

Chapter 7

DEVELOPMENT OF NON-TOXIGENIC STRAINS OF ASPERGILLUS FLAVUS FOR CONTROL OF AFLATOXIN IN MAIZE

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ABSTRACT

Aflatoxin (AF), produced by *Aspergillus flavus*, can be a major problem in Mississippi Delta maize (*Zea mays* L.) causing economic losses if levels of contamination are high. Although research has been directed at reducing maize AF contamination, no consistent control methods are available. This manuscript summarizes research approaches in development of non-aflatoxigenic *A. flavus* strains to control maize AF contamination. In a survey of *A. flavus* isolates from Mississippi Delta soil, 36% of 517 isolates produced less than 20 ppb of aflatoxin. This survey identified two non-toxigenic strains (K49 and CT3) that, when applied to maize as soil inoculants, suppress AF by competitive displacement. Four years of field testing showed these non-toxigenic isolates reduced AF contamination by 60-94%. The non-toxigenic strains displace the toxigenic *A. flavus* populations in soil. K49 may be a more suitable biocontrol agent than CT3 because K49 also does not produce cyclopiazonic acid. K49 has greater soil colonization potential, based on fast growth and sclerotia formation, than does CT3. Using a pinbar inoculation technique, K49 displayed a more rapid maize colonization than CT3. These results suggest the basic method for reducing AF contamination is by competitive exclusion. Further work to improve biocontrol efficacy in reducing AF contamination is in progress.

Key words: Aflatoxin, *Aspergillus flavus*, Biocontrol, Competitive Displacement, Fungal Ecology, Maize

SIGNIFICANCE OF AFLATOXIN CONTAMINATION IN MAIZE

Aflatoxin (AF) contamination of commercial grain is one of the most important production concerns for Mississippi maize (*Zea mays* L.). Growers throughout the southern USA experience financial loss from this problem. There is increasing demand for maize because it is used in crop rotation with other crops such as cotton and soybean, thus improving pest control, profits and yields (Reddy et al., 2006). Maize is used for animal feed of poultry and catfish, alcohol fermentation and heating, and human consumption. However, maize production is economically hindered in the Mid South by AF contamination. Since aflatoxin is a potent carcinogen, the USDA-FAD permits no more than 20 ppb in maize for human consumption. Aflatoxin is produced by *Aspergillus flavus* with growth of this fungus is facilitated by hot and dry conditions (CAST, 2003). This problem was particularly severe in 1998, when significant portions of the corn crop were lost due to high levels of contamination (Robens and Cardwell, 2003).

Research to date has resulted in little progress toward AF reduction in maize, especially in a high incidence year. Several approaches to reduce AF and develop integrated control systems are in progress at the USDA-ARS in Stoneville, Mississippi (Table 1).

Table 1. Summary of strategies evaluated to control aflatoxin (AF) contamination in maize investigated by the USDA-ARS, Stoneville, Mississippi.

Practice	Results	Citations
Plant breeding	Commercial hybrids with lower levels of AF identified	Abbas et al., 2002 Moore et al., 2004 Abbas et al., 2006a
Fertility management	Inconclusive	Bruns & Abbas, 2004; 2005a
Planting date	Inconclusive	Bruns & Abbas, 2006a
Irrigation management	Inconclusive	Bruns & Abbas, 2003
Antifungal compounds	Messenger – no effect Glufosinate – no effect	Abbas et al., 2006c; Bruns & Abbas, 2006b
Insect control	Maize lines with resistance to AF insect vectors identified	Abel et al., 2000
Fungal ecology	Factors associated with <i>A. flavus</i> populations assessed	Abbas et al., 2005
Biological control	Non-aflatoxigenic strains reduce AF contamination	Abbas et al., 2006c

AGRICULTURAL RESEARCH SERVICE, STONEVILLE, MISSISSIPPI, APPROACHES FOR AFLATOXIN REDUCTION

Although no commercially available measures exist for pre-harvest AF control in maize, some hybrid lines of maize have been identified with a lower incidence of AF (Abbas et al., 2002; Abbas et al., 2006a). It is well documented that ear feeding insects can increase aflatoxin levels in pre-harvest maize [for a review of pertinent literature, (Dowd, 1998)]. However, demonstrating the benefit of growing maize that is resistant to ear-feeding insects to reduce aflatoxin has been inconclusive. Abel et al. (2000) developed two backcross populations with a new, native source of resistance to silk-feeding corn earworm, from donor parents Lambayeque 45 and Piura 208 that should be evaluated for their ability to reduce aflatoxin via insect resistance.

Pre-harvest aflatoxin contamination of maize grain is facilitated by unfavorable environmental factors, i.e. heat stress, drought, inadequate nutrition, pests, and other stress inducing factors (Bruns, 2003; Payne, 1992). To increase yield, plant populations for maize production have increased during the past three decades. However, higher plant populations place increased demand on available water and increase the likelihood of drought stress (Bruns and Abbas, 2003). Higher plant populations also require increased amounts of N fertilizer to avoid nutrient shortages, particularly during reproductive growth (Bruns and Ebelhar, 2006). Current research in the Mississippi Delta however has not shown a tendency towards increased mycotoxin contamination with increased plant populations in irrigated maize (Bruns and Abbas, 2003 and 2005b). Lower N-fertility levels resulted in reduced grain yields but no increase in aflatoxin in irrigated maize in this region (Bruns and Abbas, 2005a, b). Delaying maize harvests in the Mississippi Delta also did not appear to increase the incidence of mycotoxin contamination (Bruns and Abbas, 2004; Bruns and Abbas 2006). Various antimicrobial agents and fungicides have been evaluated for reduction of AF contamination. The bacterial protein Harpin (Messenger) that elicits induced resistance or the herbicide glufosinate that exhibits antifungal activity had no effect on maize AF contamination (Abbas et al., 2006b; Bruns and Abbas, 2006b). However, the use of biologically-based strategies to minimize AF level and *Aspergillus* colonization in maize with non-aflatoxigenic isolates offers much potential.

Biological control strategies to control AF contamination in maize have been investigated by several laboratories (Abbas et al., 2006c, Brown et al., 1991; Dorner et al., 1999). In peanuts (*Arachis hypogaea* L.) and cotton (*Gossypium hirsutum* L.), the use of *A. flavus* strains which do not produce AF (non-aflatoxigenic strains) have been shown to reduce AF contamination (Cole and Dorner, 2001; Dorner et al., 1992, 1998; Cotty, 1994). Successful biological control and other techniques to manage AF contamination requires an understanding of the ecology of *A. flavus*, and the ability to manipulate colonization of the crop by aflatoxigenic *A. flavus* populations (Abbas et al., 2004a; Horn and Dorner, 1998; Griffin et al., 1981, 2001; Horn, 2003; Wicklow et al., 1998). In addition to the use of non-aflatoxigenic strains of *A. flavus*, other biocontrol strategies being considered include the use of bacterial antagonists (Misaghi et al., 1995) and epiphytic yeasts (Hu et al., 1999).

The objectives of manuscript are to summarize research approaches in assessing *A. flavus* colonization in maize and highlight results from biological control research programs in the

USDA-ARS at Stoneville, Mississippi which might assist southern USA producers in achieving economic and safe maize production.

DIVERSITY OF *A. FLAVUS* IN THE MISSISSIPPI DELTA ECOSYSTEM

The distribution and diversity of *A. flavus* has been characterized in several ecosystems (Abbas et al., 2004a, Horn and Dorner, 1998, Orum et al., 1997, Wicklow et al., 1998). Under certain ecosystems, e.g., the Midwestern USA, populations of *A. flavus* are too low to typically support a high incidence of AF contamination (McGee et al., 1996; Wicklow et al., 1998). In most peanut growing regions of the Southern USA, higher *A. flavus* populations are observed (Horn and Dorner, 1998). Likewise, *A. flavus* populations associated with soils planted in cotton soils in Arizona were higher than lettuce and wheat (Orum et al., 1997).

To determine the potential for aflatoxin contamination in the Mississippi Delta, we initiated a detailed collection and characterization of *A. flavus* from soils and crops of the Mississippi Delta (Abbas et al., 2004a, 2004b, 2005, and Zablotowicz et al., this volume). The toxigenic potential of 517 Mississippi Delta *A. flavus* isolates were characterized using chemical and cultural methods (Table 2). Overall, 61% of all the isolates were aflatoxigenic (produced > 20 ppb on potato dextrose agar). This study (Abbas et al., 2004b) indicated that cultural methods were quite satisfactory for economically characterizing the AF production by *A. flavus* isolates. The overall frequency of toxigenic *A. flavus* isolates were quite similar in maize and soil, while a higher incidence of toxigenic isolates were observed in rice (*Oryzae sativa* L.). The lower incidence of aflatoxigenic *A. flavus* isolates in peanuts in our study is most likely because peanuts are not typically grown in this region.

Table 2. Distribution of aflatoxigenic *Aspergillus flavus* isolates¹ and in vitro sclerotia collected from various sources in the Mississippi Delta.

Source	Number of Isolates	Aflatoxin production ² (%)			Sclerotia production ³ (%)		
		None (<20 ppb)	Moderate (20-10,000 ppb)	High (>10,000 ppb)	None	Large (>400 µm)	Small (<400 µm)
Corn	224	42	35	23	44	56	0
Peanut	183	61	6	33	52	44	4
Rice	67	14	35	51	74	20	6
Soil	43	30	26	44	42	53	5
Average		36	27	37	47	51	2

¹ A total of 517 isolates were evaluated

² Data compiled from Abbas et al., 2004b

³ Data compiled from Abbas et al., 2005

Production of sclerotia is a morphological criteria that is useful in characterizing *A. flavus* isolates and populations (Horn and Dorner, 1998; Orum et al., 1997; Noval and Cabral, 2002). *Aspergillus flavus* strains producing sclerotia are classified as S (small sclerotia, <400 µm) and L (large sclerotia, >400 µm). In our survey, we observed that about 50% of the Mississippi *A. flavus* isolates do not readily form sclerotia on Czapek media following 14 d of

incubation at 30 °C, and that most isolates were the L morphotype. Horn and Dörner (1998) demonstrated that the L isolates were most abundant from Virginia to Mississippi, while the S isolates were most abundant in Louisiana and Texas. About 50% of the Mississippi isolates that did not produce sclerotia were non-aflatoxigenic. Most sclerotia-producing isolates produced AF, with large sclerotia isolates having the highest AF production levels (Abbas et al., 2005). Contrasting correlations were observed in Arizona where the S isolates had a higher level of AF production.

General trends of population dynamics can be ascertained from surveys; however, studies evaluating the spatial and temporal dynamics of *A. flavus* populations may be most informative in understanding the ecology of this fungus (Abbas et al., 2004a; Orum et al., 1997). The study conducted on a small section of a Mississippi Delta grower's field evaluated *A. flavus* populations following cotton, maize and wheat, as well as the levels of toxin and *A. flavus* colonization of maize (Abbas et al., 2004a). The highest propagule density of *A. flavus* was observed following maize. In this study, isolates from maize had a significantly greater frequency of AF producers than isolates from the soil. Although most AF isolates were aflatoxigenic, there was no correlation between colonized maize kernels and aflatoxin concentrations, indicating a significant role of environmental stress, especially water availability in controlling AF accumulation. The areas that had the highest organic matter content and greatest moisture holding ability had the highest density of *A. flavus* propagules and maize kernel colonization, but had little or no AF accumulation.

Several researchers have utilized characterization of *A. flavus* enzymes to understand its infectivity and AF production. Studies by Leger et al., 2000, indicated that the pectinase P2c was associated with the ability of *A. flavus* to colonize cotton bolls; however, all isolates studied exhibited similar protease isozymes.

The use of molecular techniques to understand the diversity and distribution of *A. flavus* populations was initially used in the studies by Wicklow et al., 1998. These studies used restriction fragment length polymorphism (RFLP) to explore the relatedness of various isolates from air, insects, maize and soil. Using this technique, the authors found a wide diversity of isolates from these four sources. However, they were unable to correlate a dominant source from infected maize kernels. Their conclusion was that there was no single dominant strain responsible for AF contamination. A phylogenetic study using analysis of an AF biosynthesis gene (*omt12*) was conducted on 33 S and L strains, in addition to *A. oryzae* and *A. parasiticus* (Geiser et al., 2000). This study divided the isolates into two major groups. Group I isolates produced only aflatoxin B and had both S and L phenotypes. Group II isolates produced both aflatoxin B and G, with all isolates having the S phenotype. A recent study by Baird et al. (2006) used DNA fingerprinting techniques based on amplification of mini hairpin primers to generate Arbitrary Signatures of Amplification Profiles (ASAP) of 75 isolates, with most of them isolated from Mississippi Delta surveys (Abbas et al., 2004a, Abbas et al., 2004b). These authors initially attempted to use the Internal Transcriber Spacer (ITS) region. However, results using this approach were not robust enough to differentiate among strains. Using ASAP resolution of the isolates into aflatoxigenic and non-aflatoxigenic isolates was accomplished and suggested that the ASAP techniques may be valid in determining relative colonization potential of specific isolates. This information should also assist in a sophisticated breeding program for AF resistance.

NON-TOXIGENIC ISOLATES IN COMPETITIVE BIOCONTROL OF AFLATOXIN

The use of non-aflatoxigenic isolates to control AF contamination has been considered in several cropping systems and geographical areas (Abbas et al., 2006c; Brown et al., 1991; Cole and Dorner, 2001; Cotty, 1994; Dorner et al., 1992, 1998).

BIOCONTROL OF MAIZE AF BY MISSISSIPPI NON-AFLATOXIGENIC ISOLATES

The colonization of soil by non-aflatoxigenic strains K49 and CT3 and toxigenic F3W4, applied as a wheat formulation, was assessed over four years with propagule density and proportion of aflatoxigenic isolates evaluated before inoculation and after corn harvest (Fig. 1) (data summarized from Abbas et al., 2006c). Although inoculation significantly increased propagule density of *A. flavus* in soil, the differences between inoculated and non-inoculated were typically between log (10) 0.2 and 0.5 cfu g⁻¹soil. However, inoculation with the aflatoxigenic isolate F3W4 displaced most of the non-aflatoxigenic population with typically over 80% of the propagules being aflatoxigenic, while noninoculated soil remained fairly constant ~60% aflatoxigenic. When soil was inoculated with non-aflatoxigenic strains CT3 or K49, there was a similar level of displacement of the indigenous *A. flavus* with aflatoxigenic isolates representing 0 to 31% of the population.

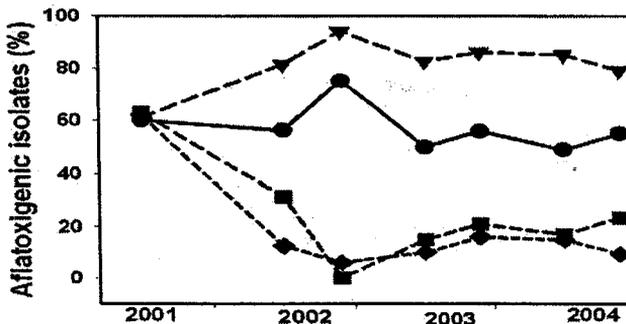


Figure 1. Effect of inoculation on the relative distribution of aflatoxigenic *Aspergillus flavus* isolates in surface soil. Non-inoculated control (●), aflatoxigenic F3W4 (▼), and non-aflatoxigenic CT3 (■), and K49 (◆) sampled one month after planting (May) and after harvest (September) as determined by fluorescence on β -cyclodextrin potato dextrose agar (Mean and standard deviation of four replicates, 10 isolates per replicate).

The potential for colonization of maize by several non-aflatoxigenic isolates was assessed using the pin-bar assay of King and Scott (1982). Four non-toxic isolates (CT3, HA516, HA542 and K49) and the toxigenic strain F3W4 were grown on PDA (7 d), the conidia were removed by scraping, suspended in aqueous Tween 20 (0.2%), and adjusted to a concentration of about 5×10^7 conidia ml⁻¹. Maize ears were inoculated at 20 d after mid-

silking using a pin bar inoculator (100 mm-long row of 36 sewing needles mounted on a wood bar, with 6 mm of the points exposed). Pins were dipped in conidia suspensions or Tween 20 formulation control, and the bars were pressed into the center of the ear. Maize (five ears per treatment) was visually assessed for kernel colonization at various times up to 12 d after inoculation. There was a 2 day lag in colonization by all strains, with less than 10% of the inoculated area colonized by the inoculated strain. The most vigorous colonization of maize was observed by the toxigenic isolate F3W4, and non-toxicogenic isolate K49 (Fig. 2), with both attaining 100% colonization of the inoculated area. The other three non-aflatoxigenic isolates (CT3, HA516 and HA542) colonized at a slower rate and attained a maximum colonization of 75 to 82% of the inoculated area. The formulation control displayed colonization of less than 20% of the inoculated area (data not shown). This data suggests that K49 has a superior ability in colonizing maize. K49 was isolated from corn kernels, while CT3, 542 and HA516 were from soil. The pin-bar technique is a useful assay to demonstrate differential colonization potential by *A. flavus* isolates.

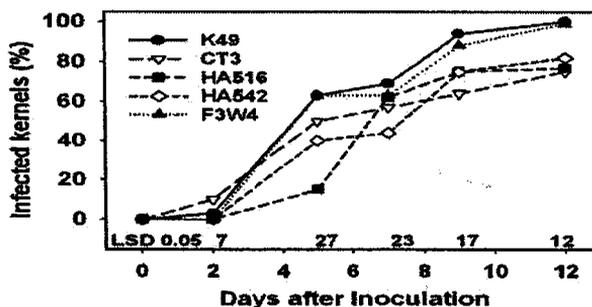


Figure 2. Colonization of corn following pin bar inoculation by toxigenic isolate F3W4, and four non-aflatoxigenic strains in 2004, LSD ($P = 0.05$) indicated in graph for each sample date five, inoculated ears were sampled per treatment at each date.

The effects of inoculation, by single strains or combinations of toxigenic isolate F3W4 with K49 or CT3 is presented in Fig. 3 A & B (data summarized from Abbas et al., 2006).

High levels of aflatoxin were observed in maize grain from control plots in 2001 and 2002. In these two years, inoculation with CT3 and K49 resulted in a decrease of AF concentration from 58 to 86%. Inoculation with the aflatoxigenic isolate F3W4 resulted in a significant increase of aflatoxin concentration compared to the control in all years but 2002, when the natural level of contamination was very high. In 2002, there was less rainfall and higher temperatures than the other three years, facilitating high levels of AF contamination. The use of mixed inoculum treatments may also give additional insight in the potential of non-aflatoxigenic isolates, as there was a 65 to 94 % reduction in AF concentration compared to F3W4. Strain K49 was significantly more effective in reducing AF levels compared to CT3 in three of the four years (2001 to 2003). The relative colonization of maize kernels by toxigenic *A. flavus* was only determined in 2004. When soil was inoculated with a single strain, colonization by K49 and CT3 were equivalent. However, when the non-aflatoxigenic strains were co-inoculated with F3W4, K49 was more effective in displacing toxigenic isolates from maize compared to CT3.

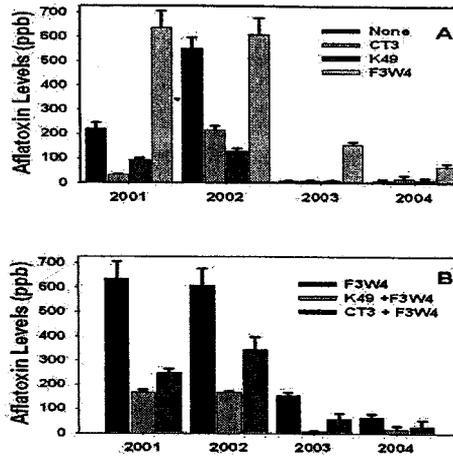


Figure 3. Aflatoxin concentrations in corn as influenced by single strain inoculation (A), and combinations of non-aflatoxigenic strains K49 and CT3 with F3W4 (B), mean and standard deviation of four replicates.

CHARACTERISTICS OF SUPERIOR NON-AFLATOXIGENIC STRAINS FOR CONTROLLING AFLATOXIN CONTAMINATION IN MAIZE

Based upon these studies, we are able to define certain phenotypic characteristics that improve the potential of a non-aflatoxigenic strain to competitively displace colonization by aflatoxigenic populations under field conditions, as summarized in Table 3. Non-aflatoxigenic isolates K49 and CT3 do not produce any aflatoxin or the precursors (averantin, norsoloronic acid, versicolorin or o-methyl-sterigmatocycin) which are produced by F3W4 (Shier et al., 2005). K49 does not produce cyclopiazonic acid which accumulates in high levels in CT3. Cyclopiazonic acid is highly toxic to animals; if high levels are present, it may pose an additional concern for mycotoxin contamination of maize and other crops (Horn and Dörner, 1999; CAST, 2003). Sclerotia are the dominant resting structure for enabling long-term *A. flavus* colonization of soil and crop debris. Although not demonstrated in our studies, the ability to form sclerotia may provide ecological competence for survival in soil during adverse environmental conditions (Wicklów et al., 1984).

Table 3. Desirable characteristics for non-aflatoxigenic isolates of *Aspergillus flavus* for competitive biocontrol of colonization of corn by aflatoxigenic isolates.

Characteristic	Ecological value
No production of aflatoxin (AF) or metabolic precursors	Reduce total AF contamination
No cyclopiazonic acid (CPA)	CPA is toxicogenic to animals
Rapid growth	Improved colonization
Wide substrate use	Improved colonization / survival
Sclerotia formation	Greater potential for soil colonization
Selective maize colonization	Adapted to colonization of maize reproductive tissue; better target control

FUTURE CONSIDERATIONS

The efficacy of biological control systems can be greatly enhanced by adoption of novel formulation / delivery systems (Burgess, 1998). Work completed to date in our group has used soil application to deliver atoxigenic strains. Development of a liquid spray system that can provide superior colonization the maize ear and successfully compete with native toxigenic isolates to exclude AF production would greatly improve opportunities for commercialization. A better understanding of the basic mechanisms of competitive exclusion may also aide in the refinement of this technology. Although biocontrol may offer a unique opportunity to control maize AF contamination, an integrated systems approach combining, resistant hybrids, cultural practices and insect control, along with biological control, would most likely be required to eliminate the problem.

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