Factors Influencing Infection by *Aspergillus flavus* in Silk-Inoculated Corn

R. K. JONES, Graduate Research Assistant, H. E. DUNCAN, Specialist in Charge, Extension Plant Pathology, G. A. PAYNE, Assistant Professor, and K. J. LEONARD, Plant Pathologist, USDA, SEA, AR, Department of Plant Pathology, North Carolina State University, Raleigh 27650

ABSTRACT

JONES, R. K., H. E. DUNCAN, G. A. PAYNE, and K. J. LEONARD. 1980. Factors influencing infection by *Aspergillus flavus* in silk-inoculated corn. Plant Disease 64:859-863.

Aspergillus flavus infected and produced aflatoxin in kernels of silk-inoculated corn grown in an environment devoid of ear-inhabiting insects. Aflatoxin concentration was linearly correlated with the number of visibly infected kernels. Infection was favored more by warm (32–38 C) than by cool (21–26 C) temperatures. Inoculation of exposed silks of two field-grown commercial dent cultivars 0, 1, 2, and 4 wk after silk emergence resulted in infection and aflatoxin production.

Infection by *Aspergillus flavus* Link ex Fries and subsequent production of aflatoxin in corn before harvest have been documented (1,14,27,29). Extensive aflatoxin accumulation in the field is more likely in the southern United States than in the corn belt states (13,15,23,24), but aflatoxin has been found in preharvest corn in Iowa (9,10) and Indiana (20). Jones (6) suggested that high temperatures (>30 C) and high relative humidity (>85%) favor infection in the field and may account for the greater incidence of aflatoxin in southern regions.

Zuber and Lillehoj (30) pointed out that many aspects of the infection process in preharvest corn have not been resolved, including the availability of inoculum, method of spore transmission to the infection site, establishment of initial infection in developing kernels, and redistribution of secondary inoculum from the original infection site to uninfected kernels in the ear.

Taubenhaus (25) first reported the occurrence of *A. flavus* on Texas field corn in 1920. He concluded that insect injury to the maturing ear was necessary for infection. Rambo et al (21) examined infection of Indiana yellow and white dent corn by silk inoculation and wound inoculation. Although 11% of the silk-inoculated ears contained kernels with bright greenish yellow fluorescence (usually one or two kernels at the tip of an ear), none contained detectable infection or aflatoxin. Infection and aflatoxin were detected in wound-inoculated treatments. Consequently, they concluded that wounding is necessary for invasion of ears by *A. flavus* in the field.

Since that time, insects have been implicated in transporting inoculum to the developing ear (2,22), moving inoculum from silks into the kernel region (4), and providing wounds for establishment of the fungus in damaged kernels (11,15). The role of insects in the epidemiology of *A. flavus* on corn was recently reviewed (26).

Observations in North Carolina in 1976, 1977, and 1978 revealed a high incidence of *A. flavus* infection in ears and kernels free of obvious insect damage (Duncan and Jones, *unpublished*). In this study, we examined the influence of temperature, humidity, and time of inoculation on the ability of *A. flavus* to colonize silk tissue and invade and produce aflatoxin in undamaged kernels.

Present address for senior author: Texas Agricultural Research and Extension Center, Garner Field Road, Uvalde, TX 78801.

Paper 6542 of the Journal Series of the North Carolina Agricultural Research Service.

Use of trade names in this article does not imply endorsement of the products named nor criticism of similar ones not mentioned.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1980.

MATERIALS AND METHODS

Inoculation. An aflatoxin-producing isolate of *A. flavus* (NRRL-3357) was grown for 10 days on Czapek-Dox agar at 25 C. Spores were harvested in sterile distilled water containing 0.01% Triton X-100, and the concentration of suspensions was adjusted to 10^6 conidia/ml. Naturally exposed portions of silks were atomized with 0.5 ml of this suspension (except in field study II where 2.0 ml was used). Ears were enclosed in a plastic bag and then covered with a cloth or a paper bag. Ears of noninoculated (control) plants were sprayed with water and covered with bags. Plants grown in the greenhouse or growth chambers were hand-pollinated; those in the field were naturally pollinated.

Infection and aflatoxin determination. After incubation, aflatoxin and/or *A. flavus* infection were determined as follows:

Plating method. Ears were harvested and shelled by hand with kernels separated according to position (tip, middle, or base) on the ear. Kernels were surface-sterilized in 0.5% sodium hypochlorite for 2 min and plated on a Botran-amended *Aspergillus*-selective medium (5). Plates were incubated at 25 C for 5–7 days and the number of infected kernels was recorded.

Visible growth method. Ears were harvested and dried at 70 C for 72 hr in a forced air oven. Hand-shelled kernels were split longitudinally with a razor blade and the number of kernels with visible growth of *A. flavus* was recorded.

Aflatoxin analysis. Whole ear or bulked samples were ground in a Wiley Mill to pass through a 20-mesh screen. Aflatoxins were extracted using a modified Pons procedure (19). Activated thin layer chromatograms (Eastman No. 6061) were spotted with 1 μl of diluted extract and developed in an unsaturated chamber containing a 90:5:5 solution of benzene, methanol, and acetic acid. Aflatoxin B_1 concentration was estimated visually under ultraviolet light (366 nm) by comparison with commercially prepared standards (Applied Sciences Laboratory, State College, PA) of known concentration.

Experimental design. *Phytotron study.* Plants of a single cross hybrid, Gaspe (flint) × C103 (dent), selected for dwarf growth and early maturity, were grown in

a controlled environment chamber of the Southeastern Plant Environment Laboratory (Phytotron) at 34 C during the day (12 hr) and 30 C at night (12 hr). Ten plants inoculated 3 wk after silking and two noninoculated plants were incubated with plastic bags on the ears for 4 wk. Infection was determined by the visible growth method, and ear samples were analyzed for aflatoxin. The experiment was repeated with the following modifications: Ears of inoculated plants were enclosed in plastic bags for only 72 hr, ears were harvested after 3 wk, and the number and location (tip, middle, or base) of infected kernels on the ear were determined by the plating method.

Greenhouse study. Plants of Seneca 60 sweet corn, chosen for its small size, early maturity, and suitability for greenhouse culture, were grown in 23-cm pots. Exposed silks were inoculated and plants were randomly assigned to a warm (32–38 C) or a cool (21–26 C) greenhouse. Plastic bags were removed 72 hr after inoculation and ears were harvested after 6 wk. The number and location of infected kernels were determined by the plating method.

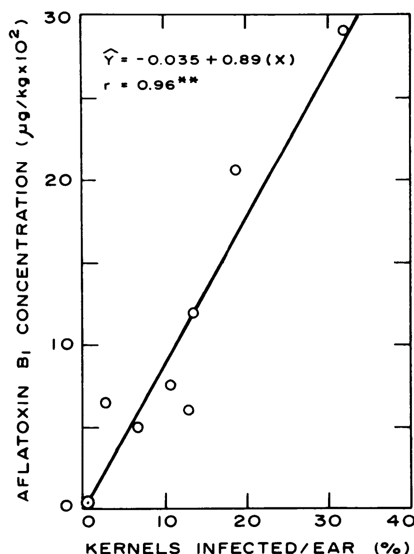

Fig. 1. Relationship between kernel infection per ear and aflatoxin B_1 concentration in Gaspe × C103. Silks inoculated with *Aspergillus flavus* 3 wk after emergence and incubated in the Phytotron. ⊙ = three ears.

Field study I. Plants of Gaspe Flint (Gaspe), a single cross of two partly inbred lines from the accession PI 214279 were seeded two per 38-cm pot on 25 April and placed outdoors on a sand transplant bed. Silks were inoculated 2 wk after emergence (15 June). Ears were harvested 8 wk after inoculation. Infection was determined by the visible growth method, and ear samples were analyzed for aflatoxin.

Field study II. Pioneer Brand 3780 (a short-season, modified, single-cross hybrid) and Pioneer Brand 3147 (a full-season, modified, single-cross hybrid) were planted 13 April and again on 13 May in 8 row × 9.1 m subplots at the Central Crops Research Station near Clayton, NC. One-hundred plants in each subplot were tagged just after silk emergence (silks 2.5–5.0 cm long). Twenty ears of each planting date and cultivar combination were inoculated 0, 1, 2, and 4 wk after silking. Noninoculated plants were included as a control. Alternate tagged plants were either covered immediately after inoculation with both a plastic and a cloth drawstring bag or with only a cloth drawstring bag. All plants were sprayed with 413 g/ha formulated carbaryl insecticide (Sevin) twice weekly from the time of silk emergence until the early dent stage.

Table 1. Effect of temperature after inoculation on the incidence of *Aspergillus flavus* infection in kernels of Seneca 60 sweet corn

Location	Ears infected[a]	Kernels infected No.	Kernels infected Percent
21–26 C greenhouse			
Tip	2/5	118	6.8
Middle	0/5	112	0.0
Base	1/5	123	0.8
32–38 C greenhouse			
Tip	4/4	105	57.1
Middle	4/4	110	69.1
Base	4/4	103	93.2

[a]Number at location/number in study.

Plots were harvested on 18 October, and six of 10 ears in each treatment were randomly selected and bulked for aflatoxin analysis. Infection in the remaining ears was determined by the visible growth method. The locations of infected kernels were recorded on schematic maps of kernel position in the ear.

RESULTS

Phytotron study. In the first experiment, aflatoxin was produced in seven of 10 ears after inoculation of the silks with *A. flavus*. Inoculated ears had a mean of 9.8% of the kernels with visible *A. flavus* growth and a mean aflatoxin concentration of 866 µg/kg. There was a positive correlation between the percent of infected kernels and the concentration of aflatoxin in each ear (Fig. 1). Noninoculated ears had no detectable infection or aflatoxin.

In the second experiment, eight of 10 inoculated ears were infected. Of 1,802 kernels plated from these ears, 13.4, 16.1, and 10.5% of the kernels from the tip, middle, and base of ears, respectively, were infected with *A. flavus*. The short time of incubation within plastic bags (72 hr) suggests that high humidity may be necessary only for spore germination. With three additional plants in this study, plastic bags were removed from the ear 24 hr after inoculation. Only one kernel from these three ears was infected, suggesting that more than 24 hr of high humidity may be required for successful infection of kernels via silk tissue.

Greenhouse study. Seneca 60 sweet corn plants incubated in a warm greenhouse (32–38 C) after inoculation of the silks showed heavy infection by *A.*

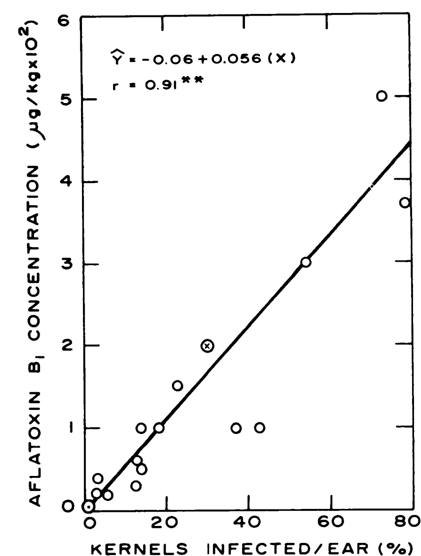

Fig. 2. Relationship between kernel infection per ear and aflatoxin B_1 concentration in Gaspe Flint. Silks inoculated with *Aspergillus flavus* 3 wk after emergence and incubated under natural conditions. ⊙ = five ears, ⊗ = two ears.

Table 2. Effect of planting date and time of inoculation on aflatoxin B_1 in field-grown dent corn inoculated by spraying silks with a spore suspension of *Aspergillus flavus* (Clayton, NC, 1979)

Bagging treatment	Planting date	Cultivar	Inoculated (wk after silking) 0	1	2	4	Control
Cloth[a]	4/13/79	3780[b]	0	20[d]	0	0	0
		3147[c]	60	30	0	0	0
	5/13/79	3780	20	40	20	0	0
		3147	0	0	0	0	0
Cloth and plastic	4/13/79	3780	0	30	60	60	0
		3147	40	100	40	50	0
	5/13/79	3780	20	100	40	40	0
		3147	15	15	15	15	0

[a]Insect damage controlled with cloth drawstring bag and Sevin insecticide.
[b]Pioneer Brand (short-season hybrid).
[c]Pioneer Brand (full-season hybrid).
[d]Aflatoxin B_1 µg/kg (ppb).

flavus (mean of 73% of kernels infected per ear). The proportion of infected kernels was greater at the base than at the middle or tip positions of these ears (Table 1). The incidence of *A. flavus* was much lower (mean 2.5%) in kernels of ears from plants incubated in a cool (21–26 C) greenhouse after inoculation of the silks. Nearly all infected kernels (eight of nine) were from the tip of the ear, suggesting that the ability of *A. flavus* to colonize silks and move down into the ear may depend on temperature.

Field study I. Fourteen of 20 (70%) ears of Gaspe plants grown in pots outdoors were infected with *A. flavus* and contained aflatoxin. A mean of 15.9% of the kernels from inoculated ears were infected, and the mean aflatoxin concentration per ear was 83.5 μg/kg. As in the phytotron study, there was a correlation between the number of infected kernels and aflatoxin accumulation (Fig. 2). One of 10 noninoculated plants had infected kernels (5.2%) and aflatoxin (40 μg/kg).

Field study II. Inoculation of exposed silks of two commercially grown dent cultivars resulted in infection and aflatoxin production. The short-season hybrid planted late and the full-season hybrid planted early contained more aflatoxin B_1 than the short-season hybrid planted early or the full-season hybrid planted late (Table 2). All noninoculated ears were free of visible infection and aflatoxin. Inoculated ears covered with plastic bags had a mean aflatoxin B_1 level of 46 μg/kg compared with 14 μg/kg in ears covered only with cloth bags. Most aflatoxin occurred in treatments inoculated earlier than 4 wk after silk emergence. In this study, silk dessication may have limited the receptive period for infection through silks since ears inoculated at 4 wk and kept humid through plastic bagging remained susceptible.

In ears of Pioneer Brand 3147 (planted 13 April) inoculated at silking, infection was observed in a broad band of kernels around the middle position of all four ears examined (Fig. 3). Visible growth of *A. flavus* within infected kernels was generally confined to the embryo region. The bright greenish yellow fluorescent (BGYF) compound, when present, was detected in the endosperm region immediately adjacent to the embryo. Lillehoj et al (14) reported similar observations. Kernels from inoculated ears were separated into five classes based on infection and the extent of diffusion of

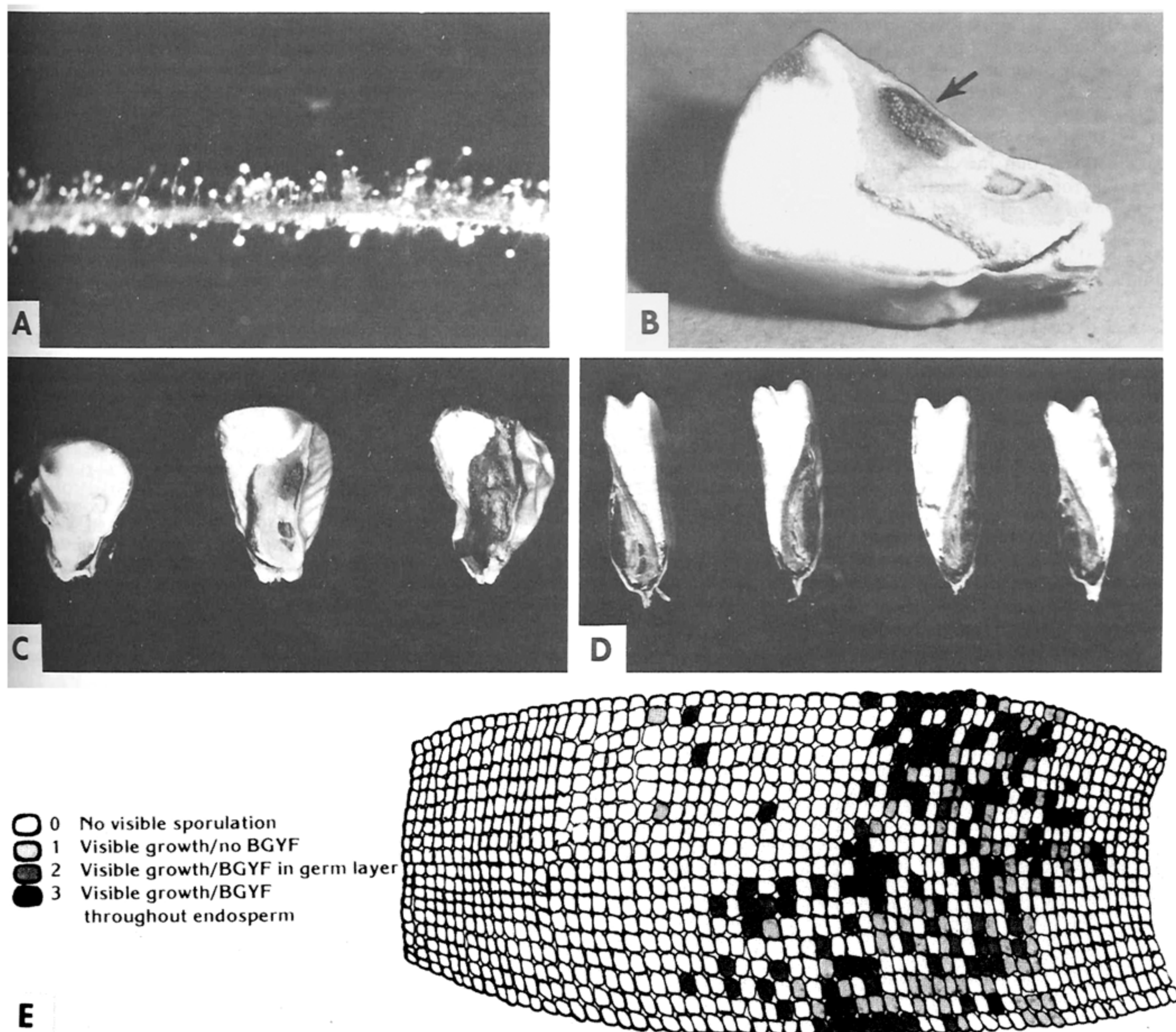

Fig. 3A–E. Infection by *Aspergillus flavus*: **(A)** Heavy colonization of detached silk. **(B)** Silk-inoculated Gaspe × C103. Note sporulation in embryo region. **(C)** Successive stages of embryo destruction of Gaspe × C103. **(D)** Split kernel of Pioneer Brand 3147 (left) silk-inoculated and (right) from naturally infected ear. **(E)** Schematic map of kernel showing infected kernels of Pioneer Brand 3147 planted 13 April and inoculated at silking; ear tip is at right.

the BGYF compound into the endosperm. Twenty-five grams of kernels from classes 0 (no visible growth), 1 (visible growth but no BGYF), 2 (visible growth and BGYF adjacent to embryo), and 3 (visible growth and BGYF throughout endosperm) contained 80, 5,000, 5,000, and 5,000 $\mu g/kg$ aflatoxin B_1, respectively. Evidently aflatoxin concentration is poorly correlated with the BGYF compound in undamaged kernels infected with *A. flavus*.

Examination of split kernels for visible growth of *A. flavus* did not reveal distinct infection patterns among ears inoculated 0, 1, 2, and 4 wk after silking. However, the presence of 80 $\mu g/kg$ aflatoxin B_1 in kernels without visible *A. flavus* illustrates the inability of this technique to accurately determine total infection within inoculated ears.

DISCUSSION

We found that when temperatures and relative humidity are high, *A. flavus* can colonize silk tissue and invade developing corn kernels. The infection of kernels on plants grown in environmental chambers devoid of ear-inhabiting insects demonstrates that insect feeding is not necessary for establishment of the fungus.

A. flavus has often been regarded as a saprophyte or a weak parasite. We suggest that at high temperatures this fungus has increased parasitic ability. The mode of entry of *A. flavus* into kernels of corn is distinct from that into agricultural crops such as cotton or peanuts. Exposed silk tissue is susceptible to colonization by the fungus and provides a suitable infection court for entry of *A. flavus* into intact seeds.

Associations between insect damage and the presence of aflatoxin in corn have frequently been reported, but a direct cause and effect relationship has not been demonstrated (3,18). LaPrade and Manwiller (8) showed that the rice weevil is not an effective vector of *A. flavus*. Widstrom et al (27) found no evidence of a significant relationship between corn earworm injury and aflatoxin B_1. In another study, Widstrom et al (28) found that Lepidopteran insect injury was associated with BGYF but that reduction of insect damage by bagging the ears did not reduce levels of aflatoxin B_1.

Evidence for a relationship between insect damage and *A. flavus* in corn may be biased by the method of detecting *A. flavus*. Damage by insects should increase peroxidase activity and subsequently may increase BGYF in damaged kernels. Peroxidases convert kojic acid (a secondary metabolite of *A. flavus*) into the BGYF compound (17). Thus, use of the BGYF test, which accounts for only 25–70% of the aflatoxin (9,12,13) is likely to overestimate the proportion of *A. flavus* infection in insect-damaged kernels and underestimate that in intact kernels. Similarly, examination of ears for visual growth and sporulation of the fungus on the surface of kernels leads to overestimation of the role of insect damage in *A. flavus* infection. We found that most intact kernels infected with *A. flavus* had no external growth or sporulation. Injury of previously infected kernels allowed for rapid growth and sporulation of the fungus when incubation conditions were suitable. Effective detection of *A. flavus* infection in intact kernels requires either plating them out, which destroys them for use in aflatoxin analysis, or splitting the kernels to reveal internal symptoms.

Recent investigations (7) support earlier claims (30) that airborne spores of *A. flavus* may be an important source of inoculum. Once deposited on silks, the spores may infect developing kernels if environmental conditions favor *A. flavus*. Insects may play a role in increasing infection by spreading the fungus from kernel to kernel within the ears or by providing sites for extensive growth and sporulation on the surface of kernels injured or killed by insect feeding. In addition, the rapid dehydration and consequent lowered moisture content of insect kernels may result in higher concentrations of aflatoxin in damaged kernels than in their undamaged counterparts.

Our research shows that a new concept of the infection process needs to be explored. The possible importance of silk infection in the epidemiology of *A. flavus* infection of corn suggests new opportunities for control of the disease through screening hybrids and inbred lines for resistance.

ACKNOWLEDGMENTS

We wish to thank W. M. Hagler and A. R. Ayers for technical assistance. This work was supported in part by a grant from the Corn Growers Association of North Carolina, Inc.

LITERATURE CITED

1. ANDERSON, H. W., E. W. NEHRING, and W. R. WICHSER. 1975. Aflatoxin contamination of corn in the field. J. Agric. Food Chem. 23:775-782.
2. CHRISTENSEN, J. J., and C. L. SCHNEIDER. 1950. European corn borer (*Pyrausta nubilalis* Hbn.) in relation to shank, stalk, and ear rots of corn. Phytopathology 40:284-291.
3. FENNELL, D. I., W. F. KWOLEK, E. B. LILLEHOJ, G. A. ADAMS, R. J. BOTHAST, M. S. ZUBER, O. H. CALVERT, W. D. GUTHRIE, A. J. BOCKHOLT, A. MANWILLER, and M. D. JELLUM. 1977. *Aspergillus flavus* presence in silks and insects from developing and mature corn ears. Cereal Chem. 54:770-778.
4. FENNELL, D. I., E. B. LILLEHOJ, W. F. KWOLEK, W. D. GUTHRIE, R. SHEELEY, A. N. SPARKS, N. W. WIDSTROM, and G. L. ADAMS. 1978. Insect larval activity on developing corn ears and subsequent aflatoxin contamination of seed. J. Econ. Entomol. 71:624-628.
5. GRIFFIN, G. J., R. H. FORD, and K. H. GARREN. 1975. Relation of *Aspergillus flavus* colony growth on three selective media to recovery from naturally infested soil. Phytopathology 65:704-707.
6. JONES, R. K. 1979. The epidemiology and management of aflatoxins and other mycotoxins. Pages 381-392 in: Plant Disease: An Advanced Treatise, Vol. IV. Academic Press, Inc., New York.
7. JONES, R. K., and H. E. DUNCAN. 1979. Airborne populations of *Aspergillus flavus* in irrigated and non-irrigated field corn. (Abstr.) Phytopathology 69:528.
8. LaPRADE, J. C., and A. MANWILLER. 1977. Relation of insect damage, vector, and hybrid reaction to aflatoxin B_1 recovery from field corn. Phytopathology 67:544-547.
9. LILLEHOJ, E. B., D. I. FENNELL, and W. F. KWOLEK. 1977. Aflatoxin and *Aspergillus flavus* occurrence in 1975 corn at harvest from a limited region of Iowa. Cereal Chem. 54:366-372.
10. LILLEHOJ, E. B., D. I. FENNELL, and W. F. KWOLEK. 1978. *Aspergillus flavus* and aflatoxin in Iowa corn before harvest. Science 193:495-496.
11. LILLEHOJ, E. B., D. I. FENNELL, W. F. KWOLEK, G. L. ADAMS, M. S. ZUBER, E. S. HORNER, N. W. WIDSTROM, H. WARREN, W.. D. GUTHRIE, D. B. SAUER, W. R. FINDLEY, A. MANWILLER, L. M. JOSEPHSON, and A. J. BOCKHOLT. 1978. Aflatoxin contamination of corn before harvest: *Aspergillus flavus* association with insects collected from developing ears. Crop Sci. 18:921-924.
12. LILLEHOJ, E. R., W. F. KWOLEK, D. I. FENNELL, and M. S. MILBURN. 1975. Aflatoxin incidence and association with bright greenish-yellow fluorescence and insect damage in a limited survey of freshly harvested high-moisture corn. Cereal Chem. 52:403-412.
13. LILLEHOJ, E. B., W. F. KWOLEK, A. MANWILLER, J. A. DU RANT, J. C. LaPRADE, E. S. HORNER, J. REID, and M. S. ZUBER. 1976. Aflatoxin production in several corn hybrids grown in South Carolina and Florida. Crop Sci. 16:483-485.
14. LILLEHOJ, E. B., W. F. KWOLEK, R. E. PETERSON, O. L. SHOTWELL, and C. W. HESSELTINE. 1976. Aflatoxin contamination, fluorescence and insect damage in corn infected with *Aspergillus flavus* before harvest. Cereal Chem. 53:505-512.
15. LILLEHOJ, E. B., W. F. KWOLEK, G. M. SHANNON, O. L. SHOTWELL, and C. W. HESSELTINE. 1975. Aflatoxin occurrence in 1973 corn at harvest I. A limited survey in the southeastern U.S. Cereal Chem. 52:603-611.
16. LILLEHOJ, E. B., W. F. KWOLEK, E. E. VANDEGRAFT, M. S. ZUBER, O. H. CALVERT, N. WIDSTROM, M. C. FUTRELL,
17. MARSH, P. B., M. E. SIMPSON, R. J. FERETTI, G. V. MEROLA, J. DONOSO, G. O. CRAIG, M. W. TRUCKSESS, and P. S. WORK. 1969. Mechanism of formation of a fluorescence in cotton fiber associated with aflatoxins in the seeds at harvest. J. Agric. Food Chem. 17:468-472.
18. McMILLIAN, W. W., D. M. WILSON, and N. W. WIDSTROM. 1978. Insect damage, *Aspergillus flavus* ear mold, and aflatoxin contamination in south Georgia corn fields in 1977. J. Environ. Qual. 7:564-566.
19. PONS, W. A., A. F. CUCULLA, L. S. LEE, J. A. ROBERTSON, A. O. FRANZ, and L. A. GOLDBLATT. 1966. Determination of aflatoxins in agricultural products: Use of aqueous acetone for extraction. J. A.O.A.C. 49:554-562.
20. RAMBO, G. W., J. TUITE, and R. W. CALDWELL. 1974. *Aspergillus flavus* and aflatoxin in preharvest corn from Indiana in 1971 and 1972. Cereal Chem. 51:848-853.
21. RAMBO, G. W., J. TUITE, and P. B. CRANE. 1974. Preharvest inoculation and infection of dent corn ears with *Aspergillus flavus* and *A. parasiticus*. Phytopathology 64:797-800.
22. SHOTWELL, O. L. 1977. Aflatoxin in corn. J. Am. Oil Chem. Soc. 54:216A-224A.
23. SHOTWELL, O. L., C. W. HESSELTINE, and M. L. GOULDEN. 1973. Incidence of aflatoxin in southern corn, 1969-1970. Cereal Sci. Today 18:192-195.
24. SHOTWELL, O. L., C. W. HESSELTINE, E. E. VANDEGRAFT, and M. L. GOULDEN. 1971. Survey of corn from different regions for aflatoxin, ochratoxin and zearalenone. Cereal Sci. Today 16:266-273.

25. TAUBENHAUS, J. J. 1920. A study of the black and the yellow molds of ear corn. Tex. Agric. Exp. Stn. Bull. 270. 38 pp.
26. WIDSTROM, N. W. 1979. The role of insects and other plant pests in aflatoxin contamination of corn, cotton, and peanuts—A review. J. Environ. Qual. 8:5-11.
27. WIDSTROM, N. W., E. B. LILLEHOJ, A. N. SPARKS, and W. F. KWOLEK. 1976. Corn earworm damage and aflatoxin B_1 on corn ears protected with insecticide. J. Econ. Entomol. 69:677-679.
28. WIDSTROM, N. W., A. N. SPARKS, E. B. LILLEHOJ, and W. F. KWOLEK. 1975. Aflatoxin production and lepidopteran insect injury on corn in Georgia. J. Econ. Entomol. 68:855-856.
29. ZUBER, M. S., O. H. CALVERT, E. B. LILLEHOJ, and W. F. KWOLEK. 1976. Preharvest development of aflatoxin B_1 in corn in the United States. Phytopathology 66:1120-1121.
30. ZUBER, M. S., and LILLEHOJ, E. B. 1979. Status of the aflatoxin problem in corn. J. Environ. Qual. 8:1-5.