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Management of Aflatoxin B1 Producing *Aspergillus flavus* on Walnuts and Pistachio Seeds.

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ABSTRACT

Aflatoxins are extremely potent natural carcinogens and a major food safety concern because of potential contamination of food commodities. Plant resistance to aflatoxin contamination is not commercially available and fungicides are not effective. The use of antagonists microorganisms against mycotoxin fungi is a promising approach to manage aflatoxin contamination via biological control. The objective of this study was to evaluate some effective biological control include *Rhodotorula glutinis*, *Penicillium oxalicum* and *Trichoderma harzianum* for inhibition aflatoxin B1 produce by *Aspergillus flavus* isolated from Walnuts and Pistachio grown in Sinai governorate. The filtrates of all isolates produced varying degrees of inhibition against *A. flavus*. Nevertheless, the highest percentage of inhibition of toxigenic *A. flavus* by the culture filtrate of *R. glutinis* at 150 mg/kg . The ability of biological control agents strains to prevent aflatoxin production have been confirmed by HPLC analysis. Certain yeast strain was found to confine the spreading and sporulation and reduce the amount of aflatoxin production. Sporulation was limited due to the inhibitory effect of yeast. There was a noticeable decrease in fungal dry mass and aflatoxin production. Treated walnuts and pistachio and seeds with biocontrol isolates were effective in controlling fungal infection and increased seeds germination . *Rhodotorula glutinis* and *Trichoderma harzianum* antagonistic isolates provided good protection against *A. flavus*.

Keywords: Biocontrol, aflatoxin B1, *Aspergillus flavus, Aspergillus flavus, Rhodotorula glutinis , Penicillium oxalicum Trichoderma harzianum,* Walnuts and Pistachio.

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INTRODUCTION

Worldwide nuts are known expensive and delicious flavor (Sindoni *et al*., 2005). Besides, Walnuts and pistachio nut are characterized by the high percentage of lipids and carbohydrates that makes them vulnerable to the attack by pre- and postharvest with field and storage fungi such as Aspergillus (Adebajo and Diyaolu, 2003). Aflatoxins are secondary metabolites produced by certain strains of *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. tamarii*, *A. bombycis* and *A. pseudotamarii* (Afsah Hejri *et al.,* 2005). Aflatoxins are one of the most potent toxic substances that occur naturally and their contamination in economic crops is a major problem all over the world (Peterson *et al.,* 2001) . The most toxic of this group, is the most potent carcinogen known. Mycotoxin contamination is a serious concern that occurs in the field before harvest or during storage, despite efforts of prevention . Various methods have been developed by deploying various microbes antagonists to degrade these mycotoxins and reduce pesticide inputs. Detoxification of mycotoxin contaminated food and feed by microorganisms has been amply documented in the literature Yin Yan-Ni *et al.,* (2008). Many strategies including biological control such as *Aspergillus niger, Fusarium* and *Trichoderma* were suppressed aflatoxin production by *A. flavus* (Dharmaputra, 2003). *Trichoderma harzianum w*as antagonistic to toxigenic *A. flavus* (Dharmaputra, 2003). *T. harzianum* can produce amylolytic, pectinolytic, proteolytic and cellulolytic enzymes for growth inhibition of *A. flavus* and *Fusarium moniliforme* (Dharmaputra, 2003). The extract of *Penicillium oxalicum* showed fungicidal activity which affected *A. candidus*, *A. versicolor*, *P. griseofulvum* , *Curvularia trifolii* and *Botrytis cinerea* (Kabak, and Dobson, 2009). Recently, it has been proposed to reduce Ochratoxin A producing *Asperigillus niger* by means of biocontrol yeast, *Rhodotorula glutinis* Harrison **(**Yuan *et al*., 2004 **) .** Several isolates of *R. glutinis* reduced sporulation and established pathogen and disease severity (Haggag Wafaa and , Abdall, 2012). Several yeast species are known to accumulate carotenoid pigments as secondary metabolites. This present study assessed the effects of certain microbes on growth and aflatoxin production by *Aspergillus flavus* infection walnuts and Pistachio in vitro and in vivo.

MATERIAL AND METHODS

Fungal isolates

Aspergillus niger was isolated from walnuts and pistachio fruits in North of Seini. *Trichoderma harzianum* and *Penicillium oxalicum* isolated from soils of walnuts. Each fungal isolate was sub cultured on Potato Dextrose Agar (PDA) medium and incubated at 28 ± 2°C for 7 days. Fungal isolates were identified by morphological studies using PDA and 2% malt extract agar. *Trichoderma harzianum* and *P. oxalicum* were grown on potato dextrose broth containing 0.01% Tween 20 to dislodge conidia for seven days at 28°C under shaker. Culture filtrates of these fungi were then filtered through sterile cotton wool and membrane filter (0.2 µm) under sterile condition to obtain cell-free filtrates.

Selected yeast *Rhodotorula glutinis* isolate was grown on Malt extract agar slants for 48 hr. The fresh cultures were used to prepare suspensions at concentrations of 10^{7} cells/ ml in Tween-80 water.

Detection of aflatoxins under fluorescence (UV)

Potato dextrose agar was used as culture media. The cultures at 25°C for 4 days, and the presence or absence of fluorescence in the agar surrounding the assayed colonies was determined under UV light (365 nm) and expressed as positive or negative according to Franco *et al.* (1998).

Preparation of *A. flavus* spore suspension

Spores of fungal isolates were prepared by growing these fungi on PDA for 7 days before harvesting and filtering through sterile cotton wool by sterile distilled water plus 0.02 % tween 80. The numbers of spores were counted by haemacytometer.

Growth inhibition

Agar diffusion test

Twenty five ml of PDA were poured into a Petri dish (15 cm diameter) and left to set and 20 ml of PDA inoculated with spores of *A. flavus* IMI (10^6 spores/ml) were poured over the surface of PDA agar plate . Sterile cylinder cups (6mm. diameter) containing 300 µl of each culture filtrate were placed onto the same plates. Cylinder cup containing broth without any culture was served as a control. The plates were then incubated for 4 days at room temperature and the inhibition zones were determined after 3 and 5 days. All experiments were repeated at least five times.

To assess the inhibitory effect of isolated bioagents on *Aspergillus flavus* growth, the dry weight of the fungal mass in the agar between two streaks of biocontrol agents was determined

Control of sporulation

The ability of the bioagents to reduce sporulation of *A. flavus* can even be visualized. Agar strips were cut from the centerline of the PDA plates and transferred to 20ml Tween-80 water, bottles were capped and shaked at 150rpm for 60min. Conidia were counted microscopically using a hemocytometer. The content of the above mentioned bottles were boiled for 10min to dissolve the agar content. The warm suspensions were filtered through Wattman paper No.40. The fungal mass was washed once and dried at 50ºC for 24 hours .

Aflatoxin analysis

High-performance liquid chromatography

The contents of individual Petri dishes including agar and fungal culture were transferred into separate glass bottles and extracted with methanol (85%). Bottles were capped and shaked on a rotary shaker at 120 rpm for 90 min and the suspensions were centrifuged at 3500 rpm for 20 min. Supernatants were transferred to glass bottles and kept at room temperature for hours. Aflatoxin levels were measured using high performance

liquid chromatography (HPLC) (model: Perkin Elmer series 200 UV/VIS) with a C18 column that had an internal diameter of 300 x 3.9 mm. The HPLC apparatus was equipped with a UV detector, and fluorescence was measured using 365-nm excitation and 430-nm emission wavelengths. The mobile phase consisted of methanol: acetic acid: water (20:20:60 v/v/v). The total run time for the separation was approximately 30 min, and the flow rate was 1 ml/min (Christian, 1990).

Effect of culture filtrates of tested bioagents on dry weight and aflatoxin B1production of toxigenic *A. flavus*

One ml of conidial suspension of toxigenic *A. flavus* (1xl0^7 conidia/ml) was inoculated into 100,150 and 200 ml of sterilized culture filtrates derived from each treatment combination in a glass bottle volume 100 ml. They were then incubated at room temperature for 10 days. As control, toxigenic *A. flavus* was grown on SMKY liquid media. Three replications were used for each treatment (including the control). Observations were made on the dry weight of mycelia of toxigenic *A. flavus* and aflatoxin production. The dry weight of mycelia was determined by drying the fungal colonies in an oven at 70C for 4 days until a constant weight was attained (Abd El-Aziz *et al*., 2012). Aflatoxin B1 content was determined using a High Performance Liquid Chromatography method (Christian, 1990).

Effect of biocontrol agents on AFB1 produced by *A. flavus* on seeds samples

Stored seeds were treated with tested biocontrol agents at the above-mentioned concentrations and subsequently, these 20 g seeds grains were inoculated with 100,150 and 200 ml/kg suspension of tested biocontrol agents and incubated at 25°C for 5 days. For aflatoxin extraction, 20 g of tested samples were mixed with 100 ml of a 4% acetonitrile aqueous solution of potassium chloride (9:1), followed by shaking for 20 min and filtration through Whatman No. 4 .For purification, 100 ml of n-hexane was added to the filtrate, and the solution was shaken for 10 min. After separation, the upper phase (n-hexane) was discarded, and 50 ml of deionized water and 50 ml of chloroform were added to the lower phase. This solution was then shaken for 10 min. The upper phase was subsequently extracted twice more with 25 ml of chloroform, and the chloroform was evaporated at 40°C in a water bath at low speed. Subsequently, 2 ml of methanol was added, and the solution was filtered through a 0.45-µl filter (Zaboli *et al*., 2011).

RESULTS

Detection of aflatoxins under fluorescence (UV)

The presence or absence of fluorescence in the agar surrounding the assayed colonies was determined using UV light (365 nm) and was expressed as positive or negative. Data provide that the isolate of *A. flavus* was toxigenic and produce aflatoxin B1 (39.20 (µg/ml) (Data not showing) .

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Growth inhibition

Biocontrol isolates exhibited a wide range of inhibitory activity against *Aspergillus flavus* growth and aflatoxin production . The growth of *A. flavus* was restricted to and within the boundary of the two streaks of zone in the biocontrol test (Table 1). The yeast isolates had prominent inhibitory effect on colony expansion. *A. flavus* produced 7.27x106 spore /ml. The number of spores reduced to $2x10^5$ by *Rhodotorula glutinis* (Table 1). The dry weight of the fungal mass depended upon the bioagents strain on Petri dish and was significantly lower than control plate. Fungal mass was observed due to the inhibitory effect of *Rhodotorula glutinis*.(Table 1). All selected isolates of bioagents were effective in inhibiting aflatoxin biosynthesis of *A. flavus*.

The same results also obtained in AFB1 production. The toxic strain *A. flavus* produced 39.20 ppb aflatoxin per plate when grown on PDA. The amount of aflatoxin produced was drastically reduced when *A. flavus* was grown between the two streaks of *Rhodotorula glutinis* (0.6).

Table 1: Effect of antagonistic microorganisms on the toxigenic strain

Bioagents	Diameter of inhibition zone (mm) *	Biomass (mg/strip)	Spore (per ml)	AFB1 (ppb)
T. harzianum	30.5	17.6	$6 x10^5$	0.6
Penicillium oxalicum	15.5	18.4	$25x10^5$	1.44
Rhodotorula glutinis	33.5	12.4	$2x10^5$	0.5
Control	0.0	87.6	$7.2x10^6$	39.20
LSD	3.3	1.2		0.2

Efficacy of biocontrol agents on inhibition of *A. flavus*

Data in Table (2) show that the effect of culture filtrates of bioagents on the percentage frequently and the percentage of inhibition of toxigenic *A. flavus* was significantly different. The highest percentage of inhibition of toxigenic *A. flavus* was 91% caused by the culture filtrate of *Rhodotorula* at 200 ml/kg while the culture filtrate of *T. harzianum* was 86%.

Table 2: Efficacy of bioagents on % frequently and % inhibition of *A. flavus*

Conc. of culture filtrates of bioagents (ml/kg)	*T. harzianum*		*Penicillium oxalicum*		*Rhodotorula glutinis*	
	% frequently	% inhibition	% frequently	% inhibition	% frequently	% inhibition
100	40.1	59.8	54.8	46.4	26.6	73.2
150	12.8	78.2	22.8	77.6	10.8	90.2
200	9.8	91.3	11.8	88.1	3.7	97.7
Control	100	0	100	0	100	0
LSD	1.23	6.1	2.3	5.6	1.3	6.4

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Effect of culture filtrates of tested bioagents on dry weight of toxigenic *A. flavus*

Data in Table (3) show that the effect of culture filtrates of bioagents on dry weight of mycelia toxigenic *A. flavus* was significantly different. % inhibition of *A. flavus* dry weight reach to (97.5) at 200 ml/kg when treated with *T. harzianum*, while reach to (99.1) at 200ml/kg when treated with *Rhodotorula glutinis.*

Table 3: Effect of culture filtrates of tested bioagents on dry weight (g) of toxigenic *A. flavus*

Conc. of culture filtrates of bioagents (ml/kg)	*T. harzianum*		*Penicillium oxalicum*		*Rhodotorula glutinis*	
	Dry weight (g)	% inhibition	Dry weight (g)	% inhibition	Dry weight (g)	% inhibition
100	5.8	94.2	8.8	91.2	2.6	97.4
150	4.8	96.1	6.4	93.2	1.7	98.2
200	2.5	97.5	3.2	96.8	0.9	99.1
Control	100		100		100	
LSD	0.8	0.4	1.3	0.5	0.5	0.4

Effect of biocontrol agents on AFB1 produced by *A. flavus*.

Effect of culture filtrates of tested bioagents on aflatoxin B1 (µg/ml) production of toxigenic *A. flavus* obtained in Table (4). The highest percentage of inhibition of aflatoxin B1 production of toxigenic *A. flavus* caused by culture filtrate of *Rhodotorula* and *T. harzianum* (100%) at 150 ml/kg while aflatoxin B1 production was **97.6** % at the same concentration of *Penicillium oxalicum* culture filtrate.

Table 4: Effect of culture filtrates of tested bioagents on aflatoxin B1 (µg/ml) production of toxigenic *A. flavus*

Conc. of culture filtrates of bioagents (ml/kg)	*T. harzianum*		*Penicillium oxalicum*		*Rhodotorula glutinis*	
	B1 (µg/ml)	% inhibition	B1 (µg/ml)	% inhibition	B1 (µg/ml)	% inhibition
100	9.1	91.8	10.8	89.2	6.6	93.3
150	0	100	2.4	97.6	0	100
200	0	100	0	100	0	100
Control	100	0	100	0	100	0
LSD	0.4	1.5	0.6	1.6	0.8	1.1

Effect of spores suspension of tested bioagents on aflatoxin B1 production of toxigenic *A. flavus* on Walnuts and Pistachio seeds

Effect of spores suspension of tested bioagents on production of aflatoxin B1(µg/kg) on walnuts and pistachio seeds obtained in Table (5 and 6). Percentage of inhibition of aflatoxin B1 production increases whenever concentration spores suspension of tested bioagents. % inhibition ranged from (87.6 to 100%) caused by spores suspension of *Rhodotorula* and *T. harzianum* .

At the same time, percentage of seeds germination increased by treated with bioagents (Table 7). The highest percent by spores suspension of *Rhodotorula* and *T. harzianum* .

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Table 5: Effect of biocontrol agents on aflatoxin B1(µg/kg) on walnut seeds samples

Conc. of spores suspension of bioagents (ml/kg)	*T. harzianum*		*Penicillium oxalicum*		*Rhodotorula glutinis*	
	B1 (µg/kg)	% inhibition	B1 (µg/kg)	% inhibition	B1 (µg/kg)	% inhibition
100	20.8	79.2	23.4	76.6	12.4	87.6
150	3.5	96.5	4.9	95.1	0	100
200	0	100	0	100	0	100
Control	100	0	100	0	100	0
LSD	2.1	2.5	2.3	2.1	1.2	2.3

Table 6: Effect of biocontrol agents on aflatoxin B1(µg/kg) on Pistachio seeds samples

Conc. of spores suspension of bioagents (ml/kg)	*T. harzianum*		*Penicillium oxalicum*		*Rhodotorula glutinis*	
	B1 (µg/kg)	% inhibition	B1 (µg/kg)	% inhibition	B1 (µg/kg)	% inhibition
100	18.8	87.2	21.7	88.3	15.8	84.2
150	2.4	97.6	4.5	95.5	0	100
200	0	100	0	100	0	100
Control	100	0	100	0	100	0
LSD	2.1	2.2	2.7	2.5	2.1	2.4

Table 7: Effect of biocontrol agents on seeds germination of Pistachio seeds samples

Conc. of spores suspension of bioagents (ml/kg)	*T. harzianum*		*Penicillium oxalicum*		*Rhodotorula glutinis*	
	Walnuts	Pistachio	Walnuts	Pistachio	Walnuts	Pistachio
100	89.8	88.8	88.6	90.9	84.5	91.7
150	2.4	97.6	4.5	95.5	100	100
200	100	100	100	100	100	100
LSD	2.1	2.2	2.7	2.5	2.1	2.4

DISCUSSIONS

Several mycotoxins in agricultural products cause health hazards to people and animals and economical problem. They are pathologically classified as hepatotoxins, nephrotoxins, vomitoxin and neuro-musculotoxin, some of which are potentially carcinogenic and mutagenic. Toxin-producing fungi may invade at pre-harvesting period, harvest-time, during post-harvest handling and in storage. According to the site where fungi infest grains, toxinogenic fungi can be divided into three groups: (a) field fungi; (b) storage fungi; and (c) advanced deterioration fungi. The first category includes species of plant pathogenic fungi, namely, genus *Fusarium, e.g. F. moniliforme, F roseus, F. tricinctum and F. nivale.* The "storage fungi" are principally the general *Aspergillius and Penicillium*, e.g. *A. flavus* and *A. parasiticus (*Cavin *et al.* 2007). Various strategies have been developed to prevent OA – food contamination. Some microorganisms have been proven to prevent the growth of ochratoxigenic fungi and OA production. They could be used as natural control material. The use of chemical fungicides has resulted in development of pest resistance and resurgence. In addition, use of fungicides in certain agricultural systems is impractical due to the expense, risk of environmental pollution, and negative effects to human health (Peterson *et al.,* 2001). Accordingly, microorganisms naturally present in agricultural ecosystems are being studied as environmentally compatible alternatives to traditional

chemical methods for controlling plant diseases and fungi associated with mycotoxin production. Biological control can reduce the harmful effect of phytopathogenic or mycotoxigenic fungi while having a minimal impact on the environment (Pal and Gardener, 2006). Contamination by mycotoxins such as aflatoxin in tree nuts, peanuts, corn and cottonseed is a serious food safety hazard to both humans and animals. Biocontrol of aflatoxin-producing strains by atoxigenic strains of *A. flavus* is being developed for corn, cottonseed, peanuts, rice kernels and wheat seed (Kabak and Dobson 2009). *Trichoderma* spp. is biological control agent for certain fungal plant diseases (Dorner *et al*., 2003). *Trichoderma spp* involves a complementary action of antibiosis, nutrient competition and cell wall degrading enzymes such as 1, 3-glucanases, chitinases and proteases (Choudary *et al*., 2003 and Sinha and Choudhary, 2008). *T. harzianum* have been reported to be inhibiting the growth of *A. flavus* and complete inhibition the production of aflatoxin B (Reddy *et al*., 2010). *Penicillium oxalicum* isolated from soil produced antifungal substances effectively suppressed the mycelia growth of *A. flavus.* It is likely that the *Rhodotorula* produce diffusible compounds which inhibit aflatoxin biosynthesis of *A. flavus*. *Rhodotorula* not only restrict the growth of the fungus but also reduce sporulation. The yeast antagonists appear to exert multiple modes of action on *A. flavus*. The development of resistance by the fungus to these yeasts should be more difficult than to those biocontrol agents having a single mode of- action such as antibiosis. The selected yeast isolates reduced the population of *A. flavus* by inhibiting conidia production and suppressing the growth of the fungus and reduced aflatoxin production. These results are compatible with the data published by Hua, (1999). Yeast species are promising biocontrol agents because they do not produce allergenic spores and they are usually nonpathogenic. A number of commercial yeast products have been developed in recent years. The use of yeasts in postharvest biocontrol formulations apparently presents advantages over other organisms. Yeasts are easy to cultivate, fast growing and are present in a variety of environmental niches (Janisiewicz, and Korsten, 2002). Biological detoxification of mycotoxins works mainly via two major processes, sorption and enzymatic degradation, both of which can be achieved by biological systems (Halász *et al*., 2009). Thus this may lead to the discovery of new biological control agents for controlling the growth of aflatoxin producing fungi. Further studies have to be conducted in order to identify these antifungal substances.

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