

***Aspergillus flavus* aflatoxin occurrence and expression of aflatoxin biosynthesis genes in soil**

Cesare Accinelli, H.K. Abbas, R.M. Zablotowicz, and J.R. Wilkinson

Abstract: The carcinogen aflatoxin B1 (AFB1) produced by *Aspergillus flavus* is a major food safety concern in crops. However, information on AFB1 occurrence in soil and crop residue is scarce. A series of experiments investigated the occurrence of AFB1 in soil and corn residues and ascertained the ecology of *A. flavus* in a Dundee silt loam soil. Samples of untilled soil (0–2 cm) and residues were collected in March 2007 from plots previously planted with a corn isolate containing the *Bacillus thuringiensis* (Bt) endotoxin gene or the parental non-Bt isolate. AFB1 levels were significantly different in various corn residues. The highest AFB1 levels were observed in cobs containing grain, with 145 and 275 ng·g⁻¹ in Bt and non-Bt, respectively ($P \geq F = 0.001$). Aflatoxin levels averaged 3.3 and 9.6 ng·g⁻¹ in leaves and (or) stalks and cobs without grain, respectively. All soils had AFB1 ranging from 0.6 to 5.5 ng·g⁻¹ with similar levels in plots from Bt and non-Bt corn. Based on cultural methods, soil contained from log₁₀ 3.1 to 4.5 *A. flavus* cfu·g⁻¹ with about 60% of isolates producing aflatoxin. Laboratory experiments demonstrated that AFB1 is rapidly degraded in soil at 28 °C (half-life ≤ 5 days). The potential of the soil *A. flavus* to produce aflatoxins was confirmed by molecular methods. Transcription of 5 aflatoxin biosynthesis genes, including *aflD*, *aflG*, *aflP*, *aflR*, and *aflS*, were detected by reverse transcription – polymerase chain reaction analysis in soil. Although AFB1 appears to be transient in soils, it is clear that AFB1 is produced in surface soil in the presence of corn residues, as indicated by *A. flavus* cfu levels, AFB1 detection, and expression of aflatoxin biosynthetic genes.

Key words: aflatoxins, aflatoxin degradation, *Aspergillus flavus*, gene expression, PCR, soil ecology.

Résumé : L'aflatoxine B1 (AFB1), un cancérogène produit par *Aspergillus flavus*, constitue une préoccupation majeure pour la sécurité alimentaire des récoltes. Cependant, l'information qui concerne l'occurrence de l'AFB1 dans le sol et les résidus agricoles est insuffisante. Une série d'expériences a été réalisée pour déterminer l'occurrence de l'AFB1 dans le sol et des résidus agricoles, et pour étudier l'écologie de *A. flavus* dans le sol limoneux fin Dundee. Des échantillons de sol non travaillé (0–2 cm) et des résidus ont été récoltés en mars 2007 à partir de lots précédemment cultivés avec une lignée isogène de maïs contenant le gène de l'endotoxine de *Bacillus thuringiensis* (Bt) ou avec la lignée parentale isogène sans Bt. Les niveaux d'aflatoxine B1 étaient significativement différents d'un type de résidu de maïs à l'autre. Les niveaux d'AFB1 les plus élevés étaient observés dans les épis comportant des grains, à 145 et 275 ng·g⁻¹, chez les échantillons avec Bt et sans Bt, respectivement ($P \geq F = 0,001$). Les niveaux d'aflatoxine moyens des tiges et (ou) feuilles et des épis sans grains se situaient à 3,3 et 9,6 ng·g⁻¹, respectivement. Tous les sols contenaient de 0,6 à 5,5 ng·g⁻¹ d'AFB1, les niveaux étant similaires entre les parcelles cultivées avec du maïs avec ou sans Bt. En se fondant sur les méthodes de culture, le sol contenait de 3,1 à 4,5 (log₁₀) ufc·g⁻¹ de *A. flavus* dont 60 % des isolats produisaient de l'aflatoxine. Des expériences en laboratoire ont démontré que l'AFB1 est rapidement dégradée dans le sol à 28 °C (demi-vie ≤ 5 jours). Le potentiel de production d'aflatoxines de *A. flavus* présent dans le sol a été confirmé par des méthodes moléculaires. La transcription de 5 gènes de biosynthèse des aflatoxines dont *aflD*, *aflG*, *aflP*, *aflR* et *aflS* a été détectée par une analyse RT-PCR du sol. Malgré le fait que AFB1 semble transitoire dans les sols, il est clair que l'AFB1 est produite à la surface du sol en présence de résidus de maïs, tel qu'indiqué par le nombre d'ufc de *A. flavus*, la détection d'AFB1 et l'expression des gènes de biosynthèse d'aflatoxine.

Mots-clés : aflatoxines, dégradation d'aflatoxine, *Aspergillus flavus*, expression génique, PCR, écologie du sol.

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C. Accinelli.¹ Department of Agro-Environmental Science and Technology, University of Bologna, 40127 Bologna, Italy.

H.K. Abbas. United States Department of Agriculture – Agriculture Research Service (USDA–ARS), Crop Genetics and Production Research Unit, Stoneville, MS 38776, USA.

R.M. Zablotowicz. USDA–ARS Southern Weed Science Research Unit, Stoneville, MS 38776, USA.

J.R. Wilkinson. Department of Biochemistry and Molecular Biology, Mississippi State University, Starkville, MS 39762, USA.

¹Corresponding author (e-mail: cesare.accinelli@unibo.it).

Introduction

Aflatoxins are carcinogenic secondary metabolites produced by several species of *Aspergillus* section *Flavi*, including *Aspergillus flavus* Link, *Aspergillus parasiticus* Speare, and *Aspergillus nominus* Kutzman, Horn and Hesseltime. Concerns for human and livestock health have led several countries to constantly monitor and regulate aflatoxin contamination of agricultural commodities (Wang and Tang 2005). Since the discovery of aflatoxins in the early 1960s (CAST 2003), many studies have been conducted to assess the occurrence and to describe the ecology of aflatoxin-producing fungi in natural and agricultural environments. *Aspergillus flavus* is the most abundant aflatoxin-producing species associated with corn (Wicklow et al. 1998; Abbas et al. 2004a, 2004b). Although *A. flavus* is readily isolated from diverse environmental samples, soil and plant tissues or residues are considered the natural habitat of this fungus (Orum et al. 1997; Geiser et al. 2000; Horn 2003; Jaime-Garcia and Cotty 2004). Soil serves as a reservoir for primary inoculum for the infection of susceptible crops. Information concerning the soil ecology of *A. flavus* is consequently considered a prerequisite for developing effective measures to prevent and to control aflatoxin contamination of crops (Horn and Dorner 1998; Abbas et al. 2006; Zabolowicz et al. 2007). Soil and crop management practices and a number of environmental factors can influence the population size and the spatial distribution of *A. flavus* in cultivated soils (Cole et al. 1982; Orum et al. 1997; Abbas et al. 2004b). The population size of *A. flavus* has been correlated with soil organic matter and nutritional status, with the most fertile soils containing the greatest concentration of aspergilli (Zabolowicz et al. 2007). Subsequently, as more soils are managed under no tillage systems, a higher inoculum of this fungus may result, which could contribute to increased preharvest aflatoxin contamination of susceptible crops.

The distribution of aflatoxin in agricultural commodities has been fairly well characterized because of its importance to the food supply. However, little is known on the occurrence and fate of aflatoxin in soil. Radiological assays conducted to assess the fate of aflatoxin B1 (AFB1) in soil indicated that a low level of mineralization of AFB1 to CO₂ was observed, with less than 1%–8% mineralized in 120 days (Angle 1986). Not surprisingly, several microorganisms have the potential to degrade aflatoxins (Ciegler et al. 1966), especially bacteria, e.g., *Flavobacterium* (Line and Brackett 1995) and *Mycobacterium* (Hormisch et al. 2004). In addition, *A. flavus* also is capable of degrading aflatoxins during later stages of mycelial growth in pure culture (Huynh and Lloyd 1984).

In recent years, molecular techniques have increased the possibilities to characterize soil microbial ecology. While molecular methods have been extensively used for studying soil bacteria, these techniques have been applied to studying soil fungi, such as the biological control agents *Colletotrichum coccodes* (Dauch et al. 2003), *Trichoderma* (Weaver et al. 2005), and mycorrhizal fungi (Ma et al. 2005). Amplification of specific DNA fragments using polymerase chain reaction (PCR) and specific gene probes is extremely sensitive and has the potential to detect the presence of *A. flavus*

in agricultural commodities (Geisen 1996; Criseo et al. 2001; Manonmani et al. 2005). Since all of the genes involved in the aflatoxin biosynthesis pathway have been identified and cloned (Bhatnagar et al. 2003; Yu et al. 2004a, 2004b) and the entire genome of *A. flavus* sequenced (Payne et al. 2006), molecular methods for the detection of *Aspergillus* should be fairly readily adapted by using biosynthetic pathway genes as probes, as evidenced by the recent work differentiating toxigenic and atoxigenic *A. flavus*-utilizing aflatoxin gene expression using the reverse transcription – polymerase chain reaction (RT-PCR) (Scherer et al. 2005; Degola et al. 2007).

Application of these molecular techniques to *A. flavus* soil ecology should greatly enhance our understanding of this fungus. *Aspergillus flavus* is commonly considered a saprophytic fungus; however, its ability to colonize growing crops and inflict economic damage clearly shows that it can and does function as an opportunistic pathogen. Despite the elucidation of many aspects influencing *A. flavus* ability to colonize crops and accumulate aflatoxins, its activity and potential to produce aflatoxins in soil and in crop residues has remained unexplored. The main objectives of this experiment were to determine the occurrence of *A. flavus* by using cultural and molecular methods and to evaluate the expression of aflatoxin genes in surface field soil 5 months after corn harvest. The occurrence of naturally occurring AFB1 in field soil and its degradation were also investigated under in vitro conditions.

Materials and methods

Soil and corn residues

Soil and corn residues were collected on 5 March 2007 from an experimental corn field located in the Mississippi Delta (Stoneville, Mississippi, 33°25'19.3"N, 90°53'54.8"W). The field experiment was designed to assess aflatoxin contamination in the corn hybrid 34B23 expressing the *Bacillus thuringiensis* gene (Bt) and in its parental non-Bt isolate 34B24 (Pioneer Hi-Bred Inc., Johnson, Iowa). The experiment was conducted in a randomized complete block design with 5 replicates. Plots (25 m × 31 m) were planted on 13 May 2006. Plots received supplemental furrow irrigation throughout the growing season to mitigate drought stress. Herbicides and fertilizer (application based on soil tests) were applied according to the standard cultural practices in corn farming for a continual production system in northern Mississippi. Plots were separated by a 31 m buffer zone planted with soybean (*Glycine max* L. Merr.).

A composite sample consisted of ten 25 g subsamples of soil from each plot collected from the upper 2.5 cm using a sterilized spatula and was stored at 4 °C until it was processed. The soil from each plot was homogenized by passing through a 4 mm sieve, and moisture was determined gravimetrically. Corn residues were collected from 5 locations in each plot and combined before drying for 72 h at 50 °C. The dried residues were ground at 20 mesh using a Romer mill (Union, Missouri).

Chemical and physical analyses were conducted on air-dried soil that was sieved (2 mm) and ground in a Romer mill. Soil textural analysis was determined using the hydrometer method (Gee and Bauder 1986), which indicated that

Table 1. Properties of a Dundee silt loam soil collected from plots planted with corn expressing the *Bacillus thuringiensis* (Bt) endotoxin gene or with corn lacking the Bt (non-Bt) endotoxin gene.

Property	Bt corn plots	Non-Bt corn plots
Sand (g·kg ⁻¹)	316±37	310±30
Silt (g·kg ⁻¹)	596±30	607±28
Clay (g·kg ⁻¹)	81±19	83±9
Electrical conductivity (mS·m ⁻¹)	82±12	76±10
pH	5.8±0.4	5.8±0.6
Total carbon (g·kg ⁻¹)	5.5±1.0	4.7±2.6
Total nitrogen (g·kg ⁻¹)	0.68±0.09	0.66±0.08

Note: Data are the mean ± standard deviation of 5 replicates.

a similar particle size distribution was present in all plots (Table 1). Electrical conductivity (EC) and pH were determined in an aqueous soil suspension (2:1 v/m). Total carbon and nitrogen content determined using a Flash EA 1112 elemental analyzer (C.E. Elantec, Lakewood, New Jersey) also showed similar organic matter and total nitrogen content in soil from Bt and non-Bt plots.

Enumeration of *A. flavus* in soil

The size of the *A. flavus* population in soil and the relative abundance of atoxigenic isolates were estimated using the method described in Abbas et al. (2004b). Briefly, soil samples (0.5 or 2.5 g) were suspended in a 10 mL agar solution (2 g·L⁻¹), vortexed twice for 1 min, and duplicate samples were plated onto modified dichloronitroaniline rose Bengal agar with 3% NaCl. Plates were incubated at 37 °C for 5 days and propagules enumerated. Colonies of *A. flavus* were randomly picked (40 colonies per plot), and transferred to β-cyclodextrin (0.3%) potato dextrose agar, and incubated at 28 °C for 5 days in the dark. Aflatoxin-producing isolates were identified following exposure to UV light at an absorbance of 365 nm. Colonies that developed the characteristic blue fluorescence were counted as toxigenic aspergilli. Aflatoxin production was further confirmed by observing the color change of isolates exposed to aqueous ammonium hydroxide (27% v/v) for 30 min. The density of *A. flavus* colony-forming units (cfu) was calculated on a soil dry mass basis and transformed on a log₁₀ scale. The detection limit of *A. flavus* was log₁₀ 1.9 cfu·g⁻¹ soil.

PCR analysis

Total DNA was isolated from soil using the commercial kit PowerSoil (MoBio Laboratories Inc., Solana Beach, California), following the instructions of the manufacturer. For each plot, duplicate samples of DNA extracts were pooled and purified with the Wizard DNA Clean-Up System (Promega, Madison, Wisconsin, USA). Eluted DNA was used for PCR analysis to detect 5 genes involved in the aflatoxin biosynthesis pathway. The primers used to amplify the 3 structural genes (*aflD*, *aflG*, *aflP*) and the 2 regulatory genes (*aflR*, *aflS*) are described in Scherm et al. (2005). Primer sequences and length of amplified products are reported in Table 2.

The PCR reaction mixture contained 25 µL of RedTaq ReadyMix (Sigma Chemicals, St. Louis, Missouri),

0.5 µmol·L⁻¹ (each) primer (Operon Biotechnologies, Inc., Huntsville, Alabama), 5–10 ng of template DNA, and water to a final volume of 50 µL. The cycling was performed with the PTC-100 programmable thermal cycler (MJ Research, Watertown, Massachusetts, USA) as follows: 1 cycle at 94 °C for 4 min; followed by 30 thermal cycles at 94 °C for 30 s, 56 °C for 30 s, 68 °C for 60 s; and a final elongation step at 72 °C for 15 min. PCR products were separated on a 1% agarose gel and visualized after staining with SYBR Green I (Sigma-Aldrich Corp., St. Louis, Missouri).

Expression of aflatoxin genes by RT-PCR

For each plot, total soil RNA was isolated from duplicate 2 g samples using the RNA PowerSoil Total RNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, California), according to the manufacturer's instructions. RNA extracts from each plot were pooled, and DNA was removed by incubating subsamples (4 µL) at 37 °C for 30 min in a solution containing 5 units of RQ1 RNase-free DNase, 1 µL of 10× reaction buffer, and nuclease-free water to a final volume of 10 µL. The reaction was terminated by adding 1 µL of stop solution. Following DNase digestion, RNA was reverse transcribed using the ImProm-II Reverse Transcription System. All the reagents for RT-PCR were purchased from Promega (Madison, Wisconsin). Approximately 1 µg of RNA was incubated at 70 °C for 5 min with 0.5 µg of Oligo(dT)15 primer in a total volume of 20 µL. After cooling in ice, 5 µL of the primer/template mix was added to 15 µL of a reaction mixture containing 4 mmol·L⁻¹ MgCl₂, 0.5 mmol·L⁻¹ (each) dNTP, 4 µL of the 5× reaction buffer, 20 units of recombinant RNasin ribonuclease inhibitor, and nuclease-free water. Reactions were performed by incubating samples at 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min. Aliquots of cDNA (approximately 5–10 ng) were amplified using the same 5 primer couples and the conditions described above (Table 2). RT-PCR products were separated by agarose gel electrophoresis as described above. RT-PCR efficiency was tested by including amplification of the housekeeping gene *tub1*. In addition, RNA isolated from 2 aflatoxigenic strains NRRL 3357 (ATCC 200026; SRRC167) and F3W4 (NRRL 30796), and 2 non-aflatoxigenic strains CT3 (NRRL 30798) and K49 (NRRL 30797) (Abbas et al. 2006) were used as additional controls for RT-PCR efficiency using the same 5 described primers and the ITS1/ITS4 primers amplifying the internal transcribed spacer (ITS) region incorporating the 5.8S rRNA (White et al. 1990). These primers were also used for sequencing analysis. After clean up with ExoSAP-IT (USB Co., Cleveland, Ohio), PCR products from the nonaflatoxigenic strains were sequenced using the ABI Dye Terminator Cycle Sequencing Ready Reaction Kit and analyzed using an ABI 310 automated sequencer (The Perkin-Elmer Co., Foster City, California). Sequences were aligned using the software DNAMAN (Lynnon Co., Quebec, Canada) and deposited in the NCBI GenBank with the following accession Nos.: EF565459, EF565460, EF565461, EF565462, EF565463, EF565464, EF565465, EF565466, and EF565467.

Occurrence and degradation of AFB1 in soil

The concentration of naturally occurring AFB1 in soil for

Table 2. Primer sequences, GenBank accession numbers, and expected length of products.

Gene		Primer sequence ^a	Acc. Nos. for strains CT3 and K49 ^b	PCR product size (bp)	RT-PCR product size (bp)
<i>aflD</i>	Forward	ACGGATCACTTAGCCAGCAC	EF565459	990	812
<i>aflD</i>	Reverse	CTACCAGGGGAGTTGAGATCC	EF565463		
<i>aflG</i>	Forward	ATGCATCTTCCGCCCTTC	EF565461	1163	1163
<i>aflG</i>	Reverse	ATAGCGAGGTTCCAGCGTAA	EF565465		
<i>aflP</i>	Forward	GCCTTGCAAACACACTTTCA	EF565460	1490	1210
<i>aflP</i>	Reverse	AGTTGTTGAACGCCCCAGT	EF565464		
<i>aflR</i>	Forward	CGAGTTGTGCCAGTTCAAAA	EF565462	999	999
<i>aflR</i>	Reverse	AATCCTCGCCACCATACTA	EF565466		
<i>aflS</i>	Forward	GAGTCCCTGAGTGTCGGCTA	NA	1450	1004
<i>aflS</i>	Reverse	TCGGTTGTCATCGTTATCCA	EF565467		
ITS1		TCCGTAGGTGAACCTGCGG			
ITS4		TCCTCCGCTTATTGATATGC			
<i>tub1</i>	Forward	GCTTTCTGGCAAACCATCTC		1498	1198
<i>tub1</i>	Reverse	GGTCGTTTCATGTTGCTCTCA			

^aPrimers ITS1 and ITS4 are described in White et al. (1990), all others primers are described in Scherm et al. (2005).

^bAccession numbers for *Aspergillus flavus* CT3 are in bold, all other accession numbers are for *A. flavus* K49. NA, not available.

the March sample collected from the 10 plots was determined by high performance liquid chromatography (HPLC). For each plot, triplicate samples of 10 g soil were extracted with 20 mL of water – ethyl acetate (1:3). After shaking overnight, samples were centrifuged at 5000g for 10 min, and a 5 mL aliquot of the supernatant was evaporated under N₂, followed by reconstitution of the residue in water-methanol (3:7). Sample cleanup was performed using a modification of Sobolev and Dorner (2002). Briefly, an aliquot (800 µL) of reconstituted sample was cleaned using a 1.5 mL extract-clean reservoir minicolumn packed with aluminum oxide (Alltech Co., Deerfield, Illinois). After elution by gravity, 20 µL of the eluate was injected on a HPLC system equipped with a Nova-Pak C18 column (150 × 3.9 mm, 4 µm) and a 474 model fluorescence detector (Waters Corporation, Milford, Massachusetts). Separation was carried out at 30 °C, with a mobile phase consisting of water – methanol – 1-butanol (60:25:1, by volume) and a flow rate of 0.9 mL·min⁻¹. Detection of AFB1 was achieved by setting the detector wavelength at 365 nm (excitation) and 440 nm (emission).

Degradation of AFB1 in soil was investigated under laboratory conditions using soil from plot 10. AFB1 was applied as concentrated methanol solutions to 5 g portions of soil. After the solvent was evaporated, untreated soil was thoroughly mixed with the aflatoxin-treated soil to obtain the final concentration of 10 ng·(g soil)⁻¹. Soil samples (10 g) were weighed into sterile 50 mL centrifuge tubes, the soil moisture was adjusted to the gravimetric content at –33 kPa using distilled water, and samples were incubated in the dark at 25 °C. Aflatoxin degradation was monitored by removing triplicate tubes for analysis at 0, 3, 6, 13, and 17 days after treatment. Collected samples were stored at –20 °C until analyzed. Soil samples were extracted and analyzed following the procedure described above.

Statistical analysis

Soil data on AFB1 concentration, *A. flavus* cfu, and aflatoxin phenotype were subjected to analysis of variance using

PROC GLM (SAS Institute Inc., version 2001, Cary, North Carolina). Treatment means were separated at the 5% level of significance using Fisher's protected least significant difference test. Aflatoxin concentration data in corn residues were subjected to analysis of variance using PROC MIXED (SAS Institute Inc.) to assess the effects of corn isolate (Bt or non-Bt), type of residue (leaves and stalks, versus cobs or cobs with grain), and the interaction of these variables. Pearson correlations among soil properties, *A. flavus* cfu, and aflatoxin genes detected by RT-PCR were conducted using PROC CORR (SAS Institute Inc.).

Results and discussion

The size of the soil population of *A. flavus* based on cultural methods in the 10 plots is presented in Table 3. *Aspergilli* propagules ranged from log₁₀ 3.1 to 4.5 cfu·(g soil)⁻¹. Soil density of *A. flavus* was not significantly different ($P \geq F = 0.53$) in plots previously planted with the Bt corn hybrid and the corresponding non-Bt isolate. Approximately 60% of the isolates had the potential to produce aflatoxins, with no differences observed in Bt and in non-Bt plots ($P \geq 0.114$). Although the occurrence of aflatoxigenic strains in agricultural soil has been widely reported in the literature (Abbas et al. 2004a), no information on aflatoxin contamination of soil is available. Aliquots of soil samples used for *aspergilli* enumeration were consequently analyzed for AFB1 concentration. All the samples from the 10 plots had detectable AFB1 (Table 3). Concentration values were highly variable, ranging from 0.6 to 5.5 ng·(g soil)⁻¹, with slightly higher AFB1 in soil from Bt plots ($P \geq 0.079$). Evaluations of the fungus *Penicillium verrucosum* (Mortensen et al. 2006) have indicated that the occurrence of ochratoxins in agricultural soil may be due to the release of mycotoxins from fungi-infested plant residues. In the present experiment, surface soil samples were collected from no-till plots beneath the remaining corn residues, with coarse plant residue material removed by sieving. There was no correlation observed between any soil property measured and *A. flavus* cfu, toxigenic isolates, or recovery of AFB1;

Table 3. *Aspergillus flavus* propagules, frequency of aflatoxigenic isolates, and aflatoxin B1 (AFB1) concentration in soil collected from plots planted with corn expressing the *Bacillus thuringiensis* (Bt) endotoxin gene or with corn lacking the Bt (non-Bt) endotoxin gene.

Soil plot	No. of <i>Aspergillus flavus</i> propagules (log cfu·(g soil) ⁻¹)	Aflatoxigenic isolates (%)	Concn. of AFB1 (ng·g ⁻¹)
Bt corn plots			
2	4.1	83	1.1
3	4.5	68	3.7
5	3.5	83	0.9
7	3.5	65	5.5
10	4.3	35	4.7
Mean ± SD*	3.9±0.5	75±8	3.1±2.1
Non-Bt corn plots			
1	4.3	35	0.6
4	3.1	55	2.2
6	4.1	54	1.1
8	3.1	53	2.2
9	3.8	83	1.0
Mean ± SD*	3.7±0.5	55±17	1.4±0.7

*Mean ± standard deviation of 5 replicates.

Table 4. Concentration of aflatoxin B1 (AFB1) in different components of corn debris collected from the soil surface in plots planted with corn expressing the *Bacillus thuringiensis* (Bt) endotoxin gene or with corn lacking the Bt (non-Bt) endotoxin gene.

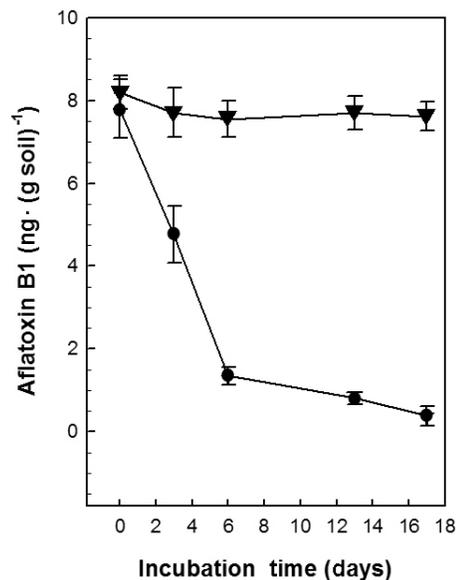
Soil plot	AFB1 concn. (ng·g ⁻¹)		
	Leaves and stalks	Cobs	Cobs with grain
Bt corn plots			
2	0.0	7.4	115.7
3	3.8	7.9	48.9
5	6.2	0.0	210.8
7	7.7	11.6	72.5
10	0.9	0.0	276.3
Mean ± SD*	3.8±3.2	5.4±5.1	144.8±9.6
Non-Bt corn plots			
1	1.5	2.7	102.1
4	2.4	7.2	152.9
6	2.8	3.9	348.8
8	0.0	34.4	240.7
9	7.3	20.3	529.7
Mean ± SD*	2.8±2.7	13.7±13.5	274.7±170.6

*Mean ± standard deviation of 5 replicates.

however, the highest level of AFB1 was found in soils with the highest proportion of aflatoxigenic isolates.

Chemical analysis of corn residues showed that AFB1 concentration was significantly different in leaves and (or) stalks, cobs, and cobs with grain. The highest concentration of AFB1 was observed in cobs with grain, with 145 and 275 ng·g⁻¹ in Bt and non-Bt corn residues ($P \geq F = 0.001$) (Table 4), respectively. AFB1 levels averaged 3.3 and 9.6 ng·g⁻¹ in leaves and (or) stalks and cobs, respectively, with no difference between residues from the 2 different corn hybrids. These findings are consistent with the assump-

Fig. 1. Concentrations of aflatoxin B1 recovered from native soil (●) and autoclaved soil (▼) during a 17 day incubation. Data are the mean and standard deviations of 3 replicates.



tion that corn debris is likely responsible for AFB1 contamination of soil.

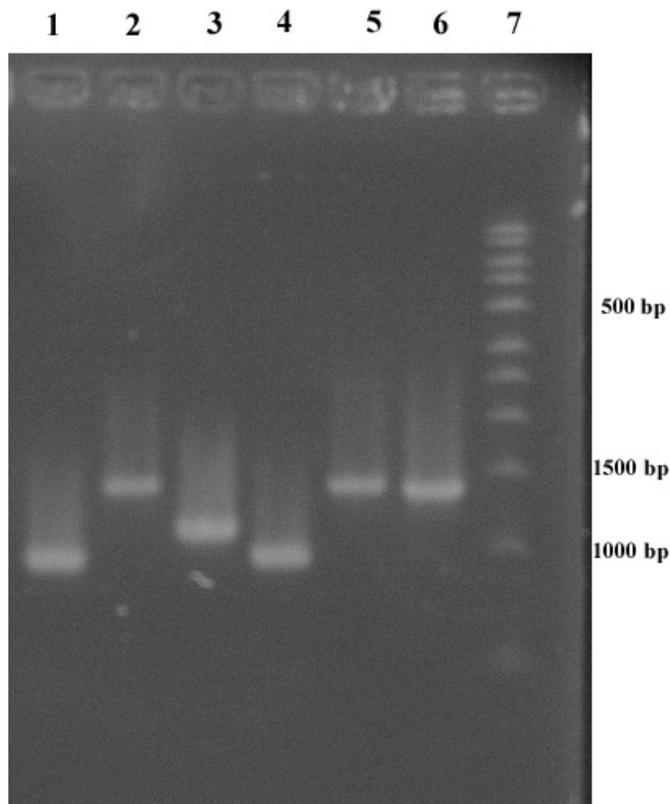
Results from the AFB1 degradation experiment showed that AFB1 rapidly dissipated in soil. More precisely, degradation of AFB1 in soil was correctly described by the first-order kinetic model ($r^2 = 0.91$), with an estimated half-life of 4.1 days (Fig. 1). Since AFB1 persisted in autoclaved soil, it is evident that AFB1 degradation in soil was mainly driven by microbial processes. The potential for aflatoxin degradation by soil microorganisms, especially bacteria, has been demonstrated by many researchers (Ciegler et al. 1966; Line and Brackett 1995; Hormisch et al. 2004). This current

Table 5. Detection (PCR) and expression (RT-PCR) of aflatoxin genes in soil collected from plots planted with corn expressing the *Bacillus thuringiensis* (Bt) endotoxin gene or with corn lacking the Bt (non-Bt) endotoxin gene.

Soil plot	PCR/RT-PCR*				
	<i>aflD</i>	<i>aflG</i>	<i>aflP</i>	<i>aflR</i>	<i>aflS</i>
Bt-corn plots					
2	+/-	+/-	+/+	+/-	+/-
3	+/+	+/-	+/+	+/+	+/+
5	+/+	+/-	+/+	+/-	+/+
7	+/+	+/+	+/-	+/-	+/-
10	+/+	+/+	+/+	+/+	+/+
Non-Bt-corn plots					
1	+/+	+/-	+/-	+/-	+/+
4	+/+	+/+	+/+	+/-	+/-
6	+/-	+/-	+/+	+/+	+/+
8	+/+	+/+	+/-	+/-	+/-
9	+/+	+/-	+/-	+/-	+/-

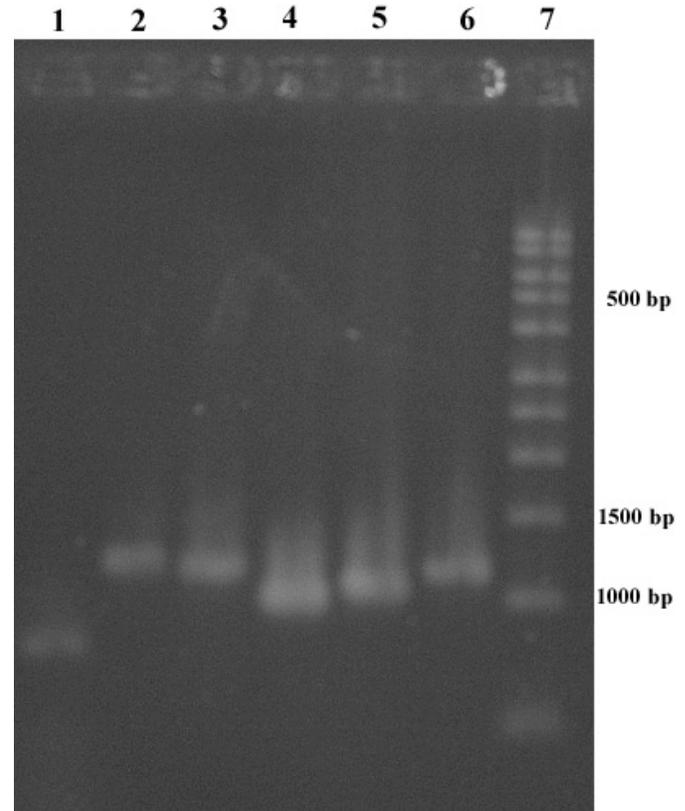
*+, signal present; -, signal absent.

Fig. 2. Amplification profiles of aflatoxin genes and the housekeeping gene *tub1* in soil of plot 10. Lanes: 1, *aflD*; 2, *aflP*; 3, *aflG*; 4, *aflR*; 5, *aflS*; 6, *tub1*; and 7, 1 kb DNA ladder.



study is one of the first to demonstrate the rapid degradation of AFB1 in soil, as compared with previous work (Angle 1986) using ^{14}C -radiolabelled AFB1, which showed that there is minimal mineralization of this important mycotoxin in soil. Even considering that our study was conducted in vitro under favorable conditions (i.e., temperature and soil

Fig. 3. Expression profiles of aflatoxin genes and the housekeeping gene *tub1* in soil of plot 10. Lanes: 1, *aflD*; 2, *aflP*; 3, *aflG*; 4, *aflR*; 5, *aflS*; 6, *tub1*; and 7, 1 kb DNA ladder.



water content) for microbial growth and AFB1 degradation, the detection of AFB1 in soil approximately 5 months after corn harvest suggests that in the warm conditions of the Mississippi Delta, corn debris gradually releases AFB1 into the soil, prolonging the time span of aflatoxin contamination.

The wide distribution of *A. flavus* in soil of the 10 plots was confirmed by PCR analysis. Using selected primer pairs to amplify 5 genes of the aflatoxin biosynthetic pathway, all 10 soil samples yielded DNA fragments of the appropriate size for these genes (Table 5 and Fig. 2). Likewise, in soil samples collected in February, *aflD*, *aflP*, *aflR*, and *aflS* were amplified in all soils tested. Three of the selected genes, *aflD*, *aflG*, *aflP*, are structural genes, the 2 remaining genes, *aflR* and *aflS*, are regulatory genes of the AFB1 biosynthetic pathway (Yu et al. 2004b). PCR-based methods have been largely used in food science for the detection of *A. flavus* in cereals and other agricultural commodities (Shapira et al. 1996; Somashekar et al. 2004). This study demonstrates that a rapid and cost-effective PCR method can be used as an alternative to cultivation-based methodologies for detecting aflatoxin-producing fungi in soil. Some authors have suggested that soil populations of *A. flavus* have high genetic diversity (Croft 1987; Horn et al. 1996). It is not surprising then, that nonaflatoxin strains of *A. flavus* used or proposed for biological control of mycotoxin contamination of agricultural crops have been isolated from soil (Cotty 1994; Dorner et al. 1999; Abbas et al. 2006).

Although major factors regulating aflatoxin production have been described, the potential of *A. flavus* to produce

aflatoxin in soil and, especially, in a mixture of soil and crop debris remains unexplored prior to our study. Among the different approaches, the level of aflatoxin gene expression determined by RT-PCR has been proposed as an accurate method to discriminate aflatoxigenic and non-aflatoxigenic isolates in agricultural commodities (Scherer et al. 2005). Since RNA molecules do not persist in soil, the RT-PCR amplification of soil RNA is also proposed for studying metabolic activities of soil fungi and other soil microorganisms (Ostle et al. 2003; Anderson and Parkin 2007). Preliminary studies with soil samples inoculated with 4 selected strains of *A. flavus*, which were grown on potato dextrose agar, showed the ability to isolate soil RNA and to transcribe the target sequences (data not shown). The efficiency of the adopted methodology was further confirmed by the successful RT of the housekeeping gene *tub1* coding for β -tubulin (Fig. 3). RT-PCR results of soil RNA extracts are summarized in Table 5. The gene expression profile of the 10 soils did not generally seem to be related to AFB1 concentration in the soil, the exception to this being a complete positive profile observed in soil from plot 10, which also contained the highest AFB1 concentration. Future studies should use real-time PCR to quantitatively estimate the density of individual aflatoxin genes. The gene transcripts for *aflD* and *aflR* were the most and least frequently expressed of evaluated genes, respectively. The *aflD* is a structural gene encoding for an enzyme that is involved in one of the first steps of the aflatoxin biosynthesis pathway; *aflR* is a pathway regulatory gene. Differences in the gene expression profile may likely be due to differences in the composition of aspergilli population and environmental conditions (corn residue content, soil temperature, etc.). In our preliminary experiment with the 2 aflatoxigenic strains (NRRL3357 and F3W4) and the 2 nonaflatoxigenic strains (CT3 and K49), there is a high degree of similarity between sequences generated from ITS-amplified products (percent identity \geq 96.8). This high degree of similarity did not reflect differences in aflatoxin production capability. For instance, the non-aflatoxigenic strain K49 did not express the *aflR* genes when grown on potato dextrose agar. Under the same conditions, the other nonaflatoxigenic strain CT3 expressed all 5 of the tested genes (data not shown). Aflatoxin production is optimum between 28 and 30 °C, with production decreasing as the temperature increases, while 37 °C is optimal for fungal growth (O'Brian et al. 2007). This study has shown that some *afl* genes can be expressed in surface soil at relatively cool temperatures (~12 °C, average daily temperature at the time of soil collection).

Conclusions

This study confirmed results of other studies (Horn and Dorner 1998; Abbas et al. 2004a; Zablutowicz et al. 2007) that *A. flavus* is abundant in agricultural soil of the southern US. Additionally, these studies suggest that corn residues may serve as a significant source of aflatoxin contamination in soil and a source of inoculum to sustain fungal growth during the noncropping season. Expression of some aflatoxin biosynthesis genes (secondary metabolism) in soil suggests that aflatoxin production may have a role in the ecology of this important class of toxin-producing fungi in soil. Use of Bt corn has been suggested as an important

tool in managing aflatoxin contamination in corn by minimizing insect damage to the corn grain (Dowd 2003). Critically, corn residues from the Bt-protected isolate exhibited lower AFB1 content, although no observable differences in soil populations of *A. flavus*, aflatoxin concentrations found in soil, or gene expression of aflatoxin synthesis were detected. More research is needed to better understand the role of Bt-engineered corn on *A. flavus* occurrence and AFB1 contamination of agro-ecosystems during the non-cropping season.

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