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Coconut as a Medium for the Experimental Production of Aflatoxin

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Fresh, grated coconut has been found to be an excellent medium for aflatoxin production by *Aspergillus flavus*. Under optimal conditions, yields of 8 mg of total aflatoxin per g of substrate were obtained. Continuous agitation of the growth medium under moist conditions at 24 C produced highest yields. Aflatoxin was assayed both biologically and chromatographically. The aflatoxin content of cultures varied biphasically with the duration of incubation. It is suggested that this pattern could result from the sequential operation of factors promoting aflatoxin formation on the one hand and a detoxifying mechanism on the other.

Large quantities of aflatoxins are needed for the extensive investigations now being carried out on these substances. Although the total synthesis of aflatoxin B_1 has been achieved (1), aflatoxins used for most experimental purposes are still obtained from laboratory cultures of selected strains of *Aspergillus flavus*. Both solid and liquid media have been used in the microbiological preparation of the aflatoxins, and the yields obtained depend to a large extent on the nature of the substrate employed. A summary of the available literature on the experimental production of aflatoxins is given in Table 1.

During the course of our work on the fungal spoilage of coconut products, we studied the growth of *A. flavus* and the production of aflatoxins by this organism, using various preparations of coconut. Preliminary experiments indicated that appreciably higher yields of aflatoxin were obtained from freshly grated coconut than from peanut, a substrate which has been used as medium by many workers in the preparation of aflatoxins.

This paper gives a detailed account of aflatoxin production with coconut as medium. Under optimal conditions, yields of about 8 mg of substrate per g were readily obtained. Variations in the aflatoxin content with age of the culture were consistently observed. These variations are discussed in relation to possible detoxification mechanisms.

MATERIALS AND METHODS

Aflatoxin standards. Quantitative standards of aflatoxins B_1 and G_1 in chloroform were kindly supplied by Leo A. Goldblatt of the U. S. Department of Agriculture.

Media. Fresh, ungerminated coconuts were grated soon after splitting; they were autoclaved and used immediately thereafter. Replicate samples of coconut were taken from the same nut to ensure uniformity in composition of the substrate. Commercial, desiccated coconut was rehydrated with distilled water to the original water content (approximately 44%) of fresh kernel, kept in contact with the water for 1 hr at room temperature, and then autoclaved. A short-grained local variety of rice was soaked in tap water for 2 hr at room temperature and then autoclaved after removal of excess water. All substrates were autoclaved at 121 C for 10 min.

Organisms. *A. flavus* strains ATCC 15546 and NRRL 2999 used in this work were maintained on Difco Potato Dextrose Agar. Strain NRRL 2999 was kindly supplied by C. W. Hesseltine of the U. S. Department of Agriculture. Strain ATCC 15546, which originally produced both aflatoxins B_1 and G_1 in potato-dextrose broth, produced only small amounts of aflatoxin G_1 on coconut; after repeated subculture, no aflatoxin G_1 was detectable in coconut cultures. This strain was therefore used by us to produce pure aflatoxin B_1. Strain NRRL 2999, however, produced relatively greater amounts of aflatoxin G_1 than B_1 on coconut.

Inocula. Inocula were prepared by suspending 10- to 14-day-old cultures on Potato Dextrose Agar sloped in "bijou" bottles. The culture on each slope was suspended in 2.5 ml of 0.1% Tween 80 in distilled water. The inoculum used, per 20 g of substrate, was 0.2 ml of this suspension.

Incubation of substrates. Sterilized substrates (20 g in each case) in 500-ml Erlenmeyer flasks were inoculated and thoroughly mixed by manual agitation. Flasks were either kept static or shaken once daily by hand to disperse the medium, or they were agitated continuously on a rotary shaking machine (Fig. 1) at about 50 rev/min during the incubation period. The contents of the flasks were kept constantly agitated at this speed, whereas at faster speeds the medium tended

TABLE 1. *Review of literature on the experimental production of aflatoxins*

Substrate	Conditions	Yield of total aflatoxin	Reference
Crushed wheat	30 C, 7 days	750 μg/g	Chang et al. (2)
Peanut	30 C, 10–13 days, shaken daily	265 μg/g	Codner et al. (5)
Rice[a]	28 C, 5 days, continuous shaking	1,510 μg/g	Shotwell et al. (25)
Wheat + methionine[a]	28 C, 6 days, shaken	1,690 μg/g	Hesseltine et al. (10)
Peanut	30 C, 8 days	650 μg/g	Schroeder (23)
Cottonseed	25 C, 8–10 days	1,100 μg/g	Schroeder and Hein (24)
Wheat	28 C, 4–5 days	1,950 μg/g	Stubblefield et al. (26)
Sucrose + salts	25 C, 3 days, aerated	200–300 mg/liter	Ciegler et al. (4)
Sucrose + yeast extract	25 C, 6 days	630 mg/liter	Davis et al. (7)
Czapek-Dox broth + cornsteep liquor	30 C, 4 days	190 mg/liter	Schroeder (23)

[a] *Aspergillus flavus* NRRL 2999 was used.

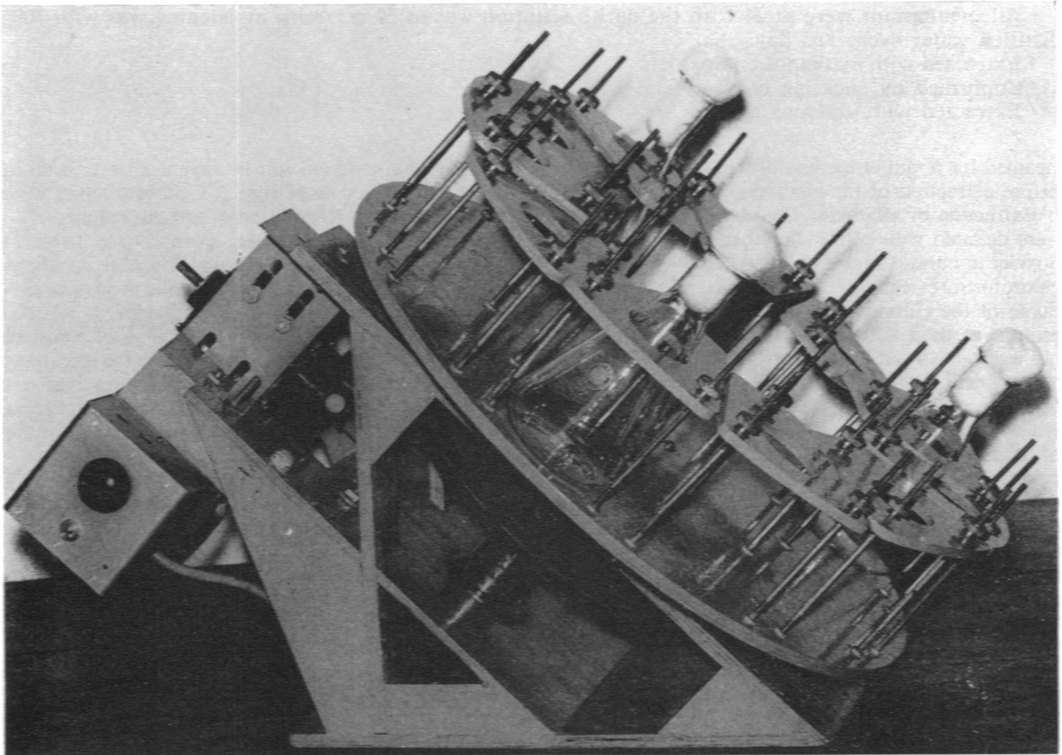

FIG. 1. *Machine for the rotary agitation of cultures in 500-ml Erlenmeyer flasks. Optimum speed 50 rev/min.*

to merely deposit on the walls of the flasks without adequate mixing. Clumps adhering to the walls were dispersed by means of a sterile glass rod. In flasks which were kept static or shaken once daily, the medium was maintained at the bottom of the flask as an even layer throughout the incubation. In some experiments, the substrates which were kept agitated continuously were moistened with 2 ml of sterile distilled water every 3rd day. Incubation was carried out in the dark at 20, 24, or 30 C. The flasks were

TABLE 2. *Aflatoxin production on coconut and rice*

Expt	Medium and strain[a]	Maximal yield of total aflatoxin ($B_1 + G_1$)[b]	Conditions[c]
4	Fresh coconut, 1	1,960[d]	Static, 12 days
5	Fresh coconut, 1	8,368	Agitated, 13 days
6	Fresh coconut, 1	6,434	Agitated, 7 days
7	Fresh coconut, 1	510[e]	Shaken once daily, 5 days
	Rice, 1	24	Shaken once daily, 5 days
8	Fresh coconut, 2	2,850	Static, 4 days
	Rice, 2	825	Static, 4 days
10	Fresh coconut, 1	225	Static, 5 days
	Rice, 1	17	Static, 5 days
11	Fresh coconut, 2	8,788[e]	Agitated, moistened, 9 days
	Rice, 2	1,563	Agitated, moistened, 9 days
12	Fresh coconut, 1	2,813	Shaken, once daily, 12 days
13	Fresh coconut, 1	7,500[e]	Agitated, moistened, 12 days
	Fresh coconut, 2	7,500	Agitated, moistened, 12 days
14	Fresh coconut, 1	2,813	Shaken once daily, 5 days
	Fresh coconut, 1	2,110[e,f]	Shaken once daily, 8 days

[a] Strain 1, ATCC 15546; strain 2, NRRL 2999.
[b] Expressed as micrograms per gram.
[c] All incubations were at 24 C in the dark; agitation was at 50 rev/min; moistening was with 10% distilled water every 3rd day.
[d] Extracted with methanol on Soxhlet.
[e] Confirmed by duckling bioassay.
[f] Extracted with aqueous acetone.

steamed for 5 min at the end of the incubation period before extraction of the aflatoxin.

Extraction of aflatoxin. The contents of the flasks were defatted with petroleum ether (40 to 60 C) in a Soxhlet apparatus and then extracted with water and chloroform essentially as described by Lee (14). Some of the cultures were extracted by the aqueous acetone method (18); Soxhlet extraction with methanol was carried out with others (27).

Purification of aflatoxin. Aflatoxins in crude extracts were purified by repeated precipitation with either 20 volumes of petroleum ether or 10 volumes of hexane followed by chromatography on silicic acid columns (25). Yellow pigments which were abundantly produced on coconut were initially removed from the column by washing with diethyl ether (18), and the aflatoxin was eluted with chloroform containing 2% ethyl alcohol.

Assay of aflatoxins. Aflatoxins in extracts were assayed both chromatographically and biologically by the biliary hyperplasia test on ducklings.

For chromatographic methods, samples of the chloroform extracts, diluted appropriately, were run alongside aflatoxin standards on 250-μm thin-layer silicic acid plates, and the intensity of fluorescence of the spots was compared under a Hanovia ultraviolet lamp with principal emission at 366 nm. Chromatography was carried out in the following solvent systems (3, 9) to eliminate possible interference by fluorescent spots due to nonaflatoxin substances which might have similar R_F values in one or more of these solvents: solvent 1, methanol-chloroform (3:97, v/v); solvent 2, methanol-chloroform-acetic acid (5:94.5:0.5, v/v); solvent 3, acetone-chloroform (1:9 v/v).

Plates with a 15-cm run in solvent 1 were rerun in the same direction in solvent 2. Certain samples were chromatographed in addition in solvent 3 alone.

For biological assay, some extracts (*see* Table 2) were tested on 1-day-old Khaki-Campbell ducklings fed with either 0.1 ml of 10% aqueous propylene glycol (controls) or 0.1 ml of 10% propylene glycol containing a calculated dose of 0.5 to 1.0 μg of aflatoxin B_1 per day for 5 days. On the 8th day, the ducklings were killed and their livers were fixed in buffered Formol-saline for sectioning and staining with hematoxylin and eosin. Aflatoxicity was confirmed by the presence of biliary hyperplasia.

Plate screening test for aflatoxin production by *Aspergillus flavus*. The hyflo-supercel plate test (8) was modified by replacing the peanut homogenate with an equal amount of coconut homogenate prepared by homogenizing freshly grated coconut with distilled water (2:1, w/w) in a Waring Blendor. Sodium nitrate in the Czapek-Dox formula was replaced by an equivalent amount of ammonium chloride. Some plates were also prepared with 50% coconut homogenate in distilled water and solidified by the addition of 2% agar. These media were autoclaved at 121 C for 10 min before layering on hyflo-supercel-coated petri dishes. Plates were incubated at 24 and 30 C and were examined daily for fluorescence under an ultraviolet lamp.

RESULTS

General observations. Rice, or fresh or desiccated coconut, when inoculated with either strain showed no visible change during the 1st day of

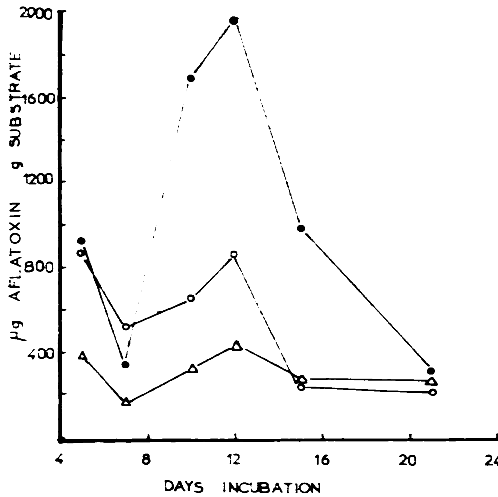

FIG. 2. *Effect of temperature of incubation on aflatoxin yield. Aspergillus flavus ATCC 15546 cultured on fresh coconut; static, unmoistened. Symbols:* O, *20 C;* ●, *24 C;* △, *30 C.*

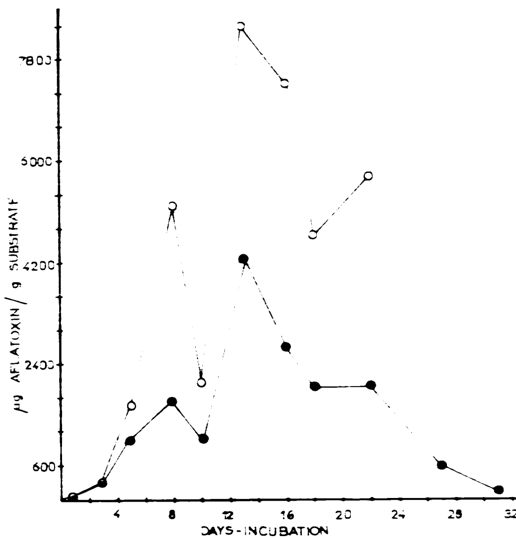

FIG. 3. *Effect of agitation on aflatoxin yield. Aspergillus flavus ATCC 15546 cultured on fresh coconut at 24 C; cultures moistened every 3rd day. Symbols:* ●, *static;* O, *agitated.*

incubation. Yellow pigmentation was noticeable on the 2nd day, deepening to a light brown color from about the 3rd day; this was especially seen on coconut subjected to rotary agitation. Sporulation was more marked on rice than on coconut. Considerable water was lost from cultures that were agitated; these cultures were drier as compared with static cultures in which sufficient moisture was retained throughout incubation as evidenced by the presence of water condensed on the walls of the flasks.

Effect of temperature. Higher yields of aflatoxin from cultures incubated in flasks were obtained at 24 C than at 20 or 30 C (Fig. 2). The yields at 30 C were appreciably lower than at 20 C. Sporulation was most marked at 30 C.

Effect of agitation. The yield of aflatoxin was markedly increased by rotary agitation of cultures under moist conditions (Fig. 3 and Table 2). High yields of aflatoxin were associated with the absence of sporulation; with strain ATCC 15546 under such conditions, no sporulation was produced even on the 12th day. Cultures in flasks which were subjected to rotary agitation were moistened with 2 ml of sterile distilled water every 3rd day to replace water lost by evaporation. Such moistened cultures gave approximately 25% higher yields than unmoistened cultures. In general, incubation at 24 C with agitation under moist conditions was best suited for aflatoxin accumulation.

Variation of aflatoxin content with time. A regular pattern of variation in the yield of aflatoxin with the age of the culture was observed (Fig. 2-4); similar variations in aflatoxin yield were observed at the three temperatures used in this study (Fig. 2). Biphasic curves were obtained with both strains in static as well as in agitated

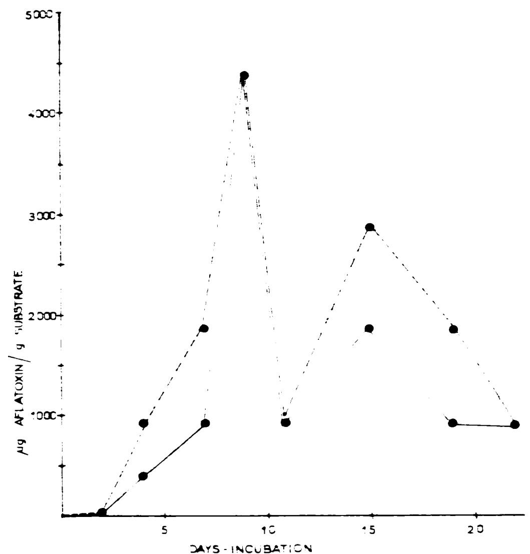

FIG. 4. *Variation of yield of aflatoxin B_1 and G_1 with age of culture. Aspergillus flavus NRRL 2999 at 24 C; continuous agitation and moistening every 3rd day. Symbols:* ●——●, *aflatoxin B_1;* ●- - -●, *aflatoxin G_1.*

cultures. Similar patterns have been observed by us on coconut medium with other strains of *A. flavus* isolated from fungus-contaminated copra. The variations in aflatoxin content are larger than can be accounted for by any errors arising in the assay procedure and probably reflect actual differences in the net content of aflatoxin arising from alterations in the relative rates of synthesis and degradation of these compounds within the culture.

Yields. The yields of aflatoxin obtained from coconut medium in a series of experiments (Table 2) were approximately fivefold greater than those reported by previous workers who used substrates other than coconut. The yields in our experiments with strain NRRL 2999 on rice as substrate were, however, comparable with those obtained by Shotwell et al. (25) who used the same strain but on a different variety of rice.

Purity of extracts. Although the Lee procedure was used in most extractions of aflatoxin in this series of experiments, it was found subsequently that aqueous acetone gave much purer extracts. Extracts of cultures on coconut obtained by the Lee procedure were brown and oily, with an aflatoxin content varying between 1 and 4%. Separation of the yellow-brown pigments, which were abundant in these extracts, was incomplete even after repeated precipitation with petroleum ether or hexane. The dry weights of the extracts from 20-g samples of coconut ranged from approximately 1 to 4 g. Aqueous acetone extracts, however, on evaporation of the solvent gave light-yellow dry solids which had an aflatoxin content of 10 to 25%. The weights of acetone extracts from 20-g samples ranged from 0.3 to 0.4 g. Replicate samples from bulk cultures in 1-liter flasks extracted in parallel by the Lee procedure and by the acetone method showed that the amount of aflatoxin extracted by the latter method was 80% of that extracted by the Lee procedure. In spite of the greater recovery of aflatoxin in Lee extracts, the acetone procedure was used in the preparation of pure aflatoxins from coconut cultures because of the purer product obtained. Extracts of cultures of strain NRRL 2999 on coconut contained aflatoxin $B_1 : G_1$ in ratios of about 1:1.7, whereas Shotwell et al. (25) obtained relatively greater amounts of aflatoxin B_1 from rice inoculated with this strain.

Plate toxigenicity test. Replacement of the peanut homogenate by coconut homogenate in the plate test produced a significantly greater intensity of blue or blue-green fluorescence in the hyflo-layer under the cultures of both strains. This indicates a greater production of aflatoxin on the coconut medium as compared with the peanut medium, assuming that the intensity of fluorescence is an index of aflatoxin content. This effect was clearly seen even on the 2nd day of incubation. This observation was in agreement with our findings of higher aflatoxin yields obtained from flask cultures on coconut, as compared with the results obtained by other workers using peanut. The intensity of fluorescence on plates incubated at 30 C was, however, more marked than in those incubated at 24 C, a result which is the reverse of that observed with respect to aflatoxin yields obtained from flask cultures grown at these two temperatures.

DISCUSSION

A factor which is of importance in aflatoxin production is the temperature of incubation. The optimum temperature for production of aflatoxin B_1 was found by us to be 24 C, as has also been shown by other workers (20, 23, 24). Many previous workers, however, have grown the fungus at temperatures of 28 to 30 C in preparing aflatoxin.

Coconut is superior to peanut and other media used so far in the preparation of aflatoxins. Some of the factors that might account for the high yields obtained on coconut may be the nature and content of neutral fat in the mature coconut kernel. The oil contents of fresh coconut and peanut kernel are 30 to 40% and 47 to 50%, respectively (12). However, the fatty acid constituents of coconut are predominantly the C_{12}-C_{14} acids, lauric and myristic acids, whereas, in peanut, the principal fatty acids are the C_{18} acids, oleic and linoleic acids (11). This results in a proportionately greater yield of glycerol from hydrolysis of the neutral fat from coconut oil than from peanut oil. Menon and Pandalai (17) noted that coconut oil gives about 40% more glycerol than most other oils. Glycerol was found by Davis and Diener (6) to be a good carbon source for growth and aflatoxin production by *A. parasiticus*. *A. flavus* is known to have a high content of lipolytic enzymes. Both strains of *A. flavus* used in these experiments have been observed by us to be lipolytic, and it is possible that the glycerol formed by the hydrolysis of fat in coconut would enhance growth and aflatoxin formation.

Glucose, sucrose, and fructose were found by Mateles and Adye (16) to be the preferred carbon sources in a medium containing ammonium salts. Davis, Diener, and Eldridge (7) found that 20% sucrose promoted optimal aflatoxin formation in a yeast extract medium; glucose and fructose were also found to be suitable though to a lesser degree. The differences between coconut and peanut in respect to their sucrose, glucose, and fructose contents are, however, too small to account for the differences in aflatoxin-forming capacity of these two substrates. Copra, which has a water

content of below 6% (fresh coconut has approximately 44%), has a carbohydrate content of about 20%, of which the sucrose, glucose, and fructose contents are 32.82, 2.72, and 2.75%, respectively (13). Peanut kernel has a sucrose content of 5.8%, with no reducing sugars (19).

A further factor which might account for the higher yields of aflatoxin obtained from coconut could be a lower degree of aflatoxin degradation. Ciegler et al. (4) demonstrated that peroxidized methyl esters of soybean oil could degrade aflatoxin in vitro. It is possible that compounds of this type might arise in medium containing unsaturated fatty acids and that degradation of aflatoxin might be more extensive in medium richer in unsaturated fatty acids. Coconut has a lower content of unsaturated fatty acids than has peanut; the oleic acid contents of the seed fat of coconut and of peanut are 5.0 to 8.2% and 40 to 64% by weight, respectively; the linoleic acid contents of coconut and of peanut are 1.0 to 2.6% and 18 to 38% by weight, respectively (11). In the case of coconut with its lower content of unsaturated fatty acids, one could expect degradation of aflatoxins by peroxidized esters to be lower than in peanut. The initial drop in aflatoxin content observed on the 6th to 8th day might conceivably be due to such a degrading mechanism or possibly also to breakdown of aflatoxin by an enzyme appearing during this phase of growth of the organism. The appearance of the second peak of aflatoxin content might be due to an increased production arising as a result of glycerol now being liberated by lipolysis, in addition to a possible exhaustion of the degrading reactions. The exact mechanism that operates to produce the biphasic curve is not yet known. Experiments both with suspensions of *A. flavus* and enzymes derived at different phases of the growth of this organism are being carried out to elucidate this mechanism.

Whatever the mechanism of degradation of aflatoxins, whether it be enzymatic or chemical breakdown, one might expect degradation to be more effective at the higher temperature of 30 C than at 24 C. One would thus expect a lower yield of aflatoxin at 30 C, as was observed experimentally in solid cultures in flasks. The observation of higher yields of aflatoxin at 30 C in the plate test may be explained as being due to the protection afforded to the aflatoxin by its absorption into the hyflo-layer and its separation from the factors promoting degradation present in the culture. Exclusion of material within the hyflo-layer from atmospheric oxygen might further help to stabilize the aflatoxin. It thus seems that the lower aflatoxin content in flask cultures incubated at 30 C than at 24 C might be due to increased breakdown rather than to a lower degree of production by the organism.

Shotwell et al. (25), using the same strain of *A. flavus* (NRRL 2999) cultured on rice, observed variations of aflatoxin content in samples incubated for periods varying from 2 to 12 days. They attributed the differences in yield to possible errors in their assay rather than to any real variations in aflatoxin content with time; their results, however, did not show a definite pattern as observed by us on coconut. The phasic nature of the variations of aflatoxin content which we have observed on coconut may be due to the sequential operation of opposing factors present in this substrate, such as the greater capacity to promote aflatoxin formation on one hand, with the development of degrading mechanisms on the other.

Shotwell et al. (25), who obtained high yields of aflatoxin on rice, suggested the use of cultures on feedstuffs for direct feeding to animals in the study of the biological effects of aflatoxins. A disadvantage of the use of such feedstuffs is the reduction of nutritive value of the foods resulting from fungus growth. For example, a reduction in the lysine content of soybean meal artificially contaminated with *A. glaucus* was found to be the cause of growth retardation in chicks and poults fed on the contaminated meal (21). Such secondary factors will need evaluation in studies on mycotoxicoses where moldy meal has been used instead of purified fungal metabolites. Nutritional deficiencies, apart from producing biological effects per se, might even enhance the effects of the mycotoxin under study (15). It might be preferable, therefore, to use purified aflatoxin added to regular diets rather than foodstuffs which have been used as a culture medium for toxigenic strains.

As for the plate test, we have found that 10% coconut homogenate with the modified Czapek-Dox medium produced more intense fluorescence than the peanut medium as early as even the 2nd day at 30 C. The coconut medium was therefore used routinely in the plate test for screening of aflatoxigenic fungi.

From the results obtained in this study, coconut is recommended as a useful substrate in the preparation of aflatoxins on a large scale.

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