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Variability among Atoxigenic *Aspergillus flavus* Strains in Ability To Prevent Aflatoxin Contamination and Production of Aflatoxin Biosynthetic Pathway Enzymes

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Five strains of *Aspergillus flavus* lacking the ability to produce aflatoxins were examined in greenhouse tests for the ability to prevent a toxigenic strain from contaminating developing cottonseed with aflatoxins. All atoxigenic strains reduced contamination when inoculated into developing bolls 24 h prior to the toxigenic strain. However, only one strain, AF36, was highly effective when inoculated simultaneously with the toxigenic strain. All five strains were able to inhibit aflatoxin production by the toxigenic strain in liquid fermentation. Thus, in vitro activity did not predict the ability of an atoxigenic strain to prevent contamination of developing bolls. Therefore, strain selection for competitive exclusion to prevent aflatoxin contamination should include evaluation of efficacy in developing crops prior to field release. Atoxigenic strains were also characterized by the ability to convert several aflatoxin precursors into aflatoxin B_1. Four atoxigenic strains failed to convert any of the aflatoxin biosynthetic precursors to aflatoxins. However, the strain (AF36) most effective in preventing aflatoxin contamination in developing bolls converted all tested precursors into aflatoxin B_1, indicating that this strain made enzymes in the aflatoxin biosynthetic pathway.

Aflatoxins are toxic, carcinogenic compounds produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* (17, 22). Aflatoxin contamination of various commodities can occur as a result of crop infection by one of these fungi. Animal and human health concerns about aflatoxin-tainted commodities have resulted in stringent regulations worldwide on aflatoxin content; these regulations on aflatoxin contamination have a significant international economic impact (26). Aflatoxin B_1 originates from a polyketide precursor according to the following scheme (4, 5): polyketide precursor → norsolorinic acid → averantin → averufanin → 1′-hydroxyversicolorone → versiconal hemiacetal acetate → versicolorin B → versicolorin A → demethylsterigmatocystin → sterigmatocystin → *O*-methylsterigmatocystin → aflatoxin B_1.

Most contamination of corn, cottonseed, and tree nuts is caused by *A. flavus* (17). The incidence of contamination is largely determined by the environment, with preharvest contamination being favored under hot, dry conditions (17, 28). The lack of reliable and practical methods to prevent contamination when environmental conditions are most conducive to *A. flavus* (13, 28) has resulted in a variety of new technologies (8). One such technology is the use of atoxigenic strains of the causal agent (i.e., strains which do not produce aflatoxins) to prevent contamination through competitive exclusion of toxigenic strains during infection (6, 10, 12). Cotton has been used as a model crop for the development of the atoxigenic strain strategy because (i) there exists an easily manipulated greenhouse disease model for cotton (24), (ii) aflatoxin contamination of cottonseed is an important economic problem (13, 17), and (iii) cottonseed is grown for feed and not food and may thus provide an easier target for regulatory approval of atoxigenic strain use.

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Although atoxigenic strains are known to vary in the ability to prevent contamination of cottonseed by toxigenic strains, all seven strains examined thus far showed some effect (12). Little else, however, is known about atoxigenic strain characteristics and the relation of various characteristics to strain efficacy.

In this study, we compared the atoxigenic strain with the greatest known efficacy (isolate AF36 [12]) with other previously identified, frequently cited (10, 20, 23) atoxigenic strains with respect to the ability to prevent toxigenesis both in liquid fermentation and during infection of developing cotton bolls. We have also characterized the atoxigenic strains according to the ability to produce enzymatic activities in the aflatoxin biosynthetic pathway. Relationships among enzymatic activities, phenotype stability, and strain efficacy are discussed.

MATERIALS AND METHODS

Organisms and media. The origins and characteristics of the *A. flavus* strains used in this study have been described (11, 20). Strain AF36 was isolated by the author (11) and shown to be very effective at reducing aflatoxin contamination of developing cotton bolls (12); strains NRRL-5918, NRRL-5565, NRRL-5917, and NRRL-1957 were supplied by S. W. Peterson of the National Center for Agricultural Utilization Research, Peoria, Ill. Isolates were maintained and stored as previously described (11). Inocula for experiments consisted of suspensions of spores from 7-day-old cultures grown on 5% V-8 vegetable juice–2% agar, pH 5.2, at 30°C.

Greenhouse tests. Greenhouse tests to assess strain efficacy were performed as previously described (12). Twenty-eight- to 32-day-old bolls were each inoculated in a single locule through a simulated pink bollworm exit hole made with a cork borer (3-mm diameter). Each wound was inoculated with a 10-μl aliquot of a spore suspension containing approximately 2,000 spores; bolls inoculated with two strains received a 10-μl aliquot of each strain. Bolls were inoculated either with toxigenic strain AF13 alone, with AF13 and an atoxigenic strain (either AF36, NRRL-5918, NRRL-5565, NRRL-5917,

TABLE 1. Effects of five atoxigenic strains of *A. flavus* on the ability of toxigenic strain AF13 to contaminate developing cottonseed with aflatoxin B_1

Atoxigenic strain	Test 1		Test 2	
	Concn of aflatoxin B_1 (μg/g)[a]	% Change[b]	Concn of aflatoxin B_1 (μg/g)[a]	% Change[b]
AF36	45 C	−94	5 B	−86
NRRL-5565	220 CB	−71	38 A	NS
NRRL-5918	327 CB	−57	59 A	NS
NRRL-5917	427 CBA	NS	30 A	NS
NRRL-1957	553 BA	NS	86 A	NS
None[c]	769 A		37 A	

[a] Values are averages of four replicates in test 1 and six replicates in test 2. Values followed by the same letter are not significantly different by Fisher's protected least significant difference test.

[b] Percent difference in aflatoxin content of bolls inoculated with both toxigenic and atoxigenic strains and bolls inoculated with the toxigenic strain alone. NS, change not statistically significant ($P = 0.05$).

[c] Plants were inoculated with the toxigenic strain AF13 alone.

or NRRL-1957) simultaneously, or with an atoxigenic strain first and then AF13 after 24 h. Randomized complete block designs were used, and experiments were performed at least twice.

At maturity (3 weeks after inoculation), bolls were harvested, dried at 60°C for 3 days, and kept at room temperature in plastic bags containing silica gel desiccant until analyzed for aflatoxin content. Aflatoxins were extracted by the method of the Association of Official Analytical Chemists (33) as previously modified (12). Briefly, intact locks were pulverized and extracted with an 85% aqueous-acetone solution. The extract was purified, concentrated, and applied adjacent to aflatoxin standards on thin-layer chromatography plates. After development, the quantity of aflatoxin B_1 was measured with a densitometer with fluorescence capabilities (33).

In vitro tests and enzyme assays. Erlenmeyer flasks (250 ml) containing 70 ml of the defined growth medium of Adye and Mateles (1) were inoculated with approximately 5,000 spores of either an atoxigenic or a toxigenic strain separately or in combination. Flasks were incubated on a rotary shaker at 30°C and 150 rpm for 5 days, at which time 70 ml of acetone was added to each flask to kill the culture and solubilize secreted and cellular aflatoxin. After filtration, equal volumes of water were added to the extracts, the resulting solutions were each extracted twice with 25 ml of methylene chloride, and the extracts were combined and evaporated to dryness. The aflatoxin B_1 content of the extracts was determined by standard thin-layer chromatography procedures as described above.

Enzyme activities were determined by adding known quantities of aflatoxin B_1 precursors to fungal cultures and measuring conversion of these precursors to aflatoxin B_1 as previously described (5, 25). Mycelia (1 g) of either AF36 or NRRL-5918 from 3-day-old cultures were transferred to 10 ml of low-sugar replacement medium containing either 2.0 μg of norsolorinic acid, 2.0 μg of averantin, 2.0 μg of averufanin, 1.0 μg of sterigmatocystin, or 0.6 μg of *O*-methylsterigmatocystin. After 6 h of incubation at 150 rpm and 37°C, metabolites were extracted and analyzed for aflatoxins. Precursor standards were chromatographed on the same plates as extracts to establish the presence or absence of spiked precursors.

RESULTS

Developing cotton bolls inoculated simultaneously with both atoxigenic strain AF36 and toxigenic strain AF13 contained significantly less aflatoxin B_1 at maturity than bolls inoculated with AF13 alone (Table 1). During the present study, strain AF36 was the only consistently effective atoxigenic strain evaluated. Over the past 5 years we have evaluated AF36 in several similar tests for various purposes. All these tests involved at least two treatments: (i) bolls were inoculated with a toxigenic strain alone and (ii) bolls were inoculated simultaneously both with the same toxigenic strain as in treatment 1 and with AF36. In each of these 16 similar greenhouse tests, contamination by a toxigenic strain was significantly ($P = 0.05$ by Fisher's least significant difference test) reduced by AF36 (an average reduction of 95.3%, with a standard deviation of 5.5%). Two atoxigenic strains (NRRL-5917 and NRRL-1957) were consistently ineffective at reducing contamination when simultaneously inoculated with toxigenic strain AF13 (Table 1), whereas two other strains (NRRL-5918 and NRRL-5565) were effective in only one test. Over the past 3 years, strain NRRL-5918 was further evaluated in an additional three similar greenhouse tests in which it was not effective. In liquid fermentations, however, atoxigenic strain NRRL-5918 greatly reduced toxin production by toxigenic strain AF13 (Table 2). This outcome held for the single test in which all five atoxigenic strains were tested and in both tests in which strains AF36 and NRRL-5918 were tested.

When developing cotton bolls were inoculated first with an atoxigenic strain and then 24 h later with a toxigenic strain, all the atoxigenic strains were effective at reducing the toxin content of seed at maturity compared with bolls inoculated with a toxigenic strain alone. Usually, bolls treated with an atoxigenic strain 24 h prior to treatment with a toxigenic strain contained no detectable toxin at maturity (Table 2).

Two atoxigenic strains were characterized by the ability to remove aflatoxin B_1 precursors from spiked cultures and convert these precursors to aflatoxin B_1. Strain AF36 removed

TABLE 2. Influence of two atoxigenic strains of *A. flavus* on toxin production by toxigenic strain AF13 in culture and in developing cotton bolls

Atoxigenic strain	Simultaneous inoculation				Prior (24 h) inoculation of bolls with atoxigenic strain	
	In culture		In cotton bolls			
	Concn of aflatoxin B_1 (μg/g)[a]	% Change[b]	Concn of aflatoxin B_1 (μg/g)[a]	% Change[b]	Concn of aflatoxin B_1 (μg/g)[a]	% Change[b]
AF36	7 B	−97	5 B	−98	0 B	−100
NRRL-5918	14 B	−98	384 A	+24	0 B	−100
None	209 A		309 A		769 A	

[a] Values are averages of four replicates. Values followed by the same letter in the same column are not significantly different by Fisher's protected least significant difference test. Flasks and cotton bolls inoculated with either NRRL-5918 or AF36 alone contained no detectable levels of aflatoxin B_1 (limit of detection, 10 ng/g).

[b] Percent difference in aflatoxin content between treatments with the toxigenic strain alone and treatments with both toxigenic and atoxigenic strains.

TABLE 3. Conversion of aflatoxin precursors to aflatoxins by 3-day-old cultures of *A. flavus* AF36

Precursor[a]	Amt of precursor (μg)	Amt of aflatoxin B_1 (μg)	Amt of aflatoxin B_2 (μg)	% Conversion[b]
None		ND[c]	ND	ND
Norsolorinic acid	2.0	0.24	0.03	12
Averantin	2.0	0.34	0.05	17
Averufanin	2.0	0.41	0.12	21
Sterigmatocystin	1.0	0.45	ND	45
O-Methylsterigmatocystin	0.6	0.32	ND	58

[a] Each precursor was fed in 10 μl of acetone to 1 g of 3-day-old fungal mycelia in low-sugar replacement medium. After 6 h of incubation at 37°C and with constant shaking at 150 rpm, metabolites were extracted and analyzed for aflatoxins. No conversion was detected with strain NRRL-5918.

[b] Efficiency of conversion of metabolites to aflatoxin B_1.

[c] ND, none detected (limit of detection, 10 ng).

all tested precursors from cultures and converted these to aflatoxin B_1. Conversion efficiency increased with precursor closeness to aflatoxin B_1 in the aflatoxin biosynthetic pathway (Table 3). Strain NRRL-5918 did not remove any tested precursor from cultures and failed to produce aflatoxin B_1 in all spiked cultures. When no conversion of an introduced precursor was observed, greater than 70% of the precursor was recovered.

DISCUSSION

Application of atoxigenic strains of *A. flavus* to agricultural fields and crops has been suggested as a potential method for preventing aflatoxin contamination (11, 15, 16). In theory, the applied atoxigenic strains will lower the potential for aflatoxin contamination by competing with aflatoxin-producing strains (10, 16). To date, field evaluation of this concept has been limited. Propagule suspensions of *A. parasiticus* strains which do not produce aflatoxins have been applied to peanuts in environmental control plots in Georgia (18), and autoclaved wheat seed colonized by an atoxigenic strain of *A. flavus* has been applied to cotton grown in field plots in Arizona (14, 16). In those studies, strain applications were associated with both fungal population changes and reductions in the quantity of aflatoxins contaminating the crop at maturity. In greenhouse and field tests, certain atoxigenic strains of *A. flavus* interfere with aflatoxin contamination of developing crops when these crops are inoculated simultaneously with both toxigenic and atoxigenic strains (6, 12). Cotton bolls naturally infected in agricultural fields become infected with multiple *A. flavus* strains at high rates (more than 50% of bolls were infected by multiple strains in one study) (2), and therefore the ability to interfere with contamination during coinfection might be of practical importance. The results reported here indicate that not all atoxigenic strains are effective at reducing contamination under these conditions. Efficacy during coinfection should be considered an important criterion when selecting strains for use in preventing aflatoxin contamination in commercial fields.

The results suggest that atoxigenic strains which fail to produce certain enzymes in the aflatoxin biosynthesis pathway (e.g., NRRL-5918) may not be more likely to reduce contamination by toxigenic strains than atoxigenic strains which do produce these enzymes. Indeed, strain AF36, which produced many of the enzymatic activities present in the pathway but did not produce aflatoxins, was the most effective atoxigenic strain at reducing contamination in the present study.

All four atoxigenic strains which lacked the ability to inhibit aflatoxin contamination of cottonseed when inoculated simultaneously with toxigenic strain AF13 did interfere with contamination when inoculated 24 h before the toxigenic strain. These strains may thus be useful in aflatoxin control strategies seeking to competitively exclude toxigenic strains prior to crop infection, providing that strain displacement is very efficient. However, because of poor competitive ability, atoxigenic strains may fail to prevent aflatoxin production by a toxigenic strain during coinfection of developing crops.

Atoxigenic strain AF36 significantly reduced aflatoxin contamination of developing cottonseed in all tests. However, in one test, even though the aflatoxin content of the seed at maturity was reduced by 94%, the seed still contained 45 μg of aflatoxin B_1 per g (Table 1). Thus, crops exposed to conditions highly conducive to aflatoxin contamination may contain unacceptable contamination levels even when effective doses of atoxigenic strains are applied. However, in most cases, a 90% reduction in contamination will result in a commercially useful cottonseed crop.

Strain NRRL-5918 interfered with aflatoxin production by toxigenic strain AF13 in liquid fermentation but not during infection of developing cotton bolls. Similarly, anthraquinone-accumulating mutants of *A. parasiticus* (19), non-aflatoxin-producing species of the *A. flavus* group (32, 34), and many other fungi (30) interfere with aflatoxin production in culture. The failure of NRRL-5918 to inhibit contamination during coinfection of developing cotton bolls indicates that in vitro interference with aflatoxin biosynthesis is not necessarily related to the ability to inhibit in vivo. Indeed, these results suggest that the mechanism of in vitro inhibition of aflatoxin biosynthesis may differ from that of in vivo inhibition.

Certain atoxigenic strains of *A. flavus* are known to be unstable and to convert to a highly toxigenic phenotype (9, 31). The stability of the aflatoxin-producing phenotype may be an important consideration in selecting strains for use in strategies to prevent aflatoxin contamination through intraspecific competition (6, 10). Neither phenotype described here can be considered more stable on the basis of current information, and each might result from a single mutation.

Strains NRRL-1957, NRRL-5565, NRRL-5917, and NRRL-5918 were previously shown to lack the ability to convert *O*-methylsterigmatocystin and sterigmatocystin to aflatoxin B_1 (23). The results presented here confirm those results. However, in the same report, Lee (23) suggested that production of aflatoxin biosynthesis enzymes by an atoxigenic strain is characteristic only of atoxigenic strains generated in the laboratory and that such enzyme-producing strains are not stable and may convert to a toxigenic form on introduction to a crop. Lee further suggested that these converted strains might cause a net increase in aflatoxin contamination. The results of the present study show that this is not the case. Strain AF36, which produces aflatoxin biosynthetic enzymes, was isolated from an agricultural field and consistently reduced contamination of developing cottonseed by toxigenic strains. Moreover, AF36 has been phenotypically stable through five serial single conidium transfers and in numerous mass transfers in our laboratory (data not shown).

The mechanisms of atoxigenicity of all five atoxigenic strains remain unknown. None of the examined strains accumulate large quantities of either anthraquinone or xanthone precursors of aflatoxins, as do certain atoxigenic strains of *A. parasiticus* (3). This is expected because although atoxigenic *A. flavus* strains are much more common than atoxigenic *A. parasiticus* strains, naturally occurring precursor-accumulating strains of *A. flavus* have not been described (3). Genes affecting aflatoxin biosynthesis occur in several linkage groups (27), and it is unknown which genes or gene clusters are lacking in NRRL-5918. However, if there is a regulatory gene controlling overall expression of the aflatoxin biosynthetic enzymes, as has

been postulated (7, 21, 29), a lesion in that gene could explain the failure of NRRL-5918 to produce pathway enzymes. Mutations in regulatory loci are potential explanations for atoxigenicity of all the examined strains. Strain AF36 converts norsolorinic acid, the earliest known aflatoxin precursor, to aflatoxin B_1. This suggests that strain AF36 either is blocked in a structural gene prior to the described portion of the pathway or is mutated at a regulatory locus governing incorporation of acetate units into the aflatoxin polyketide skeleton. The mechanism of atoxigenicity of AF36 clearly differs from that of NRRL-5918.

AF36 and similar strains may prove to be useful tools in the study of aflatoxin biosynthesis because AF36 produces more enzyme activities in the aflatoxin biosynthetic pathway than any of the previously identified atoxigenic strains of either *A. flavus* or *A. parasiticus*. Thus, AF36 may facilitate the identification of new aflatoxin precursors in feeding studies as well as facilitate studies of potential interactions between various aflatoxin precursors. The use of AF36 in such studies may prevent the occurrence of artifacts caused by model systems using unusual media to restrict toxin production in the presence of pathway enzymes (35) and may also preclude the need for radiolabeled precursors in feeding studies with aflatoxin-producing strains of *A. flavus* and *A. parasiticus* (4, 5, 25).

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