Relative Aggressiveness of *Aspergillus flavus* and *A. parasiticus* on Maize in Mississippi

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ABSTRACT

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Aspergillus flavus and *A. parasiticus* were equally aggressive for colonization of field-grown maize (*Zea mays*) kernels after inoculation of ears. The incidence of infection was higher after pinbar inoculation than after needle inoculation. Uninoculated cobs that overseasoned in the field during 1986–1987, 1987–1988, or 1988–1989 contained predominantly *A. flavus*. Moreover, sclerotia of *A. flavus*, but not of *A. parasiticus*, were found in the pith tissues of the cobs. Thus, *A. flavus* appears to have a greater capacity for survival in maize cob debris than does *A. parasiticus*. Conidia populations of *A. flavus* were higher than those of *A. parasiticus* in maize fields when ears were developing. Therefore, *A. flavus* may have greater potential than *A. parasiticus* for serving as inoculum for natural infection of maize kernels.

The colonization of maize (*Zea mays* L.) kernels by *Aspergillus flavus* Link:Fr. and the subsequent production of aflatoxin by the fungus is a serious problem in the southeastern region of the United States. Livestock and poultry losses may result from the feeding of maize contaminated with aflatoxin (1,3,20). In most reports of aflatoxin in maize, *A. flavus* has been recovered (7). *A. parasiticus* Speare is also capable of infecting maize kernels in the field (6,8–10,18), but it has generally been associated with peanuts (*Arachis hypogaea* L.) (7).

Alfatoxin was found in maize in the early 1960s but was considered to be primarily a stored grain problem until Anderson et al (2) and Lillehoj et al (14,15) demonstrated that aflatoxin also could be produced in maize before harvest. However, the role of *A. flavus* and *A. parasiticus* in the infection of, and subsequent production of aflatoxin in, maize has not been clearly defined. Lillehoj et al (16) reported that both *A. flavus* and *A. parasiticus* were associated with insects from developing ears and with soil insects from maize fields and that both were present in soils from maize fields in Iowa, Illinois, Missouri, and Georgia. Insects collected from soil contained mainly *A. parasiticus* and those collected aboveground contained mainly *A. flavus*. In contrast, only *A. flavus* was isolated from molded kernels. Hill et al (11) reported that 92% of the colonies of *Aspergillus* species isolated from naturally infected maize kernels in Georgia were *A. flavus* and 8% were *A. parasiticus*. Wilson et al (25) inoculated maize with several color mutants of *A. flavus* and one of *A. parasiticus* and found that the latter seemed to be more aggressive and survived better than those of *A. flavus*.

The production of sclerotia as a survival mechanism in *Aspergillus* species may play an important role in their dissemination (4,5,17,22). Wicklow and Horn (22) reported the formation of sclerotia by *A. flavus* in maize kernels before harvest and described the dispersal of the sclerotia during harvest (23). Wicklow and Donahue (21) described the formation of conidia on germinating sclerotia (sporogenic germination) of *A. flavus* and *A. parasiticus* and suggested that the phenomenon could be important in the dissemination of primary inoculum of these two fungi. Wicklow and Wilson (24) subsequently reported sporogenic germination of sclerotia of *A. flavus* in a maize field in Georgia.

Direct comparisons of the infectivity of the two fungi to maize ears in the past were difficult to make because reliable inoculation methods were lacking. Early inoculation techniques involved some sort of injury to the ear, and levels of colonization and toxin accumulation varied extensively (12,18). Such variability has been greatly reduced by use of a technique that deposits inoculum on uninjured kernels (19,26).

Our objectives were to determine: 1) relative aggressiveness of *A. flavus* and *A. parasiticus* on uninjured corn kernels

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on ears in the field, 2) relative populations of the two fungi in cornfields throughout the maize growing season, and 3) possible association between fungal survival and disease occurrence.

MATERIALS AND METHODS

Field plots, harvest, and handling methods. The maize assayed in this study was grown in replicated single-row plots at the Plant Science Research Center, Mississippi State, Mississippi. Each single-row plot was 5 m long and spaced 1 m from adjacent plots. Each row was overseeded and thinned to 20 plants spaced about 25 cm apart. The top ear of each plant was harvested at 60 days after midsilk, at which time the moisture content of the kernels was approximately 17%. Immediately after harvest, the ears were dried at 42 C for 7 days in a forced-air dryer (kernel moisture content was approximately 10%), then shelled mechanically or by hand. Kernels from each plot were bulked in paper bags and stored at 6 C and 45% relative humidity until assayed. Corn plants with unharvested uninoculated ears that remained in and adjacent to the test plots were chopped after harvest with a commercial stalk chopper, and the soil was disked. The kernels on cobs of unharvested ears were left in the field along with all other debris. All inoculated ears were removed from the field at harvest. All assayed kernels, cobs, and petri plates with assayed kernels or cob pieces were incinerated. Thus, any *Aspergillus* recovered from uninoculated ears or cobs represented endemic types.

Inoculum source and production, inoculation techniques, and assays. Inoculum was prepared from *A. flavus* (NRRL 3357) or *A. parasiticus* (NRRL 2999) obtained from D. T. Wicklow (Northern Regional Research Center, Peoria, IL) each year of these studies. In earlier inoculation studies (26), these strains colonized maize kernels. The isolates could be reliably differentiated in culture. When 10^4 conidia per milliliter were spread over 10 ml of Czapek solution agar (CSA) amended with 7.5% NaCl (CSA-S) in petri plates, NRRL 2999 produced a conspicuous white mycelial mat within 3 days at 28 C; NRRL 3357 did not. Also, conidia of NRRL 2999 were distinctly more echinulate than those of NRRL 3357.

In 1985, cultures were grown on CSA in petri dishes at 28 C. After 12–14 days, conidia were washed from the surface of the agar with sterile distilled water containing two drops of Tween 20 per 100 ml. In 1986 and 1989, cultures were grown on corncob grits in 500-ml Erlenmeyer flasks, each containing 50 g of grits and 100 ml of H_2O. After 12–14 days, conidia were washed from the surface of the grits with sterile distilled water containing two drops of Tween 20 per 100 ml. All inoculum for each year's inoculations was prepared at one time, diluted to 10^8 conidia per milliliter, and stored at 4 C. At the time of inoculation, the suspensions were diluted to 9×10^6 conidia per milliliter and kept on ice in the field until used. Periodically, samples of the inoculum were spread on CSA. Germination of conidia remained above 90% throughout these studies.

Some maize ears were inoculated by the pinbar technique (12). A single 100-mm row of 35 stainless-steel pins with 6 mm of their points exposed was dipped into 2×10^6 conidia per milliliter, lined up with the ear axis, and pressed through the husks into the kernels beneath. Only the husks and one row of kernels on inoculated ears were injured by the pinbar; the cob was not penetrated. Ears were hand-shelled, and only the row of kernels adjacent to the inoculated row were assayed. In the needle inoculation technique, a tree marking gun fitted with a 14-gauge hypodermic needle, 35 mm long with the tip opening plugged and three 1-mm holes drilled 6, 8, and 10 mm from the tip (26), was inserted through the husks and 3.4 ml of inoculum containing 9×10^6 conidia per milliliter was injected over the kernels without visibly damaging them. Ears were mechanically shelled, kernels were bulked, and a random sample was selected for assay.

Only undamaged kernels were plated on CSA-S. Kernels were dipped momentarily in 70% ethanol, submerged in 1.25% NaOCl for 3 min, and rinsed in sterile distilled water to eliminate surface microbes. Then, the kernels were plated on 100-mm petri dishes (13 kernels per dish). After 7 days at 28 C, plates were examined for fungal growth.

Field experiments. In 1985, 23 hybrids were compared for percentage of kernel infection when ears were inoculated with *A. flavus* or *A. parasiticus* using a needle inoculation technique. The field design was a split-split plot with four replications; hybrids were the main plots, fungi were subplots, and inoculation treatments (needle or no inoculation) were the sub-subplots.

In 1986, four maize hybrids (Mp440 × SC170, Mp496 × Mp701, Mp414 × Mp68:616, and C121 × Ga203) were compared for susceptibility to *A. flavus* or *A. parasiticus* using needle inoculation. The design was a split-plot design, with fungal species as whole plots, hybrids as subplots, and inoculum treatments as sub-subplots. Inoculum treatments included, per liter of inoculum: 1) 500 ppm of tetracycline HCl and 10 g of maize pollen, 2) 500 ppm of tetracycline, or 3) a no-additive control inoculum treatment. The corn pollen, a potential food source for the fungi, was added at the approximate rate of 0.034 g per ear. Tetracycline, which in earlier studies did not affect the growth of *A. flavus* or *A. parasiticus* but did reduce bacterial contamination, was included to reduce bacterial contamination occurring naturally on the pollen.

In a second test in 1986, the same four hybrids were compared for susceptibility to both *A. flavus* and *A. parasiticus* after inoculation by the pinbar. The test was a randomized block design with four replications of each treatment.

In 1989, two maize hybrids (GA209 × Mp339 and T226 × T232) considered susceptible to kernel infection by *A. flavus* and two hybrids (Mp420 × Mp412 and Mp313E × Mp317) considered resistant (19) were compared for susceptibility to *A. flavus* and *A. parasiticus* after needle inoculation. Ears from uninoculated plots were used as a control. The test was a split-plot design with six replications, with fungi as main plots and genotypes as subplots. Three separate tests were planted 30 days apart.

Cob samples. In 1987, cobs or pieces of cobs 5–10 cm long were collected from the surface of the soil in and around the treatment. Similarly, cobs from the 1987 crop were collected in 1988. At each sampling date, 500 cobs were collected and shaken vigorously to remove adhering soil, and five 5- to 10-mm rectangular pieces were removed from each cob with a pair of surface-sterilized cutting pliers. Pieces of cob were immersed for 2 min in an aqueous 1.5% (v/v) solution of NaOCl, rinsed in sterile H_2O, and plated directly on 10 ml of CSA-S in petri plates. The plates were examined for fungal growth after 7 days at 28 C. Sclerotia were removed from cob tissues, immersed for 2 min in a 1.5% NaOCl solution, and rinsed in sterile distilled H_2O. Then, a single sclerotium was placed in the center of a petri plate containing 10 ml of CSA-S. The plates were incubated at 28 C for 6 days, at which time the resulting cultures were examined. Conidia from each plate were examined for echinulation under the oil immersion lens of a microscope.

Spore samples. In 1988, the ratio of conidia of the two *Aspergillus* species in the field was examined. Slides coated with petroleum jelly were mounted in a modified Dust Buster Plus cordless vacuum cleaner. Several locations in and adjacent to the maize test plots were sampled at irregular intervals for airborne spores over the period of maize silking to harvest.

In 1989, a modified Kramer-Collins volumetric spore sampler (GR Manufacturing Co., Manhattan, KS) (13) was mounted on a platform within the 1.2-ha field. The sampling orifice was 1 m above the soil surface. A rotary wind vane was used to keep the sampling orifice pointing into the wind and to keep precipitation out of the sampler. The airflow of the sampler was set at 22.7 L per minute and operated for 30 sec every 30 min. The spore sampler was modified to collect spores in 100-mm petri plates containing 10 ml of CSA-S. This was

accomplished by attaching the collection orifice to the small end of a 125-mm funnel fitting over a petri plate while suction was applied to an opening in the side of the funnel. Petri plates were changed daily. Plates were incubated at 28 C for 4 days.

Analysis of data. Data were subjected to a standard analysis of variance using the percentage of 390 kernels that were infected with *A. flavus* or *A. parasiticus* as a plot mean for the appropriate experimental design. LSD values were computed.

RESULTS AND DISCUSSION

Field experiments. A significantly higher percentage of kernels was infected by *A. flavus* than by *A. parasiticus* in 1985 (3.5 vs. 2.4%). The incidence of both *A. flavus* and *A. parasiticus* was higher in kernels from inoculated ears than in those from uninoculated ears (3.0 vs. 1.3%). There were no significant differences among hybrids for resistance to infection by either pathogen. There was no interaction of hybrids with inoculation treatments. When kernels from the uninoculated plots were assayed for *Aspergillus*, all yellow-green cultures were identified as *A. flavus* based on growth in culture and conidial echinulation.

There were no significant differences in incidence among the four maize hybrids needle-inoculated in 1986 with *A. flavus* or *A. parasiticus* (4.3 vs. 6.6%). The addition of corn pollen or tetracycline to inoculum did not significantly affect kernel infection with either *A. flavus* or *A. parasiticus*. The infection rate was significantly higher with the pinbar than with the needle inoculation technique (21.8 vs. 5.4%). However, kernels assayed in pinbar treatments were closer to infection loci (kernels from rows adjacent to inoculated row), whereas in the needle inoculated treatments, the percentage of kernels was based on a bulked sample of all kernels on the ears. Overall, the incidence of *A. flavus* with pinbar inoculation did not differ significantly from that of *A. parasiticus* (22.7 vs. 21.0%).

At the first planting date in 1989, the incidence of *A. flavus* infection was significantly higher than that of *A. parasiticus* (6.3 vs. 4.6%). At the other planting dates, however, there were no significant differences between infection levels of *A. flavus* and those of *A. parasiticus* (6.6 vs. 5.9%). There were no significant differences in infection levels between resistant hybrids or between susceptible hybrids inoculated with *A. flavus* or *A. parasiticus* (Table 1). A significantly lower incidence was found in kernels from the resistant cultivars than in those from susceptible cultivars at all planting dates. All yellow-green colonies recovered from kernels from uninoculated ears were identified as *A. flavus*.

Table 1. Percentage of kernels from four maize hybrids infected with *Aspergillus flavus* or *A. parasiticus* after needle inoculation in the field at Starkville, Mississippi, in 1989

Hybrid	Inoculum		Uninoculated
	A. flavus	*A. parasiticus*	
GA209 × Mp339	11.1 a[z]	7.5 a	3.1 a
T226 × T232	8.1 a	7.4 a	1.7 a
Mp420 × Mp412	4.1 b	3.4 b	1.3 a
Mp313E × Mp317	2.5 b	3.4 b	1.0 a
Mean	6.5	5.4	1.8

[z] Each value is the average of six replications of 390 kernels in three separate tests (consecutive plantings). Means not followed by the same letter differ significantly from each other at the 0.05 level of probability according to Duncan's multiple range test.

Cob samples. When uninoculated cobs from the 1986–1989 maize crops were collected from the field and assayed for presence of maize pathogens, *A. flavus* was the predominant *Aspergillus* species (Table 2); *A. parasiticus* was recovered only a few times. *A. niger, Penicillium* sp., *Fusarium moniliforme*, and *F. chlamydosporium* were also prevalent on cobs (Table 2). When uninoculated 1-yr-old cobs were dissected in the laboratory, sclerotia of *A. flavus*, but not of *A. parasiticus*, were found in the pith tissues. Thus, we conclude that *A. flavus* may have a greater capacity for survival in cornfields on corncobs than does *A. parasiticus*. Although both *A. flavus* and *A. parasiticus* could probably survive on kernels or other crop debris, most maize debris does not persist throughout the crop year as do cobs. In Mississippi, maize kernels do not normally overwinter in the field because they are eaten by wildlife, germinate in the fall, or rot because of the relatively high moisture and temperature. Because of this, we believe that *A. flavus* could cause more natural infection of maize kernels than *A. parasiticus* could. This is consistent with our observations in 1985 and 1989, when *A. flavus*, but not *A. parasiticus*, was recovered from a portion of the kernels harvested from uninoculated ears.

Spore samples. In samples collected during 1988 and 1989, *A. flavus* was the predominant species. Although the spore samples in 1988 were not quantitative, only spores of *A. flavus* and *A. niger* were identified. In 1989, no colonies of *A. parasiticus* were identified in petri plates. Colonies of *A. flavus* per plate averaged 229 in July and 315 in August of 1989. Daily colony counts of *A. flavus* ranged from two to 1,208 (*data not shown*). We were not able to correlate spore or colony counts with rainfall on any particular day.

In summary, *A. flavus* and *A. parasiticus* may be equally aggressive in maize kernels in the field after the artificial

Table 2. Monthly averages of fungi recovered from uninoculated corncobs left on the soil surface after harvest at Starkville, Mississippi[z]

Crop year	Collection dates	Percent recovery					
		Aspergillus flavus	*A. parasiticus*	*A. niger*	*Penicillium* sp.	*Fusarium moniliforme*	*F. chlamydosporium*
1986	11 May to 19 Aug. 1987	72	1	19	68	51	···
1987	2 Sept. 1987 to 18 Mar. 1988	72	2	6	60	63	77
	13 Apr. to 29 Aug. 1988	51	3	10	51	19	96
1988	13 Sept. to 16 Dec. 1988	35	2	19	10	67	61
	11 Jan. to 13 July 1989	44	0	22	19	25	77
1989	11 Aug. to 20 Dec. 1989	36	0	10	14	32	86

[z] Based on 500 cobs sampled per month. In 1987, 1988, and 1989, ears were harvested mechanically and shelled; the cobs were left in the field, the stalks were chopped with a commercial stalk chopper, and the soil was disked.

inoculation of ears, but *A. flavus* appears to have a greater capacity for survival in the field. Thus, the naturally occurring inoculum in old cornfields would be *A. flavus*.

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