A Coconut-Agar Medium for Rapid Detection of Aflatoxin Production by Aspergillus spp.

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ABSTRACT

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An agar medium containing commercial coconut extract and with pH adjusted to 6.9 was developed for detection of aflatoxin production by *Aspergillus* spp. On this medium, aflatoxin-positive, but not -negative, isolates showed a characteristic blue or blue-green fluorescence in agar surrounding the colonies when observed under ultra-violet light. The fluorescence produced by strong aflatoxin producers began to appear within 32 hours after transfer to the medium. The intensity of fluorescence increased with prolonged incubation. An incubation time of 3 days was sufficient for detection of even very weak aflatoxin production. Several known aflatoxin promoters either had no effect or decreased the production of fluorescence. Incubation at 20 C slightly reduced the intensity of fluorescence, whereas no difference was observed at 24, 28, or 32 C. Seven of 27 Brazilian isolates of the *A. flavus* group that were tested on the medium were aflatoxin-positive; this was confirmed by thin-layer chromatographic assay of culture broths. An orange-yellow pigmentation on the coconut-agar medium consistently was associated with the production of fluorescence and the aflatoxin-producing ability of *Aspergillus* spp. The pigmentation can be used to identify aflatoxin-positive isolates without the use of ultra-violet light. The medium is easy to prepare and aflatoxin detection is rapid and simple with only minimal equipment.

In 1960, more than 100,000 young turkeys and thousands of ducklings in England died after consuming peanut meal contaminated with aflatoxin (AT), mycotoxins produced by certain isolates of *Aspergillus flavus* Link ex Fries and *A. parasiticus* Speare. The highly toxic and carcinogenic properties of AT and the omnipresence of the *Aspergillus* fungi in foodstuffs and animal feeds have encouraged numerous investigations in which AT-producing isolates must be identified.

Since not all isolates of *A. flavus* can produce AT (6), determination of AT-producing ability of a particular isolate is important in biochemical, genetical, toxicological, and epidemiological studies. The method commonly used to assay for AT production is to inoculate various liquid or solid substrates with the fungus and to incubate for a period of time. Aflatoxins then are extracted from the substrates with organic solvents, concentrated, and assayed by thin-layer chromatography; this is a time-consuming and laborious procedure.

Hara et al. (8) developed a method for detecting AT production in which fluorescence in agar medium under ultraviolet (UV) light facilitates the screening of isolates for AT production. In connection with a study to determine the incidence of AT-producing fungi in foodstuffs and animal feeds in Brazil, a simpler and quicker method was developed for screening large numbers of isolates for ability to produce AT. This method is based on the detection of UV-induced fluorescence, and a specific orange-yellow pigmentation on a coconut-agar medium. This paper describes the development and use of this medium.

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MATERIALS AND METHODS

Reference isolates.—Four AT-positive (*A. parasiticus* NRRL 2999, and *A. flavus* NRRL 3251, NRRL 3357, and NRRL 5520) and three AT-negative (*A. flavus* NRRL 1957, and *A. oryzae* NRRL 482, and NRRL 5593) isolates (6) obtained from the Northern Regional Research Laboratories (NRRL) of the U.S. Department of Agriculture, and *A. parasiticus* UNBF A12, a strong AT-producer from the collection of Universidade de Brasilia, Fitopatologia (UNBF) were used as reference isolates in the development of the medium. All stock cultures were maintained on Czapek solution agar.

Preparation of inoculum.—Conidia from a test culture were transferred to a plate containing Aspergillus-Differential Medium (ADM) (4) and incubated at 28 C for 2 days. Mycelial plugs (3-mm in diameter) from the ADM plate served as inocula. Fungi of *A. flavus* group do not sporulate on ADM; thus, secondary colonies do not develop from conidia disseminated during handling.

Furthermore, *A. flavus*-group fungi produce characteristic bright, yellow-orange pigment in this medium (4) which facilitates identification.

Cultivation and observation of fluorescence.—Each mycelial plug from ADM was placed on the center of a plate containing a test medium and incubated for a period of up to 1 week. Plates were examined visually for colony morphology and pigmentation and then with a UV lamp in a dark room for fluorescence. Intensity of fluorescence expressed by number of "+" signs was estimated subjectively.

Assay of aflatoxin in agar.—Ten grams of agar was triturated in 30 ml of chloroform, centrifuged, and the chloroform phase was checked for the presence of AT by thin-layer chromatography on a silica-gel G plate which was developed with a chloroform : acetone : *n*-hexane (85:15:20) solvent system. Aflatoxin standards were spotted on the same plate as references. Aflatoxin spots were identified under a UV lamp. Relative concentration of AT was estimated by comparing the intensity and size of the fluorescent AT spots.

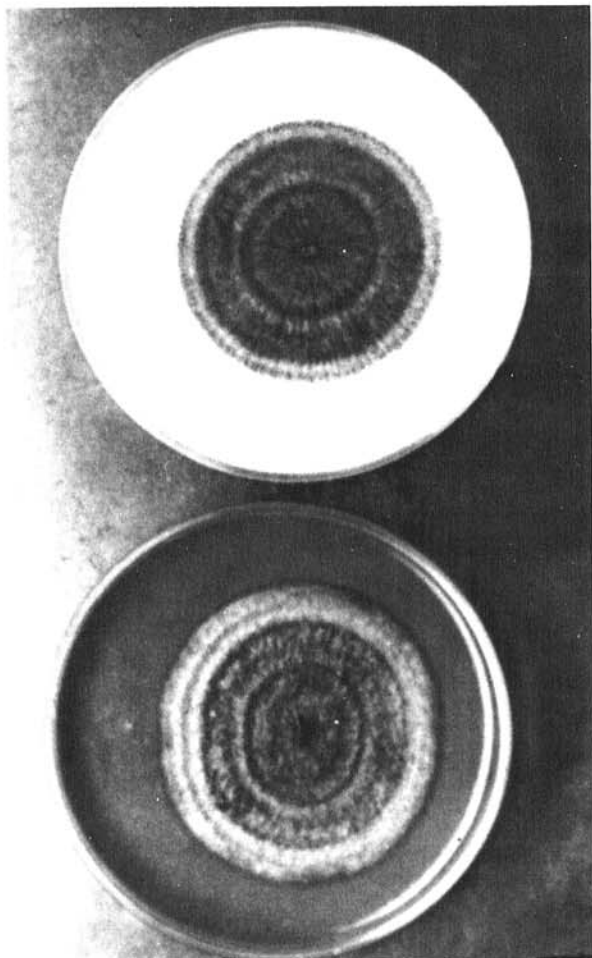

Fig. 1. Colonies of aflatoxin (AT)-positive (*Aspergillus parasiticus* UNBF A12, upper) and -negative (*A. oryzae* NRRL 482, lower) isolates on coconut-agar medium under ultraviolet light showing the presence (upper) and absence (lower) of AT-related fluorescence.

Production of aflatoxin in liquid medium.—To verify the AT-producing ability of an isolate, each of three 250-ml Erlenmeyer flasks containing 50 ml of 2% yeast extract and 20% sucrose was inoculated with a spore suspension and incubated for 1 week at room temperature (24-26 C). The culture broths were filtered through two layers of cheesecloth and extracted with two volumes of chloroform. The extracts then were checked for AT by thin-layer chromatography.

RESULTS AND DISCUSSION

Fresh coconut extract as substrate.—In the initial experiments, the APA agar medium (8) was tested. However, results were not satisfactory because of the omission of corn-steep liquor (CSL) which was not available. Coconut, reported to be an excellent substrate for AT production (2), is readily available in Brazil. Furthermore, agar plates prepared with coconut have a white background which enhances visualization of blue or blue-green fluorescence in the agar. Therefore, fresh-coconut extract (FCE), prepared by homogenizing one part of coconut meat with two parts of hot-distilled water (w/v) in a blender and filtering through two layers of cheesecloth, was tested as a possible replacement for CSL.

In the initial trial, FCE was added to APA, less CSL, to make a 10% final concentration of FCE. This medium was compared with APA medium, less CSL, or with undiluted FCE alone. After incubation for 5 days at 28 C, intense blue-green fluorescence was observed in plates containing FCE alone that were inoculated with NRRL 2999 or UNBF A12 (Fig. 1). There was less-intense fluorescence in this medium inoculated with NRRL 3357, and cultures of NRRL 482 did not show any fluorescence. No fluorescence was evident in two other media that were inoculated with any of the isolates tested. Chloroform extracts from the agar plates were assayed for AT by thin-layer chromatography. The results confirmed the previous observation of Hara et al. (8), that the fluorescence in agar is strongly indicative of AT-producing capability.

Since FCE formed a precipitate after autoclaving, and was difficult to distribute evenly in the petri plates, a test was run to determine if dilution of FCE would prevent the formation of precipitates without decreasing AT production. The results indicated that dilution of FCE to a concentration which prevented precipitation resulted in failure to detect AT production.

Commercial coconut extract as substrate.—Commercial coconut extract (CCE) trademark "Sococo" (Sococo S.A. Industrias Alimenticias, Maceio, Alagoas) is readily available in Brazil. This material, which formed much less precipitate than FCE after autoclaving, was tested for capability to support AT production. No precipitate was formed in media containing CCE at dilutions ⩾1:2 and (surprisingly) agar plates with a dilution of 1:4 supported production of more intense fluorescence by both NRRL 2999 and UNBF A12 than other dilutions. Thus CCE at dilution of 1:4 was selected as the optimal concentration for later tests. It is not known why this dilution supported the highest yield of AT, but "Sococo" contains 0.1% or less of sorbic acid added as a preservative (information from the label of

container and Bulletin No. 22 of the Brazilian Society of Food Science and Technology, 1972). Thus, we suspect that sorbic acid might have contributed to the result presented above, although there is no report on the effect of sorbic acid on AT production.

Besides "Sococo", there are three other brands of CCE in Brazil: "Tudor" (Coco Alimentar de Alagoas S.A., Pilar, Alagoas), "Serigy" (Vieira Sampaio Ind. E Comercio S.A., Aracaju, Sergipe), and "Maguary" (Industrias Alimenticias Maguary S.A., Bonito, Pernambuco). Tudor and Maguary were equally as efficent as Sococo in supporting AT production, but Serigy provided less AT production.

Effect of pH on production of fluorescence.—Medium prepared with CCE at 1:4 dilution was adjusted to pH 2.2, 3.5, 4.3, 5.3, 5.7, 6.6, 6.9, and 7.6 to test for production of fluorescence. Media at the two lowest pH values did not solidify and thus were not tested. In the 4.3 to 7.6 pH range, the growth of colonies of NRRL 2999 and UNBF A12 was proportional to the increase of pH. The production of fluorescence also increased with the increase of pH from 4.3 to 6.9 and then dropped sharply at pH 7.6. In various substrates, AT was produced at reduced pH levels (below 4.0) (6). The optimal pH for the detection of AT in APA medium was 5.5 and the intensity of fluorescence decreased markedly at pH 6.5 (8). Therefore, the optimal pH (pH 6.9) for detecting AT production in coconut-agar medium is unusually high.

Effect of aflatoxin promoters on intensity of fluorescence.—Peptone or a combination of glucose and citric acid (12), Zn^{2+} (1, 11), ethyl alcohol (3), and a combination of sucrose and yeast extract (5) have been reported to increase the production of AT. These substances were added separately to the coconut-agar medium to test for relative effect on fluorescence production by NRRL 2999 and UNBF A12. The results showed that these substances either had no effect or reduced the production of fluorescence, although most of them stimulated vegetative growth of the fungi (Table 1).

Effect of incubation temperature and time.—No significant differences in the intensity of fluorescence were observed when the inoculated coconut-agar plates were incubated at 24, 28, and 32 C for 4 days. However, there was a slight reduction in fluorescence in those incubated at 20 C.

The minimal time required to detect fluorescence in the coconut-agar medium was determined by inoculating plates with isolates known to produce different levels of AT and examining them for fluorescence after incubation for 32, 44, 53, 60, 75, 84, and 94 hours at room temperature. Fluorescence was evident within 32 hours in plates with two strong AT-producers, NRRL 2999 and UNBF A12. Two other AT-positive isolates, NRRL 3357 and 3251, produced fluorescence in 53 hours. A weak AT-producer (NRRL 5520) that required 1 week for production of detectable amounts of AT in APA medium (8), showed fluorescence beginning after 60 hours. Three AT-negative isolates, NRRL 482, NRRL 1957, and NRRL 5593, did not produce detectable fluorescence after 94 hours.

Several compounds that fluoresce blue or green, similar to AT, are reported to be produced by NRRL 482 (6) and by NRRL 5593 in APA medium (8). However, these two isolates did not produce extraneous fluorescent compounds in coconut-agar medium. It appears that coconut-agar medium specifically supported only the production of AT fluorescence and thus prevented the misinterpretation of the results.

Screening Brazilian isolates for aflatoxin production.—Twenty-seven isolates of the *A. flavus* group, including 10 from peanut, four from moldy corn, one from lima bean, and the rest obtained from various research institutes in Brazil were tested on the coconut-agar medium. These isolates also were tested for production of AT in liquid medium. The results of both tests agreed well; seven of the 27 isolates produced both fluorescence in agar and AT in liquid medium (five produced AT B_1 and B_2 and two produced AT B_1, B_2, G_1, and G_2).

Association of pigmentation with fluorescence.—During the development of the coconut-agar medium, we observed that the fluorescence-producing colonies always produced a conspicuous orange-yellow pigmentation. Twelve AT-positive and 18 AT-negative isolates were tested to determine if the pigmentation could be used for identification of AT-producing ability without the use of UV lamp for fluorescence observation. All of the AT-positive, but none of the AT-negative isolates, produced the orange-yellow pigmentation. The pigmentation was seen before the appearance of fluorescence and the degree of pigmentation seemed to be proportional to the intensity of fluorescence. Therefore, the AT-producing ability of an isolate could be estimated presumptively by the intensity of pigmentation in coconut-agar medium without the use of a UV lamp. The identity of the

TABLE 1. Effect of aflatoxin promoters on the formation of fluorescence by two strains of *Aspergillus parasiticus* on coconut-agar medium

Additive[a]	*A. parasiticus* UNBF A12[b]		*A. parasiticus* NRRL 2999[b]	
	Fluorescence[c]	Diameter[c] (mm)	Fluorescence	Diameter (mm)
None	++	30	+++	30
Peptone, 1%	−	40	−	36
Glucose, 7% and citric acid, 0.4%	−	39	−	40
Sucrose, 20% and yeast extract, 2%	−	50	++	50
Zn^{++} 0.4 mg/liter	++	31	+++	29
Ethyl alcohol, 2%	+	25	++	22

[a]Additives were added after pH of the media was adjusted to 6.9.
[b]Isolate UNBF A12 was obtained from the collection of the Universidade de Brasilia, Fitopatologia (UNBF) and NRRL 2999 was obtained from the collection of the Northern Regional Research Laboratories (NRRL) of the U.S. Department of Agriculture.
[c]Fluorescence intensities were estimated and colony diameters were measured after 3 days of incubation at 28 C.

pigment(s) is (are) not known. A mutant of *A. parasiticus,* ATCC 15517, which is impaired in AT biosynthesis, produced an orange-yellow pigment which was identified as averufin (7). Lin et al. (10) later found that the parent strain also produced a small amount of averufin and that C^{14}-labeled averufin could be converted to AT. Arseculeratne et al. (2) reported abundant production of yellow pigments and AT on freshly grated coconut inoculated with *A. flavus*. Yellow pigmentation is associated with AT production by toxic strains (13) and anthraquinone pigments are isolated from a wild, toxin-producing isolate of *A. flavus* (9).

Use and advantages of the medium.—The following procedure has been used routinely in our laboratory: one 200-ml bottle of Sococo, Tudor, or Maguary coconut extract is diluted with 600 ml of distilled water, the pH adjusted to 6.9, and 16 grams of agar is added. The medium after autoclaving, is distributed in petri dishes. A mycelial plug from a 24- to 48-hour-old colony of a test fungus grown on an ADM plate is transferred to the center of the coconut-agar plate. Three plates are inoculated with each isolate. The plates are incubated at room temperature and are examined for pigmentation and fluorescence once each day. The isolates that did not produce pigmentation and fluorescence until the third day were considered to be AT-negative.

We have used this method to determine the incidence of AT-producing fungi in agricultural products, to measure the effect of chemical substances on the production of AT, and to select for mutants of *Aspergillus* fungi impaired in AT biosynthesis.

Coconut-agar medium offers the following advantages for detecting AT production by *Aspergillus* spp.: (i) it is easy to prepare; (ii) detection of AT production by the presence of fluorescence is rapid and specific; and (ii) association of AT production with an orange-yellow pigmentation permits the quick identification of AT-positive isolates without the use of UV illumination.

LITERATURE CITED

1. ARMBRECHT, B. H., F. A. HODGES, H. R. SMITH, and A. A. NELSON. 1963. Mycotoxins. 1. Studies on aflatoxin derived from contaminated peanut meal and certain strains of Aspergillus flavus. J. Assoc. Offic. Agric. Chem. 46:805-817.
2. ARSECULERATNE, S. N., L. M. DE SILVA, S. WIJESUNDERA, and C. H. S. R. BANDUNATHA. 1969. Coconut as a medium for the experimental production of aflatoxin. Appl. Microbiol. 18:88-94.
3. BASAPPA, S. C., A. JAYARAMAN, V. SREENIVASAMURTHY, and H. A. B. PARPIA. 1967. Effect of B-group vitamins and ethyl alcohol on aflatoxin production by Aspergillus flavus. Indian J. Exp. Biol. 5:262.
4. BOTHAST, R. J., and D. I. FENNELL. 1974. A medium for rapid identification and enumeration of Aspergillus flavus and related organisms. Mycologia 66:365-369.
5. DAVIS, N. D., U. L. DIENER, and D. W. ELDRIDGE. 1966. Production of aflatoxin B_1 and G_1 by Aspergillus flavus in a semisynthetic medium. Appl. Microbiol. 14:379-380.
6. DETROY, R. W., E. B. LILLEHOJ, and A. CIEGLER. 1971. Aflatoxin and related compounds. Pages 3-178 *in* A. Ciegler, S. Kadis, and S. J. Ajl, eds., Microbial toxins, Vol. VI. Academic Press, New York, and London. 563 p.
7. DONKERSLOOT, J. A., R. I. MATELES, and S. S. YANG. 1972. Averufin production by a mutant of Aspergillus parasiticus impaired in aflatoxin biosynthesis. Biochem. Biophys. Res. Commun. 47:1051.
8. HARA, S., D. I. FENNELL, and C. W. HESSELTINE. 1974. Aflatoxin-producing strains of Aspergillus flavus detected by fluorescence of agar medium under ultraviolet light. Appl. Microbiol. 27:1118-1123.
9. HEATHCOTE, J. G., and M. F. DUTTON. 1969. New metabolite of Aspergillus flavus. Tetrahedron Lett. 25:1497-1500.
10. LIN, M. T., D. P. H. HSIEH, R. C. YAO, and J. A. DONKERSLOOT. 1973. Conversion of averufin into aflatoxin by Aspergillus parasiticus. Biochemistry 12:5167-5171.
11. MATELES, R. I., and J. C. ADYE. 1965. Production of aflatoxins in submerged culture. Appl. Microbiol. 13:208-211.
12. WILMAN, J. D., L. STOLOFF, and R. JACOBS. 1967. Aflatoxin production by a potent Aspergillus flavus Link isolate. Biotech. Bioeng. 9:429-437.
13. WISEMAN, H. G., W. C. JACOBSON, and W. C. HARMEYER. 1967. Note on removal of pigments from chloroform extracts of aflatoxin cultures with copper carbonate. J. Assoc. Offic. Anal. Chem. 50:982-983.