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Investigation of fungi accompanying wheat grains at wheat cultivation areas in Iraq and the possibility of diagnosing it

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Abstract

The aim of this study was isolating and diagnosing the fungi accompanying the harvested wheat grains for the 2021 agricultural season, at some Iraqi provinces: Erbil, Sulaymaniyah, Mosul, Najaf, Karbala, Qadisiyah, Muthanna, ThiQar and Basra. The results of the field survey showed the accompanying fungi *Alternaria* sp, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium sp*, *Mucor*, *Penicillium sp*, *Rhizopus sp* and other fungi. The results confirmed that the two genus, *A. flavus* and *Alternaria sp*, were the most prominent in all Iraqi provinces, with mean of 35.84 % for *A. flavus* and 25.36 % for *Alternaria sp*, followed by *A. niger*, and then both the *fungi Fusarium sp*, *Rhizopus sp*, *Penicillium sp* and *Mucor*, with mean 20.008, 5.20, 4.61, 2.20 and 0.63%, respectively. The incidence of other undiagnosed fungi was 6.09%, the genus *A. niger* recorded the highest occurrence in the provinces of ThiQar and Mosul with 45 and 40%, respectively, it was less visible in Erbil by 2.73%. It was also noted that the genus *Fusarium sp* recorded its highest occurrence in the provinces of Erbil and ThiQar with a percentage of 10.95 and 10%, while it was not recorded in the holy province of Karbala, the percentage of its occurrence varied in the rest of the provinces. The fungus *Mucor sp* recorded 2.94, 2.5, 1.4 and 1.35% at the provinces of Qadisiyah, ThiQar, Sulaymaniyah and Muthanna, respectively, while it was not recorded in the rest of the provinces. The genus *Penicillium sp* recorded occurrences in some Iraqi provinces by 5.47% in Erbil province and 4.83 and 4.41% in Karbala and Qadisiyah provinces, respectively, the percentage of his occurrence was 1.4% in Sulaymaniyah province and 1.26 in Najaf. The rest of the provinces did not record his occurrence. As for the genus *Rhizopus sp*, it appeared on wheat grains in most of the Iraqi provinces in different proportions, the highest percentage of its occurrence was in Basra Province, with 11.11%, did not record his occurrence in the province of Qadisiyah, while the results showed that *A. flavus* was the most frequent fungus at the level of the provinces under study, with a frequency of 22.77%. The fungus *Alternaria sp* ranked second with a frequency of 16.22%, where it was observed that the highest frequency was for *A. flavus* in the provinces of Muthanna and Najaf Al-Ashraf by 43% and 34%, respectively.The process of

molecular diagnosis using polymerase chain reaction (PCR) technology for the most frequent isolate showed that it belongs to the genus *Aspergillus flavus*, it showed a 90% concordance with global isolates. The nucleotide sequence of the isolate has been deposited in GenBank under accession number ON932490.

Keywords: Wheat, fungi, diagnosis, frequency, emergence

Introduction

Wheat *Triticum aestivum* L. is one of the most important cereal crops as it is the most important source of food, which contributed to ensuring food security for the world's population **[**1,2]. Wheat is the staple food as it is a cheap source of energy for the body, its importance as a food is due to the fact that it contains good proportions of nutritional components such as protein, as it ranges in baked wheat 11.3 gm of protein, fats, carbohydrates, fiber, salts, vitamin B1 and B2, pantothenic acid, folic acid and tocopherols, it is included in a wide range of products of economic interest [3]. Wheat is one of the basic foods that the need for it increases with the increase in the population, as studies indicated that the world's population in the year 2020 needs one billion tons of wheat, maize and rice constitute 75% of the world's cereal production [4, 5]. Wheat production in Iraq for the winter season of 2021 is estimated at 4234 thousand tons, with an estimated decrease of 32.1% from the year 2020, which was estimated to produce 6,238 thousand tons, the cultivated area throughout Iraq was estimated at 9464 thousand dunums for the winter season 2021 [6]. Cereal crops are exposed to various pathogens in the field or during transportation and storage, the fungi transmitted is one of the most important pathogens on wheat, leads to poor germination and poor quality of grain, as well as its production of mycotoxins [7, 8]. Toxigenic fungi that produce mycotoxins such as aflatoxin B1 (AFB1) grow naturally on various foods [9]. It constitutes the problem of food contamination and its effects on humans and animals, as a result of consuming food contaminated with fungi or its toxins, it is one of the most important global problems, especially in the field of nutrition, and it has serious health risks represented in the many effects that appear on living organisms, including severe effects that show clear symptoms of disease that lead to the death of infected animals and have carcinogenic effects on humans, aflatoxin B1 is the main cause of liver cancer [10]. The conditions that promote mycotoxin production are genetically controlled and dependent on fungal species, pH, substrate, relative humidity, temperature, and incubation time [11].

Therefore, the study aimed to isolate and diagnose the fungi associated with locally grown wheat grains harvested in the same season.

Materials and Methods

Sample collection

Wheat grain samples were collected from different regions of the provinces of Iraq (Erbil, Sulaymaniyah, Mosul, Najaf, Karbala, Diwaniyah, Muthanna, ThiQar and Basra), during the harvest period, the weight of 4 kg / sample. The random sampling

method was adopted with three replicates for each site, recorded collection information, collection date and location, the sample was collected and brought to the laboratory and kept at laboratory temperature**.**

Isolation and investigation of fungi associated with wheat grains:

400 grains of wheat were taken from each sample, superficially sterilized by immersion in 1% sodium hypochlorite solution, with a soft shake for two minutes, after that, the grains were washed three times with sterile distilled water, then dried with sterile filter papers, transfer 10 grains to each Petri dish containing the prepared PDA culture media by sterile forceps and under sterile conditions, the dishes were incubated in the incubator for 7 days at a temperature of 25°C, after the incubation period, the fungi were diagnosed according to their phenotypic characteristics according to the approved taxonomic keys [11,12]. The percentage of frequency and occurrence was recorded to determine the most frequent and appearing mushrooms according to the two equations:

$$Occurrence = \frac{\text{Fungus isolates No.}}{\text{Total fungi isolates No.}} \times 100$$

$$Frequency = \frac{\text{Genus contain sample No.}}{\text{Total sample No.}} \times 100$$

[14]**.**

Purification of *Aspergillus flavus* isolates

After diagnosing the fungi accompanying wheat grains phenotypically and calculating the percentage of frequency and occurrence, the isolates of *A. flavus* were purified by transferring the tip of the fungal hyphae using a sterile isolation needle, from all plates and samples in which the fungus appeared to plates containing PDA media, incubated at 25°C ±2 for 7 days, diagnosed based on phenotypic traits, according to the taxonomic keys [12,13]. the most frequent fungi were partially diagnosed by PCR technology.

Molecular diagnosis of the most frequent isolate of *Aspergillus flavus*

After the morphological diagnosis was made to isolate the fungus, the most frequent and the most toxin-producing, a single spore was taken from the colony, they were grown on PDA media and sent to Al-Musayyab Bridge Company at 7 days of age for molecular diagnosis.

DNA extraction

A. The sample was prepared by taking a small piece of the fungal colony with a range of (500-100) mg, placed in a small tube Eppendrof tube size 1.5 ml, add to it (100 ml) Gp1 buffer solution, then it was crushed with a special hammer with a pointed end until the sample was homogeneous.

B. The cell wall was destroyed by adding (400 µl of Gp1 buffer solution + 20 µl of Proteinase K enzyme), to break down proteins in the cell wall, then the mixture was

mixed with a Vortex device and then placed in a water bath at a temperature of 60 ° C, for 60 minutes, then 100 µl of Gp2 buffer solution was added and mixed with a Vortex device, keep for 30 minutes in the freezer until the temperature reaches -20°C, then put in filters to seize the impurities above the filter.

C. The filtrate was placed in the centrifugal system at laboratory temperature at 3000 rpm for a period of 5 minutes, transfer the floater to a new 1.5ml Eppendrof tube.

D. The ligation process, in step (c) 750 µl of Gp3 buffer was added to the supernatant to obtain the complete concentration of the nucleic acid, Gp3 was ethanol and isopropanol, according to the quality of the extraction kit for sedimentation and assembly of DNA, use the Vortex device to mix it and then collect it in tubes containing a mesh made of silica, it has a positive charge to bind DNA because the DNA has a negative charge, placed in the centrifugal system for one minute at 13,500 rpm, take the float associated with the network and neglect the precipitate.

E. The neutral Universal Wash Solution was added to disengage the DNA from the network and to wash, to precipitate the DNA, it was placed in the centrifuge at 12500 rpm for one minute and transferred to new Eppendrof tubes, the DNA extraction process was carried out.

Replication of the extracted DNA

Eppendorf tubes with a volume of 0.2 ml were prepared, and a master mix with a volume of 12.5 µl was placed in them, then 5 µl of the extracted DNA was added to it, then add 2 µl of the forward starter and 2 µl of the reverse starter, then 4 µl of deionized water was added, then it was centrifuged in a Vortex Centrifuge and mixed until the substances were homogenized, then it was placed in a thermal amplifier to perform DNA amplification cycles by the Polymerase chain reaction (PCR), as shown in Table (1).

Table (1): PCR reaction conditions

PCR steps	Cycle frequency	Temperature (°C)	Time (min)
DNA strand separation stage	1	94-98	1-9
DNA primer adhesion stage	30	50-60	1
DNA structure (elongation)	30	72	2
Final elongation stage	1	72	10

Electrophoresis of DNA by agarose gel

Prepare agarose gel by taking 2.5 ml of X TBE buffer and fill the volume to 25 ml by adding non-ionic distilled water for dilution, then 0.375 gm of agarose gel was added, put in the oven until it boils, then it cooled to 65°C, then ethidium bromide dye was added and mixed with light shaking.

A. Pour the agarose gel in the designated place on the electric relay device, taking into account that the place is level so that it is distributed evenly, shake gently to

prevent bubbles from forming, put the sterilized comb to make holes in the mixture, leave at room temperature for 30 minutes, the comb was removed after hardening.

B. 4 microliters of standard DNA of known molecular weight were added to the first hole, 4 µl of target DNA was placed in the remaining pits, cover the device with its cover and connect the electrodes, to be the electrical relay for 60 minutes at 70 volts.

C. The samples were sent to (Macrogen) company to obtain the sequence of the nitrogenous bases of the DNA.

Data Analysis

The statistical analysis program genstat-12 was used in the analysis of data according to the design of the complete random sectors RCBD and the averages were compared according to the lowest significant difference according to the probability level of 0.05.

Results and Discussion

Isolation and identification of fungi associated with wheat grains

The results of isolating and diagnosing the fungi associated with locally grown wheat and harvested in the same season showed the presence of a group of fungal species*Alternaria sp, Aspergillus flavus, Aspergillus niger, Fusarium sp, Mucor, Penicillium sp, Rhizopus sp and other fungi.* Figure (1).

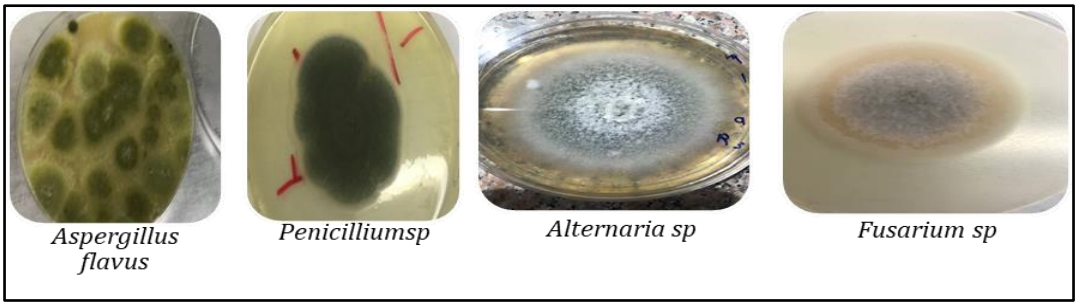

Figure (1): Some fungi isolated on PDA culture medium

The results confirmed that the two sexes, *Aspergillus flavus* and *Alternaria sp,* were the mostvisible in all Iraqi provinces, with an occurrence rate of 35.84 % for*A.flavus*and 25.36 % for *Alternaria sp,* it was followed by the fungus *A. niger,* then each of the fungi *Fusarium sp, Rhizopus sp, Penicillium sp and Mucor,* respectively, with an occurrence rate of 20.008%, 5.20, 4.61, 2.20 and 0.63%, the occurrence of other undiagnosed fungi was 6.09% (Table 2).

Table (2) The average percentage of the frequency and occurrence of fungi associated with wheat grains in Iraq for the agricultural season 2021

No.	Fungi	Frequency (%)	Occurrence (%)
1	***Alternaria sp***	16.22	25.36

2	***A.flavus***	22.77	35.84
3	***A.niger***	12.44	20.008
4	***Fusarium***	3.11	5.20
5	***Mucor***	0.44	0.63
6	***Penicillium***	1.44	2.20
7	***Rhizopus***	2.66	4.61
8	**Other fungi**	3.55	6.09
L.S.D$_{0.05}$		0.130**	1.813**

Table (3) shows that there were significant differences in the percentage of occurrence of fungi isolated from wheat grains harvested in the same season, these differences may be attributed to the different geographical location of the provinces and the method of cultivation, this was possible because the environmental conditions of temperature and humidity are suitable for the growth and spread of mushrooms in those provinces, it was noted that the genus *A.flavus* recorded the highest incidence in Muthanna, Najaf, Karbala, Sulaymaniyah and Mosul, with an occurrence rate of 58.1%, 43.03, 38.7, 32.39 and 30%, respectively, while the provinces of Qadisiyah, Basra, Erbil and ThiQar recorded a lower occurrence of 29.4, 26.66, 26.02 and 2.5, respectively. The fungus *Alternaria sp* ranked second with the most visible fungi at the level of the Iraqi provinces. The provinces of Erbil and Sulaymaniyah recorded the highest occurrence of this genus at 47.94% and 43.66%, respectively, w While the percentage of its occurrence in Basra, Najaf, Qadisiyah, ThiQar, Karbala, Mosul and Muthanna was 31.11, 29.11, 22.05, 20, 17.74, 14 and 2.7%, respectively.The genus *A. niger* recorded the highest occurrence in the provinces of ThiQar and Mosul by 45% and 40%, respectively, and it was less occurrence in Erbil province by 2.73%, it was also noted that the genus *Fusarium sp* recorded its highest occurrence in the provinces of Erbil and DhiQar by 10.95% and 10%, while it was not recorded in the holy province of Karbala and the percentage of its occurrence varied in the rest of the provinces. The fungus *Mucor sp* recorded an occurrence rate of 2.94%, 2.5%, 1.4% and 1.35% in the provinces of Qadisiyah, ThiQar, Sulaymaniyah and Muthanna, respectively, while it was not recorded in the rest of the provinces.The genus *Penicillium sp* recorded occurrences in some Iraqi provinces by 5.47% in Erbil province, 4.83 and 4.41% in Karbala and Qadisiyah provinces, respectively, and the percentage of its occurrence was 1.4% in Sulaymaniyah province and 1.26 in Najaf. The rest of the provinces did not record its occurrence. As for the genus *Rhizopus sp,* it appeared on wheat grains in most of the Iraqi provinces in different proportions, and the highest percentage of its occurrence was in Basra province by 11.11%, and it did not appear in Al-Qadisiyah province.

Table (3): The percentage of the occurrence of fungi in the Iraqi provinces for the agricultural season 2021

No.	Province	*Alternaria*	*A.Flavus*	*A.niger*	*Fusariun*	*Mucor*	*Penicillum*	*Rhizopus*	Other fungi
1	**Erbil**	47.94	26.02	2.73	10.95	-	5.47	4.10	2.73
2	**Sulaymaniyah**	43.66	32.39	12.67	1.4	1.4	1.4	2.81	4.22
3	**Mosul**	14	30	40	6	-	-	4	6
4	**Najaf**	29.11	43.03	11.39	2.53	-	1.26	6.32	6.32
5	**Karbala**	17.74	38.7	30.64	-	-	4.83	1.6	6.45
6	**Diwaniyah**	22.05	29.4	26.47	8.82	2.94	4.41	-	5.88
7	**Muthanna**	2.7	58.1	27.02	2.7	1.35	-	4.05	4.05
8	**ThiQar**	20	2.5	45	10	2.5	-	7.5	12.5
9	**Basra**	31.11	26.66	20	4.44	-	-	11.11	6.66

While the results indicated in Table (4) showed that *A.flavus* was the most frequency fungus at the level of the provinces under study, with a frequency of 22.77%. The fungus *Alternaria sp* ranked second with a frequency of 16.22%, where it was noted that the highest frequency was for *A. flavus* in the provinces of Muthanna and Najaf by 43 and 34%, respectively. These results were close to what was found by [15] , when they studied wheat samples in three Bulgarian cities, and the frequency of *A. favus* was 44.78%. The result converged with the findings of a previous study [16], where the frequency of *A. flavus* in their study was 36%, this result was close to the study that was conducted in the southern provinces of the Kingdom of Saudi Arabia when examining 25 samples of grain, it was found that the frequency of *A. flavus* was 68% and the percentage of occurrence was 22.08% [17]. Also, the result converged from [18], when they studied Pakistani wheat, the frequency of the fungi was *A. flavus* 31%, *Penicillium* 9%, *Fusarium* 8%, *Rhizopus* 3% and *Alternaria* 2%, it was found that the dominant fungi during storage of wheat grains in Ethiopia were *Aspergillus sp* and *Penicillium*, with a frequency of 45.54 and 29%, respectively [19]. The result converged with [20], when they studied 34 wheat samples in different regions of Iran, the percentage of *A. flavus* frequency was 10.7%. The results were similar to what the researchers found when studying 25 samples of wheat grains from some Libyan mills, which showed that the frequency of the fungi was *Aspergillus sp* 29.50% and *Penicillumsp* 21.06%, *Alternaria sp* came with a frequency of 23.25%, *Mucor* with 18.13%, *Rhizopus* and *Fusarium* with 5.54 and 0.77%, respectively [21, 22], showed that agricultural crops were susceptible to field fungi and storage fungi, that accompany the seeds from the field during transportation, and the most important fungal genera were *Fusarium sp*, *Aspergillus sp* and *Penicillium sp*.The fungal genera *A. alternate*, *A. flavus*, *A. fumigates*, *A. niger*, *A. terreus*, *Penicillium sp* and *Fusarium sp* were the most common fungi associated with wheat [22]. Additionally, it was found [23] numerous Fusarium species causing seed decay and damping-off disease of wheat crop in Kerbala province in 2020.

The differences in environmental factors between the Iraqi provinces and the different methods of cultivation and harvesting, led to a discrepancy in the number of fungi from one province to another, as well as the moisture content of grain and rain, which was the owner of the harvest process and primitive storage operations followed by the Iraqi farmer.

Table (4): the percentage of fungi frequency in the Iraqi provinces for the agricultural season 2021

No.	Province	*Alternaria*	*A.Flavus*	*A.niger*	*Fusarium*	*Mucor*	*Penicillum*	*Rhizopus*	Other fungi
1	**Erbil**	35	19	2	8	-	4	3	2
2	**Sulaymaniyah**	31	23	9	1	1	1	2	3
3	**Mosul**	7	15	20	3	-	-	2	3
4	**Najaf**	23	34	9	2	-	1	5	5
5	**Karbala**	11	24	19	-	-	3	1	4
6	**Diwaniyah**	15	20	20	6	2	3	-	4
7	**Muthanna**	2	43	20	2	1	-	3	3
8	**ThiQar**	8	18	1	4	-	1	3	5
9	**Basra**	14	9	12	2	-	-	5	3

Molecular diagnosis of the fungus *A. flavus*

The results of the electrophoresis of the DNA of the isolate AFBSN1 on agarose gel showed the presence of a single band with a size of 569 bp (Figure 2).

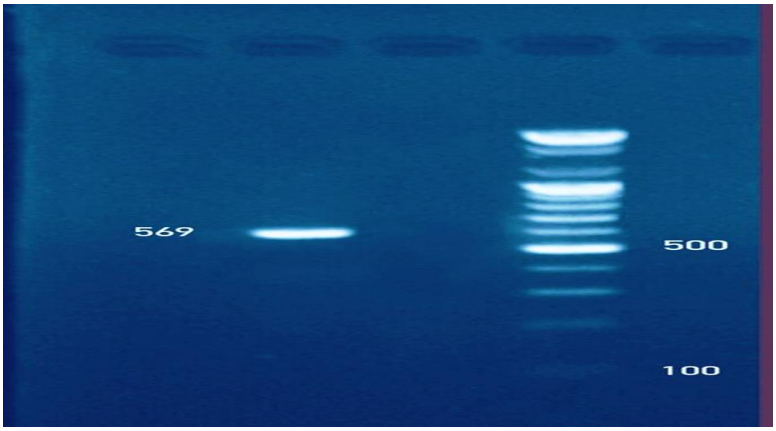

Figure (2): Result of DNA electrophoresis of *A. flavus*

The results showed that the sequence of nitrogenous bases of *A. flavus* isolates, their number was 569 pb, the result was that the tested isolate belonged to the fungus *Aspergillus flavus*, it showed an identical percentage (90%) with many global and local

isolates found in the NCBI gene bank, the nucleotide sequence of the isolate was deposited under accession number ON932490 (Figure 3).

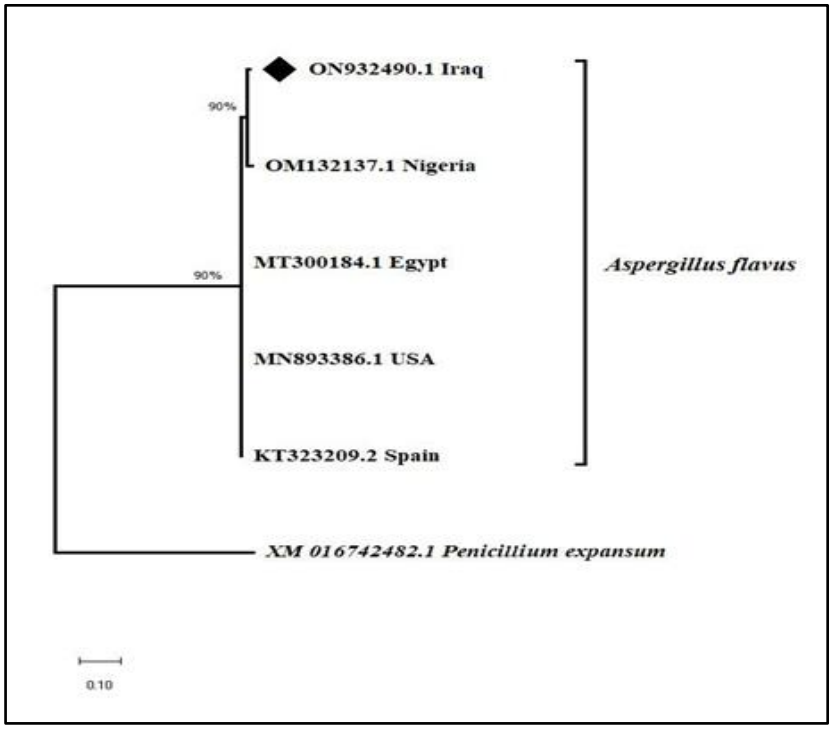

Figure (3): The germplasm tree of the fungus *A. flavus*

All wheat grain samples are under study and for the various Iraqi provinces, it was contaminated with fungi, *Alternaria sp* and *Aspergillus flavus* were the most frequent and occurrence in all provinces, the most frequent isolate was prepared for the fungus, *Aspergillus flavus*, which is the main responsible for the production of aflatoxin B1. Therefore, we recommend periodic inspection of wheat grains intended for storage, not to be received from silos unless they meet the conditions of good storage**.**

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