Effectiveness of nontoxigenic *Aspergillus flavus* and *Trichoderma harzianum* as biocontrol agents on aflatoxin B1 producing by *Aspergillus flavus* isolated from Cashew

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Abstract: The objective of this study was to evaluate biological control using atoxigenic *Aspergillus flavus* and *Trichoderma harzianum* for inhibition aflatoxin B1 produced by *Aspergillus flavus* was isolated from Cashew nuts. The effect of culture filtrates of nontoxigenic *A. flavus* and *T. harzianum* on the percentage frequently and % inhibition of toxigenic *A. flavus* was significantly different. Nevertheless, the highest percentage of inhibition of toxigenic *A. flavus* was 89% caused by the culture filtrate of nontoxigenic *A. flavus* at 200 mg/kg while *T. harzianum* was 83% and % inhibition of *A. flavus* dry weight reach to (81.8%) at 200ml/kg when treated with nontoxigenic *A. flavus*, while reach to (77.8%) when treated with *T. harzianum.* Percentage of inhibition of aflatoxin B1 production increases whenever concentration cultures filtrate of two tested bioagents. % inhibition ranged from (68.8 to 100%) caused by culture filtrate of *T. harzianum* while ranged from (63.2 to 100%) caused by nontoxigenic *A. flavus* culture filtrate.
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1. Introduction

Cashew plant (*Anacardium occidentale* L.) belonging to the family Anacardiaceae is a small tree, 7 - 20 m length, and seeds have a reniform shape (Perozo *et al*., 2006). Worldwide cashew nuts are known expensive and delicious flavor (Sindoni *et al*., 2005) Besides, cashew nut is 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 and Penicillium (Francisco *et al*., 1999; Doyle *et al*., 2001; Adebajo and Diyaolu, 2003; Acevedo *et al*., 2011).

Mycotoxins are secondary fungal metabolites that contaminate agricultural commodities and can cause diseases or death in humans and animals have a significant economic impact worldwide. *Aspergillus* is one of the most important large genus genera. More than twenty five genes are involved in pathway synthesized aflatoxins (Pearson *et al*., 1999).

The most toxic of this group, is the most potent carcinogen known. Because of their high toxicity, the presence of aflatoxins in food commodities is believed to pose a risk to human health (Leszczynska *et al*., 2000) aflatoxin B1 (AFB1) is considered as the most dangerous toxic metabolite because of its hepatotoxic, teratogenic, immunosuppressive and mutagenic nature.

The International Agency for Research on Cancer has classified AFB1 as a probable human carcinogen (IARC, 1993). Possible presence of highly toxic and carcinogenic mycotoxins in foods and foodstuffs has led to extensive research involving methods for inhibiting the synthesis of mycotoxins and of aflatoxin in particular. Mycotoxin contamination is a serious concern that occurs in the field before harvest or during storage, despite efforts of prevention .Therefore, it is important to find practical, cost effective and non-toxic methods. 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 (Dorner, 2004; Shetty and Jespersen, 2006). Many strategies including biological control such as *Aspergillus niger, Fusarium*, *Trichoderma*, and *Rhizopus* sp. were suppressed aflatoxin production by *A. flavus* (Dharmaputra, 2003). Addition of a non aflatoxigenic strain of *A. flavus* to soil around plants was inhibited aflatoxin contamination (Cotty, 1992; Dorner *et al*., 2003).

Trichoderma viride was found to inhibit the production of aflatoxin B1 (73.5%) and aflatoxin G1 (100%) when cultured with *A. flavus* (Bilgrami and Choudhary 1998). *Bacillus pumilus* is also reported to inhibit the growth and aflatoxin production by *A. flavus* to the extent of 99.2% (Sinha and Choudhary, 2008). *Trichoderma harzianum* were antagonistic to

toxigenic *A. flavus* (Dharmaputra, 2003), *Lactobacillus casei* (Chang and Kim, 2007).

This present study assessed the effects of certain microbes on growth and aflatoxin production by *Aspergillus flavus* infection cashew nuts.

2. Material and Methods

Fungal isolates

Fungal isolates used were toxigenic and nontoxigenic *A. flavus* were isolated from cashew in Riyadh, Saudi Arabia in May 2013, *Trichoderma harzianum*, isolated from soils of Palm. Each fungal isolate was sub cultured on Potato Dextrose Agar (PDA) medium and incubated at 28 ± 2°C for 7 days. The identification of isolates was confirmed by the Regional Center of Fungi and their Applications, Al-Azhar University, Cairo, Egypt.

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 biocontrol agents

Trichoderma harzianum and nontoxigenic *A. flavus* were grown on potato dextrose broth containing 0.01% Tween 20 to dislodge conidia for seven days at 28°C under shaker and the culture filtrate was collected aseptically by filtration using Whatman No 4 to get cell-free filtrate (Choudary *et al*., 2007).

Preparation of *A. flavus* spore suspension

Stock culture of aflatoxin B1 producing *A. flavus* was maintained on potato dextrose agar slants at 4°C and was harvested by adding 10 ml of sterile distilled water to get the final concentration to 10^7 spore/ml. The concentrations of conidia were counted using a haemacytometer.

Efficacy of biocontrol agents on % frequently of *A. flavus*

The stored seeds of cashew were treated with culture filtrates of tested bioagents at a concentration of 100,150 and 200 ml/ kg (Reddy *et al*., 2010). One hundred cashew seeds were treated with these culture filtrates and plated on potato dextrose agar medium containing traces of Rose Bengal (Cotty, 1994). The plates were then incubated for 5 days at 28°C and the incidence of *A. flavus* colonies was counted and percent inhibition calculated. For each treatment of biocontrol agent, 3 replications were maintained; whereas sterile broth used for the growth of biocontrol agents served as control.

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 70Cfor 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 cashew samples

Stored cashew were treated with tested biocontrol agents at the above-mentioned concentrations and subsequently, these 20 g cashew 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).

High-performance liquid chromatography

Aflatoxin levels were measured using high-performance liquid chromatography (HPLC) (model: PerkinElmer 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).

3. 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. The data presented in Figure 1 show that non-aflatoxigenic aspergilli did not display fluorescence, whereas the aflatoxigenic strains were positive for fluorescence. Data in Table (1) and figure (1) proof that the one isolate of *A. flavus* was nontoxigenic and can use it as biocontrol agent for toxigenic *A. flavus*.

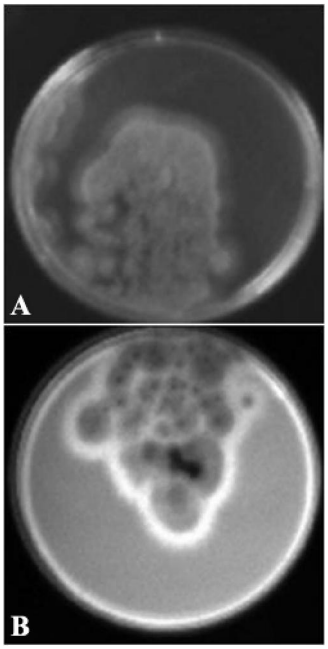

Figure 1. *A. flavus* isolate , under 365 nm UV light, (A) non-aflatoxigenic isolate (B) aflatoxigenic isolate of *A. flavus* (The white ring around the colony faint blue fluorescence)

Table 1. Detection of aflatoxin B1 produce by two tested *A. flavus* by HPLC

Isolates	B1 (µg/ml)
Nontoxigenic *A. flavus*	0.0
Toxigenic *A. flavus*	37.23

Efficacy of biocontrol agents on % frequently of *A. flavus*

Data in Table (2) show that the effect of culture filtrates of nontoxigenic *A. flavus* and *T. harzianum* on the percentage frequently and % inhibition of toxigenic *A. flavus* was significantly different. Nevertheless, the highest percentage of inhibition of toxigenic *A. flavus* was 89% caused by the culture filtrate of nontoxigenic *A. flavus* at 200 mg/kg while the culture filtrate of *T. harzianum* was 83%. It indicated that nontoxigenic *A. flavus* was more competitive to toxigenic *A. flavus* compared with *T. harzianum*.

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

Conc. of bioagents (ml/kg)	*T. harzianum*		Nontoxigenic *A. flavus*	
	% frequently	% inhibition	% frequently	% inhibition
100	57.00	43.00	68	32.00
150	22.00	78.00	31	69.00
200	11.00	89.00	17	83.00
Control	100.00	0.0	100.00	0.0

LSD at 5% for Bioagent (b) = 13
Concentration (C) = 16
BxC = 21

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 nontoxigenic *A. flavus* and *T. harzianum* on dry weight of mycelia toxigenic *A. flavus* was not significantly different. % inhibition of *A. flavus* dry weight reach to (81.8%) at 200 ml/kg when treated with nontoxigenic *A. flavus*, while reach to (77.8%) at 200ml/kg when treated with *T. harzianum*.

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

Conc. of bioagents (ml/kg)	*T. harzianum*		Nontoxigenic *A. flavus*	
	dry weight (g)	% inhibition	dry weight (g)	% inhibition
100	3.68	27.9	3.28	35.8
150	2.10	58.9	1.86	63.0
200	1.13	77.8	0.93	81.8
Control	5.11	0.0	5.11	0.0

LSD at 5% for bioagent (b) = 1.05
Concentration (C) = 0.84
bxc = 0.91

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

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 *T. harzianum* (100%) at 150 ml/kg while aflatoxin B1 production was 90.5% at the same concentration of nontoxigenic *A. flavus* culture filtrate.

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

Conc. of bioagents (ml/kg)	*T. harzianum*		Nontoxigenic *A. flavus*	
	B1 (µg/ml)	% inhibition	B1 (µg/ml)	% inhibition
100	10.4	55.2	12.1	47.8
150	0.0	100.0	2.2	90.5
200	0.0	100.0	0.0	100.0
Control	23.2	0.0	23.2	0.0

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

Effect of culture filtrates of tested bioagents on natural production of aflatoxin B1(µg/kg) on cashew samples obtained in Table (5). Percentage of inhibition of aflatoxin B1 production increases whenever concentration culture filtrate of two tested bioagents. % inhibition ranged from (68.8 to 100%) caused by culture filtrate of *T. harzianum* while ranged from (63.2 to 100%) caused by nontoxigenic *A. flavus* culture filtrate.

Table 5. Effect of biocontrol agents on aflatoxin B1(µg/kg) on cashew samples

Conc. Of bioagents (ml/kg)	*T. harzianum*		Nontoxigenic *A. flavus*	
	B1 (µg/kg)	% inhibition	B1 (µg/kg)	% inhibition
100	21.3	68.8	25.1	63.2
150	9.2	86.5	13.7	79.9
200	0.0	100.0	0.0	100.0
Control	68.3	0.0	68.3	0.0

4. Discussions

The aflatoxin biosynthetic pathway consists of at least 18 multi enzymatic (Wang *et al*., 2008). Some of the genes responsible to express the required enzymes involved in aflatoxin biosynthesis (Phillips *et al*., 2008). The aflatoxin contamination in crops can be minimized by early harvest, prevention of insect damage and proper storage, but not completely (Trowbridge *et al*., 2008; Brown *et al*., 2003).

Hence they can be used to displace toxigenic strains. Following that, various tests of competitive ability of an atoxigenic *A. flavus* strain to inhibit the aflatoxin contamination were performed under several approaches and interestingly found effective (Betran and Isakeit, 2004). The atoxigenic strains of *A. flavus* were reported to play important role in aflatoxin bio-control, this strategy is based on the application of nontoxigenic strains to competitively exclude naturally toxigenic strains in the same niche and compete for crop substrates (Yin *et al*., 2008; Hell *et al*., 2008).

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 D'Mello *et al*., 1998; 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 (Sinha and Choudhary, 2008, Bhatnagar et al., 2008; El-Katatny *et al*., 2004).

T. harzianum and nontoxigenic *A. flavus* have been reported to be inhibiting the growth of *A. flavus* and complete inhibition the production of aflatoxin B (Dharmaputra, 2003; Reddy *et al*., 2010). Biological detoxification of mycotoxins works mainly via two major processes, sorption and enzymatic degradation, both of which can be achieved by biological systems (Yin *et al*., 2008; Halász *et al*., 2009).

Live microorganisms can absorb either by attaching the mycotoxin to their cell wall components or by active internalization and accumulation. Dead microorganisms too can absorb mycotoxins.

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