Growth Characteristics of *Aspergillus flavus* on Agar Infused With Maize Kernel Homogenates and Aflatoxin Contamination of Whole Kernel Samples

N. W. Widstrom, W. W. McMillian, D. M. Wilson, D. L. Garwood, and D. V. Glover

Research geneticist and research entomologist, Southern Grain Insects Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Tifton, GA 31793; professor, Department of Plant Pathology, University of Georgia College of Agriculture Experiment Stations, Coastal Plain Station, Tifton; owner, Garwood Seed Company, Stonington, IL 62567; and professor, Department of Agronomy, Purdue University, W. LaFayette, IN 47907, respectively.

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ABSTRACT

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Methods of measurement have been a major obstacle to the detection of maize (*Zea mays* L.) genotypes resistant to infection by *Aspergillus flavus* and aflatoxin production. Mature grain of field-inoculated hybrids was evaluated for preharvest aflatoxin contamination in 1977, 1978, and 1980, and laboratory-inoculated mature kernel samples were evaluated for aflatoxin production in 1981. In each year, ground grain of eight commercial hybrids sampled 25, 40, and 56 days after anthesis was infused in antibiotic-amended agar which was placed in petri plates and inoculated with the fungus. After incubation for 7 days at 30 C, evaluations were made for colony diameter, sporulation amount, and sporulation distribution. Colony diameter was a better criterion than sporulation characteristics for detecting differences among hybrids. Grain harvested 25 days after anthesis supported greater fungal growth than that harvested at 40 and 56 days. Various kernel genotypes having endosperms classified as amylose extender, brittle, dull, floury, normal, shrunken, soft starch, sugary, or waxy in B37 × Oh43 (dent) and/or Ia453 × Ia5125 (sweet) backgrounds were similarly tested. Colony diameters and amounts of sporulation were significantly different among endosperm genotypes within these backgrounds. Differences among hybrids and endosperm variants were more consistent for aflatoxin contamination than for colony characteristics. Mycelial growth was clearly enhanced by the presence of sugary endosperm. The more restricted growth on starchy types was consistent with that found for mature kernels of the commercial hybrids. Sugar content of the kernels was an overriding factor contributing to differences among genotypes for aflatoxin contamination of kernel samples in this study.

Several commonly grown cereal crops contain chemical compounds that help protect them against disease and insects. Genetic manipulation of the quantity of these compounds could assist in the control of pests and diseases. Sitton and West (9) demonstrated that "casbene," a diterpene produced by castor bean (*Ricinus communis* L.), inhibited growth of *Zea mays* L., *Aspergillus niger*, and *Escherichia coli* when bioassayed at concentrations from 3×10^{-6}M to 7×10^{-6}M. Trypsin inhibitors isolated from maize retarded the growth of six fungi of five genera (2). Peng and Black (8) reported an association between proteinase inhibitor activity and the resistance of tomato (*Lycopersicon esculentum* Mill.) to *Phytophthora infestans* (Mont.) DBy.

Plant pigments and associated compounds have been associated with resistance to diseases in sorghum (*Sorghum bicolor* [L.] Moench) and maize (3,5,7). Glucose was reported to induce aflatoxin production by *A. flavus* on artificial medium (1). Beta-ionone, a volatile metabolite of developing maize ears, has major effects on the morphology of the asexual reproductive structures of *A. flavus* (11). Other compounds that influence aflatoxin production also may exist in the maize kernel (4).

The objectives of this study were to identify maize genotypes that may contain compounds that influence growth of *A. flavus* in vitro, to determine the association of these influences with kernel maturity, and to assess these effects on growth of *A. flavus* and compare them with aflatoxin production in vivo.

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MATERIALS AND METHODS

Commercial hybrid tests. Eight commercial maize hybrids, widely grown in the southern United States at the time, were chosen for testing based on diversity of reaction to infection by *A. flavus* and subsequent aflatoxin contamination.

The maize hybrids to be evaluated for preharvest aflatoxin contamination were planted during the third week of April in 1977 and 1978 at Tifton, GA, in a randomized complete-block design with four replications. Plots were single rows, 76 cm apart and 6.1 m long, containing about 25 plants. The top ears on 10 plants per plot were infested with 30 corn earworm larvae per silk 7 days after full silk. On the 25th day after full silk, the ears were also inoculated with *A. flavus* at the tip, middle, and base by inserting a 26-gauge, 10-mm hypodermic needle through the husk and injecting 0.1 ml of spore suspension (isolate NRRL 3357), 10^7 conidia per milliliter. The infested and inoculated ears were harvested at maturity, dried for 5 days at 60 C in a forced-air drier, and shelled. Shelled samples were coarse-ground and reduced to 500 g by using a riffle sampler. These 500-g samples were ground to pass through a 20-mesh screen, a 50-g analytical sample was taken, and aflatoxin content was determined by the method of Thean et al (10). Plot means were analyzed as $\ln(ng \cdot g^{-1} + 1)$ and are reported as geometric means.

The same commercial hybrids were also planted in a split-plot design during the second and first weeks of April in 1977 and 1978, respectively. The 1977 and 1978 experiments contained three and eight replications, respectively, in two-row plots with the same row spacing and lengths as described above. Split plots were 10-ear samples harvested 25, 40, and 56 days after full silk. Surface-disinfested kernels (3 min in 0.5% aqueous sodium hypochlorite) from these samples were homogenized with a Super Dispak (Tecmor, Inc.) and incorporated in agar solution on three plates per plot at 40 $g \cdot L^{-1}$. The agar solution contained 20 g of agar, 30 g of

NaCl, 50 mg of streptomycin sulfate, 50 mg of chlorotetracycline, and 1.0 mg of botran (2,6-dichloro-4-nitroanaline) per liter of water. The plates were inoculated by touching the center of the agar surface with a needle that had been dipped in a conidial suspension of *A. flavus* containing 10^6 spores per millilter. After the plates were incubated at 30 C for 7 days, we recorded colony diameter (mm), sporulation on a 0–5 scale (0 = none and 5 = very heavy sporulation), and sporulation distribution on a 1–3 scale (1 = sporulation only very near the point of inoculation and 3 = sporulation distant from the point of inoculation). Plot means were compared with each other and with the aflatoxin contamination values of hybrids obtained in the other experiment.

Endosperm variant tests. Seeds of 16 endosperm genotypes in a sweet corn (Ia453 × Ia5125) background (F_3) and of 13 endosperm types in a dent corn (B37 × Oh43) background (F_2) seed were tested in the laboratory for ability to support fungal growth and aflatoxin production. The field design for each group of endosperm types (Table 1) was a randomized complete block planted during the third and first week of April in 1980 and 1981, respectively.

The experiments to measure aflatoxin contamination had five replications in 1980 and six replications in 1981. Mycelial growth characteristics were measured in three replications during both years and data were recorded on sporulation levels only in 1981. In 1981, 25 g of kernels were surface-disinfected for 3 min in 0.5% aqueous sodium hypochlorite, rinsed with sterile water, placed in sterile flasks at approximately 25% moisture, and inoculated with 0.1 ml of a conidial suspension of *A. flavus* (NRRL 5520) containing 10^6 spores per milliliter. The flasks were covered with sterile cotton plugs and aluminum foil and incubated for 10 days at 30 C. Traits were evaluated and data analyzed as for the commercial hybrid tests, except that all samples were harvested at kernel maturity (56 days after silking), and when possible the variation due to differences among genotypes was subdivided into single degrees of freedom for differences between mutants or mutant combinations. All plants involving endosperm mutants were sib- or self-pollinated to maintain the carbohydrate characteristic of the mutant. All ears (about 10/plot) were harvested from the single 3-m row.

TABLE 1. Maize endosperm genes present in backgrounds of sweet (Ia453 × Ia5125) and dent (B37 × Oh43) corn tested for effects on in vitro growth of *Aspergillus flavus* and production of aflatoxin

Gene		Test background	
Symbol	Name	Ia453 × Ia5125	B37 × Oh43
ae	amylose extender	yes	yes
bt	brittle endosperm	no	yes
bt2	brittle endosperm	no	yes
du	dull endosperm	yes	yes
fl	floury endosperm	no	yes
fl2	floury endosperm	no	yes
h	soft starch	no	yes
···	normal	no	yes
sh	shrunken endosperm	no	yes
sh2	shrunken endosperm	no	yes
su	sugary endosperm	yes	yes
su2	sugary endosperm	no	yes
wx	waxy endosperm	yes	yes

RESULTS

In 1977, plants suffered drought stress about 6 wk after emergence (12-leaf stage) through maturity, and ears were heavily infested by insects. Also, the highest level of natural field contamination of grain by aflatoxin ever recorded occurred in south Georgia in 1977. The least contaminated hybrid in 1977 had three times more aflatoxin than the most contaminated hybrid in 1978 (Table 2).

Differences in mycelial characteristics among hybrids were less pronounced than differences in aflatoxin concentration. No differences were detected among hybrids for sporulation distribution, and in the 1977 experiment, differences were detected only for amount of sporulation. The colony diameters on the hybrids were significantly different in 1978.

Differences occurred among sampling dates for all cultural characteristics on maize-infused agar in 1978 but not in 1977 (Table 2). Significant differences among hybrids were detected for all cultural characteristics from maize collected 25 days after anthesis.

The 12 endosperm mutants that were tested in the dent corn (B37 × Oh43) as seed taken from sibbed F_1 plants are listed in Table 3. All five nonstarchy mutants of the shrunken, sugary, or brittle type supported the highest amounts of aflatoxin contamination. The modified starchy *su2* genotype also supported high amounts of aflatoxin production, but the amounts were low for the *ae* (60% amylose) and *du* (30–35% amylose) genotypes. Levels of fungal infection and toxin development on inoculated ears in 1980 were lower and differences among genotypes for amounts of aflatoxin were not significant.

The sugary genotypes supported higher amounts of sporulation than starchy types did, but no differences were detected among genotypes for sporulation distribution or colony diameter in 1980.

TABLE 2. Hybrid and sampling date means[v] for aflatoxin contamination and colony characteristics of *Aspergillus flavus* measured on maize grain samples taken from field plots

	Aflatoxin[w] (ng·g^{-1})			Colony diameter (mm)			Sporulation amount[x]			Sporulation distribution[y]		
Hybrid	1977	1978	Combined	1977	1978	Combined	1977	1978	Combined	1977	1978	Combined
Pioneer 3030	934 a	37 a	186 a	46 a	49 ab	48 ab	1.2 bcd	2.4 a	2.1 a	1.6 a	2.1 a	2.0 a
Asgrow RX140A	2,080 ab	29 a	246 ab	44 a	55 b	52 b	1.3 cd	2.7 a	2.3 a	1.6 a	2.1 a	2.0 a
Coker 77	1,572 ab	97 ab	391 abc	43 a	54 b	51 b	0.8 ab	2.6 a	2.1 a	1.5 a	2.1 a	1.9 a
Dekalb XL394	2,807 abc	67 ab	436 abc	44 a	46 ab	46 ab	0.9 abc	2.5 a	2.0 a	1.5 a	2.1 a	1.9 a
Dekalb XL80	6,634 bc	31 a	454 abc	47 a	42 ab	44 ab	1.0 abc	2.2 a	1.9 a	1.9 a	2.1 a	2.0 a
Northrup King PX675	4,722 abc	99 ab	683 bcd	44 a	39 a	40 a	0.7 a	2.0 a	1.7 a	1.1 a	1.9 a	1.6 a
RingAround RA1501	8,604 bc	105 ab	950 cd	46 a	37 a	40 a	1.6 d	2.0 a	1.9 a	1.7 a	1.8 a	1.8 a
Funk G-4611	14,186 c	240 b	1,851 d	46 a	45 ab	46 ab	1.3 cd	2.5 a	2.2 a	1.7 a	1.9 a	1.8 a
Sampling[z]												
25				45 a	60 a	56 a	1.1 a	2.8 a	2.3 a	1.4 a	2.9 a	2.5 a
40				46 a	45 b	45 b	1.3 a	2.1 b	1.9 b	1.5 a	1.6 b	1.6 b
56	3,668 s	69	506	44 a	33 c	36 c	0.9 a	2.1 b	1.8 b	1.7 a	1.6 b	1.6 b
Marginal mean				45	46		1.1 s	2.4		1.5 s	2.0	

[v] Means within a column grouping not followed by the same letter and marginal or year means separated by s are significantly different, $P = 0.01$, according to Duncan's multiple range test.
[w] Values are geometric means (antilogarithm of the logarithmic mean).
[x] On a 0–5 scale (0 = none and 5 = very heavy).
[y] On a 1–3 scale (1 = near and 3 = removed from point of inoculation).
[z] Number of days after anthesis when samples were removed from the field.

Significant differences in aflatoxin contamination occurred in 1981, but not in 1980, among the endosperm genotypes of Ia453 × Ia5125 as sibbed seed collected from F_2 plants (Table 4). Large differences occurred among genotypes in the analysis combined over years, even though the field and laboratory evaluations revealed highly significant differences between years. The sugary genotypes had the highest amounts of aflatoxin. Contrary to the results for B37 × Oh43 genotypes, significant differences among Ia453 × Ia5125 endosperm types were observed for colony diameter and sporulation distribution of *A. flavus*, but not for sporulation amount. The *ae wx* type had the lowest value for sporulation distribution.

The only single-degree-of-freedom comparison having a significant difference was between genotypes containing the sugary *su* gene and those not containing it (Table 5). Sugar content was the prominent factor overshadowing smaller effects among the starchy endosperm genotypes.

DISCUSSION

The dramatic difference between the 1977 and 1978 crop years is reflected by the aflatoxin amounts in samples from the eight commercial hybrids grown in our field study (Table 2). The highly significant differences in aflatoxin contamination among hybrids during both years indicate that the propensity for contamination in different environments is a heritable and measurable character.

The combined data suggest that colony diameter would be the best of the three cultural characteristics to distinguish among hybrids for factors that influence fungal growth. Of the traits studied, however, only aflatoxin concentration appears to

TABLE 3. Means for aflatoxin contamination and colony characteristics of *Aspergillus flavus* measured on endosperm genotypes in B37 × Oh43 test background[w]

Endosperm type	Aflatoxin[x] (ng·g^{-1}) 1980 (field)	1981 (lab)	Combined	Colony diameter (mm) 1980	Sporulation amount[y] 1980	1981	Combined	Sporulation distribution[z] 1980
du	67 a	26 a	40 a	80 a	2.0 abc	1.2 a	1.6 ab	3.0 a
ae	81 a	39 ab	54 a	67 a	1.3 a	1.2 a	1.2 a	2.0 a
h	110 a	65 abc	82 ab	78 a	4.3 d	1.5 ab	2.9 bcd	2.7 a
fl 2	119 a	77 abc	94 ab	63 a	3.0 abcd	2.5 ab	2.8 bcd	2.3 a
fl	126 a	198 abc	161 ab	72 a	3.3 bcd	1.8 ab	2.6 abcd	2.3 a
Normal	96 a	391 abc	206 ab	77 a	1.7 ab	1.8 ab	1.8 abc	3.0 a
wx	62 a	765 abc	244 ab	72 a	2.3 abc	1.7 b	2.0 abc	2.7 a
sh 2	156 a	440 abc	276 ab	80 a	2.0 abc	2.7 b	2.4 abcd	2.7 a
sh	104 a	2,587 bc	602 b	77 a	3.0 abcd	2.3 b	3.1 cd	3.0 a
su	140 a	2,941 c	735 b	77 a	3.3 bcd	3.7 b	3.5 d	2.7 a
su 2	207 a	2,279 bc	765 b	75 a	3.7 cd	2.7 b	3.2 cd	2.7 a
bt	148 a	3,828 c	871 b	60 a	3.0 abcd	3.5 b	3.2 cd	2.3 a
bt 2	281 a	2,372 bc	898 b	...	...	2.8 ab		
Marginal mean	120 s	473			2.7 s	2.3		

[w] Means within a column not followed by the same letter and marginal means separated by s are significantly different, $P = 0.05$, according to Duncan's multiple range test.
[x] Values are geometric means (antilogarithm of the logarithmic mean).
[y] On a scale of 0–5 (0 = none and 5 = very heavy).
[z] On a scale of 1–3 (1 = near and 3 = removed from point of inoculation).

TABLE 4. Means for aflatoxin contamination and colony characteristics of *Aspergillus flavus* measured on endosperm genotypes in Ia453 × Ia5125 test background[w]

Endosperm type	Aflatoxin[x] (ng·g^{-1}) 1980 (field)	1981 (lab)	Combined	Colony diameter (mm) 1980	Sporulation amount[y] 1980	1981	Combined	Sporulation distribution[z] 1980
Normal	206 a	515 a	340 a	90 b	3.7 a	1.2 a	2.4 a	3.0 b
du	167 a	1,437 ab	539 ab	65 ab	2.7 a	2.8 a	2.8 a	2.3 b
ae wx	849 a	489 a	626 abc	35 a	1.7 a	2.8 a	2.2 a	1.0 a
ae su	452 a	1,444 ab	854 abc	90 b	3.0 a	2.3 a	2.6 a	3.0 b
wx	644 a	883 ab	765 abcd	80 b	2.7 a	2.8 a	2.8 a	2.7 b
du wx	364 a	1,763 ab	863 abcd	58 ab	2.3 a	1.8 a	2.0 a	2.7 b
ae	307 a	2,666 ab	992 abcd	90 b	2.3 a	2.2 a	2.2 a	3.0 b
de su wx	401 a	3,123 ab	1,224 bc	78 b	3.7 a	2.7 a	3.2 a	3.0 b
ae du su	462 a	2,380 ab	1,130 bcd	73 b	3.0 a	2.8 a	2.9 a	2.3 b
ae du su wx	598 a	2,295 ab	1,249 bcd	87 b	3.3 a	2.5 a	2.9 a	3.0 b
ae su wx	564 a	3,487 b	1,525 bcd	80 b	3.0 a	2.8 a	2.9 a	3.0 b
ae du	340 a	5,560 b	1,556 bcd	67 ab	3.0 a	2.7 a	2.8 a	2.3 b
ae du wx	830 a	2,697 ab	1,572 bcd	90 b	2.7 a	2.3 a	2.5 a	3.0 b
du su	534 a	3,931 b	1,588 bc	80 b	4.0 a	2.3 a	3.2 a	2.7 b
su wx	670 a	5,635 b	2,143 c	90 b	3.0 a	2.7 a	2.8 a	3.0 b
su	944 a	5,324 b	2,416 d	90 b	4.0 a	3.2 a	3.6 a	3.0 b
Marginal mean	470 s	2,165			3.0 s	2.5		

[w] Means within a column not followed by the same letter and marginal means separated by s are significantly different, $P = 0.05$, according to Duncan's multiple range test.
[x] Values are geometric means (antilogarithm of the logarithmic mean).
[y] On a 0–5 scale (0 = none and 5 = very heavy).
[z] On a 1–3 scale (1 = near and 3 = removed from point of inoculation).

TABLE 5. Means[y] for aflatoxin contamination and colony diameter of *Aspergillus flavus* measured on grouped endosperm genotypes in Ia453 × Ia5125 test background

Mutant in genotype	Aflatoxin[z] ($ng \cdot g^{-1}$)	Colony diameter (mm)
su	1,437 a	84 a
wx	1,164 ab	75 ab
du	1,153 ab	75 ab
ae	1,141 ab	76 ab
non *ae*	1,022 ab	79 ab
non *du*	1,012 ab	81 ab
non *wx*	1,002 ab	81 ab
non *su*	812 b	72 b

[y] Means within a column not followed by the same letter are significantly different, $P = 0.01$, according to Duncan's multiple range test.

[z] Values are geometric means (antilogarithm of the logarithmic mean).

differentiate reliably among genotypes. Maize collected 25 days after anthesis in 1978 was much less capable of restricting fungal growth than maize collected 40 or 56 days after anthesis. Also, factors influencing sporulation were significantly less restrictive in 1978 than in 1977, as shown by the marginal means (Table 2). Early in the development of the kernel, there are apparent differences among hybrids for the restrictive factors, but these differences disappear as the kernel matures. This finding encouraged us to initiate the tests involving endosperm variants in the dent and sweet corn backgrounds.

The level of sugar at later stages of kernel development may be an important factor in toxin formation. The *su*2 and normal genotypes have similar low sugar levels and higher amylose levels at maturity, but the normal seeds support much lower amounts of aflatoxin production. The lower amount of aflatoxin contamination for the high-amylose genotype *ae* (Table 3) is consistent with the findings of McMillian et al (6). In this study, the *ae* (60% amylose) and *du* (30–35% amylose) genotypes also had low amounts of aflatoxin. However, if high levels of amylose directly inhibit aflatoxin production or accumulation, then the *su*2 genotypes (38–40% amylose) should also have had a low amount of aflatoxin. The difference in aflatoxin formation associated with the *su*2 genotype is unclear. The difference in amounts of aflatoxin contamination between years was probably because in 1980 ears were inoculated in the field, whereas in 1981 kernels were inoculated in the laboratory.

Of the three cultural characters, amount of sporulation was most effectively differentiated in the B37 × Oh43 background. Differences for sporulation are probably due to enhancement by sugar content rather than chemical inhibition by some other compound in the nonsugary genotypes. Differences for colony diameter are not as easily interpreted, but the general trend seems to be that sugary genotypes support larger fungal colonies.

The 16 genotypes of Ia453 × Ia5125 constitute a balanced set of genotypes for the four endosperm mutants (*ae*, *du*, su, and *wx*). Several single gene effects were thus evaluated as single-degree-of-freedom comparisons in the analysis of variance. For example, all genotypes having one mutant can be compared with those having another, or all genotypes having a mutant can be compared with all genotypes not having that mutant (Table 5). In 10 such comparisons for aflatoxin contamination and colony diameter, only genotypes containing *su* differed from those not containing *su*. Other factors undoubtedly affect mycelial characters and aflatoxin development, but we conclude that sugar content is the dominating influence.

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