Comparison of cultural and analytical methods for determination of aflatoxin production by Mississippi Delta *Aspergillus* isolates

Hamed K. Abbas, R.M. Zablotowicz, M.A. Weaver, B.W. Horn, W. Xie, and W.T. Shier

Abstract: This study compared cultural and analytical methods for detecting aflatoxin production by *Aspergillus* species. *Aspergillus* isolates were obtained from various Mississippi Delta crops (corn, peanut, rice, cotton) and soils. Most of the isolates (99%) were *A. flavus* and the remainder comprised *A. parasiticus* and *A. nomius*. The following three cultural methods were evaluated on potato dextrose agar: fluorescence (FL) on β-cyclodextrin-containing media (CD), yellow pigment (YP) formation in mycelium and medium, and color change after ammonium hydroxide vapor exposure (AV). Aflatoxins in culture extracts were confirmed by thin-layer chromatography (TLC) and quantified by enzyme-linked immunosorbent assay (ELISA). Of the 517 isolates, 314 produced greater than 20 ng/g of total aflatoxin based on ELISA, and 180 produced greater than 10 000 ng/g of aflatoxin in the medium. Almost all the toxigenic isolates (97%) were confirmed by TLC as producers. Of the toxigenic isolates, as determined by ELISA, 93%, 73%, and 70% gave positive FL, YP, and AV responses, respectively. Of the 203 isolates producing less than 20 ng/g of aflatoxin, 20%, 6%, and 0% of respective FL, YP, and AV methods gave false-positive responses. The 9% false-positive results from TLC fall within this range. This study showed good agreement among all tested cultural methods. However, these cultural techniques did not detect aflatoxin in all cultures that were found to produce aflatoxins by ELISA, LC/MS, and TLC. The best results were obtained when the AV color change and CD fluorescence methods were used together, yielding an overall success rate comparable to TLC but without the need for chemical extraction and the time and expense of TLC.

Key words: aflatoxins, analytical methods, *Aspergillus flavus*, *Aspergillus parasiticus*, cultural methods, β-cyclodextrin, fluorescence enhancers, mycotoxins, yellow pigment.

Résumé : La présente étude a comparé les méthodes culturales et analytiques pour la détection de la production d'aflatoxine par les espèces d'*Aspergillus*. Des isolats d'*Aspergillus* ont été obtenus à partir de divers sols et cultures (maïs, arachide, riz, coton) du Delta du Mississippi. La plupart des isolats (99 %) étaient *A. flavus* et le reste était composé de *A. parasiticus* and *A. nomius*. Les trois méthodes culturales suivantes ont été évaluées sur des gélose dextrose-pomme de terre: fluorescence (FL) sur du milieu contenant de la β-cyclodextrine (CD), formation d'un pigment jaune (PJ) dans le mycélium et le milieu, et changement de couleur après une exposition à de la vapeur d'hydroxyde d'ammonium (VH). La présence d'aflatoxines dans les extraits de culture a été confirmée par chromatographie en couche mince (CCM) et quantifiée par la méthode un test immuno-enzymatique (ELISA). Des 517 isolats, 314 ont produit plus de 20 ng/g d'aflatoxine totale tel que déterminé par ELISA, et 180 ont produit plus de 10 000 ng/g d'aflatoxine dans le milieu. La CCM a confirmé que presque tous les isolats toxigènes (97 %) étaient des producteurs. Parmi les isolats toxigènes, tel que déterminé par ELISA, 97 %, 73 % et 70 % ont donné respectivement des réponses positives de FL, PJ et VH. Des 203 isolats produisant mois de 20 ng/g d'aflatoxine, 20 %, 6 % et 0 % de ceux-ci étaient des faux positifs par les méthodes FL, PJ et VH, respectivement. Les 9 % de faux positifs obtenus par CCM se situent dans cet écart. Cette étude a démontré une bonne concordance entre toutes les méthodes culturales. Toutefois, ces techniques culturales n'ont pas retrouvé d'aflatoxine dans toute les cultures dont ont avait détecté une production par ELISA, CL/SM et CCM. Les meilleurs résultats furent obtenus lorsque les méthodes mesurant le changement de couleur de la vapeur d'ammoniaque et la fluorescence de la CD étaient utilisées en combinaison, fournissant un taux de succès généralement comparable à la CCM, mais sans extraction chimique et sans l'investissement en temps et en argent de la CCM.

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H.K. Abbas.[1] USDA–ARS, Crop Genetics and Production Research Unit, Stoneville, MS 38776, USA.
R.M. Zablotowicz and M.A. Weaver. USDA–ARS, Southern Weed Science Research Unit, Stoneville, MS 38776, USA.
B.W. Horn. USDA–ARS, National Peanut Research Laboratory, Dawson, GA 31742, USA.
W. Xie. Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108, USA.
W.T. Shier. College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, USA.

[1]Corresponding author (e-mail: habbas@ars.usda.gov).

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Mots clés : aflatoxines, méthodes analytiques, *Aspergillus flavus*, *Aspergillus parasiticus*, méthodes culturales, β-cyclodextrin, amplificateurs de la fluorescence, mycotoxines, pigment jaune.

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Introduction

Aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2) produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* are a global food safety concern (Dorner 2002; Leitao et al. 1988; Scott 1987; Yiannikouris and Jouany 2002). These fungi can be isolated from soil, air-borne dust, plants, and insects. Corn, peanuts, tree nuts, and cottonseed are often contaminated with aflatoxins, making them unfit for human and animal consumption (Payne 1992, 1998; Widstrom 1996; Yiannikouris and Jouany 2002). Aflatoxins are carcinogens that cause disease in livestock (CAST 2003; Georggiett et al. 2000; Yiannikouris and Jouany 2002) and possibly humans (Hussein and Brasel 2001; Peraica et al. 1999).

There are many highly specific and sensitive methods for determining aflatoxin concentration in commodities or in culture, such as high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), thin-layer chromatography (TLC), and fluorescence polarization assay (Maragos and Thompson 1999; Nasir and Jolley 2002; Seitz 1975; Sobolev and Dorner 2002; Stroka and Anklam 2000; Trucksess et al. 1994; Whitaker et al. 1996). Usually these methods are expensive and time-consuming. Chromatographic methods require extraction procedures to remove interfering substances, by using a mixture of water and a polar organic solvent. Commercially available ELISA kits provide a relatively easy assay for quantification of total aflatoxin concentration but do not identify individual aflatoxins present in the sample.

Many areas of the world with a serious aflatoxin problem have difficulty screening potentially toxigenic *A. flavus* cultures because of the expense and expertise required to maintain an analytical laboratory. There has been much interest in developing and using cultural methods for detecting aflatoxins in fungal cultures. Several applied research approaches in the ecology of *A. flavus* and the potential for biocontrol in the agroecosystem are dependent on screening numerous isolates for the ability to produce aflatoxin. The time and expense associated with traditional analytic methods makes this research prohibitively expensive.

Aflatoxin production by *Aspergillus* isolates can be visualized under long-wave UV light (365 nm) when grown on media suitable for aflatoxin production, such as potato dextrose agar and coconut agar (Gupta and Gopal 2002; Hara et al. 1974; Lemke et al. 1989). The fluorescence emission of AFB_1 and AFG_1 is substantially improved when treated with enhancer agents, such as cyclodextrins (CDs). Fente et al. (2001) showed that adding β-CD to a suitable agar medium enhanced detection of aflatoxin production by *A. flavus* and *A. parasiticus* in the blue fluorescent zone under 365 nm. A second cultural method involves yellow pigment production by *A. flavus* colonies that correlates with aflatoxin production (Lin and Dianese 1976). Gupta and Gopal (2002) found that aflatoxin-producing *A. flavus* cultures isolated from insect pests on coconut produced a bright yellow pigmentation. Finally, ammonia vapor can detect aflatoxin production by changing the color of toxigenic colonies from yellow to pink upon exposure (Saito and Machida 1999).

Various analytical methods have been compared for the detection and determination of aflatoxins (Nilufer and Boyacioglu 2002; Seitz 1975; Trucksess et al. 1990, 1994; Whitaker et al. 1996). However, cultural methods and analytical methods have not been compared in detail. The major objective of this study was to compare three cultural methods (use of fluorescent-enhanced cyclodextrin derivatives (FL), production of yellow pigmentation (YP) in the medium, and response to ammonia vapor (AV) exposure) with two analytical methods (TLC and a widely used, commercially available ELISA method). The cultural and analytical methods were compared in the course of screening a large number of fungal isolates from the Mississippi Delta region of the United States for aflatoxin production. TLC and ELISA were selected as analytical methods because they both have relatively high throughput, and they complement each other in that TLC is specific for aflatoxin type and ELISA is quantitative. Extensive studies in this laboratory on aflatoxins in corn (Abbas et al. 2002*b*) and in many other laboratories have validated ELISA, in comparison with very accurate, but expensive, low throughput research-oriented techniques, including HPLC and LC/MS.

Materials and methods

Aspergillus spp. cultures

The 517 *Aspergillus* isolates used in this study were obtained from soil, corn, rice, and peanuts collected from various fields in Mississippi and Arkansas in 2000–2001 (Abbas et al. 2000, 2001, 2002*a*). Briefly, surface soils (0–5 cm) were collected from 12 commercial production sites and four experimental sites under various crop management practices in Washington and Sunflower counties at Mississippi in the Mississippi Delta region of Mississippi. Four to nine replicate samples were collected from each site and sampling points were georeferenced to enable precise resampling. Soils were sieved (2-mm screen mesh) and stored at 5 °C until processed. Soil samples were diluted in a solution of 2 g of agar/L of water and plated on modified dichloronitroaniline rose bengal (MDRB) agar, according to Horn and Dorner (1998). Corn samples (10 ears per site) were collected from the same sites, as described above, in 2000–2001 according to Abbas et al. (1988). Unpolished rice grain samples (n = 86) were obtained from Rick D. Cartwright, Cotton Branch Experimental Station, University of Arkansas, Little Rock, Ark., in 2000, and 16 additional unpolished rice samples were collected from four rice fields in the Mississippi Delta in 2001. Unshelled peanut samples (n = 44) were obtained from Hector Portillo, BASF, Greenville, Miss., in 2000, and three peanut samples were

Table 1. Characteristics of *Aspergillus* spp. isolates included in this study, and agreement between aflatoxin production detected by thin-layer chromatography (TLC) with fluorescent detection and by enzyme-linked immunosorbent assay (ELISA) measurements on extracts from *Aspergillus* spp. isolates grown on potato dextrose agar.

Characteristics of *Aspergillus* isolates					% agreement of aflatoxin levels (ng/g) between TLC and ELISA[a]					
		Aflatoxin levels (ng/g)[b]			<20		20 – 10 000		>10 000	
Source	Number	<20	20 – 10 000	>10 000	Agreement	False-positive	Agreement	False-negative	Agreement	False-negatives
Corn	183	77	64	42	96	4	95	5	100	0
Peanut	67	41	4	22	92	8	100	0	100	0
Rice	43	6	15	22	83	17	94	6	100	0
Soil	224	67	58	99	87	13	91	9	100	0
Total	517	191	141	185	91	9	94	6	100	0

[a]Measurements were made in methanol–water (70:30, *v*/*v*) extracts of fungal mycelium scraped from agar surfaces.
[b]Average of four independent experiments.

obtained from J. Ethridge, Valent USA Corp., Mid-South Agricultural Research Center, Greenville, Miss., in 2001. Depending on the sample size, 50 or 100 kernels or seeds from each crop were surface sterilized by soaking in 2.5% sodium hypochlorite for 1 min, rinsed three times in sterile tap water, and transferred to MDRB agar. When possible, three to five discrete colonies were selected from each sample from MDRB plates and cultured on potato dextrose rose bengal media. Cultures were maintained on potato dextrose agar (PDA, Difco Laboratories, Detroit, Mich.) slants and silica gel (Windels et al. 1988). Most of the isolates (99%) were *A. flavus*, and the remainder comprised *A. nomius* and *A. parasiticus*. Identification of *Aspergillus* species was based on the morphological criteria of Klich (2002). Ten aflatoxin-producing *A. flavus* strains from the ARS Culture Collection, Peoria, Ill., were used as reference strains for determining the limit of detection for aflatoxins, using cultural methods.

Aflatoxin extraction and determination

Each isolate was grown in duplicate on PDA and PDA enriched with 0.3% β-CD (CD-PDA) (Cavasol®W7M, Wacker-Chemie GMBH, Burghausen, Germany) in 9-cm Petri dishes. Cultures were incubated for 5 days at 28–30 °C with a 12 h dark : 12 h light photoperiod. PDA and CD-PDA plates were scraped (separately) using a rubber policeman to collect fungal biomass. Policemen were washed with bleach, absolute ethanol, and three sterile water rinses between plates. Fungal biomass (mycelia, conidia heads, conidia) was placed in glass scintillation vials (20 mL) and fresh weights were recorded (typically 0.5–1 g). Methanol–water (70:30, *v*/*v*) was added (10:1, *v*/*m*) to vials, and the vials were shaken for 30 min at high speed with a reciprocal shaker. A 1-mL aliquot of extract was removed and centrifuged (12 000*g*, 10 min) with a MicroSpin 12S centrifuge (Sorvall Instruments, Kendro, Ashville, N.C., USA). The supernatant was assayed for the presence of aflatoxins, using ELISA kits ("Vertox", Neogen Corp., Lansing, Mich.) and TLC (Abbas et al. 2002*b*). All experiments were carried out at least two times.

Cultural methods for aflatoxin detection

YP formation in mycelium and medium (PDA) was recorded prior to scraping the plates for analysis, as described above (Davis et al. 1987; Lin and Dianese 1976; Gupta and Gopal 2002). In addition, each isolate was plated on PDA and CD-PDA (Fente et al. 2001) and incubated for 5–7 days at 28–30 °C. Cultures were examined for fluorescence after exposure to UV light (365 nm). Five-day-old PDA cultures were exposed to 27% ammonium hydroxide (Sigma, St. Louis, Mo.) for 30 min., and color changes in the mycelium and medium after exposure to ammonia vapor were recorded (Saito and Machida 1999).

Toxin confirmation

The presence of AFB_1, AFB_2, AFG_1, and AFG_2 was confirmed by TLC, using a modification of the techniques of Horn et al. (1996). Aliquots of the methanol extract (20 and 40 μL) were spotted in duplicate on gypsum–silica gel plates (SIL G-25 HR, Alltech Associates, Deerfield, Ill.) and developed using chloroform–methanol (93:7, *v*/*v*). Chromatographs were visualized under UV light (365 nm); a mixture of aflatoxin standards (AFB_1, AFB_2, AFG_1, and AFG_2; Sigma) was included in each run.

Statistical analysis

Results obtained by ELISA were categorized as "none" for isolates producing less than 20 ng/g of aflatoxin in culture, "low" for isolates producing between 20 and 10 000 ng/g in culture, and "high" for isolates producing 10 000 ng/g or more in culture. Results by the cultural methods and TLC were scored as positive or negative, with isolates that were initially ambiguous eventually scored as positive. Differences between sources or between methods were evaluated using the maximum likelihood statistic derived from a chi-square test.

Results and discussion

In the ELISA analysis, 62% of the 517 *Aspergillus* spp. isolates produced greater than 20 ng of methanol-extractable aflatoxins per gram of fungal structures (Table 1). Although there were minor discrepancies between TLC and ELISA, there was a 97% agreement with these methods (Table 1). The limit of detection in the ELISA assay (recommended by the supplier) was 5 ng/g of total aflatoxins, and the lowest concentration of AFB_1 discernable

Table 2. Agreement among three cultural methods and enzyme-linked immunosorbent assay (ELISA) measurements from *Aspergillus*

	% agreement of aflatoxin levels (ng/g) among culture-based aflatoxigenicity assessments and ELISA[a]							
	Ammonium vapor color change						Fluorescence on β-cyclodextrin agar	
	<20		20 – 10 000		>10 000		<20	
Source	Agreement	False-positive	Agreement	False-negative	Agreement	False-negative	Agreement	False-positive
Corn	100	0	78	22	64	36	76	24
Peanut	100	0	80	20	75	25	84	16
Rice	100	0	71	29	75	25	80	20
Soil	100	0	56	44	73	27	81	19
Total	100	0	69	31	71	29	80	20

Note: *, significant deviation by maximum likelihood χ^2 test from the overall distribution at 0.05 level. **, significant deviation by maximum likelihood χ^2 test from the overall distribution at 0.001 level.

[a]Aflatoxins were measured by ELISA in methanol–water (70:30, *v*/*v*) extracts of fungal mycelium scraped from agar surfaces.

Table 3. Limit of detection of aflatoxin by various cultural methods using aflatoxigenic *Aspergillus flavus* strains.

			Response to cultural methods					
	ELISA		Fluorescence (UV light)		Yellow pigment		Ammonium hydroxide vapor color change	
A. flavus strain	PDA (ng/g)	CD-PDA (ng/g)	PDA	CD-PDA	PDA	CD-PDA	PDA	CD-PDA
NRRL 29495	0	0	–	–	–	–	–	–
NRRL 29504	(2.7)[a]	(2.1)[a]	–	±	±	±	–	–
NRRL 20024	7.1	5.1	–	+	±	+	±	±
NRRL 29512	10	9.1	–	+	±	+	+	+
NRRL 29513	21	17.5	–	+	+	+	+	+
NRRL 29463	42.1	38.4	±	+	±	±	±	–
NRRL 29501	71.7	61.2	+	+	+	+	+	+
NRRL 29531	90.7	91.6	+	+	+	+	+	+
NRRL 29480	180	161.0	+	+	+	+	+	+
NRRL 29489	197	201.0	+	+	+	+	+	+

Note: –, no reaction; ±, partial reaction; and +, full positive reaction. Cultures were grown on agar media (solid media). PDA, potato dextrose agar; CD-PDA, 0.3% β-cyclodextrin in potato dextrose agar.

[a]Numbers in parentheses are extrapolated concentrations, which are below detection limits recommended by the ELISA kit manufacturer.

on TLC was about 2.5 ng/g. The Food and Drug Administration (FDA) guideline regulatory level is 20 ng/g of methanol-extractable aflatoxins in most foods for human consumption. This FDA regulatory level was selected as the base detection level for the cultural assays. The advantage of the TLC method was that it enabled qualitative identification of the four major aflatoxins. With the very low detection limits of TLC, some of the false-positives detected by TLC could be isolates that produced less than 5 ng/g of aflatoxin and were thus identified as "atoxigenic" by ELISA.

There were significant differences in the reliability of the three cultural methods in differentiating between toxigenic and nontoxigenic isolates (Table 2). The suitability of the different methods is dependent on the particular application. For example, the color response to ammonia vapor was in 100% agreement with ELISA in the identification of the nontoxigenic isolates (i.e., a false-positive rate of 0%). However, it was the method with the lowest agreement with ELISA for identifying toxigenc isolates, for which it had a false-negative rate of about 30% (Table 2). In contrast, the fluorescent response on CD-PDA had the highest agreement rate with ELISA for identifying toxigenic isolates, with agreement rates of 87% and 98% for isolates producing low and high levels of aflatoxin, respectively (i.e., a false-positive rate of 20% and a false-negative rate of 13% for low aflatoxin producers and 2% for high aflatoxin producers) (Table 2). This false-positive rate may actually indicate the sensitivity of the fluorescence response on CD-PDA to detect isolates producing low levels of aflatoxin.

The observation of yellow pigment on PDA had a 94% agreement rate with ELISA in identifying nontoxigenic strains, but it was not in agreement in 26% and 29% of the strains producing low and high aflatoxin levels, respectively (Table 2). The best agreement with ELISA might be obtained with the ammonia vapor color change and CD-PDA fluorescence methods used together, yielding an overall success rate comparable to that of TLC, but without the need for chemical extraction and the time and expense of TLC.

The agreement between each method and ELISA was considered separately for *A. flavus* strains isolated from different sources (Table 2). In the cases of fluorescence on β-CD and yellow pigment production, there were differences larger

spp. isolates grown on potato dextrose agar.

				Yellow pigment production					
20 – 10 000		>10 000		<20		20 – 10 000		>10 000	
Agreement	False-negative	Agreement	False-negative	Agreement	False-positive	Agreement	False-negative	Agreement	False-negative
97*	3	98	2	92	8	84*	16	71	29
100	0	100	0	97	3	80	20	70	30
47**	53	100	0	100	0	82	18	70	30
85	15	97	3	95	5	58*	42	70	30
87	13	98	2	94	6	74	26	71	29

than would be expected by chance. For example, fluorescence on β-CD was an unexpectedly poor indicator for the strains producing low levels of aflatoxin from rice but was a generally good indicator for strains from other sources.

In the development of the yellow color and fluorescent cultural assays, most researchers (Fente et al. 2001; Gupta and Gopal 2002; Lemke et al. 1989) used only a limited number of isolates (3–20). However, Saito and Machida (1999) used a larger number of isolates (80 *A. flavus* and 37 other species) with the AV technique and noted a low frequency of false-positives and false-negatives (4% and 10%, respectively). Certain *Aspergillus* species that belong to the section *Flavi*, e.g., *A. caelatus* (= *A. tamarii* type B), produce a yellow pigment(s) but do not produce aflatoxins (Horn et al. 1996).

The present study confirms observations (Fente et al. 2001; Wilson et al. 2002) that qualitative fluorescence-based screening methods can be improved through the use of CD fluorescence enhancers, not just through quantitative methods, such as liquid chromatography. Successful application of fluorescence enhancers in quantitative analysis has so far been limited to β-CDs, which are thought to act by complexing fluorescent mycotoxins in a hydrophobic hole in the center of the saccharide ring structure (Vazquez et al. 1992). CD fluorescence enhancers have been extensively used in quantitative liquid chromatography methods for aflatoxins (Vazquez et al. 1991; Hongyo et al. 1992; Franco et al. 1998; Chiavaro et al. 2001), as well as for two other mycotoxins, ochratoxins and zearalenones (Seidel et al. 1993).

Ten reference strains of *A. flavus* from the National Peanut Research Laboratory, Dawson, Ga., which produce aflatoxins at the low end of the normal range (Horn and Dorner 1998; Abbas et al. 2002*b*), were used in duplicate to estimate the limits of detection of the three cultural techniques on PDA and on CD-PDA (Table 3). On CD-PDA, all *A. flavus* strains capable of producing over 5 ng/g of aflatoxin in fungal structures were fluorescent. In contrast, all cultures capable of producing greater than 61 ng/g of aflatoxin in fungal structures were fluorescent on PDA without β-CD. It should be noted that *A. flavus* NRRL 29463, which produced >31 ng/g of aflatoxin in fungal structures exhibited fluorescence on CD-PDA but gave variable results in both the ammonia and yellow pigment cultural assays.

Conclusions

This study demonstrates that inexpensive methods for detecting aflatoxin production in cultures may be suitable when resources are limited. An overall success rate comparable to that of TLC, but with less time and expense, could be obtained by combining ammonia vapor color change and CD-PDA fluorescence. Ideally, all three cultural methods can be used on the same media, PDA + β-CD. Based upon assays with reference cultures, the fluorescence assay appears more sensitive in identifying the cultures producing low levels of aflatoxin. The cultural assays would be ideal for ecological and genetic studies where a large population of *Aspergillus* isolates is characterized for aflatoxin production (Abbas et al. 2000)

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