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ORIGINAL ARTICLE

Effects of cultivar, storage period, and seed-borne fungi on aflatoxin content of cotton seeds

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ABSTRACT

Abd-Elsalam, K.A.; Habeeb, M.M.; Asran, A.A. **Effects of cultivar, storage period, and seed-borne fungi on aflatoxin content of cotton seeds** ***Summa Phytopathologica,*** v.49, p.1-16, 2023.

Non-sterilized seeds from three commercial cultivars of cotton (*Gossypium barbadense* L.) were examined for qualitative and quantitative estimates of seed-borne fungi. The observed fungi were *Aspergillus* sp.1, *Aspergillus* sp.2, *Penicillium* sp., *A. flavus*, *Alternaria* sp., *A. niger*, *Fusarium* sp., *Rhizopus* sp. and *A. ochrecous*. According to the quantitative estimates, *A. niger* (36.02%), *A. flavus* (19.29%) and *Penicillium* sp. (16.74%) were the most predominant fungi isolated from the seeds. Other fungi occurred at frequencies ranging from 0.21% to 10.44%. Analysis of variance showed that each aflatoxin type (A) and cotton cultivar (V) was a significant source of variation in the seed aflatoxin content , while storage period (P) was a nonsignificant source of variation. The first-order interactions A×V and A×P were always nonsignificant sources of variation. In general, aflatoxin B_1 content was greater than that of B_2. Two regression models, derived from stepwise multiple regression analysis, were constructed to describe the effects of the isolated fungi (independent variables or predictors) on aflatoxin content (dependent variables). The first one-variable model (R^2 = 34.8%) was used to predict B_1 content, while the second five-variable model (R^2 = 98.2%) was used to predict B_2 content. It is worth noting that species of the genus *Aspergillus* alone accounted for 53.6% of the total variation in B_2 content. In conclusion, cottonseed is susceptible to infection with toxigenic fungi that can be harmful during storage. The study demonstrated the deleterious impacts of *A. flavus* and aflatoxins on the assessed seed quality measures, highlighting the need to monitor toxigenic fungi and their aflatoxins. The findings of this study might aid in the development of techniques for reducing aflatoxins in oily seeds.

Keywords: Fungal community structure, aflatoxin, Egypt, cattle feed, cotton seed

RESUMO

Abd-Elsalam, K.A.; Habeeb, M.M.; Asran, A.A. **Efeitos de cultivar, período de armazenamento e fungos transmitidos por sementes no teor de aflatoxina em sementes de algodão.** ***Summa Phytopathologica,*** v.49, p.1-16, 2023.

Sementes não esterilizadas de três cultivares comerciais de algodão (*Gossypium barbadense* L.) foram analisadas quanto a estimativas qualitativas e quantitativas de fungos transmitidos por sementes. Os fungos observados foram *Aspergillus* sp.1, *Aspergillus* sp.2, *Penicillium* sp., *A. flavus*, *Alternaria* sp., *A. niger*, *Fusarium* sp., *Rhizopus* sp. e *A. ochrecous*. De acordo com estimativas quantitativas, *A. niger* (36.02%), *A. flavus* (19.29%) e *Penicillium* sp. (16.74%) foram os fungos predominantes isolados das sementes. Outros fungos ocorreram com frequência variando de 0.21% a 10.44%. Análise de variância mostrou que cada tipo de aflatoxina (A) e cultivar de algodão (V) constituiu uma fonte significativa de variação no teor de aflatoxina nas sementes, enquanto que o período de armazenamento (P) não se mostrou uma fonte significativa de variação. As interações de primeira ordem A×V e A×P resultaram sempre como fontes de variação não significativas. Em geral, o teor de aflatoxina B1 foi maior que o de B2. Dois modelos de regressão, derivados de análise de regressão múltipla stepwise, foram elaborados para descrever os efeitos dos fungos isolados (variáveis ou preditores independentes) no teor de aflatoxina (variáveis dependentes). O primeiro modelo de uma variável (R2= 34.8%) foi usado para prever o teor de B1, enquanto que o segundo modelo de cinco variáveis (R2= 98.2%) foi usado para prever o teor de B2. Vale destacar que as espécies do gênero *Aspergillus* foram responsáveis por 53.6% da variação total no teor de B2. Em conclusão, sementes de algodão são susceptíveis a infecção por fungos toxigênicos que podem ser prejudiciais durante o armazenamento. Este estudo demonstrou os impactos negativos de *A. flavus* e aflatoxinas nas medidas de qualidade das sementes avaliadas, ressaltando a necessidade de se monitorar fungos toxigênicos e suas aflatoxinas. Os resultados do presente estudo podem ajudar no desenvolvimento de técnicas para reduzir as aflatoxinas em sementes oleaginosas.

Palavras-chave: Estrutura da comunidade fúngica, aflatoxina, Egito, alimentação para gado, semente de algodão

Due to their propensity to negatively affect the growth and development of crops, aflatoxins cost the global economy around $270 million every year (14, 15, 16). At least 16 distinct aflatoxins are estimated to occur, although the six most common ones (AB1, AB2, AG1 AG2, M1 and M2) are those that most typically contaminate food and agricultural food commodities (8, 14). Aflatoxins are most dangerous to human and animal health, and two *Aspegilius* species, *Aspergillus flavus* and *Aspergillus parasiticus* , are the principal producers of aflatoxins. Some oily crops, such as cotton, used for animal feed, are susceptible to fungal contamination during growth, harvest or storage, leading to production of mycotoxins (9). Under favorable preharvest and postharvest conditions, aflatoxins can infect cottonseed, and cottonseed contaminated with aflatoxin at a concentration of more than 20 ppb is prohibited for use in animal feed in the United States. (4). Aflatoxins in contaminated seeds may easily be transmitted to milk of dairy cows such as aflatoxin M1 ; therefore, aflatoxin contamination has long been a worry for the cottonseed business (11). Although cottonseed has significant levels of protein, aflatoxin contamination is a major problem since cottonseed is utilized as preferred feed for dairy cattle, as well as for oil production. Another concern is that cows fed on infected cottonseed would eventually convert aflatoxin B1 to M1 (a carboxylated derivative of aflatoxin B1) in their milk, which will then potentially damage human health (14).

When Egyptian cotton seeds were surveyed for their fungal flora and aflatoxin generation, there were 11 genera and 31 species isolated from stored cotton seeds from different sites, along with two species variations. B2 was generated by *A. flavus*, and the ideal conditions for aflatoxin production in synthetic agar medium were evaluated by El-Naghy et al. (7). Considering the potential for aflatoxin production and the frequency at which it occurs in infected plants, it is important to determine the risk that a specific mold group poses to aflatoxin contamination (20, 23). For example, the prevalence of mycotoxigenic species, as well as their mycotoxigenic potential, was studied in the agroecosystems of pioneer grain corn plantations in Malaysia (30).

Since mycotoxin levels in dairy feed samples are high and data on the real mycotoxin contamination of feed are scarce, more focus should be placed on routine mycotoxin testing of dairy feed and milk. The current study aimed to investigate the presence of mycoflora and aflatoxins in stored seeds of three Egyptian cotton cultivars. In addition, the current study investigated the effect of the storage period on cottonseed aflatoxin content between 2019-2021.

MATERIALS AND METHODS

Fungal profiles in stored seeds of cotton cultivars

Seeds of three commercial cultivars of Egyptian cotton (*Gossypium barbadense* L.) were received from the Cotton Research Institute, Agricultural Research Center, Giza, Egypt, and preserved at the end of 2018 for one, two and three years before isolation. For isolation, a random subsample of 100 seeds from each cultivar for each storage period, under the same conditions, was employed. The usual blotter method was used to test the presence of seed-borne fung i (10). Ten non-sterilized seeds were randomly selected from each cultivar and deposited on three layers of moist 9-cm Whatman No. 1 filter paper in Petri dishes. Each duplicate was then repeated ten times. For eight days, the plates were incubated at 20°C under a 12-hour photoperiod (cold white light). Fungi were isolated and purified using either single spore or hyphal tip techniques before being placed on slanted potato dextrose agar (PDA). Taxonomic systems suggested by Pitt & Hocking were used to identify *Aspergillus* species (21). *Penicillium* isolates were identified morphologically using the approach published by Visagie et al. (28). *Aspergillus* isolates were identified to the genus level on PDA , yeast extract sucrose agar (YES) and Sabouraud dextrose agar (SDA). After incubation, each colony was investigated under a light microscope, and the morphological traits of every fungal species were studied and photographed (A Nikon Eclipse E200 microscope). Each fungal isolation frequency was calculated as a proportion of seeds from which it developed. If the same seed produced more than one fungus, they were all counted.

Aflatoxins assay in cotton seeds

Aflatoxins extraction

Toxins found in cotton seeds were purified utilizing a Neogen Corporation-recommended AOAC-approved method (AOAC-RI 050901). Using a mill grinder, a small sample of seeds or cakes (about 200 g) was completely ground into a fine powder (IKA, Wilmington, USA). Next, the powdered sample (10 g) was added to the mycotoxin extraction cup (250 mL) together with 50 mL methanol/deionized water (70:30 vol./vol.) to create a suspension, which was vigorously agitated for 3 minutes. The suspension was left to incubate until every particle was at the bottom. Then, the supernatant solution was decanted and filtered through a cotton wool filter in a sample tube.

Aflatoxin Quantification

The high-performance liquid chromatography (HPLC) system used for detection of aflatoxins included a Shimadzu liquid chromatography (Shimadzu, Kyoto, Japan) with a Shimadzu SPD-M10Avp UV Fluorescent detector set at excitation and emission wavelengths of 362 and 460 nm, respectively. The analytical column was an ODS C18 (4.6 x 250 mm, 5 m diameter). SIGMA (St. Louis, MO, USA) provided the aflatoxin standards, which were used for the calibration and determination of aflatoxins . A 60-40 v/v methanol-water solution was employed as the mobile phase, and it was pumped at a 1 mL min flow rate (14).

Statistical analysis of the data

The study used a randomized complete block design with three replicates in a factorial arrangement as an experimental setup. To compare treatment means, the least significant difference (LSD) statistic was employed. The effect of the isolated fungi on the aflatoxin content of seeds was studied using stepwise regression analysis, regarding the largest increase in R^2 as the decision criterion. SPSS 10.0 was used for the statistical analysis.

RESULTS

Fungal species isolated from cottonseed

Using conventional mycological procedures, various fungal species were isolated and quantitatively enumerated on solid medium. Species determination indicated a tremendous deal of variability. The colony morphology of some *Aspergillius* species are shown in Figures 1 and 2. The *A. flavus* group was morphologically identified based on yellow-green conidia , globose to sub-globose vesicles and biseriate seriations. In addition, Table 1 shows the identification of 10 genera and 29 species of mo lds. The most frequently isolated fungi were *Aspergillus* and *Pencillium* species, in decreasing order: *A. niger. A. flavus, A. ochraceous P. citrinum, P. chrysogenum, P. verrucosum, P. expansum, P. paneum,* and *Alternaria alternate*.

Table 1. Toxicogenic fungal species isolated from cottonseed and their mycotoxins.

Isolate Number	Fungal species	Major Mycotoxins
1	*Alternaria alternata*	Tenuazonic acid, alternariol, alternariol monomethyl ether, altenuene and altertoxin I
2	*Aspergillus flavus*	Aflatoxins (AFB1, AFB2, AFG1, AFG2)
3	*Aspergillus clavatus*	Patulin
4	*Aspergillus ochraceous*	Ochratoxin A, Penicillic acid
5	*Aspergillus niveus*	Ochratoxin A
6	*Aspergillus terreus*	Citrinin
7	*Aspergillus fumigatus*	Viriditoxin, fumigaclavines A, B, C, and D, spinulosin and Gliotoxin
8	*Aspergillus versicolor*	Cyclopiazonic acid
9	*Penicillium paneum*	penipanoid A, B, C, patulin and roquefortine C
10	*Penicillium crustosum*	Penitrem A
11	*Penicillium chrysogenum*	Roquefortine C, PR-*toxin*
12	*Penicillium roqueforti*	Roquefortine, PR-*toxin*
13	*Penicillium expansum*	Patulin and citrinin
14	*Penicillium citrinum*	Citrinin
15	*Penicillium griseofulvum*	Patulin
16	*Penicillium verrucosum*	Ochratoxin A, Citrinin
17	*Penicillium atrovenetum*	β-nitropropionic acid
18	*Penicillium marneffei*	Unknow n
19	*Penicillium* spp.	Unknown
20	*Epicoccum nigrum*	---
21	*Stemphylium* sp.	---
22	*Acremonium* sp.	---
23	*Ulocladium* sp.	---
24	*Chaetomium globosum*	Chaetoglobosins A and C
25	*Chaetomium funicola*	---
26	*Fusarium solani*	T-2 toxin
27	*Fusarium oxysporum*	Zearalenone
28	*Rhizopus* sp.	---
29	*Chetomium* spp.	---

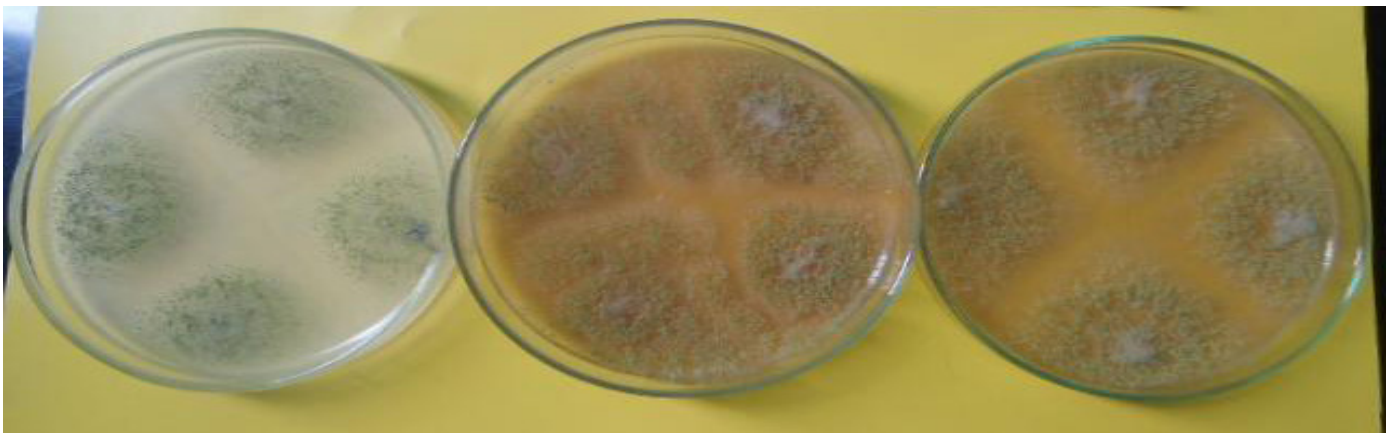

Figura 1. Cultural characteristics of *Aspergillius flavus* grown on three types of culture media: from left to right, potato dextrose agar (PDA), yeast extract sucrose agar (YES), and Sabouraud dextrose agar (SDA) .

Figura 2. from left to right Cultural characteristics of *Aspergillus ochraceous*, from left to right, grown on potato dextrose agar (PDA), yeast extract sucrose agar (YES) and Sabouraud dextrose agar (SDA) .

The most common fungi recovered from non-sterilized cottonseed were *A. niger* (36.02%), *A. flavus* (19.29%) and *Penicillium* sp. (16.74%), according to the mean percentage of fungal recovery (Table 2). Other fungi were found at the range from 0.21% to 10.44% . Nine fungal species were identified among the nine tested treatments . Five of these species (55.56%) belonged to the genus *Aspergillus*. No single treatment yielded all 10 species. Treatment no. 5 yielded the highest number of species (eight species), while treatment no. 3 yielded the lowest number (three species).

Aspergillus niger was the only fungus which was isolated from all tested treatments. Toxigenic potential test of *A. flavus* isolates from cottonseed evidenced that 5 treatments generated aflatoxin types B1 and B2. The treatments Giza92 and Giza90 had the greatest proportion of *A. flavus* toxigenic strains : eight and nine, respectively, and maximal aflatoxin B1 levels: 55.29 and 41.46 ppb, respectively. After two years of cottonseed storage , the maximum quantity of aflatoxin was 55.29 ppb, measured from Giza 92. Five cotton seed samples exceeded one of the European Union (EU)'s regulatory limits (5 ng g^{-1}), four samples were above EU's maximum limit and FDA's safety standard (20 ppb), and four samples exceeded Iran's regulatory limits (50 ppb) for total aflatoxin. Aflatoxin B1 levels in cotton seeds were found to be highest in all positive samples. Among the positive concentrate feed samples, 5 had aflatoxin B1 concentrations greater than the permitted threshold for concentrates, which is 5 ppb, as suggested by the European Communities.

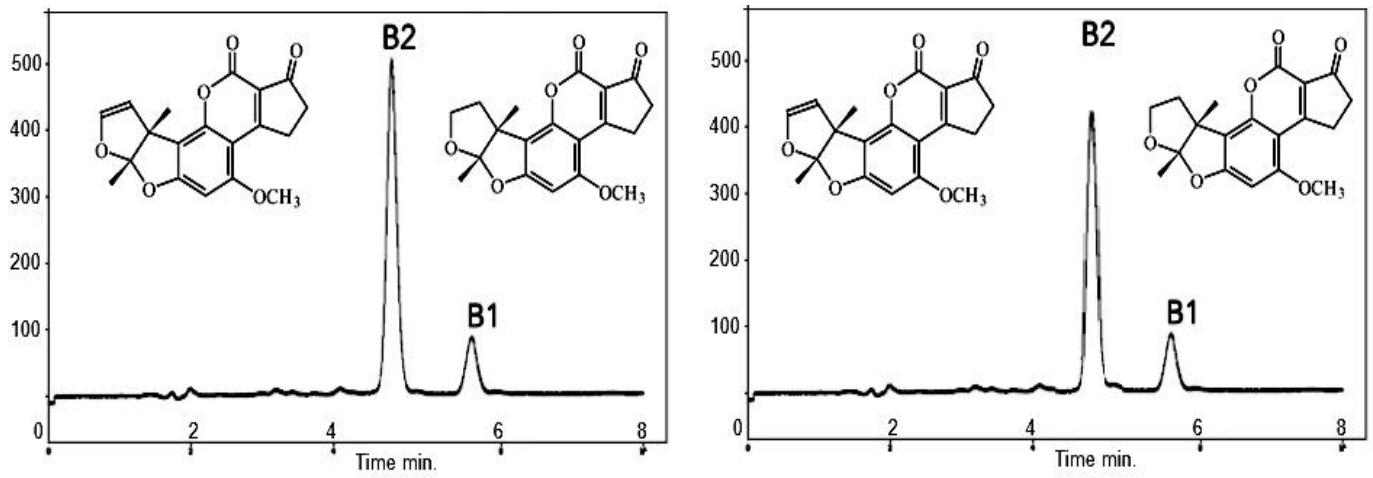

Figura 3. Representative HPLC chromatogram of naturally contaminated cotton seeds for treatments 8 and 9 stored for two and three years on cotton cultivars Giza 92 during 2020-2021, respectively.

According to the results of analysis of variance (ANOVA) shown in Table 3, aflatoxin type (A) and cotton cultivar (V) were significant sources of variation in the aflatoxin concentration of cottonseed . On the other hand, the interaction AxV was a nonsignificant source of variance. Since the interaction was not significant, the general means were used to compare between aflatoxin types and cotton cultivars.

These comparisons (Table 4) revealed that, regardless of the cultivar, aflatoxin B1 content was considerably higher than aflatoxin B2 content. The aflatoxin content of Giza 92 was significantly greater than that of Giza 90. However, the difference was not significant between Giza 90 and Giza 87. The difference between Giza 92 and Giza 87 was not significant either (Table 5). ANOVA showed that aflatoxin type was the only significant source of variation, while each of the storage periods and the interaction were nonsignificant sources of variation. Aflatoxin B_1 did not significantly differ from aflatoxin B_2 , regardless of the storage period (Table 6).

Table 2. Effect of storage period and cultivar on aflatoxin content of cotton seeds and on the frequency of fungi isolated from these seeds.

Treatment	Cultivar	Date	Storage period (year)	Aflatoxin concentration (ppb)		Isolation frequency (%) of							
				B_1 (y_1)	B_2 (y_2)	Asp.1 (x_1)	Pen. (x_2)	AF. (x_3)	Alt. (x_4)	AN. (x_5)	F. (x_6)	R. (x_7)	AO. (x_8)	Asp.2 (x_9)
1	Giza 87	2019	one	0.00	0.00	25.00	36.53	5.77	1.92	26.92	0.00	3.85	0.00	0.00
2		2020	two	0.00	0.00	0.00	10.00	23.33	0.00	56.67	0.00	10.00	0.00	0.00
3		2021	three	**38.57**	6.26	3.23	9.68	16.13	3.23	29.03	0.00	0.00	6.45	0.00
4	Giza 90	2019	one	0.00	0.00	8.70	21.74	23.91	0.00	34.78	0.00	4.35	10.87	0.00
5		2020	two	0.00	0.00	3.85	23.08	15.38	1.92	36.54	0.00	5.77	11.54	1.92
6		2021	three	2.33	0.30	13.64	0.00	0.00	0.00	45.45	0.00	40.91	0.00	0.00
7	Giza 92	2019	one	16.36	3.76	9.61	15.38	32.69	0.00	23.08	0.00	0.00	19.23	0.00
8		2020	two	**55.29**	8.77	0.00	11.36	36.36	0.00	43.18	0.00	9.09	0.00	0.00
9		2021	three	41.46	7.18	2.86	22.86	20.00	0.00	28.57	2.86	20.00	2.86	0.00
Mean				17.11	2.92	7.43	16.74	19.29	0.79	36.02	0.32	10.44	5.72	0.21

The isolated fungi were *Aspergillus* sp.1 (Asp.1), *Penicillium* sp. (P en.), *A. flavus* (AF.), *Alternaria* sp. (Alt.), *Aspergillus niger* (AN.), *Fusarium* sp. (F.), *Rhizopus* sp. (R.), *A. ochrecous* (AO.), and *Aspergillus* sp.2 (Asp.2).

Table 3. Analysis of variance of the effect of aflatoxin type, cotton cultivar and their interaction on aflatoxin content of cotton seeds.

Source of variation	D.F.	M.S.	F.value	P>F.
Replicate	2	242.507	1.825	0.211
Aflatoxin type (A)	1	906.528	6.823	0.026
Cotton cultivar (V)	2	735.348	5.535	0.024
A×V	2	361.037	2.717	0.114
Error	10	132.860		

Table 4. Effect of aflatoxin type, cotton cultivar and their interaction on aflatoxin content of cotton seeds.

Aflatoxin type (ppb)	Giza 87	Giza 90	Giza 92	Mean
B_1	12.86	0.78	37.70	17.11
B_2	2.09	0.10	6.57	2.92
Mean	7.48	0.44	22.14	

LSD for aflatoxin type ($p \leq 0.05$) =12.49. LSD for cotton cultivar ($p=0.05$) =15.30.

Table 5. Analysis of variance of the effect of aflatoxin type, storage period and their interaction on aflatoxin content of cotton seeds.

Source of variation	D.F.	M.S.	F.value	P>F.
Replicate	2	735.348	4.119	0.050
Aflatoxin type (A)	1	906.528	5.078	0.048
Storage period(P)	2	242.507	1.358	0.301
A×P	2	132.700	0.743	0.500
Error	10	178.528		

Table 6. Effect of aflatoxin type, storage period and their interaction on aflatoxin content of cotton seeds.

	Storage period (year)[a]			
Aflatoxin type	**one**	**two**	**three**	Mean
$\mathbf{B_1}$	5.45	18.43	27.45	17.11
$\mathbf{B_2}$	1.25	2.92	4.58	2.92
Mean	3.35	10.68	16.02	

LSD for aflatoxin type ($p \leq 0.05$) =14.36.

[a] The required storage dates were 2019, 2020, and 2021, respectively.

Table 7. Stepwise regression models describ ing the effects of frequencies (X_S) of fungi isolated from cottonseed on aflatoxin content (Y_S) of these seeds.

Aflatoxin content	Stepwise linear regression model	R^2 (%)	F. value	P>F
$\mathbf{B_1}$	$Y=21.411-0.423X_2$	34.8	5.865	0.034
$\mathbf{B_2}$	$Y=44.384-1.228X_4-7.723X_3+0.172X_8-0.263X_5-0.338X_6$	98.2	77.265	0.000

Table 8. Predictors (fungi isolated from seed frequencies) included in the stepwise regression models, as well as their relative contributions (percentages) to the overall variation in aflatoxin content of seeds.

Aflatoxin and predictor	Predictor symbol	Relative contribution (%) of the predictor to the total variation
$\mathbf{B_1}$		
Penicillium sp.	X_2	34.8
$\mathbf{B_2}$		
Alternaria sp.	X_4	43.1
A. flavus	X_3	40.1
A. ochrecous	X_8	8.6
A. niger	X_5	4.9
Fusarium sp.	X_6	1.5

Data for concentrations of aflatoxins and frequencies of the fungi isolated from non-sterilized seeds of three cotton cultivars were obtained and a computerized stepwise multiple regression analysis was used to enter cultivars. The study created a predictive model by adding predictors to the model in order of their contribution to R2, in this example, frequencies of the isolated fungus. Including only those factors in the model made it an acceptable substantial contribution to the R2 value of the model; the study was effective in removing variables with little or no predictive value. Two models were created to predict types B1 and B2 using the predictors provided by stepwise regression (Table 7).

It is worth noting that species of the genus *Aspergillus* alone accounted for 53.6% of the total variation in B_2 content (Table 8).

DISCUSSION

Aflatoxins are found in a variety of foods on a global scale; however, cereals, oilseeds, spices, peanuts and tree nuts are all recognized to be high-risk foods for aflatoxin contamination. (18, 22). *Aspergillus* section f lavi strains are widely dispersed in soils and seeds, and they are highly transmitted to the storage environment. The degree of fungal contamination rises not only in the field, but also in the kernel development, harvesting, drying, shipping and storage processes (25). On the other hand, formation of aflatoxins in animal foo ds is affected by different variables, including storage conditions and feeding practices (26).

The most common fungi identified from non-sterilized cottonseed in this study were *A. niger* (36.02%), *A. flavus* (19.29%) and *Penicillium* sp. (16.74%). Other fungi were found at the range from 0.21% to 10.44% . Toxicogenic *Alternaria* and *Fusarium* species are frequently classified as field fungi, whereas storage fungi include *Aspergillus* and *Penicillium* species. The prevalence of *A. niger* over other cottonseed fungi is consistent with the findings of Simpson et al. (19, 27), who reported that *A. niger* was a prominent fungus at several sites in their study, infecting up to 23% seeds. *Penicillium* is one of the fungi implicated in cotton boll rot and may induce fiber quality deterioration under favorable climate circumstances (1). Davis and his team (1977) identified *Alternaria* genus as a major component of the cottonseed mycoflora (6). However, *Alternaria* was classified as an uncommon fungus by Roncadori et al. (24) and was found in more than 10% seeds from one single area by Simpson co-workers (27). Klich (15) detected *A. alternata* in over 10% seeds. *Alternaria* sp. was detected in 0.79% seeds in this study. Previously, *Fusarium* spp. constituted prominent components of the fungal flora. *Fusarium* sp. was detected in 0.32 % seeds in this study (24, 27).

Seeds of Giza 87 and Giza 90 yielded the highest concentrations of B1 and B2 in 2021 after a three- year storage period. On the other hand, seeds of Giza 92 yielded the highest concentrations of B1 and B2 in 2020, after two- year storage. The concentration of aflatoxins in 140 cotton seed samples was measured by high-performance liquid chromatography. Aflatoxin B1 had the greatest rate of contamination, appearing in 129 of the 139 samples (9). If cottonseed contains more than 20 ppb aflatoxin B1, it is forbidden as feed for dairy cows because

aflatoxins are transferred from the feed to the milk. Nonetheless , milk will not be contaminated with aflatoxin quantities higher than the legal limit of 0.5 ppb , and aflatoxin B1 levels below 300 ppb in cottonseed can be given to mature cattle. Considering that cottonseed cannot be used as a feed because of high aflatoxin levels, aflatoxin concentration is the most critical element influencing the value of whole cottonseed when it does not match dairy standards. Since crop-associated fungal populations remain in the crops until consumption and may create aflatoxins throughout handling, storage and processing, the levels of aflatoxins after harvest and at markets may not completely represent the risk of aflatoxin exposure by the crop (5). Each of Zambia's fungal species on crops has a high average of aflatoxin-producing capacity. Aflatoxins accumulate in crops that are not properly stored after harvest (5, 12).

Protection may be provided not only before harvest but also during storage by changing the fungal community composition in the field to enhance the proportions of atoxigenic L strain morphotype fungus (2, 3, 13).

Two models were generated using the predictors provided by stepwise regression analysis. The models' R^2 values were 34.8% and 98.2% for types B1 and B2, respectively. It is worth noting that the genus *Aspergillus* alone was responsible for 53.6% of the entire variation in B2 content. Agents previously reported to be linked to real contamination in maize and peanuts in markets in Zambia were investigated using regression analysis (13).

In conclusion, according to th e current study, the risk of aflatoxin contamination in cottonseed after three years of storage may be accurately predicted. Stepwise regression and precise models were elaborated under the specified simulated granary storage conditions. The results of this study will help regulatory authorities in Egypt create strategies for monitoring aflatoxins in animal feed . Furthermore, the high average aflatoxin-producing potentials identified in this study must be considered in safety management methods. Further research is needed to determine if storage fungi other than *A. flavus* and storage insects have a role in the aflatoxin levels found in cotton seeds used as animal feed.

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