

A Study of *Aspergillus flavus/parasiticus* in Iowa Crop Fields: 1988-1990

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ABSTRACT

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Fungi belonging to the *Aspergillus flavus/parasiticus* group were consistently isolated from soil collected in fields of corn (*Zea mays*) and other crops in Iowa over a 3-yr period. The population recovered declined from an estimated 1,231 colonies per gram of dry soil in fall 1988 to 396 colonies per gram in fall 1990. Aflatoxin-producing isolates declined from 65% in 1988 to 14% in 1990, whereas sclerotia-producing isolates increased from 23 to 68% over the same period. Similar trends were observed in the isolates obtained from corn debris sampled from the fields in 1988 and 1990.

Aspergillus flavus Link:Fr. occurs worldwide in diverse habitats on a variety of plant and animal substrates (9). In the United States, contamination with aflatoxin, a carcinogenic mycotoxin produced by *A. flavus*, particularly in corn (*Zea mays* L.), peanuts, cottonseed, and tree nuts (8), has become a major health concern (4). Despite extensive research

into all aspects of the biology of *A. flavus* over the past 20 yr, there are still many unanswered but very basic questions about the ecology of this important organism.

Presence of *A. flavus* in soils has been well documented by reports from tropical and subtropical regions (9). Only in recent years have researchers tried to quantify *A. flavus* populations in soils in the United States. Griffin and Garren (10) found that *A. flavus* populations in Virginia field soils averaged 0.5-57.3 propagules per gram of dry soil. Using selective medium, Bell and Crawford (2) reported significantly higher numbers of

propagules in naturally infested soils in Georgia, where as many as 1.5×10^4 propagules per gram of dry soil were recovered. Martyniuk and Wagner (16) found a maximum of 2.8×10^3 propagules per gram from soils that were manured and cropped to continuous corn in Missouri. Angle (1) found that soil populations of *A. flavus* and *A. parasiticus* Speare in Missouri soils ranged from zero propagules in native prairie soils to 265 propagules per gram of soil from cultivated fields on a 3-yr rotation of wheat, red clover, and corn. In Iowa, there have been no published reports documenting *A. flavus* occurrence in crop soils or in native prairie soils.

In studies over a 4-yr period in Mississippi, Zummo and Scott (27) found that uninoculated cobs collected from the field were colonized more frequently by *A. flavus* than by *A. parasiticus*. Sclerotia of *A. flavus*, but not of *A. parasiticus*, were found in 1-yr-old cob pith tissue. They hypothesized that *A. flavus* had a better capacity than *A. parasiticus* for survival on cob debris and could cause more natural infection of corn kernels.

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The primary inoculum responsible for infection of corn is thought to start from sporulating colonies that originate from mycelium in plant debris and litter in the soil and/or from spores borne on wind currents (8). The spores of *A. flavus* have been shown to infect corn in the field through the silks (18). Insects have been implicated as vectors in the infection process by transporting spores to developing ears and by facilitating ingress by damaging kernels (17).

It has long been established that many isolates of *A. flavus* readily produce sclerotia in culture (9). In 1984, Wicklow et al (24) reported for the first time evidence of naturally occurring sclerotia on corn in a Georgia crop field. These sclerotia may not only disperse the fungus at harvest time via debris from combines (24) but also remain as long-term survival structures in the soil (22). Because surviving sclerotia were shown to germinate sporogenically in the laboratory, Wicklow and Donahue (23) suggested they may be an important source of primary inoculum in the field. Wicklow and Wilson (25) later demonstrated that sclerotia placed in a cornfield in Georgia were able to germinate sporogenically before silking time.

Cultural practices have been suggested as contributing factors in the development of preharvest aflatoxin contamination of corn (1,13). In North Carolina, early planting, early harvest, and irrigation of cornfields were associated with reduced aflatoxin levels at harvest time (14). Payne et al (19) found that irrigation and subsoiling indirectly reduced aflatoxin contamination of preharvest corn by reducing water stress in the plants. Reduction in numbers of propagules of *A. flavus* and *A. parasiticus* were associated with different tillage practices and crop rotations in soil samples taken from selected fields in Missouri (1). Other studies have implicated crop sequences as factors in the buildup of *A. flavus* in soil and subsequent aflatoxin contamination of crops in the field (10,11). However, environmental conditions, particularly maximum and minimum daily temperatures and net daily evaporation rates during the growing season, seem to be the overriding factors affecting the

amount of aflatoxin that will develop in a standing crop in any given year (26).

In years when unusually high temperatures are combined with low moisture availability, *A. flavus* has been observed growing on kernels in field-collected ears in the Midwest (15,21). Optimum climatic conditions for the fungus in Iowa in 1988 resulted in the development of *A. flavus* on the standing crop and an unusually high rate of aflatoxin-contaminated grain, particularly in the eastern half of the state. This report describes attempts to recover *A. flavus/parasiticus* from field soil and crop debris over a 3-yr period in Iowa.

MATERIALS AND METHODS

Study sites. Forty crop fields in eight counties in eastern Iowa (Fig. 1) were selected because in 1988 each was the source of corn that tested higher than 20 ppb of aflatoxin. Samples of soil and corn debris were collected from each field after the 1988 harvest. The sites were revisited to collect soil and/or debris samples after spring planting in 1989 and 1990 and after harvest in the fall of 1989 and 1990. The crop rotations in the study sites for 1989 and 1990 were corn-corn (14), soybeans-corn (19), oats/alfalfa-alfalfa (1), corn-oats/alfalfa (2), Conservation Reserve Program-Conservation Reserve Program (1), corn-soybeans (2), and soybeans-soybeans (1).

Collection procedures. Soil samples were collected after planting in the spring (mid-May to 1 June) and after harvest in the fall (mid-October to 1 November). For each soil sample, surface debris was removed and an approximately 20-g subsample was collected with a sterilized trowel from the top 3 cm of soil at about 20 locations within each field. The subsamples were pooled and thoroughly mixed to yield one composite soil sample per field. The samples were transported to the laboratory and frozen at -4°C until analyzed.

Stalks and cobs were collected from each field in fall 1988 and spring 1989. Similar debris samples were collected after harvest from fields cropped to corn in 1990. Because less than one-half of the fields were planted to corn in 1989, no debris samples were collected in fall 1989 or spring 1990.

Laboratory procedures. Approximately 0.5 g of soil was sprinkled onto the surface of selective medium plates (10) amended with 10 ppm of dicloran (Botran) (M3S10B). Five replicate plates were inoculated from each soil sample. The plates were then incubated at 37°C for 4 days. Plates were visually examined and rated for presence of *A. flavus*, and a percent cover on each plate was estimated.

The fall 1990 soil samples were analyzed by two additional methods. A dilution plate technique modified from Pitt et al (20) was used to determine

colony numbers and to examine colony origin. A 10-g soil sample was added to 250 ml of sterile 0.5% peptone in distilled water. The suspension was shaken thoroughly for 5 min, pipetted in 1-ml aliquots onto the agar surface, and spread evenly over the medium. After incubation at 37°C for 4 days, colonies were counted and examined to determine if they originated from sclerotia. Soil subsamples were also carefully hand-sifted on sterile paper, and 50 small pieces of organic debris were picked from each sample. The pieces were surface-sterilized for 30 sec with a 0.5% sodium hypochlorite solution, rinsed in sterile deionized water, placed on the surface of the selective medium, and processed as described above.

A maximum of five sporulating colonies of *A. flavus* on the soil or debris plates was transferred to PDA slants. From each plate in a sample that had *A. flavus*, the colony closest to the center point was picked to avoid bias in selection. If a plate lacked *A. flavus*, another was picked from those that had more than one colony, and again the colony nearest the center point was selected. If five or fewer colonies were present per sample, all were transferred to PDA slants.

Aflatoxin-producing ability was determined with the coconut agar test (7). Each isolate was plated on coconut agar and grown for 4 days at 28°C . The appearance of blue-green fluorescence under long-wave (365 nm) ultraviolet light was presumptive evidence that the culture could produce aflatoxin.

Sclerotia producers were determined by visual examination of the PDA slant and by growing each isolate on a plate of Czapek-Dox medium amended with 0.5% sodium nitrate and 0.5% sucrose. Inoculated plates were incubated at 28°C for 7 days, then visually examined for presence of sclerotia. Isolates of *A. flavus* on plates of Czapek-Dox amended medium were incubated at 10, 15, 20, 25, 30, and 35°C for 14 days to determine optimum temperatures for sclerotia production.

Debris samples were analyzed for the presence of *A. flavus* by placing either 10 kernels or 10 1-cm pieces of stalk or cob pith with a sterile forceps onto the surface of M3S10B plates and incubated for 4 days at 37°C . Sporulating colonies typical of *A. flavus* were transferred onto PDA slants, grown for 7 days at room temperature to assure clean, uncontaminated cultures, and analyzed for aflatoxin- and sclerotia-producing ability as described above.

Dilution plating was used to determine fungal community composition of the soils of the 40 study fields in spring 1990. A 1:2,000 dilution of field-collected soil suspended in sterile water was used for isolation of fungi on a soil extract agar amended with rose bengal and strepto-

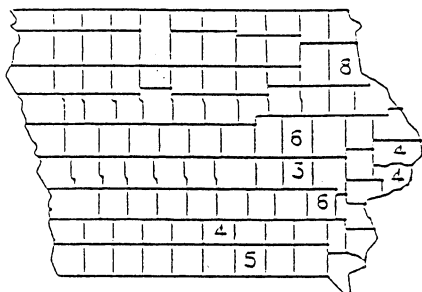


Fig. 1. Eight counties, with number of fields per county, in eastern Iowa selected as *Aspergillus flavus/parasiticus* study sites.

mycin as described by Clarke and Christensen (5). For each dilution series, 30 hyphal tip transfers provided a random sample of the common soil fungi in each crop field.

Isolates were examined visually during the study to determine the presence of *A. flavus*. Because colony color was not supplemented with microscopic examination for definitive separation of *A. flavus* from *A. parasiticus*, the fungus is reported here as *A. flavus/parasiticus*.

RESULTS AND DISCUSSION

A. flavus/parasiticus was found in every soil sample collected from the study fields during 1988 and 1989. In 1990, only six samples from two sampling periods failed to produce colonies on M3S10B agar plates. *A. flavus/parasiticus*, although readily isolated with selective media, actually occurs with very low incidence in Iowa crop soils. Only two of the 1,170 fungal isolates retrieved from the dilution plate series used to analyze the spring 1990 soil samples were *A. flavus/parasiticus*. The number of fungal colonies for crop fields is often estimated to be over 3×10^6 propagules per gram of dry soil. Thus, *A. flavus/parasiticus* constitutes a very small portion of the total fungal community.

For the study period 1988–1990, the percentage of soil plates that contained colonies of *A. flavus/parasiticus* was higher in fall collections and decreased the subsequent spring (Fig. 2A). The percent decline of occurrence in spring can probably be attributed to loss of spore viability during adverse winter conditions. During the growing season, however, even if conditions do not result in contamination of corn by *A. flavus/parasiticus* or aflatoxin, the fungus seems able to grow and reproduce, although not at levels characteristic of an epidemic year.

For fall collections during 1988–1990, the area covered on the soil plates by *A. flavus/parasiticus* was visually estimated. This procedure was used in 1988 because *A. flavus/parasiticus* was present in such high numbers that an accurate colony count was not possible. Percent cover declined from an average of 41% in 1988 to 13% in 1990. In the fall of 1990, counts in the 40 fields ranged from zero to 2×10^3 , with an average of 396 colonies per gram of dry soil. On the basis of correlations of plate coverage with counts in 1990, over 7×10^2 and 1.2×10^3 colonies of *A. flavus/parasiticus* were recovered per gram of dry soil in 1989 and 1988, respectively.

The percentage of isolates that appeared toxigenic remained consistently high for the year following the aflatoxin outbreak in 1988 but have declined substantially since fall 1989 (Fig. 2B). By fall 1990, only 14% of the isolates were toxigenic. The percentage of *A. flavus/parasiticus* isolates that produced sclerotia increased

from 23% in 1988 to 68% in 1990 (Fig. 2C). Bennett et al (3) found no correlation between aflatoxin and sclerotia production. Our data would seem to support that finding, as only 20% of the isolates from the 1988 sampling period and 4% of the isolates from the 1990 sampling period produced both sclerotia and aflatoxin in laboratory cultures.

Percent recovery of *A. flavus/parasiticus* from cob pieces after the 1988 epidemic in Iowa was 70%, similar to rates (72%) found in Mississippi in 1986 and 1987 (27). Although the recovery rates in Mississippi declined during 1988–1989, they were still substantially higher than the 1% recovery of the fungus from cob samples in Iowa in 1990.

The apparent population shift in toxic vs. atoxigenic strains of *A. flavus/parasiticus* was also reflected in field debris samples over the 3 yr of our study. *A. flavus/parasiticus* grew out of 56 and 0.5% of the corn debris pieces in 1989 and 1990, respectively (Table 1). Of the *A. flavus/parasiticus* isolates from debris in 1988, 55% produced the blue-green fluorescence on coconut agar. In contrast, no toxin producers were found among the 12 colonies that sporulated and were isolated from debris pieces in 1990. Sclerotia-producing isolates over that same time period increased from 32 to 92%. The percentage of toxigenic vs. atoxigenic strains showed a similar pattern in isolates from small debris pieces picked from soil samples collected during fall 1990. *A. flavus/parasiticus* grew from only 26 (1%) of the 1,950 debris pieces. Two of the 26 isolates were toxin producers and 25 were sclerotia producers. Thus, atoxigenic strains of *A. flavus/parasiticus* may be better competitors during average growing seasons in the Midwest and toxin-producing strains may be better adapted to drought-related conditions of high temperatures and low water availability. The interaction of *A. flavus/parasiticus* and stressed corn plants may stimulate population explosions of toxigenic strains.

In southeastern corn-growing regions, sclerotia have been observed on insect-damaged and moldy ears (24). Sclerotia have been demonstrated to be survival structures for many fungi (6). Because sclerotia of *A. flavus* and *A. parasiticus* can germinate sporogenically, potentially they could serve as a source of primary inoculum (22,25). Cob and stalk debris collected during the study was dissected and carefully examined for sclerotia, but none were observed. Wet sieving and density gradient centrifugation techniques used to process the soil samples also failed to yield sclerotia. Development of sclerotia in kernels, cobs, or stalk pieces in the Midwest appears to be uncommon.

Sclerotia of *A. flavus/parasiticus* have been shown to develop optimally in the laboratory between 28 and 35 C (9). The

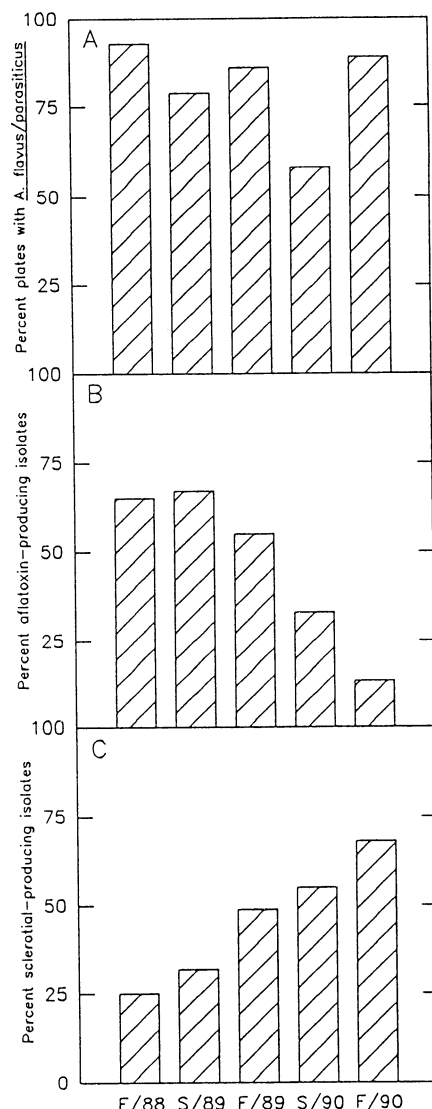


Fig. 2. Results of studies of soil collected during the fall (F) and spring (S) of 1988–1990 from 40 crop fields in Iowa: (A) Percentage of M3S10B agar plates inoculated with soil that yielded *Aspergillus flavus/parasiticus*, (B) percentage of aflatoxin-producing isolates, and (C) percentage of sclerotia-producing isolates.

Table 1. Recovery of *Aspergillus flavus/parasiticus* from corn debris collected after harvest from 40 crop fields in Iowa during 1988 and 1990

	1988	1990
Cob pieces	561/800 ^a	7/940
Stalk pieces	333/800	2/950
Kernels	...	3/310
Average cob, stalk debris, and kernels colonized	56 ^b	0.5%
Aflatoxin-producing isolates	55 ^c	0%
Sclerotia-producing isolates	32 ^d	92%

^aNumber of isolates recovered/number of samples.

^bPercent total units yielding *A. flavus* per units examined.

^cPercent isolates producing blue-green fluorescence on coconut agar.

^dPercent isolates producing sclerotia on Czapek-Dox amended medium.

Iowa strains also produced sclerotia optimally over these temperature ranges. Fewer sclerotia were produced at 25 C than at higher temperatures, and although none were produced at 20 C, sclerotial initials were observed on the plates. So, temperatures may not remain high enough long enough during September and October for sclerotia to develop in the field in Iowa. Average soil temperatures in Iowa decline from 25 C on 1 September to 8 C by 1 November. Because sclerotial initials will develop at lower temperatures, these thick-walled hyphal units may become the principal survival units of the fungus in the Midwest corn-growing region.

The method of survival of *A. flavus/parasiticus* in midwestern soils still is not well understood. Dilution plating of a 1:25 soil suspension of each sample onto M3S10B agar plates provided evidence that the fungus survives either as mycelium or spores in the soil. Colonies that developed on the selective media were critically examined under a dissecting scope for origin. Whether colony growth originated from hyphae or spores was impossible to ascertain, but none developed from sclerotia. Jones's (12) suggestion that the fungus survives as mycelium in debris pieces in the soil, although not well supported by our data, may be the most likely possibility. The critical factor may be the size of the debris pieces. Most of the debris pieces picked from the soil during our study were small, less than 1 cm. Most of the 26 colonies that developed from debris pieces picked out of soil came from larger pieces of debris in the samples.

The population changes of *A. flavus/parasiticus* in Iowa crop soils appear not to be influenced by crop rotation. Of the 40 fields, 35% were cropped continuously to corn during the course of the study, 48% were alternately cropped to soybeans in 1989, and the remaining were planted to crops such as oats and alfalfa. Populations of *A. flavus/parasiticus* recovered from fields cropped in continuous corn covered 41, 27, and 13% of the soil plates in 1988, 1989, and 1990, respectively. Samples from fields alternately cropped to soybeans in 1989 also yielded fewer colonies, with cover of 28 and 15% in 1989 and 1990, respectively. These results support our hypothesis that environmental conditions near harvest, particularly temperature and moisture levels, instead of crop rotation are the

most important factors that determine the incidence of *A. flavus/parasiticus* in crop fields.

Preharvest aflatoxin contamination of corn is of annual concern in the southeastern United States but occurs sporadically in the Midwest. Our ability to obtain useful field information is complicated by the intermittent occurrence of aflatoxin contamination. Years may pass between outbreaks. To date, our understanding of the nature of aflatoxin contamination in corn has been gained primarily from research conducted in the southeastern states. However, conditions that bring about contamination in one part of the country may not necessarily be applicable in a geographically separated area, for example, Iowa, where the environment and agricultural practices are substantially different.

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