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Antagonistic Activities of Some Fungal Strains against the Toxigenic *Aspergillus flavus* Isolate and its Aflatoxins Productivity

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Antagonistic activities of 14 fungal isolates were evaluated *in vitro* against the toxigenic *Aspergillus flavus* strain isolated form wheat in Saudi Arabia using dual culture technique. Eight of the fourteen fungal strains showed an inhibitory effect to *Aspergillus flavus*. Non-toxigenic strain of *A. flavus* and *Trichoderma harzianum* proved to be highly antagonistic and completely inhibited the aflatoxin B_2 produced by the toxigenic *A. flavus* isolate. Non-toxigenic *A. flavus* isolate, *T. harzianum, T. viride*, *T. sp* and *Fusarium moniliforme* showed a strong antagonism and inhibited aflatoxins production by 94.13, 91.2, 82.91, 80.1 and 77.68%, respectively. The aggressive behavior towards the toxigenic *A. flavus* demonstrated by *T. harzianum* could be explained by mycoparasitism while the inhibitory effect of *T. viride* and other antagonistic isolates could be explained by liberation of extracellular enzymes secreted by these fungi or production of an inhibitory volatile compounds. Non-toxigenic *A. flavus* isolate was able to inhibit aflatoxins production by interruption the aflatoxins biosynthetic pathway enzymes in the toxigenic *A .flavus* strain. These fungal strains which proved to be effective and environmentally safer are useful in limiting or preventing toxigensis of *A.flavus* isolate and may be applicable as biological control avoiding unwanted health and environment risks of chemical fungicides.

Key words: Antagonistic fungi, Biological control, Toxigenic *Aspergillus flavus*.

Aflatoxins are polypeptide-derived secondary metabolites which are highly toxic, mutagenic and carcinogenic to animal and human. They are stable under normal food processing conditions and can therefore be present not only in food and feed but also in processed products threaten both human and animal health (Reddy *et al*., 2010). The outbreak of mycotoxins all over the world is considered a major risk factor to public health and according to (FAO), 25% of the world commodities are annually affected by known mycotoxins (Schalzmayr *et al*., 2006). In order to minimize potential human exposure to aflatoxins, a stringent regulations of maximum aflatoxins levels permitted in foods and feeds have been set as low as 4 ppb. According to WHO and FAO regulation, the content of aflatoxin B_1 (AFB_1) should be less than 0.5 µg/Kg (Chang and Hua, 2007). United State federal guide lines for food and feed set a limit of 20 ng/gm total aflatoxins, while the European Union guide lines are more strict, with a limit of 1.0 ng/gm for AFB_1 and 4.0 ng/gm for total aflatoxins (Van Egmond and Jonker, 2004). Aflatoxins are maninly produced by the fungal species *Aspergillus flavus* and *A. parasiticus*. Species of *A. flavus* occur as saprophytes in soil and

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contaminate a wide variety of agricultural products in the field, processing plants and storage areas. *A. flavus* strains are varied greately in their aflatoxins productivity. Some *A. flavus* strains produce both aflatoxin B_1 and B_2 whereas other strains are non-toxigenic (Horn, 2003 and Dorner, 2004). Contaminated foods with aflatoxins and toxigenic fungi received world wide attention due to their deleterious effect on human and animal health as well as their importance in international food trade. Prevention of aflatoxins contamination in food and feed stuffs has a vital concern to minimize their hazards to human health. Strategies have been established to control aflatoxins contamination. Traditional control strategies (using certain cultural practices, pesticides and resistant varieties) which effectively reduces populations of many plant pests in field have not been effective in controlling aflatoxins producing fungi (Garland and Barr, 1998). Biological control using plant extracts and microbial antagonists either alone or as a part of integrated control strategy has a promising approach for aflatoxins control. Results of previous studies (Bueno *et al*., 2006; Zhang *et al*., 2008; Shetty &Jespersen, 2006; Shetty *et al*., 2007; Sidhu *et al*., 2009; Al-Rahmah *et al*.,2011 and Mostafa *et al*., 2011) indicated that bacteria, yeast and certain plant extracts may have potential as biocontrol agent for reducing aflatoxins contamination. Two biological control strategies using microbial antagonists are involved in *vitro*. One of the main strategies involves competitive co-inhabitant of different fungal strains suppressing aflatoxins produced by the toxigenic *A. flavus* isolates. Several microorganisms including yeast, bacteria and fungi have been tested for their ability to reduce both fungal infection and aflatoxins contamination. Many researchers have applied fungal strains as biocompetitive agents to prevent colonization of wheat grains by the toxigenic *A. flavus* and inhibit their aflatoxins biosynthensis (Dorner *et al*., 1999, Cvetnic & Pepeljnjak, 2007; Misra *et al*., 2010; Reddy *et al*., 2009 & 2010; Degola *et al*., 2011 and Atayde *et al*., 2012). Another approach is based on the displacement of toxigenic *Aspergillus flavus* isolate using atoxigenic aspergilli isolates of the same species. Non-toxigenic strains of *A. flavus* are being used widely as biological control agents to prevent pre-harvest aflatoxins contamination of the crops. Early studies by Brown *et al*. (1991), Choudhary (1992), Cotty & Bhatnagar (1994) and Garber & Cotty (1997) showed that five nonaflatoxigenic strains of *A. flavus* were able to inhibit aflatoxins production by the toxigenic isolate in liquid medium at different levels, when inoculated before the toxigenic strain and some of nonaflatoxigenic strains interrupted the aflatoxins biosynthetic pathway enzymes. Other researchers have applied atoxigenic *A. flavus* to the planted soil to assess the ability of the atoxigenic strain to competitively exclude aflatoxin-producing strains during crops plantation and thereby prevent aflatoxins contamination of crops (Abbas *et al*.,2006; Chang & Hua, 2007; Abbas *et al*., 2011 and Degola *et al*.,2011). The aim of this study, was to evaluate microbial interaction between fourteen fungal strains isolated from wheat seeds in respect to their ability to reduce or inhibit aflatoxins production by the toxigenic *A.flavus* isolate *in vitro* using liquid medium.

MATERIALS AND METHODS

Fungal strains

The toxigenic *Aspergillus flavus* and fourteen nontoxigenic fungal strains used in this study (Table, 1) were provided from the culture collection of Botany and Microbiology dept. King Saudi University, Riyadh, K.S.A. The toxigenic *A. flavus* was isolated from *Triticum* spp. and identified with other Aspergilli on the basis of colony and morphological characteristics (Raper and Fennell, 1977) while other fungal strains isolated from wheat grains were identified according to their macro- and microscopic characteristics (Barnett & Hunter, 1972 and Nelson *et al*., 1983).

Detection of fungal aflatoxins productivity

The toxigenicity of all tested fungal strains (15 strians) was detected using coconut milk agar medium (CMA) (Ordaz *et al*., 2003). The fungal strains were cultivated in CMA medium and incubated at $25 \pm 2\ ^0$C for 5 days to detect their aflatoxins productivity through visualization of blue or greenish blue fluorescence zone surrounding colonies under UV light (365 nm). All experiments were duplicated and the results were reported as positive (+ ve) or negative (- ve) productivity.

Evaluation of fungal antagonistic activity

Initial screenings of the antagonistic activity of the non-toxigenic *A. flavus* and thirteen fungal strains to compete with the toxigenic isolate *A. flavus*, were achieved on potato dextrose agar plates using dual culture technique (Table, 1). Mycelia discs (6 mm) of the toxigenic *A. flavus* isolate and the other antagonistic fungal strains were simultaneously inoculated in sterile plates containing (PDA) at a distance of 4mm from each other. PDA plates inoculated with the toxigenic *A. flavus* alone served as control. Three replicates were maintained for each treatments and the plates were incubated at 25 ± 2 ^{0}C. When the mycelial growth in the control treatment covers all medium surface, all plates were examined and the percentage of reduction in mycelial growth of the toxigenic *A. flavus* were calculated and recorded. Among several fungal species screened for their antagonistic activities, the fungi listed in Table (2) were chosen as the most effective antagonistic fungi and their bioactivity were re-evaluated. The tangent areas between the most effective antagonistic fungal isolates and the toxigenic *A. flavus* were examined for detection of mycoparasitism.

Effect of culture filtrate of biocontrol agents

In this investigation, we have utilized cell free filtrates which suggests the presence of more than a single antifungal compound (Reddy *et al*.,2009). The most effective antagonistic fungal strains were grown on 50 ml of potato dextrose broth with shaking at 25 ± 2 ^{0}C for seven days. The fungal cultures were filtered though double layers of muslin, centrifuged at 9000 rpm for 10 min and finally sterilized through Millipore filter (0.22). An equal volumes of both sterilized fungal antagonistic filtrates and sterilized double strength potato dextrose broth were mixed to obtain final concentration of 50% of each antagonistic fungal filtrates. Control set with dist water was kept parallel to the treatment sets. The flasks were inoculated with discs of 6 mm diameter of the toxigenic *A. flavus* isolate and incubated at 25 ± 2 ^{0}C for 7 days. After incubation, content of each flask was filtered (Whatman paper No. 1) and biomass of the filtered mycelium was determined after drying at 80 ^{0}C for 3 days till their weights remain constant. The percentage of mycelia inhibition was calculated (Table,) using the following formula:

Percentage of mycelial inhibition = [C – T / C] x 100

(Where, C and T are the mycelial dry weight (mg) in control and treatment respectively)

Fungal strains and spore suspensions

Stock cultures of the toxigenic *A. flavus* with the antagonistic fungal strains were maintained on potato dextrose agar slant, harvested by adding 5 ml of strile saline solution then filtered through double layers of strerile muslin. For quantitification of the fungal spores, haemocytometer cell counting method described by Mahlo *et al*., (2010) was used for counting spore suspensions of each fungal strain. Appropriate dilutions were used and the number of fungal spores per ml were made by microscopic enumeration using haemocytometer. The final spore concentration was adjusted to approximately 10^6 spores/ml.

Mycological co-inhabitant effect of the antagonistic fungal strains on aflatoxins productivity

For detecting the influence of the antagonistic fungal isolates on aflatoxins production by the toxigenic *A. flavus* strain, yeast extract sucrose broth (YES) medium was prepared, adjusted at pH; 5.6 and dispensed into 150 ml flasks each containing 50 ml. Flasks were autoclaved and inoculated with 1ml of spore suspensions (10^6 spores/ml) of the antagonistic fungi and re-inoculated with 1ml of spore suspensions (10^6 spores/ml) of the toxigenic *A. flavus* strain. A positive control flasks were inoculated with 1ml of spore suspensions of the toxigenic *A. flavus* alone. Three flasks were prepared for each treatment and incubated at 25 ± 2 ^{0}C for 7 days. For aflatoxins extractions, the filtrates of each flask was treated three times with 50 ml of chloroform in a separating funnel. The chloroform extract was separated and dehydrated with anhydrous sodium sulfate and evaporated till dryness on water bath at 50 ^{o}C under vacuum. The residues were dissolved in 1 ml chloroform and 50 ul of chloroform extract spotted on TLC plates (20 x 20 cm^2) coated with 0.25 mm thick silica gel (Alugram, Germany). The plates were developed with benzene-methanol- acetic acid (95 : 5 : 5 v/v/v) solvent system described by (AOAC, 1995) and aflatoxin B_1 & B_2 were detected by visual examination of TLC plates under UV lamp at 365 nm and comparison of the fluorescent band

with that of the standard aflatoxins. The presence of aflatoxins were confirmed chemically by spraying trifluoroacetic acid (Bankole and Joda, 2004). Quantification of aflatoxins B_1 and B_2 were done by photodensitometry (Bio-metera- Germany) comparing the area and density of the spot samples with aflatoxins standard (Supelco - USA). Aflatoxin B_1 & B_2 content were expressed in terms of μgl^{-1} and aflatoxins inhibition was calculated as follow; percentage of inhibition = [Y – X / Y] x 100, where X" is the concentration of aflatoxins in treated sample and Y" is the concentration of aflatoxins in control.

RESULTS

The screening of aflatoxins producing abilities of all fungal strains used in this experiment were achieved using coconut milk agar through visualization under U.V light (365 nm). The toxigenic *A. flavus* colony showed blue fluorsence zone in CMA after 5 days of incubation indicating its ability to produce aflatoxins. None of the fungal strains isolated from wheat seeds nor atoxigenic *A. flavus* isolate showed a fluorescence zone. These results were recorded in Table (1) and ascertained that all fungal strains and atoxigenic *A.flavus* isolate failed to produce aflatoxins and that was easily detected by absence of blue fluorescence zones surrounding their colonies. Also, screening of antagonistic activities of the same fungal strains isolated from wheat grains represented in same table indicated that the highest antagonistic activities were detected when *Trichoderma viride, Mucor hiemalis, T. harazianum, T.* sp, *Fusarium moniliforme* and *Rhizopus nigricans* were applied against the toxigenic *A.flavus* strain. Moderate activities were also detected when *Phoma glomerata* and atoxigenic *A. flavus* strains were used while all other isolated fungal strains showed slight antagonistic activities against that toxigenic *A. flavus* isolate. Among several fungal species isolated from wheat grains, the fungal strains listed in Table (2) were chosen as the most effective antagonistic fungi screened against the toxigenic *A. flavus* isolate. Re-evaluation of the antagonistic activities of the most effective fungal strains against the mycelial growth of the toxigenic *A. flavus* strain was presented in Table, 2 and illustrated in Fig (1).

Table 1. Screening of antagonistic activities and aflatoxins producing abilities of the fungal strains isolated from wheat grains

Antagonistic isolates	Potency of antagonism using dual culture technique	Aflatoxins producing ability using coconut milk agar medium
Aspergillus flavus (tox)	-	+ ve
A. flavus (Atox)	++	- ve
A. niger	+	- ve
A. tamarii	+	- ve
Alternaria alternata	+	- ve
Fusarium culmorum	+	- ve
F. moniliforme	+++	- ve
F. oxysproum	+	- ve
Mucor hiemalis	+++	- ve
Penicillium roquefortii	+	- ve
Phoma glomerata	++	- ve
Rhizopus nigricans	+++	- ve
Trichoderma sp.	+++	- ve
T. harzianum	+++	- ve
T. viride	+++	- ve

Comparative antagonistic activity: (-) no activity; (+) slight activity; (++) moderate activity and (+++) high activity
Aflatoxins producing ability: (+ ve) aflatoxins producer and (- ve) non-aflatoxins producer

The dual culture plates showed initial rapid growth of *T. viride, M. hiemalis, T. harazianum* and *F. moniliforme* overgrow the toxigenic *A. flavus* isolate resulting into completely inhibition of the latter. The highest percentage of reduction in mycelial growth of the toxigenic *A. flavus* isolate (89.72, 88.14, 83.62, 80.34 and 73.79%) were detected when *T. viride, M. hiemalis, T. harzianum, T.* sp. and *F. moniliforme* were used respectively. Isolates of *A. niger* and *F. oxysporum* showed the least inhibitory effect reducing mycelia growth of the toxigenic *A. flavus* to (21.47 and 16.05%) while *Rhizopus nigricans, Phoma glomerata* and nontoxigenic *A.flavus* isolate reduced mycelial growth of the toxigenic *A.flavus* by 68.02, 62.6 and 56.27% respectively. The examination of tangent areas between the most effective antagonistic fungal isolates and the toxigenic *A. flavus* strain showed the mycoparastic activity of *T. harzianum* only against the toxigenic strain. *T. harzianum* showed parasitic behavior against the toxigenic *A. flavus* strain (Fig.2), by coiling round the host hyphae (Fig.,2-A),by entangling the hyphae of *A. flavus* with infection

Tabel 2. Effect of antagonistic fungi on the reduction of mycelail growth of the toxigenic *A. flavus* isolate using double culture technique

Antagonistic isolates	Mean of colony diameter of the toxigenic *A. flauvs* (mm)	Percentage of mycelial growth inhibition
*Aspergillus flavus (Atox)**	38.70* ± 0.06	56.27
A. niger	69.50* ± 0.03	21.47
Fusarium moniliforme	23.20* ± 0.06	73.79
F. oxysproum	74.30* ± 0.02	16.05
Mucor hiemalis	10.50* ± 0.05	88.14
Phoma glomerata	33.10*± 0.08	62.60
Rhizopus nigricans	28.30* ± 0.06	68.02
Trichoderma sp.	17.40* ± 0.07	80.34
T. harzianum	9.10* ± 0.04	83.62
T. viride	14.50* ± 0.05	89.72
A. flavus (+ve control)	88.50* ± 0.03	00.00

Values in the same column followed by asterisk (*) are significantly different at (P=0.05)
Data are means (n=3) ± standard error of three replicates (*Atox*)*; non-toxigenic

Tabel 3. Effect of Millipore sterilized culture filtrates of the antagonistic fungi on mycelail growth of the toxigenic *A. flavus* isolate

Antagonistic isolates	Mycelial dry weights of the toxigenic *A. flauvs* (mg)	Percentage of mycelial growth inhibition
*Aspergillus flavus (Atox)**	423.2*± 0.009	0.00
A. niger	377.6*± 0.006	9.51
Fusarium moniliforme	99.7*± 0.004	76.11
F. oxysproum	402.9*± 0.005	3.45
Mucor hiemalis	161.3*± 0.008	61.35
Phoma glomerata	147.4* ± 0.002	64.68
Rhizopus nigricans	195.2* ± 0.005	53.22
Trichoderma sp.	78.6* ± 0.004	81.17
T. harzianum	131.9*± 0.002	68.39
T. viride	48.6* ± 0.005	88.35
A. flavus (+ve control)	417.3*± 0.007	00.00

Values in the same column followed by asterisk (*) are significantly different at (P = 0.05)
Data are means (n=3) ± standard error of three replicates (*Atox*)*; non-toxigenic

Table 4. Mycological co-inhabitants effect of antagonistic fungal strains on afaltoxins production by the toxigenic *A. flavus* isolate

Antagonistic isolates	Aflatoxins production (µg/50ml)				Total aflatoxin production (µg/50ml)	(%) of aflatoxins inhibition
	B_1		B_2			
	R_f	Conc.	R_f	Conc.		
*Aspergillus flavus (Atox)**	3.78	4.6	—	N.D	4.6	94.13
A. niger	3.78	41.7	3.51	31.9	73.6	6.12
Fusarium moniliforme	3.76	12.8	3.53	4.7	17.5	77.68
F. oxysporum	3.77	42.4	3.54	35.7	78.1	00.0
Mucor hiemalis	3.76	22.8	3.52	10.1	32.9	58.04
Phoma glomerata	3.78	15.3	3.51	7.1	22.4	71.43
Rhizopus nigricans	3.75	19.4	3.52	8.2	27.6	64.80
Trichoderma sp.	3.77	12.1	3.54	3.5	15.6	80.10
T. harzianum	3.76	6.9	3.53	N.D	6.9	91.20
T. viride	3.76	11.3	3.53	2.1	13.4	82.91
A. flavus (+ve control)	3.77	42.6	3.54	35.8	78.4	—-

Values in the same column followed by asterisk (*) are significantly different at (P=0.05) Data are means (n =3) ± standard error of three replicates (*Atox*)*; non-toxigenic
N.D; Not detected

pegs (Fig,2-B) and by coiling with pentration leading finally to its degradation (Fig., 2-C)**.** None of the other antagonistic fungi showed any mycoparasitic activity against the toxigenic *A. flavus* isolate as no sign of parasitic behavior were detected.

Millipore sterilized culture filtrates of the most effective antagonistic fungal strains were evaluated to compare their effects with those recorded by antagonistic isolates themselves against the linear growth of the toxigenic *A. flavus* strain and the results were recorded in Table, 3. It was obvious from Table, 3 that filtrates of the three *Trichoderma* isolates (*Trichoderma viride, T.* sp. and *F. moniliforme*) were the most effective in reducing the mycelial growth of the toxigenic *A. flavus* isolate compared with the other tested antagonistic filtrates. Highest reduction percentages (88.35, 81.17 and 76.11% respectively) in growth of toxigenic *A. flavus* were detected when filtrates of *Trichoderma viride*, *T.* sp. and *F. moniliforme* were used. On the other hand, filtrates of *M. hiemalis*, *T. harzianum* and *R. nigricans* showed a decreased inhibitory effects against the toxigenic *A. flavus* compared with their previous results obtained by dual culture technique and recorded inhibitions (61.35, 58.09 and 53.22%) respectively. On contrast, the filterate of *Phoma glomerata* showed slight increased inhibitory effects (64.68%) agaisnt the toxigenic *A. flavus* strain while filterate of non-toxigenic *A. flavus* strain enhanced the growth of the toxigenic *A. flavus* and increased its mycelial accumulation compared with control and those recorded in dual culture technique.

The ability of antagonistic fungal strains to inhibit aflatoxins produced by the toxigenic *A. flauvs* strain in (YES) broth was investigated and the results were presented in Table, 4. It is noticed that ability of the antagonistic fungal strains to inhibit aflatoxins production was variable where non-toxigenic strain of *A. flavus* and *T. harzianum* were highly effective and completely inhibited production of aflatoxin B_2 and decreased total aflatoxins level produced by the toxigenic *A. flavus* to 4.6 and 6.9µg /50ml. Non-toxigenic *A. flavus* strain and *T. harzianum* inhibited aflatoxins production with 94.13 and 91.2% respectively compared with aflatoxins level in control. Moreover, *T. viride*,, *T. sp*. and *F. moniliforme* showed a high ability to inhibit aflatoxins production by 82.91, 80.10 and 77.68% respectively whereas aflatoxins production was reduced with 71.43% by *Phoma glomerata*. On the other hand, *R. nigricans* and *M. hiemalis*., showed a moderate inhibitory effect and decreased the aflatoxins level

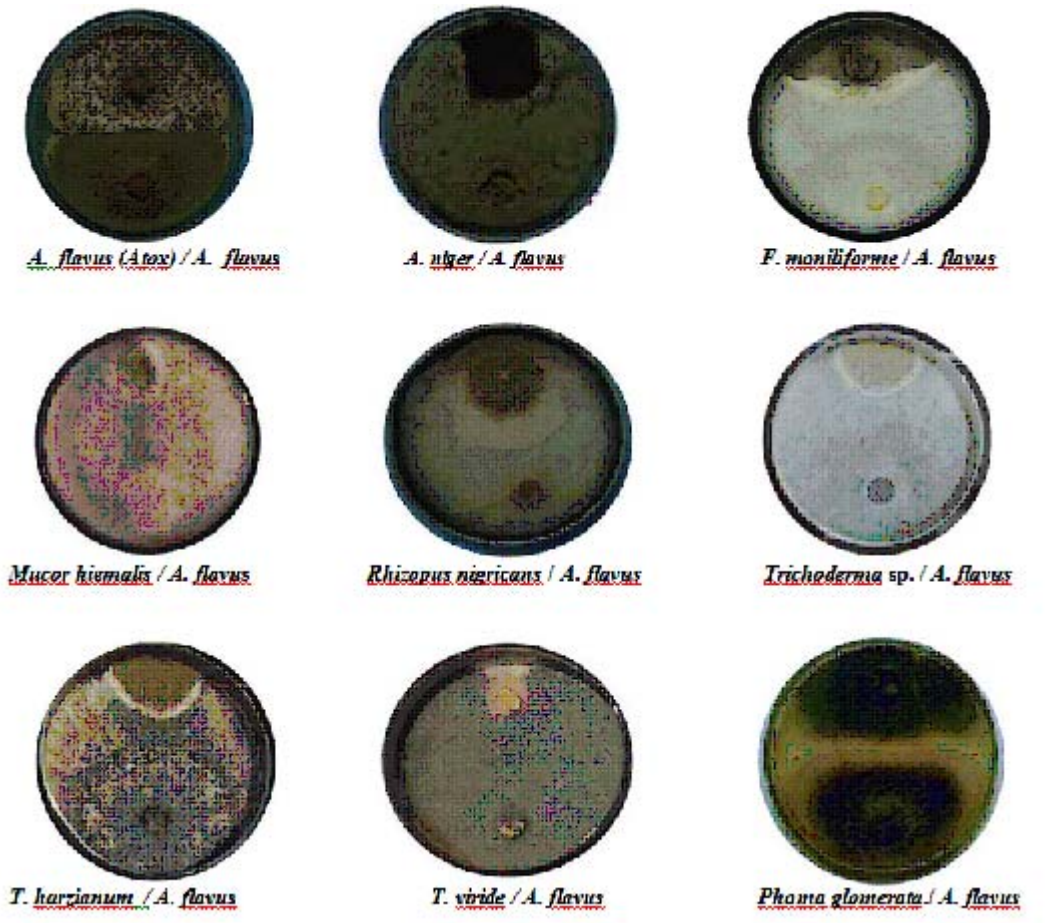
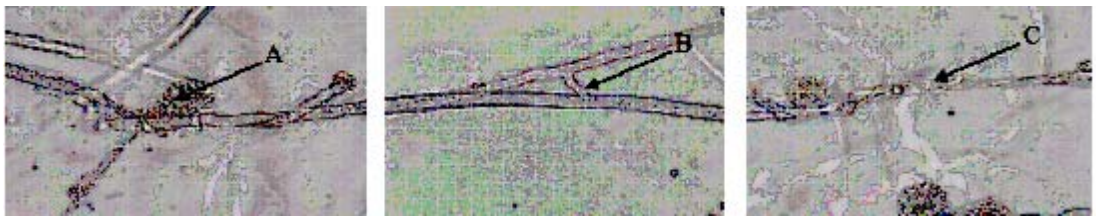


Fig. 1. Effect of the most effective antagonistic fungal strians against mycelial growth of the toxigenic *A. flavus* using dual culture technique



A; showing coiling of *T. harzianum* hyphae over the toxigenic *A. flavus*
B; showing infection pegs of *T. harzianum*
C; showing coiling of *T. harzianum* with disintegrated hyphae of toxigenic *A. flavus*

Fig. 2. Micrograph of light microscope showing mycoparasitic activity of *T. harzianum*:

by 64.80 and 58.04% respectively compared with that of control.

The least aflatoxins reduction was observed in case of *A. niger* decreasing the aflatoxins level by 6.12% while no inhibitory effect was detected when *F. oxysporum* was used.

DISCUSSION

For biological control of aflatoxins produced by the toxigenic *A. flavus* strain, 14 fungal isolates were isolated from wheat grains in Saudi Arabia and evaluated *in vitro* against the toxigenic

Aspergillus flavus using dual culture technique. Eight of the 14 fungal strains showed an inhibitory effect to mycelial growth of the toxigenic *A. flavus* strain. The most effective bioagents including *T. viride, M. hiemalis, T. harazianum, T.* sp, *F. moniliforme*, *R. nigricans*, *P. glomerata* and nontoxigenic *A. flavus* were used to suppress aflatoxins production as there is a strong debate about the safety aspects of fungicides in use since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity. The mycological co-inhabitant of antagonistic isolates with the toxigenic *A. flavus* strain showed that non-toxigenic isolate of *A. flavus* was highly effective in inhibition of aflatoxins production by the toxigenic *A. flavus*. These finding were in accordance with those of Dorner *et al* (1998), and Abbas *et al* (2011) who reported that nontoxigenic isolates of *A. flavus* were able to inhibit aflatoxins production by the toxigenic *A. flavus* strain in liquid medium and interrupt the aflatoxins biosynthetic pathway enzymes in the toxigenic strains.

The highly antagonistic effect of *T. harzianum* against the toxigenic fungi was explained by Claydon *et al*., (1999), El-Katatny *et al*. (2006) and Choudary *et al*., (2007) who ascertained that strong antagonistic effect of *T. harzianum* is positively correlated with the ability of this fungus to produce antifungal substances (Alkyl pyrones) like harzianolide and butenolide that inhibit fungal growth of the other fungi. Other researchers attributed the antagonistic effect of *T. harzianum* to its ability to produce lytic enzymes as chitobiosidase, endochitinase, glucan-1,3 β-glucosidase that degradate the consitituents of the toxigenic fungal cell wall leading to permeation of penetration pegs and rupture of cell constituents causing death (Roco and Perez, 2001).

On the other hand, Calistru (1997) showed that the culture filtrates of the *T. harzianum* and *T. viride* were inhibitory to growth and aflatoxins production of the toxigenic *A. flavus* strain. *T. viride* was found to be highly effective and completely suppressed aflatoxin G_1 production and inhibit production of aflatoxin B_1 with (73.5%) when they were co-cultured with *A. flavus*. Moreover, *T.viride* exerted several aflatoxins degradation substances such as dioctyl phthalate, methyl jasmonate, butabarbitol and cyclopentanione which were detected in medium filtrate and fungal mycelium. The presence of these degradation substances especially cyclopentanione indicated cleavage of cyclopentane ring of aflatoxins.

F. moniliforme, *P. glomerata* and the fast grower fungi as *M. hiemalis* and *R. nigricans* showed inhibitory effect on both mycelial growth and aflatoxins production when compared to control. The growth restriction and aflatoxins reduction in co-cultures may be caused by nutritional competition (as they considered as fast grower fungi) or by metabolites produced by these fungi which inhibit aflatoxins productivity of the toxigenic *A. flavus* isolate. *M. hiemalis* and *R. nigricans* has ability to degrade aflatoxins and that was confirmed by cleavage of aflatoxins structure through the presence of cyclopentane moiety, furan moieties and dioctyl phthalate in the medium filtrates and mycelium of the previous fungi

The results of the present study revealed that certain fungal strains isolated from agricultural commodity can effectively control mycelial growth and aflatoxins productivity of the toxigenic *A. flavus* isolate. On the basis of the these findings, nontoxigenic *A. flavus* strain, *T. harazianum*, *T. viride*, *M. hiemalis*, *F. moniliforme*, *R. nigricans*, *P. glomerata* can be recommended as potentially effective and environmently safer alternative fungicide to protect spoilage wheat seeds from the toxigenic *A.flavus* isolate. The development of a biological control trend can also be constructed on displacement of toxigenic isolate using non-toxigenic isolate of the same species. Finally, the application of fungal strains as biological control was found to be effective and can be considered as an attractive and alternative for biological control of aflatoxigenic fungi avoiding unwanted health and environmental risks of chemical fungicides.

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REFERENCES

1. A.O.A.C., Association of Official Analytical Chemistry., Official methods of analysis, 16th Ed. Washington, D.C. 20044 Sections. 49.1. 01; 49. 3. 04., 1995; P. 32.
2. Abbas, H.K., Weaver, M.A., Horn, B.W., Carbone, I., Monacell, J.T. and Shier, W.T., Selection of *Aspergillus flavus* isolates for biological control of aflatoxins in corn. Toxin Reviews, 2011; **30**(2-3): 59-70.
3. Abbas, H.K., Zablotowicz, R.M., Bruns, H.A. and Abel, C.A., Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillus flavus* isolates. *Biocont. Sci. & Technol*., 2006; **16**(5): 437-449.
4. Al-Rahmah, A. N., Mostafa, A. A. and Abel-Megeed, A., Antifungal and antiaflatoxigenic activities of some plant extracts. *Afr. J. Microbiol. Res*.; 2011; **5**(11): 1342 – 1348.
5. Atayde, D.D., Reis, T. A., Godoy, I.J., Zorzete, P., Reis, G.M. and Correa, B., Mycobiota and aflatoxins in a peanut variety grown in different regions in the state of Soa Paulo, Brazil. Crop Prot., 2012; **33**: 7-12.
6. Bankole, S.A. and Joda, A.O., Effect of lemon grass (*Cymbopgon citrates* Stapf.) powder and essential oil on mould deterioration and aflatoxin contamination of melon seeds (*Colocynthis citrullus* L.). *Afr. J. Biotechnol.*, 2004; **3**: 52-59.
7. Barnett, H.L. and Hunter, B.B., Illustrated Genera of Imperfect Fungi. 3rd Ed., Burgess publishing Company, Minnesota, USA. 1972; 241 pp.
8. Brown, R.L., Cotty, P.J. and Cleveland, T.E., Reduction in aflatoxin content of maize by atoxigenic strains of *Aspergillus flavus. J. Food. Protect*., 1991; **54**(8): 623 – 626.
9. Bueno, D.J., Silva, J.D., Oliver, G. and Gonzalez, S.N., *Lactobacillus casei* CRL431 and *Lactobacillus rhamnosus* CRL1224 as biological control for *Aspergillus flavus* strains. *J. Food Prot*., 2006; **69**: 2544-2548.
10. Calistru, C., Mclean, M. and Berjak, P., In *vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. A study of the production of extracellular metabolites by *Trichoderma* species. *Mycopathologia,* 1997; **137**(2): 115 –124.
11. Chang, P-K. and Hua, S-S.T., Nonaflatoxigenic *Aspergillus flavus* TX9-8 competitively prevents aflatoxin accumulation by *A. flavus* isolates of large and small sclerotial morphotypes. *Int. J. Food Microbiol*., 2007; **114**: 275 – 279.
12. Choudary, D.A., Reddy, K.R.N and Reddy, M.S., Antifungal activity and genetic variability of *Trichoderma harzianum* isolates. *J. Mycology & Plant Pathol*., 2007; **37**(2): 295-300.
13. Choudhary, A.K., Influence of microbial co-inhabitants on aflatoxin synthesis of *Aspergillus flavus* on maize kernels. *Letters. in Applied Microbiol*., 1992; **14**(4): 143 – 147.
14. Cotty, P.J. and Bhatnagar, D., Variability among atoxigenic *Aspergillus flavus* strains in ability to prevent aflatoxin contamination and production of aflatoxins biosynthetic pathway enzymes. *Appl. Environ. Microbiol*., 1994; **60**(7): 2248 – 2251.
15. Cvetnic, Z. and Pepeljnjak, S., Interaction between certain moulds and aflatoxin B_1 producer *Aspergillus flavus* NRRL3251. *Arh. Hig. Roda. Tokiskol*., 2007; **58**: 429-434.
16. Degola, F., Berni, E. and Restivo, F.M., Laboratory tests for assessing efficacy of Atoxigenic *Aspergillus flavus* strains as biocontrol agents. *Int. J. Food Microbiol*., 2011; **146**: 235- 243.
17. Dorner, J.W., Biological control of aflatoxin contamination of crops. *J. Toxicol. Toxin Rev.;* 2004; **23**: 425-450.
18. Dorner, J.W., Cole, R.J. and Balnkenship, P.D., Effect of inoculation rate of biological control agents on preharvest aflatoxin contamination of pea nuts. *Biological Control*, 1998; **12**(3): 171–176.
19. Dorner, J.W., Cole, R.J. and Wicklow, D.T., Aflatoxin reduction in corn through field application of competitive fungi. *Journal of Food Protection*, 1999; **62**(6): 650 – 656.
20. El-Katatny, M.H., Abdelzaher, H.M.A. and Shoulkamy, M.A., Antagonistic actions of *Pythium oligandrum* and *Trichoderma harzianum* against phytopathogenic fungi (*Fusarium oxysporum* and *Pythium ultimum* var. *ultimum*). *Archives of Phytopathology and Plant Pathology* 2006; **39**(4): 289-301.
21. Garber, R. K. and Cotty, P.J., Formation of sclerotia and aflatoxins in developing cotton bolls infected by the S-strain of *Aspergillus flavus* and potential for biocontrol with an atoxigenic strain. *Phytopathology*, 1997; **87**: 940-945.
22. Garland, T. and Barr, A.C., Toxic plants and other natural toxicants. Published by CAB international New York, USA, 1998; 585 pp.
23. Horn, B.W, Ecology and population biology of aflatoxigenic fungi in soil. *J. Toxicol. Toxin Rev.;* 2003; **22**: 351-379.
24. Mahlo, S. M., Mc-Gaw, L.J. and Eloff. J.N., Antifungal activity of leaf extracts from south

African trees. *Crop Prot.*, 2010; **29**: 1529 – 1533.
25. Misra, T., Dixit, J. and Singh, S., Effect of some co-existing moulds on aflatoxin production in wheat grains under competitive environment. *Ind. J. Sci. Res*., 2010; **1**(2):75 – 77.
26. Mostafa, A. A., Al-Rahmah, A.N. and Abdel-Megeed, A., Evaluation of some plant extracts for their antifungal and antiaflatoxigenic activities. *J. Med. Plant Res*., 2011; **5**(17): 4231 – 4238.
27. Nelson, P.E., Tousson, T.A. and Marasas, W.F.O., *Fusarium* species. An illustrated manual for identification. The Pennsylvania State University press, U.S.A. 1983; 193 pp.
28. Ordaz, J.J., Fente, C.A., Vazquez, B.I., Franco, C.M. and Cepeda, A., Development of a method for direct visual determination of aflatoxin production by colonies of the *Aspergillus flavus* group. *Int. J. Food Microbiol*., 2003; **83**: 219 – 225.
29. Raper, K.B. and Fennell, D. P., The genus *Aspergillus*, Krieger Publishing Co., Huntington, New York, 1977.
30. Reddy, K.R.N., Raghavender, C.R., Reddy, B.N. and Salleh, B., Biological control of *Aspergillus flavus* growth and subsequent aflatoxin B_1 production in sorghum grains. Afr. J. Biotechnol., 2010; **9**(27): 4247-4250.
31. Reddy, K.R.N., Reddy, C.S. and Muralidharan, K., Potential of botanicals and biocontrol agents on growth and aflatoxin production by *Aspergillus flavus* infecting rice grains. *Food Control*, 2009; **20**: 173-178.
32. Roco, A. and Perez, L.M., *In Vitro* biocontrol analysis of *Trichoderma harzianum* on *Alternaria alternata* in the presence of growth regulators. *Plant Biotechnology* 2001; **4**(2): 68-73.
33. Schalzmayr, G., Zehner, F., Taubel, M., Schatzmayr, D., Klimitsch, A. Loibner, A.P. and Binder, E.M., Microbiologicals for deactivating mycotoxins. *Mol. Nutr. Food Res*., 2006; **50**: 543 – 551.
34. Sidhu, O.P., Chandra, H. and Behl, H.M., Occurrence of aflatoxins in mahua (*Madhuca indica Gmel*) seeds: synergistic effect of plant extracts on inhibition of *Aspergillus flavus* growth and aflatoxins production. *Food Chem. Toxicol.*, 2009; **47**: 774-777.
35. Shetty, P.H., Hald, B. and Jespersen, L., Surface binding of aflatoxin B_1 by *Saccharomyces cerevisiae* strains with potential decontaminating abilities in indigenous fermented foods. *Int. J. Food Microbiol.*, 2007; **113**: 41 – 46.
36. Shetty, P.H. and Jespersen, L., *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontamination agents. *Trends Food Sci. Technol*., 2006; **17**: 48-55.
37. Van-Egmond, H.P. and Jonker, M.A, Worldwide regulation on aflatoxins- the situation in 2002. *J. Toxicol. Toxin Rev*., 2004; **23**: 273-293.
38. Zhang, T., Shi, Z. O., Hu, L.B., Cheng, L.G. and Wang, F., Antifungal compounds from *Bacillus subtilis* BFSO6 inhibiting the growth of *Aspergillus flavus*. *World J. Microbiol. Biotechnol*., 2008; **24**: 783-788.