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Toxigenicity of *Fusarium* isolates and fumonisin B_1 contamination in rainy season sorghum (*Sorghum bicolor*)

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ABSTRACT

Fusarium grain infection and resultant contamination by fumonisins in sorghum [*Sorghum bicolor* L. (Moench)] grains hamper the quality of rainy season sorghum in enhancing its use as food, feed and value-added products. Laboratory and glasshouse experiments were conducted during 2007 to determine the toxigenicity of *Fusarium* isolates with respect to fumonisin B_1 production on sorghum grains. Field trials were conducted at 4 locations to assess fumonisin B_1 contamination in elite sorghum cultivars. *Fusarium* isolates from different sorghum locations in India showed great variations in their ability to produce fumonisin B_1 (range: 0–82 143 µg/kg) on sorghum grain. Four out of 65 isolates (around 6%) were highly toxigenic with ability for fumonisin B_1 production both on stored grain (>5 000 µg/kg) as well as on fresh grain (>200 µg/kg) samples. Around 32% of the isolates were non-toxigenic. Morphological characters of the *Fusarium* isolates and their relation with fumonisin B_1 production are discussed. Fumonisin B_1 contamination in field samples ranged from 5 to 1 398 µg/kg grain and varied over locations and genotypes. Few genotypes showed toxins below safety limits.

Key words: Fumonisin B_1, *Fusarium moniliforme*, Grain sorghum, Mycotoxins

Contamination of sorghum grain and fodder with *Fusarium* toxins is a global problem. *Fusarium* species synthesize a wide range of mycotoxins of diverse structure and chemistry such as fumonisins, moniliformin, fusarenon-X, T-2 and deoxynivalenol. Of these, fumonisins (especially fumonisin B_1), synthesized by *Fusarium moniliforme*, are the most commonly occurring and potentially dangerous mycotoxins in sorghum and maize. Occurrence of fumonisins is higher in sorghum than in maize (Waliyar *et al.* 2008). The change of the staple diet of Black South Africans from sorghum to maize was because of contamination of sorghum with mycotoxins (Isaacson 2005). Fumonisins were first described and characterized in 1988 from *F. moniliforme*. Fumonisins are produced by a number of *Fusarium* species, notably *F. moniliforme*, *F. proliferatum*, and *F. nygamai*, as well as *Alternaria alternata* f. sp. *lycopersici* (Rheeder *et al.* 2002). Unlike most known mycotoxins, which are soluble in organic solvents, fumonisins are hydrophilic. This makes them difficult to study. Fumonisins may contribute to liver and kidney damage and cause esophageal cancer in human being. These toxins do not decompose in the animal organs but are transferred to products – eggs, meat and milk, thus jeopardizing human health also. In addition to *Fusarium* toxins, mycotoxins produced by *Aspergillus* spp (eg, aflatoxins, ochratoxins) are also important in sorghum because of their deleterious effects on human and livestock health as well as trade.

Fusarium is the most predominant genus among the grain mold fungi that severely affects grain quality of sorghum produced during rainy (*kharif*) season in India. As *kharif* grain is mostly used for food and feed purpose, *Fusarium* infection and concomitant fumonisin contamination in sorghum assume significance in relation to food and feed safety. Earlier studies have reported fumonisin contaminations in rainy season sorghum grains (Waliyar *et al.* 2008). Little information is available on fumonisin B_1 contamination in elite and released varieties and hybrids. Further, production of fumonisins by *F. moniliforme* is influenced by a diverse array of factors, broadly divisible into biological, physical and chemical factors. Literature is replete with information on physical and chemical factors required for production of mycotoxin by different fungi. But information is scanty on how isolates of *Fusarium* from sorghum grains vary in fumonisin production. Hence, a study was conducted to

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determine the toxigenicity of *Fusarium* isolates of grain sorghum and to assess fumonisin B_1 contamination in elite sorghum cultivars.

MATERIALS AND METHODS

A total 104 grain samples were collected from various markets (each 500 g), farmers' fields and research farms (each 250 g) in Andhra Pradesh (63), Maharashtra (10), Karnataka (10), Gujarat (15) and Madhya Pradesh (6) for isolation of *Fusarium* spp. Another set of 144 grain samples (12 genotypes × 4 locations × 3 replications) (each 250 g) were collected from 9 varieties ('CSV 15', 'CSV 17', 'SPV 462', 'SPV 1600', 'SPV 1616', 'SPV 1742', 'SPV 1746', 'SPV 1774', and 'SPV 1786') and 3 hybrids ('CSH 16', 'CSH 18', and 'CSH 23',) grown in 4 locations (Coimbatore, Dharwad, Akola and Udaipur) under All India Coordinated Sorghum Improvement Project (AICSIP) during rainy (*kharif*) season 2007 for assessment of fumonisin B_1 contamination.

For isolation of *Fusarium* spp, the samples from a particular location (or market) were mixed together to form a composite sample representative of that location. A sub-sample of 200 g from each composite sample was washed aseptically with sterile distilled water, surface-sterilized using 4% sodium hypochlorite solution (NaOCl), and then rinsed with sterile distilled water 3 times. The samples were then air-dried in a laminar flow hood. Twenty-five surface sterilized grains were placed on pre-sterilized blotter paper inside a Petri-dish. Four Petri-dishes (ie 100 seeds) were used per sample. Plates were incubated at 28 ± 1°C in an incubator, with 12 hr alternate cycles of light and dark for 5-days. Each seed in a plate was observed for growth of *Fusarium* under stereo-binocular microscope. Fifteen to twenty colonies resembling *Fusarium* spp were randomly picked from each composite sorghum sample and placed on potato dextrose agar (Hi media) (¼ th strength) plates. Each isolate was purified through single spore isolation (Fig 1 A) on Rose Bengal agar (Hi Media) and were preserved in potato dextrose agar slant for further studies.

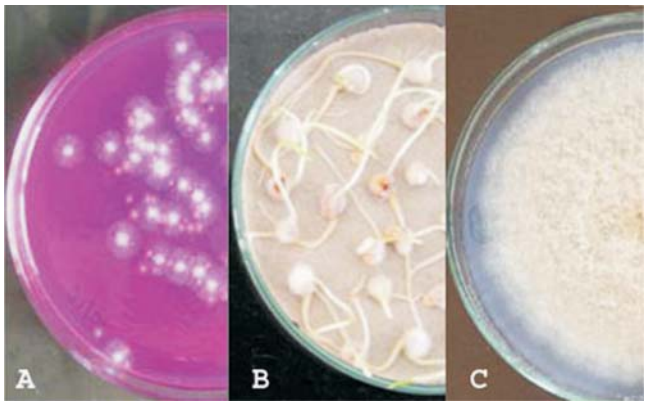

Fig 1 (A) *Fusarium* colony developed from single spore on Rose Bengal Agar; (B) Growth of pure culture of *Fusarium* on sorghum grain on artificial inoculation; and (C) colony characters of highly toxigenic *F. moniliforme* SF035 on PDA

Table 1 Sorghum grain samples used for isolation of *Fusarium* spp

Source of sample	Location of collection (state)	Grain type	Sample[a] pooled	*Fusarium* spp selected
Grain from field	Akola (MS)	White	10	9
	Dharwad (KA)	White	10	7
	Mahaboobnagar (AP)	White	5	2
	Mahaboobnagar (AP)	Yellow	5	3
	Ranga Reddy (AP)	White	8	5
	Indore (MP)	White	5	2
	Surat (GU)	White	15	12
Grain from market	Guntur (AP)	White	5	2
	Guntur (AP)	Yellow	5	5
	Hyderabad (AP)	White	20	11
	Hyderabad (AP)	Yellow	5	2
	Ranga Reddy (AP)	White	5	2
	Ranga Reddy (AP)	Yellow	5	2
Grain from brewery	Indore (MP)	White	1	1

MS, Maharashtra; KA, Karnataka; MP, Madhya Pradesh; GU, Gujarat; AP, Andhra Pradesh

[a]Samples from a particular location (or market) were mixed together to form a composite sample representative of that location. A sub-sample of 200 g from each composite sample was used for isolation of fungi

Scotch-tape method was used for preliminary identification of fungi using a compound microscope (Olympus B×50). *Fusarium* spp appeared as white to light orange powdery colony with abundant microconidia that were formed in short or long chain, or in false head on conidiophores; clamidospores were either present or absent. Confirmatory identification of the cultures was done by Indian Type Culture Collection (New Delhi).

For study of morphological characters, each isolate was grown on potato dextrose agar plate (¼ th strength) and incubated at 28 ± 1°C in an incubator, with 12 hr alternate cycles of light and dark for 7 days. Colony characters like growth rate, aerial growth, texture, edge and color on under surface of the plate (pigmentation) were recorded. Details about source of samples and location for 65 isolates of *Fusarium* spp that were isolated from 104 grain samples are given in Table 1. The qualitative morphological characters (eg aerial growth, texture, pigmentation) were quantified by assignining score on a 1–3 scale. These scores were used for correlation studies.

Toxigenicity of the *Fusarium* isolates with respect to fumonisin B_1 accumulation in sorghum grain was studied by artificial inoculation under laboratory (*in vitro*) and glasshouse (*in vivo*) conditions. Two-hundred grams of clean surface-sterilized sorghum grains (cv. 'CSH 16') were spread

in thin layer on a sterile blotting paper in a laminar flow chamber and spray-inoculated with conidial suspension (10^4/ ml) of a *Fusarium* isolate. Conidia from few mycelial discs from 7-day old fungal culture were harvested and serially diluted with sterile water to obtain desired concentration of spore suspension. The inoculated grains were air-dried for 1 hr in the flow, divided into 2 equal parts, and each part (treated as one replication) was transferred to a paper bag and incubated at 28 ± 1°C for 7-days. After establishment of infection the grain samples were stored at room conditions for 30-days and were analyzed for fumonisin B_1 content. Four highly toxigenic (SF018, SF019, SF035 and SF036) and 2 mildly toxigenic (SF049 and SF161) isolates of *Fusarium* selected based on *in vitro* study were further evaluated for fumonisin B_1 production in developing sorghum grains in panicles under glasshouse conditions. Spore suspension (10^6 conidia/ ml) of each of the above 6 *Fusarium* isolates was spray-inoculated on 6 panicles ('CSH 16') at 80% anthesis for infection and mold development. Panicles sprayed with sterile water served as controls. Panicles were harvested at grain maturity. Six panicles obtained from each treatment were randomly divided into 2 groups (treated as replications) and threshed separately. Fumonisin B_1 was estimated in grains in each replication.

Around 100 g grain sample was powdered in a blender, and a 20 g sub-sample was used for extraction of fumonisin B_1. The sub-sample was mixed with 100 ml of 70% methanol containing 0.5% KCl in a blender until the seed powder was thoroughly grounded. The emulsion was thoroughly shaken on a rotary shaker (300 rpm) for 30 min. The extract was filtered through Whatman filter paper No 41 and stored at 4°C. Estimation of fumonisin B_1 in sample extracts was done by indirect ELISA Method. Regression curve was drawn with the help of computer by plotting $\log_{10}$ values of concentration of fumonisin B_1 standards on Y-axis and corresponding optical density values on X-axis. Using the values of the regression equation, the toxin content in the sample was determined.

Data on fumonisin B_1 contamination, morphological characters of *Fusarium* isolates and their correlations were analyzed statistically, using a statistical software package (*Statistix*, version 8.1).

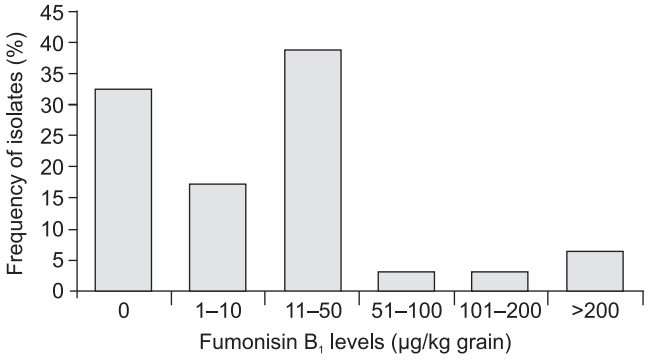

Fig 2 Frequency distribution of toxigenic and non-toxigenic isolates of *Fusarium* spp for fumonisin B_1 production on sorghum grain

RESULTS AND DISCUSSION

Fumonisin B_1 production by Fusarium isolates

Variability of *Fusarium* isolates with respect to production of fumonisin B_1 in sorghum grain under *in vitro* conditions is represented in Fig 2. The isolates were highly variable in their ability to produce fumonisin B_1 (range: 0–82 143 µg/ kg). Fumonisin B_1 production by majority of the isolates (94%) was within safety limit (200 µg/kg) prescribed by CODEX Committee. Around 6% isolates (4 out of 65) were highly toxigenic (fumonisin B_1 >500 µg/kg) and 32% (21 isolates) non-toxic and could not produce any detectable fumonisin B_1. The variation in level of toxin production was purely due to variation among fungal isolates as the substrate (sorghum grain) and growing environments were same for all the isolates. *Fusarium* spp isolated from sorghum is known for such variation in toxigenicity (Leslie *et al*. 2005, Ratnavathi and Das 2008). Under in vitro conditions *Fusarium* isolate SF035 accumulated the highest amount of fumonisin B_1 on sorghum grain (82 143 µg/kg), followed by SF019 (41 789 µg/kg), SF018 (37 447 µg/kg) and SF036 (5 028 µg/kg). Accumulation of high concentration of fumonisin B_1 (226 000 µg/kg) by artificial inoculation of *Fusarium* was reported on maize (Bacon *et al.* 2001). The highly toxigenic isolates (SF018, SF019, SF035 and SF036) when further evaluated for fumonisin B_1 production in developing sorghum grains under glasshouse conditions (*in vivo*), there was great reduction in fumonisin B_1 production (Table 2). This was due to difference in growing conditions which were more congenial for infection and colonization (high spore density, optimum temperature,

Table 2 Fumonisin B_1 production by selected isolates of *Fusarium* spp on artificial inoculation under *in vitro* and *in vivo* conditions

Fusarium isolate	Fumonisin B_1 (µg/kg) ± SE	
	in vitro[a]	*in vivo*[b]
Fusarium sp. SF018	37 447 ± 2 447[c]	468 ± 68[c]
Fusarium sp. SF019	41 789 ± 2 889	268 ± 88
F. moniliforme SF035	82 143 ± 4 523	1 393 ± 140
F. moniliforme SF036	5 028 ± 502	731 ± 161
F. moniliforme SF049	21 ± 9	33 ± 7
F. moniliforme SF161	51 ± 11	126 ± 32
Control (sterile water)	15 ± 6	40 ± 7

[a]Fumonisin B_1 accumulation on sorghum grains artificially inoculated with *Fusarium* isolate under laboratory conditions;

[b]Fumonisin B_1 accumulation on sorghum grains obtained from panicles artificially inoculated with *Fusarium* isolate under glasshouse conditions; [c]Standard error

Table 3 Distribution of toxigenic and non-toxigenic isolates of *Fusarium* spp in different locations in India

Location	Number of isolates			Mean fumonisin B_1 (µg/kg)
	Total tested	Toxigenic	Non-toxigenic	
Akola (MS)	9	8	1[a]	40 (0)[b]
Dharwad (KA)	7	2	5	10 (0)
Indore (MP)	3	2	1	13 (0)
Surat (GU)	12	11	1	38 (0)
Guntur (AP)	7	6	1	16 (0)
Hyderabad (AP)	13	5	8	6 098 (2)
Mahaboobnagar (AP)	5	3	2	16 432 (1)
Ranga Reddy (AP)	9	7	2	575 (1)

MS, Maharashtra; KA, Karnataka; MP, Madhya Pradesh; GU, Gujarat; AP, Andhra Pradesh

[a]Number of isolates that did not produce any detectable amount of fumonisin B_1 (non-toxigenic)

[b]Number of isolates that produced fumonisin B_1 above safety limit (200 µg/kg) (highly toxigenic)

physiologically inactive grain) and longer duration (30-days) under laboratory (*in vitro*) than under glasshouse (*in vivo*). However, there was a strong correlation between fumonisin B_1 production by the toxigenic isolates under the above two conditions (r= 0.79, $P< 0.05$).

Distribution of fumonisin B_1 positive isolates varied over location. Out of every 10 isolates from Akola (Maharashtra), Hyderabad (Andhra Pradesh), Ranga Reddy (Andhra Pradesh) and Surat (Gujarat) at least 5 were fumonisin positive (Table 3). Whereas 5 isolates out of 7 in Dharwad were non-toxigenic. Fumonisin B_1 accumulation by most of the positive isolates was below safety limits (200 µg/kg) and 4 were highly toxigenic (fumonisin B_1 >200 µg/kg). Interestingly all the highly toxigenic isolates (SF018, SF019, SF035 and SF036) were obtained from grain samples collected from various markets in Andhra Pradesh (2 from Hyderabad and 1 each from Mahaboobnagar and Ranga Reddy). Presence of highly toxigenic *Fusarium* isolates in sorghum sample poses threat to food and feed safety for human, animal and poultry and indicates the need for checking the samples from poultry and animal feed industries.

Correlations

Fusarium isolates were characterized for range of cultural and morphological characters including growth, pigmentation, sporulation and spore characteristics (data not sown). Correlation studies of colony characters of 65 isolates with fumonisin B_1 production ability revealed that colony texture and pigmentation on potato dextrose agar had strong positive relations with fumonisin B_1 productions ($P<0.01$) (Table 4). Isolates with powdery to cottony growth and pink to yellow pigmentation generally had toxigenic properties. While isolates with dense (compact) growth and no pigmentation were non-toxigenic (non-producer of fumonisin B_1). Other morphological characters such as aerial growth pattern and growth rate, however, had little relation with fumonisin production. Though mycotoxins production is highly related to substrate and growth conditions, these morphological characters might give possible indications for presence of a toxigenic isolate that would be useful for preliminary screening of large number of isolates.

Table 4 Correlations between fumonisin B_1 (FB_1) production and morphological characters of *Fusarium* spp isolated from sorghum grain

	FB_1 production	Pigmentation	Aerial growth	Texture
Pigmentation	0.388**			
Aerial growth	0.182	–0.213		
Texture	0.393**	–0.012	0.451**	
Growth rate	–0.052	–0.122	0.058	0.099

Qualitative morphological characters were quantified on a 1–3 scale as: Aerial growth: 1= compact with no aerial growth, 2= loose and thin with minimum aerial mycelium, 3=loose with maximum aerial mycelium; Texture: 1= dense, 2= powdery, 3= cottony; Pigmentation: 1=no pigmentation, 2= light pink to pink, 3= yellow to orange. **P=0.01

Fumonisin B_1 contamination in elite sorghum cultivars

Fumonisin B_1 contamination in freshly collected field samples of elite varieties and hybrids during *kharif* 2007 is presented in Table 5. Most of the samples were positive for the presence of fumonisin and in around 6% of the samples contamination was above safety limit (200 µg/kg). Contamination varied over locations and genotypes. Among 4 locations Coimbatore recorded the maximum fumonisin contamination (Loc. mean 493 µg/kg). Out of 11 genotypes, 9 registered high level of fumonisin contamination in Coimbatore (>200 µg/kg). Remaining one variety ('SPV 1774') and one hybrid ('CSH 16') recorded fumonisin within the safe limit. In other locations, viz Dharwad (44 µg/kg), Udaipur (24 µg/kg) and Akola (20 µg/kg) contamination was minimum and no genotype recorded fumonisin level above the safety limit. Incidence of grain mold (or *Fusarium* mold) varied over locations depending on rainfall and relative humidity. Coimbatore had more weekly mean rainfall (32.2 mm) and relative humidity (88.6%) as compared to Udaipur (rainfall, 18.7 mm; relative humidity, 77.8%), and Dharwad (rainfall, 16.0 mm; relative humidity, 86%) during flowering to maturity (August to October). This is one of the reasons for high fumonisin contamination in Coimbatore. Low to high level of fumonisin contamination (range: 0–441 µg/kg) was reported in samples from farmer's field in Andhra Pradesh and Maharashtra (Waliyar *et al.* 2008). Mycotoxin (fumonisin) contamination is mostly attributed to the post-harvest handling of grain and storage conditions. In the

Table 5 Fumonisin B_1 contamination (µg/kg seed) in elite grain sorghum cultivars at different locations in India during rainy season 2007

Cultivar	Fumonisin B_1 (µg/kg seed)					
	Akola (MS)	Coimbatore (TN)	Dharwad (KA)	Udaipur (RJ)	Cultivar range	Cultivar mean
Variety						
'CSV 15'	8	310	13	12	8–310	86
'CSV 17'	40	260	36	42	40–260	95
'SPV 462'	5	294	19	12	5–294	82
'SPV 1600'	39	491	15	20	15–491	141
'SPV 1616'	23	365	19	26	19–365	108
'SPV 1742'	20	1398	28	9	9–1398	364
'SPV 1746'	29	357	98	59	29–357	136
'SPV 1774'	6	36	42	17	6–36	25
'SPV 1786'	21	na	30	9	9–30	20
Hybrid						
'CSH 16'	21	191	44	21	21–191	69
'CSH 18'	26	365	13	13	13–365	104
'CSH 23'	10	739	51	38	10–739	210
Mean	20	493	40	24		
CD (*P*= 0.05)	6	194	13	8		
CV (%)	60[a]	90[a]	74[a]	64[a]		

MS, Maharashtra; TN, Tamil Nadu; KA, Karnataka; RJ, Rajasthan; na, data not available
[a]Probable reasons for high CV are discussed in the Results and Discussion part

present study, in spite of using fresh grains, contamination level varied among locations (especially in Coimbatore it was high) within genotype. This indicated that crop-growing environments play important role for fumonisin B_1 contamination. A few possible reasons for such differential response of a genotype over locations might be random occurrence of toxigenic *Fusarium* isolates, their spore density and prevailing weather conditions across location. The frequency of the occurrence of *Fusarium* spp contributing to fumonisin B_1 production depended on the season (year) and the grain species. There was wide variability in fumonisin B_1 concentration among replicates within a genotype (in all locations CV> 60%) (Table 5). This reflected random nature of contamination by an air-borne pathogen under natural field conditions. Apart from *Fusarium* strain variability, variable spore density that actually land on panicle and ability to establish infection on spikelet may vary under microclimate in field. Screening genotypes with assured inoculums of toxigenic *Fusarium* strains under controlled conditions will be useful. The observed genotypic variations in fumonisin B_1 production within a location indicated existence of differential *Fusarium* resistance (*Fusarium* mold) among sorghum genotypes. Such genotypic variations were also observed in maize where *Fusarium* infection (*Fusarium* ear rot) and fumonisin B_1 production was strongly correlated (Robertson *et al*. 2007).

It is concluded that there is variability among Indian isolates of *Fusarium* in terms of fumonisin B_1 production and a few isolates are highly toxigenic for fumonisin B_1 and sorghum grains produced during rainy season often get contaminated with fumonisin B_1. Level of contamination depends on sorghum genotypes, location and environment. Only 2 genotypes ('SPV 1774'and 'SPV 1786') had toxins below safety limits. Therefore, breeding and selection for low toxins might be useful. As *Fusarium* infection in sorghum grain mostly takes place in between anthesis and grain development stages, reduction of pre-harvest *Fusarium* load from grain is important for management of mycotoxins. Management techniques for grain mold *per se* (mold complex) for improvement of grain and seed quality in rainy season sorghum are well documented (Audilakshmi *et al*. 2005, Kannababu *et al*. 2009). It is necessary to incorporate resistance against *Fusarium* in new varieties and hybrids for improving the quality of sorghum grain to increase acceptability and to enhance its use as feed and value-added products.

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