Bacterial Antagonists of Aspergillus flavus

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In order to search for bacteria capable of reducing the aflatoxin contamination of cottonseed, 892 indigenous bacterial isolates, including 11 that were endophytic to cotton, were screened for their ability to inhibit the growth of Aspergillus flavus on cottonseed in an in vitro bioassay. Only six isolates partially or totally inhibited fungal growth. All antagonistic isolates were recovered from bolt, lint or seed surface or from the lint of mature bolls. One was retrieved from mature seeds. None of the endophytic isolates showed activity. In four field trials, the incidence of A. flavus-induced damage to locules inoculated simultaneously with A. flavus plus the most effective antagonistic isolate (D1) was reduced by 41–100% relative to locules inoculated with A. flavus alone. The severity of damage to locules inoculated simultaneously with A. flavus and with D1 was reduced by 60–100% relative to locules inoculated with A. flavus alone. Isolate D1, identified as Pseudomonas cepacia, completely inhibited the growth of A. flavus on synthetic media.

Keywords: aflatoxins, biocontrol, bacterial antagonists, cotton, Aspergillus flavus

INTRODUCTION

Aflatoxins are potent carcinogens produced by certain fungal strains belonging to the Aspergillus flavus group (Diener et al., 1987). In many countries, the presence of these toxins in foods and feeds is regulated, and these regulations can have a significant impact on the profitability of agricultural operations (Stoloff et al., 1991). Aflatoxin contamination of cottonseed is of a particular concern because cottonseed is commonly fed to dairy cows which, in turn, produce aflatoxin-contaminated milk (Stoloff et al., 1991). Indeed, the first identified aflatoxin contamination problem in the US was in cottonseed meal (Goldblatt & Stoloff, 1983). Cottonseed is contaminated with aflatoxins both during boll development and after boll maturation and opening (Cotty, 1991). In the desert valleys of Arizona, cottonseed contamination is a perennial problem (Diener et al., 1987). In these valleys, most contamination occurs through the infection of pink bollworm exit holes (Russell et al., 1976; Heneberry et al., 1978; Cotty & Lee, 1989). Prevention of A. flavus colonization of pink bollworm wounded tissue is one approach to preventing infection of the developing seed and accumulation of large quantities of aflatoxin.

No economical method of preventing A. flavus infection has been developed (Cotty & Lee, 1989). For certain diseases, biological control strategies have been effective where conventional disease control approaches have failed (Cook & Baker, 1983). Thus, the present study was initiated to seek preliminary information that would be useful in developing a biological strategy
to prevent aflatoxin contamination of cottonseed. The objectives were: (1) to identify bacterial isolates capable of inhibiting growth of \textit{A. flavus}; and (2) to determine the ability of such isolates to prevent both colonization of mature cottonseed and damage to developing bolls by the fungus.

**MATERIALS AND METHODS**

**Bacterial Isolates**

In this study, 892 bacterial isolates were used. Of these, 834 isolates were recovered from field soils and from the surfaces of plants, including leaves, stems, seeds and immature as well as opened bolls of cotton. Eleven endophytic isolates were recovered from the interior parts of stems, cottonseed and unopened flowers, and from the mesocarp and endocarp of immature cotton boll surfaces. A total of 47 isolates were recovered from cottonseed surfaces from 35 cottonseed samples collected from fields in Alabama, Arkansas, Tennessee, Mississippi, Texas and California. About 2 g of seeds or plant tissue or 3 g of soil were placed in 10 ml of 0.05 m-phosphate buffer, pH 7.0, in a 20-ml tube. The content was shaken vigorously for 20 min, and different dilutions of the extract were plated on King’s Medium B agar (KB) (2.0% proteose peptone No. 3, 1.0% glycerol, 0.15% MgSO₄, 0.15% K₂HPO₄ and 1.5% agar). Discrete bacterial colonies were selected after 24–48 h at 25°C and their purity was insured by streaking and re-isolation (Misaghi, 1990). Endophytic isolates were recovered by the procedures described earlier (Misaghi & Donnelly, 1990). All isolates were maintained in sterile water at 5°C prior to screening for their ability to prevent \textit{A. flavus} growth on cottonseed. Effective isolates were stored in 40% glycerol at −80°C.

**Mass Screening of Bacteria for Antagonistic Activity**

A simple, rapid method was developed for the initial screening of bacterial isolates for their antagonistic activity against \textit{A. flavus}, isolate AF36 (Cotty, 1989). Bacterial suspensions were prepared by adding bacterial growth from 48-h-old solid KB cultures to sterile water in a test tube, shaking the content vigorously and adjusting the concentration to \(1 \times 10^6\) colony-forming units (CFU) ml⁻¹ by serial dilutions using sterile water. A suspension of \textit{A. flavus} conidia was prepared by adding two 5-mm-diameter disks from a 4-day-old potato dextrose agar (PDA) plate to 5 ml of sterile water in a test tube. The tube content was shaken vigorously for about 30 s; the conidial count was determined by a hemocytometer, and adjusted to contain 8000 conidia ml⁻¹. Two dry, non-surface-sterilized cottonseeds were placed into each 15-mm wide, 18-mm deep well of a 24-well titer plate. A total of 400 μl of a suspension of one of the bacterial isolates and 20 μl of the conidial suspension was added to each well. Positive controls received 20 μl of conidial suspension and 400 μl of sterile water. Negative controls received 420 μl of sterile water. The titer plates were covered and incubated at 30°C for 3–4 days. Seeds were then examined for fungal colonization. The protocol of the bioassay chosen, including the number of seeds, the size of the titer plate wells, the amount of water, the use of dry versus imbibed seeds and the concentration of fungal and bacterial inocula were initially optimized empirically.

Mass screening of the bacterial isolates using the above bioassay was performed twice, each time with four observations. A well of a titer plate containing two cottonseeds treated with fungal inoculum, bacterial and fungal inocula or water served as one observation. Six bacterial isolates that were found to be effective antagonists in the initial screenings were tested an additional six times, each with four observations, according to the above procedure except for the use of bacterial suspension containing \(1 \times 10^8\) CFU ml⁻¹.

**Field Tests**

For field studies, four bacterial isolates were selected from among six isolates which exhibited antagonistic activity against \textit{A. flavus} in the cottonseed bioassay. The selection was based on the results of cursory greenhouse tests, in which the effective isolates were compared for their ability to reduce \textit{A. flavus}-induced boll damage in cotton plants.

Cotton (\textit{Gossypium hirsutum}), cv. Deltapine 61, was planted at the Campus Agricultural
Experiment Station, University of Arizona, Tucson, in March 1992 and April 1993. Plants were furrow-irrigated and maintained according to commercial agricultural practices in Arizona. Flowers were date-tagged at opening (July through September) and 28–32-days-old bolls were inoculated with A. flavus alone, with A. flavus plus one of the four antagonists or with sterile water, according to a modified version of a previously published procedure (Cotty, 1989). Two oppositely faced locules on each boll were wounded to a depth of 3 mm with a cork borer (3 mm in diameter) in order to simulate pink bollworm exit holes. Each hole was inoculated first with 10 μl of a suspension of one of the four bacterial antagonists and shortly after with 10 μl of a suspension of A. flavus conidia. Bacterial and fungal suspensions were prepared as described earlier, and were diluted to contain respectively 1 × 10⁸ CFU ml⁻¹ and 40,000 conidia ml⁻¹. Locules receiving 20 μl of sterile water in each hole and those receiving 10 μl of the conidial suspension and 10 μl of sterile water in each hole served as negative and positive controls respectively. In the first, second and third field trials, unopened bolls were harvested either 7 or 14 days after inoculation. Inoculated locules were cut open and rated for the amount of A. flavus-induced damage, including discoloration of the endocarp and immature fibers and the presence of fungus-induced bright green yellow fluorescence (BGYF) under UV light (Ashworth & McMeans, 1966). In the fourth field trial, bolls were rated for damage at maturity, and were examined for color and fuzziness of the loccks. A. flavus sporulation and the presence of BGYF. Isolate D1 (the most effective isolate) was included in four field trials with 38, 22, 104 or 106 replicates; isolates D2, D3 and D4 were included in two trials with 6, 34, 14, 32, and 34, 31 replicates respectively. Replicates consisted of individual locules. The severity of the damage in both treated and control locules was calculated as the average amount of damage on a scale from 0 (no damage) to 5 (extensive damage). The severity data were subjected to analysis of variance. The incidence of A. flavus-induced damage to locules was calculated as a percentage of the damaged locules. Locules with a rating of 2–5 were considered to be damaged and those with a 0–1 rating were considered undamaged. The incidence data were then statistically analyzed using the C test.

Antifungal Activity of the Bacterial Isolates
Culture plates (of diameter 86 mm) containing either PDA or KB, were inoculated by streaking each of the 892 bacterial isolates in a circular band, 28 mm in diameter. After 3 days of incubation at 30°C, a 5-mm diameter disk from the edge of a 5-day-old colony of A. flavus on PDA was placed in the middle of the circle. Plates were incubated for 3–5 additional days at 30°C, and compared with control plates (inoculated with the fungus alone) for inhibition of fungal growth.

RESULTS

Mass Screening of Bacteria for Antagonistic Activity
Ninety five percent of seeds inoculated with A. flavus alone were colonized by the fungus within 7 days. Of the 892 isolates (including 11 endophytes) tested, six (D1, D2, D3, D4, D5 and D6) reduced or totally inhibited seed colonization by the fungus. All other isolates were ineffective. Isolate D1 performed most consistently, and inhibited colonization by A. flavus in 30 of 32 observations in eight tests. The respective values for isolates D2, D3, D4, D5 and D6 were 13, 22, 16, 17 and 8 of 32 observations respectively. None of the isolates showed enhanced seed colonization by the fungus.

Five of the bacterial isolates (D1, D3, D4, D5 and D6) which exhibited antagonism against A. flavus in cottonseed bioassay were recovered from the surfaces of immature bolls or from the lint of mature bolls. Isolate D2 was retrieved from mature de-linted seeds removed from a cotton boll.

Field Trials
In four field trials, 100, 75, 39 and 58% of locules inoculated with A. flavus alone were heavily
### TABLE 1. Incidence (%) and severity (score) of *A. flavus*-induced damage to cotton boll locules inoculated with *A. flavus* alone or with *A. flavus* plus one of the bacterial antagonists (D1, D2, D3 or D4) in the field

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Field trial</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Mean</th>
<th>Field trial</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF³ alone</td>
<td>100</td>
<td>75</td>
<td>39</td>
<td>58</td>
<td>68</td>
<td>3.5</td>
<td>2.6</td>
<td>1.1</td>
<td>2.0</td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF + D1</td>
<td>27</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>29</td>
<td>0.8</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF + D2</td>
<td>50</td>
<td>29</td>
<td>—</td>
<td>—</td>
<td>40</td>
<td>1.7</td>
<td>0.7</td>
<td>—</td>
<td>—</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF + D3</td>
<td>75*</td>
<td>53*</td>
<td>—</td>
<td>—</td>
<td>64</td>
<td>2.4*</td>
<td>1.3</td>
<td>—</td>
<td>—</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF + D4</td>
<td>30</td>
<td>59*</td>
<td>—</td>
<td>—</td>
<td>45</td>
<td>1.0</td>
<td>1.8</td>
<td>—</td>
<td>—</td>
<td>1.4</td>
<td></td>
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</tbody>
</table>

³Two oppositely situated locules on each 28-32-day-old intact boll were punctured with a 3-mm diameter cork borer. Each hole was inoculated, first with 10 μl of a suspension (1 × 10³ CFU ml⁻¹) of one of the bacterial antagonists; this was then followed immediately with 10 μl of a suspension containing about 400 conidia of *A. flavus*. The incidence of *A. flavus*-induced damage to locules is represented as a percentage, based on the following numbers of inoculations: isolate D1, 37, 104, 76 and 106 in tests 1-4; isolate D2, 6 and 34; isolate D3, 12 and 30; isolate D4, 44 and 32. The severity of damage is the mean of ratings on a scale from 0 (no damage) to 5 (extensive damage). In all cases (except those marked by asterisks), the incidence and severity of damage in locules inoculated with *A. flavus* plus antagonist were significantly (P = 0.001) lower than with *A. flavus* alone.

³AF = *A. flavus*.

damaged (Table 1). Locules inoculated with *A. flavus* showed discoloration of endocarp, immature fibers and immature seeds within 5-7 days after inoculation. The fungus sporulated on 70% of infected locules. None of the locules injured and inoculated with sterile water (negative control) showed damage. In all field trials, both the incidence and severity of damage to locules inoculated simultaneously with *A. flavus* and with either D1 or D2 were significantly (P = 0.05) lower than those of locules inoculated with *A. flavus* alone (Table 1). The incidence and severity of damage were also lower in the presence of D3 and D4. However, not all differences were significant for these two isolates. Isolate D1 was the most effective in the field. In two of four trials, none of the locules inoculated with *A. flavus* plus D1 showed damage, while 39 and 58% of those inoculated with *A. flavus* alone were damaged (Table 1). The incidence of damage to locules in treatments receiving *A. flavus* plus D1 in field trials was an average of 57% lower than in those receiving *A. flavus* alone. The respective average percentage reduction values for D2, D3 and D4 were 41, 6 and 34. The severity of damage to locules in treatments receiving *A. flavus* plus D1 in field trials was an average of 65% lower than in those receiving *A. flavus* alone. The average percentage reduction values for D2, D3 and D4 were 48, 37 and 39 respectively.

### Antifungal Activity of Bacteria
Isolate D1 completely inhibited the growth of *A. flavus* on both KB and PDA. Isolates D2, D3, D4, D5 and D6 did not exhibit antifungal activity in vitro.

### Characterization of D1
Isolate D1, the most effective of those tested, was identified as *Pseudomonas cepacia* on the basis of standard biochemical tests (Misaghi & Grogan, 1969), Biolog (Biolog Inc., Hayward, CA, USA), API (API Analytab Products, Plainview, New York, NY, USA), and fatty acid profile (Microbe Inotech Laboratories Inc., St Louis, MO, USA). Isolates D2, D3, D4, D5 and D6 were not identified. Isolate D1:

- grew readily on synthetic media;
- did not grow at 4 or 40°C;
- was oxidase and arginine dihydrolase negative;
- produced levan;
- caused pitting on pectate media;
- produced fluorescent compounds on KB medium;
- was sensitive to kanamycin and chloramphenicol;
- was resistant to tetracycline, streptomycin, nalidixic acid, and rifampicin;
- utilized adonitol, inositol, sorbitol, erythritol and L-lactate, but not betaine, quinate, L-tartrate and mannotrol;
- produced acid from inositol and sorbitol, but not from erythritol and sucrose;
- did not cause disease in either young or mature cotton plants, mature onion plants or pea seedlings; and
- induced a hypersensitive reaction on tobacco.

**DISCUSSION**

The screening of 892 bacterial isolates resulted in the recovery of six isolates that were antagonistic against *A. flavus*. One of these isolates (D1), identified as *P. cepacia*, was the most effective and consistent antagonist. Isolate D1 reduced the incidence and severity of *A. flavus*-induced damage to locules in the field. A number of fungi (Wilson, 1988), bacteria (Kimura & Hirano, 1988; Karunaratne et al., 1990) and a yeast (Paster et al., 1993) have been shown to inhibit the growth of *A. flavus* in vitro. However, as far as is known, this is the first report of a bacterial antagonist that is capable of reducing *A. flavus*-induced damage to a plant in the field.

In the field trials, none of the locules inoculated with sterile water (negative control) showed damage. Therefore, the damage to the locules, inoculated with the fungus, must have been the result of infection by *A. flavus* introduced into the bolls artificially, and not by naturally occurring boll-decaying microorganisms which are present in some fields.

The mode of action of isolate D1 has not yet been elucidated. However, the isolate exhibited antibiotic activity against *A. flavus* in vitro. Studies are underway to characterize D1 antibiotics, and to assess the potential of D1 for reducing *A. flavus* infection in the field.

The cottonseed bioassay method developed for the screening of bacteria against *A. flavus* is quite simple, reproducible, rapid and accurate. With no exception, all isolates that were active against *A. flavus* in the cottonseed bioassay reduced *A. flavus*-induced boll damage in the field at statistically significant (*P* = 0.05) levels. The assay is also safe, and does not pose health hazards to laboratory personnel. The sealed microtiter plates used in the bioassay prevent dissemination of fungal conidia in the work environment during the course of experiments. The bioassay method is currently being used to search for additional antagonists against *A. flavus*.

All six antagonistic isolates were recovered from surfaces of immature bolls, from the lint of mature bolls or from seeds. It seems likely that cotton boll surfaces provide ecological niches for bacteria that are potentially antagonistic to *A. flavus*. These bacteria may have evolved to compete with other microbial occupants of boll surfaces. The screening of niches used by the target pathogens may increase the prospect of the discovery of effective antagonists in other plant-pathogen combinations. Biocontrol may be a promising strategy for reducing the aflatoxin contamination of cottonseed. Presently, there are no registered products for prevention of aflatoxin contamination of cottonseed. Fungicides may be ineffective, because infection sites inside pink bollworm exit holes are inaccessible to foliarily applied products. In contrast, application of *A. flavus* antagonists to bolls would allow them to colonize insect-damaged sites and would prevent the fungus from establishing itself at these sites. The *P. cepacia* strain D1 is a potential candidate for use as an antagonist against *A. flavus*. However, application techniques must be developed to overcome ecological limitations of any bacterial strain used for biocontrol. The development of such techniques may require insights into the ecological adaptation of both the antagonist and the target pest.
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REFERENCES


