Cultural Methods for Aflatoxin Detection

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ABSTRACT

Aflatoxins present important food safely problems in both developed and
developing countries. Contamination is monitored in developed countries
using enzyme-linked immunusorbent assay (ELISA)- and high-perfor-
mance liquid chromatography (HPLC)-based assays, both of which may
be too expensive for routine use in many developing countries. There is a
need for inexpensive alternative approaches to detect aflatoxins in lots of
foods and feeds. Reviewed here are culture-based methods that determine
if a sample is contaminated with aflatoxigenic fungi. These approaches
include 1) blue fluorescence of aflatoxin B_1, particularly when enhanced
by including β-cyclodextrin in the culture medium, 2) yellow pigment
production, and 3) color change on exposure to ammonium hydroxide
vapor. The presence of aflatoxin B_1 can be detected by its blue
fluorescence, which is enhanced when the toxin complexes with the
hydrophobic pocket of β-cyclodextrin. The yellow pigment and
ammonium hydroxide vapor tests are based on the production of yellow
anthraquinone biosynthetic intermediates in the aflatoxin pathway. These
compounds act as pH indicator dyes, which are more visible when they
have turned red at alkaline pH. Because these tests are based on two
different mechanisms, it has been possible to combine them into a single
test. In a study of 517 A. flavus isolates from the Mississippi Delta, the
combined assay reduced false positives for aflatoxigenicity to 0%, and
false negatives to 7%. The increased predictive power of the combined

cultural assay may enable its use for inexpensively identifying potential aflatoxin contamination in feeds and foods.

I. INTRODUCTION

Current events have focused attention on the role of microbes and their toxins in food safety and terrorism, and they have stimulated considerable research activity on the characterization of toxigenic fungi and the development of techniques for detection and quantitation of their mycotoxins. Aflatoxins have been a particular focus of research studies. Aflatoxins are produced primarily by *Aspergillus flavus, A. parasiticus*, and *A. nomius*, and they contaminate many commodities used for human food and animal feed (Dorner, 2002; Leitao et al., 1988; Scott, 1987; Yiannikouris and Jouany, 2002). Aflatoxins are known to be mutagenic, teratogenic, carcinogenic, and immunosuppressive in animals and possibly in humans (Alpert et al., 1971; CAST, 1979, 1989, 2003; DiPaolo et al., 1967; Georggiett et al., 2000; Hussein and Brasel, 2001; Ong, 1975; Peraica et al., 1999; Pier, 1973; Yiannikouris and Jouany, 2002). Economic losses due to aflatoxin contamination occur in four major commodities grown in the southern United States (corn, peanut, cottonseed, and tree nuts), and those losses are extremely high, with annual estimates of many millions of dollars (CAST, 1989, 2003; Payne, 1992, 1998; Robens and Cardwell, 2003; Widstrom, 1996).

The cost of analytical services to monitor aflatoxin levels in both contaminated and uncontaminated crops is a substantial part of the overall cost of aflatoxin contamination in the United States (Robens and Cardwell, 2003). The analytical methods used in developed countries are sensitive, quantitative, and reliable, but sufficiently expensive to preclude their use in many developing countries, except in crops intended for the export market. This situation has created a longstanding need for aflatoxin detection methods that do not require major expenditures for equipment, have low unit costs for assay supplies, and can be conducted with a level of technical skill readily available in developing countries. These needs have led us to focus on cultural methods for aflatoxigenicity testing methods. There is currently an additional need for these types of methods in developed countries to prescreen large numbers of *A. flavus* isolates to identify nonaflatoxigenic strains that may be evaluated as potential biocontrol agents for use in limiting aflatoxin production in agricultural crops. In this

review, we describe the use of three cultural methods for detecting aflatoxigenicity, as well as techniques for the isolation of *Aspergillus* species and the media used for aflatoxin production.

II. ANALYTICAL METHODS VS. CULTURAL METHODS: ADVANTAGES AND DISADVANTAGES

A. Analytical Methods

Numerous publications describe analytical and cultural methods for the detection and quantification of aflatoxins in agricultural commodities and in cultures of fungi isolated from them. These methods vary in accuracy and precision, depending on the end goal of the analysis. Analytical methods for aflatoxin detection and quantification (Nilufer and Boyacioglu, 2002; Whitaker et al., 1996) include 1) thin layer chromatography (TLC) (Stroka and Anklam, 2000), 2) high-performance liquid chromatography (HPLC) (Seitz, 1975; Sobolev and Dorner, 2002; Trucksess et al., 1994), 3) liquid chromatography/ mass spectroscopy (LC/MS), 4) enzyme-linked immunosorbent assay (ELISA) (Patey et al., 1989; Trucksess et al., 1990), and (v) immunoaffinity with fluorescence (VICAM) (Nasir and Jolley, 2002; Maragos and Thompson, 1999).

Analytical methods have a number of advantages that include the following:

- They are sensitive.
- They are reliable.
- They are quantitative.
- They may be specific for mycotoxin type.
- They may measure multiple mycotoxin subtypes.
- They are definitive.
- They are accepted for export markets.

Analytical methods also have a number of disadvantages that include the following:

- The necessary equipment for HPLC and LC/MS is expensive to purchase and to maintain.
- The unit costs for ELISA immunoaffinity columns are high and they have short shelf lives.
- The methods are time-consuming and require highly skilled technicians.
- The methods require extraction with solvents, which create waste disposal problems.

- The methods measure toxins not toxicity, so that novel or otherwise unregulated toxins that may contribute to toxicity (e.g., cyclopiazonic acid) are not measured.

B. Cultural Methods

Cultural methods include: 1) blue fluorescence, particularly in the presence of an enhancer in the medium such as β-cyclodextrin (FL) (Fente et al., 2001; Ordaz et al., 2003); 2) yellow pigmentation, particularly on the undersides of colonies (YP) (Gupta and Gopal, 2002; Lin and Dianese, 1976); and 3) color change of the yellow pigment to plum-red on exposure of the culture to ammonium hydroxide vapor (AV) (Saito and Machida, 1999).

Cultural methods have a number of advantages that include the following:

- They are inexpensive.
- No major equipment is required, and minor equipment that is needed is available in developed and developing countries.
- The technical skill level that is needed to conduct the assays is available in developed and developing countries.
- Negative assessment by cultural methods is expected to be definitive; that is, if there is no viable *A. flavus*, it is expected that there will be no aflatoxin either.

Cultural methods also have a number of disadvantages that include the following:

- Positive assessments are not specific with respect to mycotoxin type.
- Positive assessments may be quantitative with respect to the amount of fungus in the sample, but the amount of mycotoxin present will not necessarily correlate with the infestation level.
- They are generally regarded as being less sensitive than analytical methods.
- They vary with respect to limits of detection, accuracy, requirements, and applications.

Many developing countries in the world that have serious aflatoxin contamination problems have difficulty screening all lots of foods and feeds, because of the expense and lack of expertise required to maintain an analytical laboratory. This situation has created much interest in developing and using cultural methods as a prescreen to identify those lots of agricultural products that are the best candidates for the export market, and

to identify which of those lots of commodities intended for direct human consumption locally have the lowest amount of *A. flavus* contamination. Cultural methods are also of considerable interest in the United States for use in prescreening large numbers of *A. flavus* field isolates to identify atoxigenic candidate isolates for possible development as biocontrol strains.

III. ISOLATION OF AFLATOXIGENIC FUNGI AND MEDIA FOR AFLATOXIN PRODUCTION

Species of *Aspergillus* that produce aflatoxins are ubiquitous and easily isolated from nature. Soil samples are usually diluted with known volumes of sterile water, and soil suspensions are plated on agar media (''dilution plated'') to obtain quantitative estimates of the population density expressed as colony-forming units (CFUs)/g in soil. The same technique can be used for agricultural commodities by grinding the sample before suspending in water or by homogenizing the sample in water with a blender (Dorner, 2002; Hartog and Notermans, 1988; Horn and Dorner, 1998; Klich et al., 1992). Alternatively, grains and seeds can be plated whole after surface sterilization to obtain the percentage infection. This technique does not measure the extent of fungal colonization of grains, and surface-sterilization techniques are not 100% effective (Andrews, 1996; Andrews et al., 1997; Sauer and Burroughs, 1986). Therefore, for precise population studies such as vegetative compatibility group (VCG) analysis (Horn and Greene, 1995), seeds must be individually surface-sterilized to avoid cross contamination during sterilization and the subsequent rinses.

A. flavus and *A. parasiticus* are not fastidious in their nutritional requirements and will grow on nearly all commonly prepared media for fungi. Isolation of these fungi on agar media instead relies on their sensitivity to certain antibiotics relative to other fungi, their ability to grow at relatively high temperatures (37°C), and their tolerance of low moisture content in the growth medium. The most commonly used media for dilution plating contain the antibiotics dichloran and/or rose bengal for restricting fungal colony diameter (Cotty, 1994; Frisvad et al., 1992; King et al., 1979; Pitt, 1992). Antibiotics against bacteria are also added, and incubation at 37°C inhibits the growth of many soil fungi that would interfere with the detection of aflatoxigenic species. Various formulations of dichloran-rose bengal medium permit the accurate identification of many *Aspergillus* species, including *A. flavus* and *A. parasiticus*, directly from the dilution plates (Horn and Dorner, 1998; Pitt, 1992). Other media rely on low water activity for selection of aflatoxigenic *Aspergillus* species. Sodium chloride is often used to adjust the water activity (Griffin et al., 1975, 2001). However, *A. flavus, A. parasiticus*, and other members of *Aspergillus*

section *Flavi* cannot be identified to species in the presence of NaCl and must be subcultured to another medium such as Czapek agar for final identification (Horn et al., 1995).

When growing fungi in culture for aflatoxin production, single-spore isolates of *A. flavus* and *A. parasiticus* are necessary to ensure that mixed cultures do not compromise the results. Aflatoxigenic strains may be grown on solid substrates or in liquid media. Agricultural commodities that are susceptible to aflatoxin contamination in nature, such as corn, peanuts, and rice, are excellent substrates for aflatoxin production in the laboratory (Diener and Davis, 1969; Hesseltine et al., 1970). It is necessary to determine beforehand that agricultural substrates are free of aflatoxins. Following fermentation, solvent extracts often require extensive cleanup procedures before aflatoxin analysis. For these reasons, either defined (Davis et al., 1967; Mateles and Adye, 1965; Reddy et al., 1971) or undefined (El-Kady et al., 1994; Ren et al., 1999) liquid media are more commonly used to assess aflatoxin production. Of the undefined media, various formulations of yeast extract-sucrose broth (YES) are most commonly used (Horn and Dorner, 1999; Wei and Jong, 1986). Large numbers of isolates can be examined by growing them in small vials of YES, manually macerating the mycelium, then adding chloroform and vortex mixing. The chloroform phase that separates can be analyzed directly by TLC or HPLC without further cleanup (Horn and Dorner, 1999; Horn et al., 1996).

IV. TYPES OF CULTURAL METHODS

Aflatoxins B_1 and B_2 produce an intense blue fluorescence visible at approximately 450 nm, when exposed to long-wavelength (365 nm) ultraviolet (UV) light. This property has been useful for developing a variety of qualitative and quantitative analytical methods for aflatoxins (Pons and Goldblatt, 1969), such as TLC and HPLC with fluorescence detection systems. These procedures are generally used to analyze extracts prepared from the samples, and they provide a quantitative, sensitive assessment of aflatoxins in the low ppb (ng/g) range. Immunoaffinity methods such as VICAM also use fluorescence to measure the amount of aflatoxin. Cultural methods for detecting aflatoxins produced by fungal isolates are based on observing either the fluorescence of the aflatoxins they produce, or the visible color of pigments the colonies produce.

A. Blue Fluorescence

The blue fluorescence of aflatoxins has been used for developing qualitative cultural methods for detecting aflatoxin production by *Aspergillus*

species grown on suitable media. Some of the methods use solid media, such as potato dextrose agar and coconut agar (Davis et al., 1987; Gupta and Gopal, 2002; Lemke et al., 1988, 1989; Lin and Dianese, 1976; Saito and Machida, 1999; Wei et al., 1984), whereas other methods use liquid media, including aflatoxin-producing ability medium (APA) and a medium containing corn steep liquor (Hara et al., 1974; Wicklow et al., 1981). Cotty (1988) developed a simple fluorescence method for aflatoxins in culture using a solid medium. With Cotty's technique, aflatoxins were quantified in culture tubes by measuring fluorescence with a scanning densitometer. Hybrid cultural/analytical methods have also been developed. Filtenborg and Frisvad (1980) developed GY-agar medium, which contains glucose and yeast extract, for aflatoxin production by *Aspergillus* species. Small plugs were cut from the agar medium and directly placed on the TLC plate. After development with a suitable solvent, aflatoxin spots were detected under long-wave UV light. Yabe et al. (1987) used GY-agar medium as a simple method for screening aflatoxin-producing species by UV photography. It was possible to detect production of aflatoxins even by small colonies that were only 36 hr old, and many colonies could be examined on a single plate. Ultraviolet photography could also be used as a rapid method for the identification of aflatoxin-producing isolates. The aflatoxin-producing isolates appeared as gray or black colonies in the UV photographs, whereas nonproducing isolates appeared as white colonies. Aflatoxins B1 and G1 were primarily responsible for the absorbance of UV light.

B. Cyclodextrin-Enhanced Blue Fluorescence

The blue fluorescence of aflatoxin B1 under UV light is apparent in both aqueous and solid media, and the fluorescence is enhanced significantly by treatment with various fluorescence enhancers such as iodine and cyclodextrins. Davis and Diener (1979) developed a photo-fluorometric (PFM) procedure using iodine to enhance aflatoxin fluorescence for use in HPLC (Davis and Diener, 1980; Davis et al., 1987). Later, Lemke et al. (1988) developed a simple mini-assay for detecting aflatoxin-producing isolates grown for 3–10 days in coconut extract broth using the PFM procedure with iodine. The results of the mini-assays were confirmed by HPLC and TLC. The PFM procedure showed an approximate 100-fold increase in sensitivity for aflatoxin detection with iodine derivatization compared to that for nonderivatized aflatoxin B1.

The fluorescence emission of aflatoxins B1 and G1 also is substantially improved when treated with enhancer agents such as cyclodextrins (CDs). The CD fluorescence enhancers have improved various analytical methods for the detection of aflatoxins and other mycotoxins (Franco et al., 1998;

Hongyo et al., 1992; Seidel et al., 1993; Vazquez et al., 1991, 1992). These methods include liquid chromatography in which CDs are added to either the column eluent (Chiavaro et al., 2001) or to the unpurified aflatoxin extract for screening as well as quantitative methods for fluorometry analysis (Wilson et al., 2002). Fente et al. (2001) showed that adding ß-CD to a suitable agar medium enhances detection of aflatoxin production by *A. flavus* and *A. parasiticus* by blue fluorescence under 365-nm light. The enhancement of aflatoxin fluorescence by precolumn derivatization with Trifluoro-Acetic Acid (TFA) has been developed for HPLC and TLC (Asao et al., 1963; DeVries and Chang, 1982; Garner, 1975; Philipps and Cauderay, 1979; Pons and Goldblatt, 1969; Seitz, 1975; Trucksess, 1976). The enhancement of aflatoxin fluorescence by postcolumn derivatization with a photochemical technique called "FRED" was developed by Joshua (1993).

C. Yellow Pigment

Production of yellow to orange pigments by aflatoxigenic *A. flavus* was first identified by Wiseman et al. (1967) because they caused interference in the quantitative measurement of aflatoxin levels by absorbance. They developed a method for removing the pigments from solvent extracts by treatment with insoluble basic copper carbonate. Production of abundant amounts of yellow pigment by *A. flavus* was also reported by Arseculeratne et al. (1969), who observed that pigment production was noticeable by the second day in colonies on coconut agar medium. Lin and Dianese (1976) were the first to associate bright yellow pigment production with aflatoxigenicity in *A. flavus* as detected by blue fluorescence of aflatoxin in the medium. The yellow pigmentation was observed earlier than the blue fluorescence and was not observed in colonies that did not produce aflatoxins. Yellow pigment was secreted into the medium, but it was most easily visualized on the reverse side of colonies grown on semitransparent agar media such as potato dextrose agar. Lin and Dianese (1976) reported that the degree of yellow pigmentation was proportional to blue fluorescence in all media tested. However, Davis et al. (1987) concluded that yellow pigment production was not a reliable predictor of the amount of aflatoxin in all media. This conclusion was confirmed by Gupta and Gopal (2002), who identified semiquantitiative differences between media.

D. Ammonium Hydroxide Vapor-Induced Color Change

Saito and Machida (1999) introduced a novel method for rapid, sensitive identification of aflatoxin-producing and nonproducing strains of *A. flavus* and *A. parasiticus*. The method was developed empirically and

was validated using a collection of 120 strains of *A. flavus, A. parasiticus, A. oryzae,* and *A. sojae* that were characterized with respect to aflatoxin production by HPLC and UV fluorescence methods. For this method a single colony is grown in the center of a Petri dish containing medium such as potato dextrose agar. The dish was inverted and 1 or 2 drops of concentrated ammonium hydroxide solution are placed on the inside of the lid. The undersides of aflatoxin-producing colonies quickly turn plum-red after the bottom of the Petri dish has been inverted over the lid containing the ammonium hydroxide. Essentially no color change occurs on the undersides of colonies that are not producing aflatoxins. Saito and Machida (1999) observed the greatest color change in colonies grown on yeast extract-sucrose and coconut media, a less intense color change on potato dextrose agar, and the least color change on glucose-mineral salts media, all of which favor aflatoxin production. However, no color change was observed on peptone-mineral salts and Czapek solution media, which do not support aflatoxin production.

We have investigated the biochemical basis of the Saito and Machida test by using methanol to extract pigments from lyophilized cultures of aflatoxigenic *A. flavus* grown on potato dextrose agar. The extracts contained yellow pigments, which are presumably the same yellow pigments that are the basis of the aflatoxigenicity test of Lin and Dianese (1976). These pigments turned plum-red upon mixing with ammonium hydroxide solution or a solution of any other base (e.g., sodium hydroxide, potassium hydroxide, sodium carbonate, sodium bicarbonate). Addition of an excess of any acid converted the color back to yellow, indicating that the pigments act as pH indicator dyes. The pH indicator dye function could be replicated under the conditions of the Saito and Machida test by replacing the drops of concentrated ammonium hydroxide solution with a few drops of glacial acetic acid and again inverting the bottom of the Petri dish with the *A. flavus* colony over it. The underside of the colony reverted from plum-red back to the original yellow or grey. It was possible to cycle colonies through multiple color changes like an isolated pH indicator dye.

A total of 14 yellow pigments were purified from the methanol extract of aflatoxigenic *A. flavus* cultures. The purification of pigments by preparative thin layer chromatography was facilitated by color changes on exposure to ammonium hydroxide vapor. The structures of seven of the pigments were identified using spectroscopic and chromatographic methods; they include norsolorinic acid, averantin, averufin, versicolorin C, versicolorin A, versicolorin A hemiacetal, and nidurufin (Aucamp and Holzapfel, 1970; Cole, 1981; Heathcote and Dutton, 1969). All turn from yellow to red at about pH 6.5 as the pH is raised, except norsolorinic acid, which turns from red to purple. All of these pigments are anthraquinone intermediates in the aflatoxin biosynthetic pathway (Bhatnagar et al., 2003;

Sinz and Shier, 1991) except nidurufin, which is a known side product of the pathway in several species. The seven identified pigments constitute the large majority of the total yellow pigment in extracts of aflatoxigenic *A. flavus* cultures. When extracts were prepared in an identical manner from cultures of nonaflatoxigenic *A. flavus,* and examined by high performance liquid chromatography using the method of McCormick et al. (1988), no yellow pigments were detected, although they were readily detected in extracts from aflatoxigenic *A. flavus* cultures.

Several early studies reported the production of yellow anthraquinone pigments by aflatoxigenic *A. flavus*. For example, Donkersloot et al. (1972) showed that averufin was produced by a nonaflatoxigenic mutant of *A. parasiticus*. Similarly, Lin et al. (1973) showed that nonmutated *A. parasiticus* also produced averufin, and that the averufin could be converted to aflatoxin. However, the present study is the first to examine all the yellow pigments produced by an aflatoxigenic *A. flavus* strain, and to use assay-guided purification to provide evidence that the same yellow pigments form the basis of both the yellow pigment test of Lin and Dianese (1976) and the ammonium hydroxide vapor-induced color change test of Saito and Machida (1999). The identification of the yellow pigments as aflatoxin biosynthetic intermediates provides both a rationale for the effectiveness of these two empirical tests, and a basis for predicting some conditions under which false positives and false negatives might be expected to occur. False positives will result from mutations in the enzymes that catalyze the last steps of the biosynthetic pathway after the anthraquinone ring has been destroyed (Sinz and Shier, 1991). Because these mutations are expected to be rare, false positives are expected to occur by this mechanism in only limited numbers. Similarly, knowing the molecular basis of the tests makes it possible to predict that false negatives will occur when production of both aflatoxins and yellow pigments are at such low levels that sensitive, fluorescence-based assays can detect traces of aflatoxin, but yellow pigments are produced at levels too low to be detected with the naked eye. In practice, very few false negatives were observed (Abbas et al., 2004), suggesting that aflatoxin production may be regulated by a mechanism such that the fungus either produces aflatoxin at levels detectable with ELISA assays, or it does not produce aflatoxin at all.

E. Cyclodextrin-Enhanced Blue Fluorescence Combined with Ammonium Hydroxide Vapor-Induced Color Change

Abbas et al. (2004) demonstrated that three of the cultural aflatoxigenicity tests described above can be carried out in the same Petri

dish (Fig. 1), and still allow sampling by two analytical methods. In one method, ß-cyclodextrin is incorporated into potato dextrose agar (ß-CD-PDA, 0.3% wt/vol) (Cavasol W7M, Wacker-Chemie GMBH, Burghausen, Germany) in 9-cm Petri dishes. Isolates are grown at 28° to 35°C for 4 to 7 days in continuous darkness or on a 12-hr dark/light cycle. The underside of the colony is examined under natural light for bright-yellow pigmentation in the medium (Gupta and Gopal, 2002; Lin and Dianese, 1976). Aflatoxin production is also visualized by the presence of blue fluorescent zones under long wavelength (365 nm) UV light. Small plugs are cut from the agar medium using a clean plastic tube, transferred to TLC plates (SIL G-25 HR, Alltech Associates, Deerfield, IL), and removed within 10 seconds following the appearance of the liquid front migrating into the silica gel. The spots are allowed to dry and the TLC plate is developed in chloroform:acetone (93:7, vol/vol) under vapor-saturated conditions. Aflatoxins B1, B2, G1, and G2 are detected directly under long-wave ultraviolet light by their fluorescence. Approximately 10 plugs (~1 gram each) are weighed, placed in plastic scintillation vials, extracted with 70% MeOH for 30 min on a high speed shaker, and centrifuged. An aliquot of the supernatant is tested for the presence of aflatoxins by any suitable analytical method such as a commercially available enzyme-linked immunosorbant assay (ELISA) kit (Patey et al., 1989), high performance liquid chromatography, thin layer chromatography, or liquid chromatography with mass spectrometric detection. Finally, the same Petri dishes are inverted over 0.5 mL of 25–27% ammonium hydroxide on the lid of the dish. Aflatoxin production is detected as a color change on the undersides of colonies from yellow to plum-red.

V. CULTURAL METHODS VS. ANALYTICAL METHODS: A COMPARISON AMONG ISOLATES FROM THE MISSISSIPPI DELTA

A study of 517 isolates of *A. flavus*, *A. parasiticus*, and *A. nomius* from corn (maize), peanut (groundnut), rice, cotton, and soil in the Mississippi Delta region, Mississippi, U.S., was conducted to compare cultural methods with established analytical methods, and to determine if combinations of cultural methods increase their power to predict aflatoxigenicity (Abbas et al., 2004). For each isolate, aflatoxigenicity was assessed by analyzing potato dextrose agar culture extracts by thin layer chromatography as described above, and by a commercially available ELISA kit ("Vertox" purchased from Neogen Corp., Lansing, MI; limit of detection, 0.5 µg/kg), which was considered definitive for aflatoxigenicity. Aflatoxigenicity was

Grow fungal isolate on **CD-PDA** for four to seven days.

•Score for yellow pigment in medium

Yellow pigment correctly identified 94% of atoxigenic strains and 71 to 74% of aflatoxigenic strains.

•Score for fluorescence under 365-nm UV light

Blue fluorescence is greatly enhanced by addition of ß-cyclodextrin. Fluorescence correctly identified 80% of nontoxigenic strains 87% of low-producing strains and 98% of high-producing strains.

•Remove plugs from medium

•Add plugs to TLC plate, develop, view under 365-nm UV light

•Extract plugs, assay by ELISA, HPLC or other analytic method

TLC correctly identified 92% of nontoxigenic isolates and 100% of high-producing strains.

•Expose culture to ammonia vapor, observe development of pink pigment

Response to ammonia vapor correctly identified 100% of nontoxigenic strains and about 70% of aflatoxigenic strains.
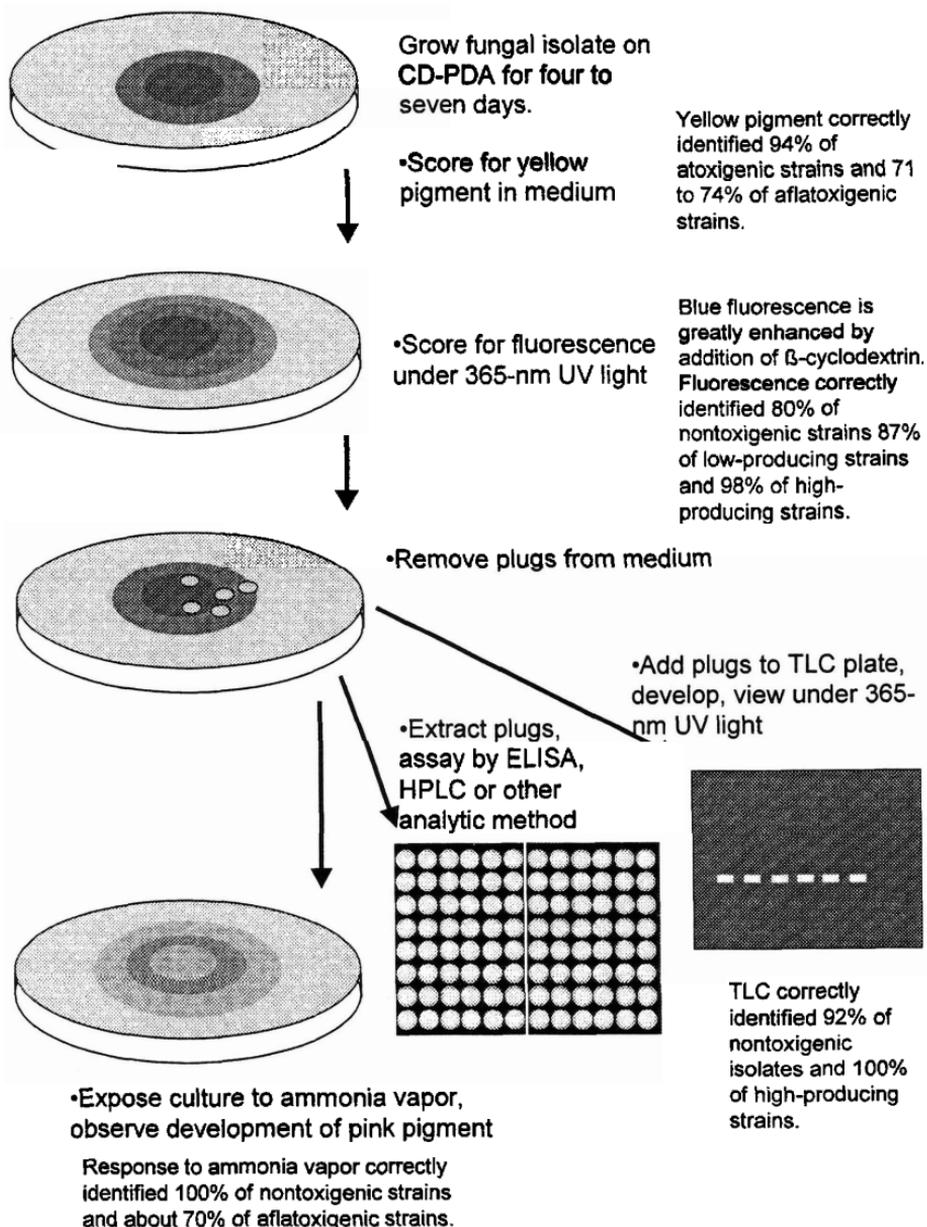
Figure 1. Combined cultural methods for identifying aflatoxigenic isolates of *Aspergillus* species.

also assessed by three cultural methods on potato dextrose agar as described above: 1) fluorescence on ß-cyclodextrin-containing agar; 2) yellow pigment formation; and 3) color change on exposure to ammonium hydroxide vapor. About 60% of the isolates produced > 20 ng/g aflatoxins, and were considered aflatoxigenic, and the remainder were considered nonaflatoxigenic. Almost all (99%) of the isolates found to be toxigenic by ELISA were confirmed to be producing aflatoxin by thin layer chromatography. For the aflatoxin-producing isolates, the cyclodextrin-enhanced fluorescence test gave the strongest correlation with ELISA among the cultural methods tested. Cyclodextrin-enhanced fluorescence confirmed 93% of aflatoxin-producing cultures, whereas the yellow pigment and ammonium hydroxide-induced color change confirmed only 73% and 70%, respectively. However, among the isolates not producing aflatoxins, the ammonium hydroxide-induced color change test gave the best correlation with aflatoxigencity determined by ELISA. It gave no false positive responses, whereas the fluorescence and yellow pigment tests gave 20% and 6% false positives, respectively. Combining into a single cultural test the low false negative results (7%) in the fluorescence test with the absence of false positives in the ammonium hydroxide-induced color change test results in a reliability comparable to thin layer chromatography. As described above, these two cultural methods are readily conducted on the same culture dish.

All of the cultural methods listed above are considerably less expensive and faster than traditional analytical methods. Appropriate selection of a cultural technique for the identification of aflatoxigenic isolates may depend on the application. For example, if one were to screen large numbers of field isolates to identify candidate isolates for development as biocontrol strains, it would be very undesirable to falsely identify a strain as nontoxigenic when it was actually toxigenic. For this purpose, the ammonium hydroxide vapor-induced color change test would be appropriate because it was the most reliable cultural method for identifying nontoxigenic strains. Alternatively, it is necessary to correctly identify aflatoxigenic strains in studies on the ecological role of aflatoxin production. For this purpose, fluorescence on ß-cyclodextrin-containing medium would be most appropriate because of its high reliability in identifying aflatoxigenic isolates (Abbas et al., 2004).

VI. CONCLUSIONS

Inexpensive cultural methods for detecting contamination by aflatoxigenic *A. flavus* may be a suitable alternative when limited resources

preclude the use of analytical methods. This conclusion is further supported by observations that combining two cultural methods on the same medium, ammonium hydroxide vapor-induced color change and blue fluorescence on β-cyclodextrin-containing medium, can provide an overall detection rate comparable to TLC but with less time and expense. The cultural assays should also be ideal for characterizing aflatoxin production in ecological and genetic studies of large populations of *Aspergillus* isolates, as well as for prescreening *A. flavus* isolates for nontoxigenicity in applications such as biocontrol or food processing.

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