Effect of Atoxigenic Strains of *Aspergillus flavus* on Aflatoxin Contamination of Developing Cottonseed

P. J. COTTY, Research Plant Pathologist, Southern Regional Research Center, U.S. Department of Agriculture, Agricultural Research Service, New Orleans, Louisiana 70179

ABSTRACT

Cotty, P. J. 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Dis. 74: 233-235.

Simultaneous inoculation of wounded 28- to 32-day-old cotton bolls with toxigenic and atoxigenic strains of *Aspergillus flavus* led to lower levels of aflatoxin B_1 (B1) in the cottonseed at maturity than in bolls inoculated with the toxigenic strain alone. Six of seven atoxigenic strains tested reduced the level of contamination produced by toxigenic strains. Less B1 was detected when the atoxigenic strain was introduced into the wound 1 day before inoculation with a toxigenic strain than when atoxigenic and toxigenic strains were coinoculated. In contrast, toxin levels at maturity were not reduced when the atoxigenic strain was introduced 1 day after the toxigenic strain. Use of an atoxigenic strain at 10-fold higher spore concentration led to significant reduction in B1 if the atoxigenic strain was introduced within 16 hr after the toxigenic strain. Atoxigenic strains of *A. flavus* may be useful in biological control of aflatoxin contamination.

Aflatoxins are toxic metabolites of the fungi *Aspergillus flavus* Link:Fr. and *A. parasiticus* Speare (6). These toxins are potent carcinogens that frequently contaminate agricultural commodities and pose a serious threat to humans and domestic animals (2). There is great variation among strains of *A. flavus* in the quantity of aflatoxins produced (5,7); this quantity is independent of a strain's ability to infect and colonize developing cottonseed. Strains of *A. flavus* that do not produce aflatoxins in developing cottonseed can be selected from fungal populations in agricultural fields (5).

Atoxigenic strains of *A. flavus* may have potential as biological control agents for reducing aflatoxin contamination. Several plant diseases have been controlled by applying certain strains of the causal organism. Strains of *Pseudomonas syringae* van Hall or *Erwinia herbicola* (Lohnis) Dye that are without genes for ice nucleation can be used to exclude ice-nucleation active strains and prevent frost injury (10). The cross-protection phenomenon has been used to control several viral diseases (14); nonpathogenic strains of *Fusarium oxysporum* Schlechtend.:Fr. can competitively exclude pathogenic strains from infection courts in celery (12). Similarly, atoxigenic strains of *A. flavus* may be able to exclude toxigenic strains from cotton bolls (5).

The objective of this study was to evaluate atoxigenic strains of *A. flavus* for their ability to reduce contamination by aflatoxin B_1 (B1) in cottonseed maturing in bolls inoculated with toxigenic strains.

Accepted for publication 15 September 1989.

MATERIALS AND METHODS

Fungal strains and growth conditions. Strains of *A. flavus* were isolated from agricultural soil and cottonseed collected in Arizona. The origins and aflatoxin-producing capabilities of the strains have been described previously (5). Strains 13 and 42 produced large quantities of aflatoxins both in culture and in developing cottonseed; strains 19, 36, 40, 51, 53, 55, and 63 did not produce detectable levels (10 ng/g) (5). Active cultures were grown in the dark at 30 C on a medium containing 5% V-8 juice and 2% agar (5). For long-term storage, plugs (3 mm in diameter) of sporulating cultures were maintained at 8 C in 4-dram vials containing 5 ml of distilled water (4). Inoculum was prepared by suspending conidia from 7- to 10-day-old cultures in distilled deionized water.

Infection of developing cottonseed. Plants of *Gossypium hirsutum* L. 'Deltapine 90' were grown in a greenhouse in 3-L pots containing a 1:1 mixture of Promix and sand (4). Plants were fertilized weekly with 100 ml of 2,000 ppm Miracle-Gro beginning 3 wk after emergence. Plants were maintained at all times in complete randomized blocks. At 29–31 days after anthesis, pink bollworm damage was simulated in developing cotton bolls by wounding them once in a single lock using a cork borer (3-mm diameter) to a depth of 3–4 mm (4,8). Each boll was inoculated by placing a 10-μl aliquot of an aqueous suspension of conidia into the wound (4). Bolls inoculated with two strains received a 10-μl aliquot of each strain.

To determine how coinoculation of wounds with toxigenic and atoxigenic strains affects aflatoxin contamination at maturity, each boll was inoculated either with approximately 20,000 conidia of a single strain or with 20,000 conidia of the toxigenic strain followed immediately by 20,000 conidia of the atoxigenic strain. To evaluate how prior colonization of wounds by atoxigenic or toxigenic strains affects the ability of the toxigenic strain to contaminate developing cottonseed, wounds inoculated with one strain were subsequently (after 24 hr) inoculated with 20,000 conidia of a second strain. To test the ability of an atoxigenic strain to influence boll contamination after brief initial infection by a toxigenic strain, bolls were inoculated with 2,000 conidia of a toxigenic strain and then reinoculated in the same wound site after various periods (2, 4, 8, or 16 hr) with 20,000 conidia of an atoxigenic strain.

In all tests, bolls were harvested at maturity (3 wk after inoculation) and dried at 60 C for 2 days. After drying, bolls were kept at room temperature in sealed plastic bags containing silica gel desiccant. Treatments were replicated six to eight times; each replicate consisted of one or two plants (one to three bolls). Experiments were performed twice.

Aflatoxin analyses. The B1 content of intact inoculated locules was determined by a modification of the method of the Association of Official Analytical Chemists (13) as previously described (5). Intact locks were hammered to pulverize the seed and added to 200 ml of acetone and water (85:15). The mixture was shaken for 15 sec, allowed to set overnight, and then filtered through Whatman No. 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 $AlCl_3$. Diatomaceous earth (5 g) was added to the mixture, which was shaken and left to settle for 1–2 hr. The liquid phase was filtered (Whatman No. 4 filter paper) and 100 ml of the filtrate was extracted twice with 25 ml of methylene chloride. The hydrophobic fractions were pooled and dried; residues were dissolved in methylene chloride. B1 was purified by thin-layer chromatography and quantified with a densitometer with fluorescence capabilities (13).

Statistical analysis. Analyses were performed either manually or with the

Statistical Analysis System (SAS Institute, Inc., Cary, NC). All multiple comparisons were first subjected to analysis of variance. Toxin values were log-transformed (log $x + 1$) when necessary to homogenize variances among treatments.

Treatment replicates from two experiments were ranked, and the ranks were subjected to split-plot analyses as follows. In tests comparing atoxigenic strains, the test was the main plot and the strain was the subplot. In tests evaluating the effect of challenge with an atoxigenic strain after brief infection by a toxigenic strain, the test was the main plot and the treatment (no challenge or challenge after 2, 4, 8, or 16 hr) was the subplot. Significant differences among treatment means were determined with the LSD test for split-plot analyses (11).

RESULTS

Very high concentrations of B1 were detected in seed from bolls inoculated with strain 13 or strain 42 (Table 1). However, strain 13 produced significantly more toxin than strain 42. In contrast, bolls coinoculated with conidia of toxigenic and atoxigenic strains in equal proportions had markedly reduced quantities of B1 in their seed at maturity. The magnitude of the reduction in toxin associated with coinoculation with strain 36 appeared proportionally greater with strain 42 than with strain 13 (Table 1). The occurrence of aflatoxin was prevented almost completely by introducing strain 36 into wounds 1 day before inoculation with an equal quantity of conidia of strain 13 or strain 42 (Table 1). Seeds from bolls inoculated with a toxigenic strain 1 day before inoculation with strain 36 contained B1 levels equal to that of seed from bolls inoculated with the toxigenic strain alone.

In both the test of different atoxigenic strains and the test of delayed challenge, the test variable was not significant ($P = 0.05$), and it did not interact with the treatment variable (Tables 2 and 3). Consequently, data from the two tests were pooled for each experiment. In both cases, the treatment variable was significant ($P = 0.05$).

Six of seven atoxigenic strains significantly reduced accumulation of aflatoxin in bolls inoculated with the highly toxigenic strain 13 (Table 2). Strain 36 was the most effective at limiting contamination by strain 13. Inoculation of developing cotton bolls with strain 36 alone usually resulted in aflatoxin-free cottonseed at maturity. However, low levels (<50 ng/g) of B1 were occasionally extracted from such seed (*data not shown*).

When bolls were inoculated with toxigenic strain 13 and then reinoculated (in the same wound site after various time periods) with 10-fold more conidia of atoxigenic strain 36, they developed lower aflatoxin levels than bolls inoculated with strain 13 alone (Table 3). The quantity of B1 in cottonseed at maturity was significantly ($P = 0.05$) reduced when strain 36 was inoculated into bolls up to 16 hr after inoculation with strain 13 (Table 3).

DISCUSSION

Atoxigenic strains of *A. flavus* appear to have potential as biological control agents for reducing aflatoxin contamination in cottonseed. Atoxigenic strains are endemic to agricultural fields and should be equally adapted to the hot, dry conditions that favor host colonization and infection by toxigenic strains. Typically, *A. flavus* comes in contact with crops before harvest and remains associated with the crop throughout harvest and storage (9). Thus, seed can become contaminated with B1 both before and after harvest (9). Atoxigenic strains should be able to proliferate under the same conditions as toxigenic strains and, once applied in sufficient quantity, they should have activity proportional to need throughout the season and during storage. These characteristics indicate a potential use for atoxigenic strains of *A. flavus* in a biocontrol strategy for managing aflatoxin contamination.

Populations of *A. flavus* in agricultural fields are composed of strains that vary widely in aflatoxin-producing ability, sclerotial size, and virulence (5). Atoxigenic strains of *A. flavus* also appear to vary in their ability to prevent aflatoxin contamination of cottonseed. Screening of field populations of *A. flavus* may result in strains more efficient at preventing aflatoxin contamination.

The occasional occurrence of low levels of aflatoxins in bolls inoculated only with atoxigenic strain 36 may have been caused by chance introduction of a toxigenic strain into the wounded boll before or during inoculation. Such introduction is likely because *A. flavus* sporulates profusely on inoculated bolls and several experiments were performed simultaneously in the same greenhouse. However, we cannot rule out the possibility that some atoxigenic strains are unstable or that some strains can produce toxin under certain conditions. In a given crop, relatively few seeds contain large concentrations of toxin; these seeds typically account for the majority of toxin within a sample (1). Therefore, occasional low levels (<50 ng/g) of toxin produced by biocontrol strains should not prevent them from reducing these high aflatoxin levels and being useful in the management of aflatoxin contamination. Strain stability, however, should be an important criterion in selection of bio-

Table 1. Aflatoxin content of cotton bolls inoculated with toxigenic and atoxigenic *Aspergillus flavus* strains individually and in combination

Strain	Toxigenicity	Aflatoxin B_1 content of cottonseed ($\mu g/g$)[a]			
		Inoculated alone	Coinoculated with strain 36	Inoculated 24 hr after strain 36	Inoculated 24 hr before strain 36
13	+	72 w	6 x	0.4 z	96 w
42	+	17 y	0 z	0.0 z	15 y
36	−	0 z	...	...	...

[a] Limit of detection: 10 ng/g. Values are means of eight replicates. Means followed by the same letter do not differ significantly ($P = 0.05$) by Fisher's least significant difference test. Data was log-transformed before analysis.

Table 2. Effect of various atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of cottonseed by a toxigenic strain[y]

Atoxigenic strain	Aflatoxin B_1 ($\mu g/g$)[z]
None	66.24 a
53	35.47 ab
51	20.32 b
19	12.52 bc
55	6.71 bc
63	5.86 bc
40	3.31 bc
36	0.65 c

[y] Developing cotton bolls were inoculated first with toxigenic strain 13 and 30 min later with an atoxigenic strain.

[z] Values are averages of eight observations made during two tests. Values followed by the same letter are not significantly different by the LSD test for split-plot analyses (11). Analyses were performed on ranks assigned to values within tests before analysis.

Table 3. Effect of challenge with an atoxigenic strain on production of aflatoxin in developing cottonseed by a toxigenic strain of *Aspergillus flavus*[a]

Time between inoculation and challenge (hr)	Aflatoxin B_1 ($\mu g/g$)[b]
2	1.40
4	1.51
8	3.69
16	6.89
No challenge	30.35

[a] Developing cotton bolls were inoculated first with toxigenic strain 13 and then after various periods with a 10-fold greater quantity of conidia of atoxigenic strain 36.

[b] Values are averages of eight observations made during two tests. Values for 2, 4, 8, and 16 hr differ significantly ($P = 0.05$) from no challenge but not from each other. Analyses were performed on ranks assigned to values within tests before analysis.

control strains. Efforts to produce genetically altered strains without potential to produce aflatoxins should be encouraged.

ACKNOWLEDGMENTS

I thank Lisa A. Williams for technical assistance and Bryan Vinyard for statistical assistance.

LITERATURE CITED

1. Ashworth, L. J., Jr., McMeans, J. L., Pyle, J. L., Brown, C. M., Osgood, J. W., and Ponton, R. E. 1968. Aflatoxins in cotton seeds: Influence of weathering on toxin content of seeds and on a method for mechanically sorting seed lots. Phytopathology 58:102-107.
2. CAST. 1979. Aflatoxins and other mycotoxins: An agricultural perspective. Counc. Agric. Sci. Technol. Rep. 80. Ames, IA. 56 pp.
3. Cotty, P. J. 1988. Simple fluorescence method for rapid estimation of aflatoxin levels in a solid culture medium. Appl. Environ. Microbiol. 54:274-276.
4. 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.
5. Cotty, P. J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79:808-814.
6. Diener, U. L., Cole, R. J., Sanders, T. H., Payne, G. A., Lee, L. S., and Klich, M. A. 1987. Epidemiology of aflatoxin formation by *Aspergillus flavus*. Annu. Rev. Phytopathol. 25:249-270.
7. Joffe, A. Z. 1969. Aflatoxin produced by 1626 isolates of *Aspergillus flavus* from groundnut kernels and soils in Israel. Nature 221:492.
8. Lee, L. S., Lacey, P. E., and Goynes, W. R. 1987. Aflatoxin in Arizona cottonseed: A model study of insect-vectored entry of cotton bolls by *Aspergillus flavus*. Plant Dis. 71:997-1001.
9. Lillehoj, E. B. 1987. The aflatoxin-in-maize problem: The historical perspective. Pages 13-32 in: Aflatoxin in Maize: Proceedings of the Workshop. M. S. Zuber, E. B. Lillehoj, and B. L. Renfro, eds. CIMMYT, Mexico, DF.
10. Lindow, S. E. 1987. Competitive exclusion of epiphytic bacteria by ice-*Pseudomonas syringae*. Appl. Environ. Microbiol. 53:2520-2527.
11. Milliken, G. A., and Johnson, D. E. 1984. Analysis of Messy Data. Van Nostrand Reinhold, New York. 468 pp.
12. Schneider, R. W. 1984. Effects of nonpathogenic strains of *Fusarium oxysporum* on celery root infection by *F. oxysporum* f. sp. *apii* and a novel use of the Lineweaver-Burk double reciprocal plot technique. Phytopathology 74:646-653.
13. 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, Arlington, VA.
14. Yeh, S.-D., Gonsalves, D., Wang, H.-L., Namba, R., and Chiu, R.-J. 1988. Control of papaya ringspot virus by cross protection. Plant Dis. 72:369-460.