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Potential Biocontrol Agents Used For Management of Aflatoxin Contamination in Corn Grain Crop.

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ABSTRACT

Oilseed crops is an imperative standing in agriculture and industrial economy in Egypt as they are one of the main staple food. So, management of crop problems using novel technologies to prevent pre- and post harvest loss could increase the quality and quantity of the products. Aflatoxins produced primarily by two closely related fungi, *Aspergillus flavus* and *Aspergillus parasiticus* are carcinogenic in animals and humans. Of many approaches investigated to manage aflatoxin contamination, biological control method has shown great promise. Numerous organisms, including bacteria, yeasts and fungal, have been tested for their ability in controlling aflatoxin contamination. *Bacillus subtilis*, *Bacillus pumilus, Pseudomonas putida*, *Streptomyces aureofaciens* and *Pichia anomala* have shown the ability to inhibit fungal growth and production of aflatoxins by *Aspergillus flavus* and *Aspergillus parasiticus* in laboratory experiments and they give good efficacies for protection of corn grain crops.

Keywords. Aflatoxins, *Aspergillus flavus*, *Aspergillus parasiticus*, *Bacillus subtilis*, *Bacillus pumilus, Pseudomonas putida*, *Streptomyces aureofaciens* and *Pichia anomala* and corn

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INTRODUCTION

Corn (*Zea mays*) is the second most important crop (750,000 feddans) in Egypt . Its main use is in cooking, where its high smoke point makes refined corn oil valuable frying oil. The grain is vulnerable to degradation by mycotoxigenic fungi which include *Aspergillus, Fusarium and Penicillium.* The major aflatoxin-producing fungi are *Aspergillus flavus* and *Aspergillus parasiticus.* The aflatoxins are secondary fungal metabolites for which there is no known function within the fundamental life processes of the organism. They are, however, extremely toxic to other forms of life and cause through fungal contamination of food and feedstuffs mycotoxicosis in both man and animals.

Toxic moulds may invade agricultural products during plant growth, and during storage and processing. The aflatoxins occur in the sub ug/kg to the mg/kg range in groundnuts and groundnut products, corn and other grains, such as rice, wheat, sorghum and millet. In many countries, tolerance levels for aflatoxins in foodstuffs are in the range of 5-50 Mg/kg. Maize contamination by fungi does not only reduce its quality through discolouration and reduction of nutritional value but also lead to mycotoxin production. The most important mycotoxins are aflatoxins, ochratoxins, fumonisin, T-2 toxin and T-2 toxins. The mycotoxins are produced by fungal action during production, harvest, transportation, storage and food processing (Murphy *et al.,* 2006).

Mycotoxins produced by fungi that infect the kernels may result in feed that is unsafe for consumption by humans or livestock. Mycotoxins are chemically and biologically active by-products produced by fungal (mold) growth naturally in a range of plant products. Mycotoxins have received considerable attention due to their significance in agricultural loss and livestock / human health. Aflatoxin contamination of crops is a worldwide food safety concern. Aflatoxins refer to a group of four mycotoxins (B1and B2) produced primarily by four closely related fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. Strains of *A*. *flavus* show a great variation in their ability to produce aflatoxins. Toxigenic strains of *A*. *flavus* typically produce only two aflatoxins, B1 and B2, but most strains of *A*. *parasiticus* could produce all the four toxins (Dorner, 2004 and 2008). The major aflatoxin-producing fungus, *Aspergillus flavus* is ubiquitous in agricultural soils and has a broad ecological niche. The use of bacteria and yeast as antagonists against Mycotoxin fungi is a promising approach to manage aflatoxin contamination via biological control.

Research to enhance the effectiveness and mass production of biocontrol yeast will facilitate commercial product development for practical application. Biological control appears to be the most promising approach for control of aflatoxin in both pre- and post-harvested crops. Several bacterial species, such as *Bacillus subtilis*, *Bacillus pumilus , Streptomyces aureofaciens and Pseudomonas putida* have shown the ability to inhibit fungal growth and production of aflatoxins by *Aspergillus* spp. in laboratory experiments. Several strains of *B*. *subtilis* isolated from the non-rhizophere of maize soil were also able to inhibit aflatoxin accumulation (Nesci et al., 2005). This inhibitory result from many factors including competition for space and nutrients in general, competition for nutrients required for aflatoxin production but not for growth, and production of antiaflatoxigenic metabolites by co-existing microorganisms. Yeast species can develop quickly in leaf, fruit and flower surfaces, excluding the other microorganism growth by means of competition for space and nutrient. 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 (Walker 2011).

In this paper, inhibition of aflatoxin production of *Aspergillus flavus* and *A. parasiticus by Bacillus pumilus, Pseudomonas putida , Streptomyces aureofaciens* and *Pichia anomala* and on its antiaflatoxigenic activity in corn grains are presented.

MATERIALS AND METHODS

Corn grains were randomly collected for analysis of *Aspergillus* producing aflatoxin. For isolation of the mycoflora in whole seeds, subsamples of seeds from each sample were surface-disinfected in a commercial 5% aqueous solution of sodium hypochlorite for 1 minute, rinsed twice with sterile distilled water and dried in a sterile laminar flow cabinet. One hundred seeds per sample were placed, 10 seeds per plate, on Yeast Extract- Glucose-Chloramphenicol Agar, Biokar Diagnostic BK007 (González et al, 1995). The plates were incubated in the dark at 28°C for 4-7 days and the resulting fungal colonies subcultured onto Potato Dextrose

Agar, and identified. Where several different fungi were isolated from a single seed, all were recorded. Keys for fungal identification were those developed by, Samson (2004) and Simmons (2007).

Bacterial isolates and aflatoxin-producing mold

Bacillus subtilis, *Bacillus pumilus, Pseudomonas putida* , *Streptomyces aureofaciens* and *Pichia anomala* were screened for inhibition of aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. Isolates were maintained on nutrient agar (NA) slants (Difco Laboratories, Detroit, MI) at 4^0C.

Production of inoculum and growth medium

The inoculum of *Bacillus subtilis*, *Bacillus pumilus, Pseudomonas putida* , *Streptomyces aureofaciens* and *Pichia anomala* were obtained by growing isolates in 5 ml of yeast extract (2%) sucrose (20%) broth (YES, pH 6.5) at 30 ^{0}C for 24 hours. The concentration of viable cells in the inoculum was determined by plate counts on NA medium. The inoculum of *Aspergillus flavus* and *Aspergillus parasiticus* were obtained by growing the mold at 25 ^{0}C on slants of potato dextrose agar (PDA) (Difco Laboratories,) until well sporulated (7 days). Spores were harvested by adding 10 ml of sterilized aqueous solution of Tween 80 (0.05% v/v) , filtered through 4 layers of sterile cheesecloth to remove mycelial debris. The spore concentration was determined by serial dilution in peptone-water (0.1% w/v) and plate counts on mycological agar.

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 *Aspergillus flavus* and *Aspergillus parasiticus* (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 on days 2, 3 and 4 using vernier digital caliper. All experiments were repeated at least five times.

Inhibition assays

Cell-free supernatant fluids were used in the deferred antagonism assay. Each bacterial isolate was grown at 30 ^{0}C and 100 rpm for 48 hours in 100 ml of YES broth inoculated with 0.1 ml of bacterial inoculum containing ca. 107 CFU/ml. Then, cell-free supernatant fluids were prepared by centrifuging the culture for 15 minutes. Supernatant fluids were sterilized and 25 ml were aseptically dispensed in 125-ml Erlenmeyer flasks before being inoculated with 0.1 ml of a spore suspension of *Aspergillus flavus* and *Aspergillus parasiticus* containing 10^7 CFU/ml. Cultures were incubated at 25 ^{0}C for 10 days and analyzed for aflatoxin production.

Determination of aflatoxin production

As per AOAC method by HPTLC and quantified with reference standards. The technique is screening by TLC and quantified by HPTLC(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).

Analysis of aflatoxin B1 in grains

One hundred subsamples of grains were ground to fine powder and five grams extracted with 25 ml methanol: water (50:50 v/v). The methanol extract was de-fatted with 10 ml hexane and the mixture centrifuged at 1500 g for 10 minutes and 4 ml supernatant recovered. The methanolic extract was diluted 1:5 in Phosphate Buffered Saline (PBS) and again 1:4 in methanol-PBS (9:1) and analyzed using HPLC.

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Effect of biocontrol agents on aflatoxin B1 produced by *A. flavus* on seeds samples

Stored seeds were treated with tested biocontrol agents at the above-mentioned concentrations and subsequently, these 100 g seeds grains were inoculated with 100ml/kg suspension of tested biocontrol agents and incubated at 25°C for 10 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).

Data analysis

All data were subjected to analysis of variance (ANOVA) using the PROC ANOVA procedure of Genstat and differences among the treatment means compared using Fisher's Protected LSD test at 5% probability level.

Results and discussion

Aflatoxin B1 content in maize products

Aflatoxin B1 was detected in whole maize grain from farm and trade region of Egypt (Table 1). The highest aflatoxin B1 levels (up to 146µg/kg) were detected in semi-processed grain than in Immature kernel.

Table 1: Mean aflatoxin content (µg/kg) in maize samples from different agro ecological zones of Egypt during short season harvest .

Samples	Spores counts /ml (10^{6})				Aflatoxin µg/Kg	
	Whole maize		Semi-processed grain		Whole maize	Semi-processed grain
	Aspergillus flavus	*A. parasiticus*	*Aspergillus flavus*	*A. parasiticus*		
Immature kernel	*54.6*	*21.4*	*76.8*	*43.6*	23.6	21.7
Farmes	128.0	54.6	213	143.4	35.7	146
Trade	78.4	45.8	101.3	98.6	17.8	76.8

Screen biocontrol agents strains antagonistic to *Aspergillus spp.*

A bioassay has been developed to screen for effective bioagents inhibiting both the growth of the *Aspergillus flavus* and *Aspergillus parasiticus*. Biocontrol isolates exhibited a wide range of inhibitory activity against *Aspergillus flavus and Aspergillus parasiticus* using either spores suspension or culture filtrates . Data presented in Table 2 indicated that each of the *Bacillus subtilis*, *Bacillus pumilus, Pseudomonas putida* , *Streptomyces aureofaciens* and *Pichia anomala* isolates inhibited aflatoxin production and mycelial growth of *Aspergillus flavus* and *Aspergillus parasiticus* . *Bacillus pumilus* is the most effective one against *Aspergillus flavus* and *Aspergillus parasiticus*. The yeast isolate, *Pichia anomala* had prominent inhibitory effect on colony expansion.

The same results were obtained in broth culture using culture filtrates of bioagents . Data in Table 3 also indicated that mycelium production and sporulation were inhibited in supernatant of cultured YES broth. *Bacillus pumilus* is the most effective one against *Aspergillus flavus* and *Aspergillus parasiticus*. The yeast isolate, *Pichia anomala* had prominent inhibitory effect.

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Inhibition of aflatoxin production by bioagents strains

Significantly reduction of aflatoxin biosynthesis were observed in *Bacillus pumilus* and *Pichia anomala* Aflatoxin production was moderate inhibited with *Pseudomonas putida*, *Streptomyces aureofaciens and Bacillus subtilis* (Table 3). These findings are in agreement with Reddy et al.,(2009)
Mycotoxin contaminated products cause significant economic and trade problems at almost every stage of production and marketing.

This study showed the biological control mechanism of *Bacillus subtilis*, *Bacillus pumilus, Pseudomonas putida*, *Streptomyces aureofaciens* and *Pichia anomala* against *Aspergillus flavus* and *Aspergillus parasiticus* may be the production of antifungal substances. Thus this may lead to the discovery of new biological control agents for controlling the growth of aflatoxin producing fungi.

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

Effect of spores suspension of tested bioagents on production of aflatoxin B1(µg/kg) on nature corn seeds obtained in Table (4). Significantly reduction of aflatoxin biosynthesis were observed in *Bacillus pumilus* and *Pichia anomala* . Aflatoxin production was moderate inhibited with *Pseudomonas putida , Streptomyces aureofaciens and Bacillus subtilis*

Table 2: Effect of biocontrol isolates on the growth of *Aspergillus flavus* and *Aspergillus parasiticus* and aflatoxin.

Biocontrol isolates	Spores suspension		Culture filtrates	
	Diameter of inhibition zone (mm)			
	Aspergillus flavus	*A. parasiticus*	*Aspergillus flavus*	*A. parasiticus*
Bacillus subtilis	13.240 + 5.13	15.610 + 6.23	11.320 + 4.23	16.230 + 5.35
Bacillus pumilus	34.054 + 0.58	36.044 + 0.28	30.176 + 0.43	35.032 + 0.28
Pseudomonas putida	13.133 + 0.65	15.211 + 0.64	12.126 + 0.54	14.301 + 0.74
Streptomyces aureofaciens	15.6+0.42	16.7+0.54	11.2+0.34	11.2+76
Pichia anomala	21.141 + 0.43	25.132 + 0.33	20.121 + 0.23	23.142 + 0.53

Table 3: Effect of culture filtrates of biocontrol isolates on the growth and aflatoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* .

Biocontrol isolates	Dry weight (g)		Spores counts /ml (10^{6})		Aflatoxin µg/Kg	
	Aspergillus flavus	*A. parasiticus*	*Aspergillus flavus*	*A. parasiticus*	*Aspergillus flavus*	*A. parasiticus*
Bacillus subtilis	4.5	5.8	12.0	14.6	0.9	0.6
Bacillus pumilus	2.2	2.8	5.4	9.8	0.1	0.1
Pseudomonas putida	3.3	3.5	6.7	10.9	0.8	0.6
Streptomyces aureofaciens	2.7	2.8	6.6	6.8	0.9	0.5
Pichia anomala	3.2	3.1	5.2	11.8	0.3	0.2
Control	15.7	21.4	65.7	45.7	53.5	41.8
LSD	1.2	1.1	1.3	2.3	0.3	0.2

Table 4: Effect of biocontrol agents on Aflatoxin B1 produced by *Aspergillus* on seeds samples

Isolates	Aflatoxin B1 µg/Kg
Bacillus subtilis	4.5
Bacillus pumilus	0.3
Pseudomonas putida	1.3
Streptomyces aureofaciens	1.9
Pichia anomala	0.7
Control	142.5
LSD	0.6

Aspergillus flavus is a common fungus found in soil and debris. Since it occurs so frequently in nature, particularly as airborne spores, it can be found on most grain in the field and in storage. Moisture and heat stress is commonly associated with most aflatoxin outbreaks. Aflatoxin contamination problems are minimized with management such as thoroughly grain cleaning, proper combine adjustment to reduce kernel damage, matching drying capacity to wet corn holding capacity, proper drying, removal of fines and broken kernels, proper grain cooling after drying, and sound storage practices. Physical, chemical and biological methods have been investigated in order to prevent the growth of aflatoxigenic fungi, to reduce or eliminate the toxin levels, to degrade or detoxify the toxins in foods and feeds (Pohanka, 2006 and Thanaboripat, 2002). Many groups of fungi are well known as biocontrol agents. Biological control can reduce the harmful effect of phytopathogenic or mycotoxigenic fungi while having a minimal impact on the environment (Pal and Gardener, 2006).

For example, *Trichoderma viride* and *T. harzianum* can produce amylolytic, pectinolytic, proteolytic and cellulolytic enzymes for growth inhibition of *A. flavus* [Pitt, Hocking 2006 and Gachomo and Kotchoni, 2008). This is consistent with the activity of several biocontrol agents in other systems, such as production of antifungal peptides by Bacillus strains (Pohanka, 2006) and production of aerugine, phenazine, and phloroglucinol antibiotics by Pseudomonas species . We also observed bacterial isolates that inhibited growth of *A. flavus* once the mycelial growth advanced to the edge of the bacterial colony. Production of aflatoxininhibiting compounds has been previously demonstrated in B. pumilus (Munimbazi and Bullerman, 1997). Screens for inhibitory activity of fractionated culture filtrates against *A. flavus* and other fungi will aid in characterizing the nature and spectrum of activity of antimicrobial compounds produced by those bacterial strains showing diffusible antifungal activity on agar. Similar screens using fractionated culture filtrates of strains with apparent aflatoxin-inhibiting activity and quantification of mycotoxins from *A. flavus and A. parasiticus* will be useful to define the activity of those strains. Various microbial antagonists have been investigated as potential antifungal biocontrol agents of plant diseases. Many species of actinomycetes, especially those belonging to the genus *Streptomyces* (Gram-positive, mycelia- forming soil bacteria), are well known as biocontrol agents that inhibit or lyse several soilborne and airborne plant pathogenic fungi . The antifungal potential of extracellular metabolites of *Streptomyces* strains against some fungi was previously reported from different locations of the world. The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of extracellular hydrolytic enzymes (Chellapandi and Jani, 2008).

The present study necessitates the periodical monitoring of post-harvest surveillance of mycotoxin in processed foods, studies in compliance with regulatory norms.

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