Mycoflora, Aflatoxins and Free Fatty Acids in California Cottonseed During 1967-1968

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ABSTRACT

In central California, neither fungal infections nor aflatoxins are significant problems in cottonseed during the receiving and storage seasons. However, in southern California, the 1967 harvest contained a relatively high percentage of seed which were invaded before harvest by fungi, including *Aspergillus flavus*. Seed infection and concentrations of aflatoxins in seed increased significantly during the time between harvest and storage in southern California. For a short time during storage, seed infection by *A. flavus* increased because of the moisture the seed received late in the season; however, aflatoxin concentrations in seed did not increase in storage. The aflatoxin content of the seed removed from storage was a reflection of the relative amount of aflatoxins the seed contained when they were received for storage. In 1967, the conditions that existed in the large, densely packed seed pile did not favor accummulation of aflatoxins in seed, even though *A. flavus* was active.

INTRODUCTION

The extensive literature on fungal deterioration of cereal grains has recently been summarized by Christensen and Kaufmann (1). The role of fungi in loss of certain quality characteristics has been investigated (2,3), but little is known about postharvest deterioration of cottonseed, particularly with regard to cottonseed destined for processing into oil and meal. Unlike the situation described for cereal grains (1), both field and storage type fungi invade cottonseed prior to harvest. The most notable storage fungi infecting cottonseed prior to harvest are members of the *Aspergillus flavus* group. *A. flavus* has for some years been known as a preharvest invader of lint (extensions of seed coat cells) (4) and of seed coats and embryos where aflatoxins may accumulate (5-7). Most primary infections by the fungus occur when carpels separate at maturity, exposing the seed to invasion by the fungus (5). Other data (8) indicate than an association may exist between invasion of bolls by the pink bollworm (*Pectinophora gossypiella* Saunders) and infections may occur any time seeds contain enough moisture for fungus growth.

This paper reports the results of experiments made to assess the relative importance of preharvest and postharvest development of fungal infection and occurrence of aflatoxins in the seed.

TABLE I

Fungi Infecting Cottonseed of Southern California at Harvest, November 2-4, 1967

Fungi infecting seed		Amount of seed infected by fungi, %
Genera	Species or groups	
Field fungi[a]		
Fusarium	spp.[b]	3.4
Rhizopus	spp.	0.2
Cladosporium	sp.	0.1
Alternaria	sp.	0.7
Nigrospora	*oryzae*	None
Macrophomina	*phaseoli*	Trace[c]
Storage fungi[a]		
Aspergillus	*flavus*	1.7
Aspergillus	*niger*	1.5
Aspergillus	*glaucus*	3.3
Aspergillus	*flavipes*	1.9
Aspergillus	spp.[d]	1.1
Penicillium	spp.	1.4
Total infection		15.3

[a]Classifications described by Christensen (6).

[b]Includes *Fusarium oxysporum, F. roseum* and *F. moniliforme*.

[c]Less than 0.1%.

[d]Includes *A. ochraceous* (0.5%) and *A. versicolor* (0.6%).

MATERIALS AND METHODS

Seed Quality at Harvest

Estimates of the amounts of fungal infection and of aflatoxin content of seed were obtained from 16.5 lb samples of cottonseed hand-picked from each of five fields in southern California, November 2-4, 1967. The samples were from the lowermost one third part of plants where

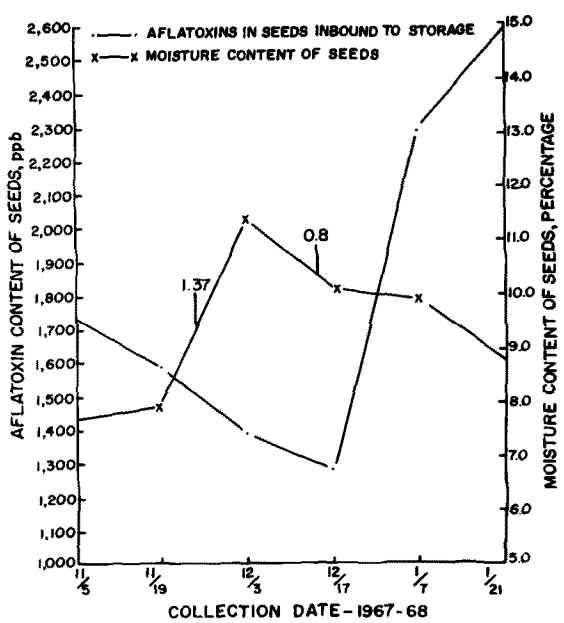

FIG. 1. Mean aflatoxin and mean moisture contents of cottonseed inbound to storage in southern California in 1967-68. The inches of rainfall received during the seed receiving season is shown on line indicating seed moisture contents.

TABLE II

Mean Amounts of Fungal Infection of Fuzzy Cottonseed Inbound to Storage and During the Storage Season of 1967-68 in Central and in Southern California

		Amount of seed infected by fungi, %			
		Central California		Southern California	
Genera	Species or groups	Inbound[a]	Storage[a]	Inbound	Storage
Field fungi[b]					
Fusarium	spp.[c]	0.5	0.1	3.3	1.2
Rhizopus	spp.	0.6	0.2	0.2	0.1
Cladosporium	sp.	0.3	Trace[d]	0.1	Trace
Alternaria	sp.	0.5	0.1	0.3	0.1
Nigrospora	*oryzae*	0.3	Trace	0.1	Trace
Macrophomina	*phaseoli*	None	None	0.1	Trace
Storage fungi[b]					
Aspergillus	*flavus*	0.2	0.1	7.4	11.2
Aspergillus	*niger*	0.6	1.2	2.5	0.8
Aspergillus	*glaucus*	2.7	0.6	5.1	5.4
Aspergillus	spp.[e]	0.1	0.2	2.2	0.6
Penicillium	spp.	0.2	0.2	0.5	1.3
Total infection		6.0	2.7	21.8	20.7

[a]Respectively, seeds enroute to storage and stored seed.

[b]Classifications described by Christensen (6).

[c]Includes *Fusarium roseum, F. moniliforme* and *F. oxysporum.*

[d]Less than 0.1%.

[e]Includes *Aspergillus flavipes, A. ochraceous, A. tamarii, A. terreus, A. wentii, A. candidus* and *A. versicolor.*

essentially all infections and aflatoxins occur (9). Three hundred seeds from each sample were cultured for fungal determinations and aflatoxin analyses were made on each sample, as described earlier (6). Estimates of the amounts of infected seed and aflatoxin content of seed were based upon the assumption that the bolls of the lowermost one third of the plant represent about 40% of the crop. The free fatty acid (FFA) content of the seed was determined by the AOCS official method (10). A portion of this work was reported earlier (11).

Quality of Cottonseed Inbound to Storage

Determinations were made on seed produced in and received for storage in both central California (CC) and southern California (SC). Three to 4 lb samples were drawn from trucks arriving at receiving docks one day each week. Seed from trucks became a part of seed piles used later for storage studies. Beginning on 10/22/67 and ending on 12/24/67, 159 samples were collected in CC. In SC 207 samples were collected between 11/5/67 and 1/21/68. Each of these samples was analyzed for moisture content, fungal infection, FFA and aflatoxin content. Moisture levels were determined by oven drying and expressed on a wet weight basis. Other methods have been discussed above. The samples were collected weekly but the data are summarized as averages for samples collected during two week intervals.

Quality of Stored Cottonseed

Determinations, as described above, also were made on stored seed at about biweekly intervals during the storage season in both CC and SC. Four to 5 lb samples of seeds were drawn from each of 30 places (stations) of the currently exposed face of seed storage piles as the piles were processed by mills. The seed piles, one each in CC and

TABLE III

Variability in the Amounts of Fungal Infection, Free Fatty Acids, and Aflatoxins Detected in Fuzzy Cottonseed Inbound to Storage During 1967-68 in Central and Southern California

	Seed infection, %			
Seed stored during the weeks	*Aspergillus flavus*	Total infection[a]	Free fatty acids, %	Aflatoxins, ppb
Central California				
10/22, 10/29	0.1	2.3	0.6	Trace[b]
11/5 , 11/12	0.4	3.3	0.5	Trace
11/19, 11/26	0.1	10.3	0.6	Trace
12/3 , 12/10	0.3	9.2	0.9	Trace
12/17, 12/24	0.1	4.9	1.0	Trace
Southern California				
11/5 , 11/12	6.2	22.2	3.0	1,735
11/19, 11/26	8.6	23.0	2.1	1,587
12/3 , 12/10	6.3	18.9	1.8	1,388
12/17, 12/31	5.5	18.3	6.3	1,277
1/7 , 1/14	7.0	23.0	5.8	2,281
1/21	10.8	30.2	10.1	2,578

[a]Includes *Aspergillus flavus* and other fungi.

[b]Only aflatoxin B, observed and the greatest amount observed was 0.5 ppb.

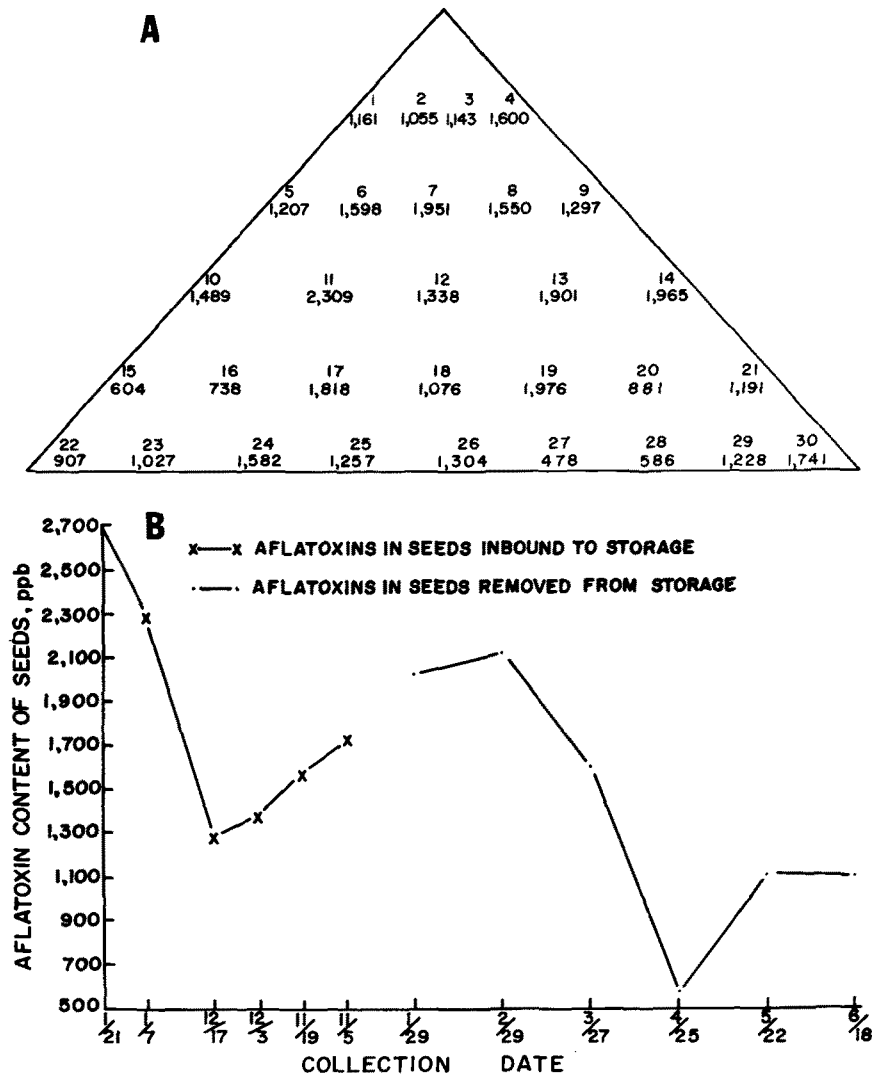

FIG. 2. (A) Diagram of a cross section of a cottonseed storage pile. The mean amounts of aflatoxins, in ppb, detected at 30 locations in a southern California seed pile during 1967-68; (B) A comparison of the mean amounts of aflatoxins detected in seed inbound to storage in southern California, plotted in the order in which the samples were removed from storage, with the mean aflatoxin content of seed during storage in 1967-68.

SC, were about 50 ft high, 80-100 ft wide at the base, and 300 or more feet long. A series of samples was collected near the base of the pile and other series of samples were collected at succeeding increments of about 10 ft elevation, as illustrated in Figure 2-A. The same relative locations were sampled each time. Sampling ceased when the seed pile diminished to the point where a full-sized face could not be sampled. Samples were collected in CC between 12/18/67 and 4/22/68 and between 1/29/68 and 7/1/68 in SC.

The data were summarized as monthly means of two sets of samples, with one exception. The first increment of storage time in southern California is represented only by the data for samples collected on 1/29/68, because 16 of 30 samples for 2/12/68 were lost in transit.

RESULTS AND DISCUSSION

Seed Quality at Harvest

This part of the study was confined to SC because epidemiological studies showed that CC cottonseed was essentially free from infection by *A. flavus* and from aflatoxins at harvest because of climatic conditions (11). On the other hand, fungal infection of unharvested cottonseed appears to be an endemic problem in SC, although the amounts of infection and aflatoxins vary considerably from year to year depending largely upon climatic conditions (6,11).

At least 10 genera of fungi may infect SC cottonseed before harvest. The comparative prevalence of fungal species infecting cottonseed at harvest in SC in 1967 is shown in Table I. *Fusarium* was the most common genus of field type fungi observed. In decreasing order of prevalence the species were, respectively, *F. oxysporum* Schlecht. ex Fr. emend. Snyder and Hansen, *F. roseum* Lk. ex Fr. emend. Snyder and Hansen, and *F. moniliforme* Lk. ex Fr. emend. Snyder and Hansen. Storage fungi were about 2.5 times more common than field type fungi. Within the group of storage fungi, *Aspergillus* spp. outnumbered *Penicillium* spp. about 7:1. The relative abundance of storage fungi in cottonseed at harvest may be explained by the fact that the lower, early maturing bolls, in which most infections occur (9), often remain on plants three to four months before being harvested. This is because of the long growing season (up to 10 months), the indeterminant fruiting habit of the cotton plant, and the current practice of harvesting once only, at the end of the growing season.

The amount of preharvest fungal infection of seed was 15.3% (Table I). Results of determinations for FFA and aflatoxin indicated considerable activity of fungi following

TABLE IV

Variability in the Amounts of Infection of Fuzzy Cottonseed by the Dominant Fungal Species and by Other Fungi During Storage in 1967-68 in Central and Southern California

Seed removed from storage	Amounts of infection, %					
		Aspergillus groups				
	flavus	*niger*	*glaucus*	*flavipes*	Other fungi	Total
Central California						
12/18, 1/2	0.1	0.5	Trace[a]	0.2	0.8	1.6
1/15 , 1/28	Trace	1.4	0.1	0.2	0.4	2.1
2/12 , 2/28	0.1	1.1	Trace	0.2	0.2	1.6
3/11 , 3/25	0.1	1.5	1.4	0.3	0.9	4.2
4/12 , 4/22	Trace	1.3	1.3	0.2	0.5	3.3
Southern California						
1/29 , 2/12	48.2	4.5	4.7	5.2	7.4	70.0
2/29, , 3/11	6.9	2.7	9.0	1.8	2.5	22.9
3/27 , 4/8	3.2	1.0	5.2	0.4	2.2	12.0
4/24 , 5/6	3.6	0.8	5.0	0.3	1.8	11.5
5/22 , 6/3	2.2	0.9	4.1	0.3	2.4	9.9
6/18 , 7/1	3.2	1.1	4.7	0.5	2.7	12.2

[a]Less than 0.05%.

infection. The FFA content of seed ranged from 0.2-25%, depending upon the sources of samples, with a mean of 3.4%. The mean amount of aflatoxins in seed at harvest was 985 ppb, which was about 50 times greater than the amounts observed in seed from the same area in 1965 and 1966. This large difference between years appears to be caused by secondary development of *A. flavus* in seed rewet during a preharvest rain in 1967 (11).

Quality of Cottonseed Inbound to Storage

Except for *Fusarium* spp. and *Macrophomina phaseoli* (Maubl.) Ashby, field fungi were more common in CC seed than in SC seed during the time seed were being received for storage (Table II). But storage fungi generally were much more common in seed inbound to storage in SC than in CC, with *A. flavus* being nearly 40 times more prevalent. Total infection of seed inbound to storage was 6% and 21.8%, respectively, for CC and SC. These results agree with earlier observations made in the two cotton growing areas (11). Data in Table II show the seasonal mean percentage infection for all fungi, whereas those in Table II show the biweekly variability in the amounts of infection, FFA and aflatoxins. Total seed infection ranged from 2.3-3% through 11/12/67 in CC (Table III). During this time, seed moisture content was low, ranging from 7.7-9.8%. Infection increased sharply in seed received between 11/19 and 12/10. This increase in infection was associated with high seed moisture resulting from 1.58 in. of rain in November beginning 11/18 and 0.57 in. in December; moisture content of seed ranged from 11.6-16.3% after 11/18. The amount of seed infection observed between 12/17 and 12/24 diminished to 4.9% (Table III), although seed moisture remained high (11.8-14.6%). This reduction in the amount of infection observed probably is related to a reduction in air temperature from November to December. The monthly mean temperature for November was 15 C with a daily minimum of 9 C and a daily maximum of 21 C. On the other hand, the monthly mean temperature for December was 7 C with a mean daily minimum of 2 C and a daily maximum of 13 C. The amount of infection by *A. flavus* appeared to be quite variable, ranging from 0.1-0.4% in CC (Table III). These differences are not associated with particular portions of the harvest season, as was total infection, and they therefore probably are not different from the season mean of 0.2% (Table II). The FFA content of CC seed appeared to increase as the seed receiving season progressed (Table III). This observation is in agreement with the moisture content data and is attributed to growth of fungi in moist seed. Aflatoxins were detected in 15 of 159 samples during the seed receiving season but never in an amount greater than 0.5 ppb (Table III).

In SC, the amount of infection by field type fungi (4.4%) at the time of harvest (Table I) was about the same (4.1%) as observed during the seed receiving season (Table II). But total infection increased from 15.3% to 21.8% (Table II). The amount of infection by *A. flavus* also increased from 1.7% (Table I) to 7.4% (Table II). The data in Table III show how infection, FFA and aflatoxin values changed as the seed receiving season progressed. The data indicate that the amount of seed infection was stable through 1/14/68, while there was an apparent increase in the FFA content of seed beginning with samples received on 12/17/67. Aflatoxins were detected in all inbound samples of seed. At harvest the aflatoxin content of seed inbound to storage was variable, ranging from 10-8,300 ppb. The mean amount of aflatoxins in these seed ranged from 1,300 to 1,700 ppb between 11/5 and 12/31/67; thereafter an increase was observed (Table III). This increase in aflatoxin and FFA content of seed followed two periods of rain during the seed receiving season, 1.37 in. from 11/20-27/67 and 0.80 in. from 12/13-20/67. Figure 1 shows the influence of rain upon seed moisture and aflatoxin content of seed during the seed receiving season. In general, these observations agree with earlier observations of Ashworth, et al. (6,12). They observed an increase in aflatoxin content of seed within about two weeks after seeds were moistened and temperatures were favorable for growth of *A. flavus*. They also observed loss of aflatoxins from seed after several wetting and drying cycles.

Quality of Stored Cottonseed

In CC total infection of seed received for storage was 6.0% and of seed after storage was 2.7%. Total infection by field fungi was also lower (0.4%) in stored seed than for inbound seed (2.2%). Like field fungi, the mean amount of infection by storage fungi decreased from 3.8% to 2.3% during storage (Table II). Three species groups of *Aspergillus* constituted the major part of the seed microflora during storage; they were *A. niger, A. glaucus,* and *A. flavipes. A. flavus* and *A. flavipes* infection was static during storage in CC. *A. niger* infection percentage was also static

following 1/2/68, but *A. glaucus* infections increased beginning in March, 1968 (Table IV). Aflatoxins were detected only in the seed taken from storage in CC on 2/28 and 3/11/68. Nine samples, or 2.2% of the total samples, contained detectable amounts of aflatoxins. Except for one sample with 50 ppb aflatoxins, the aflatoxin content of CC seed never exceeded 5 ppb.

In SC, the mean amount of infection of seed inbound to storage was about the same as the mean amount observed for the storage season, 21.8% versus 20.7% (Table II), suggesting that the amount of postharvest seed infection was static. However, changes did occur during storage, as in CC stored seed. The amount of seed infection by field fungi dropped sharply, from 4.1% to 1.4% (Table II). Greater variability was observed in the amount of infection by storage fungi than by field fungi. The seasonal mean amount of infection by *A. flavus* increased from 7.4% in seed inbound to storage to 11.2% for seed during storage (Table II). This increase occurred very early in the storage season. *A. flavus* infected 10.8% of seed received for storage on 1/21/68. Within eight days after seed were stored (on 1/29/68), however, the amount of seed infected by the fungus increased to 35.8% and to 60.5% on 2/12/68, with an average infection of 48.2% for the two collections (Table IV). Thereafter, infections by *A. flavus* declined and became static at about 2-3% by 3/27/68. Infections by the other major fungi, *A. niger* and *A. flavipes*, also declined in storage. *Penicillium* spp. were relatively abundant and increased early in the storage season (0.5% to a maximum of 6.3%). Increases in seed infection, however, occurred only in the seed that were stored late in the seed-receiving season, and which were the first seed removed from storage. While the average moisture content of these seed was 9-10% between 12/17/67 and 1/21/68 (Fig. 1), many seed obviously were sufficiently moist to allow new infections from conidia on the surface of seed or to allow seed-to-seed spread by these fungi. The average moisture content of seed in storage, as shown by Christensen and Kaufmann (1), can be misleading. In this respect, our results agree with their observations on stored grain.

As stated above, only infections by *A. flavus* and *Penicillium* spp. appeared to increase in storage, an then only early in the storage period. However, there was obvious fungal development on the surface of seed in storage. It first was apparent on May 5. From May 5 to June 3, fungal conidiophores and conidial heads were observed on seed throughout the face of the seed pile, except in the outermost 3-5 ft. The fungus primarily responsible for this sporulation appeared to be *A. flavus*, which sporulated profusely on the surface of 100 seed collected at random, and cultured without surface disinfestation on malt-salt agar. *Rhizopus* sp. (3%), *A. niger* (4%), and *A. flavipes* (2%) also sporulated on these seed. The sporulation appeared to be due only to growth of the fungi on the surface of seed because, acid delinted, surface disinfested seed showed no more internal infection than seed that were free from external fungal structures.

All except one of the 330 seed samples drawn from storage in SC contained a detectable amount of aflatoxins, which agrees with the observations made on inbound seed. Also, as with inbound seed, the amount of aflatoxins in particular samples varied considerably, from 2-8,000 ppb. For this reason, it appears that the variability between mean seasonal values, at 30 sampling stations within the seed pile, are related to condition of inbound seed and not to locations within the pile (Fig. 2-A). This interpretation is supported by the fact that the mean amount of aflatoxins observed at stations in the central core of the pile (1,367ppb) is about the same as the mean value for samples collected from stations at the periphery of the seed pile (1,280 ppb) (Fig. 2-A).

Figure 2-B shows the amounts of aflatoxins detected in seed inbound to storage, plotted in the order in which the samples were removed from storage. That is, the last seed inbound for storage were received on 1/21/68 and they were the first seed removed from storage (1/29/68). The mean amount of aflatoxins observed during storage was less (1,347 ppb) than the mean amounts of aflatoxins detected in seed inbound to storage (1,808 ppb). Aflatoxins in stored seed were 500-800 ppb less at all points during storage than in seed inbound to storage (Fig. 2-B). Experimental error appears to be the most likely explanation for the differences since they were neither associated with fungal activity (Table IV), nor with locations within the seed pile (Fig. 2-A). The strong similarity between the shapes of the two curves in Figure 2-B suggest that the amount of aflatoxins in stored seed is a reflection of the relative amounts of aflatoxins seed contain when they are stored.

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