Isolation and Identification of Fungal Propagation in Stored Maize and detection of aflatoxin B1 Using TLC and ELISA Technique

Fadia. F. Hassan[1*], Muna. H. Al- Jibouri [1] Abdul. Kareem. J. Hashim[2]
[1] Department of Biology, College of Science, University of Baghdad, Baghdad,Iraq
[2] Departments of Biotechnology, College of Science, University of Baghdad, Baghdad,Iraq

Abstract:
Aflatoxin B1 (AFB1) is a mycotoxin produced mainly by fungi *Aspergillus flavus* in food and animals feed. It is considered as a carcinogenic toxin for human and animals. The current study is designed to investigate the incidence of mycoflora in twenty four samples of local stored maize collected from Iraqi governorates; investigate the presence of aflatoxin B1 on these samples using TLC and ELISA techniques. The fungi recovered from maize samples were *Aspergillus flavus* (18.57 %), *Fusarium spp*. (12.8 %), *A. ocraceus* (9.96 %) , *A. terrus* (9.07 %), *A. fumigatus* (8.46 %) , *Alternaria* spp. (6.40 %) *Rhizopus* spp. (4.98 %), *A. niger* spp., *A. oryzae* spp. (4.80 %), *Penicillium* spp. (4.53 %) *A. versicolor* spp., *Rhizoctonia* spp. (4.27 %), *A. tamari* and *Mucor* spp. (3.20 %). Aflatoxin B1 was present in twelve samples of stored maize collected from Iraqi governorate and the concentration of toxin ranged between 2.30 to 30 ppb using TLC technique and 270 to 500 ppb using ELISA technique.

Key Words: Mycoflora, Stored Maize, TLC, ELISA.

عزل وتشخيص الفطريات المرافقة للذرة الصفراء المخزونة والكشف عن Aflatoxin B1 بأستخدام تقنيتي ELISA وTLC

فادية فلاح حسن [1*]، منى حمودي الجبوري [1] ، عبد الكريم جاسم هاشم [2]
[1] قسم علوم الحياة, كلية العلوم, جامعة بغداد, بغداد، [2] قسم التقانات الأحيائية, كلية العلوم, جامعة بغداد, بغداد

الخلاصة
يعد الأفلاتوكسين B1 احد انواع السموم الفطرية والذي ينتج بشكل اساسي من الفطر *Aspergillus flavus* في الأغذية والأعلاف , ويعتبر مادة سامة مسرطنة للأنسان والحيوانات , صممت الدراسة الحالية : للتحري عن وجود الفطريات في اربعة وعشرين عينة من عينات الذرة المحلية المخزونة التي تم جمعها من بعض المحافظات العراقية و التحري عن وجود الأفلاتوكسين B1 في هذه العينات باستخدام تقنية كروماتوكرافيا الطبقة الرقيقة وتقنية ELISA. الفطريات المعزولة من عينات الذرة هي *A.flavus* بنسبة (18.57٪), *Fusarium.spp* بنسبة (12.8٪), *A.ochraceus* بنسبة (9.96٪), *A.terrus* بنسبة (9.07٪), *A.Fumigatus*, بنسبة (8.46٪), *Alternaria*. Spp بنسبة (6.4٪), *Rhizopus*. spp بنسبة (4.98٪) , *A. niger* , *A.oryzae* بنسبة (4.8٪), *Penicillium*.spp بنسبة (4.53٪), *Rhizoctonia*.spp ,*A.versicolor* بنسبة (4.27٪), *A.tamari* و *Mucor*. spp بنسبة(3.2٪). وجود الأفلاتوكسين B1 في اثني عشر عينة من عينات الذرة المخزونة التي تم جمعها من

*Email: Fadiaalhadithy@yahoo.com

بعض المحافظات العراقية وقد تراوح تركيز السم بين 30.2 الى 30 جزء بالبليون باستخدام تقنية كروماتوكرافيا الطبقة الرقيقة و 270 الى 500 جزء بالبليون باستخدام تقنية ELISA.

Introduction:

Food and feed could be contaminated by molds. These molds develop inside or around the seeds, depending on their biological make-up and Eco physiological conditions [1].

Some of these fungi are said to be toxigenic, they can synthesis one or more metabolites which become toxic for man and certain animals when ingested in large quantities. These are called mycotoxins [2].

Toxigenic fungi do not systematically produce toxins; this depends largely on environmental conditions. The level of toxicity is very variable for different animal species there are more than 200 mycotoxins produced by a variety of common fungi, some include aflatoxins, ochratoxins, citrinin, Patulin, and *Fusarium* toxins [3].

Mycotoxins have been found in homes, agricultural settings, and food; it can cause human health problems. Mycotoxins may have toxic effects ranging from short-term mucous membrane irritation to suppression of the immune system and cancer. Almost all the information related to diseases caused by mycotoxins concerns eating contaminated food. The health effects of ingesting moldy foodstuffs might include acute (immediate) and chronic (long-lasting) damage to the liver, kidneys and gastrointestinal tract [4].

Aflatoxin is produced by *Aspergillus flavus* and *Aspergillus parasiticus*. These toxins are named for the fungus producing them, e.g. "A" from the genus name *Aspergillus*, "fla" from the species name *flavus* added to toxin to give the name aflatoxin. There are several different toxins in the aflatoxin group. They are designated aflatoxin B1 and aflatoxin B2 (because they are blue under UV light), aflatoxin G1 and G2 (because they are green under UV light) and aflatoxin M1 which may be found in milk of cows fed aflatoxin contaminated feed [5].

Aflatoxin is extremely durable under most conditions of storage, handling and processing of seeds or in foods or feeds made from contaminated seeds. It is very heat stable and will withstand temperatures up to boiling. Toxin levels in maize may decline in storage, but may still be present after 7 years [6].

Aflatoxin B1 is the most carcinogenic and best studied of the compounds. The toxic effects include acute hepatitis, immunosuppression, and hepatocellular carcinoma for human and animals [6].

Many research projects which study various aspects of mycotoxin management require the analysis of grain samples at a great expense. Thin Layer Chromatography (TLC) is widely used method for screening of mycotoxins in addition to other methods routinely used for screening such as Enzyme Linked Immune Sorbent Assay (ELISA) [7].

Therefore , the main goal of this study are :- Investigate the incidence of fungal mycoflora in locally stored maize cereals collected from some Iraqi governorate , detecting the presence of aflatoxin B1 in collected maize samples using TLC and ELISA technique and compare TLC to ELISA for determining the concentration of aflatoxin B1 in maize samples.

Materials and Methods

Samples collection:

Twenty four samples of locally stored Maize were randomly collected from some Iraqi governorates local markets and silo (ten samples from Baghdad governorate , three samples from Al-Sulaimania governorate , two samples from each of Al-Anbar , Erbil and Al-Mousel governorates , one sample from each of Tikreet , Al-Basarah , Al-Muthana , Al-Kut and Diala governorates) . Two kilogram of each sample was placed in plastic bags and stored at 5°C until analysis.

Isolation of Fungi:

Twenty eight grains from each cereal sample were surface-sterilized by immersion in 2% sodium hypochlorite solution in 250 ml conical flask for 1 minute, and washed three time with sterilized distilled water, the grains were dried with sterilized filter paper in a laminar flow hood and placed on potato-dextrose agar medium containing chloramphenicol (125 mg/L) using four petri plates for each sample (7 grain / each plate). After incubation for 5 to 7 days at 25°C, the fungi which grown out from the grains were sub cultured to another medium (Czapek Solution Agar, then incubated for 5 to 7 days

at 25°C. All fungi were identified morphologically and under light microscope according to simplified key by [8-10].

Preparation of *Aspergillus flavus* Inoculums :

Spore inoculums were prepared according to the method described by [11].

Detecting the ability of *Aspergillus flavus* isolates for aflatoxin B1 production :

To test the ability of *A. flavus* isolates for aflatoxin B1 production, coconut extract medium (CEM) and aflatoxin producing ability (APA) which prepared according to [12, 13] were used.

Both media were inoculated with (10^6) spores / ml) and then stabbing by loop contaminated with spores taken from each isolates.

The plates were incubated at 28°C for 7 days (CEM medium) and for 3 days (APA medium). After incubation the plates were examined under U .V. light at 365 nm, the formation of blue zone around the colonies on CEM and APA media were indicated for aflatoxin B1 production.

Detection of aflatoxin B1 in maize samples by TLC technique:

Extraction of aflatoxin B1 from Maize samples:

The aflatoxin B1 was extracted according to the method described by [14] as follows:

Detection of aflatoxin B1 by TLC:

Thin layer chromatography (TLC) technique was employed in 20 × 20 cm aluminum plates pre coated with silica gel 60 to detect aflatoxin B1, The plates were observed under U.V light at 365 nm. Aflatoxin B1 detected as a blue fluorescent spots were determined by visual comparison with standard spot [14, 15].

Calculation of aflatoxin B1 concentration by TLC technique:

Many methods for calculation of aflatoxin B1 concentration were used, these methods are:

1- Direct detection and estimation technique [16]:

2- Determine the concentration of aflatoxin B1:

Aflatoxin B1 concentration in µg/kg was determined according to [17, 18].

Detection of aflatoxin B1 in maize extracts by Enzyme Linked Immune Sorbent Assay (ELISA) technique:

Detection of aflatoxin B1 by ELISA technique was performed using ELISA kit which supplied by Shenzhen Lvshiyuan Biotechnology company.

Determination of aflatoxin B1 concentration:

The concentration % of aflatoxin B1 in test samples was calculated using aflatoxin B1 standard curve according to the following equation:

$$\text{Percentage of absorbance value} = \frac{B}{B0} \times 100\ \%$$

B = the average OD value of the sample or the standard solution.

B0 = the average OD value of the 0 ng/ml standard solution.

Results and discussions

Mycoflora of Maize Samples:

The incidence of fungal infection of locally maize samples was presented in figure 1, Fungi recovered from maize samples, were *Aspergillus flavus* (18.57 %), *Fusarium* spp. (12.8 %), *A. ochraceus* (9.96 %), *A. terrus* (9.07 %), *A. fumigatus* (8.46 %), *Alternaria* spp. (6.40 %) *Rhizopus* spp. (4.98 %), *A. niger* and *A. oryzae* (4.80 %), *Penicillium* spp. (4.53 %), *A. versicolor* and *Rhizoctonia* spp. (4.27 %), *A. tamari* and *Mucor* spp. (3.20%).

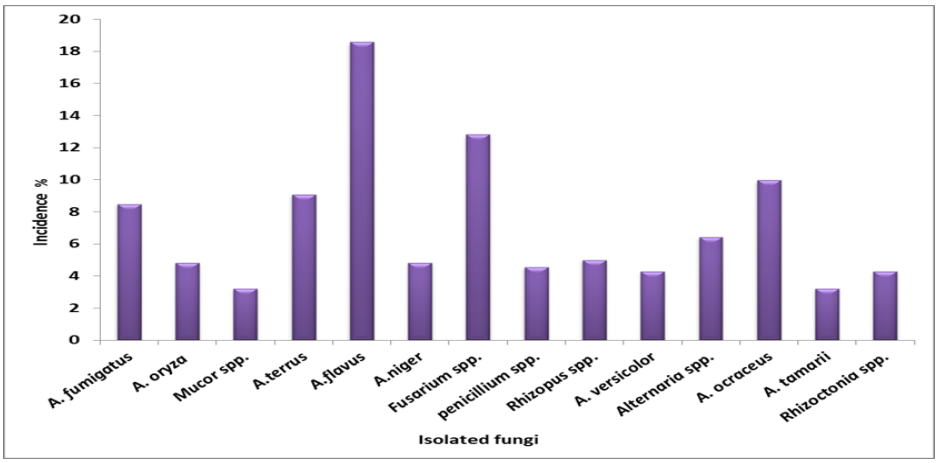


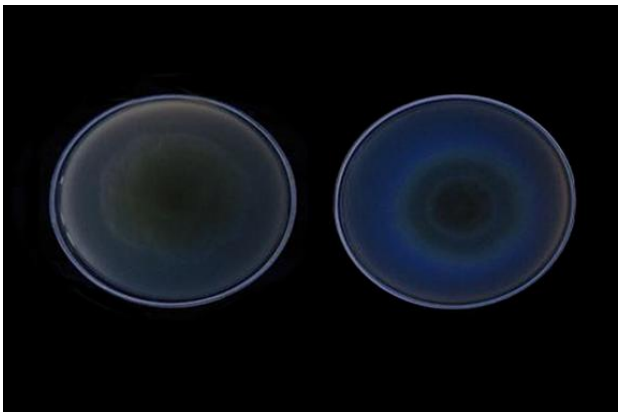
Figure 1- The incidence percentage of isolated fungi from maize Samples.

As shown in figure-1, *A. flavus* is the most prevalent among isolated fungi from maize grains followed by *Fusarium* spp., *A. ochraceus* , *A. terrus*, *A. fumigatus* , *Alternaria* spp. , *Rhizopus* spp. , *A. niger* , *A. oryzae* , *Penicillium* spp. , *A. versicolor* and *Rhizoctonia* spp. , these results agree with those of maize mycoflora in Iraq [19, 20] , Egypt [21] , Saudia Arabia [22] and in Ethiopia [23] , where *A. flavus* are prevalent between isolated fungi , and disagree with results of maize mycoflora in Nigeria [24] , and in Argentina [25] where *Fusarium* species are prevalent between isolated fungi .

The distribution *of Aspergillus* species such as *A.flavus*, *A. fumigatus*, *A. ochraceus* and *fusarium* spp. had implications for human and animal health due to their potential ability to produce mycotoxins such as aflatoxins by *A. flavus* which is primarily hepatotoxic and ochratoxin by *A. ochraceus* which is primarily nephrotoxic [4, 5]. While *fusarium* spp produce mycotoxins such as moniliformin , neosolaniol , T-2 toxin , fumonisins , deoxynnivalenol and zearalenone which the latest one is primarily oestrogenic [4, 5, 26].

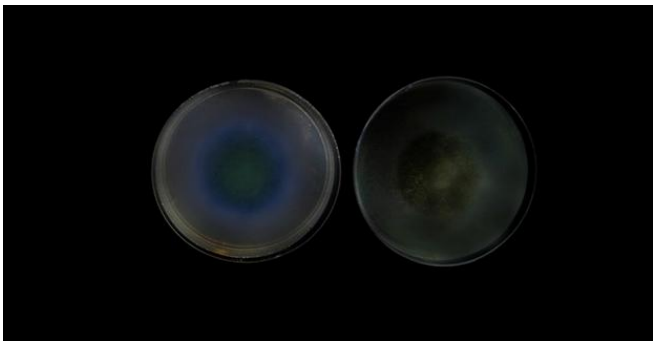
Detection of aflatoxin B1 producer isolates:

Eighteen fungal isolates of *A. flavus* were detected for their ability of aflatoxin B1 production. Fungal isolates were cultivated on CEM medium and APA medium, on CEM and APA medium the cultivated plates were observed under U.V. light at 365 nm, the producer isolates were formed a blue zone around the colonies, figure-2, 3



A **B**

Figure 2- Growth of *A. flavus* on CEM medium at 28°C for 3 days.(A): Aflatoxin B1 non producer isolate. (B): Aflatoxin B1 producer isolate.



A **B**

Figure 3- Growth of *A. flavus* on CEM medium at 28°C for 7 days.(A): Aflatoxin B1 non producer isolate. (B): Aflatoxin B1 producer isolate.

As shown in figure-2 and figure-3, aflatoxin B1 producer isolates were cultivated on CEM and APA media formed a blue zone around the colonies under U.V. light at 365 nm, these results accordance with finding of [27- 31].

Detection of aflatoxin B1 in maize samples by TLC Technique:

Twenty four samples of locally stored Maize which obtained from different Iraqi governorates (ten samples from Baghdad governorate , three samples from Al-Sulaimania governorate , two samples from Al-Anbar , Erbil and Al-Mousel governorates , one sample from Tikreet , Al-Basarah , Al-Muthana , Al-Kut and Diala governorates) were analyzed for aflatoxin B1 content (quantitatively) using TLC technique . Twelve sample of maize (six samples from Baghdad governorate , one sample from Al-Anbar governorate , Al-Sulaimania , Erbil , Al-mousel , Al-Kut and Diala governorates) were contained aflatoxin B1 when examined under U.V. light , the positive results were detected as a blue fluorescent spots and had been determined by visual comparison with standard spot.

Our results revealed that (Rf) of aflatoxin B1 for both maize samples and standard aflatoxin B1 were (0.25) when observed under U.V. light at 365nm .The result showed that twelve samples of maize were contained aflatoxin B1 using TLC samples, and the concentration of aflatoxin B1 in these samples ranged from 2.30 ppb to 33pbb, table-1.

In addition to above-mentioned results, It has been shown that not all the samples which contained aflatoxin B1 to contain *A. flavus*, table-2, on the other hand some of the samples were contained *A. flavus* without the existence of aflatoxin B1 (*A. flaves* isolates were isolated from these samples gave a negative result on both CEM and APA media.

Table 1- Quantitative determination of aflatoxin B1 in some Maize collected samples by TLC technique.

Maize samples	AflatoxinB1 concentration/ppb
Baghdad 1	2.33
Baghdad 2	33.00
Baghdad 3	10.05
Baghdad 6	13.07
Baghdad 7	17.43
Baghdad 8	2.30
Al-Anbar 1	8.88
Al-Sulaimania3	21.99
Erbil 2	4.74
Al-Mousel 1	9.62
Al-Kut	8.30
Diala	5.26

Table 2- Correlation between the presence of aflatoxin B1 and *A. flavus* in maize samples, (+) presence and (–) absence of aflatoxin B1 and *A.flavus* from maize samples.

Samples collected from some Iraqi	Aflatoxin B1	*A.flavus*
Baghdad 1	+	+
Baghdad 2	+	+
Baghdad 3	+	–
Baghdad 4	–	+
Baghdad 5	–	+
Baghdad 6	+	+
Baghdad 7	+	+
Baghdad 8	+	–
Baghdad 9	–	–
Baghdad 10	–	+
Al-Anbar 1	+	+
Al-Anbar 2	–	+
Al-Sulaimania 1	–	–
Al-Sulaimania 2	–	+
Al-Sulaimania 3	+	+
Erbil 1	–	+
Erbil 2	+	+
Al-Mousel 1	+	+
Al-Mousel 2	–	+
Tikreet	–	–
Basrah	–	–
Al-Muthana	–	+
Al-kut	+	+
Diala	+	+

We can conclude from the obtained results, that the apparently healthy grains may possible be infected in the field before harvest , and some fungi lose their vitality after seeds drought , but their secondary metabolites (mycotoxins) still remain in those seeds. While some times the grains contain fungi but free of mycotoxins , and this may be due to that the condition not available for fungi to produce mycotoxins or the reason may be genetically.

Detection of aflatoxin B1 in maize samples by Enzyme Linked Immune Sorbent Assay (ELISA) technique:

Twenty four samples of locally stored Maize were analyzed for aflatoxin B1 content (quantitatively) using ELISA kit, the extracted samples, aflatoxin B1 enzyme conjugate and aflatoxin B1 Antibody working solution were mixed and added to micro well. On removal of non-specific reactants, substrate (A & B) were added, and the microwells measured optically using microplate reader at 450 nm for yellow color or at 650 nm for unstopped blue color to determine the OD value.The result showed that twelve samples of maize were contained aflatoxin B1, and the concentration of aflatoxin B1 in these samples ranged from 270 ppb to 500 pbb, table-3.

Table 3- Quantitative determination of aflatoxin B1 in some maize collected samples by indirect ELISA technique.

Maize samples	Aflatoxin B1 concentration / ppb
Baghdad 1	280
Baghdad 2	500
Baghdad 3	440
Baghdad 6	450
Baghdad 7	480
Baghdad 8	270
Al-Anbar 1	400
Al-Sulaimania 3	470
Erbil 2	320
Al-Mousel 1	440
Al-Kut	440
Diala	330

The results of the present study show that the concentration of aflatoxin B1 in examined maize samples ranged from 2.30 ppb to 33 ppb and 270 ppb to 500 ppb using TLC and ELISA technique respectively , while [32] clarified that in parts of India 100% of maize samples have been found contaminated with aflatoxin B1 in the range of 6250 ppb – 15600 ppb , the results were obtained by [33] showed that the concentration of aflatoxin B1 in maize samples ranged from 25 ppb to 750 ppb , results by [34] showed that the maximum concentration of aflatoxin B1 in maize samples was 0.1 ppb , while [35] showed the that the concentration of aflatoxin B1 ranged from 0.2 ppb to 770 ppb in examples of market maize samples collected from some countries , [36] reached a results that the maximum concentration of aflatoxin B1 in maize was 0.99 ppb, [37] , found that concentration of aflatoxin B1 ranged from 45ppb to 62 ppb , while the concentration of aflatoxin B1 in maize samples were determined by [38] ranged from 5 ppb to 27 ppb .

The concentration of aflatoxin B1 in maize samples detected by TLC technique lower than the concentration of aflatoxin B1 in maize samples detected by ELISA technique. TLC technique was failed to measure toxin concentration as ELISA because the TLC technique estimate samples concentration by comparison to standards, the concentration determined by TLC can only be as precise as the number of standard used, and therefore the measured concentration was dependent upon the concentration difference between standards [7]. Our results were in accordance with results obtained by [39] which revealed that the concentration of aflatoxin B1 in naturally contaminated groundnut samples detected by TLC technique were ranged from 3.6-4.0 ppb, while using of ELISA technique showed that the concentration of aflatoxin B1 were ranged from 40.9-80.2 ppb , [7] showed that the comparison of TLC technique with ELISA technique were gave a very good agreement for determination of some *fusarium* toxins in some grains samples, [40-42] ; reached to results including detection of ochratoxin A in barley, malt and beer using TLC technique and ELISA techniche , the concentration of ochratoxin A in barley were 10 ppb and 1 ppb using TLC and ELISA respectively , while the concentration of ochratoxin A in malt were 0 ppb and 1ppb using TLC and ELISA respectively , the concentration of ochratoxin A in barley were 0ppb using both TLC and ELISA .

U.S Food and Drug Administration (FDA) were determined the action levels for aflatoxin-contaminated maize , the action levels were 20 ppb in animal feed and feed ingredients intended for dairy, immature poultry, and stressed animals ; 20 ppb for Human consumption ; 100 ppb in grain intended for breeding cattle, breeding swine, and mature poultry (such as laying hens or breeding birds) ; 200 ppb in grain intended for finishing swine of 100 pounds or greater and 300 ppb in grain intended for finishing beef cattle [43]

According to FDA´s action levels for aflatoxin-contaminated maize which mentioned above, and comparing with our results, we can conclude disqualification of the most of maize samples for human and animal's consumption due to the concentration of aflatoxin B1 in these samples which exceeded the allowable limit.

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