Mycology

STUDIES ON THE FUNGUS FLORA AND AFLATOXIN PRODUCTION OF COTTON SEEDS IN EGYPT

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SUMMARY: Eleven genera and 31 species in addition to 2 species varieties were isolated from stored cotton seeds collected from different localities in Egypt. The most common species encountered on 2% glucose-Czapek's agar at 28°C using the dilution and the seed plate methods were: Aspergillus niger, A. flavus, A. terreus, A. tamarii, Mucor racemosus and Rhizopus stolonifer. On 2% cellulose-Czapek's agar, A. niger, A. flavus, A. terreus, A. fumigatus, A. zonatus, Mucor racemosus and Rhizopus stolonifer were more frequently encountered. Screening of fungal species and strains isolated for aflatoxin production by the fluorescence technique revealed that, only two strains of A. flavus, two strains of A. oryzae and one strain of A. fumigatus were positive for aflatoxin production. Only aflatoxin B_2 was produced by A. flavus and the optimal conditions for toxin production on synthetic agar medium is described.

Key Words: Aflatoxin B_2, cotton seed borne fungi, glucose or cellulose Czapek's agar media.

INTRODUCTION

It is now recognized that fungi play a more important role in the deterioration of stored grains and seeds than once suspected. Seeds commonly may carry a heavy spore load from the field into storage. In addition, it is recognized that some of the storage fungi particularly certain strains of *A. flavus* and *A. parasiticus* have tremendous capacity for aflatoxin production and therefore are associated with toxicities caused by moldy animal feeds (6,18).

Literature review indicated that, although several studies have been conduced in Egypt to survey seed borne fungi of various plant seeds including cereal grains (3, 4, 7, 13, 21, 26); lupine, sweet pea (10-12); certain leguminous seeds (5); umbeliferous seeds (1,15,16, 23, 25, 31); few studies were conducted with cotton seeds (22).

This work was designed to survey the fungi of stored cotton seeds collected from different localities in Egypt. The isolated fungi were tested for their ability of produce aflatoxins.

MATERIALS AND METHODS

Nineteen samples of cotton seeds (stored for one year except sample No. 5 which was stored for about 5 years) were collected from different localities in Egypt (Figures 1a and 1b). Samples were placed directly in polyethylene bags and transferred immediately to the laboratory where they were stored in a cool place for further investigation.

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Determination of fungi of the seeds

The seed plate and the dilution plate methods were used for determination of cotton seed borne fungi essentially as described by Moubasher *et al.* (26) and Christensen (8) respectively. Glucose or cellulose (20 g/L each) Czapek's agar media to which rose-bengal (1:15000) was added as a bacteriostatic agent (30) were used for fungal isolations. Six plates for each sample, substrate and isolation technique were incubated at 28°C for 5-7 days and the developing fungi were identified and counted. Colonies of slow growing fungi were transferred to slants to ensure precise counting and then to plates for identification.

Screening of isolated fungal strains for aflatoxins production

Screening experiments: The capability of the isolated fungal strains to produce aflatoxins was tested by the fluorescence technique developed by Hara *et al.* (17) using a nutrient medium of the following composition: sucrose, 30 g; K_2HPO_4, 10 g; $MgSO_4$-$7H_2O$, 0.5 g; KCl, 0.5 g; (NH_4) H_2PO_4, 10 g; $FeSO_4$-$7H_2O$, 0.01 g; $HgCl_2$, $5x10^{-4}$ M; corn steep liquor, 0.5 g; distilled water, 1000 ml and adjusted to pH 5.5.

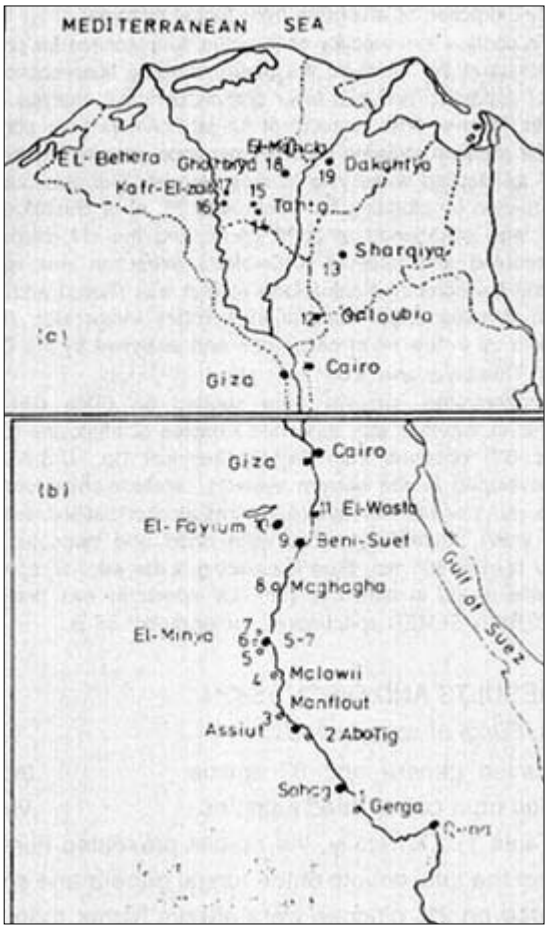

Figure 1: The places in Delta (a) and Upper Egypt (b) from which samples of cotton seeds were collected.

Four plates were inoculated with each test fungus grown on PDA plate and then incubated together with a non-inoculated control in the dark for 7 days at 28°C. At the end of the incubation period, the plates were inverted and examined under a strong ultraviolet lamp (365 nm) and the production of bright blue fluorescence under and around the fungal colonies was observed.

Extraction of aflatoxins from fungal cultures

To confirm the specify of the blue fluorescence for presence of aflatoxins in the medium, the plates showing fluorescence were used for extraction and thin layer chromatographic analysis of aflatoxins as follows: after removal of fungal colonies from plates, 30 g of agar medium showing blue fluorescence were mixed for 5 min with 75 ml distilled water in a Waring blender. The aqueous slurry was extracted by blending for 5 min with 25 ml of chloroform. The mixture was centrifuged at 2000 r.p.m. and the chloroform layer was decanted and retained. Chloroform extraction was repeated twice and the combined chloroform extract was filtered and evaporated to dryness under vacuum in a rotary evaporator. Residue was taken up in few ml of chloroform and analyzed by T.L.C.

Thin layer analysis

Concentrated samples were spotted on Silica Gel plates (Merk F254) together with authentic samples of aflatoxins (B_1, B_2, G_1 and G_2) obtained from Sigma Chemical Co., USA. Plates were developed in the solvent systems, acetone-chloroform-water (12:1.5 v/v/v) or benzene-absolute ethyl alcohol-distilled water (46:35:19 v/v/v), thereafter, plates were dried and inspected under long UV light at 365 nm. Blue fluorescing spots were scraped from plates and eluted in methanol (28). UV spectrum was determined using PERKIN ELMER spectrophotometer model 55 B.

RESULTS AND DISCUSSION

Fungi of cotton seeds

Eleven genera and 33 species were isolated and identified from cotton seed samples used in this investiga-

Table 1: Total count and number of cases of isolation (out of 19 samples) of the fungal genera and species recovered on 2% glucose and 2% celliulolase-Czapek's agar at 26°C using the dilution and seed plate technique.

Media	2% Glucose				2% Glucose			
Methods	DP		SP		DP		SP	
Species	TC	NCI	TC	NCI	TC	NCI	TC	NCI
Gross total count	5633	-	106,6	-	816	-	97,7	-
Aspergillus	5584	16	94,1	19	744	14	76,5	16
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Aspergillus	5584	16	94,1	19	744	14	76,5	16
A. niger Van Tieghem	3640	14	64,6	18	468	7	47,4	14
A. flavus Link	559	7	12,7	9	183	8	15,9	10
A. terreus Thom	95	5	0,2	1	4	2	3,2	6
A. tamarii Kita	679	4	8,9	4	-	-	-	-
A. midulans var latus Thom and Raper	78	3	1,3	2	-	-	-	-
A. tumigatus Fresenius	63	2	0,4	3	27	3	4,2	7
A. zonatus Kwon and Fennal	450	1	5,8	1	35	2	5	5
A. oryzae (Anib.) Chon	20	1	0,2	-	-	-	-	-
A. nidulans (Eidam) Winter	-	-	-	-	25	1	-	-
A. nidulans var, acristatus Fennel and Raper	-	-	-	-	-	-	0,2	1
A. ochraceus Wilhelm	-	-	-	-	2	1	0,2	1
Penicillium	25	4	-	-	9	3	1,4	2
P. nigricans (Bainier) Thom	13	1	-	-	-	-	-	-
P. steckii Zaleski	10	1	-	-	-	-	-	
P. cyclopium Westling	1	1	-	-	-	-	-	-
P. chrysogenum Thom	1	1	-	-	-	-	0,2	1
P. requeforti Thom	-	-	-	-	13	1	-	-
P. duclauxi Delacroix	-	-	-	-	2	1	-	-
P. jensenii Zaleski	-	-	-	-	2	1	-	-
P. stoloniferum Thom	-	-	-	-	2	1	1	1
P. frequantans Westling	-	-	-	-	-	-	0,2	1
Other genera	24	5	14,5	63	8	19,8	16	
Mucor racemosus Freenius	11	2	5,5	5	19	4	0,2	7
M. hiemalis Wehmer	-	-	0,4	2	-	-	-	-
Rhizopus stolonifer (Ehrenb. ex. Fr.) Lindt	-	-	7,8	6	22	2	8,6	8
Fusarium oxysporum Schlecht	10	1	-	-	3	2	1	3
F. moniliforme Sheldon	-	-	-	-	-	-	1,2	2
Paecilomyces variotii Bainier	2	1	0,2	1	14	2	0,4	1
Apiocrea chrysospermum (Tul.) Syd.	-	-	0,2	1	3	2	1	2
Microascus longristris Zukal	-	-	-	-	2	1	-	-
Curvularia lunata (Walker) Boedijn	-	-	-	-	-	-	0,2	1
C. pallescans Boedijn	-	-	0,2	1	-	-	-	-
Alternaria alternata (Fr.) Keissler	-	-	0,2	1	-	-	-	-
A. tenuissima (Kunze ex. Pers.) Wiltshire	1	1	-	-	-	-	0,4	1
Cephalosporum curtipus Saccardo	-	-	-	-	-	-	0,8	1

TC: Total count, DP: Dilution plate, SP: Seed plate
NCL: Number of cases of isolation (out of 19) and the occurrence remarks are as follows:
High occurrence: More than 9 cases out of 19
Moderate occurrence: Between 5-8 cases
Low occurrence: Between 2-4 cases
Rare occurrence: One case only out of 19

tion (Table 1). Generally, the results presented here indicate that the total counts of the fungal genera and species recovered on 2% glucose were always higher than those recorded on 2% cellulose by either the seed plate or the dilution plate method and that *Aspergillus* was the most common genus recovered in all these cases. This genus was recorded in high frequency in all the plates tested, donating 99.1, 86.7, 91.2 and 78.3% of the total count on the two media using the two methods of isolation respectively. These results are in accordance with those reported for cereal grains and other seeds (2, 4, 13, 15, 16, 19, 21, 24-26, 31).

Among the *Aspergillus* species isolated, *A. niger* and *A. flavus* were recovered in high counts from almost all the cotton seed samples on either 2% glucose or cellulose Czapek's agar. *A. terreus, A. fumigatus* and *A. zonatus* were also recovered on both substrates but with less counts and frequencies, whereas, *A. tamarii* and *A. nidulans* var. *latus* were isolated on 2% glucose but were missed on cellulose. All the previous species were also recovered previously from various seeds by other investigators in Egypt (23-26).

Penicillium was recovered from seed samples in moderate or low frequencies of occurrence. From the genus, 9 species were isolated and identified among which; *P. stoloniferum, P. frequantans, P. roqueforti, P. duclauxi* and *P. jensenii;* and *P. nigricans, P. steckii* and *P. cyclopium* were isolated on 2% cellulose or glucose Czapek's agar by one or the two methods tested. These species were previously recorded by other investigators to be isolated on glucose or cellulose Czapek's agar from cereal grains and seeds (2, 4, 12, 15, 16, 21, 22, 24-26, 31).

From Mucors; *Mucor racemosus* and *Rhizopus stolonifer* were recovered from cotton seeds mostly in moderate frequency of occurrence on the two media using the two media using the two methods tested. *M. hiemalis* occurred in low frequency on 2% glucose-Czapek's agar when using the seed plate method but was completely missing in the other plates tested.

Fusarium oxysporum was recovered by the two methods on glucose or cellulose in low or rare frequencies of occurrence whereas, *F. moniliforme* occurred only on 2% cellulose-Czapek's agar when the seed plate method was used. *Fusarium* species were reported to represent 17% of the total fungi isolated from peanut seeds (19).

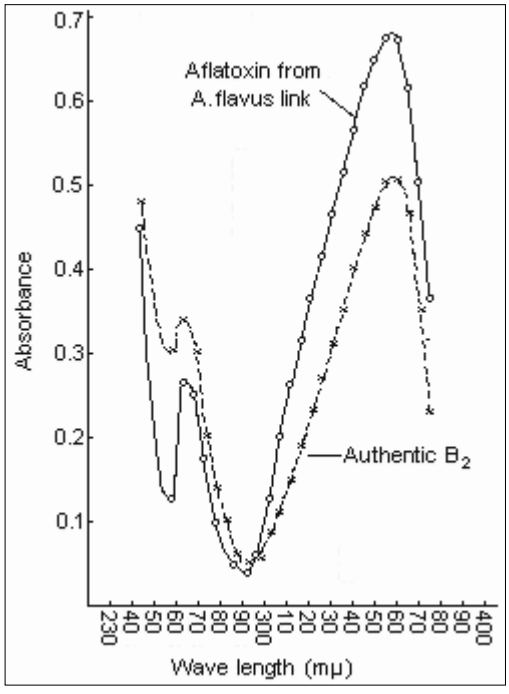
Other genera and species isolated in this study were found to occur either in low or rare frequencies on the two media tested using the two methods and these were; *Paecilomces variotii, Microascus longristris, Alternaria alternata, A. tenuissema, Curvularia palescens, C. lunata, Apiocrea chrysospermum* and *Cephalosporium curtipus*. Most of these fungi were identified at other laboratories in Egypt on cereal grains, seeds and other laboratories in Egypt on cereal grains, seeds and other plant sources as reported by Moubasher *et al.* (23-26).

Screening of fungal isolates for aflatoxins production

Screening of fungi isolated in this study for aflatoxin production by the fluorescence method on Czapek's agar medium containing corn steep liquor and $HgCl_2$ and adjusted to pH 5.5 as described by Hara *et al.* (17) revealed that among the 48 isolates tested which belong to 33 species and include 13 strains of *Aspergillus flavus*, only two strains of *Aspergillus flavus*, two strains of *A. oryzae* and one strain of *A. fumigatus* produced blue fluorescence. One of the two strains of *A. flavus* (designated strain 10) produced the most intense blue fluorescence.

Generally, it was observed that, strains of *A. flavus* and *A. oryzae* produced variuosly coloured fluorescent substances during their growth on Czapek's agar medium which interfered with observation of aflatoxin fluorescence. Experiments to eliminate this interference and enhance blue fluorescence using the two aflatoxin producing strains of each of *A. flavus* and *A. oryzae* by changing the nitrogen source supplied to the medium indicated that *A. flavus* (strain No. 10) produced the most

Figure 2: Ultraviolet absorption spectra of authentic aflatoxin B_2 and aflatoxin produced by *A.flavus* link in methanol.



intense blue colour on $(NH_4)H_2PO_4$ at 1% concentration. Experimentation to determine optimal condition for enhancement of intense blue fluorescence by this strain on synthetic agar medium was extended to include, carbon source, incubation temperature and period, pH, enzyme inhibitors and enhancers. Results revealed that the presence of 0.1% $(NH_4)H_2PO_4$, 3% sucrose, $5x10^{-4}$ M $HgCl_2$, 0.05% corn steep liquor and 0.01% methionine and adjusting the pH of the medium to 5.5 gave the maximal fluorescence after incubation for 8-10 days at 22°C.

Extraction of aflatoxins produced by *A. flavus* (strain 10) from synthetic agar medium followed by thin layer chromatographic analysis revealed the presence of a single blue fluorescence spot on thin layer plates with R_f value similar to that of aflatoxin B_2. The toxin co-chromatographed with authentic sample in different solvent systems. Elution of the toxin from thin layer chromatograms with methanol and determination of its UV absorption revealed similarity to authentic aflatoxin B_2 (Figure 2). No other aflatoxins were detected on the chromatograms.

It is known that toxigenic strains of *A. flavus* and *A. parasiticus* usually produce the four main aflatoxins (B_1, B_2, G_1, and G_2) under specific environmental conditions with aflatoxin B_1 and G_1 being the major toxins produced. The occurrence of isolates which produce only one individual toxin was rarely reported particularly with aflatoxin B_2 (27). The possibility that the cultural conditions we used to grow *A. flavus* favor the biosynthesis of aflatoxin B_2 but not others and that under different conditions other aflatoxins may be produced by this isolate cannot be excluded. Shih and Marth (29) reported that maximal production of the aflatoxins B_1, B_2, G_1 and G_2 by an isolate of *A. parasiticus* occurred when the medium contained 1% $(NH_4)_2SO_4$ and maximal production of aflatoxin G_1 was noted on the medium with 5% $(NH_4)_2SO_4$ and no G_1 and G_2 appeared when the medium contained 0.05% $(NH_4)_2SO_4$. They concluded that cultural conditions used to grow toxigenic strains affect the ratio of toxins, the partition of toxins between the mycelium and substrate, degradation of toxin during extending incubation and the amount of each individual aflatoxin that is produced. Further studies on aflatoxin production by the local strain of *A. flavus* to clarify this point would be of interest.

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