Aflatoxin and Bright Greenish-Yellow Fluorescence of Corn Kernels Inoculated with *Aspergillus Flavus* Genotypes (rflp) from a Field in Illinois

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Abstract

The objective of this study was to relate the diversity of a naturally occurring population of *Aspergillus flavus* to their ability to contaminate the grain with aflatoxin and produce bright greenish-yellow fluorescent (BGYF) kernels. Nineteen strains of *A. flavus* isolated from a corn field near Kilbourne, 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 and twenty 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, 20–24 kernels nearest 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 1416 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 ultra violet 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.

Key words: aflatoxin, *Aspergillus flavus*, bright greenish-yellow fluorescence, corn, genotype.

Introduction

The corn industry's first level of assessment for aflatoxin is the black light test for kernels exhibiting bright greenish-yellow fluorescent (BGYF) kernels.¹⁾ The fluorescence is produced by the oxidative action of heat-labile enzymes (peroxidases) in living plant tissue

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on kojic acid, which is formed with aflatoxin by *Aspergillus flavus* Link : Fr.²⁾ The detection of BGYF in undamaged kernels is evidence that *A. flavus* has infected the germ and/or endosperm. However, predicting aflatoxin levels is difficult based on the proportion of BGYF kernels or kernel fragments in grain samples.^{3,4)} 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.^{5,6)} 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 6290 ng/g.⁷⁾ Unfortunately, no attempt was made to separate BGYF kernels or to record aflatoxin concentration in these kernels. *Aspergillus flavus* isolates from corn harvested at the University of Illinois River Valley Sand Field (IRVSF, Kilbourne, IL) were characterized recently using DNA fingerprinting and the ability to produce aflatoxin in laboratory culture.⁸⁾ Naturally occurring populations of *A. flavus* may vary in their pathogenicity/virulence⁹⁾ and their ability to produce aflatoxin.¹⁰⁾ 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

The 19 *A. flavus* strains examined in this study, isolated from corn sampled at harvest at IRVSF, included 16 distinct genotypes (Table 1) and were selected to represent strains that produce or do not produce aflatoxin respectively.⁸⁾ In 1996 and 1998, a commercial corn hybrid (Pioneer 3394) was grown to maturity at IRVSF. Twenty corn ears in the late-milk to early-dough stage of kernel maturity (about 21 days after mid-silk ; 7, August 1996 ; 24, July 1998) were toothpick wound-inoculated with a conidial suspension of one of the *A. flavus* strains. Ears at 15.5% moisture content were hand harvested on 2, October 1996 and 19, October 1998. Twenty kernels nearest to each wound site⁷⁾ were separated into three categories as follows : wound-damaged kernels, intact BGYF kernels as determined with an ultra violet light at 365 nm ; and the remaining kernels. Each category of kernels was pooled within each *A. flavus* strain, weighed (see Table 1), ground using a Stein Mill (Steinlite Inc., Atchison, KS), and analyzed for aflatoxins by the Aflatest (Vicom Inc. Watertown, MA) procedure according to the manufacturer's instructions. Calculation of “total sample” aflatoxin levels for each inoculation treatment were performed using the formula : [‘wounded kernels’ (g)x aflatoxin (ng/g)+‘intact BGYF kernels’ (g)x aflatoxin (ng/g)+‘all other kernels’ (g)x 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 : % weight of BGYF 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) ; aflatoxin (ng/g) content of grain samples (n=13) before and after the removal of both the ‘wounded kernels’ and the ‘intact BGYF kernels’.

Results and Discussion

In 1996, kernels that were wound-inoculated with one 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 ($P < 0.05$), but not for grain samples harvested in 1996 ($P > 0.05$). The number of intact BGYP kernels may be associated with frequency of seed coat 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.^{6,7,11,12,13} Zuber¹⁴) 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.^{15,16} 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.¹⁷ Smart et al.¹⁷) 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

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

Grain at harvest-1996

NRRL No.	Sample weight %		Aflatoxin (ng/g)			Total sample
	Wounded kernels	Intact BGYP	Wounded kernels	Intact kernels BGYP	All other	
A-27837	0.8	2.8	13,600	3,800	3	215
26474	0.9	7.8	16,600	2,900	14	387
27473	0.5	6.6	12,200	1,500	<1	164
26491 ^x	0.5	3.0	10,800	1,200	4	93
26482	0.4	4.9	14,800	1,100	3	113
26468	0.9	8.0	13,000	900	1	191
26480	0.6	2.9	17,200	200	<1	108
26485 ^x	0.4	3.1	6,000	300	<1	40
A-27676	0.5	3.9	8,800	52	<1	45
26477	1.0	1.5	2,000	71	0	21
26476 ^y	0.4	4.4	1,060	67	0	7
26479 ^y	0.5	4.5	2	0	0	<1
26472	0.6	4.4	280	0	0	2
26641 [*]	0.6	7.4	102	1	0	<1
26639 [*]	0.6	6.2	240	4	0	2
A-27675 [*]	1.0	11.5	0	0	0	0
26640 [*]	1.0	5.3	3	0	0	<1
A-27667 ^{*z}	0.4	2.6	4	3	3	<1
A-27668 ^{*z}	0.4	4.3	6	0	<1	<1

^{*} aflatoxin negative (–) on GYES medium as determined by TLC.

^{x,y,z} NRRL strains sharing a common genotype.

Table 2 Aflatoxin contamination of corn kenels (Pioneer 3394) would-inoculated with 19 strains of *Aspergillus flavus* isolated from corn.

Grain at harvest-1996						
NRRL No.	Sample weight %		Aflatoxin (ng/g)			Total sample
	Wounded kernels	Intact BGYF	Wounded kernels	Intact kernels BGYF	All other	
A-27837	0.3	11.0	2,200	6,400	43	747
26474	0.4	10.8	1,800	8,700	19	965
27473	0.2	14.8	2,000	9,200	7	1,370
26491 ^x	0.3	12.0	2,100	5,200	5	634
26482	0.3	13.5	3,300	9,200	190	1,416
26468	0.4	13.7	1,400	9,200	12	1,283
26480	0.2	13.3	1,700	7,200	36	990
26485 ^x	0.3	9.6	4,200	8,900	42	904
A-27676	0.1	4.8	1,100	150	12	20
26477	0.2	10.2	160	610	1	64
26476 ^y	0.3	10.8	620	1,840	14	212
26479 ^y	0.1	6.5	0	990	2	66
26472	0.4	5.6	4,400	4,200	9.4	262
26641 [*]	0.2	9.0	0	<1	1.7	2
26639 [*]	0.3	7.9	<1	90	5.5	12
A-27675 [*]	0.4	9.5	0	0	0	0
26640 [*]	0.2	8.8	0	0	4.2	4
A-27667 ^{*z}	0.3	2.6	310	86	0.1	3
A-27668 ^{*z}	0.2	6.4	0	42	0	3

scattered distribution of undamaged BGYF kernels removed from the same ear^{12,18,19}) may simply mirror the distribution of kernels having torn seed coats.¹⁷⁾

Aflatoxin levels for wound-damaged kernels inoculated with one of the 13 aflatoxin producing *A. flavus* strains exceeded 1000 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 one 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 ($P < .01$). When the aflatoxin content of each fraction was combined as the weighted mean for each of the treatments inoculated with one of the 13 aflatoxin positive strains, ten grain samples harvested in 1996 and thirteen 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 ($P < .01$) 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 IRVSF for September 1996 (16.6 C) and September 1998 (20.6 C), the warmer temperatures favoring *A. flavus* parasitic ability and aflatoxin production.^{18,20)} The extent to which a corn hybrid develops symptoms of *A. flavus* kernel rot and aflatoxin accumulates within individual infected kernels is positively related to temperature during the period of grain filling and

maturation²⁰⁾ and drought stress.²¹⁾ The present results show that BGYP 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 BGYP kernels. The naturally occurring *A. flavus* population may include a majority of strains that produce no aflatoxin^{8,22)} but exhibit BGYP and are thus aflatoxin “false positives” when corn grain is examined with an ultra violet light at 365 nm.

Conclusions

Interpretations of the association between weight of BGYP kernels and aflatoxin levels in grain samples³⁾ 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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