PHENOTYPIC VARIATION WITHIN THE S STRAIN OF *ASPERGILLUS FLAVUS*

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Abstract

Variability in growth rate was observed among S strain isolates of *Aspergillus flavus* from western Arizona. Isolates were grouped into fast and slow growing biotypes and compared for radial growth, and sclerotia, spore, aflatoxin, and pectinase production. Slow and fast biotypes were distributed equally among three collection areas with 35% of 300 S strain isolates classified as the slow biotype. Fast biotype isolates tended to produce more conidia than slow biotype isolates. However, both types produced similar quantities of aflatoxin B_1 in vitro (>200,000 ng/g). Plate assays indicated that both producers and non-producers of pectinase P2c were distributed among both biotypes. The results document divergence within the S strain isolates of *A. flavus*.

Introduction

Aflatoxin contamination of cotton seed is caused by *Aspergillus flavus* Link: Fr. Isolates of *A. flavus* can be divided into S and L strains based on sclerotial morphology (Cotty, 1989). On average, S strain isolates produce greater levels of aflatoxin than L strain isolates and can be important contributors to aflatoxin contamination of cottonseed (Cotty, 1996).

However, S strain isolates produce relatively few conidia and many sclerotia suggesting adaptation to the soil environment (Cotty et al., 1994). S strain isolates also vary in ability to produce pectinase P2c, an enzyme linked to virulence in aflatoxin contamination of developing cotton bolls (Cotty et al., 1990, Cleveland and Cotty, 1991). While studying large communities of aflatoxin producing fungi we observed variability among S strain isolates in radial growth. The objectives of the current study were to characterize growth rate variability among S strain isolates and to assess the relation of this variability to other S strain characteristics.

Materials and Methods

Isolate collection and culturing

Soil samples (5 to 12 per field taken from top 2 cm) were collected from fields within three areas (A, B, and C) in southwestern Arizona. *A. flavus* communities within soils were quantified and isolates were obtained via dilution plating on a modified rose bengal medium (Cu) (Cotty, 1994). *A. flavus* colonies were transferred from Cu to 5/2 agar (5% V-8 vegetable juice, 2% agar, pH 5.2) after three days and incubated for 4 days (31◦C), after which either L or S strains were distinguished (based on colony morphology). During the process, isolates with different growth rates were distinguished and classified as the slow biotype if growth was less than 1.0 cm per day. The incidence of both S strain isolates and the fast and slow biotypes was calculated. Fifteen S strain isolates (10 fast and 5 slow) were transferred by single conidium and stored for detailed study. Isolates were from 5 different fields, with at least one field representing each area. For long term storage, 5 mm plugs were taken from all cultures and saved in 2.5 ml sterile distilled water in Wheaton vials and stored at 3◦C.

Growth rate

To directly compare growth rates of the fast and slow biotypes of the S strain, 15 S strain isolates, 10 fast and five slow, were cultured on 5/2 medium. One hundred microliters of spore suspension (about 10^6 spores/ml) for each isolate was spread on 5/2 medium and incubated (31◦C). After 18 h plugs (5 mm in dia.) were transferred from 5/2 to Czapek-Dox (CZ) agar (Difico), Potato Dextrose agar (Difico)and 5/2 agar. For each isolate a single plug was placed mycelia down onto each medium (6 plates per medium). Plates were divided into two groups incubated at either 20◦C or 30◦C. Radial growth was measured at 24 and 72 h and used to calculate growth rate per day.

Spore and Sclerotia production

Spore and sclerotia production was determined for two fast and one slow S strain isolate, and one L strain isolate from each of 5 cotton fields (16 isolates total). For each isolate conidia were initially spread onto 5/2 as in the growth rate experiments, and 5 millimeter plugs subsequently transferred to 3 CZ plates. After 5 days at 31◦C , mean spore production was calculated for each isolate using a turbidimeter (Orbeco-Hellige Co.) and standard curve. Sclerotia production was determined on a dry wt. basis.

Aflatoxin production

Aflatoxin production in liquid culture was determined for 12 S strain isolates, (eight fast and four slow). As previously described (Cotty and Bayman, 1993) 100 µl spore suspension (1.3×10^5 spores/ml) from each isolate was inoculated into a 250 ml Erlenmyer flask containing 70 ml of A&M liquid medium with 3.0g/L NH_4SO_4 (Adye and Mateles, 1964). After incubation in a rotary shaker (150 rmp, five d, 31◦C, dark), culture pH was measured, and 70 ml of acetone added to each flask to stop culture growth and extract the aflatoxin from the mycelium. Mycelia was separated by filtration, dried and weighed. Extracts and aflatoxin standards were separated on TLC plates and aflatoxin B_1 was quantified with a scanning densitometer (Cotty and Bayman, 1993).

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Pectinase Assay

Pectinase agar (Cotty et al., 1990) was inoculated as described in the growth rate experiment (above). After incubation (31◦C , 4 d, dark), plates were stained for 30 minutes with ruthenium red (0.1g) Plates positive for pectinase P2c were evident by a visible clear zone around the center colony, and negative plates had a red ring. Clear zone width was also measured.

Results

There were no differences among the 3 examined areas (A, B, C) in propagules/gram soil, incidence of S strain isolates, and the percent S strain isolates classified as the slow biotype (table 1). About 45 % *A. flavus* isolates belonged to the S strain and, of these, 35% were the slow biotype. This was consistent for all locations in all areas.

Growth rate

On all media tested fast biotype isolates grew significantly ($P<0.01$) faster than slow biotypes at 30◦C but not at 20◦C. There were no difference in growth rate at either temperature for the slow biotype except on 5% V-8 agar (figure 1). The change in growth rate between 20◦C and 30◦C was greater for the fast biotype than the slow biotype.

Spore and Sclerotia production

L strain *A. flavus* isolates produced significantly ($P<0.01$) more conidia than S strain isolates (figure 2). However, there were no differences between the fast and slow biotypes in the amount of conidia produced. There also were no differences in sclerotia mass between the L and S or between the slow and fast biotypes (figure 3). However, S strain isolates produced considerably more sclerotia than the L strain. Sclerotial and conidial were inversely correlated (figure 4).

Aflatoxin production

In liquid fermentation, significant differences occurred between isolates in aflatoxin production, pH, and mass ($P<0.01$, $P<0.01$, and $P=0.02$, respectively). However, the fast and slow biotypes did not differ significant in any variable (table 2). Aflatoxin B_1 levels were greater than 200,000 ppb in vitro for all S strain isolates. PH levels ranged from 2.13 to 2.30 and dry culture mass ranged from 0.502 g to 0.653 g.

Pectinase Assay

A clear zone developed on pectin agar with 80% of fast biotype isolates and 60% of slow biotype isolates produced (Cotty et al. 1990) (table 3). There were significant differences in the size of the clear zone produced by these isolates ($P = 0.04$). Isolate M96MC4G produced the largest clear zone (0.9cm), and M96MC4H produced the smallest clear zone (0.5cm). Isolates differed in morphology on pectinase medium. All slow biotype isolates exhibited a fluffy colony morphology whereas some fast biotype isolates exhibited flat colony morphologies.

Discussion

Genetic diversity within the S strain of *A. flavus* has been observed using both vegetative compatibility and RAPD analyses (Bayman and Cotty, 1991). In the current study, S strain isolates were found to differ in growth rate at 30◦C. Isolates were sorted into two biotypes: fast (growth rate > 1.2 cm/day on V-8 agar at 30◦C) and slow (growth rate < 1.0 cm/day on V-8 agar at 30◦C). Growth rate did not differ among the two biotypes at 20◦C, possibly indicating adaptation to high temperature environments by the fast biotype. In all test areas, the fast biotype isolates were in greater abundance than the slow biotype isolates. This also may indicate that high temperature adaptation of the fast biotype isolates is an advantage in the warm desert soils of southwestern Arizona.

The L strain isolates produced abundant conidia, and had a similar sclerotial mass to the S strain isolates, although the L strain produced fewer, larger sclerotia. These observations are supported by previous research (Cotty, 1989). This may indicate S strain isolates are adapted to the soil environment, and that L strains are adapted to aerial dispersal and crop colonization. Correlation data indicates as conidial production increases sclerotial production decreases, perhaps reflecting a trade off among strains between colonization of food sources with conidia and long term survival through production of sclerotia.

The ability to produce large quantities of aflatoxin by fast and slow biotypes may aid in the survival of the S strain in the soil environment. Aflatoxins may protect sclerotia and conidia from insect herbivory and microbial degradation (Cotty et al., 1994). Several isolates of both the fast and slow biotypes were able to produce pectinase P2c. S strain isolates that produce pectinase have an advantage in that they may infect and colonize crop components more effectively than non-pectinase producing counterparts. Failure of many S strain isolates to produce pectinase P2c may indicate S strain adaptation to a niche in which rapid utilization of pectin is not essential. Differences among S strain isolates in ability to produce pectinase P2c is another indication of divergence among S strain isolates.

Differences in pectinase production and growth rate suggest adaptation to different niches. How these adaptations benefit *A. flavus* in different situations is unknown in the desert agroecosystem. The threat of aflatoxin contamination suggests we need a greater understanding of the interactions of these biotypes. This may ultimately benefit the control of *A. flavus*. Thus, geographic distribution of the fast and slow biotypes and the divergence of these biotypes in the desert agroecosystem merits further research.

References

Adye, J., and Mateles, R. I. 1964. Incorporation of labeled compounds into aflatoxins. Biochim. Biophys. Acta. 86:418-420.

Bayman, P., and Cotty, P. J. 1991. Vegetative compatibility and genetic diversity in the *Aspergillus flavus* population of a single field. Canad. J. Bot. 69:1707-1711.

Cleveland, T. E., and Cotty, P. J. 1991. Invasiveness of *Aspergillus flavus* isolates in wounded cotton bolls is associated with production of a specific fungal polygalacturonase. Phytopathology 81:155-158.

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

Cotty P. J., Cleveland, T. E., Brown, R. L., and Mellon, J. E. 1990. Variation in polygalacturonase production among *Aspergillus flavus* isolates. Appl. Environ. Microbiol. 56:3885-3887.

Cotty, P. J., and Bayman, P. 1993. Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxogenic strain. Phytopathology 83:1283-1287.

Cotty, P. J. 1994. Comparison of four media for the isolate of *Aspergillus flavus* group fungi. Mycopathologia 125:157-162.

Cotty, P. J., Bayman, P., Egel, D. S., and Elias, K. S. 1994. Agriculture, aflatoxins and *Aspergillus*. pages 1-27 in K. A. Powell et al. ed. The Genus *Aspergillus*. Plenum Press. New York.

Cotty, P. J. 1996. Aflatoxin contamination of commercial cottonseed caused by the S strain *of Aspergillus flavus*. Phytopathology 86:S71(Abstract).

Table 1. Percentage of slow growing S strain islolates in three locations in Southwest Arizona.

Area[a]	Propagules/ gram[b]	Average S Strain Isolates[c]	% Slow S Strain Isolates[d]
A	425	45	36
B	53	50	34
C	212	39	35
Average	230	45	35

[a] Three fields were sampled for A and B and 2 fields for C. Each field was approximately 40 acres.
[b] Total amount of *Aspergillus flavus* propagules present in one gram of soil; includes both S and L strains.
[c] Percent *A. flavus* isolates belonging to the S strain.
[d] S strain isolates were classified as slow if they grew less than 1.0 cm per day.

Table 2. Aflatoxin production by fast and slow growing S strain isolates.

Biotype	Isolate[a]	pH	Aflatoxin B1 (µg/g)	Mass (g)
Fast	Y96BARKA11J	2.30 a[b]	1700 a	0.502 b
	M96MC3G	2.17 b	2000 a	0.578 a, b
	Y9695B5D	2.22 a, b	2000 a	0.546 a, b
	M962055X	2.15 b	780 a, b, c	0.630 a, b
	M96MC4H	2.17 b	1000 a, b, c	0.566 a, b
	Y96BARKA11O	2.20 b	250 c	0.568 a, b
	Y9695B1G1	2.15 b	350 b, c	0.643 a
	M962055F	2.13 b	630 c	0.653 a
Avg fast		2.18	1089	0.585
Slow	M962055K	2.17 b	1300 a, b	0.593 a, b
	Y9695B4F	2.21 a, b	1900 a	0.586 a, b
	M96MC4G	2.16 b	720 a, b, c	0.643 a
	Y96BARKA11H	2.14 b	910 a, b, c	0.582 a, b
Avg slow		2.17	1208	0.601

[a] Isolates were collected from cotton fields in southwest Arizona.
[b] Values within a column followed by the same letter do not differ significantly (P=0.05) by Tukey's HSD test.

Table 3. S strain pectin bioassay

Biotype	Isolate[a]	Colony morphology on pectin medium	Pectin assay reaction[b]
Fast	M96STUHRX33I	FLAT	0.7 b[c]
	Y9695B5D	FLAT	0.7 b
	Y96BARKA11J	FLUFFY	0.7 b
	M96MC3G	FLAT	0.5 b
	M962055X	FLUFFY	RED RING
	M96MC4H	FLUFFY	RED RING
	M96STUHRX31C	FLAT	RED RING
	Y96BARKA110	FLAT	RED RING
	M962055F	FLUFFY	RED RING
	Y9695B1G1	FLAT	RED RING
Slow	M96MC4G	FLUFFY	0.9 a
	Y9695B4F	FLUFFY	0.7 b
	M962055K	FLUFFY	0.6 b
	Y96BARKA11H	FLUFFY	RED RING
	M96STUHRX34N	FLUFFY	RED RING

[a]Isolates were collected from fields in southwest Arizona.
[b]Cells containing values are positive for pectinase P2c activity, the mean values indicate the size of the clear zone in centimeters. Cells containing 'RED RING' indicate the isolate lacks pectinase activity (Cotty, et al. 1991).
[c]Values within a column followed by the same letter do not differ signficantly (P=0.05) by Fisher's LSD test.

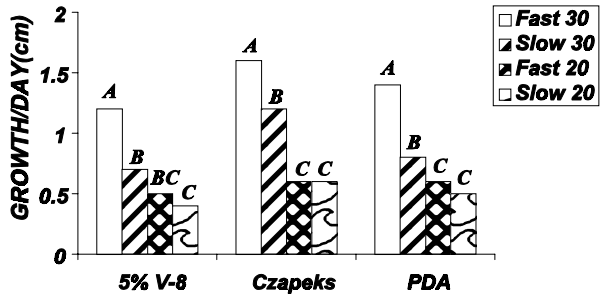

Figure 1. Difference in growth rate between the fast and slow biotypes of the S strain of *A. flavus* at 20◦C and 30◦C. Bars representing the same media with the same letter are not significantly different based on Tukey's HSD test (P=0.05).

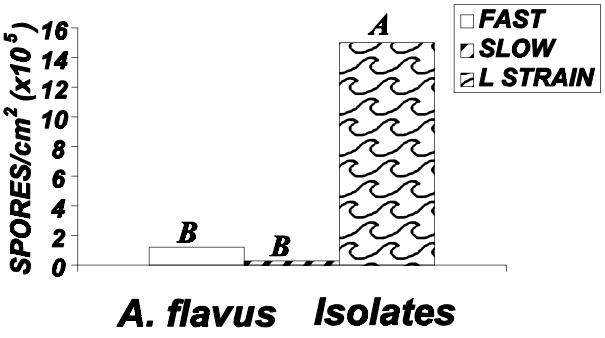

Figure 2. Difference in spore production between fast and slow biotypes of the S strain of A. flavus and L strain isolates of *A. flavus*. Bars with the same letter are not significantly different based on Tukey's HSD test (P=0.05).

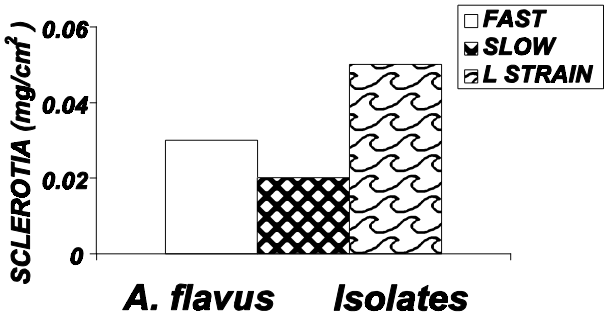

Figure 3. Difference in sclerotial production between the fast and slow biotypes of the S strain of *A. flavus* and the L strain of *A. flavus*.

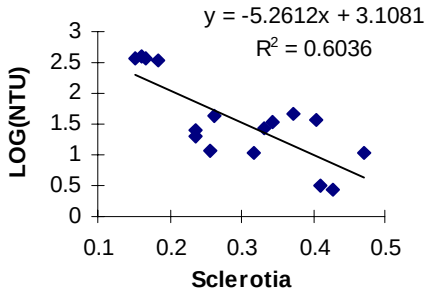

Figure 4. Relationship between conidial and sclerotial production by S and L strain isolates of *A. flavus.*