Geographical Localization and Lint Fluorescence in Relation to Aflatoxin Production in Aspergillus flavus-Infected Cottonseed

D. E. Gardner, J. L. McMeans, C. M. Brown,
R. M. Bilbrey, and L. L. Parker

Third author, Imperial Valley Conservation Research Center, ARS, USDA, Brawley, California 92227; others, Western Cotton Research Laboratory, ARS, USDA, Phoenix, Arizona 85040. Present address of senior author: National Park Service, Mississippi Test Facility, Bay Saint Louis 39520.

Accepted for publication 23 September 1973.

ABSTRACT

Aspergillus flavus boll infection in specific western regions of the cotton belt results in a characteristic greenish-yellow lint fluorescence often accompanied by aflatoxin accumulation in the seed. However, essentially no aflatoxin or fluorescence have been detected in cottonseed grown at Safford, Arizona; although most *A. flavus* isolates from infected seed produced aflatoxins in the laboratory.

Natural *A. flavus* infection probably occurs in nondamaged bolls only as bolls open at maturity. At Phoenix, Arizona, and Brawley, California, opening coincided with extended periods of elevated temp. At Safford, most bolls opened during periods of lower temp. *A. flavus* boll inoculation during the boll-opening period at each location resulted in high infection, aflatoxin, and fluorescence levels at Phoenix and Brawley and also at Safford in August. However, no more than traces of aflatoxin or fluorescence resulted from inoculation-induced infection at Safford in September when the majority of bolls at that location opened. Low temp is suggested as the principal factor limiting aflatoxin accumulation at Safford.

Considerable amounts of aflatoxin were detected throughout this study in seed from inoculated bolls containing no fluorescent lint; a significant exception to the usual association of fluorescence with high aflatoxin levels in cottonseed.

Phytopathology 64:452-455.

Infection of cottonseed by *Aspergillus flavus* (Lk.) Fr., accompanied by aflatoxin production, occurs in specific western regions of the cotton belt (3, 8, 9, 10, 11). Associated with *A. flavus* infection is a greenish-yellow fluorescence of lint, which has value as an indirect qualitative indication of aflatoxin-contaminated seed (2, 7, 8, 9, 10, 12).

In our investigations of seed harvested in 1971 at Safford, Arizona, we detected a high percentage of seed infected internally with *A. flavus*, but did not detect significant amounts of lint fluorescence or aflatoxins. The present study was conducted to determine the reasons for the absence of aflatoxin in the Safford area and to ascertain factors that favor or limit *A. flavus* infection and aflatoxin production under field conditions.

MATERIALS AND METHODS.—*Gossypium hirsutum* L. 'Delta-Pine 16' was used in both 1971 and 1972 at each location. Cultural methods were those recommended for each test locality.

Samples from the second harvest of the 1971 crop at Safford were machine-picked. Eight 2.27-kg samples from the first harvest of the 1972 crop at Safford were handpicked from entire plants and eight from the lowermost part of plants. After ginning, all seeds were examined for greenish-yellow fluorescence under a high-intensity (100 W, wavelength = 3.66×10^{-7} m) ultraviolet lamp. Seeds were then acid-delinted, surface-disinfested in 0.5% sodium hypochlorite, and plated on malt-salt agar (MSA) for determination of internal seed fungi (3). Aflatoxins B_1 and B_2 in embryos were determined by the methods of Pons and Goldblatt, which were used in all aflatoxin analyses throughout this study (13).

Each *A. flavus* isolate obtained from the 1971 seed was grown on autoclaved ground cottonseed embryos (30 g of embryos plus 10 ml of water). The embryos were from seed lots previously determined to contain no aflatoxin (13). The isolates were incubated in pure culture for 6-7 days at 27 C, then assayed for aflatoxins.

A. flavus infection was experimentally induced in cotton bolls in the field to determine whether aflatoxin production was possible in areas where it did not naturally occur. Ten highly toxicogenic *A. flavus* isolates from seed grown at Phoenix, Arizona, and at Brawley (Imperial Valley), California, were selected. Conidia from cultures grown on MSA for 5 days at 27 C were suspended in sterile water. Spore suspensions were adjusted to a concentration of approximately 7.5×10^5 conidia/ml. Approximately 1 ml of this inoculum was injected from a 6-ml syringe through a 15-gauge needle into cotton bolls.

Bolls which were unopened but would open during the following 72-h period were selected for injection. The greater uniformity in boll maturation time at Phoenix and at Brawley as compared with that at Safford permitted only two inoculation dates at the former locations as compared with three at the latter. However, all bolls selected for inoculation throughout the study were in the same stage of maturation (immediately prior to natural opening) regardless of the date or location, enabling cross-comparisons to be made.

Each boll selected for injection was visually undamaged and occurred in the lowermost part of the plants. Prior to injection, each boll was tagged and disinfested in 0.5% sodium hypochlorite. An equal number of control bolls were similarly injected with sterile water. Inoculations were made on August 15

and 30 and September 11, 1972, at Safford. Approximately 100 bolls were inoculated with *A. flavus*, and a similar number injected with water on each inoculation date. The same procedure was followed at Phoenix on July 28 and August 7, and at Brawley on July 17 and 31.

Inoculated bolls were allowed to remain on the plants until all other cotton was harvested at the end of the season. Individual locules and seeds of each inoculated boll were then removed and examined for fluorescence; and internal *A. flavus* seed infection and aflatoxin content were determined.

Rate of normal boll opening was determined at Safford by tagging 50 mature, cracked bolls and periodically measuring the gap between pericarp tips. Air temp and relative humidity (RH) among plants were measured with human hair hygrothermographs placed under the foliage as near the lower bolls as possible.

RESULTS.—*A. flavus* was isolated from seed harvested at Safford in 1971, although neither fluorescence nor aflatoxin in greater than trace amounts was detected. Isolation frequency varied among seed samples from 0 to 11.5%, with a mean of 4.5%. Seventy-four percent of 73 isolates cultured on ground cottonseed embryos produced significant levels of aflatoxin in amounts ranging from 58 to 1.58×10^5 ng/g of seed.

Only a trace of aflatoxin was detected among the 16 field cotton samples harvested at Safford in 1972, and only three seeds fluoresced among approximately 2.3×10^5 gin-run seeds examined. *A. flavus* was isolated from 0.063% of the seeds.

An average of 36% of the locules from bolls inoculated in August at Safford showed fluorescence. This frequency decreased sharply to 2% in those bolls inoculated in September. Conversely, fluorescence levels among locules from bolls inoculated on each date at Phoenix and at Brawley remained high (Table 1). Seeds of inoculated bolls that showed fluorescence from each location were found to contain substantial, though variable, quantities of aflatoxin. The single exception was the few seeds from fluorescent locules inoculated in September at Safford from which no aflatoxin could be detected (Table 1).

High percentages of seed from inoculated bolls which did not fluoresce were found to be infected with *A. flavus*. The fungus was isolated from 42, 58, and 49% of the seeds from nonfluorescing bolls inoculated at Safford on the two August dates and the September date, respectively. August inoculation at this location caused visibly severe infection; the lint was discolored (although not fluorescent), and large portions of the lint and the seed had deteriorated. In contrast, lint and seed from the September-inoculated bolls appeared sound, and isolation procedures were required for detection of infected seed.

At Phoenix and at Brawley, percentages of infected seed were similarly high in inoculated bolls that showed no fluorescence. Inoculations on all dates at these locations resulted in severe infection intensities which were comparable with those from August inoculations at Safford.

Although levels varied, considerable amounts of aflatoxin were detected in seeds from bolls that showed no fluorescence inoculated on each date at Phoenix, Brawley, and Safford except in those from bolls inoculated in September at Safford. Only a trace of aflatoxin was detected in these seeds (Table 1).

Mean numbers of hours per day above 32 C in late July and early August, when bolls in the lowermost part of plants opened, were approximately 11.4 at Phoenix and 11.7 at Brawley; the mean number of hours per day in September at Safford, when the majority of bolls at that location opened, was 1.5 (Table 2).

Boll-opening measurements at Safford indicated that 72 h was the approximate minimum time required for opening and lint drying. Relative humidity among plants at that location was above 80%

TABLE 1. Occurrence and levels of greenish-yellow fluorescent lint and total aflatoxins (B_1 and B_2) in embryos from mature unopened cotton bolls inoculated with *Aspergillus flavus* at three locations in 1972

Location	Inoculation dates	Fluorescent locules (%)	Aflatoxins B_1 and B_2 (ng/g)	
			Seed from bolls with fluorescence	Seed from bolls with no fluorescence
Phoenix	July 28	46	32,414	8,568
	August 7	47	39,321	585
Brawley	July 17	52	32,698	7,878
	July 31	60	10,930	1,062
Safford	August 15	34	45,841	5,062
	August 30	38	10,723	2,073
	September 11	2	none detected	trace

TABLE 2. Comparison of cottonfield temp data among three locations where mature, unopened bolls were inoculated with *Aspergillus flavus* in 1972

Month and location	Date	Max temp (C)	Duration (hr)	Avg hr/day/month above 32 C
July				
Phoenix	31	47	1	13.2
Brawley	3	47	1	13.3
August				
Phoenix	1	47	2	9.7
Brawley	1	46	1	10.2
Safford	1	40	1	5.4
September				
Phoenix	5	41	5	7.7
Brawley	5	41	2	7.8
Safford	18	36	2	1.5

for an average of 10 h/day during the boll-opening period, with the minimum never falling below 30%.

DISCUSSION.—The isolation of substantial amounts of potentially toxicogenic *A. flavus* from 1971 Safford-grown seed indicates that the absence of significant aflatoxin contamination in cottonseed from that area cannot be attributed to a scarcity of inoculum.

A. flavus is not thought to penetrate pericarp tissue directly, but to enter noninsect-damaged bolls as opening occurs and before the lint dries (4). Environmental conditions during the boll-opening period, therefore, may determine or influence aflatoxin accumulation, lint fluorescence, and infection itself. Halisky et al. (6) found positive correlations among severity of *A. flavus* boll rot, lint fluorescence, and the high field temp in southern (Imperial Valley) California. Marsh et al. (9) likewise attributed *A. flavus* boll rot accompanied by fluorescence and aflatoxin accumulation, to the exceptionally high field temp prevalent in areas where the disease occurs.

Boll opening in the lowermost part of plants, the site of natural *A. flavus* infection and aflatoxin accumulation (12), was found in the present study to correspond at both Phoenix and Brawley to the extended daily periods of elevated temp during late July and early August. Aflatoxin and lint fluorescence resulting from inoculation-induced infection at these locations during the boll opening period were readily detected. In contrast, even though high concns of toxicogenic inoculum were injected directly into the bolls, aflatoxin and fluorescence were not produced in more than trace amounts at Safford as a result of inoculation in September, when most bolls at that location opened and became subject to invasion by the fungus. Field temp corresponding to boll opening were elevated during markedly fewer daily hours at Safford than were those at Phoenix or Brawley (Table 2).

Although infection resulting from the September inoculation at Safford was extensive, it was notably less intense than that resulting from inoculation on either date at Phoenix or Brawley, or at Safford in August. August temp at Safford were higher than those in September. Aflatoxin and fluorescence may have therefore been produced in August following inoculation, although not following any natural infection in which the inoculum strength was less intense.

Ashworth (1) and Ashworth et al. (3, 5) presented evidence that low RH was responsible for absence of *A. flavus* cottonseed infection and aflatoxin accumulation in the San Joaquin Valley of California, compared with the extensive infection in the Imperial Valley. Boll opening and lint drying both proceed rapidly under conditions of low RH. Fungal invasion and development beyond the lint infection stage is prevented, permitting the seeds to escape infection. High RH in the Imperial Valley retards boll opening and provides sufficient time for seed infection and aflatoxin production before lint drying.

According to the results of the present study, rapid boll opening and drying apparently do not account for absence of aflatoxin at Safford. Boll-opening measurements and RH data taken at that location indicated an extended opening and drying period compared with that in the San Joaquin Valley (3). Also, the high percentage of actual seed infection (49%) (as opposed to lint infection only) adds evidence which supports this conclusion. Based upon this study, influence of low temp is suggested as the principal factor which determines the absence of aflatoxin-contaminated seed in the Safford area.

In addition to the high levels of aflatoxin detected in greenish-yellow fluorescing bolls throughout this study, considerable infection and aflatoxin production occurred in seed from inoculated bolls that did not fluoresce (Table 1). To our knowledge, this extensive and severe infection by *A. flavus* which resulted in high aflatoxin concns in the absence of any fluorescence has not previously been reported. The use of greenish-yellow lint fluorescence as an indication of aflatoxin-contaminated cottonseed, therefore, did not prove valid under the conditions of this study. Thus, the need for caution in relying upon this method alone for routine aflatoxin detection is emphasized.

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