

Review

Detection of aflatoxigenic *Aspergillus* strains by cultural and molecular methods: A critical review

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Aflatoxin contamination of food and feed commodities, caused by *Aspergillus* section *Flavi* group of fungi, is a serious problem worldwide. Exposure through consumption of contaminated food and feed has deleterious effects on human and animal health. Therefore, aflatoxin contaminated products are a barrier to international trade of agricultural commodities. Not all fungi from *Aspergillus* section *Flavi* produce aflatoxins. Hence it is important to differentiate *Aspergillus* spp. into toxigenic and atoxigenic species to better understand their population structure in a specific environment. A range of methods are available today, including cultural, analytical and molecular methods, to identify the toxin producing ability of isolates from section *Flavi*. A comprehensive review of these methods would be of great use for researchers in developing nations where fully equipped aflatoxin detection laboratories are lacking. In this paper we critically reviewed the cultural and molecular methods of detecting aflatoxigenic *Aspergillus* species and their precision.

Key words: Toxigenic *Aspergillus*, atoxigenic *Aspergillus*, cultural methods, PCR based methods.

INTRODUCTION

Aflatoxins are secondary metabolites produced by *Aspergillus* section *Flavi* group of fungi. These aflatoxins are potent carcinogenic, teratogenic, mutagenic, hepatotoxic and immunosuppressive agents that cause significant damage to human and animal health (Moss, 2002; Saleemullah et al., 2006). The *Aspergillus* section *Flavi* comprises two groups of species; first group include *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii* and *A. bombycis*. Of these, the *A. flavus* and *A. parasiticus*

are the predominant aflatoxin producers (Cary and Ehrlich, 2006; Ehrlich et al., 2007). Other *Aspergillus* spp. reported to be aflatoxigenic in this section are *A. pseudocaelatus* (B1, B2 and G1, G2 aflatoxins), *A. pseudonomius* (B1 and not G-type aflatoxins) (Varga et al., 2011) and *A. parvisclerotigenus* (Godet and Munaut, 2010). The second group comprises atoxigenic species such as *A. oryzae*, *A. sojae* and *A. tamarii* (Kumeda and Asao, 2001). The atoxigenic species such as *A. sojae*

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and *A. oryzae* are generally used as starters in fermentation of foods (Chang et al., 2007). Particularly, *A. sojae* and *A. oryzae* are perceived as atoxigenic variants of *A. flavus* and *A. parasiticus* (Klich and Pitt, 1988). Other species of *Aspergillus* not included in section *Flavi* are also reported to be aflatoxigenic (Chang et al., 2007). For example, *A. ochraceoroseus* (from section *Ochraceroesi*); the ascomycete fungi *Emericella astellata* and *E. venezuelensis* (*Aspergillus* section *Nidulantes*) also produce B1 (Cary et al., 2005; Klich et al., 2000; Frisvad et al., 1999, 2005).

The predominant aflatoxigenic species of *Aspergillus*; *A. flavus* and *A. parasiticus* produce aflatoxins, that are a group of 20 closely related secondary metabolites (Liu and Wu, 2010; Snigdha et al., 2013). These fungi are ubiquitous, they are common soil inhabitants, air-borne, and are also found in crops and foods at both pre-and post-harvest stages (Waliyar et al., 1994; Jaime-Garcia and Cotty, 2004; Williams et al., 2004). Among different aflatoxins, the naturally occurring and well-known ones are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2) (Gimeno, 2004; Saleemullah et al., 2006; Strosnider et al., 2006). Of them, AFB1 is categorized as a group 1 carcinogen (Castegnaro and McGregor, 1998), and is the most prevalent aflatoxin. Predominantly, these toxins affect peanuts, corn, cotton seed, tree nuts, pea, sorghum, rice, pistachio, oilseed rape, sunflower seed, figs, spices, meats, dairy products, and fruit juices (apple, guava) (Abdin et al., 2010). Further, to this, in occupational settings, mycotoxin exposure is one of the major health concerns. Monitoring for mycotoxin exposure is therefore mandatory so as to take adequate precautionary steps. Presently, monitoring on Aflatoxin B1 is rare at occupational settings. In this context, occupational settings with potential residents of aflatoxigenic *A. flavus* strains needs enumeration. It is also at this juncture, the application aspects of the current review would be prudent. Knowledge on differentiation of atoxigenic and toxigenic strains would finally contribute to enhancement of precautionary safety systems in various occupational settings.

There are morphological similarities between aflatoxigenic and non-aflatoxigenic *Aspergillus* species. For example, *A. sojae* is morphologically similar to *A. parasiticus* and it is believed that *A. sojae* is a domesticated strain of *A. parasiticus* (Chang et al., 2007). However, few distinguishing characters such as color, texture and conidial diameter separate these two species (Klich, 2002). Precise detection of aflatoxigenic *Aspergillus* species is important for both research and mitigation. In this paper, we have comprehensively reviewed cultural and molecular methods of detection of aflatoxigenic *Aspergillus* spp. and their differentiation using cultural and molecular methods. Current analytical methods used to validate the aflatoxin production and quantification include thin layer chromatography (TLC) (Stroka and Anklam, 2000), high-performance liquid

chromatography (HPLC) (Seitz, 1975; Sobolev and Dorner, 2002; Trucksess et al., 1991), liquid chromatography /mass spectroscopy (LC/MS), enzyme linked immunosorbent assay (ELISA) (Patey et al., 1989) and immunoaffinity with fluorescence (Nasir and Jolley, 2002). Each of these methods has advantages and limitations. For example, cultural methods though inexpensive, but are less sensitive, affecting accuracy (Abbas et al., 2004a). On the other hand, molecular techniques provide rapid diagnosis because of their high sensitivity, specificity, and are currently in use for detection of aflatoxigenic strains of *A. flavus* and *A. parasiticus* (Shapira et al., 1996; Sweeney et al., 2000).

Since, the toxigenic profiles of both *A. flavus* and *A. parasiticus* are mostly different; adopting a single method has not yet been reliable in differentiation. The compiled information may be useful in devising a polyphasic, cost effective and robust approaches using one or more methods that allow accurate differentiation between aflatoxin producing and non-producing strains of *Aspergillus*.

CULTURAL METHODS

Cultural methods for detecting aflatoxins either rely on quantification of purified extracts (Filtborg and Frisvad, 1980; Shotwell et al., 1966), or on qualitative assessments of fluorescence (Bennett and Goldblatt, 1973; de Vogel et al., 1965; Hara et al., 1974; Lin and Dianese, 1976) or UV absorption (Yabe et al., 1987). They include a) blue fluorescence (FL) (particularly in the presence of an enhancer in the medium such as β -cyclodextrin (Fente et al., 2001; Ordaz et al., 2003); b) yellow pigmentation (YP) on the undersides of colonies (Gupta and Gopal, 2002; Lin and Dianese, 1976; Odhiambo et al., 2014); and c) color change of the yellow pigment to plum-red on exposure of the culture to ammonium hydroxide vapor (AV) (Saito and Machida, 1999; Abbas et al., 2004a; Odhiambo et al., 2014).

Different media are in use for growing aflatoxigenic *Aspergillus* spp. They include *Aspergillus flavus* and *parasiticus* agar (AFPA) (Pitt et al., 1983), Czapek's yeast extract agar (CYA), yeast extract sucrose agar medium (YES), coconut agar medium (CAM), aflatoxin producing ability medium (APA) (Jaimez et al., 2003b). However, the toxin production in these media varies with extraneous factors such as pH, temperature and time. Incubation for a period of five days is necessary for toxin production in YES (Gqaleni et al., 1996; Leontopoulos et al., 2003). Aflatoxin production (AFB1) by *A. flavus* and *A. parasiticus* in cheese and rice for toxigenic isolates can be peak at 7, 10, 14, 21 and 28 days. Further, maximum production of AFB1 will be at 14 days (Park and Bullerman, 1983).

Different reports on the potential of media in supporting toxin production are available. Ritter et al. (2011) showed

that YES media is better compared to CYA, whereas Abranson and Clear (1996) proved that CYA agar was slightly better than YES agar. Reddy et al. (2009) reported that all toxigenic isolates of *A. flavus* produced AFB1 on YES media, whereas, 65%, and 53% of the same isolates produced aflatoxin on Czapek's and PDA agar, respectively. However, none of the toxigenic isolates produced AFB1 in AFPA medium. In another study, Fente et al. (2001) showed that YES media was comparatively superior in supporting aflatoxin production over Czapek's, aflatoxin producing ability (APA) media, and different coconut agars. In contrast, Desai and Ghosh (2003) reported high AFB1 production in Czapek's agar over APA and CAM.

A combination of media can be of great advantage in determining the toxigenic potential of fungi. For example, combined use of CYA and YES media is advantageous since both these media allow production of different mycotoxins (Bragulat et al., 2001; Frisvad and Filtenborg, 1983). A new media containing Czapek's yeast extract agar (CYA) and YES + 0.6% of YCSD (commercial YES medium supplemented with 0.3% M β -cyd and 0.6% sodium desoxycholate) is used to detect aflatoxigenic potential of *A. flavus* strains. When grown on this media, previously verified toxigenic strains have developed a fluorescent ring under UV light. Other media that have potential to detect aflatoxigenic *A. flavus* and *A. niger* are PDA + NaCl and APA media that produce fluorescence around the colonies. Further, the diameter of the beige ring and the intensity of its fluorescence under UV increased over time up to four days (Almoammar et al., 2013). Coconut agar medium (CAM) is also generally used for rapid detection of aflatoxin production by *Aspergillus* spp. (Lin and Dianese, 1976). Cultivation on CAM is generally a preliminary tool to detect aflatoxin production (de Vogel et al., 1965; Arseculeratne et al., 1969; Lin and Dianese, 1976; Davis et al., 1987). Liquid formulation of CAM is also used for detecting aflatoxins by a microplate fluorescent reader-based assay (Degola et al., 2011). A visible beige ring can be observed, under UV light, in cultures of aflatoxigenic fungi (Figure 1). Further, a blue fluorescence surrounding aflatoxigenic colonies under UV light is also seen on the reverse of the plates (Almoammar et al., 2013) (Figure 2). Isolates thought to be atoxigenic also produced toxins in CAM under optimal conditions (Hoeltz, 2005). However, due to the frequent false negative results, CAM is an unreliable method for detecting the toxigenic potential of *A. flavus* and *A. parasiticus* (Taniwaki, 1996). Other coconut culture media in use for detecting toxigenic strains include coconut milk agar (CMA), coconut extract agar, coconut cream agar (Fente et al., 2001; Davis et al., 1987; Dyer and McCammon, 1994), fresh coconut extract (FCE) and commercial coconut extract (CCE) as substrates (Lin and Dianese, 1976). Coconut cream agar is relatively more effective than other synthetic coconut media due to desiccated coconut as the ingredient (Dyer

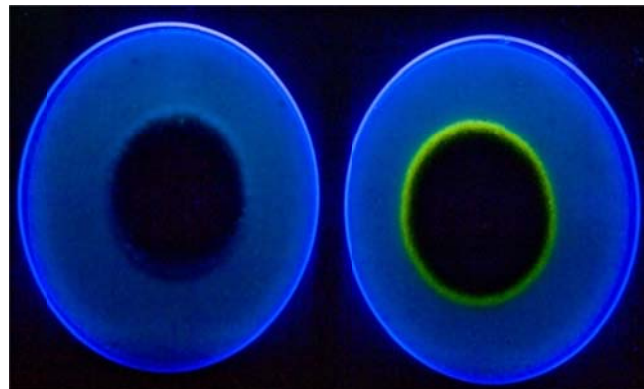


Figure 1. Characteristic beige ring shown by aflatoxigenic *Aspergillus flavus* (right side) on coconut agar medium

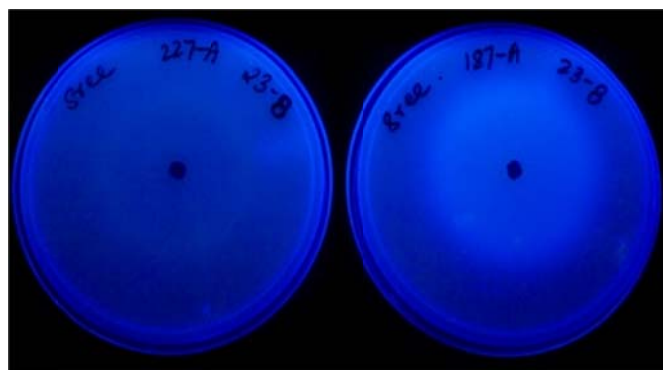


Figure 2. Characteristic blue fluorescence exhibited by aflatoxigenic *Aspergillus flavus* (right side) on reverse side of coconut agar medium.

and McCammon, 1994). Another reliable substrate for rapid detection of aflatoxigenic fungi is palm kernel. Yellow pigmentation of toxigenic isolates in palm kernel media is indicative of the presence of aflatoxins. Compared to desiccated coconut agar, the fluorescence nature, intensity of diffusion of water soluble fluorescent compounds of isolates was unique in palm kernel media (Atanda et al., 2005).

Presence of a beige ring around the colonies on media and its fluorescence under UV light is an indication of aflatoxin-producing ability of strains. Quantitative measure of agar medium fluorescence for estimating the aflatoxins are also available. Simple fluorescence is one such rapid estimation of aflatoxin levels in a solid culture medium (Cotty, 1988). In this procedure, glass test tubes containing 5 ml of agar are inoculated with spores of *Aspergillus* spp. and incubated for 3 days at 30°C. Fluorescence in the agar by UV light is observed. Further, the agar fluorescence is directly correlated with concentration of aflatoxins (Cotty, 1988). Hara et al. (1974) described in detail the fluorescence method of

detecting aflatoxigenic strains of *A. flavus* by utilizing UV induced fluorescence of toxin in a modified Czapek's solution agar containing corn steep liquor, HgCl_2 , and $(\text{NH}_4)_2\text{PO}_4$ instead of NaNO_3 . Further, the toxin presence is confirmed by thin layer chromatography (TLC) of CHCl_3 extracts of the fluorescing agar. In another study, Atanda et al. (2005) reported that aflatoxigenic isolates exhibit a characteristic blue or blue green fluorescence of agar under long wave UV light against a pink background as confirmed by TLC. However, certain non-aflatoxigenic isolates also fluorescence under UV light (Almoammar et al., 2013). For example, strains of *A. flavus* and *A. oryzae* produce several substances other than aflatoxins that give a blue fluorescence under UV light. They include asperopterin A or B (Kaneko and Sanada, 1969), flavacol and deoxy-hydroxy-aspergillilic acid (Yokotsuka et al., 1967).

Several other cultural media used for detection of toxigenic strains through observations on fluorescence or visible color of pigments are potato dextrose agar (PDA) and coconut agar (Davis et al., 1987; Gupta and Gopal, 2002; Lemke et al., 1988; Lin and Dianese, 1976; Saito and Machida, 1999), corn steep liquor (Hara et al., 1974; Wicklow et al., 1981) and glucose-yeast extract medium (GY-agar) (Filtenborg and Frisvad, 1980; Yabe et al., 1987; Cotty, 1988), Sabouraud dextrose and yeast extract agar when fortified with cyclodextrin (β -CD) was also a screening substrate for detecting aflatoxigenic strains (Fente et al., 2001). Complex agar medium containing sucrose, various salts, and an aqueous extract of aflatoxin-free groundnut (de Vogel et al., 1965); synthetic liquid medium (Abye and Mateles, 1964) and silica gel medium (Torrey and Marth, 1976) are also used (Fente et al., 2001). Dichloran 18% glycerol agar, DG18; dichloran rose bengal agar, DRBC; malt extract agar, MA; oxytetracycline glucose yeast extract agar, OGY; tryptic soy with lecithin and polysorbate 80, TSA; Dichloran chloramphenicol peptone agar, DCPA are also used in certain investigations on phosphorescence phenomenon to allow the detection of aflatoxigenic strains in culture media (Rojas-Duran et al., 2007).

Enrichment of culture media with cyclodextrins

Fluorescence enhancers such as cyclodextrins (CDs) are generally used to confirm the production of fluorescence by aflatoxigenic *A. flavus* strains. Fluorescence by AFB1 and AFG1 is greatly enhanced in presence of CDs. These CDs are cyclic oligosaccharides consisting of (α -1, 4)-linked α -D-glucopyranose units. The behaviour of AFB1 in presence of CDs was investigated by several researchers (Blais et al., 1988; Cepeda et al., 1988; Francis et al., 1988; and Vazquez et al., 1991). However, the exact mechanism behind fluorescence enhancement by aflatoxins through β -CD complexation is not clearly understood (Vazquez et al., 1992). Recently, a theoretical mechanism on fluorescence enhancement of AFB1 by β -

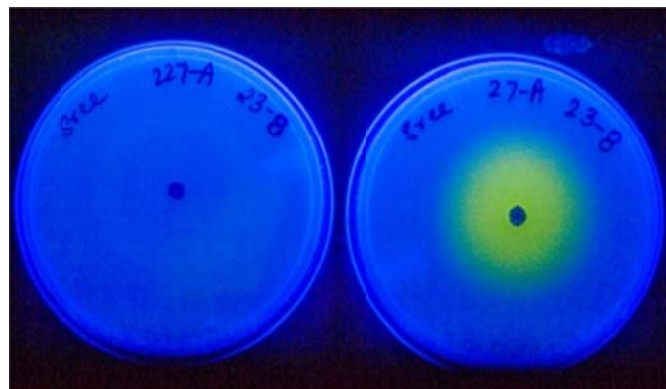


Figure 3. Characteristic golden yellow pigmentation by aflatoxigenic *Aspergillus flavus* (right side) on reverse side of coconut agar medium.

CD was proposed (Fente et al., 2001). The β -CD form is relatively more effective in the formation of aflatoxin inclusion complexes when both α and β -CD are used (Cepeda et al., 1996). However, no significant differences on intensity of color change at different concentrations of β -CD and methylated β -CD derivatives in different media were noticed. Further, the intensity of fluorescence had increased with time (Fente et al., 2001). Conjunctive use of hydroxypropyl- β -cyclodextrin (HBC) (Cavasol®) and bile salts (cholic acid, sodium taurocholate and sodium dehydrocholate) also resulted in better detection of aflatoxigenic strains (Fente et al., 2001; Jaimez et al., 2003a, b; Rojas et al., 2005).

Pigments as indicators

Production of yellow to orange pigments by aflatoxigenic *A. flavus* strains was observed for the first time by Wiseman et al. (1967). Yellow pigment formation in mycelia and media is also a basis for diagnosis of aflatoxigenic isolates (Figure 3) (Abbas et al., 2004a; Shier et al., 2005; Odhiambo et al., 2014). Aflatoxin producing *A. flavus* cultures isolated from insect pests on coconut produced a bright yellow pigmentation (Gupta and Gopal, 2002). The degree of yellow pigmentation is proportional to blue fluorescence in culture media (Lin and Dianese, 1976).

Vapor tests for color change

A new and rapid method for detecting toxigenic strains of *A. flavus* and *A. parasiticus* is through vapor tests. By exposing the aflatoxigenic colonies to ammonium hydroxide vapors using standard procedures (Abbas et al., 2004b), will result in quick color change of the reverse side from brownish/yellowish color to plum-red (Saito and Machida, 1999) (Figure 4). Biochemical basis of vapor

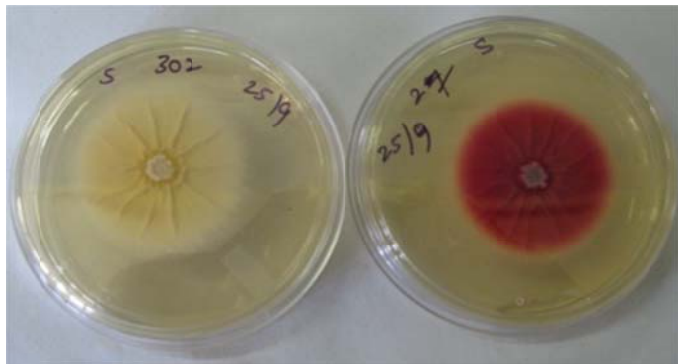


Figure 4. Characteristic plum red color change by aflatoxigenic *Aspergillus flavus* (right side) on reverse side of Yeast extract sucrose (YES) medium when exposed to ammonium hydroxide vapors.

tests was investigated by extracting pigments from lyophilized cultures of toxigenic strains grown on PDA. Further mixing of these pigments with ammonium hydroxide or other bases (sodium hydroxide, potassium hydroxide, sodium carbonate, and sodium bicarbonate) resulted in color change to plum-red (Abbas et al., 2004b). The pigments associated with color change are norsolorinic acid, averantin, averufin, versicolorin C, versicolorin A, versicolorin A hemiacetal, and nidurufin (Aucamp and Holzappel, 1970; Cole, 1981; Heathcote and Dutton, 1969). All these pigments are anthraquinone intermediates in aflatoxin biosynthetic pathway (Bhatnagar et al., 2003). Of these, averufin was produced by a non-aflatoxigenic mutant of *A. parasiticus* (Donkersloot et al., 1972).

MOLECULAR METHODS

Application of molecular techniques in aflatoxin research is multitude. For example, molecular approaches have been used for differentiating the *Aspergillus* section *Flavi* complex, determining the phylogenetic analysis, characterization of isolates, identification of aflatoxigenic isolates from different food and feeds, diversity studies and in understanding the non-aflatoxigenicity of certain *Aspergillus* spp. (Montiel et al., 2003; Almoammar et al., 2013; Chang et al., 2007; Baird et al., 2006; Hatti et al., 2010; Yin et al., 2009). Applications of such studies can be useful in bringing out effective atoxigenic strains rapidly for their further use as potential biocontrol agents (Yin et al., 2009). Differentiation of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* group through molecular approaches is important because conventional approaches are not entirely reliable (Criseo et al., 2001). Moreover, the molecular mechanisms responsible for loss of aflatoxigenicity of *Aspergillus* are not clearly understood (Schmidt-Heydt et al., 2008). Earlier reports indicated that atoxigenic *A. flavus* isolates

were found to be majorly associated with the deletions of a part or the entire aflatoxin gene cluster (Chang et al., 2005). Defects in the aflatoxin gene, *pkSA* in *A. flavus* AF36 isolate of cotton seed is also responsible for its atoxigenicity (Ehrlich and Cotty, 2004). Other reasons for atoxigenicity of *Aspergillus* can be attributed to large deletions in the aflatoxin gene cluster (Prieto et al., 1996). Jiang et al. (2009) reported a large fragment deletion in the aflatoxin gene cluster and further replacement of its location by a heterologous insert. PCR assays have revealed that *Aspergillus* isolates with entire aflatoxin gene cluster could not produce aflatoxins (Yin et al., 2009). Hence, analysis of deletion within aflatoxin gene cluster can be an effective method for rapid identification of true non-aflatoxigenic *Aspergillus* strains (Yin et al., 2009).

Based on molecular characterization studies, it is concluded that both toxigenic and atoxigenic *A. flavus* isolates are genetically similar, but some atoxigenic isolates having deletions within the aflatoxin gene cluster can be identified readily by PCR assays (Yin et al., 2009). Complete differentiation of atoxigenic and toxigenic strains is however not feasible with molecular methods such as random amplified polymorphic DNA (RAPD), Microsatellite-primed PCR (MP-PCR) profiles and DNA sequences of internal transcribed spacer (*ITS*) regions, *pkSA* and *omtA* genes (Yin et al., 2009). Previous research has also indicated that molecular techniques such as RAPD and AFLP (amplified fragment length polymorphism) could not differentiate toxigenic and atoxigenic strains (Tran-Dinh et al., 1999; Montiel et al., 2003; Barros et al., 2007). For example, the atoxigenic *A. sojae* and the toxigenic *A. parasiticus* could not be differentiated through sequencing of the ribosomal DNA *ITS* (internal transcribed spacers) (Montiel et al., 2003). However, this technique could separate the *A. flavus/A. parasiticus* subgroup from the *A. oryzae/A. flavus* subgroup. In another study, Baird et al. (2006) reported that DNA amplification fingerprinting (DAF) was not effective in distinguishing aflatoxigenic and non-aflatoxigenic isolates of *A. flavus*. However, use of mini-hairpin primers using the arbitrary signatures from amplification profiles (ASAP) technique successfully distinguished the majority of aflatoxigenic from non-aflatoxigenic isolates (Baird et al., 2006). AFLP fingerprints however can be used for genetic differentiation of *Aspergillus* section *Flavi* complex fungi. In a study by Montiel et al. (2003), analysis of AFLP data consistently and clearly separated *A. sojae/A. parasiticus* isolates from the *A. oryzae/A. flavus* isolates. Further studies by Montiel et al. (2003) have identified polymorphic sequences that could be developed into markers for aflatoxigenic *A. parasiticus* isolates.

Use of multiplex PCR with three sets of primers specific for three structural genes of the aflatoxin pathway (*nor-1*, *ver-1* and *omt-A*) could differentiate only the aflatoxin producing fungi, *A. flavus* and *A. parasiticus*

from others, but not aflatoxin producing and non-producing strains of the same species (Geisen, 1996). In another study, Rashid et al. (2008) categorized *A. flavus* and *A. parasiticus* isolates of stored wheat into aflatoxigenics and non-aflatoxigenics using multiplex PCR based assay. Their studies on detection of four genes, *AflR* (regulatory gene) and structural genes such as *Nor 1* (norsolorinic acid), *Ver 1* (Versicolorin) and *Omt* (O methylsterigmatocystin) indicated that only one of the tested strains of *A. parasiticus* (AP4) out of 36 strains has shown the presence of these four genes. Further, it was also observed that strains with presence of *aflR* gene also did not produce aflatoxins (Rashid et al., 2008). Criseo et al. (2001) worked with quadruplex PCR using primers for *aflR*, *nor-1*, *ver-1* and *omt-A* genes of the aflatoxin biosynthetic pathway for differentiating aflatoxigenic and non-aflatoxigenic strains. Their results indicated that aflatoxigenic strains gave a quadruplet pattern, indicating the presence of all the genes involved in the aflatoxin biosynthetic pathway. However, for non-aflatoxigenic strains, the results are variable with one, two, three or four banding patterns. Further, a banding pattern in few non-aflatoxigenic strains resulted in non-differentiation between these two strains.

Aflatoxin production by *A. flavus* is more stable in nature than in culture (Bayman and Cotty, 1993). Gene expression between *Aspergillus* spp. can be different despite their close relatedness at the DNA level according to microarray analysis (Wilkinson et al., 2007). Hence, understanding the aflatoxigenicity and non-aflatoxigenicity through molecular means at gene level is an important step for further rapid and precise detection of these species. Developing rapid, precise, cost-effective and less cumbersome methods in this direction will further aid in devising strategies that prevent aflatoxin contamination in agriculturally important crops.

CONCLUSIONS

For precise differentiation of toxigenic and atoxigenic strains of *A. flavus*, a polyphasic approach involving the use of different cultural and molecular methods is apt. False positives with respect to aflatoxin production are also not uncommon in aflatoxigenic strain detection. In this regard, analytical methods such as HPLC, TLC and ELISA that detect toxin production in the substrate can also be confirmative when used in polyphasic approach along with cultural and molecular methods. An elaborative and updated review in this area would be useful for aflatoxin researchers especially in detecting and differentiating atoxigenic and toxigenic *Aspergillus* spp. in crop soils that are posing severe health hazards and trade losses in several major crops.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

- Abbas HK, Shier WT, Horn BW, Weaver MA (2004a). Cultural methods for aflatoxin detection. *Toxin Rev.* 23:295-315.
- Abbas HK, Zablutowicz RM, Weaver MA, Horn, BW, Xie W, Shier WT (2004b). Comparison of cultural and analytical methods for determination of aflatoxin production by Mississippi Delta *Aspergillus* isolates. *Can. J. Microbiol.* 50(3):193-199.
- Abdin MZ, Ahmad MM, Javed S (2010). Advances in molecular detection of *Aspergillus*: an update. *Arch. Microbiol.* 192(6): 409-425.
- Abranson D, Clear RM (1996). A convenient method for assessing mycotoxin production in cultures of *Aspergilli* and *Penicillia*. *J. Food Prot.* 59:142-144.
- Adey J, Mateles RI (1964). Incorporation of labelled compounds into aflatoxins. *Biochim. Biophys. Acta* 86:418-420.
- Almoammar H, Bahkali AH, Abd-El Salam KA (2013). A polyphasic method for the identification of aflatoxigenic *Aspergillus* species isolated from Camel feeds. *Aust. J. Crop Sci.* 7(11): 1707-1713.
- Arseculeratne SN, De Silva LM, Wijesundera S, Bandunatha CHSR (1969). Coconut as a medium for the experimental production of aflatoxin. *Appl. Microbiol.* 18(1):88-94.
- Atanda I, Rati ER, Ozoja M (2005). Palm Kernel: A potential substrate for rapid detection of aflatoxigenic fungi. *Food Sci. Technol. Int.* 11(1):67-74.
- Aucamp PJ, Holzapfel CW (1970). Polyhydroxy anthraquinones from *Aspergillus versicolor*, *Aspergillus nidulans* and *Bipolaris* sp. Their significance in relation to biogenetic theories on aflatoxin B1. *J. S. Afr. Chem. Inst.* 23: 40-56.
- Baird RE, Trigiano RN, Windham G, Williams P, Kelley R, Abbas HK, Moulton JK, Scruggs ML (2006). Comparison of aflatoxigenic and non aflatoxigenic isolates of *Aspergillus flavus* using DNA amplification fingerprinting techniques. *Mycopathologia* 161: 93-99.
- Barros GG, Chiotta ML, Reynoso MM, Torres AM, Chulze SN (2007). Molecular characterization of *Aspergillus* section Flavi isolates and *Aspergillus flavus* S and L morphotype isolates. *Int. J. Food Microbiol.* 108:172-177.
- Bayman P, Cotty P (1993). Genetic diversity in *Aspergillus flavus*: association with aflatoxin production and morphology. *Can. J. Bot.* 71:23-31.
- Bennett JW, Goldblatt LA (1973). The isolation of mutants of *Aspergillus flavus* and *A. parasiticus* with altered aflatoxin producing ability. *Sabouraudia* 11: 235-241.
- Bhatnagar D, Ehrlich KC, Cleveland TE (2003). Molecular genetic analysis and regulation of aflatoxin biosynthesis. *Appl. Microbiol. Biotechnol.* 61: 233-93.
- Blais J, Prognon P, Mahuzier G, Vigny P (1988). Relative affinity of 5-methoxypsoralen and 8-methoxypsoralen towards β -cyclodextrin: a fluorescence, circular dichroism and chromatographic study. *J. Photochem. Photobiol. B* 2(4): 455-465.
- Bragulat MR, Abarca ML, Cabanes FJ (2001). An easy screening method for fungi producing ochratoxin A in pure culture. *Int. J. Food Microbiol.* 71:139-144.
- Cary JW, Ehrlich KC (2006). Aflatoxigenicity in *Aspergillus*: molecular genetics, phylogenetic relationships and evolutionary implications. *Mycopathologia* 162(3):167-177.
- Cary JW, Klich MA, Beltz SB (2005). Characterization of aflatoxin-producing fungi outside of *Aspergillus* section Flavi. *Mycologia* 97: 425-432.
- Castegnaro M, McGregor D (1998). Carcinogenic risk assessment of mycotoxins. *Rev. Med. Vet-Toulouse* 149: 671-678.
- Cepeda A, Franco CM, Fente CA, Vazquez BI, Rodriguez JL, Prognon

- P, Mahuzier G (1996). Post column excitation of aflatoxins using cyclodextrins in liquid chromatography for food analysis. *J. Chromatogr. A*. 721(1): 69-74.
- Cepeda A, Prognon P, Mahuzier G, Blais J (1988). Improvement in the fluorometric detection of 5-methoxyysoralen by using β -cyclodextrin column. *Anal. Chim. Acta* 211: 333-337.
- Chang PK, Horn BW, Dorner JW (2005). Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in non aflatoxigenic *Aspergillus flavus* isolates. *Fungal Genet. Biol.* 42: 914-923.
- Chang PK, Matsushima K, Takahashi T, Yu Jiujiang, Abe K, Bhatnagar D, Yuan GF, Koyama Y, Cleveland TE (2007). Understanding non aflatoxigenicity of *Aspergillus sojae*: a windfall of aflatoxin biosynthesis research. *Appl. Microbiol. Biotechnol.* 76: 977-984.
- Cole RJ (1981). Versicolorin group. In: *Handbook of toxic fungal metabolites*. New York: Academic Press, pp. 94-127.
- Cotty PJ (1988). Simple fluorescence method for rapid estimation of aflatoxin levels in a solid culture medium. *Appl. Environ. Microbiol.* 54: 274-276.
- Criseo G, Bagnara A, Bisignano G (2001). Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group. *Lett. Appl. Microbiol.* 33: 291-295.
- Davis ND, Iyer, SK, Diener UL (1987). Improved method of screening for aflatoxin with a coconut agar medium. *Appl. Environ. Microbiol.* 53: 1593-1595.
- De Vogel P, Van Rhee R, Koelensmid W (1965). A rapid screening test for aflatoxin-synthesizing *Aspergilli* of the *flavus-oryzae* group. *J. Appl. Bacteriol.* 28: 213-220.
- Degola F, Berni E, Restivo, FM (2011). Laboratory tests for assessing efficacy of atoxigenic *Aspergillus flavus* strains as biocontrol agents. *Int. J. Food Microbiol.* 146(3): 235-243.
- Desai MR, Ghosh S (2003). Occupational exposure to airborne fungi among rice mill workers with special reference to aflatoxin producing *A. flavus* strains. *Ann. Agric. Environ. Med.* 10(2):159-162.
- Donkersloot JA, Mateles RI, Yang SS (1972). Isolation of averufin from a mutant of *Aspergillus parasiticus* impaired in aflatoxin biosynthesis. *Biochem. Biophys. Res. Commun.* 47(5): 1051-1055.
- Dyer SK, McCammon S (1994). Detection of toxigenic isolates of *Aspergillus flavus* and related species on coconut cream agar. *J. Appl. Bacteriol.* 76: 75-78.
- Ehrlich KC, Cotty PJ (2004). An isolate *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. *J. Microbiol. Biotechnol.* 65: 473-478.
- Ehrlich KC, Kobbeman K, Montalbano BG, Cotty PJ (2007). Aflatoxin-producing *Aspergillus* species from Thailand. *Int. J. Food Microbiol.* 114: 153-159.
- Fente CA, Ordaz JJ, Vazquez BI, Franco CM, Cepeda A (2001). New additive for cultural media for rapid identification of aflatoxin producing *Aspergillus* strains. *Appl. Environ. Microbiol.* 67: 4858-4862.
- Filtborg O, Frisvad JC (1980). A simple screening method for toxigenic fungi in pure cultures. *Lebensm. Wiss-Technol.* 13:128-130.
- Francis OJ, Kirschenheuter GP, Ware GM, Barman AS, Kuan SS (1988). β -Cyclodextrin post-column fluorescence enhancement of aflatoxins for reverse-phase liquid chromatographic determination in corn. *J. AOAC Int.* 71: 725-728.
- Frisvad JC, Filtborg O (1983). Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites. *Appl. Environ. Microbiol.* 46(6): 1301-1310.
- Frisvad JC, Houbraeken J, Samson RA (1999). *Aspergillus* species and aflatoxin production: a reappraisal. In: A.C.J. Tuijelaars, R.A. Samson, F.M. Rombouts, S. Notermans (Eds.), *Food Microbiology and Food Safety into the Next Millennium*, Foundation Food Micro '99, Zeist, pp.125-126.
- Frisvad JC, Skouboe P, Samson RA (2005). Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B₁, sterigmatocystin and 3-O-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. *Syst. Appl. Microbiol.* 28(5): 442-453.
- Geisen R (1996). Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Syst. Appl. Microbiol.* 19(3): 388-392.
- Gimeno A (2004). Aflatoxina M₁ no leite. Riscos para a saúde pública, prevenção e controlo. *Alimentação Animal (Revista de la Associação Portuguesa dos Industriais de Alimentos Compostos para Animais (IACA))* 49: 32-44.
- Godet M, Munaut F (2010). Molecular strategy for identification in *Aspergillus* section *Flavi*. *FEMS Microbiol. Lett.* 304: 157-168.
- Gqaleni N, Smith JE, Lacey J (1996). Co-production of aflatoxins and cyclopiazonic acid in isolates of *Aspergillus flavus*. *Food Addit. Contam.* 13(6): 677-685.
- Gupta A, Gopal M (2002). Aflatoxin production by *Aspergillus flavus* isolates pathogenic to coconut insect pests. *World J. Microbiol. Biotechnol.* 18(4): 329-335.
- Hara S, Fenell DL, Hesseltine CW (1974). Aflatoxin producing strains of *Aspergillus flavus* detected by fluorescence of agar medium under ultraviolet light. *Appl. Microbiol.* 27: 1118-1123.
- Hatti AD, Taware SD, Taware AS, Pangrikar PP, Chavan AM, Mukadam DS (2010). Genetic diversity of toxigenic and non-toxigenic *Aspergillus flavus* strains using ISSR markers. *Int. J. Curr. Res.* 5: 61-66.
- Heathcote JG, Dutton MF (1969). New metabolites of *Aspergillus flavus*. *Tetrahedron* 25(7): 1497-1500.
- Hoeltz M (2005). Estudo da Influencia de Manejos Pos-Colheita na Incidencia de Fungos e Micotoxinas no Arroz (*Oryza sativa* L.). *Dissertacao (Mestrado em Microbiologia Agricola e do Ambiente)-Universidade Federal do Rio Grande do Sul. Porto Alegre*.
- Jaime-Garcia R, Cotty PJ (2004). *Aspergillus flavus* in soils and corn cobs in South Texas: Implications for management of aflatoxins in corn-cotton rotations. *Plant Dis.* 88: 1366-1371.
- Jaimez J, Fente CA, Franco CM, Cepeda A, Vazquez B (2003a). Application of a modified culture medium for the simultaneous counting of molds and yeasts and detection of aflatoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*. *J. Food Prot.* 66: 311-318.
- Jaimez J, Fente CA, Vazquez BI, Franco CM, Cepeda A (2003b). Development of a method for direct visual determination of aflatoxin production by colonies of the *Aspergillus flavus* group. *Int. J. Food Microbiol.* 83:219-225.
- Jiang H, Yan L, Ma Z (2009). Molecular characterization of an atoxigenic *Aspergillus flavus* strain AF051. *Appl. Microbiol. Biotechnol.* 83(3): 501-505.
- Kaneko Y, Sanada M (1969). Studies on the fluorescent substances produced by *Aspergillus* fungi. VII. Purification and isolation of asperopterin B and chemical properties of asperopterin B and A. *J. Ferment. Technol.* 47:8-19.
- Klich MA (2002). Identification of common *Aspergillus* species. *Centralbureau voor Schimmelcultures, The Netherlands*.
- Klich MA, Mullaney EJ, Daly CB, Cary JW (2000). Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by *Aspergillus tamarii* and *Aspergillus ochraceoroseus*. *Appl. Microbiol. Biot.* 53(3): 605-609.
- Klich MA, Pitt JI (1988). A laboratory guide to common *Aspergillus* species and their teleomorphs. *CSIRO Division of Food Processing, North Ryde, NSW*.
- Kumeda Y, Asao T (2001). Heteroduplex panel analysis, a novel method for genetic identification of *Aspergillus* section *Flavi* strains. *Appl. Environ. Microbiol.* 67(9): 4084-4090.
- Lemke PA, Davis ND, Iyer SK, Creech GW, Diener UL (1988). Fluorometric analysis of iodinated aflatoxin in mini cultures of *Aspergillus flavus* and *Aspergillus parasiticus*. *J. Ind. Microbiol.* 3: 119-125.
- Leontopoulos D, Siafaka A, Markaki, P (2003). Black olives as substrate for *Aspergillus parasiticus* growth and aflatoxin B₁ production. *Food Microbiol.* 20(1):119-126.
- Lin MT, Dianese JC (1976). A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. *Phytopathology* 66: 1466-1469.
- Liu Y, Wu F (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: A risk assessment. *Environ. Health Perspect.* 118(6): 818-824.
- Montiel D, Dickinson MJ, Lee HA, Dyer PS, Jeenes DJ, Robertis IN, James S, Fuller LJ (2003). Genetic differentiation of the *Aspergillus* section *flavi* complex using AFLP fingerprints. *Mycol. Res.* 107:1427-

- 1432.
- Moss MO (2002). Risk assessment for aflatoxins in food stuffs. *Int. Biodeter. Biodegradation* 50(3-4): 137-142.
- Nasir MS, Jolley ME (2002). Development of a fluorescence polarization assay for the determination of aflatoxins in grains. *J. Agric. Food Chem.* 50(11): 3116-3121.
- Odhiambo BO, Murage H, Wagara IN (2014). Screening for atoxigenic *Aspergillus* species and evaluating their inhibitory potential against growth and sporulation of aflatoxigenic *Aspergillus* species. *Egerton J. Sci. Technol.* 14: 61-80.
- Ordaz JJ, Fente CA, Vazquez BI, Franco CM, Cepeda A (2003). Development of a method for direct visual determination of aflatoxin production by colonies of the *Aspergillus flavus* group. *Int. J. Food Microbiol.* 83: 219-225.
- Park KY, Bullerman LB (1983). Effect of cycling temperatures on aflatoxin production by *Aspergillus parasiticus* and *Aspergillus flavus* in rice and Cheddar cheese. *J. Food Sci.* 48: 889-896.
- Patey AL, Sharman M, Wood R, Gilbert (1989). Determination of aflatoxin concentrations in peanut butter by enzyme linked immunosorbent assay (ELISA): study of three commercial ELISA kits. *J. Assoc. Off. Anal. Chem.* 72(6): 965-969.
- Pitt JI, Hocking AD, Glenn DR (1983). An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. *J. Appl. Bacteriol.* 52: 109-114.
- Prieto R, Yousibova GL, Woloshuk C (1996). Identification of aflatoxin biosynthesis genes by genetic complementation in an *Aspergillus flavus* mutant lacking the aflatoxin gene cluster. *Appl. Environ. Microbiol.* 62: 3567-3571.
- Rashid M, Khalil S, Ayub N, Ahmad W, Khan AG (2008). Categorization of *Aspergillus flavus* and *Aspergillus parasiticus* isolates of stored wheat grains into aflatoxinogenics and non-aflatoxinogenics. *Pak. J. Bot.* 40: 2177-2192.
- Reddy KRN, Saritha P, Reddy CS, Muralidharan K (2009). Aflatoxin B1 producing potential of *Aspergillus flavus* strains isolated from stored rice grains. *Afr. J. Biotechnol.* 8(14): 3303-3308.
- Ritter AC, Hoeltz M, Noll IB (2011). Toxigenic potential of *Aspergillus flavus* tested in different culture conditions. *Ciencia e Tecnologia de Alimentos Campinas* 31(3): 623-628.
- Rojas TR, Fente CA, Vazquez BI, Franco CM, Cepeda A (2005). Use of twelve fungal culture media with added cyclodextrin for the rapid determination of aflatoxigenic strains common in foods. *Food Control* 16: 445-450.
- Rojas-Duran TR, Fente CA, Vazquez BI, Franco CM, Sanz-Medel A, Cepeda A (2007). Study of a room temperature phosphorescence phenomenon to allow the detection of aflatoxigenic strains in culture media. *Int. J. Food Microbiol.* 115(2): 149-158.
- Saito M, Machida S (1999). A rapid identification method for aflatoxin producing strains of *A. flavus* and *A. parasiticus* by ammonia vapor. *Mycoscience* 40:205-221.
- Saleemullah AI, Khalil IA, Shah H (2006). Aflatoxin contents of stored and artificially inoculated cereals and nuts. *Food Chem.* 98: 699-703.
- Schmidt-Heydt M, Magan N, Geisen R (2008). Stress induction of mycotoxin biosynthesis genes by abiotic factors. *FEMS Microbiol. Lett.* 284: 142-149.
- Seitz LM (1975). Comparison of methods of aflatoxin analysis by high-pressure liquid chromatography. *J. Chromatogr.* 104:81-91.
- Shapira R, Paster N, Eyal O, Menasherov M, Mett A, Salomon R (1996). Detection of atoxigenic molds in grains by PCR. *Appl. Environ. Microbiol.* 62: 3270-3273.
- Shier WT, Lao Y, Steele TW, Abbas HK (2005). Yellow pigments used in rapid identification of aflatoxin-producing *Aspergillus* strains are anthraquinones associated with the aflatoxin biosynthetic pathway. *Bioorg. Chem.* 33(6): 426-438.
- Shotwell OL, Hesseltine CW, Stubblefield RD, Sorenson WG (1966). Production of aflatoxin on rice. *Appl. Microbiol.* 14(3):425-428.
- Snigdha M, Hariprasad P, Venkateswaran G (2013). Mechanism of aflatoxin uptake in roots of intact groundnut (*Arachis hypogaea* L.) seedlings. *Environ. Sci. Pollut. Res. Int.* 20 (12): 8502-8510.
- Sobolev V, Dörner J (2002). Cleanup procedure for determination of aflatoxins in major agricultural commodities by liquid chromatography. *J. AOAC Int.* 85(3):642-645.
- Stroka J, Anklam E (2000). Development of a simplified densitometer for the determination of aflatoxins by thin-layer chromatography. *J. Chromatogr. A* 904(2): 263-268.
- Strosnider H, Azziz-Baumgartner E, Banziger M, Bhat RV, Breiman R, Brune MN, DeCock K, Dilley A, Groopman J, Hell K, Henry SH, Jeffers D, Jolly C, Jolly P, Kibata GN, Lewis L, Liu X, Luber G, McCoy L, Mensah P, Miraglia M, Misore A, Njapau H, Ong CN, Onsongo MTK, Page SW, Park D, Patel M, Phillips T, Pineiro M, Pronczuk J, Rogers HS, Rubin C, Sabino M, Schaafsma A, Shephard G, Stroka J, Wild C, Williams JT, Wilson D (2006). Workgroup report: Public health strategies for reducing aflatoxin exposure in developing countries. *Environ. Health Perspect.* 114: 1898-1903.
- Sweeney MJ, Pamies P, Dobson AD (2000). The use of reverse transcription-polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in *Aspergillus parasiticus* 439. *Int. J. Food Microbiol.* 56(1): 97-103.
- Taniwaki MH (1996). Meios de cultura para contagem de fungos em alimentos. *Bol. SBCTA* 30 (2): 132-141.
- Torrey GS, Marth EH (1976). Silica gel medium to detect molds that produce aflatoxin. *Appl. Environ. Microbiol.* 32(3): 376-380.
- Tran-Dinh N, Pitt JI, Carter DA (1999). Molecular genotype analysis of natural toxigenic and nontoxigenic isolates of *Aspergillus flavus* and *A. parasiticus*. *Mycol. Res.* 103: 1485-1490.
- Trucksess MW, Stack ME, Nesheim S, Page SW, Albert RH, Hansen TJ, Donahue KF (1991). Immunoaffinity column coupled with solution fluorometry or liquid chromatography postcolumn derivatization for determination of aflatoxins in corn, peanuts and peanut butter: collaborative study. *J. Assoc. Off. Anal. Chem.* 74(1): 81-88.
- Varga J, Frisvad JC, Samson RA (2011). Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Stud. Mycol.* 69: 57-80.
- Vazquez ML, Cepeda A, Prognon P, Mabuzier G, Blais J (1991). Cyclodextrins as modifiers of the luminescence characteristics of aflatoxins. *Anal. Chim. Acta* 255: 343-350.
- Vazquez ML, Franco CM, Cepeda A, Prognon P, Mabuzier G (1992). Liquid-chromatographic study of the interaction between aflatoxins and beta-cyclodextrin. *Anal. Chim. Acta* 269: 239-247.
- Waliyar F, Ba A, Hassan H, Bonkougou S, Bosc JP (1994). Sources of resistance to *Aspergillus flavus* and aflatoxin contamination in groundnut genotypes in West Africa. *Plant Dis.* 78: 704-708.
- Wicklow DT, Shotwell OL, Adam GL (1981). Use of aflatoxin producing ability medium to distinguish aflatoxin-producing strains of *Aspergillus flavus*. *Appl. Environ. Microbiol.* 41: 697-699.
- Wilkinson JR, Yu J, Bland JM, Niernan WC, Bhatnagar D, Cleveland TE (2007). Amino acid supplementation reveals differential regulation of aflatoxin biosynthesis in *Aspergillus flavus* NRRL 3357 and *Aspergillus parasiticus* SRRC 143. *Appl. Microbiol. Biotechnol.* 74:1308-1319.
- Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am. J. Clin. Nutr.* 80: 1106-1122.
- Wiseman HG, Jacobson WC, Marmeyer WC (1967). Note on removal of pigments from chloroform extracts of aflatoxin cultures with copper carbonate. *J. Assoc. Off. Anal. Chem.* 50: 982-983.
- Yabe K, Ando Y, Ito M, Terakado N (1987). Simple method for screening aflatoxin-producing molds by UV photography. *Appl. Environ. Microbiol.* 53: 230-234.
- Yin Y, Lou T, Yan L, Michailides TJ, Ma Z (2009). Molecular characterization of toxigenic and atoxigenic *Aspergillus flavus* isolates, collected from peanut fields in China. *J. Appl. Microbiol.* 107: 1857-1865.
- Yokotsuka T, Sasaki M, Kikuchi I, Asao Y, Nobuhara A (1967). Studies on the compounds produced by moulds. 1. Fluorescent compounds produced by Japanese industrial moulds. *Nippon Nogei Kagaku Zasshi* 41:32-38.