
Occurrence of toxigenic fungi and mycotoxins in some legume seeds

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The current research was conducted to study the natural occurrence of toxigenic fungi and mycotoxins contamination in three legume seeds (i.e. beans, pea and soybean) in great Cairo governorate. The results indicated that four fungal genera were isolated from the examined seeds. These isolated fungi included *Aspergillus flavus*, *A. niger*, *A. parasiticus*, *Fusarium moniliforme*, *F. oxysporum*, *Fusarium* spp., *Penicillium* spp and *Sclerotinia sclerotiorum*. Soybean seeds were found the higher percentage of fungal infection followed by pea and beans seeds. *Aspergillus niger* was the common in beans and soybean, followed by *A. parasiticus*. Whereas, *A. parasiticus* was the common in pea, followed by *Fusarium* spp. *S. sclerotiorum* was found to be the lowest in all examined seeds. On the other hand, *A. parasiticus* and *F. moniliforme* were capable to produce aflatoxins and fumonisin in significant concentrations exceed the permit levels recommended by the Egyptian authorities. The fungal infection with *A. parasiticus*, *F. moniliforme* decreased the chemical components of the tested seeds (i.e. protein, fat, carbohydrates and ash). Furthermore, moisture content was found to be a causative factor in fungal infection. It could be concluded that fungal infection of legume seeds reduced its nutritive value as well as induced a health risk for the consumer.

Key words: Legume, beans, pea, soybean, fungi, mycotoxins, chemical components.

Introduction

Legumes "Fabaceae" is one of the most important plant in Egypt for local consumption and exportation. Legumes are generally good sources of slow release carbohydrates and are rich in proteins. Legumes are normally consumed after processing, which not only improves palatability of foods but also increases the bioavailability of nutrients, by inactivating trypsin and growth

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inhibitors and haemagglutinins (Tharanathan and Mahadevamma, 2003). It is the most important source of plant protein in human food. Several fungi attack the legume plants during growth, harvest and storage. While more than 25 different species of fungi are known to invade stored grains and legumes (Duan *et al.*, 2007), species of *Aspergillus*, *Penicillium* and *Fusarium* are responsible for most spoilage and germ damage during storage. They cause reduction in cooking or baking quality, and nutritive values, produce undesirable odors and color, and change appearance of stored food grade grains and decrease germinability and total decay (Quenton *et al.*, 2003 and Castillo *et al.*, 2004). In addition, they produce mycotoxins those are health hazard for man and animals, make products unacceptable for edible purposes or lower their market grade. Moreover, fungal infestation of seed coat decreases viability of seeds, or may cause abnormal seedlings (Selcuk *et al.*, 2008).

A large number of fungal species regularly associated with seeds and can infect developing seeds and still attached to the mother plant (Neergaard, 1979, Agrwal and Sinclair, 1993 and Mathur and Olga 2003). This has been demonstrated by the isolation of fungi from seeds collected before seed-set.

Many of these fungi have no negative impact on seeds but there are also many saprophytic and pathogenic fungi commonly isolated from seeds (Schafer and Kotanen, 2004). These include the mainly saprophytic genera *Mucor*, *Rhizopus*, *Trichoderma*, *Cladosporium*, *Penicillium*, *Chaetomium* and *Aspergillus* as well as the mainly pathogenic genera *Pythium* and *Alternaria*.

Finally, *Fusarium*, *Acremonium* and *Phoma* contain both saprophytes and pathogens (Schafer and Kotanen, 2004) While the fungal pathogens of growing plants are comparatively well-investigated (Friberg *et al.*, 2005), the knowledge on fungal seed decay and its importance for plant demographic and community processes is quite limited (Blaney and Kotanen, 2001). Five fungal genera i.e. *Alternaria*, *Aspergillus*, *Epicoccum*, *Fusarium* and *Trichoderma* were isolated from some legume seeds as beans, cowpea, and lupine (Embaby and Mona, 2006).

In recent years, there has been a notable increase in the occurrence of chronic diseases caused by the consumption of food products contaminated with mycotoxins (U.S. FDA/CFSAN, 2001). Mycotoxins are secondary metabolites produced by toxigenic fungi in contaminated foods. Aflatoxins and fumonisin are the most dangerous mycotoxins in tropical areas. They are produced, respectively, by species of the genera *Aspergillus* and *Fusarium* (Konietzny and Greiner, 2003). Regarding legumes in Egypt, very little information exists with respect to its natural contamination with toxigenic fungi and mycotoxin. Aflatoxin(s) were detected in some *Aspergillus* isolates while Fumonisin was detected in some *Fusarium* isolates (Embaby and Mona, 2006).

The main toxigenic species identified were *Aspergillus flavus*, *A. fumigatus*, *Fusarium graminearum* and *F. culmorum* in all cereals and *F. verticillioides* in maize (Tabuc *et al.*, 2009).

Changes in the protein, reducing and non-reducing sugars were observed in cowpea seeds infected with either *A. nidulans* and *A. terreus* (Maheshwari and Mathur, 1987). Chemical composition (protein, lipid, carbohydrate, crude fibre) of sesame and soybean seeds were influenced by *A. flavus* growth (Frag, 1990). Invasion of seeds by some pathogens may result in biochemical deterioration and change in quality of seed nutrient as infected in soybean seed with *A. flavus* (Agrwal and Sinclair, 1993). *Fusarium moniliforme* decreased with time with increase in the relative humidity. Protein, total and reducing sugar contents decreased gradually with increase in the RH values (Lokesh and Hiremath, 1993). There was an increase in moisture content, reduction in the fat and decrease in the available carbohydrates in all grain cowpeas analyzed. Similarly, the energy content showed a significant ($p < 0.05$) decrease in all the grains (Kungu *et al.*, 2003). *Aspergillus flavus* decrease lipids and carbohydrate contents of wheat, soybean and faba-bean seeds. *A. flavus* utilizes carbohydrates of seeds for its growth and aflatoxin production (Aziz and Mahrous, 2004). The aim of the current study was to isolate and identify the toxigenic fungi associated with some legumes included beans (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.) and soybean (*Glycine max* L.), the ability of these fungi to produce mycotoxins and the effect of *Aspergillus parasiticus* and *Fusarium moniliforme* on chemical content of seeds.

Materials and methods

Samples: Thirty samples of legume seeds, beans (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.) and soybean (*Glycine max* L.) were collected from the local markets at great Cairo Governorates, Egypt.

Isolation: Purification and identification of all fungal association were done. Seed samples were tested using two standard methods of isolation (i.e. agar plate and blotter tests) as described by Neergaard (1979), Agarwal and Sinclair, (1993) and Mathur and Olga (2003). Seed samples were divided into two groups; the first group was disinfected with sodium hypochlorite solution (1%) for 2 min, while the second group was untreated (non-disinfected). All seed samples were washed several times by sterilized water (SW), then dried between two sterilized filter papers and plated on potato dextrose agar (PDA) and/or in sterilized filter papers with enough moisture (blotter test) in sterilized Petri dishes. Five seeds/dish and three dishes were used as replicates for each treatment. All dishes were incubated for 5-7 days at $25 \pm 2^\circ\text{C}$. All fungal growth was transferred and purified using hyphal tip

and/or single spore techniques onto PDA medium in the presence of antibiotic (Streptomycin). Developing fungi were cultured on PDA slants (5-7 days old) then identified at Department of Plant Pathology, National Research Centre (NRC), El-Dokki, Egypt based on cultural characteristics using specific media and the available literature according to Raper and Funel (1965) and Maren and Johan (1988) for *Aspergillus*, Booth (1977) and Nelson *et al.* (1983) for *Fusarium*, and Barent and Hunter (1977) for the genera of imperfect fungi and Singh *et al.* (1991) for *Aspergillus*, *Fusarium* and *Penicillium*.

Mycotoxin production: Each isolate of *Aspergillus* and *Fusarium* was grown in 500 ml flask containing 100 g of each autoclaved legume seeds with enough moisture and incubated at 25 °C for 14 days for *Aspergillus* and 21 days for *Fusarium* isolates. The incubated seeds were extracted for aflatoxins and fumonisins according to the method described by AOAC (2007).

Mycotoxins determination: Mycotoxins were determined at Department of Food Toxicology and Contamination, National Research Centre (NRC). Aflatoxins and fumonisin were determined by HPLC according to the methods described by Hustchins and Hagler (1983) for aflatoxins and Shephard *et al.* (1990) for fumonisin respectively.

Effect of *Aspergillus* and *Fusarium* on chemical content of seeds: The chemical content (i.e. protein, carbohydrate, ash and moisture) of the inoculated and control legume seeds were determined as described by AOAC (2007). The results were calculated as percentage of losses or reduction in the infected seeds compared to the control seeds.

Results

The results of the total fungal count (TFC), germination and the percentage of infection for the three tested legume seeds using the two standard methods (Blotter and PDA) as presented in Table 1. These results indicated that the blotter method exhibited TFC and infection percentage in the non-disinfected and disinfected pea seeds that was higher than beans and soybean seeds.

On the other hand, in PDA method, TFC and percentage of infection in disinfected beans and pea seeds were the same and higher than soybean seeds, whereas the higher TFC was found in non-disinfected pea followed by disinfected beans than soybean. Also the results showed that agar plate (PDA medium) was better than blotter test which gave higher percentage of germinated seeds. Germination of disinfected phaseolus seeds gave 53% in blotter test and 73% in PDA medium while non-disinfected seeds gave 27% in blotter and 53% in PDA methods, 20 and 27% were the results of infection percent in disinfected and non-disinfected beans (phaseolus) seeds with blotter

test and 80 and 87% of infection percent in disinfected and non-disinfected seeds with agar plate method respectively.

Germination of disinfected and non-disinfected pea seeds resulted in 33 and 13 % in blotter test and 93 and 73 % of germination in disinfected and non-disinfected pea seeds with PDA medium. The infection percent of disinfected and non-disinfected pea seeds with blotter test were 67 and 80 % comparing with 80 and 100 % with PDA test respectively.

On the other hand, the percentage of germinated soybean seeds resulted in 12 and 13% with blotter test compared with 40 and 40 % of germination in PDA test method with disinfected and non-disinfected seeds respectively. Infection percentage of disinfected and non-disinfected soybean seeds showed 7 and 27 % with blotter test and 60 and 67% with PDA respectively.

Table 1. Total count(s), germination and infection percentage of some disinfected and non disinfected legume seeds on blotter and agar plate methods

Seed crops	Blotter method								PDA							
	Disinfected				Non-disinfected				Disinfected				Non-disinfected			
	G		I		G		I		G		I		G		I	
	N.	%	T.	%	N.	%	T.	%	N.	%	T.	%	N.	%	T.C	%
Beans	8	53	3	20	4	27	4	27	11	73	12	80	8	53	13	87
Pea	5	33	10	67	2	13	12	80	14	93	12	80	11	73	15	100
soybean	3	20	1	7	2	13	4	27	6	40	9	60	6	40	10	67

G = Germination N.G = Number of Germinated seeds I = Infected seeds T.C= Total count of fungi in 15seeds (5seeds/dish x 3 replicates)

Results indicated that the frequency of *A. falvus* was the most prominent fungi in beans and pea seeds whereas, *A. niger* was the most prominent in soybean seeds. *Fusarium moniliforme* was the lowest fungus found in beans and soybean seeds while; *Sclerotinia sclerotiorum* was the lowest fungal isolates that infected pea seeds as shown in Table 2 and Figs.1, 2 and3.



Fig. 1. a-*Fusarium* associated with non disinfected and disinfected beans seeds (blotter test and PDA method). b-*Aspergillus flavus* with non disinfected beans seed.

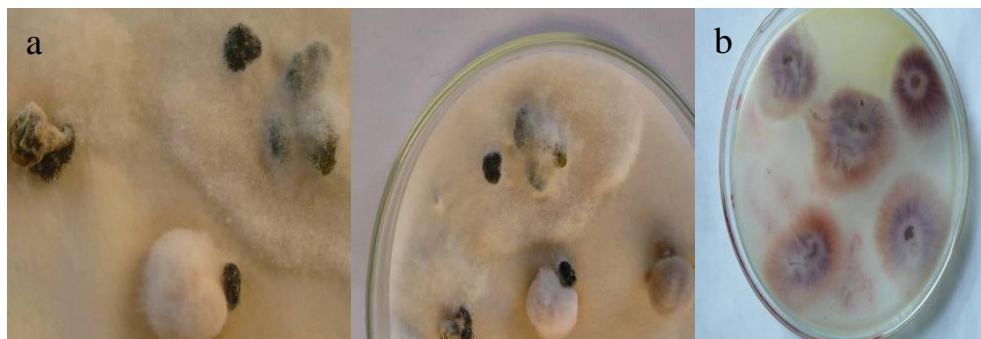


Fig. 2. a-*Sclerotinia sclerotiorum* and sclerotia associated with disinfected pea seeds (blotter test). b-*Fusarium* with disinfected pea seeds.

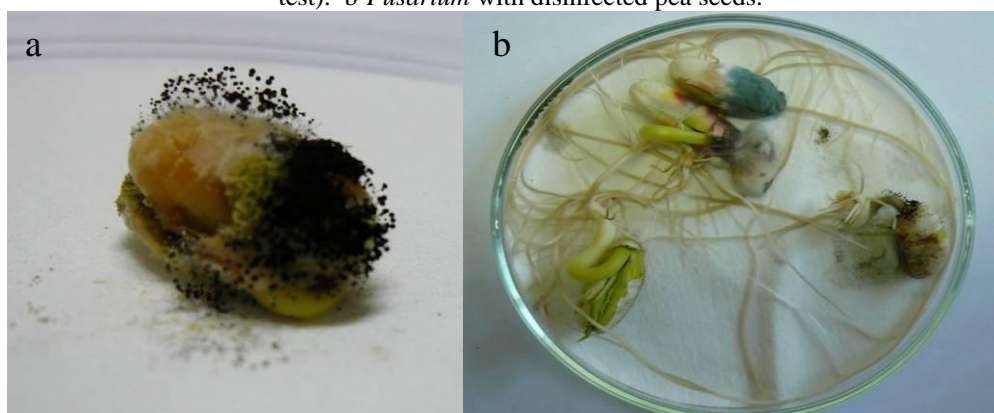


Fig. 3. a- *Aspergillus niger* and *A. flavus* associated with disinfected soybean seed, b- *Penicillium* sp. with bean seed.

Table 2. Frequency of some legume seed-borne Fungi

Fungi	Beans		Pea		Soybean		Total	%
	T.C	%	T.C	%	T.C	%		
<i>Aspergillus flavus</i>	8	6.3	9	7.1	10	7.8	27	21.3
<i>A. niger</i>	9	7.1	4	3.2	18	14.2	31	24.4
<i>A. parasiticus</i>	4	3.2	2	1.6	5	3.9	11	8.7
<i>Fusarium moniliforme</i>	1	0.8	4	3.2	1	0.8	6	4.7
<i>F. oxysporum</i>	5	3.9	2	1.6	7	5.5	14	11.0
<i>Fusarium</i> . spp	8	6.3	6	4.7	6	4.7	20	15.7
<i>Penicillium</i> spp	5	3.9	1	0.8	6	4.7	12	9.5
<i>Sclerotinia sclerotiorum</i>	0	0.0	6	4.7	0	0.0	6	4.7
Total	40	31.5	34	26.8	53	41.7	127	100%

T.C = Total count of fungi.

Results showed that mycotoxins concentration was produced by the toxigenic fungi which isolated from legume seeds are presented in Table 3. It is clearly demonstrated that both *A. parasiticus* (No.59) isolated from beans seeds and *F. moniliforme* (No.8) isolated from soybean had the ability to produce mycotoxins in significant concentrations. The total aflatoxins concentration was 196.58 μ /kg whereas, fumonisin concentration was 198 mg/kg seeds. It is of interest to mention that *A. flavus* isolated from the legume seeds was not able to produce aflatoxin. (Fig 5. a,b) showing HPLC chromatogram for aflatoxin and fumonisin. HPLC Chromatograph of Aflatoxins sample showing that, AFB₁ eluted at 10.8 , AFB₂ at 21.4, AFG₁ at 9.0 and AFG₂ at 14.3 min. HPLC Chromatograph for Fumonisin showing that AFB₁ eluted at 6.957min.

Table 3. Concentration of mycotoxins production by the toxigenic *A. parasiticus* and *F. moniliforme* isolated from some legume seeds

Aflatoxins (μ /kg)					Fumonisins (mg/kg)
AFB ₁	AFB ₂	AFG ₁	AFG ₂	Total	
44.74	1.4	15.24	135.2	196.58	198

AFB_{1,2} = Aflatoxin B₁, B₂

AFG_{1,2} = Aflatoxin G₁, G₂

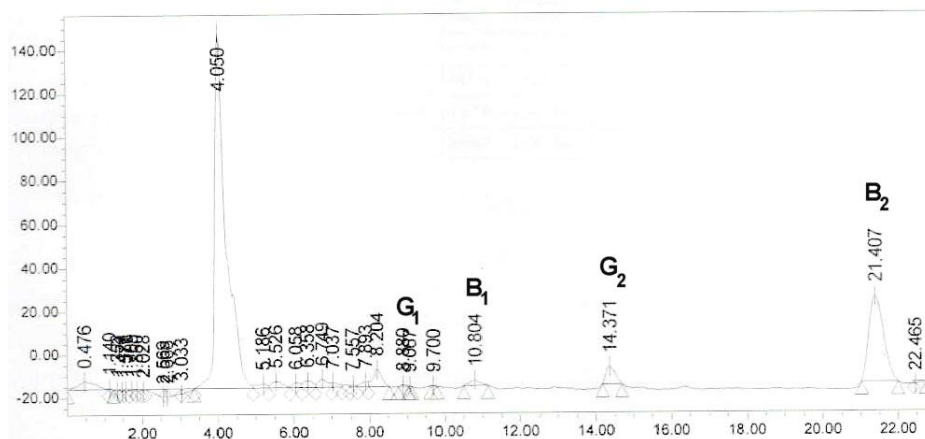


Fig. 5a. HPLC Chromatograph for Aflatoxins.

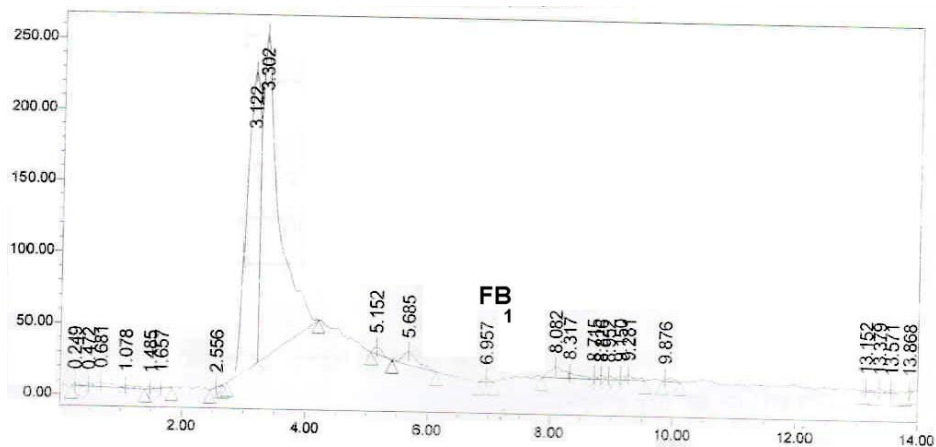


Fig. 5b. HPLC Chromatograph for Fumonisin.

The results showed that chemical composition of *Aspergillus parasiticus* contaminated and healthy legume seeds are presented in Table 4. It indicated that *A. parasiticus* infection resulted in 8.7, 2.0 and 3.4 % loss in protein content in beans, pea and soybean respectively. Whereas, the loss in carbohydrates reached 20.38, 18.0 and 6.1% for beans, pea and soybean respectively. The loss in fat content in the same seeds reached 20.4, 2.29 and 11.9% meanwhile; the loss in ash reached 3.8, 2.7 and 4.74% for the same seeds respectively. The main factor in *A. parasiticus* infection was found to be higher in the infected seeds compared to the healthy seeds. These percentages of moisture content in the infected seeds recorded 37.4, 52.7 and 28.8% which higher than the healthy beans, pea and soybean respectively.

Table 4. Effect of infection with *Aspergillus parasiticus* on the chemical composition of legume seeds

Seed crops Chemical composition	Beans				Pea				Soybean			
	H	I	L%	R%	H	I	L%	R%	H	I	L%	R%
Protein	31.1	22.4	8.7	27.9	32.2	30.2	2	6.2	45.0	41.6	3.4	7.5
Carbohydrate	38.7	18.32	20.38	52.6	30.95	12.88	18.0	58.3	30.7	24.6	6.1	19.86
Fat	21.9	1.5	20.4	93.1	3.99	1.7	2.29	57.3	22.8	10.9	11.9	52.1
Ash	7.2	3.4	3.8	52.7	5.0	2.3	2.7	54	6.1	1.36	4.74	77.7
Moisture	8.49	45.9	37.4	81.4	8.43	61.3	52.7	85.9	7.7	36.5	28.8	78.9

H= Healthy I= Infected L= Loss% R= Reduction%

On the other hand, the effects of *F. moniliforme* infection on chemical composition of legume seeds are presented in Table 5. It is clearly shown that *F. moniliforme* infection also affected the nutritive values of the legume seeds. The infection with this species resulted in the loss of the chemical components

of beans, pea and soybean reached 4.3, 9.5 and 4.1% for protein, protein, 17.7, 13.59 and 5.5% for carbohydrates, 19.2, 1.09 and 6.3% for fat, 2.1, 0.8 and 0.8% for ash respectively. However, moisture content was found to be higher in the infected seeds than the healthy seeds. These percentages reached 38.7, 43.87 and 22.4% higher in the infected seeds than the healthy beans, pea and soybean respectively.

Table 5. Effect of infection with *Fusarium moniliforme* on the chemical composition of legume seeds

Seed crops Chemical composition	Beans				Pea				Soybean			
	H	I	L%	R%	H	I	L%	R%	H	I	L%	R%
Protein	31.1	26.8	4.3	13.8	32.2	22.7	9.5	29.5	45.0	40.9	4.1	9.1
Carbohydrate	38.7	21.0	17.7	43.7	30.95	17.36	13.59	43.9	30.76	25.25	5.5	17.9
Fat	21.9	2.7	19.2	87.6	3.99	2.9	1.09	27.3	22.8	16.5	6.3	27.8
Ash	7.2	5.1	2.1	29.1	5.0	4.2	0.8	16	6.1	5.3	0.8	13.1
Moisture	8.49	47.2	38.7	82	8.43	52.3	43.87	83.8	7.77	30.1	22.4	74.4

H= Healthy I= Infected L=Loss% R= Reduction %

Discussion

There are many fungi associated with legume seeds. In the current study, both blotter method and PDA method showed that pea seeds were found to be highly infected with different fungal species followed by beans and soybean. TFC recorded in disinfected legume seeds were lower than the non disinfected seeds in both applied methods. Four major fungal genera were isolated including *A. parasiticus*, *A. flavus*, *A. niger*, *Penicillium* spp., *F. moniliforme*, *F. oxysporum*, *Fusarium* spp. and *S. sclerotiorum*. Similar to the current observation, Pepeljnjak and Cvetnic (1986) reported that the frequency of *Penicillium* spp. and *Aspergillus* spp. was 67 and 33%, respectively in beans samples. Moreover, Tseng *et al.* (1995) indicated that *F. oxysporum*, *Fusarium* spp., *F. solani*, *Ascochyta pisi*, *A. pinodes* (*Mycosphaerella pinodes*), *Phoma medicaginis* var *pinodella*, *Alternaria alternata*, *F. poae*, *F. sporotrichioides*, *F. sambucinum* (*Gibberella putearis*), *F. culmorum*, *F. avenaceum* (*G. avenacea*), *F. equiseti*, *S. sclerotiorum*, *Botrytis cinerea* and *Rhizoctonia solani* were found in beans samples collected from Ontario and Taiwan. They reported that the fungi most frequently isolated from the diseased Ontario beans were *Alternaria* (51.1%) *Fusarium* (18.0%), *Rhizoctonia* (65.1%) *Penicillium* (5.2%), *Rhizopus* (3.2%) *Sclerotinia* (3.0%), *Gliocladium* (2.2%) and *Mucor* (1.7%), however, *Aspergillus*, *Penicillium*, *Euriotium*, *Rhizopus* and *Nularia* were the most fungal isolates from diseased Taiwan beans which recorded 48.5, 6.7, 5.3 and 2.4% frequently respectively.

In the current study, PDA medium was found to be a better method than blotter test. Moreover, the total fungal count isolated on PDA medium was found to be higher in both disinfected and non disinfected seeds compared with blotter test. Similar results were reported by Neergaard (1979), Agarwal and Sinclair, (1993), Mathur and Olga (2003) and Kumud *et al.* (2004). Also, El-Nagerabi *et al.* (2000) found that *Aspergillus* was the most common genus isolate followed by *Rhizopus*, *Alternaria*, *Fusarium*, *Emericella*, *Drechslera*, *Cladosporium*, *Pencillium* and *Pythium*. In the same concern, Rauf (2002) isolated *A. alternata*, *Ascochyta* spp, *Colletotrichum* spp. *Fusarium* spp., and *Macrophomina phaseolina* from major legume crops in Pakistan. Moreover, Henning (2005) reported that the main seed-transmitted pathogens affecting soybean are *Phomopsis* sp, *Fusarium semitectum*, *S. sclerotiorum*, *Sclerotium rolfsii*, *Aspergillus* spp. *A. flavus* which cause germination problems and mycotoxins accumulation.

On the other hand, percentage of seed germination with PDA medium was found to be higher in both disinfected and non-disinfected seeds than blotter test method. Also, percentage seed germination of disinfected legume seeds were found to be higher than the non-disinfected seeds in both applied methods. Similar results were reported by Neergaard (1979), Agarwal and Sinclair, (1993) and Mathur and Olga (2003), Kumud *et al.*, (2004) and Embaby and Mona (2006). Pathogenic seed-borne fungi caused decreased in the germination ability and emergence weight of 1000 seeds, plant healthiness, number of yielding plants and seed yield (Czyzewska, 1983).

In the present study, the isolated fungal genera were tested for their ability to produce mycotoxins. *A. parasiticus* and *F. moniliforme* were found to have the ability for aflatoxins and fumonisin production in significant concentrations exceed the save limits recommended by the Egyptian authorities (Embaby and Mona, 2006). It is well documented that aflatoxins have a carcinogenic effects (I.A.R.C, 1993) whereas; fumonisin causes lipid peroxidation, sphingolipid disturbances and developmental toxicity as well as its role as a cancer promoter (Abdel-Wahhab *et al.*, 2004). In this regards, Tseng *et al.* (1995, 1996) reported that aflatoxin B₁, B₂, G₁ and G₂ and fumonisins were found in the infected beans that collected from Taiwan. Furthermore, Ruiz *et al.* (1996) and Vaamonde *et al.* (2003) reported that *A. flavus* isolated from green beans and soybean had capacity synthesise aflatoxins.

The effects of fungal infection on chemical components of legume seeds were also investigated. Our results showed that infection with the tested fungi reduced all chemical components in the legume seeds including protein, carbohydrates, fat and ash consequently reduced the nutritive values of the

infected seeds. The same result reported by Embaby and Mona (2006) and these results were in accordance with those reported by Lokesh and Hiremath (1993) and Ushamalini *et al.* (1998) they reported that, biochemical content of seed-borne fungi were changed by *A. flavus*, *A. niger*, *F. oxysporum* and *Macrophomina phaseolina*. Also, fungal infection resulted in the decrease in protein, total and reducing sugar contents. The reduction of these chemical components in the infected legume seed may be due to the utilization of these components by the fungi in its growth (Azize and Mahrous 2004). Inoculated lupine seeds with *F. oxysporum* f. sp. *lupine* resulted in a considerable decline in soluble carbohydrates between 24 and 72h. (Morkunas *et al.*, 2005).

Moisture content was found to be higher in the infected seeds compared to the healthy seeds. These percentages reached 38.7, 43.87 and 22.4% higher in the infected seeds than the healthy beans, pea and soybean respectively. Similar results were reported by Embaby and Mona (2006) and many investigators reported that, the increase in moisture content in the legume seeds reported in the current study which considered the causative factor in the infection rate. It is well documented that a higher seed moisture content increased fungal infection particularly, by *Aspergillus* spp., of which *A. flavus* and *A. niger* were predominant at higher and lower moisture contents respectively (Maheshwari and Mathur, 1987). *F. moniliforme* decreased with time with increase in the relative humidity. Protein, total and reducing sugar contents decreased gradually with increase in the RH values (Lokesh and Hiremath, 1993). There was an increase in moisture content, reduction in the fat and decrease in the available carbohydrates in all grain cowpeas analyzed. Similarly, the energy content showed a significant ($p < 0.05$) decrease in all the grains (Kungu *et al.*, 2003).

It concluded that legume seeds collected from Cairo and Kalubia Governorates were found to be infected with numerous fungal genera. Moisture content was found to be the most important factor in fungal infection. The isolated fungi were capable to produce mycotoxins in significant concentrations exceed the save limits recommended by the Egyptian authorities and may cause health risk for consumers. Moreover, the fungal infection resulted in the decrease of the chemical components of the seeds and consequently reduces its nutritive value.

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