Aflatoxin in Arizona Cottonseed: Field Inoculation of Bolls by *Aspergillus flavus* Spores in Wind-Driven Soil

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Conditions typical of an Arizona monsoon were mimicked in the field to inoculate cotton plants with *Aspergillus flavus*. Spores, mixed with autoclaved local soil, were blown onto cotton plants having bolls at all stages of maturity, using a modified commercial leaf blower. Half the plants were sprayed with water following inoculation. After one month, plants were pulled and the position of bolls mapped. All bolls were examined for bright-green-yellow fluorescence (BGYF) of lint, and ginned seeds from each boll were assayed for aflatoxin. Control non-wetted, non-inoculated bolls had no BGYF lint and no aflatoxin-containing seed. In contrast, 15% of the bolls from wetted, inoculated plants exhibited BGYF; 18% of these BGYF bolls had toxin. Only 3% of the non-wetted bolls had BGYF lint and none contained toxin. Lower bolls (fully fluffed at inoculation) were not infected, nor were upper bolls (flower stage at inoculation). Infection occurred only in bolls that had opened during the 30 days following inoculation. While the position of BGYF bolls on naturally contaminated plants was the same as for the inoculated, the ratio of toxic bolls to BGYF bolls was different. All BGYF bolls from plants naturally contaminated with *A. flavus* contained aflatoxin.

Aflatoxins are sometimes formed in cottonseed following *Aspergillus flavus* infection of cotton grown in the desert regions of California and Arizona. Contamination occurs in the field (1). Entry of the fungus traditionally has been considered a secondary infection, possibly following insect damage to the carpel wall (2). However, there has been a recent report of increased incidence of *A. flavus* in seed from bolls following inoculation of the involucral nectaries of flowers or of immature bolls where no visible damage to the carpel wall was incurred (3).

Toxin levels are highest in seed from bolls opening in August (4), when storms disturb the usually dry and tranquil desert. This is the monsoon season during which rain usually follows strong winds that cause "sand-devils" or small cyclonic currents of air that pick up sandy soil and carry it at high rates of speed. It is possible that *A. flavus* propagules, driven along with such soil, could penetrate into developing cotton bolls. Rain could facilitate the entry of the fungus into natural openings on the boll. To test this hypothesis, we attempted to simulate these environmental conditions. We determined the levels of *A. flavus* propagules in the soil and air of specific Arizona cotton fields and investigated the effect of water treatments following wind-driven soil inoculations with *A. flavus*. We used both toxin in seed and the presence of a bright-green-yellow fluorescence (BGYF) of lint, a property associated with *A. flavus* in cotton (5,6), to assess fungal infection. The positions of BGYF bolls on plants and the ratio of BGYF bolls with no toxin to those containing toxin were compared between inoculated plants and those with naturally incurred *A. flavus*.

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EXPERIMENTAL PROCEDURE

Environmental spore count. Levels of *A. flavus* propagules in soil and on leaves were determined in 1983. Leaf samples were 12.6 cm^2 plugs cut from leaf surfaces. Soil samples were collected in cotton fields close to the plants, and propagules per 0.01 g of soil determined. Both leaf and soil samples were plated onto Rose Bengal differential medium. Air samples drawn through the same medium were taken with an Anderson Air Sampler run for 30 min, usually in mid-morning.

Simulated monsoon. Experiments were conducted at the University of Arizona farm at Maricopa in August, 1984. Wind was provided by a generator-powered commercial leaf blower rated capable of producing a 125-mph air current. The blower was modified by inserting a plastic funnel into a 3/4" hole drilled into the barrel. A dowel of slightly smaller diameter than the funnel was used to aid flow of a soil-spore mixture fed through the funnel into the barrel. The blower, hand held about 3 ft from the cotton plants, was moved vertically and horizontally to spread the soil-spore mixture over the entire plant. Squares, buds and flowers, as well as developing and fully opened bolls, were treated. Inoculum consisted of soil taken from local fields, autoclaved and mixed with spores from a toxigenic strain of *A. flavus* (SRRC 1000) isolated from Arizona cotton. A spore count of the mixture determined the inoculation potential. Approximately 400 g of the soil-spore mixture was blown onto each plant. Monsoon season rain was simulated by water application from a small plastic atomizer (spray bottle). Eight plants were treated with wind-driven soil inoculation followed by water. Eight others were wind inoculated but received no water treatment. Approximately 200 ml of water was applied to each water-treated plant. Control plants in the near vicinity but isolated from air blower effects received neither treatment. All plants, controlled and treated, were exposed to the ambient weather conditions that prevailed during the duration of the trial.

After 30 days, plants were pulled and air dried. A map of each boll's position on a plant was made for every plant; then bolls were excised. A long wave UV lamp was used to examine bolls for lint that exhibited BGYF. Lint was removed from all seed with a small laboratory gin (7). Bolls were ginned individually and the seeds (20 to 30) from each boll were assayed together as a composite sample by a scale-up of the method of Cucullu et al. (8). Whole seed were weighed, placed between folds of waxed weighing paper and crushed. Crushed seeds (hulls included) were transferred to a 150-ml beaker and soaked in 50 ml of 70% aqueous acetone for ca. 1 hr, then 5 ml of 20% lead acetate solution was added. Contents were transferred to a 250-ml separatory funnel, and the beaker was washed with ca. 80 ml of water. The washings, which

were added to the initial extract, were partitioned into ca. 50 ml methylene chloride, and the organic phase was drained through sodium sulfate held in folded filter paper hung in the top of a 100-ml beaker. The sulfate was washed with ca. 25 ml of additional methylene chloride, after which the solvent was evaporated. Aflatoxins were quantitated by visual comparison with a standard using thin layer chromatography (TLC) (8).

A search of cotton fields in the valley region of Arizona yielded eight plants with bolls visibly infected with *A. flavus*. These plants were pulled and the bolls examined and assayed in the same manner used for bolls from the control and treated plants.

RESULTS AND DISCUSSION

A. flavus propagules in soil (Table 1), in the air (Fig. 1) and on leaves (Table 2) were highest in August. Propagules in the air increased more dramatically from July to August than those either in soil or on leaves. As soil

TABLE 1

Soil Populations[a] of *Aspergillus flavus* as Related to Date, Location and Canopy Closing (Arizona)

Date	Stanfield	Maricopa	Laveen	Tolleson	Cosmo	Paloma
6/17	10	3	13	2	12	4
6/21	4	5	12	30	43	40
7/06	7	6	65	17	16	100
7/18	4	5	50[b]	21	50	90[b]
7/25	5	10	93	10	51	190
8/01	4	10	43	8	48	364
8/11	28[b]	67	30	20[b]	70[b]	300
8/22	500	40[b]	60	42	110	180
8/30	150	7	85	20	180	320
9/01	120	15	42	12	185	632
9/06	250	20	60	8	112	420

[a]Mean of 4 replications. Propagules/0.01 g soil.
[b]Approximate date at canopy closure.

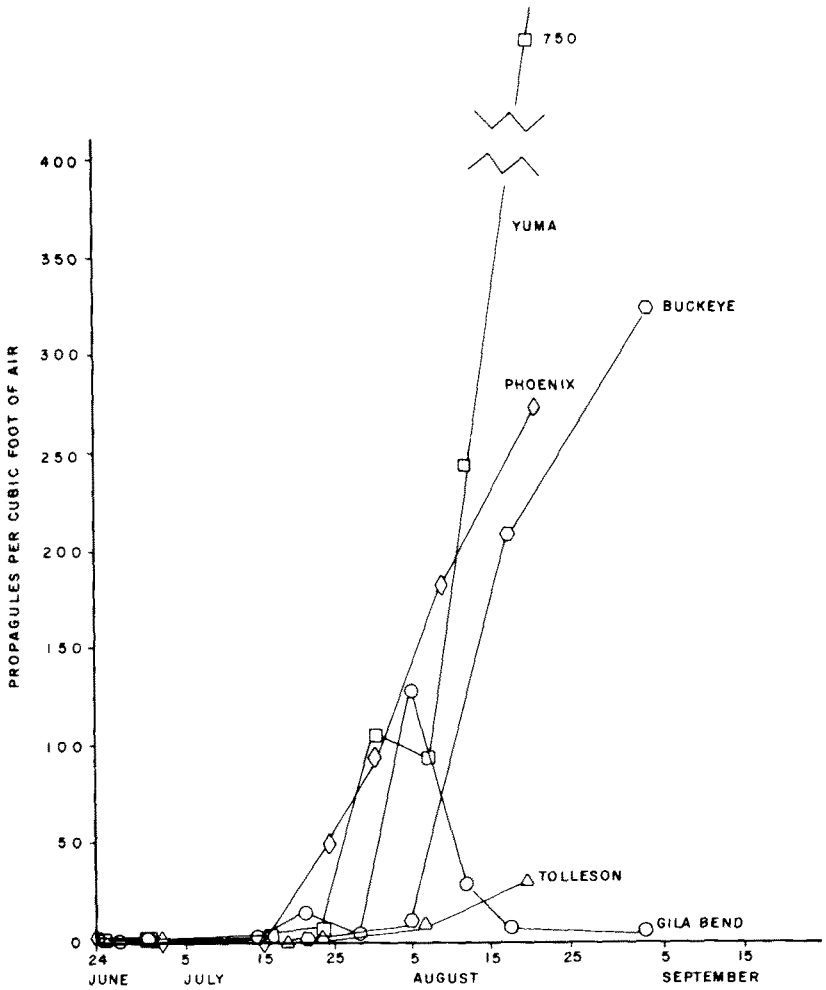

FIG. 1. Typical population of airborne propagules of *Aspergillus flavus* in selected Arizona fields (1980).

TABLE 2

Leaf Surface Populations[a] of *Aspergillus flavus* as Related to Date, Location and Canopy Closing (Arizona)

Date	Stanfield	Maricopa	Laveen	Tolleson	Cosmo	Paloma
6/17	<1	<1	2	<1	2	1
7/06	<1	<1	3	<1	<1	<1
7/18	<1	1	6[b]	1	2	1[b]
7/25	<1	2	15	<1	5	<1
8/01	25	2	5	1	2	75
8/11	9[b]	4	7	2[b]	11[b]	92
8/22	5	10[b]	12	5	8	107
8/30	5	3	22	4	13	96
9/01	10	23	42	6	8	60
9/06	18	7	39	2	8	104

[a]Mean of 10 replications. Propagules per 12.6 cm^2.

[b]Approximate date at canopy closure.

TABLE 3

BGYF Bolls and Aflatoxin Bolls from Treated Plants and Plants Naturally Contaminated with *Aspergillus flavus*

Treatment	Bolls assayed	BGYF bolls	Toxin bolls
Soil with *A. flavus*[a] no water	140	4	0
Soil with *A. flavus* followed by water	150	22	4
Control: no treatment	140	0	0
Plants naturally contaminated with *A. flavus*	180	8	8

[a]Spore count: 23 × 10^7/g of soil.

and leaves are constantly exposed to the atmosphere, *A. flavus* on leaf surfaces and soil are an indication of air contamination. The dramatic increase in fungal propagules in air indicates an influx of the fungus on prevailing winds. Both soil and air-borne *A. flavus* could be blown onto cotton plants by heavy winds during the summer monsoon. Our experiments, therefore, merely mimicked possible natural exposure of plants to *A. flavus*.

Experiments initially were planned for a time after the monsoon season is usually over, but due to the unusually long period that rain fell, even those plants intended to be non-wetted were rained on. No rain fell during the week just following treatments. Only bolls (Table 3) harvested from plants receiving the water treatment contained toxin. Since no toxin was detected in bolls from non-wetted plants, we assume that the water treatment immediately following inoculation aided entry of the fungus into developing bolls. All toxin-containing bolls were from those exhibiting some BGYF on lint; this fluorescence often appeared in the placental tissue where peroxidase concentration is great (9). However, linters on ginned seed from these bolls with BGYF lint did not always exhibit BGYF, indicating fungal invasion of the lint but not of the seed. No BGYF linters were observed on seed from 18 of the bolls with BGYF lint from the treatment with inoculum plus water or on seed from any of the four bolls from the treatment with inoculum alone. In contrast, all of the toxin-containing bolls from naturally contaminated plants had both BGYF lint and BGYF seed linters. When natural fungal invasion occurred as determined by BGYF lint, it caused toxin in the seed. Toxin levels ranged from 1,000 ng/g to 25,000 ng/g, and usually only one boll per plant was contaminated. For treated plants, several bolls on a plant exhibited BGYF lint but levels of toxin were less than 1,000 ng/g of seed. All bolls invaded by the fungus, from both natural and treated plants, were in the middle third of those plants. This observation was consistent with that reported in a seasonal sampling study (4). Bolls fully fluffed located at the bottom of the plants were never infected, nor were those high on the plant that were in the flower stage when treated. Infection occurred in bolls that fluffed during the 30 days following treatment. Bolls could have been infected just as the sutures opened, or fungal invasion could have been through natural openings such as the involucral nectaries (3) or through an injury to the carpel wall caused by abrasion from the wind-blown sand.

Related facts emerge from this study. *A. flavus* propagules in air and soil increase in August, the monsoon season when wind and rain occur. Plants treated with water following the fungal-soil inoculation had bolls with BGYF lint and seed that contained toxin, while plants receiving the fungal-soil treatment with no water had bolls with limited BGYF lint and no toxigenic seed. It is clear that water administered immediately following the inoculation aided fungal invasion of developing bolls. However, the pattern of contaminated bolls and level of toxin in seed was different between inoculated plants and plants with naturally incurred *A. flavus* contamination. Contamination could occur from *A. flavus* inoculation as indicated by the results in our study. These results do not preclude other entrances, possibly insect vectored following injury to the carpel wall. They merely validate one hypothesis concerning the mode of entry of *A. flavus* into cotton bolls.

ACKNOWLEDGMENTS

The National Cottonseed Processing Association provided travel funds.

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[Received October 10, 1985]