Aflatoxin B_1 and G_1 Production in Developing Zea mays Kernels from Mixed Inocula of Aspergillus flavus and A. parasiticus

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ABSTRACT

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Yields of aflatoxin B_1 for various fungal conidial proportions used as inocula (0/100% *A. flavus* only, 25/75, 50/50, 75/25, and 100/0% *A. parasiticus* only) were relatively constant for all inoculation treatments. But the yields of aflatoxin G_1, produced only by *A. parasiticus* in the mixed inocula showed marked, significant decreases from that produced by *A. parasiticus* alone. Ratios calculated from the aflatoxin G_1 and B_1 produced, remained relatively constant for each inoculum regardless of the inoculation treatment, but G_1/B_1 ratios for each treatment decreased significantly as the proportion of *A. flavus* conidia in the inocula increased. Production of aflatoxin G_1 and B_1 was significantly greater in the thin- than in the thick-pericarp hybrid. The highest amounts of aflatoxin G_1 and B_1 were observed when the husks of the ears were pulled back and the kernels either razor- or pinboard-injured before inoculation. The lowest amounts were observed for the widely-used hypodermic-syringe method. The results suggest that *A. parasiticus* in mixtures is significantly limited from developing fully by *A. flavus* in competing for the same corn substrate. This may help explain why *A. flavus* routinely is found present in naturally aflatoxin-contaminated corn and *A. parasiticus* only rarely.

Aspergillus flavus Link ex Fries and *A. parasiticus* Speare are capable of synthesizing B_1, B_2, G_1, and G_2 aflatoxins on many commodities both before and after harvest (1, 3, 5, 8, 9, 10, 11). Strains of *A. flavus* exhibit wide variation in aflatoxin synthesis, ranging from no production to high yields of aflatoxin on the same substrate (4, 7). With the exception of a few atypical, morphological isolates, the *A. flavus* strains produce only aflatoxin B_1 and B_2 (7). However, *A. parasiticus* strains consistently produce all four aflatoxins; i.e., B_1, B_2, G_1, and G_2 (7). Further, the relative amount of aflatoxin produced by each species should be in proportion to its number of conidia in the inoculum. However, previous studies showed that *A. flavus* usually was present in aflatoxin-contaminated corn whereas *A. parasiticus* occurred only rarely (5, 6, 8).

Rambo et al. (11) corroborated the observation of Taubenhaus (13) that wounding of developing kernels of corn was necessary for infection by either *A. flavus* or *A. parasiticus*. More recent studies have shown that *A. flavus* infection of corn before harvest is associated with insect damage of developing kernels (1, 8, 9).

In this study we examined aflatoxin production in developing corn to determine the relationship between relative yields and degrees of mechanical damage in two hybrids with thick and thin pericarps and determined the effect of inocula with varying proportions of conidia from *A. flavus* and *A. parasiticus* by comparing aflatoxin yields and the ratios of G_1 to B_1 produced under field conditions.

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MATERIALS AND METHODS

In 1975 a field experiment was conducted in a split plot design with 12 combination treatments of two hybrids, one with a thick pericarp, B37 × B14A, and the other with a thin pericarp, H49 × CI44 with six different injury treatments as whole plots and six conidial inocula proportions and a noninoculated water control, as subplots. Two replications were used.

The husks of test ears either were left intact or were pulled back to expose the developing kernels 20 days after 50% of the ears had visible silks. Kernel injury was accomplished by the use of (i) three 123-mm long razor blades, set 11 mm apart and mounted in a plastic holder; (ii) 85 steel sewing pins arranged in five rows of 17 pins to form a slightly concave surface to fit the shape of the ear in an area 25 mm × 102 mm; or (iii) a 3-ml B-D plastic syringe with 0.1-ml graduations and equipped with a 0.64-mm diameter (22-guage), 25-mm needle (Becton, Dickinson and Co., Rutherford, NJ 07070). The inoculum was prepared from conidial suspensions of *A. parasiticus* (NRRL 2999) and *A. flavus* (NRRL 3357) grown on potato-dextrose agar in Roux flasks for 14 days at 28 C. Conidia (10^8 conidia/ml) were washed from the

surface of the agar with sterile distilled water containing 0.01% Triton X (Rohm and Haas Co., Philadelphia, PA 19105). Inocula containing different proportions of conidia of the two species were prepared by diluting a 1×10^8 suspension with sterile distilled water to make stock suspensions containing 4×10^7 conidia/ml. By blending the two stock suspensions separately, 1:1 with water, the 100/0 or 0/100 conidial proportions contained 2×10^7 conidia; or by blending the stock solutions with each other, the 50/50 conidial proportion contained 2×10^7 conidia/ml of *A. parasiticus* and 2×10^7 conidia/ml of *A. flavus*; or by blending one fourth of one stock suspension with three fourths of the other, the 25/75 conidial proportion contained 1×10^7 conidia/ml of *A. parasiticus* and 3×10^7 conidia/ml of *A. flavus*; and the 75/25 conidial proportion by blending three fourths of one stock suspension and one fourth of the other. Approximately 1.5 ml of each prepared conidial suspension or water control were atomized over the injured kernels with a Model 15 DeVilbiss atomizer (The DeVilbiss Co., Somerset, PA 15501) or 0.3 ml/ear of each prepared conidial suspension or water control was syringe-injected beneath the husk. In treatments involving pulling back the husk, the husk was repositioned over the ear and secured with rubber bands after the kernels were injured and inoculated. After inoculation all ears except those inoculated with the hypodermic syringe were covered for seven days with a plastic bag secured with rubber bands to maintain a high humidity favorable for conidial germination. The ears were harvested 30 days after inoculation (at physiological maturity), dried at 60 C for 4 days to less than 13% moisture, shelled, and the kernels ground in a 30.5-cm Raymond hammer mill having screens with 3.2-mm perforations (Raymond Pulverizer Division, Combustion Engineering Co., Inc., 200 West Monroe, Chicago, IL 60606). Ground corn samples were assayed for aflatoxin as described in the official first action of the Association of Official Analytical Chemists (2). Quantities of aflatoxin present in the extracts were determined on thin-layer chromatographic plates coated with 0.5 mm Adsorbosil-1 (Applied Science Lab., Inc., Box 440, State College, PA 16801). The plates were developed with chloroform:acetone:water (88:12:1.5, v/v), and fluorescent zones were measured densitometrically. Since a wide range of experimental values (0 to more than 18,000 ng/g) was obtained, some results are expressed as geometric means. Analysis of the data utilized log of $B_1 + 1$ and $G_1 + 1$ levels, but G_1/B_1 ratios were not transformed (12).

RESULTS AND DISCUSSION

The effects of the several kernel damage treatments and use of inocula containing various proportions of *A. flavus* and *A. parasiticus* conidia on the production of aflatoxin

TABLE 1. Effect of different methods of preharvest injury on aflatoxin production in corn kernels simultaneously inoculated with different proportions of *Aspergillus parasiticus* and *A. flavus* conidia in the inocula

Inoculation treatment	Aflatoxin type	Aflatoxin level (geometric means in ng/g)[a]					
		A. parasiticus/*A. flavus* conidial proportions used as inoculum[b]					Water
		0/100	25/75	50/50	75/25	100/0	0/0
Husk pulled back, razor damaged, inoculum sprayed	B_1	4,691	2,574	6,066	2,407	10,162	2,830
	G_1	10	76	303	430	6,406	547
Husk pulled back, pinboard damaged, inoculum sprayed	B_1	5,434	4,388	3,978	6,440	7,128	128
	G_1	3	116	405	1,338	6,133	1
Husk intact, razor damaged, inoculum sprayed	B_1	1,215	2,617	2,723	2,090	2,472	73
	G_1	2	598	958	1,037	2,573	4
Husk intact, pinboard damaged, inoculum sprayed	B_1	894	422	850	403	876	291
	G_1	36	42	220	154	752	11
Husk intact, inoculum inserted with hypodermic syringe	B_1	169	463	265	435	342	92
	G_1	2	8	33	90	209	9
Husk pulled back, not damaged, inoculum sprayed	B_1	80	142	310	156	161	37
	G_1	2	16	34	43	116	3

[a]Least significant ratio (LSR) for aflaxtoxin B_1 values = 3.27 at P=0.05. LSR for aflatoxin G_1 values = 9.09 at $P = 0.05$.
[b]Number of conidia in the percentage proportions: 0/100 (2×10^7 *A. flavus*), 25/75 (1×10^7 *A. parasiticus* and 3×10^7 *A. flavus*), 50/50 (2×10^7 for each fungus), 75/25 (3×10^7 *A. parasiticus* and 1×10^7 *A. flavus*), and 100/0 (2×10^7 *A. parasiticus*).

B_1 and G_1 are shown in Table 1. The inoculation treatments with the husks pulled back and the kernels damaged before being sprayed with each of the conidial proportions were significantly higher in the production of aflatoxin B_1 than the inoculation treatments with the husks left intact. Both treatments had significantly higher yields of aflatoxin B_1 than in the hypodermic syringe method in which the ears were inoculated through the husk and in the inoculated control in which the husks of the ears were pulled back but the kernels not injured.

The data also show that the yields of aflatoxin B_1 produced by using the five conidial proportions as inocula for each inoculation treatment did not differ significantly with only minor exceptions in the first inoculation treatment, and in the last (Table 1, 10,162 ng/g aflatoxin B_1 versus 2,574 and 2,407 ng/g, and 310 ng/g aflatoxin B_1 versus 80 ng/g). However, the data show that the yields of aflatoxin G_1 were significantly less than the amounts of aflatoxin G_1 produced by the nonmixed *A. parasiticus* conidial inoculum. Conidia from both species should have an equal chance of developing to produce aflatoxin B_1 but only *A. parasiticus* produces G_1. The results, however, on the basis of the conidial proportions as inocula, showed that the amounts of aflatoxin B_1 produced were relatively constant for the inoculation treatments, but the amount of G_1 changed significantly.

The amounts of aflatoxin B_1 and G_1 in the inoculated ears in a majority of the comparisons were significantly higher for the first two inoculation treatments than in the last two (Table 1). In addition, the apparent growth of the fungi on the ears was denser on ears of the first two inoculation treatments than on the last two. The conidial and mycelial growth on the ears by *A. flavus* was less dense than that of *A. parasiticus*. Certainly with the marginal growth of both species in the last two inoculation treatments, the aflatoxin B_1 and G_1 production was at a minimum.

If *A. flavus* and *A. parasiticus* produced 100% of their aflatoxin B_1 in the corn inoculated with the mixed conidial proportions as they did with the nonmixed conidial proportions used as inoculum, and assuming that aflatoxin G_1 would be produced in corn in the same porportion as when inoculated with 100/0 conidial proportion, the amounts of aflatoxin G_1 observed showed a significant, marked decrease. In the mixed conidial proportions used as inocula, instead of producing a quarter, or a half or three-quarters of that amount of aflatoxin G_1 produced by the nonmixed conidia of *A. parasiticus*, only marginal amounts of aflatoxin G_1 were produced. *Aspergillus parasiticus* was not completely prevented from producing aflatoxin G_1, since G_1 was produced in corn from each of the conidial proportions containing conidia of *A. parasiticus*.

Aflatoxin contamination was detected in about half of the test ears sprayed with water as a control, but particularly high amounts of aflatoxin B_1 and G_1 were observed in the first inoculation treatment in which the husks were pulled back and the kernels razor damaged (Table 1). Relatively high numbers of *A. parasiticus* and *A. flavus* conidia dispersed in the air probably furnished the inoculum and the plastic-bag moist chambers furnished a suitable environment for germination and infection. Isolations were not made to determine the species present in these ears except to visually note that mostly *A. flavus* was usually present on the basis of the color and the compactness of the conidial heads. The *A. flavus* strain was light green and formed diffuse heads; the *A. parasiticus* strain was dark green and formed compact heads on kernels.

Ratios were calculated from the aflatoxin G_1 and B_1 produced from corn inoculated by using the various conidial inocula proportions (Table 2). The G_1/B_1 ratios for a specific conidial-proportion inoculum remained relatively constant regardless of the inoculation treatment. But for each inoculation treatment the ratio of G_1 to B_1 decreased significantly as the proportion of *A. flavus* conidia in the inocula was increased. The large decrease in the aflatoxin G_1/B_1 ratios associated with the 100% *A. parasiticus* inoculum and the 75/25 conidial-proportion inoculum for each of the six inoculation treatments was exceptional and significant. The G_1/B_1 ratios produced in corn from the 50/50 and 25/75 conidial-proportion inocula were not significantly different from each other. If we can assume that the inoculum containing 75% *A. parasiticus* and 25% *A. flavus* conidia should produce aflatoxin G_1 in approximately the same proportions as in the nonmixed inocula, then the G_1/B_1 ratios should be only slightly lower than those calculated for the 100% *A. parasiticus* inoculum. But the observed ratios were far lower than G_1/B_1 ratios calculated for the 100/0 conidial-inoculum proportion, falling to 0.21 to 0.52, or falling an average of 38% for all inoculation treatments (Table 2). The aflatoxin G_1/B_1 ratios showed that G_1 is selectively decreased, but clearly was not completely prevented from occurring.

Production of aflatoxins B_1 and G_1 was significantly greater in the thin- than in the thick-pericarp hybrid in all of the inocula combinations (Table 3). These results averaged from all types of the inoculation treatments indicate that structural differences of the seed influence fungal development and aflatoxin production. Since the inoculated kernels were injured through the pericarp, integumentary thickness may affect spread of the fungus from the inoculated kernels to adjoining, nondamaged seed. However, nonmorphological characteristics also could be responsible for reduced aflatoxin yields in the thick-pericarp hybrid.

A summary of aflatoxin production from the damage-inoculation treatments and of all inocula combinations is shown in Table 4. Higher levels of aflatoxin B_1 were produced from damaged ears in which the husks were pulled back than when the husks remained intact before inoculation with these species. No significant differences in aflatoxin G_1 or B_1 were observed between inoculation treatments in which ears were either razor-damaged or pinboard-damaged. These inoculation treatments also produced the highest amounts of aflatoxin G_1 and B_1. The inoculation treatment in which the inoculum was inserted under the husks with a hypodermic needle produced significantly less aflatoxin G_1 and B_1 than the other inoculation treatments except when the husks were pulled back and the kernels not damaged (Table 4). The aflatoxin G_1/B_1 ratios, with two exceptions (0.36 and 0.43), were not significantly different between the inoculation treatments and were lower than that obtained

TABLE 2. The effect of types of preharvest injury and inoculum proportion (*Aspergillus parasiticus*/*A. flavus*) on ratios of G_1 to B_1 aflatoxin production in developing corn

Inoculation treatment	Aflatoxin G_1/B_1 ratio (arithmetic means)[a]					
	Aspergillus parasiticus/*A. flavus* conidial proportions used as inoculum[b]					Water
	0/100	25/75	50/50	75/25	100/0	0/0
Husk pulled back, razor damaged, inoculum sprayed	.01	.04	.05	.21	.64	0.26
Husk pulled back, pinboard damaged, inoculum sprayed	.01	.03	.11	.21	.90	0.01
Husk intact, razor damaged, inoculum sprayed	.01	.24	.35	.52	1.05	0.16
Husk intact, pinboard damaged, inoculum sprayed	.16	.13	.27	.40	.86	0.08
Husk intact, inoculum inserted with hypodermic syringe	.02	.04	.14	.22	.67	0.18
Husk pulled back, not damaged, inoculum sprayed	.03	.13	.13	.32	.74	0.18

[a]Least significant difference (LSD) = 0.18 at P 0.05 between any two arithmetic means, each averaged over two replications and both hybrids.

[b]Number of conidia in the percentage proportions: 0/100 (2×10^7 *A. flavus*), 25/75 (1×10^7 *A. parasiticus* and 3×10^7 *A. flavus*), 50/50 (2×10^7 for each fungus), 75/25 (3×10^7 *A. parasiticus* and 1×10^7 *A. flavus*), and 100/0 (2×10^7 *A. parasiticus*).

TABLE 3. Aflatoxin production in corn kernels of hybrids with thin and thick pericarps inoculated with different proportions of *Aspergillus parasiticus* and *A. flavus* conidia in the inocula

Hybrid[c]	Aflatoxin type	Aflatoxin level (geometric means in ng/g)[a]					Pericarp mean[b]
		A. parasiticus/*A. flavus* conidial proportions used as inoculum[d]					
		0/100	25/75	50/50	75/25	100/0	
Thick pericarp	B_1	707	552	1,285	934	910	843
	G_1	4	23	138	218	681	70
Thin pericarp	B_1	1,016	1,689[e]	1,293	1,029	2,250[e]	1,387[e]
	G_1	6	135[e]	227	327	1,796	162[e]

[a]Least significant ratio (LSR) for aflatoxin B_1 values = 1.98; LSR for aflatoxin G_1 values = 3.57. Values based on all values from two replicates and five damage treatments.

[b]LSR for aflatoxin in B_1 values = 1.36; LSR for aflatoxin G_1 values = 1.77.

[c]Thick pericarp hybrid is B37 × B14A; thin pericarp hybrid is H49 × CI44.

[d]Number of conidia in the percentage proportions: 0/100 (2×10^7 *A. flavus*), 25/75 (1×10^7 *A. parasiticus* and 3×10^7 *A. flavus*), 50/50 (2×10^7 for each fungus), 75/25 (3×10^7 *A. parasiticus* and 1×10^7 *A. flavus*), and 100/0 (2×10^7 *A. parasiticus*); the inoculation treatments were combined: husk pulled back, razor damaged, inoculum sprayed; husk pulled back, pinboard damaged, inoculum sprayed; husk intact, razor damaged, inoculum sprayed; husk intact, pinboard damaged, inoculum sprayed; and husk intact, inoculum inserted with hypodermic syringe.

[e]Significantly different ($P = 0.05$) from the corresponding thick pericarp value.

TABLE 4. Summary of aflatoxin production in damaged corn kernels inoculated with inocula containing different proportions of *Aspergillus parasiticus* and *A. flavus* conidia

Inoculation treatment	Aflatoxin type	Aflatoxin level (geometric mean in ng/g)[a]	Aflatoxin ratio (arithmetic mean of G_1/B_1)[a]
Husk pulled back, razor damaged, inoculum sprayed	B_1	4,473	0.19
	G_1	231	
Husk pulled back, pinboard damaged, inoculum sprayed	B_1	5,343	0.25
	G_1	271	
Husk intact, razor damaged, inoculum sprayed	B_1	2,138	0.43
	G_1	333	
Husk intact, pinboard damaged, inoculum sprayed	B_1	647	0.36
	G_1	130	
Husk intact, inoculum inserted with hypodermic syringe	B_1	315	0.22
	G_1	39	
Husk pulled back, not damaged, inoculum sprayed	B_1	154	0.27
	G_1	23	
	B_1	1.70 (LSR)[b]	0.082 (LSD)[b]
	G_1	2.79 (LSR)	

[a]Significant $P = 0.05$–least significant ratio (LSR), and least significant difference (LSD) for geometric and arithmetic means, respectively.

[b]Geometric and arithmetic means based on two replications, two hybrids and five combinations of inoculum of *A. parasiticus* and *A. flavus* conidia.

using only *A. parasiticus* conidia as inoculum. This suggests the use of separate species as inoculum for the determination of relative susceptibility of different hybrids and as indispensable for measuring the production of aflatoxins by different strains of the fungi.

The results of this study suggest that in mixtures of *A. parasiticus* and *A. flavus* conidia as inocula for corn, *A. flavus* has a decided advantage. This is reflected in the low levels of aflatoxin G_1 observed in conidial mixtures of *A. parasiticus* and *A. flavus* used as inocula and the low aflatoxin G_1/B_1 ratios obtained. We suggest that *A. parasiticus* is less aggressive than *A. flavus* as a species and so its production of aflatoxin G_1 is limited by *A. flavus*. We contend that the latter species is a better competitor for the available corn substrate than *A. parasiticus* and this is why G_1 is limited. This may explain why *A. flavus* is routinely found present in naturally aflatoxin-contaminated corn and *A. parasiticus* only rarely (5, 6, 8). We point out, however, even small amounts of aflatoxin G_1 suggest that *A. parasiticus* was present in the inoculum. Also, we cannot rule out the possibility that *A. parasiticus* in conidial mixtures with *A. flavus* cannot produce as much aflatoxin G_1 as we assume can be produced with the 100% *A. parasiticus* inoculum.

We have shown that the widely-used hypodermic-syringe method of inserting inoculum into ears to determine relative susceptibility of hybrids results in significantly lower B_1 and G_1 levels than other procedures demonstrated in this experiment. As to ways to lower the incidence of aflatoxin-contaminated corn, certainly hybrids having tight, intact husks, resistant to insects that injure kernels, and those lines with thick pericarps should be sought out. Hybrid combinations that can keep aflatoxin G_1 and B_1 at a minimum under conditions that are optimum for aflatoxin production should be the first consideration in seeking aflatoxin-free corn strains.

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