Preharvest Inoculation and Infection of Dent Corn Ears with Aspergillus flavus and A. parasiticus

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Purdue University Agricultural Experiment Station Journal Paper No. 5245.

The authors acknowledge with appreciation the assistance of Arnold J. Ullstrup, and financial assistance from CPC International and Corn Refiners Association.

Accepted for publication 27 December 1973.

ABSTRACT

Ears of yellow and white dent corn were inoculated with *Aspergillus flavus*,*A. flavus* var *columnaris*, and *A. parasiticus*. Twelve of the 14 isolates were parasitic and the three white and two yellow dent varieties inoculated were susceptible. Ears inoculated at later stages of maturity, late milk and early dough, were more susceptible to infection than ears inoculated at the silking or early milk stages. *Aspergillus* growth was usually observed on injured kernels.

The bright greenish-yellow fluorescence (BGYF) was observed in 52% of the kernels which yielded aflatoxin. A false-positive "BGY" was occasionally observed in sound corn, and in cobs of all hybrids tested. This "BGYF" compound was insoluble in water and was not associated with kernels which yielded aflatoxin.

Phytopathology 64:797-800.

Members of the *Aspergillus flavus* group are occasionally found in dent corn prior to harvest in some areas of the United States (3,7,10,11). Surveys of Indian dent corn indicated that *A. flavus* was rare. Although a slightly higher incidence occurred in the southern counties (12). Wichser (*personal communication*) suggested that preharvest invasion by *A. flavus* occurs frequently in southern areas of the U.S. and is associated with aflatoxin.

FDA seizures of aflatoxin-contaminated white corn have increased interest in the significance of preharvest invasion and the development of fast methods for aflatoxin detection in corn (13). A bright greenish yellow or greenish-gold fluorescence (BGYF) in corn kernels is often associated with aflatoxin (2,9) and may be identical to the one described in cotton (1, 4, 5). We report here the extent of *Aspergillus* invasion in white and yellow dent corn in Indiana after artificial inoculation, the effect of ear maturity on susceptibility, and the production of BGYF and aflatoxins.

MATERIALS AND METHODS.–Two hybrids of yellow dent corn, 'Ind 814', 'Funk G-4446' and three white dent hybrids; 'Kamp 916', 'Taylor Evans M20W' and 'Moews 101N' were used. Ind. 814 was grown at Lafayette and the other hybrids at Washington, Indiana, in 1972. Fourteen isolates of *Aspergillus flavus*, Link, *A. flavus* var *columnaris* Raper and Fennel and *A. parasiticus* Speare were used. Isolates were maintained by lyophilization. They were evaluated for aflatoxin production after incubation for 2 wk at 28C on autoclaved popcorn (8). The isolates were combined into groups, designated A,B,C,D, and E, for inoculation (Table 1), however, in one test they were inoculated separately.

The methods of inoculation were; (i) spore atomization, the silks were sprayed until runoff with the spore suspension using a chromatograph spray atomizer. Inoculations were at 0900 and at 1630, 1 and 2 wk after the initiation of silking. (ii) Ear injection; 0.2 ml of the spore suspension was injected into the ear, using a syringe with an 18-gauge needle. Injections were approximately at the early milk, milk, late milk and early dough stages of Ind 814, and at the milk stage of the other hybrids. Ind 814 was also inoculated with individual isolates at the early milk and late milk stages. (iii) Cotton swab; a swab dusted with spores was inserted into a hole drilled into the cob of the hybrids at Washington. For each technique 12 ears were selected and, except for method iii, a spore suspension of 10^5 spores/ml was used. The ears were inoculated at the tip and butt for the injection and cotton swab techniques.

The corn was hand-harvested on 27 September and stored at 2-3 C until examined, usually less than 3 days. Each ear was examined for mold growth under X3 magnification, and for fluorescence, particularly BGYF, in a chromatavue chamber with long wave ultraviolet light (Brinkman Instruments). The ears were labeled, placed in large mesh bags, dried rapidly at 40 C for 3-4 days in a forced-air drier, and stored at 2-3 C until evaluated for aflatoxins and mycoflora.

Kernels showing growth of *A. flavus* and kernels immediately adjoining them were removed from the ear. The moldy kernels were split longitudinally to observe internal fluorescence and analyzed for aflatoxins using the chloroform: water extraction of Shotwell et al (9). Kernels adjoining moldy ones were disinfected with 1% $HgCl_2$ for 1 min, washed twice with sterile water, then split longitudinally. Half of the split kernel was plated on potato-dextrose agar containing 100 μg/ml Tergitol NPX (Union Carbide) and 30 μg/ml chlortetracycline (PDTC); the other half was extracted for aflatoxins. This was done to evaluate spread of infection and aflatoxin to kernels next to the original point of infection.

After the removal of moldy and fluorescing kernels the ears from each inoculation group were machine shelled and 100 kernels were submerged in 5% NaClO for 1 min, then washed twice with sterile water. They were plated on PDTC, 10 per plate, to determine the fungal flora. Incubation was usually at 22-24 C for 7 days.

RESULTS.–Three of the 14 isolates failed to

TABLE 1. Species, source, grouping, and aflatoxin production of isolates used in inoculations

Isolate designation	*Aspergillus* sp.	Inoculation group	Aflatoxin production	Source & year obtained
254	*A. parasiticus*	A	++[a]	corn, 1966
228	*A. flavus*	A	+++	lasagna, 1966
441	*A. flavus* var. *columnaris*	A	-	macaroni, 1968
446	*A. parasiticus*	B	+++	NRRL 3145, 1968
447	*A. flavus* var. *columnaris*	B	-	macaroni, 1969
15517	*A. parasiticus*	B	++	ATCC 15517, 1970
78-5	*A. flavus*	C	++	corn, 1972
Warren	*A. flavus*	C	++	corn leaves, 1972
256	*A. parasiticus*	C	+++	corn, 1966
438	*A. flavus*	D	-	corn, 1968
627	*A. parasiticus*	D + E	+++	NRRL 2999, 1965
WC-2	*A. flavus*	D	+	white corn, 1972
2221	*A. parasiticus*	E	++	peanuts, 1970
Wichser	*A. flavus*	E	+	corn, 1972

[a]+++ = high producer 50,000 μg/kg average of 2 replicates for each isolate on popcorn at 28 C.
++ = less than 50,000 μg/kg
+ = less than 5,000 μg/kg
- = none detected.

produce aflatoxin and these were distributed into different inoculation groups (Table 1).

Inoculation of the silks by atomization did not result in any visible infection but 11% of the ears inoculated and 4% of the control ears had BGYF kernels, usually one or two kernels at the tip of an ear. Among the thirty-eight fluorescing kernels detected, none contained aflatoxins. When the samples were shelled and plated on PDTC three samples yielded 1.0% *A. flavus*.

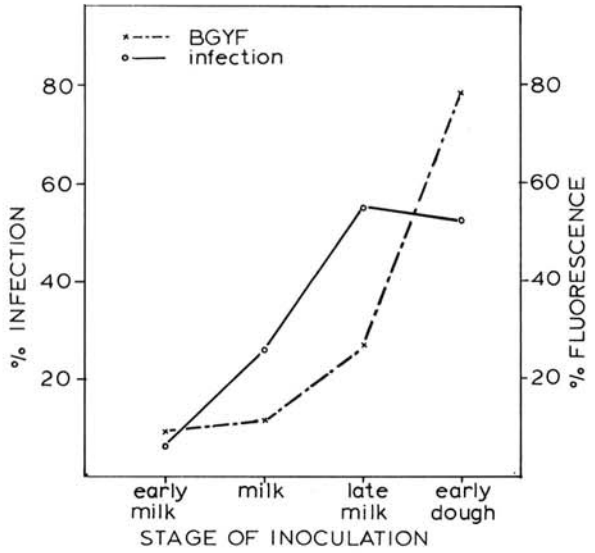

Fig. 1 Percent of ears of corn cultivar 'Ind. 814' having visible signs of *Aspergillus* growth and bright greenish-yellow fluorescence (BGYF) of kernels after inoculation by injection. Percent infection values are based on the total number of moldy spots possible for 12 ears (two per ear) and percent fluorescence is calculated as number of ears with fluorescing kernels. Values represent the average for all the group inoculums for that date.

Visible infection of the corn was obtained by injection at all inoculation dates, but more infection was found in corn inoculated at the late milk and early dough stage (Fig. 1). Ears of Ind 814 inoculated at the early dough stage had the greatest number of fluorescing kernels (Fig. 1). Again, 4% of the control ears had fluorescing kernels. After inoculations with individual isolates, only three of the 14, including an isolate from each species tested, failed to give infection (Table 2).

Results from inoculations by injection and cotton swab in the milk stage at Washington, indicate that the white and yellow dent hybrids inoculated were susceptible (Table 3). Again control ears had BGYF kernels (Table 3).

Aspergillus growth and aflatoxin production.—The kernels injured by inoculation had extensive growth on them, were dark, underdeveloped, and many exhibited BGYF. In several ears, the fungus grew to one or two adjacent kernels, but rarely spread more than this. However, some ears had either superficial growth over several kernels, or had growth between kernels, adjacent to the point of original infection. When growth was superficial there was no apparent damage to the kernels and plating of these kernels did not yield *Aspergillus*. *Aspergillus* growth between the kernels appeared heaviest around the base of the kernels. Also, several of the kernels associated with this growth had breaks in the pericarp adjacent to the germ. Of the 14 kernels damaged in this manner, 12 had BGYF associated with the split area, 7 yielded *Aspergillus*, from plated half kernels, and 8 yielded aflatoxins.

To assess the spread of *A. flavus* in the ear and its significance, 500 uninjured kernels adjacent to visible molded kernels were removed. After surface disinfection half of each kernel was plated and the other half assayed for aflatoxin. Approximately 16%

of the kernels were infected with *A. flavus:* 54% in the germ, 38% in the endosperm, and 8% in both germ and endosperm. BGYF and aflatoxins were not detected in any of these split kernels. Thus the occasional spreading of *Aspergillus* to adjoining kernels was not accompanied by aflatoxin formation.

To determine the levels of aflatoxin in moldy and/or BGYF kernels, 150 kernels were selected from ears of all the inoculation groups on Ind 814. Ninety-seven kernels contained aflatoxin, of these only three had BGYF alone, 44 had visual *A. flavus* growth only, and 50 had the mold plus BGYF. Aflatoxin quantities varied from 0.6 μg/g to 208 μg/g with an average of 187 μg/g. The maturity of the ear at the time of inoculation seemed to have little effect on the aflatoxin levels. From ears inoculated at Washington, 210 moldy or BGYF kernels were selected and 94 yielded aflatoxin; 50 had mold plus BGYF while the remaining 44 had mold but no fluorescence. Aflatoxin amounts varied from 0.3 μg/g to 408 μg/g with an average of 37.6 μg/g.

BGYF was observed on kernels and cobs from uninoculated ears of all the hybrids used. This fluorescence was usually associated with the glumes, and often externally on the kernel tip. However, the BGYF was never found internally in these seed. Aflatoxin was not detected by analysis of 50 kernels and 11 cob tips from the uninoculated ears. The "BGYF" material of these kernels and cobs, was not water-soluble, while the BGYF substance of *A. flavus* infected kernels is water-soluble (9).

DISCUSSION.–Our tests indicate that wounding is probably needed for invasion of ears by *A. flavus* or *A. parasiticus* in the field. Inoculations by syringe, or by insertion of cotton swabs, resulted in infected kernels, BGYF, and aflatoxin. In 1920, Taubenhaus (10) concluded that injury to the ear was associated with preharvest invastion of *A. flavus* in Texas. Although he did not test all stages, he found the early milk stage the most susceptible.

In our early inoculations (early milk-milk), the amount of visible infection was appreciably lower than the amounts observed in the later inoculations,

TABLE 2. Infection incidence in Indiana 814 injected with isolates of *Aspergillus*

Isolate No.	*Aspergillus* spp.	Inoculation Date and Stage: 3 Aug Early Milk Infection[a] (%)	Inoculation Date and Stage: 9 Aug Milk Infection (%)
447	*A. flavus* var *columnaris*	0	33
441	*A. flavus* var *columnaris*	0	15
256	*A. parasiticus*	0	0
2221	*A. parasiticus*	16	16
254	*A. parasiticus*	0	16
15517	*A. parasiticus*	33	16
627	*A. parasiticus*	0	33
608	*A. parasiticus*	0	16
446	*A. parasiticus*	0	0
WC2	*A. flavus*	0	16
438	*A. flavus*	0	16
78-5	*A. flavus*	0	28
228	*A. flavus*	0	0
Warren	*A. flavus*	0	16

[a]No. of moldy spots/12 ears. Two spots possible per ear.

TABLE 3. Incidence of visible *Aspergillus flavus* growth and bright greenish-yellow fluorescence (BGYF) in dent corn cultivars grown at Washington, Indiana and inoculated 3 August at milk stage

Method of Inoculation	White corn: Kamp *A. flavus* (%)	White corn: Kamp BGYF (%)	White corn: Taylor Evans M20W *A. flavus* (%)	White corn: Taylor Evans M20W BGYF (%)	Yellow corn: Moews 101 N *A. flavus* (%)	Yellow corn: Moews 101 N BGYF (%)	Yellow corn: Funk G-4446 *A. flavus* (%)	Yellow corn: Funk G-4446 BGYF (%)
Inoculation group								
A	48	77	63	60	32	100	44	75
B	18	91	22	72	3	91	21	92
C	16	69	27	67	10	100	23	71
D	54	0	63	48	37	84	40	47
E	48	4	0	4	0	10	0	0
Avg.	36.8	48.2	35.0	50.2	16.4	77.0	25.6	57.0
Cotton Swab group								
E	21	35	23	23	19	33	75	25
Control	0	5.8	0	10	0	0	0	7.0

[a]Percent of visible *A. flavus* possible in 12 ears.
[b]Percent of ears, 12 per sample, having fluorescing kernels classified as BGY.

approximately late milk and early dough. Although BGYF and aflatoxin positive kernels were present on ears from all inoculation times, the amounts of aflatoxin varied greatly. These variations may reflect the aflatoxin-producing abilities of the different isolates, and not be due to the stage of maturity at inoculation.

The limited spreading of *A. flavus* in the ear indicates limited parasitic ability, under Indiana conditions. On occasion *A. flavus* grew over or between kernels adjacent to the point of infection. Moldy areas of more than two kernels were considered an indication of spreading, but these may have resulted from multiple injury during inoculation or from initial dissemination of inoculum by moisture present in the ear or supplied with the inoculum. Only in rare instances (a total of 14 kernels) did there appear to be physical damage to the kernel as a result of spreading fungal growth and this did not always result in BGYF or aflatoxin.

The correlation between the presence of BGY and aflatoxin was approximately 52%. Frequently BGYF and visible mold were not observed in the same kernel. From the kernels with BGYF, or *A. flavus* with BGYF, 60% yielded aflatoxin. Aflatoxin was not detected in kernels not having either mold or BGYF. There were many kernels and cob tips from non-inoculated ears which fluoresced "BGY" similar to the BGYF in *A. flavus* infected kernels. The presence of this "BGY" gave false positives, since no aflatoxin was detected from these fluorescing kernels. False positives have been reported by other researchers in cotton seed and corn (2,6). This fluorescence could conceivably cause problems in using BGYF as a presumptive test for presence of *Aspergillus* and aflatoxin.

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