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Biological Control of Aflatoxin in Maize Grain at Ambient Storage Conditions

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A B S T R A C T

Aflatoxins are potent carcinogenic and mutagenic metabolites mainly produced by *Aspergillus flavus*. A study was conducted to manage the aflatoxin production in maize grains in ambient storage conditions using bioagents *i.e., Trichoderma asperellm, T. viride* and *Aspergillus niger* isolated from maize grain itself and initially tested for antagonistic ability against radial growth of *Aspergillus flavus. T. asperellum* was found most effective in suppressing the growth of test fungi. Inhibitory effect of these antagonists on aflatoxin production in ambient storage conditions was also studied on maize cultivars HQPM-1, HM-4 and Bio- 9681 under artificially inoculated condition. The formulation of *T. asperellum* @8gm/kg of grains was found most effective for 100% reduction in aflatoxin build up in cultivar HQPM-1, 96.32% in HM-4 and 93.04% in Bio-9681 after 4 month storage period.

Introduction

Aflatoxin contamination of crop is a worldwide food safety concern. Aflatoxins are a group of approximately 20 related fungal metabolites with major ones as B_1, B_2, G_1, and G_2. Aflatoxin B_1 (AFB_1) is the most potent naturally occurring chemical liver carcinogen (IARC, 1993).

Long term exposure from moderate to low concentration of aflatoxin causes chronic toxicity. It is one of the most important causes for stunting in children and immune system disorders (Turner *et al.,* 2003). Hence, their quantity in food and feed is closely monitored and regulated in most of the countries (Egmond Van, 1995). Almost 40% of crop products are lost yearly due to aflatoxin contamination in developing world (Miller, 1996). Fungal deterioration of stored seed and grain is a chronic problem in the Indian storage system because of tropical hot and humid climate. These conditions lead to colonization of harvested grains by various species of *Aspergillus* leading to deterioration in quality of food/feed due to aflatoxin production. *Aspergillus* is the main fungus responsible for aflatoxin contamination. Mycotoxins are well known for their health hazardous effect in human beings and animals (Probest *et al.*, 2007; Reddy and Raghavender, 2007). Among all the aflatoxin, particularly AFB_1 is the most hazardous, causing damage

such as toxic hepatitis, hemorrhage, edema, immune suppression and hepatic carcinoma.

In India, human disease outbreaks attributable to consumption of aflatoxin-contaminated maize have been reported from district of Banswara in Rajasthan and Panchmahal in Gujarat (Krishnamachari *et al.*, 1975). Also**,** the occurrence of aflatoxins and ochratoxin A has been reported in poultry feeds from India (Gong *et al.,* 2003; Thirmula Devi *et al.,* 2002).

There are strict regulations on chemical pesticide use and there is a political pressure to remove the most hazardous chemicals from market. Although restrictions are being imposed to protect food quality and the environment, chemicals are still our only recourse at present to prevent disease of food crops. Therefore, it is important to find a practical, cost effective and non-toxic method to prevent fungal contamination of stored maize. Use of biocontrol agents provides an opportunity to avoid various chemical preservatives and fungicides. Wide arrays of organisms have been tested for biological control of aflatoxin contamination including bacteria, yeasts, actinomycetes and algae (Mishra and Das 2003). Keeping this in a view, studies were undertaken to elucidate the potential of biological agents isolated from maize grains to minimise the buildup of aflatoxin due to contamination of *Aspergillus flavus* in post-harvest maize.

Materials and Methods

Grains from three genotypes *viz*., HQPM-1, HM-4 and Bio- 9681 were surface sterilized for 1 minute in 2.5% NaOCl, washed in three changes of sterile distilled water and plated 10 kernels on each culture plates containing Potato Dextrose Agar (PDA) medium aseptically. Three replicates from each sample plated and incubated at 28°C $\pm 2°C$ for three days. Fungal colonies on maize kernels visualized in stereo-binocular microscope (Olympus BH 2) counted and identified up to genus level as *Fusarium*, *Trichoderma* (Nelson *et al.,* 1983); *Aspergillus*, *Penicillium*, and other fungi (Pitt and Hocking, 1997). Further from each sample, only fungi *A. flavus*, was selected (Singh *et al.,* 1991). To obtain pure culture sub culturing was done for further study. These pure *A. flavus* isolates were enumerated AF-1 to AF-50, which were characterized on the basis of their toxicity as highly toxic; moderately toxic and non-toxic, with the help of Ammonia vapour test. Petri dishes were placed upside down and a drop (2 ml) of concentrated ammonia solution (SRL Extra pure AR Grade) was poured into the lid of each inverted culture plate and kept for 10-15 minutes to release ammonia vapour (Kumar *et. al* 2006).

Laboratory assessments of antagonistic effect of biocontrol agents with Dual culture technique

To assess the antagonistic effect of biocontrol agents; *Trichoderma asperellum*, *T. viride* and *A. niger* isolated from maize grains were purified and maintained on culture plate. Dual culture of bioagents and test pathogens were established to test the antagonistic activity. The PDA was inoculated at 1cm distance from the edge of the Petri dish with a 5mm mycelial disc cut from the leading edge of an active 5days-old colony of *A. flavus* and these biocontrol agents seperately. The plates were incubated at 28 ± °C for 5-6 days and the diameter of the inhibition zones of *A. flavus* growth, due to antagonistic activity, was measured in centimetres. The growth inhibition percentage (GIP) was expressed in terms of inhibition percentage of radial growth of the phytopathogen, i.e. by comparing with control plates without the presence of *T. asperellum* disc (Fig. 1 and 2).

Effect of seed treatment of biocontrol agents in minimizing the aflatoxin in post-harvest maize

Mass multiplication of biocontrol agents were accomplished on sorghum grains, prior soaked and autoclaved in a flask and incubated at 28 ±°C for a week (Fig. 3). Talcum formulations of these biocontrol agents were prepared after shade drying and grinding of these impregnated grains in to powder form and CFU adjusted at 10^9 by adding talcum powder. The formulation of these biocontrol agents were tested on fresh harvest maize samples of genotypes HQPM–1; HM–4; and Bio 9681 @4 and 8g/kg grains. The sample size of each genotype was 2½ kg.

Initial estimation of concentration of aflatoxin B_1 (AFB_1) of these grain samples were also done prior to start the experiment. The total grain samples of these genotypes were artificially inoculated with toxic isolate of *A. flavus*, (*Af* no. 8), before 24 hours to start the experiment @10mg spore biomass of *A. flavus*/kg grains. After 24 hours each inoculated grain sample (2½ kg) kept in a separate gunny bags and treated with above biocontrol agents separately and kept in ambient storage condition for 4 months duration. Stirring of grains was done once in a month to maintain the uniformity in the samples with individual glass rod. At the end a total of 100g of grain sample was taken from each replication for estimation of aflatoxin. The experiment was started in the month of August and estimation of aflatoxin from these maize grain samples was done by using Enzyme-Linked Immunosorbant Assay (ELISA) method after four month's storage period. The direct competitive ELISA method was followed. (A Training Course ICRISAT 2005). The statistical analysis was done by using the OD values obtained for AFB1 standards, taking AFB1 concentrations on the X-axis and OD values on the Y-axis (Fig. 5)

AFB1 (µg/kg): (A x D x E)/G

A = AFB1 concentration in sample extract (ng/ml)

D= Times dilution with buffer

E = Extraction solvent volume used (ml)

G = Sample weight (g)

Results and Discussion

All the bioagents were found effective in minimising the radial growth of *A. flavus*. Among all *T. asperellum* overgrew the test pathogen when grown together in a single plate and found more effective in suppressing the growth of test pathogen (Fig. 4). In this study the potential of these biocontrol agents *i.e., T. asperellum, T. viride* and *A. niger* were studied in reducing the synthesis of AFB_1 in post-harvest maize for four months duration. They were evaluated in two doses 4 and 8 g/kg. Among them *T. asperellum* was found most effective in inhibition the synthesis of AFB_1 by 100 % in *cv.* HQPM -1; 96.32 % in *cv*. HM-4 and 93.04 in *cv.* Bio 9681@ 8 g/kg whereas *T. viride* could inhibit the synthesis of AFB_1 in *cv.* HQPM-1 by 88.24 %; in *cv.* HM-4, 91.83 % and in *cv.* Bio 9681 93.79 (Table 1) as compared to check.

The initial level of AFB_1 concentration of these maize genotypes were 0.016ppb in *cv.* HQPM; 0.012ppb in *cv.* HM–4; and 0.031ppb in *cv.* Bio 9681 (Table 1 A and B). This indicated that grains were not free from aflatoxin contamination and already carrying very small amount of infection from field itself and these grains may spoil in storage due to poor storage condition and consequently the aflatoxin level may increase in due course. To avoid further spoilage of grains, application of these biocontrol agents in post-harvest storage were found very effective.

Table.1 Evaluation of biocontrol agents (isolated from maize grains) for reducing aflatoxin synthesis in maize grains (inoculated with AF no. 8 strain) after four months stored period

A. Effect of Biocontrol agents @4 gm/kg seeds inoculated by *A. flavus* on production of AFB_1 after 4 months storage period

Biocontrol agents used @ 4 gm/kg seeds	Grain Moisture %			Germination %			AFB1 (ppb)			% Reduction in AFB1		
	HQPM-1	HM-4	Bio 9681	HQPM-1	HM-4	Bio-9681	HQPM-1*	HM-4*	Bio-9681*	HQPM-1	HM-4	Bio 9681
T. asperellum	13.0	13.1	13.3	2.0	50	100	1.978	9.789	1.1949	96.86	85.91	**87.79**
T. virede	12.5	12.8	13.3	10	50	90	21.985	9.789	1.598	65.105	85.91	**83.67**
A. niger	12.6	13.5	13.2	20	20	80	41.370	22.967	9.789	34.35	66.95	**0.0**
A. flavus[1]	12.3	13.5	12.9	10	40	100	63.005	69.496	9.789	-	-	-
Check (No treatment)	**13.0**	**13.7**	**13.1**	**10**	**43**	**100**	**64.00**	**18.57**	**3.21**	-	-	-

B. Effect of Biocontrol agents @ 8 gm/kg seeds inoculated by *A. flavus* on production of AFB_1 after 4 months storage period

Biocontrol agents used @ 8 gm/kg seeds	Moisture %			Germination %			AFB1 (ppb)			% Reduction in AFB1		
	HQPM-1	HM-4	Bio 9681	HQPM-1	HM-4	Bio-9681	HQPM-1*	HM-4*	Bio-9681*	HQPM-1	HM-4	Bio 9681
T. asperellum	11.8	13.3	13.3	10	60	100	0.009	3.371	0.938	100.0	96.32	**93.04**
T. viride	12.5	13.3	13.2	20	20	95	12.554	7.499	0.836	88.24	91.83	**93.79**
A. niger	12.9	13.3	12.8	30	10	85	45.422	28.425	11.231	57.46	69.04	**16.67**
A. flavus[1]	13.1	13.3	13.1	10	30	100	106.785	91.825	13.478	-	-	-
Check (No treatment)	**13.0**	**13.7**	**13.1**	**10**	**43**	**100**	**64.00**	**18.57**	**3.21**	-	-	-

Conc. at initial level * HQPM-1 -- 0.016 ppb; HM-4 -- 0.012 ppb; Bio 9681-- 0.031 ppb

[1]Untreated Grain samples artificially inoculated with toxic strains of *A. flavus* @10mg/kg grains

Grain sample size of each genotype - 2½ kg

The storage experiment was started in the month of August for the four months duration

Fig.1 A. Healthy maize grains B. *Aspergillus* contaminated grains

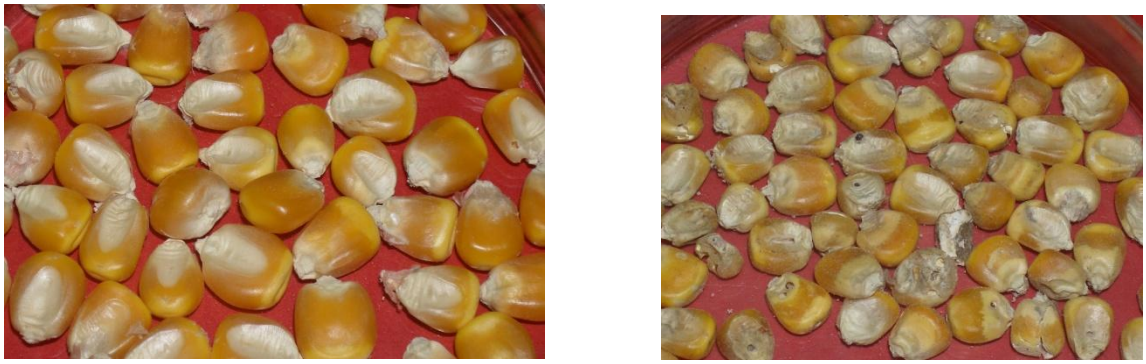
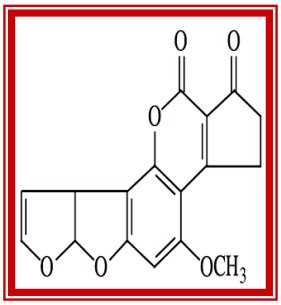


Fig.2 Aflatoxin molecule

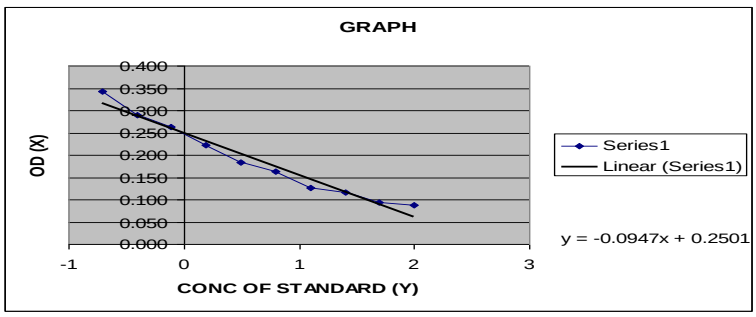


AFB1

Fig.3 Mass multiplication of biocontrol agents

Fig.4 *Trichoderma asperellum* and *Aspergillus niger* inhibiting the growth of *A. flavus*

Fig.5 Graph based on the different concentration of standard



T. asperellum inhibited the synthesis of AFB_1 up to 100% in *cv.* HQPM-1 @8gm/kg grain as compared to check. The initial concentration of AFB_1 in *cv.* HQPM-1 was 0.016ppb which goes up to 106.78ppb when artificially inoculated with toxic strain of *A. flavus* in four months duration which is above the permissible limits (20ppb) whereas the grain treated with *T. asperellum* exhibited concentration of AFB_1 0.009ppb and 0.938ppb in Bio 9681. 100% reduction in aflatoxin build-up was observed when treated with *T. asperellum* and E.L. Katatny *et al.*, (2001) was also observed that the inhibition activity of *T. asperellum* was likely due to antimicrobial effects when co-cultured with other organism. It is therefore hypothesized that the *T. asperellum* and *T. viride* considered in this study probably produced an array of enzymes with antimicrobial effect against the *A. flavus*. Gachoma and Kotchoni (2008) suggested that the *Trichoderma* sp. can effectively control aflatoxin contamination of aflatoxin in peanut kernels. The production of volatile and extracellular enzymes by *T. asperellum* isolates may have been sufficient to suppress the growth of maize molds, responsible for AFB1 production and consequently a lower production of aflatoxin synthesis.

The study showed that the aflatoxin concentration is minimized, which may be an indirect effect of the suppressed growth of aflatoxigenic fungi *A. flavus* by *T. asperellum* leading to assumption that fewer colonies produced less aflatoxin. The antimicrobial effect of *T. asperellum* might be a potential source of anti-pathogenic activity that can be used to control aflatoxin build up in post-harvest maize. This may be linked to the chitinase activity displayed by *T. harzianum*. Chitinase is believed to be a key enzyme in mycoparasitism hypothesis presented by Howell (2003). The bio control agent, *Trichoderma* species have been successfully used in minimising aflatoxin build-up due to their high reproductive capacity, efficient utilization of nutrients, strong aggressiveness against other phytopathogens, efficiency in promoting plant growth and defence mechanism and ability to modify the rhizosphere (Kleifeld and Chet, 1992; El-Katatny *et al.*, 2001; Benítez *et al.*, 2004). Based on our findings we concluded that the likely mechanisms by which *Trichoderma* species suppressed A. flavus in maize might involve production of volatiles and/or production of extracellular enzymes. This is an important finding because it opens the opportunities for further study the underlying antagonistic mechanisms of the genus *Trichoderma* to efficiently minimise aflatoxin contamination of crop products.

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