Regional Differences in Production of Aflatoxin B_1 and Cyclopiazonic Acid by Soil Isolates of *Aspergillus flavus* along a Transect within the United States

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Soil isolates of *Aspergillus flavus* from a transect extending from eastern New Mexico through Georgia to eastern Virginia were examined for production of aflatoxin B_1 and cyclopiazonic acid in a liquid medium. Peanut fields from major peanut-growing regions (western Texas; central Texas; Georgia and Alabama; and Virginia and North Carolina) were sampled, and fields with other crops were sampled in regions where peanuts are not commonly grown. The *A. flavus* isolates were identified as members of either the L strain ($n = 774$), which produces sclerotia that are >400 μm in diameter, or the S strain ($n = 309$), which produces numerous small sclerotia that are <400 μm in diameter. The S-strain isolates generally produced high levels of aflatoxin B_1, whereas the L-strain isolates were more variable in aflatoxin production; variation in cyclopiazonic acid production also was greater in the L strain than in the S strain. There was a positive correlation between aflatoxin B_1 production and cyclopiazonic acid production in both strains, although 12% of the L-strain isolates produced only cyclopiazonic acid. Significant differences in production of aflatoxin B_1 and cyclopiazonic acid by the L-strain isolates were detected among regions. In the western half of Texas and the peanut-growing region of Georgia and Alabama, 62 to 94% of the isolates produced >10 μg of aflatoxin B_1 per ml. The percentages of isolates producing >10 μg of aflatoxin B_1 per ml ranged from 0 to 52% in the remaining regions of the transect; other isolates were often nonaflatoxigenic. A total of 53 of the 126 L-strain isolates that did not produce aflatoxin B_1 or cyclopiazonic acid were placed in 17 vegetative compatibility groups. Several of these groups contained isolates from widely separated regions of the transect.

Peanuts, corn, and cottonseed are often invaded before harvest by *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare, fungi that produce carcinogenic aflatoxins. Aflatoxins are highly regulated for both animal feed and food destined for human consumption (37). Of the naturally occurring aflatoxins, aflatoxin B_1 is the most toxic (10). *A. flavus* also may produce cyclopiazonic acid (CPA), which is toxic to a variety of animals and has been implicated in human poisoning (4, 32). CPA and aflatoxins commonly occur together in contaminated agricultural commodities (25, 36). In corn and cottonseed, aflatoxin contamination is largely attributable to *A. flavus* (11, 35). Although peanuts are invaded by *A. parasiticus* more often than other crops, *A. flavus* also is the dominant aflatoxigenic species in peanuts (15, 17).

The following two strains of *A. flavus* are recognized: the L strain, which produces sclerotia that are >400 μm in diameter; and the S strain, also described as *A. flavus* var. *parvisclerotigenus* (34), which is characterized by numerous small sclerotia that are <400 μm in diameter (6). Populations of both strains comprise numerous subpopulations called vegetative compatibility groups (VCGs) (1, 18, 31). VCG 1 of *A. parasiticus* appears to be widely distributed in peanut-growing regions throughout the United States (16), but little is known about the distribution of *A. flavus* VCGs. Because isolates within a VCG are similar in their production of aflatoxins and CPA (2, 20), isolates belonging to the same VCG can be detected among isolates that have the same mycotoxin profile.

Regional differences in aflatoxin contamination of crops may be attributable to climatic conditions and to agricultural practices that increase the susceptibility of plants to invasion by *A. flavus*. Drought stress accompanied by elevated temperatures during seed development promotes *A. flavus* invasion and subsequent aflatoxin contamination of peanuts, corn, and cottonseed (14, 23, 24). However, the toxigenicity of *A. flavus* isolates within a region also may influence the severity of aflatoxin contamination of crops. *A. flavus* populations are extremely diverse genetically, and L-strain isolates vary considerably in their capacity to produce aflatoxins and CPA, with many isolates producing only one mycotoxin or neither mycotoxin (20, 21, 28, 33). Surveys of *A. flavus* isolates from various geographic regions have revealed differences in the proportions of isolates that produce low, medium, and high amounts of aflatoxins (8, 29). The factors responsible for the toxigenicity profile of *A. flavus* populations in a region are not understood (9), but the dominance of particular crops may be important in determining the relative proportions of *A. flavus* genotypes. Schroeder and Boller (35) examined aflatoxin production by *A. flavus* isolates from peanuts, cottonseed, rice, and sorghum in Texas. These workers found differences among crops in the percentage of aflatoxin producers, as well as in the concentrations of aflatoxins produced. They reported that the percentage of peanut isolates that produced aflatoxins was high and that these isolates produced high levels of aflatoxins. Other researchers also have reported that the proportion of aflatoxin-producing isolates of *A. flavus* from peanuts and peanut field soils is high (20, 22, 28).

Agricultural soil serves as a reservoir for populations of *A. flavus* (1, 8, 18, 19, 29). Peanuts are in direct contact with soil populations, whereas above-ground crops, such as corn and

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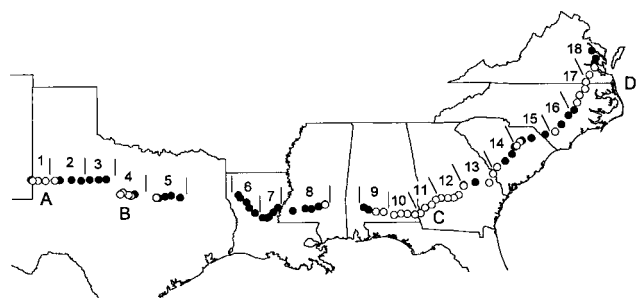
FIG. 1. Transect showing fields from which *A. flavus* soil isolates were obtained. The crops present in the fields included peanuts (○) (*n* = 40) and corn, cotton, or soybeans (●) (*n* = 43). The transect was divided into segments 1 to 18, each of which was 150 to 200 km long. The following major peanut-growing regions are indicated: western Texas (region A); central Texas (region B); Georgia and Alabama (region C); Virginia and North Carolina (region D).

TABLE 1. Production of aflatoxin B_1 and CPA by L- and S-strain isolates of *A. flavus* from the entire transect

Aflatoxin B_1 production	CPA production	% of isolates	
		L strain (*n* = 774)	S strain (*n* = 309)
+	+	70.7	98.7
ND[a]	ND	16.3	0.0
ND	+	12.4	0.3
+	ND	0.6	1.0

[a] ND, not detected.

cottonseed, may be infected with conidia from soil through dispersal by wind or insects (26, 27). Previously, we (16) examined soil populations of *Aspergillus* species belonging to section *Flavi* that were distributed along a transect extending from eastern New Mexico through Georgia to eastern Virginia. Peanut fields from four major peanut-growing regions in the United States (western Texas; central Texas; Georgia and Alabama; and Virginia and North Carolina) were included in the transect, as were fields with other crops in regions where peanuts are not commonly grown. *A. flavus* was the dominant species belonging to section *Flavi* along most of the transect, and both L-strain and S-strain isolates were present. There were significant differences among the peanut-growing regions in terms of the density and incidence of both strains in the soil.

Knowledge of regional differences in the toxigenicity of *A. flavus* populations, as well as knowledge of the association of these populations with the dominant crops in a region, may be important in determining which control measures are most effective in reducing preharvest aflatoxin contamination. For example, biological control of *A. flavus* in agricultural fields through application of an atoxigenic *A. flavus* strain to the soil (7, 9, 12) might be preferentially used in regions where the populations are most toxigenic. In this study, L- and S-strain isolates of *A. flavus* were obtained from the soil populations described by us previously (16) from a transect across the United States. These isolates were examined with the following objectives: (i) to determine whether *A. flavus* populations from different geographic regions differ in production of aflatoxin B_1 and CPA; and (ii) to characterize the distribution of several *A. flavus* VCGs over a wide geographic area.

MATERIALS AND METHODS

Transect fields, soil collection, and *A. flavus* isolation. Isolates of *A. flavus* were obtained during a study of soil populations of *Aspergillus* species belonging to section *Flavi* along a transect through major peanut-growing regions of the United States (16). The transect extended from eastern New Mexico through Georgia to eastern Virginia and comprised 83 fields (40 peanut fields, 22 cotton fields, 15 corn fields, and 6 soybean fields) (Fig. 1). In the peanut-growing regions, which included western Texas, central Texas, Georgia and Alabama, and Virginia and North Carolina, peanut fields were preferentially sampled along the transect at 15- to 25-km intervals; in the regions where peanuts are not commonly cultivated, corn, cotton, and soybean fields were sampled at 25- to 40-km intervals. Soil samples were collected from 17 to 28 June 1996 and were processed and dilution plated onto a modified dichloran-rose bengal medium as previously described (16). All crops were immature at the time of soil collection.

We prepared 40 dilution plates per field, and 10 colonies of *A. flavus* L strain and 10 colonies of *A. flavus* S strain (if available) were randomly selected from different plates. Conidia were transferred to 8 ml of sterile water containing 100 μl of Tween 20 per liter. After vortexing, each spore suspension (0.1 ml) was spread onto a plate containing modified dichloran-rose bengal medium, and the

plate was incubated for 25 to 30 h at 30°C. Germlings were transferred to Czapek agar slants and grown in the light to encourage sporulation.

The transect was divided in two ways in order to examine regional differences in mycotoxin production by the L strain of *A. flavus* (Fig. 1). First, the fields were grouped by defining 18 transect segments consisting of 150 to 200 km each. Second, the major peanut-growing regions were compared; these regions consisted of western Texas (portions of transect segments 1 and 2) (4 peanut fields, 28 isolates), central Texas (a portion of transect segment 4) (4 peanut fields, 40 isolates), Georgia and Alabama (transect segments 10 through 12) (13 peanut fields, 130 isolates), and Virginia and North Carolina (portions of transect segments 17 and 18) (6 peanut fields, 51 isolates).

VCGs. The 126 isolates that did not produce detectable aflatoxin B_1 or CPA were tested for vegetative compatibility. Five plates of Czapek agar supplemented with potassium chlorate (25 g/liter) were inoculated with a spore suspension of each isolate as described by Horn and Greene (18). One non-nitrate-utilizing sector on each plate was identified as a *niaD*, *nirA*, or *cnx* mutant. *cnx* mutants provide the strongest reactions between compatible isolates and are recommended as testers for identifying VCGs (5). *cnx* mutants were paired on a nitrate medium with complementary *niaD* and *nirA* mutants of all atoxigenic isolates. Formation of a stable prototrophic heterokaryon at the zone of interaction indicated that two isolates were members of the same VCG. The number designations used for VCGs were a continuation of previously described VCGs 1 to 63 of *A. flavus* (18, 31).

Mycotoxin analyses. Approximately 10^5 dry conidia of isolates of the *A. flavus* L strain (*n* = 774) and the *A. flavus* S strain (*n* = 309) were used to inoculate 4-ml vials containing 1 ml of liquid medium containing 150 g of sucrose, 20 g of yeast extract, 10 g of soytone, and 1 liter of distilled water; the pH of the medium was adjusted to 6.0 with HCl. One vial was inoculated per isolate; the atoxigenic isolates tested for vegetative compatibility were reexamined with an additional independent set of vial cultures. The cultures were incubated for 7 days at 30°C in the dark.

Vial cultures were analyzed by high-performance liquid chromatography for production of aflatoxin B_1 and CPA as previously described (20), except that aflatoxin B_1 was quantified with a Shimadzu Class VP chromatography laboratory automated software system instead of the data module. The limits of quantification were 0.5 ng of aflatoxin B_1 per ml of culture medium and 2 μg of CPA per ml of culture medium.

Statistics. To compare transect segments or major peanut-growing regions, the mycotoxin concentrations in vial cultures were analyzed by using the Kruskal-Wallis one-way analysis on ranks test (H statistic) and then Dunn's nonparametric multiple comparison test. The analyses were based on the number of isolates in each transect segment or peanut-growing region. The correlation coefficient (*r*) for aflatoxin B_1 production and CPA production was determined by using the Pearson product moment correlation method; the 126 L-strain isolates that did not produce detectable levels of either mycotoxin were not included in the correlation. The statistical analyses were performed by using SigmaStat, version 1.0 (Jandel Scientific, San Rafael, Calif.).

RESULTS

Isolates of the *A. flavus* L strain (*n* = 774) and the *A. flavus* S strain (*n* = 309) from field soils over a wide geographic area (Fig. 1) were examined for production of aflatoxin B_1 and CPA. A large percentage of the L-strain isolates produced both mycotoxins (Table 1). However, a sizable percentage of isolates were atoxigenic (there was no detectable aflatoxin B_1 or CPA) or produced only CPA; isolates that produced aflatoxin B_1 but not CPA were rare (0.6%). Nearly all of the S-strain isolates produced both aflatoxin B_1 and CPA. No atoxigenic S-strain isolates were observed. There was a positive correlation between production of aflatoxin B_1 and production
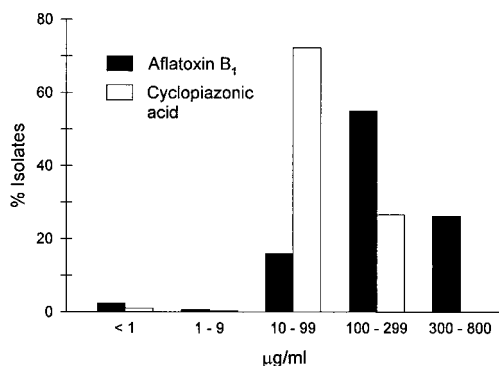
FIG. 2. Production of aflatoxin B_1 and CPA by S-strain isolates of *A. flavus* ($n = 309$) from fields along the entire transect.

of CPA for both the L strain ($r = 0.54$; $n = 648$; $P < 0.0001$) and the S strain ($r = 0.29$; $n = 309$; $P < 0.0001$).

Nearly all of the S-strain isolates from the entire transect produced >10 µg of aflatoxin B_1 and CPA per ml of culture medium, and 26% of the S-strain isolates produced >300 µg of aflatoxin B_1 per ml (Fig. 2). In contrast, the aflatoxin B_1 and CPA concentrations produced by the L-strain isolates varied considerably more, and many L-strain isolates produced <10 µg of the mycotoxins per ml (Fig. 3 and 4). No L-strain isolate produced >300 µg of aflatoxin B_1 per ml.

The transect was divided into segments consisting of 150 to 200 km to assess regional differences in the production of aflatoxin B_1 and CPA by L-strain isolates of *A. flavus* (Fig. 1). There were significant differences among transect segments in aflatoxin B_1 production by the L-strain isolates (H = 219; 17 df; $P < 0.0001$) (Fig. 3). In the western half of Texas (transect segments 1 through 4) and the peanut-growing region of Georgia and Alabama (transect segments 10 through 12), 62 to 94% of the L-strain isolates produced >10 µg of aflatoxin B_1 per ml. In other regions along the transect, 0 to 52% of the L-strain isolates produced >10 µg of aflatoxin B_1 per ml, and the remaining isolates produced low levels of aflatoxin B_1 or were nonaflatoxigenic. Significant differences among transect segments also were detected for CPA production by the L-strain isolates (H = 141; 17 df; $P < 0.0001$) (Fig. 4). The differences in CPA production were less apparent than the differences in aflatoxin B_1 production, but the same transect segments in western Texas and the peanut-growing region of Georgia and Alabama tended to have higher percentages of isolates that produced >10 µg of CPA per ml.

Statistical analyses also revealed significant differences among the four major peanut-growing regions (Fig. 1) in production of aflatoxin B_1 (H = 64; 3 df; $P < 0.0001$) and production of CPA (H = 47; 3 df; $P < 0.0001$) by L-strain isolates. The aflatoxin B_1 production by L-strain isolates from Georgia and Alabama was significantly greater ($P < 0.05$) than the aflatoxin B_1 production by isolates from central Texas and from Virginia and North Carolina but not the aflatoxin B_1 production by isolates from western Texas. The L-strain isolates from Virginia and North Carolina produced significantly less aflatoxin B_1 than the L-strain isolates from the other three peanut-growing regions. The CPA production by isolates from Virginia and North Carolina was significantly less than the CPA production by isolates from western Texas, central Texas, and Georgia and Alabama.

Of the 774 L-strain isolates of *A. flavus* examined from the transect, 126 were atoxigenic. All of the atoxigenic isolates
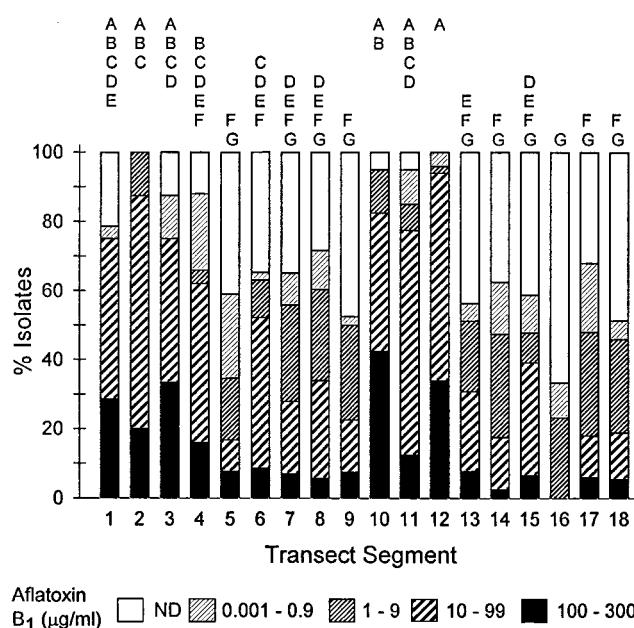

FIG. 3. Production of aflatoxin B_1 by L-strain isolates of *A. flavus* ($n = 774$) from fields in 18 transect segments, each of which was 150 to 200 km long. The transect segments are shown in Fig. 1. The numbers of fields and numbers of L-strain isolates examined were as follows: transect segment 1, four fields and 28 isolates; transect segment 2, four fields and 40 isolates; transect segment 3, three fields and 24 isolates; transect segment 4, five fields and 50 isolates; transect segment 5, eight fields and 78 isolates; transect segment 6, five fields and 46 isolates; transect segment 7, five fields and 43 isolates; transect segment 8, six fields and 53 isolates; transect segment 9, four fields and 40 isolates; transect segment 10, four fields and 40 isolates; transect segment 11, four fields and 40 isolates; transect segment 12, five fields and 50 isolates; transect segment 13, four fields and 39 isolates; transect segment 14, four fields and 40 isolates; transect segment 15, five fields and 46 isolates; transect segment 16, three fields and 30 isolates; transect segment 17, five fields and 50 isolates; and transect segment 18, five fields and 37 isolates. Transect segments not sharing a common letter are significantly different ($P \leq 0.05$) according to Dunn's test on ranks of aflatoxin B_1 concentrations. ND, not detected.

produced *nirA* and/or *niaD* mutants, but only 23 produced *cnx* mutants. The *cnx* mutants were paired with complementary mutants of all isolates, which resulted in 53 isolates that were distributed among 17 VCGs (Table 2). The remaining 73 isolates that were incompatible with the *cnx* mutants were not examined further for vegetative compatibility. VCGs 24 and 64 to 70 were detected in fields in different states along the transect. VCG 24, an atoxigenic group, had previously been found in a Georgia peanut field (18, 20). VCGs 71 to 79 were detected in only one field. Two or more compatible isolates from the same field were observed for VCGs 64, 66, and 71 to 74.

DISCUSSION

Regional differences in production of aflatoxin B_1 and CPA by soil isolates of the *A. flavus* L strain were evident along the transect (Fig. 3 and 4). Surveys done in Japan (29) and in cotton-growing regions of the United States (8) also have revealed geographic differences in production of aflatoxin B_1 by *A. flavus*. In this study, the toxigenicity of the L-strain isolates of *A. flavus* exhibited little association with dominant crops over most of the transect. Although the crop histories of the fields sampled were not known, the crops present were considered indicators of the types of crops traditionally grown in a region. For transect segments 1 through 4 (Fig. 1), the per-
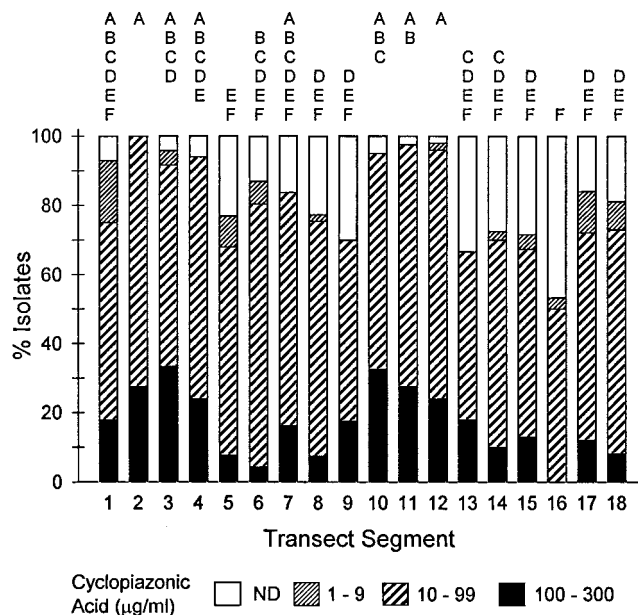
FIG. 4. Production of CPA by L-strain isolates of *A. flavus* ($n = 774$) from fields in 18 transect segments, each of which was 150 to 200 km long. The transect segments are shown in Fig. 1. The numbers of fields and numbers of isolates examined are given in the legend to Fig. 3. Transect segments not sharing a common letter are significantly different ($P \leq 0.05$) according to Dunn's test on ranks of CPA concentrations. ND, not detected.

centages of isolates that produced >10 μg of aflatoxin B_1 per ml were relatively high (Fig. 3) despite differences in the major crops, which were largely peanuts for transect segments 1 and 4 and cotton for transect segments 2 and 3. Transect segments 13 through 18 also were characterized by different dominant crops, including peanuts for transect segment 17 and varying proportions of corn, cotton, soybeans, and peanuts for transect

segments 13 through 16 and 18; however, the mycotoxin production data for all of these segments were similar. A possible exception to these findings was the data obtained for the major peanut-growing region of Georgia and Alabama (transect segments 10 through 12). In this region, the high percentages of L-strain isolates that produced >10 μg of aflatoxin B_1 and CPA per ml were associated with extensive peanut cultivation. This region also had a higher mean soil density of *A. flavus* L-strain isolates (247 CFU/g) than the other major peanut-growing regions, including western Texas (7 CFU/g), central Texas (103 CFU/g), and Virginia and North Carolina (29 CFU/g) (16).

The dominance of highly toxigenic L-strain isolates and the occurrence of these isolates at relatively high soil densities in the peanut-growing region of Georgia and Alabama may not be coincidental. Drought stress accompanied by elevated soil temperatures, conditions that are conducive to *A. flavus* invasion of peanuts and subsequent aflatoxin contamination (13), is not uncommon in nonirrigated fields in Georgia and Alabama (14). The soil densities of *A. flavus* in these fields most likely reflect crop colonization over years of cultivation due to the dispersal of spores and infected crop debris to the soil (19, 38). In addition, the high percentages of aflatoxigenic isolates of *A. flavus* observed previously in peanuts and peanut field soils (20, 22, 28, 35) suggest that peanut cultivation selects for aflatoxin producers. The combined climatic and crop selection pressures in Georgia and Alabama could account for the dominance of highly aflatoxigenic isolates in this region.

The results of our examination of *A. flavus* isolates from a wide geographic area supported previous findings which showed that the S strain produces higher levels of aflatoxin B_1 and shows less variation in aflatoxin B_1 production than the L strain (6, 8). CPA production also was less variable in the S strain than in the L strain, and very few S-strain isolates produced <10 μg of CPA per ml (Fig. 2). The S strain has been associated with cotton cultivation in portions of the southern United States (8, 30). In the fields along the transect described here, the S strain was most prevalent in west central Texas and

TABLE 2. Geographic distribution of VCGs containing atoxigenic isolates of the *A. flavus* L strain[a]

VCG[b]	No. of soil isolates	No. of fields							
		Texas	Louisiana	Mississippi	Alabama	Georgia	South Carolina	North Carolina	Virginia
24	2					1		1	
64	11			1	1	1	1	2	3
65	7	1			1	1	1	1	2
66	7	1						1	
67	5	1		1		2	1		
68	4		1				1	2	
69	3			1		1		1	
70	2	1			1				
71	2	1							
72	2			1					
73	2				1				
74	2							1	
75	1	1							
76	1	1							
77	1				1				
78	1						1		
79	1							1	

[a] The isolates produced no detectable aflatoxin B_1 or CPA. VCGs were determined by pairing *cnx* mutants obtained from 23 isolates with *niaD* and/or *nirA* mutants obtained from all 126 atoxigenic isolates. Seventy-three of these isolates were not placed in VCGs.

[b] VCG 24 from a Georgia peanut field was described previously (18, 20), and the one soil isolate obtained in the previous studies was included in this VCG along with one North Carolina isolate obtained in the present study. The designations used for the other VCGs were a continuation of previously determined VCGs 1 to 63 of *A. flavus* (18, 31).

Louisiana, where cotton is grown extensively (16). Because of the small amount of variability in aflatoxin B_1 and CPA production by the S strain, as well as its restricted distribution, regional differences in mycotoxin production were not examined.

Significant positive correlations between aflatoxin B_1 production and CPA production were detected for both the L strain and the S strain. Horn et al. (20) previously reported a positive correlation between the two mycotoxins for L-strain isolates from a peanut field in Georgia, in contrast to other studies that showed either no correlation or a negative correlation (3, 21). In the L strain, the positive correlation accounts for the similar regional patterns of production of aflatoxin B_1 and CPA (Fig. 3 and 4). Although the correlations were statistically significant for samples containing a large number of isolates, individual isolates exhibited considerable differences in production of the two mycotoxins, and in some instances, a correlation was not evident. This was best illustrated by the L strain; 12% of the L-strain isolates produced CPA but not aflatoxin B_1.

Populations of *A. flavus* are extremely diverse genetically and include numerous VCGs (1, 18, 31). Since isolates belonging to the same VCG are similar in production of mycotoxins (2, 20), L-strain isolates that did not produce detectable aflatoxin B_1 or CPA were specifically examined for vegetative compatibility in order to identify VCGs among the large number of isolates along the transect. Even within this chemotype of *A. flavus*, there was considerable diversity among the 53 isolates placed in VCGs, which was reflected by a diversity value (number of VCGs divided by number of isolates) of 0.32. This diversity also was indicated by the presence of many single-isolate VCGs, as well as by the 73 isolates that were not compatible with any of the 17 VCGs identified in this study (Table 2). VCGs of *A. flavus* were often detected in geographically distant portions of the transect. Previously, we (16) showed that VCG 1 of *A. parasiticus* was present in peanut fields along most of this transect. These data may indicate that *A. flavus* and *A. parasiticus* genotypes are widely dispersed, but this interpretation assumes that isolates belonging to the same VCG are clonal and genetically identical. *A. flavus* isolates belonging to the same VCG occasionally differ in morphology (20) and in random amplified polymorphic DNA (2). The geographically separated representatives of a VCG in this study may have diverged from one another while still maintaining the same alleles at loci that govern vegetative compatibility.

Application of atoxigenic strains of *A. flavus* and/or *A. parasiticus* to agricultural soil has been used to control aflatoxin contamination of crops by native populations of these two species. Aflatoxin reduction has been reported for peanuts and cottonseed (7, 12). The results of the present study suggest which regions along the transect might benefit most from soil application of an atoxigenic L-strain isolate of *A. flavus*. The majority of the L-strain isolates from the western half of Texas and the peanut-growing region of Georgia and Alabama produced moderate to high levels of aflatoxin B_1 (Fig. 3), and it is in these regions that introduction of an atoxigenic strain might result in the greatest reduction in aflatoxin contamination in nonirrigated fields. In contrast, regions along the transect from central Texas to south central Alabama, as well as north of south central Georgia, contain soil populations with sizable percentages of L-strain isolates that do not produce aflatoxin B_1 (Fig. 3). Native nonaflatoxigenic strains in such regions may provide some degree of control of aflatoxin contamination due to toxigenic strains.

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