# Virulence and Cultural Characteristics of Two *Aspergillus flavus* Strains Pathogenic on Cotton

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I thank J. P. Evans for performing the video image analysis, B. Vinyard for assistance with the statistical analysis, and L. A. Williams and L. G. Fortune for technical assistance.

Accepted for publication 22 March 1989 (submitted for electronic processing).

## ABSTRACT

Cotty, P. J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79:808-814.

Seventy *Aspergillus flavus* isolates from Arizona desert valleys were sorted into two distinct strains on the basis of sclerotial size, cultural characteristics, and virulence to cotton. Strain L isolates produced large sclerotia (over 400 μm in diameter), and strain S isolates produced small sclerotia (less than 400 μm in diameter). Strain S isolates produced greater quantities of sclerotia in a wider assortment of media and a broader temperature range than strain L isolates. Isolates of both strains exhibited pH homeostasis in culture. However, the strains maintained different pH values. Strain S isolates produced more aflatoxin than strain L isolates in culture; however, the strains produced similar aflatoxin levels in developing cottonseed. Strain L isolates were more aggressive than strain S isolates at deteriorating cotton boll locks (locules) and spreading within bolls, and this tendency partly explains the difference between in vitro and in vivo production of aflatoxin. The aflatoxin level in infected seeds was not correlated with either the aflatoxin level in vitro, intraboll fungal spread, or lock deterioration, but it was correlated with the product of the aflatoxin level in vitro and the intraboll spread parameter ($r$ = 0.75–0.91). The correlation increased slightly ($r$ = 0.78–0.93) when lock deterioration was factored into the equation. These results indicate that pathogenic aggressiveness contributes to the ability of an isolate to contaminate cottonseed with aflatoxins. The lack of correlation between pathogenic aggression and aflatoxin production in vivo suggests aflatoxin does not enhance virulence in the cotton-*A. flavus* interaction.

*Aspergillus flavus* is the primary cause of aflatoxin contamination of corn, cottonseed, and tree nuts (10). *A. flavus* populations consist of individuals that vary widely in their ability to produce sclerotia or aflatoxins in vitro (13,18,19), and isolates are often grouped on the basis of the quantities of aflatoxins and sclerotia produced (12,17). Aflatoxin production in vitro is thought to reflect the ability of an isolate to contaminate plant tissues (23,26). The relationship between *A. flavus* isolates and preharvest aflatoxin contamination of crops is complicated both by changes that occur during culture in the abilities of isolates to produce aflatoxins (9,23) and by a lack of experimental evidence on the relation of toxin production in vitro to virulence on host plants.

Several studies correlated sclerotial production in vitro with aflatoxin production (9,14,16,22), and a single study found that cottonseed infected with *A. flavus* contained higher aflatoxin levels when sclerotia were present than when the seed contained no sclerotia (25). Furthermore, aflatoxin biosynthesis and sclerotial morphogenesis may have interrelated regulation (7).

"Atypical" isolates of *A. flavus* from agricultural soils in Thailand have recently been reported (21). These isolates produced abundant small sclerotia and large quantities of aflatoxins. In my laboratory, similar *A. flavus* isolates were obtained from cottonseed and soil collected in Arizona, where aflatoxin contamination of cottonseed is a perennial problem. It was speculated that the atypical isolates represent a distinct *A. flavus* strain. The repeated association of aflatoxin production with sclerotial production led to further speculation that this strain may be an important causative agent of aflatoxin contamination of cottonseed. The purpose of this study was to determine if Arizona desert valleys bear two *A. flavus* strains distinguishable by cultural characteristics and virulence to cotton. The relationship of aflatoxin production to the virulence of *A. flavus* on cotton was also investigated.

## MATERIALS AND METHODS

**Cultures and strains.** The origins of the isolates studied are described in Table 1. All isolates examined produced sclerotia in culture. For convenience, isolates producing numerous small sclerotia were designated strain S ("small") isolates, which are synonymous with the atypical isolates of Saito et al (21); isolates with any sclerotia greater than 400 μm in diameter were designated strain L ("large") isolates. Soil isolates were obtained on a dicloran-amended medium by the dilution plate technique (3). Distinct colonies were transferred to 5/2 agar (5% V-8 juice and 2% agar, adjusted to pH 5.2 prior to autoclaving) and utilized directly in the studies. Seed isolates were obtained on 5/2 agar either directly from the lint or from pieces of manually delinted seed disinfected for 2 min with 95% ethyl alcohol; stock cultures of seed isolates originated from single spores. The fungi were maintained in the dark on 5/2 agar at 25–30 C. For long-term storage, plugs of mature cultures (3 mm in diameter) were maintained in sterile distilled water (7).

**Sclerotial production.** Conidial suspensions (10 μl) were seeded into the centers of petri dishes (9 cm in diameter) containing 30–35 ml of Czapek solution agar (CZ) with 3% $NaNO_3$ and incubated at either 25, 30, or 38 C for 12 days in the dark. Temperatures were maintained within 1°. Subsequently, conidia were washed from the plates with 95% ethyl alcohol, and the number of sclerotia per plate was estimated: 0 = no sclerotia, and 1 = 1–20, 2 = 50–100, 3 = 200–400, 4 = 500–1,000, and M = more than 1,000 sclerotia. Each test evaluated five to 12 isolates, and isolates were tested two to four times. Sclerotial production by several typical isolates of each strain was evaluated at 30 C on 5/2 agar, potato-dextrose agar (PDA), and CZ with either 0, 2, 3, or 6% $NaNO_3$. Sclerotia in three portions (each 4 $cm^2$) of each plate were counted after 12 days. The two tests each contained two replicates and compared four to six isolates.

**Sclerotial size.** Diameters of sclerotia produced on CZ with 3% $NaNO_3$ at 30 C were determined by video image analysis. Conidia were washed from the plates with 95% ethyl alcohol, and sclerotia were dislodged with a spatula and fixed in a mixture of ethanol and glacial acetic acid (1:1, v/v). This procedure reduced human exposure to conidia. Sclerotia were blotted dry and stuck to clear plastic plates with two-sided tape. Sclerotial silhouette areas were determined by video image analysis, and diameters were calculated on the assumption that the sclerotia were spherical.

**Aflatoxin production in vitro.** Aflatoxin production was assessed qualitatively by seeding conidial suspensions (10 μl) in petri dishes (9 cm in diameter) containing 30–35 ml of solidified Adye and Mateles medium (A&M agar) (6,15). After 10 days of growth at 30 C agar cultures were transferred to 250-ml jars with 25 ml of acetone and shaken for 1 min. Methylene chloride (25 ml) was added, the mixture was shaken for 1 min and filtered through 25 g of sodium sulfate, and the filtrate was evaporated to dryness. Residues were solubilized in methylene chloride for thin-layer chromatography (TLC). Extracts and aflatoxin standards were separated on TLC plates (silica gel 60, 250 μm thick) by development with a mixture of diethyl ether, methanol, and water (96:3:1, v/v) (24) and examined under ultraviolet light. Isolates negative for aflatoxin production in initial tests were grown on three plates containing A&M agar, which were combined prior to extraction.

Quantitative estimates of aflatoxin production in vitro were made by the rapid fluorescence method (6). Culture tubes containing 5 ml of A&M agar (6,15) were seeded with 100-μl spore suspensions containing approximately 100 spores per microliter. After 3 days of incubation at 30 C, agar fluorescence 5 mm beneath the mycelial mat was measured with a scanning densitometer. Agar fluorescence measured in this manner is directly correlated with the quantity of aflatoxin in the culture (6). Aflatoxin was extracted with solvents from representative tubes and quantified by TLC in order to construct a standard curve (fluorescence vs. toxin concentration) for each experiment (6). Three experiments were performed, containing eight, 24, and 44 isolates replicated six, seven, and four times, respectively.

**Fungal influence on culture pH.** *A. flavus* modifies culture pH in an isolate-specific manner (12,20); this characteristic has been used to group *A. flavus* isolates (12). Therefore, potential differences in culture pH between strains were evaluated. Conidial suspensions (10 μl) containing about 10,000 spores were seeded in the centers of culture plates (9 cm in diameter) containing 25 ml of CZ with 0.05 M Hepes buffer adjusted to pH 6, 7, or 8 prior to autoclaving. After 10 days of growth at 30 C in the dark, the agar pH was measured with a flat electrode in the center of the plate on the reverse side of the culture.

**Aflatoxin production in vivo.** Infection of cotton bolls by *A. flavus* and the subsequent aflatoxin contamination of cottonseed occur in the field via pink bollworm exit holes (2). Therefore, the ability of isolates to infect simulated pink bollworm exit holes and contaminate cottonseed with aflatoxins was determined in the greenhouse, as previously described (8). *Gossypium hirsutum* 'Deltapine Acala 90' was grown in 3-L pots containing a 1:1 mixture of Pro-mix (Premier Brands, New Rochelle, NY) and sand. After 21 days, the plants were fertilized weekly with 100 ml of Miracle-Gro (Stern's Miracle-Gro Products, Port Washington, NY) at 2,000 ppm. Bolls 25–28 days old were wounded with a cork borer (3 mm in diameter) and inoculated with 10 μl of a conidial suspension containing approximately 10,000 spores. Bolls were harvested after 4 wk and dried at 60 C for 72 hr.

The aflatoxin content of intact inoculated locks (locules) was determined by a modification (8) of the method of the Association of Official Analytical Chemists (24). Intact locks were hammered to pulverize the seed and added to 200 ml of a mixture of acetone and water (85:15). The mixture was shaken for 15 sec, set overnight, and filtered with number 4 Whatman paper. The filtrate (100 ml) was mixed with 20 ml of a solution of zinc acetate and aluminum chloride (1.1 M $(CH_3COO)_2Zn$ and 0.04 M $AlCl_3$), together with 80 ml of water and 5 g of diatomaceous earth. The mixture was shaken, left to settle for 1–2 hr, and passed through number 4 Whatman filter paper. The filtrate (100 ml) was extracted twice with 25 ml of methylene chloride. Fractions were pooled and concentrated to dryness, and the residues were solubilized in methylene chloride for TLC. Aflatoxins were separated and quantified by TLC (24).

The plants were maintained at all times in complete randomized blocks. Each experiment evaluated eight isolates, which were replicated eight times. The replicates consisted of one plant with one or two bolls in the first experiment and two plants with two to five bolls in the second experiment.

**Evaluation of pathogenic aggression.** Intralock fungal spread was used as one measure of pathogenic aggression. During growth on cotton lint, *A. flavus* produces kojic acid, which is converted by host peroxidase into a compound with bright green-yellow fluorescence (BGYF) (1). The presence of BGYF on cotton lint is a reliable indicator of the activity of *A. flavus* (1). BGYF on lint of uninoculated locks, therefore, indicates fungal spread from inoculated to uninoculated locks. Uninoculated locks were examined under ultraviolet light after drying, and BGYF on lint of each lock was rated as follows: 0 = no BGYF; 1 = BGYF on less than 50% of the lint; 2 = BGYF on more than 50% of the lint. The intraboll spread parameter was the BGYF value; for correlations, the values were transformed (0.1 was added to each value) in order to compensate for the low sensitivity of the technique and to avoid multiplying by 0.

After the locks were dried, the weight per lock of inoculated and uninoculated locks was determined. The weight of each lock was divided by the number of seeds within the lock, because the number of seeds per lock varied within bolls; the inoculated lock weights were then divided by the uninoculated lock weights to compensate for variance among bolls. For correlations, the resulting value was subtracted from 1 and squared; this produced a value positively related to aggression.

**Statistical analysis.** Analyses were performed with the Statistical Analysis System (SAS Institute, Cary, NC). All multiple comparisons were first subjected to analysis of variance and then to Fischer's least significant difference test. Toxin and fluorescence

TABLE 1. Origins and sclerotial characteristics of *Aspergillus flavus* isolates

| Isolate[v] | Origin | | | Sclerotial diameter ($\mu$m)[y] | | | Sclerotial production[z] | | |
|---|---|---|---|---|---|---|---|---|---|
| | Crop[w] | Substrate | Locale[x] | Sample size | Average ± SD | Percentage over 400 $\mu$m | 25 C | 30 C | 38 C |
| 24 S | Alf | Soil | NGV | 192 | 250 ± 42 | 0 | M | M | M |
| 69 S | | | | 132 | 235 ± 49 | 0 | M | M | M |
| 37 S | | | SGV | 104 | 212 ± 55 | 0 | M | M | M |
| 61 L | | | | 91 | 481 ± 109 | 82 | 2 | 4 | 0 |
| 10 S | | | YV | 117 | 217 ± 51 | 0 | M | M | M |
| 18 S | | | | 90 | 199 ± 40 | 0 | M | M | M |
| 33 S | | | | 219 | 196 ± 43 | 0 | M | M | M |
| 19 L | Bg | Soil | YV | 125 | 505 ± 119 | 82 | 1 | 3 | 0 |
| 5 S | | | | 274 | 211 ± 47 | 0 | M | M | M |
| 21 L | | | | 20 | 325 ± 64 | 10 | 0 | 1 | 0 |
| 23 L | | | | 62 | 543 ± 101 | 92 | 1 | 2 | 0 |
| 26 S | | | | 405 | 184 ± 37 | 0 | M | M | M |
| 60 L | | | | 54 | 514 ± 82 | 94 | 2 | 3 | 0 |
| 65 S | | | | 163 | 191 ± 38 | 0 | M | M | M |
| 66 S | | | | 167 | 189 ± 41 | 0 | M | M | M |
| 20 S | Cit | Soil | NGV | 141 | 210 ± 54 | 0 | M | M | M |
| 25 S | | | | 480 | 238 ± 62 | 0 | M | M | M |
| 27 S | | | | 190 | 243 ± 43 | 0 | M | M | M |
| 39 L | | | | 61 | 436 ± 67 | 75 | 2 | M | 0 |
| 7 S | | | YV | 107 | 200 ± 45 | 0 | M | M | M |
| 13 L | | | | 50 | 548 ± 103 | 94 | 0 | 2 | 0 |
| 15 S | On | Soil | NGV | 268 | 216 ± 51 | 0 | M | M | M |
| 59 S | | | | 148 | 209 ± 41 | 0 | M | M | M |
| 31 S | | | YV | 127 | 239 ± 43 | 0 | M | M | M |
| 46 S | | | | 220 | 203 ± 44 | 0 | M | M | M |
| 63 L | | | | 44 | 670 ± 110 | 98 | 0 | 4 | 0 |
| 64 L | | | | 80 | 486 ± 79 | 86 | 0 | 2 | 0 |
| 68 L | | | | 54 | 554 ± 108 | 91 | 2 | M | 0 |
| 1 L | PC | Soil | YV | 10 | 650 ± 92 | 100 | 1 | 1 | 0 |
| 6 L | | | | 61 | 603 ± 108 | 93 | 1 | 2 | 0 |
| 55 L | | | | 46 | 451 ± 89 | 67 | 2 | 3 | 0 |
| 70 S | | | | 159 | 163 ± 31 | 0 | M | M | M |
| 35 L | UC | Seed | MC | 77 | 396 ± 93 | 49 | 3 | M | 0 |
| 16 L | | | | 86 | 542 ± 108 | 92 | 0 | 1 | 0 |
| 17 L | | | | 47 | 691 ± 103 | 96 | 0 | 3 | 0 |
| 38 L | | | | 63 | 433 ± 57 | 65 | 3 | M | 0 |
| 41 L | | | | 101 | 447 ± 82 | 73 | 1 | M | 0 |
| 43 L | | | | 87 | 471 ± 76 | 82 | 4 | M | 0 |
| 45 L | | | | 47 | 447 ± 121 | 62 | 2 | 4 | 0 |
| 47 L | | | | 50 | 549 ± 61 | 98 | 2 | 4 | 0 |
| 48 L | | | | 88 | 431 ± 75 | 70 | 4 | M | 0 |
| 49 L | | | | 57 | 549 ± 77 | 98 | 0 | 4 | 0 |
| 51 L | | | | 2 | 480 ± 21 | 100 | 0 | 1 | 0 |
| 52 L | | | | 67 | 493 ± 96 | 84 | 2 | 4 | 0 |
| 53 L | | | | 73 | 409 ± 75 | 58 | 0 | 1 | 0 |
| 56 L | | | | 58 | 548 ± 81 | 97 | 1 | 3 | 0 |
| 57 L | | | | 60 | 628 ± 82 | 98 | 0 | 1 | 0 |
| 28 L | | | YV | 3 | 821 ± 91 | 100 | 0 | 1 | 0 |
| 29 S | | | | 134 | 211 ± 43 | 0 | M | M | M |
| 36 L | | | | 16 | 503 ± 117 | 75 | 1 | 4 | 0 |
| 40 L | | | | 70 | 506 ± 126 | 80 | 2 | 4 | 0 |
| 42 S | | | | 170 | 281 ± 36 | 0 | M | M | M |
| 50 L | | | | 74 | 521 ± 71 | 95 | 4 | M | 0 |
| 54 L | | | | 25 | 692 ± 130 | 92 | 1 | 2 | 0 |
| 58 S | | | | 342 | 263 ± 47 | 0 | M | M | M |
| 2 L | | Soil | NGV | 125 | 515 ± 103 | 89 | 2 | 4 | 0 |
| 3 S | | | YV | 127 | 193 ± 59 | 0 | M | M | M |
| 4 L | | | | 131 | 433 ± 52 | 73 | 2 | 2 | 0 |
| 8 L | | | | 90 | 466 ± 83 | 79 | 2 | 1 | 0 |
| 9 L | | | | 36 | 595 ± 130 | 94 | 0 | 3 | 0 |
| 11 L | | | | 52 | 642 ± 102 | 98 | 0 | 1 | 0 |
| 12 S | | | | 156 | 217 ± 49 | 0 | M | M | M |
| 22 L | | | | 147 | 488 ± 147 | 80 | 0 | 1 | 0 |
| 34 L | | | | 55 | 430 ± 121 | 53 | 2 | 4 | 0 |
| 30 S | | | | 115 | 200 ± 53 | 0 | M | M | M |

[v] Isolate numbers are followed by letters denoting the strain. Strain S isolates produce numerous small sclerotia (average diameter less than 300 $\mu$m); strain L isolates produce larger sclerotia, some of which have a diameter exceeding 400 $\mu$m.

[w] Alf = alfalfa; Bg = bermudagrass; Cit = citrus; On = onion; PC = Pima cotton; UC = upland cotton.

[x] NGV = North Gila Valley; SGV = South Gila Valley; YV = Yuma Valley; MC = Maricopa County.

[y] Silhouette areas were measured by video image analysis; average diameters were calculated from the areas on the assumption that the sclerotia were spherical.

[z] Sclerotia per plate after 12 days on Czapek solution agar with 3% $NaNO_3$: 1 = 1–50, 2 = 50–100, 3 = 200–400, 4 = 500–1,000, and M = more than 1,000 sclerotia per plate.

data were log-transformed prior to analysis.

Pearson product-moment correlations were calculated for relationships between the aflatoxin level in vivo and either the aflatoxin level in vitro, intraboll fungal spread, or lock deterioration; correlations between the aflatoxin level in vivo and equations containing the three other parameters were also tested.

## RESULTS

**In vitro comparisons.** All strain L isolates produced at least some sclerotia with diameters greater than 400 μm, whereas strain S isolates produced no sclerotia of that size (Table 1). The average diameters of strain S sclerotia were 150–250 μm; strain L sclerotia averaged 300–700 μm in diameter (Fig. 1). Two strain L isolates (21 and 53) produced sclerotia in only two of four tests. Strain S isolates produced abundant sclerotia at 25–38 C, whereas no strain L isolate produced sclerotia at 38 C, and only 65% of strain L isolates produced sclerotia at 25 C (Table 1).

Strain S isolates produced sclerotia on a wider variety of media than strain L isolates. L isolates produced no sclerotia on 5/2 agar and CZ lacking $NaNO_3$, whereas the S isolates produced at least four sclerotia per square centimeter on all media (Table 2). Certain L isolates did not produce sclerotia on PDA and tended to produce more sclerotia on CZ with 3% $NaNO_3$ than on CZ with 2% $NaNO_3$.

The initial pH value of the culture medium (pH 6, 7, or 8) did not significantly influence the final pH of cultures of either strain L or strain S isolates (Table 3). The L and S isolates differed significantly in their effect on culture pH. L isolates altered the culture pH to 5.8–6.7 in the two experiments, whereas S isolates changed it to 6.7–7.8 (data shown are from one experiment).

Culture extractions indicated that all isolates produced aflatoxin $B_1$ in culture except isolates 19, 36, 40, 51, 53, and 55; many isolates also produced small quantities of aflatoxin $B_2$. No isolate produced aflatoxin $G_1$ or $G_2$. Quantitative differences in aflatoxin production in vitro between isolates and between strains were detected with the rapid fluorescence method (Table 4 and Fig. 2). In the three experiments L isolates produced only 2, 3, and 8% as much aflatoxin as S isolates. All S isolates produced over 500 ng of aflatoxin $B_1$ per gram of culture (Fig. 2), whereas most L isolates produced less. The fluorescence method is less sensitive than the extraction method, and several isolates produced quantities of aflatoxins detectable only by extraction.

**In vivo comparisons.** Most isolates produced large concentrations of aflatoxin $B_1$ (5–152 μg/g) in developing cottonseed (Table 5). One isolate, however, produced no aflatoxins in vivo, and isolates that did produce aflatoxins varied significantly in the quantity produced. The average levels of aflatoxin produced by L and S strains in vivo were not significantly different (Table 5).

Pathogenic aggression varied significantly, both between isolates and between strains (Table 5). BGYF on lint of uninoculated locks, an indication of intraboll fungal spread, was significantly greater in bolls inoculated with strain L isolates than in bolls inoculated with strain S isolates. L isolates also caused

TABLE 2. Production of sclerotia by isolates of *Aspergillus flavus* on various media incubated at 30 C for 12 days

| Isolate[y] | Number of sclerotia per square centimeter[z] | | | | | |
|---|---|---|---|---|---|---|
| | PDA | 5/2 | CZ-0% | CZ-2% | CZ-3% | CZ-6% |
| 13 L | 0 | 0 | 0 | 0.7 ± 0.1 | 4.3 ± 1.0 | 6.7 ± 4.5 |
| 36 L | 0 | 0 | 0 | 4.9 ± 6.2 | 19.7 ± 7.6 | 22.2 ± 18.3 |
| 11 L | 0.4 ± 0.3 | 0 | 0 | 7.9 ± 0.9 | 13.4 ± 2.6 | 14.7 ± 0.6 |
| 6 L | 1.7 ± 1.0 | 0 | 0 | 20.3 ± 4.7 | 31.0 ± 12.5 | >50 |
| 22 L | 1.7 ± 0.1 | 0 | 0 | 11.4 ± 3.0 | 42.0 ± 17.6 | 39.3 ± 18.5 |
| 42 S | >50 | 32.5 ± 5.0 | 9.5 ± 1.0 | >50 | >50 | >50 |
| 12 S | >50 | 16.0 ± 3.0 | 4.2 ± 0.4 | >50 | >50 | >50 |
| 3 S | >50 | >50 | 14.3 ± 0.9 | >50 | >50 | >50 |
| 25 S | >50 | >50 | 30.0 ± 3.1 | >50 | >50 | >50 |
| 29 S | >50 | 24.1 ± 5.8 | 6.1 ± 3.6 | >50 | >50 | >50 |

[y] Isolate numbers are followed by letters denoting the strain. Strain S isolates produce numerous small sclerotia (average diameter less than 300 μm); strain L isolates produce larger sclerotia, some of which have a diameter exceeding 400 μm

[z] PDA = potato-dextrose agar; 5/2 = 5% V-8 juice and 2% agar; CZ = Czapek solution agar with either 0, 2, 3, or 6% sodium nitrate. Values are means of two replicates, plus or minus standard deviations.

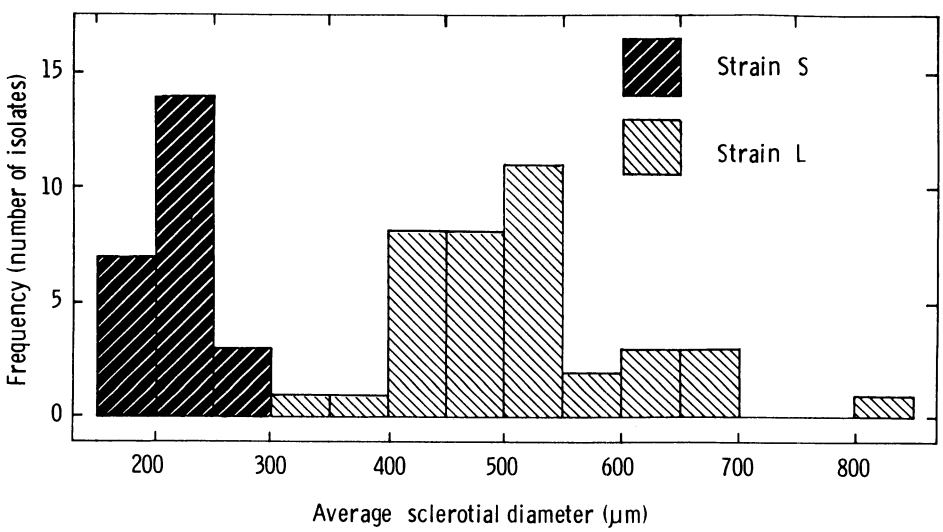


**Fig. 1.** Distribution of average sclerotial diameters of isolates of the S and L strains of *Aspergillus flavus*. Sclerotial diameters were determined by video image analysis as described in the text.

significantly larger reductions in inoculated lock weights than S isolates (Table 5). The two measurements of pathogenic aggression were significantly correlated with each other ($r = -0.85$ to $-0.89$).

Aflatoxin levels in vivo did not correlate with aflatoxin levels in vitro (Table 6). Similarly, aflatoxin levels in vivo did not correlate with either intraboll fungal spread or inoculated lock deterioration. Aflatoxin levels in vivo were, however, correlated with the product of the intraboll spread parameter and the aflatoxin level in vitro ($r$ = 0.75–0.91); factoring in the lock deterioration parameter slightly improved the correlation ($r$ = 0.78–0.93) (Table 6).

## DISCUSSION

Two *A. flavus* strains that differ both morphologically and physiologically apparently coexist in Arizona desert valleys. Strain S isolates produce abundant small sclerotia (less than 400 μm in diameter), whereas strain L isolates produce fewer, larger sclerotia. Strains S and L also have different requirements for sclerotial production. Strain S isolates produce sclerotia at 38 C and on 5/2 agar and CZ lacking $NaNO_3$; strain L isolates do not. Isolates belonging to both strains apparently have been described previously (12,18), and Saito et al (21) recently described typical and atypical isolates, which clearly belong to the L and S strains, respectively. Saito et al (21) described atypical (strain S) isolates that produced either only B aflatoxins or B and G aflatoxins. Isolates examined in the current study produced only B aflatoxins.

*A. flavus* modifies culture pH in an isolate-distinct manner (12,20). Variance between isolates may be attributed in part to differences between L and S strains. Both strains exhibited pH homeostasis by altering agar pH to certain values regardless of the initial pH. Strain L isolates, however, made cultures more acidic than strain S isolates. This may indicate a significant physiologic difference between strains.

All *A. flavus* isolates tested produced sclerotia during this study. This contrasts with studies in which numerous isolates failed to produce sclerotia (5,11,17,22). Although *A. flavus* isolates unable to produce sclerotia may exist, a lack of sclerotial formation is probably more often attributable to one or more of the following: an attenuation of sclerotial production in culture (18), an unfavorable medium (i.e., PDA) (17), unfavorable temperature (12), the differential sensitivity of isolates to light (4), or other environmental constraints in culture. Strain L isolates require more precise conditions to produce sclerotia than strain S isolates. If sclerotial production had been assessed in the current study on PDA only (17) or at 25 C only (12), 40 and 35% of the L isolates would have been nonsclerotial, respectively (Tables 1 and 2). Differentiating strains by the occurrence of sclerotia is thus at least partly ambiguous. Disagreements between studies correlating the sclerotial production of isolates with aflatoxin production (5,9,14,16,22) may have resulted from this ambiguity. Sclerotial size, however, has been demonstrated in the current study to correspond to important physiologic traits.

Results presented here agree with the finding by Saito et al (21) that strain S isolates produce far greater quantities of aflatoxins in vitro than strain L isolates. In three tests, aflatoxin production in vitro by strain S isolates was 10 times greater than

TABLE 3. Modification of culture pH by *Aspergillus flavus* isolates belonging to strains L and S

| Isolate[y] | Final pH of agar medium[z] | | |
|---|---|---|---|
| | Initial pH 6 | Initial pH 7 | Initial pH 8 |
| 13 L | 5.99 ± 0.07 | 5.91 ± 0.20 | 5.77 ± 0.01 |
| 22 L | 6.37 ± 0.04 | 6.65 ± 0.06 | 6.67 ± 0.01 |
| 34 L | 6.44 ± 0.09 | 6.42 ± 0.06 | 6.43 ± 0.05 |
| 36 L | 6.51 ± 0.14 | 6.70 ± 0.02 | 6.68 ± 0.03 |
| 9 L | 6.63 ± 0.21 | 6.53 ± 0.15 | 6.39 ± 0.01 |
| 2 L | 6.66 ± 0.04 | 6.67 ± 0.01 | 6.67 ± 0.01 |
| 12 S | 6.96 ± 0.02 | 6.95 ± 0.01 | 7.00 ± 0.04 |
| 10 S | 6.98 ± 0.01 | 7.02 ± 0.04 | 6.95 ± 0.05 |
| 46 S | 6.99 ± 0.07 | 6.93 ± 0.13 | 6.95 ± 0.05 |
| 15 S | 7.04 ± 0.01 | 7.05 ± 0.04 | 7.16 ± 0.01 |
| 59 S | 7.10 ± 0.03 | 6.95 ± 0.12 | 7.07 ± 0.06 |
| 31 S | 7.20 ± 0.04 | 7.17 ± 0.01 | 7.05 ± 0.01 |
| Average for strain L | 6.43 ± 0.24 | 6.48 ± 0.30 | 6.44 ± 0.35 |
| Average for strain S | 7.05 ± 0.09 | 7.00 ± 0.09 | 7.03 ± 0.08 |

[y] Isolate numbers are followed by letters denoting the strain. Strain S isolates produce numerous small sclerotia (average diameter less than 300 μm); strain L isolates produce larger sclerotia, some which have a diameter exceeding 400 μm.

[z] Average pH (plus or minus standard deviation) of Czapek solution agar plus 0.05 M Hepes buffer after 10 days of growth at 30 C. For each initial pH, the average value for strain L isolates differs significantly ($P = 0.01$) from the average value for strain S isolates by Student's $t$-test. Similar results were obtained when the experiment was repeated with different isolates.

TABLE 4. Aflatoxin production in vitro by *Aspergillus flavus* isolates belonging to strains L and S

| Strain[x] | Test | Number of isolates | Fluorescence[y] | | Aflatoxin (ng/g)[z] | |
|---|---|---|---|---|---|---|
| | | | Average | Range | Average | Range |
| L | 1 | 4 | 5 | 3–8 | 73 | 0–260 |
| S | | 4 | 54 | 14–130 | 2,743 | 562–6,794 |
| L | 2 | 17 | 4 | 3–10 | 112 | 0–659 |
| S | | 6 | 55 | 31–70 | 6,807 | 3,554–8,911 |
| L | 3 | 24 | 8 | 2–47 | 519 | 0–4,900 |
| S | | 19 | 58 | 16–123 | 6,261 | 1,350–13,899 |

[x] Strain S isolates produce numerous small sclerotia (average diameter less than 300 μm); strain L isolates produce larger sclerotia, some of which have a diameter exceeding 400 μm.

[y] Agar fluorescence was measured on a linear scale of 0–150 with a scanning densitometer (7). Tests were replicated four to seven times. In all tests the average value for strain L isolates differs significantly ($P = 0.01$) from the average value for strain S isolates by Student's $t$-test. Values were log-transformed prior to analysis.

[z] Nanograms of aflatoxin $B_1$ per gram of culture. Aflatoxin contents of cultures were predicted for each tube from agar fluorescence by means of standard curves generated for each test (7). Tubes with negative predicted values were assigned the value 0.

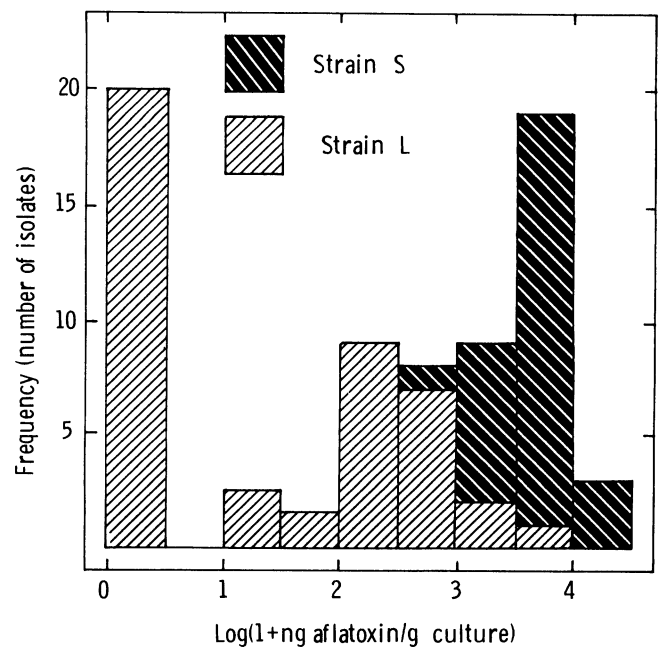


**Fig. 2.** Distribution of levels of aflatoxin production by isolates of the S and L strains of *Aspergillus flavus*. Aflatoxin production was determined on solid medium with the rapid fluorescence method as described in the text.

that by strain L isolates. No strain S isolate produced less than 500 ng/g in culture, whereas 80% of the strain L isolates produced less than 500 ng/g. The presence of several strain S isolates in a study would produce results indicative of an association between sclerotial production and aflatoxin production and may partly explain such correlations (9,14,16,22). Sclerotial morphogenesis and aflatoxin biosynthesis are interrelated (7), however, and differences between strains in sclerotial regulation may partly explain observed differences in aflatoxin production.

TABLE 5. Production of aflatoxin $B_1$ and bright green-yellow fluorescence (BGYF) by isolates of *Aspergillus flavus* during boll infection and effect of infection on lock weight

| Test | Isolate[w] | Aflatoxin[x,y] ($\mu$g/g) | BGYF[y,z] | Inoculated lock weight as percentage of uninoculated lock weight[y] |
|---|---|---|---|---|
| A | 13 L | 152 ± 91 a | 1.11 ± 0.5 a | 47 ± 7 cd |
| | 12 S | 119 ± 92 a | 0.26 ± 0.36 c | 57 ± 10 ab |
| | 6 L | 101 ± 58 a | 0.93 ± 0.59 a | 49 ± 9 bcd |
| | 11 L | 74 ± 36 a | 0.79 ± 0.68 ab | 53 ± 7 abcd |
| | 3 S | 38 ± 48 ab | 0.30 ± 0.57 bc | 57 ± 8 ab |
| | 42 S | 27 ± 32 b | 0 ± 0 c | 55 ± 13 abc |
| | 25 S | 8 ± 14 c | 0 ± 0 c | 62 ± 8 a |
| | 36 L | 0 ± 0 d | 0.79 ± 0.65 a | 45 ± 8 d |
| | Average for strain L | 82 ± 63 | 0.91 ± 0.15* | 49 ± 3* |
| | Average for strain S | 48 ± 49 | 0.14 ± 0.16* | 58 ± 3* |
| B | 70 S | 129 ± 56 a | 0.84 ± 0.49 c | 60 ± 8 bc |
| | 65 S | 101 ± 56 ab | 0.64 ± 0.26 c | 61 ± 6 b |
| | 60 L | 59 ± 29 bc | 0.88 ± 0.44 bc | 56 ± 7 bc |
| | 58 S | 44 ± 29 c | 0.19 ± 0.22 d | 63 ± 7 ab |
| | 41 L | 43 ± 20 c | 0.88 ± 0.21 bc | 55 ± 7 bc |
| | 16 L | 40 ± 27 c | 1.18 ± 0.26 ab | 52 ± 6 c |
| | 54 L | 39 ± 22 c | 1.24 ± 0.19 a | 56 ± 4 bc |
| | 66 S | 5 ± 4 d | 0 ± 0 d | 70 ± 5 a |
| | Average for strain L | 45 ± 9 | 1.05 ± 0.19* | 55 ± 3* |
| | Average for strain S | 69 ± 56 | 0.42 ± 0.39* | 64 ± 5* |

[w] Isolate numbers are followed by letters denoting the strain. Strain S isolates produce numerous small sclerotia (average diameter less than 300 $\mu$m); strain L isolates produce larger sclerotia, some of which have a diameter exceeding 400 $\mu$m.

[x] Aflatoxin $B_1$ content of inoculated locks. Aflatoxin was extracted with organic solvents, purified by thin-layer chromatography, and quantified by fluorescence, as described in the text.

[y] Average plus or minus standard deviation. For each experiment, values in the same column followed by different letters differ significantly ($P = 0.05$) by Fischer's LSD test. * = Average value for strain L isolates differs significantly ($P = 0.01$) from the average value for strain S isolates by Student's $t$-test.

[z] BGYF per uninoculated lock: 2 = BGYF on more than 50% of the lint; 1 = BGYF on less than 50% of the lint; 0 = no BGYF.

TABLE 6. Correlation coefficients and probabilities of correlations between aflatoxin level in vivo and various parameters

| Parameter correlated with toxin level in vivo[z] | Test 1 | | Test 2 | |
|---|---|---|---|---|
| | $r$ | Probability | $r$ | Probability |
| $A$: toxin level in vitro | 0.3964 | 0.3309 | 0.4780 | 0.2309 |
| $B$: lock deterioration | 0.2783 | 0.5045 | 0.1590 | 0.7069 |
| $C$: intraboll spread | 0.5298 | 0.1768 | 0.2560 | 0.5405 |
| $D$: $A \times B$ | 0.5064 | 0.2003 | 0.7262 | 0.0414 |
| $E$: $A \times C$ | 0.7530 | 0.0220 | 0.9101 | 0.0017 |
| $F$: $A \times B \times C$ | 0.7843 | 0.0204 | 0.9291 | 0.0008 |

[z] $A$ = quantity of toxin produced in culture medium during 3 days of growth at 30 C; $B = [1 - (\text{average inoculated lock weight}/\text{average uninoculated lock weight})]^2$; $C$ = average quantity of bright green-yellow fluorescence on lint of uninoculated locks plus 0.1.

Although aflatoxin production by the L and S strains differed in culture, aflatoxin production in vivo was not correlated with production in vitro, and the strains produced similar aflatoxin levels in vivo. Strain L, however, exhibited more pathogenic aggression by spreading within bolls and deteriorating locks to a greater extent. Neither measure of pathogenic aggression alone correlated with the level of aflatoxin produced in vivo. However, the product of the intraboll fungal spread parameter and the aflatoxin level in vitro did correlate with the aflatoxin level in vivo ($r$ = 0.75–0.91). The correlation improved slightly ($r$ = 0.78–0.93) when lock deterioration was factored into the term. Pathogenic aggression and its role in the *A. flavus*-cotton disease syndrome thus apparently explains some disparities between aflatoxin levels in vitro and in vivo.

Aflatoxin production in vitro is thought to reflect the ability of isolates to contaminate crops (23,26). Therefore, levels of aflatoxin produced in vitro by *A. flavus* isolates have repeatedly been quantified and compared (11,13,19). The lack of correlation between in vitro and in vivo aflatoxin levels in the current study indicates that the measurement of aflatoxin production in vitro alone is of little value in assessing the potential of an isolate to contaminate cottonseed.

Aflatoxin production in developing cottonseed is not correlated with pathogenic aggression; aflatoxin thus apparently does not contribute to virulence to cotton. This contention is further supported by the behavior of isolate 36, which produced no detectable aflatoxin in vivo and was highly aggressive. Similar isolates may have uses as biological control agents directed toward outcompeting toxigenic strains.

The relationship of the S and L strains is not clear. The strains may represent components of the same genetic pool or genetically distinct strains.

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