

Biological Control of Aflatoxin Contamination in Agricultural Commodities by Atoxigenic Strains of *Aspergillus*

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Abstract: Atoxigenic strains of *Aspergillus* are promising biocontrol agents to inhibit the pre-harvest aflatoxins (AFs) contamination in vulnerable crops. In this study, the effect of atoxigenic strains of *Aspergillus* on the growth of toxigenic strains and AFs production were evaluated, using well diffusion and culture filtrate methods. The AFs contamination was quantified, using HPLC technique with post column derivatization. It was observed that atoxigenic strains reduced the growth of toxigenic *Aspergillus (A.) flavus* and *A. parasiticus* from 55 to 65%. Moreover, the culture filtrates derived from atoxigenic strains of *A. niger* and *A. flavus* reduced the AFB₁ and AFB₂ contamination up to 100%. The results further showed that AFB₁ and AFB₂ production gradually decreased with the increase of conidial concentrations of atoxigenic strains of *A. niger* and *A. flavus*. It was concluded that the atoxigenic *A. flavus* was the most effective strain inhibiting the growth of toxigenic *A. flavus* and *A. parasiticus* and AFs production in both on agar medium and on culture filtrates. In general, these atoxigenic strains can be used to control toxigenic strains at pre- and post-harvest levels.

Key words: *Aspergillus*, Aflatoxins, Biological control.

INTRODUCTION

Food safety is one of the most essential components of consumer protection. Many cereals, nuts, fruits and other important food crops are susceptible to infection by toxigenic fungi, such as, *Aspergillus*, *Penicillium*, *Fusarium*, etc. These fungi pose serious phyto-sanitary and mycotoxicological risks, due to their ability to produce several mycotoxins at different stages of harvesting, storage, transportation as well as in food processing (Zain, 2011). Mycotoxins can affect both human and animal health when they are consumed with contaminated food and feed at sufficient levels. However, toxic effects may differ according to different factors, e.g., the intake levels, duration of exposure, toxin species, metabolism and immune system status (Hussein and Brasel, 2001).

Aflatoxins (AFs) are the best known and most intensively reported mycotoxins that are produced by fungi of genus *Aspergillus* species, particularly, *A. flavus* and *A. parasiticus* during different stages of harvesting and storage (Creppy, 2002). AFs are considered as carcinogenic, hepatotoxic, teratogenic and immunosuppressive fungal metabolites (Wen *et al.*, 2004). To date, at least 18 different types of AFs

have been identified of which AFB₁ has been recognized as the most important in a range of food commodities from various parts of the world (Khan *et al.*, 2014; Asghar *et al.*, 2016a; Asghar *et al.*, 2016b). Bonsi *et al.* (1999) demonstrated that AFB₁ could repress the cyclic nucleotide phosphodiesterase activity in brain, liver, heart and kidney tissues. AFs production might be affected by temperature, relative humidity or moisture, CO₂ and water activities (Maren, 2007). Generally, AFs are produced at temperature between 24-30 °C due to some variations in fungal strains and substrate (Zheng *et al.*, 2015).

In order to improve the nutritional status and food safety, it is important to increase the capacity to control AFs contamination levels in food supply. Several physical, chemical, and biological techniques have been developed to control the aflatoxigenic moulds and AFs production (Guessas *et al.*, 2005; Tripathi and Mishra, 2009; Khan *et al.*, 2011; Khan *et al.*, 2013). However, the use of many methods is restricted, due to problems concerning safety issues, possible losses in the nutritional quality of treated commodities, limited efficacy and cost implications.

Therefore, it is suggested that strategies should be taken right from the field, where commodities are

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cultivated. In these circumstances, a promising strategy might be the adaptation of biological control approach which, not only, reduces the AFs content, but also retains the nutritional values along with the organoleptic properties of the commodities. Several organisms, including atoxigenic *Aspergillus* species, yeasts, bacteria and fungi, have been tested for their ability to reduce both fungal infection and AFs contamination (Yan-ni *et al.*, 2008). Currently, atoxigenic fungal strains are being used widely to prevent pre-harvest AFs contamination in several parts of the world. Recent advancements in the use of biocontrol strategies have led to the registration of commercial products with increased practical applications for the benefit of growers.

This study was designed to get the information on the type of interactions between each of the two fungal isolates, i.e., atoxigenic (*A. flavus* and *A. niger*) and toxigenic (*A. flavus* and *A. parasiticus*), using Well diffusion method. Furthermore, toxigenic cultures were grown in liquid broth medium to investigate the production of AFs in the presence and absence of atoxigenic strains.

MATERIALS AND METHODS

Chemicals and reagents: Aflatoxin B₁ (2 mg/ml; cat. # BMR 002017), B₂ (0.5 mg/ml; cat. # BMR 002018), G₁ (2 mg/ml; cat. # BMR 002019) and G₂ (0.51 mg/ml; cat. # BMR 002020) standards for HPLC were purchased from Biopure (Vienna, Austria). All other chemical and solvents were purchased from Oxoid (Hampshire, UK), Sigma-Aldrich (St. Louis-MO, USA) and Merck (Darmstadt, Germany). Highly purified water (18 MΩ•cm) was obtained from a Purelab Ultra system (ELGA, UK).

Apparatus: The HPLC system consisted of a pump (Model # L-2130; VWR-Hitachi, Tokyo, Japan), an auto-sampler (Model # L-2200; Merck-Hitachi, Tokyo, Japan) and a fluorescence detector (Model # L-2480; VWR-Hitachi, Tokyo, Japan). A LiChroCART® 100 Å RP-18 (5 µm, 250 × 4.0 mm) column from Merck (Darmstadt, Germany) and column thermostat were purchased from Jones-Chromatography (Wales, United Kingdom). Kobra Cell from R-Biopharm (Glasgow, Scotland) and AflaStar™ IACs (cat. # COIAC1001) were procured from Romer Labs. (Tulln, Austria). Micro-centrifuge machine from Hettich (Buckinghamshire, UK) and autoclave were purchased from Rexall (Kaohsiung, Taiwan).

Aspergillus Strains: A total of 102 samples of food and feed commodities were tested, for the presence of *Aspergillus* species (Ibrahim *et al.*, 2016). These strains were isolated and screened for their ability to produce AFs using polymerase chain reaction (PCR) and thin layer chromatography (TLC) (Ibrahim *et al.*, 2016). Out of 102 *Aspergillus* strains, 9 strains were positive for AFs production. The competitive inhibition was carried out using two atoxigenic strains, i.e., *A. flavus* (FH21) and *A. niger* (FH60) and

two toxigenic strains *A. flavus* (FH25) and *A. parasiticus* (FH44). The toxigenic strains were reconfirmed for their potential to produce AFs by HPLC.

Evaluation of antagonism between toxigenic and satoxigenic strains: Atoxigenic strains of *A. flavus* and *A. niger* were evaluated for their antagonistic property against toxigenic strains of *A. flavus* and *A. parasiticus*, using Well diffusion method. The spores' suspension of toxigenic strains was inoculated in 9 mm well (made by sterilized borer) on Petri dish containing potato dextrose agar medium (Oxoid, UK). At the same time, spore suspension of atoxigenic was also inoculated in another well at a distance of 3cm from toxigenic strains. The plates were incubated at 25 ± 2 °C for 7 days in dark. All experiments were performed in triplicates. Radius of toxigenic strains was measured from both sides, away from the atoxigenic strain (*r1*) and towards the atoxigenic strain (*r2*). The percentage of inhibition was calculated from the following formula as reported by Fokkema (1973).

$$\text{Inhibition (\%)} = \frac{r1-r2}{r2} \times 100 \quad \dots \text{Eq. 1}$$

Furthermore, each test of atoxigenic strains was performed on separate dish with toxigenic strains of *Aspergillus*.

Effect of atoxigenic strains on AFs production: AFs inhibition was observed by the competition growth of toxigenic and atoxigenic strains of *Aspergillus* in cultural filtrates. Briefly, 1 ml of each toxigenic strain (10⁶ spores/ml) were inoculated with different concentrations of atoxigenic strains (0-1.0 ml) in 100 ml czepak dox liquid medium (Oxoid, UK) and incubated at 25 °C ± 2 °C for 15 days in dark. In parallel, positive control medium inoculated with toxigenic strain and negative control with atoxigenic strain were also studied. AFs were quantified, using HPLC. All experiments were performed in triplicate.

AFs detection by HPLC: The process for the quantification of AFs could be divided into two major steps as described below:

Sample extraction: After incubation period, the spore suspension was blended at 5000 rpm, using an explosion-proof blender (Ebach, USA) for 2 min. The blended extract was filtered through Whatman No. 1 filter paper. Ten milliliter filtrate was diluted with 100 ml of Phosphate buffer saline (PBS) (pH 7.4) and applied to AflaStar™ aflatoxin immunoaffinity columns (IAC_s) at a flow rate of 1 drop/s. IAC_s were washed with 20 ml of PBS (pH 7.4) at a flow rate of approximately 5 ml/min and rapidly dried by passing air. AFs were eluted in an amber vial with 1.5 ml of methanol followed by 1.5 ml of deionized water.

Chromatographic analysis: Chromatographic analysis of AFs was carried out, using HPLC system with post-column derivatization and fluorescence detector (Asghar *et al.*, 2014). The operating conditions of HPLC are presented in Table 1.

Briefly, 99 µl of each standard and/or sample were injected to HPLC system through an auto-sampler and performed in an isocratic mode. All four AFs were properly resolved within total run time about 20 min.

Statistical analysis: The data analysis was performed using a randomized factorial design with 2 factors. The first and second factors were different concentration of atoxigenic fungi and the isolates of toxigenic fungi, respectively. All values were expressed as the means ± SD.

Table 1: HPLC operating conditions for the quantification of aflatoxins (B₁, B₂, G₁ and G₂).

Mobile phase	Flow rate	Inject volume	Column temperature	Fluorescence detector	Kobra cell	Detection limit	Quantification limit
65/17.5/17.5 H ₂ O/ACN/MeOH with 119 mg/l of KBr and 154 µl/l of HNO ₃ (65%; w/v)	1 mL/min	99 µl	40 °C	λ _{ex} = 365 nm λ _{em} = 435 nm	Current source = 100 µA	0.142	0.381

ACN: acetonitrile; MeOH: methanol; KBr: potassium bromide; HNO₃ nitric acid; λ_{ex}: excitation wavelength; λ_{em}: emission wavelength.

RESULTS AND DISCUSSION

Effect of atoxigenic strains on the growth of toxigenic strains of *Aspergillus*: Growth inhibition of toxigenic *A. flavus* and *A. parasiticus* by atoxigenic *A. flavus* and *A. niger* strains were observed, using well diffusion method. The percentage of growth inhibition was calculated, using Fokkema formula (Table 2).

The results showed that the inhibition of toxigenic strains of *A. parasiticus* by atoxigenic strains of *A. niger* was found 61.0%, as compared to atoxigenic *A.*

flavus (55.0%). Moreover, the growth inhibition of toxigenic strains of *A. flavus* was higher by atoxigenic strains of *A. flavus* (65.2%) in comparison to atoxigenic *A. niger* (54.5%). Dharmaputra *et al.* (2003) reported that the percentage of growth inhibition of toxigenic *A. flavus* by culture filtrate of atoxigenic *A. flavus* and *A. niger* were 69.44% and 72.51%, respectively. The achieved results of the present study were close to the reported study by Dharmaputra *et al.* (2003).

Table 2: Growth inhibition of toxigenic strains of *A. flavus* and *A. parasiticus* by atoxigenic strains of *A. flavus* and *A. niger* grown on PDA medium.

S. No.	Toxigenic strains	Atoxigenic strains	Growth inhibition (%)
1.	<i>A. parasiticus</i>	<i>A. flavus</i>	55.0 ± 1.2
		<i>A. niger</i>	61.0 ± 1.6
2.	<i>A. flavus</i>	<i>A. flavus</i>	65.2 ± 0.9
		<i>A. niger</i>	54.5 ± 1.0

PDA: potato dextrose agar

Effect of atoxigenic strains of *Aspergillus* on Aflatoxins (AFs) production: Inhibition of aflatoxins (AFB₁ and AFB₂) production was also investigated by growing both types of strains in liquid medium. One mL of each toxigenic strain (10⁶ spores/ml) was inoculated with different concentrations of atoxigenic strains. It was observed that atoxigenic *Aspergillus* inhibited the synthesis of AFs by toxigenic strains of

Aspergillus in Czepak Dox liquid broth (CZ). The effect of atoxigenic strains of *A. niger* and *A. flavus* on the growth of toxigenic strains of *A. parasiticus* and AFs production is presented in Table 3, while the effect of atoxigenic strains of *A. niger* and *A. flavus* on the growth of toxigenic strains of *A. flavus* and AFs production is presented in Table 4.

Table 3: Effect of different concentration of atoxigenic strains of *A. niger* and *A. flavus* on the production of AFB₁ and AFB₂ (produce by toxigenic *A. parasiticus*). All experiments were performed in triplicate (the results are reported in mean ± SD).

Toxigenic <i>A. parasiticus</i> (ml/100ml CZ broth)	Atoxigenic <i>A. niger</i> (ml/100ml CZ broth)	AFB ₁		AFB ₂		Atoxigenic <i>A. flavus</i> (ml/100ml CZ broth)	AFB ₁		AFB ₂	
		C (µg/g)	I (%)	C (µg/g)	I (%)		C (µg/g)	I (%)	C (µg/g)	I (%)
1.0	0	500 ± 20	0	50 ± 5	0	0	500 ± 22	0	50 ± 4	0
1.0	0.10	480 ± 15	4 ± 1	48 ± 2	4 ± 1	0.10	410 ± 14	18 ± 2	46 ± 2	9 ± 1
1.0	0.25	380 ± 12	24 ± 3	45 ± 5	11 ± 2	0.25	312 ± 11	38 ± 2	33 ± 2	34 ± 2
1.0	0.50	210 ± 11	58 ± 3	23 ± 4	54 ± 4	0.50	140 ± 8	72 ± 3	16 ± 3	68 ± 3
1.0	0.75	115 ± 8	77 ± 4	14 ± 1	73 ± 3	0.75	85 ± 6	84 ± 4	9 ± 1	81 ± 4
1.0	1.0	0	100 ± 4	0	100 ± 4	1.0	0	100 ± 4	0	100 ± 4

CZ; czepak dox liquid medium; AFB₁: aflatoxin B₁; AFB₂: aflatoxins B₂; C: concentration; I: inhibition.

Table 4: Effect of different concentration of atoxigenic strains of *A. niger* and *A. flavus* on the production of AFB₁ and AFB₂ (produce by toxigenic *A. flavus*). All experiments were performed in triplicate (the results are reported in mean ± SD).

Toxigenic <i>A. flavus</i> (ml/100ml CZ broth)	Atoxigenic <i>A. niger</i> (ml/100ml CZ broth)	AFB ₁		AFB ₂		Atoxigenic <i>A. flavus</i> (ml/100ml CZ broth)	AFB ₁		AFB ₂	
		C (µg/g)	I (%)	C (µg/g)	I (%)		C (µg/g)	I (%)	C (µg/g)	I (%)
1.0	0	460 ± 20	0	46 ± 4	0	0	460 ± 21	0	46 ± 5	0
1.0	0.10	320 ± 14	30 ± 2	34 ± 2	27 ± 2	0.10	280 ± 11	39 ± 3	31 ± 4	32 ± 3
1.0	0.25	225 ± 11	51 ± 4	26 ± 3	44 ± 3	0.25	165 ± 10	64 ± 3	19 ± 2	58 ± 4
1.0	0.50	140 ± 6	70 ± 3	18 ± 2	67 ± 4	0.50	98 ± 4	79 ± 4	10 ± 1	77 ± 4
1.0	0.75	85 ± 4	81 ± 3	9 ± 1	79 ± 3	0.75	43 ± 3	90 ± 4	4 ± 0.8	90 ± 3
1.0	1.0	0	100 ± 4	0	100 ± 4	1.0	0	100 ± 4	0	100 ± 4

CZ: czepak dox liquid medium; AFB₁: aflatoxin B₁; AFB₂: aflatoxins B₂; C: concentration; I: inhibition.

The results showed that a very high concentration of AFB₁ (500 µg/g) and AFB₂ (50 µg/g) was produced by the toxigenic strains of *A. flavus* and *A. parasiticus* when grown alone in CZ medium. However, these strains when inoculated with atoxigenic strains, culture filtrates have shown the reduction of AFB₁ and AFB₂ concentration up to 100%. Furthermore, the results also showed that the

inhibition rate of AFB₁ and AFB₂ production gradually increased with the increment of conidial concentrations of atoxigenic strains of *A. niger* and *A. flavus*. The effect of different concentrations of atoxigenic strains (*A. niger* and *A. flavus*) on the production of total aflatoxins (AFB₁ + AFB₂) production by toxigenic strains (*A. parasiticus* and *A. flavus*) is presented in Fig. 1 (A-B).

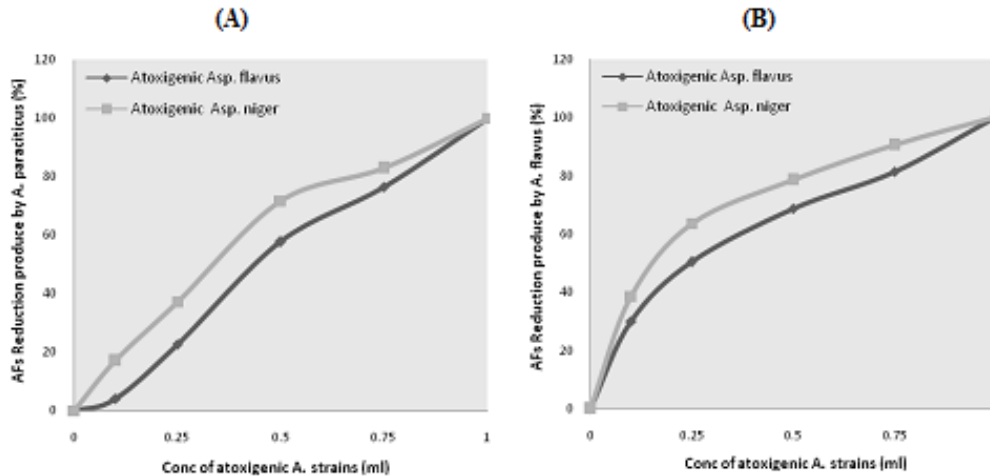


Fig. 1 (A-B): Effect of different concentration of atoxigenic strains (*A. niger* and *A. flavus*) on the production of total aflatoxins (AFB₁ + AFB₂) produce by toxigenic strains of (A) *A. parasiticus* (B) *A. flavus*.

It was observed that atoxigenic *A. flavus* was the most effective fungus in inhibiting the growth of toxigenic *A. flavus* and *A. parasiticus*. Culture filtrate of atoxigenic *A. flavus* was also the most efficient filtrate in inhibiting AFB₁ and AFB₂ production of toxigenic *A. flavus* and *A. parasiticus*. Brown *et al.*, (1991) reported that the atoxigenic strains of *A. flavus* reduced pre-harvest AFs contamination by 80 to 95% in maize. The atoxigenic strains were also effective in reducing postharvest AFs contamination caused by both an introduced toxigenic strain and by strains resident on the kernels. These finding are similar to the earlier studies that atoxigenic is the most potential

test fungus in inhibiting the growth of toxigenic and AFs contamination up to 100%.

Extensive studies have been reported on the biological control of toxigenic fungi and subsequent AFs contamination in both pre- and post-harvest levels. Cotty and Bhatnagar (1994) reported that the application of atoxigenic strains of *A. flavus* (on grain seeds) to soils of cotton fields was very effective for controlling AFs production in cottonseed. In many field experiments with peanut and cotton, significant reductions in AFs contamination in the range of 70~90% have been observed consistently by the use of atoxigenic *Aspergillus* strains (Pitt and Hocking, 2006; Dorner, 2008). Dorner and Cole (2002)

reported that the application of atoxigenic strains of *A. flavus* and *A. parasiticus* on soils of peanut fields at different doses, significant reduction reduced AF contamination by 74.3–99.9%. These results are in line with those of previous reports (Domer and Cole, 2002; Pitt and Hocking, 2006).

In theory, the atoxigenic strain is applied once per growing season when the overall *A. flavus* inocula levels in the field are low, thereby providing the applied strain the time and preferential exposure to be established in the crop, as well as, a subsequent advantage when competing for crop resources with the toxigenic strains (Cleveland *et al.*, 2003; Cotty and Mellon, 2006). Two atoxigenic *A. flavus* strains, AFs36 and NRRL 21882 are currently being used in USA, to minimize AFs contamination in two important crops. AFs36 has been registered as a bio-pesticide by the U.S. Environmental Protection Agency and is being used for the management of AFs producing fungi in cotton fields. However, NRRL 21882 is being used to prevent AFs contamination in peanut fields (Chang and Hua, 2007). Many countries also conducted similar studies, e.g., in Africa, atoxigenic strain BN30 was very effective in reducing the amount of toxin produced in maize when co-inoculated with the highly toxigenic S-strain (Cardwell and Henry, 2004). Further, a study in Australia revealed that the application of atoxigenic strains could reduce AFs formation in peanuts by 95% (Pitt and Hocking, 2006).

Different authors have reported a possible interaction mechanism of toxigenic and atoxigenic strains of *Aspergillus*. For instance, Jeffries and Young (1994) reported that the *Aspergillus* strain produced some extracellular metabolites, such as, antibiotics and lytic enzymes, which were antagonistic to toxigenic strains. As a result, toxigenic strains of different *Aspergilla* reduce the AFs production. Different factors influence the reduction of AFs contamination using atoxigenic strains of *Aspergillus*, such as, (i) the formulation in the combination of competitive strain and carrier/substrate, (ii) optional time for application of atoxigenic strain and (iii) inoculum rate (Yan-ni *et al.*, 2008). Further studies are required to evaluate the potential efficacy of various biological agents, including studies focusing on the dose, formulation and timing of the applications as well as molecular studies that elucidate impact of biocontrol agents in alterations of plant physiology or plant innate immune system.

CONCLUSION

Biological control holds a promise of offering a long-term solution for the growth of aflatoxigenic fungi and AFs production. Atoxigenic *A. flavus* was the most efficient test fungus in inhibiting the growth of toxigenic *A. flavus* and *A. parasiticus* and AFs production. This technology may improve food production and the income of small farmers as they

will be able to participate in local and formal trade initiatives. The technology addresses a serious food safety issue and benefits will accrue to the entire value chain to small producers, their families, food and feed processors and food consumers, among groups with increased vulnerability to diseases, particularly the people of Pakistan.

CONFLICTS OF INTEREST

Authors declared they have no conflicts of interest.

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