Fungi That Infect Cottonseeds Before Harvest

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As a part of an investigation of aflatoxins and other mycotoxins in cottonseeds at harvest, samples of seeds collected from the 1971 crop at locations across the U.S. Cotton Belt were examined to determine the kinds of microorganisms causing internal or seed-coat infection in the field. Aspergillus flavus infection was absent from all seeds examined from most areas but was present in some samples from Arizona, California, and Texas. Fusarium spp., Alternaria sp., and A. niger caused internal infection at many locations; Colletotrichum gossypii and Rhizopus stolonifer were present in seeds from some areas but were generally much less common. Many of the infections with A. niger were in the seed coat. Bacterial infections were fairly frequent. In a series of commercial samples from Arizona, A. flavus infection was found in 61% of seeds, with fiber showing the bright, greenish-yellow (BGY) fluorescence that is diagnostic for A. flavus boll rot. Aflatoxin contamination was also concentrated in the same seeds. The above findings agree with previous data showing that aflatoxin contamination of cottonseeds before harvest occurs rarely, if at all, in most parts of the U.S. Cotton Belt and that when such contamination does occur, it tends to be concentrated in seeds with the BGY fluorescence in their fiber and seed fuzz.

The aflatoxin-producing fungus Aspergillus flavus Link grows frequently in humid storage situations, but also causes a boll rot of cotton in the field and, in some cases, an accompanying contamination of the seeds with aflatoxins before harvest. A. flavus boll rot can be detected by a characteristic bright, greenish-yellow (BGY) fluorescence caused by A. flavus in spots in the fiber (18) and is relatively uncommon. It has been noted particularly in certain western parts of the Cotton Belt (30). Aflatoxin contamination in the seeds at harvest appears to be similarly uncommon but has been found in some samples in the same areas as the boll rot (19). Aflatoxins have been detected most frequently and at highest levels in seeds whose fiber and seed fuzz display the BGY fluorescence (19, 20). Aflatoxin contamination of U.S.-grown cottonseeds at harvest thus appears from previously available data to be significant only in localized areas. However, because of the present and anticipated future importance of cottonseed products in both animal and human nutrition and the unusual hazards associated with the aflatoxins (15), we considered it desirable to make further observations.

Fungi that infect cottonseeds before harvest have been known only incompletely and uncertainly. Fusarium spp., Colletotrichum gossypii Southworth, Diplodia gossypina Cooke, and Alternaria sp. have been reported to cause infections in U.S.-produced cottonseeds (1, 2, 8, 26, 27, R. B. Streets, A. M. Boyle, and H. Simonsen, Phytopathology 47:535, 1957); and A. flavus has been observed in seeds from the Imperial Valley of California (3, 19). Seed infection with A. flavus has been noted in association with the characteristic BGY fluorescence in the fiber (19). As a whole, however, investigations of preharvest infections of cottonseeds have involved examination of only a relatively small number of seeds from limited cotton-producing areas. Mayne (21) reported that 17 of 41 fungal isolates from 28 samples of cottonseeds from six southern states were A. flavus, but the history of most of her seed samples was not known, and infection may have occurred in damp storage after harvest. Further background information on the cottonseed-aflatoxin problem has been detailed and documented with references elsewhere (19).

This paper reports results of examinations for fungal infection of cottonseeds grown across the U.S. Cotton Belt under widely variable condi-
tions of culture and harvested at known dates. Confirmatory aflatoxin analyses on certain samples are also reported.

MATERIALS AND METHODS

Bolls from commercial cotton varieties were collected directly from plants on four to eight picking dates at 17 locations across the U.S. Cotton Belt (Table 1). The maximum weathering period before harvest was not accurately known, but probably it was not in excess of that common with commercial cotton. The bolls were dried at the point of origin and sent to Beltsville. At Beltsville, each boll was ginned individually by hand, and the seeds were delinted in concentrated sulfuric acid, washed first in 1% sodium carbonate solution and then in water, dried, and treated overnight in chlorine gas as described previously to surface disinfect (20). Seeds that were immature or obviously damaged were avoided for the examinations summarized in Table 1. For each picking date, fifty seeds were examined. Five seeds from each boll were planted on 2% water agar in a petri dish and were incubated for 4 days at 30 C in a room with a 12-h light cycle provided by daylight fluorescent lamps, by which they were examined by microscope to detect any fungi or bacteria growing out of them. No distinction was made between A. flavus and A. parasiticus Speare. The 161 commercial seedcotton samples from fields in unspecifed parts of Arizona were kindly provided by B. B. Taylor. The samples, as received, weighed in the range of 400 to 2,600 g. Seedcotton consists of seeds with fiber still attached and occurs in locks, one lock for each of the four or five segments of the boll and each lock containing about eight seeds.

Analyses for aflatoxin B1 were by a mini-plate screening procedure which detects aflatoxin B1 at levels above 100 parts per billion (ppb), followed by a quantitative assay on 20- by 20-cm Schleicher and Schuell silica gel plates. The mini-plate method, to be detalled elsewhere, consisted essentially of the following. A seed meat sample was shaken with three times its weight of chloroform-acetonitrile (1:2) and one-half of its weight of 10% aqueous ferric chloride, after which the extract was chromatographed on a 2- by 3-inch (5.08- by 7.62-cm) thin-layer chromatography plate with a 0.75-inch (1.905-cm) band of Al2O3 at the bottom and a 2.25-inch (5.7-cm) band of silica gel on the remainder of the plate. Development was for 8 min with diethyl ether-methanol-water (96:3:1). For a more quantitative estimate of the aflatoxin level, a sample of the above extract was diluted with benzene-acetonitrile (98:2), and this diluted extract was then spotted on a Schleicher and Schuell plate and developed in an unlined tank with diethyl ether-methanol-water (96:3:1).

RESULTS

Table 1 records data on fungal and bacterial infection of seeds from 17 locations across the Cotton Belt. A. flavus was detected only in the collections from Tulare County, California, and Vernon, Texas, and at low levels at these locations, even though the overall level of microbial infection in most of the samples was

| Location          | No. of pickings | Date of | Total no. of seeds examined | Alternaria sp. | A. flavus | A. niger | Bacteria | C. gossypii | F. moniliforme | Fusarium spp. | R. stolonifer | Other | Total |
|-------------------|-----------------|---------|-----------------------------|----------------|-----------|---------|----------|------------|---------------|---------------|--------------|--------|-------|
| Blackville, S.C.  | 6               | 8/27    | 11/29                       | 300            | 1         | 0       | 10       | 2          | 17            | 1             | 27           | 0      | 1     | 59    |
| College Station, Tex. | 4          | 8/27    | 10/1                        | 200            | 1         | 10      | 0        | 1          | 0             | 2            | 17            | 0      | 1     | 29    |
| Experiment, Ga.   | 6               | 9/27    | 12/6                        | 300            | 0         | 0       | 1        | 0          | 7             | 0            | 16           | 0      | 1     | 25    |
| Hartsville, S.C.  | 6               | 10/11   | 12/20                       | 300            | 4         | 0       | 0        | 1          | 0             | 1            | 7             | 0      | 1     | 25    |
| Jackson, Tenn.    | 6               | 9/22    | 12/4                        | 300            | 2         | 0       | 0        | 1          | 0             | 1            | 7             | 0      | 1     | 20    |
| Kern County, Calif. | 6            | 9/17    | 11/25                       | 300            | 0         | 0       | 1        | 8          | 0             | 0            | 0             | 0      | 0     | 10    |
| Las Cruces, N. Mex. | 5            | 9/16    | 11/11                       | 250            | 0         | 0       | 4        | 7          | 0             | 0            | 1             | 0      | 1     | 12    |
| Laurinburg, N.C.  | 6               | 10/29   | 11/18                       | 300            | 1         | 1       | 1        | 8          | 1             | 2            | 32           | 0      | 6     | 51    |
| Lubbock, Tex.     | 8               | 10/4    | 1/7                         | 400            | 2         | 0       | 4        | 2          | 0             | 0            | 0             | 0      | 1     | 9     |
| Portageville, Mo. | 6               | 9/23    | 11/30                       | 300            | 8         | 0       | 1        | 0          | 1             | 2            | 2             | 1      | 2     | 17    |
| Raleigh, N.C.     | 6               | 9/13    | 10/25                       | 200            | 1         | 0       | 2        | 14         | 0             | 1            | 20           | 0     | 6     | 44    |
| Stillwater, Okla. | 6               | 9/22    | 11/12                       | 300            | 5         | 0       | 1        | 1          | 0             | 0            | 1             | 1     | 3     | 11    |
| Stoneville, Miss. | 8               | 9/15    | 12/22                       | 400            | 2         | 0       | 1        | 1          | 0             | 1            | 14           | 0     | 1     | 20    |
| Tallassee, Ala.   | 4               | 9/28    | 11/8                        | 200            | 0         | 0       | 1        | 4          | 0             | 0            | 6             | 0     | 0     | 11    |
| Tifton, Ga.       | 5               | 9/7     | 11/2                        | 250            | 0         | 0       | 1        | 1          | 2             | 1            | 7             | 0     | 9     | 21    |
| Tulare County, Calif. | 6         | 9/27    | 12/17                       | 300            | 1         | 1       | 21       | 6          | 0             | 1            | 0             | 1     | 0     | 33    |
| Vernon, Tex.      | 5               | 10/21   | 12/18                       | 250            | 14        | 1       | 23       | 1          | 0             | 0            | 1            | 1     | 1     | 42    |
moderate to high. Fusarium was responsible for almost one-third of the observed infections. Except for F. moniliforme Sheldon, inadequate information is available on the Fusarium species present in the seeds, but six isolates of unknown species were sent to W. C. Snyder, and all were identified as F. roseum (Link) Fries. Alternaria was detected in nearly all samples. A. niger was common in seeds from locations west of the Mississippi River Valley but was also found, although usually at lower levels, in the other sections of the Cotton Belt. Almost all of the isolates of Aspergillus with black spores were within the description of A. niger van Tieghem. As with previously reported infections of fiber (29), infections of seeds with C. gossypii occurred only in the Southeast and mid-South. Infections of seeds with Rhizopus stolonifer (Ehrenberg ex Fries) Vuillemin were relatively uncommon. Microorganisms listed as "other" in Table 1 comprised a wide variety of types, but included essentially no species of Penicillium, Aspergillus, or other organisms generally categorized as "storage fungi." Cladosporium herbarum (Link) Fries and actinomycetes, frequent in preharvest infections of fiber (29), were not observed. The average percentage of infected seeds for all locations, including all organisms, was approximately 25%.

In almost all cases, infections of the seeds of Table 1 appeared to involve only a single fungus or bacterium per seed. The infecting fungus generally grew luxuriantly out of the seed at either the chalazal or funicular end. An exception to this was A. niger, which caused both internal (as above) and seed-coat infections. In the latter case, a sparse sporulating outgrowth of the fungus appeared over much or all of the surface of the seed and hyphae could be seen inside the seed coat but not in the seed meats when the infected seeds were cut open. More than half of the observed infections with A. niger were in the seed coat.

The general absence of detectable A. flavus infection in seeds from the samples of Table 1 was believed to be real and not a result of any inadequacy in the detection method. The same method was used successfully to detect A. flavus in cottonseeds in previous work (19). Furthermore, it was successfully applied to a series of 161 commercial samples of the 1971 crop in Arizona in the present investigation, with results as shown in Table 2. All locks with BGY-fluorescing fiber were picked out of 25 samples, and 5 seeds from each fluorescent subsample were delinted and examined for fungal infection; of the 125 such seeds, 76 (61%) were infected with A. flavus. For comparison, 25 seeds from locks with non-BGY-fluorescing fiber were examined from each of the 161 samples; of the 4,025 such seeds, only 48 (approximately 1%) yielded A. flavus. Thus, the A. flavus infections were highly concentrated in seeds with the BGY-fluorescing fiber. The BGY-fluorescing locks were low in number, constituting only 0 to 1.2% of the weight of the total seed cotton samples. The high general level of fungal infection in the seeds from non-fluorescing locks may have been related to breaks in the seed coat, readily seen in many of them, possibly caused by mechanical harvesting. A. niger was the predominant fungus isolated from these seeds (Table 2).

The fact that A. flavus infections in the 161 Arizona samples were highly concentrated in seeds with BGY-fluorescing fiber suggested that aflatoxins might also be similarly concentrated in these seeds. Aflatoxin analyses were made to check this point. Of the 161 seed cotton samples, 65 had no BGY-fluorescing locks and no detectable aflatoxin B₁ by the mini-plate procedure. Of the 96 samples with BGY-fluorescing locks, 51 contained detectable aflatoxin B₁ in the seeds from the fluorescing locks and 45 did not. From the same 96 samples, a random selection of non-BGY-fluorescing locks was made for each sample, and only three showed detectable aflatoxins present in their seeds. In each of these three cases, aflatoxins had also been detected in the seeds from the BGY-fluorescing locks in the same samples.

Extracts from seeds of the BGY-fluorescing locks were spotted on 20- by 20-cm Schleicher and Schuell thin-layer chromatography plates, and the levels of aflatoxin B₁ were estimated (Table 3). Obviously, a wide range in aflatoxin B₁ content occurred from one sample to the next, but with a very high average level, i.e., 28,206 ppb aflatoxin B₁.

Among the 51 samples that contained BGY-fluorescing locks with detectable aflatoxins an approximate minimum aflatoxin B₁ content was calculated by multiplying the parts per billion of aflatoxin B₁ in the seeds from the BGY-fluorescing locks by the fractional weight of

| BGY fluorescence in lock | Total examined | Total infected | Percent seed infected with: |
|-------------------------|----------------|----------------|-----------------------------|
|                         |                |                | A. flavus | A. niger | Other fungi |
| Present                 | 125            | 116            | 61        | 20       | 22          |
| Absent                  | 4,025          | 2,210          | 1         | 34       | 19          |

*Thirteen seeds had infections with two different fungi.

Table 2. Level of certain fungal infections in seeds from commercial fields in Arizona (crop of 1971)
such seeds in the total sample. Values of more than 100 ppb were obtained for 18 samples; the individual values in parts per billion were 363, 447, 738, 1,180, 794, 323, 192, 1,080, 369, 633, 206, 427, 528, 293, 532, 502, 365, and 324 (average = 516). Thus, about 11% of the 161 samples collected had minimum aflatoxin B₁ levels above 100 ppb.

**DISCUSSION**

The results here reported on *A. flavus* infections of cottonseeds are in accord with and substantiate previous evidence indicating that *A. flavus* boll rot and aflatoxin contamination of cottonseeds at harvest involve only a small fraction of the total U.S. cotton crop (19). Data indicate that all of these phenomena are very uncommon in the Southeast and mid-South. All have been obviously present in the Imperial Valley of California and occur at detectable levels in some still-undeveloped areas of Arizona. *A. flavus* boll rot has also been detected repeatedly at low to moderate levels in parts of Texas, especially in the region near Brownsville, but also in an area around Dallas. Evidence indicates that *A. flavus* boll rot is typical of hot, dry climates and that its incidence may be materially increased by insect attack on the bolls (19).

Aflatoxin contamination of the seeds at harvest appears to be very uncommon, if present at all, in any growing area where the BGY fluorescence, caused by *A. flavus*, is not also easily detectable. Such contamination occurs especially in seeds whose fiber or seed fuzz, or both, exhibit the BGY fluorescence. The above observations provide no evidence regarding *A. flavus* infection and aflatoxin contamination of the seeds during humid transit and storage, matters still very inadequately investigated.

The following comments may be made about the several fungi other than *A. flavus* which were found in the present work to infect cottonseeds before harvest.

**Alternaria.** Cotton may now be added to wheat (4, 6, 17, 22), barley (7, 12, 17), oats (17, 25), rye (17), soybeans (10, 24), corn (17), and the very many other plants (23) whose seeds are known to be infected frequently with this fungus in the field. Some isolates under some circumstances produce mycotoxins (5), but no information on such mycotoxins in the cotton crop is known to have been reported.

**A. niger.** This fungus seems to be present in cotton particularly in the western parts of the U.S. Cotton Belt. This conclusion is based more on previously published fiber-infection data (29) than on the seed-infection data here presented. The fungus appears to be more common in field infections of cottonseeds than in field infections of seeds of other plants.

**Fusarium spp.** These fungi are geographically widespread as a common cause of infection not only of cottonseed but also of fiber (29). *F. roseum*, apparently common in cottonseeds in the field, produces the estrogenic mycotoxin zearalenone (11). Other species of *Fusarium* also produce mycotoxins (5), but their significance to cotton culture is unknown. In exhibiting field infections of its seeds with species of *Fusarium*, cotton is similar to wheat (9, 34), oats (9), rye (9, 34), barley (9, 34), rice (32), corn (31), pearl millet (33), soybeans (24), and sorghum (16).

**Nigrospora oryzae (Berkley and Broome)**

Petch. This fungus has been reported by others to be a common cause of cotton boll rot in California (19) but was found rarely in the present study.

If we were to select any major infecting fungus as distinguishing the fungal flora of cottonseeds at harvest from that of most other plants, it would be *A. niger*. We cannot, however, ascribe infection with this fungus to the oil component of cottonseed nor to any other chemical component. The fact that *A. niger* is a vigorous wound parasite capable of penetrating from wounded into living tissue of the boll wall may be relevant. In the peanut, also an oil-bearing seed, the fungal population before harvest includes numerous species of *Penicillium* and *Aspergillus* (14), but the flora in this case are probably influenced strongly by soil contact. The

**TABLE 3. Aflatoxin B₁ levels in seeds from BGY-fluorescing locks picked out of 96 commercial seed cotton samples from Arizona (crop of 1971)**

| Level of aflatoxin B₁ (ppb) | No. of samples |
|-----------------------------|----------------|
| <100                        | 45             |
| 101-250                     | 4              |
| 251-500                     | 4              |
| 501-1,000                   | 1              |
| 1,001-3,000                 | 14             |
| 3,001-6,000                 | 2              |
| 6,001-9,000                 | 1              |
| 9,001-15,000                | 2              |
| 15,001-30,000               | 3              |
| 30,001-60,000               | 7              |
| 60,001-90,000               | 4              |
| 90,001-150,000              | 5              |
| 150,001-300,000             | 4              |
tendency for A. flavus to occur on small grains, especially under conditions of heating in storage, and the semixerophytic nature of the organism have been noted by Semeniuk (28).

Because species of Alternaria and Fusarium cause field infections in the seeds of so many crops that have important food and feed uses, further investigations of mycotoxins produced by these fungi are especially important to U.S. agriculture.

Some readers might wonder whether our surface sterilization of cottonseeds in chlorine gas, after delinating in sulfuric acid (20), might kill some fungi found internally in the seeds or whether the chlorine might selectively kill A. flavus. Actually, bolls incubated for several days with A. flavus, as previously described, (18), and then examined by this method have shown a high internal seed infection with the fungus; the 61% infection of the seeds from BGY-fluorescing locks recorded in Table 2 also suggests no major killing of the fungus internally in the seeds. Uninfected seeds germinate in high percentage after the chlorine treatment and produce seedlings of normal appearance. Some insight may also be gained by comparing the microbial population of the seeds with that of field-exposed cotton fiber (29) because, in the latter case, no sterilization was used. With the fiber, the microbial population consisted mainly of actinomycetes, Alternaria sp., A. niger, bacteria, C. herbarum, Fusarium spp., and R. stolonifer. In the seeds, all of these were detected except the actinomycetes and C. herbarum. We believe, however, that the absence of these two forms from the detected seed population did not result from their elimination during the sulfuric acid-chlorine treatment. Rather, we think that internal infection of the seeds occurs primarily through the chalazal opening, that the rapidly growing fungi tend to preempt the infection court, and that the slower growing actinomycetes and C. herbarum are eliminated by selective competition. We would agree that a real question about the adequacy of our methods for detection of microorganisms in both cotton fiber and seed may exist in respect to D. gossypina, which probably does not produce recognizable fruiting bodies during the relatively short incubation periods used.

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