Molecular characterization of toxigenic *Aspergillus flavus* strains isolates from animal feed stuff in Egypt

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Abstract: The objective of this study was to categorize *Aspergillus flavus* strains isolated from feed stuff from El-Gharbia government, Egypt, into aflatoxigenic and non aflatoxigenic ones by cultural as well as PCR methods. *Aspergillus flavus* isolated from different animal feed were tested for their aflatoxigenic and non aflatoxigenic by plating experiments. The effect of chitosan at concentration (0.0, 0.2, 0.4 and 0.6 mg/ml) on the growth and aflatoxin production was also studied. Seven from seventeen of the *Aspergillus flavus* isolated from feed stuff produced aflatoxin. chitosan treatment decreased the growth and aflatoxin production for *A. flavus 2* and *A. flavus 9* .The higher dose of chitosan 0.6 mg/ml was the most effective on *A.flavus2and flavus 9*. Using D2R and D&R specific primer for 28s rRNA a DNA fragment of approximately 1500bp was amplified when their total nucleic acid extracted from the local *Aspergillius flavus* was used as template. The 1500 bp PCR product indicated that our strain is *Aspergillus flavus*. The 900 bp PCR product indicated the strain number (2) and (9) have aflatoxins. These confirm the UV and fluorescent for production of aflatoxins by these two strains.
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1. Introduction:

Fungal contamination of animal feeds, with the consequent mycotoxin production, is one of the major threats to human and animal health Castillo *et al.* (2004). Livestock feed quality may however be affected by various microorganisms such as bacteria and fungi growing in different parts of the worlds. Cereals, concentrate, hay and the other animal feeds have been reported as substrates for fungal growth and mycotoxin production Scudamore and Patel (2000). . Foods and feeds especially in warm climates are susceptible to invasion by aflatoxigenic *Aspergillus* species and the subsequent production of aflatoxins during preharvesting, processing, transportation, or storage. Over the last few years, means for mycotoxin detection have been simplified. This is mainly because of the official recognition of immunological methods. However, the level of mold infestation and the identification of governing species are still important parameters which could give an indication of the quality of the material as well as of the future potential for the presence of mycotoxins. Mold counts are therefore included in the quality control assurance of many foods. Aflatoxin (AF) producing fungi belong to several *Aspergillus* species including *A. flavus* and *A. parasiticus*, the major species of concern for aflatoxins contamination, and other species like *A,nomius*, *A.pseudotamarii*, *A. bombycis, A. ochraceoroseus* (Varga *et al.,*2003; Cary *et al.,* 2005; Frisvad *et al.,* 2005). Ability to produce AF is highly conserved in some species but is highly variable in others. Almost all *A.parasticus* strains produce AFs. With only 3 to 6% non –aflatoxigenic strains (Horn *et al.,* 1996; Vaamonde *et al.,* 2003; Barros *et al.,* 2006), whereas toxigenicity in populations of *A.flavus* varies considerably with strain, substrate and geographic origin. Toxigenic *A.flavus* strains produce aflatoxin B1, aflatoxinB2, and often cyclopiazonic acid with AFB1, being the most relevant in food safety.

Mycotoxins are secondary metabolites that have adverse animal farms of suspected or potential mycotoxicosis effects on human, animals and crops, resulting in illness and economic losses (Hussein and Brasel, 2001). They include aflatoxins, fumonisins, trichothecenes and zearalenone that produce by some toxigenic fungi such as *Aspergillus*, *Penicillium Fusarium* in tropical or warmer parts of the world(Abarca *et al.,* 2001; Rosa *et al (* 2002).

Aflatoxins are potent carcinogenic, mutagenic, and teratogenic metabolites produced primarily by the fungal species *Aspergillus flavus* and *Aspergillus parasiticus*. Lillehoy *et al.* (1976) studied aflatoxin production in case of corn seeds due to association of *A. flavus* and *A. parasiticus*. They also stated that simply presence mould growth was not found to be an indicative for the presence of aflatoxin. While, Diener and Davis (1966) stated that all strains of *Aspergillus flavus* were not found to be aflatoxin produces as they had screened nearly 1400 strains which were isolated from different sources but only 58% strains were reported to be aflatoxigenic, Boller and Shcroeder (1966) found 94% among 284 isolates of *A. flavus*

were found to be capable of aflatoxin production in rough rice. *Aspergillus flavus* populations have been studied extensively in order to find a correlation with diversity in aflatoxigenic ability.

Recently a potential approach in biological control involves the use of the natural bioactive substance chitosan, which inhibits fungal growth and also activates the biological efficiency of the antagonistic microorganisms(El-Ghaouth, *et al.,* 1994; and Spandaro *et al. (*2003). Chitosan has a fungistatic activity demonstrated against some plant diseases, i.e., virus infection Chirkov *et al.* (1998) and pathogenic fungi(Zeidan,1998, Rhoades, and Roller, 2000).

The aflatoxin biosynthetic pathway involves approximately 25 genes clustered in a 70 kb DNA region (Yu *et al.,* 2004). *A. flavus*, *A. parasiticus*, and other *Aspergillus* Sect. *Flavi* species share nearly identical sequences and conserved gene order in the cluster. In recent years PCR detection of aflatoxin biosynthetic gene presence or expression has been used as diagnostic tool for aflatoxigenic fungi in selected food commodities (Geisen, 2007). Sequence variability and deletions in various genes/regions of the aflatoxin biosynthetic cluster have also been used to determine the polyphyletic assemblage of *A. flavus* group/species (Chang *et al.,* 2005; Chang *et al.,* 2006).

In our work, a part of this population of *Aspergillus* Sect. *flvus* was subjected to molecular identification to establish the *Aspergillus* species occurrence. Furthermore the strains were tested for presence in their genome of genes of the characterized aflatoxin biosynthetic in relation to aflatoxin production. To this aim primer pairs designed in this study and targeting the two regulatory genes D2R and D7R and the identification of *Aspergillus flavus* genes LR3 and CS3.

2.Material and Methods

1.Collection of the animal feed samples:

In the present study eleven animal feed samples were collected from El-Gharbia Government, Egypt. These samples included wheat, Chopped rice (1&2), Wheat flour, Wheat bran (1&2), maize flour (1&2), Rice bran, and poultry feed (1&2)**.**

2.Isolation and identification of fungi in animal feed:

Microorganisms were isolated from all animal feed samples by dilution-plate method, by gentle shaking of 10 g from each feed with 100 ml of sterile water for 30 min. after sedimentation, the supernatant suspension was used to prepare serial dilutions and a 1 ml a liquot of appropriate dilution were spread-plated onto czapek agar medium. The plates were incubated at 28˚C for 7 days (Johnson *et al*., 1959, Ismail *et al*., 2009). Identifications of pure fungal isolates were made on the basis of their cultural, morphological and microscopically charactristic according to the keys of Ainsworth (1971), Pitt (1979), and Moubasher (1993).

3- *Determination of toxigenic potential of Aspergillus flavus*

Aspergillus flavus isolated from different animal feed were tested for their toxicity by plating experiments. Toxigenic potential of pure cultures of *Aspergillus flavus* following inoculation on yeast extract sucrose medium (20g Sucrose, yeast extract 20g made up to 1L with distilled water) (YES) were determined by their fluorescence character in UV light at 365 nm wavelength Yabe *et al* (1987). These plates were examined at 12, 24, 36, 48, 72hrs, and 4th, 5 th and 7 th day post inoculation. Both observed and reverse sides of the plates were examined under UV (Black Ray model C50; Ultra- violet product, San Gabriel, Calif).

3-Extraction of aflatoxin from *Aspergillus flavus*:

The method adopted in the present investigation was carried out according A.O.A.C (1984). *Aspergillus flavus* were grown on slants Czapeks agar medium and adjusted with sterile 0.05 % tween to give a concentration of approximately 1x10^6 conidia/ml using a heamocytometer. Fungal isolates were grown separately in 250 ml conical flasks containing 50 ml of yeast extract sucrose medium YES (20g Sucrose, yeast extract 20g made up to 1L with distilled water). Each flask was inoculated with 1 ml of the prepared spore suspension. The inoculated flasks were incubated stationary in the dark at 25-28^{o}C for 9 days. At the end of the incubation period the content of each flask were homogenized for 5 min, in a high speed blender (1600r.p.m.) with 100 ml chloroform, filtered and the mycelial which was washed with equal volume of distilled water, dried over anhydrous sodium sulfate, concentrated under vacuum to near dryness and diluted to 1 ml with chloroform and then maintained for further determination.

3-a Aflatoxin analysis

Nine circular plugs were cut uniformly across the whole surface of yeast extract sucrose medium, to be representative of each colony. Plugs were weighed in a plastic tube; then 3 mL of methanol were added and aflatoxin was extracted by shaking for 2 h at room temperature. The extraction solution was filtered through a syringe filter (RC 0.45 µm, Alltech, Deerfield, IL, USA). One mL of the filtered extract from yeast extract sucrose medium or 5 mL of YES broth were diluted with PBS (1:6 v/v and 1:1 v/v, respectively). The diluted extracts were applied to the immunoaffinity clean up column AflaTest© (VICAM, Milford, MA, USA). The column was then washed with 5 mL of distilled water. Developing by 1 ml of diluted developer (1 ml conc. Developer + 9ml

distilled water). Reading by the following the steps on the screen of the flurometer.

4- Effect of Chitosan on the growth and aflatoxins production by *A. flavus*:

Purified Chitosan (obtained from Sigma chemical steinham, Germany) was prepared by the method which described by El-Ghaouth *et al*.(1994)

To study the effect of different doses of chitosan on the production of aflatoxin by *Aspergillus flavus*, four concentrations of chitosan were used. one ml of the prepared spore suspension ($1x10^6$conidia/ml) from the tested fungi was placed in 250 ml conical flasks containing 50 ml of yeast extract sucrose medium YES containing; 0.00, 0.2, 0.4 and 0.60 mg/ml of chitosan. The inoculated flasks were incubated stationary in the dark at 25-28C for 9 days. At the end of the incubation period the aflatoxin was measured as above.

5- Molecular study:

5.1-DNA extraction from *Aspergillus flavus*:

Five days old colony of *Aspergillus flavus* were collected by vacuum filtration and ground to a fine powder in liquid N2. Fifty milligrams of the powder was transferred to 1.5 ml Eppendrof tube and mixed with 700 ml CTAB BUFFER (0.1m Tris-Hcl, pH8.0; 20mM EDTA; 1.4 MNacl, 2% cetyltrimethylammonium bromide and 0.2% B-mercaptoethanol). The tube were incubated at 65˚C for 30 mim, and then 700 ml of chloroform was added and the mixture was vortexed briefly. The resulting mixture was centrifuged at a maximum speed of 15,000 rpm for 30 min and the cleared supernatant was mixed with 600 ml of isopropanol chilled to -72˚ C/ 1h. The mixture was centrifuged at the maximum speed for 5 min and the resulting pellet washed twice with 1 ml of 70% ethanol. The pellet was dried under Vacuum and dissolved in 100 ml TE buffer (10 µm Tris, 1 mM EDTA, pH7.5). The DNA was observed by electrophoresis in 1% agarose gel in TBE buffer{(89 mM Tris –Hcl (pH8,3), 89 mM boric acid, and 20mM EDTA (Maniatis etal., 1982)}. Agarose gels were stained with ethidium bromide. Examined under ultraviolet light and photographed Gherbawy and Hussein, (2010).

5.2 PCR analysis:

Polymerase chain reaction (PCR) condition and separation of random amplified polymorphic DNA (PAPD)-PCR fragment were carried out according to the techniques of Messner et.al. (1994). PCR was carried out using A 1500bp fragment from the 28s RNA region primer pair D2R [5 TTG GTC CGT GTT TCA AGA CG3]- D7R [GAG ACC TGC GG3] or A 900bP fragment corresponding to the ITS2-LSU region (primer pair CS3 (5CGA ATC TTT GAACGCACATTG3)- LR3 [5CCG TGT TTC AAC ACG GG3] primers manufactured by (Bioneer.Inc, South Korea). The PCR reaction was performed in a total volume of 75 µl by using 12.5µl of 5x PCR Master mix (4 prime, Bioneer. Inc, south Korea), 2,5µl from each), 25µl nuclease free water and µl DNA (equivalent to 10 mg). A negative control was included without template DNA. The following temperature protocol; was used: initial denaturation step of 3 min at 94 C , 35 cycles of denaturation for 1 min at 94C, annealing for 1 min at 55C and extension for 1 min at 72 ^{0}C. The PCR products were resolved by electrophoresis by using 1.5% agarose gel in 0.5xtris Borate EDTA buffer, at 125V for 2hrs. Gels were stained with ethidium bromide and viewed under UV light using UVP BioImaging CDS 8000 system (UVP, Cambridge, UK). Dendrograms for RAPD result were constructed by the unweighted pair group method with arithmetic average based on Jaccard,s Similatarity Coefficient by using phoretix ID Advanced v 5,20 soft ware (Non-linear Dynamics).

3. Results and Discussion

The mycoflora associated with 11 animal feed samples was shown in Table 1. Among the isolated fungi, four filamentous genera were obtained. These genera were *Alternaria, Penicillium, Aspergillus* and *Rhizopus*. The genus *Aspergillus* was represnted by four species belonging to four groups,these groups were *Aspergillus flavus, A.fumigatus, A. niger* and *A. ochraceous.*

Quality livestock feed is necessary for the maintenance of physiological functions and animal defense systems against diseases and parasites. Traditionally, feed quality has been specified on basis of the nutritional value of every individual feed component Fink- Gremmels (2004). In the present investigation, the occurrence of species of *Aspergillus* in higher percentage is particularly important, because these is known to be toxin producers Bankole and Kpodo (2005).These results are similar to those obtained by other researchers (Domsch and Gams, 1980; Kurata and Ueno, 1984; Marsilio and Spottie, 1987; Bragulat *et al.,* 1995; Accensi *et al.,* 2004). *Aspergillus* species increased over all studied sampling periods. This finding is also in agreement with Zimmerli and Dick (1996) and Pitt and Hocking (1997) who had earlier established *Aspergillus* genera predominance over other genera in tropical environments. In addition, *A. flavus* predominates in all kinds of feed ingredients under any storage conditions in our tropical climate. This result agrees with the findings of (Adebajo *et al.,* 1994, Dalcero *et al.,* 1998, Magnoli and Dalcero, 2002, and Accensi *et al.,* 2004) obtained the same *A. flavus* isolation frequencies. It is suggested that the majority of this genus representatives such as *A. flavus* are thermophilic and thermo-resistant and distribute

abundantly in tropical to subtropical climates, (Lebars Bailly *et al.,* 1999).

Screening of *Aspergillus flavus* isolates for Aflatoxin producing ability

From Table 2 it is clearly observed that 17 *Aspergillus flavus* strains were isolated from different feed stuff, out of which ten isolate were found to nontoxic (*A. flavus* 1,4,5,17,6,10, 16, 11, 13 and 14) and seven isolates were found to be toxic. From these seven toxic isolates, *A. flavus* 2 and 9 are found to be highly toxic, *A. flavus* 15 are found to be moderate toxic, where isolates *A. flavus* 3, 7 and 8 are mildly toxic. On the other hand the highest aflatoxin produced by *A. flavus2* (770ppb) isolated from chopped rice2 followed by *A. flavus* (720 ppb) isolated from maize flour2.This results similar to Muhammad *et al.* (2012) who isolate and identify toxigenic mycoflora of maize and maize-gluten meal and found that percentage of toxigenic fungi among *Aspergillus* isolates was 52%. Aflatoxigenic isolates of *A. flavus* and *A. parasiticus* were 43 and 67% respectively and *Aspergillus parasiticus* produced higher concentrations of AFB1 (maximum 1374.23 ng g-1) than *A. flavus* (maximum 635.50 ng g-1).

Table (3) shows the effect of different concentration (0.0,0.2, 0.4 and 0.6 mg/ml) from chitosan on aflatoxin produced by *A. flavus2* and *A. flavus* 9 which produced the highest amount of aflatoxin. In this work three doses of chitosan 0.2,0.4 and 0.6 in addition to control were used to study the effect of the chitosan on the growth and aflatoxin production by *A.flavus* 2 and *A.flavus* 9 which produced the highest aflatoxins in this study. Data in Table (3) showed that chitosan treatment decreased the growth and aflatoxin production for *A.flavus*2 and *A.flavus* 9 .The higher dose of chitosan 0.6 mg/ml was the most effective with two strain of *A.flavus,* these results were in agreement with Ryan (1988) who reported that chitosan induced multiple biological reactions including induction of phytoalin synthesis of B-1-3 gluconase and chitinase. Abou Sereih *et al.* (2007) found that the two doses of chitosan 3.00 and 4.50 mg/ml were lethal with *Fusarium* while in case of *Trichoderma* they resulted in the change in growth color from green to yellow**.**

Table (1) Total counts of fungi isolated from different feed stuff (colony/g)

Source	Fungi encoutered	Total count
Wheat	*Alternaria humicola* *Penicillium sp.* *Aspergillus ochraceous* *Aspergillus niger*	55 150 15 30
Chopped rice1	*A.niger* *Rhizopus sp.*	160 10
Chopped rice2	*A.niger* *A.flavus* *A.fumigatus* *Rhizopus sp.*	20 10 10 10
Wheat flour	*A.niger* *A.flavus* *Penicillium sp..*	20 10 10 10
Wheat bran1	*A.flavus* *A.niger* *Rhizopus sp.*	30 120 10
Wheat bran2	*A.flavus* *A.niger* *Penicillium sp.*	25 100 15
Maize flour1	*A.niger* *A.flavus* *Rhizopus sp* *Penicillium sp*	300 260 10 10
Maize flour2	*A.flavus* *A.niger*	150 50
Rice bran	*A.flavus* *A.niger* *Rhizopus sp* *Penicillium spp*	350 120 10 10
Poultry feed (1)	*A.flavus* *Penicillium spp*	30 10
Poultry feed (2)	*A.flavus* *A.niger* *Rhizopus sp*	30 20 10

Table (2) Aflatoxins detection by plating experiment and the amount of aflatoxins produced by *A. flavus* isolated from different feed stuff

Source	Isolates	Colour intenisty	Aflatoxin (ppb)
Chopped rice 2	*A.flavus* (1) *A.flavus* (2)	- ++++	 770
Rice bran	*A.flavus* 3 *A.flavus* 4 *A.flavus* 5 *A.flavus* 17	++ - - -	390
Maize flour1	*A.flavus* 6 *A.flavus* 7 *A.flavus* 8	++ - ++	380 370
Maize flour2	*A.flavus* 9	++++	720
Wheat bran 1	*A.flavus* 10	-	

Wheat bran 2	*A.flavus* 15 *A.flavus* 16	+ -	
Poultry feed (1) Poultry feed (2)	*A.flavus* 11 *A.flavus* 12	- +++	410
Wheat flour	*A.flavus* 13 *A.flavus* 14	- -	

Non fluorescence = **- , Moderate** fluorescence = **+, Mildly** fluorescence = **++, Highly** fluorescence = ++++

Table 3.Effect of Chitosan on the growth and aflatxoin production by *A.flavus*.

Conc. Of chitosan(mg/ml)	***A.flavus* 2**		***A.flavus* 9**	
	Aflatoxin(ppb)	Mcelial dry weight	Aflatoxin(ppb)	Mcelial dry weight
0.0	770	0.73±0.1	720	0.7±0.1
0.2	0.7	0.5±0.02	1.27	0.537±0.021
0.4	0.65	0.07±0.01	0.8	0.23±0.03
0.6	0.52	0.023±0.002	0.6	0.03±0.002

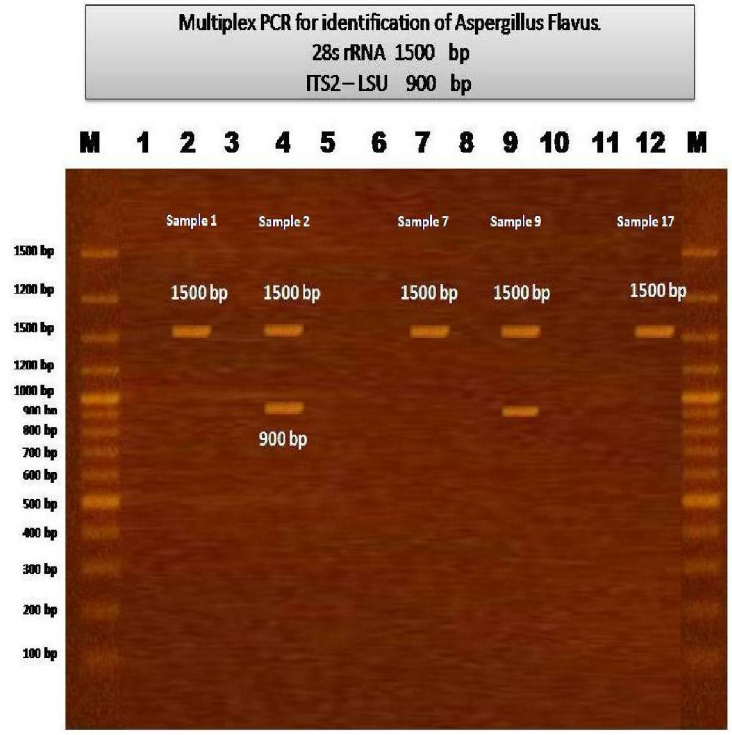

Fig. 1. Agarose gel electrophoresis of PCR product of the 18S ribosomal RNA. Lanes (2,4,7,9,12) PCR products of *A.flavus* isolated from animal feed,M;1500bpfor confirm identification of *A.flavus*; Lanes (4,9) PCR product of *A.flavus* aflatoxin, M,900b pladder.

Molecular identification of aflatoxinogenic and non-aflatoxinogenic isolates:

Using D2R and D&R specific primer for 28s rRNA a DNA fragment of approximately 1500bp was amplified when their total nucleic acid extracted from the local *Aspergillius flavus* was used as template (Fig 1).The 1500 bp PCR product indicated that our strain is *Aspergillus flavus.* The 900 bp PCR product indicated the strain number (2) and (9) have aflatoxin gene. These confirm the UV and fluorescent for production of aflatoxin by these two strains.

Multiplex PCR with the aflatoxin pathway genes aflatoxin, to clearly differentiate aflatoxin producers from non-producers *A.flavus* strains.

In this study *A.flavus* was the only species detected in connection with aflatoxin contamination of animal feed in Egypt. Predominance of *A.flavus* as an aflatoxigenic species in animal feed in worldwide**.**

Aspergillus flavus is reported to be extremely diverse in terms of morphology and toxigenicity and incidence of non toxigenic-strains is dependent on

geographic origin and substrate ,Vaamonde *et al.* (2003) Pildain *et al.* (2004).

Molecular characterization also facilitated proper identification as *A.flavus* of four isolates which had been previously described as *A.parasiticus* on the basis of morphological parameters, Giorni *et al.* (2007). In the last decate, development of molecular methods for distinction of aflatoxigenic and non-aflatoxigenic strains of *A .flavus* and *A. prasiticus* has been focused on aflatoxin biosynthesis genes.

In our work, a population of *A.flavus* including both aflatoxigenic and non-afltoxigenic strains was screened for the presence of two genes of the aflatoxin. The result was the grouping of strain into two different amplification patterns, characterized by one or no DNA bands. All the aflatoxin –producing strains exhibited the presence of aflatoxin gene, whereas the non-aflatoxigenic strain lacked the PCR products corresponding to this gene. Interestingly, two strains were not able to produce Aflatoxin.

To date, lack of amplification of aflatoxin biosynthetic genes has been consistently linked to non-aflatoxigenicity of strains. Chng *et al.* (2005) investigated and characterized deletions of a part or the entire gene cluster in non-aflatoxigenic *A,flavus* strains supporting the hypothesis that the loss of aflatoxin-producing ability could be associated with deletions or mutations in the related genes. The results obtained in our study confirmed that lack of amplification of afltoxin biosynthetic genes is correlated with non-aflatoxigenicty. Criseo *et al.* (2008) also reported that variable DNA banding patterns with one to four genes (aflR,aflD, aflM, aflP) missing typical of the non-aflatoxigenic strains o A.flavus.These finding do not completely clarify if non-afltoxigenicity is due to complete deletion of a gene, part of or the entire biosynthetic cluster or to the presence of changes at the primer binding sites, It is likely that one or more of the other genes involved in aflatoxin biosynthesis are lacking or carry some deletions in these three strains.

Monitoring of the expression of aflatoxin genes has been applied for detection and differentiation of aflatoxigenic strains. To this aim, various regulatory and structural aflatoxin pathway genges in *A.parasiticus* and *A.flavus* have been targeted (Sweeney *et al.,* 2000; Scherm *et al.,* 2005; Degola *et al.,* 2007). However, it must be considered that the pathway is highly complex and only some genes can be regarded as key genes directly coupled to afltoxin biosynthesis. Antonic *et al.* (2012)study Molecular characterization of an *Aspergillus flavus* population isolated from maize during the first outbreak of aflatoxin contamination in Italy.

The known biochemical steps and genes involved in the aflatoxin biosynthesis is shown in Fig. 1. Amplification products, regardless of the primer set used, were obtained from *A. flavus* DNA. Each primer pair yielded a single DNA fragment of the expected size: 900, 1500 bp for D2R and *D7R* respectively. Nonspecific products were never observed, probably because of the relatively high annealing temperature.

Several recent studies on the use of PCR technology for the detection and diagnosis of fungi have been published Henson and French (1993). Quantitation of phytopathogenic fungi in diseased plants is important, especially with the ubiquitous phytopathogens that are present on healthy plants, such as *Verticillium dahliae* and *Verticillium albo-atrum* Hu *et al.* (1993). Likewise, PCR enabled the detection and identification of obligate biotrophic vesicular arbuscular mycorrhizal fungi, Wyss and Bonfante (1993). Nevertheless, the use of PCR to identify specific organisms obtained from a variety of food samples has been problematic because of the presence of various interfering substances, Atmar *et al.* (1993). Moreover, molds are found on dry food mostly as asexual spores or dried mycelia, which contain only small amounts of DNA and are resistant to cellular disruption for DNA extraction.

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