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IMPACT OF AFLATOXINS ON GROWTH OF NITROGEN FIXING BACTERIA

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ABSTRACT

Aflatoxins produced by *Aspergillus* are the potent carcinogenic compounds frequently found in foods contaminated with *Aspergillus* species of section *Flavi*. The purpose of this study was to determine the type(s) of aflatoxins in strains isolated from different food sources grown on different growth media; and to assess the impact of aflatoxins in reducing growth of soil nitrogen fixing bacteria. Three fungal strains; *Aspergillus flavus* (FCBP0616 and FCBP0862) and *A. oryzae* (FCBP1202) were assessed for aflatoxins production when grown in coconut cream and malt extract agar/broth. Preliminary screening indicated stronger signal of aflatoxins by *A. flavus* strains than *A. oryzae* under UV light. Mycotoxigenic assays through Thin Layer Chromatography (TLC) revealed presence of aflatoxins B1, G1 and G2 in all three stains grown on coconut cream agar with aflatoxin B1 as the most pronounced. Coconut broth medium showed maximum toxin production ability as compared to other media. Afterwards, extracted aflatoxins were screened for their efficacy against three bacteria; *Azospirillum lipoferum*, *Azomonas agilis* and *Azotobacter insignis*. Results revealed that toxins produced by *A. flavus* (FCBP0616) depicted maximum inhibitory effect on the growth of *A. agilis* and *A. lipoferum* while *A. oryzae* (FCBP1202) maximally influenced A. *insignis*. Results suggested that under different growth conditions ability of strains to produce aflatoxin(s) varies and presence of aflatoxigenic strains in soil reduces its fertility.

Key words: Aflatoxins, *Aspergillus* species, Thin Layer Chromatography, soil fertility, Nitrifying Bacteria.

INTRODUCTION

Aflatoxins are the well-known mycotoxins being potent carcinogenic compounds produced mainly by strains of *Aspergillus flavus* and *A. parasiticus*. It has been previously established that strains of *Aspergillus oryzae* which is generally regarded as safe are not safe and can produce aflatoxins in detectable amounts (Fente *et al*., 2001; Jorgensen, 2007; Kim *et al*., 2014). Due to the high risk of toxic effects of aflatoxins on human and animal health, the quantity of these compounds is monitored carefully all over the world. Cream Agar (CCA) has been previously used as a simple and inexpensive chromogenic growth medium for screening the aflatoxin production ability of fungi (Dyer and McCammon, 1994). In addition of visual screening of aflatoxin, CCA has also been used for the detection of ochratoxin (Heenan *et al*., 1998). Reverse colonies of aflatoxigenic fungi growing on CCA when visualized under UV produce florescence while the stronger the florescence indicates higher amounts of aflatoxins (Mohamed *et al*., 2013).

Species of Aspergillus in section flavi have known antibacterial potential. *Aspergillus flavus* is also capable of producing an antibiotic substance, designated as aspergillic acid. This isolated substance had about the same effect against gram-positive as against gram-negative bacteria (White and Hill, 1943). The production of antibiotics by a strain of *A. flavus* was aspergillic acid (Wintersteiner *et al*., 1943) and hydroxyaspergillic acid (Perry *et al*., 1984). Isolation of antibiotics had also been done by the cultured filtrate of *A. oryzae*, responsible against *Hiochi* bacteria. It had been proved that this antibiotic was identical with hydroxyaspergillic acid (Nakamura *et al*., 1959). Some other strains of *A. flavus* group were found to produce an antibacterial agent named as flavicin, which is active largely against gram-positive bacteria (Milton *et al*., 1945). It has been previously reported that *A. flavus* had inhibitory effects on nitrogen fixation (Al-Maadhidi and Henriksson, 1980). *A. flavus* also produces bioactive compounds that inhibits the growth of bacteria like *Escherichia coli* (Mohammed *et al*., 2016) and *A. flavus* also showed inhibition due to its ability to produce aflatoxins (Hina *et al*., 2015). Mycotoxin disturbs various metabolisms of bacterial strain i.e. DNA synthesis, protein synthesis, thuricin excretion and inhibition of endotoxin formation, specific activities (bacterial motility, flagellar arrangement and sporulation) (Auffray and Boutibonnes, 1986). By keeping the above constrains in view, the present study was designed to detect and evaluate the mycotoxigenic fungi like *A. flavus* and its biocontrol potential. Aflatoxins were selected among mycotoxins because of its wide occurrence in raw products and its high toxicity to other organisms including nitrogen fixing bacteria.

MATERIALS AND METHODS

Species of genus *Aspergillus* section Flavi were selected to assess their aflatoxin production potential. Pure cultures of selected *Aspergillus* species were obtained from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan (Table 1). These isolates were revived and maintained on 2% Malt Extract Agar medium; pH 6.5.

Table 1. Detail of fungal strains used for present study.

Sr No.	*Aspergillus* sp.	Substrate	Accession No.
1	*A. flavus*	*Malus domestica* fruit	FCBP0616
2	*A. flavus*	*Arbutus unedo* fruit	FCBP0862
3	*A. oryzae*	*Zea mays* seeds	FCBP1202

Fungal Growth Media for Determining Aflatoxigenic Potential

A variety of growth media recommended for the growth of aflatoxigenic strains of *Aspergillus* were used to detect their comparative ability of toxin production. These growth media include Malt Extract Broth (MEB), Coconut Cream Agar (CCA) and Coconut Cream Broth (CCB) (Dyer and McCammon, 1994).

CCA media was prepared by homogenizing 200 mL of coconut cream in 300 mL of hot distilled water. To this mixture, 7g agar was added. However Coconut Cream broth (CCB) was prepared by mixing 400 mL of coconut cream in 600 mL of distilled water. Finally Malt Extract broth was prepared by dissolving 25g Malt extract in 1.5 L distilled water. To avoid bacterial contamination, streptomycin was added to cooled molten media at the rate of 200 mg/L medium. Media were sterilized by autoclaving and poured into pre-sterilized Petri plates under aseptic conditions. The media plates were inoculated in by spores of selected strains while broth media with agar discs of 5 mm from actively growing fungus culture. Inoculated flasks and Petri plates were incubated for 15 and 7 days, respectively, at 26 $\pm$ 2 °C in dark.

Study of Aflatoxin Production

To know the aflatoxin production ability, 36 h old culture plates were exposed to ultra-violet (UV) light (365 nm) and observed for the presence or absence of aflatoxins fluorescence surrounding the growing colonies (Abbas *et al.*, 2004). Then aflatoxins extraction was carried out to detect the toxins via thin layer chromatography (TLC). Agar medium was cut after gently removing the upper mycelium of fungi, put into 50 mL chloroform, incubated at 27 °C and 200 rpm. After 3 h of gentle shaking, contents were filtered through Whatman No 1 filter paper. Chloroform extracts were allowed to dry completely. For qualitative analysis of aflatoxin(s) by TLC, (Davies *et al.*, 1987), chloroform extracts were dissolved in 2 mL commercial methanol. From these samples, 20 µL of each sample was spotted on TLC plates (silica gel 60 F254 coated on aluminium sheet, 20×20 cm in size) along with aflatoxins standard. Samples were run in chloroform and acetone mixture prepared in a ratio of 9:1 (Reddy *et al*., 2004) up to 3/4 of the plate height. TLC plates were dried in dark, observed under UV light (365 nm) and compared with aflatoxin standard.

Aflatoxin Extraction for Anti-bacterial Assays

Broth medium was inoculated with an agar disc from the culture of each *Aspergillus* isolate. Strains were allowed to grow for 15 days 26 $\pm$ 2 °C in dark. Mycelia were filtered and filtrate was dried at 40 °C in an oven until it converted into thick paste like material. Such material was dissolved in 50 mL chloroform and left for mixing in an orbital shaker for 3 hrs. Chloroform soluble fractions were separated by filtration through Whatman filter paper No 1 and evaporated to collect the crude chloroform soluble fraction. Toxigenicity of *Aspergillus* species was evaluated by TLC to confirm the presence or absence of aflatoxins (B1, B2, G1, and G2).

For the preparation of stock solutions, 2 g of each extracted toxin was mixed with 2 mL of distilled water. Stock solution was diluted for the preparation of, 0, 10, 20, 30, 40 and 50 % (w/v) concentrations.

Experimental Set up of Anti-bacterial Assay

Three nitrogen fixing bacteria namely *Azospirillum lipoferum* (FCBP0356), *Azomonas agilis* (FCBP0385) and *Azotobacter insignis* (FCBP0416) were procured from First Fungal Culture Bank of Pakistan, (FCBP), Institute of Agricultural Sciences, University of Punjab, Lahore, Pakistan. Bacteria were maintained on LBA medium (Yeast

Extract 5 g, Agar 10 g, Trypton 10 g, NaCl 5 g, in 1 L of distilled water; pH 6.85). Antibacterial activity was screened by agar well diffusion method against selected bacterial strains (Okeke *et al.*, 2001). The 10^5 bacterial cells were spread on LBA plates under aseptic conditions. Three wells each of 0.5 cm diameter were bored in agar medium and 50 µL of appropriate aflatoxin treatment was loaded on all three wells to serve as replicate. Pure water was used as control treatment. Each treatment was carried out in triplicate (Plate 1). Petri plates were incubated at 37 °C for 24 h and the diameter of inhibition zones around the wells was measured. Standard errors of means of replicates of each treatment were computed using computer software Microsoft Excel .

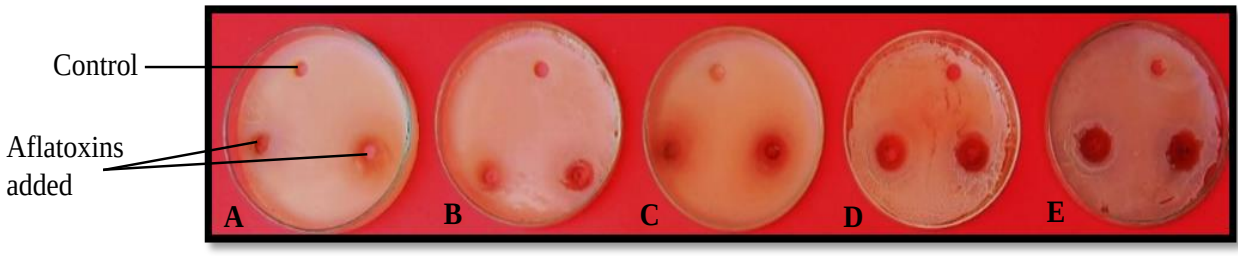

Plate 1. A representation of experimental set up to study antibacterial potential of extracted aflatoxins. Out of the three 6 mm wells in each petriplate, top well of each plate demonstrated control treatment (No aflatoxins added) while two other wells received the same amount of aflatoxins. **A:** 10 mg; **B:** 20 mg; **C:** 30 mg; **D:** 40 mg and **E:** 50 mg toxins added.

RESULTS

Toxigenic Potential of Strains

Initially all the three strains were grown on Coconut Cream Agar (CCA) that allowed the detection of aflatoxins in agar medium by direct visualization of a yellow colour around the growing colony after 7 days of incubation at 30 °C and on the reverse side under UV light green or blue fluorescence on CCA culture plate. The blue fluorescence indicated the production of AFGs (G1, G2) and green fluorescence for AFBs (B1, B2) (Fig. 1).

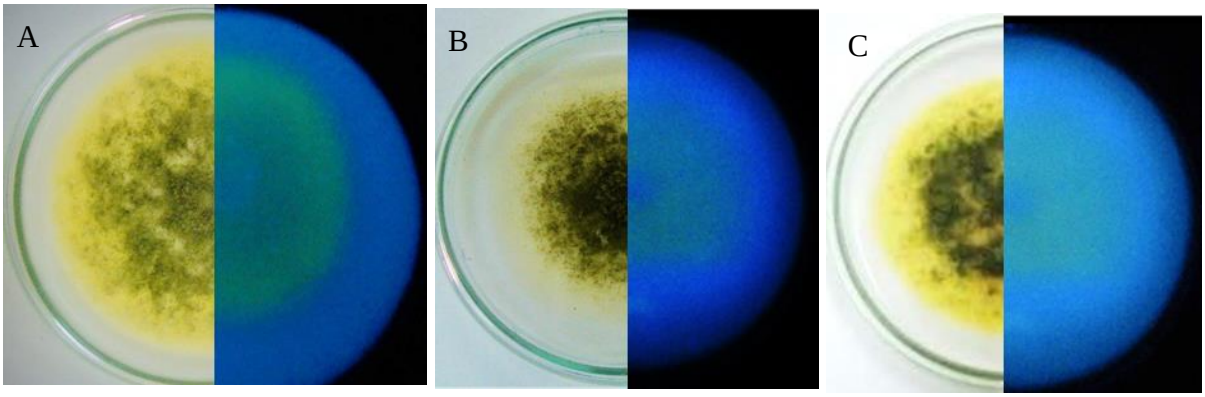

Fig. 1. **(A)** *Aspergillus flavus* (FCBP0616); **(B)** *Aspergillus flavus* (FCBP0862) and **(C)** *Aspergillus oryzae* (FCBP1202) colony on CCA. Half plate shows the front side of colony while the other half under UV light.

Thin Layer Chromatography (TLC) of extracted aflatoxins from the strains grown on CCA was performed to confirm the type(s) of aflatoxins produced by each strain. TLC results showed consistent mycotoxigenic profile of all selected strains. All the three selected stains produced aflatoxin B1, G1 and G2 when grown in CCA. Aflatoxin B2 was not showed. In all cases, production of aflatoxin B1 was more pronounced than the aflatoxin G1 and G2 (Fig. 2).

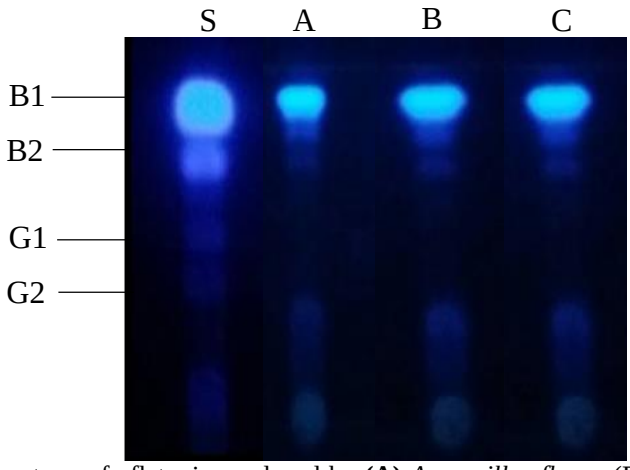

Fig. 2. Chromatogram showing type of aflatoxin produced by **(A)** *Aspergillus flavus* (FCBP0616); **(B)** *Aspergillus flavus* (FCBP0862) and **(C)** *Aspergillus oryzae* (FCBP1202) colony on CCA. S represented aflatoxins standard.

On the other hand when the strains were grown on CCB, toxin production ability was found to be stronger than CCA. All tested strains produced two clear and highly strong bands one each for aflatoxins B1, B2, G1 and G2 was slightly visible. The strain *A. flavus* (FCBP0616) produced only aflatoxin B1 and B2 (Fig. 3).

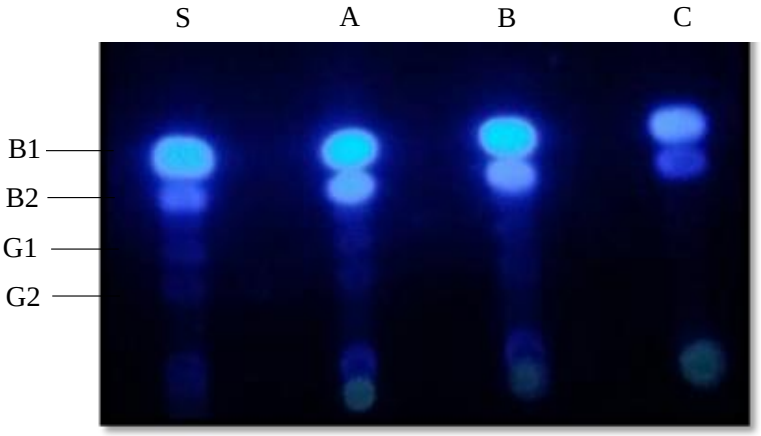

Fig. 3. Qualitative analysis of aflatoxin production by **(A)** *Aspergillus flavus* (FCBP0616); **(B)** *Aspergillus flavus* (FCBP0862) and **(C)** *Aspergillus oryzae* (FCBP1202) colony on CCB. S represented aflatoxins standard. Malt Extract Broth (MEB) indicated aflatoxins B1 and B2 in *A. oryzae* (FCBP1202).

While only aflatoxin B1 in *A. flavus* (FCBP0862) and (FCBP0616) were detected in TLC plates under UV light (Fig. 4).

Bactericidal Effect of Aflatoxins

During the present study, aflatoxins produced by *A. flavus* and *A. oryzae* were screened for their potential to limit the growth of three nitrogen fixing bacteria namely; *Azospirillum lipoferum*, *Azomonas agilis* and *Azotobacter insignis*. Aflatoxins were extracted from the test *Aspergillus* species by growing these species in Malt Extract Broth. The effect of different concentrations of these crude aflatoxin extracts in aqueous was evaluated against the growth of these bacteria. Aqueous extracts of all *Aspergillus* species significantly reduced the growth of the target bacterial isolates.

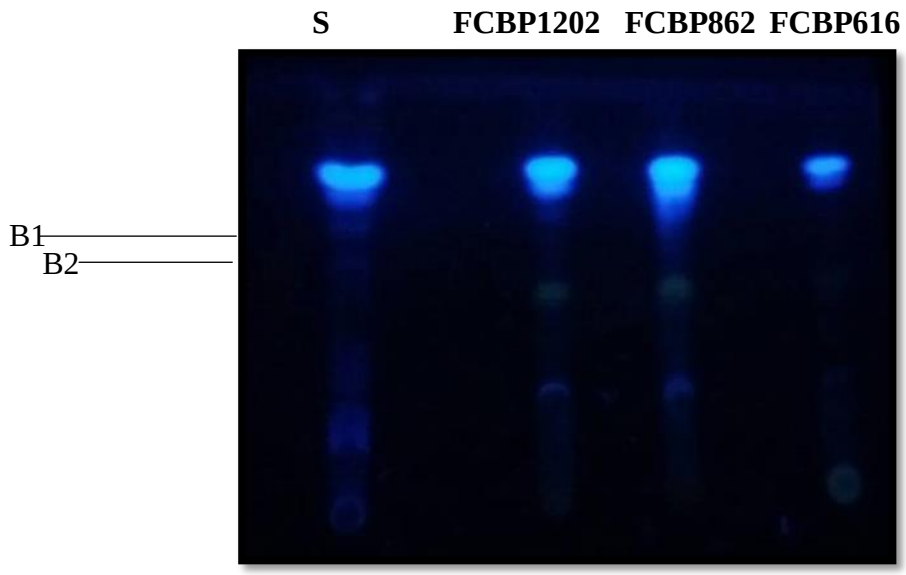

Fig. 4. Qualitative analysis of aflatoxin production by the strains of *A. flavus* and *A. oryzae* by TLC in Malt Extract Broth (MEB). S represented aflatoxin standard.

Effect of *A. flavus* (FCBP0616) Aflatoxins on Growth of *Azospirillum lipoferum*:

The results obtained from the anti-bacterial assays of *A. lipoferum* in different concentrations of aflatoxins are presented in Table 1. It was depicted from the data that an increase in extract concentration of aflatoxins, resulted in a significant decrease in the growth of *A. lipoferum*. The lowest concentration of (10 mg) exhibited around the lowest inhibition growth zone of *A. lipoferum* that was 4 mm. At 20 mg, 30 mg and 40 mg of aflatoxins concentrations, the inhibitory zones of 12 mm, 13 mm and 16 mm diameters were recorded, respectively. Finally, the highest concentration (50 mg) employed caused maximum suppression of growth and the growth zone inhibition was reached to 21 mm in diameter.

Effect of *A. flavus* (FCBP0616) Aflatoxins on Growth of *Azomonas agilis*:

Extracted aflatoxins of *A. flavus* exhibited potential anti-bacterial activity against *Azomonas agilis* (Table 2). An increase in extract concentration of aflatoxins resulted in a significant decrease in the growth of *A. agilis* due to inhibitory effect of aflatoxins. The lowest concentration (10 mg) exhibited the lowest growth inhibition zone of *Azomonas agilis* that was recorded around 6 mm. At 20 mg, 30 mg, 40 mg and 50 mg of aflatoxins, concentration zone of inhibition was increased gradually and 12 mm, 14 mm, 19 mm and 24 mm diameter of zone was observed, respectively.

Effect of *A. flavus* (FCBP0616) Aflatoxins on Growth of *Azotobacter insigns*:

Antibacterial efficiency of various concentrations of aflatoxins against *A. insigns* is also demonstrated in Table 1. Inhibition in the growth of *A. insigns* was observed as the concentration of *A. flavus* (FCBP0616) aflatoxins increased. At the little doses of aflatoxins (10 mg and 20 mg), bacterial strain exhibited the maximum growth and diameter of its inhibition zone was found to be 14 mm. As the dose increased from 30 to 50 mg, inhibition zone was gradually increased from 17 mm to 21 mm.

Effect of *A. flavus* (FCBP0862) Aflatoxins on Growth of *Azospirillum lipoferum*:

A. flavus (FCBP0862) aflatoxins showed significant inhibitory effect on the *A. lipoferum* growth due to employed concentration of aflatoxins. Inhibition pattern of the bacterial growth was concentration dependent. Inhibition of *A. lipoferum* at the maximum amount of aflatoxins (50 mg) was 22 mm and at the 10 mg of aflatoxins treatment, inhibition zone diameter was 5 mm. At 20 mg, 30 mg and 40 mg aflatoxins concentration, 8 mm, 13 mm and 15 mm of zone of inhibition was recorded, respectively (Table 2).

Effect of *Aspergillus flavus* (FCBP0862) on Growth of *Azomonas agilis*:

Aspergillus flavus (FCBP0862) aflatoxins inhibited *Azomonas agilis* growth. It was noted that growth of bacterial isolate was inhibited in a dose-dependent manner. Minimum concentrations tried (10 mg), resulted in 6 mm

diameter of growth inhibition zone. At 20 mg, 30 mg, 40 mg and 50mg aflatoxins concentration, recorded diameter of inhibition zone was 10 mm, 15 mm, 17 mm and 22 mm, respectively (Table 2).

Effect of *Aspergillus flavus* (FCBP0862) on Growth of *Azotobacter insignis*:

Results of increasing concentration of aflatoxins on growth of *A. insignis* showed similar growth pattern as recorded for *A. flavus* (FCBP0616). Zone of inhibition of growth became more visible at the maximum concentration of aflatoxins. Concentration dependent inhibition pattern of *A. insignis* demonstrated the increase in diameter of zone. Maximum diameter of zone of inhibition was 21.443 at 50 mg of aflatoxins extract and minimum zone was about 6 mm when employed 10 mg treatment to bacterial cells. At 20 mg, 30 mg, and 40 mg aflatoxins, recorded diameters of zones were 6 mm, 9 mm, 12 mm and 14 mm, respectively (Table 2).

Effect of *A. oryzae* (FCBP1202) on Growth of *Azospirillum lipoferum*:

The effect of *A. oryzae* toxins on growth of *A. lipoferum* also depicted the same pattern of growth inhibition but its zones of inhibition were smaller than *A. flavus* aflatoxins. At 50 mg treatment of toxins, zone was 17 mm. Graph represented the linear relationship from lowest toxin concentration to highest with gradually increasing the value of zone of inhibition. The 10 mg, 20 mg, 30 mg and 40 mg of aflatoxins concentrations resulted in 6 mm, 10 mm, 14 mm and 17 mm diameter zone of inhibition, respectively (Table 2).

Table 2. Antibacterial activity of different concentrations of aflatoxins on the growth of selected nitrifying bacteria.

	10	20	30	40	50	R^2
			Aspergillus flavus (FCBP0616)			
A. lipoferum	4.61±0.316 e	12.50±0.331 d	13.50±0.255 c	16.72±0.303 b	21.83±0.419 a	0.9436
A. agilis	6.22±0.340 e	12.77±0.186 d	14.99±0.271 c	19.66±0.271 b	24.50±0.142 a	0.9834
A. insignis	5.27±0.304 e	14.61±0.233 d	17.99±0.192 c	19.72±0.291 b	21.89±0.141 a	0.9557
			Aspergillus flavus (FCBP0862)			
A. lipoferum	5.76±0.404 e	8.94±0.956 d	13.28±0.657 c	15.83±0.437 b	22.71±0.412 a	0.9874
A. agilis	6.88±1.307 e	10.83±0.115 d	15.11±0.782 c	17.94±0.460 b	21.44±0.444 a	0.9983
A. insignis	6.49±0.342 e	9.88±0.306 d	12.49±0.318 c	14.28±0.767 b	21.44±0.819 a	0.9781
			Aspergillus oryzae (FCBP1202)			
A. lipoferum	8.99±0.271	11.72±0.379	14.13±0.430c	16.66±0.709	17.83±1.210	0.9968
A. agilis	7.83±0.330	11.71±0.443	12.77±0.251	13.39±0.417	14.50±0.550	0.9569
A. insignis	6.49±0.569 e	10.71±0.540 d	14.83±0.411 c	17.49±0.074 b	20.55±0.294	0.9673

Effect of *A. oryzae* (FCBP1202) on Growth of *Azomonas agilis*:

Antibacterial assay of *Azomonas agilis* by *A. oryzae* aflatoxins showed the peculiar pattern of growth inhibition (Table 1). Its lowest zone of inhibition was of 7.83 mm diameter resulted by the lowest concentration of aflatoxins i.e. 10 mg and led to 14.5 mm diameter of zone formation at the highest concentration of aflatoxins (50 mg). At 40 mg, 30 mg and 20 mg of aflatoxins concentration produced 13 mm, 12 mm and 11 mm diameter of inhibition zone.

Effect of *A. oryzae* (FCBP1202) on Growth of *Azotobacter insignis*:

Toxins of *A. oryzae* showed little inhibition of growth at lower concentrations. At the concentrations of 10 mg, 20 mg, 30 mg, 40 mg and 50 mg, inhibition zones for *A. insignis* were 6 mm, 10 mm, 14 mm, 17 mm and 20 mm, respectively (Table 1).

DISCUSSION

The current study was conducted to identify the toxigenic importance of *Aspergillus* isolates belonging to section *Flavi*. The study was started from the characterization of fungi on the basis of aflatoxigenic or non-aflatoxigenic potential. Finally role of these toxins was studied with regard to their ability to inhibit growth of soil beneficial bacteria.

The isolated species were analysed for their mycotoxigenic pattern of aflatoxins producing ability (Vaamonde *et al*., 2003; Pildain *et al*., 2004) by growing on Coconut Cream Agar (Lin and Dianese, 1976; Davis *et al*., 1987), Coconut Broth and Malt Extract broth media. Toxigenic properties of isolates were confirmed by thin layer chromatography (TLC) (Yassin *et al*., 2010; Gherbawy *et al*., 2012). It was recorded that all selected isolates of *Aspergillus* (FCBP1202, FCBP862, and FCBP0616) were aflatoxigenic and produced B1 and/or B2 in Coconut Cream agar, Coconut Broth and Malt Extract media under UV light. Present findings are in accordance with previous findings where one strain of *A. flavus* showed tremendous ability of producing aflatoxins (Fente *et al*., 2001; Abbas *et al*., 2005; Guezlane- Tebibel *et al*., 2013). Whereas one strain of *A. flavus* (FCBP0616) produced only B1. Isolates of *A. flavus* producing B1 are most toxic and potent hepato-carcinogenic ever characterized (Samson *et al*., 2004; Hedayati *et al*., 2007).

A. oryzae had variety related to toxicity or non-toxicity. Non-toxigenic strains of *A. oryzae* carry various mutation in aflatoxins producing genes resulting inability to producing aflatoxins (Tominaga *et al*., 2006) while some strains of *A. oryzae* produced aflatoxins (Codner *et al*., 1963). Strain of *A. oryzae* (FCBP1202) also showed toxicity on TLC plates.

A. flavus is responsible for huge losses in agricultural sanitary and nutritional quality in grains, through the production of aflatoxins, responsible for poisoning several species of animals, as well as being carcinogenic (Luo *et al.*, 2009; Roze *et al*., 2011; Sajid *et al.*, 2009). Aflatoxins also inhibit the growth of plants at a particular concentration. This inhibition mainly focuses on roots and in elongation of hypocotyls. *Lepidium sativum* was the most susceptible to aflatoxin B_1 and exhibited the maximal inhibitory response (Crisan, 1973). Higher concentration of aflatoxins also inhibits the growth of crops beneficial microorganisms like nitrogen fixing bacteria. *A. flavus* also produces bioactive compounds that inhibit the growth of bacteria like *E. coli* (Mohammed *et al*., 2016).

Plant growth promoting (PGP) bacteria may promote growth directly, e.g. by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, or production of plant growth regulators (hormones) (Barnawal *et al*., 2017). Some bacteria support plant growth indirectly, by improving growth restricting conditions either via production of antagonistic substances or by inducing resistance against plant pathogens. Some important nitrogen-fixing bacteria include, *Achromobacter, Acetobacter, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Azomonas, Bacillus, Beijerinckia, Clostridium, Corynebacterium, Derxia, Enterobacter, Herbaspirillum, Klebsiella, Pseudomonas, Rhodospirillum, Rhodopseudomonas* and *Xanthobacter* (Saxena *et al*., 1986). *Bacillus thuringiensis* has been used for the determination of antibacterial activity of various mycotoxins (Boutibonnes, 1975). Toxins of *A. flavus* and *A. oryzae* were evaluated for their anti-bacterial activity against the selected bacteria. These strains were also resistant towards compounds with a double furan system (aflatoxins B1, B2, G1, G2 and sterigmatocystin). Cyclopiazonic acid (CPA) is a neurotoxin produced by some of the strains of *A. flavus* that produce aflatoxins and by some *A. oryzae* strains (Chang *et al*., 2009). Similarly, flavicin was also an antibacterial substance that inhibits the growth of nitrogen fixing bacteria (Milton *et al*., 1945). Results of anti-bacterial activity from present study revealed that *A. flavus* (FCBP0862 and FCBP0616) and *A. oryzae* (FCBP1202) had similar pattern of inhibition of growth of selected nitrogen fixing microorganisms; *Azospirillum lipoferum, Azomonas agilis* and *Azotobacter insignis*. Zone of inhibition was dependent on aflatoxins dose. Higher concentration of aflatoxins was more prominent reason of growth reduction.

The present study has identified antibacterial activity of selected *Aspergilli*. Extract of aflatoxins effective against bacterial isolates may further be investigated to avoid such type of contamination in crops. Thus identifying toxicity of *Aspergillus* group which can inhibit bacterial production at reasonably high concentrations is of particular importance.

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