

## Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillus flavus* isolates

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### Abstract

The ability of two non-aflatoxigenic *Aspergillus flavus* Link isolates (CT3 and K49) to reduce aflatoxin contamination of corn was assessed in a 4-year field study (2001–2004). Soil was treated with six wheat inoculant treatments: aflatoxigenic isolate F3W4; two non-aflatoxigenic isolates (CT3 and K49); two mixtures of CT3 or K49 with F3W4; and an autoclaved wheat control, applied at 20 kg ha<sup>-1</sup>. In 2001, inoculation with the aflatoxigenic isolate increased corn grain aflatoxin levels by 188% compared to the non-inoculated control, while CT3 and K49 inoculation reduced aflatoxin levels in corn grain by 86 and 60%, respectively. In 2002, the non-toxigenic CT3 and K49 reduced aflatoxin levels by 61 and 76% compared to non-inoculated controls, respectively. In 2001, mixtures of aflatoxigenic and non-aflatoxigenic isolates had little effect on aflatoxin levels, but in 2002, inoculation with mixtures of K49 and CT3 reduced aflatoxin levels 68 and 37% compared to non-inoculated controls, respectively. In 2003 and 2004, a low level of natural aflatoxin contamination was observed (8 ng g<sup>-1</sup>). However, inoculation with mixtures of K49 + F3W4 and CT3 + F3W4, reduced levels of aflatoxin 65–94% compared to the aflatoxigenic strain alone. Compared to the non-sclerotia producing CT3, strain K49 produces large sclerotia, has more rapid *in vitro* radial growth, and a greater ability to colonize corn when artificially inoculated, perhaps indicating greater ecological competence. Results indicate that non-aflatoxigenic, indigenous *A. flavus* isolates, such as strain K49, have potential use for biocontrol of aflatoxin contamination in southern US corn.

**Keywords:** Corn (*maize*, *Zea mays* L.), *Aspergillus flavus*, biological control, biocompetition, non-aflatoxigenic strains, aflatoxigenic strains

### Introduction

Aflatoxins are a class of mycotoxins produced by *Aspergillus flavus* Link and *A. parasiticus* Speare. There are four closely related aflatoxins: B1, B2, G1, and G2 (Diener et al. 1987; CAST 2003). Toxigenic strains of *A. flavus* produce mainly B1 and B2, while *A. parasiticus* produces all four aflatoxins (Diener et al. 1987;

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CAST 2003). Aflatoxin contamination of corn (maize, *Zea mays* L.) is a significant problem worldwide because aflatoxins are potent carcinogens and hepatotoxins (Cullen et al. 1994; CAST 2003). United States federal guidelines for food and feed set a limit of 20 ng g<sup>-1</sup> total aflatoxins, while the European Union guidelines are more strict, with a limit of 1.0 ng g<sup>-1</sup> limit for B1 and a 4.0-ng g<sup>-1</sup> limit for total aflatoxins (van Egmond & Jonker 2004). Cyclopiazonic acid (CPA) is another mycotoxin also produced by various *Aspergillus* species that may cause toxicological problems in animals (Bryden 1991). CPA has been found to occur in maize and other foods (Trucksess et al. 1987), and can further increase the health risks of food and feed contaminated with *A. flavus* (Sosa et al. 2002; Mphande et al. 2004; Takahashi et al. 2004). Residues of CPA from feed can be transferred into milk and eggs (Dorner et al. 1983; CAST 2003). Strains of *A. flavus* vary greatly in aflatoxin production, with some producing copious amounts and others none (Horn 2003; Abbas et al. 2004a,b). Many *A. flavus* strains produce both CPA and aflatoxins, whereas other strains are non-toxigenic (Horn & Dorner 1999; Geiser et al. 2000; Dorner 2004).

Because aflatoxin contamination is a major economic and food safety concern, strategies have been developed to control aflatoxin in crops (Robens & Riley 2002; Abbas 2003, 2004; Robens & Brown 2004). Non-toxigenic strains of *A. flavus* have been suggested as biological control agents in the hope that they might compete with naturally occurring toxigenic *A. flavus*. Early studies by Erlich (1987) showed that co-inoculation of corn with a mixture of certain non-toxigenic mutants and the toxigenic wild-type significantly reduced aflatoxin contamination under *in vitro* conditions. The potential for biological control of aflatoxin has been demonstrated under field conditions in cotton (*Gossypium hirsutum* L.) (Cotty 1994a,b), peanut (*Arachis hypogaea* L.) (Dorner & Cole 2002) and corn (Brown et al. 1991; Dorner et al. 1999). These authors have typically applied the non-aflatoxigenic strains to soil as infested grain cultures (e.g., barley, rice, or wheat), while Brown et al. (1999) inoculated corn ears directly by injection. In the cotton studies conducted by Cotty (1994a), application of the *A. flavus* biocontrol strain was not as effective when directly sprayed on the forming cotton bolls as when applied to soil in a wheat formulation. Corn is frequently infected by *Aspergillus* species which can result in significant aflatoxin accumulation, especially when heat and drought stress occur (Payne 1992; Abbas et al. 2002). When aflatoxin concentration exceeds regulatory levels, this contamination causes a severe economic impact on growers, the grain industry, and may be a significant health risk (Robens & Cardwell 2003). Thus, suitable strategies to control aflatoxin contamination of corn would reduce the risks in corn production in certain geographical regions, such as the southern United States.

In a survey of *A. flavus* from the Mississippi Delta region of the USA, over 500 isolates were characterized from soil and various crops including corn, rice and peanuts (Abbas et al. 2004a). Of these isolates, about 36% produced <20 ng g<sup>-1</sup> total aflatoxins when grown on potato dextrose agar. One phenotypic variation within *A. flavus* used in subdividing species or strains into groups is sclerotial size, the large (L > 400 µm in diameter) sclerotia strains and the small sclerotia (S < 400 µm) strains (Horn 2003). A survey conducted by Abbas et al. (2005) observed that isolates that did not produce sclerotia were significantly less likely to be toxigenic than strains that produced large sclerotia.

We have identified non-aflatoxigenic strains of *A. flavus* that show great promise for reduction of aflatoxin contamination in corn. Field studies in cotton and

peanuts have shown that non-aflatoxigenic strains applied to soil are capable of competing and displacing naturally occurring aflatoxigenic strains (Cotty 1994a,b; Dorner & Cole 2002). This study was conducted to evaluate the potential of two non-aflatoxigenic *A. flavus* strains, K49 and CT3, in reducing aflatoxin contamination in corn grown in the Mississippi Delta.

## Materials and methods

### *Aspergillus flavus* strains and characterization studies

*A. flavus* strains used in this study were isolated as described by Abbas et al. (2004a). These strains originated from single spores, and were maintained on Czapek agar (CZA) slants and stored on silica gel (Windels et al. 1988; Horn 2003). Non-aflatoxigenic *A. flavus* strain K49 was isolated from corn grain from Sunflower County, Mississippi in 2001 (Abbas et al. 2004b). Non-aflatoxigenic *A. flavus* strain CT3 was isolated in 2000 from Dundee silt loam soil, Washington County, Mississippi, in a field that had been continuously cropped in cotton for over 10 years. The aflatoxigenic *A. flavus* strain F3W4 was isolated from Dundee silt loam soil in Sunflower County, Mississippi, in 2001 (corn/cotton rotation). These strains were selected based on their growth characteristics, sclerotia production and toxin profiles (Table I). Toxin production patterns have been stable in cultures transferred over 20 cycles. When multiple single spores are isolated from stock cultures, toxin production patterns are also similar to their original culture (Abbas, unpublished).

Aflatoxin production on potato dextrose agar (PDA) after (7 days) was determined using high performance liquid chromatography (HPLC) as described elsewhere (Sobolev & Dorner 2002). Cyclopiazonic acid was determined on yeast extract sucrose agar as described by Sobolev et al. (1998). Production of both toxins was confirmed by

Table I. Characteristics of *A. flavus* strains used in this study.

Characteristics	<i>A. flavus</i> strains		
	CT3	K49	F3W4
Sclerotia <sup>1</sup>	None	Large	Large
Aflatoxin B1 <sup>2</sup>	ND <sup>6</sup>	ND	32,000–47,500
Aflatoxin B2	ND	ND	300–510
Aflatoxin G1	ND	ND	ND
Aflatoxin G2	ND	ND	ND
Cyclopiazonic acid <sup>2</sup>	800–1100	ND	3450–5100
Radial growth rate <sup>3</sup>	0.39 b	0.54 a	0.53 a
Pigmentation <sup>4</sup>	Off-white	White	Yellow
Fluorescence on CD-PDA <sup>5</sup>	None	None	Blue

<sup>1</sup>Sclerotia production determined on CZA, 14 days after incubation in continuous darkness; Large >400 µm in diameter. <sup>2</sup>Aflatoxin and cyclopiazonic acid concentrations in fungal biomass (ng g<sup>-1</sup>, fresh weight) determined by HPLC; limits of detection = 4 ng g<sup>-1</sup>; ranges based on five separate experiments. <sup>3</sup>Radial growth rate (mm day<sup>-1</sup>) determined on dilute (1/10th strength) PDA, 3–5 days incubation in darkness at 30°C. Means of five replicates; means followed by the same letter do not differ significantly at the 95% confidence level. <sup>4</sup>Pigmentation of fungal biomass grown on PDA after 3–5 days incubation at 28°C in the dark. Results of at least 10 separate experiments. <sup>5</sup>Fluorescence produced by the culture grown on CD-PDA for 3–5 days incubation at 28°C in the dark and assayed by exposure to UV light 350 nm. Results of at least 10 separate experiments. <sup>6</sup>ND, none detected.

liquid chromatography–mass spectroscopy (LC–MS) (Abbas et al. 2002). Sclerotium production was determined according to Horn et al. (1996). Briefly, isolates were grown on CZA slants for 2 weeks at 28–30°C in darkness. Sclerotium types S (<400 µm diameter) and L (>400 µm) were confirmed for each culture by measuring 100 sclerotia as described by Abbas et al. (2005), and sclerotium assessment was conducted at least three times for each strain. Radial growth rate was determined on dilute PDA (Difco potato dextrose broth, 3 g L<sup>-1</sup>; 18 g L<sup>-1</sup> agar). Five replicate Petri dishes (9 cm) were inoculated in the centre with a 2-mm plug of a 3-day-old culture, incubated at 30°C, based on three measurements between 3 and 5 days. Radial growth rates during this period were linear for all three strains, and avoided lag periods in initial growth or reduced growth at later stages due to limitation of Petri dish size. Colony pigmentation was recorded on Petri dish plates visually after 3–5 days of incubation on PDA at 28–30°C. Fluorescence was determined on cultures grown on β-cyclodextrin (0.3%) PDA (CD-PDA) for 3–5 days at 28–30°C. Fluorescence was observed after exposure to UV light (365 nm) as described by (Abbas et al. 2004a). Aflatoxin producing strains produced a blue fluorescent halo around the colony, while non-aflatoxigenic strains produced no fluorescence following UV exposure.

A pin bar inoculation technique (King & Scott 1984; Windham et al. 2003) was used to determine the relative colonization abilities of the three *A. flavus* strains on corn in the field in 2004. *Aspergillus* cultures were grown on PDA for 7 days. Conidia and mycelium were removed by scraping, suspended in aqueous Tween 20 (0.2%), and adjusted to a concentration of about 5 × 10<sup>7</sup> conidia mL<sup>-1</sup>. Corn ears (50/treatment) were inoculated at mid-silking stage using a pin bar inoculator (100-mm long row of 35–36 sewing needles mounted on wood or a plastic bar, each with 6 mm of the points exposed). Pin bars were dipped in spore suspensions, and the bars were pressed into the ear. At 2, 5, 7, 9, and 12 days after pin-bar inoculation, five inoculated ears were harvested per treatment, and the number of infected kernels and total number of kernels was determined based on visual assessment of sporulation. After assessing infected kernels, aflatoxin concentration in the infected kernels at 12 days after inoculation was determined by methanol extraction and HPLC according to Sobolev and Dorner (2002).

#### *Field study*

*Inoculum preparation.* Wheat was used as the inoculant carrier. Wheat seed was soaked in water overnight, drained, placed in autoclavable bags (1 kg/bag with 200 mL water), and autoclaved for 55 min at 121°C. Starter cultures of *A. flavus* were grown on PDA in 9-cm Petri dishes at 28–30°C for 5 days under continuous darkness. The autoclaved wheat was inoculated with the appropriate fungal culture (one 3-cm<sup>2</sup> portion of a 5-day-old culture on PDA plate/bag) and incubated at 28–30°C. *Aspergillus flavus* inoculum added to wheat on the PDA plate possessed abundant conidia. After 24 h growth, the inoculated wheat was manually shaken and incubated for another 24 h and further homogenized by manual shaking. Colonization by the inoculant strain was confirmed by determining aflatoxin concentration in the inoculants based on HPLC analysis and enumeration based on plating on modified MDRB agar with aflatoxin production assessed by subculture on β-cyclodextrin (0.3%) PDA (CD-PDA). All inoculated wheat contained >log 8.0 cfu *A. flavus*/g with appropriate aflatoxin production phenotype.

*Field study experimental design.* Corn was planted 23 March 2001, 6 April 2002, 1 April 2003, and 15 April 2004 in a randomized complete block design. The treatments were: (1) non-infected application of autoclaved wheat seed; (2) *A. flavus* K49-treated wheat; (3) *A. flavus* CT3-treated wheat; (4) *A. flavus* F3W4-treated wheat; (5) mixture of *A. flavus* K49- and F3W4-treated wheat; and (6) mixture of *A. flavus* CT3- and F3W4-treated wheat. The six inoculation treatments were applied to the same experimental units over the 4 years of the study. Inoculations were made immediately after cultivation at growth stage V6 (Ritchie et al. 1997), by scattering the treated wheat seed by hand in the middle furrows at rate of 20 kg ha<sup>-1</sup> for each strain.

Individual plots consisted of eight rows that were 9.1 m long and spaced 1 m apart. Buffer plots consisted of four rows of non-treated corn separating each plot. In addition, inoculated plots and buffer plots were staggered to also reduce contamination. Inoculation treatments and subsequent data were collected from the four center rows of each plot. Plots were geo-referenced (GPS coordinates) to enable precise sampling and replanting.

The cultivars Pioneer 3225 (2001 and 2002) and Pioneer 32R25 (2003 and 2004) were planted at a rate of 70,000 plants ha<sup>-1</sup>. Soil tests were conducted by a commercial laboratory each year to determine N, P, and K fertility requirements for a yield goal of 12.5 Mg ha<sup>-1</sup>. A pre-plant application of the required P and K as super phosphate and muriate of potash was made along with 112 kg N ha<sup>-1</sup> as urea. An additional application of 112 kg N ha<sup>-1</sup> (urea: NH<sub>4</sub>NO<sub>3</sub> solution) was made just prior to cultivation at growth stage V6. Weed control was achieved with the application of the herbicide acetochlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide] and atrazine [2-chloro-4-ethylamine-6-isopropylamine-5-triazine] in each year. In 2003, nicosulfuron [3-pyridimeccarboxamide, 2-[[4,6-dimethoxy-pyrimidin-2-yl] amino carbonyl] aminosulfonyl]-*N*-*N*-dimethyl] was also applied for control of johnsongrass [*Sorghum halpense* (L.) pers.]. Plots were furrow irrigated at growth stages R1 and R2 (approximately 25 mm of water/irrigation). Climatic data for the Stoneville experimental farm was obtained on line from the Mississippi State Experiment Station (Mississippi Weather Station Data, accessed 28 August, 2005).

*Experimental parameters.* Aflatoxin concentration in corn was quantitatively determined using a commercial ELISA kit (Neogen Corp., Lansing MI) according to Abbas et al. (2002). Quantification of aflatoxin using these ELISA kits correlated well with quantification by HPLC (Sobolev & Dorner 2002). Corn was hand harvested at maturity (20 top ears/plot) from the centre rows. The harvested corn was shelled, dried, and ground (20 mesh) using a Romer mill (Union, MO). Triplicate subsamples (20 g) were extracted in 100 mL of methanol (70%) for 30 min on a high speed reciprocal shaker. The methanol extracts were filtered (Whatman #1 filter paper), and the filtrate analyzed by ELISA. The limit of detection in this assay is 5 ng g<sup>-1</sup>.

In 2002–2004, *A. flavus* populations were enumerated from soils using modified dichloronitroaniline rose bengal (MDRB) agar according to Horn and Dorner (1998), amended with 3.0% sodium chloride (Griffin et al. 1975). Soil samples consisted of a composite of nine surface (0–2.5 cm) subsamples taken from between the two centre rows from each plot. Soils were sieved to pass a 2.25-mm mesh screen, moisture content was determined gravimetrically and soils were stored at 4°C until plating

(within 2 days of collection). Isolated colonies were plated on  $\beta$ -cyclodextrin (0.3%) PDA (CD-PDA) to assess aflatoxin production based on blue fluorescence of the colonies (Abbas et al. 2004a).

In 2004, whole corn grain and ground corn samples were assessed for *A. flavus* colonization and frequency of toxigenic isolates. Whole kernels were surface sterilized in 0.3% sodium hypochlorite solution for 2 min and seeds were rinsed three times in sterile distilled water. Forty kernels from each experimental plot were plated on MDRB agar and incubated for 5 days, and recovery of *A. flavus* colonies was recorded. Forty colonies/plot were transferred to CD-PDA and incubated for 5 days under continuous darkness at 28–30°C for determining aflatoxin production. The CD-PDA assay reliably detects aflatoxin below 5 ng g<sup>-1</sup> of fungal mycelia (Abbas et al. 2004a). In addition, ground grain samples were homogenized in 0.2% water agar, serially diluted and plated on MDRB agar. Colony forming units (cfu) were counted following 5 days incubation, and 30 colonies/plot were transferred to CD-PDA for assessment of aflatoxin production as described above.

### Statistical analysis

For all field and laboratory data analysis of variance (ANOVA) was conducted using PROC GLM of the Statistical Analysis System (SAS 2001). Mean separation was performed using Fisher's Least Significant Difference.

## Results

### Characterization of *Aspergillus flavus* strains

The characteristics of the *A. flavus* strains used in this study are summarized in Table I. Based upon HPLC, ELISA and LC-MS confirmation of several repeated assays (> five assays on duplicate cultures), CT3 or K49 did not produce any aflatoxins (detection limit < 4 ng g<sup>-1</sup> of fungal biomass), while F3W4 produced high levels of aflatoxins B1 and B2. Both CT3 and F3W4 produced cyclopiazonic acid, while K49 did not produce cyclopiazonic acid. Both the aflatoxigenic strain F3W4 and the non-aflatoxigenic isolate K49 produced abundant large sclerotia, whereas CT3 did not produce sclerotia, under our conditions. On dilute PDA (1/10th strength), strain CT3 grew slower than either K49 or F3W4 (Table I). Colonization of corn following pin point inoculation is presented in Figure 1. The non-aflatoxigenic strain K49 displayed a similar level of colonization of corn kernels as the aflatoxigenic strain F3W4, while non-aflatoxigenic strain CT3 displayed significantly reduced colonization ( $P < 0.05$ ) at 9 and 12 days after inoculation. Total aflatoxin concentration in infected kernels from pin-bar inoculation with F3W4 was 34,184 compared to 6998 ng g<sup>-1</sup> in non-inoculated corn. However, aflatoxin concentration was only 190 and 4 ng g<sup>-1</sup> in CT3 and K49 inoculated corn, respectively. The reduced levels of aflatoxin observed in K49 and CT3 pin-bar inoculated corn under field conditions is indicative of the capacity of the isolates to inhibit aflatoxin contamination by indigenous aspergilli.

### Corn aflatoxin levels

The effects of soil inoculation with aflatoxigenic and non-aflatoxigenic *A. flavus* strains on aflatoxin levels in corn grain are summarized in Table II. In 2001 and 2002,

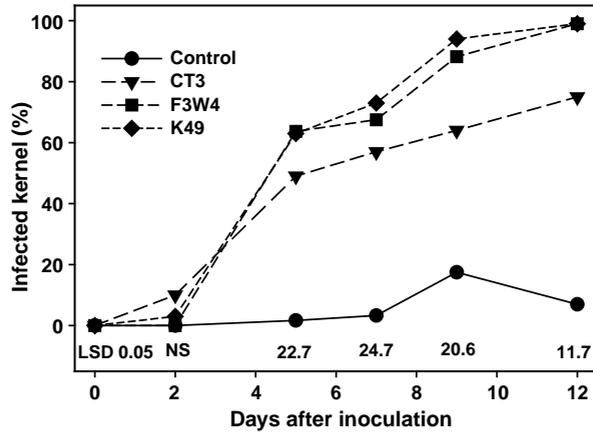


Figure 1. Effect of pin-bar inoculation with *A. flavus* strains on the development of *Aspergillus* infected corn kernels in field trials in 2004. Mean separation by Fishers LSD test at the  $P < 0.05$  level. Note control was pin-bar wounded.

there were high endemic levels of aflatoxin contamination in untreated corn (221 and 552  $\text{ng g}^{-1}$ , respectively), while in 2003 and 2004 natural levels of aflatoxin were very low (8  $\text{ng g}^{-1}$ ). In 2002, there was the lowest average rainfall between inoculation and harvest (0.279  $\text{cm/day}$ ) compared to 0.356, 0.300 and 0.483  $\text{cm/day}$  for 2001, 2003, and 2004, respectively. Average maximum temperature for the same period was highest in 2001 (32.9°C) compared to 32.3, 31.6 and 31.5°C for 2002, 2003, and 2004, respectively. In both 2001 and 2002, there was a significantly lower aflatoxin concentration in corn kernels ( $P < 0.05$ ) from plots inoculated with CT3 or K49 compared to the untreated control, or plots inoculated with aflatoxigenic strain F3W4. Inoculation with K49 significantly ( $P < 0.05$  level) decreased aflatoxin by 58 and 76% in 2001 and 2002, respectively, while inoculation with CT3 decreased aflatoxin concentration by 86 and 61% in 2001 and 2002, respectively, relative to the non-inoculated controls. In 2001, aflatoxin levels in corn from plots inoculated with the aflatoxigenic strain F3W4 were 188% greater than corn from uninoculated plots, while in 2002 aflatoxin levels in F3W4 and control treatments were similar. In both 2001 and 2002, aflatoxin levels in corn from plots inoculated with mixtures of

Table II. Effect of soil treatment with toxigenic *A. flavus* F3W4, non-aflatoxigenic strains CT3 and K49, and mixtures of aflatoxigenic and non-aflatoxigenic strains on aflatoxin concentrations in corn.

Treatment/Isolate	Aflatoxin levels <sup>1</sup> (ppb)			
	2001	2002	2003	2004
Untreated	221 b <sup>2</sup>	552 a	8 c	8 c
F3W4	637 a	610 a	157 a	65 a
CT3	32 e	215 c	8 c	15 bc
K49	94 d	131 d	8 c	10 c
CT3+F3W4	251 b	347 b	58 b	28 b
K49+F3W4	167 c	167 cd	8 c	17 bc

<sup>1</sup>Mean aflatoxin concentration of four replicates in corn kernels determined by ELISA. <sup>2</sup>Values followed by the same letter in a given column do not differ significantly at the 95% confidence level using Fisher's LSD test.

inoculum were lower than those inoculated with the toxigenic isolate alone. In 2003 and 2004 when there were low levels of natural infection ( $8 \text{ ng g}^{-1}$ ), inoculation with F3W4 increased aflatoxin levels to 157 and  $65 \text{ ng g}^{-1}$ , respectively. Corn from plots inoculated with mixtures of either K49 or CT3 with F3W4 had significantly lower levels of aflatoxin compared to F3W4 alone ( $P < 0.05$ ). Aflatoxin levels in corn from plots inoculated with a mixture of strains K49 and F3W4 was significantly lower than that in plots inoculated with a mixture of CT3 and F3W4 strains in 3 of 4 years, indicating that K49 is perhaps a more aggressive competitor than CT3 (Table II).

#### *Aspergillus flavus* propagules in soil and corn

There was no effect of prior inoculation in 2001 on *Aspergillus* propagule density in May 2002 (Table III). However, inoculation with aflatoxigenic strains, non-aflatoxigenic strains or mixtures of *A. flavus* strains increased *A. flavus* soil propagule density in September of 2002 and both sampling times in 2003 and 2004 ( $P < 0.05$ ).

Table III. Effect of soil treatment with aflatoxigenic and non-aflatoxigenic *A. flavus* on soil propagule density of *Aspergillus* and recovery of aflatoxin-producing isolates from soil before inoculation and following corn harvest (2002–2004).

Year/Treatment	Propagule density <i>Aspergillus</i> ( $\log_{10} \text{ cfug}^{-1}$ ) <sup>1</sup>		Aflatoxigenic Isolates (%) <sup>2</sup>	
	May 2002	September 2002	May 2002	September 2002
Untreated	2.34 a <sup>3</sup>	3.67 d	56.3 b	75 ab
F3W4	2.38 a	3.90 bc	81.3 a	94 a
CT3	2.22 a	3.83 c	31.3 c	0 c
K49	2.24 a	3.83 c	12.5 c	6 c
CT3+F3W4	2.34 a	3.97 a	68.8 ab	69 ab
K49+F3W4	2.28 a	3.94 ab	75.0 a	50 b
Probability	NS <sup>4</sup>	0.05	0.05	0.05
	May 2003	September 2003	May 2003	September 2003
Untreated	3.39 c	3.26 b	50.0 a	56 b
F3W4	3.64 bc	3.63 a	82.5 a	86 a
CT3	3.68 ab	3.58 a	15.0 b	21 c
K49	3.67 ab	3.62 a	10.0 b	16 c
CT3+F3W4	3.79 a	3.72 a	70.0 a	62 b
K49+F3W4	3.64 ab	3.55 a	62.5 a	63 ab
Probability	0.05	0.05	0.05	0.05
	May 2004	September 2004	May 2004	September 2004
Untreated	2.77 c	3.49 c	49 b	55.0 b
F3W4	3.28 b	4.00 a	85 a	79.3 a
CT3	3.77 a	3.94 ab	17 c	23.5 cd
K49	3.53 ab	3.96 ab	15 c	9.3 d
CT3+F3W4	3.68 a	3.89 b	47 b	34.3 c
K49+F3W4	3.81 a	3.95 ab	58 b	37.5 c
Probability	0.05	0.05	0.05	0.05

<sup>1</sup> $\log_{10} \text{ cfu } Aspergillus \text{ g}^{-1}$  soil. Mean of four replicates determined by serial dilution and plating on modified MDRB agar amended with 3% sodium chloride; colonies were counted after 5 days incubation.

<sup>2</sup>In 2002, 16 isolates (four/plot) and in 2003 and 2004, 120 isolates (30/plot), were assayed for aflatoxin production based on fluorescence on CD-PDA. <sup>3</sup>Values followed by the same letter in a given column do not differ significantly at the 95% confidence level using Fisher's LSD test. <sup>4</sup>NS, not significantly different at the  $P < 0.05$  level.

The lowest recovery of aflatoxigenic *A. flavus* isolates was found in soil inoculated with strains K49 and CT3.

The distribution of aflatoxigenic strains present in corn grain at harvest was determined in 2004 (Table IV). *Aspergillus flavus* was isolated from 100% of all kernels tested, regardless of treatment. The propagule density of *A. flavus* estimated in ground corn grain, however, was 10-fold higher in grain from inoculated plots compared to non-inoculated plots regardless of treatment. A similar frequency of aflatoxigenic isolates was observed in whole kernels and ground grain samples. Greater than 90% of *A. flavus* isolates recovered from plots inoculated with F3W4 were aflatoxigenic, while the lowest frequency of aflatoxigenic isolates (<11%) was observed in K49 and CT3 inoculated plots. A similar frequency of aflatoxigenic isolates was observed in corn from non-inoculated plots and plots inoculated with a mixture of CT3 and F3W4, while a lower frequency of aflatoxigenic isolates was found in corn from plots inoculated with mixtures of K49 and F3W4.

## Discussion

Results from this 4-year study consistently demonstrate that soil inoculation with mixtures of non-aflatoxigenic *A. flavus* strains with an aflatoxigenic *A. flavus* strain, had significantly lower levels of aflatoxin contamination compared to inoculation with the aflatoxigenic *A. flavus* strain F3W4 alone (43–63% reduction when co-inoculated with CT3, and 74–95% reduction when co-inoculated with K49). Although both CT3 and K49 were shown to reduce aflatoxin contamination in corn, K49 may be more desirable as an inoculant strain, because it produces neither aflatoxin nor CPA. However, reduced aflatoxin contamination compared to non-inoculated controls attributable to inoculation with non-aflatoxigenic strains was only observed in the first 2 years of the study with a high incidence of natural aflatoxin contamination. The potential for aflatoxin contamination of corn is generally enhanced by drought and high temperatures after silking (Payne 1992;

Table IV. Effect of soil treatment with toxigenic and non-aflatoxigenic *Aspergillus flavus* on recovery of toxigenic *A. flavus* from corn grain samples in 2004.

Treatment	Whole kernel Infected <sup>1</sup> (%)	Whole kernel aflatoxigenic isolates <sup>2</sup> (%)	Ground grain cfu (log <sub>10</sub> g <sup>-1</sup> ) <sup>3</sup>	Ground grain aflatoxigenic isolates <sup>2</sup> (%)
Untreated	100	36.9 b <sup>4</sup>	3.39 b	41.8 b
F3W4	100	93.6 a	4.38 a	91.7 a
CT3	100	0.0 d	4.37 a	10.8 de
K49	100	0.0 d	4.39 a	3.3 e
CT3+F3W4	100	33.1 b	4.37 a	31.7 bc
K49+F3W4	100	14.4 c	4.37 a	25.0 d
Probability	NS <sup>5</sup>	0.05	0.05	0.05

<sup>1</sup>Forty surface sterilized corn kernels plated on modified MDRB agar amended with 3% sodium chloride; assessed for colonies of *Aspergillus* 5 days after plating. <sup>2</sup>Isolates assessed for aflatoxin production based on fluorescence on  $\beta$ -cyclodextrin PDA; 40 colonies/plot assessed from whole kernel isolates and thirty isolates per plot from ground corn. <sup>3</sup>CFU determined by serial dilution and plating on modified MDRB agar amended with 3% sodium chloride; colonies counted after 5 days incubation. <sup>4</sup>Values followed by the same letter in a given column do not differ significantly at the 95% confidence level using Fisher's LSD test. <sup>5</sup>NS, not significantly different at the  $P < 0.05$  level.

Dorner et al. 1999; Abbas et al. 2002), with considerable variation from year to year. The reduction in aflatoxin contamination observed in this study (57–86% under high levels of natural contamination) is in a similar magnitude to reductions observed in studies by others using soil inoculation of corn and other crops. In Georgia corn field studies (Dorner et al. 1999), aflatoxin contamination was significantly reduced when the natural contamination level was high (above  $80 \text{ ng g}^{-1}$ ). In the year with the highest level of natural contamination ( $188 \text{ ng g}^{-1}$ ), inoculation of soil with  $225 \text{ kg ha}^{-1}$  of a mixture of non-aflatoxigenic *A. flavus* and *A. parasiticus* reduced aflatoxin contamination by 87%. However, with lower natural contamination ( $87.5 \text{ ng g}^{-1}$ ), inoculating the soil with  $25 \text{ kg ha}^{-1}$  of the non-aflatoxigenic strains only reduced aflatoxin contamination by 66%. In cotton field trials, application of non-aflatoxigenic *A. flavus* strain reduced aflatoxin B1 from 75 to 99.9%. However, application rates were extremely high  $>8.0 \text{ g per meter of crop row}$  (Cotty 1994a). Inoculation of soil with non-aflatoxigenic strains of *A. flavus* and *A. parasiticus* reduced aflatoxin levels in peanuts from 74.3 to 99.9% depending on rate of soil inoculation (Dorner et al. 1998). Peanut produces fruiting structures below ground improving the opportunities for biological control of aflatoxin.

Good survival of introduced non-aflatoxigenic and aflatoxigenic *A. flavus* inoculants in soil was observed in our studies based on characteristics of aflatoxin production of the soil *Aspergillus* community after inoculation, although total propagule densities of *Aspergillus* were typically only 1–3-fold higher in soil from inoculated compared to non-inoculated plots. After 2 years of inoculation with non-aflatoxigenic strains  $<23\%$  of the *Aspergillus* community was characterized as aflatoxigenic compared to about 50% of the control soil. In studies by Dorner et al. (1999), inoculation with mixtures of *A. flavus* and *A. parasiticus* increased the propagule density of the *Aspergillus* community from 10- to 100-fold, however, these researchers used 10-fold higher application rates than our studies. Their studies also demonstrated that the *A. parasiticus* strain was more aggressive in displacing the *Aspergillus* soil community compared to the *A. flavus* strain. However, the more aggressive soil colonizer *A. parasiticus* was not as effective in colonizing the corn as the *A. flavus* strain. Dorner et al. (1999) concluded that soil inoculation with a non-aflatoxigenic strain could provide control for soil-borne infestation and aflatoxin contamination of crops such as peanut, while control of aflatoxin in a crop such as corn, where infestation occurs from airborne contamination, would be difficult using a soil applied inoculant.

Based upon growth rate, colonization of corn ears and sclerotium production, K49 is expected to have a greater degree of ecological competence compared to CT3 in colonizing soil and competing with other *A. flavus* in corn. The pin bar inoculation technique indicates that K49 is a more aggressive colonizer of corn compared to CT3 and also agrees with *in vitro* studies of K49 having a more rapid growth rate compared to CT3. CT3 does not produce sclerotia, thus it is expected that K49 would have a greater over-wintering potential and survive on organic debris in soil. *A. flavus* sclerotia serve as a survival structures in soil and plant residues, and upon germination will release conidia under appropriate conditions (Wicklow et al. 1982, 1993).

Non-aflatoxigenic strains can successfully compete with indigenous *Aspergillus* present in infection sites of the reproductive tissue of corn resulting in reduced aflatoxin contamination. There are three basic mechanisms of competition among plant pathogens for infecting specific plant organs: competitive exploitation

(Wicklow 1981); interference competition (Wicklow 1981); and parasitic fitness (Reid et al. 1999). The exact mechanisms for reducing aflatoxin contamination in corn by non-aflatoxigenic *A. flavus* isolates CT3 and K49 in corn needs further research. *Aspergillus flavus* introduced to the soil surface can release airborne conidia, and perhaps be more abundant than background levels of aspergilli propagules (conidia) present in air. Field experiments have been established to quantify aerial dispersal of introduced inoculum and the native levels of airborne aspergilli present in the Mississippi Delta.

These current studies demonstrate that *Aspergillus* inoculants introduced to the soil on a wheat carrier can colonize aerial parts of the corn plant as occurred for both aflatoxigenic and non-aflatoxigenic strains. Colonization by augmented aflatoxigenic strains increases aflatoxin concentration in corn grain, while inoculation with non-aflatoxigenic strains reduces aflatoxin concentration only when there is a significant potential for aflatoxin concentration or when co-inoculated with a aflatoxigenic strain. Secondly, this study indicates that strain K49 is perhaps a more effective competitor than CT3 against native aspergilli or when competing against F3W4. In conclusion, non-aflatoxigenic, indigenous *A. flavus* isolates, such as strain K49, have potential use for biocontrol of aflatoxin contamination in southern US corn.

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