Factors Affecting Aflatoxin Contamination of Cottonseed I. Contamination of Cottonseed with *Aspergillus flavus* at Harvest and during Storage

THOTA A.P. HAMSA and **JOHN C. AYRES,** Department of Food Science, University of Georgia, Athens, GA 30602

ABSTRACT AND SUMMARY

Examination of cottonseed production has shown that boll weevils (*Anthonomus grandis*), boll rots, and improper handling and storage conditions are critical factors in *Aspergillus flavus* contamination of cottonseed. *A. flavus* cultures were isolated from both field-collected and laboratory emerged boll weevils, as well as from boll weevil emergence holes. Diseased cotton bolls have been found to contain *A. flavus* conidia. Infection by *A. flavus* was limited to the surface of cottonseeds collected from gin and from the gin blower. Seeds improperly stored outside the gin were infected internally with *A. flavus*. Observation of bright greenish-yellow fluorescence was not useful as a diagnostic procedure to detect contaminated seeds. Aflatoxin-producing potential of *A. flavus* isolates is being investigated. When *A. flavus* conidia were artificially inoculated onto the surface of the seeds, 87% of the seeds from Athens, GA, were internally infected whereas only 29% of the seeds from Macon, GA, were internally infected. *A. flavus* invade the cottonseed embryo through the chalazal region, the micropylar region, or cracks developed in the seed coat during ginning. Invasion of cottonseed by *A. flavus* was predominant at 28, 30, and 37 C while at 15 and 20 C other fungi dominated in surface and internal invasions of cottonseed. At relative humidities of 75% and 80%, *Chaetomium* spp. successfully competed in growth on the surface, while at a relative humidity of 90% and above, fungi belonging to the order *Mucorales* outgrew *A. flavus*.

INTRODUCTION

Cottonseed is one of the major oilseeds in the world, with an annual production of 24 million metric tons (1). The proximate composition of cottonseed is carbohydrates 23%, oil 22%, protein 20%, crude fiber 20%, moisture 10%, and ash 5%. Food uses of cottonseed in the past have been limited by the presence of pigment glands containing gossypol. Development of glandless varieties of cottonseed and of the liquid cyclone process of removing gossypol from the seed offer reliable means of introducing cottonseed proteins into the nation's food supply (2). Many opportunities exist for utilizing cottonseed products as meat extenders, for the production of meat analogues, nondairy protein beverages, cheeses and spreads, whipped topping, gels, and desserts (3). One of the most promising uses for liquid cyclone process cottonseed flour is in the form of a 95% protein isolate (4). This isolate shows excellent promise for protein fortification of carbonated citric acid beverages (5,6).

A major limitation in the use of cottonseed is the ability of some strains of *Aspergillus flavus* to grow and produce aflatoxins. Results of experiments in California between 1965 and 1969 showed that the aflatoxin problem in cottonseed is the result of field infection of the seed (7). Aflatoxins are a problem in cottonseed produced in Southern California, Arizona, and parts of New Mexico, Texas, Oklahoma. In the Southeast, the most frequently involved pathogens are *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp. *Diplodia gossypina*, and *Pellicularia filamentosa* (8-10). In the Southwest and West, *Aspergillus* spp., *Fusarium* spp., *Nigrospora* spp. (11), and *Rhizopus* spp. (12) are frequently detected.

Aflatoxins in cottonseeds from Experiment, GA, in 1969 and 1970 were nondetectable (13). Cotton fiber frim the 1971 crop from Atlanta and Augusta, GA, was infected with Actinomycetes, *Alternaria* spp., *Cladosporium* spp., *Collectotrichum* spp., and *Fusarium* spp., but not with *A. flavus* and *A. niger* (14). Although *A. flavus* is present in the humid and hot areas of the Southeast, Aflatoxin contamination of cottonseed has not been reported as a serious problem (15). Since peanuts grown in this region were affected by the problem, natural escape from infection of cottonseed in the Southeast is an unexplained anomaly.

The purpose of our study was to determine the critical factors that lead to *A. flavus* infection of cottonseed at harvest and during storage. The present paper provides information on the occurrence of *A. flavus* conidia on cotton boll weevils, occurrence of *A. flavus* infections in bollrots, fungi associated with gin-run cottonseed, internal infections of cottonseed during storage, and the conditions of temperature and relative humidity that favor the invasion of cottonseed by *A. flavus*. Also, this paper illustrates points of internal invasion of cottonseed by *A. flavus* using light and electron microscopy techniques.

MATERIALS AND METHODS

Four collections of adult boll weevils (*Anthonomus grandis*) were made from cotton crops during July and August, 1974, in the vicinity of Athens, GA. The individual boll weevils moving on cotton buds, squares, flowers, and bolls were collected in sterile petri dishes. In the event more than one weevil was present, all the weevils were collected in one petri dish. Boll weevils were handled under aseptic conditions until plated. Each boll weevil was dissected into three parts: (a) head, (b) legs, and (c) body. Each of the insect fragments was plated on a primary isolation medium. For the determination of fungi parasitic on boll weevils, the weevils were surface disinfected by immersion in 5.25% sodium hypochlorite for 5 min, washed in several rinsings of sterile tap water, blotted dry, immersed in 95% ethyl alcohol for 5 sec and flame sterilized. Fungal colonies developing on and around the insect fragments were isolated in pure culture using 2,6-dichloro-4-nitroaniline (Botran)-Rose Bengal-Streptomycin agar (BRBS) (16) as the primary isolation medium. Identification of pure cultures to the genus level was based on morphological and colony characteristics of the cultures grown on Malt Extract (ME) agar, Czapek Solution (CS) agar, Sabouraud Dextrose (SD) agar, and Potato Dextrose (PD) agar media. Identification of *Aspergillus* cultures to species level was based on morphological and colony characteristics on ME agar and CS agar media.

Boll weevil oviposited cotton squares were surface disinfected in sodium hypochlorite for 5 min, washed with several rinsings of sterile tap water, and incubated at room temperature using one cotton square per petri plate. Upon

TABLE I

Predominant Fungi Isolated from Boll Weevils

Date of Collection	No. of weevils	*Alternaria* spp.	*Aspergillus* spp.	*Cladosporium* spp.	*Fusarium* spp.	*Geotrichum* spp.	*Harpographium* spp.	*Humicola* spp.	*Oidiodendron* spp.	*Penicillium* spp.	*Rhizopus* spp.	*Ascogenous* yeast	*Aspergillus niger*
7/23/74	94	24	49	50	31	12	6	0	11	31	7	0	44
7/28/74	92	48	13	63	19	0	0	0	0	11	30	0	18
8/07/74	102	0	0	101	55	0	0	77	0	0	0	34	0
8/12/64	97	34	0	42	33	0	0	0	0	17	0	0	15

emergence from the cotton squares, fungi were isolated from adult boll weevils as described above.

Young cotton squares attacked by boll weevils and containing boll weevil emergence holes were carefully collected from the field. Boll weevil emergence holes were washed with 100μl of sterile distilled water containing .01% sodium lauryl sulfate, and 50μl of the resulting spore suspensions were plated on BRBS agar medium. Fungi isolated in pure cultures were identified to genus level.

Fungi were isolated from gin-run cottonseeds collected from commercial ginning in October and from ginned seeds sampled from warehouse in November by washing ca. 100 seeds with 5 ml of sterile distilled water. One ml of the resulting slurry containing spores was plated on BRBS agar for the isolation of fungi. For the determination of internal fungi, the seeds were surface disinfected by acid-delinting in concentrated sulfuric acid followed by washing with dilute solution of sodium bicarbonate and sterile distilled water and then dried at room temperature. The acid-delinted seeds were disinfected, washed, blotted dry in the same manner as used for the insects, and then plated on BRBS agar. Fungi which invade the seeds were allowed to grow out. Observations were taken after incubation for 10 days at 28 C.

Fungal invasion of cottonseed during storage was investigated by inoculation of four batches of 5-lb samples of cottonseed with 20 ml conidial suspension of *A. flavus* containing 1 x 10^6 conidia per ml. One batch of seeds from Athens, GA, and one from Macon, GA, were surface sterilized in sodium hypochlorite solution. Conidial suspensions of *A. flavus* were harvested from freshly grown slants of CS agar by washing with sterile distilled water containing .01% sodium lauryl sulfate and by making appropriate dilutions to adjust the conidial concentration. The moisture content of the seeds was determined by drying the seeds to constant weight at 100 C. Appropriate amounts of sterile distilled water were added to raise the moisture content of the seeds to 20%. The conidial suspensions were dispersed in the water to be added to the seeds and the diluted conidial suspension was then added to the seeds. After the inoculated seeds were placed in plastic bags, the bags were tied and incubated for 60 days at 28 C. At the end of storage, ca. 100 seeds from each batch were plated for checking internal contaminations as described earlier.

To determine the conditions of temperature and relative humidity that favor fungal invasion of cottonseed, seeds were placed in 100 x 15 mm petri dishes containing solid medium with low nutrient qualities. This medium had the following composition: K_2HPO_4:0.5 g, KH_2PO_4:0.5 g, $MgSO_4$:0.5 g, $NaNO_3$:0.3 g, agar:20 g, distilled water:1 liter. The petri dishes were then placed in larger petri dishes (150 x 25 mm) containing saturated salt solutions of known relative humidity (17). The seeds were equilibrated for 24 hr and inoculated with *A. flavus* suspensions as described earlier. After 10 days the seeds were observed for visual fungal growth. To determine the percentage of seeds with internal infection, the seeds were surface disinfected in sodium hypochlorite solution and plated on agar in petri dishes, and observations were taken after 7-10 days.

Cottonseeds infested with *A. flavus* were prepared for scanning electron microscopy by the critical point drying method (18). Dried specimens were mounted on holders with conductive cement, coated with gold in a vacuum evaporator, and examined with a Cambridge Mark 2A scanning electron microscope operating at 10KV. The seeds were prepared for transmission electron microscopy by removing a thin membrane layer immediately beneath the seed coat and placing it in 2% glutaraldehyde in 0.1 M cacodylate buffer. The tissues were cut into 1 mm cubes and fixed in buffered glutaraldehyde for 1 hr followed by

TABLE II

Frequency of Isolations of *A. flavus* from 100 Boll Weevils

Collection Date	Climatological Data[a] Av. Temp. °F	Av. % RH at 7 AM	Boll weevils with nonsterile surfaces containing *A. flavus*	Boll weevils with sterile surfaces containing *A. flavus*
July 23	74	93	52	0
July 28	77.5	93	14	0
Aug. 7	73	93	0	0
Aug. 12	75	93	0	0

[a]From local climatological data, U.S. Department of Commerce, National Oceanic and Atmospheric Administration Environmental Data Service.

fixation in 2% osmium tetroxide in 0.1 M cacodylate buffer. Following fixation, the tissues were washed in 0.2 M sucrose in 0.1 M cacodylate buffer at pH 7.2-7.4, dehydrated in a series of graded alcohols, and embedded in Spurr-Low viscosity medium (19). Silver sections 500-700 Å thick were cut on Reichert Om U_2 ultramicrotome. The sections were stained in 2% uranyl acetate for 20 min followed by staining in Reynolds lead citrate solution (20) for 3 min. Observations and electron micrographs were made with a Phillips EM-200 microscope.

RESULTS AND DISCUSSIONS

Table I records data on the predominant fungi isolated from adult boll weevils collected during July and August, 1974, from the University of Georgia Experimental Farm located in Athens, GA. Predominant cultures isolated were soil borne fungi while others have long been shown to be associated as pathogens of insects. Fungal cultures of *Cladosporium* spp., *Fusarium* spp., *Alternaria* spp., *Humicola* spp., *Aspergillus* spp., and *Penicillium* spp., were isolated in decreasing order of frequency. *Geotrichum* spp., *Oidiodendrun* spp., and *Harpographium* spp., were less frequently isolated.

The reported figures for *Rhizopus* spp. are low considering the ubiquitous nature of the distribution of fungi belonging to the order *Mucorales*. Usually mycelia of *Rhizopus* spp. overgrow solid media in petri dishes and mask and fungi that grow under the mycelial mat. By use of BRBS agar, growth of this fungus is restricted while the recovery of other fungal species increases. This medium also decreased the number and colony size of other fungi, thus increasing the chances of recovery of *A. flavus*. While investigating test materials for contamination with *A. flavus*, use of a medium that restricts the growth of other fungi and selectively detects *A. flavus* is necessary. It has long been shown that insects contain large numbers of microorganisms, including millions of bacteria and thousands of Actinomycetes, yeasts, and fungi. When weevils are investigated for contamination with *A. flavus*, preferential detection of this fungus can be made by using BRBS agar medium.

The data from Tables I and II show that the numbers and kinds of fungi associated with boll weevils vary among collections. There was high incidence of *A. flavus* contamination from weevils collected in July while little or no contamination occurred on weevils collected in August. Possible factors responsible for this variability are temperature, relative humidity, and frequency of pesticide applications on the cotton field at the time that boll weevils were collected. Average temperature and % relative humidity values (Table III) are similar for July and August. It was interesting to note that boll weevils were collected in July when pesticides were not sprayed on the field, while in August they were collected 24 hr after pesticides were sprayed. We have not determined the effect of pesticide sprays on the association of *A. flavus* with boll weevils. While others (21) show that *A. flavus* parasitizes insects visiting cotton squares and cotton bolls, our results (Table II) show that boll weevils were not internally invaded by *A. flavus*.

TABLE III

Climatological Data at the University of Georgia Experimental Farm[a]

Year 1974	Monthly average values: Temperature °F	% Relative humidity at 7 AM	Total precipitation inches
July	88.6	93	3.65
August	87.1	93	6.14

[a]From local climatological data, U.S. Department of Commerce, National Oceanic and Atmospheric Administration Environmental Data Service.

Female boll weevils oviposit one to several eggs in a deep puncture in developing cotton flower buds or squares. The larvae feed in the square and in about 3 wk adult boll weevils emerge. Boll weevils that emerged from surface disinfected cotton squares were contaminated with *A. flavus*. The developing cotton squares are thought to be free of internal fungal contamination. If this is so, the source of inoculum comes from the female boll weevils while they oviposit eggs.

There was a high incidence of *A. flavus* contamination in the boll weevil emergence holes of field-collected cotton squares (Table IV). While still on the cotton plant, the

TABLE IV

Frequency of Isolations of Various Fungi

Collection Date	Source	Numbers plated	*Alternaria* spp.	*A. flavus*	*A. niger*	*Cladosporium* spp.	*Fusarium* spp.	*Penicillium* spp.	*Rhizopus* spp.
Sept. 19	Boll weevils emerged in laboratory	45[a]	18	13	0	16	11	4	0
Aug. 20	Boll weevile emergence holes	114	0	47	4	13	3	0	0
Oct. 14	Seed from diseased bolls	100	8	21	5	11	4	4	17

[a]Forty-five boll weevils emerged from 79 boll weevil oviposited cotton squares.

TABLE V

Fungi Isolated from Cottonseed from Athens, GA.

Source	No. of seeds	*Acremoniella* spp.	*Actinomucor* spp.	*Alternaria* spp.	*A. flavus*	*A. niger*	*A. ochraceous*	*Botryotrichum* spp.	*Chalara* spp.	*Chalaropsis* spp.	*Cladosporium* spp.	*Eipcoccum* spp.	*Fusarium* spp.	*Penicillium* spp.	*Phialophora* spp.	*Rhizopus* spp.	*Trichoderma* spp.	*Ulocladium* spp.
Continental Gin-300 B	250	1	4	16	2	0	1	0	2	1	18	3	13	1	5	0	0	0
Continental Gin-141	130	0	8	10	2	0	0	0	0	0	10	3	5	0	0	0	0	0
Gin blower	140	2	6	11	5	0	0	1	0	0	12	6	8	0	0	0	0	0
Seed stored in the gin	130	0	0	15	3	1	1	0	0	0	15	0	11	8	1	5	1	0

TABLE VI

A. flavus Invasion of Cottonseed[a], Stored for 60 Days at 28 C

Source of seed	Seeds/sample	No. of visibly Contaminated seeds	No. of internally contaminated seeds	
			A. flavus	Other fungi
Athens	100	100	7	22
Macon	100	100	0	45
Athens-surface sterilized	100	100	87	6
Macon-surface sterilized	100	100	29	4

[a]Initial moisture content adjusted to 20%.

infested cotton squares provide a breeding place for the fungi and serve as a source of inoculum of *A. flavus* contamination of developing bolls. Airborne *A. flavus* conidia can cause contamination of neighboring cotton bolls when they open at maturity. From July through September, wind speeds recorded in the field ranged from 1.3-10.4 MPH.

There was also high incidence of boll rots in the field. As a preliminary screening test for the detection of *A. flavus* contamination of cottonseed, the fiber from the diseased bolls was scanned for bright-greenish-yellow (BGY) fluorescence under long wave ultraviolet (UV) light. The fluorescence of the fiber ranged from yellow to orange and *Fusarium* spp. were predominantly associated with orange fluorescing fiber. Fiber from one hundred diseased bolls scanned did not fluoresce BGY, yet 21% of the seeds taken from the diseased bolls were contaminated with *A. flavus*. Peroxidases present in the cotton fiber degrade kojic acid produced by *Aspergillus* spp. to a compound which fluoresces BGY under long wave UV light (22, 23). If the peroxidases in the fiber were inactivated, no BGY fluorescence was produced (22). Association of BGY fluroescence with *A. flavus* contamination was repeatedly demonstrated (22-24). However, *A. flavus* contamination of cotton fiber may not always be accompanied by BGY fluorescence, and in our experiments BGY fluorescence was not useful as a diagnostic procedure for the detection of the contaminated seed.

Table V records data on fungi isolated from cottonseed collected from the gin, the gin blower, and the seed stored inside the gin. Most commonly isolated fungal cultures were *Alternaria* spp., *Cladosporium* spp., and *Fusarium* spp. Less frequently isolated fungi include *Epicoccum* spp., *Actinomucor* spp., *A. niger, A. ochraceous, Botryotrichum* spp., *Chalara* spp., *Chalaropsis* spp., *Penicillium* spp., *Phialophora* spp., *Rhizopus* spp., *Trichoderma* spp., and *Ulocladium* spp. *A. flavus* cultures were isolated from all the sources but in fewer numbers. The seeds sampled were not internally contaminated with *A. flavus*. Seven percent of a sample of 350 seeds taken from seeds kept in storage outside the gin were internally contaminated with *A. flavus*. Field samples of cottonseed free from internal infections may be internally contaminated during storage after ginning if the spores of *A. flavus* are present on the surface of the seed at the time of storage. Certain varieties of cottonseed may be more prone to infection by *A. flavus* than others (Table VI). Seven percent of cottonseeds artificially inoculated with *A. flavus* were internally contaminated in a sample of seeds from Athens, while none of the seeds sampled from Macon was internally contaminated at the end of storage. Also, 87% of the surface sterilized seeds from Athens were internally infected whereas only 29% of the seeds from Macon were internally contaminated.

Morphology of the seed plays a very important role in its susceptibility to fungal invasion. The conidia present on the seed lints germinate under favorable conditions and invade the seed embryo through the chalazal region (Fig. 1), the micropylar region (Fig. 2), or the cracks developed in the seed coat during ginning. Fungi may also penetrate the intact seed coat. After the fungus becomes established within the seed, it grows luxuriantly inside the cavities and sporulates profusely. The mycelium also spreads between the innerside of the seed coat and the seed embryo. Figure 3 shows a transmission electronmicrograph of longitudinal section of hypha of *A. flavus* resting on the inner membrane layers of cottonseed. The seed coat protects the internal fungi from the chemicals used for their control. Possibilities of apparently healthy looking cottonseed being internally invaded by *A. flavus* do exist. The cottonseed coat is a less favorable substrate for fungal growth than is the seed embryo. Yet, when inoculated onto the surface of the seed coat, *A. flavus* can grow heavily and sporulate profusely. Figure 4 shows a scanning electronmicrograph of

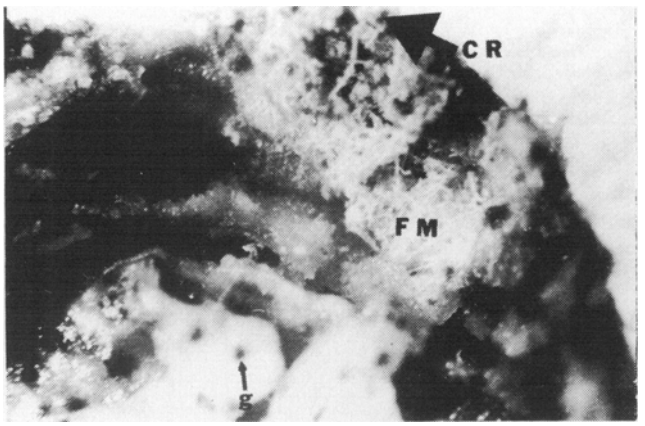

FIG. 1. Invasion of cottonseed embryo by *A. flavus* through chalazal region (X40). Cr, chalazal region; FM, fungal mycelia; g, gossypol pigment gland.

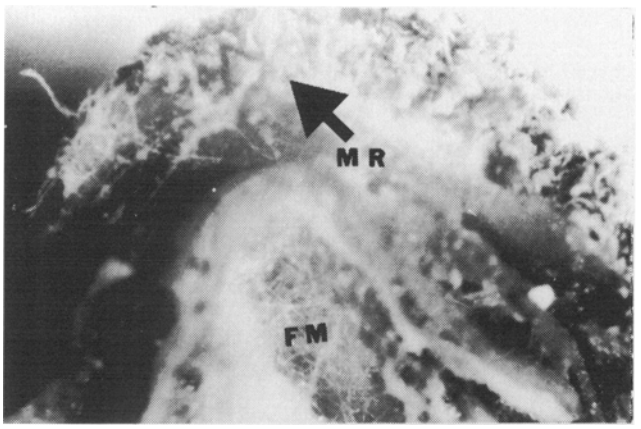

FIG. 2. Invasion of cottonseed embryo by *A. flavus* through micropylar region (X40). MR, micropylar region; FM, fungal mycelia.

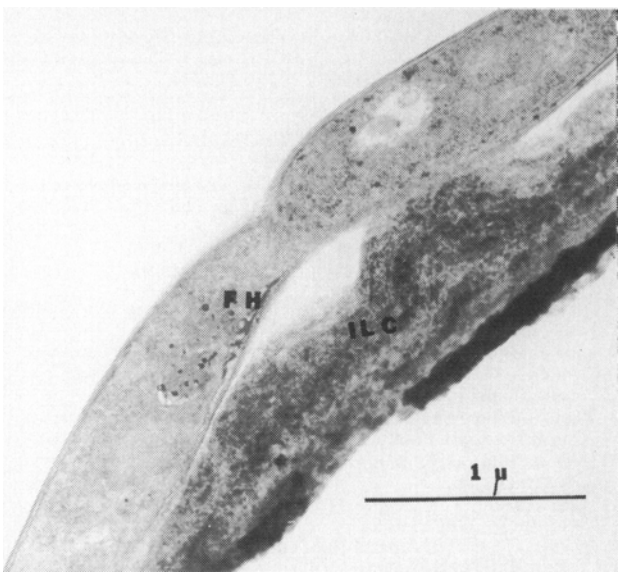

FIG. 3. Electronmicrograph (X 52000) of *A. flavus* associated with cottonseed. FH, fungal hypha; ILC, inner layer of cottonseed coat.

A. flavus growing on cottonseed coat, while Figure 5 shows the conidiophores of *A. flavus* growing on the innerside of the seed coat. Whether *A. flavus* penetrates deep inside the seed coat or is restricted to the surface layers has not been

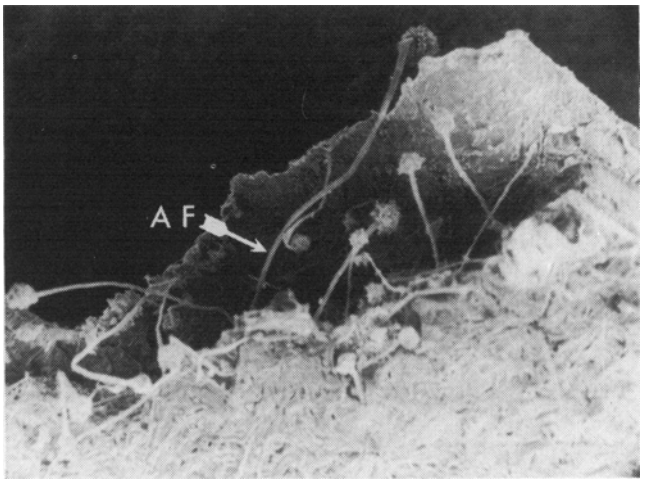

FIG. 4. Electronmicrograph of *A. flavus* (X250) growing on the cotton coat. AF, *A. flavus* conidiophore.

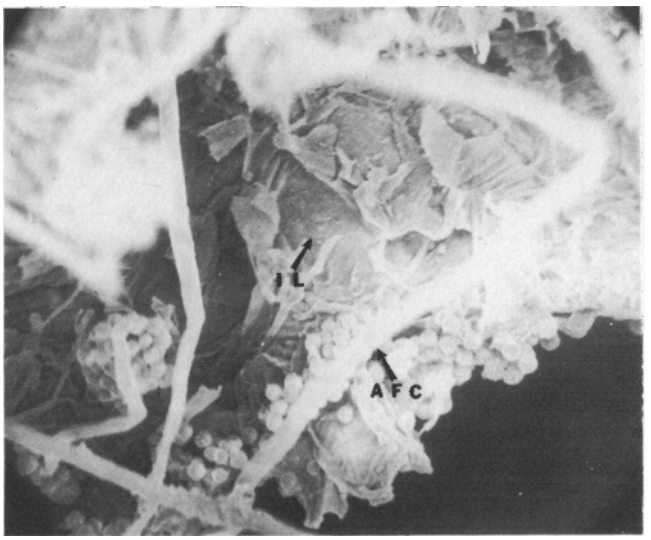

FIG. 5. Electronmicrograph of *A. flavus* (X 1025) growing on the inner membrane layers of cottonseed coat. IL, inner membrane layers of cottonseed coat; AFC, *A. flavus* conidiophores.

established. Figure 6 shows microbial interactions known to occur in cottonseed stored under conditions favorable for fungal invasion. From an ecological standpoint, the fungi invading seeds can be divided into field fungi and storage fungi (25). Field fungi invade seeds as they develop on the plants in the field, or after seeds have matured but before they are harvested, while storage fungi invade seeds during storage. Common genera of field fungi are *Alternaria* spp., *Cladosporium* spp., *Fusarium* spp., and *Helminthosporium* spp. while several species of *Aspergillus* and a few of *Penicillium* comprise storage fungi. *A. flavus* is predominantly a storage fungus. Also, it is associated with the field contamination of cottonseed before harvest and under the broad definition of field fungi, it can be considered as a field fungus. Invasion of cottonseed by *A. flavus* is influenced by the enormous variety of microorganisms inhabiting seeds before harvest and during storage. Other fungi and bacteria may be antagonistic to *A. flavus* or may compete with it in invading cottonseed. Cottonseed should not be considered as an inert substrate for fungal invasion. The cottonseed may excrete substrates that can be utilized by microorganisms while at low vigor (26). Also, seeds may have defense mechanisms which may be responsible for species or varietal resistance. Chemical antibiosis with the

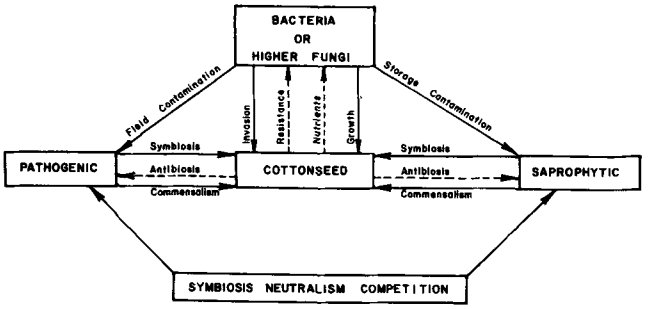

FIG. 6. Microbial interactions on cottonseed.

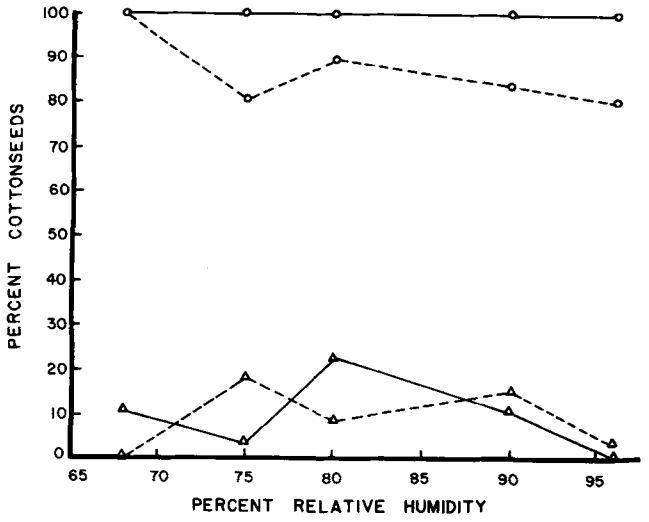

FIG. 7. Microbial interactions on cottonseed stored at different percent relative humidities; 28 C; 10 days. –△– surface contamination with other fungi; –○– surface contamination with *A. flavus*; –⊕– internal contamination with other fungi; –○– internal contamination with *A. flavus*.

induced production of phytoalexins is one of the negative effects of the seed on the invading fungi (27). Also, the storage environment can alter the ecological balance between fungi dominating in the invasion of cottonseed (Fig. 7 and 8). Higher temperatures appear to be required for invasion of cottonseed by *A. flavus*. Invasion of cottonseed was predominant at 28, 30, and 37 C while at 15 and 20 C other fungi dominated in surface and internal invasions of cottonseed. Temperatures above 40 C were unfavorable for fungal growth. At lower temperatures, fungi belonging to the order *Mucorales* overgrew *A. flavus*. At low relative humidities, *A. flavus* dominated in surface and internal contamination of cottonseed. At relative humidities of 75% and 80%, *Chaetomium* spp. successfully competed in growth on the surface, while at a relative humidity of 90% and above, fungi belonging to the order *Mucorales* outgrew *A. flavus*. Although *A. flavus* invaded the seeds over a wide range of moisture contents, competing fungal flora varied. At moisture contents between 11 and 13%, *Chaetomium* competed with *A. flavus* while at 18% moisture content and above, *Rhizopus* spp. overgrew this organism. At all of the relative humidities tested, all seeds artifically inoculated in the laboratory were internally infected with *A. flavus* while fewer seeds were visibly contaminated.

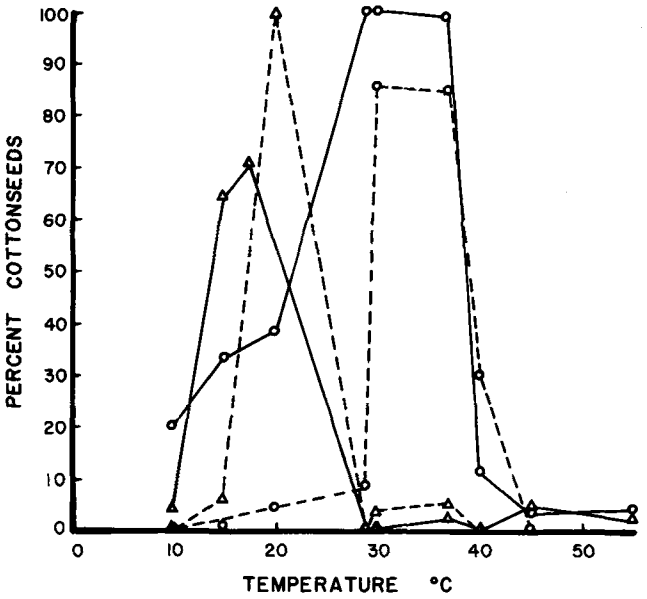

FIG. 8. Microbial interactions on cottonseed stored at different temperatures; 95% relative humidity; 10 days. –△– surface contamination with other fungi; –○– surface contamination with *A. flavus*; –△– internal contamination with other fungi; –○– internal contamination with *A. flavus*.

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