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PRODUCTION OF AFLATOXINS BY *ASPERGILLUS FLAVUS* AND OF FUMONISINS BY *FUSARIUM* SPECIES ISOLATED FROM BRAZILIAN SORGHUM

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ABSTRACT

Fifty-nine *Aspergillus flavus* and 35 *Fusarium verticillioides* strains, isolated from freshly harvested (10) and stored (130) Brazilian sorghum samples, were tested regarding their ability to produce aflatoxins (coconut milk agar) and fumonisins (rice culture), respectively. Aflatoxins B_1 and B_2 were detected by TLC, and fumonisins B_1 and B_2 were analyzed by HPLC. Thirty-eight (64.4%) *A. flavus* strains produced detectable levels of aflatoxins at concentrations ranging from 12.00 to 3282.50 µg/kg ($AFB_1 + AFB_2$), while thirty two (91%) *F. verticillioides* strains produced FB_1 at concentrations ranging from 0.12 to 5.38 µg/g. Two *F. proliferatum* strains produced low fumonisin levels. The toxigenic potential of *A. flavus* (64.4%) and *F. verticillioides* (91.5%) strains observed in sorghum samples indicates that rigorous control should be directed at the storage conditions of these products to minimize contamination with toxigenic deteriorating fungi, preventing further hazard to human and animal health.

Key words: toxigenicity, *Aspergillus flavus*, *Fusarium verticillioides*, mycotoxins, sorghum, aflatoxin, fumonisin

INTRODUCTION

Sorghum (*Sorghum bicolor* L., Moench) is a worldwide grass originating from the African and Asian continents, which has spread to other temperate and tropical regions. Sorghum has been ranked as the seventh most cultivated grain in the world and the fourth in Africa (32).

Sorghum grains are used as raw material for poultry, swine and bovine feeds, but are also destined for human use (37), constituting the staple food in India, China, and some African and Asian countries.

The presence of deteriorative fungi with ability to produce mycotoxin in grains and food represents a great hazard for human and animal health, and it has been reported for sorghum in many countries with a high frequency of *Aspergillus* and *Fusarium* genera (1,7,11,10,25).

Aflatoxins are bifuranocumarin mycotoxins produced by *A. flavus* and *A. parasiticus*, with aflatoxin B_1 (AFB_1) being the most hepatotoxic, showing mutagenic and carcinogenic and, probably, teratogenic properties in animals (34,35). According to the International Agency for Research on Cancer, AFB_1 is classified as a human carcinogen class 1.

Fumonisins are mycotoxins produced mainly by *F. verticillioides* Sacc Nirenberg (= *F. moniliforme* Sheldon), and *F. proliferatum* in several agricultural products worldwide, especially maize and sorghum (1,5,18). The toxic effects of fumonisins depend on the animal specie and the toxigenicity of *Fusarium* strains (26). This toxin causes leukoencephalomalacia in equines (18) and rabbits, pulmonary edema in swine (3,12), and it has been reported as a probable cause of esophageal cancer in humans (19,36).

Taking into account the lack of mycotoxigenicity studies of *Aspergillus* and *Fusarium* strains isolated from Brazilian

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sorghum, the objective of the present study was to determine the toxigenic potential of *A. flavus*, *F. verticillioides* and *F. proliferatum* strains isolated from both freshly harvested and stored sorghum in São Paulo State, Brazil.

MATERIALS AND METHODS

Aspergillus and *Fusarium* strains

Fifty-nine *A. flavus*, 35 *F. verticillioides*, and 3 *F. proliferatum* isolates, obtained from freshly harvested (10) and stored (130) sorghum grains cultivated in Nova Odessa, São Paulo State, were evaluated. Samples (10 g) were collected monthly, and the grains were ground and homogenized in 90 mL water. Decimal dilutions of up to 10^{-6} were accomplished and 1-mL aliquots of the dilutions were inoculated onto potato dextrose agar. After incubation (5 days at 25ºC), the colonies were counted, isolated, and identified. *Aspergillus* and *Fusarium* strains were identified according to Rapel and Fennell (27) and Nelson *et al*. (21,23) methods, and stored on Sabouraud dextrose agar (SDA) slants at 4-8ºC.

Production and determination of aflatoxins

Culture Preparation: A small fragment of *A. flavus* colony activated in SDA at 25ºC was inoculated onto the centre of a coconut milk agar plate (17), and incubated at 25ºC for 10 days.

Extraction and Analytical Method: Aflatoxins were extracted with methanol / 4% KCl (9:1), followed by clarification with ammonium sulphate and partition with chloroform. AFB_1 and AFB_2 were detected by thin layer chromatography as described by Soares and Rodriguez-Amaya (33), followed by confirmation using trifluoroacetic acid (31). The detection limit was 2 µg/kg for both AFB_1 and AFB_2.

Production and determination of fumonisins

Culture preparation: fumonisin production by 35 *F. verticillioides* and 3 *F. proliferatum* strains was carried out in 50 g polished rice grains humidified with 50 mL distilled water (121ºC for 15 min). The rice medium was inoculated with an aqueous suspension of conidia (2 mL), containing 10^7 spores obtained from potato dextrose agar, and incubated in the dark at 25ºC for 3 weeks. Then, rice cultures were dried, ground finely with a laboratory mill and stored at 4ºC until fumonisin analysis.

Extraction and analytical method: fumonisins were extracted and determined according to Ross *et al*. (29) with some modification. Ten grams of rice culture were added to 50 mL acetonitrile/water (1:1) and stirred for 30 min, and the extract was filtered through Whatman No. 1. Following, 2 mL of the filtrate were added to 5 mL water, and the mixture was applied onto a Sep-Pak C_{18} cartridge (Waters, Division Millipore Corp., Milford, MA), preconditioned with 2 mL methanol and washed with 2 mL Milli Q water (Millipore, Belford, MA, USA). The cartridge was washed with 2 mL acetonitrile/water (20:80), and the toxin was eluted with 2 mL of the same solvent, but at a ratio of 70:30. The final extract was collected in Eppendorf tubes and stored at -20ºC until use.

Two hundred microliters of the final extract were derivated with 50 µL *o*-phthaldialdehyde (OPA) solution prepared by dissolving 40 mg OPA in 1 mL methanol and diluted in 5 mL 0.1 M sodium tetraborate, with 50 µL mercaptoethanol. The derivated product was analysed by reverse-phase isocratic HPLC system (Shimadzu SCL-6B pump, RF55 fluorescent detector with excitation and wavelength emission of 355 and 400 nm, respectively), using a 150 x 4.6 mm C_{18} column (50 ODS-20, O-Phenomenex-ultracarb). The mobile phase consisted of methanol/sodium borate acetate buffer (77:23), pH 3.6.

Calibration was carried out with fumonisin standard solutions (Sigma) prepared with 0.0125, 0.025, and 0.05 µg FB_1, and 0.005, 0.01, and 0.02 µg FB_2 per mL. In the recovery experiment, four samples of polished rice grains (10 g each contaminated with 12.5 to 75.0 µg/g FB_1, and 25.0 to 175.0 µg/g FB_2) were analysed. The coefficients of variation were 4.8 (FB_1) and 7.5 (FB_2), and the recovery rate was 88% for FB_1 and 94% for FB_2. The detection limit was approximately 50 ng/g for both FB_1 and FB_2.

RESULTS AND DISCUSSION

Aflatoxin analysis showed that 38 (64.4%) of 59 tested *A. flavus* strains produced detectable levels of aflatoxins at concentrations ranging from 12.00 to 3282.50 µg/kg (AFB_1 + AFB_2). Fifteen strains produced only AFB_1, while 23 produced both AFB_1 and AFB_2 (Table 1). Aflatoxin group B (AFB_1 and AFB_2), producing *A. flavus* strains, has also been described by Pier (24) and Pitt (25), who identified 10% AFB_1 producer strains and 90% strains producing both AFB_1 and AFB_2. In addition, other researchers (13,15) have also been reported higher AFB_1 levels compared to AFB_2. Our results agree with those ones reported by Kichou *et al*. (14), who demonstrated that 23% of *A. flavus* strains isolated from sorghum in Morocco produced AFB_1 and AFB_2. In India, Sashidhar *et al*. (30), analysing 150 sorghum grain samples, found high rates of contamination by *A. flavus* (67%) and *Fusarium* (59%); however, only two strains produced AFB_1 at concentrations of 16 and 40 µg/kg. Production of AFB_1 and AFB_2 in sorghum and wheat inoculated with *A. flavus* was also reported (39).

Fumonisin analysis showed that 32 (91.5%) of 35 tested *F. verticillioides* strains produced detectable levels of fumonisins at concentrations ranging from 0.12 to 5.38 µg/g (FB_1 + FB_2). Twenty-three strains produced only FB_1 and 9 produced FB_1 + FB_2 (Table 2). The mean recovery rate for fumonisins was approximately 85%. Fumonisin production by almost every *F. verticillioides* strains (28,38) has been observed in 100% of *F. verticillioides* strains isolated from corn.

Table 1. Aflatoxin production by *Aspergillus flavus* strains isolated from freshly harvested and stored sorghum kernels in Brazil.

A. flavus	Aflatoxin concentration (µg/kg)		
Strain	B_1	B_2	Total
FH-06[a]	63.70	33.00	96.70
S7-393[b]	ND	ND	ND
S7-397	ND	ND	ND
S7-400	ND	ND	ND
S7-402	788.40	23.30	811.70
S7-404	467.00	173.00	640.00
S7-405	29.00	ND	29.00
S7-408	27.00	ND	27.00
S7-409	ND	ND	ND
S7-413	320.00	9.50	329.50
S7-415	559.00	207.00	766.00
S7-417	12.00	ND	12.00
S8-420	89.00	ND	89.00
S8-421	1139.00	84.50	1223.50
S8-423	22.00	ND	22.00
S8-425	1422.00	528.00	1950.00
S9-426	94.00	ND	94.00
S8-427	25.00	ND	25.00
S8-429	750.00	16.00	766.00
S8-436	723.50	5.40	728.90
S9-440	ND	ND	ND
S9-441	ND	ND	ND
S9-446	3258.00	24.50	3282.50
S9-449	769.00	57.00	826.00
S9-452	ND	ND	ND
S9-456	ND	ND	ND
S10-462	439.00	6.50	445.50
S10-463	591.00	ND	591.00
S10-464	52.00	ND	52.00
S10-465	568.00	ND	568.00
S10-468	320.00	9.50	329.50
S10-469	ND	ND	ND
S10-470	198.00	ND	198.00
S10-471	ND	ND	ND
S10-473	527.00	86.00	613.00
S10-477	72.00	ND	72.00
S10-479	ND	ND	ND
S10-480	ND	ND	ND
S10-483	ND	ND	ND
S11-484	ND	ND	ND
S11-485	878.50	326.00	1204.50
S11-494	ND	ND	ND
S11-505	ND	ND	ND
S12-511	559.00	ND	559.00
S12-515	615.00	9.00	624.00
S12-517	27.00	ND	27.00
S12-518	45.50	ND	45.50
S12-519	ND	ND	ND
S12-520	56.50	21.00	77.50
S12-521	35.50	7.50	43.00
S12-527	42.00	ND	42.00
S12-529	ND	ND	ND
S12-533	ND	ND	ND
S13-536	574.00	42.50	616.50
S13-539	439.00	163.00	602.00
S13-541	ND	ND	ND
S13-547	94.50	35.00	129.50
S13-552	189.00	42.00	231.00
S13-556	ND	ND	ND

[a] Freshly harvested; [b] stored samples. ND = not detected.

Fumonisin-producing *F. verticillioides* strains have also been analyzed by other investigators (6,16), who detected high fumonisin producer strains in corn, but low producers in sorghum. According to Nelson e*t al*. (22), the low production of fumonisins by *F. verticillioides* strains from sorghum grains may be related to the substrate and/or to the geographical area.

The higher production of FB_1, when compared to FB_2, has also been reported (4,8,9), with FB_1 accounting for 70% of all fumonisins both in culture and in naturally contaminated corn. FB_2 and FB_3 concentrations detected in foods or produced in culture by *F. verticillioides* strains are approximately 15 to 25% of the produced FB_1. However, Apsimon (2) isolated *F. verticillioides* strains producing more FB_2 than FB_1.

Moretti *et al*. (20) concluded all strains isolated from sorghum belonged to the "F" mating population characterized by little or no FB_1 and FB_2 production. In contrast, majority of strains isolated from maize belonged to the "A" mating population, which produces moderate to high levels of FB_1 and FB_2.

Two strains of the 3 *F. proliferatum* isolates produced FB_1 + FB_2 at concentrations of 0.12 and 0.18 µg/g (Table 2). Fumonisin production by other *Fusarium* species, mainly *F. proliferatum*, has been reported (23,38); however, *F. verticillioides* continues to be the main producer of this toxin.

In the present study, a small number of *A. flavus* strains was isolated from freshly harvested sorghum samples (1 strain), although a larger number of toxigenic strains were isolated from stored sorghum (S7-S13). This result might be explained by the fact that *Aspergillus* is classified in the literature as a storage fungus, which has already been detected in the field. Concerning *F. verticillioides*, which is typically considered to be a field fungus, a larger number of strains was detected in freshly harvested samples. Nevertheless, this fungus was isolated until the seventh month of storage.

Table 2. Fumonisin production by *Fusarium verticillioides* and *F. proliferatum* strains isolated from freshly harvested and stored sorghum kernels in Brazil

Fusarium	Fumonisin concentration (µg/g)		
Strain	FB_1	FB_2	Total
FH-17[a]	0.15	ND	0.15
FH-20	0.76	0.09	0.85
FH-29	0.16	ND	0.16
FH-31	0.83	ND	0.83
FH-32	0.67	0.39	1.06
FH-34	0.82	0.13	0.95
FH-35	0.52	0.15	0.67
FH-36	0.24	ND	0.24
FH-39	4.38	1.00	5.38
FH-41	0.37	ND	0.37
FH-42	1.79	0.36	2.15
FH-47	0.29	ND	0.29
FH-49	0.16	ND	0.16
FH-54	0.15	ND	0.15
FH-55	0.12	ND	0.12
FH-57	0.13	ND	0.13
FH-61*	0.12	ND	0.12
FH-74	0.12	0.06	0.18
FH-75	0.40	0.08	0.48
S1-80[b]	0.12	ND	0.12
S1-81*	0.16	0.02	0.18
S1-83*	ND	ND	ND
S1-84	0.65	ND	0.65
S1-85	0.19	ND	0.19
S1-86	0.14	ND	0.14
S1-87	0.15	ND	0.15
S1-89	0.15	ND	0.15
S2-143	0.06	ND	0.06
S2-145	0.19	ND	0.19
S2-169	0.67	ND	0.67
S4-259	0.51	0.11	0.62
S4-260	0.23	ND	0.23
S4-293	ND	ND	ND
S4-295	0.23	ND	0.23
S4-296	ND	ND	ND
S5-319	ND	ND	ND
S7-407	1.18	ND	1.18
S7-416	0.35	ND	0.35

[a] Freshly harvested; [b] stored samples. ND = not detected.
* *Fusarium proliferatum*.

The occurrence of toxin production by strains isolated from foods and animal feed does not necessarily imply the presence of mycotoxins. However, it indicates a potential risk for a possible contamination with mycotoxins. Furthermore, if these foods represent a good substratum for mycotoxin production and if the abiotic factors (especially moisture and temperature) are appropriate, the contaminant hazard tends to increase.

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RESUMO

Avaliação da toxigenidade das cepas de *Aspergillus flavus* e *Fusarium* spp. isoladas de amostras de sorgo

A produção de aflatoxinas por 59 cepas de *Aspergillus flavus* e fumonisinas por 35 cepas de *Fusarium verticillioides* isoladas de amostras de grãos de sorgo recém colhido (10 amostras) e armazenado (130 amostras), foram avaliadas. A detecção de aflatoxinas (AFB_1 e AFB_2) foi efetuada por Cromatografia em Camada Delgada (CCD) e fumonisinas (FB_1 e FB_2) foram analisadas por Cromatografia Líquida de Alta Eficiência (CLAE). Os resultados demonstram a produção de AFB_1 e AFB_2 em 38 cepas (64,4%) de *A. flavus* cujos níveis variaram de 12,00 a 3282,50 µg/kg. Referente às cepas de *F. verticillioides*, 32 (91%) produziram FB_1, nas concentrações de 0,12 a 5,38 µg/g. Baixos níveis de fumonisinas foram detectados em 2 cepas de *F. proliferatum*. A constatação da potencialidade toxígena das cepas de *A. flavus* (64,4%) e de *F. verticillioides* (91,5%) nesta investigação, revelam a importância da pesquisa de aflatoxinas e fumonisinas nas amostras de sorgo. Diante disto, sugere-se o controle rigoroso das condições de armazenamento de sorgo, visando minimizar a contaminação por fungos deteriorantes toxígenos, evitando riscos à saúde humana e animal.

Palavras-chave: toxigenicidade, *Aspergillus flavus*, *Fusarium verticillioides*, micotoxinas, sorgo, aflatoxina, fumonisina

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