Aflatoxigenic Isolates of *Aspergillus flavus* from Pecans

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Of 120 isolates of the *Aspergillus flavus* group from pecans used in bakery products, 85 were shown to produce aflatoxin on yeast extract sucrose medium. Extracts from moldy sections of raw pecans obtained commercially at the retail level showed aflatoxin-like spots on thin-layer chromatography. Cooked (autoclaved) pecans inoculated with toxigenic isolates of *A. flavus* were also good substrates for aflatoxin production.

While investigating a bakery mold problem involving pecans, it was noted that a number of pecan halves (meats) being cultured for internal fungi yielded colonies of *Aspergillus flavus* Link ex Fries. Inasmuch as the presence of aflatoxin in foods may constitute a health hazard, a study was made to determine the frequency and toxigenic potential of these strains as well as the suitability of pecans as a substrate for aflatoxin production.

**MATERIALS AND METHODS**

The presence of *A. flavus* in pecans was determined by plating the seed on either 6% malt-salt agar (1) or rose bengal-streptomycin agar (RBM-2) after surface sterilization by immersion for 2 min in a solution of sodium hypochlorite (10 ml of bleach, 10 ml of 95% ethyl alcohol, and 80 ml of water). The RBM-2 medium was prepared as described by Tsao (9), except that the streptomycin sulfate was added at the rate of 0.06 g/liter. The plates were incubated at room temperature and examined every other day for 3 weeks. Colonies of *A. flavus* were isolated onto malt extract agar slants (7) for identification and toxin analysis.

A total of 2,061 pecan pieces were plated out during this study; the source and nature of each sample are shown in Table 1. Samples 1–10 were taken from pecan lots used in the production of commercial bakery products; samples 11–16 were packaged pecans purchased in local supermarkets.

**Screening for aflatoxin.** For screening of mold isolates, 50 ml of 2% yeast extract plus 20% sucrose [YES medium (3)] in a 250-ml Erlenmeyer flask was inoculated with 10⁶ spores, incubated at room temperature for 7 days, and extracted with two 100-ml portions of chloroform (CHCl₃) on a gyratory shaker.

The pooled extracts were filtered and evaporated to dryness on a flash evaporator; the residue was cooled and resuspended in 5 ml of CHCl₃. Visual estimates of aflatoxin content were made by comparing thin-layer chromatograms of appropriate dilutions of the extracts with aflatoxin standard obtained from the Southern Utilization Research and Development Laboratory, USDA, New Orleans, La. Thin-layer chromatograms (20 by 20 cm, 0.25 mm thickness of silica gel G-HR) were developed in acetone-chloroform (1:9, v/v) in an unequilibrated tank. Chloroform extracts were purified by column chromatography and precipitation with hexane in the first part of this study. However, thin-layer chromatography (TLC) results indicated that visual estimates could be made easily and accurately without further purification. Consequently, column chromatography and hexane precipitation were discontinued in the latter part of this study.

For screening batches of pecans, pecans were extracted directly by the method of Pons et al. (6) and extracts were examined by TLC to determine the aflatoxin content.

For screening individual nuts, moldy pecans were extracted by the method of Cucullu et al. (2) for determining aflatoxins in individual peanuts and peanut sections.

**Presumptive TLC results** were confirmed by spectrophotometric analysis and chick embryo bioassay. Spectrophotometric determinations of aflatoxin were made by the method of Nabney and Nesbitt (5) on a Shimadzu model MPS-50L recording spectrophotometer. The method of Verrett et al. (10) was used for chick embryo bioassays. Further confirmation of aflatoxins was obtained by administering extracts per os to 1-day-old Peking ducklings and examining for bile duct cell proliferation (8). Extracts for duckling bioassays and spectrophotometric analyses were purified further by preparative TLC.

**RESULTS AND DISCUSSION**

The level of *A. flavus* in the bakery pecans (lots 1–10) ranged from 2 to 21% (average 9%)
whereas in market pecans the level ranged from 0 to 85% (Table 1).

Of the 120 colonies isolated and identified, 105 (87.5%) were A. flavus and 15 (12.5%) were A. parasiticus Speare. Table 2 shows the results of screening mold isolates for aflatoxin production in YES medium. Presumptive TLC results showed that 29.1% of the isolates were negative; 13.4% produced aflatoxins B1, B2, G1, and G2, and 57.5% produced only aflatoxins B1 and B2.

Diener and Davis (4) reported that 90% of their toxigenic isolates of A. flavus from agricultural commodities produced primarily aflatoxin B. In this study, 80% of the toxigenic isolates produced only aflatoxins B1 and B2. Almost all the negative isolates and those producing only the B toxins were A. flavus; most of the isolates producing all four aflatoxins were A. parasiticus. Isolates were considered to be negative when aflatoxin was not detected in 3 μl of undiluted chloroform extract.

In all but one instance, when four toxins were produced in YES medium there was more aflatoxin G produced than B. The one exception was A. parasiticus ( # PC101) which produced more aflatoxin B than G.

Table 3 gives the number of isolates producing aflatoxin within several arbitrarily chosen ranges. All isolates of A. parasiticus produced aflatoxin in amounts of 10 μg/ml or more, whereas the majority of A. flavus isolates produced aflatoxin in lower ranges (less than 10 μg/ml). Though A. parasiticus isolates seem to produce more total aflatoxin (B plus G) than do A. flavus isolates, this does not necessarily indicate a greater potential hazard. As has already been pointed out in Table 2, most of the A. parasiticus isolates produce more aflatoxin G than B and, to date, aflatoxin B1 is regarded as the most carcinogenic of the aflatoxins.

Spectrophotometric determinations on chloroform extracts of 20 randomly selected samples confirmed preliminary positive TLC results. Maximum absorption typical of aflatoxins was obtained at 363 nm from samples that were positive on TLC but not from those that were negative.

Toxicity of randomly selected strains of A. flavus and A. parasiticus was further confirmed by bioassay. Culture extracts from eight strains of A. flavus that were negative by TLC were also negative by the chick embryo bioassay. Two of these cultures were shown to be negative by duckling bioassay.

Extracts of six cultures that were suspected to produce four aflatoxins by TLC screening were shown to be toxic to chick embryos. Toxicity of three of these cultures was further confirmed by duckling bioassay in which administration per os resulted in bile duct cell proliferation typical of aflatoxin.

Extracts of 18 randomly selected cultures that produced only aflatoxins B1 and B2 were also toxic to chick embryos. Confirmation of the toxigenicity of 5 of these 18 isolates was obtained by duckling bioassay.

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**Table 1. Source and characteristics of pecan samples utilized during study**

| Sample no. | No. of pecan pieces per sample | Product type | % moisture | Medium | % with A. flavus |
|------------|-------------------------------|--------------|------------|--------|-----------------|
| 1          | 100                           | Chopped      | 2.8        | RBM-2a | 5.0             |
| 2          | 100                           | Chopped      | 2.8        | RBM-2  | 10.0            |
| 3          | 100                           | Chopped      | 2.4        | RBM-2  | 12.0            |
| 4          | 100                           | Chopped      | 2.8        | RBM-2  | 5.0             |
| 5          | 100                           | Chopped      | 2.4        | RBM-2  | 4.0             |
| 6          | 50                            | Chopped      | 3.0        | MSA-6b | 2.0             |
| 7          | 25                            | Chopped      | 3.2        | MSA-6  | 8.0             |
| 8          | 360                           | Whole        | 3.0        | RBM-2  | 11.9            |
| 9          | 190                           | Whole        | 3.2        | MSA-6  | 21.0            |
| 10         | 560                           | Chopped      | 3.2        | RBM-2  | 10.7            |
| 11         | 112                           | Pieces       | 3.2        | RBM-2  | 21.4            |
| 12         | 16                            | Whole        | 3.2        | RBM-2  | 0               |
| 13         | 114                           | In shell     | 3.6        | RBM-2  | 5.2             |
| 14         | 19                            | Whole        | 3.2        | RBM-2  | 5.2             |
| 15         | 60                            | Pieces       | 3.2        | RBM-2  | 85.0            |
| 16         | 55                            | Pieces       | 3.2        | RBM-2  | 67.2            |

* Rose-bengal streptomycin agar.
  
* Six per cent malt-salt agar.

**Table 2. Toxin-producing characteristics of 120 A. flavus and A. parasiticus isolates from pecans**

| Aflatoxin production | No. of A. flavus isolates | No. of A. parasiticus isolates |
|----------------------|---------------------------|-------------------------------|
| Negative             | 34                        | 1                             |
| B1 and B2           | 68                        | 1                             |
| B1, B2, G1, G2      | 3a                        | 13b                           |

* All isolates produced more aflatoxin G than B.
  
* Twelve isolates produced more aflatoxin G than B.
Eighty-five isolates were found to be aflatoxicogenic when grown on YES medium. Since many of these isolates were obtained from pecans used commercially in bakery products, the ability of randomly selected isolates to produce aflatoxin on cooked pecans was also determined. Nine isolates of *A. flavus* and four of *A. parasiticus* were grown on 25 g of crushed, autoclaved pecans. The five isolates that were negative in YES medium were also negative on autoclaved pecans. The eight isolates showing positive TLC results after growth on YES medium also gave positive TLC results after growth on autoclaved pecans.

*A. flavus* and *A. parasiticus* were isolated from moldy, raw pecans obtained at retail commercial outlets. Results of direct extraction for aflatoxin of 22 of these pecan samples (50 to 100 g each) were inconclusive. Strong presumptive evidence of aflatoxin in commercial pecans was obtained by extracting individual moldy raw pecans by the method of Cucullur et al. (2). Fluorescent compounds with \( R_f \) values identical to standard aflatoxin were extracted from several individual sections of moldy pecans. However, due to the small amount extracted, confirmation by bioassay or ultraviolet spectroscopy was not possible.

In view of the known carcinogenic properties of aflatoxin, the detection of these compounds in market pecans poses a potential health hazard to the consumer. Consideration should be given to the conditions under which pecans are stored and processed. Additional research is needed to determine the conditions which would minimize mold growth and the threat of aflatoxin contamination of pecans.

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