Original Article

Illustration of Pre-harvest Peanut Seeds Mycoflora and Mycotoxins

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ABSTRACT

Of pre-harvest peanut seeds (folded seeds), on two isolation media (1 % glucose and 1 % cellulose Czapeck's agar), the gross fungal counts on glucose (325 colonies) was higher than on cellulose (272 colonies) per 20 samples (each, 10 seeds). Where, the average number of colonies per 10 seeds was higher on glucose (7-32 colonies) than on cellulose (3-21 colonies). Also, 6 % and 7.5 % of the seeds were free of filamentous fungi on the two isolation media, respectively. Using seed-plate method and without surface disinfection, 7 species of 5 genera were isolated and identified from peanut seeds on glucose (6 species of 5 genera) and cellulose (7 of 5) Czapeck's agar at 18 ± 10 C. Of isolated genera, *Aspergillus* was superior in count (56 % and 53.3 % of fungal count, respectively) and frequency (85 % and 90 % of samples, respectively) on the isolation media. Of the genus, *A. flavus* had the highest count (34.5 % and 29.4 %) and frequency (each, 80 %). *A. niger* was less in count (21 % and 17.4 %) and in occurrence (each, 55 %) on the two media. Two species (*Fusarium oxysporum* and *Penicillium citrinum*) were detected in moderate frequencies (40 % and 30 % of the samples on the two media, respectively). *F. oxysporum* had the higher count (14.2 % and 16.2 % on the two media) compared with *P. citrinum* (each, 11.4 %). *Cladosporium cladosporioides* was low in occurrence (each, 20% of the samples) with counts 12 % and 11.4 %. *A. fumigatus* appeared on cellulose medium only whereas, *Nectria haematococca* on glucose only. Based on biological assay (brine shrimp test) and chemical detection (TLC and UV spectra), the ethyl acetate extracts of pre-harvest peanut seeds (approx. 15-20 days before harvest) proved to be non-toxic and mycotoxin free.

KEYWORDS

Peanut,
Seeds,
Fungi,
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INTRODUCTION

Peanuts have been grown and consumed for approximately 4000 years. They are an important cash crop, with current production of over 40 million metric tons annually. Recent cultivation of high-oleic varieties has recently increased to improve stability and health benefits. Additionally, peanuts are an important source of nutrition worldwide, as they are rich source of proteins, fibers, micronutrients and phytochemicals.

Moderate intake has not been associated with weight gain, but is associated with glucose tolerance and reduced cardiovascular disease rick (**Jones *et al*. 2016**).

Different studies of developing countries showed that the threat of fungi and mycotoxins contamination of foods and feeds resulting in human and livestock poisoning is really a major problem (**Wild 2007**; **Shehpard 2008**). Soil is a

source of primary inoculum of *Aspergillus flavus* and *A. parasiticus*, fungi that produce highly carcinogenic aflatoxins in peanut. Aflatoxigenic fungi commonly invade peanut seeds during maturation, and the highest concentrations of aflatoxins are found in damage seeds. Aerial crops such as corn and cotton seeds are infected predominantly with *A. flavus*, whereas peanut with their subterranean growth habit are invaded by both *A. flavus* and *A. parasiticus* (**Horn 2003**).

Toxin-producing moulds may invade plant material in the field before harvest, during post-harvest handling, storage and during processing into food and feed products. Thus, toxigenic fungi have been classified into two groups (i) field fungi; (ii) storage fungi. *Fusarium* species are ubiquitous soil organisms which may infect cereals directly in the field, thereby producing fumonisins, trichothecenes and zearalenone (depending on the species) during growth, ripening of grains and at harvest (**Jouany 2007**).

The present work was designed for studying the filamentous fungi associated with peanut seeds before harvest (approx. 15-20 days) in addition to natural occurrence of mycotoxins of peanut seeds.

MATERIALS AND METHODS

1. Collection of pre-harvest peanut fruits

Pre-harvest (approx. 15-20 days before harvest) peanut fruits (20 samples) from farmer's field in Upper Egypt (5 governorates, El-Giza, Assiut, Sohag, Qena and Aswan) were collected. The samples were placed in sample bags, sealed and placed in other bags which were also sealed. The samples were kept cool during transfer (3-50C) to the laboratory.

2. Isolation and identification of mycoflora

In laboratory, the peanut fruits were subjected to series of washing with sterile dist. water. The seeds were unfolded, divided to two segments (two cotyledons), dried between sterilized filter paper. The segments (10 cotyledons) were inserted on the surface of two isolation (1 % glucose and 1 % cellulose Czapeck's agar) media (2 segments per Petri dish) supplemented by chloramephenicol 1/30000 as reported by **Al-Doory** (**1980**) . The dishes were incubated at 18± 10C for 7-10 days The growing colonies were examined, counted (per segment), isolated and identified based on morphological features according the keys of **Raper and Thom 1949**; **Raper & Fennell 1965**; **Booth 1977**; **Nelson *et al*. 1983** and **Moubasher 1993**.

3. Extraction of mycotoxins

Twenty-five g of peanut seeds were continuously defatted by extraction with n-hexane for 10 h using soxhlet-type extractor. The defatted residue was re-extracted by ethyl acetate (75 ml) in a 250 ml Erlenmeyer flask, shaken using a rotary shaker (200 rpm, 24 h) and filtered through Whatman No. 1 filter paper. Then, the residue was washed twice with ethyl acetate (each, 25 ml). The ethyl acetate extracts were combined, dried over anhydrous sodium sulphate (5 g), concentrated in vacuo, transferred to glass vial and evaporated in air.

4. Clean up of crude extract:-

For cleanup of crude extract, a silica gel column (14x0.4 cm) containing 2.5 g Kiesel gel 60, 70/230 (Merck) was used as follows: Aflatoxins, ochratoxins A & B sterigmatocystin, and zearalenone were cleaned up according to **AOAC** (**1984**), whereas trichothecenes were processed according **Jarvis *et al***. (**1986**).

5. Biological assay:-

For bioassay of mycotoxins, brine shrimp (*Artemia salina* L.) larvae (3^{th} nauplii) were used based on the **korpinen** (**1974**) method. A filter paper disc (1 cm^2) was saturated with 50 µg of crude extracts, air dried and the discs were added to sea water (2 ml) containing the larvae (40-60

larvae). The mortality of the larvae was estimated after 16 h.

6. Chemical detection of mycotoxin

Thin layer chromatography (TLC) was carried out on silica gel plates (Merck). Aflatoxins B1, B2, G1 & G2, ochratoxins A & B, sterigmatocystin, zearalenone, T-2 toxin, diacetoxyscirpenol (DAS) were applied as standards. The developing solvent system was methanol-chloroform (v/v, 3:97), and the developing plates were viewed under 254 and 366 nm irradiation or spread with reagents (**AOAC 1984** and **Jarvis *et al.* 1986**).

Spectrophotometer (Cecil model 703) was used for detection the mycotoxins using the molecular coefficient of 21.800 at 254 and 366 nm. The fluorescent zones, including standards were removed from TLC plates and dissolved in methanol-chloroform (v/v, 5: 95).

RESULTS

I. Filamentous Fungi of Pre-harvest Peanut Seeds

Of filamentous fungi associated with pre harvest peanut seeds (20 samples) on two isolation media (1 % glucose and 1 % cellulose Czapeck's agar) revealed that, glucophilic (325 colonies/200 cotyledons) were higher than cellulose-decomposer (272 colonies) fungi. Where, the number of colonies per sample fluctuated between 7-32 and 3-21 colonies on glucose and cellulose agar, respectively. On other side, the percentage of uninfected (health) seeds was 6 % and 7.5 % on the two isolation media, respectively (Table 1).

Table (1) Total count (TC) calculated per 20 peanut seed samples (each, 10 cotyledons), total count percentage per gross count (TC %), number of cases of isolation (NCI) and occurrence remarks (OR) of fungal genera and species isolated on glucose- and cellulose-Czapeck's agar at 18 ±1 0C.

Genera and species	Glucose-Czapek,s agar			Cellulose-Czapek,s agar		
	TC	TC %	NCI & OR	TC	TC%	NCI & OR
Aspergillus	182	56 %	17 H	145	53.3%	18 H
A. flavus Link	112	34.5 %	16 H	80	29.4%	16 H
A. niger Van Teighem	70	21.5 %	11 H	48	17.4%	11 H
A. fumigatus Fresenius	-	-	-	17	6.4%	6 M
Fusarium oxysporum Schlecht ex Fr.	46	14.2 %	8 M	44	16.2%	8 M
Penicillium citrinum Thom	37	11.4 %	6 M	31	11.4%	6 M
Cladosporium cladosporioies (Fr) de Vries	39	12 %	4 L	31	11.4%	4 L
Nectria haematococca Berkely & Brown	21	6.5 %	-3 L	-21	7.7%	3 L
Gross total count	325			272		
Number of fungal species and genera	6 species/5 genera			7 species/5 genera		
Number of colonies (per 10 cotyledons)	7-32 colonies			3-21 colonies		
Percentage of healthy seeds	6 %			7.5%		

Concerning to isolated fungal species and their genera, a total of 7 species of 5 genera were isolated and identified on glucose (6 species of 5 genera) and cellulose (7 of 5) agar media. *Aspergillus* was quite the dominant genus on the two isolation media (56 % & 53.3 % of gross fungal count, respectively).

Of the genus, *A. flavus* was superior in counts (34.5 % and 29.4 % of gross counts on the two isolation media, respectively) and frequencies (80 % of the samples on both media), followed by *A. niger* which was less in counts (21.1 % and 17.4 % of total fungi, respectively) and frequencies (each, 55 % of samples). *A. fumigatus* was only observed on

cellulose medium in less count (6.3 %) and frequency (30 %). Of moderate frequency of occurrence, two species of two genera (*Fusarium oxysporum* and *Penicillium citrinum*) were isolated and identified on the two media tested. *F. oxysporum* had the higher counts (14.2 % and 16.2 % of gross fungi) with the same frequency (each, 40 % of samples), whereas *P. citrinum* was less in counts (each, 11.4 %) and occurrence (each, 30 %). Finally, *Cladosporium cladosporioides* and *Nectria haematococca* had low frequencies (20 % & 15 % of peanut seed samples) and the counts were higher of the first species (12 % & 11.4 % of gross fungal counts on the two media) than the second (6.5 % & 7.7 %, respectively).

II. Mycotoxins Analysis

Of pre-harvest peanut seeds (15-20 days before harvest), the ethyl acetate extracts (20 samples, each 25 g) after de-fatted by n-hexane proved that, all samples tested were non-toxic (less than 25 % dead larvae) to brine shrimp (*Artemia salina* L.) larvae (3^{th} stage of nauplii). Based on chemical analysis (Thin layer chromatography "TLC" and UV spectrophotometric analyses), there is no mycotoxins could be detected.

DISCUSSION

Of isolated filamentous fungi with regarding the total fungal counts and detected genera and species on two isolation media (glucose- and cellulose-Czapeck's agar), the gross fungal count on glucose was more than on cellulose, whereas the number of species on cellulose was higher compared with glucose. These results accepted with **El-Sherbeny *et al.* (2020)** through isolation filamentous fungi from damping off and wilting off diseases of peanut plants. Also, glucose is easy and quickly utilizing mono-saccharides in glycolysis process than other carbohydrates (**Sequeira *et al.* 2019**). Therefore, cellulose (polysaccharide) especially with pH 8 has the ability to restrict the growth of heavy sporulating filamentous fungi to give good chance for growing non-heavy sporulating fungi (**El-Maghraby & El-Maraghy 1988**).

Based on identified fungal species, *Aspergillus flavus* and *A. niger* were quite the most dominant based on frequency and count. In this respect, A. flavus was amongst toxigenic storage fungi (**Horn 2003**), the previous two species in addition **A. fumigatus** were the commonest species in stored peanut seeds (**El-Maghraby & El-Maraghy 1987, 1988**) and the only species isolated from peanut plants (**El-Khadem 1975**). Two *Fusarium* species namely, *F. oxysporum* and *F. solani* (*Nectria haematococca*) were detected in moderate and low frequencies with moderate and low counts, respectively. Concerning these results, *Fusarium* amongst toxigenic field fungi (**Horn 2003**), widely detected in Egyptian damping off and wilting off diseases of Egyptian peanut plants (**El-Sherbeny 2020**). Whereas, six *Fusarium* spp. were isolated and identified from stored peanut seeds (40 samples) as reported by **El-Maghraby & El-Maraghy (1988)**. The remaining two species, *Penicillium citrinum* and *Cladosporium cladosporioides*, were observed in moderate and low frequencies of occurrence, respectively with moderate counts. In this respect, the previous two species disappeared in damping off and wilting off diseases of peanut plants (**El-Sherbeny *et al.* 2020**), whereas widely detected as endophytes of peanut shoots (leaves and stems) as reported by **El-Maghraby *et al.* (2009)**. The authors in aware, the source of underground fruits filaments fungi related mainly to the soil and less to endophytic fungi which transfer from shoot to root.

Concerning to toxicity and mycotoxin of pre-harvest peanut seeds, all samples tested were non-toxic and free of mycotoxins. But, of stored peanut seeds in Egypt, 47 % of chloroform extracts proved to be toxic to brine shrimp larvae (**El-Maghraby & El-Maraghy 1987**) and 20 %, 10 % and 0 % of un-treated, roasted and roasted with salts were toxic to

the larvae (**Youssef *et al.* 2009**). Aflatoxins (B1, B2, G1 & G2), trichothecenes (T-2 toxin & diacetoxysripenol) were superior in occurrence followed by, zearalenone and citrinin which were detected in toxigenic samples. The aflatoxins are a group of toxic and carcinogenic polyketide secondary metabolites, which are produced by strains of *Aspergillus flavus* group (**Ito *et al.* 2001**). The international Agency for Research on Cancer (IARC) has classified aflatoxins as a group I carcinogen affected liver (**IARC 1993**), contaminated various agricultural commodities with highest risk of oil seeds and/or grains including corn, peanut, cottonseed, nut (Pitter, 1998). Trichothecene toxins are a group of related mycotoxins that possess a tetracyclic 12, 13-epoxytrichothec-9-ene skeleton. These biologically active metabolites are associated predominantly with *Fusarium* species, but they also are produced by several other fungi including Myrothecium, Stachybotrys, Trichothecium, Acremonium (Cephalosporium), Cylindrocapon and Trichoderma (**Ueno 1983**). Although more than 190 derivatives of trichothecenes have been detected in the laboratories, only 11 (T-2 toxin, Ht-2 toxin, AcHT-2 toxin, T-2 tetraol, diacetoxyscirpenol, tri-acetoxyscirpenol, nivalenol, deoxynivalenol, fusarin C, fusarenone X, and neosolaniol) have been detected occurring naturally in feedstuffs, grains and seeds (**Ueno 1983; El-Magraby *et al.* 1995; El-Maghraby 1996; Desjardins 2006; McCormick *et al.* 2011**). Zearalenone is an important mycotoxin, has estrogenic properties, as it causes a variety of reproductive disorder in female farm animals. It is found in maize as well as small grains and mainly produced by *Fusarium* spp. associated with deoxynivalenol (Pitt, 2014), T-2 toxin and diacetoxyscirpenol (**El-Maghraby & El-Maraghy 1987, 1988**). Citrinin (one derivative) is nephrotoxic mycotoxin produced by several strains belonging to *Aspergillus*, *Penicillium* and *Monoascus*. It contaminates various commodities of plant origin, cereal is particular and it usually found together with another nephrotoxic mycotoxin, ochratoxin A (**Doughari 2015**). The toxin is not widely detected in Egyptian cereal grains or oil seeds (**El-Maghraby & El-Maraghy 1987; El-Magraby *et al.* 1995; El-Maghraby 1996; Youssef *et al.* 2009**).

In conclusion, of underground peanut fruits (seeds), there are several aspects:

- There is no specific filamentous fungus of peanut seeds.
- The isolated fungi were mainly amongst soil fungi and less to endophytes of peanut (leaf and stem) plant.
- The presence of toxigenic fungi is not meaning the presence of their mycotoxins.

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