GLOBAL HEALTH ISSUES OF AFLATOXINS IN FOOD AND AGRICULTURE: CHALLENGES AND OPPORTUNITIES

EDITED BY: Mehdi Razzaghi-Abyaneh, Perng-Kuang Chang, Masoomeh Shams-Ghahfarokhi and Mahendra Rai

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GLOBAL HEALTH ISSUES OF AFLATOXINS IN FOOD AND AGRICULTURE: CHALLENGES AND OPPORTUNITIES

Topic Editors:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran
Perng-Kuang Chang, Southern Regional Research Center, USA
Masoomeh Shams-Ghahfarokhi, Tarbiat Modares University, Iran
Mahendra Rai, Sant Gadge Baba Amravati University, India

Aflatoxins are a group of polyketide mycotoxins that are produced mainly by members of the genus Aspergillus. Production of these toxic secondary metabolites is closely related to fungal development (Keller et al., 2005; Jamali et al., 2012). Contamination of food, feed and agricultural commodities by aflatoxins poses enormous economic and serious health concerns because these chemicals are highly carcinogenic and can directly influence the structure of DNA. The resulting genetic defects can lead to fetal misdevelopment and miscarriages; aflatoxins are also known to suppress immune systems (Razzaghi-Abyaneh et al., 2013). In a global context, aflatoxin contamination is a constant concern between the 35N and 35S latitude where developing countries are mainly situated. With expanding boundaries of developing countries, aflatoxin contamination has become a persistent problem to those emerging areas (Shams-Ghahfarokhi et al., 2013). The continuing threat by aflatoxin contamination of food, feed and agricultural commodities to the world population has made aflatoxin research one of the most exciting and rapidly developing study areas of microbial toxins.

The present research topic includes six review articles, three mini reviews and four original research articles. Contributors highlight current global health issues arising from aflatoxins and aflatoxigenic fungi and cover important aspects of aflatoxin research including contamination of crops, epidemiology, molecular biology and management strategies. Special attention is given to fungus-plant host interactions, biodiversity and biocontrol, sexual recombination in aflatoxigenic aspergilli, potential biomarkers for aflatoxin exposure in humans and safe storage programs.

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Global health issues of aflatoxins in food and agriculture: challenges and opportunities

Mehdi Razzaghi-Abyaneh[1]*, Perng-Kuang Chang[2], Masoomeh Shams-Ghahfarokhi[3] and Mahendra Rai[4]

[1] *Research Laboratory of Fungal Metabolites, Department of Mycology, Pasteur Institute of Iran, Tehran, Iran*
[2] *Food and Feed Research Unit, Southern Regional Research Center, New Orleans, LA, USA*
[3] *Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
[4] *Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati, India*
**Correspondence: mrab442@yahoo.com*

Edited and reviewed by:
Giovanna Suzzi, Università degli Studi di Teramo, Italy

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Aflatoxins are a group of polyketide mycotoxins that are produced mainly by members of the genus *Aspergillus*. Production of these toxic secondary metabolites is closely related to fungal development (Keller et al., 2005; Jamali et al., 2012). Contamination of food, feed and agricultural commodities by aflatoxins poses enormous economic and serious health concerns because these chemicals are highly carcinogenic and can directly influence the structure of DNA. The resulting genetic defects can lead to fetal misdevelopment and miscarriages; aflatoxins are also known to suppress immune systems (Razzaghi-Abyaneh et al., 2013). In a global context, aflatoxin contamination is a constant concern between the 35N and 35S latitude where developing countries are mainly situated. With expanding boundaries of developing countries, aflatoxin contamination has become a persistent problem to those emerging areas (Shams-Ghahfarokhi et al., 2013). The continuing threat by aflatoxin contamination of food, feed and agricultural commodities to the world population has made aflatoxin research one of the most exciting and rapidly developing study areas of microbial toxins.

The present research topic includes six review articles, three mini reviews and four original research articles. Contributors highlight current global health issues arising from aflatoxins and aflatoxigenic fungi and cover important aspects of aflatoxin research including contamination of crops, epidemiology, molecular biology and management strategies. Special attention is given to fungus-plant host interactions, biodiversity and biocontrol, sexual recombination in aflatoxigenic aspergilli, potential biomarkers for aflatoxin exposure in humans and safe storage programs.

Perrone et al. (2014) reported the expected risk of a shift in aflatoxin problems toward new territories particular in South East of Europe due to increasing average temperatures. Giving an overview on genetic diversity of *A. flavus* populations in Europe, the authors stressed the importance of selecting stable atoxigenic *A. flavus* strains as biocontrol agents. In the review of climate change on *A. flavus* growth and aflatoxin production, Medina et al. (2014) focused on the potential impact of key environmental factors, such as water activity (a_w), temperature and atmospheric CO_2, and their interactions on ecology, growth and aflatoxin production by the *A. flavus* both *in vitro* and on maize. The authors showed that while such interacting abiotic factors have little effect on fungal growth, they however have a significant impact on aflatoxin biosynthetic gene expression and can stimulate the production of aflatoxins. In the insightful mini-review on sexual recombination in aflatoxin-producing *Aspergillus* species, Moore (2014) concisely summarized the potential negative impact of sexual recombination on the feasibility of using biological controls to reduce aflatoxin contamination of field crops. The author discussed specifically the implication of sexual recombination on the fate of two commercially available biopesticides: AF36 and Afla-Guard®. In the excellent review on the characteristics of *A. flavus* as well as the biocontrol strategy using non-toxigenic *A. flavus* strains, Ehrlich (2014) described the current state and outlook of this application in agricultural field. The author concluded that understanding genetic variations among *A. flavus* strains is critical for developing a robust biocontrol strategy, and it is unlikely that a "one size fits all" strategy will work for preharvest aflatoxin reduction.

Host resistance is a very attractive area on aflatoxin research, and various aspects of *A. flavus*-plant host interaction were investigated with special focuses on mechanisms resistant to fungal growth and aflatoxin production (Dolezal et al., 2014; Fountain et al., 2014; Hruska et al., 2014; Scarpari et al., 2014; Shan and Williams, 2014). In the up-to-date review on environmental influences on aflatoxin production on maize, Fountain et al. (2014) detailed the history of research on this complex interaction and pointed out future directions for elucidating host resistance and susceptibility to *A. flavus* colonization in relation to abiotic stress such as drought and heat stresses, and oxidative stress in which aflatoxin may function as an antioxidant to the producing fungus. Utilizing an aflatoxigenic Green Fluorescence Protein (GFP) *A. flavus* strain, Hruska et al. (2014) investigated invasion, colonization and competition in maize kernels by this engineered strain. The authors showed that a decrease in aflatoxin production is correlated with depression of the aflatoxigenic population by the biocontrol strain, AF36, supporting the theory of competitive exclusion. Using a lipidomic approach to investigate *A. flavus*-maize interactions, Scarpari et al. (2014) suggested that *A. flavus* elicits the production of oxylipins in host plants, which function as signals for regulating aflatoxins biosynthesis, conidiogenesis

and sclerotia formation. Their results highlighted the important role of maize oxylipins in driving secondary metabolism in *A. flavus*. In the microarray study to identify maize genes expressed during pathogen infection, Dolezal et al. (2014) found that metabolic processes are linked to defense responses, which include physical changes within the kernel as well as a disruption in kernel development. Shan and Williams (2014) provided a concise but clear overview of current knowledge about quantitative trait loci of corn related to aflatoxin contamination and ongoing efforts in the development of resistant corn lines. The authors concluded that the "phenotypic traits/data" established based on transcriptomics and proteomics approaches could be translated into the practices for improving corn resistance.

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, Mohd-Redzwan et al. (2013) described the historical problems related to aflatoxins in Malaysia, and how these problems have influenced the Malaysian population by highlighting the aflatoxin concentrations in basic food products and their comparison with established aflatoxin limits. The authors emphasized the importance of the legislation of law for a more controlled food production, legal enforcement to meet the set regulatory standards, and the improvement of pre and post-harvest techniques to reduce aflatoxin amounts in food and hence to decrease diseases in Malaysian population. In the comprehensive review about using microRNAs as specific molecular biomarkers in populations exposed to aflatoxins and as early markers for evidence of presence of or damage by hepatocellular carcinoma (HCC), Valencia-Quintana et al. (2014) described differential expression of microRNAs under specific conditions related not only to chemical and environmental pollutants but also to biological pollutants such as the presence of aflatoxins in humans and animals, and consequently, their influence over HCC. The authors provided findings that are important to toxicological research because microRNAs can be used to predict the toxicity of some compounds and will help to explore new treatments.

In a well designed study aimed at enhancing antimycotic activities of known antifungal chemicals by natural compounds, Kim et al. (2014) successfully increased chemosensitization of a kresoxim methyl (Kre-Me), a natural fungicide from strobilurin class by chemically-synthesized benzo analogs. The authors found that among tested benzo analogs, octylgallate (OG) inhibits both growth and aflatoxin production by toxigenic aspergilli more efficiently. The study provided good evidence of remarkable synergism between OG and Kre-Me, which enhances the effectiveness of Kre-Me considerably. The efficient chemosensitizing capability of OG in increasing the efficacy of Kre-Me could reduce effective dosages of strobilurins and alleviate negative side effects associated with the current antifungal treatment.

Finally, as one of the most important and practical issues on aflatoxin research, aflatoxin prevention and elimination, Villers (2014) presented laboratory and field data on an Ultra Hermetic™ storage system, which creates an unbreatheable atmosphere through insect and microorganism respiration alone, in preventing the exponential production and accumulation of aflatoxins. This system is proven useful during multi-month post-harvest safe storage tests of maize, rice and peanuts in hot, humid countries. The author further stressed the need for research on post-harvest protection against aflatoxin contamination by determining the frequency at which excessive aflatoxin levels are reached in the field vs. after months of post-harvest storage using this system.

In conclusion, this research topic opens exciting perspectives on global health issues related to aflatoxins in the food chain and on the development of suitable strategies for preventing toxigenic fungal growth in field and storage, thereby reducing or eliminating subsequent aflatoxin contamination of our food supplies.

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REFERENCES

Dolezal, A. L., Shu, X., O'Brian, G. R., Nielsen, D. M., Woloshuk, C. P., Boston, R. S., et al. (2014). *Aspergillus flavus* infection induces transcriptional and physical changes in developing maize kernels. *Front. Microbiol.* 5:384. doi: 10.3389/fmicb.2014.00384

Ehrlich, K. C. (2014). Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops: advantages and limitations. *Front. Microbiol.* 5:50. doi: 10.3389/fmicb.2014.00050

Fountain, J. C., Scully, B. T., Ni, X., Kemerait, R. C., Lee, R. D., Chen, Z. Y., et al. (2014). Environmental influences on maize-*Aspergillus flavus* interactions and aflatoxin production. *Front. Microbiol.* 5:40. doi: 10.3389/fmicb.2014.00040

Hruska, Z., Rajasekaran, K., Yao, H., Kincaid, R., Darlington, D., Brown, R. L., et al. (2014). Co-inoculation of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus* to study fungal invasion, colonization, and competition in maize kernels. *Front. Microbiol.* 5:122. doi: 10.3389/fmicb.2014.00122

Jamali, M., Ebrahimi, M. A., Karimipour, M., Shams-Ghahfarokhi, M., Dinparast-Djadid, N., Kalantari, S., et al. (2012). Distribution, genetic diversity and mycotoxin production of *Aspergillus* section *Flavi* from soils of pistachio orchards. *Folia Microbiol.* 57, 27–36. doi: 10.1007/s12223-011-0090-5

Keller, N. P., Turner, G., and Bennett, J. W. (2005). Fungal secondary metabolism—from biochemistry to genomics. *Nat. Rev. Microbiol.* 3, 937–947. doi: 10.1038/nrmicro1286

Kim, J. H., Mahoney, N., Chan, K. L., Campbell, B. C., Haff, R. P., and Stanker, L. H. (2014). Use of benzo analogs to enhance antimycotic activity of kresoxim methyl for control of aflatoxigenic fungal pathogens. *Front. Microbiol.* 5:87. doi: 10.3389/fmicb.2014.00087

Medina, A., Rodriguez, A., and Magan, N. (2014). Effect of climate change on *Aspergillus flavus* and aflatoxin B_1 production. *Front. Microbiol.* 5:348. doi: 10.3389/fmicb.2014.00348

Mohd-Redzwan, S., Jamaluddin, R., Abd-Mutalib, M. S., and Ahmad, Z. (2013). A mini review on aflatoxin exposure in Malaysia: past, present, and future. *Front. Microbiol.* 4:334. doi: 10.3389/fmicb.2013.00334

Moore, G. G. (2014). Sex and recombination in aflatoxigenic Aspergilli: global implications. *Front. Microbiol.* 5:32. doi: 10.3389/fmicb.2014.00032

Perrone, G., Logrieco, A. F., and Gallo, A. (2014). Biodiversity of *Aspergillus* section *Flavi* in Europe in relation to the management of aflatoxin risk. *Front. Microbiol.* 5:377. doi: 10.3389/fmicb.2014.00377

Razzaghi-Abyaneh, M., Saberi, R., Sharifan, A., Rezaee, M. B., Seifili, R., Hosseini, S. I., et al. (2013). Effects of *Heracleum persicum* ethyl acetate extract on the growth, hyphal ultrastructure and aflatoxin biosynthesis in *Aspergillus parasiticus*. *Mycotox. Res.* 29, 261–269. doi: 10.1007/s12550-013-0171-1

Scarpari, M., Punelli, M., Scala, V., Zaccaria, M., Nobili, C., Ludovici, M., et al. (2014). Lipids in *Aspergillus flavus*-maize interaction. *Front. Microbiol.* 5:74. doi: 10.3389/fmicb.2014.00074

Shams-Ghahfarokhi, M., Kalantari, S., and Razzaghi-Abyaneh, M. (2013). "Terrestrial bacteria from agricultural soils: versatile weapons against aflatoxigenic fungi," in *Aflatoxins: Recent Advances and Future Prospects*, ed M. Razzaghi-Abyaneh (Rijeka: InTech), 23–40.

Shan, X., and Williams, W. P. (2014). Toward elucidation of genetic and functional genetic mechanisms in corn host resistance to *Aspergillus flavus* infection and aflatoxin contamination. *Front. Microbiol.* 5:364. doi: 10.3389/fmicb.2014.00364

Valencia-Quintana, R., Sánchez-Alarcón, J., Tenorio-Arvide, M. G., Deng, Y., Montiel-González, J. M. R., Gómez-Arroyo, S., et al. (2014). The microRNAs as potential biomarkers for predicting the onset of aflatoxin exposure in human beings: a review. *Front. Microbiol.* 5:102. doi: 10.3389/fmicb.2014.00102

Villers, P. (2014). Aflatoxins and safe storage. *Front. Microbiol.* 5:158. doi: 10.3389/fmicb.2014.00158

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Biodiversity of *Aspergillus* section *Flavi* in Europe in relation to the management of aflatoxin risk

Giancarlo Perrone[1], Antonia Gallo[2] and Antonio F. Logrieco[1]*

[1] *Institute of Sciences of Food Production, National Research Council, Bari, Italy*
[2] *Unit of Lecce, Institute of Sciences of Food Production, National Research Council, Lecce, Italy*

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Massimo Reverberi, Università La Sapienza, Italy
Sofia Noemi Chulze, Universidad Nacional de Rio Cuarto, Argentina

***Correspondence:**
Antonio F. Logrieco, Institute of Sciences of Food Production, National Research Council, via Amendola 122/O, 70126 Bari, Italy
e-mail: antonio.logrieco@ispa.cnr.it

Aflatoxins and the producing fungi *Aspergillus* section *Flavi* are widely known as the most serious and dangerous mycotoxin issue in agricultural products. In Europe, before the outbreak of aflatoxins on maize (2003–2004) due to new climatic conditions, their contamination was confined to imported foods. Little information is available on molecular biodiversity and population structure of *Aspergillus* section *Flavi* in Europe. Preliminary reports evidenced the massive presence of *Aspergillus flavus* L-morphotype as the predominant species in maize field, no evidence of the highly toxigenic S-morphotype and of other aflatoxigenic species are reported. The risk of a shift in traditional occurrence areas for aflatoxins is expected in the world and in particular in South East of Europe due to the increasing average temperatures. Biological control of aflatoxin risk in the field by atoxigenic strains of *A. flavus* starts to be widely used in Africa and USA. Studies are necessary on the variation of aflatoxin production in populations of *A. flavus* to characterize stable atoxigenic *A. flavus* strains. The aim of present article is to give an overview on biodiversity and genetic variation of *Aspergillus* section *Flavi* in Europe in relation to the management of aflatoxins risk in the field.

Keywords: *Aspergillus*, aflatoxin, genetic variation, climate change, atoxigenic strains, biocontrol

INTRODUCTION

The relatively recent outbreak of aflatoxins risk contamination in Europe has driven the EU researchers to investigate on this topic usually confined to tropical and sub-tropical areas. In this respect, aflatoxins and aflatoxigenic fungi are by far the most serious, dangerous and studied mycotoxin issue in agricultural products.

Aflatoxins are secondary metabolites synthesized by several *Aspergillus* species and are highly toxic to humans and animals when ingested at high concentrations. They may cause severe liver damage accompanied by jaundice, hepatitis and death, especially in developing countries (Probst et al., 2007). In addition, they are genotoxic, carcinogenic, and teratogenic for both humans and animals. Daily consumption of foods contaminated with low levels of aflatoxin B_1 (AFB_1) can result in chronic aflatoxicosis with stunting in children, immune suppression, cancer, and reduced life expectancy (Shephard, 2008).

Aflatoxins could extensively contaminate pre-harvest corn, cotton, soybean, peanuts, and tree nuts, and in addition residues from contaminated feed may appear in milk (Bennett and Klich, 2003).

In general, and in most of the published studies, *A. flavus* and *A. parasiticus* are the major prominent species associated in aflatoxin contamination of agricultural crops. However, recently additional species of Section *Flavi* have been reported to be responsible of aflatoxin contamination, i.e., *A. arachidicola* in peanuts and *A. nomius* in corn, nuts, and brazil nuts, especially in certain geographical area (Varga et al., 2011). New aflatoxigenic species *A. mottae*, *A. sergii* and *A. transmontanensis* have been described from maize and almonds in Portugal, they all belong to *A. parasiticus* group (Soares et al., 2012). *A. flavus* isolates produce AFB_1 and AFB_2 and often cyclopiazonic acid (CPA), while most *A. parasiticus* strains produce AFG_1 and AFG_2 in addition to AFB_1 and AFB_2 but never produce CPA (Horn and Dorner, 1999). Production in *A. flavus* is highly variable and depends on genotype, substrate and geographic origin, climate change and agronomic practice. Moreover, *A. flavus* is considered the predominant contaminating organism in soil and in agricultural areas and this species is more invasive and out competes *A. parasiticus* when both species are together in soil. So, most of the extensive researches have been conducted on this species and on its diverse assemblage of strains: different vegetative compatibility groups (VCGs), sclerotial type variants, toxigenic and atoxigenic strains (Ehrlich, 2014). In fact, *A. flavus* populations include isolates with two morphologically distinct sclerotial size variants, L strains with average sclerotial size >400 μm and S strains with sclerotial size <400 μm. Both these morphotypes are found in maize regions around the world; L strains are usually less toxigenic than S strains and produce only AFBs, while S strains are often high producer of aflatoxins and could be divided in two chemotypes: the S_{BG} producing both AFBs and AFGs, and the S_B producing only AFBs. The closely related new species *A. parvisclerotigenus* and *A. minisclerotigenes* were potentially associated to the S_{BG} and S_B taxons of *A. flavus*, respectively (Saito and Tsuruta, 1993; Pildain et al., 2008). Isolates that share morphological characters with S_{BG} have been reported from Thailand, Argentina, and Australia, but the exact taxonomic designation of S_{BG} remains unclear (Cotty and Cardwell, 1999; Donner et al., 2009). More recently, Probst et al. (2012) assigned four phylogenetic distinct groups to the S strains: one seems to belong to *A. minisclerotigenes* (S_{BG} from Australia, Nigeria, Argentina, USA); the second is the highly toxic Kenyan S_B

group (Probst et al., 2007), and a third is the S_B group from elsewhere in the world, e.g., Thailand, United States, and Philippines. Finally, there is the S_{BG} strain group from Nigeria. In general, members of the S_{BG} group are found in locations where high levels of aflatoxin contamination occur.

It is important to underline that, based on actual surveys, the Europe population of *A. flavus* seems constituted only by L strains, none of the studies conducted have found *A. flavus* S strains. In Europe, the occurrence of *A. parasiticus* in maize seems to be very rare, while it resulted more distributed in almond especially in Portugal with an atypical chemotype producing only AFBs toxins (Rodrigues et al., 2011).

Incidence of aflatoxin outbreaks are most severe in tropical and subtropical areas around the world and also temperate regions, such as the United States Midwest are subject to occurrence of aflatoxin contamination. Until 2004, the European perspective regarding aflatoxin contamination was confined to imported foods such as peanut cake, palm kernel, copra, and corn gluten meal (depending of origin; European Food Safety Authority [EFSA], 2004). Several surveys have been conducted for detection of AFs in feed samples in Europe founding a small percentage of materials contaminated with AFB_1 concentration above the regulatory limit. In fact, a survey of 110 maize samples in Northern Italy in 2003, initially planned to monitor the occurrence of fumonisins, showed 75% positive samples to AFBs with a mean of 4.4 and a maximum of 154.5 μg/kg (Piva et al., 2006). In 2006, aflatoxin contaminated rice meal used in dairy cattle feed production had been identified as the cause of elevated AFM_1 levels in Swedish milk.

However, a big survey conducted by European Food Safety Authority [EFSA] (2007) evidenced the emerging issue of potential aflatoxin contamination of corn, almonds, pistachios grown in areas of Southern Europe, due to the subtropical climate occurring in some recent years. In the study of Ibáñez-Vea et al. (2012), detectable levels of AFB_1 were reported for all the 123 Spanish barley samples from 2007 to 2008, and it was found to co-occur with other mycotoxins like ochratoxin A and zearalenone. In the recent work of Streit et al. (2013), samples of feed and feed raw materials from over the world were analyzed during an 8 year period for contamination with different mycotoxins. Regarding aflatoxin contamination, European samples originated primarily from Central Europe showed a high percentage of around 30% positive samples, albeit the pool of samples tested was made up by finished feeds or imported feedstuffs for more than half. A recent warning for maize contamination in Northern Italy was issued in 2012–2013 in consequence of drought conditions favorable to *A. flavus* infection (Andreotti, 2013; Causin, 2013).

In the following chapters of this mini-review we intend to analyze the most important critical points that should be studied and keep under audit to prevent and reduce the spreading of aflatoxin risk in Europe in the next decades.

CLIMATE CHANGE AND RISK OF AFLATOXIN CONTAMINATION IN EUROPE

A wide body of evidence demonstrates that the ability of fungi to grow, survive and interact with a large variety of crop species and to produce mycotoxins is greatly influenced by environmental factors, mainly temperature, relative humidity, insects. These factors are greatly related to climate change and to the variation of temperature and rainfalls. In this sense, food safety has become a very important issue worldwide and the potential effects of climate change on yields and quality of food crops, especially for mycotoxins, have received special attention in the last years, in particular from a risk analysis perspective (Miraglia et al., 2009; Magan et al., 2011). A shift in traditional occurrence areas for mycotoxins is to be expected due to the increasing average temperatures. In this respect, the Mediterranean zones have been identified as a climate change hotspot where extreme changes in temperature, CO_2 levels and rainfall patterns are predicted. Regarding aflatoxins, their contamination events are more prevalent during times of high heat and drought, which may stress the host plant thereby facilitating *A. flavus* infection (Schmidt-Heydt et al., 2009; Mohale et al., 2013).

In 2003 and sporadically in the following years a hot and drying season led to severe *A. flavus* infection of maize in Northern Italy, as mentioned above (Piva et al., 2006; Battilani et al., 2008). The use of this maize as feedstuff for dairy cattle led to a widespread AFM_1 contamination in milk and several thousands of tons of milk exceeding the EU legal limit of 0.05 μg/kg had to be discarded. In the 2 years following this incidence from the study of Decastelli et al. (2007) the presence of AFM_1 in milk and AFB_1 in feed was higher than the maximum allowable in 1.7% of raw milk samples and in 8.1% of feed samples. In 2005, the presence of these aflatoxins was below the limits of EU regulations. So, because of the very dry conditions in those years, *A. flavus* became a significant problem.

Under heat/drought stress also peanuts and pistachio can develop cracking in pods or hull splitting resulting in a significant increase in aflatoxin contamination (Cotty and Jaime-Garcia, 2007). Drought is also a major stress for the plants and undermines their natural immunity against pathogens like mycotoxin-producing fungi (Bircan et al., 2008; Kebede et al., 2012).

New strategies to monitor and predict mycotoxin contamination, either in specific foods or in geographical regions are of recent development, they could be useful in the next years for identify and predict environmental conditions present in regions that may favor mycotoxin proliferation. In this regard, some models have been created to predict aflatoxin for pistachio nuts (Marín et al., 2012), peanuts (Boken et al., 2008), and other crops (Masuoka et al., 2010). More recently, as in Northern Europe *A. flavus* became a dominant pathogen in maize, Battilani et al. (2013) have developed a mechanistic model "AFLA-maize" for the prediction of *A. flavus* infection and AFB_1 contamination in this crop.

Another potential consequence of climate change is that the biocontrol strain could be an inadvertent cause of increased damage to the plant, especially if growing conditions are less favorable for cultivation. Changes in soil environment and its microbiome due to temperature increase, could also subject the crop to amplified damage. So, across Europe, it is relevant to improve harmonization of surveillance and monitoring of aflatoxins; improve database on the geographical distribution and prevention methods for aflatoxin; develop models for the prediction of aflatoxin contamination in the new biogeographical agricultural scenarios.

BIODIVERSITY AND GENETIC DIVERSITY OF *Aspergillus* SECTION *Flavi* IN EUROPE

Soil populations of *A. flavus* are typically composed of isolates from hundreds of different VCGs which reflect phenotypic differences (or similarity) among individuals (Leslie, 1993). Individuals (genotypes) of a fungal species having the same heterokaryon or vegetative incompatibility loci can fuse and undergo genetic exchange through parasexuality (Glass et al., 2000). Fungal isolates that form stable heterokaryons are considered to belong to same VCGs. In *A. flavus* populations, most of variations in morphology and mycotoxin production can be attributed to differences among VCGs. Vegetative compatibility group was believed to be a strong barrier to genetic exchange but recent studies found that VCGs are able to outcross, leading to new VCGs and thereby increased diversity (Olarte et al., 2012).

Recently, sexual reproduction was demonstrated in *A. flavus* which resulted to be an heterothallic fungus with two mating type loci, MAT1-1 and MAT1-2 maintained separately in homokaryotic isolates (Ramirez-Prado et al., 2008; Horn et al., 2009). Recombination can occur within conidia or sclerotia when they harbor multiple nuclei of different mating type and thereby capable of recombination.

Sexual recombination occurs in *A. flavus* through the meiotic process of independent assortment and crossing over that may influence the toxin phenotype of *A. flavus* strains, with a reduction or a complete loss of toxicity (Olarte et al., 2012). The majority of the genetic variation in mycotoxin production arises from mutations in the aflatoxin biosynthetic gene cluster (Chang et al., 2005, 2006), including gene loss, recombination, DNA inversions, partial deletions, translocations, and other genomic rearrangements of the cluster likely due to proximity of the cluster to the telomeric region of chromosome (Carbone et al., 2007).

Comparative analyses of the aflatoxin cluster in various *Aspergillus* species have underlined the complex evolutionary history of this cluster and its role in species adaptation and diversification (Ehrlich et al., 2003; Moore et al., 2009). From analyses of *Aspergillus* populations, several distinct deletions within aflatoxin cluster have been described that may each be responsible for atoxigenicity in various isolates. Either part or the entire biosynthetic cluster resulted deleted, or the non-aflatoxigenicity was associated to inability to amplify selected aflatoxin genes (Chang et al., 2005; Criseo et al., 2008; Donner et al., 2010).

Not many studies on the molecular diversity of *Aspergillus* populations isolated in Europe are available. Among these, the work of Gallo et al. (2012) was about an *A. flavus* population isolated from maize in 2003, during the first outbreak of aflatoxin contamination documented in Northern Italy (Piva et al., 2006; Giorni et al., 2007). The strains were analyzed for the presence of seven aflatoxin biosynthesis genes, including the regulatory genes *aflR* and *aflS*, in relation to their capability to produce AFB_1. All aflatoxin producing isolates exhibited the complete set of amplification products, whereas non-producing isolates did not yield amplified products for three, four or all seven tested genes. The genetic diversity of *A. flavus* populations collected from maize kernels in Northern Italy from 2003 to 2010 was assessed by analysis of VCG and presence or absence of several aflatoxin genes by Mauro et al. (2013). Forty-eight VCGs were identified by means of complementation between nitrate non-utilizing mutants. Twenty-five of these VCGs contained only atoxigenic isolates and the remaining 23 only aflatoxin producers. In addition six deletion patterns of genes in aflatoxin cluster were detected. Regarding the atoxigenic isolates, 12 of them had no deletion in the cluster, 10 had the entire cluster deleted and only one had a deletion pattern only seen once before in Nigeria, with only two genes amplified out of the thirteen tested.

The genetic variability of aflatoxin cluster in non-aflatoxigenic isolates appears diversified and complex but its understanding is important for the selection of safe and effective non-producing strains potentially usable in biocontrol for limiting aflatoxin contamination.

ATOXIGENIC STRAIN AND GENETIC VARIATION IN AFLATOXIN CONTROL STRATEGY

Interest in the variation of aflatoxin production by strains of *Aspergillus* section *Flavi* has increased recently because atoxigenic strains of *A. flavus* are being used as biological control agents to reduce the risk of aflatoxin contamination (Atehnkeng et al., 2008; Wu and Khlangwiset, 2010). Atoxigenic strains may displace wild-type aflatoxigenic strains in crop environments so only the non-aflatoxigenic population of fungi would be present in the field.

The effectiveness of pre-harvest biocontrol strategies using atoxigenic strains is based on competition for substrate, the potential production of inhibitory metabolites, and on their inability to recombine with native toxigenic strains, thus preventing the reacquisition of aflatoxigenicity (Ehrlich and Cotty, 2004; Abbas et al., 2011). Anyway, the choice of a candidate biocontrol atoxigenic *A. flavus* strain could not be based only on the phenotypic characteristic of atoxigenicity; it is necessary to investigate the genotypic condition. The long-term effect of atoxigenic biocontrol strains on native population depends on possibility of sexual recombination, in presence of which the high aflatoxin heritability will induce the regaining of ability to produce aflatoxins. There is never complete inheritance of the atoxigenic phenotype in the offspring of a biocontrol parent, so the use of atoxigenic strains biocontrol with lacking cluster genes would be preferable to one with intact biosynthetic cluster (Olarte et al., 2012; Moore, 2014).

Other factors should be considered for a successful application of biocontrol strategy such as the better understanding of natural diversity of *A. flavus* populations in agricultural soil, the ability of the introduced non-aflatoxigenic strains to recombine with the existing aflatoxigenic strains, the adaptation of *A. flavus* isolates for growth on the plant, the potential damage to the plant from the introduced strain, the potential effect on the soil microenvironment, the timing and the economical cost of application of biocontrol isolates, the potential production of other toxic metabolites, in addition to aflatoxins, which could affect animal health (Ehrlich, 2014).

This form of competitive exclusion of toxigenic strains by non-aflatoxigenic biocontrol strains has been demonstrated under field conditions in cotton (Cotty, 1994), peanuts (Dorner, 2005; Alaniz Zanon et al., 2013) and maize (Abbas et al., 2006).

The aflatoxigenic isolate AF36, which is unable to produce aflatoxin because of a point mutation in the polyketide synthase gene (*pksA*) necessary for aflatoxin biosynthesis (Ehrlich and Cotty, 2004), is registered to be used in USA for the

management of aflatoxin contamination in cottonseed fields. The strain NRRL21882 is the active ingredient of Afla-Guard, a biocontrol formulation consisting of spore-coated barley seeds and used in peanut fields (Chang and Hua, 2007); the isolate is missing the entire aflatoxin and CPA gene clusters and therefore is unable to produce both mycotoxins (Chang et al., 2005, 2009). Recently the possibility to replace grain seeds in this formulation with bioplastic based granules has been explored in field experiments conducted in Northern Italy showing that bioplastic formulations are effective in reducing aflatoxin contamination in corn (Accinelli et al., 2014). In some cases the competing fungi are used as cocktails that include application of multiple strains of non-aflatoxigenic *A. flavus* (Wu et al., 2013).

CONCLUSION

The occurrence of AFB_1 at high levels in Europe in the years 2003–2004 and 2012–2013 underlines the fact that the climate change will entail a change in the mycotoxin distribution patterns observed today. Global trade of plant products can also contribute to the spread of aflatoxigenic fungi and to the increase of diversity of local fungal populations. The study of diversity of aflatoxigenic fungi occurring in maize in Europe, under different points of view-morphological, molecular, metabolic, and plant pathological is essential for the development of strategies for the control of aflatoxin contamination. In this regard, the molecular characterization of native atoxigenic strains, acting through competitive exclusion of aflatoxin producers, with superior adaptation to a geographical region, should provide benefit of long-term displacement of toxigenic strains in maize environment. Additional information on the behavior of these atoxigenic isolates in the target agro-ecosystem will be needed to choose the best biological control agents. Finally, the development of predictive models for aflatoxins occurrence based on regional weather data would be a valuable tool to estimate the risk of contamination after a given growing season, together with using biopesticides in the frame of an integrated pest management (IPM).

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REFERENCES

Abbas, H. K., Zablotowicz, R. M., and Abel, C. A. (2006). Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillus flavus* isolates. *Biocontrol. Sci. Techn.* 16, 437–449. doi: 10.1080/09583150500532477

Abbas, H. K., Zablotowicz, R. M., Horn, B. W., Phillips, N. A., Johnson, B. J., Jin, X., et al. (2011). Comparison of major biocontrol strains of non-aflatoxigenic *Aspergillus flavus* for the reduction of aflatoxins and cyclopiazonic acid in maize. *Food Addit. Contam.* 28, 198–208. doi: 10.1080/19440049.2010.544680

Accinelli, C., Abbas, H. K., Vicari, A., and Shier, W. T. (2014). Aflatoxin contamination of corn under different agro-environmental conditions and biocontrol applications. *Crop Prot.* 63, 9–14. doi: 10.1016/j.cropro.2014.04.021

Alaniz Zanon, M. S., Chiotta, M. L., Giaj-Merlera, G., Barros, G., and Chulze, S. (2013). Evaluation of potential biocontrol agent for aflatoxin in Argentinean peanuts. *Int. J. Food Microbiol.* 162, 220–225. doi: 10.1016/j.ijfoodmicro.2013.01.017

Andreotti, L. (2013). Torna la minaccia aflatossine per il mais 2013. *L'Informatore Agrario* 32, 15.

Atehnkeng, J., Ojiambo, P. S., Ikotun, T., Sikora, R. A., Cotty, P. J., and Bandyopadhyay, R. (2008). Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. *Food Addit. Contam.* 25, 1264–1271. doi: 10.1080/02652030802112635

Battilani, P., Camardo Leggieri, M., Rossi, V., and Giorni, P. (2013). AFLA-maize, a mechanistic model for *Aspergillus flavus* infection and aflatoxin B1 contamination in maize. *Comput. Electron. Agric.* 94, 38–46. doi: 10.1016/j.compag.2013.03.005

Battilani, P., Pietri, A., Barbano, C., Scandolara, A., Bertuzzi, T., and Marocco, A. (2008). Logistic regression modeling of cropping systems to predict fumonisin contamination in maize. *J. Agric. Food Chem.* 56, 10433–10438. doi: 10.1021/jf801809d

Bennett, J. W., and Klich, M. A. (2003). Mycotoxins. *Clin. Microbiol. Rev.* 16, 497–516. doi: 10.1128/CMR.16.3.497-516.2003

Bircan, C., Barringer, S. A., Ulken, U., and Pehlivan, R. (2008). Increased aflatoxin contamination of dried figs in a drought year. *Food Addit. Contam.* 25, 1400–1408. doi: 10.1080/02652030802163414

Boken, V. K., Hoogenboom, G., Williams, J. H., Diarra, B., Dione, S., and Easson, G. L. (2008). Monitoring peanut contamination in Mali (Africa) using the AVHRR satellite data and a crop simulation model. *Int. J. Remote Sen.* 29, 117–129. doi: 10.1080/01431160701264250

Carbone, I., Ramirez-Prado, J. H., Jakobek, J. L., and Horn, B. W. (2007). Gene duplication, modularity and adaptation in the evolution of the aflatoxin gene cluster. *BMC Evol. Biol.* 7:111. doi: 10.1186/1471-2148-7-111

Causin, R. (2013). Cosa sapere per prevenire il rischio aflatossine nel mais. *L'Informatore Agrario* 3, 46–49.

Chang, P. K., Ehrlich, K. C., and Hua, S. S. (2006). Cladal relatedness among Aspergillus oryzae isolates and *Aspergillus flavus* S and L morphotypes. *Int. J. Food Microbiol.* 108, 172–177. doi: 10.1016/j.ijfoodmicro.2005.11.008

Chang, P. K., Horn, B. W., and Dorner, J. W. (2005). Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. *Fungal Genet. Biol.* 42, 914–923. doi: 10.1016/j.fgb.2005.07.004

Chang, P. K., Horn, B. W., and Dorner, J. W. (2009). Clustered genes involved in cyclopiazonic acid production are next to the aflatoxin biosynthesis gene cluster in *Aspergillus flavus*. *Fungal Genet. Biol.* 46, 176–182. doi: 10.1016/j.fgb.2008.11.002

Chang, P. K., and Hua, S. S. (2007). Nonaflatoxigenic *Aspergillus flavus* TX9-8 competitively prevents aflatoxin accumulation by A. *flavus* isolates of large and small sclerotial morphotypes. *Int. J. Food Microbiol.* 114, 275–279. doi: 10.1016/j.ijfoodmicro.2006.09.017

Cotty, P. J. (1994). Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. flavus* infecting cottonbolls and on the aflatoxin content of cottonseed. *Phytopathology* 84, 1270–1277. doi: 10.1094/Phyto-84-1270

Cotty, P. J., and Cardwell, K. F. (1999). Divergence of West African and North American communities of *Aspergillus* section *Flavi*. *Appl. Environ. Microb.* 65, 2264–2266.

Cotty, P. J., and Jaime-Garcia, R. (2007). Influences of climate on aflatoxin producing fungi and aflatoxin contamination. *Int. J. Food Microbiol.* 119, 109–115. doi: 10.1016/j.ijfoodmicro.2007.07.060

Criseo, G., Racco, C., and Romeo, O. (2008). High genetic variability in non-aflatoxigenic *Aspergillus flavus* strains by using Quadruplex PCR-based assay. *Int. J. Food Microbiol.* 125, 341–343. doi: 10.1016/j.ijfoodmicro.2008.04.020

Decastelli, L., Lai, J., Gramaglia, M., Monaco, A., Nachtmann, C., Oldano F., et al. (2007). Aflatoxins occurrence in milk and feed in Northern Italy during 2004–2005. *Food Control* 18, 1263–1266. doi: 10.1016/j.foodcont.2006.08.006

Donner, M., Atehnkeng, J., Sikora, R. A., Bandyopadhyay, R., and Cotty, P. J. (2009). Distribution of *Aspergillus* section *Flavi* in soils of maize fields in three agroecological zones of Nigeria. *Soil Biol. Biochem.* 41, 37–44. doi: 10.1016/j.soilbio.2008.09.013

Donner, M., Atehnkeng, J., Sikora, R. A., Bandyopadhyay, R., and Cotty, P. J. (2010). Molecular characterization of atoxigenic strains for biological control of aflatoxins in Nigeria. *Food Addit. Contam.* 27, 576–590. doi: 10.1080/19440040903551954

Dorner, J. W. (2005). "Biological control of aflatoxin crop contamination," in *Aflatoxin and Food Safety*, ed. H. K. Abbas (New York, NY: Taylor & Francis), 333–352. doi: 10.1201/9781420028171.ch16

Ehrlich, K. C. (2014). Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops: advantages and limitations. *Front. Microbiol.* 5:50. doi: 10.3389/fmicb.2014.00050

Ehrlich, K. C., and Cotty, P. J. (2004). An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase

gene. *Appl. Microbiol. Biotechnol.* 65, 473–478. doi: 10.1016/S1087-1845(02)00509–501

Ehrlich, K. C., Montalbano, B. G., and Cotty, P. J. (2003). Sequence comparison of aflR from different *Aspergillus* species provides evidence for variability in regulation of aflatoxin production. *Fungal Genet. Biol.* 38, 63–74. doi: 10.1016/S1087-1845(02)00509-1

European Food Safety Authority [EFSA] (2004). Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to Aflatoxin B1 as undesirable substance in animal feed. *EFSA J.* 39, 1–27.

European Food Safety Authority [EFSA] (2007). Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. *EFSA J.* 446, 1–127.

Gallo, A., Stea, G., Battilani, P., Logrieco, A. F., and Perrone, G. (2012). Molecular characterization of an *Aspergillus flavus* population isolated from maize during the first outbreak of aflatoxin contamination in Italy. *Phytopathol. Mediterr.* 51, 198–206.

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., and Battilani, P. (2007). Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. *Int. J. Food Microbiol.* 113, 330–338. doi: 10.1016/j.ijfoodmicro.2006.09.007

Glass, N. L., Jacobson, D. J., and Shiu, P. K. T. (2000). The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. *Annu. Rev. Genet.* 34, 165–186. doi: 10.1146/annurev.genet.34.1.165

Horn, B. W., and Dorner, J. W. (1999). Regional differences in production of aflatoxin B1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. *Appl. Environ. Microbiol.* 65, 1444–1449.

Horn, B. W., Moore, G. G., and Carbone, I. (2009). Sexual reproduction in *Aspergillus flavus*. *Mycologia* 101, 423–429. doi: 10.3852/09-011

Ibáñez-Vea, M., González-Peñas, E., Lizarraga, E., and López de Cerain, A. (2012). Co-occurrence of aflatoxins, ochratoxin A and zearalenone in barley from a northern region of Spain. *Food Chem.* 132, 35–42. doi: 10.1016/j.foodchem.2011.10.023

Kebede, H., Abbas, H. K., Fisher, D. K., and Bellaloui, N. (2012). Relationship between aflatoxin contamination and physiological responses of corn plants under drought and heat stress. *Toxins* 4, 1385–1403. doi: 10.3390/toxins4111385

Leslie, J. F. (1993). Fungal vegetative compatibility. *Annu. Rev. Phytopathol.* 31, 127–150. doi: 10.1146/annurev.py.31.090193.001015

Magan, N., Medina, A., and Aldred, D. (2011). Possible climate-change effects on mycotoxin contamination of food crops pre-and postharvest. *Plant Pathol.* 60, 150–163. doi: 10.1111/j.1365-3059.2010.02412.x

Marín, S., Ramos, A. J., and Sanchis, V. (2012). Modeling *Aspergillus flavus* growth and aflatoxins production in pistachio nuts. *Food Microbiol.* 32, 378–388. doi: 10.1016/j.fm.2012.07.018

Masuoka, P., Chamberlin, J., and Elias, M. (2010). Modeling the distribution and probability of aflatoxin occurrence using environmental data. *International Food Policy Research Institute (IFPRI)*. Available at: http://www.ifpri.org/sites/default/files/publications/aflacontrol_wp02.pdf

Mauro, A., Battilani, P., Callicott, K. A., Giorni, P., Pietri, A., and Cotty, P. J. (2013). Structure of an *Aspergillus flavus* population from maize kernels in northern Italy. *Int. J. Food Microbiol.* 162, 1–7. doi: 10.1016/j.ijfoodmicro.2012.12.021

Miraglia, M., Marvin, H. J. P., Kleter, G. A., Battilani, P., Brera, C., Coni, E., et al. (2009). Climate change and food safety: an emerging issue with special focus on Europe. *Food Chem. Toxicol.* 47, 1009–1021. doi: 10.1016/j.fct.2009.02.005

Mohale, S., Medina, A., and Magan, N. (2013). Effect of environmental factors on in vitro and in situ interactions between atoxigenic and toxigenic *Aspergillus flavus* strains and control of aflatoxin contamination of maize. *Biocontrol Sci. Technol.* 23, 776–793. doi: 10.1080/09583157.2013.794895

Moore, G. G. (2014). Sex and recombination in aflatoxigenic *Aspergilli*: global implications. *Front. Microbiol.* 5:32. doi: 10.3389/fmicb.2014.00032

Moore, G. G., Singh, R., Horn, B. W., and Carbone, I. (2009). Recombination and lineage-specific gene loss in the aflatoxin gene cluster of *Aspergillus flavus*. *Mol. Ecol.* 18, 4870–4887. doi: 10.1111/j.1365-294X.2009.04414.x

Olarte, R. A., Horn, B. W., Dorner, J. W., Monacell, J. T., Singh, R., Stone, E. A., et al. (2012). Effect of sexual recombination on population diversity in aflatoxin production by *Aspergillus flavus* and evidence for cryptic heterokaryosis. *Mol. Ecol.* 21, 1453–1476. doi: 10.1111/j.1365-294X.2011.05398.x

Pildain, M. B., Vaamonde, G., and Cabral, D. (2008). Analysis of population structure of *Aspergillus flavus* from peanut based on vegetative compatibility, geographic origin, mycotoxin and sclerotia production. *Int. J. Food Microbiol.* 93, 31–40. doi: 10.1016/j.ijfoodmicro.2003.10.007

Piva, G., Battilani, P., and Pietri, A. (2006). "Emerging issues in Southern Europe: aflatoxins in Italy," in *The Mycotoxin Factbook, Food and Feed Topics*, eds D. Barug, D. Bhatnagar, H. P. van Egmond, J. W. van der Kamp, W. A. van Osenbruggen and A. Visconti (Wageningen: Wageningen Academic Publishers), 139–153.

Probst, C., Callicot, K. A., and Cotty, P. J. (2012). Deadly strains of Kenyan Aspergillus are distinct from other aflatoxin producers. *Eur. J. Plant Pathol.* 132, 419–429. doi: 10.1007/s10658-011-9887-y

Probst, C., Njapau, H., and Cotty, P. J. (2007). Outbreak of an acute aflatoxicosis in Kenya in 2004: identification of the causal agent. *Appl. Environ. Microbiol.* 73, 2762–2764. doi: 10.1128/AEM.02370-2376

Ramirez-Prado, J. H., Moore, G. G., Horn, B. W., and Carbone, I. (2008). Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. *Fungal Genet. Biol.* 45, 1292–1299. doi: 10.1016/j.fgb.2008.06.007

Rodrigues, P., Santos, C., Venâncio, A., and Lima, N. (2011). Species identification of *Aspergillus* section *Flavi* isolates from Portuguese almonds using phenotypic, including MALDI-TOF ICMS, and molecular approaches. *J. Appl. Microbiol.* 111, 877–892. doi: 10.1111/j.1365-2672.2011.05116.x

Saito, M., and Tsuruta, O. (1993). A new variety of *Aspergillus flavus* from tropical soil in Thailand and its aflatoxin productivity. *Proc. Jpn. Assoc. Mycotoxicol.* 37, 31–36.

Schmidt-Heydt, M., Abdel-Hadi, A., Magan, N., and Geisen, R. (2009). Complex regulation of the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* in relation to various combinations of water activity and temperature. *Int. J. Food Microbiol.* 135, 231–237. doi: 10.1016/j.ijfoodmicro.2009.07.026

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Addit. Contam.* 25, 146–151. doi: 10.1080/02652030701567442

Soares, C., Rodrigues, P., Peterson, S. W., Lima, N., and Venâncio, A. (2012). Three new species of *Aspergillus* section *Flavi* isolated from almonds and maize in Portugal. *Mycologia* 104, 682–697. doi: 10.3852/11-088

Streit, E., Naehrer, K., Rodrigues, I., and Schatzmayra, G. (2013). Mycotoxin occurrence in feed and feed raw materials worldwide: long-term analysis with special focus on Europe and Asia. *J. Sci. Food Agric.* 93, 2892–2899. doi: 10.1002/jsfa.6225

Varga, J., Frisvad, J. C., and Samson, R. A. (2011). Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Stud. Mycol.* 69, 57–80. doi: 10.3114/sim.2011.69.05

Wu, F., and Khlangwiset, P. (2010). Evaluating the technical feasibility of aflatoxin risk reduction strategies in Africa. *Food Addit. Contam. A* 27, 658–676. doi: 10.1080/19440041003639582

Wu, F., Stacy, S. L., and Kensler, T. W. (2013). Global risk assessment of aflatoxins in maize and peanuts: are regulatory standards adequately protective? *Toxicol. Sci.* 135, 251–259. doi: 10.1093/toxsci/kft132

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Effect of climate change on *Aspergillus flavus* and aflatoxin B_1 production

Angel Medina , Alicia Rodriguez and Naresh Magan*

Applied Mycology Group, Cranfield Soil and AgriFood Institute, Cranfield University, Cranfield, Bedford, UK

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Paula Cristina Azevedo Rodrigues, Polytechnic Institute of Braganca, Portugal
Russell Paterson, University of Minho, Portugal

***Correspondence:**
Naresh Magan, Applied Mycology Group, Cranfield Soil and AgriFood Institute, School of Applied Science, Cranfield University, Vincent Building, College Road, Cranfield, Bedford MK43 0AL, UK
e-mail: n.magan@cranfield.ac.uk

This review considers the available information on the potential impact of key environmental factors and their interactions on the molecular ecology, growth and aflatoxin production by *Aspergillus flavus in vitro* and in maize grain. The recent studies which have been carried out to examine the impact of water activity × temperature on aflatoxin biosynthesis and phenotypic aflatoxin production are examined. These have shown that there is a direct relationship between the relative expression of key regulatory and structural genes under different environmental conditions which correlate directly with aflatoxin B1 production. A model has been developed to integrate the relative expression of 10 biosynthetic genes in the pathway, growth and aflatoxin B_1 (AFB_1) production which was validated under elevated temperature and water stress conditions. The effect of interacting conditions of a_w × temperature × elevated CO_2 (2 × and 3 × existing levels) are detailed for the first time. This suggests that while such interacting environmental conditions have little effect on growth they do have a significant impact on aflatoxin biosynthetic gene expression (structural *aflD* and regulatory *aflR* genes) and can significantly stimulate the production of AFB_1. While the individual factors alone have an impact, it is the combined effect of these three abiotic factors which have an impact on mycotoxin production. This approach provides data which is necessary to help predict the real impacts of climate change on mycotoxigenic fungi.

Keywords: climate change factors, water activity, temperature, elevated CO_2, growth, gene expression, aflatoxin production, ecology

INTRODUCTION

Food security has become a very important issue world-wide and the potential effects of climate change on yields and quality of food is now receiving significant attention by scientists, especially from a risk analysis perspective. The moldy contamination of staple foods such as cereals has received attention because of their acute and chronic effects in humans and animals. Indeed, the increasing use of staple crops, especially maize for biofuel production, has put further pressure on such key food crops. There is particular interest in maize because it is a key staple food in both developed and developing regions world-wide. Maize is prone to infection by *Aspergillus flavus* and *Aspergillus parasiticus*, especially via insect damage during silking and contamination with aflatoxins. Aflatoxins have been rated as class 1A carcinogens by the International Agency for Research of Cancer (IARC, 2012). They are heat stable and difficult to destroy during processing. Thus exposure, both acute and chronic, can have significant impacts on vulnerable groups, especially babies and children. This has resulted in strict legislative limits in many parts of the world for aflatoxins and mycotoxins in a wide range of foodstuffs (European Commission., 2006). However, in African countries where legislation is often applied to export crops only, consumption of mycotoxin contaminated staple foods is a significant risk, with rural populations exposed to aflatoxins throughout their lives, with serious impacts on their health (Wagacha and Muthomi, 2008). This is exemplified by the relatively recent acute outbreak of severe aflatoxicosis in Kenya (Lewis et al., 2005).

Climate change is expected to have a profound effect on our landscape world-wide. For some areas, climatic models have projected a marked decrease in summer precipitation and increases in temperature, which would result in concomitant drought stress episodes. The environment in which crops will be grown in the next 10–25 years may change markedly with atmospheric CO_2 concentrations expected to double or triple (from 350 to 700 or 900–1000 ppm). Thus, there has been a lot of interest in the impact that climate change scenarios may have on economically important crops/mycotoxigenic fungal infection and contamination with mycotoxins (Paterson and Lima, 2010, 2011; Magan et al., 2011; Wu et al., 2011). Indeed, climate change conditions may impact on the interactions between different mycotoxigenic species and indeed other mycobiota and determine the relative mycotoxin composition contaminating staple foods/feeds (Magan et al., 2010; Paterson and Lima, 2012). Because of this increase and that of other greenhouse gases, the global temperature is expected to increase by between +2 and +5°C.

The EU green paper on climate change in Europe also suggests that effects will be regional and be either detrimental or advantageous depending on geographical area. Thus, in Southern Europe, changes may equate to an increase of 4–5°C with longer drought periods, resulting in increasing desertification, and a

decrease in crop yields. In areas of Western and Atlantic Europe, changes of 2.5–3.5°C with dryer and hotter summers are envisaged. In Central Europe, an increase of 3–4°C, higher rainfall and floods are forecast, although longer growing periods may benefit crop yields. Northern Europe would expect a mean temperature increase of 3–4.5°C, with a significant increase in precipitation of 30–40%. This may lead to increases in crop yields and perhaps new crop cultivation patterns (European Commission., 2007; Solomon et al., 2007). Similar impacts have been described in other areas of the world, especially parts of Asia and Central and South America which are important producers of staple crops (IPCC, 2007). A recent study has predicted that, on a global scale, pests, and diseases are moving to the poles at the rate of 3–5 km/year (Bebber et al., 2013). This could have further impacts on contamination of staple foods such as maize, as increases in pest reproduction rates will lead to more damage and facilitate more infection by *A. flavus* and contamination with aflatoxins. However, in the recent predictions by Bebber et al. (2013) no focus on spread of mycotoxigenic fungi or mycotoxins or interactions between pathogens and pests were considered in the context of climate change.

In developing countries drought stress may be particularly important in terms of food security. For example, marginal land where stress tolerant sorghum was previously grown has now been replaced with maize in both West and East Africa. Maize as well as ground nuts are particularly prone to infection when water stress periods occur. This leads to increased aflatoxin contamination of such crops pre-harvest and post-harvest and can significantly impact on the ability to export the crop and also on the nutritional quality when consumed in rural subsistence communities.

Magan et al. (2011) suggested that climate change factors may result in xerophilic fungi such as *Wallemia sebi*, *Xeromyces bisporus*, and *Chrysosporium* species becoming more important as colonizers of food commodities, as they can grow under very dry conditions [0.65–0.75 water activity (a_w)] where there is much less competition from the majority of mesophilic fungi (Magan, 2006; Magan and Aldred, 2007). For example, *W. sebi* can produce metabolites such as walleminol and walleminone which can be toxic to animals and humans (Piecková and Kunová, 2002). Studies also suggest that there are competitive interactions between these xerophilic fungi in dry and hot conditions and that secondary metabolites may play a role (Leong et al., 2010). This will certainly have an impact on agricultural productivity, especially of essential/staple food crops such as maize and nuts and also influence the interface between plants, insect pests and fungal infection of staple foods (Miraglia et al., 2009). This could have a profound effect on pre- and post-harvest mycotoxin contamination, especially aflatoxins in developing countries, where food quality and security issues are critical.

Examples of modified weather regimes impacting on mycotoxins were demonstrated by the 2003/2004 and subsequently in 2012 summer seasons in the Mediterranean region such as Northern Italy where drought and elevated temperatures resulted in a switch from *Fusarium verticillioides* and contamination with fumonisins to significant contamination of maize grain with *A. flavus* and aflatoxins and entry of aflatoxin M_1 into the dairy chain via the animal feed chain (Giorni et al., 2007). More recently, a survey of Serbian maize samples in 2009–2011 contained no aflatoxins. However, prolonged hot and dry weather in 2012 resulted in 69% of samples containing aflatoxins (Kos et al., 2013). Similarly in Hungary it has also been shown that an increase in aflatoxins may be due to climate change conditions (Dobolyi et al., 2013). However, previous to these examples there are only a few concrete examples of such incidences where climate change factors have been implicated (Magan et al., 2011).

EFFECT OF WATER STRESS × TEMPERATURE STRESS EFFECTS ON AFLATOXIN CLUSTER GENE EXPRESSION, GROWTH, AND AFLATOXIN PRODUCTION

Generally, the aflatoxin biosynthesis genes of *A. flavus* and *A. parasiticus* are highly homologous and the order of the genes (approx. 30) within the cluster has been shown to be the same (Yu et al., 1995, 2004). These include key regulatory genes (*aflR* and *aflS*) and a series of up and downstream structural genes. It has been shown that both water availability and temperature modifications affect the expression of these clusters of genes, relative growth rate and aflatoxin production in both *A. flavus* and *A. parasiticus* (Schmidt-Heydt et al., 2010, 2011). It was shown that there was a good correlation between the expression of an early structural gene (*aflD*) and aflatoxin B_1 (AFB_1) (Abdel-Hadi et al., 2010). It has also been shown that temperature × a_w interactions were related to the ratio of the two key regulatory genes (*aflR/aflS*). The higher the ratio, the higher the relative AFB_1 production (Schmidt-Heydt et al., 2009, 2010; **Figure 1**). This suggests that under certain interacting conditions of two environmental stress factors significantly influences on the relative amounts of AFB_1 produced.

The study by Abdel-Hadi et al. (2010) also showed that when examining the relationship between temporal AFB_1 production and the relative expression of the *aflD* structural gene involved early in the biosynthetic pathway then the relative expression could be mapped over time (**Figure 2**). This also suggests that the optimum a_w for *aflD* expression was at 0.90 a_w, which is different from that for growth (0.95 a_w).

More recent detailed studies using a mycotoxin microarray (Schmidt-Heydt and Geisen, 2007) have been useful in elucidating the relationship between both key structural genes and the regulatory genes and interacting conditions of a_w × temperature and integrated the data on relative expression of 10 genes with growth and AFB_1 data (Abdel-Hadi et al., 2012). **Figure 3** shows the effect of these factors on growth and AFB_1 production. They were able to model and validate this relationship under elevated temperature and drought stress conditions but elevated CO_2 was not included in these studies. However, the relative relationship between the regulatory genes and key structural genes were examined using relative expression data under conditions of changing temperature and water stress to better understand the relationships between the regulatory and structural genes (**Figure 4**). This development of such ternary diagrams can help to evaluate the relationships between 3 key regulatory and structural genes at a time under different temperature and water stress conditions to help identify which are critical in the biosynthetic pathway as environmental factors change.

Ratio of *aflS/aflR* in relation to aflatoxin biosynthesis and different parameter combinations

Parameter combination	Ratio *aflS/aflR*	*Aflatoxin [ng/g]*
25°C/0.90	0.5	3.7
35°C/0.90	0.3	4.7
25°C/0.95	7.4	830.2
30°C/0.95	7.1	3016.9
25°C/0.99	1.5	1957.3
30°C/0.99	2.7	2758.7

FIGURE 1 | The key structural and regulatory genes involved in aflatoxin production and the effect of temperature × water activity conditions, ratio of the two regulatory genes and relative amounts of aflatoxin B_1 production (adapted from Schmidt-Heydt et al., 2010).

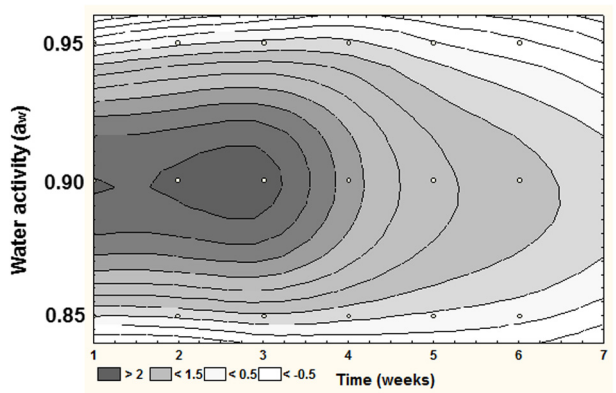

FIGURE 2 | Relative expression of *aflD (nor1)* gene during colonization of peanuts by *Aspergillus flavus* during storage. Optimum expression occurred at 0.90 a_w during the first 2–3 weeks of storage at 25°C (from Abdel-Hadi et al., 2010).

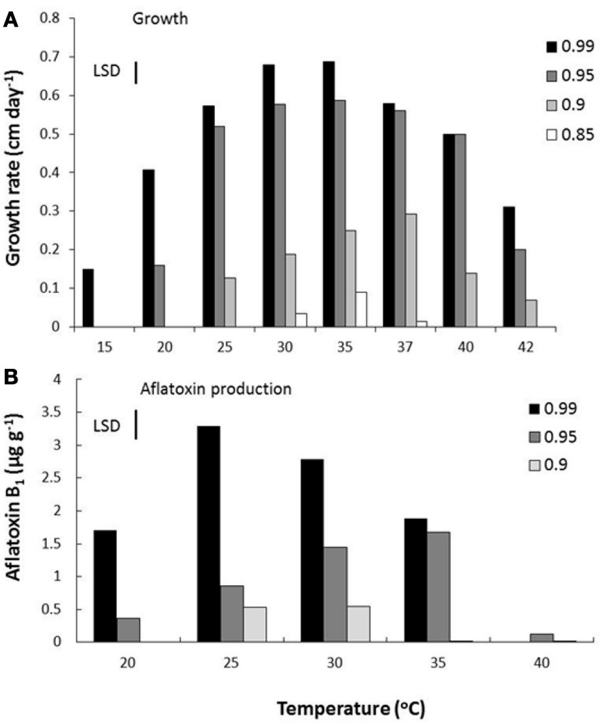

FIGURE 3 | Effect of water activity and temperature on (A) growth and (B) aflatoxin B_1, production by a strain of *A. flavus* (Abdel-Hadi et al., 2012). Bars indicate least Significant Differences.

Schmidt-Heydt et al. (2008) also demonstrated that there was a stimulation of toxin biosynthetic gene expression in different mycotoxigenic fungi when exposed to interacting a_w × temperature stresses including *A. flavus*. They suggested that there was two peaks of expression one under optimum abiotic interacting conditions and one when water and temperature stress was applied. Studies by Yu et al. (2011) examined the effect of elevated temperature on the relative expression of the whole genome of a type strain of *A. flavus* to identify groups of up and down regulated genes. However, these studies were carried out over short time periods and did not include interactions with environmental stresses or with elevated CO_2.

A significant amount of data exists on the effect of interactions between water availability and temperature on the life cycle of mycotoxigenic fungi and mycotoxin production (Sanchis and Magan, 2004; Magan and Aldred, 2007). This includes the ecological conditions of a_w × temperature which will facilitate growth and AFB_1 production. The a_w × temperature boundary conditions for toxin production are slightly different from that for growth. Based on this information it is possible to predict the effect of increased temperature (e.g., 37°C) and water stress

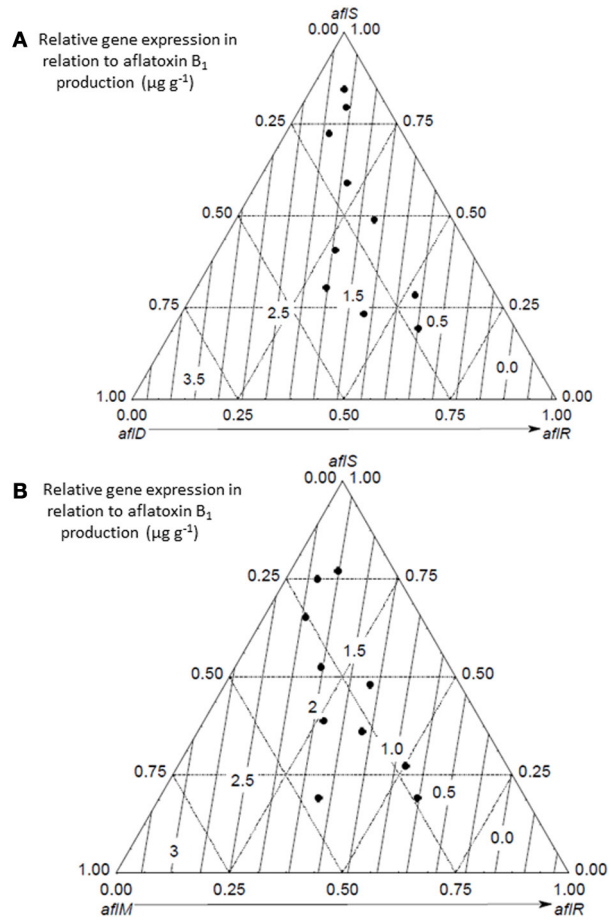

FIGURE 4 | Ternary diagrams of the relationship between the relative expression of the two regulatory genes *(aflS,aflR)* structural genes *(aflD,aflM)* on aflatoxin B_1 production (μg g^{-1}) (from Abdel-Hadi et al., 2012).

(0.95, 0.90 a_w) on growth and aflatoxin production (**Table 1**). This shows that as you increase the temperature to 37°C you get significantly less AFB_1 produced although the *A. flavus* is able to grow. However, this excludes the interaction with CO_2 which is necessary to examine in more detail the impact of predicted climate change scenarios.

CLIMATE CHANGE IMPACTS (a_w × TEMPERATURE × CO_2) ON AFLATOXIN GENE CLUSTER EXPRESSION, GROWTH AND TOXIN PRODUCTION

Recently, Medina et al. (in press) examined the effect of existing environmental conditions and when conditions were changed from 34 to 37°C, with drought stress and CO_2 was increased to 650 and 1000 ppm. They examined the effects on growth of *A. flavus* and on the relative expression of the structural *aflD* and the regulatory *aflR* genes, as well as AFB_1 production for the first time. These studies have shown that for growth of *A. flavus* there was relatively little effect of these interacting climate change conditions (**Table 2**). However, there were significant differences between growth in relation to a_w, but no effect of a_w × temperature × CO_2 on growth rate.

Table 3 summarizes the results of this study and shows the effects of the three-way interacting conditions on relative gene expression (*aflD*, *aflR*) and AFB_1 production. This clearly shows that under slightly elevated CO_2 conditions there was a stimulation of AFB_1 production, especially under drought stress at 37°C and 650 and 1000 ppm CO_2 exposure. It seems that the interactions between these three factors together are critical in the impact that slightly elevated CO_2 has. This is clear from the results obtained at 0.92 and 0.95 a_w × 37°C and 650 or 1000 ppm CO_2 where a statistically significant increase in AFB_1 was observed.

Table 1 | Changes in growth and toxin production by *Aspergillus flavus* due to increase in temperature by +3 and +5°C at different water stress conditions.

Growth				Aflatoxin B_1 production			
a_w	μ max range/T	μ+3	μ+5	a_w	τ max range/T	τ+3	τ+5
0.95	6.9/35	5.6	5.0	0.95	3082–2278/37	102–138	6.1-NP
0.90	2.9/37	1.4	0.7	0.90	448.5–331.5/37	1-NP	NP

Key: μ max, Maximum growth rate (mm day^{-1}); μ+3, Growth rate increasing 3° C; μ+5, Growth rate increasing 5° C; τ max, Maximum toxin production (μg g^{-1}); τ+3° C, Predicted toxin increasing +3° C; τ+5° C, Predicted toxin increasing +5° C, NP, No toxin production.

Table 2 | Comparison of growth of *Aspergillus flavus* under different interacting conditions of elevated temperature, drought stress, and elevated CO_2 *in vitro* on a conductive yeast-glucose medium (adapted from Medina et al., in press).

		Diametric growth (mm/day)					
Temperature		**34°C**			**37°C**		
Water activity		0.97	0.95	0.92	0.97	0.95	0.92
Carbon dioxide (ppm)	350	12.4	11.7	6.8	10.2	9.8	7.3
	650	12.1	11.6	6.9	11.3	10.7	7.8
	1000	12.1	11.3	6.3	10.9	10.5	7.8

This was compared with the effect of these three-way interactions on the relative expression of both a structural gene and a regulatory gene (aflD, aflR) in modified a_w × temperature × 650 and 1000 ppm CO_2.

DISCUSSION AND CONCLUSIONS

This review has considered the impact of different key environmental factors on the growth, gene expression and AFB_1 production by *A. flavus*. This has shown that while there are some examples of the impact that changes in climatic weather conditions may have resulted in a switch with contamination from fumonisins to aflatoxins in maize, there have been few studies to examine the three-way interactions of the key environmental factors. Previous studies have examined water stress × temperature interactions on relative biosynthetic genes involved in aflatoxin production and that by other mycotoxigenic fungi

Table 3 | Summary of the impact that interactions between the three climate change variables have on relative expression of the structural and regulatory genes (*aflD, aflR*), and aflatoxin B_1 production (from Medina et al., in press).

Temperature (°C)	a_w	CO_2 (ppm)	*aflD*	*aflR*	AFB_1
34	0.97	650	=	=	=
		1000	=	=	=
	0.95	650	=	=	=
		1000	=	↑(×3.6)	=
	0.92	650	=	↑↑(×24.4)	↑(×2.6)
		1000	=	↑(×2.0)	↑(×2.0)
37	0.97	650	↑(×4.6)	=	↑↑(×30.7)
		1000	↑(×6.5)	=	↑↑(×23.8)
	0.95	650	↑(×6.4)	↑↑(×14.6)	↑↑↑(×79.2)
		1000	↑(×3.2)	↑↑(×43.9)	↑↑↑(×78.5)
	0.92	650	=	↑↑(×40.4)	↑↑(×15.1)
		1000	↑↑(×22.5)	↑↑↑(×1680)	↑↑(×23.8)

=, variation lower than 2-fold. Numbers between brackets refer to the fold-variation with respect to the control.

(Abdel-Hadi et al., 2010; Schmidt-Heydt et al., 2010; Medina et al., 2013). However, there has been little detailed evidence of the impact that three-way interacting factors may impact on aflatoxin production.

The recent study by Medina et al. (in press) is the first to attempt to quantify the effects of interacting factors of water stress × temperature × elevated CO_2 on growth, biosynthetic gene expression and AFB_1 production. This new study suggests that when the three climate change factors are interacting there are responses which are not obtained when examining a_w × temperature conditions only. Thus, while growth is relatively unaffected by the addition of 2× and 3× existing CO_2 levels at 37°C under the different water stress treatments used, this is not the case with mycotoxin production. The relative increased expression of both the structural *aflD* and the regulatory *aflR* genes in this recent study suggests that there is a significant impact on the biosynthetic genes involved in secondary metabolite production by strains of *A. flavus*. This was especially so at 37°C and under water stress (0.95, 0.92 a_w) where more changes were observed. This study showed that there is a strong stimulation of mycotoxin production (from ×15.1 to ×79.2 depending on the climate exposure conditions used).

Additional studies are now required to evaluate whether this is a general stress response or whether the presence of elevated CO_2 results in its incorporation into the biosynthetic pathways for enzyme production and secondary metabolite production. Perhaps new studies need to be carried out with the cell wall integrity (CWI) and high-osmolarity glycerol (HOG) pathways to examine whether they are triggered by stimuli of the three interacting factors of water stress × temperature × elevated CO_2 or if this is a general stress response *per se* (Hayes et al., 2014). Work is in progress with maize grain to compliment the data obtained by Medina et al. (in press). A recent study by Vaughan et al. (2014) showed that twice the existing CO_2 concentrations (400 and 800 μmol $CO_2 mol^{-1}$) increased the susceptibility of maize to *Fusarium verticillioides* proliferation although fumonisin B1 mycotoxin production was not affected. They showed that inoculation at silking the accumulation of sugars, free fatty acids, lipoxygenase transcripts, phytohormones, and downstream phytoalexins were reduced in the maize grown at elevated CO_2 conditions. Further studies using this approach are required where maize is grown under such conditions and then examining the host-pathogen interaction under the climate change scenarios described here.

Abdel-Hadi et al. (2012) with the aim of forecasting the AFB_1 production by *Aspergillus flavus* examined the integration of growth, gene expression of multiple aflatoxin genes and AFB_1 production by using a mixed secondary metabolite model. This model was validated at 37 and 40°C and different water stress levels and predicted AFB_1 production at 37°C under water stress conditions, but none at 40°C. However, CO_2 was not included in this model. The results obtained suggest that this model could be extended to include CO_2 as a parameter and that this could be a very interesting tool to help in predicting the impact of climate change scenarios with experimental data sets as opposed to being based on historical data sets. This would be beneficial in quantifying impacts of climate change scenarios on economically important staple food crops.

There are some examples of previous studies using data on drought stress × temperature effects on *A. flavus* to predict impacts of interacting environmental factors. Work by Chauhan et al. (2008, 2010) demonstrated that it is possible to utilize an Agricultural Production Systems Simulator to calculate an Aflatoxin Risk Index (ARI) in both maize and peanuts in Australia. For maize they related seasonal temperature and soil moisture during the critical silking period to determine the ARI. They showed that both dry and hot climates made maize prone to a much higher aflatoxin contamination risk. For peanuts, they used the fractional amounts of available soil water during the crucial pod-filling period to determine the ARI. This showed that historically there has been an increase in aflatoxin contamination of peanuts in Australia related to increases in ambient temperature and decreases in rainfall. This has been developed into a web-interface tool for practically real-time use of this model. This approach is very valuable to predict low and high risk years in relation to climatic fluxes and may have application in West Africa where maize is also an important staple crop. However, these models may need modification to provide accurate predictions under climate change scenarios. Recently, Battilani et al. (2013) developed a mechanistic weather-driven model based on the infection cycle of *A. flavus* on maize to predict the risk of aflatoxin contamination in field on a daily basis from silk emergence to harvest. This included a probability index to exceed the legal limit of 5 μg/kg maize for aflatoxin. They suggested that this approach can be used for prediction of *A. flavus* infection and aflatoxin contamination during the growing season and at harvest. It may be possible to input the type of data from the present study to make this approach more accurate and improve the predictions of relative risk to take account of climate changes.

Many of the recent reviews which have examined aspects of the impact of climate change have focused on plant breeding, plant diseases and mycotoxins in Europe, Australia, Africa, and the USA (Boken et al., 2008; Chauhan et al., 2008, 2010; Wu et al., 2011). These have predominantly examined the existing or historical information and tools relevant to the impacts on crop yield, the impact of drought episodes and lack of water or elevated temperatures. Magan et al. (2011) examined the impacts of a_w × temperature stress on potential changes in mycotoxin production when the temperature is changed by +3 and +5°C and under different water stress regimes. Other reviews have used this same data (Paterson and Lima, 2010, 2011, 2012) to make their prediction of potential impacts. However, these previous studies did not include the three way interactions between a_w × temperature × elevated CO_2. The laboratory based studies now available and those being done in FACE based systems need to be combined to be able to obtain more accurate information which can be used to predict on a regional basis the real impact that climate change scenarios may have on exposure to aflatoxins. This is especially important in at risk regions such as parts of Africa and Asia where the risks of exposure may increase under these predicted climate change conditions and threaten food security.

REFERENCES

Abdel-Hadi, A., Carter, D., and Magan, N. (2010). Temporal monitoring of the nor-1 (*aflD*) gene of *Aspergillus flavus* in relation to aflatoxin B1 production during storage of peanuts under different environmental conditions. *J. Appl. Microbiol.* 109, 1914–1922. doi: 10.1111/j.1365-2672.2010.04820.x

Abdel-Hadi, A., Schmidt-Heydt, M., Parra, R., Geisen, R., and Magan, N. (2012). A systems approach to model the relationship between aflatoxin gene cluster expression, environmental factors, growth and toxin production by *Aspergillus flavus*. *J. R. Soc. Interface* 9, 757–767. doi: 10.1098/rsif.2011.0482

Battilani, P., Camardo Leggieri, M., Rossi, V., and Giorni, P. (2013). AFLA-maize, a mechanistic model for *Aspergillus flavus* infection and aflatoxin B1 contamination in maize. *J. Comp. Electron. Agric.* 94, 38–46. doi: 10.1016/j.compag.2013.03.005

Bebber, D. P., Ramotowski, M. A. T., and Gurr, S. J. (2013). Crop pests and pathogens move poleward in a warming world. *Nat. Clim. Change* 3, 985–988. doi: 10.1038/nclimate1990

Boken, V. K., Hoogenboom, G., Williams, J. H., Diarra, B., Dione, S., and Easson, G. L. (2008). Monitoring peanut contamination in Mali (Africa) using the AVHRR satellite data and a crop simulation model. *Int. J. Remote Sens.* 29, 117–129. doi: 10.1080/01431160701264250

Chauhan, Y. S., Wright, G. C., and Rachaputi, N. C. (2008). Modelling climatic risks of aflatoxin contamination in maize. *Aust. J. Exper. Agric.* 48, 358–366. doi: 10.1071/EA06101

Chauhan, Y. S., Wright, G. C., Rachaputi, R. C. N., Holzworth, D., Broome, A., Krosch, S., et al. (2010). Application of a model to assess aflatoxin risk in peanuts. *J. Agric. Sci.* 148, 341–351. doi: 10.1017/S002185961000002X

Dobolyi, C. S., Sebok, F., Varga, J., Kocsube, S., Szigeti, G., Baranyi, N., et al. (2013). Occurrence of aflatoxin producing *Aspergillus flavus* isolates in maize kernels in Hungary. *Acta Alimentria* 42, 451–459. doi: 10.1556/AAlim.42.2013.3.18

European Commission. (2006). Commission Regulation 1881/2006 of 19 December, 2-006. Setting maximum levels for certain contaminants in food stuffs. *Off. J. Eur. Union L* 364, 5–24.

European Commission (2007). Adapting to climate change in Europe – options for, EU action. *Green paper from the Commission to the Council, the European, Parliament, the European, Economic and Social Committee of the Regions, COM. (2007). 254 final, SEC (2007) 849*. European Commission Brussels.

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., and Battilani, P. (2007). Studies on *Aspergillus* Section *Flavi* isolated in northern Italy from maize. *Int. J. Food Microbiol.* 113, 330–338. doi: 10.1016/j.ijfoodmicro.2006.09.007

Hayes, B. M. E., Anderson, M. A., Traven, A., van der Weerden, N. L., and Bleackley, M. R. (2014). Activation of stress signalling pathways enhances tolerance of fungi to chemical fungicides and antifungal proteins. *Cell. Mol. Life Sci.* 71, 2651–2666. doi: 10.1007/s00018-014-1573-8

IARC. (2012). *Agents Classified by the IARC Monographs.* Vol. 1e104. International Agency for Research on Cancer. Available online at: http://monographs.iarc.fr/ENG/Classification/index.php

IPCC. (2007). "Intergovernmental panel on climate change report. Climate Change 2007," in *Synthesis Report* (Geneva), 52.

Kos, J., Mstilovic, J., Hajnal, E. J., and Saric, B. (2013). Natural occurrence of aflatoxins in maize harvested in Serbia during 2009-2012. *Food Control* 34, 31–34. doi: 10.1016/j.foodcont.2013.04.004

Leong, S., Pettersson, O. V., Rice, T., Hocking, A. D., and Schnurer, J. (2010). The extreme xerophilic mould *Xeromyces bisporus* – growth and competition at various water activities. *Int. J. Food Microbiol.* 145, 57–63. doi: 10.1016/j.ijfoodmicro.2010.11.025

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., et al. (2005). Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. *Environ. Health Perspect.* 113, 1763–1767. doi: 10.1289/ehp.7998

Magan, N. (2006). Mycotoxin contamination of food in Europe: early detection and prevention strategies. *Mycopathologia* 162, 245–253. doi: 10.1007/s11046-006-0057-2

Magan, N., and Aldred, D. (2007). Post-harvest control strategies: minimizing mycotoxins in the food chain. *Int. J. Food Microbiol.* 119, 131–139. doi: 10.1016/j.ijfoodmicro.2007.07.034

Magan, N., Aldred, D., Hope, R., and Mitchell, D. (2010). Environmental factors and interactions with mycoflora of grain and grapes: effects on growth and deoxynivalenol and ochratoxin production by *Fusarium culmorum* and *Aspergillus carbonarius*. *Toxins* 2, 353–366. doi: 10.3390/toxins2030353

Magan, N., Medina, A., and Aldred, D. (2011). Possible climate change effects on mycotoxin contamination of food crops pre- and post-harvest. *Plant Pathol.* 60, 150–163. doi: 10.1111/j.1365-3059.2010.02412.x

Medina, A., Rodriguez, A., and Magan, N. (in press). Climate change factors and *A. flavus*: effects on gene expression, growth and aflatoxin production. *World Mycotoxin J.*

Medina, A., Schmidt-Heydt, M., Cárdenas-Chávez, D. L., Parra, R., Geisen, R., and Magan, N. (2013). Integrating toxin gene expression, growth and fumonisin B1 and B2 production by a strain of *Fusarium verticillioides* under different environmental factors. *J. R. Soc. Interface* 10, 20130320. doi: 10.1098/rsif.2013.0320

Miraglia, M., Marvin, H. J. P., Kleter, G. A., Battilani, P., Brera, C., Coni, E., et al. (2009). Climate change and food safety: an emerging issue with special focus on Europe. *Food Chem. Toxicol.* 47, 1009–1021. doi: 10.1016/j.fct.2009.02.005

Paterson, R., and Lima, N. (2010). How will climate change affect mycotoxins in food? *Food Res. Int.* 43, 1902–1914. doi: 10.1016/j.foodres.2009.07.010

Paterson, R., and Lima, N. (2011). Further mycotoxin effects from climate change. *Food Res. Int.* 44, 2555–2566. doi: 10.1016/j.foodres.2011.05.038

Paterson, R., and Lima, N. (2012). "Climate change, fumonisins and animal feed," in *Nutrieconomics: Balancing Global Nutrition and Productivity,* ed E. M. Binder (Tulln: Biomin World Nutrition Forum), 241–247.

Piecková E., and Kunová Z. (2002). Indoor fungi and their ciliostatic metabolites. *Ann. Agric. Environ. Med.* 9, 59–63.

Sanchis, V., and Magan, N. (2004). "Environmental profiles for growth and mycotoxin production, Chapter 8," in *Mycotoxins in Food: Detection and Control*, eds N. Magan and M. Olsen (Cambridge, MA: Woodhead Publishing Ltd.), 174–189.

Schmidt-Heydt, M., Abdel-Hadi, A., Magan, N., and Geisen, R. (2009). Complex regulation of the aflatoxin biosynthesis gene cluster of *A. flavus* in relation to various combinations of water activity and temperature. *Int. J. Food Microbiol.* 135, 231–237. doi: 10.1016/j.ijfoodmicro.2009.07.026

Schmidt-Heydt, M., and Geisen, R. (2007). A microarray for monitoring the production of mycotoxins in food. *Int. J. Food Microbiol.* 117, 131–140. doi: 10.1016/j.ijfoodmicro.2007.01.014

Schmidt-Heydt, M., Magan, N., and Geisen, R. (2008). Stress induction of mycotoxin biosynthesis genes in relation to abiotic factors. *FEMS Microbiol. Lett.* 284, 142–149. doi: 10.1111/j.1574-6968.2008.01182.x

Schmidt-Heydt, M., Parra, R., Geisen, R., and Magan, N. (2011). Modelling the relationship between environmental factors, transcriptional genes and deoxynivalenol mycotoxin production by two Fusarium species. *J. Royal Soc. Interface* 8, 117–126. doi: 10.1098/rsif.2010.0131

Schmidt-Heydt, M., Rüfer, C. E., Abdel-Hadi, A., Magan, N., and Geisen, R. (2010). The production of aflatoxin B1 or G1 by *Aspergillus parasiticus* at various combinations of temperature and water activity is related to the ratio of *aflS* to *aflR* expression. *Mycotoxin Res.* 26, 241–246. doi: 10.1007/s12550-010-0062-7

Solomon, S., Quin, D., Manning, M., Alley, R. B., Berstein, T., Bindoff, N. I., et al. (2007). "Technical summary," in *Climate Change 2007: The Physical Science Basis. Contribution of Working Group 1 to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, eds S. Solomon, D. Quin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor and H. L. Miller (Cambridge, NY: Cambridge University Press), 996.

Vaughan, M. M., Huffaker, A., Schmelz, E. A., Dafoe, N. J., Christensen, S., Sims, J., et al. (2014). Effects of elevated [CO2] on maize defence against mycotoxigenic *Fusarium verticiilioides*. *Plant Cell Environ.* doi: 10.1111/pce.12337. [Epub ahead of print].

Wagacha, J. M., and Muthomi, J. W. (2008). Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. *Int. J. Food Microbiol.* 124, 1–12. doi: 10.1016/j.ijfoodmicro.2008.01.008

Wu, F., Bhatnagar, D., Bui-Klimke, T., Carbone, I., Hellmich, R., Munkvold, G., et al. (2011). Climate change impacts on mycotoxin risks in US maize. *World Mycotoxin J.* 4, 79–93. doi: 10.3920/WMJ2010.1246

Yu, J., Chang, J. W., Wright, M., Bhatnagar, D., Cleveland, T. E., Payne, G. A., et al. (1995). Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. *Appl. Environ. Microbiol.* 61, 2365–2371.

Yu, J., Chang, P.-K., Ehrlich, K. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E., et al. (2004). Clustered pathway genes in aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 70, 1253–1262. doi: 10.1128/AEM.70.3.1253-1262.2004

Yu, J., Fedorova, N. D., Montalbano, B. G., and Bhatnagar, D. (2011). Tight control of mycotoxin biosynthesis gene expression in *Aspergillus flavus* by temperature as revealed by RNA-Seq. *FEMS Microbiol. Lett.* 322, 145–149. doi: 10.1111/j.1574-6968.2011.02345.x

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Sex and recombination in aflatoxigenic Aspergilli: global implications

Geromy G. Moore*

Southern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, New Orleans, LA, USA

Edited by:
Perng-Kuang Chang, United States Department of Agriculture, USA

Reviewed by:
Massimo Reverberi, Sapienza – Università di Roma, Italy
Edwin Rene Palencia, United States Department of Agriculture, USA

***Correspondence:**
Geromy G. Moore, Southern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 1100 Robert E. Lee Blvd, New Orleans, LA 70124, USA
e-mail: geromy.moore@ars.usda.gov

For most of the half century that aflatoxigenic species have been intensively studied, these molds were known only to reproduce asexually, with parasexuality found only in the laboratory between certain mutant strains. Therefore, the fairly recent discovery of their sexual (teleomorphic) states creates a new wrinkle in our understanding of the field behavior of these agriculturally significant fungi. Sex within populations of these fungi, and attendant genetic recombination, eventually may create difficulties for their control; and subsequently for the protection of important human and animal food supplies. Moreover, if fungal sex is a form of response to ecological and environmental stressors, then perhaps human influence and climate change could accelerate this phenomenon. This article will explore scientific research into sexuality and recombination in aflatoxigenic *Aspergillus* species; the potential impacts these phenomena could have on a popular method of pre-harvest prevention of aflatoxin contamination (i.e., use of non-aflatoxigenic *A. flavus* for biocontrol); and the outlook for maintaining control of aflatoxin contamination in an era of changing global climate.

Keywords: *Aspergillus*, aflatoxin, sexuality, recombination, climate change, biocontrol

INTRODUCTION

Within the expansive community of agriculturally significant fungi are species that pose health risks to animals and humans through the production of mycotoxins; consequently, these mycotoxigenic fungi are important to understand and to control (Bennett and Klich, 2003). Aflatoxins are the most serious agricultural mycotoxins. They are mostly produced by a group of fungal species within the genus *Aspergillus*' section *Flavi* (Bennett, 2010), although a few other species outside of this section produce aflatoxins, and carry homologs of the genes for aflatoxin (AF) synthesis (Bradshaw, 2006; Cary et al., 2009; Bradshaw et al., 2013). *Aspergillus flavus* and *A. parasiticus* are the most prominent aflatoxigenic (AF+) species with agricultural significance, contaminating cereal and oilseed crops as well as tree nuts; *A. nomius* has also been reported in agricultural fields (Feibelman et al., 1998; Ehrlich et al., 2007). The global economic losses due to contamination by these fungi are in the billions of dollars (Wu et al., 2008). AF contamination causes negative impacts on health and life across the globe, especially in many developing nations where inhabitants lack the education regarding the risks of consuming aflatoxin-contaminated foods, lack understanding of the importance for proper food storage, or perhaps would rather risk eating contaminated crops than go hungry (Williams et al., 2004; Shephard, 2008). The burden of illness and death associated with aflatoxin consumption is a constant reminder that efficient AF control measures are in need of globalization.

Researchers are working to develop a food supply that is free of AF contamination through implementation of pre-harvest and/or post-harvest strategies (Hell et al., 2008; Abbas et al., 2011). One of the most appealing control measures involves the use of naturally occurring, non-aflatoxigenic (AF−) *A. flavus* as pre-harvest biological control instead of chemical fungicides. This method involves the field dispersal of a high volume of inocula composed of a highly competitive AF− strain. The presence of the AF− strains interferes with the proliferation of indigenous AF+ fungi (Abbas et al., 2011). Studies on the genetic background of these AF− strains show that they result from either random mutations in, or absence/loss of, genes necessary for AF synthesis (Ehrlich and Cotty, 2004; Chang et al., 2005; Moore et al., 2009). Since their introduction a decade ago, two commercially available biopesticides have been in active use throughout the United States known as AF36 and Afla-Guard®. Currently, other strains are being studied and developed as potential biocontrol agents, not only in the U.S. but also in other parts of the world (Pitt and Hocking, 2006; Abbas et al., 2011; Probst et al., 2011). This method of control is gaining favor due to its proven success at reducing AF contamination in the field, but our confidence in using AF− *A. flavus* strains as biopesticides is based largely on the logic that they are predominantly asexual and genetically stable (Ehrlich and Cotty, 2004).

RECOMBINATION AND SEXUALITY IN SECTION *FLAVI*

Ken Papa explored parasexual recombination in *A. flavus* and *A. parasiticus* 40 years ago, but his findings were limited to laboratory experiments between mutant strains (Bennett, 1985). David Geiser was one of the first to report evidence for genetic recombination in *A. flavus*, due to a cryptic sexual state, and hence the potential risk for using *A. flavus* strains as biocontrol (Geiser et al., 1998). His findings inspired further studies by Ignazio Carbone's research group to uncover supportive evidence of recombination within the AF gene clusters of *A. parasiticus* and *A. flavus* populations sampled from within the same peanut field in the U.S.

(Carbone et al., 2007; Moore et al., 2009). Recombination breakpoints within the AF gene clusters of *A. parasiticus* and *A. flavus* were observed, even though the *A. parasiticus* population exhibited less historical recombination compared to the *A. flavus* population in the same field (Moore et al., 2009). Moore et al. performed a global scale study of recombination for *A. flavus* and *A. parasiticus* field populations, representing five separate continents, and observed evidence of recombination within each *A. flavus* population examined and for two of the *A. parasiticus* populations (Moore et al., 2013a). Similar patterns of linkage disequilibrium (LD) could be observed for global *A. flavus* L-strain populations, although the same could not be observed for all of the sampled *A. parasiticus* or *A. flavus* S-strain populations. Ramirez-Prado et al. (2008) characterized mating alleles in *A. flavus* and *A. parasiticus*, and determined that since only one mating-type idiomorph (*Mat1-1* or *Mat1-2*) could be amplified for each isolate it could be concluded that both species exhibit a heterothallic mating system. They developed a PCR diagnostic to quickly identify the idiomorphs, and using this diagnostic test they investigated the distribution of mating-types for the U.S. populations of *A. flavus* and *A. parasiticus*. Global recombination rates appear to correlate with the distribution of mating-type idiomorphs within certain field populations – an equal distribution of *Mat1-1* and *Mat1-2* will yield higher incidences of recombination as well as yield greater diversity of aflatoxigenicity among individuals (Moore et al., 2009). Recombination generates more individual offspring with genomes that differ from both parents, meaning that sex in fungi may increase the number of vegetative compatibility groups (VCGs; Olarte et al., 2011; Moore et al., 2013a). When fungi like *A. flavus* out-cross, they overcome barriers such as vegetative incompatibility (likely to prevent cell death) and allow the exchange of novel genetic material between two fertile strains (Pál et al., 2007; Horn et al., 2009a). Supportive evidence for this circumvention of vegetative incompatibility exists through mating studies in *A. flavus* and *A. parasiticus* whereby the only successful pairings involved strains from different VCGs and opposite mating types (Horn et al., 2009a,b).

Investigations into the sexuality of three AF+ species (*A. parasiticus*, *A. flavus*, and *A. nomius*) eventually led to the discovery and characterization of their teleomorph states using the taxonomic nomenclature of genus *Petromyces* (Horn et al., 2009a,b, 2011). The teleomorph discoveries for the AF+ Aspergilli occurred in the laboratory, and there are some who might argue that experimental crosses in a controlled environment are not representative of natural field conditions. However, experimental field studies by Horn et al. (2013) demonstrated *A. flavus* sexuality in the field. Ears of corn were inoculated with compatible parental strains and later found to contain sclerotia. The sclerotia harvested from the ears of corn were not cleistothecium-bearing stromata; however, sclerotia collected from the corn and further incubated in and on non-sterile soil eventually contained cleistothecia. Reportedly, when these sclerotia fall onto the soil out-crossing is stimulated (Horn et al., 2013). Another teleomorph discovery is the evidence of hybridization reported between *A. flavus* and *A. parasiticus* (Worthington et al., 2011). This may mean that the biological species recognition concept, which defines a species as reproductively isolated (Taylor et al., 2006; Samson and Varga, 2009), cannot be applied to these aflatoxigenic Aspergilli.

A. flavus, *A. nomius*, and *A. parasiticus* exhibit self-incompatible (heterothallic) mating systems (Horn et al., 2009a,b, 2011). Of particular interest for *A. nomius* is that both mating-type idiomorphs may exist within a single strain, whereby certain isolates PCR-amplified as both *Mat1-1* and *Mat1-2*, and either idiomorph may be functional when out-crossing (Horn et al., 2011). A possible explanation for the presence of both mating-type idiomorphs may relate to the fact that *Aspergillus* species possess multi-nucleic conidiospores and hyphal cells, and are potentially heterokaryotic (Olarte et al., 2011; Runa et al., 2011). Olarte et al. (2011) examined progeny from out-crossing *A. flavus* strains that have either a full, partial, or absent AF gene cluster. In one generation of experimental crosses, most of the offspring exhibited recombinant genomes, having specific locus similarity to one or the other parent, while others were genetically distinct from both parents. They reported absence of cyclopiazonic acid (CPA) and AF loci in array Comparative Genomic Hybridization (aCGH) analysis for progeny, despite both parents having full and present CPA and AF clusters within their genomes, and yet the loci could be amplified using PCR. They posit that cryptic alleles influence genome variability, since some offspring were observed to amplify portions of the CPA and AF clusters when both parents exhibited the partial/absent AF cluster configuration (Olarte et al., 2011). Olarte's research suggests that copy numbers of examined loci may influence the genomic condition observed, whereby low copy, ancestral alleles are often masked, but still present among heterokaryotic nuclei (Olarte et al., 2011).

POTENTIAL IMPACTS OF SEXUAL RECOMBINATION ON *A. FLAVUS* BIOCONTROL

The effectiveness of pre-harvest biocontrol strategies using AF− *A. flavus* strains is based on their aggressiveness as competitors coupled with their inability to recombine with native AF+ strains, thereby preventing the re-acquisition of aflatoxigenicity (Ehrlich and Cotty, 2004; Abbas et al., 2011). Indeed, the biocontrol strains may be incapable of reinstated AF production, but not all of the offspring that result from their out-crossing will inherit the AF− phenotype. Through annual inundation of fields with biocontrol strains, the potential, over many generations, to increase the load of "super-competitors" with AF+ properties increases (Moore et al., 2013b). Given the possibility of obtaining multiple VCGs in a single generation, sexually promiscuous offspring could recombine with each other and further increase the population of AF+ individuals, thereby rendering the current biocontrol methodology ineffective in the future.

According to Olarte et al. (2011), the incidence of AF+ offspring observed was higher for crosses involving AF36 (58%) than for the Afla-Guard strain® (36%). For offspring with an AF36 parent, replacement with a functional *pksA* gene can promote AF synthesis, but with the component strain in Afla-Guard® (NRRL 21882) lacking the entire AF gene cluster, a simple replacement is less likely. Since the re-acquisition of AF cluster genes in offspring is less likely, use of a biocontrol strain lacking cluster genes such as NRRL 21882 would be preferable to one with an

intact AF gene cluster. There is never complete inheritance of the AF− phenotype in the offspring of a biocontrol parent, unless both parents exhibit partial- or absent-cluster genotypes (Olarte et al., 2011). In field populations where AF+ strains are present, sex may yield toxigenic progeny. At this time, no studies have been reported that test colonization aggressiveness for progeny resulting from AF+ and biocontrol strain pairings, though there is evidence that AF levels are not significantly higher in the offspring from these out-cross events (Olarte et al., 2011). However, Moore et al. (2013a) reported that higher incidences of toxin diversity exist in actively recombining populations of *A. flavus* and *A. parasiticus*. For example, balancing selection in *A. flavus* seeks to maintain the AF− phenotype, but active recombination will alter the overall AF load of the population by reducing the numbers of AF− individuals (Moore et al., 2009, 2013a).

With regard to hybridization between *A. flavus* and *A. parasiticus*, perhaps the impact from their recombination may be important to agriculture beyond their mycotoxigenic potential. Firstly, *A. parasiticus* is predominately a soil inhabitant (Angle et al., 1982), whereas *A. flavus* is more ubiquitous (Zuluaga-Montero et al., 2010). Hybridization may allow *A. parasiticus* to alter or increase its niche through genetic modification. Hybrid offspring could exhibit far more diversity than recombinant offspring within each species (Olarte et al., 2011; Worthington et al., 2011). Perhaps, the most recently described AF+ "species" such as *A. parvisclerotigenus* and *A. minisclerotigenes* are actually hybrids, between *A. flavus* and B+G AF producers such as *A. parasiticus* or *A. nomius,* which have persisted due to selective advantages resulting from their hybridization events. Current molecular techniques will allow us to refute or support this hypothesis.

In the future, when seeking to use AF− *A. flavus* as pre-harvest biocontrol, there should be more diligence in researching the field ecology where biocontrol strain dispersal is intended for use or is currently in use. Specifically, the population density of native, potentially fertile AF+ species should be ascertained first by thorough field sampling; and additionally, the mating-type distribution of these native field strains should be determined since this will influence the stability of biocontrol (Moore et al., 2013a). If the field is predominately skewed to one mating type or the other, then using a biocontrol strain of the majority mating type could further restrict recombinant opportunities and slow the progression of both strain and toxin diversity (Moore et al., 2013a). In addition, any AF− strains sampled in a field should undergo extensive phenotypic and genomic investigations for consideration as biocontrol in the field where sampled (Moore et al., 2013a). Merely exhibiting the AF− phenotype is no longer a sufficient phenotypic criterion to warrant consideration of a strain as a candidate agent for biocontrol. Given its genotypic condition, the opportunity to generate AF+ offspring with a biocontrol strain such as NRRL 21882 is less likely than that with a strain such as AF36 (Olarte et al., 2011). Not only does NRRL 21882 exhibit the AF− phenotype because of it lacks AF cluster genes, but it also is incapable of producing CPA, another mycotoxin that is considered by some to have contributed to the severity of the Turkey X outbreak in the 1960s (Richard, 2008), while the AF36 biocontrol strain is AF− but produces CPA (Abbas et al., 2011), and CPA production has been observed in all offspring resulting from out-crossing with the AF36 parent (Olarte et al., 2011).

FUNGAL SEX AND RECOMBINATION IN A CHANGING GLOBAL CLIMATE

There is increasing evidence that climate change is causing more unpredictability in global weather patterns. High heat and drought conditions stress plants and facilitate infection by aflatoxigenic species such as *A. flavus* (Scheidegger and Payne, 2003). Agricultural areas experiencing drought often suffer outbreaks of AF contamination. Moreover, diminished water availability limits the ability to irrigate and thereby mitigate the effects of drought (Kebede et al., 2012). At this time, incidences of AF outbreaks are most severe in tropical and sub-tropical areas (between latitudes 40°N and 40°S) around the world (Williams et al., 2004), and even temperate regions such as the United States Midwest are subject to occurrences of AF contamination. However, if current scientific reports are accurate, the average global surface temperature has been increasing by 0.15 °F each year since 1901 (United States Environmental Protection Agency [U.S.E.P.A], 2013). If temperatures continue to increase then the ideal climate for outbreaks of AF contamination will encompass more of our "temperate" agricultural regions and also become more frequent in occurrence. Therefore, it is imperative that as the research establishments continue to seek ways to control AF that researchers be aware of the potential impacts of climate change on the pathogenicity of AF+ fungi and the basic biology of these fungi. Sexual recombination often results from environmental stressors these fungi must overcome in order to adapt and survive. There is an extensive history of recombination in *A. flavus* (Moore et al., 2009). If global climate events assert constant negative pressures on AF+ Aspergilli, then this may accelerate the frequency of recombination in natural populations and lead to unfavorable outcomes for crop protection.

CONCLUSION

The ultimate goal for using AF− *A. flavus* as biocontrol agents is long-term crop protection. Although biocontrol strains are reported to persist for years in inoculated fields (Cotty, 2013), current strategies require annual re-application of biocontrol strains. If the signature of the biocontrol strain is lost then perhaps recombination is to blame. Potentially, even a low rate of recombination between native AF+ fungi and introduced AF− fungi is significant when one considers future food safety. Generations from now, the aflatoxin problem may become more intractable because of the short-term method currently being used to prevent pre-harvest contamination. These fungi are – and have long been – sexually active. Their ability to evolve new phenotypes and genotypes via sexual recombination is a fact that cannot be ignored.

Sometimes, pathologists refer to plant: pathogen interactions as an evolutionary arms race (Anderson et al., 2010). Perhaps the same could be said for the use of non-aflatoxigenic strains for aflatoxin control. Hopefully, with continued research and understanding, we can maintain a consistent level of control without future risk of exacerbating the aflatoxin problem.

REFERENCES

Abbas, H. K., Zablotowicz, R. M., Horn, B. W., Phillips, N. A., Johnson, B. J., Jin, X., et al. (2011). Comparison of major biocontrol strains of non-aflatoxigenic *Aspergillus flavus* for the reduction of aflatoxins and cyclopiazonic acid in maize. *Food Addit. Contam. A.* 28, 198–208. doi: 10.1080/19440049.2010.544680

Anderson, J. P., Gleason, C. A., Foley, R. C., Thrall, P. H., Burdon, J. B., and Singh, K. B. (2010). Plants versus pathogens: an evolutionary arms race. *Funct. Plant Biol.* 37, 499–512. doi: 10.1071/FP09304

Angle, J. S., Dunn, K. A., and Wagner, G. H. (1982). Effect of cultural practices on the soil population of *Aspergillus flavus* and *Aspergillus parasiticus*. *Soil Sci. Soc. Am. J.* 46, 301–304. doi: 10.2136/sssaj1982.03615995004600020017x

Bennett, J. W. (1985). "Taxonomy of the fungi and biology of the Aspergilli," in *Biology of Industrial Microorganisms*, eds A. L. Demain and N. A. Solomon (Boston, MA: Addison-Wesley), 359–406.

Bennett, J. W., and Klich, M. A. (2003). Mycotoxins. *Clin. Microbiol. Rev.* 16, 497–516. doi: 10.1128/CMR.16.3.497-516.2003

Bennett, J. W. (2010). "An overview of the genus *Aspergillus*," in *Aspergillus*: *Molecular Biology and Genomics*, eds M. Machida and K. Gomi (Portland, OR: Caister Academic Press), 1–17.

Bradshaw, R. E. (2006). From protoplasts to gene clusters. *Mycologist* 20, 133–139. doi: 10.1016/j.mycol.2006.09.012

Bradshaw, R. E., Slot, J. C., Moore, G. G., Chettri, P., de Wit, P. J. G. M., Ehrlich, K. C., et al. (2013). Fragmentation of an aflatoxin-like gene cluster in a forest pathogen. *New Phytologist* 198, 525–535. doi: 10.1111/nph.12161

Carbone, I., Jakobek, J. L., Ramirez-Prado, J. H., and Horn, B. W. (2007). Recombination, balancing selection and adaptive evolution in the aflatoxin gene cluster of *Aspergillus parasiticus*. *Mol. Ecol.* 16, 4401–4417. doi: 10.1111/j.1365-294X.2007.03464.x

Cary, J. W., Ehrlich, K. C., Beltz, S. B., Harris-Coward, P., and Klich, M. A. (2009). Characterization of the *Aspergillus ochraceoroseus* aflatoxin/sterigmatocystin biosynthetic gene cluster. *Mycologia* 101, 352–362. doi: 10.3852/08-173

Chang, P.-K., Horn, B. W., and Dorner, J. W. (2005). Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. *Fungal Genet. Biol.* 42, 914–923. doi: 10.1016/j.fgb.2005.07.004

Cotty, P. J. (2013). *Frequently Asked Questions about AF36 on Cotton*, FAQ – ARS Cotty Lab, The University of Arizona. Available at: http://ag.arizona.edu/research/cottylab/faq.htm#seventeenth [accessed December 30, 2013]

Ehrlich, K. C., and Cotty, P. J. (2004). An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. *Appl. Microbiol. Biotechnol.* 65, 473–478. doi: 10.1007/s00253-004-1670-y

Ehrlich, K. C., Kobbeman, K., Montalbano, B. G., and Cotty, P. J. (2007). Aflatoxin-producing *Aspergillus* species from Thailand. *Int. J. Food Microbiol.* 114, 153–159. doi: 10.1016/j.ijfoodmicro.2006.08.007

Feibelman, T. P., Cotty, P. J., Doster, M. A., and Michailides, T. J. (1998). A morphologically distinct strain of *Aspergillus nomius*. *Mycologia* 90, 618–623. doi: 10.2307/3761221

Geiser, D. M., Pitt, J. I., and Taylor, J. W. (1998). Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proc. Natl. Acad. Sci. U.S.A.* 95, 388–393. doi: 10.1073/pnas.95.1.388

Hell, K., Fandohan, P., Bandyopadhyay, R., Kiewnick, S., Sikora, R., and Cotty, P. J. (2008). "Pre- and postharvest management of aflatoxin in maize: an African perspective," in *Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade* eds J. F. Leslie, R. Bandyopadhyay, and A. Visconti (Oxfordshire, UK: CAB International), 219–229.

Horn, B. W., Ramirez-Prado, J. H., and Carbone, I. (2009a). Sexual reproduction and recombination in the aflatoxin-producing fungus *Aspergillus parasiticus*. *Fungal Genet. Biol.* 46, 169–175. doi: 10.1016/j.fgb.2008.11.004

Horn, B. W., Moore, G. G., and Carbone, I. (2009b). Sexual reproduction in *Aspergillus flavus*. *Mycologia* 101, 423–429. doi: 10.3852/09-011

Horn, B. W., Moore, G. G., and Carbone, I. (2011). Sexual reproduction in aflatoxin-producing *Aspergillus nomius*. *Mycologia* 103, 174–183. doi: 10.3852/10-115

Horn, B. W., Sorensen, R., Lamb, M., Sobolev, V., Olarte, R., Worthington, C., et al. (2013). Sexual reproduction in *Aspergillus flavus* sclerotia naturally produced in corn. *Phytopathology* 104, 75–85. doi: 10.1094/PHYTO-05-13-0129-R

Kebede, H., Abbas, H. K., Fisher, D. K., and Bellaloui, N. (2012). Relationship between aflatoxin contamination and physiological responses of corn plants under drought and heat stress. *Toxins* 4, 1385–1403. doi: 10.3390/toxins4111385

Moore, G. G., Elliott, J. L., Singh, R., Dorner, J. W., Stone, E. A., Chulze, S. N., et al. (2013a). Sexuality generates diversity in the aflatoxin gene cluster: evidence on a global scale. *PLoS Pathog.* 9:e1003574. doi: 10.1371/journal.ppat.1003574

Moore, G. G., Mack, B. M., and Beltz, S. B. (2013b). Testing the efficacy of eGFP-transformed *Aspergillus flavus* as biocontrol strains. *Food Nutr. Sci.* 4, 469–479. doi: 10.4236/fns.2013.44060

Moore, G. G., Singh, R., Horn, B. W., and Carbone, I. (2009). Recombination and lineage-specific gene loss in the aflatoxin gene cluster of *Aspergillus flavus*. *Mol. Ecol.* 18, 4870–4887. doi: 10.1111/j.1365-294X.2009.04414.x

Olarte, R. A., Horn, B. W., Dorner, J. W., Monacell, J. T., Singh, R., Stone, E. A., et al. (2011). Effect of sexual recombination on population diversity in aflatoxin production by *Aspergillus flavus* and evidence for cryptic heterokaryosis. *Mol. Ecol.* 21, 1453–1476. doi: 10.1111/j.1365-294X.2011.05398.x

Pál, K., van Diepeningen, A. D., Varga, J., Hoekstra, R. F., Dyer, P. S., and Debets, A. J. M. (2007). Sexual and vegetative compatibility genes in the Aspergilli. *Stud. Mycol.* 59, 19–30. doi: 10.3114/sim.2007.59.03

Pitt, J. I., and Hocking, A. D. (2006). Mycotoxins in Australia: biocontrol of aflatoxin in peanuts. *Mycopathologia* 162, 233–243. doi: 10.1007/s11046-006-0059-0

Probst, C., Bandyopadhyay, R., Price, L. E., and Cotty, P. J. (2011). Identification of atoxigenic *Aspergillus flavus* isolates to reduce aflatoxin contamination of maize in Kenya. *Plant Dis.* 95, 212–218. doi: 10.1094/PDIS-06-10-0438

Ramirez-Prado, J. H., Moore, G. G., Horn, B. W., and Carbone, I. (2008). Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. *Fungal Genet. Biol.* 45, 1292–1299. doi: 10.1016/j.fgb.2008.06.007

Richard, J. L. (2008). Discovery of aflatoxins and significant historical features. *Toxin Rev.* 27, 171–201. doi: 10.1080/15569540802462040

Runa, F., Carbone, I., and Payne, G. A. (2011). "Nuclear condition in *Aspergillus flavus* during growth and conidiation," in *Genetics of Maize Disease Workshop*, Raleigh. Available at: http://www.pngg.org/maize/abstract.html [accessed February 20–23, 2011].

Samson, R. A., and Varga, J. (2009). What is a species in *Aspergillus*? Medical Mycology 47 (S1), S13–S20. doi: 10.1080/13693780802354011

Scheidegger, K. A., and Payne, G. A. (2003). Unlocking the secrets behind secondary metabolism: a review of *Aspergillus flavus* from pathogenicity to functional genomics. *Toxin Rev.* 22, 423–459. doi: 10.1081/TXR-120024100

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Addit. Contam. A.* 25, 146–151. doi: 10.1080/02652030701567442

Taylor, J. W., Turner, E., Townsend, J. P., Dettman, J. R., and Jacobson, D. (2006). Eukaryotic microbes, species recognition and the geographic limits of species: examples from the kingdom Fungi. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361, 1947–1963. doi: 10.1098/rstb.2006.1923

United States Environmental Protection Agency. [U.S.E.P.A]. (2013). "Climate Change Indicators in the United States" in *EPA*. Available at: http://www.epa.gov/climatechange/science/indicators/weather-climate/temperature.html [accessed Sepetember 20, 2013 and November 21, 2013].

Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M., and Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am. J. Clin. Nutr.* 80, 1106–1122.

Worthington, C. J., Horn, B. W., Moore, G. G., Monacell, J. T., Singh, R., Stone, E. A., et al. (2011). "Hybridization between *Aspergillus flavus* and *Aspergillus parasiticus*," in *Fungal Genetics Conference Proceedings*, Pacific Grove, CA.

Wu, F., Liu, Y., and Bhatnagar, D. (2008). Cost-effectiveness of aflatoxin control methods: economic incentives. *Toxin Rev.* 27, 203–225. doi: 10.1080/15569540802393690

Zuluaga-Montero, A., Ramírez-Camejo, L., Rauscher, J., and Bayman, P. (2010). Marine isolates of *Aspergillus flavus*: denizens of the deep or lost at sea? *Fungal Ecol.* 3, 386–391. doi: 10.1016/j.funeco.2010.05.003

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Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops: advantages and limitations

Kenneth C. Ehrlich*

Southern Regional Research Center, United States Department of Agriculture – Agricultural Research Service, New Orleans, LA, USA

Edited by:
Mahendra Kumar Rai, Sant Gadge Baba Amravati University at Amravati, India

Reviewed by:
Ebrahim Hadavi, Islamic Azad University, Iran
Zhao Chen, Clemson University, USA

***Correspondence:**
Kenneth C. Ehrlich, Southern Regional Research Center, United States Department of Agriculture – Agricultural Research Service, New Orleans, LA 70124, USA
e-mail: ken.ehrlich@ars.usda.gov

Aspergillus flavus is a diverse assemblage of strains that include aflatoxin-producing and non-toxigenic strains with cosmopolitan distribution. The most promising strategy currently being used to reduce preharvest contamination of crops with aflatoxin is to introduce non-aflatoxin (biocontrol) *A. flavus* into the crop environment. Whether or not introduction of biocontrol strains into agricultural fields is enough to reduce aflatoxin contamination to levels required for acceptance of the contaminated food as fit for consumption is still unknown. There is no question that biocontrol strains are able to reduce the size of the populations of aflatoxin-producing strains but the available data suggests that at most only a four- to five-fold reduction in aflatoxin contamination is achieved. There are many challenges facing this strategy that are both short term and long term. First, the population biology of *A. flavus* is not well understood due in part to *A. flavus*'s diversity, its ability to form heterokaryotic reproductive forms, and its unknown ability to survive for prolonged periods after application. Second, biocontrol strains must be selected that are suitable for the environment, the type of crop, and the soil into which they will be introduced. Third, there is a need to guard against inadvertent introduction of *A. flavus* strains that could impose an additional burden on food safety and food quality, and fourth, with global warming and resultant changes in the soil nutrients and concomitant microbiome populations, the biocontrol strategy must be sufficiently flexible to adapt to such changes. Understanding genetic variation within strains of *A. flavus* is important for developing a robust biocontrol strategy and it is unlikely that a "one size fits all" strategy will work for preharvest aflatoxin reduction.

Keywords: aflatoxin, *Aspergillus flavus*, biocontrol, food safety, recombination, maize, cottonseed, population diversity

DEVELOPMENT OF THE BIOCONTROL STRATEGY

Fungal growth on agricultural commodities, with or without mycotoxin production, does not occur in pure culture. Early studies found that aflatoxin production by *Aspergillus flavus* is reduced when it is cultivated with certain other fungi and bacteria (Ashworth et al., 1965; Weckbach and Marth, 1977; Wicklow et al., 1980, 1988; Horn and Wicklow, 1983; Ehrlich et al., 1985). The soil microbiome, mainly fungi and bacteria, affects the ability of the fungi to produce secondary metabolites (Ashworth et al., 1965). It has long been known that some plants are never contaminated with aflatoxin even though aflatoxin-producing species are present in the soil (Wogan, 1966). However, when tissues of these resistant plants are sterilized, *A. flavus* has no trouble producing aflatoxin on the tissue (Ehrlich and Ciegler, 1985). The ability of a fungus to compete for a host depends on many factors including pH, soil type, nitrogen and carbon availability, and water and mineral content (Eugenio et al., 1970). In 1975 an outbreak of *A. flavus* contamination of field corn in Iowa led to an unexpectedly low level of aflatoxin contamination of the crop (Ehrlich et al., 1985). Wicklow and others noticed that co-culture of *A. flavus* with *A. niger* caused a marked reduction in the formation of aflatoxin beyond a simple displacement of one fungus by the other (Wicklow et al., 1980). We found that co-cultivation of *A. flavus* with *P. oxalicum* had a similar effect on aflatoxin production but, in this case, not only were aflatoxin amounts reduced to a level not accountable by simple displacement, but a metabolite of *P. oxalicum*, secalonic acid, was also reduced (Ehrlich et al., 1985). We speculated that the co-cultivation of the two organisms caused a competition for ATP that is needed for one or more of the oxidation steps in secondary metabolite formation. The order of inoculation was also important for determining which of the two competing fungi was successful in reducing mycotoxin production. Inoculation with the *P. oxalicum* first greatly inhibited *A. flavus* production of aflatoxins even when eventual growth was similar. We also showed that *A. flavus* mutants reduced in their abilities to produce aflatoxins also showed similar competitor ability to that of *P. oxalicum* (Ehrlich, 1987). Recent work (see below) has offered a different interpretation of the mechanism behind these competition results (Sweany et al., 2011).

This early work supported the concept that competition with *A. flavus* isolates incapable of aflatoxin production could remediate aflatoxin contamination. This mode of biocontrol is currently the most widely used biocontrol method for reducing aflatoxin contamination of cereal crops in maize and cottonseed where

aflatoxin contamination is a persistent problem for human and animal health (Wu and Khlangwiset, 2010a). Cotty and co-workers in the 1990s found that one particular non-aflatoxigenic *A. flavus* isolate (AF36) isolated from Arizona cottonseed, is an especially good competitor for reduction of aflatoxin content of cottonseed (Cotty and Bhatnagar, 1994). They determined that isolates from this VCG group were generally good as competitors against aflatoxin-producing isolates (Chang et al., 2012). We subsequently found that this isolate was unable to produce aflatoxin because of a point mutation in the polyketide synthase gene that is necessary for aflatoxin biosynthesis (Ehrlich and Cotty, 2004).

Cotty and co-workers developed a method to apply the non-aflatoxigenic strain to cotton-growing fields to prevent aflatoxin production by the wild-type aflatoxigenic populations present in the growing regions (Cotty, 2006; Cotty and Mellon, 2006). The method involves spreading non-aflatoxigenic *A. flavus* spores onto the field at particular times prior to harvest (Jaime-Garcia and Cotty, 2007). They assumed that addition of the non-aflatoxin producing strain would then allow it to out-compete the wild-type populations for access to the cottonseed and thereby displace the wild-type fungus. Therefore, based on this concept they called this strategy a "displacement" strategy for biocontrol of aflatoxin contamination (Jaime-Garcia and Cotty, 2009). Assuming this is true than the resulting treated field should never have to be treated again because, then, only the non-aflatoxigenic population of fungi would be present in the field. This method to prevent aflatoxin contamination is now in widespread use in Arizona for cotton fields and in other places in the southeast U.S for treatment of maize-growing areas. In other countries where aflatoxin contamination of maize is an endemic problem such as Kenya and parts of China, other strains have been discovered that are being used for aflatoxin remediation. In some cases the competing fungi are used as "cocktails" that include application of multiple strains of non-aflatoxigenic *A. flavus* (Wu et al., 2013).

There is a growing awareness that *A. flavus* also produces an indole tetramic acid mycotoxin, cyclopiazonic acid (CPA), under the same conditions that it produces aflatoxin (Chang and Ehrlich, 2011). CPA is a specific inhibitor of sarcoplasmic and endoplasmic reticulum calcium-dependent ATPase, an enzyme necessary for proper muscle contraction and relaxation. Therefore, non-aflatoxigenic competitor isolates incapable of production of this metabolite are being used instead of AF36 for reduction of aflatoxin levels in maize (Abbas et al., 2011). In a subsequent section we will discuss the possibility that other mycotoxins are produced by both the non-aflatoxigenic and aflatoxigenic populations that could contribute to mycotoxin contamination and cause toxic effects in humans and animals upon consumption.

Although the biocontrol strategy for aflatoxin remediation is increasingly being adopted world wide, there are several potential pitfalls that should be addressed These include the need to better understand the natural diversity of *A. flavus* populations in agricultural soil, the effects of climate change on both this diversity and on plant susceptibility, the ability of the introduced biocontrol strain to outcross with existing aflatoxin-producing *A. flavus*, the adaptation of certain *A. flavus* isolates for predominant growth on the plant rather than in the soil, the difficulty in timing the application or controlling the stability of the inoculum, how the introduction of the biocontrol strain affects the soil microenvironment, the potential damage to the plant from the introduced strain, and the need to better understand the entire *A. flavus* toxin burden that may result from *A. flavus* contamination beyond that of aflatoxin. In addition the cost of the biocontrol method and the potential need to continue reapplication seasonally must also be considered in weighing the benefits of biocontrol *A. flavus* as a means of reducing food and feed contamination with aflatoxin.

EFFICACY OF BIOCONTROL BY NON-AFLATOXIGENIC ISOLATES OF *A. flavus*

More than 100 countries have enforced or proposed regulations for levels of aflatoxin in feeds and foods (Wu et al., 2013). Because these levels are so low, the regulations place a strong burden on grain intended for export. Some of these requirements are listed in **Table 1**. Normally maize contains only low levels of aflatoxin and usually meets these requirements (Yu et al., 2008), but in years with severe outbreaks of *A. flavus*, contamination levels can exceed 100–200 ppb (Johansson et al., 2006). In cottonseed grown in Arizona levels of aflatoxin frequently exceed the levels permitted for commerce and remediation by either diluting the contaminated meal with less contaminated grain or by chemical treatment to destroy aflatoxins is often necessary. **Table 2** presents a summary of results from several laboratories showing aflatoxin concentrations after treatments with several different biocontrol *A. flavus* and of several different crops. In some studies the reported reduction in aflatoxin content in treated versus untreated fields is as much as 20-fold (Dorner et al., 2003; Dorner, 2009, 2010; Abbas et al., 2011; Zanon et al., 2013). These data are for experimental laboratory studies where treatments were presumably done under optimized and highly controlled conditions. Generally a 5- to 20-fold reduction in aflatoxin levels would be sufficient for allowing the crop to meet the standards for consumption, but if the starting levels are particularly high, even a 20-fold reduction may not be enough. Recently, in several maize-growing regions in Kenya there have been reports of aflatoxin poisoning in humans (Probst et al., 2012; Wagacha et al., 2013; Yard et al., 2013). In these cases, ingestion was of maize that was contaminated after harvest by improper storage. It is not clear that a pre-harvest biocontrol strategy would be able to prevent such exposure and there may be simpler and more cost-effective methods to prevent ingestion of post-harvest contaminated maize.

LIFE CYCLE AND DIVERSITY OF *A. flavus*

Aspergillus flavus is the most common species associated with aflatoxin contamination of agricultural crops (Cotty et al., 1994; Cotty, 1997). *A. flavus* is found in temperate and tropical regions in soil and, in agricultural areas, most commonly, on maize, cotton, tree, and ground nuts (Samuel et al., 2013) and less frequently on rice (Chen et al., 2013). *A. flavus* populations are highly diverse and their stability in the soil and on the plant is not well understood. An atoxigenic relative of *A. flavus*, *A. oryzae*, is widely used in soybean and rice fermentation (Chang and Ehrlich, 2010). It is now increasingly clear that *A. oryzae* is not a separate species but actually is only one of many examples of atoxigenic variants of *A. flavus* (Geiser et al., 2000; Chang et al., 2006). Other aflatoxin-producing

Table 1 | Allowable levels of aflatoxins in foods and feeds.

Country	Limit in PPB
France	0.1–10
Netherlands	0.02–5
Germany	5
Japan	10
Austria	0.2–1
United Kingdom	10
India	30
Malaysia	35
Mexico	20
United States	20

Table 2 | Efficacy of biocontrol treatments.

Crop	Non-AF agent	Range of AF reduction % (treated control)	Reference
Maize	K49	83–98	Abbas et al. (2012)
	Afla-guard	9–75	Dorner (2009)
	Afla-guard	85–88	Dorner (2010)
Peanut	Afla-guard	89–96	Dorner et al. (2003)
	AFCHG2	75	Zanon et al. (2013)
Cotton	AF36	20–88	Cotty and Bhatnagar (1994)

fungi have been implicated in contamination of agricultural commodities. *A. parasiticus* has been associated with contaminations of peanuts in the United States (Horn, 2005), Argentina (Vaamonde et al., 2003), and West Africa (Ismail, 2001), but generally, the predominant contaminating organism is *A. flavus* (Cotty et al., 1994). *A. flavus* appears to be more invasive and out-competes *A. parasiticus* when both species are together in the soil. *A. nomius* is more rarely found in the soil, and usually is not associated with agricultural contamination episodes (Cotty et al., 1994; Bhatnagar et al., 2001; Cardwell and Cotty, 2002). Mis-identification of the contaminating organism, in some cases is possible. For example, in Thailand, some aflatoxin B-and G-producing organisms, found to be common in the soil resemble *A. flavus*, but have been conclusively identified as a new clade of *A. nomius* (Ehrlich et al., 2007a).

A. flavus is a diverse assemblage of strains which include toxin-producing and non-toxigenic strains, sclerotial type variants, strains with variability in response to light, strains residing in multiple vegetative compatibility groups (VCGs), and strains with variable ability to colonize living plant tissue. A cladogram showing *A. flavus* diversity is shown in **Figure 1**. As a predominantly saprophytic fungus, *A. flavus* resides in the soil, but as an opportunist it is readily able to colonize most environments whenever there is a rich source of carbon and nitrogen. *A. flavus*'s diversity, therefore, appears to be an evolutionary response to its cosmopolitan distribution. Its main mode of replication is by asexual sporulation but under some conditions, *A. flavus* forms sclerotia, hardened masses of desiccated and melanized mycelia that are able to survive adverse environmental and nutritional conditions.

Aspergillus flavus soil populations contain isolates from two morphologically distinct sclerotial size variants, termed the L-strain [also called *A. flavus* Group IB (Geiser et al., 2000)] for isolates with average sclerotial size >400 μm and the S-strain (Group IA) for isolates with sclerotial size less that 400 μm (Cotty, 1997). Both S-and L-strains of *A. flavus* are found globally in maize-growing regions of the world. On typical laboratory growth media, when grown in the dark, S-strain isolates produce higher levels of aflatoxins, more abundant sclerotia, and fewer conidia. Atoxigenic S-strain isolates are very rarely found in natural environments (Orum et al., 1997). *A. flavus* lacks the ability to produce G-aflatoxins due to a gap in the cluster that includes a required cytochrome P450-encoding gene, *cypA*. The size of the deletion that causes loss of a portion of *cypA* is 1.5 kb for S-strain isolates and 0.8 kb for L-strain isolates. Differences in sclerotial morphology correlate with the differences between the S- and L-strain *A. flavus* in the size of the deletion in the *norB-cypA* gene (Ehrlich et al., 2004). Soil populations of *A. flavus* are typically composed of isolates from hundreds of different VCGs. Although frequent genetic exchange among these groups has not been observed, historical recombination in populations probably has occurred. Because the 0.8 kb deletion in S-strain isolates is identical to the deletion in those *A. oryzae* isolates that possess most of the aflatoxin cluster, such isolates may have descended from a common ancestor that had the S-strain-type *norB-cypA* gene deletion (Chang et al., 2005). On average 30% of the *A. flavus* soil isolates in Arizona were identified as belonging to the S-strain (Cotty, 1997; Orum et al., 1997). Because S-strain isolates consistently produce more aflatoxin than L-strain isolates and aflatoxin production in this strain is not as strongly affected by nitrogen source, the concentration of S-strain isolates in the soil appears to be better correlated with major outbreaks of aflatoxin contamination in cotton-growing areas in Arizona and Texas (Orum et al., 1997; Jaime-Garcia and Cotty, 2006). Furthermore, up to 40% of the L-strain soil isolates of *A. flavus* found in Arizona and other regions of the United States (Horn and Dorner, 1999) were incapable of producing aflatoxins while S-strain isolates rarely were atoxigenic (Cotty et al., 1994). Interestingly, Two of the biocontrol strains used in the United States, AF36 and K49, have the S-strain type *norB-cypA* deletion which may correlate with their competitor abilities (Chang et al., 2012).

POPULATION DYNAMICS OF *A. flavus* IN AGRICULTURAL ENVIRONMENTS

Aspergillus flavus was considered to be incapable of forming a sexual state and therefore was expected to maintain an entirely asexual life-style. Populations are divided into VCGs. Vegetative compatibility was believed to be a strong barrier to genetic exchange and in *A. flavus* was thought to be controlled by as many as 12 genetic loci (Bayman and Cotty, 1991; Ehrlich et al., 2007b). We found that

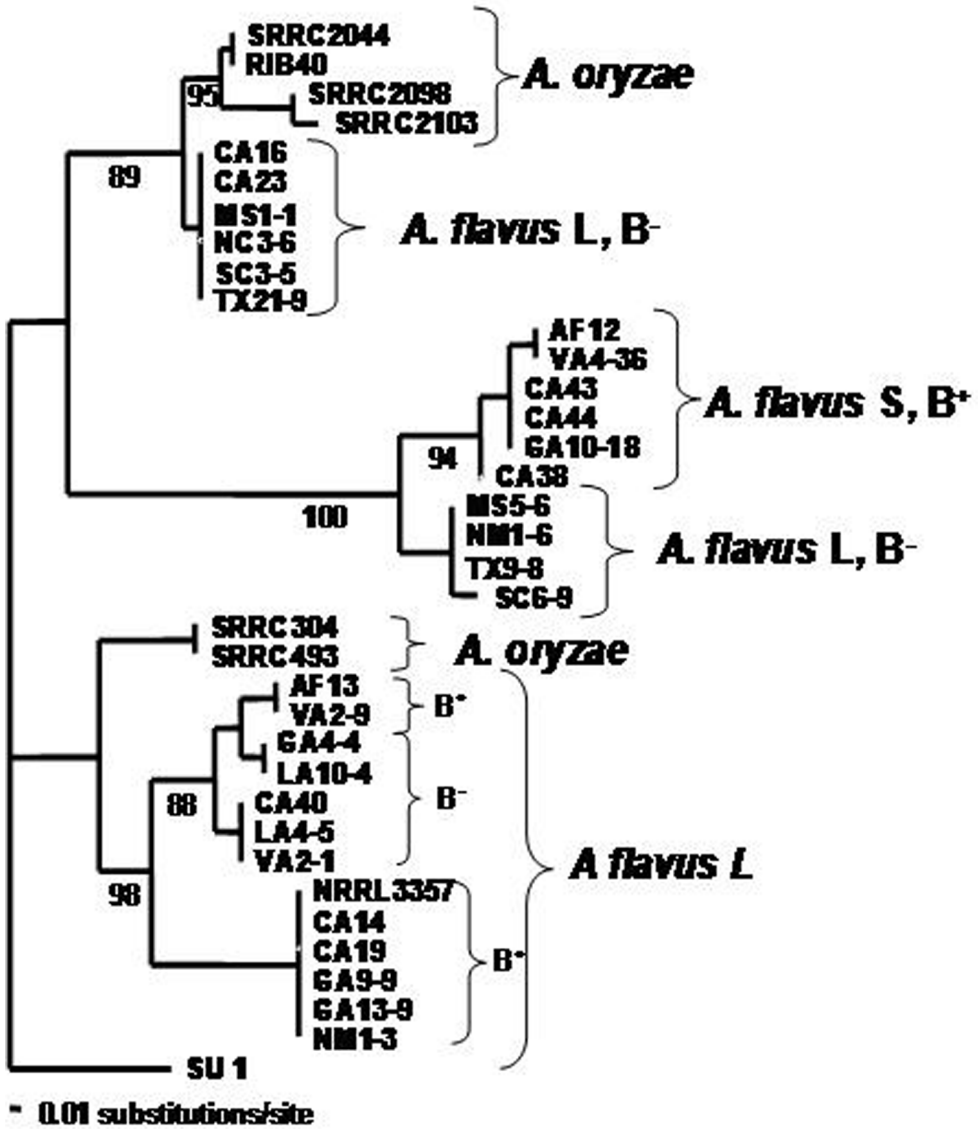

FIGURE 1 | Cladogram illustrating the diversity of *A. flavus* isolates selected from several different cotton and maize-growing areas in the United States. RIB40 is the Japanese *A. oryzae* strain used for soy and rice fermentations.

A. flavus isolates from different VCGs formed genetically distinct groups suggesting that recombination is at most an infrequent event (Ehrlich et al., 2007b). Genetic isolation has been suggested by an additional study that found no evidence of gene flow between VCGs, including VCGs of opposite mating-type. Their results suggest that the VCGs diverged before domestication of agricultural hosts (> 10,000 year before the present; Grubisha and Cotty, 2009).

Recently *A. flavus* and many other presumed asexual fungi have been found to be capable of sexual reproduction, when grown in the dark under nutrient deprived conditions. *A. flavus*, as a heterothallic fungus, has two mating type loci, Mat1-1 and Mat1-2, maintained separately in homokaryotic isolates (Ramirez-Prado et al., 2008). Early evidence from genetic analysis suggested that *A. flavus* populations are able to undergo recombination (Geiser et al., 1998). Recent studies found that *A. flavus* in different VCG are able to outcross, and that VCG is not a strong barrier to sexual recombination (Olarte et al., 2012a). In fact such outcrossing among VCGs leads to new VCGs, and thereby, increased diversity (Olarte et al., 2012b). Most of these recombination studies have been done under laboratory conditions. Recombination can occur within conidia or sclerotia when they harbor multiple nuclei of different mating type. Fusion of nuclei containing different fluorescent markers revealed that, while conidial populations are predominantly homokaryotic, a small percentage can become heterokaryotic and, thereby, capable of recombination. The frequency of mating-type genes in the population was found to be correlated with recombination in the aflatoxin gene cluster.

Recombination has been detected between aflatoxigenic and non-aflatoxigenic *A. flavus* with some of the offspring regaining the ability to produce aflatoxins (Olarte et al., 2012a; Horn et al., 2013). Clearly, such recombination is a source of diversity within *A. flavus*. Because of this ability to recombine, it is critical to assess the frequency of such events in agricultural environments where atoxigenic biocontrol *A. flavus* have been introduced. A recent study found that, under an agricultural environment, a small percentage of the sclerotia that can form on contaminated maize can be heterokaryotic if the seed is contaminated with isolates of both mating types. Upon contact with non-sterile soil, these sclerotia can develop into ascocarps, the sexual reproductive developmental forms (Horn et al., 2013). A separate study found that soil populations in agricultural environments that were not treated with biocontrol *A. flavus* had approximately equal populations of fungi of both mating types. The population of fungi obtained from the plant (maize) was skewed to overrepresent isolates with Mat1-2 loci fungi (Sweany et al., 2011). These recent studies show that both asexual/sexual reproduction and ecological factors influence recombination.

It has been shown that the populations of *A. flavus* in an agricultural environment contain abundant amounts of non-aflatoxigenic *A. flavus* (Horn and Dorner, 1999). This suggests that loss of aflatoxin-producing ability in *A. flavus* could be a consequence of adaptation to a carbon-rich environment that makes the aflatoxin cluster less genetically stable. The ability to produce aflatoxins (and other mycotoxins) may give the fungi a long-term advantage over a non-aflatoxigenic biocontrol strain for survival in the soil, but in agricultural environments this adaptive pressure may be partially lost. Larger effective population sizes tend to increase mean population mutation and recombination rates (Hartl and Clark, 1997), further driving the evolution of new VCGs, some of which have lost aflatoxin-producing ability due to mutations within the biosynthetic cluster or due to large chromosomal deletions resulting in losses of entire telomeric regions (Chang et al., 2006). Since the aflatoxin and CPA clusters reside near the telomere of chromosome 3 in *A. flavus*, such mutations result in a high frequency of loss of aflatoxin and CPA-producing ability. The detection of linkage disequilibrium blocks in partial clusters indicates that recombination has played a large a role in cluster disassembly, and multilocus coalescent analyses of cluster and non-cluster regions indicate lineage-specific gene loss in *A. flavus* (Moore et al., 2007).

The long-term fate of the non-aflatoxigenic biocontrol strain in the agricultural environment has not yet been fully addressed. A preliminary report found that in cotton fields treated with biocontrol *A. flavus* the introduced biocontrol isolate while the highly toxigenic strain S increased to reach an equilibrium in which the population of the biocontrol strain was about 10% that of the aflatoxin-producing isolate after 4 years. After only 1 year, the soil of treated fields had *A. flavus* populations with greater than 50% of the biocontrol isolate. This result suggests that long-term longevity of the biocontrol *A. flavus* could be an important consideration in establishing treatment protocols (Jaime-Garcia and Cotty, 2013). Survival of sclerotia in soil has been studied (Wicklow et al., 1993). After 36 months exposure to an agricultural soil, 68–100% of the sclerotia survived, with the main loss being due to nematode fungivory (Wicklow et al., 1993; McCormick, 2013). While soil sclerotia are largely stable in soil, the conidial inoculum is less stable and is subject to losses due to ingestion and degradation by soil bacteria and that formulations not using wheat or barley as a carrier are desirable (Accinelli et al., 2009).

OTHER SECONDARY METABOLITE GENE CLUSTERS IN *A. flavus*

Aspergillus flavus is able to produce toxic secondary metabolites in addition to aflatoxins. This suggests that caution is needed in considering what isolates should be used as non-aflatoxigenic biocontrol agents (Rank et al., 2012). Among these secondary metabolites are the indole-diterpenes, aflatrem, paxillenes, paspalicines, and aflavinines, as well as is the ergot-like alkaloids CPA and pseurotin (**Figure 2**). While none of these metabolites is currently regulated as a food or feed contaminant, toxicity studies indicate that they could have neurotoxic and nephrotoxic effects on animals. Other metabolites are also frequent metabolites of *A. flavus*, including the Substance P neurotransmitter antagonist, ditryptophenaline. We have recently determined that some of these metabolites are produced in greater quantities in S strain *A. flavus* than in L strain and are produced by some of the non-aflatoxigenic competitor strains. The reported toxic effects on humans of ingestion of *A. flavus*-contaminated maize was growth retardation, immune suppression, and liver damage, the latter being manifested the most in people with hepatitis C infection (Probst et al., 2007; Probst et al., 2010; Wu and Khlangwiset, 2010b). These toxic effects have usually been ascribed to ingestion of aflatoxins. We suggest that simultaneous ingestion of other toxic *A. flavus* metabolites may contribute to these observed toxicities in people who have eaten aflatoxin-contaminated maize. The S morphotype *A. flavus*, the *Aspergillus* strain is most associated with the recent outbreaks of toxicity to humans in Kenya and Nigeria and may be far more toxic than the L strain (Donner et al., 2009; Mehl and Cotty, 2010; Probst et al., 2010). *A. flavus* is also able to produce metabolites that are usually not considered to be particularly toxic but could affect animal health. These include metabolites such as orcellinic acid, aspergillic acid and kojic acid (Varga et al., 2012) as well as iron-chelating siderophores similar to ferricrocin (Wallner et al., 2009). What effect these additional metabolites might have on animal and plant health is unknown.

GLOBAL WARMING AND BIOCONTROL

Global warming has increased daily high temperatures in the mid west and northern maize-growing regions of the United States and Canada. The resulting temperatures are predicted to eventually resemble those in the southern United States where aflatoxin contamination of maize is a frequent problem. Aflatoxin contamination of mid west maize has not been recognized as a problem as yet. Besides temperature shifts, global warming can cause climate changes which result in more unpredictable weather problems for agricultural areas. Aflatoxin contamination events are more prevalent during times of high heat and drought,

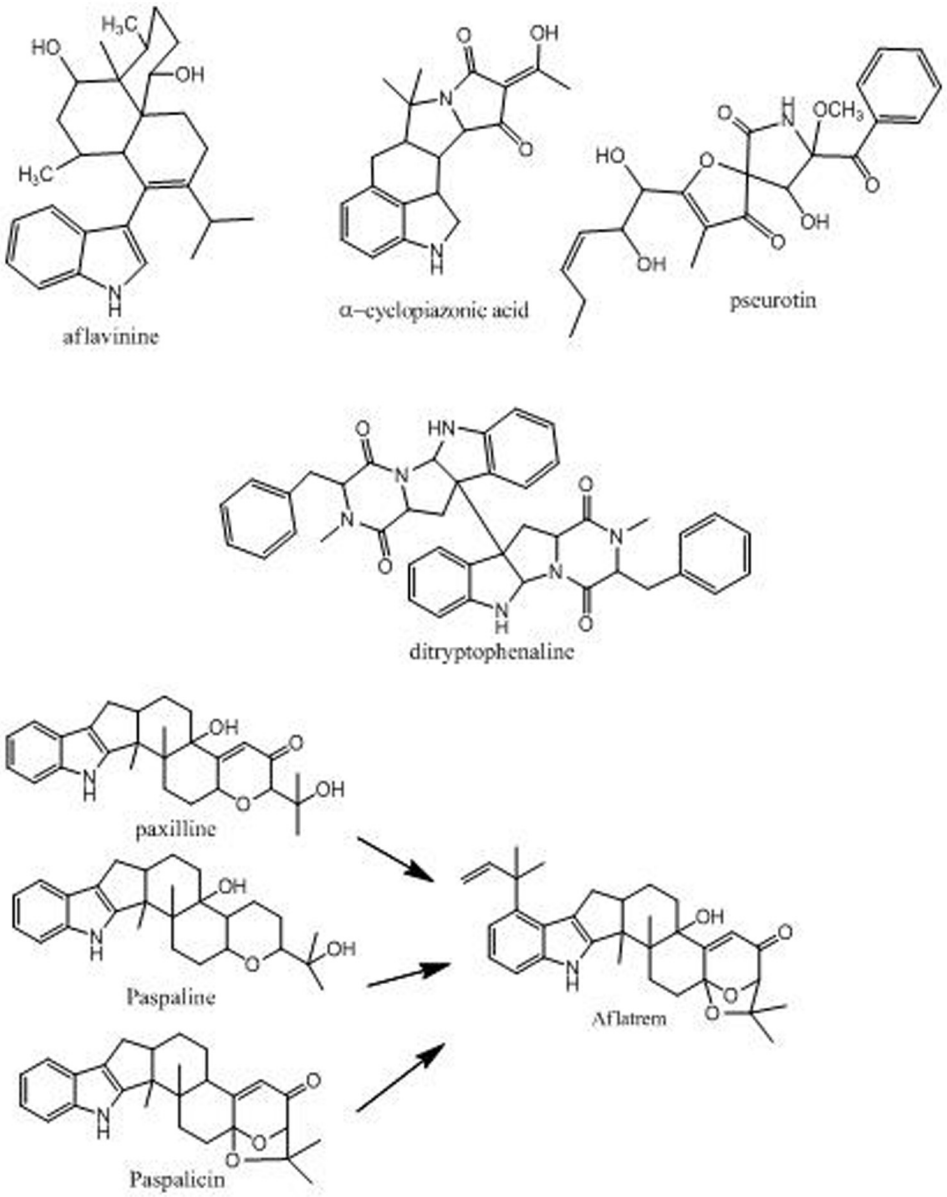

FIGURE 2 | Some known non-aflatoxin metabolites from *A. flavus*.

which may stress the host plant thereby facilitating *A. flavus* infection (Schmidt-Heydt et al., 2009; Roze et al., 2012; Mohale et al., 2013; Reverberi et al., 2013). Fungal stress has been correlated with increased expression of genes involved in both secondary metabolism production and sexual recombination as discussed above. Agricultural areas experiencing drought often suffer aflatoxin contamination outbreaks, and unpredictable changes in climate that result in drought may occur with increased frequency. Currently, incidences of aflatoxin contamination of crops are limited to tropical and sub-tropical areas (between latitudes 40°N and 40°S) around the world (Samuel et al., 2013). Because the average global surface temperature has increased by 0.8°C since 1901, with most of that increase occurring in the last 30 years, it is possible that by the end of the 21st century the favorable climate for aflatoxin contamination may encompass more of the maize-growing regions of the U.S. and outbreaks will become more frequent in occurrence.

Another potential consequence of climate change is that the biocontrol strain could be an inadvertent cause of increased damage to the plant, especially if growing conditions are less favorable for cultivation. Concomitantly, changes in the soil environment and its microbiome due to temperature elevation, could also subject the crop to increased damage. Understanding genetic variation within strains of *A. flavus* is important for developing a robust biocontrol strategy and it is unlikely that a "one size fits all" strategy will work.

CONCLUSION

The ultimate goal for using non-aflatoxigenic *A. flavus* as a biocontrol agent should be the long-term protection of crops against aflatoxin contamination. Current strategies utilize a program of annual re-application of biocontrol strains, and the fate of the biocontrol strains after one growing season is still unknown. Even a low rate of recombination for aflatoxigenic fungi could be significant for future food safety. There exist other challenges to the biocontrol strategy for remediation of aflatoxin contamination. The inherent diversity of *A. flavus* populations makes a biocontrol strategy more difficult because *A. flavus* populations differ in their abilities to produce aflatoxins and other toxic secondary metabolites. Some of these other secondary metabolites could be important for assessing the full toxic burden when grains contaminated with *A. flavus* are ingested. Climate change could increase stress on the plant and the fungus and environmental stress could increase plant susceptibility to the fungus and is a known inducer of secondary metabolite production. Stress could also affect the ability of the fungus to outcross with native populations of *A. flavus*. Also, in use of the biocontrol *A. flavus* care must be taken to prevent undue crop damage or damage to the soil microflora that might result.

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REFERENCES

Abbas, H. K., Weaver, M. A., Horn, B. W., Carbone, I., Monacell, J. T., and Shier, W. T. (2011). Selection of *Aspergillus flavus* isolates for biological control of aflatoxins in corn. *Toxin Rev.* 30, 59–70. doi: 10.3109/15569543.2011.591539

Abbas, H. K., Zablotowicz, R. M., Horn, B. W., Phillips, N. A., Johnson, B. J., Jin, X., et al. (2012). Comparison of major biocontrol strains of non-aflatoxigenic *Aspergillus flavus* for the reduction of aflatoxins and cyclopiazonic acid in maize. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 28, 198–208. doi: 10.1080/19440049.2010.544680

Accinelli, C., Sacca, M. L., Abbas, H. K., Zablotowicz, R. M., and Wilkinson, J. R. (2009). Use of a granular bioplastic formulation for carrying conidia of a non-aflatoxigenic strain of *Aspergillus flavus*. *Bioresour. Technol.* 100, 3997–4004. doi: 10.1016/j.biortech.2009.03.010

Ashworth, L. J., Schroeder, H. W., and Langley, B. C. (1965). Aflatoxins: environmental factors governing occurrence in Spanish peanuts. *Science* 148, 1228–1229. doi: 10.1126/science.148.3674.1228

Bayman, P., and Cotty, P. J. (1991). Vegetative compatibility and genetic diversity in the *Aspergillus flavus* population of a single field. *Can. J. Bot.* 69, 1707–1711. doi: 10.1139/b91-216

Bhatnagar, D., Cotty, P. J., and Cleveland, T. E. (2001). "Genetic and biological control of aflatoxigenic fungi," in *Microbial Food Contamination*, eds. C. L. Wilson and S. Droby (Boca Raton: CRC Press), 208–240.

Cardwell, K., and Cotty, P. J. (2002). Distribution of *Aspergillus* Section Flavi among field soils from the four agroecological zone of the Republic of Benin, West Africa. *Plant Dis.* 86, 434–439. doi: 10.1094/PDIS.2002.86.4.434

Chang, P. K., Abbas, H. K., Weaver, M. A., Ehrlich, K. C., Scharfenstein, L. L., and Cotty, P. J. (2012). Identification of genetic defects in the atoxigenic biocontrol strain *Aspergillus flavus* K49 reveals the presence of a competitive recombinant group in field populations. *Int. J. Food Microbiol.* 154, 192–196. doi: 10.1016/j.ijfoodmicro.2012.01.005

Chang, P. K., and Ehrlich, K. C. (2010). What does genetic diversity of *Aspergillus flavus* tell us about *Aspergillus oryzae*? *Int. J. Food Microbiol.* 138, 189–199. doi: 10.1016/j.ijfoodmicro.2010.01.033

Chang, P.-K., and Ehrlich, K. C. (2011). Cyclopiazonic acid biosynthesis by *Aspergillus flavus*. *Toxin Rev.* 30, 79–89. doi: 10.3109/15569543.2011.576795

Chang, P.-K., Ehrlich, K. C., and Hua, S.-S. H. (2006). Cladal relatedness among *Aspergillus oryzae* isolates and *Aspergillus flavus* S and L morphotypes. *Int. J. Food Microbiol.* 108, 172–177. doi: 10.1016/j.ijfoodmicro.2005.11.008

Chang, P. K., Horn, B. W., and Dorner, J. W. (2005). Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. *Fungal Genet. Biol.* 42, 914–923. doi: 10.1016/j.fgb.2005.07.004

Chen, J. G., Egner, P. A., Ng, D., Jacobson, L. P., Munoz, A., Zhu, Y. R., et al. (2013). Reduced aflatoxin exposure presages decline in liver cancer mortality in an endemic region of China. *Cancer Prevent. Res.* 6, 1038–1045. doi: 10.1158/1940-6207.CAPR-13-0168

Cotty, P. J. (1997). Aflatoxin-producing potential of communities of *Aspergillus* section *flavi* from cotton producing areas in the United States. *Mycol. Res.* 101, 698–704. doi: 10.1017/S0953756296003139

Cotty, P. J. (2006). "Biocompetitive exclusion of toxigenic fungi," in *The Mycotoxin Factbook, Food and Feed Topics*, eds D. Barug, D. Bhatnagar, H. P. Van Egmond, J. W. Van der Kamp, W. A. Van Osenbruggen, and A. Visconti (Wageningen: Wageningen Academic Publishers), 179–197.

Cotty, P. J., Bayman, D. S., Egel, D. S., and Elias, K. S. (1994). "Agriculture, aflatoxins and *Aspergillus*," in *The Genus Aspergillus*, ed. K. Powell (New York: Plenum Press), 1–27.

Cotty, P. J., and Bhatnagar, D. (1994). Variability among atoxigenic *Aspergillus flavus* strains in ability to prevent aflatoxin contamination and production of aflatoxin biosynthetic pathway enzymes. *Appl. Environ. Microbiol.* 60, 2248–2251.

Cotty, P. J., and Mellon, J. E. (2006). Ecology of aflatoxin producing fungi and biocontrol of aflatoxin contamination. *Mycotoxin Res.* 22, 110–117. doi: 10.1007/BF02956774

Donner, M., Atehnkeng, J., Sikora, R. A., Bandyopadhyay, R., and Cotty, P. J. (2009). Distribution of *Aspergillus* section *flavi* in soils of maize fields in three agroecological zones of Nigeria. *Soil Biol. Biochem.* 41, 37–44. doi: 10.1016/j.soilbio.2008.09.013

Dorner, J. W. (2009). Biological control of aflatoxin contamination in corn using a nontoxigenic strain of *Aspergillus flavus*. *J. Food Prot.* 72, 801–804.

Dorner, J. W. (2010). Efficacy of a biopesticide for control of aflatoxins in Corn. *J. Food Prot.* 73, 495–499.

Dorner, J. W., Cole, R. J., Connick, W. J., Daigle, D. J., McGuire, M. R., and Shasha, B. S. (2003). Evaluation of biological control formulations to reduce aflatoxin contamination in peanuts. *Biol. Control* 26, 318–324. doi: 10.1016/s1049-9644(02)00139–131

Ehrlich, K. (1987). Effect on aflatoxin production of competition between wild-type and mutant strains of *Aspergillus parasiticus*. *Mycopathologia* 97, 93–96. doi: 10.1007/BF00436844

Ehrlich, K., and Ciegler, A. (1985). Effect of phytate on aflatoxin formation by *Aspergillus parasiticus* grown on different grains. *Mycopathologia* 92, 3–6. doi: 10.1007/BF00442651

Ehrlich, K., Ciegler, A., Klich, M. A., and Lee, L. (1985). Fungal competition and mycotoxin production on corn. *Experientia* 41, 691–693. doi: 10.1007/BF02007725

Ehrlich, K. C., Chang, P.-K., Yu, J., and Cotty, P. J. (2004). Aflatoxin biosynthesis cluster gene cypA is required for G aflatoxin formation. *Appl. Environ. Microbiol.* 70, 6518–6524. doi: 10.1128/AEM.70.11.6518-6524.2004

Ehrlich, K. C., and Cotty, P. J. (2004). An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase. *Appl. Microbiol. Biotechnol.* 65, 473–478. doi: 10.1007/s00253-004-1670-y

Ehrlich, K. C., Kobbeman, K., Montalbano, B. G., and Cotty, P. J. (2007a). Aflatoxin-producing *Aspergillus* species from Thailand. *Int. J. Food Microbiol.* 114, 153–159. doi: 10.1016/j.ijfoodmicro.2006.08.007

Ehrlich, K. C., Montalbano, B. G., and Cotty, P. J. (2007b). Analysis of single nucleotide polymorphisms in three genes shows evidence for genetic isolation of certain *Aspergillus flavus* vegetative compatibility groups. *FEMS Microbiol. Lett.* 268, 231–236. doi: 10.1111/j.1574-6968.2006.00588.x

Eugenio, C., Christensen, C., and Mirocha, C. (1970). Factors affecting production of the mycotoxin F-2 by Fusarium roseum. *Phytopathology* 60, 1055–1057. doi: 10.1094/Phyto-60-1055

Geiser, D. M., Dorner, J. W., Horn, B. W., and Taylor, J. W. (2000). The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. *Fungal Genet. Biol.* 31, 169–179. doi: 10.1006/fgbi.2000.1215

Geiser, D. M., Pitt, J. I., and Taylor, J. W. (1998). Cryptic speciation and recombination in the aflatoxin-producing fungus, *Aspergillus flavus*. *Proc. Natl. Acad. Sci. U.S.A.* 95, 388–393. doi: 10.1073/pnas.95.1.388

Grubisha, L. C., and Cotty, P. J. (2009). Twenty-four microsatellite markers for the aflatoxin-producing fungus *Aspergillus flavus*. *Mol. Ecol. Resour.* 9, 264–267. doi: 10.1111/j.1755-0998.2008.02378.x

Hartl, D., and Clark, A. (1997). *Principles of Population Genetics*. Sunderland, MA: Sinauer Associates.

Horn, B., Sorensen, R., Lamb, M., Sobolev, V., Olarte, R., Worthington, C., et al. (2013). Sexual reproduction in *Aspergillus flavus* sclerotia naturally produced in corn. *Phytopathology* doi: 10.1094/PHYTO-05-13-0129-R [Epub ahead of print].

Horn, B. W. (2005). Colonization of wounded peanut seeds by soil fungi: selectivity for species from *Aspergillus* section *flavi*. *Mycologia* 97, 202–217. doi: 10.3852/mycologia.97.1.202

Horn, B. W., and Dorner, J. W. (1999). Regional differences in production of aflatoxin B1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. *Appl. Environ. Microbiol.* 65, 1444–1449.

Horn, B. W., and Wicklow, D. T. (1983). Factors influencing the inhibition of aflatoxin production in corn by *Aspergillus niger*. *Can. J. Microbiol.* 29, 1087–1091. doi: 10.1139/m83-167

Ismail, M. A. (2001). Deterioration and spoilage of peanuts and desiccated coconuts from two sub-Saharan tropical East African countries due to the associated mycobiota and their degradative enzymes. *Mycopathologia* 150, 67–84. doi: 10.1023/A:1010863507652

Jaime-Garcia, R., and Cotty, P. J. (2006). Spatial distribution of *Aspergillus flavus* and its toxigenic strains on commercial cottonseed from south Texas and its relationship to aflatoxin contamination. *Plant Pathol.* 55, 358–366. doi: 10.1111/j.1365-3059.2006.01358.x

Jaime-Garcia, R., and Cotty, P. J. (2007). Influences of application timing on sporulation of the biocontrol product *Aspergillus flavus* AF36 in cotton fields of Arizona. *Phytopathology* 97, S168–S168.

Jaime-Garcia, R., and Cotty, P. J. (2009). Effect of application time on displacement of aflatoxin producers by the atoxigenic strain *Aspergillus flavus* AF36. *Phytopathology* 99, S58–S58.

Jaime, R., and Cotty, P. J. (2013). Temporal dynamic of *Aspergillus flavus* community structure in soils of fields treated with the atoxigenic biocontrol *A. flavus AF*36 in Arizona. *Phytopathology* 103, 66.

Johansson, A. S., Whitaker, T. B., Hagler, W. M. Jr., Bowman, D. T., Slate, A. B., Payne, G., et al. (2006). Predicting aflatoxin and fumonisin in shelled corn lots using poor-quality grade components. *J. AOAC Int.* 89, 433–440.

McCormick, S. P. (2013). Microbial detoxification of mycotoxins. *J. Chem. Ecol.* 39, 907–918. doi: 10.1007/s10886-013-0321-0

Mehl, H. L., and Cotty, P. J. (2010). Variation in competitive ability among isolates of *Aspergillus flavus* from different vegetative compatibility groups during maize infection. *Phytopathology* 100, 150–159. doi: 10.1094/phyto-100-2-0150

Mohale, S., Medina, A., and Magan, N. (2013). Effect of environmental factors on in vitro and in situ interactions between atoxigenic and toxigenic *Aspergillus flavus* strains and control of aflatoxin contamination of maize. *Biocontrol Sci. Technol.* 23, 776–793. doi: 10.1080/09583157.2013.794895

Moore, G. G., Ramirez-Prado, J. H., Horn, B. W., and Carbone, I. (2007). Evidence of extensive recombination in the aflatoxin gene cluster of *Aspergillus flavus*. *Phytopathology* 97, S79–S79.

Olarte, R. A., Horn, B. W., Dorner, J. W., Monacell, J. T., Singh, R., Stone, E. A., et al. (2012a). Effect of sexual recombination on population diversity in aflatoxin production by *Aspergillus flavus*. *Phytopathology* 103, 8.

Olarte, R. A., Horn, B. W., Dorner, J. W., Monacell, J. T., Singh, R., Stone, E. A., et al. (2012b). Effect of sexual recombination on population diversity in aflatoxin production by *Aspergillus flavus* and evidence for cryptic heterokaryosis. *Mol. Ecol.* 21, 1453–1476. doi: 10.1111/j.1365-294X.2011.05398.x

Orum, T. V., Bigelow, D. M., Nelson, M. R., Howell, D. R., and Cotty, P. J. (1997). Spatial and temporal patterns of *Aspergillus flavus* strain composition and propagule density in Yuma County, arizona, soils. *Plant Dis.* 81, 911–916. doi: 10.1094/PDIS.1997.81.8.911

Probst, C., Callicott, K. A., and Cotty, P. J. (2012). Deadly strains of Kenyan *Aspergillus* are distinct from other aflatoxin producers. *Eur. J. Plant Pathol.* 132, 419–429. doi: 10.1007/s10658-011-9887-y

Probst, C., Njapau, H., and Cotty, P. J. (2007). Outbreak of an acute aflatoxicosis in Kenya in 2004: identification of the causal agent. *Appl. Environ. Microbiol.* 73, 2762–2764. doi: 10.1128/aem.02370-06

Probst, C., Schulthess, F., and Cotty, P. J. (2010). Impact of *Aspergillus* section *flavi* community structure on the development of lethal levels of aflatoxins in Kenyan maize (*Zea mays*). *J. Appl. Microbiol.* 108, 600–610. doi: 10.1111/j.1365-2672.2009.04458.x

Ramirez-Prado, J. H., Moore, G. G., Horn, B. W., and Carbone, I. (2008). Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. *Fungal Genet. Biol.* 45, 1292–1299. doi: 10.1016/j.fgb.2008.06.007

Rank, C., Klejnstrup, M. L., Petersen, L. L., Kildgaard, S., Frisvad, J. C., Gotfredsen, C. H., et al. (2012). Comparative Chemistry of *Aspergillus oryzae* (RIB40) and *A. flavus* (NRRL 3357). *Metabolites* 2, 39–56. doi: 10.3390/metabo2010039

Reverberi, M., Punelli, M., Smith, C. A., Zjalic, S., Scarpari, M., Scala, V., et al. (2013). How peroxisomes affect aflatoxin biosynthesis in *Aspergillus flavus*. *PLoS ONE* 7:e48097. doi: 10.1371/journal.pone.0048097

Roze, L. V., Chanda, A., Wee, J., Awad, D., and Linz, J. E. (2012). Stress-related transcription factor AtfB integrates secondary metabolism with oxidative stress response in aspergilli. *J. Biol. Chem.* 286, 35137–35148. doi: 10.1074/jbc.M111.253468

Samuel, S. M., Aiko, V., Panda, P., and Mehta, A. (2013). Aflatoxin B-1 occurrence, biosynthesis and its degradation. *J. Pure Appl. Microbiol.* 7, 965–971.

Schmidt-Heydt, M., Abdel-Hadi, A., Magan, N., and Geisen, R. (2009). Complex regulation of the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* in relation to various combinations of water activity and temperature. *Int. J. Food Microbiol.* 135, 231–237. doi: 10.1016/j.ijfoodmicro.2009.07.026

Sweany, R. R., Damann, K. E. Jr., and Kaller, M. D. (2011). Comparison of soil and corn kernel *Aspergillus flavus* populations: evidence for niche specialization. *Phytopathology* 101, 952–959. doi: 10.1094/PHYTO-09-10-0243

Vaamonde, G., Patriarca, A., Fernandez Pinto, V., Comerio, R., and Degrossi, C. (2003). Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *flavi* from different substrates in Argentina. *Int. J. Food Microbiol.* 88, 79–84. doi: 10.1016/S0168-1605(03)00101-6

Varga, J., Frisvad, J. C., and Samson, R. A. (2012). Two new aflatoxin producing species, and an overview of *Aspergillus* section *flavi*. *Stud. Mycol.* 69, 57–80. doi: 10.3114/sim.2011.69.05

Wagacha, J. M., Mutegi, C., Karanja, L., Kimani, J., and Christie, M. E. (2013). Fungal species isolated from peanuts in major Kenyan markets: emphasis on *Aspergillus* section *flavi*. *Crop Protect.* 52, 1–9. doi: 10.1016/j.cropro.2013.05.004

Wallner, A., Blatzer, M., Schrettl, M., Sarg, B., Lindner, H., and Haas, H. (2009). Ferricrocin, a siderophore involved in intra- and transcellular iron distribution in *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 75, 4194–4196. doi: 10.1128/AEM.00479-09

Weckbach, L. S., and Marth, E. H. (1977). Aflatoxin production by *Aspergillus parasiticus* in a competitive environment. *Mycopathologia* 62, 39–45. doi: 10.1007/BF00491994

Wicklow, D. T., Hesseltine, C. W., Shotwell, O. L., and Adams, G. L. (1980). Interference competition and aflatoxin levels in corn. *Phytopathology* 70, 761–764. doi: 10.1094/Phyto-70-761

Wicklow, D. T., Horn, B. W., Shotwell, O. L., Hesseltine, C. W., and Caldwell, R. W. (1988). Fungal interference with *Aspergillus flavus* infection and aflatoxin contamination of maize grown in a controlled environment. *Phytopathology* 78, 68–74. doi: 10.1094/Phyto-78-68

Wicklow, D. T., Wilson, D. M., and Nelsen, T. C. (1993). Survival of *Aspergillus flavus* sclerotia and conidia buried in soil in Illinois and Georgia. *Phytopathology* 83, 1141–1147. doi: 10.1094/Phyto-83-1141

Wogan, G. (1966). "Mycotoxin contamination of foodstuffs," in *Advances in Chemistry, World Protein Resources*, (Washington, DC: American Chemical Society), 195–215.

Wu, F., and Khlangwiset, P. (2010a). Evaluating the technical feasibility of aflatoxin risk reduction strategies in Africa. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 27, 658–676. doi: 10.1080/19440041003639582

Wu, F., and Khlangwiset, P. (2010b). Health economic impacts and cost-effectiveness of aflatoxin-reduction strategies in Africa: case studies in biocontrol and post-harvest interventions. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 27, 496–509. doi: 10.1080/19440040903437865

Wu, F., Stacy, S. L., and Kensler, T. W. (2013). Global risk assessment of aflatoxins in maize and peanuts: are regulatory standards adequately protective? *Toxicol. Sci.* 135, 251–259. doi: 10.1093/toxsci/kft132

Yard, E. E., Daniel, J. H., Lewis, L. S., Rybak, M. E., Paliakov, E. M., Kim, A. A., et al. (2013). Human aflatoxin exposure in Kenya, 2007: a cross-sectional study. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 30, 1322–1331. doi: 10.1080/19440049.2013.789558

Yu, J., Payne, G. A., Campbell, B. C., Guo, B. Z., Cleveland, T. E., Robens, J. F., et al. (2008). "Mycotoxin production and prevention of aflatoxin contamination in food and feed," in *The ASPERGILLI: Genomics, Medical Aspects, Biotechnology, and Research Methods*, eds S. Osmani and G. Goldman. (Boca Raton, FL: CRC Press), 457–472.

Zanon, A., Chiotta, M., Giaj-Merlera, G., and Barros, G. (2013). Evaluation of potential biocontrol agent for aflatoxin in Argentinean peanuts. *Int. J. Food Microbiol.* 162, 220–225. doi: 10.1016/j.ijfoodmicro.2013.01.017

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Aspergillus flavus infection induces transcriptional and physical changes in developing maize kernels

Andrea L. Dolezal[1], Xiaomei Shu[2], Gregory R. OBrian[2], Dahlia M. Nielsen[3], Charles P. Woloshuk[4], Rebecca S. Boston[5] and Gary A. Payne[2]*

[1] Monsanto Company, Waterman, IL, USA
[2] Department of Plant Pathology, North Carolina State University, Raleigh, NC, USA
[3] Department of Genetics, North Carolina State University, Raleigh, NC, USA
[4] Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN, USA
[5] Department of Plant and Microbiological Sciences, North Carolina State University, Raleigh, NC, USA

Edited by:
Perng-Kuang Chang, Southern Regional Research Center, USA

Reviewed by:
Ann E. Stapleton, University of North Carolina Wilmington, USA
Jeffrey William Cary, United States Department of Agriculture, ARS, SRRC, USA

***Correspondence:**
Gary A. Payne, Department of Plant Pathology, North Carolina State University, 851 Main Campus Drive, Raleigh, NC 27695-7567, USA
e-mail: gary_payne@ncsu.edu

Maize kernels are susceptible to infection by the opportunistic pathogen *Aspergillus flavus*. Infection results in reduction of grain quality and contamination of kernels with the highly carcinogenic mycotoxin, aflatoxin. To understanding host response to infection by the fungus, transcription of approximately 9000 maize genes were monitored during the host-pathogen interaction with a custom designed Affymetrix GeneChip® DNA array. More than 4000 maize genes were found differentially expressed at a FDR of 0.05. This included the up regulation of defense related genes and signaling pathways. Transcriptional changes also were observed in primary metabolism genes. Starch biosynthetic genes were down regulated during infection, while genes encoding maize hydrolytic enzymes, presumably involved in the degradation of host reserves, were up regulated. These data indicate that infection of the maize kernel by *A. flavus* induced metabolic changes in the kernel, including the production of a defense response, as well as a disruption in kernel development.

Keywords: ***Aspergillus flavus*, maize, transcription, genetic, aflatoxins, pathogenesis**

INTRODUCTION

Aspergillus flavus is an opportunistic fungal pathogen that infects developing maize kernels, attacking plants that are weakened by environmental stresses such as drought and heat. Disease reduces grain quality and contaminates the kernel with the carcinogenic mycotoxin aflatoxin (Scheidegger and Payne, 2003; Payne and Yu, 2010; Dolezal et al., 2013; Hruska et al., 2013; Kew, 2013). The development of resistant maize lines has proven difficult although there is evidence for sources of resistance (Brown et al., 1999; Windham and Williams, 2002; Mylroie et al., 2013; Warburton et al., 2013; Mideros et al., 2014). The lack of reliable resistance phenotyping markers, the inconsistency of disease development each year, and an insufficient understanding of host resistance mechanisms, all have made the selection of resistance difficult.

Advances in technology, such as microarrays, have enabled researches the ability to monitor transcription on a genome-wide level and provided a better understanding of how organisms respond to their environment on a cellular level. Studies investigating plant gene expression during pathogen attack have found the defense response goes beyond PR-proteins and involves transcription changes in both primary and secondary plant metabolic pathways and detoxification pathways (Boddu et al., 2007; Doehlemann et al., 2008; Alessandra et al., 2010). Phytohormones like salicyclic acid (SA), jasmonic acid (JA), ethylene (ET) have long been known to be an integral part of the defense response (Glazebrook, 2005; Jones and Dangl, 2006; Robert-Seilaniantz et al., 2011). Yet carbohydrate metabolism pathways, though not typically associated with resistance, may be an important component of the plant defense response including in maize (Berger et al., 2007; Bolton, 2009). Higher maize stalk carbohydrate levels have been associated with increased resistance to stalk infecting fungi, many of which are also capable of infecting the ear and kernel (Dodd, 1980).

Transcriptional changes of maize kernels during infection by *A. flavus* have been studied using microarrays (Luo et al., 2011; Kelley et al., 2012) and qPCR (Jiang et al., 2011). Kelley et al. (2012) compared maize varieties that were either susceptible or resistant to aflatoxin accumulation. They found 16 genes highly expressed in the resistant variety and 15 in the susceptible variety and concluded that multiple mechanisms are likely involved in resistance to aflatoxin accumulation. Jiang et al. (2011) reported higher levels of gene expression in stress related genes in resistant lines of maize. Luo et al. (2011) found that more maize genes were induced by *A. flavus* in susceptible kernels compared with resistant kernels. In all these studies, defense-related, and regulatory genes were associated with the response to *A. flavus*. To provide a clearer understanding of maize kernel resistance to *A. flavus* we monitored the transcriptional response of maize kernels during infection by *A. flavus* in the field using a custom DNA microarray. We report changes in expression of well-characterized defense signaling pathways and defense related genes as well as striking changes in expression of genes related to carbohydrate metabolism.

There are several stages in the infection process that host resistance could restrict fungal growth and aflatoxin contamination. Kernel infection with *A. flavus* begins through silk colonization. Conidia germinate and grow on senescing silks, moving down the silk channel to the developing kernels, which can take as little as 8 days (Marsh and Payne, 1984; Payne et al., 1988b). Subsequent steps in the infection process are less defined, but data suggest that *A. flavus* can attack kernels during their six stages (Ritchie et al., 1997) of their development: silking (R1), blister (R2), milk (R3), dough (R4), dent (R5), and physiological maturity (R6). Recently, Reese et al. (2011) inoculated detached kernels at stages R2–R5 in the lab and found that kernels at these four stages were susceptible to infection by *A. flavus*. Fungal infection has been observed in injured kernels as young as the milk (R3) stage (Taubenhaus, 1920; Anderson et al., 1975). These young kernels tend to accrue high concentrations of aflatoxin because of prolonged colonization by the pathogen (Lillehoj et al., 1980; Payne et al., 1988a). Infection in non-injured kernels in the field is thought to take place later, during the dent (R5) developmental stage just prior to physiological maturity (R6) (Koehler, 1942; Marsh and Payne, 1984; Payne et al., 1988a; Smart et al., 1990; Windham and Williams, 1998). Once inside, *A. flavus* preferentially colonizes the oil-rich germ tissue (Fennell et al., 1973; Jones et al., 1980; Smart et al., 1990; Keller et al., 1994). Fungal growth within endosperm tissue, more specifically the nutrient-rich starchy endosperm, has been observed, but there are discrepancies in the literature as to the extent of colonization (Lillehoj et al., 1976; Smart et al., 1990; Keller et al., 1994; Brown et al., 1995; Dolezal et al., 2013).

Our studies focused on the transcriptional response of developing kernels that were inoculated with *A. flavus* through a wound. We realize that this approach could overlook some resistance mechanisms, but it results in more consistent disease development. Resistance to infection of wounded kernels is also relevant as it mimics insect injury, which is important in the epidemiology of the disease. Furthermore, to capture the response in the different stages of kernel development, we evaluated *A. flavus* infection of four kernels stages, R2–R5. We also chose a specific time of 4 days after inoculation to examine gene expression based on previous histological studies by Dolezal et al. (2013) who showed that within 4 days after inoculation *A. flavus* mycelium reached the aleurone, endosperm, and germ tissue. Thus, sampling at 4 days allowed assessment of host response in several tissue types within the kernels.

EXPERIMENTAL PROCEDURE

FUNGAL STRAIN AND CULTURE CONDITIONS *ASPERGILLUS FLAVUS*

NRRL 3357 was grown on potato dextrose agar (PDA) at 28°C for 7–10 days. Conidia were dislodge with 0.05% (v/v) Triton X-100 and diluted to a working solution of 1×10^6 spores mL^{-1}.

MAIZE KERNEL INOCULATION AND HARVESTING

Inbred maize genotype B73 was grown at the Central Crops Research Station in Clayton, NC. Ears were hand pollinated and the date recorded on the bag. Ears at the blister (R2), milk (R3), late milk (R3)–early dough (R4), dough (R4), and dent (R5) stages of development were either mock-inoculated or inoculated with *A. flavus* as outline in Dolezal et al. (2013). Briefly, ears selected for inoculation had the husk pulled back to exposed the developing kernels below. The protruding portion of the pins of the pinbar was dipped into the *A. flavus* conidial suspension and inserted into the crown of the kernel. The husk was repositioned and secured around the ear with a rubber band, and a paper bag placed over the inoculated ear. Ears inoculated at the blister (R2), milk (R3), dough (R4), and dent (R5) stages of development were removed from the plant 4 days after inoculation (dai), and the kernels flash frozen immediately after removal. Harvested kernels were stored at −80°C until RNA was extracted using the protocol outlined in Smith et al. (2008). Additional ears inoculated at the late milk (R3)–early dough (R4) stage of development were left in the field and picked at end of the growing season. Kernels adjacent to the pinbar-inoculated rows were harvested. Kernels on non-inoculated ears, pollinated the same day as the inoculated ears, were also collected and used as controls. Adjacent diseased kernels and control kernels were cut-in-half and visually compared to assess for physical changes in kernel structure resulting from *A. flavus* infection.

MICROARRAY PROCESSING AND ANALYSIS

Custom-designed *A. flavus* Affymetrix GeneChip DNA microarrays were used to identify genes differentially expressed in maize during *A. flavus* kernel colonization. This multi-species array, in addition to being capable of monitoring genome-wide transcription of *A. flavus*, has close to 9000 probe sets representing maize genes. This pairing of *A. flavus* and maize genes onto a single array allowed for simultaneous detection of disease-associated transcript in the plant-pathogen interaction. The majority (83%) of maize genes selected for the array came from seed-specific cDNA libraries. The remaining genes were chosen based on recommendations from members of the maize community and prior association with disease resistance. The quality of RNA extracted from the mock-inoculated and *A. flavus*-inoculated kernels was assessed before processing. All array work was carried out at the Purdue Genomic Core Facility (http://www.genomics.purdue.edu) in West Lafayette, IN, and standard Affymetrix protocols were followed.

CEL files generated from the GeneChip DNA microarray scans were imported into JMP Genomics and $\log_2$ transformed. Mismatched probes were not used in the calculation of the expression values. The expression profiles of *A. flavus* and maize genes were examined for each array, and arrays for the mock-inoculated treatment that had moderate-to-strong *A. flavus* signal intensities were removed from further analysis. While these kernels did not visually appear infected, they were likely inadvertently contaminated with *A. flavus*. Data were then normalized using Loess Normalization. Normalized data from arrays generated from blister (R2), milk (R3), dough (R4), and dent (R5) inoculated kernels stages were grouped into either a mock-inoculated or *A. flavus* inoculated treatment group. The assemblage of the different developmental stages into a single treatment group allowed for the identification of maize genes that consistently responded to *A. flavus* infection regardless of what age infection initiated. An analysis of variance (ANOVA) was performed comparing the mock- and *A. flavus*-inoculated treatment groups. To account for multiple testing, a significance threshold based on

a false discovery rate (FDR) of 0.05 was used (Benjamini and Hochberg, 1995). The data were deposited into Gene Expression Omnibus. The series record number is GSE57629 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57629). A volcano plot was generated showing significance on the y-axis and fold change on the x-axis using JMP 11 (**Figure 1**). Gene names were assigned by using Tophat to align the affy probe sequences to the ZmB73_RefGen_v2 reference genome. AgriGO was used to perform Singular Enrichment Analysis (SEA) on differentially expressed genes (Du et al., 2010).

For SEA, the AGRIGO toolkit was used (http://bioinfo.cau.edu.cn/agriGO/analysis.php). Default values were used for the advanced options including the Yekutieli (FDR under dependency) multi-test adjustment method at a significance level of 0.05.

VALIDATION OF MICROARRAY DATA BY qRT-PCR

For each of the developmental stages used for the microarray study, a second set of RNA isolations was performed. RNA was treated with DNase (Promega) and cDNA was synthesized using a First Strand cDNA Synthesis Kit (Fermentas). Quantitative real-time RT-PCR (qRT-PCR) was performed using a SYBR® Green kit (Applied Biosystems) according to the manufacturer's instructions. The expression levels of a ribosome gene were used for normalization. Data were analyzed by the comparative CT method with the amount of target given by the calibrator $2^{-\Delta\Delta CT}$. The primers used for qRT-PCR analysis are listed in **Table 1**.

RESULTS

DIFFERENTIALLY EXPRESSED GENES

Maize kernels were inoculated at the blister (R2), milk (R3), dough (R4), and dent (R5) stages, and harvested 4 days later. Transcriptional changes for 8875 maize genes were monitored with an Affymetrix GeneChip® DNA array. Data were grouped into either mock-inoculated or *A. flavus*-inoculated treatment groups. An ANOVA comparing mock-inoculated with *A. flavus*- inoculated treatment groups ($\alpha \leq 0.05$ FDR) identified 912 and 3737 of the Affymetrix GeneChip probe-sets up- and down-regulated, respectively, (Table S1). Each probe-set represents a unique maize gene, except for those with a suffix attached to the probe-set name (e.g., ZM_a_at). These probe-sets may contain probes that represent more than one gene within a gene family or contains conserved sequence common to multiple genes. Some maize genes are represented by multiple probe-sets. Consult http://www.affymetrix.com/estore/support/help/faqs/mouse430/faq8.jsp for more detail on the different suffixes. This analysis showed the differential expression of genes associated with host resistance and defense signaling pathways, and of genes associated with sugar metabolism.

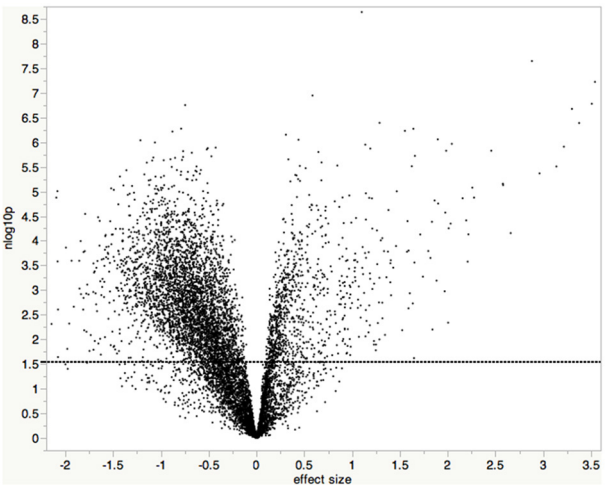

FIGURE 1 | Volcano plot of test results showing statistical significance vs. fold change. Each point represents the results of one gene, where the x-axis is the difference in expression between *A. flavus*-infected samples and mock-infected samples ($\log_2$ [*A. flavus*]—$\log_2$ [mock]). The y-axis is the $-\log_{10}$ transformed *p*-value. The dashed line indicates the significance threshold based on an FDR of 0.05 (Benjamini and Hochberg, 1995); all points above the line are considered statistically significant.

GENE ENRICHMENT ANALYSIS (SEA)

To gain additional insight into the collective biological function of proteins whose genes showed differential expression during infection, we performed annotation enrichment using SEA (Du et al., 2010) on the genes listed in Table S1. The resulting list of enriched Gene Ontology terms is shown in Table S2. Notable are transcriptional changes in several genes associated with carbohydrate metabolism. Representative examples include GO:0005975, GO:0006006, GO:0034637, GO:0044262, and GO:0019318.

CHANGES IN EXPRESSION OF GENES ASSOCIATED WITH CARBOHYDRATE METABOLISM

Infection of maize kernels with *A. flavus* resulted in transcriptional changes of several maize genes involved in primary and secondary metabolism, particularly those associated with the synthesis and hydrolysis of starch, and the mobilization of hexoses (**Figure 2**; **Table 2**; Table S2). As an example, genes encoding

Table 1 | Primers used in qRT-PCR.

Gene annotation (gene name)	Primers 5′–3′
Structural constituent of ribosome (LOC100285698)	Ribosome F: GGCTTGGCTTAAAGGAAGGT Ribosome R: TCAGTCCAACTTCCAGAATGG
PRms (Pathogenesis related protein, maize seed) (AC205274.3_FG001)	PRms F: TACAATGGAGGCATCCAACA PRms R: CTGTTTTGGGGAGTGAGGTA
β-fructofuranosidase (invertase cell wall1) (GRMZM2G139300)	CWINV1 F: CGGCAAGATCACCCTTAGAA CWINV1 R: CGTAGAGGTGAGCGTCCTTC
1,4-alpha-glucan branching enzyme (GRMZM2G088753)	SBE F: TAGCCCTGGACTCTGATGCT SBE R: CCGGTTGTTGAAGTTCGTTT
Lipoxygenase4 (GRMZM2G109056)	LOX4 F: ATCGAGATCCTCTCCAAGCA LOX4 R: CTGATCCGCTTCTCGATCTC
Lipoxygenase9 (GRMZM2G017616)	LOX9 F: CCTCATGGCATCAGACTCCT LOX9 R: GAGCTGCACATACGACTCCA

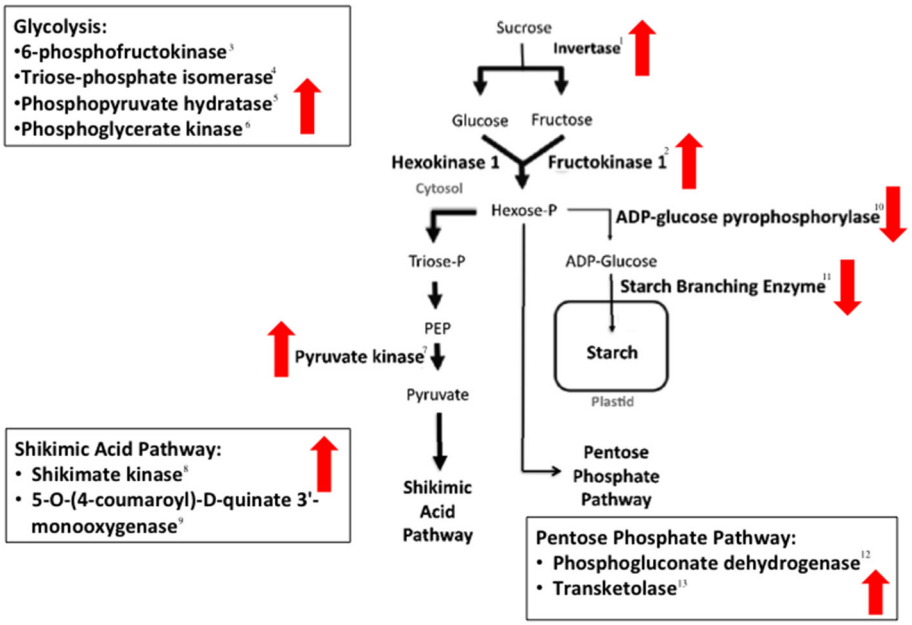

FIGURE 2 | Enzymes in maize seed carbohydrate metabolism whose biosynthetic genes are differentially expressed in the *A. flavus*-maize interaction. Black arrows denote described carbohydrate metabolic pathways. Up or down directed block arrows indicate increased or decreased expression of genes, respectively. Numbers beside the enzymes corresponds to their description in **Table 2**.

Table 2 | Statistically significant differentially expressed genes referenced in Figure 2.

Figure 2 References	Probe ID	Gene name	Putative protein	Fold change
1	TC302492_ZM_at	GRMZM2G394450	Sucrose:sucrose fructosyltransferase (invertase1)	2.3
1	TC281577_ZM_s_at	GRMZM2G139300	β-fructofuranosidase (invertase cell wall1)	6.0
1	TC309652_ZM_at	GRMZM2G123633	β-fructofuranosidase (invertase cell wall3)	1.3
2	TC292194_ZM_at	GRMZM2G08037	6-phosphofructokinas	2.2
3	TC292194_ZM_at	GRMZM2G080375	6-phosphofructokinase	1.7
4	TC279919_ZM_x_at	GRMZM5G852968	Triose-phosphate isomerase	1.3
5	TC310338_ZM_x_at	GRMZM2G046679	Phosphopyruvate hydratase	1.4
6	TC289234_ZM_at	GRMZM2G051806	Phosphoglycerate kinase	1.8
7	TC285970_ZM_s_at	GRMZM2G150098	Pyruvate kinase	2.1
8	TC311287_ZM_at	GRMZM2G161566	Shikimate kinase	1.6
9	TC299754_ZM_at	GRMZM2G138074	5-O-(4-coumaroyl)-D-quinate 3′-monooxygenase	3.2
10	TC310488_ZM_at	GRMZM2G429899	Glucose-1-phosphate adenylyltransferase (Sh2)	−1.6
11	TC311334_ZM_at	GRMZM2G088753	1,4-alpha-glucan branching enzyme	−3.5
12	TC311531_ZM_s_at	GRMZM2G127798	Phosphogluconate dehydrogenase (decarboxylating)	1.5
13	TC305088_ZM_at	GRMZM2G033208	Transketolase	2.0

starch biosynthetic enzymes, including two starch branching enzymes, (GRMZM2G088753 and GRMZM2G032628), were down regulated during infection as was ADP-glucose pyrophosphorlase (GRMZM2G429899), which catalyzes a key metabolic step in the synthesis of starch in higher plants (Greene and Hannah, 1998). In addition to the apparent down regulation of starch synthesis, there was an increase in transcription of genes involved in starch hydrolysis. The transcription of a β-amylase-like genes, GRMZM2G025833, was upregulated during *A. flavus* pathogenesis.

Associated with changes in starch accumulation were changes in the mobilization of hexoses. Three maize invertases (GRMZM2G139300, GRMZM2G394450, GRMZM2G123633) were more highly expressed in the *A. flavus* infected kernels than in non-infected kernels (**Table 2**; **Figure 2**). Invertases are responsible for hydrolyzing sucrose into glucose and fructose (Cheng et al., 1996; Chourey et al., 2006), and are important in maize kernel development (Weber et al., 1997; Roitsch et al., 2003). Up regulation of invertases in the maize kernel is predicted to cause an increase in free hexose

levels in the kernel and affect seed storage reserves such as starch.

The conversion of sucrose to hexoses also was associated with the down regulation of six genes involved with starch biosynthesis. Genes in the starch biosynthetic pathway [*wx1* (GRMZM2G024993), *su1* (GRMZM2G138060), *ss1* (GRMZM2G129451), *sbe1* (GRMZM2G088753), *su2* (GRMZM2G348551), and *ae1* (GRMZM2G032628)] as well as over 20 zein-annotated genes (Table S1) and the gene encoding the transcription factor that regulates 22-kD zein expression, *opaque2* (GRMZM2G015534), were all down-regulated during infection.

It was not possible to determine the exact pathways of hexose remobilization in our studies, but gene expression in both the glycolytic pathway and the Pentose Phosphate Pathway (PPP) was altered by infection (**Figure 2**; **Table 2**). Before glucose can be utilized by either pathway, it must be phosphorylated by a hexokinase (**Figure 2**; Spielbauer et al., 2006). The fructokinase (GRMZM2G080375) was up regulated during pathogenesis. Hexose kinases have been associated with sugar sensing in plants and are potentially involved in the plant defense response (Granot et al., 2013). Glucose-6-phosphate also can be involved in starch synthesis, but because starch biosynthesis genes are down regulated it is likely used by other pathways for the production of energy or defense-related compounds during pathogenesis.

Up regulation of genes in the shikimate pathway supports the premise that hexoses are shunted away from starch synthesis in *A. flavus* infected kernels (**Figure 2**; **Table 2**). Several bioreactive compounds from this pathway are known to be involved in host defense (Daayf and Lattanzio, 2009). The shikimate pathway is an entry to aromatic secondary metabolism (Herrmann, 1995) and chorismate synthesized from this pathway is used to make the aromatic amino acids Phe, Tyr, and Trp. These amino acids are precursors for aromatic secondary metabolites including flavonoids and phytoalexins (Herrmann, 1995). Genes in the flavonoid pathway, *fht1* [(GRMZM2G062396), *c2* (GRMZM2G422750) (Bruce et al., 2000)] and genes from other phenypropanoid pathways (Table S1) increased in transcription after *A. flavus* inoculation.

The shikimic acid pathway also provides precursors for the biosynthesis of lignins (Herrmann, 1995), compounds associated with basal resistance to pathogens. Genes involved in lignin biosynthesis have been reported to be induced after *A. flavus* infection in both susceptible (VA35) (Kelley et al., 2012) and resistant varieties (Eyl25) (Luo et al., 2011) of maize. Liang et al. (2006) found lignin concentrations to increase in response to infection by *A. flavus*, and they found a negative correlation between lignin content of peanut cultivars and infection by *A. flavus*. Magbanua et al. (2013) following colonization by a GFP expressing strain of *A. flavus,* found less colonization of maize cob tissue in the resistant inbred Mp313e than in cobs of SC212m, a more susceptible genotype. They attributed the more restricted growth in Mp313e to the highly cross-linked lignin found in Mp313e. In our study, infection of maize kernels by *A. flavus* led to higher expression of three genes (GRMZM2G099420, GRMZM2G131205, GRMZM2G090980) involved in lignin biosynthesis (Table S1).

The carbohydrate metabolic methylerythritol phosphate (MEP) pathway was also found differentially expressed during infection. The following genes from this pathway, which utilizes pyruvate from glycolysis to produces an assortment of isoprenoids including the hormone abscisic acid (ABA) were likewise up-regulated during *A. flavus* infection: (GRMZM2G056975, GRMZM2G493395, GRMZM2G172032, GRMZM2G027059, GRMZM5G859195). We found one gene, 9-cis-epoxycarotenoid dioxygenase (GRMZM2G014392), involved in ABA synthesis up-regulated. The MEP pathway expression has been found induced in maize root colonized by arbuscular mycorrhizal fungi (Lange et al., 2000; Walter et al., 2000).

DEFENSE SIGNALING PATHWAYS

Phytohormones are chemical compounds synthesized by the plant that regulate biochemical processes necessary for growth, reproduction, and survival. The plant defense response is hormonally regulated predominantly by the phytohoromones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Niu et al., 2011; Mengiste, 2012; Derksen et al., 2013). Each hormone likely activates different components of the defense response system that are effective against specific pathogens. The JA/ET pathways are often induced in resistance to necrotrophic pathogens, whereas the SA pathway is typically induced by biotrophic and hemibiotrophic pathogens (Glazebrook, 2005; Derksen et al., 2013).

In this study kernel infection by *A. flavus* resulted in increased expression of the 12-oxo-phytodienoic acid reductases (OPR) encoding *ZmOPR3* (GRMZM2G000236), and the alcohol dehydrogenase encoding *ts2* (GRMZM5G840653). Expression of these genes has been linked with JA biosynthesis in maize (Vick and Zimmerman, 1984; Browse, 2009), and *Ts2* has been associated with the hypersensitive response and resistance to Northern Leaf Blight in maize (Delong et al., 1993; Wisser et al., 2011). Our finding suggests that JA may be involved in the kernel-*A. flavus* interaction.

Other lipid-derived defense-related compounds besides JA are generated from enzymes generally associated with the JA-biosynthesis pathway. As an example, plants contain multiple LOX and OPR genes. In maize, the exact copy number of functional LOX genes varies between maize genotypes (De La Fuente et al., 2013). The function for most LOX and OPR isoenzymes is independent from JA biosynthesis. LOXs catalyze the formation of various oxidized lipids called oxylipins that can act as signaling molecules separate from JA and are thought to have antimicrobial properties (Blée, 2002; Prost et al., 2005). Oxylipins are known to effect fungal growth, including that of *A. flavus*, and mycotoxin production in seeds (Burow et al., 1997; Wilson et al., 2001; Brodhagen and Keller, 2006). The functional role for most OPR isoenzymes is unknown. *ZmLOX* and *ZmOPR* genes were previously found expressed in response to *A. flavus* and other maize fungal pathogen infections (Wilson et al., 2001; Zhang et al., 2005). In accordance with these findings, we observed *LOX* and *OPR* genes differentially expressed during *A. flavus* kernel colonization. *ZmLOX4*, *7*, and *9* (GRMZM2G109056, GRMZM2G070092, GRMZM2G017616) were up-regulated during *A. flavus* infection, whereas *ZmLOX11* (GRMZM2G009479) was down-regulated. *ZmOPR1*, *2*, *3*, and *5* (GRMZM2G106303, GRMZM2G000236, GRMZM2G156712,

GRMZM2G087192) were up-regulated in the diseased kernel with *OPR1* and *OPR3* having a 18 and 16 fold-change, respectively, (Table S1).

Whether JA and the other lipid-derived compounds increase maize resistance against pathogen attack may depend on the pathogen and which isoenzyme is expressed. Disruption of the *ZmLOX3* results in enhanced resistance to *F. verticillioides* (Gao et al., 2009), *Colletotrichum graminicola* (Gao et al., 2007), *Cochliobolus heterostrophus* (Gao et al., 2007), and *Exserohilum pedicellatum* (Isakeit et al., 2007). However, the maize *lox3* mutant shows increased susceptibility to *A. flavus* and *A. nidulans*, indicating this gene regulates disease resistance in a pathogen-specific manner (Gao et al., 2009).

DEFENSE-ASSOCIATED GENES IN *A. FLAVUS* INFECTED SEEDS

Pathogenesis-related (PR) proteins are the hallmark of the induced defense response and their expression has been associated with resistance (van Loon et al., 2006; Luo et al., 2011). Several genes annotated as encoding for PR-proteins including those for chitinases [GRMZM2G112538, GRMZM2G477128, *PR-10* (GRMZM2G075283).

GRMZM2G051943, GRMZM2G129189, GRMZM2G133781, *chn2* (GRMZM2G145461), GRMZM2G145518, GRMZM2G 162359] were up-regulated in the *A. flavus* infected kernels (Table S1). The expression of *chitinase2* and *PR-10* genes has been reported to be induced in fungal infected maize seed (Cordero et al., 1994). Furthermore, studies by Chen et al. (2006) showed that PR-10 has antifungal activity against *A. flavus in vitro*, and its production is increased upon *A. flavus* infection in the resistance line GT-MAS: gk, but not in the susceptible line Mo17. They also showed that repression of maize *PR-10* by RNAi gene silencing resulted in increased susceptibility to *A. flavus* and aflatoxin production (Chen et al., 2010). A Bowman-Birk-like proteinase inhibitor (GRMZM2G156632), which encodes a PR-like protein showed a 2.5 fold increase in gene expression during infection (Rohrmeier and Lehle, 1993). This gene has been associated with the maize hypersensitive response (Simmons et al., 2002; Chintamanani et al., 2010).

The oxidative burst is an integral part of early plant immunity and is associated with reactive oxygen species, programmed cell death, and the hypersensitive response (Lamb and Dixon, 1997; Dickman and Fluhr, 2013). This defense cascade leads to the production of antimicrobial compounds. Associated with these defense responses are the production of peroxidases and gluathione-S-transferases (GST). Several genes encoding peroxidase-annotated genes (AC197758.3_FG004GRMZM2G080183, GRMZM2G089959, GRMZM2G095404, GRMZM2G103342, GRMZM2G108207, GRMZM2G138918, GRMZM2G149273, GRMZM2G173195, GRMZM2G320269, GRMZM2G321839, GRMZM2G382379, GRMZM2G419953, GRMZM2G441541, GRMZM2G471357) were up-regulated in the diseased kernels.

Four additional peroxidase-encoding genes (GRMZM2G 034896, GRMZM2G089895, GRMZM2G103169, GRMZM2G 315176) were down-regulated, implying that only certain peroxidase-isozymes are needed during *A. flavus* infection. Gluathione-S-transferases (GST) reduce host cellular damage by detoxifying toxins and xenobiotics commonly encountered during periods of disease and abiotic stress. Wisser et al. (2011) recently correlated ZmGST23 (NP_001104994.1) with moderate resistance to multiple maize pathogens. Though *ZmGST23* was not differentially expressed in this study, the expression of GRMZM2G01909, predicted to encode a GST, had increased expression during *A. flavus* infection.

VALIDATION OF MICROARRAY DATA BY qRT-PCR

In order to validate the results of the microarray study, the expression levels of five selected genes were monitored by qRT-PCR: (AC205274.3_FG001, GRMZM2G139300, GRMZM2G088753, GRMZM2G109056, GRMZM2G017616). The fold changes of these genes as determined by qRT-PCR were highly correlated with the results obtained from the microarrays (**Table 3**).

PHYSICAL CHANGES WITHIN KERNEL IN RESPONSE TO NATURAL INFECTION WITH *A. FLAVUS*

The molecular analysis of maize gene expression during pathogenesis indicated major metabolic effects within kernels in response to infection by *A. flavus*. To determine if such effects could be manifest in the physical structure of kernels, we examined naturally infected kernels at the end of the growing season. Maize kernels adjacent to wound-inoculated kernels were harvested at maturity, dissected, and examined for growth of *A. flavus* and structural integrity. These kernels did not show any obvious wounds or cracks within their pericarp. **Figure 3** shows a comparison of three representative infected kernels and non-infected kernels. The most striking modification was the reduced size of the zein-filled hard [horny] endosperm in infected kernels (**Figure 3**e). In diseased kernels the hard endosperm had been replaced with starchy endosperm (**Figure 3**d), but the consistency of the entire starchy endosperm was different from that of the non-infected kernel. Instead of being firm and intact, the starchy endosperm of the diseased kernel was fragile, friable, and filled with tiny air pockets. *A. flavus* could be discerned in some of these pockets including the gap between endosperm and germ (**Figure 3**d, l). Mycelium was also observed in the embryo around the plumule (**Figure 3**g) and primary root (**Figure 3**h), and the germ was discolored and shriveled (**Figure 3**f).

Table 3 | qRT-PCR results for select differentially expressed genes are consistent with microarray results.

Gene name	Annotation	Fold change using microarray	Fold change using qRT-PCR
AC205274.3_FG001	PRms (Pathogenesis related protein, maize seed)	3.1	8.8
GRMZM2G139300	β-fructofuranosidase (invertase cell wall1)	4.0	8.2
GRMZM2G088753	1,4-alpha-glucan branching enzyme	−3.5	−2.3
GRMZM2G109056	Lipoxygenase4	1.9	4.4
GRMZM2G017616	Lipoxygenase9	1.2	2.1

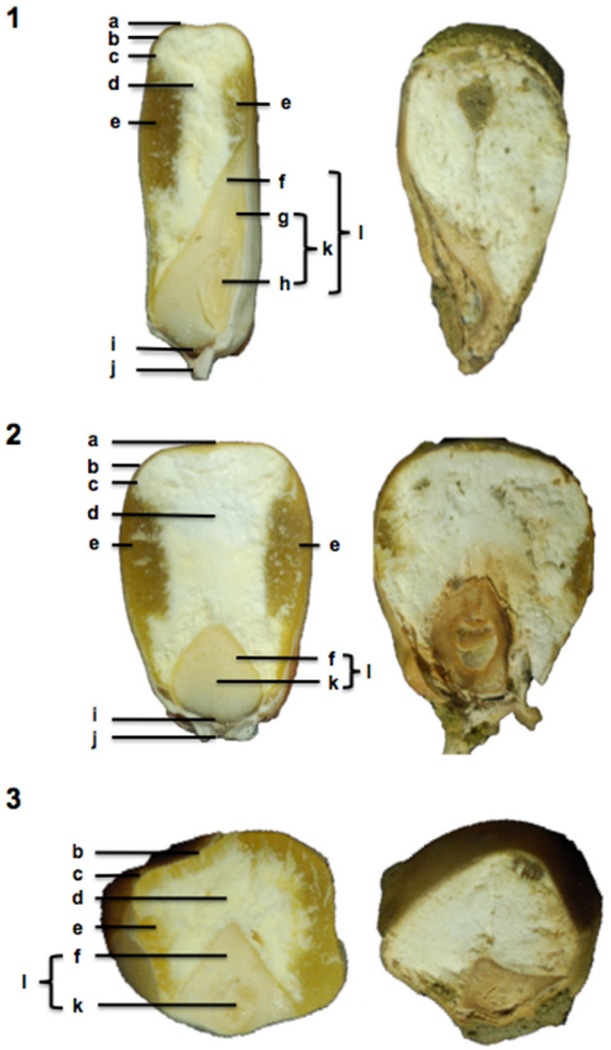

FIGURE 3 | Mature B73 kernels naturally infected with *Aspergillus flavus*. A sagittal (1) and frontal (2) and transversal (3) section of healthy B73 kernels (left) were compared to disease kernels (right) to discern any physical changes that occurred as a result of *A. flavus*. Bolded letters denote kernel parts and tissues: **a**—crown; **b**—pericarp; **c**—aleurone; **d**—starchy endosperm; **e**—hard endosperm; **f**—scutellar tissue; **g**—leaf primordia (plumule); **h**—primary root; **i**—transfer cells; **j**—pedicel; **k**—embryo; and **l**—germ.

DISCUSSION

A. flavus kernel colonization is most aggressive on maize plants that have been subjected to heat or water stress (Tubajika and Damann, 2001; Scheidegger and Payne, 2003; Widstrom et al., 2003; Guo et al., 2008). Such conditions frequently occur throughout the world on rain-fed fields, and thus *A. flavus* colonization of kernels and subsequent contamination with aflatoxins is a concern internationally. Resistance to aflatoxin accumulation shows low heritability in the field, owing to the quantitative nature of resistance, the lack of reliable phenotyping, and strong genotype by environment (GxE) interactions. Thus, any approach that facilitates the identification of genes contributing to host resistance could accelerate the development of resistant maize genotypes.

The overall goal of this study was to better characterize host response to *A. flavus* opportunistic infection by identify maize genes differentially expressed during infection that could have applications as genetic markers in future breeding programs. Our previous research (Dolezal et al., 2013) showed that *A. flavus* follows a predictable pattern of colonization after inoculation of maize kernels. While all tissue types of the kernel can be colonized, growth of the fungus into scutellum tissue appears to follow the formation of a biofilm-like structure by *A. flavus* (Dolezal et al., 2013). The scutellum is metabolically active and known to synthesize numerous hydrolytic enzymes and defense-associated compounds (Casacuberta et al., 1991, 1992). Based on these studies, we chose 4 days after inoculation as the time to evaluate host response to *A. flavus*. Data presented in this study indicate that infected seed at this time to be transcriptionally responsive and express genes known to be involved in host defense.

Our observations show that B73 kernels mount a multi-pronged defense response to *A. flavus* typical of that associated with plant basal resistance. Many of the maize genes induced by *A. flavus* are important in resistance against maize foliar pathogens, underscoring a possible commonality of the resistance response in seeds and leaves. These data further suggest that *A. flavus* infection invokes defense tactics used against more aggressive maize pathogens including increased transcription of defense signaling pathways and genes several genes known to be involved in the host's defense response.

We also observed striking changes in the transcription of genes associated with carbohydrate utilization (Table S2). An analysis of these transcriptional changes leads us to conclude that infection by *A. flavus* decreases starch synthesis, increases starch degradation, and mobilizes hexoses into pathways associated with plant defense (**Figure 2**; **Table 2**). A physical examination of kernels naturally infected with *A. flavus* (**Figure 3**) showed changes in the structure of the maize endosperm that could reflect the remobilization of hexoses in the seed in response to infection.

Physical changes in seeds infected with fungi have been observed before (Fennell et al., 1973; Koltun et al., 1974; Huff, 1980; Shetty and Bhat, 1999; Cardwell et al., 2000; Pearson and Wicklow, 2006). Most researchers have speculated that hydrolytic enzymes secreted by infecting fungi are responsible for this loss in grain quality. However, maize mutants with abnormal expression levels of carbohydrate and protein biosynthetic pathway genes can also develop atypical endosperm tissue (Neuffer et al., 1997; Black et al., 2006). Our findings showing changes in kernel primary metabolism during *A. flavus* infection challenges the assumption that fungal produced enzymes are solely responsible for changes in kernel structure, and suggests the plant may also contribute to these changes through starch degradation and hexose mobilization away from starch synthesis.

While these metabolic changes could represent a defense response by the kernel to infection by *A. flavus*, the changes could instead promote host susceptibility to the pathogen. Fungi, particularly fungal plant pathogens, are capable of manipulating the plant's metabolism to create an environment advantageous for

fungal growth (Govrin and Levine, 2000; Doehlemann et al., 2008). Increased invertase transcription in *A. flavus* infected kernels could indicate a higher-than-normal accumulation of free-hexoses within diseased tissue. Because glucose is the preferred carbon source of *A. flavus,* the up-regulation of sucrose hydrolyzing enzymes would presumable promote disease development by providing a steady supply of nutrients to the pathogen. IVR1 was previously found induced in sugar-poor environments, and its expression associated with tumor formation in *Ustilago maydis* infected maize (Xu et al., 1996; Doehlemann et al., 2008). Simple carbohydrates are also known to promote aflatoxin in maize kernels. Woloshuk et al. (1997) found an *A. flavus* α-amylase to play an important role in the production of aflatoxin by providing simple sugars conducive for aflatoxin production. Thus, sugar status in kernels could condition increased susceptibility as well as aflatoxin contamination.

In contrast, other studies have noted increased levels of hexoses in-and-around the site of pathogen infection and have hypothesized that these starch-derived sugars are an integral component of the host defense response (Berger et al., 2007; Bolton, 2009). Free-hexoses are thought to be used in the generation of reducing agents [NAD(P)H], energy [ATP], and pathway intermediates needed to synthesize secondary metabolite compounds. Their presence may also help trigger the synthesis of defense-related compounds.

Data from these studies, along with previous transcriptional studies (Luo et al., 2009, 2011; Jiang et al., 2011; Kelley et al., 2012), lay the groundwork for future studies investigating *A. flavus* resistance in maize. Under normal growth conditions, inducible defenses of B73 genotype may be adequate in inhibiting or at least slowing down *A. flavus* disease development. However, external and internal factors could affect this response. Abiotic stress, such as drought, can have a negative impact on the defense response (Wotton and Strange, 1987; Duke and Doehlert, 1996; Luo et al., 2010). Also, inherently low expression of defense and defense-associated genes may predispose the plant to greater infection (Chen et al., 2001; Alessandra et al., 2010). Genes expressed during infection may not necessarily be involved in resistance and could be causing increased susceptibility to fungal disease. Knowing which genes are typically expressed in response to pathogen attack is useful when examining how genotype and abiotic stress influence the infection process. Progress on more fully understanding disease development will ultimately leads to the development of genetically resistant cultivars.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00384/abstract

REFERENCES

Alessandra, L., Luca, P., and Adriano, M. (2010). Differential gene expression in kernels and silks of maize lines with contrasting levels of ear rot resistance after *Fusarium verticillioides* infection. *J. Plant Physiol.* 167, 1398–1406. doi: 10.1016/j.jplph.2010.05.015

Anderson, H. W., Nehring, E. W., and Wichser, W. R. (1975). Aflatoxin contamination of corn in the field. *Food Chem.* 23, 775–782. doi: 10.1021/jf60200a014

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B.* 57, 289–300.

Berger, S., Sinha, A., and Roitsch, T. (2007). Plant physiology meets phytopathology: plant primary metabolism and plant pathogen interactions. *J. Exp. Bot.* 58, 4019. doi: 10.1093/jxb/erm298

Black, M. J., Bewley, J. D., and Halmer, P. (2006). *The Encyclopedia of Seeds*: *Science, Technology and Uses*. Wallingford: CABI.

Blée, E. (2002). Impact of phyto-oxylipins in plant defense. *Trends Plant Sci.* 7, 315–322. doi: 10.1016/S1360-1385(02)02290-2

Boddu, J., Cho, S., and Muehlbauer, G. J. (2007). Transcriptome analysis of trichothecene-induced gene expression in barley. *Mol. Plant Microbe Interact.* 20, 1364–1375. doi: 10.1094/MPMI-20-11-1364

Bolton, M. (2009). Primary metabolism and plant defense- fuel for the fire. *Mol. Plant Microbe Interact.* 22, 487–497. doi: 10.1094/MPMI-22-5-0487

Brodhagen, M., and Keller, N. P. (2006). Signalling pathways connecting mycotoxin production and sporulation. *Mol. Plant Pathol.* 7, 285–301. doi: 10.1111/j.1364-3703.2006.00338.x

Brown, M. P., Brown-Jenco, C. S., and Payne, G. A. (1999). Genetic and molecular analysis of aflatoxin biosynthesis. *Fungal Genet. Biol.* 26, 81–98. doi: 10.1006/fgbi.1998.1114

Brown, R. L., Cleveland, T. E., Payne, G. A., Woloshuk, C. P., Campbell, K. W., and White, D. G. (1995). Determination of resistance to aflatoxin production in maize kernels and detection of fungal colonization using an *Aspergillus flavus* transformant expressing *Escherichia coli* β-glucuronidase. *Phytopathology* 85, 983–989. doi: 10.1094/Phyto-85-983

Browse, J. (2009). Jasmonate: preventing the maize tassel from getting in touch with his feminine side. *Sci. Signal.* 2. 9. doi: 10.1126/scisignal.259pe9

Bruce, W., Folkerts, O., Garnaat, C., Crasta, O., Roth, B., and Bowen, B. (2000). Expression profiling of the maize flavonoid pathway genes controlled by estradiol-inducible transcription factors CRC and P. *Plant Cell* 12, 65–80. doi: 10.1105/tpc.12.1.65

Burow, G. B., Nesbitt, T. C., Dunlap, J., and Keller, N. P. (1997). Seed lipoxygenase products modulate *Aspergillus* mycotoxin biosynthesis. *Mol. Plant Microbe Interact.* 10, 380–387. doi: 10.1094/MPMI.1997.10.3.380

Cardwell, K. F., Kling, J. G., Maziya-Dixon, B., and Bosque-Pérez, N. A. (2000). Interactions between *Fusarium verticillioides*, *Aspergillus flavus*, and insect infestation in four maize genotypes in Lowland Africa. *Phytopathology* 90, 276–284. doi: 10.1094/PHYTO.2000.90.3.276

Casacuberta, J. M., Puigdomenech, P., and San Segundo, B. (1991). A gene coding for a basic pathogenesis-related (PR-like) protein from *Zea mays*. Molecular cloning and induction by a fungus (*Fusarium moniliforme*) in germinating maize seeds. *Plant Mol. Biol.* 16, 527–536. doi: 10.1007/BF00023419

Casacuberta, J. M., Raventos, D., Puigdomenech, P., and San Segundo, B. (1992). Expression of the gene encoding the PR-like protein PRms in germinating maize embryos. *Mol. Genet. Genomics* 234, 97–104.

Chen, Z. Y., Brown, R. L., Cleveland, T. E., Damann, K. E., and Russin, J. S. (2001). Comparison of constitutive and inducible maize kernel proteins of genotypes resistant or susceptible to aflatoxin production. *J. Food Prot.* 64, 1785–1792.

Chen, Z. Y., Brown, R. L., Damann, K. E., and Cleveland, T. E. (2010). PR10 expression in maize and its effect on host resistance against *Aspergillus flavus* infection and aflatoxin production. *Mol. Plant Pathol.* 11, 69–81. doi: 10.1111/j.1364-3703.2009.00574.x

Chen, Z. Y., Brown, R. L., Rajasekaran, K., Damann, K. E., and Cleveland, T. E. (2006). Identification of a maize kernel pathogenesis-related protein and evidence for its involvement in resistance to *Aspergillus flavus* infection and aflatoxin production. *Phytopathology* 96, 87–95. doi: 10.1094/PHYTO-96-0087

Cheng, W. H., Taliercio, E. W., and Chourey, P. S. (1996). The miniature1 seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. *Plant Cell* 8, 971–983. doi: 10.1105/tpc.8.6.971

Chintamanani, S., Hulbert, S. H., Johal, G. S., and Balint-Kurti, P. J. (2010). Identification of a maize locus that modulates the hypersensitive defense response, using mutant-assisted gene identification and characterization. *Genetics* 184, 813–825. doi: 10.1534/genetics.109.111880

Chourey, P. S., Jain, M., Li, Q. B., and Carlson, S. J. (2006). Genetic control of cell wall invertases in developing endosperm of maize. *Planta* 223, 159–167. doi: 10.1007/s00425-005-0039-5

Cordero, M., Raventos, D., and Segundo, B. (1994). Differential expression and induction of chitinases and β-1, 3-glucanases in response to fungal infection during germination of maize seeds. *Mol. Plant Microbe Interact.* 7, 23–31. doi: 10.1094/MPMI-7-0023

Daayf, F., and Lattanzio, V. (2009). *Recent Advances in Polyphenol Research*. Vol. 1. New York, NY: Wiley InterScience, 47–56.

De La Fuente, G. N., Murray, S. C., Isakeit, T., Park, Y. S., Yan, Y., Warburton, M. L., et al. (2013). Characterization of genetic diversity and linkage disequilibrium of ZmLOX4 and ZmLOX5 loci in maize. *PLoS ONE* 8:e53973. doi: 10.1371/journal.pone.0053973

Delong, A., Calderon-Urrea, A., and Dellaporta, S. L. (1993). Sex determination gene TASSELSEED2 of maize encodes a short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. *Cell* 74, 757–768.

Derksen, H., Rampitsch, C., and Daayf, F. (2013). Signaling cross-talk in plant disease resistance. *Plant Sci.* 207, 79–87. doi: 10.1016/j.plantsci.2013.03.004

Dickman, M. B., and Fluhr, R. (2013). Centrality of host cell death in plant-microbe interactions. *Annu. Rev. Phytopathol.* 51, 543–570. doi: 10.1146/annurev-phyto-081211-173027

Dodd, J. L. (1980). The role of plant stresses in development of corn stalk rots. *Plant Dis.* 64, 533–537. doi: 10.1094/PD-64-533

Doehlemann, G., Wahl, R., Horst, R. J., Voll, L. M., Usadel, B., Poree, F., et al. (2008). Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph *Ustilago maydis*. *Plant J.* 56, 181–195. doi: 10.1111/j.1365-313X.2008.03590.x

Dolezal, A. L., Obrian, G. R., Nielsen, D. M., Woloshuk, C. P., Boston, R. S., and Payne, G. A. (2013). Localization, morphology and transcriptional profile of *Aspergillus flavus* during seed colonization. *Mol. Plant Pathol.* 14, 898–909. doi: 10.1111/mpp.12056

Du, Z., Zhou, X., Ling, Y., Zhang, Z., and Su, Z. (2010). agriGO: a GO analysis toolkit for the agricultural community. *Nucleic Acids Res.* 38, W64–W70. doi: 10.1093/nar/gkq310

Duke, E., and Doehlert, D. (1996). Effects of heat stress on enzyme activities and transcript levels in developing maize kernels grown in culture. *Environ. Exp. Bot.* 36, 199–208. doi: 10.1016/0098-8472(96)01004-0

Fennell, D., Bothast, R., Lillehoj, E., and Peterson, R. (1973). Bright greenish-yellow fluorescence and associated fungi in white corn naturally contaminated with aflatoxin. *Cereal Chem.* 50, 404–413.

Gao, X., Brodhagen, M., Isakeit, T., Brown, S. H., Gobel, C., Betran, J., et al. (2009). Inactivation of the lipoxygenase ZmLOX3 increases susceptibility of maize to *Aspergillus* spp. *Mol. Plant Microbe Interact.* 22, 222–231. doi: 10.1094/MPMI-22-2-0222

Gao, X., Shim, W. B., Gobel, C., Kunze, S., Feussner, I., Meeley, R., et al. (2007). Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin. *Mol. Plant Microbe Interact.* 20, 922–933. doi: 10.1094/MPMI-20-8-0922

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Govrin, E. M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* 10, 751–757. doi: 10.1016/S0960-9822(00)00560-1

Granot, D., David-Schwartz, R., and Kelly, G. (2013). Hexose kinases and their role in sugar-sensing and plant development. *Front. Plant Sci.* 4:44. doi: 10.3389/fpls.2013.00044

Greene, T. W., and Hannah, L. C. (1998). Maize endosperm ADP–glucose pyrophosphorylase SHRUNKEN2 and BRITTLE2 subunit interactions. *Plant Cell* 10, 1295–1306.

Guo, B., Chen, Z. Y., Lee, R. D., and Scully, B. T. (2008). Drought stress and preharvest aflatoxin contamination in agricultural commodity: genetics, genomics and proteomics. *J. Integr. Plant Biol.* 50, 1281–1291. doi: 10.1111/j.1744-7909.2008.00739.x

Herrmann, K. M. (1995). The shikimate pathway as an entry to aromatic secondary metabolism. *Plant Physiol.* 107, 7–12. doi: 10.1104/pp.107.1.7

Hruska, Z., Yao, H., Kincaid, R., Darlington, D., Brown, R. L., Bhatnagar, D., et al. (2013). Fluorescence Imaging Spectroscopy (FIS) for comparing spectra from corn ears naturally and artificially infected with aflatoxin producing fungus. *J. Food Sci.* 78, T1313–T1320. doi: 10.1111/1750-3841.12202

Huff, W. (1980). A physical method for the segregation of aflatoxin-contaminated corn. *Cereal Chem.* 57, 236–238.

Isakeit, T., Gao, X., and Kolomiets, M. (2007). Increased resistance of a maize mutant lacking the 9-lipoxygenase gene, *ZmLOX3*, to root rot caused by *Exserohilum pedicellatum*. *Phytopathology* 155, 758–760. doi: 10.1111/j.1439-0434.2007.01301.x

Jiang, T., Zhou, B., Luo, M., Abbas, H. K., Kemerait, R., Lee, R. D., et al. (2011). Expression analysis of stress-related genes in kernels of different maize (Zea mays L.) inbred lines with different resistance to aflatoxin contamination. *Toxins (Basel)* 3, 538–550. doi: 10.3390/toxins3060538

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Jones, R. K., Duncan, H. E., Payne, G. A., and Leonard, K. J. (1980). Factors influencing infection by *Aspergillus flavus* in silk-inoculated corn. *Plant Dis.* 64, 859–863. doi: 10.1094/PD-64-859

Keller, N. P., Butchko, R., Sarr, B., and Phillips, T. D. (1994). A visual pattern of mycotoxin production in maize kernels by *Aspergillus* spp. *Phytopathology* 84, 483–488. doi: 10.1094/Phyto-84-483

Kelley, R. Y., Williams, W. P., Mylroie, J. E., Boykin, D. L., Harper, J. W., Windham, G. L., et al. (2012). Identification of maize genes associated with host plant resistance or susceptibility to *Aspergillus flavus* infection and aflatoxin accumulation. *PLoS ONE* 7:e36892. doi: 10.1371/journal.pone.0036892

Kew, M. C. (2013). Aflatoxins as a cause of hepatocellular carcinoma. *J. Gastrointestin. Liver Dis.* 22, 305–310.

Koehler, B. (1942). Natural mode of entrance of fungi into corn ears and some symptoms that indicate infection. *J. Agric. Res.* 64, 421–442.

Koltun, S. P., Gardner, H. K., Dollear, F. G., and Rayner, E. T. (1974). Physical properties and aflatoxin content of individual cateye fluorescent cottonseeds. *J. Am. Oil Chem. Soc.* 51, 178–180. doi: 10.1007/BF02639734

Lamb, C., and Dixon, R. (1997). The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 251–275. doi: 10.1146/annurev.arplant.48.1.251

Lange, B. M., Rujan, T., Martin, W., and Croteau, R. (2000). Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. *Proc. Natl. Acad. Sci. U.S.A.* 97, 13172–13177. doi: 10.1073/pnas.240454797

Liang, X. Q., Luo, M., and Guo, B. Z. (2006). Resistance mechanisms to *Aspergillus flavus* infection and aflatoxin contamination in peanut (*Arachis hypogaea*). *Plant Pathol. J.* 5, 115–124. doi: 10.3923/ppj.2006.115.124

Lillehoj, E., Kwolek, W., Horner, E., Widstrom, N., Josephson, L., Franz, A., et al. (1980). Aflatoxin contamination of preharvest corn: role of *Aspergillus flavus* inoculum and insect damage. *Cereal Chem.* 57, 255–257.

Lillehoj, E. B., Kwolek, W. F., Peterson, R. E., Shotwell, O. L., and Hesseltine, C. W. (1976). Aflatoxin contamination, fluorescence, and insect damage in corn infected with *Aspergillus flavus* before harvest. *Cereal Chem.* 53, 505–512.

Luo, M., Brown, R. L., Chen, Z. Y., and Cleveland, T. E. (2009). Host genes involved in the interaction between *Aspergillus flavus* and maize. *Toxin Rev.* 28, 118–128. doi: 10.1080/15569540903089197

Luo, M., Brown, R. L., Chen, Z. Y., Menkir, A., Yu, J., and Bhatnagar, D. (2011). Transcriptional profiles uncover *Aspergillus flavus*-induced resistance in maize kernels. *Toxins (Basel)* 3, 766–786. doi: 10.3390/toxins3070766

Luo, M., Liu, J., Lee, R. D., Scully, B. T., and Guo, B. (2010). Monitoring the expression of maize genes in developing kernels under drought stress using oligo-microarray. *J. Integr. Plant Biol.* 52, 1059–1074. doi: 10.1111/j.1744-7909.2010.01000.x

Magbanua, Z., Williams, W. P., and Luthe, D. (2013). The maize rachis affects *Aspergillus flavus* spread during ear development. *Maydica* 58, 182–188.

Marsh, S., and Payne, G. (1984). Preharvest infection of corn silks and kernels by *Aspergillus flavus*. *Phytopathology* 74, 1284–1289. doi: 10.1094/Phyto-74-1284

Mengiste, T. (2012). Plant immunity to necrotrophs. *Annu. Rev. Phytopathol.* 50, 267–294. doi: 10.1146/annurev-phyto-081211-172955

Mideros, S. X., Warburton, M. L., Jamann, T. M., Windham, G. L., Williams, W. P., and Nelson, R. J. (2014). Quantitative trait loci influencing mycotoxin contamination of maize: analysis by linkage mapping, characterization of near-isogenic lines, and meta-analysis. *Crop Sci.* 54, 127–142. doi: 10.2135/cropsci2013.04.0249

Mylroie, J. E., Warburton, M. L., and Wilkinson, J. R. (2013). Development of a gene-based marker correlated to reduced aflatoxin accumulation in maize. *Euphytica* 194, 431–441. doi: 10.1007/s10681-013-0973-4

Neuffer, M., Coe, J. E., and Wessler, S. (1997). *Mutants of Maize*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Niu, D. D., Liu, H. X., Jiang, C. H., Wang, Y. P., Wang, Q. Y., Jin, H. L., et al. (2011). The plant growth-promoting rhizobacterium bacillus cereus AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways. *Mol. Plant Microbe Interact.* 24, 533–542. doi: 10.1094/MPMI-09-10-0213

Payne, A. G., and Yu, J. (2010). *Ecology, Development and Gene Regulation in Aspergillus flavus. Aspergillus: Molecular Biology and Genomics.* Norfolk: Masayuki Machida and Katsuya Gomi Caister Academic Press.

Payne, G., Hagler, W. Jr, and Adkins, C. (1988a). Aflatoxin accumulation in inoculated ears of field grown maize. *Plant Dis.* 72, 422–424. doi: 10.1094/PD-72-0422

Payne, G., Thompson, D., Lillehoj, E., Zuber, M., and Adkins, C. (1988b). Effect of temperature on the preharvest infection of maize kernels by *Aspergillus flavus*. *Phytopathology* 78, 1376–1380. doi: 10.1094/Phyto-78-1376

Pearson, T., and Wicklow, D. (2006). Detection of corn kernels infected by fungi. *Trans. ASABE* 49, 1235–1245. doi: 10.13031/2013.21723

Prost, I., Dhondt, S., Rothe, G., Vicente, J., Rodriguez, M. J., Kift, N., et al. (2005). Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. *Plant Physiol.* 139, 1902–1913. doi: 10.1104/pp.105.066274

Reese, B. N., Payne, G. A., Nielsen, D. M., and Woloshuk, C. P. (2011). Gene expression profile and response to maize kernels by *Aspergillus flavus*. *Phytopathology* 101, 797–804. doi: 10.1094/PHYTO-09-10-0261

Ritchie, S., Hanway, J., and Benson, G. (1997). *How a Corn Plant Develops. Special Report Number 48*. Ames, IA: Iowa State University of Science and Technology Cooperative Extension Service.

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. G. (2011). Hormone crosstalk in plant disease and defense: more than just JASMONATE-SALICYLATE antagonism. *Annu. Rev. Phytopathol.* 49, 317–343. doi: 10.1146/annurev-phyto-073009-114447

Rohrmeier, T., and Lehle, L. (1993). WIP1, a wound-inducible gene from maize with homology to bowman-birk proteinase inhibitors. *Plant Mol. Biol.* 22, 783–792. doi: 10.1007/BF00027365

Roitsch, T., Balibrea, M. E., Hofmann, M., Proels, R., and Sinha, A. K. (2003). Extracellular invertase: key metabolic enzyme and PR protein. *J. Exp. Bot.* 54, 513–524. doi: 10.1093/jxb/erg050

Scheidegger, K. A., and Payne, G. A. (2003). Unlocking the secrets behind secondary metabolism: a review of *Aspergillus flavus* from pathogenicity to functional genomics. *Toxin Rev.* 22, 423–459. doi: 10.1081/TXR-120024100

Shetty, P., and Bhat, R. (1999). A physical method for segregation of fumonisin-contaminated maize. *Food Chem.* 66, 371–374. doi: 10.1016/S0308-8146(99)00052-7

Simmons, C., Tossberg, J., Sandahl, G., Marsh, W., Dowd, P., Duvick, J., et al. (2002). Maize pathogen defenses activated by avirulence gene avrRxv. *Maize Genet. Coop. News Lett.* 76, 40–41.

Smart, M. G., Wicklow, D. T., and Caldwell, R. W. (1990). Pathogenesis in *Aspergillus* ear rot of maize: light microscopy of fungal spread from wounds. *Phytopathology* 80, 1287–1294. doi: 10.1094/Phyto-80-1287

Smith, C. A., Robertson, D., Yates, B., Nielsen, D. M., Brown, D., Dean, R. A., et al. (2008). The effect of temperature on Natural Antisense Transcript (NAT) expression in *Aspergillus flavus*. *Curr. Genet.* 54, 241–269. doi: 10.1007/s00294-008-0215-9

Spielbauer, G., Margl, L., Hannah, L. C., Römischb, W., Ettenhuber, C., Bacher, A., et al. (2006). Robustness of central carbohydrate metabolism in developing maize kernels. *Phytochemistry* 67, 1460–1475. doi: 10.1016/j.phytochem.2006.05.035

Taubenhaus, J. J. (1920). A study of the black and yellow molds of ear corn. *Texa Agric. Exp. Station Bull* 290, 3.

Tubajika, K. M., and Damann, K. E. (2001). Sources of resistance to aflatoxin production in maize. *J. Agric. Food Chem.* 49, 2652–2656. doi: 10.1021/jf001333i

van Loon, L. C., Rep, M., and Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44, 135–162. doi: 10.1146/annurev.phyto.44.070505.143425

Vick, B. A., and Zimmerman, D. C. (1984). Biosynthesis of jasmonic acid by several plant species. *Plant Physiol.* 75, 458–461. doi: 10.1104/pp.75.2.458

Walter, M. H., Fester, T., and Strack, D. (2000). Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the yellow pigment and other apocarotenoids. *Plant J.* 21, 571–578. doi: 10.1046/j.1365-313x.2000.00708.x

Warburton, M. L., Williams, W. P., Windham, G. L., Murray, S. C., Xu, W., Hawkins, L. K., et al. (2013). Phenotypic and genetic characterization of a maize association mapping panel developed for the identification of new sources of resistance to *Aspergillus flavus* and aflatoxin accumulation. *Crop Sci.* 53, 2374–2383. doi: 10.2135/cropsci2012.10.0616

Weber, H., Borisjuk, L., and Wobus, U. (1997). Sugar import and metabolism during seed development. *Trends Plant Sci.* 2, 169–174. doi: 10.1016/S1360-1385(97)85222-3

Widstrom, N. W., Guo, B. Z., and Wilson, D. M. (2003). Integration of crop management and genetics for control of preharvest aflatoxin contamination of corn. *Toxin Rev.* 22, 199–227. doi: 10.1081/TXR-120024092

Wilson, R. A., Gardner, H. W., and Keller, N. P. (2001). Cultivar-dependent expression of a maize lipoxygenase responsive to seed infesting fungi. *Mol. Plant Microbe Interact.* 14, 980–987. doi: 10.1094/MPMI.2001.14.8.980

Windham, G., and Williams, W. (1998). *Aspergillus flavus* infection and aflatoxin accumulation in resistant and susceptible maize hybrids. *Plant Dis.* 82, 281–284. doi: 10.1094/PDIS.1998.82.3.281

Windham, G. L., and Williams, W. P. (2002). Evaluation of corn inbreds and advanced breeding lines for resistance to aflatoxin contamination in the field. *Plant Dis.* 86, 232–234. doi: 10.1094/PDIS.2002.86.3.232

Wisser, R. J., Kolkman, J. M., Patzoldt, M. E., Holland, J. B., Yu, J., Krakowsky, M., et al. (2011). Multivariate analysis of maize disease resistances suggests a pleiotropic genetic basis and implicates a GST gene. *Proc. Natl. Acad. Sci. U.S.A.* 108, 7339–7344. doi: 10.1073/pnas.1011739108

Woloshuk, C. P., Cavaletto, J. R., and Cleveland, T. E. (1997). Inducers of aflatoxin biosynthesis from colonized maize kernels are generated by an amylase activity from *Aspergillus flavus*. *Phytopathology* 87, 164–169. doi: 10.1094/PHYTO.1997.87.2.164

Wotton, H., and Strange, R. (1987). Increased susceptibility and reduced phytoalexin accumulation in drought-stressed peanut kernels challenged with *Aspergillus flavus*. *Appl. Environ. Microbiol.* 53, 270.

Xu, J., Avigne, T. W., McCarty, D. R., and Koch, K. E. (1996). A similar dichotomy of sugar modulation and developmental expression affects both paths of sucrose metabolism: evidence from a maize invertase gene family. *Plant Cell* 8, 1209–1220. doi: 10.1105/tpc.8.7.1209

Zhang, J., Simmons, C., Yalpani, N., Crane, V., Wilkinson, H., and Kolomiets, M. (2005). Genomic analysis of the 12-oxo-phytodienoic acid reductase gene family of *Zea mays*. *Plant Mol. Biol.* 59, 323–343. doi: 10.1007/s11103-005-8883-z

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Environmental influences on maize-*Aspergillus flavus* interactions and aflatoxin production

Jake C. Fountain[1], Brian T. Scully[2], Xinzhi Ni[3], Robert C. Kemerait[1], Robert D. Lee[4], Zhi-Yuan Chen[5], and Baozhu Guo[1,2]*

[1] *Department of Plant Pathology, University of Georgia, Tifton, GA, USA*
[2] *Crop Protection and Management Research Unit, Agricultural Research Service – United States Department of Agriculture, Tifton, GA, USA*
[3] *Crop Genetics and Breeding Research Unit, Agricultural Research Service – United States Department of Agriculture, Tifton, GA, USA*
[4] *Department of Crop and Soil Sciences, University of Georgia, Tifton, GA, USA*
[5] *Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA, USA*

Edited by:
Perng-Kuang Chang, Southern Regional Research Center, USA

Reviewed by:
Chuck Niblett, Venganza, Inc., USA
Ahmad Fakhoury, Southern Illinois University Carbondale, USA

***Correspondence:**
Baozhu Guo, Research Plant Pathologist, Crop Protection and Management Research Unit, Agricultural Research Service – United States Department of Agriculture, 2747 Davis Road, Tifton, GA 31793, USA
e-mail: baozhu.guo@ars.usda.gov

Since the early 1960s, the fungal pathogen *Aspergillus flavus* (Link ex Fr.) has been the focus of intensive research due to the production of carcinogenic and highly toxic secondary metabolites collectively known as aflatoxins following pre-harvest colonization of crops. Given this recurrent problem and the occurrence of a severe aflatoxin outbreak in maize (*Zea mays* L.), particularly in the Southeast U.S. in the 1977 growing season, a significant research effort has been put forth to determine the nature of the interaction occurring between aflatoxin production, *A. flavus,* environment and its various hosts before harvest. Many studies have investigated this interaction at the genetic, transcript, and protein levels, and in terms of fungal biology at either pre- or post-harvest time points. Later experiments have indicated that the interaction and overall resistance phenotype of the host is a quantitative trait with a relatively low heritability. In addition, a high degree of environmental interaction has been noted, particularly with sources of abiotic stress for either the host or the fungus such as drought or heat stresses. Here, we review the history of research into this complex interaction and propose future directions for elucidating the relationship between resistance and susceptibility to *A. flavus* colonization, abiotic stress, and its relationship to oxidative stress in which aflatoxin production may function as a form of antioxidant protection to the producing fungus.

Keywords: maize, aflatoxin contamination, environment effects, oxidative stress, host resistance

INTRODUCTION

Aspergillus flavus (Link ex Fr.; Teleomorph: *Petromyces flavus*; Horn et al., 2009a,b) is a facultative, plant parasitic pathogen, which has the ability to colonize a number of common crop species including corn, cotton, peanuts, and many other crops (Diener et al., 1987). Economic losses due to the infection of grain crops such as maize (*Zea mays* L.) by *A. flavus* is not primarily due to the expression of symptoms known as *Aspergillus* ear rot but, rather, is due to the subsequent contamination of the grain with the fungal metabolite aflatoxin. Aflatoxins are a group of polyketide-derived furanocoumarin secondary metabolites produced by certain species of fungi, including the genus *Aspergillus* (Bennett and Klich, 2003; Chanda et al., 2009). Aflatoxins are highly carcinogenic and can be acutely toxic or fatal if ingested in sufficient quantities for both livestock and humans (Shephard, 2008).

Although this species was first described by Link in 1809 (Amaike and Keller, 2011), major research on the biology and pathogenicity of *A. flavus* did not commence until the mid 1960s with the incidence of Turkey X disease which killed over 100,000 turkey poults due to aflatoxin contaminated feed associated with *A. flavus* infected peanuts (Wogan, 1966). Shortly thereafter broad screening of feed and food was initiated, the chemical structures of the major aflatoxins (B1, B2, G1, and G2) were elucidated, and research was conducted to prevent post-harvest contamination of grain crops through the modulation of storage conditions (Asao et al., 1965; Trenk and Hartman, 1970). However, it was found during a particularly severe outbreak of aflatoxin contamination in maize in the late 1970s in the U.S. that it was possible for *A. flavus* to both colonize and produce aflatoxin on developing maize kernels prior to harvest (Diener et al., 1983, 1987).

Since the extensive losses from the 1977 growing season due to aflatoxin contamination (Diener et al., 1983), research efforts have focused on determining the source of host plant resistance to prevent *A. flavus* colonization and subsequent aflatoxin production pre-harvest and before transportation to storage. These efforts have employed numerous techniques and approaches including modern plant breeding and genetics tools such as proteomic, transcriptomic, and biochemical analyses in an effort to discover the underlying mechanism of host plant resistance and the interaction between the two organisms. To date, these efforts have revealed that resistance is quantitatively inherited with a strong genotype by environment component. It is a complex interaction with a high degree of environmentally induced variability with abiotic and biotic stress strongly influencing resistance or susceptibility. Here we review the results of research into the

mechanism of host resistance to both *A. flavus* colonization and abiotic stress, and propose future research directions for determining the relationship between oxidative stress and aflatoxin contamination.

HOST RESISTANCE AGAINST *A. flavus*: GENE-FOR-GENE VS. GENOTYPE × ENVIRONMENT INTERACTION?

From a gene-for-gene perspective, host resistance against *A. flavus* colonization and subsequent aflatoxin contamination has been approached as a single virulence factor produced by the invading pathogen that would be countered by a single avirulence or resistance protein in the host. This results in compatible or incompatible reactions based on specific recognition (Keen, 1990). The particular virulence mechanisms of these plant-microbe interactions are utilized to classify plant pathogens into groups such as biotrophic, necrotrophic, and hemibiotrophic pathogens (Glazebrook, 2005).

The classification of *A. flavus* into a particular class of plant pathogens has yet to be determined. A microscopy study of the growth of *A. flavus* in maize kernel tissue by Smart et al. (1990) showed that cellular components such as cell walls were broken down in advance of mycelia. This has been interpreted as being indicative of necrotrophic pathogenicity in the literature (Mideros et al., 2009), however, not all research concurs with this conclusion. Magbanua et al. (2007) found that the colonization of kernel tissue from resistant maize lines exhibited increased levels of salicylic acid (SA) and unchanged levels of jasmonic acid (JA). Such patterns are commonly associated with resistance to biotrophic pathogens in various plant species (Glazebrook, 2005). As a facultative parasite, which naturally exists as a saprophyte, *A. flavus* may possess a unique pathogenicity mechanism that does not categorically fit into this classification scheme.

In keeping with the concept of gene-for-gene resistance, virulence factors produced by *A. flavus* during the colonization of maize tissues as well as maize kernel avirulence proteins have been the focus of multiple studies. These efforts have helped to better characterize the nature of this plant–pathogen interaction, and have sought to identify pathogen and host-derived proteins encoded by potential single-gene sources of host resistance or susceptibility. Proteomics-based techniques have identified several virulence proteins produced by *A. flavus*, most of which being hydrolytic enzymes. Examples of these enzymes are amylases, cellulases, chitinases, cutinases (e.g., phyto-cutinase), lipases, pectinases (P2c), proteases such as alkaline protease, and xylanases (Cleveland and Cotty, 1991; Guo et al., 1995; Chen et al., 1998, 1999; Fakhoury and Woloshuk, 1999; Mellon et al., 2000; Brown et al., 2001; Cleveland et al., 2004; Chen et al., 2009a; Pechanova et al., 2013). These enzymes are consistent with the biology and classification of *A. flavus* as a saprophyte since they typically catabolize decaying plant materials as a source of nutrition.

Several constitutively expressed and inducible proteins have been described in the literature, which have been shown to counter the function of other hydrolytic virulence proteins produced by *A. flavus*. For example, Chen et al. (1998) described a 14-kDa trypsin inhibitor (TI) which functions as an inhibitor of α-amylase, a protein utilized by *A. flavus* for the catabolism of complex carbohydrates. The same group also showed that silencing the expression of the TI gene in maize increases the susceptibility of maize kernel tissue to *A. flavus* infection and aflatoxin contamination (Chen et al., 2009b). In addition, β-1,3-glucanases, chitinases, pathogenesis-related proteins 10 and 10.1, ribosome inactivating proteins (RIPs), and zeamatin have also been shown to be involved in the resistance of maize against *A. flavus* (Mauch et al., 1988; Walsh et al., 1991; Huynh et al., 1992; Guo et al., 1997; Lozovaya et al., 1998; Chen et al., 2006; Chen et al., 2010; Xie et al., 2010). In peanut (*Arachis hypogaea*), Liang et al. (2005) reported that the increase of β-1,3-glucanases among resistant lines was higher than in the susceptible lines after infection with *A. flavus*.

Accumulation of such antifungal and avirulence proteins has been shown to contribute to the resistance observed in several maize lines along with morphological characteristics of resistant kernels such as thickened wax cuticles (Guo et al., 1995). Various breeding techniques have been employed to develop varieties with enhanced resistance to *A. flavus* and aflatoxin contamination on the basis of utilizing phenotypic screenings and molecular markers associated with known avirulence genes to identify quantitative trait loci (QTL) for selection (Brown et al., 2013). These efforts have met with some success such as in the case of Willcox et al. (2013) who identified 20 QTL explaining 22–43% of phenotypic variation within a F_2 mapping population derived from Mp313E × Va35. This study, along with others (Paul et al., 2003; Brooks et al., 2005; Kelley et al., 2012), illustrate two important challenges in breeding resistant maize varieties, including: (1) that resistance to *A. flavus* is a quantitative trait involving multiple genes rather than single-gene forms of gene-for-gene resistance, and (2) a large genotype x environment (G × E) interaction conditions the expression of this trait. For example Willcox et al. (2013) determined that only 11 of the 20 identified QTL were consistently expressed across different environments with these accounting for 2.4–9.5% of phenotypic variance.

Environmental factors can have a significant effect on maize resistance to *A. flavus* and aflatoxin production, particularly abiotic stresses such as drought and heat stress. This was clearly demonstrated as early as 1977 when widespread and intense drought conditions in the Midwestern and Southeastern United States resulted in a high degree of aflatoxin contamination of maize kernels pre-harvest (Zuber and Lillehoj, 1979; Diener et al., 1987). However, maize genotypes possessing drought tolerance, such as Lo964 and Tex6, tend to be less susceptible to aflatoxin contamination (Guo et al., 2008; Luo et al., 2008; Jiang et al., 2012).

RELATIONSHIP OF DROUGHT TOLERANCE, AFLATOXIN CONTAMINATION AND *A. flavus* RESISTANCE

It has been hypothesized that there is an underlying relationship among various molecular mechanisms of drought stress adaptation and resistance to *A. flavus* infection and subsequent aflatoxin contamination. This connection has been demonstrated experimentally in a number of studies. Chen et al. (2007) found a number of drought stress-related proteins that were induced in response to *A. flavus* colonization in maize endosperm tissue including late embryogenesis abundant proteins LEA 3,

14, peroxiredoxin (PER1), and a 17.9-kDa heat shock protein (HSP17.9). Pechanova et al. (2011) found that these proteins along with several antioxidant proteins, such as ascorbate peroxidase and superoxide dismutase (SOP), were up-regulated in resistant maize rachis tissue earlier in development and formed the basis, when combined with increased expression of antifungal and pathogenesis-related proteins, for resistance to *A. flavus* colonization.

It has also been shown that global defense regulators have the potential to modulate maize resistance to drought stress, oxidative stress, and possibly *A. flavus* resistance. The WRKY transcription factors have been shown to regulate the responses of multiple plant species to both biotic and abiotic stresses (Rushton et al., 2010). The transcription factor *Zm*WRKY33 was recently shown to enhance abscisic acid (ABA) signaling and enhance osmotic stress tolerance in maize seedlings (Li et al., 2013). *Zm*WRKY33 along with *Zm*WRKY19, whose homolog in *Arabidopsis thaliana* (*At*WRKY53) is known to induce a response to oxidative stress and regulate the expression of antioxidant enzymes like catalase (Miao et al., 2004; Eulgem and Somssich, 2007; Miao et al., 2007; Rushton et al., 2010). These factors were found to be up-regulated earlier in resistant maize varieties in response to *A. flavus* inoculation in whole kernel tissues (Fountain et al., 2013).

OXIDATIVE RESPONSES OF PLANTS TO HERBIVORY

Oxidative responses of plants to feeding damage by both chewing and piercing-sucking insects and nematodes have been described in recent years. Walling (2000) and Kaloshian and Walling (2005) described the piercing-sucking hemipteran insect feeding on crop plants as resembling plant pathogen infections that they are often associated with chitosan (oligogalacturonides produced by pectinases) and reactive oxygen species (ROS)-activated wound or defense signaling pathways in host plants. Kessler and Baldwin (2002) reviewed the molecular mechanisms underlying plant responses to insect herbivory and how they differ from pathogen infections. They concluded that insect herbivores are physiologically independent from their host plants, whereas pathogens are physiologically dependent on their host plants for their growth and development. Oxidative enzyme-mediated wounding responses also play a critical role in understanding plant responses to insect herbivory, although the insect-specific elicitors frequently modify the responses of their host plants, and allow the host plants to optimize their defenses against a specific insect pest (Kessler and Baldwin, 2002). Das et al. (2008) studied the accumulation of ROS in cowpea–root-knot nematode interaction and confirmed that the induction of resistance is relatively late in this system. Typically, hypersensitive response is closely associated with an oxidative burst in infected tissue.

Bi and Felton (1995) reported that corn earworm (*Helicoverpa zea* Boddie), herbivory caused significant increases in lipid peroxidation and hydroxyl radical formation in the soybean leaves. The activities of several oxidative enzymes (i.e., lipoxygenases, peroxidase, diamine oxidase, ascorbate oxidase, and NADH oxidase I) were also increased following *H. zea* herbivory on soybean (*Glycine max*) leaves. They concluded that oxidative responses in the soybean plants may have led to a decrease in herbivory and an increase in oxidative damage to the plant. Ni et al. (2000) described the salivary enzyme profiles of the leaf-chlorosis-eliciting Russian wheat aphid (*Diuraphis noxia* Mordvilko), and the non-leaf-chlorosis-eliciting bird cherry-oat aphid (*Rhopalosiphum padi* L.), which differ in oxidative enzyme activities. While only peroxidase activity was detected in *R. padi*, catalase activity was only detected in *D. noxia*. The oxidative responses of four cereal plants (i.e., susceptible "Arapahoe" and resistant "Halt" wheat, susceptible "Morex" barley, and resistant "Border" oat) to the feeding of the two species of aphid differed (Ni et al., 2001a). The chlorosis-eliciting *D. noxia* feeding caused a three-fold increase in peroxidase activity in the resistant Halt wheat, and nine-fold increase in the susceptible Morex barley 9 days after infestation when compared to the control leaves. In contrast, *R. padi* did not cause any changes in peroxidase activity in any of the cereal leaves. At the same time, *D. noxia* feeding did not elicit any change in either catalase or polyphenol oxidase activity in comparison with either the *R. padi*-infested or the control cereal leaves (Ni et al., 2001a). Furthermore, oxidative bleaching in leaf chlorosis elicited by *D. noxia* was not detected, but Mg-dechelatase activity was increased in the *D. noxia*-elicited chlorosis in wheat leaves (Ni et al., 2001b).

Zavala et al. (2013) also proposed a cellular mechanism to decipher the influence of elevated CO_2 on insect herbivory. They highlighted that oxidative enzyme activities in the subcellular organelles, such as peroxisomes and chloroplasts, via the jasmonic signaling pathway are likely to be critical factors in dissecting the molecular mechanisms of plant defenses against both biotic and abiotic stresses. In general, the oxidative responses of plants to pathogen infection and insect herbivory under varying environmental conditions (e.g., drought and the elevated CO_2) are critical for the management of pest outbreaks and the reduction of mycotoxin contamination in agricultural crops.

THE ROLE OF OXIDATIVE STRESS IN AFLATOXIN BIOSYNTHESIS

The presence of increased expression of antioxidant mechanisms in resistant maize tissues leads to the hypothesis that increased resistance to ROS-induced oxidative stress may correlate to resistance to *A. flavus* and aflatoxin contamination. Although this conclusion seems credible due to the high degree of correlative evidence present in the literature, the exact mechanism of how this phenomenon functions has yet to be elucidated completely. More recent studies into the biology of *A. flavus* and the mechanisms regulating the production of aflatoxin may illuminate this issue (Magbanua et al., 2007; Roze et al., 2013).

Aflatoxin biosynthesis is a complex process involving multiple gene products and regulatory mechanisms coded for by an approximately 70-kb cluster of 25 genes (Yu et al., 2004). This pathway is responsible for the biosynthesis of five major mycotoxins: sterigmatocystin, and aflatoxins B_1, B_2, G_1, and G_2 (Yu et al., 2004), and is the focus of intensive research into methods of negatively regulating its function. Although the structure and biochemical characteristics of aflatoxins have been known since the 1960s (Asao et al., 1965; Wogan, 1966), the specific purpose of their

production by *A. flavus* or other aflatoxigenic fungi has remained a mystery. Prior research exploring the roles of oxidative stress in regulating aflatoxin biosynthesis as well as recent discoveries into the upstream regulation of major pathway regulatory factors have begun to elucidate the biological function of aflatoxins (Roze et al., 2013). It has been shown that aflatoxin production by *A. flavus* is higher in maize kernel tissues containing higher levels of lipids, such as embryo tissues (Earle et al., 1946; Fabbri et al., 1980; Brodhagen and Keller, 2006). The roles of lipids in regulating aflatoxin biosynthesis in *Aspergillus spp.* have been investigated, particularly oxylipins (Reviewed in Gao and Kolomiets, 2009). Earlier research by Fabbri et al. (1983) found that seeds of high-oil crops such as peanut support higher levels of aflatoxin production by *A. parasiticus* than seeds of graminaceous plants such as maize or wheat, which contain higher levels of starch. In addition, they found that culturing *A. parasiticus* amended with peroxidized lipids resulted in significantly elevated aflatoxin production with no significant effect on fungal biomass (Fabbri et al., 1983).

Lipid peroxidation is a byproduct of lipid metabolism in peroxisomes as well as the reaction of naturally produced free fatty acids with ROS (Reverberi et al., 2012). Therefore, it is possible that excessive peroxisome function in the fungal mycelia or oxidative stress may be a causative factor in the production of aflatoxin. *In vitro*, Jayashree and Subramanyam (2000) showed that toxigenic strains of *A. parasiticus* have increased oxygen requirements, which they postulate to be a potential source of ROS accumulation, in comparison to non-toxigenic strains. In addition, they showed that higher levels of glutathione and thiobarbituric acid-reactive substances (TBARS), as well as antioxidant enzyme activities, were present in toxigenic strains in comparison to non-toxigenic strains. This indicated that oxidative stress may be a pre-requisite for aflatoxin production (Jayashree and Subramanyam, 2000). Also, Reverberi et al. (2012) found that bezafibrate and transformation of the *Cymbidium ringspot virus* P33 gene into *A. flavus* induced peroxisome proliferation resulting in an increase in aflatoxin production both *in vitro* and when cultured on maize kernel tissues in addition to increased levels of antioxidant enzyme gene expression, lipid metabolism, oxylipin biosynthesis, and ROS accumulation in the *A. flavus* mycelia. Recent studies have also found that cAMP and G-protein-mediated quorum sensing signaling pathways based on oxylipin perception can play a vital role in growth regulation and aflatoxin production in *A. nidulans* (Affeldt et al., 2012). Such G-protein mediated signaling has also been reported in other *Aspergillus spp.* including *A. fumigatus* (Grice et al., 2013). Therefore, it may be concluded that oxidative stress in *A. flavus* induced by ROS and/or oxylipins in the growth environment/medium will result in increased aflatoxin production from a biochemical perspective.

Recent studies have also shown that ROS can play a role in the transcriptional regulation of aflatoxin and sterigmatosystin biosynthesis pathway genes. Reverberi et al. (2008) found that a putative binding site for the Ap*yapA* gene, which regulates oxidative stress tolerance and conidiogenesis, was present in the promoter of the regulatory gene *aflR* in *A. parasiticus*, and that silencing Ap*yapA* results in an increase in aflatoxin biosynthesis. It has also been shown that the basic leucine zipper (bZIP) transcription factor AtfB regulates the aflatoxin biosynthesis genes *fas-1*, *ver-1*, and *omtA* as well as antioxidant genes encoding for catalase and SOP (Hong et al., 2013). They also found that the promoter regions associated with AtfB also contained cAMP-responsive elements implicating cAMP in the regulation of aflatoxin biosynthesis (Hong et al., 2013).

POTENTIAL REACTIVE OXYGEN SPECIES-MEDIATED CROSSTALK BETWEEN MAIZE AND *A. flavus*

Given the apparent role of oxidative stress in the promotion of aflatoxin biosynthesis, the hypothesis has been proposed that aflatoxin may function as a form of antioxidant protection to Aspergilli (Reverberi et al., 2010). This would provide an explanation to the long standing question, rather the mystery, as to the biological significance of aflatoxin, although the potential antioxidant mechanism of action of aflatoxin has yet to be fully elucidated. In addition, this explanation also provides for the potential role of ROS and oxylipins cross-kingdom communication between maize and *A. flavus*.

Cross-kingdom communication between plants and various fungi through the use of oxylipins has been previously documented (Eckardt, 2008; Christensen and Kolomiets, 2011). Specifically, the role of oxylipins has been clearly illustrated in the specific interaction between maize and *A. flavus* (Gao and Kolomiets, 2009). In a recent study, it was found that maize lipoxygenase-3 (LOX3) is required for resistance to *A. flavus* indicating that certain 9-oxylipins can play important roles in suppressing aflatoxin biosynthesis while other oxylipins may promote aflatoxin biosynthesis (Gao et al., 2009). The role of LOX-1 in resistance mechanisms against *A. flavus*, *A. nidulans,* and *A. parasiticus* has also been implied in soybean (Doehlert et al., 1993; Burow et al., 1997), although there are contradicting reports on the subject in the literature. Mellon and Cotty (2002) found that soybeans lacking LOX activity were just as resistant to *A. flavus* and aflatoxin contamination as those possessing LOX activity. This seems to imply some degree of specificity in the role of LOX enzymes or their products in resistance to certain *Aspergillus* species.

In addition to oxylipins, other host-derived compounds may influence oxidative stress including ROS and phytohormones. It was found that 2-chloroethyl phosphoric acid (CEPA), the metabolic precursor to ethylene, was capable of reducing the expression of *aflR* and *aflD* (two key genes in the aflatoxin biosynthetic pathway), reducing the accumulation of oxidative compounds, and regulating glutathione redox in *A. flavus* mycelia (Huang et al., 2009). Therefore, host-derived ethylene may result in the reduction of ROS accumulation in *A. flavus* mycelia and reduce aflatoxin biosynthesis. This hypothesis seems plausible since the expression of the maize Ethylene Responsive Factor 1 (*Zm*ERF1), a key transcription factor involved in ethylene and JA signaling, was found to be higher in the immature kernel tissues of the resistant maize inbred TZAR101 (Menkir et al., 2008) in comparison to the susceptible maize inbred B73 following *A. flavus* inoculation (Fountain et al., 2013).

Previous research has shown that maize varieties resistant to *A. flavus* tend to accumulate antioxidant enzymes, such as peroxidase and SOP, and tend to be more resistant to drought and heat stress than varieties susceptible to *A. flavus* (Guo

et al., 2008; Pechanova et al., 2011). Given the reported role of oxidative stress in *Aspergillus spp.* biology, it may be possible for host-derived antioxidant proteins, phytohormones, and oxylipins to negatively regulate the production of aflatoxin in infecting *A. flavus* by reducing the level of oxidative stress endured by both the host and the fungus, particularly during drought or heat stress. Such an interaction may explain several observed phenomena in the literature. For example, Guo et al. (1996, 1997) observed that the pre-incubation of maize kernels in high-humidity conditions for 3 days prior to inoculation with *A. flavus* results in a significant reduction in aflatoxin contamination in comparison with kernels inoculated without pre-incubation. Given the fact that ROS such as hydrogen peroxide accumulates to maximum quantities two days post-imbibition (DPI) followed by increased catalase activity beginning at three DPI in maize kernels (Hite et al., 1999), a combination of host-derived resistance and antioxidant proteins and reduced ROS production at the time of inoculation may have contributed to the reduction in aflatoxin production (Guo et al., 1996).

CONCLUSION

Determining the role of oxidative stress in the regulation of aflatoxin biosynthesis as well as the role of host defenses against both *A. flavus* infection and mycotoxin biosynthesis are critical areas of research for the mitigation of aflatoxin contamination in maize. Maize resistance to *A. flavus* is a complex, quantitative trait which is the culmination of the interaction of numerous resistance-associated proteins and antioxidant enzymes which have been the subject of more than 50 years of rigorous research. Solutions to the problem of aflatoxin contamination of crops, particularly maize, have been elusive given the high level of environmental influence on the interaction and the lack of stable resistance in maize germplasm across multiple environments. By better understanding the role of oxidative stress and its remediation by the host and the pathogen, additional tools will be made available to counter the threat aflatoxin poses to food safety and security and further enhance the knowledge of cross-kingdom interactions which may be applied to other mycotoxin producing pathogens in various agricultural commodities.

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REFERENCES

Affeldt, K. J., Brodhagen, M., and Keller, N. P. (2012). *Aspergillus oxylipin* signaling and quorum sensing pathways depend on G protein-coupled receptors. *Toxins* 4, 695–717. doi: 10.3390/toxins4090695

Amaike, S., and Keller, N. P. (2011). *Aspergillus flavus. Ann. Rev. Phytopathol.* 49, 107–133. doi: 10.1146/annurev-phyto-072910-095221

Asao, T., Büchi, G., Abdel-Kader, M., Chang, S., Wick, E. L., and Wogan, G. (1965). The structures of aflatoxins B1 and G1. *J. Am. Chem. Soc.* 87, 882–886. doi: 10.1021/ja01082a031

Bennett, J. W., and Klich, M. A. (2003). Mycotoxins. *Clin. Microbiol. Rev.* 16, 497–516. doi: 10.1128/CMR.16.3.497-516.2003

Bi, J., and Felton, G. (1995). Foliar oxidative stress and insect herbivory: primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. *J. Chem. Ecol.* 21, 1511–1530. doi: 10.1007/BF02035149

Brodhagen, M., and Keller, N. P. (2006). Signalling pathways connecting mycotoxin production and sporulation. *Mol. Plant Pathol.* 7, 285–301. doi: 10.1111/j.1364-3703.2006.00338.x

Brooks, T. D., Williams, W. P., Windham, G. L., Willcox, M. C., and Abbas, H. K. (2005). Quantitative trait loci contributing resistance to aflatoxin accumulation in the maize inbred Mp313E. *Crop Sci.* 45, 171–174. doi: 10.2135/cropsci2005.0171

Brown, R. L., Chen, Z. Y., Cleveland, T. E., Cotty, P. J., and Cary, J. W. (2001). Variation in in vitro alpha-amylase and protease activity is related to the virulence of *Aspergillus flavus* isolates. *J. Food Protect.* 64, 401–404.

Brown, R. L., Menkir, A., Chen, Z. Y., Bhatnagar, D., Yu, J., Yao, H., et al. (2013). Breeding aflatoxin resistant maize lines using recent advances in technologies – a review. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 30, 1382–1391. doi: 10.1080/19440049.2013.812808

Burow, G., Nesbitt, T., Dunlap, J., and Keller, N. (1997). Seed lipoxygenase products modulate *Aspergillus* mycotoxin biosynthesis. *Mol. Plant Microbe Interact.* 10, 380–387. doi: 10.1094/MPMI.1997.10.3.380

Chanda, A., Roze, L. V., Kang, S., Artymovich, K. A., Hicks, G. R., Raikhel, N. V., et al. (2009). A key role for vesicles in fungal secondary metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 106, 19533–19538. doi: 10.1073/pnas.0907416106

Chen, Z. Y., Brown, R. L., Cary, J. W., Damann, K. E., and Cleveland, T. E. (2009a). Characterization of an *Aspergillus flavus* alkaline protease and its role in the infection of maize kernels. *Toxin Rev.* 28, 187–197. doi: 10.1080/15569540903089221

Chen, Z. Y., Brown, R. L., Guo, B. Z., Menkir, A., and Cleveland, T. E. (2009b). Identifying aflatoxin resistance-related proteins/genes through proteomics and RNAi gene silencing. *Peanut Sci.* 36, 35–41. doi: 10.3146/AT07-005.1

Chen, Z. Y., Brown, R. L., Damann, K. E., and Cleveland, T. E. (2007). Identification of maize kernel endosperm proteins associated with resistance to aflatoxin contamination by *Aspergillus flavus. Phytopathology* 97, 1094–1103. doi: 10.1094/PHYTO-97-9-1094

Chen, Z. Y., Brown, R. L., Damann, K. E., and Cleveland, T. E. (2010). PR10 expression in maize and its effect on host resistance against *Aspergillus flavus* infection and aflatoxin production. *Mol. Plant Pathol.* 11, 69–81. doi: 10.1111/j.1364-3703.2009.00574.x

Chen, Z. Y., Brown, R. L., Lax, A. R., Guo, B. Z., Cleveland, T. E., and Russin, J. S. (1998). Resistance to *Aspergillus flavus* in corn kernels is associated with a 14-kDa protein. *Phytopathology* 88, 276–281. doi: 10.1094/PHYTO.1998.88.4.276

Chen, Z. Y., Brown, R. L., Rajasekaran, K., Damann, K. E., and Cleveland, T. E. (2006). Identification of a maize kernel pathogenesis-related protein and evidence for its involvement in resistance to *Aspergillus flavus* infection and aflatoxin production. *Phytopathology* 96, 87–95. doi: 10.1094/PHYTO-96-0087

Chen, Z. Y., Brown, R. L., Russin, J. S., Lax, A. R., and Cleveland, T. E. (1999). A corn trypsin inhibitor with antifungal act inhibits *Aspergillus flavus* alpha-amylase. *Phytopathology* 89, 902–907. doi: 10.1094/PHYTO.1999.89.10.902

Christensen, S. A., and Kolomiets, M. V. (2011). The lipid language of plant–fungal interactions. *Fungal Genet. Biol.* 48, 4–14. doi: 10.1016/j.fgb.2010.05.005

Cleveland, T. E., and Cotty, P. J. (1991). Invasiveness of *Aspergillus flavus* isolates in wounded cotton bolls is associated with production of a specific fungal polygalacturonase. *Phytopathology* 81, 155–158. doi: 10.1094/Phyto-81-155

Cleveland, T. E., Yu, J. J., Bhatnagar, D., Chen, Z. Y., Brown, R. L., Chang, P. K., et al. (2004). Progress in elucidating the molecular basis of the host plant – *Aspergillus flavus* interaction, a basis for devising strategies to reduce aflatoxin contamination in crops. *Toxin Rev.* 23, 345–380. doi: 10.1081/TXR-200027892

Das, S., DeMason, D. A., Ehlers, J. D., Close, T. J., and Roberts, P. A. (2008). Histological characterization of root-knot nematode resistance in cowpea and its relation to reactive oxygen species modulation. *J. Exp. Bot.* 59, 1305–1313. doi: 10.1093/jxb/ern036

Diener, U. L., Asquith, R. L., and Dickens, J. W. (1983). Aflatoxin and *Aspergillus flavus* in corn. *So. Coop. Ser. Bull.* 279, 112 pp.

Diener, U. L., Cole, R. J., Sanders, T. H., Payne, G. A., Lee, L. S., and Klich, M. A. (1987). Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annu. Rev. Phytopathol.* 25, 249–270. doi: 10.1146/annurev.py.25.090187.001341

Doehlert, D. C., Wicklow, D. T., and Gardner, H. W. (1993). Evidence implicating the lipoxygenase pathway in providing resistance to soybeans against *Aspergillus flavus*. *Phytopathology* 83, 1473–1477. doi: 10.1094/Phyto-83-1473

Earle, F. R., Curtis, J. J., and Hubbard, J. E. (1946). Composition of the component parts of the corn kernel. *Cereal Chem.* 23, 504–511.

Eckardt, N. A. (2008). Oxylipin signaling in plant stress responses. *Plant Cell* 20, 495–497. doi: 10.1105/tpc.108.059485

Eulgem, T., and Somssich, I. E. (2007). Networks of WRKY transcription factors in defense signaling. *Curr. Opin. Plant Biol.* 10, 366–371. doi: 10.1016/j.pbi.2007.04.020

Fabbri, A., Fanelli, C., Panfili, G., Passi, S., and Fasella, P. (1983). Lipoperoxidation and aflatoxin biosynthesis by *Aspergillus parasiticus* and *A. flavus*. *J. Gen. Microbiol.* 129, 3447–3452. doi: 10.1099/00221287-129-11-3447

Fabbri, A., Fanelli, C., and Serafini, M. (1980). Aflatoxin production on cereals, oil seeds and some organic fractions extracted from sunflower. *Rendiconti Accademia Nazionale delle Sciencze detta dei XL* 98, 219–228.

Fakhoury, A. M., and Woloshuk, C. P. (1999). Amy1, the alpha-amylase gene of *Aspergillus flavus*: involvement in aflatoxin biosynthesis in maize kernels. *Phytopathology* 89, 908–914. doi: 10.1094/PHYTO.1999.89.10.908

Fountain, J. C., Raruang, Y., Luo, M., Brown, R. L., and Chen, Z. Y. (2013). Maize WRKY transcription factors and their potential roles in regulating defense gene expression during *Aspergillus flavus* infection. *Phytopathology* 103(Suppl. 1), S1.4.

Gao, X., Brodhagen, M., Isakeit, T., Brown, S. H., Göbel, C., Betran, J., et al. (2009). Inactivation of the lipoxygenase ZmLOX3 increases susceptibility of maize to *Aspergillus* spp. *Mol. Plant Microbe Interact.* 22, 222–231. doi: 10.1094/MPMI-22-2-0222

Gao, X., and Kolomiets, M. V. (2009). Host-derived lipids and oxylipins are crucial signals in modulating mycotoxin production by fungi. *Toxin Rev.* 28, 79–88. doi: 10.1080/15569540802420584

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Grice, C., Bertuzzi, M., and Bignell, E. (2013). Receptor-mediated signaling in *Aspergillus fumigatus*. *Front. Microbiol.* 4:26. doi: 10.3389/fmicb.2013.00026

Guo, B. Z., Chen, Z. Y., Brown, R. L., Lax, A. R., Cleveland, T. E., Russin, J. S., et al. (1997). Germination induces accumulation of specific proteins and antifungal activities in corn kernels. *Phytopathology* 87, 1174–1178. doi: 10.1094/PHYTO.1997.87.11.1174

Guo, B. Z., Chen, Z. Y., Lee, R. D., and Scully, B. T. (2008). Drought stress and preharvest aflatoxin contamination in agricultural commodity: genetics, genomics and proteomics. *J. Int. Plant Biol.* 50, 1281–1291. doi: 10.1111/j.1744-7909.2008.00739.x

Guo, B. Z., Russin, J. S., Brown, R. L., Cleveland, T. E., and Widstrom, N. W. (1996). Resistance to aflatoxin contamination in corn as influenced by relative humidity and kernel germination. *J. Food Protect.* 59, 276–281.

Guo, B. Z., Russin, J. S., Cleveland, T. E., Brown, R. L., and Widstrom, N. W. (1995). Wax and cutin layers in maize kernels associated with resistance to aflatoxin production by *Aspergillus flavus*. *J. Food Protect.* 58, 296–300.

Hite, D. R., Auh, C., and Scandalios, J. G. (1999). Catalase activity and hydrogen peroxide levels are inversely correlated in maize scutella during seed germination. *Redox Rep.* 4, 1–2. doi: 10.1179/135100099101534710

Hong, S. Y., Roze, L. V., Wee, J., and Linz, J. E. (2013). Evidence that a transcription factor regulatory network coordinates oxidative stress response and secondary metabolism in Aspergilli. *MicrobiologyOpen* 2, 144–160. doi: 10.1002/mbo3.63

Horn, B. W., Moore, G. G., and Carbone, I. (2009a). Sexual reproduction in *Aspergillus flavus*. *Mycologia* 101, 423–429. doi: 10.3852/09-011

Horn, B. W., Ramirez-Prado, J. H., and Carbone, I. (2009b). The sexual state of *Aspergillus parasiticus*. *Mycologia* 101, 275–280. doi: 10.3852/08-205

Huang, J.-Q., Jiang, H.-F., Zhou, Y.-Q., Lei, Y., Wang, S.-Y., and Liao, B.-S. (2009). Ethylene inhibited aflatoxin biosynthesis is due to oxidative stress alleviation and related to glutathione redox state changes in *Aspergillus flavus*. *Int. J. Food Microbiol.* 130, 17–21. doi: 10.1016/j.ijfoodmicro.2008.12.027

Huynh, Q. K., Hironaka, C. M., Levine, E. B., Smith, C. E., Borgmeyer, J. R., and Shah, D. M. (1992). Antifungal proteins from plants – purification, molecular cloning, and antifungal properties of chitinases from maize seed. *J. Biol.Chem.* 267, 6635–6640.

Jayashree, T., and Subramanyam, C. (2000). Oxidative stress as a prerequisite for aflatoxin production by *Aspergillus parasiticus*. *Free Rad. Biol. Med.* 29, 981–985. doi: 10.1016/S0891-5849(00)00398-1

Jiang, T., Fountain, J. C., Davis, G., Kemerait, R., Scully, B. T., Lee, R. D., et al. (2012). Root morphology and gene expression analysis in response to drought stress in maize (*Zea mays*). *Plant Mol. Biol. Rep.* 30, 360–369. doi: 10.1007/s11105-011-0347-9

Kaloshian, I., and Walling, L. L. (2005). Hemipterans as plant pathogens. *Annu. Rev. Phytopathol.* 43, 491–521. doi: 10.1146/annurev.phyto.43.040204.135944

Keen, N. T. (1990). Gene-for-gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* 24, 447–463. doi: 10.1146/annurev.ge.24.120190.002311

Kelley, R. Y., Williams, W. P., Mylroie, J. E., Boykin, D. L., Harper, J. W., Windham, G. L., et al. (2012). Identification of maize genes associated with host plant resistance or susceptibility to *Aspergillus flavus* infection and aflatoxin accumulation. *PLoS ONE* 7:e36892. doi: 10.1371/journal.pone.0036892

Kessler, A., and Baldwin, I. T. (2002). Plant responses to insect herbivory: the emerging molecular analysis. *Annu. Rev. Plant Biol.* 53, 299–328. doi: 10.1146/annurev.arplant.53.100301.135207

Li, H., Gao, Y., Xu, H., Dai, Y., Deng, D., and Chen, J. (2013). ZmWRKY33, a WRKY maize transcription factor conferring enhanced salt stress tolerances in Arabidopsis. *Plant Growth Reg.* 70, 207–216. doi: 10.1007/s10725-013-9792-9

Liang, X. Q., Holbrook, C. C., Lynch, R. E., and Guo, B. Z. (2005). β-1,3-glucanase activity in peanut seed (*Arachis hypogaea*) is induced by inoculation with *Aspergillus flavus* and copurifies with a conglutin-like protein. *Phytopathology* 95, 506–511. doi: 10.1094/PHYTO-95-0506

Lozovaya, V. V., Waranyuwat, A., and Widholm, J. M. (1998). β-l,3-glucanase and resistance to *Aspergillus flavus* infection in maize. *Crop Sci.* 38, 1255–1260. doi: 10.2135/cropsci1998.0011183X003800050024x

Luo, M., Liu, J., Lee, R., and Guo, B. Z. (2008). Characterization of gene expression profiles in developing kernels of maize (*Zea mays*) inbred Tex6. *Plant Breed.* 127, 569–578. doi: 10.1111/j.1439-0523.2008.01538.x

Magbanua, Z. V., De Moraes, C. M., Brooks, T. D., Williams, W. P., and Luthe, D. S. (2007). Is catalase activity one of the factors associated with maize resistance to *Aspergillus flavus*? *Mol. Plant Microbe Interact.* 20, 697–706. doi: 10.1094/MPMI-20-6-0697

Mauch, F., Mauchmani, B., and Boller, T. (1988). Antifungal hydrolases in pea tissue: 2. Inhibition of fungal growth by combinations of chitinase and beta-1,3-glucanase. *Plant Physiol.* 88, 936–942. doi: 10.1104/pp.88.3.936

Mellon, J., and Cotty, P. (2002). No effect of soybean lipoxygenase on aflatoxin production in *Aspergillus flavus*-inoculated seeds. *J. Food Protect.* 65, 1984–1987.

Mellon, J. E., Cotty, P. J., and Dowd, M. K. (2000). Influence of lipids with and without other cottonseed reserve materials on aflatoxin B1 production by *Aspergillus flavus*. *J. Agric. Food Chem.* 48, 3611–3615. doi: 10.1021/jf0000878

Menkir, A., Brown, R. L., Bandyopadhyay, R., and Cleveland, T. E. (2008). Registration of six tropical maize germplasm lines with resistance to aflatoxin contamination. *J. Plant Reg.* 2, 246–250. doi: 10.3198/jpr2008.01.0028crg

Miao, Y., Laun, T., Zimmermann, P., and Zentgraf, U. (2004). Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis. *Plant Mol. Biol.* 55, 853–867. doi: 10.1007/s11103-004-2142-6

Miao, Y., Laun, T. M., Smykowski, A., and Zentgraf, U. (2007). Arabidopsis MEKK1 can take a short cut: it can directly interact with senescence-related WRKY53 transcription factor on the protein level and can bind to its promoter. *Plant Mol. Biol.* 65, 63–76. doi: 10.1007/s11103-007-9198-z

Mideros, S. X., Windham, G. L., Williams, W. P., and Nelson, R. J. (2009). *Aspergillus flavus* biomass in maize estimated by quantitative real-time polymerase chain reaction is strongly correlated with aflatoxin concentration. *Plant Dis.* 93, 1163–1170. doi: 10.1094/PDIS-93-11-1163

Ni, X., Quisenberry, S. S., Heng-Moss, T., Markwell, J., Sarath, G., Klucas, R., et al. (2001a). Oxidative responses of resistant and susceptible cereal leaves to symptomatic and nonsymptomatic cereal aphid (Hemiptera: Aphididae) feeding. *J. Econ. Entomol.* 94, 743–751. doi: 10.1603/0022-0493-94.3.743

Ni, X., Quisenberry, S. S., Markwell, J., Heng-Moss, T., Higley, L., Baxendale, F., et al. (2001b). In vitro enzymatic chlorophyll catabolism in wheat elicited by

cereal aphid feeding. *Entomol. Exp. Appl.* 101, 159–166. doi: 10.1046/j.1570-7458.2001.00900.x

Ni, X., Quisenberry, S. S., Pornkulwat, S., Figarola, J. L., Skoda, S. R., and Foster, J. E. (2000). Hydrolase and oxido-reductase activities in Diuraphis noxia and Rhopalosiphum padi (Hemiptera: Aphididae). *Ann. Entomol. Soc. Am.* 93, 595–601. doi: 10.1603/0013-8746(2000)093[0595:HAORAI]2.0.CO;2

Paul, C., Naidoo, G., Forbes, A., Mikkilineni, V., White, D., and Rocheford, T. (2003). Quantitative trait loci for low aflatoxin production in two related maize populations. *Theor. Appl. Genet.* 107, 263–270. doi: 10.1007/s00122-003-1241-0

Pechanova, O., Pechan, T., Rodriguez, J. M., Williams, W. P., and Brown, A. E. (2013). A two-dimensional proteome map of the aflatoxigenic fungus *Aspergillus flavus*. *Proteomics* 13, 1513–1518. doi: 10.1002/pmic.201100659

Pechanova, O., Pechan, T., Williams, W. P., and Luthe, D. S. (2011). Proteomic analysis of the maize rachis: potential roles of constitutive and induced proteins in resistance to *Aspergillus flavus* infection and aflatoxin accumulation. *Proteomics* 11, 114–127. doi: 10.1002/pmic.201000368

Reverberi, M., Punelli, M., Smith, C. A., Zjalic, S., Scarpari, M., Scala, V., et al. (2012). How peroxisomes affect aflatoxin biosynthesis in *Aspergillus flavus*. *PLoS ONE* 7:e48097. doi: 10.1371/journal.pone.0048097

Reverberi, M., Ricelli, A., Zjalic, S., Fabbri, A. A., and Fanelli, C. (2010). Natural functions of mycotoxins and control of their biosynthesis in fungi. *Appl. Microbiol. Biotech.* 87, 899–911. doi: 10.1007/s00253-010-2657-5

Reverberi, M., Zjalic, S., Ricelli, A., Punelli, F., Camera, E., Fabbri, C., et al. (2008). Modulation of antioxidant defense in *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for the ApyapA gene. *Eukaryot. Cell* 7, 988–1000. doi: 10.1128/EC.00228-07

Roze, L. V., Hong, S.-Y., and Linz, J. E. (2013). Aflatoxin biosynthesis: current frontiers. *Annu. Rev. Food Sci. Tech.* 4, 293–311. doi: 10.1146/annurev-food-083012-123702

Rushton, P. J., Somssich, I. E., Ringler, P., and Shen, Q. J. (2010). WRKY transcription factors. *Trends Plant Sci.* 15, 247–258. doi: 10.1016/j.tplants.2010.02.006

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Addit. Contam. Part A Chem. Anal. Control. Expo. Risk Assess.* 25, 146–151. doi: 10.1080/02652030701567442

Smart, M. G., Wicklow, D. T., and Caldwell, R. W. (1990). Pathogenesis in *Aspergillus* ear rot of maize – light microscopy of fungal spread from wounds. *Phytopathology* 80, 1287–1294. doi: 10.1094/Phyto-80-1287

Trenk, H. L., and Hartman, P. A. (1970). Effects of moisture content and temperature on aflatoxin production in corn. *Appl. Microbiol.* 19, 781–784.

Walling, L. L. (2000). The myriad plant responses to herbivores. *J. Plant Growth Reg.* 19, 195–216. doi: 10.1007/s003440000026

Walsh, T. A., Morgan, A. E., and Hey, T. D. (1991). Characterization and molecular cloning of a proenzyme form of a ribosome-inactivating protein from maize: novel mechanism of proenzyme activation by proteolytic removal of a 2.8 kilodalton internal peptide segment. *J. Biol. Chem.* 266, 23422–23427.

Willcox, M. C., Davis, G. L., Warburton, M. L., Windham, G. L., Abbas, H. K., Betrán, J., et al. (2013). Confirming quantitative trait loci for aflatoxin resistance from Mp313E in different genetic backgrounds. *Mol. Breed.* 32, 15–26. doi: 10.1007/s11032-012-9821-9

Wogan, G. N. (1966). Chemical nature and biological effects of the aflatoxins. *Bacteriol. Rev.* 30, 460.

Xie, Y.-R., Chen, Z. Y., Brown, R. L., and Bhatnagar, D. (2010). Expression and functional characterization of two pathogenesis-related protein 10 genes from *Zea mays*. *J. Plant Physiol.* 167, 121–130. doi: 10.1016/j.jplph.2009.07.004

Yu, J., Chang, P.-K., Ehrlich, K. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E., et al. (2004). Clustered pathway genes in aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 70, 1253–1262. doi: 10.1128/AEM.70.3.1253-1262.2004

Zavala, J. A., Nabity, P. D., and Delucia, E. H. (2013). An emerging understanding of mechanisms governing insect herbivory under elevated CO2. *Annu. Rev. Entomol.* 58, 79–97. doi: 10.1146/annurev-ento-120811-153544

Zuber, M., and Lillehoj, E. (1979). Status of the aflatoxin problem in corn. *J. Environ. Qual.* 8, 1–5. doi: 10.2134/jeq1979.00472425000800010001x

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Co-inoculation of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus* to study fungal invasion, colonization, and competition in maize kernels

Zuzana Hruska[1], Kanniah Rajasekaran[2]*, Haibo Yao[1], Russell Kincaid[1], Dawn Darlington[1], Robert L. Brown[2], Deepak Bhatnagar[2] and Thomas E. Cleveland[2]

[1] *Geosystems Research Institute, Mississippi State University, Stennis Space Center, Starkville, MS, USA*
[2] *Southern Regional Research Center, Agricultural Research Service – United States Department of Agriculture, New Orleans, LA, USA*

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Geromy G. Moore, Agricultural Research Service – United States Department of Agriculture, USA
Fuguo Xing, Institute of Agro-Products Processing Science and Technology – Chinese Academy of Agricultural Science, China
Hamed Abbas, Agricultural Research Service – United States Department of Agriculture, USA

****Correspondence:***
Kanniah Rajasekaran, Southern Regional Research Center, Agricultural Research Service – United States Department of Agriculture, 1100 Robert E. Lee Boulevard, New Orleans, LA 70124, USA
e-mail: rajah.rajasekaran@ars.usda.gov

A currently utilized pre-harvest biocontrol method involves field inoculations with non-aflatoxigenic *Aspergillus flavus* strains, a tactic shown to strategically suppress native aflatoxin-producing strains and effectively decrease aflatoxin contamination in corn. The present *in situ* study focuses on tracking the invasion and colonization of an aflatoxigenic *A. flavus* strain (AF70), labeled with green fluorescent protein (GFP), in the presence of a non-aflatoxigenic *A. flavus* biocontrol strain (AF36), to better understand the competitive interaction between these two strains in seed tissue of corn (*Zea mays*). Corn kernels that had been co-inoculated with GFP-labeled AF70 and wild-type AF36 were cross-sectioned and observed under UV and blue light to determine the outcome of competition between these strains. After imaging, all kernels were analyzed for aflatoxin levels. There appeared to be a population difference between the co-inoculated AF70-GFP+AF36 and the individual AF70-GFP tests, both visually and with pixel count analysis. The GFP allowed us to observe that AF70-GFP inside the kernels was suppressed up to 82% when co-inoculated with AF36 indicating that AF36 inhibited progression of AF70-GFP. This was in agreement with images taken of whole kernels where AF36 exhibited a more robust external growth compared to AF70-GFP. The suppressed growth of AF70-GFP was reflected in a corresponding (upto 73%) suppression in aflatoxin levels. Our results indicate that the decrease in aflatoxin production correlated with population depression of the aflatoxigenic fungus by the biocontrol strain supporting the theory of competitive exclusion through robust propagation and fast colonization by the non-aflatoxigenic fungus.

Keywords: *Aspergillus flavus*, biocontrol, green fluorescent protein (GFP), fungal competition, aflatoxin, fluorescence microscopy, corn (*Zea mays*)

INTRODUCTION

The opportunistic, soil-borne fungus *Aspergillus flavus* is the main producer of aflatoxin, a mycotoxin known for its carcinogenic properties and other deleterious effects on health in both human and animal populations. Aflatoxins have been found in many agricultural commodities including corn, cottonseed, groundnut, and tree nuts (CAST, 2003), and contamination can occur at any time point between pre- and post- harvest; specifically, plant growth through crop storage. Aflatoxin affects food production and international commerce, prompting enforcement of regulations regarding acceptable aflatoxin limits in many countries around the world [Food and Agriculture Organization (FAO), 2004].

Different pre- and post-harvest strategies have been tested for the prevention and control of aflatoxin in order to protect the integrity of corn. Pre-harvest strategies include traditional and molecular breeding approaches to develop resistant germplasm (Rajasekaran et al., 2006; Cary et al., 2009), and biological control by utilizing various microorganisms including bacteria, yeasts, and non-aflatoxigenic fungi (Yin et al., 2008). The most promising pre-harvest biocontrol method involves field inoculation with non-aflatoxigenic *A. flavus*, a strategy that has proven successful at reducing aflatoxin contamination in the field (Cotty, 1994, 2001; Dorner, 2009a,b). Two bio-pesticides, AF36 and Afla-Guard®, have been approved by the Environmental Protection Agency (EPA) for pre-harvest application to control aflatoxin contamination in the U.S. (Moore et al., 2013). Neither AF36 (NRRL 18543) nor the component strain in Afla-Guard® (NRRL 21882) can produce aflatoxin due to genetic mutations in, or lack of, critical genes along the aflatoxin synthesizing pathway, respectively, (Ehrlich and Cotty, 2004; Chang et al., 2005; Moore et al., 2009). This method of control has been effectively tested on several commodities including cottonseed (Cotty, 1994, 2001), corn, (Brown et al., 1991; Dorner, 2009a), and peanuts (Dorner, 2009b). The suppression of measurable aflatoxin has been attributed to competitive exclusion of aflatoxigenic strains by non-aflatoxigenic strains; however, the exact mechanism of suppression has yet to be elucidated. Exclusion of the aflatoxin producing strain may be achieved by a more robust propagation and faster growth of the competing non-aflatoxigenic fungal strain (Chang and Hua, 2007). Alternatively, the inhibition of aflatoxin contamination/production could be due to a close physical proximity between the competing strains

which may initiate a signal to down-regulate or inhibit synthesis of aflatoxin in the aflatoxigenic strain by the non-aflatoxigenic strain (Huang et al., 2011).

Dispersion of conidial inocula in the corn field is presumed to occur through a direct transfer by insects, or via colonization of silks, and subsequent movement toward the developing kernels (Payne, 1998; Mehl and Cotty, 2011). Entry into individual kernels is thought to occur primarily through the pedicel (tip cap) of intact kernels (Lillehoj, 1983; Rajasekaran et al., 2013). However, it is difficult to determine the mode of action taken by the competing fungal strains, particularly during the infection and colonization process inside individual kernels.

The use of fungal strains that express fluorescent proteins in the cytoplasm greatly enhances the potential for visual tracking of biological processes without markedly affecting fungal growth or pathogenicity (Rajasekaran et al., 2008; Crespo-Sempere et al., 2011). Enhanced forms of the green fluorescent protein (eGFP) were recently developed to tag *A. flavus* for study of fungal growth, mode of entry and colonization of cottonseeds (Rajasekaran et al., 2008), to track the progress of fungal infection within developing corn ears (Magbanua et al., 2013), to study infection, fungal growth, colonization, and aflatoxin production in intact corn kernels (Rajasekaran et al., 2013), and also to tag *A. carbonarius* for monitoring fungal colonization in grapes (Crespo-Sempere et al., 2011).

To better understand the mechanisms involved during competition between co-inoculated *A. flavus* isolates in corn, the current *in situ* study focuses on tracking the invasion and colonization by aflatoxigenic AF70, labeled with GFP, in the presence of the AF36 biocontrol strain.

MATERIALS AND METHODS

FUNGAL STRAINS AND PREPARATION OF INOCULA

Two *A. flavus* strains were used as competitors. Toxin producing AF70 labeled with GFP (AF70-GFP; Rajasekaran et al., 2008), and a non-aflatoxigenic strain of *A. flavus* (AF36) fungal cultures were obtained from the SRRC fungal collection (ARS-USDA, New Orleans, LA, USA), and grown on potato dextrose agar (PDA) media for 7 days at 30°C. Bright green fluorescence was observed in the AF70-GFP culture from both the conidiophores and the mycelia. Harvest of conidia was accomplished by flooding a single culture for each strain with 20 ml of 0.01% (v/v) sterile Triton X-100 solution and scraping the surface mycelia with a sterile scraper. Conidial suspensions were adjusted to 4×10^6 cells/ml using sterile distilled water. The inocula were kept in separate containers at 4°C. Immediately before use, 50 ml of each inoculum, at 1:1 ratio, was combined to make up the AF70-GFP+AF36 co-inoculation mixture.

IN SITU INOCULATION

Corn kernels (N78B-GT, Syngenta NK Brand Seeds, Laurinburg, NC, USA), collected in 2010 from the ARS Field Station in Stoneville, MS, USA, were utilized in all experiments. Whole, undamaged kernels of roughly uniform size were randomly assigned into four treatment groups and processed according to a modified kernel screening assay (KSA; Brown et al., 1995). All kernels were surface sterilized in 70% ethanol and rinsed in dH_2O. Ten kernels per treatment per day were inoculated by immersion and stirred for 1 min. The treatments included:(1) kernels co-inoculated with AF70-GFP+AF36, (2) kernels inoculated with AF70-GFP only, (3) kernels inoculated with AF36 only, and (4) kernels inoculated only with dH_2O as control for non-specific fluorescence, and/or inherent fungal contamination. Each group of kernels was incubated in a humidity chamber using a plastic tray with individual compartments (**Figure 1**). Kernels were incubated at 30°C and examined at several time points (3, 4, 5, 7, and 9 days) after inoculation.

GFP imaging and analysis

At each specified time interval, kernels were removed from the incubator, wiped free of visible exterior mold growth, sliced longitudinally, and mounted on a slide with double sided tape. A total of 20 kernel cross-sections per treatment per day were analyzed for GFP fluorescence. Images were taken in the dark with an Olympus SZH10 GFP stereomicroscope (Olympus, Center Valley, PA, USA) equipped with 480 nm excitation and 535 nm emission filters for GFP fluorescence, and also with UV filters. Digital images were acquired with a Nikon Digital Camera DXM1200 (Nikon Instruments, Melville, NY, USA). Sectioned samples were then processed for aflatoxin analysis.

In order to determine the extent of suppressed expression of the GFP signal from the AF70-GFP by AF36 in the co-inoculated corn kernels, as compared with those kernels inoculated with AF70-GFP alone, all GFP-expressing images were analyzed with pixel count analysis in ENVI (ENVI software v 4.7, ITT Exelis, Boulder,

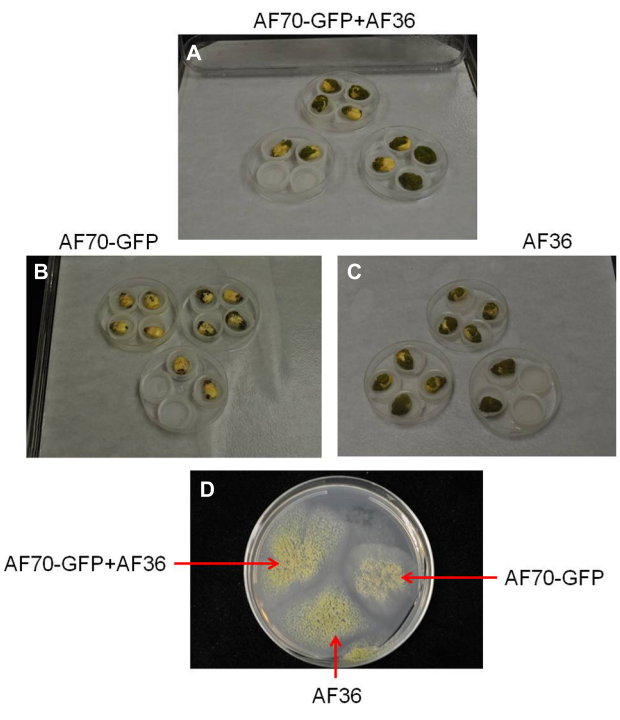

FIGURE 1 | Images of *A. flavus* fungal isolates AF70-GFP and AF36 in a mixture (A) or grown separately (B,C) on inoculated corn kernels and in culture (D); compared on the seventh day of growth.

CO, USA). Pixel count analysis has been employed previously as a useful tool for quantifying fluorescence signals in digital images (Waters, 2009). Each 8-bit image contained 3200 x 2560 pixels with brightness values ranging from 0–255. In order to quantify the number of pixels exhibiting fluorescence within each image, a region of interest (ROI) was created by utilizing a band thresholding process. A threshold brightness value of 20 was selected to separate the pixels exhibiting fluorescence (>20) from the background (<20) and minimize non-specific background fluorescence. Regions of interest were drawn around all pixels exhibiting values above the set threshold, and totals for each cross section of each sample were recorded for statistical analysis.

AFLATOXIN ANALYSIS

Imaged kernels were dried at 60°C for 2 days and analyzed for aflatoxin with the VICAM AflaTest assay (VICAM, Milford, MA, USA). Dried kernels were processed following the single kernel assay protocol of Yao et al. (2010), modified from the VICAM AflaTest instruction manual for corn, milo, grains, and feeds. Briefly, kernels from each day were first uniformly, semi-coarsely ground in a coffee grinder (KitchenAid® BCG100). Next, aflatoxin B from each ground sample was extracted with sodium chloride (NaCl) in methanol:water (80:20 v/v) maintaining the proportion of 1 g of sample plus 0.1 g NaCl in 2 ml of methanol:water, and filtered through a fluted filter. Filtered extract was diluted 1:5 with ultrapure water; 2 ml of each sample were passed through a glass filter and pushed through the high affinity AflaTest column. Samples were washed with ultrapure water, eluted with pure methanol (HPLC grade), and fluorescence was measured using the VICAM Series-4EX fluorometer (VICAM, Milford, MA, USA). Raw data values were expressed as ppb or μg/kg equivalents.

STATISTICAL ANALYSES

Pixel counts from the images, as well as the aflatoxin concentrations from the aflatoxin extractions, were analyzed in a two-way ANOVA with repeated measures using the Excel Data Analysis package (Excel 2007, Microsoft Office, Microsoft). Graphs were created in the R statistical program (R statistics software, http://www.r-project.org).

RESULTS

GFP IMAGING AND POPULATION (PIXEL) COUNT

On intact corn kernels the observed external fungal growth was predominantly AF36 for the co-inoculated groups; in some instances AF36 completely covered the pericarp (**Figure 1**). Internally, the spread of AF70-GFP was evident by the presence of fluorescence (**Figure 2**). In corn kernels inoculated solely with AF70-GFP, the fungus spread unimpeded, filling the whole kernel from the basal transfer layer through the embryo and into the endosperm, eventually compromising the kernel's integrity. Although the point of entry was predominantly at the pedicel, it was not unusual to find GFP fluorescence in other areas of a kernel

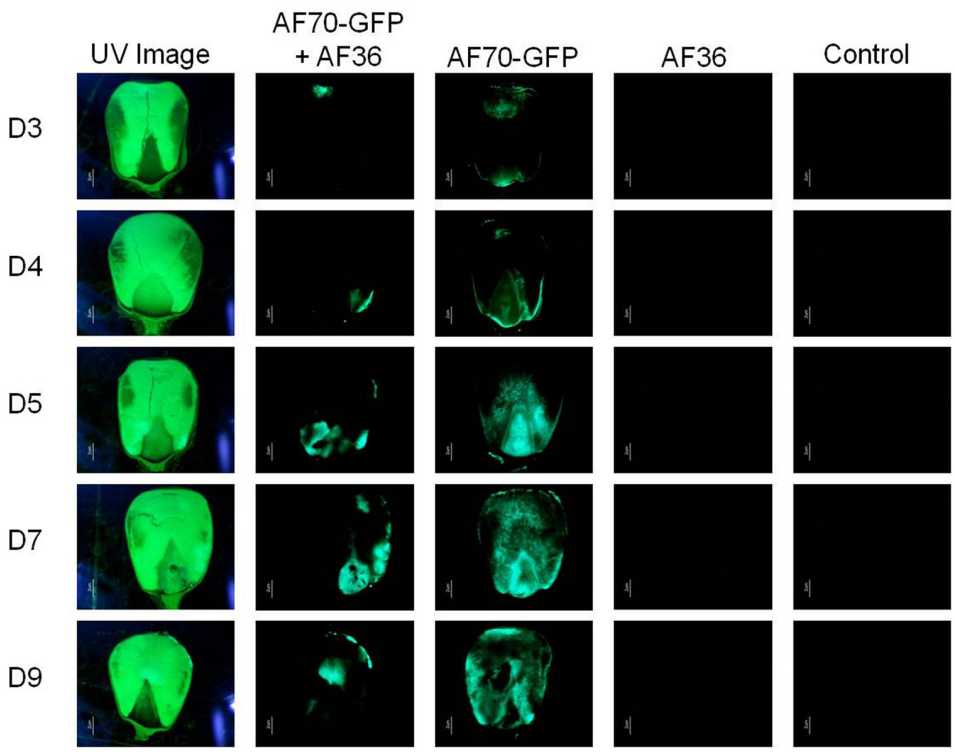

FIGURE 2 | Fluorescence images representing inoculation treatments observed using UV light (first column) and using a GFP filter (columns two through five) for each day of examination (day 3–day 9; D3–D9). Columns one and two show co-inoculated kernels of AF70-GFP+AF36; columns three through five shows kernels inoculated with AF70-GFP alone, AF36 alone, and a negative control using sterile water, respectively.

appearing first. The co-inoculated kernels exhibited a radically different fungal spread (**Figure 2**). The AF70-GFP growth appeared limited to the edges of the corn kernel, presumably outcompeted by AF36. For kernels inoculated with AF36 alone, or with the sterile water control, there was no GFP fluorescence observed; therefore, they were not included in the pixel count analysis. No GFP fluorescence could be observed externally on co-inoculated kernels which supports the premise that AF36 predominated.

Statistical analysis of the fluorescent pixels occupied by AF70-GFP is presented in **Figure 3**. Data was analyzed in a two way ANOVA using each treatment (AF70-GFP+AF36 and AF70-GFP) with repeated measures of day (post-inoculation). There was a significant main effect of treatment with $p < 0.001$, and a statistically significant interaction of treatment by day $p < 0.001$, $\alpha = 0.01$. These results indicate that there was a significant difference between co-inoculated kernels and those treated with AF70-GFP alone, and this difference changed over time. Suppression of AF70 by AF36 is presented in terms of percentages in **Figure 4**. The peak difference between the two groups appeared to be on day nine whereby the highest suppression of AF70-GFP by AF36 was observed to be 82%. Average suppression over the duration of the study was 66%.

AFLATOXIN CONTENT

Similar to the population count, the aflatoxin data was analyzed in a two way factorial design of each treatment (AF70-GFP+AF36 and AF70-GFP) with repeated measures of day (**Figure 5**). A significant main effect of treatment with $p < 0.001$, and a statistically significant interaction of treatment by day $p < 0.001$ with $\alpha = 0.01$ were revealed, indicating a significant difference in aflatoxin concentration between the co-inoculated kernels and those treated with AF70-GFP alone that changed over time. The peak difference between the two groups appeared to be on day five (73%), dipped by day seven (25%), and recovered to a lesser degree on day nine (47%). The observed suppression of AF70-GFP by AF36 presented in **Figure 4** is also observed in the aflatoxin content data presented in **Figure 5**. Maximum decrease in aflatoxin production was determined by calculating the percent difference between means on a particular day and was 73% on day five.

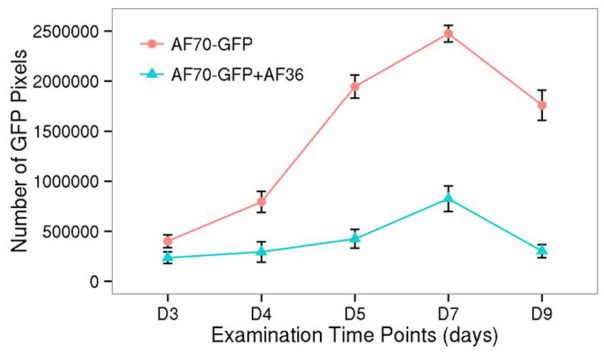

FIGURE 3 | GFP fluorescence at each time point (day 3–day 9 after inoculation), collected as pixel counts, from images of corn kernels inoculated with AF70-GFP+AF36 or the AF70-GFP isolate alone. The AF70-GFP+AF36 mixture is represented by the green line and AF70-GFP alone is represented by the red line. There was a significant treatment effect $p < 0.001$, and a statistically significant interaction of treatment by day $p < 0.001$, $\alpha = 0.01$.

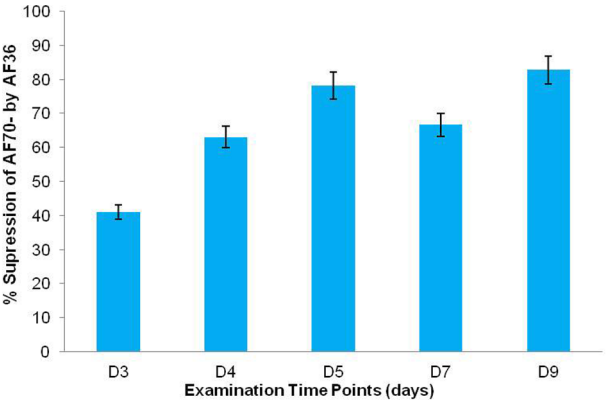

FIGURE 4 | Percent (%) growth suppression of AF70-GFP by AF36 in co-inoculated corn kernels as determined by calculating the percent difference between treatment means on a particular day. Over 5% difference in suppression was observed between consecutive time points (day 3–day 9).

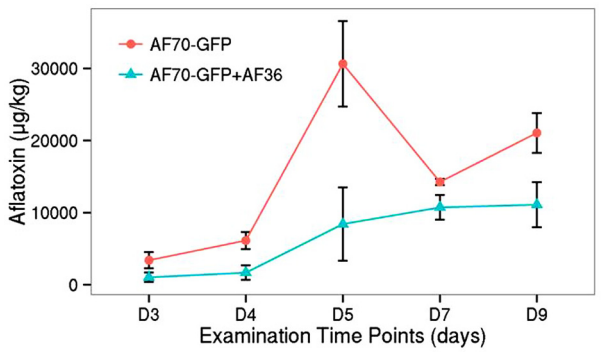

FIGURE 5 | Aflatoxin concentration for co-inoculated corn kernels treated with AF70-GFP+AF36 (green) or AF70-GFP (red) alone from day 3 to day 9 after inoculation. There was a significant treatment effect $p < 0.001$, and a statistically significant interaction of treatment by day $p < 0.001$ with $\alpha = 0.01$.

DISCUSSION

The current study used an *A. flavus* strain, expressing the GFP gene, to investigate its invasion and colonization potential in the presence of a non-aflatoxigenic biocontrol strain in single corn kernels. Although all co-inoculated corn kernels exhibited growth on day three, external fungal growth over the entire growth period was predominantly attributed to AF36, confirmed by lack of fluorescence combined with aggressive growth. Furthermore, the fluorescence was very specific to AF70-GFP and was not present externally or internally in the AF36 controls. It appears the competitive edge exhibited by AF36 may be in its propensity for rapid growth and aggressive colonization of the host compared to AF70-GFP (**Figure 1**). Aflatoxin production requires expending energy in the form of ATP (Ehrlich et al., 2011), and perhaps this affects a

toxigenic strain's ability to rapidly colonize, since it may be diverting energy toward the secondary metabolism pathway. Because this does not appear to be an effective competitive strategy targeted against non-aflatoxigenic strains, it appears that aflatoxigenic and non-aflatoxigenic strains are not in competition with each other, under natural conditions, and may coexist without interfering with each other's life cycles unless their natural proportions are disturbed, for example, by inundating fields with excess biocontrol (Yin et al., 2008).

Although the initial entry of the fungus through the pedicel agrees with previous research (Rajasekaran et al., 2013), there were instances where the GFP fluorescence initially appeared in other areas of the corn kernel, particularly when AF70-GFP and AF36 were co-inoculated. The AF70-GFP fluorescence in the co-inoculated kernels was significantly reduced and limited to the edges of the kernels, presumably because of the more aggressive growth of the AF36 biocontrol strain. Entry points were randomly distributed along the hull, possibly created by peripheral damage to the pericarp by the robust growth of AF36 where the extensive hyphal growth may have weakened the external kernel tissue. Alternately, invisible weak points in the external tissue may have been created during kernel harvest. Greatest population difference observed for GFP fluorescence between the AF70-GFP and AF70-GFP+AF36 treatments are in agreement with the literature, whereby population, aflatoxin production was decreased by aflatoxin non-producers between 70 and 90% (Brown et al., 1991). The average suppression of aflatoxin production in the current study was 50% compared to results reported in previous studies involving cotton (Cotty, 1990, 1994). The discrepancy may be attributed to the shorter duration and a smaller sample size of the laboratory experiment, compared to field experiments. The fact that AF36 was first isolated from cotton and may have a host preference could be another reason for the difference. The timing of the inoculation may have contributed to the reduced inhibition of aflatoxin production observed in this study. Greater reduction (80–90%) of aflatoxin concentration in corn (Brown et al., 1991), and cottonseed (Cotty, 1990), was shown when the non-aflatoxigenic fungal strain was introduced 24 h prior to the aflatoxin producing strain rather than simultaneously. However, this may not be a reflection of what is actually happening in the field. Studies that explore pre-inoculation with aflatoxigenic strains would better demonstrate the field environment.

The observed population differences between the co-inoculated and the sole AF70-GFP treatments support the premise that growth suppression of AF70-GFP is due to competitive exclusion by AF36 (Brown et al., 1991; Mehl and Cotty, 2010). The exclusion could be due to the initial acquisition of nutrients or tissue space by the biocontrol strain, or something unknown that the fungus requires for survival in a particular situation (Mehl and Cotty, 2011, 2013). Since spore concentrations for inocula in our competitive study were approximately the same, faster growth, and a more robust propagation of AF36 effectively excludes the aflatoxin producing strain and consequently decreases measurable aflatoxin levels. An additional possibility for decrease in aflatoxin production is presented by the presence of specific volatiles. Aflatoxigenic fungi reportedly produce volatile compounds that differ from non-aflatoxigenic fungi (De Lucca et al., 2012). Certain terpenes found in essential oils, such as alpha-pinene have known antifungal properties (Moghtader et al., 2011) and have been discovered among the volatile compounds produced by some non-aflatoxigenic *A. flavus* isolates (De Lucca et al., 2012). Furans are another class of anti-microbial volatiles produced by *A. flavus*; however, in non-aflatoxigenic strains they are released several days earlier than in aflatoxin producing strains (De Lucca et al., 2012). A possible competitive strategy may involve the production of specific volatiles by the non-aflatoxigenic fungal strains to inhibit growth progression of the aflatoxin producers which would affect the population size of the aflatoxigenic fungi, and consequently reduce the amount of aflatoxin produced. Because the non-aflatoxigenic strains do not appear affected by close proximity to aflatoxin producers, it seems unlikely non-aflatoxigenic strains target aflatoxin production specifically. Therefore, the decrease in aflatoxin may be an indirect result of the suppressed population size of the aflatoxin-producing strain.

This strategy would help explain the apparent success shown by using non-aflatoxigenic fungi to effectively control aflatoxin contamination under field conditions. Unfortunately the long-term consequences of this form of treatment have not been studied. Our study has demonstrated that although AF36 decreased aflatoxin production, the kernels colonized by the biocontrol strain were severely damaged by day nine. There may not have been aflatoxin produced by the AF36 fungus; however, by the end of the study most of the kernels lost their integrity and were overwhelmed by the fungus which may not benefit the crop should the biocontrol strain be allowed to remain during post-harvest storage. Another potential caveat to use AF36 as biocontrol is that it produces cyclopiazonic acid (CPA) which is a mycotoxin that reportedly targets some internal organs as well as the skeletal muscles (Chang et al., 2009; Abbas et al., 2011; Moore et al., 2013). Although the toxic effects of CPA are not as well-documented as the effects of aflatoxin (Chang et al., 2009), overuse of the *A. flavus* isolates that produce CPA may lead to unintended effects on animal and human health. Additionally, when using non-aflatoxigenic fungal strains for applications in biocontrol, it is also important to take into account the potential course the suppressed aflatoxigenic strains may take to overcome inundation and suppression by the competing non-aflatoxigenic strains. For example, the constantly suppressed strain may call upon its latent ability for sexual reproduction demonstrated under laboratory conditions resulting in generations of aflatoxigenic offspring far more virulent than anticipated (Moore et al., 2013). All of these concerns regarding potential effects on crop integrity and/or unanticipated health risks need to be carefully evaluated when considering specific non-aflatoxigenic strains for aflatoxin biocontrol applications.

The present study has offered *in situ* evidence that the AF36 biocontrol strain is successful at suppressing tissue proliferation, and subsequently, aflatoxin contamination by an aflatoxigenic *A. flavus* isolate. Use of a GFP-labeled, aflatoxin-producing isolate allowed us to easily track invasion and colonization for a better understanding of the competitive relationship between the two strains in corn kernels. Our findings support the theory of competitive exclusion, in favor of the biocontrol strain, based on its robust

growth and proliferation. However, this study also points to valid concerns regarding the long-term use of non-aflatoxigenic fungi for suppression of native toxigenic strains in biocontrol strategies.

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REFERENCES

Abbas, H. K., Zablotowicz, R. M., Horn, B. W., Phillips, N. A., Johnson, B. J., Jin, X., et al. (2011). Comparison of major biocontrol strains of non-aflatoxigenic *Aspergillus flavus* for the reduction of aflatoxins and cyclopiazonic acid in maize. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 28, 198–208. doi: 10.1080/19440049.2010.544680

Brown, R. L., Cleveland, T. E., Payne, G. A., Woloshuk, C. P., Campbell, K. W., and White, D. G. (1995). Determination of resistance to aflatoxin production in maize kernels and detection of fungal colonization using an *Aspergillus flavus* transformant expressing *Escherichia coli* β-glucuronidase. *Postharvest Pathol. Mycotoxins* 85, 983–989.

Brown, R. L., Cotty, P. J., and Cleveland, T. E. (1991). Reduction in aflatoxin content of maize by atoxigenic strains of *Aspergillus flavus. J. Food Prot.* 54, 623–626.

Cary, J. W., Rajasekaran, K., Yu, J., Brown, R. L., Bhatnagar, D., and Cleveland, T. E. (2009). Transgenic approaches for pre-harvest control of mycotoxin contamination in crop plants. *World Mycotoxin J.* 2, 203–214. doi: 10.3920/WMJ2009.1138

CAST. (2003). "*Mycotoxins": Risks, in Plant, Animal and Human Systems.* CAST Report, No. 139. Ames, IA: Council for Agricultural Science and Technology.

Chang, P.-K., Ehrlich, K., and Fujii, I. (2009). Cyclopiazonic acid biosynthesis of *Aspergillus flavus* and *Aspergillus oryzae. Toxins* (*Basel*) 1, 74–99. doi: 10.3390/toxins1020074

Chang, P.-K., Horn, B. W., and Dorner, J. W. (2005). Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. *Fungal Genet. Biol.* 42, 914–923. doi: 10.1016/j.fgb.2005.07.004

Chang, P.-K., and Hua, S. S. T. (2007). Nonaflatoxigenic *Aspergillus flavus* TX9-8 competitively prevents aflatoxin accumulation by *A. flavus* isolates of large and small sclerotial morphotypes. *Int. J. Food Microbiol.* 114, 275–279. doi: 10.1016/j.ijfoodmicro.2006.09.017

Cotty, P. J. (1990). Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. *Plant Dis.* 74, 233–235. doi: 10.1094/PD-74-0233

Cotty, P. J. (1994). Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. flavus* infecting cotton bolls and on aflatoxin content of cottonseed. *Phytopathology* 84, 1270–1277. doi: 10.1094/Phyto-84-1270

Cotty, P. J. (2001). "Part 1. Infectious diseases, cottonseed losses and mycotoxin," in *Cotton Compendium,* 2nd Edition, eds T. L. Kirkpatrick and C. S. Rothrock (St. Paul, Minnesota: American Phytopathological Society), 9–13.

Crespo-Sempere, A., Lopez-Perez, M., Martfnez-Culebras, P. Y., and Gonzalez-Candelas, L. (2011). Development of a green fluorescent tagged strain of *Aspergillus carbonarius* to monitor fungal colonization in grapes. *Int. J. Food Microbiol.* 148, 135–140. doi: 10.1016/j.ijfoodmicro.2011.05.021

De Lucca, A. J., Boue, S. M., Carter-Wientjes, C. H., and Bhatnagar, D. (2012). Volatile profiles and aflatoxin production by toxigenic and non-toxigenic isolates of *Aspergillus flavus* grown on sterile and non-sterile cracked corn. *Ann. Agric. Environ. Med.* 19, 91–98.

Dorner, J. W. (2009a). Biological control of aflatoxin contamination in corn using a nontoxigenic strain of *Aspergillus flavus. J. Food Prot.* 72, 801–804.

Dorner, J. W. (2009b). Development of biocontrol technology to manage aflatoxin contamination in peanuts. *Peanut Sci.* 36, 60–67. doi: 10.3146/AT07-002.1

Ehrlich, K. C., and Cotty, P. J. (2004). An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. *Appl. Microbiol. Biotechnol.* 65, 473–478. doi: 10.1007/s00253-004-1670-y

Ehrlich, K., Wei, Q., Brown, R. L., and Bhatnagar, D. (2011). Inverse correlation of ability to produce aflatoxin and *Aspergillus* colonization of maize seed. *Food Nutr. Sci.* 2, 486–489. doi: 10.4236/fns.2011.25070

Food and Agriculture Organization (FAO). (2004). *Worldwide Regulations for Mycotoxins in Food and Feed in 2003. Food and Nutrition Paper* 81. Rome: FAO Press.

Huang, C., Jha, A., Sweany, R., DeRobertis, C., and Damann, K. E. Jr. (2011). Intraspecific aflatoxin inhibition in *Aspergillus flavus* is thigmoregulated, independent of vegetative compatibility group and is strain dependent. *PLoS ONE* 6:e23470. doi: 10.1371/journal.pone.0023470

Lillehoj, E. B. (1983). "Effect of environmental and cultural factors on aflatoxin contamination of developing corn kernels," in *Aflatoxin and Aspergillus in corn. Southern Cooperative Series Bulletin 279,* eds U. L. Diener, R. L. Asquith, and J. W. Dickens (Auburn, AL: Alabama Agricultural Experiment Station), 27–34.

Magbanua, Z. V., Williams, W. P., and Luthe, D. S. (2013). The maize rachis affects *Aspergillus flavus* spread during ear development. *Maydica* 58, 182–188.

Mehl, H. L., and Cotty, P. J. (2010). Variation in competitive ability among isolates of *Aspergillus flavus* from different vegetative compatibility groups during maize infection. *Phytopathology* 100, 150–159. doi: 10.1094/PHYTO-100-2-0150

Mehl, H. L., and Cotty, P. J. (2011). Influence of the host contact sequence on the outcome of competition among *Aspergillus flavus* isolates during host tissue invasion. *Appl. Environ. Microbiol.* 77, 1691–1697. doi: 10.1128/AEM.02240-10

Mehl, H. L., and Cotty, P. J. (2013). Nutrient environments influence competition among *Aspergillus flavus* genotypes. *Appl. Environ. Microbiol.* 79, 1473–1480. doi: 10.1128/AEM.02970-12

Moghtader, M., Salari, H., and Farahmand, A. (2011). Evaluation of the antifungal effects of rosemary oil and comparison with synthetic borneol and fungicide on the growth of *Aspergillus flavus. J. Ecol. Nat. Environ.* 3, 210–214.

Moore, G. G., Mack, B., and Beltz, S. B. (2013). Testing the efficacy of eGFP-transformed *Aspergillus flavus* as biocontrol strains. *Food Nutri. Sci.* 4, 469–479. doi: 10.4236/fns.2013.44060

Moore, G. G., Singh, R., Horn, B. W., and Carbone, I. (2009). Recombination and lineage-specific gene loss in the aflatoxin gene cluster of *Aspergillus flavus. Mol. Ecol.* 18, 4870–4887. doi: 10.1111/j.1365-294X.2009.04414.x

Payne, G. A. (1998). "Process of contamination by aflatoxin-producing fungi and their impact on crops,"in *Mycotoxins in Agriculture and Food Safety,* eds K. K. Sinha and D. Bhatnagar (New York, NY: Marcel Dekker), 279–306.

Rajasekaran, K., Cary, J. W., and Cleveland, T. E. (2006). Prevention of preharvest aflatoxin contamination through genetic engineering of crops. *Mycotoxin Res.* 22, 118–124. doi: 10.1007/BF02956775

Rajasekaran, K., Cary, J. W., Cotty, P. J., and Cleveland, T. E. (2008). Development of a GFP-expressing *Aspergillus flavus* strain to study fungal invasion, colonization, and resistance in cottonseed. *Mycopathologia*, 165, 89–97. doi: 10.1007/s11046-007-9085-9

Rajasekaran, K., Sickler, C. M., Brown, R. L., Cary, J. W., and Bhatnagar, D. (2013). Evaluation of resistance to aflatoxin contamination in kernels of maize genotypes using a GFP-expressing *Aspergillus flavus* strain. *World Mycotoxin J.* 6, 151–158. doi: 10.3920/WMJ2012.1497

Waters, J. (2009). Accuracy and precision in quantitative fluorescence microscopy. *J. Cell Biol.* 185 , 1135–1148. doi: 10.1083/jcb.200903097

Yao, H., Hruska, Z., Kincaid, R., Brown, R. L., Cleveland, T. E., and Bhatnagar, D. (2010). Correlation and classification of single kernel fluorescence hyperspectral data with aflatoxin concentration in corn kernels inoculated with *Aspergillus flavus* spores. *J. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 27, 701–709. doi: 10.1080/19440040903527368

Yin, Y. N., Yan, L. Y., Jiang, J. H., and Ma, Z. H. (2008). Biological control of aflatoxin contamination of crops. *J. Zhejiang Univ. Sci. B.* 9, 787–792. doi: 10.1631/jzus.B0860003

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Lipids in *Aspergillus flavus*-maize interaction

Marzia Scarpari[1], Marta Punelli[1], Valeria Scala[1], Marco Zaccaria[1], Chiara Nobili[2], Matteo Ludovici[3], Emanuela Camera[3], Anna A. Fabbri[1], Massimo Reverberi[1]* and Corrado Fanelli[1]

[1] *Dipartimento di Biologia Ambientale, Università Sapienza – Roma, Roma, Italy*
[2] *Unità Tecnica Sviluppo Sostenibile ed Innovazione del Sistema Agro-industriale, Laboratorio Innovazione Agroindustriale, ENEA C.R. Casaccia, Roma, Italy*
[3] *IFO-S.Gallicano, Roma, Italy*

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Xueyan Shan, Mississippi State University, USA
Gary L. Windham, USDA Agricultural Research Service, USA

***Correspondence:**
Massimo Reverberi, Dipartimento di Biologia Ambientale, Università Sapienza, L.go Cristina di Svezia 24, 00165 Roma, Italy
e-mail: massimo.reverberi@uniroma1.it

In some filamentous fungi, the pathways related to the oxidative stress and oxylipins production are involved both in the process of host-recognition and in the pathogenic phase. In fact, recent studies have shown that the production of oxylipins in filamentous fungi, yeasts and chromists is also related to the development of the organism itself and to mechanisms of communication with the host at the cellular level. The oxylipins, also produced by the host during defense reactions, are able to induce sporulation and to regulate the biosynthesis of mycotoxins in several pathogenic fungi. In *A. flavus*, the oxylipins play a crucial role as signals for regulating the biosynthesis of aflatoxins, the conidiogenesis and the formation of sclerotia. To investigate the involvement of an oxylipins based cross-talk into *Z. mays* and *A. flavus* interaction, we analyzed the oxylipins profile of the wild type strain and of three mutants of *A. flavus* that are deleted at the Af*lox1* gene level also during maize kernel invasion. A lipidomic approach has been addressed through the use of LC-ToF-MS, followed by a statistical analysis of the principal components (PCA). The results showed the existence of a difference between the oxylipins profile generated by the WT and the mutants onto challenged maize. In relation to this, aflatoxin synthesis which is largely hampered *in vitro*, is intriguingly restored. These results highlight the important role of maize oxylipin in driving secondary metabolism in *A. flavus*.

Keywords: maize kernels, aflatoxins, lipoxygenase, lipidomic, reverse genetic

INTRODUCTION

Zea mays is one of the most cultivated grain crop. The yield, quality and safety of its kernels are continuously challenged by pathogens. Amongst these, the mycotoxigenic fungi are actually the most health hazardous. *A. flavus* under suitable conditions infect maize kernels both during development in the field as well as during the storage (Sheidegger and Payne, 2003). In most cases *A. flavus* produces the harmful and carcinogenic aflatoxins, among which B1 is considered by IARC (International Agency for Research on Cancer) as belonging to group 1, i.e., carcinogenic to humans and animals. Seed composition, notably lipid composition, may affect susceptibility to fungal infection and mycotoxins production (Reddy et al., 1992; Dall'Asta et al., 2012). Unsaturated fatty acids (FAs) have been frequently described as modulators of plant resistance pathway upon pathogen attack, even though their profile is strongly influenced by the environmental conditions experienced by plants during the entire growing season (Kachroo and Kachroo, 2009; Dall'Asta et al., 2012). Polyunsaturated FAs, released from membranes by lipases in response to attacks by biotic agents, play a key role in plant-pathogen interaction either directly as free FAs or as precursors of oxylipins (Walley et al., 2013). As an example, linoleic acid levels contribute to the regulation of development, seed colonization and mycotoxin production in *Aspergillus* spp. (Calvo et al., 1999). FAs may also serve as precursors in oxylipins synthesis. These oxidized FA-derived compounds are involved in plant-fungi interaction. It is possible to suggest the presence of an "oxylipins signature profile" typical for every pathogen (Tsitsigiannis and Keller, 2007; Reverberi et al., 2010). Similarly, plants contain phyto-oxylipin pools conferring an oxylipin signature on a given organelle, tissue or plant (Blée, 2002; Camera et al., 2004). Lipoxygenases (LOX) are amongst the main oxylipin-producing enzymes in all organisms. LOX are widespread in animals, plants and fungi, and their products have a variety of biological functions, involved in important physiological processes, for instance, the events of infection (in plants) or inflammatory events (in animals) and the structural and metabolic changes in animal cells. LOX enzymes have been found in plants, animals, fungi, and in all organisms that possess the required substrates, i.e., PUFAs (Polyunsaturated Fatty Acids) (Feussner and Wasternack, 2002).

Lipoperoxidation seem to play an important role in the induction of the production of certain mycotoxins including aflatoxins. *In vitro*, synthetic inducers of lipid peroxidation, such as cumene hydroperoxide, stimulate the production of aflatoxins, when added to culture media inoculated with toxigenic strains of *A. flavus* and *A. parasiticus* (Passi et al., 1985). *In vivo*, the amount of toxin produced on sunflower seeds differs according to the age of seeds and the amount of peroxides found in the oil obtained from them; older will be the seeds, the greater the number of peroxides, and will be higher the amount of aflatoxin produced by these fungi (Passi

et al., 1984). Several studies have shown how oxylipins in plants are capable of inhibiting the mycelial growth and germination of spores (Prost et al., 2005). The similar structure between the oxylipins of fungi and plants suggest the hypothesis that these molecules are very important for the host-pathogen communication.

According to Tsitsigiannis and Keller (2007), oxylipins are able to modulate the sporulation through specific transcription factors related to cleistothecia (NsdD) and conidiospores (BrlA) formation; they may control homeostasis through the lipogenic transcription factors SREBP -1 and SREBP -2 and induce the development of the fungus and the production of secondary metabolites, such as penicillin, through the pathway of G proteins involving protein kinase A (PkaA). Studies on *A. nidulans* and *A. fumigatus* has led to hypothesize that oxylipins are able to escape from the cell through specific transport proteins, act as autocrine or paracrine ligand with membrane receptors, GPCRs, both of the fungal cell itself than that of the host, and activate a downstream signaling cascade (Tsitsigiannis and Keller, 2007).

Linoleic acid host-derived oxylipins may drive aflatoxin synthesis, as widely demonstrated (Burow et al., 1997; Brodhagen et al., 2008; Reverberi et al., 2012). The oxylipin-driven actions are further complicated by the evidence that even fungal pathogens produce oxylipin during the interaction with the host. A recent evidence of the central role of oxylipins in the regulation in the biosynthesis of mycotoxins and fungal sporulation was obtained in *A. ochraceus* interacting with viable seeds of wheat (Reverberi et al., 2010). In this study, a mutant defective for a *lox*-like gene produces minimal amounts of 13S-HPODE and show a strong decrease in the production of ochratoxin A, a delayed formation of conidia and an increase in the production of sclerotia. Furthermore, seeds infected with this mutant are not able to accumulate normal levels of 9S-HPODE nor to induce the expression of the gene of defense PR1, suggesting that the fungal oxylipins may modulate the response of the host defense. These data demonstrate that, the plants oxylipins influence the processes of development of *Aspergillus* because of their similarity with those of fungi. It has been observed that the PSIB α oxylipins of *A. nidulans*, derived from linoleic acid, are very similar to those produced from fatty acids of seeds and that these, after infection, are able to regulate the development of the fungus mimicking and/or interfering with the signals that regulate the processes sporogenesis (Prost et al., 2005).

In this paper we demonstrate that combining previous microarray data (Reverberi et al., 2013) with reverse genetic approach and with a lipidomic procedure may provide evidence of the key role played by lipids and oxylipins in the interaction between *A. flavus* and maize kernels, notably in influencing aflatoxin biosynthesis.

MATERIALS AND METHODS

FUNGAL STRAINS AND CULTURE CONDITIONS

Aspergillus flavus NRRL 3357, aflatoxin B_1 producer and an argD/uracil double auxotroph mutant (AFC-1) (Zhu-Mei et al., 2007) were used in these studies. An A*flox1*Δ transformant was generated from AFC-1 as described below. The fungal strains were maintained on Czapek Dox Agar (CDA) (Difco), amended with $ZnSO_4$ (5 mg/L) and $NaMoO_4$ (1 mg/L), for 7 days at 30°C.

FUNGAL GROWTH, CONIDIOGENESIS, AND AFLATOXIN PRODUCTION

In vitro assays

Fifty ml of Potato Dextrose Broth (PDB) (Difco, USA) in 100 mL Erlenmeyer flasks were inoculated with the WT or the 3 strains of A*flox1*Δ (1.1; 3.2; 5.1), using 0.1 mL of conidial suspension (~10^6 conidia) for each flask ($n = 3$); the cultures were then incubated at 30°C for different time periods (0–14 days post inoculation; total $n = 5$ time intervals). $N = 2$ biological replica were carried out. At each point in time, fungal growth was determined by weighting the mycelium after filtration (Millipore filters, 0.45 μm) and drying it for 48 h at 80°C (d.w.). Conidia formation was determined at each time interval by washing the mycelia with a solution of triton 0.01% w/v, taking 0.5 ml of this solution and calculating conidia's number using a hemacytometer. The filtered mycelia were lyophilized and weighed for determining the amount of oxylipins and performing molecular analyses.

In vivo assays

Each stock of maize seeds (30 g) was superficially sterilized (2% v/v NaClO solution), rinsed threefold by sterilized distilled water, moistened up to 0.90 a_w and then inoculated with 250 μL of *A. flavus* conidia ($n = 3$ Erlenmeyer flasks for each test) suspension (10^4 conidia/mL) at 30°C. Each biological replica ($n = 2$) consisted in non-contaminated (control) and contaminated seeds of maize kernels, harvested at $n = 5$ different time intervals (from 0 up to 14 days post inoculation, dpi).

Aflatoxin production was analyzed from cultures of the WT and A*flox1*Δ strains grown both in PDB and onto viable maize seeds following extraction with chloroform:methanol (2:1 v/v) procedure. The extracts were collected, the volume was reduced under a stream of nitrogen and the quantitative analyses were carried out by HPLC, as previously reported (Reverberi et al., 2008).

PLASMIDS AND TRANSFORMATION

To obtain the disruption cassette for the A*flox1* gene,5′- and 3′- flanking ends of the gene have been amplified, inserting in the primers (see below) a complementary tail to the 5′-end (*primer* #3) and 3′ (*primer* #4) of the *argD* gene; *argD* either has been amplified by inserting into the primers tails complementary to the 3′-end of 5′-flanking region (*primer* #2) and to the 5′-end of the 3′-flanking region (*primer* #5)

#1 (*for*) 5′-AAAGGCTGGCACGTAGAAGA-3′

#2 (*for*) 5′-CCTAGTCGAGACGGAAAACGTAATTGCGGAGC AAATCACA 3′

#3 (*rev*) 5′-TGTGATTTGCTCCGCAATTACGTTTTCCGTCT CGACTAGG-3′

#4 (*for*) 5′-GAATCCCTGCATCAGAGGAATCAATTCCATCA TTCCACGA-3′

#5 (*rev*) 5′-TCGTGGAATGATGGAATTGATTCCTCTGATGC AGGGATTC-3′

#6 (*rev*) 5′-CTACTGTGGCCTTTCCCAAA-3′

DNA extracted (50 ng) from *A. flavus* NRRL 3357 was amplified in a thermal mastercycler gradient (Eppendorf, Germany) following amplification steps (95°C × 2 min; 95°C × 30 s, 55°C × 45 s, 72°C × 1 min × 35 times; 72°C × 8 min). 5′-flanking region (1386 bp) has been amplified by *primers* #1 and #3; *argD* gene fragment (2217 bp) by *primers* #2 e #5; 3′-flanking region (1321 bp) by *primers* #4 and #6. After the obtainment of the 3 fragments, a PCR with external primers, #1 and #6, was carried on for obtaining gene disruption cassette; a DNA *mix* composed by 104 ng of 5′ *-flank* + 168 ng of argD + 100 ng of 3′*-flank* was used as template for the amplification. Protoplast transformations of *A. flavus* were performed by the polyethyleneglycol method as described elsewhere (Woo et al., 1999).

SELECTION OF TRANSFORMANTS

The selection of A*flox1*Δ transformant strains was conducted at 30°C on CDA containing 30 mM uracyle; putative transformants were selected, transferred to fresh selective medium, and allowed to sporulate. To obtain homokaryons, single spores were isolated from each selected heterokaryotic transformant and transferred to fresh selective medium. This monoconidial transfer was conducted three times. Finally, 20 monoconidial progenies were selected and further sub-cultured to determine the occurrence of abortive transformants. The stability of these transformants was also tested by two additional single-spore transfers on non-selective medium and then again on selective medium, and by several mycelial transfers on selective plates.

SOUTHERN BLOT HYBRIDIZATION

For Southern blot analysis, 10 μg of genomic DNA from *A. flavus* NRRL 3357 and A*flox1*Δ was completely restricted with *Eco*RI (10 U) at 37°C for 4 h in the manufacturer's buffer at the recommended concentrations (Fermentas, Germany). *Eco*RI-digested DNA fragments were separated by electrophoresis for 3 h and 30 min at 40 V on 0.8% w/v agarose gel in TAE buffer. DIG-labeled *Hin*dIII cut lambda (λ) (Roche, Swiss) was used as MW standard. Fluorescent DNA probes were prepared according to the PCR DIG-labeling mix method (Roche, Swiss). The membranes were pre-hybridized according to the instructions of the manufacturer of the DIG- detection kit, at 64°C in DIG-easy buffer (Roche, Swiss); they were then hybridized for 12–16 h in the same buffer containing 250 ng of freshly denatured digoxigenin *argD* probe at 65°C.

RT-PCR ANALYSES

Total RNA from 100 mg of freeze-dried mycelia was extracted using the Tri-Reagent protocol (Sigma-Aldrich, USA) and was quantified by spectrophotometry, determining the optical density at 260 nm. RNA was treated with RNAse-free DNAse I and then re-suspended in 20 μL of DEPC-treated water. RNA was extracted at different points in time (from 24 to 168 hpi; 3 tubes for each point in time) from *A. flavus* WT and A*flox1*Δ CDB cultures and was used to develop *lox1* SYBR green RT-PCR assay, as previously reported (Reverberi et al., 2010). Gene expression in the WT strain and the A*flox1*Δ transformants were also measured by comparing mRNA levels in the different time intervals with their own basal expressions at the baseline, i.e., after conidia germination (time 0). *A. flavus* β-tubulin RNA was used as the housekeeping gene to normalize the differences in total RNA target input and quality and in RT efficiency, using specific primers as Afβtub (Afβtub_for GGAAGTCAGAAGCAGCCATC; Afβtub_rev GTGACCACCTGTCTCCGTTT).

LIPOXYGENASE ASSAY

The LOX activity in the WT strain and in the A*flox1*Δ transformants grown in PDB medium was assayed 0 to 14 days after inoculation using a Beckman DU 530 spectrophotometer by following up the formation of conjugate dienes at 234 nm as previously reported (Reverberi et al., 2008). In order to exclude the possible interference of laccase activity, KCN 1 mM was added to the reaction mixture before the spectrophotometric assay (Gülçin et al., 2005).

LIPIDOMIC UNTARGETED ANALYSIS

Free, conjugated and modified fatty acids were extracted as described by Stumpe et al. (2005) with slight modifications. An amount of maize kernels (100 g) and/or mycelia (20 mg) were lyophilized and ground in liquid nitrogen. An aliquot of 20 mg was collected in a clean tube and added of 1 mL of the extraction medium (hexane: 2-propanol 3:2 v/v, containing 0.0025% butylated hydroxytoluene w/v) and 5 μL heptadecanoic acid standard solution 2 mg/ml in EtOH added as the internal standard reference as reported in Scala et al. (2013). Separation and accurate mass measurements of lipid compounds was performed with a 1200 series rapid resolution HPLC coupled with a G6220A series time of flight mass spectrometer (ToF-MS, Agilent Technologies, CA, USA) equipped with an electrospray (ESI) interface operating in the negative ion mode. LC/MS-ESI ToF data were acquired and deconvoluted into individual chemical peaks using the Mass Hunter™ acquisition software. Untargeted and semi-targeted mining of the HPLC/MS-ESI ToF data were performed with the molecular feature extraction (MFE) algorithm in the Mass Hunter™ software. All the analyses were performed as previously described in Scala et al. (2013).

STATISTICS

All the experiments were carried out in three replicates of two biological replica. The values presented in figures and tables are the mean ± SE of 6 different results. The mean values were compared by using the Mann-Whitney test; *p*-values above 0.05 were considered not significant. Analysis of variance (ANOVA) was applied in the comparison of the treatments, and significance of differences were tested at 95% confidence by Fisher's LSD test which is a least significant difference (LSD) method consisting in a two-step testing procedure for pair wise comparisons of several treatment groups. Calculations were performed using XLSTAT Addinsoft software [45]. Concerning the lipidomic approach, the MFE algorithm was used to extract individual molecular species by their accurate mass detected with the ToF

MS. The detected species were characterized by accurate mass, isotopic pattern and absolute abundance and lists of molecular features were generated from each analyzed sample and converted into compound exchange files (CEF), which were then processed with Mass Profiler Professional (MPP) 12 (see below). Molecular features detected in the HPLC-MS system were aligned by their retention time (RT) in the chromatographic runs, and accurate mass axis in order to compare their expression across the different maize hybrids in different growth stages. Compounds detected in the different samples and presenting consistent RT (shift below 6 % of the RT) and accurate mass (mass error below 6 ppm) were assigned as the same molecular species. Relative abundance of individual features was obtained by normalizing their peak area by the area of the ISTD. Among all the metabolites, only those features consistently detected throughout all the analyzed maize samples were selected for further statistical analysis. ANOVA with Tukey's *post-hoc* test was performed on the entities detected with 100% frequency in the different samples. Fold changes of filtered entities were compared between WT, mutant strains and maize alone and significance was determined by Student's *T*-test. Differential expression was evaluated at each sampling time, including harvest, and visualized by Volcano plots. Changes higher than 2-folds, with $p < 0.05$ after the Benjamini-Hochberg correction, were considered as significant. Principal components analysis (PCA) was then performed on entities filtered following grouping of samples according fumonisins amounts. Compound identification and annotation was performed using the METLIN Personal Metabolite Database by means of the ID browser tool, and the Molecular Formula Generator algorithm. The LIPIDMAPS (http://www.lipidmaps.org/) database was used to infer compound identity. The annotation of free fatty acids (Fas) reported the number of carbon atoms and of double bonds. Other lipids were annotated consistently with the names reported on LIPIDMAPS.

RESULTS

A*flox1* DELETION: MOLECULAR AND PHYSIOLOGICAL CHARACTERIZATION OF DELETED STRAINS

Microarray analysis performed in previous study (Reverberi et al., 2013), indicated that a *lox* gene (corresponding to the affy probe 2911_m00089), coding for a Mn-dependent lipoxygenase (BLASTX result), was up-regulated in *A. flavus* during the pathogenic exploitation of maize kernels. To uncover the relation of oxylipin production in the fungus and in the maize kernels with aflatoxin synthesis A*flox1*Δ strains have been generated by inserting a deletion cassette containing *argD* as selectable marker (AFC-1 strain is auxotroph for arginine—see Methods section) (**Figure 1**) and used for maize kernel infection.

Transformants selected as indicated in the Methods section, showed striking differences with the native strain AFC-1. These differences concern the growth (30–52% slower both in rich - PDB—and minimal—CD- media) and conidiogenesis (−90% compared to AFC-1) (**Figure 2**). This latter was completely recovered when A*flox1*Δ strains were inoculated onto viable maize kernels (data not shown).

To confirm and quantify deletion cassette insertion into A*flox1*Δ strains a southern blot analysis has been performed by using *Eco*RI-digested genomic DNA of the putative A*flox1* deleted mutants, AFC-1 and the WT with which AFC-1 was previously originated (i.e., *A. flavus* NRRL3357). Hybridization was performed by using an *argD* probe. Hybridization pattern confirm a single insertion of the deletion cassette into A*flox1*Δ 1.1 and 3.2 strains whereas, a putative double insertion event can be suggested for A*flox1*Δ 5.1 strain (**Figure 3**).

A*flox1* mRNA expression was analyzed into AFC-1 and in the 3 mutant strains selected for further analysis. All the fungal strains were grown in PDB and harvested at different time post inoculation (0–14 dpi). Results shown how *lox1* mRNA expression is under the detection limit into the A*flox1*Δ strains also compared to gene expression into AFC-1 strain (**Figure 4**).

Since *lox1* should be present in multiple copies in *A. flavus* as well as in other toxigenic fungi (Reverberi et al., 2010) we quantify the amount of LOX activity, by quantifying diene conjugates at λ_{234}, into AFC-1 and A*flox1*Δ mutant strains. It emerges that even if severely unpaired, a residual LOX activity is still present into deleted strains even if its amount is significantly ($P < 0.001$) to AFC-1 (**Figure 5A**). LC-TOF analysis of lipids extracted indicated that the synthesis of HPODEs (the main LOX-related oxylipins—Feussner and Wasternack, 2002) is consistently hampered in all A*flox1*Δ mutant strains compared to AFC-1 strain (**Figure 5B**).

Aflatoxin synthesis has been monitored at different time after inoculation (0-14 dpi) in PDB medium of the 4 fungal strains (AFC-1, Af*lox1*Δ_1.1, Af*lox1*Δ_3.2, Af*lox1*Δ_5.1). It emerged that A*flox1* deleted strains are unable to synthesize AFB1 in this culture conditions whereas AFC-1 has an AFB1 production similar to the native wild type NRRL 3357 (102 ± 15.2 ppb at 14 dpi) (**Figure 6**).

MAIZE KERNELS INOCULATION WITH AF*lox1* DELETED STRAINS

To study the behavior of A*flox1*Δ mutant strains *in vivo* compared to the AFC-1 strain, an inoculum was performed directly on viable corn seeds in environmental conditions that mimicked the natural condition of pathogenesis. Since the mutants do not produce conidia, *in vitro,* the inoculation of was performed by a small portion (200 mg) of fresh mycelium into 10 g of viable seeds of maize, to make the test more homogeneous as possible, even the mycelium of the AFC-1 strain was inoculated in the same way, i.e., by inoculating the same portion of mycelium that had not differentiated conidia into maize seeds. The three mutant strains, already at early time of analysis (3 dpi), intriguingly recover the ability to produce conidia although the overall growth appeared anyway to be slower compared to the AFC-1 strain (data not shown). Aflatoxins biosynthesis was then evaluated in times of analysis similar to the *in vitro* test (3, 7, 10, and 14 dpi). The results of this analysis have highlighted the recovery, and in some cases the stimulation (in strain A*flox1*Δ_3.2), of the capacity for synthesizing aflatoxin B1 in the three A*flox1* deleted strains examined (**Figure 7**).

OXYLIPIN PROFILE INTO MAIZE KERNELS CHALLENGED WITH *A. flavus*

Lipidome analysis and the characterization of specific oxylipin has been performed into maize kernels challenged with the 4 strains—1 native and 3 A*flox1* deleted strains—of *A. flavus*

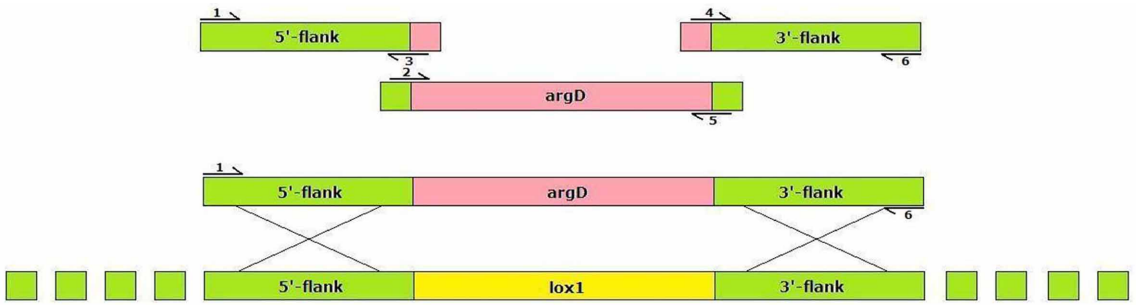

FIGURE 1 | Af*lox1* disruption cassette.

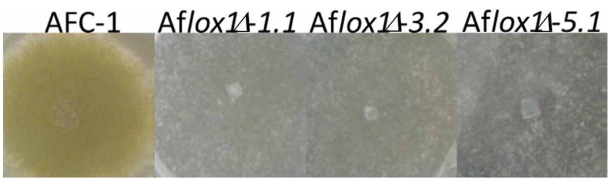

FIGURE 2 | Phenotypic comparison among Af*lox1*Δ strains with the native AFC-1 strain.

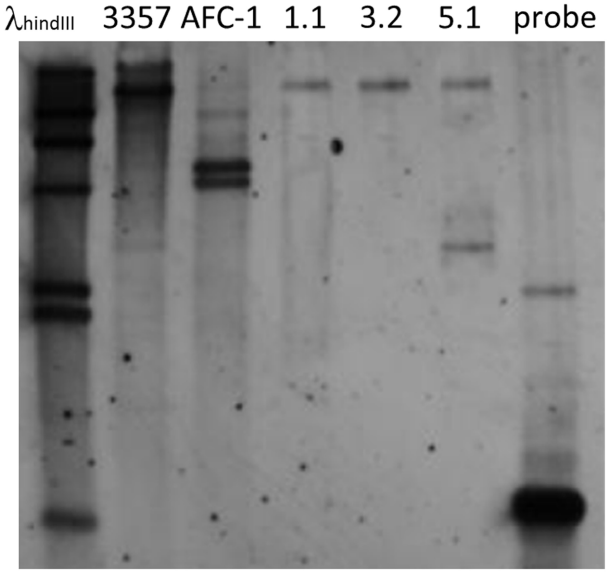

FIGURE 3 | Gel blot analysis of Af*lox1* gene replacement mutants. Genomic DNA was isolated from the WT strain, the arginine auxotroph AFC-1 strain and gene replacement transformants (Af*lox1*Δ). The blot was hybridized at 65°C with a 0.65-Kb *argD* DIG-labeled probe. Lane 1 DIG-labeled $\lambda_{hindIII}$ (Roche) used as molecular weight marker; lane 2 *EcoR*I-restricted genomic DNA of *A. flavus* WT strain; lane 3 *EcoR*I-restricted genomic DNA of *A. flavus* AFC-1; lane 4–6 *EcoR*I-restricted genomic DNA of Af*lox1*Δ strains (clone number 3 out of a set of 20 transformants screened); lane 7 *argD* 0.65 Kb PCR fragment.

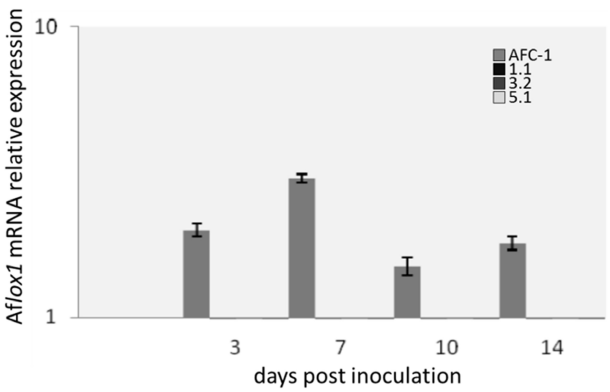

FIGURE 4 | Af*lox1* mRNA relative expression normalized to Af*lox1* expression at inoculation time 0 in *A. flavus* AFC-1 and Af*lox1* deleted strains (1.1; 3.2; 5.1) in PDB at 30°C at different days after inoculation (3–14 dpi). Results are the mean (± SE) of a total of six replications deriving from two independent experiments.

(**Figure S1**). Through *Mass Hunter* software (Agilent tech, USA) it has been possible to pinpoint 5 oxylipins amongst the *plethora* of lipid compounds highlighted by lipidomic approach (**Figures S2, S3**). Notably, HPODEs, as described elsewhere (Burow et al., 1997), and di-HODEs result produced at a different amount by pathogen- challenged and unchallenged maize kernels. By simply comparing the chromatograms it is possible to highlight, concerning HPODEs, striking difference among the different samples, with particular regard to HPODEs produced by maize kernels inoculated with AFC-1 compared to those produced when the seeds were challenged with the 3 Af*lox1* deleted strains. Furthermore, the same analysis was carried out on maize seeds un-inoculated in the same experimental conditions. The HPODE profile of the not inoculated seeds is considerably lower compared to preceding, to indicate that the presence of the fungus can effectively elicit in somehow the production of oxylipin in maize too (**Figure 8**).

At this point, we evaluated the level of production of di-HODE and HPODE in inoculated relative to un-inoculated maize kernels. In both cases oxylipin production in the seeds infected by mutant strains results higher than that in the seeds infected with the WT (**Figures 9A,B**).

DISCUSSION

One of the principal concern related to the production of cereal seed is the contamination with mycotoxins produced by *Aspergillus* spp. and *Fusarium* spp. In particular, Aspergilli are

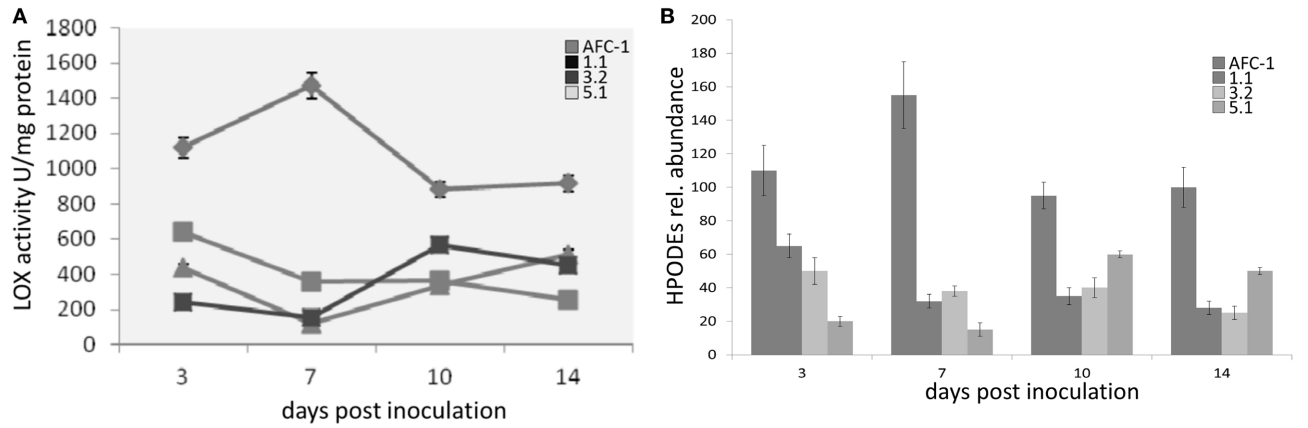

FIGURE 5 | (A) LOX activity (U/mg protein) measured as diene conjugates formation at 234 mn in AFC-1 and Af*lox1* deleted strains in PDB at 30°C at different days after inoculation (3–14 dpi). **(B)** HPODE synthesis (expressed as relative abundance) in AFC-1 and Af*lox1* deleted strains in PDB grown at 30°C at different days after inoculation (3–14 dpi). Results are the mean (±SE) of a total of six replications deriving from two independent experiments.

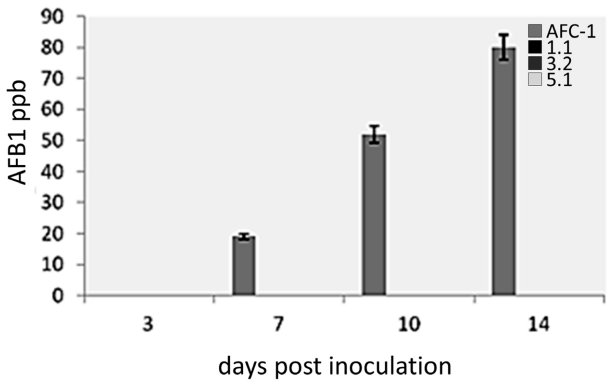

FIGURE 6 | Aflatoxin (AFB1) production measured by HPLC in culture filtrate (ppb), in the AFC-1 and Af*lox1*Δ strains grown in PDB at different time intervals of incubation (0–14 dpi) at 30°C. Results are the mean (±SE) of a total of six replications deriving from two independent experiments.

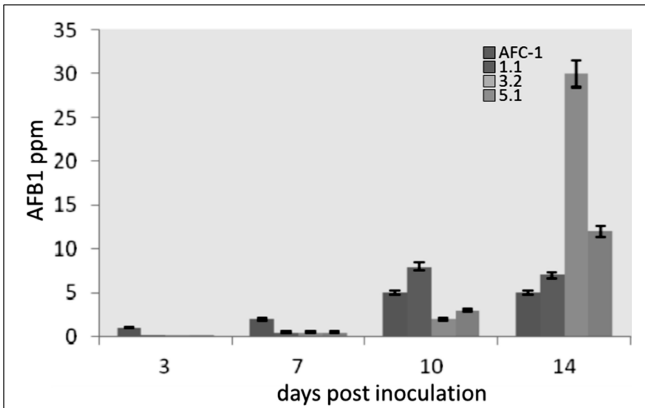

FIGURE 7 | Aflatoxins biosynthesis measured by HPLC (ppm) in viable seeds of maize by AFC-1, Af*lox1*Δ_1.1, Af*lox1*Δ_3.2, and Af*lox1*Δ_5.1 strains incubated at 30°C for different days post inoculation (3–14 dpi). Results are the mean (± SE) of a total of six replications deriving from two independent experiments.

commonly associated with crops such as maize, wheat, cotton, peanut, characterized by seeds rich in lipids, suggesting an important role played by the host lipids in influencing the ability of the pathogen to synthesize mycotoxins.

The role of lipids in the recognition and plant-pathogen communication has recently been revised and re-evaluated. Lipids and oxylipins currently represent one of the most effective control signals for morphogenesis, development and virulence of pathogens (Gao and Kolomiets, 2009; Christensen and Kolomiets, 2010; Reverberi et al., 2010).

Several studies have shown a direct correlation between the lipoperoxidative processes that occur in the seeds of various plant species (maize, sunflower) and the production of aflatoxins by *A. flavus* and *A. parasiticus* (Fabbri et al., 1983; Burow et al., 1997; Gao et al., 2009). In particular, experiments on maize have shown that the processes of lipoperoxidation in the seeds induce an alteration of the balance between oxidants and antioxidants, in favor of oxidants accumulation into the fungal cell thus stimulating biosynthesis of mycotoxins in *A. parasiticus* (Reverberi et al., 2007). The production of the toxins could be interpreted as the result of a fungal cell response to an incomplete scavenging of reactive oxygen species at the intracellular level (Reverberi et al., 2008; Hong et al., 2013).

Several studies showed that plants oxylipins are able of inhibiting the mycelial growth and germination of fungal spores (Prost et al., 2005). The similar structure found between fungal oxylipins and those of plants has led to the hypothesis that the latter can "mimic" the corresponding oxylipins acting directly on the physiology of the fungal pathogen try to alter its pathogenic behavior. Indeed, *in vitro* experiments have shown that linoleic acid and two of its oxidation products, namely 9S- and 13S-HPODE, play a significant role in differentiation processes in *A. nidulans*, in *A. flavus* and *A. parasiticus* (Burow et al., 1997; Gardner, 1998; Calvo et al., 1999; Gao et al., 2009).

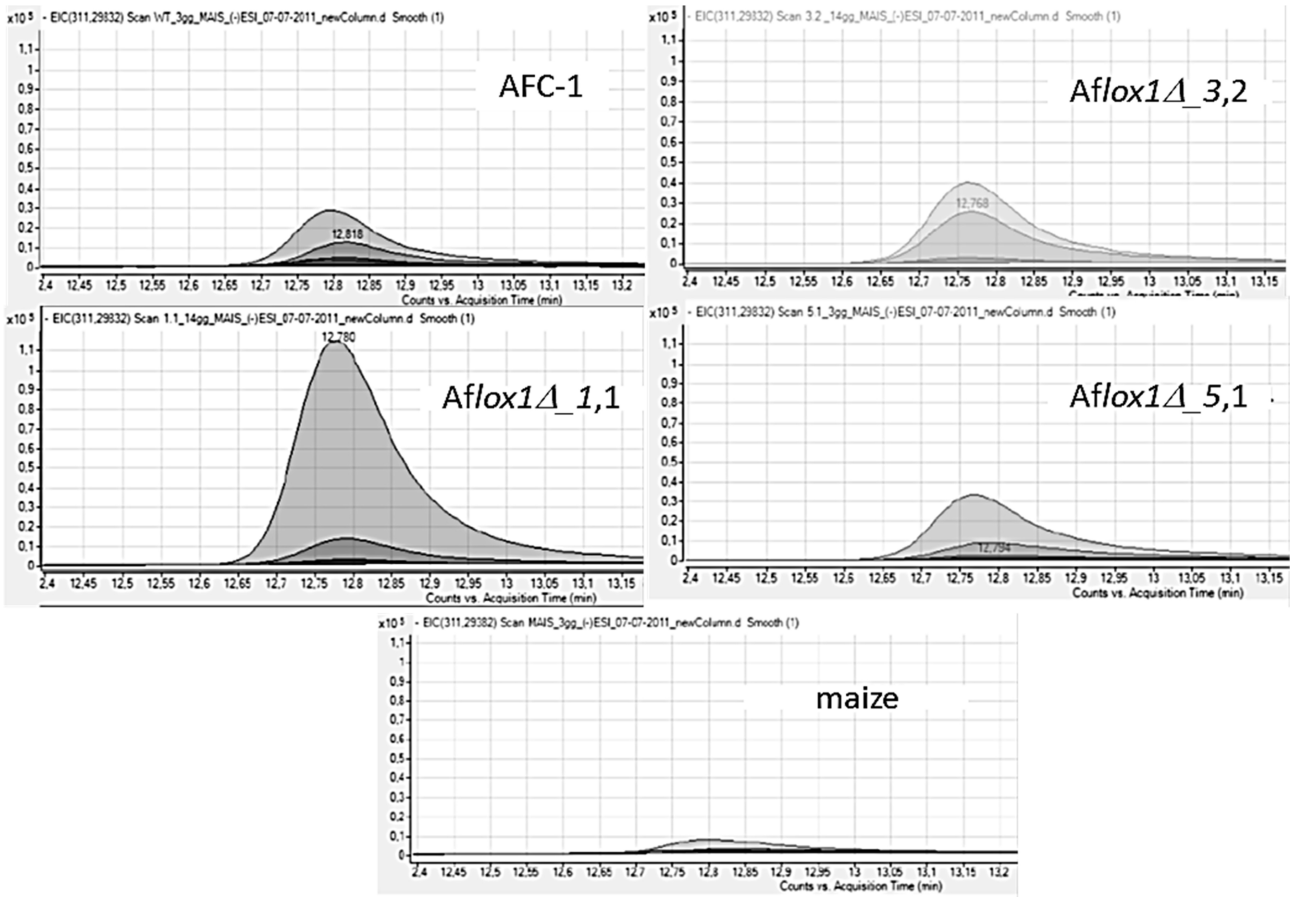

FIGURE 8 | Chromatogram (of the lipid compound at 311.2983 m/z corresponding to HPODE) in overlap mode deriving by LC-TOF analysis of lipid extract of maize kernels non-inoculated or inoculated with AFC-1 and the 3 deleted Af*lox1* strains (1.1/3.2/5.1) at 3, 7 and 14 dpi.

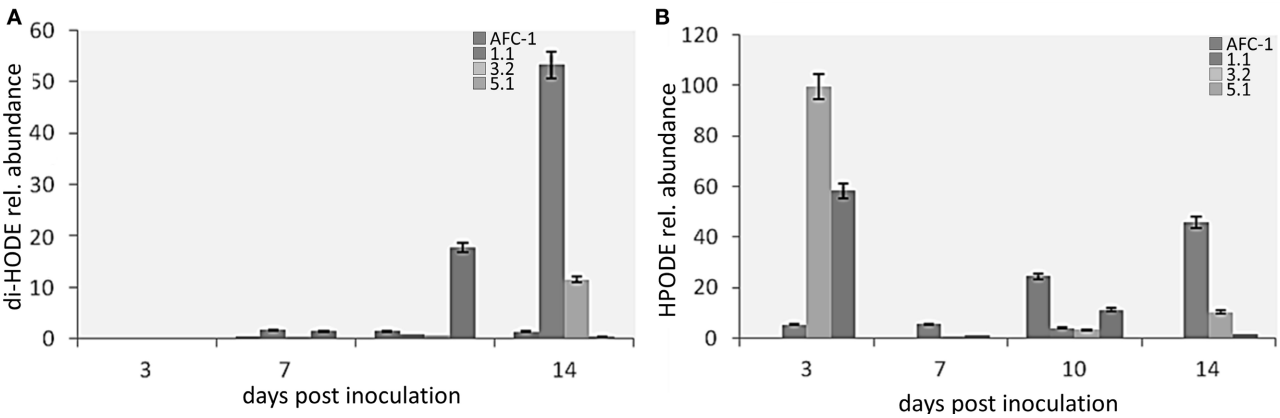

FIGURE 9 | Results in the production of (A) di-HODE and (B) HPODE in maize inoculated with the mutant strains or with AFC-1, 3, 7, 10, and 14 days post inoculation, compared to non-inoculated maize. The results represent the average of six repetitions arising from two different experiments ± SE.

Af*lox1* gene, Mn-lipoxygenase (LOX) encoding, knock-out mutants showed a significant reduction of mycelial growth and a complete inability to differentiate conidia and produce aflatoxins, if grown *in vitro* conditions. As shown by recent studies (Tsitsigiannis and Keller, 2007) in *A. nidulans* and *A. fumigatus*, the change in lipid metabolism has a strong influence on the production of sexual spores and the ability to produce toxins (Tsitsigiannis and Keller, 2006). The data obtained

herein are therefore in line with the others reported in the literature. Intriguingly, inoculating Af*lox1*Δ mutants on viable and germinating maize seeds, it was possible to highlight the almost total recovery of conidiogenesis and production of aflatoxins. Oxylipins released from the seed, probably simulating/substituting fungal ones, induce the activation of secondary metabolism and changes in morphogenesis, as also suggested in Brodhagen et al. (2008). In these studies maize 9-LOX (Zm*LOX3*) was cloned in a mutant of *A. nidulans* unable to produce conidia. The oxylipins produced by the Zm*LOX3* gene are able to induce again the conidiogenesis, suggesting a strong correlation between host-oxylipins and production of conidia and providing a clear proof of the mutual cross-talk between plants and fungi mediated by oxylipins.

The LC-TOF and chemometric based lipidomic analysis of the maize kernels infected with the mutants and the wild-type strain of *A. flavus* showed a complete modification of the lipid and oxylipin profile. A higher production of HPODE and di-HODE in maize seeds inoculated with Af*lox1*Δ strains compared to those inoculated with the wild-type strain may be evidenced. The comparison with the production of the same oxylipins in non-inoculated maize seeds makes it possible to demonstrate not only that production is higher in seeds inoculated with the mutants, but also that the production of these particular oxylipins, is greatly increased if the host is interacting with the pathogen. To confirm this, it was also evaluated the production of HPODE and di-HODE in mycelia of the strains grew on media containing maize seeds (data not shown). The production was significantly reduced, showing that synthesis of these oxylipins is specifically charged to the seed. Recent studies have, in fact, show that LOX host genes expression is driven by the pathogen in *Aspergillus* infections leading to changes in the profile of the plant oxylipins (Tsitsigiannis and Keller, 2007). These results suggest that some derivatives of 9 -LOX may act as signal molecules for the production of mycotoxins and conidia. Experiments based on a knock-out mutant of the 9 -LOX gene of maize (Zm*LOX*3) confirm this hypothesis, showing that the lack of derivatives in the seed of the 9-LOX affect the pathogenesis and production of conidia and mycotoxins (Gao et al., 2007) and therefore, the importance of the role played by the host oxylipins for its exploitation by some pathogenic fungi. It can be suggested that the presence of the fungus (PAMP?) elicits the production of such oxylipins in the plant, which, in turn may influence the development of the fungus itself by stimulating certain physiological processes such as the production of conidia and the biosynthesis of toxin. The way in which these process aid pathogenesis is not yet elucidated.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00074/abstract

Figure S1 | Representative Total ion chromatogram of lipid compounds of AFC-1 grown on maize kernels after 7 dpi. The different lipid compounds were sorted for exact mass and RT. A total of 1217 different lipid compounds were separated.

Figure S2 | PCA score plot of data generated by HPLC-ESI/TOF-MS analysis of of lipid extract of maize kernels non-inoculated or inoculated with AFC-1 (WT) and the 3 deleted Af*lox1* strains (1.1/3.2/5.1) at 3, 7, 10 and 14 dpi. The results of the PCA analysis referred to three separate experiments performed in duplicate.

Figure S3 | Eulero–Venn's diagram showing compounds differences present in maize kernels inoculated with AFC-1 (WT) or the 3 deleted Af*lox1* strains. The labels of each circle report the number of compounds selected on the basis of significant difference and 100% frequency in at least one group.

REFERENCES

Blée, E. (2002). Impact of phyto-oxylipins in plant defence. *Trends Plant Sci.* 7, 315–321. doi: 10.1016/S1360-1385(02)02290-2

Brodhagen, M., Tsitsigiannis, D. I., Hornung, E., Goebel, C., Feussner, I., and Keller, N. P. (2008). Reciprocal oxylipin-mediated cross-talk in the *Aspergillus*–seed pathosystem. *Mol. Microbiol.* 67, 378–391. doi: 10.1111/j.1365-2958.2007.06045.x

Burow, G. B., Nesbitt, T. C., Dunlap, J., and Keller, N. P. (1997). Seed lipoxygenase products modulate *Aspergillus* mycotoxin biosynthesis. *Mol. Plant Microbe Interact.* 10, 380–387. doi: 10.1094/MPMI.1997.10.3.380

Calvo, A. M., Hinze, L. L., Gardner, H. W., and Keller, N. P. (1999). Sporogenic effect of polyunsaturated fatty acids on development of Aspergillus spp. *Appl. Environ. Microbiol.* 65, 3668–3673.

Camera, S. L., Gouzerh, G., Dhondt, S., Hoffmann, L., Fritig, B., Legrand, M., et al. (2004). Metabolic reprogramming in plant innate immunity: the contributions of phenylpropanoid and oxylipin pathways. *Immunol. Rev.* 198, 267–284. doi: 10.1111/j.0105-2896.2004.0129.x

Christensen, S. A., and Kolomiets, M. V. (2010). The lipid language of plant-fungal interactions. *Fungal Genet. Biol.* 48, 4–14. doi: 10.1016/j.fgb.2010.05.005

Dall'Asta, C., Falavigna, C., Galaverna, G., and Battilani, P. (2012). Role of maize hybrids and their chemical composition in Fusarium infection and fumonisin production. *J. Agr. Food Chem.* 60, 3800–3808. doi: 10.1021/jf300250z

Fabbri, A. A., Fanelli, C., Panfili, G., Passi, S., and Fasella, P. (1983). Lipoperoxidation and aflatoxin biosynthesis by *Aspergillus parasiticus* and *A. flavus*. *J. Gen. Microbiol.* 129, 3447–3452.

Feussner, I., and Wasternack, C. (2002). The Lipoxygenase Pathway. *Annu. Rev. Plant Biol.* 53, 275–297. doi: 10.1146/annurev.arplant.53.100301.135248

Gao, X., Brodhagen, M., Isakeit, T., Brown, S. H., Göbel, C., Betran, J., et al. (2009). Inactivation of the lipoxygenase ZmLOX3 increases susceptibility of maize to *Aspergillus* spp. *Mol. Plant Microbe Interact.* 22, 222–231. doi: 10.1094/MPMI-22-2-0222

Gao, X., Shim, W. B., Göbel, C., Kunze, S., Feussner, I., Meeley, R., et al. (2007). Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin. *Mol. Plant Microbe Interact.* 20, 922–933. doi: 10.1094/MPMI-20-8-0922

Gao, X. Q., and Kolomiets, M. V. (2009). Host-derived lipids and oxylipins are crucial signals in modulating mycotoxin production by fungi. *Toxin Rev.* 28, 79–88. doi: 10.1080/15569540802420584

Gardner, H. W. (1998). Soybean lipoxygenase is active on nonaqueous media at low moisture: a constraint to xerophilic fungi and aflatoxins? *J. Am. Oil Chem. Soc.* 75, 1801–1808. doi: 10.1007/s11746-998-0334-y

Gülçin, I., Küfrevioğlu, O. I., and Oktay, M. (2005). Purification and characterization of polyphenol oxidase from nettle (Urtica dioica L.) and inhibitory effects of some chemicals on enzyme activity. *J. Enzyme Inhib. Med. Chem.* 20, 297–302. doi: 10.1080/1475636032000141890

Hong, S. Y., Roze, L. V., and Linz, J. E. (2013). Oxidative stress-related transcription factors in the regulation of secondary metabolism. *Toxins (Basel)* 5, 683–702. doi: 10.3390/toxins5040683

Kachroo, A., and Kachroo, P. (2009). Fatty acid-derived signals in plant defense. *Ann. Rev. Phytopathol.* 47, 153–176. doi: 10.1146/annurev-phyto-080508-081820

Passi, S., Fanelli, C., Fabbri, A. A., Finotti, E., Panfili, G., and Nazzaro-Porro, M. (1985). Effect of halomethanes on aflatoxin induction in cultures of *Aspergillus parasiticus*. *J. Gen. Microbiol.* 131, 687–691.

Passi, S., Nazzaro-Porro, M., Fanelli, C., Fabbri, A. A., and Fasella, P. (1984). Role of lipoperoxidation in aflatoxin production. *Appl. Microbiol. Biotechnol.* 19, 186–190. doi: 10.1007/BF00256452

Prost, I., Dhondt, S., Rothe, G., Vicente, J., Rodriguez, M. J., Kift, N., et al. (2005). Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. *Plant Physiol.* 139, 1902–1913. doi: 10.1104/pp.105.066274

Reddy, M. J., Shetty, H. S., Fanelli, C., and Lacey, J. (1992). Role of seed lipids in *Aspergillus parasiticus* growth and aflatoxin production. *J. Sci. Food Agric.* 59, 177–181. doi: 10.1002/jsfa.2740590207

Reverberi, M., Punelli, F., Scarpari, M., Camera, E., Zjalic, S., Ricelli, A., et al. (2010). Lipoperoxidation affects ochratoxin A biosynthesis in *Aspergillus ochraceus* and its interaction with wheat seeds. *Appl. Microbiol. Biotechnol.* 85, 1935–1946. doi: 10.1007/s00253-009-2220-4

Reverberi, M., Punelli, M., Scala, V., Scarpari, M., Uva, P., Mentzen, W. I., et al. (2013). Genotypic and phenotypic versatility of *Aspergillus flavus* during maize exploitation. *PLoS ONE* 7:e68735. doi: 10.1371/journal.pone.0068735

Reverberi, M., Punelli, M., Smith, C. A., Zjalic, S., Scarpari, M., Scala, V., et al. (2012). How peroxisomes affect aflatoxin biosynthesis in *Aspergillus flavus*. *PLoS ONE* 7:e48097. doi: 10.1371/journal.pone.0048097

Reverberi, M., Zjalic, S., Punelli, F., Ricelli, A., Fabbri, A. A., and Fanelli, C. (2007). *Apyap1* affects aflatoxin biosynthesis during *Aspergillus parasiticus* growth in maize seeds. *Food. Add. Contam*. 24, 1070–1075. doi: 10.1080/02652030701553244

Reverberi, M., Zjalic, S., Ricelli, A., Punelli, F., Camera, E., Fabbri, C., et al. (2008). Modulation of antioxidant defense in *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for the Ap*yapA* gene. *Eukaryot. Cell* 7, 988–1000. doi: 10.1128/EC.00228-07

Scala, V., Camera, E., Ludovici, M., Dall'Asta, C., Cirlini, M., Giorni, P., et al. (2013). *Fusarium verticillioides* and maize interaction *in vitro*: relation between oxylipin cross-talk and fumonisin synthesis. *World Mycotox. J.* 6, 343–351. doi: 10.3920/WMJ2012.1527

Sheidegger, K. A., and Payne, G. A. (2003). Unlocking the secrets behind secondary metabolism: a review of *Aspergillus flavus* from pathogenicity to functional genomics. *Toxin Rev.* 22, 423–459. doi: 10.1081/TXR-120024100

Stumpe, M., Carsjens, J. G., Stenzel, I., Gobel, C., Lang, I., Pawlowski, K., et al. (2005). Lipid metabolism in arbuscular mycorrhizal roots of Medicago truncatula. *Phytochem* 66, 781–791. doi: 10.1016/j.phytochem.2005.01.020

Tsitsigiannis, D. I., and Keller, N. P. (2006). Oxylipins act as determinants of natural product biosynthesis and seed colonization in *Aspergillus nidulans*. *Mol. Microbiol.* 59, 882–892. doi: 10.1111/j.1365-2958.2005.05000.x

Tsitsigiannis, D. I., and Keller, N. P. (2007). Oxylipins as developmental and host-fungal communication signals. *Trends Microbiol.* 15, 109–118. doi: 10.1016/j.tim.2007.01.005

Walley, J. W., Kliebenstein, D. J., Bostock, R. M., and Dehesh, K. (2013). Fatty acids and early detection of pathogens. *Curr. Opin. Plant Biol.* 16, 520–526. doi: 10.1016/j.pbi.2013.06.011

Woo, S. L., Donzelli, B., Scala, F., Mach, R., Harman, G. E., Kubicek, C. P., et al. (1999). Disruption of the ech42 (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. *Mol. Plant Microbe Interact.* 12, 419–429. doi: 10.1094/MPMI.1999.12.5.419

Zhu-Mei, H., Price, M. S., O'Brian, G. R., Georgianna, D. R., and Payne, G. A. (2007). Improved protocols for functional analysis in the pathogenic fungus *Aspergillus flavus*. *BMC Microbiol.* 7:104. doi: 10.1186/1471-2180-7-104

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Toward elucidation of genetic and functional genetic mechanisms in corn host resistance to *Aspergillus flavus* infection and aflatoxin contamination

***Xueyan Shan*[1]* and *W. Paul Williams*[2]**

[1] *Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University, Mississippi, MS, USA*
[2] *Agricultural Research Service, United States Department of Agriculture, Corn Host Plant Resistance Research Unit, Mississippi, MS, USA*

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Perng-Kuang Chang, Southern Regional Research Center, USA
Jong H. Kim, United States Department of Agriculture – Agricultural Research Service, USA

***Correspondence:**
Xueyan Shan, Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University, Mississippi, MS 39762, USA
e-mail: xshan@bch.msstate.edu

Aflatoxins are carcinogenic mycotoxins produced by some species in the *Aspergillus* genus, such as *A. flavus* and *A. parasiticus*. Contamination of aflatoxins in corn profusely happens at pre-harvest stage when heat and drought field conditions favor *A. flavus* colonization. Commercial corn hybrids are generally susceptible to *A. flavus* infection. An ideal strategy for preventing aflatoxin contamination is through the enhancement of corn host resistance to *Aspergillus* infection and aflatoxin production. Constant efforts have been made by corn breeders to develop resistant corn genotypes. Significantly low levels of aflatoxin accumulation have been determined in certain resistant corn inbred lines. A number of reports of quantitative trait loci have provided compelling evidence supporting the quantitative trait genetic basis of corn host resistance to aflatoxin accumulation. Important findings have also been obtained from the investigation on candidate resistance genes through transcriptomics approach. Elucidation of molecular mechanisms will provide in-depth understanding of the host–pathogen interactions and hence facilitate the breeding of corn with resistance to *A. flavus* infection and aflatoxin accumulation.

Keywords: *Aspergillus flavus*, aflatoxin, corn, host resistance, transcriptomics

INTRODUCTION

Aflatoxins are produced by *Aspergillus flavus*, an ear rot fungus that infects corn. Aflatoxins are carcinogenic to humans and animals. Consequently, they are of great concern as food contaminants and are closely monitored in oily-seeded crops and dairy products (Payne, 1992; Gourama and Bullerman, 1995). Aflatoxins are designated as B1, B2, G1, G2, M1, and M2 types based on their molecular structures. Aflatoxin B1 is the most toxic form which is found in fungal-infected corn kernels (Bhatnagar et al., 2006). The production of aflatoxin B1 starts soon after the colonization of *A. flavus* in the developing corn kernels (Thompson et al., 1983). Accumulation of aflatoxin B1 continues during the maturation of corn. Commercial corn hybrids are generally susceptible to *A. flavus* infection. The Food and Drug Administration (FDA) has imposed a strict limit of 20 ppb on aflatoxin concentration present in corn (Aflatoxin Guidelines, http://www.admcrs.com/Aflatoxin.html). Aflatoxins remain stable under food processing conditions. Once corn is contaminated with aflatoxins, few detoxification options are available. Therefore, the prevention of aflatoxin accumulation should start in pre-harvest corn stage.

Aspergillus flavus infection and aflatoxin accumulation in pre-harvest corn largely depend on the local weather and field conditions. High temperature and drought conditions are in favor of aflatoxin production, thus posing the high risk of an aflatoxin outbreak. The colonization of *A. flavus* also frequently occurs at sites damaged by insects. In many locations of the southeastern United States, including Mississippi, *A. flavus* infection in corn is a chronic problem. In 1998, a severe aflatoxin outbreak in corn fields occurred in Mississippi and throughout the Southeast, resulting in considerable economic loss to corn farmers (Robens and Cardwell, 2005). In Mississippi, efforts to reduce aflatoxin contamination in corn at the pre-harvest stage have focused on multiple strategies including bio-control using atoxigenic *A. flavus* strains, optimal agronomic practices (irrigation, fungicides, planting dates), and breeding for host resistance (Larson, 1997; Cleveland et al., 2004). While notable improvement can be achieved through optimizing cultural practices for control of pre-harvest aflatoxin contamination, combination strategy by including breeding for corn host resistance would be the most promising and effective avenue.

RESISTANT CORN GERMPLASM DEVELOPED BY USDA–ARS AT MISSISSIPPI STATE

As early as in the 1970s, USDA–ARS scientists at Mississippi State University initiated breeding programs for screening and developing corn germplasm with resistance to *A. flavus* infection and aflatoxin accumulation. Reliable techniques (such as side-needle inoculation) were first developed to assure that all germplasm lines were treated with the same amount of fungal inoculums (Zummo and Scott, 1989; Windham et al., 2005, 2009; Buckley et al., 2006). Numerous germplasm and breeding lines were screened with such consistent methods. The results of these evaluations established the foundation of today's collection of Mississippi corn inbred lines that possess naturally occurring resistance to aflatoxin accumulation (Windham and Williams, 1998, 1999, 2002; Windham et al., 1999, 2010; Williams et al., 2002, 2005a,b, 2008; Williams, 2006). Resistant corn inbred lines were developed by consecutive

selfing and selection against aflatoxin accumulation. In 1988, the first inbred line Mp313E with resistance to *A. flavus* infection was released (Scott and Zummo, 1990). Mp313E was developed from germplasm Tuxpan. Since the release of Mp313E, additional resistant corn germplasm lines (Mp420, Mp715, Mp717, Mp718, and Mp719) have also been released; all exhibit significantly low levels of aflatoxin accumulation under artificial inoculation conditions (Scott and Zummo, 1992; Williams and Windham, 2001, 2006, 2012). Mp715, Mp718, and Mp719 also have lineages tracking back to germplasm Tuxpan. Tuxpan is a US derivative of Tuxpeño, one of the most productive and successful Mexican races of corn.

In the continual field studies conducted under artificial inoculation conditions, aflatoxin levels were evaluated for selected resistant corn inbred lines, single-cross hybrids from the selected resistant corn inbred lines, and a set of commercial corn hybrids. In 1999, aflatoxin level in the resistant corn inbred line Mp715 was 24 ppb which was contrasted to the level of 1622 ppb in SC212m, a susceptible corn inbred line. In 1996, aflatoxin level in the single-cross hybrid Mp715 × Mp313E was 18 ppb compared to 1532 ppb in the single cross of GA209 × SC212m. In 2001, aflatoxin level for Mp715 was 14 ppb compared to 9289 ppb for SC212M. In 2008, aflatoxin level was as low as 17 ppb for the single-cross hybrid of Mp313E × Mp717, whereas it was as high as 10800 ppb for a commercial hybrid. Similar results were observed in 2009, where aflatoxin levels ranged from 5 ppb for the single-cross hybrid of Mp313E × NC388 to 1992 ppb for a commercial hybrid. From these studies, the lowest aflatoxin levels were found in the single-cross hybrids of the resistant inbred lines developed by the USDA–ARS Corn Host Plant Resistance Research Unit (CHPRRU) at Mississippi State. Four of the single-cross hybrids had aflatoxin levels lower than the FDA action level of 20 ppb in the 2008 and 2009 field studies. Commercial corn hybrids generally contained consistently high levels of aflatoxins in the studies conducted in 2008 and in 2009 at Mississippi State (Daves et al., 2010).

THE IDENTIFICATION OF MAJOR RESISTANCE QUANTITATIVE TRAIT LOCI (QTLs)

The resistant corn inbred lines exhibited significant general combining ability for reduced aflatoxin accumulation in a diallel cross, indicating good breeding values for the resistance trait (Williams et al., 2007). To incorporate the resistance into commercially acceptable lines for corn farmers, CHPRRU has made important progress in combining traditional and molecular breeding methods to develop resistant corn lines. Among the resistant corn germplasm lines developed by CHPRRU at Mississippi State, Mp313E and Mp715 are two of the most important parental lines used for breeding of *A. flavus* resistance and aflatoxin reduction.

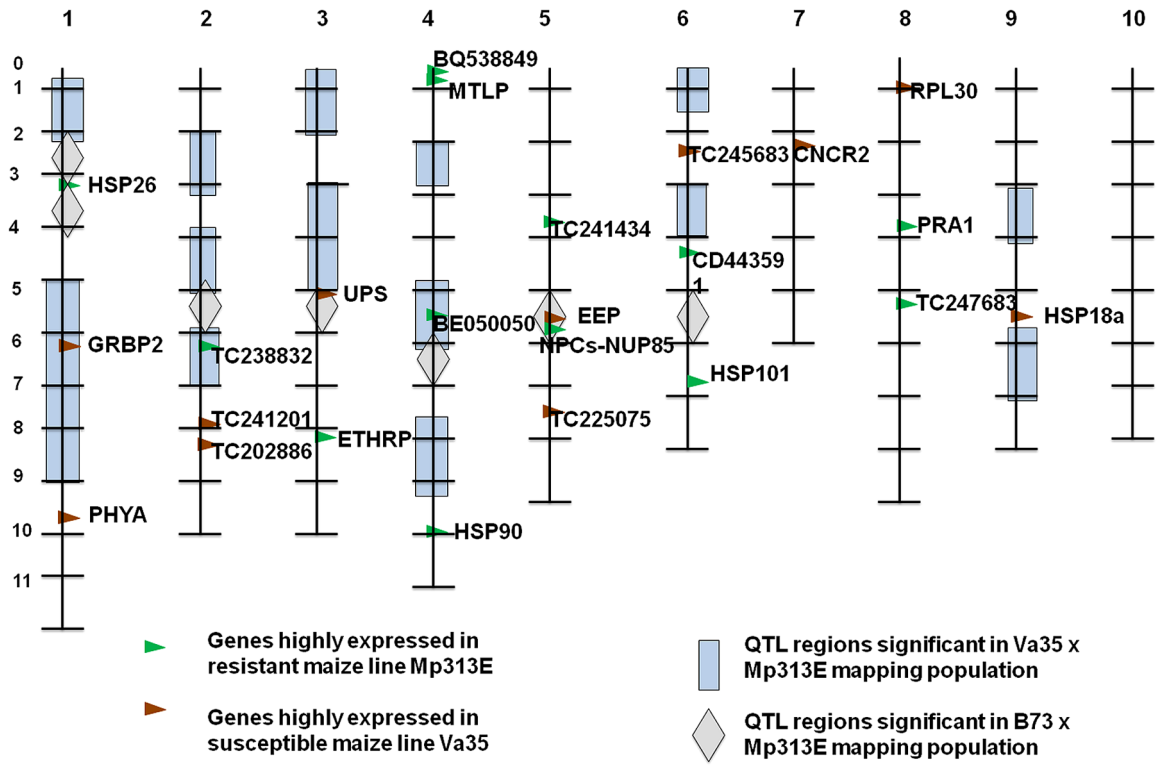

FIGURE 1 | Chromosome bin map showing the positions of the identified QTL regions from the two Mp313E-derived QTL mapping populations. The significant genes marked in the map were from a separate genome-wide microarray study. Eight genes were located to seven most significant QTL regions based on the microarray study. A significant candidate gene, NPCs–NUP85, is located to the chromosome 5 QTL region (Chr 5, bin 5.05) which is from Mp313E (Kelley et al., 2012).

They have been used as resistance donors for many of the new experimental lines in the CHPRRU breeding programs. Two quantitative trait loci (QTL) mapping populations with Mp313E as a parental line were developed and investigated. The population from Mp313E × Va35 included 216 $F_{2:3}$ families and was evaluated for aflatoxin accumulation over 3 years (Davis et al., 2000; Willcox et al., 2013). A total of 15 QTL regions were identified (**Figure 1**). Four QTLs on chromosomes 1, 4, and 9 were above a significance level of 23.58 in likelihood ratio. The chromosome 4 (bin 4.04–4.08) QTL was associated with Mp313E. The Mp313E × B73 population contains 210 $F_{2:3}$ families and was also evaluated for 3 years (Brooks et al., 2005). A total of seven QTL regions were identified (**Figure 1**). The two QTLs on chromosomes 2 and 4 were most significant and were associated with Mp313E. From these studies, up to 48% of the genotypic effects of the resistance can be explained by the QTLs identified on chromosomes 2, 3, and 4 from Mp313E. Significant progress has been made to increase corn host plant resistance by DNA marker-assisted breeding. However, a major obstacle has been that the genomic regions containing the QTLs are large and indecipherable without the knowledge of the underlying genetic and molecular information. Closely linked molecular markers to the QTLs, ideally from resistance genes, are needed to expedite the breeding process and to reduce the breeding cycles.

COMPARATIVE GENOME-WIDE GENE EXPRESSION STUDY ON SELECTED CORN INBRED LINES

Corn host resistance to *A. flavus* infection and aflatoxin accumulation is a quantitative trait potentially controlled by many genes. Characterization of the corresponding genes and their effects is essential for the breeding of resistant corn lines. To characterize genes involved in the corn host plant resistance, a microarray study was conducted to investigate gene expression patterns in two resistant corn inbred lines (Mp313E and Mp04:86) and two susceptible corn inbred lines (Va35, B73) under artificial inoculation in field conditions (Kelley et al., 2012).

Developing kernels of corn were collected from field for RNA preparation. The field experimental plan was a randomized, complete block design with split plots and three replications. The average aflatoxin levels in mature corn kernels from the inoculated primary ears of each genotype were measured as 195 ppb for Mp04:86, 140 ppb for Mp313E, 1243 ppb for Va35, and 3791 ppb for B73. Mp04:86 was a resistant recombinant inbred line derived from the cross of Mp715 (resistant) × Va35 (susceptible), and it was selected by the phenotypic trait of low-level aflatoxin accumulation. The microarrays (from the NSF Maize Oligonucleotide Array Project) used in this experiment contained 57,452 maize gene probes (Pontius et al., 2003).

The log2 fold changes (inoculation/un-inoculation) for each gene probe were used as gene expression values, resulting in a high-dimensional (57,452 × 4) data matrix. Principal component analysis (PCA) was used to analyze the resulting gene expression values. PCA is a commonly used technique in finding patterns and relationships between variables in data analysis and the results can be displayed in biplots. The direction and length of the arrows in the biplots represented the larger variances in the gene expression values of each corn inbred line (**Figures 2A,B**). By using PCA analysis, the resistant corn inbred lines (Mp313E and Mp04:86) were separated from the susceptible

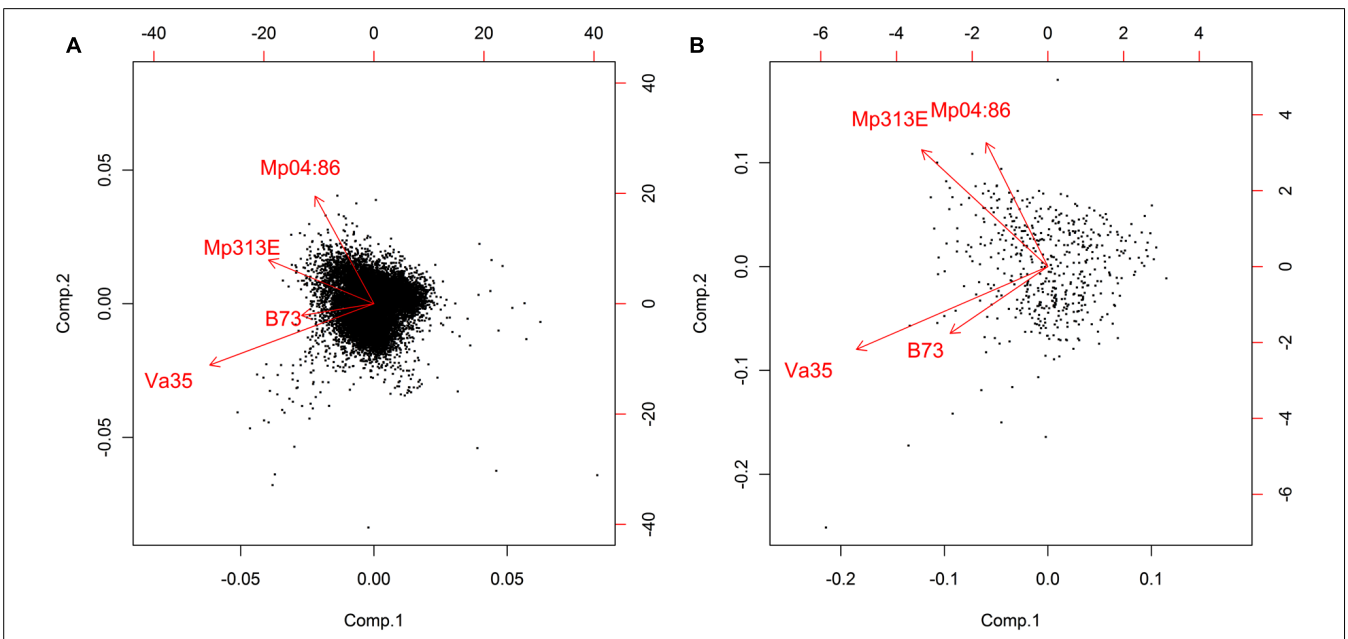

FIGURE 2 | Biplots of PCA on the log2 ratios (*I/U*) of expressed genes in corn inbred lines. The eigenvectors evidently separated into resistant and susceptible groups, respectively. Genes coordinately expressed in resistant lines located near the eigenvectors for Mp313E and Mp04:86. Genes coordinately expressed in susceptible lines located near the eigenvectors for Va35 and B73. **(A)** PCA of 13,107 expressed genes and **(B)** PCA of a subset of 500 expressed genes. The arrows represent eigenvectors. The direction and length represent the larger variance of the fold changes (Kelley et al., 2012).

corn inbred lines (Va35 and B73). It had important implications. The patterns of gene expression appeared to be similar in Mp313E and Mp04:86 in contrast to those of Va35 and B73. The fact that Mp04:86 was clustered with Mp313E (resistant, not a parent) but not with the susceptible parent Va35 strongly indicated that Mp313E and Mp04:86 might have shared similar resistance mechanisms. It is worth mentioning that the PCA analysis was directly applied to all gene expression values without any pre-selection by statistical significance levels. These findings demonstrated that all levels of the gene effects collectively contributed to the grouping of resistant genotypes versus susceptible genotypes.

The microarray data were also analyzed statistically to determine the significance levels and the significant genes were selected for further validation by quantitative real-time PCR (qRT-PCR) experiments. Fifty genes that were statistically significant from microarray study were selected for a qRT-PCR analysis in a separate time course experiment using developing kernel samples of Mp313E and Va35. Thirty-one of the 50 genes were found to be significantly differentially expressed ($P < 0.05$) by analysis of qRT-PCR data. Among these genes, eight were mapped to seven previously identified QTL regions (**Figure 1**; Kelley et al., 2012).

Three of the mapped significant genes (**Figure 1**) that were highly expressed in Va35 were known plant stress responsive genes. AI664980 encodes a glycine-rich RNA-binding protein (GRBP2; Naqvi et al., 1998; Govrin and Levine, 2000; Singh et al., 2011). TC234808 encodes a protein that governs the large and small ribosomal subunits assembly. Both AI664980 and TC234808 genes have RNA-binding domains and are likely to be involved in the post-transcriptional regulation in plant defense systems. BG266083 encodes stress-induced small heat shock proteins (sHSPs). The development of ear rot caused by *A. flavus* infection in the susceptible corn inbred line Va35 showed hypersensitivity. AI664980 (GRBP2) protein was reported to be associated with plant–pathogen hypersensitivity interactions (Naqvi et al., 1998).

The resistant corn inbred line Mp313E appeared to have highly expressed genes encoding RNA transport regulators, molecular chaperones, and detoxification proteins. TC231674 is homologous to the human nucleoporin NUP85 which is a component of the nuclear pore complexes (NPCs). Studies have strongly suggested that components of NPCs regulate the transport of R proteins (Cheng et al., 2009; Garcia and Parker, 2009; Faris et al., 2010). TC231674 gene was found highly expressed in the resistant line Mp313E. BM498943 encodes ethylene responsive protein (ETHRP) which belongs to the universal stress responsive protein family. BM379345 encodes a metallothionein-like protein (MTLP) involved in the detoxification of heavy metal ions. It was found that increased aflatoxin production was associated with high levels of certain trace metal elements (Lillehoj et al., 1974).

EXPLORATION OF COMPUTATIONAL AND STATISTICAL METHODS ON ANALYSIS OF RNA PATHWAY GENE EXPRESSION DATA

Evidence from the microarray study showed that TC231674, which encodes a possible RNA transport regulator, was highly expressed in the resistant corn inbred line Mp313E. A number of previous studies have demonstrated that RNA transport pathway genes play direct roles in plant defense (Piffanelli et al., 1999; Chisholm et al., 2006; Walley et al., 2007). RNA transport pathways are made up from a number of different protein complexes. For example, the cap binding complex (CBC), spliceosome, transcription-export complex (TREX), exon-junction complex (EJC), and translation initiation factors (eIFs) are involved in the transport of mRNA (Nakielny et al., 1997). Importins, exportins, Ran-GTP-related protein complex, and the survival motor neuron complex (SMN) are for the transport of rRNA, tRNA, and snRNA molecules (Kindler et al., 2005; Jambhekar and Derist, 2007; Vazquez-Pianzola and Suter, 2012). All RNAs are transported across the nuclear membrane through NPCs (Köhler and Hurt, 2007; Meier and Brkljacic, 2009; Deslandes and Rivas, 2011; Rivas, 2012). Several reports have shown that nucleoporins are directly involved in the regulation of R protein activities.

The expression patterns of corn RNA transport pathway genes and their relations were studied by a qRT-PCR experiment in the selected resistant and susceptible corn inbred lines (Asters et al., 2014). The advance of qRT-PCR technique makes it possible to precisely describe and compare the level of gene expression changes (Heid et al., 1996; Kubista et al., 2006; Rieu and Powers, 2009). In contrast to the comprehensive genome-wide microarray or RNA sequencing techniques, qRT-PCR provides the flexibility to measure gene expression levels on more samples across a wide range of experimental conditions as "phenotypic traits." This is an important and novel strategy since it presents a way to overcome the commonly seen "phenotyping bottleneck" due to the limitation of available measureable traits compared with the huge amount of genomic single nucleotide polymorphisms (SNPs) data. We aimed at the exploration on using gene expression values as the "phenotypic data" in addition to the levels of aflatoxin accumulation to conduct future genetic marker analysis.

Experiments were conducted to establish a computational pipeline for analysis of qRT-PCR gene expression data (Asters et al., 2014). This research aimed at the qRT-PCR gene expression analysis of more corn inbred lines, focused pathways, with an experimental design in parallel to a typical breeding project, so that statistical analysis over potential DNA markers, candidate gene expression levels, and aflatoxin levels could directly apply. Resistant (Mp718, Mp719, and Mp04:104) and susceptible (Va35, Mp04:85, and Mp04:89) corn inbred lines were used in this experiment. Experimental conditions consisted of two treatments (inoculated and un-inoculated with *A. flavus*), six corn inbred lines with three replications for each, and two sample collection time points at 2 and 7 days after inoculation (DAI). Since these inbred lines were offspring from a single cross of Mp715 and Va35, only up to two possible alleles for each gene were involved in all samples. The gene expression variations observed in these samples were likely related to gene regulating patterns associated with the resistance or susceptibility.

Forty RNA transport pathway genes and 16 candidate genes identified from the previous microarray experiment were investigated. Trends of gene expression patterns were observed

among the test corn inbred lines (**Figure 3**). AI664980 showed significant variations among samples with down-regulation patterns in resistant lines. TC231674 was found highly expressed in the resistant corn line Mp718. Of the 56 genes analyzed, 17 were significantly differentially expressed between the resistant and the susceptible groups. Most of the significant genes were from the NPC and SMN protein complexes. Resistance candidate genes BE050050, TC231674, and BM498943 were found positively correlated. Whereas the susceptibility-related gene AI664980 was found negatively correlated with BE050050 (−0.97; Asters et al., 2014). The inclusion of previously identified candidate genes in this research provided another way of validation. The observation was consistent with the previous findings on TC231674 where it was found highly expressed in a different resistant corn inbred line Mp313E.

The genes from the RNA transport pathways were considered as being selected from a static gene network. However, in contrast to the methods excessively used in the functional clustering by matching with Gene Ontology (GO) terms, the analysis in this study aimed to explore a method on determination of gene relationships from the empirical gene expression data. Susceptibility-related gene AI664980 was found clustered with six test genes (Nup88, eIF2, CD443591, CA399536, SPN1, and MAGOH) by a network analysis method, suggesting a similar expression pattern of these genes in response to *A. flavus* infection. Resistance candidate genes, such as TC231674 and BE050050, appeared as isolates in the network. Further experiments with more genes included are needed to reveal positions of these genes in the empirical network regarding corn defense against *A. flavus* infection and toxin production (Asters et al., 2014).

CONCLUSION

Many studies have shown that significantly low levels of aflatoxin accumulation have been achieved in a number of resistant corn inbred lines. However, an efficient transfer of such resistance to

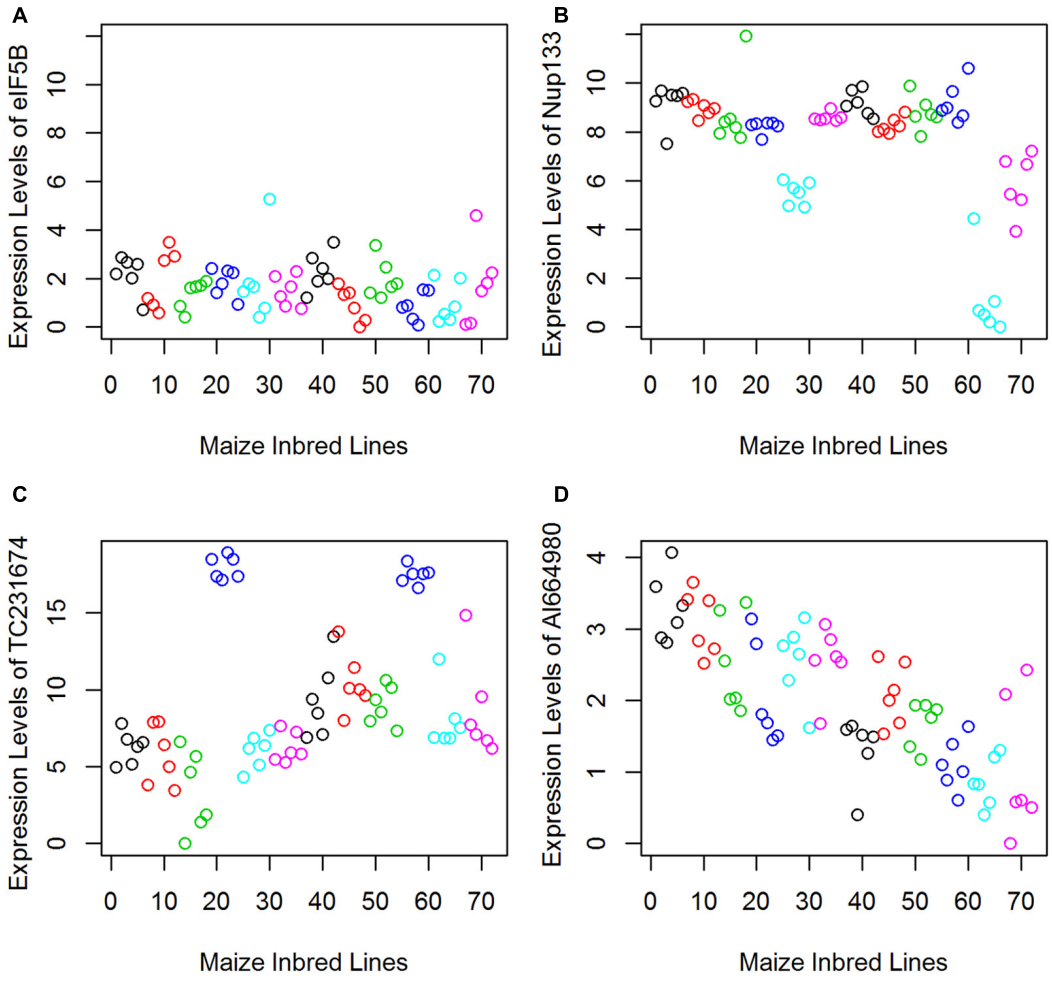

FIGURE 3 | Examples (A–D) showing that qRT-PCR gene expression values can be used as "phenotypic data" for statistical analysis. Each scatterplot represents gene expression values measured for one gene across all samples. The horizontal axis represents the 72 samples with different colors coded for the six corn inbred lines. Samples 1–36 were collected at 2 DAI and samples 37–72 were at 7 DAI. The vertical axis represents the relative gene expression values (Asters et al., 2014).

commercially available corn lines has been proven difficult due to the complex nature of this quantitative trait. Many genes were proposed to be involved in the corn host plant resistance to the aflatoxin reduction. The exploration for effective methods to identify and prioritize resistance genes will expedite the discovery of DNA markers and facilitate the breeding for corn host resistance.

For future work, we propose the use of multi-environmental gene expression data as "phenotypic data" for calculation of the DNA marker genetic effects in performing genomic selection for the breeding of corn host resistance. Parallel quantitative proteomics and metabolomics approaches will also be explored to identify proteins and metabolites related to *A. flavus* responsive pathways. Using transcriptomics, proteomics, and metabolomics data as "phenotypic data" will facilitate the identification of connections among genome regions, resistance-related genes and proteins, aflatoxin contents, and other metabolites for statistical analysis and modeling. The application of the resulting statistical models will be very helpful on estimating marker genetic effects and predicting the breeding values for aflatoxin resistance in corn.

REFERENCES

Asters, M. C., Williams, W. P., Perkins, A. D., Mylroie, J. E., Windham, G. L., and Shan, X. (2014). Relating significance and relations of differentially expressed genes in response to *Aspergillus flavus* infection in maize. *Sci. Rep.* 4, 4815. doi: 10.1038/srep04815

Bhatnagar, D., Cary, J. W., Ehrlich, K., Yu, J., and Cleveland, T. E. (2006). Understanding the genetics of regulation of aflatoxin production and *Aspergillus flavus* development. *Mycopathologia* 162, 155–166. doi: 10.1007/s11046-006-0050-9

Brooks, T. D., Williams, W. P., Windham, G. L., Willcox, M. C., and Abbas, H. K. (2005). Quantitative trait loci contributing resistance to aflatoxin accumulation in the maize inbred Mp313E. *Crop Sci.* 45, 171–174. doi: 10.2135/cropsci2005.0171

Buckley, P. M., Williams, W. P., and Windham, G. L. (2006). Comparison of two inoculation methods for evaluating corn for resistance to aflatoxin contamination. *Miss. AFES Bull.* 1148.

Cheng, Y. T., Germain, H., Wiermer, M., Bi, D., Xu, F., García, A. V., et al. (2009). Nuclear pore complex component MOS7/Nup88 is required for innate immunity and nuclear accumulation of defense regulators in *Arabidopsis*. *Plant Cell* 21, 2503–2516. doi: 10.1105/tpc.108.064519

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006). Host–microbe interactions: shaping the evolution of the plant immune response. *Cell* 124, 803–814. doi: 10.1016/j.cell.2006.02.008

Cleveland, T. E., Yu, J., Bhatnagar, D., Chen, Z. Y., Brown, R. L., Chang, P. K., et al. (2004). Progress in elucidating the molecular basis of the host plant–*Aspergillus flavus* interaction, a basis for devising strategies to reduce aflatoxin contamination in crops. *J. Toxicol.* 23, 345–380.

Daves, C. A., Windham, G. L., and Williams, W. P. (2010). Aflatoxin Accumulation in commercial corn hybrids artificially inoculated with *Aspergillus flavus* in 2008 and 2009. *Miss. AFES Res. Rep.* 24:9.

Davis, G. L., Windham, G. L., and Williams, W. P. (2000). QTL for aflatoxin reduction in maize. *Maize Genet. Conf.* 41, 22.

Deslandes, L., and Rivas, S. (2011). The plant cell nucleus, a true arena for the fight between plants and pathogens. *Plant Signal. Behav.* 6, 42–48. doi: 10.4161/psb.6.1.13978

Faris, J. D., Zhang, Z. C., Lu, H. J., Lu, S. W., Reddy, L., Cloutier, S., et al. (2010). A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13544–13549. doi: 10.1073/pnas.1004090107

Garcia, A. V., and Parker, J. E. (2009). Heaven's gate: nuclear accessibility and activities of plant immune regulators. *Trends Plant Sci.* 14, 479–487. doi: 10.1016/j.tplants.2009.07.004

Gourama, H., and Bullerman, L. B. (1995). *Aspergillus flavus* and *Aspergillus parasiticus*: aflatoxigenic fungi of concern in foods and feeds. *J. Prot. Ecol.* 58, 1395–1404.

Govrin, E. M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* 10, 751–757. doi: 10.1016/S0960-9822(00)00560-1

Heid, C. A., Stevens, J., Livak, K. J., and Williams, M. P. (1996). Real time quantitative PCR. *Genome Res.* 6, 986–994. doi: 10.1101/gr.6.10.986

Jambhekar, A., and Derist, J. L. (2007). Cis-acting determinants of asymmetric, cytoplasmic RNA transport. *RNA* 13, 625–642. doi: 10.1261/rna.262607

Kelley, R. Y., Williams, W. P., Mylroie, J. E., Boykin, D. L., Harper, J. W., Windham, G. L., et al. (2012). Identification of maize genes associated with host plant resistance or susceptibility to *Aspergillus flavus* infection and aflatoxin accumulation. *PLoS ONE* 7:e36892. doi: 10.1371/journal.pone.0036892

Kindler, S., Wang, H. D., Richter, D., and Tiedge, H. (2005). RNA transport and local control of translation. *Annu. Rev. Cell. Dev. Biol.* 21, 223–245. doi: 10.1146/annurev.cellbio.21.122303.120653

Köhler, A., and Hurt, E. (2007). Exporting RNA from the nucleus to the cytoplasm. *Nat. Rev. Mol. Cell Biol.* 8, 761–773. doi: 10.1038/nrm2255

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jona, J., Lind, K., et al. (2006). The real-time polymerase chain reaction. *Mol. Aspects Med.* 27, 95–125. doi: 10.1016/j.mam.2005.12.007

Larson, E. (1997). Minimizing aflatoxin in corn. *Miss. Cooper. Ext. Serv. Info. Sheet* 1563.

Lillehoj, E. B., Garcia, W. J., and Lambrow, M. (1974). *Aspergillus flavus* infection and aflatoxin production in corn: influence of trace elements. *Appl. Microbiol.* 28, 763–767.

Meier, I., and Brkljacic, J. (2009). The nuclear pore and plant development. *Curr. Opin. Plant Biol.* 12, 87–95. doi: 10.1016/j.pbi.2008.09.001

Nakielny, S., Fischer, U., Michael, W. M., and Dreyfuss, G. (1997). RNA transport. *Annu. Rev. Neurosci.* 20, 269–301. doi: 10.1146/annurev.neuro.20.1.269

Naqvi, S. M. S., Park, K. S., Yi, S. Y., Lee, H. W., Bok, S. H., and Choi, D. (1998). A glycine-rich RNA-binding protein gene is differentially expressed during acute hypersensitive response following Tobacco Mosaic Virus infection in tobacco. *Plant Mol. Biol.* 37, 571–576. doi: 10.1023/A:1006031316476

Payne, G. A. (1992). Aflatoxins in maize. *Crit. Rev. Plant Sci.* 10, 423–440. doi: 10.1080/07352689209382320

Piffanelli, P., Devoto, A., and Schulze-Lefert, P. (1999). Defence signaling pathways in cereals. *Curr. Opin. Plant Biol.* 2, 295–300. doi: 10.1016/S1369-5266(99)80052-1

Pontius, J. U., Wagner, L., and Schuler, G. D. (2003). "UniGene: a unified view of the transcriptome," in *The NCBI Handbook*, Bethesda, MD: National Center for Biotechnology Information.

Rieu, I., and Powers, S. J. (2009). Real-time quantitative RT-PCR: design, calculations, and statistics. *Plant Cell* 21, 1031–1033. doi: 10.1105/tpc.109.066001

Rivas, S. (2012). Nuclear dynamics during plant innate immunity. *Plant Physiol.* 158, 87–94. doi: 10.1104/pp.111.186163

Robens, J., and Cardwell, K. F. (2005). "The cost of mycotoxin management in the United States," in *Aflatoxin and Food Safety*, ed. H. K. Abbas (Boca Raton, FL: CRC Press), 1–12. doi: 10.1201/9781420028171.ch1

Scott, G. E., and Zummo, N. (1990). Registration of Mp313E parental line of maize. *Crop Sci.* 30:1378. doi: 10.2135/cropsci1990.0011183X003000060080x

Scott, G. E., and Zummo, N. (1992). Registration of Mp420 germplasm line of maize. *Crop Sci.* 32:1296. doi: 10.2135/cropsci1992.0011183X003200050054x

Singh, U., Deb, D., Singh, A., and Grover, A. (2011). Glycine-rich RNA binding protein of *Oryza sativa* inhibits growth of M15 *E. coli* cells. *BMC Res. Notes* 4:18. doi: 10.1186/1756-0500-4-18

Thompson, D. L., Payne, G. A., Lillehoj, E. B., and Zuber, M. S. (1983). Early appearance of aflatoxin in developing corn kernels after inoculation with *Aspergillus flavus*. *Plant Dis.* 67, 1321–1322. doi: 10.1094/PD-67-1321

Vazquez-Pianzola, P., and Suter, B. (2012). Conservation of the RNA transport machineries and their coupling to translation control across eukaryotes comparative and functional genomics. *Comp. Funct. Genomics* 2012, 287852. doi: 10.1155/2012/287852

Walley, J. W., Coughlan, S., Hudson, M. E., Covington, M. F., Kaspi, R., Banu, G., et al. (2007). Mechanical stress induces biotic and abiotic stress responses via a novel cis-element. *PLoS Genet.* 3:e172. doi: 10.1371/journal.pgen.0030172

Willcox, M. C., Davis, G. L., Warburton, M. L., Windham, G. L., Abbas, H. K., Betran, J., et al. (2013). Confirming QTL for aflatoxin resistance from Mp313E in different genetic backgrounds. *Mol. Breed.* 32, 15–26. doi: 10.1007/s11032-012-9821-9

Williams, W. P. (2006). Breeding for resistance to aflatoxin accumulation in maize. *Mycotoxin Res.* 22, 27–32. doi: 10.1007/BF02954554

Williams, W. P., Krakowsky, M. D., Windham, G. L., Balint-Kurti, P., Hawkins, L. K., and Henry, W. B. (2008). Identification maize germplasm with resistance to aflatoxin accumulation. *Toxin Rev.* 27, 319–345. doi: 10.1080/15569540802399838

Williams, W. P., and Windham, G. L. (2001). Registration of maize germplasm line Mp715. *Crop Sci.* 41, 1374–1375. doi: 10.2135/cropsci2001.4141374-ax

Williams, W. P., and Windham, G. L. (2006). Registration of maize germplasm line Mp717. *Crop Sci.* 46, 1407. doi: 10.2135/cropsci2005.09-0330

Williams, W. P., and Windham, G. L. (2012). Registration of Mp718 and Mp719 Germplasm lines of maize. *J. Plant Regist.* 6, 200–202. doi: 10.3198/jpr2011.09.0489crg

Williams, W. P., Windham, G. L., and Buckley, P. M. (2005a). "Enhancing maize with resistance to *Aspergillus flavus* infection and aflatoxin accumulation," in *Aflatoxin and Food Safety*, ed. H. K. Abbas (New York, NY: CRC Press), 379–394.

Williams, W. P., Windham, G. L., Buckley, P. M., and Perkins, J. M. (2005b). Southwestern corn borer damage and aflatoxin accumulation in conventional and transgenic corn hybrids. *Field Crops Res.* 91, 329–336. doi: 10.1016/j.fcr.2004.08.002

Williams, W. P., Windham, G. L., and Buckley, P. M. (2007). Diallel analysis of aflatoxin accumulation in maize. *Crop Sci.* 48, 134–138. doi: 10.2135/cropsci2007.05.0306

Williams, W. P., Windham, G. L., Buckley, P. M., and Daves, C. A. (2002). Aflatoxin accumulation in conventional and transgenic corn hybrids infested with southwestern corn borer (Lepidoptera: Crambidae). *J. Agric. Urban Entomol.* 19, 227–236.

Windham, G. L., Hawkins, L. K., and Williams, W. P. (2010). Aflatoxin accumulation and kernel infection of maize hybrids inoculated with *Aspergillus flavus* and *Aspergillus parasiticus*. *World Mycotoxin J.* 3, 89–93. doi: 10.3920/WMJ2009.1183

Windham, G. L., and Williams, W. P. (1998). *Aspergillus flavus* infection and accumulation in resistant and susceptible maize hybrids. *Plant Dis.* 82, 281–284. doi: 10.1094/PDIS.1998.82.3.281

Windham, G. L., and Williams, W. P. (1999). Aflatoxin accumulation in commercial corn hybrids in 1998. *Miss. AFES Res. Rep.* 22:8.

Windham, G. L., and Williams, W. P. (2002). Evaluation of corn inbred lines and advanced breeding lines for resistance to aflatoxin contamination in the field. *Plant Dis.* 86, 232–234. doi: 10.1094/PDIS.2002.86.3.232

Windham, G. L., Williams, W. P., Buckley, P. M., Abbas, H. K., and Hawkins, L. K. (2005). "Techniques used to identify aflatoxin resistant corn," in *Aflatoxin and Food Safety*, ed. H. K. Abbas (New York, NY: CRC Press), 407–421.

Windham, G. L., Williams, W. P., and Davis, F. M. (1999). Effects of the southwestern corn borer on *Aspergillus flavus* kernel infection and aflatoxin accumulation in maize hybrids. *Plant Dis.* 83, 535–540. doi: 10.1094/PDIS.1999.83.6.535

Windham, G. L., Williams, W. P., Hawkins, L. K., and Brooks, T. D. (2009). Effect of *Aspergillus flavus* inoculation methods and environmental conditions on aflatoxin accumulation in corn hybrids. *Toxin Rev.* 28, 70–78. doi: 10.1080/15569540802450037

Zummo, N., and Scott, G. E. (1989). Evaluation of field inoulation teqhniques for screening maize genotypes against kernel infection by *Aspergillus flavus* in Mississippi. *Plant Dis.* 73, 313–316. doi: 10.1094/PD-73-0313

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A mini review on aflatoxin exposure in Malaysia: past, present, and future

Sabran Mohd-Redzwan[1], Rosita Jamaluddin[1]*, Mohd Sokhini Abd.-Mutalib[1] and Zuraini Ahmad[2]

[1] Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Malaysia
[2] Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Malaysia

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Eva-Guadalupe Lizárraga-Paulín, Universidad Nacional Autónoma de México, Mexico
Perng-Kuang Chang, Southern Regional Research Center, USA

***Correspondence:**
Rosita Jamaluddin, Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
e-mail: rosita@medic.upm.edu.my

This mini review article described the exposure of aflatoxin in Malaysia, including its presence in the foodstuffs and the detection of aflatoxin biomarkers in human biological samples. Historically, the exposure of aflatoxin in Malaysia can be dated in 1960s where an outbreak of disease in pig farms caused severe liver damage to the animals. Later, an aflatoxicosis case in Perak in 1988 was reported and caused death to 13 children, as up to 3 mg of aflatoxin was present in a single serving of contaminated noodles. Since then, extensive research on aflatoxin has been conducted in Malaysia. The food commodities such as peanuts, cereals, spices, and their products are the main commodities commonly found to be contaminated with aflatoxin. Surprisingly, some of the contaminated foods had levels greater than the permissible limit adopted by the Malaysian Food Regulation 1985. Besides, exposure assessment through the measurement of aflatoxin biomarkers in human biological samples is still in its infancy stage. Nevertheless, some studies had reported the presence of these biomarkers. In fact, it is postulated that Malaysians are moderately exposed to aflatoxin compared to those high risk populations, where aflatoxin contamination in the diets is prevalent. Since the ingestion of aflatoxin could be the integral to the development of liver cancer, the incidence of cancer attributable by dietary aflatoxin exposure in Malaysia has also been reported and published in the literatures. Regardless of these findings, the more important task is to monitor and control humans from being exposed to aflatoxin. The enforcement of law is insufficient to minimize human exposure to aflatoxin. Preventive strategies include agricultural, dietary, and clinical measures should be implemented. With the current research on aflatoxin in Malaysia, a global networking for research collaboration is needed to expand the knowledge and disseminate the information to the global scientific community.

Keywords: aflatoxin, aflatoxin B_1, aflatoxin exposure, Malaysia, Southeast Asia

INTRODUCTION

There are many toxic compounds produced by fungi and one of them is mycotoxin. The occurrence of mycotoxin in the food commodities can be dated as early as in the tenth century. The infection of fungi in the diets caused the outbreak of disease known as St. Anthony's or Holy Fire in many European countries (Paterson and Lima, 2010). Paterson and Lima (2010) reported the outbreak was intensified with the contamination of rye by ergot alkaloid, produced by *Claviceps purpurea*. Since then, many cases have been reported and the discovery of "Turkey X" disease caused by aflatoxin, in 1950s and early 1960s had opened new prospectus on the scientific research on the etiology of mycotoxicosis and preventive strategies in foods, animals, and humans (Kensler et al., 2011). Kensler et al. (2011) described the epidemic disease as the major cause of death of numerous poultry animals including ducklings and chicks due to the consumption of diet containing contaminated peanuts. Further investigations at that time revealed the toxicity was associated with *Aspergillus flavus*, a pathogenic fungus and extracts from the culture of the fungus isolated from the meal were found to have the capability to induce the "Turkey X" syndromes (Kensler et al., 2011). Due to this, most of the reported cases on mycotoxicosis focused on specific species of fungi that are found in many contaminated foods and feeds. Indeed, four major species of fungi have been discovered belonging to the species of *Aspergillus*, *Fusarium*, *Penicillium*, and *Claviceps* that produced some major mycotoxins such as aflatoxin, ochratoxin A, fumonisim, and zearalenone (Paterson and Lima, 2010). Of these four mycotoxins, research on aflatoxin has been extensively conducted as aflatoxin represents the global main threat due to its toxicity (Paterson and Lima, 2010; Kensler et al., 2011)

Aflatoxin is produced by *A. flavus*, *A. paraciticus*, and *A. nomius* and ubiquitously found in foodstuffs (Reddy et al., 2011). Since aflatoxins are visible under ultraviolet (UV) light, the first attempt to detect aflatoxin is mainly based on this criteria (Groopman et al., 2005). Later the isolation of purified aflatoxin metabolites with identical physical and chemical properties formed the core to the scientific research on aflatoxin (Kensler et al., 2011). These findings have stimulated numerous research efforts, which transcend to the present, to assess possible hazards of aflatoxin contamination in the human food sources and finally to reduce the exposure through various preventive strategies. In one of many instances, the development of analytical methods to detect and

quantify aflatoxin in foods and feeds is significance (Kensler et al., 2011) as more studies are able to be conducted to determine the association of aflatoxin ingestion with diseases in human population, especially with the incidence of hepatocellular carcinoma (HCC). Moreover, as the science is moving forward in parallel to the advancement of technology, the development of method for structural characterization, and synthesis of the major aflatoxins has led to better understanding of the mechanistic studies of their toxicology and metabolism (Groopman et al., 2005; Paterson and Lima, 2010; Kensler et al., 2011). For instance, the isolation of aflatoxin biomarkers in human biological samples such as serum AFB_1-DNA adduct, AFB_1-lysine adduct, and other metabolites of AFB_1 in urine and feces (Wang et al., 1999; Mykkänen et al., 2005; Polychronaki et al., 2008) can give a better indication on the extent of human exposure to aflatoxin. These data are essential for the risk assessment as more concrete evidence can be used to evaluate human exposure to aflatoxin in a population.

PAST-HISTORY OF AFLATOXIN IN MALAYSIA

Malaysia is one of the Southeast Asia countries and geographically located in the equatorial area. Due to that, Malaysia experiences tropical climate with high temperature around 28–31°C (Abdullah et al., 1998). On average, the relative humidity ranged from 70 to 80% during wet season and 50–60% during the dry season (Sulaiman et al., 2007). Furthermore, commodities stored under these conditions are easily deteriorated and susceptible to fungal infections (Abdullah et al., 1998; Sulaiman et al., 2007). With these conditions, the most prominent contamination is the infection of foods by mycotoxin-producing fungi. Aflatoxin, one of the mycotoxin is produced by *Aspergillus* species of fungi and this toxin has been classified by the International Agency for Research on Cancer (IARC) as Group 1 carcinogen and is linked to the development of liver cancer (International Agency for Research on Cancer [IARC], 1993).

In Malaysia, the first indicator of problem associated with aflatoxin occurred around 1960s due to the outbreak of disease in two pig farms in Malacca (Hamid, 1997). Lim and Yeap (1966) reported the presence of aflatoxin in the feed ingredients, supported the evidences of severe liver damage to the animals. However, an episode in Perak, Malaysia around 1988 reminded the hazard of aflatoxin to human health, as the public was shocked by the death of 13 children as a result of consuming contaminated noodles called *loh shi fun*. The post-mortem was performed and the cause of death was associated with acute hepatic encephalopathy (Lye et al., 1995). The culprit was aflatoxin as Lye et al. (1995) reported up to 3 mg of aflatoxin was detected in a single serving of *loh shi fun*. Furthermore, the wheat flour used to make the noodles was reported to contain aflatoxin due to the poor storage and processing, which in turn promoted the growth of pathogenic *Aspergillus* fungi and subsequently the production of aflatoxin. Since then, extensive research on aflatoxin has been carried out and the first monitoring was performed by the Institute of Medical Research (IMR), followed by other agencies such as The Food Technology Center (FTC), MARDI, and local universities (Hamid, 1997). Up till know, various studies in Malaysia have reported the presence of aflatoxin in the foodstuffs. Moreover, the biomarkers of aflatoxin have also been reported recently in serum and urine samples, which can provide additional information for assessing aflatoxin exposure in Malaysia.

PRESENT – OCCURRENCE OF AFLATOXIN IN THE FOOD COMMODITIES, HUMAN EXPOSURE AND RISK ASSESSMENT

The contamination of foodstuffs by aflatoxin has been documented in the literatures. Peanuts, cereals, spices, and their products are the commodities most susceptible to aflatoxin contamination. These food commodities are generally used in many Malaysian delicacies either as a main ingredient or as a base material (Reddy et al., 2011). In fact, they are available almost in all retail shops in Malaysia and are inexpensive. For example, various studies have detected aflatoxin in the peanuts and peanut-based products as shown **Table 1**. Sulaiman et al. (2007) indicated that raw shelled peanut and its products are the good substrate for the growth of aflatoxin by *A. flavus* and *A. paraciticus*. Even though peanuts are not extensively produced in Malaysia, the occurrence of aflatoxin in this food commodity is frequently reported. Moreover, Leong et al. (2011) postulated that high level of AFB_1 in nuts can result in the high potential risk of AFB_1 exposure in Malaysia. In fact, Malaysians probably consume higher amount of AFB_1 than Europeans and Americans but lower than those in Africa and China (Leong et al., 2011).

Cereals such as rice are one of the main agricultural products in Malaysia. The presence of aflatoxin in the rice could pose serious health issues. For example, in a study by Soleimany et al. (2012a), 33.3% of analyzed rice from the retail market had detectable level of aflatoxin ranging from 0.19 to 3.96 ng/g. Even though the levels are not considered dangerous and harmful to humans, it was an unexpected finding as rice is the main staple food consumed by majority of Malaysian. Statistically, Malaysians consume rice about 289.68 g/day (Ministry of Health [MOH], 2006). Thus it is assumed that dietary aflatoxin exposure from rice is somewhere between 55.04 ng/day and 1.15 μg/day [288.68 g/day × 0.19 or 3.96 ng/g] for an adult of 60 kg. Given that the aflatoxin-contaminated rice is consumed on a regular basis, this small amount of aflatoxin at the end will be accumulated in the body and can be detrimental to the health as previously reported.

As highlighted in **Table 1**, varieties of food samples were found to be contaminated with aflatoxin. The detection rate ranged from 16% up to 92% showing the ubiquitous presence of aflatoxin in the human food resources. Although the findings do not represent the whole scenario of aflatoxin contamination in Malaysia, such information shows the pervasiveness of human exposure to this food contaminant. In an instance, Reddy et al. (2011) detected aflatoxin in the foods normally consumed by Malaysian, where they are used and consumed on a daily basis. The presence of aflatoxin in the food chain is a serious matter but not knowing its impact to the health is a big problem and should be a public concern. Indeed in Malaysia, a recent survey reported low awareness and knowledge among the public on the problems associated with fungal and aflatoxin contamination in the diets (Mohd Redzwan et al., 2012a). Furthermore, the similar observations were also reported by several studies in African countries (Jolly et al., 2006, 2009; Ilesanmi and Ilesanmi, 2011).

Table 1 | Occurrence of aflatoxin in the foodstuffs in Malaysia.

Food commodity	Samples	Positive samples, *n* (%)	Aflatoxin levels	Important findings	Reference
Spices and herbs	Whole and ground, black and white peppercorn	70/126 (55.5)	0.1–4.9 ng/g[a]	None of the positive had levels exceeded the regulatory limit set by the Malaysian Food Regulation 1985. Black peppers were more contaminated than white pepper	Jalili et al. (2009)
	Commercial dried chili	52/80 (65.0)	0.2–56.61 ng/g[b]	6 (7.5%) and 9 (11.3%) of positive samples had AFB_1 and total AFs levels greater than 5 ng/g for AFB_1 and 10 ng/g for total AFs	Jalili and Jinap (2012)
	Spices from Penang markets	14/15 (93.3%)	0.58–4.64 μg/kg[b]	Two cumin powders contained highest levels of AFB_1 ranging from 1.89 to 4.64 μg/kg. Besides, four pepper samples were contaminated with AFB_1 ranging from 0.65 to 2.1 μg/kg	Reddy et al. (2011)
	Chili	9/10 (90.0%)	5.85–44.2 ng/g[a]	Samples were contaminated mainly by AFB_1. Samples that contained AFB_1 had levels exceeded the legal limits established by European. In fact, one of the positive samples had AFB_1 level of 33.2 ng/g, about seven times higher than the regulatory limit of 5 ng/g by Malaysia Food Regulation, 1985	Khayoon et al. (2012)
Cereals	Starch-based foods (wheat flour)	18/83 (21.7)	11.25–436.25 μg/kg[a]	Of 18 positive samples, 11 had total AFs concentration higher than permitted level adopted by the Malaysian Food Regulation 1985 (i.e., 35 μg/kg), ranging from 41.88 to 436.25 μg/kg	Abdullah et al. (1998)
	Rice, wheat, barley, oat, and maize-meal	40/80 (50.0)	0.12–4.42 ng/g[a]	Although the levels detected were not high, the highest level was found in rice samples. Moreover, 60 out of 80 samples analyzed had at least one mycotoxin	Soleimany et al. (2012b)
	Rice, wheat, oat, barley, and maize-meal	60/100 (60.0)	0.12–4.54 ng/g[a]	More than 50% of sample analyzed contained aflatoxin. Two rice samples were contaminated with aflatoxin at levels of 4.32 and 4.54 ng/g. In fact, 77 of 100 cereal samples were detected to have at least one myctoxin	Soleimany et al. (2012a)
	Cereal-based foods	29/45 (64.4)	0.65–8.95 μg/kg[b]	High incidence of AFB_1 was found in corn-based product (75%), followed by rice-(69.2%), wheat-(64.2%) and oats-(50%) based foods	Reddy et al. (2011)
	Corn-based products	5/11 (45.5%)	2.5–12.2 ppb (AFB_1), 5.8–101.8 ppb (AFB_2)	Two popcorn products exceeded the Malaysia permissible level of 5 ppb	Hong et al. (2010)

(Continued)

Table 1 | Continued

Food commodity	Samples	Positive samples, *n* (%)	Aflatoxin levels	Important findings	Reference
	Red rice	46/50 (92%)	0.61–77.3 μg/kg[a]	*Aspergillus flavus* was isolated in 44% samples. In addition, 35 red rice samples had AFs level higher than the Malaysian standard of 5 μg/kg and European standard of 4 μg/kg	Samsudin and Abdullah (2013)
	Black and white rice	4/5 (80.0%)	1.10–5.28 ng/g[a]	One black and white rice respectively had total AFs greater than 5 ng/g set by the Malaysia Food Regulation, 1985	Khayoon et al. (2012)
Peanuts and nuts	Peanut and nuts	13/20 (65.0%)	0.66–15.33 μg/kg	Of 13 peanuts samples, 11 were positive with AFB_1 with the mean contamination of 4.25 μg/kg	Reddy et al. (2011)
	Nuts and commercial nutty products	32/196 (16.3)	16.6–711 μg/kg[a]	The raw groundnut (shell) and coated nut product had total concentration of aflatoxin of 711 and 514 μg/kg, respectively	Leong et al. (2010)
	Raw shelled peanuts	73/145 (50.0)	0.8–762.1 μg/kg[a]	45% of positive samples exceeded the maximum permitted levels of 35 μg/kg adopted by the Malaysian Food Regulation, 1985	Sulaiman et al. (2007)
	Nuts and nut products	73/128 (57.0)	0.4–222 μg/kg[b]	17 of the 128 samples (13.3%) analyzed had AFB_1 greater than permissible set by the European Commission. The highest mean value of AFB_1 was detected in fried peanuts (58.9 μg/kg), followed by bakery products (12 μg/kg)	Leong et al. (2011)
	Raw peanut kernels	66/84 (78.6)	2.76–97.28 ng/g[a]	Of these positive samples, 10.7% had levels exceeded the maximum tolerable of 15 ng/g for total aflatoxin set by Codex. AFB_1 was the most prevalent aflatoxin detected, followed by AFB_2 and AFG_1	Arzandeh et al. (2010)
	Peanut	4/9 (44.4%)	2.15–6.36 ng/g[a]	At least one metabolite of aflatoxin was detected in the positive samples, except for AFB_2. The level of AFB_1 (6.00 ng/g) was the highest among the positive samples	Khayoon et al. (2012)
	Peanuts-based product	5/9 (55.6%)	19.5–33.4 ppb (AFB_1), 0.2 ppb (AFB_2)	AFB_1 was found predominantly in the positive samples	Hong et al. (2010)

AFs, Aflatoxins; [a] *Total aflatoxin;* [b] *AFB_1 .*

Exposure assessment by measuring aflatoxin levels in the food samples and extrapolating to estimate average intake at the population level is of low reliability (Kensler et al., 2011). The reason is such data cannot provide information on the individual's exposure as different individuals may have different exposure to aflatoxin. Nevertheless, the expansion of metabolomic study such as the use of aflatoxin biomarkers could provide the tools for molecular epidemiology to evaluate aflatoxin exposure among individuals within human population. Besides, measuring aflatoxin biomarkers could improve estimation of aflatoxin exposure (Liu and Wu, 2010). The aflatoxin biomarkes such as AFB_1-DNA adduct, AFB_1-lysine adduct, and urinary AFM_1 have been documented in the literatures especially in African countries (Gong et al., 2002; Jolly et al., 2006; Shuaib et al., 2010) and China (Mykkänen et al., 2005; Xu et al., 2010), where aflatoxin contaminations are ubiquitous. These biomarkers are useful for epidemiologist and public health workers to make a better postulation on the extent and severity of aflatoxin exposure in a population. For example, the detection of AFB_1-DNA adduct such as AFB_1-N^7-guanine in the urine samples could be an indicator of mutagenic effect of aflatoxin.

In Malaysia, exposure assessment by measuring aflatoxin biomarkers in human biological samples is still in its infancy. Nevertheless, several studies have reported the presence of these biomarkers among the Malaysia population. Indeed, it is believed that study by Zulhabri et al. (2009) is the first one in Malaysia that reported AFB_1-albumin adduct in the HCC patients. Zulhabri et al. (2009) indicated that the HCC patients had significantly higher level of AFB_1-albumin adduct compared to the healthy subjects. Aflatoxin can be an integral to the development of liver cancer (International Agency for Research on Cancer [IARC], 1993), thus the finding could provide an interesting insight on the synergistic effect of aflatoxin exposure. On the other hand, a recent study by Leong et al. (2012) found AFB_1-lysine adduct in 97% of 170 subjects in Penang, with the levels ranging from 0.2 to 23.16 pg/mg albumin. The study also reported that Chinese and Indian respectively were 3.05 and 2.35 times more likely to have high AFB_1-lysine adduct compared to the Malay (Leong et al., 2012). As a matter of fact, it was corroborated with a report from the Ministry of Health, Malaysia that showed high incidence of liver cancer among Chinese compared to other main ethnicities (Zainal Ariffin and Nor Saleha, 2011). Statistically in 2007, the report listed liver cancer as number 5 out of 10 most frequent cancers among male Chinese with the incidence rate of 5.9% (Zainal Ariffin and Nor Saleha, 2011). Besides, the other aflatoxin biomarkers have also been investigated. A preliminary study involving 22 adult subjects found the presence urinary AFM_1 ranging from 0.029 to 0.15 ng/ml (Sabran et al., 2012). Moreover, a significant and positive association was detected between urinary AFM_1 with the consumption of milk and dairy products among 98 subjects with detectable level of AFM_1 in Malaysia (Mohd Redzwan et al., 2012b). Since AFM_1 is a metabolite of AFB_1, such findings indicated by Mohd Redzwan et al. (2012b) and Sabran et al. (2012) are useful to estimate human exposure to aflatoxin as a study has found good correlation between dietary intake of AFB_1 and urinary excretion of metabolite AFM_1 (Zhu et al., 1987).

Given that ingestion of aflatoxin can be linked to the development of liver cancer, a risk assessment can provide additional information on the extent of human exposure to this food contaminant. Risk assessment is the process of estimating the magnitude and the probability of a harmful effect to individuals or populations for certain agents or activities (Liu and Wu, 2010). Several studies have reported Malaysian population could be exposed as high as 140 ng/kg body weight/day (**Table 2**). In one of the examples given in **Table 2**, Chin et al. (2012) indicated that Malaysian exposed to AFB_1 ranging from 24.3 to 34 ng/kg body weight/day and such exposure could contribute to 12.4–17.3% of liver cancer in Malaysia. It was noteworthy as Liu and Wu (2010) described that more than 55 billion people worldwide facing uncontrolled exposure of aflatoxin and the burden of aflatoxin-induced liver cancer still remained unclear. Although it seems impossible to remove and eliminate aflatoxin completely from the food chain, these data are useful to develop plans to control and monitor aflatoxin in the foodstuffs. In fact, interventions could be done to prevent human exposure to aflatoxin and reduce the incidence of aflatoxin-related diseases in Malaysia.

Table 2 | Risk assessment of aflatoxin exposure in Malaysia.

Dietary AFB_1 exposure (ng/kg body weight/day)	Estimated liver cancer risk (Cases/100, 000 population/year)	Cancer incidence attributable to dietary aflatoxin (%)	Reference
24.37–34.00	0.61–0.85	12.4–17.3	Chin et al. (2012)
28.81–58.02[a]	0.72–1.45	14.7–29.6	
0.36–8.89	0.03–0.73	0.61–14.9[c]	Leong et al. (2011)
26.2	0.66[b]	13.5[b]	Sabran et al. (2012)
15–140	4.5–42[d]	91–857[c]	Liu and Wu (2010)
10.69	0.27[b]	5.5[b]	Arzandeh et al. (2010)

[a] *Dietary aflatoxin exposure.*
[b] *The value was calculated based on formula given by Chin et al. (2012) – (Dietary exposure × 0.025 cancers/100, 000 in Malaysia).*
[c] *The value was computed from formula by Chin et al. (2012) – (Estimated liver cancer risk per 10,000 population/incidence rate of liver cancer in Malaysia of 4.9 per 100,000 × 100%).*
[d] *Data obtained from estimated annual HCC per 100,000 population for HBsAG-positive.*

FUTURE – MONITORING AND CONTROLLING THE OCCURRENCE OF AFLATOXIN AND INTERVENTION STRATEGIES

It is a very important task to monitor and control the presence of aflatoxin in the human food resources as it involves many aspects. The legislation of law for instance by setting a certain limit can be considered as the "first defense" to prevent aflatoxin exposure to human. This rule was established following the massive outbreak of diseases associated with aflatoxin back in 1960s as mentioned earlier. Prior to that in Malaysia, aflatoxin was not considered to be a significant problem as it was not covered by the Sale of Food and Drug Ordinance 1952. In fact, the ruling on aflatoxin-associated cases was referred from countries such as in UK and USA (Hamid, 1997). However, around 1980s, Malaysia has promulgated stricter standards to prevent the "flow" of aflatoxin in the food chain by the implementation of Malaysia Food Regulation 1985, through the Food Act 1983. Initially, a maximum limit of 35 μg/kg for all mycological contaminants was implemented as reported by studies back in 1990s (Hamid, 1997; Abdullah et al., 1998; Ali et al., 1999). Later, it was revised and the new legislation limit was set to 5 μg/kg for all mycological contaminants including aflatoxin. Moreover, a limit of 15 and 0.5 μg/kg was set for groundnut for further processing and milk products respectively.

With the enforcement of law, it will be meaningless if no proper actions are taken by the public to remove the aflatoxin-contaminated foodstuffs from the line of production. Although Malaysia has joined Codex Alimentarius since 1971 and strengthened the law on the occurrence of aflatoxin in the foodstuffs, it is still believed that some of the contaminated foods might "escape" during the surveillance process and persist in the food chain. A few studies explained that some contaminated foods may be perceived as safe and edible if there are no sign of defects and contaminations (Jolly et al., 2006; Mohd Redzwan et al., 2012b). Since the main route of human exposure to aflatoxin is through the diets, humans can be directly and/or indirectly exposed. Predicated upon that, interventions strategies have been carried out globally. Liu and Wu (2010) classified the strategies into three categories namely agricultural, dietary, and clinical. The agricultural interventions can be applied during pre- or post-harvest. This strategy is considered as the main primary intervention whereas dietary and clinical interventions are regarded as the secondary intervention (Liu and Wu, 2010).

Since then, various research around the globe have come up with measures to limit human exposure to this food contaminant. The use of adsorbents such as activated carbon, hydrated sodium calcium alumino silicate, zeolite, bentonite, and certain clays are beneficial as they are proven to prevent aflatoxin absorption (Denli and Okan, 2006; Thieu and Pettersson, 2008; Gallo et al., 2010). Recently, the use of probiotic has been studied as one the potential adsorbents of aflatoxin in the gastrointetstinal tract. The *in vitro* and animal studies found the potential of certain probiotic lactic acid bacteria in reducing the bioavailability of aflatoxin (El-Nezami et al., 1998; Haskard et al., 2000; Lahtinen et al., 2004; Gratz et al., 2006; Hernandez-Mendoza et al., 2009; Nikbakht Nasrabadi et al., 2013). In fact, probiotics have many beneficial health effects (Oelschlaeger, 2010), thus its use to counteract the toxicity of alfatoxin could be further examined as one the preventive strategies.

CONCLUSION

Malaysia is a country that is shifting to become a fully developed nation by 2020 and agricultural sector is one of the driving forces for the economy. The occurrence of aflatoxin in the agricultural commodities can be a big loss to the economy and consequently affect the nation. Aflatoxin is dangerous and potent toxin as a number of expert groups have reviewed the health effect of aflatoxin (International Agency for Research on Cancer [IARC], 1993; Joint FAO/WHO Expert Committee on food additives [JECFA], 1998). In Malaysia, reports and publications on the level of aflatoxin in the foodstuffs are abundant. Nevertheless, from the scarce literatures of human exposure to aflatoxin, it is clear that more works need to be done on this very important matter. This is because the information is useful to estimate the magnitude of aflatoxin exposure in Malaysia. On the global scale, a worldwide networking with other nations is essential to gather data and information for harmonization in order to prevent this problem continuing to happen in the future.

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REFERENCES

Abdullah, N., Nawawi, N., and Othman, I. (1998). Survey of fungal counts and natural occurrence of aflatoxins in Malaysian starch-based food. *Mycopathologia* 143, 53–58. doi: 10.1023/A:1006945514876

Ali, N., Hashim, N. H., and Yoshizawa, T. (1999). Evaluation and application of a simple and rapid method for analysis of aflatoxins in commercial foods from Malaysia and the Phillipines. *Food Addit. Contam.* 16, 273–280. doi: 10.1080/026520399283939

Arzandeh, S., Selamat, J., and Lioe, H. (2010). Aflatoxin in raw peanut kernels marketed in Malaysia. *J. Food Drug Anal.* 18, 44–50.

Chin, C. K., Abdullah, A., and Sugita-Konishi, Y. (2012). Dietary intake of aflatoxins in the adults Malaysian population – an assessment of riks. *Food Addit. Contam. Part B Surveill.* 5, 286–294. doi: 10.1080/19393210.2012.713028

Denli, M., and Okan, F. (2006). Efficacy of different adsorbents in reducing the toxic effects of aflatoxin B1 in broiler diets. *S. Afr. J. Anim. Sci.* 36, 222–228.

El-Nezami, H., Kankaapaa, P., Salminen, S., and Ahokas, J. (1998). Ability of dairy strains of lactic acid bacteria to bind a common food cacrcinogen, aflatoxin B1. *Food Chem. Toxicol.* 36, 321–326. doi: 10.1016/S0278-6915(97)00160-9

Gallo, A., Masoero, F., Bertuzzi, T., Piva, G., and Pietri, A. (2010). Effect of the inclusion of adsorbents on aflatoxin B1 quantification in animal feedstuffs. *Food Addit. Contam.* 27, 54–63. doi: 10.1080/02652030903207219

Gong, Y. Y., Cardwell, K., Hounsa, A., Egal, S., Turner, P. C., Hall, A. J., et al. (2002). Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: cross sectional study. *BMJ* 325, 20–21. doi: 10.1136/bmj.325.7354.20

Gratz, S., Taubel, M., Juvonen, M. T., Viluksela, M., Turner, P. C., Mykkanen, H., et al. (2006). Lactobacillus rhamnosus strain GG modulates intestinal absorption, fecal excretion and toxicity of aflatoxin B1 in rats. *Appl. Environ. Microbiol.* 72, 7398–7400. doi: 10.1128/AEM.01348-06

Groopman, J. D., Johnson, D., and Kensler, T. W. (2005). Aflatoxin and hepatitis B virus biomarkers: a paradigm for complex environmental exposures and cancer risk. *Cancer Biomark.* 1, 5–14.

Hamid, A. B. (1997). "Present status and future prospects of research on the groundnuts aflatoxin problem in Malaysia," in *Aflatoxin Contamination Problems in Groundnut in Asia: Proceeding of the First Asia Working Group Meeting*, eds V. K.

Mehan and C. L. L. Gowda (Hyderabad: International Crops Research Institute for the Semi-Arid Tropics), 32–35.

Haskard, C., Binnion, C., and Ahokas, J. (2000). Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. *Chem. Biol. Interact.* 128, 39–49. doi: 10.1016/S0009-2797(00)00186-1

Hernandez-Mendoza, A., Guzman-de-Pena, D., and Garcia, H. S. (2009). Key role of techoic acids on aflatoxin B1 binding by probiotic bacteria. *J. Appl. Microbiol.* 107, 395–403. doi: 10.1111/j.1365-2672.2009.04217.x

Hong, L. S., Mohd Yusof, N. I., and Ling, H. M. (2010). Determination of Aflatoxin B1 and B2 in peanuts and corn based products. *Sains Malays.* 39, 731–735.

"IARC, International Agency for Research on Cancer." (1993). "Aflatoxin," in *Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, Vol. 56 (Lyon: IARC), 245–395.

Ilesanmi, F. F., and Ilesanmi, O. S. (2011). Knowledge of aflatoxin contamination in groundnut and risk of its ingestion among health workers in Ibadan, Nigeria. *Asian Pac. J. Trop. Biomed.* 1, 493–495. doi: 10.1016/S2221-1691(11)60108-1

Jalili, M., and Jinap, S. (2012). Natural occurrence of aflatoxins and ochratoxin A in commercial dried chili. *Food Control* 24, 160–164. doi: 10.1016/j.foodcont.2011.09.020

Jalili, M., Jinap, S., and Adzahan, N. (2009). Survey of aflatoxin in retail samples of whole and ground black and white peppercorns. *Food Addit. Contam. Part B Surveill.* 2, 178–182. doi: 10.1080/19440040903384190

JECFA, Joint FAO/WHO Expert Committee on food additives. (1998). "Safety evaluation of certain food additives and contaminants in foods: aflatoxin. WHO Food Additives Series 40," in *49th meeting of the Joint FAO/WHO Expert Committee on Food Additives*, (Geneva: World Health Organization), 359–469.

Jolly, C. M., Bayard, B., Awuah, R. T., Fialor, S. C., and Williams, J. T. (2009). Examining the structure of awareness and perceptions of groundnuts aflatoxin among Ghanaian health and agricultural professionals and its influence on their actions. *J. Socioecon.* 38, 280–287. doi: 10.1016/j.socec.2008.05.013

Jolly, P., Jiang, Y., Ellis, W., Awuah, R., Nnedu, O., Phillips, T., et al. (2006). Determinants of aflatoxin levels in Ghanaians: sociodemographic factors, knowledge of aflatoxin and food handling and consumption practices. *Int. J. Hyg. Environ. Health* 209, 345–358. doi: 10.1016/j.ijheh.2006.02.002

Kensler, T. W., Roebuck, B. D., Wogan, G. N., and Groopman, J. D. (2011). Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. *Toxicol. Sci.* 120, S28–S48. doi: 10.1093/toxsci/kfq283

Khayoon, W. S., Saad, B., Lee, T. P., and Salleh, B. (2012). High performance liquid chromatographic determination of aflatoxins in chili, peanut and rice using silica based monolithic column. *Food Chem.* 133, 489–496. doi: 10.1016/j.foodchem.2012.01.010

Lahtinen, S. J., Haskard, C. A., Ouwehand, A. C., Salminen, S. J., and Ahokas, J. T. (2004). Binding of aflatoxin B1 to cell wall components of *Lactobacillus rhamnosus* strain GG. *Food Addit. Contam.* 21, 158–164. doi: 10.1080/02652030310001639521

Leong, Y. H., Ismail, N., Latiff, A. A., and Ahmad, R. (2010). Aflatoxin occurrence in nuts and commercial nutty products in Malaysia. *Food Control* 21, 334–338. doi: 10.1016/j.foodcont.2009.06.002

Leong, Y. H., Rosma, A., Latiff, A. A., and Ahmad, N. I. (2011). Exposure assessment and risk characterization of aflatoxin B1 in Malaysia. *Mycotoxin Res.* 27, 207–214. doi: 10.1007/s12550-011-0097-4

Leong, Y. H., Rosma, A., Latiff, A. A., and Nurul Izzah, A. (2012). Associations of serum aflatoxin B1-lysine adduct level with socio-demographic factors and aflatoxin intake from nuts and related nut products in Malaysia. *Int. J. Hyg. Environ. Health* 215, 368–372. doi: 10.1016/j.ijheh.2011.12.005

Lim, H. K., and Yeap, G. S. (1966). The occurrence of aflatoxin in Malayan imported oil cakes and groundnut kernels. *Malayan Agric. J.* 45, 232–244.

Liu, Y., and Wu, F. (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environ. Health Perspect.* 118, 818–824. doi: 10.1289/ehp.0901388

Lye, M. S., Ghazali, A. A., Mohan, J., Alwin, N., and Nair, R. C. (1995). An outbreak of acute hepatic encephalopathy due to severe aflatoxicosis in Malaysia. *Am. J. Trop. Med. Hyg.* 53, 68–72.

MOH, Ministry of Health. (2006). *Food Consumption Statistic of Malaysia 2002/2003 for Adult Population Aged 18-59 Years.* Vol. 1, Putrajaya: Ministry of Health, Malaysia.

Mohd Redzwan, S., Rosita, J., Mohd Sokhini, A. M., and Nurul Aqilah, A. R. (2012a). Socio-demographic and socio-economic determinants of adults knowledge on fungal and aflatoxin contamination in the diets. *Asian Pac. J. Trop. Biomed.* 2, S1835–S1841. doi: 10.1016/S2221-1691(12)60504-8

Mohd Redzwan, S., Rosita, J., Mohd Sokhini, A. M., and Nurul Aqilah, A. R. (2012b). Association between aflatoxin M1 excreted in human urine samples with the consumption of milks and dairy products. *Bull. Environ. Contam. Toxicol.* 89, 1115–1119. doi: 10.1007/s00128-012-0853-y

Mykkänen, H., Zhu, H., Salminen, E., Juvonen, R. O., Ling, W., Ma, J., et al. (2005). Fecal and urinary excretion of aflatoxin B1 metabolites (AFQ1, AFM1, and AFB-N7-guanine) in young Chinese males. *Int. J. Cancer* 115, 879–884. doi: 10.1002/ijc.20951

Nikbakht Nasrabadi, E., Jamaluddin, R., Abdul Mutalib, M. S., Khaza'ai, H., Khalesi, S., and Mohd Redzwan, S. (2013). Reduction of aflatoxin level in aflatoxin-induced rats by the activity of probiotic *Lactobacillus casei* strain Shirota. *J. Appl. Microbiol.* 114, 1507–1515. doi: 10.1111/jam.12148

Oelschlaeger, T. A. (2010). Mechanisms of probiotic actions – a review. *Int. J. Med. Microbiol.* 300, 57–62. doi: 10.1016/j.ijmm.2009.08.005

Paterson, R. R. M., and Lima, N. (2010). "Toxicology of mycotoxins," in *Molecular, Clinical and Environmental Toxicology. Volume 2: Clinical Toxicology*, ed. A. Luch (Zürich: Birkhauser Verlag), 31–63.

Polychronaki, N., Wild, C. P., Mykkänen, H., Amra, H., Abdel-Wahhab, M., Sylla, A., Diallo, M., et al. (2008). Urinary biomarkers of aflatoxin exposure in young children from Egypt and Guinea. *Food Chem. Toxicol.* 46, 519–526. doi: 10.1016/j.fct.2007.08.034

Reddy, K. R. N., Farhana, N. I., and Salleh, B. (2011). Occurrence of *Aspergillus* spp. and aflatoxin B1 in Malaysian foods used for human consumption. *J. Food Sci.* 76, T99–T104. doi: 10.1111/j.1750-3841.2011.02133.x

Sabran, M. R., Jamaluddin, R., and Abdul Mutalib, M. S. (2012). Screening of aflatoxin M1, a metabolite of aflatoxin B1 in human urine samples in Malaysia: a preliminary study. *Food Control* 28, 55–58. doi: 10.1016/j.foodcont.2012.04.048

Samsudin, N. I. P., and Abdullah, N. (2013). A preliminary survey on the occurrence of mycotoxigenic fungi and mycotoxins contaminating red rice at consumer level in Selangor, Malaysia. *Mycotoxin Res.* 29, 89–96. doi: 10.1007/s12550-012-0154-7

Shuaib, F. M., Jolly, P. E., Ehiri, J. E., Yatich, N., Jiang, Y., Funkhouser, E., et al. (2010). Association between birth outcomes and aflatoxin B1 biomarker blood levels in pregnant women in Kumasi, Ghana. *Trop. Med. Int. Health* 15, 160–167. doi: 10.1111/j.1365-3156.2009.02435.x

Soleimany, F., Jinap, S., and Abas, F. (2012a). Determination of mycotoxins in cereals by liquid chromatography tandem mass spectrometry. *Food Chem.* 130, 1055–1060. doi: 10.1016/j.foodchem.2011.07.131

Soleimany, F., Jinap, S., Faridah, A., and Khatib, A. (2012b). A UPLC-MS/MS for simultaneous determination of aflatoxins, ochatoxin A, zearenone, DON, dumonisims, T-2 toxin and HT-2 toxin, in cereals. *Food Control* 25, 647–653. doi: 10.1016/j.foodcont.2011.11.012

Sulaiman, M. R., Yee, C. F., Hamid, A., and Yatim, A. M. (2007). The occurrence of aflatoxins in raw shelled peanut samples from three districts of Perak, Malaysia. *Electron. J. Environ. Agric. Food Chem.* 6, 2045–2052.

Thieu, N. Q., and Pettersson, H. (2008). In vitro evaluation of the capacity of zeolite and bentonite to adsorb aflatoxin B1 in simulated gastrointestinal fluids. *Mycotoxin Res.* 24, 124–129. doi: 10.1007/BF03032338

Wang, J. S., Shen, X., He, X., Zhu, Y. R., Zhang, B. C., Wang, J. B., et al. (1999). Protective alteration in Phase 1 and 2 metabolism of aflatoxin B1 by oltipraz in residents of Qidong, People's Republic of China. *J. Natl. Cancer Inst.* 91, 347–354. doi: 10.1093/jnci/91.4.347

Xu, L., Qian, C., Tang, L., Su, J., and Wang, J.-S. (2010). Genetic variations of hepatitis B virus and serum aflatoxin-lysine adduct on high risk of hepatocellular carcinoma in Southern Guangxi, China. *J. Hepatol.* 53, 671–676. doi: 10.1016/j.jhep.2010.04.032

Zainal Ariffin, O., and Nor Saleha, I. T. (2011). *National Cancer Registy Report 2007. Malaysia Cancer Statistics – Data and Figure.* Putrajaya: National Cancer Registry, Ministry of Health Malaysia.

Zhu, J. Q., Zhang, L. S., Hu, X., Xiao, Y., Chen, J. S., Xu, Y. C., et al. (1987). Correlation of dietary aflatoxin B1 levels with excretion of aflatoxin M1 in human urine. *Cancer Res.* 47, 1848–1852.

Zulhabri, O., Jamal, R., Isa., M. R., Harjit, S., and Wang Ngah, W. Z. (2009). Hotspot Ser-249 TP53 mutations, aflatoxin B1-adducts and hepatitis B infection in human hepatocellular carcinoma from Malaysia. *Med. J. Mal.* 64 (Suppl B), 88.

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The microRNAs as potential biomarkers for predicting the onset of aflatoxin exposure in human beings: a review

Rafael Valencia-Quintana[1]*, Juana Sánchez-Alarcón[1], María G. Tenorio-Arvide[2], Youjun Deng[3], José M. R. Montiel-González[1], Sandra Gómez-Arroyo[4], Rafael Villalobos-Pietrini[4], Josefina Cortés-Eslava[4], Ana R. Flores-Márquez[4] and Francisco Arenas-Huertero[5]*

[1] *Evaluación de Riesgos Ambientales, Facultad de Agrobiología, Universidad Autónoma de Tlaxcala, Tlaxcala, México*
[2] *Departamento de Investigación en Ciencias Agrícolas, Benemérita Universidad Autónoma de Puebla, Puebla, México*
[3] *Department of Soil and Crop Sciences, Texas AgriLife, Texas A&M University, College Station, TX, USA*
[4] *Departamento de Ciencias Ambientales, Centro de Ciencias de la Atmósfera, Universidad Nacional Autónoma de México, Distrito Federal, México*
[5] *Laboratorio de Patología Experimental, Hospital Infantil de México Federico Gómez, Distrito Federal, México*

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Eva-Guadalupe Lizárraga-Paulín, Universidad Nacional Autónoma de México, Mexico
Jing Shen, Columbia University, USA

****Correspondence:***
Rafael Valencia-Quintana, Evaluación de Riesgos Ambientales, Facultad de Agrobiología, Universidad Autónoma de Tlaxcala, Av. Universidad No.1, Col. La Loma Xicohténcatl, 90070 Tlaxcala de Xicohténcatl, Tlaxcala, México
e-mail: prvq2004@yahoo.com.mx;
Francisco Arenas-Huertero, Laboratorio de Patología Experimental, Hospital Infantil de México Federico Gómez, Dr. Márquez 162, Col. Doctores, 06720 Ciudad de México, Distrito Federal, México
e-mail: farenashuertero@yahoo.com.mx

The identification of aflatoxins as human carcinogens has stimulated extensive research efforts, which continue to the present, to assess potential health hazards resulting from contamination of the human food supply and to minimize exposure. The use of biomarkers that are mechanistically supported by toxicological studies will be important tools for identifying stages in the progression of development of the health effects of environmental agents. miRNAs are small non-coding mRNAs that regulate post-transcriptional gene expression. Also, they are molecular markers of cellular responses to various chemical agents. Growing evidence has demonstrated that environmental chemicals can induce changes in miRNA expression. miRNAs are good biomarkers because they are well defined, chemically uniform, restricted to a manageable number of species, and stable in cells and in the circulation. miRNAs have been used as serological markers of HCC and other tumors. The expression patterns of different miRNAs can distinguish among HCC-hepatitis viruses related, HCC cirrhosis-derivate, and HCC unrelated to either of them. The main objective of this review is to find unreported miRNAs in HCC related to other causes, so that they can be used as specific molecular biomarkers in populations exposed to aflatoxins and as early markers of exposure, damage/presence of HCC. Until today specific miRNAs as markers for aflatoxins-exposure and their reliability are currently lacking. Based on their elucidated mechanisms of action, potential miRNAs that could serve as possible markers of HCC by exposure to aflatoxins are miR-27a, miR-27b, miR-122, miR-148, miR-155, miR-192, miR-214, miR-221, miR-429, and miR-500. Future validation for all of these miRNAs will be needed to assess their prognostic significance and confirm their relationship with the induction of HCC due to aflatoxin exposure.

Keywords: AFB1, aflatoxin exposure, microRNAs, HCC, potential biomarkers

INTRODUCTION

The aflatoxins were structurally identified in the early 1960s and over the last 50 years have been extensively studied with respect to their mechanisms of action, including their mutagenic and carcinogenic activity. This work has been paralleled by developments in biomarkers of aflatoxin metabolism, DNA adducts, and mutations applied to exposed human populations. The improvements in exposure assessment in epidemiological studies and the demonstration of a specific mutation in the *TP53* gene have contributed significantly to the identification of aflatoxins as human carcinogens. In addition, the studies of animal and human aflatoxin metabolism have provided opportunities to develop chemoprevention approaches in human populations (Wild and Turner, 2002; Valencia-Quintana et al., 2012). These findings stimulated extensive research efforts, which continue to the present, to assess potential health hazards resulting from contamination of the human food supply and to minimize exposure (Kensler et al., 2011).

The use of biomarkers that are mechanistically supported by toxicological studies will be important tools for identifying stages in the progression of development of the health effects of environmental agents. Since the development of a general paradigm for molecular epidemiology and biomarkers nearly 20 years ago, progress has been made in applying these tools to specific environmental situations that may be hazardous to humans, as exemplified by AFB1 studies. The major goals of molecular epidemiology research are to develop and to validate biomarkers that reflect specific exposures, their interactions, and predictions of disease risk in individuals. Presumably, after an environmental exposure each person has a unique response to both dose

and time to disease onset. These responses will be affected both by genetic, host and environmental modifiers. It is assumed that biomarkers that reflect the mechanisms of action of the etiologic agents will be strong predictors of an individual's risk of disease. It is also expected that these biomarkers can more clearly classify the status of exposure of individuals and general populations (Groopman et al., 2005).

Biomarkers can be used as outcome measures in these and primary prevention studies. Overall, the integrated, multidisciplinary research on aflatoxins has provided the scientific platform on which to base decisions regarding acceptable exposures and priorities for interventions to reduce human risk in a public health context (Wild and Turner, 2002).

AFLATOXIN BIOMARKERS

AFB1 requires metabolic activation to its ultimate carcinogenic form, a reactive epoxide (aflatoxin-8,9-epoxide), primarily by the cytochrome P450 (CYP) monooxygenase system. Epoxidation is catalyzed by CYP1A2 and CYP3A4 in humans (Gallagher et al., 1994; Ueng et al., 1995). Many other oxidation products, including aflatoxin M1, are also formed. The epoxide can react further by interacting with DNA to produce a promutagenic aflatoxin-N7-guanine adduct. This adduct is unstable in DNA, rapidly undergoes depurination, and is excreted in urine (Bennett et al., 1981). The epoxide can also form products that react with serum albumin to form long-lived lysine adducts (Sabbioni et al., 1987). In addition, the epoxide can be conjugated by certain glutathione S-transferases (GSTs), which are further metabolized to form aflatoxin-mercapturic acid detoxification products that can be excreted in urine (Scholl et al., 1997). Urinary measures of aflatoxin M1, aflatoxin-mercapturic acid, and the aflatoxin-albumin adduct are used as biomarkers of internal dose. Aflatoxin-N7-guanine in urine serves as an elegant biomarker of biologically effective dose because it is clear that formation of this adduct lies on the causal pathway to aflatoxin-induced HCC (Kensler et al., 2011).

An objective in development of AFB1 biomarkers is to use them as predictors of past and future exposure status in people (Kensler et al., 2011). However, two key attributes, one biological (tracking) and the other chemical (stability), need to be confirmed to successfully use biomarkers for these purposes. miRNAs are good biomarkers because they are well defined, chemically uniform, restricted to a manageable number of species, and stable in cells and in the circulation (Wang et al., 2012a).

microRNAs AND ENVIRONMENTAL POLLUTANTS

Exposure to environmental chemicals is well known to increase risks for various diseases (Crinnion, 2010; Newbold, 2010), and gene expression can be changed as a response to these exogenous stressors (Ueda, 2009; Patel and Butte, 2010; Hou et al., 2012), like tobacco and polycyclic aromatic hydrocarbons in urban air of megacities (Arenas-Huertero et al., 2011). Such changes may be regulated by specific miRNAs and emerged as a gene expression regulatory factor that may link environmental chemicals and their related diseases.

Secreted miRNAs have many requisite features of good biomarkers. miRNAs are stable in various bodily fluids, the sequences of most miRNAs are conserved among different species, the expression of some miRNAs is specific to tissues or biological stages, and the level of miRNAs can be easily assessed by various methods, as polymerase chain reaction (PCR), which allows for signal amplification. The changes of several miRNA levels in plasma, serum, urine, and saliva have already been associated with different diseases (for review see Etheridge et al., 2011).

Growing evidence has demonstrated that environmental chemicals can induce changes in miRNA expression (Hou et al., 2011). Arsenite exposure induced significant decrease in miR-19a expression in human lymphoblast cells line TK-6, resulting in cell growth arrest and apoptosis (Marsit et al., 2006). Metal sulfates have been shown to generate reactive oxygen species (ROS) and trigger the expression of specific miRNAs (Lukiw and Pogue, 2007). Bollati et al. (2010) found an increased expression of miR-146a related to inhalation of Cd-rich air particles in steel workers, and induced rapid changes in the expression of two inflammation-related miRNAs, miR-21 and miR-222. Aluminum exposure may induce genotoxicity via miRNA-related regulatory elements, for example, miR-146a, miR-9, miR-125b, and miR-128 (Lukiw and Pogue, 2007; Pogue et al., 2009).

Jardim et al. (2009) have shown extensive alterations of miRNA expression profiles in human bronchial epithelial cells treated with diesel exhaust particles. Schembri et al. (2009) have identified 28 miRNAs that were differentially expressed in smokers when compared to non-smokers, changes in miRNA expression were suggested to contribute to altered regulation of oncogenes, tumor suppressor genes, oxidative stress, xenobiotic metabolism, and inflammation. Izzotti et al. (2009a,b) have monitored the expression of 484 miRNAs in the lungs of mice exposed to cigarette smoking, the most remarkably downregulated miRNAs belonged to several miRNA families, such as let-7, miR-10, miR-26, miR-30, miR-34, miR-99, miR-122, miR-123, miR-124, miR-125, miR-140, miR-145, miR-146, miR-191, miR-192, miR-219, miR-222, and miR-223. These miRNAs regulate expression of genes involved in stress responses, apoptosis, proliferation, and angiogenesis.

Zhang and Pan (2009) have evaluated the effects of Hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (also known as hexogen or cyclonite) (RDX) on miRNA expression in mouse brain and liver, most of the miRNAs that showed altered expression, including let-7, miR-17-92, miR-10b, miR-15, miR-16, miR-26, and miR-181, were related to toxicant-metabolizing enzymes, and genes related to carcinogenesis, and neurotoxicity, in addition, consistent with the known neurotoxic effects of RDX, the authors documented significant changes in miRNA expression in the brains of RDX-treated animals, such as miR-206, miR-30a, miR-30c, miR-30d, and miR-195. STS (sodium thiosulfate) treatment also resulted in differential expression of miR-124a and miR-133a in the treated embryos (Choudhuri, 2010). Fukushima et al. (2007) have demonstrated that rat exposed to acetaminophen or carbon tetrachloride showed down-regulation of miR-298 and miR-370 in the liver that was accompanied by hepatocyte necrosis and inflammation. Wang et al. (2009a) found increase serum concentration of hepatocyte-specific miRNAs including miR-122 and miR-192 within 1 h after acetaminophen exposure. In

mouse exposure to Wy-14,643, peroxisome proliferator-activated receptor alpha (PPARα) agonist, up-regulate let-7C (Shah et al., 2007). Ethanol exposure down-regulate miR-21, miR-335, miR-9, and miR-153 (Sathyan et al., 2007). In rats, tamoxifen up-regulate miR-17-92 cluster, miR-106a, and miR-34 (Pogribny et al., 2007). 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco carcinogen, down-regulate miR-34, miR-101, miR-126, and miR-199 (Kalscheuer et al., 2008). In humans, 1 alpha, 25-dihydroxyvitamin D(3) [1,25(OH)(2)D(3)], major vitamin D metabolite, up-regulate miR-125b (Mohri et al., 2009), and 5-fluorouracil (5-FU) an antineoplasic drug, up-regulate miR-200b (Rossi et al., 2007). Bisphenol A (Avissar-Whiting et al., 2010), dioxin (Elyakim et al., 2010), and diethylstilbestrol (DES) (Hsu et al., 2009) desregulate expression of miR-146a, miR-191, and miR-9-3, respectively. Moffat et al. (2007) looked at the effects of dioxin treatment on miRNA in mice, dioxin-resistant rats (Han/Wistar; Kuopio) and dioxin-sensitive rats (Long–Evans; Turku/AB), it is interesting to note that the dioxin sensitive rats had more affected miRNAs. X-ray exposure resulted in down regulation of miR-7 (Ilnytskyy et al., 2008). The ROS induction resulted in up-regulation of a specific set of miRNAs, including miR-9, miR-125b, and miR-128 (Lukiw and Pogue, 2007). Lin et al. (2009), and Cheng et al. (2009), found that hydrogen peroxide induce up-regulation of miR-21.

The increasing evidence that the expression of miRNAs is affected by several known toxicants as well as oxidative and other forms of cellular stress certainly suggest an important role of miRNAs in toxicology, which could provide a link between environmental influences and gene expression (Lema and Cunningham, 2010). Analysis of the resulting molecular signatures provides new tools to identify mechanisms of toxicity, as well as to classify compounds based on the biological response they elicit and to identify cluster of genes detective or predictive of certain type of toxic response, which are employed as biomarkers (Gatzidou et al., 2007). miRNAs are one of the main mechanisms of epigenetic regulation of gene expression. Conversely to stress-related miRNAs, the toxicological research of miRNAs associated to specific toxicants has started few years ago. Therefore, available publications are focused in a broad spectra of toxicants and compiled research about a particular miRNA is yet very limited (Lema and Cunningham, 2010).

microRNA IN HCC AS POTENTIAL BIOMARKERS OF AFLATOXIN EXPOSURE

In the recent years, several studies revealed that the expression of miRNAs is deregulated in human HCC in comparison with matched non-neoplastic tissue (Lu et al., 2005; Gramantieri et al., 2008; Law and Wong, 2011; Borel et al., 2012a; Wang et al., 2012a,b; Sun and Karin, 2013; Wong et al., 2013). Some miRNAs identified in HCC are reported in **Table 1**.

Cellular miRNAs can be released into the circulation, and circulating miRNA levels are also affected in HCC. Circulating plasma miRNA signatures may provide a novel diagnosis method for early, pre-symptomatic HCC patients, and may prove useful as prognosis biomarkers (Borel et al., 2012a).

In HCC has been reported up-expression of miR-21, miR-221, miR-22, miR-15, miR-517a, and down-expression of miR-122, miR-29 family, miR-26a, miR-124, let-7 family members, and miR-199a/b-3p (Szabo et al., 2012). Other miR- reported as markers involved in HCC have been miR-15b, miR-16, miR-17-5p, miR-18, miR-18a, miR-20, miR-23b, miR-26a, miR-29, miR-34a, miR-92, miR-101, miR-106a, miR-125b, miR-130b, miR-143, miR-146a, miR-195, miR-203, miR-223, miR-224, miR-338, miR-378, miR-422b, miR-500 (Murakami et al., 2006; Budhu et al., 2008; Jiang et al., 2008; Chen, 2009; Zhang et al., 2009; Kerr et al., 2011; Qu et al., 2011; Liu et al., 2012; Singhal et al., 2012; Wong et al., 2013).

In a review, Gramantieri et al. (2008) show miRNAs aberrantly expressed in HCC compared to non-tumorous liver tissue (up-expression of miR-33, miR-130, miR-135a, miR-210, miR-213, miR-222, miR-331, miR-373, miR-376a, and down-expression of miR-130a, miR-132, miR-136, miR-139, miR-143, miR-145, miR-150, miR-200a, miR-200b, miR-214). However, specific markers and their reliability are currently lacking as occur with aflatoxin-exposure.

Singhal et al. (2012), review molecular and serum markers in HCC as predictive tools for prognosis and recurrence. Aberrant expression of miR-21 can contribute to HCC by modulating PTEN expression and PTEN-dependent pathways (Meng et al., 2007). A significantly high expression of miR-224 in HCC patients promotes proliferation and inhibits apoptosis inhibitor-5 (API-5) transcript expression (Wang et al., 2008). An inverse correlation between miR-221 and both CDKN1B/p27 and CDKN1C/p57, suggested miR-221 oncogenic function in hepatocarcinogenesis (Fornari et al., 2008). Also miR-221 has been involved in the modulation of Bmf, a proapoptotic BH3-only protein, regulating the cell proliferation and apoptosis proteins (Gramantieri et al., 2009).

HCC cells showed highly deregulated miR-223 expression and a strong inverse relationship with its downstream target Stathmin 1 (Wong et al., 2008). Murakami et al. (2006) and Li et al. (2008), found that miR-125b that suppress the cell growth and phosphorylation of Akt to be a prognostic marker of HCC. miR-122 found up to 70% of total miRNA in the liver, modulates cyclin G1, thus influences p53 protein stability and transcriptional activity and reduces invasion capability of HCC-derived cell lines (Fornari et al., 2009). Bcl-w is a direct target of miR-122 that functions as an endogenous apoptosis regulator in these HCC-derived cell lines (Lin et al., 2008). miR-122 is under the transcriptional control of HNF1A, HNF3A and HNF3B and loss of miR-122 results in an increase of cell migration and invasion. From a clinical point of view, miR-122 can be used as a diagnostic and prognostic marker for HCC progression (Coulouarn et al., 2009).

In HCC cell line, miR-34a directly targeted c-Met and reduced both mRNA and protein levels of c-Met, thus blocking cell migration (Li et al., 2009a). miR-18a high expression in HCC tumors (Liu et al., 2009). miR-101 has a downstream target of v-fos oncogene and it is involved in cell invasion and migration in overexpressed HCC cell lines (Li et al., 2009b). Xu et al. (2009) show that miR-195 may block the G(1)/S transition by repressing Rb-E2F signaling through targeting multiple molecules, including cyclin D1, CDK6, and E2F3. Upregulation of miR-143 expression transcribed by NF-kappa B in HBV-HCC promotes cancer cell invasion/migration and tumor metastasis by repression of

Table 1 | microRNA deregulated in HCC.

miR-	Effects over	References
miR-9/9*/-2	Promote HCC migration and invasion through regulation of KLF17	Budhu et al., 2008; Wang et al., 2008; Sun et al., 2013; Xu et al., 2013
miR-10b	Promoted cell migration and invasion	Ladeiro et al., 2008; Li et al., 2010
miR-15b	Molecular mechanisms and roles in HCC remain largely unknown	Liu et al., 2012; Wong et al., 2013
miR-17/-5p	Proliferation and migration	Kutay et al., 2006; Huang et al., 2009; Yang et al., 2010; Chen et al., 2012a; Zheng et al., 2013
miR-17-92	Induce proliferation and anchorage-independent growth	Pogribny et al., 2007; Wang et al., 2012a
miR-18/a/p-18	High expression in HCC tumors. Promote cell growth. Proliferation	Murakami et al., 2006; Jiang et al., 2008; Liu et al., 2009; Kerr et al., 2011
miR-19a	Proliferation	Budhu et al., 2008; Connolly et al., 2008; Wong et al., 2008; Li et al., 2009c
miR-20/a	Proliferation, recurrence, and prognosis	Kutay et al., 2006; Fan et al., 2013
miR-21	Modulating PTEN expression and PTEN-dependent pathways. Enhanced AKT pathway. Promote cell cycle progression, reduce cell death and favor angiogenesis and invasion. Able to differentiate HCC from chronic hepatitis	Kutay et al., 2006; Volinia et al., 2006; Meng et al., 2007; Gramantieri et al., 2008; Huang et al., 2008; Jiang et al., 2008; Ladeiro et al., 2008; Wang et al., 2008; Chen, 2009; Garofalo et al., 2009; Li et al., 2009b; Pogribny et al., 2009; Pineau et al., 2010; Wong et al., 2010, 2013; Xu et al., 2011; Tomimaru et al., 2012; Karakatsanis et al., 2013
miR-22	Enhanced NF-kB signaling	Takata et al., 2011
miR-23a/b	Repress the expression of uPA and c-met decreasing migration and proliferation abilities in HCCcells	Kutay et al., 2006; Huang et al., 2008; Salvi et al., 2009
miR-24	Promote cell growth and inhibit apoptosis	Kutay et al., 2006; Huang et al., 2008, 2009
miR-25	Apoptosis inhibition	Li et al., 2008, 2009c; Wang et al., 2008; Huang et al., 2009
miR-26a	Regulates the expression of cyclin D2 and E2 and induces G1 arrest of human liver cancer cells. Reduced expression in HCC. Systemic administration inhibe cancer cell proliferation and induced apoptosis in HCC	Chang et al., 2008; Ji et al., 2009a; Braconi et al., 2011; Kerr et al., 2011; Szabo et al., 2012
miR-27a	Promote cell growth and inhibit apoptosis	Huang et al., 2008, 2009
miR-29c	Apoptosis inhibition	Li et al., 2008; Xiong et al., 2010; Wang et al., 2011
miR-34a	Stimulation of HCC proliferation. Targeted c-Met and reduced both mRNA and protein levels of c-Met, thus blocking cell migration. Reduce invasion	Meng et al., 2007; Pogribny et al., 2007; Budhu et al., 2008; Gramantieri et al., 2008; Li et al., 2008, 2009a; Wong et al., 2008; Chen, 2009; Pineau et al., 2010
miR-92	The physiological significance of deregulation is still unknown	Meng et al., 2007; Shigoka et al., 2010
miR-93	Prevention of E2F1 acumulation. Proliferation	Kutay et al., 2006; Wong et al., 2008; Li et al., 2009c; Su et al., 2009; Pineau et al., 2010
miR-101/b-2	Downstream target of v-fos oncogene. Apoptosis inhibition. Inhibits cell proliferation and colony formation. Inhibits invasion and migration	Kutay et al., 2006; Gramantieri et al., 2008; Jiang et al., 2008; Li et al., 2008, 2009b; Su et al., 2009
miR-106a/b	Prevention of E2F1 acumulation. Proliferation	Kutay et al., 2006; Pogribny et al., 2007; Shah et al., 2007; Jiang et al., 2008; Ji et al., 2009a; Li et al., 2009c; Pineau et al., 2010
miR-122/a	Stimulation of HCC proliferation. Enhanced cell cycle progression. Modulates cyclin G1, influences p53 protein stability, and transcriptional activity and reduces migration and invasion capability of HCC-derived cell lines. Also Bcl-w is its direct target. Apoptosis inhibition	Kutay et al., 2006; Gramantieri et al., 2007; Meng et al., 2007; Budhu et al., 2008; Ladeiro et al., 2008; Lin et al., 2008; Wong et al., 2008; Bai et al., 2009; Chen, 2009; Coulouarn et al., 2009; Fornari et al., 2009; Huang et al., 2009; Liu et al., 2009; Tsai et al., 2009; Fan et al., 2011; Kerr et al., 2011; Li et al., 2011; Qi et al., 2011; Xu et al., 2012; Karakatsanis et al., 2013
miR-124/a-2	Stimulation of EMT. Supress cell proliferation	Gramantieri et al., 2007, 2008; Budhu et al., 2008; Huang et al., 2009; Furuta et al., 2010; Lang and Ling, 2012; Zheng et al., 2012a
miR-125a/b/b-2	Inversely correlated with aggressiveness and poor prognosis. Ectopic expression can inhibit the proliferation, invasion, and metastasis	Murakami et al., 2006; Meng et al., 2007; Budhu et al., 2008; Gramantieri et al., 2008; Li et al., 2008; Wong et al., 2008, 2010; Huang et al., 2009; Su et al., 2009; Kerr et al., 2011; Bi et al., 2012

(Continued)

Table 1 | Continued

miR-	Effects over	References
miR-130/a/a-1/b	It is still unknown if contribute to HCC development and tumor progression	Kutay et al., 2006; Gramantieri et al., 2007; Jiang et al., 2008; Wong et al., 2008; Liu et al., 2009, 2012
miR-143	Promotes cancer cell invasion/migration and tumor metastasis by repression of fibronectin type III domain containing 3B (FNDC3B) expression	Gramantieri et al., 2007, 2008; Huang et al., 2009; Zhang et al., 2009
miR-145	Invasion and development	Gramantieri et al., 2007, 2008; Varnholt et al., 2008; Wang et al., 2008; Wong et al., 2008, 2010; Huang et al., 2009; Liu et al., 2009; Borel et al., 2012b; Karakatsanis et al., 2013
miR-146	Promote cell growth	Gramantieri et al., 2007; Xu et al., 2008; Karakatsanis et al., 2013
miR-148a/b	Metastasis	Budhu et al., 2008; Li et al., 2008, 2009b; Wong et al., 2008
miR-150	Cell differentiation and survival	Gramantieri et al., 2007, 2009; Jiang et al., 2008; Fornari et al., 2009; Zhang et al., 2009, 2012; Pineau et al., 2010
miR-151	Migration and invasion	Wang et al., 2008; Wong et al., 2008; Ding et al., 2010
miR-155	Development and invasion	Gramantieri et al., 2007; Wang et al., 2008; Huang et al., 2012
miR-181a/a-1/a-2/b/c/d	Migration. Enhanced MMP2 and MMP9	Gramantieri et al., 2007; Meng et al., 2007; Li et al., 2008; Garofalo et al., 2009; Ji et al., 2009a; Pogribny et al., 2009; Wang et al., 2010; Song et al., 2013
miR-182	Metastasis	Wang et al., 2008, 2012b; Wong et al., 2008, 2010
miR-183	Onset and progression, Apoptosis	Wang et al., 2008; Wong et al., 2008, 2010; Liang et al., 2013
miR-185	Metastasis	Budhu et al., 2008; Wong et al., 2008, 2010; Huang et al., 2009; Zhi et al., 2013
miR-192	Inhibition of DNA excision repair	Xie et al., 2011
miR-194	Metastasis	Budhu et al., 2008; Huang et al., 2009; Meng et al., 2010; Xu et al., 2013
miR-195	Proliferation, colony formation. Repressing Rb-E2F signaling. Enhanced G1-S transition	Murakami et al., 2006; Gramantieri et al., 2007, 2008; Wong et al., 2008, 2010; Huang et al., 2009; Liu et al., 2009; Xu et al., 2009
miR-199a/a*/a-1/a-2/b/-3p/-5p	MET, the tyrosine kinase HGF receptor, is post-transcriptionally regulated	Murakami et al., 2006; Gramantieri et al., 2007, 2008; Meng et al., 2007; Jiang et al., 2008; Wong et al., 2008, 2010; Chen, 2009; Liu et al., 2009; Su et al., 2009; Wang et al., 2009a; Kerr et al., 2011; Borel et al., 2012b
miR-200a/b/c	Stimulation of EMT	Murakami et al., 2006; Gramantieri et al., 2007; Jiang et al., 2008; Huang et al., 2009; Wong et al., 2010; Kim et al., 2011; Zhou et al., 2012; Karakatsanis et al., 2013
miR-203	Progression	Ladeiro et al., 2008; Chen et al., 2012b
miR-205	Proliferation	Huang et al., 2009; Wei et al., 2013a
miR-207	Metastasis	Budhu et al., 2008; Huang et al., 2009
miR-210	Metastasis	Meng et al., 2007; Wong et al., 2008; Su et al., 2009; Pineau et al., 2010; Ying et al., 2011
miR-214	Cell growth and invasion	Gramantieri et al., 2007; Jiang et al., 2008; Li et al., 2008; Wang et al., 2008; Wong et al., 2008, 2010
miR-221	Proliferation, colony formation, apoptosis, migration. Down-regulation of p27 and p57. Involved in the modulation of CDKN1B/p27 and CDKN1 C/p57, cell cycle proteina, and Bmf, a proapoptotic BH3-only protein. Promote cell cycle progression, reduce cell death and favor angiogenesis and invasion. TSC1/2 complex inhibition and enhanced AKT pathway. Enhanced MMP2 and MMP9. Inhibition of caspases 3, 6, 7, and 8	Volinia et al., 2006; Gramantieri et al., 2007, 2008, 2009; Meng et al., 2007; Fornari et al., 2008; Jiang et al., 2008; Li et al., 2008, 2011; Wang et al., 2008; Wong et al., 2008; Chen, 2009; Garofalo et al., 2009; Huang et al., 2009; Liu et al., 2009; Pogribny et al., 2009; Pineau et al., 2010; Wang et al., 2010; Kerr et al., 2011; Karakatsanis et al., 2013
miR-222	Enhanced AKT pathway. Enhanced MMP2 and MMP9. Inhibition of caspases 3, 6, 7, and 8. Migration, invasion	Gramantieri et al., 2007; Meng et al., 2007; Ladeiro et al., 2008; Li et al., 2008; Wang et al., 2008, 2010; Wong et al., 2008, 2010; Garofalo et al., 2009; Huang et al., 2009; Liu et al., 2009; Pogribny et al., 2009; Su et al., 2009; Pineau et al., 2010; Karakatsanis et al., 2013
miR-223	Proliferation. Inhibit cell viability. Inverse relationship with its downstream target Stathmin 1. Microtubules stabilization (G1-M transition)	Gramantieri et al., 2007, 2008; Jiang et al., 2008; Wong et al., 2008; Liu et al., 2009; Xu et al., 2011; Karakatsanis et al., 2013

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Table 1 | Continued

miR-	Effects over	References
miR-224	Promotes proliferation and inhibits apoptosis inhibitor-5 (API-5) transcript expression	Murakami et al., 2006; Meng et al., 2007; Gramantieri et al., 2008; Ladeiro et al., 2008; Li et al., 2008; Wang et al., 2008; Chen, 2009; Huang et al., 2009; Liu et al., 2009; Su et al., 2009; Pineau et al., 2010; Wong et al., 2010
miR-296-5p	It is still unknown if contribute to HCC development and tumor progression	Borel et al., 2012b; Katayama et al., 2012; Vaira et al., 2012; Wei et al., 2013b
miR-338/-3p	Associated with clinical HCC aggressiveness. Stimulation of HCC proliferation	Budhu et al., 2008; Gramantieri et al., 2008; Huang et al., 2009, 2011
miR-373	Invasion and metastasis	Meng et al., 2007; Bartels and Tsongalis, 2009; Wu et al., 2011
miR-374	Development	Wang et al., 2008; Wong et al., 2008, 2010; Koh et al., 2013
miR-375	Stimulation of HCC proliferation	Liu et al., 2010; He et al., 2012
miR-376a	Proliferation and apoptosis	Meng et al., 2007; Zheng et al., 2012b
miR-423	Enhanced CDK2 activity	Lin et al., 2011
miR-491-5p	Inhibition of TNF-α-related apoptosis	Yoon et al., 2010
miR-500	Elevated in HCC, returned to physiologic level after surgical intervention	Yamamoto et al., 2009
miR-637	Active STAT3	Zhang et al., 2011
let-7a/a-1/a-2/b/c/d/e/f/f-2/g	Development. Enhanced HCC proliferation, colony formation, and cell migration	Gramantieri et al., 2007, 2008; Budhu et al., 2008; Li et al., 2008; Wong et al., 2008; Huang et al., 2009; Liu et al., 2009; Ji et al., 2010; Pineau et al., 2010; Kerr et al., 2011; Lan et al., 2011; Sukata et al., 2011; Zhou et al., 2012

Potential biomarkers of aflatoxin exposure.

fibronectin type III domain containing 3B (FNDC3B) expression (Zhang et al., 2009).

The level of miR-338 expression can be associated with clinical aggressiveness of HCC (Huang et al., 2009). miR-23b can recognize target sites in the 3-UTR of uPA and of c-met mRNAs and translationally repress the expression of uPA and c-met decreasing migration and proliferation abilities in HCC cells (Salvi et al., 2009).

MiR-126 down-regulation has been suggested to be directly linked to alcohol-induced hepatocarcinogenesis (Morgan et al., 2004). Microarray profiling studies showed reduction in miRNAs expression specific of HCV and HBV-associated cases: down-regulation of miR-190, miR-134, and miR-151 occurs in HCV cases, and of miR-23a, miR-142-5p, miR-34c, in HBV cases (Ura et al., 2009). MiR-96 was reported to be distinctively upregulated in HBV-associated HCC (Ladeiro et al., 2008), whereas miR-193b upregulation has been found upon transfection of HCV genome (Braconi et al., 2010). As quoted above, up-regulation of miRNAs, including miR-17-92 cluster, miR-106a, and miR-34, occurs during tamoxifen-induced hepatocarcinogenesis in female rats (Pogribny et al., 2007), also long-term-administration of 2-AAF resulted in disruption of regulatory miR-34a-p53 feed-back loop (Pogribny et al., 2009). In mice administered a choline-deficient and amino acid-defined diet, in which steatohepatitis precedes HCC development, microarray analysis identified that miR-155 was consistently up-regulated (Wang et al., 2009b).

Ross et al. (2010) analyzed the miRNA expression levels in control and conazole-treated mice. Conazol exposure induced many more changes in miRNA expression. All but one of the altered miRNAs were downregulated compared to controls. The authors suggest that this pattern of the altered miRNA expression represents a signature for tumorigenic conazole exposure in mouse liver.

This newly emerging area of research should unravel novel biomarkers of diagnostic as well as prognostic value in HCC.

microRNA AND AFLATOXIN B1

Exposure to environmental carcinogens may affect miRNAs expression in liver cells. While this concept is largely acceptable in principle, the specific miRNAs that are deregulated by various toxic and/or carcinogenic agents are yet to be fully documented. What we know at best today is the end-point of the process: the miRNAs whose expression is altered in HCCs (**Table 1**). However, results from some reports suggest that changes in expression of miRNAs may occur early in the process (Jiang et al., 2008), and these changes may be related to specific etiological factors, such as AFB1. These still preliminary evidences suggest the possibility of using miRNAs as early markers for aflatoxins exposure.

Exposure to natural or chemical environmental agents contributes to HCC development (Wild, 2009). In this context, uncovering relationships between exposure to environmental carcinogens and expression of miRNAs may reveal practical and sensitive biomarkers of toxic exposures and/or carcinogenicity testing (Wang et al., 2009a). A few reports addressed this hypothesis and revealed the existence of differential miRNAs expression patterns in HCCs in accordance with specific risk factors suggesting that exposure to specific risk factors could be responsible for the appearance of characteristic pathogenetic miRNA signatures (Elamin et al., 2011).

Although the precise roles of miRNA in the response to xenobiotics, drugs and chemical toxicants, remain to be established, there is little doubt that miRNAs are important in the cellular

and *in vivo* responses to xenobiotics (Taylor and Gant, 2008). At this time, no specific studies on the effect of AFB1 on miRNA expression have been reported.

The field of miRNA and toxicology, particularly as it pertains to AFB1 toxicological outcome, is still in its beginnings. Nonetheless, there seems to be an increasing interest among toxicologists trying to understand the contribution of miRNA in regulating various toxicological outcomes through regulation of gene expression. A number of questions need to be addressed, such as a global role of miRNAs in cellular toxicity and disease; how miRNA biogenesis and expression affect susceptibility/resistance to xenobiotic-induced toxicity or disease (Taylor and Gant, 2008); whether cellular miRNAs form a regulatory networks and how perturbations of such network can cause toxicity/disease including developmental toxicity; how miRNAs may regulate transgenerational toxicological response through epigenetic regulation of gene and genome expression; as well as whether homologous miRNAs can be identified in an animal species based on known miRNA species and their action in other species or even in plant kingdom (Choudhuri, 2010).

Until today specific microRNAs as markers for aflatoxin-exposure and their reliability are currently lacking. The following are some potential candidates based on their elucidated mechanisms of action.

The high expression of miR-122 in the liver appears to correlate with a central role in various functions of normal and diseased livers (Lewis and Jopling, 2010; Negrini et al., 2011). It provides a very attractive target for aflatoxins. Rather surprisingly, given the high intracellular levels and numerous targets of miR-122, inactivation of the miRNA does not have any apparent adverse effects on liver physiology. However, reduced miR-122 expression does show an association with hepatocellular carcinoma, and further work will be necessary. In HCC, miR-122 is downregulated in approximately 70% of cases, suggesting a tumor suppressor function for this miRNA (Bai et al., 2009; Fornari et al., 2009; Ma et al., 2010; Callegari et al., 2013). In addition, loss of miR-122 expression in patients with liver cancer is correlated with the presence of metastasis and a shorter time to recurrence (Coulouarn et al., 2009; Fornari et al., 2009; Tsai et al., 2009). The role of miR-122 in liver cancer has been demonstrated directly by the generation of miR-122 knockout mice (Hsu et al., 2012; Tsai et al., 2012) These mice were characterized by hepatic inflammation, fibrosis, and development of spontaneous tumors similar to HCC, demonstrating the tumor-suppressor function of this miRNA and its important role in liver metabolism and differentiation of hepatocytes (Jensen et al., 2003; Gramantieri et al., 2007; Lin et al., 2008; Bai et al., 2009; Fornari et al., 2009; Tsai et al., 2009; Callegari et al., 2013).

On the other hand, up-regulation of miR-221, may be involved from the very early stage of hepatocarcinogenesis, and expression of the miRNA may progressively increase during malignant transformation. Especially, high expression of miR-221 can be used to predict local recurrence of HCC, and fold changes in miR-221 less than 1 can be used as a predictive marker of metastasis after curative surgical resection in patients with HCC (Yoon et al., 2011). Thus, among the miRNAs that are upregulated in HCC, there is evidence in support of the tumor-promoting activity of miR-221. It is upregulated in 70–80% of HCC samples (Fornari et al., 2008). From a functional point of view, HCC cells overexpressing miR-221 show increased growth, proliferation, migration, and invasion capability (Fornari et al., 2008; Medina et al., 2008; Garofalo et al., 2009; Gramantieri et al., 2009; Pineau et al., 2010; Callegari et al., 2012). Additionally, high level of miR-221 positively correlated with cirrhosis, tumor size and tumor stage, and negatively correlated with overall survival. miR-221 serum level monitoring could be of clinical relevance as a potential diagnosis tool and biomarker of treatment efficacy. It remains to be established which miRNA can sensitively and reliably be correlated with the presence of HCC at early stages of disease development and prognosis (Borel et al., 2012a).

miR-429 expression increased AFB1-DNA adducts in the SMMC-7721 Cells. To explore the effects of miR-429 expression on AFB1-DNA formation, Huang et al. (2013), accomplished a toxin experiment of AFB1 in the SMMC-7721 cells transfected by different mimics. Results showed that group with overexpression of miR-429 had elevated levels of AFB1-DNA adducts compared with control group. MiR-429 is classified as a member of miR-200 family and may play an important role in tumor prognosis. Overexpression of miR-429 induces cell proliferation and inhibits cell apoptosis. On the contrary, the suppression of miR-429 expression hindered cell proliferation and promoted cell apoptosis. These data suggest that this microRNA plays an important role in liver tumorigenesis, and functionally acts as an oncogene in HCC. Increasing evidence has shown that the levels of AFB1-DNA adducts correlate with HCC risk and prognosis, whereas the formation process of AFB1-DNA adducts can be modified by some factors such as detoxifying enzymes and DNA repair enzymes (Long et al., 2006, 2011; Xia et al., 2013). Is possibly that miR-429 can target some detoxification enzyme genes and/or DNA repair genes and reduce their detoxification capacity or DNA repair capacity and subsequently increase DNA damage and promote AFB1-DNA adducts formation. These results provided new insights into the mechanism of HCC induced by AFB1 (Huang et al., 2013).

The maintenance of genomic integrity through efficient DNA repair is essential for propagation of cellular life (Natarajan and Palitti, 2008). Nucleotide excision repair (NER) is one of the most versatile DNA repair system for elimination of bulky DNA adducts caused by environmental agents (Nouspikel, 2009) as AFB1 and other carcinogens. A possibility is that AFB1 could interfere with cellular NER through the regulation of microRNAs. Several miRNAs involved in DNA repair have been identified (Crosby et al., 2009; Yan et al., 2010; Hu and Gatti, 2011). A recent study showed that miR-192 directly targets a NER-associated protein (Georges et al., 2008). A bioinformatic analysis of miRNAs which potentially played a role in NER, show that miR-192, was the most differentially upregulated miRNA. The expression of ERCC3 and ERCC4 were reduced when miR-192 was overexpressed. Also has been observed that the relative repair capacity of damage by HepG2 and HeLa cells was reduced (Xie et al., 2011). Since of AFB1 is an important risk factor of HCC and AFB1-DNA adducts are known to be repaired by NER, dietary AFB1 exposure could impaired NER mediated by miRNAs like miR-192.

miR-500 is an oncofetal miRNA, which is highly expressed in fetal liver, more than in adult normal liver, and aberrantly expressed in HCC. This miRNA was associated with liver maturation in a mouse model of liver development. Levels tended to be higher in HCC lines and tumor samples when compared with matched normal tissue. Importantly, significant difference in miR-500 expression was found between normal liver and liver cirrhosis samples, suggesting that miR-500 expression was upregulated during cirrhosis development. An increased amount of miR-500 was found in the sera of 3 out of 10 HCC patients, which means that liver cancer-specific miRNA such as miR-500 is circulating in the peripheral blood and can be a novel diagnostic marker. These results show that the miR-500 abundance profile in serum of the HCC patients might reflect physiological and/or pathological conditions. However, although results are promising for miRNA-based HCC screening, further validation is suggested (Yamamoto et al., 2009).

miR-148, another candidate. There are also reports suggesting that drug-metabolizing enzymes such as CYP family genes are targeted by certain miRNAs. The expression of drug- and xenobiotic-metabolizing enzymes and nuclear receptors and their regulation by miRNA could be important factors for the outcomes of toxicity (Yokoi and Nakajima, 2011). Members of the CYP family are the most important enzymes catalyzing the metabolism of xenobiotics including drugs, environmental chemicals, and carcinogens. The different profiles of the expression of P450 isoenzymes determine the amount of reactive intermediates formed and the resulting toxic response. P450s are also known to bioactivate many procarcinogens to their ultimate carcinogens as in the case of AFB1. Recently, some P450s and nuclear receptors have been found to be post-transcriptionally regulated by miRNAs. Aflatoxin B1 and G1 are known to be oxidized efficiently to genotoxic metabolite(s) by CYP3A (Shimada et al., 1989; Forrester et al., 1990), epoxidation of AFB1 is catalyzed by CYP1A2 and CYP3A4 in humans (Gallagher et al., 1994; Ueng et al., 1995). The role of miRNA in the regulation of the expression of CYP3A4 has been reported, Takagi et al. (2008), found that miR-148 modulated inducible and/or constitutive levels of CYP3A4 in human liver cancer.

miRNAs are important regulators for CYP3A. Among these differentially regulated miRNAs, miR-155 appears to be the most prominent regulator as it was significantly associated with lower hepatic CYP3A activity (Vuppalanchi et al., 2013). CYP3A4 is the most abundant hepatic and intestinal CYP enzyme in humans, contributing to the metabolism of various drugs (Gonzalez and Yu, 2006), as AFB1. Pan et al. (2009) suggest that intervention of miRNA pathways may modify CYP3A4 expression and alter CYP3A4-catalyzed drug activation. Of particular note, miR-148a has been shown to control post-transcriptional regulation of PXR and, consequently, affect the expression of CYP3A4 (Takagi et al., 2008). Another study suggests that miR-27a and miR-27b may target RXR and regulated of CYP3A4 transcriptional expression (Ji et al., 2009b). The results indicate that intervention of miRNA pathways can be translated into an altered sensitivity of cells to xenobiotics. These findings may provide increased understanding of the complex regulation of CYP3A4 expression, as well as determine the role of miRNAs in drug metabolism and disposition (Pan et al., 2009).

Aflatoxin B1 (AFB1) is carcinogenic due its potential in inducing the oxidative stress and distortion of the most antioxidant enzymes (Abdel-Wahhab et al., 2007; El-Agamy, 2010; Alm-Eldeen et al., 2013). Recently, the role of miRNAs in oxidative stress-mediated etiology is emerging. Dong et al. (2013) found that miR-214 directly bound to 3′-UTR of the GSR and POR genes, and repressed their endogenous expressions and activities. These findings suggested miR-214 mediating down-regulation of glutathione reductase and CYP oxidoreductase genes might play an important role in oxidative stress in live cells. Wang et al. (2008, 2013) reported that miR-214 is one of the most significantly downregulated miRNAs in HCC patients. Extensive research has suggested that continued oxidative stress is a common pathologic pathway for most chronic diseases including cancer, and liver diseases. Therefore, Dong et al. (2013) postulated that miR-214 could be a key post-transcriptional regulator in oxidative stress-mediated human diseases. This microRNA will be also an important molecule to study in oxidative stress induced by AFB1 in liver.

Future validation for all of these miRNAs will be needed to assess their prognostic significance. It is notable that only a few miRNA signatures could potentially be used for diagnosis and prognosis, and even for these there is still a long way to go before they can be used in clinics. To achieve this goal, the miRNA signatures need to be further validated with high accuracy in prospective studies (Ji and Wang, 2009).

FUTURE PERSPECTIVES IN TOXICOLOGICAL RESEARCH

There is a need for novel markers that would combine the less invasiveness of a blood test and serve as a reliable early detection method. miRNAs definitely have this potential because not only they can be detected in plasma, but their sensitivity and stability are suitable for a clinical setting. Depending on the method, as little as one copy can be detected. The discovery of circulating miRNAs offers interesting clinical perspectives but this field of research is quite recent and more work has to be done.

Recently, measurement of circulating miRNAs has shown promise in identification of new biomarkers of liver injury. Further studies are needed to evaluate the sensitivity and specificity as well as validate the omics biomarkers of hepatotoxicity-xenobiotic exposure related.

It is difficult to establish the precise cause-effect relationships among environmental chemicals, miRNA alterations, and diseases. Future studies will need to demonstrate the contribution of environment-miRNA interaction to environmental human disease. The rapidly growing evidence linking miRNAs and environmental chemical, coupled with the unique regulatory role of miRNAs in gene expression, makes miRNAs potential biomarkers for elucidating the mechanisms and developing more effective prevention strategies for environmental diseases (Hou et al., 2011).

Currently, over five billion people worldwide experience uncontrolled exposure to aflatoxin (Strosnider et al., 2006). What remains unknown is how many liver cancer cases can be attributed to this aflatoxin exposure worldwide. Recently Liu and Wu (2010) have developed a risk assessment for the contribution

of aflatoxin to the global burden of HCC. Of the 550,000–600,000 new HCC cases worldwide per year, they estimate about 25,200–155,000 (4.6–28.2%) may be attributable to aflatoxin exposure alone. The broad range in the estimate reflects limitations in determining levels of aflatoxin exposures, uncertainties in the nature of the dose-response curve, uncertainties in the mode of interaction between aflatoxins and viruses, and incomplete data on the prevalence of HBV in different regions of the world. Data driven estimates of the noncarcinogenic health effects of aflatoxins in humans have not been undertaken (Kensler et al., 2011).

The understanding of miRNA biology has advanced greatly in recent years, and the continuous technological advances in accurate miRNA detection, prospect a very promising role for miRNAs as novel biomarkers of environmental chemical exposure-related diseases Identifying chemical-specific miRNAs will not only help our understanding of environmental disease, but may open the way to novel biomonitoring and preventive strategies. Therefore, it is critically important to be able to identify and validate miRNAs that can be induced by specific environmental chemicals and regulate gene expression (Hou et al., 2011).

Understanding the miRNAs roles in toxicological processes requires overall a toxicogenomic approach. On the other hand, miRNA profiling data looks promising as a tool to predict the potential toxicity of unknown compounds. Thus, miRNA signatures of a known toxic compound may include miRNAs related to cellular response to stress, xenobiotic metabolism, and/or DNA repair. These signatures derived from supervised classification algorithms may effectively identify potential toxic compounds. Several examples of miRNAs active in cellular stress as well as in interactions of a number of toxicants. miRNA profiling may lead to the discovery of miRNA exposure biomarkers, which might work as sentinel molecules to better predict both efficacy and safety. The miRNA field in toxicology is still in its early stages. However, progress is occurring at a fast pace and the numbers of publications featuring miRNAs are increasing. As the roles of miRNAs in cellular response to xenobiotic stress and the development of physiological changes and other toxicological phenomenon such susceptibility and resistance are gradually uncovered, the coming years promise to be full of exciting avenues of miRNA research in toxicogenomics (Lema and Cunningham, 2010).

Potential microRNAs that could serve as possible markers of HCC by exposure to aflatoxins are miR-27a, miR-27b, miR-122, miR-148, miR-155, miR-192, miR-214, miR-221, miR-429, and miR-500. Future studies should include some of these microRNAs and confirm their relationship with the induction of HCC due to aflatoxin exposure.

REFERENCES

Abdel-Wahhab, M. A., Omara, E. A., Abdel-Galil, M. M., Hassan, N. S., Nada, S. A., Saeed, A., et al. (2007). *Zizyphus spina-christi* extract protects against aflatoxin B1-initiated hepatic carcinogenicity. *Afr. J. Tradit. Complement. Altern. Med.* 4, 248–256.

Alm-Eldeen, A. A., Mona, M. H., Shati, A. A., and El-Mekkawy, H. I. (2013). Synergistic effect of black tea and curcumin in improving the hepatotoxicity induced by aflatoxin B1 in rats. *Toxicol. Ind. Health.* doi: 10.1177/0748233713491807. [Epub ahead of print].

Arenas-Huertero, F., Apátiga-Vega, E., Miguel-Pérez, G., Villeda-Cuevas, D., and Trillo-Tinoco, J. (2011). "Molecular markers associated with the biological response to aromatic hydrocarbons from urban air in humans," in *Air Pollution: New Developments, Chapter 4.* ed A. M. Moldoveanu (Croacia: INTECH), 87–112.

Avissar-Whiting, M., Veiga, K. R., Uhl, K. M., Maccani, M. A., Gagne, L. A., Moen, E. L., et al. (2010). Bisphenol A exposure leads to specific microRNA alterations in placental cells. *Reprod. Toxicol.* 29, 401–406. doi: 10.1016/j.reprotox.2010.04.004

Bai, S., Nasser, M. W., Wang, B., Hsu, S. H., Datta, J., Kutay, H., et al. (2009). MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J. Biol. Chem.* 284, 32015–32027. doi: 10.1074/jbc.M109.016774

Bartels, C. L., and Tsongalis, G. J. (2009). microRNAs: novel biomarkers for human cancer. *Clin. Chem.* 55, 623–631. doi: 10.1373/clinchem.2008.112805

Bennett, R. A., Essigmann, J. M., and Wogan, G. N. (1981). Excretion of an aflatoxin-guanine adduct in the urine of aflatoxin B1-treated rats. *Cancer Res.* 41, 650–654.

Bi, Q., Tang, S., Xia, L., Du, R., Fan, R., Gao, L., et al. (2012). Ectopic expression of MiR-125a inhibits the proliferation and metastasis of hepatocellular carcinoma by targeting MMP11 and VEGF. *PLoS ONE* 7:e40169. doi: 10.1371/journal.pone.0040169

Bollati, V., Marinelli, B., Apostoli, P., Bonzini, M., Nordio, F., Hoxha, M., et al. (2010). Exposure to metal-rich particulate matter modifies the expression of candidate microRNAs in peripheral blood leukocytes. *Environ. Health Perspect.* 118, 763–768. doi: 10.1289/ehp.0901300

Borel, F., Han, R., Visser, A., Petry, H., van Deventer, S. J., Jansen, P. L., et al. (2012b). Adenosine triphosphate-binding cassette transporter genes up-regulation in untreated hepatocellular carcinoma is mediated by cellular microRNAs. *Hepatology* 55, 821–832. doi: 10.1002/hep.24682

Borel, F., Konstantinova, P., and Jansen, P. L. (2012a). Diagnostic and therapeutic potential of miRNA signatures in patients with hepatocellular carcinoma. *J. Hepatol.* 56, 1371–1383. doi: 10.1016/j.jhep.2011.11.026

Braconi, C., Henry, J. C., Kogure, T., Schmittgen, T., and Patel, T. (2011). The role of microRNAs in human liver cancers. *Semin. Oncol.* 38, 752–763. doi: 10.1053/j.seminoncol.2011.08.001

Braconi, C., Valeri, N., Gasparini, P., Huang, N., Taccioli, C., Nuovo, G., et al. (2010). Hepatitis C virus proteins modulate microRNA expression and chemosensitivity in malignant hepatocytes. *Clin. Cancer Res.* 16, 957–966. doi: 10.1158/1078-0432.CCR-09-2123

Budhu, A., Jia, H. L., Forgues, M., Liu, C. G., Goldstein, D., Lam, A., et al. (2008). Identification of metastasis related microRNAs in hepatocellular carcinoma. *Hepatology* 47, 897–907. doi: 10.1002/hep.22160

Callegari, E., Elamin, B. K., Giannone, F., Milazzo, M., Altavilla, G., Fornari, F., et al. (2012). Liver tumorigenicity promoted by microRNA-221 in a mouse transgenic model. *Hepatology* 56, 1025–1033. doi: 10.1002/hep.25747

Callegari, E., Elamin, B. K., Sabbioni, S., Gramantieri, L., and Negrini, M. (2013). Role of microRNAs in hepatocellular carcinoma: a clinical perspective. *Onco Targets Ther.* 6, 1167–1178. doi: 10.2147/OTT.S36161

Chang, T. C., Yu, D., Lee, Y. S., Wentzel, E. A., Arking, D. E., West, K. M., et al. (2008). Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat. Genet.* 40, 43–50. doi: 10.1038/ng.2007.30

Chen, H. Y., Han, Z. B., Fan, J. W., Xia, J., Wu, J. Y., Qiu, G. Q., et al. (2012b). miR-203 expression predicts outcome after liver transplantation for hepatocellular carcinoma in cirrhotic liver. *Med. Oncol.* 29, 1859–1865. doi: 10.1007/s12032-011-0031-9

Chen, L., Jiang, M., Yuan, W., and Tang, H. (2012a). miR-17-5p as a novel prognostic marker for hepatocellular carcinoma. *J. Invest. Surg.* 25, 156–161. doi: 10.3109/08941939. 2011.618523

Chen, X.-M. (2009). MicroRNA signatures in liver diseases. *World J. Gastroenterol.* 15, 1665–1672. doi: 10.3748/wjg.15.1665

Cheng, Y., Liu, X., Zhang, S., Lin, Y., Yang, J., and Zhang, C. (2009). MicroRNA-21 protects against the H(2)O(2)-induced injury on cardiac myocytes via its target gene PDCD4. *J. Mol. Cell. Cardiol.* 47, 5–14. doi: 10.1016/j.yjmcc.2009.01.008

Choudhuri, S. (2010). Small noncoding RNAs: biogenesis, function, and emerging significance in toxicology. *J. Biochem. Mol. Toxicol.* 24, 195–216. doi: 10.1002/jbt.20325

Connolly, E., Melegari, M., Landgraf, P., Tchaikovskaya, T., Tennant, B. C., Slagle, B. L., et al. (2008). Elevated expression of the miR-17-92 polycistron and

miR-21 in hepadnavirus-associated hepatocellular carcinoma contributes to the malignant phenotype. *Am. J. Pathol.* 173, 856–864. doi: 10.2353/ajpath.2008.080096

Coulouarn, C., Factor, V. M., Andersen, J. B., Durkin, M. E., and Thorgeirsson, S. S. (2009). Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. *Oncogene* 28, 3526–3536. doi: 10.1038/onc. 2009.211

Crinnion, W. J. (2010). The CDC fourth national report on human exposure to environmental chemicals: what it tells us about our toxic burden and how it assist environmental medicine physicians. *Altern. Med. Rev.* 15, 101–109.

Crosby, M. E., Kulshreshtha, R., Ivan, M., and Glazer, P. M. (2009). MicroRNA regulation of DNA repair gene expression in hypoxic stress. *Cancer Res.* 69, 1221–1229. doi: 10.1158/0008-5472.CAN-08-2516

Ding, J., Huang, S., Wu, S., Zhao, Y., Liang, L., Yan, M., et al. (2010). Gain of miR-151 on chromosome 8q24.3 facilitates tumour cell migration and spreading through downregulating RhoGDIA. *Nat. Cell Biol.* 12, 390–399. doi: 10.1038/ncb2039

Dong, X., Liu, H., Chen, F., Li, D., and Zhao, Y. (2013). MiR-214 promotes the alcohol-induced oxidative stress via down-regulation of glutathione reductase and cytochrome P450 oxidoreductase in liver cells. *Alcohol. Clin. Exp. Res.* 38, 68–77. doi: 10.1111/acer.12209

Elamin, B. K., Callegari, E., Gramantieri, L., Sabbioni, S., and Negrini, M. (2011). MicroRNA response to environmental mutagens in liver. *Mutat. Res.* 717, 67–76. doi: 10.1016/j.mrfmmm.2011.03. 015

El-Agamy, D. S. (2010). Comparative effects of curcumin and resveratrol on aflatoxin B1-induced liver injury in rats. *Arch. Toxicol.* 84, 389–396. doi: 10.1007/s00204-010-0511-2

Elyakim, E., Sitbon, E., Faerman, A., Tabak, S., Montia, E., Belanis, L., et al. (2010). hsa-miR-191 is a candidate oncogene target for hepatocellular carcinoma therapy. *Cancer Res.* 70, 8077–8087. doi: 10.1158/0008-5472.CAN-10-1313

Etheridge, A., Lee, I., Hood, L., Galas, D., and Wang, K. (2011). Extracellular microRNA: a new source of biomarkers. *Mutat. Res.* 717, 85–90. doi: 10.1016/j.mrfmmm.2011.03.004

Fan, C. G., Wang, C. M., Tian, C., Wang, Y., Li, L., Sun, W. S., et al. (2011). miR-122 inhibits viral replication and cell proliferation in hepatitis B virus-related hepatocellular carcinoma and targets NDRG3. *Oncol. Rep.* 26, 1281–1286. doi: 10.3892/or.2011.1375

Fan, M. Q., Huang, C. B., Gu, Y., Xiao, Y., Sheng, J. X., and Zhong, L. (2013). Decrease expression of microRNA-20a promotes cancer cell proliferation and predicts poor survival of hepatocellular carcinoma. *J. Exp. Clin. Cancer Res.* 32, 21. doi: 10.1186/1756-9966-32-21

Fornari, F., Gramantieri, L., Ferracin, M., Veronese, A., Sabbioni, S., Calin, G. A., et al. (2008). MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. *Oncogene* 27, 5651–5661. doi: 10.1038/onc.2008.178

Fornari, F., Gramantieri, L., Giovannini, C., Veronese, A., Ferracin, M., Sabbioni, S., et al. (2009). MiR-122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human hepatocarcinoma cells. *Cancer Res.* 69, 5761–5767. doi: 10.1158/0008-5472.CAN-08-4797

Forrester, L. M., Neal, G. E., Judah, D. J., Glancey, M. J., and Wolf. C. R. (1990). Evidence for involvement of multiple forms of cytochrome P-450 in aflatoxin B1 metabolism in human liver. *Proc. Natl. Acad. Sci. U.S.A.* 87, 8306–8310. doi: 10.1073/pnas.87.21.8306

Fukushima, T., Hamada, Y., Yamada, H., and Horii, I. (2007). Changes of microRNA expression in rat liver treated by acetaminophen or carbon tetrachloride–regulating role of micro-RNA for RNA expression. *J. Toxicol. Sci.* 32, 401–409. doi: 10.2131/jts.32.401

Furuta, M., Kozaki, K. I., Tanaka, S., Arii, S., Imoto, I., and Inazawa, J. (2010). MiR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. *Carcinogenesis* 31, 766–776. doi: 10.1093/carcin/bgp250

Gallagher, E. P., Wienkers, L. C., Stapleton, P. L., Kunze, K. L., and Eaton, D. L. (1994). Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B1. *Cancer Res.* 54, 101–108.

Garofalo, M., Di Leva, G., Romano, G., Nuovo, G., Suh, S. S., Ngankeu, A., et al. (2009). miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. *Cancer Cell* 16, 498–509. doi: 10.1016/j.ccr.2009.10.014

Gatzidou, E. T., Zira, A. N., and Theocharis, S. E. (2007). Toxicogenomics: a pivotal piece in the puzzle of toxicological research. *J. Appl. Toxicol.* 27, 302–309. doi: 10.1002/jat.1248

Georges, S. A., Biery, M. C., Kim, S. Y., Schelter, J. M., Guo, J., Chang, A. N., et al. (2008). Coordinated regulation of cell cycle transcripts by p53-Inducible microRNAs, miR-192 and miR-215. *Cancer Res.* 68, 10105–10112. doi: 10.1158/0008-5472.CAN-08-1846

Gonzalez, F. J., and Yu, A. M. (2006). Cytochrome P450 and xenobiotic receptor humanized mice. *Annu. Rev. Pharmacol. Toxicol.* 46, 41–64. doi: 10.1146/annurev.pharmtox.45. 120403.100007

Gramantieri, L., Ferracin, M., Fornari, F., Veronese, A., Sabbioni, S., Liu, C. G., et al. (2007). Cyclin G1 is a target of miR-122a, a microRNA frequently downregulated in human hepatocellular carcinoma. *Cancer Res.* 67, 6092–6099. doi: 10.1158/0008-5472.CAN-06-4607

Gramantieri, L., Fornari, F., Callegari, E., Sabbioni, S., Lanza, G., Croce, C. M., et al. (2008). MicroRNA involvement in hepatocellular carcinoma. *J. Cell. Mol. Med.* 12, 2189–2204. doi: 10.1111/j.1582-4934.2008.00533.x

Gramantieri, L., Fornari, F., Ferracin, M., Veronese, A., Sabbioni, S., Calin, G. A., et al. (2009). MicroRNA-221 targets Bmf in hepatocellular carcinoma and correlates with tumor multifocality. *Clin. Cancer Res.* 15, 5073–5081. doi: 10.1158/1078-0432.CCR-09-0092

Groopman, J. D., Johnson, D., and Kensler, T. W. (2005). Aflatoxin and hepatitis B virus biomarkers: a paradigm for complex environmental exposures and cancer risk. *Cancer Biomark.* 1, 5–14.

He, X. X., Chang, Y., Meng, F. Y., Wang, M. Y., Xie, Q. H., Tang, F., et al. (2012). MicroRNA-375 targets AEG-1 in hepatocellular carcinoma and suppresses liver cancer cell growth *in vitro* and *in vivo*. *Oncogene* 31, 3357–3369. doi: 10.1038/onc.2011.500

Hou, L., Wang, D., and Baccarelli, A. (2011). Environmental chemicals and microRNAs. *Mutat. Res.* 714, 105–112. doi: 10.1016/j.mrfmmm.2011.05.004

Hou, L., Zhang, X., Wang, D., and Baccarelli, A. (2012). Environmental chemicals exposure and human epigenetics. *Int. J. Epidemiol.* 41, 79–105. doi: 10.1093/ije/dyr154

Hsu, P. Y., Deatherage, D. E., Rodriguez, B. A., Liyanarachchi, S., Weng, Y. I., Zuo, T., et al. (2009). Xenoestrogen-induced epigenetic repression of microRNA-9-3 in breast epithelial cells. *Cancer Res.* 69, 5936–5945. doi: 10.1158/0008-5472.CAN-08-4914

Hsu, S. H., Wang, B., Kota, J., Yu, J., Costinean, S., Kutay, H., et al. (2012). Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. *J. Clin. Invest.* 122, 2871–2883. doi: 10.1172/JCI63539

Hu, H., and Gatti, R. A. (2011). MicroRNAs: new players in the DNA damage response. *J. Mol. Cell. Biol.* 3, 151–158. doi: 10.1093/jmcb/mjq042

Huang, S., He, X., Ding, J., Liang, L., Zhao, Y., Zhang, Z., et al. (2008). Upregulation of miR-23a approximately 27a approximately 24 decreases transforming growth factor-beta-induced tumor-suppressive activities in human hepatocellular carcinoma cells. *Int. J. Cancer* 123, 972–978. doi: 10.1002/ijc.23580

Huang, X. H., Chen, J. S., Wang, Q., Chen, X. L., Wen, L., Chen, L. Z., et al. (2011). miR-338-3p suppresses invasion of liver cancer cell by targeting smoothened. *J. Pathol.* 225, 463–472. doi: 10.1002/path.2877

Huang, X. H., Wang, Q., Chen, J. S., Fu, X. H., Chen, X. L., Chen, L. Z., et al. (2009). Bead-based microarray analysis of microRNA expression in hepatocellular carcinoma: miR-338 is downregulated. *Hepatol. Res.* 39, 786–794. doi: 10.1111/j.1872-034X.2009.00502.x

Huang, X. Y., Yao, J. G., Huang, H. D., Wang, C., Ma, Y., Xia, Q., et al. (2013). microRNA-429 modulates hepatocellular carcinoma prognosis and tumorigenesis. *Gastroenterol. Res. Pract.* 2013: 804128. doi: 10.1155/2013/804128

Huang, Y. H., Lin, K. H., Chen, H. C., Chang, M. L., Hsu, C. W., Lai, M. W., et al. (2012). Identification of postoperative prognostic microRNA predictors in hepatocellular carcinoma. *PLoS ONE* 7:e37188. doi: 10.1371/journal.pone.0037188

Ilnytskyy, Y., Zemp, F. J., Koturbash, I., and Kovalchuk, O. (2008). Altered microRNA expression patterns in irradiated hematopoietic tissues suggest a sex-specific protective mechanism. *Biochem. Biophys. Res. Commun.* 377, 41–45. doi: 10.1016/j.bbrc.2008.09.080

Izzotti, A., Calin, G. A., Arrigo, P., Steele, V. E., Croce, C. M., and De Flora, S. (2009b). Downregulation of microRNA expression in the lungs of rats exposed to cigarette smoke. *FASEB J.* 23, 806–812. doi: 10.1096/fj.08-121384

Izzotti, A., Calin, G. A., Steele, V. E., Croce, C. M., and De Flora, S. (2009a). Relationships of microRNA expression in mouse lung with age and exposure to cigarette smoke and light. *FASEB J.* 23, 3243–3250. doi: 10.1096/fj.09-135251

Jardim, M. J., Fry, R. C., Jaspers, I., Dailey, L., and Diaz-Sanchez, D. (2009). Disruption of microRNA expression in human airway cells by diesel exhaust particles is linked to tumorigenesis-associated pathways. *Environ. Health Perspect.* 117, 1745–1751. doi: 10.1289/ehp.0900756

Jensen, M. R., Factor, V. M., Fantozzi, A., Helin, K., Huh, C. G., and Thorgeirsson, S. S. (2003). Reduced hepatic tumor incidence in cyclin G1-deficient mice. *Hepatology* 37, 862–870. doi: 10.1053/jhep.2003.50137

Ji, J., Shi, J., Budhu, A., Yu, Z., Forgues, M., Roessler, S., et al. (2009a). MicroRNA expression, survival, and response to interferon in liver cancer. *N. Engl. J. Med.* 361, 1437–1447. doi: 10.1056/NEJMoa0901282

Ji, J., and Wang, X. W. (2009). New kids on the block: diagnostic and prognostic microRNAs in hepatocellular carcinoma. *Cancer Biol. Ther.* 8, 1686–1693. doi: 10.4161/cbt.8.18.8898

Ji, J., Zhang, J., Huang, G., Qian, J., Wang, X., and Mei, S. (2009b). Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation during rat hepatic stellate cell activation. *FEBS Lett.* 583, 759–766. doi: 10.1016/j.febslet.2009.01.034

Ji, J., Zhao, L., Budhu, A., Forgues, M., Jia, H. L., Qin, L. X., et al. (2010). Let-7g targets collagen type I alpha2 and inhibits cell migration in hepatocellular carcinoma. *J. Hepatol.* 52, 690–697. doi: 10.1016/j.jhep.2009.12. 025

Jiang, J., Gusev, Y., Aderca, I., Mettler, T. A., Nagorney, D. M., Brackett, D. J., et al. (2008). Association of microRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. *Clin. Cancer Res.* 14, 419–427. doi: 10.1158/1078-0432.CCR-07-0523

Kalscheuer, S., Zhang, X., Zeng, Y., and Upadhyaya, P. (2008). Differential expression of microRNAs in early-stage neoplastic transformation in the lungs of F344 rats chronically treated with the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Carcinogenesis* 29, 2394–2399. doi: 10.1093/carcin/bgn209

Karakatsanis, A., Papaconstantinou, I., Gazouli, M., Lyberopoulou, A., Polymeneas, G., and Voros, D. (2013). Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. *Mol. Carcinog.* 52, 297–303. doi: 10.1002/mc.21864

Katayama, Y., Maeda, M., Miyaguchi, K., Nemoto, S., Yasen, M., Tanaka, S., et al. (2012). Identification of pathogenesis-related microRNAs in hepatocellular carcinoma by expression profiling. *Oncol. Lett.* 4, 817–823. doi: 10.3892/ol.2012.810

Kensler, T. W., Roebuck, B. D., Wogan, G. N., and Groopman, J. D (2011). Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. *Toxicol. Sci.* 120, S28–S48. doi: 10.1093/toxsci/kfq283

Kerr, T. A., Korenblat, K. M., and Davidson, N. O. (2011). MicroRNAs and liver disease. *Transl. Res.* 4, 241–252. doi: 10.1016/j.trsl.2011.01.008

Kim, T., Veronese, A., Pichiorri, F., Lee, T. J., Jeon, Y. J., Volinia, S., et al. (2011). p53 regulates epithelial-mesenchymal transition through microRNAs targeting ZEB1 and ZEB2. *J. Exp. Med.* 208, 875–883. doi: 10.1084/jem.20110235

Koh, Y. S., Kim, J. H., Cai, H., Li, L.-H., Kim, H.-S., Kim, K., et al. (2013). Dysregulated microRNAs in non-cirrhotic hepatocellular carcinoma. *Genes Genom.* 35, 759–765. doi: 10.1007/s13258-013-0126-0

Kutay, H., Bai, S., Datta, J., Motiwala, T., Pogribny, I., Frankel, W., et al. (2006). Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. *J. Cell. Biochem.* 99, 671–678. doi: 10.1002/jcb.20982

Ladeiro, Y., Couchy, G., Balabaud, C., Bioulac-Sage, P., Pelletier, L., Rebouissou, S., et al. (2008). MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. *Hepatology* 47, 1955–1963. doi: 10.1002/hep.22256

Lan, F. F., Wang, H., Chen, Y. C., Chan, C. Y., Ng, S. S., Li, K., et al. (2011). Hsa-let-7g inhibits proliferation of hepatocellular carcinoma cells by downregulation of c-Myc and upregulation of p16(INK4A). *Int. J. Cancer* 128, 319–331. doi: 10.1002/ijc.25336

Lang, Q., and Ling, C. (2012). MiR-124 suppresses cell proliferation in hepatocellular carcinoma by targeting PIK3CA. *Biochem. Biophys. Res. Commun.* 426, 247–252. doi: 10.1016/j.bbrc.2012.08.075

Law, P. T., and Wong, N. (2011). Emerging roles of microRNA in the intracellular signaling networks of hepatocellular carcinoma. *J. Gastroenterol. Hepatol.* 26, 437–449. doi: 10.1111/j.1440-1746.2010.06512.x

Lema, C., and Cunningham, M. J. (2010). MicroRNAs and their implications in toxicological research. *Toxicol. Lett.* 198, 100–105. doi: 10.1016/j.toxlet.2010.06.019

Lewis, A. P., and Jopling, C. L. (2010). Regulation and biological function of the liver-specific miR-122. *Biochem. Soc. Trans.* 38, 1553–1557. doi: 10.1042/BST0381553

Li, N., Fu, H., Tie, Y., Hu, Z., Kong, W., Wu, Y., et al. (2009a). miR-34a inhibits migration and invasion by down-regulation of c-Met expression in human hepatocellular carcinoma cells. *Cancer Lett.* 275, 44–53. doi: 10.1016/j.canlet.2008.09.035

Li, R., Qian, N., Tao, K., You, N., Wang, X., and Dou, K. (2010). MicroRNAs involved in neoplastic transformation of liver cancer stem cells. *J. Exp. Clin. Cancer Res.* 29, 169. doi: 10.1186/1756-9966-29-169

Li, S., Fu, H., Wang, Y., Tie, Y., Xing, R., Zhu, J., et al. (2009b). MicroRNA-101 regulates expression of the v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) oncogene in human hepatocellular carcinoma. *Hepatology* 49, 1194–1202. doi: 10.1002/hep.22757

Li, W., Xie, L., He, X., Li, J., Tu, K., Wei, L., et al. (2008). Diagnostic and prognostic implications of microRNAs in human hepatocellular carcinoma. *Int. J. Cancer* 123, 1616–1622. doi: 10.1002/ijc.23693

Li, Y., Tan, W., Neo, T. W., Aung, M. O., Wasser, S., Lim, S. G., et al. (2009c). Role of the miR-106b-25 microRNA cluster in hepatocellular carcinoma. *Cancer Sci.* 100, 1234–1242. doi: 10.1111/j.1349-7006.2009.01164.x

Li, Y. P., Gottwein, J. M., Scheel, T. K., Jensen, T. B., and Bukh, J. (2011). MicroRNA-122 antagonism against hepatitis C virus genotypes 1-6 and reduced efficacy by host RNA insertion or mutations in the HCV 5′ UTR. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4991–4996. doi: 10.1073/pnas.1016606108

Liang, Z., Gao, Y., Shi, W., Zhai, D., Li, S., Jing, L., et al. (2013). Expression and significance of microRNA-183 in hepatocellular carcinoma. *Sci. World J.* 2013:381874. doi: 10.1155/2013/381874

Lin, C. J., Gong, H. Y., Tseng, H. C., Wang, W. L., and Wu, J. L. (2008). miR-122 targets an anti-apoptotic gene, Bcl-w, in human hepatocellular carcinoma cell lines. *Biochem. Biophys. Res. Commun.* 375, 315–320. doi: 10.1016/j.bbrc.2008.07.154

Lin, J., Huang, S., Wu, S., Ding, J., Zhao, Y., Liang, L., et al. (2011). MicroRNA-423 promotes cell growth and regulates G(1)/S transition by targeting p21Cip1/Waf1 in hepatocellular carcinoma. *Carcinogenesis* 32, 1641–1647. doi: 10.1093/carcin/bgr199

Lin, Y., Liu, X., Cheng, Y., Yang, J., Huo, Y., and Zhang, C. (2009). Involvement of MicroRNAs in hydrogen peroxide-mediated gene regulation and cellular injury response in vascular smooth muscle cells. *J. Biol. Chem.* 284, 7903–7913. doi: 10.1074/jbc. M806920200

Liu, A. M., Poon, R. T., and Luk, J. M. (2010). MicroRNA-375 targets Hippo-signaling effector YAP in liver cancer and inhibits tumor properties. *Biochem. Biophys. Res. Commun.* 394, 623–627. doi: 10.1016/j.bbrc.2010.03.036

Liu, A. M., Yao, T. J., Wang, W., Wong, K. F., Lee, N. P., Fan, S. T., et al. (2012). Circulating miR-15b and miR-130b in serum as potential markers for detecting hepatocellular carcinoma: a retrospective cohort study. *BMJ Open* 2:e000825. doi: 10.1136/bmjopen-2012-000825

Liu, W. H., Yeh, S. H., Lu, C. C., Yu, S. L., Chen, H. Y., Lin, C. Y., et al. (2009). MicroRNA-18a prevents estrogen receptoralpha expression, promoting proliferation of hepatocellular carcinoma cells. *Gastroenterology* 136, 683–693. doi: 10.1053/ j.gastro.2008.10. 029

Liu, Y., and Wu, F. (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environ. Health Perspect.* 118, 818–824. doi: 10.1289/ehp.0901388

Long, X.-D., Ma, Y., Wei, Y. P., and Deng, Z. L. (2006). The polymorphisms of GSTM1, GSTT1, HYL1*2, and XRCC1, and aflatoxin B1-related hepatocellular carcinoma in Guangxi population, China. *Hepatol. Res.* 36, 48–55. doi: 10.1016/j.hepres.2006.06.004

Long, X.-D., Yao, J.-G., Zeng, Z., Huang, C.-H., Liao, P., Huang, Z.-S., et al. (2011). "DNA Repair capacity-related to genetic polymorphisms of DNA repair genes and aflatoxin B1-related hepatocellular carcinoma among Chinese population, chapter 25," in *DNA Repair*, ed I. Kruman (Rijeka: InTech), 505–524.

Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., et al. (2005). MicroRNA expression profiles classify human cancers. *Nature* 435, 834–838. doi: 10.1038/nature03702

Lukiw, W. J., and Pogue, A. I. (2007). Induction of specific micro RNA (miRNA) species by ROS-generating metal sulfates in primary human brain cells. *J. Inorg. Biochem.* 101, 1265–1269. doi: 10.1016/j.jinorgbio.2007.06.004

Ma, L., Liu, J., Shen, J., Liu, L., Wu, J., Li, W., et al. (2010). Expression of miR-122 mediated by adenoviral vector induces apoptosis and cell cycle arrest of cancer cells. *Cancer Biol. Ther.* 9, 554–561. doi: 10.4161/cbt.9.7.11267

Marsit, C. J., Eddy, K., and Kelsey, K. T. (2006). MicroRNA responses to cellular stress. *Cancer Res.* 66, 10843–10848. doi: 10.1158/0008-5472.CAN-06-1894

Medina, R., Zaidi, S.K., Liu, C.G., Stein, J.L., van Wijnen, A.J., Croce, C.M., et al. (2008). MicroRNAs 221 and 222 bypass quiescence and compromise cell survival. *Cancer Res.* 68, 2773–2780. doi: 10.1158/0008-5472.CAN-07-6754

Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S. T., and Patel, T. (2007). MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* 133, 647–658. doi: 10.1053/j.gastro.2007.05.022

Meng, Z., Fu, X., Chen, X., Zeng, S., Tian, Y., Jove, R., et al. (2010). miR-194 is a marker of hepatic epithelial cells and suppresses metastasis of liver cancer cells in mice. *Hepatology* 52, 2148–2157. doi: 10.1002/hep.23915

Moffat, I. D., Boutros, P. C., Celius, T., Linden, J., Pohjanvirta, R., and Okey, A. B. (2007). microRNAs in adult rodent liver are refractory to dioxin treatment. *Toxicol. Sci.* 99, 470–487. doi: 10.1093/toxsci/kfm189

Mohri, T., Nakajima, M., Takagi, S., Komagata, S., and Yokoi, T. (2009). MicroRNA regulates human vitamin D receptor. *Int. J. Cancer* 125, 1328–1333. doi: 10.1002/ijc.24459

Morgan, T. R., Mandayam, S., and Jamal, M. M. (2004). Alcohol and hepatocellular carcinoma. *Gastroenterology* 127, S87–S96. doi: 10.1053/j.gastro.2004.09.020

Murakami, Y., Yasuda, T., Saigo, K., Urashima, T., Toyoda, H., Okanoue, T., et al. (2006). Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and nontumorous tissues. *Oncogene* 25, 2537–2545. doi: 10.1038/sj.onc.1209283

Natarajan, A. T., and Palitti, F. (2008). DNA repair and chromosomal alterations. *Mutat. Res.* 657, 3–7. doi: 10.1016/j.mrgentox.2008.08.017

Negrini, M., Gramantieri, L., Sabbioni, S., and Croce, C. M. (2011). microRNA involvement in hepatocellular carcinoma. *Anticancer Agents Med. Chem.* 11, 500–521. doi: 10.2174/ 187152011796011037

Newbold, R. R. (2010). Impact of environmental endocrine disrupting chemicals on the development of obesity. *Hormones (Athens)* 9, 206–217. doi: 10.14310/horm.2002.1271

Nouspikel, T. (2009). DNA repair in mammalian cells: nucleotide excision repair: variations on versatility. *Cell. Mol. Life Sci.* 66, 994–1009. doi: 10.1007/s00018-009-8737-y

Pan, Y. Z., Gao, W., and Yu, A. M. (2009). microRNAs regulate CYP3A4 expression via direct and indirect targeting. *Drug Metab. Dispos.* 37, 2112–2117. doi: 10.1124/dmd.109.027680

Patel, C. J., and Butte, A. J. (2010). Predicting environmental chemical factors associated with disease-related gene expression data. *BMC Med. Genom.* 3:17. doi: 10.1186/1755-8794-3-17

Pineau, P., Volinia, S., McJunkin, K., Marchio, A., Battiston, C., Terris, B., et al. (2010). miR-221 overexpression contributes to liver tumorigenesis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 264–269. doi: 10.1073/pnas. 0907904107

Pogribny, I. P., Muskhelishvili, L., Tryndyak, V. P., and Beland, F. A. (2009). The tumor-promoting activity of 2-acetylaminofluorene is associated with disruption of the p53 signaling pathway and the balance between apoptosis and cell proliferation. *Toxicol. Appl. Pharmacol.* 235, 305–311. doi: 10.1016/j.taap.2008.12.021

Pogribny, I. P., Tryndyak, V. P., Boyko, A., Rodriguez-Juarez, R., Beland, F. A., and Kovalchuk, O. (2007). Induction of microRNAome deregulation in rat liver by long-term tamoxifen exposure. *Mutat. Res.* 619, 30–37. doi: 10.1016/j.mrfmmm.2006.12.006

Pogue, A. I., Li, Y. Y., Cui, J. G., Zhao, Y., Kruck, T. P., Percy, M. E., et al. (2009). Characterization of an NF-kappaB-regulated, miRNA-146a-mediated down-regulation of complement factor H (CFH) in metal-sulfate-stressed human brain cells. *J. Inorg. Biochem.* 103, 1591–1595. doi: 10.1016/j.jinorgbio.2009.05.012

Qi, P., Cheng, S. Q., Wang, H., Li, N., Chen, Y. F., and Gao, C. F. (2011). Serum microRNAs as biomarkers for hepatocellular carcinoma in Chinese patients with chronic hepatitis B virus infection. *PLoS ONE* 6:e28486. doi: 10.1371/journal.pone.0028486

Qu, K. Z., Zhang, K., Li, H., Afdhal, N. H., and Albitar, M. (2011). Circulating microRNAs as biomarkers for hepatocellular carcinoma. *J. Clin. Gastroenterol.* 45, 355–360. doi: 10.1097/MCG.0b013e3181f18ac2

Ross, J. A., Blackman, C. F., Thai, S. F., Li, Z., Kohan, M., Jones, C. P., et al. (2010). A potential microRNA signature for tumorigenic conazoles in mouse liver. *Mol. Carcinog.* 49, 320–323. doi: 10.1002/mc.20620

Rossi, L., Bonmassar, E., and Faraoni, I. (2007). Modification of miR- gene expression pattern in human colon cancer cells following exposure to 5-fluorouracil *in vitro*. *Pharmacol. Res.* 56, 248–253. doi: 10.1016/j.phrs.2007.07.001

Sabbioni, G., Skipper, P. L., Buchi, G., and Tannenbaum, S. R. (1987). Isolation and characterization of the major serum albumin adduct formed by aflatoxin B1 *in vivo* in rats. *Carcinogenesis* 8, 819–824. doi: 10.1093/carcin/8.6.819

Salvi, A., Sabelli, C., Moncini, S., Venturin, M., Arici, B., Riva, P., et al. (2009). MicroRNA-23b mediates urokinase and c-met downmodulation and a decreased migration of human hepatocellular carcinoma cells. *FEBS J.* 276, 2966–2982. doi: 10.1111/j.1742-4658.2009.07014.x

Sathyan, P., Golden, H. B., and Miranda, R. C. (2007). Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: evidence from an *ex vivo* model of the fetal cerebral cortical neuroepithelium. *J. Neurosci.* 27, 8546–8557. doi: 10.1523/JNEUROSCI.1269-07.2007

Schembri, F., Sridhar, S., Perdomo, C., Gustafson, A. M., Zhang, X., Ergun, A., et al. (2009). MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2319–2324. doi: 10.1073/pnas. 0806383106

Scholl, P. F., Musser, S. M., and Groopman, J. D. (1997). Synthesis and characterization of aflatoxin B1 mercapturic acids and their identification in rat urine. *Chem. Res. Toxicol.* 10, 1144–1151. doi: 10.1021/tx960161+

Shah, Y. M., Morimura, K., Yang, Q., Tanabe, T., Takagi, M., and Gonzalez, F. J. (2007). Peroxisome proliferator-activated receptor alpha regulates a microRNA-mediated signaling cascade responsible for hepatocellular proliferation. *Mol. Cell. Biol.* 27, 4238–4247. doi: 10.1128/MCB.00317-07

Shigoka, M., Tsuchida, A., Matsudo, T., Nagakawa, Y., Saito, H., Suzuki, Y., et al. (2010). Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development. *Pathol. Int.* 60, 351–357. doi: 10.1111/j.1440-1827.2010.02526.x

Shimada, T., Martin, M. V., Pruess-Schwartz, D., Marnett, L. J., and Guengerich, F. P. (1989). Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo(a)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Res.* 49, 6304–6312.

Singhal, A., Jayaraman, M., Dhanasekaran, D. N., and Kohli, V. (2012). Molecular and serum markers in hepatocellular carcinoma: predictive tools for prognosis and recurrence. *Crit. Rev. Oncol. Hematol.* 82, 116–140. doi: 10.1016/j.critrevonc.2011.05.005

Song, M. K., Park, Y. K., and Ryu, J. C. (2013). Polycyclic aromatic hydrocarbon (PAH)-mediated upregulation of hepatic microRNA-181 family promotes cancer cell migration by targeting MAPK phosphatase-5, regulating the activation of p38 MAPK. *Toxicol. Appl. Pharmacol.* 273, 130–139. doi: 10.1016/j.taap.2013.08.016

Strosnider, H., Azziz-Baumgartner, E., Banziger, M., Bhat, R. V., Breiman, R., Brune, M. N., et al. (2006). Workgroup report: public health strategies for reducing aflatoxin exposure in developing countries. *Environ. Health Perspect.* 114, 1898–1903. doi: 10.1289/ehp.9302

Su, H., Yang, J. R., Xu, T., Huang, J., Xu, L., Yuan, Y., et al. (2009). MicroRNA-101, down-regulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. *Cancer Res.* 69, 1135–1142. doi: 10.1158/0008-5472.CAN-08-2886

Sukata, T., Sumida, K., Kushida, M., Ogata, K., Miyata, K., Yabushita, S., et al. (2011). Circulating microRNAs, possible indicators of progress of rat hepatocarcinogenesis from early stages. *Toxicol. Lett.* 200, 46–52. doi: 10.1016/j.toxlet.2010.10.013

Sun, B., and Karin, M. (2013). Inflammation and liver tumorigenesis. *Front. Med.* 7, 242–254. doi: 10.1007/s11684-013-0256-4

Sun, Z., Han, Q., Zhou, N., Wang, S., Lu, S., Bai, C., et al. (2013). MicroRNA-9 enhances migration and invasion through KLF17 in hepatocellular carcinoma. *Mol. Oncol.* 7, 884–894. doi: 10.1016/j.molonc.2013.04.007

Szabo, G., Sarnow, P., and Bala, S. (2012). MicroRNA silencing and development of novel therapies for liver disease. *J. Hepatol.* 57, 462–466. doi: 10.1016/j.jhep.2012.01.030

Takagi, S., Nakajima, M., Mohri, T., and Yokoi, T. (2008). Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the

expression of cytochrome P450 3A4. *J. Biol. Chem.* 283, 9674–9680. doi: 10.1074/jbc.M709382200

Takata, A., Otsuka, M., Kojima, K., Yoshikawa, T., Kishikawa, T., Yoshida, H., et al. (2011). MicroRNA-22 and microRNA-140 suppress NF-κB activity by regulating the expression of NF-κB coactivators. *Biochem. Biophys. Res. Commun.* 411, 826–831. doi: 10.1016/j.bbrc.2011.07.048

Taylor, E. L., and Gant, T. W. (2008). Emerging fundamental roles for non-coding RNA species in toxicology. *Toxicology* 246, 34–39. doi: 10.1016/j.tox.2007.12.030

Tomimaru, Y., Eguchi, H., Nagano, H., Wada, H., Kobayashi, S., Marubashi, S., et al. (2012). Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. *J. Hepatol.* 56, 167–175. doi: 10.1016/j.jhep.2011.04.026

Tsai, W. C., Hsu, P. W., Lai, T. C., Chau,.G. Y., Lin, C. W., Chen, C. M., et al. (2009). MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma. *Hepatology* 49, 1571–1582. doi: 10.1002/hep.22806

Tsai, W. C., Hsu, S. D., Hsu, C. S., Lai, T. C., Chen, S. J., Shen, R., et al. (2012). MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. *J. Clin. Invest.* 122, 2884–2897. doi: 10.1172/JCI63455

Ueda, K. (2009). Effect of environmental chemicals on the genes and the gene expression. *Yakugaku Zasshi* 129, 1501–1506. doi: 10.1248/yakushi.129.1501

Ueng, Y. F., Shimada, T., Yamazaki, H., and Guengerich, F. P. (1995). Oxidation of aflatoxin B1 by bacterial recombinant human cytochrome P450 enzymes. *Chem. Res. Toxicol.* 8, 218–225. doi: 10.1021/tx00044a006

Ura, S., Honda, M., Yamashita, T., Ueda, T., Takatori, H., Nishino, R., et al. (2009). Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. *Hepatology* 49, 1098–1112. doi: 10.1002/hep.22749

Vaira, V., Faversani, A., Dohi, T., Montorsi, M., Augello, C., Gatti, S., et al. (2012). miR-296 regulation of a cell polarity-cell plasticity module controls tumor progression. *Oncogene* 31, 27–38. doi: 10.1038/onc.2011.209

Valencia-Quintana, R., Sánchez-Alarcón, J., Tenorio, M. G., Deng, Y., Waliszewski, S. M., and Valera, M. A. (2012). Preventive strategies aimed at reducing the health risks of Aflatoxin B1. *Toxicol. Environ. Health Sci.* 4, 71–79. doi: 10.1007/s13530-012-0119-4

Varnholt, H., Drebber, U., Schulze, F., Wedemeyer, I., Schirmacher, P., Dienes, H. P., et al. (2008). microRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. *Hepatology* 47, 1223–1232. doi: 10.1002/hep.22158

Volinia, S., Calin, G. A., Liu, C. G., Ambs, S., Cimmino, A., Petrocca, F., et al. (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2257–2261. doi: 10.1073/pnas.0510565103

Vuppalanchi, R., Liang, T., Goswami, C. P., Nalamasu, R., Li, L., Jones, D., et al. (2013). Relationship between differential hepatic microRNA expression and decreased hepatic cytochrome P450 3A activity in cirrhosis. *PLoS ONE* 8:e74471. doi: 10.1371/journal.pone.0074471

Wang, B., Hsu, S. H., Majumder, S., Kutay, H., Huang, W., Jacob, S. T., et al. (2010). TGFbeta-mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3. *Oncogene* 29, 1787–1797. doi: 10.1038/onc.2009.468

Wang, B., Majumder, S., Nuovo, G., Kutay, H., Volinia, S., Patel, T., et al. (2009b). Role of microRNA-155 at early stages of hepatocarcinogenesis induced by choline-deficient and amino acid-defined diet in C57BL/6 mice. *Hepatology* 50, 1152–1161. doi: 10.1002/hep.23100

Wang, C. M., Wang, Y., Fan, C. G., Xu, F. F., Sun, W. S., Liu, Y. G., et al. (2011). miR-29c targets TNFAIP3, inhibits cell proliferation and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma. *Biochem. Biophys. Res. Commun.* 411, 586–592. doi: 10.1016/j.bbrc.2011.06.191

Wang, J., Li, J., Shen, J., Wang, C., Yang, L., and Zhang, X. (2012b). MicroRNA-182 downregulates metastasis suppressor 1 and contributes to metastasis of hepatocellular carcinoma. *BMC Cancer* 12:227. doi: 10.1186/1471-2407-12-227

Wang, J., Li, J., Wang, X., Zheng, C., and Ma, W. (2013). Downregulation of microRNA-214 and overexpression of FGFR-1 contribute to hepatocellular carcinoma metastasis. *Biochem. Biophys. Res. Commun.* 439, 47–53. doi: 10.1016/j.bbrc.2013.08.032

Wang, K., Zhang, S., Marzolf, B., Troisch, P., Brightman, A., Hu, Z., et al. (2009a). Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4402–4407. doi: 10.1073/pnas.0813371106

Wang, X. W., Heegaard, N. H. H., and Ørum, H. (2012a). microRNAs in liver disease. *Gastroenterology* 142, 1431–1443. doi: 10.1053/j.gastro.2012.04.007

Wang, Y., Lee, A. T., Ma, J. Z., Wang, J., Ren, J., Yang, Y., et al. (2008). Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target. *J. Biol. Chem.* 283, 13205–13215. doi: 10.1074/jbc.M707629200

Wei, L., Gong, X., Martinez, O. M., and Krams, S. M. (2013a). Differential expression and functions of microRNAs in liver transplantation and potential use as non-invasive biomarkers. *Transpl. Immunol.* 29, 123–129. doi: 10.1016/j.trim.2013.08.005

Wei, R., Huang, G. L., Zhang, M. Y., Li, B. K., Zhang, H. Z., Shi, M., et al. (2013b). Clinical significance and prognostic value of microRNA expression signatures in hepatocellular carcinoma. *Clin. Cancer Res.* 19, 4780–4791. doi: 10.1158/1078-0432.CCR-12-2728

Wild, C. P. (2009). Environmental exposure measurement in cancer epidemiology. *Mutagenesis* 24, 117–125. doi: 10.1093/mutage/gen061

Wild, C. P., and Turner, P. C. (2002). The toxicology of aflatoxins as a basis for public health decisions. *Mutagenesis* 17, 471–481. doi: 10.1093/mutage/17.6.471

Wong, K. F., Xu, Z., Chen, J., Lee, N. P., and Luk, J. M. (2013). Circulating markers for prognosis of hepatocellular carcinoma. *Expert Opin. Med. Diagn.* 7, 319–329. doi: 10.1517/17530059.2013. 795146

Wong, Q. W., Ching, A. K., Chan, A. W., Choy, K. W., To, K. F., Lai, P. B., et al. (2010). MiR-222 overexpression confers cell migratory advantages in hepatocellular carcinoma through enhancing AKT signaling. *Clin. Cancer Res.* 16, 867–875. doi: 10.1158/1078-0432.CCR-09-1840

Wong, Q. W., Lung, R. W., Law, P. T., Lai, P. B., Chan, K. Y., To, K. F., et al. (2008). MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin 1. *Gastroenterology* 135, 257–269. doi: 10.1053/j.gastro.2008.04. 003

Wu, N., Liu, X., Xu, X., Fan, X., Liu, M., Li, X., et al. (2011). MicroRNA-373, a new regulator of protein phosphatase 6, functions as an oncogene in hepatocellular carcinoma. *FEBS J.* 278, 2044–2054. doi: 10.1111/j.1742-4658.2011.08120.x

Xia, Q., Huang, X.-Y., Xue, F., Zhang, J.-J., Zhai, B., Kong, D.-C., et al. (2013). "Genetic polymorphisms of DNA repair genes and DNA repair capacity related to aflatoxin B1 (AFB1)-induced DNA damages," in *New Research Directions in DNA Repair*, *Chapter 15, 1st Edn.*, ed C. Chen (Rijeka: InTech), 377–412. doi: 10.5772/53967

Xie, Q. H., He, X. X., Chang, Y., Sun, S. Z., Jiang, X., Li, P. Y., et al. (2011). MiR-192 inhibits nucleotide excision repair by targeting ERCC3 and ERCC4 in HepG2.2.15 cells. *Biochem. Biophys. Res. Commun.* 410, 440–445. doi: 10.1016/j.bbrc.2011.05.153

Xiong, Y., Fang, J. H., Yun, J. P., Yang, J., Zhang, Y., Jia, W. H., et al. (2010). Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. *Hepatology* 51, 836–845. doi: 10.1002/hep.23380

Xu, J., Wu, C., Che, X., Wang, L., Yu, D., Zhang, T., et al. (2011). Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. *Mol. Carcinog.* 50, 136–142. doi: 10.1002/mc.20712

Xu, J., Zhu, X., Wu, L., Yang, R., Yang, Z., Wang, Q., et al. (2012). MicroRNA-122 suppresses cell proliferation and induces cell apoptosis in hepatocellular carcinoma by directly targeting Wnt/β-catenin pathway. *Liver Int.* 32, 752–760. doi: 10.1111/j.1478-3231.2011.02750.x

Xu, T., Zhu, Y., Wei, Q. K., Yuan, Y., Zhou, F., Ge, Y. Y., et al. (2008). A functional polymorphism in the miR-146a gene is associated with the risk for hepatocellular carcinoma. *Carcinogenesis* 29, 2126–2131. doi: 10.1093/carcin/bgn195

Xu, T., Zhu, Y., Xiong, Y., Ge, Y. Y., Yun, J. P., and Zhuang, S. M. (2009). MicroRNA-195 suppresses tumorigenicity and regulates G1/S transition of human hepatocellular carcinoma cells. *Hepatology* 50, 113–121. doi: 10.1002/hep.22919

Xu, Y., Xie, Y., Wang, X., Chen, X., Liu, Q., Ying, M., et al. (2013). Identification of cancer stem cells from hepatocellular carcinoma cell lines and their related microRNAs. *Oncol. Rep.* 30, 2056–2062. doi: 10.3892/or.2013.2703

Yamamoto, Y., Kosaka, N., Tanaka, M., Koizumi, F., Kanai, Y., Mizutani, T., et al. (2009). MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma. *Biomarkers* 14, 529–538. doi: 10.3109/13547500903150771

Yan, D., Ng, W. L., Zhang, X., Wang, P., Zhang, Z., Mo, Y. Y., et al. (2010). Targeting DNA-PKcs and ATM with miR-101 sensitizes tumors to radiation. *PLoS ONE* 5:e11397. doi: 10.1371/journal.pone.0011397

Yang, F., Yin, Y., Wang, F., Wang, Y., Zhang, L., Tang, Y., et al. (2010). miR-17-5p promotes migration of human hepatocellular carcinoma cells through the p38

mitogen-activated protein kinase-heat shock protein 27 pathway. *Hepatology* 51, 1614–1623. doi: 10.1002/hep.23566

Ying, Q., Liang, L., Guo, W., Zha, R., Tian, Q., Huang, S., et al. (2011). Hypoxia-inducible microRNA-210 augments the metastatic potential of tumor cells by targeting vacuole membrane protein 1 in hepatocellular carcinoma. *Hepatology* 54, 2064–2075. doi: 10.1002/hep.24614

Yokoi, T., and Nakajima, M. (2011). Toxicological implications of modulation of gene expression by microRNAs. *Toxicol. Sci.* 123, 1–14. doi: 10.1093/toxsci/kfr168

Yoon, S., Kim, T. H., Natarajan, A., Wang, S. S., Choi, J., Wu, J., et al. (2010). Acute liver injury upregulates microRNA-491-5p in mice, and its overexpression sensitizes Hep G2 cells for tumour necrosis factor-alpha-induced apoptosis. *Liver Int.* 30, 376–387. doi: 10.1111/j.1478-3231.2009.02181.x

Yoon, S. O., Chun, S. M., Han, E. H., Choi, J., Jang, S. J., Koh, S. A., et al. (2011). Deregulated expression of microRNA-221 with the potential for prognostic biomarkers in surgically resected hepatocellular carcinoma. *Hum. Pathol.* 42, 1391–1400. doi: 10.1016/j.humpath.2010.12.010

Zhang, B., and Pan, X. (2009). RDX induces aberrant expression of microRNAs in mouse brain and liver. *Environ. Health Perspect.* 117, 231–240. doi: 10.1289/ehp.11841

Zhang, J., Luo, N., Luo, Y., Peng, Z., Zhang, T., and Li, S. (2012). microRNA-150 inhibits human CD133-positive liver cancer stem cells through negative regulation of the transcription factor c-Myb. *Int. J. Oncol.* 40, 747–756. doi: 10.3892/ijo.2011.1242

Zhang, J. F., He, M. L., Fu, W. M., Wang, H., Chen, L. Z., Zhu, X., et al. (2011). Primate-specific microRNA-637 inhibits tumorigenesis in hepatocellular carcinoma by disrupting signal transducer and activator of transcription 3 signaling. *Hepatology* 54, 2137–2148. doi: 10.1002/hep.24595

Zhang, X., Liu, S., Hu, T., He, Y., and Sun, S. (2009). Up-regulated microRNA-143 transcribed by nuclear factor kappa B enhances hepatocarcinoma metastasis by repressing fibronectin expression. *Hepatology* 50, 490–499. doi: 10.1002/hep.23008

Zheng, F., Liao, Y. J., Cai, M. Y., Liu, Y. H., Liu, T. H., Chen, S. P., et al. (2012a). The putative tumour suppressor microRNA-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 and EZH2. *Gut* 61, 278–289. doi: 10.1136/gut.2011.239145

Zheng, J., Dong, P., Gao, S., Wang, N., and Yu, F. (2013). High expression of serum miR-17-5p associated with poor prognosis in patients with hepatocellular carcinoma. *Hepatogastroenterology* 60, 549–552.

Zheng, Y., Yin, L., Chen, H., Yang, S., Pan, C., Lu, S., et al. (2012b). miR-376a suppresses proliferation and induces apoptosis in hepatocellular carcinoma. *FEBS Lett.* 586, 2396–2403. doi: 10.1016/j.febslet.2012.05.054

Zhi, Q., Zhu, J., Guo, X., He, S., Xue, X., Zhou, J., et al. (2013). Metastasis-related miR-185 is a potential prognostic biomarker for hepatocellular carcinoma in early stage. *Biomed. Pharmacother.* 67, 393–398. doi: 10.1016/j.biopha.2013.03.022

Zhou, Y. M., Cao, L., Li, B., Zhang, R. X., Sui, C. J., Yin, Z. F., et al. (2012). Clinicopathological significance of ZEB1 protein in patients with hepatocellular carcinoma. *Ann. Surg. Oncol.* 19, 1700–1706. doi: 10.1245/s10434-011-1772-6

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Use of benzo analogs to enhance antimycotic activity of kresoxim methyl for control of aflatoxigenic fungal pathogens

Jong H. Kim*, Noreen Mahoney, Kathleen L. Chan, Bruce C. Campbell, Ronald P. Haff and Larry H. Stanker

Foodborne Toxin Detection and Prevention Research Unit, Western Regional Research Center, USDA-ARS, Albany, CA, USA

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Masoomeh Shams-Ghahfarokhi, Tarbiat Modares University, Iran
Eva-Guadalupe Lizárraga-Paulín, Universidad Nacional Autónoma De México, Mexico

****Correspondence:***
Jong H. Kim, Foodborne Toxin Detection and Prevention Research Unit, Western Regional Research Center, USDA-ARS, 800 Buchanan Street, Albany, CA 94710, USA
e-mail: jongheon.kim@ars.usda.gov

The aim of this study was to examine two benzo analogs, octylgallate (OG) and veratraldehyde (VT), as antifungal agents against strains of *Aspergillus parasiticus* and *A.flavus* (toxigenic or atoxigenic). Both toxigenic and atoxigenic strains used were capable of producing kojic acid, another cellular secondary product. *A. fumigatus* was used as a genetic model for this study. When applied independently, OG exhibits considerably higher antifungal activity compared to VT. The minimum inhibitory concentrations (MICs) of OG were 0.3–0.5 mM, while that of VT were 3.0–5.0 mM in agar plate-bioassays. OG or VT in concert with the fungicide kresoxim methyl (Kre-Me; strobilurin) greatly enhanced sensitivity of *Aspergillus* strains to Kre-Me. The combination with OG also overcame the tolerance of *A. fumigatus* mitogen-activated protein kinase (MAPK) mutants to Kre-Me. The degree of compound interaction resulting from chemosensitization of the fungi by OG was determined using checkerboard bioassays, where synergistic activity greatly lowered MICs or minimum fungicidal concentrations. However, the control chemosensitizer benzohydroxamic acid, an alternative oxidase inhibitor conventionally applied in concert with strobilurin, did not achieve synergism. The level of antifungal or chemosensitizing activity was also "compound—strain" specific, indicating differential susceptibility of tested strains to OG or VT, and/or heat stress. Besides targeting the antioxidant system, OG also negatively affected the cell wall-integrity pathway, as determined by the inhibition of *Saccharomyces cerevisiae* cell wall-integrity MAPK pathway mutants. We concluded that certain benzo analogs effectively inhibit fungal growth. They possess chemosensitizing capability to increase efficacy of Kre-Me and thus, could reduce effective dosages of strobilurins and alleviate negative side effects associated with current antifungal practices. OG also exhibits moderate antiaflatoxigenic activity.

Keywords: aflatoxin, antioxidant system, *Aspergillus*, cell wall integrity, chemosensitization, octylgallate, strobilurin, veratraldehyde

INTRODUCTION

Controlling fungi that produce hepato-carcinogenic aflatoxins in crops, such as tree nuts, corn, peanuts, etc., is problematic as effective commercial fungicides for treating aflatoxigenic fungi are very limited (Roze et al., 2013). Aflatoxins are secondary metabolites produced mainly by the filamentous fungi *Aspergillus flavus* and *A. parasiticus*. Significant amounts of harvested crop can be made unsuitable for sale and consumption as a result of aflatoxin contamination. Very low level (parts per billion) of aflatoxin contamination can have a perniciously negative effect on food safety and economic value of a number of crops from year to year (Campbell et al., 2003). Therefore, effective methods are continually needed for control of aflatoxigenic fungal pathogens.

Strobilurins are widely used agricultural fungicides. Strobilurins were initially identified in the fungus *Strobilurus tenacellus*, and were synthetically developed into several subgroups, such as kresoxim methyl (Kre-Me), azoxystrobin, pyraclostrobin, etc. (Bartlett et al., 2002 and references therein). The molecular target for strobilurins is the bc_1 complex (complex III; ubiquinol-cytochrome *c* oxidoreductase, EC 1.10.2.2) in the mitochondrial respiratory chain (MRC). Strobilurins specifically bind to the Q_P (Q_O) center of cytochrome *b* (Bartlett et al., 2002 and references therein), resulting in the inhibition of MRC.

Development of fungal resistance to conventional antifungal agents is a global agricultural issue (Ghannoum and Rice, 1999; Wood and Hollomon, 2003; Possiede et al., 2009; Cools and Hammond-Kosack, 2013). For example, agricultural fields receiving continuous applications of the fungicide, strobilurin, resulted in the development of insensitivity of fungi to this fungicide (Keinath, 2009). Noteworthy is that if strobilurin-containing fungicides are applied at suboptimal time-points of fungal growth, these fungicides can actually potentiate mycotoxin production in fungi (Ellner, 2005).

Similar type of fungicide-potentiation of mycotoxin production was documented in *Penicillium verrucosum*, a filamentous fungal pathogen producing the mycotoxin, citrinin

(Schmidt-Heydt et al., 2013). When *P. verrucosum* was treated with the fungicide "Rovral" (Iprodione), an inhibitor of DNA/RNA biosynthesis and cell division, the growth of fungi was decreased. However, a concomitant strong induction of citrinin biosynthesis also occurred in fungi with the same treatment (Schmidt-Heydt et al., 2013). Altogether, studies indicated that certain conventional fungicides could stimulate secondary metabolism, especially mycotoxin production, in fungal pathogens. Therefore, effective strategies are urgently needed to overcome counterproductive repercussions of fungicides currently in use.

Recent studies have shown that safe, natural phenolic compounds or their structural derivatives (e.g., benzo derivatives) could act as potent antifungal or antimycotoxigenic agents (Beekrum et al., 2003). For example, vanillic or caffeic acid not only inhibited the growth of *Fusarium verticillioides*, but also reduced its production of the mycotoxin, fumonisin (Beekrum et al., 2003). Natural benzo derivatives could also effectively inhibit the growth of *A. fumigatus*, *A. flavus*, *A. terreus,* and *Penicillium expansum* (Kim et al., 2011). These fungi are causative agents of human invasive aspergillosis or are producers of mycotoxins, including aflatoxin, patulin, gliotoxin, etc. The redox-active natural phenolic agents can be potent redox cyclers that inhibit fungal growth by disrupting cellular redox homeostasis (and thus triggering cellular oxidative stress) (Guillen and Evans, 1994; Jacob, 2006). For defense, the fungal antioxidant system, such as oxidative signaling pathway, plays an important role for fungal tolerance to those phenolic agents (Kim et al., 2011).

Genes involved in stress-signaling pathways are also important for fungal virulence, pathogenesis and protection from oxidative burst exerted by the host (Washburn et al., 1987; Hamilton and Holdom, 1999; Clemons et al., 2002; de Dios et al., 2010). Oxidative stress signals sensed by a fungal cell are incorporated into the upstream mitogen-activated protein kinase (MAPK) pathway, which regulates the expression of the downstream response genes (such as antioxidant enzyme genes) detoxifying the stress (Miskei et al., 2009). In *A. fumigatus*, SakA and MpkC are orthologous MAPKs to *Saccharomyces cerevisiae* Hog1p, which plays a key role in countering oxidative stress (Toone and Jones, 1998; Lee et al., 2002; Xue et al., 2004; Reyes et al., 2006; Miskei et al., 2009).

Chemosensitization is a strategy where combined application of certain types of compounds along with a conventional fungicide/drug enhances the effectiveness of the conventional agents (Niimi et al., 2004; Agarwal et al., 2012; Campbell et al., 2012). Noteworthy is that certain benzo derivatives possessed antifungal chemosensitizing capability. In our prior study, co-application of antifungal agents with chemosensitizing benzo derivatives, such as 2-hydroxy-5-methoxybenzaldehyde, greatly enhanced the efficacy of antifungal agents (Kim et al., 2011). Thus, chemosensitization could lead to lowering dosages of conventional fungicides/drugs required for control of pathogens, especially drug-resistant strains. Collectively, these studies showed the potential for safe, natural phenolics to serve as effective antifungal and/or antimycotoxigenic agents.

In nature, in addition to oxidative stress, high temperature (heat) stress is another type of environmental challenge that many microbes face, which also triggers "signaling cascades" in fungal cells (Morano et al., 2012). Heat treatment is also a strategy to prevent contamination by food spoilage fungi in foods (Dagnas and Membré, 2013). In corn, artificial drying of maize kernels with high temperatures is one of the postharvest practices to prevent fungal growth and aflatoxin production (Hawkins et al., 2005). A prior study showed that heat treatment at 70°C significantly reduced the maize kernel infection of *A. flavus*. However, heat treatment had no effect on aflatoxin concentration, which reflects the heat stability of the mycotoxin (Hawkins et al., 2005). Also, heat treatment can result in deterioration of the quality of the crop (seed breakage, viability, etc.). Therefore, development of new alternative strategies, which warrant early intervention of mycotoxin production/fungal growth as well as the quality of harvested crops, is necessary.

In this study, we investigated the role of two benzo derivatives, octylgallate (OG) and veratraldehyde (VT), currently used as food additives, as antifungal agents against strains of *A. flavus, A. parasiticus,* or *A. fumigatus*. OG and VT are generally regarded as safe (GRAS) reagents (FDA, 2011). We also evaluated antifungal chemosensitizing capacities of OG and VT, especially for overcoming strobilurin resistance of *Aspergillus* MAPK gene deletion mutants (*sakA*Δ, *mpkC*Δ). Kre-Me, containing (*E*)-methyl methoxyiminoacetate group in the structure, was tested as an exemplary strobilurin. Kre-Me is currently applied in the agricultural field for control of various fungal diseases caused by ascomycete, basidiomycete, oomycete, etc., while it exhibited high toxicity to the agriculturally important insects, bees (Bartlett et al., 2002). Our results showed that OG could serve as a potent antifungal chemosensitizer to Kre-Me for controlling *Aspergillus* strains.

MATERIALS AND METHODS

MICROBIAL STRAINS AND CULTURE CONDITIONS

Microbial strains used in this study are summarized in **Table 1**. *Aspergillus* strains were cultured on potato dextrose agar (PDA) at 30°C, except otherwise noted in the text. Yeast strains, wild type (WT) and gene deletion mutants of *Saccharomyces cerevisiae* (**Table 1**), were cultured on Synthetic Glucose (SG; Yeast nitrogen base without amino acids 0.67%, glucose 2% with appropriate supplements: uracil 0.02 mg mL^{-1}, amino acids 0.03 mg mL^{-1}) or Yeast Peptone Dextrose (YPD; Bacto yeast extract 1%, Bacto peptone 2%, glucose 2%) medium at 30°C.

CHEMICALS

Antifungal compounds, kresoxim methyl (Kre-Me; strobilurin), octylgallate (octyl 3,4,5-trihydroxybenzoic acid; OG), veratraldehyde (3,4-dimethoxybenzaldehyde; VT), benzohydroxamic acid (BHAM) (**Figure 1**), were procured from Sigma Co. (St. Louis, MO, USA). Each compound was dissolved in dimethylsulfoxide (DMSO; absolute DMSO amount: <2% in media) before incorporation into culture media (except for those plates used in aflatoxin assays; see below). Throughout this study, control plates (No treatment) contained DMSO at levels equivalent to that of cohorts receiving antifungal agents, within the same set of experiments.

Table 1 | Microbial strains used in this study.

	Characteristics	Source/references
Aspergillus		
A. flavus 3357	Plant pathogen (aflatoxin), Human pathogen (aspergillosis), Reference toxigenic (aflatoxin-producing) strain used for genome sequencing	NRRL[a], *Aspergillus* Comparative Database
A. flavus 4212	Plant pathogen (aflatoxin), Human pathogen (aspergillosis)	NRRL
A. flavus 21882	Atoxigenic (aflatoxin non-producing) strain, A pesticide active ingredient displacing toxigenic fungus	NRRL, *Aspergillus flavus* NRRL 21882 Fact Sheet
A. flavus 18543	Atoxigenic strain, A pesticide active ingredient displacing toxigenic fungus	NRRL, Ehrlich and Cotty (2004)
A. parasiticus 5862	Plant pathogen (aflatoxin)	NRRL
A. parasiticus 2999	Plant pathogen (aflatoxin)	NRRL
A. fumigatus AF293	Human pathogen (aspergillosis), Parental strain, Reference clinical strain used for genome sequencing	Xue et al. (2004); *Aspergillus* Comparative Database
*A. fumigatus sakA*Δ	Human pathogen (aspergillosis), Mitogen-Activated Protein Kinase (MAPK) gene deletion mutant derived from AF293	Xue et al. (2004)
*A. fumigatus mpkC*Δ	Human pathogen (aspergillosis), MAPK gene deletion mutant derived from AF293	Reyes et al. (2006)
Saccharomyces		
S. cerevisiae BY4741	Model yeast, Parental strain (Mat a *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*)	(SGD)[b]
*S. cerevisiae bck1*Δ	MAPK kinase kinase mutant derived from BY4741	SGD
*S. cerevisiae slt2*Δ	MAPK mutant derived from BY4741	SGD

[a] *NRRL, National Center for Agricultural Utilization and Research, USDA-ARS, Peoria, IL, USA.*

[b] *Saccharomyces Genome Database. Available online: http://www.yeastgenome.org (accessed on 10 January 2014).*

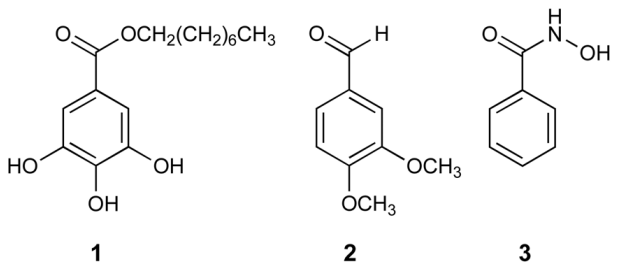

FIGURE 1 | Structures of benzo analogs used in this study. (1) octylgallate (OG); (2) veratraldehyde (VT); (3) benzohydroxamic acid (BHAM).

SUSCEPTIBILITY TESTING

Agar plate-bioassay in Aspergillus

In agar plate-bioassays, susceptibility of filamentous fungi to OG, VT, or Kre-Me, alone or in combination, were measured based on percent radial growth of treated compared to control fungal colonies (see Figures and Tables for test concentrations). The percent inhibition of growth was calculated using the Vincent equation (Vincent, 1947) [% inhibition $= 100\,(C - T)/C$; where $C =$ diameter of fungal colony on control plate (receiving only DMSO), and $T =$ diameter of fungal colony on the treated plate]. Minimum inhibitory concentration (MIC) values on agar plates were based on triplicate assays and defined as the lowest concentration of agent where no fungal growth was visible on the plate. For the above assays, fungal conidia (5×10^3) were diluted in phosphate buffered saline and applied as a drop onto the center of PDA plates with or without antifungal compounds. Growth was observed for 5–7 days.

CLSI liquid bioassay in Aspergillus

To determine the precise level of chemosensitizing activities of OG or BHAM (0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 mM) to Kre-Me (0.5, 1, 2, 4, 8, 16, 32 $\mu g\,mL^{-1}$) in the strains of *Aspergillus*, checkerboard bioassays (triplicate) (0.4×10^4–5×10^4 CFU mL^{-1}) were performed in microtiter wells using a broth microdilution method (in RPMI 1640 medium; Sigma Co., St. Louis, MO, USA), according to protocols outlined by the Clinical and Laboratory Standards Institute (CLSI) M38-A (CLSI, 2008). OG was chosen since this compound showed much higher antifungal activity than VT (see Results). For comparison, BHAM, an alternative oxidase (AOX) inhibitor conventionally used in combination with strobilurin, was also included as a reference chemosensitizer in this test. RPMI 1640 medium was supplemented with 0.03% L-glutamine and buffered with 0.165 mM 3-[*N*-morpholino] propanesulfonic acid. MICs, lowest concentration of agents showing no visible fungal growth in microtiter wells (200 μL per well), were assessed after 48 and 72 h. Minimum fungicidal concentrations (MFCs), lowest concentration of agents showing $\geq$ 99.9% fungal death, were determined following completion of MIC assays by spreading entire volumes of microtiter wells (200 μL) onto individual PDA plates, and culturing for another 48 and 72 h. Compound interactions, Fractional Inhibitory Concentration Indices (FICIs) and Fractional Fungicidal Concentration Indices (FFCI), were calculated as follows: FICI or FFCI = (MIC or MFC of compound

A in combination with compound B/MIC or MFC of compound A, alone) + (MIC or MFC of compound B in combination with compound A/MIC or MFC of compound B, alone). Interactions were defined as: "synergistic" (FICI or FFCI ≤ 0.5) or "indifferent" (FICI or FFCI > 0.5–4) (Odds, 2003).

GROWTH RECOVERY TEST IN *ASPERGILLUS* STRAINS TREATED WITH HIGH TEMPERATURES: AGAR PLATE-BIOASSAY

Agar plate-based bioassay was performed to evaluate differential susceptibility of *Aspergillus* strains to high temperatures. First, fungal conidia (5×10^3) were spotted on PDA (see above for method), and were initially incubated at four different temperatures (moderate: 30, 35°C; high: 45, 55°C). Triplicate PDA plates were then removed from each temperature (30, 35, 45, or 55°C) at day 1, 2, 3, and 4, and were transferred to 30°C for additional 6, 5, 4, and 3 days of growth, respectively, resulting in a total of 7 days of incubation for each treatment (i.e., 1 day growth at 55°C + 6 days growth at 30°C = Total 7 days growth, 2 days growth at 55°C + 5 days growth at 30°C = Total 7 days growth, 3 days growth at 55°C + 4 days growth at 30°C = Total 7 days growth, 4 days growth at 55°C + 3 days growth at 30°C = Total 7 days growth). For controls, *Aspergillus* strains were grown solely at respective temperature (30, 35, 45, or 55°C) for 7 days. The level of growth recovery at 30°C was evaluated based on fungal radial growth as described above.

GROWTH RECOVERY TEST IN CELL WALL INTEGRITY MUTANTS OF *SACCHAROMYCES CEREVISIAE* TREATED WITH OG: YEAST DILUTION BIOASSAY

To determine the effect of OG on the cell wall-integrity system of fungi, sorbitol recovery tests were performed using Petri plate-based yeast dilution bioassays. Ten-fold diluted, i.e., 10^0 (1×10^6 cells), 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, strains of *S. cerevisiae* BY4741 (WT), *bck1*Δ and *slt2*Δ were spotted on: (1) SG only, (2) SG + caffeine (5 mM; a positive control for cell wall disruption) or SG + OG (0.03, 0.04, 0.05, 0.06 mM) (Testing sensitivity of *bck1*Δ and *slt2*Δ mutants to compounds), and (3) SG + sorbitol (0.5 M) + caffeine or OG (Testing growth recovery of *bck1*Δ and *slt2*Δ mutants by sorbitol). Cell growth was monitored for 5–7 days. If the growth score on the sorbitol-containing medium was higher than that on the "no sorbitol" medium, OG was considered to negatively affect cell wall integrity.

AFLATOXIN ASSAYS

For aflatoxin analysis, spore suspensions (200 spores) were inoculated onto the center of PDA plates. Cells were grown at 30°C for 7 days with or without OG (OG concentration: 0.025, 0.05, 0.1 mM; added directly into PDA), with each treatment in triplicate. Aflatoxins were quantitated as described previously (Rodriguez and Mahoney, 1994). Aflatoxin standard solutions (AFB_1, AFB_2, AFG_1, AFG_2), used for the quantification of aflatoxins from OG-treated samples, were prepared as described in AOAC 971.22 (AOAC International, 2005).

KOJIC ACID ASSAYS

To examine whether the cellular secondary-product metabolism other than aflatoxin production is normal in the atoxigenic strains used in this study (namely, *A. flavus* 21882, 18543), we determined kojic acid (KA) production in these atoxigenic strains. *A. flavus* 3357 and 4212, toxigenic strains, were used as controls for KA production. Fungal spores (1×10^5 spores per 100 μL, 0.05% Tween 80) were inoculated into 50 mL potato dextrose broth, and were incubated at 30°C without shaking. KA production was measured from the culture media using an HPLC system consisting of a degasser, autosampler, quaternary pump, and photodiode array detector (Agilent 1100, Santa Clara, CA USA). Culture media aliquots of 1 mL were filtered through 0.45 μm sterile nylon 25 mm syringe filters (Fisherbrand, Thermo Fisher Scientific, Waltham, MA, USA), with injection volumes of 20 μL on a 4.6 × 250 mm Inertsil 5 μm ODS-3 column (GL Sciences, Torrance, CA, USA) using an isocratic mobile phase consisting of MeOH/0.1% H_3PO_4 (25:75, v/v) at a flow rate of 1 mL per minute. KA quantitation was based on UV detection at 265 nm. HPLC quantification of KA was linear in the range of 0.02–4.00 μg per 20 μL, with a retention time of 5.4 min.

STATISTICAL ANALYSIS

Statistical analysis (student's *t*-test) was performed based on "Statistics to use" (Kirkman, 2014), where $p < 0.05$ was considered significant.

RESULTS

SUSCEPTIBILITY OF *ASPERGILLUS* STRAINS TO OG OR VT: AGAR PLATE-BIOASSAY

As shown in **Table 2**, OG possessed much higher antifungal activity compared to VT. The average MICs of OG was 0.4 mM, while that of VT was higher than 4.8 mM (viz. more than 10 times higher antifungal activity of OG).

Table 2 | Differential sensitivity of *Aspergillus* strains to OG, VT, or high temperature (55°C).

Strains	OG (mM)	VT (mM)	High temperature recovery from 55°C (Days)[a]
A. flavus 3357	0.40	>5.0[b]	0
A. flavus 4212	0.40	>5.0	0
A. flavus 21882	0.50	>5.0	0
A. flavus 18543	0.45	>5.0	1
A. parasiticus 5862	0.40	>5.0	1
A. parasiticus 2999	0.50	>5.0	1
A. fumigatus AF293	0.35	4.5	4
*A. fumigatus sakA*Δ	0.30	4.5	4
*A. fumigatus mpkC*Δ	0.30	4.5	4
Average	0.40	>4.8	1.7
t-test	$p < 0.005$[c]	–	–

Concentrations (mM) are MICs examined on PDA plates.

[a] Maximum day(s) of incubation at 55° C, which can result in growth recovery of fungi at 30° C.

[b] VT was tested up to 5.0 mM. For statistical calculation (student's t-test) purpose, 10.0 mM (doubling of 5.0 mM) was used.

[c] Student's t-test for paired data (MIC_{OG}) was vs. MIC_{VT} determined in nine strains.

Results also indicated differential susceptibility (namely, different MIC level) of *Aspergillus* strains to the treatments, where: (1) All *A. fumigatus* strains (WT, *sakA*Δ, *mpkC*Δ) were more sensitive to either OG or VT compared to other *Aspergillus* strains ($p < 0.05$ for OG, $p < 0.005$ for VT), (2) *A. fumigatus* MAPK mutants (*sakA*Δ, *mpkC*Δ) were more sensitive to OG compared to the WT ($p < 0.005$). Although MICs of VT for all *A. fumigatus* strains were similar (MIC_{VT}: 4.5 mM), the level of growth of MAPK mutants was also lower than the WT (see below; **Table 3**). Thus, results indicated MAPK mutants lack the defense mechanism to protect fungal cells from the toxicity generated by redox-active benzo derivatives, (3) *A. flavus* 21882 and *A. parasiticus* 2999 were more tolerant to OG compared to other *Aspergillus* strains ($p < 0.05$), (4) *A. flavus* 3357, 4212 and *A. parasiticus* 5862 showed similar levels of susceptibility to OG, and (5) *A. flavus* 3357, 4212, 21882, 18543 and *A. parasiticus* 5862, 2999 also showed similar levels of susceptibility to VT (up to 5 mM).

SUSCEPTIBILITY OF *ASPERGILLUS* TO HIGH TEMPERATURES

Heat responses of *Aspergillus* strains were compared to their responses to benzo derivatives. *Aspergillus* strains were treated with moderate (30, 35°C) to high (45, 55°C) temperatures, and were then transferred to 30°C for growth recovery. From the pathogenicity perspective, *A. flavus* shares agro-infectivity with *A. parasiticus*, while *A. flavus* shares human-infectivity with *A. fumigatus* (**Figure 2A**). As shown in **Figure 2B**, *A. fumigatus* strains, both WT and MAPK mutants, were much more tolerant to high temperature (55°C), compared to other *Aspergillus* strains examined. For example, *A. fumigatus* strains did not germinate on PDA when they were maintained at 55°C for 7 days. However, *A. fumigatus* treated with heat (55°C) for 1–4 days could recover the growth after incubation at 30°C, while *A. flavus* 3357, 4212 and 21882 could not recover their growth even after 1 day-heat treatment at 55°C (see **Table 2** for summary). The remaining *Aspergillus* strains (18543, 5862, 2999) showed growth recovery only with 1 day-heat treatment at 55°C. *A. fumigatus* MAPK mutants were marginally more sensitive to heat (55°C) (i.e., 2–10% less radial growth during recovery w/ 3–4 day-treatment at 55°C) compared to the WT (**Figure 2B**).

The order of susceptibility of *Aspergillus* strains to high temperature (55°C) (higher susceptibility to lower susceptibility) was: *A. flavus* 3357, 4212, 21882 > *A. flavus* 18543, *A. parasiticus* 5862, 2999 > *A. fumigatus* strains. Therefore, like in benzo treatments, *Aspergillus* strains showed differential susceptibility to high temperature (55°C), where *A. fumigatus* strains were the most heat-tolerant species among *Aspergillus* strains tested. However, there was no direct correlation in fungal responses between benzo and heat treatments, indicating the mechanism of antifungal action of benzo analog(s) is considered to be different from that of heat treatment (see **Table 2**). [*Aspergillus* strains were also sensitive to

Table 3 | Responses of *A. fumigatus* WT and MAPK mutants to the treatments of benzo derivatives w/o or w/Kre-Me (25 μM)[a].

VT (mM)	AF293 %	*sakA*Δ %	*mpkC*Δ %	OG (mM)	AF293 %	*sakA*Δ %	*mpkC*Δ %
w/o Kre-Me							
0.0	100	100	100	0.000	100	100	100
0.5	100	100	96	0.025	86	70	74
1.0	100	98	96	0.050	70	60	66
1.5	100	96	94	0.100	54	48	52
2.0	100	92	92	0.150	46	38	42
2.5	100	92	92	0.200	36	24	28
3.0	96	84	86	0.250	28	few	few
3.5	75	69	72	0.300	few	**0**	**0**
4.0	few[b]	few	few	0.350	**0**	**0**	**0**
4.5	**0**	**0**	**0**	0.400	**0**	**0**	**0**
5.0	**0**	**0**	**0**	0.450	**0**	**0**	**0**
w/Kre-Me							
0.0	100	104	109	0.000	100	104	109
0.5	83	96	96	0.025	52	few	few
1.0	70	78	83	0.050	**0**	**0**	**0**
1.5	61	74	74	0.100	**0**	**0**	**0**
2.0	52	65	70	0.150	**0**	**0**	**0**
2.5	43	57	65	0.200	**0**	**0**	**0**
3.0	**0**	48	48	0.250	**0**	**0**	**0**
3.5	**0**	few	few	0.300	**0**	**0**	**0**
4.0	**0**	**0**	**0**	0.350	**0**	**0**	**0**
4.5	**0**	**0**	**0**	0.400	**0**	**0**	**0**
5.0	**0**	**0**	**0**	0.450	**0**	**0**	**0**

[a] *Data shown in the table are % radial growth of fungi compared to control (no treatment). SD < 5%. No visible cell growth (0%) is in bold.*

[b] *Few: Only few colonies appeared.*

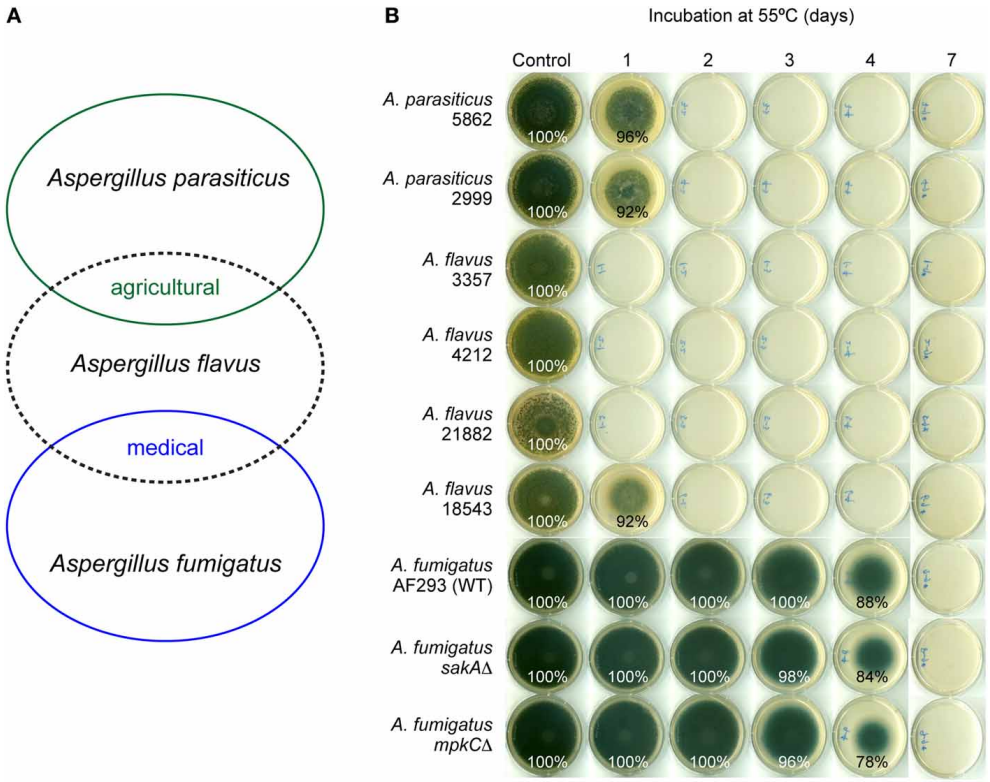

FIGURE 2 | Differential sensitivity of *Aspergillus* strains to high temperature (55°C). (A) Scheme showing *A. flavus* shares agro-infectivity with *A. parasiticus*, while it shares human-infectivity with *A. fumigatus*. **(B)** Fungal plate bioassay showing *A. fumigatus* strains were more tolerant to high temperature (55°C) compared to *A. flavus* or *A. parasiticus*. Control, Incubation at 30°C for 7 days; 1–7, Incubation at 55°C for 1, 2, 3, 4, or 7 days, then growth recovery at 30°C for 6, 5, 4, 3, or 0 days, respectively. Fungal growth is indicated as percent relative to control. Unlabeled plates showed no growth (0%).

45°C. However, all *Aspergillus* strains recovered their growth after transfer from 45 to 30°C (see Figure S1 for exemplary bioassay with *A. flavus*). As expected, *Aspergillus* strains grew normally at the moderate temperatures (30 and 35°C) (Figure S1)].

SUSCEPTIBILITY OF *A. FUMIGATUS* TO CHEMOSENSITIZATION

Chemosensitizing capability of OG or VT to Kre-Me was tested in *A. fumigatus* strains by using agar plate-bioassays. Results showed that: (1) When Kre-Me was co-applied with VT, the MIC of VT was lowered from 4.5 mM to 3.0 mM_{MAPK} or 4.0 mM_{WT}, respectively (**Table 3**), and (2) When Kre-Me was co-applied with OG, the MIC of OG was lowered from 0.3 mM_{MAPK} or 0.35 mM_{WT} to 0.05 $mM_{MAPKandWT}$. Therefore, both OG and VT possessed chemosensitizing capability to Kre-Me in *A. fumigatus* strains, where OG possessed much higher chemosensitizing capacity (viz. needed ∼10 times lower concentration for chemosensitization; $p < 0.005$) than VT (**Table 3**).

Noteworthy is that the *A. fumigatus* MAPK mutants were more tolerant to Kre-Me (**Table 3**), where: (1) The level of radial growth of MAPK mutants with independent treatment of Kre-Me was marginally greater (4–9% more growth) than the WT, and (2) When VT was co-applied with Kre-Me (25 μM), MIC_{VT} was 3.0 mM for the WT, while that of MAPK mutants was 4.0 mM (namely, higher concentration of VT is needed compared to the WT) during this chemosensitization (**Table 3**). Therefore, although VT-mediated chemosensitization was achieved in all *A. fumigatus* strains, results showed relatively higher tolerance of MAPK mutants to "Kre-Me + VT" compared to the WT [see below (**Table 4**) for relative tolerance of MAPK mutants to "Kre-Me + BHAM" compared to the WT]. However, Kre-Me tolerance of *A. fumigatus* MAPK mutants was completely abolished by OG (**Table 3**).

Co-application of OG or VT with Kre-Me also enhanced growth inhibition in toxigenic strains of *Aspergillus* (*A. flavus* 3357, *A. parasiticus* 5862) (viz. either complete growth inhibition or reduced radial growth, depending on types of strains/combination of compounds; **Figure 3**). For example, the growth of *A. parasiticus* 5862 was completely inhibited by Kre-Me + VT, while a similar level of growth inhibition in *A. flavus* 3357 could be achieved by Kre-Me + OG (**Figure 3**). Independent treatment of each compound alone at the same concentration did not achieve such a level of growth inhibition. Thus, results indicated differential susceptibility of each strain to different combinations of antifungal agents, where 3357 showed higher susceptibility (complete growth inhibition) to Kre-Me + OG, whereas 5862 was more susceptible to Kre-Me + VT (complete growth inhibition). As observed in *A. fumigatus*, OG possessed much higher antifungal potency in toxigenic *Aspergillus* strains

Table 4 | Antifungal chemosensitization of OG (mM) or BHAM (mM) to Kre-Me ($\mu g\ mL^{-1}$), tested against *Aspergillus* strains: summary of CLSI-based microdilution bioassays (48 h)[a].

	Compounds	MIC alone	MIC combined	FICI	Compounds	MFC alone	MFC combined	FFCI
***Aspergillus* STRAINS OG**								
A. flavus 3357	Kre-Me	>32[b]	2	**0.2**	Kre-Me	>32	0.5	**0.3**
	OG	0.2	0.025		OG	0.8	0.2	
A. flavus 4212	Kre-Me	>32	2	**0.2**	Kre-Me	>32	0.5	**0.3**
	OG	0.2	0.025		OG	0.8	0.2	
A. flavus 21882	Kre-Me	>32	2	**0.2**	Kre-Me	>32	0.5	**0.1**
	OG	0.2	0.025		OG	>0.8	0.2	
A. flavus 18543	Kre-Me	>32	2	**0.2**	Kre-Me	>32	2	**0.1**
	OG	0.2	0.025		OG	>0.8	0.1	
A. parasiticus 5862	Kre-Me	>32	4	**0.2**	Kre-Me	>32	4	**0.2**
	OG	0.2	0.025		OG	>0.8	0.2	
A. parasiticus 2999	Kre-Me	>32	4	**0.2**	Kre-Me	>32	1	**0.1**
	OG	0.2	0.025		OG	>0.8	0.2	
A. fumigatus AF293	Kre-Me	>32	1	**0.1**	Kre-Me	>32	0.5	**0.5**
	OG	0.1	0.0125		OG	0.2	0.1	
*A. fumigatus sakA*Δ	Kre-Me	>32	1	**0.1**	Kre-Me	>32	0.5	**0.5**
	OG	0.1	0.0125		OG	0.1	0.05	
*A. fumigatus mpkC*Δ	Kre-Me	>32	1	**0.1**	Kre-Me	>32	0.5	**0.5**
	OG	0.1	0.0125		OG	0.1	0.05	
Mean	Kre-Me	64.00	2.11	**0.1**	Kre-Me	64.00	1.11	**0.2**
	OG	0.17	0.02		OG	0.93	0.14	
t-test[c]	Kre-Me	–	$p < 0.005$	–	Kre-Me	–	$p < 0.005$	–
	OG	–	$p < 0.005$	–	OG	–	$p < 0.005$	–
***Aspergillus* STRAINS BHAM**								
A. fumigatus AF293	Kre-Me	>32	4	0.6	Kre-Me	>32	>32	2.0
	BHAM	>0.8[d]	0.8		BHAM	>0.8	>0.8	
*A. fumigatus sakA*Δ	Kre-Me	>32	16	0.8	Kre-Me	>32	>32	2.0
	BHAM	>0.8	0.8		BHAM	>0.8	>0.8	
*A. fumigatus mpkC*Δ	Kre-Me	>32	16	0.8	Kre-Me	>32	>32	2.0
	BHAM	>0.8	0.8		BHAM	>0.8	>0.8	
All other strains	Kre-Me	>32	>32	2.0	Kre-Me	>32	>32	2.0
	BHAM	>0.8	>0.8		BHAM	>0.8	>0.8	
Mean	Kre-Me	64.00	46.67	1.6	Kre-Me	64.00	64.00	2.0
	BHAM	1.60	1.33		BHAM	1.60	1.60	
t-test	Kre-Me	–	$p < 0.1$	–	Kre-Me	–	ND[e]	–
	BHAM	–	$p < 0.1$	–	BHAM	–	ND	–

[a] *MIC, Minimum inhibitory concentration; MFC, Minimum fungicidal concentration; FICI, Fractional Inhibitory Concentration Indices; FFCI, Fractional Fungicidal Concentration Indices. Synergistic FICIs and FFCIs are in bold.*

[b] *Kre-Me was tested up to 32 $\mu g\ mL^{-1}$. For calculation purpose, 64 $\mu g\ mL^{-1}$ (doubling of 32 $\mu g\ mL^{-1}$) was used.*

[c] *Student's t-test for paired data (combined, i.e., chemosensitization) was vs. mean MIC or MFC of each compound (alone, i.e., no chemosensitization) determined in strains.*

[d] *BHAM was tested up to 0.8 mM. For calculation purpose, 1.6 mM (doubling of 0.8 mM) was used.*

[e] *ND, Not determined (neutral interaction).*

compared to VT (namely, 50 μM_{OG} vs. 3 mM_{VT}, respectively, for achieving chemosensitization).

In CLSI liquid bioassay, co-application of Kre-Me with OG completely inhibited the growth of *Aspergillus* strains (100% killing in MFC testing), where independent treatment of each compound alone at the same concentrations allowed the survival of fungi (**Figures 4**, **5**). For MICs, "synergistic" FICIs (FICI $\leq$ 0.5; see Materials and Methods for calculations) were found between OG and Kre-Me for all *Aspergillus* strains (**Table 4**). For MFCs, "synergistic" FFCIs (FFCI $\leq$ 0.5) were also achieved by co-application of OG with Kre-Me for all *Aspergillus* strains tested (**Table 4**). Although there was increased antifungal activity of BHAM and Kre-Me when co-applied in *A. fumigatus* strains (WT, *sakA*Δ, *mpkC*Δ) (FICI = 0.6–0.8; **Table 4**), no calculated synergism was found in any of the *Aspergillus* strains tested with BHAM + Kre-Me (**Table 4**). As with VT + Kre-Me

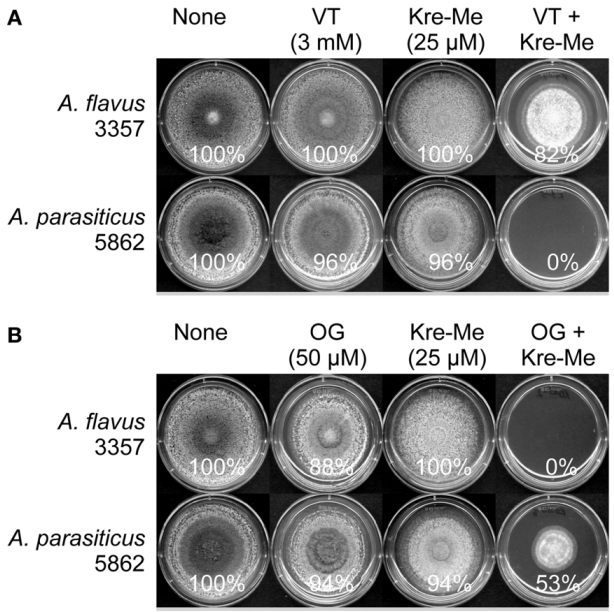

FIGURE 3 | Differential sensitivity of *A. flavus* 3357 and *A. parasiticus* 5862 to co-treatment of Kre-Me with VT or OG. (A) *A. parasiticus* 5862 was more sensitive to "Kre-Me + VT" compared to *A. flavus* 3357. **(B)** *A. flavus* 3357 showed higher sensitivity to "Kre-Me + OG" compared to *A. parasiticus* 5862. Results show differential sensitivity of each strain to different combinations of compounds.

(see above), *A. fumigatus* MAPK mutant also showed higher tolerance to "BHAM + Kre-Me" chemosensitization, where the value of $MIC_{COMBINED}$ of Kre-Me was 4 μg mL^{-1} for WT, while that for MAPK mutants was 16 μg mL^{-1}, respectively, (viz. four times higher tolerance to Kre-Me) (**Table 4**). Noteworthy is that certain fungi with mutation(s) in the MAPK signaling pathway can also escape toxicity of the commercial fungicide fludioxonil (Kojima et al., 2004).

GROWTH RECOVERY TEST IN CELL WALL INTEGRITY MUTANTS OF *S. CEREVISIAE* TREATED WITH OG

In sorbitol remediation bioassay, sensitivity of *slt2*Δ and *bck1*Δ to OG was alleviated by sorbitol. The growth rate of *slt2*Δ and *bck1*Δ on sorbitol-containing media was 100–1000 times higher for caffeine or OG, respectively, compared to controls without sorbitol (**Figure 6**). Thus, the remediation by sorbitol indicates disruption of cell wall/membrane integrity in fungi is one contributing mechanism of how OG generates chemosensitization.

EFFECT OF OG ON AFLATOXIN PRODUCTION

When toxigenic strains of *A. flavus* and *A. parasiticus* were treated with OG (tested at 25, 50, 100 μM), the highest reduction in aflatoxin production was observed at 25 or 50 μM of OG, depending on types of strains. Aflatoxin production in OG-treated plates was reduced 4–30% compared to non-treated control plates (**Table 5**).

DISCUSSION

In this study, nine *Aspergillus* strains, namely, four toxigenic strains of *A. flavus* and *A. parasiticus*, two atoxigenic strains of *A. flavus*, and three *A. fumigatus* strains, WT and MAPK mutants (*sakA*Δ, *mpkC*Δ), were examined for their responses to different treatments. The *A. fumigatus sakA*Δ (*sakA* gene deletion) is an osmotic/oxidative stress sensitive mutant, while the *mpkC*Δ (*mpkC* gene deletion) is a mutant of the polyalcohol sugar utilization system (Xue et al., 2004; Reyes et al., 2006). In a prior study, both *sakA*Δ and *mpkC*Δ mutants showed higher sensitivity to certain benzo derivatives compared to the WT (Kim et al., 2011). The molecular biological/genetic resources (such as gene deletion mutants) for *A. flavus* or *A. parasiticus* available are few in number. Hence, *A. fumigatus sakA*Δ and *mpkC*Δ mutants could serve as model strains for investigating potential modes of antifungal responses in congeners, such as *A. flavus* or *A. parasiticus*. Except for the incapability to produce aflatoxins, the atoxigenic strains *A. flavus* 21882 and 18543 could produce KA (Table S1), a different type of secondary metabolite produced by *A. flavus* and *A. parasiticus*. Thus, the cellular secondary-product metabolism other than aflatoxin production is thought to be normal in the atoxigenic strains examined in this study.

Results showed that OG- or VT-based chemosensitization could enhance antifungal activity of Kre-Me in *Aspergillus* strains, where *Aspergillus* strains tested were sensitive to Kre-Me + OG or VT. OG was a more potent chemosensitizing agent than VT or BHAM to Kre-Me, where the concentration of OG necessary to achieve antifungal "synergism" was much lower (≥10 times lower) than the other compounds. When fungi are treated with Kre-Me, cellular AOX allows completion of electron transfer and ATP production via the MRC, thus resulting in overcoming the toxicity triggered by Kre-Me (or other MRC inhibitors) (Costa-de-Oliveira et al., 2012; Inoue et al., 2012). Therefore, AOX inhibitors, such as BHAM, have the effect to enhance the activity of Kre-Me (or other MRC inhibitors) when co-applied. The OG-based chemosensitization to Kre-Me, performed in this study, indicated that the AOX-inhibitory activity of OG (Sierra-Campos et al., 2009; Robles-Martinez et al., 2013) is much higher than that of the conventional AOX inhibitor, BHAM. Noteworthy is that the efficacy of OG-based chemosensitization to Kre-Me (which was "synergistic") was higher compared to the prior chemosensitization test with "2-hydroxy-5-methoxybenzaldehyde (phenolic) + antimycin A (MRC inhibitor)" (Kim et al., 2011), in which the level of compound interaction was "additive/indifferent" but not "synergistic."

The inhibition of MRC not only disrupts cellular ATP production, but also triggers oxidative stress, which results from abnormal leakage of electrons from the MRC (Fujita et al., 2004; Ruy et al., 2006). The escaped electrons cause oxidative damage to cellular components, such as cell membranes. Therefore, the enhanced oxidative stress generated by both MRC inhibitor (Kre-Me) and the redox-active phenolic derivative (OG) would result in increased growth inhibition of fungi.

Caffeine (a "control" reagent used in the cell wall/membrane integrity bioassay) tends to disorganize cell surface in fungi. Thus, fungi having abnormalities in cell surface integrity show increased sensitivity to caffeine treatment (Lussier et al., 1997). Caffeine also activates the protein kinase C (PKC) pathway, where the MAPK pathway genes *SLT2* and *BCK1* play key roles for maintaining cell wall integrity (Martin et al., 2000). Therefore, *SLT2* and *BCK1*

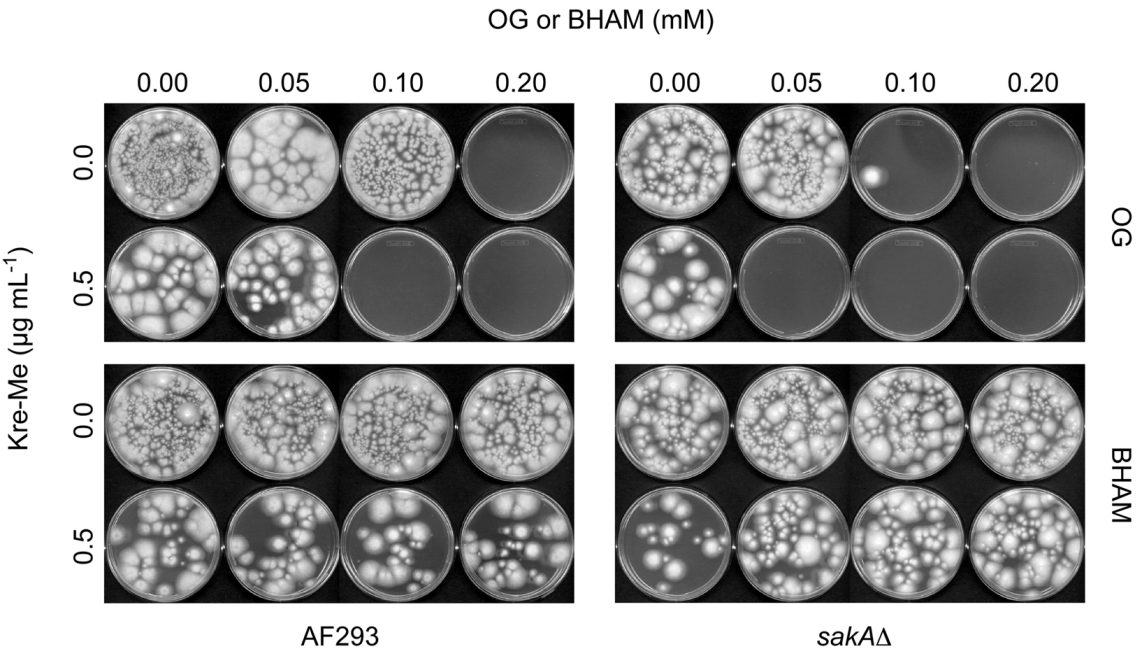

FIGURE 4 | Chemosensitization test in *A. fumigatus*: Kre-Me + OG or BHAM. Results shown here are the determination of MFCs of antifungal agents. Co-application of Kre-Me (0.5 μg mL^{-1}) with OG (0.10 or 0.05 mM for *A. fumigatus* WT or *sakA*Δ, respectively), completely inhibited the growth of *A. fumigatus*, while individual treatment of each compound, alone, at the same concentrations allowed the growth of fungi. The *sakA*Δ mutant was also more sensitive (viz. required lower concentration of OG) to the chemosensitization than the WT (AF293), indicating that the antioxidant system of *Aspergillus* plays an important role for fungal tolerance to the chemosensitization. On the other hand, co-application of Kre-Me with BHAM (conventional antifungal chemosensitizer disrupting AOX) at the same concentrations resulted in survival of *A. fumigatus* strains. Similar result was observed in *A. fumigatus mpkC*Δmutant (Figure data not shown).

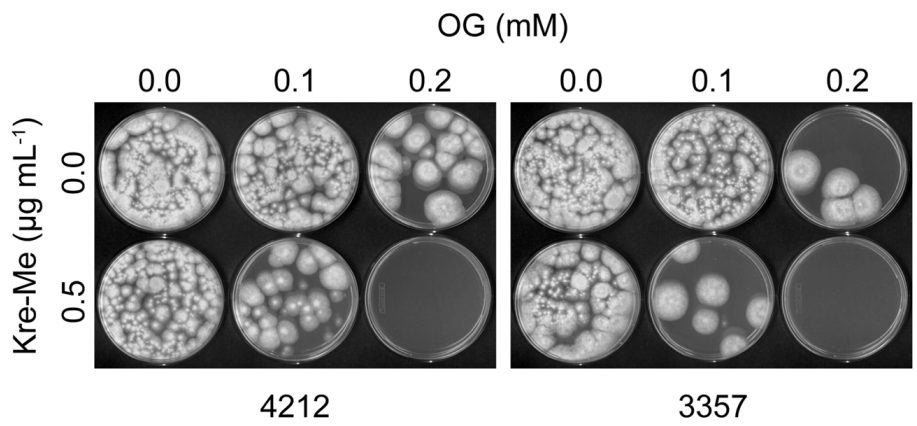

FIGURE 5 | Chemosensitization test in aflatoxigenic *Aspergillus*: Kre-Me + OG. Exemplary MFC bioassay showing co-application of Kre-Me (0.5 μg mL^{-1}) with OG (0.2 mM) completely inhibited the growth of aflatoxigenic *A. flavus* 3357 and 4212.

gene deletion mutants (*slt2*Δ and *bck1*Δ) are hypersensitive to caffeine treatment, while caffeine sensitivity of *slt2*Δ and *bck1*Δ could be alleviated by sorbitol (Martin et al., 2000; Ferreira et al., 2006). As shown in this study, OG also disrupted cell wall integrity, where *S. cerevisiae* MAPK pathway mutants (*slt2*Δ, *bck1*Δ) showed enhanced sensitivity to OG, while this sensitivity was remedied by sorbitol.

OG possessed some moderate level of antiaflatoxigenic activity, where 4–30% of reduction in aflatoxin production was achieved depending on types of toxigenic *Aspergillus* strains. In a prior study, more than 95% of inhibition in aflatoxin production could be achieved with caffeic acid, which is another type of phenolic compound (Kim et al., 2008). Modulation of the expression of antioxidant genes, such as alkyl hydroperoxide reductases (Ahp1) that detoxify organic peroxides, has been the mechanism of antiaflatoxigenic activity of caffeic acid (Kim et al., 2008). However, caffeic acid did not exhibit potent antifungal activity in the same study, indicating antimycotoxigenic activity

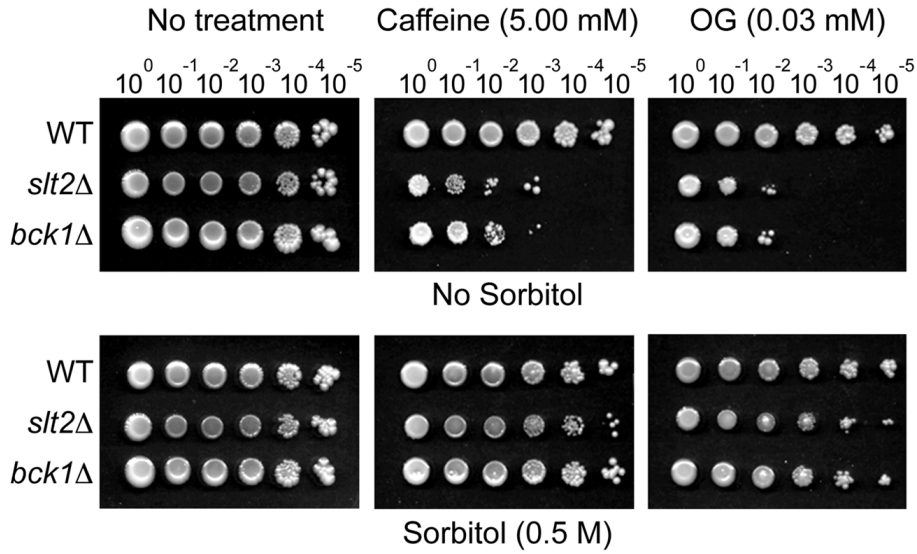

FIGURE 6 | Yeast cell-dilution bioassay showing sensitivity of *S. cerevisiae slt2*Δ and *bck1*Δ mutants to caffeine (5 mM; control) and OG (0.03 mM) was remediated by sorbitol. Similar remediation by sorbitol was found from treatment with higher concentration (0.04, 0.05, 0.06 mM) of OG (results not shown).

Table 5 | Antiaflatoxigenic activity of OG in *A. flavus* 3357, 4212, *A. parasiticus* 2999 (OG at 25 μM) and *A. parasiticus* 5862 (OG at 50 μM).

		A. flavus 3357	*A. flavus* 4212	*A. parasiticus* 2999	*A. parasiticus* 5862
AFB_1	No OG	5.920 ± 0.816	6.170 ± 0.816	16.100 ± 1.325	16.100 ± 2.904
	OG	5.260 ± 0.663	5.460 ± 0.561	14.900 ± 1.119	15.200 ± 1.271
	% Reduction	−11%	−12%	−7%	−6%
AFB_2	No OG	0.090 ± 0.020	0.031 ± 0.010	0.290 ± 0.046	0.290 ± 0.112
	OG	0.066 ± 0.015	0.026 ± 0.005	0.260 ± 0.036	0.240 ± 0.033
	% Reduction	−27	−16	−10	−17
AFG_1	No OG	ND[a]	ND	4.030 ± 0.612	4.030 ± 1.581
	OG	ND	ND	3.720 ± 0.510	3.870 ± 0.608
	% Reduction			−8	−4
AFG_2	No OG	ND	ND	0.090 ± 0.015	0.100 ± 0.040
	OG	ND	ND	0.070 ± 0.010	0.070 ± 0.016
	% Reduction			−22	−30

Aflatoxin amount produced: μg per cm^2 of fungal mat.

[a] *ND, Not detectable.*

of phenolic compounds is not always parallel to their antifungal activity or *vice versa*. Although OG possessed potent antifungal activity, as determined in this study, its antiaflatoxigenic capacity is not comparable to other types of phenolic agents, such as caffeic acid (e.g., reduction of aflatoxin production: 4–30% w/ OG vs. >95% w/ caffeic acid). Therefore, the intervention of either aflatoxin production or the growth of fungal pathogens could be achieved by treating or modulating different cellular targets, such as antioxidant enzymes (Ahp1) for mycotoxin control or MRC and cell wall/membrane integrity, etc., for fungal growth control, respectively.

"Compound or chemosensitization—strain specificity" exists with OG, reflecting differential susceptibility of *Aspergillus* strains to the treatments. Of note, cellular signaling system, such as histidine kinase receptors, MAPK, etc., was involved in differential susceptibility of fungi to fungicide or drugs (Ochiai et al., 2002; Kojima et al., 2004; Chapeland-Leclerc et al., 2007). For example, (1) Three histidine kinase receptors were differentially involved in drug sensitivity or stress adaptation of the opportunistic yeast *Candida lusitaniae* (Chapeland-Leclerc et al., 2007), (2) The pathogenic yeast *C. albicans* (containing multiple histidine kinase genes) were sensitive to the fungicides iprodione and fludioxonil, while the model yeast *S. cerevisiae* (having only one histidine kinase gene) showed insensitivity to these fungicides (Ochiai et al., 2002), and (3) Intact MAPK signaling system is required for the effective antifungal activity of fludioxonil, where MAPK mutants of the fungal pathogen *Colletotrichum lagenarium* exhibited increased resistance to the fungicide. Thus, it is thought

that (1) different set-up of signaling components in various species of *Aspergillus*, and/or (2) modification or mutation in cellular signaling system during evolution/stress adaptation could be possible mechanisms of actions for differential susceptibility of *Aspergillus* strains to fungicides or environmental stresses (such as Kre-Me tolerance of *A. fumigatus* MAPK mutants or heat responses, as determined in this study). Determination of precise mechanism of differential susceptibility to the treatments warrants future study.

As with agricultural application, the MRC is recently considered as a new antifungal target for clinical antimycotic therapy. Examples include: (1) MRC inhibitors increased fluconazole susceptibility of both patient and laboratory isolates of *C. albicans* (Sun et al., 2013); (2) co-application of antimycin A (another MRC-inhibitory drug) and BHAM significantly enhanced antifungal potency of posaconazole and itraconazole (Shirazi and Kontoyiannis, 2013), where the antifungal activity of the drugs was determined as "fungicidal" in mucormycosis-causing *Rhizopus oryzae*; (3) inhibition of MRC of *C. parapsilosis*, causative agent for neonatal and device-related infections, enhances susceptibility of the pathogen to caspofungin, which is a cell wall-disrupting drug [(Chamilos et al., 2006) and references therein].

Application of chemosensitizing agents, such as OG, would lower the effective dosage of MRC-inhibitory drugs or fungicides (e.g., strobilurins). This approach thus, lowers negative side effects of MRC inhibitors and/or prevents fungal tolerance to strobilurins, as described in this study. Consequently, lowered dosage level of antifungal agents would render treatment or agricultural practice, such as control of aflatoxin-producing *Aspergillus* strains, less expensive and safer.

In conclusion, OG and/or VT exhibit potential to serve as safe antifungal chemosensitizers that in concert with Kre-Me greatly potentiate antifungal activity. This capacity was shown to be effective in most of the *Aspergillus* strains tested in this study. Moreover, OG was shown to also have moderate antiaflatoxigenic activity.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00087/abstract

REFERENCES

Agarwal, A. K., Tripathi, S. K., Xu, T., Jacob, M. R., Li, X. C., and Clark, A. M. (2012). Exploring the molecular basis of antifungal synergies using genome-wide approaches. *Front. Microbiol.* 3:115. doi: 10.3389/fmicb2012.00115

AOAC International. (2005). *Official Methods of Analysis of AOAC International*. 18th Edn. Official method 971.22. Gaithersburg, MD: AOAC International.

Aspergillus Comparative Database. Available online at: www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html (Accessed: 9 January 2014).

Aspergillus flavus NRRL 21882 (006500) Fact Sheet. Available online at: www.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-006500_25-Nov-09.pdf (Accessed: 8 January 2014).

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. (2002). The strobilurin fungicides. *Pest Manag. Sci.* 58, 649–662. doi: 10.1002/ps.520

Beekrum, S., Govinden, R., Padayachee, T., and Odhavet, B. (2003). Naturally occurring phenols: a detoxification strategy for fumonisin B_1. *Food Addit. Contam.* 20, 490–493. doi: 10.1080/0265203031000098678

Campbell, B. C., Chan, K. L., and Kim, J. H. (2012). Chemosensitization as a means to augment commercial antifungal agents. *Front. Microbiol.* 3:79. doi: 10.3389/fmicb.2012.00079

Campbell, B. C., Molyneux, R. J., and Schatzki, T. F. (2003). Current research on reducing pre- and post-harvest aflatoxin contamination of U. S. almond, pistachio and walnut. *J. Toxicol. Toxin Rev.* 22, 225–266. doi: 10.1081/TXR-120024093

Chamilos, G., Lewis, R. E., and Kontoyiannis, D. P. (2006). Inhibition of *Candida parapsilosis* mitochondrial respiratory pathways enhances susceptibility to caspofungin. *Antimicrob. Agents Chemother.* 50, 744–747. doi: 10.1128/AAC.50.2.744-747.2006

Chapeland-Leclerc, F., Paccalletm, P., Ruprich-Robert, G., Reboutier, D., Chastin, C., and Papon, N. (2007). Differential involvement of histidine kinase receptors in pseudohyphal development, stress adaptation, and drug sensitivity of the opportunistic yeast *Candida lusitaniae*. *Eukaryot. Cell* 6, 1782–1794. doi: 10.1128/EC.00155-07

Clemons, K. V., Miller, T. K., Selitrennikoff, C. P., and Stevens, D. A. (2002). *fos-1*, a putative histidine kinase as a virulence factor systemic aspergillosis. *Med. Mycol.* 40, 259–262. doi: 10.1080/714031112

Clinical and Laboratory Standards Institute (CLSI). (2008). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi: Approved Standard-Second Edition. CLSI Document M38-A2*. Wayne, PA: Clinical and Laboratory Standards Institute.

Cools, H. J., and Hammond-Kosack, K. E. (2013). Exploitation of genomics in fungicide research: current status and future perspectives. *Mol. Plant Pathol.* 14, 197–210. doi: 10.1111/mpp.12001

Costa-de-Oliveira, S., Sampaio-Marques, B., Barbosa, M., Ricardo, E., Pina-Vaz, C., Ludovico, P., et al. (2012). An alternative respiratory pathway on *Candida krusei*: implications on susceptibility profile and oxidative stress. *FEMS Yeast Res.* 12, 423–429. doi: 10.1111/j.1567-1364.2012.00789.x

Dagnas, S., and Membré, J. M. (2013). Predicting and preventing mold spoilage of food products. *J. Food Prot.* 76, 538–551. doi: 10.4315/0362-028X.JFP-12-349

de Dios, C. H., Román, E., Monge, R. A., and Pla, J. (2010). The role of MAPK signal transduction pathways in the response to oxidative stress in the fungal pathogen *Candida albicans:* implications in virulence. *Curr. Protein Pept. Sci.* 11, 693–703. doi: 10.2174/138920310794557655

Ehrlich, K. C., and Cotty, P. J. (2004). An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. *Appl. Microbiol. Biotechnol.* 65, 473–478. doi: 10.1007/s00253-004-1670-y

Ellner, F. M. (2005). Results of long-term field studies into the effect of strobilurin containing fungicides on the production of mycotoxins in several winter wheat varieties. *Mycotoxin Res.* 21, 112–115. doi: 10.1007/BF02954432

Ferreira, C., Silva, S., van Voorst, F., Aguiar, C., Kielland-Brandt, M. C., Brandt, A., et al. (2006). Absence of Gup1p in *Saccharomyces cerevisiae* results in defective cell wall composition, assembly, stability and morphology. *FEMS Yeast Res.* 6, 1027–1038. doi: 10.1111/j.1567-1364.2006.00110.x

Fujita, K., Tani, K., Usuki, Y., and Taniguchi, M. (2004). Growth inhibition dependent on reactive oxygen species generated by C9-UK-2A, a derivative of the antifungal antibiotic UK-2A, in *Saccharomyces cerevisiae*. *J. Antibiot. (Tokyo)* 57, 511–517. doi: 10.7164/antibiotics.57.511

Ghannoum, M. A., and Rice, L. B. (1999). Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* 12, 501–517.

Guillen, F., and Evans, C. S. (1994). Anisaldehyde and veratraldehyde acting as redox cycling agents for H_2O_2 production by *Pleurotus eryngii*. *Appl. Environ. Microbiol.* 60, 2811–2817.

Hamilton, A. J., and Holdom, M. D. (1999). Antioxidant systems in the pathogenic fungi of man and their role in virulence. *Med. Mycol.* 37, 375–389. doi: 10.1046/j.1365-280X.1999.00208.x

Hawkins, L. K., Windham, G. L., and Williams, W. P. (2005). Effect of different postharvest drying temperatures on *Aspergillus flavus* survival and aflatoxin content in five maize hybrids. *J. Food Prot.* 68, 1521–1524.

Inoue, K., Tsurumi, T., Ishii, H., Park, P., and Ikeda, K. (2012). Cytological evaluation of the effect of azoxystrobin and alternative oxidase inhibitors in *Botrytis cinerea*. *FEMS Microbiol. Lett*. 326, 83–90. doi: 10.1111/j.1574-6968.2011.02438.x

Jacob, C. (2006). A scent of therapy: pharmacological implications of natural products containing redox-active sulfur atoms. *Nat. Prod. Rep.* 23, 851–863. doi: 10.1039/b609523m

Keinath, A. P. (2009). Sensitivity to azoxystrobin in *Didymella bryoniae* isolates collected before and after field use of strobilurin fungicides. *Pest Manag. Sci*. 65, 1090–1096. doi: 10.1002/ps.1797

Kim, J. H., Chan, K. L., Mahoney, N., and Campbell, B. C. (2011). Antifungal activity of redox-active benzaldehydes that target cellular antioxidation. *Ann. Clin. Microbiol. Antimicrob*. 10:23. doi: 10.1186/1476-0711-10-23

Kim, J. H., Yu, J., Mahoney, N., Chan, K. L., Molyneux, R. J., Varga, J., et al. (2008). Elucidation of the functional genomics of antioxidant-based inhibition of aflatoxin biosynthesis. *Int. J. Food Microbiol*. 122, 49–60. doi: 10.1016/j.ijfoodmicro.2007.11.058

Kirkman, T. W. (2014). *Statistics to Use*. Available online at: http://www.physics.csbsju.edu/stats/ (Accessed: 8 January 2014).

Kojima, K., Takano, Y., Yoshimi, A., Tanaka, C., Kikuchi, T., and Okuno, T. (2004). Fungicide activity through activation of a fungal signalling pathway. *Mol. Microbiol*. 53, 1785–1796. doi: 10.1111/j.1365-2958.2004.04244.x

Lee, J., Kwon, E. S., Kim, D. W., Cha, J., and Roe, J. H. (2002). Regulation and the role of Cu, Zn-containing superoxide dismutase in cell cycle progression of *Schizosaccharomyces pombe*. *Biochem. Biophys. Res. Commun.* 297, 854–862. doi: 10.1016/S0006-291X(02)02290-8

Lussier, M., White, A. M., Sheraton, J., di Paolo, T., Treadwell, J., Southard, S. B., et al. (1997). Large scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. *Genetics* 147, 435–450.

Martin, H., Rodríguez-Pachón, J. M., Ruiz, C., Nombela, C., and Molina, M. (2000). Regulatory mechanisms for modulation of signaling through the cell integrity Slt2-mediated pathway in *Saccharomyces cerevisiae*. *J. Biol. Chem*. 275, 1511–1519. doi: 10.1074/jbc.275.2.1511

Miskei, M., Karányi, Z., and Pócsi, I. (2009). Annotation of stress-response proteins in the aspergilli. *Fungal Genet. Biol.* 46(Suppl. 1), S105–S120. doi: 10.1016/j.fgb.2008.07.013

Morano, K. A., Grant, C. M., and Moye-Rowley, W. S. (2012). The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. *Genetics* 190, 1157–1195. doi: 10.1534/genetics.111.128033

Niimi, K., Harding, D. R., Parshot, R., King, A., Lun, D. J., Decottignies, A., et al. (2004). Chemosensitization of fluconazole resistance in *Saccharomyces cerevisiae* and pathogenic fungi by a D-octapeptide derivative. *Antimicrob. Agents Chemother.* 48, 1256–1271. doi: 10.1128/AAC.48.4.1256-1271.2004

Ochiai, N., Fujimura, M., Oshima, M., Motoyama, T., Ichiishi, A., Yamada-Okabe, H., et al. (2002). Effects of iprodione and fludioxonil on glycerol synthesis and hyphal development in *Candida albicans*. *Biosci. Biotechnol. Biochem*. 66, 2209–2215. doi: 10.1271/bbb.66.2209

Odds, F. (2003). Synergy, antagonism, and what the chequerboard puts between them. *J. Antimicrob. Chemother.* 52:1. doi: 10.1093/jac/dkg301

Possiede, Y. M., Gabardo, J., Kava-Cordeiro, V., Galli-Terasawa, L. V., Azevedo, J. L., and Glienke, C. (2009). Fungicide resistance and genetic variability in plant pathogenic strains of *Guignardia citricarpa*. *Braz. J. Microbiol.* 40, 308–313. doi: 10.1590/S1517-83822009000200018

Reyes, G., Romans, A., Nguyen, C. K., and May, G. S. (2006). Novel mitogen-activated protein kinase MpkC of *Aspergillus fumigatus* is required for utilization of polyalcohol sugars. *Eukaryot. Cell* 5, 1934–1940. doi: 10.1128/EC.00178-06

Robles-Martinez, L., Guerra-Sanchez, M. G., Flores-Herrera, O., Hernandez-Lauzardo, A. N., Velazquez-Del Valle, M. G., and Pardo, J. P. (2013). The mitochondrial respiratory chain of *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill. *Arch. Microbiol.* 195, 51–61. doi: 10.1007/s00203-012-0845-7

Rodriguez, S. B., and Mahoney, N. E. (1994). Inhibition of aflatoxin production by surfactants. *Appl. Environ. Microbiol*. 60, 106–110.

Roze, L. V., Hong, S. Y., and Linz, J. E. (2013). Aflatoxin biosynthesis: current frontiers. *Annu. Rev. Food Sci. Technol*. 4, 293–311. doi: 10.1146/annurev-food-083012-123702

Ruy, F., Vercesi, A. E., and Kowaltowski, A. J. (2006). Inhibition of specific electron transport pathways leads to oxidative stress and decreased *Candida albicans* proliferation. *J. Bioenerg. Biomembr.* 38, 129–135. doi: 10.1007/s10863-006-9012-7

Saccharomyces Genome Database. Available online at: http://www.yeastgenome.org (Accessed: 10 January 2014).

Schmidt-Heydt, M., Stoll, D., and Geisen, R. (2013). Fungicides effectively used for growth inhibition of several fungi could induce mycotoxin biosynthesis in toxigenic species. *Int. J. Food Microbiol*. 166, 407–412. doi: 10.1016/j.ijfoodmicro.2013.07.019

Shirazi, F., and Kontoyiannis, D. P. (2013). Mitochondrial respiratory pathways inhibition in *Rhizopus oryzae* potentiates activity of posaconazole and itraconazole *via* apoptosis. *PLoS ONE* 8:e63393. doi: 10.1371/journal.pone.0063393

Sierra-Campos, E., Valdez-Solana, M. A., Matuz-Mares, D., Velazquez, I., and Pardo, J. P. (2009). Induction of morphological changes in *Ustilago maydis* cells by octyl gallate. *Microbiology* 155, 604–611. doi: 10.1099/mic.0.020800-0

Sun, N., Fonzi, W., Chen, H., She, X., Zhang, L., Zhang, L., et al. (2013). Azole susceptibility and transcriptome profiling in *Candida albicans* mitochondrial electron transport chain complex I mutants. *Antimicrob. Agents Chemother*. 57, 532–542. doi: 10.1128/AAC.01520-12

Toone, W. M., and Jones, N. (1998). Stress-activated signalling pathways in yeast. *Genes Cells* 3, 485–498. doi: 10.1046/j.1365-2443.1998.00211.x

U.S. Food and Drug Administration (FDA). (2011). *Everything Added to Food in the United States*. Available online at: http://www.fda.gov/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/ucm115326.htm (Accessed: 8 January 2014).

Vincent, J. M. (1947). Distortion of fungal hyphae in the presence of certain inhibitors. *Nature* 159:850. doi: 10.1038/159850b0

Washburn, R. G., Gallin, J. I., and Bennett, J. E. (1987). Oxidative killing of *Aspergillus fumigatus* proceeds by parallel myeloperoxidase-dependent and -independent pathways. *Infect. Immun.* 55, 2088–2092.

Wood, P. M., and Hollomon, D. W. (2003). A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of complex III. *Pest Manag. Sci.* 59, 499–511. doi: 10.1002/ps.655

Xue, T., Nguyen, C. K., Romans, A., and May, G. S. (2004). A mitogen-activated protein kinase that senses nitrogen regulates conidial germination and growth in *Aspergillus fumigatus*. *Eukaryot. Cell* 3, 557–560. doi: 10.1128/EC.3.2.557-560.2004

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Aflatoxins and safe storage

Philippe Villers*

GrainPro, Inc., Concord, MA, USA

Edited by:
Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran

Reviewed by:
Theo Varzakas, Technological Educational Institute of Peloponnese, Greece
David Okello Kalule, National Semi Arid Resources Research Institute, Serere, Uganda

****Correspondence:***
Philippe Villers, GrainPro, Inc., 200 Baker Avenue, Suite 309, Concord, MA 01742, USA
e-mail: Pvillers@grainpro.com

The paper examines both field experience and research on the prevention of the exponential growth of aflatoxins during multi-month post-harvest storage in hot, humid countries. The approach described is the application of modern safe storage methods using flexible, Ultra Hermetic™ structures that create an unbreatheable atmosphere through insect and microorganism respiration alone, without use of chemicals, fumigants, or pumps. Laboratory and field data are cited and specific examples are given describing the uses of Ultra Hermetic storage to prevent the growth of aflatoxins with their significant public health consequences. Also discussed is the presently limited quantitative information on the relative occurrence of excessive levels of aflatoxin (>20 ppb) before vs. after multi-month storage of such crops as maize, rice, and peanuts when under high humidity, high temperature conditions and, consequently, the need for further research to determine the frequency at which excessive aflatoxin levels are reached in the field vs. after months of post-harvest storage. The significant work being done to reduce aflatoxin levels in the field is mentioned, as well as its probable implications on post-harvest storage. Also described is why, with some crops such as peanuts, using Ultra Hermetic storage may require injection of carbon dioxide, or use of an oxygen absorber as an accelerant. The case of peanuts is discussed and experimental data is described.

Keywords: post harvest, aflatoxin, hermetic, safe storage, grain storage, Cocoons™, pesticide free, Ultra Hermetic

INTRODUCTION

Aflatoxins (*Aspergillus flavus and Aspergillus parasiticus*) are widely recognized as a major health problem, especially in hot, humid countries. This is a particular serious problem in such crops as maize, rice, peanuts, tree nuts, and dried fruits. Aflatoxin production normally occurs in the field, particularly when stimulated by drought, stress, and high temperatures or during prolonged drying. Aflatoxin-producing molds grow exponentially in conventional multi-month storage as a result of a combination of heat and high humidity (Hell et al., 2010).

Figure 1 (Villers et al., 2008) shows the graphic relationship between mold growth and relative humidity at equilibrium. It explains the mold growth during storage, when sustained relative humidity is beyond 65%. **Table 1** shows the optimum conditions and ranges for *Aspergillus* growth and therefore aflatoxin development. When temperatures are below 65 degrees F, and the moisture of the maize is below 12–13%, development of the fungus usually stops (Sumner and Lee, 2012).

In East Africa post-harvest losses in maize, as shown in **Figure 2**, often reach 25%, or more (World Bank Report, 2011).

AFLATOXIN EFFECTS

Dr. J. H. Williams of the University of Georgia, USA, cites a survey of local African markets showing that 40% of the commodities found there exceeded permissible aflatoxin levels (in excess of the international standard of 10–20 ppb) and that an estimated 4.5 billion people in developing countries are at risk of uncontrolled or poorly controlled exposure to aflatoxins, and up to 40% of commodities in local African markets exceed allowable levels of aflatoxins in foods (Williams, 2011).

It is known that high aflatoxin levels in the bloodstream depresses the immune system, thereby facilitating cancer, HIV, and stunting the growth of children. A cross-sectional study conducted in Ghana and cited by Dr. Williams shows that immune systems of recently HIV-infected people are significantly modified if they have above-median levels of natural exposures to aflatoxins (Williams et al., 2004). Referring to another study, Dr. Williams notes, "People with a high aflatoxin biomarker status in the Gambia and Ghana were more likely to have active malaria" (Williams, 2011).

In 2014, the Global Forum for Innovations in Agriculture (GFIA) convened a high level meeting in Abu Dhabi, UAE, on revolutionizing global agriculture through innovations. Frank Rijsberman, the CEO of the CGIAR Consortium, in his report based on a Benin study (Gong et al., 2004) on the post-weaning exposure to aflatoxin, concludes that aflatoxins have impaired growth in children and are costing African farmers over $450 million USD per year in lost exports (Rijsberman, 2014). In 2010, 10% of the Kenyan maize crop was condemned because of excessive aflatoxin levels. One of the laboratories in Kenya that year tested 130 maize samples out of which only 47 samples had aflatoxin levels less than 10 ppb. The highest level of aflatoxin recorded in that year was 830 ppb (FAO, 2011).

Excessive aflatoxin levels also cause failure to thrive (or even death) in farm animals such as chickens and turkeys. According to Dr. Oladele Dotun, a Veterinarian at the Animal Care Laboratory

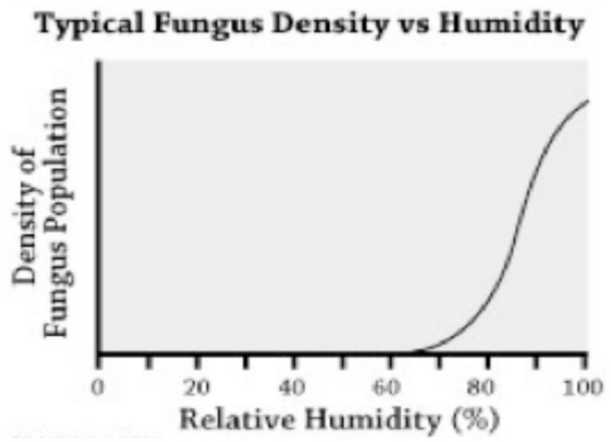

FIGURE 1 | Typical fungus density vs. humidity.

Table 1 | Conditions favoring *Aspergillus flavus* development.

Factor	Optimum	Range
Temperature	86°F	80–110°F
Relative humidity	85%	62–99%
Kernel moisture	18%	13–20%

Table shows the optimum conditions for Aspergillus growth and aflatoxin development. When temperatures are below 65° F and the moisture of the corn is below 12–13%, development of the fungus usually stops.

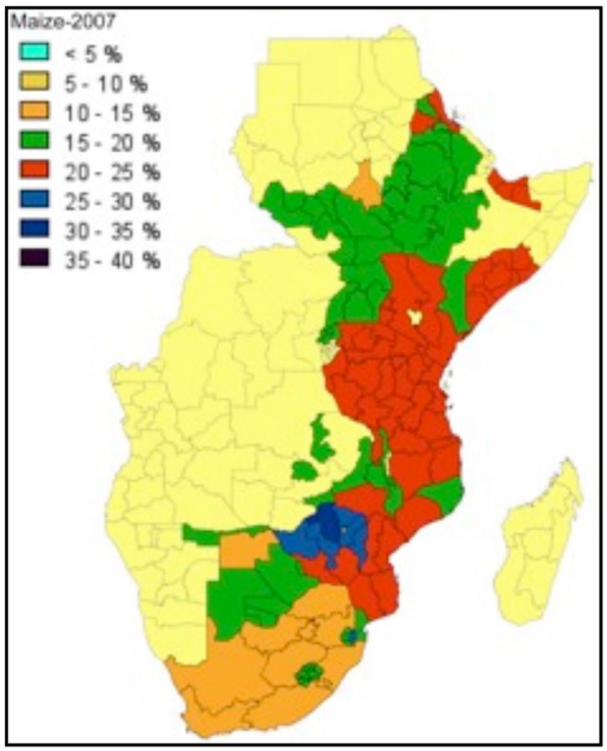

FIGURE 2 | 2011 World Bank Report on post-harvest losses of maize in East Africa.

Increase in Aflatoxin Concentration During Storages in the Farmers Fields-Ground Nuts Icrisat, Mali data
(Courtesy of Baozhu Gou, Ph.D. -USDA-ARS Tifton,GA, USA) (May 2013)

Village	Aflatoxin content (ppb)		
	At harvest	1 month in storage	2 months in storage
Bamba (5)	101.3	168.9	275.5
Gouak (5)	61.4	118.0	174.7
Kolokani (5)	119.2	352.6	400.0
Sido (5)	53.7	93.6	166.2

FIGURE 3 | Increase in aflatoxin concentration during storage in the farmers' fields.

in Nigeria, research has shown that aflatoxins cause infertility, abortions, and delayed onset of egg production in birds as well as sudden losses in egg production in actively laying birds. Furthermore, loss of appetite, skin discoloration, or even yellowish pigmentation on skin can be observed in fish (Oladele, 2014).

An increasing amount of scientific research has been devoted to learning more about aflatoxin growth problems and possible solutions, including using genetically modified or hybridized seeds formulated for mold resistance or through use of products such as AflaSafe, now used in Africa. AflaSafe's "biological approach" uses a closely related, non-aflatoxin-producing mold to out-compete the aflatoxin-producing molds. In temperate climates, aflatoxin problems have been controlled largely with ventilation during cooler nights and through lower winter temperatures (CAES, 2014).

A neglected part of the aflatoxin problem has been the rapid growth of post-harvest aflatoxin levels when stored for prolonged periods in the conditions of high temperature and relative humidity above 65% found commonly in all tropical climates (See **Figure 1**). In the tropics, field studies by Icrisat in Mali show that conventional storage for more than 2 months causes rapid growth of aflatoxins (Waliyar et al., 2002) (**Figure 3**).

In Guatemala, a field survey of maize sold in rural markets by Martinez-Herrera in 1968 found that considerable contamination by several fungi, including *Aspergillus* species were frequently present. His evidence suggests that maximum aflatoxin contamination of maize in Guatemala occurs during the rainy season. Maize samples analyzed 20 days after harvest had levels of 130 μg aflatoxin per kg of total maize. The same samples analyzed 60 days later showed rapid increase, up to 1 680 μg per kg (FAO, 1992).

This data, as well as data from several other studies, strongly indicate the need to dry maize before storage. Drying is an important step in ensuring good quality grain that is free of fungi and microorganisms and that has desirable quality characteristics for marketing and final use. Diverse drying systems and equipment are available that use various sources of energy, including solar energy. Choice of system requires consideration of a number of factors: temperature and air velocity, rate of drying, drying efficiencies, kernel quality, air power, fuel source, fixed costs, and management (FAO, 1992).

An interesting question is what happens to aflatoxin growth in Ultra Hermetic Storage™ following the use of biological controls such as AflaSafe, which introduces non-aflatoxin-producing molds to compete with aflatoxin-producing molds. However, biological competition between mold populations alone will not totally eliminate aflatoxin mold prior to storage. We believe that storage under tropical conditions in classical containers such as silos, bags, and tarps will continue to produce the exponential aflatoxin growth shown in **Figure 1**, probably from a lower starting population.

CHEMICAL APPROACHES TO POST-HARVEST CONTROL

Many chemical and additive methods for controlling aflatoxin growth have been proposed, but used only to a limited extent. For instance, as noted by Lunven at FAO, "Chakrabarti showed that aflatoxin levels could be reduced to less than 20 ppb using separate treatments with 3% hydrogen peroxide, 75% methanol, 5% dimethylamine hydrochloride, or 3% perchloric acid. These treatments, however, induced losses in weight and also in protein and lipids. Other methods include the use of carbon dioxide plus potassium sorbate and the use of sulphur oxide." Lunven also noted another process that had received some attention, namely the use of calcium hydroxide, a chemical used for lime cooking of maize. Regarding this process, Lunven stated, "Studies have shown a significant reduction in aflatoxin levels, although the extent of reduction is related to the initial levels. Feeding tests with moldy maize treated with calcium hydroxide have shown a partial restoration of its nutritional value" (FAO, 1992).

However, to date, no chemical or additive method has gained general acceptance. Concerns about using chemicals or additives is still growing, encouraging the expanded use of methods that have no contamination potential.

SAFE STORAGE USING ULTRA HERMETIC AIRTIGHT CONTAINERS

Ultra Hermetic Storage™ is in use now at a varying scale in 103 countries. It has been shown that WHEN sufficiently airtight, it drastically inhibit mold growth and hence aflatoxin expression. Since molds need oxygen and high humidity, the natural respiration of the contained insects and other microorganisms, and sometimes respiration of the commodity itself, will use up the available oxygen faster than any residue leakage. After 10 days to 2 weeks at room temperature or above, this results in an unbreatheable atmosphere–typically 3% oxygen and 15% carbon dioxide. This does not kill the molds, but as shown in **Table 2**, in the case of peanuts, the unbreatheable atmosphere arrests mold development of Colony Forming Units (CFUs) so that even after several months, the levels of mold products, and therefore aflatoxins do not rise significantly. The same table shows inhibition of Free Fatty Acids (FFAs), which cause rancidity (Navarro et al., 2012).

Martin Gummert, post-harvest rice specialist at the International Rice Research Institute (IRRI), wrote about storage of rice and rice seed, "Hermetic Storage of rice is becoming increasingly popular across Asia, and for good reason—as well as being transportable, it is better than air-conditioned storage and almost as good as a cold room, at a fraction of the cost" (Villers and Gummert, 2009).

THE SPECIAL CASE OF PEANUTS

Peanuts often are often contaminated with aflatoxins before storage, but (unlike most grains) may take as long as 30 days or so to reach a 3% oxygen level in Ultra Hermetic storage (Navarro et al., 2012). This is too long a period to prevent major increase of aflatoxin levels. For this reason, one of two forms of accelerant is used. For field operation and portable bags, an oxygen-absorbing sachet weighing 65 g per 69 kg capacity is sufficient. For large storage units or volume production, injecting carbon dioxide to drive out the air is adequate. **Table 2** shows mold growth densities measured in CFUs after 90 days (Navarro et al., 2012). The table shows two orders of magnitude difference in mold density for

Table 2 | FFA and CFU mold levels in peanuts.

Moisture content (%)	Test parameters	Initial	After 90 days				
			Hermetic clean peanuts	Hermetic with 3% broken peanuts	CO_2 with 3% broken peanuts	Control clean peanuts	Control with 3% broken peanuts
7	% Moisture content	5.97 ± 0.03	6.80 ± 0.20	7.20 ± 0.21	6.60 ± 0.40	6.33 ± 0.53	6.60 ± 0.26
	FFA (% oleic acid)	0.36 ± 0.01	0.63 ± 0.53	0.70 ± 0.17	0.43 ± 0.07	0.57 ± 0.03	1.50 ± 0.12
	Aflatoxin (μg/kg)	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
	CFU molds	$3*10_2$	$1.8*10_3 \pm 1.2*10_3$	$1.7*10_3 \pm 7*10_2$	$9.7*10_1 \pm 28$	$1.3*10_4 \pm 9*10_3$	$4*10_4 \pm 3*10_3$
8	% Moisture content	7.53 ± 0.07	6.87 ± 0.15	6.37 ± 0.2	7.10 ± 0.32	6.63 ± 0.19	7.30 ± 0.17
	FFA (% oleic acid)	0.42 ± 0.09	0.67 ± 0.17	2.13 ± 0.07	0.77 ± 0.03	2.57 ± 0.47	4.00 ± 0.42
	Aflatoxin (μg/kg)	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3

crops stored in conventional (the Control) vs. hermetic storage, using carbon dioxide injection. Hermetic storage, even without injected carbon dioxide, still shows a five-fold improvement vs. the Control. Unfortunately, in this particular study, aflatoxin-producing molds turned out to be largely absent in the mold mix, and therefore the data of **Table 2** does not allow direct measurement of the growth of aflatoxin, as distinct from all molds (CFU). **Table 2** shows the significant improvement with hermetic storage alone and even greater improvement with the early addition of CO_2 in controlling the growth of rancidity-producing FFAs.

AN UNSOLVED QUESTION

Needing further study is the question of exactly how frequently aflatoxin levels exceed the international standard of 10–20 ppb at initial storage time vs. after long-term storage. The unanswered question is how frequently initial tolerable levels of aflatoxins become intolerable after improper drying and or improper post-harvest storage. We believe it is quite likely, that the levels of aflatoxin in grains registering above 10–20 ppb in peanuts before storage may well be relatively infrequent but only exceed the international safety standard after long-term storage. The absence of a definitive answer to this question may well contribute to scientific attention still being devoted almost entirely to the problem of controlling aflatoxin in the field. To date, the demonstrated use of Ultra Hermetic storage, although widespread and available, has not yet resulted in much scientific work being focused on existing ways of preventing aflatoxin growth post-harvest and in longer-term storage. We believe such study is overdue.

AVAILABLE FORMS OF ULTRA HERMETIC AIRTIGHT STORAGE

Ultra-Hermetic™ (airtight) enclosures without pesticides or fumigants use the respiration of infesting insects and other microflora as their "engines" to produce an unbreatheable atmosphere. Available sizes of such airtight plastic enclosures vary over a wide range depending on the application. They range from the patented man-portable SuperGrainbags™, made from thin (0.078 mm) coextruded, multi-layer plastic with capacities from 25 kg to 2.8 tons (**Figure 4**), to flood- and tsunami-resistant Cocoons™ made from 0.8 mm PVC with capacities of 5- to 1000-tonnes (**Figure 5**). Their massive air and watertight bulk and geometry help explain their survival when full in several Philippine typhoons and in one flood.

Cocoons are also highly rodent-resistant, as a result of intentionally stretching their tough exterior surface to prevent rodents from gaining a tooth hold. Cocoons can be erected without specialized tools or equipment and can be rapidly installed either directly on the ground or on pavement, either indoors or outdoors, and have a 10–15 year useful life.

The SuperGrainbag, man-portable Ultra Hermetic storage shown in **Figure 4** has permeability to oxygen of only 3 cc/m^2/per day, 500 times more airtight than ordinary plastics such as polyethylene. This results from the use of a proprietary barrier layer inside its 3-layer, coextruded design.

Figure 5 shows 100-tonne Cocoons enclosing bags of grains stacked on the bottom of the Cocoon. After the bags are stacked, the Cocoon is closed by mounting its upper section over the pile.

FIGURE 4 | Nepalese woman with SuperGrainbags, Mulpani Village near Kathmandu, Nepal.

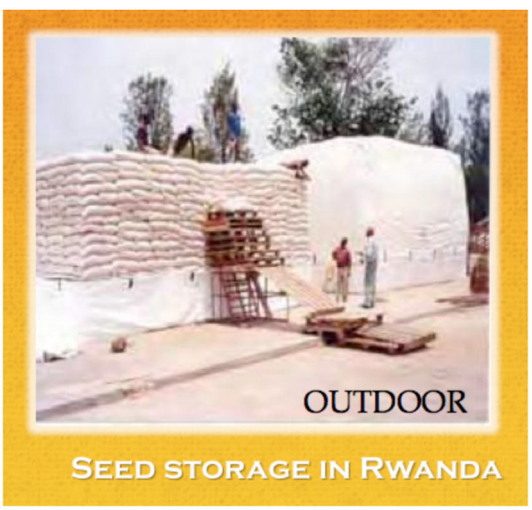

FIGURE 5 | Outdoor seed storage in Rwanda.

Then the two sections are zipped together with an airtight zipper, originally designed for astronauts.

Other designs, such as the 1- to 2-tonne capacity GrainSafe™ shown in **Figure 6**, are used for storing bulk grain, and are designed for continuous in-and-out access while maintaining hermeticity and avoiding empty headspace when partly empty.

The largest existing Ultra Hermetic storage units are known as Hermetic Bunkers™ and store 10,000- to 20,000-tonnes (**Figure 7**) (Navarro et al., 1993). They are designed for multi-year strategic storage. Hermetic Bunkers are used for up to 5-year storage of barley, maize or wheat, such as in Cyprus, Jordan, and Israel (Navarro et al., 1993).

FIGURE 6 | GrainSafe in Guatemala storing maize.

FIGURE 8 | Collapsible solar dryer in Ghana.

FIGURE 7 | Bunker in Jordan storing wheat.

Rice and other grain seeds now are among the leading commodities stored in Ultra Hermetic enclosures instead of air-conditioned or refrigerated facilities (Villers and Gummert, 2009).

SAFE SOLAR DRYING

All forms of longer-term storage historically have required proper drying before storage. Various strategies have been used, but in most developing countries the capital and consumption costs of using non-renewable fuels are unaffordable, and so solar drying is used. However, much solar drying is done on paved patios, paved roads, or even dirt roads, with no protection from rewetting when it rains. The Collapsible Solar Dryer (CDC)™ (**Figure 8**) has the special property of being manually reclosed on itself when it rains, creating a rainproof cover like a suitcase lid that keeps the seeds or grains dry until the rain stops.

CONCLUSION

Programs to control aflatoxin-producing molds in the field through improved seeds or biological control have yielded encouraging results, but they do not protect commodities from exponential aflatoxin growth in post-harvest storage under hot, humid conditions, which in many cases may be the dominant growth factor. Ultra Hermetic storage is an available, important answer to keeping aflatoxin levels at (or below) the International Standards of a maximum of 10- to 20-ppm, now so often exceeded in hot, humid climates. It is already widely proven in the field but not yet utilized on a sufficient scale. To date, the importance of post-harvest protection against aflatoxin growth remains not widely understood by the scientific community. Existing literature on aflatoxin control focuses on means of reducing aflatoxin levels during the growing period, and more work is needed to prove the full public health significance of aflatoxin growth during conventional drying and in non-hermetic, post-harvest storage vs. levels of aflatoxin already existing by harvest time.

REFERENCES

CAES. (2014). *Reducing Aflatoxins in Corn During Harvest.* Atlanta, GA: CAES Publication, University of Georgia, B 1231.

FAO. (1992). *Maize in Human Nutrition*. Food and Nutrition Series, No. 25, Chapter 3, Rome: Food Policy and Nutrition Division.

FAO. (2011). "Situation analysis: improving food safety in the maize value chain in Kenya," in *Report prepared for FAO by Prof. Erastus Kang'ethe College of Agriculture and Veterinary Science*, University of Nairobi.

Gong, Y. Y., Hounsa, A., Egal, S., Turner, P. C., Sutcliffee, A. E., Hall, A. J., et al. (2004). Post weaning exposure to aflatoxin results in impaired child growth: a longitudinal study in Benin, West Africa. *Environ. Health Perspect.* 112, 1334–1338. doi: 10.1289/ehp.6954

Hell, K., Mutegi, C., and Fandohan, P., (2010). "Aflatoxin control and prevention strategies in maize for Sub-Saharan Africa," in *10th International Working Conference on Stored Product Protection*, Vol. 2 (Estoril), 534–540; *Green. J. Agricult. Sci.* 2, 280, October 2012.

Navarro, H., Navarro, S., and Finkelman, S. (2012). "Hermetic and modified atmosphere storage of shelled peanuts to prevent free fatty acid and Aflatoxin formation," in *Proceedings of the Conf. Int. Org. Biol. Integrated Control of Noxious Animals and Plants (IOBC), Work Group on Integrated Prot. Stored Prod. Bull.* (Volos).

Navarro, S., Varnava, A., and Donahaye, E. (1993) "Preservation of grain in hermetically sealed plastic liners with particular reference to storage of barley in Cyprus," in *Proceedings International Conference on Controlled Atmosphere and Fumigation in Grain Storages, Winnipeg, Canada, June 1992*, eds S. Navarro and E. Donahaye (Jerusalem: Caspit Press Ltd.), 223–234.

Oladele, D., (2014). "The effects of aflatoxins on animals," Partnership for Aflatoxin Control in Africa (Meridian Institute, Washington, DC), *Aflatoxin Partnership Newsletter*, Vol. II (Accessed, February 2014), 4.

Rijsberman, F. (2014). PACA (Partnership for Aflatoxin Control in Africa) presents at the global forum for innovations: middle east and Africa focus (Meridian Institute, Washington, DC), *Aflatoxin Partnership Newsletter*, Vol. II (Accessed February, 2014).

Sumner, P. E., and Lee, D. (2012). *Reducing Aflatoxin in Corn During Harvest and Storage*. Atlanta, GA: The University of Georgia, Georgia College of Agriculture and Environmental Sciences.

Villers, P., and Gummert, M. (2009). Seal of approval. *Rice Today* (Los Banos, CA) 8, 26–27.

Villers, P., Navarro, S., and DeBruin, T. (2008). "Development of hermetic storage technology in sealed flexible storage structures," in *CAF 2008 Conference Paper* (Chengdu).

Waliyar, F., Natre, B. R., Traore, A., Diarra, B., Kodio, O., and Lava Kumar, P. (2002). *Pre and Postharvest Management of Aflatoxin Contamination in Groundnut*. Bamako: ICRISAT (International Crops Research Institute for the Semi-Arid Tropics).

Williams, J. H. (2011). *Aflatoxin as a Public Health Factor in Developing Countries and its Influence on HIV and Other Diseases*. Peanut Collaborative Research Support Program, University of Georgia, World Bank Report #60371-AFR, 1–95.

Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M., and Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am. J. Clin. Nutr.* 80, 1106–1122.

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