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Mycoflora Colonization and Mycotoxin Accumulation in Cotton Seeds

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Cottonseed borne fungi were isolated from five cotton (*Gossypium barbadense* L.) cultivars; Giza-80, Giza-86, Giza-88, Giza-90 and Giza-92. *Alternaria* spp., *Aspergillus flavus*, *A. niger*, *Aspergillus* spp. *Fusarium* spp., *Penicillium* spp., *Rhizopus stolonifer* and *Trichoderma* spp. were occurred in both non-strilized and sterilized seeds. *A. niger* was generally the most predominant fungus with isolation frequency range of 20.95% to 73.17%. Mycotoxin analysis were performed by high performance liquid chromatography (HPLC). HPLC analysis of cottonseed revealed the presence of mycotoxins that were varied according to storage periods and genotypes. Significant correlations between the frequency of isolated fungi and the mycotoxin production was recorded in this study. Seven models were constructed using the predictors supplied by stepwise regression with R^2 values ranged from 0.433 to 0.578%. Since the stepwise multiple regression was effectively constructed 7 predictive models; this study suggested that the contents of some mycotoxins in cotton seeds could be predicated depending on the fungal isolation frequencies.

Key words: HPLC, *Gossypium barbadense*, Mycotoxins, Storage.

Cotton seeds (*Gossypium barbadense* L.) are extensively used as a raw material in oil production as well as in animal feed manufacturing. Those seeds are vulnerable to the fungal attack due to the high levels of fat and protein content[1, 2]. Cotton seed infection with mycotoxigenic fungi could be occurred at pre and/or post-harvest stages[3]. During these periods, the mycoflora contamination and whole conditions will determine the further injuries and mycotoxin occurrence[4,5].

Numerous fungi could be isolated from cotton seeds during the different stages. Aspergillus, Penicillium, Fusarium and other fungal genera may be involved in cotton seed spoilage[6,7]. *Aspergillus flavus* was generally the most dominant fungus, capable of deteriorating seeds, reducing seed viability, and seedling growth[8,9]. In addition to previous injuries this mould has a capability of producing aflatoxins, which are harmful toxins, carcinogenic and mutagenic agents[10,11].

Fungal contamination of stored cotton seeds, particularly those used for oil manufacturing, is the main factor affecting the taste and flavor of the oil produced. In addition to the reduction of fine product quality due to the probable mycotoxin accumulation[2,12].

Natural co-occurrence of mycotoxins in stored cotton seeds, particularly those produced by Aspergillus and Fusarium species had previously been reported[13,14].

The present study aimed to investigate the natural occurrence of mycotoxigenic fungi in the stored cotton seeds and the effect of storage periods on the mycotoxin accumulation in the stored seeds.

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MATERIALS AND METHODS

Mycological analysis

Seed borne fungi were isolated from cotton seed samples of five cultivars; Giza-80, Giza-86, Giza-88, Giza-90 and Giza-92 obtained from Cotton Research Institute, Agri. Rec. Center, Giza Egypt. Subsamples of 100 cotton seeds/cultivar were randomly selected for fungal isolation. Such cotton seeds were surface sterilized using 5.2% sodium hypochlorite solution for 3 minutes then washed several times in sterilized water and blotted between filter paper. Ten seeds of surface sterilized or non-sterilized for each treatment and cultivars were seeded on the surface of petri dishes in 10 replicates for each cultivar [15]. Petri dishes were incubated at 28±2°C for 7 days after which growing fungal colonies were examined counted and purified. The isolated fungi was identified according to Mycological Center, Assiut University, Egypt and then fungi occurrence percentage was calculated for each cultivars.

Mycotoxins assays

Aflatoxin

One hundred grams of random representative samples were blended for 2 min using a high speed homogenizer and filtered though Whatman's filter paper. Aflatoxins were extracted from the homogenized filtrates using methanol solution (80:20 methanol/isolate filtrate). Solvents were evaporated under vacuum at 35°C; dried residues containing aflatoxin were dissolved in 1 ml of the same liquid mobile phase solution which contained methanol: acetic acid: water and stored in dark vials. The method of Christian (1990)[16] was used to detect and determine aflatoxin production. The extract was passed through a 0.45 ¼m micro-filter. Analysis of compounds was performed on HPLC model PerkinElmer® Brownlee™ validated C18, 100 mm×4.6 mm, 3 micron. The HPLC was equipped with an UV detector and the wave length in the UV detector was 365 nm. The mobile phase consisting of methanol: acetic acid: water (20.0/20.0/60.0 v/v/v). The total run time for the separation was approximately 25 min at a flow rate of 1 ml/min.

Ochratoxin A analysis

One hundred grams of random representative samples were extracted with a solution of methanol: sodium bicarbonate at 1% (50:50, v/v) and blended at high speed for one minute. The pure extract was filtered to remove particulate matter, and 20 ml of extract was taken and diluted with 20 ml of PBS containing 0.01% Tween 20. The diluted extract was filtered through a microfibre filter. A ten-milliliter portion was taken and added to an immunoaffinity column (OchraTest™, Vicam, Digen Ltd., Oxford, UK), and allowed to elute under gravity. The column was washed with 10 ml PBS containing 0.01% Tween 20 and then with 10 ml double-distilled water. OTA was eluted from the column with methanol (HPLC grade), at a flow rate of one to two drops per second. OTA detection was performed by HPLC, following the methodology proposed by Scudamore and McDonald (1998)[17], with some modifications. The HPLC apparatus used for OTA determination was a Hewlett Packard chromatography with a loop of 100 ¼l, equipped with a spectrofluorescence detector (excitation, 330 nm; emission, 460 nm) and a C18 column (Supelcosil LC-ABZ, Supelco; 150×4.6 mm, 5 ¼m particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20×4.6 mm, 5 ¼m particle size). The mobile phase was pumped at one ml/min and consisted of an isocratic system as follows: 57% acetonitrile, 41% water, and 2% acetic acid. OTA was quantified on the basis of HPLC fluorometric response compared with OTA standard (Sigma Aldrich Co., St. Louis, MO, USA, purity >99%). The lowest limit of detection was 1.0 ng/g.

Fusarium toxins

Fumonisin toxins were determined according to the method described by Mazzani et al. (2001)[18]. One hundred grams of random representative samples were blended with 5 g sodium chloride and 100 ml of methanol: water (80:20) solution at a high speed for one min, then filtered through glass microfiber filter paper. Ten ml of the filtrate was diluted with 40 ml of wash buffer and filtered again through 1 ¼m micro-fiber filter. Ten ml of the diluted extract were passed through a fumontest column (VICAM Company) and the column was washed using 10 ml of the same dilute solution. The fumonisin was eluted by passing one ml of HPLC grade methanol through the column and then elutes were re-collected. One ml of each of developer A (VICAM product No. G5005) and developer B (VICAM product No. G5004) were added to the elute and placed in a

calibrated fluorometer (Series-4/VICAM). The zearalenone and vomitoxin concentration was determined as described above for fumonisin, but the dilution was made with 49 ml distilled water, which were passed through a Zearatest and/or vomitoxin column (VICAM Company) and then measured in a calibrated fluorometer model (Series-4/VICAM).

Statistical analysis

ANOVA, correlation, regression and cluster analyses of the collected data were performed with SPSS 16 statistical package. The least significant difference (LSD) was used to compare isolate means. Linear correlation coefficient (r) was calculated to evaluate the degree of association between frequencies of the isolated fungi. Stepwise regression technique with greatest increase in R^2 as the decision criteria was used to describe the effect of frequencies of the isolated fungi on mycotoxin production.

RESULTS

Mycological analysis

Analysis of variance of the fungal isolation frequency (Table 1) revealed that fungus and cultivar × fungus interaction were highly significant sources of variation in frequencies of isolated fungi from both sterilized and non-sterilized cotton seeds after, 3 months storage, and those isolated from only serialized seeds after 6 months storage. Five fungal genera included several species were obtained from the tested samples. *Alternaria* spp., *Aspergillus flavus*, *A. niger*, *Aspergillus* spp. *Fusarium* spp., *Penicillium* spp., *Rhizopus stolonifer* and *Trichoderma* spp. were occurred in both non-strilized and sterilized seeds. *A. niger* was generally the most predominant fungus with isolation frequency range of 10.48% to 73.17% (Table 2). The highly significant interaction (Cultivar x fungus) refers to variant association of fungal genera with cotton seeds according to the tested cultivars.

Significant positive and negative associations were observed between the incidences of some fungal species when compared with the frequency of the others. In the case of non-sterilized seeds; highly significant negative correlation was found among *A. niger* & *Fusarium* spp. and significant negative correlations were among *Alternaria* spp. & *A. flavus* and among *Penicillium* spp. & *Trichoderma* spp. These results referred to different colonization condition of these fungi (Table 3). In the case of sterilized seeds significant positive correlation only was found among *R. stolonifer* & *Trichoderma* spp. (Table 4).

Similar patterns of fungal distribution; based on average linkage cluster analysis of isolation frequencies (%); were obtained in both sterilized and non-sterilized seeds. Isolated fungi appear to form main distinct group including all

Table 1. ANOVA of the isolation frequencies of mycotoxigenic fungi in cotton seeds

		Source	D.f	Mean Square	F	Sig.
After 3 months storage	Non sterilized	Cultivar	4	146.069	0.825	0.511
		Fungi	7	5337.455	30.150	0.000
		Cultivar x Fungi	28	525.978	2.971	0.000
		Error	200	177.029		
	sterilized	Cultivar	4	253.445	2.119	0.080
		Fungi	7	1904.508	15.925	0.000
		Cultivar x Fungi	28	393.027	3.286	0.000
		Error	200	119.596		
After 6 months storage	Non sterilized	Cultivar	4	131.080	0.719	0.580
		Fungi	7	10927.639	59.910	0.000
		Cultivar x Fungi	28	222.992	1.223	0.214
		Error	200	182.399		
	sterilized	Cultivar	4	216.846	1.847	0.121
		Fungi	7	8698.106	74.091	0.000
		Cultivar x Fungi	28	311.614	2.654	0.000
		Error	200	117.397		

Table 2. Comparison of the isolation frequencies of fungi isolated fromsterilized and non-sterilized cotton seeds.

	After 3 months storage									
	Giza-80		Giza-86		Giza-88		Giza-90		Giza-92	
	Non-ster	Sterilized	Non-ster	Sterilized	Non-ster	Sterilized	Non-ster	Sterilized	Non-ster	Sterilized
Alternaria spp.	7.13	10.30	28.40	9.50	9.62	6.12	12.51	11.74	7.07	5.25
A. flavus	15.74	17.85	4.75	2.72	14.49	15.00	9.81	7.20	12.98	7.49
A. niger	41.65	27.85	22.64	20.95	68.55	10.48	46.83	24.08	61.53	58.60
Aspergillus spp.	13.08	13.89	4.30	0.98	19.38	4.41	17.37	7.54	11.17	13.99
Fusarium spp.	23.17	15.56	19.82	13.33	8.10	11.43	15.67	8.33	7.08	0.00
Penicillium spp.	17.92	12.93	13.09	13.88	0.00	0.00	11.93	5.79	17.59	13.21
R. stolonifer	2.71	0.98	11.67	3.60	9.40	7.58	10.09	2.51	10.55	1.28
Trichoderma spp.	1.52	0.00	6.25	7.41	19.91	5.98	2.08	0.00	0.00	0.00
L.S.D 0.05 For (FxC) interaction		Non-Sterelized = 21.410			Sterilized = 17.601					
	After 6 months storage									
	Non-ster	Sterilized	Non-ster	Sterilized	Non-ster	Sterilized	Non-ster	Sterilized	Non-ster	Sterilized
Alternaria spp.	2.08	3.02	18.89	8.48	12.27	4.17	6.02	4.33	0.00	0.00
A. flavus	30.42	48.74	19.61	22.71	30.30	10.91	23.98	28.61	37.22	17.59
A. niger	55.91	45.21	50.05	37.49	66.83	58.85	61.31	60.92	73.17	55.41
Aspergillus spp.	8.39	12.50	8.20	5.04	16.67	1.85	17.92	4.38	14.31	14.58
Fusarium spp.	8.25	6.24	15.22	6.17	4.23	2.50	5.00	6.02	7.21	3.66
Penicillium spp.	8.81	14.72	12.50	9.02	0.00	0.00	10.74	9.72	13.69	14.58
R. stolonifer	14.22	4.31	18.47	5.56	17.36	4.85	11.66	2.08	13.29	1.96
Trichoderma spp.	0.00	0.00	2.83	5.00	9.58	7.07	0.00	0.00	0.00	0.00
Mean	16.01		18.22		19.66		17.08		19.86	
L.S.D 0.05 For (FxC) interaction =17.435			for cultivar (C)=13.308							

fungi while, *A. niger* was in the separate group (Figure 1). Within the particular group, fungi were associated strongly and positively in their distribution patterns over samples, whereas between groups, they were associated weakly or negatively. This result implies the potential existence of sample (environment) related fungi.

Mycotoxin analysis

High performance liquid chromatography revealed that all stored cotton seeds found to be contaminated with aflatoxins, ochratoxins, fumonisin (Fum), deoxynivalenol (DON) and zearalenone (Zen). The quantities of toxin contamination in the cotton seed samples were

Table 3. Correlation among frequencies of fungi isolated from non-sterilized cotton seeds

Fungi	F1	F2	F3	F4	F5	F6	F7	F8
F1	1	-0.892*	-0.677	-0.486	0.514	-0.143	0.793	0.318
F2		1	0.796	0.403	-0.611	-0.082	-0.538	0.003
F3			1	0.751	-0.965**	-0.403	-0.134	0.307
F4				1	-0.811	-0.596	-0.222	0.370
F5					1	0.471	-0.042	-0.375
F6						1	-0.307	-0.955*
F7							1	0.515
F8								1

F1=*Alternaria spp.*, F2 =*A. flavus*, F3=*A. niger*, F4=*Aspergillus* spp., F5=*Fusarium* spp., F6= *Penicillium* spp., F7= *R.* stolonifer, F8=*Trichoderma* spp

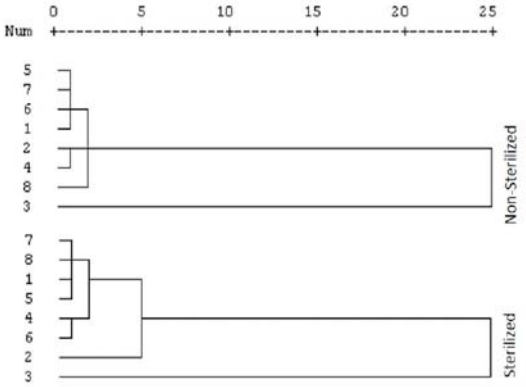
Table 4. Correlation among frequencies of fungi isolated from sterilized cotton seeds

Fungi	F1	F2	F3	F4	F5	F6	F7	F8
F1	1	0.190	-0.770	-0.594	0.800	-0.024	0.225	0.264
F2		1	-0.164	0.495	0.591	0.386	-0.329	-0.517
F3			1	0.704	-0.876	0.344	-0.724	-0.684
F4				1	-0.340	0.711	-0.786	-0.809
F5					1	-0.004	0.344	0.260
F6						1	-0.765	-0.598
F7							1	0.941*
F8								1

F1=Alternaria spp., F2 =A. flavus, F3=A. niger, F4=Aspergillus spp., F5=Fusarium spp., F6= Penicillium spp., F7= R. stolonifer, F8=Trichoderma spp.

Table 5. Mycotoxins content (µg/kg) of stored cotton seeds at 3 & 6 months after storage under ordinary condition.

Toxins	After 3 months storage					After 6 months storage				
	Giza-80	Giza-86	Giza-88	Giza-90	Giza-92	Giza-80	Giza-86	Giza-88	Giza-90	Giza-92
B1	3.0	1.0	4.0	9.0	2.5	7.0	3.5	6.0	21.5	11.0
B2	4.0	-	4.0	2.0	1.0	5.0	2.0	8.5	5.5	7.5
G1	5.0	2.0	2.0	1.0	1.5	8.5	3.0	6.0	4.0	9.0
G2	2.5	-	3.5	2.5	2.5	4.0	1.5	5.0	-	3.5
OTA	2.0	0.5	14.0	3.5	5.0	1.0	-	9.5	1.0	2.0
FUM	861.0	171.0	15.0	205.0	11.0	134.5	27.0	-	30.0	2.0
DON	1363.0	1673.0	703.0	1313.0	1750.0	524.0	614.0	270.0	502.0	673.0
ZEN	318.0	121.0	331.0	118.0	312.0	84.0	32.0	79.0	31.0	88.0



cotton seeds: F1=*Alternaria spp.*, F2 =*A. flavus*, F3=*A. niger*, F4=*Aspergillus* spp., F5=*Fusarium* spp., F6= *Penicillium* spp., F7= *R.* stolonifer, F8=*Trichoderma* spp.

Fig. 1. Phenogram based on average linkage cluster analysis of frequencies fungi isolated from non-sterilized and sterilized

varied among and/or within storage periods. An amount of 2 µg/kg and 1.5 µg/kg of B2 and G2 aflatoxins were recorded in Giza-86 samples 6 months after storage although, no detectable amount of both were found in 3 months after storage. Giza-90 had no detectable amounts of aflatoxins G2, Giza-86 had no ochratoxin and Giza-88 had no fumonisin 6 months after storage (Table 5).

Significant and highly significant positive correlations were observed between zearalenone (Zen) production and the frequencies of *Fusarium* spp. isolated from non-sterilized, sterilized cotton seeds and their mean respectively. Highly significant positive correlation of the frequency of *A. flavus* isolated from non-sterilized cotton seeds and the production of B2 & G1 aflatoxins (Table 6).

Stepwise multiple regression analysis (Table 7) using the frequencies of fungi isolated from cotton seeds and contents of toxins produced by mycotoxigenic fungi, shows the contribution

Table 6. Correlations between mycrotoxin content of cotton seeds (ys) and frequency of fungi (Xs) isolated from these seeds.

Fungi	Mycotoxin content	Frequency of fungus isolated from		
		Non-sterilized seeds (X1)	Sterilized seeds (X2)	Mean (X3)
Fusarium spp	Zen (Y_1)	0.761*[a]	0.698**	0.777**
	Fum (Y_2)	0.594	0.296	0.495
	Don (Y_3)	0.222	0.314	0.276
A. niger	OTA (Y_1)	0.519	0.327	0.420
	B1 (Y_1)	0.454	0.365	0.453
A. flavus	B2 (Y_2)	0.880**	0.362	0.658*
	G1 (Y_3)	0.897**	0.626	0.834**
	G2 (Y_4)	0.489	0.164	0.342

a pearson's correlation coefficient(r) is significant at p< 0.01 (**) or p < 0.05 (*) .

Table 7. Regression equations that describe the effect of frequency of fungi isolated from non-sterilized seeds (X1), sterilized seeds (X2), and their means (X3) on cottonseed content of mycotoxins (y_s) .

Fungus	Mycotoxin	Regression equation	r[a]	R^{2b}	F. value	P>F
Fusarium spp	Zen (Y_1)	Y1= -197.061+30.192 X1	0.760	0.578	10.940	0.011
	Fum (Y_2)	Y1= -128.099+37.382 X 2	0.698	0.487	7.594	0.025
	Don (Y_3)	Y1= -205.446+37.570 X 3	0.777	0.604	12.200	0.008
A. niger	OTA (Y_1)	Y2= -0.739+0.235 X 1	0.880	0.774	27.465	0.001
	B1 (Y_1)	Y2= 0.730 +0.171 X 3	0.658	0.433	6.098	0.039
A. flavus	B2 (Y_2)	Y3= -0.765 +0.249 X 1	0.897	0.805	33.075	0.000
	G2 (Y_4)	Y3= -0.039 +0.225 X 3	0.834	0.695	18.269	0.003

a correlation coefficient.; b coefficient of determination.

of isolation frequencies in the R^2. In this analysis; factors with little or no predictive value in the R^2 were eliminated and only the significant contributed factors were incorporated. The addition of predictors into the model by the stepwise multiple regression resulted in 7 predictive models.

DISCUSSION

This study indicated that *A. niger* was the most predominant fungus and could be found in all stored cotton seeds up to 6 months followed by *A. flvaus*. This result is consistent with the documents demonstrated that *A. niger* was among the dominant fungi of cotton seeds[1,6,7]. Recovering of variant fungal genera could be attributed to the fat and protein contents of the oil seeds, as well as inappropriate post-harvest and storage conditions, which may lead to increasing fungal population[2,19]. The existence of mycotoxigenic fungi in the cotton seeds could be found at the pre and/or post-harvest stages; leading to seed deterioration and mycotoxin contamination[1,3,12].

Aflatoxins B_1, B_2, G_1 and G_2 secreted by some different species of *Aspergillus* fungi as *A. flavus* and *A. parasiticus*[20]. Aflatoxin B_1 and G_1 were found in all tested varieties under different storage conditions on the contrary of another aflatoxins B_2, and G_2 and this may be due to the Aflatoxin B2 and G2 are dihydro derivatives of the parent compounds B_1 and G_1[21].

Aflatoxins contamination increased their rates in seeds stored for a period of 6 months than the seeds stored for 3 months which can be explained that *Aspergillus* which detachment of these toxins considered storage fungi and have ability to survive and adapt with the hard and different storage conditions[22].

The ochratoxin produced by some species of *Aspergillus* and *Penicillium*[23]. The concentration of this toxin was decreased with the length of storage period with all tested cultivars; meanwhile the concentrations of ochratoxin for all treatment were under the regulation limit[24] (European Commission No 123/2005). *Fusarium* toxins concentration (Fumonisin, Deoxynivalenol and Zearalenone) decreased after increased seed storage period to 6 months compared with seeds stored for 3 months only. This may be due to *Fusarium* is one of field fungi which can't bear the difficult storage conditions. All concentrations of Fumonisin and Zearalenone were under regulation limit (1250 ppb) while the deoxynivalenol was out of this limits with varieties Giza 80, Giza 86, Giza 90, Giza 92 after 3 months storage according to CAC[25].

The significant correlations between the frequency of isolated fungi and the mycotoxin production was recorded in this study. This result could be attributed to the importance of the seed oils and lipid-rich tissues in supporting of the fungal growth, sporulation and mycotoxin production[26,27]. Cotton seed content of G1 and B2 aflatoxins were significantly correlated with fungal isolation frequency because the Cotton seed oils is important to aflatoxin production by *Aspergillus*. Moreover, extracting the lipids from cottonseed meal had been reported to reduce aflatoxin production by *A. flavus* approximately 1000-fold[28].

Since the stepwise multiple regression was effectively constructed 7 predictive models using the factors that made a statistically significant contribution to the R^2 values; isolation frequencies of some cotton seed fungi could be used to predict the mycotoxin content in those seeds. In this respect; good relationship had previously been found between the fungal development and secondary metabolism[10,29].

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