Competitive Exclusion of a Toxigenic Strain of *Aspergillus flavus* by an Atoxigenic Strain

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ABSTRACT

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Several experiments were employed to test the role of competition in the ability of an atoxigenic strain of *Aspergillus flavus* to inhibit the aflatoxin contamination of developing cotton bolls. In initial tests, nitrate-nonutilizing mutants were used to follow seed infection by toxigenic and atoxigenic strains of *A. flavus* in coinoculated bolls. Competitive exclusion was found to contribute to the effect of the atoxigenic strain on contamination, but results suggested a second mechanism may also have been in effect. Aflatoxin contamination by the toxigenic strain was similarly inhibited by an atoxigenic strain in vivo and in liquid fermentation, and the atoxigenic strain was equally effective when applied at spore concentrations either equal to those of the toxigenic strain or one-half those of the toxigenic strain. The atoxigenic strain reduced aflatoxin production in vitro when mycelial balls of the two strains were mixed after a 48-h fermentation period, which suggested that close intertwining of mycelia was not required and that aflatoxin biosynthesis could be interrupted even after initiation. The atoxigenic strain did not degrade aflatoxins in vitro, and both culture filtrates and mycelial extracts of the atoxigenic strain stimulated aflatoxin production by the toxigenic strain. The results suggest that the atoxigenic strain may interfere with the contamination process both by physically excluding the toxigenic strain during infection and by competing for nutrients required for aflatoxin biosynthesis.

Aflatoxins are toxic, carcinogenic fungal metabolites that occur in foods and feeds worldwide (25). Health concerns and regulations that limit the uses of contaminated commodities greatly influence the profitability of several important crops in the United States. (25). Aflatoxins are produced by *Aspergillus flavus* Link:Fr. and *A. parasiticus* Speare when these fungi infect and decay either developing or mature crops (17). Conventional methods for the prevention of contamination are not reliable on a commercial scale for any of the crops affected; this has resulted in several attempts to develop novel control methods (9). One such method is to displace toxigenic strains of *A. flavus* from agricultural fields with strains of *A. flavus* that do not produce aflatoxins (atoxigenic strains) (10). This strategy is possible because of the great diversity of phenotypes of *A. flavus* in agricultural fields and the common occurrence of atoxigenic strains (13,18). Furthermore, toxigenicity is apparently unrelated to a strain's ability to colonize and/or infect living or dead plant tissues (13). These observations led us to speculate that atoxigenic strains might be adapted to conditions that favor aflatoxin contamination, and therefore atoxigenic strains might be used to displace toxigenic strains (13,14). In theory, competitive exclusion of toxigenic strains from crops might reduce the overall toxigenicity of *A. flavus* populations and might even interfere with the contamination process on an infection-by-infection basis (8,10,14).

Aflatoxin contamination of cottonseed is severe in the desert valleys of Arizona and southern California, where most aflatoxin occurs in seed from bolls damaged by the pink bollworm (15). Several atoxigenic strains of *A. flavus* isolated from agricultural fields in Arizona can reduce the aflatoxin contamination of developing cotton bolls caused by toxigenic strains (14). In the previous experiments, the atoxigenic strains greatly reduced contamination when inoculated into developing bolls either prior to or simultaneously with toxigenic strains. In those studies, however, it was not clear whether the atoxigenic strains prevented contamination by physically excluding the toxigenic strains from infected tissues or by directly inhibiting toxigenesis. The studies described here sought a better understanding of the mechanism through which atoxigenic strains reduce contamination of developing bolls.

MATERIALS AND METHODS

Fungal isolates and mutants. *A. flavus* isolates AF36 and AF13 collected from the Yuma Valley of Arizona and previously shown to be pathogenic to cotton were used in all tests (13). AF36 is atoxigenic and reduces aflatoxin contamination caused by the highly toxigenic AF13 (14). AF36 and AF13 were previously shown to belong to different vegetative compatibility groups (3). Cultures were maintained at 31 C in the dark on 5/2 agar (5% V8 vegetable juice, 2% agar adjusted to pH 5.2 prior to autoclaving) (13). For long-term storage, plugs of sporulating cultures were submerged in sterile distilled water and maintained at 8 C (13).

Two different mutants of both AF13 and AF36 were isolated on Czapek-Dox (CD) agar supplemented with chlorate as previously described (2). Mutants were maintained in the same manner as the wild types and characterized by the method of Cove (16). One mutant of each isolate was deficient in the structural nitrate reductase gene, *niaD*$^-$ mutant, and the other was defective in the pathway specific regulatory gene, *nirA*.

Greenhouse tests. To determine isolate behavior, developing cotton bolls were inoculated through simulated exit holes of the pink bollworm as previously described (13). *Gossypium hirsutum* cv. Deltapine acala 90 was grown in 3-L pots containing a 50:50 mixture of Pro-mix (Premier Brands Inc., New Rochelle, NY) and sand. Plants were fertilized weekly with about 100 ml of 2,000 ppm 15-30-15 fertilizer. Flowers were dated at opening. Bolls 29–31 days old were wounded in a single locule with a cork borer (3 mm in diameter) and inoculated with 20 μl of an aqueous spore suspension of each isolate tested. Spore concentrations and number of bolls per treatment are indicated below for each experiment. After boll opening (about 45 days after flowering), bolls were picked, dried in a forced-air oven at 45 C for 3 days, and stored at room temperature in plastic bags containing silica gel desiccant.

To determine whether the AF13 *niaD*$^-$ mutant retained wild-type ability to infect and contaminate developing cottonseed with aflatoxins and whether the *niaD*$^-$ mutant of AF36 retained wild-

type ability to interfere with contamination, wild types and mutants were compared in an initial test with six treatments and three replicates. In four of the treatments, each boll was inoculated with 10,000 spores of either wild types or mutants of either AF13 alone or AF36 alone. In two of the treatments, each boll was inoculated with 10,000 spores each of either mutants or wild types of both AF13 and AF36. After the initial test demonstrated that wild types and mutants behaved similarly, the following tests were performed with the *niaD*$^-$ mutants alone in order to assess competition between the two strains during boll infection. Bolls were inoculated with 5,000 spores of the *niaD*$^-$ mutants of isolate AF13 alone, isolate AF36 alone, or both isolates AF13 and AF36. Tests were randomized and replicated three times. Replicates consisted of two bolls each; the inoculated locule from one boll of each replicate was analyzed for aflatoxin content, and the inoculated locule from the second boll was used for fungal isolations. This test was performed three times.

In greenhouse tests used to compare in vivo activity with in vitro activity, bolls were inoculated with wild-type strains. In the four treatments, bolls were inoculated with 1) 10,000 spores of AF13, 2) 10,000 spores of AF36, 3) 10,000 spores each of both isolates, or 4) 10,000 spores of AF13 and 5,000 spores of AF36. Locules from this experiment were analyzed only for aflatoxin content. Treatments were randomized and replicated four times; each replicate consisted of a single inoculated boll, and the experiment was performed twice.

Fungal isolations and isolate identification. Isolations from seed were made to determine the relative success of AF36 and AF13 in infecting developing cottonseed in coinoculated locules. Seeds were manually separated from the long lint fibers, wetted with 50% ethanol, delinted, surface-sterilized in concentrated H_2SO_4 for 2–5 min, rinsed twice in sterile water, and plated on CD agar amended with antibiotics (4). Five isolations were made from colonies growing from each of four seeds from each inoculated locule for a total of 20 isolations per locule. Isolates were then paired with *nirA*$^-$ mutants of both AF13 and AF36 on CD agar, and complementation reactions after 10 days at 31 C were used to identify the isolate (3). The test was performed three times. In the first two tests, isolations were made from bolls inoculated with AF13 and AF36 alone and from bolls inoculated with both isolates. In the third test, isolations were made only from bolls inoculated with both isolates.

In vitro interactions. Interactions of strains AF36 and AF13 in vitro were assessed in the liquid medium of Adye and Matales (1) with either 3 g/L of NH_4SO_4 (NH_4 medium) or 3 g/L of $NaNO_3$ (NO_3 medium) as the sole nitrogen source as previous described (11). Erlenmeyer flasks (250-ml) containing 50 ml of either NO_3 medium or NH_4 medium were inoculated with either 15,000 spores of AF13 per milliliter, 15,000 spores of AF36 per milliliter, 15,000 spores of AF13 and 15,000 spores of AF36 per milliliter, or with 15,000 spores of AF13 and 7,500 spores of AF36 per milliliter. Flasks were incubated in the dark on an orbital shaker (150 rpm) for 4 days, after which 50 ml of acetone was added to each flask to lyse fungal cells and extract the aflatoxins from the mycelium.

To ascertain potential inhibitory effects of AF36 on growth of AF13, isolates were paired on 5/2 agar and on CD agar by the inoculation of wells (3 mm in diameter) in the agar. The wells were spaced 2 cm apart. Two wells on each plate were inoculated with either the same isolate or with different isolates. Plates were incubated at 31 C and visually examined after 5 days for zones of inhibition.

To determine the influence of actively growing mycelia of AF36 on aflatoxin production by mycelia of AF13, flasks containing NO_3 medium were inoculated with each isolate individually and shake-incubated as described above. After incubation periods of 0, 5, 10, 24, and 48 h, the contents of a flask containing AF13 and another containing AF36 were combined and mixed by swirling (2 min). The contents were then divided into approximately 50 ml per flask and shake-incubated for the remainder of 5 days from the initial inoculation; i.e., cultures mixed after 24 h were incubated for an additional 4 days. Control flasks inoculated with AF13 alone were incubated for 1, 2, or 5 days and included in the randomized experimental design. After the incubation period, fungal growth was stopped by the addition of 50 ml of acetone, and aflatoxin analyses were performed as described below. This test was performed three times and replicated four times; the four replicates resulted from two separate mixings of paired flasks each consisting of one flask with AF13 and one flask with AF36.

Filtrates, mycelial extracts, and degradation. To test the involvement of factors that interfere with aflatoxin biosynthesis, AF36 in NO_3 medium was shake-cultured as described above for 4 days at 31 C; the filtrate was then recovered and substituted for the water component of the NO_3 and NH_4 media. The influence of mycelial extracts of AF36 on aflatoxin biosynthesis by AF13 was also tested. Mycelial balls (15–20 g) from 4-day-old NO_3 medium shake cultures were blended at low speed for 30 s in 100 ml of distilled water. The aqueous extract was then filter-sterilized and added to NO_3 medium at a 10% rate (v/v). To test for degradation of aflatoxin by AF36, filtrates from 4-day-old shake cultures of AF13 in NO_3 medium were substituted for the water component of NO_3 medium as described above. In all three experiments, the media were filter-sterilized, dispensed into 50-ml Erlenmeyer flasks (30 ml per flask), inoculated, and incubated as described. Mycelial extract and filtrate experiments were inoculated with approximately 5,000 spores of AF13 and incubated for 4–5 days. Aflatoxin degradation tests were inoculated with approximately 5,000 spores of AF36 and incubated for 7 days. The experiments were performed at least twice and contained two to four replicates.

Aflatoxin analyses. Culture filtrates containing 50% acetone (v/v) were filtered through #4 Whatman filter paper. Fifty milliliters of filtrate was added with an equal volume of water to a 250-ml separatory funnel, and the solution was extracted twice with 25 ml of methylene chloride. The methylene chloride extracts were filtered through 50 g of anhydrous sodium sulfate to remove residual water, and the sodium sulfate was rinsed with an additional 25 ml of methylene chloride after filtration. The rinse and extracts were combined, evaporated at room temperature, and the residual was dissolved in 4 ml of methylene chloride. The extracts and aflatoxin standards were separated on thin-layer chromatography plates (silica gel 60, 250 mm) by development with diethyl ether-methanol-water (96:3:1) (32). Extracts were either concentrated or diluted to permit accurate densitometry (27), and aflatoxin B_1 was quantified with a scanning densitometer (model cs-930, Shimadzu Scientific Instruments, Inc., Tokyo) after development (27).

Infected cotton locules were extracted as previously described (12). Dried intact locules were hammered to pulverize the seed and added to 200 ml of acetone and water (85:15). The mixture was shaken for 15 s, allowed to set overnight, and filtered through a #4 filter paper. A 100-ml portion of the filtrate was mixed with 100 ml of an aqueous solution of 0.22 M $Zn(CH_3COO)_2$ and 0.008 M $AlCl_3$, allowed to set for 1–2 h, and filtered again. A 100-ml portion of the filtrate was added to a 250-ml separatory funnel, and aflatoxin extraction and analysis were performed as described for culture filtrates.

Statistical analysis. Analyses were performed with CSS:Statistica (Statsoft, Inc., Tulsa, OK) and Excel (Microsoft Corporation, Redmond, WA). Treatments were randomized within experiments. Analysis of variance was used to test differences among treatments prior to application of multiple comparison techniques. Comparisons of proportions were made with the *Z* test (29).

RESULTS

Toxin content of inoculated bolls. Bolls inoculated with *niaD*$^-$ mutants of both AF13 and AF36 had 0–20% of the aflatoxin content of bolls inoculated with the *niaD*$^-$ mutant of AF13 alone (Table 1). The AF13 *niaD*$^-$ mutant retained wild-type ability to infect and contaminate developing cottonseed with aflatoxins, and the AF36 *niaD*$^-$ mutant retained wild-type ability to inhibit aflatoxin contamination. In three experiments, locules inoculated

with the AF36 *niaD*$^-$ mutant alone contained no detectable aflatoxins, and locules inoculated with the AF13 *niaD*$^-$ mutant alone had 75–440 μg of aflatoxin B_1 per gram. Locules inoculated with wild-type AF13 typically contained similar toxin levels (14). In the one head-to-head test performed, locules inoculated with the AF13 *niaD*$^-$ mutant alone contained 159 ± 39 μg/g, and locules inoculated with AF13 wild-type contained 176 ± 22 μg/g.

Seed isolations from inoculated bolls. Results of isolations from locules inoculated with both *niaD*$^-$ mutants varied among the three experiments. In test 1, 70% of all isolates were AF36, and AF36 alone was isolated from one locule (five isolates from each of four seeds) and from three of eight seeds from two other locules. Only one seed (8%) was infected by AF13 alone. One-third of the seeds from the three locules were infected with both AF13 and AF36 (Table 1). In test 2, AF36 alone was isolated from one locule and from three of four seeds in a second locule. However, AF13 alone was isolated from one locule. Data from test 2 is presented in Table 1 with and without the locule containing only AF13 (explained further in Discussion). In test 3, both AF13 and AF36 were isolated from all three locules, although 80% of all isolates in test 3 were AF36. AF36 alone infected seven of 12 seeds (58%), AF13 alone infected one of 12 seeds (8%), and four of 12 seeds were infected by both isolates. When isolations were made from seeds produced in locules inoculated with only one mutant, only that mutant was recovered.

Mutants of AF13 remained stable throughout the tests. However, in two of the in vivo tests, the *niaD*$^-$ mutant of strain 36 partially reverted to wild type in all bolls sampled. These revertants exhibited growth on CD agar intermediate between that of the wild type and the mutant. However, the growth remained sparse enough to permit detection of complementation between the revertant and the *nirA*$^-$ tester mutant.

In vivo versus in vitro activity. At maturity, locules inoculated with AF13 alone contained aflatoxin B_1 levels above 50 μg/g in all experiments. Locules inoculated with both AF13 and AF36 contained less than 10% of the aflatoxin in locules inoculated with AF13 alone. However, the quantity of toxin did not differ significantly ($P = 0.05$ according to Fisher's protected least significant difference test) between locules inoculated with equal quantities of AF13 and AF36 and those inoculated with twice as much AF13 as AF36 (Table 2). Similar results were obtained in liquid fermentation in both NO_3 and NH_4 media. High toxin levels were produced in fermentations inoculated with AF13 alone, and low toxin levels occurred in fermentations inoculated with AF13 and AF36 together. The quantity of toxin in fermentations inoculated with twice as much AF13 as AF36 was equal to the toxin content of fermentations inoculated with equal quantities of AF13 and AF36.

On both CD and 5/2 agar media, mycelia of each strain grew until it met the mycelia of the other strain. The strains did not overgrow each other, and no zones of inhibition formed. Sporulation and mycelia were less dense where the colonies met, but this also occurred in self-confrontations.

When AF36 and AF13 were fermented individually, mixed after various periods, and refermented for the remainder of the 120-h test period, significantly less aflatoxin B_1 was produced by AF13 than in 120-h fermentations of AF13 alone (Table 3). In tests 1 and 3, linear regressions of aflatoxin content of mixed cultures with hours of growth until mixing were significant ($P = 0.049$–0.026). However, the R^2 values were low ($R^2 = 0.26$–0.34). In tests 2 and 3, the aflatoxin content of cultures mixed after 48 h was significantly less than that of unmixed controls, even though aflatoxin production by AF13 had already begun at 48 h (Table 3).

Filtrates, mycelial extracts, and degradation. Culture filtrates of toxigenic AF13 that were filter-sterilized, supplemented with the nutrients of NO_3 medium, and incubated at 31 C for 5 days did not have significantly different toxin levels than similar filtrates inoculated with AF36 prior to the incubation. All filtrates contained over 15 μg of aflatoxin B_1 per gram at the end of the experiments. Degradation of aflatoxin B_1 was thus not observed.

Supplementation of NO_3 medium with either filtrates or

TABLE 1. Seed infected (%) with two strains of *Aspergillus flavus* at maturity after immature bolls were coinoculated with both strains and percent reduction of aflatoxin in bolls coinoculated compared to bolls inoculated with strain AF13 alone[a]

Test	Percent reduction[b]	AF36[c]	Seed infected by inoculated strain(s)[d]		
			AF36 alone	AF13 alone	AF13 and AF36
1	82	70*	58*	8	33
2	100	64	58	33	8
2B[e]	100	96*	88*	0	13
3	99	80*	58*	8	33

[a] Toxin levels and seed infection were measured in parallel samples within each replicate.

[b] Percent reduction in aflatoxin B_1 content of seed from bolls coinoculated with atoxigenic strain AF36 and toxigenic strain AF13 compared to bolls inoculated with strain AF13 alone. Bolls inoculated with strain AF13 alone contained 76, 176, and 444 μg of aflatoxin B_1 per gram in tests 1, 2, and 3, respectively.

[c] Percentage of total isolates (55–60 isolates per test) from infected seed from locules inoculated with both AF13 and AF36 and identified as AF36. * = Values significantly ($P = 0.05$) greater than 50 by the Z test for proportions (29).

[d] At maturity, seeds were acid delinted, washed, and plated on selective medium; five isolations were made from each seed, and isolates were classified as AF36 or AF13 as described. Values represent the percentage of total seed infected by *A. flavus* that were infected by individual strains. There were 12 seeds per test, four seeds per replicate, five isolations per seed. * = AF36 values significantly ($P = 0.05$) greater than AF13 values by the Z test for proportions (29).

[e] In test 2, 57 isolates from 12 seeds from three bolls were evaluated; four seeds contained AF13 but not AF36. These all occurred in one boll (boll x) from which AF36 was not recovered. Test 2B is the data from test 2 minus boll x.

TABLE 2. Growth, toxin production, and alteration of culture pH by two strains of *Aspergillus flavus* grown individually and in combination

Strain ratio[a] (13:36)	Aflatoxin B_1			Final pH[b]		Fungal mass (g)	
	In vivo[c]	NH_4[d]	NO_3[d]	NH_4	NO_3	NH_4	NO_3
1:0	501 x[e]	445 x	2.34 x	2.13 x	6.10 x	0.48 y	0.38 y
0:1	0 z	0 z	0 z	2.28 x	5.96 x	0.43 y	0.45 yx
1:1	8 y	30 y	3 y	2.30 x	5.71 x	0.50 xy	0.54 x
2:1	7 y	45 y	9 y	2.24 x	5.68 x	0.52 x	0.53 x

[a] Flasks containing 50 ml of medium were seeded with 16 conidia per microliter of either AF13 or AF36. For two treatments, flasks that were seeded with AF13 were also seeded with either 16 or eight conidia per microliter of AF36. Flasks were incubated at 28 C for 4 days prior to being analyzed for aflatoxin content.

[b] Final pH of the culture medium; initial pH was 5.0.

[c] Cotton bolls 28–30 days old were inoculated with 10,000 conidia of either AF13 or AF36; plants in two treatments were inoculated with both AF13 and either 10,000 or 5,000 conidia of AF36. At maturity, seed from all inoculated bolls were analyzed for aflatoxin content, which is expressed as microgram per gram of whole seed.

[d] NH_4 = the NH_4 medium; NO_3 = the NO_3 medium. Aflatoxin is expressed as micrograms per gram of mycelium.

[e] Values for in vivo tests are averages of four replicates; values for in vitro tests are averages of three replicates. Values within a column followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test.

mycelial extracts of AF36 stimulated aflatoxin production by AF13 as compared to unsupplemented NO_3 medium in every experiment. In one test, AF36 filtrates increased total aflatoxin B_1 production nine-fold, and mycelial extracts increased production 68-fold (to 199 μg per 70 ml fermentation), although in another test, toxin production was stimulated only four-fold by the mycelial extract.

DISCUSSION

The *niaD*$^-$ mutants of AF36 and AF13 were useful in discerning strain prevalence during infection of developing cottonseeds in locules inoculated with both strains. Nitrate-nonutilizing mutants have previously been used to study plant pathogen population structure (3), to follow survival of *Fusarium oxysporum* in soil under field conditions (22), and to differentiate between phenotype switching and contaminants during host-passaging experiments with *Septoria nodorum* (26). Both the *Septoria* and *Fusarium* mutants retained virulence (22,26). Similarly, in the tests reported here, the *niaD*$^-$ nitrate-nonutilizing mutants retained virulence to developing cotton bolls. Furthermore, the *niaD*$^-$ mutant of AF13 retained the ability to contaminate developing cottonseed with aflatoxins, and the *niaD*$^-$ mutant of AF36 retained the ability to interfere with the process of contamination when coinoculated into developing cotton bolls. Similar mutants may have further uses in the investigation of interactions between strains in soils and on crop surfaces.

Although the *niaD*$^-$ mutant of AF36 was stable through 10 serial passages in culture, during infection of developing cotton bolls this mutant partially reverted to wild type in two of three experiments. Reversion did not occur with several strains of *Septoria nodorum* (26). The partially reverted AF36 mutants were still useful in complementation tests. However, reversion may have confused mutant identification as nitrate-nonutilizing if this was the sole criterion for identification. Thus, caution should be exercised when similar mutants of *A. flavus* are used to monitor strain migration in agricultural fields, as was done with *F. oxysporum* (22).

In all three boll inoculation tests, AF36 infected a greater percentage of the developing seeds than did AF13. Thus, the ability of AF36 to inhibit contamination by AF13 may partly be due to competitive exclusion of AF13 during infection of the developing cottonseed. In test 2, apparently one developing boll was inadvertently inoculated with only AF13; if AF13 had dominated in any of the locules analyzed for toxin, aflatoxin B_1 would have been detected. The data for test 2 without the outlier boll (the boll infected with AF13 alone) probably better represents the true situation. Although competitive exclusion apparently is one mechanism through which AF36 reduces boll contamination, a second mechanism may also occur. This is suggested by the percent reduction in aflatoxin content that results from simultaneous inoculation with AF36 exceeding the percentage of total isolates identified as AF36 in each test (Table 1).

TABLE 3. Influence of atoxigenic strain AF36 on toxin production by toxigenic strain AF13 when strains are cultured separately for 0–48 h prior to mixing[a]

Treatment	Aflatoxin B_1[b] (μg/g of mycelium)		
	Test 1	Test 2	Test 3
Mix after			
0 h	1.4	8.5	5.2
5 h	2.4	11.4	0.5
10 h	17.7	28.8	2.7
24 h	ND	10.4	22.8
48 h	ND	5.0	30.8
AF13 control[c]	76.0	64.2	138.5

[a]Flasks containing 70 ml of NO_3 medium were inoculated with either AF13 or AF36. After various periods, media containing AF36 were mixed with equal quantities of media containing AF13. The mixed contents were then divided into equal portions and returned to the original number of flasks. The resulting flasks were shake-incubated for the remainder of the 120-h incubation period and analyzed for aflatoxin content.

[b]Values are means of four replicates. Linear regressions (aflatoxin content of mixed cultures vs. hours growth until mixing) were significant for tests 1 and 3 ($R^2 = 0.34$, $P = 0.049$ and $R^2 = 0.26$, $P = 0.026$, respectively) but not for test 2. For each test, all mix treatments differed significantly ($P = 0.05$) from the unmixed controls according to Fisher's protected least significant difference test. ND = not determined.

[c]Control flasks were incubated with AF13 alone for 120 h. In tests 2 and 3, flasks with AF13 alone were also analyzed after 24 and 48 h. In both cases, no toxin was detected after 24 h and between 1 and 2 μg/g of mycelium was detected after 48 h.

A second mechanism of action is also suggested by the ability of AF36 to interfere equally with contamination of developing cotton bolls by AF13 when either coinoculated at equal spore concentrations or at one-half the spore concentration of AF13. This phenomenon also occurred in liquid fermentation, and yet on solidified agar media at the same temperature as the fermentations, AF13 and AF36 did not appear to inhibit the growth of each other. Similar inhibition of aflatoxin production in liquid fermentation was observed when *A. parasiticus* was cofermented with *A. parasiticus* mutants blocked at specific steps in the aflatoxin biosynthetic pathway (20). Such mutants accumulate intermediates in the biosynthetic pathway and still exert a negative influence on aflatoxin biosynthesis without interfering with wild-type growth (21). In those studies, the influence of the mutants was not attributable to the mutants merely outgrowing the wild types. In experiments reported here, we tested whether the influence of AF36 on aflatoxin production was attributable to either degradation of aflatoxins, production of compounds inhibitory to aflatoxin biosynthesis, or competition for nutrients required for aflatoxin biosynthesis.

Degradation of aflatoxin by several fungi has been demonstrated, and even some strains of *A. flavus* and *A. parasiticus* partially degrade aflatoxin B_1 after biosynthesis has stopped (5,7). In the current studies, AF36 did not degrade aflatoxin B_1. Degradation experiments were performed in medium with NO_3 as the sole nitrogen source to prevent degradative effects attributable to low pH (11). Degradation is apparently not a mechanism through which AF36 acts. These observations are not necessarily in conflict with previous observations of degradation of aflatoxins by *A. flavus*, because the ability of a strain to degrade aflatoxin is thought to be correlated with strain ability to produce aflatoxins; atoxigenic strains are thought to have little ability to degrade aflatoxins (19).

AF36 exerted influence on aflatoxin production by AF13 even when AF13 mycelium was actively growing prior to exposure to AF36. Significant reductions occurred even when AF13 had initiated aflatoxin biosynthesis prior to exposure. Thus, inhibition of aflatoxin biosynthesis by AF36 differs from inhibition caused by both fungistatic and fungicidal agents that also inhibit growth; these chemical inhibitors are ineffective after initiation of aflatoxin biosynthesis (6,24,32). When spores are mixed in shake fermentation, the resulting mycelial balls are agglomerations of numerous germlings. Thus, mycelia of the two strains become closely intertwined. Cultures mixed after 12 h have already formed mycelial balls. Inhibition in these mixtures indicates that mycelial intertwining is not required for activity of AF36. Similarly, intertwining of mycelium was not required for *A. niger* interference with aflatoxin production (31). These observations, taken together, suggest diffusible factors may be involved in the inhibitory ability of AF36.

Many bacteria and fungi can interfere with aflatoxin production (21,24,30). However, microbes that are as effective as AF36 are rare. Reduction in aflatoxin production in corn substrates coinoculated with *A. niger* and *A. flavus* have been attributed to alterations in substrate pH (23); no significant influence of AF36 on pH was observed in any of the tests reported here, suggesting alteration in pH is not a significant mechanism. Similarly, Shantha and coworkers found that pH was not involved in inhibition of aflatoxin production by *A. niger* and *A. tamarii* in liquid fermentation (31). These workers provided evidence for chemical inhibitors of aflatoxin biosynthesis. Results of the current study lead us to conclude that inhibition caused by AF36 probably does not

involve inhibitors. This conclusion is supported by stimulation of aflatoxin production by both culture filtrates and mycelial extracts of AF36. These observations do not rule out the possibility that short-lived inhibitors, i.e., the volatiles described by Zeringue and McCormick (33), might play a role. However, stimulation of toxigenesis by filtrates and mycelial extracts of AF36 suggests AF36 may reduce toxin production merely by competition for nutrients.

In one study of naturally infected cotton bolls, at least 50% were infected with multiple strains of *A. flavus* at maturity (3). In the current study, bolls inoculated at wounding with two strains often became predominantly infected by a single strain. Competition between strains initially infecting bolls may therefore cause an underestimation in the frequency of multiple infections. This competition may be an important determinant of the extent of contamination of naturally infected bolls. The ability of an atoxigenic strain to compete during colonization and infection of wounded locules may be a prerequisite for strain efficacy in the reduction of contamination in locules with multiple infecting strains.

Most studies on the physiology of aflatoxin formation have used only one isolate at a time. However, part of the possible range of phenotypes of a fungus may be expressed only when a mycelium confronts another genetic individual (28). The genotypic diversity of *A. flavus* in cotton fields and even in cotton locules ensures that competition between strains of *A. flavus* occurs in developing bolls. The data presented here show that this competition may have a complex effect on aflatoxin contamination and also suggests creative solutions for its control.

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