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MOLECULAR DETECTION OF MYCOBIOTA AND AFLATOXIN CONTAMINATION OF CHILI

Youssuf A. Gherbawy[1, 2, *], Yassmin M. Shebany[1, 2], Mohmaed A. Hussein[2] and Thanaa A. Maghraby[2]

[1] *Biological Sciences Department, Faculty of Science, Taif University,* Taif, Saudi Arabia
[2] *Botany Department, Faculty of Science, South Valley University,* Qena, Egypt

[*]Corresponding author: youssufgherbawy@yahoo.com

Abstract - Capsicum annuum grows in warm areas. Pepper production conditions require the drying of fruits by sunlight. During the drying processes, the crop is exposed to contamination by microorganisms, especially fungi. In this article, the isolation of mycobiota from retail markets and food restaurants of Taif city was studied. Crushed chili showed a high fungal load compared to chili sauce and chili powder, while chili powder showed a high occurrence of total aflatoxins (AFs). *Aspergillus*, *Eurotium* and *Penicillium* were the most common genera isolated from chili samples. Thirty-four samples (out of 60) were naturally contaminated with AFs ranging from 20 to 200 ppb. The total aflatoxin potential of 35 isolates of *A. flavus*, *A. parasiticus* and *A. tamarri* were studied. Seventy percent of *A. flavus* isolates were aflatoxigenic. The frequencies of aflatoxin biosynthesis genes *aflR, nor-1, ver-1* and *omtA* were studied in aflatoxigenic and non-aflatoxigenic isolates of *Aspergillus* species collected in this study. All aflatoxigenic isolates (21) and 1 non-aflatoxigenic isolate of *A. flavus* showed DNA fragments that correspond to the complete set of the targeted genes. In conclusion, the high co-occurrence of *Aspergillus* species capable of producing aflatoxins, particularly in chili samples, suggests the need for more efficient control during processing and storage to reduce fungal contamination.

Key words: *Aspergillus flavus*; aflatoxin genes; fluorometer; co-occurrence; molecular markers

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INTRODUCTION

The pepper (*Capsicum annuum* L. Solanaceae) is a vegetable crop grown in warm climates (Sreedhara et al., 2013). It constitutes about 34% of the spice trade in the world and the demand and consumption of the pepper increases more than 5.2% annually. The total world production of pepper was 496.19 million metric tons. 10.35 thousand tons have been imported to Saudi Arabia since 2000. India is the largest producer of chili. Its production level hovers around 1.1 million tons a year. India also has the maximum area dedicated to the production of this crop. Chili is an important commodity used as a vegetable, spice, medicinal herb and ornamental plant by billions of people every day (Rajeev, 2010). It is also used as an ingredient in industrial products. The world production of chili crops comes to around 7 million tons that is cultivated on approximately 1.5 million hectares of land.

Spoilage caused by fungi during plant growth and the storage of agricultural products is highly undesirable. Not only is the quality of the products decreased

but also there is a risk to human and animal health from mycotoxins (Erdogan, 2004; Venâncio and Paterson, 2006; Salem and Ahmad, 2010; Zain, 2011).

Aflatoxins are toxic metabolites produced by some species of fungi. Common aflatoxins are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2). AFB1 is the most potent of all aflatoxins known to date and is generally found in the highest concentration in food and animal feeds (Lee et al., 2004). These toxins are highly carcinogenic and elicit a wide spectrum of toxic effects when foods and feeds contaminated with aflatoxins are ingested (Peskta and Bonday, 1990).

Chilies have been reported as one of the crops with the highest aflatoxin contamination (Marin et al., 2009). Contamination of chili with aflatoxins and ochratoxins may take place in the field (pre-harvest) or during drying, storage or processing stages (post-harvest). Aflatoxin contamination was found to be higher in summer chili samples and hence winter chilies may provide a better quality product with respect to aflatoxin contamination. The European Union limit for aflatoxin B1 and total aflatoxins in whole chilies is 48 and 36%, respectively, in winter, and 52 and 38%, respectively, in summer (Iqbal et al., 2011). Because of the toxic and carcinogenic potential of mycotoxins, there is an urgent need to develop detection methods that are rapid and highly specific (Konietzny and Greiner, 2003). At present, monitoring of agricultural commodities, foods and animal feeds for the presence of fungal contamination includes cultivation and taxonomic identification at the morphological level (Liewen and Bullerman, 1999). This approach is, however, very time consuming, labor-intensive, requires the expertise of mycologist and above all possesses the inherent possibility of misclassification, since morphological characters could be highly variable depending on the media and culture conditions. Therefore, more rapid and more objective methods for the identification of mycotoxigenic fungi in human foods and animal feeds are needed for evaluating the microbiological risks of a given product (Konietzny and Greiner, 2003). In addition, the most important aflatoxin producers from a public health point of view are members of the *Aspergillus* section *Flavi*, in particular *A. flavus* and *A. parasiticus*. Originally, several isolates of *A. parasiticus* were misidentified as *A. flavus*, for example NRRL 2999, 3000 and 3145 (Hesseltine et al., 1966; Applegate and Chipley, 1973).

Molecular techniques have been introduced as powerful tools for detecting and identifying fungi (Geisen, 1998). For this reason, rapid methods, including polymerase chain reaction (PCR), have been developed for direct testing of food for the presence of pathogens, as well as for confirmation and genotyping of isolates from foods. PCR-based assays for the detection of common foodborne pathogens are available from several manufacturers. The main advantage of PCR is that organisms need not be cultured, at least not for long periods prior to their detection. PCR-based methods that target DNA are considered a good alternative for rapid diagnosis because of their high specificity and sensitivity (Accensi et al., 1999; Rath and Ansorg, 2000; Schmidt et al., 2003; Perrone et al., 2004), especially when multi-copy sequences are used to develop species-specific primers (Bluhm et al., 2002).

The biosynthetic pathway for aflatoxin production by *A. flavus* has been elucidated, and genes in the aflatoxin biosynthetic pathway have been identified (Payne et al., 1993; Trail et al., 1995; Yu et al., 1995, 2004). The *AflR* gene plays an important role in the aflatoxin biosynthetic pathway by regulating the activity of other structural genes such as *omt-A, ver-1,* and *nor-1* (Woloshuk et al., 1994; Chang et al., 1999).

This study aimed to (i) determine the mycobiota of chili offered for sale to consumers in retail stores at the Taif region in Saudi Arabia, paying special attention to the mycotoxigenic moulds; (ii) evaluate the contamination levels of aflatoxins in chili samples; (iii) detect aflatoxigenic abilities of the collected aspergilli; and (iv) evaluate the presence and frequencies of the PCR products corresponding to amplification of *aflR, nor-1, ver-1* and *omtA* genes in *Aspergillus Flavi* section isolated from chili samples.

MATERIALS AND METHODS

Sampling

Sixty chili samples including chili sauce (n = 20), crushed chili (n= 20) and chili powder (n = 20) were purchased randomly from retail markets and restaurants in Taif city. The sample size of each powdered chili and crushed chili was at 500g while each chili sauce sample was 200 mL. The samples were saved in plastic bags and stored in freezer at 4°C until further analysis.

Mycobiota determination

Total fungal counts were performed on dichloran 18% glycerol (DG18) agar (glucose 10 g; peptone 5 g; KH_2PO_4 1 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; glycerol 220 g; agar 15 g, dichloran 2 mg; chloramphenicol 100 mg; and H_2O 1 L), a medium that has lower water activity (*aw* = 0.955) and favors xerophilic fungi development (Pitt and Hocking, 1997). Quantitative enumeration was performed by the surface-spread method. Ten grams of each sample were homogenized in 90 mL of peptone water solution. Serial dilutions were made and 0.1 mL aliquot was inoculated in duplicate onto the culture media. All the plates were incubated in the dark at 25°C for 5-7 days. Only plates containing 15-150 colony forming units (CFU) were used for counting and the results were expressed as CFU per gram of sample. All colonies were transferred for subculturing to plates of MEA. Taxonomic identification of the different genera and species was made according to macroscopic and microscopic criteria with appropriate keys (Nelson et al., 1983; Pitt and Hocking, 1997; Samson et al., 2000; Klich, 2002). After taxonomic identification, all *Aspergillus Flavi* were subjected to confirmation by molecular analysis with specific primers for these species described in previous studies (González-Salgado et al., 2008).

Detection of natural occurrence of total aflatoxins in chili samples

The samples were analyzed for total aflatoxins using a slightly modified immunoaffinity method based on the Association of Official Analytic Chemists (AOAC) method (Trucksess et al., 1991). As previously described by Lewis et al. (2005), the whole sample was ground and a 100 g subsample was removed for analysis. Methanol:water (80:20) solvent (100 ml) and 5 g NaCl were added to each sample and the mixture was blended at high speed for 3 min. The mixture was then filtered through fluted filter paper (Whatman 2V, Whatmanplc, Middlesex, UK), and the filtrate was diluted (1:4) with water and refiltered through glass-fiber filter paper. Two milliliters of the glass-fiber filtrate were placed on an AflaTest®WB SR Column (VICAM, Watertown, MA, USA) and allowed to elute at 1-2 drops/s. The columns were washed two times with 5 ml water, and aflatoxin was eluted from the column with 1 ml high performance liquid chromatography (HPLC)-grade methanol. A bromine developer (1 ml) was added to the methanol extract, and the total aflatoxin concentration was read in a recalibrated VICAMSeries-4 fluorometer set at 360 nm excitation and 450 nm emissions.

Determination of total aflatoxin abilities of Aspergillus flavus, A. parasiticus and A. tamarii isolates

The aflatoxin-producing ability of the isolates was examined by cultivating fungal strains in Czapek Yeast extract agar (Ben Fredj et al., 2009) medium for 5 days at 25±2°C. Three replications were maintained for each isolate for each media. Total AFs were extracted by grinding the moldy agar (20 g) in a Waring blender for 5 min with methanol (100 ml) containing 0.5% NaCl and the above-mentioned procedure was applied.

Molecular detection of mycotoxin-producing genes

The isolation of DNA from mycelia was performed according to the method described by Farber et al. (1997). Four published primer sets were used for the specific detection of *nor-1, ver-1, omt-A* and *aflR* genes (Criseo et al., 2008). The 400, 537, 797 and 1032 bp fragments were amplified, respectively. A typical PCR was carried out under the following condition: 5 µl of genomic DNA were used as tem-

plate (2 μg ml^{-1}), 0.5U EuroTaq polymerase (Euroclone, Pero-Milan, Italy), 1 × reaction buffer, 2.5 mM $MgCl_2$, 200 μM each dNTP and 7.5 pmol each primer in a total reaction volume of 50 μl. A total of 35 PCR cycles with the following temperature regimen was performed: 95°C, 1 min; 65°C, 30 s; 72°C, 30 s for the first cycle; and 94°C, 30 s; 65°C, 30 s; 72°C, 30 s for the 34 left (Criseo et al., 2008). PCR products were separated on a 1.3% (wt/vol) agarose gel, stained with ethidium bromide.

RESULTS AND DISCUSSION

Mycobiota of different chili samples

Fungal growth may occur before or during drying, during storage or shipping of spices as reported by several researchers (Freire et al., 2000; Elshafie et al., 2002; Hashem and Alamri, 2010). The mycobiota of different chili products marketed in Taif city (Saudi Arabia) were investigated in this study. In general, crushed chili samples were more contaminated than other samples ($11.335X10^3$ mean total CFU/g of crushed chili) as shown in Table 1. Santos et al. (2011) reported that smoked paprika samples were more contaminated with fungi than other samples (1.9×10^3 CFU/g in DG18). In addition, they reported that paprika and chili samples were less contaminated (3.8×10^2 CFU/g of paprika, 1.3×10^2 CFU/g of chili).

Eleven genera comprised of a total of 26 species were recovered from 60 samples of chili collected from retail markets and restaurants in Taif city during 2013 (Table 2). The predominant mycobiota of chili samples, taking into account their occurrence and abundance, respectively, were *Aspergillus* sp. (65.49%, 70%), *Eurotium* sp. (9.92%, 40%), *Penicillium* sp. (7.80%, 30%), *Mucor racemosus* (3.78%, 15%), *Alternaria alternata* (3.55%, 16.67 %), *Mycosphaerella tassiana* (3.31%, 13.33 %) and the remaining genera were isolated in low or moderate frequencies that comprised collectively 6.15%. The same results were obtained by Hashem and Alamri (2010) in their studies of mycobiota of chili in the Aseer region (Saudi Arabia); they isolated *Aspergillus awamori, A. flavus, A. niger , A. ochraceus, A. amarii, Eurotium repens, Paecilomyces lilacinus, Penicillium arenicola, P. corylophilum, P. dunkii, P. funiculosum, P. oxalicum, P.waksmani, Rhizopus stolonifer, Scopulariopsis brevicaulis, Trichoderma harzianum and Ulocladium botrytis from pepper samples in PDA medium*. Atanda et al. (1990) isolated seven mold species from dry 'tatase' pepper and *Aspergillus niger, Aspergillus flavus* and *Geotrichum candidum* were the most prevalent species. Adegoke et al. (1996) isolated *Rhizopus oryzae, Aspergillus niger, A. flavus, Geotrichum candidum* and *Saccharomyces* sp. as dominant fungi from *Capsicum annum* and *C. frutescens*.

The isolated fungal species in the present work were isolated previously from chili fields in four different districts (Thiruvarur, Nannilam, Kudavasal and Valangaiman) in India (Gomathi et al. 2011). In addition, they were recovered as field and storage fungi from black and white pepper from Amazonia (Freire et al., 2000).

Table 3 shows the co-occurrence of *Aspergillus* species in chili samples, where 30% of the samples were not contaminated with *Aspergillus* species, but 70% were. 13.33% of the samples were contaminated by 1 species, 18.33% by 2 species, and 18.33% by 3 species. The percentage of samples contaminated by 4 species was 16.66 %, while 3.33% of the samples were contaminated by 5 species. The high co-occurrence of aspergilli may increase the chances of AF contamination. In Spain, Sardiñas et al. (2011) reported that the percentage of chili samples not contaminated with aspergilli was 11 (35.5%), while 9 (29.0%) and 11 (35.5%) samples were contaminated with 1 and 2 *Aspergillus* species, respectively.

Among these *Aspergillus* species, only three (*A. flavus, A. parasiticus*, and *A. tamari*) were well known as aflatoxin producers. They were isolated from 30, 2 and 3 out of 60 samples of different chili products, respectively (Table 4). Using PCR technique, Santos et al. (2011) proved that *A. flavus* DNA was detected in 82% of chili, 60% of paprika and in all smoked paprika samples, while the *A. parasiticus* DNA was detected in only one chili sample.

Table 1. Mean values of total fungal counts, occurrence of *Aspergilli* on three different chili products and incidence range of total aflatoxins (AFs) in chili products.

Sample types	Total fungal counts (CFU/g) Mean value ± Standard deviation	*Aspergillus* spp. occurrence (%)	Positive samples *	Range of AFs (PPB)	Mean of AFs (PPB) ± Standard deviation
chili sauce	49 ± 11.0	5	0	0	0
crushed chili	11335 ± 65.0	35	16	20 - 170	15± 0.9
chili powder	9771 ± 42.0	27	18	35 - 200	17± 2.1
Total	21155 ± 74	67	34	0 - 200	16 ± 3

* Out of 20 samples from each type of chili product

Table 2. Occurrence, average total counts (calculated per g sample), abundance, number of cases of isolation and frequency of collected species from 60 of different chili samples.

Fungal genera & species	Chili products*	ATC	Abundance	NCI	F%
Alernaria alternata	C+P	750	3.55	10	16.67
Aspergillus	S+C+P	13855	65.49	42	70
A. candidus	S+C+P	150	0.71	5	8.33
A. carbonarius	C+P	600	2.84	8	13.33
A. flavus	S+C+P	11500	54.36	30	50
A. niger	S+C+P	850	4.02	13	21.67
A. parasiticus	C+P	150	0.71	2	3.33
A. niveus	C	5	0.02	1	1.67
A. ochraceus	S+C	150	0.71	2	3.33
A. tamarii	C+P	250	1.18	3	5
A. terreus	C	50	0.24	1	1.67
A. ustus	C	150	0.71	2	3.33
Botrytis cinirea	C+P	100	0.47	2	3.33
Eurotium	S+C+P	2100	9.92	24	40
E. amstelodami	S+C+P	900	4.25	12	20
E. chevalieri	S+C+P	500	2.36	6	10
E. repens	C+P	400	1.88	3	5
E. rubrum	C+P	300	1.42	5	8.33
Fusarium oxysporum	S+C+P	550	2.60	7	11.67
Mucor racemosus	S+C+P	800	3.78	9	15
Mycosphaerella tassiana	C+P	700	3.31	8	13.33
Penicillium	S+C+P	1650	7.80	18	30
P.chrysogenum	S+C+P	1000	4.73	10	16.67
P.corylophilum	C+P	300	1.42	4	6.67
P. citrinum	C	150	0.71	2	3.33
P. griseofulvum	C+P	200	0.95	3	5
Rhizopus stolonifer	C+P	400	1.88	5	8.33
Scopulariopsis brevicaulis	C	200	0.95	2	3.33
Ulocladium chartarum	C	50	0.24	1	1.67
Total		21155	100		

*S = chili sauce, C = crushed chili and P = chili powder

Natural occurrence of total aflatoxins in chili samples

Thirty-four samples out of 60 were naturally contaminated with aflatoxins (Table 1). The percentages of contaminated samples were 80 and 90% of crushed and powdered chili, respectively. The concentration of AFs in crushed chili samples ranged from 20-170 ppb, while this range was from 35 to 200 ppb from

Table 3. Co-occurrence of *Aspergillus* species in chili samples. Values in brackets are percentages of the total samples analyzed.

Samples	No. species per sample (%)					
	0	**1**	**2**	**3**	**4**	**5**
chili sauce	15 (75)	2 (10)	3(15)	0	0	0
crushed chili	1 (5)	3 (15)	4 (20)	5 (25)	6 (30)	1 (5)
chili powder	2 (10)	3 (15)	4 (20)	6 (30)	4 (20)	1 (5)
Total	18 (30.00)	8(13.33)	11(18.33)	11 (18.33)	10 (16.66)	2 (3.33)

Table 4. The frequencies of occurrence of mycotoxigenic species detected in this study.

Samples	Mycotoxigenic species		
	A. flavus	***A. parasiticus***	***A. tamarii***
chili sauce	1	0	0
crushed chili	14	1	1
chili powder	15	1	2
Total	30	2	3

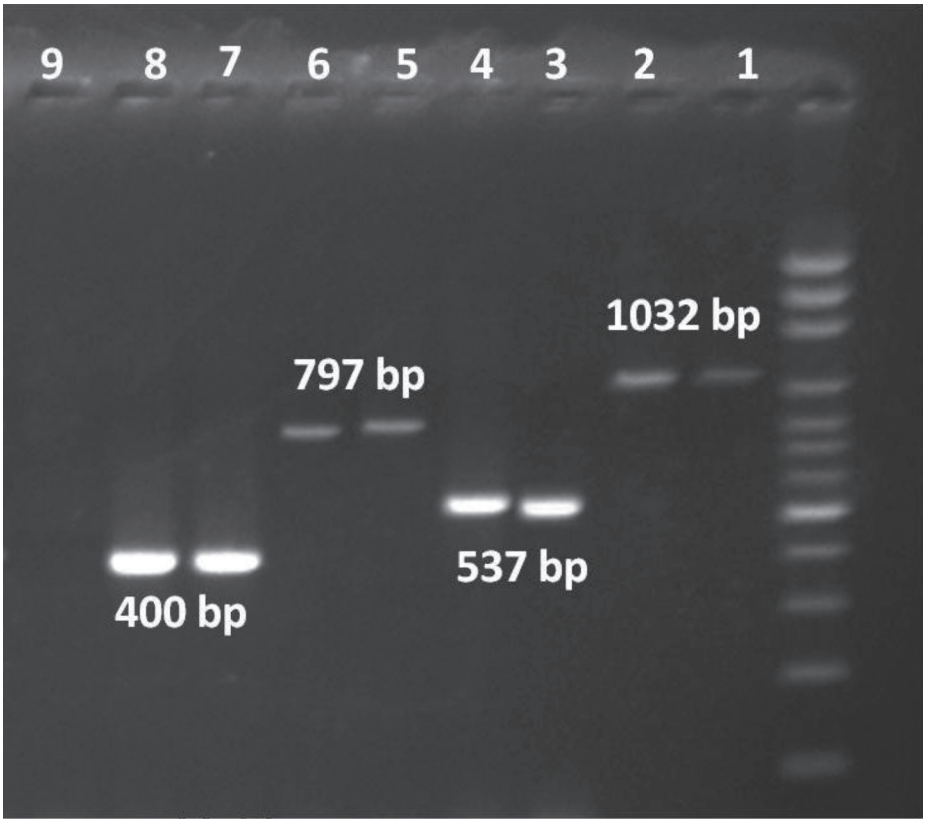

Fig. 1. Agarose gel electrophoresis of PCR products of four DNA fragments specific for *aflR, nor-1, ver-1,* and *omt-A* genes. Lane 1: *Aspergillus flavus* (*aflR* gene); Lane 2: *Aspergillus parasiticus* (*aflR*); Lane 3: *A. flavus* (*ver-1*); Lane 4: *A. parasiticus* (*ver-1*); Lane 5: *A. flavus* (*omt-A*); Lane 6: *A. parasiticus* (*omt-A*); Lane 7: *A. flavus* (*nor-1*); lane 8: *A. parasiticus* (*nor-1*), Lane 9: *A. tamarii.*

Table 5. Frequency of single genes in *Aspergillus flavus*, *A. parasiticus* and *A. tamarii* isolates collected from chili samples.

Strains code	Source of isolation	Total Aflatoxins PPM	Aflatoxin genes			
			aflR	omt-A	ver-1	nor-1
TUAf1	chili powder	23.0	+	+	+	+
TUAf2	crushed chili	34.5	+	+	+	+
TUAf3	chili powder	35.6	+	+	+	+
TUAf4	chili powder	37.3	+	+	+	+
TUAf5	chili powder	25.5	+	+	+	+
TUAf6	crushed chili	-	-	+	-	+
TUAf7	chili powder	42.3	+	+	+	+
TUAf8	crushed chili	-	+	-	-	-
TUAf9	crushed chili	36.5	+	+	+	+
TUAf10	chili powder	41.3	+	+	+	+
TUAf11	chili powder	43.5	+	+	+	+
TUAf12	chili powder	36.5	+	+	+	+
TUAf13	crushed chili	-	+	-	+	-
TUAf14	chili powder	40.3	+	+	+	+
TUAf15	chili sauce	-	+	+	+	-
TUAf16	crushed chili	23.7	+	+	+	+
TUAf17	chili powder	33.6	+	+	+	+
TUAf18	crushed chili	31.5	+	+	+	+
TUAf19	chili powder	45.8	+	+	+	+
TUAf20	crushed chili	-	-	+	-	+
TUAf21	crushed chili	-	+	+	-	-
TUAf22	chili powder	42.5	+	+	+	+
TUAf23	chili powder	48.2	+	+	+	+
TUAf24	crushed chili	33.5	+	+	+	+
TUAf25	crushed chili	-	-	+	-	-
TUAf26	crushed chili	36.1	+	+	+	+
TUAf27	crushed chili	-	-	-	-	+
TUAf28	chili powder	49.5	+	+	+	+
TUAf29	chili powder	41.3	+	+	+	+
TUAf30	crushed chili	-	+	+	+	+
TUAp1	crushed chili	173	+	+	+	+
TUAp2	chili powder	210	+	+	+	+
TUAt1	crushed chili	2.3	-	-	-	-
TUAt2	chili powder	3.6	-	-	-	-
TUAt3	chili powder	2.5	-	-	-	-

+ = PCR amplification signal present.
- = PCR amplification signal absent.

powdered chili samples. The results of this study indicated that there is no aflatoxin contamination in sauce samples, while mean AF levels in powdered and crushed chilies were 17 and 15 ppb, respectively (Table 1). Hell et al. (2009) reported that chili samples were naturally contaminated with aflatoxin B1 and aflatoxin B2, at concentrations of 3.2 μg/kg in Benin, Mali and Togo. In Pakistan, Russell and Paterson (2007) studied aflatoxin contamination in chili samples from Pakistan. They reported that all investigated chili samples contained with high levels of aflatoxin B1. Zinedine et al. (2006) analyzed spice samples for aflatoxins (AFs) and the average contaminations found for AFB_1 were 0.09, 0.63, 2.88 and 0.03 μg/kg for black pepper, ginger, red paprika and cumin, respectively. The effect of chili season on contamination with aflatoxins was studied by Iqbal et al. (2011) in Pakistan. Their results reported that limits of detection and quantification for AFB1 and AFG1 were 0.05 μg/kg and 0.50 μg/kg, whilst

for AFG2 and AFB2 they were 0.10 µg/kg and 0.60 µg/kg. In the winter samples, AFs were detected in 18 (72%) whole and 14 (60%) ground chilies, with concentration ranges of 0.00-52.30 µg/kg and 0.00-74.60 µg/kg, respectively. Recently, Khan et al. (2014) collected 331 red chili samples (226 whole, 69 powdered and 36 crushed) from all over Pakistan for the estimation of total aflatoxin contamination by thin layer chromatography (TLC). Their results indicated that mean AF levels in whole, powdered and crushed chilies were 11.7, 27.8 and 31.2 µg/kg, respectively.

Total aflatoxin potentials of Aspergillus flavus, A. parasiticus and A. tamarii isolates

Thirty-five isolates of *A. flavus* (30 isolates), *A. parasiticus* (2) and *A. tamarii* (3) were subjected to detection of their aflatoxigenic potentials. Analysis of the toxigenic potentials of the tested isolates revealed that they varied in their abilities to produce total aflatoxins. Thus, although some isolates were toxigenic, others exhibited no detectable toxin production (Table 5). Twenty-one isolates (70%) of *A. flavus* isolated from powdered (15 isolates) and crushed (6 isolates) chilies showed aflatoxin potentials. The level of AF productions by *A. flavus* fluctuated from 5 to 12 ppm. All *A. parasiticus* (2 isolates) and *A. tamarii* (3) were aflatoxigenic. The ranges of AFs produced by *A. parasiticus* and *A. tamari* were 173-210 and 2.3-3.6 ppm, respectively (Table 5). In the Sultanate of Oman, Elshafie et al. (2002) recorded that nine isolates (45%) of twenty *A. flavus* strains isolated from species screened for aflatoxins were aflatoxigenic. Rajasinghe et al. (2009) reported that some *A. flavus* strains isolated from chili powder in Sri Lanka showed aflatoxin potentials. Approximately 68% of *A. flavus* isolates from different pepper types in Nigeria produced aflatoxins in neutral red desiccated coconut agar (Ezekiel et al., 2013). Saadullah (2013) reported that 91 of 113 isolates of *A. flavus* and all isolates of *A. parasiticus* (80 isolates) isolated from dried vine fruits in Iraq were aflatoxigenic, while none of the *A. tamari* isolates (18) was aflatoxigenic. In Iraq, Mohammed et al. (2010) showed that 81.8% of isolates of *A. flavus* from different agricultural commodities had aflatoxigenic ability. In Saudi Arabia, Gashgari et al. (2010) reported that 7 isolates of 29 *A. flavus* strains isolated from wheat flour were aflatoxigenic. In addition, seven out of 18 *A. flavus* isolates from date palm in Saudi Arabia had AF potentials (Gherbawy et al., 2012).

PCR amplification of the aflatoxin structural genes

Twenty-five identified genes clustered within a 70-Kb DNA region in the chromosome are involved in the biosynthesis of AFB1 and their DNA sequences have been published (Yu et al., 2004, Criseo et al., 2001a,b, Scherm et al., 2005). PCR was used for the detection of aflatoxigenic aspergilli based on the intermediated enzymes including the norsolorinic acid reductase-encoding gene *nor-1*, the versicolorin A dehydrogenase-encoding gene *ver-1*, the sterigmatocystin 0-methyltransfrase encoding gene *omt-1* and the regulatory gene *aflR* (Erami et al., 2009).

Polymerase chain reaction (PCR) was applied using four sets of primer for different genes involved in aflatoxin biosynthetic pathway. Bands of the fragments of *aflR, omt-1, ver-1* and *nor-1* genes can be visualized at 1032, 797, 537 and 400 bp, respectively (Fig. 1). All aflatoxigenic and non-aflatoxigenic examined isolates yielded a different DNA banding patterns with a number of bands ranging from zero to four (Table 5).

Twenty out of thirty aflatoxigenic *A. flavus* isolates (46.66%) showed DNA fragments that corresponded to the complete set of genes. In addition, *A. parasiticus* isolates (2) showed the complete set of investigated genes (Table 5). The presence of four targeted genes confirmed the abilities of isolates to produce aflatoxins as previously mentioned by other researchers (Geisen, 1996; Criseo et al., 2001a, b; Scherm et al., 2005; Rashid et al., 2008; Gherbawy et al., 2012).

Isolate TUAf30 isolated from crushed chili showed the complete set of investigated genes, although this isolate is non-aflatoxigenic. These results in agreement with the results of Criseo et al. (2001b) who detected some *A. flavus* strains that showed a

complete set of genes but do not produce aflatoxins. Yang et al. (2004) reported different results by multiplex-PCR and enzyme-linked immunosorbent assay (ELISA). They stated that although *norA, ver1, omtA* and *avfA* (*aflR*) genes had been detected in all tested strains, some of these samples were negative for aflatoxin detection.

The results of this work indicated clearly that the presence of four tested genes is not a sufficient marker for the differentiation between aflatoxigenic and non-aflatoxigenic isolates. Other studies (Flaherty and Payne, 1997; Chang et al., 1999 a,b, 2000; Cary et al., 2002; Takahshi et al., 2002; Ehrlich et al., 2003) suggest that the regulation of aflatoxin biosynthesis in *Aspergillus* spp. involves a complex pattern of positive- and negative-acting transcriptional regulatory factors, which are affected by environmental and nutritional parameters. Geisen (1996) suggests that the lack of aflatoxin production could also be due to simple mutations, including the substitution of some bases, and Liu and Chu (1998) suggested that a variety of different physiological conditions affect aflatoxin biosynthesis.

A second group constituted of one isolate (12.5%) of non-aflatoxigenic *A. flavus* isolates showed three DNA banding pattern corresponding to *aflR, omt-A* and *ver-1* (Table 5). A third group of 4 (20%) non-aflatoxigenic *A. flavus* isolates yielded two DNA banding pattern. *Omt-A* and *ver-1* were amplified from 2 isolates, TUAf13 contained *aflR* and *ver-1,* while TUAf21 showed *aflR* and *omt-1* genes. The last group contained 2 isolates, TUAf8 and TUAf27, that showed the presence of *aflR* and *nor-1* genes, respectively (Table 5). Criseo et al. (2001b) reported that aflatoxin biosynthesis in *A. flavus* is strongly dependent on the activities of regulatory proteins and enzymes encoded by four genes – *aflR, nor-1, ver-1* and *omt-A*. By using specific PCR-based methods, they proved that the aflatoxigenic *A. flavus* isolates always show the complete gene set, whereas non-aflatoxigenic isolates lacking one, two, three or four PCR products indicated that the genes do not exist in these strains or that the primer binding sites changed.

Generally, *omt-A* was the most prevalent gene in the isolates tested in this study. This gene was recovered from 27 isolates of *A. flavus* (out of 30) and 2 isolates of *A. parasiticus*. *Nor-1, aflR* and *ver-1* were recovered from 25, 26 and 24 isolates of aflatoxigenic and non-aflatoxigenic isolates of *A. flavus*, respectively. In Italy, Criseo et al. (2008) used 134 non-aflatoxin producing strains of *A. flavus* isolated from food, feed and officinal plants to study the different genes involved in the aflatoxin biosynthetic pathway. Their results indicated that *nor-1* was the most representative (88%) of the four aflatoxin genes, followed by *ver-1* and *omt-A* that were found with the same frequencies (70.1%); a lower incidence (61.9%) was found for *aflR*.

The PCR protocol used in this study failed to amplify any DNA pattern for the targeted genes in *Aspergillus tamari* isolates (Fig. 1). On the contrary, under the high stringency conditions, *A. tamarii* DNA hybridized to all four of the *A. flavus* and *A. parasiticus* gene probes, indicating strong similarities in the biosynthetic pathway genes of these three species (Klich et al., 2000).

The presence of aflatoxigenic aspergilli in chili products with aflatoxins indicates that further investigation is needed for monitoring and routine analysis. Furthermore, proper harvesting, drying, handling, storage and transport conditions need to be implemented.

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