Fungal Interference with *Aspergillus flavus* Infection and Aflatoxin Contamination of Maize Grown in a Controlled Environment

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ABSTRACT

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The ability of 'competing fungi' in preharvest maize to interfere with *Aspergillus flavus* infection and aflatoxin contamination of developing kernels was examined. Experiments were conducted in a plant growth room to eliminate insect damage of the ear, contaminating fungi, and unpredictable climatic events. Silk inoculation of uninjured ears resulted in fewer than 2% *A. flavus*-infected kernels and no detectable aflatoxin. Sterile toothpick-wounding of several kernels on ears that were similarly silk-inoculated promoted substantial *A. flavus* infestation and aflatoxin contamination of the wounded kernels. Many of the uninjured kernels also became infected with *A. flavus*. Kernel wounding by toothpick also promoted ear infection by *Fusarium moniliforme, Acremonium strictum*, and *Aspergillus niger*. When kernels were directly wound-inoculated with *A. flavus* and competing fungi, high levels of aflatoxins were detected in both the wounded kernel tissues and the first surrounding circle of uninjured kernels. However, the competing fungi, particularly *F. moniliforme*, inhibited *A. flavus* from further infecting and contaminating the other uninjured kernels on the same ear with aflatoxin.

It has been known for decades that *Aspergillus flavus* Link:Fr. can invade maturing maize (*Zea mays* L.) ears in the field (33). The more recent discovery that *A. flavus* also contaminates preharvest maize with aflatoxin (1) has served as a stimulus for ecological studies aimed at understanding the environmental conditions that promote *A. flavus* infection and aflatoxin formation in maize (8). The present research was organized to examine the ability of common fungal colonists of maize to interfere with *A. flavus* infection and aflatoxin contamination of maturing kernels. Evidence that fungal colonists of maize kernels may interact negatively with *A. flavus* is sparse and largely circumstantial. Rambo et al (29) attempted to explain different levels of *A. flavus* in maize samples from Indiana by suggesting that "a large amount" of *Fusarium moniliforme* Sheldon in some of the samples may have acted as a barrier to *A. flavus* invasion. Shortly thereafter, Lillehoj et al (20) noted that on one maize ear with a "high occurrence" of *F. moniliforme, A. flavus* was restricted to the ear tip. Hill et al (10) found a negative correlation between the percentage of kernels infected by *A. flavus* and the percentage infected by *F. moniliforme* in naturally infested commercial maize hybrids. However, a second study of naturally infected maize kernels failed to show a consistent correlation between the occurrence of *F. moniliforme* in kernels from test hybrids and the level of aflatoxin detected in those kernels (5). Significantly lower levels of aflatoxin have been detected in maize ears inoculated with *A. flavus* and two potential competitors, *F. moniliforme* and *Penicillium oxalicum* Currie & Thom (22).

The present research recognizes that individual fungal colonists of maize ears can have independent origins and infect kernels at different stages in ear maturation (9,12,37). The ability of 'competing fungi' to interfere with *A. flavus* (37) and the sequence in which these fungi colonize the ear should affect both the extent of kernel infection by *A. flavus* and the amount of aflatoxin contamination. To control confounding experimental variables associated with field plot trials (e.g., temperature, relative humidity, availability of soil water and nutrients, insect damage, and fungal contaminants), commercial dent hybrids were grown to maturity in a specially modified plant growth room.

MATERIALS AND METHODS

Commercial hybrids: growth environment. A loose-husked dent hybrid (DeKalb XL-12) used commercially in the upper midwest corn belt was grown to maturity in a controlled environment room (photoperiod 14 hr; temperature 30 ± 1 C day/20 ± 1 C night; humidity 82 ± 3%) in the Biotron, University of Wisconsin,

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Madison (3). As determined by the formula given by Thompson et al (34), this temperature regime is equal to 15.8 C thermal units/day and adequately meets the temperature requirements for substantial aflatoxin production in preharvest corn. The maize silked at 42–44 days and was harvested 105 days after planting.

Fungal inoculum: culture conditions. *A. flavus* inoculum consisted of a mixture of 10 strains, each isolated from corn and known to produce aflatoxins: NRRL 6536, NRRL 6537, NRRL 6539, NRRL 6540, NRRL 6576, NRRL 6577, NRRL 6578, NRRL 6579, NRRL 6580, and NRRL 6581. Conidia from each strain were harvested separately from 14-day-old Czapek's agar slants that were incubated at 25 C. Five milliliters of 0.01% Triton X-100 was added to each slant. Cultures were then agitated, and the spore suspensions were filtered through glass wool and adjusted to 10^6 spores per milliliter with distilled water. Equal portions of spore suspension from each of the 10 isolates were combined to provide the final inoculum. The suspension was stored at 4 C between applications.

Inoculum of maize isolates used as potentially competing fungi was prepared as above except that only three strains of each species were used and, in some cases, different culture media were selected to promote sporulation. The competing fungi and the medium they were grown on were: *Acremonium strictum* W. Gams NRRL 6415, NRRL 13540, NRRL 13541, malt extract agar; *Aspergillus niger* V. Tiegh. NRRL 6408, NRRL 6411, NRRL 13542, Czapek's agar; *F. moniliforme* NRRL 6413, NRRL 13543, NRRL 13544, potato-dextrose agar (PDA); *P. funiculosum* Thom NRRL 6420, NRRL 13547, NRRL 13548, PDA; *P. oxalicum* NRRL 6416, NRRL 13545, NRRL 13546, Czapek's agar. In experiments with *A. flavus* and one competing fungus (e.g., *Acremonium strictum* or *F. moniliforme*), equal volumes of the conidial suspension for each organism were used. In experiments with *A. flavus* and five competing fungi, equal volumes of the five potential competitors were combined to provide a final mixed inoculum (1×10^6 spores per milliliter), which was then combined with an equal volume of *A. flavus* conidial inoculum (1×10^6 spores per milliliter).

Inoculation procedure. Eleven inoculation procedures were used in this study. They are numbered below as they appear in Tables 1–4. To determine the importance of ear wounding in promoting kernel infection by *A. flavus* and five potential fungal competitors, the following four treatments were applied: 1) Silks of five ears were inoculated at 7, 14, and 21 days after silking. Each ear was inoculated with approximately 2 ml of a spore suspension of *A. flavus* containing 1×10^6 spores per milliliter, applied with a chromatography sprayer. 2) Eleven ears were silk-inoculated as above, but on each of the three inoculation dates five sterile wooden toothpicks were inserted through the husk 4 cm apart and in a vertical row. The three vertical rows were spaced an equal distance from one another. 3) Silks of each of five ears were inoculated at 7, 14, and 21 days following silking with approximately 2 ml of a spore suspension containing 1×10^6 spores per milliliter of *A. flavus* and five potential fungal competitors. 4) Eleven ears were silk-inoculated as in treatment 3, but on each of the three inoculation dates each ear was wounded by inserting sterile toothpicks through the husk (see treatment 2).

Immediately after harvest, husks were removed from naturally dried-down ears, and the kernels were segregated as follows: wounded kernels; first circle = four nonwounded kernels immediately adjacent to a wounded kernel; second circle = nonwounded kernels immediately surrounding the first circle kernels; and all other kernels = remaining kernels on the ear. The pooled kernel samples in each segregation category, from individual ears, constituted replicates that were later subsampled to provide data on percent fungal infection and aflatoxin concentration. Samples of dried kernels were stored in capped jars at room temperature (22–24 C) before being examined for molds (<2 days) or aflatoxins (<60 days).

To examine the ability of *A. flavus* and five potential fungal competitors to infect kernels of different maturity, two treatments were applied: 5) At 7, 14, or 21 days after silking, four ears each were wound-inoculated with *A. flavus* by inserting the tip of a sterile wooden toothpick through the husk and into the underlying kernel(s). The toothpick was removed, dipped in the *A. flavus* conidial suspension, and reinserted into the wound. Five toothpicks were thus inserted, 4 cm apart, in two vertical rows on opposite sides of the five ears representing a particular maturation state. The toothpicks remained in place for the entire experiment. 6) The same procedure was repeated using a mixed conidial

TABLE 1. Kernel infection and aflatoxin contamination of maize ears silk-inoculated with *Aspergillus flavus* and five common fungal colonists of preharvest maize

	Kernel infection (%)[v]						Aflatoxin analyses[w]		
Treatment & kernel samples[x,y]	*Penicillium oxalicum*	*Acremonium strictum*	*P. funiculosum*	*Fusarium moniliforme*	*A. niger*	*A. flavus*	Mean sample wt (g)	Aflatoxin (ppb $B_1 + B_2$) Range	Mean
1 *Aspergillus flavus* silk-inoculations	...	...	...	...	...	<1	163	ND[z]	ND
2 *Aspergillus flavus* silk-inoculation & sterile wounding									
Wounded kernels	...	...	...	...	...	...	3.84	27,500–60,700	39,400 a
First circle	...	...	...	...	31	73	33	176–1,070	655 b
Second circle	...	...	...	...	20	67	70	48–784	295 b
All other kernels	...	...	...	...	22	68	65	7–728	95 b
3 *Aspergillis flavus* & fungal competitors silk inoculation	<1	2	9	2	1	2	161	ND	ND
4 *Aspergillus flavus* & fungal competitors silk-inoculation & sterile wounding									
Wounded kernels	...	...	...	...	...	...	5.19	18,400–56,900	33,200 a
First circle	2	0	17	18	32	48	71	57–3,580	1,965 b
Second circle	0	2	19	22	16	31	136	ND	ND c
All other kernels	<1	2	10	24	19	26	85	ND	ND c

[v] Kernel infection (%) based on 250–550 surface-sterilized kernels (50 kernels × *n* ears).
[w] Within treatments, aflatoxin values assigned the same letter (a or b) do not differ significantly ($P > 0.05$), whereas values showing different letters are significantly different ($P > 0.05$).
[x] Kernels representing each category were pooled for individual ears, irrespective of wounding date.
[y] Treatments 1 and 3 = five replicate ears; treatments 2 and 4 = 11 replicate ears.
[z] ND = No aflatoxin detected.

inoculum consisting of *A. flavus* and five potential fungal competitors. Here each maturation state was represented by eight ears.

In treatments 5 and 6 the kernels were segregated as above and like categories from all ears were pooled. Each pool of kernels was then subsampled to provide mycological data and to determine aflatoxin levels.

To learn whether initial colonization of ears by *F. moniliforme* or *Acremonium strictum* interferes with the establishment of *A. flavus*, treatments 7 through 11 were applied: 7) Five ears were wounded with sterile toothpicks at 7 days after silk. The toothpicks were inserted, 4 cm apart, in two vertical rows on opposite sides of the ear. Then, 14 days later (21 days post-silk), the same ears were wound-inoculated with *A. flavus* in two vertical rows of equal distance between the rows of sterile toothpicks. 8) By means of the approach outlined in treatment 7, five ears were wound-inoculated with *F. moniliforme* 7 days after silk and then wound-inoculated with *A. flavus* 14 days later. 9) *Acremonium strictum* was substituted for *F. moniliforme* and the procedure outlined in treatment 8 was repeated. 10) *F. moniliforme* and *A. flavus* were simultaneously inoculated into the same toothpick wounds (two vertical rows) made 21 days following silking. 11) The procedure used in treatment 10 was repeated substituting *Acremonium strictum* for *F. moniliforme*. Preliminary studies revealed that when *F. moniliforme* was inoculated by wounding ears 7 days post-silk, nearly every kernel (>90%) was infected at harvest. The rationale for wound-inoculating *A. flavus* at 21 days post-silk was based on numerous published reports that ears in the late milk to early dough stage of maturation are most susceptible to *A. flavus* infestation (1,12,15).

TABLE 2. Distribution of kernel-infecting fungi within uninjured maize kernels[a]

Fungal taxa	Dissected kernels[b] (no.)	Tip cap (rachilla)	Pedicel & hilum scar	Proximal one-half & germ	Distal one-half & silk scar
Fusarium moniliforme	29	28(97)	27(93)	19(66)	2(7)
Acremonium strictum	21	9(43)	10(48)	5(24)	1(4)
Penicillium funiculosum	33	32(97)	27(82)	15(45)	4(12)
Aspergillus flavus	45	33(73)	43(96)	36(80)	7(16)
A. niger	46	33(72)	40(87)	26(56)	6(13)

[a] Number of dissected kernel sections infected with percent occurrence (in parentheses).
[b] Number of dissected kernels infected with the fungus.

TABLE 3. Kernel infection and aflatoxin contamination of maize ears following wound inoculation with *Aspergillus flavus* and five common fungal colonists of preharvest maize

Treatment & kernel samples[c,d]	Kernel infection (%)[a]						Aflatoxin analyses[b]	
	Penicillium oxalicum	*Acremonium strictum*	*P. funiculosum*	*Fusarium moniliforme*	*A. niger*	*A. flavus*	Sample wt (g)	Aflatoxin (ppb B_1 & B_2)
No. 5 *Aspergillus flavus*								
A. Inoculation 7 days post-silk								
Wounded kernels	...	...	...	...	...	...	0.31	23,500
First circle	...	...	...	...	...	96	29.14	842
Second circle	...	...	...	...	...	95	64.40	1,920
All other kernels	...	...	...	...	...	89	280.0	632
B. Inoculation 14 days post-silk								
Wounded kernels	...	...	...	...	...	...	0.33	1,890
First circle	...	...	...	...	...	96	34.28	1,700
Second circle	...	...	...	...	...	88	59.91	20
All other kernels	...	...	...	...	...	79	223.0	1,050
C. Inoculation 21 days post-silk								
Wounded kernels	...	...	...	...	...	...	1.01	15,600
First circle	...	...	...	...	...	98	42.34	1,010
Second circle	...	...	...	...	...	82	72.12	1,810
All other kernels	...	...	...	...	...	75	210.0	929
6 *Aspergillus flavus* & fungal competitors								
A. Inoculation 7 days post-silk								
Wounded kernels	...	...	...	...	...	...	0.81	ND
First circle	0	0	41	87	3	<1	71.42	670
Second circle	1	1	22	47	4	<1	127.71	1
All other kernels	0	0	11	18	2	<1	431.0	ND
B. Inoculation 14 days post-silk								
Wounded kernels	...	...	...	...	...	...	1.50	11,600
First circle	0	0	27	92	74	27	78.17	1,420
Second circle	0	0	26	34	47	2	135.22	ND
All other kernels	0	0	10	26	29	<1	387.0	ND
C. Inoculation 21 days post silk								
Wounded kernels	...	...	...	...	...	...	2.68	14,500
First circle	0	0	32	95	88	37	76.28	1,760
Second circle	0	0	24	56	68	9	144.14	1
All other kernels	0	0	14	32	52	1	425.0	ND

[a] Kernel infection (%) based on 200–400 surface-sterilized kernels (50 kernels × *n* ears).
[b] A single aflatoxin value was determined for each treatment; ND = no aflatoxin detected.
[c] Treatment No. 5-A, B, or C each with 4 ears; treatment No. 6-A, B, or C each with 8 ears.
[d] Kernels representing each category were pooled for each treatment.

In treatments 7–11 the kernels were segregated into categories described above for individual ears.

Fungal occurrence in kernels. Kernels were surface-sterilized in a 2% sodium hypochlorite solution for 1 min, washed twice in sterile water, plated (five per plate) on petri dishes of malt extract agar, and incubated for 6 days at 25 C. The occurrence of *A. flavus* or other fungi growing from the kernels was recorded. Only the uninjured kernels from wound-inoculated ears were plated, since *A. flavus* consistently showed visible sporulation on kernels wound-inoculated with this fungus.

To obtain information on the distribution of *A. flavus* and competing fungi within maize kernels, uninjured kernels from silk-inoculated and sterile toothpick-wounded ears (treatments 2 and 4) were dissected. Thirty uninjured kernels from samples showing 10% or more kernel infection by a particular fungus were soaked in distilled water for 12 hr at 5 C to soften the kernels for dissection. Kernels were surface-sterilized as previously described, then individually dissected with a sterile scalpel on a separate square (5 × 5 cm) of sterile cardboard. Kernels were sectioned transversely at three points to provide four kernel fragments or zones, including: the tip cap (rachilla), a 2-mm-thick fragment (pedicel) that included the hilum scar, the remaining proximal one-half of the kernel that included the germ, and the distal one-half of the kernel that included the crown and point of silk attachment. Kernel fragments were plated on malt extract agar and incubated 7 days at 25 C, and the fungal colonies developing from each fragment were identified.

Aflatoxin analyses. All samples were analyzed by the CB Methods approved by the Association of Official Analytical Chemists (2). Samples of kernels weighing 15–75 g were analyzed by the procedure as written; samples weighing more than 75 g were separated into appropriately sized portions, and results were averaged. Samples weighing 5–15 g were also assayed by the CB Method with one exception: The entire extract was collected and filtered, and the filter paper was washed. The combined extract and washes were concentrated in vacuo for the silica gel chromatography. Samples weighing less than 1 g were steeped overnight with 25 ml of $CHCl_3$, 2.5 ml of water, and 2.5 g of Celite. The mixture was then transferred to a blender with chloroform, and vials were washed with 7.5 ml of chloroform. The material was blended for 3 min and filtered, and the filter paper was washed three times with 100-ml portions of chloroform. Combined washes and extract were concentrated for chromatography on a Silica Gel 60 (0.063–0.2 mm) column (0.6 cm i.d. × 20 cm). Quantities of aflatoxins were measured by thin-layer chromatography (2).

Statistical analyses. Mean aflatoxin values for kernel locations were compared within each treatment by analysis of variance (ANOVA) after transformation of aflatoxin values to log (aflatoxin + 1). Transformations were done to equalize variance structures among location classes. Individual comparisons between means were done by least significant differences (LSD) where a significant ($P<0.05$) F-test was found in the ANOVA.

RESULTS

Neither *A. flavus* nor any of the five other common fungal isolates from maize infected more than 10% of the uninjured kernels when conidial inoculum was applied to silks of nonwounded ears (Table 1; treatments 1 and 3). *P. funiculosum* infected 9% of the kernels sampled, while *A. flavus* infected less than 2% of the kernels sampled; in the latter experiment no aflatoxins were detected. When silk inoculation with *A. flavus* alone was accompanied by kernel wounding through the husk with sterile toothpicks (treatment 2), *A. flavus* infected 68–73% of the three categories of uninjured kernels and contaminated the ears with aflatoxin (Table 1). Some of the kernels from these ears also became infected with *A. niger* (20–31% infection among three kernel categories), one of five potential competing fungi applied to the silks of neighboring ears used in parallel treatments (treatments 3 and 4). When silk-inoculation with the potential competing fungi

TABLE 4. *Aspergillus flavus* infection and aflatoxin contamination of maize ears inoculated with *Acremonium strictum* or *Fusarium moniliforme*

	Kernel infection (%)[v]			Aflatoxin analyses[w,x]		
Treatment & kernel samples[y]	*F. moniliforme*	*A. strictum*	*A. flavus*	Mean sample wt (g)	Aflatoxin (ppb $B_1 + B_2$)	
					Range	Mean
7 Sterile toothpick 7 days; *A. flavus* 21 days						
Wounded kernels	...	...	...	0.41	6,300–35,300	14,100 a
First circle	...	...	75	11.16	ND–1,030[z]	470 b
Second circle	...	...	48	17.08	ND–1,300	280 c
All other kernels	...	...	53	46.41	8–1,420	430 b,c
8 *F. moniliforme* 7 days; *A. flavus* 21 days						
Wounded kernels	...	...	...	0.34	435–43,700	14,000 a
First circle	98	...	6	9.97	ND–548	180 b
Second circle	98	...	2	15.10	ND	ND c
All other kernels	100	...	0	37.50	ND	ND c
9 *A. strictum* 7 days; *A. flavus* 21 days						
Wounded kernels	...	...	...	0.45	1,550–30,500	9,400 a
First circle	...	35	75	11.07	ND–586	260 b
Second circle	...	52	20	16.37	ND	ND c
All other kernels	...	64	7	38.79	ND–28	5 c
10 *F. moniliforme* & *A. flavus* 21 days						
Wounded kernels	...	...	...	0.47	ND–2,920	1,200 a
First circle	89	...	56	7.61	ND–3,340	760 a,b
Second circle	96	...	13	11.16	ND	ND b
All other kernels	81	...	14	38.2	ND	ND b
11 *A. strictum* & *A. flavus* 21 days						
Wounded kernels	...	...	...	0.59	290–3,560	1,850 a
First circle	...	40	94	10.4	2–4,410	1,700 a
Second circle	...	34	74	16.2	ND–25,400	6,000 a
All other kernels	...	35	69	48.9	ND–5,230	1,100 a

[v] Kernel infection (%) based on 125–250 surface-sterilized kernels (25 or 50 kernels × five ears).
[w] Range and mean values representing the total aflatoxin in 5 replicates (ears) per treatment.
[x] Within treatments, aflatoxin values assigned the same letter (a, b, or c) do not differ significantly ($P>0.05$), whereas values showing different letters are significantly different ($P>0.05$).
[y] Kernels representing each category were pooled for individual ears.
[z] ND = No aflatoxin detected.

and *A. flavus* was accompanied by sterile toothpick-wounding (treatment 4), the three categories of uninjured kernels became infected as follows: *A. flavus*, 26–48% infection; *A. niger*, 16–32%; *F. moniliforme* 18–24%; *P. funiculosum* 10–19%. *P. oxalicum* and *Acremonium strictum* infected <2% of the uninjured kernels. Aflatoxin levels in samples of the wounded kernels from this treatment (range = 18,400–56,900 ppb) or first circle (range = 57–3,580 ppb) were similar to those recorded for wounded ears that were silk-inoculated only with *A. flavus* (treatment 2). However, in treatment 4 aflatoxins were not detected in kernels from the second circle and all other kernel categories. Wounded kernels were contaminated with significantly more aflatoxin than adjacent uninjured kernels ($P<0.05$). In treatments 2 and 4 an invasion route through the husk perforations cannot be excluded.

A summary of fungal distribution patterns within selected samples of uninjured maize kernels is presented in Table 2. The internal distribution of *A. flavus* and four other kernel-infecting molds was restricted primarily to the proximal one-half of the kernel (i.e., tip cap, hilum scar, and germ).

Fungal interference limited *A. flavus* infection of uninjured kernels when *A. flavus* and five potentially competing fungi were inoculated directly through toothpick wounds (Table 3). *A. flavus* infected 75% or more of the uninjured kernels from different categories when the fungus represented the sole source of inoculum in treatment 5. However, when spores of five potential fungal competitors composed one-half of the fungal inoculum in treatment 6, *A. flavus* was recorded from <1 to 37% of the uninjured kernel samples. The maturity of maize ears at the time of wound inoculation was important to the establishment of *A. flavus* and *A. niger*. Neither fungus infected more than 4% of the uninjured kernels when inoculated 7 days post-silk (treatment 6A), but their presence in kernels increased sharply when wound-inoculations were performed at either 14 or 21 days post-silk (treatments 6B and C). Aflatoxin levels in wound-inoculated kernels were substantial in all but one sample (Table 3). No aflatoxin was detected in wounded kernels inoculated with *A. flavus* and potential fungal competitors at 7 days post-silk (treatment 6A).

Interference with *A. flavus* infection of dent kernels by *F. moniliforme* and *Acremonium strictum* is indicated by the data in Table 4. When ears were wound-inoculated with *A. flavus* in the absence of other fungi (treatment 7), *A. flavus* infected 75% of the first circle of uninjured kernels and approximately 50% of the remaining kernels from the same ear. Ears inoculated with *F. moniliforme* at 7 days following silking were not as extensively colonized by *A. flavus* when the latter was introduced through separate wounds made 14 days later (treatment 8). In this treatment, only 6% of the uninjured kernels from the first circle and less than 2% of the remaining kernels on the ear became infected with *A. flavus*. At the same time, nearly all of the kernels from these ears (>98%) were infected with *F. moniliforme*. When *A. flavus* and *F. moniliforme* were simultaneously inoculated into the same wounds (21 days), *F. moniliforme* was less effective in limiting kernel infection by *A. flavus*. In treatment 10, 56% of the uninjured kernels from the first circle, 13% of the second circle kernels, and 14% of all other kernels became infected with *A. flavus*. From these same points of inoculation, *F. moniliforme* spread to and infected 80% or more of the uninjured kernels and apparently interfered with *A. flavus* infection.

The range and mean values for total aflatoxin concentrations in both wound-inoculated and uninjured kernels are also presented in Table 4. Ears inoculated only with *A. flavus* showed substantial aflatoxin contamination in all samples of the wound-inoculated kernels (range = 6,300–35,300 ppb), and aflatoxins were detected in 11 of 15 samples of the uninjured kernels. Detection of aflatoxin in samples of all other kernels (range 8–1,420 ppb) can be explained by the fact that these ears were wounded with sterile toothpicks (7 days post-silk) and *A. flavus* had infested these wounds.

Prior inoculation of ears with *F. moniliforme* (7 days post-silk) did not prevent aflatoxin contamination in kernels that were later wound-inoculated with *A. flavus* (Table 4). Levels of aflatoxin contamination in these *A. flavus*-infested kernels (range 435–43,700) were similar to aflatoxin levels recorded for *A. flavus*-inoculated controls (Table 4). Aflatoxins were also detected in uninjured kernels from the first circle (range = ND–548 ppb), but none were detected in any other kernel samples from these ears.

Kernels simultaneously wound-inoculated with *A. flavus* and *F. moniliforme* (treatment 10) showed mean aflatoxin values 12 times less (mean = 1,200 ppb) than values recorded for kernels wound-inoculated with *A. flavus* alone (treatment 7) and for treatment 8, where *A. flavus* followed inoculation with *F. moniliforme* (Table 4). Aflatoxins were also detected in adjacent uninjured first circle kernels from three of the five ears receiving this treatment (range = ND–3,340 ppb) but not in the remaining 12 samples of uninjured kernels (Table 4).

Acremonium strictum infected 64% or less of the uninjured kernels when wound-inoculated into ears at 7 days following silking (Table 4; treatment 9). Inoculation of ears with *Acremonium strictum* limited *A. flavus* infection of uninjured kernels within the second circle (20% infection) and all other kernels located further from the wounds (7%). This same group of 10 samples produced only one aflatoxin-positive sample (28 ppb). In simultaneous wound-inoculations with *Acremonium strictum* and *A. flavus* (treatment 11), *Acremonium strictum* infected only 34–40% of the uninjured kernels among three categories, whereas *A. flavus* infected 69–94%. There was an eightfold reduction in the mean aflatoxin level of the wounded kernels when *Acremonium strictum* comprised 50% of the conidial inoculum as contrasted with the *A. flavus*-inoculated control (Table 4). At the same time, *Acremonium strictum* did not prevent *A. flavus* infection of uninjured kernels; aflatoxins were detected in 13 of the 15 samples.

Contrasts among mean aflatoxin values for individual treatments were calculated using LSD within the ANOVA. Treatments 7 and 11 composed one group, and treatments 8–10 formed a second group. Mean aflatoxin values of treatments within each group were not different ($P>0.05$), but between-group differences were significant ($P<0.05$). The results indicate that *F. moniliforme* interfered with aflatoxin formation by *A. flavus* when inoculated into wounds with *A. flavus* (treatment 10) and through wound inoculation 14 days before wound-inoculation with *A. flavus* (treatment 8). With *A. strictum*, significant interference with aflatoxin contamination was found only when *A. strictum* was inoculated 14 days in advance of *A. flavus* (treatment 9).

DISCUSSION

Results indicate that kernel infection by *A. flavus*, *A. niger*, and *F. moniliforme* is promoted by the wounding of kernels. Inoculation of silks with *A. flavus*, alone or in combination with other fungi isolated from maize ears, produced only a few *A. flavus*-infected kernels and no aflatoxin at the temperature regime tested (Table 1). More than 60 yr ago Taubenhaus (33) reported that ear infection with *A. flavus* occurred only when spores were introduced by puncturing the husk and wounding kernels in the milky stage, as is brought about by tunneling earworm larvae. In the absence of injury, *A. flavus* developed only superficially at the site where Taubenhaus deposited conidia. Others have since provided evidence that wound-inoculation is necessary for invasion of ears by *A. flavus* (15,30). Observations in North Carolina in 1976, 1977, and 1978 revealed a high incidence of *A. flavus* infection in ears and kernels free of obvious insect damage. This prompted studies on the ability of *A. flavus* to colonize silk tissue and then invade and produce aflatoxin in undamaged kernels (13). Because it is difficult to prevent insect damage to maize grown in field plots, experiments were conducted in a controlled environment chamber (Phytotron) (13,24,25). In those experiments, a conidial suspension of *A. flavus* NRRL 3357 (1×10^6 spores per milliliter) was applied to exposed silks (21 days post-silk), and the ears were enclosed in plastic bags to produce a high humidity for conidium germination and mycelial growth. Approximately 15% of the kernels became infected with *A. flavus*, and there was a positive correlation between the percent infected kernels and the concentration of aflatoxin. Despite these high levels of inoculum and ideal conditions for *A. flavus* spore

germination and growth, no infected kernels were found in five of 20 silk-inoculated ears. Payne (28) offers evidence that temperature greatly influences infection of silk-inoculated kernels. Sweet corn plants grown in the greenhouse and held at 21–26 C after silk-inoculation had 2.5% *A. flavus*-infected kernels, whereas silk-inoculated plants held at 32–38 C had 73% infection. Temperatures used in the present study (20–30 C) may have been too low to provide the optimal conditions for infection of uninjured kernels through a silk invasion route. Payne (28) and others have argued that elevated temperatures in the southern United States are critical to *A. flavus* infection and aflatoxin contamination of preharvest maize because these temperatures also encompass the optimal growing conditions for *A. flavus*. At the same time, however, Thompson et al (35) found that aflatoxin concentrations in wound-inoculated ears were not significantly affected by temperatures that ranged from 13.5 to 21.5 C thermal units per day. When *A. flavus* inoculum is carried to damaged maize ears grown in the upper midwest, aflatoxin contamination can be substantial (29). However, this rarely occurs because *A. flavus* inoculum is less common at higher latitudes (8). When a correlation is found between high temperatures and high *A. flavus* occurrence in preharvest maize, the biological explanation could rest in the effect of such climatic differences on *A. flavus* inoculum buildup in soil and crop debris.

Our results from kernel dissections suggest that infection is through the tip cap (rachilla) and that fungal invasion in these symptomless infections is limited primarily to the proximal one-half of the kernel that includes that rachilla-tip cap, pedicel-hilum scar, and germ (Table 2). Marsh and Payne (24,25) offered evidence that *A. flavus* can colonize the exposed yellow-brown silks and follow their senescence down into the ear. They proposed that *A. flavus* hyphae, after arriving at the silk attachment site on the kernel, grow onto the adjacent pericarp, then over the kernel surface to penetrate the tip-cap region of the kernel. This mode of infection has been reported for other kernel-infecting fungi (11,16,23). Marsh and Payne (25) also suggested that *A. flavus* spreads from kernel to kernel by mycelial growth on the pericarps or glumes, and if insects were to injure such kernels, *A. flavus* could readily infest the damaged tissues and contaminate them with aflatoxin. In the present study we have demonstrated that when *A. flavus* is inoculated onto the silks of ears that were wounded to simulate insect damage, *A. flavus* does colonize these wounds, produces aflatoxins, and infects a majority of the uninjured kernels from the same ear. The incidence of kernel infection typically decreases with increasing distance from the wounded kernels. Wounding also promoted ear colonization by *F. moniliforme*, *A. niger*, and *Acremonium strictum*, each of which demonstrated little or no ability to infect kernels when applied to the silks of nonwounded ears. *F. moniliforme* occurs throughout the world as a pathogen of maize. Establishment of *Fusarium* spp. on maize ears is favored by a break in the pericarp of maize kernels (7,36). Windels et al (38) suggested that the sap beetle *Glischrochilus guadrisignatus* (Say) serves as a vector of *Fusarium* spp., including *F. moniliforme*, from rotted ears overwintered in the field to developing maize ears. It is difficult to assess claims that maize ears infected with *A. flavus* and contaminated with aflatoxins can be free of insect damage (13). When examining ears for insect damage, investigators typically record tunneling by lepidopteran caterpillars. Less obvious breaks in the pericarp can serve as entry points for *A. flavus* or other kernel-rotting fungi. McMillian (26) pointed out that under field conditions insect damage is often prevalent in varying degrees, and the preponderance of published studies confirm that there is a higher incidence of *A. flavus* contamination and higher levels of aflatoxin in insect-damaged grain than in undamaged grain.

This study indicates that common fungal colonists of corn kernels interfere with the ability of *A. flavus* to infect preharvest maize. *F. moniliforme* was particularly effective in inhibiting kernel infection by *A. flavus*, while *Acremonium strictum* also limited *A. flavus* infection of uninjured kernels. *F. moniliforme* and *Acremonium strictum* commonly infect maize kernels in the southeastern United States where aflatoxin contamination of maize is a recurrent problem (14). Beginning 1 wk after silking and for up to 10 wk, King (14) assayed sound ears for internal kernel infection by *F. moniliforme* and *Cephalosporium acremonium* (= *Acremonium strictum*). *F. moniliforme* infected >60% and *Acremonium strictum* >40% of the kernels sampled on the 10th week. This ratio of kernels infected by *F. moniliforme* to *Acremonium strictum* is similar to what we recorded for wound-inoculated ears grown in the Biotron (Table 4). *Acremonium strictum* grows slowly on malt extract agar and simply may not have reached every kernel that was susceptible to infection. It would be interesting to know the importance of timing in kernel infection by *A. flavus* against this background of ear colonization by *Acremonium strictum* and *F. moniliforme* under field conditions. Lawrence et al (17) and Salama and Mishricky (31) offered evidence that *F. moniliforme* infects kernels through the placento-chalazal region or hilum. This is also believed to be the invasion route used by *A. flavus* (25). Kernels initially infected with *F. moniliforme* may be resistant to later infection by *A. flavus* if the *Fusarium* hyphae induce metabolic resistance (e.g., papillae, cell wall thickening, phytoalexins). The presence of competing fungi may explain why, in our data (Tables 1, 3, and 4) and in ears mapped by Lee et al (18), some kernels with high levels of aflatoxin were located next to kernels that were toxin-free.

A. flavus produced high levels of aflatoxin in wounded kernel tissues, even when competing fungi were introduced with *A. flavus* during toothpick wounding (Tables 1, 3, and 4). Even so, there was a 12-fold difference in the aflatoxin levels between kernels simultaneously wound-inoculated with *A. flavus* and *F. moniliforme*, and kernels inoculated only with *A. flavus* (Table 4). *F. moniliforme* is capable of rapidly colonizing these wounded kernel tissues, thus reducing the resource pool available to *A. flavus* or interfering in some other way with its ability to produce aflatoxin (36). Jones et al (13) made the important observation that when the pericarp of a kernel is broken, the contents are exposed to invasion by microorganisms and the moisture content drops rapidly to levels (<35% moisture) where *A. flavus* can compete successfully with other microorganisms. In mature maize kernels, *A. flavus* exhibits extensive growth and produces aflatoxin when kernel moisture contents are in equilibrium with 85% RH (= 18–18.5% kernel moisture; [4]) but not at lower moisture readings (32). Therefore, *A. flavus* would have a competitive advantage over most other kernel-rotting fungi in wounded kernel tissues but not in uninjured kernels with higher moisture readings. It is not surprising that attempts to relate data on aflatoxin contamination in wound-inoculated ears to percent kernel moisture of the developing kernels from those ears have been inconclusive (19,21). Data from this research suggest that the bulk of the aflatoxin is produced in wounded kernels that no longer mirror the moisture content of intact kernels from the same ear. It could not be determined whether all of the aflatoxin detected in uninjured kernels was produced by the *A. flavus* mycelium infecting those kernels or was translocated from the adjacent wounded and *A. flavus*-infested kernel. Mertz et al (27) demonstrated the uptake and translocation of aflatoxin by maize seedlings.

Our results help to explain why efforts to identify maize genotypes resistant to *A. flavus* kernel rot and aflatoxin contamination have been so unsuccessful (6). No maize genotype has a complete defense against ear-feeding insects and no insect-damaged kernel is resistant to *A. flavus* infestation. Our research has shown that it is in these damaged kernels where the bulk of the aflatoxin is produced. Fungal interference with *A. flavus* infection and aflatoxin contamination in preharvest maize was associated primarily with the uninjured kernels.

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