

Full Length Research Paper

Incidence of *Aspergillus flavus* and extent of aflatoxin contamination in peanut samples of Pothwar region of Pakistan

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Incidence of aflatoxin producing fungi and level of aflatoxin was examined in peanut samples from main markets of Pothwar region that is, districts of Chakwal, Rawalpindi, Attock and Jehlum. Total number of collected samples was 40 having 10 samples from main market of each district. Seeds were plated on PDA, water agar and filter paper and incubated at 29°C. *Aspergillus parasiticus* was not found on any of the samples, thus, only isolated aflatoxin producing fungus *Aspergillus flavus* was identified on the basis of morphological characteristics. The results (14 out of 40 samples showed incidence on PDA, 4 on water agar and no results on filter paper) indicated that PDA was the best substrate amongst three. Mean location wise incidence on PDA and water agar on samples collected from Chakwal was 21.1 and 7.7% respectively. In case of samples from Jehlum, incidence on PDA and water agar was 14.44 and 6.6% respectively, whereas 21.1 and 6.6% incidence (only on PDA) was found to be in samples from Attock and Rawalpindi respectively. Aflatoxin contents in peanut samples of these four districts ranged from 2.01 to 3.54 ppb ($\mu\text{g}/\text{kg}$), 2.09 to 2.86 ppb, 2.00 to 2.78 ppb and 2.33 to 2.58 ppb in the order earlier mentioned when detected through enzyme linked Immunosorbent Assay. This study depicts a clear picture about the current incidence of *A. flavus* on peanut samples and extent of aflatoxin contamination in them. The highest value of aflatoxin (3.54 ppb) was found within safe limit (20 ppb, regulatory limit of FDA/WHO) and hence, freshly collected peanut of Pothwar region may be said to be non-hazardous for human consumption.

Key words: Peanut, aflatoxin, pothwar, Pakistan.

INTRODUCTION

Peanut (*Arachis hypogaea*) is a good source of edible oil as it contains about 50 to 53 percent oil of good quality use in ghee, margarine and salad. Rich in protein (21 to 30%) fat (41 to 52%) and carbohydrates (11 to 27%), some percentage of Ca, K, P, Mg and vitamin E is also present, that's why peanut plays important role in the diets of rural population. Worldwide production of peanut is approximately 25.7 million tons annually from about 21

million hectares of cropped land; seventy percent of global production (17.9 million tons) is from Asia (Reddy and Waliyar, 2009). Peanut occupies the second largest area among the oilseed crops after rapeseed and mustard in Pakistan (Rao, 1982). During 2007 to 2008, the total area was 94.7 thousand hectares with the production of 73.6 thousand tons with an average yield of 879 kg/ha. About 84% of the total peanut area lies in Punjab 13% in NWFP and 3% in Sindh (Anonymous, 2008). It is prone to infestation by aflatoxins producing fungi, *A. flavus* and rarely *A. parasiticus*. Aflatoxins are carcinogenic mutagenic immunosuppressive substances. The blood tests have shown that very high percentage of

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population of several countries in Asia is exposed to Aflatoxin that is cause of cancer (hepatocellular carcinoma), liver cancer and Hepatitis B and C. It is worldwide most important food quality problem. The WFO categories sample with over 30 µg/kg as hazardous for human consumption and the FAO with over 20 µg/kg while European Union has banned the import of peanut with Aflatoxin B1 content above 6 µg/kg. Strict quality regulations by many countries resulted in severe decline in international peanut trade and severe impact on economic development of those exporting peanut (Reddy and Waliyar, 2002). As aflatoxin contamination in peanut poses a serious issue concerning food safety and human health and there is little information of incidence of aflatoxin producing fungi in peanut of Pakistan, therefore the present study was initiated with the objective of the isolation and characterization of Aflatoxins producing fungi in peanut under laboratory conditions.

MATERIALS AND METHODS

Research studies were accomplished at Crop Disease Research Programme (CDRP), National Agricultural Research Centre (NARC) Islamabad. Laboratory facilities available at CDRP and IPEP (Institute of Plant and Environmental Protection) at NARC Islamabad and PMAS Arid University Rawalpindi were utilized for isolation, detection and quantification of aflatoxin producing fungi based on their morphology.

Sampling

Samples were collected randomly from the pothwar region of Punjab that is, Chakwal, Rawalpindi, Attock and Jehlum. The total number of collected samples was 40 which included 10 samples from main market in each district.

Isolation, culture preparation and identification

The fungus associated with aflatoxin in peanut was isolated by the technique of Ricker and Ricker (1936). At the time of isolation the samples were surface sterilized with 1% aqueous solution of Clorox for 1 min. The sterilized seeds rinsed in sterilized distilled water were then blotted and plated on PDA (three seeds per 90 mm Petri-plate), agar water medium (three seeds per Petri-plate) and also on sterilized autoclaved moist filter paper lined in Petri plates. Later all the plates were incubated at 29°C for alternate light and dark periods for 24 hr each. Upon the completion of 48 hr incubation all the Petri plates having fungal growth were observed to identify the *Aspergillus* from other seed-borne fungi on the basis of colony characters. For further confirmation slides were prepared and observed under compound microscope (Nikon eclipse, E200) at low as well as high magnifications (10, 40, 60 and 100x) for the presence of conidiophores and conidia. Incidence was recorded by the following formula:

$$\text{Incidence \%} = \frac{\text{No. of infested seeds}}{\text{Total no. of plated seeds}} \times 100$$

Pure culture was prepared by single spore technique by picking a single conidium under stereo-microscope placed in laminar flow

hood and transferred on PDA. Later the plates were incubated at 29°C in dark for one week till the fungus attained maximum growth in the Petri-plates. Then these cultures were used for identification based on their following morphological characteristics, namely, colony color, colony texture, conidiophores (stipe) length, color and septation, vesicle shape, length and presence of seriation (uni or biseriate, metulae covering), conidium surface and length and sclerotium color and length. *A. flavus* was preserved on PDA slants. For this, PDA slants were inoculated with fungus by touching the tips of needle to the selected fungal colony. Slants were incubated at 29°C for a week. Photography of Petri plates was carried out with digital camera while Microphotography of slides was done at 40x magnification with auto fixed digital camera on microscope (Nikon eclipse, E200).

Detection and quantification of aflatoxin contents

Aflatoxin contents were detected and quantified through ELISA. Samples were ground and mixed thoroughly prior to proceeding with extraction and then passed through mesh sieve to obtain fine ones. Briefly, 5 g of the sample were mixed in 25 ml of methanol (70%) by shaking for 3 min. 5 ml of the extract was filtered by passing through Whatman filter paper #1.

In the first step 100 µl conjugate was added to red marked mixing wells. Then 100 µl controls and samples were added to red marked wells and the contents were mixed by pipetting up and down 5 times. After mixing, 100 µl were transferred to antibody wells and incubated for 2 min. After completion of incubation time liquid from these antibody wells was dumped and these were washed 5 times with deionized water. Then, water was taped out on absorbent paper towel and the 100 µl substrate was transferred from reagent boat to antibody wells using 8 channels pipette and incubated for 3 min. Red stop (100 all) was transferred from reagent boat to these antibody wells. Reading was taken within 20 min on microwell reader with a 650 nm filter.

Statistical analysis

The data of incidence was subjected to analysis of variance (ANOVA) using statistical analysis software Gen Stat Release 12.1. For the conversion of optical density of aflatoxin contents obtained through ELISA reader into ppb (part per billion) following regression equation was used:

$$Y = 0.152X + 0.135$$

$$X = (Y - 0.135) / 0.152$$

Where X and Y are aflatoxin contents (ppb) and optical density of the samples respectively.

RESULTS AND DISCUSSION

Isolation and morphological characterization of *A. Flavus*

Ten samples of peanut seeds collected from the main markets of each district were plated on three different substrates for isolation of Aflatoxin producing fungi. On the basis of various characters, discussed below, the only aflatoxin producing fungus isolated was *A. flavus*. The present section contains morphological data of colonies of the fungus incubated at 29°C on two of three

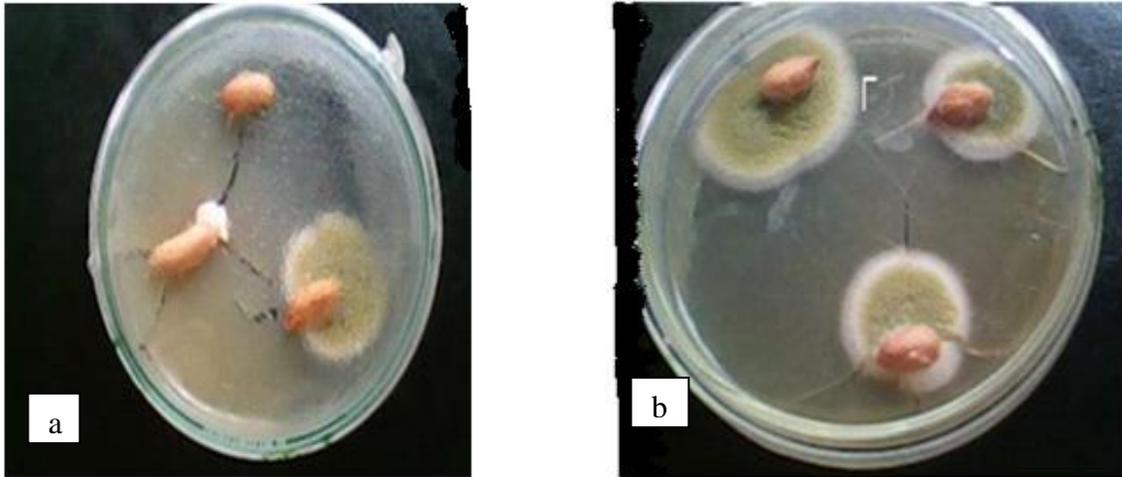


Figure 1. Incidence of *A. flavus* on two peanut samples (a) only one seed has the fungal incidence while in (b) 3 seeds have incidence of *A. flavus*.



Figure 2. Conidiophores of *A. flavus* with conidial head (400 x).

substrates for alternate 24 h light and dark periods. Colonies on PDA and water agar were yellowish green in color and the growth was linear (Figures 1a and b). While on filter papers (third substrate) no incidence of the fungus was noticed. Young conidial head of *A. flavus* was found columnar ranging from 36.89 to 41.82 μm (aver. =39.36 μm). Stipe (conidiophores) 282.9 to 750 μm (aver. =516.45 μm) \times 9.84-12.3 μm (aver. =11.07 μm) walls were smooth and uncolored, aseptate conidiophores distinct from hyphae, erect swollen at the top to from vesicle. Vesicle was spherical or globule (Figure 2). Variation in seriation was noticed and most of the isolates

were uniseriate (that is, borne directly), while some found biseriate that borne on short growths, metulae.

It was difficult to detect metulae because the primary phialides (sterigmata) were tiny and easily obscured by the spores of other sterigmata. Vesicles ranging from 22.814 to 61.5 μm (aver. =41.82 μm). Metulae were found covering 2/3 of the entire surface of vesicle and the phialides were 9.84 μm at 40x. Conidia were round 3-9.84 μm with smooth surface (Figures 2 and 3). Sclerotia produced in fresh isolates sometime dominated the colony color appearance. These were variable in form, dimension and pigmentation, arising as mycelia growth.

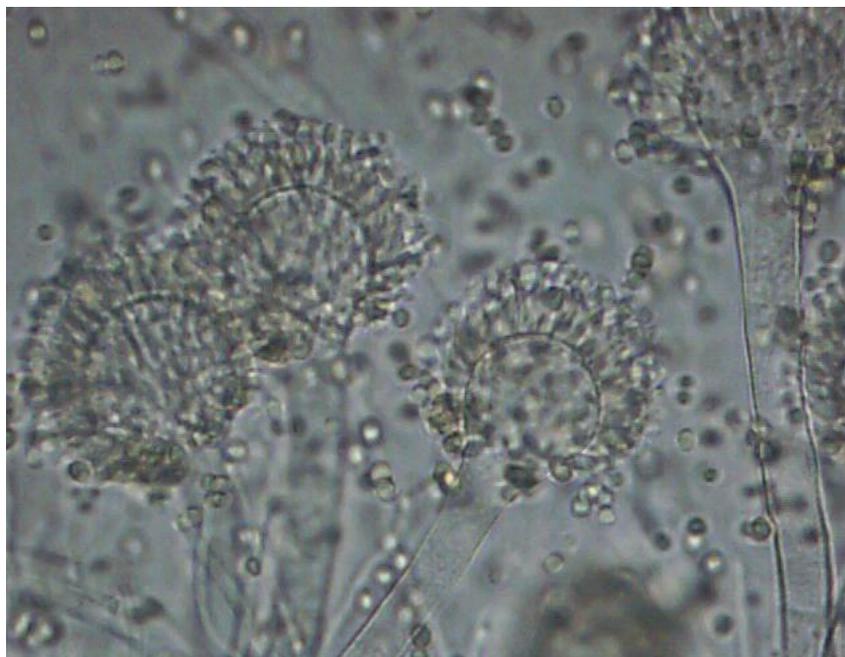


Figure 3. Vesicles and sterigmata of *A. flavus* (400 ×).

Table 1. Presence of *A. flavus* and concentration of aflatoxin in the infected peanut samples collected from 4 districts of Pothwar region.

Sample no.	Chakwal		Rawalpindi		Attock		Jhelum	
	Infection Rate (%)*	Aflatoxin (ppb)	Infection Rate (%)	Aflatoxin (ppb)	Infection Rate (%)	Aflatoxin (ppb)	Infection Rate (%)	Aflatoxin (ppb)
1	33.3	2.01	33.3	2.33	33.3	2.78	33.3	2.09
2	66.7	2.45	33.3	2.58	44.4	2.07	66.6	2.86
3	-	-	-	-	33.3	2.10	-	-
4	33.3	3.25	-	-	-	-	-	-
5	-	-	-	-	-	-	33.3	2.81
6	44.4	3.51	-	-	100.0	2.00	44.4	2.75
7	44.4	3.54	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-
9	33.33	3.48	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-

*Each value is the mean of 09. seeds.

Sclerotic were globule and gradually changing from white through dark reddish brown color, commonly about 600 to 740 μm . The results were confirmed from the manual (Identification of common *Aspergillus* species) reported by Klich (2002).

Only *A. flavus* has been found in pothwar region among the aflatoxin producing fungi *A. flavus* and *A. parasiticus*. It is well established that in Asia and Africa *A. flavus* is found and *A. parasiticus* is predominant in America (Reddy et al., 2009). Results regarding incidence of *A. parasiticus* reported by Arc (2001) also support the rare occurrence of the fungus where only 2

samples from 60 samples of peanut had *A. parasiticus*.

3.2. Incidence of *A. Flavus*.

Incidence of *A. flavus* was calculated by dividing the number of infested seeds on total number of plated seeds. Mean incidence on the samples collected from Chakwal on PDA was found to be 33.33- 66.66 % (Table 1). While maximum and minimum mean incidences on samples from Attock were 33.33 and 100% respectively as shown in Table 1. The seeds collected from Rawalpindi were found healthier and only two samples were found infected. On the samples from Jhelum the mean incidence of fungus was found to be same as it was in Chakwal i.e.,

33.33 -66.66% (Table 1). As far as the incidence of *A. flavus* on water agar is concerned, it was recorded 33.33 and 44.44% on samples of Chakwal (Table 1). Whereas, in case of Attock and Rawalpindi districts there were no incidence on the substrate. In the samples collected from Jehlum, the mean incidence of fungus was found 33.33% (Table 1)

The above results (14 out of 40 samples showed incidence on PDA, 4 on water agar and no results on filter paper) indicate that PDA was the best substrate amongst three, because it was rich nutrient media as compared to water agar which was only a solidifying agent and not a nutrient medium. Same was in case of filter paper where no incidence of any fungus reported. Three tested media, PDA supported vigorous growth of *A. flavus* at the temperatures of 27 and 30°C (Achar et al., 2009). The mean location wise incidence on PDA and water agar in case of Chakwal was recorded 21.1 and 7.7% respectively. In Attock and Rawalpindi the overall incidence on PDA was found to be 21.1 and 6.6% respectively, while there was no incidence on water agar. Whereas, it was 14.44% on PDA and 6.6% on water agar in Jehlum. There was a significant difference among the substrates and locations. The significance difference in incidence was found districts might be due to improper storage conditions and faulty procedure of drying.

The percentage incidence recorded in this study was high in spite of fresh samples were collected from the market and the storage period was short. Increase in humidity and prolonged storage of nuts (walnut, almond and peanut) for 12-18 months resulted in faster and high *Aspergillus* growth compared to 2-3 months short storage periods (Saleemullah et al., 2006). Youssef et al. (2008) found that the most prevalent fungi in pre-storage and post-storage (stored at normal conditions for 3, 6, 12 and 24 months) were *Aspergillus* (*A. niger*, *A. flavus* and *A. fumigatus*), *Fusarium* (*F. oxysporum*) and *Penicillium* (*P. citrinum*) and their counts increased with lengthening of storage period.

Detection and quantification of aflatoxin contents

Aflatoxin contents were detected and quantified following Veratox procedure for aflatoxins through ELISA (www.neogen.com). The results indicated that aflatoxin contents in peanut samples of Chakwal ranged from 2.0 to 3.54 ppb. This quantity (3.54 ppb) was found to be highest amongst four locations. In Attock, aflatoxin contents in peanut ranged from 2.0 to 2.78 ppb and the lowest value amongst four locations was 2.0 ppb. While in Rawalpindi, the contamination of aflatoxin ranged from 2.33 to 2.58 ppb and it was 2.09 to 2.86 ppb in Jehlum. Prolonged storage for 18 months significantly increased

aflatoxin contents of seeds compared to short storage (2 to 3 months) periods (Saleemullah et al., 2006). Level of detection of aflatoxins changes with storage material as 1.35 to 3.93 µg/kg of aflatoxins were detected in Chakori in jute bag while 0.80 to 1.19 µg/kg were detected in HDPE (High density polyethylene) bag after 60 days. Similarly in BARI-89 and BARD-479 total aflatoxins varied from 1.18 to 2.12 µg/kg and 1.21 to 3.38 µg/kg, respectively in jute bags and ranged from 0.52 to 0.85 µg/kg and 0.61 to 0.93 µg/kg when stored in HDPE bags (Unpublished data).

Conclusion

This study depicts a picture about the current incidence (40%) of *Aspergillus flavus* on peanut samples from major chickpea growing area of Pakistan. The highest value of aflatoxin (3.54 ppb) was found within safe limit (20 ppb, regulatory limit of FDA/WHO). All samples were taken afresh from the current year produce but this high incidence is alarming. Low values of aflatoxin contents (ppb) might be due to presence of less mycotoxic strains of the fungus on peanut of Pothwar region of Pakistan and/or short storage time which needs further investigations about pathogenic variability among the isolates of the fungus and various storage durations.

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