

Full Length Research Paper

The role of rhizospheric *Aspergillus flavus* in standing maize crop contamination in different ecological zones of Khyber Pakhtunkhwa, Pakistan

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Soil and un-husked maize samples were collected from 29 different locations belonging to three distinct ecological zones (Swat, Hazara and Peshawar) of Khyber Pakhtunkhwa, Pakistan. The samples were evaluated for the incidence of aflatoxigenic strains of *Aspergillus flavus*. The soil samples collected from Peshawar (100%) and Hazara (66%), and grain samples collected from Swat (64%) and Peshawar (55%) were severely infected with aflatoxigenic strains of *A. flavus*. The strains isolated from maize kernels of Manyar, Jalala (Swat zone), Palosi and Takkar (Peshawar zone) produced the highest amount of aflatoxin B1 (324 to 514 $\mu\text{g g}^{-1}$) and B2 (23 to 486 $\mu\text{g g}^{-1}$). Similarly, the strains isolated from soils of Bannu (Peshawar) and Huripur (Hazara) were prominent in B1 (662 to 1323 $\mu\text{g g}^{-1}$) and B2 (145 to 826 $\mu\text{g g}^{-1}$). Microbial analysis of the surface sterilized grains showed that the strains isolated from Jalala (Swat) and Palosi (Peshawar) samples were high in B1 (62 to 79 $\mu\text{g g}^{-1}$) and B2 (21 to 36 $\mu\text{g g}^{-1}$). It was concluded, therefore, that *A. flavus* from soil might contaminate maize crop, but not directly from the same field. The most probable contamination occurred through air borne spores. It is thus recommended that protection from air borne spore should be devised.

Key words: *Aspergillus flavus*, aflatoxin, ecology, maize crop, field, soil toxicology.

INTRODUCTION

Aspergillus flavus is a well documented fungus prevalent in humid and warm regions (80 to 90% and 25 to 30°C, respectively) of the world (Gupta et al., 1993; Gibson et al., 1994; Adhikari et al., 2004). Due to its infective nature, the fungus is predominantly found in soil where it mostly contaminates agriculture and related vegetation (Scheidegger and Payne, 2003; Masayuki and Katsuya, 2010; Shah et al., 2010). A plethora of research confirmed that consumption of infected victuals cause weight loss, liver damage, hepatitis, cancer and interfere protein and other nutrients metabolism. *A. flavus* can be identified as green or yellowish colonies, round or oblong shape conidia in soil and grains of various crops that can be easily isolated by various techniques (Sutton et al., 1998; Hell et al., 2000; Cardwell and Cotty, 2002).

Various studies have reported that *A. flavus* infection occurs during yielding or maturity, harvest and storage

stage in agricultural crops (Silva et al., 2000; Magan et al., 2004; Alam et al., 2009). Likewise, recent studies confirmed increased amount of toxins during storage (Marin et al., 2004; Waliyar et al., 2009). However, due to the presence of the fungus in the rhizosphere, there must be some chance of infection of the standing crops (Jeffrey et al., 2010). Whatsoever the mechanism, green *Aspergillus* mycelium can be seen on freshly uncovered cobs of maize. This and other observations relate the standing crops infection especially of maize crop with soil *A. flavus*. Considering the importance of maize crop for the populace of Khyber Pakhtunkhwa, Pakistan, a project was design to assess the soil as a possible inoculum of standing maize crop and weigh up its potential role of aflatoxins contaminations during storage.

MATERIALS AND METHODS

Study area and Sampling

Khyber Pakhtunkhwa Province of Pakistan varies from dry rocky

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areas in the south to forests and green plains in the north. Its climate immensely varies from intensely hot summers to freezing cold winters. It is a wide arable land with irrigation facilities. The area is well known for maize production. In the present study, the subject area is divided into three different ecological zones on the basis of temperature, humidity and rainfall.

Soils (1 kg each) and un-husked maize cobs samples (3 kg each) were obtained after harvest from 29 different sites of the three distinct ecological zones comprising Swat, Hazara and Peshawar of Khyber Pakhtunkhwa province of Pakistan. There was only one composite sample each of soil and maize cobs per region and each was collected from five different places per region. Each representative sample of maize cobs was threshed and systematically mixed in a composite sample (200 g each). They were air dried in shade for at least 42 h under protected conditions to avoid any foreign contaminations. Whereas the air-dried soil materials (100 g each) were pulverized and were sieved from 80 mm mesh sieve. Both maize and soil samples were packed in sealed plastic bags and stored at 4°C for subsequent fungal enumeration analysis. All the research work pertaining to this project was carried out in the Toxicology Laboratory of the Department of Agricultural Chemistry, Khyber Pakhtunkhwa Agricultural University, Peshawar, Pakistan and the Laboratory of Applied Mycology Group, Cranfield Health, Cranfield University, Bedford, U.K.

Media preparation and malt extract agar (MEA) plates

About 45 g of malt extract agar media (MEA, Oxoid, Basingstoke, Hampshire, U.K.) was prepared in 900 ml of double distilled water in 1000 ml capacity bottle. The media was sterilized in autoclave at 121°C for 25 min. The MEA was then spread in disposable Petri dishes and were placed in 4°C temperature for fungal culturing (Ali et al., 2009).

Serial dilution of soil samples

Soil sample (about 1 g each) was dissolved in 10 ml of Tween-20 mixed sterile water in a 25 ml capacity bottle, and then a dilution range of 10^{-2} to 10^{-5} g ml⁻¹ was obtained by transferring 1 ml to 9 ml of sterile water in five serially placed bottles for each sample. Afterward, 10^{-3} and 10^{-4} were plated (0.4 µL) in duplicate on already prepared MEA plates. The plates were incubated at 25°C room for about seven days (Sharma, 2010). The *A. flavus* was then identified from the color of mycelium, shape of the colony and structure of spore head etc (Pitt and Hocking, 2009) and to type strains of *A. flavus* (SRKC-G1907; USDA, USA) and *Aspergillus parasiticus* (SSWT 2999, USA). Subsequently, they were subcultured by taking a patch of the mycelium from the mixed culture and transferring under sterile condition to a clean Petri dish of MEA. The plates were incubated at 25°C for seven days. Consequently, clean *A. flavus* strains were obtained.

Isolation of *A. flavus* from maize grains

Five grains were randomly selected from 100 grains of each maize composite sample and were incubated on MEA plates at 25°C under sterile condition. Grains were also sterilized with 70% isopropanol for 3 min, rinsed thoroughly with double distilled water, dried with ballot paper and then placed on MEA Petri dishes for internal *A. flavus* infection. The *A. flavus* colonies were identified and counted. The data was converted to percentage of grain infection.

Aspergillus flavus toxicity and aflatoxin content

Various strains of *A. flavus* isolated from soil and grain samples were grown on coconut agar media (Dyer and McCammon, 1994). The plates were inoculated with 2 or 3 pinches of each isolated *A. flavus* strain and kept at 25°C for six days. Plates on reverse sides were examined for blue or green fluorescence.

Quantification of aflatoxin

The isolated strains were cultured on yeast extract agar media (YEA, Fisher, Loughborough, Leicestershire, U.K.) for their aflatoxin production. About 4 µL of the suspended spores in 20 ml of Tween-20 water of each isolated strain were spread on YEA plates and kept for 10 days at 25°C. Five patches, each about 4mm in diameter, was taken from YEA with a circular cutter (Farid et al., 2009) and smashed in 1 ml of chloroform in a plastic vial. The clear liquid was pipetted out and dried at ambient temperature in a hood. Furthermore, the dried extract was redissolved in 200 µL of hexane and 50 µL trifluoroacetic acid (TFA) agitated mechanically for 30 s. After waiting for 5 min, 950 µL of water-acetonitrile mixture with a ratio of 9:1 was added and vortexed again for 30 s and kept for 10 min. The hexane layer was decanted and the rest was transferred in 1 ml amber vial for high performance liquid chromatography (HPLC) analysis (AOAC, 2000).

HPLC conditions

The HPLC system used for aflatoxins determination was an Agilent 1200 series system (Agilent, Berks., Germany) with a fluorescence detector (FLD G1321A), an auto sampler ALS G1329A, FC/ALS therm G1330B, Degasser G1379B, Bin Bump G1312A and a C18 (Phenomenex, Luna 5 micron, 150 × 4.6 mm) column joined to a pre-column (security guard, 4 × 3 mm cartridge, Phenomenex Luna). The mobile phase was methanol : water : acetonitrile (30:60:10, v/v/v) using an isocratic flow rate of 1 ml min⁻¹ at 360 nm excitation and 440 nm emission wavelengths and a 25 min run time for aflatoxin determination (Figure 1). The aflatoxin content of the strains was calculated using the following formula (AOAC, 2000).

$$\text{Aflatoxin B1 (ng/g)} = \frac{\text{Area of sample peak} \times \text{quantity of aflatoxin in standard injection volume} \times 1000}{\text{Area of standard peak} \times \text{injection volume of sample} \times \text{weight of sample}}$$

RESULTS

A. flavus was isolated from 17 samples of soil (Figure 2) and 16 samples of maize kernels (Figure 3), while four samples of sterilized maize grains (Figure 4) were found infected with *Aspergillus* strains. The zonal distribution of *A. flavus* strains showed that 100% of soil samples of Peshawar zone were found infected, followed by Hazara Zone (66%), whereas minimum infection (28%) was found in Swat. The highest colonies strength was found in Mardan (29.30×10^3 CFUs g⁻¹) and Palosi (1.00×10^3 CFUs g⁻¹) regions of Peshawar zone, Abattabad (30.70×10^3 CFUs g⁻¹) and Shenkiari (11.7×10^3 CFUs g⁻¹) regions of Hazara zone and in Ghalagay (7.00×10^3 CFUs g⁻¹) of Swat zone. In case of maize kernels, the zonal distribution of *A. flavus* strains were different than that of soil samples; the highest isolation was done from Swat Zone (64%), followed by Peshawar zone (55%) and

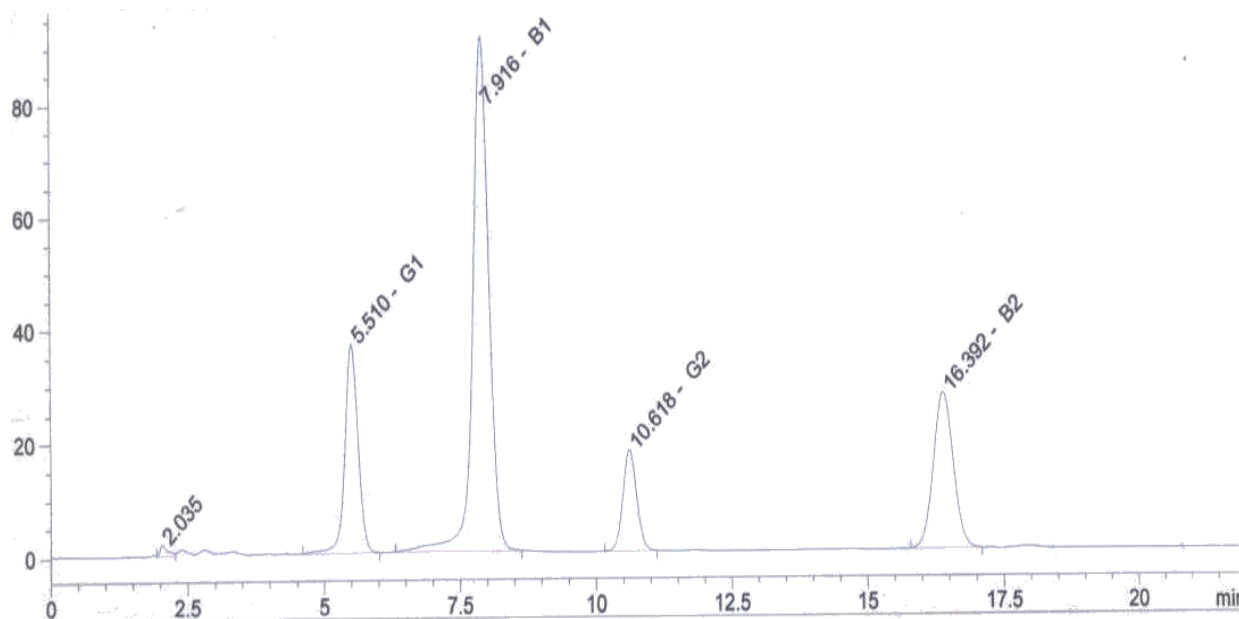


Figure 1. Chromatogram of aflatoxin standards (60 ng B1, G1 and 200 ng B2, G2/ml, runtime 20 min).

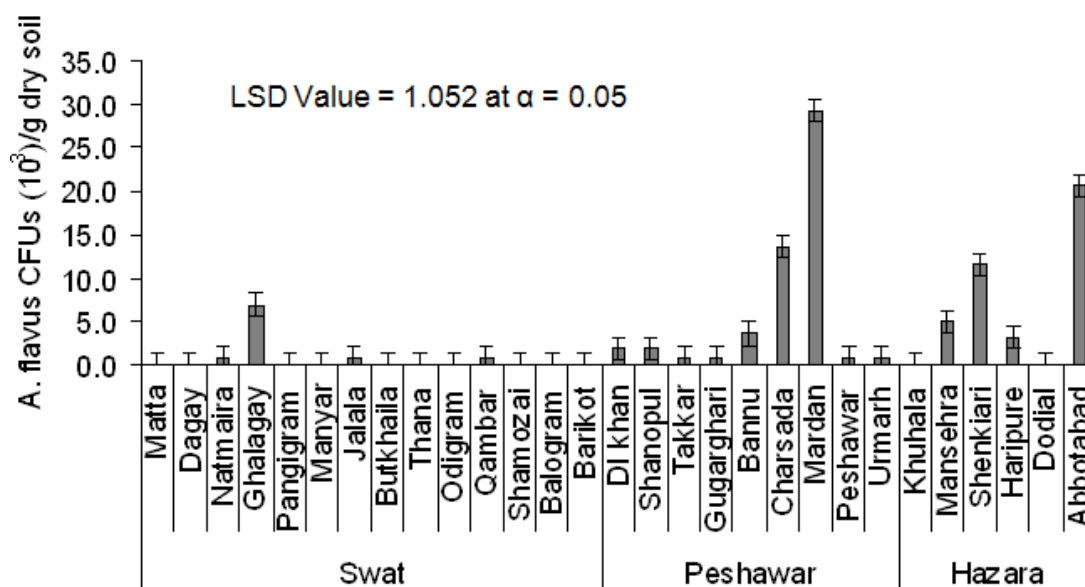


Figure 2. *A. flavus* infection CFUs (10³ g⁻¹) of soil samples belonging to three different ecological zones of Khyber Pakhtunkhwa. The bars represent the standard errors of means.

Hazara zone by 33% of different regions (Figure 3). The fungal density denoted as %grain infection was highest (16 and 11.67%) in Natmaira and Jalala regions of Swat zone, Palosi region (12.33%) of Peshawar zone and Dodial region (14.67%) of Hazara zone. The strains of *A. flavus* of sterilized grain showing internal infection of maize kernels, was found in one sample of Jalala region of Swat zone (14.67%), and in one sample of Palosi region (2.67%) of Peshawar zone, while also Dodial

region sample (10.33%) of Hazara zone was found to be infected.

The aflatoxin production rates of the various strains are presented in Figures 5 to 8. Almost all the strains produced aflatoxin B1 and B2, which indicated that all the strains were *A. flavus* because one of its subspecies, *A. parasiticus*, produced four type of toxin (B1, B2, G1 and G2) such as in the case of strains isolated from soil sample of Haripure region and from grain sample of

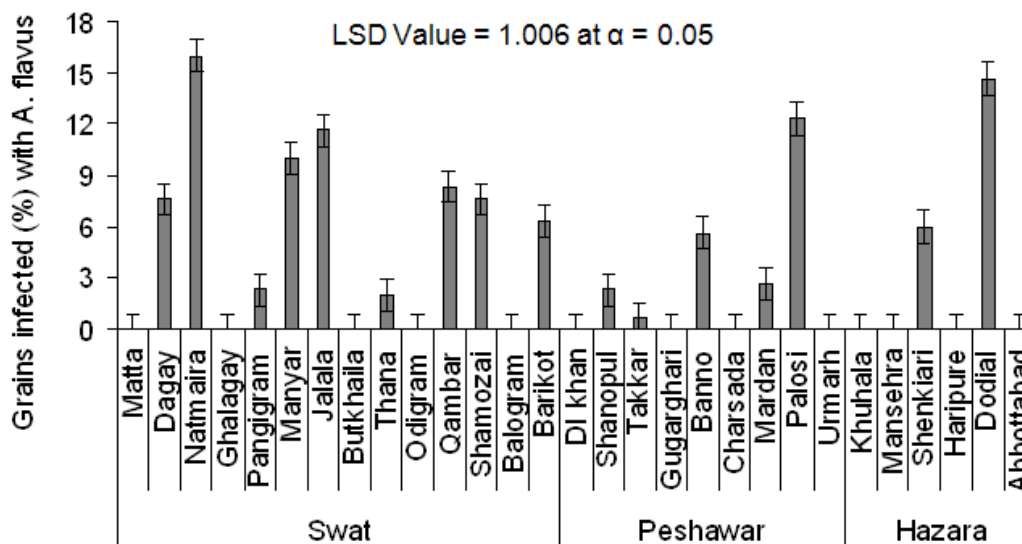


Figure 3. *A. flavus* infection of maize kernels (%) samples belonging to three different ecological zones of Khyber Pakhtunkhwa. The bars represent the standard errors of means.

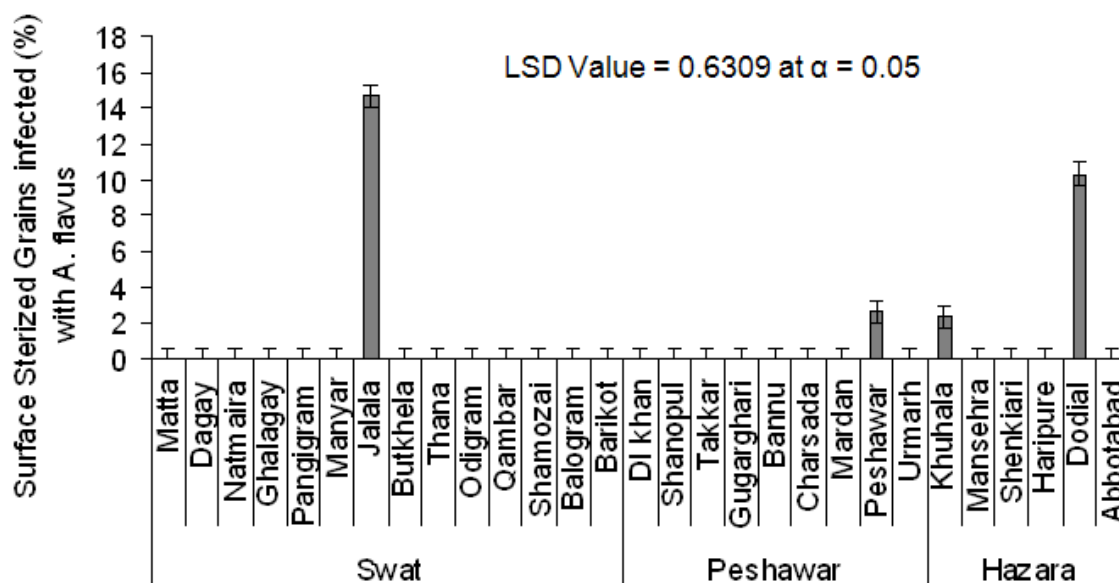


Figure 4. *A. flavus* infection of sterilized maize kernels (%) belonging to three different ecological zones of Khyber Pakhtunkhwa. The bars represent the standard errors of means.

Palosi Peshawar regions. In soils samples (Figure 6), maximum quantities of aflatoxin B1 and B2 (1323 and $828 \mu\text{g g}^{-1}$) were produced by strain isolated from the soil of Haripure region of Hazara zone, followed by 668 and $416 \mu\text{g g}^{-1}$ of Banu region of Peshawar zone. In Swat zone, maximum production was observed for strain obtained from Qumber region, which was B1 $215 \mu\text{g g}^{-1}$ and B2 $13 \mu\text{g g}^{-1}$. On the other hand, in grain samples (Figure 7), the highest amounts of B1 (504.17 and $451.87 \mu\text{g g}^{-1}$) and B2 (478.09 and $76.03 \mu\text{g g}^{-1}$) were produced by sample from Palosi and Takkar regions of Peshawar

zone, followed by Jalala and Manyar regions of Swat which produced 335.77 and $319.09 \mu\text{g g}^{-1}$ of B1 and 29.39 and $22.81 \mu\text{g g}^{-1}$ of B2, respectively. Of the Hazara regions, no appreciable amount of aflatoxin was produced by the isolated strains. In sterilized maize grain samples, maximum amounts of B1 and B2 (78.11 and $36.09 \mu\text{g g}^{-1}$) were produced by strain isolated from Jalala region of Swat Zone, whereas minimum levels (26.11 and $18.66 \mu\text{g g}^{-1}$, respectively) were found in samples from Dodial of Hazara region.

Figure 8 shows the comparative production rate of

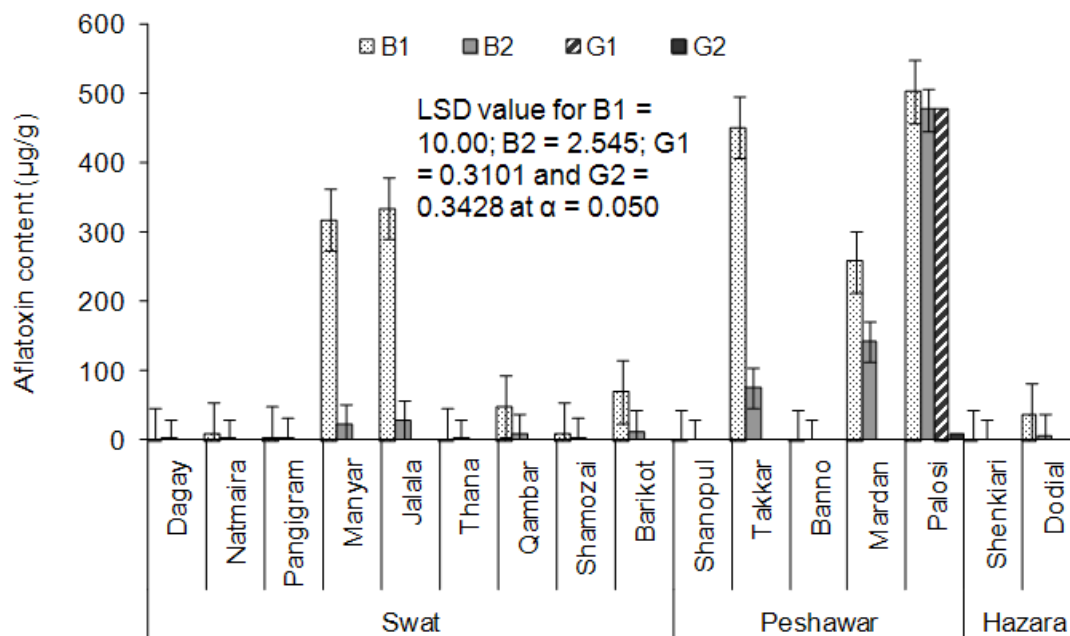


Figure 5. Aflatoxin production ($\mu\text{g g}^{-1}$) of *A. flavus* strains isolated from soil samples belonging to three different ecological zones of Khyber Pakhtunkhwa. The bars represent the standard errors of means.

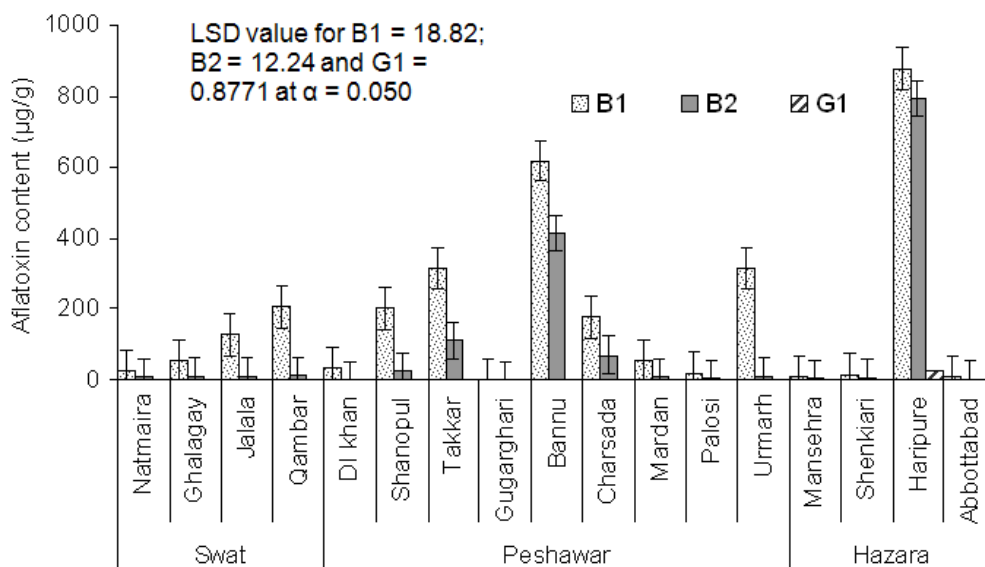


Figure 6. Aflatoxin production ($\mu\text{g g}^{-1}$) of *A. flavus* strains isolated from maize kernels belonging to three different ecological zones of Khyber Pakhtunkhwa.

aflatoxin by various isolated strains. Although almost all the species were able to produce both B1 and B2, however, the amount was quite different for soil stains versus maize kernels strains isolated from the same regions.

DISCUSSION

A. flavus produces aflatoxin when it attacks crops,

especially in the final agricultural products. In this context, maize is the most susceptible crop for this fungus as historical out-breaks are connected to the consumption of the moldy corn. This fungus is present in the soil and might infect the crops when cultivated, and if this assumption is true, then it will be a great risk to cultivate crops in infected soils or where there is a prevalence of *A. flavus*. From various research studies, it is evaluated that the *A. flavus* from soil may be the possible cause of

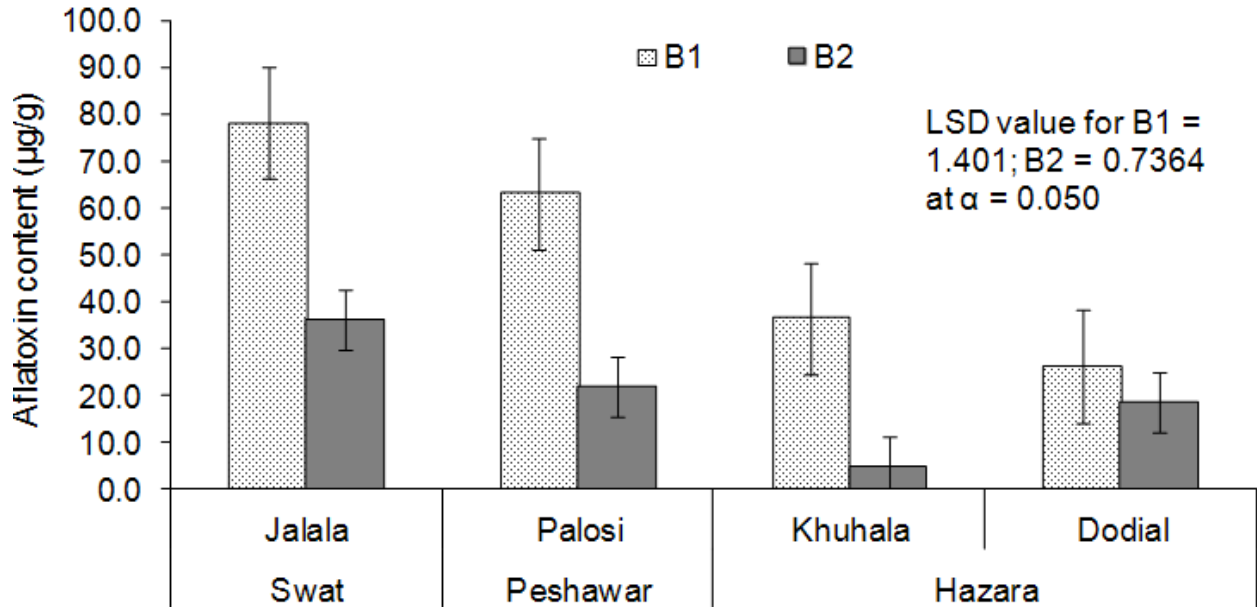


Figure 7. Aflatoxin production ($\mu\text{g g}^{-1}$) of *A. flavus* strains isolated from sterilized maize kernels belonging to three different ecological zones of Khyber Pakhtunkhwa. The bars represent the standard errors of means.

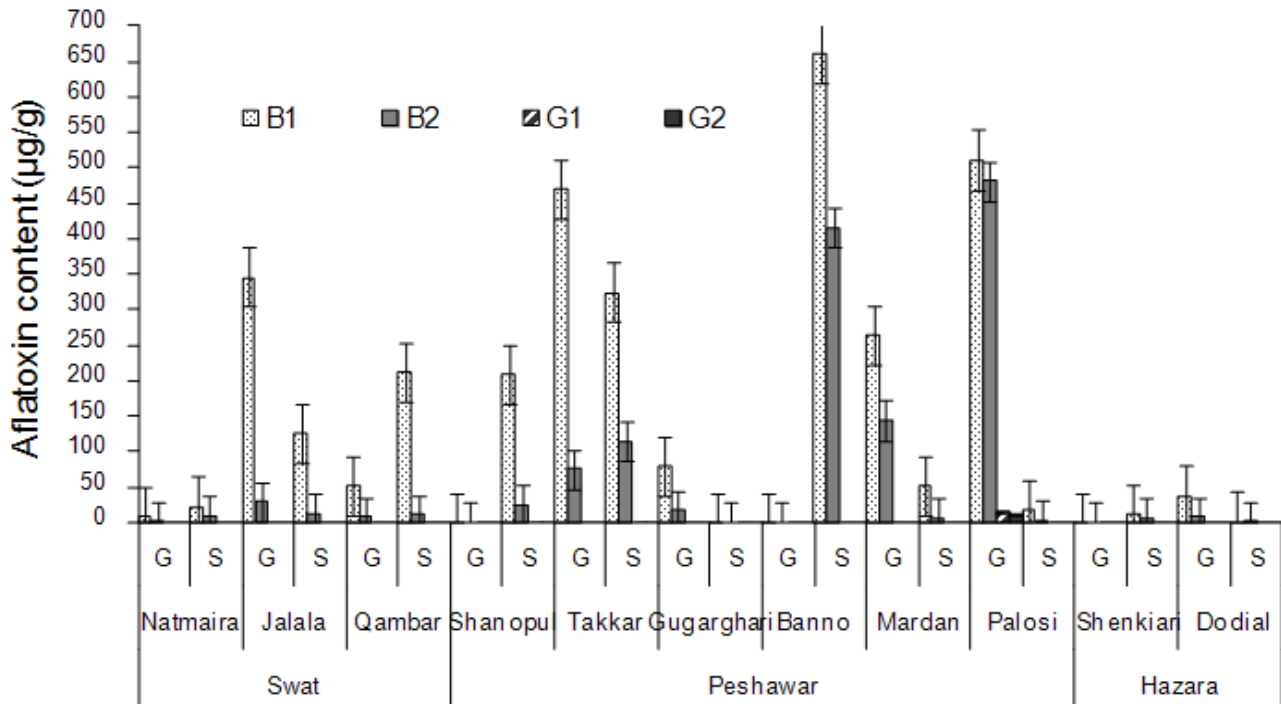


Figure 8. Comparative aflatoxin production ($\mu\text{g g}^{-1}$) of *A. flavus* strains isolated from soil and maize kernels belonging to three different ecological regions. The bars represent the standard error of means. G, Grain; S, soil samples.

infection of standing maize crops or the field laying crop immediately after harvest (Abbas et al., 2008; Miguel et al., 2007). This view could also be clarified by the work of Nesci and Etcheverry (2002) who pointed out that maize crop could possibly be contaminated by the *A. flavus* in

the rhizosphere. Lillehoj et al. (1980), Angle et al. (1982) and Horn et al. (1994) also suggested the soil as the main source of inoculation of maize and peanut crops, especially when drought conditions prevail. For this purpose, *A. flavus* from the soil and from the standing

maize crops of the same field was isolated and checked for their aflatoxin production. The similarity in aflatoxin quantity could possibly be an indication that the rhizospheric *A. flavus* contaminate the standing or the field laying crops

In the present study, the data obtained shows that 17 samples of soil, 16 samples of maize grains and four samples of sterilized maize grains were found infected with *A. flavus* strains. This constituted 64% infection of maize samples from Swat region, 55% of Peshawar and 33% of Hazara region. The soil samples infection was 100% from Peshawar zone, 66% from Hazara zone and 28% of Swat zone. The data also indicates that *A. flavus* is prevalent in the study area both in the soil and maize grains samples. This means that the grain infection may be from the *A. flavus* present in the soil of the same field. However, aflatoxins B₁ and B₂ produced by the *A. flavus* strains isolated from soil and maize grains samples of the same fields were different, indicating that strains present on grains might not come from the same soil because the variation in aflatoxin is related to difference in the strains. This could be explained by the work of Miguel et al. (2007), Leema et al. (2010) and Niles et al. (1985), Faraj et al., (1991), Miguel et al., (2007), Giorni et al., (2009), Leema et al., (2010), and who reported that *A. flavus* strains were different in their aflatoxin production due to their genetic variation. The difference in the infected *A. flavus* strains found in the present study could possibly illustrate that the *A. flavus* may not come from the soil of the same field because the spores of the *A. flavus* from old crops debris in the soil blow in the air and this could contaminate crops anywhere in the nearby area, and so crops standing in one field might get contaminated with spores from the other field. This view is supported by Abbas et al. (2004), who reported that difference in strains might be due to the difference in sources.

Conclusions and recommendation

The present study confirmed the prevalence of *A. flavus* in the three ecological regions of Swat, Hazara and Peshawar. Majority of the strains were found to be toxigenic in nature. However, the soil and grain samples collected from same fields showed that the *A. flavus* present in the same field was not the absolute contaminant of the standing crop. The crop might get contaminant from any other source, which might be from the airborne spore. It was also concluded that the aflatoxin production rate were strain-related and they produced unequal amount of aflatoxin.

In the light of above study, we therefore recommended that fields must be cleared from any contaminant present in the form of fungus or any other dangerous micro-organisms. However care should be taken to control airborne spores' contamination, which might cause greater damage as compared to the microbes present in

the same field of crops.

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