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, Kalobiya, Gharbia, Alexandria, Assute, and Sohage). Isolation and identification of fungi and the determination of mycotoxins (i.e. Aflatoxins Afs and Fumonisins FBs) 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 Kalobiya were found to be the lesser infected. All Aspergillus parasiticus and Fusarium moniliforme isolated from sorghum grains were found to have the ability to produce Afs and FBs. 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 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.

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. The isolated fungi were found to have the ability to produce Aflatoxin B, AFB, and T, 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 Aspergillus were Alternaria, Penicillium sorghuma, Penicillium fumiculosis and Aspergillus flavus, respectively. Diener et al., Gonzalez et al., 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. In animals, ingested aflatoxins may be metabolically degraded. Aflatoxin B, may be converted into aflatoxin M, which may occur in the milk. The concentration of aflatoxin M in the milk of cows is about 300 times lower than the concentration of Aflatoxin B consumed in the feed. In certain experimental animals, only small amounts of administered aflatoxins have been found in tissues, 24h after injection. Aflatoxin B 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, of 1µg/kg, and 0.1µg/kg, respectively. In some studies, carcinomas of the colon and kidney have been observed in rats treated with aflatoxins. Aflatoxin B, causes chromosomal aberrations and DNA breakage in plant and animal cells and after microsomal activation, gene mutations in several bacterial test systems. In high doses, it may be teratogenic. Fumonisin B, 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. Available correlation studies from the Transkei, South Africa, suggested a link between dietary fumonisin exposure and oesophageal cancer. 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 Fumonisins B and toxigenic fungi in Egyptian sorghum grain. This study attempted to assess some crop situation with respect to Aflatoxin and Fumonisins through detecting the residues of these toxins as well as the incidence of toxicogenic and other fungi in Egyptian sorghum grain. However attention was focused on Fusarium moniliforme and Aspergillus parasiticus being the most common fungi responsible of Fumonisin and Aflatoxin production. In addition the Toxigenicity of isolated Fusarium moniliform and Aspergillus 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 Fumonisins B.
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The purified strains maintained

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. 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 on 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.

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 Stubblefield et al.

Production of FB, 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 27 days at 25°C (flasks were shaken 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.

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, B, G, and G 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 shaken 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 elute 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 shaken vigorously to dissolve. The stopper was punctured to accommodate needle of 10ul syringe. In subdued incandescent light and as rapidly as possible 5ul was spotted on the imaginary line 3cm from bottom of TLC plate. The vial was kept for quantitative analysis. On the same plate 5ul 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 dry film of standard and samples and the mixture was let to stand for 15min followed by 450µl H2O: 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, and AFG were converted into other derivatives, AFB,a and AFG,a, respectively (Aflatoxins G, AFB, and AFG, have low florescence properties, therefore, they were converted to Aflatoxin G,a and B,a, which have high fluorescence properties, using trifluoroacetic acid).

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 (H2O: CH3OH: CH3CN, 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: One mg of FB1 standards was dissolved in 1ml of Acetonitrile ACN: H2O (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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Table 1

| 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                        |

ACN:H₂O (1:1,v/v) were added respectively. The obtained standard solutions contained 100µg/ml, 50µg/ml, 25µg/ml, and 10µg/ml FB₁, respectively.

**Extraction and cleanup of FB₁:** The extraction and cleanup of FB₁ from sorghum grain samples was carried out according to. 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 Alexandria 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 species, 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 Aspergillus parasiticus represented 4.7% and 4.1 respectively. The other species included Penicillium, Aspergillus ochraceus, Helminthosporum sativum, Aspergillus terreus, Aspergillus chevalier, Aspergillus 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.

The significance of the differences among results was determined by Waller-Duncan k-ratio. 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, Kaliobia, Gharbia, Alexandaria, 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 Alexandria 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 species, 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 Aspergillus parasiticus represented 4.7% and 4.1 respectively. The other species included Penicillium, Aspergillus ochraceus, Helminthosporum sativum, Aspergillus terreus, Aspergillus chevalier, Aspergillus 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.

### Chromatography conditions

**Stationary phase:** Column Hyper Clone 5µ ODS (C18) 120A⁰, DIM: 250x4.60mm. (Phenomenex).

**Mobile phase:** Methanol: 0.1M NaH₂PO₄ (75:25, v/v) isocratic eluants were discarded. Fumonisin B₁ from sorghum grain samples was carried out according to. The mixed solutions of standard as well as sample extract after derivatization and HPLC analysis.

**Preparation of derivatization reagent:** Forty mg of o-phtaldialdehyde OPA was 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 FB₁ 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 system. Fluorescence of OPA- Fumonisin beginning to decrease after 2min.

**Preparation of solorgh 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 FB₁ by HPLC:** The method described by Shephard et al. was used for the determination of FB₁ in samples by HPLC.

**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. FB₁ 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. The significance of the differences among results was determined by Waller-Duncan k-ratio. All statements of significance were based on probability of P<0.05.

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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 equesi | 2                    | 0.63          |
| Fusarium solani | 2                    | 0.63          |
| Fusariumavenaceum | 2                    | 0.63          |
| Penicillium sp | 8                    | 2.55          |
| Aspergillus parasiticus | 15                | 1.2           |
| Aspergillus ochraceus | 4                   | 1.2           |
| Helminthosporium Sativium | 4        | 1.2           |
| Aspergillus terreus | 2                    | 0.63          |
| Aspergillus chevalier | 2                  | 0.63          |
| Aspergillus humicolata | 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 aflatoxin 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, harvested, and stored. 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. The results of AFs concentration in the all samples collected from the six governorates (Cairo, Kaliobia, Gharbia, Alexandria, 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, Alexandria, 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 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., 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    | FB1                                 | 1.0519                      | Kaliobia    |
| Fusarium moniliform    | FB1                                 | 0.526                       | Kaliobia    |
| Fusarium moniliform    | FB1                                 | 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 (µg/kg)

The results of FB1 residues which detected in all sorghum grain samples collected from the six selected governorates (Cairo, Kaliobia, Gharbia, Alexandria, 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, Alexandria and Sohage governorates respectively. It is of interest to mention that

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Conclusion and recommendations

From the obtained results concluded that sorghum grains infected by several fungal spieces 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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Table 4 Natural occurrence of Aflatoxins in sorghum grain samples collected from different governorates (µg/kg)

| Replicates | Governorates |
|------------|--------------|
|            | Cairo | Kaliobia | Gharbia | Assute | Sohage |
| 1          | ND    | ND       | ND      | 0.821   | ND     |
| 2          | ND    | ND       | ND      | 1.02    | ND     |
| 3          | ND    | ND       | ND      | ND      | ND     |
| 4          | ND    | ND       | ND      | ND      | ND     |
| 5          | 1703  | 0.174    | 54.25   | 17.16   | ND     |
| 6          | 30.07 | 21.58    | ND      | 1.59    | ND     |
| 7          | 49910 | 2.811    | 0.3412  | ND      | 0.6413 |
| 8          | ND    | 13.614   | ND      | 1.1315  | 0.8316 |

Mean ± SE

233 ± 13.73 ± 27.27 ± 6.58 ± 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 | Assute | Sohage |
| 1          | ND    | ND       | ND      | ND     | ND     |
| 2          | ND    | ND       | ND      | ND     | ND     |
| 3          | ND    | ND       | ND      | ND     | ND     |
| 4          | ND    | ND       | ND      | ND     | ND     |
| 5          | ND    | 7.2      | 23.9    | 52.6   | ND     |
| 6          | ND    | ND       | ND      | ND     | ND     |
| 7          | 39.32 | 37.3     | 96.1    | ND     | ND     |
| 8          | ND    | 129.5    | 98.1    | ND     | ND     |

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