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Improved Method of Screening for Aflatoxin with a Coconut Agar Medium†

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Nine isolates of *Aspergillus flavus* and *Aspergillus parasiticus* were screened for aflatoxin production on a coconut extract agar medium. Aflatoxin-producing colonies were detected under long-wave UV light (365 nm) by blue fluorescence on the reverse side after 2 to 5 days of growth. Aflatoxin production was verified by chemical analysis. Several types of shredded coconut available in the United States were tested and found to be satisfactory. No additives were required. Various parameters affecting the test were investigated.

Our recent research in genetics required screening vast numbers of single-spore isolates of *Aspergillus flavus* rapidly and economically. Such screening differs from programs which survey agricultural commodities, mixed feeds, and raw and processed foods for contamination with aflatoxin (4–6, 8) in that our interest is in the aflatoxin-producing potential of the isolated spores rather than the amount of aflatoxin per se in a given substrate. In this regard, we found the screening methods described previously to be unsuitable for our purposes.

De Vogel et al. (3) described a screening test with a complex agar medium containing sucrose, various salts, and an aqueous extract of aflatoxin-free groundnuts (peanuts). A thin layer of Hyflo Supercel was spread on the bottom of the dish before the medium was poured into plates. Following inoculation and incubation for 48 to 72 h, plates were examined under UV radiation (350 to 370 nm), and a bright blue fluorescence indicated the presence of aflatoxin. Hara et al. (5) described a somewhat similar method that did not require the groundnut extract. They used a modified Czapek agar medium containing corn steep liquor, named aflatoxin-producing-ability (APA) medium. APA medium was less laborious to prepare than the De Vogel et al. medium. However, we have found that after prolonged storage, corn steep liquor may lose its aflatoxin-promoting ability and that different batches of corn steep liquor vary in effectiveness (unpublished data). Lin and Dianese (7) described a coconut agar medium (CAM) for rapid detection of aflatoxin. Blue fluorescence under long-wave UV light was observed on the reverse side of aflatoxin-producing colonies. This method was faster and simpler than those previously reported. However, they used commercial coconut extracts that are not available in the United States. We could not prepare the medium used in Brazil. Thus, we have found previously described techniques to be unsuitable for one or more of the following reasons: (i) the medium was laborious to prepare, (ii) the ingredients were not readily available, (iii) the medium did not support vigorous growth of *A. flavus* Link and *Aspergillus parasiticus* Speare, or (iv) the medium was not consistently reliable or sufficiently accurate. Thus, we have modified a previously reported procedure (7) to satisfy our requirements for a simple, rapid method for large-scale screening for aflatoxin-producing strains of *A. flavus* and *A. parasiticus*.

MATERIALS AND METHODS

Isolates. All isolates used in this study were obtained from the Northern Regional Research Center, except for *A. flavus* SI5565, which was produced in our laboratory from NRRL 5565 following treatment with emetine (S. K. Iyer, M. S. thesis, Auburn University, Auburn, Ala., 1986).

Cultivation and observation of fluorescence. Stock cultures were maintained on PDA tubes and plates. Inoculations were done by mass conidial transfer to the center of inverted plates, which were then incubated upside down at 26 to 28°C. Each plate contained a single, large, central colony, the reverse side of which was periodically observed under long-wave (365 nm) UV light for blue fluorescence. An uninoculated plate was observed for reference.

CAM. Readily available brands of shredded coconut were obtained locally (Baker's in plastic bags and cans and Tropical brand shredded coconut). Typically, coconut marketed for home use consists of the following: moisture, 17%; protein, 3.7%; fat, 28.6%; carbohydrates, 49.6%; sugars (mostly sucrose), 32%; fiber, 4.2%; and ash content, 0.8% (USDA standard analysis). In addition, unimproved Baker's Gem coconut was obtained from General Foods in bulk. The Gem coconut had the following composition: moisture, 3.5%; fat, 67 to 71%; free fatty acids, 0.15%; invert (reducing) sugar, 0.6%; pH, 6.1 to 6.7 (data supplied by General Foods).

For the Baker's and Tropical brand coconut purchased locally, 100 g of shredded coconut was homogenized for 5 min with 200 ml of hot distilled water. For Baker's Gem coconut, 100 g of shredded coconut was homogenized for 5 min with 300 ml of hot distilled water. In all cases, the homogenate was filtered through four layers of cheese cloth, and the pH of the clear filtrate was adjusted to pH 7 with 2 N NaOH. Agar was added (20 g/liter), and the mixture was heated to boiling and cooled to about 50°C. The pH was checked and adjusted to 7 when necessary. The mixture was then autoclaved for 18 min at 15 lb/in^2, cooled to about 40 to 45°C, and poured while being stirred into sterile petri dishes. Generally, 12 to 15 petri dishes of CAM were prepared from each 100-g portion of shredded coconut.

Assay of aflatoxin. Analyses were conducted by published methods (1) modified as follows. The entire content of each CAM plate (approximately 25 g) was scraped into an explo-

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TABLE 1. Production of blue fluorescence and aflatoxin B_1 by isolates of *A. flavus* and *A. parasiticus* on CAM and YES media

Fungal isolate	Blue fluorescence visible at 365 nm	Aflatoxin B_1 production	
		CAM (μg/g)	YES broth[a] (μg/ml)
A. flavus			
NRRL 5565	−	0	0
NRRL 5941	−	0	0
NRRL 6107	+	2.20	20.00
NRRL 27465[b]	+	0.11	0.10
NRRL 27466[b]	+	0.31	0.07
NRRL 27470[b]	+	0.21	0.20
SI5565[c]	+	0.15	0.10
A. parasiticus			
NRRL 3240	+	2.00	27.00
NRRL 6109	−	0	0

[a] Data are for 100 ml of filtrate of fungal cultures grown on YES broth (2% yeast extract–20% sucrose) for 13 days.

[b] Albino strains.

[c] Produced by treating normally nontoxigenic *A. flavus* NRRL 5565 with emetine.

sion-proof blendor with 100 ml of chloroform-acetone (85:15, vol/vol) and blended for 3 min. Contents of cultures grown on 100 ml of 2% yeast extract–20% sucrose (YES) broth per 250-ml flask (2) were filtered through Whatman no. 4 filter paper, and the filtrates were extracted with chloroform in a separatory funnel. Chloroform extracts from broth and agar plate cultures were dried with sodium sulfate, evaporated to dryness under an air stream, and quantitatively analyzed for aflatoxins by thin-layer chromatography (1).

RESULTS AND DISCUSSION

Results of tests with YES broth and CAM without any additives are presented in Table 1. Strains NRRL 5565, 5941, and 6109 did not produce blue fluorescence on the reverse side of the colony under UV light. Toxigenic strains produced blue fluorescence on CAM but not on YES agar plates. Chemical analysis confirmed the presence of aflatoxin in blue-fluorescing agar plates and its absence in nonfluorescing agar plates. In *A. flavus* NRRL 6107 and *A. parasiticus* 3240, both rapid growers and relatively potent aflatoxin producers, the blue fluorescence produced on the reverse side of the colonies was detected in 2 days. In the lower aflatoxin producers (*A. flavus* SI5565 and NRRL 27465, 27466, and 27470), the 2-day cultures produced a weak blue fluorescent ring surrounding the colony, but it was necessary to incubate these cultures for 3 to 5 days to observe an intense background of bright blue fluorescence. When cultures were incubated longer than 7 days, they became difficult to evaluate because mycelial growth reached the margin of the plate and obscured the blue fluorescence. Generally it was best to evaluate the plates after 2 days, but before a plate was judged to be negative it was examined carefully for the presence of a thin blue fluorescent ring just external to the colony (reverse side); if it was present, the plate was incubated an extra 2 to 3 days.

Lin and Dianese (7) observed the production of a conspicuous orange-yellow pigmentation of the mycelium prior to the appearance of blue fluorescence. This allowed them to judge the aflatoxin-producing ability of their cultures sooner and without the use of a UV lamp. Some of our cultures also produced the yellow pigment. *A. parasiticus* 6109 was a copious producer of yellow pigment, and *A. flavus* NRRL 27470 and 6107 were intermediate producers, whereas *A. flavus* NRRL 27465, 27466, and 27469 (albino strains), *A. flavus* NRRL 5941, and *A. parasiticus* NRRL 3240 produced only small amounts of yellow pigment, which was obscured after about 2 days. *A. flavus* NRRL 5565 and SI5565 did not produce any discernible yellow pigment. Thus, we found that the production of yellow pigment was not a reliable indicator of aflatoxin-producing ability.

Tropical and Angel Flake shredded coconut, the only brands readily available locally, were equally satisfactory in supporting growth, blue fluorescence, and aflatoxin production when incorporated into CAM. These products contained appreciable levels of added sugar and unknown quantities of various preservatives such as propylene glycol, metabisulfite, and sorbitol. Nevertheless, all fungi tested grew extremely well on CAM prepared with these coconut products.

The use of Gem coconut purchased in bulk greatly lowered the cost of screening on this medium. However, Gem coconut differed from coconut purchased locally (see Materials and Methods). Gem contained only 3.5% moisture and 0.6% sugar. Experimentally, it was not beneficial to supplement the coconut with sugar, since growth and aflatoxin production by *A. flavus* was just as rapid on the low-sugar medium as on medium supplemented with 5 to 20% dextrose. Blue fluorescence was also just as pronounced on low-sugar as on high-sugar CAM.

Because of its lower moisture, the basic Gem coconut medium was prepared with extracts consisting of 3 parts hot distilled water to 1 part coconut rather than only 2 parts water to 1 part coconut. Best results were obtained when this extract was used without further dilution or when the extract was diluted by addition of an equal volume of hot water (a 1:1 dilution). A dilution of 1:2 resulted in slightly less growth and slightly less pronounced blue fluorescence. Dilutions of 1:3 or greater resulted in failure of the test, even when sucrose or yeast extract was added to CAM at each dilution to maintain a constant level. Yeast extract quenched fluorescence. Contrary to the work of Lin and Dianese (7), we found that using undiluted extract or extract diluted no more than 1:1 was preferable; a dilution of 1:2 was acceptable, but is not recommended. Precipitation was not a problem, as discussed by Lin and Dianese (7), except when the coconut extract was diluted more than 1:2, in which case a precipitate was conspicuous in the medium. Thus, the test seemed to work best with undiluted and unamended Gem coconut extract containing 2% agar, and the addition of sugar, nitrogen, or other ingredients to CAM was neither necessary nor desirable. We found, as did Lin and Dianese (7), that excellent results were obtained in 2 to 5 days, rather than 7 to 8 days as on APA medium (5, 9), probably because the fungi grew more rapidly on CAM than on APA medium. Also, we found disposable plastic petri dishes to be satisfactory and economical, whereas Hara et al. (5) specified that glass petri dishes should be used for screening on APA medium.

Although we found screening on CAM to be preferable in most cases, there are circumstances under which it would be preferable to use APA for screening. The ultimate intensity of the blue fluorescence obtained on APA is significantly greater than that obtained on CAM, although growth on APA is slower than on CAM, on which colony growth more quickly fills the plate and obscures fluorescence. However, for most purposes, we found the CAM screening method to be simpler, faster, and less expensive than any of the other methods tested.

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