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Determination of Fungi and Aflatoxin Contaminating Wheat Stored in Silos of Salah Al-Den Governorate–Iraq

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Abstract

The study was conducted in the laboratories of Food Sciences Department in College of Agriculture at Tikrit University with the aim of isolating and diagnosing of fungalspecies which contaminated the wheat samples that stored in the silos of Salah Al-Din Governorate, The samples were included T1:local wheat from the Sharqat silo, T2: local wheat from the Al-Alamsilo, T3: imported wheat From Tikrit silo, T4:Wheat imported from Al-Sharqat silo. The fungal was isolation by inoculation the wheat samples after dilution with saline on the media of potato dextrose agar (PDA), Czapek Dox agar and Rose Bengal agar, and incubated on 30 ºC for 5 days then diagnosis used the morphological and microscopic characterized and confirming using specific primer in the PCR technique.

The results showed the diagnosis of the species of fungi contaminated with stored wheat, whether imported or locally used in the production of flour, which was supplied within the ration card items, which were the species *Aspergillus flavus*, *A.niger*, *A.parasiticus*, *Peniciliumexpansum*, and *Rhizopusstolinifer*, as their accounts were the total logarithmic in the samples. T1, T2, T3, and T4 are at logs of 6.72, 6.66, 6.78 and 6.70 colonies/g, respectively, and *A. flavus* prevailed with a frequency rate of 45.5, 51.3, 37.1 and 41.2%, respectively, also the AFIR2 gene was appeared at 500 bp. The aflatoxin B1 were detected in the wheat samples at 192, 169.6, 100,8 and 100 µg/g respectively.

Key words: fungi, wheat, Aflatoxins, PCR reaction.

Introduction

Grains are considered to be the mainstay in the manufacture of bread and cereal products, as it has been classified as the main and important component of human food for thousands of years. Grains provide a person with nearly three quarters of his energy needs and more than half of his protein needs.

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Triticumaestivum is one of the most important cereal crops in terms of its cultivation capacity and spread and the unique characteristic of some of its varieties in the production of bread. It is also the cheapest source of energy and protein, followed by the crops of yellow corn, rice and oats [(1)]. It is also the second most productive grain in the world and occupies a large position in the global agricultural economy, as global production of wheat in 2016 reached approximately 749.5 million tons [(2)].From the foregoing, grains in general, and wheat in general, are distinguished in their occupation of an important role in both the human food system and economic development, due to the various possibilities of use they contain in the manufacture of grains and their products. Therefore, the state of its infection with fungal diseases has a great impact on its productivity and quality, and thus in the effect on the sources of people's nutrition, and wheat is considered one of the most vulnerable types of grain to infection with fungi and their toxins [(3)].

The contamination of grains with types of fungi and their toxins (Mycotoxins) no longer has its effects on reducing the sources of food and its quality, but goes beyond that to be one of the main problems affecting human health that results in the case of direct nutrition from contaminated cereal crops or in indirect cases after feeding field animals on the types of contaminated grains and their transmission to humans after feeding on the products of these animals [(4)].

Cereal crops, especially wheat grains, during agricultural production and after harvest are exposed to pollution by micro-organisms, especially fungi, as the moisture content of these crops after harvest remains high or in cases of imperfect storage, lack of ventilation and stirring in silos and grain stores all give the opportunity for different types of fungi to grow and reproduction and production of toxins.

Among these fungi are the genera Aspergillus, Fusarium, and Penicillium, which are able to produce mycotoxins at high concentrations. The species *A.flavus* and *A.parasiticus* are considered to be the two most dangerous species among the species of fungi mentioned in their production of aflatoxin, especially type B1 which is highly toxic. Studies have confirmed that it is a carcinogenic agent of the liver and kidneys and an inhibitor of the immune system and has significant effects on genetic factors through the development of a defect. In cell DNA, and various other health effects in cells and organs of the body [(5)].

Based on the above, and to increase the concentrations of mycotoxins, especially aflatoxins, which are contaminated with wheat grains in grain stores in Iraq, the research was designed to identify the species of fungi and determine the amount of aflatoxin B1 contaminating the wheat grains stored in the stores in Salah al-Din Governorate.

Materials and methods

Preparation of samples and isolation of fungi: Samples of wheat stored in silos in Salah Al-Din Governorate were collected from both imported and local types and prepared for bread in the ration card program for the distribution of flour to Iraqi citizens, which included T1: local wheat from the Sharqat silo, T2: local wheat from Al-Alam complex, T3: wheat. Imported from Tikrit silo, T4: Wheat imported from Al Sharqat silo.

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Sampling was at a rate of 1 kg per sample and by 5 replications of each type in clean and sterile paper bags. It was taken into consideration that it was taken randomly from separate areas so that the samples were representative of the stored grains. Samples were transferred to a laboratory refrigerator and kept at 4 ° C until use in laboratory experiments.

Many researchers[6] used The method to isolate the fungi from the grains by taking 100 tablets from each sample by three replicates and sterilizing it with 2% sodium hypochlorite for 2 minutes. Then the seeds were washed with sterile distilled water three times. They were dried using filter paper and transferred to dishes that contain the culture medium (PDA) with 10 pills per dish. Part of the pill was inserted into the medium with sterile forceps and the plates were incubated at 28 ˚C for 7 days [7] .The method of dilution of samples was also used to determine the total numbers of fungi that contaminated the wheat samples, as in [8].

Diagnosis of fungi isolated from the grain of wheat: The shapes, colony colors, phenotypic, and Microscopic characteristics of each fungal isolate were observed. The optical microscope was used to observe the microscopic characteristics as well as to clarify the phenotypic characteristics in diagnosing the types of fungi contaminating the stored wheat samples after making swabs of purified fungi isolates on the appropriate food media on glass slides using Lacto Phenol Cotton Blue Stain.The obtained traits were compared with the approved taxonomic keys for fungi [9], through which the isolated fungi species were obtained.

The percentage of isolates of molds was calculated after diagnosis through the following equation:

Percentage of fungi isolates% = number of colonies of the fungus species / total number of fungi species x 100

Purification and preservation of fungal isolates growing on wheat grains: After the fungi were diagnosed, a swab of the fungus spores was taken and then transferred to dishes containing culture media and incubated at 25 ° C for 7 days. The process was repeated to obtain pure isolates. The purified isolates were kept diagonally in tubes and glass dishes and were kept in the refrigerator at 4˚C for later tests [10].

Molecular diagnostics of *Aspergillus flavus:*

DNA extraction: DNA isolation of the fungal species *Aspergillus flavus* was performed using CTAB, according to [11]. fungus DNA was extracted by weighing 1gm of fungus mycelium, which was washed with running water and then with distilled water and dried, after which it was cut into small pieces. The cut parts were placed in a medium-sized ceramic mortar, then liquid nitrogen was added to it. The fungus parts were crushed continuously until it was transformed into a white powder. Then the powder was transferred to sterile glass tubes and directly added 8ml of the previously prepared extraction solution that was taken directly from The water bath was at a temperature of 65 ° C and the tube was turned over until the mixture was homogeneous. Then it was transferred to incubation in the water bath at 65 ° C for a period of (60 - 90) minutes. The tubes were removed from the water bath and left for a period of 10 minutes to gain room temperature, after which 5ml of a chloroform solution: isoamyl alcohol was added 1:24 and the tubes were shaken quietly for 15-20 minutes.

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After stirring, the tubes were placed in a centrifuge and rotated at a speed of 4000 cycles/ mint. for 15mints, after the end of the centrifugation process, the upper thin aqueous layer was withdrawn from the tubes by a micropipette and placed in new sterile tubes, to which the same previous amount was added from chloroform:isoamyl solution and the same centrifugation stage was repeated under the same conditions above, The aqueous layer was withdrawn by pipette to new sterile tubes after the end of centrifugation to which a similar volume of cooled isopropanol alcohol was added to each sample, then turned the tubes quietly to mix the alcohol well with the sample, in the meantime a white mass was formed, which is a DNA strand, this mass was raised by hook to another tube containing 5 ml of washing solution.The DNA strands were kept for a period of 20-30 minutes in the washing solution, and the process could be repeated more than once, after which the DNA strings were lifted from the washing solution to new tubes containing 300 - 400 microliters of TE Buffer and left until the complete melting of the strings. The stock samples were then stored at -20 ° C for later use.

Determination of DNA concentration and purity: The process of measuring the concentration of DNA was carried out as an estimation of its purity using a (Nano Drop) device, as one drop of the genomic DNA extracted previously was taken and then placed in the location of the sample, then an order was given to the device to measure after calibrating the device on the same dissolution solution, and then the device gave the concentration in ng/ µL and purity accurately Then the sample was diluted to 50ng/µL and stored by freezing until use.

Electrophoresis on agarose gel: The solutions were prepared in the electrophoresis process according to the method mentioned by [(12)].

Agarose gel was prepared at 1% concentration for electrophoresis of Genomic DNA as well as genomic DNA sectioned by restriction enzymes. To obtain a concentration of 1%.One gram of agarose powder was dissolved in 100 ml of TAE 1X using a heat source (microwave), then cooled the solution to 55 ° C, then the gel solution was poured into the special basin (Tray) with an electrophoresis device. After fixing a special comb to form pits (Wells) at one end of the gel. The pouring was done quietly to avoid bubbles, and if they had formed, they should be removed using a pipette. Then the gel was left until it hardened, after which the comb was lifted and (Tray) was placed in an electrophoresis chamber containing an appropriate amount of TAE (X1) solution.

The electrophoresis samples were prepared by mixing 5 microliters of DNA sample with 3 microliters of loading buffer solution using this micropipette of the genomic DNA as well as the volumetric index of lambda DNA in a special well on one side of the gel. Then the electrophoresis device was started by passing the electric current with a voltage difference of 3 volts/ cm from the size of the electrophoresis chamber, and after adjusting the electrodes, the direction of the samples electrophoresis should be towards the positive electrode to the point where the samples arrive before the end and the process takes 1.5- 2 hours.

Specific Primers Interactions: Specific Markers interactions were performed on DNA samples extracted from *Aspergillus flavus* and isolated from wheat included in the study (Ismail, 2015). The Specificprimer Afir2, which has a molecular size of 500bp (Table 1), was used and the process was carried out using the ZR Fungal / Yeast / Bacterial DNA Extraction Kit MiniPrepD6005 ZYMO / USA.

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Table (1): The sequence and source of specific primer used in the study

Primer name		Primer sequence 3-5	Source
Afir2	Forward Reverse	GCACCCTGTCTTCCCTAACA ACGACCATGCTCAGCAAGTA	Ismail, (2015)

The Specific Primers reaction program was applied according to the kit described above (Table 1). After the end of the reaction time, the tubes were removed from the thermoplastic device and kept in the freeze. Then5 microliters were withdrawn from the tubes and the mixture was loaded onto a pre-prepared agarose gel at a concentration of 1.5% with the volumetric index(Marker). Then the samples were electrophoresed on the agarose gel. Then, the gel was dyed by immersing it with ethidium bromide dye for one hour while stirring and exposed to the UV source on a UV-transilluminator and photographing the gel.

Table (2) Afir2 Specific primer doubling PCR reaction program

Stage	The temperature˚ C	Time required	Rotation number
Primary metamorphosis	94	7 minutes	1
Normal metamorphosis	93	45 seconds	40
Primer Link	36	1 minute	
Elongation	72	1.5 minutes	
Final elongation	72	10 minutes	1

Aflatoxin's assay: Aflatoxin B1 was estimated in wheat samples taken from wheat grain samples from each group, using the ELISA device (Bio Tech Company of Korean origin). The estimation kit for each toxin was used, manufactured by Shen Zhen Lvshiyuan Biotechnology Co. Ltd of China. The method for each kit was followed according to the manufacturer's instructions.

Results and discussion

Isolation and diagnosis of fungi: Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA), which are considered suitable cultures media, were used to fully display the phenotypic characteristics of molds from the Deuteromycetes family because of the nutrients they contain in their contents. In addition to the use of the medium Rose Bengal Agar (RBA) that determines the growth of fungi when growing on it and leads to preventing its rapid spread, allowing sufficient time to reach its diagnosis, given that it contains Rose Bengal that leads to this in fungi, especially for the types of molds of the Zygomycetes family.

The isolated fungi colonies appeared in different numbers on the used culture media after incubation at 28 ° C for 7 days and according to their later diagnosed types after purification by regrowing them individually on the same culture media. The outcome was calculated on both PDA and MEA media plates.

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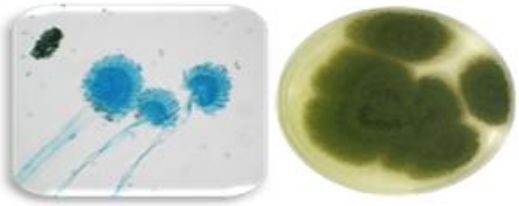

Figure (1): *A. parasiticus*A: After growing on MEA medium at 30 ° C for 7 days. B: Fungus parts under a light microscope at magnification power of 400.

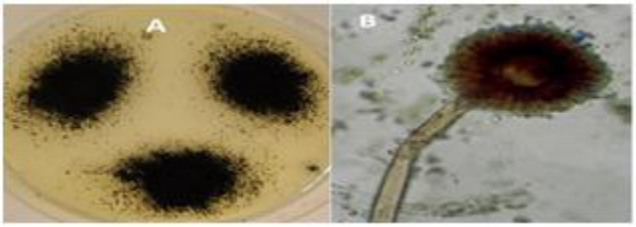

Figure (2): *A. nige*A: After growing on MEA medium at 30 ° C for 7 days. B: Fungus parts under a light microscope at magnification power of 400.

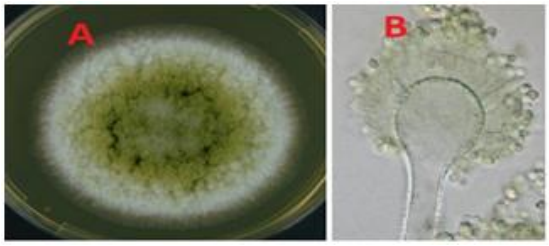

Figure (3): *A. flavus* A: After growing on MEA medium at 30 ° C for 7 days. B: Fungus parts under a light microscope at magnification power of 400.

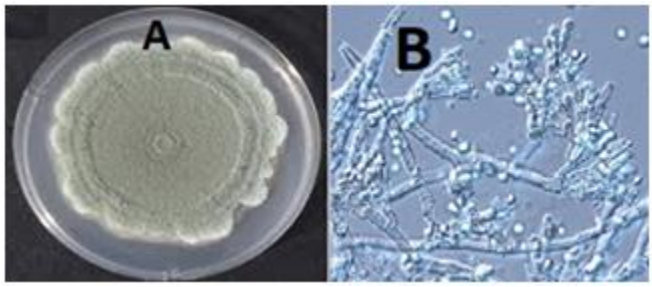

Figure (4): *P.expansum* A: After growing on MEA medium at 30 ° C for 7 days. B: Fungus parts under a light microscope at a magnification of 400.

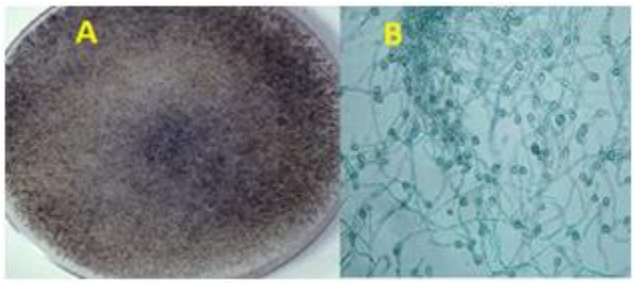

Figure (5): *R. stolinfer* A: After growing on MEA medium at 30 ° C for 7 days. B: Fungus parts under a light microscope at magnification power of 400.

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The forms of fungal isolates growing and isolated on culture media PDA, MEA, and RBA, as well as the forms of conidiophore and Conidia, the type of branches in conidiophore, as well as the sizes of the colonies, their colors and the upside-down colors of each of them are shown that they included the fungi *Aspergillus parasiticus* (Fig. 1) and *A. niger* (Fig. 2), *Aspergillus flavus* (Fig. 3), *Peniciliumexpansum* (Fig. 4), and *Rhizopusstolinifer* (Fig. 5), after comparing the mentioned traits with those mentioned in the taxonomic keys [9],[13].

The percentage of isolation of fungi species: After diagnosing the fungal species contaminated with the wheat samples, it was found that the diagnosed fungi species were in repeat ratios in the examined samples when preparing a totalcount in the samples of T1, T2, T3 and T4 which are at log 6.72, 6.66, 6.78 and 6.70 colonies/g respectively (Table 3). *Aspergillus flavus* prevailed in all the examined samples compared with other fungi species contaminated the samples, and it appeared in the samples T1, T2, T3 and T4 with a frequency of 45.5, 51.3, 37.1 and 41.2%, respectively.

Then came Aspergillus niger with a frequency of 22, 32, 35 and 24%, respectively, and the Aspergillus parasiticus which was 12.3, 15.6, 11.4 and 13.0%, respectively, while the Peniciliumexpansum was present at 13.8, 1.1, 9.2 and 16.6% respectively. The species Rhizopusstolinifer was with numbers of 6.4, 0.0, 7.3 and 5.2, respectively.

Table (3) the number of species of fungi contaminated with stored wheat samples.

Samples	**Account of fungi** log (CFU/g)	**%of the number of fungi contaminated with stored wheat samples** log (CFU/g)				
		R. stolinifer	***P. expansum***	***A.parasiticus***	***A. niger***	***A.flavus***
T1	6.72	6.4	13.8	12.3	22	45.5
T2	6.66	0.0	1.1	15.6	32	51.3
T3	6.78	7.3	9.2	11.4	35	37.1
T4	6.70	5.2	16.6	13.0	24	41.2

*** The different letters within one column indicate the presence of significant differences at the 0.05 probability level.**

T1: local wheat from the Al-Sharqat silo, T2: local wheat from the Al-Alam complex, T3: wheat imported from the Tikrit silo, and T4: wheat imported from the Al-Sharqat silo.

The results are in agreement with other studies [14] who found that *A.flavus* was more frequent than other fungi species in dry crops stored in Iraq.The reason why this fungus is the most frequent one could come from its ability to grow and multiply in storage conditions in addition to its ability to grow in field conditions, which led to this fungus being the most frequent of the other species in the studied wheat grains, contaminated with species of fungi.

Molecular diagnosis of *A.flavus* using PCR: The diagnosis of *A.flavus*, contaminated with the studied wheat samples, was confirmed using molecular diagnostics through the use of PCR technology and inferred it through its Specific primer. The DNA extraction of the above species of fungus using the extraction kit provided by the (ZR Fungal / Yeast / Bacterial DNA Miniprep) kit (D6005 ZYMO / USA) revealed that the extracted DNA was of high purity at 2% as in the Figure (6). The concentration, was also typical, with evidence that was efficient in subsequent tests.

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Figure (6) Results of genome electrophoresis of *A. flavus*

The results of the polymerase chain reaction showed that the *AFIR2* gene appeared in *A.flavus* isolates. As indicated by the results of the electrophoresis using a 2% agarose gel, the visible beams were at a size of 500 bp indicating the presence of the *AFIR2* gene above and thus confirming the return of the two isolates to the*A.flavus* species under test (Fig. 7). These results coincide with that found in variant studies [(8),(11)] , who confirmed that the *AFIR2* primer was a specific for inferring *A.flavus*.



Figure (7) Agarose gel electrophoresis of PCR product using 500bp *AFIR2* primer

The concentration of aflatoxin B1 in wheat samples: The results in Fig. (8) showed that the concentration of the toxin in the local wheat samples from the Sharqat silo R1, the local wheat samples from the science complex R2, the wheat imported from the Tikrit silo R3, and the wheat samples imported from the Sharqat silo R4 at a concentration of 4.8, 4.24, 2.52, 2.5 mg/ Kg. From the results, it was found that the highest concentration of toxin was in treatment R1 and the lowest concentration in treatment R4. The results are consistent with that found in[(15)].

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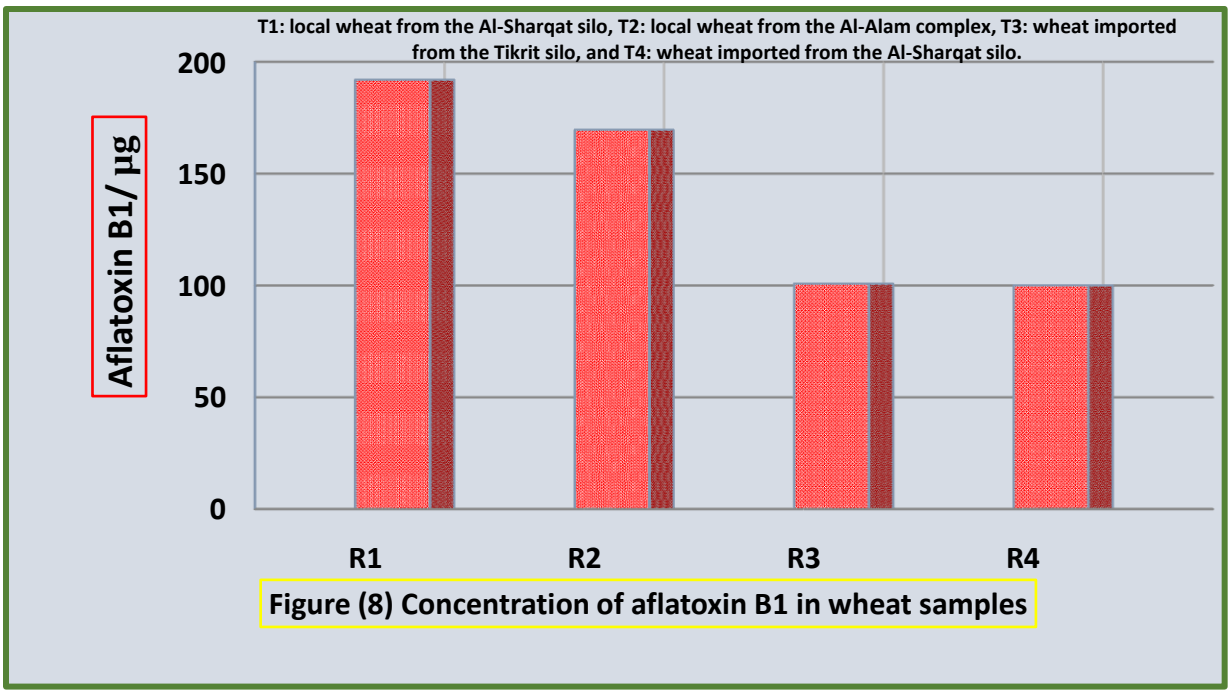

Figure (8) Concentration of aflatoxin B1 in wheat samples

The reason for the presence of aflatoxin in wheat samples may be due to its production in the field after being infected with *A.flavus* in the field, as mentioned by [12],[17] *A.flavus* is one of the fungi that infects wheat in the field and accompanies it in stores, and due to changing environmental conditions, its metabolic processes are directed to the production of aflatoxin .

The more production lies in the ability of the above fungi to produce toxins after infecting grains in stores, which showed from previous studies that the conditions for storing these grains from temperature or relative humidity are suitable for the production of these toxins from fungi as mentioned by [16],[18]The concentration of aflatoxin B1 increased with the increase in temperature and relative humidity in three models of economic crops (wheat, rice and corn) taken from different silos.

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