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Effect of competition and adverse culture conditions on aflatoxin production by *Aspergillus flavus* through successive generations

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Abstract: Strains of *Aspergillus flavus* often degenerate with serial transfers on culture media, resulting in morphological changes and loss of aflatoxin production. However, degeneration does not readily occur in nature as indicated by the wild-type morphological characters of newly isolated strains and the high percentage of aflatoxigenic *A. flavus* from soil and crops in some geographic regions. In this study, three aflatoxin-producing strains of *A. flavus* were serially transferred using conidia for 20 generations (three independent generation lines per strain) on potato dextrose agar at 30 C. The rate of degeneration was compared to that of cultures grown in the presence of competing fungi (*A. terreus*, *Penicillium funiculosum*, and the yeast, *Pichia guilliermondii*) and under adverse conditions of elevated temperature, reduced water activity, low pH, and nutrient deprivation. Formation of morphological variants and the associated loss of aflatoxin production over generations varied considerably according to strain and the generation line within each strain. In the strain most sensitive to degeneration on potato dextrose agar, aflatoxin-producing ability was maintained to varying degrees under adverse culture conditions, but not when *A. flavus* was competing with other fungi.

Key Words: conidium, sclerotium, serial transfer

INTRODUCTION

Peanuts, corn, cottonseed, and tree nuts are commonly invaded by *Aspergillus flavus* and *A. parasiticus*, fungi that contaminate agricultural commodities with acutely toxic and carcinogenic aflatoxins. Aflatoxins primarily affect the liver, and several outbreaks of aflatoxicosis have been documented in rural human populations in tropical countries (Peraica et al 1999). Of the four major aflatoxins (B_1, B_2, G_1, G_2), aflatoxin B_1 is the most toxic to animals (Cullen and Newberne 1994). Although *A. flavus* and *A. parasiticus* are easily grown under laboratory conditions, they are notorious for their genetic instability when repeatedly transferred in culture (Kale and Bennett 1992). Serial transfers often result in loss of aflatoxin production and in associated morphological changes such as reduced sporulation, proliferation of aerial hyphae, and an inability to produce sclerotia (Mayne et al 1971, Torres et al 1980, Bennett 1981, Bennett et al 1981, Bilgrami et al 1988, Clevström and Ljunggren 1985, Kale et al 1994).

Conidia of aflatoxigenic fungi are readily dispersed by wind and insects (Lillehoj et al 1980, Holtmeyer and Wallin 1981). Through dispersal, individual genotypes are repeatedly transferred to new substrates over time yet show no evidence of the degeneration resulting from serial transfers in the laboratory. Freshly isolated strains from nature always exhibit wild-type morphological characters (Wicklow 1983), and some regions of the United States have a high percentage (>95%) of *A. flavus* isolates that produce aflatoxins (Horn and Dorner 1999). In all geographic regions examined, *A. parasiticus* strains are high aflatoxin producers and nonproducers are rare (Horn et al 1996, McAlpin et al 1998). Therefore, the wild-type morphological characters of natural populations as well as the occurrence of populations that are predominantly aflatoxigenic argue against the degeneration of genotypes with a long history of dispersal.

Bilgrami et al (1988) have postulated that aflatoxin production and wild-type morphological characters are maintained in nature by competition with other microorganisms and by exposure to suboptimal growth conditions and that the degeneration of cultures in the laboratory is due to an absence of these factors. To test this hypothesis, three aflatoxin-producing strains of *A. flavus* were serially transferred using conidia for 20 generations on a nutrient-rich medium. The rate of degeneration was compared to that of cultures serially transferred in the presence of competing fungi and under adverse conditions of elevated temperature, reduced water activity, low pH, and nutrient deprivation.

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MATERIALS AND METHODS

Fungal strains.—*A. flavus* NRRL 29459, NRRL 29474, and NRRL 29499 originated from soil collected in a peanut field (Terrell Co., Georgia, USA) and belong to vegetative compatibility groups 6, 23, and 28, respectively (Horn and Greene 1995). All strains were derived from single conidia. The fungal competitors consisted of *A. terreus* NRRL 31139 and *Penicillium funiculosum* NRRL 31138, both isolated from a peanut seed in Georgia, and the yeast *Pichia guilliermondii* NRRL Y-2075 (*ex type*) (anamorph = *Candida guilliermondii*). The three competitor species commonly cooccur with *A. flavus* on preharvest crops (Hesseltine et al 1976, Horn 1985, Horn et al 1995).

Culture conditions.—Fungi were grown on slants consisting of 20 × 125-mm screw-cap test tubes containing 7 mL of agar medium. Potato dextrose agar (PDA) (Difco Laboratories, Detroit, Michigan), which has a water activity (A_w) of 0.99 ± 0.001 (± SD; n = 10), was used for the control and the fungal competition treatments. For treatments involving adverse growth conditions, preliminary experiments were conducted to determine the limits of growth that still allowed for sporulation within a 14-d period. The high temperature treatment consisted of PDA slants incubated at 42 C. The low pH treatment comprised PDA that was adjusted to pH 2.5 with HCl after sterilization (precooled to 50 C) and then added to sterile test tubes. For the low A_w treatment, PDA was supplemented with additional glucose (676.4 g/L final concentration; A_w = 0.85 ± 0.004). Water agar (1.5%) was used for the low nutrients treatment. All cultures were grown in darkness and with the exception of the high temperature treatment, at 30 C.

Serial transfers.—The three strains of *A. flavus* were revived from silica gel by inoculating three Czapek agar (Cz) slants per strain (= generation 0). The three slants of a strain represented separate generation lines (A, B, C). For generation 1, dry conidia were obtained from three different areas of each Cz slant and combined in 5 mL of water containing 50 μL/L of Tween 20 (less volume when sporulation was sparse during subsequent generations) to give approximately 10^6 conidia/mL. Slants containing treatment media were then inoculated by spreading the conidial suspension (approximately 8 μL) over the entire medium surface with a transfer loop. Cultures were grown for 14 d before slants of treatment media for the next generation were inoculated as described for generation 1. The generation lines were carried through 20 generations.

To prepare inoculum of the competitors, *A. terreus* and *P. funiculosum* were grown on Cz slants for 14 d at 30 C. Conidia were scraped from the slants in sterile water with Tween 20, filtered through glass wool, and adjusted to three concentrations (5×10^4, 5×10^5, and 5×10^6 conidia/mL) with distilled water. A loopful of each suspension was evenly spread on the slants of PDA. It was previously determined that when these slants were immediately inoculated with *A. flavus*, the two competitors were excluded from the medium by *A. flavus*. Therefore, *A. terreus* and *P. funiculosum* were given a competitive advantage by first incubating the slants at 30 C for 16 and 13 h, respectively, before inoculation with *A. flavus* conidia. The slant with the competitor conidial concentration that resulted in <40% area of *A. flavus* sporulation after 14 d was used for the next generation. *Pichia guilliermondii* was grown for 3 d on PDA (30 C) and the cells were suspended in 5 mL of water (approximately 5×10^7 cells/mL). The yeast suspension was spread on PDA slants with a loop immediately before *A. flavus* inoculation.

The above inoculation method of spreading conidia over the entire medium surface was compared with two other methods for a period of 10 generations. *Aspergillus flavus* NRRL 29459 and NRRL 29499 (three generation lines each) were grown on PDA slants at 30 C and serially transferred at 14-d intervals. In one method, slants were single-point inoculated with a conidial suspension using a transfer needle. The other method involved single-point inoculation with sclerotia. Approximately 10 sclerotia were removed from a slant, vortexed 30 s in 10 mL of water with Tween 20 to remove conidia, filtered onto filter paper, and rinsed with water. The outer surface of sclerotia with adhering conidia and mycelium was then abraded by vortexing for 15 min in a 25 × 150-mm test tube containing 5 mL of water with Tween 20 and 10 g of glass beads (4-mm diameter). Sclerotia were surface-sterilized with 0.25% sodium hypochlorite (2 min), rinsed 3× with sterile water, and placed on sterile filter paper. A single sclerotium was transferred to the center of a PDA slant.

Colony characters.—Conidia from slant cultures representing generations 0, 2, 4, 7, 10, 15, and 20 were transferred to vials containing 0.2 mL of 0.2% water agar with Tween 20. Two Cz plates were three-point inoculated with each conidial suspension and incubated for 7 d at 30 C in darkness. After colonies were photographed and notes on their appearance were made, the plates were sprayed with 70% ethanol to remove conidia and expose the sclerotia. Two colonies from each plate were then delimited by a 3.45-cm diameter circle that was centered at the point of inoculation. All sclerotia were counted within the circle boundary (area = 9.3 cm^2).

Aflatoxin analyses.—Two replicate vials containing 1 mL of modified YES broth (mYES) (150 g sucrose, 20 g yeast extract, 10 g soytone, 1 L distilled water; pH adjusted to 6.0 with HCl) were inoculated with dry conidia from two different areas in the slant cultures (generations 0, 2, 4, 7, 10, 15, and 20) and incubated for 7 d at 30 C in darkness as described by Horn and Dorner (1999). Vial cultures were analyzed for aflatoxin B_1 using high performance liquid chromatography according to the techniques of Horn et al (1996) except that aflatoxin B_1 was quantified with a Shamadzu Class VP chromatography laboratory automated system instead of the data module. The limit of quantification was 0.5 nm of aflatoxin B_1 per mL of culture medium.

Aflatoxin analyses also were performed on single-spore isolates to determine the frequency of aflatoxin-producing abilities at different generations for NRRL 29474 (low A_w, generation line A; high temperature, generation line C) and NRRL 29499 (low nutrients, generation line B; PDA control, generation line B). Slant cultures were flooded with water containing Tween 20, and conidia were scraped

from the surface with a transfer loop. A series of conidial dilutions in water were plated on mYES with 1.5% agar (0.1 mL/plate) and incubated at 30 C until germlings were detected under the stereomicroscope (12–19 h). Thirty randomly selected germlings were transferred to vials containing 1 mL of mYES with 0.2% agar added to keep germlings on the medium surface. Vials were incubated for 7 d at 30 C. Immediately before aflatoxin extraction, conidia from vial cultures were transferred to Cz plates to examine the colony morphology. Aflatoxin B_1 was extracted and quantified as previously described.

Statistics.—Nonparametric Spearman correlation coefficients (r_s) for aflatoxin B_1 concentration (μg/mL) verses sclerotium number were determined using SAS statistical package version 8 (SAS Institute, Cary, North Carolina). Mean values for aflatoxin B_1 concentration (n = 2) and sclerotium number (n = 4) were used for the correlations.

RESULTS

Colony morphology.—Growth and sporulation by *A. flavus* were greatly restricted on slants during treatments at high temperature, low pH, and low A_w compared to the control (PDA at 30 C). For the treatment involving low nutrients (1.5% water agar), mycelial growth was extremely diffuse and conidial heads were sparse. The colonization of PDA slants by *A. flavus* in the competition treatments differed according to the species of fungal competitor. Conidial heads of *A. flavus* and *A. terreus* were often intermixed when the two species were grown together. In contrast, *A. flavus* typically was restricted to one or two homogeneous patches, often at the bottom of the slant, when competing with *P. funiculosum*. *Pichia guilliermondii* formed a continuous yeast layer on the medium surface from which *A. flavus* sporulated in an unrestricted manner.

Morphological changes in *A. flavus* during serial transfers were assessed by inoculating Cz plates with conidia from the various treatment slants. The three *A. flavus* strains at generation 0 all formed sclerotia. NRRL 29474 and 29499 produced 231 ± 59.3 and 177 ± 46.7 sclerotia per 9.3 cm^2 of medium surface (± SD; n = 96), respectively, and showed considerable sporulation. NRRL 29459 was predominantly sclerotial (393 ± 36.8) and sporulation was sparse (FIG. 1). Several variant colony types were associated with the loss of aflatoxin B_1 production in subsequent generations (FIGS. 1, 2). The following generalized variant colony types were observed: type *m,* membranous and radially furrowed with sparse to moderate sporulation; type *f,* floccose with sparse to moderate sporulation; type *v,* velvety and densely sporulating. In colony type *f,* sporulation occasionally increased during serial transfers (FIG. 1; generation line C). The three variant colony types were all characterized by a loss of sclerotium production. In instances where sporulation was heavy enough to assess color, conidia *en masse* often were browner than those of wild-type colonies. *Aspergillus flavus* NRRL 29474 converted mostly to colony type *f* whereas NRRL 29459 and 29499 formed all three colony types (TABLE I).

TABLE I. Number of generation lines showing colony types during 20 generations

A. flavus strain	Colony type[a]			
	No change[b]	Type *m*	Type *f*	Type *v*
NRRL 29459[c]	4	3	8	11
NRRL 29474[d]	3	1	21	0
NRRL 29499[e]	5	8	5	8

[a] Twenty-four generation lines (includes control and all treatments) per strain. See Results for description of colony types.

[b] Retained wild-type (generation 0) morphology through 20 generations.

[c] One generation line changed from type *m* to *v* and another comprised both types *f* and *v* at generation 20.

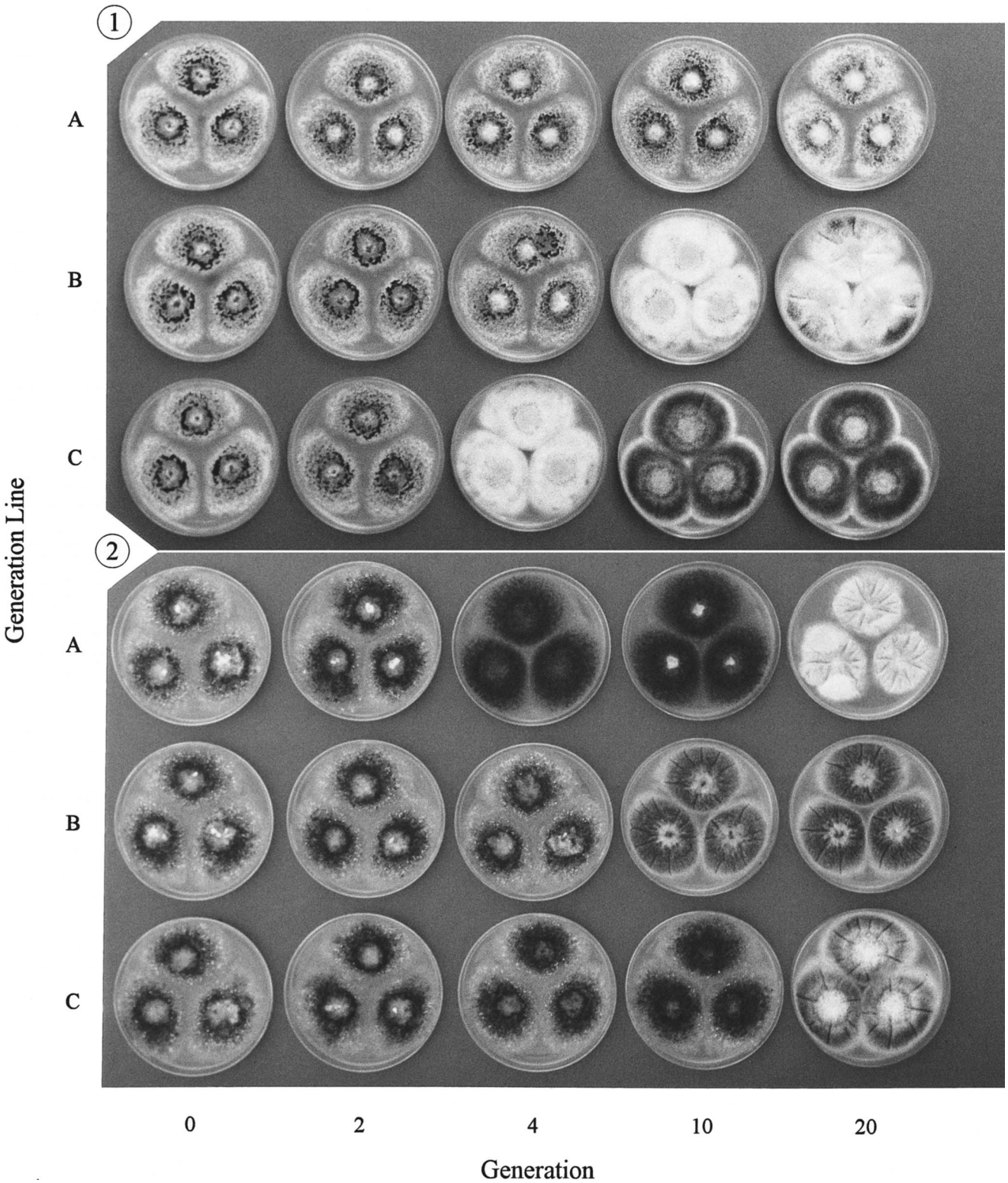
[d] One generation line changed from colony type *f* to *m.*

[e] One generation line changed from colony type *v* to *m* and another from type *v* to *f.*

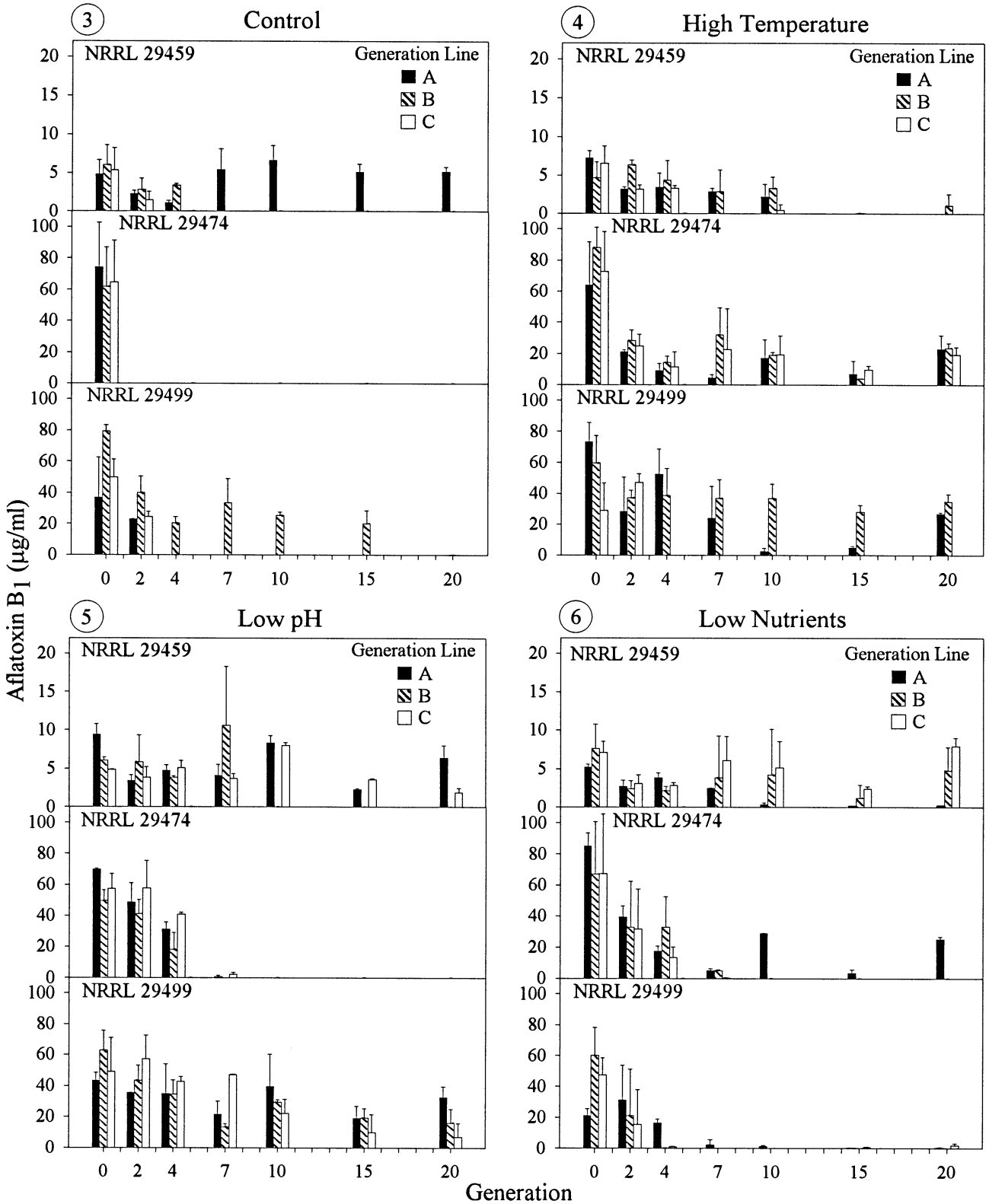
Sectoring was often observed in generations that were transitional from the wild-type morphology to a variant colony type or from one variant colony type to another. A second variant colony type occasionally developed from the initial variant during the experiment (4 out of 72 generation lines) (FIG. 2, generation line A). In no instance did colony type *m, f* or *v* revert to a wild-type morphology.

Aflatoxin production.—Loss of aflatoxin production and the associated formation of morphological variants over generations varied considerably according to the *A. flavus* strain and the generation line within each strain (FIGS. 3–10). NRRL 29474 was more sensitive to degeneration than NRRL 29459 and NRRL 29499 and lost the ability to produce aflatoxin B_1 by the second generation on PDA at 30 C (control) (FIG. 3). Generation lines within a strain often showed different rates of loss of aflatoxin production. For example, in NRRL 29459 under control conditions, generation line A showed no loss in aflatoxin production after 20 generations whereas aflatoxin B_1 was not detectable in generation lines B and C by 7 generations (FIG. 3). Similarly, generation line B in NRRL 29499 produced aflatoxin B_1 through 15 generations in the control whereas generation lines A and C quit producing aflatoxin B_1 in 4 generations.

When compared to the control (FIG. 3), aflatoxin-producing ability was maintained for more genera-



FIGS. 1, 2. Change in colony morphology of *A. flavus* during serial transfers on slants for 20 generations. Colonies shown are on Cz plates (7 d at 30 C). 1. NRRL 29459 serially transferred on PDA at 30 C (control). Generation line A, wild-type colonies throughout; generation line B, colony type *f* (generations 10 and 20); generation line C, colony type *f* with increasing sporulation (generations 4, 10 and 20). 2. NRRL 29499 serially transferred on PDA when competing with *P. guilliermondii*. Generation line A, colony type *v* (generations 4 and 10) and colony type *m* with sparse sporulation (generation 20); generation line B, colony type *m* with moderate sporulation (generations 10 and 20); generation line C, colony type *f* (generation 20). See Results for description of colony types.



FIGS. 3–6. Production of aflatoxin B_1 by *A. flavus* NRRL 29459, 29474 and 29499 during serial transfers for 20 generations. Three separate generation lines (A, B, C) are shown for each strain. Bars represent the mean (± SD; n = 2) of aflatoxin B_1 concentration. Scale for aflatoxin B_1 in NRRL 29459 has a lower range than in NRRL 29474 and 29499. 3. Control (PDA at 30 C). 4. High temperature (PDA at 42 C). 5. Low pH (PDA at pH 2.5). 6. Low nutrients (1.5% water agar).

tions under adverse culture conditions of high temperature, low pH, and low nutrients (FIGS. 4–6). This was most apparent in NRRL 29474. The effects of adverse conditions were less definitive for NRRL 29459 and NRRL 29499 because in the control (FIG. 3), the loss of aflatoxin production was more gradual and one generation line in each strain still produced aflatoxin B_1 through at least 15 generations. Low A_w (FIG. 7) and competition with *A. terreus*, *P. funiculosum* and *P. guilliermondii* (FIGS. 8–10) had little effect on extending aflatoxin production over generations.

Correlation of aflatoxin production and sclerotium formation.—Sclerotium production was generally an accurate measure of aflatoxin-producing ability within a generation line. Aflatoxin B_1 concentration and sclerotium number were positively correlated ($P < 0.0001$; n = 168) for *A. flavus* NRRL 29459 ($r_s = 0.54$), NRRL 29474 ($r_s = 0.89$), and NRRL 29499 ($r_s = 0.75$).

Conidial populations.—Four generation lines were examined to determine whether changes in aflatoxin production were associated with a shift in the composition of conidial populations. Single-spore isolates of *A. flavus* NRRL 29474 and NRRL 29499 at generation 0 produced high levels of aflatoxin B_1 and exhibited a wild-type morphology (FIGS. 11, 12). Subsequent serial transfers resulted in heterogeneous mixtures of high producers, low producers, and nonproducers of aflatoxin B_1.

NRRL 29474 (generation line A) in the low A_w treatment became nonaflatoxigenic in four generations (FIG. 7). Conidial populations in this generation line shifted to a mixture of aflatoxigenic and nonaflatoxigenic isolates (generation 2) and finally to nearly all nonaflatoxigenic isolates (generation 4) (FIG. 11). In contrast, the high temperature treatment for NRRL 29474 (generation line C) in which aflatoxin production was maintained at a reduced level through 20 generations (FIG. 4) showed a mixture of high and low producers through 20 generations (FIG. 11). Nonaflatoxigenic isolates of the low A_w treatment and low producers of the high temperature treatment comprised colony types *f* and *v*, respectively.

NRRL 29499 (generation line B) in the low nutrients treatment produced very low levels of aflatoxin B_1 (0.14 ± 0.090 μg/mL) by the fourth generation (FIG. 6) and showed a concomitant increase in the proportion of nonaflatoxigenic isolates (FIG. 12). Generation line B of NRRL 29499 in the control had a more gradual loss of aflatoxin production (FIG. 3) and by 20 generations, still showed a mixture of isolates dominated by low producers and nonproducers (FIG. 12). Nonaflatoxigenic isolates of the low nutrients treatment and low producers/nonproducers in the control comprised colony types *v* and *m*, respectively.

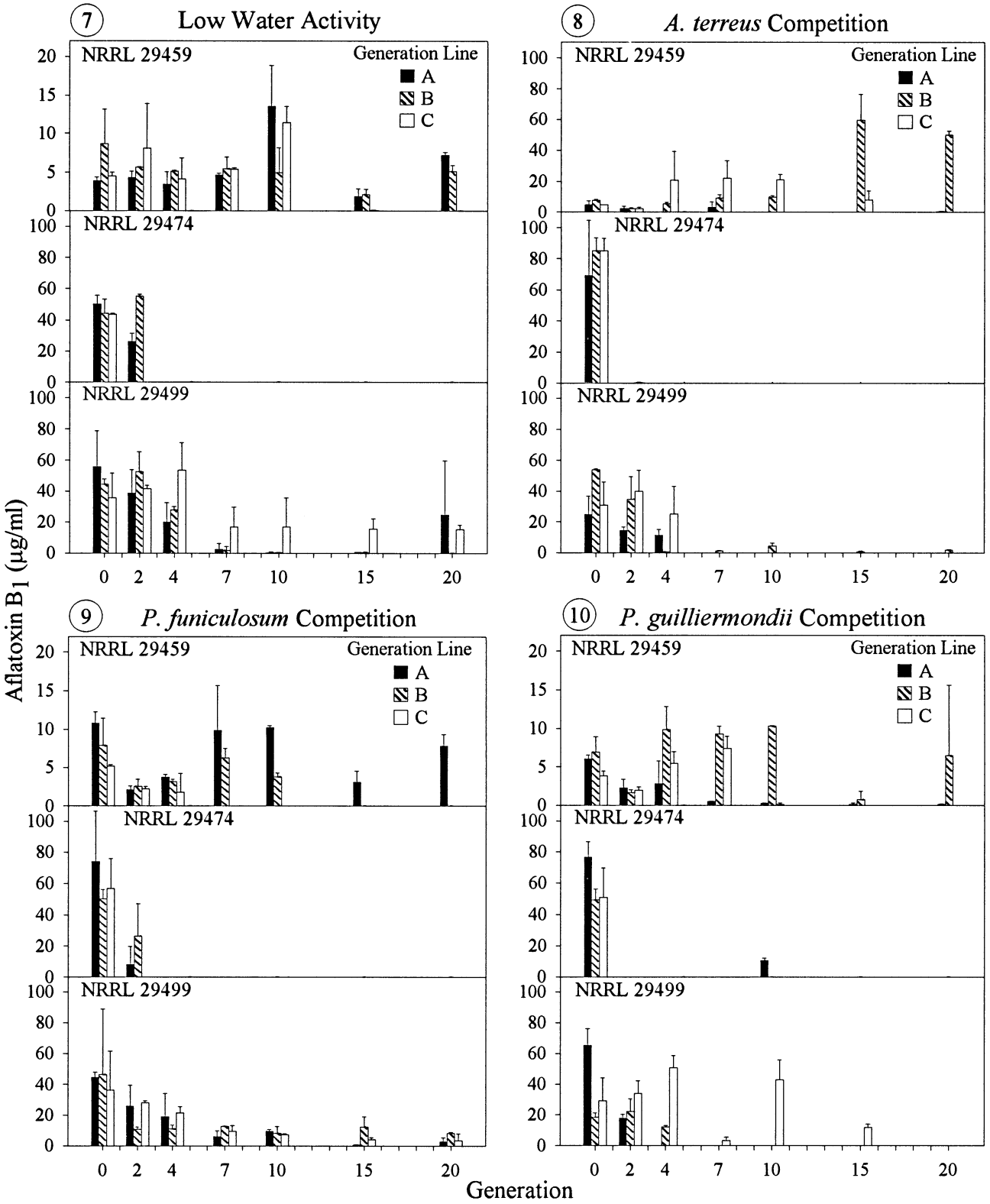
Method of inoculation.—When PDA slants were single-point inoculated with either conidia or sclerotia, *A. flavus* NRRL 29459 and 29499 retained their wild-type characters of aflatoxin production and sclerotium formation for 10 generations (TABLE II). In contrast, generation lines often degenerated by the tenth generation when conidia were spread over the agar medium surface.

DISCUSSION

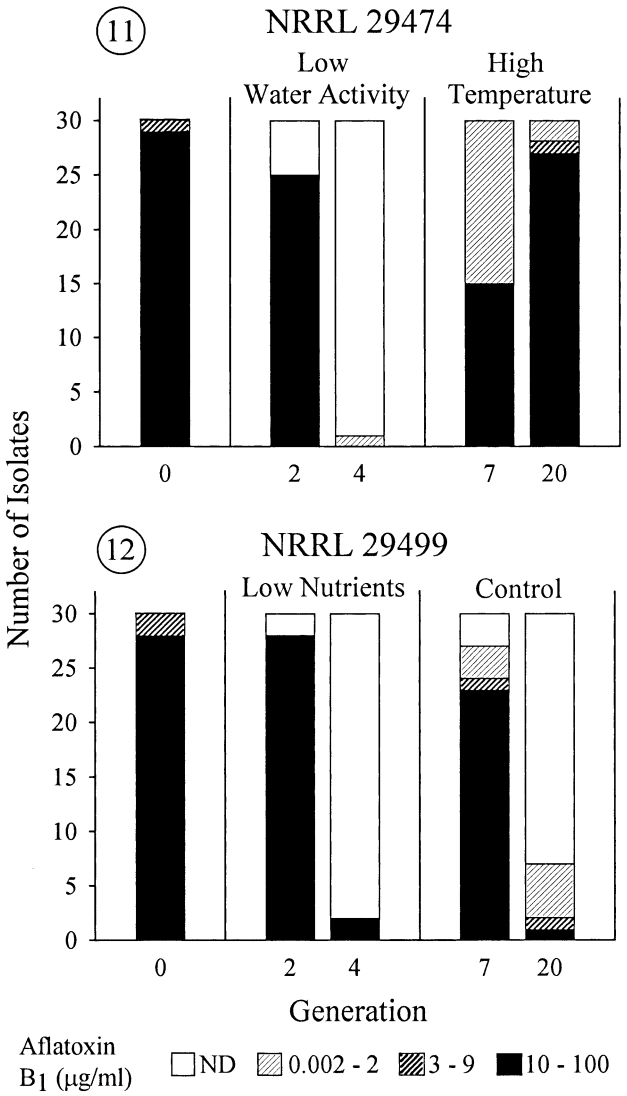
Effect of competition and adverse conditions.—Bilgrami et al (1988) postulated that loss of aflatoxin production by *A. flavus* in the laboratory is due to a lack of exposure to competitive and stressed conditions in nature. Data from this study suggest that in some strains of *A. flavus*, adverse environmental conditions have a stabilizing effect in reducing the rate of culture degeneration. Adverse conditions (high temperature, low pH, and low nutrients) extended the capacity of *A. flavus* NRRL 29474 to produce aflatoxin B_1 during the course of 20 generations. However, contrary to expectations, competition with other fungi had little effect on preventing loss of aflatoxin production with serial transfers.

Serial transfers under conditions optimal for growth appear to select for nonaflatoxigenicity and a variety of morphological characters not exhibited by wild-type strains. A similar situation is illustrated by the nonaflatoxigenic species *A. oryzae* and *A. sojae*, domesticated koji molds used in Oriental food fermentations. Molecular research (Kurtzman et al 1986, Kumeda and Asao 1996, Geiser et al 2000) has shown that *A. oryzae* and *A. sojae* are nearly identical to *A. flavus* and *A. parasiticus*, respectively. Koji molds and some variants of *A. flavus* and *A. parasiticus* obtained through serial transfers share many morphological characters, including floccose growth, reduced sporulation, olive-brown conidial color and an absence of sclerotia, and degenerated strains of *A. flavus* frequently have been misidentified as *A. oryzae* (Wicklow 1983). These observations suggest that koji molds arose from *A. flavus* and *A. parasiticus* through repeated subculturing on fermentation substrates. Under the artificial conditions of the koji environment, aflatoxin production and morphological features such as profuse sporulation and sclerotium formation have little adaptive value and, therefore, have been lost over time (Wicklow 1983).

The reduction in mycotoxin production by strains of *A. flavus* and *A. parasiticus* when repeatedly sub-



FIGS. 7–10. Production of aflatoxin B_1 by *A. flavus* NRRL 29459, 29474 and 29499 during serial transfers for 20 generations. Three separate generation lines (A, B, C) are shown for each strain. Bars represent the means (± SD; n = 2) of aflatoxin B_1 concentration. Scale for aflatoxin B_1 in NRRL 29459 has a lower range than in NRRL 29474 and 29499 for all figures except Fig. 8. 7. Low A_w (PDA with added glucose). 8. Competition with *A. terreus* (PDA at 30 C). 9. Competition with *P. funiculosum* (PDA at 30 C). 10. Competition with *P. guilliermondii* (PDA at 30 C).



FIGS. 11, 12. Production of aflatoxin B_1 by single-spore isolates of *A. flavus* during serial transfers for 20 generations. 11. NRRL 29474 at generation 0 and under conditions of low A_w (PDA with added glucose) (generations 2 and 4 of generation line A) and high temperature (PDA at 42 C) (generations 7 and 20 of generation line C). 12. NRRL 29499 at generation 0 and under conditions of low nutrients (1.5% water agar) (generations 2 and 4 of generation line B) and the control (PDA at 30 C) (generations 7 and 20 of generation line B). ND, not detected.

cultured is not unique and has been widely reported for other toxigenic fungi. Serial transfers have resulted in a reduced production of alkaloids by *Claviceps purpurea* and *C. paspali* (Kobel 1969, Kobel and Sanglier 1978), sporidesmin by *Pithomyces chartarum* (Dingley et al 1962), and zearalenone by *Fusarium graminearum* (Duncan and Bu'Lock 1985). In all of these examples, reduction in mycotoxin production was associated with changes in colony morphology.

Heterogeneous conidial populations.—Single-spore isolates from cultures representing four generation lines indicated that conidial populations following serial transfers comprise a mixture of wild-type aflatoxin-producers and variant colony types that are low producers or nonproducers of aflatoxin B_1. The proportion of wild-type colonies to variant colonies decreased in generation lines that showed an overall reduction in aflatoxin production, yet even after aflatoxin production had been largely lost, low levels of wild-type aflatoxigenic individuals were often still present in the conidial population. Heterogeneous conidial populations during serial transfers also have been reported in *A. parasiticus* (Mayne et al 1971, Bennett 1981). Therefore, the loss of aflatoxin production over successive generations in the laboratory may be interpreted on a population level in which selection favors variant nonaflatoxigenic individuals. In populations from nature, adverse environmental conditions may instead select for wild-type individuals and remove individuals that are observed only in the laboratory.

The effects of competition and adverse conditions on aflatoxin production by *A. flavus* were often difficult to detect because of the variability among generation lines during serial transfers. This within-strain variability was demonstrated not only by the rate of aflatoxin loss but also by the different colony types that developed during the experiment. Variation among replicate generation lines has been similarly reported for *A. parasiticus* (Mayne et al 1971, Bennett 1981) and *Fusarium* species (Wing et al 1995).

Genetic drift due to transferring small samples from a heterogeneous conidial population may be responsible for the variability among generation lines in *A. flavus*. Cowen et al (2000) examined the evolution of fluconazole resistance in *Candida albicans* when 12 replicate populations were serially transferred for 330 generations. In the presence of fluconazole, each population followed a different trajectory during selection for drug resistance. It was postulated that the diverse patterns in the evolution of resistance were due to chance mutations during the experiment and that genetic drift was minimal because of the high populations ($>10^6$ individuals). In the current study, however, sporulation was often sparse in variant colony types and relatively few conidia were available for transfer to the next generation. Furthermore, conidia were selected from three different regions of the slant. Slants contained mixed conidial populations and the spatial distribution of the different colony types was not known. A slant culture is not a homogeneous environment and gradients in nutrient availability and water activity are likely present due to decreasing thickness of the agar

TABLE II. Effect of inoculation method on aflatoxin production and sclerotium formation by *A. flavus* after 10 generations

		Generation 10[b]			
		NRRL 29459[c]		NRRL 29499[d]	
Inoculation method[a]	Generation line	Aflatoxin B_1 (μg/mL)	No. sclerotia	Aflatoxin B_1 (μg/mL)	No. sclerotia
Conidia: entire agar surface	A	6.6 ± 1.89	1188 ± 133.1	0.0 ± 0.00	0 ± 0.0
	B	0.0 ± 0.00	1 ± 2.0	25.5 ± 1.94	189 ± 50.9
	C	0.0 ± 0.00	0 ± 0.0	0.0 ± 0.00	0 ± 0.0
Conidia: single point	A	7.0 ± 2.71	726 ± 30.8	81.8 ± 11.47	259 ± 58.1
	B	7.3 ± 3.99	767 ± 91.3	76.9 ± 15.16	269 ± 70.7
	C	8.2 ± 1.60	789 ± 96.5	56.4 ± 8.28	293 ± 42.3
Single sclerotium	A	6.2 ± 0.59	958 ± 113.8	54.6 ± 15.55	337 ± 34.8
	B	9.0 ± 0.83	696 ± 55.4	54.1 ± 7.04	307 ± 46.2
	C	7.3 ± 0.04	767 ± 38.3	61.0 ± 12.03	331 ± 42.9

[a] Grown on PDA slants at 30 C and serially transferred at 14-d intervals.

[b] Mean ± SD for aflatoxin B_1 (n = 2) from mYES culture vials and for sclerotium number (n = 4) per 9.3 cm^2 of Cz plate surface.

[c] Initial (generation 0) aflatoxin B_1 concentration and sclerotium number were 8.9 ± 0.94 μg/mL and 773 ± 26.6 sclerotia, respectively.

[d] Initial (generation 0) aflatoxin B_1 concentration and sclerotium number were 71.5 ± 9.01 μg/mL and 372 ± 21.0 sclerotia, respectively.

medium from the base of slant toward the mouth opening. Therefore, it is possible that the colony types occurred in patches and that selection of conidia did not reflect overall proportions of colony types in the population.

Association between aflatoxin biosynthesis and morphology.—The loss of aflatoxin production during serial transfers in this study was always associated with morphological changes. Bennett (1981) described two variant colony types in *A. parasiticus* as *fan* and *fluff* and these appear similar to respective colony types *m* and *f* for *A. flavus* in this study, with the exception that colony type *m* often had sparser sporulation than that of *fan*. The heavily sporulating colony type *v*, however, was not reported.

Experimental studies indicate that aflatoxin biosynthesis and fungal development may share regulatory elements (Kale et al 1996, Guzmán-de-Peña and Ruiz-Herrera 1997). Several genes that regulate hyphal growth and asexual sporulation have marked effects on aflatoxin production (Hicks et al 1997, Zhou et al 2000). The precise mechanisms underlying these observations have not yet been determined. Nonaflatoxigenic variant colonies of *A. flavus* and *A. parasiticus* obtained through serial transfers have intact genes involved in aflatoxin biosynthesis, including *afl*R, which regulates the transcription of aflatoxin pathway genes (Klich et al 1995, Kale et al 1996). These pathway genes appear to be transcriptionally blocked, possibly by the lack of expression of *afl*R (Kale et al 1996, Klich et al 1997). Nonaflatoxigenic variants typically do not revert back to aflatoxigenic forms (Bennett 1981, Bilgrami et al 1988, Kale et al 1994).

Maintenance of aflatoxin production in nature.—The effect of single-point inoculation in addition to adverse culture conditions on slowing the degeneration of *A. flavus* cultures suggests that the maintenance of wild-type phenotypes of aflatoxigenic fungi in nature is complex. It is not understood why single-point inoculations (conidia or a single sclerotium) preserve aflatoxin-producing ability whereas the spreading of conidia over the medium surface results in culture degeneration. Intraspecific interactions involving competition for space and nutrients as well as the frequency of hyphal anastomosis may differ according to the inoculation method. Interactions would be intense when conidia are densely spread over the medium surface but may be reduced in single-point inoculations where the radial growth favors fewer individuals. Sporulation by many individuals as a result of spreading conidia also may increase the likelihood of transferring mutants to the next generation. In contrast to the degeneration of cultures with conidial transfers in this study, Bennett et al (1981) and Kale et al (1994) reported that conidia (but not macerated mycelium) prevented *A. parasiticus* from degenerating during serial transfers. Differences in the method of inoculation with conidia may explain the lack of degeneration in these studies. In nature, conidia are the primary dispersal units for the inoculation of new substrates (Lillehoj et al 1980, Holt-

meyer and Wallin 1981). The low density of conidia in wind dispersal and the directed feeding activity of insects may favor single-point inoculations.

Adverse culture conditions slowed but did not completely stop the degeneration of *A. flavus* strains during serial transfers in this study. Suboptimal conditions of temperature, pH, water activity, and available nutrients as well as fungal competition were examined individually, but in nature aflatoxigenic fungi would be exposed to combinations of these and other variables. Hence, simultaneous exposure to parameters that restrict growth and sporulation may be more effective in maintaining aflatoxigenicity and wild-type morphological characters during successive dispersal events.

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