

Use of Aflatoxin-Producing Ability Medium to Distinguish Aflatoxin-Producing Strains of *Aspergillus flavus*

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Aflatoxin-producing ability medium was tested for its ability to distinguish aflatoxin-positive from aflatoxin-negative strains of *Aspergillus flavus* in naturally occurring populations from corn at harvest. All of the aflatoxin-positive strains and some of the aflatoxin-negative strains produced aflatoxins when cultured on cracked corn. Although the data indicate that aflatoxin-producing ability medium is not entirely reliable in distinguishing potential aflatoxin-producing strains of *A. flavus* from nontoxigenic strains, it is significant that the medium did not yield false-positives.

Aflatoxin-producing ability (APA) medium was developed to simplify the screening of large numbers of *Aspergillus flavus* isolates for aflatoxin production (2). The detection of an ultraviolet-induced (366-nm) intense-blue fluorescence was linked to the production of aflatoxin in APA medium as determined by thin-layer chromatography of CHCl_3 extracts of the fluorescing agar. Since strains belonging to the *A. flavus* group may also produce substances other than aflatoxin that give a blue fluorescence (false-positives), the authors reasoned that the fluorescence test should be performed on several known aflatoxin-positive and aflatoxin-negative strains to determine the validity of APA test results. Of the 12 aflatoxin-negative strains selected for study by Hara et al. (2), 10 included isolates of *Aspergillus oryzae* and *Aspergillus sojae*. These are domesticated molds used in oriental food fermentations; aflatoxin production has never been documented for strains assigned to these taxa. A more rigorous test of the ability of APA medium to distinguish aflatoxin-positive and aflatoxin-negative strains seemed justified. An isolate of *A. flavus* NRRL 6412 from corn harvested in North Carolina was aflatoxin negative on APA medium but produced aflatoxin when grown on autoclaved corn (4). Cultivation of individual isolates on a natural substratum, such as autoclaved corn, has proven to be a more reliable test of the aflatoxin-producing potential of an organism than has cultivation on a semisynthetic medium (e.g., APA medium) (3).

Before the APA test can be used to distinguish between aflatoxin-producing and nonproducing strains obtained from natural sources, it should be applied to a randomly selected, naturally occurring population of *A. flavus* group isolates

whose APA is unknown when the fluorescence test is performed.

MATERIALS AND METHODS

During the course of a mycological study of corn kernels sampled at harvest from North Carolina fields (C. W. Hesseltine, R. Rogers, and O. L. Shotwell, *Mycologia*, in press), we isolated approximately 200 strains of *A. flavus*. They were obtained from colonies growing on or around individual surface-sterilized kernels incubated for 5 days at 28°C on malt extract agar in petri dishes. The initial isolates were cultured on slants of Czapek agar, and 2-mm³ blocks of agar-containing mycelium were transferred to slants of APA medium. The slants were examined after 5, 7, and 10 days for a blue fluorescence in the medium. A total of 12 fluorescent and 12 nonfluorescent cultures were selected to represent corn samples in which levels of aflatoxin B₁ ranged from not detected to over 800 ppb (800 µg/g) (Table 1).

The following fermentations were performed to determine whether any of the 24 isolates could produce aflatoxins on a solid substrate (e.g., cracked corn). Inoculum was obtained by harvesting conidia from 14-day-old slant cultures of each isolate on Czapek agar. Cultures were incubated at 25°C. Spore inoculum of individual strains was suspended in a volume of sterile 0.05% Triton X-100 to give a final spore concentration of approximately 1×10^6 /ml.

Fermentations were carried out in 300-ml Erlenmeyer flasks containing 50 g of aflatoxin-free cracked corn. Distilled water (25 ml) was added to each flask before autoclaving at 15 lb/in² for 25 min. After the flasks had cooled to room temperature, they were inoculated with 1.0 ml of the appropriate spore inoculum and incubated for 8 days at 25°C.

After incubation, samples were assayed for aflatoxin by the Association of Official Analytical Chemists approved method for corn (1). They were extracted with chloroform-water, and the extracts were partially purified on a Silica Gel 60 column. The column was washed separately with hexane and ether, and aflatox-

TABLE 1. Aflatoxins produced by isolates of *A. flavus* that differ in their ability to cause fluorescence under ultraviolet light (8-day incubation at 25°C)^a

Aflatoxin level (ppb) in 10-lb (ca. 4.54-kg) corn sample from which isolate was obtained ^c	Aflatoxin (ppb) produced during 8-day fermentation on cracked corn							
	APA positive ^b				APA negative ^b			
	B ₁	B ₂	G ₁	G ₂	B ₁	B ₂	G ₁	G ₂
ND	2,956 ^d	64	ND	ND	5,675 ^e	105	ND	ND
	2,017	39	ND	ND	5	1	ND	ND
1-25	1,894 ^f	26	ND	ND	ND ^g	ND	ND	ND
	3,175	53	ND	ND	ND	ND	ND	ND
	1,740	6	ND	ND	ND	ND	ND	ND
26-50	3,480	8	ND	ND	4	1	ND	ND
51-100	4,481	8	ND	ND	ND	ND	ND	ND
101-200	1,664	22	ND	ND	ND ^h	ND	ND	ND
	4,272 ^d	55	ND	ND	3,014 ⁱ	84	ND	ND
201-400	1,414	7	ND	ND	3	1	ND	ND
	4,794	109	ND	ND	31	1	ND	ND
800+	8,362	470	2,786	71	ND	ND	ND	ND

^a Cultures given in footnotes c-h are maintained in the Agricultural Research Culture Collection, Peoria, Ill.

^b Total number of isolates, 12.

^c North Carolina corn sampled at harvest in 1977. ND, Not detected.

^d NRRL 6539.

^e NRRL 6536.

^f NRRL 6540.

^g NRRL 6541.

^h NRRL 6538.

ⁱ NRRL 6537.

ins were eluted with methanol-chloroform. The corn extracts were spotted onto Adsorbosil-1 thin-layer chromatography plates developed in acetone-chloroform-water (12:88:1.5), and aflatoxins were determined by densitometry.

To obtain single-spore isolates of selected *A. flavus* strains, we distributed 0.5 ml of a given conidial suspension (1×10^4) over Czapek agar in petri dishes and incubated the plates at 25°C for 18 h. Individual conidia showing germ tubes were excised on an agar block (2 mm³) supporting just one spore and transferred to a slant of Czapek agar. After 7 days of incubation at 25°C, a portion of the mycelium was transferred to slants of APA medium for fluorescence testing. Finally, single-spore isolates of NRRL 6536 and NRRL 6537 were examined for their ability to produce aflatoxins on cracked corn.

RESULTS AND DISCUSSION

Aflatoxin B₁ was produced in substantial quantities (>1,400 ppb) on cracked corn by each of the 12 isolates giving aflatoxin-positive test results on APA medium (Table 1). Furthermore, aflatoxin B₁ production by the isolate was uncorrelated with the aflatoxin level in the corn sample from which the isolate was obtained. Surprisingly, two isolates (NRRL 6536 and NRRL 6537) that were initially classified as aflatoxin negative based on the APA test produced aflatoxin at levels of greater than 3,000 ppb. In addition, four other APA-negative isolates produced detectable levels of aflatoxin (3 to 31 ppb). The remaining APA-negative isolates

(six strains) produced no detectable aflatoxins on cracked corn. We next wanted to determine whether the isolates that produced conflicting results on APA medium and cracked corn might represent genetically heterogeneous cultures.

Thirty single-spore isolates from each of seven selected *A. flavus* strains were grown on APA medium and examined under black light for a blue fluorescence (Table 2). These included APA-negative strains that failed to produce detectable quantities of aflatoxins on cracked corn (NRRL 6538 and NRRL 6541) and both APA-negative strains (NRRL 6537, NRRL 6536, and NRRL 6412) and APA-positive strains (NRRL 6539 and NRRL 6540) that produced substantial quantities of aflatoxin on cracked corn. Single-spore isolates from strains that initially were APA negative and that produced no detectable aflatoxins on cracked corn were all APA negative. Likewise, single-spore isolates from strains that initially were APA positive and that produced substantial quantities of aflatoxin on cracked corn were all APA positive. However, single-spore isolates from strains that produced aflatoxin in cracked corn but that were aflatoxin negative on APA medium produced varied test results. In the case of NRRL 6537, all 30 single-spore isolates were aflatoxin negative on APA medium. Single-spore isolates from NRRL 6536 or NRRL 6412 were either aflatoxin positive or aflatoxin negative on APA medium. The latter results provided evidence that our initial isolates

TABLE 2. Distribution of APA-positive (APA +) and APA-negative (APA -) test results among 30 single-spore isolates from selected strains of *A. flavus*^a

Initial isolate	Initial APA test	Aflatoxin produced on cracked corn	Single-spore isolates	
			APA +	APA -
NRRL 6538	APA -	ND ^b	0	30
NRRL 6541	APA -	ND	0	30
NRRL 6537	APA -	+	0	30
NRRL 6536	APA -	+	26	4
NRRL 6412	APA -	+	13	17
NRRL 6539	APA +	+	30	0
NRRL 6540	APA +	+	30	0

^a Development of a blue fluorescence under black light (ultraviolet, 366 nm) after a 7-day incubation at 28°C (= APA +).

^b ND, Not detected.

were genetically heterogeneous. When single-spore isolates of NRRL 6536 (including APA-negative and APA-positive strains) were cultured on cracked corn, aflatoxin was detected in every instance (Table 3).

Our results demonstrate that APA medium will not identify every aflatoxin-producing strain of *A. flavus*. Some APA-negative strains produced aflatoxin when cultured on cracked corn. It is apparent that APA medium is not a suitable substrate for aflatoxin production by all aflatoxin-producing strains of *A. flavus*. However, because all of the APA-positive strains also produced aflatoxin in corn, false-positives do not appear to be a problem and the fluorescence test does have value as a presumptive test in screening for aflatoxin-producing molds.

A survey procedure that might be adopted in examining naturally occurring *A. flavus* populations for numbers of toxigenic versus nontoxigenic individuals would be to (i) first screen all isolates on APA medium and record the number of aflatoxin-positive strains and (ii) culture the

TABLE 3. Aflatoxin production in corn by single-spore (SS) isolates of *A. flavus* representing both aflatoxin-positive and aflatoxin-negative strains on APA medium^a

Parent strain	Fluorescence in APA medium	Aflatoxin (ppb) in fermented corn			
		B ₁	B ₂	G ₁	G ₂
NRRL 6536	-	128	ND ^b	ND	ND
SS-1	+	122	ND	ND	ND
SS-2	-	211	1	ND	ND
SS-11	-	191	1	ND	ND
SS-18	+	238	2	ND	ND
NRRL 6537	-	389	2	ND	ND
SS-14	-	520	5	ND	ND
SS-17	-	274	2	ND	ND
SS-18	-	430	6	ND	ND
SS-29	-	336	3	ND	ND

^a Inoculum from 7-day-old subculture of initial isolate grown on Czapek agar slant.

^b ND, Not detected.

APA-negative strains on autoclaved cracked corn or some other cereal and assay for the presence of aflatoxins by using chemical analytical methods.

LITERATURE CITED

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