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Investigation of fungi isolated from stored yellow maize (*Zea mays* L.) in different regions of Iraq, and evaluation of aflatoxin B1 production by *Aspergillus flavus* isolates

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Abstract:

Stored yellow maize (*Zea mays* L.) provides a favorable substrate for fungal colonization, particularly under warm and humid conditions, which can lead to contamination with toxigenic species and aflatoxins. This study aimed to survey the mycobiota associated with stored yellow maize grains from different regions of Iraq and to assess the aflatoxin B1(AFB1)–producing potential of *Aspergillus flavus* isolates. Fungi were isolated and identified based on their macroscopic and microscopic characteristics using standard taxonomic keys. The fungal community was dominated by *Aspergillus* spp. 31.2% , followed by *Penicillium 20.9%*, *Fusarium* 18.7% and Alternaria 17.6% whereas Trichoderma and *Rhizopus* were less frequent 7.5% and 4.1% respectively. Isolates of *A. flavus* were subsequently screened for AFB1 production using thin-layer chromatography, and toxin levels in representative isolates were quantified by high-performance liquid chromatography. Aflatoxin B1 production was confirmed in a high proportion of the *A. flavus* isolates, and some strains exhibited relatively elevated AFB1 concentrations reaching 126.9 ppb and 112.6 ppb. Overall, these findings indicate a substantial prevalence of potentially toxigenic *A. flavus* in stored maize in Iraq and highlight the need for improved post-harvest handling, better storage conditions and regular monitoring of aflatoxin contamination to safeguard food and feed safety.

Keywords: *Zea mays* , *Aspergillus flavus*, Aflatoxin B1, Stored maize, TLC , HPLC.

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Introduction:

Grains are considered a staple food and represents a major source of energy and nutrients for humans and animals. Cereal crops such as corn, wheat and rice are grown in larger quantities globally than any other agricultural commodity. Cultivation and management of these crops are crucial to global food security (Farooq *et al*., 2023; Yamini et al., 2025). However, climate change and the emergence of pathogens can compromise the sustainable production of these crops (Lahlali *et al*., 2024). Throughout the production chain, grains are exposed to many sources of contamination, including fungi and bacteria, fungi are common agents of food and feed contamination, leading to deterioration of quality and nutritional properties (Wu *et al*., 2014; Ochieng *et al*., 2021). This subsequently leads to the production of mycotoxins especially in yellow corn as a result of their ideal composition of nutrients needed by the fungus. Many fungal species such as *Aspergillus* , *Alternaria*, *Penicillium* ,*Fusarium* and *Claviceps* are known to cause infections and produce mycotoxins in a wide range of plants and cereal crops (Lahlali *et al*. 2024; Haider and Hussein 2022). Thes toxic secondary metabolites pose significant health risks to humans and animals, so the prevalence of mycotoxins is a global concern necessity effective detection and mitigation strategies. Morphological characteristics used for identification involves macroscopic features like colony diameters, elevation, margins, texture and color as well as microscopic features such as conidia and conidiophore vesicles appearance and hyphae shape and size (Okayo *et al*.,2020).

Different *Aspergillus* species demonstrate rapid growth on a wide range of culture media, forming thick, velvety to cottony colonies. Colony color is species-dependent and is also influenced by medium composition. *A. flavus* typically produces yellowish-green to olive-green, granular colonies due to abundant conidia, whereas *A. niger* forms dense black colonies as a result of heavy conidial production and melanin synthesis (Mangal *et al*., 2014; Gherbawy *et al*., 2021). *A. fumigatus* usually develops blue-green to gray-green colonies with a velvety surface (Diba *et al*., 2007; Siqueira, 2017). The reverse side of *Aspergillus* colonies can show colors ranging from yellow to brown, which may assist in species differentiation
(Sugui *et al*., 2015; Suleiman, 2023).

A. flavus is of particular concern because it produces aflatoxins that pose serious health risks to humans and animals (Luis *et al*., 2020; Okayo *et al*., 2020; Faye *et al*., 2022; Saber *et al*., 2022). This species can form sclerotia that develop into stromata during sexual reproduction, starting from hyphal aggregation and leading to ascospore formation

B. (Zen , 2017; Cherif *et al*., 2022).

Materials and methods:

Collection of yellow corn samples:

Zea mays sample were collected from 3 different regions representing the governorates of Iraq (Northern Region, Central Region, Southern Region) at harvest time with a weight of 1 kg/sample. The random method was adopted in taking samples from several locations in each governorate of the study, each of which represents a location with a different environment and climatic conditions from the other location. 9 bags were collected with three replicates for each location. Information was recorded (sample collection date and collection area). It was brought to the laboratory and stored at the laboratory temperature until the tests were conducted, 200 seeds were taken from each sample and superficially sterilized by immersing them in 1% sodium hypochlorite solution, shaking gently for 2 minutes. The seeds were then washed three times with sterile deionized water and dried with sterile filter paper. Potato dextrose agar (PDA) was prepared according to the manufacturer's instructions and autoclaved at 121 °C for 20 min. After cooling to about 45 °C, tetracycline was added to a final concentration of 500 mg/L, and the medium was poured into 9 cm Petri dishes. Five seeds were transferred to each Petri dish using sterile forceps under sterile conditions. The dishes were incubated in an incubator for 7 days at 25°C. After the incubation period, the fungi were identified based on morphological characteristics according to the approved taxonomic keys (Klich Hocking Pitt, 2002, 2009). The percentage of frequency and occurrence were recorded.

Evaluation the ability of *A. flavus* isolates to produce aflatoxin in yeast extract–sucrose (YES) broth

Aflatoxin production by *A. flavus* isolates was evaluated in yeast extract–sucrose (YES) broth prepared by dissolving 20 g yeast extract and 200 g sucrose in 1 L distilled water. The medium (100 mL) was dispensed into 250 mL Erlenmeyer flasks and autoclaved at 121 °C (1.5 kg/cm², 20 min). After cooling, flasks were inoculated with four 0.5 cm mycelial plugs from actively growing cultures, with three replicate flasks per isolate, and incubated at 25 ± 2 °C for 21 days (Davis et al., 1967; Yousaf et al., 2017).

The resulting cultures were then used for aflatoxin extraction.

Aflatoxin was extracted from ground maize samples according to the AOAC (2005) method with slight modifications. Briefly, 25 g of dried, milled sample were mixed with equal volumes of methanol and chloroform and shaken for 60 min. The extract was filtered and subjected to successive liquid–liquid partitioning using n-hexane and 90% methanol in a separatory funnel. The methanolic phase was collected and evaporated to dryness in a water bath, and the residue was re-dissolved in chloroform. The chloroform layer was washed twice with distilled water, dried over anhydrous sodium sulfate, filtered and finally evaporated to dryness to obtain the aflatoxin extract.

Aflatoxin B1 was analyzed by thin-layer chromatography (TLC) on silica gel 60 F_{254} glass plates (20 × 20 cm; Sigma), activated at 120 °C for 15 min. Sample extracts (10 µL) and an AFB_1 standard solution (5 µL; Lab Standard) were spotted onto the plates using a microsyringe. Development was carried out in a chloroform:methanol (3:97, v/v) mobile phase in a glass chamber until the solvent front reached approximately 2 cm from the upper edge. Plates were air-dried at room temperature and examined under UV light at 370 nm in a UV viewing cabinet (AOAC, 2005).

Determination of aflatoxin concentration by high-performance liquid chromatography (HPLC):

Aflatoxin B1 concentration in the fungal extracts was determined by high-performance liquid chromatography (HPLC were carried out at the Environment and Water Directorate, Ministry of Science and Technology (Iraq), using a SYKAM HPLC system. following the method of Liu *et al*. (2012). Separation of AFB1 was performed on a C18-ODS column (25 cm × 4.6 mm). The mobile phase consisted of acetonitrile:distilled water (70:30, v/v), delivered at a flow rate of 0.7 mL/min. Detection was carried out using a fluorescence detector with an excitation wavelength of 365 nm and an emission wavelength of 445 nm.

Results and Dissection:

The results of isolation and morphological identification of fungi associated with yellow corn samples collected from several Iraqi governorates showed the presence of six main genera of fungi with varying frequencies. *Aspergillus* spp. was the most common, accounting for 31.2% of the total isolates, followed by *Penicillium* spp. at 20.9%, *Fusarium* spp. at 18.7%, and *Alternaria* spp. at 17.6%. *Trichoderma* sp. and *Rhizopus* sp. were recorded at lower frequencies of 7.5% and 4.1%, respectively.as shown in table (1).

Table1 :Frequency of fungal species associated with yellow maize kernels.

Fungi	Frequency ratio (%)
Aspergillus spp.	31.2 %
Penicillium spp.	20.9 %
Fusarium spp.	18.7 %
Alternaria spp.	17.6 %
Trichoderma sp.	7.5 %
Rhizopus sp.	4.1 %

These findings confirm that the genus *Aspergillus* spp. represents the predominant fungal component in stored maize grains, recording the highest frequency compared with other fungal genera. This is consistent with numerous local and international studies that have reported the wide distribution of *Aspergillus* spp. in stored yellow maize grains under similar environmental conditions. This predominance is also attributed to the fact that yellow maize grains provide a highly suitable nutritional substrate for the growth of these fungi (Abe *et al*., 2015; Haider and Hussein, 2022).

In particular, the high frequency of *Aspergillus flavus* is of special concern, as its widespread occurrence is linked to its strong preference for the nutritional components of yellow maize grains, which contain high levels of carbohydrates and sugars. This confers a high growth capacity and enables the fungus to proliferate over a wide range of temperatures (11–45 °C). Previous studies have also demonstrated the ability of this fungus to tolerate elevated temperatures, as well as fluctuations in moisture levels within the grains, and to survive under conditions of drought and reduced oxygen concentration. Moreover, *A. flavus* is capable of producing large numbers of easily disseminated spores, which together provide it with a strong competitive advantage, allowing it to dominate the grains and exploit their nutritional content. This, in turn, exacerbates the risk associated with its production of mycotoxins (Bennett and Klich, 2003; Rushing and Selim, 2019). By contrast, the lower frequency of *Aspergillus fumigatus* in samples from the central and southern regions may be attributed to its requirement for higher moisture levels or to its relatively limited presence in the storage environment compared with other species (Zhou *et al*., 2023).

During the isolation process, it was observed that one of the samples collected from the northern region, specifically from Nineveh (Mosul) Governorate, was almost completely contaminated with *Rhizopus* spp. This may be due to the biological nature of this genus, which is characterized by

extremely rapid growth and a high capacity to rapidly consume available nutrients, thereby conferring a strong competitive advantage over other fungal genera. Furthermore, the requirement of *Rhizopus* spp. for high humidity and moderate temperatures may indicate that the grains were exposed to storage conditions with elevated moisture levels, which are considered ideal for its growth and for outcompeting other fungi (Samson , 2011 ; Pitt and Hocking, 2009).

Table 2: Fungi associated with yellow maize kernels, their macroscopic and microscopic characteristics, and their frequency and incidence.

Fungal species	Microscopic & Macroscopic description	Frequency (%)
Aspergillus flavus	A cottony growth with a yellowish-green color was observed, and microscopic examination revealed spherical conidia (conidiospores) with a regular, rounded shape. The conidium-bearing cells (phialides) were arranged in a biseriate pattern at the apex of the conidiophore (Gonu, 2015).	23.3
Aspergillus niger	The colony is characterized by a dark black color and dense growth, with the center becoming progressively darker as it ages. Microscopically, the colonies are distinguished by large conidial heads covered with numerous small, black conidia. The microscopic images also revealed dense black conidia with a spiny (echinulate) surface (Matsumoto, 1991).	13.4
Aspergillus fumigatus	The fungus forms greyish-green colonies, and the centre of the colony becomes more compact as growth progresses. The conidia are arranged in a monophialidic pattern. Microscopic images showed hemispherical conidia and clearly revealed the arrangement of the single phialides (Sugui,*et al.*, 2015).	3.4
Penicillium spp.	The fungus is characterized by dense, cottony growth with a blue-green coloration. Microscopic examination revealed brush-like conidial structures arising from multiple branched phialides, and the micrographs showed a distinctly radial arrangement of the conidia (Pitt, 2000).	20.1
Alternaria spp.	The colonies exhibited a dark to black coloration with a woolly appearance. Microscopically, the conidia appeared oval to elongate and were divided by both transverse and longitudinal septa (muriform), giving them a multi-celled appearance. The micrographs showed well-defined, distinctly septate (multicellular) conidia (Simmons, 2007).	13.1

Fusarium spp.	The fungus forms rapidly growing, white, cottony colonies that gradually turn pink, violet, or orange. Under microscopic examination, the macroconidia appear fusiform, curved and sickle-shaped, while the smaller microconidia are oval in shape and borne on short phialides. Thick-walled chlamydospores are produced singly or in pairs on the hyphae. The micrographs clearly show both macroconidia and microconidia (Leslie and Summerell, 2006).	16.7
Trichoderma spp.	The fungus forms rapidly growing colonies with a light green to yellowish coloration. Microscopic examination revealed small, globose conidia produced on highly branched fungal hyphae, and the micrographs showed a dense network of conidiophore branches bearing these conidia (Gams and Anderson, 2007).	6.7
Rhizopus spp.	The fungus is characterized by white, cottony growth that turns black at maturity as a result of spore formation. Microscopic examination revealed the presence of sporangiophores bearing spherical spores (Gryganskyi *et al*., 2018).	3.3

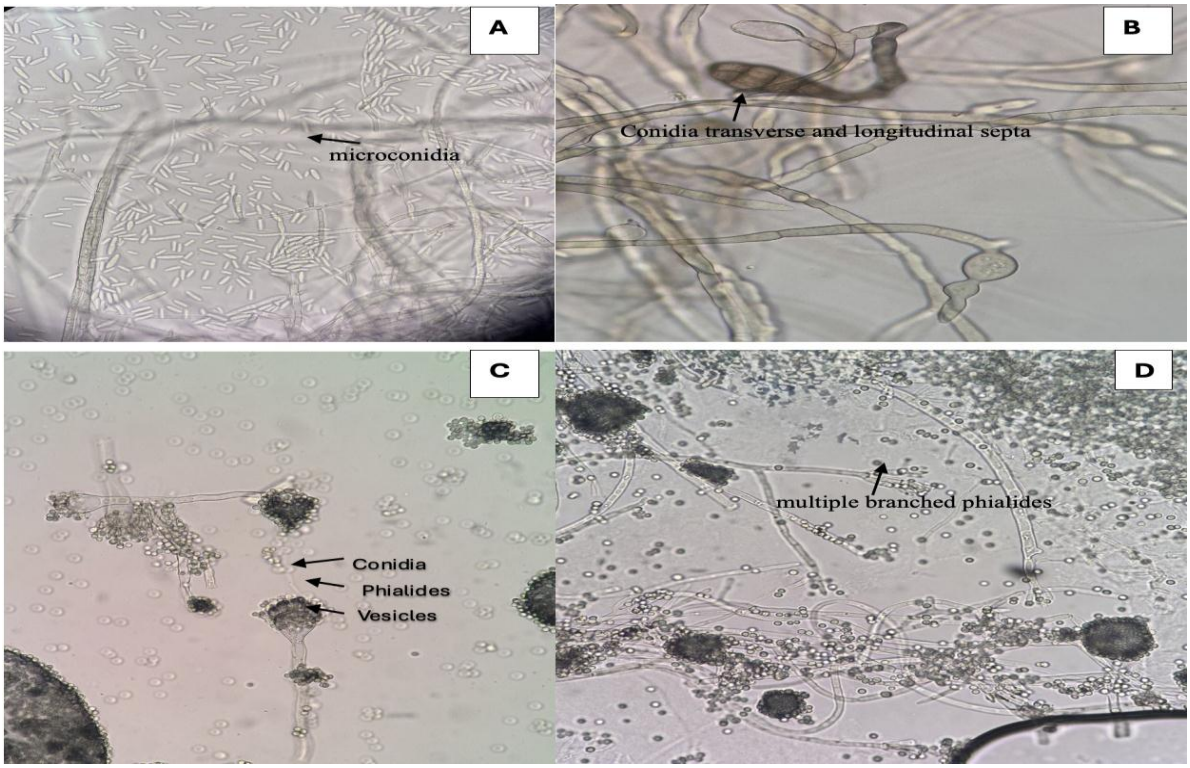

Figure 1: Microscopic description of isolates (A) Fusarium spp. (B) Alternaria spp. (C) Aspergillus spp. (D) Pencillum spp.

Detection of Aflatoxin Using Thin-Layer Chromatography (TLC) Plates

TLC was used to verify aflatoxin synthesis of aflatoxin B 1(AFB1) using isolates of Aspergillus flavus by extracting them. They were detected using ultraviolet light with wavelength 365nm. The blue fluorescent spots were observed on the chromatogram at the retention factor (RF) of the spots corresponding to the AFB 1 reference and, therefore, confirmed the toxigenic ability of the isolates under examination. Aflatoxin was detected in 18 out of 25 of the isolates, implying that 72 % of the purified isolates could produce toxins. Isolate no. (8) had the highest toxicity level implying there are highly aflatoxigenic strains in the samples of the northern region and proved the circulation of the high-producing aflatoxigenic strains in the northern region. The isolates in the central area were mainly positive, and the level of toxicity was between the range of (+) to (+++) thus showing that the majority of the isolates were toxin-producing, although at moderate levels as compared to the southern area. In the latter, only one isolate (no.13) had a ++++ toxicity level, which indicates that there are possibly dangerous isolates in mycotoxin production.

Table 3: Efficiency of fungal isolates in producing aflatoxin B1.

Isolate ID	Presence/absence of toxin	Isolation region
1	+	North regoin
2	-	Middle region
3	++	North regoin
4	-	South region
5	+++	North regoin
6	++	North regoin
7	+	Middle region
8	+++++	North regoin
9	-	North regoin
10	+	Middle region
11	++	South region
12	+	Middle region
13	++++	South region
14	+	Middle region
15	-	South region
16	-	South region
17	-	North regoin

18	++++	North regoin
19	++	South region
20	+	Middle region
21	+++	South region
22	++	Middle region
23	++	North regoin
24	+++	North regoin
25	-	South region
(-) no toxin detected (+)low toxicity (++) moderate toxicity (+++) High toxicity (++++) very high toxicity (+++++) highest toxin production		

The high proportion of toxin-producing isolates, particularly in the northern region, may be attributed to environmental conditions that induce the expression of genes responsible for mycotoxin biosynthesis (Amaike and Keller, 2011), in addition to delayed harvest, poor storage practices, and prolonged exposure of grains to humid conditions that favor fungal growth and toxin production. Furthermore, genetic variability among isolates may explain why certain strains exhibit a greater capacity for toxin production, which accounts for the occurrence of highly toxigenic isolates (++++) in both the northern (isolate 8) and southern (isolate 13) regions.

Quantification of Aflatoxin by High-Performance Liquid Chromatography (HPLC)

The results of the sample using High performance liquid chromatography demonstrate the retention time values of the detector responses of 25 -985.98 and 10 -254.90 -MAU/s at retention times of 4.35 and 5.80 respectively showed that the isolate (8) in the northern region had at least two chromatographic peaks. The aflatoxin concentration of this isolate was the highest one, it was 127.9 ppb. Isolates (13), in turn, of the southern area had 4.37 and 5.85 min peaks with the corresponding areas of 28225.65 and 11224.08 mAU s. Its aflatoxin level was 112.6 part per billion, which is 8.9 per cent higher compared to the isolate of the north. The HPLC profiles are represented in figure 2. The findings are in line with the earlier source suggesting that the storage facilities and the moisture present in harvests do affect aflatoxin levels (Amaike and Keller, 2011; Gnonlonfin et al., 2013).

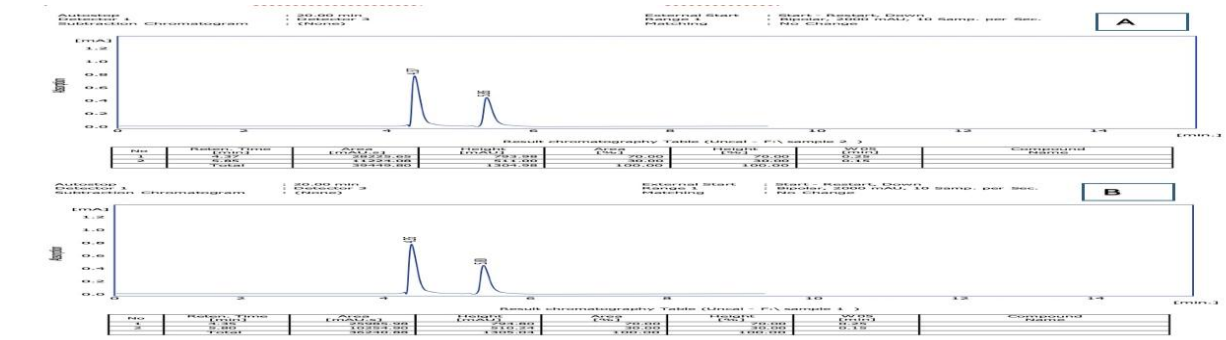

Figure 2: HPLC chromatograms showing AFB1 concentration in isolate (3) (A) and isolate (13) (B).

Conclusion:

This study has revealed that stored grains of yellow maize in various parts of Iraq have a rich mycobiota with *Aspergillus spp*. representing the most common genus of fungi. The commonest species isolated was *A. flavus* reflecting its strong ecological adaption to maize as good nutrient source and to local storage conditions. Morphological identification together with TLC screening discovered that a substantial number of *A. flavus* isolates were capable of producing aflatoxin B 1 and the presence of AFB 1 at a level of concern was confirmed in some representative northern and southern isolates using HPLC. These results emphasize the possibility of aflatoxin contamination in post-harvest stored maize in sub-optimal post- harvest handling and storage. The findings highlight the necessity of constant monitoring of fungal levels and mycotoxins in maize and the introduction of better storage conditions and risk prevention measures to establish food and feed safety.

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مستخلص :
تمثل حبوب الذرة الصفراء المخزونة (*Zea mays* L.) بيئة غذائية ملائمة للفطريات، ولا سيما تحت الظروف الدافئة والرطبة، مما يزيد من احتمالية تلوثها بالفطريات السامة وإنتاج الافلاتوكسينات. هدفت هذه الدراسة إلى مسح الفطريات المرافقة لحبوب الذرة الصفراء المخزونة في مناطق مختلفة من العراق، وتقويم القدرة الإنتاجية لعزلات *Aspergillus flavus* على إنتاج سم الافلاتوكسين B1 (AFB1) جرى عزل الفطريات وتشخيصها اعتماداً على الصفات المظهرية والصفات المجهرية بالاستعانة بالمفاتيح التصنيفية القياسية. أظهرت النتائج أن المجتمع الفطري كان مهيمناً بشكل رئيسي بأجناس *Aspergillus* 31.2%، تلاه *Penicillium* 20.9% و*Fusarium* 18.7% و Alternaria 17.6% في حين كان Trichoderma وRhizopus أقل بنسبة 7.5% و4.1% على التوالي. وتم بعد ذلك فحص عزلات *A. flavus* للكشف عن قابليتها على إنتاج الافلاتوكسين B1 باستعمال تقنية كروماتوغرافيا الطبقة الرقيقة(TLC) ، في حين اجري تقدير تراكيز السم في عدد من العزلات المنتخبة باستعمال كروماتوغرافيا السائل عالية الأداء (HPLC) وأكدت النتائج أن نسبة مرتفعة من عزلات *A. flavus* كانت قادرة على إنتاج الافلاتوكسين B1 ، إذ أظهرت بعض السلالات مستويات مرتفعة نسبياً من السم بتركيزات تصل إلى 126.9 ppb و112.6 ppb. وبصورة عامة، تشير هذه النتائج إلى وجود انتشار ملحوظ لعزلات *A. flavus* ذات القدرة السمية في حبوب الذرة الصفراء المخزونة في العراق، وتسلط الضوء على الحاجة إلى تحسين ممارسات ما بعد الحصاد وظروف الخزن، واعتماد برامج رصد منتظم لتلوث الافلاتوكسين بهدف تعزيز سلامة الغذاء والعلف.

الكلمات المفتاحية : الذرة الصفراء، Aspergillus Flavus، الافلاتوكسين B1، الذرة المخزونة ، HPLC، TLC
ملاحظة : هل البحث مستل من رسالة ماجستير او اطروحة دكتوراه ؟ نعم : ✓ كلا :