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


**ABSTRACT**


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 B1 (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 co-inoculated. 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); non-pathogenic 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 B1 (B1) in cottonseed maturing in bolls inoculated with toxigenic strains.

**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. 'Delta-pine 90' were grown in a greenhouse in 3-L pots containing a 1:1 mixture of Pro-mix 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 co-inoculation 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 re-inoculated 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 settle 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 (CH3COO)2 and 0.008 M AlCl3. 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. Toxic 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 bolts inoculated with strain 13 or strain 42 (Table 1). However, strain 13 produced significantly more toxin than strain 42. In contrast, bolts inoculated 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 bolts inoculated with a toxigenic strain 1 day before inoculation with strain 36 contained B1 levels equal to that of seed from bolts 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 bolts 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 bolts 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 bolts 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.

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

<table>
<thead>
<tr>
<th>Atoxigenic strain</th>
<th>Aflatoxin B1 content of cottonseed (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>66.24 a</td>
</tr>
<tr>
<td>53</td>
<td>35.47 ab</td>
</tr>
<tr>
<td>51</td>
<td>20.32 b</td>
</tr>
<tr>
<td>19</td>
<td>12.52 bc</td>
</tr>
<tr>
<td>55</td>
<td>6.71 bc</td>
</tr>
<tr>
<td>63</td>
<td>5.86 bc</td>
</tr>
<tr>
<td>40</td>
<td>3.31 bc</td>
</tr>
<tr>
<td>36</td>
<td>0.65 c</td>
</tr>
</tbody>
</table>

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

2 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

<table>
<thead>
<tr>
<th>Time between inoculation and challenge (hr)</th>
<th>Aflatoxin B1 content of cottonseed (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.40</td>
</tr>
<tr>
<td>4</td>
<td>1.51</td>
</tr>
<tr>
<td>8</td>
<td>3.69</td>
</tr>
<tr>
<td>16</td>
<td>6.89</td>
</tr>
<tr>
<td>No challenge</td>
<td>30.35</td>
</tr>
</tbody>
</table>

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