Aflatoxin in Arizona Cottonseed: A Model Study of Insect-Vectored Entry of Cotton Bolls by *Aspergillus flavus*

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ABSTRACT

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Insects have been implicated in the aflatoxin problem of cotton, but model studies have not been conducted to study insect-vectored entry of the *Aspergillus flavus* into bolls. Carpel walls of green bolls 12, 19, 26, 33, and 40 days after flowering were punctured to simulate damage of sucking insects or drilled to simulate the hole caused by exit of pink bollworm larvae. *A. flavus* was dusted on wound sites. Treated bolls were harvested 4, 6, 10, and 30 days after injury-inoculation. Microscopic examination followed fungal progression from wound sites to seeds, and chemical assays on individual seeds determined the pattern of aflatoxin-contaminated seeds to uncontaminated seeds. Six days were required for fungal penetration to seed surfaces. Drying was necessary for fungal entry into seed. The pattern of toxin to nontoxin seeds in locks from bolls injured by drilling and inoculated 33 days after flowering most closely resembled that pattern found in locks from naturally contaminated bolls. Results indicate that insect-vectored *A. flavus* entry and subsequent toxin infection are primarily of green bolls close to maturity and reinforce existing knowledge that control of insects lowers aflatoxin potential.

Aflatoxin in Arizona cottonseed causes severe losses to both growers and processors. Toxin-containing seeds are usually found in tight locks, which are caused by fungal hyphae that weaken fibers so that they fail to fluff out (1). Tight locks that do not fall from bolls are harvested along with fully fluffed bolls (11,12). In Arizona, *Aspergillus flavus* is often associated with tight locks. *A. flavus* has been isolated from one insect, *Euschistus impictiventris* (stink bug) (13), and has been associated with boll damage by another, *Pectinophora gossypiella* (pink bollworm) (2). Both insects are prevalent in areas of Arizona where cotton is grown and have been indirectly associated with aflatoxin contamination (4,13). Boll injury by the stink bug is caused when the mature bug inserts its proboscis through the carpel wall to obtain the sugar-rich juice that surrounds the lint in green bolls. Injury by the pink bollworm is due to damage by its larvae. The adult moth deposits her eggs on the bases of maturing green bolls under the calyx, eggs hatch within a few days, and the tiny larvae bore into the boll. Mature larvae (usually fourth or fifth instars) cut their way out of the maturing green boll, drop to the ground, form a cocoon in the dirt and litter under the plant, and pupate (10). Depending on temperature, a generation will take 22–32 days during the cotton-growing season. Secondary fungal infection occurs through the larvae exit holes (2), possibly vectored by windborne spores (5) or by other insects.

No studies have been conducted to determine whether the type or time of insect injury of the boll affects toxin levels in seed (15). Our study simulated infection of green bolls by *A. flavus* following damage caused by either the stink bug (puncture) or a pink bollworm larva (drill) and undertook to correlate boll age at time of injury with aflatoxin content of seeds. Levels of aflatoxin in seeds and the pattern of toxin to nontoxin seeds from bolls inoculated with *A. flavus* were compared with similar levels and patterns found in locks from naturally contaminated bolls. This information is needed to predict the probable stage in boll development when fungal invasion following damage causes maximum toxin formation in seed and to

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predict the most advantageous times for insect control.

MATERIALS AND METHODS

Naturally contaminated seeds were obtained from bolls with tight locks harvested in Arizona in 1983, 1984, and 1985. In 1985, inoculation experiments were conducted in a commercial field in the Phoenix area. About 100 flowers were tagged at 1-wk intervals in late July and early August. Nearly 2 wk after completion of tagging, bolls that were 12, 19, 26, 33, and 40 days from flowering were injured and inoculated. All treatments were done the same day. The carpel wall was injured to simulate the small puncture wound caused by a stink bug or the larger exit hole of a pink bollworm larva. A dissecting needle was used for the puncture wound, and a cork borer (3 mm) was used for the drill wound that simulated the exit hole of the pink bollworm. Inoculation was done with spores of SRRC 2002, an isolate of *A. flavus* obtained from Arizona cotton. Spores, maintained on potato-dextrose agar (PDA) slants, were applied dry at the wound site with a small artist's brush. One lock from each boll was inoculated. A spore count indicated the application of about 6×10^3 spores per wound site. Bolls were harvested after an additional development period of 4, 6, 10, or 30 days. Harvested bolls were dried in individual small paper bags in a screened insectary at the Cotton Research Center (CRC) in Phoenix. Two or three bolls from each treatment were chilled over ice in the field and transported to CRC and frozen. Still frozen, they were sent to New Orleans for microscopic examination.

Microscopic examination. Bolls used for light microscopy were thawed and examined immediately. Sections that contained lint were stained with lactophenol and cotton blue by the technique described by deGruy and Carra (3). Fibers appear transparent while fungal mycelia stain dark blue. For scanning electron microscopy (SEM), bolls were dried overnight in a vacuum oven with no heat. Seeds were separated from lint, then acid-delinted in concentrated sulfuric acid and rinsed in deionized water. Delinted seeds were sliced longitudinally, and the internal surfaces of seed coats and meats were examined. A few seeds from bolls that had been dried in the paper bags were also acid delinted and similarly examined by SEM.

Aflatoxin assays. Both inoculated and naturally contaminated seed were treated in the same manner. Nearly 600 individual seeds from inoculated bolls and 400 individual seeds from naturally contaminated bolls were examined. Long lint fibers were removed by hand, and the seeds with the short linters attached were observed under ultraviolet (UV) for the bright-green-yellow fluorescence (BGYF) associated with *A. flavus* infection of cotton (7). A map recorded the positions of individual seeds in *A. flavus*-infected locks. These seeds were assayed individually. Assay and mapping procedures were identical to those used by Lee and Russell (6). The seven or eight seeds from each of the three or four fluffed (apparently *A. flavus*-free) locks from both inoculated and naturally contaminated bolls were assayed as composite seed samples on a lock-to-lock basis by the procedure used by Lee et al (5).

Microbial examination. One hundred seeds from bolls inoculated with *A. flavus* and seeds from naturally contaminated bolls were acid-delinted with concentrated sulfuric acid. Seeds were surface-sterilized in a solution of 2% (w/v) sodium hypochlorite and 0.001% (v/v) Triton X-100 for 2 min under agitation, then seeds were rinsed three times in deionized water and placed on PDA plates. After a week, seeds were observed for *A. flavus* emergence.

RESULTS

Because bolls that were visibly *A. flavus*-contaminated were the only naturally contaminated samples examined, the study did not compare overall toxin levels in Arizona over a 3-yr period. Results show that, when contamination occurs, the pattern of toxin to nontoxin seed was similar from year to year, but levels of toxins varied (Table 1). Occasionally, a trace of aflatoxin B_2 was detected; only aflatoxin B_1 was reported in this study. Toxin was contained in 26% of the seeds (37 seeds) examined in 1983, only 21% (11 seeds) in 1984, and 20% of the seeds examined in 1985 (34 seeds). Five of the toxin-containing seeds (1983) had levels higher than 100,000 ng/g. In 1984, three seeds, and in 1985, four seeds were among those with the highest levels of toxin. Most of the toxin-containing seeds had levels lower than 100,000 ng/g. In these naturally contaminated bolls, aflatoxins were never detected in seed from the fluffed locks of bolls adjacent to the tight, *A. flavus*-contaminated lock (Fig. 1). In only one case was more than one lock per boll contaminated with *A. flavus*; the general observed pattern was one tight *A. flavus* lock and three or four fluffed locks. These *A. flavus*-contaminated bolls were most often found in the middle one-third of the plant, usually one contaminated boll to about 30 uncontaminated bolls. Close examination of these naturally contaminated bolls showed an injury to the carpel wall corresponding to injury of each tight lock, indicating that the carpel wall covered the lock when the injury took place. Exit of the bollworm larva or puncture injury (stink bug), then, must have taken place before boll opening.

Because of dropoff either before or after injury and inoculation, an unequal number of bolls was harvested for each time period for inoculated samples (Table 2). The number of seed examined reflects this. Bolls injured and inoculated 12 days after flowering fell from the plant without further development. Seeds with aflatoxin were confined to the lock that was inoculated. Seeds from the adjacent

Table 1. Aflatoxin levels in individual cottonseeds from locks naturally contaminated with *Aspergillus flavus*

	Year seed were examined					
	1983		1984		1985	
Aflatoxin B_1 (ng/g)	Seed (%)	BGYF[a] seed (%)	Seed (%)	BGYF seed (%)	Seed (%)	BGYF seed (%)
>100,000	3.1	10.4	5.4	10.0	2.3	11.1
5,000–80,000	7.4	25.0	8.9	13.3	9.3	44.4
200–4,000	12.3	37.5	7.1	13.3	8.1	27.8
None detected[b]	77.2	27.13	80.4	63.3	80.2	16.7
Total no. seed	162.0	48.0	56.0	31.0	172.0	36.0

[a] Bright-green-yellow fluorescence of linters.
[b] Because of the small sample size (0.1 g), the limit of detection is 100 ng/g.

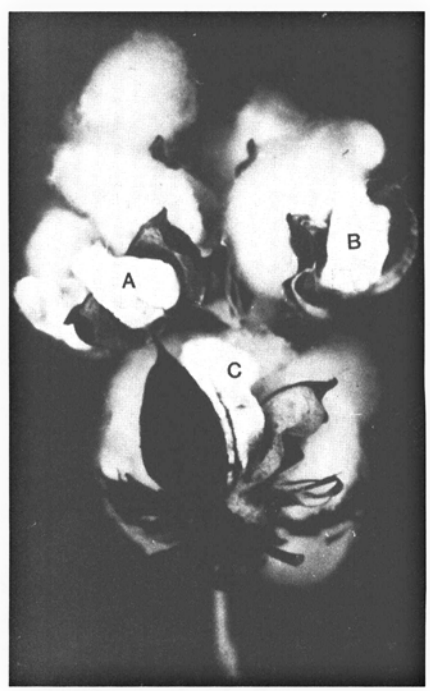

Fig. 1. Ultraviolet photograph of bolls. A and B = bolls naturally contaminated with *Aspergillus flavus* and C = boll inoculated to simulate insect damage.

Table 2. Aflatoxin levels[a] in individual cottonseed from locks in bolls of specified ages from flower; locks injured, inoculated at injury site, and harvested at specified times after inoculation

Days after flowering	Toxin seed/ total seed		BGYF[b] seed with toxin/ total BGYF seed		Toxin levels (ng/g) in seed from locks remaining on plants after inoculation for (days)			
					4	6	10	30
			Puncture inoculation					
12	...[c]	...	...	...	...	...	...	...
19	2/40	(0.5)[d]	2/36	(0.6)	ND	ND	115	200
26	10/49	(2.4)	10/40	(2.9)	ND	ND	220	660
33	56/218	(13.5)	55/168	(16.2)	ND	ND	530	1,800
40	34/108	(8.2)	30/96	(8.8)	ND	ND	150	100
Total no. seed	415		340					
			Drill inoculation					
12	...[c]	...	...	...	...	...	...	...
19	3/33	(0.8)[d]	3/30	(1.0)	ND	ND	260	240
26	8/46	(2.0)	8/38	(2.7)	ND	ND	1,300	2,400
33	63/216	(16.0)	62/147	(20.7)	ND	ND	2,200	4,560
40	27/98	(6.9)	27/84	(9.0)	ND	ND	210	215
Total no. seed	393		299					

[a] Average values obtained on all toxin-positive seed examined.
[b] Bright-green-yellow fluorescence of linters.
[c] Bolls fell from plant without further development.
[d] Percentage of toxin seed in total seed and total BGYF seed for all specified ages after flowering for both puncture and drill inoculations.

fluffed locks (assayed as composite samples) were always toxin-negative, a pattern duplicating that found in bolls naturally contaminated with *A. flavus*. Levels of toxins in inoculated bolls (Table 2), however, were never as high as those found in bolls naturally contaminated with *A. flavus*. An 800-fold difference exists between the lowest level of toxin detected in an inoculated sample (110 ng/g) and the highest level detected in seed from bolls naturally contaminated with aflatoxin (higher than 100,000 ng/g).

No toxins were detected in seed harvested after 4 or 6 days following treatment, whether bolls were inoculated after injury by puncture or injury by drilling. Inoculation following drill injury usually resulted in higher toxin levels than did inoculation following puncture wounding. Toxins were detected only after bolls remained on plants for 10 days. Initiation of toxin formation was between 6 and 10 days after treatment of bolls at all ages after flowering. Bolls that developed 33 days after flowering before the injury-inoculation contained higher levels of toxin than did those injured and inoculated either earlier or later. The same trend in toxin levels was reported by Sun et al (14). The pattern of toxin to nontoxin seed within locks was random for both naturally contaminated bolls and inoculated bolls. Never more than one or two seeds per lock had toxin. This was true at all levels of toxin formation in seed from bolls at all ages from flower.

A direct correlation existed between seed with BGYF linters and seed with aflatoxin for both inoculated and naturally contaminated seed. In seeds examined from bolls naturally contaminated with *A. flavus* (Table 1), of the 82 seeds with aflatoxin, 77 (94%) had BGYF linters. Similarly, in seeds from inoculated bolls (Table 2), of the 203 seeds with aflatoxin, 97% were seeds with BGYF linters. In both seed from bolls naturally contaminated and seed from inoculated bolls, however, all seeds with BGYF linters did not contain aflatoxin. Ten percent of the toxin-negative seeds from bolls naturally contaminated with *A. flavus* in 1983 had BGYF linters; 42% of such seed in 1984 and 4% in 1985 had BGYF linters. The non-BGYF seed (five from puncture inoculation and one from drill inoculation, Table 2) that contained toxins were from bolls that remained on the plant for 30 days before harvest.

Results of the microbial examination corroborated the results of observations of BGYF linters, i.e., that the fungus can be present in seeds without toxin formation. Of the 100 naturally contaminated seed incubated on PDA, *A. flavus* emerged from 61. *A. flavus* emerged from all of the 100 seeds from inoculated bolls.

SEM of seeds that had been frozen immediately on return from the field (Fig. 2) showed no fungal penetration of the seed coat after 6 days of boll development; however, SEM examination of a boll harvested at the same time but dried in a paper bag (Fig. 3) indicated fungal penetration into the air space above the chalazal cap.

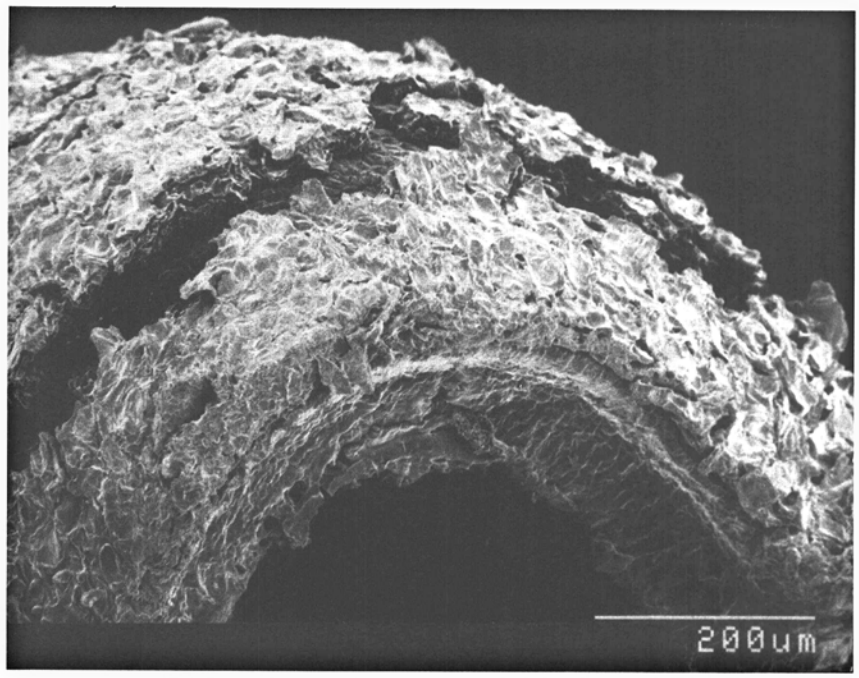

Fig. 2. Scanning electron micrograph of nondried, sliced seed 6 days after inoculation.

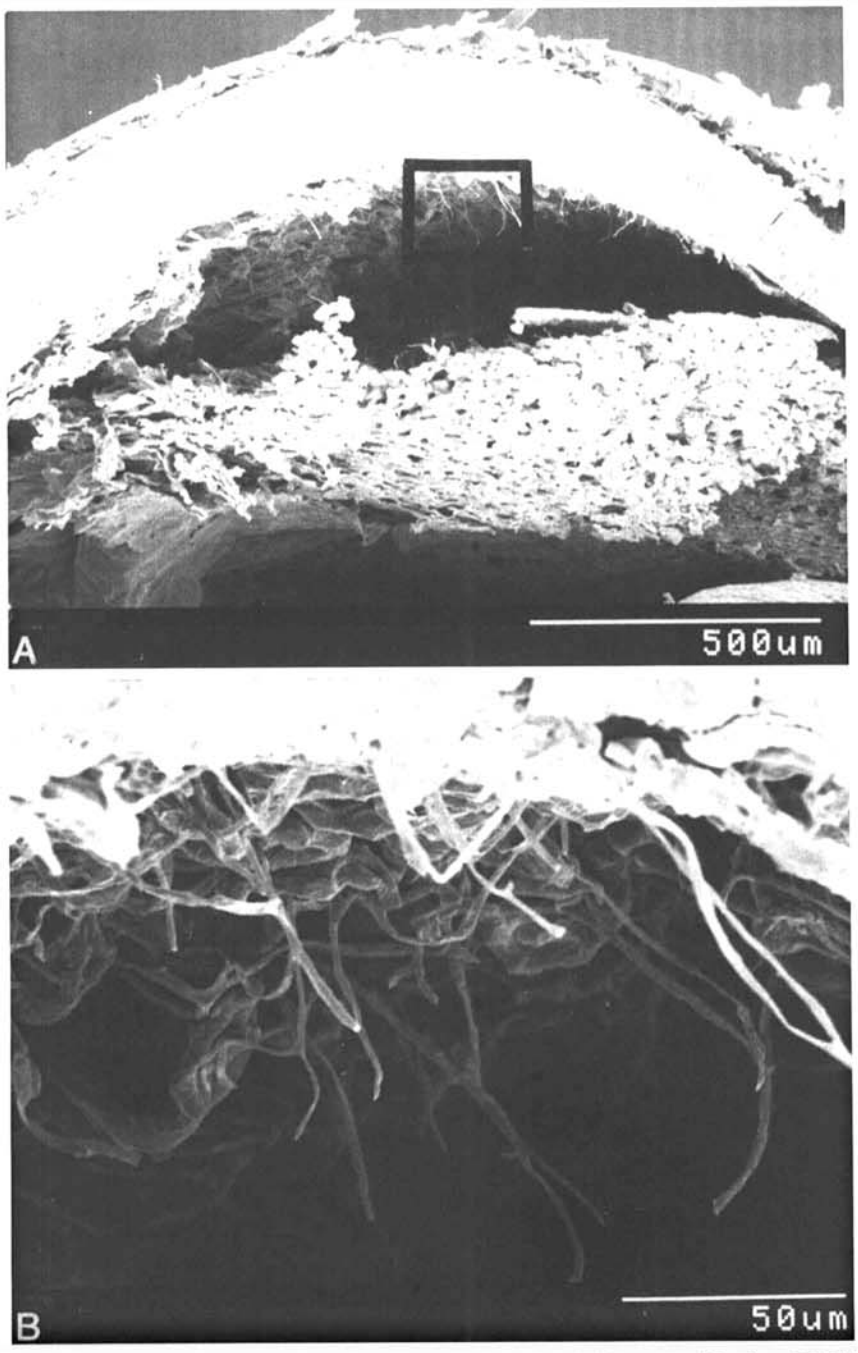

Fig. 3. Scanning electron micrograph of dried, sliced seed at **(A)** low magnification; **(B)** high magnification of framed area in A.

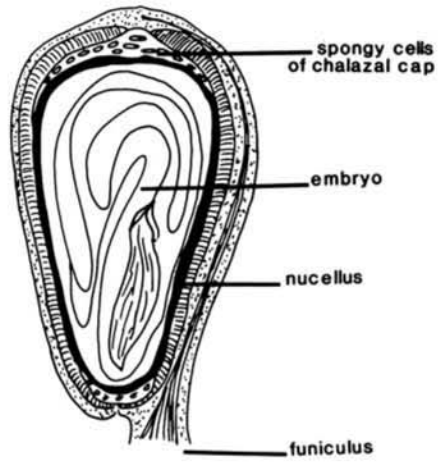

Fig. 4 Diagram of a longitudinal section through the layers of the cottonseed coat. Styled from: Leahy, J., Structure of cottonseed. Page 110 in: *Cottonseed.* A. E. Bailey, ed. Interscience Publishers, New York, 1948.

DISCUSSION

Both naturally contaminated and inoculated seed could have had levels of toxin below the detection limit of the analytical method. Although no toxin would have been detected in seed with aflatoxin levels lower than 100 ng/g, detection of small microbial infections were possible when tissue was cultured on PDA. We found that *A. flavus* emerged from all of the 100 seeds from inoculated locks, yet in the seeds assayed for aflatoxin, only 24% were toxin-positive. Limit of detection of the assay could also explain the lack of toxin detection in some BGYF-lintered seeds. Fungal presence on linters but not in seed could, of course, explain BGYF with no toxin. A recent report by McCormick et al (8) offers a different and interesting explanation for this presence of the fungus without toxin production. They describe a component of seed from bolls harvested before 25 or 40 days after flowering that inhibits toxin formation but not fungal growth. Our study reports lower toxin levels in bolls inoculated 19, 26, and 40 days after flowering than were detected in bolls inoculated 33 days after flowering.

Drying seems to be an important factor in fungal entry into seeds. The scanning electron micrographs of seed with mycelium inside the seed after drying and no mycelium in seed frozen immediately after harvest (Figs. 2 and 3) offer convincing new evidence that drying enhances fungal entry. Fungal presence in the cavity above the chalazal cap and the lack of toxin detection in some seeds indicate a physical barrier to fungal entry into the embryo where toxin formation is maximal (9). The nucellus membrane that surrounds the embryo seems a likely candidate (Fig. 4). Drying could affect its integrity. As seed dry in the lock, the fungus, already present in the chalazal cap, could break through the nucellus membrane, enter the embryo, and form the toxin. The time that elapsed during drying was not addressed in this study. Extended slow drying that would allow the fungus to continue to colonize tissue may have been as important as the dry-down itself. The differential effects of drying on seed metabolism compared with fungal metabolism is then an important, yet unexplored factor in toxin formation. Such biochemical interactions as well as the physical aspects of fungal penetration need further research. Although the plant/fungal interactions are not yet understood, the results of this study show that insect-vectored entry of *A. flavus* and subsequent toxin infection are probably of green bolls and that control of insects throughout the period of maximum boll development is necessary for toxin control.

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