Interference Competition and Aflatoxin Levels in Corn

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ABSTRACT

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An experiment was designed to determine whether certain fungi commonly isolated from corn at harvest can affect aflatoxin development when inoculated with *Aspergillus flavus* (NRRL 6412) onto individual sterilized corn kernels (incubation 8 days at 28 C). No aflatoxins were detected when *Aspergillus niger* (NRRL 6411) or *Trichoderma viride* (NRRL 6418) were paired with *A. flavus*. Aflatoxin contamination was substantial (mean values for B_1 ranging from 676 ppb to 3,765 ppb) in 11 other simultaneous pairings. When inoculation with *A. flavus* followed by 5 days that of the other test fungi, aflatoxin was detected only in pairings with *Candida guilliermondii* (B_1, 16 ppb; B_2, 1 ppb). However, *A. flavus* invaded a number of the other preinoculated kernels in this series, as evidenced by its abundant sporulation on kernel surfaces and/or the formation of a bright greenish-yellow (BGY) fluorescence in kernel tissues examined under black light (365 nm). The status of individual fungal colonists as interference competitors, and the order in which species colonize a kernel (ie, prior to, or simultaneous with, the introduction of *A. flavus*), are examined as factors contributing to variation in aflatoxin levels among field samples.

The discovery that *Aspergillus flavus* produces aflatoxin in corn before harvest (1) has forced mycologists and plant pathologists to abandon the previously established viewpoint that mycotoxin-producing aspergilli, penicillia, and other molds are limited to the postharvest stages of a cereal agroecosystem (3).

Regional surveys have shown that there is considerable variation in the occurrence of *A. flavus* and aflatoxin among individual fields, rows, ears, kernels, and seasons (7,14). Extensive research is being conducted to provide biological explanations for such variation through studies of suspected causal factors contributing to aflatoxin development in corn. Such studies include the role of host genotype and genetic factors of resistance (15); arthropods as vectors in the mechanical dispersal of spore inoculum of *A. flavus* and in predisposing certain kernels to invasion by *A. flavus* through tissue damage during egg deposition or larval/adult feeding (5); and moisture stress during drought (8).

An important missing component in these ecological investigations is the failure to recognize that *A. flavus* coexists or competes with other microbial inhabitants of corn kernels and that the outcome of such species interactions may be important in determining if and to what extent aflatoxin develops in the kernels (12). If *A. flavus* is principally introduced into developing corn by arthropods originating in stored corn, then one should expect that the inoculum of other so-called storage molds is likely to be introduced as well. The status of individual fungal colonists as interference competitors, and the sequence in which these colonists become established within the kernel, may contribute to the considerable variation in aflatoxin contamination among field samples. The present study was designed to investigate the potential effects of different fungal colonists of corn on aflatoxin development in sterilized (autoclaved) kernels. Ideally, experiments such as these should be conducted by using gnotobiotically grown corn representing different stages of kernel maturity. However, we wanted first to examine the potential effects of these fungi on the production of aflatoxins by *A. flavus* under conditions otherwise known to be optimal for the biosynthesis of these toxigenic metabolites.

MATERIALS AND METHODS

Aspergillus flavus (NRRL 6412) and the 13 species of fungi used in these experiments were isolated from aflatoxin-contaminated corn sampled at harvest in 1977 from a field in North Carolina. Evaluation of cultural antagonism was accomplished by inoculating triplicate plates of buffered 3% malt extract agar (pH 6.0) with the 13 fungal isolates in all possible combinations. Conidial suspensions, representing a given test pair, were streaked over the agar surface according to predesignated markings on the underside of a petri dish. Four 5-cm lines were positioned to form a square that was not joined at the corners. The distance from the point where a line ended and the actual corner of the square was 1.0 cm. The test fungi comprising a pairing were streaked along the opposite facing (parallel) lines. Fungi with rapid rates of growth were streaked onto the agar surfaces 4 days after the slower growing isolates. Plates were incubated at 25 C and examined daily (10 days) in order to characterize the type of cultural reaction involving a given test pair. Reaction types listed by Johnson and Curl (6) were ranked and numerical values were assigned according to the following scheme:

Reaction type	Points
A. Mutual intermingling of the two organisms.	0
B. Mutual inhibition on contact; the space between the two colonies is small, but clearly marked.	1
C. Mutual inhibition at a distance.	2
D. Inhibition of one organism on contact; the antagonist continues to grow, unchanged or at a reduced rate, through the colony of the inhibited organism.	3
E. Inhibition of one organism at a distance; the antagonist continues to grow through the resulting clear zone at an unchanged or reduced rate.	4

An index of antagonism (IA) was calculated for each organism by examining cultural reaction(s) from all tested fungi and by applying the following formula: IA = reaction type B(n·1) + C(n·2) + D(n·3) + E(n·4); where the types of reactions elicited by a species on all potential competitors are assigned points and the totals for each category are summed.

The ability of fungal isolates to affect aflatoxin synthesis by *A. flavus* was determined in the following manner. Cell and conidial suspensions (propagule density = 1×10^6/ml) of each fungal isolate, prepared from 14-day-old slants of either potato dextrose agar or corn meal agar, represented the inoculum. Individual test plates were prepared by placing 12 undamaged and aflatoxin-free corn kernels on a double layer of Whatman No. 1 filter paper in a glass petri dish. Five milliliters of distilled H_2O were added, and the kernels were allowed to soak for 2 hr before autoclaving for 30 min at 121 C. A small wound (1–2 mm^2) was made in the germ of each kernel with a sterile microscalpel. In one series of test plates, *A. flavus* was simultaneously inoculated onto kernels with each of the 13 fungi as the sole partner. This was accomplished by micropipetting 1 drop (0.05 ml) of the appropriate mixed conidial suspensions onto the wounded area of each kernel. A second series consisted of incubation (5 days) of kernels inoculated with each of the 13 fungal species, followed by inoculation with *A. flavus*. The test plates were then incubated an additional 8 days. Controls consisted of kernels inoculated with *A. flavus* alone and incubated 5, 8, and 13 days. All treatments were carried out in triplicate. Test plates were placed in a closed plastic container to prevent moisture loss and incubated at 28 C. Following incubation, kernels from individual test plates were dried in a forced-air oven at 80 C for 4 hr, cooled, transferred to tared 50-ml beakers, and weighed to the nearest tenth of a milligram.

Kernels were transferred to a Waring Blendor container with 100 ml chloroform, 10 ml of distilled water, and 10 g of celite and then comminuted (extracted) for 5 min. The entire sample was recovered by washing the Pyrex container twice with 50 ml chloroform. The recovered contents of the container were filtered through Whatman

TABLE 1. Evaluation of cultural antagonism between *Aspergillus flavus* (NRRL 6412) and other fungal colonists of corn kernels, with levels of aflatoxin detected in autoclaved corn simultaneously inoculated with each fungus and *Aspergillus flavus* (NRRL 6412)

Taxa	Growth rate (mm per day)[a]	Distributed according to reaction type[b]				Index of antagonism[c]	Reaction of *A. flavus* to potential antagonist	Aflatoxin (ppb)[d,e]	
		B	C	D	E			B_1	B_2
Candida guillermondii (Castellani) Langeronet Guerra (NRRL Y-11, 6240)	1.0	-	-	-	-	0	A	773 (3,562) 6,046	17 (55) 112
Cladosporium cladosporioides (Fresen.) de Vries (NRRL 6421)	3.7	1	-	-	-	1	*f*	2,074 (2,816) 3,859	34 (45) 56
Curvularia lunata (Wakker) Boedijn (NRRL 6409)	11.5	3	-	-	-	3	B	464 (676) 1,069	4 (10) 14
Alternaria alternata (Fr.) Keissler (NRRL 6410)	10.0	2	-	1	-	5	B	2,085 (3,765)	37 (56) 92
Aspergillus niger V. Tiegh. (NRRL 6411)	12.0	5	-	1	-	8	B	ND	ND
Aspergillus flavus Link (NRRL 6412)	10.5	5	1	1	-	10	-	5 d 1,540 (2,273) 3,135 8 d 1,310 (2,861) 5,106 13 d 2,983 (4,892) 7,026	19 (37) 55 16 (45) 66 67 (92) 119
Fusarium moniliforme Sheldon (NRRL 6413)	11.0	2	-	3	-	11	B	144 (961) 2,041	3 (18) 37
Nigrospora oryzae (Berk. & Br.) Petch. (NRRL 6414)	22.5	1	-	4	-	13	D	1,764 (2,656) 3,390	47 (55) 63
Acremonium strictum W. Gams (NRRL 6415)	2.5	2	1	3	1	17	C	895 (1,295) 1,538	2 (21) 36
Penicillium oxalicum Currie and Thom (NRRL 6416)	5.5	4	-	2	3	22	D	849 (1,034) 1,244	8 (16) 20
Penicillium funiculosum I Thom (NRRL 6417)	4.0	3	-	6	2	29	D	2,180 (3,665) 6,542	41 (95) 151
Trichoderma viride Pers. ex S. F. Gray (NRRL 6418)	19.0	-	-	10	-	30	D	ND	ND
Penicillium variabile Sopp (NRRL 6419)	3.3	2	-	5	4	33	D	205 (2,186) 5,217	4 (82) 222
Penicillium funiculosum Thom (NRRL 6420)	5.0	2	-	5	5	37	D	650 (840) 1,279	11 (18) 30

[a] Colony growth rate on 3% malt extract agar buffered at pH 6.0.
[b] Reaction types designated by Johnson and Curl (6); see text.
[c] Index of antagonism (IA) = reaction type B(n·1) + C(n·2) + D(n·3) + E(n·4); values obtained by examining all possible combinations of species listed in this table; see text.
[d] Values in parentheses represent the mean aflatoxin levels of three replicates/treatment and the range. ND = not detected.
[e] *A. flavus* inhibits test organism (reaction type B, C, or D).

2 V filter paper; the filtrate then was evaporated to about 20 ml, restored to 50 ml with chloroform, and applied to the top of a silica gel chromatography column. The CB procedure was used to quantify levels of aflatoxin (2). The limit of detection of the analysis was 5 ppb.

A one-way analysis of variance (ANOVA) with a partitioning of species variation was performed on the aflatoxin values recorded for pairings involving species found to be less antagonistic than *A. flavus* (Group 1), for the *A. flavus* controls (Group 2), and for the speices found to be more antagonistic than *A. flavus* (Group 3).

RESULTS

In Table 1, fungi have been listed in descending order according to increasing potential to interfere with the growth of other fungi isolated from corn kernels. There was no consistent relationship between the status of individual fungi as interference competitors and their growth rate and ability to affect aflatoxin development in corn kernels simultaneously inoculated with *A. flavus*. When *A. flavus* was paired with *A. niger* or *Trichoderma viride*, no aflatoxins were detected. However, mean levels of aflatoxin B_1 in the other pairings were greater than 676 ppb in all simultaneously inoculated kernels and controls.

Variation in aflatoxin levels among the three replicates of several treatments, including that of the *A. flavus* control, was substantial; therefore, it was not possible to find significant differences among the means of individual treatments. However, there was significant variation between overall means of treatments assigned to one of the following three groups (Table 2). Group 1—species found to be less antagonistic than *A. flavus* (eg, *Candida guilliermondii, Cladosporium cladosporioides, Curvularia lunata, Alternaria alternata*); Group 2—*A. flavus* control (eg, 5-, 8-, and 13-day incubation); Group 3—species found to be more antagonistic than *A. flavus* (eg, *Fusarium moniliforme, Nigrospora oryzae, Acremonium strictum, Penicillium oxalicum, P. funiculosum, Penicillium funiculosum* I, *Penicillium variabile*).

As anticipated, aflatoxin B_1 contamination was greater in pairings with fungi in Group 1 (mean 1,410 ppb) than in Group 3 (mean 848 ppb). In cultural tests for antagonism, all but one of the fungi assigned to Group 3 could inhibit the growth of *A. flavus* and overrun the *A. flavus* colony. Fungi comprising Group 3 include suspected systemic pathogens of corn, *F. moniliforme* and *A. strictum*, as well as a number of molds recognized as soil or storage organisms. By contrast, the fungi listed in Group 1 are commonly recorded from leaf surfaces (phylloplane).

When inoculation with *A. flavus* followed 5 days of incubation of the other fungal colonists, aflatoxin was consistently detected in only one cultural pairing (*Candida guilliermondii*).

DISCUSSION

We recognize that ecologically specialized fungal pre- and postharvest colonists of cereal grains may affect the susceptibility of kernels to *A. flavus* group infection and subsequent aflatoxin contamination. Some of the fungi listed in Table 1 are recognizable for their ecological specializations: *Early Phylloplane*—fungi that rapidly colonize newly emerging leaves (*Cladosporium cladosporioides*); *Late Phylloplane*—fungi that colonize living leaf surfaces several weeks after leaf emergence and, along with the early colonists, persist through stages of leaf senescence and litter fall (*Alternaria alternata, Curvularia lunata*); *Seedborne* Systemic—fungi suspected of originating from the F_1 hybrid seed and growing through the vascular bundles, sometimes causing late wilt, into the newly forming F_2 kernels (*Fusarium moniliforme, Acremonium strictum*); *Storage Molds*—fungi capable of growth, sporulation, or survival in microenvironments associated with stored seed caches and transported by insect vectors to corn growing in the field (*Aspergillus flavus, Aspergillus niger, Nigrospora oryzae, Penicillium oxalicum, Penicillium funiculosum, Penicillium variabile, Trichoderma viride*); and of uncertain origin (*Candida guilliermondii*).

Our results suggest that successful establishment of *A. flavus* in preharvest corn and subsequent aflatoxin contamination may depend upon the sequence in which *A. flavus* inoculum reaches the kernels in relation to their colonization by other fungi and the biological properties of the co-invading fungal partner. *Trichoderma viride* (reaction type D) and *A. niger* (reaction type B) interact quite differently with *A. flavus*, yet both prevent it from forming aflatoxin in autoclaved corn kernels. *Trichoderma viride* grows rapidly, is a strong antagonist of *A. flavus*, and could be expected to restrict or prevent establishment of the latter in individual kernels. Indeed, there was no visual evidence (sporulation; bright greenish yellow fluorescence = BGYF) that *A. flavus* was able to colonize these kernels. In contrast, although sporulation of *A. niger* equaled that of a *A. flavus* on individual kernel surfaces, no aflatoxin was detected. *A. niger* and *A. flavus* commonly co-occur in kernels sampled from the field (4,11).

When inoculation with *A. flavus* followed (5 days) inoculation of other molds tested, aflatoxin (average $B_1 = 16$ ppb) was detected in only one instance (ie, *Candida guilliermondii*). However, *A. flavus* had successfully invaded some of the other kernels. In several of the treatment combinations, *A. flavus* conidial structures were observed. Furthermore, pairings with *Cladosporium cladosporioides, Alternaria alternata, Curvularia lunata,* and *Nigrospora oryzae* each produced a typical BGY fluorescence (13), even though no aflatoxin was detected. Quantitative determinations of aflatoxin in individual BGY fluorescent kernels and kernel fragments from corn sampled at harvest have consistently yielded detectable, although highly variable, levels of aflatoxin (9,10). The present results indicate that microenvironmental conditions favoring kojic acid biosynthesis by *A. flavus* may not necessarily be those favoring aflatoxin development in kernels. This represents the first indirect evidence demonstrating that biosynthesis of kojic acid by *Aspergillus flavus* can occur apart from aflatoxin synthesis in a solid substrate fermentation. Moreover, these results indicate the potential importance of co-occurring fungal species in determining which biosynthetic pathways are available to *A. flavus*.

Our rationale for examining the effects of different fungal species combinations on aflatoxin development in autoclaved, mature corn kernels is based on the premise that fungal populations sharing a common resource over evolutionary time will become ecologically specialized (genetically adjusted) in response to a history of interactions. Arthropods that carry *A. flavus* inoculum into corn fields carry the inoculum of associated microbial/fungal colonists as well. One can visualize a closed system in which functional communities of storage microbes are dispersed by individual insects. Following maturation and harvest, the corn again serves as a reservoir of microbial inoculum for subsequent corn crops.

TABLE 2. Aflatoxin levels in corn. Comparison of treatment means for total aflatoxins within and among groups of treatments: Analysis of variance of log (aflatoxin $B_1 + B_2$)

Treatment groups	Degrees of freedom	Log (aflatoxin $B_1 + B_2$) mean square	F-ratio
Replicates[a]	2	0.20586	
Group 1	3	0.34435	3.60[b]
Group 2	2	0.09361	0.98
Group 3	6	0.20909	2.18
Between groups	2	0.43164	4.51[c]
Remainder	26	0.09572	
Total	41		

[a] Group 1—*Candida guillermondii, Cladosporium cladosporioides, Curvularia lunata, Alternaria alternata*; Group 2—*Aspergillus flavus* controls (eg, 5-, 8-, and 13-day incubation); and Group 3—*Fusarium moniliforme, Acremonium strictum, Nigrospora oryzae, Penicillium oxalicum, P. funiculosum* I, *P. variabile, P. funiculosum*.

[b] Variation within group was significant, $P = 0.05$.

[c] Variation between groups significant, $P = 0.05$.

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