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Fungi and mycotoxins associated with egyptian sorghum grains

Abstract

The aim of the present work was to study the infected fungi and the mycotoxins contamination in Egyptian sorghum grains collected from different governorates. Forty eight sorghum grain samples were collected from six governorates (Cairo, Kaliobia, Gharbia, Alexandaria, Assute, and Sohage). Isolation and identification of fungi and the determination of mycotoxins (i.e. Aflatoxins Afs and Fumonisin FB_1) were carried out. The results revealed that all samples were infected with at least one of fungi species. Nineteen fungi species belonging to five genera were isolated and identified from the investigated sorghum grain samples. Moreover, the result indicated that samples collected from Assute governorate were found to be more infected compared with the other governorates, meanwhile; samples collected from Kaliobia were found to be the lesser infected. All Aspergellus parasiticus and Fusarium moniliform isolated from sorghum grains were found to have the ability to produce Afs and FB_1. In the same respect, results showed that aflatoxins were detected in 33.3 % (16 out of 48) of the investigated samples with levels ranged from 0.17µg/kg to 499µg/kg, while FB_1 was detected in 16.6 % (8 out of 48) of investigated samples with levels ranged from 7.2 to 129.5µg/kg. It is worthy to mention that the contamination levels of the investigated samples with aflatoxins were found below the Maximum Residue Limits (MRLs) established by International agencies or by the Egyptian standards.

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Introduction

Mycotoxin contamination of agriculture commodities has become a natural phenomenon in many parts of the world. This may be due to favorable environmental condition prevalent in those regions coupled with the traditional method of crop cultivation, harvesting, handling and storage, all of which ultimately lead to severe mold growth and mycotoxin production in these agriculture commodities. The most frequently contaminated foods with mycotoxins producing molds include sorghum, corn, and wheat grains.[1] The isolated fungi were found to have the ability to produce Aflatoxin B_1 AFB_1 and T_2 toxin. The risk of sorghum contamination by mycotoxins is related to mycoflora associated with the sorghum grain. The genus Fusarium was the most prevalent component of the internal seed borne mycoflora, genera Alternaria, phoma, penicillium and Aspergillus were also isolated whereas, the predominant Fusarium was Fusarium moniliforme and the most frequently isolated species of Alternaria, Phoma. Penicillium and Aspergillius were Alternaria, Penicillium sorghuma, Penicillium funiculosum and Aspergillus flavus, respectively. Diener et al.,[2] Gonzalez et al.[3] Outbreaks of aflatoxicosis in farm animals have been reported from many areas of the world. The liver is mainly affected in such outbreaks and also in experimental studies on animals, including nonhuman primates. The acute liver lesions are characterized by necrosis of the hepatocytes and biliary proliferation, and chronic manifestations may include fibrosis. A feed level of aflatoxin as low as 300µg/kg can induce chronic aflatoxicosis in pigs within 3-4 months Kusak et al.[4] In animals, ingested aflatoxins may be metabolically degraded. Aflatoxin B_1 may be converted into aflatoxin M_1 which may occur in the milk. The concentration of aflatoxin M_1 in the milk of cows is about 300 times lower than the concentration of Aflatoxin B_1 consumed in the feed. In certain experimental animals, only small amounts of administered aflatoxins have been found in tissues, 24h after injection.[5,6] Aflatoxin B_1 is a liver carcinogen in at least 8 species including nonhuman primates. Dose-response relationships have been established in studies on rats and rainbow trout, with a 10% tumour incidence estimated to occur at feed levels of aflatoxin B_1 of 1µg/kg, and 0.1µg/kg, respectively.[7] In some studies, carcinomas of the colon and kidney have been observed in rats treated with aflatoxins.[8] Aflatoxin B_1 causes chromosomal aberrations and DNA breakage in plant and animal cells and after microsomal activation, gene mutations in several bacterial test systems.[9] In high doses, it may be teratogenic.[10] Fumonisin B_1 has been detected in maize and maize-based products worldwide at mg/kg levels, sometimes in combination with other mycotoxins. Concentrations at mg/kg levels have also been reported in food for human consumption.[11] Available correlation studies from the Transkei, South Africa, suggested a link between dietary fumonisin exposure and oesophageal cancer.[12] It is worthy to report that sorghum grain is one of the most popular feed and food in Egypt, questions arise concerning the detection and the prevalence of Aflatoxins and Fumonisin B1 and toxigenic fungi in Egyptian sorghum grain. This study attempted to assess some crop situation with respect to Aflatoxin and Fumonisin B_1 through detecting the residues of these toxins as well as the incidence of toxigenic and other fungi in Egyptian sorghum grain. However attention was focused on Fusarium moniliform and Aspergillis parasiticus being the most common fungi responsible of Fumonisin and Aflatoxin production. In addition the Toxigenicity of isolated Fusarium moniliform and Aspergillis parasiticus were studied.

Materials and methods

Materials

Sorghum grain samples: Total of 48 sorghum grain samples were collected from different Egyptian Governorates (i.e. Cairo, Kaluobia. Al-Gharbia, Alexandria, Assute and Sohag) One kilogram of each sample was stored in polyethylene bag for the isolation of fungi and determination of Aflatoxins and Fumonisin B_1.

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Standard: Fumonisin B_1 (FB_1) and Aflatoxins B_1 (AFB_1), (AFB_2), (AFG_1) and (AFG_2) standards were purchased from Sigma Chemical Co. (St. Louis, Mo. U.S.A.).

Thin layer chromatography (TLC) plates: TLC aluminum sheets (20 X20 cm) with 0.2 mm thickness of silica gel G60, without fluorescent indicator were purchased from Merck Co, (Darmstadt, Germany).

Sep- pack cartridge C18 columns: Sep- pack cartridge C18 columns were purchased from Waters Company (Water Company USA).

Media used: Potato dextrose agar (PDA) was purchased from Sigma-Aldrich France.

Methods

Isolation and identification of fungi associated with Egyptian sorghum grain: Potato dextrose agar medium (PDA), according.[13] Culture media of Potato Dextrose Agar (PDA) was dissolved in distilled water by bringing to boil and autoclaved for 15min at 121°C. each sorghum grain samples (25 grains) were immersed in 2.5% sodium hypochlorite solution. After 2 min the sodium hypochlorite solution was drained off and the sterilized grains washed twice with sterilized distilled water. Distilled water was drained off, and then the grains were dried. Disinfected grains of each sample were plated Petri dishes (five grains / dish), and incubated for 5 day at 25°C and reported as mold count per ml of product.

Identification of the isolated fungi: The purified strains maintained on PDA slants were identified according to Nelson et al.[14]

Production of Aflatoxin by isolated Aspergillus parasiticus strains: Corn grains were moistened to 18% and artificially infected with isolated Aspergillus parasiticus. The infected substrate was incubated at 25°C for 15 days as recommended by Stubblefied et al.[15]

Production of FB_1 by isolated fusarium moniliforme strains: One hundred gram of corn grains were transferred into Erlenmeyer flask, 29ml distilled water were added to the flask to adjust the moisture content to 43% and autoclaved at 121°C for 15min. The autoclaved corn grains were then inoculated with 1ml of spore suspension of the tested Fusarium moniliforme isolates and incubated in the dark for 28 days at 25°C (flasks were shaked daily during the first incubating week). After incubation period, corn grains cultures were dried over night at 50°C and finally ground with a blender and stored at 4°C for analysis.[16]

Detection and determination of aflatoxins in sorghum grain samples

Preparation of working solution of aflatoxins: Diluted portions of stock solution to spotting concentration (0.5µg /ml) using the same solvent used to prepare Aflatoxins standards. Standard solutions of Aflatoxins B_1, B_2, G_1 and G_2 were stable for more than one year.

Extraction method

Fifty grams of blended sorghum grains powdered representative sample was taken in a 1L conical flask. Twenty five ml water, 25gm diatomaceous earth (celite) and 250ml chloroform were added. The flask was shaked for 30min to extract the toxin. The content of the flask was filtered through filter paper. The first 50ml of filtrate were collected.

Clean-up procedures

Preparation of column chromatography: A ball of glass wool loosely placed in bottom of 22x300mm chromatographic column and 5gm of sodium sulphate anhydrous was added to give base for silica gel. The glass column was filled by 40-50ml of chloroform and 10g of activated silica gel was added to the column. Finally 15g of sodium sulphate anhydrous was added to be the top surface of the column. Then, 50ml of the sample extract was applied to the column. One hundred fifty ml of n hexane was used for de-fating followed by 150ml diethyl ether for de-pigmentation at a flow rate of 5ml/min. One hundred fifty ml of chloroform: methanol (97:3) was used to eluate the Aflatoxins from the column at a flow rate of 5ml/min. The elution was concentrated using a rotary evaporator to 1ml and directly transferred to a vial and dryness at 40°C to dry film.

Preliminary TLC: The vial containing dray extract residue was uncapped and 200ul benzene - acetonitrile (98+2) was added and reseal with stopper. The vial containing extract was shaked vigorously to dissolve. The stopper was punctured to accommodate needle of 10ul syringe. In subdued incandescent light and as rapidly as possible 5µl was spotted on the imaginary line 3cm from bottom of TLC plate. The vial was kept for quantitative analysis. On the same plate 5µl of Aflatoxin standards were spotted.

Fifty ml acetone: CHCl3 (1:9) were placed in the trough of the unlined developing tank. Only 1 plate per tank was used. Immediately the plate was into the tank and was sealed. The chamber was saturated with solvent before use. The plate was developed 40 minutes or until Aflatoxins reach top of the plat and then it was removed from the tank, the solvent was evaporated at room temperature and the plate was viewed under long wave UV lamp in a viewing chamber.

Determination of aflatoxins by HPLC technique

Derivatization: The derivatives of samples and standards were done as follow: Fifty µl trifluoracetic acid(TFA) were added to the dray film of standard and samples and the mixture was let to stand for 15min followed by 450µl H_2O: cH3cN (9:1 v/v) and they were mixed well by vortex for 30s and the mixture was left to stand for 5min. In this step of reconstitution of the dry film, AFB_1 and AFG_1 were converted into other derivatives, AFB_2a and AFG_2a, respectively (Aflatoxins G_1 and B_1 have low florescence properties, therefore, they were converted to Aflatoxin G_2a and B_2a, which have high fluorescence properties, using trifluoracetic acid).[17]

HPLC conditions: The HPLC instrument used for Aflatoxins determination was waters (474) system, equipped with quaternary pump fluorescence detector set system at 360nm excitation and 440nm emission wavelengths. The chromatography column was phenomenex C18. The mobile phase system (H_2O: CH_3OH:CH_3CN, 30:60:10 v/v/v) was isocratically at a flow rate of 1ml/min. Data were collected and integrated using (Totalchrom Navigator) Chromatography Manager Software.

Detection and determination of FB1 in sorghum grain samples

Stock standard of FB_1: One mg of FB_1 standards was dissolved in 1ml of Acetonitrile ACN: H_2O (1:1, v/v).

Working standard solution: The working standard solution was prepared from stock standard solution by transferring 100µl, 50µl, 25µl, and 10µl to 4 vial then 900µl, 950µl, 975µl, and 990µl, of

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ACN:H_2O (1:1,v/v) were added respectively. The obtained standard solutions contained 100µg/ml, 50µg/ml, 25µg/ml, and 10µg/ml FB_1, respectively.

Extraction and cleanup of FB_1: The extraction and cleanup of FB1 from sorghum grain samples was carried out according to.[17] Fifty grams of finely ground sorghum sample were transferred into an Erlenmeyer flask with 100ml ACN: H_2O (1:1, v/v). The flask was shaked for 30min to extract the toxin. The content of the flask was filtered through filter paper. Two ml of the filtered extract was combined with 5ml aqueous 1% KCL and applied to a preconditioned C18 Sep-Pak column. The column was washed with 5ml 1% aqueous KCL followed by 5ml acetonitrile. Applied extract on C18 was washed with 5ml KCL 1%, followed by 2ml aqueous AcN: KCL (1:9), and the eluants were discarded. Fumonisin B_1 was eluted with 4ml AcN: H_2O (7:3) and evaporated to dryness.

Derivatization and HPLC analysis

Preparation of derivatization reagent: Forty mg of o-phtaldialdehide OPA were dissolved in 1ml methanol and diluted with 4ml disodium tetra borate (0.1M) then 50µl 2-mercaptoethanol were added. The reagent solution was stored in aluminum foil covered vial and was stored for no more than 1 week at room temperature in dark.

Preparation of standard derivatives: Fifty µl of FB_1 working standard solution were transferred to base of small vial, then mixed with 225µl OPA reagent and 10µl of reacted mixture were injected to HPLC within 1min (It was critical to adhere to reproducible time between addition of OPA reagent and injection into HPLC system). Fluorescence of OPA- Fumonisin beginning to decrease after 2min.

Preparation of sorghum extract derivatives: The purified dry film residue of sample extract was dissolved in 200µl methanol. Fifty µl of this extract were transferred to base of small vial, then 225µl OPA reagent were added, mixed, and 10µl derivative was injected to HPLC within 1min of adding OPA reagent.

Determination of FB_1 by HPLC: The method described by Shephard et al.[18] was used for the determination of FB_1 in samples by HPLC.

Chromatography conditions

Stationary phase: Column Hyper Clone 5µ ODS (C18) 120A°, DIM: 250x4.60mm. (Phenomenex).

Mobile phase: Methanol: 0.1M NaH2PO4 (75:25, v/v) isocratic system adjusted to pH 3.35 by the addition of phosphoric acid and was filtered through membrane filter and it was pumped at 1ml/min flow rate.

Detector: Fluorescence detector (Excision 335 nm and Emission 450nm).

Quantitation

The mixed solutions of standard as well as sample extract after derivatization were filtered through a 0.22µm membrane filter and loaded (50µl) into a 200µl injection loop. FB_1 contents in sample were calculated from chromatographic peak areas using the standard curve.

Statistical analysis

All data were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System.[19] The significance of the differences among results was determined by Waller-Duncan k-ratio.[20] All statements of significance were based on probability of $P<0.05$.

Results and discussion

The total fungal counts and percentages of fungal infection in sorghum grain samples

The occurrence and infection fungi of sorghum grains collected from different governorates (Cairo, Kaluobia, Gharbia, Alaxandaria, Assute and Sohage) were carried out. The results presented in (Tables 1&2) indicated the total fungal counts and percentage infection of sorghum grain samples. The total fungal count isolated from the sorghum grains collected from the aforementioned governorates were 313 isolates. Nineteen species belonging to five genera were isolated and identified from 48 samples of sorghum grains. Aspergillus, Alternaria, and Fusarium genera were the most frequently and were abundance greater than the other genera of fungi. The present results indicated that sorghum samples collected from Assute governorate were the most infected with molds since the percentage of infection was 19.8%, followed by Alexandaria 18.5%, this may be due to the increase of moisture content and or bad storage in these governorates, while, the infection of Cairo, Sohage, Gharbia and Kaliobia governorates were 16.9, 15.9, 14.3, 14.3% respectively. Data presented in (Table 2) percentage of isolated fungi species which occurred in sorghum grain samples collected from different governorates and the results indicated that among the detected fungi, Aspergillus group was the most prevalent fungi in all examined samples. Within these speacies, Aspergillus Niger was the predominant and comprised 54.3% of the total fungal count of the isolates, followed by Aspergillus flavus, which was comprised 14.6%, Alternaria was comprised 14.6%. Whereas, Fusarium spp and Aspergellus parasiticus represented 4.7% and 4.1 respectively. The other species included Penicillium, Aspergillus ochrecus, Helminthosporium sativum, Aspergillus terreus, Aspergellus chevalier, Aspergellus humicola, Nigrospora sphaerica and Cephalosporium acremonium represented 2.5%, 1.2%, 1.2%, 0.36%, 0.36%, 0.31%, 0.31% of the total fungal count respectively. These data revealed that the percentage infection of sorghum grains with fungi was high. This may be probably attributed mainly to the bad storage condition. These results were coincided with those reported by many investigators Diener et al.,[2] Gonzaleze et al.,[3] Bhat et al.[21]

Table 1 %Number of isolated fungi spices from sorghum grains collected from different governorates

Governorates	Fungi isolate	% of TFC of each governorate
Cairo	53	16.9
Kaliobia	45	14.3
Gharbia	45	14.3
Alexandaria	58	18.5
Assute	62	19.8
Sohage	50	15.9

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Table 2 Isolated fungal species and percentage of occurrence in sorghum grains collected from different governorates

Type of fungi	No. of isolate fungi	% of infection
Aspergillus Niger	170	54.3
Aspergillus flavus	46	14.6
Alternaria spp	46	14.6
Fusarium moniliform	3	4.6
Fusarium semiteetum	2	0.95
Fusarium graminearum	2	0.63
Fusarium equseti	2	0.63
Fusarium solani	2	0.63
Fusarium avenaceum	2	0.63
Penicillium sp	8	2.55
Aspergillus parasiticus	15	4.1
Aspergillus ocracues	4	1.2
Helminthosporium Sativium	4	1.2
Aspergillus terreus	2	0.63
Aspergillus chevalier	2	0.63
Aspergillus humicola	1	0.31
Nigrospora sphaerica	1	0.31
Sephalosporium acremonium	1	0.31

Toxin production by some toxigenic fungi isolated from sorghum grains collected from different governorates

The ability of the isolated fungi to produce mycotoxins was examined in the current study. The results presented in (Table 3) indicated that Aspergillus parasiticus isolated from sorghum grain samples collected from Cairo has the ability to produce aflatoxin in a concentration ranged from 0.03 to 0. 80µg/kg sorghum grains. Whereas other strain was found to produce aflatoxins in concentrations reached 0.02, 0.26, 0.01 and 2.98µg/kg in the samples collected from Kaliobia, Gharbia, and Assute and Sohage governorates respectively. On the other hand, three species of Fusarium moniliform isolated from the samples collected from Kaliobia governorate was found to produce Fumonisin in a concentrations of 0.52, 1.36 and 1.05µg/kg respectively.

The natural occurrence of Aflatoxins in sorghum grain samples collected from different governorates

Aflatoxins are secondary metabolites produced by certain strains of Aspergillus flavus and Aspergillus parasiticus. These fungi are ubiquitous and the potential for contamination of foodstuffs and animal feeds is widespread. The occurrence and magnitude of aflatoxin contamination varies with geographical and seasonal factors, and also with the conditions under which a crop is grown, harvest, and storage.[22] Crops in tropical and subtropical areas are more subject to contamination than those in temperate regions, since optimal conditions for toxin formation are prevalent in areas with high humidity and temperature. producing fungi can infect growing crops as a consequence of insect or other damage, and may produce toxins prior to harvest, or during harvesting and storage.[23] The results of AFs concentration in the all samples collected from the six governorates (Cairo, Kaliobia, Gharbia, Alexandaria, Assute and Sohage) as determined by the HPLC are summarized in (Table 4). It is clear from these results that the AFs levels were ranged from (30 to 499), (0.17 to 13.6), (0.34 to 54.2), (1.13 to 17.1) and (0.64 to 1.0) µg/kg for Cairo, Kliobia, Gharbia, Alexandaria, Assute and Sohage. However, the presented data also revealed that AFs was detected in 33.3% (16 out of 48) of the total samples analyzed with a total range from 0.17 to 499µg /kg. These results agreed with[24] who reported that the sorghum grains are often damaged by the infected mold, such as Aspergillus and consequently aflatoxin contamination. The contamination with AFs in some collected sorghum grain samples can be probably attributed to the bad storage condition of sorghum grains in some regions of the examined governorates. In this regards, Ayalew et al.,[25] who reported that higher mycotoxin contamination in sorghum, may be related to the widespread storage of sorghum grain in the underground and the occurrence of pits may leading to elevated of seed moisture contents.

Table 3 Toxin production by some toxigenic fungi isolated from sorghum grain samples collected from different governorates

Fungi	Type of produced toxin by each fungi	Amount of fungal toxin ug/kg	Governorate
Aspergillus parasiticus	AFs	0.0332	Cairo
Aspergillus parasiticus	AFs	0.8023	Cairo
Fusarium .moniliform	F B_1	1.0519	Kaliobia
Fusarium .moniliform	F B_1	0.526	Kaliobia
Fusarium .moniliform	FB_1	1.365	Kaliobia
Aspergillus parasiticus	AFs	0.022	Kaliobia
Aspergillus parasiticus	AFs	0.26	Tanta
Aspergillus parasiticus	AFs	0.0115	Assute
Aspergillus parasiticus	AFs	2.98	Sohage

Occurrence of Fumonisin B1 in sorghum grain samples collected from different governorates (ug/kg)

The results of FB_1 residues which detected in all sorghum grain samples collected from the six selected governorates (Cairo, Kaliobia, Gharbia, Alexandaria, Assute and Sohage) and analyzed by HPLC are summarized in (Table 5). It is clear from these results that the FB1 was found in concentration reached 39.3, 7.2, (23.9 to 37.3), (52.6 to 129.5), and 98.1µg/kg for Cairo, Kaliobia, Gharbia, Alexandaria and Sohage governorate respectively. It is of interest to mention that

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sorghum samples collected from Assute governorate were found to be negative for FB1. The presented data revealed that FB1 was detected in 16.6% (8 out of 48) of the total samples analyzed with a total range from (7.2 to 129.5) µg/kg. These results may be attributed to the good transport and handling condition of sorghum grains and storage temperature. These results are also in agreement with those reported by Nair et al.,[26] who stated that Fumonisins are mycotoxins produced by Fusarium moniliforme that are prevalent in corn, sorghum, millet and other agricultural products. Silva et al.,[27] reported that the levels of Aflatoxin and Fumonisin contamination detected in the sorghum grains depended on the prevailing a biotic factors (moisture content, water activity, temperature, relative humidity, and mean rainfall) at the time of sampling. The effect of location and /or geographical distribution on the level of FB_1 is shown in (Table 5). The level of FB_1 was found to be in the range between (7.2 and 129.5) µg/kg in Kaliobia and Alexandaria samples. Comparable results were performed in Assute, Kaliobia, Cairo and Sohage which reached (zero, 7.2, 39.3, and 98.1) µg/kg but with a relatively lower percentage of incidence being (zero, 12.5% and 12.5%) respectively.

Table 4 Natural occurrence of Aflatoxins in sorghum grain samples collected from different governorates (µg/kg)

Replicates	Governorates					
	Cairo	Kaliobia	Gharbia	Alex	Assute	Sohage
1	ND	ND	ND	ND	0.821	ND
2	ND	ND	ND	ND	1.02	ND
3	ND	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	ND	ND
5	1703	0.174	54.25	17.16	ND	ND
6	30.07	21.58	ND	1.59	ND	ND
7	49910	2.811	0.3412	ND	0.6413	ND
8	ND	13.614	ND	1.1315	0.8316	ND
Mean ± SE	233 ±139	13.73 ±3.98	27.27 ±26.93	6.58 ±5.26	0.82 ±0.07	-

Table 5 Natural occurrence of Fumonisin B_1 in sorghum grain samples collected from different governorates (µg/kg)

Replicates	**Governorates**					
	Cairo	**Kaliobia**	**Gharbia**	**Alex.**	**Assute**	**Sohage**
1	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	ND	ND
5	ND	7.2	23.9	52.6	ND	ND
6	ND	ND	ND	ND	ND	ND
7	39.32	ND	37.3	96.1	ND	ND
8	ND	ND	ND	129.5	ND	98.1

Conclusion and recommendations

From the obtained results concluded that sorghum grains infected by several fungal spices and their mycotoxins produced by these toxigenic isolated fungi and therefore we recommended surveys of AFs and FB_1 in sorghum grains which should be continued.

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None.

Conflict of interest

The author declares no conflict of interest.

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