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ASPERGILLUS FLAVUS ISOLATES ASSOCIATED WITH WHEAT GRAINS INFECTED WITH KHAPRA BEETLE *TROGODERMA GRANARIUM* AND THEIR ABILITY TO PRODUCE AFLATOXINS B1

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Abstract

A series of experiments were carried out to isolate and identify the fungal species associated with wheat grains that infected with Khapra beetle *Trogoderma granarium* in addition to determine the *Aspergillus flavus* isolates ability to produce aflatoxins. The results showed that the infection of wheat grains with *T. granarium* was accompanied by appearance of different fungal species. Also, the number of *A. flavus* isolates that isolated from wheat grains which infected with *T. granarium* or the body of *T. granarium* larvae was significantly higher than other isolated fungal species, where 38 isolates of *A. flavus* were isolated from *T. granarium* and infected grains of wheat. The results of Ammonia test and Spectrophotometer test were showed that 24 and 36 out of 38 isolates had ability to produce aflatoxins.

Key words*: Trogoderma granarium*, *Aspergillus flavus*, Ammonia test, Spectroscopy test

Introduction

Wheat (*Triticum* spp.) is the main source of food calories and proteins in the world. In storage, wheat grains are attacked by various arthropods causing quantitative and qualitative losses (Adams, 1998; Ahmad and Ahmad, 2002). Furthermore, insect contamination represents a crucial problem for food industries and for export commodities (Rajendran, 2002). The khapra beetle *Trogoderma granarium* Everts (Coleoptera: Dermestidae) is the most widespread and destructive primary insect pests of stored wheat grains and its products, especially in the tropical and sub-tropical regions (Burges, 2008).

The most important losses caused by stored insect pests including *T. granarium* is due to it has 9 generations a year, its larval stage is destructive, feeding on the germ portion of the grains and it is able to resist inappropriate environmental conditions as well as the larval stages may survive several years under diapause condition (Anonymous, 2001; Athanassiou *et al.*, 2009). Another important damage caused by storage insect pests is their transfer to fungal spores. Al–Saedy (2015) reported the ability of *Sitotroga cerealella* to transfer and spread the conidia of *Aspergillus flavus*. Welson (1991) was also found that insects help to transfer the fungal spores in storage through the adhesion of fungal conidia on their bodies. The chances of contact between stored insects and fungal conidia in the presence of mucous substances on the insect's body which help attaching the fungal conidia to their bodies (Eilenberg *et al*, 1986).

Some of these fungi spread by stored insects has been found to be able to produce dangerous compounds known as Mycotoxins which are secondary metabolites produced by filamentous fungi. Mycotoxins can lead to acute and chronic toxicity, mutagenicity and teratogenicity in human and animal health (Barkai-Golan, 2008). The mycotoxin-producing fungi are the genera *Aspergillus*, *Penicillium*, *Alternaria*, and *Fusarium* species (Richard, 2007). Aflatoxins and Ochratoxins are the most important groups of acutely toxic compounds which produced by species of *Aspergillus* such as *A. flavus*, *A. niger* and *A. parasiticus*. The main types of aflatoxins found in agricultural products are B1, B2, G1 and G2 (Lee and Hagler, 1991). The International Organization for Cancer Research (IRC) was identified and placed Aflatoxin B1 in the first group of chemical compounds carcinogen which is one of the liver cancer tumors causes (Karaca *et al.*, 2010). Several studies have been isolated the conidia of *A. flavus* from stored insect bodies and grains infected with them (Mcmillian *et al.*, 1990).

There is a little information about the ability of *T. granarium* to spread of *A. flavus* species and detect their capacity to produce aflatoxin B1. Therefore, the present study was aimed to (1) find the relationship between the infection of wheat grains with *T. granarium* and infecting them with *A. flavus* and other fungal species; (2) test the effect of storage periods of wheat grains infected with *T. granarium* on the quantitative contamination of the accompanying fungi; (3) test the effect of the numerical level of *T. granarium* population on the quantitative contamination of the accompanying fungi, (4) detect the ability of *A. flavus* isolates to produce aflatoxin B1 using both ammonia and

Materials and Methods

Trogoderma granarium rearing

The stock culture of *T. granarium* originated from wheat grain stores in Najaf, Iraq, in 2017, and was maintained in the Entomology Laboratory, Faculty of Agriculture, University of Kufa, Najaf, Iraq. Insects (50 male and female pairs) were reared on whole sterilized wheat grains (200 g per jar) placed in 300-ml plastic jars secured with a muslin cloth and rubber bands, and maintained at 30 ± 2°C and 65 ± 3% RH in continuous darkness.

Wheat grains

Wheat grains were obtained from the local markets in Najaf, Iraq. The samples were taken to the laboratory in sterilized polyethylene bags. They were sterilized at -20^{o}C for 48 hours and then with 70% ethanol to eliminate any insect or fungal infections.

Culture media

In this study, the following culture media were used:

Potato Dextrose agar (Potato Dextrose)

200 g of potato tubers, were cut into small pieces and boiled with 500ml distilled water for 20-30 minutes in a glass beaker. After the boiling period, then filtered content by a piece of gauze. 10 g of sucrose and 17 g of agar were dissolved in 500 ml of distilled water and then the potato leach and full size to the liter. The media were distributed in flasks according to its use, and their vents were sealed with cotton bolts and sterilized with the sprinkler device at 121°C and 15 lb / kg for 20 minutes. After that the flasks were left to cool and then placed in the refrigerator until use. This medium was used to isolate and grow the fungi.

Coconut Extract Agar

The media were prepared by mixing 100 g of coconut powder with 300 ml of distilled water and then the mixture was heated for 20 min. After the heating period, the mixture was filtered using a piece of gauze and then 1.5 g of agar was added and full size to 300 ml. The media were sterilized using the same procedure described above. This medium was used to develop *A. flavus* and to detect its ability to produce aflatoxin.

Effect of different storage periods of infected wheat grains with *T. granarium* on the level of contamination with fungal conidia

In this experiment, samples of whole sterilized wheat grains (200 g per jar) placed in 300-ml plastic jars secured with a muslin cloth and rubber bands were infected with 10 third instar larvae of *T. granarium*. Jars in control treatment were left with whole sterilized wheat grains only. Then, all jars were incubated at 30°C and 75% humidity for storage periods 1, 2, 3 or 4 months. At the end of each storage period, 15 dead of *T. granarium* larvae were collected randomly from each replicate and surface sterilized by rinsing with 70% ethanol for 1 min and placed on PDA in Petri dishes at 25°C for 7 days. In addition, 1 g of wheat grains of each jar was taken after the end of each storage period and placed on the center of PDA in Petri dishes and incubated at 25°C for 7 days. After 7 days, growing fungi were identified in the Laboratory of Fungi / Faculty of Agriculture / University of Kufa based on the taxonomic keys mentioned by Hoching and Pitt (2009) using a compound microscope by making glass slides for each isolate. The number of isolates or colonies for each growing fungus were calculated from samples taken from wheat grains or dead insects.

The effect of density levels of *T. granarium* on the contamination level of wheat grains with fungi

Samples of whole sterilized wheat grains (200 g per jar) placed in 300-ml plastic jars secured with a muslin cloth and rubber bands were infected with different levels of third instar larvae of *T. granarium* including 10, 20, 30 or 40 instars with 4 replicates for each numerical level of *T. granarium* larvae. All jars were incubated at 30 ° C and 75% humidity for 3 months. After this storage period, 15 dead of *T. granarium* larvae were collected randomly from each replicate of each numerical level and surface sterilized by rinsing with 70% ethanol for 1 min and placed on PDA in Petri dishes at 25°C for 7 days. In addition, 1 g of wheat grains of each jar in each treatment was taken and placed on the center of PDA in Petri dishes and incubated at 25°C for 7 days. After 7 days, growing fungi were identified as described above. The number of isolates or colonies for each growing fungus were calculated from samples taken from wheat grains or dead insects.

Detection of the ability of *A. flavus* isolates to produce aflatoxin B1 using Ammonia test

The ability of *A. flavus* isolates that isolated from wheat grain and the body of insect larvae in both experiments above on producing of Aflatoxin B1 was tested using the method described by Danes and Lin (1976). The coconut media was used to cultivate *A. flavus* isolates using 9-cm Petri dishes by placing 5 mm tablet from each isolate which already grows on PDA. Where each isolate was replicated three times. The dishes were sealed by a parafilm and incubated at 25°C for 7 days. Afterward, the ability of *A. flavus* isolates to produce aflatoxins was detected using ammonia solution 20% by placing filter papers saturated with ammonia solution in the lid of the dish containing fungal strain grew on the coconut media. The plates are incubated upside down at 25°C for 2 days. Changing the color of fungal strain from a transparent color to pink or red indicates that this strain was able to produce Aflatoxins.

Detection of the ability of *A. flavus* isolates to produce aflatoxin B1 using Spectrophotometer

The isolates of *A. flavus* were developed on PDA media by placing tablets with 5 mm diameter (one-week old) of each isolate at the center of each dish with three replicates for each isolate. Then, the Petri dishes were incubated at 25 ± 2°C for 7 days. After that, one dish of each isolate was selected and cut with a sterile knife to small pieces. They were then transferred by a sterile needle to an electric mixture containing 20 ml of chloroform for 10 minutes. The mixture was filtered with filter paper and then taken into a clean, sterile flask and placed in an electric oven at a temperature of 50°C to dry. And then, the mixture was dissolved in 1 ml of chloroform. To detect the presence of Aflatoxin, B1 using Spectrophotometer method which depends on the properties of the compound to absorb light in the ultraviolet or infrared fields. Where there was a direct correlation between the absorption time and the concentration of the mycotoxins as the wavelength is 365.

Statistical analysis

Statistical analyses were carried out using complete random design (CRD) as a single-factor experiment. Mean comparisons were performed using LSD test at the 5 % level of significance ($P \leq 0.05$).

Results and Discussion

Effect of different storage periods of infected wheat grains with *T. granarium* on the level of contamination with fungal conidia

The results showed that the number of fungal species isolated from wheat grains which infected with *T. granarium* was significantly affected by the storage period ($P \leq 0.05$). Where, the storage periods of 2 and 3 months were recorded 47 fungal isolates, compared with 28 and 41 fungal isolates after 1 and 4 months of storage period, respectively (Table 1). However, only 4 fungal isolates were isolated in the control treatment. This confirms that the stored insects when

present in the stores, they can carry the fungal conidia on their bodies and thus facilitate the transfer and spread of fungi. The high number of isolated fungal species during the storage period of 2 and 3 months may due to spread of *T. granarium* and increase its population. However, decreasing the number of fungal species after 4 months of the storage period may be due to the lack of nutrient availability and the accumulation of insect residues which led to the dominance some fungal species compared with other fungal species. The results are consistent with Al–Saedy (2015) who found that the number of fungal species isolated from different stored grains infected with *Sitotroga cerealella* were decreased with increasing the storage period.

In addition, most of isolated fungal species belonged to class Deuteromycota, especially *A. flavus* and *A. niger* which isolated from all wheat grains and insect samples in all storage periods. Where, 20 isolates of *A. flavus* and 16 isolates of *A. niger* were isolated after 3 months of storage period. This may be due to their ability to produce a large number of asexual reproduction unit, their high competition with other fungal species for food sources (Roy and Chourasia, 1989) and/or they have a high enzyme capacity that enables them to exploit many materials for energy. These results are in agreement with Tizaki and seyed (2011) who reported that *A. flavus* and *A. Niger* were the most fungal species are found in stored white maize grains infected with storage insect pests.

The storage period also had a significant effect on the total number of fungal isolates isolated from the body of third instar larvae of *T. granarium*. Where, the total number of fungal isolates after 3 months were significantly higher (9 isolates), compared with 1, 2 and 4 months of storage (Table 2). This is in agreement with Okwuelchie (2004) who found that stored insects attacking the stored products were highly infected with found to be infected with *A. flavus*, *A. niger* and *Penicillium* sp.

Table 1: Number of fungal species and their isolates which isolated from wheat grains infected with *T. granarium* in different storage periods.

Storage period	A number of fungal isolates								Total
	A. flavus	***A. niger***	***Mucor sp.***	***Alternaria sp.***	***A. oryza***	***Fusarium sp***	***Rhizopus sp.***	***Penicillium sp.***	
One month	2	4	2	5	0	1	2	12	28
Two months	16	16	8	0	6	0	1	0	47
Three months	20	14	8	1	0	2	2	0	47
Four months	16	7	5	0	2	4	7	0	41
$L.S.D_{(0.05)}$	1.129	0.824	0.467	0.299	0.467	0.554	0.401	0.537	

Table 2: Number of fungal species and their isolates which isolated from *T. granarium* larvae in different storage periods.

Storage period	A number of fungal isolates				Total
	A. flavus	***A. niger***	***Mucor sp.***	***Penicillium sp.***	
One month	0	1	0	0	1
Two months	3	0	0	0	3
Three months	0	1	2	6	9
Four months	2	1	3	0	6
$L.S.D_{(0.05)}$	0.400	0.277	0.401	0.339	

The effect of density levels of *T. granarium* on the contamination level of wheat grains with fungi

The results showed that there were no significant differences in the number of fungal species isolated from wheat grains infected with different levels of *T. granarium*. Where, 5, 7, 6 and 7 fungal species were recorded from grains infected with 10, 20, 30 and 40 larvae after 3 months of storage period (Table 3). However, there was a significant difference in the total number of isolates for each fungal species, where 37 and 36 fungal isolates were isolated from wheat grains infected with 40 and 30 *T. granarium* larvae respectively, compared with 24 fungal isolates were isolated from wheat grains infected with 10 *T. granarium* larvae. For example, 15 isolates of *A. flavus* were isolated from wheat grains infected with 40 *T. granarium* larvae, compared with 3 isolates in case of wheat grains infected with 10 *T. granarium* larvae (Table 3). The positive relationship between the number of *T. granarium* larvae and the diversity of fungal isolates may due to the role of *T. granarium* in carrying the fungal conidia and spreading them between the grains during its movement. The current results are consistent with Beti *et al*. (1995) who found that increasing the density of *sitophilus zeamais* infecting corn grains has led to increase the relative humidity level by 20-15%, thus providing favorable conditions for the growth of mycotoxin-producing fungal species.

The results showed that there were no significant differences in the number of fungal species isolated from the body of third instar larvae of *T. granarium* of different treatments ($P \leq 0.05$). However, there was a significant difference in the total number of isolates for each fungal species in different treatments. For example, 10 isolates of *A. flavus* were isolated from insect body collected from wheat grain jars infected with 40 *T. granarium* larvae, compared with no isolate was isolated from insect body collected from wheat grain jars infected with 20 *T. granarium* larvae (Table 4).

Table 3: Number of fungal species and their isolates which isolated from wheat grains infected with different density level of *T. granarium* larvae after 3 months of storage periods.

Density of *T. granarium*	A number of fungal isolates							Total
	A. flavus	*A. niger*	*Mucor sp.*	*Alternaria sp.*	*Geotrochum sp.*	*Rhizopus sp.*	*Penicillium sp.*	
10 larvae	3	0	2	3	9	0	7	24
20 larvae	3	12	1	2	8	2	8	36
30 larvae	6	5	3	1	4	0	12	31
40 larvae	15	7	3	5	3	0	4	37
L.S.D$_{(0.05)}$	1.018	1.334	0.430	0.438	1.989	0.211	0.999	

Table 4: Number of fungal species and their isolates which isolated from *T. granarium* larvae after 3 months of storage periods.

Density of *T. granarium*	A number of fungal isolates					Total
	A. flavus	*A. niger*	*Alternaria sp.*	*Rhizopus sp.*	*Penicillium sp.*	
10 larvae	7	2	1	0	2	12
20 larvae	0	10	0	0	0	10
30 larvae	6	3	0	1	0	10
40 larvae	10	1	0	3	0	14
L.S.D$_{(0.05)}$	0.688	0.571	0.160	0.288	0.320	

Detection of the ability of *A. flavus* isolates to produce aflatoxin B1 using Ammonia test

The results of this test showed the ability of 24 isolates out of 38 isolates of *A. flavus* that isolated from infected wheat grains and body of *T. granarium* larvae to produce aflatoxin by changing the color of coconut media that they were cultured on, while 14 isolates gave negative test results (Table 5). Furthermore, the isolates were deferred in producing aflatoxin depending on the intensity of colony color change. The most important isolates in producing Aflatoxin were AF4, AF5, AF10, AF13, AF21, AF26 and AF33 (Photo 1). Similarly, Ali (2017) found that 23 isolates out of 38 isolates of *A. flavus* were able to produce aflatoxin B1. In addition, Davis and Diener (1970) reported that 86% of *A. flavus* isolates were able to infect the field pistachios and contaminate them with aflatoxins. Differences in the ability of *A. flavus* isolates to produce aflatoxin B1 may be related to their genetic differences, which can explain the gradient in red color. Where isolates with dark red indicate their ability to produce large amounts of aflatoxins.

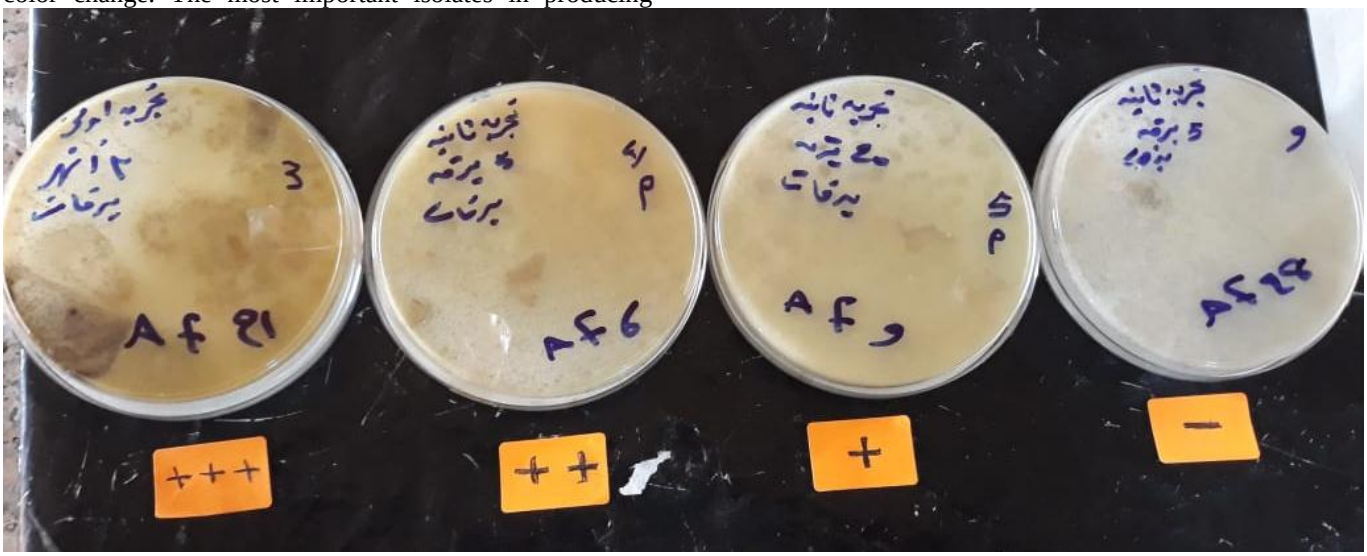

Photo 1: Ammonium test to detect the ability of *A. flavus* isolates to produce aflatoxin B1

Detection of the ability of *A. flavus* isolates to produce aflatoxin B1 using Spectrophotometer

The results of this test showed the capacity of 36 isolates out of 38 (94.73%) isolates of *A. flavus* on the production of aflatoxin B1 (Table 5). However, these isolates were varied in their production of aflatoxin B1. Analysis of Spectrophotometer showed that the isolates AF3, AF9, AF13 and AF31 were the most productive of aflatoxin B1 based on their intensity. The results obtained in the present study are consistent with those of Yu *et al*. (2004) who indicated that 75% of *A. flavus* isolates were able to produce aflatoxin B1 using Spectrophotometer test.

It can be concluded that the infection of wheat grains with *T. granarium* was accompanied by appearance of different fungal species, especially *A. flavus*. Where the number of *A. flavus* isolates were increased with increasing the population density of *T. granarium*. In addition, the number of *A. flavus* isolates which were able to produce Aflatoxin B1, using Ammonia test was less than the number of isolates that have been detected to produce Aflatoxin B1 using Spectrophotometer. Therefore, Ammonia test can be more accurate in the identification of *A. flavus* isolates that able to produce mycotoxins in general, including Aflatoxin B1.

Table 5: Testing the ability of *A. flavus* isolates that isolated from wheat grains and bodies of *T. Granarium* larvae on production of aflatoxin B1 using Ammonia and Spectrophotometer tests. (+) Isolate had ability to produce aflatoxin B1, (-) Isolate had no ability to produce aflatoxin B1.

Isolate No.	Source of isolate	Ability to produce Aflatoxin B1	
		Ammonia test	Spectrophotometer
AF1	*T. granarium* larvae	-	+
AF2	*T. granarium* larvae	+	++
AF3	*T. granarium* larvae	+	+++
AF4	*T. granarium* larvae	+++	++
AF5	*T. granarium* larvae	+++	++
AF6	*T. granarium* larvae	++	+++
AF7	*T. granarium* larvae	-	++
AF8	*T. granarium* larvae	-	++
AF9	*T. granarium* larvae	+	+++
AF9	*T. granarium* larvae	+++	-
AF10	*T. granarium* larvae	++	+
AF11	*T. granarium* larvae	++	++
AF12	*T. granarium* larvae	+++	+++
AF13	*T. granarium* larvae	-	++
AF14	*T. granarium* larvae	-	++
AF15	*T. granarium* larvae	-	+
AF16	*T. granarium* larvae	+	+
AF17	Wheat grains	++	++
AF18	*T. granarium* larvae	++	++
AF19	*T. granarium* larvae	+++	++
AF20	*T. granarium* larvae	-	+
AF21	*T. granarium* larvae	-	+
AF22	Wheat grains	+	++
AF23	Wheat grains	++	++
AF24	Wheat grains	+++	++
AF25	Wheat grains	++	++
AF26	Wheat grains	-	+
AF27	Wheat grains	-	+
AF28	Wheat grains	-	++
AF29	Wheat grains	++	++
AF30	Wheat grains	++	+
AF31	*T. granarium* larvae	+++	++
AF32	*T. granarium* larvae	-	-
AF33	*T. granarium* larvae	-	++
AF34	Wheat grains	++	+
AF35	Wheat grains	-	++
AF36	*T. granarium* larvae	++	+++
AF37	Wheat grains	-	+
AF38	*T. granarium* larvae	++	++

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