Aflatoxin-Producing Strains of *Aspergillus flavus* Detected by Fluorescence of Agar Medium Under Ultraviolet Light

S. HARA, D. I. FENNELL, AND C. W. HESSELTINE

Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

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The fluorescence method of detecting aflatoxin-producing strains of *Aspergillus flavus* and related species utilizes the ultraviolet-induced fluorescence of aflatoxin produced in a modified Czapek's solution agar containing corn steep liquor, HgCl₂, and (NH₄)₂HPO₄ instead of NaNO₃. The presence of aflatoxin is confirmed by thin-layer chromatography of CHCl₃ extracts of the fluorescent agar.

Since the first report (8) that "turkey X" disease was caused by a toxic metabolite of *Aspergillus flavus*, hundreds of strains belonging to that group have been isolated from food and feed materials and surveyed for their aflatoxin-producing abilities. Both liquid and solid (rice, wheat, corn, and peanuts) substrates have been used (4). Since testing large numbers of isolates on a variety of substrates requires much time and effort, a simplified method is needed. This paper reports a series of experiments that led to the development of a method based on ultraviolet (UV) detection of aflatoxin diffused into an agar medium.

**MATERIALS AND METHODS**

**Strains used.** The representatives of *A. flavus* and *A. parasiticus* listed in Table 1, known from mycotoxin investigations at the Northern Regional Research Laboratory (NRRL) to be either aflatoxin-positive or aflatoxin-negative (on agricultural commodities; analyzed by official AOAC methods [Sect. 26]; sensitivity limit approximately 20 ppb), were selected for use in preliminary experiments.

Later, the reproducibility of the method was tested with additional strains of these and other species in the *A. flavus* group. All strains had been tested previously for aflatoxin production at either NRRL or the Research Institute of Brewing. All test organisms are now maintained in the ARS Culture Collection at NRRL.

**Cultivation.** Strains were inoculated at the center of solidified agar medium in glass petri dishes (fluorescence cannot be detected in plastic dishes) and incubated at 28°C in the dark. The effect of various N sources and their concentration, enzyme inhibitors and enhancers, and pH were investigated in a basal medium composed of K₂HPO₄, 1 g; MgSO₄.7H₂O, 0.5 g; KCl, 0.5 g; FeSO₄.7H₂O, 0.01 g; sucrose, 30 g; agar, 20 g per liter of distilled water (i.e., Czapek's solution agar without N source). The pH was adjusted with 1 N NaOH as required.

**Observation of fluorescence.** Plates were examined under UV (366 nm) illumination from the seventh through the 10th day of incubation for the presence or absence of blue fluorescence in the agar surrounding the colonies (Fig. 1). Intensity of the fluorescence was determined subjectively.

**Extraction of aflatoxins from fluorescent medium.** A 30-g amount of agar medium showing blue fluorescence was mixed for 5 min with 75 ml of water in a Waring blender. The aqueous slurry was extracted by blending for 5 min with 25 ml of chloroform; the mixture was centrifuged, and the CHCl₃ layer was decanted and retained. Chloroform extraction of the aqueous layer was repeated. The two chloroform fractions were combined, filtered, and concentrated to dryness under nitrogen. Residues were taken up in 10 ml of chloroform for thin-layer chromatography (TLC).

**Determination and confirmation of aflatoxins.** Aflatoxin B₁, B₂, G₁, and G₂ were determined quantitatively by TLC with a recording densitometer. The developing solvent was acetone-chloroform-water (12:88:1.5, vol/vol/vol). Confirmative tests for aflatoxin B₁ were performed by preparing B₁ derivatives according to AOAC's official method of analysis (2).

**RESULTS AND DISCUSSION**

Generally, strains of *A. flavus* produce variously colored fluorescent substances during their growth on standard Czapek's solution agar adjusted to pH 5.0 with 1 N NaOH. Since these would interfere with the observation of aflatoxin fluorescence, preliminary experiments were directed toward their elimination and enhancement of the blue fluorescence. NRRL 1957, an aflatoxin-negative strain of *A. flavus*, and

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1. Present address: Research Institute of Brewing, Tokyo, Japan.
NRRL 3145 an aflatoxin-positive strain, were selected as test organisms to determine the effect of substituting N sources (0.3%) other than NaNO₃ in Czapek's agar. Both produced other diffusible fluorescent substances when the organic compounds shown in Table 2, or NaNO₃, were the N source. When nitrogen was supplied in the form of the inorganic ammonium salts, NRRL 1957 produced no fluorescent substance, whereas NRRL 3145 yielded only the blue fluorescence characteristic of aflatoxin. Maximum intensity was observed on the medium containing (NH₄)H₂PO₄.

Zn²⁺ (1, 6), methionine (4), and corn steep liquor (3, 9) have been reported to promote the production of aflatoxin. These and other compounds which might enhance the accumulation of aflatoxin precursors by blocking various enzyme systems were tested (Table 3) for their effect on the intensity of the blue fluorescence in the basal medium with (NH₄)H₂PO₄ (0.3%). Fluorescence produced by NRRL 465, an aflatoxin-producing strain of *A. parasiticus*, was increased by the addition of either HgCl₂ (5 × 10⁻⁴ M) or corn steep liquor (0.02%).

Since the observed influence of corn steep liquor might be attributable to the additional N it supplies, various concentrations of (NH₄)H₂PO₄ in the basal medium plus HgCl₂ (5 × 10⁻⁴ M) but without corn steep liquor were examined. Three known aflatoxin-producing strains (NRRL 2999 *A. parasiticus*, NRRL 3251, and 5520 *A. flavus*) were used in this test (Table 4). Results with the one strain of *A. parasiticus* were similar to those obtained with the representative of this species tested on corn steep liquor in the absence of HgCl₂ (Table 3). Although one of the two strains of *A. flavus* (NRRL 3251) showed fairly intense fluorescence, the second (NRRL 5520) failed to respond on this medium.

The same three organisms were tested to

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**Table 1. Strains used**

| Strain   | Organism          |
|----------|-------------------|
| Aflatoxin-positive |                   |
| NRRL 465          | *A. parasiticus*  |
| NRRL 2999         | *A. parasiticus*  |
| NRRL 3251         | *A. flavus*       |
| NRRL 5520         | *A. flavus*       |
| Aflatoxin-negative |                  |
| NRRL 1957         | *A. flavus*       |

* Classified as *A. toxicarius* by Murakami (7).
Table 2. Color and intensity of fluorescence produced by two strains of A. flavus with various N sources

| N source        | NRRL 1957 A. flavus (aflatoxin negative) | NRRL 3145 A. parasiticus (aflatoxin positive) |
|-----------------|-----------------------------------------|-----------------------------------------------|
|                 | Color | Intensity | Color | Intensity |
| Urea            | Yellow | ++       | Blue yellow | +++   |
| Peptone         | Yellow | +        | Blue yellow | ++    |
| Asparagine      | Yellow | ±        | Blue yellow | +++   |
| Glutamic acid   | Yellow green | +++ | Yellow green | +++   |
| Aspartic acid   | Yellow green | +++ | Yellow green | +++   |
| Alanine         | Yellow | ++       | Blue yellow | ++    |
| NaNO₃          | Yellow green | ++ | Blue yellow | ++    |
| NH₄Cl          | Blue |       | Blue |     |
| NH₄NO₃        | Blue |       | Blue |     |
| (NH₄)₂SO₄      | Blue | +++     | Blue | +++   |
| (NH₄)H₂PO₄     | Blue | +++     | Blue | +++   |

*Culture conditions: basal medium plus N sources 0.3%, pH 5.0; 30 C for 7 days.

(+) None, (±) not clear, < + < +++ < ++++ < ++++ < ++++++.

Table 3. Intensity of blue fluorescence produced by NRRL 465 in the presence of various enzyme inhibitors and nutritional supplements

| Concn of test compounds | 7 Days* | 14 Days* |
|------------------------|---------|----------|
|                        | Intensity | Colony diam. (cm) | Intensity | Colony diam. (cm) |
| 10⁻⁴ M HgCl₂            | ++       | 2.0       | +++       | 3.0       |
| 5 × 10⁻⁴ M HgCl₂        | ++++     | 1.6       | ++++      | 2.9       |
| 10⁻⁴ M AgNO₃           | −        | 1.7       | −         | 3.0       |
| 5 × 10⁻⁴ M AgNO₃       | −        | 1.2       | ±         | 2.0       |
| 10⁻⁴ M CH₃COOH        | −        | 2.5       | ±         | 3.0       |
| 5 × 10⁻⁴ M CH₃COOH    | −        | 2.2       | −         | 3.0       |
| 10⁻⁴ M NaN₃           | +        | 2.3       | +++       | 3.0       |
| 5 × 10⁻⁴ M NaN₃       | −        | No growth | −         | No growth |
| 10⁻⁴ M α, α'-dipyridyl | −       | 2.2       | +         | 3.1       |
| 5 × 10⁻⁴ M α, α'-dipyridyl | −   | No growth | −         | No growth |
| 10⁻⁴ M CuSO₄         | +        | 2.3       | +         | 3.0       |
| 5 × 10⁻⁴ M CuSO₄     | ±        | 1.8       | +         | 3.0       |
| 5 × 10⁻⁴ M ZnSO₄     | +        | 2.0       | +++       | 2.7       |
| 0.02% Methionine      | +        | 2.0       | +++       | 3.1       |
| 0.02% Corn steep liquor | ++++    | 3.1       | ++++      | 4.5       |
| 0                      | +        | 2.5       | +++       | 3.2       |

*Culture conditions: basal medium plus (NH₄)₂H₂PO₄ 0.3% plus various chemicals; 30 C; pH 5.0.

Table 4. Effect of (NH₄)₂H₂PO₄ concentration on intensity of blue fluorescence

| Concn of (NH₄)₂H₂PO₄ (%) | NRRL 2999 | NRRL 3251 | NRRL 5520 |
|--------------------------|-----------|-----------|-----------|
|                          | Intensity | Colony diam. (cm) | Intensity | Colony diam. (cm) | Intensity | Colony diam. (cm) |
| 0.3                      | ++        | 1.5       | ++        | 1.5       | −         | 1.5       |
| 1.0                      | ++++      | 1.7       | +++       | 2.0       | ±         | 1.5       |
| 3.0                      | +++       | 1.0       | ++        | 1.8       | ±         | 1.4       |
| 5.0                      | ++        | 0.8       | +         | 1.6       | ±         | 1.2       |

*Culture conditions: basal medium plus (NH₄)₂H₂PO₄ (0.3 ~ 5.0%) plus HgCl₂ (5 × 10⁻⁴ M); pH 5.0; 28 C; 7 days.
determine whether these reduced responses could be improved by altering the pH of a medium containing 1% (NH₄)₂HPO₄ and HgCl₂ at $5 \times 10^{-4}$ M. As can be seen in Table 5, the fluorescence of A. parasiticus remained the same at pH 5 and 5.5, whereas that of one of the two A. flavus cultures was enhanced at pH 5.5. NRRL 5520 again failed to respond under these conditions. Intensity of fluorescence decreased markedly at pH 6.5 where growth was most rapid. All subsequent tests were run at pH 5.5.

Because A. flavus NRRL 5520 repeatedly failed to respond under the conditions tested thus far, the effect of different concentrations of HgCl₂ was reexamined. Results are seen in Table 6. As in preliminary experiments with NRRL 465, $5 \times 10^{-4}$ M concentration of this inhibitor promoted development of maximum fluorescence by NRRL 2999 and 3251. Again, fluorescence of NRRL 5520 was questionable. Higher concentrations were not investigated, since growth of the fungi was depressed at $5 \times 10^{-4}$ M HgCl₂.

The possibility that the combined effects of HgCl₂ and corn steep liquor might enhance fluorescence of NRRL 5520 was explored (Table 7). Limited but unequivocal fluorescence was observed in NRRL 5520 at 7 days with 0.03 and 0.05% concentrations of corn steep liquor in a medium containing $5 \times 10^{-4}$ M HgCl₂. Higher concentrations of corn steep liquor were unsatisfactory because steep liquor itself contains some unknown substance that shows a similar fluorescence under UV light. Fluorescence of NRRL 2999 and 3251 at 7 days was similar to that observed with HgCl₂ alone. The cultures on 0.05% corn steep liquor were retained at room temperature and reexamined at 10 and 13 days (Table 7). Fluorescence increased in all three strains at 10 days but decreased in NRRL 2999 and 3251 at 13 days.

Since the plates were held at room temperature (approximately 21 to 22°C) after 7 days, it was not clear whether the increased fluorescence reflected a time or temperature effect. Subsequent tests at 20, 24, and 28°C showed that toxin production by NRRL 5520 is delayed at all three temperatures (Table 8). Therefore, observations should be made at 10 days on cultures incubated at 28°C.

As a result of these studies, a medium of the composition shown in Table 9 is recommended for detection of aflatoxin-producing strains.

Fluorescent APA medium surrounding colonies of NRRL 2999 and 3251 was extracted as described in Materials and Methods. By TLC of the chloroform extracts, aflatoxins B₁, B₂, G₁, G₂, B₁a, and G₂a were detected in NRRL 2999. Only aflatoxin B₁, B₂, and B₁a were detected in NRRL 3251. Aflatoxin B₁ from both strains was

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**Table 5. Effect of pH on the intensity of blue fluorescence**

| pH | NRRL 2999 | NRRL 3251 | NRRL 5520 |
|----|-----------|-----------|-----------|
|    | Intensity | Colony diam. (cm) | Intensity | Colony diam. (cm) | Intensity | Colony diam. (cm) |
| 4  | +++       | 1.3       | +++       | 2.0       | –        | 1.7       |
| 5  | +++       | 1.7       | +++       | 2.0       | ±        | 1.5       |
| 5.5| +++      | 2.2       | +++       | 3.2       | ±        | 3.5       |
| 6.5| +        | 4.7       | +         | 4.7       | –        | 4.5       |

*Culture conditions: basal medium plus (NH₄)₂HPO₄ (1%) plus HgCl₂ ($5 \times 10^{-4}$ M); pH 4.5–6.5, adjusted with 1 N NaOH; 28°C; 7 days.*

**Table 6. Effect of HgCl₂ concentration on the intensity of blue fluorescence**

| Conc of HgCl₂ (M)* | NRRL 2999 | NRRL 3251 | NRRL 5520 |
|-------------------|-----------|-----------|-----------|
|                   | Intensity | Colony diam. (cm) | Intensity | Colony diam. (cm) | Intensity | Colony diam. (cm) |
| 0                 | +++       | 3.3       | +         | 4.0       | –        | 4.0       |
| $10^{-4}$         | +++       | 2.4       | +++       | 3.5       | ±        | 3.8       |
| $5 \times 10^{-4}$| +++      | 2.2       | +++       | 3.2       | ±        | 3.5       |

*Culture conditions: basal medium plus (NH₄)₂HPO₄ (1%) plus HgCl₂ (0 ~ $5 \times 10^{-4}$ M); pH 5.5; 28°C; 7 days.*
TABLE 7. Effect of corn steep liquor concentration and incubation time on the intensity of blue fluorescence

| Conc of corn steep liquor (%) | Incubation (days) | NRRL 2999 | NRRL 3251 | NRRL 5520 |
|------------------------------|-------------------|-----------|-----------|-----------|
|                              | Intensity | Colony diam. (cm) | Intensity | Colony diam. (cm) | Intensity | Colony diam. (cm) |
| 0                            | ++       | 1.8         | ++       | 2.4         | ±         | 1.6         |
| 0.01                         | ++       | 2.0         | +++      | 3.0         | ±         | 2.4         |
| 0.03                         | ++       | 3.5         | +++      | 3.5         | +         | 3.2         |
| 0.05                         | +++      | 3.6         | +++      | 4.2         | +         | 4.7         |
| 0.05                         | +++      | 4.5         | +++      | 5.0         | +++       | 5.6         |
| 0.05                         | +++      | 4.9         | +++      | 5.5         | +++       | 6.1         |

*Culture conditions: basal medium plus (NH₄)₂H₂PO₄ (1.0%) plus HgCl₂ (5 × 10⁻⁴ M) plus corn steep liquor (0 to 0.05%); pH 5.5; 28 C for 7 days; room temperature (±22 C) thereafter.

TABLE 8. Influence of incubation time and temperature on fluorescence of two strains of A. flavus

| NRRL no. | Time (days) | 20 | 24 | 28 |
|----------|-------------|----|----|----|
| 3251     | 7           | + + (2.4)* | + + (3.2) | + + + + + (4.3) |
|          | 10          | + + + (2.5) | + + + + (4.5) | + + + + + (5.5) |
|          | 14          | + + + + (3.0) | + + + + (4.0) | + + + + + (5.9) |
| 5520     | 7           | ± (1.7) | ± (3.8) | + (4.5) |
|          | 10          | + (2.0) | + + + (4.5) | + + + + + (5.5) |
|          | 14          | + + (2.5) | + + + + (5.0) | + + + + + (6.0) |

*Numbers in parentheses equal diameter (cm) of colony.

TABLE 9. Medium recommended for detection of aflatoxin-producing strains

| Aflatoxin-producing ability medium* | Quantity |
|-----------------------------------|----------|
| Distilled H₂O                     | 1 liter  |
| (NH₄)₂H₂PO₄                       | 10 g     |
| K₂HPO₄                            | 1 g      |
| MgSO₄·7H₂O                        | 0.5 g    |
| KCl                               | 0.5 g    |
| FeSO₄·7H₂O                        | 0.01 g   |
| Sucrose                           | 30 g     |
| HgCl₂                             | 5 × 10⁻⁴ M |
| Corn steep liquor*                | 0.5 g    |
| Agar                              | 20 g     |

*e.g., asperperiner A or B (5), flavacol (10), and deoxy-hydroxy-aspergillic acid (10). To determine the extent to which these substances might interfere with the test proposed here, 13 known aflatoxin-positive strains and 12 strains that had failed to produce aflatoxin in earlier surveys on solid substrates were compared on APA. The intensity of the blue fluorescence in the agar was recorded, and CHCl₃ extracts of the agar were prepared and were examined under UV light. In all strains, the intensity of the fluorescence in the CHCl₃ extract duplicated that recorded for the agar.

TABLE 10. Aflatoxin concentrations in fluorescent APA agar surrounding colonies of NRRL 2999 and 3251

| Aflatoxin | NRRL 2999 (µg/g) | NRRL 3251 (µg/g) |
|-----------|------------------|------------------|
| B₁        | 4.6              | 2.7              |
| B₂        | 0.57             | 0.57             |
| G₁        | 2.6              | 0.3              |
| G₂        | 0.3              | 0.3              |

* pH adjusted to 5.5 with 1 N NaOH before the addition of agar.
* Corn Products Co., Argo, Ill.

Further identified by preparation of its derivative according to the AOAC method (2).

Aflatoxins B₁, B₂, G₁, and G₂ in the fluorescent APA agar surrounding colonies of NRRL 2999 and 3251 were determined quantitatively (Table 10) by methods described above.

Reportedly, strains of A. flavus and A. oryzae produce several substances other than aflatoxin that give a blue fluorescence under UV light;
gave a slight blue fluorescence under the colony but not in the agar. No aflatoxin was detected in the CHCl₃ extract by TLC. The fluorescent material may be flavacol or a related compound.

Evidently, blue fluorescence in APA agar surrounding a colony is strongly indicative of aflatoxin-producing capability. However, extraction by CHCl₃ and identification by TLC are required for positive identification of aflatoxin as the fluorescing substance.

Despite this limitation, the three-step method facilitates screening many isolates for aflatoxin production, since it provides a simple and reliable means of eliminating nonproducing strains. The method also avoids difficulties encountered in extracting aflatoxin from complex natural substrates.

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**Table 11. Comparison of aflatoxin-positive and aflatoxin-negative strains on APA medium**

| Strain no. | Name* | Intensity of blue fluorescence in agar |
|------------|-------|---------------------------------------|
| Aflatoxin-negative | | |
| RIB 23 | A. oryzae var. brunnneus | – |
| RIB 25 | A. oryzae var. brunnneus | – |
| RIB 40 | A. oryzae var. viride | – |
| RIB 178 | A. oryzae (Ahlburg) Cohn | – |
| RIB 331 | A. oryzae (Ahlburg) Cohn | ± |
| RIB 1040 | A. sojae Saka. and Yamada | – |
| RIB 1041 | A. sojae Saka. and Yamada | – |
| RIB 1042 | A. sojae Saka. and Yamada | – |
| RIB 1046 | A. sojae Saka. and Yamada | – |
| RIB 1047 | A. sojae Saka. and Yamada | – |
| RIB 69 | A. flavus Link ex Fr. | – |
| RIB 1427 | A. flavus Link ex Fr. | – |
| Aflatoxin-positive | | |
| NRRL 465 | A. parasiticus Speare | ++++ |
| NRRL 2389 | A. parasiticus Speare | ++++ |
| NRRL 3000 | A. parasiticus Speare | ++++ |
| NRRL 3145 | A. parasiticus Speare | ++++ |
| NRRL 3240 | A. parasiticus Speare | ++++ |
| NRRL 5835 | A. parasiticus Speare | ++++ |
| RIB 4032 | A. parasiticus Speare | ++++ |
| RIB 4033 | A. parasiticus Speare | ++++ |
| RIB 4025 | A. flavus Link ex Fr. | +++ |
| NRRL 5520 | A. flavus Link ex Fr. | + |
| A-11610 | A. flavus Link ex Fr. | + |
| A-12353 | A. flavus Link ex Fr. | + |
| A-12569 | A. flavus Link ex Fr. | + |

*Names cited for RIB numbers are those furnished by the Research Institute of Brewing.