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The prevalence of aflatoxin and *Aspergillus parasiticus* in Egyptian sesame seeds

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Abstract : Sesame is usually contaminated with many fungi where some of them are mycotoxigenic causing economic and health problems. Therefore, the aim of this study was to detect and identify the mycoflora associated with Egyptian sesame seeds and to detect the aflatoxigenic isolates genetically as well as to evaluate some measures to decrease fungal load and mycotoxin contamination. Results indicated that several fungal genera were isolated, whereas *Aspergillus* was the most predominant genera. Results also indicated that higher increase in fungal counts was recorded for samples stored at higher water activity (a_w0.95) compared with those stored at lower (a_w 0.90). The molecular identification was carried out to detect the presence of *aflR* genes in fifty *A. parasiticus* isolates, and results revealed thatfourteenisolates were able to produce aflatoxin B_1(AFB_1).The highest level of AFB_1(42.37 and 66.74 µg/kg)in sesame was detected in Dakahlia and El-Behera governorates respectively. Meanwhile, roasting and microwave treatments showed the potential way in the elimination of mycotoxins. It could be concluded that sesame seeds are susceptible to invasion by several fungal species where some of them are mycotoxigenic.
Keywords: Sesame seeds; *Aspergillus parasiticus*; *aflR* genes; aflatoxins; detoxification; microwave; roasting.

Introduction

Sesame, (*Sesamum indicum*), which belongs to the *Pedaliaceae* family, is rich in different unsaturated fatty acids and minerals[1], and it is probably the most ancient oilseed cultivated in several countries. Most of the sesame seeds are used for oil extraction and the rest are used for edible purposes[2] and play an important role in human nutrition. It contains about 50-52% oil, 17-19% protein and 16-18% carbohydrate[3]. It plays an important role in the natural economy due to export and local industry[4]. In Egypt, sesame is one of the important cereals used in the industry; it represents a chief constituent and is seen spread on top of bread or ground into creamy butter.

Sesame is attacked by many fungi such as *F. oxysporum, F. sesame* and *M. phaseolina*[5]. These fungi may cause seed deterioration with a reduction in quality and quantity. Water activity (a_w) is an important ecological factor for the growth of microorganisms such as fungi since they contaminate various foods at a wide range of water activity[6]. The need for a meaningful term to describe the behaviour of fungi in the environment with reduced moisture helped to establish the term water activity[7]. Labuza *et al*.[8] reported earlier that reducing water activity below 0.7 prevented food from fungal spoilage. Moreover, some of the contaminated fungi *such*

as Fusarium, *Aspergillus* and *Penicillium* may produce several mycotoxins[9]. One of the most important mycotoxins is aflatoxins which are produced by several species of *Aspergillus*[10, 11, 12, 13].

There are many methods for the determining aflatoxin concentration in commodities or in culture and include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC) [14]. However, these methods are relatively expensive and time-consuming. Nowadays the ability of toxigenic *A. parasiticus* species to produce aflatoxins is identified by molecular methods since there are at least 23 enzymatic reactions with 15 structurally defined aflatoxin intermediates required for aflatoxin biosynthesis and 29 genes for these enzymes have been cloned[15, 16]. It was previously reported by Liu and Chu[17] that *aflR* genes play an important role in the aflatoxin biosynthesis pathway by regulating the activity of other structural genes. The analyses of the aflatoxin expression genes of *A. parasiticus* are greatly needed and it usually depends on many factors such as transcriptional regulatory factors, physiological response and environmental factors[18, 19].

In Egypt, sesame hulled seeds are used extensively in many food industries such as bakery, tahini, and halva which are heavily consumed by the Egyptian consumer, therefore many safety issues should be considered before the use of sesame seeds. Thus, the aim of this study was to evaluate fungal and aflatoxin contaminants in sesame seeds collected from different regions of Egypt and to detect the aflatoxigenic isolates by studying the existence of an *aflR* gene in aflatoxin biosynthesis by PCR method. Also to investigate the effect of storage at different water activities on the fungal load and to determine the effect of heat treatments on aflatoxin levels.

Materials and Methods

Collection of samples

Twenty-eight samples of sesame seeds were collected from food stores in different governorates of Egypt (Figure 1) and were preserved in plastic bags and stored at 4°C until used.

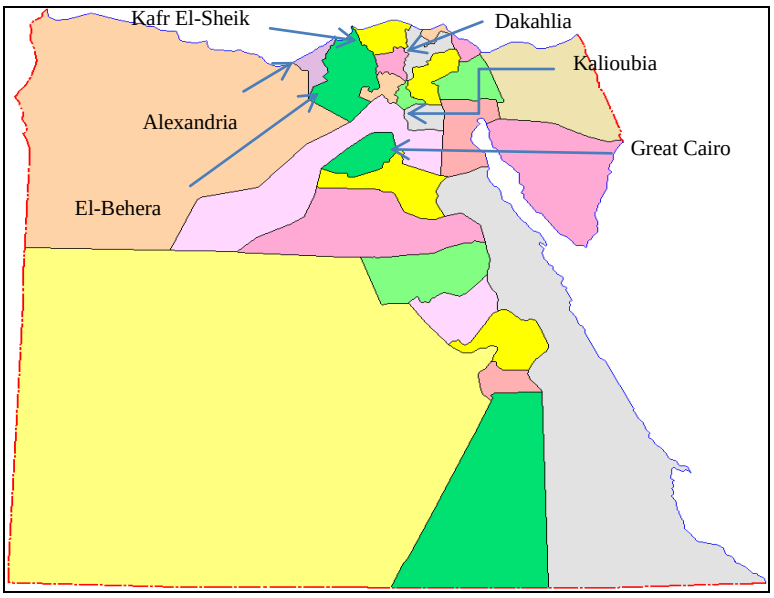


Figure 1. Sesame sampling sites from different governorates in Egypt

Storage conditions and isolation of fungi

Fungal mycoflora was isolated directly after collections of samples and also after storage. Samples of 50gm in a set of three replicates were prepared and stored in specific Jar to maintain the water activity (a_w) 0.90 and 0.95 stored for one month at 25 ±2°C. Agar plate method was used for the isolation of fungi from sesame samples before and after storage[20]. Seeds were surface disinfected for 2 minutes with 0.2% sodium hypochlorite solution and rinsed three times with sterile distilled water and placed on Potato Dextrose Agar (PDA, corda,

Madrid, Spain) medium at the rate of 10 seeds per plate for each sample. Then the plates were incubated at 25±2°C for five days. After the incubation period, the fungal colonies developed were recorded in term of percentage frequency. Fungal and *Aspergillus* load in all samples were calculated as $\log_{10}$CFU/g.

Morphological identification

The pure culture of fungi was characterized and identified based on their morphological, macroscopic and microscopic characteristics using the keys of Raper and Fennel[21], and Pitt and Hocking[22]. The major and remarkable macroscopic features in species identification were the colony diameter, colour (conidia and reverse), exudates and colony texture. Microscopic characteristics for the identification were conidial heads, stipes, colour and length vesicles shape and serrations, metula covering, conidia size, shape and roughness.

Ability of Aspergillus parasiticus isolates to produce aflatoxins

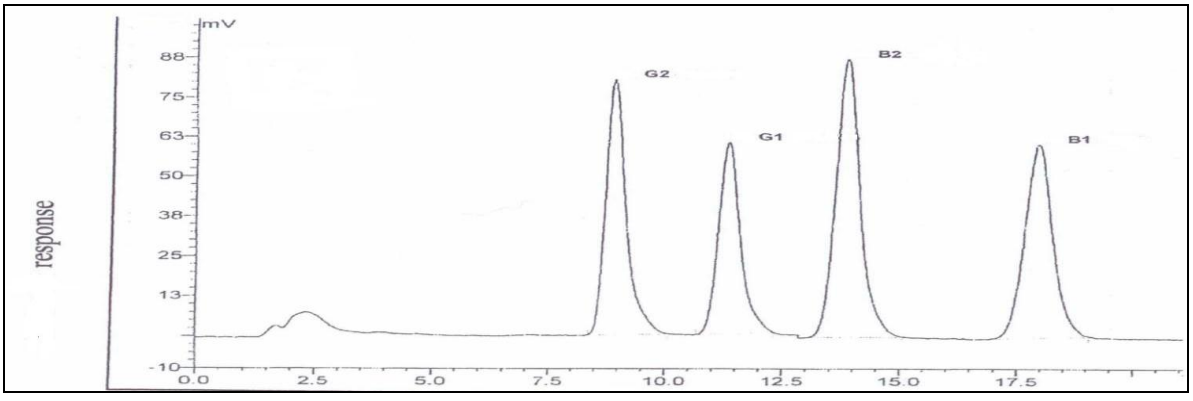
Amplification of aflR gene

Genomic DNA was extracted from pure mycelia by DNA Kit (Sigma-Aldrich, St. Louis, MO, USA). Fifty *Aspergillus parasiticus* isolates were tested for amplification of *aflR* gene. The oligonucleotide primers in the PCR were as follow: *aflR* forward 5′-TATCTCCCCCCGGGCATCTCCCGG-3′ and *aflR* reverse 5′-CCGTCAGACAGCCACTGGACA CGG 3′. Primers were used for the specific detection of *aflR* genes which are specific for aflatoxin biosynthetic pathway and used for the detection of aflatoxin production[23, 24, 25]. PCR amplification was performed using a PTC-100 thermal cycler (MJ Research Inc, Watertown, MA, USA). Amplified products were isolated with a silica matrix (Geneclean II Kit; Bio 101). Aliquots (8 µL) of each PCR product were analysed by electrophoresis in a TBE buffer in 1.0% agarose gels and ethidium bromide staining and visualized by UV illumination.

Aflatoxin determination in sesame seeds

Extraction: Fifty grams of ground sesame samples, 5g NaCl and 100 mL of a methanol: water (80:20, v/v) were blended at high speed for 1 min. The samples were then filtered through Whatman filter paper and 10 mL of the filtrates were diluted with 40 mL purified water[26].

Clean-up: Ten mL of filtered diluted extract was completely passed through immune-affinity column (AflaTest® HPLC, VICAM, Ma 01757 USA) at a rate of about 1-2 drops/second. After passing the samples, the immune-affinity column was washed twice with 10 mL purified water at a rate of about 2 drops/second. Elution was performed with 1.0 mL HPLC grade methanol after which 1.0 mL of purified water was added to elute.



Time (min)

Figure 2. Auto-Scaled chromatogram of aflatoxins standard (20µg/ml) using High Performance Liquid chromatography (HPLC)

HPLC analyses: The HPLC system used for AF analyses was an Agilent 1200 series system (Agilent, Berks, UK) with a fluorescence detector (FLD G1321A), an auto sampler ALS G1329A, FC/ALS therm G1330B, Degasser G1379B, Bin Bump G1312A and a C18 (Phenomonex, Luna 5 micron, 150 × 4.6 mm) column joined to a pre-column (security guard, 4 × 3-mm cartridge, Phenomenex Luna). The mobile phase was

water: acetonitrile: methanol (3:1:1, v/v/v) using an isocratic flow rate of 1 mL/min at 360 nm excitation and 420 nm emission wavelengths and a 20-min run time for AFs analyses (Figure 2).

Effect of roasting and microwave radiation on aflatoxin B_1 in sesame seeds

Artificially contaminated sesame seeds (20 µg/kg) were roasted in an oven (Sheldon, England) at 100 and 150ºC for 20 and 30 minutes, and were microwave radiated (Smart SM/3021MH, China) at 20Kgy with a maximum time of 5 minutes. After treatments aflatoxin B_1 was extracted from the contaminated sesame as previously reported.

Statistical analysis

All experiments were performed in replicates. Results were expressed as mean values ± standard deviation. Duncan Multiple Range Tests (DMRT) was used to estimate significant differences among mean values at the 5% probability level.

Results and Discussion

Sesame is one of the important cereals used in the food industry in Egypt as it represents a chief constituent in many food products. There is limited data about the contamination of sesame with *Aspergillus* species and mycotoxins in general. Therefore, in this study, we gathered twenty-eight sesame samples from Cairo, Kalioubia, Alexandria, El-Behera, Kafr El-Sheik, and Dakahlia governorates to assess the level of contamination with different fungal species before and after storage and frequency occurrence of aflatoxins

Morphological identification of fungal isolates

Aspergillus parasiticus isolate grown on PDA produced aerial mycelium (dense and floccose), yellow-dark green colony appearance, and whitish- yellowish on the reverse. The isolate also had globose conidial head and globose to subglobose conidia. *Aspergillus flavus* isolate grown on PDA produced aerial mycelium, olive-lime green parrot colony colour, and yellowish on the reverse. The isolate also had radiate splitting into the poorly defined column conidial head and globose to subglobose conidia. *Aspergillus parasiticus* belong to section *Flavi* and is closely related to *A. flavus* but could be separated by DNA sequence and AFLP fingerprint analyses[27, 28], and are also easily distinguished phenotypically. *Aspergillus parasiticus* produces AFB_1, AFB_2, AFG_1, and AFG_2; whereas *A. flavus* produce cyclopiazonic acid[29]. Also, *Aspergillus parasiticus* can be separated from *A. flavus* by the darker green conidial heads and by the more pronounced conidial enhancement according to Klich and Pitt[30]. Raper and Fennell[21] and Horn *et al*.[31] reported that many species in section *Flavi*, including *A. parasiticus*, produce sclerotia, thus *Aspergillus* species such as *A. parasiticus* was considered to be strictly asexual by means of sclerotia and lost their ability to undergo meiosis[32]. The data obtained in this study are considered consistent with the features of *A. parasiticus*[29, 33]. Other fungal isolates identified in this study were as *A. niger*, *Alternaria* sp., *Cladosporium* sp., *Fusarium* sp. and *Penicillium* sp. according to Sutton *et al*.[34].

Frequency occurrence of fungi in sesame seed samples

Fungal isolates belonging to five genera were isolated from sesame samples. Data in Table (1) showed that all the sesame samples were contaminated with differentgenera of fungi including *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, and *Cladosporium*, whereas *Aspergillus* was the dominant genera ranging between 60.40 and 91.66% for kalioubia and kafrEl-Sheik governorates respectively. Data in Table (2) revealed that *A. parasiticus* was isolated from all governorate with a percentage of occurrence ranging between 42.62% in Kafr El-Sheik to 78.95% in Alexandria governorates followed by *A. niger* which ranged from 18.18% in Kalioubia to 57.38%in Kafr El-Sheik governorates. Meanwhile, *A. flavus* was isolated in lower incidence from Cairo and Kalioubia governorates. Data also indicated that samples from El-Behera, showed higher fungal (1.21 $\log_{10}$ CFU/g) followed by Kafr El-Sheik, Dakahlia, Kalioubia and Alexandria governorates respectively. *Aspergillus* genera were found to be the most predominant fungi in sesame samples obtained from various governorates in Egypt, whereas *A. parasiticus* was the dominant fungi among the *Aspergillus* genera. One of the alarming findings was that *A. parasiticus* was quite dominant and it is known for its high ability to produce aflatoxins[35]. These aflatoxins are carcinogenic mycotoxins and can cause various hazards in terms of food safety and human

health[36, 37]. In this respect, Mbah and Akueshi[38] reported that only *Aspergillus*, *Fusarium*, and *Penicillium* species invaded and colonized post-harvest sesame in Nigeria.

Table 1. Frequency occurrence of fungal genera in sesame obtained from different governorates in Egypt

Governorate	NC/ TNS	Fungal load (log_{10} CFU/g)		Percentage occurrence of fungal genera				
		Range	Mean ± SD	*Alternaria*	*Aspergillus*	*Fusarium*	*Penicillium*	*Cladosporium*
Great Cairo	4/4	1.72 –2.32	1.91±0.77[a]	34.21	60.53	2.63	ND	2.63
Kalioubia	3/3	1.72 –2.32	1.97±1.03[a]	4.17	91.66	ND	4.17	ND
Alexandria	6/6	1.72 –2.67	1.99±1.63[a]	4.35	82.61	6.52	ND	6.52
El-Behera	5/5	1.72 –2.87	2.15±1.63[b]	ND	71.82	0.91	17.27	10.00
Kafr El-Sheik	5/5	1.72 –2.80	2.26±2.72[c]	3.96	60.40	1.98	17.82	15.84
Dakahlia	5/5	1.72 –3.02	2.52±0.70[d]	ND	79.68	1.59	6.37	12.35

NC: Number of contaminated samples; TNS: Total number of samples
Mean with different superscript letters are significantly different

Table 2. Frequency occurrence of *Aspergillus* sp. in sesame obtained from different governorates in Egypt

Governorate	NC/ TNS	*Aspergillus* load (log_{10}CFU/g)		Percentage occurrence of *Aspergillus* species		
		Range	Mean ± SD	*Aspergillus parasiticus*	*Aspergillus flavus*	*Aspergillus niger*
Great Cairo	1/4	1.72 –2.32	1.96±0.86[a]	69.56	8.69	21.75
Kalioubia	1/3	1.72 –2.32	2.02±1.09[a]	77.27	4.54	18.18
Alexandria	1/6	1.72 –2.67	2.02±1.77[a]	78.95	ND	21.05
El-Behera	3/5	1.72 –2.87	2.23±3.09[b]	67.08	ND	32.91
Kafr El-Sheik	3/5	1.72 –2.80	2.37±3.41[b]	42.62	ND	57.38
Dakahlia	3/5	2.20 –3.02	2.69±0.75[c]	71.00	ND	29.00

NC: Number of contaminated samples; TNS: Total number of samples
Mean with different superscript letters are significantly different

On the other hand, Nayyar *et al*.[39] reported that a total number of 36 species belonging to 10 genera of fungi were isolated from sesame, whereas the prevalent genera were *Penicillium*, *Alternaria*, *Fusarium*, *Cladosporium*. Our results are in agreement with Altaf *et al*.[40] who isolated *A. niger*, *Fusarium* sp., *Alternaria* sp., and *Penicillium sp*. from sesame samples obtained from Pakistan. Our results also revealed that *A. parasiticus* was the dominant fungi isolated from sesame samples, unlike previous studies that reported the high incidence *of A. flavus* in sesame seeds along with other fungi[38, 41].Recently, different species of *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium* were isolated from sesame in varying proportions[42]. Sahab *et al*.[43] also isolated seven genera, and the most prevalent fungi were *Aspergillus* (55.6 %), followed by *Alternaria* (15.0 %), *Penicillium* (10.9 %) and *Fusarium* (5.7 %) from fresh harvest maize. The difference in the fungal species isolated from sesame than other authors could be due to the different environmental conditions in the different regions samples was obtained from.

Effect of storage at different water activity on fungal count

Data in Figure (3) indicated that stored sesame samples at the higher water activity (a_w 0.95) showed an increase of total fungal count compared with samples stored at a lower water activity (a_w 0.90).A significant increase of total fungal count was noticed for samples obtained from El-Behera governorates at 0.95 (a_w) followed by Dakahalia.

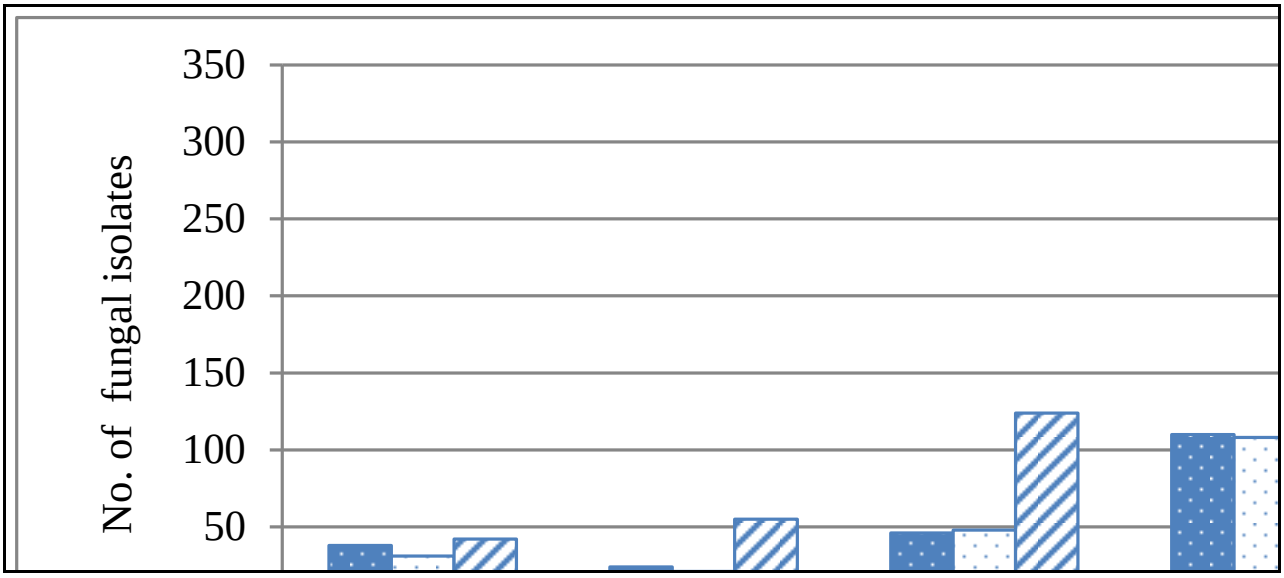


Figure 3. Fungal counts before and after sesame samples storage.

On the other hand, similar results were noticed for the growth of *A. parasiticus* isolates under storage at different water activities (Figure 4). Sesame samples stored at a lower water activity (a_w 0.90) showed a slight decrease of total fungal and *A. parasiticus* counts. Data clearly indicated that fungal counts increased by storage at 0.95 a_w and decreased by storage at 0.90 a_w in sesame samples (Figure 5). Spadaro *et al*.[44] revealed that *Aspergillus carbonarius* grew and produced ochratoxin A at water activity levels of 0.98 to 0.90 but not at 0.88. On the other hand, Marin *et al*.[45] found that above 0.90 a_w linear growth was noticed for all tested *Aspergillus* and *Penicillium* species (*A. ochraceus*, *A. flavus*, *A. niger*, *P. aurantiogriseum* and *P. hordei*).

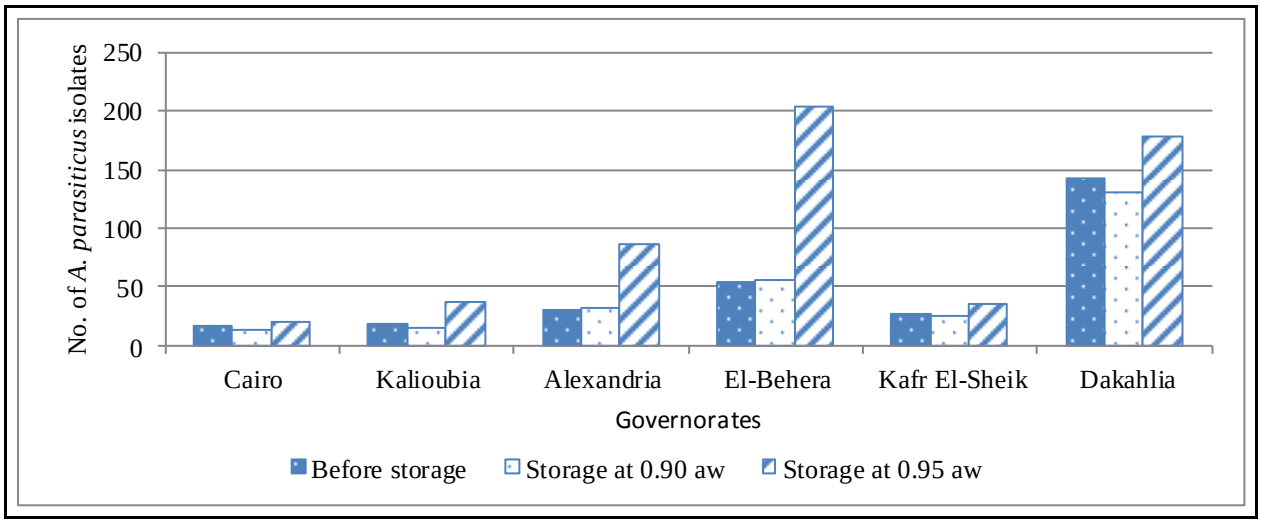


Figure 4. *Aspergillus parasiticus* counts before and after sesame samples storage.

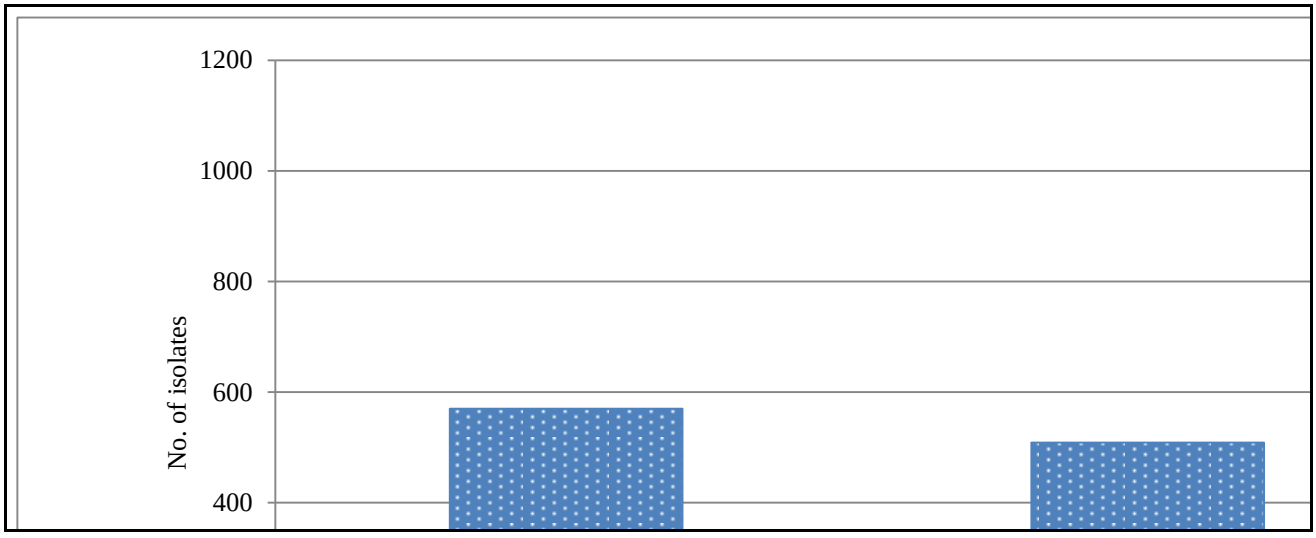


Figure 5.Total fungal counts before and after storage of sesame samples.

Molecular detection of aflatoxin producing isolates

Aspergillus parasiticus is not always correlated with the ability to produce aflatoxins. Detection of aflatoxin genes in *A. parasiticus* isolates was carried out using *aflR* genes involved in the aflatoxin biosynthetic pathway. Using PCR analysis, a banding pattern which matched *aflR* primers in this study have been amplified with an approximate size of 798 bp in fifty studied isolates and compared with the negative control. Molecular sizes of the DNA of fungal species were estimated by the fluorescence intensity and comparison of the distance travelled with that of the molecular weight of marker standard as measured using gel electrophoresis (Figure 6). Results revealed that *aflR* genes were successfully amplified in fourteen out of fifty isolates.

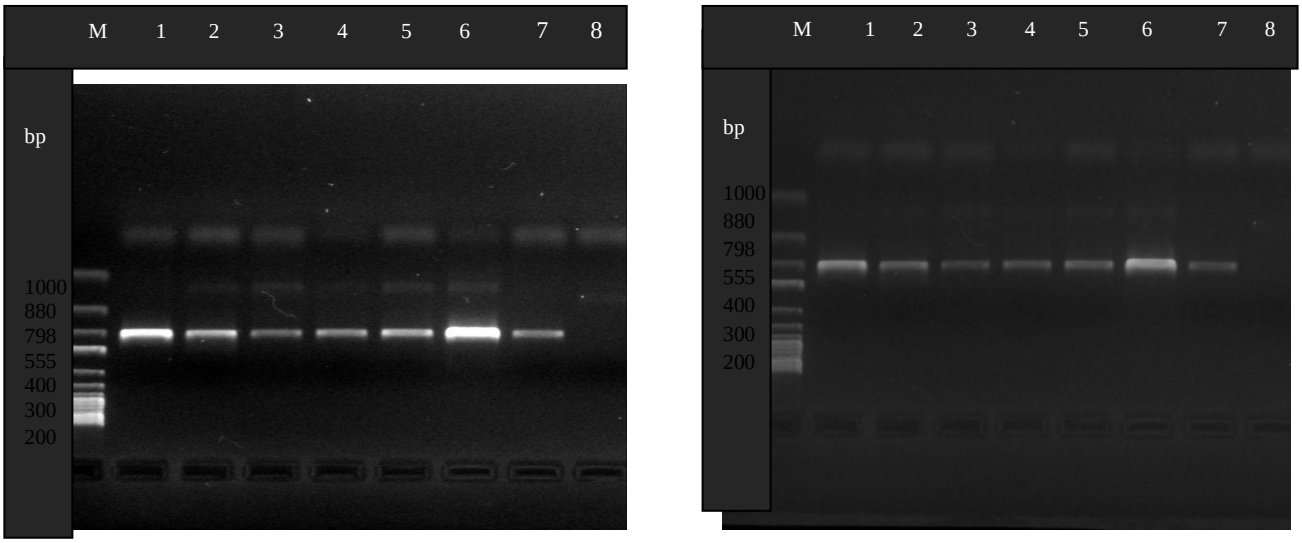


Figure 6. Gel electrophoresis analysis PCR products using primers and DNA extracted from strains of *A. parasiticus*. (A): Lanes 1-7 DNA of *A .parasiticus* strains using an *aflR* primer with 798 bp. Lane 8 is a negative control (B): Lanes 1-7 DNA of *A. parasiticus* strains using an *aflR* primer with 798 bp. Lane 8 is a negative control. DNA molecular weight marker Band sizes: 200bp, 300bp, 400bp, 555bp, 798bp, 880bp, 1000bp.

Our results revealed that *A. parasiticus* is not always correlated with the ability to produce aflatoxins. Somashekar *et al*.[46] previously reported that *aflR* genes appeared on 80% of *Aspergillus* sp., whereas Pham and Dam[47] indicated that the *aflR* gene was represented in 67% of *A. flavus* isolated from peanut. Chang[48] added that the *aflJ* gene which is adjacent to *aflR* is necessary for expression of other genes in the aflatoxin cluster. It has

also been reported that the genes (*aflR* and *aflJ*) play 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[16, 49]. The *aflR* regulatory gene was named *afl*-2 in *A. flavus*[50] and *apa*-2 in *A. parasiticus* previously[51], and later named *aflR* in both *A. flavus* and *A. parasiticus* for its role as a transcription activator[52].

Aflatoxin levels in sesame samples

Twenty-eight sesame samples from different governorates were analysed to evaluate the presence of aflatoxins B_1, B_2, G_1, and G_2. Data in Table (3) showed that AFB_1 and AFG_1 were detected in 100% of sesame samples obtained from Cairo Governorate, with mean values of 18.63 and 18.27µg/kg respectively. In El-Behera governorate, the concentration of AFB_1 and AFG_1 was high with a mean value of 66.74 and 43.81µg/kg and a percentage of contamination reaching 60 and 80% respectively. Both Dakahlia and Alexandria governorates followed with high aflatoxin level. It was also noticed that sesame samples obtained from these three governorates Alexandria, El-Behera and Dakahlia contained all four aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2), whereas sesame samples obtained from Great Cairo, Kalioubia and Kafr El-Sheik governorates contained AFB_1 and AFG_1 only. Results in Figure (7) showed the total aflatoxins concentration (µg/kg) in sesame samples and it was noticed that all samples exceeded the safe limits for human consumption (i.e. 20 µg/kg). Data also revealed that samples from Alexandria, El-Behera, and Dakahlia showed the highest aflatoxin content compared to other governorates.

Table 3. Percentage occurrence and level of aflatoxins (µg/kg) contaminants in Egyptian sesame samples

Governorates	TNS	Aflatoxin B_1		Aflatoxin B_2		Aflatoxin G_1		Aflatoxin G_2	
		µg/kg	%	µg/kg	%	µg/kg	%	µg/kg	%
Great Cairo	4	18.63 ± 0.79	100	ND	ND	18.27 ± 1.31	100	ND	ND
Kalioubia	3	23.25 ± 0.93	100	ND	ND	21.33 ±1.22	66.66	ND	ND
Alexandria	6	21.04 ± 2.32	66.66	0.28 ± 0.10	33.33	51.47 ± 2.18	33.33	1.55 ± 0.59	16.66
El-Behera	5	66.74 ± 1.71	60.00	0.42 ± 0.07	40.00	43.81 ± 2.10	80.00	ND	ND
Kafr El-Sheik	5	29.94 ± 1.02	100	ND	ND	14.88 ± 1.55	80.00	ND	ND
Dakahlia	5	42.37 ± 1.34	100	0.19 ± 0.10	20.00	27.51 ±1.07	100	0.12 ± 0.13	20.00

Results are mean ± SD (n=3); TNS: Total number of samples

These findings are in agreement with several previous studies[53, 54, 55, 56] who indicated that sesame seed is sensitive to aflatoxin producing fungi, and may, therefore, be contaminated with AFs. Our results revealed that all samples obtained from various governorates exceeded the safe limits for human consumption (i.e. 20 µg/kg) as recommended by WHO (World Health Organization) and FDA (Food and Drug Administration, of United States) [57]. These results are in good harmony with those reported by Li *et al*.[58] who found AFs detected in 37 % of sesame paste samples obtained from China, some of which containing exceptionally high levels of up to 20 µg/kg.

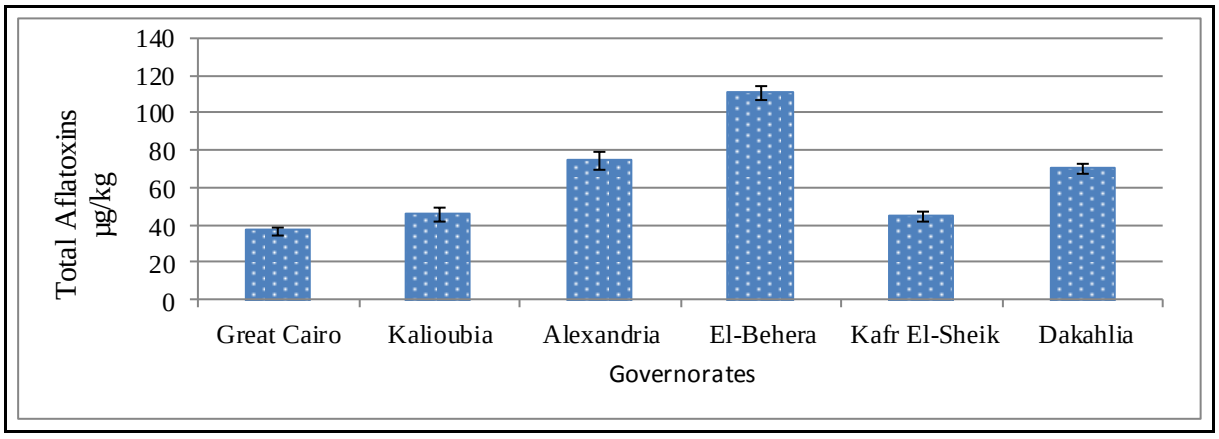


Figure 7. Total aflatoxins level (µg/kg) in sesame samples collected from different governorates. Results are mean ± SD (n=3);

Effect of roasting and microwave radiation on aflatoxin B_1 in sesame seeds

Data in Table (4) revealed that AFB_1 degradation was increased by the increase of time of exposure. Our results also showed that microwave treatment increased AFB_1 degradation more than roasting. Roasting has been reported in some cases to reduce aflatoxin levels, but in no case, the total destruction has been achieved[59]. This might be due to aflatoxins which are quite stable to dry heating at temperatures below its thermal decomposition temperature of 267 °C[60]. Our results are similar to those reported by Mobeen *et al*.[61] who indicated that microwave heating showed to be much better in reducing the aflatoxin levels than any ordinary dry heating.

Table 4. Percentage of degradation (%) of aflatoxin B_1 in sesame seeds affected by roasting and microwave radiation

Treatment Time	Roasting		Microwave (20 kgy)
	100ºC	150ºC	
5	-	-	18.14±0.024
20	5.33±0.026	11.50±0.079	-
30	7.21±0.011	14.14±0.090	-

Results are mean ±SD (n=3)

Conclusion

Our results concluded that sesame which is an essential cereal crop in Egypt and used extensively in the food industry could represent a major source of health hazards and therefore sanitation measures should be considered before storage as well as several treatments should be applied to decrease the level of contamination before use in food industry.

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