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Isolation and identification of fungi produced aflatoxin B1 from iraqi wheat for the agricultural season 2015

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ABSTRACT The current study included the isolation and identification of contaminated fungi of wheat grains collected from the Iraqi provinces covered by the agricultural plan for growing season of 2015 and the detection of ability of fungi to produce aflatoxin B1. The results showed the existence of five different species of fungi, which were identification after their development on the media. These species were belonged to *Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger, Penicillium* spp., *Trichoderma* spp., *Mucor* spp., *Fusarium* spp. The occurrence and frequency percentage of fungi in the local *A. flavus* and *A. parasiticus* were significantly higher (p <0.05) in the provinces of Dohuk, Sulaymaniyah, Kirkuk, Karbala, Dhi Qar and Missan in comparison with other studied governorates. *A.parasiticus* did not register any appearance in the province of Muthanna. However, the occurrence of *A. flavus* have exceeded in the same provinces mentions above, which reached 14.28% and it did not register any appearance in the holy Karbala. In terms of frequency ratio, Dahuk governorate was significantly higher with 46.66% for *A. parasiticus*, while *A. flavus* had the highest value in Baghdad governorate (42.79%). The ability of isolates (*A.flavus* and *A. parasiticus*) to produced Afatoxin B1 was detected by using ammonia vapor and ultraviolet radiation and the results were 92.59 and 60.97%, respectively.

KEYWORDS : wheat, *Aspergillus,* HPLC, Aflatoxin B1, ammonia detection

Introduction

Cereals are one of the most important food sources that have contributed to human nutrition for thousands of years. Grains and their products represent an important base of human food resources around the world. Wheat (*Triticum* spp.) is the most important grain crops, which represents the most essential sources of food (FAO, 2007 and USDA, 2013). The contamination of wheat and its products is a source of concern to many food organizations, commercial companies and food industries in terms of detection of contaminated food products with fungi and its toxins (Aflatoxin). Aflatoxin has caused loss of grain and other food. The world food organization (FAO) (2007) estimates that 25% of the world's crops are contaminated with fungal toxins, with losses reaching 1 billion tons per year. The process of controlling microorganisms in the food production series has been a continuing challenge. Although modern applications of food safety in progress, contamination is a serious challenge may lead to infect grain with harmful fungi. The detection methods of fungi are still continuing process in particular those product fungal toxins and cause diseases with a large scale (Olewnik, 2012 and Scauflaire *et al.*, 2012). Those aflatoxin toxins have contaminated with a large number of foods, particularly in regions which have high temperature and humidity. With regard to these areas, there are seventeen of these compounds, including B1, B2, G1 and G2, have been identified (Eaton and Gallagher, 1994). The amount of aflatoxin of (B1, B2, G1 and G2 combined) that has been allowed by the legal limits for direct human consumption is approximately 4 ppm, while aflatoxin B1 alone has been permitted by about 2 ppm (Ramesh *et al.*, 2013). Regarding Iraq, the amount of aflatoxin toxin has limited by 5 ppm as a maximum of Iraqi standard (Iraqi Standard Specifications, 1998). The harmful ingredients of vegetable food could be man-made sources or by action of microorganisms such as Ascomycota fungi that produce fungal toxins that contaminate many crops within the food chain over all countries without exception (Reddy *et al.*, 2010).

Wheat (*Triticum aestivum L.*) is the most important episode of the main food chain, which represents the main food of most population around the world. It is also inexpensive to equip human body with energy. Furthermore, its importance as food is due to the fact that it contains good proportions of food ingredients such as protein 11.3 (N×6.25), Moisture 12.6, Lipids 1.8, Carbohydrates 59.4, Fiber 13.2, Minerals 1.7 g/100 g wheat grain, Thiamine B1 4.6, Riboflavin B2 0.9, Nicotinamide 51.0, Pantothenic acid 12.0, B6 2.7, Folic acid 0.9 and Total tocopherols 41.0 mg/ kg wheat which are found in a wide range of wheat products as economic importance (Koehler and Wieser, 2013).

Iraq imported amount of wheat from various origin countries reaching to 20.398.700 million tons at a cost of 6.389.376.917 billion US dollars from 2006 to 2016. Recently, Iraq has tended to grow this crop at a level almost equivalent to that of the importer. According to the Planning and Follow-up Department (2017), its production of local wheat reached to 22.140.313 million tons from 2006 to 2016. The aim of this study is to conduct a survey of fungi present in wheat grain of the Iraqi provinces that were covered by agricultural plan of the growing season 2015 as well as the detection of ability of fungi to produce aflatoxin B1.

MATERIALS AND METHODOLOGY

1.Sampling:

Wheat samples were randomly collected from silos, marketing centers of the Iraqi Ministry of Trade and wheat growing fields of 16 Iraqi governorates that were covered by the official agricultural plan in 2015. From each province, three samples weighing (5 kg for each) were collected to obtain fungal isolates that produce aflatoxin.

2.Culture media:

A.Potato dextrose agar (PDA) medium:

This medium was prepared as following steps:
First of all, 39g of PDA powder was added to 1 liter distilled water at pH 5.6 ±0.2. Then, it was sterilized by using autoclave at 121°C, 15 psi for 20 minutes. After finishing sterilization process, antibiotic (chloramphenicol at a concentration of 250 mg/liter) was added to liquid of PDA at 45°C.

The steps that mention above were followed as instructions of the manufacturer. This medium was used to process of isolation and diagnose of fungi.

B.Malt extract Agar medium:

This medium was prepared as following steps:
To begin with, 39g of medium powder was added to 1 liter distilled water at pH 5.4 ±0.2. After that, it was sterilized by using autoclave at 121°C, 15 psi for 20 minutes. After finishing sterilization process, antibiotic (chloramphenicol at a concentration of 250 mg/liter) was added to liquid of medium at 45°C.

The steps that mention above were followed as instructions of the manufacturer. This medium was also used to process of isolation and diagnose of fungi.

C.Coconut Extract Agar (CEA) Medium:
This medium was used to detect aflatoxin toxins produced by some kinds of fungi. It was prepared according to the method used by Saito and Machida (1999) as the following steps:

First of all, 100g coconut powder was added to 300 ml of hot distilled water then it was mixed by using blender. Suspend was then filtered by using piece of texture. The volume of solution was completed by adding distilled water and 1.5% Agar to 600 ml. After that, it was sterilized at 121° C and 15 psi for 15 minutes. Finally, antibiotic (Chloramphenicol) was added to medium (250 mg/L) to prevent any bacterial growth.

3.Isolation and diagnosis of fungi associated with local wheat:
Samples of the wheat materials were sterilized with sodium hypochlorite solution at a concentration of 2% for 2 minutes. After that, they were washed with distilled water and then placed on filter papers to get rid to dry. Next, they were placed on petri dishes of media and then incubated all dishes at 25 ° C ± 2° C for seven days. After the purification of isolates process, fungi were diagnosed depending on morphologic traits and appearance of fungal isolates according to method of Pitt and Hocking (1997).

The steps that mention above were applied at the Laboratory of Biotechnology at the Department of Food Science/ College of Agriculture/ University of Basrah.

The proportion of occurrence and frequency was calculated according to the following formulas:

Occurrence proportion % :
The percentage of occurrence of fungi that produced aflatoxin B1 and isolated from wheat was calculated as the following formula:

$$\text{Occurrence proportion} = \frac{\text{Number of specimens showing genus or species of fungi}}{\text{Total number of samples}} \times 100$$

Frequency proportion %:
The percentage of frequency of fungi that produced aflatoxin B1 and isolated from wheat was calculated as the following formula:

$$\text{Frequency proportion} = \frac{\text{Number of colonies of genus or species of fungi}}{\text{Total number of colonies of genus or species of fungi}} \times 100$$

4.Detection of aflatoxin B1 by ammonia vapor method:
According to Saito and Machida (1999), Ammonia Vapor method was used to test the ability of fungal isolates that were isolated from different sources to produce aflatoxin. These isolates were activated (5 days before applying the experiment) by culturing in petri dishes of (CEA) Medium. After that, plug of mycelia was taken from edge of fungal isolate colony and placed in the center of petri dishes of (CEA) Medium. These dishes were then incubated at 25° C for 5 days. This followed by placing wet filter papers with 20% ammonia solution on covers of dishes. These dishes were then placed upside down in incubator at 25° C. After an hour and 24 hours of incubation, the dishes were examined. Results showed that isolates produced aflatoxin through changing color of fungal culture from white to pink (Saito and Machida, 1999).

5.Determination of quality and quentity of Aflatoxin B1 by high performance liquid chromtography (HPLC):
High-efficiency liquid chromatography (HPLC) technique was used to diagnose fungal toxins produced from fungal isolates and wheat grains. This method was applied to assist in qualitative and quantitative assessment process. Steps of separation and estimation process of were applied following procedure of Pierre *et al* (2011):

A highly efficient liquid chromatographic device, which belongs to the Center of Marine Sciences/ Ministry of Higher Education/ Basrah University, was used to estimate the type of toxins through using the inverse phase column C18 ODS2 with dimensions 4.6 x 250 mm and the type of column used PWXL-CP3000 for organic acids. The volume of the injected specimens was 5 µl and a wavelength at 365 nm. The separation process was carried out by using a movable phase consisting of (water: methanol: acetonitrile) with proportions (50: 40: 10) (volume: volume: volume) respectively. The separation was undertaken by using HPLC device at room temperature and at inflow speed of 0.7 ml/ min. The aflatoxin toxin B1 was estimated according to the method used by Cataldi *et al*. (2000) as the following formula:

$$\text{Concentration of unidentified material} = \frac{\text{Space of specimen bound}}{\text{Space of bound measurement}} \times \text{Standard concentration} \times \text{Number of dilution}$$

6.The statistical analysis:
The statistical analysis was carried out by designing completely randomized design experiment (CRD). A comparison of the means of treatments was performed using a less significant analysis LSD. Finally, the mean at a significant level of (0.05) was carried out using ready statistical analysis systems (SPSS 2012) (issue 22).

Results and discussion

1.Isolation of fungi from local wheat of the growing season 2015:
The results indicate that five different genus of fungi were isolated from local wheat marketed in 2015 as in Table (1). These genus were identified after culturing on media, which mentioned previously, depending on methods used by (Raper, and Fennell, 1973; Pitt, 1979 and Pitt and Hocking, 1997) as keys of diagnosis. It was confirmed that fungi are one of the microorganisms associated with wheat and its products. Also, most of these fungi are Ascomycetes (Filamentous fungi) that produce fungal toxins, which cause damage and waste in the economies of countries in addition to the first cause of cancer, liver disease and death (Adeyeye, 2016).

Table (1) Species and genus of fungi present in Iraqi grain wheat collected from the growing season 2015.

No.	Genus and species of fungi
1	Aspergillus flavus
2	Aspergillus parasiticus
3	Aspergillus niger
4	Penicillium sp
5	Trichoderma sp
6	Mucor
7	Fusarium

The results also indicate the frequency of *Aspergillus flavus* and *Aspergillus parasiticus* in wheat samples of growing season of the Iraqi provinces in 2015. These two species are considered as harmful fungi because of their ability to secrete aflatoxin B1.

2.The occurrence and frequency of fungi in the local wheat of growing season in 2015
Table (2) shows the occurrence of fungi in various Iraqi governorates. It was found that there was a difference between the occurrence of *A. flavus* and *A. parasiticus*. In the governorates of Dohuk, Sulaymaniyah, Kirkuk, Karbala, Dhi Qar and Maysan, it was significantly higher ($P < .05$) than in other governorates with a proportion occurrence at 14.28% for *A. parasiticus*; however, there was no occurrence of *A. parasiticus* recorded in the province of Muthana samples. Regarding percentage of occurrence of A. flavus, it was the highest recorded at 14.28% in the governorates of Dohuk,

Sulaymaniyah, Kirkuk, Dhi Qar and Maysan, whereas, it was not recorded in the holy governorate of Karbala.

With regard to table (3), it indicates to the frequency of the fungi of the study. It was found that there was a difference between the frequency of *A. flavus* and *A. parasiticus* at a significant level ($p < 0.05$) among various provinces. The percentage of frequency of *A. parasiticus* in Dohuk governorate was more significant recorded at 46.66% then other governorates. In the governorate of Baghdad, the highest frequency of *A. flavus* was 42.79%, while no frequency was observed in the holy governorate of Karbala. Also, the mean difference RLSD of both *A. flavus* and *A. Parasiticus* was the lowest with 2.43 in the holy governorate of Karbala.

This research indicated most results are almost similar to other studies. First of all, it was found that frequency of *A. flavus* in the wheat samples studied in three Bulgarian cities was 44.78% (Faria *et al.*, 2017), while it was recorded at 36% by Del Palacio *et al.* (2016). In addition, Hamed *et al.* (2016) reported that 68% of the frequency of the *A. flavus* resulted in 22.08% of 25 samples of grain which collected from the southern governorates of Saudi Arabia. In their study, Pacin *et al.* (2002) also found that the frequency of *A. Flavus* and *A. parasiticus* was 60% and 20% respectively. Furthermore, it was recorded that the percentage of occurrence of *A. flavus* was 10.7% of 34 wheat grain samples collected from various regions of Iran (Joshaghani *et al.*, 2013). However, the results were not consistent with the findings of other studies. In their study, Al-Muthomi and Muttu (2003) found that the percentage of frequency of *Aspergillus* was 6%. This was due to different conditions of storage, type of wheat and trading processes. Also, it was observed that a high percentage of the seeds were infected by fungi. It was believed that the main cause was the presence of assistant factors that increased the proportion of fungal infection such as Insects, spiders, quality of the crop and mixing crop with other contaminated materials during either harvesting or storage (Abbas, 1983). Moreover, other studies confirmed that *Aspergillus* spread into many crops, including maize, wheat, field pistachio and rice through low moisture content conditions (Eaton and Groopman, 1993; Pitt *et al.*, 1993).

The results of this research are also consistent with the findings of other previous studies. For example, Eaton and Groopman (1993) indicated that grain components were appropriate to the growth of *Aspergillus* as well as the relative density of production of fungal spores. In their study, Abu-Taleb *et al.* (2012) found that the most frequent genus in the grain samples (including barley, rice, wheat, maize and maize), which were collected from various regions of Riyadh, was *Aspergillus* which had the ability to produce aflatoxin. Furthermore, it was reported that the frequency of ten fungal genus found in Pakistani wheat were *Aspergillus* 31%, *Penicillium* 9%, *Fusarium* 8%, *Rhizopus* 3% and *Alternaria* 2% (Asghar *et al*., 2016). Moreover, Senbeta and Gure (2014) found that the dominant fungi during the storage of wheat grains in Ethiopia were *Aspergillus* and *Penicillium* considered as a storage fungi with frequency 45.54%, 29% respectively. It was also confirmed that the positive storage conditions should be with good relative humidity, temperature, ventilation and continuous cleanliness in the wheat stores.

According to Bartosik *et al.* (2008), there is a strong association between the amount of carbon dioxide and the presence of fungi in wheat grain when stored in closing places such as silos. If the amount of carbon dioxide is at the level of 400-500 ppm (volume / volume) there is no problem in the grain stores. However, if the amount of CO_2 increases to 1000 ppm, it may cause a problem. Moreover, if the quantity of CO_2 exceeds 3000 ppm or more with moisture in wheat grain 14.5%, there is certainly fungal infection in the stored grain.

Food and Agriculture Organization (FAO) (2007) point out that fungal damage may occur when aquatic activity (a_w) is greater than critical level. In addition, grains stored within moderate atmosphere conditions are lesser infected with fungus than those stored at hot and humid atmosphere. Furthermore, infection of fungi is much more in grains packed with bags because of breathing, temperature of stored grain and grain without cleaning before storage.

The difference in the number of fungal isolates among various regions of Iraq is to the differences in the environmental factors that affect the presence of fungi in wheat, whether in the field or stored in the silos. These factors are the relative humidity of grain stores, open places where amount of grains was received during agricultural marketing. They are also grain moisture and the rains that fall during the harvest. Another important factor is the primitive storage process followed by Iraqi farmer by covering the high-moisture crop grain with plastic covers until marketed to the silos.

Table (2) shows the occurrence of fungi during growing season of various Iraqi provinces in 2015.

No.	Provinces	*A. flavus*	*A. parasiticus*	*A. niger*	*Penicillium spp.*	*Trichoderma spp.*	*Mucor spp.*	*Fusarium spp.*
1	Nineveh	4.76	4.76	4.76	4.76	2.38	4.76	7.14
2	Dahuk	14.28	14.28	0	0	0	0	7.14
3	Arbil	12.85	10	2.85	0	1.42	2.85	4.28
4	Sulaymaniyah	14.28	14.28	14.28	0	0	7.14	0
5	Kirkuk	14.28	14.28	0	7.14	7.14	0	14.28
6	Salah Din	8.57	5.71	1.42	4.28	2.85	2.85	5.71
7	Baghdad	9.52	7.14	4.76	1.19	2.38	0	2.38
8	Wasit	5.55	3.96	6.34	10.31	6.34	3.17	4.76
9	Babil	2.85	5.71	1.42	4.28	2.85	8.57	5.71
10	Qadisiyah	7.14	12.5	1.78	8.92	3.57	5.35	14.28
11	The holy Karbala	0	14.28	0	7.14	7.14	14.28	14.28
12	The holy Najaf	7.14	7.14	0	3.57	7.14	7.14	7.14
13	Muthanna	7.14	0	4.76	1.19	2.38	9.52	2.38
14	Dhi Qar	14.28	14.28	14.28	14.28	14.28	0	0
15	Maysan	14.28	14.28	10	2.85	5.71	1.42	5.71
16	Basrah	5.71	7.14	5.71	11.42	4.28	5.71	0
	RLSD	1.79						

The association of *A. flavus* and *A. parasiticus* was significant at a probability level ($P<0.05$).

Table (3) shows percentage of fungal frequency during growing season in various Iraqi governorates in 2015

No.	Provinces	*A. flavus*	*A. parasiticus*	*A. niger*	*Penicilliumspp.*	*Trichoderma sp.*	*Mucor spp.*	*Fusarium spp.*
1	Nineveh	17.77	16.66	11	13.48	5.55	6.66	28.88
2	Dahuk	36.68	46.66	0	0	0	0	16.66
3	Arbil	41.68	28.33	10	0	2.5	6.66	10.83
4	Sulaymaniyah	28.57	28.57	28.58	0	0	14.28	0
5	Kirkuk	18.18	36.37	0	18.18	9.09	0	18.18
6	Salah Din	26	16	4	12	12	8	22
7	Baghdad	42.79	21.95	9.72	3.33	5.55	0	16.66

8	Wasit	14.42	8.27	15.09	35.36	9.79	5.26	11.81
9	Babil	8	22	4	12	12	26	16
10	Qadisiyah	10.09	21.66	3.12	19.59	6.92	8.84	29.78
11	The holy Karbala	0	18.18	0	18.18	9.09	36.37	18.18
12	The holy Najaf	12.5	12.5	0	10	40	12.5	12.5
13	Muthanna	21.95	0	9.72	3.33	5.55	42.79	16.66
14	Dhi Qar	27.27	18.19	18.18	18.18	18.18	0	0
15	Maysan	32.34	29.31	13.31	3.76	8.81	2.86	9.61
16	Basrah	14	18.54	10.91	30.22	10.7	15.63	0
	RLSD	2.43						

A. flavus and A. parasiticus were significantly correlated at a probability level (P <0.05).

3.Detection of fungal produced by aflatoxin using ammonia vapor method

In this study, 81 isolates of *A. flavus* and 82 isolates of *A. parasiticus* that were isolated from local wheat during the growing season 2015 and cultured in media of the coconut extract agar (CEA), were tested their ability to produce aflatoxin B1 (AFB1). The test was undertaken by using ammonia steam with UV rays assay. The results showed the ability of fungal isolates to produce aflatoxin B1. It was found that percentage of aflatoxin B1 production by isolates was 92.59% of *A. flavus* and 60.97% of *A. parasiticus*. It was also observed that the range of ability of each isolate to produce AFB1 differed from others through a change of color of fungal colonies. This gradual color belonged to the quantities of AFB1 production. Dark red isolates showed a higher ability to produce AFB1 than pink or light pink isolates. Figure (1) shows that color of dish A was not changed due to the inability of the isolates to produce fungal toxins. However, color of dishes B, C and D was changed because of the ability of the isolates to produce AFB1. This color change also was occurred through using ammonia for isolate detection. With regard to dishes E and F, color indicates to the ability of these isolates to produce AFB1 after exposure culturing media to the UV ray.

In addition, the study also showed the ability of 85% of *A. flavus* isolates that gave a positive result at an hour. 95% of *A. parasiticus* isolates also gave a positive result when it was incubated at 25 ° C for an hour. However, the rest of isolates were responded to the test after 12 hours. These differences occurred because of the genetic differences of fungal isolates produced AFB1 and the differences of samples extracted from the same grains as well as differences of climatic conditions and storage conditions. These results are also consistent with the findings of a number of studies. Abarca *et al*. (1988) illustrated that medium of coconut extract agar (CEA) could encourage the growth of fungal isolates mentioned previously compared to other culturing media such as media of yeast and sucrose extract. It was confirmed that this method has had importance because its application has been rapid and easy to detect AFB1 (Saito and Machida, 1999).

Moreover, Qahtan and Abdullah (2002) point out that the fungal strains varied in their ability to secrete AFB1. Some of them have not secreted AFB1 while other strains could produce more than one type of fungal toxins depending on kind of fungal strain. Furthermore, Al-Mariani (2006) indicated a rapid change of isolate color from yellowish brown to purple red due to the chemical effect of evaporation assay. This was caused by dyes secreted from fungal strains that produce Aflatoxin B1.

Sudini *et al*. (2015) demonstrated mechanism of color change of culturing media was due to reaction of pigments (including norsolorinic acid, averantin, averufin, versicolorin C, versicolorin A, versicolorin A hemiacetal and nidurufin) with ammonium hydroxide or other base solutions such as sodium carbonate, sodium hydroxide and sodium bicarbonate resulting in color change to purple red. This color change indicated the intermediate pathways of aflatoxin synthesis.

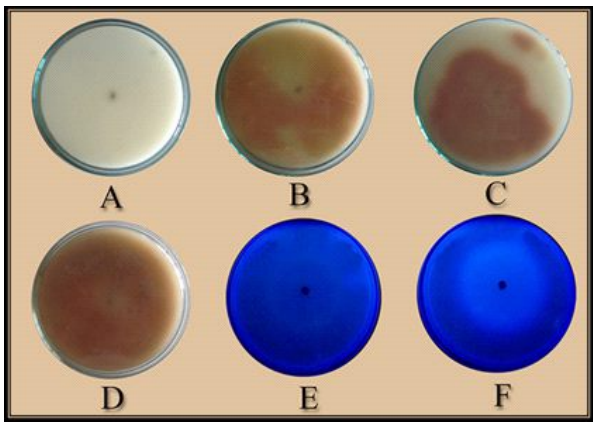

Figure (1) Determination of ability test of *A. flavus* and *A. parasiticus* to produce of aflatoxin B1 on medium of coconut extract agar (CEA) by using ammonia vapor and ultraviolet ray assay along 360 nm of wavelengths. (A) isolate was non-productive aflatoxin B1 using ammonia vapor, (B + C + D) isolates produced Aflatoxin B1 using ammonia vapor, (E + F) isolates produced aflatoxin B1 using UV ray.

With regard to the effect of incubation period on increasing the ability of isolates to produce fungal toxins, Al-Sherba (2007) confirmed the number of isolates that gave positive examination after an hour of incubation was lower than the isolates that responded to the test after 24 hours of incubation.

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