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Mycobiota and Mycotoxins of Egyptian Peanut (*Arachis hypogeae* L.) Seeds

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Abstract: Sixty-three species in addition to 3 varieties of 21 genera were isolated from 20 samples of each of untreated (51 species + 3 varieties of 21 genera and 51.24×10^3 cfu g^{-1} dry weight seeds), roasted (28 + 2 of 12 and 11.5×10^3 cfu) and roasted with salt (28 + 2 of 7 and 7.5×10^3 cfu) on dextrose-Czapek's agar at 28°C using dilution-plate method. The dominant fungal genera with their respective species on three types of seeds were *Aspergillus* (*A. niger*, *A. flavus* and *A. ficuum*), *Penicillium* (*P. citrinum*) and *Fusarium* (*F. oxysporum*). Based on biological, TLC, spectrophotometeric and ELISA assays, fourteen samples (23.3%) out of 60 tested proved to be toxic with different mycotoxins; aflatoxins, sterigmatocystin, ochratoxins, diacetoxyscirpenol and zearalenone. Also, mycoflora and myctoxins of six cultivars, widely cultivated in Upper Egypt were studied as pre-storage and post-storage in normal store for 3, 6, 12 and 24 months. A total of 28 species belonging to 14 genera were identified on dextrose-Czapek's agar medium (25 species of 12 genera) and cellulose-Czapek's agar medium (24 of 12) using dilution-plate method at 28°C. *Aspergillus* (*A. niger*, *A. flavus* and *A. fumigatus*), *Fusarium* (*F. oxysporum*) and *Penicillium* (*P. citrinum*) were the most prevalent fungal genera and species and their counts increased with lengthening of storage period. Cultivars were non-toxic, but toxins production appeared after 12 and 24 months of storage on two and three cultivars, respectively.

Key words: Peanut seeds, cultivars, mycobiota, mycotoxins, storage fungi

INTRODUCTION

Peanuts are unique among cultivated crops in that produce seed-bearing pods below the soil surface. Pods are in direct contact with soil populations and the seeds are frequently invaded by soil fungi before harvest (Horn and Greene, 1995).

Infection of peanut by *Aspergillus*, especially members of *A. flavus* group and *A. niger* group occurs under both pre-harvest and post-harvest conditions. Pre-harvest infection by *A. flavus* and *A. parasiticus* and consequent aflatoxin contamination is a major problem in the semi-arid tropic environment. These fungi are widespread in light sandy soils most suitable for peanut cultivation. Peanut pods when in direct contact with spores of *A. flavus* in soil are frequently invaded before harvest. The mode and extent of invasion by *A. flavus* depend on soil population density of *A. flavus*, soil moisture content and soil temperature during the pod development to maturity period (Horn *et al*., 1995). Post-harvest contamination may occur when stored products are not maintained at a safe moisture level. Also, these fungi can invade and produce toxins in peanut kernels before harvest, during drying and in storage. Owing to the toxicity and carcinogenicity of aflatoxins contaminated commodities destined for human or animal consumption pose a serious health hazards and are therefore, closely monitored and regulated. Apart from its effect on health, aflatoxins contamination also impacts the agricultural economy, through the loss of produce and thus time and costs involved in monitoring and decontamination (Craufurd *et al*., 2006; Kumar *et al*., 2008).

The average aflatoxin concentration accepted by PAC (aflatoxin testing program) in the United States depends on the conditions of peanut crop after shelling (rejected lots with over 25 ppb and accepted lots with 25 ppb aflatoxin or less). In the European Union, the aflatoxin B and the total aflatoxins level in peanut products are regulated with Maximum Residue Levels (MRLs) that cannot be greater than 2 and 4 ng g^{-1}, respectively (EU Commission Regulation, 2002).

Egypt exports shelled nuts at about 7 millions US$ and in shell nuts at about 4.4 millions US$, yearly (FAO, 2006). The major mycotoxins found in Egyptian peanuts were aflatoxins (El-Maghraby and El-Maraghy, 1987). Aflatoxin concentration is the most important quality problem in peanut worldwide with serious health implications for human as well as livestock (Sinha *et al*., 1999; D'Mello, 2003). The climatic conditions as well as the food production chains are characteristic in most parts of Africa and the largest mycotoxin-poisoning epidemic in a decade was reported in Africa during the last 5 years (Wagacha and Muthomi, 2008).

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The present investigation was designed to study the mycoflora and mycotoxins of 20 samples of each of untreated, roasted and roasted with salt peanut seeds prepared for human consumption, collected from different localities in Egypt. Also, fungal contamination and natural occurrence of mycotoxins on six peanut cultivars, widely cultivated in Upper Egypt were studied as post-harvest (1-3 months) and stored in normal store for 3, 6, 12 and 24 months.

MATERIALS AND METHODS

Collection of peanut seed samples: Twenty fruit samples, 500 g, each were collected after harvest of 2004 production, from the Egyptian markets in different Governorates, put in a sterile polyethylene bags, transferred to the laboratory, then the pericarps were unfolded and the seeds were released under sterile conditions, put in other sterile polyethylene bags and kept in a cool place (3-5°C). As well as, 20 samples of each of roasted and roasted with salt (500 g, each) were collected from the peanut roasters in different Governorates of Egypt, transferred to the laboratory, shelled in sterile conditions and put the seeds of each sample in a sterile polyethylene bag sealed and put in another one, which was also sealed to minimize the loss of water-content and kept in a cool place (3-5°C) till fungal cultivation, isolation, identification and mycotoxins assay.

Also, the 6 cultivars ($Ismaila_1$, $Giza_4$, $Giza_5$, $Giza_6$, $Local_{262}$ and R_{92}) were kindly supplied by Oil Crops Department of Agriculture Research Center, Shandaweel Station for Agriculture Researches, Sohag Governorate, Upper Egypt. The seed samples (500 g, each) were put in sterile polyethylene bags, transferred to the laboratory and kept in cool (3-5°C) conditions till different assays. Storage of cultivars for 3, 6, 12 and 24 months was carried out in oil crops store (Shandaweel Station for Agriculture Researches). The fungal flora and mycotoxins investigations of cultivars were performed pre-storage and after each storage period.

Determination of moisture content of peanut seeds: Twenty grams of each seed sample were milled and dried in an oven at 105°C for 24 h, then cooled in a desiccator and re-weighted to a constant weight. The moisture content was calculated as percentage of the dry weight.

Determination of seed-borne fungi: Dilution-plate method as described by Johnson and Curl (1972) was used for isolation of fungi. Modified 1% dextrose-Czapek's agar medium (g L^{-1}; sodium nitrate 3.0, magnesium sulphate 0.5, potassium chloride 0.5, di-potassium hydrogen phosphate 1.0, iron sulphate 0.01, dextrose 10.0, agar agar 15.0-20.0, pH 6.6±0.1) was used as cultivation and isolation medium. In case of seed cultivars fungal flora, modified 1% dextrose-Czapek's agar and 2.1% cellulose-Czapek's agar media were used. Chloramphenicol (0.5 mg mL^{-1}) as bacteriostatic agent and rosebengal (30 ppm) to restrict for widespreading fungi because of stimulate slow growing fungi, were added to the medium (Al-Doory, 1980).

Five plates were used for each sample tested and each cultivar after every storage period, in addition to different control samples as post-harvest (pre-storage). The plates were incubated at 28±2°C for 7-15 days and the developing fungi were identified, counted and calculated per gram dry weight of each tested sample. The colonies of slow growing fungi, as well as mycelial bits were transferred to slants with special media to ensure precise counting, then to plate for identification.

Taxonomic identification of fungi (based on purely morphologically macro- and microscopic characteristics) was carried out according to (Booth, 1971, 1977; Raper and Fennell, 1977; Christensen and Raper, 1978; Pitt, 1979, 1991; Domsch *et al*., 1980; Moubasher, 1993; Klich, 2002; Samson *et al*., 2002; Summerell *et al*., 2003).

Sample preparation for mycotoxins analysis

Extraction procedures: Fifty grams of each sample were defatted by extraction with cyclohexane (150 mL) for 10 h using Soxhlet type extractor. The defatted residue was extracted with ethyl acetate (three times, 50 mL/each). The extracts were combined, dried over anhydrous sodium sulphate, filtered and then concentrated under vacuum to near dryness, transferred into a brown glass vial and evaporated under nitrogen stream.

Clean up of crude extracts: For cleaning up the crude extracts (purified from interfering compounds), it was suspended in 1 mL chloroform and applied to 14×0.8 cm column containing 2.5 g kiesel gel 60, 70/230 silica gel (MERCK. Germany). The washing and eluting solvents (8 mL, each) for aflatoxins (n-hexane followed by ether and 3: 97 methanol-chloroform, respectively), sterigmatocystin (n-hexane and 3: 97 methanol-chloroform, respectively), ochratoxins and citrinin (n-hexane and 5: 95 methanol-chloroform, respectively), trichothecenes (dichloromethane and 5: 95 methanol-dichloromethane, respectively), zearalenone (n-hexane and 5: 95 acetone-benzene, respectively), fusarin C (dichloromethane and 10: 90 methanol-dichloromethane, respectively) and moniliformin (chloroform and 5: 3: 2 toluene-acetone-methanol, respectively) were carried out according to AOAC (1984), Jarvis *et al*. (1986) and Dorner (1998).

Bioassay of mycotoxins: Three bioassay tests for mycotoxins detection were used; brine shrimps (*Artemia salina* L.) larvae, *Chlorella vulgaris* Bejerinck and *Bacillus subtilis* according to Korpinen (1974), Bean *et al.* (1992) and Földes *et al.* (2000), respectively.

Thin Layer Chromatography (TLC): For qualitative detection of mycotoxins, thin layer chromatography technique was employed using precoated silica gel plates type 60 F_{254} TLC (E, MERCK, Germany). Aflatoxins B_1, B_2, G_1 and G_2, ochratoxins A and B, sterigmatocystin, citrinin, diacetoxyscirpenol (DAS), T-$_2$ toxin, zearalenone, moniliformin and fusarin C were used as standard references (Sigma). The plates were developed using the following solvent systems; methanol-chloroform (v/v, 3/97) for aflatoxins, ochratoxins, sterigmatocystin and citrinin, ethyl alcohol-chloroform (v/v, 5/95) for zearalenone, ethyl acetate-n-hexane (v/v, 70/30) for trichothecenes and toluene-acetone-methanol (v/v/v, 50/30/20) for moniliformin and fusarin C. The developed plates were viewed under short and/or long wave length UV (254 and/or 366 nm) light and sprayed with reagents according to Gimeno (1976), Takitani *et al.* (1979), AOAC (1984), Farber and Sanders (1986), Vesonder (1986) and Dorner (1998).

For quantitative determination of mycotoxins, spectrophotometeric (Cecil, model 703) technique was used at molecular coefficient of 21800 at 260 and/or 366 nm UV light according to the method described by Bean *et al.* (1972).

Enzyme Linked Immuno-Sorbent Assay (ELISA): For quantitative determination of aflatoxin B_1 (AFB_1), ELISA technique was employed according to Gathumbi *et al.* (2001) and Rodriguez *et al.* (2003) because World Health Organization (WHO, 2006) has cited aflatoxins as the most potent naturally occurring carcinogens, as well as International Agency for Research on Cancer (IARC) placed aflatoxin B_1 on the list of human carcinogens (Wu, 2004) and presence of aflatoxin B_1 kits.

RESULTS AND DISCUSSION

The moisture content of untreated, roasted and roasted with salt peanut seed samples (on oven dry basis) was low and ranged between 3.03-4.35, 0.81-1.75 and 1.03-1.63, respectively.

Mycological analysis of peanut seed samples tested based on dilution-plate method using 1% dextrose-Czapek's agar medium at 28±2°C revealed that 63 species in addition to 3 varieties belonging to 21 genera were isolated and identified from untreated (51 species + 3 varieties of 19 genera), roasted (28 + 2 of 12) and roasted with salt (28 + 2 of 7) peanut seeds. The gross total viable count of fungi as well as the mean of fungal contamination of untreated seeds (72.9% of general total fungi, 51.24×10^3 and 2.56×10^3 cfu g^{-1} dry peanut seed sample) was remarkably high in comparison of roasted (16.4%, 11.5×10^3 and 0.6×10^3 cfu) and roasted with salt (10.7%, 7.5×10^3 and 0.4×10^3 cfu) as recorded in Table 1. These results are in harmony with that obtained by El-Maghraby and El-Maraghy (1987, 1988), who isolated (64 species + 2 varieties of 19 genera) on 1% dextrose-Czapek's agar and (43 species + 1 variety of 16 genera) on 2.1% cellulose-Czapek's agar medium at 28°C from untreated peanut seeds, respectively.

Generally, the most dominant fungal genera on the three seed types tested were *Aspergillus* (20 species + 2 varieties, 57 cases out of 60 tested, 95% of total samples and 38.03% of general total fungi) and *Penicillium* (17 species, 34 cases, 56.7 and 20.4%), respectively. *A. niger* (22 cases out of 60 tested and 36.7% of total samples), *A. flavus* (20 and 33.3%), *A. ficuum* (16 and 26.7%), *A. oryzae* (11 and 18.3%), *A. parasiticus* (11 and 18.3%) and *P. citrinum* (9 and 15%) were the most prevalent species. Also, the high degrees of occurrence of *Aspergillus* and *Penicillium* were detected on different seed types tested; that on untreated (90 and 75% of the samples and 20.9 and 24.3% of total fungi), roasted (95, 35, 85.3 and 7.3%) and roasted with salt (100, 60, 81.4 and 13.9%) peanut seed samples, respectively. On the other hand, *Fusarium* (4 species, 12 cases, 20 and 12.2%) isolated from untreated (3 species, 50 and 16.4%) and roasted (2 species, 10 and 1.2%) seed samples only, respectively and disappeared on roasted with salt samples as recorded in Table 1.

Also, the order of occurrence frequency of isolated species varied on different seed types, that on untreated seeds *A. flavus* (40% of the samples and 4.9% of total fungi), followed by *A. aculeatus* (40 and 4.7%), *A. niger* (35 and 1.3%), *A. flavus* var. *columnaris* (25 and 1.1%), *P. citrinum* (30 and 15.1%) and *F. oxysporum* (30 and 0.7%) were the most dominant, respectively. While, on roasted seeds, *A. ficuum* (45 and 27.8%), *A. niger* (35 and 30.8%), *A. parasiticus* (30 and 10.4%), *A. flavus* (25 and 4.3%) and *P. chrysogenum* (15 and 2.3%), whereas, on roasted seeds with salt, *A. niger* (40 and 28.1%), *A. flavus* (35 and 4.9%), *A. oryzae* (25 and 18.1%), *A. ficuum* (25 and 5.4%) and *P. roquefortii* (20 and 4.3%) were the most frequent, respectively as shown in Table 1.

These obtained results are in full agreement with those previously recorded by El-Maghraby and El-Maraghy (1988), and Gonçales *et al.* (2008) that *Aspergillus* and *Penicillium* were the most dominant genera in Egyptian peanut, chickpea seeds and Brazilian peanut seeds, respectively. Also, Christensen

Table 1: Total count (TC, calculated per g dry weight peanut seeds) in each peanut sample, percentage (TC%, per total count of fungi), number of cases of isolation (NCI, out of 20 samples) of fungal genera and species isolated from peanut sample types (untreated, roasted and roasted with salt seeds) on 1% dextrose-Czapek's agar medium at 28±2°C using dilution-plate method

	Untreated seed samples (1-20)				Roasted seed samples (21-40)				Roasted with salt seed samples (41-60)			
Fungal genera and species	TC	TC (%)	NCI	OR	TC	TC (%)	NCI	OR	TC	TC (%)	NCI	OR
Total count	51235				11505				7505			
Aspergillus Link	10730	20.9	18	H	9815	85.3	19	H	6110	81.4	20	H
A. niger van Tieghem	655	1.3	7	M	3545	30.8	7	M	2110	28.1	8	M
A. flavus Link	2540	4.9	8	M	495	4.3	5	L	365	4.9	7	M
A. ficuum (Reich.) Hennings	130	0.3	2	R	3200	27.8	9	M	405	5.4	5	M
A. oryzae (Ahlb.) Cohn	280	0.5	3	L	280	2.4	3	L	1360	18.1	5	L
A. parasiticus Speare	140	0.3	3	L	1200	10.4	6	M	240	3.1	2	R
A. alutaceus Berk. and Curt.	1540	3.0	4	L	20	0.2	1	R	140	1.9	4	L
A. flavus var. *columnaris* Raper and Fennell	555	1.1	5	L	320	2.8	2	R	400	5.3	2	R
A. foetidus var. *pallidus* Nakazawa, Simo and Watanabe	480	0.9	3	L	300	2.6	4	L	320	4.2	2	R
A. aculeatus Iizuka	2415	4.7	8	M	-	-	-	-	-	-	-	-
A. carbonarius (Bainier) Thom	330	0.6	3	L	180	1.6	2	R	205	2.7	3	L
A. sydowi (Bainier and Sartory) Thom and Church	280	0.5	5	L	-	-	-	-	160	2.1	1	R
A. fumigatus Fresnius	5	0.01	1	R	40	0.3	2	R	200	2.6	2	R
A. tubingensis (Schober) Mosseray	455	0.8	2	R	60	0.5	1	R	40	0.5	1	R
A. pulverulentus (McAlpine) Thom	320	0.6	2	R	-	-	-	-	-	-	-	-
A. viride-nutans Ducker and Thrower	275	0.5	2	R	-	-	-	-	-	-	-	-
A. flavo-furcatis Batista and Maia	-	-	-	-	120	1.0	2	R	-	-	-	-
A. duricaulis Raper and Fennell	300	0.6	1	R	-	-	-	-	-	-	-	-
A. parvulus Smith	-	-	-	-	-	-	-	-	160	2.1	1	R
A. japonicus Saito	-	-	-	-	50	0.4	1	R	-	-	-	-
A. phoenicis (CDS.) Thom	20	0.03	1	R	-	-	-	-	-	-	-	-
A. terreus Thom	10	0.02	1	R	-	-	-	-	-	-	-	-
A. foetidus (Naka.) Thom and Raper	-	-	-	-	-	-	-	-	5	0.1	1	R
Penicillium Link	12425	24.3	15	H	835	7.3	7	M	1050	13.9	12	H
P. citrinum Thom	7700	15.1	6	M	280	2.4	2	R	100	1.3	1	R
P. roquefortii Thom	-	-	-	-	80	0.7	1	R	325	4.3	4	L
P. chrysogenum Thom	5	0.01	1	R	260	2.3	3	L	20	0.3	1	R
P. funiculosum Thom	1335	2.6	3	L	75	0.7	1	R	-	-	-	-
P. rugulosum Thom	420	0.8	3	L	140	1.2	1	R	-	-	-	-
P. purpurogenum Stoll	240	0.5	2	R	-	-	-	-	30	0.4	2	R
P. verruculosum Peyronel	320	0.6	3	L	-	-	-	-	-	-	-	-
P. spinulosum Thom	1680	3.2	2	R	-	-	-	-	-	-	-	-
P. puberulum Bainier	70	0.1	1	R	-	-	-	-	140	1.9	1	R
P. corylophilum Dierckx	100	0.2	1	R	-	-	-	-	5	0.1	1	R
P. aurantiogriseum Dierckx	-	-	-	-	-	-	-	-	10	0.1	2	R
P. simplicissimum (Oud.) Thom	455	0.9	1	R	-	-	-	-	-	-	-	-
P. piceum Raper and Fennell	-	-	-	-	-	-	-	-	220	2.9	1	R
P. griseo-roseum Dierckx	-	-	-	-	-	-	-	-	160	2.1	1	R
P. aculeatum Raper and Fennell	80	0.2	1	R	-	-	-	-	-	-	-	-
P. paxilli Bainier	-	-	-	-	-	-	-	-	40	0.5	1	R
P. brevi-compactum Dierckx	20	0.03	1	R	-	-	-	-	-	-	-	-
Fusarium Link	8420	16.4	10	M	135	1.2	2	R	-	-	-	-
F. oxysporum Schlecht	380	0.7	6	M	-	-	-	-	-	-	-	-
F. sporotrichioides Sherb, Mem	8020	15.7	4	L	-	-	-	-	-	-	-	-
F. sulphureum Schlecht	20	0.03	1	R	35	0.3	1	R	-	-	-	-
F. fusarioides (Frag and Gif.) Booth	-	-	-	-	100	0.9	1	R	-	-	-	-
Emericella nidulans var. *acristata* (Fennell and Raper) Subram	65	0.1	2	R	65	0.6	4	L	45	0.6	2	R
Cladosporium cladosporioides (Fresen.) de Vries	40	0.1	2	R	45	0.4	2	R	70	0.9	4	L
Ulocladium alternariae (Cke) Simmons	50	0.1	3	L	40	0.3	2	R	45	0.6	2	R
Scopulariopsis (Bainier) Thom	95	0.2	4	L	80	0.7	2	R	-	-	-	-
S. costantini Bainier	30	0.1	3	L	20	0.2	1	R	-	-	-	-
S. brevicaulis (Sacc.) Bainier	40	0.1	1	R	60	0.5	1	R	-	-	-	-
S. communis Bainier	25	0.04	2	R	-	-	-	-	-	-	-	-
Gibberella fujikuroi (Sawada) Ito	14320	27.9	5	L	-	-	-	-	-	-	-	-
Acremonium Link	520	1.0	4	L	120	1.0	1	R	-	-	-	-
A. implicatum Gilman and Abbott	500	0.9	3	L	-	-	-	-	-	-	-	-

Table 1: Continued

Fungal genera and species	Untreated seed samples (1-20)				Roasted seed samples (21-40)				Roasted with salt seed samples (41-60)			
	TC	TC (%)	NCI	OR	TC	TC (%)	NCI	OR	TC	TC (%)	NCI	OR
A. strictum W.Gams	-	-	-	-	120	1.0	1	R	-	-	-	-
A. retilum W.Gams	20	0.03	1		-	-	-	-	-	-	-	-
Alternaria Nees	80	0.2	2	R	-	-	-	-	150	1.9	3	L
A. alternata (Fr.) Keissler	60	0.1	1	R	-	-	-	-	130	1.6	2	R
A. tenuissima (Kunze ex Pers.) Wiltshire	20	0.03	1	R	-	-	-	-	20	0.3	1	R
Talaromyces. luteus (Zukal) Benjamin	85	0.16	2	R	15	0.1	1	R	35	0.5	2	R
Nectria haematococca Berkely and Brown	290	0.6	4	L	-	-	-	-	-	-	-	-
Emericillopsis terricola van Beyma	-	-	-	-	80	0.7	3	L	-	-	-	-
Rhizopus stolonifer (Ehrenb.) Lind	15	0.02	1	R	140	1.2	1	R	-	-	-	-
Trichoderma viride Pers. Ex S.F. Gray	20	0.03	1	R	40	0.3	1	R	-	-	-	-
Verticillium chlamydosporium Goddard	2600	5.1	1	R	-	-	-	-	-	-	-	-
Actinomucor repens Schostakowitsch	740	1.4	1	R	-	-	-	-	-	-	-	-
Apocrea chrysosperma (Bulliard) Fries	680	1.3	1	R	-	-	-	-	-	-	-	-
Chaetomium globosum Kunze ex Fries	-	-	-	-	100	0.9	1	R	-	-	-	-
Humicola grisea Traaen	40	0.1	1	R	-	-	-	-	-	-	-	-
Paecilomyces lilacinus (Thom) Samson	20	0.03	1	R	-	-	-	-	-	-	-	-
Total count (colonies g^{-1} dry sample)		51235				11505				7505		
Mean of fungal contamination (colonies g^{-1} dry sample)		2561.8				575.3				375.3		
No. of genera (21)		19				12				7		
No. of species and varieties (63 + 3)		51 + 3				28 + 2				28 + 2		

- : No fungus isolated, OR: Occurrence remarks; H: High occurrence, (more than 10 samples out of 20 tested), M: Moderate occurrence, (6-10 samples), L: Low occurrence (3- 5 samples), R: Rare occurrence, (less than 3 samples)

(1991) reported that *Aspergillus* and *Penicillium* species are the main components of storage fungi play an important role in seed deterioration and they have competitive ability against other fungi due to heavy spores and mycotoxins production of their diversity species. Creppy (2002) reported that seeds and grains are more liable to fungal infection particularly *Aspergillus*, *Penicillium* and *Fusarium* species in tropical and sub-tropical regions dependent on high levels of moisture content. Costa and Scussel (2002) reported that the problem of food contamination with mycotoxins has led to an increasing concern of toxigenic fungi contamination, mainly *Aspergillus*, *Penicillium* and *Fusarium* genera.

The toxicity test using three biological assays (*Artemia salina* L. larvae, *Chlorella vulgaris* Bejerinck and *Bacillus subtilis*) revealed that 14 samples (23.3%) out of 60 peanut seed samples tested proved to be toxic. Based on thin layer chromatography (TLC), spectrophotometeric and ELISA analyses, aflatoxins B_1 and B_2 or B_1, B_2, G_1 and G_2 were detected in 20, 10 and 15% of untreated, roasted and roasted with salt samples, respectively. These toxic samples were heavily contaminated with many members of *Aspergillus flavus* group (*A. flavus*, *A. parasiticus*, *A. flavus* var. *columnaris*, *A. oryzae* and *A. flavo-furcatis*) as aflatoxins-producers as shown in Table 2.

Similarly, 42.5% of Egyptian peanut, 35% of soybean and 32% of Brazilian peanut seed samples were proved to be contaminated by aflatoxins B_1 and B_2 or B_1, B_2, G_1 and G_2 (El-Maghraby and El-Maraghy, 1987; El-Kady and Youssef, 1993; Gonçales *et al*., 2008), respectively. Aflatoxins are potent hepatotoxic, carcinogenic metabolites produced by *A. flavus* Link, *A. parasiticus* Spear and *A. nomius* Kurtzman, Hort and Hesseltine as reported by Kurtzman *et al.* (1987) and Richard (2007). Species of *A. flavus* group have been responsible for production of carcinogenic aflatoxins in peanuts (*Arachis hypogeae* L.) in soils worldwide. The mode and extent of invasion by *A. flavus* depend on high levels of *A. flavus* group colonization of peanut fruit in soil, moisture content and temperature of soil during the pod development to maturity period. These fungi can also, invade and produce toxins in peanut kernels before harvest, during drying and in storage. Aflatoxins contamination of peanut does not affect yield only, but also, causes serious health risks to human and cattle (Craufurd *et al*., 2006; Kumar *et al*., 2008).

Sterigmatocystin was recorded in 15% of roasted seeds and 5% of roasted with salt seed samples. These samples were contaminated by *Emericella nidulans* var. *acristata* as sterigmatocystin-producing fungus as stated in Table 2. This toxin was naturally reported in 8.5% of Egyptian sunflower seeds and 8% of peanut seeds (El-Maraghy and El-Maghraby, 1986; El-Maghraby and El-Maraghy, 1987), respectively. This toxin was also detected by Egyptian thermotolerant fungal isolates including *Emericella nidulans*. *E. nidulans* var. *lata* and *E. quadrilineata* (El-Maraghy and El-Maghraby, 1986).

Ochratoxins A and B occurred naturally in 5% of each of untreated and roasted peanut seed samples.

Table 2: Biological assay and natural occurrence of mycotoxins in the toxic peanut (untreated, roasted and roasted with salt) seed samples

SN	MC (%)	Biological assays: *Bacillus subtilis* (mm)	Biological assays: *Chlorella vulgaris* (mm)	Biological assays: Brine shrimp (% dead larvae)	Mycotoxins identified (μg kg^{-1})	Mycotoxin-producing fungi
Untreated seed samples						
2	3.78	6	6	L	Aflatoxins B_1, B_2, G_1 and G_2 (6.2-8.8)	*A. flavus*, *A. flavus* var. *columnaris*, *A. oryzae*
3	3.95	8	8	L	Aflatoxins B_1, B_2, G_1 and G_2 (7.4-10.6)	*A. flavus*, *A. flavus* var. *columnaris*, *A. oryzae*
14	3.75	14	18	L	Aflatoxins B_1, B_2, G_1 and G_2 (9.5-16.4)	*A. flavus*, *A. flavus* var. *columnaris*, *A. oryzae*
18	3.59	15	18	L	Aflatoxins B_1, B_2, G_1 and G_2 (10.2-18.3)	*A. flavus*, *A. flavus* var. *columnaris*
20	3.67	22	28	M	Ochratoxins A and B (8.2 and 6.6), zearalenone (9.8) and Diacetoxyscirpenol (DAS) (10.2)	*A. alutaceus*, *A. niger*, *A. carbonarius* *Fusarium oxysporum*, *F. sporotrichioides*, *F. sulphureum*
Roasted seed samples						
22	1.34	17	18	L	Aflatoxins B_1 and B_2 (8.8 and 7.9)	*A. flavus*, *A. parasiticus*, *A. oryzae*
27	1.41	21	23	L	Aflatoxins B_1, B_2, G_1 and G_2 (10.4-16.5)	*A. flavus*, *A. parasiticus*, *A. flavus* var. *columnaris*, *A. flavo-furcatis*
29	1.28	18	23	L	Ochratoxins A and B (14.8 and 10.6)	*A. alutaceus*, *A. niger*, *A. carbonarius*
32	1.16	18	20	L	Sterigmatocystin (16.8)	*Emericella nidulans* var. *acristata*
34	1.17	9	14	L	Sterigmatocystin (12.2)	*Emericella nidulans* var. *acristata*
35	1.75	14	18	L	Sterigmatocystin (14.8)	*Emericella nidulans* var. *acristata*
Roasted with salt seed samples						
45	1.63	17	21	L	Aflatoxins B_1, B_2, G_1 and G_2 (10.2-17.4)	*A. flavus*, *A. flavus* var. *columnaris*
46	1.03	9	14	L	Aflatoxins B_1, B_2, G_1 and G_2 (8.4-10.6)	*A. flavus*, *A. flavus* var. *columnaris*, *A. oryzae*
47	1.37	22	29	M	Aflatoxins B_1, B_2, G_1 and G_2 (12.4-20.8) and sterigmatocystin (12.2)	*A. flavus*, *A. oryzae*, *A. parasiticus*, *Emericella nidulans* var. *acristata*

SN = Sample No, % MC = Percentage moisture content, M = Moderate toxicity, 50-75% of dead larvae, L = Low toxicity, 25-50% of dead larvae

These samples were rich in *A. ochraceus* (*A. alutaceus*), *A. niger* and *A. carbonarius* as ochratoxins-producing fungi (Table 2). Ochratoxin A (OA), a phenylalanyl derivative of a substituted isocoumarin with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties, is mainly produced by *Aspergillus ochraceus*, also by *A. niger*, *A. carbonarius* and some species of *Penicillium* (Serra *et al*., 2003; Wagacha and Muthomi, 2008). This toxin was recorded as a contaminant in plant products worldwide (Creppy, 2002; Ghitakou *et al*., 2006; Wagacha and Muthomi, 2008; Kumar *et al*., 2008). The toxin was also found frequently and in high average concentrations in blood samples obtained from people living in Balkan countries and affected by Balkan Endemic Nephropathy disease (Pfohl-Leszkowicz *et al*., 2002).

Zearalenone combined with diacetoxyscirpenol (simple microcyclic trichothecene) were detected in one untreated seed sample (5% of samples tested). This sample heavily contaminated by *Fusarium* species (*F. oxysporum*, *F. sporotrichioides* and *F. sulphureum*) as diacetoxyscirpenol and zeralenone-producers as recorded in Table 2. Zearalenone can co-occur with simple microcyclic trichothecenes synthesized by *Fusarium* species (El-Maghraby, 1996; Schollenberger *et al*., 2006; Kumar *et al*., 2008). These toxins were detected in Egyptian oil seeds (El-Maraghy and El-Maghraby, 1986; El-Maghraby and El-Maraghy, 1987) and cereal grains (El-Maghraby *et al*., 1995; El-Maghraby, 1996; Al-Abssy, 2002).

Zearalenone has been implicated in numerous mycotoxicoses in farm animals, causing infertility and reproductive problems such as abortions, false heat, recycling, reabsorption of fetuses and mummies and vulval uterine prolapse (Schollenberger *et al*., 2006). Doses of ZEN that are much greater than concentrations, which have hormonal effect may have genotoxic and carcinogenic effects (Mitterbauer *et al*., 2003). In blood, zearalenone and its metabolite, zearalenol bind to human sex hormone-binding globulin to some extent (Eriksen and Alexander, 1998). As well as, trichothecenes are well-known strong irritants and have been associated with naturally occurring outbreaks of vomiting feed refusal and possibly gastric ulcers when consumed. Also, trichothecenes inhibit protein synthesis which followed by a secondary disruption of DNA and RNA synthesis. They affect the actively dividing cells and can decrease antibody levels, immunoglobulins and certain other humoral factors such as cytokines (Eriksen and Pettersson, 2004; Richard, 2007).

Effect of storage on mycoflora and mycotoxins of peanut cultivars: Based on dilution-plate method, 28 species belonging to 14 genera were isolated and identified on 1% dextrose-Czapek's (25 species of 12 genera) and 2.1% cellulose-Czapek's agar media (24 species of 12 genera) from six peanut seed cultivars (Ismailia$_1$, Giza$_4$, Giza$_5$, Giza$_6$, Local$_{262}$ and R$_{92}$) in pre-storage and after storage for 3, 6, 12 and 24 months. The most dominant fungal genera and their respective species were; *Aspergillus* (*A. niger*, *A. flavus* and *A. fumigatus*), *Fusarium* (*F. oxysporum*) and *Penicillium* (*P. citrinum*) as recorded in Table 3. These results are in harmony with those obtained by

Table 3: Total count (TC, calculated per g dry weight peanut seeds), number of cases of isolation (NCI, out of 6 samples) of fungal genera and species isolated from 6 peanut seed cultivars, in pre-storage and stored for 3, 6, 12 and 24 months on 1% dextrose- and 2.1% cellulose-Czapek's agar media at 28±2°C

	Dextrose-Czapek's agar medium									
	Control (pre-storage)		Storage periods: 3 months		6 months		12 months		24 months	
Genera and species	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
Total count	1490		1545		1775		5180		17160	
Aspergillus Link	825	6	825	6	550	6	900	6	13580	6
A. niger van Tieghem	630	6	630	6	340	6	190	6	7260	6
A. flavus Link	140	5	140	5	200	5	420	3	6140	6
A. fumigatus Fresnius	55	5	55	5	-	-	210	4	80	3
A. ustus (Bain.) Thom and Church	-	-	-		-	-	30	2	-	-
A. terreus Thom	-	-	-	-	-	-	10	1	60	2
A. flavo-furcatis Batista and Maia	-	-	-	-	-	-	-	-	40	1
A. alutaceus Berk. and Curt.	-	-	-	-	10	1	-	-	-	-
A. versicolor (Vuill.) Tiraboschi	-	-	-		-	-	30	1	-	-
A. parasiticus Speare	-	-	-		-	-	10	1	-	-
Fusarium oxysporum Schlecht	245	6	245	6	250	6	3750	5	940	2
Penicillium Link	255	6	255	6	805	6	430	5	20	1
P. citrinum Thom	160	5	160	5	495	5	210	3	-	-
P. funiculosum Thom	80	4	80	4	255	3	200	4	-	-
P. puberulum Bainier	15	2	15	2	15	1	-	-	-	-
P. purpurogenum Stoll	-	-	-	-	-	-	-	-	20	1
P. duclauxi Delacroxi	-	-	-	-	40	2	20	1	-	-
P. chrysogenum Thom	-	-	-	-	-	-	-	-	-	-
Cladosporium cladosporioides (Fresen.) de Vries	85	3	85	3	100	4	20	1	-	-
Alternaria alternata (Fr.) Keissler	30	4	45	6	20	1	-	-	-	-
Emericella nidulans (Eidam) Vuillemin	25	5	25	5	20	2	10	1	-	-
Trichoderma viride Pers. Ex S.F. Gray	-	-	-	-	-	-	-	-	2260	6
Stachybotrys chartarum (Ehrenb. ex Link) Hughes	15	1	15	1	-	-	-	-	-	-
Gibberella fujikuroi (Sawada) Ito	10	1	10	1	10	1	50	1	-	-
Myrothecium Tode	-	-	-	-	20	2	20	2	-	-
M. verrucaria (Albertini and Schweinitz) Ditmar	-	-	-	-	10	1	20	2	-	-
M. roridum Tode ex Steudel	-	-	-	-	10	1	-	-	-	-
Apocrea chrysosperma (Bulliard) Fries	-	-	-	-	-	-	-	-	300	2
Rhizopus stolonifer Ehrenb. ex Fr. Lindt	-	-	-	-	-	-	-	-	80	2
Mucor racemosus Fresenius	-	-	-	-	-	-	-	-	-	-
Cochliobolus spicifer Nelson	5	1	40	1	-	-	-	-	-	-
Total count (colonies g^{-1} dry sample)	1490		1545		1775		5180		17160	
No. of genera	9		9		8		7		6	
No. of species	13		13		14		15		10	
Total No. of genera and species (14 genera and 28 species)	12 genera and 25 species									

	Cellulose-Czapek's agar medium									
	Control (pre-storage)		Storage periods: 3 months		6 months		12 months		24 months	
Genera and species	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
Total count	1460		1500		1610		4470		16520	
Aspergillus Link	515	6	540	6	675	6	1720	6	12180	6
A. niger van Tieghem	290	5	300	5	365	6	160	2	4300	6
A. flavus Link	65	5	70	5	205	5	460	3	7160	6
A. fumigatus Fresnius	125	6	130	6	60	3	1070	5	600	5
A. ustus (Bain.) Thom and Church	35	2	40	2	35	1	20	1	-	-
A. terreus Thom	-	-	-	-	-	-	10	1	80	2
A. flavo-furcatis Batista and Maia	-	-	-	-	-	-	-	-	40	1
A. alutaceus Berk. and Curt.	-	-	-	-	10	1	-	-	-	-
A. versicolor (Vuill.) Tiraboschi	-	-	-	-	-	-	-	-	-	-
A. parasiticus Speare	-	-	-	-	-	-	-	-	-	-
Fusarium oxysporum Schlecht	280	6	310	6	350	6	1410	6	1120	5

Table 3: Continued

Genera and species	Cellulose-Czapek's agar medium									
	Control (pre-storage)		Storage periods							
			3 months		6 months		12 months		24 months	
	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
Penicillium Link	380	6	300	6	445	5	1250	5	500	5
P. citrinum Thom	305	5	215	4	345	5	1160	4	420	4
P. funiculosum Thom	75	2	85	2	40	4	20	1	40	2
P. puberulum Bainier	-	-	-	-	15	1	-	-	-	-
P. purpurogenum Stoll	-	-	-	-	-	-	70	2	40	1
P. duclauxi Delacroxi	-	-	-	-	5	1	-	-	-	-
P. chrysogenum Thom	-	-	-	-	40	2	-	-	-	-
Cladosporium cladosporioides (Fresen.) de Vries	35	3	55	3	55	5	40	3	-	-
Alternaria alternata (Fr.) Keissler	60	2	80	2	40	1	-	-	-	-
Emericella nidulans (Eidam) Vuillemin	-	-	-	-	20	2	-	-	-	-
Trichoderma viride Pers. Ex S.F. Gray	-	-	-	-	-	-	-	-	2040	6
Stachybotrys chartarum (Ehrenb. ex Link) Hughes	135	4	155	4	-	-	10	1	-	-
Gibberella fujikuroi (Sawada) Ito	10	1	-	-	10	1	40	1	280	3
Myrothecium Tode	15	2	30	2	15	2	-	-	-	-
M. verrucaria (Albertini and Schweinitz) Ditmar	10	2	15	1	15	2	-	-	-	-
M. roridum Tode ex Steudel	5	1	15	1	-	-	-	-	-	-
Apocrea chrysosperma (Bulliard) Fries	-	-	-	-	-	-	-	-	300	2
Rhizopus stolonifer Ehrenb. ex Fr. Lindt	-	-	-	-	-	-	-	-	100	2
Mucor racemosus Fresenius	30	2	-	-	20	2	-	-	-	-
Cochliobolus spicifer Nelson	-	-	-	-	-	-	-	-	-	-
Total count (colonies g^{-1} dry sample)	1460		1500		1610		4470		16520	
No. of genera	9		7		9		6		7	
No. of species	14		12		17		12		13	
Total No. of genera and species (14 genera and 28 species)	12 genera and 24 species									

- = No fungus isolated

El-Maghraby and El-Maraghy (1988), who surveyed 40 groundnut seed samples collected throughout Egypt and isolated 43 fungal species belonging to 16 genera. The most dominant genera with respective species were *Aspergillus* (*A. fumigatus* matching in 62% of the samples, *A. flavus*, 57%, *A. niger*, 55%), *Fusarium* (*F. oxysporum*, 55%) and *Penicillium* (*P. chrysogenum*, 52%). Also, several reports on mycoflora of peanut seeds worldwide are in agreement with the previous results (Chisholm and Coates-Beckford, 1997; Gonçales *et al*., 2008).

With regarding the results obtained, the gross fungal count of the stored peanut six cultivars under normal conditions, faintly increased than control samples in pre-storage on both of dextrose- and cellulose-Czapek's agar media (103.7 and 102.7%), respectively after 3 months of storage, followed by relatively increased (119.1 and 110.3%) after 6 months, sharply increased (347.7 and 306.2%) after 12 months and extremely increased (1151.7 and 1131.5%) after 24 months of storage period. The sharply increase after 12 months is up to promotion of *Fusarium* (*F. oxysporum*) by 72.4% on dextrose-Czapek's and *Aspergillus* and *Fusarium* counts (*A. flavus*, *A. fumigatus* and *F. oxysporum*) by totally 65.8% on cellulose-Czapek's agar. As well as, the extremely increase after 24 months of storage is due to flourishing of *Aspergillus* (79.1 and 73.7%) on the two media used, respectively in addition to appearing of *Trichoderma viride*, *Apocrea chrysosperma* and *Rhizopus stolonifer* (collectively, 15.4 and 14.8%) on the two isolation media, respectively (Table 3).

These results are in harmony with those obtained by Youssef (1987), who studied the effect of different relative humidity (~0, ~75, ~80, ~85, ~92 and ~100%) levels for 1, 2, 3, 4 and 5 months of incubation periods at 20°C on mycoflora of soybean seeds at 28 and 45°C, as well as Abdel-Hafez *et al*. (1992) studied the effect of different moisture content (11.5, 17, 22.7 and 28%) levels at 8, 18 and 28°C on paddy grain fungi. They reported that an increase in moisture content of seeds and grains, increase in incubation temperature in addition to lengthening of storage period led to flourishing of some fungi especially storage fungi which, consequently led to extremely in gross total fungal count.

Concerning mycotoxins, the different cultivars ((Ismailia$_1$, Giza$_4$, Giza$_5$, Giza$_6$, Local$_{262}$and R$_9$) tested proved to be non-toxic and no mycotoxins could be

Table 4: Effect of storage periods (3, 6, 12 and 24 months) on mycotoxins production on different peanut cultivars tested

	Storage periods									
	Control (pre-storage)		3 months		6 months		12 months		24 months	
Cultivars	Brine shrimp test	Mycotoxins identified (μg kg^{-1})	Brine shrimp test	Mycotoxins identified (μg kg^{-1})	Brine shrimp test	Mycotoxins identified (μg kg^{-1})	Brine shrimp test	Mycotoxins identified (μg kg^{-1})	Brine shrimp test	Mycotoxins identified (μg kg^{-1})
Chloroform extract										
Ismailia1	NT	ND	NT	ND	NT	ND	NT	ND	NT	ND
Giza 4	NT	ND	NT	ND	NT	ND	NT	ND	L	Aflatoxins B_1, B_2, G_1 and G_2 (6.8-10.6)
Giza 5	NT	ND	NT	ND	NT	ND	L	Aflatoxins B_1, B_2, G_1 and G_2 (6.2-8.8)	L	Aflatoxins B_1, B_2, G_1 and G_2 (8.4-15.6)
Giza 6	NT	ND	NT	ND	NT	ND	NT	ND	NT	ND
$Local_{262}$	NT	ND	NT	ND	NT	ND	L	DAS and ZEN (8.6 and 9.3)	H	DAS and ZEN (26.4 and 28.8)
R_{92}	NT	ND	NT	ND	NT	ND	NT	ND	NT	ND
Methanol re-extract										
Ismailia1	NT	ND	NT	ND	NT	ND	NT	ND	NT	ND
Giza 4	NT	ND	NT	ND	NT	ND	NT	ND	NT	ND
Giza 5	NT	ND	NT	ND	NT	ND	NT	ND	NT	ND
Giza 6	NT	ND	NT	ND	NT	ND	NT	ND	NT	ND
$Local_{262}$	NT	ND	NT	ND	NT	ND	NT	ND	L	UTF
R_{92}	NT	ND	NT	ND	NT	ND	NT	ND	L	UTF

Brine shrimp test: H = High toxicity; > 75% dead larvae, M = Moderate toxicity; 50-75% dead larvae, L = Low toxicity; 25-50% dead larvae, NT = Non toxic; < 25% dead larvae, Mycotoxins detected: DAS = Diacetoxyscirpenol, ZEN = Zearalenone, UTF = Unidentified toxin factor, ND = None detected

detected in control samples (pre-storage) and after 3 and 6 months of storage period. But, after 12 months of storage, two cultivars had low toxicity with producing aflatoxins B_1, B_2, G_1 and G_2 on ($Giza_5$) cultivar and diacetoxyscirpenol combined with zearalenone on ($Local_{262}$) cultivar. Also, after 24 months of storage, three cultivars proved to be toxic; two cultivars ($Giza_4$ and $Giza_5$) had low toxicity with producing aflatoxins B_1, B_2, G_1 and G_2 and the third one ($Local_{262}$) recorded high toxicity with producing diacetoxyscirpenol and zearalenone (Table 4). Similarly, USDA, RMA (2005) stated that, post-harvest aflatoxin contamination can increase during storage and if crop drying is delayed. Insects or rodent infestations may also facilitate mould invasion of some stored commodities. Corn, peanuts and cotton seeds are the commodities with the high risk of contamination during storage.

CONCLUSION

In conclusion, it is clearly evident that peanut seeds is a good substrate for mold infection and production of dangerous mycotoxins; aflatoxins, ochratoxins, sterigmatocystin, trichothecenes and zearalenone with potentially hazards to the health of both humans and animals. So, for human public health, peanuts and their products in different stages of production chain from the field to the final consumer must be subjected to quality control and good testing protocol for molds and mycotoxins contamination to ensure a food supply free of toxic levels of mycotoxins. Also, genetic modification resistant cultivars to fungal invasion and mycotoxin production is effective means beside to, biological control and others in prevention or minimizing mycotoxin synthesis on agricultural commodities in field, post-harvest and in storage. Therefore cultivation of suitable and selective resistant cultivars seeds and grains in different climatic and environmental conditions must be applied all over the world.

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