Inhibition of Aflatoxin Production in *Aspergillus flavus* Infected Cotton Bolls After Treatment with Neem (*Azadirachta indica*) Leaf Extracts

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In separate treatments, a spore suspension of *A. flavus* (control), an aqueous leaf extract of the subtropical neem tree plus a spore suspension of *A. flavus*, or an aqueous neem leaf extract followed by an *A. flavus* spore suspension were injected 48 hr later onto the surfaces of locks of developing cotton bolls (30-day post anthesis). Thirteen days after the treatments, the seeds from the locules were harvested and both fungal growth and aflatoxin production were determined. Fungal growth was unaffected by the treatments but the seeds from locules receiving both neem leaf extracts and *A. flavus* simultaneously exhibited 16% inhibition of aflatoxin production, while the seeds in locules receiving *A. flavus* spores 48 hr after neem extract was added exhibited >98% inhibition in aflatoxin production. Neem leaf extracts contain an aflatoxin inhibiting factor, however, the neem leaf extract may need to translocate from the fibrous locule surface to the seed, prior to the fungal inoculation, for maximal effect.

Neem, *Azadirachta indica* A. Juss. is a subtropical tree native to the drier areas of Pakistan, India, Indonesia, Thailand, Burma, Sri Lanka, Malaysia, and East Africa. Several tetranortriterpenoids, mainly found in the seeds of the neem tree, have been described to be quite active as insect feeding deterrents, toxicants, and/or disruptants of growth and development against a large variety of insect species and nematodes (1).

Aflatoxins are secondary metabolites produced by the two fungi, *Aspergillus flavus* Link ex. Fries and *Aspergillus parasiticus* Speare, which are carcinogenic to both man and animals (2). Aflatoxin contamination of cottonseed is prevalent in cotton *Gossypium hirsutum* L., which is grown in the desert regions of the Southwestern United States. Wounds caused by insects on the carpel surfaces of developing green cotton bolls have been implicated in the aflatoxin problem of cotton (3). The pink bollworm *(Pectinophora gossypiella)* is common in Arizona cotton fields, and the 3 mm exist hole it makes in the developing cotton boll is believed to be one of the major causes of *A. flavus* infection of cottonseed (4,5). Recently, we have demonstrated that neem leaf extract added to submerged cultures of aflatoxin producing strains of *A. flavus* at the time of spore inoculation could essentially block aflatoxin biosynthesis without affecting fungal growth (6).

The purpose of the present investigation was to test neem extract *in vivo* on a natural substrate. We attempted to determine: (i) the effect of neem leaf extract on *A. flavus* growth on seeds contained in developing cotton bolls; (ii) if neem leaf extract could inhibit aflatoxin production in cottonseeds contained in developing cotton bolls; and (iii) the set of conditions which might favor aflatoxin inhibition in the developing cotton boll.

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MATERIALS AND METHODS

A wild-type aflatoxigenic isolate of *A. flavus* (SRRC 1100), obtained from Arizona cotton, was cultured on potato-dextrose-agar (PDA) Petri plates. The spores were harvested after seven days incubation at 28°C, and a spore suspension (10^6 spores/ml in sterile, distilled water with 1% Triton 100) was used in the cotton boll inoculations.

Fresh neem leaves were obtained from the ARS/USDA Subtropical Horticulture Research Station (Miami, FL). After washing the leaves thoroughly with sterile, distilled water, extracts were prepared by blending 100 g (wet weight) of fresh leaves in 1 l sterile, distilled water. Extracts were filtered through several layers of cheesecloth, and the filtrate was centrifuged for 15 min at 7,000 × *g*. The pellet was discarded and the supernatant extract was stored at 4°C.

Thirty days post anthesis developing cotton bolls obtained from approximately 4-month-old Acala SJ-2 cotton plants growing under greenhouse conditions were utilized throughout the experiment. Two 3 mm holes were produced on the surface of all treated bolls, simulating the exit holes of the pink boll worm, *Pectinophora gossypiella*, one of the main sources of *A. flavus* cotton boll infection (3). All inoculations were introduced through these surface holes. The holes were produced approximately 1 mm from one side of the exterior capillary suture line. This position allowed the treatment area to be located in the center of the surface area of the developing fibers and, therefore, confined the treatment to an individual lock. Ten μl of *A. flavus* spore suspension and 50 μl neem leaf extract, each inoculated in separate bored holes, were used in the treatments. The controls included inoculations of 10 μl *A. flavus* spore suspension and 50 μl of sterile, distilled water. The developing bolls were harvested 13 days after spore inoculation.

The seeds in the treated locks from bolls harvested 13 days after treatment were delinted and extracted for aflatoxin B_1, according to previously described procedures (7). Aflatoxins were separated on silica gel thin-layer chromatographic (TLC) plates in ether:methanol: water (96:3:1) (v/v/v), the toxins were quantitated by fluorometric scans (excitation wavelength 360 nm) of TLC plates containing extracts from the cotton seeds, and aflatoxin standards were co-chromatographed on the same plate (8).

Cottonseeds were separated from either neem leaf extract and *A. flavus*-treated or *A. flavus*-treated locks to assay the fungal growth within the seeds. These seeds were acid delinted with concentrated sulfuric acid (1 min), rinsed in distilled H_2O, treated in 10% NaOCl (1 min)

and rinsed several times with sterile, distilled H_2O. The surfaced sterilized seeds were positioned on PDA Petri plates, and after incubation for four days at 28°C the radial growth of emerging developing cultures were compared to radial growth of control seeds (6).

RESULTS AND DISCUSSION

Neem has been reported to contain various components with insecticidal and fungicidal properties (1). In an earlier report from our laboratory (6), we have demonstrated that aqueous extracts of neem leaves inhibited aflatoxin production by submerged cultures of *A. parasiticus* and *A. flavus* without affecting fungal growth. The inhibitory component(s) in these extracts were nonvolatile and affected the regulation of the synthesis of the secondary metabolic enzymes involved in aflatoxin biosynthesis. In that study, however, no practical application of use of neem leaf extracts in controlling pre-harvest aflatoxin contamination was demonstrated. In this report we have carried out greenhouse experiments to measure the inhibitory effects of neem leaf extracts on aflatoxin production by *A. flavus* in developing cotton bolls.

Treating developing cotton bolls (30 day post anthesis) with the neem leaf extract 48 hr prior to infecting the bolls with a spore suspension of *A. flavus* produced almost total (>98%) inhibition of aflatoxin synthesis measured in cottonseeds from treated cotton boll locks 13 days after spore inoculation (Table 1). Similar cotton bolls, which received a simultaneous application of both *A. flavus* spore suspension and neem leaf extract treatment, exhibited only a 16% inhibition of aflatoxin production in the cottonseeds after the 13 day incubation period. Thus, maximum inhibition of aflatoxin synthesis resulted when there was a lag period between neem leaf extract application and fungal spore infection. This delay period may possibly be needed for the active factor in the neem leaf extract to translocate from the developing fibrous lint surface of the boll to the seed.

When delinted, surface-sterilized seeds from control or treated locks were placed on potato-dextrose-agar PDA, Petri plates and the fungus was allowed to grow for four days, the radial fungal growth in the case of seeds from treated locks was 93-96% of that of controls. Neem leaf extract apparently did not kill the fungus, for we were able to recover the fungus from the neem-treated locules.

Experiments are underway using individual, separated components from the neem leaf extract in order to determine the component(s) responsible for the bioactivity described. Delivery systems (e.g., foliar spray or soil application) also are being developed in greenhouse studies for effectively utilizing the neem components in controlling aflatoxin contamination. The practical application of this discovery could only then be utilized in field trials to eliminate the preharvest contamination of cottonseed with aflatoxin.

TABLE 1

Effect of Neem Leaf Extracts on Aspergillus flavus Growth and Aflatoxin B_1 Production in Developing Cottonseed[a]

Treatment	Experiment	Aflatoxin B_1 (% of control)	*A. flavus* growth (% of control)[e]
Neem extract and *A. flavus* spores inoculated at the same time	I[b]	79.60 ± 4.35[d]	96 ± 2[d]
	II[c]	87.83 ± 5.27	94 ± 1
A. flavus spores inoculated 48 hr after neem extract injection	I	2.18 ± 0.82	94 ± 3
	II	0.32 ± 0.12	93 ± 2

[a] Determined 13 days after fungal spore suspension inoculation.

[b] Experiment I represents mean % of control of total seeds of at least 10 individual treated locules; 100% of control = 38,692 ng B_1/g seed.

[c] Experiment II represents mean % of control of total seeds of at least 10 individual treated locules; 100% of control = 44,731 ng B_1/g seed.

[d] ± Standard deviation.

[e] Four-day radial growth of emerging *A. flavus* from seeds of treated locules; 100% of control = 25 cm.

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