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Mycoflora Association and Contamination with Aflatoxins in Sunflower (*Helianthus annuus* L.) Seeds

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Authors' contributions

This work was carried out in collaboration between all authors. Author AAK managed the literatures, researches, performed statistical analysis and wrote the first draft of the manuscript. Author DAE designed the study and wrote the protocol. Author MIG manage the analysis of study. Read and approved the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The study aimed to identify sunflower seed- borne fungi associated with sunflower *(Helianthus annuus* L.), and also to explain the effect of environmental factors on increasing or decreasing the levels of aflatoxins in sunflower seeds. Seeds were collected from two governorates from season 2009. The sunflower seed- borne fungi was screened by using blotter and agar plate methods in two cultivars seeds. Fungi associated with sunflower seeds under different storage temperature degrees for different periods were determined using blotter and agar methods. It was found that sunflower seeds contained different fungal genera in both Sakha. 53 and Giza. 102 sunflower cultivars studied. The main species (spp.) found were *Aspergillus flavus* at the highest frequency, followed by *Alternaria alternata* and then *Penicillium* spp, *Drechlera* spp were found at the lowest frequency. A positive correlation between frequency of fungal infection, storage period and temperature was found. The presence of aflatoxin was determined in contaminated sunflower seeds using flow through strips and Vicam immunoaffinity column methods. The effect of environmental factors plays an important role on increasing or decreasing the levels of aflatoxins in sunflower seeds.

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1. INTRODUCTION

Sunflower (*Helianthus annuus L.*) is an important member of the family Asteraceae and is one of the major oil seed crops. The area cultivated with sunflower in Egypt, reached about 9346 acres in season 2011 producing around 19 tons which is assuming increasing importance because the seed contains 25-32% edible oil, a rich source of polyunsaturated fatty acids used for human consumption [1]. Sunflower seeds are highly contaminated with fungi, which attack the plants at different stages of development and subsequently during harvesting and storage [2,3]. The deleterious effects of seed-borne fungi include: Biodeterioration of sunflower seeds when used as feed, reduce seed viability, germination and seedling vigor of the crop in the field and causing low yields. Infection of seeds with *Alternaria alternata* and *Aspergillus flavus* decreased the iodine number of the sunflower oil [4]. Many fungal pathogens, some of which are seed transmitted, often reduce the germination ability or kill the infected plants or substantially reduce the productive capacity [5].

Mycotoxins can be produced in the growing crop and during storage depending on the specie of mycotoxigenic fungus present and the prevailing condition of the substrate. More than 300 fungal species are known to produce mycotoxin with variable toxic effects [6]. The toxin can be produced in the growing crop and during storage. It has been established that strains of several fungal spp., i.e., *Alternaria alternata, Aspergillus flavus, A. niger, A. parasiticus, Fusarium oxysporum, Fusarium moniliforme,* and *F. equiseti,* are able to produce mycotoxins in various commodities including sunflower seeds [7]. These include: alternariol, aflatoxins B_1 B_2, G_1 and G_2, beauvericin, bikaverin, fusaric acid, fusarin C, moniliformin, sambutoxin and zearalenone, which affected crops such as maize, cereals, peanuts and beans [8,9]. Several phytopathogenic and saprotrophic fungal species have been reported on sunflower seeds. The most important seed-borne pathogens represented the genera *Aspergillus flavus*, *Alternaria alternata, Fusarium moniliforme* and *Macrophomina phaseolina* [10,11,12,13,14].

As aflatoxins are mutagenic, carcinogenic and teratogenic and toxic to most experimental and domesticated animals and humans [15] this study is aimed to: (a) isolate, classify and identify fungi associated with sunflower seeds using two methods. b) determine the effect of storage periods at different temperature degrees. c) determine aflatoxin in contaminated sunflower seeds using a flow through strip method (Aflacheck, Vicam) and immunoaffinity column system (Vicam apparatus).

The present investigation was undertaken to find out the relation between seed borne fungi associated with the seeds and the effect of contamination on production of aflatoxin.

2. MATERIALS AND METHODS

2.1 Collection of Seed Samples

Seed samples of Sakha 53 and Giza. 102 sunflower cultivars were obtained from Oil Crops Department, Field Crops Research Institute, Agriculture Research Center, Giza, Egypt. Samples of sunflower seeds collected from khalyobia as a north governorate (Sakha 53) and El-Minia as a south governorate (Giza 102) during season 2009, were used to detect the seed-borne mycoflora. Sample of two kilograms of each cultivar was examined.

2.1.1 Isolation and Identification of fungi

The standard Blotter and Agar plate methods [16] were employed. The selected seeds were thoroughly washed with running tap water and surface sterilized by immersing in 3% sodium hypochlorite for 2 minutes, then rinsed in sterilized water and dried between two sterilized filter papers.

2.1.1.1 Blotter method

For the standard blotter method, untreated seeds and seeds were placed on three layers of moistened blotter [17]. To determine the seed-borne mycoflora of sunflower, 25 seed were plated on sterile Petri dishs (9 cm diameter). 400 seeds with total of eight replicates were examined.

2.1.1.2 Agar plate method

The treated seeds were plated on define medium to detect the seed-borne mycoflora. 400 seeds from each sample were plated on the surface of PDA in sterile Petri dishes [16]. The plates with a total of four replicates of each treatment were incubated at 25°C for 7 days. The fungi growing on seeds were identified depending on their morphological features according to various taxonomic keys [18,19,20,21]. Identification was carried out at the laboratory of Seed Pathology Research Department, Plant Pathology Research Institute, Agriculture Research Center., Giza, Egypt. The percentages of incidence and frequency of seed mycoflora were calculated.

2.1.2 Storage conditions and fungal Identification

The seeds were placed in sterilized paper bags and stored in different temperatures at 0°C, 10°C, 20°C and 30°C and different periods (4,8 and 12 months). Samples after these treatments were examined for fungi by plating on blotter and agar plate methods, 25 seed per Petri dish and the dishes were incubated at 25°C for 7 days.

2.2 Detection of Aflatoxins

Aflatoxins were detected by using two new methods, Vicam strips and Vicam immunoaffinity column.

2.2.1 Flow through strip method

The method used was the Vicam Aflacheck system, which is a qualitative one step test kit for detection of aflatoxin. Alfacheck uses highly specific reactions between antibodies and aflatoxin to detect total aflatoxin in variety of samples. The test strips was used to detect the presence of aflatoxin at two different levels: 10 ppb or 20 ppb, depending on the protocol of Aflatest Manual [22].

2.2.1.1 Sample extraction

Sample (5g) was placed in a 40ml extraction tube to which was added 20 ml of extraction solvent (methanol 70%). The tube was stoppered and manually shaken for three minutes and then rested for 3 minutes to allow the solid to settle.

2.2.2 Alfacheck procedure

A strip test dilution tube was placed in a provided cardboard rack and 250µl of sample extract was pipetted into it. To this 250µl of distilled water was added and the solution was manually mixed after capping the tube. The Aflacheck test strip was then inserted in to the solution as directed and the strip was allowed to develop until a negative results was recorded (<20ppb) after 3 minutes or a positive one after 5 minutes (=/>20ppb) total aflatoxins. The test strip was read on a digital fluorometer, which gave a total aflatoxin concentration in ppb derived from the positive coloured band on the strip.

2.2.3 VICAM apparatus

Vicam apparatus, is an immunoaffinity column method. Total aflatoxin (B_1,B_2 G_1 and G_2) were detected in the two cultivars (Sakha 53 and Giza 102) in Central Lab of Biotechnology, Plant Pathology Research Institute, Agriculture Research Center, following the Vicam Aflatest method as per the Vicam manual (Vicam, Waterton USA) .

2.2.4 Statisistical Analysis

Data analysis was statistically performed using Spss [23] and [24] software. Analysis of variance was used to test the difference between the groups. Least Significant Difference (LSD) test was used to determine the significant differences ranking among the mean values at *P*= .05.

3. RESULTS AND DISCUSSION

3.1 Occurrence and Detection of Seed-Borne Mycoflora of Sunflower

Data in Table 1 shows the fungi associated with sunflower seeds. It was found that 14 genera and 12 species of fungi were isolated and identified from sunflower seed samples. In general, Sakha 53 cv. recorded a higher frequency of fungi than Giza 102 cv. in both blotter and agar plate methods. Moreover, detection of fungi was more frequent in the blotter method than the agar plate method, as was found for the Sahka 53 cv. colony count and not that in the case of the Giza 102 cv. The fungi found to have the highest incidence was *Aspergillus flavus* followed by *Alternaria alternata* and *Penicillium* spp. Nevertheless, variations between the two tested cultivars and the two methods of detection were observed. For example, *Stemphylium* spp., *Cladosporium* spp. and *Nigrospora spp* were not detected in Sakha 53 cv. either in the blotter or agar plate methods. While, *Tricotheium roseum, Macrophominia phaseolina* and *Rhizoctonia solani* was not detected in the blotter method and detected with very low frequency by the agar plate method. Correlation between storage period and stored temperatures are presented in Table 2 and Table 3. For Sakha 53 cv. a positive correlation between temperature degrees and strored period. Fungi were found using the blotter method Table 2, *Aspergillus flavus* followed by *Alternaria alternate* and *Aspergillus niger* were the most frequently occurring fungi when stored at 30°C for 12 months. The incidence of the fungi was decrease as the temperature and time of storage was reduced. However, *Drechlera* spp. was not detected using the blotter method in any of the treatments, whereas *Rhizopus nigricans* was only found in the 4 months storage samples held at 10°C and 20°C (Table 2). In the case of Giza 102 cv., a Similar trend of the results as Sakha 53 cv. Table 3 although *Drechlera* spp. and *Rhizopus nigricans* were found in most of the treatments of this cultivar. In both cultivars tested, it was noted that increase temperature and storage periods increased subsequently frequency of the fungi associated with

sunflower seeds. These results are in general agreement with those reported earlier [25,26] [27,28,29]. [30] where it was observed that the standard blotter method was better for isolation of large number of fungal species. The results obtained in close conformity with those of limonard [31] and Afzal et al. [32] who reported that chloral disinfection effectively reduced the microbial contamination. Reduction of frequency rate of fungi from sterilized sunflower seeds was also found by other workers [33,14]. Similar results have also been reported from seeds other than sunflower eg., groundnut seed [34] and legume seeds [35].

3.2 Detection of Aflatoxin

3.2.1 Flow through strips (Aflacheck)

Detection of aflatoxin in sunflower contaminated seeds using the strip method is presented in Table 4. Aflatoxins was detected in both sunflower cultivars (Sakha 53) and (Giza 102). Moreover, the amount of aflatoxin detected was increased by increasing storage period from 8 month up to twelve months. Whereas, aflatoxin was not detected at 4 months storage periods.

3.2.2 Vicam apparatus

Measurement of total aflatoxin using the Vicam method showed a greater increase in the Sakha 53 cv. seed than in the Giza 102 cv. with increasing storage period (Fig 1).Such increases in aflatoxin level was positively correlated with storage period at maximum level after 12 months. This result was confirmed in both tested cultivars [36].

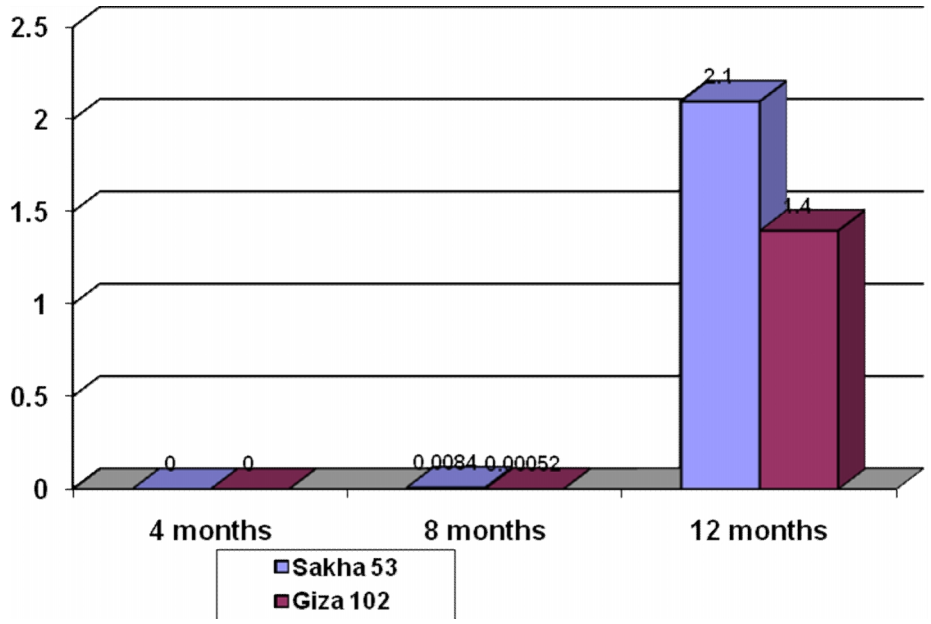

Fig. 1. Measurement of total aflatoxin in two cultivars (Sakha 53 and Giza 102) using the Vicam apparatus

Table 1. Percentage of incidence and frequencies of seed–borne mycoflora associated with sunflower seeds collected from two cultivars (Sakha 53 and Giza 102) in Egypt in season 2009 using two methods

Sunflower Cultivars	Sakha 53 Khalyobia.				Giza 102 El-Minia.			
Treatment / Fungi	Blotter Incide)nce (%)	Frequency (%)	Agar plat Incidence (%)	Frequency (%)	Blotter Incidence (%)	Frequency (%)	Agar plate Incidence (%)	Frequency (%)
Alternaria alternate	5.0	10.2	6.5	19.9	8.0	20.9	6.6	19.6
Aspergillus flavus	24.0	48.9	9.0	26.7	6.0	15.7	11.0	32.6
Aspergillus niger	3.0	0.1	8.3	24.6	4.0	10.5	6.0	17.8
Aspergillus ochraceus	2.2	4.5	1.0	3.0	2.0	5.2	2.6	7.7
Stemphylium spp.	0.0	0.0	0.0	0.0	2.0	5.2	0.0	0.0
Trichotheium rosum	0.0	0.0	1.0	3.0	2.0	5.2	2.0	5.9
Fusarium moniliforme	0.0	0.0	3.4	10.1	1.0	2.6	0.4	1.2
Cladosporium spp.	0.0	0.0	0.0	0.0	1.0	2.6	0.0	0.0
Penicillium spp.	11.0	22.4	0.0	0.0	9.0	23.6	0.2	0.6
Nigrospora spp.	0.0	0.0	0.0	0.0	1.0	2.6	0.0	0.0
Rhizopus nigricans	3.0	6.1	0.0	0.0	2.0	5.2	0.9	2.7
Drechlera spp.	0.0	0.0	1.6	4.7	0.0	0.0	0.2	0.6
Macrophomina phaseolina	0.0	0.0	1.5	4.5	0.0	0.0	2.4	7.1
Rhizoctonia solani	0.0	0.0	0.8	3.8	0.0	0.0	1.3	3.9
LSD .05	1.47		0.82		1.46		1.34	

Table 2. Precentage of occurrence of different fungi in sunflower seeds using the blotter method in Sakha 53 cv. stored for 4, 8 and 12 months

Storage periods time	4 months				8 months				12 months			
Temperature	**0°C**	**10°C**	**20°C**	**30°C**	**0°C**	**10°C**	**20°C**	**30°C**	**0°C**	**10°C**	**20°C**	**30°C**
Pathogen												
Alternaria alternate	0.0	1.8	3.2	5.1	1.0	1.7	3.5	5.8	1.2	5.3	7.8	10.9
Aspergillus flavus	2.0	13.2	18.6	17.0	11.6	8.1	19.0	18.9	16.0	14.1	19.4	20.2
Aspergillus niger	1.2	1.0	3.0	0.0	0.0	4.0	9.0	7.0	0.0	2.8	2.0	7.0
Stemphylium spp.	0.0	0.0	0.0	0.0	0.0	4.0	0.0	0.0	0.0	4.0	0.0	0.0
Trichotheium roseum	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	1.0	0.0	0.0
Fusarium moniliforme	0.9	0.7	2.5	4.0	0.0	6.0	0.0	0.0	0.0	7.2	1.3	0.0
Cladosporium spp.	0.0	0.0	0.0	6.0	0.1	0.0	0.0	1.0	0.1	0.0	0.0	1.0
*Penicillum s*pp.	0.1	1.4	11.0	5.0	0.0	0.0	7.0	16.0	0.0	0.8	21.3	17.1
Nigrospora spp.	0.0	0.0	0.0	1.0	0.0	0.0	0.5	4.3	0.0	0.0	0.9	5.1
Rhizopus nigricans	0.0	1.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Drechlera spp.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	4.2	19.1	41.3	38.1	12.7	24.8	39.0	53.0	17.3	35.2	52.7	61.3

Table 3. Percentage of occurrence of different fungi in sunflower seeds using the blotter method in Giza 102 cv. stored after 4, 8 and 12 months

Storage period time	4 months				8 months				12 months			
Temperature	**0°C**	**10°C**	**20°C**	**30°C**	**0°C**	**10°C**	**20°C**	**30°C**	**0°C**	**10°C**	**20°C**	**30°C**
Pathogen												
Alternaria alternate	0.2	0.0	8.0	19.3	0.5	1.6	10.1	12.7	0.7	11.2	15.1	17.2
Aspergillus flavus	0.3	0.7	6.0	8.2	1.1	0.9	8.2	14.1	1.3	1.6	15.4	18.3
Aspergillus niger	0.1	1.2	5.0	6.1	0.9	6.0	6.3	9.2	0.4	4.3	8.1	10.7
Stemphylium spp.	0.0	0.0	2.0	3.4	0.0	0.9	3.1	4.1	0.5	0.8	3.3	2.0
Trichotheium roseum	0.0	1.2	1.5	1.9	0.5	0.3	0.6	2.1	0.0	0.0	0.8	2.0
Fusarium moniliforme	0.1	2.3	3.1	2.5	0.5	0.3	4.2	3.1	0.3	6.2	7.3	7.7
Cladosporium spp.	0.3	0.6	1.6	1.1	0.3	0.3	2.1	1.0	0.0	1.7	2.1	2.2
*Penicillium s*pp.	0.2	1.9	2.4	2.7	1.1	0.7	3.5	1.4	1.1	3.9	5.5	7.9
Nigrospora spp.	0.0	0.0	0.1	1.4	0.2	0.9	0.0	0.4	0.0	1.2	1.3	1.0
Rhizopus nigricans	0.0	0.0	0.0	0.1	0.1	2.1	0.4	0.6	0.7	5.3	12.9	10.9
Drechlera spp.	0.0	0.0	0.6	0.2	0.0	0.0	0.1	0.3	0.0	0.4	0.2	2.0
Total	1.2	7.9	30.3	46.9	5.2	14.0	38.6	49.0	5.0	36.6	72.0	81.9

Table 4. Detection of total aflatoxin of two sunflower cultivars (Sakha 53 and Giza 102) by using strips (Alfachek)

Sunflower Cultivars Storage time	Sakha 53	Giza 102
4 months	-	-
8 months	+	+
12 months	++	+

(+):Moderate (++):Present

Fungal contaminated sunflower seeds can affect seed germination and greatly affected seed quality by producing toxins [7,36]. The results obtained by using Vicam apparatus [37] showed that sunflower seed which were stored for 4 months showed no evidence of contamination by aflatoxin but after 8 months of storage aflatoxin was found at 0.0084 ppm in Sakha 53 cv. and 0.00052 ppm in Giza 102 cv. After 12 months of storage, total aflatoxin (B_1,B_2,G_1 and G_2) were detected as 2.1 and 1.4 ppm in Sakha 53 and Giza 102 cvs.). Maximum levels in both cultivars was attained at a maximum storage time of 12 months. The deleterious biological effect of these toxins contaminated sunflower seed materials on these results raise concerns with respect to the health of human consumers of sunflower seeds after prolonged storage. This including immune system disorder, permanent damage of spleen, liver, kidney and brain functions [38].

4. CONCLUSION

It can be recommended that the soundness of sunflower seeds can be maintained (free of fungal contamination) by reducing storage periods and/or high temperature of storage. This avoids reduction in oil quality and limits the accumulation of aflatoxin in the seeds. Sound seeds play a vital role in the production of healthy crops and are the foundation of healthy plant; a necessary condition for good yields [39].

COMPETING INTERESTS

Authors AAK, DAE and MIG are co-operative in this investigation through their experience and honoraria. Also authors have wide personal relationships with other insititutes and orangizations related. Finally, authors have a lot of publications in field.

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