Influence of Field Application of an Atoxigenic Strain of *Aspergillus flavus* on the Populations of *A. flavus* Infecting Cotton Bolls and on the Aflatoxin Content of Cottonseed

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ABSTRACT

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An atoxigenic strain of *Aspergillus flavus* was applied to soils planted with cotton in Yuma, Arizona, to assess the ability of the atoxigenic strain to competitively exclude aflatoxin-producing strains during cotton boll infection and thereby prevent aflatoxin contamination of cottonseed. In both 1989 and 1990, the atoxigenic strain displaced other infecting strains during cotton boll development. Displacement was associated with significant reductions in the quantity of aflatoxins contaminating the crop at maturity. Although frequency of infected locules differed between years (1% versus 25%), in both years displacement occurred without increases in the amount of infection as measured by the quantity of locules with bright-green-yellow-fluorescence (BGYF). In the low infection year (1990), locules exhibiting BGYF were analyzed individually for both incidence of the applied strain and aflatoxin content. In the high infection year (1989), infected seed from each replicate plot (32 total) were pooled and analyzed for both aflatoxin and incidence of the released strain. Results of the latter analyses indicate an inverse relationship ($r = 0.71$, $P < 0.001$) between aflatoxin content and the percent seed infected by the applied strain. In 1990, quantities of *A. flavus* on mature crop surfaces did not differ between treated and untreated plots. When reisolated from the infected crop the applied atoxigenic strain retained the atoxigenic phenotype. Most infecting strains belonging to other vegetative compatibility groups did produce detectable quantities of aflatoxin B_1 in liquid fermentation. The applied atoxigenic strain spread from treated plots to untreated controls at different rates in the two years and accounted for 7 and 25% of *A. flavus* strains isolated from infected locules in untreated control plots in 1990 and 1989, respectively. The results suggest that the aflatoxin-producing potential of *A. flavus* populations associated with crop production can be reduced in order to reduce aflatoxin contamination.

Additional keywords: biocompetition, biological control, population displacement.

Aflatoxins are toxic, carcinogenic fungal metabolites produced by certain isolates of the species *Aspergillus flavus* Link:Fr., *A. parasiticus* and *A. nomius* (37). Concern for human and animal health has led to regulatory limitations on the quantity of aflatoxins permitted in foods and feeds throughout most of the world (40). The most toxic and highly regulated aflatoxin is B_1 (40,33). Aflatoxin contamination has long been a concern for several U.S. crops and for animal industries that depend on susceptible crops for feed (33). Whole cottonseed and cottonseed products are commonly fed to various livestock, including dairy cows. Aflatoxins in contaminated seed can be readily transferred to milk in slightly modified form (32,35). U.S. regulations prohibit aflatoxin concentrations over 0.5 μg/kg in milk. Dairies producing milk tainted with unacceptable aflatoxin levels can have milk destroyed and entire operations temporarily shut down and quarantined (26). To prevent unacceptable aflatoxin levels in milk, the regulatory threshold for aflatoxin B_1 in cottonseed fed to dairy cows is 20 μg/kg (32,33).

Populations of the primary causal agent of aflatoxin contamination of cottonseed, *A. flavus,* are highly complex and composed of strains that differ morphologically, physiologically, and genetically (4,6,14). Differences among strains in ability to produce aflatoxins are well known (24) and aflatoxin-producing ability is not correlated with strain ability to colonize and infect cotton, *Gossypium hirsutum* L. (14). These observations led to the suggestion that atoxigenic strains of *A. flavus* might be used to exclude toxigenic strains through competition during infection of developing crops and thereby prevent aflatoxin contamination (14,20). In both greenhouse and field experiments, wound inoculation of developing cotton bolls and corn ears with toxigenic and atoxigenic strains simultaneously led to reductions in aflatoxin contamination of the developing crop parts as compared with controls inoculated with only the toxigenic strains (9,15). Atoxigenic strains were effective at preventing postharvest aflatoxin contamination both when the crop was infected naturally in the field and when inoculated after harvest (9). Similarly, in special environmental control plots, peanuts were protected from preharvest aflatoxin contamination by irrigating the developing crop with conidial suspensions of *A. parasiticus* strains that accumulate specific aflatoxin precursors (i.e. *O*-methylsterigmatocystin and versicolorin-A) but not aflatoxins (25).

Aflatoxin contamination of cottonseed can be minimized by early harvest, prevention of insect damage, and proper storage (17,18). However, even under careful management, unacceptable aflatoxin levels may occur from unpreventable insect damage to the developing crop (22) or from exposure of the mature crop to moisture either prior to harvest (18), or during storage in modules (36), handling, transportation, or even use (17). Competitive exclusion of aflatoxin-producing strains of *A. flavus* with atoxigenic strains of the same fungal species may provide a single method for preventing aflatoxin accumulation throughout crop production and utilization (11,14,15,20).

In the United States, aflatoxin contamination of cottonseed is most consistent and severe in the irrigated western desert valleys where most contamination is associated with pink bollworm dam-

age (17,22). Contamination levels are highly variable within fields, plants, and even bolls (17,22,30) and contamination is often associated with bolls exhibiting bright-green-yellow-fluorescence (BGYF) on the lint under ultraviolet light (2). BGYF occurs when kojic acid produced by *A. flavus* reacts with peroxidases in developing cotton bolls (31); therefore, BGYF indicates boll infection by *A. flavus* prior to boll maturity via wounds (i.e., pink bollworm exit holes) or infection of partially open bolls (18,28). Because bolls infected through wounds during development accumulate very high aflatoxin levels (13,22), when BGYF is detected, most aflatoxin contamination is associated with the component of the crop exhibiting BGYF (2,18,36). During seasons when aflatoxin contamination is severe, *A. flavus* populations increase as the cotton crop is produced (29). In theory, application of an atoxigenic *A. flavus* strain early in the season should permit the atoxigenic strain to compete with resident toxigenic strains both during crop infection and during population increases associated with cultivation (11). Results of greenhouse studies suggest that the end result of this competition might be reduced aflatoxin in the crop (9,15,21). The current study sought to determine efficacy of an atoxigenic strain in preventing aflatoxin contamination of cottonseed produced in an irrigated desert valley in western Arizona. Summaries of preliminary aspects of this work have been published (16,19).

MATERIALS AND METHODS

Cultures and inoculum preparation. Atoxigenic *A. flavus* strain AF36, previously shown in greenhouse tests to exclude aflatoxin-producing strains competitively during infection of developing cotton bolls was used in all field tests (15,21). Active cultures were maintained in the dark at 32 C on a modified V8 vegetable juice medium (5/2 agar, 5% V-8 vegetable juice, 2% agar, pH 5.2) (14). For long-term storage, plugs (3 mm in diameter) of sporulating cultures were submerged in 5 ml of sterile distilled water and kept at 4 C (14).

Two types of inoculum were produced. Conidial suspensions were produced from 14-day-old cultures grown as above. Plates were flooded with 0.01%, w/v, Triton X-100, the colony surface was agitated with a rubber policeman to dislodge the spores, and spore concentrations were determined with a hemacytometer. Suspensions were diluted to 2×10^7 spores/ml in 0.01% Triton X-100. The second form of inoculum was autoclaved wheat seed that had been colonized by AF36 (20). Whole red winter wheat was purchased from a health food store, autoclaved (1 h, 120 C), allowed to set at room temperature for 18 h, and autoclaved again. Wheat was dried in culture bottles with loose caps in a forced air oven at 60 C for 2 days. The wheat was then seeded with AF36 (approximately 200,000 spores per milliliter) in sufficient water to bring the moisture level of the wheat to between 20 and 25% (w/w). Subsequently, 220 g of wheat was incubated in each 490-cm^2 roller bottle (Corning, Inc., Corning, NY) on a roller drum (5 RPM, 28 C, 7 days). During this incubation the fungus grew in the folds of the seed and under the seed coat but very few or no spores were produced and the appearance of the wheat remained unchanged.

Field plots. In both 1989 and 1990, at the Yuma Valley Agricultural Center near Yuma, AZ, cotton (cv. Deltapine 90) was planted in mid-March (9 March 1989 and 14 March 1990) on a silty clay loam soil in rows on 1-m centers. In both years, fields were furrow-irrigated eight times including a preplant irrigation. The experimental design in 1989 was a randomized complete block design augmented with an additional untreated control and

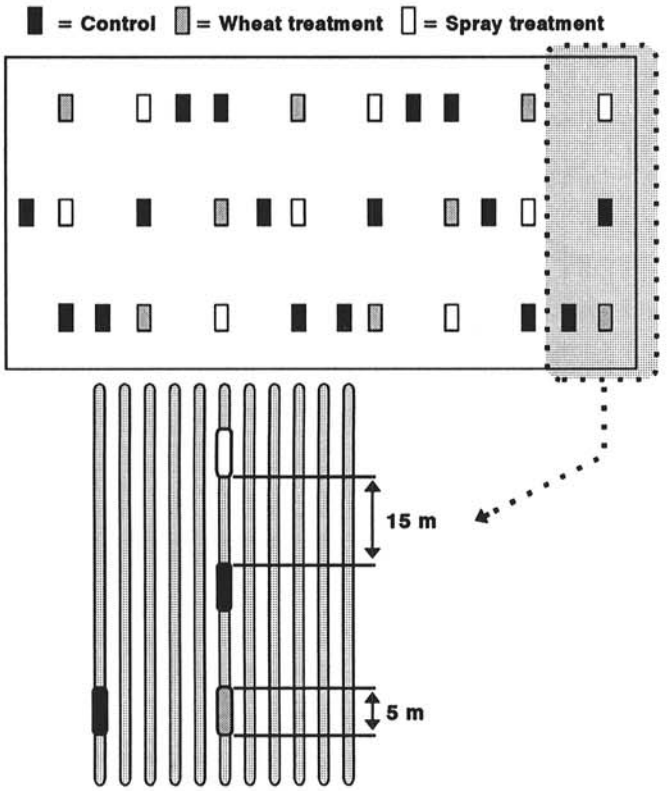

Fig. 1. Field plot design of the 1989 experiment, which contained two untreated controls and eight replicate blocks. Each replicate block was 76 m long and 11 rows wide (1 m centers). Wheat = area where autoclaved wheat seed colonized by *A. flavus* strain AF36 was applied; Spray = area where a conidial suspension of AF36 was applied; Control = sampling area for untreated control.

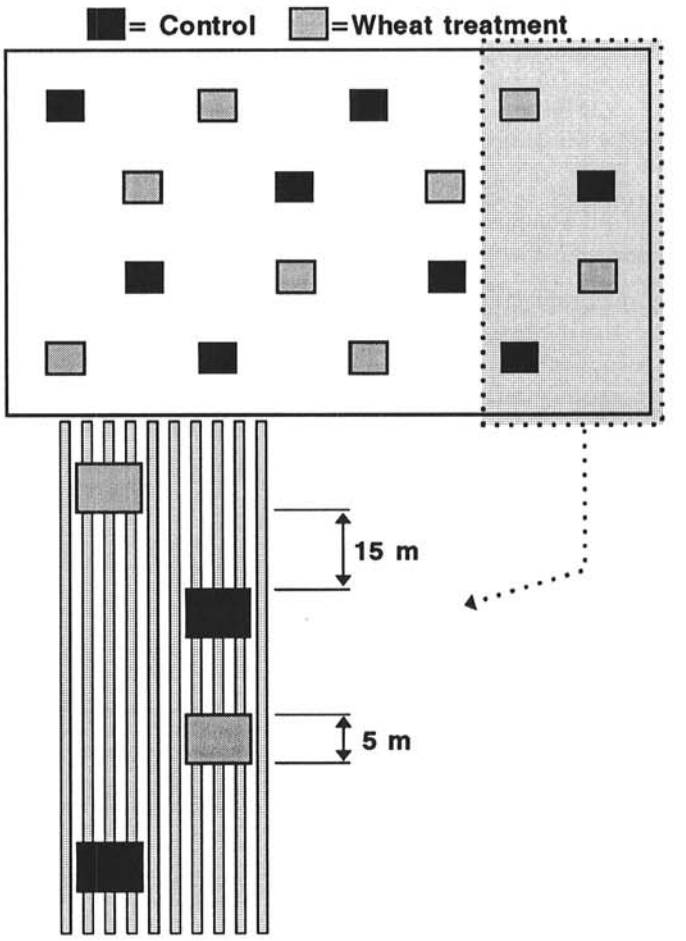

Fig. 2. Field plot design of the 1990 experiment, which contained only one treatment and one untreated control. Each replicate block was 75 m long and 8 rows wide (1 m centers). Wheat = area where autoclaved wheat seed colonized by *A. flavus* strain AF36 was applied; Control = sampling area for untreated control.

replicated eight times (Fig. 1). Each block contained 11 76-m rows of cotton and only the center row was treated. Treatments were applied to 5 m of row and were separated within the row by 15 m of untreated cotton. A second untreated control, designated control 2, was positioned in the first row of each block (Fig. 1). In 1990 the blocks were reduced to three rows and only the wheat treatment was used (Fig. 2). The treated areas were 5 m long and three rows wide and only the center row of each treatment was sampled. The blocks were separated by two untreated rows. Treatments in blocks 1, 3, 5, and 7 started 5 m into the field and were separated by 55 m of untreated cotton; treatments in replicate blocks 2, 4, 6, and 8 started 25 m into the field and were separated by 15 m of untreated cotton (Fig. 2).

Fields used in the 2 yr were 1.2 km apart. The field used for the 1989 test had been planted with cotton for 2 yr immediately prior to the test and a winter fallow was maintained. In both prior years greater than 15% of the bolls were infested with pink bollworms and harvest was delayed, permitting pink bollworm diapause. The average aflatoxin B_1 content of the cottonseed crop produced in this field exceeded 1,000 μg/kg in both prior years. The field used for the 1990 test was planted to winter vegetables immediately prior to the test. Practices typical of commercial operations in the Yuma area were followed except, in order to increase both the incidence and the homogeneity of aflatoxin contamination, insecticidal sprays to control the pink bollworm were not applied, as previously described (18,22). The test organism, AF36, was applied prior to first bloom (24 May 1989 and 13 June 1990) when the plants were 30–60 cm in height. The atoxigenic strain was distributed either by spraying plants (spray treatments) with a conidial suspension (2×10^7 spores/ml at a rate of 130 ml/m row length) or by spreading colonized wheat seed (wheat treatments) on the soil beneath the canopy at rates of 110 g and 8.4 g oven-dry weight per meter of row length in 1989 and 1990, respectively. On 14 September 1989 and 25 October 1990, approximately 2 kg of the mature crop per treatment per replicate was harvested by hand from a continuous segment of the treated area. All bolls on each plant were harvested, dried in a forced-air oven at 60 C for 3 days, and stored in sealed plastic bags at room temperature until analyzed.

Sorting and quantification of locules infected prior to maturation. The percentage of the crop infected prior to maturation was based on the percentage (by weight) of locules (there were three to five locules per boll) with BGYF (14). To reduce variability among determinations of aflatoxin content, the aflatoxin contents of locules with BGYF and locules without BGYF were determined separately (18). In 1989, seeds from the BGYF locules were delinted with a small laboratory gin and sound seeds exhibiting BGYF on the linters (small hairs not removed by ginning) were removed and divided into two portions, one for aflatoxin analyses and one for determination of the incidence of AF36. In 1990 there was a very low incidence of BGYF locules due to low pink bollworm damage. Therefore, BGYF locules were not processed with a gin. Instead, a single sound seed was removed from each BGYF locule for fungal isolations and the remainder of each locule was analyzed individually for aflatoxin content.

Aflatoxin content of the crop. In 1989, 25-g portions of whole, ginned cottonseed were pulverized and extracted as previously described (13,18). Seed was pulverized with a hammer 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 number 4 Whatman 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 1–2 h and filtered again. A 100-ml portion of the filtrate was added to a 250-ml separatory funnel; aflatoxin extraction and analysis were performed as described for culture filtrates. Cottonseed exhibiting BGYF on the linters and cottonseed without BGYF were analyzed separately. To reduce variability, two separate analyses (25 g of seed each) of the non-BGYF seed were performed for each replicate of each treatment and the results were averaged to determine the value for that replicate. For the 1990 test, the same technique was used as for the 1989 test, except that infected whole locules (minus the single seed used to isolate the infecting strain) were extracted individually.

Monitoring strain distribution. The incidence of the vegetative compatibility group (VCG) of AF36 was determined to infer the distribution of that strain. To determine which isolates belonged to the VCG of AF36, nitrate-nonutilizing (*nit*) mutants of each isolate were generated using modifications of the previously described techniques (5). These modifications yielded *nit* mutants of all isolates tested whereas the previous technique yielded mutants from only 88% of tested isolates. Most fungal isolates spontaneously sectored into nitrate-nonutilizing auxotrophs within 30 days at 32 C after being transferred to a well in the center of the modified selection medium (Czapek-Dox broth (Difco) with 25 g/L $KClO_3$, 50 mg/L rose bengal and 20 g/L agar, pH 7.0). A few isolates had to be transferred to the selection medium as many as four times. Auxotrophic sectors were transferred from the modified selection medium to Czapek-Dox broth with 15 g/L $KClO_3$ and 20 g/L agar (pH 6.5, 7 days, 32 C) in order to stabilize the mutants. Mutants were subsequently grown on 5/2 agar and stored in sterile water, as described above, until used in complementation tests. Assignment of isolates to the VCG of AF36 was made on the basis of complementation tests (4) between *niaD*$^-$ (deficient in the structural gene for nitrate reductase) and *cnx* (deficient in a molybdenum cofactor) tester mutants (characterized by the method of Cove)(23) of AF36 and an uncharacterized *nit* mutant of the isolate to be assigned. Due to difficulties caused by the conidial nature of *A. flavus* and the instability of some mutants, only one complementation test was performed on each plate. Three wells (3-mm-dia), 2 cm apart, were cut in a triangular pattern in the center of the medium (20–25 ml) contained in 9-cm plastic petri dishes. The complementation medium consisted of Czapek-Dox broth adjusted to pH 6.0 with 2 N HCl, solidified with 2% agar (Bacto-Agar, Difco) and supplemented after autoclaving with Nitsch and Nitsch vitamin solution (Sigma) at twice the recommended concentration. For each complementation test, one well each was seeded with the AF36 tester mutants and one isolate mutant. Complementations occurred within 10 days at 32 C. A total of 544 and 166 isolates were assessed with this method for the 1989 and 1990 tests, respectively.

The isolates used in the vegetative compatibility tests were obtained as follows. In 1989, 10–12 isolates of *A. flavus* from each replicate-treatment (384 total) were obtained from ginned seed with BGYF linters. In 1990, isolates were obtained from one seed each of 34 BGYF locules (38 BGYF locules were harvested and four did not yield an *A. flavus* isolate; 16 of the 34 were from wheat treatment replicate plots; 18 of the 34 were from control replicate plots). Seed was wetted with a few drops of 95% ethanol, delinted for 3 min in concentrated sulfuric acid, washed three times in deionized water (2 min each), surface sterilized with 95% ETOH for 3 min, plated on the modified rose bengal medium described under quantification of fungal populations and incubated at 32 C 5–10 days. *A. flavus* colonies were transferred to 5/2 agar and stored in sterile water, as described under cultures and inoculum preparation (above), until used to generate *nit* mutants. In 1990, *A. flavus* strains resident on the surfaces of the mature crop were also isolated. Three isolates per treatment per replicate (48 total) were picked from the dilution plates used to quantify these populations (below). In both 1989 and 1990, strains resident in soils both prior to application of AF36 and after harvest of the crop were isolated (two isolates per replicate treatment) from dilution plates used to quantify these populations (see below). To remove bias from colony selection, discrete colonies closest to plate centers were chosen.

Quantification of fungal populations. Populations of *A. flavus* in the soil were enumerated both 1 day before to application of treatments and 1 day after harvest in 1989 and 1990. Soil samples (35–50 g) from the top 2 cm of soil beneath the canopy were taken from each treatment-replicate. Two samples (48 total, no soil samples were taken from second untreated control) were taken on each date in 1989 and one sample (16 total) was taken on each date in 1990. Soils were dry (powdery) at sampling and

were sealed dry in plastic vials at room temperature until assayed within 1 mo. Populations in samples were enumerated by dilution plating on a modification of the rose bengal medium of Bell and Crawford (7). The modified rose bengal medium contained the following per liter: 10.0 g of NaCl, 3.0 g of sucrose, 3.0 g of $NaSO_4$, 0.3 g of KH_2PO_4, O.7 g of K_2HPO_4, 0.5 g of $MgSO_4$ $7H_2O$, 0.5 g of KCl, 0.7 mg of $Na_2B_4O_7$ $10H_2O$, 0.5 mg of $(NH_4)_6Mo_7O_{24}$ $4H_2O$, 10.0 mg of $Fe_2(SO_4)_3$ $6H_2O$, 0.3 mg of $CuSO_4$ $5H_2O$, 0.11 mg of $MnSO_4$ H_2O, 17.5 mg of $ZnSO_4$ $7H_2O$, 50 mg of chloramphenicol, 10 mg of dichloran, 25 mg of rose bengal, 50 mg of streptomycin sulfate, and 20 g of agar. In 1990, the quantity of *A. flavus* superficially associated with the mature crop at harvest was also determined. Samples of seed-cotton (25 g) without BGYF were placed in culture bottles (1 L) containing 250 ml of 0.01% Triton X-100. Subsequently the bottles were shaken vigorously 1 min, allowed to settle 5 min and shaken again. The quantity of *A. flavus* propagules suspended in the liquid was then determined by dilution plate technique on the modified rose bengal agar.

Aflatoxin-producing phenotypes. An estimate of the spectrum of aflatoxin-producing phenotypes among isolates infecting the 1989 crop was made by determining the ability of infecting isolates to produce aflatoxin in liquid fermentation. From each replicate block, ten isolates in the VCG of AF36 and 10 isolates not in this VCG (160 isolates total) were checked for aflatoxin production in the liquid medium of Adye and Mateles (1) with 3 g/L NH_4SO_4 as the nitrogen source as previously described (21). For each isolate approximately 3.5×10^4 spores were added to a single Erlenmeyer flask (250 ml) containing 70 ml of medium. Flasks were incubated in the dark on an orbital shaker (150 rpm) for 5 days, after which time 70 ml of acetone was added to each flask to extract the aflatoxins from the mycelium. Culture filtrates containing 50% acetone (v/v) were filtered through number 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, then evaporated at room temperature, and the residual was dissolved in 4 ml of methylene chloride. Extracts and aflatoxin standards (aflatoxins B_1, B_2, G_1, and G_2) were separated on TLC plates (silica gel 60, 250 mm) by development with diethyl ether-methanol-water (96:3:1) (39). Extracts were either concentrated or diluted to permit accurate densitometry (34) and aflatoxin B_1 was quantified with a scanning densitometer after development (model cs-930, Shimadzu Scientific Instruments, Inc., Tokyo) (34). The limit of detection was 1 μg/kg culture medium.

Statistical analyses. Analyses were performed with the Statistical Analysis System (SAS Institute, Inc., Cary, NC) and Microsoft Excel. ANOVA was used to test differences among treatments prior to application of multiple comparison techniques.

RESULTS

Incidence of BGYF. In 1989, there was a great deal of pink bollworm damage (over 30% of the bolls were infested) and subsequent infection of developing bolls by *A. flavus* resulted in a high percentage of locules (22 ± 2% by weight; Table 1) that were positive for BGYF. In 1990, there was little pink bollworm damage (less than 5% of bolls were infested) and there were relatively few locules with BGYF (0.9 ± 0.1%). In both the 1989 and 1990 tests, the percentage of locules infected prior to boll maturity (BGYF locules) did not differ significantly ($P = 0.05$) among treatments.

Aflatoxin content of the crop. In both years, BGYF seed from plots treated with colonized wheat seed contained significantly less aflatoxin B_1 than BGYF seed from untreated control plots (Table 1). The aflatoxin B_1 content of the BGYF seed was 75–82% lower than the controls in 1989 and 99.6% lower in 1990. In 1989, the quantity of toxin in the seed not exhibiting BGYF was also determined. Only 2.6% of the detected aflatoxin occurred in seed not exhibiting BGYF and the quantity did not differ significantly among treatments.

The quantity of aflatoxin B_1 in the BGYF seed from the 1989 crop was inversely correlated with the percentage of isolates from that seed belonging to the applied VCG (Fig. 3). Replicate blocks containing high incidences of the applied VCG had low aflatoxin content and vice versa. Complete analyses were successfully performed on a total of 34 locules exhibiting BGYF on the lint in 1990. Only one of 18 locules from which an isolate belonging to the AF36 VCG was isolated contained detectable quantities of aflatoxins (Fig. 4). However, aflatoxin was detected in 13 of 16 locules (81%) from which an isolate not belonging to the applied VCG (the AF36 VCG) was isolated. Locules from which the applied VCG was isolated contained significantly ($P = 0.05$ by Student's *t*-test) less aflatoxin than locules from which other VCGs were isolated (0.2 μg/g versus 65.9 μg/g). Most locules (63%) from which other VCGs were isolated contained over 10 μg/g (Fig. 4).

Strain distribution. Nitrate-nonutilizing mutants were generated for all isolates examined (710 total). Prior to application of AF36, the incidence of its VCG in test field soils was one

TABLE 1. Influence of atoxigenic *Aspergillus flavus* AF36 on incidence of bright-green-yellow-fluorescence (BGYF), aflatoxin content of harvested seed cotton, and incidence of *A. flavus* strains infecting and resident on surface of crop

	BGYF (%)[t]		Aflatoxin B_1 (μg/g)[u]			Isolates in applied VCG (%)[v]			Quantity of *A. flavus*[w] on harvested crop (propagules/g)
						Infecting isolates[x]		Surface isolates[y]	
Treatment[s]	1989	1990	1989 BGYF	1989 Non-BGYF	1990 BGYF	1989	1990	1990	1990
Control 1	22 a[z]	0.85 a	39.0 a	0.7 a	81.8 a	25 c	7 b	4 b	28,059 a
Control 2	24 a	ND	53.5 a	0.8 a	ND	25 c	ND	ND	ND
Wheat	25 a	1.03 a	9.7 b	0.5 a	0.3 b	67 a	100 a	75 a	23,949 a
Spray	20 a	ND	36.8 a	1.6 a	ND	45 b	ND	ND	ND

[s] Control 1 = untreated control in the same row as treated plots; Control 2 = untreated control separated from the row with treated plots by four untreated rows; Wheat = application of colonized wheat to the soil beneath the canopy (110 g and 8.4 g dry weight per meter row length in 1989 and 1990, respectively); Spray = canopy sprayed with 130 ml per meter of 2×10^7 spores/ml in 0.01% Triton X-100.

[t] Percent seed-cotton exhibiting BGYF on a weight basis. Entire locules of seed-cotton were sorted into the BGYF category. Locules exhibiting even small amounts of BGYF were considered positive.

[u] Aflatoxin content is expressed per gram whole seed. Contents of cotton exhibiting BGYF and cotton not exhibiting BGYF were determined separately.

[v] Percent isolates assigned to the applied vegetative compatibility group (VCG) to which AF36 belongs, on the basis of auxotroph complementation. Two isolates per replicate per treatment were assessed. ND = not determined.

[w] Quantity of *A. flavus* propagules washed from seed-cotton with 0.01% Triton X-100.

[x] Isolates from internal seed isolations.

[y] Isolates from surface washes of seed-cotton. Three isolates per replicate per treatment (48 total) were assessed.

[z] Values are averages of eight replicates. Values followed by the same letter are significantly different by Fisher's protected LSD test. ND = not determined.

of 48 isolates in 1989 and one of 36 isolates in 1990 (Table 2). By contrast, the overall frequency of the AF36 VCG within *A. flavus* soil populations increased by harvest ($P = 0.05$ by the paired *t*-test) to 42 and 63% in 1989 and 1990, respectively (Table 2). However, differences ($P = 0.05$ by analysis of variance) in the incidence of the AF36 VCG did not occur among treatments in either year and the VCG occurred in untreated control plots at a rate of 19 and 56% in 1989 and 1990, respectively (Table 2). After harvest, in 1989 the incidence of the applied VCG increased with distance ($R^2 = 0.77$, $P < 0.01$) from the south border of the test field (Fig. 5). A skewed pattern of distribution was not evident at harvest in 1990.

The applied VCG was also a major component of the *A. flavus* population infecting the crop during boll maturation (identified by BGYF) (2,18) (Table 1). Although the applied VCG was isolated from a greater percentage of the infected bolls from treated plots than from infected bolls from untreated controls, the applied VCG was isolated from portions (25 and 7% in 1989 and 1990, respectively) of infected bolls from untreated plots in both years (Table 1). In 1989, the colonized wheat seed treatment resulted in the greatest level of the applied VCG in the infecting population (67 vs. 45% for the spray treatment) and, therefore, in 1990 only the colonized wheat seed treatment was used. In 1990, the applied VCG was isolated from all bolls exhibiting BGYF and harvested from the plots treated with colonized wheat seed. The incidence of the applied VCG within populations of *A. flavus* resident on the surfaces of seed-cotton at harvest was also determined in 1990. Seventy-five percent of isolates from seed-cotton surfaces from plots treated with colonized wheat seed in 1990 were assigned

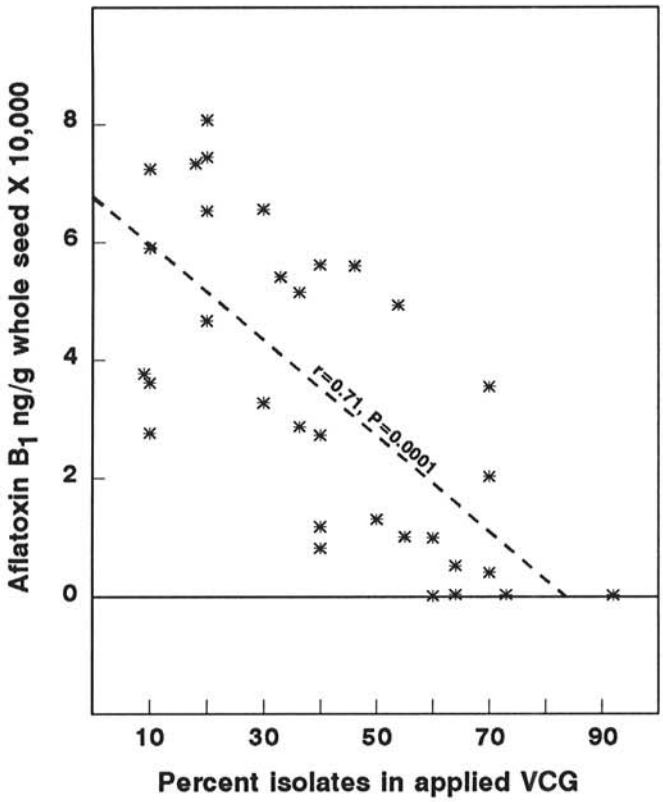

Fig. 3. Correlation of the incidence of the applied VCG among isolates internally infecting BGYF seed in 1989 and the quantity of aflatoxin B_1 detected within that seed. Each point represents the average for a replicate plot (4 treatments × 8 replicates = 32 replicate plots).

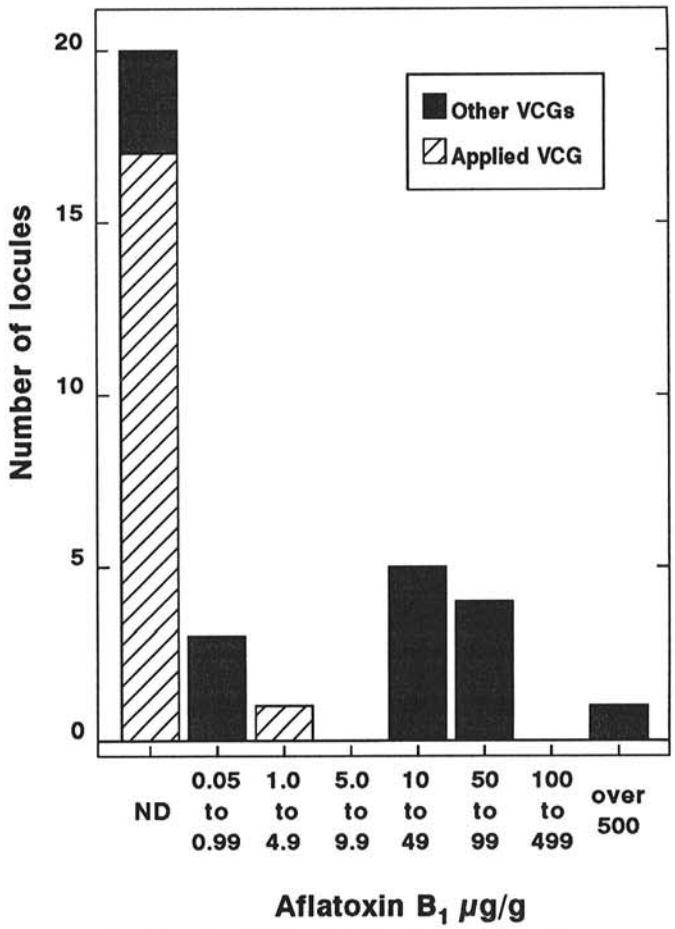

Fig. 4. Number of locules exhibiting BGYF and containing various concentrations of aflatoxin B_1. One *A. flavus* strain was isolated from one seed of each locule and determined to either belong to the applied atoxigenic VCG or not, through vegetative compatibility tests.

TABLE 2. Population density of *Aspergillus flavus* in soil and incidence of an applied vegetative compatibility group (VCG)

	A. flavus (propagules/g)				Isolates in applied VCG (%)[x]			
	Before[y]		After[y]		Before		After	
Treatment[w]	1989	1990	1989	1990	1989	1990	1989	1990
Control	2,979[z]	1,100	4,288	11,038	6	6	19	56
Wheat	7,822	1,583	48,217*	55,858	0	0	69	69
Spray	5,596	ND	6,408	ND	0	ND	38	ND

[w] Control = untreated control in the same row as treated plots; Wheat = application of colonized wheat to the soil beneath the canopy (110 g and 8.4 g dry weight per meter row length in 1989 and 1990, respectively); Spray = canopy sprayed with 130 ml per meter of 2×10^7 spores/ml in 0.01% Triton X-100.

[x] Percent isolates assigned to the applied VCG on the basis of auxotroph complementation. Two to three isolates per replicate (16 total/replicate/treatment) were assessed. ND = not determined.

[y] 'Before' samples were taken prior to first bloom on the day prior to treatment application (24 May 1989 or 13 June 1990) and 'After' samples were taken the day after harvest (14 September 1989 and 25 October 1990).

[z] Values are averages of 8 replicates. Overall before and after values differ significantly ($P = 0.01$) for both 1989 and 1990 by the paired *t*-test. This holds for both *A. flavus* propagules/gram and for percent applied VCG. The value denoted by "*" differs significantly from other values in the same column ($P = 0.05$) by Fischer's protected LSD test.

to the applied VCG on the basis of complementation tests, whereas only 7% of surface isolates from untreated plots were in the applied VCG.

Magnitude of fungal populations. The quantity of *A. flavus* on the surface of the seed at harvest was only quantified in 1990. High counts of propagules of *A. flavus* (over 20,000 per gram) were recovered from seed harvested from both treated and control plots in 1990 (Table 1). In 1989 and 1990, soil populations exceeded 1,000 propagules per gram prior to application of treatments and increased ($P = 0.05$ by paired t-test) in all treatments between application and harvest (Table 2). Differences ($P = 0.05$) among treatments were detected only in 1989 when soils from wheat treated plots had over 10-fold more propagules after harvest than did soils from the untreated control plots.

Aflatoxin production by field isolates. None of 80 isolates from the harvested seed belonging to the applied VCG produced detectable aflatoxin B_1 levels in liquid fermentation. However, 80% of isolates not in the applied VCG produced detectable aflatoxin B_1 and 65% of these isolates produced greater than 10 μg/g of culture (Fig. 6).

DISCUSSION

In 2 yr of field tests in Yuma, Arizona, soil application of atoxigenic *A. flavus* AF36 on colonized wheat seed resulted in a reduced quantity of aflatoxins in the cottonseed crop at maturity without an increase in the incidence of infection, as measured by BGYF. Vegetative compatibility analysis of fungal populations infecting the crops in both years provided evidence that these reductions were associated with displacement of the resident *A. flavus* population by the applied atoxigenic strain. In 1989, the atoxigenic strain was applied by spray, as well as on colonized wheat seed, but the spray application was not as effective as the colonized wheat seed in either displacement of the infecting population or prevention of the aflatoxin B_1 accumulation in the infected portion of the crop. Greater efficacy of the colonized wheat seed treatment probably stems from a far greater quantity of conidia being released for a longer period of time by the applied wheat than by the spray application.

The quantity of aflatoxin in plots treated with colonized wheat seed was 75–82% less than in untreated controls in 1989 and 99.6% less than in 1990. However, the applied VCG was isolated from 25 and 7% of infected seed in the untreated control plots in 1989 and 1990, respectively. Infection by AF36 or coinfection by AF36 and a strain not in the same VCG would be expected to result in lower aflatoxin levels than infection by most other VCGs alone (15,21). Therefore, aflatoxin levels in control plots were probably lowered by atoxigenic strain applications, and the control of aflatoxin B_1 contamination associated with the application of colonized wheat seed is probably underrepresented, especially for 1989. The correlation between incidence of the applied VCG and aflatoxin content of infected seed (Fig. 3) may better describe the impact of the atoxigenic strain on contamination.

Although the rate of application of wheat infested with the biocontrol agent in 1989 was greater than in 1990 (110 g/m row length versus 8.4 g/m row length), the percentage of the applied strain in infected locules from treated plots was only 67% in 1989 versus 100% in 1990. Lower displacement in treated plots in 1989 may have resulted from failure to treat rows adjacent to rows sampled at harvest; in 1990 rows on each side of the sampled rows were treated. Higher rates of displacement in 1990 with lower application rates more broadly dispersed may indicate that useful displacement and associated aflatoxin reductions can be achieved with much lower rates uniformly applied over larger contiguous areas.

The incidence of the applied VCG in infected seed from untreated control plots was much greater in 1989 than in 1990 (25 versus 7%). The crop was treated later with less material in 1990

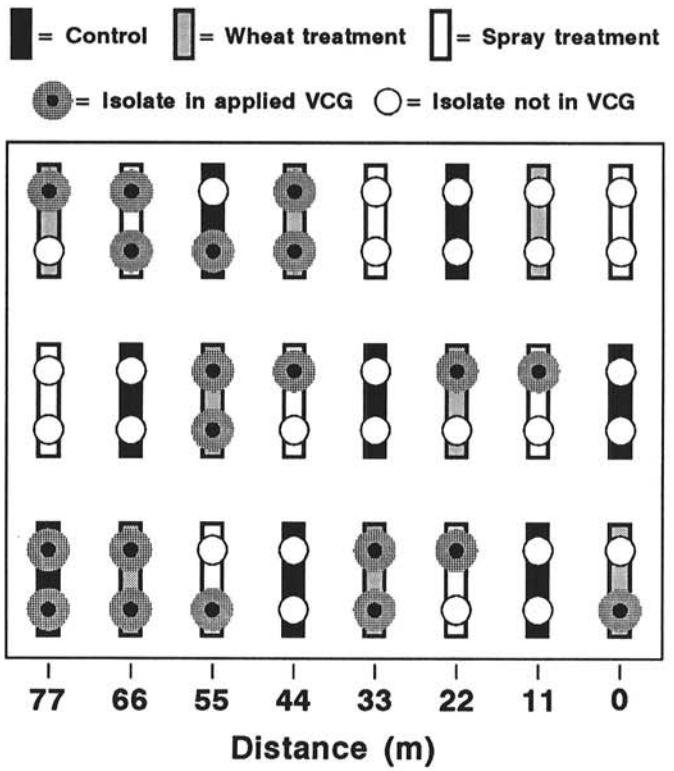

Fig. 5. Distribution of the applied vegetative compatibility group at harvest in 1989. Position of all 48 isolates is indicated. Predominant winds blew from right to left and the incidence of the applied strain increased with increased distance from the right border of the plot ($R^2 = 0.77$, $P < 0.01$).

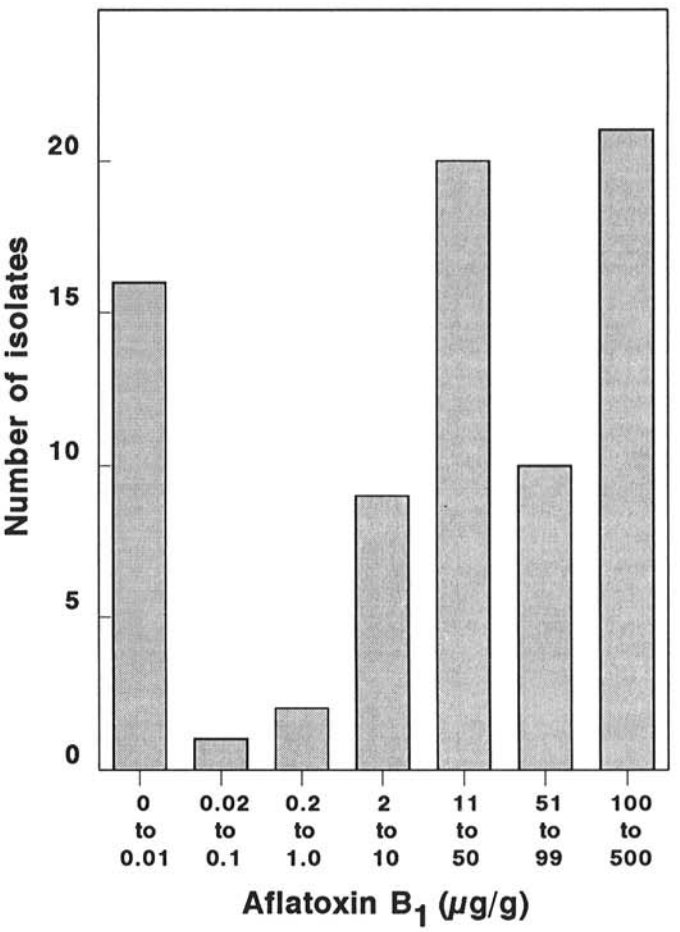

Fig. 6. Concentrations of aflatoxin B_1 produced in liquid fermentation by isolates of *A. flavus*, not in the applied VCG, from internal infections of seed exhibiting BGYF in 1989. The same quantity of isolates in the applied VCG were also tested but none of those isolates produced detectable quantities (0.01 μg/g) of aflatoxin B_1.

than in 1989 and reduced spread may have resulted from a combination of lower inoculum, a larger canopy at application, and environmental differences. It is surprising that in 1989, even though only 1.2% of the experimental field was treated with both spray and wheat treatments combined (if the amount of wheat applied had been dispersed over the entire plot, the application rate would have been 6.6 kg/ha), the average incidence of the applied strain was over 25% at the points most distant from applications.

There was a low incidence of the applied VCG infecting the developing crop (7%) and on the surfaces of the mature crop (4%) in untreated controls in 1990, but a high incidence in the soil of untreated plots after harvest. This differs from 1989 and a mechanism for this differential movement is unknown.

The rate of displacement by the applied VCG in both years suggests initial colonization of developing crops may greatly influence which fungal strains predominate during crop development. Introduction of new, uncolonized resources in the form of a crop uniformly developing may provide the opportunity for rapid swings in the composition of certain fungal populations associated with crops through colonization and establishment by relatively few initial strains. This phenomenon of epidemic increases in a few fungal types may occur frequently in agricultural fields. Such increases have been observed in unmodified *A. flavus* populations (4).

Strain application may increase the quantity of *A. flavus* inocula, at least initially. However, incidence of infection of developing cotton bolls did not differ between treated plots and untreated controls in either year. Predisposition of developing bolls (i.e., through insect activity) (22) may be a greater determinant of infection rate than the quantity of inoculum to which the crop is exposed. This may be particularly true in the desert valleys of Arizona where crops frequently are dusted by soil dispersed by agricultural activities and wind. This dust contains large quantities (at times exceeding 5,000 propagules/m^3 of air) (29) of *A. flavus* inocula. Furthermore, during the cotton season, very large proportions of dead and necrotic plant and animal tissue become colonized by *A. flavus* (3,38) and these contribute to inoculum levels. Thus, cotton bolls produced in these areas become exposed to large concentrations of *A. flavus* inocula.

Overall, *A. flavus* populations in the top 2 cm of the soil profile increased during the cotton season in both years (Table 2). These population increases occurred in both treated and control plots and the applied VCG composed significant portions (19 and 56%, in 1989 and 1990, respectively) of populations in control plot soils in both years. In 1989, the population in the soil of plots treated with colonized wheat contained significantly greater numbers of *A. flavus* propagules than untreated plots at harvest. Because the colonized wheat was delivered to the assayed sites 3 mo earlier, these differences might be expected. It is more surprising that in 1990 differences between treated and control plots were not significantly different. Nutrient sources other than the applied wheat must fuel *A. flavus* increases in these surface soils. Whether strain applications impact the quantity of *A. flavus* overwintering has not been determined. Overwintering populations may be determined to a greater extent by colonized organic matter than by the number of propagules resident at harvest.

The population of *A. flavus* on seed cotton surfaces at harvest in 1990 did not differ between treated and control plots and the applied VCG contributed only a minor portion (4%) of the propagules in the control plots and most (75%) of the propagules in the treated plots. It may, therefore, be possible to apply sufficiently low quantities of colonized matter to exclude resident strains without causing overall population increases. Exclusion apparently occurred during the *A. flavus* population increase that resulted in high propagule counts (over 20,000 propagules per gram) on the crop at harvest. Apparently the quantity of the fungus associated with the crop was dependent on a factor other than the quantity of fungus present early in the season when the colonized wheat was applied. Resources available for exploitation by this aggressive saprophyte and environmental conditions may dictate ultimate sizes of populations resident on the crops to a greater extent than the magnitude of the initial fungal population, providing a certain minimal level of fungus is present. In both the 1989 and 1990 tests, over 1,000 propagules of *A. flavus* per gram of soil were present at the time of atoxigenic VCG application.

In 1990, aflatoxin was detected in one locule from which the applied VCG was isolated. Previous work has shown that many locules exhibiting BGYF are infected by multiple *A. flavus* strains (4) and that locules coinfected by toxigenic and atoxigenic strains contain less toxin (90–100% less) than locules infected by toxigenic strains alone (15,21). Analyses used in 1990 only permitted detection of a single infecting strain from each locule and that strain was isolated from a seed not used to determine the locule's aflatoxin content. The aflatoxin content of the locule in which both the applied VCG and aflatoxin were detected was probably attributable to infection by a second undetected strain and not to conversion of the atoxigenic strain to an aflatoxin-producing phenotype. Similarly, aflatoxin concentrations in locules either lacking detectable aflatoxin or with very low aflatoxin levels, but infected by strains other than the applied strain may be partially attributable to inhibition of toxigenesis by undetected coinfection by the applied strain. Possible conversion of atoxigenic strains to toxigenic strains after application has been suggested by several critics of the use of atoxigenic strains to prevent aflatoxin contamination (27). Aflatoxin-producing ability of certain *A. flavus* strains has been reported to be variable in culture (8,10) and certain strains apparently increase toxigenicity during boll infection (28). In the present study, no instability in atoxigenicity was detected among 80 isolates of the applied VCG from infected seed harvested 5 mo after strain application. This result, and the impact of applications on crop aflatoxin contents, suggest strain instability was not a problem during the course of experiments reported here. Furthermore, a recent study on the relationship of aflatoxin-producing ability to vegetative compatibility group suggested that aflatoxin-producing ability is relatively stable in individual strains and among groups of strains recently diverged (4). The authors further suggested that instability noted in previous studies might, at least in some cases, be attributable to in vitro culture methods.

The use of atoxigenic strains of *A. flavus* to prevent aflatoxin contamination is an unusual concept for the prevention of a plant disease problem. Like a few other biocontrol strategies (12,15), this strategy utilizes a strain of the species that incites the problem to be contained. However, unlike other strategies, for the atoxigenic strains to be effective during infection of the developing crop, the applied strains probably need to be at least as virulent as the strains they are directed at displacing. This may not be a requirement if strains are applied during periods in which the saprophytic habit of *A. flavus* is dominant. During such periods, atoxigenic strains with reduced virulence might be able to displace more virulent toxigenic strains during saprophytic utilization of crop and insect debris and thus reduce the incidence of highly virulent toxigenic strains.

Regardless of the means of intervention employed, there will be fungi associated with our crops. Dead, weakened, and partially decayed plant tissues are readily available and it is not feasible to prevent utilization of these resources by fungi. A level of control over which fungi become associated with crops may be permitted by the seeding of select fungal strains into agricultural fields in a manner similar to the seeding of plants. Such strains may be selected for adaptation to the crop ecosystem, reduced quantities of traits detrimental to human activity, and increased traits considered beneficial. This process of fungal domestication may permit minimization of certain problems caused by fungi (i.e., mycotoxin contamination) and optimization of beneficial fungal traits (i.e., degradation of crop debris). This is most likely to succeed where an undesirable fungal trait (e.g., mycotoxin production) is not necessary for fungal growth and multiplication on the crop or other substrate. However, many plant pathogenic fungi have large saprophytic phases. Deliberate introduction, during rotations to nonsusceptibles, of isolates with reduced virulence but greater saprophytic competitiveness may permit reductions in pathogenic potential in a manner similar to that

in which the reductions in aflatoxin-producing potential were achieved in the current study.

LITERATURE CITED

1. Adye, J., and Mateles, R. I. 1964. Incorporation of labeled compounds into aflatoxins. Biochim. Biophys. Acta 86:418-420.
2. Ashworth, L. J., Jr., and McMeans, J. L. 1966. Association of *Aspergillus flavus* and aflatoxins with a greenish yellow fluorescence of cotton seed. Phytopathology 56:1104-1105.
3. Ashworth, L. J., Jr., McMeans, J. L., and Brown, C. M. 1969. Infection of cotton by *Aspergillus flavus,* epidemiology of the disease. J. Stored Prod. Res. 5:193-202.
4. Bayman, P., and Cotty, P. J. 1991. Vegetative compatibility and genetic variation in the *Aspergillus flavus* population of a single field. Can. J. Bot. 69:1707-1711.
5. Bayman, P., and Cotty, P. J. 1991. Improved media for selecting nitrate-nonutilizing mutants in *Aspergillus flavus.* Mycologia 83:311-316.
6. Bayman, P., and Cotty, P. J. 1993. Genetic diversity in *Aspergillus flavus:* Association with aflatoxin production and morphology. Can. J. Bot. 71:23-31.
7. Bell, D. K., and Crawford, J. L. 1967. A Botran-amended medium for isolating *Aspergillus flavus* from peanuts and soil. Phytopathology 57:939-941.
8. Boller, R. A., and Schroeder, H. W. 1974. Production of aflatoxin by cultures derived from conidia stored in the laboratory. Mycologia 66:61-66.
9. Brown, R. L., Cotty, P. J., and Cleveland, T. E. 1991. Reduction in aflatoxin content of maize by atoxigenic strains of *Aspergillus flavus.* J. Food Prot. 54:623-626.
10. Clevstrom, G., and Ljunggren, H. 1985. Aflatoxin formation and the dual phenomenon in *Aspergillus flavus* Link. Mycopathologia 92:129-139.
11. Cole, R. J., and Cotty, P. J. 1990. Biocontrol of aflatoxin production by using biocompetitive agents. Pages 62-68 in: A Perspective on Aflatoxin in Field Crops and Animal Food Products in the United States. J. F. Robens, ed. Agricultural Research Service, Beltsville, ND.
12. Cook, R. J., and Baker, K. F. 1983. The Nature and Practice of Biological Control of Plant Pathogens. American Phytopathological Society, St. Paul, MN.
13. Cotty, P. J. 1989. Effects of cultivar and boll age on aflatoxin in cottonseed after inoculation with *Aspergillus flavus* at simulated exit holes of the pink bollworm. Plant Dis. 73:489-492.
14. Cotty, P. J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79:808-814.
15. Cotty, P. J. 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Dis. 74:233-235.
16. Cotty, P. J. 1991. Prevention of aflatoxin contamination of cottonseed by qualitative modification of *Aspergillus flavus* populations. (Abstr.) Phytopathology 81:1227.
17. Cotty, P. J. 1991. Aflatoxin contamination: Variability and management. Series P-87. Pages 114-118 in: Cotton—A College of Agriculture Report. J. Silvertooth and M. Bantlin, eds. University of Arizona, Tucson.
18. Cotty, P. J. 1991. Effect of harvest date on aflatoxin contamination of cottonseed. Plant Dis. 75:312-314.
19. Cotty, P. J. 1992. *Aspergillus flavus,* wild intruder or domesticated freeloader. Page 28 in: Aflatoxin Elimination Workshop. J. F. Robens, ed. Agricultural Research Service, Beltsville.
20. Cotty, P. J. 1992. Use of native *Aspergillus flavus* strains to prevent aflatoxin contamination. U. S. patent 5,171,686.
21. Cotty, P. J., and Bayman, P. 1993. Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxigenic strain. Phytopathology 83:1283-1287.
22. Cotty, P. J., and Lee, L. S. 1989. Aflatoxin contamination of cottonseed: Comparison of pink bollworm damaged and undamaged bolls. Trop. Sci. 29:273-277.
23. Cove, D. J. 1976. Chlorate toxicity in *Aspergillus nidulans:* the selection and characterization of chlorate resistant mutants. Heredity 36:191-203.
24. Davis, N. D., and Diener, U. L. 1983. Biology of *A. flavus* and *A. parasiticus,* some characteristics of toxigenic and nontoxigenic isolates of *Aspergillus flavus* and *Aspergillus parasiticus.* Pages 1-5 in: Aflatoxin and *Aspergillus flavus* in Corn. U. L. Diener, R. L. Asquith, and J. W. Dickens, eds. Auburn University, Auburn.
25. Dorner, J. W., Cole, R. J., and Blankenship, P. D. 1992. Use of a biocompetitive agent to control preharvest aflatoxin in drought stressed peanuts. J. Food Prot. 55:888-892.
26. Emnett, J. 1989. Aflatoxin contamination problems in milk caused by cottonseed products. Feedstuffs 61:1-22.
27. Kilman, S. 1993. Food-safety strategy pits germ vs. germ. The Wall Street Journal, March 16. pp. B9.
28. Lee, L. S., Lax, A. R., Mellon, J. E., and Klich, M. A. 1986. Stability for the character for aflatoxin production by *Aspergillus flavus* obtained from Arizona cotton. J. Am. Oil Chem. Soc. 4:694A.
29. Lee, L. S., Lee, L. V., and Russell, T. E. 1986. Aflatoxin in Arizona cottonseed, field inoculation of bolls by *Aspergillus flavus* spores in wind-driven soil. J. Am. Oil Chem. Soc. 63:530-532.
30. Lee, L. S., Wall, J. H., Cotty, P. J., and Bayman, P. 1990. Integration of ELISA with conventional chromatographic procedures for quantitation of aflatoxin in individual cotton bolls, seeds, and seed sections. J. Assoc. Off. Anal. Chem. 73:581-584.
31. Marsh, P. B., Simpson, M. E., Ferretti, R. J., Merola, G. V., Donoso, J., Craig, G. O., Truchsess, M. W., and Work, P. S. 1969. Mechanism of formation of a fluorescence in cotton fiber associated with aflatoxins in the seeds at harvest. J. Agric. Food Chem. 17:468-472.
32. Park, D. L., Lee, L. S., Price, R. L., and Pohland, A. E. 1988. Review of the decontamination of aflatoxins by ammoniation: Current status and regulation. J. Assoc. Off. Anal. Chem. 71:685-703.
33. Park, D. L., and Stoloff, L. 1989. Aflatoxin control—how a regulatory agency managed risk from an unavoidable natural toxicant in food and feed. Regul. Toxicol. Pharmacol. 9:109-130.
34. Pons, W. A., Jr., Robertson, J. A., and Goldblatt, L. A. 1966. Collaborative study on the determination of aflatoxins in cottonseed products. J. Am. Oil Chem. Soc. 43:655-669.
35. Robens, J. F., and Richard, J. L. 1992. Aflatoxins in animal and human health. Rev. Environ. Contam. Toxicol. 127:69-94.
36. Russell, T. E., and Lee, L. S. 1985. Effect of modular storage of arizona seed cotton on levels of aflatoxins in seed. J. Am. Oil Chem. Soc. 62:515-517.
37. Samson, R. S., and Frisvad, J. C. 1990. Taxonomic species concepts of hyphomycetes related to mycotoxin production. Proc. Jpn. Assoc. Mycotoxicol. 32:3-10.
38. Stephenson, L. W., and Russell, T. E. 1974. The association of *Aspergillus flavus* with hemipterous and other insects infesting cotton bracts and foliage. Phytopathology 64:1502-1506.
39. Stoloff, L., and Scott, P. M. 1984. Natural poisons. Pages 477-500 in: Official Methods of Analysis of the Association of Official Analytical Chemists. S. Williams, ed. Association of Official Analytical Chemists, Inc., Arlington, VA.
40. Stoloff, L., van Egmond, H. P., and Park, D. L. 1991. Rationales for the establishment of limits and regulations for mycotoxins. Food Add. Contam. 8:213-222.