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MYCOTOXIGENICITY OF *ASPERGILLUS, PENICILLIUM* AND *FUSARIUM* SPP. ISOLATED FROM STORED SORGHUM

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Seedborne storage fungi namely *Aspergillus, Penicillium* and *Fusarium* were evaluated on the 72 sorghum seed samples collected from different "godowns" of traders in rainy season, winter and summer. *Aspergillus flavus, A. niger, A. fumigatus, A. nidulens, A. versicolor, A. ochraceus, A. parasiticus, Penicillium oxalicum, P. citrinum, P. purpurogenum, P. frequentans, P. chrysogenum, Fusarium verticilloides, F. proliferatum, F. oxysporum and F. semitectum* were isolated from sorghum seeds. Mycotoxigenicity of these species was also studied by thin layer chromatography. Retardation factors of different spots relative to griseofulvin (Rfg) were compared with published data and secondary metabolites were identified. Aflatoxin B1, Aflatoxin B2, Ochratoxin A, Citrinin, rubratoxin B, Cyclopiazonic acid, Sterigmatocystin, T-2 toxin, Penicillic acid, Roquefortine C, Mitorubrinic acid, Brefeldin A, Zearalenone, Equisetin, Moniliformin and Fusarin C were produced by different isolates of storage fungi namely *Aspergillus, Penicillium* and *Fusarium* species.

Mycotoxigenicity of these storage fungi on sorghum suggests potential hazards. In light of this report, further investigations for these mycotoxins, particularly quantitative estimations directly in the grain samples needs to be undertaken by the researchers, which can play an important role in formulating food safety guidelines.

Key words: *Aspergillus, Fusarium*, Mycotoxins, *Penicillium*,

Sorghum, often referred to as 'poor people's crop', is grown in harsh environments where other crops grow or yield poorly. Rainy season sorghum is mostly used for food and feed purpose (Das *et al.* 2010). As per Food and Agricultural Organization's data of 2010, India is the world's second largest producer of sorghum by quantity (FAO statistics web site). The demand for sorghum for feed purposes has been the main driving force in raising global production. In developing countries, it is usually stored in small quantities in traditional containers, often on the farm for future consumption. It is also stored by commercial traders for resale, usually on the home market.

No safety measures and guidelines are followed in storage conditions leading to growth of storage fungi. High temperature and high humidity particularly in monsoon (rainy) season in India adds to vulnerability of the stored products for fungal growth. (Singh *et al.* 1988). High concentrations of storage fungi in the air of grain shops by causing increased inoculation on the grain seeds may be responsible storage losses and production of mycotoxins (Sawane and Saoji 2005). Grain mould including Fusarium severely affects grain quality of sorghum produced during rainy season in India (Das *et al.* 2010). Once infected with the storage fungi, mycotoxins accumulate in the grains and may be responsible for mycotoxicoses of the humans and animals. (Wyllie and Morehouse 1978)

There are substantial studies on field fungi associated with sorghum in India; however, studies of storage fungi associated with sorghum particularly at the consumer end are conspicuous by their absence with very meager research data on mycotoxigenicity of storage fungi isolated from sorghum in Vidarbha, Central India.

The aim of the present investigation, therefore, was to study prevalence of storage fungi associated with sorghum in samples

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collected from grain markets and to evaluate their mycotoxin producing potential.

MATERIALS AND METHODS

Seventy two rainy season (kharif) sorghum (*S. bicolor* (L.) Moench) seed samples were collected from different "godowns" of traders in rainy season, winter and summer. Usually rainy season sorghum is harvested in September- October in India and is stored by the traders in gunny bags in their small godowns for up to 1 year. The evaluation of storage fungi on the sorghum was done by standard blotter test (Mathur and Kongsdal 2003). Sub-cultures were made from developing colonies and pure cultures of *Aspergillus, Fusarium* and *Penicillium* were obtained for subsequent studies by three point inoculation technique on Czapek agar (CA) for *Aspergillus*, potato sucrose agar (PSA) for *Fusarium*, Czapek yeast autolysate agar (CYA) for *Penicillium.* Cultures were incubated for 7 days at 25°C without illumination in an upright position.

Identification of different species of storage fungi namely *Aspergillus, Penicillium* and *Fusarium* was done up to species level with the help of standard literature (Gilman 1945 Raper and Fennell 1965; Pitt 1988 and 1989, Singh *et al.* 1991, Leslie *et al.* 2006). The isolation frequencies (Fr) of different fungal species were calculated according to Gonzalez *et al.* (1997).

Seven days old cultures of *Aspergillus* spp growing on CA, *Fusarium* spp. on PSA and *Penicillium* on CYA were used for detection of mycotoxins by thin layer chromatography (agar plug method) as described by Singh *et al.* (1991).

Seven days old cultures of *Aspergillus, Fusarium* and *Penicillium* spp. grown on these media were incubated for 7 days at 25°C without illumination in an upright position. Small agar plugs were cut from the fungal colonies grown on CYA using a cork borer with inner diameter 4 mm from the centre of the colony. The plugs were wetted by a drop of chloroform/methanol (2:1, v/v), and mycelial side of the plugs were immediately applied on to TLC plates (Silicagel 60, 20x20 cm, Merck) for few seconds, 2.5 cm from the bottom line. The diameter of the application spot was always less than 0.6 cm.

As an external standard, griseofulvin solution was applied in the centre lane of the TLC plate. After the run in TEF (Toluene/ethylacetate/90% formic acid, 5:4:1, v/v/v) solvent, developed plates were allowed to dry and then viewed in daylight, under long wave (366nm) and short wave (254 nm) ultraviolet (UV) light (Figure 1). Developed TLC plates were also treated with spraying agent 0.5% p-anisaldehyde in methanol/acetic acid/conc. sulphuric acid (17:2:1), heated for 8 minutes at 130^{0}C and again viewed in daylight, long and short wave UV light. Some mycotoxins appeared only on treatment with spraying agent. The retardation factors (Rfg values) of the spots were calculated relative to griseofulvin as:

Rfg = Distance travelled by a spot in mm / Distance travelled by Griseofulvin in mm.

The Rfg values of the observed spots were compared with the Rfg values for different mycotoxins published by Frisvad and Thrane (1987) and Singh *et al.* (1991).

Composition of media used:

Czapek agar:

Sucrose……..... .....30 g
$NaNO_3$……..3 g
K_2HPO_4.....1g
KCl…….. .....0.5g
$MgSO_4.7H_2O$…….....0.5 g
$FeSO_4.7H_2O$-…….....0.01 g
Agar-…….....15g
Distilled water-…….....1000 ml
Trace metal solution ……..... 1 ml
(Trace metal solution-$ZnSO_4.7H_20$-1g $CuSO_4.5H_2O$, Water-100 ml)Potato sucrose agar –Potato extract from 300 g of potato, boiled for 1 hour in 900 ml of water and filtered-230 ml
Sucrose-…….....20 g
Agar…….....15 g
Add distilled water to make the volume 1 liter.
Adjust pH to 6.5-7

The ingredients of CYA are:

NaNo3…….....…… 3 gm

Table 1 - Prevalence of seed borne fungi on sorghum samples obtained during different seasons.

S.No.	Fungus	Rainy		Winter		Summer	
		No. of samples infected (out of 24)	Isolation frequency	No. of samples infected (out of 24)	Isolation frequency	No. of samples infected (out of 24)	Isolation frequency
1.	*Aspergillus niger*	14	58.33	4	16.67	9	37.50
2.	*A. flavus*	21	87.50	18	75.00	20	83.33
3.	*A. versicolor*	6	25.00	2	8.33	3	12.50
4.	*A. nidulens*	7	29.17	4	16.67	5	20.83
5.	*A. ochraceus*	4	16.67	6	25.00	-	-
6.	*A. fumigatus*	10	41.67	9	37.50	5	20.83
7.	*A. parasiticus*	3	12.50	-	-	2	8.33
8.	*Penicillum citrinum*	5	20.83	4	16.67	2	8.33
9.	*P. oxalicum*	13	54.17	12	50.00	6	25.00
10.	*P.purpurogenum,*	4	16.67	4	16.67	1	4.17
11.	*P. chrysogenum,*	3	12.50	4	16.67	-	-
12.	*P. janthinellum,*	1	4.17	-	-	-	-
13.	*P. frequentans*	5	20.83	3	12.50	-	-
14.	*Fusarium verticilloides*	7	29.17	8	33.33	4	16.67
15.	*F. semitectum*	2	8.33	4	16.67	-	-
16.	*F. proliferatum*	3	12.50	6	25.00	1	4.17
17.	*F. oxysporum*	3	12.50	5	20.83	2	8.33
18.	*Fusarium spp.*	2	8.33	3	12.50	2	8.33

Table 2: Secondary metabolites produced by some storage fungi isolates obtained from sorghum.

Species	Rfg values of spots on TLC	Secondary metabolites after comparison with published data	No. of Isolates tested	No. of Isolates producing sec. metabolites
Aspergillus niger	1.39.	Ochratoxin A	6	1
A. flavus	0.56	Aflatoxin B1	6	5
	1.37	Cyclopiazonic acid		2
A. versicolor	1.75	Sterigmatocystin	6	3
A. nidulens	1.75	Sterigmatocystin	6	2
	1.0	T-2 toxin		3
A. ochraceus	1.39	Ochratoxin A	6	3
	1.03	Penicillic acid		2
A. fumigatus	0.99	Unknown	6	2
A. parasiticus	0.56	Aflatoxin B1	3	2
	0.39	Aflatoxin B2		1
Penicillum citrinum	1.23	Citrinin	6	5
P. oxalicum	0.19	Roquefortine C	6	3
	1.57	Unknown		2
P. purpurogenum,	0.32	Rubratoxin B,	6	2
	1.03	Mitorubrinic acid		3
				4
P.chrysogenum	0.19	Roquefortine C	6	1
P. janthinellum,	1.03	Penicillic acid,	1	1
	0.73	Brefeldin A		1
	1.318	Unknown		1
P. frequentans	0.95	Unknown	6	3
Fusarium verticilloides	0.58	Fusarin C	6	2
F. semitectum	1.77	Zearalenone	6	3
	1.45	Equisetin		1

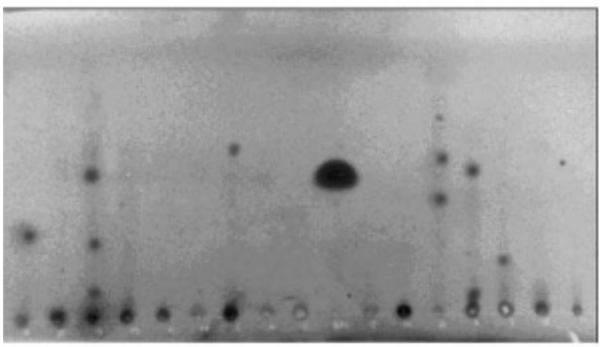

Figure 1: Photograph of the TLC plate along with mycotoxins. Large spot in the centre lane is the Griseofulvin, an external standard. Retardation factors of other spots were calculated relative to retardation factor

K_2HPO_4 ……..…….. 1 gm
KCl …… 0.5 gm
$MgSO_4.7H_2O$ …….....…….. 0.5 gm
$FeSO_4.7H_2O$ ……..... 0.01 gm
Yeast Extract ……..... 5 gm
Sucrose …….....… 30 gm
Agar …….....…… 15 gm
Distilled Water ……..... 1000 ml.
Trace Metal Solution …….…..... 1.0 ml

RESULTS AND DISCUSSION

Storage fungi isolated from sorghum

In all 72 samples of sorghum collected from "godowns" of traders were tested for the isolation of *Aspergillus, Penicillium* and *Fusarium.* Isolation frequency of different species from sorghum samples in three different seasons (rainy, winter and summer) are given in Table I.

F. moniliforme with isolation frequency of 16.2% was the second most predominant fungus on sorghum seeds in Bangladesh. *Aspergillus niger* (8.9%), *A. flavus* (4.3%) and *Penicillium oxalicum* (6.1%) were also reported to be present on sorghum seeds (Islam *et al.* 2009).

Seven species of fungi detected in sorghum seeds obtained from different locations of Punjab, India were- *A. flavus, A. niger, A. tenuis, C. lunata, F. moniliforme, Helmintho-sporium (Bipolaris) sativum* and *Penicillium* spp. *F. moniliforme* was found to be the most devastating fungus in seed germination trials by Randhawa *et al.* (1998).

Aspergillus niger, A. flavus, A. fumigatus, A. parasiticus, A. nidulens, A. ochraceus, A. versicolor, A. glaucus, Fusarium equiseti, F. oxysporum, F. semitectum, F. solani, F. spp., Penicillium citrinum, P. notatum, P. rubrum, P. verrucosum and *P*. spp. were the different species of these three genera isolated from stored sorghum in a study carried out in Nigeria (Hussaini *et al.* 2009). *F. moniliforme, A. flavus* and *P. funiculosum* has been reported to be the predominant species of *Fusarium, Aspergillus* and *Penicillium* isolated from sorghum in Argentina (Gonzalez *et al*. 1997).

F. moniliforme, F. proliferatum, F. semitectum, F. oxysporum, in addition to F. sporotrichiodes, F. poae, F. graminearum, F. subglutinans, F. avenaceum were reported by Hossain *et al.* (2002) from 3 months laboratory stored sorghum with 38.02% occurrence of Fusarium from sorghum.

A. flavus (16.98%), *A. niger* (12.28%), *Penicillium* spp. (16.57%), *Fusarium moniliforme* (2.97%) and *Fusariun subglutinans* (2.43%) were isolated from sorghum seeds in Karachi, Pakistan (Fakhrunnisa *et al.* 2006).

F. moniliforme and *F. proliferatum* were isolated from sorghum collected from markets and household samples in Andhra Pradesh, India (Bhat *et al*. 2000). *Alternaria alternata* and *Fusarium moniliforme* were the most dominant fungi isolated from farmer's stored sorghum seeds in Karanataka, India (Raj 2007).

A. flavus, A. japonicus, A. nidulens, A. niger, A. candidus, A. versicolor were isolated from stored sorghum seeds in different seasons in a study in India (Gupta Rajni 1996). In the same investigation, *F. semitectum, F. moniliforme, F. oxysporum* and *F. sporotrichiodes* were the *Fusarium* species while *P. citrinum, P. chrysogenum* and *P. expansum* were the different *Penicillium* species isolated from stored sorghum. *Fusarium* was isolated from sorghum seeds collected only in winter season while *Penicillium* was conspicuous by its absence in samples collected in summer. On the

other hand, *Aspergillus* was always isolated from stored sorghum seeds.

Mycotoxin potential of different species of storage fungi

Mycotoxin potential of all the species of *Aspergillus, Penicillium* and *Fusarium* isolates obtained during this investigation are depicted in Table II. As mycotoxins were identified by comparing Rfg values with the published Rfg values for different mycotoxins, evaluation of mycotoxins potential of different fungal species became preliminary in nature and requires confirmation by using different standards. However, availability, cost and safety measures of standard mycotoxins justify the comparative method for preliminary study.

Out of 94 isolates belonging to 17 species of *Aspergillus, Penicillium* and *Fusarium*, 47 isolates produced one or more of the 16 known and 4 unknown secondary metabolites. Aflatoxin B1, Aflatoxin B2, Ochratoxin A, Citrinin, Rubratoxin B, Cyclopiazonic Acid, Sterigmatocystin, T-2 toxin, Penicillic Acid, Roquefortine C, Mitorubrinic acid, Brefeldin A, Zearalenone, Equisetin, Moniliformin and Fusarin C were produced by different isolates of storage fungi namely *Aspergillus, Penicillium* and *Fusarium* species.

In India, occurrence of T-2 toxin in Fusarium infested sorghum has been reported by Cheruvanky and Bhat (1978). Isakeit *et al.* (2008) have reported production of fumonisin, zearalenone and fusaric acid by *F. proliferatum, F. semitectum, F. thapsinum* and *F. verticilliodes* isolated from sorghum seeds.

In an investigation by Bhat *et al.* (2000), analysis of sorghum samples collected from markets and households in sorghum-growing regions of Andhra Pradesh, India showed that the extent of fumonisin contamination was higher in rain-affected and moldy samples compared to that in normal samples. *F. moniliforme* and *F. proliferatum* were isolated from sorghum and were found to produce fumonisin. Though aflatoxins were found to co-occur no correlation was observed between fumonisin B1and aflatoxin B1 indicating that both the toxins exist independently in the sample (Bhat *et al.* 2000).

In a study in Nigeria, Aflatoxin B1 and Zearalenone was found respectively in 91 and 60 out of 168 sorghum samples while 23 of 112 samples were found to have ochratoxin A (Hussaini *et al.* 2009).

Thirty one species belonging to 17 fungal genera were found to be associated with sorghum (*Sorghum bicolor*) grain samples imported to the Kingdom of Saudi Arabia. An agar plate method was used to screen 24 samples collected from different markets located in Riyadh region. Comparisons of fungal isolation frequencies revealed that *Aspergillus niger, Penicillium funiculesum* and *Rhizopus stolonifer* were most frequently isolated from untreated grains, while *A. niger, P. funiculesum* and *F. semitectum*, were most dominant in surface-sterilized sorghum (*Sorghum bicolor*) grain samples. *A. niger* isolates were the highest producers of aflatoxins, followed by *A. flavus* var. columnaris and *A. terreus, P. funiculosum* was the highest producer of the aflatoxins, *Penicillium* toxin Patulin (41 ppb), while *P. oxalicum* was the highest producer of citreoviridin (10 ppb). *F. verticillioides* was the highest producer of the *Fusarium* toxins fumonisin (19.1 ppb) and Zearalenone (21.4 ppb). *F. nygamai* was the highest producer of vomitoxin (31.3 ppb). Two of the three *Alternaria* isolates were altenuene producers (Yassin *et al.* 2010).

"In India, warm humid climate provides congenial atmosphere for the growth of fungi and production of toxins. Foodgrains are normally harvested at higher moisture content and then dried to bring down the moisture content up to safe level before storage. Delay in drying to safe moisture levels increases risks of mould growth and mycotoxin production. Natural calamities like floods or torrential unseasonal rains during pre, mid or post-harvest stages may render the crops vulnerable to microbial attack. Faulty storage conditions may also enhance the chances of microbial attack and production of mycotoxins. Thus, there are chances of microbial invasion/fungal attack at each and every stage starting from harvesting of the crop till the food or food products are

consumed by the consumers," (Goyal 1989).

In developed countries, sophisticated equipment and procedures are used for post-harvest storage, mycotoxin prevention and control; whereas in developing countries, substandard foodgrains may be consumed without any form of sorting or inspection. Mycotoxin ingestion remains far too high in many countries, especially in rural areas (Pit, 1989).

The production of mycotoxins by the storage fungi isolates obtained from sorghum seed samples in the present investigation has demonstrated the hazards associated with the presence of these species in stored sorghum. In light of this report, further confirmatory investigations for different mycotoxins, particularly quantitative estimations directly in the grain samples collected in different seasons needs to be undertaken by the researchers, which can play an important role in formulating food safety guidelines.

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