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Characterization of fungal contaminants from wheat and the speculation of mycotoxin with reference to aflatoxin

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Thirty wheat samples procured from storage units in different zones of Coimbatore, Tamilnadu, India were processed to isolate the predominant fungal contaminants. Heterogeneous group of fungi were enumerated by standard plate count, among which four predominant organisms namely *Aspergillus flavus, Aspergillus tamorii, Rhizopus* spp. *and Fusarium* spp. were identified by macroscopic and microscopic observations. Since reputed journal reports, continuously highlight the impact of mycotoxin production in wheat by *Aspergillus flavus*, the isolate was chosen and processed to examine the production of aflatoxin and further analysis and confirmation was done using Albino rats and analytical techniques such as thin layer chromatography, immunodiffusion and Immunoelectrophoresis. TLC revealed the presence of G2 type of mycotoxin at a concentration of 15 ppb.

Key words : *Aspergillus flavus*, Mycotoxin, Aflatoxin, Immunodiffusion

INTRODUCTION

Wheat, the second largest cereal crop cultivated worldwide is a staple food used to make flour, live stock feed and for fermentation to make alcohol. Once a cereal crop is harvested, it may have to be stored for a period of time before it can be marketed or used as feed or seed. The length of time cereal can be safely stored will depend on the condition it was harvested and the type of storage facility being utilized.

Conditioning of grain has the single purpose of preserving the quality of grain. Low moisture content and temperature have been shown to be essential for successful storage of grain for a long period of time. A total of the fungal species belonging to several genera were recorded on wheat seeds stored under farm condition (Basak *et al*., 1987). The survey on wheat storage mould in Government food storage and ration shops in Dhaka and Joydebpur, reported that all seed samples were infected with storage fungi like species of Aspergillus, *Rhizopus* and *Fusarium*. Among these Aspergillus species is highest (47%) followed by *Rhizopus* (30%) and *Fusarium* (20%) (Basak *et al*., 1987). Apart from environmental pollution, the fungus causes several human and animal diseases (Khasim *et al*., 2004).

Aspergillus is the most important fungi both medically and agriculturally. It infects important food and feed crops before and after harvest. It causes Aspergillosis in humans. *Aspergillus flavus, A. parasiticus* etc. produce aflatoxin, which is carcinogenic. Aflatoxin belongs to a family of decaketides that are produced as secondary metabolites by *Aspergillus flavus* and *Aspergillus parasiticus* (Maggon *et al*., 1977). The performance of different *in vitro* diagnostic tests for the diagnosis of Invasive Aspergillosis (IA) was investigated using a rat model (Gursoy *et al*., 2008).

There are reports of outbreak of aflatoxin toxicity in many parts of the world, where people consume many common food items that contain aflatoxin even in small doses. So it is of paramount importance to detect and control these contaminants in food items (Koirala, 2005).

Keeping in mind the importance of wheat grain storage, the present investigation was attempted to screen the predominant fungal species that cause spoilage of stored wheat and special attention was made to characterize aflatoxin producers and its effect using Albino rats.

MATERIALS AND METHODS

Sample collection:

Thirty wheat samples were procured from storage units in different zones of Coimbatore, Tamilnadu, India were brought to the laboratory for processing.

Processing of the sample:

20 g of the sample collected from each unit was analyzed to identify the predominant fungal species causing

spoilage of stored wheat. Standard plate count method (Aneja, 2003) using Sabouraud Dextrose Agar was adopted for study. After incubation at 28ºC for 3-5 days, the agar plate showing maximum number of colonies belonging to the same macroscopic nature was selected for microscopic observation.

Macroscopic observation:

The colonies were observed for their pigmentation and texture on both dorsal and ventral side.

Microscopic observation:

The slide culture technique (Aneja, 2003) was used to observe morphologic characteristics of mould without disturbing the arrangement of spores and conidiogenous cells.

Extraction of protein:

50ml of coconut broth was processed to extract the mycotoxin using chloroform and sodium bisulphate (Sadasivam and Manickam, 2003).

Animal inoculation:

The protein extracted was injected in to Albino rat in its tail vein using standard protocol and labeled with color dye so as to differentiate it from control. The symptomatic observation of rat such as feed intake, movement and morphological changes were examined for 4 days after inoculation with protein. Also the pyrogen test was conducted between one-hour intervals for 10 hours successively and the raise in rectal temperature was noted.

Estimation of multi-mycotoxins (TANUVAS, Namakkal, Tamilnadu):

The multitoxin from contaminated wheat was extracted with acetonitrile, potassium chloride and hydrochloric acid, filtered and defatted with hexane twice .The fat free extract was further extracted using chloroform and subjected to thin layer chromatography.

TLC:

Multi mycotoxins obtained by above method was dissolved in 0.2 ml chloroform and 10ml was spotted on an activated TLC plate together with standards. Chloroform, Acetone, Toluene, Ethyl acetate and Formic acid were used in development of chromatogram. Finally plates were viewed under long and short wavelength to observe the spectrum of fluorescence. The Estimation of toxin was carried using the formula:

Multitoxin (ppb) = ((S*C*D) / (T*E)*100, where

S = Standard volume which matches with test volume in fluorescence intensity

C = Concentration of standard

D = Dilution factor

T = Test volume which matches with standard volume in fluorescence intensity

E = Effective weight of sample

Immunodiffusion technique (Genei, Bangalore):

Immunodiffusion technique is one of the simplest techniques extensively used to check antiserum for the presence of antibodies for a particular Antigen. The antisera raised in Albino rat were used in the assay.

Immunoelectrophoresis:

For further confirmation of toxin, an immunoelectrophoresis technique was followed. Immunoelectrophoresis is a powerful immunological tool to characterize antibodies. The technique is based on the principle of electrophoresis of antigens and immunodiffusion of the electrophorized antigens with a polyspecific antiserum to form precipitin bands. As that of immunodiffusion, the antisera raised in Albino rat were used in the assay.

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below :

Processing of the sample:

Standard plate count method revealed the predominant occurrence of *Aspergillus flavus* followed by *Fusarium* spp.(Table 1).

Table 1 : Prevalence pattern of molds in spoiled wheat

Sr. No.	Organism name	Percentage of occurrence
1.	*Aspergillus flavus*	43 %
2.	*Aspergillus tamorii*	07 %
3.	*Fusarium* spp.	35 %
4.	*Rhizopus* spp.	15 %

Macroscopic observation:

The fungal colonies enumerated from contaminated wheat were observed for their pigmentation and texture on both dorsal and ventral side (Table 2 and 3).

Microscopic observation:

The hyphal and spore structures of the isolates were

Table 2 : Pigmentation of fungal isolates from spoiled wheat

Sr. No.	Organism	Colony pigmentation	
		Dorsal view	Ventral view
1.	*Aspergillus flavus*	Yellow to green	Light yellow
2.	*Fusarium* spp.	Rose	White
3.	*Rhizopus* spp.	Gray to black	Grey
4.	*Aspergillus tamorii*	Dark yellow colony	Brown

Table 3 : Colony texture of molds from spoiled wheat

Sr. No.	Organism	Colony texture	
		Dorsal view	Ventral view
1.	*Aspergillus flavus*	Granular colony	Wrinkled appearance
2.	*Fusarium* spp.	Buffy cottony colony	No wrinkles or lines
3.	*Rhizopus* spp.	Buffy cottony colony	No wrinkles or lines
4.	*Rhizopus tamorii*	Buffy cottony colony	No wrinkles or lines

examined by lacto phenol cotton blue (LPCB) staining method, which showed heterogeneous reproductive structures of fungi (Table 4).

Table 4 : Characterization of isolates by lactophenol cotton blue staining

Sr. No.	Organism	Reproductive structure
1.	*Aspergillus flavus*	Conidiospores surrounding the vesicle
2.	*Fusarium* spp.	Hyphae bearing sickle shaped macroconidia
3.	*Rhizopus* spp.	Sporangiospores within sporangium
4.	*Aspergillus tamorii*	Conidiospores surrounding the vesicle

Animal inoculation:

The protein extracted was injected in to Albino rat's tail vein and its pyrogenic effect, morphological and behavioral changes were observed successively. Gradual increase in body temperature and prominent morphological and behavioral changes were noted (Table 5 and 6).

Table 5 : Pyrogenic effect of aflatoxin in albino rat

Sr. No.	Time (between 1 hour intervals)	Temperature (in °C)
1.	9am	36
2.	10	36
3.	11	37
4.	12pm	38
5.	1	38
6.	2	39
7.	3	40
8.	4	38
9.	5	36
10.	6pm	36

Estimation of Multi-mycotoxins:

The multitoxin from contaminated wheat was extracted with chemicals and subjected to thin layer chromatography, which showed 15ppb of G2 type of aflatoxin. Multitoxin (ppb) = ((S*C*D) / (T*E)*100 = ((2x5x20) / (30x4.9)) x 100 = 5ppb.

Immunoelectrophoresis and immunodiffusion:

A line of precipitation was formed in between the trough and the well due to the antigen antibody interaction.

In the present investigation, stored wheat samples procured from different zones of Coimbatore were investigated for microbial quality. Isolates were characterized using standard protocol. Later aflatoxin was extracted from *Aspergillus flavus*, type G2 was confirmed by TLC at a concentration of 15ppb followed

Table 6 : Symptomatic observation of rat (due to the impact of Aflatoxin)

No. of days	Feed intake		Movement		Morphological change	
	Control	Test	Control	Test	Control	Test
Day1	Normal	Medium	Normal	Partially	Normal	Normal
Day2	Normal	Less	Normal	Arrested	Normal	Tail burnt up appearance
Day 3	Normal	Less	Normal	Sluggish	Normal	Tail burnt up appearance
Day4	Normal	Less	Normal	Sluggish	Normal	Tail burnt up appearance

by testing the effect of aflatoxin using lab animal Albino rat.

The study revealed that the predominant fungal species that cause spoilage in stored wheat samples includes *Aspergillus flavus*, *A.tamorii*, *Rhizopus* spp. and *Fusarium* spp. Similar correlations were observed in the earlier studies (Basak *et al.*, 1987). In the studies of Prema *et al.* (2008), Aspergillus was the dominant organism and similar conditions exist in present investigation (Table 3)

Aflatoxin production on Coconut Extract Broth was studied by Paul (1988), in the present study Coconut Extract Agar was used to produce aflatoxin and was observed blue colored fluorescent zone around the colonies when subjected to UV light. Aflatoxin quantification was done by TLC method, (Protocol by Veterinary Medical and Research Center, Namakkal). Based on that TLC was performed to isolate mycotoxin G2 from *Aspergillus flavus* and produce 15ppb of toxin were isolated.

Agnes and Akbarsha (2003) studied the effect of mycotoxin on rat and observed results. The present study revealed the pyrogenic, physiological and psychological changes in the test animal, mice (Table 4 and Table 5). Cavit (2005), determined presence of aflatoxin by immunoaffinity column extraction using HPLC. In the current investigation an approach was made to study the toxin both quantitatively and qualitatively using TLC and immunotechniques such as immunoelectrophoresis and immunodiffusion, respectively.

The primary factor responsible for fungal contamination is the moisture content, in other words water activity (a_w) in microbiological terms. Unless preserved properly the contaminant like *Aspergillus flavus* can utilize wheat as a best substrate and produce mycotoxins (Aflatoxin). The consumption of such contaminated product will open the door for food borne disease, mainly intoxications. Hence, adequate drying of wheat after harvesting and future storage in dry climatic condition can help us to retard fungal growth.

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