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Original Article

Efficacy of corn and rice seed-borne mycoflora in controlling aflatoxigenic *Aspergillus flavus*

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Abstract

Food commodities such as cereals are subjected to spoilage and bio-deterioration during storage by mycotoxigenic fungi such as *Aspergillus flavus*. Efforts are done to biologically control toxigenic *A. flavus* and subsequently prevent or at least minimize its aflatoxin production ability, without the need of using synthetic fungicides. Antifungal activity of corn and rice seed-borne mycoflora was tested against aflatoxigenic *A. flavus in vitro*, using bioassays such as dual culture technique; ability to produce volatile and non-volatile metabolites; ability to inhibit germination and reduce germ tube length of *A. flavus* conidia; *in vivo* reduction of aflatoxins level in corn seeds co-inoculated with the pathogen and antagonists. *Penicillum crustosum*, *Aspergillus giganteus*, *Fusarium verticillioides* and *Aspergillus fumigatus* isolates showed promising antifungal activities and varying efficiencies of reducing aflatoxins level; however, only *A. fumigatus* isolate was non-aflatoxigenic. It could be concluded that *A. fumigatus* could be used effectively as a biopreservative to increase shelf life of cereals during storage, but after testing its tendency to produce other mycotoxins or causing human Aspergillosis.

Keywords: Biocontrol, cereals, mycoflora, *Aspergillus flavus,* aflatoxins, biopreservative

Eficácia da microbiota fúngica transmitida por sementes de milho e arroz no controle aflatoxigênicos *Aspergillus flavus*

Resumo

Os produtos alimentares , como cereais estão sujeitos à deterioração e bio- deterioração durante o armazenamento por fungos micotoxigênico como o Aspergillus flavus . Esforços são feitos para controle biológico toxigênico A. flavus e, posteriormente, evitar ou pelo menos minimizar a sua capacidade de produção de aflatoxina , sem a necessidade do uso de fungicidas sintéticos. Atividade antifúngica de milho e arroz microbiota fúngica transmitida por semente foi testado contra aflatoxigênicos A. flavus in vitro, utilizando bioensaios , como técnica de cultura dual; capacidade de produzir metabólitos voláteis e não- voláteis; capacidade de inibir a germinação e reduzir o comprimento do tubo germinativo de A. flavus conídios , na redução do nível de aflatoxinas vivo em sementes de milho co- inoculados com o patógeno e antagonistas. Penicillum crustosum , Aspergillus giganteus , Fusarium verticillioides e Aspergillus fumigatus isola mostrou promissora atividade antifúngica e variando a eficiência de redução de nível de aflatoxinas , no entanto, somente A. fumigatus foi isolado não- produtoras de aflatoxinas . Pode-se concluir que o A. fumigatus pode ser utilizado eficazmente como um biopreservative para aumentar a vida de prateleira de cereais durante o armazenamento , mas depois de testar a sua tendência para produzir outras micotoxinas ou causando Aspergilose humano.

Palavras-chave: controle biológico , cereais, microbiota fúngica , Aspergillus flavus , as aflatoxinas , biopreservative

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Introduction

Food and feed spoilage moulds cause great economic losses worldwide. According to Pitt & Hocking (1997), it is estimated that between 5 and 10% of the world's food production is wasted due to fungal deterioration. The hazardous effects of chemicals used in plant disease management have diverted plant pathologists to find out the alternative techniques of plant disease control which may cause little or no adverse effect on environment (Agarwal et al., 2011). Biological control of fungal plant pathogens appears as an attractive and realistic approach, and numerous microorganisms have been identified as effective biocontrol agents. A considerable role in limiting the populations of these pathogenic fungi inhabiting the above ground parts of plants is played by antagonistic microorganisms. Such antagonistic properties were first of all expressed by the fungi *Trichoderma* and *Gliocladium* (Massart & Jijakli, 2007; Sempere & Santamarina, 2007; Bartmanska & Gladysz, 2006; El-Katatny et al., 2006). A sizeable portion of the world population living below poverty line in the developing and under developed countries of Asia and Africa are suffering from health problems associated with consuming mycotoxin-contaminated grains and cereals (Majumder et al., 1997). When animals consume AFB_1-contaminated feedstuff, the toxin is metabolized in the liver and is excreted as AFM_1 via milk, urination and feces (Battacone, et al., 2005; Stoloff et al., 1991). AFM_1 is bound to milk proteins, especially casein, which leads to its presence in dairy products (Prandini et al., 2009). The International Agency for Research on Cancer (IARC), WHO introduced Aflatoxins B_1 and M_1 as primary and secondary groups of carcinogenic compounds, respectively (Dragacci et al., 1992).

Aflatoxins mycotoxins are secondary metabolites produced by certain strains of *Aspergillus flavus, A. parasiticus*, *A. nomius*, *A. tamarii*, *A. bombycis* and *A. pseudotamarii* (Peterson et al., 2001; Ito, 2001; Godet & Manaut, 2010). Aflatoxins are one of the most potent toxic substances that occur naturally (Bommakanti & Waliyar, 2007) and their contamination in economic crops is a major problem all over the world. Among all the aflatoxins, particularly AFB_1 is the most toxic form for mammals and presents hepatotoxic, teratogenic and mutagenic properties, causing damage such as toxic hepatitis, hemorrhage, edema, immunosuppression and hepatic carcinoma (Abdel-Wahhab et al., 2006, 2010). Thus, there is a need to search for alternative approaches to store grains/cereals for human consumption without toxicity problems that are eco-friendly and not capital intensive (Satish et al., 2007). Of the many research approaches, biological decontamination of mycotoxins using microbes is one of the attractive strategies for the management of these deadly fungal toxins in food and feed (Shetty & Jespersen, 2006). Great successes in reducing aflatoxin contamination have been achieved by application of non-toxigenic strains of *A. flavus* and *A. parasiticus* in fields of cotton, peanut, maize and pistachio. In accordance, Bata & Lasztity (1999); Pereira et al., (2010) emphasized that many species of bacteria, fungi and yeasts have been shown to enzymatically degrade mycotoxins, thus some microorganisms have traditionally been used as biopreservatives in food and feed. Biopreservation allows prolonged shelf life and enhanced safety of foods through natural or supplementary microflora and their antimicrobial products (Schnürer & Magnusson, 2005). These promising biocontrol agents have to be mass produced on economic substrates, and then formulated for ease of use, wide scale of application in addition to prolonged shelf life. A commercial biopesticide product (called afla-guard) has been developed based on the *A. flavus* strain NRRL21882 (Yin et al., 2008). Additionally, the non-toxigenic *A. flavus* strains CT3 and K49 have been tested in the USA and showed good efficacies in reduction of aflatoxin contamination in corn (Abbas et al., 2006). The aims of the present work are to screen number of cereals borne mycoflora as biocontrol agents to prevent the growth of the aflatoxigenic *A. flavus* strain, and subsequently eliminate or at least minimize the levels aflatoxins produced. These promising fungal strains could be used as biopreservatives to increase the shelf life of cereals during storage, and hence displace the use of the deleterious synthetic preservatives.

Material and Methods

Collection of cereals seed samples

Twenty samples (250 g each) of stored corn and rice seeds were collected from local markets of different districts of Cairo, Egypt during January 2010.

Isolation of seed-borne mycoflora

Associated fungi were isolated from stored corn and rice seeds. The seeds were surface-sterilized in 1% NaOCl for 2 min and rinsed in two changes of sterile distilled water. The seeds were blotted dry in between sterile Whatman No. 1 filter papers and plated on sterile potato dextrose agar (PDA) (4 g/l of potato infusion from 200g of potatoes, dextrose 20 g/l, agar 15 g/l, pH 5.6) at the rate of 10 grains per plate. Thirty seeds were plated per crop and incubated at room temperature of 25 ± 1°C. Sub-cultures were made from emerging colonies and pure single spore colonies were obtained for subsequent studies (Amadi & Adeniyi, 2009).

Mycological identification of fungal isolates

Mycological identification of the fungal isolates was carried out according to the morphological and microscopical characteristics which were compared with literature (Pitt & Hocking, 1997; Samson et al., 2004).

In-vitro detection of antifungal activity of seed-borne mycoflora

The effectiveness of the native seed-borne fungal isolates against mycelial growth of pathogenic *A. flavus* isolate was tested by a dual culture technique (Raju & Murthy, 2000). A mycelial disc (5mm) obtained from the peripheral region of 5-7-day-old cultures of *A. flavus* on PDA, was placed on a fresh PDA plate (3 cm from the center) and then a 5mm-mycelial disc, obtained from the periphery of a 5-7-day-old culture of antagonist isolate was placed 3 cm away from the inoculum of the pathogen, the plates were incubated at 28 °C and measurements were taken after 7 days. In the control experiment, a sterile agar disc was placed in the dish. At the end of the incubation period, radial growth was measured. The percentage inhibition of growth of tested pathogen in presence of antagonist isolate was calculated over control. The growth inhibition was calculated by using the formula:

100x (r1 –r2)\ r1

Where, r1 = diameter of fungal colony in control,

r2 = diameter of fungal colony in dual inoculation

Interactions were assayed by giving ranking according to Bell's ranking scale (Bell et al., 1982) where; R1= complete over growth of pathogen by antagonist; R2= 75 % over growth; R3= 50% over growth; R4= growth inhibition at line of contact; R5= pathogen over growing antagonist.

In-vitro detection of the presence of non-volatile metabolites in the fungal antagonist's culture filtrates

a- Preparation of 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 determined by using a haemacytometer (Thanaboripat et al., 2009).

b- Culture filtrate preparation

Yeast extract sucrose (YES) (2% yeast extract, 20% sucrose) was used for culturing each fungal isolate. The medium was prepared by adding 45 ml broth into 125 ml flask and autoclaved. Spore suspension (10^6 spores/ ml) of each isolate was inoculated into each flask and incubated at static condition for 14 days. 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 (Calistru et al., 1997).

c- Inhibitory effect of the antagonist's culture filtrates

According to the method of Mishra (2010), four ml of the antagonist's culture filtrates were placed in sterilized Petri dishes which were immediately followed by pouring 16 ml of PDA, so as to make the final concentration of culture filtrates 20%. After the agar solidified, mycelial discs of the pathogenic *A. flavus* isolate (5 mm

in diameter) obtained from actively growing colonies were placed in the centre of the agar plates. The Petri dishes were incubated at 25°C for 4 days. The percent inhibition in the radial colony growth of the pathogen was calculated by the following formula:

Percent inhibition = C – T\ C ×100

Where, C = Radial growth in control set;

T = Radial growth in treated set

Detection of the production of volatile metabolites by the fungal antagonists

Fungal antagonists were grown on 2% malt extract agar (malt extract 30 g/l, mycological peptone 5 g/l, agar 15 g/l, pH 5.4) in Petri dishes (15 ml medium per each) and incubated for up to 15 days as described by (Dennis & Webster, 1971). Incubation was carried out under light at 22-25°C. After 3-4 days, the lid of each plate was replaced by a bottom of plate containing 2% malt extract agar (15 ml per dish) inoculated with a pathogenic fungal disk (*A. flavus*) with a sterile cellophane membrane between them. The 2 dishes were taped together with adhesive tape. The lids of the control plates which were empty were also replaced in the same way. Test plates and control plates were set up in triplicate. After 3-5 days of incubation, the colony diameter of the pathogenic fungus was measured and compared to that of the control plates.

Molecular identification of the selected antagonist's isolates

The selected promising fungal isolates and the aflatoxigenic isolate of *A. flavus* (pathogen) were identified by the ITS molecular technique.

DNA Extraction

A small amount of mycelium grown on Sabouraud's dextrose agar (dextrose 40 g/l, peptone 10 g/l, agar 20 g/l, pH 5.6) was suspended in 200 µl of TE buffer (100mM Tris-Hcl, pH 8.0, 1mM EDTA) in an Eppendorf tube (1.5 ml). DNA extraction was carried out according to the procedure described by Sandhu et al. (1995).

Oligonucleotides

The oligonucleotide primers used for amplification and sequencing of the ITS regions were those described by White et al. (1990). ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 84-(5'-TCCTCCGCTTATTGATATGC-3') were purchased from Pharmacia 85-Biotech CO., LTD. (Tokyo, Japan).

PCR and DNA sequencing of ITS1-5.8S-ITS2 region rDNA of fungal strains

Amplification reactions were performed in 25 µl reactions containing 2.5 µl of each primer (20 pm), 2.5 µl of genomic DNA (5 µg/ml) and one Pure Taq Ready-To-Go PCR bead (Amersham Biosciences, Buckinghamshire, UK). The bead contains buffers, dNTPs, enzyme, stabilizers and BSA. Amplification was performed with a PCR Thermal Cycler MP (Ta Ka Ra Shuzo, Tokyo, Japan) using the initial denaturation at 94 C for 4 min, followed by 35 cycles at 94°C for 2 min, 55°C for 2 min and 72 °C for 2 min and a final extension at 72 °C for 10 min. The PCR reaction products sequenced directly using a big dye terminator reagent kit including *Taq* polymerase and the protocol recommended by the manufacturer (Model 3010 automated DNA sequencer, Perkin-Elmer/Applied Biosystems, Japan).

Detection of the ability of the selected fungal antagonist's culture filtrates to inhibit the germination and reduce germ tube length of A. flavus conidia

Of the 20 fungal isolates tested by dual culture, four isolates showed some activity. Culture filtrates of these isolates were tested to determine their potency to inhibit germination and decrease germ tube length of conidia of the test pathogen. The four isolates were grown separately for l week at 28 ± 2 °C in 250 ml conical flasks containing potato dextrose broth. The fungal cells were then removed from each culture by filtering first through two layers of muslin cloth and then through a 0.2 µm Millipore filter. One ml of filtrate from each isolate was placed on a cavity microscope slide. Then 1 ml of a conidial suspension (10^6 conidia/ mL) of pathogenic *A. flavus* in sterile distilled water was added to the slide and mixed thoroughly. The slides were kept in Petri dishes on a glass bridge chamber and incubated at 25 °C. Conidial suspensions

of pathogenic *A. flavus* in sterile distilled water served as the controls. Conidial germination and germ tube length of the pathogen were assessed for up to 4 days at 4 h intervals and then percent germination of the conidia and germ tube length were calculated by scoring 100 conidia for germination and germ tube length reduction. The experiments were replicated four times to obtain an average percent inhibition (Sangeetha et al., 2009).

In-vivo estimation of reduction of aflatoxins levels in corn seeds co-inoculated with the selected antagonists and aflatoxigenic A. flavus isolate

To examine the reduction of aflatoxins production in solid substrates, 50 g of commercial corn were soaked with 15 ml of distilled water in a 300 ml Erlenmayer flask overnight before sterilization run for 30 min at 121°C and then it was inoculated with *aflatoxigenic A. flavus* and the tested fungi (antagonists). Inoculation was made with four 4 mm diameter discs of each fungus from the edge of 1-week-old culture on PDA and 2 ml of sterile water. Cultures were incubated at 25 °C and shaken each day to support a uniform growth of mycelium. In the control, each fungus was cultured separately. After 21 days, corn seeds colonized by fungi were dried in a thin layer at room temperature and mycotoxin content was analyzed. Two replications were made for each combination of paired cultures and for each control (Popiel et al., 2008).

Extraction of aflatoxins:

i)- Sample extraction:

Five grams of sample were placed with 10g salt sodium chloride in blender jar and 200 ml methanol: water (80:20) was added to the jar. The content was blended at high speed for 1 minute then the pour was extracted into fluted filter paper and the filtrate was collected in a clean vessel.

ii)-Extract dilution:

Ten ml of the filtered extract were pipetted into a clean vessel and were diluted with 40 ml of purified water then mixed well. The dilute extract was filtrated through glass microfiber filter into a glass syringe barrel using markings on barrel to measure 4 ml.

iii)- Immunoaffinity Chromatography:

Four ml filtered diluted extract (4 ml = 0.25g sample equivalent) was passed completely through AflaTest ®-P affinity column (VICAM, Milford, MA, USA) at a rate of about 1-2 drops/ second until air comes through column. Five ml of purified water were passed through the column at a rate of about 2 drops/second. The affinity column was eluted by passing 1.0 ml HPLC grade methanol through the column at a rate of 1-2 drops/second and all of the sample elute (1ml) was collected in a glass vial, evaporated to dryness under stream of nitrogen.

Determination of aflatoxins by HPLC:
Derivatization:

The derivatives of samples and standard were done according to Madbouly et al. (2012) by the addition of 100 µl of trifluoroacetic acid (TFA) to the sample and mixed well for 30 s. The mixture stand for 15 min then 900 µl of water: acetonitrile (9:1 v/v) were added, mixed well by vortex for 30 s and the mixture was used for HPLC analysis.

The HPLC system consisted of Waters Binary Pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Watres 2475 Multi- Wavelength Fluorescence Detector and a data workstation with software Breeze 2 and A phenomenex C_{18} (250 x 4.6 mm i.d), 5 µm from Waters corporation (USA). An isocratic system with water: methanol: acetonitrile 6:3:1. The separation was performed at ambient temperature at a flow rate of 1.0 ml/min. The injection volume was 20 µl for both standard solutions and sample extracts. The fluorescence detector was operated at wavelength of 360 nm for excision and 440 nm for emission.

Statistical analysis

The data were represented as average of 3 replicates otherwise mentioned and the statistical evaluation was performed using the SPSS® (Statistical Package for the Social Sciences) version for Windows (SPSS Inc., Chicago, Illinois) and GraphPad PrismTM (San Diego, CA) statistical

software programs. The significance level was set at P≤ 0.05.

Results

Isolation and identification of seed-borne mycoflora

Isolation and identification of the mycoflora of collected samples of corn and rice seeds led to the recovery of the following isolates, *A. flavus, A. niger, A. candidus, A. tenuis, A. wentii, A. flavipes, A. giganteus, A. fumigatus, Rhizopus nigricans, Rhizopus oryzae, Fusarium verticillioides, Fusarium semitectum, Gliocladium fimbriatum, Homodendrum hordei, Macrophomonapha seolina, Mucormucedo, Penicillium funiculosum, Penicillium crustosum, Penicillium viridicatum, Cheatomella* species. Aflatoxigenic isolate of *A. flavus* NRRL 3251 was provided from Northern Regional Research Laboratory.

In-vitro detection of antifungal activity of seed-borne mycoflora

In-vitro antagonism of the fungal isolates against the toxigenic isolate of *A. flavus* showed that all isolates have an antifungal activity but with varying efficiencies (Table 1). Maximum antifungal activities (Ranking scale 1) were observed in the isolates of *A. fumigatus* and *A. giganteus,* where they completely surrounded the colony of *A. flavus* and prevented it from any radial spread (Figures 1a, b). This is followed by isolates of *P. crustosum* and *F. verticillioides* with a decreasing Ranking scale of 2 and 3, respectively; compared with the control (Figures 1c, d). On the other hand, the other fungal isolates showed a lower antifungal potential (Ranking scale 4).

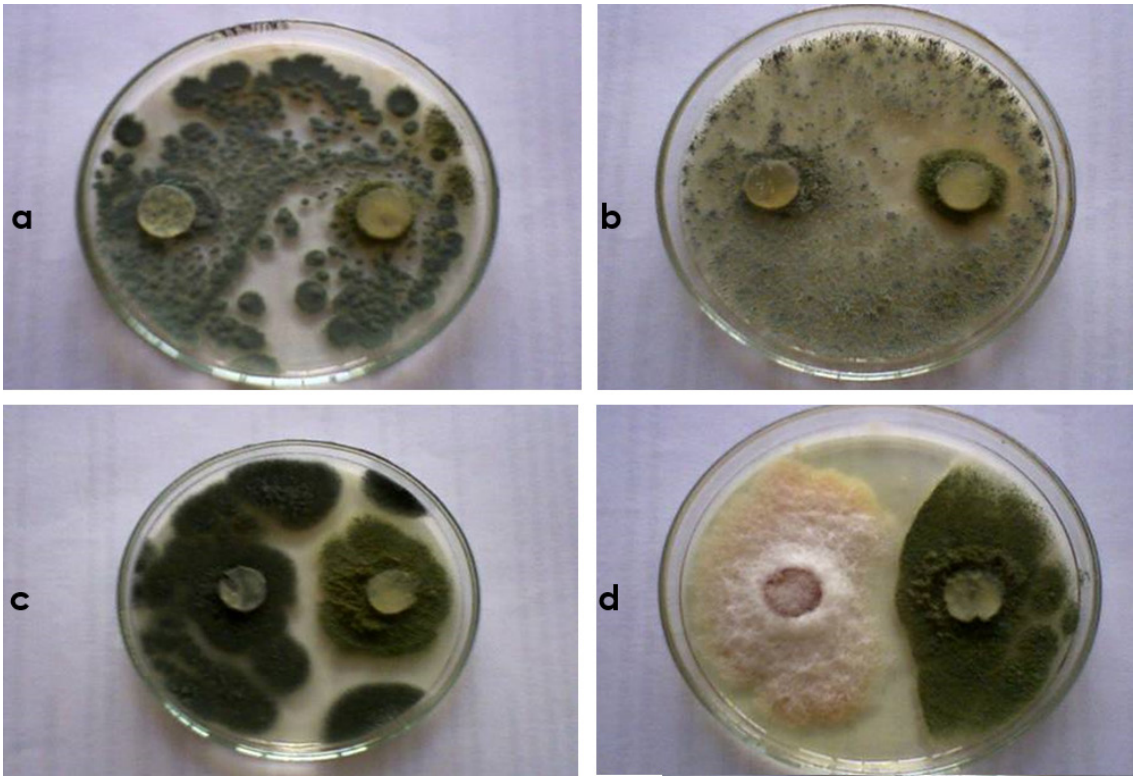

Figure 1. Dual culture technique (Antagonism *in-vitro*) **(a)**, *A. fumigatus* growing disc (left), aflatoxigenic *A. flavus* growing disc (right). *A.* fumigatus growth completely surrounded the aflatoxigenic *A. flavus* disc and prevented it from any radial spread (competition for space and nutrients); **(b)**, *A. giganteus* growing disc (left), aflatoxigenic *A. flavus* growing disc (right). *A. giganteus* growth completely surrounded the disc of aflatoxigenic *A. flavus* and prevented it from any radial spread (competition for space and nutrients); **(c)**, *Penicillium crustosum* growing disc (left), aflatoxigenic *A. flavus* growing disc (right). *P. crustosum* growth completely surrounded the growth of the aflatoxigenic *A. flavus* isolate and prevented it from radial spread (competition for space and nutrients) and **(d)**, Fusarium verticillioides growing disc (left), aflatoxigenic A. flavus growing disc (right). Fusarium verticillioides caused noticeable inhibition of the growth of aflatoxigenic *A. flavus* (lysis or antibiosis).

In-vitro detection of the presence of non-volatile metabolites in the fungal antagonist's culture filtrates

The fungal isolates culture filtrates showed moderate to weak capability to produce non-volatile or diffusible metabolites against the aflatoxigenic *A. flavus* isolate (Table 1). The percent inhibition in colony diameter of the pathogen was observed in media inoculated with culture filtrates of *P. crustosum, A. fumigatus, A. giganteus, F. verticillioides*, *Gliocladium fimbriatum* and *Aergillus candidus* with decrease in percentages of about 8, 6, 6, 4, 4 and 4%, respectively; compared with the control. The other fungal antagonist's culture filtrates did not show any appreciable inhibitory effect of colony diameter.

Table 1. In vitro antifungal activities of the fungal antagonists against the pathogenic Aspergillus flavus isolate

Name of fungal antagonists	Ranking scale of antifungal activity	Decrease of pathogen colony diameter due to non-volatile metabolites (mm)	Decrease of pathogen colony diameter due to volatile metabolites (mm)
Aspergillus niger	R4	0	0
Aspergillus flavus	R4	0	0
Aspergillus candidus	R4	4 ± 0.20	1 ± 0.13
Aspergillus tenuis	R4	0	0
Aspergillus wentii	R4	0	0
Aspergillus favipes	R4	0	0
Aspergillus giganteus	R1	6 ± 0.30	1± 0.11
Aspergillus fumigatus	R1	6 ± 0.18	1 ± 0.12
Rhizopus nigricans	R4	0	0
Rhizopus oryzae	R4	0	0
Fusarium verticillioides	R3	6 ± 0.42	0
Fusarium semitectum	R4	0	0
Gliocladium fimbriatum	R4	4 ± 0.16	1± 0.11
Homodendrum hordei	R4	0	0
Macrophomona phaseolina	R4	0	0
Mucor mucedo	R4	0	0
Penicillium funiculosum	R4	0	0
Penicillium crustosum	R2	8 ± 0.62	1± 0.08
Penicillium viridicatum	R4	0	0
Cheatomella spp.	R4	0	0

Results are averages of 3 replicates

In vitro detection of the production of volatile metabolites by the fungal antagonists

The current results revealed the potentiality of number of the antagonists to produce inhibitory volatile metabolites (Figures 2a, b and c), such as *A. candidus, A. giganteus, A. fumigatus, Gliocladium fimbriatum* and *Penicillium crustosum* compared with the control, however, their activities were very weak (Table 1). The other antagonists were not able to produce these volatile inhibitory metabolites.

Molecular identification of the selected antagonist's isolates

All the selected four fungal species and the aflatoxigenic *Aspergillus flavus* isolate yielded a unique PCR amplification. The sequence of the ITS1-5.8S-ITS2 rDNA region was used. The NCBI GenBank was accessed to identify the isolated species through BLAST homology search using the obtained ITS data. The ITS data of the isolated *Fusarium verticillioides*, *Aspergillus giganteus*, *Penicillium crustosum*, *Aspergillus flavus*, and *Aspergillus fumigatus* were respectively, highly similar (99-100 % identical) to the ITS data of *Fusarium verticillioides* (Genbank Acc. No. JX028194), Aspergillus giganteus (Genbank Acc. No. JX028195), *Penicillium crustosum* (Genbank Acc. No. JX028196), *Aspergillus flavus* (Genbank Acc. No. JX028197) and *Aspergillus fumigatus* (Genbank Acc. No. JX028198).

Inhibition of germination and reduction in germ tube length of A. flavus conidia by the selected fungal antagonists culture filtrates

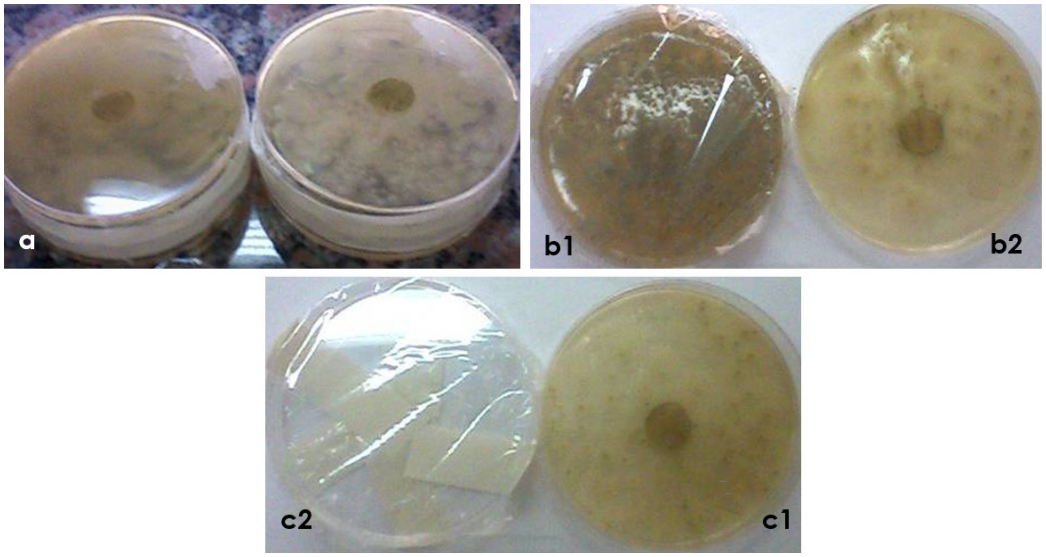

Figure 2. Detection of the production of volatile metabolites by the fungal antagonists against the aflatoxigenic *A. flavus* **(a)**, Two halves of Petri dishes taped together, with a sterile cellophane membrane between them. Lower half contains growing fungal antagonist disc, while the upper half contains the growing aflatoxigenic *A. flavus* disc; **(b1)**, Lower half of Petri dish contains growing *A. fumigatus* disc and **(b2)**, Upper half of Petri dish contains the growing aflatoxigenic *A. flavus* disc, with a sterile cellophane membrane between the two halves; **(c1)** Lower half of Petri dish empty (negative control) and **(c2)**, Upper half of Petri dish contains the growing aflatoxigenic *A. flavus* disc with sterile cellophane membrane between them.

The results of the ability of the selected antagonists culture filtrates to inhibit germination and reduce germ tube length of *A. flavus* conidia demonstrated that culture filtrate of *P. crustosum* isolate was highly effective in inhibiting germination of 90% of conidia and reducing germ length of 85% of them, compared with 10% and 0% of conidia of the negative control, respectively (Table 2). *A .fumigatus* had a lower activity as it inhibited germination of 30% of the pathogen conidia. On the other hand, although *A. giganteus* and *F. verticillioides* inhibited the germination of 25% and 10% of conidia, their culture filtrates significantly reduced the germ tube length of 70% and 60% of *A. flavus* conidia, respectively, compared with the negative control. *A. fumigatus* was the least effective as it reduced the germ tube length of only 30% of *A. flavus* conidia.

In-vivo estimation of reduction of aflatoxins level

Comparing the level of aflatoxins in corn seeds infested with the pathogenic *A. flavus* in combination with the four selected antagonists and those of controls (Table 3), revealed that all antagonists' treatments have reduced the levels of aflatoxins but with varying degrees. *A. fumigatus* and *P. crustosum* isolates were the most effective as they reduced the level of total aflatoxins by about 73.5%, 68% and AFB_1 (the most potent) by 63%, 60.5%, respectively. No aflatoxins at all were detected in *A. fumigatus* treated corn seeds (negative control); however aflatoxins were detected in seeds treated with *P. crustosum* isolate. On the other hand, the *A. giganteus* and *F. verticillioides* isolates had a lower reducing capabilities, thus they decreased the level of total aflatoxins by 38%, 37.9% and AFB_1 aflatoxin by 34%, 37%, respectively. However, both isolates had the ability to produce aflatoxins.

Table 2. Effect of the four selected fungal antagonists on inhibiting the germination and reduction of germ tube length of *Aspergillus flavus* conidia.

Name of fungal antagonist's treatments	Conidia of *A. flavus*	
	% inhibition of germinated conidia	% of conidia with reduced germ tube length
Negative control	10	0
A. giganteus	25	70
P. crustosum	90	85
A. fumigatus	30	30
F. verticillioides	10	60

Results are averages of 3 replicates

Table 3. Effect of the four selected fungal antagonists on decreasing the aflatoxins level of *A. flavus* in co-inoculated corn seeds.

Name of fungal treatments	Aflatoxins level (ng\ g of corn)				
	AFG_1	AFG_2	AFB_1	AFB_2	Total Aflatoxins
A. flavus	0.12 ± 0.02	6.1± 0.01	65.1± 3.3	5 ± 0.4	76.32 ± 4.42
Negative control	ND	ND	ND	ND	ND
A. giganteus	0.04 ± 0.01	0.1 ± 0.01	2.4 ± 0.2	2 ± 0.13	4.54 ± 0.34
A. giganteus + *A. flavus*	0.1 ± 0.01	3.3 ± 0.11	30.8 ± 3.2	4.1± 0.41	38.3 ± 2.42
P. crustosum	ND	1.1± 0.04	2.3 ± 0.12	1 ± 0.04	4.4 ± 0.34
P. crustosum + *A. flavus*	ND	1.8 ± 0.05	4.6 ± 0.33	1.8 ± 0.12	8.2 ± 1.2
A. fumigatus	ND	ND	ND	ND	ND
A. fumigatus + *A. flavus*	ND	0.4 ± 0.02	2.01 ± 0.11	0.4 ± 0.04	2.81 ± 0.13
F. verticillioides	0.1 ± 0.01	ND	2 ± 0.14	ND	2.1 ± 0.13
F. verticillioides + *A. flavus*	0.1 ± 0.01	5.6 ± 0.61	27.9 ± 1.7	4.8 ± 0.62	38.4 ± 2.25

Results are averages of 2 replicates; AFG1=aflatoxins G1, AFG2=aflatoxins G2, AFB1=aflatoxins B1, AFB2=aflatoxins B2; ND= not detectable

Discussion

Isolation of mycoflora from the seeds of stored corn and rice, led to the recovery of nine fungal genera represented by 26 species. Most of the isolated species belong to *Aspergelli*, *Fusaria* and *Penicilli*. Seed treatment by surface disinfestation with sodium hypochlorite removed saprophytic organisms found on the seed surface and decreased the overall incidence of *Fusarium* species, but allowed better recovery of species that were primary invaders like *Aspergilli*. Due to the variable antagonistic potential of individual isolates, the first *in vitro* screening was used to select the most active antagonists against the pathogenic *A. flavus* before a species or a particular isolate can be considered as a biocontrol agent. Most fungi showed *in vitro* antifungal activity against the aflatoxigenic *A. flavus* strain, but with varying efficiencies. *A. fumigatus* and *A. giganteus*, followed by *P. crustosum* showed the highest antifungal activity as they spread in the plate and prevented the pathogen from radial spread compared with the control. This study has shown also that, these colonies of *A. fumigatus*, *A. giganteus* and *P. crustosum* grew faster than that of *A. flavus* isolate. This rapid growth gave these antagonists an important advantage in the competition for space and nutrients with the pathogenic *A. flavus*, even before it deploys its arsenal of mycotoxin, similar results were reported by Barbosa et al. (2001). This competition was not due to antibiosis or enzyme production as there were no clear inhibition zones. Thus these highly competitive strains might be applied to cereal fields as biocompetitive agents (Egel et al., 1994; Cotty & Cardwell, 1999; Horn & Dorner, 1999). On the other hand, *Fusarium verticillioides* also showed moderate antifungal activity, presumably through antibiosis as the pathogen growth was retarded and the inhibition zone was noticeable. The other fungal antagonists showed weak antifungal activity, this may be due to poor competitive ability, composition of culture media or resistance of the pathogen to their antagonism.

P. crustosum strain culture filtrate's contains antifungal non-volatile metabolites, this means that this strain produced antifungal antibiotics but their activities were not strong, or the composition of the media might affect their activities. *A. fumigatus* and *A. giganteus* culture filtrates also contain non-volatile metabolites as it was clear from the inhibition of *A. flavus* colony diameter, but their activities were less than that of *P. crustosum*. *F. verticillioides*, *Gliocladium fimbriatum* and *A. candidus* filtrates also had these inhibitory metabolites but with noticeable lower activity. The remaining antagonists, although decreased the growth of *A. flavus* colony, were inferior to the ones listed above in performance, similar results were obtained on different *Trichoderma* isolates by Rini & Sulochana (2007). Some isolates showed very weak ability to produce inhibitory volatile metabolites, as there was no appreciable difference in pathogen colony diameter compared with that of the control. In accordance, Dennis & Webster (1971) stated that it was not possible to select one stage of growth which is optimal for detecting the presence of volatile metabolites. It is considered that up to 8% inhibition or stimulation of mycelial

growth could be accounted for by the variability in growth of the test fungus. Any inhibition (or stimulation) greater than this is considered to be due to volatile metabolites produced by the fungal isolate. Variations in the inhibitory potential may be due to the differences in the quantity and quality of the inhibitory substances (volatiles and non- volatiles) produced by the antagonists. This is consistent with the reports of (Bell et al., 1982). However, positive results obtained from these *in vitro* studies were only indicative, as experimental conditions do not take all ecological and endemic factors into account. For this reason, field studies are essential to test the selected competitive biocontrol agent under field conditions (Schubert et al., 2008).

We can realize that the isolates of *A. fumigatus*, *A. giganteus*, *P. crustosum* and *F. verticillioides* were the most promising with respect to their antifungal activity against the mycotoxigenic *A. flavus*. Thus, these isolates were selected for further research and were identified by the ITS molecular technique. In the present study, the culture filtrate of *P. crustosum* showed a very high potential to reduce the number of germinating and germ tube length of *A. flavus* conidia. This might be due to its ability to produce a number of hydrolytic enzymes or inhibitory metabolites. Similar results were obtained by Ghisalberti & Rowland (1993); Iqbal et al. (1994); Horvath et al. (1998) on *Trichoderma* TV9 and *T. harzianum* 3 isolate's culture filtrates. This obtained result of *P. crustosum* strain was in accordance with our early results of its *in vitro* ability to prevent the radial spread of *A. flavus* colony and produce diffusible metabolites. *A. fumigatus* had a lower activity to reduce the number of germinating conidia and their germ tube length. This result confirmed that it had completely colonized the culture media and prevented the spread of *A. flavus* growth *in vitro* due to competition for space and nutrients and not due to antibiosis or enzyme production. On the other hand, although *A. giganteus* and *F. verticillioides* did not reduce the number of *A. flavus* germinating conidia significantly, however, they reduced their germ tube length; this might be due to their ability to produce very weak antifungal antibiotics in accordance with our previous discussed results. All the four selected isolates were able to reduce the levels of aflatoxins in infested corn seeds compared with the control. *A. fumigatus* gave very promising results as it reduced the total aflatoxins by more than 70%, and the most potent AFB_1 by 63%, in addition, this isolate did not show any ability to produce aflatoxins (non-aflatoxigenic strain). This meant that it competed with the pathogenic *A. flavus* strain for nutrients and for the sites on corn seeds, hence decreased significantly its aflatoxins production potential, it might also affected the metabolism of *A. flavus* hence minimized the aflatoxins biosynthesis in accordance with Rashid et al. (2008). It might be able also to produce enzymes which degraded the aflatoxins. This showed that this non-aflatoxigenic strain could be an organism of interest as a biocontrol strategy for reducing aflatoxins contamination.

P. crustosum reduced the level of total aflatoxins and AFB_1 by 68% and 60%, respectively, however this isolate produced aflatoxins in the treated corn seeds. *A. giganteus* and *F. verticillioides* also reduced the level of total aflatoxins and the AFB_1, but they showed ability to produce aflatoxins as well. Thus, we conclude that, *P. crustosum* is able to reduce and inhibit the growth of *A. flavus in vitro*, reduce germination and germ tube length of its conidia and degrade the aflatoxins as well, but it can't be used as a biocontrol agent because it showed ability to produce aflatoxins even in minute levels. Although, *A. giganteus* significantly prevented the growth of *A. flavus in vitro*, while *F. verticillioides* strain showed moderate ability to inhibit it and both reduced its aflatoxins level, however, they could not be used also as bioagents because they are aflatoxigenic strains. Similarly, Misra et al. (2010) reported 66.84% inhibition of aflatoxin B production in wheat grains by *Aspergillus fumigatus* and complete aflatoxin G production inhibition by *Aspergillus fumigatus*, *Penicillium citrinum* and atoxigenic strain of *Aspergillus flavus*. In the current study, *A. fumigatus* gave the best and most promising results, as it was very efficient in preventing the growth of *A. flavus in vitro* and reduced the level of aflatoxins significantly as well, in addition to being a non-aflatoxigenic strain. However, further research is

required before using this isolate commercially to control *A. flavus*, in order to detect its tendency to produce other mycotoxins or cause human Aspergillosis. Results obtained in this study are particularly useful for identifying likely candidates for biocontrol of pathogenic *A. flavus* and for making educated guesses concerning the mechanisms by which they reduce its damage.

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