The Relationship of Insects to Infection of Cotton Bolls by Aspergillus flavus

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ABSTRACT

First instar larvae of the pink bollworm do not transmit *Aspergillus flavus* to the interior of the cotton fruit (boll). Nor do the small entrance tunnels of larvae appear to be avenues of entry for either *A. flavus* or other fungi, including *A. niger* and *Rhizopus* sp., which infest the surface of bolls. Exit holes made by mature larvae, however, predispose bolls to infection by fungi; and an increase in severity of infestation results in increased amounts of infection and of the aflatoxin content of seed. Infections by *A. niger* and *Rhizopus* sp., which induce carpel necrosis, further enhance infection by *A. flavus* by inducing premature separation of carpels which exposes lint to infection. Three species of Nitidulidae beetles associated with larval-damaged bolls failed to transmit fungi to bolls. The importance of the pink bollworm-*A. flavus* complex overshadows the influence of previously reported cultural practices for control of *A. flavus* infection of cotton. Phytopathology 61:488-493.

Additional key words: Pectinophora gossypiella (Saunders), *Carpophilus hemipterous* (L.), *C. dimidiatus* (Fab.), *Urophorus humeralis* (Fab.), skip-row planting.

Results of 11 experiments made in southern California cotton (*Gossypium hirsutum* L.) fields during 1966 and 1967 indicated that infection of seed by the *Aspergillus flavus* group of fungi could be greatly reduced by altering currently used cultural practices. The changes consisted of reducing the number of planted rows in fields and of chemically inducing defoliation of the lowermost one-third part of the plant. These practices called, respectively, skip-row planting (7) and bottom defoliation (10), were not innovations. They have been used before to reduce incidence of disease by increasing the amt of air movement, and by decreasing the amt of moisture in air surrounding fruit (bolls) in the lower part of plants, where most boll rot disease occurs. The amt of seed infection and aflatoxins in seeds were reduced by as much as 90% during 1967, when four planted rows were bounded by four unplanted rows (a 4 × 4 skip-row planting) (4). But in 1968, skip-row planting, alone or in combination with bottom defoliation, did not affect disease incidence or aflatoxins in seed, even though the season was free from rains which can affect both infection and aflatoxin content of seed (5). Instead. our observations indicated that the pink bollworm, *Pectinophora gossypiella* (Saunders), contributed to infection by *A. flavus* to the extent that previously favorable treatments failed. This paper reports the details of the latter observations and also the results of experiments made in 1969 to determine the relationship of the pink bollworm to infection of the cotton boll by fungi. It also reports on observations made on the possible connection of beetles of the family Nitidulidae with boll disease.

MATERIALS AND METHODS.—Five each, of solid plantings and 2 × 1- and 4 × 4-skip-row plantings were made in commercial cotton fields in Imperial County, Calif., in 1968. Superimposed upon these plantings were control and bottom defoliation treatments. The general procedures used in these experiments, including aflatoxin analyses, were the same as those followed earlier (4). In these experiments, we also determined the amt of damage caused by the pink bollworm, by inspection of seeds following ginning of the seed cotton.

Studies for determining the relation between the pink bollworm and *A. flavus* were made with in situ bolls of a planting at Brawley, Imperial County, Calif. Controlled infestations of the pink bollworm were induced with eggs of the insect from adults reared and furnished to us by the Department of Entomology, Univ. of Calif., Riverside. Eggs which hatched within 24 hr after being placed on the bolls were used in the tests. Infestations were induced by slipping small pieces of paper bearing 2, 5, or 10 eggs under the calyx of bolls 17 to 19 days old, which had been covered with small muslin bags when they were 5 to 7 days old. This was done because previous experiences at the Univ. of Calif., Riverside (J. McLaughlin, *unpublished data*) indicated that 5- to 7-day-old bolls usually are not suitable for invasion by pink bollworm larvae while 17- to 19-day-old bolls are more prone to successful invasion. Control bolls and infested bolls were covered with small muslin bags to prevent further infestation during the course of experiments. Conidia of our *A. flavus* isolate No. 606 were used for experiments in which controlled inoculations were made. Other, more specific, methods will be described in the later sections of the report.

RESULTS AND DISCUSSION.—*Results of experiments to control infection from A. flavus during 1968.*—Unlike results of experiments made in 1966 and 1967 (4), the cultural practices of skip-row planting and bottom defoliation were of no value in 1968. During 1968, the mean amt of seed infection was 0.30% with a range of 0.21-0.35%. This was a 430-fold increase over that observed in 1966 (0.0007%), a growing season essentially free from rain, and a 60-fold increase over that observed in 1967 (0.005%), a season in which preharvest rains favored seed to seed spread of the fungus

(4). In an attempt to reconcile the conflicting results, we determined the amt of nontoxic and toxic seed, from 1968 experiments, damaged by pink bollworm larvae in order to learn whether the insect might be associated with seed infection. The insect previously was associated with development of lint infection from *A. flavus* in Texas (9). We found that 3.5 times more toxic seed (a mean of 1.83%) than nontoxic seed (a mean of 0.51%) were damaged by pink bollworm larvae. Furthermore, there was an association between distribution and prevalence of the insect with increased prevalence of disease in Imperial County, Calif. That is, the pink bollworm was new to the area in 1966 and occurred only sporadically in 1967, but caused widespread damage in 1968.

Avenues of entry for fungi to locules.—We determined the amt of infection that developed in bolls free from infestation by the pink bollworm and the amt of infection that developed in bolls infested with five *A. flavus*-contaminated eggs/boll. Observations also were made on the number of larvae that penetrated bolls following hatching of eggs. We determined whether first instar larvae transmit *A. flavus* to the interior of bolls. Eggs were infested with *A. flavus* by spraying egg-bearing paper discs with a dense water suspension of conidia of the fungus. The discs were air-dried before they were inserted under calyces. Observations on these bolls were made before emergence of larvae (after 12 days), to exclude infections that could arise after appearance of exit holes and after bolls cracked at maturity.

The possible role of entrance tunnels of larvae as avenues of entrance for the fungus was determined. In this test, bags were removed from bolls 17 to 19 days of age. Then bolls were disinfested with a 0.5% sodium hypochlorite solution, infested with five eggs/boll, and rebagged. Five days later, after eggs had been allowed to hatch and larvae to penetrate bolls, the bolls were infested with a suspension of conidia of the fungus. Then, after 7 days, observations were made for locule infection and for the number of larvae that penetrated bolls. Control bolls for the test described above served as a control in this experiment.

The possible role of exit holes of larvae as avenues of entrance for the fungus also was determined. As above, 17- to 19-day-old bolls were infested with five eggs/boll. Following appearance of exit holes of larvae, covers were removed from the bolls, the bolls were atomized with a suspension of conidia, then bolls were recovered. These bolls were allowed to mature in the bags, then determinations were made for locule infections.

The data indicate that first instar larvae neither transmit the fungus to the interior of bolls, nor do their entrance tunnels provide avenues of entry for the fungus. These observations on infection by *A. flavus* and other fungi differ from those of Brazzel (6). He reported 1-11% and 10-28% infection through entrance tunnels of the pink bollworm larvae by *Penicillium* sp. and *Rhizopus* sp., respectively. Results of our tests, however, showed that bolls are prone to infection after larvae emerge from bolls; i.e., when exit holes of larvae were atomized with conidia of the fungus, 15.7% of the total locules were infected by *A. flavus* (Table 1). Other fungi, principally *A. niger* and *Rhizopus* sp., also infected bolls (Table 1), inducing necrosis and premature separation of carpels (Fig. 1). The type of necrosis illustrated in Fig. 1-A usually, but not always, bore some sporophores of *Rhizopus* sp. This observation is the basis of the identification made above; however, this is not a specific type of necrosis, like that induced by *A. niger* (Fig. 1-B). Instead, it is a type associated with other organisms, especially *Erwinia* spp. (1).

Severity of pink bollworm infestations and infection.—We determined whether multiple penetrations of bolls by larvae increases disease proneness of bolls. A total of 1,500 bolls, 5-7 days old, were covered with muslin bags on 17 July 1969. Approximately one-third of the bolls were allowed to mature in the bags. When the remaining bolls were 18 to 19 days old (29-30 July), about half were infested with two eggs/boll, and about half were infested with 10 eggs/boll. These bolls also were allowed to mature in the bags as natural infections occurred. When the bolls were mature, we determined locule infections, lint quality (as determined by the U.S. Cotton Classing Office, Bakersfield, Calif.), and aflatoxin content of seeds.

Bolls protected from infestation by the bollworm

TABLE 1. The time of infection of cotton bolls by *Aspergillus flavus* and other fungi, as influenced by controlled infestations of the pink bollworm

Infection experiments	Bolls		Locules		
			Fungal infection		
	Total	Larval penetrations/boll	Total	*A. flavus*	Other
	no.	*no.*	*no.*	%	%
Bolls covered to prevent infestation from pink bollworm	89	0.2	418	none	none
Bolls with *Aspergillus flavus*-infested eggs	102	2.5	489	none	none
Bolls infested with *A. flavus* after penetration by larvae	69	2.3	324	none	none
Pink bollworm exit holes sprayed with conidia of *A. flavus*	88		394	15.7	3.10[a]

[a] Principally *A. niger* and *Rhizopus* sp.

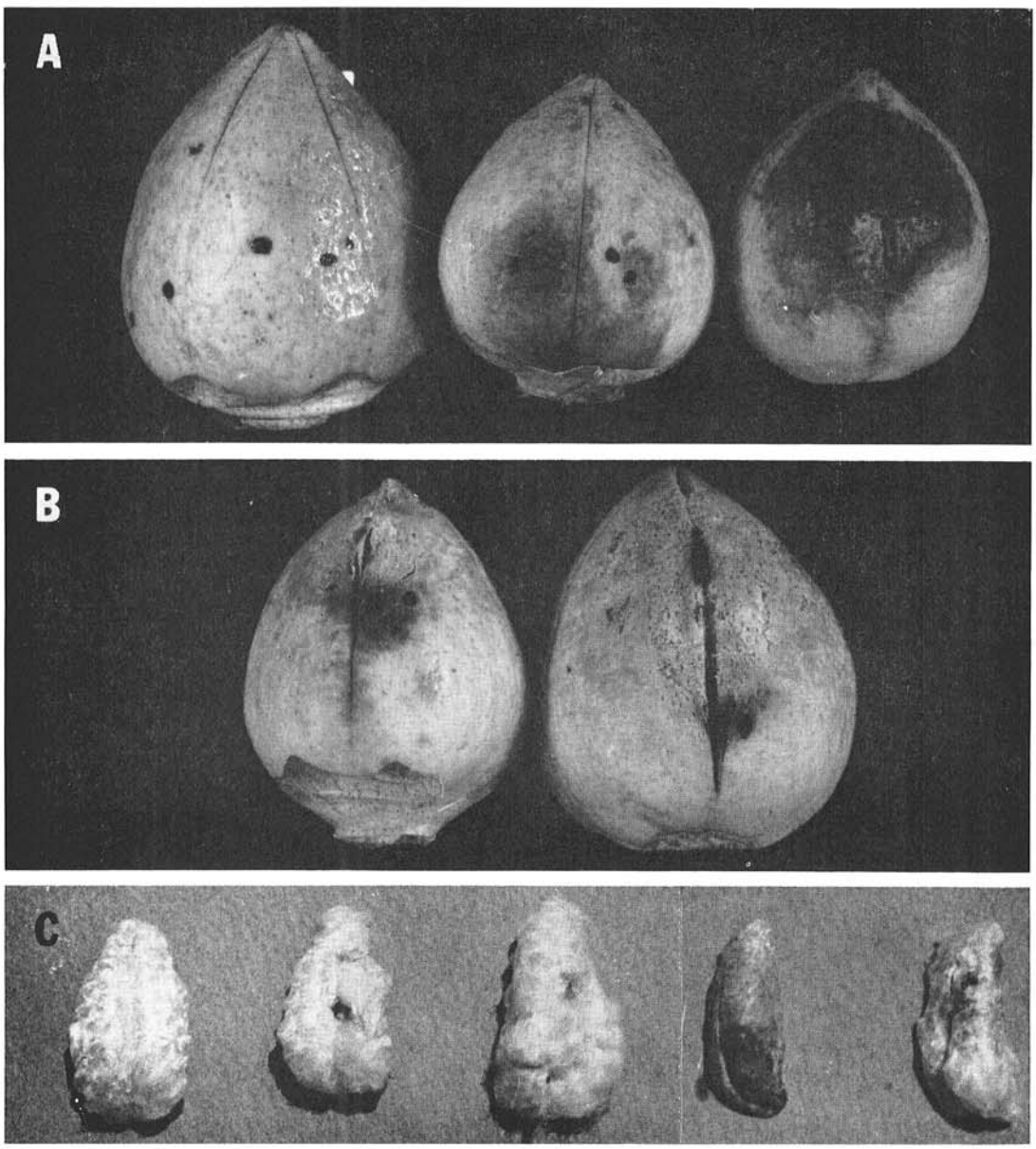

Fig. 1. Immature cotton bolls with exit holes of pink bollworm larvae; **A)** a necrosis-free boll and bolls infected with *Rhizopus* sp.; **B)** bolls infected with *Aspergillus niger;* **C)** immature cotton locules; an undamaged locule, far left, compared with two pink bollworm-damaged locules, center, and with two discolored locules, right, affected with pink bollworm damage and fungal infections.

were free from apparent infection by *A. flavus,* although the fungus obviously infected some seeds because a small amt of aflatoxins (12 ppb) was detected (Table 2). However, progressively greater amt of infection by *A. flavus* and other fungi occurred with infestations of two eggs and 10 eggs/boll. Likewise, seeds from bolls infested with 10 eggs contained considerably more aflatoxins than seeds from bolls infested with two eggs/boll (Table 2).

Lint quality was reduced as a result of fungal activity and by injury caused by larvae of the bollworm. Lint from bolls protected from bollworm infestation, and which were essentially free from fungal infection, was classed as low middling plus, with a staple length of 1$^{3}/_{32}$ inches, and with a micronaire of 3.8 (a measure of coarseness; the greater the micronaire, the more coarse the lint). Lint from bolls infested with two eggs was classed as low middling light "spot" (a measure of discoloration) with a staple length of 1$^{1}/_{16}$ inches and a micronaire reading of 3.4. Lint from bolls infested with 10 eggs also was classed as low middling light spot, and had the same staple length as lint from bolls infested with two eggs, but it had the lowest micronaire reading, 3.2. These data show that combined damage of

TABLE 2. The influence of severity of infestation of cotton bolls by the pink bollworm upon locule infection by fungi and upon aflatoxin content of seeds

Treatments	Total bolls	Locules			Aflatoxins in seeds
		Total	Fungal infection		
			Aspergillus flavus	Other	
	no.	*no.*	%	%	*ppb*[c]
Bolls protected from infestation	92	432	0	1[a]	12
Bolls infested with 2 eggs	372	1,748	7	21[b]	1,914
Bolls infested with 10 eggs	354	1,689	9	35[b]	5,337

[a] *Rhizopus* sp.
[b] Principally *A. niger* and *Rhizopus* sp.
[c] Parts per billion.

the bollworm and fungi decreases the quality of lint by inducing discoloration (spotting) (Fig. 1-C), and by reducing both staple length and micronaire. They agree with an earlier report on lint disease induced by *A. flavus,* which indicated that severity of disease increased with the severity of pink bollworm infestation (9). A direct quantitative comparison between damage due to the bollworm and damage due to fungi was not made. Figure 1-C, however, shows that bollworm damage to lint of immature locules is negligible compared to damage where fungal decay also is present.

Influence of fungal-induced carpel necrosis upon development of locule infection.—Besides locule infection by *A. flavus,* which does not induce carpel necrosis (3), we observed infection of pink bollworm-damaged immature bolls by two fungi which induce carpel necrosis, *A. niger* and *Rhizopus* sp. We determined whether exposure of lint as a result of premature carpel separation caused by *A. niger* and *Rhizopus* sp. enhances infection of locules by *A. flavus.* About 500 immature pink bollworm-damaged bolls were collected on 6 August 1969. In the laboratory, the bolls were separated into three groups as follows: bolls free from carpel necrosis; bolls necrotic as a result of infection by *A. niger;* and bolls necrotic as a result of infection by *Rhizopus* sp. Then for each group we determined the number of locules damaged by pink bollworm larvae, and the number of locules damaged by *A. flavus* and other fungi. *Aspergillus flavus* infection was determined by examining locules for occurrence of the characteristic greenish yellow fluorescence associated with lint infection (2). Other obviously discolored locules were assumed to be infected by other fungi. The fungi associated with these discolored locules were determined by inspection of 1,268 discolored locules following incubation for 1 week in petri plate moist chambers. At about the same time this experiment was made (12 August 1969), approx 350 in situ immature bolls both without and with carpel necrosis were tagged. When mature, these bolls were harvested and examined in the laboratory for fungal infection to determine whether prolonged exposure of disease-prone bolls results in increased amt of infection.

Results show that necrosis-free immature bolls were about 3 times more prevalent than bolls with necrotic carpels in a collection of 484 bolls examined on 6 August 1969, with *Rhizopus* sp. and *A. niger* accounting for, respectively, two-thirds and one-third of the carpel necroses. In this test, none of 359 bolls free from carpel necrosis suffered early separation of carpels. But about 50% of the remaining bolls, whether infected by *A. niger* or *Rhizopus* sp., split prematurely (Fig. 1).

The openings between carpels induced by A. *niger* and *Rhizopus* sp. provide ready access to lint by organisms unable to directly penetrate the carpel tissue. As indicated in Table 3, about 4 times more infection by *A. flavus* and other fungi occurred in immature bolls having carpel necrosis than in immature bolls free from carpel necrosis. The difference was not so great in bolls examined at maturity. Nevertheless, about twice as much infection by *A. flavus* occurred in bolls having necrotic carpels than in bolls free from necrosis (Table 3). The data also indicate that pink bollworm-damaged bolls were prone to infection for an extended period of time. That is, 4% of locules were infected by *A. flavus* on 6 August 1969, but the amt of infection increased to 23% in the 15-20 days between 6 August and the time of boll maturity (Table 3). In addition, we observed 55% of bolls free from carpel necrosis opened fully at maturity (Fig. 2), as compared with 6% of the bolls

TABLE 3. A comparison of the amount of locules of immature and mature cotton bolls, without and with carpel necrosis, infected by fungi

Cotton bolls	Total bolls	Locule infection		
		Aspergillus flavus	Other	Total
	no.	%	%	%
Immature bolls[a]				
Carpel necrosis absent	359	4	17	21
Carpel necrosis present	125	18	73	91
Means		8	31	39
Mature bolls[b]				
Carpel necrosis absent	353	23	38	61
Carpel necrosis present	356	47	46	93
Means		35	42	77

[a] Observations made 6 August 1969.
[b] Tagged as immature bolls on 12 August 1969 and harvested when all bolls were mature, 2-5 September 1969.

which had carpel necrosis. As shown earlier (3), locules of bolls that fail to fully open dry more slowly and thus are prone to infection longer than locules of bolls that open normally. Several other fungal species besides *A. flavus* are associated with discolored locules. Results of an examination of 1,268 discolored locules following incubation in moist chambers illustrate this point. The percentage of locules bearing various fungi were *A. flavus*, 31%; *A. niger*, 24%; *Rhizopus* sp., 31%; *Fusarium* spp., 79%; other species, including *Alternaria* sp., *A. glaucus*, *A. flavipes*, and *Penicillium* spp., 14%. The latter five fungi, included as "other" fungi (Table 3), also were more prevalent in bolls with carpel necrosis than in necrosis-free bolls.

The avenues of entry for *A. flavus* provided by exit holes of the pink bollworm and of prematurely separated carpels, which result from necrosis by other fungi, probably account for the greater amt of infected locules observed after the insect became generally distributed. The differences follow: 0.005% and 0.43% of the locules were infected by *A. flavus* in, respectively, 1965 and 1966 (4), when the insect was of no importance (assessments of locule infection were not made in 1967 and 1968). In contrast with 1965 and 1966, a mean of 35% of the locules of mature bolls were infected with the fungus in 1969 (Table 3), an 80-fold increase over the amt observed in 1966. Unpublished data for 1970 also indicate that about 35% of locules are infected by *A. flavus*.

The possible role of beetles in transmission of fungi to the interior of pink bollworm-damaged bolls.—We observed three species of the beetle family Nitidulidae, *Carpophilus hemipterous* (L.), *C. dimidiatus* (Fab.), and *Urophorus humeralis* (Fab.) infesting all pink bollworm-damaged bolls from July to September 1969 except those covered with muslin bags. These small beetles occurred in large numbers, with often 20 or more emerging from exit holes in a single boll when it was disturbed by shaking. Seven collections of beetles from melons and from soil were cultured, without surface disinfestation, on malt-salt agar to determine fungal infestation, during August-September 1969. No attempt was made to determine the relative prevalence of individual species. About 10 days after culturing, we determined the kinds and amt of fungi associated with the insects.

We found that numerous fungi, including *A. flavus*, *Rhizopus* sp., and *A. niger*, are associated with the beetles, with *A. flavus* being the only fungus found in all seven samples (Table 4).

A test was made to determine whether the Nitidulids transmit *A. flavus* to the interior of immature bolls. Four groups of 5- to 7-day-old bolls were covered with muslin bags. When about 15 days old (on 9-10 September), each boll was infested with five eggs of the bollworm, then rebagged. A control group of bolls was neither infested with *A. flavus* nor with beetles. Following emergence of pink bollworm larvae, one group of

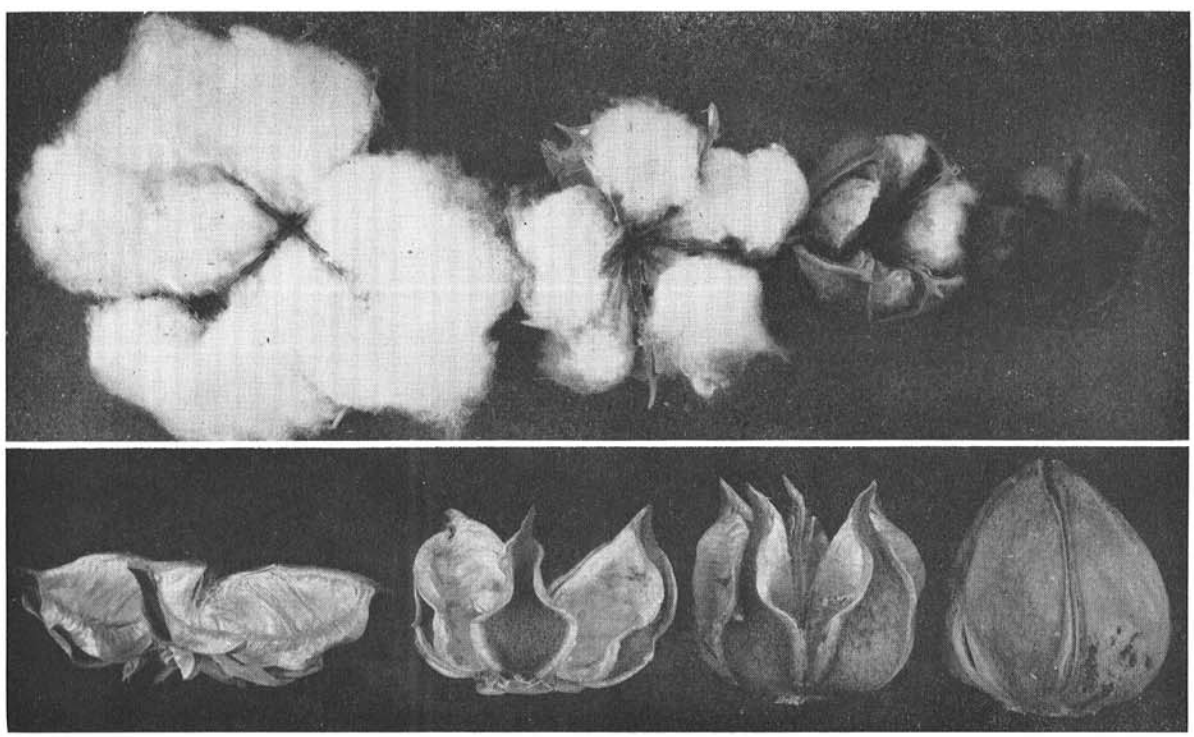

Fig. 2. Mature cotton bolls. (Above) A healthy boll (far left) is compared with bolls affected by pink bollworm larvae and with fungal-induced carpel necrosis. (Below) Bolls show how boll opening is affected by carpel necrosis; a healthy boll (far left) is compared with bolls with carpel necrosis.

TABLE 4. Fungi associated with Nitidulidae beetles in Imperial County, Calif.

Fungi	% Infestation by fungi						
	Insect collections[a]						
	I	II	III	IV	V	VI	VII
Aspergillus flavus	12	1	2	8	9	14	5
A. niger	23	14	10	3	0	2	0
A. glaucus	0	45	4	4	12	4	5
A. flavipes	0	69	39	1	0	0	0
Rhizopus sp.	6	11	100	22	0	2	20
Penicillium spp.	0	0	0	0	7	6	0
Fusarium spp.	46	100	0	9	0	0	0
Alternaria sp.	0	0	0	27	0	0	45
Cladosporium sp.	0	0	0	0	10	0	0
Geotrichum candidum	9	0	0	0	0	0	0
Total individuals	200	200	100	100	110	50	20

[a] Collections and isolations made during August and September 1969.

bolls was infested with a population of beetles, 9% of which were infested with *A. flavus,* at the rate of five beetles/boll. Each boll of another series was infested with five beetles which were first dusted with conidia of *A. flavus.* In each of the above treatments, bolls were allowed to mature in the muslin bags. Larval exit holes of a fourth series of bolls were sprayed with a conidial suspension of *A. flavus* and covered first with small plastic bags to create a water-saturated atmosphere; then muslin bags were replaced. After 3 days, the plastic bags were removed, the muslin bags replaced, and the bolls allowed to mature in the bags. Following maturation, bolls were harvested and determinations were made for the amt of infection by *A. flavus* and other fungi.

Where bollworm-damaged bolls were protected from beetle infestation with muslin bags, we observed that the amt of infection by *A. flavus* and other fungi was about the same (6%) as that observed in earlier similar tests (7-9%) (Table 2). The amt of infection by *A. flavus* tripled, and infection by other fungi increased by one-half when 9% and 100%, respectively, of the beetles were infested with *A. flavus.* We do not consider the latter data significant, however, as the amt of infection with beetles did not exceed the amt observed in absence of beetles (Table 2). Thus, it may be that beetles are attracted only to already rotting locules, in the manner that they are attracted to rotting fruit (8, 11). Tests designed to measure movement of beetles from boll to boll in response to population pressure or various attractants may alter this conclusion.

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