Published March, 1969

Retardation of the Elaboration of Aflatoxin in Cottonseed by Impermeability of the Seedcoats[1]

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ABSTRACT

Impermeable cottonseed were compared with permeable seed to determine their value in resisting the elaboration of aflatoxin. Undelintered seed were inoculated with a toxigenic strain of *Aspergillus flavus* and stored for periods up to 49 days under conditions of moisture and temperature conducive to fungal growth and to production of aflatoxin. When stored for 21 days under these conditions, in all cases except one, "selected-impermeable" seed of 1964 and 1966 experimental crops developed less than 0.2 mg aflatoxin/kg. Permeable seeds (commercial and experimental) treated under the same conditions for the same time contained from 78 to more than 500 mg aflatoxin/kg. The toxigenic fungus grew on or in fewer of the selected-impermeable than the permeable seed. However, the available lots of impermeable seed which had been grown in 1965 contained a higher percentage of permeable seed than did the lots from other years because of poor storage. As much as 22 to 200 mg aflatoxin/kg were produced in 21 days even in selected-impermeable seed obtained from improperly stored lots grown in 1965. Apparently cottonseed stored under different moisture conditions differed in their degree of impermeability and susceptibility to the action of *A. flavus*. When impermeability was maintained, the seed were protected from significant development of aflatoxin.

Additional index words: **Impermeable seedcoats, *Aspergillus flavus*, Hard cottonseed, Mycotoxin in cottonseed.**

COTTONSEED having a heritable impermeable seed coat (sometimes called "hard seed") were shown by Christiansen and Justus (1963) to be protected against rapid field deterioration, and by Christiansen, Moore, and Rhyne (1960) to be protected against rapid deterioration in storage at high humidities. Stephens (1958) credited impermeability as the characteristic for salt water tolerance belonging to certain wild species of cotton. This characteristic, plus buoyancy, enabled the wild species of *Gossypium* to be spread over coastal and insular regions. Christiansen et al. (1960) found that 85% of the seed of the 16B7 breeding line (a selection that normally produces seed with an impermeable seed coat) used in their studies were impermeable when subjected to their testing procedures. Storage at extremely high humidity gradually induced permeability into some seed, but not into all seed at the same time (Christiansen et al., 1960; and Davis[5]). Davis found that, in samples of 16B7 cottonseed grown at Knoxville, Tenn., in 1965, there was a considerable variation in impermeable seed content. He concluded that the relative humidity level of the atmosphere during post-harvest handling and storage of impermeable 16B7 cottonseed had a greater effect on the percentage of impermeable seed than any other factor studied. Walhood (1956) suggested hot water treatment as a means of eliminating the "hardness" or impermeability of seed when germination is desired, and R. B. Stone, S. O. Nelson, M. N. Christiansen, and N. E. Justus (Anon. 1965) induced permeability in dry cottonseed by using a glow-discharge treatment.

Impermeability seems to be caused by certain structural characteristics of the seed coat. Christiansen and Moore (1959) found in impermeable cottonseed a disk-like deposit within the core of the chalazal aperture. This deposit was brittle and unaffected by cold water, but could be softened by hot water, ether, or ethanol. The disrupted disk would then allow the imbibition of water. The seed must be dried to 11% moisture to establish the impermeable condition (Christiansen et al., 1960).

[1] Joint contribution from the Southern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, and the National Cottonseed Products Association. Received March 30, 1968.

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[4] The authors are indebted to Dr. Meryl N. Christiansen, Mrs. Alva Cucullu, Dr. Norman Justus, Mrs. Wilda Martinez, and Dr. Robert J. Miravalle for assistance and suggestions, and to John D. Tallant for statistical treatment of the data.

[5] Davis, L. E., Jr., 1967. Some sources of variation in sampling plants of 16B7 strain of cotton for proporation of impermeable seed. M. S. Thesis. University of Tennessee, Knoxville.

Among the agents of deterioration of cottonseed when it is left at high humidity during storage is the fungus *Aspergillus flavus*. This lipolytic organism was found by Mayne (1956) to be associated with deteriorated stored cottonseed and to be present in nine of the 28 random samples of cottonseed examined. Marsh et al. (1955) found *A. flavus* associated with fluorescent spots on raw cotton. Along with lipolysis and other deteriorative processes, this organism is capable of producing a substance toxic to animals. The toxin, now called aflatoxin, was first noted in peanut meal by English workers, and Sargeant et al. (1961) identified the causative organism as *A. flavus*. Ashworth et al. (1968) found that fluorescence was associated with the presence of aflatoxin, in that gin-run cottonseed lots free from a greenish-yellow fluorescence were not likely to contain aflatoxin, and that a much higher percentage of fluorescent seed than nonfluorescent seed had measurable amounts of aflatoxin.

Since it was concluded by Christiansen and Justus (1963) that "a heritable impermeable seedcoat characteristic can materially reduce the loss of cottonseed quality under unfavorably high moisture conditions in the field or in storage," it was considered that impermeable cottonseed might also offer resistance to the growth of *A. flavus* and aflatoxin production in storage. The work described in this paper explores this possibility. It also reveals a difference in the percentage of impermeable seed in several lots of the 16B7 breeding line of cottonseed obtained in different years and stored under different moisture conditions.

EXPERIMENTAL PROCEDURE

Four separate experiments were conducted in which one or more of four types of cottonseed were used: prime commercial seed, which may be assumed to be almost entirely permeable; the M8 breeding line, which is permeable; the 16B7 breeding line, which contains about 85% impermeable seed (called here unselected-impermeable); and the impermeable seed fraction selected from the 16B7 breeding line (called here selected-impermeable). The various samples of M8 and 16B7 cottonseed (obtained from National Cottonseed Products Association, Dr. Meryl Christiansen, and Dr. Robert J. Miravalle) had been grown at five State Agricultural Experiment Stations and the Iguala Winter Increase Station near Taxco, Mexico. The year and the place the seed were grown, the seed breeding line, and the proportion of impermeable seed as defined below, are listed in Table 1. After the experiments had been performed, it was found that the seed grown in 1965 had not been stored under controlled conditions: they had been placed in paper bags inside burlap sacks on a concrete floor for several months (personal communication from Dr. Robert J. Miravalle). On the other hand, seed grown in 1964 and in 1966 had been protected from moisture during storage.

The selected-impermeable seed were obtained by separation from unselected-impermeable seed as follows. In all but one experiment, some of the 16B7 seed as received were soaked in water at 32C for 2 days, and then squeezed between the thumb and finger (Christiansen and Moore, 1959). Those seed which were permeable and had imbibed water, broke open under the treatment and were discarded. The seed which remained intact were considered selected-impermeable seed. They were dried, leaving the seed open to the air for 3 to 6 hours and then in a desiccator over calcium chloride overnight. In the experiment in which seeds of 1964 and of 1965 were used, they were first soaked at 25C for 4 days, and then at 32C for 2 days. After squeezing, the intact selected-impermeable seed were dried.

Moisture content of the seed was determined by the method of the American Oil Chemists' Society (1964). Aflatoxin contents were determined by the method of Pons and Goldblatt (1965) after the samples were prepared as described by Mayne et al. (1966).

All experiments were performed by placing 10-g amounts of the undelintered cottonseed into sterile 250-ml widemouth erlenmeyer flasks plugged with cotton, adding the required amount of sterile tap water to adjust the seed to 25 or 27% moisture content, and inoculating. The fungus used was a known toxigenic strain of *Aspergillus flavus* Link, obtained from the U.S. Food and Drug Administration, designated as "M-3, English strain 3734/10." The fungus was cultured on potato dextrose agar for 7 days. One milliliter of a suspension, which consisted of 5×10^6 spores per ml of 0.05% Tween 20®, was placed into each flask and distributed as evenly as possible on the cottonseed. The samples were incubated at 30C for periods up to 49 days as indicated in the description of the individual experiments.

Table 1. Proportion of impermeable cottonseed in the seed lots used.

Year grown	Breeding lines*	Impermeable seed present, % †	Place grown
1964	16B7	80.3 ± 2.1	Stoneville, Miss.
1964	M8	--	Stoneville, Miss.
1964	16B7	81.5 ± 2.3	Iguala, Mex.
1965‡	16B7	78.2 ± 2.[illegible]	Tempe, Ariz.
1965‡	M8	--	Tempe, Ariz.
1965‡	16B7	65.7 ± 2.[illegible]	Brawley, Calif.
1965‡	M8	--	Brawley, Calif.
1965‡	16B7	61.7 ± 3.1	Chickasha, Okla.
1965‡	M8	--	Chickasha, Okla.
1965‡	16B7	75.0 ± 2.7	Knoxville, Tenn.
1965‡	M8	--	Knoxville, Tenn.
1965	Unknown§	--	Mississippi
1966	16B7¶, //	92.6 ± 0.9	Knoxville, Tenn.
1966	16B7 ¶,**	89.2 ± 1.4	Knoxville, Tenn.

* 16B7 breeding line contains a large percentage of impermeable seed; M8 breeding line consists of permeable seed. † Mean ± 95% confidence interval. ‡ Seed stored in paper bags until August 1966. § Commercial prime seed. ¶ Received as seed cotton. // Seed heat treated before separation. ** Seed not heated before separation.

Cottonseed Grown in 1964

In the first experiment, cottonseed grown at Stoneville, Miss., in 1964 and a control of shredded wheat were incubated for periods up to 21 days. The shredded wheat was used to demonstrate the amount of toxin produced by the fungus on an excellent substrate. The cottonseed were samples of 16B7 seed as received, selected-impermeable seed from the same lot and M8, permeable seed. The seed were adjusted to 27% moisture and the shredded wheat to 37% moisture because in earlier experiments, good aflatoxin production had occurred at these moisture levels. The temperature was maintained at 30 ± 1C for the first 14 days, but because of mechanical malfunction, varied between 27 and 33C during the last 7 days. Two flasks of each set of cottonseed samples were examined after 7, 14, and 21 days for the numbers of germinating seed, fungal growth, and aflatoxin content. The number of germinating seed were counted as well as possible by inspecting the clump of 90 to 100 seed within each flask. In this experiment, the number of seed internally infected with fungi was determined as follows. A group of 25 seed was taken at random, i.e., without visual selection, from each of the two flasks examined. The seed were acid delinted, washed in tap water, surface sterilized in 0.5% sodium hypochlorite for 2 min, rinsed with sterile water, cracked with sterile pliers, and implanted five to a plate on potato dextrose agar. The plates were incubated at 30C and observed daily for 7 days.

Cottonseed Grown in 1964 and Improperly Stored Seed Grown in 1965

In a subsequent experiment, comparison was made of the production of aflatoxin on two different lots of the 16B7 breeding line and a prime commercial seed. One of the lots of 16B7 seed was grown in 1965 at Brawley, Calif., and as discovered later, had not been protected from moisture during storage. The other lot was grown in 1964 at Iguala, Mexico and had been kept very dry. Both unselected and selected-impermeable seed were used from each of the two lots of the 16B7 breeding line. One flask of each set of seed samples was examined after 7, 14, 21, 28, and 49 days for the number of germinating seed, number of moldy seed, and aflatoxin content. Germinating seed were removed from eight of the total of 20 flasks of 16B7 seed and analyzed for aflatoxin separately.

® Use of a company and/or product named by the USDA does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Selected-Impermeable Cottonseed from Improperly Stored Seed Grown in 1965

In the third experiment, selected-impermeable seed of four lots of the 16B7 breeding line, grown at different localities in 1965 and improperly stored, were compared with permeable seed of the M8 breeding line, grown in the same year and in the same localities. Two flasks of each type of seed (except permeable seed from Arizona) were examined for germinating seed, moldy seed, and aflatoxin content after incubation for 7, 14, and 21 days.

Selected-Impermeable Cottonseed Grown in 1966

In the last experiment, selected-impermeable seed from a lot of 16B7 breeding line grown in 1966 in Tennessee were compared with permeable seed, M8, grown in the same area in 1965. The 16B7 seed were obtained as seed cotton which had been hand picked in November 1966, and kept very dry. One portion of the seed cotton was hand ginned and the seed were heat treated in a forced draft oven at 45C for 24 hours before the soft seed were removed. This was done to insure the establishment of the impermeable condition in case the seed had not dried to below 11% moisture. Another portion was roller-ginned and the seed were not heat treated before the soft seed were removed. Two flasks of each of the three sets of seed were examined for germinating seed, moldy seed, and aflatoxin after 3, 7, 14, and 28 days of incubation.

RESULTS AND DISCUSSION

Cottonseed Grown in 1964

The fungal activity in selected-impermeable, unselected impermeable, and permeable (M8) cottonseed, all grown in 1964, was compared. Results of aflatoxin analyses are shown in Table 2. Although temperature varied between 27 and 33C during the last 7 days, it is considered that comparisons within the experiment are still valid. Aflatoxin was not produced as quickly in the permeable cottonseed as it was in shredded wheat, but after 21 days the amounts produced were of the same magnitude. However, after 21 days, only one of the four samples of either type of impermeable seed contained an appreciable amount of toxin. In this experiment there was little difference in toxin production in the selected and unselected-impermeable seed samples, although toxin production was much less than in the permeable seed.

Observations of germinating and internally infected seed were revealing. Germination counts showed a few seedlings in each of the flasks of selected-impermeable seed. This would indicate that even after selection, a few partially or borderline permeable seed remained. In Table 3 is shown the extent of internal infection as percentage of seed with *A. flavus,* with any mold, and 95% confidence intervals. It is apparent that both the selected and unselected-impermeable seed were protected more than the permeable seed from the development of *A. flavus.* The upper confidence limit for the impermeable seed after 21 days was less than the lower confidence limit after 7 days for the permeable seed. However, at the end of 21 days, 32% of the impermeable seed contained *A. flavus.* With this amount of infection, the question arises as to why the aflatoxin content was so low. The percentages of seed infected do not reflect the extent to which the fungus had grown within each seed. Possibly within 21 days the fungus had penetrated the seed coat, which is a relatively poor substrate for aflatoxin production (Mayne et al., 1966) but had not yet proliferated inside the kernel.

Table 2. Aflatoxin elaboration in inoculated cottonseed grown in 1964.

Type of sample*	Separation	Incubation time, days†	Aflatoxin, mg/kg‡
16B7	Yes	7	0.01
		14	0.02
		21	0.02§
			8.96§
16B7	No	7	0.0
		14	0.27
		21	0.03
M8	--	7	14
		14	83
		21	545
Shredded wheat	---	7	580
		14	480
		21	460

* Seed at 27% moisture, shredded wheat at 37% moisture. † 30 ± 1C for first 14 days; 27 to 33C last 7 days. ‡ Each value for the seeds is an average of two samples unless otherwise noted. § Values for individual samples.

Table 3. Internally infected seed in inoculated cottonseed grown in 1964.

Breeding lines	Separation	Incubation time, days*	Seed with mold, %†			
			A. flavus		Any mold	
			Mean	95% C.I.‡	Mean	95% C.I.‡
16B7	Yes	0	0	0-7	18	8-30
		7	16	8-30	26	14-40
		14	37	20-47	73	61-85
		21	32	17-47	98	92-100
16B7	No	0	0	0-7	37	20-47
		7	26	14-40	98	92-100
		14	10	4-23	98	92-100
		21	32	17-47	100	93-100
M8	--	0	0	0-7	68	53-83
		7	68	53-83	98	92-100
		14	74	60-83	100	93-100
		21	100	93-100	100	93-100

* 27% moisture; 30 ± 1C first 14 days, 27 to 33C last 7 days. † 45 to 50 seed examined. ‡ Confidence interval.

Table 4. Aflatoxin elaboration in inoculated cottonseed* grown in 1964 and improperly stored seed grown in 1965.

Place, year grown	Breeding line	Separation	Total aflatoxin, mg/kg				
			7 days	14 days	21 days	28 days	49 days
Brawley, 1965	16B7	Yes	0.05	2.0	85	18	31
		No	0.02	49	13	60	39
Iguala, 1964	16B7	Yes	0.01	0.0	0.17	2.1	9.5
		No	0.04	3.0	6.0	18	40
Mississippi, 1965	Commercial unknown	---	1.6	4.3	78	51	14

* Seed at 25% moisture.

Fungi other than *A. flavus* were present originally in fewer of the selected-impermeable seed than in the other two types of seed, and during the incubation, these fungi spread less among the selected-impermeable seed. This observation negated a theory that the delay in toxin production in permeable cottonseed as compared to shredded wheat might be due to a competition between *A. flavus* and the other fungi present. It also corroborated the findings of Christiansen et al. (1960) that impermeability retarded rapid deterioration in storage at high humidities.

Cottonseed Grown in 1964 and Improperly Stored Seed Grown in 1965

The effect of the fungus on selected and unselected-impermeable seed of two lots of the 16B7 breeding line stored differently, was compared with the effect on prime commercial seed. After incubation for 7 days, the aflatoxin contents (Table 4) of all the 16B7 seed were less than those of the commercial seed. But only the selected-impermeable seed grown in 1964 did not develop large amounts of toxin for as much as 21 days.

Some germinated seed were observed in all flasks of the 16B7 breeding line, indicating the presence of borderline permeable seed. However, there were only

Table 5. Aflatoxin elaboration in inoculated selected-impermeable and permeable cottonseed† from improperly stored lots grown in 1965.

Breeding lines	Place grown	Total aflatoxin‡, mg/kg		
		7 days	14 days	21 days
Selected-impermeable, 16B7	Ariz.	0.0	20	210
Permeable, M8		150*	1,000*	1,800*
Selected-impermeable, 16B7	Calif.	0.10	67	100
Permeable, M8		185*	1,500*	1,850*
Selected-impermeable, 16B7	Okla.	0.05	7.5	53
Permeable, M8		57*	600*	1,000*
Selected-impermeable, 16B7	Tenn.	0.19	21	22
Permeable, M8		82	933*	1,670*

* Significantly higher at 5% level than impermeable seed grown in the same state. † At 27% moisture. ‡ All values are averages of two samples except for the soft seed samples from Arizona.

a few more seedlings in the flasks containing seed which developed aflatoxin quickly than there were in the flasks containing seed which had little aflatoxin. Germinated seed removed from eight randomly chosen flasks of the 16B7 seed were analyzed separately for aflatoxin. Six of these samples of germinated seed contained much more toxin per gram than did the larger remaining portions of seemingly nongerminated seed. Thus in these six samples, the toxin was concentrated in the germinated seed. There were more molded seed in flasks in which aflatoxin developed quickly than in flasks containing little aflatoxin. In this experiment, less toxin was produced in samples which showed least germination and least moldiness. It was very noticeable that the seed of the 1965 crop, which had been improperly stored, were not as well protected from aflatoxin production as were the properly stored seed from the 1964 crop.

Selected-Impermeable Cottonseed from Improperly Stored Seed Grown in 1965

In the experiment conducted with selected-impermeable seed of the improperly stored lots of 16B7 grown in 1965, four lots from different localities were compared with permeable seed grown in the same areas. Results are shown in Table 5. Although at every interval, the selected-impermeable seed contained less aflatoxin than its permeable counterpart, within 14 days large amounts of aflatoxin were found in these poorly stored lots of the selected-impermeable seed. There were a number of moldy seed, and as many as four to seven germinated seed in every flask of the 16B7 seed, indicating the presence of borderline permeable seed. All of the lots of 16B7 grown in 1965 contained a significantly smaller proportion of impermeable seed than did those grown in 1964 or 1966 (Table 1). It appears that improper storage affected the subsequent protection of the seed against the fungus by decreasing the number of impermeable seed and by increasing the number of borderline permeable seed.

Selected-Impermeable Cottonseed Grown in 1966

A comparison was made of the selected-impermeable seed grown in 1966 with permeable seed grown in 1965 in the same locality. The amounts of aflatoxin which developed in heat-treated selected-impermeable seed, unheated selected-impermeable seed, and permeable seed are shown in Table 6. Only a few of the selected-impermeable seed, whether heat treated or not, germinated or became moldy. On the other hand, about three-fourths of the permeable seed molded in 28 days. Selected-impermeable seed were protected for 28 days against the development of aflatoxin, but the permeable seed began to deteriorate within 3 days.

Table 6. Aflatoxin elaboration in inoculated selected-impermeable cottonseed† grown in 1966.

Breeding lines	Treatment before separation	Total aflatoxin‡, mg/kg			
		3 days	7 days	14 days	28 days
Selected-impermeable, 16B7	heated	0.0§	0.04	0.06	0.0
	unheated	0.05	0.01	0.03	0.0
Permeable, M8	---	1.3	23*	397*	553*

* Significantly higher at 5% level that either selected impermeable seed. † Seed at 27% moisture. ‡ All values are averages of two samples. § One obviously germinating seed which was removed and analyzed separately contained 110 mg/kg.

The results of all experiments show a definite resistance of selected-impermeable cottonseed against the growth of *A. flavus* and the production of aflatoxin compared with the susceptibility of permeable experimental or commercial seed. The best protection was found in the seed, harvested in 1966, which had been grown under good conditions and which had been stored so as to keep the impermeability characteristic intact. Apparently, if impermeability can be produced and maintained, cottonseed can be protected against significant development of aflatoxin. Thus, a genetic approach to the control of aflatoxin, at least for cottonseed, is possible.

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