Influence of *Aspergillus flavus* Strains on Aflatoxin and Bright Greenish Yellow Fluorescence of Corn Kernels

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ABSTRACT

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The objective of this study was to relate the diversity of a naturally occurring population of *Aspergillus flavus* to their ability to contaminate grain with aflatoxin and produce bright greenish yellow fluorescent (BGYF) kernels. A total of 19 strains of *A. flavus* isolated from a corn field near Kilbourn, Illinois were used as inoculum, including 16 genotypes (DNA fingerprinting), and representing both aflatoxin producers and non-producers. A commercial corn hybrid (Pioneer 3394) was grown in this field in 1996 and 1998. A total of 20 ears in the late-milk to early-dough stage of maturity were inoculated with each *A. flavus* strain using a toothpick-wound procedure. At harvest, 20 to 24 of the kernels nearest to each wounded site were separated into three categories: wound-inoculated kernels, intact BGYF kernels, and all other intact kernels. Sample weights of intact BGYF kernels in 1996 and 1998 grain samples averaged 5.0 and 9.5% of the total sample weight, respectively. Aflatoxin-producing strains were associated with a higher frequency ($P < 0.05$) of BGYF kernels for grain samples harvested in 1998. Removal of the individual wound-inoculated kernels and the intact BGYF kernels from corn ears inoculated with 13 aflatoxin-producing strains of *A. flavus* lowered mean aflatoxin values from 115 ng/g (range = <1 to 387 ng/g) to 2 ng/g for 1996 grain samples and from 744 ng/g (range = 20 to 1,416 ng/g) to 33 ng/g for 1998 grain samples. Results indicated substantial variation among *A. flavus* genotypes in their ability to produce aflatoxin in the germ and endosperm of infected BGYF kernels. The naturally occurring *A. flavus* population may include a majority of strains that produce no aflatoxin but exhibit BGYF and are thus aflatoxin "false positives" when corn grain is examined with an ultraviolet light at 365 nm. Intraspecific competition between aflatoxin-producing and non-producing strains would be expected to naturally suppress the severity of aflatoxin outbreaks within the Midwestern corn belt.

Additional keywords: kojic acid, maize, mycotoxin, resistance, seed coat, temperature

The corn industry's first level of assessment for aflatoxin is the black-light test for kernels exhibiting bright greenish yellow fluorescence (BGYF; 14). The fluorescence is produced by the oxidative action of heat-labile enzymes (peroxidases) in living plant tissue on kojic acid, which is formed with aflatoxin by *Aspergillus flavus* Link:Fr. (10). The detection of BGYF in

undamaged kernels is evidence that *A. flavus* has infected the germ or endosperm.

However, predicting aflatoxin levels is difficult based on the proportion of BGYF kernels or kernel fragments in grain samples (3,8). One reason for this is that kernels infected with *A. flavus* strains that produce kojic acid but do not produce aflatoxin exhibit BGYF and thus are aflatoxin "false positives" when corn grain is examined with a black light. Quantities of aflatoxins are greater when *A. flavus* grows saprotrophically in damaged kernel tissues than when the germ and endosperm of intact kernels are infected (12,19). Aflatoxin concentrations in wound-inoculated corn kernels grown in the University of Wisconsin Biotron ranged from 13,200 to 82,000 ng/g (mean, 36,700 ng/g), while the sample means for intact kernels surrounding each wound site did not exceed 2,100 ng/g (= 2,100 ppb) and concentration ranged from 154 to 6,290 ng/g (18). Unfortunately, no attempt was made to separate BGYF kernels or to record aflatoxin concentration in these kernels.

Naturally occurring populations of *A. flavus* may vary in their pathogenicity or virulence (2) and their ability to produce

aflatoxin (1). *A. flavus* isolates from corn harvested at the University of Illinois River Valley Sand Field (IRVSF, Kilbourn, IL) were characterized recently using DNA fingerprinting and the ability to produce aflatoxin in laboratory culture (20). The objective of this study was to determine whether the naturally occurring diversity in this collection of *A. flavus* isolates from Illinois corn is related to their ability to produce BGYF kernels and aflatoxin following wound inoculation.

MATERIALS AND METHODS

Fungal inoculum and culture conditions. All *A. flavus* strains examined in this study were isolated from corn sampled at harvest from an experimental planting (<1.5 ha) under continuous (1988 to 1991) corn cultivation at IRVSF (20). The ability of 121 *A. flavus* strains from IRVSF to form sclerotia and to produce aflatoxin in laboratory culture was described previously. The genotype of each strain was characterized based on DNA fingerprinting (20). On the bases of these data (20), a total of 19 strains were selected to represent strains that produce or do not produce aflatoxin in our current study (Table 1). Conidia from each strain were harvested from 14-day-old Czapek agar slants incubated at 25°C, after adding 5 ml of 0.01% Triton X-100 to each slant. Cultures then were agitated, and the spore suspensions transferred to sterile screw-capped culture tubes and adjusted to 1×10^6 spores/ml with sterile distilled water. The spore suspensions were stored at 4°C for 3 days prior to corn inoculations.

Commercial hybrid and field location.

In 1996 and 1998, a commercial corn hybrid (Pioneer 3394) was grown to maturity in a 16-ha field at IRVSF, Kilbourn, Illinois. An area within the center-pivot-irrigated portion of this field was selected for *A. flavus* inoculations. The plot (30 by 40 m) consisted of 10 rows of corn, each 40 m in length. Eight border rows separated the plot from the nearest access road. A total of 20 ears on plants within a single 7-m length of row were inoculated with one of the *A. flavus* strains. Each of the inoculated 7-m row lengths were separated by four noninoculated plants.

Inoculation procedure. Corn ears in the late-milk to early-dough stage of kernel maturity (about 21 days after mid-silk; 7 August 1996 and 24 July 1998) were wound inoculated with a conidial suspen-

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sion of *A. flavus*. Ears were inoculated at three points separated by 4 cm in a vertical row. A sterile wooden toothpick was inserted through the husk to wound an underlying kernel. A second toothpick, contaminated with *A. flavus* spore suspension, was then inserted into each wound site and left until ears at 15.5% moisture content were hand harvested on 2 October 1996 and 19 October 1998.

The 20 kernels nearest to each wound site (18) were separated into three categories: wound-damaged kernels, intact BGYP kernels as determined with an ultraviolet light at 365 nm, and the remaining kernels. All *A. flavus* strains sporulated on wound-inoculated kernels, evidence of successful fungal establishment at the site of wounding. Kernels were not sampled from portions of ears with visible insect damage. Kernels molded by fungi other than *A. flavus* also were excluded. Each category of kernels was pooled within each *A. flavus* strain, weighed (Table 1), and stored in plastic bags at -7°C prior to aflatoxin analyses.

Aflatoxin analyses. All of the grain sampled from ears inoculated with each *A. flavus* strain and separated according to BGYP kernels versus non-BGYP kernels was first ground using a Stein Mill (Steinlite Inc., Atchison, KS). All samples were analyzed for aflatoxins by the Aflatest (Vicom Inc. Watertown, MA) procedure according to the manufacturer's instructions. A total of 5 g of NaCl was added to 50 g of ground corn sample (approximately 150 kernels), which was then blended for 1 min with 100 ml of 80% MeOH in water (vol/vol) and filtered through Whatman 2V filter paper (filtrate no. 1 = 0.5 g equivalent/ml).

Next, 10 ml of filtrate no. 1 was diluted with 40 ml of distilled water (= 5 g equivalent/50 ml) and filtered through a microfiber filter, after which 10 ml of filtrate no. 2 (= 1 g equivalent/10 ml) was passed through an Aflatest affinity column. The column was washed twice with distilled water and the aflatoxin concentrated in 1 ml of 100% MeOH. Following the addition of 1 ml of bromine developer, aflatoxin concentration was determined with a Vicam Series-4 fluorometer calibrated with Vicam test solutions. For samples of intact BGYP kernels weighing 3.7 to 22.2 g (10 to 60 kernels), the method was adjusted to scale for quantity of sample. NaCl (0.1 g) and 2 ml of 80% MeOH were added to each 1 g of sample in a stoppered 125-ml Erlenmeyer flask. Flasks were shaken vigorously on a wrist-action shaker for 5 min, and the contents filtered through Whatman 2V filter paper. Filtrate no. 1 was diluted (1:4 vol/vol) with distilled water and filtered through a microfiber filter, then a measured volume of filtrate no. 2 was passed through an affinity column. Samples of wound-inoculated kernels, appearing damaged and shriveled and weighing 0.5 to 3.0 g (20 to 40 kernels), were repeatedly steeped in 10 ml of chloroform on 3 successive days, and 30 ml of total extract was combined in a sealed vial stored at -21°C. Following dry-down of chloroform under air, several grains of NaCl and appropriate volumes (1.0 to 6.0 ml) of 80% MeOH were added to individual sample vials and vortexed for 5 min. Non-filtered MeOH extract (0.5 to 3.0 ml) was diluted (1:4 vol/vol) with distilled water (2 to 12 ml), passed through a 0.4-µm pore syringe filter, passed through

an affinity column, concentrated in 1 ml of 100% MeOH, developed, and read in the fluorometer. Total sample aflatoxin levels for each inoculation treatment were calculated using the formula: [wounded kernels (g) × aflatoxin (ng/g) + intact BGYP kernels (g) × aflatoxin (ng/g) + all other kernels (g) × aflatoxin (ng/g)]/total sample weight (g).

A Student's *t* test was applied at a 5 or 1% level of probability to compare the following sample means: percent weight of BGYP kernels for *A. flavus* strains that produce aflatoxin (*n* = 13) and those that do not (*n* = 6); aflatoxin (ng/g) content of grain samples for *A. flavus* strains that produce aflatoxin harvested in 1996 (*n* = 13) and 1998 (*n* = 13); and aflatoxin (ng/g) content of grain samples (*n* = 13) before and after the removal of the wounded kernels and the intact BGYP kernels.

RESULTS AND DISCUSSION

In 1996, kernels that were wound inoculated with 1 of 19 *A. flavus* strains and intact BGYP kernels comprised 0.4 to 1.0% (mean, 0.6%) and 1.5 to 11.5% (mean, 5.0%) of the total sample based on weight, respectively (Table 1). In 1998, wound-inoculated and intact BGYP kernels comprised 0.1 to 0.4% (mean, 0.3%) and 2.6 to 14.8% (mean, 9.5%) of the total sample, respectively, based on weight. The ability of *A. flavus* strains to produce aflatoxin was associated with a significantly greater frequency of BGYP kernels (as determined by sample weight) for grain samples harvested in 1998, but not for grain samples harvested in 1996. The number of intact BGYP kernels may be associated with frequency of seed coat

Table 1. Aflatoxin contamination of corn kernels (hybrid Pioneer 3394) wound-inoculated with 19 strains of *Aspergillus flavus* isolated from corn

Strains ^a	Grain at harvest, 1996						Grain at harvest, 1998					
	Sample weight (%)		Aflatoxin (ng/g)				Sample weight (%)		Aflatoxin (ng/g)			
	Wounded	BGYP ^b	Wounded	BGYP	Other ^c	Total ^d	Wounded	BGYP ^b	Wounded	BGYP	Other ^c	Total ^d
A-27837	0.8	2.8	13,600	3,800	3	215	0.3	11.0	2,200	6,400	43	747
26474	0.9	7.8	16,600	2,900	14	387	0.4	10.8	1,800	8,700	19	965
26473	0.5	6.6	12,200	1,500	<1	164	0.2	14.8	2,000	9,200	7	1370
26491 ^x	0.5	3.0	10,800	1,200	4	93	0.3	12.0	2,100	5,200	5	634
26482	0.4	4.9	14,800	1,100	3	113	0.3	13.5	3,300	9,200	190	1416
26468	0.9	8.0	13,000	900	1	191	0.4	13.7	1,400	9,200	12	1,283
26480	0.6	2.9	17,200	200	<1	108	0.2	13.3	1,700	7,200	36	990
26485 ^x	0.4	3.1	6,000	300	<1	40	0.3	9.6	4,200	8,900	42	904
A-27676	0.5	3.9	8,800	52	<1	45	0.1	4.8	1,100	150	12	20
26477	1.0	1.5	2,000	71	0	21	0.2	10.2	160	610	1	64
26476 ^y	0.4	4.4	1,060	67	0	7	0.3	10.8	620	1,840	14	212
26479 ^y	0.5	4.5	2	0	0	<1	0.1	6.5	0	990	2	66
26472	0.6	4.4	280	0	0	2	0.4	5.6	4,400	4,200	9.4	262
26641 [*]	0.6	7.4	102	1	0	<1	0.2	9.0	0	<1	1.7	2
26639 [*]	0.6	6.2	240	4	0	2	0.3	7.9	<1	90	5.5	12
A-27675 [*]	1.0	11.5	0	0	0	0	0.4	9.5	0	0	0	0
26640 [*]	1.0	5.3	3	0	0	<1	0.2	8.8	0	0	4.2	4
A-27667 ^{*z}	0.4	2.6	4	3	3	<1	0.3	2.6	310	86	0.1	3
A-27668 ^{*z}	0.4	4.3	6	0	<1	<1	0.2	6.4	0	42	0	3

^a NRRL strain numbers; ^{*} = aflatoxin negative (-) on glucose yeast extract soytone (GYES) medium (20) as determined by thin-layer chromatography; ^x, ^y, and ^z indicate NRRL strains sharing a common genotype.

^b BGYP = intact, bright greenish-yellow fluorescent kernels.

^c Other intact kernels.

^d Total sample.

tearing. *A. flavus* colonizes the wound-inoculated kernels and uses them as a food base from which to spread and infect the surrounding intact kernels (6,15,18,19,22). Zuber (21) and others have observed that *A. flavus* is a weak pathogen of maize and that infection of the grain usually requires a break in the pericarp (4,7). However, the fungus can easily enter the air spaces of the pericarp but cannot infect the germ or endosperm unless a break occurs in the internal seed coat or testa (16). Smart et al. (16) showed that *A. flavus* gains entry to the germ through random microscopic breaks in the testa when the surrounding pericarp was intact. Aflatoxin is produced after the fungus invades the embryo or endosperm. The scattered distribution of undamaged BGYF kernels removed from the same ear (5,9,15) may simply mirror the distribution of kernels having torn seed coats (16).

Aflatoxin levels for wound-damaged kernels inoculated with 1 of the 13 aflatoxin-producing *A. flavus* strains exceeded 1,000 ng/g for 11 samples in 1996 and 10 samples in 1998 (Table 1). These light-weight, wound-damaged, and shriveled kernels did not exceed 1% of the total sample weight for grain harvested from any inoculation treatment or year. Aflatoxin levels for samples of intact BGYF kernels infected with 1 of the 13 aflatoxin positive strains varied from 0 to 3,800 ng/g in 1996 and from 150 to 9,200 ng/g in 1998. The 1998 values proved to be significantly higher ($P < 0.01$). When the aflatoxin content of each fraction was combined as the weighted mean for each of the treatments inoculated with 1 of the 13 aflatoxin positive strains, 10 grain samples harvested in 1996 and 13 grain samples harvested in 1998 had unacceptable aflatoxin concentrations (>20 ng/g). The removal of individual wound-inoculated kernels and intact BGYF kernels from grain samples harvested in 1996 and 1998 resulted in a significant ($P < 0.01$) reduction of the mean aflatoxin values from 115 to 2 ng/g and from 744 to 33 ng/g, respectively.

Differences in aflatoxin levels and the frequency of BGYF kernels between years may be explained by differences in the average daily temperatures recorded at Illinois Climate Network, Champaign, IL (IRVSF) for September 1996 (16.6°C) and September 1998 (20.6°C), with the warmer temperatures favoring *A. flavus* parasitic ability and aflatoxin production (5,17). The extent to which a corn hybrid develops symptoms of *A. flavus* kernel rot and the

accumulation or aflatoxin within individual, infected kernels is positively related to temperature during the period of grain filling and maturation (17) and drought stress (11). The present results show that BGYF kernels become contaminated with different amounts of aflatoxin because *A. flavus* strains differ in their basic ability to produce aflatoxins. Both aflatoxin-positive and aflatoxin-negative strains produced BGYF kernels. The naturally occurring *A. flavus* population may include a majority of strains that produce no aflatoxin (13,20) but exhibit BGYF and are thus aflatoxin "false positives" when corn grain is examined with an ultraviolet light at 365 nm. Interpretations of the association between weight of BGYF kernels and aflatoxin levels in grain samples (3) should recognize the aflatoxin-producing potential of the *A. flavus* population in regions where corn is grown. Intraspecific competition between aflatoxin-producing and non-producing strains would be expected to naturally suppress the severity of aflatoxin outbreaks within the Midwestern corn belt.

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