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Occurrence of Mycotoxins in Storage Fungi

Ashok K.Shukla

Department of microbiology, Holy Cross Women's College Ambikapur Chhattisgarh, India

Abstract: ***Mycotoxins are the secondary metabolites, produced by filamentous fungi and cause toxic response when ingested by higher animals. Cereal grain may be contaminated by mycotoxins by the fungi growing on plants as pathogens or associated with post-harvest transportation and storage. Temperature and water activity have boosted the growth and development of fungi in stored seed/grains. Detection and control of fungi is a continuous concern since the fungi can become established and remain with the commodity anywhere along the production, storage, transportation and consumption. Mycotoxins are toxic by product of mould infestations affecting as much as one fourth global food and feed output. Food grains contaminated with mycotoxins particularly aflatoxins, a subcategory can cause fatal acute illness and associated with increased cancer risk. Although, a few fungal strain are toxigenic under the natural conditions, and about 30% isolates of* Aspergillus flavus *and* A. terreus *are capable of elaborating aflatoxins on suitable substrate such as sorghum and wheat grains. Developing countries like India, where a large population survive below poverty line are likely to require modern technology and economic support to implement these strategies and increase awareness among the farmers, traders and consumers, which would minimize mycotoxins contamination and adopt good agricultural practices (GAPs), good trader practices (GTPs) and good consumer practices (GCPs).***

Keywords: Mycotoxins, Mycotoxicosis, Cereal grain, Aflatoxin and Aspergillus flavus.

1. Introduction

The moulds are capable of producing various kinds of secondary metabolites, one of which are the mycotoxins. The mycotoxins are possesses high structural complexity and unique biological properties. These metabolites are also known as strain and species specific (Wyllie and Morehouse, 1977). Amongst mould produced toxins, 'Aflatoxins' seems tobe the most important ones. A number of workers have contributed a great amount of information regarding the mycotoxins producing fungi (Austwick, 1975), and Aflatoxins contamination in various materials of human interest (Goldbalt, 1966; Ciegler et al.,1971; Bilgrami, 1983). In addition to these, a number of investigations have established the clinical problems encountered as a result of consumption of mycotoxins. Hepatotoxic and carcinogenic effects of aflatoxins on livestock have been reported by many workers (Krishnamachary, et.al.,1975) and effect on poultry production by Butler et al., (1974). Although, a wide variety of fungi and their major mycotoxins with their numerous derivatives are now known, but scrutinize to characterize their biological properties (Turner, 1971; Keeler and Tu, 1983; Binder, 2007). The capacity of moulds to produce mycotoxins depends on the chemical nature of substrate and also on environmental conditions such as temperature and moisture content (Scott, 1976; Hesseltine, 1974; Diener, 1976; Bhatt et al., 1980; Wyllie and Morehouse, 1977; Prasad,1983; Sahay, 1983). In the developing countries such as India where nutritive level of about 48% people is below poverty line, increasing prices and fuel crisis gradually forcing the poorer to switch over for consumption of uncooked and water soaked food grains.

Besides, a large number of fungal strains are toxigenic under the natural conditions, and about 30% isolates of *Aspergillus flavus* are known capable of elaborating Aflatoxins on suitable substrates. Rice, Wheat, Maize, Millet, Barley, Sorghum, Gram and Lentil are the important cereal of India. Agrawal et al., (1983) stated that in a large scale storage, fungal spoilage of grain is a challenging problem as mould bring about deterioration of substrate and produce mycotoxins, which are absorbed in grains and ultimately harm to the consumer. The toxins also exert a wide range of effects on biological activities. Inhibitory effects of mycotoxins on the permeability of various crop seeds have been studied by many workers (Crisan, 1973; Tripathi, 1973, 1974; Tripathi and Mishra, 1983; Bilgrami and Sinha, 1965). Aflatoxins B1 is reported as strong inhibitor of protein synthesis (Jones et al., 1967), produced and varying effect of different concentrations of aflatoxins B1 on mitotic index of plant cell and adverse cytological effects on Patulin are also known (Bilgrami, 1986). In view of the above, present studies are planned to investigate the effect of fungal metabolites on the germination of wheat and sorghum grain, and to identify the mycotoxins produced by some strains of *Aspergillus* during their *invitro* growth under the suitable conditions.

2. Materials & Methods

Effect of fungal metabolites on seed germination and production of mycotoxins in cultures was studied using two strains of *Aspergillus flavus* (M 8 and M 61) and *Aspergillus terreus* (TH 19 and TH 22). These strains were isolated during the survey of mesophilic and thermophilic fungi from stored wheat and sorghum grains. For this, the test strains were grown in broth cultures. Erlenmeyer flask of 250 ml capacity containing 100 ml YES medium (15% sucrose and 2% yeast extract) were used as culture vessels. Appropriate amount of medium was dispended in the flasks and were then autoclaved at 121 °C under 15 lbs pressure. After autoclaving, flasks were inoculated with spore suspension (equivalent to 10x 6 spores/ml in 0.1% Tween-80 solution) prepared with the help of spore of each organisms, obtained from seven days old fungal colonies on malt salt agar medium. Flasks inoculated with strains of *Aspergillus flavus* were incubated at 28°C±2°C temperature. Besides, *Aspergillus terreus* inoculated flasks were incubated at 45°C±2°C for 14 days. After the said incubation period, flasks were taken out and the content of each flask was filtered through Whatman No. 41 filter paper.

The culture filtrates were collected separately for further studies.

2.1 Effect of fungal metabolites on seed germination:

Culture filtrates of test organisms were filtered through Millipore filtration using 0.4 nm size filter (Borosil grade 4) which were then used as crude fungal metabolites to study their effect on germination of wheat and sorghum grains. For this, ten gram grains were soaked in 25 ml culture filtrates for 12 and 24 hours. After soaking the grains additional liquid drained off and soaked seeds were shown on agar plates containing 2% agar. Distilled water soaked seeds were run as control (ISTA, 1976 and Shukla, 1991). All the Petri plates were incubated in seed germinator with maintained 75% Relative humidity. The results were recorded after five days of incubation.

2.2 Production of mycotoxins:

To study the mycotoxins production in culture filtrates, the following procedures were adopted.

2.2.1 Extraction of mycotoxins

For the extraction of mycotoxins, sixty ml of culture filtrate was taken in a separating funnel of 250 ml capacity, to this an equal amount of ethyl acetate was added and shaken it gently for 30 minutes. The content in funnel was then allowed to separate into two layers. Content of upper transparent layer (ethyl acetate layer) was collected in a beaker of 500 ml capacity and allowed to evaporate at 60°C in waterbath until the volume reduced to 0.1-0.2 ml. The residue was then dissolved in known volume of chloroform to make its final volume equivalent to 1 ml so obtained extract was then purified by column chromatography (Shukla, 1991)

2.2.2 Purification of mycotoxins:

Extracted toxins sample were purified by using chromatographic column prepared in a glass tube (22 X 300 mm size) having a glass stopper at its bottom. A small plug of glass wool is loosely packed at the bottom of the column and is covered with 5 gm anhydrous sodium sulphate. The column then filled with chloroform up to half of its length and then salary of 10 g silica gel (0.05-0.2 mm) prepared in chloroform. After proper settling of silica gel in the column the slurry of 15 g anhydrous sodium sulphate in chloroform was poured in to the column. Extra chloroform then drained off to the top of the sodium sulphate layer. This prepared column, then used for purification of toxin samples. One ml of condensed extract of toxin sample was then poured into the column and chloroform is drained off and then the column was washed with 150 ml hexane followed by 150 ml anhydrous diethyl ether. Finally the toxins were eluted from the column with 150 ml of chloroform: methanol mixture (97: 3 V/v). The fraction was collected from the time when chloroform-methanol is added until the flow stops. Then elute is evaporated by warming at 60° C in waterbath, the residue was dissolved in 1 ml chloroform and was used for chromatographic analysis. (Shukla, 1991)

2.2.3 Identification of toxins

Thin layer chromatographic plates (20 X 20 cm) having homogenous coating of 03 mm was prepared by using slurry of silica gel 25 HR grade (30 gram silica gel in 55 ml distilled water). The plates were dried at room temperature and desiccated. Before use the plates were activated at 110 ° C FOR 30 minutes (Gimeno, 1980& Shukla, 1991) toxins sample (50 µl/ 100 µl) were spotted on TLC plates. Standard solution of aflatoxins B&G mixture (Sigma chemicals co., USA) was also spotted separately on each plate. The plates were then developed ascending by dipping in a chromatographic tank contains a solvent system, chloroform: methanol (95:5 v/v) to a solvent path length of 12 cm from the base line (Wyllie and Morehouse, 1977). Developed plates were air dried at room temperature in horizontal position and then in the oven for few minutes at 60°C and viewed under long wave UV chamber (Widson Scientific works, New Delhi). The spots were identified by comparing the spots in standard mixture of aflatoxins B and G (Sigma chemicals, USA).

3. Results and Discussion

3.1 Effect of fungal metabolites on seed germination:

Metabolites of all the fungal strains exhibited appreciable inhibitory effect on the germinability of wheat and sorghum grains. Twenty four hours soaked grains have indicated greater loss of germinability then 12 hours soaked grains in almost all the cases, except control. Twenty four hours soaking of wheat grains in culture filtrates of *Aspergillus flavus* (M 08), *Aspergillus flavus* (M61)*Aspergillus terreus* (TH19) and *Aspergillus terreus* (TH 22) cultures caused abnormal germination in 4%, 2%, 8% & 2% seed grains. In addition to these 16%, 18%, 24% & 22% seed grains have showed no germination respectively. Net loss of germinability in sorghum grains has not exceeded 25 percent in any of the cases. Metabolites of the two strains Aspergillus terreus (TH19 & TH22) showed no germination in 15% and 19% seeds, respectively. Inhibitory effect of various fungi isolated from soils and plants have been reported by some workers (Mannozzi , 1932; Tyner and Broadfoot, 1943; Stille, 1957 and Shukla 1991). Effect of metabolites of some of their test fungi has caused both inhibitory and stimulatory effect on wheat seed germination. In the present experiment culture filtrates of all the test strains have caused inhibitory effect on germination of wheat and sorghum seeds. Inhibition of seed germination by fungal metabolites may be due to the presence of certain toxic substances in them, which might be inhibiting various physiological and biochemical processes of seed during seedling emergence.

3.2 Production of mycotoxins

Production of mycotoxins in the culture filtrates of test fungi was studied by thin layer chromatography. All the test fungi have indicated the production of aflatoxin B2 in cultures. Production of aflatoxin B1 was noted only in cultures of *A. flavus*. However, *A.flavus* (M61) found to produce aflatoxin G1 while, A. flavus (M8) culture have indicated the presence of aflatoxin G2 . Production of Patulin was noted in cultures of A.terreus. Presence of toxins in culture filtrates

of test fungal strains might be one of the causes which bring about the inhibition of seed germination and emergence of normal seedling in germinating seeds (Table 1). The cause of abnormally germinated seeds may be due to the mutagenic effect of aflatoxins present in culture filtrates of test fungi.

Mutagenic effects of aflatoxins on both plant and animal system have been reported by various workers (Binder et. al.,2007; Fink-Gremmels & Malekinejad, 2007; Glenn, 2007; Goldblatt, 1969; Huwig, et. al., 2001; Pestka, 2007; Scudamore& Livesey, 1998; Shukla, 1991; Shukla, 2014 a,b,c & d; Voss & Haschek, 2007; Wu, 2004; Wu, 2006 etc.). In addition to these reports other researchers also indicated inhibitory effect of aflatoxins on seed germination in *Lepidium sativum* , cowpea and sorghum (Crisan 1973; 1974; Adekunle & Bassir,1973 and Tripathi, 1973, 1974). Mechanism of inhibition of maize seed germination by aflatoxin B1 have been studied by Tripathi and Mishra (1983), and found that it inhibits the respiration in germinating maize seeds. Whatever, the mode of action of mycotoxins in germination of seeds and with a greater percentage cause complete inhibition of seed germination.

Table 1: Effect of culture filtrates of some *Aspergilli*, on seed germination of Wheat (var. WH 147) and Sorghum (var. CHS O5).

S.No.	Treatment (soaked in culture filtrates)	Categories*	Seed germination (%)			
			Wheat seeds		Sorghum seeds	
			12 hrs.	24 hrs.	12 hrs.	24 hrs.
01.	*Aspergillus flavus* (M 08)	(A)	90.00	80.00	80.00	75.00
		(B)	08.00	16.00	18.00	23.00
		(C)	02.00	04.00	02.00	02.00
02	*Aspergillus flavus* (M 61)	(A)	88.00	80.00	78.00	73.00
		(B)	10.00	18.00	20.00	25.00
		(C)	02.00	02.00	02.00	02.00
03	*Aspergillus terreus* (TH19)	(A)	94.00	70.00	83.00	81.00
		(B)	04.00	24.00	13.00	15.00
		(C)	02.00	08.00	04.00	04.00
04	*Aspergillus terreus* (TH 22)	(A)	94.00	76.00	80.00	77.00
		(B)	04.00	22.00	16.00	19.00
		(C)	02.00	02.00	04.00	04.00
05	**Control** (soaked in) distilled water	(A)	98.00	100.0	96.00	100.0
		(B)	02.00	00.00	04.00	00.00
		(C)	00.00	00.00	00.00	00.00

Categories*: (A) seed showing normal germination; (B) seed showing abnormal germination; (C) seed showing no germination.

Table 2: Production of mycotoxins in cultures of some Aspergilli

S. No	Mycotoxins	*Aspergillus flavus* (M8)	*Aspergillus flavus* (M61)	*Aspergillus terreus* (TH19)	*Aspergillus terreus* (TH22)
1	Aflatoxin B1	Positive	Positive	Negative	Negative
2	Aflatoxin B2	Positive	Positive	Positive	Positive
3	Aflatoxin G1	Negative	Positive	Positive	Positive
4	Aflatoxin G2	Positive	Negative	Negative	Negative
5	Patulin	Negative	Negative	Positive	Positive

4. Conclusion

Globalisation of trading practices especially in agricultural commodities have contributed a lot of knowledge significantly, to focus their attention about potential hazards present in them, and have increased particular awareness of world community, in the field of mycotoxins. Safety and awareness in food and feed production, transportation, storage and trading also rose due to the advancement in science and technology for rapid detection of undesirable and harmful substance. Due to the modern laboratory testing method and growing interest of researchers in the field of mycotoxins research, more than 300 different mycotoxins have been identified. However, for a particular consideration in seed/grain production storage and trading processes, only a small number of mycotoxins are of relevance, with aflatoxins, trichothecenes, zearalenone, ochratoxins fusaric acid and fumonisins being of prime importance and require special attention.

Approaches reveals the preventive measures of mycotoxins production including field and post harvest management, and strategies which offers the right way for the prevention of mycotoxins formation in the field level than would be at storage level. Besides, the scientific researchers suggested various information on the effects of individual mycotoxins in different animal species, and contamination of single or multiple mycotoxins on livestock. Consumption of mycotoxin contaminated diet may induce acute diseases and long term chronic effect in the form of teratogenic, carcinogenic, oestrogenic and immuno suppresive effects. In this way, the cost of losses caused by mycotoxins is not easy to determine accurately. While mycotoxin associated loss in the growing countries such as India cause market loss as a rejected cereals grains/ seeds and their disposal in cases of severe contamination, chronic exposure to the mycotoxins, often combined with malnutrients that may cause serious level of mycotoxicosis, and even death.

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