

Spatial variability of *Aspergillus flavus* soil populations under different crops and corn grain colonization and aflatoxins

H.K. Abbas, R.M. Zablotowicz, and M.A. Locke

Abstract: Aflatoxin contamination in corn caused by *Aspergillus flavus* Link is a serious constraint on economical corn (*Zea mays* L.) production in the Mississippi Delta. The ecology of *A. flavus* was evaluated in a 3-year study assessing the spatial variability of soil populations of *A. flavus* in a Mississippi Delta field under different crops. A 1.07-ha section of the field was laid out in 126 9.2-m² plots, and soil was sampled in May 2000, March 2001, and April 2002. *Aspergillus flavus* populations were determined by plating on selective media, and *A. flavus* colonization was assessed in corn during 2000. *Aspergillus flavus* populations in soil were significantly ($P < 0.01$ level) influenced by previous crop. The highest propagule density (794 cfu·g⁻¹) was found following the corn crop in 2001 versus 251 cfu·g⁻¹ soil in 2000 following cotton and 457 cfu·g⁻¹ following wheat in 2002. *Aspergillus flavus* populations in 2001 and 2002 exhibited a moderate degree of spatial structure, described by spherical and exponential models, respectively, but populations in 2000 exhibited little spatial structure. Colonization of corn kernels by *A. flavus* in 2000 ranged from 0% to 100% (mean = 15% colonized kernels), and aflatoxin levels ranged from 0 to 1590 ppb (mean = 57 ppb). Aflatoxin levels were randomly distributed in the field and not correlated with *A. flavus* colonization. Aflatoxin production was found in 43% to 59% of *A. flavus* soil isolates with the highest incidence in soil populations following corn in 2001. However, 84% of *A. flavus* isolated from corn kernels produced aflatoxin. Results indicate that within a single field there was a wide range of *A. flavus* soil propagule densities varying in potential to produce aflatoxin.

Key words: *Aspergillus flavus*, aflatoxins, soil, corn (*Zea mays*), cotton, wheat, spatial variability.

Résumé : La contamination du maïs causée par l'*Aspergillus flavus* Link est une contrainte économique sérieuse pour la production de maïs (*Zea mays* L.) dans le Delta du Mississippi. Les auteurs ont suivi l'écologie de l'*A. flavus*, au cours de 3 années d'études, afin d'évaluer la variabilité spatiale des populations du sol de l'*A. flavus*, dans les champs du Delta du Mississippi, sous différentes cultures. Les auteurs ont divisé une surface de 1,07 ha en 126 parcelles de 9,2 m², et ils ont échantillonné le sol en mai 2000, mars 2001 et avril 2002. Ils ont déterminé les populations de l'*A. flavus* par mise en plaque sur milieux sélectifs, et la colonisation du maïs a été évaluée au cours de l'an 2000. Les populations de l'*A. flavus* dans le sol sont significativement affectées par la culture précédente ($P < 0,01$). On observe la plus forte densité de population (794 cfu·g⁻¹) suite à la récolte de maïs de 2001, versus 251 cfu·g⁻¹ en 2000 suite à du coton, et 457 cfu·g⁻¹ suite à du blé. On observe un degré modéré de structure spatiale des populations de l'*A. flavus* dans les échantillons de 2001 et 2002, suivant respectivement un modèle sphérique ou exponentiel, mais les populations de 2000 montrent peu de structure spatiale. La colonisation des grains de maïs par l'*A. flavus* observée en 2000, va de 0 % à 100 % (moyenne de 15 % des grains colonisés), et les teneurs en aflatoxine, de 0 à 1590 (moyenne = 57 ppb). Ces teneurs en aflatoxine sont distribuées au hasard dans le champ et ne sont pas corrélées avec la colonisation par l'*A. flavus*. On a décelé la production d'aflatoxine chez 43 % à 59 % des isolats de l'*A. flavus* provenant des sols, l'incidence la plus élevée étant survenue dans les populations du sol après la culture de maïs de 2001. Cependant, 84 % des isolats de l'*A. flavus* obtenus des grains de maïs produisaient de l'aflatoxine. Les résultats indiquent que pour un champ donné, on retrouve un large éventail de densités des propagules de l'*A. flavus*, ayant un potentiel variable de produire de l'aflatoxine.

Mots clés : *Aspergillus flavus*, aflatoxine, sol, maïs (*Zea mays*), coton, blé, variabilité spatiale.

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Introduction

The fungus *Aspergillus flavus* Link, which produces aflatoxin, is widespread in the environment and can colonize crops such as corn (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) (Horn 2003). Aflatoxins (AF) are toxic, mutagenic, and carcinogenic secondary metabolites produced by several *Aspergillus* species including *A. flavus*, *A. parasiticus* Speare, *A. tammarii* Kita, and *A. nominus* Kurtzman, Horn & Hesseltime (CAST 2003; Payne 1992, 1998). Products contaminated with AF can have serious effects on human and animal health when consumed (CAST 2003). *Aspergillus flavus* is highly variable in its use of substratum and its production of AF and other secondary metabolites (Bayman and Cotty 1991). Controlling AF contamination of crops is complicated further by the uncertainty regarding many aspects of *A. flavus* ecology and epidemiology (Horn and Dorner 1998; Horn 2003; Payne 1998; Scheidegger and Payne 2003; Widstrom 1996).

Aflatoxin contamination is a serious constraint for economical crop production in the Mississippi Delta. The three major crops affected are corn, cotton, and peanuts (*Arachis hypogaea* L.); all are used for both human and animal consumption. Contamination has been observed in all three crops, with high economic losses, e.g., the loss from the 1998 corn crop in the Mississippi Delta was approximately \$30 million (Robens and Cardwell 2003).

Despite the significance of *A. flavus* as a plant pathogen, storage mold, and AF producer, little is known about its population biology (Bayman and Cotty 1991; Horn 2003). For example, a survey of *Aspergillus* propagules in soil conducted in the peanut-growing region of the Southern United States (Horn and Dorner 1998) indicated high populations were present in soil, especially where peanuts were grown. Wicklow et al. (1998) used DNA fingerprinting techniques to characterize the diversity of *Aspergillus* populations from soil, corn, and insects in a single corn field in Illinois.

To formulate efficient AF control strategies, a thorough knowledge of *Aspergillus* propagules serving as inoculum for colonization of the crop is needed. Assessing the spatial variability of soil properties and processes is needed to manage soils to maintain sustainable systems and crop yield potential (Parkin 1993). Identifying the amount and distribution of genetic variation within populations is essential to an understanding of pathogenic fungi (McDonald et al. 1999). A spatial variability approach utilizing geostatistics has been applied to understanding the ecology of *A. flavus* populations (Orum et al. 1997). Both the spatial and temporal variation of *A. flavus* propagule density and strain composition based on sclerotia size was assessed for soils in Yuma county, Arizona. Isolates of *A. flavus* have been divided into two phenotypic groups based on the size of sclerotia: small (S, <400 µm) and large (L, >400 µm) (Horn 2003; Pildain et al. 2004). S forms been described as *A. flavus* var. *parvisclerotigenus* (Saito and Tsuruta 1993). These studies indicated that the largest source of spatial variance was attributable to variance of population differences observed at the field level (1–5 km) compared with larger or smaller size classifications. Recent studies by Jamie-Garcia and Cotty (2003) used geostatistics to evaluate the variability of AF contamination of cottonseed in Southern Texas. These stud-

ies indicated consistent recurrent patterns of high and low AF contamination within rather large subregions of southern Texas. The horizontal spatial variability of patterns of *A. flavus* and its antagonist *Aspergillus niger* Tiegh. were studied in soil from two peanut fields with different levels of AF contamination (Griffin et al. 2001). These studies indicated that these two *Aspergillus* species, especially for *A. niger*, have patchy populations, with population clump sizes ranging from 13 to 26 m².

The principal objectives of our study were (i) to understand the spatial variability of soil propagules of *A. flavus* within a single field managed using a no-tillage system under Mississippi Delta conditions; (ii) ascertain aflatoxin production by *A. flavus* isolated from soil and corn in this field; and (iii) explore the spatial variability of aflatoxin contamination of corn and its relationship to soil conditions and *A. flavus* propagule density.

Materials and methods

Description of the field site and sampling techniques

This study was conducted in a 1.07-ha section of a 6-ha commercial field in Sunflower County, Mississippi. The dominant soil series at the site is a Dundee silt loam. This site has been managed using no-tillage practices since 1995. Corn was initially planted on this site in 1998 and again in 2000. Cotton was planted on this field in 1995, 1996, 1997, 1999, and 2001. Winter wheat was planted in the fall of 2001. The site was not irrigated, but part of the western 21 m of the study site received irrigation drift from an adjacent field under a pivot system. The experimental site was divided using a 14 × 9 grid of 126 plots (9.23-m² grid cell consisting of eight 1-m crop rows). The center of each plot was geo-referenced for global position system (GPS) coordinates using a Pathfinder ProXR (Tremble Navigation, LTD, Sunnyvale, California) for precise resampling. Soil samples were taken from the upper 5 cm and were collected in April 2000 (prior to corn planting), May 2001 (prior to cotton planting), and April 2002 in an established wheat (*Triticum aestivum* L. ssp. *vulgare*) crop. Each sample was a composite of 12 subsamples (approx. 1 kg), with three cores taken in each of the four center rows. Soils were sieved through a 2-mm sieve and stored at 5 °C until processing for *Aspergillus* populations. Soil moisture content was determined on 50-g samples following drying at 70 °C for 72 h. Ten corn ears were harvested by hand from each plot at maturity in 2000. The corn ears were shelled and the grain was dried at 45 °C (for 5 d) and stored at 5 °C until processed. Cotton seed and wheat grain were not sampled in these studies.

Determination of *Aspergillus* populations and culture maintenance

Sieved soil (0.5 or 2.5 g, fresh weight) was diluted in 10 mL of agar solution (2 g·L⁻¹) and vortexed twice for 1 min. Duplicate samples (0.2 mL) were then spread on 9-cm Petri dishes containing modified dichloronitroaniline rose bengal (MDRB) agar (Horn and Dorner 1998). Colonies exhibiting *Aspergillus* morphology characteristics, yellowish green colonies with a rough-walled appearance, about 0.3–0.5 cm in diameter, were counted following incubation at

37 °C for 4 d, and propagule densities were reported as a $\log_{10}(\text{propagules}\cdot\text{g}^{-1} \text{ oven dry weight soil})$.

Colonization of freshly shelled and dried corn kernels by *A. flavus* was determined according to Abbas et al. (1988). One hundred corn kernels from each plot were surface-sterilized by soaking in 2.5% sodium hypochlorite for 1 min, rinsed three times in sterile water, and transferred to two MDRB agar plates, 10 kernels per plate, and incubated as previously described. *Aspergillus*-infected seed was reported as the percentage of kernels producing *Aspergillus* colonies on the media. When possible, three to five discrete colonies were selected from each soil or corn sample from MDRB plates, and recultured on MDRB media to verify isolate purity. Cultures were maintained on potato dextrose agar (PDA) (Difco Laboratories, Detroit, Michigan) slants and silica gel (Windels et al. 1988).

Aflatoxin extraction and determination

Corn collected in 2000 was analyzed for aflatoxin. Briefly, corn was ground (2 mm) in a Romer mill (Romer Labs Inc., St. Louis, Missouri) and extracted with methanol:water (70:30, v/v) in a 5:1 (v/w) ratio and shaken for 3–5 min at high speed using a reciprocal shaker. An aliquot of extract was removed and centrifuged (12 000g, 10 min.), and the supernatant was assayed for the presence of AF using ELISA kits (Vertox, Neogen Corp., Lansing, Michigan) as described by Abbas et al. (2002).

Culture methods for aflatoxin detection and fungal identification

Yellow pigment formation was recorded prior to scraping the PDA plates for analysis as described above (Abbas et al. 2004; Davis et al. 1987; Gupta and Gopal 2002; Lin and Dianese 1976). Five-day-old PDA cultures were exposed to 27% ammonium hydroxide (Sigma, St. Louis, Missouri) for 30 min, and the formation of a pink coloration after exposure to ammonia vapor was observed (Abbas et al. 2004; Saito and Machida 1999). Identification of *Aspergillus* species was based on morphological criteria of Klich (2002).

Statistical analysis

Aspergillus soil propagule density data were subjected to ANOVA to compare means between years and means were separated using Fisher's protected LSD test. A χ^2 test was used to determine the likelihood that the distribution of toxigenic isolates varied in soil over the 3 years and that the frequency in toxigenic isolates in soil differed from toxigenic isolates from corn. An ANOVA was not appropriate to compare the frequency of toxigenic isolates as there were an unequal number of colonies recovered from each sample and sample location, for example, no *A. flavus* colonies were recovered and purified from some locations.

Spatial variability data were analyzed (frequency distribution, semivariance, and kriging) and mapped using GS+ (Version 5.1, Gamma software, Plainfield, Michigan). *Aspergillus* propagule data were calculated on a \log_{10} basis, thus no further transformations were done prior to performing analysis for semivariance, a statistic based on autocorrelation. Data for both *Aspergillus*-infected seed and AF concentration were adjusted by adding +1 and then \log_{10} transforming them. The data were backtransformed prior to

Table 1. Soil *Aspergillus* propagule densities for three yearly samples taken in the spring following a cotton crop (2000) and a corn crop (2001), and during a wheat crop (2002).

Year	$\text{Log}_{10}(\text{cfu}\cdot\text{g}^{-1} \text{ soil})^*$
2000	2.40c
2001	2.94a
2002	2.66b
LSD 0.01 [†]	0.10

*Means followed by the same letter do not differ significantly at the $P = 0.01$ level.

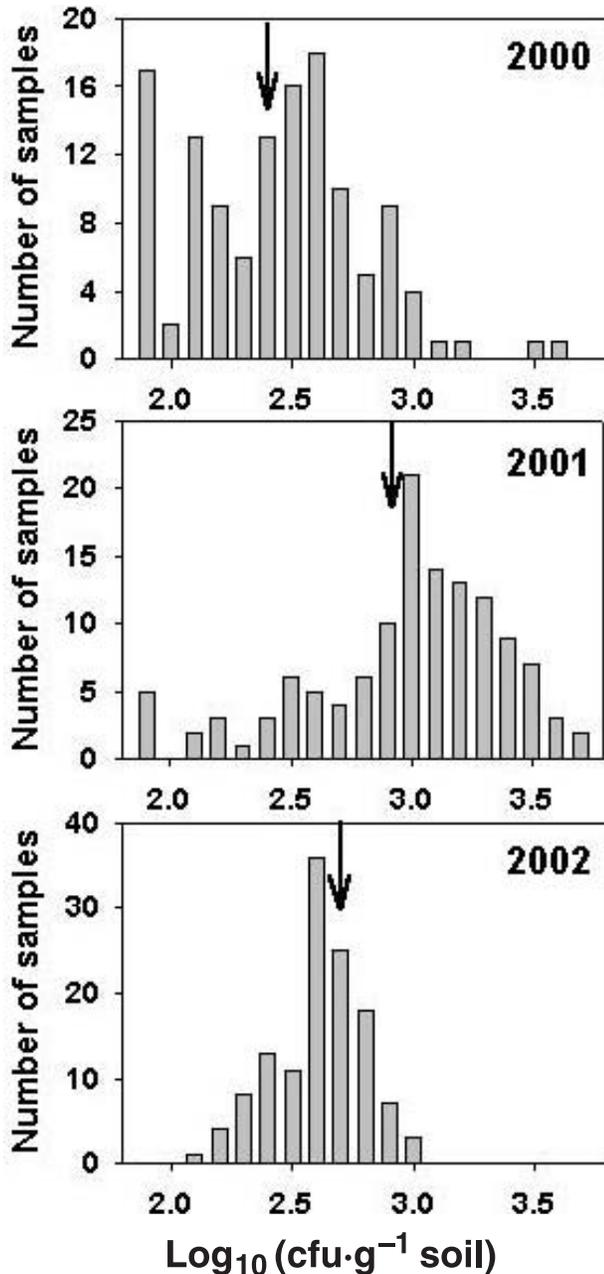
[†]Fisher's protected LSD test.

kriging and mapping. To model spatial structure, all data were initially fit for semivariance analysis with a maximum lag distance of 64 m (half the distance of the length of the field), at equal intervals of 9.2 m. The nugget (Co) or y intercept, represents spatial variability that was unaccounted for at distances smaller than the smallest lag (i.e., background variance); the sill ($C + \text{Co}$) represents the maximum variance of which C is the structural variance (i.e., the variance that is attributed to spatial position); and the range (Ao) is the distance where semivariances cease to increase and spatial variance is apparent (Rossi et al. 1992). The ratio of the nugget to the sill ($\text{Co} / (C + \text{Co})$, Cambardella et al. 1994) was used to establish the degree of spatial dependence, where a ratio less than 25% is strong, 25–50% is moderate, and greater than 75% is weak. Following the calculation of semivariance, data were block kriged in a 2×2 local grid and searched at a 16 point neighborhood, prior to generation of maps using GS+ software.

Results and discussion

A summary of the soil populations of *Aspergillus* observed in this field over the 3 years of this study is presented in Table 1 and Fig. 1. All *Aspergillus* isolates from the soil or corn in this field were identified as *A. flavus*. Mean populations of *A. flavus* observed in this Mississippi Delta field were one to two log orders of magnitude greater than those reported in Illinois (Wicklow et al. 1998) and Virginia (Griffin et al. 1981, 2001) and in a similar range to those reported in many southeastern US soils (Horn and Dorner 1998) and Arizona soils (Orum et al. 1997). In the present study, the top 0–5 cm of soil was sampled, while other investigators sampled at greater depths. *Aspergillus flavus* soil populations were lowest in the spring of 2000 after a cotton crop and intermediate in the established wheat crop in 2002 (Table 1). About 13% of the plots sampled in spring 2000 had *A. flavus* populations below the detection limit ($\log 1.9 \text{ cfu}\cdot\text{g}^{-1} \text{ soil}$), compared with 4% or none in 2001 and 2002, respectively (Fig. 1). Populations increased three-fold in the spring following a corn crop compared with those after a cotton crop. Soil *A. flavus* populations were closer to a normal distribution in 2002 samples (coefficient of skewness = -0.22) than those from soils sampled in 2000 and 2001 (coefficient of skewness = 0.42 and -0.55 , respectively; Fig. 1). Similarly soil populations of *A. flavus* increased in a Virginia minimum tilled field the year following

Fig. 1. Histogram of *Aspergillus flavus* propagule density distribution in soil sampled from 126 subplots in 2000, 2001, and 2002. Samples were collected in the spring following a cotton crop (2000) and a corn crop (2001), and during a wheat crop (2002).



a corn crop, and to a lesser extent following a peanut crop (Griffin et al. 1981).

Analysis of semivariance for soil *A. flavus* populations and corn AF levels is presented in Tables 2 and 3. For brevity, semivariograms are not shown. Soil *A. flavus* populations in the spring of 2000 and corn AF levels exhibited nugget behavior (linear model), with a random, very patchy distribution of *A. flavus* propagules having no spatial structure (linear model). A moderate degree of spatial structure (i.e., some pattern in occurrence), nugget or random occurrence about 50% of total variance, was observed for soil

A. flavus populations in 2001 (following corn) and 2002 (Table 2). In 2000 populations of *A. flavus* in soil were randomly distributed within the field (Fig. 2a), while in 2001 they were greatest in the southern part of the field (greater than one standard deviation compared with the central part of field (Fig. 2b) and in 2002 they were greatest in three of the four corners of the field (greater than one standard deviation compared with the northern part; Fig. 2c). These changes in maps of *A. flavus* soil propagule densities indicate that *A. flavus* populations were dynamic during the study, even under no-tillage practices where there is minimal disturbance to the soil surface.

Colonization of corn kernels by *A. flavus* was assessed by two methods: direct plating on MDRB (Fig. 3a) and determination of AF levels by ELISA (Fig. 3b). Aflatoxin level was highly variable in corn samples, ranging from 0 to 1590 ppb, with a mean of 57 ppb. Aflatoxin was not detected in 25% of the corn samples, while about 34% of the corn samples assayed had greater than 20 ppb AF, the level set by US Food and Drug Administration guidelines as indicating a significant risk for livestock consumption (CAST 2003). Moderate spatial structure was observed for *A. flavus* colonization of corn (Table 3). Assessment of *A. flavus*-colonized corn via direct plating on MDRB also indicated wide variability of *A. flavus* colonization (0%–100% with a mean of 15% colonized kernels). *Aspergillus flavus* was not recovered from about 37% of the samples, while about 10% of the samples had more than 50% of the seed colonized by *A. flavus*. Aflatoxin levels were greatest in the southeastern corner of the field, while *A. flavus* colonization was greatest in the western portion of the field. *Aspergillus flavus* colonization and AF levels were not correlated, as most of the samples having the highest incidence of *A. flavus* had little or no detected AF. The southeastern corner of the field had the highest sand content, while the western 25 m received some irrigation from a neighboring central pivot system. Aflatoxin levels in corn are generally greatest under drought stress (Abbas et al. 2002), thus the sandier soils would have a greater potential for AF contamination. The incidence of *A. flavus* propagules recovered from corn harvested in soils that had received additional water from the adjacent central pivot irrigation system was two- to four-fold more frequent than in kernels from other parts of the field (Fig. 3a). Based upon these observations, corn AF levels appeared to be associated with moisture status.

Aspergillus isolates were assessed for AF production based upon color formation following exposure to ammonia vapor (Table 4). Based on this assay, *A. flavus* isolates from corn had a higher frequency of being toxigenic ($P < 0.001$) than those from soils. In addition to *A. flavus* inoculum from soil, much of the inoculum for corn colonization may also have originated off site, e.g., from wind and insect vectors (Wicklow et al. 1998). As determined by the χ^2 statistic, there was a highly significant difference in frequency of toxigenic isolates during the 3 years of the study. *Aspergillus flavus* in soil isolates collected in the spring of 2002, following a corn crop, had a greater frequency of being toxigenic than it did in soil isolates collected following a cotton crop or during a wheat crop. These observations also indicate that AF-producing isolates are selectively enriched by corn (based on an analysis of 1 year of data) and may be respon-

Table 2. Parameters for spatial variogram models of *Aspergillus* soil populations for three yearly samples taken in the spring following a cotton crop (2000) and a corn crop (2001), and during a wheat crop (2002).

Year	Model	Nugget (Co)	Sill (C+Co)	Ratio Co/(C+Co)	Range Ao (m)	R ²
2000	Linear	0.116	0.129	0.899	495	0.122
2001	Spherical	0.126	0.254	0.502	136	0.957
2002	Exponential	0.0175	0.0368	0.523	19.5	0.701

Fig. 2. Kriged maps of *Aspergillus flavus* propagule density distribution in soil sampled from a 1.07-ha section of a commercial field. Samples were collected in the spring following a cotton crop in 2000 (A), following a corn crop in 2001 (B), and during a wheat crop in 2002 (C).

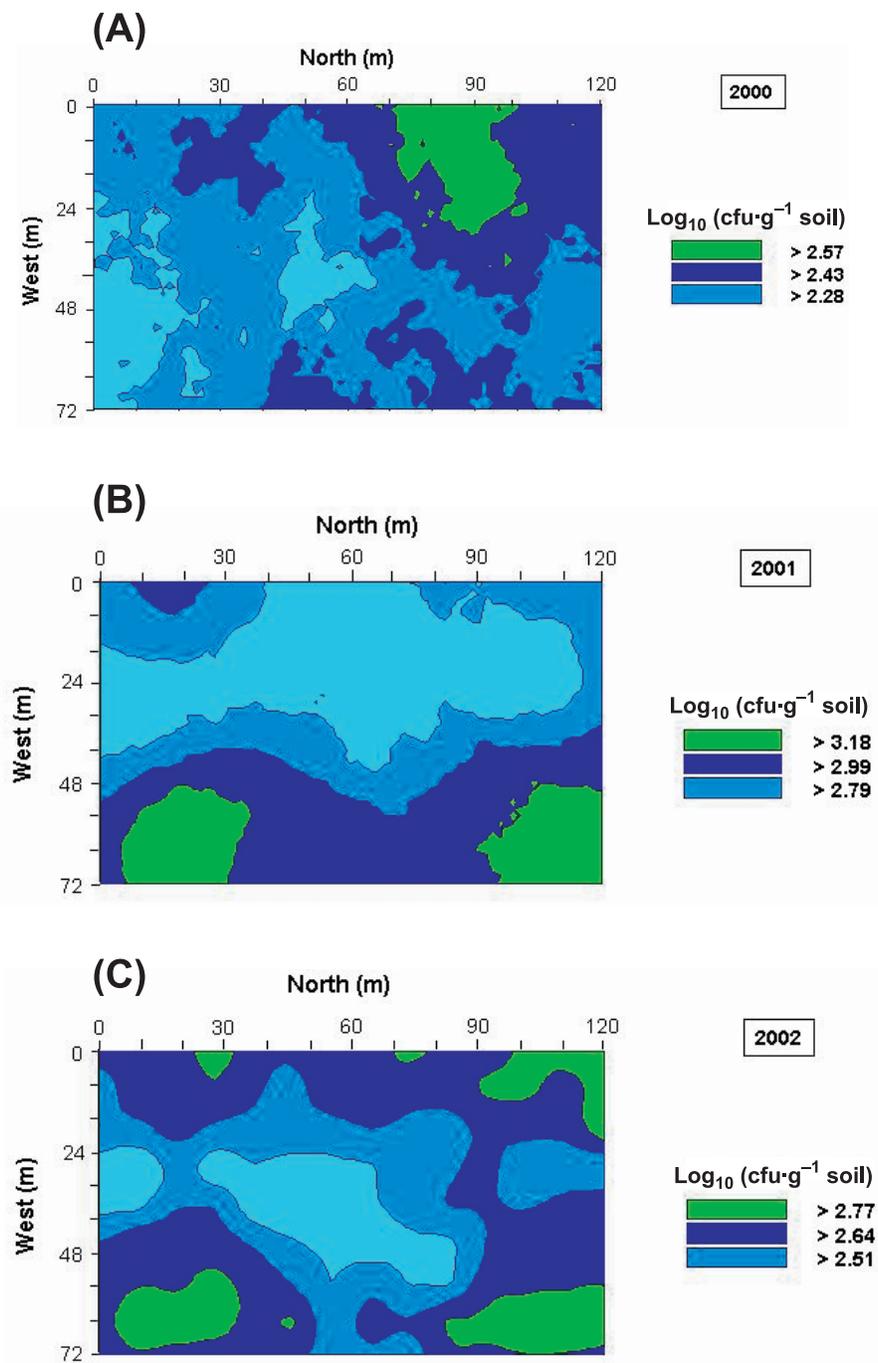


Fig. 3. Kriged maps of aflatoxin concentration (A) and incidence of *Aspergillus flavus* contamination (B) of corn sampled from a 1.07-ha section of a commercial field in 2000.

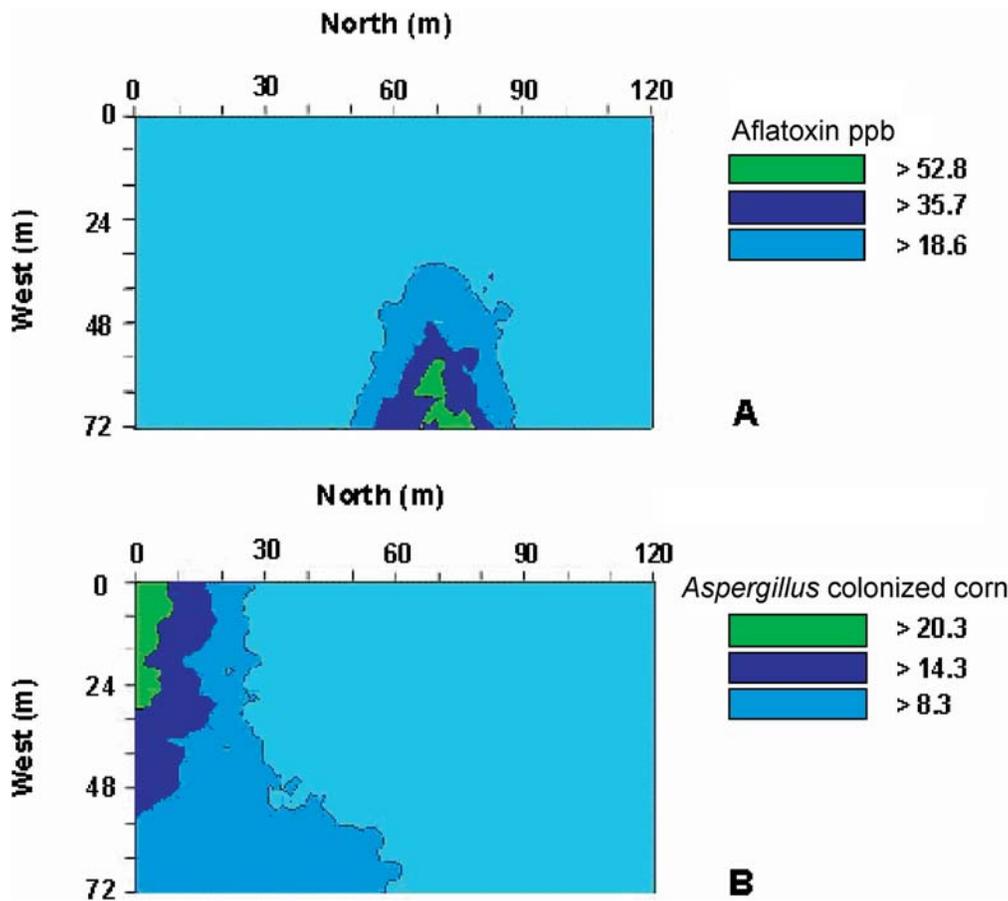


Table 3. Parameters for spatial variogram models of *Aspergillus* colonization and aflatoxin concentration in corn kernels (2000).

	Model	Nugget (Co)	Sill (C+Co)	Ratio Co/(C+Co)	Range Ao (m)	R ²
<i>Aspergillus</i> infection	Exponential	1.936	3.873	0.500	210.0	0.467
Aflatoxin	Exponential	2.6290	5.716	0.540	132	0.943

Table 4. Aflatoxin production by *Aspergillus* isolates from soil and corn kernels as determined by ammonia vapor assays.

Source	Number of isolates tested	Aflatoxin producers (%)
Soil, 2000	338	46*
Soil, 2001	444	59
Soil, 2002	482	43
Soil, 2000–2002	1123	43
Corn, 2000	186	84

*A significant deviation according to a maximum likelihood χ^2 test from the overall distribution at the $P = 0.001$ level comparing various years of soil sampling and frequency of soil isolates with frequency of corn isolates.

sible for subsequent enrichment of toxigenic isolates in soil. The temporal and spatial patterns of *A. flavus* colonization of S and L forms in Arizona soils were characterized by

Orum et al. (1997). Their research indicated that *A. flavus* soil populations increased under cotton when compared with either wheat or lettuce (*Lactuca sativa* L.) and that the S forms had a greater potential for AF production. Also the highest incidence of S forms occurred during cotton boll formation and infection by *A. flavus*.

Decreased profit margins for cotton production in the Mississippi Delta have encouraged growers to implement a rotational system, where cotton was traditionally grown in monoculture. Corn is desirable for use in a crop rotation with cotton because it reduces many pests that attack cotton, specifically reniform nematodes (*Rotylenchulus reniformis* Linford and Oliveira) (Gazaway et al. 2000; Windham and Lawrence 1992). Soil populations of *A. flavus* undergo dynamic changes that relate to cropping history. In this study, crop rotation affected the spring *A. flavus* soil populations, with the lowest populations occurring following cotton and wheat crops. All fields were sampled during the spring; however, there were differences in weather around the time

of sampling that may have contributed to these variations in populations. Cropping pattern may play an important role in reducing the source of inoculum of this microorganism, since the soil population plays an important role in *A. flavus* colonization of hosts (Horn 2003). Dorner et al. (1999) showed that the combination of non-aflatoxigenic isolates of *Aspergillus* species and crop rotation reduced host contamination by AF significantly (reduction in AF ranged from 66% to 87%). Biological control of *A. flavus* by inoculating the soil with nontoxicogenic *A. flavus* strains is one strategy under consideration for cotton, corn, and peanut production (Cotty 1990; Brown et al. 1991; Abbas et al. 2003; Dorner et al. 1998). Information from studies like this may provide a better understanding of the relationships among cropping systems, the *A. flavus* toxigenicity, and propagule density of *A. flavus* in order to implement biological control strategies to reduce colonization of crops by this fungus.

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