Toxin Reviews, 2009; 28(2–3): 142–153

informa
healthcare

REVIEW ARTICLE

Ecology of *Aspergillus flavus*, regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn

HK Abbas[1], JR Wilkinson[2], RM Zablotowicz[3], C Accinelli[4], CA Abel[5], HA Bruns[1], and MA Weaver[3]

[1]USDA-ARS, Crop Genetics and Production Research Unit, Stoneville, MS, [2]Mississippi State University, Department of Biochemistry and Molecular Biology, MS, [3]USDA-ARS, Southern Weed Science Research Unit, Stoneville, MS, [4]University of Bologna, Department of Agro-Environmental Science and Technology, Bologna, Italy, and [5]USDA-ARS, Southern Insect Management Research Unit, Stoneville, MS

Abstract

The contamination of corn (maize) by fungi and the accumulation of mycotoxins are a serious agricultural problem for human and animal health. One particular devastating group of mycotoxins, called aflatoxins, has been intensely studied since the 1960s. Studies of *Aspergillus flavus*, the agriculturally relevant producer of aflatoxins, have led to a well-characterized biosynthetic pathway for aflatoxin production, as well as a basic understanding of the organism's life cycle. Unfortunately, these efforts have not resulted in corn production practices that substantially reduce aflatoxin contamination. Similarly, the use of agrochemicals (e.g., fungicides) results in very limited reduction of the fungus or the toxin. Thus, cultural management (fertility and irrigation) coupled with aggressive insect management is current recommendation for integrated aflatoxin management. The development of resistant hybrids appears to be a very promising technology, but commercial hybrids are still not available. Thus, biocontrol appears to be the most promising available avenue of reducing aflatoxin accumulation. Biocontrol utilizes nontoxigenic strains of *Aspergillus* to reduce the incidence of toxin-producing isolates through competitive displacement. To maximize the effectiveness of biocontrol, a thorough knowledge of the environmental factors influencing colonization and growth of *Aspergillus* is needed. *A. flavus* not only colonizes living plant tissue, but it also grows saprophytically on plant tissue in the soil. These residues serve as a reservoir for the fungus, allowing it to overwinter, and under favorable conditions it will resume growth and release new conidia. The conidia can be transmitted by air or insects to serve as new inoculum on host plants or debris in the field. This complex ecology of *Aspergilli* has been studied, but our understanding lags behind what is known about biosynthesis of the toxin itself. Our limited understanding of *Aspergilli* soil ecology is in part due to limitations in evaluating *Aspergilli*, aflatoxin, and the biosynthetic genes in the varying aspects of the environment. Current methods for assessing *Aspergillus* and aflatoxin accumulation rely heavily on cultural and analytical methods that are low throughput and technically challenging. Thus to understand *Aspergillus* ecology and environmental effects in contamination to maximize biocontrol efforts, it is necessary to understand current treatment effects and to develop methodologies capable of assessing the fungal populations present. In this manuscript we discuss the current knowledge of *A. flavus* ecology, the application of selected molecular techniques to field assessments, and crop practices used to reduce aflatoxin contamination, focusing on chemical treatments (fungicides and herbicides), insect management, and crop management.

Keywords: *Fungal ecology; aflatoxin; insect; corn; mycotoxin; biological control; environmental manipulation; molecular biology; fungal biology*

Introduction

Aspergillus flavus is a fungus of economic and toxicological importance due to the production of aflatoxins and other chemicals with deleterious properties (e.g., aspergillic acid, cyclopiazonic acid, kojic acid, helvulic acid, etc.). This fungus is ubiquitous in the environment, being readily isolated

Address for Correspondence: Dr. HK Abbas, USDA-ARS, Crop Genetics and Production Research Unit, Stoneville, MS 38776, USA. E-mail: Hamed.Abbas@ars.usda.gov

(Received 06 March 2009; revised 03 April 2009; accepted 06 April 2009)

ISSN 1556-9543 print/ISSN 1556-9551 online
DOI: 10.1080/15569540903081590

http://www.informapharmascience.com/txr

from plants, air, soil, and insects (Wicklow et al., 2003). The presence of *Aspergillus* species (*A. flavus*, *A. fumigatus*, and *A. vesicolor*) in dust can compromise individuals with elevated allergies to the fungus or its products (Benndorf et al., 2008; Sharnma et al., 2007). Of more concern is the colonization of certain food or feed crops (corn, cottonseed, peanuts, and some tree nuts) by the fungus, where it may produce a high enough concentration of these chemical products, specifically aflatoxin, to cause them to be considered contaminated and unfit for their intended use.

Aflatoxin was initially identified as toxic after investigations of the death of 100,000 turkeys in the United Kingdom in 1960 (Blout, 1961). This prompted a major revolution in mycotoxin research resulting in intensive testing for mycotoxins in any moldy products. Since then several *Aspergilli* have been identified as capable of producing aflatoxins. The two most agriculturally important species are *Aspergillus flavus* and *A. parasiticus*, which are found throughout the world, being present in both the soil and the air (Abbas et al., 2005; Klich, 2002; Horn and Dorner, 1998; Wicklow et al., 1998). When conidia (spores) encounter a suitable nutrient source and favorable environmental conditions (hot and dry conditions) the fungus rapidly colonizes and produces aflatoxin (Payne, 1992).

Contamination of agricultural commodities by aflatoxin is a serious problem due to the substantial health effect it has on humans and animals. Several strategies that have been employed to minimize aflatoxin contamination are listed in Table 1. The use of agrochemicals (fungicides), timely irrigation, and alternate cropping systems have independently shown limited success in preventing aflatoxin contamination. Integration of several of these tactics will be required to manage such a difficult problem (Cleveland et al., 2003). The use of these management techniques will be further discussed in the following sections of this article. A more recent and promising technology is the use of nontoxigenic strains of *Aspergillus* as biocontrol agents. However, to maximize this methodology and to prevent the colonization of multiple crops by *A. flavus* and related species (*A. parasiticus* and *A. nominus*), it is critical that a complete understanding of the ecology of these unique fungi be developed.

Through the use of sophisticated molecular biology approaches, our knowledge of *Aspergillus* biology and aflatoxin management continues to improve. As we understand the genes and gene expression of these fungi and their interactions with the plant host, we will improve control mechanisms on several fronts (Table 2). Thus, new solutions to reduce aflatoxin contamination will result through the coupling

Table 1. Crop, insect, and soil management practices to manage aflatoxin contamination.

Strategy	Method	Rationale
Avoidance	Early planting, supplemental irrigation, short season hybrids	Reduce heat and moisture stress
Fertility management	Provide adequate nutrition	N-deficient corn more susceptible
Insecticide application	Appropriate timing of application to control insect damage to ears	Insects responsible for enhanced ingress into grain
Bt Hybrids	Hybrids engineered with resistance to ear-damaging insects	Insects responsible for penetration into grain
Natural resistance to insects	Breeding and selection hybrids for resistance	
Biological control	Use of nontoxigenic isolates of *A. flavus*	Competitive displacement of toxigenic isolates
Fungicides	Control phylosphere fungi	Reduce inoculum density
Soil management	Incorporation of crop residues	Reduce inoculum density

Table 2. Application of molecular methods in aflatoxin research.

Goal	Methods	Rationale
Identification of aflatoxin biosynthetic and regulatory genes	EST sequence, whole genome sequence, cDNA microarrays, whole genome microarrays	Reduce aflatoxin production by manipulating factors that induce aflatoxin biosynthesis
Identification of *Aspergillus* pathogenicity factors and maize resistances factors to *Aspergillus*	*A. flavus* microarrays, *Z. mays* microarrays, qRT-PCR, digital gene expression,	Develop markers for selection of hybrids with resistance to *Aspergillus* infection and aflatoxin accumuation
Identification of maize resistances factors to Fall Army Worm	*Z. mays* microarrays, qRT-PCR, digital gene expression	Develop markers for selection of hybrids with resistance to ear-damaging insects to reduce *Aspergillus* vectoring
Develop molecular identification methods	SSR fingerprinting, PCR identification of rRNA, RT-PCR of biosynthetic genes	Track soil populations during biocontrol to improve displacement of toxigenic strains

of traditional agronomic research with these modern tools.

Aflatoxin and biosynthesis

Aflatoxins are toxic compounds chemically related to bisfuranocoumarin that are produced by *A. flavus* and *A. parasiticus* strains (Abbas et al., 2004b; Payne, 1992). These two aflatoxigenic species have been frequently studied due to their impact on agricultural commodities and their devastating effects on livestock. The name "aflatoxin" comes from the genus *Aspergillus*, which is where the letter "a" in aflatoxin is derived and "fla" from the species name *flavus*. In agricultural grains the fungi *A. flavus* and *A. parasiticus* are capable of producing four major aflatoxins (AfB1, AfB2, AfG1, and AfG2). *A. flavus* typically produces only the B toxins (Abbas et al., 2004b; Fortnum, 1986; Heathcote and Hibbert, 1978; Hill et al., 1985; Jones et al., 1981; Manwiller and Fortnum, 1979; Payne, 1998). Corn and cottonseed are typically contaminated with the aflatoxin B1, produced after colonization by *A. flavus* (Hill et al., 1985; Klich, 1986). *A. parasiticus* is more prevalent in peanuts than any other crop; however, it is typically outcompeted by *A. flavus* when the two fungi are both present (Horn et al., 1995).

These toxins were named based on their physical characteristics of fluorescence under longwave ultraviolet light ($\lambda = 365$ nm) when initially isolated using thin-layer chromatography; AfB1 and AfB2 produce a blue fluorescence, whereas AfG1 and AfG2 produce a green fluorescence (Klich, 2007). There are close to 25 analogs of aflatoxins; among these the most important to animals are aflatoxin B1-2,3-oxide (AfB1-2/3-oxide) and aflatoxin M1 (AfM1, which is a derivative of AfB1, formed in dairy cattle milk) (Heathcote and Hibbert, 1978; Bhatnagar et al., 2002, 2006a, 2006b).

The biosynthetic pathway in *A. flavus* consists of approximately 23 enzymatic reactions and at least 15 intermediates (reviewed in Bhatnagar et al., 2003, 2006a; Minto and Townsend, 1997; Payne and Brown, 1998; Yu, 2004; Yu et al., 2002) encoded by 25 identified genes clustered within a 70-kb DNA region on chromosome III (Bhatnagar et al., 2006a; Cary and Ehrlich, 2006; Smith et al., 2007; Townsend, 1997; Yu et al., 1995, 2004, 2005). The initial substrate acetate is used to generate polyketides with the first stable pathway intermediate being the anthraquinone norsolorinic acid (NOR) (Bennett, 1981; Bennett et al., 1997). This is followed by anthraquinones, xanthones, and ultimately aflatoxins synthesis (Bhatnagar et al., 2003, 2006a; Yabe, 2003; Yu et al., 2002, 2004). Few regulators of this process have been identified (reviewed in Cary and Calvo, 2008), and no complete regulatory model has been validated.

Biology and ecology of *Aspergillus flavus*

Generally, *Aspergillus flavus* is a competent saprophyte and can readily survive and colonize soil and organic debris associated with plant residues (Abbas et al., 2008a; Jamie-Garcia and Cotty, 2004). The overwintering reservoir of *A. flavus* propagules in soils and plant debris can serve as a primary inoculum for infestation of below-ground plant parts, especially in peanuts. Although soil can serve as the primary habitat for *A. flavus*, *A. parasiticus*, and *A. nominus,* little is known about the life cycle of these fungi in soil. *A. flavus* is capable of surviving and overwintering in plant residues as mycelium (hypha) or sclerotia (Abbas et al., 2008a; Horn, 2007; Payne, 1998; Wicklow et al., 1993) that in turn serve as the source of new conidia (Figure 1, life cycle of *A. flavus*).

Soil populations of *A. flavus* in soils under maize cultivation can range from 200 to >300,000 colony-forming units (cfu) g^{-1} soil (Abbas et al., 2004a, Zablotowicz et al., 2007) and can constitute from ≤0.2% to ≤8% of the culturable soil fungi population. The major soil property associated with maintaining soil populations of *A. flavus* is soil organic matter. As shown in Figure 2, a highly significant correlation between soil populations and organic matter is observed. The four major outlier points consist of one soil that had several-fold higher organic matter content compared with the other soils. This soil was part of a comparison of intensive crop residue management practices. From this study it was also evident that higher populations of *A. flavus* are maintained in the soil surface of no-till compared to conventional-till soils (Zablotowicz et al., 2007).

Following harvesting of a maize crop, *A. flavus* propagules range from greater than log 3 to ~log 6 cfu g^{-1} tissue (Table 3) (Abbas et al., 2008a). Similar levels are seen in vegetative tissue from soybean and cotton residues as observed in corn stover (Abbas, unpublished). However, typically >log 5 cfu g^{-1} tissue are associated with maize cobs and maize cobs containing grain 3 months after harvest, while levels of ~log 2.5 cfu g^{-1} or lower are observed in soybean seed (Table 3). Clearly, abundant propagules of *A. flavus* can be maintained on the soil surface of no-till corn fields. Although it is wise, from a soil conservation standpoint, to utilize no-till practices to maintain soil resources, such practices may enhance the potential for aflatoxin contamination. The data on *A. flavus* propagule levels in nonhosts such as soybean suggest that maintaining residues of other

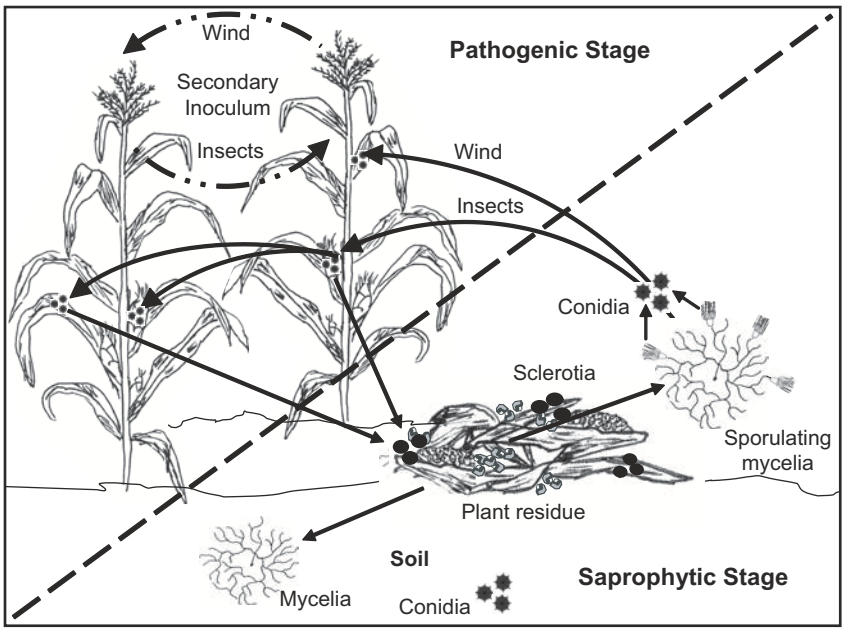

Figure 1. Life cycle of *A. flavus* in a corn cropping system saprophytic and pathogenic stages of fungal ecology.

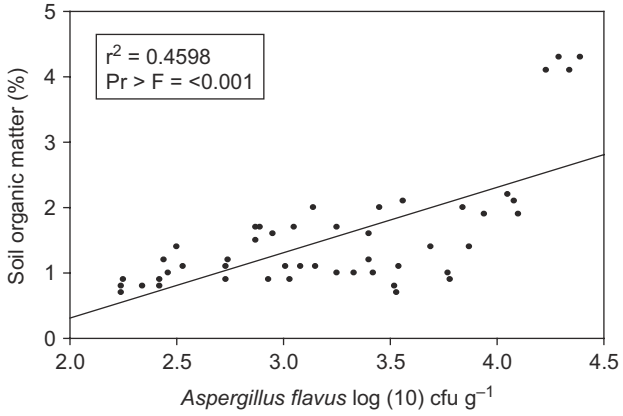

Figure 2. Relationship between soil organic matter content and populations associated with Mississippi maize-producing soils (Zablotowicz et al., 2007).

crops on the soil surface may be suitable to reduce inoculum potential of *A. flavus*. Before consideration of alternative cover crop management with crops that may be allelopathic to *A. flavus,* further investigation into *Aspergillus* antagonism is necessary.

Corn samples mean and standard deviation of five replicates, cotton and soybean mean and standard deviation of four replicates.

There are few methodologies, all of which are quite tedious, available to ascertain if propagules of *Aspergillus* are actively growing in soil or present in quiescent propagules, e.g., conidia or sclerotia. The majority of studies that evaluate *A. flavus* soil ecology have traditionally used selective plating techniques (Abbas et al., 2004a; Horn and Dorner, 1998; Zablotowicz et al., 2007). When using cultural techniques for enumeration such as serial dilution technique and plating, it is difficult to ascertain whether propagules enumerated are present as hyphal fragments or as spores. Sieving techniques can be used for the recovery of sclerotia from soil or plant tissue homogenates; however, the actual identification of species can be difficult as sclerotia of a given species can vary tremendously in size and shape (Klich and Pitt, 1988; Abbas et al., 2005; Horn, 2003).

When suitable environmental conditions arise, sclerotia and conidia germinate into mycelia that produce numerous conidiophores and release conidia into the air that can be available for colonizing plants.

Table 3. *A. flavus* propagules, toxigenic isolates, and aflatoxin concentration in various residue fractions of Bt and non-Bt corn, Bt cotton, and soybean residues, collected in December 2006.

Source	Colony-forming units (log 10 propagules $g^{-1)}$	Toxigenic isolates (% of total)	Total aflatoxin content ($\mu g\ kg^{-1}$)
Bt corn cobs with grain	5.7 ± 0.3	51 ± 24	542 ± 266
Bt corn cobs	5.1 ± 0.4	34 ± 25	17 ± 10
Bt corn stover	3.7 ± 0.1	64 ± 16	3.3 ± 1.7
Non-Bt corn cobs with grain	5.6 ± 0.3	46 ± 19	774 ± 195
Non-Bt corn cobs	5.5 ± 0.7	33 ± 21	105 ± 96
Non-Bt corn stover	3.9 ± 0.3	47 ± 15	3.9 ± 2.5
Cotton Bt	3.5 ± 0.4	73 ± 19	0.7 ± 0.5
Soybean (non-Bt)	3.5 ± 0.4	22 ± 14	0.2 ± 0.4
Soybean grain (non-Bt)	1.8 ± 0.3	15 ± 14	0.2 ± 0.4

Although *A. flavus* colonizes a plant structure, it doesn't necessarily produce aflatoxin to excessive levels. In this manner, *A. flavus* is an opportunistic pathogen in a similar context to the opportunistic human pathogens *Pseudomonas fluorescens* and *Burkholderia cepacia*. These bacteria may colonize in low levels in compromised individuals, such as burn patients or the immunocompromised, and become pathogenic. In the same context, healthy plant tissues are less prone to be extensively colonized by *A. flavus*. However, under heat stress and moisture deficit, corn reproductive structures are readily susceptible to high levels of aflatoxin contamination (O'Brian et al., 2007). Therefore, inoculum potential modified by life cycle of the fungus is as critical as the environment and host.

The *A. flavus* life cycle can be divided into two major phases: the colonization of plant residues in soil, and the infection of crop tissues, including grain and seeds of actively growing plant tissues. At the beginning of the growing season, usually in spring and sometimes at the end of winter, when sclerotia are exposed to the soil surface, they quickly germinate and form new conidial inoculum. This new inoculum will be vectored by insects or carried by the wind to begin the colonization and infection of the freshly planted crops (Horn, 2007; Payne, 1992). During the growing season, infected plant tissues can serve as sources of secondary conidial inoculum, which colonize new noninfected plant tissues (Figure 1, life cycle). Despite our understanding of how the initial and secondary inocula occur for plant infection, little information is available about the saprotrophic activities of these fungi in soil. Recently, Accinelli et al. (2008c) confirmed the presence of *A. flavus* in the soil actively synthesizing aflatoxins. Accinelli et al. reported that transcription of 5 aflatoxin biosynthesis genes, including *afl*D, *afl*G, *afl*P, *afl*R, and *afl*S, were detected by reverse transcription-polymerase chain reaction (RT-PCR) analyses in soil. However, not all *A. flavus* and *A. parasitcus* isolates produce aflatoxins (Abbas et al., 2004b).

Fungi are classified as nonaflatoxigenic if they do not produce aflatoxins but produce other toxins. If fungi produce no toxins at all, they are classified as nontoxigenic. Generally, in any environment, the frequency of aflatoxigenic isolates can range from 50% to 80% (Abbas et al., 2004a, 2004b, 2005; Pildain et al., 2004). The relative distribution of aflatoxigenic versus nonaflatoxigenic isolates is modulated by many factors including plant species present, soil composition, cropping history, crop management, and environment conditions, including rain fall and temperature (Abbas et al., 2004b; Horn et al., 1995; Zablotowicz et al., 2007). Each of these factors can reduce the levels of *A. flavus*, for example, noncultivated fields near cultivated land are observed to have very low populations of *A. flavus* (Angle et al., 1982; Horn, 2007). Similarly, the frequency of drought is a factor in populations of fungi, with significant drops in soil populations of *A. flavus* after several years without drought (Horn and Dorner, 1998; Horn, 2007). The conidia remain dormant in soil and only germinate when nutrient sources are present. This is supported by several studies where the *A. flavus* population densities increased with residue managed systems (Abbas et al., 2004b; Griffin et al., 1981; Horn et al., 1995; Zablotowicz et al., 2007).

Based on the observation of natural variability in aflatoxigenic strains, nonaflatoxigenic isolates have been used as biological control agents to control aflatoxin contamination (Abbas et al., 2006, 2008c; Cotty, 1994; Dorner et al., 1999;). If the introduced nonaflatoxigenic isolate can adequately colonize and competitively displace the toxigenic isolates, the level of contamination is reduced (Abbas et al., 2008b and c). Although biological control treatments may alter the levels of toxigenic and nontoxigenic isolates, the exact nature of these interactions is unknown. A summary of mechanisms to exploit various biocontrol strategies in reducing aflatoxin contamination is presented in Table 4. The behavior of *Aspergilli* structures in soil needs to be investigated and evaluated thoroughly, especially in agricultural soils, due to fungal structures serving as the primary inoculum resulting in aflatoxin contamination in agricultural commodities.

Table 4. Biological control approaches to reduce aflatoxin contamination in corn and other crops.

Approach	Crop	Results	Citation
Use of grain colonized by nontoxigenic *A. flavus* strains	Corn	Up to 86% reduction in aflatoxin contamination of maize	Abbas et al., 2006
Use of grain colonized by non-toxigenic *A. flavus* strains	Cotton	EPA registration, production and area-wide deployment of AF36	Cotty et al., 2007
Use of grain coated with non-toxigenic *A. flavus* strains	Peanuts	Competitive exclusion of toxigenic strains and reduction of aflatoxin in peanut both pre- and post-harvest.	Dorner, 2008; Dorner et al., 2003
Use of foliar application of non-toxigenic *A. flavus* strains	Corn	Greater than 90% colonization of maize kernels by non-toxigenic strain when applied as conidia or as a formulated conidial product	Lyn et al., 2009
Yeast	Nuts	Identification of antagonists of aflatoxin production	Campbell et al., 2003

Molecular biology applications to *Aspergillus* ecology and aflatoxin contamination

The dependence of *A. flavus* on crops to maintain soil populations emphasizes the importance of understanding the relationship between stress physiology of the crop and this opportunistic fungus. To achieve a more complete picture of *Aspergillus* spp. ecology, rapid methods of identification are needed so that a broader array of conditions can be investigated. Unfortunately *Aspergillus* species are not suitable for molecular techniques such as fluorescent in situ hybridization (FISH). The molecular ecology of several bacteria and fungi has been advanced by FISH coupled with autoradiography; however, the nucleotide probes are unable to penetrate through the cell walls of *Aspergillus* (Teertsta et al., 2004). Thus, the majority of ecological population studies rely upon enumeration and vegetative compatibility groups to characterize *A. flavus* distributions (Bayman and Cotty, 1993; Horn and Greene, 1995; Horn et al., 1996; Papa, 1986).

These traditional methods are based upon morphological and genetic characteristics and are both time consuming and complex (Klich and Pitt 1998; Klich et al., 1992). Recently, additional molecular techniques have been developed to help identify and classify these fungi (reviewed in Wilkinson and Abbas, 2008). One of the more reliable molecular methods for distinguishing *Aspergilli* is ribosomal DNA (rDNA) comparisons. Comparisons of variations within 16S rRNA gene sequences of bacterial species and 18S rRNA gene sequences for eukaryotes are used extensively for ecological investigations. In particular, the internal transcribed spacer (ITS) regions are useful for identifications of fungal species and strains within a species (Hugenholtz and Pace, 1996; White et al., 1990). PCR amplification and sequencing has also been used for resolving filamentous fungal species, including *A. flavus* (Accinelli et al., 2008a; Brun et al., 1991; Peterson, 2008; Scully and Bidochka, 2006; White et al., 1990), using comparisons based upon the aflatoxin biosynthetic pathway, which is unique to the *Aspergilli*.

The genes involved in the aflatoxin biosynthesis pathway have been identified and sequenced (Bhatnagar et al., 2003; Scheidegger and Payne, 2003; Yu et al., 2004). These genes are highly conserved in multiple *Aspergilli*, most notably *A. parasiticus* and *A. flavus.* PCR based on selected genes from the biosynthetic pathway are slowly being adapted for detection, identification, or examination of ecological aspects of *A. flavus*. Amplification of these specific genes is extremely sensitive and has the potential to be used to detect the presence of *A. flavus* in agricultural commodities and environmental samples, including soil and insects (Accinelli et al., 2008a, 2008b; Criseo et al., 2001; Geisen, 1996; Manonmani, et al., 2005).

Recently completed and ongoing studies using molecular techniques are beginning to greatly increase our knowledge of *A. flavus* population structures in soil. PCR has been employed to identify the presence of *Aspergilli* in the soil by Accinelli et al. (2008a), while RT-PCR (reverse transcription-polymerase chain reaction) has also been employed to distinguish toxigenic and nontoxigenic *A. flavus* (Degola et al., 2006; Scherm et al., 2005), as well as to track the presence of aflatoxin biosynthetic genes (Accinelli et al., 2008a and b). The novel technique of pyrosequencing may prove useful in quantifying the presence of introduced *A. flavus* strains in mixed communities of *A. flavus* (Das et al., 2008). We anticipate future ecological tests to utilize and expand these molecular methods to examine *Aspergilli* populations and biochemical behaviors in soil. In conclusion, *Aspergillus* soil ecology is a critical area that needs to be investigated and evaluated thoroughly due to the fungal structures present serving as the primary cause of aflatoxin contamination of agricultural commodities.

Reducing aflatoxin contamination using fungicides and herbicides

Early investigation in vitro indicates that the fungicide chlobenthiazone is highly effective in inhibiting aflatoxin biosynthesis by cultures of *A. flavus*; however, aflatoxin synthesis by *A. parasiticus* was, in fact, stimulated by this fungicide (Wheeler, 1991). Various surfactants, including some used in pesticide formulations, reduced aflatoxin biosynthesis by >96% (Rodriguez and Mahoney, 1994). Use of natural oils from thyme (Kumar et al., 2008), lemongrass (Bankole and Joda, 2004), and other herbs has also been studied and shown to repress aflatoxin in certain crops in Asia.

In the Mississippi Delta, a 3-year field study has indicated that none of five fungicides (azoxystrobin, pyraclostrobin, propiconazole, tetraconazole, dithiocarbamate) or fungicide mixtures of trifloxystrobin + propiconazole and azoxystrobin + propiconazole, applied to corn at midsilking, were effective in significantly reducing aflatoxin contamination (Gabe Sciumbato, personal communication).

The herbicide glufosinate has been reported as having antifungal activity against certain phytopathogenic fungi in vitro (Uchimiya et al., 1993) and has shown activity in reducing infection of corn kernels in vitro (Tubajika and Damann, 2002). Much of the corn planted

in North America are hybrids resistant to glyphosate (Roundup Ready•), glufosinate (Liberty Link•), or stacked with resistance to both herbicides. Thus, a 4-year study in Mississippi field trials was performed to assess the potential for glufosinate and applications of urea to reduce aflatoxin in corn inoculated with *A. flavus* (Bruns and Abbas, 2006). No significant effect using glufosinate was observed, and foliar application of urea actually increased the level of aflatoxin contamination in one of the 4 years.

Similarly, the incidence of aflatoxin in glyphosate-resistant and conventional corn and cotton hybrids was compared in a separate 4-year study (Reddy et al., 2007). Higher levels of aflatoxin were observed in glyphosate-resistant corn compared wih traditional corn hybrids. Thus, effects of glyphosate on in vitro growth of *A. flavus* in pure culture and on native soil populations were examined, finding that high levels of glyphosate (>5 mM) were required for inhibition. In addition, application of several-fold greater than field rates of application were found to have no effect on *A. flavus* populations. Interestingly, *A. flavus*, when grown on glyphosate water agar media, produced 20% of the aflatoxin produced on water agar without glyphosate. However, despite billions of dollars spent annually in fungicide research, little has been gained in identifying products that are active in controlling aflatoxin contamination. Currently, CIMMYT does not recommend foliar application of fungicide to prevent aflatoxin contamination because of the lack of documented efficacy (CIMMYT, 2008).

Reducing aflatoxin contamination by insect management

A. flavus is not considered to be an aggressive invader of preharvest corn ear tissue. However, developing grain when damaged is easily contaminated by the pathogen (Diener et al., 1987). The association between insect damage and fungal infection of corn ears was first recognized when Riley (1882) reported molds appearing on corn-ear tips soon after being infested with insect larvae. Garman and Jewett (1914) reported that in years with high insect populations, the incidence of moldy ears in field corn increased. Efforts to determine the specific role of insects in the *A. flavus* infection process increased dramatically when aflatoxin was recognized as a health concern, leading to recognition that ear feeding insects (e.g., corn earworm, *Helicoverpa zea*; European corn borer, *Ostrinia nubilalis*; fall armyworm, *Spodoptera frugiperda*; western bean cutworm, *Striacosta albicosta*; and southwestern corn borer, *Diatraea grandiosella*) can increase aflatoxin levels in preharvest corn (Catangui and Berg, 2006; Hesseltine et al., 1976; Lillehoj et al., 1976, 1975, 1980; Wilson et al., 1981).

Because environmental factors can greatly affect aflatoxin production in preharvest corn, it is not surprising to find that data from a number of investigations have failed to show any relationship between insect damage and the incidence of *A. flavus* or aflatoxin (Lillehoj et al., 1980; McMillian et al., 1978, 1980; Widstrom et al., 1975). Also, kernels and ear samples have been found that were essentially free of insect damage, yet contained moderate to high amounts of aflatoxin (Lee et al., 1980; Rambo et al., 1974; Shotwell et al., 1977, Widstrom et al., 1975). The difficulty in establishing this relationship is in part due to *A. flavus* ability to colonize silks, infect kernels, and produce aflatoxins in developing ears under insect-free conditions (Jones et al., 1980), and in part due to unknown factors that result in conflicting information.

Windham et al. (1999) reported that aflatoxin levels were significantly higher in 1995 when commercial hybrids were both infested with southwestern corn borer larvae and inoculated with *A. flavus* spores than when they were inoculated with *A. flavus* spores alone. In 1996, however, aflatoxin contamination was generally lower and was not increased significantly by infesting developing ears with southwestern corn borer larvae. These studies illustrate the need for continued research and more thorough examinations of the problem in areas where corn production is relatively new.

Because the relationship between insect damage to corn ears and aflatoxin is heavily influenced by environmental conditions, success in managing aflatoxin contamination via insect control has been highly variable. Research has demonstrated that insecticides cannot be applied economically to control corn insects well enough to reduce aflatoxin to acceptable levels (reviewed in Dowd, 1998). The most successful approach has been the use of corn resistant to ear-feeding insects. Several authors have shown that *Bacillus thuringiensis* (*Bt*)–transformed corn hybrids, which are resistant to ear-feeding insects, reduce aflatoxin contamination of the grain (Dowd, 2000; Munkvold et al., 1999; Williams et al., 2002). The adoption of *Bt* corn hybrids has given producers a crop with increased insect resistance, however these hybrids may only reduce aflatoxin contamination under certain circumstances. However, commercial production of these genetically modified hybrids is not allowed in some nations. Developing lines with greater natural insect resistance will be another and perhaps more acceptable and cost-effective method of reducing losses to insects and aflatoxin. Several sources of natural resistance to insects have been identified, and crosses between insect- and aflatoxin-resistant lines have shown potential to increase resistance to both

insect damage and aflatoxin contamination (Williams et al., 2002).

Limiting mycotoxins through crop management

Control of mycotoxins in corn, especially aflatoxin, by genetic manipulation of the host plant has resulted in limited success. The primary means of reducing the incidence of contamination remains avoidance of conditions favorable for infection. Drought and heat stress during the time that corn matures are the principle contributing factors to aflatoxin contamination. Such conditions are more common in the southeastern United States, resulting in aflatoxin contamination of corn being more of a problem there than in the rest of the nation (Bruns, 2003). Similarly, in less developed countries where infrastructure and financial resources are limited, mycotoxin contamination of corn grain is also a considerable problem.

High temperatures are likely the most important environmental factor influencing preharvest infection of corn by *A. flavus* and the eventual contamination of grain by aflatoxin (Fortnum, 1986; Jones et al., 1980). As temperatures increase aflatoxin levels also increase (Manwiller and Fortnum, 1979; Williams et al., 2003). Naturally, nothing can be done to control ambient temperatures, but it is possible to avoid their full impact during the later stages of kernel filling by early planting. April plantings of corn in North Carolina had lower levels of aflatoxin B1 contamination than grain produced by May plantings (Jones and Duncan, 1981; Jones et al., 1981). However, Bruns and Abbas (2006) reported that regardless of planting date or maturity rating, corn that experienced 41 days of ambient temperatures >32° C during kernel filling had greater levels of aflatoxin contamination than subsequent years that had 30 days of ambient temperatures >32° C. In the southeast United States, high heat is generally accompanied by low rainfall or drought.

Drought stress can occur in corn rather quickly, especially during reproductive growth, if exposed to a combination of high ambient temperatures and low relative humidity. In early corn hybrids, yield reductions of more than 50% could result when brief periods of wilting occurred at 50% silking (Denmead and Shaw, 1960; Robins and Domingo, 1953). Corn hybrids produced in the 1980s were more tolerant to drought than those from earlier times (Duvick, 1984) and do not appear to suffer the adverse affects of drought as soon as older hybrids.

When corn can be irrigated, drought stress may be lowered and aflatoxin levels appear to be reduced. Jones et al. (1981) reported a reduction in *A. flavus* infection and aflatoxin contamination in irrigated versus nonirrigated corn and observed these differences in years of below-normal rainfall. However, Bruns (2003) did not observe a difference in aflatoxin or fumonisin (produced by *Fusarium verticillioides*) contamination levels between irrigated and nonirrigated corn. With both mycotoxins being observed to have extremely low levels for all years of the experiment and in both irrigation treatments, it is possible that other complicating environmental factors, such as plant nutrition, were more influential.

Adequate plant nutrition, especially nitrogen, is necessary to prevent aflatoxin contamination of preharvest corn grain. Insufficient levels of mineralized nitrogen in the root zone, due to either drought stress or leaching due to excessive rain, may predispose corn to aflatoxin contamination (Jones, 1979). Anderson et al. (1975) reported that higher levels of aflatoxin contamination occurred in corn grain produced with 80 kg ha^{-1} of nitrogen compared with grain produced using 120 kg ha^{-1}. Bruns and Abbas (2005a), however, observed no differences among irrigated early-season corn hybrids grown in the mid-South with respect to aflatoxin or fumonisin contamination when nitrogen fertilizer levels varied between 112 kg ha^{-1} and 224 kg ha^{-1}. Subsequent research at the same location using a full-season hybrid and varying plant populations and nitrogen fertility levels produced similar results (Bruns and Abbas, 2005b).

Conclusions

It is clear that traditional control approaches, such as fungicides, have shown minimal success in managing aflatoxin. Fungal biocontrol, insect control, and novel germplasm appear to hold the most promise in mitigating *Aspergillus* infection and aflatoxin contamination. No single technology will be the answer, but these technologies combined with more effective crop management will contribute to reducing this important limitation to economical corn production. An increased understanding of the relationships between *A. flavus*, the host, the environment, and aflatoxin is critical to advancing these management strategies and practices. Although our knowledge is still incomplete in understanding this fungus, advancements in understanding biosynthesis and regulation, *Aspergilli* life cycle, and environmental effects will continue to increase as additional molecular techniques are developed and specifically applied to *Aspergillus* ecology. The integration of these approaches in a cost-effective

manner may be the greatest challenge we will face in providing safer food and feed production in the 21st century.

Acknowledgments

The authors are grateful to Ms. Lynn Libous-Bailey and Ms. Bobbie J. Johnson, USDA-ARS, Stoneville, Mississippi, for their technical assistance in this project. This work was approved for publication as Journal Article No. J-11489 of the Mississippi Agricultural and Forestry Experiment Station, Mississippi State University.

***Declaration of interest*:** The authors report no conflicts of interest.

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