SHORT COMMUNICATION

Inhibition of Aflatoxin Production in *Aspergillus flavus* by Cotton Ovule Extracts

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The effect of cotton ovule extracts on *Aspergillus flavus* growth and aflatoxin biosynthesis was investigated. Extracts derived from either noninoculated cotton ovule tissue or cotton ovule tissue challenged with *A. flavus* inhibited aflatoxin (B_1) synthesis by fungal cultures of *A. flavus*. When added to fungal cultures in concentrations of 50 μg per mL of medium, extracts derived from fungus-challenged ovule tissue inhibited aflatoxin synthesis by as much as 93%. The ED_{50} for this type of extract was 18 μg per mL of medium ($P < 0.01$; $r^2 = 0.46$). Similar experiments with a noninoculated ovule extract (50 μg/mL medium) produced aflatoxin inhibition levels of up to 77%. The ED_{50} for noninoculated extracts was 35 μg per mL of medium ($P < 0.01$; $r^2 = 0.66$). These extracts did not inhibit the growth of *A. flavus* in culture. Cultures of *A. flavus* that contained pure gossypol (10–50 μg/mL medium) also showed significantly reduced levels of aflatoxin production. Gossypol may account for the aflatoxin-inhibitory activity observed in the extracts derived from noninoculated cotton ovule tissue.

KEY WORDS: Aflatoxin inhibition, *A. flavus*, cotton ovule culture, cotton stress metabolites, fungus, gossypol, phytoalexins.

Developing cottonseed is a target for the toxigenic fungus *Aspergillus flavus* Link ex Fries. Plant fungus interactions that influence the fungal production of the potent carcinogen aflatoxin (B_1) are poorly understood. A whole family of antimicrobial sesquiterpenoids has been isolated and characterized from cotton stele and leaf tissue (1). Cotton ovule cultures offer a convenient model system for the study of fungus-cottonseed interactions (2).

Developing cottonseed tissue has the capacity to produce antifungal stress metabolites (phytoalexins) in response to *A. flavus* infection (3). Extracts derived from *A. flavus*-inoculated cotton ovule tissue produced pronounced inhibition zones on a standard thin-layer bioassay plate with a *Cladosporium* sp. as a test organism. However, similar experiments with *A. flavus* resulted in no inhibition zones. Since the fungal strain used in these bioassay plates was atoxigenic, toxin production could not be monitored (2). Thus, an investigation was initiated to determine the effects of cotton ovule extracts on *A. flavus* growth and the production of aflatoxin. This paper reports the results of that investigation. A preliminary report has been presented (4).

EXPERIMENTAL PROCEDURES

Biological materials. Cotton (*Gossypium hirsutum* L. "Stoneville 208 glandless") plants were maintained in standard greenhouse conditions (minimum temperature of 20°C, maximum temperature of 35°C). Cotton ovule cultures were initiated from fertilized ovules (2 d post-anthesis) and grown on a defined medium at 30°C without light (5). Each culture flask contained ovules from one boll (24–32 developing seeds). *A. flavus* NRRL 2061 was maintained on potato dextrose agar. *A. flavus* 13L was isolated from Southern Arizona and maintained on a 5% V-8 juice agar medium (6) at 28°C. Conidia were resuspended in sterile deionized water at concentrations of 10^7 to 10^8 spores per mL for use in fungal incubations.

Ovule extract preparation. Cotton ovule cultures were grown for 30 days, and the liquid medium was discarded. The tissue was washed twice with sterile deionized water. The ovule tissue was inoculated with dry conidia of *A. flavus* (NRRL 2061) by means of a small, sterile brush and incubated at 28°C in darkness for 6 d. The tissue was extracted by a procedure similar to that described by Lee *et al.* (7). Inoculated tissue in cold ethyl acetate (4°C, 5 mL/gfw tissue) containing 0.1% (vol/vol) acetic acid was homogenized in a Waring blender at full speed for 1 min. The organic extract was filtered *in vacuo*, and the remaining tissue was extracted again with the ethyl acetate solution. The ethyl acetate extracts were pooled and dried over anhydrous Na_2SO_3. The extract pool was concentrated to 5 mL on a rotary evaporator and to dryness under a stream of N_2. The residue was immediately dissolved in acetonitrile (2 gfw/mL) and sonicated for 5 min to aid in the solubilization process. The sonicated extract solution was filtered through a 0.45-μm membrane (Millex-HV, Millipore, Millford, MA) to remove insoluble material. The ovule extracts were stored at −20°C in the dark.

Fungal incubations. Fungal incubations were conducted in 50-mL flasks containing 25 mL of a modified Adye and Mateles medium (8), in which $NaNO_3$ was substituted for NH_4NO_3. The medium pH was adjusted to 5.0 before sterilization. Different aliquots of cotton ovule extract solutions were concentrated under a stream of N_2 and redissolved in 100 μL of dimethylsulfoxide (DMSO) before addition to the fungal incubation medium. The carrier solvent (DMSO) did not affect fungal growth or display aflatoxin inhibition properties. Gossypol acetate was a standard (prepared by W.A. Pons of the Southern Regional Research Center). It was dissolved in DMSO and filtered through a 0.45-μm membrane (see above) before addition to the fungal medium. Each incubation flask was inoculated with 0.1 mL of a conidial suspension containing 10^8 spores per mL (10^7 spores/flask). The fungal cultures were incubated at 28°C on a rotary shaker (150 rpm) for six days.

Aflatoxin analysis. Following the incubation period, each flask received 25 mL of acetone and was allowed to soak for 18 h. The mycelial mass for each sample was separated by filtration *in vacuo*. The mycelial fractions were dried in an oven at 50°C for 2 d. After an addition of water (about 20 mL), the medium-acetone solution was extracted three times with 25 mL of methylene chloride. The methylene chloride extracts were pooled and concen-

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trated by evaporation. Each dried fraction was subsequently re-solvated in methylene chloride and analyzed by thin-layer chromatography with silica gel G plates developed in diethyl ether/methanol/water (96:3:1). Aflatoxin B_1 was quantitated by a standard plate densitometry method (9).

RESULTS

The effects of cotton ovule extracts on fungal growth and aflatoxin production were determined after six days of fungal exposure to the extracts. The pH values of the fungal culture media were measured following the six-day growth period to evaluate any abnormal growth trends. The pH values observed were within a range of 5.9–6.4 for fungal cultures, which either did or did not receive ovule culture extracts. The presence of cotton ovule extracts in the fungal cultures did not appear to affect the growth of the fungus either positively or negatively. This observation was valid for extracts derived from *A. flavus*-inoculated cotton ovule tissue and also for noninoculated extracts.

Although cotton ovule extracts did not affect *A. flavus* growth, they did affect aflatoxin production in fungal cultures. The presence of extracts derived from fungal-inoculated cotton ovule tissue in the fungal incubations significantly reduced aflatoxin production (Table 1). The substantial inhibitory effect of noninoculated cotton ovule extracts on aflatoxin production was an unexpected result. A more detailed study verified that extracts derived from either inoculated or noninoculated cotton ovule tissue inhibited aflatoxin production in fungal cultures (Fig. 1). Careful comparison revealed that the inoculated extracts were more potent than the noninoculated extracts in aflatoxin inhibition. Concentrations of the inoculated cotton ovule extract of 50 μg per mL of fungal medium resulted in up to 93% inhibition of aflatoxin production in fungal incubations. Noninoculated ovule extracts resulted in up to 77% toxin inhibition at levels of 50 μg per mL of fungal medium. Interpolation from Figure 1 yielded an ED_{50} value for the inoculated extract of 18 μg per mL medium ($P < 0.01$; $r^2 = 0.46$). The ED_{50} for the noninculated extract was 35 μg per mL medium ($P < 0.01$; $r^2 = 0.66$).

Because of the toxin inhibitory properties of the noninoculated cotton ovule extracts, a search was initiated for materials with such properties in developing cottonseed. One possible candidate was gossypol, a tetraterpene normally found in developing cottonseed tissues. Gossypol was detected in these extracts with a qualitative thin-layer chromatographic system. The addition of pure gossypol to fungal cultures did result in significant inhibition of aflatoxin production (Table 2). However, a plateau was attained with respect to the dose-response relationship of aflatoxin production. Above 10 μg per mL, increased concentrations of gossypol resulted in only small increments of increased aflatoxin inhibition (Table 2). It also was observed that at concentrations greater than 30 μg per mL, gossypol additions caused the medium to become cloudy.

DISCUSSION

The presence of cotton ovule extracts in liquid cultures of *A. flavus* did not appear to affect the general metabolism of the fungus, as demonstrated by similar mycelial growth rates observed in fungal cultures with or without ovule extract. This observation confirmed an earlier observation that cotton ovule extracts did not produce inhibition zones in a thin-layer plate bioassay with *A. flavus* (2). In addition, the pH profiles obtained from fungal culture media were similar for cultures grown in the presence of cotton ovule extract or without it.

Extracts derived from *A. flavus*-inoculated cotton ovule tissue did cause a significant inhibition of aflatoxin syn-

TABLE 1

Effect of Cotton Ovule Extracts on Aflatoxin Production

Treatment	[Toxin][a]
A. flavus	657 ± 292
A. flavus + ninoc[b], 10 μg/mL[c]	306 ± 116
A. flavus + ninoc, 100 μg/mL	40.5 ± 33
A. flavus + inoc[b], 10 μg/mL	519 ± 192
A. flavus + inoc, 50 μg/mL	239 ± 71

[a]Mean [toxin] is expressed as μg aflatoxin (B_1)/g dry wt of mycelia (n=5).
[b]Ninoc is noninoculated extract; inoc, inoculated extract.
[c][Extract] expressed as μg/mL of culture medium.

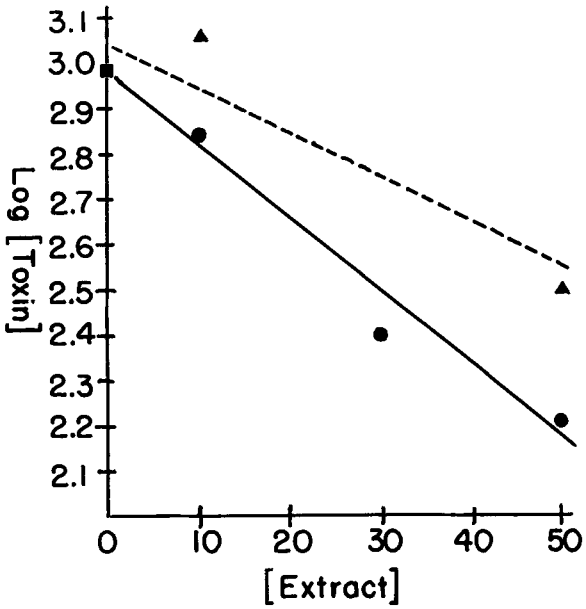

FIG. 1. Effect of cotton ovule-derived extracts on aflatoxin (B_1) production. [Toxin] is expressed as μg/g dry wt mycelia. [Extract] is given in μg/mL culture medium. Noninoculated tissue extract ($P < 0.01$; $r^2 = 0.66$), ▲---▲; inoculated tissue extract ($P < 0.01$; $r^2 = 0.46$), ●——●; no extract added to medium, ■ (n=4).

TABLE 2

Effect of Gossypol on Aflatoxin Production in *A. flavus*

Treatment	[Toxin][a]
A. flavus	506 ± 160
A. flavus + Gos, 10 μg/mL[b]	191 ± 69
A. flavus + Gos, 35 μg/mL	156 ± 29
A. flavus + Gos, 50 μg/mL	130 ± 58

[a]Mean [toxin] expressed as μg aflatoxin/g dry wt of mycelia (n = 4).
[b][Gossypol] expressed as μg/mL of culture medium. Gos, gossypol.

thesis in fungal cultures. These extracts contain considerable concentrations of sesquiterpene antifungal stress metabolites whose synthesis is elicited by *A. flavus* and other fungi (3). The stress metabolite component of the inoculated cotton extract may well be contributing to the aflatoxin inhibition potential. However, the demonstration of the specific role of these cotton stress metabolites (phytoalexins) in aflatoxin inhibition remains to be determined. If the sesquiterpene antifungal components in the cotton ovule extracts are responsible for the aflatoxin inhibition activity, they may play an important role in the regulation of the aflatoxin biosynthetic pathway.

The aflatoxin inhibitory characteristics of the noninoculated cotton ovule extracts were an unexpected result. The antifungal stress metabolites are present in very low concentrations in these extracts. Thus, a different component(s) must be responsible for this inhibitory activity. A possible metabolite that may have aflatoxin inhibitory properties and that is normally present in developing cottonseed tissue is the tetraterepene gossypol. Gossypol was detected in these extracts, although accurate concentrations could not be determined. Indeed, the presence of gossypol in the fungal incubations resulted in a significant reduction of aflatoxin synthesis (Table 2). The plateau effect of the dose-response relationship seen with increasing gossypol concentrations may be a solubility problem. Fungal culture media that received the higher gossypol dosages (>30 μg/mL) turned cloudy upon addition of the tetraterpene, an indication of possible precipitation of the added component. Although the involvement of gossypol in the toxin inhibition of the noninoculated cotton ovule extracts remains a clear possibility, other components in combination with gossypol may be responsible for this activity.

Inhibition of aflatoxin synthesis by plant-derived factors previously has been observed. A family of peanut phytoalexins, the arachidins (stilbenes), causes inhibition of aflatoxin synthesis through growth inhibition of *A. flavus* (10). Extracts of neem leaves result in a pronounced retardation of aflatoxin synthesis by *A. parasiticus* (11) without affecting fungal growth. This observation is similar to the results obtained with cotton ovule extracts in this study. However, the neem leaf extracts were prepared with an aqueous buffer and may include macromolecules, as well as low molecular-weight materials. The cotton ovule extracts were prepared with ethyl acetate, which would select for nonpolar low molecular-weight substances. Thus, the neem extract and the cotton ovule tissue extracts may well be causing aflatoxin inhibition by different mechanisms of action. An inhibitor of aflatoxin synthesis in *A. flavus* has been partially characterized from developing cottonseed (12). This factor also causes toxin inhibition without affecting fungal growth, but was determined to be a macromolecule, possibly a glycoprotein. Again, this cottonseed inhibition factor would appear to be different from the active components of the cotton ovule tissue extracts used in this study. An aflatoxin inhibitory factor in cottonseed meal reported by Hensarling *et al.* (13) was dialyzable, but was not further characterized. The soybean phytoalexin glyceollin, when added to liquid cultures of *A. flavus*, has little effect on mycelial growth, but inhibits toxin accumulation by more than 70% (14). This result may explain the absence of field contamination of soybeans with aflatoxin. The action of the soybean phytoalexin on *A. flavus* appears similar to the cotton ovule tissue extracts used in this study.

Since there are apparently components contained within developing cottonseed tissues, which result in reduced aflatoxin formation when challenged by aflatoxigenic fungi, the cause of serious field contamination events remains enigmatic. A number of other important factors may be playing a role in the aflatoxin contamination problem. Firstly, *A. flavus* appears to most successfully colonize weakened or nonviable cottonseed (15). Thus, these seeds may be weakened physiologically, producing insufficient levels of toxin inhibitory factors to prevent toxin formation. In addition, environmental factors, such as water stress, may select for conditions favorable to toxin formation and unfavorable for production of inhibitory factors. This apparently is the case with aflatoxin contamination of peanuts (16). Since areas of the Southwest United States, where aflatoxin contamination is a problem, also experience water stress problems, this phenomenon may be an important factor for aflatoxin contamination of cottonseed.

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