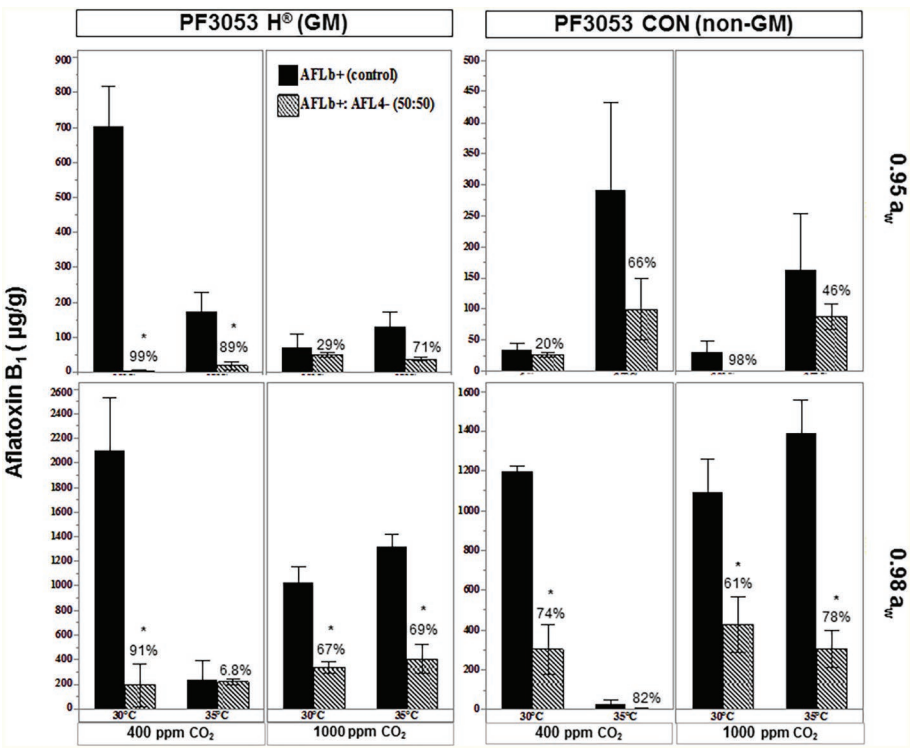

FIGURE 7 | Effect of temperature (30, 35°C), CO_2 (400, 1,000 ppm), and water activity (a_w, 0.98, 0.95) on aflatoxin B_1 contamination by the Brazilian toxigenic strain AFLb^{+} (control) and biocontrol mixture of AFLb^{+}:AFL4^{-} conidial ratio 50:50 pathogen:antagonist in conventional (P30F53 CON) and isogenic GM (P30F53 H®) stored maize kernels. Values above bars represent relative control (%) of aflatoxin B_1; *represent significant reduction ($p < 0.05$) of aflatoxin B_1 from the toxigenic control (AFLb^{+}).

biosynthetic genes involved in aflatoxin production. In their study, the *aflR* expression was decreased by a *Streptomyces* strain, but *aflD* expression was unaffected by all the biocontrol strains examined. However, these studies did not include the impact of water availability, which may have affected the relative control achieved. In the study by Al-Saad et al. (2016), the efficacy of bacterial biocontrol strains showed significant efficacy with a decrease in the *aflD* and *aflR* relative expression, although this was not always translated into effective phenotypic toxin control.

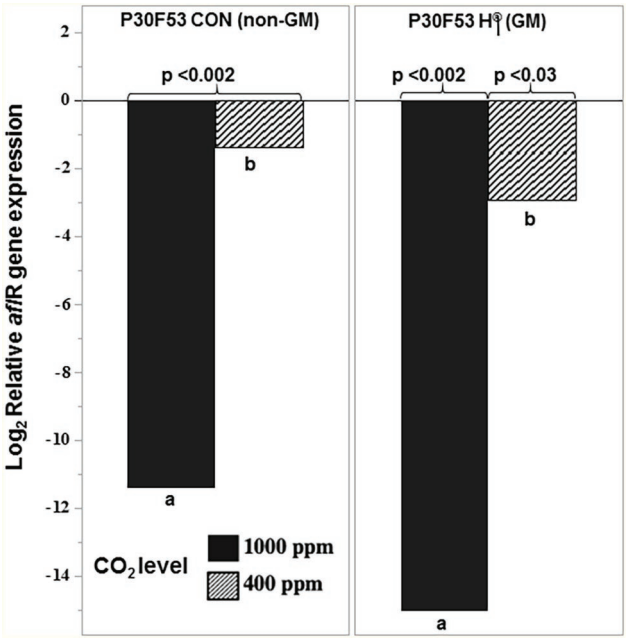

FIGURE 8 | Effect of the non-toxigenic *A. flavus* strain AFL4⁻ when applied as a conidial inoculum in the ratio of 50:50 with the toxigenic strain (AFL4⁻:AFLb⁺) on relative expression of the regulatory gene *aflR* in the conventional (P30F53 CON) and GM (P30F53 H®) stored maize grain at 0.98 a_w after 10 days at 35°C in 400 and 1,000 ppm CO_2 levels. The expression was normalized for the toxigenic control treatment (AFLb⁺) in each condition. The values of *p* represent significant differences from the control sample; different letters indicate effects of the CO_2 treatment levels ($p < 0.05$).

The non-toxigenic strain AFL4⁻ was able to significantly reduce the AFB_1 content *in situ* when paired with two different toxigenic strains, AFLb⁺ and the type strain NRRL3357 (AFLe⁺) in a 50:50 inoculum ratio. The relative AFB_1 control ranged from 58 to 100% under different a_w (0.98 and 0.95) conditions for up to 20 days storage. Furthermore, the present study has shown for the first time the comparison between impacts of such biocontrol in the reduction of AFB_1 in conventional (non-GM) and isogenic GM maize cultivars. The overall AFB_1 control indicated that the non-toxigenic strains applied were less resilient in non-GM stored maize cultivars, with the final control achieved being lower than that in the isogenic GM cultivars.

The question is whether this is due to differences in the biochemical composition of non-GM and GM cultivars due to the manipulation of the genes, e.g., the presence of the Cry1Ab gene. There are few studies, which have examined this. There is a study, which has suggested that Cry1Ab protein in maize residues had no direct effect on *F. graminearum* and *Trichoderma atroviride*. However, some corresponding BT/non-BT maize hybrids differed more in composition than that due to the Cry gene alone. However, this may affect the saprophytic growth of such fungi on crop residues (Naef et al., 2006). Changes in free fatty acids (FFA) in maize are an indicator of fungal infections and a deterioration in quality. Thus, fatty acids (FAs) in the maize could play a role in pathogen susceptibility and seed colonization (Dall'Asta et al., 2012). FFAs, especially of linoleic acid levels, partly regulate development, colonization, and mycotoxin production by *Aspergillus* spp. (Scarpari et al., 2014). Thus, changes in nutritional or environmental factors or both may also influence secondary metabolite production (Magan and Aldred, 2007).

The efficacy of the non-toxigenic strains in controlling AFB_1 production was supported by the gene expression of the target structural and regulatory genes (*afl*D, *afl*R). The results showed that competition in the maize grain niche inhibited the relative expression of both these genes. The correlation with phenotypic AFB_1 production showed that the lowered expression of these genes resulted in less toxin after 10 days storage. Previously, Al-Saad et al. (2016) when screening bacterial antagonists as biocontrol agents of toxigenic *A. flavus* strains sometimes found that while relative inhibition of *afl*D and *afl*R expression was evident, sometimes there was concomitant stimulation of AFB_1 production. Thus, the resilience of such potential biocontrol strains may well be influenced by water and temperature stress limitations, as well as inoculum ratio × nutritional parameters to avoid such effects. Recently, Venkatesh and Keller (2019) suggested that there are complex interactions between bacteria and mycotoxigenic fungi and focused on key ecological factors being light, nutrients, and pH. Two probably more important abiotic factors are water availability and temperature. Bacteria require almost freely available water (>0.98–0.99 a_w) for growth and have significantly less resilience to water stress than many mycotoxigenic fungi, which are either xerotolerant or xerophilic, the only exceptions being some of the toxigenic Fusaria. However, stored cereals are seldom harvested or stored under wet conditions, and thus, the interactions with bacteria may in fact be relatively limited, although pre-harvest interactions certainly must occur. Thus, the competitive exclusion of toxigenic strains of *A. flavus* is more likely to be successful with non-toxigenic strains or relatively xerotolerant species that may have the temperature and water stress tolerance to compete effectively. Indeed, Kohl et al. (2019) suggested that biocontrol agents use a cascade of mixed mechanisms of action to control plant pathogens, and this needs to be borne in mind when developing biocontrol strategies, including for toxigenic fungal pathogens.

It was noted that the relative gene expression of biosynthetic genes of the toxigenic *A. flavus* strains in non-GM and isogenic GM-maize was similar for the *afl*D and *afl*R at 30°C after 10 days storage. However, the phenotypic AFB_1 production was different in the two types of cultivars. For example, in the conventional M20-A78 CON cultivar, the relative reduction in AFB_1 was lower, and this was supported by the less pronounced reduction in the gene expression of the two genes examined. Furthermore, the a_w level also had an effect on the relative gene expression when the non-toxigenic strain was applied, under the same storage conditions. Previously, Abdel-Hadi et al. (2010) reported that under water stress levels (e.g., 0.90 a_w), the *afl*D expression can

be increased, although it is optimally expressed at 0.98 a_w on a conducive medium.

It has been shown previously that the ratio of the two regulatory genes *aflR* and *aflS* changes with a_w × temperature conditions (Abdel-Hadi et al., 2012). The *afl*R gene encodes for a positive regulator (AFLR), which activates the pathway gene transcription (Chang et al., 1995), whereas the *afl*S (=*afl*J) gene encodes for a protein factor (AFLS), which is involved in the regulation of transcription. These key genes are adjacent to and associated with the expression of a number of structural genes, e.g., *afl*C, *aflD* (*nor*-1), *afl*M (*ver*-1), and *afl*P (*omt*A; Chang and Hua, 2007). The capacity for disrupting the functioning of either or both of these two genes (*afl*S, *afl*R) perhaps by competition from non-toxigenic strains can reduce or completely inhibit aflatoxin production (Meyers et al., 1998; Yu et al., 2004).

This study has explored the effects of elevated CO_2 and temperature on potential for control by non-toxigenic fungi, both pre- and post-harvest with both non-GM and GM cultivars. Pre-harvest results suggest that the non-toxigenic strain used ($MEX02^-$) was not as resilient under elevated climate change interacting factors with no effective control of AFB_1 production in maize cobs of the dent stage. The post-harvest studies were able to examine the relative efficacy of the non-toxigenic strain ($AFL04^-$) on control of AFB_1 in related non-GM and isogenic GM cultivars for the first time. The effect of increasing CO_2 at 30 and 35°C varied depending on the type of maize used (conventional or GM). This was the first attempt to analyze whether using these two types of cultivars under climate-related abiotic factors can cause differences in AFB_1 production and the level of control achieved when using non-toxigenic biocontrol strains.

The action of the non-toxigenic *A. flavus* strain ($AFL4^-$) as a biocontrol agent was significant in the elevated CO_2 treatments, although the overall efficacy was lower than in non-climate change abiotic conditions. The use of the GM cultivar (P30F53 H®) showed better results in terms of relative biocontrol under abiotic stress (0.95 a_w) and increased CO_2.

There are limited previous studies to examine the effect of three-way interacting abiotic factors of elevated temperature × elevated CO_2 × drought stress effects on biocontrol of mycotoxin production (Verheecke-Vaessen et al., 2019). Previously, three-way interacting climate change abiotic factors were shown to stimulated AFB_1 production by the type strain of *A. flavus* (NRRL strain) both *in vitro* and in stored maize grain under increased temperature (30 vs. 34–37°C), 350 vs. 650/1,000 ppm, CO_2, and different a_w stress levels (Medina et al., 2015, 2017b). While growth of *A. flavus* was relatively unaffected, expression of key genes such as the *aflR* and *aflD* was significantly increased and translated into a stimulation of AFB_1 production. Indeed, Vaughan et al. (2014) demonstrated that elevated CO_2 (800 μmol CO_2 mol^{-1} air) enhanced maize susceptibility to *F. verticillioides* infection, but the increase in fungal biomass did not result in higher FB_1 toxin levels. Although subsequent studies suggested that there was an interaction between drought stress and elevated CO_2, which increased FB_1 production (Vaughan et al., 2016).

More recently, Gilbert et al. (2018) using RNA-Sequencing demonstrated that AFB_1 production in stored maize grain was altered by a_w × temperature × elevated CO_2. Also, several genes involved in the biosynthesis of secondary metabolites exhibit different responses to a_w or temperature stress depending on the atmospheric CO_2 content. At 37°C and 1,000 ppm CO_2, the transcription factor *afl*R was decreased. After 10 days incubation, the expression of biosynthetic genes in maize stored at 30°C generally decreased. However, the effects of high CO_2 (1,000 ppm) and water stress (0.91 a_w) showed decreased values, possibly in response to elevated AFB_1 levels (Gilbert et al., 2018).

Other imposed chemical stresses may also result in physiological impacts on toxigenic fungi such as *A. flavus*. Recent studies by Hanano et al. (2019) have shown that the most toxic congener of dioxin, the 2,3,7,8-tetrachlorinated dibenzo-p-dioxin, reduced growth but stimulated both conidial sporulation and AFB_1 production supported by levels of biosynthetic gene expression. Indeed, such exposure to chemical stress was shown to increase the production of superoxide dismutase and catalase. Of particular interest was the activity of caleosin/peroxygenase enzyme, which was activated in the presence of such recalcitrant compounds. This suggests that exposure to elevated levels of atmospheric particulates and gaseous stresses such as CO_2 and interaction with elevated temperatures may impact on physiological functioning of *A. flavus* and influence the toxin contamination levels.

CONCLUSIONS

This study has shown that the relationship between pre-harvest ripening stage of maize cobs and their inherent water availability will influence both colonization and AFB_1 production by toxigenic *A. flavus* strains and also influence the potential for effective control of toxin contamination. Thus, by using a 50:50 ratio of BCA:pathogen composition, the efficacy of the non-toxigenic strain was more effective at the R3 and R4 ripening stages, supported by the downregulation of the two toxin genes (*aflD, aflR*) relative expression, accompanied by a significant reduction in AFB_1 contamination. At the dent stage, perhaps a higher inoculum of the non-toxigenic strain is necessary for effective control.

Post-harvest, biocontrol of AFB_1 production in non-GM and GM cultivars was affected by interacting variables: type of cultivar, *T* °C, CO_2 levels, and water availability conditions. These interactions may also significantly affect BCA resilience and relative action. This study suggests that in GM cultivars, the relative control was slightly more effective than in the equivalent isogenic non-GM maize cultivars post-harvest. The resilience of the non-toxigenic strains appeared to vary between these. Overall, it is very important to include resilience to climate-related abiotic factors to ensure that

the identified strains of non-toxigenic strains and indeed other biocontrol candidates have the necessary ecological competence to compete effectively and reduce toxin contamination, whether the approach is for controlling pre- or post-harvest. Formulation approaches for such biocontrol agents may also play an important role in conserving resilience under a range of interacting abiotic conditions in the maize agroecosystem.

DATA AVAILABILITY STATEMENT

All the data sets are deposited with Cranfield University *via* the senior author and can be openly accessed *via* him.

AUTHOR CONTRIBUTIONS

AG and AR-S carried out the research work. EG-C and CV-V assisted with molecular work and quantification of toxins. AM and NM supervised the research and drafted the manuscript.

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Differential Expression of Signaling Pathway Genes Associated With Aflatoxin Reduction Quantitative Trait Loci in Maize (*Zea mays L.*)

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The roles of signaling pathway genes related to the aflatoxin reduction trait in maize were studied for the improvement of maize resistance to the fungal pathogen *Aspergillus flavus* (*A. flavus*). In this study, 55 maize genes in plant–pathogen interaction signaling pathways were investigated among 12 maize near-isogenic lines (NILs) that carry maize quantitative trait loci (QTL) associated with aflatoxin reduction. These maize NILs were developed from maize inbred lines Mp313E (resistant donor parent) and Va35 (susceptible recurrent parent). The quantitative RT-PCR (qRT-PCR) technique was used to study the gene expression patterns. Seven calcium-dependent protein kinases and one respiratory burst oxidase displayed significant differential expression levels among the maize QTL-NILs. In addition, the gene expression profiles of WRKY transcription factors were also examined. Maize WRKY 52, WRKY 71, and WRKY83 genes displayed significantly differential expression levels among the QTL-NILs. The elucidation of differentially expressed signaling pathway genes involving maize resistance to *A. flavus* can provide insights into maize disease resistance and enhance maize molecular breeding.

Keywords: signaling pathway genes, maize, *Aspergillus flavus*, aflatoxin reduction, quantitative trait loci

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INTRODUCTION

Aspergillus flavus (*A. flavus*) is a serious pathogen to developing maize ears. *A. flavus* is of particular interest because it is involved with the production of the carcinogenic aflatoxins. Aflatoxins are secondary metabolites of *A. flavus* and the contamination they cause greatly affects the quality and safety of maize products. A variety of health issues are associated with ingestion of aflatoxins with both humans and animals. Aflatoxin B_1 is a potent liver toxin and carcinogen (Eaton and Gallagher, 1994). The United States Food and Drug Administration established explicit guidelines to prevent hazardous amounts of aflatoxin exposure. However, high temperatures and drought provide favorable conditions for *A. flavus* contamination in maize. Researchers have proposed models estimating monetary losses in the range of $52.1 million to $1.68 billion dollars annually related to aflatoxin contamination of maize grain (Diener et al., 1987; Mitchell et al., 2016). These findings emphasized the need for intervention to reduce aflatoxin contamination in maize production.

The identification of host plant resistance genes and genetic factors associated with aflatoxin reduction is an important strategy to increase effectiveness of maize yield. The elucidation and utilization of maize resistance QTL represent a significant advancement in mitigating this plant pathogen. The maize QTL regions involved in this study have previously been described as contributing to aflatoxin reduction in maize inbred line Mp313E (Brooks et al., 2005; Warburton et al., 2009; Willcox et al., 2013). Twelve maize QTL-NILs were selected by backcrossing the QTL regions at maize chromosome bin locations 2.05, 3.05, 4.06, and 4.09 from maize inbred line Mp313E (resistant) into maize inbred line Va35 (susceptible). These breeding efforts can be enhanced with knowledge of maize defense mechanisms and genes in signal transduction pathways. In plants, the first steps in molecular responses of a pathogenic attack are to perceive the pathogen patterns and turn on the signaling pathways. These signaling pathways eventually lead to the expression of defense genes and the inhibition of pathogenic processes. Several signaling pathways have been characterized to initiate immune responses in plant when under attack by pathogens. These signaling pathway components are mainly protein kinases and transcription factors. Analysis of the relationships of plant signaling pathways with the resistance QTL regions present in the maize QTL-NILs will provide insights into the underlying mechanisms of maize resistance to *A. flavus*.

The plant immune responses consist of complex, cell-mediated defense patterns against a multitude of invading pathogens. Fungi such as *A. flavus* have the characteristic ability to invade plant epidermal cells and surround the exterior of the cells with hyphae (Jones and Dangl, 2006). The downstream signal transduction triggered by the detection of the fungus is important to maize resistance. Signaling pathways are initiated upon interaction of a transmembrane pattern recognition receptor (PRR) with a fungal pathogen-associated molecular pattern (PAMP) (Newman et al., 2013). PAMPs can be defined as conserved, specific patterns located on the pathogen that elicit a specific response from the appropriate PRR interaction on the host. Plants utilize PRRs present on the plasma membrane to detect PAPMs. PAMP detections by these receptors trigger the basal resistance response called pathogen-triggered immunity (PTI) (Cui et al., 2015). However, pathogens have developed the ability to counteract the basal PTI response by releasing effectors and thereby weakening the effects displayed by this initial response. Plants have developed a vigorous response to the pathogen effectors produced as a result of the initial basal response. This wave of robust defense has been termed the effector-triggered immunity (ETI). ETI is deployed by the plant when pathogenic effectors are recognized by pathogenic related proteins (PR proteins) characterized by nucleotide-binding and leucine-rich repeat domains (NB-LRR). These proteins potentiate the second branch of the plant immune system (Jones and Dangl, 2006). The effector response provides an amplification of the original PTI response and induces the hypersensitive response.

The genes involved in the fungal PAMP recognition and other signal transduction pathways have been characterized from a number of plant species and curated in major databases such as the Kyoto Encyclopedia of Genes and Genomes (Kanehisa and Goto, 2000). The PAMP-triggered immunity pathway includes genes of calcium-dependent protein kinase (CDPK), respiratory burst oxidase (RBO/NADPH), and reactive oxygen species (ROS) production. The DNA and protein sequences of these signaling pathway genes in the KEGG database provide the starting material for conducting a comprehensive genome-wide data mining for the corresponding maize genes in the maize genome database MaizeGDB. The objectives of this study were to conduct a genome-wide survey of maize genes involving maize and fungus interaction and signaling pathways and investigate the gene expression levels among the 12 maize QTL-NILs that carry maize resistance QTL regions.

MATERIALS AND METHODS

Plant Materials and Aflatoxin Test

The maize QTL regions were designated as QTL1 (bin 2.05), QTL2 (bin 3.05), QTL3 (bin 4.06), and QTL4 (bin 4.09), respectively. The 12 maize QTL-NILs were the advanced generations originally generated from backcrossing the maize inbred line Mp313E (resistant) into the Va35 (susceptible) background. Each QTL-NIL genotype carries either a single QTL region (e.g., NIL-QTL4) or multiple QTL regions in the genome (e.g., NIL-QTL1,2,3), in the latter case the QTL-NILs were developed by introducing two to three resistance QTL regions from Mp313E into Va35 genome. The 12 maize QTL-NILs as well as the two parental maize lines were planted at R. R. Plant Science Research Center at Mississippi State University, MS. The ears were inoculated 14 days after hand-pollination with the *A. flavus* strain NRRL3357 (ATCC # 200026; SRRC 167) utilizing the side-needle technique described by Windham et al. (2003). The field experimental design was a randomized complete block with split plot and three replications for each genotype. The developing kernel samples were collected 7 days after the inoculation and ground to a powder with liquid nitrogen (N_2) for future analysis. The remaining primary ears were harvested at maturity and measured for aflatoxin levels. Aflatoxin concentration was determined using the Vicam AflaTest method. Values for aflatoxin concentration were transformed [$\ln(y + 1)$] before statistical analysis. Tests of significance were performed before converting values to geometric means expressed in the original units of measure.

RNA Extraction

Total maize RNA was extracted from the ground kernel samples with the Aurum^{TM} Total RNA Fatty and Fibrous Tissue Kit from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, United States). The extraction followed the manufacturer's protocol with the following minor modifications. The ground kernel samples were placed into sterile 2 mL tubes with 100 mg per sample. 1 mL of cold Trizol was added to each sample and vigorously vortexed to ensure a complete suspension of kernel powder. The resulting lysate was incubated at room temperature for 5 min. The tubes

containing the lysate were centrifuged at 12,000 rpm for 5 min at 4°C. The Aurum™ Total RNA Fatty and Fibrous Tissue Pack instructions were followed from this point forward.

cDNA Synthesis

cDNA synthesis was conducted with the Invitrogen Thermoscript™ RT-PCR System (Invitrogen Life Technologies, Carlsbad, CA, United States) adhering to the manufacturer protocol. A total of 10 μL from each sample of previously extracted total RNA was used for the cDNA synthesis. The RNA was denatured at 65°C for 5 min then kept at 4°C. The cDNA synthesis was conducted at 50°C for 60 min, followed by 85°C for 5 min. The synthesized cDNA samples were stored at −20°C until further qRT-PCR analysis.

Data Mining, Primer Design, and Primer Efficiency

The DNA sequences of genes in the plant pathogen interaction pathways were obtained from the KEGG Pathway Database[1]. More specifically, the DNA sequences of the Plant-Pathogen Interactions 5.10 pathway genes from KEGG were used to blast the MaizeGDB database and search for all potential orthologous maize gene sequences present in the maize (*Zea mays L.*) genome. Some of these genes were found to locate within the resistance QTL regions. All primers were designed with software Primer3. The PCR amplification efficiencies for the designed primers were analyzed before the gene expression analysis. The PCR amplification efficiencies were determined with a standard curve analysis technique. The sample dilution for the standard curve analysis utilized a 5-stage 3-fold dilution series and the mean ΔCp values vs. log of the dilution factors were plotted. The efficiencies of primers were calculated applying the equation $\mathrm{Log}_2(r+1)$, where r is the coefficient of determination of the linear standard curve equation. Only primers with efficiency above 0.9 were used for further gene expression analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the control housekeeping gene for gene expression data normalization.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed with Roche Light-Cycler 480 (Roche Diagnostics Operations, Indianapolis, IN, United States). The Roche Light-Cycler 480 SYBR Green 1 Master kit was used for the qRT-PCR analysis. A total of 45 inoculated maize samples (with *A. flavus* strain 3357) were analyzed for gene expression, including 12 QTL-NILs, the two parental inbred lines, and one F_1 hybrid of Mp313E and Va35. The qRT-PCR program was: (1) 1 cycle of 95°C for 5 min; (2) 45 cycles of 95°C for 10 s, 60°C for 15 s, 72°C for 15 s; (3) 1 cycle of 95°C for 5 s, 65°C for 1 min, 97°C at continuous; (4) 1 cycle of 40°C for 10 s.

Data Analysis

Phylogenetic trees were constructed with the program MUSCLE and displayed with program MEGA7. The R statistical programing language was used to perform analysis of variance (ANOVA). The significance level was determined at $p < 0.05$. Pearson's coefficients were calculated on the gene expression data between all pairs of genes for the correlation analysis. The R package "Corrgram" was used to display the Pearson's coefficients. Network analysis was performed following the manual of the R packages "sna" and "network."

[1]https://www.genome.jp/kegg

RESULTS

Aflatoxin Reduction Levels in the Selected Maize QTL-NILs

The presence of QTL regions in the 12 maize QTL-NILs were confirmed by genotyping the presence of the DNA markers flanking the QTL1, QTL2, QTL3, and QTL4 chromosome bin regions. To quantify the effects of the resistance QTLs on aflatoxin reduction, the primary ears from the QTL-NILs as well as the two parental maize lines were artificially inoculated, collected at maturity, and measured for aflatoxin concentration. **Table 1** shows the average aflatoxin levels (ng/g) determined on per 50 g ground mature kernels for each QTL-NIL with three replications. Generally speaking, greater effects in aflatoxin reduction were observed from QTL-NILs carrying multiple QTLs in each line than those carrying a single QTL in each line. However, exceptions from the above observation were also revealed, indicating interactions exist between genes and QTLs. The parental inbred line Mp313E (resistant genotype) exhibited lowest level of aflatoxin at 11 ng/g, whereas, the parental inbred line Va35 (susceptible genotype and the recurrent parent) exhibited highest aflatoxin level of 805 ng/g. The 12 QTL-NILs exhibited aflatoxin levels between the parental lines (**Table 1**). Since the aflatoxin reduction effects were resulted from the introduction of the QTL regions from Mp313E to Va35, the QTL-NILs were validated suitable and valuable materials for resistance gene studies.

TABLE 1 | Mean aflatoxin concentrations measured in the mature kernels for maize QTL-NILs and inbred parents.

Genotype	ln(y+1)	ng/g
Va35 (Susceptible)	6.69	805
Mp313E (Resistant)	3.08	21
NIL-QTL #2, #3, #4	6.13	456
NIL-QTL #1, #2, #3	4.51	90
NIL-QTL #2	5.04	154
NIL-QTL #2, #3	5.66	287
NIL-QTL #1, #2	3.73	41
NIL-QTL #1, #2, #4	5.01	150
NIL-QTL #4	6.62	750
NIL-QTL #1	5.56	284
NIL-QTL #1, #4	5.77	318
Mp313E x Va35	3.7	39
NIL-QTL #3, #4	5.13	168
NIL-QTL #3	5.06	157

LSD = 1.34.

Data Mining of Candidate Maize–Fungus Interaction Pathway Genes

Using DNA sequences and protein sequences of genes in the Plant-Pathogen Interaction Pathway 5.10 from KEGG to blast the MaizeGDB database, 117 potential orthologous maize genes were identified in the maize (*Zea mays*) genome (**Supplementary Table 1**). **Figure 1** depicts the plant–pathogen interaction signaling pathways as well as the possible crosstalk that exist between these pathways. Two major pathways were focused on for selection of genes for expression analysis. The first pathway is the PAMP-triggered immunity pathway which involves CDPK, respiratory burst oxidase (RBO), and ROS production. The second pathway involves a calcium related signaling pathway which includes CDPK, calmodulin/calmodulin-like proteins (CaM/CML), cyclic nucleotide-gated channels (CNGC), and WRKY transcription factors (**Figure 1**). Data mining of maize pathway genes resulted in 35 CDPK genes, 59 CaM/CML genes, 11 RBO genes and 12 WRKY genes for further analysis (**Supplementary Table 1**).

Phylogenetic Analysis

Phylogenetic trees were constructed to assess the genetic similarities and relationship in the selected maize pathway genes with the program MUSCLE and the phylogenetic trees were displayed with program MEGA7. Phylogenetic analysis was conducted through examination of the phylogenic trees by gene families, which are associated with the plant pathogen interaction pathways. **Figure 2** displays a phylogenetic tree constructed for the CDPKs. Five major groups were observed from 35 CDPK genes. The selected CDPK genes for the subsequent gene

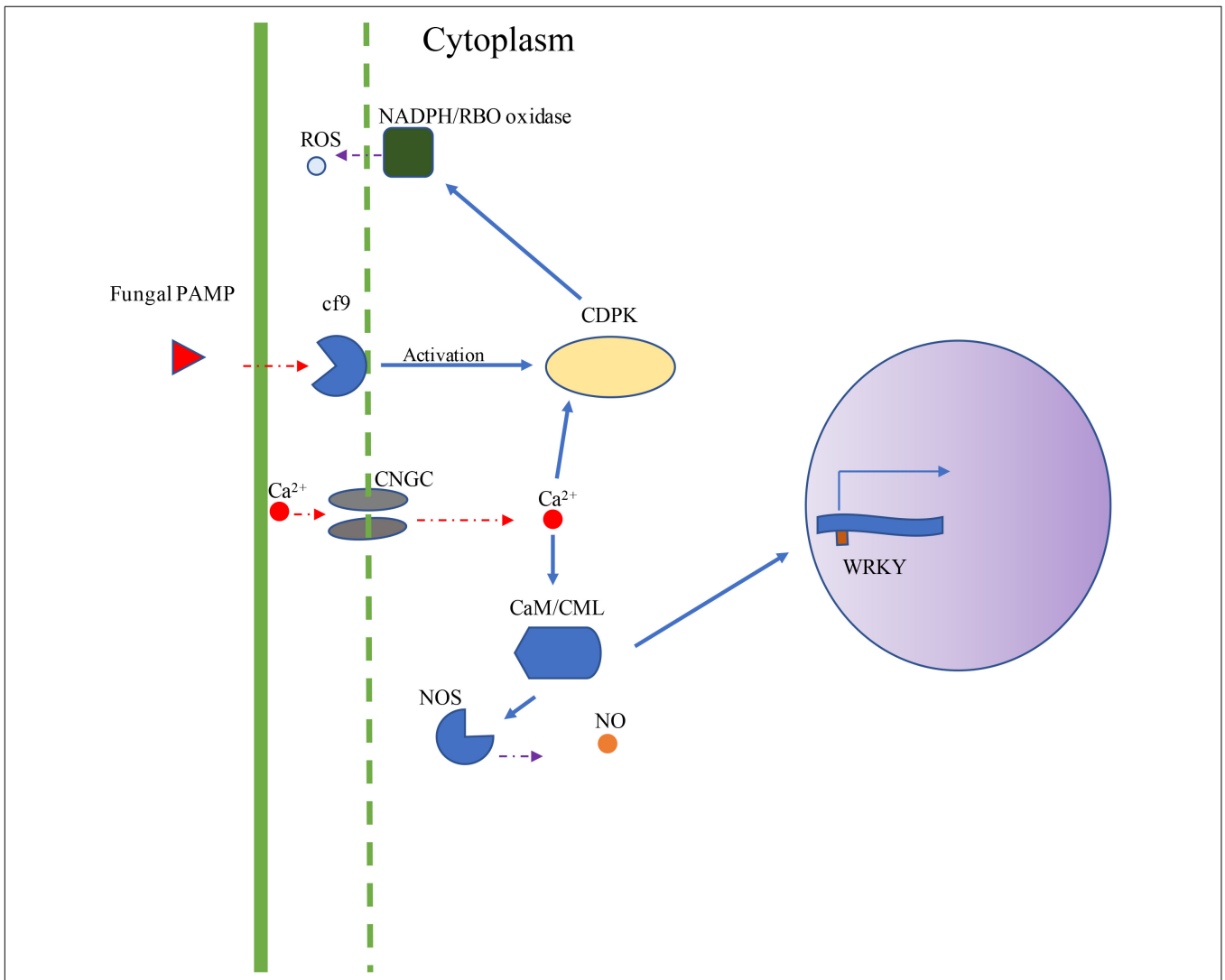

FIGURE 1 | Visual depiction of KEGG Plant–Pathogen Interaction Pathways. The visual depiction summarizes the major signaling pathways obtained from KEGG representing the plant cell interaction with fungal pathogen. Two major pathways were studied for gene expression analysis. The first pathway is the PAMP-triggered immunity pathway (Cf9, CDPK, RBO, and ROS). The second pathway involves CDPKs, CaM/CMLs, CNGCs, and WRKY transcription factors. In this study, maize genes were selected from CDPK, RBO, CaM/CML, CNGC, and WRKY gene families.

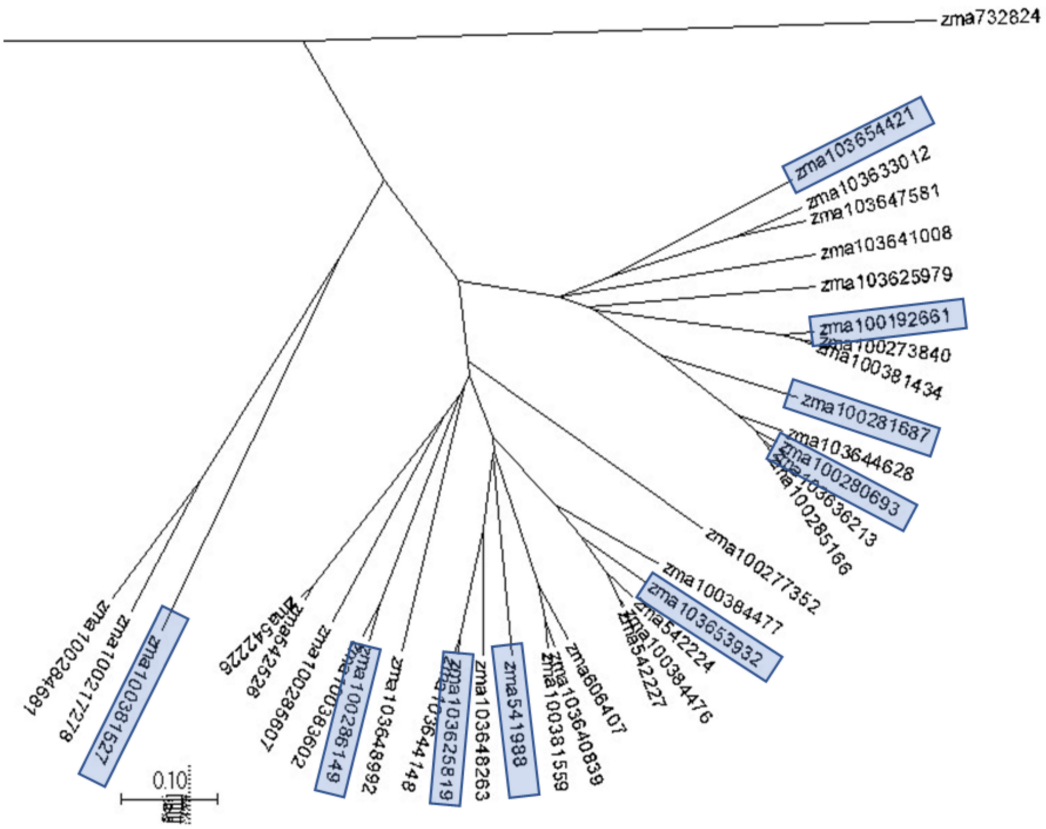

FIGURE 2 | A phylogenetic tree for maize calcium-dependent protein kinases (CDPKs). A phylogenetic tree were constructed to assess the genetic similarities and relationship in maize CDPKs using MUSCLE and displayed with MEGA7. Five major clades were observed from 35 CDPK genes. CDPK genes selected for the subsequent gene expression analysis are highlighted and represented the major clades.

expression analysis represented the major groups. Therefore, each major group in the phylogenetic tree was represented by at least one CDPK gene (**Figure 2**). Phylogenetic trees were constructed for all gene families to select the genes for primer design and qRT-PCR gene expression analysis.

Gene Expression Analysis

A total of 55 genes from four gene families were selected as pathway representing genes for the primer design and further qRT-PCR gene expression analysis (**Supplementary Tables 1, 2**). The PCR efficiency for each pair of gene primers was determined (**Supplementary Tables 1, 2**). Gene primers that showed efficiencies above 0.9 were selected for further qRT-PCR analysis. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) had a PCR efficiency of 0.98. Out of the 55 qRT-PCR gene primer evaluation assays, 20 gene primers had a PCR efficiency > 0.9 (**Supplementary Tables 1, 2**) and were used in the subsequent qRT-PCR gene expression analysis. Quantitative RT-PCR gene expression analysis revealed a total of 11 differentially expressed genes in calcium related signaling pathways based on *p*-value at significant level of 0.05, including CDPK, CNGC, and WRKY transcription factors (**Table 2**). No significant deferentially expressed genes were identified in CaM/CML (**Table 2**).

The genes *zma103653932, zma1036544221, zma100192661, zma100281687, zma100280693, zma100286149, zma103625819* displayed the significant differential gene expression ($p < 0.05$)

TABLE 2 | Differentially expressed signaling pathway genes identified in maize QTL-NILs.

	KEGG	Maize GDB	*p*-Value
CDPK	zma103653932	GRMZM2G081310	0.03
	zma103654421	GRMZM2G2332660	0.02
	zma100192661	GRMZM2G311220	0.05
	zma100281687	GRMZM2G104125	0.002
	zma541988	GRMZM2G320506	0.16
	zma100280693	GRMZM2G099425	0.008
	zma100286149	GRMZM2G025387	0.0038
	zma103625819	GRMZM2G027351	0.025
CaM/CaML	zma100285141	GRMZM2G115628	0.17
	zma100282040	GRMZM2G155822	0.1
	zma100193164	GRMZM2G142693	0.09
RBO	zma10010532	GRMZM2G043435	0.0055
WRKY	zma100275623/WRKY52	GRMZM2G151407	0.0017
	zma100383070/WRKY72	GRMZM5G816457	0.14
	zma100384128/WRKY71	GRMZM2G052671	0.01
	WRKY83	GRMZM2G012724	0.05

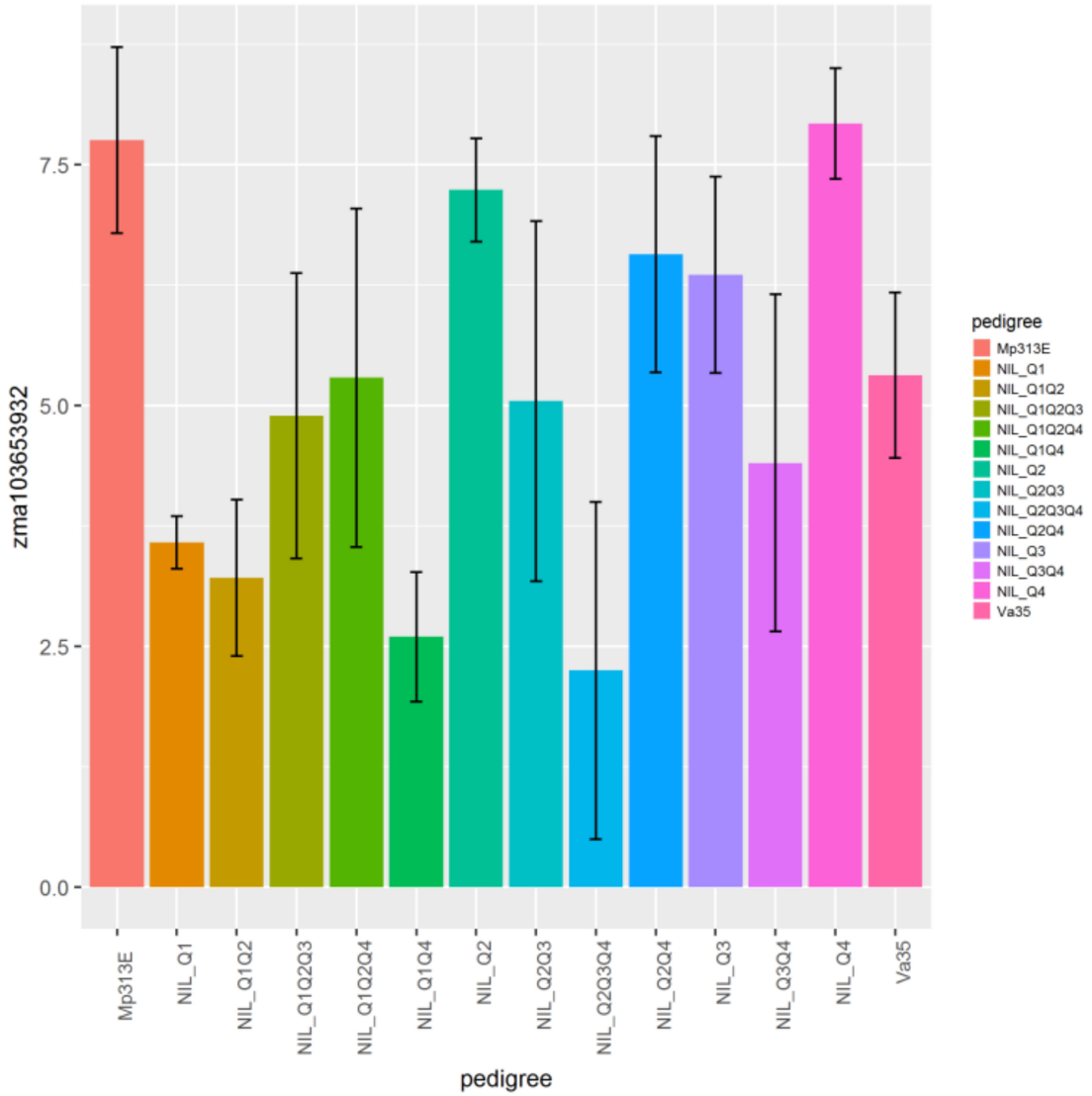

FIGURE 3 | Relative gene expression levels of a calcium-dependent protein kinase gene, zma103653932 (GRMZM2G081310) in maize QTL-NILs. A plot showing the relative expression levels of gene *zma103653932* (*GRMZM2G081310*) in maize QTL-NILs. The relative gene expression levels were presented in terms of the average ΔΔCp values obtained from qRT-PCR analysis. This data represent summarization of ΔΔCp values from samples of a field experimental design that was a randomized complete block with split plot and three replications for each QTL-NIL. The significance level of differential gene expression of the CDPK *zma103653932* gene is determined at $p < 0.05$.

(**Table 2**). Gene *zma103653932* is a CDPK based on sequence alignment with the maize B73 genome sequences in MaizeGDB. More specifically this gene is categorized as cdpk7. *Zma103653932* is located within the QTL 3 region on chromosome 4. **Figure 3** shows the expression levels of *zma103653932* expressed among the QTL-NILs. It is highly expressed in the QTL-NILs carrying single QTL2, QTL3, QTL4, and a combined QTL2 and QTL4. Computational protein structure analysis revealed unique characteristics of *zma103653932* protein such as possessing both a kinase domain and a calmodulin domain in one gene product (data not shown). The calmodulin domain of *zma103653932* protein is found to be highly related to the soybean CDPK regulatory region. Gene *zma100281687* is a second CDPK with significant differential gene expression with a *p*-value of 0.002. Gene *zma100280693* is a differentially expressed CDPK located on chromosome 2. Gene *zma100286149* is a CDPK designated on chromosome 8. The overall trend for all differentially expressed CDPKs was very similar with the highest gene expression showed in the QTL-NILs carrying the single QTL2, QTL3, and QTL4 regions.

One RBO gene, *zma10010532,* was found to be significantly differentially expressed among the QTL-NILs at $p < 0.05$. The gene *zma1001532* encodes a respiratory burst NADPH oxidase family enzyme. Upon further protein sequence analysis, this enzyme contains a ferric reductase-like transmembrane component, a FAD-binding domain (FAD binding 8), and

a ferric reductase NAD binding domain (NAD binding 6). Interestingly, this RBO gene showed higher levels of expression in the QTL-NILs carrying the single QTL2, QTL3, and QTL4 regions, similar to the trend observed in CDPKs. The genes of *WRKY 52*, *WRKY 71*, *WRKY72*, and *WRKY 83* contain the WRKY DNA binding domains. The expression of gene *WRKY72* showed similar patterns to CDPK genes with NIL-QTL2, NIL-QTL4, and NIL-QTL2,4 lines exhibiting higher expression levels. However, the highest expression for *WRKY 52* gene was found in NIL-QTL1,2,3 with a *p*-value of 0.001. *WRKY 83* exhibited a slight difference with the NIL-QTL2 line showing higher gene expression.

Gene Expression Analysis by Correlogram and Network

Given similar gene expression patterns observed among some of the signaling pathway genes, further statistical correlation studies were conducted using the gene expression data. A correlogram was generated to display the matrix of Pearson's coefficients calculated from the gene expression data between all pairs of the signaling pathway genes (**Figure 4**). All the significantly expressed signaling pathway genes investigated in this study showed positively correlation based on the gene expression patterns among the maize QTL-NILs. The qRT-PCR gene expression data were also analyzed with principal component analysis. The Euclidean distance values were calculated from the scores of the first two principal components. The empirical gene expression network was constructed based on the Euclidean distance matrix. **Figure 5** displays the network of the genes by expression analysis. The genes connecting in the network were potentially closely interacting genes in the signaling pathways. The network analysis showed that a RBO gene *zma100101532* was potentially involved with the expression of seven CDPK genes. Two WRKY factors *zma100383070* and WRKY83 were connected in the expression of a CDPK gene, *zma10365441*. All the CaM/CML genes appeared not to be connected (isolates) in the network.

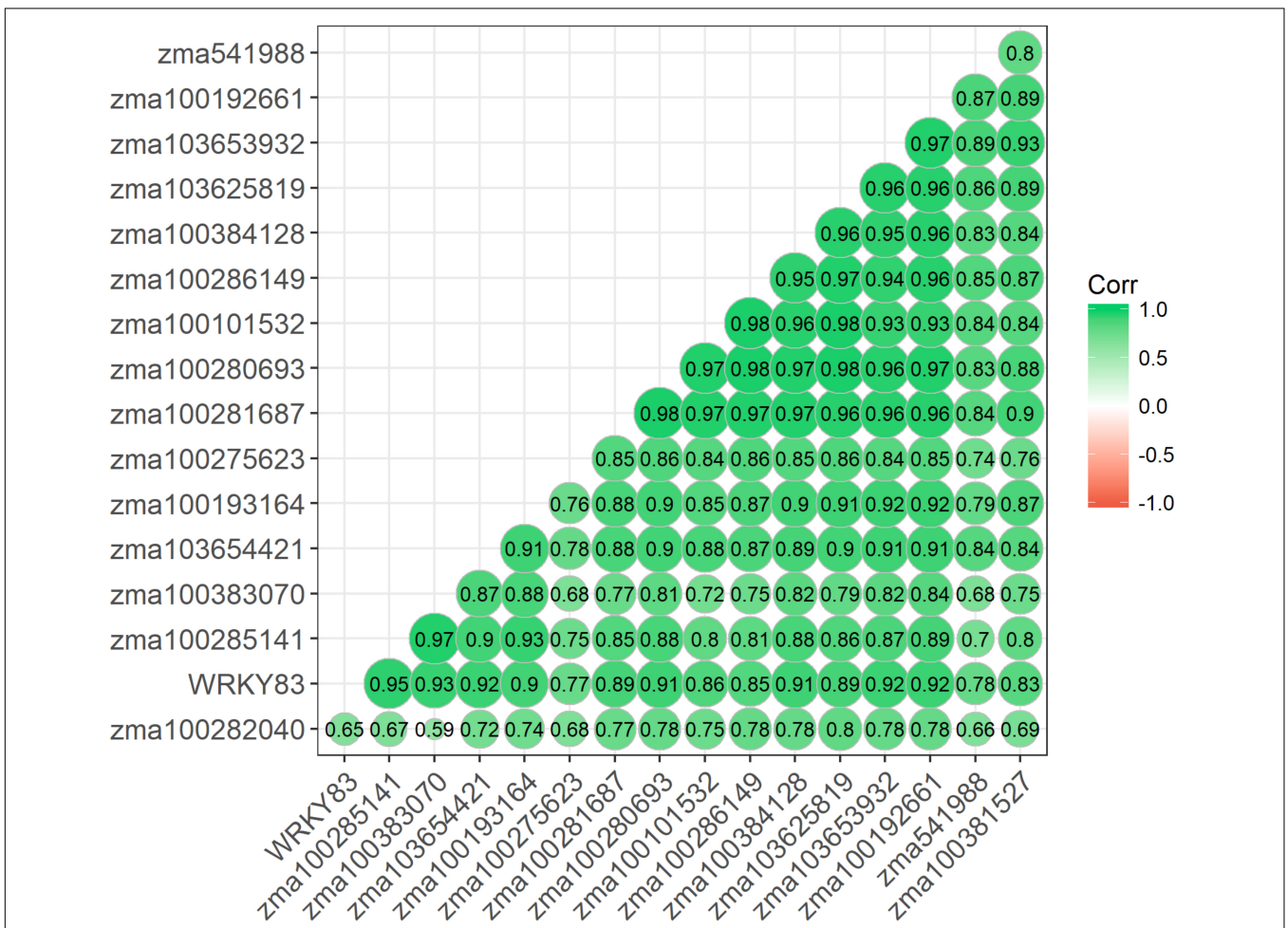

FIGURE 4 | A Correlogram displaying the Pearson's coefficients on all gene pairs using expression data of the significant candidate genes. Pearson's coefficients were calculated based on the gene expression data between all pairs of the signaling pathway genes studied. A correlogram is a direct visual display of the matrix of Pearson's coefficients. By this method, correlations between genes are displayed by grouping genes that have similar expression patterns, and the size of the color-coded circles proportionally represents the values of Pearson's coefficients. Green color represents positive correlations.

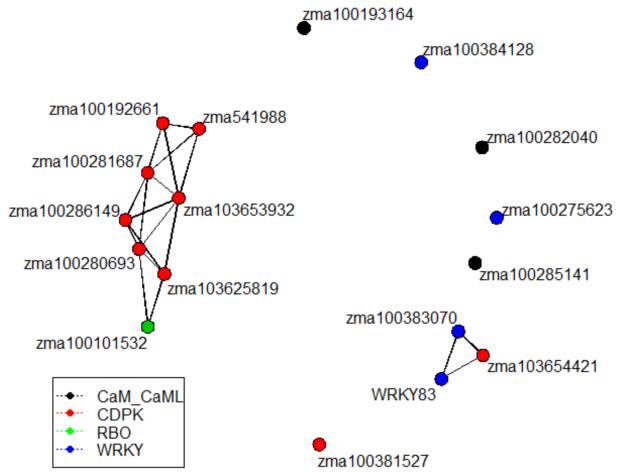

FIGURE 5 | The empirical gene network constructed from the gene expression data. Network was constructed using the Euclidean distance from the first two principal component scores of gene expression data analysis. Network were generated to display the empirical relations among signaling pathway genes. The vertices were color-coded to represent different gene families. The genes connecting in the network were potentially closely interacting genes in the maize fungus interaction signaling pathways.

DISCUSSION

The maize signaling pathway genes involve the activation of downstream proteins that lead to the defense responses in plant immune system. Upon the detection of the fungal PAMP pattern, an influx of calcium ions into the cytosol can be a result of activation of CNGCs by receptor kinase-bound nucleotidyl cyclases (Ma et al., 2009; Fountain et al., 2015a). These PTI responses increase cAMP or cGMP signaling molecules. The influx of Ca^{2+} ions activate the CDPKs which in turn active NADPH oxidase. CDPKs constitute a group of protein kinases with activity within plant extracts (Knight and Knight, 2001). These kinases along with calmodulin and calcium-regulated phosphatases are referred to as calcium sensors. These enzymes hold several roles within plant biology systems. CDPKs exhibit activation and function associated with hormone-related signaling, pathogen attack, and signaling during periods of environmental stress (Chen et al., 2004; Ludwig et al., 2004; Fountain et al., 2015a). CDPKs can also exhibit extensive cross-talk among differing signaling pathways in response to these stimuli (Ludwig et al., 2004). Specific CDPKs such as *ZmCDPK10* have previously been described to become activated significantly upon elicitation by fungal effectors (Murillo et al., 2001). In this study, seven CDPKs displayed a statistically significant difference among the maize QTL-NILs. The elucidation of signaling gene expression patterns of CDPKs associated with the resistance QTL-NILs can provide an avenue to the understanding of which genes are involved with PTI response signaling pathways.

Calmodulin/calmodulin-like proteins represent a wide array of functions within the plant. CaM/CMLs are calcium sensors and contribute to functions pertaining to metabolism, kinases, and phosphatases (Perochon et al., 2011). Previous studies have indicated that these proteins play pivotal roles in expression of plant immune responses (Perochon et al., 2011). However, analysis of the gene expression of three CaM/CML proteins in this study didn't show significant differential expression. Furthermore, these CaM/CML genes (*zma100193164, zma100282040,* and *zma100285141*) appeared to be isolates and were not connected any other genes in the empirical gene expression network (**Figure 5**). Given the findings of a maize CDPK gene, *zma103653932,* in this study has both a CDPK domain and a CaM/CML domain in one gene, it represents a point for future analysis pertaining to the roles of such 2 in 1 genes (carrying both CDPK and CaM/CML functions in one gene) in potential plant defense against *Aspergillus flavus* infection.

NADPH oxidase converts O_2 to the superoxide anion (O_2). Eventually, the superoxide dismutase (SOD) converts O_2^- to hydrogen peroxide (H_2O_2). This molecule functions in cytosolic defense signaling (Fountain et al., 2015a). Respiratory burst oxidases or NADPH oxidases constitute an essential enzyme involved with ROS production and signaling. Several studies have been conducted to continue the knowledge of these enzymes. Proels et al. (2010) developed transgenic barley plants with knock down expression of a RBO homologous NADPH oxidase gene called HvRBOHF2. The results indicated that these knock-down plants exhibited increased susceptibility to fungal invasion of leaf segments. RBO genes have been implicated with the model organism *Arabidopsis* (Torres et al., 2002). Two genes were under investigation for their roles in ROS production during the defense response. These results concluded that *ArtbohD* and *ArtbohF* are required for ROS (reactive oxygen intermediate) production in *Arabidopsis*. These results provide evidence for effectiveness of ROS production in plant cell defenses. However, a delicate balance of ROS production must be maintained to prevent fungal invasion as well. The accumulation of excess amounts of H_2O_2 can lead to detrimental results to the plants as well. In response, the plant has the ability to efficiently remove excess H_2O_2 and thereby alleviate oxidative stress involved with damage to the plant. These processes can be accomplished by the activity of catalase enzymes. The *A. flavus* resistant lines Mp313E and Mp420 have been determined to exhibit lower steady-state levels of H_2O_2 than susceptible lines (Magbanua et al., 2007). Upon analysis of this research, one RBO, *zma10010532* provided a significant difference among the maize QTL-NILs. NIL-QTL4 displayed a significantly high expression of this RBO enzyme. The processes by which plant–fungus interaction affect ROS presence is a complex dynamic (Segal and Wilson, 2018). Further analysis could point to the direction of susceptibility of the plants versus the amount of ROS present as well as the neutralization of the ROS balance.

The production of ROS is the eventual result of fungal PAMP recognition signaling. As previously stated, the hypersensitive response and cell wall reinforcement are the conclusion following ROS production. The RBO ROS production pathway is a tightly regulated signaling mechanism working in conjunction with calcium signaling and phosphorylation events (Dietz et al., 2016). These signaling pathways will provide fundamental insights to *A. flavus* initiated signaling in experimental maize crops.

Another heavily studied topic of plant immune defense strategies involves the complex network of WRKY transcription factors. WRKY transcription factors can be best described by a highly conserved 60 amino acid sequence region. This conserved region is characterized as displaying a WRKYGQK peptide sequence at the terminal end of the protein and a novel zinc-finger DNA binding motif at the C terminal end. Furthermore, this conserved region will preferentially bind to a *W* box with the specific sequence TTGAC(C/T) (Eulgem et al., 2000; Rushton et al., 2010). WRKY transcription factors have three distinct groups to which they are classified based on the designated numbers of WRKY domains present and the structural characteristics of the zinc-finger motif) (Eulgem et al., 2000; Rushton et al., 2010). WRKY transcription factors play essential roles in biotic and abiotic stresses of the plant innate immune system. The activity of these factors can be described as both positive and negative regulators of these signaling pathways (Pandey and Somssich, 2009; Rushton et al., 2010). Several plant species have shown to require WRKY transcription factors in response to abiotic and biotic stresses and include *Arabidopsis* (Deslandes et al., 2002; Imran et al., 2018), rice (Qiu et al., 2007; Xie et al., 2017), cotton (Zhou et al., 2015) and tomato (Li and Luan, 2014; Liu et al., 2014; Karkute et al., 2018). The maize ZmWRKY33 has been reported to be induced and potentially utilized in the abscisic acid defense signaling pathway (Li et al., 2013). ZmWRKY19, ZmWRKY53, and ZmWRKY67 have been reported to exhibit higher expression levels in a resistant maize line, TZAR101 potentially via salicylic acid and ethylene defense signaling pathways (Fountain et al., 2015b). Of the 55 genes analyzed in this study, WRKY52, WRKY71, and WRKY83 displayed differential gene expression with a *p*-value significant at 0.05 level among the maize QTL-NILs. Further analysis of these maize WRKY transcription factors will provide the elucidation of complete signaling pathways involved with maize resistance to *A. flavus*.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

XS and WW conceived and designed the experiments. FP, WW, GW, and XS performed the experiments. FP and XS analyzed the data and wrote the manuscript. WW and GW contributed and edited the manuscript. All authors have reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02683/full#supplementary-material

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Influence of Neighboring Clonal-Colonies on Aflatoxin Production by *Aspergillus flavus*

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Aspergillus flavus is an ascomycete fungus that infects and contaminates corn, peanuts, cottonseed, and treenuts with acutely toxic and carcinogenic aflatoxins. The ecological function of aflatoxin production is not well understood; though not phytotoxic, aflatoxin may be involved in resisting oxidative stress responses from infection or drought stress in plants. Observation of aflatoxin stimulation in 48-well plates in response to increasing inoculated wells sparked an investigation to determine if *A. flavus* volatiles influence aflatoxin production in neighboring colonies. Experiments controlling several culture conditions demonstrated a stimulation of aflatoxin production with increased well occupancy independent of pH buffer, moisture, or isolate. However, even with all wells inoculated, aflatoxin production was less in interior wells. Only one isolate stimulated aflatoxin production in a large Petri-dish format containing eight small Petri dishes with shared headspace. Other isolates consistently inhibited aflatoxin production when all eight Petri dishes were inoculated with *A. flavus*. No contact between cultures and only shared headspace implied the fungus produced inhibitory and stimulatory gases. Adding activated charcoal between wells and dishes prevented inhibition but not stimulation indicating stimulatory and inhibitory gases are different and/or gas is inhibitory at high concentration and stimulatory at lower concentrations. Characterizing stimulatory and inhibitory effects of gases in *A. flavus* headspace as well as the apparently opposing results in the two systems deserves further investigation. Determining how gases contribute to quorum sensing and communication could facilitate managing or using the gases in modified atmospheres during grain storage to minimize aflatoxin contamination.

Keywords: *Aspergillus flavus*, volatile chemical, volatile sensing, aflatoxin, quorum sensing

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INTRODUCTION

Aspergillus flavus is an ascomycete fungus that impacts agriculture, public and environmental health due to production of acutely toxic, carcinogenic aflatoxins in oil seed crops (Diener et al., 1987; Wicklow, 1991; Horn, 2003). Aflatoxins are toxic to humans and most animals including mammals, fish, and insects (Diener et al., 1987; Wicklow, 1991; Horn, 2003). Peanuts and corn are especially prone to aflatoxin contamination in tropical and sub-tropical climates (Diener et al., 1987; Wicklow, 1991; Horn, 2003). Aflatoxin production is favored in developing seeds infected

with *A. flavus* when crops experience heat and drought stress (Diener et al., 1987; Wicklow, 1991; Horn, 2003).

The ecological function of aflatoxin production in both the soil and phytobiome is not fully understood. Several exogenous factors influence aflatoxin production including: light, pH, water activity, reactive oxygen species (ROS), nitrogen, inorganic, and organic salts etc. (Joffe and Lisker, 1969; Brakhage, 2013; Roze et al., 2013; Woloshuk and Shim, 2013). It is suggested aflatoxin synthesis provides catalase and ROS consumption activity which increases tolerance to oxidative stress from plants during the infection process and under drought conditions (Fountain et al., 2015; Roze et al., 2015). Strains of *A. flavus* without aflatoxin production capabilities (atoxigenic) are also tolerant of H_2O_2 oxidative stress and are commonly isolated from plants, demonstrating aflatoxin is not necessary for infection (Diener et al., 1987; Wicklow, 1991; Horn, 2003; Abbas et al., 2005; Giorni et al., 2007; Atehnkeng et al., 2008; Sweany et al., 2011; Fountain et al., 2015) and *A. flavus* may have other mechanisms to limit oxidative stress during plant tissue invasion.

Aflatoxin is toxic to insects and may be important for competition against insects in both the soil and plant ecosystems (Zeng et al., 2006; Rohlfs and Obmann, 2009; Drott et al., 2017). Aflatoxin is toxic to *Drosophila* spp. (Rohlfs and Obmann, 2009; Drott et al., 2017), though some species are less sensitive to aflatoxin (Rohlfs and Obmann, 2009). Greater aflatoxin production in the presence of drosophila larvae and additional growth of both toxigenic and atoxigenic *A. flavus* due to greater larval mortality from supplemental aflatoxin suggest aflatoxin production gives a competitive advantage to the fungus in the enotomopathogenicity vs. fugivory interaction (Drott et al., 2017). In contrast, aflatoxin has limited effects on potential insect vectors that typically occupy similar plant niches as *A. flavus* (Zeng et al., 2006; Drott et al., 2017). Maize weevils are not killed by aflatoxin (Drott et al., 2017). Both toxigenic and atoxigenic *A. flavus* kill corn earworm larvae within a day (Poole and Damann, unpublished data). High concentrations of aflatoxin kill corn earworm larvae (Zeng et al., 2006), but in the presence of several plant compounds larvae produce cytochrome P450 monooxygenases that detoxify aflatoxin (Zeng et al., 2007; Niu et al., 2008). The ecological relationships between insects, aflatoxin production, fungal fitness, entomopathogenicity, and plant are complex with evidence of both toxicity to insects and evolving mechanisms to limit toxicity.

Aflatoxin is speculated to be important for survival in soil microbial communities. Aflatoxin is toxic to some common soil gram+ bacteria including *Bacillus*, *Streptomyces*, and *Nocardia* spp., but has limited toxicity to several fungi and other gram+ and gram− bacteria (Burmeister and Hesseltine, 1966; Arai et al., 1967). Volatiles produced by *Aspergillus nidulans* both stimulate and reduce aflatoxin production in *Aspergillus parasiticus* (Roze et al., 2007). Volatiles emitted from the soil-borne plant pathogenic bacterium *Ralstonia solanacearum* induce chlamydospore production and increase aflatoxin production (Spraker et al., 2014). Intensive research investigated the ability of atoxigenic *A. flavus* strains to competitively exclude toxigenic strains from crops and reduce aflatoxin contamination (Cotty, 1990; Brown et al., 1991; Dorner et al., 1992). Several lines of evidence indicate that there is inhibition of aflatoxin production, but the chemical/biochemical interaction is still not understood (Wicklow, et al. 2003; Mehl and Cotty, 2010; Abbas et al., 2011; Huang et al., 2011). Altering the concentration of conidia in culture modulates aflatoxin production in a density dependent manner similar to quorum sensing in bacteria (Clevström et al., 1983; Brown et al., 2009; Affeldt et al., 2012). At lower concentrations 10^3 conidia/ml medium, *A. flavus* produces sclerotia, aflatoxin, and limited conidia, in contrast at higher concentrations 10^7 conidia/ml medium, *A. flavus* shifts from sclerotial to dense conidial production and lower aflatoxin production (Brown et al., 2009; Affeldt et al., 2012). Deletion of oxylipin-generating dioxygenase genes, especially *ppoC*, restores sclerotial production at high inoculum densities and maintains aflatoxin production implicating oxylipins in the quorum sensing developmental shift (Brown et al., 2009; Affeldt et al., 2012). Cessation of aflatoxin production at higher conidial concentrations, suggests that aflatoxin is more important to survival of *A. flavus* at a low population. At low population sizes, there is more competition from surrounding microbes but as the population increases and *A. flavus* successfully colonizes the soil habitat there is less need to produce toxin presumably to compete with other organisms. Alternatively when fewer conidia are present, fewer colonies occupy the same space which results in better hyphal development and colony establishment potentially leading to secondary metabolism and sclerotization.

Initial experiments to determine if there is density-dependent stimulation of aflatoxin production from gases produced by the fungus within 24-well plates found no statistical differences in aflatoxin production if *A. flavus* grew in 6, 12, 18 or 24 wells, though there was a small increase that coincided with an increase in the number of wells with *A. flavus*. However if either 12, 24, 36 or 48 wells were inoculated with a single strain of *A. flavus* in 48-well plates, no aflatoxin was produced when *A. flavus* grew in only 12-wells, but ∼1000, 1500, and 2500 ppb aflatoxin B_1 was produced if 24, 36 or 48 wells were inoculated, respectively. Therefore, a series of experiments was conducted to determine if there is a density-dependent stimulation of aflatoxin production in *A. flavus*. Evidence is presented that volatile chemicals produced during growth of *A. flavus* both stimulate and inhibit aflatoxin production in a location and density-dependent manner. Understanding the volatile interactions between *A. flavus* has the potential to improve grain storage either by removing stimulatory compounds or modifying the atmosphere with inhibitory gases.

MATERIALS AND METHODS

Fungal Isolates

Several different experiments employed *A. flavus* isolates 53, Tox4 and Af70s. All isolates produce both aflatoxin B_1 and B_2, but do not produce any G toxins. Isolate 53 was isolated from corn in Louisiana in 2003 and produces large sclerotia >400 μm

(Huang et al., 2011). Tox4 (syn. 07-C-1-1-1) was isolated from corn in 2007, rarely produces large sclerotia and belongs to the same vegetative compatibility group as isolate 53 (Sweany et al., 2011). Af70s was isolated from a cotton field soil in Arizona and produces small sclerotia <400 μm (Cotty, 1989). Tox4 and 53 are both *Mat1-2* and Af70s is *Mat1-1*. All isolates were stored in 50:50 glycerol: water vol/vol at −20°C at Louisiana State University. Af70s was acquired from the USDA-SRRC fungal collection in New Orleans and 53 and Tox4 are deposited in the collection. For each experiment, a fresh batch of conidia grew on 5% V8 agar (pH 5.2) for 5 days and harvested in 2 mL of water by dislodging them with a glass rod.

Media

Liquid glucose salts (L) medium contained: 3.5 g $(NH_4)_2SO_4$, 750 mg KH_2PO_4, 350 mg $MgSO_4{\cdot}7H_2O$, 75 mg $CaCl_2{\cdot}2H_2O$, 10 mg $ZnSO_4{\cdot}7H_2O$, 5 mg $MnCl_2{\cdot}4H_2O$, 2 mg $NH_4Mo_7O_{24}{\cdot}4H_2O$, 2 mg $Na_2B_4O_7{\cdot}10H_2O$ and 2 mg $FeSO_4{\cdot}7H_2O$ and 50 g D-glucose per liter (Wicklow et al., 2003). The salts solution and glucose were autoclaved separately. Citrate buffer (0.053 M citric acid and 0.027 M sodium citrate) was added to medium (L-buffered) to buffer at pH4, maximize aflatoxin production, minimize degradation and prevent extreme acidification of medium to less than 2 (Doyle and Marth, 1978a,b; Cotty, 1988). Agar (2% wt/vol) was added to either non-buffered (S) or buffered (S-buffered) glucose salts medium.

Examination of *A. flavus* Well Occupancy Effects on Aflatoxin Production

A series of experiments investigated stimulation and inhibition of aflatoxin production by gases produced from increasing the number of *A. flavus* cultures in 48-well plates (a closed system). To rule out possibilities other than gaseous production by *A. flavus*, culture conditions were manipulated within 48-well plates.

(A) Initial experimental design (**Figure 1**). To determine if the number of cultures within a closed system alters aflatoxin production of an individual strain, within four Costar flat bottom 48-well plates (3548 Corning, New York, NY, United States) either 12, 24, 36 or 48 wells were filled with 500 μl 1×10^5 *A. flavus* 53 conidia/ml L-buffered glucose-salts medium; the other wells remained empty. 48-well plates consisted of 8 columns with six wells. All wells within either columns 1 and 5; 1, 4, 5, and 8; 1, 2, 4, 5, 6, and 8 or all 8 columns were filled with inoculated medium. Fungal colonies within wells were considered independent samples because the fungus and medium did not have contact with other wells and therefore should be independent resulting in either 12, 24, 36 or 48 replicated samples per plate. Individual wells (11.3 mm diameter) were attached to neighboring wells but separated by well walls with 1 mm thickness. The 48-well plates were wrapped in Parafilm, placed in individual boxes atop two wet paper towels and incubated for 4 days in the dark at 25°C. The fungus produced hyphal mats that primarily grew 0–3 mm on and below the medium-air interface. Aflatoxin was extracted from individual wells by mixing 240 μl of remaining medium below the hyphal mat with 240 μl acetonitrile. Aflatoxin B_1 was quantified with HPLC as described below. To determine if the differences in aflatoxin production were due to gases produced by the fungus or culture conditions, several questions were investigated as follows:

(B) Does the lack of fungus and/or medium in empty wells cause the changes in aflatoxin production, i.e., do volatiles released from additional medium or fungal growth change aflatoxin production? Since there were empty wells in the initial experimental design, all non-inoculated wells were filled with L-buffered medium. As in the first experiment, either 12, 24, 36 or 48 wells were filled with 500 μl 1×10^5 *A. flavus* 53 conidia/ml L-buffered medium, but now the remaining wells were filled with L-buffered medium. Plates were incubated for 4 days and aflatoxin extracted as described above. Aflatoxin B_1 was quantified by HPLC as described below.

(C and D) Does solidified medium minimize the differences between wells? Agar was added to the medium to minimize 20–40 μl moisture loss in exterior wells and determine if differences in aflatoxin production were in response to more fungal growth within a closed system or moisture loss. All wells were filled with 500 μl of either S-buffered or non-buffered S-medium. As described above, either 12, 24, 36 or 48 wells were center-point inoculated with 1.25×10^7 *A. flavus* 53 conidia/ml in 1 μl. Plates were incubated for 4 days as above. To extract aflatoxin, medium and spores from each well were placed in a screwtop 3.7 ml glass vial filled with chloroform and incubated overnight in a fume hood. The chloroform was filtered through Whatman filter paper and evaporated overnight. The aflatoxin was resuspended in 0.5 ml of 80:20 vol/vol methanol: water and then mixed with 0.5 ml acetonitrile. Aflatoxin B_1 was quantified by HPLC as described below.

(E and D) Are the differences in aflatoxin production maintained without citrate pH buffer? Since citric acid is an important component of metabolism (Buchanan et al., 1985), 53 was grown in both S and L non-buffered medium. As described in (A), *A. flavus* suspended in liquid glucose salts medium was grown in 12, 24, 36 or 48 wells of 4–48 well plates without medium in non-inoculated wells. Additionally, all wells of 5th 48-well plate were filled with L-medium but only 12 wells contained conidia. The design for non-buffered S-medium was described in (C and D). Plates were incubated for 4 days and aflatoxin extracted as described above. Aflatoxin B_1 was quantified by HPLC as described below.

(F) Does reducing airflow with Parafilm remove differences between wells? To reduce exchange or release of chemicals from individual wells, all wells of a 48-well plate where filled with 500 μl 1×10^5 *A. flavus* 53 conidia/ml L-buffered medium. Four layers of Parafilm was placed flush atop the wells, the lid placed on top, wrapped in Parafilm, incubated for 4 days and aflatoxin extracted as described above. Aflatoxin B_1 was quantified by HPLC as described below.

(G) Does adsorbing gases to activated charcoal remove differences between wells? Activated charcoal was pipetted in the spaces between the wells filled with buffered L-medium. As

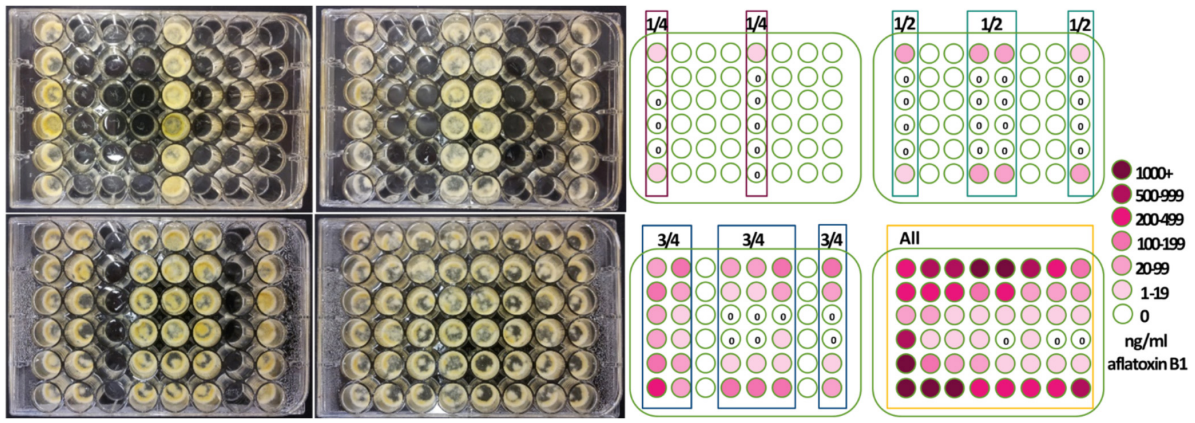

FIGURE 1 | Number of inoculated wells and well-location changed aflatoxin production by *Aspergillus flavus*. 48-well plates were inoculated with a single isolate 53 of *A. flavus* in the four arrays depicted. Centrally located wells produced more conidia and growth appeared less dense when all wells were inoculated. Aflatoxin production was less in the interior wells and greatest when all wells were inoculated with *A. flavus*.

in the second experiment, either 12, 24, 36 or 48 wells were filled with 500 μl 1×10^5 *A. flavus* 53 conidia/ml L-buffered medium; the remaining wells were filled with L-buffered medium. Plates were incubated for 4 days and aflatoxin extracted as described above. Aflatoxin B_1 was quantified by HPLC as described below.

(H and I) Does changing the isolate change the influence of the number of cultures on aflatoxin production? To determine if alteration in aflatoxin production due to changing the number of wells with *A. flavus* was isolate specific; a second isolate, Af70s was used. Either 12, 24, 36 or 48 wells were filled with 500 μl 1×10^5 *A. flavus* Af70s conidia/ml L-buffered medium; wells without conidia were either empty (H) or filled with L-buffed medium (I). Plates were incubated for 4 days and aflatoxin extracted as described above. Aflatoxin B_1 was quantified by HPLC as described below.

Rationale for Staggered Experiments

The above experiments were conducted serially due to incubator and sample processing constraints. The incubator could easily accommodate four plates in individual boxes and up to eight plates. Four plates consisted of 120 samples. Aflatoxin B_1 slowly degraded in the samples. To avoid confounding effect of aflatoxin degradation, only single variables were manipulated at a given time and compared to the first experiment A. The HPLC machine became irrepairable, therefore experiments in 48-well plates were not repeated and changing to a different pH buffer was not possible. Experiment A was repeated and the results were similar.

Examination of *A. flavus* Petri-Dish Occupancy on Aflatoxin Production

Though the chances of chemical diffusion between the wells is low, independent interior wells within 48-well have 4- (2 mm deep by 3 mm long) junction points. A method employed to investigate the influence of volatile production by *A. nidulans* on *A. parasiticus* was modified (Roze et al., 2007) to determine if gases produced by *A. flavus* grown in separate Petri-dishes with shared headspace affect aflatoxin production. Eight open 35 mm wide small Petri-dishes (Falcon 351058, Corning, New York, NY, United States) were nestled within a larger 150 mm diameter Petri-dish separated by ∼2 mm. All small Petri-dishes were filled with 4 ml of non-buffered S-medium. Either 1, 4 or 8 plates were center point inoculated with 1 μl of 1×10^8 conidia/ml of a single *A. flavus* isolate and the large dish was wrapped in two layers of Parafilm. Each inoculated dish was considered an independent sample and the experiment was replicated three times resulting in either 3, 12 or 24 replicated samples per condition. The dishes were incubated for 5 days at 25°C in the dark. Initially only isolate 53 was used. Aflatoxin was extracted from each plate by combining solid medium and spores in a 20 ml scintillation vial with chloroform and incubated overnight in a fume hood. The chloroform was filtered through Whatman filter paper and evaporated overnight. The aflatoxin was resuspended in 0.5 ml of 80:20 vol/vol methanol: water and then mixed with 0.5 ml acetonitrile. Aflatoxin B_1 was quantified by HPLC as described below.

Three grams of activated charcoal was added to the bottom of the large Petri dishes to adsorb volatile gases produced by *A. flavus*. Three different isolates (53, Tox 4 and Af70s) were independently center-point pipetted onto either 1, 4 or 8 plates with and without activated charcoal added to the base of the large dish and all treatments were replicated three times. Dishes were incubated for 5 days and aflatoxin extracted as described above. Aflatoxin B_1 was quantified by HPLC as described below.

Aflatoxin B_1 Quantification

All extracts were filtered through 1.5 ml polypropylene columns with 20 μm polyethylene frits, packed with 200 mg basic aluminum oxide (58Å, 60-mesh powder, 11503-A1, Alfa Aesar, Tewksbury, MA, United States) into an auto-sampler vial (Sobolev and Dorner, 2002). The aflatoxin was quantified with

reversed-phase high performance liquid chromatography using a Summit HPLC System (Dionex Corporation, Sunnyvale, CA, United States) with a P580 pump, ASI-100 automated sample injector, RF2000 fluorescence detector, and Chromeleon software version 6.20 (Joshua, 1993). A post-column derivatization step was conducted by exposing the extract to a UV light in a PHRED cell (Aura Industries Inc., New York, NY, United States) (Joshua, 1993). The mobile phase was 22.5 parts HPLC grade methanol: 22.5 parts HPLC grade acetonitrile: 55 parts distilled water mixture at 1 ml/min. The stationary phase was a Syncronis C18, 3 × 150 mm long column (Thermo Fisher Scientific Inc., Waltham, MA, United States). Aflatoxin B_1 was detected at 11.8 min and quantified by Chromeleon software using to pure 1, 10, 100, and 1000 ng aflatoxin B_1/ml standards.

Data Analysis

Aflatoxin B_1 means and standard error were calculated using Excel (Microsoft Corp., Redmond, WA, United States). Statistical analysis was conducted using SAS version 9.4 (SAS Institute, Cary, NC, United States). In the 48-well plates, individual wells were considered the sampling unit; $n = 1020$ across all individual experiments (including the different isolates). To partition the variance and account for heterogeneity within single 48-well plates, wells belonging to rows A and F (outter-most), B and E (intermediate) or C and D (inner-most) were treated as reps (4 reps/row location for 12 inoculated wells, 8 reps for 24, 12 reps for 36 and 16 reps for 48 inoculated wells within individual 48-well plates). Linear models approximated MANOVAs using proc Mixed. For the 48-well plate experiments, three full models were evaluated. The first model assessed the fixed categorical effects: media (L and S-medium and L and S- buffered medium), isolate (53 and Af70s), well location (rows A and F, B and E, and C and D), and number of inoculated wells (12, 24, 36 or 48) on log (aflatoxin concentration +1). A second full model assessed the fixed categorical effects activated charcoal, number of inoculated wells and location on log (aflatoxin concentration +1). The final full model tested the fixed categorical effects of Parafilm and location on aflatoxin concentration. Interactions between fixed effects were statistically significant if the *p*-value of *F*-test for type III fixed effects was less than 0.05. Due to significant interaction terms of full models, separate MANOVAs were calculated for each of the 48-well plate experiments described above (A–I) with fixed effects of well location and number of inoculated wells on log (aflatoxin +1). For the Petri-dish design two models were conducted. Individual Petri dishes were considered the sampling unit and each experiment was replicated three times. An initial model for isolate 53 accessed the fixed effects of location and number of inoculated dishes (1, 4 or 8) on aflatoxin concentration ($n = 39$). A second full model assessed the fixed effects of number of inoculated plates, strains (Tox4, 53 and Af70s) and activated charcoal on aflatoxin ($n = 234$). Due to significant interaction terms, separate MANOVAs by isolate were calculated. *Post hoc* comparison of means were calculated using Tukey-Kramer adjusted means and considered different if the *p*-value was 0.05 or less. Where appropriate, aflatoxin values were adjusted to log (toxin +1) to meet the assumption of normality.

RESULTS

Neighbors and Well-Location Alter Aflatoxin Production

Aspergillus flavus colony morphologies and aflatoxin production differed both when the number of neighboring wells within 48-well plates filled with isolate 53 conidia (in liquid buffered glucose salts medium i.e., L-buffered medium) and well-location changed (**Figure 1**). Mycelial and conidial production appeared less dense when *A. flavus* grew in all 48 wells especially in comparison to growth of *A. flavus* in 12 wells. This contrasted with a stimulation of aflatoxin production (**Table 1**, A). Increasing the number of wells with *A. flavus* stimulated aflatoxin production by as much as 900 ppb produced if *A. flavus* grew in all 48 vs. 12-wells. Regardless if grown in 12 to 48 wells, *A. flavus* in centrally located wells produced more conidia. In contrast to gain of conidial production, aflatoxin production was inhibited in the interior wells.

Filling Empty Wells Does Not Alter Location and Neighbor Effect

Overall, filling the non-inoculated wells with medium resulted in higher levels of aflatoxin production, which was most pronounced in the outer wells (**Table 1**, B). Regardless, inoculating more wells with *A. flavus* still stimulated aflatoxin production by at least 2000 ppb if more than 12 wells were inoculated, suggesting volatiles from both the identical fungal cultures and buffered liquid medium stimulated aflatoxin production. The addition of medium did not change the location effect; aflatoxin production was inhibited substantially within the interior wells, suggesting volatiles from the fungus (not additional medium) also inhibited aflatoxin production within the center of the plates.

Solid Medium Can Minimize Location and Neighbor Effect

Overall, *A. flavus* produced more aflatoxin on solidified medium. Growth on S-buffered medium minimized the location effect when all wells were inoculated, but not when 12 or 24 were inoculated (**Table 1**, C & D). The location effect was still pronounced on non-buffered S-medium, with the exception of 36 inoculated wells. Increasing the number of inoculated wells on both S-media stimulated aflatoxin production. On S-buffered medium, more inoculated wells resulted in a minimal increase in aflatoxin production, though in the innermost wells there was still a trend for increasing aflatoxin production. On non-buffered S-medium, there was an overall increase of 865 ppb as the number of inoculated wells increased from 12 to 36, followed by a decrease when all 48 wells were inoculated. Controlling for moisture loss with agar did not completely remove interior inhibition and stimulation by neighbors which suggests fungal volatiles also contribute to these effects.

TABLE 1 | Variable aflatoxin production within and between single isolates grown in individual wells of 48-well plates.

Experimental variables within 48-well plates[Z]					Number of wells inoculated with a single strain: mean aflatoxin production in wells (ppb)[Y]			
Strain (exp).[X]	**Medium**	**Non-inoculated wells filled?**	**Activated charcoal?**	**Well-row location[W]**	**12**	**24**	**36**	**48**
53 (A)	L-buffered	empty	none	Outer	11.4 ± 9.1 cd[V]	33.5 ± 6.0 bc	119 ± 15 b	914 ± 198 a
				Inner	0.10 ± 0.10 d	0 d	41 ± 15 bc	222 ± 97 b
				Inner most	0 d	0 d	17 ± 11 d	75 ± 62 cd
(B)	L-buffered	filled	none	Outer	130 ± 21 βγ	2288 ± 454 a	2343 ± 354 a	1098 ± 254 aβ
				Inner	23 ± 6 γδ	200 ± 122 γ	125 ± 18 γ	119 ± 21 γ
				Inner most	3.8 ± 2.7 δ	22 ± 10 δ	9.3 ± 3.2 δ	18 ± 3.6 δ
(C)	S-buffered	filled	none	Outer	5610 ± 181 aβ	5346 ± 154 aβ	6308 ± 75 a	5932 ± 75 a
				Inner	4433 ± 497 βγ	3527 ± 419 γ	5965 ± 163 a	5923 ± 64 a
				Inner most	1062 ± 462 δ	664 ± 189 δ	4583 ± 426 βγ	5670 ± 104 a
(D)	S	filled	none	Outer	15 ± 8.6 de	652 ± 117 a	832 ± 78 a	124 ± 19 b
				Inner	5.0 ± 3.6 e	504 ± 109 a	1046 ± 58 a	49 ± 8.3 cd
				Inner most	9.0 ± 4.6 e	264 ± 91 bc	750 ± 86 a	13 ± 1.7 e
(E)	L	empty	none	Outer	123 ± 75 b	1.4 ± 0.67 c	211 ± 28 a	83 ± 33 b
				Inner	1.5 ± 0.65 c	0 c	1.4 ± 0.60 c	1.2 ± 0.54 c
				Inner most	0 c	0 c	0 c	0.05 ± 0.03 c
	L	filled	none	Outer	0 c	n.a.u	n.a.	n.a.
				Inner	0 c	n.a.	n.a.	n.a.
				Inner most	0 c	n.a.	n.a.	n.a.
(F)	L-buffered	n.a.	Parafilm	Outer	n.a.	n.a.	n.a.	1.2 ± 0.60 γ
			reduced air	Inner	n.a.	n.a.	n.a.	4.5 ± 1.5 β
			between wells	Inner most	n.a.	n.a.	n.a.	9.7 ± 1.5 a
(G)	L-buffered	filled	charcoal	Outer	486 ± 73 δ	2040 ± 120 c	2943 ± 102 a	2805 ± 42 a
				Inner	440 ± 44 δ	837 ± 70 δ	2734 ± 134 ab	2596 ± 54 ab
				Inner most	340 ± 134 d	266 ± 89 d	2444 ± 138 abc	2306 ± 161 bc
Af70s (H)	L-buffered	empty	none	Outer	1965 ± 370 βγ	2674 ± 386 β	6297 ± 476 a	1064 ± 115 γd
				Inner	363 ± 54 eγ	2979 ± 474 β	2972 ± 425 β	793 ± 89 de
				Inner most	266 ± 65 γ	3468 ± 381 aβ	567 ± 104 eγ	710 ± 66 de
(I)	L-buffered	filled	none	Outer	4475 ± 662 bc	4793 ± 567 b	6909 ± 502 ab	1064 ± 115 d
				Inner	4838 ± 1135 ab	6002 ± 573 ab	6316 ± 432 ab	793 ± 89 d
				Inner most	5348 ± 575 ab	6755 ± 479 a	2210 ± 476 cd	710 ± 66 d
Aflatoxin scheme[t]		0 ppb	0-19 ppb	20-99 ppb	100-199 ppb	200-499 ppb	500-999 ppb	1000+ ppb

*To determine if increasing number of A. flavus cultures stimulates aflatoxin production, aflatoxin was extracted from individual wells of 48-well plates with variable number of identical A. flavus cultures. Plates incubated separately in boxes for 4 days in the dark at 25°C. [Z] Several different variables were manipulated to determine consistency of aflatoxin differences between wells in 48-well plates, including: isolates (53 and Af70s), media [(liquid (L) vs. solid (S) glucose salts medium), (citrate buffered vs. non-buffered glucose salts medium)], medium-filled vs. empty non-inoculated wells, activated charcoal between wells, and row (six total) location of cultures within plates. [Y] To determine the influence of the number of cultures on aflatoxin production within 48-well plates, A. flavus conidia were pipetted into all wells of columns 1 and 5 (1/4 plate or 12 wells); 1, 4, 5, and 9 (1/2 plate or 24 wells); 1, 2, 4, 5, 6, and 8 (3/4 plate or 36 wells) or all 48 wells of the plate. [X] Individual experiments denoted by capitalized letters investigated several different questions: (A) Does number of identical A. flavus cultures within 48-well plates alter aflatoxin production? (B) Is heterogeneity in aflatoxin production caused by lack of medium in the empty wells or more A. flavus growth? (C) and (D) Does solidified medium minimize the differences between wells? (E) and (D) Are the differences in aflatoxin production maintained without citrate pH buffer? (F) Does reducing air-flow with Parafilm remove differences between wells? (G) Does adsorbing gases to activated charcoal remove differences between wells? (H) and (I) Is isolate Af70s aflatoxin production influenced by the number of cultures? [W] Well location refers to the row a well belonged to within an individual 48-well plate. The outer rows are closest to the exterior of the plate (rows A and F), inner rows are the next rows inward from the edge (rows B and E) and the inner-most rows are in the middle of the plate (rows C and D). [V] Different letters indicate different mean aflatoxin B_1 ppb ± standard error values based Tukey-Adjusted Least Significant Differences at $\alpha < 0.05$. For simplification, differences of means are only reported within an individual experiment and not across experiments. Letters alternated from the English to Greek alphabet between individual experiments. [U] n.a. means not applicable because no experiment conducted. [t] Colors of the cells represent a heat map of aflatoxin production the colors are the same in **Figure 1**. Values above 1000 ppb are the same color, therefore, variation in exp. (C) and (I) are not visible.*

Buffering pH Enhances the Response to Neighbors but Not Location

Removing the citrate pH buffers decreased the sensitivity to neighboring colonies only in L-medium and did not change the location effect. The pH decreased from 4 to 2 in the non-buffered L-medium, which coincided with less aflatoxin, sparse mycelia and conidial production compared to buffered L-medium. There was an inconsistent aflatoxin response to the number of wells inoculated with *A. flavus* in liquid medium (**Table 1**, E). Regardless, the highest aflatoxin production within

an individual well (473 ppb) occurred when all 48 wells were inoculated. On S-medium (**Table 1**, D), removing pH buffers did not alter the stimulatory effect of neighboring cultures. Aflatoxin production was smallest in the interior of plates on both non-buffered media.

Minimizing Gas Exchange Limits Differences and Aflatoxin Production

Aflatoxin levels were essentially zero in all the wells when 4-layers of Parafilm were placed across the tops of the wells to reduce air exchange (**Table 1**, F). This was a stark contrast to 900 ppb aflatoxin production in the exterior rows with no Parafilm. In addition to minimal aflatoxin production, the location effect was lost, and when Parafilm was used there was slightly more aflatoxin production in the interior wells. Limiting gas exchange between wells removed both the stimulation caused by more inoculated wells and the inhibition in the interior of the plate.

Activated Charcoal Removes Inhibitory Gas(es)

Addition of activated charcoal between the wells of 48-well plates to adsorb volatile compounds resulted in an overall increase in aflatoxin production (**Table 1**, G). Regardless of stimulation by volatile adsorption, aflatoxin was further stimulated if *A. flavus* grew in more than 24 wells. With the exception of 24 inoculated wells, there was no longer a significant loss of aflatoxin production in the interior wells of the 48-well plates. The inhibition of aflatoxin production in the interior of the plate was 30% (12 wells), 90% (24 wells), 17% (36 wells), and 18% (48 wells) when activated charcoal was placed in between the wells, whereas if no activated charcoal was added the inhibition in the interior was 97, 99, 99, and 98% indicating the activated charcoal removed an inhibitory volatile compound.

Neighbors and Location Also Affect Af70s

A second isolate Af70s increased aflatoxin production as the number of inoculated wells increased from 12 to 36 wells by 2–4000 ppb (**Table 1**, H & I) regardless of whether the non-inoculated wells were empty or filled with buffered L-medium. Inoculation of all wells resulted in a reduction in aflatoxin production. Af70s produced more aflatoxin than isolate 53. The location effect changed for Af70s. Aflatoxin production increased in the interior of the plate when 12 or 24 wells were inoculated, but decreased in the interior of the plate when 36 and 48 wells were inoculated. More inoculated wells stimulated aflatoxin production regardless of isolate, but the location effect was different between strains.

Location Effect Is Design Dependent

Neighboring wells are actually attached; there was a chance chemicals can permeate and pass between the polystyrene wells, though not likely due to 2 mm thick plastic at the junction points. A second experimental design did not allow the culture containers (small Petri dishes) to touch physically (**Figure 2**). In the previous design, cultures were separated by 2 mm and each culture completely filled the 11.3 mm diameter wells. The second design, 35 mm Petri dishes were separated by only 1–2 mm and colony diameters were approximately 20 mm after 5 days growth. Aflatoxin production increased as the number of dishes with isolate 53 increased from 1 to 4 by 200 ppb, followed by a decrease when eight dishes were inoculated, indicating the stimulatory and inhibitory effects were likely a response to gases emitted from the fungus (**Figure 2**). Unlike within the 48-well plates, Petri dish location did not significantly alter aflatoxin production, although there were fewer locations to analyze ($p = 0.592$).

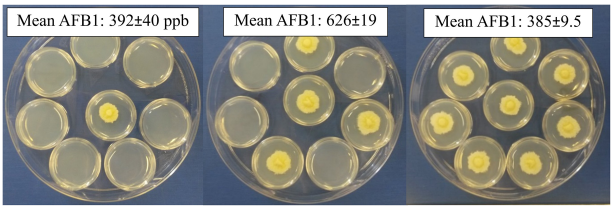

FIGURE 2 | Changing the number of inoculated open Petri-dishes within a closed dish alters aflatoxin production. Either 1, 4 or 8 small Petri-dishes were center-point inoculated and enclosed in a larger dish. All produced similar colonies with dense hyphal growth and minimal conidia; occasionally aerial hyphae could be observed for those with eight inoculated dishes.

Adding activated charcoal to the larger Petri-dish significantly increased aflatoxin production by at least 1000 ppb (**Table 2**). Unlike the previous experiment, there was only a marginal increase (75 ppb) in aflatoxin when four compared to one dish inoculated with isolate 53 and no activated charcoal used, but aflatoxin production still significantly decreased when all eight dishes were inoculated. Aflatoxin production if eight dishes were inoculated was restored to same amount as if one or four dishes were inoculated when activated charcoal was added to the large Petri-dish, suggesting an inhibitory volatile compound produced by the fungus was adsorbed by the activated charcoal.

TABLE 2 | Variable aflatoxin production in 8-Petri-dish system.

Strain[z]	Activated charcoal?[y]	Number dishes inoculated with a single strain: mean aflatoxin production/dish (ppb)		
		1[x]	4	8
53	none	1225 ± 274 cd	1299 ± 80 d	587 ± 23 e
	charcoal	3476 ± 98 a	2440 ± 119 b	1689 ± 63 c
Af70s	none	3481 ± 511 αβ	2464 ± 131 βγ	1579 ± 105 δ
	charcoal	2988 ± 462 αβγ	3565 ± 152 α	2078 ± 159 γδ
Tox4	none	2707 ± 180 ab	1675 ± 144 c	763 ± 49 d
	charcoal	3331 ± 743 a	2401 ± 138 b	1312 ± 84 c

Different number of eight small Petri-dishes were center point inoculated with the same A. flavus isolates and sealed with in a larger Petri-dish to determine if aflatoxin production changed if more cultures grew near one another. [z] Three different isolates were tested to determine if each responded similarly. [y] Activated charcoal was added to the bottom of the large Petri-dish to adsorb gases produced by A. flavus. [x] Different letters indicate different mean aflatoxin B_1 ppb ± standard error values based Tukey-Adjusted Least Significant Differences of less than $\alpha < 0.05$. For simplification, differences of means are only reported across rows (number of dishes inoculated) and between columns (activated charcoal added) within an individual isolate and not across isolates. Letters alternated from the English to Greek alphabet between isolates.

Aflatoxin production decreased for Tox4 and Af70s as more dishes were inoculated with the fungus (**Table 2**). Both Tox4 and Af70s produced more aflatoxin than 53 with Af70s producing the most aflatoxin. For isolates Af70s and Tox4, adding activated charcoal resulted in an increase in aflatoxin and restored the aflatoxin production to the same amount as if three or four fewer plates were inoculated. Unlike 53 and Tox4, Af70s produced less, though not statistically significant, aflatoxin when activated charcoal was applied to the large dish with only one inoculated dish.

Common Themes

Across all the experiments in 48-well plates, inoculating more wells with *A. flavus* isolate 53 increased aflatoxin production consistently. The increase was independent of pH buffering, solidified vs. liquid medium and adsorption of volatile compounds by activated charcoal. Only preventing air movement between the wells with Parafilm lowered aflatoxin production. Inoculating more wells also stimulated aflatoxin production for isolate Af70s, but inoculating all 48 wells decreased aflatoxin production. The stimulation was isolate independent, in spite of the final inhibition. In the Petri-dishes, isolate 53 also stimulated aflatoxin production as the number of inoculated dishes increased, but inoculating all eight dishes inhibited aflatoxin production. Isolates Tox4 and Af70s only inhibited aflatoxin if more dishes were inoculated. For each strain, the addition of activated charcoal resulted in an increase in aflatoxin comparable to having three or four fewer inoculated Petri-dishes, suggesting the adsorption of an inhibitory volatile organic compound produced by the fungi.

The location of a well within 48-well plates changed the amount of aflatoxin production. Growth in the center of 48-well plates consistently reduced aflatoxin production independent of pH-buffer for strain 53. Addition of activated charcoal minimized differences between interior and exterior wells, suggesting the adsorption of inhibitory volatile compounds. Addition of agar to buffered medium (not filling empty wells with liquid buffered medium) also minimized the location effect, suggesting an interaction between matric potential and buffering capacity on inhibition. Location of isolate Af70s in the 48-well plates had a different effect on aflatoxin production. Inoculating a few wells increased aflatoxin production in the inner wells, but inoculating more wells decreased aflatoxin production in the inner wells. Location of the dishes within a larger Petri-dish did not affect aflatoxin production and this was consistent between strains.

DISCUSSION

Neighboring colonies (or cultures) of identical *A. flavus* isolates grown within a closed system altered the aflatoxin production of one another in the absence of physical contact. Different responses occurred, either inhibition and or stimulation of aflatoxin production, which were attributable to growth of other *A. flavus* cultures, not culture conditions. By controlling different variables, results suggested different volatile organic compounds or inorganic gases likely contributed to the inhibition and stimulation of aflatoxin production. Understanding how these volatile interactions influence aflatoxin regulation, competition by *A. flavus* and aflatoxin accumulation both pre and post-harvest deserves further attention.

In general, the number of independent cultures of a single *A. flavus* isolate grown within a closed system altered the aflatoxin production within physically separated wells or petri-dishes. This was an interaction between genetically identical *A. flavus* changing aflatoxin production with only a shared headspace and without direct physical interaction or sharing the same medium suggesting heterogeneity in the gaseous environment from fungal growth altered aflatoxin production. Conidial concentration within either liquid or solid cultures regulates aflatoxin production in single *A. parasiticus* and *A. flavus* isolates, usually aflatoxin production decreases with increased concentration (Clevström et al., 1983; Brown et al., 2009; Affeldt et al., 2012). The decrease in aflatoxin production is associated with a developmental shift away from sclerotial production [sclerotia can serve as stromata for sexual ascospore production (Horn et al., 2009; Horn et al., 2016)] to increased asexual conidial production (Brown et al., 2009; Affeldt et al., 2012). This is similar to the inhibition observed by increasing the number of identical *A. flavus* cultures growing on small Petri-dishes with a shared headspace or the increase in culture density experienced in the center of 48-well plates, though there was only observed changes in conidial or sclerotial production within 48-well plates. In contrast, herein is reported a novel increase in aflatoxin production and decrease in conidia in response to increasing number of identical *A. flavus* cultures from 12 to 48 within 48-well plates with only shared headspace (i.e., more conidia in same space). Atoxigenic *A. flavus* can inhibit aflatoxin production if grown in the same medium (including corn) but only when there is direct contact with toxigenic isolates (Wicklow et al., 2003; Mehl and Cotty, 2010; Abbas et al., 2011; Huang et al., 2011). Neighboring Petri-dish cultures of both *A. nidulans* and *R. solanacearum* that share headspace with *A. parasiticus* and *A. flavus* affect aflatoxin production (Roze et al., 2007; Spraker et al., 2014). In closely related *A. parasiticus*, an increase in the number of Petri-dishes inoculated with *A. nidulans* inhibits aflatoxin production and conidiation of *A. parasiticus* within a closed larger Petri-dish (Roze et al., 2007). The inhibition is attributed to *A. nidulans*, but there is also a decrease in the number of dishes inoculated with *A. parasiticus*, perhaps some of the inhibition in toxin production could have been due to a lack of stimulation from neighbors of the same species (Roze et al., 2007). *R. solanacearum* grown in separate petri-dishes increases aflatoxin production in *A. flavus* (Spraker et al., 2014). Aflatoxin production is regulated by chemicals produced in media and released as gases from other members of the same isolate, other isolates of the same species, other *Aspergilli* and bacteria.

Culture-system design and to a lesser extent isolate altered the aflatoxin production response to *A. flavus* growing in close proximity to one another. On Petri-dishes mostly one phenomenon was observed, an inhibition of aflatoxin production as the number of cultures increased. In contrast, two contradictory phenomena were observed in 48-well plates. Increasing the number of cultures stimulated aflatoxin

production, whereas cultures in the center of the plate inhibited aflatoxin production. The difference between wells in the center of 48-well plates vs. outer rows is similar to an edge effect, suggesting a gradient of volatile chemicals can alter the aflatoxin production potential of a colony. It would have been expected if volatiles from neighboring cultures stimulate aflatoxin production, both a stimulation from increasing the number of inoculated wells and in the center of plates (where extensively there is an increase in well density and build up of chemicals). Potentially those gases that are stimulatory become inhibitory at higher concentrations or there are both stimulatory and inhibitory gases produced and stoichiometrically more inhibitory gas molecules are produced or the fungus is more sensitive to the inhibitory gases. There were also two other seemingly contradictory phenomena, when non-inoculated wells were filled with liquid medium or agar was added to medium to control moisture loss, only solidified buffered medium minimized the location effect and number of culture effect. Potentially, adding agar not only controlled moisture loss, but also changed the matric potential of the medium and affected the ability of gases to be adsorbed into the medium and therefore minimized the effect of volatile gases on fungal growth. The different responses of 53, Af70s and Tox4 to neighbors is not surprising, since aflatoxin production, biocontrol capacity and volatile production varies among isolates (Cotty, 1989; Zeringue et al., 1993; Mehl and Cotty, 2010; Abbas et al., 2011; Huang et al., 2011; De Lucca et al., 2012). It deserves further review to determine if mixing isolates within 48-well plates or Petri-dishes alters aflatoxin production especially to determine if atoxigenic isolates' volatiles can inhibit or stimulate aflatoxin production. Altering culture system design can alter aflatoxin production; *A. flavus* produces much less aflatoxin on bottom of stacks of dishes filled with peanuts (Xue et al., 2003). Spreading dishes on trays and adding spacers between trays and rotating trays during incubation ameliorates the location effect (Xue et al., 2003). All together these results suggest when screening for anti-fungal and anti-aflatoxin potential of compounds and fungi or genetic resistance it is essential to tailor culture systems that maximize phenotypic homogeneity among *A. flavus* cultures and minimizes the interaction caused by changes in the gaseous environment from fungal growth.

Since activated charcoal adsorbs both inorganic and organic gases, and adding activated charcoal resulted in greater aflatoxin production, it is likely *A. flavus* produced inhibitory volatile organic or inorganic compounds (Smíšek and Černý, 1967). The addition of activated charcoal to the bottom of Petri-dishes resulted in higher aflatoxin production and removed the inhibition from adding more cultures within the larger Petri-dishes. Activated charcoal also minimized the reduction of aflatoxin in the center of the 48-well plates. This suggests the same volatile chemicals could be responsible for the aflatoxin reduction in the 48-well plates and Petri-dishes. If the volatile chemicals are organic they may be related to the oxylipins or psi factors produced by *A. flavus* and *A. parasiticus* that have been reported to be responsible for quorum sensing and reduction of aflatoxin with increased inoculum concentrations (Brown et al., 2009; Affeldt et al., 2012). It is unknown if psi factors are volatile chemicals, but they are in the same biosynthetic pathway as methyl jasmonate, which may suggest volatility (Affeldt et al., 2012). In the interior rows of the 48-well plates, there was typically more conidial production and those conidia were greener than the outer rows, which is consistent with the psi-factor quorum sensing phenotype changes (Brown et al., 2009; Affeldt et al., 2012). Other possible organic volatiles have been demonstrated to lower aflatoxin production in *A. flavus* and *A. parasiticus* include: ethylene (from *A. nidulans* and *A. parasiticus*), *trans*-2-hexanol (from soybean), and at high concentrations 2-buten-1-ol (from *A. nidulans*) (Roze et al., 2004, 2007; De Lucca et al., 2011). Inorganic CO_2, H_2O, and O_2 gases involved in respiration would likely be adsorbed to activated charcoal. High levels of CO_2 (20%+) have been demonstrated to dramatically lower aflatoxin production, but lower levels at 3% can be a stimulatory (Davis and Diener, 1968; Sanders et al., 1968; Roze et al., 2004). In addition, reduction in O_2 below atmospheric levels can reduce aflatoxin production but the magnitude is not as great as the increase in CO_2 (Davis and Diener, 1968; Clevström et al., 1983; Ellis et al., 1993). The effect of H_2O, either as buildup of free moisture or moisture loss should have been controlled by filling non-inoculated wells or using solidified medium, respectively. There was more aflatoxin production on solidified medium, but there was still inhibition in the interior of 48-well plates and if more Petri-dishes were inoculated. Additionally there was limited change in aflatoxin production in the interior of 48-well plates if non-inoculated wells empty or filled with liquid medium. Consistent inhibition during experiments to control H_2O suggest volatilized H_2O was not solely responsible for the aflatoxin production inhibition. In both the 48-well plates and Petri-dishes, addition of activated charcoal did not fully restore the aflatoxin production to the highest aflatoxin level, this suggests either the adsorption of the activated charcoal was not sufficient to collect all of the inhibitory organic compound(s), or other gases are involved in the reduction in aflatoxin production.

Stimulation of aflatoxin production by increasing the number of cultures within the 48-well plates could not be attributed to any of the environmental variables manipulated. Stimulation was not eliminated by controlling pH, controlling moisture loss (by switching from liquid to solid medium or filling un-inoculated wells with liquid medium) or adsorbing volatile chemicals. Only covering with Parafilm, which presumably blocked exchange of gases between wells, prevented a stimulation of aflatoxin production. Although Parafilm could just as easily have trapped the volatile inhibitory substance. It is unlikely the stimulation was caused by changes in gases involved in respiration or large volatile organic compounds because activated charcoal readily adsorbs O_2, CO_2, and H_2O and complex organic gases (Smíšek and Černý, 1967). Water is a gaseous product of respiration that could lead to stimulation in aflatoxin production, though this is inconsistent (Davis and Diener, 1968; Sanders et al., 1968; Ellis et al., 1993; Schmidt-Heydt et al., 2009). Changing to solid medium, filling the non-inoculated wells with liquid media, and activated charcoal should control moisture levels

which all increased aflatoxin production. Regardless increasing the number of inoculated wells still resulted in an incremental increase of aflatoxin, making water unlikely the sole candidate for aflatoxin stimulation by greater culture density. As mentioned previously, extreme increases in CO_2 and decreases in O_2 both reduce aflatoxin production, so these would not directly cause the observed increases in aflatoxin production (Davis and Diener, 1968; Sanders et al., 1968; Clevström et al., 1983). Carbon dioxide at 3% compared to 0.1 and 0.7% resulted in a stimulation in aflatoxin, making it impossible to rule out CO_2 accumulation as a candidate to stimulate aflatoxin production (Roze et al., 2004). Additionally, it is possible, as more cultures are growing within a 48-well plate, the atmospheric conditions become hypoxic and the fungus switched from aerobic respiration to anaerobic respiration and produced alcohols. Ethanol and other alcohols, including 2-ethyl-1-hexanol (produced by *A. nidulans*) and at low levels 2-buten-1-ol (produced by *A. nidulans*) have been demonstrated to stimulate aflatoxin synthesis (Reddy et al., 1979; Keller et al., 1994; Roze et al., 2007). Alcohol dehydrogenase 1 is up-regulated on conducive medium concurrent with the start of aflatoxin synthesis and throughout aflatoxin synthesis (Woloshuk and Payne, 1994). In several studies investigating volatile head space of *A. parasiticus* and *A. flavus* growing on conducive medium and corn, ethanol and other alcohols were produced (Roze et al., 2004, 2007, 2010; De Lucca et al., 2012; Spraker et al., 2014). Conceivably, 3% CO_2 stimulates aflatoxin production because of a switch to anaerobic fermentation and ethanol caused the stimulation (Roze et al., 2004). It is likely activated charcoal did not absorb alcohols, because they are not efficiently bound by activated charcoal (Smíšek and Černý, 1967), which may explain why there was still an increase in aflatoxin production when activated charcoal was added. One explanation for lack of a substantial increase in aflatoxin production in the non-buffered L-medium is due to medium acidification (pH 1–2 after 4 days of growth). Dissolved CO_2 from respiration should acidify medium by H^+ disassociation from bicarbonate and other acids produced during fungal growth acidify medium (Cotty, 1988; Keller et al., 1997). Under highly acidic conditions, the hydroxyl group of alcohols can be protonated and then substituted with chlorine ions in the medium producing alkyl halides, thus functionally compromising the alcohol (Bruice, 1998).

We propose a model illustrated in **Figure 3**, where increasing well density both stimulates and inhibits aflatoxin production. The stimulation is likely a response to volatilized CO_2 and H_2O from respiration and alcohols during fermentation, whereas the inhibition is likely a response to greater concentrations of CO_2 within the plate interior and volatilized oxylipins. Ecologically, inhibition of aflatoxin production at low well occupancy confounds the hypothesis that aflatoxin production is an important competitive advantage in soil environments. The lack of aflatoxin production at low "population" levels was only observed when *A. flavus* was grown in a smaller culture container (11 mm vs. 35 mm diameter), which would be closer to the expected colony size in soil. Additionally, in 24-well plates there was only a marginal increase of aflatoxin production as the number of inoculated wells increased (data not shown). A possible explanation for differential aflatoxin stimulation is energy conservation. If aflatoxin synthesis is energetically expensive, an effective conservation strategy would be to produce aflatoxin only after detection of other microorganisms. This strategy would be more important for smaller colonies, as would be expected in nature, where energy resources are more limited. Inhibition of aflatoxin production when the *A. flavus* population level becomes high, supports the hypothesis that aflatoxin is synthesized to aid in competition against other microbes and is not important if the population is well established. The inhibitory compound may be specific organic compounds related to oxylipins rather than products of respiration. Determining the volatility of oxylipins that regulate the transition from sclerotial and aflatoxin production to no toxin and conidial production

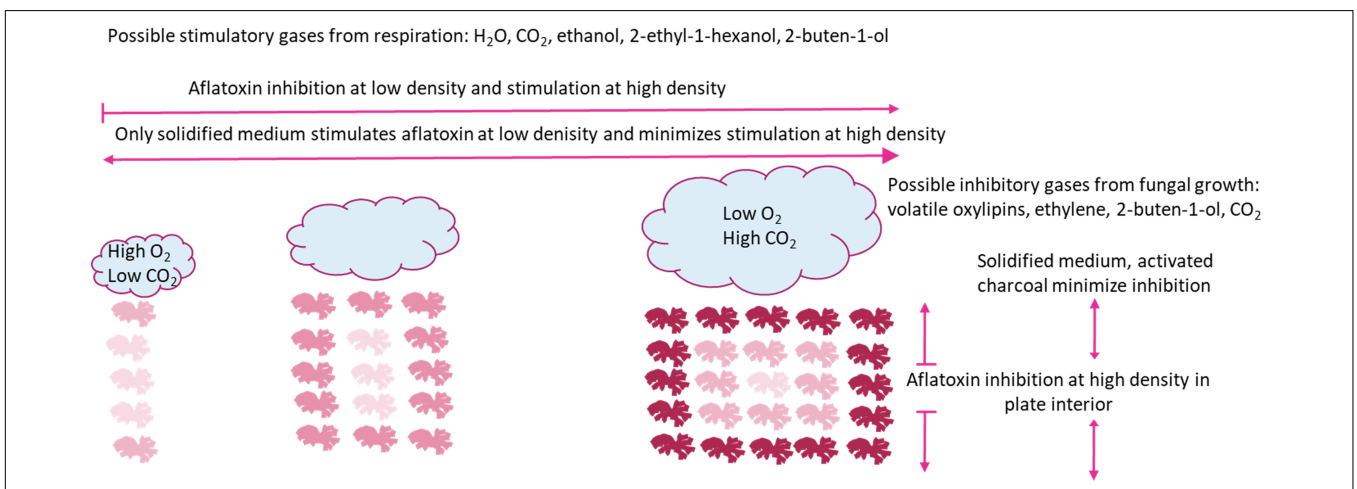

FIGURE 3 | Model for aflatoxin inhibition and stimulation caused by changing density of identical *Aspergillus flavus* cultures with/in a closed system. Individual fungal mycelial cultures are represented by line drawings. Relative aflatoxin production is represented by red shade, darker red indicate greater aflatoxin production. Clouds represent gas production during fungal growth. Arrows represent aflatoxin production, blunt ends represent blocked aflatoxin production and pointed ends represent production.

deserves further review. If indeed stimulation is less specific and a response to products of respiration such as CO_2, it stands to reason aflatoxin synthesis is switched on not only in response to other *Aspergilli*, but also other microbes and insects that are in competition for resources and potentially fungivorous.

The influence of volatiles produced by *A. flavus* on aflatoxin production warrant further attention. At this point there is evidence volatiles produced by *A. flavus* changed aflatoxin production, but no definitive information about the type of chemical cues produced by the fungus based on previous research. In the future, it will be important to test more volatiles produced by *A. flavus* to determine if these have inhibitory or stimulatory effects on aflatoxin production. It should be determined how mixtures of different volatiles effect aflatoxin to see if there are some chemicals that synergize to increase aflatoxin production or interfere with one another to inhibit aflatoxin production. Inhibitory and stimulatory volatiles could be utilized to monitor grain storage for conditions conducive to a potential aflatoxin outbreak or suggest gases to modify the storage atmospheres to be suppressive to aflatoxin production. It will be important to determine the impact *A. flavus* competition has on aflatoxin production in crops both pre and post-harvest. In spite of determining location and number of cultures affected aflatoxin production, the variability within these categories remained high, so there are more factors within an incubator leading to the variability of aflatoxin production that still needs to be understood. In the same study that found it was important to avoid excessive stacking of peanuts, location within the incubator could also affect aflatoxin production (Xue et al., 2003). Here, inoculating every well and using buffered solid medium to reduced the appearance of phenotypic variability caused by gases from neighboring colonies and is a demonstration of the need to account for and minimize the confounding effects of fungal growth when trying to understand the biology of aflatoxin production.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

Both authors contributed to the conception and design of the study, manuscript revision, read, and approved the submitted version. RS conducted the experiments, organized the data, performed the statistical analysis, and wrote the manuscript.

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Spectral-Based Screening Approach Evaluating Two Specific Maize Lines With Divergent Resistance to Invasion by Aflatoxigenic Fungi

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In an effort to control aflatoxin contamination in food and/or feed grains, a segment of research has focused on host resistance to eliminate aflatoxin from susceptible crops, including maize. To this end, screening tools are key to identifying resistant maize genotypes. The traditional field screening techniques, the kernel screening laboratory assay (KSA), and analytical methods (e.g., ELISA) used for evaluating corn lines for resistance to fungal invasion, all ultimately require sample destruction. A technological advancement on the basic BGYF presumptive screening test, fluorescence hyperspectral imaging offers an option for non-destructive and rapid image-based screening. The present study aimed to differentiate fluorescence spectral signatures of representative resistant and susceptible corn hybrids infected by a toxigenic (SRRC-AF13) and an atoxigenic (SRRC-AF36) strain of *Aspergillus flavus*, at several time points (5, 7, 10, and 14 days), in order to evaluate fluorescence hyperspectral imaging as a viable technique for early, non-invasive aflatoxin screening in resistant and susceptible corn lines. The study utilized the KSA to promote fungal growth and aflatoxin production in corn kernels inoculated under laboratory conditions and to provide actual aflatoxin values to relate with the imaging data. Each time point consisted of 78 kernels divided into four groups (30-susceptible, 30-resistant, 9-susceptible control, and 9-resistant control), per inoculum. On specified days, kernels were removed from the incubator and dried at 60°C to terminate fungal growth. Dry kernels were imaged with a VNIR hyperspectral sensor (image spectral range of 400–1000 nm), under UV excitation centered at 365 nm. Following imaging, kernels were submitted for the chemical AflaTest assay (VICAM). Fluorescence emissions were compared for all samples over 14 days. Analysis of strain differences separating the fluorescence emission peaks of resistant from the susceptible strain indicated that the emission peaks of the resistant strain and the susceptible strains differed significantly ($p < 0.01$) from each other, and there was a significant difference in fluorescence intensity between the treated and control kernels of both strains. These results indicate a viable role of fluorescence hyperspectral imaging for non-invasive screening of maize lines with divergent resistance to invasion by aflatoxigenic fungi.

Keywords: fluorescence hyperspectral imaging, susceptible and resistant hybrids, aflatoxin screening, toxigenic and atoxigenic *Aspergillus flavus*, maize

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INTRODUCTION

Aflatoxins are highly toxic and carcinogenic secondary metabolites predominantly produced by the *Aspergillus flavus* (*A. flavus*) and *A. parasiticus* fungi. When a susceptible crop (e.g., maize) is colonized by a toxigenic *Aspergillus* fungus, aflatoxins are produced and contaminate the grain and grain products, threatening human and animal health worldwide (Unnevehr and Grace, 2013). *A. flavus* is an opportunistic pathogen occurring with higher incidence on maize grown under stressed conditions preharvest, including late-season drought and high night temperatures during kernel filling and ear maturation, and under poor storage conditions post-harvest (Widstrom et al., 1987; Mahuku et al., 2013). Because of the ubiquitous nature of the *Aspergillus* fungus, aflatoxin contamination may occur at any point along the maize production line and in storage. Different pre- and post-harvest strategies for controlling aflatoxin in food and feed production have been explored and implemented over the years since its initial discovery in the 1960s; however, no permanent solution has yet been attained.

A noteworthy preventive strategy in the continued effort of mitigating aflatoxin contamination in food and/or feed grains focuses on developing host–plant resistance to eliminate aflatoxin from susceptible crops, including maize (Cary et al., 2011). In order to inhibit fungal colonization and subsequent toxin production, host resistance is a cost-effective approach, which preserves the environment in terms of harmful residue, and is compatible with other control measures, including biocontrol and appropriate storage practices (Mahuku et al., 2013). Natural resistance to *A. flavus* infection and subsequent aflatoxin production in maize was first discovered during the early 1980s (King and Scott, 1982; Gardner et al., 1987; Widstrom et al., 1987), with ongoing research adapting new technologies including next-generation sequencing and association mapping to identify gene sequences associated with aflatoxin resistance which would assist in developing aflatoxin-resistant varieties (Scott and Zummo, 1988; Campbell and White, 1995; Widstrom et al., 2003; Brown et al., 2013; Mahuku et al., 2013). The infection of maize kernels by *A. flavus* is subject to natural variability which may lead to inaccurate classification of resistant plants. Therefore, when isolating resistant germplasms, the selection of resistant genes depends on the even distribution of artificially induced fungal infection over a test field, and on the availability of high-throughput screening (Mahuku et al., 2013). To this end, screening tools are key to identifying resistant maize genotypes. In addition to the traditional field screening techniques, the kernel screening laboratory assay (KSA) has been an invaluable technique developed to study resistance to aflatoxin production in maize. The KSA measures seed-based (maize-host) genetic resistance in mature kernels and can effectively separate susceptible kernels from resistant maize (Brown et al., 1993, 1995). The assay is simple to perform in the laboratory, is independent of outdoor weather conditions, requires few kernels, and correlates favorably with field findings. Ultimately, the kernels must be crushed for aflatoxin determination and field trials are required for confirmation of resistance (Brown et al., 2013). Other screening methods used for evaluating corn lines for resistance to fungal invasion include analytical methods [e.g., enzyme-linked immunosorbent assay (ELISA) or high performance liquid chromatography (HPLC)] which require sample destruction, and more recently, non-invasive, optical-imaging and spectral-based techniques (e.g., near-infrared spectroscopy (NIR) and hyperspectral imaging (HSI)].

Near-infrared spectroscopy is based on absorption of electromagnetic radiation in the 780–2500 nm wavelength range (Nicolaï et al., 2007; Huang et al., 2008; Xia et al., 2019). The NIR spectra consist of broad wave bands from overlapping absorptions corresponding to combinations of C–H, O–H, and N–H bonds present in organic compounds, allowing for detection of biological material (Xia et al., 2019). Several studies reported the potential of NIR for rapid detection in different applications. In agricultural research, NIR was used for quality evaluation of farm products in various fruits and vegetables (Pasquini, 2018), and testing of seeds (Zhu et al., 2015). The seed research included testing seeds for constituents, such as starches, sugars, and proteins, for vigor, insect infestation, disease, seed viability (Zhu et al., 2015; Xia et al., 2019), and for variety discrimination of grass (Ren et al., 2008) and rice (Attaviroj et al., 2011) seed. NIR spectroscopy was also used for detecting kernel rot and mycotoxins in maize (Berardo et al., 2005) and it was suggested the methodology may be applicable as a screening tool in large-scale breeding programs for selecting genotypes resistant to fungal and fumonisin contamination (Lanubile et al., 2017).

Hyperspectral imaging systems integrate NIR spectroscopy with digital imaging, adding a spatial component to the spectral information provided by NIR spectroscopy, resulting in a three-dimensional "hypercube" dataset consisting of high spectral and high spatial information of a specific target (Xia et al., 2019). In the past decade, hyperspectral imaging expanded the potential of NIR technology and opened up new application opportunities for the innovative technology, particularly in agriculture (Pasquini, 2018). Allowing for simultaneous multi-kernel acquisition, hyperspectral imaging systems dramatically increased analysis of single kernels including wheat, cotton, and maize seeds. Near infrared HSI was used to detect insect damaged kernels in wheat (Singh et al., 2009), and to classify individual cotton seeds with respect to variety (Soares et al., 2016). Application of HSI systems for classification of maize seed varieties was reported in several recent studies. Feng et al. (2017) applied NIR–HSI with multivariate data analysis to discriminate between transgenic maize kernels. Zhang et al. (2012), Yang et al. (2015), and Wang et al. (2016) used HSI and chemometrics to discriminate different maize varieties.

Fluorescence hyperspectral imaging offers yet another option for non-invasive and rapid image-based screening. It is a technologically advanced take on the basic bright green-yellow fluorescence (BGYF) presumptive screening test originally introduced in the 1970s to the grain industry. The method employs a HSI system with an ultraviolet fluorescence excitation source. Fluorescence hyperspectral imaging has been extensively researched as a non-invasive tool in agriculture for assessing the quality and safety of food and feed in commodities exhibiting fluorescence properties (Kim et al., 2001; Chen et al., 2002; Carrasco et al., 2003; Kong et al., 2004; Ariana et al., 2006;

Gowen et al., 2007; Zhang et al., 2012; Liu et al., 2015). In maize, the method was applied for detecting and classification of kernels contaminated with aflatoxins (Yao et al., 2006, 2010). Fluorescence imaging of maize plants was also utilized for detecting diseases in genetic disease resistance studies (Rascher et al., 2009).

We hypothesize that fluorescence hyperspectral imaging may be a viable technique for early, non-invasive aflatoxin screening of resistant and susceptible corn lines. The present study utilized the earlier mentioned KSA to promote fungal growth and aflatoxin production in corn kernels inoculated under laboratory conditions and to provide actual aflatoxin values, determined by chemical analysis, to relate with the image data. The specific objective of the study aimed to differentiate fluorescence spectral signatures of a representative resistant and a representative susceptible corn hybrid infected by a toxigenic and an atoxigenic strain of *A. flavus*, at several time points, in order to evaluate fluorescence hyperspectral imaging as a rapid and non-destructive screening technique of resistant and susceptible maize.

MATERIALS AND METHODS

Maize Strains

To determine the viability of the use of fluorescence hyperspectral imaging to differentiate resistant and susceptible corn hybrids, one resistant and one susceptible corn line were utilized in this feasibility study. The resistant maize strain TZAR 104 obtained from FFS-SRRC-ARS-USDA, New Orleans, LA, United States, is a combination of African and southern US lines released in 2008. The N83-N5 (Liberty non-linked) hybrid seed, obtained from the LSU Ag Center, Baton Rouge, LA, United States, was chosen as the susceptible maize strain for its propensity to readily colonize maize kernels in the laboratory and in the field (Hruska et al., 2013). Typical differences of colonization between resistant and susceptible corn kernels inoculated with a toxin producing *A. flavus* are presented in **Figure 1**.

Preparation of Inocula

A toxin producing (SRRC-AF13) and a non-toxin producing (SRRC-AF36) strains of *A. flavus* were cultured on potato dextrose agar (PDA) media at 30°C in the dark. Several replicates of each fungus were cultured on separate plates. Conidia were harvested on 5th day of growth and suspended in buffer at a dilution of 4×10^6 conidia/mL, determined with a hematocytometer. The inocula were stored in separate containers at 4°C.

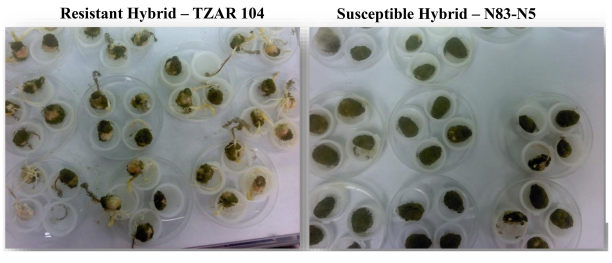

FIGURE 1 | Examples of AF13 treated resistant and susceptible kernels showing differences in colonization. The susceptible hybrid exhibits a more robust fungal growth compared to the resistant hybrid.

Sample Preparation – *In situ* Inoculation

The resistant and the susceptible maize kernels were inoculated with both strains of *A. flavus* (AF13 and AF36) and placed with their respective controls, into an incubator at 31°C. The kernels were surface sterilized in 70% ethanol (4 min) followed by three washes (1 min each) in distilled water before being placed into an inoculum made from each *A. flavus* culture at a standard dilution of 4×10^6 spores/mL and stirred for 1 min. The experiment was conducted in two parts. The first part involved only the toxigenic fungus and the second part involved the atoxigenic fungus. Each part of the experiment consisted of four trays per treatment day (susceptible, resistant, susceptible control, and resistant control). Each treatment tray contained 10 dishes with 3 kernels/dish, 30 kernels total. Each control tray contained three small plates with three kernels/plate, nine kernels total. Treatment was terminated on Days 5, 7, 10, and 14. On the specified days, the four trays designated for that day (78 kernels/treatment) were removed from the incubator, each plate from all trays was transferred into pre-labeled coin envelopes (one bag/dish/three kernels) and placed into a 60°C oven for 2 days to terminate fungal growth.

Imaging Procedure

Dry kernels were removed from the oven, wiped off from excess mold, and placed on a pre-labeled ceramic tray identifying each individual kernel with a grid. The tray contained 30 shallow indentations, each to accommodate a single corn kernel. It was painted with a flat, black paint to reduce reflection and to enhance the contrast between the background and the samples. The kernels were imaged using the VNIR hyperspectral sensor (VNIR 100E, MSU, Stennis Space Center, MS, United States) with image spectral range of 400–1000 nm, under UV (Model XX-15A; Thermo Fisher Scientific Inc., Waltham, MA, United States) excitation centered at 365 nm (Yao et al., 2010). A dark current calibration image was also taken. Following imaging, kernels were returned into the specified coin envelopes and chemically analyzed using the AflaTest assay (VICAM).

Image Analysis

The fluorescence hyperspectral images were preprocessed where the sensor background noise was removed through dark current subtraction, image band wavelengths were assigned, and random noise was removed via Savitzky–Golay filtering. Each preprocessed image had a range from 400 to 700 nm. The corn fluorescence spectra were extracted from individual corn kernels and averaged for each three-kernel sample. In order to detect differences in the spectral pattern of toxin producing and non-toxin producing *A. flavus* on susceptible and resistant kernels over a 14-day growth period, mean fluorescence spectra were obtained through spatial subset of each image (region of interest) along with standard deviations. Fluorescence emissions

were compared for all three-kernel samples over 14 days. Spectral features including peak locations (wavelengths which correspond with the maximum fluorescence intensity values) and average fluorescence intensities were extracted and used in statistical analyses.

Chemical Analysis

Imaged corn samples were chemically analyzed for aflatoxin content using the AflaTest from VICAM (VICAM, Milford, MA, United States). The AflaTest is an approved method by the United States Department of Agriculture Federal Grain Inspection Service, for quantitative analysis of aflatoxin in maize. Details of the method were described previously (Hruska et al., 2013). Briefly, the three-kernel samples were weighed, crushed, and extracted with methanol/water (80/20%). Extracts were diluted, filtered, and passed through AflaTest columns. The columns were washed with distilled water and eluted with 100% methanol. Eluted samples were mixed with developer (1:1) and aflatoxin (ppb) was measured in the EX-4 series Fluorometer (VICAM).

Statistical Analysis

Two sets of image data based on side (germ or embryo side, and the opposite endosperm side) were analyzed separately, using a 2 × 2 factorial design (resistant, susceptible) and (toxigenic, atoxigenic). Spectral emission peak locations were extracted from the preprocessed fluorescence data and group differences between spectral peaks over a 14-day incubation period were analyzed using analysis of variance (ANOVA) in MATLAB followed by *post hoc* *t*-test analyses. Results were plotted in Microsoft Excel 360. Results of the chemical analysis were also analyzed with an ANOVA to determine group differences, in MATLAB and plotted in Microsoft Excel.

RESULTS

The treatment analysis results of the entire image data set, irrespective of side (germ, endosperm), are presented in **Figure 2**. A main effect of treatment ($p < 0.001$) with *post hoc* analysis shows that the fluorescence emission peaks of both AF13 and AF36 inoculated kernels were significantly different from the untreated controls ($p < 0.001$), but not from each other.

Similar results were revealed when each side of kernels was analyzed separately. A main effect of treatment ($p < 0.001$) with *post hoc* analysis shows that in both treatment groups (AF13 and AF36) the emission peaks can be differentiated from the peaks of the controls, but not from each other on both the germ and the endosperm sides of imaged kernels (**Figures 3A,B**). Therefore, the data were pooled and no further distinction, with respect to side, was made in the subsequent analyses.

Effects of the inoculation treatment on the resistant and susceptible kernels are illustrated by the image data (**Figure 4**). In the resistant corn group, the mean peak difference between the AF13 inoculated and the AF36 inoculated kernel emissions was 3 nm, while the mean peak difference between the AF13 inoculated and AF36 inoculated kernel and their respective

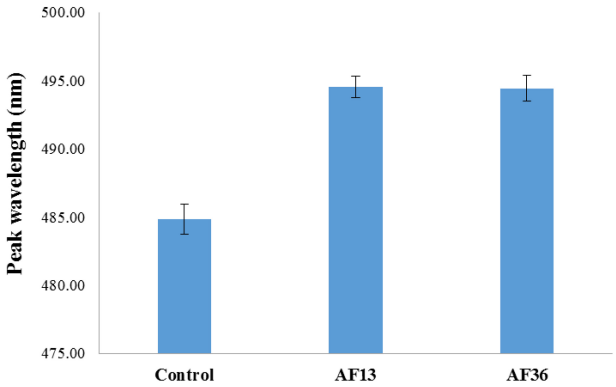

FIGURE 2 | Bar graph representation of entire treatment image data set including standard error bars. Fluorescence emission peaks of both AF13 and AF36 inoculated kernels were significantly different from the uninoculated controls ($p < 0.001$), but not from each other.

control emissions was 10 and 6 nm (**Figure 4A**). The mean peak difference between AF13 inoculated and AF36 inoculated kernel emissions was <3 nm, while the mean peak difference between both inoculated groups and their respective controls was 17 nm in the susceptible kernels (**Figure 4B**).

Statistical analysis of strain differences separating the fluorescence emission peaks of resistant and the susceptible strain (**Figure 5**) indicates a main effect of strain ($p < 0.01$) where the emission peaks of the resistant strain and the susceptible strain are significantly different from each other. Although emission peaks from both corn varieties inoculated with both fungi differed from the uninoculated controls, the difference between the controls and the resistant kernels was not significant.

The next step in the analysis involved the growth of the fungus and aflatoxin accumulation at four different time points post inoculation (Days 5, 7, 10, and 14). Mean spectral curves of image data over the 14-day incubation period are presented in **Figure 6**. An expected fluorescence peak shift toward longer wavelengths was observed in the spectra from both corn varieties inoculated with each fungal strain from Days 5 to 14 post-inoculation. In the resistant corn, both the AF13 (**Figure 6A**) and the AF36 (**Figure 6B**) inoculated kernels exhibited a 14-nm fluorescence peak shift over the incubation period. In the susceptible corn, the peak shifted 11 nm in the AF13 (**Figure 6C**) inoculated samples and 12 nm in the AF36 (**Figure 6D**) inoculated corn over the same 14-day period.

Statistical analysis of AF13-inoculated kernels (**Figure 7A**) revealed that peak locations of the susceptible kernels were significantly different from peak locations of both the control and resistant kernels on Days 5 and 7, $p < 0.01$; however, the difference between the peaks of the control and the resistant kernels was not significant. By Day 10, persisting through Day 14, the peak location of each group was significantly different from the other two groups, $p < 0.01$. Analysis of the AF36-inoculated kernels (**Figure 7B**) found that although the peak location of the resistant and susceptible groups differed early on

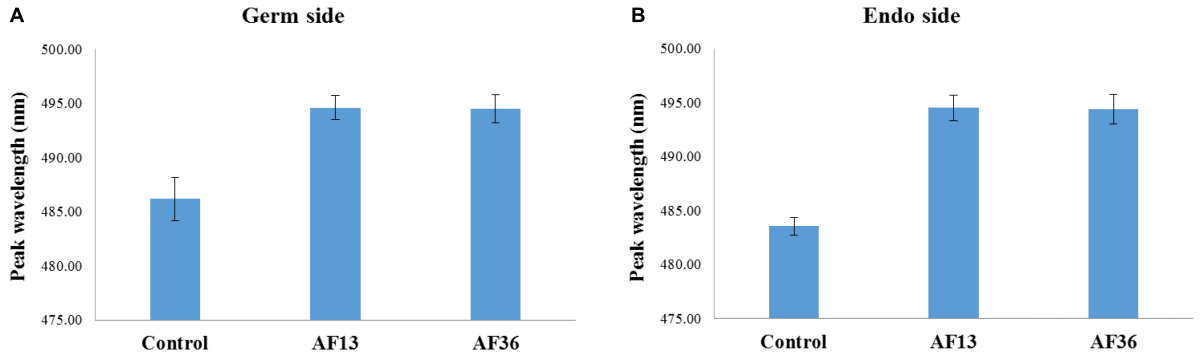

FIGURE 3 | Bar graph representation of germ **(A)** and endosperm **(B)** image data sets, with respect to treatment. Fluorescence emission peaks of both AF13 and AF36 inoculated kernels were significantly different from the controls ($p < 0.001$), but not from each other.

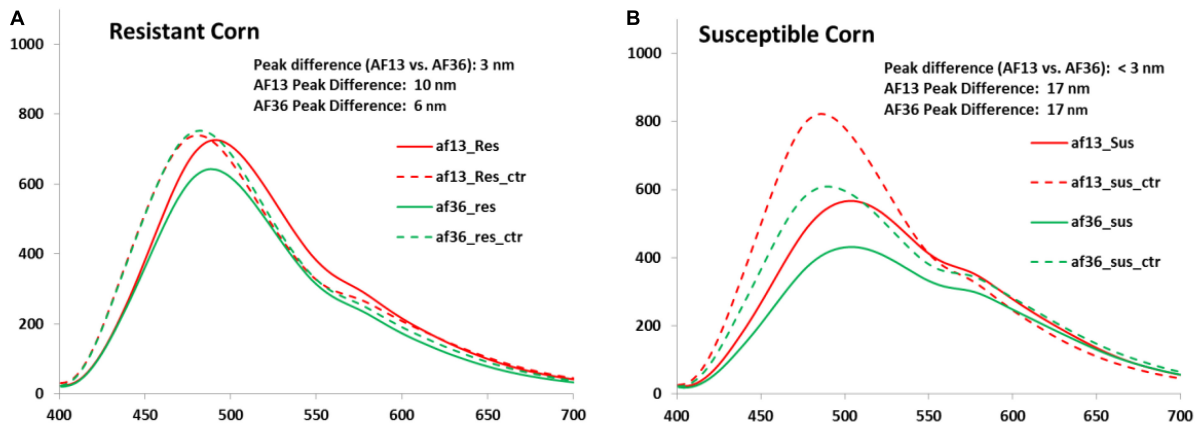

FIGURE 4 | Mean spectra of **(A)** resistant kernels and **(B)** susceptible kernels inoculated with AF13 and AF36 with their respective controls. A significant fluorescence peak shift is evident in both the resistant and susceptible kernels between the inoculated kernels and the controls.

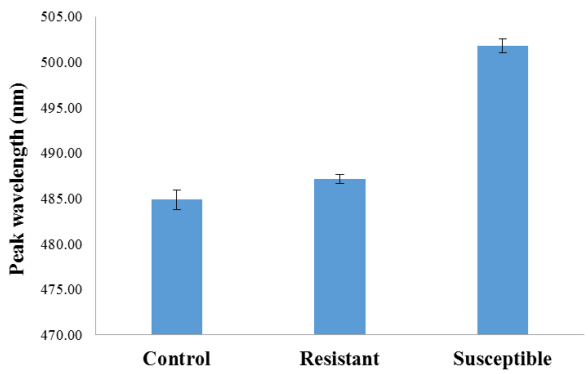

FIGURE 5 | Bar graph representation of strain differences (susceptible and resistant). There was a main effect of strain $p < 0.01$ separating the two inoculated strains from each other. The difference between the uninoculated controls and the inoculated resistant kernels was not significant.

(Day 5), $p < 0.01$, the peaks of the resistant group did not differ from those of the control group of kernels through the 14-day incubation period.

Figure 8 summarizes the analyses of the four major groups (AF13-resistant AF13R; AF13-susceptible AF13S; AF36-resistant AF36R; AF36-susceptible AF36S) at each time point post-inoculation in terms of mean peak locations. An overall main "Day" effect was found with $p < 0.01$. On Day 5, peak locations of both resistant groups, AF13R and AF36R, were significantly different from both susceptible groups, AF13S and AF36S, $p < 0.01$. And the mean peak location of each susceptible group, AF13S and AF36S, was significantly different from all the other three groups, $p < 0.01$. On Day 7, peak locations of both resistant groups, AF13R and AF36R, were significantly different from both susceptible groups, AF13S and AF36S, $p < 0.001$. However, there was no significant difference between the mean peak location of the two resistant or the two susceptible groups on Day 7 post-inoculation. Interestingly, by Day 10, a significant difference was found between the peak location means of all four treatment groups. Fourteen days post-inoculation, statistical analysis found similar differences seen previously on Day 5. The peak means of both resistant groups, AF13R and AF36R, were significantly different from both susceptible groups, AF13S and AF36S, $p < 0.01$. And the peak location of each susceptible group, AF13S and AF36S, was significantly different from all the other

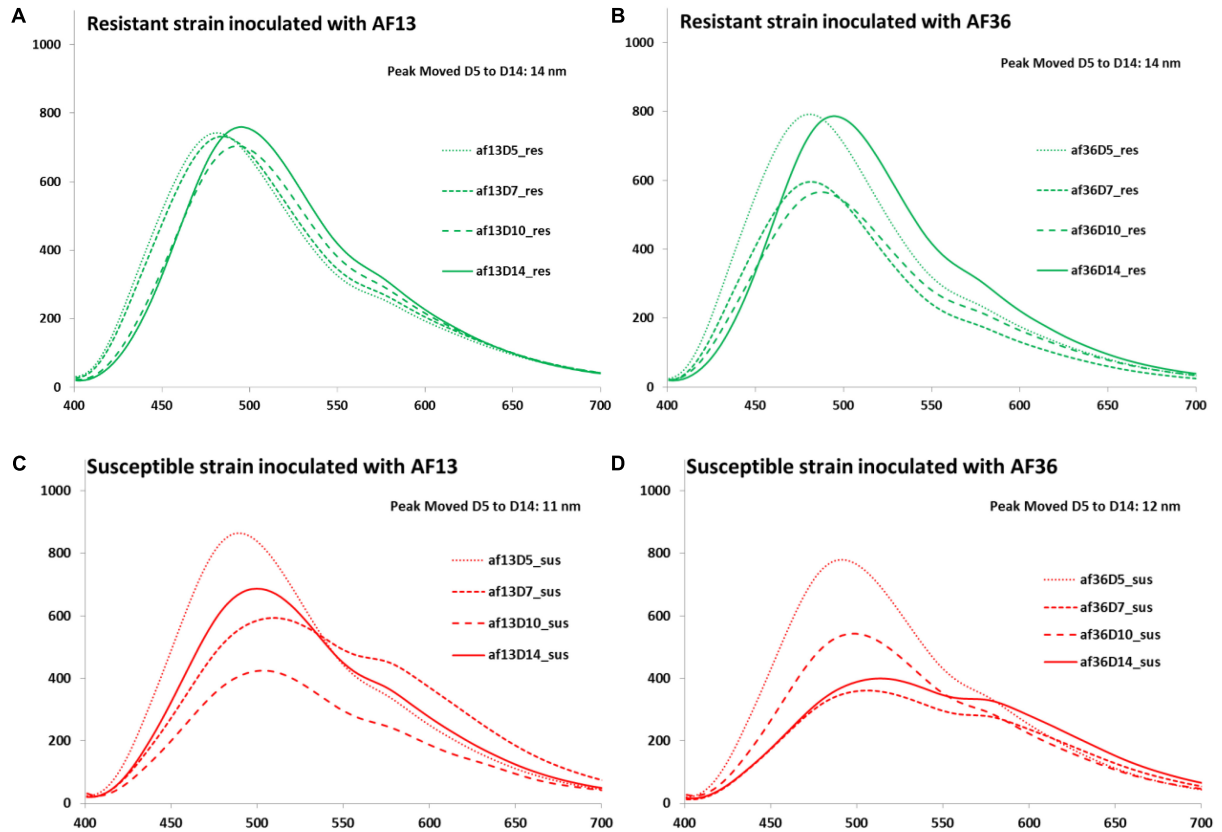

FIGURE 6 | Mean spectral curves of image data over the 14-day incubation period: **(A)** AF13 inoculated resistant corn strain **(B)** AF36 inoculated resistant corn strain **(C)** AF13 inoculated susceptible corn strain and **(D)** AF36 inoculated susceptible corn strain. A fluorescence peak shift toward longer wavelengths was observed in the spectra from both corn varieties inoculated with each fungal strain from Days 5 to 14 post-inoculation. In addition to the shift in the peak location, there was a marked difference in the general appearance of the spectral curves between the AF13 and the AF36 inoculated kernels on different days post-inoculation particularly in terms of the intensity of the fluorescence.

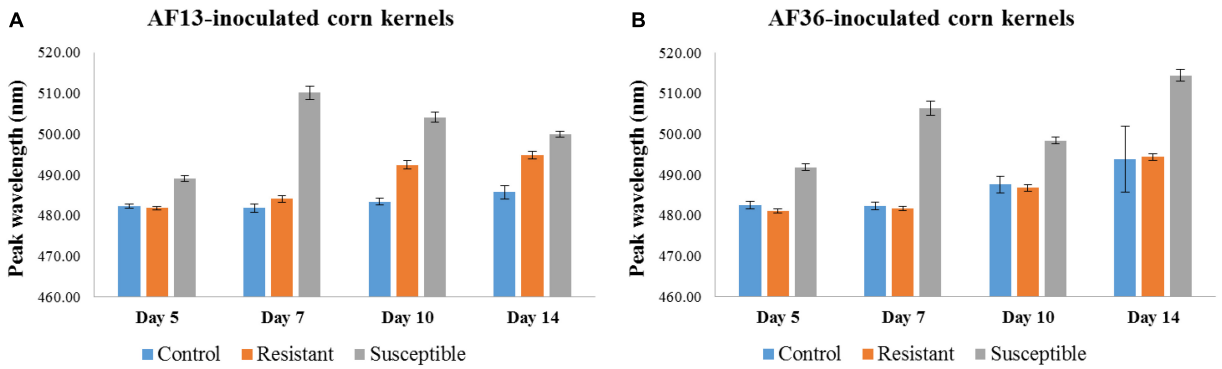

FIGURE 7 | Bar graph representation of mean peak locations of control, resistant, and susceptible corn kernels inoculated with **(A)** AF13 and **(B)** AF36 fungal strains. A significant peak shift was apparent in the AF13-inoculated kernels, differentiating the susceptible group from the resistant and control groups on Days 5 and 7. By Day 10 peak locations of all three groups were significantly different from each other $p < 0.01$. In the AF36-inoculated kernels, the peaks of the susceptible group were significantly different from the other two groups $p < 0.01$; however, the peaks of the resistant and control groups were not significantly different from each other at any of the four time points.

three groups, $p < 0.01$. The point graph representation of the data (**Figure 8B**) revealed a time-dependent interaction most apparent in the two susceptible groups, where the wavelength of the peak mean of the AF36S group gradually increased with time, while that of the AF13S increased to Day 7 and then decreased through Day 14.

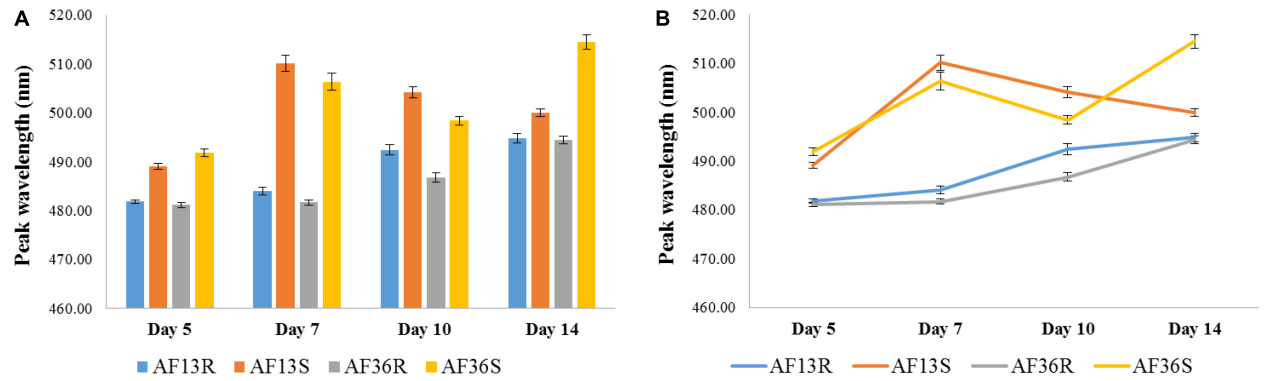

FIGURE 8 | Bar **(A)** and point **(B)** graph representation of mean peak locations of the four major groups (AF13-resistant AF13R; AF13-susceptible AF13S; AF36-resistant AF36R; AF36-susceptible AF36S) at each time point post-inoculation. An overall main effect of Day was found with $p < 0.01$. A significant difference between the peak location means of all four treatment groups was found only on Day 10. The point graph **(B)** revealed a time-dependent interaction in the two susceptible groups, where the wavelength of the peak mean of the AF36S group gradually increased with time, while that of the AF13S increased to Day 7 and then decreased through Day 14.

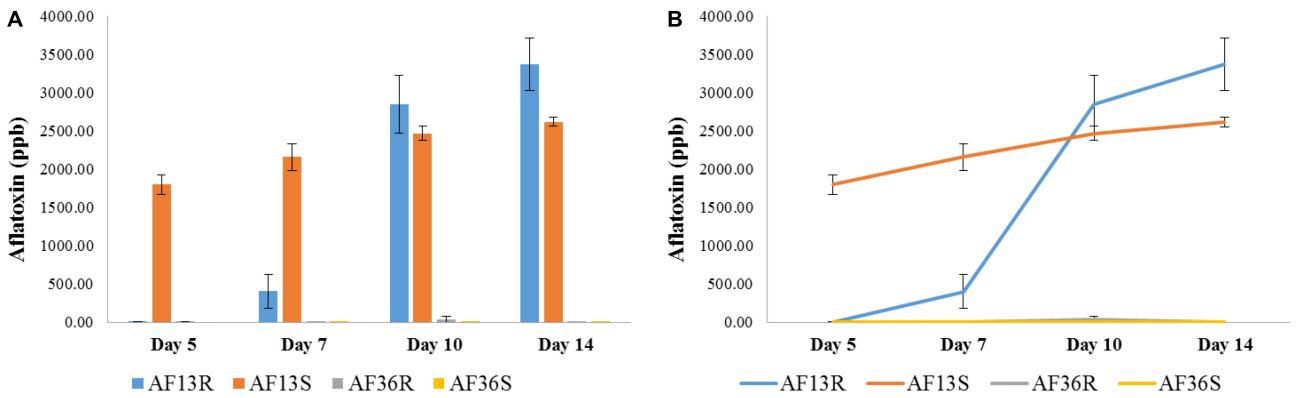

FIGURE 9 | Bar **(A)** and point **(B)** graph representing results of the AflaTest chemical analysis. An overall main effect of strain (resistant, susceptible) was revealed with $p < 0.001$. A time-dependent interaction between the AF13R and AF13S groups was found **(B)** where the aflatoxin concentration of AF13S started high and increase gradually with time, while that of the AF13R had a slow start through Day 7 and then increased exponentially through Day 14.

The results of the AflaTest chemical analysis are presented in **Figure 9**. The two groups of kernels inoculated with the atoxigenic AF36 fungal strain (AF36R and AF36S) were not expected to test positive for aflatoxin; therefore, positive aflatoxin reading in any of those kernels was attributed to internal contamination naturally acquired in the field. Statistical analysis involved mainly the two AF13-inoculated groups of kernels, AF13R and AF13S, with the other two groups serving as controls and referred to as such, only in this section. An overall main effect of strain (resistant, susceptible) was revealed with $p < 0.001$ (**Figure 9A**). On Day 5, the aflatoxin level of AF13S was already significantly different from AF13R, which at this point did not differ from the control (AF36) groups. Similar results were noted on Day 7. Although the AF13R group showed some aflatoxin contamination, it was not significantly different from the control groups. By Day 10, aflatoxin levels in the AF13R group surpassed the levels found in the AF13S group; however, the difference was not significantly different until Day 14 ($p < 0.01$). The point graph (**Figure 9B**) revealed a time-dependent interaction between the AF13R and AF13S groups, where the aflatoxin concentration of AF13S started high and increase gradually with time, while that of the AF13R had a slow start through Day 7 and then increased exponentially through Day 14, significantly overtaking the aflatoxin concentration of the susceptible group.

DISCUSSION

Aflatoxins in food and/or feed pose acute and chronic risks to health of people and animals. In human populations, consumption of high levels of aflatoxins can result in acute illness or even death (Strosnider et al., 2006). Although aflatoxin contamination is a global problem, the areas most affected are poverty stricken tropical countries south of the Sahara in Africa, and Southern Asia where maize and groundnuts are the diet staples most often contaminated with aflatoxins. In addition to acute effects, chronic exposure to aflatoxins were found to be associated with liver cancer (Wu, 2013), childhood

growth stunting (Gong et al., 2002, 2004; Khlangwiset et al., 2011), and immune suppression (Williams et al., 2004; Jiang et al., 2005). The global burden of liver cancer attributed to aflatoxin exposure was estimated to be approximately 23% of all liver cancer cases per year (Liu and Wu, 2010; Liu et al., 2012). This is significant, considering most of the people will die within 3 months of diagnosis. In animal studies, adverse effects were also found on health, growth, and productivity when animals were fed feed contaminated with mycotoxins.

General aflatoxin exposure can be reduced by improved field, harvesting, and storage practices, and by switching to crop hybrids less prone to aflatoxin contamination. Together with pre- and post-harvest strategies, and effective screening tools, host plant resistance is considered to be a practical and effective approach in reducing aflatoxin contamination in maize (Brown et al., 2016).

In this study we took the opportunity to evaluate fluorescence HSI as a viable technique for early, non-invasive aflatoxin screening of resistant and susceptible maize hybrids infected by a toxigenic and an atoxigenic strain of *A. flavus*, for potential in-field domestic as well as international applications.

Hyperspectral imaging successfully differentiated the resistant from the susceptible maize kernels based on the location of the fluorescence emission peaks regardless of the treatment received. The resistant kernels could not be differentiated from the uninoculated controls when all data were collapsed across treatments and duration of the study. Similarly, both treatment groups were differentiated from the untreated controls, when the data were collapsed across strain and time, but the treatments could not be separated from each other. However, data analysis with respect to days revealed interesting insights of the general trends which were obscured when the data were analyzed as a whole.

In terms of the maize variety data, in the AF13-inoculated kernels, both the resistant and the susceptible kernels could be separated from the controls and from each other by day 10. Although the susceptible kernels were separable from the resistant and the control kernels as early as Day 5, the resistant kernels were not separable from the controls until Day 10. In the AF36 inoculated maize, the susceptible kernels were separable from the resistant and the control kernels on Day 5, but the resistant kernels were not separable from the controls over the duration of the experiment. These results revealed a temporal window where it was possible to differentiate AF13 inoculated resistant kernels from the AF36 inoculated resistant kernels, which was Day 10 post-inoculation. Of course, these results are specific to the two strains (resistant and susceptible) used in the current experiment, and additional strains must be tested before a more generalized conclusion may be inferred. Interestingly, Day 10 was also significant when the four groups (two resistant and two susceptible) were analyzed on different days post-inoculation. Day 10 was the only day where all four groups differed from each other.

Aflatoxin-resistant variety characteristics may include direct resistance to fungus and aflatoxin accumulation, indirect resistance or tolerance to biotic and abiotic stresses, or morphological traits such as ear, kernel, and husk characteristics that impede or delay fungal access or growth. Sources of resistance to many of these factors have been identified and are now being combined to develop aflatoxin-resistant maize germplasm adapted to various agricultural ecosystems (Brown et al., 2013; Mahuku et al., 2013). The resistant TZAR104 maize variety used in the current study is one of the six maize germplasm lines released by the International Institute of Tropical Agriculture-Southern Regional Research Center (IITA-SRRC) maize breeding collaboration for use in African National Programs and U.S. maize breeding programs (Menkir et al., 2006). TZAR104 was extracted from a backcross involving GT-MAS:gk (U.S. inbred line, with proven resistance to aflatoxin contamination) as a recurrent parent and KU1414-SR (tropical elite African inbred line with some level of resistance to aflatoxin production) as a non-recurrent parent (Brown et al., 2016). Therefore, the resistance exhibited by the TZAR104 kernels is a combination of resistant traits from both the U.S. and African lines to aflatoxin production and contamination based on direct resistance to diseases such as *Aspergillus* ear rot (Brown et al., 2001). In the present experiment, it appears that the resistant maize was resistant to the AF36 infection over the course of the study (**Figure 7B**). The resistant strain also resisted the AF13 infection, where it took longer for the infection to occur compared to the susceptible strain, but by Day 10 the resistant kernels were infected, as revealed by the spectral data (**Figure 7A**). The chemical data also supported these findings. The aflatoxin contamination levels in the resistant kernels reached those of the susceptible kernels by Day 10 and surpassed them by Day 14 (**Figures 9A,B**). The enhanced resistance may be partially attributed to the rounder shape and flinty texture of the TZAR104 kernels (Brown et al., 2016) which may have been harder to penetrate than the usual dent corn seed coat. It appears that the resistance is limited to penetrating the pericarp (Guo et al., 1995), and once the barrier is breached the infection spreads rapidly.

Fluorescence intensities in the image data were also examined in the different treatment groups for each variety. There was a noticeable difference in intensities between the resistant and the susceptible corn. In the resistant corn, the difference in intensities between the AF13 and the AF36 treated groups compared to their respective controls was insignificant. Although there was a slight difference in fluorescence intensity between the AF13 treated and the AF36 treated groups, with the intensity of the AF36 group being lower than that of the AF13 treated group, this too was not significant (**Figure 4A**). In the susceptible corn, however, this difference was much greater, where fluorescence intensities of the treatment groups were between ∼25 and 30% lower than their respective controls (**Figure 4B**). Additionally, the intensity of the AF13 treated group was ∼20–25% higher than that of the AF36 treated group. This agrees with a previous study which examined internal fluorescence emissions associated with aflatoxin contamination from corn

kernel cross-sections inoculated with toxigenic and atoxigenic *A. flavus*. The study found that on Day 9 post-inoculation, it was not possible to separate the AF13 inoculated kernels from the uninoculated controls based on fluorescence peak locations. However, they noticed that the intensity of the AF13-induced fluorescence in the endosperm was approximately double that of AF36 (Hruska et al., 2017).

Over the course of the study, the two maize strains were easily distinguished from the early days of the experiment, where fluorescence signals from the susceptible corn line were different from the resistant line on Day 5 in terms of the peak location and on Day 7 in terms of both the peak location and the fluorescence intensity. It was also possible to differentiate the inoculated resistant line from the uninoculated controls from the seventh day post-inoculation based on fluorescence intensity, which was not possible until day 10, in the current study, when taking only the change in peak location into consideration. This indicates that fluorescence intensity has a relevant, or even a crucial role in HSI-based aflatoxin detection, along with the difference in peak location, when evaluating contaminated and uncontaminated maize from diverse susceptible and resistant lines. Follow up fluorescence HSI studies need to evaluate additional resistant and susceptible maize varieties focusing on the fluorescence emission intensities along with the fluorescence peak shifts to estimate the earliest time point of detecting aflatoxin contamination in maize in order to protect food and feed streams from potential adverse effects on global health.

CONCLUSION

Using non-invasive HSI technology, the present *in situ* study found that the resistant strain, TZAR 104 was resistant to the atoxigenic AF36 fungal inoculum over the 14 days of the study, while it only temporarily resisted the entry of the toxin-producing AF13 fungus, based on spectral data. The resistance appeared limited to penetrating the seed-coat, because once the barrier was breached the infection and associated toxin accumulation spread rapidly. The study also revealed a significant role for the intensity of fluorescence when using fluorescence hyperspectral imaging for early detection of maize kernels infected with toxigenic and atoxigenic *A. flavus* in resistant and susceptible corn varieties. While the study confirmed the usefulness of fluorescence HSI as a rapid and non-destructive tool for screening different varieties of maize infected with aflatoxins, it is important that follow-up studies focus on the relationship between the fluorescence peak shifts and the fluorescence intensities to determine the optimum timing indicators for the earliest detection of aflatoxins in maize, with hyperspectral fluorescence.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors participated in planning the experiments and edited the manuscript. ZH designed and executed the experiments, interpreted the data, and wrote the manuscript. HY and RB oversaw the implementation of the experiments. RK collected and processed the spectral images. FT performed the statistical analysis of the image and aflatoxin data.

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Mapping Quantitative Trait Loci Associated With Resistance to Aflatoxin Accumulation in Maize Inbred Mp719

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Aflatoxins are carcinogenic and toxic compounds produced principally by fungal species *Aspergillus flavus* (Link: Fries) and *A. parasiticus* (Speare), which are common contaminants of food and feed. Aflatoxins can be found at dangerously high levels and can readily contaminate pre-harvest maize (*Zea mays* L.) grain. Sources of resistance to aflatoxin accumulation in maize have been identified, however, the highly quantitative nature and complex inheritance of this trait have limited the introgression of aflatoxin accumulation resistance into agronomically desirable lines. Mapping of quantitative trait loci (QTL) was performed on a bi-parental population comprised of 241 F2:3 families derived from the cross of inbred lines Mp705 (susceptible) × Mp719 (resistant). The mapping population was phenotyped in replicated field trials in three environments for resistance to aflatoxin accumulation under artificial inoculation with an *A. flavus* spore suspension. The genetic linkage map was constructed with 1,276 single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) molecular markers covering a total genetic distance of 1,642 cM across all ten maize chromosomes. Multiple interval mapping revealed that majority of the aflatoxin-reducing alleles and the source for the larger effect QTL identified in this study were contributed from Mp719, the resistant parent. Two QTL identified on chromosome 1 (bin 1.06–1.07) and chromosome 3 (bin 3.09) were the most stable across different environments and when combined, explained 24.6% of the total phenotypic variance across all three environments. Results from the study showed that these chromosomal regions harbor important QTL for influencing aflatoxin accumulation, which is consistent with previous reports with other different mapping populations. These stable QTL were the most promising for controlling aflatoxin accumulation in maize grain. Identifying beneficial alleles derived from Mp719 and closely linked molecular markers through QTL analysis for implementation of MAS could accelerate breeding efforts to reduce aflatoxin accumulation in maize.

Keywords: quantitative trait locus, maize, *Aspergillus flavus*, aflatoxin, host-plant resistance

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INTRODUCTION

Maize (*Zea mays* L.), cultivated worldwide, is an agronomically important grain crop that plays a significant role in food security. Global maize production in 2017 totaled 1.1 billion tons (FAOSTAT, 2019) and the United States, the largest producer of maize, produced over 30% of the total world supply and contributed about $52 billion to the economy (FAOSTAT, 2019; National Corn Growers Association [NCGA], 2019). Abiotic and biotic stresses pose a serious threat to maize production that can lead to major yield losses and diminished grain quality causing significant impacts to the economy and threatening the livelihood of millions. The most important "biotic stresses on maize are primarily pathogens" (Gong et al., 2014). *Aspergillus*, an ear-rot fungal pathogen that produces mycotoxin, is especially problematic as the mycotoxin can be present at dangerously high levels in the grain with or without fungal growth (Thompson and Raizada, 2018). Discovered nearly 60 years ago, aflatoxin, a type of mycotoxin, has become known as a common contaminant of animal feed and human food supply. Aflatoxins are highly toxic secondary metabolites produced mainly by fungal species *Aspergillus flavus* (Link: Fries) and *A. parasiticus* (Speare) (Lin and Dianese, 1976). In developing countries, most households consume crops that they produce including maize and as a result, may be chronically exposed to aflatoxin (Strosnider et al., 2006). Regrettably, aflatoxins are linked to many negative health consequences that occur from consumption of contaminated food, including immunosuppression, teratogenic and carcinogenic effects (Bennett and Klich, 2003).

Many countries worldwide have established regulatory guidelines for maximum tolerable levels of food and feed to minimize exposure to aflatoxin. The United States Food and Drug Administration (FDA) imposed action levels for human food and animal feed. The safety range for aflatoxin in foodstuff is 20 and 0.5 ng g^{-1} for milk. To maintain a safe level of contaminants in feed for breeding animals, the action level is 100–200 ng g^{-1} (US Food and Drug Administration [FDA], 2000). The regulation of aflatoxin contamination is associated with economic losses that include the cost of preventative and mitigation measures using inspections, sampling, and analyses; the reduced value or disposal of contaminated food and feed; and losses caused by the reduction in animal productivity (Wu, 2015). Maize is one of the major crops with a high economic risk for aflatoxin contamination resulting in an estimated loss of up to $225 million annually in the United States (Wu, 2006), and as much as a billion dollars globally (Mitchell et al., 2016). Aflatoxin contamination has historically been a recurrent annual problem in the southern and southeastern regions of the United States (Mitchell et al., 2016). Regulatory guidelines to reduce the risk of aflatoxin exposure have little relevance in many food-insecure populations where they often rely on maize for daily nutrition and income (Strosnider et al., 2006).

Pre-harvest management is a critical practice for minimizing aflatoxin contamination of maize (Mahuku et al., 2019). Resistance to aflatoxin accumulation can be achieved by reducing the fungal infection in the grain, reducing the amount of toxin produced by the fungus, or both (Warburton et al., 2011). Strategies including development of acceptable agronomic practices, insect management, and biological control have been exploited to improve maize resistance to aflatoxin accumulation (Widstrom, 1996; Atehnkeng et al., 2008). Native host-plant resistance through conventional breeding strategies offers a more attractive, safe, and cost-effective solution for controlling aflatoxin production in maize (Brown et al., 1999). Most of the known resistance to aflatoxin accumulation in maize has been found in a limited number of temperate and several sources of tropical germplasm. Early breeding efforts identified *A. flavus*-resistant inbred lines Mp420 and Mp313E (Scott and Zummo, 1990, 1992). Additional germplasm lines developed for resistance to aflatoxin accumulation included GT-601–GT-603 developed from the GT-MAS:gk population, and Mp715, Mp717–Mp719, Tx736, Tx739, Tx740, Tx772, TZAR101–TZAR106 (McMillian et al., 1993; Williams and Windham, 2001, 2006, 2012; Llorente et al., 2004; Guo et al., 2007, 2011; Menkir et al., 2008; Mayfield et al., 2012). Despite steady development of genetic sources of resistance to aflatoxin accumulation in maize germplasm, attempts to transfer resistance to more agronomically adequate hybrids have been challenging (Abbas et al., 2002; Warburton and Williams, 2014). The identification of quantitative trait loci (QTL) and the discovery of associated molecular markers to facilitate the transfer of beneficial alleles to elite lines through marker-assisted selection (MAS) could contribute to traditional breeding efforts (Jiang, 2013).

Many studies have investigated genomic regions using QTL analysis for maize resistance to aflatoxin accumulation to identify molecular markers for use in MAS (Paul et al., 2003; Widstrom et al., 2003; Brooks et al., 2005; Alwala et al., 2008; Warburton et al., 2009, 2011; Willcox et al., 2013; Yin et al., 2014; Dhakal et al., 2016; Smith et al., 2019). One germplasm line, Mp719, released as a source of resistance to aflatoxin accumulation, has not been previously mapped for aflatoxin accumulation and is included in the present study. The objective of this study is to identify QTL associated with the reduction of aflatoxin accumulation in maize in a bi-parental mapping population, comprising 241 F2:3 families derived from a cross between Mp719 (resistant) and Mp705 (susceptible).

MATERIALS AND METHODS

Population Development

The mapping population was derived from a cross between maize inbred lines, Mp719 and Mp705. Mp719 (PI 662046) is a breeding inbred line that was developed and released by the USDA's Agricultural Research Service (USDA-ARS, Mississippi) as a source of resistance to aflatoxin accumulation (Williams and Windham, 2012). During the trial performed by Williams and Windham (2012), Mp719 had a geometric mean of 74 ng g^{-1} and the means for the susceptible checks in the experiment were 1,153 (Va35), 3,452 (Ga209), and 5,615 ng g^{-1} (SC212m). Mp719 was developed from a cross between inbred lines Mp715 and Va35. Mp715 (PI 614819) was released as a source of resistance for aflatoxin accumulation and developed from Tuxpan, an open-pollinated Southern Dent derived from the Mexican dent

germplasm, Tuxpeño (Williams and Windham, 2001). Va35 (PI 587150) was developed and released by the Virginia Agricultural Experiment Station by self-pollination of the backcross, (C103 x T8)T8 (Gracen, 1986). Susceptible to many diseases including aflatoxin accumulation but with good agronomic qualities, Va35 is a non-stiff stalk southern United States maize inbred line derived from Lancaster Surecrop. Mp705, the susceptible parent of the mapping population used in this study, was released in 1984 (registration number GP-130) by the USDA-ARS as a source of resistance to insect leaf-feeding damage (Williams and Davis, 1984). Mp705 was derived from MpSWCB-4, a population that was developed as a source of resistance to leaf-feeding damage caused by southwestern corn borer (SWCB), *Diatraea grandiosella* (Dyar) (Scott and Davis, 1981).

Pre-harvest aflatoxin contamination field trials were planted in three environments: 2017 and 2018 at the R. R. Foil Plant Science Research Center, Mississippi State, Mississippi (MS) and 2017 at the Quaker Research Farm, Texas A&M AgriLife Extension Center, Lubbock, Texas (TX). The population was developed by self-pollinating the F1 plant to produce F2 seed and the F2 plants were self-pollinated to produce 241 F2:3 families. The tests were phenotyped for aflatoxin accumulation in three replications and included 241 F2:3 families, the inbred parents, Mp719 and Mp705, and their F1 hybrid. This mapping population was also phenotyped, in a separate field experiment, for fall armyworm *Spodoptera frugiperda* leaf-feeding damage by Womack et al. (2020). Experiments were sown in a single 5.1 m long row plot spaced 0.96 m apart and thinned to 20 plants in a randomized complete block design. Plants were maintained with local standard cultural practices and irrigation.

Inoculum Preparation and Phenotyping

Inoculum was prepared from the *A. flavus* isolate NRRL 3357, which is known to produce aflatoxin in maize grain (Windham and Williams, 1999, 2002). The fungal inoculum was increased on 40 mesh, sterilized corn-cob grits (Grit-O-Cobs, The Andersons Inc., Maumee, OH, United States) in 500 mL flasks, each containing 50 g of grits and 100 mL of sterile, distilled water, and incubated at 28°C for 3 weeks. The conidia were washed from the grits using 500 mL sterile distilled water containing 20 drops L^{-1} of Tween 20 (Sigma-Aldrich, St. Louis, MO, United States) and filtered through four layers of sterile cheesecloth. Concentrations of conidia were determined with a hemacytometer and diluted with sterile, distilled water to 9 × 107 *A. flavus* conidia mL^{-1}. Inoculum not immediately used was stored at 4°C. Developing ears were inoculated using the side-needle technique according to Zummo and Scott (1989). Seven days after silks had emerged from 50% of the 20 plants in a plot, the top ear of each plant was inoculated with the *A. flavus* spore suspension. Using an Idico tree-marking gun fitted with a 14-gauge needle, a 3.4 mL suspension containing 3 × 108 *A. flavus* conidia was injected through the husks into the side of the ear. There was an approximate range of 14 days between the first set of genotypes that flowered until the last set of genotypes that flowered in every year. The inoculated ears in each plot were hand-harvested in bulk approximately 60 days after inoculation of each plot and dried at 53°C for 7 days. Ears were mechanically shelled and the grain was mixed thoroughly before grinding with a Romer subsampling mill (Union, MO, United States). The concentration of aflatoxin from a 50 g ground sample was determined using VICAM AflaTest (Watertown, MA, United States) per manufacturer's instructions.

Statistical Analysis of Phenotypic Data

The concentrations for aflatoxin were log-transformed to convert skewed data to conformed, normalized values using $\ln(y + 1)$, where y is the concentration of the aflatoxin in a sample. The three check genotypes (Mp705, Mp719, and the F1 hybrid) of the experiment were analyzed in SAS 9.4 (SAS Institute, 2014, Cary, NC, United States). The genotypes were subjected to analysis of variance (ANOVA) using PROC MIXED. Genotype, environment (location and year), and genotype-by-environment interaction were the fixed effects of the model and block nested in environment was the random effect.

The best linear unbiased predictors (BLUPs) for each F2:3 family (genotype) mean were calculated using the PROC MIXED function in SAS 9.4 (SAS Institute, 2014, Cary, NC, United States). Genotype and block were estimated as random effects of the model within an environment. When combined over all environments, genotype, environment (location and year), and genotype-by-environment interaction were the fixed effects of the model and block nested in environment was the random effect. The BLUP value of each family mean was used for QTL analysis. Estimates of the variance components of the F2:3 family means were obtained with restricted maximum likelihood (REML). Family mean broad-sense heritability (h2) estimates, within and across environments, were calculated according to Holland et al. (2003).

Genotyping and QTL Mapping

The genotyping and QTL analysis used in this study have been previously described in detail by Womack et al. (2020). Briefly, leaf tissue samples were collected from the all F2:3 plants and the check genotypes and DNA was extracted from each sample using a modified cetyltrimethylammonium bromide (CTAB) method described by Saghai-Maroof et al. (1984). For genotyping analysis, SSR and SNP markers were run on the extracted DNA of the mapping population and were used for genetic linkage mapping construction in the JoinMap 4.0 (Van Ooijen, 2006) computer program. The dataset consisted of a total of 1,276 molecular markers, 1,247 SNP and 29 SSR markers, run on 241 F2:3 families. Markers were omitted from analysis if data was missing (>10%), if markers disrupted marker order and significantly deviated from the Mendelian 1:2:1 ratio, if markers were identical to other markers, and if a marker had a strong linkage outside of its own group. The recombination frequencies were converted to genetic distances (centiMorgans, cM) using the Haldane (1919) mapping function.

Quantitative trait loci analysis was conducted using Windows QTL Cartographer v. 2.5 software (Wang et al., 2012). A MIM preferred model of each environment was selected according to guidelines of Silva et al. (2012). Briefly, composite interval mapping (CIM) was performed to initiate model terms for multiple interval mapping (MIM) (Kao and Zeng, 1997;

Kao et al., 1999). Main effect QTL were searched, and significant terms were added to the model only if the Bayesian information criterion (BIC) decreased. The interactions between main effect QTL were searched. Epistasis was identified when there was a significant interaction between two QTL. The position of the QTL was optimized after significant terms were added to the model. The process was repeated until no additional parameters could be added. Quantitative trait loci position and genetic effects (additive, dominance, and epistasis) were estimated and the observed phenotypic variance was obtained (Kao et al., 1999). The signs of the genetic effect of the QTL in a model were used to identify the origin of the aflatoxin-reducing alleles (Lübberstedt et al., 1997).

RESULTS

Phenotypic Performance of Parental Lines and F2:3 Families

The resistant parent, Mp719, the susceptible parent, Mp705, and their F1, were planted as checks in each environment. There were many missing plots in TX 2017; therefore, this data was not included in analyses. When the data was combined across MS 2017 and MS 2018 environments, the ANOVA results indicated that block nested in environment ($p = 0.1234$) and the variability due to the interaction between genotype and environment ($p = 0.1854$) were not significant sources of variance (**Table 1**). Genotype ($p = 0.0002$) and environment ($p = 0.0438$), treated as main effects, were shown to have a significant effect on aflatoxin production. When analyzed for total aflatoxin accumulation, the resistant parent (Mp719) had significantly lower aflatoxin levels than the susceptible parent (Mp705) ($p < 0.05$) but, did not significantly differ from the F1 in MS 2017 and MS 2018 ($p > 0.05$) (**Table 2**).

The mean aflatoxin concentrations for the F2:3 families were lower in the MS 2017 environment at a value of 47.1 ± 3.5 ng g^{-1} ($\bar{x} \pm$ s.e.) than in the MS 2018 (172.2 ± 8.7 ng g^{-1}) and TX 2017 (181.6 ± 9.9 ng g^{-1}) environments (**Table 3**). The means of the transformed data of aflatoxin concentrations for the F2:3 families in each environment, calculated as BLUPs, were 2.56 ± 0.07 (MS 2017), 4.19 ± 0.06 (MS 2018), and 4.15 ± 0.07 (TX 2017) (**Table 3**). Variance components were estimated using REML and used to calculate heritability estimate across all environments (**Table 4**). The broad-sense heritability combined across the three environments was 0.56, and within each environment the repeatability estimates ranged from 0.43 to 0.54.

Linkage Map Construction

The linkage map was constructed as in Womack et al. (2020) which included 1,276 SSR and SNP markers that resolved all maize chromosomes. The 10 linkage groups, corresponding to the 10 maize chromosomes, were identified with a LOD score of 3.0 and remained associated even at LOD = 10.0. There were 40 to 233 markers per linkage group (**Supplementary Table S1**). These markers spanned a total genetic distance of 1,642 cM with an average interval between markers of 1.3 cM and the largest interval was 16.8 cM. Chromosome 3 displayed some significant segregation distortion ($\alpha = 0.05$) from bin 3.00 to 3.06, with markers deviating from the expected 1:2:1 Mendelian ratio. In this region, the allele from Mp719 appeared more frequently than the allele contribution from Mp705.

QTL Analysis

Initial MIM models were assembled based on the models obtained from CIM analysis (**Supplementary Table S2**). Using MIM, a stepwise search for QTL identified genetic models containing six QTL and two epistatic interactions in the MS 2017 environment; four QTL and one epistatic interaction in MS 2018; and four QTL in TX 2017. When the data was combined over all environments, seven QTL and three interactions were identified (**Table 5**). Quantitative trait loci were identified on chromosomes 1, 3, 5, 8, and 10 in MS 2017; chromosomes 1, 2, 3, and 9 in MS 2018; and 1, 3, 4, and 6 in TX 2017. When combined over all environments, QTL were identified on chromosomes 1, 2, 3, 5, 8, and 10. The MIM models explained 42.3% (MS 2017), 27.4% (TX 2017) and 24.6% (MS 2018) phenotypic variances within each environment and 53.6% when combined overall environments. Four major QTL accounted for more than 10% of the total phenotypic variance in MS 2017 (11.3%, bin 1.06), TX 2017 (12.7%, bin 1.07), MS 2018 (10.5%, bin 3.09) and when combined across all environments (15.4%, bin 1.06).

Quantitative trait loci genetic effects were estimated by MIM that allowed for the identification of beneficial or aflatoxin-reducing alleles. In every environment, both parents contributed the beneficial allele, but the aflatoxin-reducing alleles have been passed down more stably from Mp719 in all environments (**Table 5**). When data was combined over all environments, Mp719 (resistant parent) contributed most beneficial alleles. The largest effects of each individual environment were found in bin 1.06 (additive, −0.294) in MS 2017, bin 3.07 (additive, −0.304) in TX 2017, bin 3.09 (additive, −0.304) in MS 2018, and bin 1.06 (additive, −0.274) combined across all environments and the source of the beneficial allele was Mp719 in every case.

DISCUSSION

Aflatoxin accumulation in maize is greatly influenced by the environment and the genetic background (Wang et al., 2019). The current study included the resistant parent, Mp719, the susceptible parent, Mp705, and their F1, planted as checks in each environment. The parents of the F2:3 population showed significant differences in aflatoxin cumulation when the data was transformed in the MS 2017 and MS 2018 environments. The F1 closely resembled Mp719, the resistant parent, but this is likely due to heterosis. For the F2:3 families, The MS 2017 environment had the lowest mean aflatoxin concentration compared to MS 2018 and TX 2017. Additionally, the coefficient of variability (standard deviation relative to the mean) was slightly higher for this environment. It is not clear if the difference in variance for MS 2017 compared to MS 2018 and TX 2017 was due to weather patterns. A mixed linear model was constructed and yielded the BLUPs for the F2:3 families. The mixed model also yielded estimates for the variance components and these were used

TABLE 1 | ANOVA results of the three check genotypes across two environments.

Source of variance	df	Mean square	*F* value	*P* value
Genotype	2	8.89	28.45	0.0002
Environment	1	6.67	8.46	0.0438
Genotype × environment	2	0.65	2.10	0.1854
Block (environment)	4	0.79	2.52	0.1234
Residual	8	0.31		

TABLE 2 | Multiple comparisons of the mean aflatoxin concentrations of the check genotypes.

MS 2017			MS 2018		
	lnAF†	Aflatoxin‡ (ng g^{-1})		lnAF	Aflatoxin (ng g^{-1})
Mp705	5.86[a]	353.53	Mp705	6.32[a]	560.00
Mp719	3.20[b]	30.27	Mp719	4.83[b]	129.70
F1	3.17[b]	37.17	F1	4.72[b]	123.33

†*lnAF = log (total aflatoxin concentration + 1).* ‡*Mean concentration of aflatoxin followed by the same letter are not significantly different (α = 0.05).*

TABLE 3 | Phenotypic descriptive statistics for the raw and log transformed aflatoxin values of the F2:3 families by year.

Env	N Obs	Variable	Mean	Std error	Min	Median	Max	Std dev	CV
MS 2017	720	Aflatoxin (ng g^{-1})	47.15	3.46	0.00	15.00	960.00	91.83	194.75
		lnAF†	2.56	0.07	0.00	2.77	6.87	1.78	69.73
MS 2018	720	Aflatoxin (ng g^{-1})	172.25	8.67	0.00	82.00	1720.00	230.16	133.62
		lnAF†	4.16	0.06	0.00	4.42	7.45	1.70	41.01
TX 2017	720	Aflatoxin (ng g^{-1})	181.62	9.93	0.00	90.00	1840.00	262.22	144.38
		lnAF†	4.11	0.07	0.00	4.51	7.52	1.84	44.70

†*lnAF = log (total aflatoxin concentration + 1). Env, environment; N Obs, number of observations; Std Error, standard error; Min, minimum; Max, maximum; CV, coefficient of variability; MS, Mississippi; TX, Texas.*

to calculate the mean broad-sense heritability. The heritability of aflatoxin contamination in this study was low to moderate in agreement with those previously reported by Busboom and White (2004) and Brooks et al. (2005). Low heritability, as well as, high genotype-by-environment interaction, and the highly quantitative nature of this trait, has made the transfer of resistance to elite cultivars difficult to achieve.

Contamination of maize grain with aflatoxin has major economic implications and negative health consequences (Strosnider et al., 2006). The development of host-plant resistance as an approach to reduce aflatoxin contamination in maize has been met with challenges owing to the highly quantitative nature of this trait. Quantitative trait loci mapping used to identify closely-linked molecular markers, is proposed to aid in genetic improvement through marker-assisted breeding programs. Several bi-parental QTL mapping studies for resistance to aflatoxin accumulation have been conducted and QTL across all maize chromosomes have been detected. However, most of these mapping studies have a limited number of molecular markers covering the maize genome mainly because more highly dense maps were harder to come by in the past. In this study, a linkage map of Mp705 x Mp719 was mapped with a considerable amount of coverage at a relatively low resolution with average interval between markers of 1.3 cM. This genome coverage leads to precision QTL mapping needed for marker-assisted breeding.

TABLE 4 | Restricted maximum likelihood (REML) analysis used to estimate variance components of the F2:3 families by year and across all environments.

Variance components	All environments	Variance components	MS 2017	MS 2018	TX 2017
Genotype	0.47	Genotype	0.89	0.82	0.75
Environment	0.81	Block	0	0	0.04
Genotype x environment	0.35	Error	2.29	2.09	2.59
Block (env)	0.01				
Error	2.33				

MS, Mississippi; TX, Texas.

TABLE 5 | Multiple interval mapping results within and across environments.

Env*	Chr bin	QTL peak position	2-LOD interval	Marker§	Effect		Phenotypic variance		
					A†	D‡	A	D	Total
		cM					%		
MS 2017	1.06	124.4	114.4–130.9	PZE-101154189	−0.294	0.021	11.3	0.0	11.3
	1.10	183.2	179.1–185.4	PZA00364-2	−0.258	−0.171	7.6	1.2	8.8
	3.09	156.7	151.3–161.8	PZE-103165542	−0.226	−0.091	5.6	0.3	5.9
	5.01	31.6	22.8–39.7	PHM3137-17	0.161	−0.141	2.4	1.1	3.5
	8.01	12.6	6.1–14.5	PZE-108002941	−0.154	0.195	1.6	2.3	3.9
	10.04	44.2	29.3–55.0	PZE-110067110	−0.198	0.141	4.5	0.6	5.1
	1.10 × 8.01				0.241 (AA)		2.4		2.4
	3.08 × 10.04				0.215 (AA)		1.4		1.4
									42.3
MS 2018	1.06	116.6	103.7–130.9	PHM5622-21	−0.180	0.145	3.9	0.9	4.8
	2.05	85.7	85.7–85.7	PHM4880-179	−0.148	0.238	2.2	2.2	4.4
	3.09	161.4	155.4–169.4	PZE-103169160	−0.304	−0.090	10.3	0.2	10.5
	9.04	63.0	59.3–63.6	PZE-109061001	0.024	−0.256	0.1	3.2	3.3
	1.06 × 3.09				0.214 (AA)		1.6		1.6
									24.6
TX 2017	1.07	141.4	138.9–147.2	PZE-101171655	−0.304	−0.095	12.6	0.1	12.7
	3.07	128.0	110.8–138.4	PZE-103144159	−0.186	0.032	4.7	0.2	4.9
	4.07	101.2	93.1–111	PZE-104101251	−0.197	−0.099	4.6	0.3	4.9
	6.04	49.6	31.6–55.9	PZE-106057733	−0.151	0.122	3.6	1.3	4.9
									27.4
Combined									
	1.06	118.6	104.6–123.3	PZE-101145417	−0.274	0.085	14.7	0.7	15.4
	1.09	177.9	173.9–181.8	PZE-101210110	−0.151	−0.117	5.4	1.4	6.8
	2.03	59.0	55.5–61.7	PZE-102036053	−0.139	0.113	2.8	0.8	3.6
	3.09	159.9	153.5–164.1	PZE-103169160	−0.209	−0.047	9.2	0.0	9.2
	5.00	3.0	0.0–17.7	PZE-105002166	0.141	0.006	4.1	0.0	4.1
	8.03	72.5	66.7–87.5	PZE-108057442	−0.102	−0.156	1.3	2.5	3.8
	10.06	78.6	64.6–92.8	PZE-110091181	−0.177	0.079	5.1	0.4	5.5
	1.09 × 2.03				−0.305 (DD)		1.5		1.5
	3.09 × 5.00				−0.301 (DD)		1.9		1.9
	1.09 × 8.03				0.167 (AA)		1.8		1.8
									53.6

**Environments: MS, Mississippi; TX, Texas. §Marker closely associated with QTL peak position. †Negative additive QTL effect indicate Mp719 is the source of the beneficial (aflatoxin-reducing) allele, and positive effects indicate the resistance allele is contributed by Mp705. ‡A negative dominance effect indicates that dominance is in the direction of the aflatoxin-reducing allele, regardless of the parental source, and a positive dominance effect indicates that dominance is in the direction of the aflatoxin-increasing allele. Epistatic interactions: A × A, additive × additive effect; D × D, dominance × dominance effect.*

Traditionally, plant breeders have utilized QTL-MAS for maize line improvement. This method has been used to introgress favorable alleles into an elite background. It was shown to speed up breeding efforts compared to conventional breeding methods. Marker-assisted selection based on genomic selection (GS) has been highlighted as a more novel approach in maize breeding in the last decade (Meuwissen et al., 2001). In GS, individuals with the most favorable estimated breeding values, which can lead to the identification of individuals harboring favorable alleles, are selected (Meuwissen et al., 2001). Both methods require high-density genetic markers; but, "GS-MAS requires a higher number of markers adequately covering the entire genome resulting in higher genotyping cost for GS-MAS" (Platten et al., 2019).

The current study identified two QTL in bins 1.06–1.07 (116.6–141.4 cM) and 3.09 (156.7–159.9 cM) (**Supplementary Figure S1**) that had the largest and most consistent genetic effects. The QTL on chromosome 3 was not in the region where makers were deviating from the Mendelian 1:2:1 ratio in bins 3.00–3.06. The aflatoxin reducing allele for these QTL came from Mp719, the resistant parent. When combined over all environments, these QTL, together, explained 24.6% of the phenotypic variance and 15.4% was contributed by the individual QTL found on 1.06. Because Mp719 was developed from the cross Mp715 × Va35, Mp719 likely acquired its aflatoxin-reducing alleles from its parents. In previous bi-parental QTL mapping studies involving Mp715, QTL were also found

on chromosomes 1 and 3, and the aflatoxin reducing-alleles came mostly from Mp715 (Warburton et al., 2011; Dhakal et al., 2016; Smith et al., 2019). In a QTL mapping study of the bi-parental population Mp715 × Va35, Smith et al. (2019) reported a QTL on the short arm of chromosome 1 that was contributed by Va35, the susceptible parent of Mp719, and this QTL was consistently the source of the beneficial alleles in that region.

Breeding for a highly quantitative trait such as maize resistance to aflatoxin accumulation has been met with difficulties. Depending on the environment, multiple QTL in a common background must be used to reduce aflatoxin accumulation. Traditional breeding approaches complemented with QTL identification and associated molecular markers present an opportunity to increase understanding into the genetic basis of aflatoxin accumulation resistance in maize. Results in this study suggest that the aflatoxin-reducing QTL in the chromosomal regions bin 1.06 and 3.09 are critically important for developing aflatoxin-resistant maize lines and hybrids and should be the primary targets for transferring from Mp719 or Mp715 to elite lines with marker-assisted breeding.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

EW and WW designed and planned the experiments. GW and WX performed the phenotyping. WW, GW, and WX contributed substantially to the writing and critical revision of the manuscript and approved its final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00045/full#supplementary-material

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Aflatoxin Contamination of Milk Produced in Peri-urban Farms of Pakistan: Prevalence and Contributory Factors

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Aflatoxin M_1 contamination of milk in Pakistan, like many developing countries, is poorly understood. The present study was therefore conducted to determine AFM_1 contamination of milk and its contributory factors in Pakistan. We sampled milk and feedstuffs from 450 peri-urban dairy farms in seven major cities following a cross-sectional study design. Analysis of milk using ELISA revealed high contamination with an overall average of 3164.5 ng of AFM_1/L, and significant differences ($p < 0.001$) between cities. The milk sampled from Gilgit, in northern hilly areas, had an average AFM_1 level of 92.5 ng/L. Milk from other cities had 3529.7 ng/L average contamination, with only 5.7% samples qualifying the maximum tolerable limit of 500 ng of AFM_1/L. Heavy mean aflatoxin contamination was found in bakery waste (724.6 μg/kg), and cottonseed cake (600.8 μg/kg). Rest of the other feedstuffs had moderate to low mean aflatoxin contamination, ranging from 66.0 μg/kg in maize stover to 3.4 μg/kg in wheat bran. The mean aflatoxin level in commercial dairy concentrates was 32.7 μg/kg. About 80% of the total aflatoxin intake of dairy animals was contributed by cottonseed cake alone due to its high aflatoxin contamination and proportion in dairy rations. On-farm storage time of oilseed cakes varied ($p < 0.01$) in different cities but was not associated with aflatoxin contamination. The exceptionally high AFM_1 contamination suggests that milk from peri-urban dairy farms is a serious public health threat in Pakistan. This situation can be mitigated by reducing aflatoxin contamination in cottonseed cake and promoting the use of commercial concentrates and other feedstuffs with low contamination.

Keywords: aflatoxin, cottonseed, dairy, feed, milk

INTRODUCTION

Aflatoxins are toxic secondary metabolites of various *Aspergillus* spp. that commonly contaminate agricultural produce worldwide. The four main forms of aflatoxins encountered in grains and other commodities are B_1, B_2, G_1, and G_2. Once ingested by animals, the aflatoxin B_1 and G_1 are excreted in the form of aflatoxin M_1 (AFM_1) in milk and eggs. Majority of the aflatoxins in food and feed occur in the form of B_1 and therefore AFM_1 is the mainly encountered form in animal products (Yunus et al., 2011). All these forms of aflatoxins are hepatotoxic and carcinogenic in nature. Therefore, their levels in food and feed are regulated in over 100 countries (Food and Agriculture Organization, 2004). The toxicity of aflatoxins is higher in younger age groups, and stunted growth due to aflatoxin contamination of foods has been suggested (Khlangwiset et al., 2011). Monitoring of aflatoxins in baby foods and milk is therefore more critical.

Pakistan has a climate that typically favors development of aflatoxins in foods. Several authors have reported aflatoxin contamination of various foods in the country during the last two decades (review, Ashiq, 2015). The government of Pakistan however only recently introduced legislation on aflatoxin levels in foods and feeds. Pakistan Quality and Standards Control Authority now allows 500 ng/L as the maximum allowed limit of AFM_1 in milk (amendment 2 in standard PS-5344-2016). In practice, this restriction is only followed by milk processors which have merely 5% share in the total milk marketed in the country.

Research on AFM_1 contamination of milk in Pakistan shows high variations in contamination levels. In this regard, Maqbool et al. (2009) and Iqbal et al. (2011) found mean AFM_1 levels to be 41 and 46 ng/L, respectively in milk sampled from major cities in two provinces. Similarly, Hussain (2009) found the average AFM_1 levels to vary from 199 to 503 ng/L in different seasons in milk sampled from 14 districts of Punjab province. In the latter study, only 3% samples were found to exceed the 500 ng of AFM_1/L limit, while the former two authors did not find any sample to exceed this limit. Contrary to these studies, Aslam et al. (2016) found 87% of milk samples to exceed the 500 ng/L limit in three districts of Punjab province. Likewise, Akbar et al. (2019) found 69% milk samples from different regions of the Punjab province to exceed the allowed limit. In one study, mean AFM_1 contamination in Lahore city was found to be as high as 17,380 ng/L with 81% samples exceeding the 500 ng/L limit. Similar to the case of Pakistan, high variability in aflatoxin levels has been noted in some other countries. In Kenya, a country where aflatoxins have been extensively studied, variation in AFM_1 contamination has been reported depending upon agroecological zones, seasons, and even type of milk product (Senerwa et al., 2016; Lindahl et al., 2018). In case of Pakistan, the studies conducted to date differ not only in the sampling area and season, but also in the methods employed to quantify AFM_1. Some of these studies were conducted on a very limited scale, which limits the application of their results to the overall situation in the country. In this scenario, we recently investigated seasonal variation in levels of AFM_1 in processed and raw milk in Pakistan (Yunus et al., 2019). The results indicated that raw milk is routinely contaminated with high levels of AFM_1 and that the levels are in general higher during winter months. The present study was therefore conducted as the first nationwide investigation on AFM_1 contamination of raw milk in peri-urban dairy farms of Pakistan during the high season (winter months). The aim was to not only identify areawise AFM_1 contamination of milk, but to also identify the factors that contribute to milk contamination.

MATERIALS AND METHODS

Sampling

A cross-sectional survey was conducted and pooled milk samples were collected from peri-urban dairy farms in all provincial/regional capitals in Pakistan from October to mid-December 2016. Assuming a simple random sampling, the calculated sample size was 384 milk samples with 50% proposed prevalence, 95% confidence interval, and 80% power/precision of the study (http://www.winepi.net). However, sample size was increased by approximately 10% to accommodate for any losses of milk samples during storage/transportations. Further, sample size for each city was calculated using stratified random sampling using probability proportional to size (PPS). A peri-urban dairy farm was defined as a farm located within the boundaries of identified city districts with a minimum herd size of 2 milch animals intended for sale of milk. This inclusion criterion was followed for all the cities except Gilgit, where it was relaxed to one milking animal due to small herd sizes in the city. The livestock population data (including herd size in different cities) and expert opinion of the Provincial Livestock and Dairy Development departments were considered about the number of peri-urban dairy farms in the city for sample size calculations. Finally, we planned sampling from 450 farms all across Pakistan (Islamabad = 75, Karachi = 70, Lahore = 90, Quetta 50, Peshawar = 75, Muzaffarabad = 50, and Gilgit = 40) (**Figure 1**). In case of the 40 samples from Gilgit, 13 samples were collected from Hunza valley, which is situated further toward north. Although information was collected from all the farms, only 372 raw milk samples could be tested.

Information on the relevant husbandry practices regarding feeding, and procurement and storage of feed was recorded from the farmers. From each farm, 500 ml of milk was sampled from the bulk milk tank. From this 500 ml sample, an aliquot of 50 ml and two further aliquots of 15 ml were separately taken in falcon tubes. Milk samples were kept refrigerated, and without any addition of preservatives, until reaching the lab where these were frozen at −20°C until analysis. On the day of milk sampling, samples of all the dry feedstuffs being used at the farms were also collected. These included wheat straw, maize stover, oilseed cakes (cotton, canola, palm, coconut, and maize), legumes (various pulses), grains (maize, and wheat), brans (wheat bran, and pulse bran), rice polish, corn gluten, dates, commercial concentrates, waste bread, and bakery waste. For each feedstuff, 6–10 incremental samples of 100 g each were collected using sampling probes.

FIGURE 1 | Geographical distribution of sampling sites within Pakistan.

The aggregate sample thus obtained was reduced to 250 g laboratory sample after thorough mixing, and carried in paper bags to the laboratory.

During the same time, all the brands of UHT milk (n = 15), pasteurized milk (n = 13), local milk powder (n = 4), and imported milk powder (n = 13) were also sampled from Islamabad. Except for the imported milk powder, the results regarding processed milk have already been published (Yunus et al., 2019). In the present report, these results are being presented for comparison purposes.

Aflatoxin Analyses

Milk samples were analyzed for AFM_1 contamination using ELISA kits (AFM-E01, Immunolab GmbH, Kassel, Germany) following protocols specified by the manufacturer. The kit had a quantification range of 10–1,000 ng of AFM_1/L. Milk samples were analyzed several times in different dilutions until the AFM_1 levels in the diluted samples fell within the quantification range of the ELISA kit, as detailed earlier for a sister study (Yunus et al., 2019). The samples were first analyzed at either 1X or 2X dilution, and then depending upon the optical density (OD) values, these were analyzed again after dilution at 3, 4, 5, 6, 8, 10, 12 15, or 20X. Analysis of some samples had to be repeated up to five times in different dilutions to get OD values within the range of the ELISA kit.

The feed samples were analyzed for total aflatoxin levels using ELISA kits (AFT-E01, Immunolab, Kassel, Germany) following protocols specified by the manufacturer. The kit for total aflatoxin analysis had a quantification range of 1.75–52.5 μg of aflatoxins/kg sample. Samples were extracted in 70% methanol and appropriately diluted to fall within the quantification range of the kit.

Kits were read on an ELISA reader (BDSL, Immunoskan MS 355, Labsystems, Vantaa, Finland). Aflatoxins in milk and feedstuffs were quantified using a software based on four parametric curve estimations provided by the manufacturer of the ELISA kits.

Quality Control in Aflatoxin Analyses

The ELISA kit for AFM_1 analysis was validated before start of this study (Imtiaz and Yunus, 2019). In addition, 4.4, and 44 ng of AFM_1/L external standards made using a reference skim milk powder (RMBD-248, EU Joint Res Center, IRMM, Geel, Belgium), and 50, and 500 ng of AFM_1/L external standards made using a purified 9.786 μg of AFM_1/ml solution (46,319 U, Supelco, Bellefonte, PA, USA) were run on each microtiter plate as described earlier (Yunus et al., 2019). For quality control during total aflatoxin analysis in feedstuffs, external standards were prepared diluting a certified standard having 3.228 μg of AFB_1/ml (catalogue numb 46,323 U, Supelco, USA).

Recovery of AFM_1 was 86.9% at 500 ng of AFM_1/L, while recovery of total aflatoxins at a reference value of 22.4 μg/kg of cornmeal was 109.4%. Results were not corrected for recovery.

Data Analyses

The percent contribution of feedstuffs to total aflatoxin exposure of animals in each city was calculated using following equation:

Percent contribution of a feedstuff in a city = (Average aflatoxin intake from a specific feedstuff per animal ÷ average of total aflatoxin intake from all feedstuffs per animal) × 100, where the aflatoxin intake from a specific feedstuff per animal was calculated as a multiple of average daily consumption of the feedstuff per animal and its average aflatoxin contamination for each city.

The data are presented as arithmetic means, and were statistically analyzed by applying ANOVA and least significant difference test. Differences were considered significant at $p < 0.050$. The Pearson correlation coefficients (r) between AFM_1 level in milk and use of various feed ingredients were determined. All statistical analyses were conducted using IBM SPSS Statistics 20 (IBM Corp., Armonk, New York, NY, USA, 2011).

RESULTS AND DISCUSSION

Aflatoxin M_1 Levels in Milk

The AFM_1 levels in milk collected from peri-urban dairy farms in different cities are presented in **Table 1**. Differences between AFM_1 contaminations in different cities were significant ($p < 0.001$). The milk sampled from Islamabad, Lahore, and Muzaffarabad was found to have a mean concentration of 4,799.6 ± 3,945.5 ng of AFM_1/L. The levels of AFM_1 in these cities were higher ($p = 0.006$) than the levels of the toxin in milk sampled from other cities. Milk sampled from Karachi, Peshawar, and Quetta was found to have an average of 1946.8 ± 1562.9 ng of AFM_1/L. These three cities had higher ($p = 0.019$) AFM_1 contamination than the milk sampled from Gilgit.

While only 3% samples in Islamabad and Lahore qualified the 500 ng of AFM_1/L limit, no sample in Muzaffarabad could qualify this limit. Also, over 80% sample in these cities were higher than 1,000 ng of AFM_1/L. Despite a lower level of contamination in Karachi, none of the samples in this city qualified the 500 ng/L limit, and over 80% samples had levels higher than 1,000 ng/L. Compared to these cities, 10–15% samples in Peshawar and Quetta had AFM_1 levels lower than 500 ng/L, and 40% samples had lower than 1,000 ng/L. The average level of AFM_1 in milk sampled from Himalayan city of Gilgit was 92.5 ± 178.6 ng/L and 46% samples here were even lower than the limit of 50 ng/L followed by the EU. Only 7.7% samples in Gilgit exceeded the 500 ng/L limit. In this regard, it would be worth mentioning that as the sampling area went further north in Hunza valley, the mean AFM_1 content decreased to only 10.9 ng/L and all the samples here were below the 50 ng of AFM_1/L limit followed by the EU.

During the same time (Oct–Nov), all the major brands of processed milk were collected for comparison. Results presented in **Table 2**, and as also partially reported earlier by us (Yunus et al., 2019), indicated that 54% of the milk powder brands based on imported milk powder were below the 50 ng of AFM_1/L limit, while the remaining others qualified the 500 ng/L limit. On the contrary, none of the local brands qualified the 50 ng/L limit. Only one out of four samples in this latter group qualified the 500 ng/L limit. These results are alarming as the end users of these milk powders are infants, who are more sensitive to AFM_1 contamination compared to the older age groups. It is interesting to note that UHT milk during the same time had a mean AFM_1 contamination level of 366 ng/L with 73% samples qualifying the 500 ng/L AFM_1 limit. Ironically, the UHT brands of the companies, whose baby milk powder brand exceeded the accepted standards, qualified the 500 ng AFM_1/L limit. These results indicate that either there is lack of knowledge or the raw milk with higher AFM_1 contamination is being channeled to production of milk powder due to lesser quality control on such products. The pasteurized milk collected during this time exceeded the accepted standards and had higher ($p \leq 0.007$) AFM_1 levels than UHT and imported milk powder. On an overall basis, the liquid processed milk (UHT and pasteurized combined) had an average AFM_1 contamination of 738.0 ng/L, which was found to be lower ($p < 0.001$) than the contamination in raw milk from Islamabad, Lahore, and Muzaffarabad. The liquid processed milk was also found to have lower ($p = 0.023$) AFM_1 contamination than Karachi. Statistically, the AFM_1 contamination in processed

TABLE 1 | Aflatoxin contamination of milk produced in various cities.

City	*n*	AFM_1 level (ng/L)				>50 ng/L	>500 ng/L	>1,000 ng/L
		Mean ± SD	Min	Max	Median			
Islamabad	69	4935.3 ± 3468.7[a]	417.7	15636.1	4123.1	100%	97.1%	89.9%
Lahore	83	4842.6 ± 4310.5[a]	311.8	15994.2	3194.6	100%	96.4%	83.1%
Muzaffarabad	35	4436.1 ± 4068.2[a]	554.3	13525.7	2990.2	100%	100%	82.9%
Karachi	32	2435.9 ± 1740.3[b]	772.3	7966.4	1853.1	100%	100%	81.2%
Peshawar	69	1930.8 ± 1626.9[b]	138.9	8789.5	1376.0	100%	85.5%	60.9%
Quetta	45	1623.5 ± 1242.5[b]	254.2	4997.1	1247.8	100%	91.1%	62.2%
Gilgit	39	92.5 ± 178.6[c]	<LOD	796.1	26.1	35.9%	7.7%	0.0%
Overall	372	3164.5 ± 3405.3	<LOD	15994.2	1904.6	93.3%	85.2%	68.8%

Means bearing different superscripts "[abc]" differ ($p \leq 0.01$) within the column. LOD < 4.4 ng AFM_1/L; LOQ = 10 ng AFM_1/L.

TABLE 2 | Aflatoxin contamination of processed milk during October to November.

Type of processed milk	*n*	AFM_1 level (ng/L)[1]				>50 ng/L	>500 ng/L	>1,000 ng/L
		Mean ± SD	Min	Max	Median			
Imported milk powder[1]	13	58.4 ± 31.5[b]	7.3	121.9	56.5	46.1%	0.0%	0.0%
Local milk powder[1,2]	4	922.5 ± 690.5[abc]	412.5	1935.0	671.4	100.0%	75.0%	25.0%
UHT[2]	15	365.7 ± 168.0[b]	145.5	642.9	346.5	73.3%	26.7%	0.0%
Pasteurized[2]	13	1167.5 ± 1333.7[a]	56.9	3935.5	454.5	100.0%	36.4%	45.5%
Overall	45	558.1 ± 857.3	7.3	3935.5	395.8	66.7%	24.4%	13.3%

Means bearing different superscripts "[abc]" differ ($p \leq 0.01$) within the column. [1]Milk powder reconstituted at 15 g per 115 ml for AFM_1 analysis; [2]Monthwise averages for local milk powder, UHT, and pasteurized milk have been reported previously (Yunus et al., 2019).

milk was not found to be different ($p \geq 0.064$) than the contamination in milk sampled from Quetta, Peshawar, and Gilgit.

The presently reported AFM_1 levels in milk are very high compared with some of the previous reports (Raza, 2006; Iqbal et al., 2011, 2014; Younus et al., 2013; Ahmad et al., 2019), but lower than the levels reported by Muhammad et al. (2010) for Lahore city. The contamination levels are also higher compared to what has been found in milk in different parts of Africa (Ayalew et al., 2016). It should be noted in this regard that the samples in the present study were collected in a season that is associated with higher contamination of milk. Secondly, samples were collected from peri-urban dairy farms, which use lesser fodder compared with the farmers in villages. Such practices are associated with higher levels of AFM_1 in milk (Iqbal et al., 2014). In addition, some of the previous authors from Pakistan such as Ahmad et al. (2019) and Maqbool et al. (2009) relied on methods that only allow AFM_1 quantification up to 100 ng/L. Similarly, Younus et al. (2013) who reported median AFM_1 concentration of 333 and 416 ng/L in summer and winter months, respectively, in district Jhang used a method (snap AFM1, by IDEXX) that only allows quantification up to 500 ng/L. Such methods may not be appropriate for accurate quantification of AFM_1 contamination.

Usage of Dairy Feedstuffs

Data regarding share of different feedstuffs in total dry matter (DM) fed to the dairy animals in different cities are presented in **Tables 3, 4**. All feed ingredients were used at different proportions ($p \leq 0.018$) in different cities. The share of cottonseed cake in total DM correlated positively ($r = 0.31$; $p < 0.001$) with the AFM_1 levels in milk in different cities. Number of farms using cottonseed cake were less in the cities with low AFM_1 contamination (Giglit). Statistically, there was a positive association ($r = 0.36$; $p < 0.001$) between incidence of cottonseed cake use and the AFM_1 levels in milk.

The amount of cottonseed cake fed per animal was also positively ($r = 0.31$; $p < 0.001$) associated with AFM_1 contamination of milk (data not shown here). The highest AFM_1 levels were found in milk from Islamabad, Lahore, and Muzaffarabad, where cottonseed cake was the sole oilseed cake used. As cottonseed cake was replaced with other oilseed cakes in other cities, the AFM_1 levels decreased in milk. In this regard, the amount of commercial concentrates fed per animal was negatively correlated ($r = -0.12$; $p = 0.026$) with AFM_1 level of milk. The combined amount fed per animal of Brassica meals, maize oilcake, palm oilcake, and coconut oilcake was also found to have low and negative correlation ($r = -0.14$; $p = 0.008$) with the AFM_1 level of milk.

In case of energy sources, the amount of waste bread fed per animal was found to have positive but low correlation ($r = 0.16$; $p = 0.002$), while amount of wheat and maize grains was found to have negative correlation ($r = -0.20$; $p < 0.001$) with AFM_1 contamination. The share of grains (wheat and maize) in total DM also showed a low but negative ($r = -0.27$; $p < 0.001$) correlation with the AFM_1 levels in milk. Besides grains, the share of wheat bran in total DM also had a low negative correlation ($r = -0.16$; $p = 0.003$) with AFM_1 contamination. Its share was lowest, i.e., 8.0 and 6.0% of DM in the two cities with highest AFM_1 levels while highest, i.e., 19.4% of DM in Gilgit, which had lowest AFM_1 contamination. In case of brans and polish, the share of pulse bran in total DM fed to animals was found to have positive correlation ($r = 0.10$; $p = 0.049$), while rice polish was found to have negative correlation ($r = -0.14$; $p = 0.007$) with AFM_1 in milk.

Overall, significant but low correlations of some feed ingredients were found with AFM_1 contamination of milk, indicating that each ingredient contributed to AFM_1 levels according to its percentage in the total DM fed to the animal. Another interesting feature found from these data was that the more the number of ingredients used in dairy ration formulation, the less was the AFM_1 contamination (except for Gilgit).

Aflatoxin Contamination of Dairy Feedstuffs

Data regarding the aflatoxin contamination of dairy feedstuffs in different cities are presented in **Tables 5, 6**. Bakery waste was the most contaminated feedstuff with an average level of 724.6 μg of aflatoxins/kg. However, only 17 farmers (3.8% of total 448) in Karachi, Quetta, and Peshawar were using bakery waste and that too at an average 0.6 to 1.1% of the total ration's DM. The second highest aflatoxin contamination, 595.9 μg/kg, was recorded in cottonseed cake, which was being used by 64.7% of the farmers and at an average rate of 9.3% of the total DM. In Islamabad, Lahore, Muzaffarabad, Peshawar, and Quetta, the average use of cottonseed cake was 17.0, 8.4, 18.5, 7.9, and 9.2% of the total DM, respectively. In these cities, use of cottonseed cake could explain the variation in AFM_1 levels

TABLE 3 | Percentage (%) of various protein sources in total DM in different cities.

City	Oilseed cakes					Legumes				Mixed concentrate	
	Cotton cake	Brassica cakes	Maize cake	Palm cake	Coconut cake	Pulses	Cowpea	Mung beans	Waste pulses	Comr.	Home mix
Islamabad	16.7^{a}	1.9^{a}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	2.2^{c}	0.0
Lahore	8.1^{b}	0.2^{c}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	1.5^{c}	0.0
Muzaffar.	18.5^{a}	0.0^{c}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	8.3^{a}	0.0
Karachi	1.3^{c}	0.6^{bc}	0.0^{b}	7.7^{a}	0.8^{a}	1.2^{a}	0.0^{b}	0.3^{a}	3.6^{a}	6.7^{a}	3.9^{a}
Peshawar	8.1^{b}	0.3^{c}	2.3^{a}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	6.1^{ab}	0.0
Quetta	9.4^{b}	2.9^{a}	0.0^{b}	0.2^{b}	0.0^{b}	0.5^{b}	1.5^{a}	0.0^{b}	0.4^{b}	0.6^{c}	0.0
Gilgit	0.9^{c}	1.8^{ab}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	0.0^{b}	3.3^{bc}	0.0
Total:	9.27	1.00	0.41	1.17	0.12	0.24	0.18	0.49	0.60	4.03	0.59
SD	9.39	3.32	1.86	3.42	1.02	1.49	1.50	0.53	2.38	8.57	3.89
p	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
n	429	429	429	429	429	429	429	429	429	429	429

Means with different superscripts "^{abc}" differ significantly within a column at $p \leq 0.05$. Muzaffar. = Muzaffarabad; Brassica cakes = mustard oilseed cake, taramira (Eruca sativa) seed cake, or canola meal; Pulses = any out of chick pea, or other Indian pulses; cowpea = local variety of cow pea (matar dana); mong beans = mong kata; waste pulses = mix ati; home mix = pala; Comr. = commercial.

TABLE 4 | Percentage (%) share of various energy sources, brans, and roughages in total dry matter.

City	Energy sources						Polish and brans			Roughages*			Misc. stuffs
	Wheat	Maize	Waste bread	Bakery waste	Dates	Oil	Rice polish	Wheat bran	Pulses bran	Straw	Fodd.	Stov.	
Islamabad	1.8^{cd}	0.1^{c}	15.0^{a}	0.0^{c}	0.1^{b}	0.03^{b}	0.0^{c}	8.0^{de}	2.1^{a}	36.4^{b}	15.7^{c}	0.1^{b}	0.0^{c}
Lahore	2.7^{c}	0.1^{c}	9.5^{b}	0.0^{c}	0.0^{b}	0.0^{b}	0.0^{c}	6.0^{e}	0.1^{b}	17.1^{d}	47.3^{a}	2.8^{a}	1.5^{c}
Muzaffar.	0.4^{d}	0.4^{bc}	2.4^{c}	0.0^{c}	0.0^{b}	0.0^{b}	0.0^{c}	13.6^{bc}	0.0^{b}	44.5^{a}	0.0^{d}	2.1^{ab}	9.8^{b}
Karachi	7.0^{b}	0.9^{b}	3.4^{c}	1.1^{a}	0.3^{a}	0.13^{a}	1.6^{b}	14.0^{b}	0.3^{b}	31.7^{b}	12.7^{c}	0.0^{b}	0.1^{c}
Peshawar	0.5^{d}	0.1^{c}	3.4^{c}	0.6^{b}	0.0^{b}	0.001^{b}	0.0^{c}	11.0^{bc}	0.0^{b}	36.1^{b}	30.9^{b}	0.0^{b}	0.5^{c}
Quetta	2.9^{c}	2.6^{a}	12.7^{a}	0.3^{bc}	0.3^{a}	0.01^{b}	8.2^{a}	10.4^{cd}	0.0^{b}	31.7^{b}	10.7^{c}	0.0^{b}	2.5^{c}
Gilgit	14.3^{a}	0.0^{c}	2.8^{c}	0.0^{bc}	0.0^{b}	0.0^{b}	0.0^{c}	19.4^{a}	0.0^{b}	23.5^{c}	0.0^{d}	3.0^{a}	30.9^{a}
Total	3.63	0.55	7.45	0.30	0.09	0.03	1.22	11.02	0.42	31.37	19.76	1.03	4.61
SD	6.99	2.37	9.48	1.57	0.70	0.16	3.57	10.03	2.17	16.49	21.43	6.88	14.88
p	0.001	0.001	0.001	0.001	0.009	0.001	0.001	0.001	0.001	0.001	0.001	0.018	0.001
n	429	429	429	429	429	429	429	429	429	429	429	429	429

*Means with different superscripts "^{abcd}" differ significantly within a column at $p \leq 0.05$. Muzaffar. = Muzaffarabad; Fodd. = Fodder; Stov. = stover; straw = wheat straw; Pulse bran = sorhi. *Share of grazing from Lahore not included.*

TABLE 5 | Total aflatoxin level (μg/kg) in different protein sources in different cities.

City	Oilseed cakes					Legumes				Mix concentrate	
	Cotton cake	Brassica cakes	Maize cake	Palm cake	Coconut cake	Pulses	Cowpea	Mung beans	Waste pulses	Commr.	Home mix
Islamabad	599.7^{b}	10.7^{b}	—	—	—	—	—	—	—	37.9	—
Lahore	1174.9^{a}	777.9^{a}	—	—	—	—	—	—	—	23.9	—
Muzaff.	397.3^{b}	—	—	—	—	—	—	—	—	30.9	—
Karachi	183.0^{b}	5.7^{b}	—	8.0	4.0	19.3	—	15.1	40.5	28.3	15.6
Peshawar	488.8^{b}	ND	45.9	—	—	—	—	—	—	33.9	—
Quetta	424.7^{b}	7.1^{b}	—	5.0	—	5.7	4.7	—	—	38.4	—
Gilgit*	31.0	8.3^{b}	—	—	—	—	—	—	—	28.3	—
Total	600.84	54.87	45.87	7.68	3.97	15.94	4.75	15.09	40.55	32.68	15.59
STD	627.00	263.33	45.45	10.37	4.22	14.50	1.03	18.27	31.99	25.45	5.97
p	0.001	0.001	—	0.710	—	0.531	—	—	—	0.980	—
n	189	39	14	21	5	4	2	2	8	52	2

*Means with different superscripts "^{ab}" differ significantly within a column at $p \leq 0.01$. ND = not determined due to unavailability of sample; Muzaff = Muzaffarabad; Brassica cakes = mustard oilseed cake, taramira (Eruca sativa) seed cake, or canola meal; Pulses = any out of chick pea, or other Indian pulses; cowpea = local cowpea variety; commr. = commercial.*Only one farmer in Gilgit used cottonseed cake, and therefore Gilgit was not included in post hoc test.*

TABLE 6 | Total aflatoxin level (μg/kg) in different energy sources and roughages.

City	Energy sources			Polish and brans				Roughages		Misc. feedstuffs
	Wheat	Maize	Waste bread	Bakery waste	Rice polish	Wheat bran	Pulses bran	Straw	Stover	
Islamabad	7.4	8.9	46.4	—	—	1.9[b]	49.1	4.4	ND	—
Lahore	34.7	ND	15.5	—	—	2.6[b]	ND	6.0	156.7	351.0[a]
Muzaffar.	—	ND	192.1	—	—	2.3[b]	—	5.0	25.5	32.5
Karachi	21.0	61.6	28.5	31.8	5.3	ND	4.7	ND	—	<LOD
Peshawar	51.5	ND	155.2	1556.1	—	1.8[b]	—	4.6	—	—
Quetta	16.0	27.7	4.6	—	25.4	3.8[b]	—	12.0	—	<LOD
Gilgit	6.4	—	8.0	—	—	6.2[a]	—	—	2.3	5.7[b]
Total	17.48	28.93	61.61	724.63	22.01	3.37	45.36	6.29	66.00	67.93
SD	33.31	49.32	219.74	1957.96	31.56	3.21	34.30	10.74	135.97	184.46
p	0.127	0.773	0.079	0.215	0.101	0.001	0.232	0.595	0.396	0.001
n	57	12	124	11	47	87	12	42	8	29

Means with different superscripts "[ab]" differ significantly within a column at $p \leq 0.01$. Aflatoxin not analyzed in dates, oil, and fodder. Muzaffar. = Muzaffarabad; Pulses bran = sorhi; ND = not determined.

in milk. The share of cottonseed cake in total ration DM in Lahore was only around half of that in Islamabad (**Table 3**) but its contamination level was around double of that in Islamabad. These figures offer reasonable explanation of the comparable milk contamination in Lahore and Islamabad.

Cottonseed cake in Lahore had higher ($p \leq 0.005$) aflatoxin contamination compared to other cities. The other oilseed being used in Lahore was canola meal and it was also found to have higher ($p \leq 0.001$) levels of aflatoxins than in other cities. This trend suggests that the environmental and management conditions in Lahore may not be suitable for storage of oilseed cakes, at least during the study year. Same was true for maize stovers and crushed wheat samples from Lahore as both of these ingredients had higher levels of aflatoxins than in other cities. The other ingredients collected from Lahore such as wheat bran, wheat straw, and waste bread were found to have aflatoxin levels comparable to other cities. Presently reported results regarding aflatoxin contamination of cottonseed cake are in line with our previous report (Yunus et al., 2015). In that study, 556–5,574 μg of total aflatoxins/kg was reported using LC–MS/MS methods. Similarly, Ullah et al. (2016) reported mean aflatoxin B_1 levels in cottonseed cake as 137.1 μg/kg using IAC-HPLC-Flu method. As found by these authors, the levels in cottonseed cake were 11 times higher than the levels in commercial feed for goats, while 24 times higher than the levels in wheat bran.

Present results on aflatoxins in cottonseed cake are however not in line with the study of Hussain (2009) who reported AFB_1 levels in dairy feeds in various cities of Punjab. Using HPLC-Flu methods, he found the AFB_1 levels in cottonseed cake to vary between 11 and 861 μg/kg with a mean of 242 μg/kg. The author reported AFB_1 levels in dairy concentrate, wheat bran, and waste bread to be 176.3, 98.4, and 23.4 μg/kg, respectively. In the present study, the levels of aflatoxins in cottonseed cake are higher than found in the study of Hussain (2009) but the overall trend is similar. The levels of aflatoxins in concentrate mixes were lower in the present study, which could be an indication of improved quality control by feed manufacturers in the recent years.

Among all the tested ingredients, wheat bran was found to be the safest regarding aflatoxin contamination. Wheat bran in Gilgit was found to have 8.6 μg/kg average aflatoxin level, which was higher ($p \leq 0.027$) than other cities. However, these levels are still within the acceptable limit of 20 μg/kg for any dairy feed ingredient. These results are not consistent with the earlier reports in which wheat bran was reported to have higher levels of mycotoxins than wheat grains (Bandara et al., 1991; Schatzmayr and Streit, 2013). It may be possible that the source of wheat bran in the present study was flour mills that produce it for human consumption and therefore might use better quality grains. The wheat grains used by farmers for dairy animal feeding are on the other hand usually from the produce that is not bought by the government due to quality issues.

The other safer feedstuffs were legumes/pulses, i.e., mung beans, cow peas (*matar dana*), and miscellaneous pulses (including chick pea etc). Contrary to these, the waste pulses (*mix ati*) used specifically for dairy animals in Karachi were found to have high levels of aflatoxins. The reason could once again be the quality and intended use of the feedstuff. While, the mung beans and other miscellaneous pulses are basically intended for human use, the pulse waste comprises of the leftover portions of various legumes/pulses and is rated not-fit for human consumption. This includes damaged legume grains and fiber portions of the legumes, besides frequent contamination with soil.

It is a general opinion of the farming community and the field veterinary officers that maize oilcake is safe for dairy animal feeding, and has very low toxin content. In the present study, maize oilcake was found to have lower levels of aflatoxins compared to cottonseed cake, but these levels were still not safe for dairy animal feeding, i.e., almost double than the allowed limit of 20 μg/kg. The palm and coconut oilcakes were however found to be good choices for dairy feeding. These are imported from Malaysia and are only used in Karachi for dairy feeding. It is therefore recommended that these two ingredients are introduced in

other cities as partial replacement of cottonseed cake. Other oilseed meals/cakes like soybean can be included in rations to improve the quality.

Crushed maize grains were found to be used only by the farmers in Karachi, Islamabad, and Quetta. Except for Karachi and few contaminated samples in Quetta, maize grains were found to be within the safe limits. Not much samples could be collected from Karachi and therefore the data regarding Karachi may not represent the true picture in the city.

On-Farm Storage of Feedstuffs

The data regarding on-farm storage of various feedstuffs in different cities are presented in **Table 7**. Significant differences in on-farm storage times were noted for oilseed cakes, mixed concentrates, waste bread, wheat bran, and wheat straw. In general, the storage time was least in case of Peshawar while longest in case of Gilgit. The longer storage duration in Gilgit could be due to closure of roads in winter in the region.

Despite the differences in on-farm storage time, association between the length of storage and aflatoxin levels in feedstuffs could not be established. The only case of positive association was of pulse bran, for which a longer on-farm storage in Islamabad compared to Karachi was associated with higher aflatoxin levels. The number of observations in this feedstuff were not enough to explore within city variations.

Longer storage under conducive conditions is positively correlated with aflatoxin development in foodstuffs (Achaglinkame et al., 2017). In this regard, relative humidity (Giorni et al., 2012; Pratiwi et al., 2015) and temperature (Schindler et al., 1967) during storage are important factors in affecting aflatoxin development. Maximum aflatoxin production occurs at 28–30°C (OBrian et al., 2007), and it ceases when temperature drops below 18°C (Schindler et al., 1967). In the present study, the lack of positive associations between storage length and aflatoxin contamination levels in feedstuffs could be because we collected samples in winter months. In particular, the cold weather of the Himalayan region (Gilgit

TABLE 7 | On-farm storage time (days) of different feedstuffs in different cities.

City	Cotton cake	Brassica cakes	Misc. Cakes[1]	Pulses[2]	Waste pulses[3]	Conc.	Grains[4]	Waste bread	Wheat bran	Pulse bran	Wheat Straw	Stover
Islamabad	17.8^{c}	20.4^{b}	—	—	—	23.0^{a}	21.9^{b}	18.5^{a}	18.9^{b}	17.5	25.9^{cd}	20.0
Lahore	4.6^{d}	1.0^{b}	—	—	—	13.0^{bc}	17.9^{ab}	1.3^{c}	5.0^{e}	—	54.9^{b}	1.0
Muzaff.	9.1^{cd}	—	—	20.0	—	8.8^{c}	23.7^{ab}	7.0^{bc}	8.3^{de}	—	44.1^{bc}	10.0
Karachi	15.7^{bcd}	14.3^{b}	11.2^{b}	9.2	8.9^{b}	7.6^{c}	10.7^{b}	5.6^{c}	10.1^{d}	9.7	12.1^{d}	—
Peshawar	4.6^{d}	6.7^{b}	6.9^{b}	—	—	7.7^{c}	28.2^{ab}	2.6^{c}	5.5^{e}	—	15.4^{d}	—
Quetta	33.2^{ab}	20.4^{b}	53.3^{a}	36.1	45.0^{a}	14.4^{ac}	19.2^{b}	10.9^{b}	14.6^{c}	—	26.5^{c}	—
Gilgit	—	102.9^{a}	—	—	—	16.8^{ab}	54.2^{a}	4.7^{c}	23.5^{a}	—	115.8^{a}	70.0
Total:	13.82	30.45	11.98	26.69	14.72	10.95	23.99	9.29	11.40	15.69	33.78	42.44
SD	33.60	60.93	15.37	28.83	17.48	9.88	49.93	10.21	11.14	8.60	54.13	55.44
p	0.001	0.019	0.000	0.240	0.000	0.000	0.044	0.000	0.000	0.176	0.000	0.492
n	245	40	63	16	25	100	124	167	305	13	324	9

Means with different superscripts "[abcde]" differ significantly within a column at $p < 0.05$. Muzaff. = Muzaffarabad; Brassica cakes = mustard oilseed cake, taramira (Eruca sativa) seed cake, or canola meal; Conc. = Mixed commercial concentrates. [1]Includes maize, palm, and coconut oilcakes; [2]Includes Mung beans, cowpeas, or other single pulses; [3]Includes maize ad wheat grains.

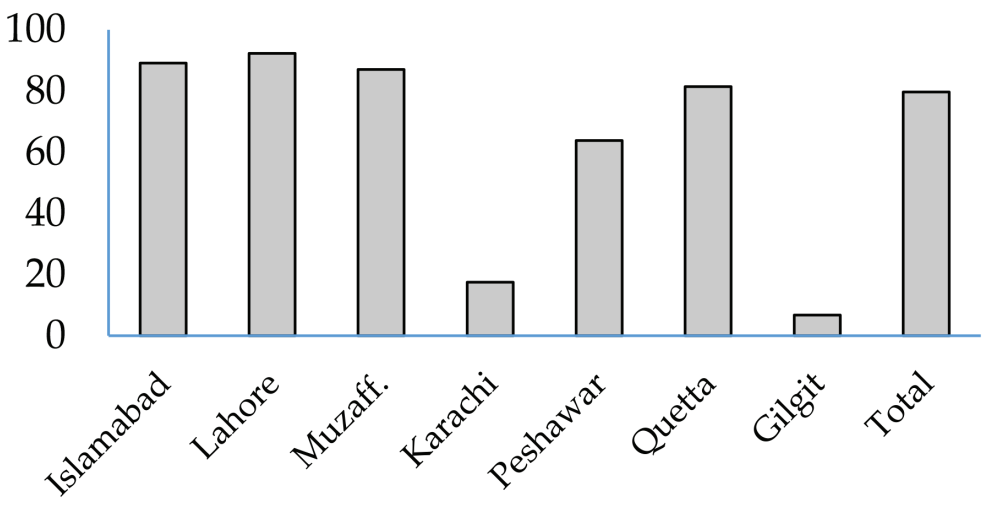

FIGURE 2 | Share of aflatoxins from cottonseed cake in daily exposure to total aflatoxins in dairy animals in different cities.

and Hunza valley) explains the lack of effects of even more than 100 days of storage. In case of Quetta, where some feedstuffs were stored for over 1 month, the dry weather of the city seems to play a preventive role in aflatoxin development. The present results indicate that the storage time and conditions in the commercial feed stores might be more relevant in studying aflatoxin development in feedstuffs and should be included in future studies.

Share of Feedstuffs in Aflatoxin M_1

Share of different feedstuffs in total daily aflatoxin exposure of animals in different cities were calculated. Overall, 79.7% of the aflatoxins consumed by dairy animals was coming from cottonseed cake. Cottonseed cake contributed 81–92% of daily aflatoxin exposure of animals in the peri-urban farms located in Islamabad, Lahore, Muzaffarabad, and Quetta (**Figure 2**). The share of cottonseed cake in cities of Karachi and Peshawar was 20 and 64%, respectively. In case of Peshawar, 21% of total daily aflatoxin consumed by animals was contributed by bakery waste. Cottonseed cake waste and bakery waste were together responsible for 90.2% of the daily aflatoxin exposure of animals in Peshawar. The aflatoxin exposure of dairy animals in the city of Karachi was from many feed ingredients including grains (19%), waste pulses (13%), and waste bread (8%).

CONCLUSIONS

It may be concluded from the present results that the AFM_1 levels in milk produced in peri-urban dairy farms, except in Gilgit, are exceptionally high. Around 80% of the AFM_1 in milk was found to be contributed by cottonseed cake in dairy rations. The high milk contamination can therefore be reduced by replacing cottonseed cake with feedstuffs lower in aflatoxin contamination such as canola meal, and commercial concentrate feeds. Long-term mitigation strategies should focus on reducing aflatoxin contamination in cottonseed cake and discouraging use of bakery waste as dairy animal feed.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AY and MI contributed to conceptualization, visualization, funding acquisition, and supervision. AY, AmU, and JL contributed to methodology. AY contributed to questionnaire formulation, data curation, project administration, and original draft preparation. AY, MI, AmU, ZA, AtU, SS, AZ, HI, SJ, ZF, AH, BA, ZB, and IK contributed to sample collection. AY, NI, and ZA contributed to method validation. AY, ZA, AtU, MA, UF, and AA contributed to laboratory analysis. AY, AmU, and JL contributed to writing-review and editing.

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Alternative Oxidase: A Potential Target for Controlling Aflatoxin Contamination and Propagation of *Aspergillus flavus*

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Aflatoxins are among the most hazardous natural cereal contaminants. These mycotoxins are produced by *Aspergillus* spp. as polyketide secondary metabolites. Aflatoxigenic fungi including *A. flavus* express the alternative oxidase (AOX), which introduces a branch in the cytochrome-based electron transfer chain by coupling ubiquinol oxidation directly with the reduction of O_2 to H_2O. AOX is closely associated with fungal pathogenesis, morphogenesis, stress signaling, and drug resistance and, as recently reported, affects the production of mycotoxins such as sterigmatocystin, the penultimate intermediate in aflatoxin B_1 biosynthesis. Thus, AOX might be considered a target for controlling the propagation of and aflatoxin contamination by *A. flavus*. Hence, this review summarizes the current understanding of fungal AOX and the alternative respiration pathway and the development and potential applications of AOX inhibitors. This review indicates that AOX inhibitors, either alone or in combination with current antifungal agents, are potentially applicable for developing novel, effective antifungal strategies. However, considering the conservation of AOX in fungal and plant cells, a deeper understanding of fungal alternative respiration and fungal AOX structure is needed, along with effective fungal-specific AOX inhibitors.

Keywords: aflatoxin, alternative oxidase, mitochondria, respiration, antiaflatoxigenic activity

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INTRODUCTION

Aflatoxin contamination is a food safety concern worldwide, affecting both the marketability and safety of multiple food crops such as maize, peanuts, and tree nuts (Kumar et al., 2016). Aflatoxins are primarily produced by *Aspergillus* spp., including *A. flavus*, as polyketide secondary metabolites. These opportunistic fungi are commonly detected as contaminants in cereal crops at both the pre- and post-harvest stages (Umesha et al., 2017). Although aflatoxigenic fungi more commonly grow in tropical and sub-tropical climates, aflatoxin contamination has always been a global concern owing to globalized trade; moreover, zones with a perennial aflatoxin contamination risk have expanded owing to climate change (Marroquín-Cardona et al., 2014; Baranyi et al., 2015). Current methods

of preventing aflatoxin contamination in food cereals primarily depend on the continued application of synthetic fungicides, which, although effective, target a limited number of cellular phenomena and also have side effects such as toxicity among humans and other animals, environmental pollution, and development of resistance in phytopathogens (Panáček et al., 2009). Therefore, new antifungal and antiaflatoxigenic strategies are urgently needed.

The respiratory chain is an effective target for fungicides to control fungal contamination in food crops. The presence of fungal-specific respiration components and the recent discovery of the association between respiration and pathogenesis in several phytopathogenic species have fostered the development of new mitochondria-targeted fungicides. However, the emergence of rapid resistance to currently used inhibitors, toxicity concerns raised from the conservation of the respiratory machinery in eukaryotes, and the limited understanding of the physiological roles of mitochondria have largely deterred the application of respiration inhibitors as fungicides.

The alternative oxidase (AOX) is an integral monotopic membrane protein localized on the matrix side of the inner mitochondrial membrane (**Figure 1**). The enzyme is ubiquitous in the plant kingdom and is present in numerous pathogenic and agriculturally important fungi including *Aspergillus* spp., *Candida* spp., *Hansenula* spp., and *Magnaporthe* spp. (Joseph-Horne et al., 2001; Tudella et al., 2004). AOX introduces a branch in the cytochrome-based electron transfer chain by coupling ubiquinol oxidation directly with the reduction of O_2 to H_2O. Consequently, fewer protons migrate across the inner mitochondrial membrane, generating a proton gradient, leading to markedly lesser ATP production through oxidative phosphorylation (Joseph-Horne et al., 2001; Young et al., 2013). AOX activity is associated with reactive oxygen species (ROS) control, metabolic homeostasis, cellular energy demand, the redox state, and the stress response (Li et al., 2011; Vanlerberghe, 2013). Furthermore, AOX affects mycotoxin production, such as sterigmatocystin, which is the penultimate intermediate in aflatoxin B_1 biosynthesis (Leiter et al., 2016; Molnár et al., 2018a). As AOX is absent in mammals, it has been investigated as a potential drug target for pathogenic fungi (Tudella et al., 2004; Ebiloma et al., 2019). Genomic sequence analyses have predicted the presence of at least one AOX in each complete *Aspergillus* genome sequence including *A. clavatus*, *A. flavus*, *A. fumigatus*, *A. nidulans*, and *A. niger* (**Table 1**). AOX exhibits high levels of conservation and genome synteny across *Aspergillus* spp. (Li et al., 2011).

Although AOX is a promising target for the development of novel antifungal strategies, further studies are required to understand the physiological function of AOX and its association with fungal pathogenesis, morphogenesis, stress signaling, drug resistance, environment adaption, and secondary metabolism. Hence, this review addresses these issues by summarizing the current understanding of AOX and the alternative respiration pathway in pathogenic and agriculturally important fungi, and progress in studies on AOX inhibitors as antifungal agents.

AOX AND FUNGAL VIRULENCE

Similar to other facultative parasites, the virulence of *A. flavus* is multifactorial and is closely connected with the cellular development, secondary metabolism, adaption to stress conditions, and interaction with host defense molecules (Amaike and Keller, 2011). An intact and functional electron transport system is important for fungal virulence (Chatre and Ricchetti, 2014). Numerous pathogenic fungi depend on oxidative phosphorylation for virulence. Respiratory activity is crucial for energy generation and for adaptation to the host environment. For example, through the life cycle of phytopathogen *Moniliophthora perniciosa*, the causal agent of the witches' broom disease of cocoa, AOX is overexpressed in the biotrophic phase, when live cocoa cells produce large amounts of nitric oxide, which serve as respiration inhibitors. Ruiz et al. (2011) reported that in *Paracoccidioides brasiliensis*, AOX is important in the fungal defense against oxidative stress imposed by immune cells and is relevant to the virulence of this human pathogenic fungus. Akhter et al. (2003) reported that AOX contributes to the virulence composite of *Cryptococcus neoformans*, potentially by improving survival within phagocytic cells. Furthermore, defects in the electron transport system in *C. albicans* affect the hyphal morphological switch, an important determinant of virulence (McDonough et al., 2002; Khamooshi et al., 2014).

Furthermore, the role of AOX in stress signaling potentially contributes to the survival of fungal pathogens in the host environment. AOX is suggested to contribute to the regulation of the ROS balance, maintenance of the redox state, and response to various types of stress (Millenaar and Lambers, 2008). Oxidative stress specifically inhibits some key metabolic enzymes including glyceraldehyde-3-phosphate dehydrogenase (Li et al., 2011) and aconitase (Murakami and Yoshino, 1997), which are essential for fungal primary metabolism. Owing to the non-proton pumping nature of AOX, the fungal alternative respiration pathway is suggested to reduce ROS generation. AOX is apparently induced under oxidative stress to minimize the negative effects caused by excess ROS; however, this in turn led to major metabolic changes in fungal cells upon a reduction in the ATP supply.

Fungal respiration and virulence may also be associated with cellular remodeling. For example, Grahl et al. (2015) reported that in *C. albicans*, the disease-associated morphological switch from yeast to hyphal growth is affected by mitochondrial functions, as mitochondrial inhibitors potently suppress the Ras1-Cyr1-PKA pathway, a major regulator of *C. albicans* morphogenesis, biofilm formation, and white-opaque switching. Furthermore, Silao et al. (2019) reported that induced respiration is critical for morphogenesis during the catabolism of morphogenic amino acids, which is an important feature for *C. albicans* to evade macrophages. *C. albicans* cells are highly adaptive to the inhibition of classical respiration; however, a recent study reported that a combination of AOX inhibitor salicylhydroxamic acid (SHAM) and sodium nitroprusside lead to fitness defects and the loss of viability in *C. albicans* (Duvenage et al., 2019). The thickness of the outer cell wall was reportedly thinner than that of untreated cells; however, no significant changes were

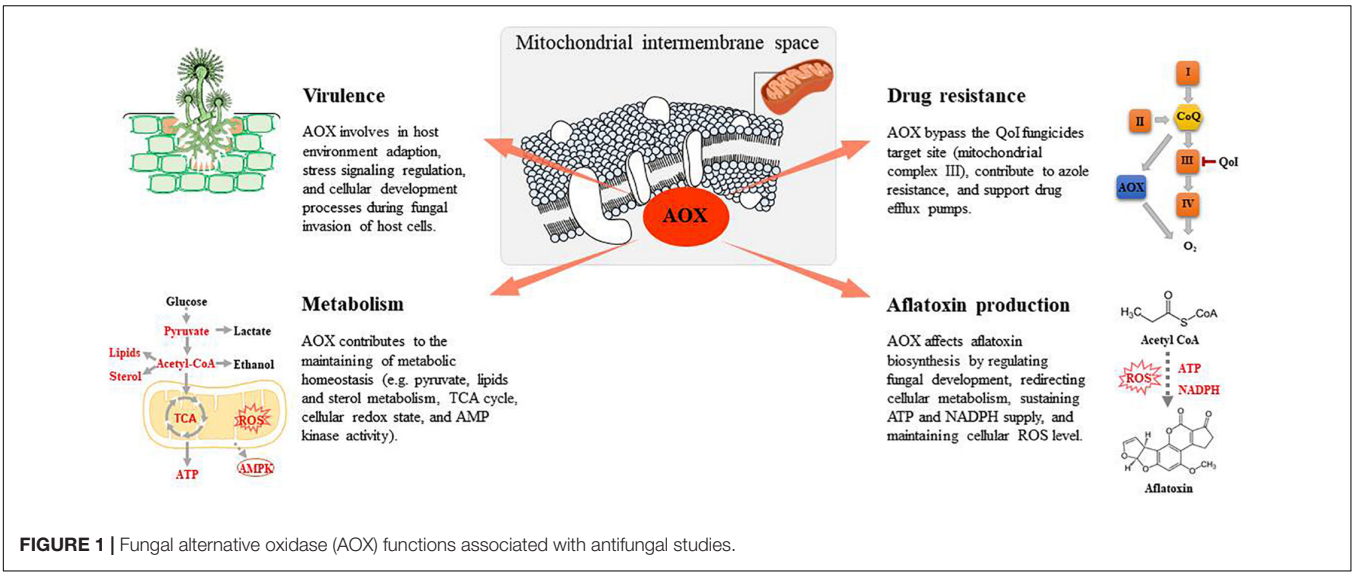

FIGURE 1 | Fungal alternative oxidase (AOX) functions associated with antifungal studies.

TABLE 1 | The existence and reported functions of alternative oxidase (AOX) in *Aspergillus spp.*

Species	Number of AOX	GenBank accession number	Reported functions	References
Aspergillus bombycis	3	XM_022535135, XM_022530731, XM_022527953	No report	–[1)]
A. caelatus	3	XM_032076675, XM_032072264, XM_032065812	No report	–
A. clavatus	2	XM_001273619, XM_001272692	No report	–
A. flavus	2	XM_002377259, XM_002374976	No report	–
A. fumigatus	1	XM_744544	Oxidative stress resistance; oxygen uptake; pathogenesis; macrophage susceptibility.	Magnani et al., 2007; Grahl et al., 2012
A. nidulans	1	XM_654611	Sterigmatocystin production regulation; oxygen uptake; conidiospores production and conidia viability.	Leiter et al., 2016; Molnár et al., 2018a
A. niger	2	XM_001394435, XM_001394775	Heat, oxidative and osmotic stress response; metabolic flow regulation; energy metabolism.	Honda et al., 2012; Hou et al., 2018
A. nomius	2	XM_015556206, XM_015548398	No report	–
A. oryzae	2	XM_001819459, XM_023232744	No report	–
A. terreus	2	XM_001216061, XM_001215177	Oxygen uptake; limiting ROS generation; defense against oxidative stress; metabolic flow regulation.	Pérez-Sánchez et al., 2017; Molnár et al., 2018b

[1)] *Not applicable.*

observed in the relative levels of cell wall components including chitin, glucan, or mannan, suggesting that the inhibition of classical and alternative modes of respiration in *C. albicans* lead to organizational changes rather than the relative levels of cell wall components.

AOX AND DRUG RESISTANCE

The respiratory chain is an effective target for fungicides to control fungal contamination in food crops. Quinone outside inhibiting (QoI) fungicides represent the most important group of fungicides developed on the basis of mitochondrial inhibition (Bartlett et al., 2002). QoI fungicides inhibit fungal pathogens by blocking the transfer of electrons at the quinone outer binding site of the mitochondrial complex III. Development of resistance to QoI fungicides in fungal cells is a growing issue (Fernández-Ortuño et al., 2008). The acquisition of QoI resistance among numerous pathogens results from mutations in their cytochrome *b* or cytochrome *c* genes (Sierotzki et al., 2000). However, the most damaging emerging resistance to QoI fungicides is associated with AOX, which offers fungal

cells the alternative respiration pathway that can bypass the target site for QoI fungicides. AOX leads to the flow of excess electrons when the cytochrome respiratory chain is inhibited or saturated, thus increasing the metabolic flexibility of fungal cells when exposed to biotic or abiotic stress potentially limiting the activity of the respiratory pathway (Juarez et al., 2006; Xu et al., 2012).

Along with ATP production, fungal mitochondrial function is associated with other important cellular functions including ergosterol biosynthesis and cell wall maintenance (Dagley et al., 2011). Specific inhibitors of fungal respiratory metabolism can reverse azole resistance (Vincent et al., 2016) and increase the sensitivity to fluconazole in *C. albicans* (Guo et al., 2014). Furthermore, AOX potentially contributes to fluconazole resistance in *C. albicans*, as combinatorial treatment with SHAM and fluconazole resulted in synergistic antifungal activity (Yan et al., 2009). Decreased ATP production may inhibit the activity of drug efflux pumps, thus decreasing drug resistance among fungal cells.

AOX AND AFLATOXIN PRODUCTION

Current methods of controlling aflatoxin contamination in food primarily depend on chemical and physical approaches usually focused on inhibiting the development of spores and mycelia, and/or inactivation of aflatoxins by their transformation to non-toxic compounds. Commonly used methods include the use of synthetic fungicides, irradiation, ozone fumigation, dehulling or cooking processes, regulation of environmental factors during harvest and storage, and the introduction of non-aflatoxin-producing *A. flavus* into the field to compete with the naturally occurring aflatoxin-producing strains (Ehrlich et al., 2014; Udomkun et al., 2017). These strategies are usually expensive, time-consuming, and inefficient, and some of them majorly alter the physical properties of food and cause serious loss of nutritive value; therefore, they are inappropriate for the elimination of aflatoxins from food. Synthetic fungicides are still the most widely used recourse to prevent fungal contamination of food crops. However, in addition to strict regulations regarding the use of synthetic compounds in food, the application of synthetic fungicides may result in notable drug resistance and serious environmental and health issues (Panáček et al., 2009). Awareness of these issues has led to an urgent need to develop novel antifungal and antiaflatoxigenic strategies.

Despite a dearth of knowledge of how AOX affects aflatoxin biosynthesis, AOX activity seems to affect sterigmatocystin, the penultimate intermediate in the biosynthesis of aflatoxin B_1. Molnár et al. (2018a) investigated the association between AOX and sterigmatocystin synthesis in *A. nidulans* by both deleting and overexpressing the gene encoding AOX. Compared with the wild-type, the overexpressing mutant produced up to 70% more sterigmatocystin and the deletion mutant produced 50% less sterigmatocystin when grown in the dark. However, when the cultures were illuminated, sterigmatocystin productions were greatly reduced and exhibited no significant difference among the wild-type and the mutants. However, Leiter et al. (2016) reported that both the deletion and overexpression of AOX in *A. nidulans* negatively affected sterigmatocystin production. Nevertheless, these observations clearly indicate the importance of AOX in the regulation of sterigmatocystin and aflatoxin production.

Alternative oxidase potentially affects aflatoxin biosynthesis through the following different mechanism: (1) regulating fungal development; (2) redirecting cellular metabolism; (3) sustaining ATP and NADPH supply; and (4) maintaining cellular ROS levels. Most fungal secondary metabolites, including aflatoxin, are produced after the fungus completes its initial growth phase and is beginning a stage of development, represented by sporulation (Calvo et al., 2002; Amare and Keller, 2014). AOX is suggested to contribute to fungal physiology, morphology, and development (Osiewacz, 2011). AOX inhibitors have reportedly prevented *M. perniciosa*, *M. grisea*, and *Botrytis cinerea* spore germination *in vitro* (Inoue et al., 2012; Barsottini et al., 2019), suggesting that AOX activity is a common feature needed for spore germination-related pathways in these fungi. A previous study reported that on initiation of aflatoxin production, the external carbon source was greatly consumed, thus resulting in aflatoxin biosynthesis from the breakdown of reserve carbon sources such as lipids and fatty acids (Molnár et al., 2018a). AOX overexpression in *Podospora anserine* increased fatty acid production and decreased 2-oxoglutarate concentrations, suggesting a redirection in cellular metabolism (Bovier et al., 2014). It was hence suggested that AOX is accessory for increased aflatoxin production fueled by reserve lipids in the late stationary phase of growth. Meanwhile, it has been suggested that lipid molecules were crucial signals affecting the interaction between plant cells and mycotoxigenic fungi (Christensen and Kolomiets, 2011; Giorni et al., 2015). Studies on *A. flavus* have revealed the important role of oxylipins in the regulation of aflatoxin biosynthesis, conidia production, and sclerotia formation (Amaike and Keller, 2011; Scarpari et al., 2014). Thus, AOX, by modulating lipid metabolism, may also play an important role in regulating these fungal activities. Furthermore, aflatoxin synthesis requires considerable amounts of ATP and NADPH (Yabe and Nakajima, 2004). However, after the initial growth phase, the energy demand of fungal cells is reduced and carbon catabolism is inhibited through oxidative phosphorylation (Cárdenas-Monroy et al., 2017; Molnár et al., 2018a). To maintain cellular homeostasis, fungal cells may rely on alternative respiratory pathways for reoxidation of NADH for aflatoxin synthesis without concomitant ATP production. In addition, AOX activity may also affect aflatoxin production via a ROS-related mechanism. Previous studies have shown that aflatoxin biosynthesis implies a boost in oxygen uptake followed by an increase of ROS generation. This change occurred at the turning point between trophophase and idiophase when different secondary metabolites began to be prevalently produced (Zaccaria et al., 2015). AOX was also found to affect oxygen uptake in *Aspergillus* spp. (**Table 1**). It has been reported in many studies that ROS-induced oxidative stress stimulates aflatoxin production (Reverberi et al., 2008; Roze et al., 2015; Fountain et al., 2016; Umesha et al., 2017).

The presence of multiple cytochrome p450 monooxygenases and monooxygenases in the aflatoxin biosynthesis pathway suggest the occurrence of both oxygen consumption and ROS production in this system.

AOX INHIBITORS AND THEIR APPLICATION

Although alternative respiration produces markedly lesser ATP and appears dispensable for virulence in some fungal pathogens (Grahl et al., 2015), it maintains respiration and essential metabolic functions of the mitochondria in fungal cells when the classical electron transport chain is inhibited, thus enhancing fungal growth and viability. Therefore, a combination of classical and alternative respiration inhibitors is potentially the most effective strategy to control fungal contamination and limit the development of resistance (Duvenage et al., 2019). AOX inhibitors potentially exert synergistic antifungal effects with classical respiration inhibitors and other antifungal agents, which induce oxidative stress. However, owing to the lack of highly effective and fungal-specific AOX inhibitors, such a combination has not yet been tested *in vivo*.

Alternative oxidase of human parasite *Trypanosoma brucei* is currently the only alternative oxidase protein structure available (Shiba et al., 2013). AOX inhibitors appear promising for treating trypanosomiasis. The discovery of antibiotic ascofuranone and the optimization of existing inhibitors were indeed stronger AOX inhibitors and controlled trypanosome infections at very low doses (Ott et al., 2006; Ebiloma et al., 2019). The development of effective AOX inhibitors has received increasing interest. Unfortunately, owing to their low efficiency and selectivity, no advanced AOX inhibitors for practical, clinical and agricultural application have been reported. Our current understanding regarding the biological connections between AOX and the classical respiration of fungal cell has raised concerns that specific inhibitors of AOX may limit effects on the mitochondrial respiration, and thus cannot inhibit fungal propagation efficiently. However, Barsottini et al. (2019) reported a novel AOX inhibitor of optimized N-phenylbenzamide derivative that has strong AOX inhibitory effects, potentially prevents spore germination in the phytopathogen *M. perniciosa in vitro* and alleviates witches' broom disease in infected plants. It suggests that further studies on the structure and physiological activity of AOX in fungal cells and the structure-activity relationship of current AOX inhibitors will surely promote the development of effective fungal AOX inhibitors to control fungal propagation. Besides, fungal mitochondrial function is associated with other important cellular functions including ergosterol biosynthesis and cell wall maintenance (Dagley et al., 2011), suggesting that respiration inhibitors potentially enhance the effects of current fungicides targeting those cellular phenomena. AOX is suggested to contribute to fluconazole resistance in *C. albicans*, as combinatorial treatment with SHAM and fluconazole displayed synergistic antifungal activity (Yan et al., 2009). The links between fungal cell walls and respiration are currently unclear. Nevertheless, combinatorial treatment with AOX and complex III inhibitors enhances the susceptibility of *C. parapsilosis* to caspofungin, a fungicide functioning by inhibiting β-D-glucan synthase (Chamilos et al., 2006). Furthermore, Duvenage et al. (2019) reported that the inhibition of classical and alternative respiration in *C. albicans* lead to changes in cell wall organization; however, the authors reported decreased susceptibility to caspofungin, highlighting the requirement for a deeper understanding of the physiological functions of fungal AOX and fungal mitochondria. Nevertheless, these studies indicated that AOX inhibitors, though they may be not efficient antifungal agents when working alone, still can contribute to fungal inhibition by working with current antifungal agents.

Another concern regarding the conservation of the respiratory machinery in pathogenic fungi and plants has also deterred the application of AOX inhibitors, since these inhibitors also act on plant AOX potentially and yield unexpected results. AOX is applicable in developmental plasticity and is associated with yield stability of food crops (Selinski et al., 2018; Barsottini et al., 2019). During soybean and cocklebur seed germination, AOX is associated with germination initiation, seedling growth, and chlorophyll synthesis. The inhibitory effect of SHAM on rooting has been observed among olives (Macedo et al., 2012; Porfirio et al., 2016). This highlights the requirement for AOX inhibitors that selectively act on fungal pathogens without disrupting normal plant activity, if AOX inhibitors are to be developed as successful antifungal agents to secure food production. Barsottini et al. (2019) presented the first study on the development of novel fungal AOX inhibitors and reported that an N-Phenylbenzamide derivative is more potent and selective than SHAM and inhibits *M. perniciosa* spore germination and prevents the appearance of the symptoms of witches' broom disease in infected plants without obvious effects on the plant itself. This study suggests the possibility of developing AOX inhibitors acting specifically on fungal cells. Further studies on the structure and physiological differences of AOX in fungal and plant cells will surely promote the development of novel fungal-specific AOX inhibitors. Another strategy to ease this concern is to use natural compounds of vegetal origin, such as flavone, quercetin, resveratrol, and curcumin, which can potentially inhibit fungal respiration. These natural compounds modulate mitochondrial function through different methods including the inhibition of mitochondrial enzymes, suppression of oxidative phosphorylation, and alteration of the mitochondrial redox balance (Basile et al., 2009; Gibellini et al., 2010). In *Pichia stipitis*, and *M. grisea*, AOX induction by QoI fungicide was suppressed by flavonoid components (Wood and Hollomon, 2003). In *B. cinerea*, flavone reportedly inhibited respiration in whole cells treated with potassium cyanide. It is reasonable to expect that some plant-based natural compounds may inhibit fungal AOX without affecting plant cells. Alternatively, plant-based inhibitors of the classical respiration complexes might function synergistically

with synthetic AOX inhibitors, thus effectively inhibiting fungal respiration with minimal or no impact on plant cells.

CONCLUSION

In conclusion, fungal virulence composite AOX is potentially a suitable target owing to its association with pathogenesis, morphogenesis, environment adaption, fungicide resistance, cell wall regulation, lipid metabolism, and probably mycotoxin metabolism in studies on aflatoxin contamination and the propagation of *A. flavus*. AOX inhibitors, either alone or along with current antifungal agents, are potentially applicable for developing novel effective antifungal strategies. However, the application of AOX inhibitors in food production is currently limited by the low efficiency and selectivity, and concerns raised from the conservation of AOX in fungal and plant cells. To overcome these limitations, a deeper understanding of fungal alternative respiration and fungal AOX structure, and screening of effective fungal-specific AOX inhibitors are required.

AUTHOR CONTRIBUTIONS

FT, SL, and SW curated all references. FT drafted the manuscript. HC revised and finalized the manuscript. All authors read and approved the final version of the manuscript.

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Prevalence and Exposure Assessment of Aflatoxins Through Black Tea Consumption in the Multan City of Pakistan and the Impact of Tea Making Process on Aflatoxins

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Aflatoxins are the highly toxic secondary metabolites of certain fungi, being mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are classified as group 1 category carcinogens by the International Agency for Research on Cancer (IARC). A large number of food commodities are reported to be contaminated with aflatoxins. Tea is the world's second most consumed beverage and the consumption of tea is increasing day by day. Besides being a source of several health promoting substances, tea leaves are also reported to be contaminated with aflatoxins. However, not a single study is reported from Pakistan regarding the level of aflatoxins in commercially available black tea samples. The current study aimed to quantify the level of aflatoxins in commercially available branded and non-branded black tea samples. The estimated daily intake (EDI) of aflatoxins through branded and non-branded black tea consumption and the health risk assessment based on margin of exposure (MOE) approach was assessed. Furthermore, the impact of local tea making processes on the concentration of aflatoxins in tea beverage (filtrate) was also investigated.

Keywords: aflatoxins, tea, health, cancer, estimated daily intake, beverage

INTRODUCTION

Tea, the world's second most commonly consumed beverage after water is produced by the infusion of *Camellia sinensis* leaves that are native to Southeast Asia. China, India, Kenya, Sri Lanka, Vietnam, Turkey, and Indonesia are the leading tea producers (Heck and de Mejia, 2009). The world's total tea production is estimated to rise from 2.9 million tons in 1994 to 6.1 million tons in the year 2017. The annual consumption of tea is estimated to be around 273 billion liters and is forecasted to rise to 297 billion liters by the year 2021 (FAOSTAT, 2017; Statista, 2019). Depending on the degree of fermentation, tea is generally categorized into three major categories, i.e., black, green, and oolong. Black tea is the most commonly consumed tea worldwide and estimated to be around 78% of total tea produced (Soni et al., 2015). Black tea is a rich dietary source of polyphenols such as theaflavins, catechins, and thearubigin, which have the

potential to influence the pathogenesis of several chronic disorders owing to their antioxidant, anti-proliferative, anti-inflammatory, antiviral, antibacterial, anti-mutagenic, cardio-protective, and neuroprotective effects (Da Silva Pinto, 2013). Moreover, black tea is also reported to contain appreciable quantities of some essential micronutrients such as fluoride, copper, and manganese (Zhang et al., 2018b). However, despite being a source of several health-promoting components, tea samples are also reported to be contaminated by a number of toxicants, the most critical among which are the mycotoxins, especially aflatoxins (Viswanath et al., 2012; Pouretedal and Mazaheri, 2013).

Mycotoxins are the secondary metabolites of certain species of fungi that may infect the crops intended for human consumption or use as animal feed. More than 450 different types of mycotoxins are reported to date, but the most toxic among all mycotoxins are aflatoxins. The major producers of aflatoxins are of the genus *Aspergillus*, primarily *Aspergillus flavus* and *Aspergillus parasiticus*. More than 17 different types of aflatoxins are reported, the most toxic as well as the most reported in the food and feed items are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2). The sum of these four naturally existing aflatoxins is collectively known as total aflatoxins (TAF). Aflatoxins are reported to be hepatotoxic, teratogenic, mutagenic, neurotoxic, immuno-retardant, and growth retardant (Pallarés et al., 2017; Sedova et al., 2018). The International Agency for Research on Cancer (IARC) has declared both AFB_1 and the TAF as group 1 category carcinogens (IARC, 2012). Based on the toxicity of aflatoxins, most of the countries around the world have established their own maximum permissible limits (MPLs) for aflatoxins in various foodstuffs ranging between 0.05 and 100 μg/kg (Ismail et al., 2018).

The warm and wet climate that is suitable for tea cultivation is also favorable for the growth of aflatoxin producing fungi and the production of their metabolites. Drying of tea leaves is usually performed above 80°C that is also sufficient to decrease the fungal load; however, the already produced aflatoxins will remain as heating is ineffective for the degradation of aflatoxins (Viswanath et al., 2012). Furthermore, the recontamination of aflatoxin producing fungi may occur due to improper handling, storage, packaging, and transportation (Sedova et al., 2018). Aflatoxins in tea are rarely regulated in most countries. The Custom Union countries (Armenia, Belarus, Kazakhstan, Kyrgyzstan, and Russia) have established an MPL of AFB_1 in raw tea as 5 μg/kg (TRCU, 2011). Argentina has established the MPL of AFB_1 and TAF in herbal tea infusion as 5 and 20 μg/kg, respectively (Zhang et al., 2018a).

Pakistan is the seventh largest country in terms of per capita black tea consumption. During, 2007–2016, a massive increase of 35.8% per capita tea consumption was recorded in Pakistan mainly due to increase in population and urbanization. The current tea consumption in Pakistan is estimated to be around 172,911 tons, which is expected to reach 250,755 tons in 2027 (Hassan, 2018). Previously, aflatoxin contamination of various foodstuffs including cereals (Lutfullah and Hussain, 2012), chillies (Iqbal et al., 2010), milk and milk products (Iqbal and Asi, 2013), infant formulas (Akhtar et al., 2017), and poultry meat and eggs (Iqbal et al., 2014) have been reported from Pakistan indicating a suitable environment for the growth of fungus responsible for the production of aflatoxins.

Despite being the most popular beverage, no information is currently available to consumers on the level of aflatoxins in black tea in Pakistan. Therefore, the current study aimed to quantify the level of aflatoxins in commercially available branded and non-branded black tea samples and to calculate the estimated daily intake (EDI) and potential health risks of aflatoxins through consumption of black tea. Furthermore, the impact of local tea making process on the transfer of aflatoxins from tea leaves to the tea beverage was also evaluated.

MATERIALS AND METHODS

Sample Collection

A total of 120 black tea samples (90 branded and 30 non-branded) were analyzed for the quantification of TAF. The branded samples were collected in their available commercial packaging and the non-branded samples (available in open form) were collected in clean glass bottles and immediately transported to the laboratories of Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan. The samples were collected from different retail shops (n = 23), supermarkets (n = 8), and local tea café (n = 13) of Multan city of Pakistan during the period of March 2018–July 2018. Non-branded tea samples being cheaper and even illegal (due to unpacked form) therefore are available only in the small scale local tea café and small retail shops, in supermarkets only branded tea samples are available. All the black tea samples were ground with a Waring blender (West Point, France, WF-7901) to obtain homogenous particle size, dried and stored in air tight glass containers at 4°C until analysis.

Quantification of Aflatoxins in Black Tea

Sample Extraction and Immunoaffinity Clean-Up

Test portions of finely grounded black tea (25 g) were extracted with 100 ml methanol/water (60/40 v/v) in an orbital shaker at 200 r/min for 4–5 h, and the extract was filtered through Whatman filter paper No. 42. The filtrate (4 ml) was diluted with 16 ml 50 Mm PBS (pH = 7.4). The diluted extract was passed through immunoaffinity columns (Eurofins) under gravity with a flow rate of 1–3 ml/min. The column was rinsed with 5 ml of 10 Mm PBS/methanol (90/10 v/v). The bounded toxins were released by elution with a total of 2 ml of methanol. The column elute was dried under nitrogen stream.

Derivatization

Post-column derivatization of aflatoxins was performed according to the AOAC official method 2005.08 (AOAC, 2005). Briefly, the dried sample extracts/standards were dissolved in 200 μl hexane. TFA (50 μl) was added and the vial was closed and placed in dark for 5 min. The solution containing aflatoxins was mixed with 1.95 ml mixture of double distilled water and acetonitrile (9:1) and vortexed for 1 min. The aqueous layer

(lower layer) containing aflatoxins was removed and filtered through a 0.45 μm syringe filter before loading on HPLC (Sykam, Germany).

Chromatographic Analysis

HPLC method for aflatoxins quantification was optimized according to the method given by Santos et al. (2010). An isocratic mobile phase of acetonitrile/methanol/water (15/25/65 v/v/v) was found most optimum with a flow rate of 1.0 ml/min. The temperature of column oven was set at 37°C. The excitation and emission wavelengths of fluorescent detector (Sykam, RF-20A) were set at 365 and 440 nm, respectively. The stationary phase used was a reverse phase silica gel C-18 column. The injection volume was 20 μl and the rum time for each sample and standard was 20 min. The retention times for AFG_1, AFB_1, AFG_2, and AFB_2 were 4.28, 5.09, 6.66, and 8.78 min, respectively.

Method Validity

The recovery percentage of the adopted procedure was computed by spiking aflatoxins free tea samples with three different concentrations of aflatoxins, i.e., 6, 12, 24, 48 μg/kg, the ratios of AFB_1, AFG_1, AFB_2, and AFG_2 were 1:1:0.5:0.5, respectively. The standards were run independently in six replicates and allowed to stand overnight for its adsorption within the samples. The spiked samples were extracted, passed through immunoaffinity column, derivatized, and run on HPLC according to the procedure as described above and the recovery percentages were calculated by using the following equation:

$$Recovery\,(\%) = \frac{measured\ concentrations}{spiked\ concentration} \times 100$$

Impact of Local Tea Making Process on the Transfer of Aflatoxins

Black tea locally known as "*Chai*" is made in Pakistan by boiling grounded tea leaves in water, then milk and sugar are added and again boiled. The tea is boiled four to five times to give the proper taste of tea leaves. Finally, the tea is filtered through tea strainers and poured in tea cups of normally 150 ml serving per cup. On an average, 5 g tea leaves are added to make one serving of tea (150 ml); however, it may vary person to person based on liking and disliking, ranging between 2.5 and 8 g/serving. Black tea samples detected negative for aflatoxins were used to make tea beverage. Aflatoxins free black tea samples (5 g) were spiked with three different levels of aflatoxins (50, 100, and 200 μg/kg) and allowed to stand for 3 h for complete adsorption of aflatoxins in the tea leaves and for the evaporation of solvent (methanol). The ratio of AFB_1, AFB_2, AFG_1, and AFG_2 in the spiked samples was 2:1:2:1, respectively (commercially available standard ratio). Tea leaves (5 g) were boiled in 50 ml of water and added with 200 ml of milk and were added with 5 g of sugar. The whole mixture was again boiled four times and then filtered with tea strainer (30 mesh size). The boiling was performed in air tight electric kettle and the kettle was opened after 5–7 min to avoid the removal of water vapors. The tea residues and the tea beverage (filtrate) were dried and analyzed to quantify the concentration of aflatoxins. Non-spiked and spiked unprocessed samples were run as −ive and +ive controls, respectively. Milk and sugar samples were also quantified for the presence of TAF.

Exposure of Aflatoxins and Health Risk Assessment

A survey was conducted to estimate the daily intake of tea by the male (n = 125) and female (n = 125) residents of Multan city of Pakistan, aging above 20 years. For exposure assessment of aflatoxins through tea consumption, the "consumer only" scenario for both male and female groups of consumers was considered, i.e., only tea lover male and females were added in the study. A *pro forma* was designed in which individual weight and intake level of tea on daily basis were inquired. The mean values of intake rate and average weight were computed for male and female groups. The EDI of aflatoxins through tea consumption was computed by using the following equation of the Food and Agriculture Organization [FAO]/World Health Organization [WHO] (2014):

$$EDI = \frac{(IR \times C)}{W_{AB}}$$

Where EDI = ng/kg bw/day; IR = intake rate of black tea (kg/person/day), C = the average concentration of aflatoxins detected in tea samples (μg/kg), and W_{AB} = the average body weight of an individual (kg).

To estimate the potential health risk related to the consumption of non-branded and branded black tea, the margin of exposure (MOE) using benchmark dose (BMD) approach of the European Food Safety Authority was used. For dose–response modeling of aflatoxins, the BMD lower confidence limit ($BMDL_{10}$) for a 10% increase in cancer incidence obtained from animal study data modeling (170 ng/kg bw/day) was considered (European Food Safety Authority [EFSA], 2007). The value of MOE for both adult male and female population groups was calculated using the following equation:

$$MOE = \frac{BMDL_{10}}{Exposure}$$

MOE ≥ 10,000 indicates a low public health risk associated with exposure to a genotoxic carcinogen (European Food Safety Authority [EFSA], 2005; Wang et al., 2018).

Statistical Analysis

Statistical analysis of data was performed by using Statistix 8.1 software (Informer Tech. Inc., United States). The probability level of <0.05 was considered as statistically significant. Mean and standard deviation (±) values were computed by using Microsoft Excel, 2013.

RESULTS AND DISCUSSION

Occurrence of Aflatoxins in Non-branded and Branded Tea

The efficiency of the HPLC method adopted for the quantification of aflatoxins in black tea samples is presented in **Table 1**.

The recovery percentages for different types of aflatoxins ranged between 85.0 and 96.3%, while the variation coefficient for aflatoxins ranged from 3.9 to 12.5%. The LOD values for AFB_1 and AFG_1 were 0.06 and 0.02 μg/kg for AFB_2 and AFG_2, while the LOQ value for AFB_1 and AFG_1 were 0.18 and 0.06 μg/kg for AFB_2 and AFG_2.

Results for TAF concentrations in non-branded and branded tea samples are represented in **Table 2**. Aflatoxins were recorded as positive in 94 samples of black tea (78.3%). TAF concentration in the positive samples ranged between 0.11 and 16.17 μg/kg. AFB_1 was detected as positive in 76.7% (n = 92) samples of black tea. The concentration of AFB_1 in positive samples ranged between 0.08 and 8.24 μg/kg. Statistical analysis (factorial design) revealed significant difference between the concentration of aflatoxins in different types of branded and non-branded tea samples ($P < 0.05$). Aflatoxins were found positive in 93% non-branded samples while Brands A, B, and C were found to have 73, 90, and 57% samples positive for aflatoxins. As there is no set limit for the levels of aflatoxins in tea, the results of the study were compared with the MPL of TAF (10 μg/kg) and AFB_1 (5 μg/kg) as set by European Union in spices (EU, 2006). Seven black tea samples (five non-branded and two branded) were found to have AFB_1 level above 5 μg/kg, while six samples of black tea (four non-branded and two branded) were found to have TAF above 10 μg/kg. However, the mean concentration of aflatoxins in all types of branded samples as well as in non-branded samples was below 5 μg/kg, indicating a low level of aflatoxins in black tea samples from Pakistan.

Prevalence of aflatoxins in different types of tea leaves and tea beverages has been reported by a number of researchers from around the world. However, only a few reports are available regarding the levels of aflatoxins in black tea. Pouretedal and Mazaheri (2013) analyzed the concentration of aflatoxins in 40 samples of raw black tea collected from Iranian markets and found 27.5% (n = 30) samples positive for aflatoxins, while the mean concentrations of AFB_1 and TAF were 10 and 12.07 μg/kg, respectively. In China, the mean concentration of AFB_1 in the samples of raw pu-erh tea and ripe pu-erh tea was 8.33 and 20.149 μg/kg, respectively (Li et al., 2015). Mannani et al. (2019) investigated the concentration of aflatoxins in herbal green tea samples collected from the Moroccan market. Out of 129 analyzed samples, 76 samples were found positive for aflatoxins ranging between 1.8 and 116.2 μg/kg. Pallarés et al. (2017) quantified the levels of aflatoxins in 12 samples of black tea bags from Spain and found that AFB_1, AFB_2, and AFG_1 were absent in all of the analyzed samples while only two samples were contaminated with AFG_2 with concentration below LOQ (0.5 μg/kg). Comparing our results with the already published literature, it can be stated that the levels of aflatoxins in black tea samples obtained in the current study are lower than those reported from Morocco, in line with the findings from Iran and China but higher than those reported from Spain. Lower levels of aflatoxins in the branded black tea samples as compared to non-branded samples indicate the adoption of good hygienic practices, proper implementation of rules and regulations, and provision of proper storage and transportation facilities. Higher levels of aflatoxins in non-branded spices as compared to branded spices are reported by Naz et al. (2016) and Akhtar et al. (2020). The relatively higher levels of aflatoxins in non-branded tea samples might be due to their sale in unpacked/open form that provides more chances for the fungus to grow and to produce aflatoxins in black tea. The unpacked tea samples are usually stored in bulk until being supplied to markets, the bulk storage facilitates moisture migration and as a result increases the chances of mold growth any mycotoxins production (Chen et al., 2018). The prevalence of aflatoxins in line with this study is already reported in various foodstuff of Pakistan, for instance, in milk samples (Ismail et al., 2016), in animal feed samples (Ismail et al., 2017), and in mother milk samples (Khan et al., 2018) indicating that the environmental conditions in Pakistan are quite favorable for the growth of fungus and the production of aflatoxins.

Exposure Assessment and Health Risk Characterization of Aflatoxins

The EDI values for AFs due to the consumption of non-branded and branded black tea are given in **Table 3**. Male group of consumers was found to be more susceptible to the exposure of aflatoxins through consumption of contaminated tea than the female group of consumers due to the higher consumption rate of tea beverage by male group of consumers (0.009 kg/person/day) compared to the female group (0.006 kg/person/day). The consumption rate of non-branded tea (0.008 kg/person/day) was higher than the branded tea (0.007 kg/person/day). The estimated mean exposure to TAF through consumption of non-branded and branded tea beverage were 0.544 and 0.129 ng/kg bw/day, respectively. Owing to the genotoxic and carcinogen nature of aflatoxins, there is no consensus for tolerable daily intake of AFs and therefore the approach of "As Low as Reasonably Achievable" (ALARA) is usually adopted against aflatoxins. However, the provisional maximum tolerable daily intake (PMTDI) value of aflatoxins for children and adults without hepatitis is 1 ng/kg bw/day (Kuiper-Goodman, 1998; Jecfa., 2017). Based on the results obtained from the present study, none of the exposure levels exceeded the reported limit. Sedova et al. (2018) calculated the dietary exposure to AFs through consumption of tea based on combined data from several countries (including Iran, Korea, and Russia) using lower bound approach. The estimated dietary exposure to aflatoxins through tea consumption was reported ranging between 0.4 and 2.6 ng/kg bw/day (mean 0.16 ng/kg bw/day), that is almost in line with our findings. The findings of this study indicate that there is a clear difference in the concentration of aflatoxins in non-branded (< LOD—16.17 μg/kg) and branded tea samples (< LOD—11.07 μg/kg). The non-branded samples were found to have higher aflatoxins level as compared to any of the analyzed brands of tea. Although the EDI values through intake of non-branded tea are less than the PMTDI value but still, the chances of toxicity are there especially for the consumers having large intake of tea on a regular basis.

The MOE values obtained from mean dietary exposure to aflatoxins were used to estimate the possible health concerns

TABLE 1 | HPLC method efficiency and validity (n = 6 for each spiking level).

Type of AF	Level of spiking (μg/kg)	Mean values found (μg/kg)	Standard deviation	Recovery (%)	Variation coefficient (%)
AFB_1	2	1.9	0.1	95.1	5.3
	4	3.7	0.2	92.5	5.4
	8	7.7	0.3	96.3	3.9
	16	15.1	0.8	94.4	5.3
AFG_1	2	1.8	0.1	90.0	5.6
	4	3.7	0.3	92.5	3.9
	8	7.6	0.3	95.0	4.0
	16	15.2	0.7	95.0	4.6
AFB_2	1	0.9	0.1	91.1	8.9
	2	1.7	0.2	85.0	11.7
	4	3.6	0.2	90.0	5.6
	8	7.1	0.6	88.8	8.5
AFG_2	1	0.8	0.1	85.0	12.5
	2	1.7	0.2	88.1	11.7
	4	3.5	0.3	88.5	8.6
	8	7.2	0.5	90.6	6.9

TABLE 2 | Concentration of total aflatoxins (μg/kg) in branded and non-branded tea samples (n = 30 for each type).

Type	AFB_1 (mean/% + ive)	AFB_2 (mean/% + ive)	AFG_1 (mean/% + ive)	AFG_2 (mean/% + ive)	TAF (mean/% + ive)
Brand A	0.41 ± 0.39^{bc}/73	0.12 ± 0.25^{bc}/40	0.01 ± 0.05^{b}/7	0.01 ± 0.04^{b}/10	0.55 ± 0.51^{c}/73
Brand B	1.67 ± 1.75^{b}/90	0.38 ± 0.70^{b}/47	0.11 ± 0.33^{ab}/20	0.17 ± 0.56^{ab}/13	2.32 ± 2.94^{b}/90
Brand C	0.48 ± 0.63^{c}/53	0.05 ± 0.11^{c}/17	0.01 ± 0.03^{b}/10	0.01 ± 0.04^{b}/10	0.55 ± 0.67^{c}/57
Non-branded	2.67 ± 2.20^{a}/90	0.91 ± 0.97^{a}/80	0.24 ± 0.55^{a}/27	0.40 ± 0.92^{a}/37	4.22 ± 4.08^{a}/93

Means values having different letters within a column are significantly different from each other ($P < 0.05$); values after ± indicate standard deviation.

TABLE 3 | Estimated dietary exposure (ng/kg bw/day) and MOE values of total AFs for the general adult population.

Consumer Groups	Non-branded tea				Branded tea			
	Number of consumers	Average CR[a]	Exposure[b]	MOE	Number of consumers	Average CR[a]	Exposure[b]	MOE
Male	75	0.010	0.680	250	50	0.008	0.147	1156.5
Female	75	0.006	0.408	416.7	50	0.006	0.110	1545.5

[a]*Consumption rate.*[b]*Based on mean body weight of the general population of 62 kg.*

TABLE 4 | Aflatoxins recovery in tea residues and filtrate (beverage).

Level of spiking (μg/kg)	TAF in tea residues (μg/kg)	TAF in filtrate (μg/kg)	Mean recovery (%) through tea residues and filtrate
50	1.4 ± 0.2	26.9 ± 2.2	56.6
100	4.6 ± 0.3	55.8 ± 3.2	60.4
200	12.4 ± 1.8	98.9 ± 4.3	55.7

TAF = total aflatoxins.

arising from the consumption of black tea. The MOE values for consumers of both non-branded and branded tea obtained in the current study (range 250–1545.5) are much lower than the safe limit of ≥10,000, thus indicating a potential public health risk. Mean maximum MOE value (1545.50) was recorded for the female group of branded tea consumers while mean minimum MOE value (250) was recorded for the male group of non-branded tea consumers, thus indicating a high health risk in all groups of consumers. To the best of our knowledge, not a single study is published on the estimation of MOE values through consumption of aflatoxin contaminated black tea. In a study conducted by Sakin et al. (2018), the EDI of aflatoxins through Surk (a Turkish dairy food) was estimated to be 0.057 and the mean MOE values for AFB_1 was reported as 2982. Andrade et al. (2013) reported the MOE values for Brazilian population based on 942 food items, the MOE values ranged between 6 and 25. Heshmati et al. (2017) reported MOE values ranging between 1417 and 4250 from Iran based on the intake of aflatoxins through consumption of dried fruits. MOE values obtained in the current study are in line with the other studies reported from Turkey, Brazil, and Iran that are far below the safe limit of 10,000, thus indicating a severe health risk that needs proper consideration by the health and regulatory agencies.

Effect of Processing on Aflatoxins in Tea

TAFs were spiked in three different levels (50, 100, 200 μg/kg) to evaluate the impact of tea making process on aflatoxins. The concentration of TAFs in tea residues (collected on tea strainer) and in tea filtrate (beverage) are described in **Table 4**. All four aflatoxins in milk and sugar were found below the detection limits. The mean recovery percentages of aflatoxins in the analyzed three spiking levels ranged between 55 and 60%. The percentages of aflatoxins recorded in the tea residues obtained on the surface of tea strainer ranged between 3 and 6% while TAF% in the filtrate ranged between 50 and 55%. The results clearly indicate that more than half of the aflatoxins found in tea leaves get dissolved in the water and become a part of the beverage, to be consumed by the tea drinkers. A small percentage of aflatoxins were retained by the tea leaves while around 40–45% of the aflatoxins could not be detected. It is not clear why this is but the results show a large proportion of aflatoxins reach the final beverage.

Aflatoxins are water soluble (10–20 mg/mL) and so get easily dissolved in water when the tea leaves are brewed (Sedova et al., 2018). However, a lower percentage of aflatoxins in tea leaves (about 30%) were transferred to the water in a study by Viswanath et al. (2012). The possible reasons behind the differences in results might be due to the difference of tea varieties used in our study as compared to India, or due to the difference in tea making processes, as in this study we added milk and sugar also (local tea recipe), while in the study of Viswanath et al. (2012), the tea leaves were boiled in water only. Low recovery rates of TAFs in case of tea processing might be due to the conversion of aflatoxins in some other metabolites/degradation products due to four to five times boiling of tea. Reduction in aflatoxins level due to heating is reported by a number of authors. Lee et al. (2015) reported 42–81% reduction in aflatoxins level by heating the soybeans at temperature ranging between 100 and 150°C. Rastegar et al. (2017) reported 93% reduction in aflatoxins level (spiking level was 268 μg/kg) of roasted pistachio nuts. However, a number of other researchers have reported the ineffectiveness of heating on aflatoxin (Awasthi et al., 2012; Bohloli-Oskoii and Mohamadi, 2013; Hassan and Kassaify, 2014). Therefore, further studies are required to investigate the presence of possible aflatoxin breakdown products in black tea.

CONCLUSION

The current study is the first of its kind that provides information regarding the levels of aflatoxins in commercially branded and non-branded black tea samples available in the local markets of Pakistan. Among the analyzed samples of tea, non-branded black tea samples were found to be more contaminated with aflatoxins as compared to branded samples indicating the adoption of poor post-harvest practices in case of non-branded black tea. Based on the limited number of samples especially of non-branded tea samples, it is not possible to state that the non-branded tea samples are always more contaminated than the branded ones but it is clear that the contamination of aflatoxins in both types of tea samples is common in Pakistan. The MOE values for aflatoxins through black tea consumption indicate severe health risks through black tea consumption. Male consumers are more at risk as compared to female consumers. The study on the impact of the local tea making process on aflatoxins content indicates the transfer of more than 50% aflatoxins from tea leaves to the tea beverage and possible production of aflatoxins degradation products probably due to four to five times boiling of tea in the local tea making method, while only 3–6% aflatoxin are left in the remaining tea residue on the tea strainer. Current findings indicate that the health and regulatory agencies must put strenuous efforts to reduce the load of aflatoxins in commercially available black tea. However, further studies are needed to clearly understand the mechanism involved in the possible degradation of aflatoxins in local tea making processes.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

All the authors actively participated in research work and write up of the article. Planning of research was done by AI, YG, SA, and MR. Experimentation was performed by AI and IN. The write up performed mainly by AI, YG, and MR while some assistance was also provided by SA, MR, and IN.

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Acoustic-Based Screening Method for the Detection of Total Aflatoxin in Corn and Biological Detoxification in Bioethanol Production

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Aspergillus spp. are widely occurring fungi in nature; they produce toxic compounds such as aflatoxins (AFs) and mainly target plant products such as corn and nuts. The development of prevention strategies is challenging because AFs are highly toxic and have been regulated to small concentrations. This study proposes a new strategy of AF prevention through the application of rapid methods using acoustic techniques in combination with fermentation for the elimination of contaminated corn from bioethanol production processes. An acoustic device was used for the analysis of model systems consisting of corn and nuts (hazelnuts and peanuts) contaminated with different amounts of AFs. High correlations were obtained between penetrated acoustic signal amplitude (Ap) and corn sample density, and between Ap and AF content. Also, relationships were found between changes in Ap values and AF contamination in the nuts model systems. The results of biotreatment of contaminated corn during bioethanol production confirmed that AFs cannot be completely eliminated in dried distiller's grains with solubles, a valuable by-product for animal feed. Microbially, contamination of the raw material has a negative impact on bioethanol quality by increasing the content of volatile compounds. Therefore, the application of methods such as acoustic screening is a promising alternative for rapid AF detection in corn and nuts (it can handle multi-layers of grain). With the application of acoustic techniques, the prevention of AFs in contaminated raw plant materials could be achieved.

Keywords: *Aspergillus* spp., corn, nuts, rapid method, aflatoxins screening, acoustic sensors, bioethanol, detoxification

INTRODUCTION

Aspergillus flavus and *A. parasiticus* are one of the most common fungal strains in the agricultural sector, producing the aflatoxins (AFS) AFB1 and AFB2, and AFG1 and AFG2, which are chemically related to bisfuranocoumarin (Udomkun et al., 2017; He et al., 2018) and found worldwide in soil, air, and plants (Bandyopadhyay et al., 2016; Rushing and Selim, 2019). Aflatoxins are potential carcinogens that frequently contaminate food raw materials such as corn, cottonseed,

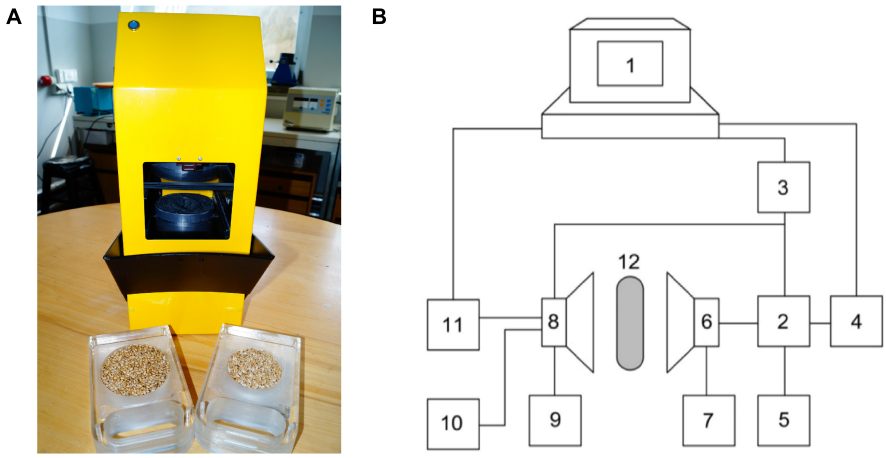

FIGURE 1 | The acoustic spectrometer **(A)** and its schematic drawing **(B)** (1 – computer; 2 – sine-wave generator; 3 – pulse generator; 4 – frequency converter; 5 – frequency meter; 6 – transmitting acoustic aerial; 7 – power supply; 8 – receiving acoustic aerial; 9 – power supply; 10 – oscilloscope; 11 – digital voltmeter; 12 – grain seeds sample under test).

peanuts, and some tree nuts in high concentrations, representing a high risk to food and feed chains and lowering the nutritional quality (Santini et al., 2009; Mikusova et al., 2010; Ritieni et al., 2010; Weaver et al., 2015; Moretti et al., 2019). Therefore, for the food and feed industry to avoid economical losses, a major task is to control the mycotoxin contamination levels in the end products. The prevention of fungal contamination and the development of methods for the decontamination of foods from mycotoxins are important strategies to protect human and animal health (Ehrlich, 2014; Reinholds et al., 2016; Ismail et al., 2018; Pankaj et al., 2018; Mwakinyali et al., 2019).

The common practice to determine mycotoxins is to use laboratory- and time-intensive fundamental chemical, physical, and enzyme immunoassay analyses (Santos Pereira et al., 2019). Considering the fact that these mycotoxin detection methods are complex and expensive, special attention should be given to innovative mycotoxin determination technology, that will allow quick and cheap detection of mycotoxins in the raw materials. Fungal infection not only results in the accumulation of mycotoxins, but also causes grains to shrivel and become more porous. This phenomenon is known as head blight or scab, one of the indicators of poor wheat grain quality (Juodeikiene et al., 2011; Ropelewska et al., 2019; Zhang and Ji, 2019). Due to changes in grain microstructure a rapid and non-destructive method to evaluate the quality and safety of grains is therefore required to detect and subsequently eliminate these toxins from the food chain. The first portable acoustic device equipped with a wide-range capacity ultrasonic transducer was developed at Kaunas University of Technology (Lithuania) during the implementation of the EUREKA ITEA2 project ACOUSTICS for the prediction of deoxynivalenol (DON) contamination levels in wheat grains. Project results showed that the acoustics method, applied for the first time to grain safety monitoring, is innovative and important in ensuring the safety of grains (ITEA 2 Magazine, 2013; Juodeikiene et al., 2014b). However, until now, no studies of the influence of *Aspergillus* spp. and their metabolites on corn grain and nut microstructure, their technological properties and the use of an acoustic method for the detection of AFS, have been performed.

TABLE 1 | The corn samples with different contamination levels, used for bioethanol production.

Corn No.	Aflatoxin Concentrations, μg/kg
C-0	0,00
C-14	14,17
C-15	15,24
C-39	38,55
C-50	50,00
C-54	53,68
C-57	56,70

To avoid the detrimental effects of feed and food contaminated by AFs, not only prevention of contamination but also decontamination of toxic compounds during processing should be applied (Taheur et al., 2019). Fungal infection not only results in the accumulation of AFs, but due to contamination corn raw materials are no longer available for food or feed consumption and can be used as biomass. One of the possible applications of corn biomass is for the production of bioethanol. It is known that fermentation processes could eliminate grain contamination, with the possibility of using the by-products obtained as feed for cattle (Čolović et al., 2019). For this reason, the degradation or decontamination of mycotoxins using appropriate biological microorganisms have been used in the last decade (Juodeikiene et al., 2012; Oliveira et al., 2013; Peles et al., 2019). Recently, the novel aspects of the biological detoxification of mycotoxins (Vila-Donat et al., 2018) included a strategy that relies on mycotoxin inactivation or transformation to non-toxic products by applying low-cost and economically feasible decontamination technologies, retaining the nutritive quality of feed or food, remaining palatable, and

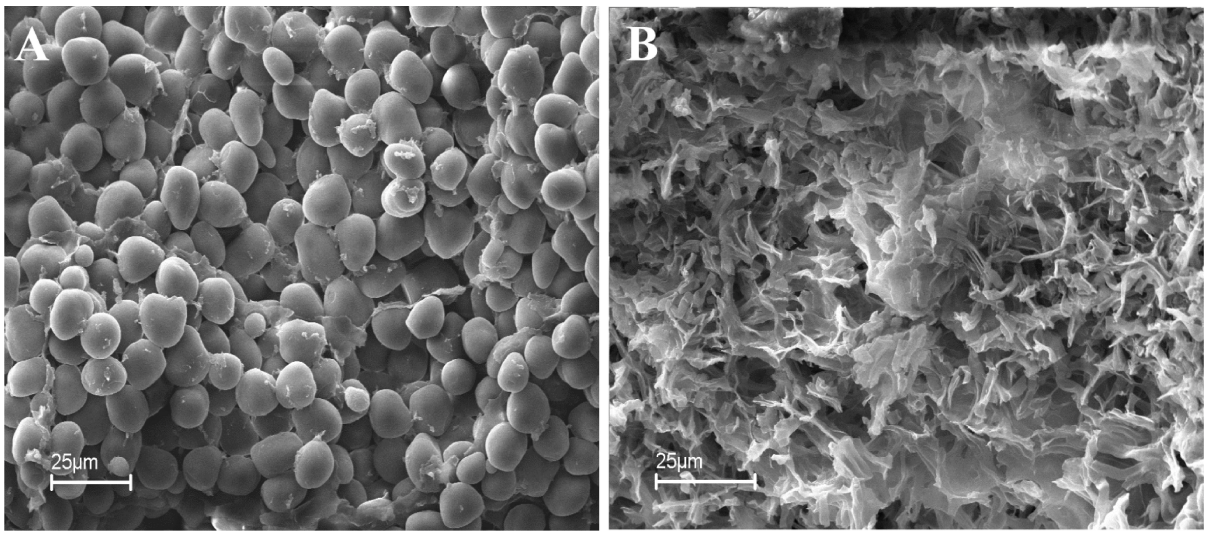

FIGURE 2 | Microscopic view of healthy kernels with intact starch granules **(A)** and kernels damaged by fungi showing stripped starch granules **(B)**.

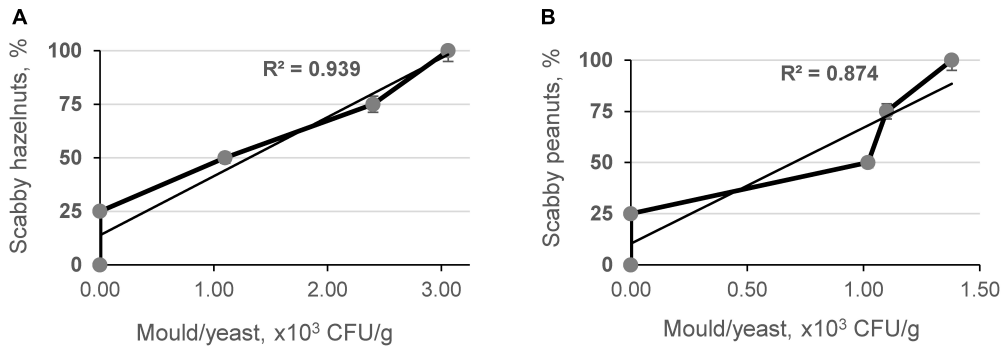

FIGURE 3 | Relationship between content of mold/yeast in scabby hazelnut **(A)** and peanut **(B)** model systems.

not changing significantly the physical properties of the raw material (Haque et al., 2020). A number of microbial species of bacterial and fungal origin have shown the capability to degrade mycotoxins via sorption/enzymatic degradation (Risa et al., 2017; Wang et al., 2018, 2019).

In the present study, the efficiency of corn biomass bioprocessing was explored by using a multi-step prevention system: in the first step, acoustic screening of grains with the elimination of contaminated corn from the production chain was used, and in the second one, a detoxification approach (e.g., fermentation with selected bio-tools) for bioethanol production was applied.

MATERIALS AND METHODS

Plant Material

Corn Samples

Uncontaminated corn grains and grains artificially infected with *Aspergillus flavus* with a high level of total AFs (59.2 μg/kg) were obtained from the USDA (United States). The corn model systems were prepared by mixing the uncontaminated corn kernels with 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% of contaminated kernels.

Nut Samples

Additionally, this study analyzed different nuts (peanuts and hazelnuts) obtained from a Lithuanian supermarket. The two model systems (peanuts and hazelnuts) were prepared by mixing whole-appearance nuts (peanuts and hazelnuts) with mold-damaged nuts (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%). The infected nuts were smaller and more shriveled and were selected according to their degree of shriveling.

Methods of Analysis

Determination of Chemical Composition and the Qualitative Characteristics of Grain or Nuts

Humidity was determined by the weight loss on drying of the grain or nuts (130 ± 3°C) to constant weight [AACC method 44-15 (2000)].

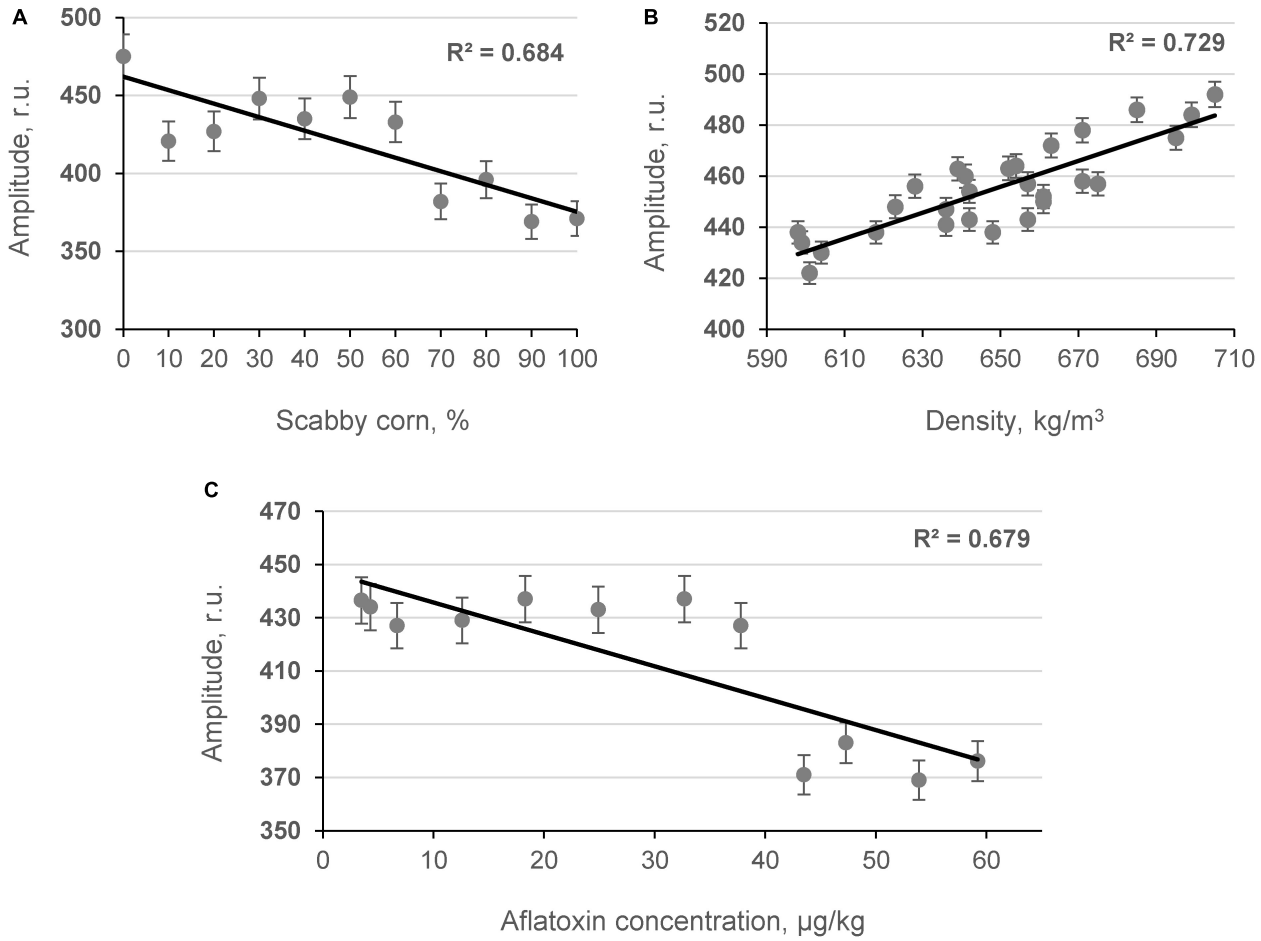

FIGURE 4 | Relationship between content of scabby corn in model systems **(A)**, density of samples **(B),** AF content in samples **(C)**, and amplitude of the penetrated acoustic signal (Ap, frequency 24.3 kHz; n = 10).

The bulk density of the corn or nuts (ρ, kg/m^3) was calculated using the equation ρ = G/V, where G is mass and V is volume.

Grain Microstructure Evaluation

Cross-sectional images of infected and healthy grains were taken by scanning electron microscope EVO 50 (LEO Electron Microscopy Ltd., Cambridge, United Kingdom) equipped with a second electron (SE) detector.

Microbiological Contamination of Nuts

Microbiological tests on both types of nuts were carried out using five mixtures: (1) 100% whole nuts, (2) 75% whole nuts + 25% contaminated nuts, (3) 50% whole nuts + 50% contaminated nuts, (4) 25% whole nuts + 75% contaminated nuts, and (5) 100% infected nuts. The total numbers of aerobic microorganisms in the mixtures were determined using the plate-count agar (CM0325, Oxoid, United Kingdom). Nuts (10 g) were mixed with 100 ml of distilled water, and after serial dilution, the obtained homogenate was mixed with the agar medium and incubated at 24°C for 5 days in aerobic conditions. The count of microorganisms was expressed in CFU (colony-forming units) per gram of nuts.

Acoustic Technique

Samples of the corn grain, corn grain model system, and different nuts model systems were screened using a recently developed portable acoustic spectrometer (**Figure 1**) with penetration (Juodeikiene et al., 2014b).

The spectrometer measures in relative units the amplitude of the acoustic signal (Ap) that penetrates the sample matrix over the frequency range 10–80 kHz. The 15–40 kHz interval was selected as the optimum frequency range. The duration of each measurement was ∼10 s. The test was carried out by placing the test portion of 200 g of sample into a plastic vessel whose base was covered with sound-transmitting material. The thickness of the sample layer was 50 mm and diameter 80 mm.

Determination of AFs

The quantitative analysis of the total AFs (B1, B2, G1, and G2) in corn samples and corn samples after bioethanol production (the stillage obtained after drying at 50°C for 24 h) was performed

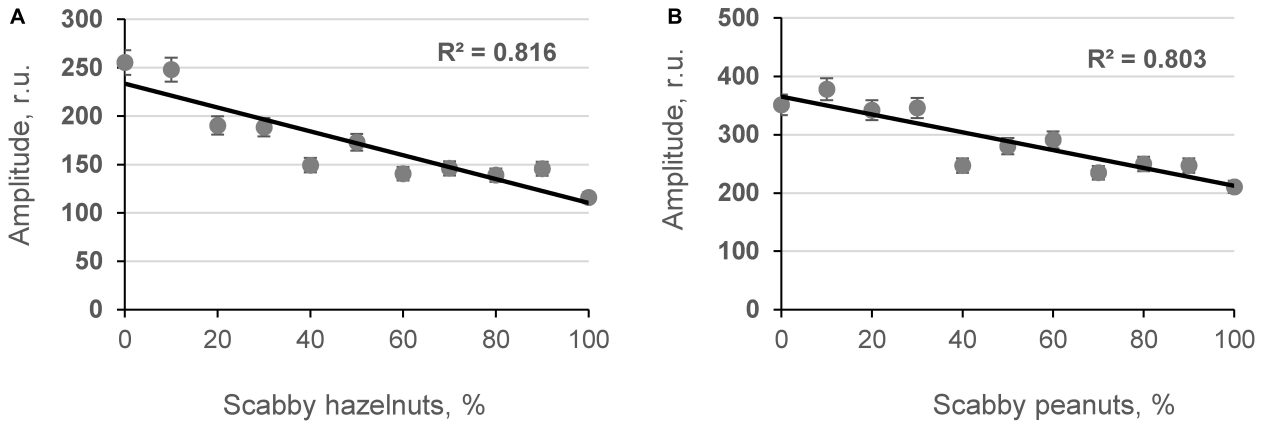

FIGURE 5 | Relationship between content of scabby hazelnuts and peanuts in model systems (**A,B**, respectively) and amplitude of the penetrated acoustic signal (Ap, frequency 18.1 and 27.0 kHz; $n = 10$).

by a competitive enzyme linked immunosorbent assay (ELISA) according to the total AF test (AgraQuant®, Romer Labs Ltd., Germany) procedure. The ground test sample amount used in the ELISA assay was 100 g. Mycotoxin extraction and testing was carried out according to the manufacturer's instructions.

Acidity analysis of fermented broth in bioethanol production was performed according to our previous study (Juodeikiene et al., 2012). The concentration of ethanol was determined using direct distillation and pycnometry.

Volatile compound determination was completed by gas chromatography (GC). Corn samples with different contamination levels were used for bioethanol production (**Table 1**). The bioethanol production was performed by using the low-temperature process according to Juodeikiene et al. (2014a). A Hewlett-Packard 5890 gas chromatograph equipped with an FID detector was used for the quantitative analysis of volatile compounds as described by Juodeikiene et al. (2014a).

RESULTS AND DISCUSSION

The Changes in the Microstructural Composition and Microbiological Contamination of Corn Grains and Nuts Damaged by *Aspergillus* spp.

Microscopic analysis of the grains contaminated by *Aspergillus* and the wholesome corn shows visible damage on the surface of the contaminated grain kernels (**Figure 2B**) and shows what happens to the structure of the grain kernels when attacked by *Aspergillus* spp. In **Figure 2A**, the structure of the grain kernel walls is healthy and wholesome; in **Figure 2B**, the starch granules have been "consumed" by the fungus and a more skeleton-like type of landscape appears. Endosperm cells of healthy kernels (**Figure 2A**) were filled with regular-shaped starch granules and distributed in the unfolded protein matrix. On the other hand, the endosperm in the *Aspergillus*-damaged kernels were restructured, fractured, and of irregular form and formed single agglomerates that are influenced by the amylolytic degradation of the starch granules (**Figure 2B**).

Additionally, the microbiological contamination effect was studied on the hazelnut and peanut model systems, which were prepared by mixing visually damaged and contaminated nuts with wholesome ones. Statistical analysis showed a significant positive relationship between the number of scabby hazelnuts ($R^2 = 0.939$, $p < 0.05$) and peanuts ($R^2 = 0.874$, $p < 0.05$) in model samples and mold/yeast counts, respectively (**Figures 3A,B**).

Fungal infection not only results in the accumulation of mycotoxins, but could also considerably influence the structure and the physical criteria of corn grains and nuts. These changes in the structure influence the porosity of the kernels as well as change the packing factor of kernels in the matrix and could be the basis for the development of a screening method for detection of microbial contamination in this type of raw material.

The Application of the Acoustic Screening Technique for the Detection of Microbiological Contamination in Corn Grains and Nuts Damaged by *Aspergillus* spp.

The influence of contaminated grains on grain bulk density was studied by determining the relationship between the content of damaged grains and the amplitude of the penetrating (Ap) acoustic signal measured by the acoustic spectrometer (**Figure 4**). As shown in **Figures 4A,C**, strong inverse linear relationships were obtained between the number of contaminated corn grains in model samples, the AF content in the samples measured by ELISA [AFL(ELISA)], and the amplitude of the acoustic signal in the model samples ($R^2 = -0.684$ and $R^2 = -0.679$, $p < 0.05$, respectively). A strong positive correlation was observed between the density of model corn samples and the Ap values recorded using the acoustic spectrometer ($R^2 = 0.729$, $p < 0.05$) as shown in **Figure 4B**. Further acoustic analyses were performed with contaminated hazelnut and peanut samples (**Figures 5A,B**).

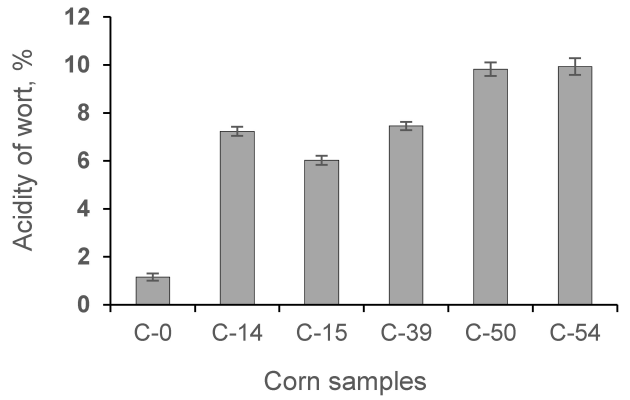

FIGURE 6 | The acidity of wort obtained during fermentation of whole corn grains and contaminated grain samples (C-0, C-14, C-15, C-39, C-50, C-54).

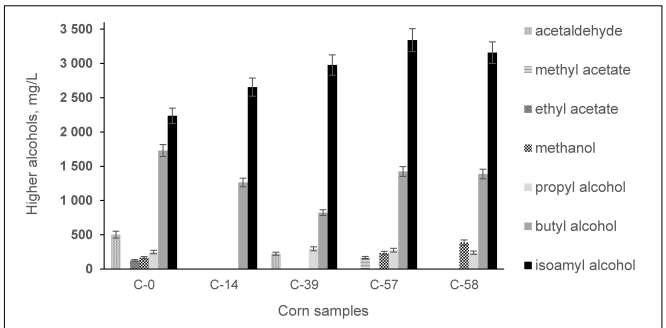

FIGURE 7 | Concentrations of metabolic products in bioethanol during fermentation of corn contaminated with AFs.

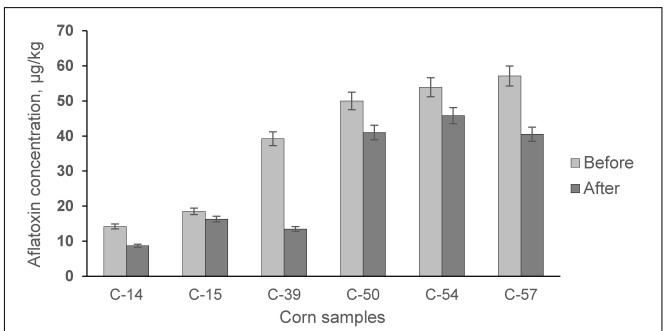

FIGURE 8 | Concentration of AF in corn samples before fermentation versus after fermentation.

Results indicated that as microbiological contamination of nuts increased, the amplitude of the acoustic signal penetrating the nut sample decreased. The obtained results are in agreement with previous studies in which a strong dependence between DON and the content of scabby kernels in wheat matrix was found by Juodeikiene et al. (2008, 2014b) and Tutelyan (2004). The results of the fungal invasion are that the attacked grains shrivel (become scabby in the case of wheat) and become more porous. The same is true for corn kernels affected by *A. flavus*, although the shriveling is less manifested compared to wheat grains because the pericarp of the corn kernel is sturdier. At the point of harvest, a mixture of wholesome and shriveled grains (or more porous kernels) is seen. The differences between the wholesome (less scabby nuts) and contaminated nuts (more scabby nuts) can also be detected by screening acoustic techniques (**Figure 5**). Experiments have shown that the acoustic behavior of porous granular materials can be characterized essentially in terms of porosity and airflow resistance (Guo et al., 2019; Pereira et al., 2019). Furthermore, it was found (Guo et al., 2005) that in beads of cereal grains, the absorption of the acoustic signal depends on the size and shape of the particles. Therefore, it is advisable to use developed equipment at point of harvest where one strain of cereal usually dominates (with one particle size and shape).

The Effect of Microbial Contamination by *Aspergillus* spp. on the Fermentation Processes During Bioethanol Production

The influence of corn biomass being contaminated with AFs at different levels on alcoholic fermentation was evaluated by chromatographic analysis of the yield ethanol and fusel oils.

The process of fermentation of corn contaminated with AFs resulted in a higher organic acid formation (on average 7.03 times) in wort compared to the control sample (**Figure 6**).

Qualitative and quantitative analysis of bioethanol showed (**Figure 7**) that fermentation of AF-contaminated corn produced, on average, higher levels of higher alcohols: isoamyl alcohol (35.57%), propyl alcohol (8.63%), and methanol (88.62%). During the experiment, the levels of AF in the infected corn before and after fermentation were examined (**Figure 8**).

Our study showed that contamination of corn with AFs has a negative influence on the fermentation process by increasing the acidity profile (**Figure 6**) and increasing secondary metabolites in the final product (**Figure 7**).

However, the positive detoxification effect of fermentation was achieved by reducing the AF content by ∼29,71% in the by-product of ethanol production (DDGS) (**Figure 8**). DDGS is characterized by its high contents of protein, fiber, and various minerals and vitamins (Chatzifragkou and Charalampopoulos, 2018; Chen et al., 2019), which are valuable as animal nutrition in feed. Increasing supply and demand for DDGS (Wu and Munkvold, 2008) is expected to be driven by increased bioethanol production (Mohanty and Swain, 2019), which will allow DDGS to be used as a renewable source (Kumar and Singh, 2019). There is still no information offering biological tools for the complete elimination of mycotoxins from fermentation media, including raw materials.

The obtained results of the possible biological decontamination are in agreement with other reviewed papers (Mahmood Fashandi et al., 2018; Chiocchetti et al., 2019). Fermentation is influenced by microorganisms occurring naturally in the raw materials or by the addition of starter cultures of microorganisms. Yeasts, such as *S. cerevisiae* and various lactic acid bacteria (LAB), occur naturally

and spontaneously as a natural part of fermentation in the food industry. Detoxification of mycotoxins usually occurs in two stages: sorption and enzymatic degradation of mycotoxins (Perczak et al., 2018; Pereyra et al., 2018).

CONCLUSION

A portable acoustic penetration spectrometer was applied for high-throughput monitoring of contaminated corn and nuts (hazelnuts and peanuts). Strong correlation coefficients were achieved between acoustic results and corn grain density and AF concentrations in model systems (R^2 = 0.729 and −0.684, $p < 0.05$, respectively). The relationships between the amplitude of the acoustic signal penetrating the samples and AF contamination of hazelnuts and peanuts presented strong relationships (R^2 = 0.816 and 0.803, $p < 0.05$, respectively). Our results show that bioprocesses such as bioethanol production cannot completely eliminate AF contamination of dried distillers' grains with solubles (AF removal was on average 29.71%). In addition, there is a problem with bioethanol quality (lower ethanol content and more volatile metabolites). Therefore, the development and use of rapid methods, such as the use of broadband capacitive acoustic transducers, are still very attractive solutions for AF prevention. Because of its speed, non-invasive character, and quantification ability, this method is comparable in precision to wet-chemistry methods such as ELISA and is far faster and cheaper per analysis to set up than wet-chemistry methods. It lends itself to the monitoring and high-throughput detection of AFs in corn and nuts and eliminates their contamination of the food chain.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

AUTHOR CONTRIBUTIONS

GJ, EB, and AS conceived and designed the experiments. GB and KT-R performed the experiments. DC and DZ analyzed the data. GJ and EB contributed the reagents, materials, and analysis tools. GJ, DC, and AS wrote the manuscript. All authors read and approved the final version of manuscript.

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Host Induced Gene Silencing Targeting *Aspergillus flavus aflM* Reduced Aflatoxin Contamination in Transgenic Maize Under Field Conditions

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Maize (*Zea mays* L.) is one of the major crops susceptible to *Aspergillus flavus* infection and subsequent contamination with aflatoxins, the most potent naturally produced carcinogenic secondary metabolites. This pathogen can pose serious health concerns and cause severe economic losses due to the Food and Drug Administration (FDA) regulations on permissible levels of aflatoxins in food and feed. Although biocontrol has yielded some successes in managing aflatoxin contamination, enhancing crop resistance is still the preferred choice of management for long-term sustainability. Hence, host induced gene silencing (HIGS) strategy was explored in this study. The *A. flavus* gene *aflM* encoding versicolorin dehydrogenase, a key enzyme involved in the aflatoxin biosynthetic pathway, was selected as a possible target for suppression through HIGS. An RNAi vector containing a portion of the *aflM* gene was constructed and introduced into immature B104 maize zygotic embryos through *Agrobacterium* transformation. PCR analysis of the genomic DNA from T0 leaf tissue confirmed the presence of the transgene in six out of the seven events. The seeds from the lines that showed reduced aflatoxin production in laboratory aflatoxin kernel screening assay (KSA) have been increased from T1 to T4 generation in the past four years. Changes in aflatoxin resistance in these transgenic kernels have been evaluated under both field and laboratory conditions. The T2 generation kernels containing the transgene from two events out of four examined had less aflatoxin ($P \leq 0.01$ and $P \leq 0.08$) than those without the transgene. Field-inoculated homozygous T3 and T4 transgenic kernels also revealed lower levels of aflatoxins ($P \leq 0.04$) than kernels from the null (segregated non-transgenic samples) or B104 controls. A similar result was observed when the harvested T3 and T4 homozygous transgenic kernels were evaluated under KSA conditions without inoculation ($P \leq 0.003$–0.05). These two events were crossed with LH195, LH197, LH210, and PHW79 elite breeding lines and the resulting crosses

supported less aflatoxin ($P \leq 0.02$) than the crosses made with non-transgenic lines. In addition, significantly higher levels of *aflM* gene-specific small RNAs were detected in the transgenic leaf and kernel tissues, indicating that the enhanced aflatoxin resistance in the homozygous transgenic kernels is likely due to suppression of *aflM* expression through HIGS.

Keywords: *Aspergillus flavus*, RNAi, host induced gene silencing, *aflM*, aflatoxin, transgenic, maize, droplet digital PCR

INTRODUCTION

Maize (*Zea mays L.*) is one of the major agricultural crops grown worldwide on about 191.2 million ha of land in 2018 with the United States accounting for 17.3%. Global maize production reached 1,078 million metric tons (MMT) in 2017 and was expected to reach 1,123 MMT in 2018, with United States maize production accounting for 32.6–34.4%, according to the latest released report release by USDA-Foreign Agricultural Service[1]. However, global maize production is under constant threat of various diseases. One of them is infection by *Aspergillus flavus* and subsequent contamination with aflatoxins, the most potent naturally occurring toxic secondary metabolites, which are known to cause liver cancer in humans (Squire, 1981; Robens and Richard, 1992; IARC, 2012; Moradi et al., 2015).

Aflatoxin contamination has led to public outbreaks of aflatoxicosis. In 2004, hundreds of people died from consuming aflatoxin contaminated maize in Kenya and hundreds of dogs in the United States died in 2006 from eating aflatoxin contaminated feed (Richard, 2008). Currently there are no effective controls that can completely eliminate aflatoxin contamination in maize and other susceptible crops. The use of chemicals to control *A. flavus* infection and subsequent aflatoxin contamination is ineffective (Wheeler et al., 1991; Bruns and Abbas, 2006). Biocontrol is the only measure known to reduce aflatoxin contamination in the field, but its efficacy varies depending on moisture and timing of application (Moore et al., 2011). Although conventional breeding has greatly improved yields, elite breeding lines remain susceptible to aflatoxin contamination. Transferring polygenic resistance currently available in maize into elite breeding lines has been met with limited success due to linkage drag and incomplete resistance (Warburton et al., 2011; Mylroie et al., 2013).

Several studies have found that small RNAs, including both small interfering RNA (siRNA) and micro RNA (miRNA), travel between cells via plasmodesmata and systemically throughout the plant as mobile silencing signals, to regulate cellular processes, host defense, transcription and translation (Dunoyer et al., 2010; Pyott and Molnar, 2015; Rosas-Diaz et al., 2018). Several studies have also found that siRNA in the diet or medium can be transported across cellular membranes and affect target gene expression in *Caenorhabditis elegans* (Tabara et al., 1998) or *Aspergillus nidulans* (Khatri and Rajam, 2007), respectively. Further studies demonstrated movement of siRNA molecules between a parasite and its host plant (Tomilov et al., 2008), or between herbivorous insects and the host plant engineered to express dsRNAs targeting vital insect genes (Baum et al., 2007).

The major breakthrough in applying RNAi to control plant fungal diseases came from two studies. Tinoco et al. (2010) reported the suppression of *gus* gene expression in a GUS-transformed fungus *Fusarium verticillioides* when it was inoculated onto transgenic tobacco plants expressing an RNAi construct targeting the *gus* gene. Another study was by Nowara et al. (2010) who reported reduced infection by the powdery mildew fungus *Blumeria graminis* by expressing a silencing construct targeting the fungal effector gene *Avra10* in susceptible barley and wheat. This cross-kingdom RNAi based gene silencing phenomenon is called host induced gene silencing (HIGS), which has been demonstrated to successfully suppress disease development caused by fungi (including biotrophs, hemibiotrophs, and necrotrophs) as well as oomycetes (Govindarajulu et al., 2015; Park et al., 2016; Song and Thomma, 2016). Panwar et al. (2013) reported suppression of the wheat leaf rust fungus, *Puccinia triticina* when genes involved in pathogenicity were targeted. Later, Ghag et al. (2014) showed that transgenic banana producing siRNAs targeting vital fungal genes increased its resistance against *Fusarium oxysporum* f.sp. *cubense*. Jahan et al. (2015) further demonstrated successful control of *Phytophthora infestans* in potato using the same strategy. This strategy also improved plant resistance in a recent study against verticillium wilt, an economically important and notoriously hard to control disease that affects a wide range of host plants (Song and Thomma, 2016). These studies convincingly demonstrated that small RNA trafficking between plants and fungal pathogens provides a new and powerful tool to control plant diseases. In addition, some limited success of using HIGS to suppress aflatoxin production in maize by targeting *aflR* (encoding a key regulator of aflatoxin biosynthetic pathway) or *aflC* (encoding a polyketide synthase involved in the initial steps of aflatoxin biosynthesis) or *amy1* (encoding an alpha-amylase involved in fungal infection) of *A. flavus* has been reported (Masanga et al., 2015; Thakare et al., 2017; Gilbert et al., 2018). The lack of field confirmation and/or possible off-target effects of these studies, however, weakened the validity of these RNAi-based gene silencing strategies in managing aflatoxin contamination in maize.

Therefore, the objectives of the present study are to suppress through HIGS a different aflatoxin biosynthetic pathway gene

[1] www.fas.usda.gov/psdonline

ver-1 (*aflM*), which was highly expressed and was involved in the later steps of aflatoxin biosynthesis (Yu, 2012), to (a) determine its ability in reducing aflatoxin contamination in progenies under both laboratory and field conditions, (b) determine whether the transgene can reduce aflatoxin production when transferred to elite inbred lines, and (c) determine whether the reduced aflatoxin contamination was due to the presence of gene specific small RNA from the HIGS construct.

MATERIALS AND METHODS

Construction of HIGS Vector for Suppressing *aflM* Gene Expression

AflM (*ver1*) from *A. flavus* AF13 (gene accession number XM_002379900) was selected in this work. This gene encodes a versicolorin dehydrogenase that is involved in the conversion of versicolorin to demethyl-sterigmatocystin in later steps of aflatoxin biosynthesis (Yu et al., 2004; Yu and Ehrlich, 2011). To clone the gene into a Gateway-based vector (Chen et al., 2010), the 5′ and 3′ arms were selected from the coding region of the versicolorin dehydrogenase gene and were amplified using PCR with homologous recombination sites (italicized) attached to the end of the gene-specific primers (**Supplementary Table 1**). Briefly, the 5′ arm was amplified with attB4-Ver1F and attB1-Ver1R using the *A. flavus ver1* cDNA clone as a template, and the 3′ arm was amplified with attB2-Ver1F and attB3-Ver1R in a similar manner. The 5′ and 3′ arms were then ligated into pDONR P4-P1R and pDONR P2R-P3 (Invitrogen, Carlsbad, CA, United States), respectively, through BP clonase reactions, according to the manufacturer's instruction. The resulting vectors were named pENTR-L4-5′arm-R1 and pENTR-R2-3′armL3, respectively. A MultiSite Gateway LR recombination reaction was performed with the four vectors pBS-d35S-attR4-attR3, pENTRL4-5′arm-R1, pDONR221-PR 10-intron-CmR (Chen et al., 2010), and pENTR-R2- 3′arm-L3, according to the manufacturer's instructions. The reaction mixture was transformed into TOP10 *Escherichia coli* cells and selected on LB plates containing 100 mg/mL ampicillin and 30 mg/mL chloramphenicol. The resulting vector pBS-aflM-RNAi (pBS-d35S-attB4-5′arm-attB1-PR 10 intronCmR-attB2-3′arm-attB3) was then verified through restriction digestion and sequencing before digesting the vector with *Eco*RI and *Sac*I to remove the DNA region containing the aflM-RNAi cassette, which was then ligated into the corresponding sites of pTF102 (Frame et al., 2002), to generate the final RNAi vector pTF102-aflM-RNAi, which was further verified through digestion, before being used in maize transformation.

Transformation of HIGS Vector Into Maize

Genetic transformation of maize inbred B104 was performed by plant transformation facility (PTF) of Iowa State University as described by Frame et al. (2000). Regenerable type I calli were subcultured, and fertile transgenic plants were recovered following selection on bialaphos-containing medium (Frame et al., 2000; Chen et al., 2010). The regenerated transgenic plants were pollinated with pollen from B104 between April and May of 2013, and ears from all seven independent transgenic events were harvested in June 2013.

Confirmation of Transformation and Target Gene Expression

Genomic DNA was isolated from ground leaf tissues (100 mg) developed from transgenic cali or kernels of all seven independent transformation events using a modified CTAB method as describe by Doyle and Doyle (1987). The quality and quantity of the isolated total DNA was determined using a Nano-Drop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). DNA was diluted to the same concentration (50 ng/μL) and used as a template for PCR using specific primers corresponding to the *aflM* gene (Ver-1-F and Ver-1-R, **Supplementary Table 1**). The reaction was prepared at 1× final concentration in a 20 μL volume containing 0.4 μM of each primer and 1 μL of template. Expression of the target gene in developing leaves of young transgenic maize plants was confirmed using reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from plants of all 7 events and B104 wild type, which was used as a negative control. A reverse transcription cDNA RT kit (Life Technologies, Carlsbad, CA, United States) was used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis follow the manufacture's protocol. cDNA was used as a template for real time PCR using RT-Ver-F and RT-Ver-R (**Supplementary Table 1**). The expression level of the maize 18S rRNA gene (accession AF168884) was used as an internal control to normalize the level of target gene expression. The amplification efficiency of each primer pair used in this study was determined through serial dilutions, and this was taken into account in calculating target gene expression if it was outside the ideal efficiency range. The transgenic events confirmed positive for transformation were used in the studies described below.

Evaluation of Aflatoxin Resistance in Different Generations of Transgenic Maize Kernels

Of the seven dependent transformation events received from Iowa, three events with the highest (aflM10, aflM14, and aflM16) and one event with the lowest (aflM13) levels of *aflM* gene expression were selected for the initial screening of the T1 generation of transgenic maize kernels. After surface sterilization of the kernels as described in the kernel screening assay (KSA) by Brown et al. (1993), 10–15 kernels per event were inoculated with 4×10^6 conidia/mL of *A. flavus* toxigenic strain AF13 (ATCC 96044, SRRC 1273), and incubated at 30°C under 100% humidity. After seven days of incubation, kernels were dried at 65°C for 72 h to stop the fungal growth and ground for aflatoxin extraction using MeOH as described by Sobolev and Dorner (2002). Aflatoxin was quantified using a high performance liquid chromatography (HPLC) according to Joshua (1993). Genomic DNA was also isolated from ground powder of individual kernels

after aflatoxin extraction to determine whether it contained the target gene or not.

Another fifteen T1 kernels from each of the above four events were sown in pots filled with potting mix (Marysville, OH, United States) in a greenhouse for seed increase in spring of 2015. Five to eleven seedlings from each event that were verified by PCR to contain the *aflM* gene were transplanted to a field for self-pollination by hand. Twenty-five kernels per event from the resulting T2 ears were tested for aflatoxin accumulation using KSA as described above. DNA isolation and verification for presence of the transgene was conducted on individual kernels for which aflatoxin data were obtained. T2 seeds (45 kernels/event) were increased to T3 in the field in spring of 2016, and from T3 to T4 in the field (60 kernels/event) in 2017 for two of the events (aflM14 and aflM16). In 2018, homozygous lines of these two events were crossed with four elite inbred lines (LH195, LH197, LH210, and PHW79) to determine whether the transgene can reduce aflatoxin production in the resulting crosses.

Transgene Copy Number Assessment Using Real Time PCR and Droplet Digital PCR

Besides PCR confirmation of the presence of the target gene in genomic DNA extracted from transgenic seedling leaf tissues, transgene copy number (C) as described below was also determined for T0 leaf tissues collected from each transformation events that contained the target gene determined using real time PCR. TaqMan real-time PCR primers (**Supplementary Table 1**) specific to the *aflM* and to the endogenous single copy alcohol dehydrogenase gene (*adh1*) as a reference were used to quantify the relative ratios of *aflM*/*adh1* with the fluorogenic TaqMan probes. Real-time PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, United States) in a final volume of 25 μL containing 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 200 nM of each primer, 100 nM of probe and 150 ng of genomic DNA under the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 amplification cycles of 95°C for 15 s, and 55–60°C for 1 min depending on primers. Three technical replicates were included for each sample. Copy number was calculated as $C = 2^{T0\ Ct(adh1) - T0\ Ct(aflM)}$. Here, T0 *Ct* (*adh1*) is the threshold cycle number of the *adh1* reference gene in T0 leaf tissue. To distinguish between heterozygous and homozygous plants among the T2 seedlings, Zygosity (*Z*) was calculated by comparing the *Ct* values of T2 plants to T0 plants from the same events using the following equation: $Z = 2^{[T2\ Ct(adh1) - T2\ Ct(aflM)] - [T0\ Ct(adh1) - T0\ Ct(aflM)]}$ (Bubner and Baldwin, 2004).

To obtain more precise assessment of transgene copy number, the droplet digital PCR was also performed on the genomic DNA extracted from following samples: aflM14 (T0), aflM14 (T4), aflM16 (T0), aflM16 (T4), and aflM17 (T0) using the same real-time PCR primer and probe sets with the *bar* gene as the target gene and the *adh1* gene as a reference to quantify the transgene copy number in the T0 and T4 (homozygous) transgenic plants at the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL (Hindson et al., 2011; Głowacka et al., 2016; Xu et al., 2016; Collier et al., 2017).

Transfer of the Transgene Into Elite Inbred Lines for Field Evaluation

To further verify whether the reduced aflatoxin production observed in the homozygous transgenic lines was due to the presence of the transgene, two non-stiff stock (LH210 and PHW79) and two stiff-stock (LH195 and LH197) elite inbred lines were pollinated with pollen from T4 generation homozygous and null aflM14 and aflM16 plants, and the resulting ears were inoculated 2 weeks after pollination at the Louisiana State University Agricultural Center Botanic Gardens, Baton Rouge, LA.

For field evaluation of the self-pollinated T3 and T4 generation of aflM14 and aflM16 events, homozygous, heterozygous, and non-transgenic plants grown in 2016 and 2017, respectively, and of crosses of the above four elite lines with homozygous plants and null of aflM14 and aflM16 in 2018, 8–10 ears from each line were inoculated with 3.4 mL per ear of *A. flavus* AF13 conidial suspension at four injection sites in the mid-ear using an Indico tree-marking gun (Forestry Suppliers, Jackson, MS, United States) with a 15-gauge hypodermic needle. Kernels from non-inoculated ears in the field were also collected each year and used as controls. The inoculum concentration used for field inoculation in 2016 was 4×10^6 conidia/mL in 0.01% (w/v) SDS, which was adjusted to 1×10^5 conidia/mL for 2017 and 2018 due to extremely high levels of aflatoxins detected in inoculated kernels from 2016. Four intact kernels surrounding the inoculation sites were recovered after maturing and used for aflatoxin extraction and analysis for 2016 and 2017. For crosses in 2018, at least eight ears per treatment were collected. Kernels from half of each ear were mixed and ground, and three subsamples were analyzed for aflatoxin levels using HPLC.

Aflatoxin Extraction and Quantification Using HPLC

When multiple maize kernels were used for aflatoxin analysis, they were first ground into a fine powder with a coffee mill (Mr. Coffee) and then ∼60 to ∼1000 mg of ground powder was weighed and added to a 50 mL flask containing 25 mL of an 80: 20 methanol: water (HPLC grade) mixture, which was shaken at approximately 112 rpm at room temperature for 1 h. The extract was then filtered through 100-mm No. 1 Whatman filter paper into a 50-mL glass beaker. One hundred microliter of the extract was then diluted 10 fold with 100% methanol in a 1.5 mL tube and mixed well before being filtered through a 1.5-mL alumina-basic column (Sobolev and Dorner, 2002) and used for injection into HPLC for aflatoxin analysis.

The aflatoxin was quantified by reversed-phase HPLC as described in Sweany et al. (2011). Ten microliters of each sample was separated using a Waters e2695 HPLC (Waters Corp., Milford, MA, United States) with a Nova-Pak C18 4 μm 3.9 × 150 mm column at 38°C. The mobile phase

was methanol: water (37.5: 62.5) at a 0.8 mL/min flow rate. Each sample was run for 16 min with the aflatoxin B_1 (AFB_1) peak emerging at approximately 13.5 min. The detection and quantification of aflatoxin was achieved through an in-line post-column derivatization using a UV right in a Photochemical Reactor for Enhanced Detection (Aura Industries Inc., New York, United States) followed by excitation at 365 nm wavelength and 440 nm emission with a Waters 2475 FLR Detector (Waters Corp., Milford, MA, United States) (Joshua, 1993). Empower software (Waters Corp., Milford, MA, United States) was used to calculate the area under the AFB_1 peak. The peaks were manually assigned and aflatoxin quantity was calculated based on a calibration curve calculated from 4 replications of serial diluted AFB_1 standards at 1, 5, 50, 500, and 1000 ng/mL. The average of AFB_1 from the three injections of each dilution was used for standard curve calculation.

Small RNA Library Construction, Sequencing and Bioinformatics for Detecting Gene Specific Small RNA

Total RNAs were isolated from T0 leaf tissues collected in 2013 of aflM14, aflM16, and aflM11 (null), T3 leaf tissues of aflM14H, aflM16H, and B104, and the immature maize kernels of the T4 plants collected from homozygous and null of aflM14 and aflM16 as well as B104 14 days after self-pollination. After grinding into powder, maize kernel RNA was extracted by TRIzol reagent according to the manufacturer's instructions and then cleaned with RNAeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The total RNA from maize leaf tissues was isolated by RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The total RNA was checked for quality using a Nanodrop for small RNA library construction. Indexed sRNA libraries were constructed from the enriched sRNA fractions with the TruSeq Small RNA Library Preparation Kits (RS-200-0012, Illumina, San Diego, CA, United States) according to the manufacturer's instructions. Indexed sRNA libraries were sequenced on the Illumina HiSeq 2500 platform at the Genomic Science Laboratory at North Carolina State University (Raleigh, NC, United States) in 2016 (T3 leaf tissues) and on Illumina HiSeq 4000 the Genomic Sequencing Core at UC Davis (Davis, CA, United States) in 2017 (T0 leaf tissues and T4 kernels), respectively. The adapters and indexes were trimmed using Cutadapt (Martin, 2011) version 1.12, and the reads were mapped to the maize and *A. flavus* genome sequences using Bowtie (Langmead et al., 2009; Langmead and Salzberg, 2012) to identify sRNAs with a perfect match. Awk command lines were used to extract small RNA specific to the targeted gene *aflM*. R (R Core Team, 2013) was used to generate a sRNA mapping figure.

Statistical Analysis

Standard error was calculated using Excel (Microsoft Corp., Seattle, WA, United States). Statistical analysis was conducted using SAS version 9.4 (Statistical Analysis System, SAS Institute, Cary, NC, United States). Analyses of variance (ANOVAs) were calculated using Proc Mixed. *Post hoc* comparison of means was calculated using Turkey's LSD means (Saxton, 1998). Significance in this study was defined by a confidence interval ≥95% ($\alpha = 0.05$). Raw aflatoxin data were used directly in statistical analysis without transformation except those data from KSA of T1 and T2 generation and from PHW79 × aflM16H and PHW79 × aflM16N, which were log transformed to equalize variation between samples of the experiment.

RESULTS

Construction and Transformation of HIGS Vector Into Maize

The HIGS vector was constructed as described in **Supplementary Figure 1** and the final construct with inverted repeats of *aflM* fragment inserted was verified through digestions with *Eco*RV, *Mfe*I, and *Kpn*I restriction enzymes (**Supplementary Figure 2**). The fragment sizes estimated based on DNA markers were in agreement with the expected fragment sizes of the correctly assembled vector when it is digested with these enzymes, which were 2447 and 9085 bp; 299, 2447, and 8786 bp; and 1328 and 10204 bp, respectively. In addition, the correct assembly of the *aflM* inverted repeats in all four clones was also verified through sequencing with d35S-F, RNAi-R and PR10-F primers

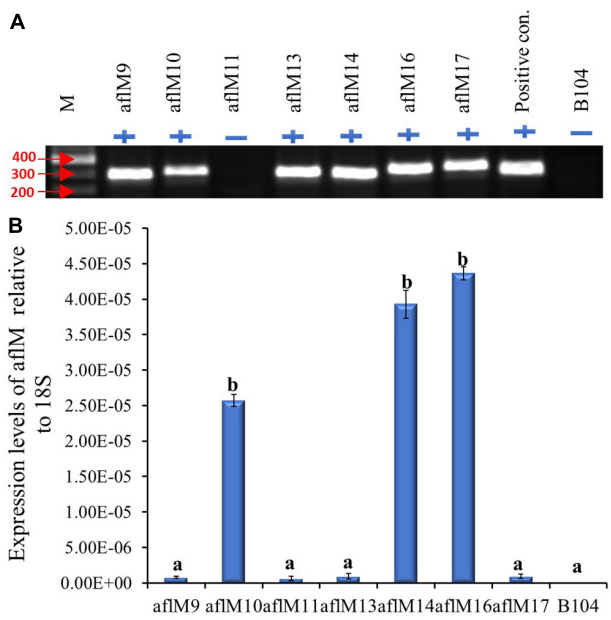

FIGURE 1 | Determining the presence and level of expression of the target gene in the T0 transgenic leaf tissues. **(A)** PCR confirmation of the presence (+) or absence (–) of target gene in versicolorin (*ver-1, aflM*) RNAi vector transformed T0 leaf tissues. AflM RNAi plasmid DNA was used as a positive control and the genomic DNA from commercial maize line B104 was used as a negative (–) control. **(B)** Expression of transgene *aflM* in the T0 leaf tissue of various transformation events relative to 18S rRNA using real time PCR. AflM11 is negative for the transgene.

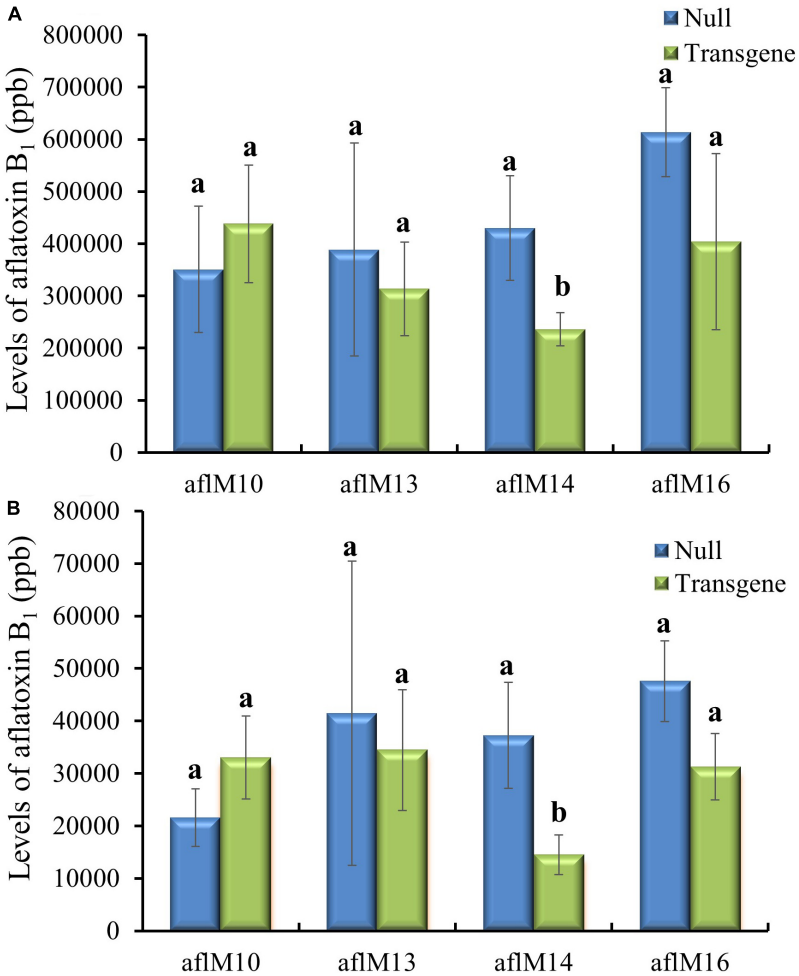

FIGURE 2 | Aflatoxin production of the T1 **(A)** and T2 **(B)** generation of transgenic seeds containing *aflM* from four different events compared to null seeds under kernel screening assay (KSA) conditions. Data presented are the mean and standard errors of ten replicates for each event. Bars labeled with the same letters are not significantly different at $P \leq 0.05$. Transgene represents the kernels that contain *aflM*. Null seeds for T1 are kernels from the same transformation events without the presence of *aflM*, and for T2 are segregating non-transgenic kernels from the same transformation events.

(**Supplementary Table 1**). This construct is capable of producing a 325-bp *aflM* dsRNA transcript with a 130-bp single-strand loop in the middle, once the transcript is processed in the host plant.

The construct was transformed into immature embryos of maize inbred line B104 through *Agrobacterium* infection in October of 2012. Twenty-three transgenic plants regenerated from seven independent transformation events were pollinated from April to May of 2013, and mature kernels were harvested in June 2013. Each event had one to six plants with a total of one to five ears per event. All events except aflM11 were confirmed positive for the presence of the target gene when genomic DNA from T0 plant leaf tissues was used as template (**Figure 1A**). Three of the positive transformation events (aflM16, followed by aflM14 and aflM10) showed significantly higher *aflM* target gene expression than the other events when the RNA extracted from T0 plant leaf tissues was examined using qRT-PCR (**Figure 1B**). AflM9, aflM13, and aflM17 had the lowest level of target gene expression (**Figure 1B**).

Characterization of T1 and T2 Generations of Transgenic Seeds

Twenty-one ears were produced from the six events that were confirmed positive for the transgene. The number of kernels ranged from 13 to 145 per ear, and average kernel weight ranged from 0.15 to 0.22 g (**Supplementary Table 2**). Ten to fifteen T1 generation transgenic kernels from each of the three events (aflM10, aflM14, and aflM16) with high levels of target gene expression and one event (aflM13) with very low level of target gene expression in the leaf tissue were selected for aflatoxin resistance analysis through KSA. Only the transgenic kernels from the aflM14 produced significantly less aflatoxin than the kernels without the transgene (null) (**Figure 2A**). T1 seeds from the aflM10, aflM13, aflM14, and aflM16 events were increased in the field in 2015 through self-pollination to the T2 generation for further analysis. Up to 60% less aflatoxin B_1 production was observed in kernels from the T2 generation of aflM14 compared

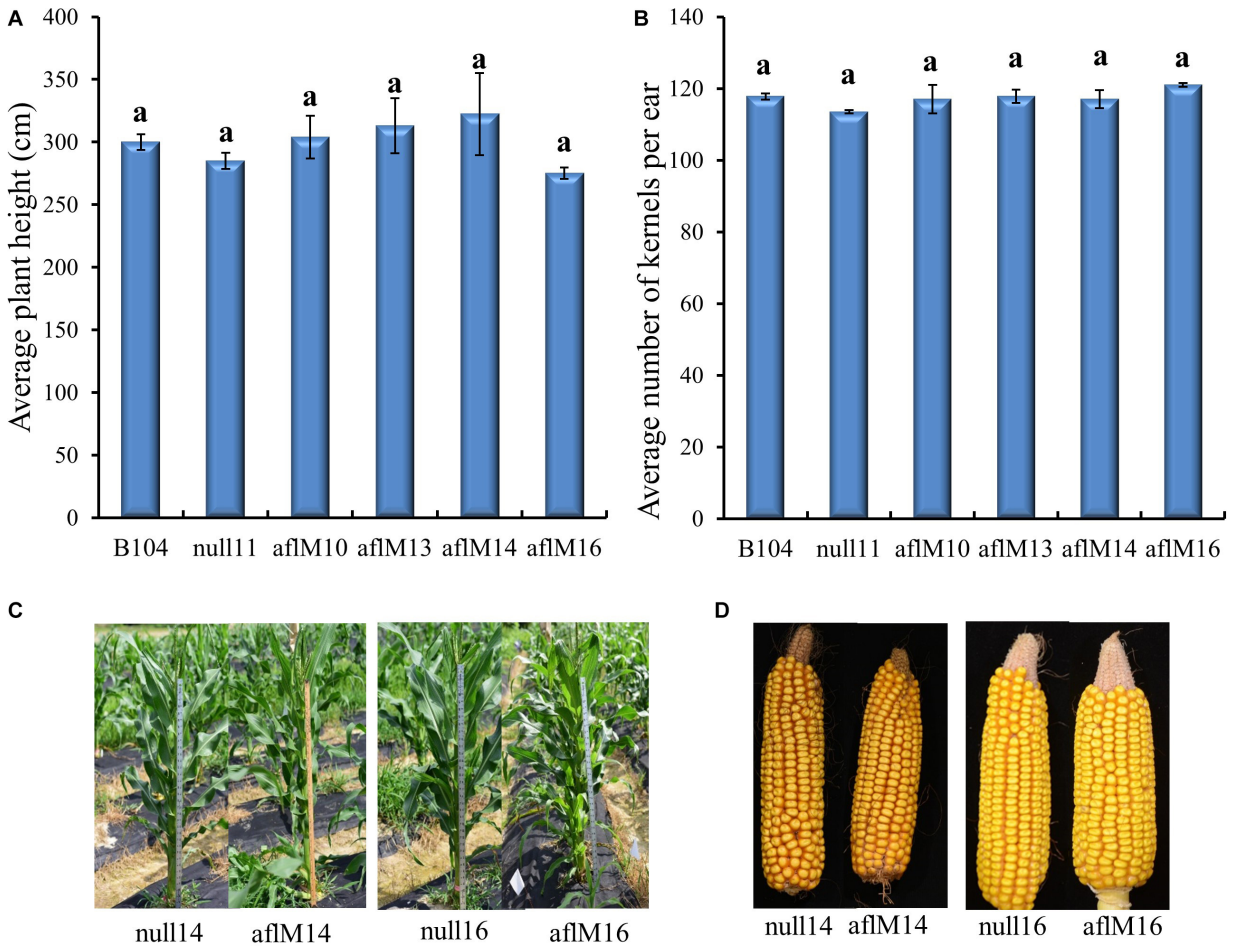

FIGURE 3 | Phenotypic assessment of transgenic plants and mature ears. **(A)** Average plant height in aflM10, aflM13, aflM14, and aflM16 in comparison to the null11 and B104 (wild type) controls at 50 days after planting at T1 generation. **(B)** Average number of T2 generation kernels per ear of aflM10, aflM13, aflM14, and aflM16 compared to null11 and B104 (wild type). Vertical bars represent standard errors of the means. Means with the same letters are not significantly different between treatments at $P \leq 0.05$. **(C)** Representative appearance of plant height of null14, aflM14, null16 and aflM16 in T3 generation at 50 days after planting. **(D)** Representative appearance of dehusked mature maize ears from null14, aflM14, null16, and aflM16 at harvest (T4).

with the null (segregating non-transgenic) when analyzed using KSA (**Figure 2B**). The kernels from the T2 generation of aflM16 also produced less aflatoxin ($P = 0.08$) than the kernels from the null control (**Figure 2B**).

Phenotypic Assessment of Transgenic Plants

T1 to T2 generation plants from each of the four transgenic events were evaluated for height and kernel number per ear. Five to eleven plants per event were measured for height at the silk stage. Three to ten ears per event were counted to determine seed number. Plant height (T1) and number of T2 kernels per ear were not significantly different between transgenic and non-transgenic plants and also among events (**Figures 3A,B**). The T3 generation transgenic plants and the resulting mature ears (T4) harvested from these plants of aflM14 and aflM16 events also showed no phenotypic differences compared to null14 and null16 (**Figures 3C,D**).

AFB_1 Production in T3 and T4 Generation Homozygous Seeds

Mature kernels from non-inoculated ears grown in the field in 2016 (T3) and in 2017 (T4) were ground and analyzed for aflatoxin levels in these kernels under natural infection. Only very low levels (<0.6 ppb for aflM16 in 2016 and <0.04 ppb for both lines in 2017) of aflatoxin was detected in those kernels, and there was no difference between transgenic lines and the null controls (**Figures 4A,B**). However, significantly high levels of aflatoxin were detected after these kernels were surface-sterilized and incubated under 100% humidity at 30°C for 7 days without inoculation (**Figures 4C,D**), indicating the presence of sufficient levels of *A. flavus* inoculum inside the naturally infected kernels. It is also clear that the transgenic kernels had significantly lower levels of aflatoxin than their null controls for both events from both years ($P \leq 0.003$–0.05), with 54.2–95.3% reduction in aflatoxin production compared to that in null kernels (**Figures 4C,D**).

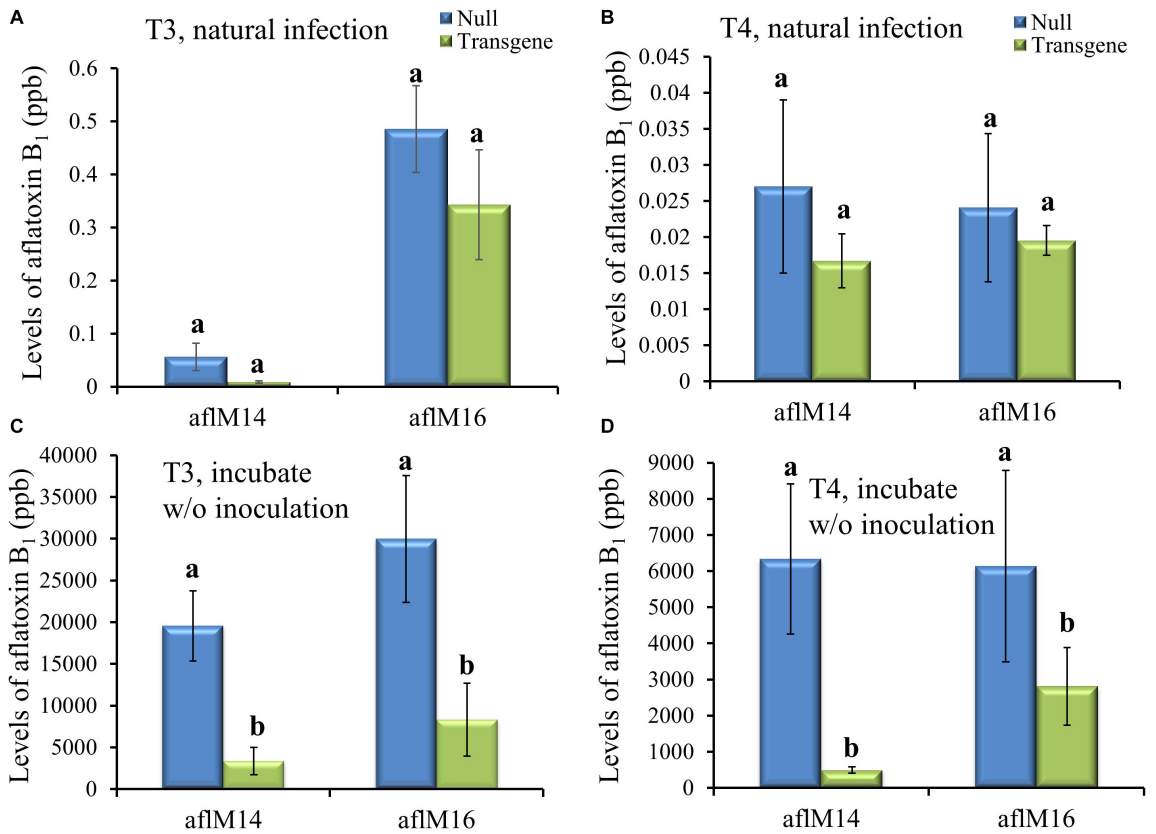

FIGURE 4 | Aflatoxin production in the transgenic and null kernels of two different events in 2016 and 2017. Aflatoxin production in T3 (2016) generation **(A, C)** and T4 (2017) generation **(B, D)** transgenic and null kernels without *aflM* under field natural infection **(A, B)** and after incubation under laboratory Kernel Screening Assay conditions without inoculation **(C, D)**. Data are the mean and standard errors of 12–36 replicates of each event. Bars with different letters are significantly different at $P \leq 0.05$. Transgene represents the kernels that contain *aflM* gene. Null represents the segregating non-transgenic kernels from the same event.

Mature kernels from field inoculated T3 and T4 generation ears were also analyzed for aflatoxin levels. The field-inoculated T3 generation kernels homozygous for *aflM* showed significantly reduced (up to 42.2–76.4%) aflatoxin contamination compared to kernels from the null (segregating non-transgenic) ($P \leq 0.04$) for both aflM14 and aflM16 events (**Figure 5A**). Homozygous transgenic kernels from T4 generation also contained significantly less (68.0% reduction) aflatoxin than the null control under field inoculation conditions in 2017 ($P \leq 0.04$) (**Figure 5B**). Overall, significant reduction in aflatoxin production was observed for transgenic maize lines in field inoculation and in incubation of naturally infected kernels under KSA conditions. These results demonstrated clearly that HIGS targeting of the *aflM* gene significantly reduced aflatoxin production in the homozygous transgenic kernels.

Zygosity and AflM Transgene Copy Number Estimation in Different Transformation Events

In order to identify homozygous T2 seedlings from the segregating T2 population for seed increase, genomic DNA from T2 seedlings and T0 leaf tissues of the two events was used to determine the threshold number of cycles of *adh1* reference gene and the target *aflM* gene in each of the samples, which were then used to estimate zygosity of the T2 plants based on the ratio of aflM copy number in T2 vs in T0: $2^{[(T2Ct(adh1)-T2Ct(aflM)]-[T0Ct(adh1)-T0Ct(aflM)]}$ (**Table 1**). The ratio for homozygous seedlings from AflM14 event ranged from 1.90 to 2.03 and for the AflM16 event was from 6.40 to 6.55. This high value indicated the possible presence of multi-copies of target gene in the AflM16 event. However, our number of transgene integrations based on target gene segregation in T2 seedlings and Chi-square analysis indicated that both events have a single integration (**Supplementary Table 3**). To resolve this apparent conflicting information, the more accurate, Southern blot hybridization equivalent, droplet digital PCR was also performed to verify the copy number of the above transgenic lines using genomic DNA from T0 and T4 seedlings. The ratio of calculated gene copy number of *bar*/*adh1* for genomic DNA samples from aflM14(T0), aflM16(T0), aflM17(T0) ranged from 0.5 to 0.54 (**Table 2**), confirming these three events are single-copy hemizygous for the transgene. The droplet digital PCR also confirmed that aflM14(T4) and aflM16 (T4) are homozygous for the transgene based on the ratio of calculated gene copy number of *bar*/*adh1* (ranging from 0.86 to 0.96) (**Table 2**).

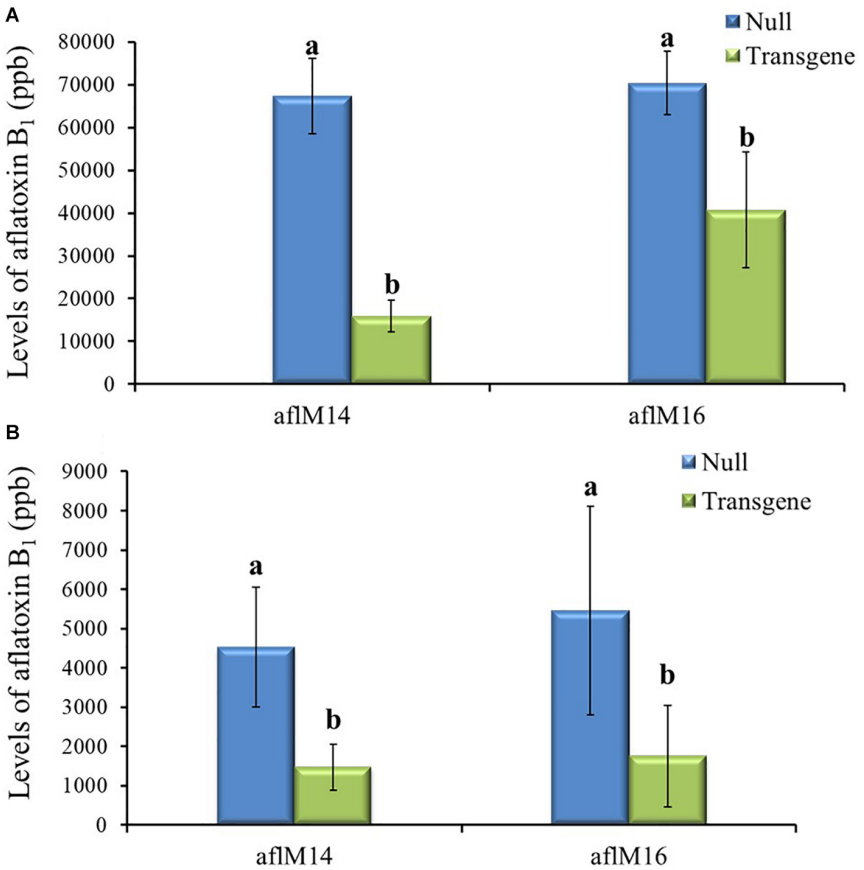

FIGURE 5 | Aflatoxin production in the transgenic and null kernels of two different events in 2016 and 2017 under field inoculation conditions. Aflatoxin production in T3 (2016) generation **(A)** and T4 (2017) generation **(B)** transgenic and null kernels without *aflM* under field inoculation condition. Data are the mean and standard errors of 28–36 replicates of each event. Bars with different letters are significantly different at $P \leq 0.05$. Transgene represents the kernels that contain *aflM* gene. Null represents the segregating non-transgenic kernels from the same event.

Crossing of the Transgene Into Elite Inbred Lines Resulted in Reduced AFB_1 Production in the F1 Crosses

In crosses with non-stiff stock elite inbred lines, the resulting kernels of LH210 × aflM14H (homo) or aflM16H produced significantly less aflatoxin (60–80% reduction) compared to those in the kernels of LH210 × aflM14N (null) or aflM16N with $P = 0.0056$ and $P = 0.0452$, respectively, under field inoculation conditions (**Figure 6A**). The kernels of PHW79 × aflM14H (homo) or aflM16H crosses also supported significantly less aflatoxin compared to those in the kernels of PHW79 × aflM14N (null) or aflM16N with $P = 0.0023$ and $P = 0.02$, respectively (**Figure 6B**). In crosses with stiff stock lines (LH195 and LH197) and under field inoculation conditions, the resulting kernels of LH195 and LH197 × aflM14H or aflM16H crosses supported significantly less aflatoxins compared to those in the kernels of LH195 and LH197 × aflM14N (null) or aflM16N crosses with P values ranging from 0.0001 to 0.0183 (**Figures 6C,D**). In addition, among the four inbred lines used in the crosses, LH197 appeared to be the most susceptible one and supported 10 times more aflatoxin production than PHW79, 15 times more than LH210, and 30 times more than LH195, which is the most resistant one (**Figure 6**).

TABLE 1 | Zygosity estimation of T2 seedling population from both aflM14 and aflM16 events using real time PCR.

Independent transgenic plant	$2^{(T2Ct(adh)-T2Ct(aflM)}/2^{(T0Ct\ (adh1)-T0Ct(aflM)}$		
	Heterozygous	Homozygous	Ratio
aflM14-1	0.87 ± 0.14	1.90 ± 0.11	1:2.2 ± 0.22
aflM14-2	1.05 ± 0.07	2.03 ± 0.05	1:1.9 ± 0.14
aflM16-1	3.09 ± 0.02	6.55 ± 0.46	1:2.1 ± 0.13
aflM16-2	3.12 ± 0.01	6.40 ± 0.04	1:2.04 ± 0.02

Detection of High Levels Gene-Specific Small RNAs in the Transgenic Leaf and Kernel Tissues

In order to determine whether the enhanced aflatoxin resistance in the homozygous transgenic kernels compared to the null was due to the presence of *aflM* specific small RNA produced from the introduced RNAi vector, small RNAs from

TABLE 2 | Transgene copy number analysis through droplet digital PCR of genomic DNA from leaf tissues of T0 and T4 transgenic plants.

Event	Bar copy/20 μL	Adh1 copy/20 μL	Bar/Adh1	Copy number
aflM14 (T0)	278	514	0.54	1 (hemi)
aflM16 (T0)	414	828	0.5	1 (hemi)
aflM17 (T0)	342	660	0.52	1 (hemi)
aflM14 (T4)	1110	1154	0.96	1 (homo)
aflM16 (T4)	666	774	0.86	1 (homo)

The target gene copy number was calculated based on the ratio of number of target bar gene (phosphinothricin acetyltransferase gene) molecules in the construct compared to maize single copy reference alcohol dehydrogenase gene (adh1) in the genomic DNA samples.

T0 and T3 leaf tissues and from T4 kernel tissues were sequenced and analyzed. The total number of reads from the libraries of aflM14 homo and aflM14 null T3 leaf tissues was about 40 and 30 million (**Table 3**), respectively, and the total number of reads for B104 was over 62 million. After filtering out the reads that were aligned to the maize genome, 3,164 reads from the leaf tissue of aflM14 homo transgenic plants were specifically aligned to the *aflM* target gene, whereas only 4 and 1 reads from the aflM14 null and B104 controls were aligned to the *aflM* gene (**Table 3**), respectively. The total number of small RNA reads derived from the immature kernel tissues of T4 generation aflM14 and aflM16 were 1,532,829 and 1,800,225 (**Table 3**), respectively. Three hundred fifty-nine and 197 reads were *aflM* specific for the aflM14 and aflM16 events, respectively (**Table 3**), compared to 1 and 2 *aflM*-specific reads observed for the null aflM14 and null aflM16 controls (**Table 3**). These results are consistent with data obtained from T0 leaf tissue of alM14 and aflM16 that were collected in 2013 from greenhouse grown plants (**Table 3**).

Furthermore, the distribution of *aflM*-specific small RNAs on the target gene was also examined in aflM14 and aflM16 (**Figures 7A,C**). Based on the small RNA distribution map, most of the small RNA appeared to be generated from a few hot spots in the 330 bp target sequence. The results of the small RNA distribution in the target gene were similar for both events (**Figures 7A,C**). However, the most abundant small RNA in aflM14 was 21 nt in length, followed by one that was 24 nt long (**Figure 7B**), whereas in aflM16, the most abundant small RNA was 24 nt in length, followed by one that was 21 nt long (**Figure 7D**).

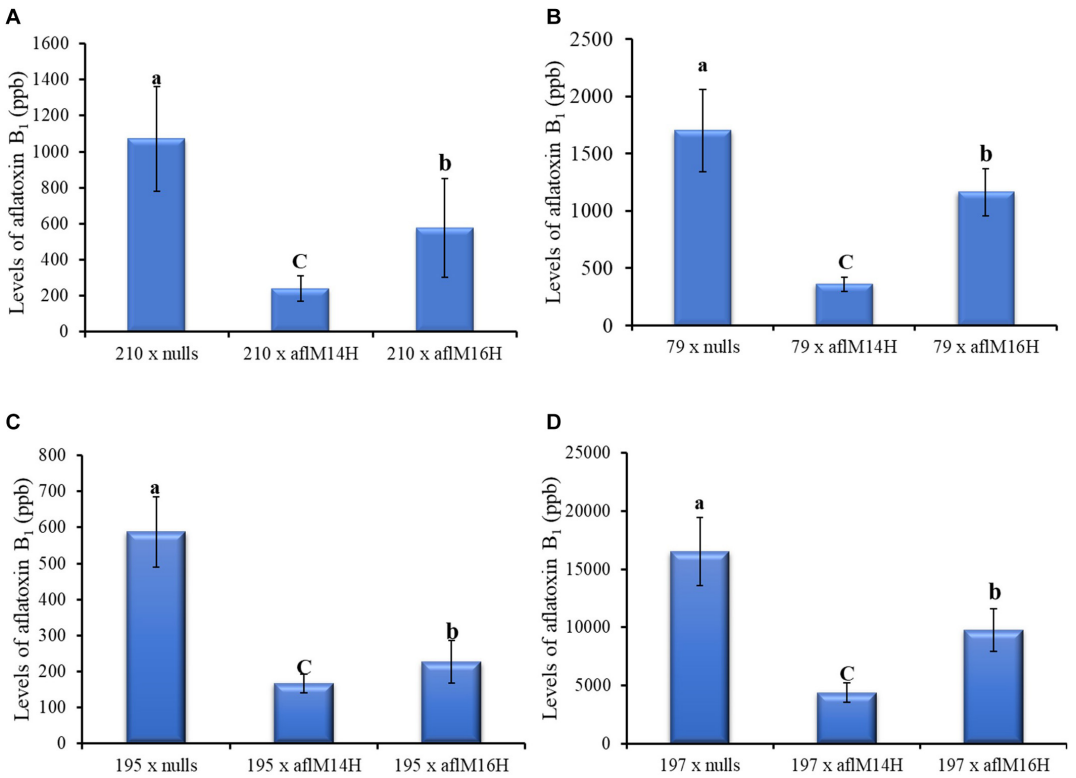

FIGURE 6 | Aflatoxin production in crosses of two non-stiff stalk (**A**, PHW79 or 79; and **B**, LH210 or 210) and two stiff stalk (**C**, LH195 or 195; and **D**, LH197 or 197) elite inbred lines with aflM14 (homozygous and null) or aflM16 (homozygous and null) lines under field inoculation conditions. At least eight ears per treatment were collected. Kernels from each half ear were ground and three subsamples were analyzed for aflatoxin levels using HPLC. The aflatoxin data of elite line crossing with nulls were a combined data from elite line crossing with both aflM14N and aflM16N. Data presented here are the mean and standard errors of at least 24 replicates of each cross. Bars with the same letter were not significantly different at $P \leq 0.05$. All of the analyses were done using non-transformed raw data except those from PHW79 × aflM16H and PHW79 × aflM16N, which were log transformed to reduce sample variation.

TABLE 3 | Number of small RNA reads in leaf tissues and immature kernel tissues of transgenic and non-transgenic maize lines.

Tissue type*	Events	Total read	Reads aligned to maize genome	Reads aligned to *A. flavus* > 1 times	Reads aligned to *A. flavus* 1 time	Reads aligned to *aflM*
Leaf tissue (T0) collected in 2013	aflM14	1,300,823	834,079	107	2,076	1,372
	aflM16	1,254,164	963,063	74	1,706	1,256
	aflM11 (null)	1,203,478	869,307	60	1,301	3
Leaf tissue (T3) collected in 2016	aflM14 homo	40,222,099	30,003,837	1,233	5,894	3,164
	aflM14 null	30,795,339	29,030,160	17,516	26,750	4
	B104 (WT)	62,902,688	61,179,007	5,285	6,236	1
Immature kernels (T4) collected in 2017	aflM14 homo	1,532,829	989,008	86	670	359
	aflM14 null	1,552,692	1,111,600	446	86	1
	B104 (WT)	1,367,547	796,105	670	732	0
	aflM16 homo	1,800,225	1,163,932	88	599	197
	aflM16 null	1,588,892	1,294,348	45	321	2

**The small RNA libraries from T3 leaf tissues collected in 2016 were sequenced on Illumina HiSeq 2500 Platform at NC State University and the small RNA libraries from T0 leaf tissues collected in 2013 and T4 immature kernel tissues collected in 2017 were sequenced on Illumina HiSeq 4000 Platform in 2017 at UC Davis.*

DISCUSSION

The present study investigated the changes in aflatoxin resistance in transgenic maize lines containing HIGS construct targeting *aflM* of *A. flavus* in two independent events and found that both homozygous transgenic lines produced significantly less aflatoxins under repeated field inoculation studies. This enhanced aflatoxin resistance in the transgenic lines coincides with the presence of high levels of gene- specific small RNAs in their leaf and kernel tissues. Transferring this gene into elite inbred lines through crossing also led to enhanced aflatoxin resistance in the resulting F1 crosses containing the transgene. This study demonstrates that reduction of aflatoxin production through HIGS targeting the *A. flavus* aflatoxin biosynthesis pathway genes can be a practical and sustainable approach to manage aflatoxin contamination in maize and other susceptible crops.

During initial evaluation of different independent transformation events and later characterization of the progenies of aflM14 and aflM16 events, it was clear that transgenic lines developed from different events had different efficacy in reducing aflatoxin production. Homozygous transgenic kernels from the aflM14 event always produced less aflatoxin than those from the aflM16 event. One possible reason could be the dosage (copy number) effect. Therefore, real time PCR was first attempted to determine the target *aflM* gene copy number using the single copy *adh1* gene as a reference, which suggested that aflM16 could have multiple copies of the target gene (**Table 1**). However, due to the well-known varying accuracy (ranging from 14 to 100%) of real time PCR in gene copy number assessment (Bubner and Baldwin, 2004) and the apparent contradiction to our number of transgene integration loci calculation based on chi-square analysis (**Supplementary Table 3**), the droplet digital PCR was performed using the same genomic DNA samples, which confirmed both events to have a single copy of integration. The accuracy of droplet digital PCR in comparison to Southern blot analysis in determining gene copy number has been well established (Głowacka et al., 2016; Collier et al., 2017). Another possible explanation of such differences is the result of random integration of the T-DNA into the maize genome during the initial *Agrobacterium* transformation process. The position of the T-DNA insertion in chromosome and the chromatin structure of the area surrounding the transgene insertion can influence transgene expression (Dean et al., 1988; Peach and Velten, 1991; Breyne et al., 1992). Such a "chromosomal position effect" has been widely reported (Alberts and Sternglanz, 1990; Kumpatla et al., 1998; Matzke and Matzke, 1998), even though not all event-to-event variation can be explained by such an effect, according to Petolino and Kumar (2016).

A 42–76% reduction under field condition and 54.2–95.3% reduction under KSA in aflatoxin production was observed in the two events compared to the controls, which is similar to what has been reported in earlier studies in transgenic maize and peanut using similar approaches (Masanga et al., 2015; Thakare et al., 2017; Sharma et al., 2018). However, our data were based on much larger sample sizes and on multi-year field studies with additional laboratory KSA confirmations as well as highly sensitive HPLC analysis of aflatoxin B_1 production.

The intrinsically high variation of aflatoxin production among different maize kernels of the same line makes evaluating changes in aflatoxin resistance of the HIGS construct-containing transgenic maize lines a challenge. In order to reduce such variations under field inoculation conditions and get a true assessment of aflatoxin levels, four different sites per ear were inoculated with *A. flavus*, multiple kernels surrounding each inoculation site were collected and up to 15 ears per line were inoculated for analysis of toxin production. This field inoculation study was also conducted over a period of three years to further rule out any possible impact caused by environmental differences, which have been known to affect aflatoxin production in maize (Cotty et al., 2007;

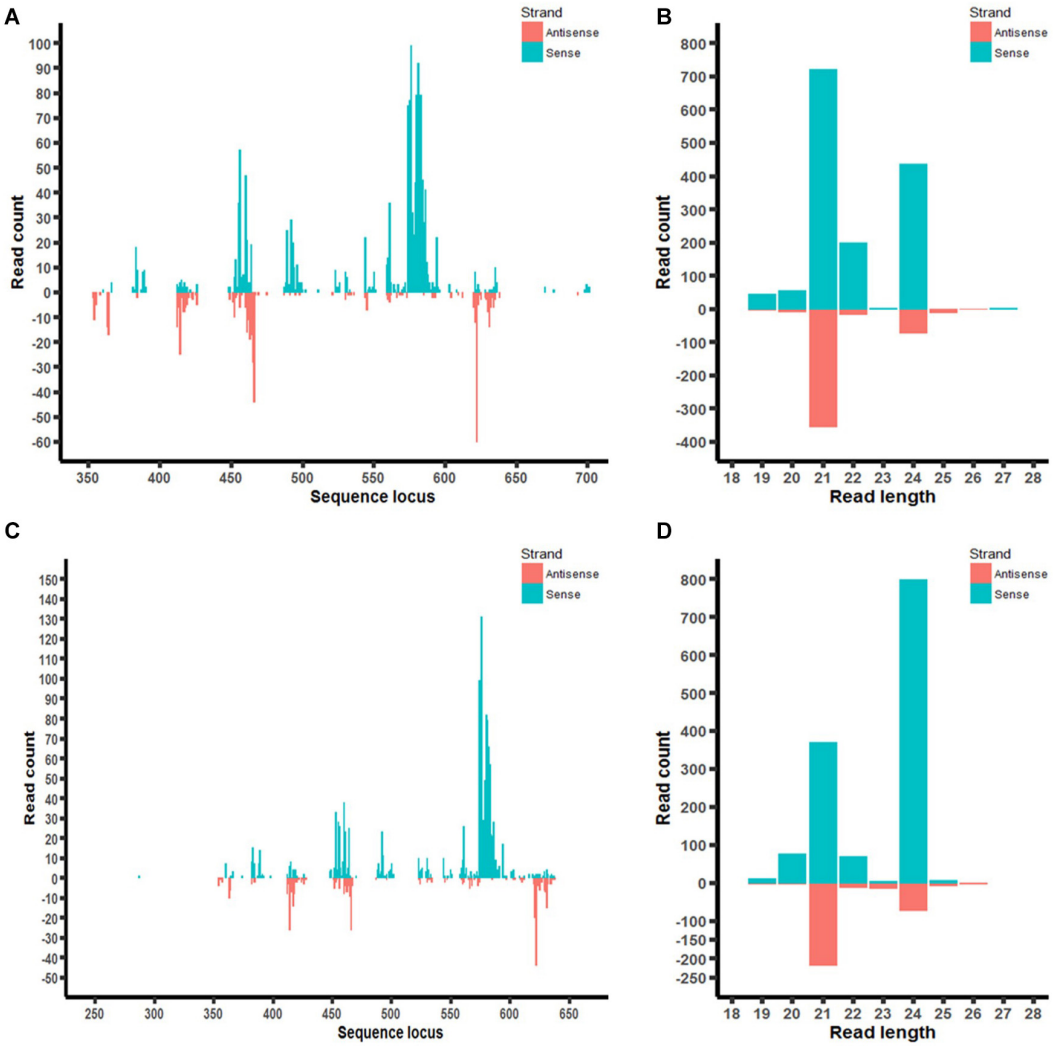

FIGURE 7 | Small RNA profiling (RNAseq analysis) of *aflM* target gene in transgenic maize leaf tissue. **(A,C)**: Distribution of *aflM* specific small RNAs isolated from T0 leave tissue of aflM14 **(A)** and aflM16 **(C)** aligned to the target gene sequence. **(B,D)**: Read length distribution of sRNAs mapped to *aflM* from leaf tissue of aflM14 **(B)** and aflM16 **(D)**.

Fountain et al., 2014). In addition, KSA was performed to verify the levels of toxin production under more uniform inoculation and more controlled environmental conditions. The toxin data from both the field and KSAs showed good agreement among them. To the best of our knowledge, this is the first report demonstrating the efficacy of HIGS in reducing aflatoxin contamination through both repeated field and laboratory studies.

Analysis of naturally infected kernels only detected very low levels of aflatoxin in both transgenic and control kernels, indicating the necessity of performing artificial inoculations to separate the resistance between transgenic and control line. Our field inoculations and KSAs subjected the kernels to extremely high inoculum concentrations, under which the transgenic plants still had significantly less aflatoxin than the controls. The aflatoxin levels in the inoculated transgenic lines, however, were still much higher than the 20 ppb limit set by FDA (Park and Liang, 1993). These plants are unlikely to encounter such extremely high inoculum concentration under natural infection conditions. Therefore, it is reasonable to speculate that the toxin levels in these transgenic lines under natural infection conditions would be much lower than under artificial inoculation conditions. Field inoculation studies conducted in 2017 and 2018 also supported the above speculation. Overall aflatoxin production was much lower in both control and transgenic lines in 2018 when the inoculum concentration was reduced from 4×10^6 in 2016 to 1×10^5 conidia/mL.

Sequencing of small RNA libraries constructed from T0 and T3 leaf tissues as well as from T4 kernel tissues confirmed the presence of high levels of gene-specific small RNAs in the homozygous transgenic leaf and kernel tissues compared to B104 and null controls. These high levels of gene specific small RNAs

can only come from the HIGS RNAi vector since all the samples used for small RNA sequencing study were from field-grown or greenhouse (T0 leaf) plants without inoculations, indicating the observed enhanced aflatoxin resistance in the transgenic lines was due to the siRNAs produced from the transformed HIGS vector. The RNA sequencing study also revealed that the double 35S promoter used in the present study drove more gene specific small RNAs expression in the leaf tissues than in the kernel tissues. Future studies should use a seed-specific and stress- or infection-inducible promoter to reduce the energy cost and possible yield reduction due to constant expression of the transgene in the whole transgenic plants.

Several recent studies have reported suppression of fungal diseases through direct applications of dsRNA (Koch et al., 2016; Wang and Jin, 2017; McLoughlin et al., 2018; Song et al., 2018). In addition, small RNAs have been reported to be transported locally from cell to cell through plasmodesmata and over long distances through plant phloem systems (Liu and Chen, 2018). Therefore, future studies could also examine the feasibility of direct application of *in vitro* synthesized dsRNA targeting *aflM* as a more practical and effective way of managing aflatoxin contamination in maize and other susceptible crops. Although this GMO-free RNAi approach is appealing (Dalakouras et al., 2019), one factor that may limit the direct application of dsRNA as a practical disease control approach is the lack of sufficient secondary amplification (Song et al., 2018). Successful disease control may require frequent reapplication of dsRNA to maintain a high level of dsRNA on leaf surface for this to work (Song et al., 2018). In comparison with external application of dsRNAs, genetic transformation to suppress the fungal target genes through HIGS is likely to result in more consistent presence of high levels of siRNA and to offer a more sustainable approach in managing aflatoxin contamination in maize and other susceptible crops.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI SRA database under the following accession number: PRJNA577960.

AUTHOR CONTRIBUTIONS

YR and Z-YC designed the research. YR, OO, QW, and Z-QH performed the research. YR and DH analyzed the data. YR and Z-YC wrote the manuscript. KW directed the production of the maize transgenic lines; JC and KR provided technical support.

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ORIGINAL RESEARCH
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Use of Dual RNA-seq for Systems Biology Analysis of *Zea mays* and *Aspergillus flavus* Interaction

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The interaction between *Aspergillus flavus* and *Zea mays* is complex, and the identification of plant genes and pathways conferring resistance to the fungus has been challenging. Therefore, the authors undertook a systems biology approach involving dual RNA-seq to determine the simultaneous response from the host and the pathogen. What was dramatically highlighted in the analysis is the uniformity in the development patterns of gene expression of the host and the pathogen during infection. This led to the development of a "stage of infection index" that was subsequently used to categorize the samples before down-stream system biology analysis. Additionally, we were able to ascertain that key maize genes in pathways such as the jasmonate, ethylene and ROS pathways, were up-regulated in the study. The stage of infection index used for the transcriptomic analysis revealed that *A. flavus* produces a relatively limited number of transcripts during the early stages (0 to 12 h) of infection. At later stages, in *A. flavus*, transcripts and pathways involved in endosomal transport, aflatoxin production, and carbohydrate metabolism were up-regulated. Multiple WRKY genes targeting the activation of the resistance pathways (i.e., jasmonate, phenylpropanoid, and ethylene) were detected using causal inference analysis. This analysis also revealed, for the first time, the activation of *Z. mays* resistance genes influencing the expression of specific *A. flavus* genes. Our results show that *A. flavus* seems to be reacting to a hostile environment resulting from the activation of resistance pathways in *Z. mays*. This study revealed the dynamic nature of the interaction between the two organisms.

Keywords: interactome, maize, *Aspergillus flavus*, aflatoxin, gene regulatory network

INTRODUCTION

Zea mays is one of the three largest cereal crops in the world (*Z. mays*, *O. sativa*, and *T. aestivum*) in terms of annual production and in the United States alone 13.8 billion bushels of corn was produced for the year 2020. It is also crucial as a staple crop that feeds millions of people and animals daily. However, corn yields are affected by diseases caused by pests, including fungal pathogens such as *Aspergillus flavus*. *A. flavus* is an opportunistic pathogen that adopts a necrotrophic lifestyle, causing cell death in the host and feeding on dead host tissue. *A. flavus*

has also been shown to infect multiple crops such as *Gossypium hirsutum*, *Arachis hypogaea*, and *Prunus dulcis* (Bedre et al., 2015; Fountain et al., 2015a). In addition to its effect on plant health, *A. flavus* can also affect animal and human health due to the production of aflatoxins in infected crops (Nesbitt et al., 1962; Cole, 1986, Ostry et al., 2017). Aflatoxins, notably aflatoxin B1, are potent mycotoxins and potential carcinogens when consumed by animals. Hence, many countries have adopted laws that restricting the marketing of crops that are contaminated with aflatoxins in certain national or international markets. Additionally, an intensive effort has been made to study the *A. flavus - Z. mays* interaction at multiple levels to identify factors involved in crop yield loss. Similarly, extensive research has been conducted to study the life cycle and biology of the pathogen *A. flavus* itself in genes such as VeA and LaeA (Amare and Keller, 2014; Fountain et al., 2015a, Tang et al., 2015).

The results from genome comparison with other *Aspergillus* species have led to the identification of several genes that are keys to the production of secondary metabolites and other pathogenicity factors in *A. flavus*, (Rokas et al., 2007; Ehrlich and Mack, 2014). The *A. flavus* aflatoxin gene cluster has been elucidated by mutational studies. Some genes in this cluster have also been linked to multiple developmental processes (Yu et al., 2004; Price et al., 2006). At the cellular level, the velvet complex is made up of multiple developmental genes that also have an effect on secondary metabolism (Calvo et al., 2004). Likewise, *LaeA*, another developmental gene in *A. flavus,* linked to pathogenesis and secondary metabolism, was identified through comparison to *A. nidulans* (Kale et al., 2008; Amaike and Keller, 2009, Chang et al., 2013). Other studies have highlighted the importance of hydrolytic processes and cellular transport at the cellular level during pathogenesis, including the development of aflatoxisomes, special organelles that harbor enzymes essential for the biosynthesis of aflatoxins in *A. flavus* (Kistler and Broz, 2015). Early work attempting to understand the crosstalk between *A. flavus* and *Z. mays* led to the identification of lipoxygenases in both *A. flavus* and *Z. mays* as key genes in this process (Brown et al., 2009). Since then, the girth of *Aspergillus* genomic data has allowed the discovery of many new interactions highlighting the involvement of proteins such as the *A. flavus* small ubiquitin-like modifiers *PdeH* in the interaction between *A. flavus* and *Z. mays* (Nie et al., 2016; Yang et al., 2017).

Genomic studies with *Z. mays* have also been successful in elucidating mechanisms controlling resistance to fungal pathogens. Unlike *A. flavus*, there are multiple post-genomics resources available to *Z. mays* researchers, such as publicly available interactomes, that can be used to associate complexes and pathways with interacting proteins (Musungu et al., 2015). Nevertheless, recent studies investigating resistance of *Z. mays* to *A. flavus* almost exclusively involved utilizing singular genomics approaches (using information from one genome at a time) such as RNA-seq and DNA-seq. These have been able to identify and analyze key maize disease resistance proteins such as PR10, PR5, chitinases, trypsin inhibitors, and a vast array of other genes that contribute to the resistance in *Z. mays* to *A. flavus* and other pathogens (Brown et al., 1995; Chen et al., 2007). Moreover, recent "single genomics" transcriptomic studies have shown that the activation of key pathways, such as the jasmonate, ethylene biosynthesis and several other signaling pathways, are implicated in the resistance of *Z. mays* to *A. flavus* (Burow et al., 1997; Scarpari et al., 2014, Christensen et al., 2015). Breeding and genetic marker analysis efforts using genome wide association and quantitative trait loci studies indicate that the *A. flavus* – *Z. mays* interaction involves multiple genes for resistance. These studies also show that there is a strong environmental influence on resistance, which complicates breeding for resistance to *A. flavus* in *Z. mays* (Wisser et al., 2006).

A significant limitation of the current body of genomic work that tackles the *A. flavus* – *Z. mays* interaction, is the tendency to analyze data from the pathogen and the host separately. Although many co-expression networks can be found in the literature, they describe transcription in *A. flavus* or *Z. mays* individually (Sekhon et al., 2013; Asters et al., 2014). Thus, in those studies, interactions between pathogenicity factors in *A. flavus* and resistance genes in *Z. mays* merely denote inferences of association. To gain a better understanding of these interactions in the early stages of the infection, dual transcriptomic analysis of both host and pathogen was undertaken in this study using high depth RNA sequencing. Previous work involved understanding *A. flavus* and *Z. mays* in a small study where there was limited replication reducing the power for making statistical inferences. The study did, however, find that there was significant correlation between pathways in *Z. mays* and *A. flavus* (ref). The experimental design was that of a high-density time series transcriptomic study that allowed the use of casual inference to predict gene regulatory interactions, and to identify key pathways that are active during the early stages of the infection. This provides insight into different gene regulators that are activated at specific times during the infection process, and thus allows for reverse engineering of the entire regulatory pathway. When combined into a gene regulatory network (GRN), the inference of cause-effect relationship between co-regulated genes in pathways within and across species can be comprehensively mapped. Two algorithms were used; GeneNet, a partial correlation/partial variance-based algorithm, and TDARACNE, a time delay algorithm. Both algorithms can determine cause and effect (Opgen-Rhein and Strimmer, 2007; Schaefer et al., 2010, Schaefer et al., 2015). In this work, we used this systems approach to describe the underlying genetics of the molecular interactions between a host and a pathogen in the early stages of infection (SI). Likewise, we provide the first attempt to infer regulatory connections between *Z. mays* and *A. flavus*.

MATERIALS AND METHODS

Growth and Inoculation of Maize

The maize inbred line B73 was grown in the field in Clayton, NC at the Central Crops Research Station at North Carolina State University, during the years 2011 and 2013. Both years were planted on April and grown according to standard practices. Ears were hand pollinated on July 5–8 and covered with a paper bag. *A. flavus* NRRL *3357*, was grown on potato dextrose agar (PDA) and collected from plates with 0.05% (v/v) Triton X-100. In July,

a time course study was performed by pin bar inoculating one ear (per time point) from eight maize B73 with *A. flavus* NR3357, and harvesting at 0, 6, 12, 18, 24, 30, 36, 42, 48, 72, 96, 120, and 140 h post inoculation. Samples were frozen in liquid nitrogen, placed on dry ice, and stored at −80°C until RNA was isolated.

RNA Isolation

Eight kernels per ear were grounded using a mortar and pestle in order to isolate RNA. Approximately one hundred milligrams of ground tissue was homogenized (Virtis, Gardiner, NY, United States) in saturated phenol, pH 6.6 for 2 min. Samples were then dissolved in Tris EDTA buffer, pH 8.0 (ACROS Organics, Morris Plains, NJ, United States), extracted with 5:1 acid phenol: chloroform, pH 4.5 (Fisher), and the RNA precipitated with ice-cold 100% ethanol (ACROS Organics) overnight. Total RNA was purified again with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality (RIN > 8) and concentration of RNA was analyzed using an RNA Pico chip on an Agilent Bioanalyzer (Agilent, Santa Clara, CA, United States).

Sequencing

cDNA library construction and sequencing was performed at the Genomic Sciences Laboratory, North Carolina State University. Individual libraries were made from each time point, pooled and run on a single lane. Sequencing was performed on an Illumina HiSeq 2500 platform. The data from the RNA-seq can be accessed at NCBI using the accession (GSE101899).

Bioinformatics Analysis

For both *Z. mays* and *A. flavus,* mapping, trimming and fastqc quality control of the reads was done with CLC workbench 4.9 (Workbench, 2010). CLC genomics workbench default parameters were used to perform the mapping and trimming similar to previous publication (Musungu et al., 2016). The reference genomes used in the study where *Z. mays* (AGPv3, INSDC Assembly GCA_000005005.5, Apr 2013) and *A. flavus* (JCVI-afl1-v2.0, INSDC Assembly GCA_000006275.1, Jan 2009). Reads that had a total gene count less than 1 were removed from the counts table. The unique reads from CLC genomics workbench were then used with Deseq2 package in R statistical program (**Table 1**) with the default settings (Love et al., 2014). For contrast selection in DESeq2, SI-1 was considered to be the control. Whereas for the time-course control, the time point samples 0 h were used as the control (**Table 1**). For *A. flavus,* only samples from SI-8 to 18 where used in the differential expression analysis. The heat maps of the differential expressed genes were analyzed using K-means clustering and hierarchal clustering using Tcluster3 (de Hoon et al., 2004).

Gene Network Generation

For gene network analysis, the samples fold changes = [$gene_i$ – $gene_{i+n}$ (average)] were calculated by using the DESeq2 regularized log transformed data (**Supplementary R Script 2**) and then using R (**Supplementary R Script 1**). The R package (GeneNet) was then used for network generation. The criteria for selection into the high confidence networks was a significant partial correlation q-value (0.05) and a significant direction *q*-value (0.05) initially and an additional filtering of the *q*-val.dir $< 10^{-5}$. The gene network also included a "low confidence set" of genes. This set consisted of edges that only had a significant direction *q*-value. The cytoscape 3.0.1 visualization software was used to visualize and display the gene expression network. The edges were depicted as directed graphs to display the causal inference between genes.

TABLE 1 | The sum of unique reads that aligned to the Maize and *A. flavus* genomes.

Time	Maize	*Aspergillus*
0 h	183546459	16774
4 h	175225669	3700771
6 h	165052452	7223
12 h	191346756	18611
18 h	189130692	119690
24 h	177574520	127124
30 h	149353775	210432
36 h	175423737	765709
42 h	173900923	3029592
48 h	168062408	2716143
72 h	167752678	21747211
96 h	167752678	21747211
120 h	129154376	45150333
144 h	88430362	69915184

RESULTS

RNA-seq Analysis During Infection of *Z. mays* Kernels by *A. flavus*

Zea mays kernels were infected by pinning with a conidial suspension of *A. flavus*. Pinned kernels were harvested for RNA extraction immediately after pinning (time point 0), and at different time points up to 6 days post inoculation. To analyze gene expression in both *Z. mays* and *A. flavus,* eight biological replicates of each of the time points were sequenced. The Illumina HiSeq reads were separated by organism by simultaneous mapping to both *Z. mays* and *A. flavus* genomes. RNA sequences of *Z. mays* and *A. flavus* were processed by quantile normalization of counts per million of counts uniquely mapping to each gene model. Only unique reads were retained to calculate normalized gene expression as RPKM (Reads Per Kilobase of transcript per Million mapped reads). On average, 20862468.45 reads where mapped uniquely to each of the genomes.

The total amount of sequenced RNA reads in this study, a total of 2303938848 unique reads for maize and 157832429 for *A. flavus*, exceeded what has been reported in other dual RNA-seq studies that have characterized the transcriptomes of host and pathogen simultaneously (Yazawa et al., 2013; Rudd et al., 2015). In the early stages of infection, most of the RNA in each sample was found to be host (*Z. mays*) RNA. This was observed in other dual RNA-seq studies as well and was reported to be

simply due to the small number of pathogen cells in the initial inoculum, and the slow initial growth of the pathogen (Musungu et al., 2016). The subsequent growth and spread of the pathogen resulted in increasing the fraction of *A. flavus* RNA in later samples. Interestingly, the number of total unique reads mapping to *Z. mays* did not change significantly from sample to sample until 24 hours post inoculation (hpi) (**Table 1**). Since the total amount of tissue in each sample was the same for all samples across all time points, the absolute decrease in host RNA reads may also reflect a per gram decrease in living *Z. mays* tissue due to necrosis caused by the pathogen in plant tissue at the later stages of infection, as well as increased amount of pathogen mycelia per gram tissue as the infection progresses.

Within our data we delve into the link between the standard time course vs our stage of infection which represents grouped by *A. flavus* infection dependence. In **Figure 1** we highlight stage of infection as opposed to time because it represents a correlation in the activity of transcription for *A. flavus* and *Z. mays* interaction. MA-plots that graphically illustrate the relationship of m-value ($\log_2$ fold change) vs. absolute expression were used to determine the mean expression distribution and differential expression characteristic of each biological sample (**Figure 1**). Read counts showed a significant skewing of data, especially for *A. flavus*, that would affect the reliability of differentially expressed gene calling; low counts, especially at early time points, and large dynamic range of RNA-seq data, made log-fold change-based analysis quite noise-prone (Love et al., 2014). DESeq2 regularized log normalization was used during the analysis of the differential expression of genes to reduce the amount of false positives that could be present due to low counts. Additionally, genes that had a total count less than 10 could be removed from the analysis. Consequently, the *A. flavus* transcripts from the early stages of infection (SI1–6) were excluded from the analysis because of the low overall reads. Thus, SI7 was used as the denominator in calculating log fold changes and differential gene expression for *A. flavus*. Even though crucial, many of these early infection stages, had too few overall counts to be considered reliable estimates of relative expression for the pathogen. Similar observations have been reported in other host-pathogen studies (Teixeira et al., 2014).

Infection Index and Principle Components Analysis

There was noticeable variability in the progress of infection over time, with significant discrepancies occurring between 12 and 42 hpi. Not all time points in replicate experiments overlapped in terms of the ratio of *A. flavus* to maize RNA, indicating that our time-ordered samples may be out of order in terms of the stage and progression of infection and disease development. Given the presence of a significant number of outliers in our data, outliers that are typically ignored during differential expression analysis, we developed an additional index to better gauge the progress of infection. The index was built using the ratio of *A. flavus* to *Z. mays* unique RNA, rather than using the time of tissue collection, as the sole criterion to assess infection progress. Consequently, the individual biological replicates were reordered and grouped into 18 Stages of Infection (numbered SI1 to SI18) based on similar infection index values. This reordering was used alongside a time-based ordering of data for subsequent normalization and downstream analyses of the experiments. The effect of this reordering reduced the gene expression variance among replicates significantly. In fact, using the stage of infection index, the maize MA-plots revealed a large change in differential expression relative to the early stage of infection (SI 0 compared SI18 in **Figure 2**). The time course ordered samples also showed a significant difference in expression as early as 4 h after inoculation. Using the stage of infection index data, it took a few stages before detecting significant differences in expression in maize. For example, SI 2-4 had 36 total genes that were found to be differentially expressed. The difference between time ordered data and stage of infection data is likely resulting from variability in pathogen success at very early time points, leading some biological replicates to reach advanced stages of infection more quickly than others.

When time points and stages of infection were both analyzed using Principal Component Analysis (PCA), there was some difference between replicates of samples using either grouping, but replicates of the mid to late infection stages, SI–7 to 15, were more tightly clustered together when grouped by stage (**Figure 3**). Further analysis of the two components revealed a similarity of variance in the components for SI7 and SI8, which are the stages where the transcripts of the pathogen begin to accumulate significantly. Moreover, SI9 and SI10 shifts had similar variances in the components based on PCA analysis. Therefore, SI progression was accompanied by an increase in the *A. flavus*/*Z. mays* expression ratio.

This analysis also showed that, for both host and pathogen, most of the variation in the different biological replicates occurred between samples collected at the same hpi. For *A. flavus,* the clustering of the experiments grouped by SI was more consistent, and tight clustering of experimental replicates was observed. Additionally, the time ordered data displayed a variance where most of the variance was explained by the *x*-axis component. The hpi time points had multiple samples with similar overlap between samples. For example, hpi 0, the point after inoculation which was considered to be the control, shared a tight cluster and overlapped with hpi 4 and 6. Similar results were found with the later timepoints. For instance, this is reflected in the difficulty to distinguish transcriptomes from the 5 and 6 days post inoculation samples. Similarly, the maize component data was ordered by time with multiple samples showing overlap between different hpi.

Differential Expression Analysis for *Z. mays*

The differential expression analysis of *Z. mays* was performed utilizing the (Stage of Infection) SI index to organize the samples for the downstream analysis. Multiple genes were found to be differentially expressed in at least one infection stage in *Z. mays* when DESeq2 was used to compare later infection stages to

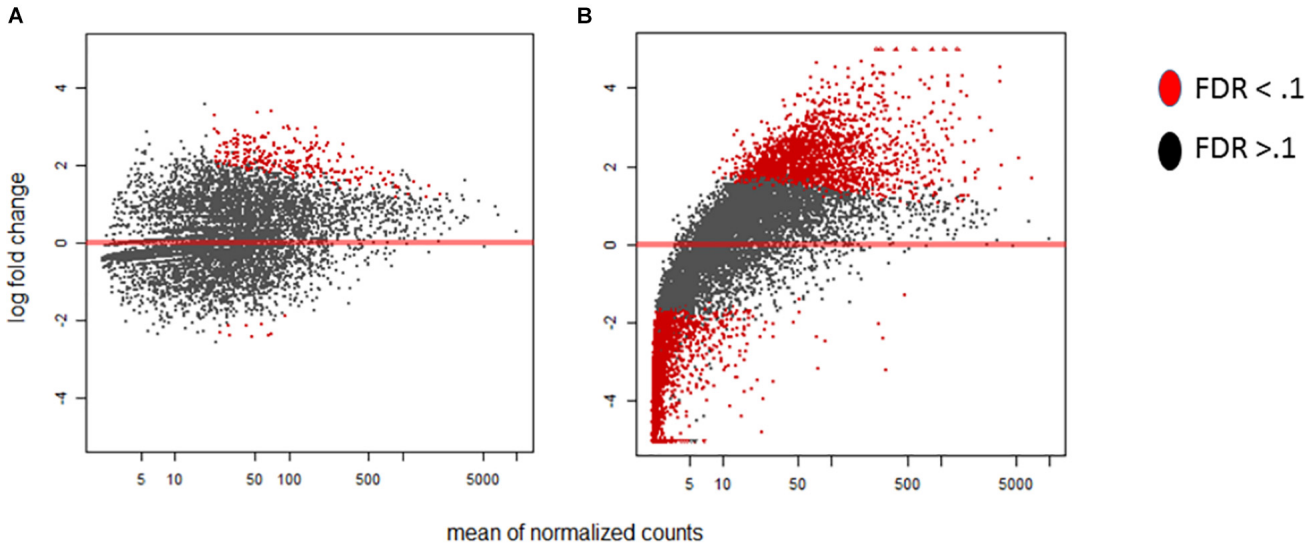

FIGURE 1 | MA-plots were generated for the DESeq2 normalized RNA-seq samples of *Aspergillus flavus*. For this Stage of infection index (SI) analysis, SI7 was used as the background (control). **(A)** MA-plot displays the SI8 treatment relative to SI7. **(B)** MA-plot displays the SI18 treatment relative to SI7. Significant genes are denoted by the red color whereas non-significant genes are colored black.

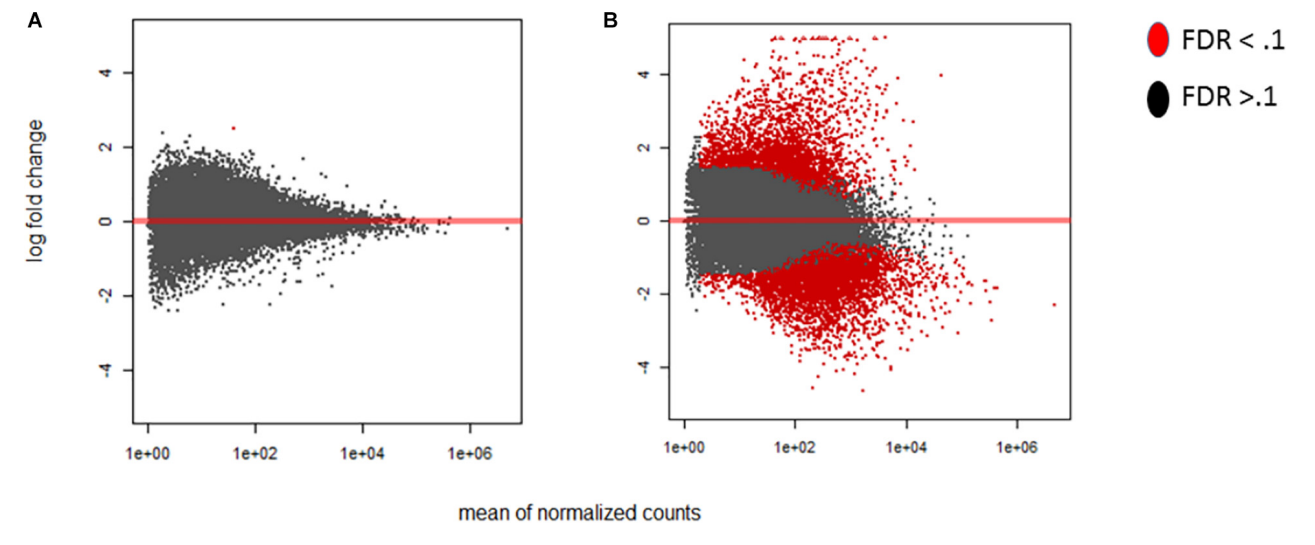

FIGURE 2 | MA-plots were generated for the early and late maize infection stages. The MA-plots illustrate the distribution of the log fold change of the differentially expressed genes. Genes that are significantly differentially expressed are highlighted in red, while those that are not, are displayed in black. For each of the graphs the *Y* axis shows the log fold change, and *X* axis shows the mean normalized counts. The fold change is displayed for the stage of infection index (SI) data to determine the distribution effect on differentially expressed genes in maize. **(A)** SI2 compared to the control SI0. **(B)** SI18, the latest stage, compared to SI0. Differentially expressed genes were determined using DESeq2 (False discovery rate; FDR < 0.1).

SI-1 (**Supplementary Table S1**). It should be noted that the samples at the early stage of infection showed very low pathogen RNA levels. However, at SI-3, there was an increase in both the number of significantly differentially expressed up/down regulated maize genes (7 genes) as well as higher levels of pathogen RNA. The largest increase from SI-2 in terms of the number of detected differentially expressed genes did not occur until SI-6 when the activation of multiple genes that are known to be involved in the resistance to *A. flavus* was observed (**Table 2**). The one gene (GRMZM2G175574) detected in SI-2 that had a significant differential expression (p-adj < 0.1) probably indicating a lack of response to the pathogen at this stage (**Table 2**). For SI-3, there were only 7 additional differentially expressed genes (**Table 2**). Genes in *Z. mays* kernels began to show significant differential regulation around 30 hpi when the datasets were arranged by time, or at SI-7 when evaluated by infection index. Notably, this is the same stage at which *A. flavus* RNA became abundant enough to be analyzed

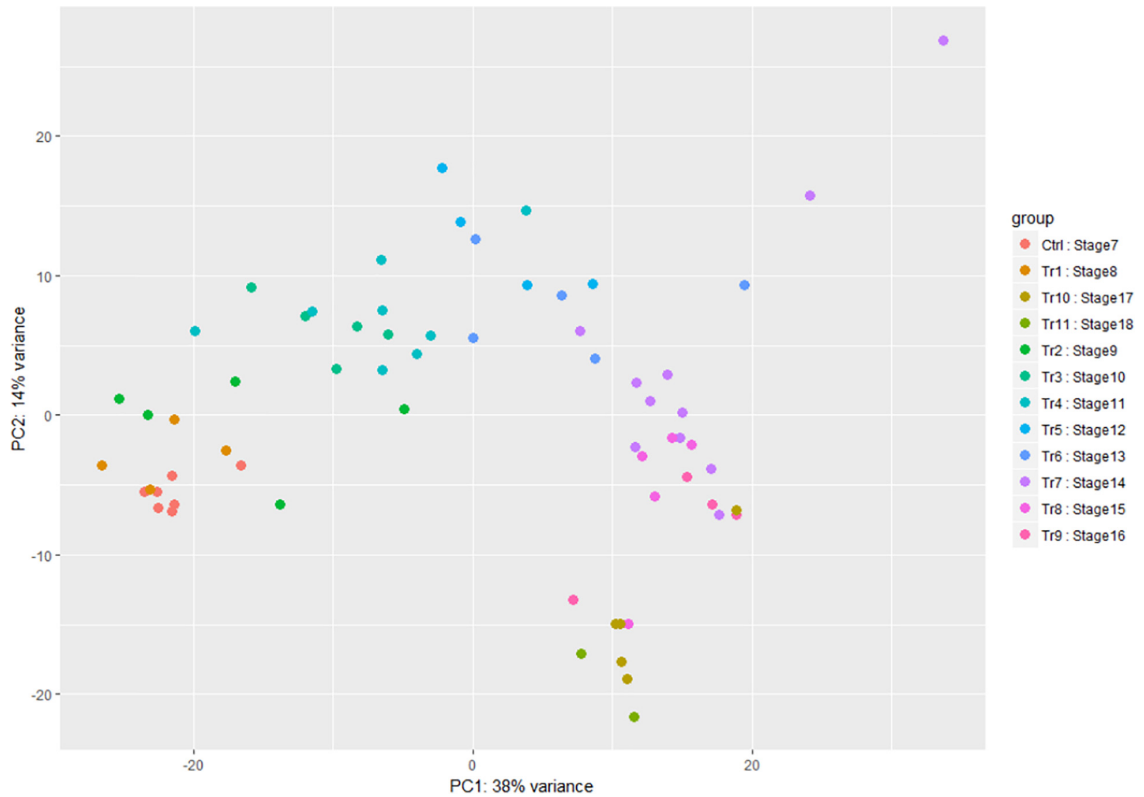

FIGURE 3 | Principal component analysis of the *A. flavus* transcriptomic data. SI7 to SI18 were used to observe variation between samples. Each circle in the graph represents a RNA-seq sample and each color represent a stage of infection (Tr represents Treatment and Ctrl represents Control).

reliably. At the crux of the infection (144 h, infection stage 18), 2345 maize genes were significantly up-regulated, and another 4057 were suppressed. Many of these genes were components of pathogen resistance pathways.

Gene Ontology Enrichment Analysis Using SI for *Z. mays* and *A. flavus*

Functional enrichment analysis was used to reveal pathways in both the host and the pathogen, involved in the interaction between the two organisms. Analysis of data from the early infection stage (SI-2) identified the *Z. mays* gene GRMZM2G175574 to be the only differentially expressed gene in the SI-2 dataset (**Supplementary Table S1**) therefore no geno ontology analysis was possible during those early SI's. When observing the groups of SI genes found in the data set many of the common resistance genes were found. These genes included the late embryogenesis abundant 3 gene (GRMZM2G072890), sucrose synthase 4 (GRMZM2G008507), as well as multiple chitinase-related genes.

At SI-9, 235 genes were differentially expressed. These included key marker genes involved in pathogen resistance such as 12-oxophytodienoate reductase 2 (GRMZM2G156712), a gene involved in the biosynthesis of jasmonate that has been implicated in resistance to necrotrophs (Robert-Seilaniantz et al., 2011; Mur et al., 2012, Lyons et al., 2013), and endochitinase A (GRMZM2G051943), an antifungal enzyme implicated in resistance to *A. flavus* (Dolezal et al., 2014). Later stages showed a dramatic increase in the number of differentially

TABLE 2 | Differentially expressed genes of maize at each of the Stages of Infection.

Stage of Infection	Differential expression count	Up	Down
SI 2	1	1	0
SI 3	7	6	1
SI 4	28	24	4
SI 5	50	50	0
SI 6	359	279	80
SI 7	39	32	7
SI 8	159	56	103
SI 9	235	200	35
SI 10	470	270	200
SI 11	872	664	208
SI 12	2173	1611	562
SI 13	1851	1309	542
SI 14	5157	3499	1658
SI 15	4267	2094	2173
SI 16	6274	2910	3364
SI 17	9416	4539	4877
SI 18	6402	2345	4057

expressed maize genes at SI-12, and a corresponding increase in the number of *A. flavus* genes at the final stage SI-18. Common *A. flavus* resistance gene markers were observed in both mid- and late-infection stages. For *Z. mays*, there was also a considerable overlap between genes in the late stages. Notably, only one of the early stages genes, the uncharacterized GRMZM2G175574, was still differentially expressed at the later stages of infection. The overlap that was observed from SI-10 to SI-18 consisted of 60 genes including resistance genes, such as (GRMZM2G117942) and (GRMZM2G117971) which are chitinases, (GRMZM2G475059) and (GRMZM2G156877) glutathione S-transferase, and (GRMZM5G894619) 1-Aminocyclopropane-1 carboxylate synthase.

GO-term enrichment analysis of maize genes differentially expressed at SI-18 revealed multiple pathogen resistance pathways. Up-regulated genes detected by SI-18 included Endochitinase A (GRMZM2G051943) and Coronatine-insensitive protein 1 (GRMZM2G151536) (Naumann et al., 2009; Hawkins et al., 2015). Several of the genes were linked to the following GO pathways: GO:0055114- oxidation-reduction process, GO:0016491-oxidoreductase activity and GO:0004497-monooxygenase activity. Analysis of this stage of infection also revealed multiple unique genes that were not detected in any of the earlier stages. In fact, 1890 genes were unique to SI-18. Many of these genes did not significantly correlate to any known pathways. Fifteen of the genes that became active between S-18 and S-I9 are known resistance genes previously implicated in resistance to *A. flavus*. Most of the GO-terms that were highlighted at these later stages of the infection were primarily involved in responses to stimuli and in the production of secondary metabolites. This could be linked to the progression of *A. flavus* at these late stages. By SI-18, down regulated defense GO-terms began to appear. These included known resistance genes like lipoxygenase (GRMZM2G109056), PR-10 genes (GRMZM2G112524, GRMZM2G112538, GRMZM2G112488) and chitinase (GRMZM2G145518) which are involved in signaling and combating fungal infection (Chen et al., 2010; Christensen et al., 2015, Hawkins et al., 2015). Many processes were initially upregulated in the early stages of the infection index and appeared to peak in the later (SI12–18) (Hawkins et al., 2013).

Although only a few transcripts of the pathogen genes were detectable at the early stages of infection (SI1–6), many shared orthologs with developmental genes found in other fungi. For instance, the most abundant *A. flavus* gene detected at SI-1 was (AFLA_090780), the translation elongation factor EF-1 alpha subunit. This gene retained one of the highest mean values for absolute expression throughout the study, although it was not significantly differentially expressed. This makes this gene a useful marker to assess the relative abundance of *A. flavus* in a sample. The total abundance of reads for *A. flavus* in the complete data set was 169272008 reads, most occurring in the later infection stages.

Due to the low coverage of *A. flavus* reads at early stages of infection, SI-1 to 6 were not included in the analysis for differential gene expression in *A. flavus*. SI-7 was chosen as the starting point for differential gene expression analysis rather than using RNA from a conidial suspension, because it is more likely that the infection started by already germinated conidia. Throughout (SI 8-18), shifts in gene expression in *A. flavus,* reflected more up-regulation of genes rather than down-regulation (**Figure 4**); 159 *A flavus* genes were found to be differentially expressed (157 up-regulated and 2 down-regulated) at SI-8 (**Table 3**). Many of the genes observed at this initial stage involved primary metabolism and were enriched for primary metabolism - related GO terms. Gene Ontology pathways involving the carboxylic acid metabolic process GO:0019752 and the monocarboxylic acid metabolic process pathway GO:0032787 were significantly enriched. At SI-9, 857 *A. flavus* genes were found to be differentially expressed and GO-TERMS involving nitrogen metabolism, such as the nitrogen compound metabolic process GO:0006807, proteolysis GO:0006508, organ nitrogen compound metabolic process GO:1901564, and the cellular nitrogen compound biosynthetic process GO:0044271. At SI-10 there was a large increase in differentially up-regulated *A. flavus* genes detected in the data (**Table 3**). Interestingly, this stage was marked by a significant increase in the expression of aflatoxin cluster genes. The global regulators *AflR* and *AflS* were detected within this dataset to be differentially expressed. There were also biological processes that were enriched at SI-10 including GO:0032502 (developmental processes), GO:0006897 (endocytosis) and GO:0043436 (oxoacid metabolic process). As *A. flavus* progressed at later SI's, there was significant overlap with biological processes reported in other studies involving terms such as GO: 0005975 (carbohydrate metabolic process), GO:0019538 (metabolic processes), and GO:0016192 (vesicle-mediated transport) (Bai et al., 2015).

Clustering Analysis

Clustering is widely used to determine correlations between pathways. Therefore, differentially expressed *A. flavus* and *Z. mays* genes were clustered using K-means. The genes clustered into 100 groups capturing "unions" and "intersections" between *Z. mays* and *A. flavus*. It is to be noted that *Z. mays* genes reported to be involved in disease resistance often clustered together, even when their presumed biological roles are different. Furthermore, K-means clustering revealed the polygenic nature of the interaction between *Z. mays* and *A. flavus*.

Further analysis of the clusters uncovered maize defense genes such as (GRMZM2G156006) *AP2/EREBP* transcription factor, (GRMZM2G088765) Peroxidase 54 and other uncharacterized genes. Moreover, one of *Z. mays* co-clusters included genes that were initially up-regulated, but later displayed depleted levels of expression (**Supplementary File S1**). This group of genes may comprise resistance genes that are part of the earlier responders to *A. flavus* infection and to the exposure to mycotoxins.

The analysis of differential gene expression in *A. flavus* with K-means clustering revealed shifts in gene expression patterns from SI-7 to SI-18. The *A. flavus* transcriptome transitioned from an inactive state in SI-6 to an active state in SI-7 as revealed by a dramatic shift in gene expression. Multiple pathways were initially activated and later down-regulated (**Supplementary Table S3**). Further analysis revealed multiple pathways that were significantly enriched and down-regulated; the oxidative

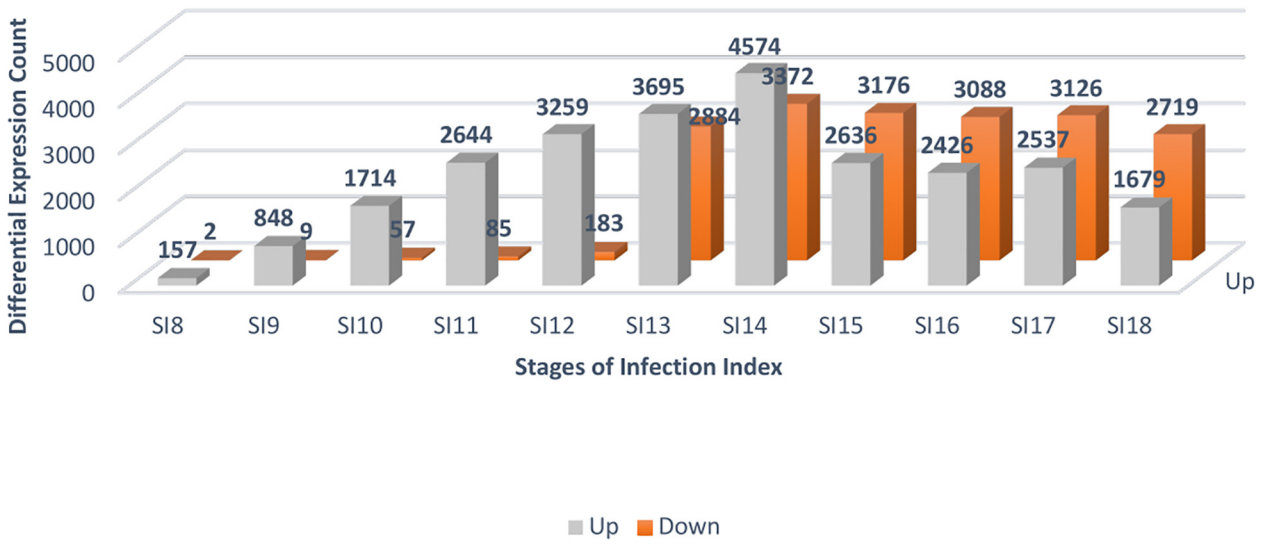

FIGURE 4 | Differential expression analysis was conducted with DESeq2 to identify genes that were significantly differentially expressed at the stages of infection. The color gray denotes genes that were up-regulated, and the color orange indicates genes that were down-regulated. The FDR cutoff used to call differentially expressed genes was (*p* adjusted value < 0.05).

phosphorylation pathway (**Figure 5**), multiple genes involved in RNA transport, and genes involved in ribosome biogenesis, were among the pathways that were significantly down-regulated (**Supplementary File S1** and **Figure 6**). Interestingly, the oxidative phosphorylation pathway includes genes that have previously been implicated in the biosynthesis of mycotoxins (Grintzalis et al., 2014).

In some cases, gene expression was highly elevated during the later stages of infection. For example, cluster 32 contained *A. flavus* and maize genes that were activated at the mid stage of infection. The *A. flavus* arsenal of hydrolytic enzymes, such as (AFLA_007720) pectin lyase, (AFLA_124660) pectin lyase precursor and (AFLA_023340) pectinesterase precursor, were found to be upregulated at the later stages of infection. This cluster also surprisingly contained pectinesterase 11 (GRMZM2G070913) of maize.

The aflatoxin cluster gene *AflR* clustered with aflatoxin cluster genes *AflMA*, *AflQ*. Our data did not find all the aflatoxin cluster genes to be located in a single cluster in the analysis. The jasmonate biosynthesis 12-oxophytodienoate reductase 2 maize gene (GRMZM2G000236) was included in the same cluster, and appeared to be co-expressed with *AflR*, *AflMA* and *AflQ*. Additionally, that same cluster contained the maize genes for chalcone synthase (*C2*), an *AP2/EREBP* type transcription factor (GRMZM2G159592), and multiple reactive oxygen species domain containing genes.

TABLE 3 | Differentially expressed genes identified following the DESeq2 analysis of stages 7 to 18.

Stages of Infection *Aspergillus*	Total	Up	Down
SI8	159	157	2
SI9	857	848	9
SI10	1771	1714	57
SI11	2729	2644	85
SI12	3442	3259	183
SI13	6579	3695	2884
SI14	7946	4574	3372
SI15	5812	2636	3176
SI16	5514	2426	3088
SI17	5663	2537	3126
SI18	4398	1679	2719

KEGG Analysis for *Z. mays* and *A. flavus*

When the dual transcriptomic SI data was analyzed using Kegg Pathview package in R, the first pathway found to be significantly enriched was the DNA replication pathway for *Z. mays* (Luo et al., 2017). In the earlier stages, there was not a significant enrichment in the pathways usually involved in resistance to *A. flavus*. The detection of these enriched pathways did not occur until SI-6 when the flavonoid biosynthesis and the glutathione metabolism pathways were found to be significantly enriched (**Figure 7**). This result was also reported in other GWAS metabolic analysis studies involving the *A. flavus* and

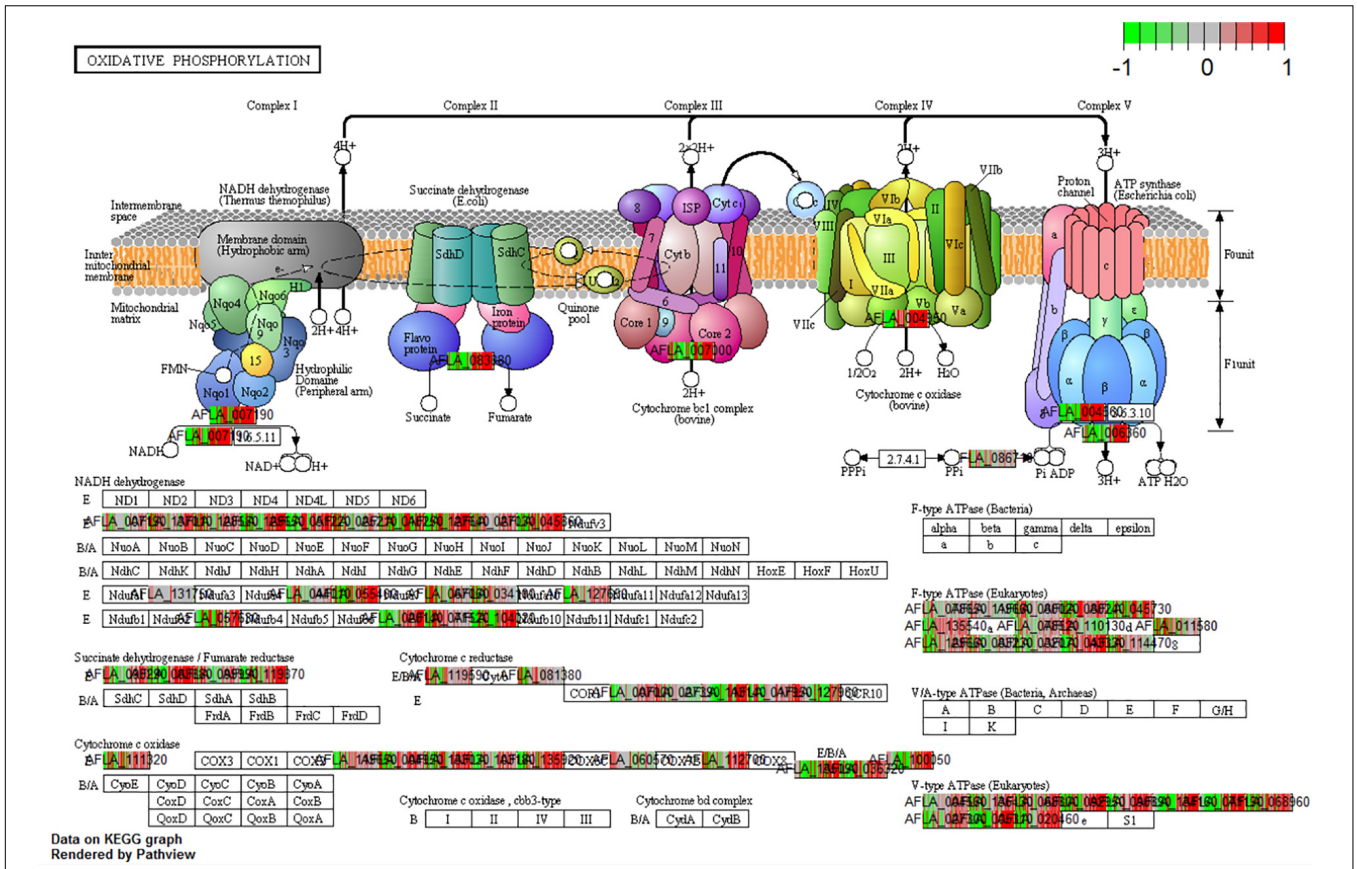

FIGURE 5 | KEGG pathway analysis on the different Stages of Infection (SI) was done to observe the enrichment of pathways in the pathogen *in vivo*. The current pathway was a significant enriched and differentially expressed within the dataset (q-val < 0.1) when comparing SI8 vs. SI7.

Z. mays interaction (Tang et al., 2015; Warburton et al., 2015). Our results also agree with the identified pathways, like amino acid metabolism, which was found to be significantly enriched as early as S-I5 in the data set (Tang et al., 2015). The ethylene biosynthesis pathway was found not to be significantly enriched in the KEGG pathway analysis, whereas the building blocks for the pathways regulating cysteine and methionine metabolism were enriched in the GO-analysis for the data set. Furthermore, α-linolenic acid metabolism (jasmonate) was significantly enriched at the SI-14.

Further investigation using KEGG pathway analysis for *A. flavus* revealed additional pathways that were initially activated and later down-regulated (Luo and Brouwer, 2013). When SI-9 was analyzed, pathways such as the biosynthesis of secondary metabolites, biosynthesis of amino acids and carbohydrate metabolism were up-regulated and significantly enriched. The aflatoxin pathway was found to be significantly enriched in the SI-10 data set. The analysis also revealed multiple pathways that were significantly up-regulated and enriched; the oxidative phosphorylation pathway (SI-10-18), and multiple genes involved in RNA transport (SI-10-18). One pathway that was significantly enriched in the analysis of the early stages of infection and that was later depleted, was the ribosome biogenesis pathway (**Figure 6**). Interestingly, it was the only pathway to deplete significantly in the later stages of infection.

Interactome Analysis

The differentially expressed genes for *A. flavus* and *Z. mays*, were then analyzed in their respective predicted protein interactomes to allow for inference for of novel protein protein interactions (Musungu et al., 2015; Szklarczyk et al., 2015). There was a total of 1451 proteins that were found to be in the *Z. mays* interactome. Some of the highest connective proteins that were identified was GRMZM2G030299 (Protein-ribulosamine 3-kinase chloroplastic). This transcript was highly down-regulated in the DESeq2 analysis, with a log fold change of −0.54 (DeSeq2 Fold Changes). This was followed by GRMZM2G097878 serine-threonine protein kinase, which had 299 connections. The corresponding gene was also down-regulated in the data set. The next gene GRMZM2G004356 (transcription factor *UNE12*-related) had 254 connections and was up-regulated through the initial stages of infection and down-regulated from stage 13 to 18. At each stage of infection, the differentially expressed genes had different amounts of interacting partners in the interactome of *Z. mays*. The largest subnetwork for the infection study was produced from the later stages that had the largest number of differentially expressed genes. Additionally, analysis from the

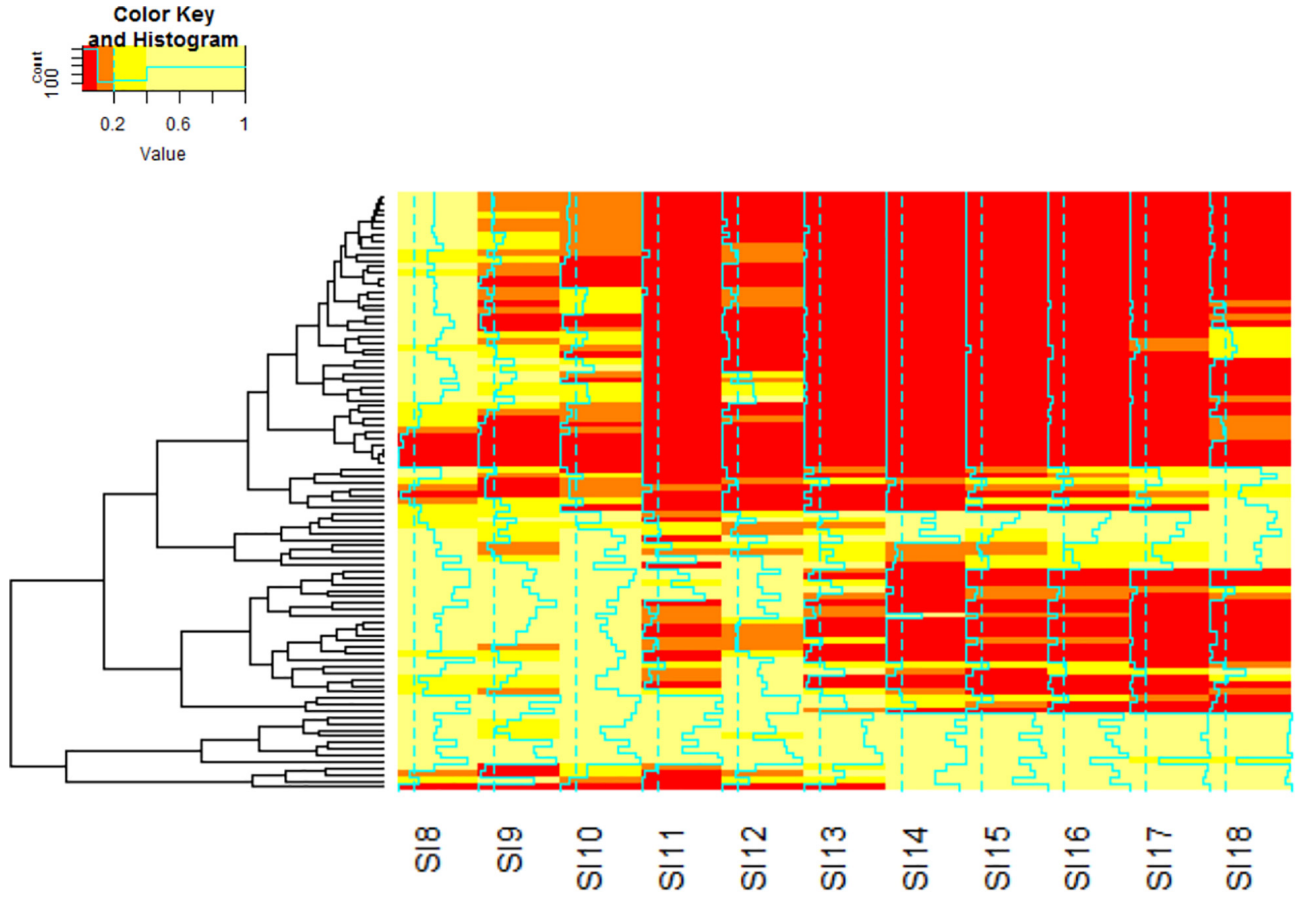

FIGURE 6 | Hierarchal clustering analysis of the KEGG Pathways by *q*.value was performed to identify the pathways that are significantly involved in the interaction. The color red denotes genes with the lowest *q*.value. Orange indicates genes with moderate significance within the data set. Yellow indicates genes that do not exhibit significant differential expression. The blue lines through each stage indicate the strength of the clustering by the *q*.value. The order of the matrix is stored in **Supplementary Table S1**.

PiZeaM disease subnet differential identified 400 common targets that are predicted to be involved in biotic response. From the hormonal response network, 23 genes were identified within the interactome with 31 interacting partners. With many of the genes involving reactive oxygen species pathways, heat shock proteins and jasmonate pathway genes. The multiple proteins identified and pathways determined demonstrate the polygenic nature of the resistance to *A. flavus*.

With *A. flavus,* the network was highly dense including 927 differentially expressed genes with 3584 interacting protein partners. The network contains proteins that are involved in development, which were also shown to play a role in mycotoxin and aflatoxin production. Networks also contained carbohydrate and nitrogen metabolism subnetworks. The *A. flavus* subnetworks followed a pattern similar to that seen in *Z. mays* subnetworks, where the connectivity or interacting protein partners increased over time.

Gene Regulatory Network Analysis

The multiple time points allowed the generation of a stage of infection index for the transcriptomic study. The GeneNet (Schaefer et al., 2015) module was utilized to develop a partial correlation network where both host and pathogen transcriptomes were present. The criteria for cutoff in edge selection was done using familywise false discovery corrected *p*-values (*q*-val and *q*-val.dir $< 10^{-5}$) (**Figure 8**). As expected, this produced a large amount of edges which agrees with what was reported in many gene regulatory network identification studies. Therefore, to determine if the edges were significant in the network connectivity, genome wide association data, and quantitative loci information involved with resistance to *A. flavus* were used to mine for significant genes in the directed network. The edges for the network can be retrieved using (**Supplementary Code S1**). The network was generated from 47,801 genes. We did not find any significant enrichment within the unused genes from the transcriptome for both *Z. mays* and *A. flavus*. This network adds to previous work that only utilized gene co-expression from Pearson Correlation and utilizes partial correlation to determine significant genes within the network.

First, the differentially expressed genes were evaluated for hub enrichment to determine connectivity. The connectivity (or Degree) has previously been shown to be an indicator

of biological importance (Horvath and Dong, 2008). Second, publicly available data from chip-seq and GWAS (genome-wide association studies) studies was used to determine coverage of resistance marker genes and transcription factors (Bolduc et al., 2012; Eveland et al., 2014, Tang et al., 2015). The connectivity of *A. flavus* genes highest interactors were found to include genes from the up-regulated KEGG pathway involved in endocytosis (**Supplementary Table S4**). Of the *Z. mays* genes found in the network to contain the most connection was a predicted translation initiation factor SUI1 (GRMZM2G017966) which according to qteller (Schnable, 2014) has strong expression in the pericarp of the tissue (**Table 4**). When looking at the DESeq2 $\log_2$Fold changes, the gene was activated in the earlier stages (SI-2-9), but expression was not detected at later stages of infection. Early expression was observed for many of the top 20 highly connected genes within the network. Included in this group of highly connected genes were *RPM1*, *SAM* and a leucine rich repeat receptor gene previously implicated in resistance.

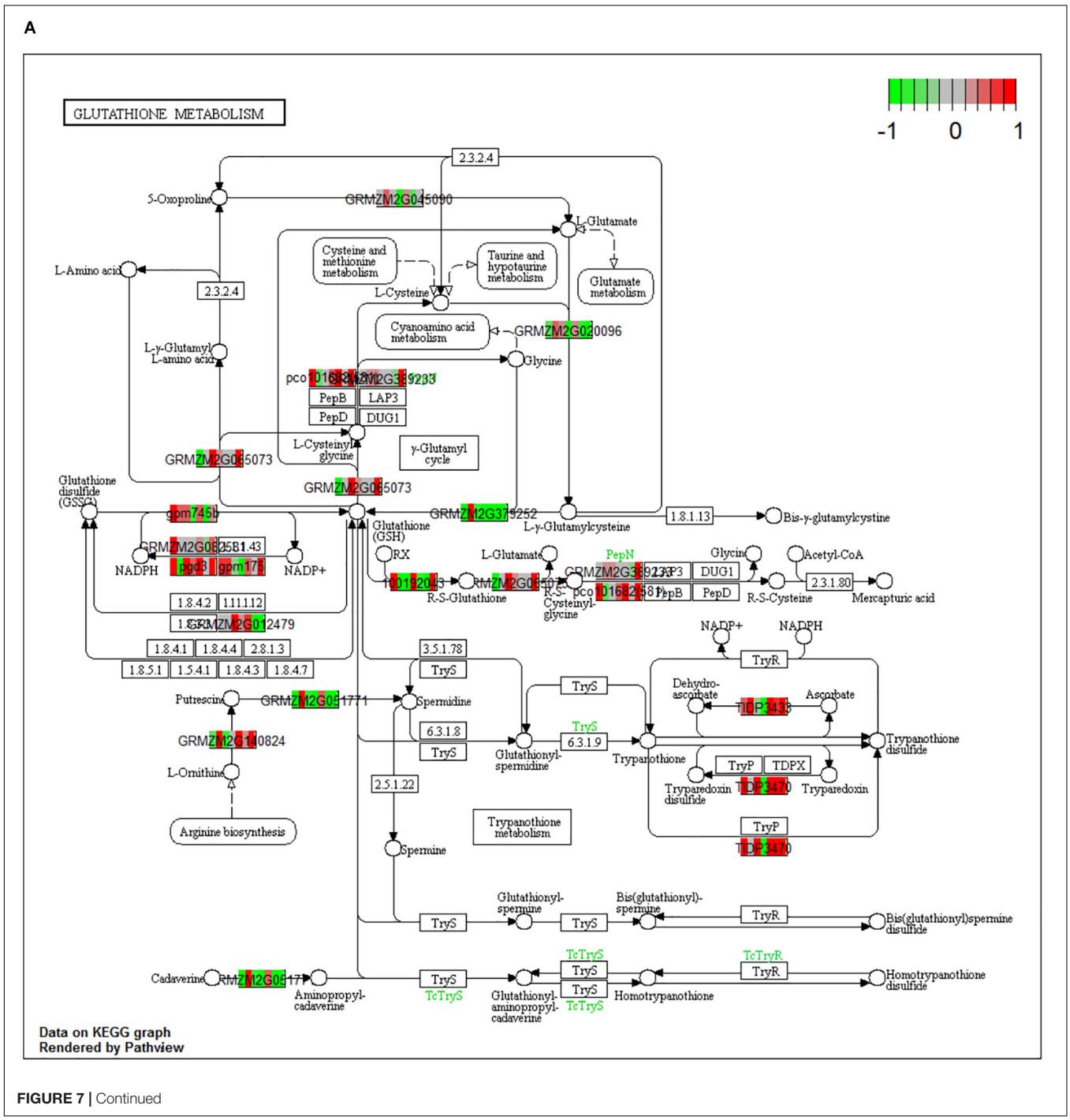

FIGURE 7 | Continued

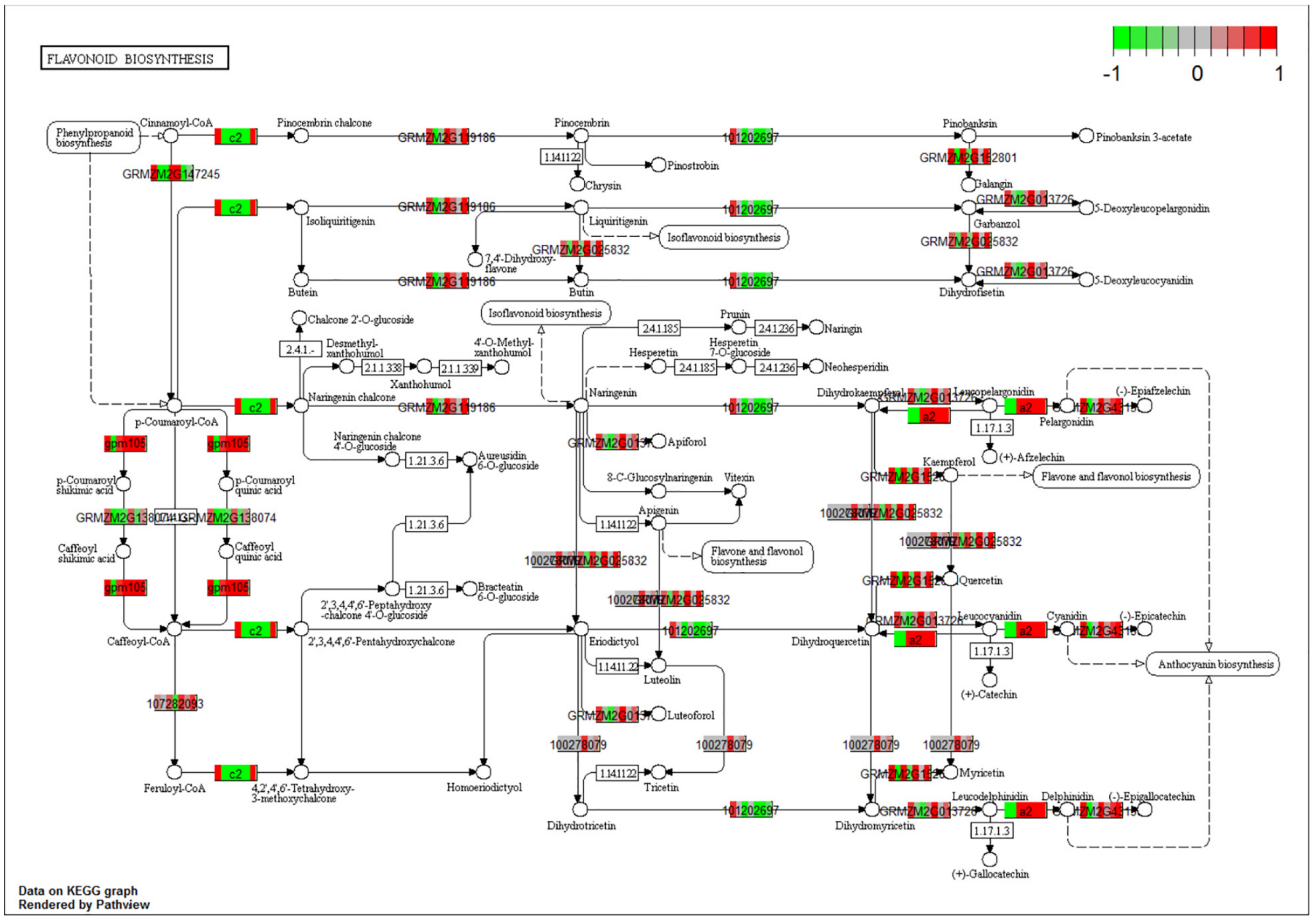

FIGURE 7 | (A,B) Kegg analysis was done on the Stage of Infection (SI) 6 index to determine pathways that were significantly enriched within the *Zea mays* data set. For the boxes that are white indicate genes where it was unable to find a match in the pathway. **(A)** Signifies the gluthatione pathway (KEGG pathway id: ko00480) and **(B)** signifies the flavonoid pathway (KEGG Pathway: ko00941). The cutoff for a significant pathway was signified with a (qval < 0.1) for each of the SI.

The transcription factors that were present in the network are the *WRKYs*, *AP2*, *MYB* and *NAC* that were previously found in the gene ontology enrichment analysis. The largest *WRKY* transcription factor found within the network was (GRMZM2G383594) which has already been implicated in resistance studies with other pathogens as well as with *A. flavus* (Gao et al., 2014) (**Supplementary Table S3**). There was also *WRKY* genes that were targeted by *A. flavus* genes; the network contained 107 different WKRY genes based on UniProt Consortium (2015).

Z. mays genes identified from previous GWAS analysis studies formed a network where multiple GWAS genes were correlated with differentially expressed genes (**Figure 8**). Hub genes were likely to occur more for the target genes compared to the source genes. This indicates that the network can capture hypothetical upstream targets as well as downstream targets. The latter can be used as likely candidates for marker assisted breeding, Further analysis was performed utilizing the genes identified through GWAS studies involved in resistance to *A. flavus*. This list contained 66 genes that were originally found to be associated with aflatoxin resistance in grain (Warburton et al., 2015). When mining the differentially expressed genes, 56 genes were inferred to be involved with 30 other maize genes. The gene from the GWAS study with the largest connectivity within our total network was GRMZM2G162233, which is still predicted to be uncharacterized according to the latest maize genomics data. It however, shares orthology with AT1G18730 which is predicted to be a NAD(P)H gene. Overall the network analysis was able to capture 56 genes out of the 66 highlighted in recent GWAS studies (**Figure 9**).

DISCUSSION

To uncover some of the complexity of the interaction between *Z. mays* and *A. flavus*, a systems biology approach was undertaken (**Figure 10**; Saito and Matsuda, 2010). This has been demonstrated to beneficial in reusing available data build

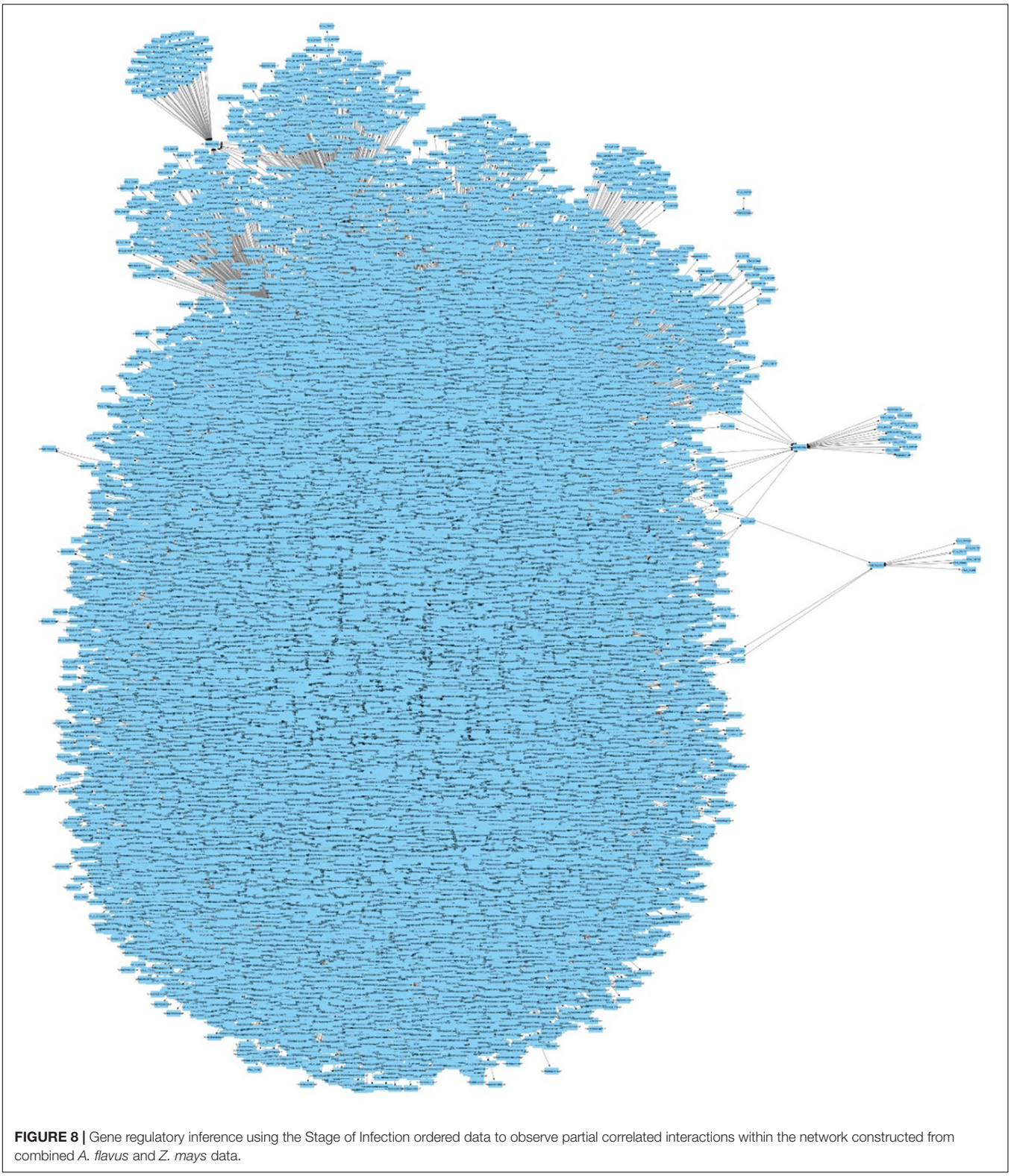

FIGURE 8 | Gene regulatory inference using the Stage of Infection ordered data to observe partial correlated interactions within the network constructed from combined *A. flavus* and *Z. mays* data.

complex models and networks (Brandl and Andersen, 2017; Rodenburg et al., 2018). In addition, a classical qualitative epidemiological approach of categorically ranking samples by the detection of *A. flavus* was utilized for the first time to group samples for differential expression analysis and gene regulatory network inference. This was beneficial in that the traditional time series analysis assumes that the host pathogen interaction is theoretically synchronous upon the point of

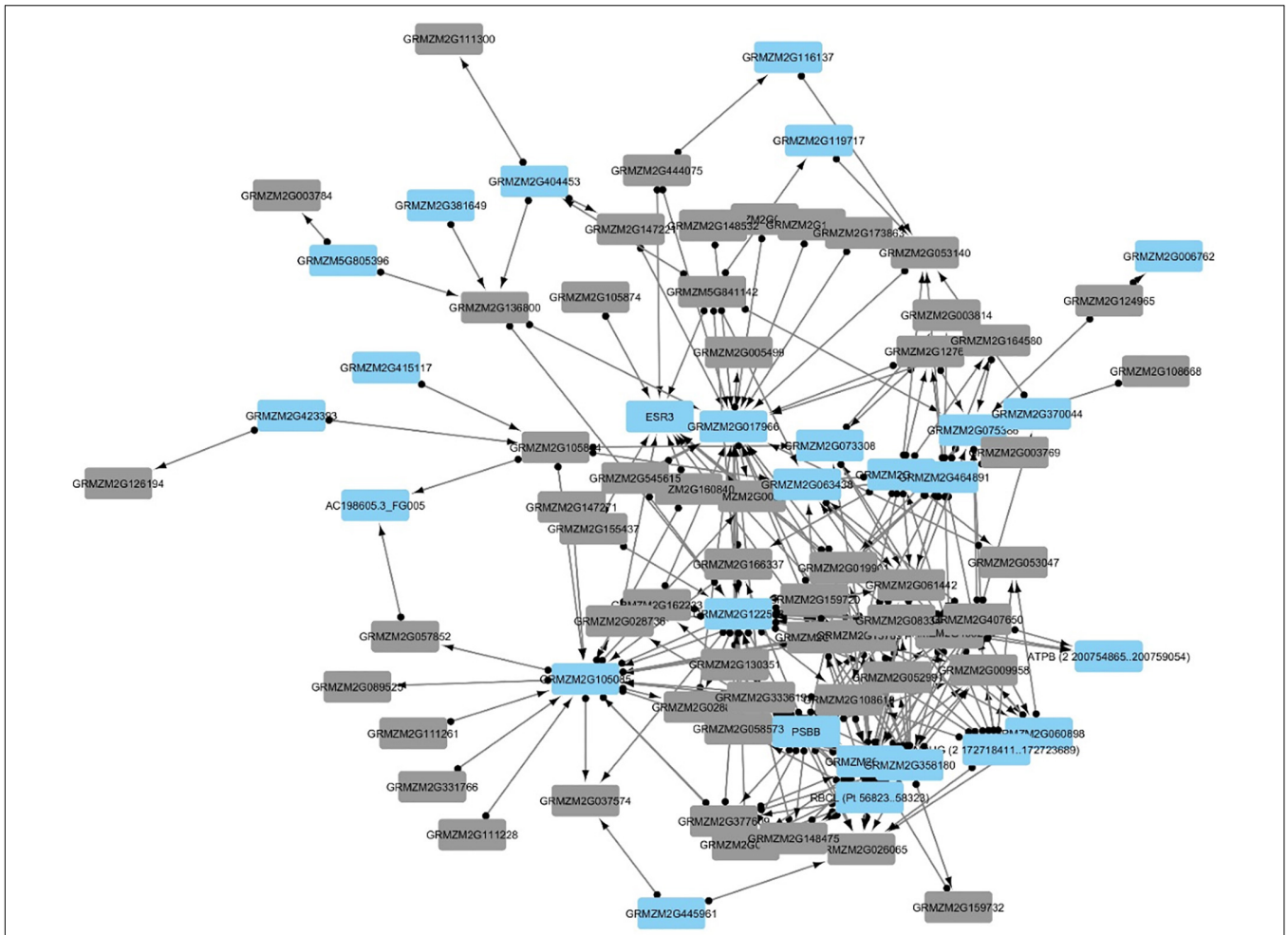

FIGURE 9 | The network represents 83 genes, which were inferred from the Stage of Infection transcriptomic analysis. Since the network was large, only edges with a FDR $< 10^{-5}$ were used in building the network. Additionally, the edges in the network connect genes that were found to be partially correlated and that have a downstream effect. The blue nodes indicate genes that have not been described in previous aflatoxin/ear rot- related genome wide association studies (GWAS). Gray nodes indicate genes that have been previously associated in resistance from GWAS to *A. flavus*.

infection (OBrian et al., 2003; Chang et al., 2007). However, our data for *Z. mays* and *A. flavus* suggested that that is not the case as revealed by the PCA analysis; the samples showed variability with confounding factors which are common *in vivo* studies, justified the development and adoption of the novel stage of infection index. This was noted upon observing the reads values for *A. flavus* genes that were identified in the initial time points and stages of infection.

The amount of *A. flavus* genes detected in the study was highly variable depending on the sample, and it ranged from 158 to 11961 genes. This is probably due to the low levels of detected pathogen RNA in the earlier stages of the infection, when only highly expressed genes could be detected. The subset of *A. flavus* genes in these samples represent the pathogen beginning to be able to establish itself in the kernel tissue. This has been attributed to background amounts of pathogen originating from the field or the soil used in those experiments. For example, the pathogen *Moniliophthora perniciosa* was detected with less than 1000 transcripts using RNA-seq libraries with close to 80 million reads (Teixeira et al., 2014).

Our data also shows a unique increase/decrease in the amount of gene expression activity between the host and pathogen. The progressive increase of unique *A. flavus* RNA reflected the progression of disease; the increase of the relative amount of *A. flavus* RNA with time indicates fungal growth. On the other hand, the level of *Z. maize* RNA remained unchanged and eventually dropped. This is likely due to significant cell death from necrosis, which is one of the reported outcomes of infection by *A. flavus* (Dolezal et al., 2013).

Our approach of using stages of infection captures the divergent properties observed in the transcriptomes of the host and the pathogen during the early, mid and late stages of infection based on our study of early infection. The gene regulatory network approach was then further utilized to infer causal relationships between genes within host and

TABLE 4 | Connectivity/Degree for genes identified in the host pathogen network. Gene ID refer to the maizegdb annotated gff3 file.

Gene ID	Annotation	Node1	Node2	Total
GRMZM2G017966	Uncharacterized protein	3112	14556	17668
GRMZM2G073308	Uncharacterized protein	6009	10045	16054
GRMZM2G122563	ribulose bisphosphate carboxylase	7535	8212	15747
ESR3	embryo surrounding region	1740	13816	15556
GRMZM2G105085	Porin_dom	6646	8867	15513
PSBB	Photosystem II CP47 reaction center protein	15258	59	15317
GRMZM2G464891	Uncharacterized protein	13153	2079	15232
GRMZM2G063438	S-adenosyl-L-methionine-dependent methyltransferases	4566	10544	15110
GRMZM2G397788	(RPM1, RPS3) NB-ARC disease resistance protein	11611	3241	14852
GRMZM2G119717	leucine-rich repeat receptor-like protein kinase family protein	7189	7651	14840
GRMZM2G358180	PS_antenna-like	10817	3880	14697
GRMZM2G075386	Acanthoscurrin-2	4732	9860	14592
RBCL	Ribulose bisphosphate carboxylase large chain	14511	45	14556
GRMZM2G096792	PSI_PsaC	5689	8816	14505
GRMZM2G445961	predicted pleiotropic drug resistance protein	11950	2170	14120
GRMZM2G116137	Transcription factor PCF6	6664	7184	13848
GRMZM2G404453	Uncharacterized protein	10778	2835	13613
GRMZM2G148605	Flavin_mOase	7532	5815	13347
GRMZM2G370044	Uncharacterized protein	10126	3190	13316
NDHG	NAD(P)H-quinone oxidoreductase subunit 6, chloroplastic	13228	70	13298

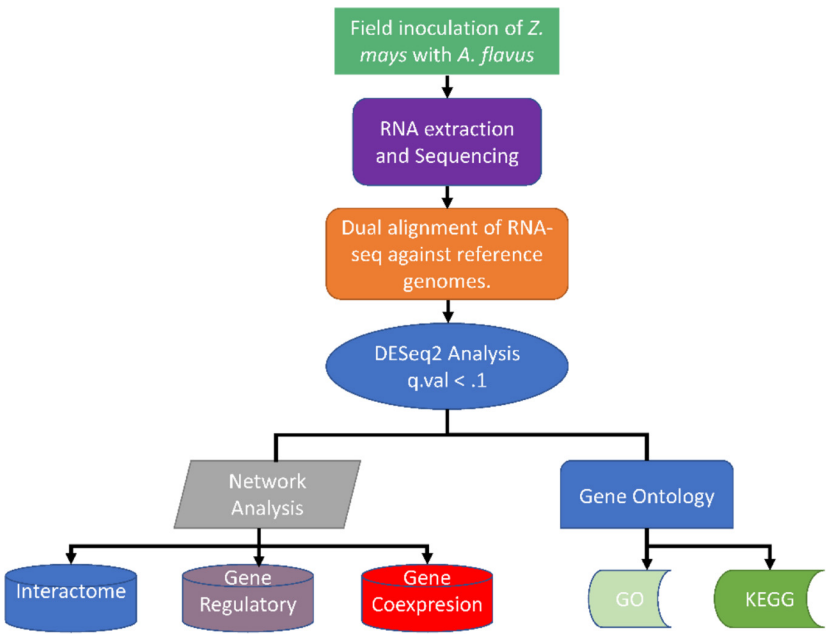

FIGURE 10 | Summary of the different methods used for analyzing the dual RNA-seq study for *Zea mays* and *Aspergillus flavus*.

pathogen, and between host and pathogen (Guo et al., 2016; Banf and Rhee, 2017). This novel approach expands on the previous Pearson correlation analysis which studied the two organisms independently, and revealed a significant dependence between the interactions of the two organisms (Musungu et al., 2016; Zheng and Huang, 2018). In addition, genes were modeled into correlative interactions groups using linear correlation methods. Connections between expression patterns and expression clusters in transcriptomes were drawn by mapping them to biological functions. Similar approaches were previously used in research were transcriptomes of hosts and pathogens were studied separately (Musungu et al., 2016). Moreover, we examined correlations and clusters between host and pathogen genes that may indicate inter-organismal interactions of biological functions or pathways. This study captured all known regulators and identified several new

regulators that activate their targets at time points not covered in previous studies.

An *A. flavus* and *Z. mays* gene regulatory network, containing 47,801 genes and multiple regulatory connections, was constructed to observe causal relationships. The network can be used to study many pathways not directly involved in host-pathogen response, but that are active in this 7-day time series. We focused on subnetworks and key pathways involved in resistance in *Z. mays* and in affecting the mechanisms of pathogenicity that *A. flavus* utilizes to infect susceptible hosts. Our initial selection of the SI-1 point is key for capturing possible circadian genes that were likely to be affected throughout the SI's. This was confirmed with the maize KEGG analysis. This was also reflected by the significance of down-regulation of multiple *Z. mays* circadian rhythm genes.

Upon assessing the differential expression data, the *A. flavus* transcriptome seemed to have a progressive effect on the *Z. mays* transcription. A similar conclusion was reached in a previous study using Pearson correlation exclusively (Musungu et al., 2016). Until SI-2, *A. flavus* did not have any differentially expressed genes that were up-regulated. This could have been due to *A. flavus* utilizing preformed transcripts as it has been suggested in other studies with different *Aspergillus spp.*(van Leeuwen et al., 2013). SI-6 was when an increase in transcriptional activity in *A. flavus* was noted. Moreover, multiple resistance marker genes such as PR1, OPR2 and PR10 were activated by SI-4. Although none of these marker genes were found to be statistically significant in the differential expression analysis, they were captured within the gene regulatory network. The GRN was able to visualize genes activated at these early stages for *A. flavus,* with many of these genes serving as downstream targets within the network.

The aflatoxin cluster was significantly differently expressed for many of the genes in the cluster. However, we were unable to capture the complete aflatoxin cluster in the GRN network. This is most likely due to limitations of the genes that were kept for the analysis as well as the partial correlation significance values. This could be also due to the complexity effects on the expression of the aflatoxin pathway as shown in studies involving temperature, pH and carbohydrates (Yu et al., 2004; Amare and Keller, 2014, Grintzalis et al., 2014; Medina et al., 2017, Gilbert et al., 2018). There is also the possibility when dealing with partial correlation that the connection was lost due to hidden confounders within the dataset. *AflR* was the only aflatoxin cluster gene not to be detected during the gene regulatory analysis. We however, found the rest of the cluster to be present including *AflS* which has been implicated to be a co-regulator in the cluster.

A shift in many of the pathways, including starch and sucrose metabolism as well as the oxidative phosphorylation pathway, was observed at SI-9. These pathways were down-regulated by SI-18 for *A. flavus*. This likely reflects the abundance of cytochrome P450s that were present in the differentially expressed gene set. The presence of these oxidative stress genes is most likely related to the abundance of reactive oxygen species causing genes, such as the peroxidase (GRMZM2G177792, AC210003.2_FG004, GRMZM2G410175, GRMZM2G408963, and GRMZM2G089982) to be activated in the kernel at this infection stage. Interestingly, it has been reported that in *A. fumigatus,* the loss of cytochrome expression can lead to an increase in pathogenesis *in vivo* (Grahl et al., 2012). This was the case in our study at SI-18. A similar process pattern was noted for *A. fumigatus in vivo* studies involving aspergillosis (Grahl et al., 2012). In our study, this switch in *A. flavus* was probably induced by the activation of resistance genes in *Z. mays*.

Our data also agrees with the previous work showing the importance of the WRKY TF family (Fountain et al., 2015b). For example, WRKY TFs which have been shown to be involved in abiotic and biotic stress, were found in our study targeting multiple jasmonate induced proteins (GRMZM2G020423, AC206425.3_FG002) and heat shock proteins that were conserved in the maize interactome. With our GRN, *Z. mays* genes, orthologous to biological stress pathway genes in *Arabidopsis thaliana*, being also initially targeted by specific *WRKY* genes. Moreover, the induction of *WRKY* TFs affected primarily resistance genes, but *A. flavus* oxidative stress genes such the O-methyltransferase group, lipase, and hydrolase were observed in the network as well. This was interesting because our data showed that the differentially expressed genes such as alternative oxidase (AOX2) and Cytochrome P450 (GRMZM2G147774) to be correlated with the aflatoxin cluster genes. This agrees with previous work involving environments rich in reactive oxygen species and their effect on *A. flavus* (Reverberi et al., 2012; Zaccaria et al., 2015). This subset of the network identified targets that were induced in the expression study and that share functional similarity to genes involved in resistance to pathogens.

In conclusion, this system biological approach utilized the available body of information to determine gene regulatory networks as well as motifs for co-regulated partners. The produced information can improve the broad understanding about early processes that are involved in resistance to *A. flavus*. However, it is important to note that one of the limitation of the data-set was that it was not complete by not encompassing the analysis of every possible gene that was expressed in the transcriptome. It is likely that even though many genes were unable to pass the threshold of detection for selection as a node, they could still make up the motifs with the network intermediates. Another observation made during the analysis of the GRN was that there were not many of the linear relationships typically seen in co-expression analyses given the partial correlation preferential detection of motifs. This is to be expected given the inclusion of multiple variables, once considered to be governed by a common regulator that are now partially explained by multiple regulators. Previously these partial regulations would have been masked using analyses that rely solely on Pearson correlation. To date, this analysis provides one of the first comprehensive transcriptomic dual RNA-seq studies in a plant-pathogen system.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GSE101899.

AUTHOR CONTRIBUTIONS

BM conceived the original research plans. SQ, RB, MG, and AF supervised the experiments. GO'B and GP carried out sequencing. BM wrote the manuscript with contributions from all the authors.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00853/full#supplementary-material

TABLE S1 | (A,B) DESeq2 outputs for all the stages of infection (1 to 18). Differentially expressed genes found in *Zea mays* after using DESeq2.

TABLE S2 | KEGG Gene Set Enrichment for *A. flavus*. Pathway enrichment analysis was done for Stages of Infection 8 to 18. Each column has a *q*-val which was considered to be significant ($x < 0.1$).

TABLE S3 | WRKY Transcription Factor Network. This data set contains the subnetwork gene net for the WRKY transcription factors found in the network.

TABLE S4 | Connectivity for Gene Regulatory Network. This dataset contains the degree/connectivity for each of the nodes within the network.

TABLE S5 | DESeq2 output for stages 8 to 18. Differentially expressed genes found in *A. flavus* after using DESeq2.

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