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MYCOFLORA AND INCIDENCE OF AFLATOXIN IN WHEAT SEEDS FROM DUHOK PROVINCE, KURDISTAN REGION OF IRAQ

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ABSTRACT:
Identification of fungi that contaminated wheat grains and their aflatoxins production were investigated in Thirty-three samples collected from different sources in Duhok province, Kurdistan region of Iraq during 2014-2015. In this study a total of twenty-five species belonged to 12 genera was isolated and identified on DRBC, MEA and PDA media. Nine distinct species were identified within *Aspergillus* which revealed highest diversity among all isolated genera. Followed by five species of *Penicillium*. *Alternaria* came after which showed only two species. Two teleomorphic ascomycota *Emericella*, and *Eurotium* were recorded. The results showed that *A. niger* and *A. flavus* were the most isolated species. Aflatoxin production potential detected by *A. flavus* and *A. parasiticus* were screened using ELISA method. Aflatoxin potential detected in culture of *Aspergillus flavus* isolates was ranged from 57 to 310 ppb, while, the potential production aflatoxin for *A. parasiticus* isolates were ranged from 189 – 450 part per billion.

KEYWORDS: Mycolflora, *Aspergillus*, Aflatoxins, Wheat, Duhok province.

1. INTRODUCTION

Contamination of agricultural commodities by Fungi considered an important caused as spoilage of food. Fungi are not only causes large losses of economy, but represented a high risk and harmful effects for both animal and human health through the composition and producing of mycotoxins (MacDonald *et al.*, 2004; Pitt and Hocking, 1997 and Tutelyan, 2004).Wheat is one of the major primary foods of human and is overripe in nearly all temperature and sub-tropical area in the world.

Wheat is very sensitive to infection by fungi and contamination by different kinds of mycotoxin. Fungal contamination mostly occurred during post-harvest as a result of improper harvesting procedures, and inadequate storage conditions combined with unfavourable climate. These moulds develop inside or around the seeds, depending on their biological make-up and Eco physiological conditions (Bryden, 2012).

Mycotoxins are toxic metabolites produced naturally by diverse filamentous fungi or moulds on certain conditions of temperature, humidity, moisture, drought, damage by insect, harvesting performance, handling transport and storage (Hussein and Brasel, 2001; Marin, *et al.*, 2013; Rodríguez-Carrasco, *et al.*, 2013 and Zain, 2011).

Many mycoflora seed borne of wheat newly notify includes

Seed-borne mycoflora of wheat reported latterly including *Alternaria alternata*, *Drechslira sorokiniana*, *Fusarium avenaceum*, *F. moniliforme*, *F. nivale*, *F. graminearum*, *F. equiseti*, *F. culmorum* ,*F. sporotrichioides*, *Cladosporium herbareum*, *Stemphylium botryosuem* (Nirenberg, 1994 and Glazek ,1997).

The main important mycotoxin types produced by *Aspergillus*, *Penicillium* and *Fusarium* are Aflatoxins, fumonisins, ochratoxins, and trichothecenes (Mirza, and Qureshi, 1978). Which may contaminate foods including various agricultural products as cereals, vegetables and fruits.

Aflatoxins are produced by some other species of *Aspergillus* section Flavi, including *A. toxicarius*, *A.bombycis Aspergillus nomius* and *A. pseudotamarii* (Hedayati *et al.*, 2007). Furthermore, *A. oryzae*, an industrially useful fungus, is recognized as safe since its biosynthesis pathway of aflatoxin is proved to be silent and commonly come across in animal feeds and foodstuffs extensively. Amongst several types of aflatoxins, including B1, G1, M1 ,B2 and G2, Aflatoxin B1 (AFB1) is the main, prevalent and toxic member of the series (FAO, 1986; Farkas and Tannenbaum, 2005; Kobayashi, 2001), and well-studied of the compounds. The main toxic influence includes strong hepatitis, hepatocellular carcinoma and immunosuppression, for human and animals (Richard and Payne, 2003). Aflatoxin B1 enters a animal or human system via inhalation ,ingestion, or dermal contact causing an enormous range of chronic and acute toxic effects (Richard and Payne, 2003**)** .Chemical composition of Aflatoxin is difuranocoumarin derivatives produced through a pathway of polyketide (Klich , 2007).

The aims of this study were to isolate and identify mycoflora of wheat seed borne fungi and asses the ability of isolates of *Aspergillus* Section Flavi to produce Aflatoxins *In vitro* using ELISA technique.

2. MATERIALS AND METHODS

2.1 Source of wheat grains samples

During 2014 – 2015 a total of 33 samples (500 g) of wheat from two active silos in Duhok province, North of Iraq, including Shekhan and Zakho were collected in sterile polyethylene bags. Wheat seeds were submitted to Mycology Research Laboratory at the College of Science, Biology Department, in order to assess any possible fungal contaminations, aflatoxin determination, and fungal identification. The seeds were stored at 4°C and used when needed.

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2.2 Fungal isolation and identification

The samples of wheat seeds were examined using direct Plating method described by (Pitt *et al*.,1997)
For each sample, one hundred seeds was surface disinfected with 2% Sodium Hypochlorite for one to two minute at room temperature and washed twice by sterilized distilled water then placed on three culture media used ISTA techniques (10seed for each plate): and used different agar media (Dichloran rose bengal Chloramphenicol agar medium (DRBC) (Germany- Fluka), Malt Extract Agar medium (Biokar Diagnostics, France), and Potato dextrose agar medium PDA (France-Biokar Diagnostics) each media supplementing with 50 mg/L Chloramphenicol before autoclaving to inhibit growth of bacteria. Plates were incubated at 28ºC for 5 to 7 days. The fungi growing on seeds were identified either, directly or through sub culturing on another media such as Potato dextrose agar (PDA), and Czapeck'sagar.
The identification of fungal species was done according to the descriptions of previous studies (Ellis, 1976; Pitt and Hocking, 1997; Watanabe, 2002; Klich, 2002; Abarca *et al.*, 2004; Frisvad and Samson, 2004; Frisvad *et al.*, 2004; Samson *et al.*, 2004 ; Samson *et al.*, 2007).
Frequency of fungal isolates were calculated using the following equation:

$$\textbf{Isolation Frequency \% } = \frac{\textbf{Number of samples on which a fungus appeared}}{\textbf{Total Number of Samples}}$$

2.3 Sample preparation and extraction

To estimate the production potential of aflatoxins, several isolates of *Aspergillus* section Flavi were chosen randomly using the procedure of technique of Bragulat *et al.*, (2001). Petri dishes (9 cm) containing sterilized Yeast Extract Sucrose were centrally inoculated with (3 mm) disc of *Aspergillus flavus* isolates and *A. parasiticus* isolates taken from the edges of recent colonies then incubate at 25 ºC for 7 days.
From the centre and a midway between the edge and the centre of the growing colonies. The three plugs were mixed with 1 mL methanol in a small vial , shaking vigorously and left at room temperature for 1hr. , mixed again and the extracts were filtered through Millipore filter (0.22 um) diameter (Millex GP Filter Unit Coringhwohill ,Co. Ireland).

2.4 Aflatoxin analysis

The quantification of aflatoxin was achieved by Enzyme linked Immunosorbant test (ELISA), according to instructions provided by the company producer (Veratox Aflatoxins quantitateve assay, Neogin Corporation,USA). Aflatoxins produced by isolates was calculated using standard curve that the previously prepared and expressed in part per billion ppb.

3. RESULTS AND DISCUSSION

Fungi may vary in growth and resistance or susceptibility to infections due to environmental factors, culture methods, linkage between microorganism, and climates conditions (Vuianovic *et al.*, 2012). According to Ominski *et al*., (1994), fungi and insects represent a great cause of losses and deterioration of storage seeds and grains.
Twenty-five species belong to 12 genera were isolated and identified, in addition to their percentage occurrence as illustrated in Table one.

Fungal contaminations of 33 samples of wheat grains (500 gr.) collect from Duhok province were tested. Twenty-five species represented 12 genera were identified and isolate. the occurrence frequency is illustrated in Table one.

Table 1. List of identified fungi isolated from wheat grains with their % occurrence.

No.	Fungi	Frequency %
1	*Alternaria alternate (*Fr.) Keiss l	5.0
2	*Alternaria chlamydospora*	1.55
3	*Aspergillus alliaceus*	3.0
4	*A. carbonarius (*Bainier) Thom	22.3
5	*A.flavus* Link	60.0
6	*A.pseudotamarri* Sartory	1.0
7	*A.fumigatus* Fresen	2.0
8	*A.niger* Tiegh.*nom.cons*	71.4
9	*A.ochraceus* K.Wilh	6.0
10	*A.parasiticus* Speare	33.5
11	*A.terreus* Thom	3.0
12	*Aureobasidium* spp	1.0
13	*Penicillium citrinum* Thom	3.0
14	*P.brevicompactum* Dierckx	2.0
15	*P.chrysogenum* Thom	12.0
16	*P.spinulosum* Thom	1.33
17	*P.verrocosum* Dierckx	1.67
18	*Paecilomyces varioti* Bainier	1.0
19	*Cladosporium herborum* (Pers.) Link	3.5
20	*Curvularia* sp	1.0
21	*Emericella rugulosa* C.R. Benj	1.0
22	*Eurotium amestelodami* Mangin	0.5
23	*Fusarium oxysoprum* Schlecht	1.0
24	*Mucor circinelloides*Tiegh	2.33
25	*Rhizopus stolonifer* Ehrenb Vuill	2.1
26	*Mycelia sterilia with chlamydospore*	3.1
27	*Ulocladium atrum* Preus	0.5

Various fungal species were isolated as natural contaminant of wheat in local grains. The isolated genera included *Aspergillus*, *Penicillium*, *Fusarium*, and others like *Alternaria*, *Rhizopus*, *Cladosporium*, and *Mucor*.
The most mycotoxigenic fungal species which identified morphologically was reported previously in wheat seeds in different regions of the world (Abdullah and Atroshi, 2014; Habib *et al.*, 2011 ; Broggi *et al.*, 2005).
From Malt extract agar and Potato dextrose agar. *Aspergillus* was higher frequency than other genera in the three culture media, followed by *Penicillium*. Nine species belonged to *Aspergillus* was recorded revealing substantial diversity among all identified genera.
Two species of black *aspergilli* including *A. carbonarius* and *A. niger* was represented in this study.
Two species were recorded with high frequency *A. niger* (71. 4 %) and *A. flavas* (60%), followed by *A. parasiticus* (33.5%) and *Aspergillus carbonarius* (22.3 %). While the other species were relatively found at low frequencies different from one medium to another. The results of this study is in agreement with that reported by Sulaiman and Husain (1985), also Hussain *et al.*,

(2013) revealed great incidence of *Apergillus niger* and *A. flavus* in trade wheat cultivars in Pakistan.

Alternaria represent by two species including *A. alternata* and *A. chlamydospora*. Species in this genus are including saprophytic fungi and plant pathogenic this is one of the causal agents of black points infection in wheat and reduce seed germination (Mahmuda *et al.*, 1987) *Penicillium* were second isolated species from wheat that represent by 5 species *Penicillium citrinum* was the main frequently species (3.0%), followed by *P. brevicompactum* (2.00%) and *P. verrucoseum* (1.67 %), *Penicillium chrysogenum* was the most predominant fungal contaminant with more than 12%, followed by *P. citrinium* with a value of 3%. *P. spinulosum* showed less frequency of 1.33% while other species like *P. echinulatum*, found with the least frequency (2.70%). *Penicillium* were genetically identified and difficult to distinguish from each other, their taxonomy was not fully resolved and that require molecular technique to resolve precisely the identification problems for individual species.

Two teleomorph Ascomycota includes *Emericella* sp. and *Eurotium amestelodami* was detected with the frequencies percentage 1.0% and 0.5 % respectively.

The results showed (Table 2) screening 3 isolates for each of *A. flavus* and *A. parasiticus* isolates from wheat grains for their aflatoxigenic production abilities in culture medium as detected by ELISA method. Tow isolates of *Aspergillus flavus* showed positive ability while all other isolates from *A. parasitcius* were shown positive result .The tested isolates revealed remarkable variance in their aflatoxins potential. This is an agreement with different studies which indicated variability in production of aflatoxins by *Aspergillius* Section Flavi (Saadullah and Abdullah, 2015).

Table 2. *In vitro* quantitative production of aflatoxins by specific isolates of *Aspergillus* section Flavi in wheat grains by ELISA test.

Aflatoxin (ppb)	Fungal Isolate
Aspirgillus flavus (isolate I)	310.0
A.flavus (isolate II)	N.D
A.flavus (isolate III)	N.D
A.flavus (isolate VI)	57.0
Aspergillus parasiticus (isolate A)	450.0
A. parasiticus (isolate B)	189.0
A. parasiticus (isolate C)	170.0

> N .D ... Not detected (negative)

Many studies revealed that not all isolates of *A. flaveus* have ability to produce aflatoxins and the ratio of non aflatoxigenic isolates to the aflatoxigenic isolates varied according to the source and location of isolates (Schroeder and Bolla 1973 ; Abdel -Malek *et al*., 1993 ; Abdullah *et al*., 2009).

Aspergillus carbonarius A . parasiticus ,A. flavus , and to extent or less some species of the genus *Fusarium* are most important species contaminates wheat seeds because to the potential of Mycotoxin production. (Vaamonde *et al*., 2003; Senyuva *et al.*, 2008 ; Embaby *et al.*, 2012).

4. CONCLUSION

The result of the present study revealed that seeds of wheat cultivates grown in Duhok governorate, Kurdistan area in Iraq was contaminated with a variety of Fungi among them various mycotoxigenic Fungi such as *Aspergillus flavus*, *A. parasiticus* and others. Thus, accurate hygienic mycology measurement (ISTA) should be done during harvesting and storage to reduce contamination with different toxigenic fungi.

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