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Full Length Research Paper

Evaluation of the detection techniques of toxigenic *Aspergillus* isolates

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Aflatoxins are difuranocoumarin derivatives produced by some *Aspergillus* species through a polyketide pathway. Mycotoxins are considered hazardous and there is a need for accurate detection of each toxin. Several screening methods for direct visual determination of aflatoxins and ochratoxin A (OTA) production have been reported. These methods rely on using different types of coconut culture media, methylated β-cyclodextrin and ammonium hydroxide vapour tests. However, our results showed that use of these techniques were not sufficiently sensitive for all *Aspergillus* species and suggested a simple thin layer chromatography (TLC) as a sensitive and reliable technique for detection of aflatoxins and OTA produced by *Aspergillus* and *Eurotium* species.

Key words: *Aspergillus*, *Eurotium*, Mycotoxins detection, thin layer chromatography.

INTRODUCTION

Aflatoxins and ochratoxin A (OTA) are the most important naturally occurring mycotoxins in agricultural products. Aflatoxins are produced by several species of *Aspergillus* (*Aspergillus bombycis, Aspergillus flavus, Aspergillus nomius, Aspergillus ochraceoroseus, Aspergillus parasiticus* and *Aspergillus psudotamarii*), *Emericella astellata* and *Peteromyces alliaceus*, classified within the section Flavi of subgenus Circumdati (Samson et al., 2006, Varga and Samson, 2008). Aflatoxins are not produced by other fungi (Frisvad et al., 2007). Chemically, aflatoxins are difuranocoumarin derivatives produced via a polyketide pathway (Klich, 2007).

OTA was discovered as a metabolite of *Aspergillus ochraceus* in 1965. It is a nephrocarcinogenic and teratogenic mycotoxin that has been detected in several food products (Joosten et al., 2001; Nguyen et al., 2007; Kumar et al., 2008). OTA is produced by two genera of fungi: *Aspergillus* (20 species belonging to sections Circumdati, Flavi and Nigri of subgenus Circumdati) and *Penicillium* (Bayman et al., 2002; Kumar et al., 2007; Varga and Samson, 2008). The accumulation of small amounts from a variety of foods would eventually reach a toxic threshold level in the body (Richard, 2004).

Not all strains of *Aspergillus* are able to produce mycotoxins, therefore, there is a need for screening for their toxin production abilities. Davis et al. (1987) described a method using coconut agar (CA) medium for detection of aflatoxin. In this method, aflatoxigenic strains produced blue fluorescence on the reverse side of the colony under UV light. This method was also described by Varga and Samson (2008) for detecting OTA producing *Aspergillus* strains. Strong green-blue fluorescence was observed in the reverse plates under UV light.

Rapid techniques for detection of aflatoxigenic and non-aflatoxigenic *Aspergillus* using ammonia vapour (Saito and Machida 1999; Kumar et al. 2007) and methylated β-cyclodextrine (Fente et al., 2001) as indicators on *Aspergillus* colonies grown on yeast extract sucrose (YES) agar have been described. In these methods, aflatoxigenic strains produced a pink color against ammonium vapour and green-blue fluorescence under

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Abbreviations: OTA, Ochratoxin A; **CA,** coconut agar; **YES,** yeast extract sucrose; **TLC,** thin layer chromatography; **HPLC,** high performance liquid chromatography; **HPTLC,** high performance thin layer chromatography; **CYA,** Czapek yeast agar; **MEA,** malt extract agar; **CAM,** coconut agar medium; **CMA,** coconut milk agar.

Table 1. *Aspergillus* species isolated from rice collected from retailers in Malaysia.

No	Isolate	Species	Section
1	A2	*Aspergillus flavus*	Flavi
2	A4	*A. fumigatus*	Fumigati
3	A6	*Eurotium amstelodami*	Aspergillus
4	A7	*A. niger*	Nigri
5	A8	*A. flavus*	Flavi
6	A9	*A. tamari*	Flavi
7	A10	*E. tonophilum*	Aspergillus
8	A11	*E. herbariorum*	Aspergillus
9	A12	*E.amstelodami*	Aspergillus
10	A13	*E.cristatum*	Aspergillus
11	A14	*E. rubrum*	Aspergillus
12	A15	*E.amstelodami*	Aspergillus
13	A16	*A. tamari*	Flavi
14	A17	*E. tonophilum*	Aspergillus
15	A18	*A. ficum*	Nigri
16	A19	*E, tonophilum*	Aspergillus
17	A20	*A.niger*	Nigri
18	A21	*A. tubingensis*	Nigri
19	A22	*A. flavus*	Flavi

UV light on the reverse side of colonies.

Another simpler technique described by Filtenborg et al. (1983) for detecting aflatoxins and ochratoxin in pure culture is the agar plug sampling thin layer chromatography (TLC) method. TLC, also known as flat bed chromatography or planar chromatography is one of the most widely used separation techniques in mycotoxin analysis. The aflatoxins are well suited for analysis by TLC since most of the compounds fluoresce strongly under long-wave UV light. Besides TLC, many analytical and immunological methods are available for estimation of aflatoxin such as high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC). In the present paper, we compared the efficiency of the different reported techniques for detection of toxigenic *Aspergillus* isolated from rice (*Oryza sativa* L.) grains collected from retailers in Peninsular Malaysia.

MATERIALS AND METHODS

Aspergillus strains

All strains used in our experiments (Table 1) were isolated from rice grains obtained from retailers in four states (Selangor, Perak, Penang and Kedah) in Peninsular Malaysia during the period between October and November, 2008. In identification and morphological studies, fungi were cultured on different media (Czapek yeast agar (CYA), malt extract agar (MEA) and Czapek 20% sucrose). *Aspergillus* spp. were identified based on standard identification keys (Raper et al., 1973; Klich and Pitt, 1988; Samson et al., 2007).

Toxin extraction

Colony margins together with adjacent surrounding zones were scraped into a large test tube (32 × 200 mm) containing 10 ml chloroform: acetone (85:15 v/v). The suspension was incubated at room temperature (25°C) for 15 - 20 min and agitated every 5 min using a vortex stirrer. The extract was filtered through Whatman no.1 filter paper and the filtrate evaporated to dryness under 40°C in an air circulated oven dryer. The residue was resuspended in 500 µl of methanol and aseptically filtered using a 0.2 µm syringe filter. It was kept at 4°C before been used for TLC and HPLC tests.

Detection tests

Coconut based medium test

Two coconut media were prepared, coconut agar medium (CAM) and coconut milk agar (CMA) medium based on Davis et al.(1987) and Dyer and Mc Cammon (1994), respectively, with some modifycations. In CAM preparation, desiccated coconut was obtained locally (protein 2 g, carbohydrate 22.5 g, fat 17.5 g / 50 g based on company report). 100 g desiccated coconut powder were mixed with 600 ml hot water in a separation funnel. 350 ml clear extract were slowly separated from the bottom of the funnel. CMA was prepared with local coconut milk (protein 0.65 g, carbohydrate 1.05 g, fat 7.5 g / 50 ml based on company report). pH of both media was adjusted to 6.8 with 2 N NaOH. Bacto agar (15g/l) was added, the mixture heated to boiling and autoclaved for 20 min at 1.05 kg/cm^2, and poured into sterile plates. The plate centre was inoculated with 5 µl of spore suspension and incubated in the dark at 28°C.The presence or absence of a fluorescence ring in the agar surrounding the colonies under UV light after 7 days incubation was noted and the results were scored as positive or negative.

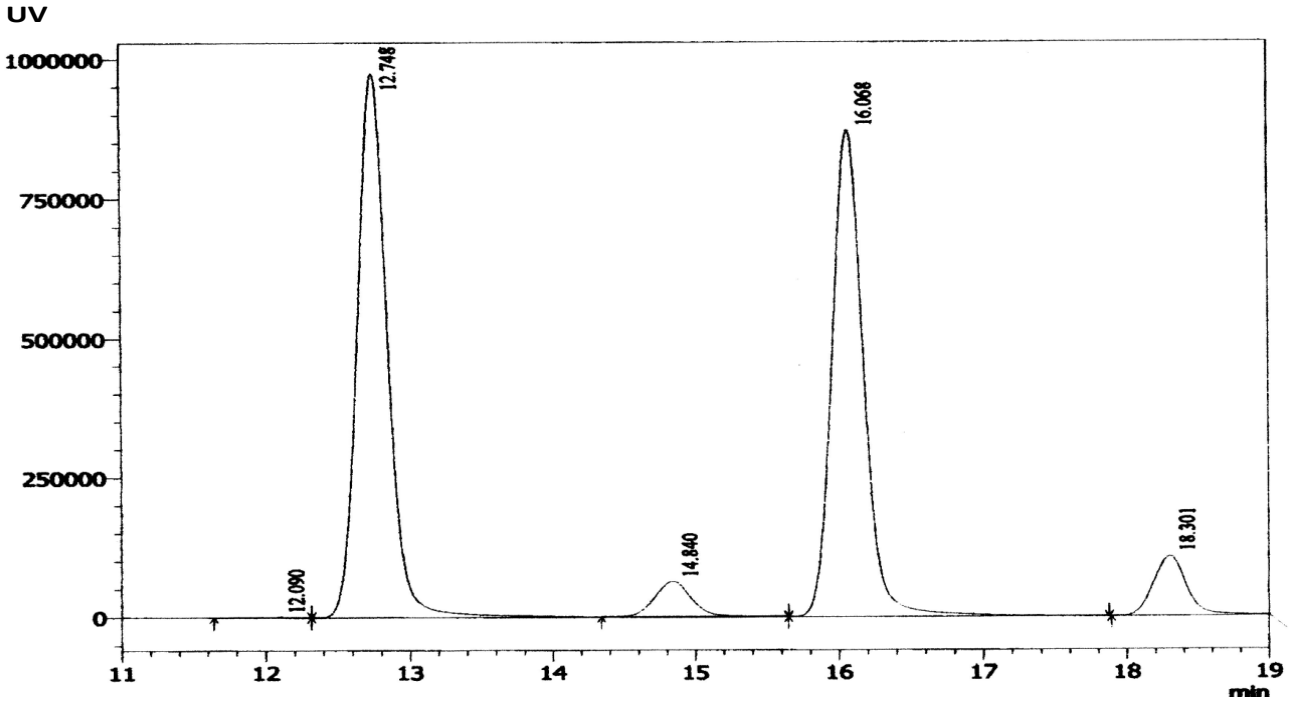

Figure 1. HPLC chromatogram of aflatoxin standards. Retention time (RT), 12.748; aflatoxin G2, RT 14.84; aflatoxin G1, RT 16.068; aflatoxin B2 and RT 18.301, aflatoxin B1.

Ammonia vapour test

Aspergillus isolates were grown on YES agar (2% yeast extract, 15% sucrose and 1.5% agar) as single colonies in the centre of plate and incubated in the dark at 28°C (Saito and Machida, 1999; Kumar et al., 2007). After 3 days, a set of plates were inverted over 2 ml of ammonium hydroxide (Sigma- Aldrich). This was repeated with another set after 7 days. A change in colour of the culture medium was used to determine the toxicity or otherwise of isolates. After ten minutes, the undersides of aflatoxin producing isolates turned into pink to red color. But no color change occurred in the non-toxic isolates.

Methyl-β-cyclodextrin test

Based on Fente et al. (2001), YES agar was used as the culture medium. 3% (w/v) methylated β-cyclodextrin (Mβ-cyd; SIGMA) sterilized using a syringe filter of 0.2 µm (Whatman GD/x 13 mm) were added to autoclaved YES agar in a Petri dish with a final concentration of 0.3%. The plate centre was inoculated with a small drop of spore suspension using a micropipette. Plates were incubated in the dark at 28°C. The presence or absence of a fluorescence ring in the agar surrounding the colonies observed under UV light was scored as positive or negative.

TLC test

TLC was carried out on a silica gel 60 plate 20 × 20 cm (Merck) using toluene : chloroform : acetone (15:75:10 v/v) for aflatoxins and acetonitrile : acetic acid : methanol (90:5:5 v/v) as mobile phases for OTA detection. 5 µl of 1 $\mu g ml^{-1}$ concentration of B_1, B_2, G_1 and G_2 aflatoxins (Supelco, USA) and OTA standards (Sigma-Aldrich, Germany) and 20 µl of test samples were spotted on TLC plates and run for 45 min in a TLC tank at room temperature. Plates were observed under UV light.

HPLC

Confirmation of TLC results was performed with HPLC. 20 µl of extracted toxin were injected into HPLC. The HPLC apparatus was a Shimadzu Liquid Chromatograph LC-20AT fitted with a fluorescence detector (Shimadzu RF-10AXL, Japan), C18 column 250 × 4.6 mm SS Wakosil II (SGE Analytical Science, Australia). The system was run with a programme for simultaneous determination of aflatoxin and OTA as described by Zabe et al. (2004) and Berente et al. (2005) using three solvents (A: 100% methanol, B: 100% acetonitrile and C: water with 0.1% acetic acid). The gradient was 0 - 12 min isocratic 25% A, 15% B, 60% C, 12 - 14 min linear gradient to 10% A, 50% B, 40% C, 14 - 20 min held at 10% A, 50% B, 40% C and immediately returned to 25% A, 15% B, 60% C at minute 24 and followed by a 2 min delay for equilibration. Flow rate was 1 ml/min. Excitation and emission wavelength settings on the fluorescence detector were 365 and 455 nm, respectively from 0 - 14 min for aflatoxins and 330 and 460 nm from 21 - 25 min for ochratoxin A. The HPLC chromatograms for aflatoxin and OTA standards are shown in Figures 1 and 2, respectively.

RESULTS AND DISCUSSION

Coconut based medium test

The constituents of coconut have an effect on fluorescent pigment production in coconut culture. The use of coconut

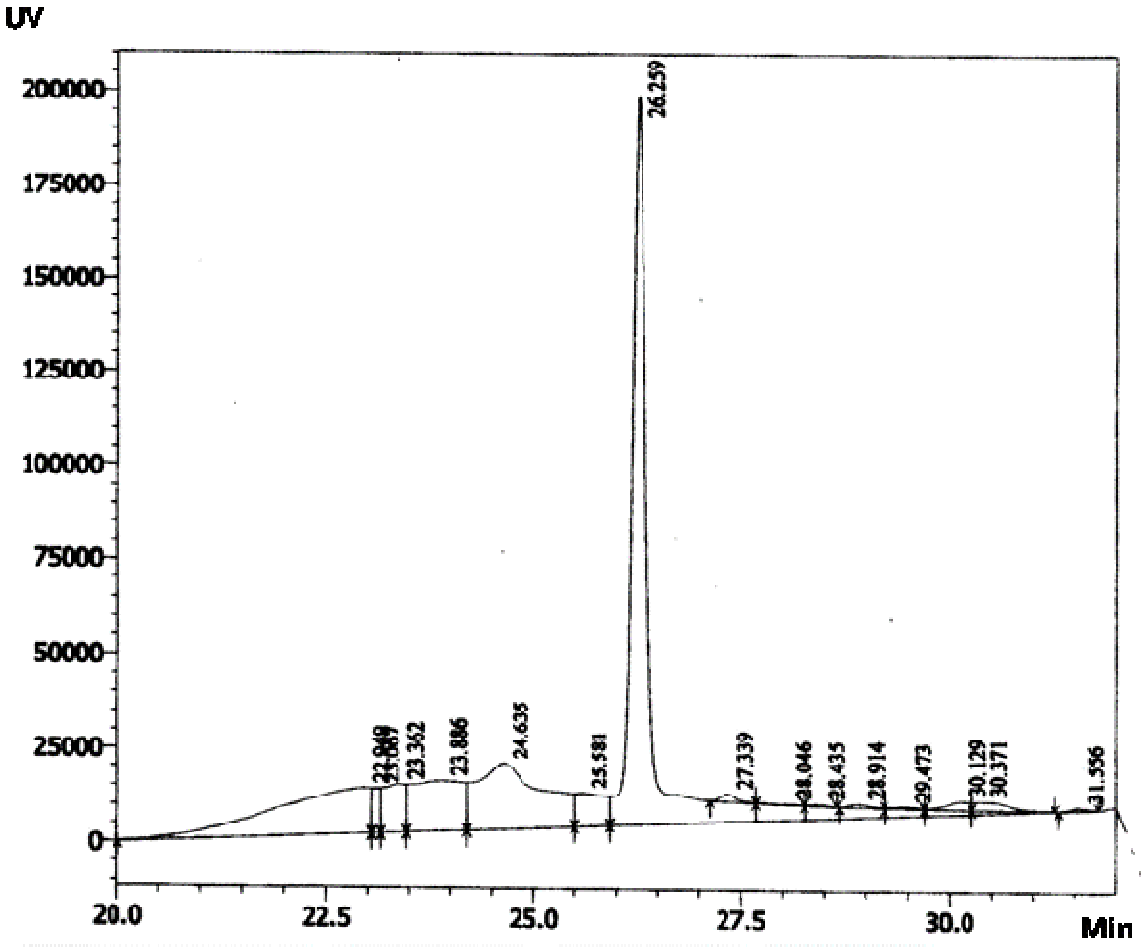

Figure 2. HPLC chromatogram of OTA standard. Retention time (RT): 26.259.

media for detection of aflatoxins is not always reliable because of the high sensitivity of *Aspergillus* to constituents of the medium. Dyer and Mc Cammon (1994) reported coconut cream agar (50% coconut cream) to be more effective than a medium made with dried coconut. Our findings showed that aflatoxins were detected only on CMA by the presence of a fluorescence ring but not on CAM. The aflatoxin producing ability of *A. flavus* isolte A2 was confirmed using TLC and HPLC (Table 2).

Ammonium hydroxide vapour test

Eurotium rubrum isolates A6, *Eurotium tonophilum* isolate A10, *Eurotium amstelodami* isolate A12 and *E. rubrum* isolate A14 produced strong pinkish pigmentation and *E. tonophilum* isolates A17 and A19 produced moderate pinkish pigmentation on the reverse of colonies with the ammonium hydroxide test (Table 2). However, no toxins were extracted or detected with TLC and HPLC. In the case of *A. flavus* A2, an opposite phenomenon was observed. There was toxin production as demonstrated with TLC and HPLC but there was no pinkish pigmentation. It can therefore be concluded that the ammonium hydroxide test was an unreliable test for detection of aflatoxins as false negatives and positives were produced. It was reported by Saito and Machida (1999) that the ammonium hydroxide vapour test are 11% false positive and 6% false negative results for aflatoxigenicity. Kumar et al. (2007) reported a 92% efficacy for the ammonium vapour test having 8% false negatives. Abbas et al. (2004) reported on using a yellow pigmentation combined with the ammonium hydroxide vapour test and reduced false negatives to 7%.

Methyl-β-cyclodextrin test

Results of methyl-β-cyclodextrin test (Table 2) corroborated the findings of the ammonium hydroxide vapour test. No toxins were produced in *E. rubrum* isolates A6, *E. tonophilum* isolate A10, *E. amstelodami* isolate A12 and *E. rubrum* isolate A14 as indicated with TLC and HPLC and false positives were obtained with the

Table 2. Detection of toxin producing ability in *Aspergillus* and *Eurotium* isolates with different techniques.

Species	Isolate	28°C					
		Ammonium test[a]	YES + β cyd[b]	CMA[b]	CA[b]	TLC[c]	HPLC[c]
A. flavus	A2	_	_	+	_	+[c]	+[c]
A. fumigatus	A4	_*	_	_	_	_	_
E. amstelodami	A6	++	+	_	_	_	_
A. niger	A7	_	_	_	_	_	_
A. flavus	A8	_	_	_	_	_	_
A. tamari	A9	_	_	_	_	_	_
E. tonophilum	A10	++	+	_	_	_	_
E. herbariorum	A11	_	_	_	_	_	_
E. amstelodami	A12	++	+	_	_	_	_
E. cristatum	A13	_*	_	_	_	_	_
E. rubrum	A14	++	+	_	_	_	_
E. amstelodami	A15	_	_	_	_	_	_
A. tamari	A16	_	_	_	_	_	_
E. tonophilum	A17	+	_	_	_	_	_
A. ficum	A18	_	_	_	_	_	_
E. tonophilum	A19	+	_	_	_	_	_
A. niger	A20	_*	_	_	_	_	_
A. tubingensis	A21	_	_	_	_	_	_
A. flavus	A22	_	_	_	_	_	_

[a]-: Negative, colourless; -*: negative, brownish; +: positive, moderate pinkish; ++: positive, strong pinkish. [b]-: negative, no fluoresce; +: positive, fluoresce. [c]-: negative; +: positive (aflatoxin B1 and B2); YES: yeast extract sucrose; CMA: coconut milk agar; CA: coconut agar.

presence of blue fluorescence in the media under UV. Fente et al. (2001) have reported a fluorescence ring with this test. Our findings showed that the methyl-β-cyclodextrin test did not have enough sensitivity for detection of aflatoxins.

TLC and HPLC test

Aflatoxin production capability of all *Aspergillus* spp. used in this experiment was analysed with two chromatographic techniques (HPLC and TLC) and the results were in agreement with each other (Table 2). The *Rf* for aflatoxins standards B1, B2, G1 and G2 were 0.55, 0.50, 0.43 and 0.40, respectively and 0.87 for OTA. Only *A. flavus* isolate A2 was found to produce toxins with TLC and HPLC. None of the other strains and species tested gave any positive results. As discussed earlier, other tests except for CMA gave false positive and negative results.

Despite several reports on aflatoxin production by *Aspergillus tamarii* (Klich et al., 2000), this isolate was not found to produce aflatoxins in this study, concurring with that reported by Frisvad et al. (2007). Although there were claims of aflatoxin production by *Eurotium* spp. (Senyuva et al. 2008), all of *Eurotium* species tested were unable to produce aflatoxins or OTA within the incubation period. This finding concurred with those of Samson et al. (2006) and Frisvad et al. (2007).

Although many techniques have been increasingly applied in the determination of certain varieties of mycotoxins, the use of cultural media for detection did not appear advisable. HPLC and TLC involving testing for actual extracts of the toxins were confirmed to be the only reliable methods for toxin detection, although other tests could be used as preliminary indicators.

REFERENCES

Abbas HK, Shier WT, Horn BW, Weaver MA (2004). Cultural methods for aflatoxin detection. Toxin Rev. 23: 295-315.

Bayman P, Baker JL, Doster MA, Michailides TJ, Mahoney NE (2002). Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. Appl. Environ. Microbiol. 68: p. 2326.

Berente B, Móricz A, H-Otta K, Záray G, Lékó L, Rácz L (2005). Determination of ochratoxin A in hungarian wines. Microchem. J. 79: 103-107.

Davis ND, Iyer SK, Diener UL (1987). Improved method of screening for aflatoxin with a coconut agar medium. Appl. Environ. Microbiol. 53: p. 1593.

Dyer SK, Mc Cammon S (1994). Detection of toxigenic isolates of *aspergillus flavus* and related species on coconut cream agar. J. Appl. Bacteriol. 76: 75-78.

Fente CA, Ordaz JJ, Vazquez BI, Franco CM, Cepeda A (2001). New additive for culture media for rapid identification of aflatoxin-producing A*spergillus* spp. Appl. Environ. Microbiol. 67: 4858-4862.

Filtenborg O, Frisvad JC, Svendsen JA (1983). Simple screening method for molds producing intracellular mycotoxins in pure cultures. Appl. Environ. Microbiol. 45(2): 581.

Frisvad JC, Thrane U, Samson RA (2007). Mycotoxin producers. In Dijksterhuis J and Samson RA (Ed.). Food mycology, A multifaceted approach to fungi and food. Boca Raton, FL: CRC Press. pp. 136-

158.
Joosten H, Goetz J, Pittet A, Schellenberg M, Bucheli P (2001). Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. Int. J. Food Microbiol. 65: 39-44.
Klich MA (2007). *Aspergillus flavus*: the major producer of aflatoxin. Mol. Plant Pathol. 8: 713-722.
Klich MA, Mullaney EJ, Daly CB, Cary JW (2000). Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by A*spergillus tamarii* and *A. ochraceoroseus*. Appl. Microbiol. Biotechnol. 53: 605-609.
Klich MA, Pitt JI (1988). A laboratory guide to common *aspergillus* species and their *teleomorphs*. North Ryde, Australia: CSIRO, Division of Food Processing.
Kumar S, Shekhar M, Ali KA, Sharma P (2007). A rapid technique for detection of toxigenic and non-toxigenic strain of *Aspergillus flavus* from maize grain. Ind. Phytopathol. 1: 31-34.
Kumar V, Basu MS, Rajendran TP (2008). Mycotoxin research and mycoflora in some commercially important agricultural commodities. Crop Prot. 27: 891-905.
Nguyen MT, Tozlovanu M, Tran TL, Leszkowicz AP (2007). Occurrence of aflatoxin B1, citrinin and ochratoxin A in rice in five provinces of the central region of Vietnam. Food Chem. 105: 42-47.
Raper KB, Fennell DI, Austwick PK (1973). The genus *Aspergillus,* Robert E. Krieger Publishing Company, New York.
Richard JL (2004). Mycotoxins and mycotoxicoses: A 2004 update. *Mycotoxins* and *phycotoxins,* Maryland, USA. pp. 21-30.
Saito M, Machida S (1999). A rapid identification method for aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus* by ammonia vapor. Mycoscience, 40(2): 205-208.
Samson RA, Hong SB, Frisvad JC (2006). Old and new concepts of species differentiation in A*spergillus*. Med. Mycol. 44: 133-148.
Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J (2007). Diagnostic tools to identify black aspergilli. Stud. Mycol. 59: p. 129.
Senyuva HZ, Gilbert J, Samson RA, Özcan S, Öztürkoğlu Ş, Önal D (2008). Occurrence of fungi and their mycotoxins in individual turkish dried figs. World Mycotox. J. 1: 79-86.
Varga J, Samson RA (2008). *Aspergillus* in the Genomic Era. Wageningen Academic Publishers. The Netherlands.
Zabe N, Wadleigh E, Cohen BA (2004). Simultaneous determination of aflatoxin, ochratoxin a and zearaleone by a new immunoaffinity column. *Mycotoxins* and *phycotoxins,* Maryland, USA. pp. 73-81.