Mycoflora and Occurrence of Aflatoxin B_1 and Fumonisin B_1 during Storage of Brazilian Sorghum

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The present study is a 1-year follow up of the mycoflora of 140 samples of Brazilian freshly harvested (10) and stored (130) sorghum, the levels of aflatoxin and fumonisin contamination detected in the grains, and the prevailing abiotic factors (grain moisture content, water activity, temperature, relative humidity, and mean rainfall) at the time of sampling. The results show a predominance of the genera *Phoma* (57.1%), *Aspergillus* (42.7%), *Fusarium* (25.0%), and *Rhizopus* (21.4%) and the presence of nine other filamentous fungi. *Fusarium*, *Aspergillus*, and *Penicillium*, the three most important genera in terms of toxicity, presented numbers of colony forming units per gram of sorghum (CFU/g) that varied from 1×10^3 to 36×10^3, from 1×10^3 to 295×10^3, and from 1×10^3 to 20×10^3 CFU/g, respectively. The species most frequently found were *Aspergillus flavus* and *Fusarium moniliforme*. Of the total samples analyzed, 12.8% were contaminated with aflatoxin B_1 (concentration mean = 7−33 μg/kg) and 74.2% with fumonisin B_1 (concentration mean = 0.11−0.15 μg/g). This paper is the first report of the natural occurrence of aflatoxins and fumonisins in sorghum grain from Brazil.

Keywords: *Fumonisin B_1; aflatoxin B_1; mycoflora; occurrence; sorghum*

INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is an old world grass originating from the African and Asian continents and has now a worldwide distribution that spans temperate and tropical regions. It has been ranked as the seventh most cultivated cereal in the world, coming fourth after rice, maize, and wheat in Africa (Simpson and Ogarzaly, 1995).

In 1997, there was a 23% increase in the world's production of sorghum with respect to that of the previous year; the United States and India were the main producers, contributing 30 and 15% of the total yield, respectively. In Brazil, the 1997 annual sorghum crop totaled 385.2 thousand tons harvested from 487.28 acres of cultivated land (Olivetti and Camargo, 1997).

Sorghum grains are used as raw material for poultry, swine, and bovine feeds but are also destined for human consumption (Veiga, 1986); in fact, they constitute the staple food of millions of people in India, China, and some African and Asian countries. Sorghum can also be employed in the manufacture of a variety of products (Simpson and Ogarzaly, 1995).

The overall world loss of foodstuff in the form of grains is considered to be 5% of the total production (FAO/WHO/UNEP, 1977). In Brazil, losses of 10−25% (Pedrosa and Dezen, 1991) have been estimated to occur throughout the trading process, partly due to contamination with toxigenic fungi and mycotoxins. Improved storage conditions would allow a 10−20% rise in the supply of foodstuffs to mankind (Christensen and Kaufmann, 1969).

Fungi proliferate in the most different environments, yet tropical climates favor the growth of toxigenic species on agricultural products, thus increasing the risk of contamination of many cereal grains and their byproducts with secreted mycotoxins (Hill et al., 1985).

These facts associated with the lack of studies of mycotoxins on sorghum in Brazil motivated us to undertake the present study. Our main objectives were to identify the mycoflora of freshly harvested and stored sorghum from the State of São Paulo, to determine the occurrence of aflatoxins and fumonisins in the cereal grains, and to correlate these results with the data on abiotic factors (temperature, moisture content, water activity, relative humidity, and rainfall).

MATERIALS AND METHODS

Samples. One hundred and forty samples of sorghum (AG-2005-E) (10 from freshly harvested grains and 130 from stored grains) were collected between June 1996 and May 1997 in Nova Odessa, State of São Paulo, Brazil, within an area of ~4 ha. After harvest, the grains were put in 60 kg jute sacks (a total of 10 sacks) commonly used for sorghum storage in this region, stacked over wooden boards, and kept for 12 months in a well-ventilated warehouse, located near the production area. Every 28 days, small portions were collected from various points of each sack (Fonseca, 1991) to make up a total sample of 1 kg of grains/sack (one sample per month per sack), resulting in 10 samples per month.

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Table 1. Relative Frequencies of Fungal Genera Isolated in Brazil from 140 Sorghum Grains, over a 1-Year Period (June 1996−June 1997)

time (days)	relative frequencies (%) of isolated genera												
	Phoma	*Fusarium*	*Aspergillus*	*Penicillium*	*Drechslera*	*Nigrospora*	*Curvularia*	*Helminthosporium*	*Cladosporium*	*Alternaria*	*Rhizopus*	*Mucor*	*Cephalosporium*
FH (0)[a]	5.7	5.7	0.7	0.7	3.6	4.3	3.6	0.7	0.7	ND[c]	ND	0.7	ND
S1 (28)[b]	5.7	5.7	ND	0.7	ND	2.9	2.1	0.7	ND	ND	ND	ND	ND
S2 (56)	7.1	3.6	ND	ND	2.1	1.4	2.9	2.1	1.4	ND	ND	0.7	ND
S3 (84)	5.7	3.6	ND	ND	ND	ND	1.4	ND	ND	ND	ND	ND	ND
S4 (112)	7.1	3.6	1.4	0.7	1.4	ND	ND	2.1	2.9	0.7	ND	ND	0.7
S5 (140)	6.4	0.7	ND	0.7	ND	ND	2.1	2.9	ND	ND	ND	ND	ND
S6 (168)	6.4	0.7	0.7	0.7	ND	ND	2.1	3.6	ND	ND	ND	ND	ND
S7 (196)	5.0	1.4	2.1	ND	ND	ND	ND	0.7	ND	ND	2.1	ND	ND
S8 (224)	4.3	ND	5.7	ND	ND	ND	ND	0.7	ND	ND	1.4	1.4	ND
S9 (252)	1.4	ND	5.7	ND	ND	ND	ND	ND	ND	ND	1.4	1.4	ND
S10 (280)	0.7	ND	6.4	ND	ND	ND	ND	ND	ND	ND	2.9	2.1	ND
S11 (308)	ND	ND	6.4	ND	ND	ND	ND	ND	1.4	ND	4.3	2.8	ND
S12 (336)	0.7	ND	7.1	2.9	ND	ND	ND	ND	ND	ND	3.6	4.3	ND
S13 (364)	0.7	ND	6.4	3.6	ND	ND	ND	ND	ND	ND	5.7	4.3	ND
total	57.1	25.0	42.7	10.0	7.1	8.6	14.3	13.5	6.4	0.7	21.4	17.7	0.7

[a] Freshly harvested grains sample. [b] Sample of stored grains. [c] Not detected.

Moisture Content and Water Activity. Ten grams of sorghum grains were dried to constant weight in a Petri dish at 105 °C. The moisture content of the samples was expressed as percentage values. Water activity was determined by automated analysis with the equipment Aqualab CX-2 (Decagon Devices Inc.).

Identification and Counting of the Mycoflora. A 10 g sample of sorghum grains was collected from each of the 140 samples and then ground and mixed with 90 mL of sterile distilled water to obtain a 10^{-1} stock dilution, from which 10-fold serial dilutions up to 10^{-6} were made using the same diluent. Duplicate 1 mL volumes of each solution were added to Petri dishes containing 10−15 mL of potato dextrose agar (Swanson et al., 1992). The plates were then incubated at 25 °C for 5 days and observed daily. Plates that were found to contain 15−150 colony forming units (CFU) were used for counting, and the results were expressed as CFU per gram of sample (Mislivec et al., 1992). The fungal colonies recovered were counted and identified according to methods recommended for each genus (Raper and Fennell, 1965; Arx, 1974; Nelson et al., 1983; Nelson, 1992; Ellis, 1993; Barnett and Hunter, 1972).

Determination of Aflatoxins. Detection and quantitation of aflatoxins B_1, B_2, G_1, and G_2 were done by thin-layer chromatography (TLC), according to the method described by Soares and Rodriguez-Amaya (1989). The procedure involved extraction with methanol/4% KCl (9:1) followed by clarification of the extract with ammonium sulfate and partitioning to chloroform. Quantitation was done by visual comparison with aflatoxin standards. Confirmation of the identified aflatoxins was carried out by derivatization reactions using trifluoroacetic acid (Scott, 1990). The TLC detection limit was 2 μg/kg.

Determination of Fumonisins. The sorghum samples and cultures were analyzed for fumonisins B_1 (FB_1) and B_2 (FB_2) following the method of Ross et al. (1991), with some modifications. Briefly, 10 g of sorghum grains was added to 50 mL of a 1:1 solution of acetonitrile/water and stirred for 30 min. The extract was then filtered through filter paper (Whatman No. 1). Next, 2 mL of the filtrate was added to 5 mL of water, and the mixture was applied to a Sep-Pak cartridge C_{18} (Waters Division, Millipore Corp., Milford, MA), preconditioned with 2 mL of methanol, and then washed with 2 mL of Milli-Q water (Millipore, Bedford, MA). The cartridge was washed with 2 mL of a 20:80 mixture of acetonitrile/water and the toxin eluted with 2 mL of a 70:30 mixture of acetonitrile/water. The final extract was collected in Eppendorf tubes and kept at −20 °C until use.

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

Calibration was done with standard solutions of fumonisins (Sigma) containing 0.0125, 0.025, and 0.05 μg of FB_1 and 0.005, 0.01, and 0.02 μg of FB_2 per milliliter. In the recovery experiment, four samples of sorghum (10 g each) were analyzed added (12.5−75 μg/g for FB_1 and 25−175 μg/g for FB_2) for each level of toxin. The coefficients of variation were 4.8 for FB_1 and 7.5 for FB_2. The recoveries of FB_1 and FB_2 were 88 and 94%, respectively. The detection limit of the method was 50 ng/g for both FB_1 and FB_2.

Climatic Data. During storage, the climatic conditions relating to temperature (°C), relative humidity (%), and rainfall (mm) were recorded, and monthly averages were determined.

Statistical Analysis. The data were analyzed through three stages: (a) simple and partial correlation analysis; (b) multiple linear regression analysis; and (c) residual analysis using a statistical software (SAS) to determine the influence of independent variables [time of storage, mean annual rainfall, relative humidity, moisture content, water activity, and temperature (minimal, average and maximal)] on the dependent variables (*Aspergillus*, *Fusarium*, and *Penicillium*, aflatoxin B_1, and fumonisin B_1) (Neter et al., 1990; Draper and Smith, 1981).

RESULTS AND DISCUSSION

The presence of fungi was observed in all of the sorghum samples analyzed (10 freshly harvested and 130 stored grains). The genera isolated, listed in decreasing order of frequency, were *Phoma* (57.1%), *Aspergillus* (42.7%), *Fusarium* (25.0%), *Rhizopus* (21.4%), *Mucor* (17.7%), *Curvularia* (14.3%), *Helminthosporium* (13.5%), *Penicillium* (10.0%), *Nigrospora* (8.6%), *Dreschlera* (7.1%), *Cladosporium* (6.4%), *Alternaria* (0.7%), and *Cephalosporium* (0.7%). Most genera occurred at higher frequencies in freshly harvestd sorghum and in grains of the first months of storage, except for *Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp., and *Mucor* spp. (Table 1), which are microorganisms known to cause spoilage of stored products (Samsom, 1995).

The predominance of filamentous fungi such as *Phoma* spp., *Aspergillus* spp., and *Fusarium* spp. found in this study agrees with other published evaluations

Table 2. Number of CFU per Gram for the Genera *Fusarium*, *Aspergillus*, and *Penicillium*; Levels of Aflatoxin and Fumonisin Recovered from Sorghum Grains over a 1-Year Period (June 1996−June 1997); and Prevailing Abiotic Factors (Grain Moisture Content, Water Activity, Mean Rainfall, Relative Humidity, and Temperature) at the Time of Sampling

time (days)	MC[c] (%)	A_w[d]	MR[e]	RH[f] (%)	temp (°C) max	temp (°C) min	temp (°C) mean	CFU/g × 10^3 *Fusarium*	CFU/g × 10^3 *Aspergillus*	CFU/g × 10^3 *Penicillium*	FB1 (μg/g) mean[h] (positive)	AFB_1 (μg/kg) mean[i] (positive)	CFU/g × 10^3 *Aspergillus* mean[j]
FH (0)[a]	16	0.74	0	78	26	12	19	29	1	1	0.13 (9)	ND	
S1 (28)[b]	14	0.65	31	72	25	13	19	36	ND	1	0.12 (9)	ND	
S2 (56)	13	0.64	1	59	25	11	18	4	ND	ND	0.11 (3)	ND	
S3 (84)	13	0.69	83	65	26	14	20	6	ND	ND	0.11 (4)	ND	
S4 (112)	13	0.68	165	70	27	15	21	6	2	1	0.11 (3)	ND	
S5 (140)	13	0.65	101	72	28	18	23	1	ND	1	0.11 (9)	ND	
S6 (168)	12	0.63	166	73	25	22	23.5	1	1	1	0.14 (9)	ND	
S7 (196)	13	0.72	203	77	29	19	24	2	3	ND	0.11 (9)	ND	
S8 (224)	13	0.72	339	82	28	20	24	ND[g]	45	ND	0.11 (9)	ND	
S9 (252)	12	0.66	258	78	30	19	24.5	ND	47	ND	0.15 (8)	33 (2)	160
S10 (280)	12	0.67	66	71	30	19	24.5	ND	140	ND	0.14 (10)	8 (3)	240
S11 (308)	12	0.66	40	70	28	17	22.5	ND	107	ND	0.13 (10)	8 (4)	188
S12 (336)	12	0.64	5	69	27	15	21	ND	267	10	0.13 (8)	7 (4)	570
S13 (364)	14	0.73	157	76	22	12	17	ND	295	20	0.13 (10)	18 (5)	348

[a] Freshly harvested grain sample. [b] Sample of stored grains. [c] Grain moisture content. [d] Water activity. [e] Mean rainfall. [f] Relative humidity. [g] Not detected. [h] Mean value of samples positive for fumonisins. [i] Mean values of samples positive for aflatoxins. [j] Mean number of CFU of *Aspergillus* spp. in samples positive for aflatoxins.

of sorghum mycoflora in Brazil (Lasca et al., 1986), Thailand (Pitt et al., 1994), India (Tripathi, 1974), and Egypt (El-Kady et al., 1982).

Within the genus *Aspergillus*, the following species were identified: *A. flavus* (42.1%), *A. amstelodami* (2.1%), *A. sydowi* (2.1%), *A. versicolor* (1.4%), *A. niger* (0.7%), and *A. terreus* (0.7%). The *Fusarium* species recovered were *F. moniliforme* (25.0%), *F. subglutinans* (7.1%), *F. semitectum* (5.7%), *F. proliferatum* (2.1%), *F. clamydosporum* (2.1%), *F. avenaceum* (2.1%), and *F. acuminatum* (0.7%). The prevalence of *A. flavus* and *F. moniliforme*, which are species commonly found in tropical and subtropical areas (Samsom et al., 1995; Pitt and Hocking, 1997), clearly indicates the importance of research work on contamination of cereal grains with aflatoxins and *Fusarium* toxins (especially the fumonisins) in such localities.

Comparing the degree of contamination (CFU/g) with the genera *Aspergillus*, *Fusarium*, and *Penicillium* (the most important ones in terms of toxicity) with grain moisture, we found that *Aspergillus* spp. were prevalent at moisture content and water activity values below 14% and 0.73, respectively. Concerning the genera *Fusarium* and *Penicillium*, the highest numbers of CFU/g were recorded at levels that ranged from 14 to 16% (moisture content) and from 0.65 to 0.74 (water activity) for the former and from 12 to 14% (moisture content) and from 0.64 to 0.73 (water activity) for the latter (Table 2).

Aspergillus spp. were the main contaminant, presenting CFU/g numbers that varied between 1×10^3 and 295×10^3 and which exceeded the tolerance limits (10^2−10^4 CFU/g) recommended by the International Commission on Microbiological Specification for Foods (Elliott, 1980). Mean temperature, time of storage, and rainfall were the variables that most influenced *Aspergillus* growth ($P < 0.0001$ and $r^2 = 0.95$).

The increase in CFU/g detected for *Aspergillus* spp. between days 196 and 336 of storage may be explained by the increase in temperature recorded during that period because this genus shows optimal growth at temperatures >20 °C (Kozakiewics and Smith, 1994). Grain spoilage may have been an additional factor that contributed to a higher fungal contamination, as it facilitates the penetration of mites and insects or even microorganisms present on the surface of the sorghum grains and hence provides better conditions for microbial growth. This possibility was also discussed by Nelson (1992) on a study on maize grains. The fact that no other genus occurred to the same extent as *Aspergillus* in the samples analyzed suggests that competition between the different microorganisms took place over the substrate.

Rainfall, relative humidity, and storage time showed a significant interaction with *Fusarium* growth ($P < 0.0003$ and $r^2 = 0.87$). The reduced frequency of isolation and low CFU/g with *Fusarium* spp (1×10^3 to 36×10^3) could be explained by the low moisture content of the grains. It is known that *Fusarium* spp., despite being able to grow on desiccated substrates, normally prefer environments with higher water availability (Christensen and Kaufmann, 1974). In addition to the low moisture content of the grains, prolonged storage also contributed to the reduction in CFU of the genus *Fusarium*, as also reported by Pozzi et al. (1995) in a study on maize hybrids.

Contamination of sorghum grains with the genus *Penicillium* was extremely low and uniform throughout the experimental period, being of the order of 1×10^3 CFU/g soon after harvest and 20×10^3 at the end of storage (day 364). Multiple linear regression analysis of the data showed a significant correlation between *Penicillium* growth and time of storage, maximum temperature, and water content of the grains ($P < 0.0001$ and $r^2 = 0.88$).

A multiple comparison of time of storage, climatic factors, and CFU numbers showed that *Fusarium* species were the most frequent isolates on recently harvested grains and stored grains up to day 196. The greatest levels of contamination were recorded for recently harvested and 28-day-stored samples (29×10^3 to 36×10^3 CFU/g); during this period, the mean temperature and relative humidity levels varied from 19 °C and 72− 78%, respectively, and mean annual rainfall reached to 31 mm. On the other hand, the highest numbers of CFU/g for the genus *Aspergillus* were recorded after 8 months of storage (224 days), when the mean temperature and relative humidity levels ranged between 17 and 24 °C and 69 and 82%, respectively, and the mean annual rainfall value reached to 339 mm. The genus *Penicillium* presented the highest

numbers of CFU/g after 336 days of storage, when the characteristic environmental conditions were 17−21 °C mean temperature, 69−76% relative humidity, and 157 mm mean monthly rainfall (Table 2).

The screening for mycotoxins indicated that fumonisin B_1 had the highest incidence; it appeared in 104 (74.2%) of the samples, followed by aflatoxin B_1, which was detected in 18 (12.8%) of the samples. The mean concentrations varied from 0.11 to 0.15 μg/g for fumonisin B_1 and from 7 to 33 μg/kg for aflatoxin B_1 (Table 2). Aflatoxins B_2, G_1, and G_2 and fumonisin B_2 were not detected. These values are somewhat lower than the ones reported by Shetty and Bhat (1997) on mycotoxin contamination of Indian sorghum.

The maximum permissible level for aflatoxins in foods in Brazil is 20 μg/kg (Ministério da Agricultura, 1996), and in this survey only four (4.5%) samples exceeded that value.

Although in our study aflatoxin B_1 concentrations in the sorghum samples were usually low, the *Aspergillus* isolates showed a high incidence, especially *A. flavus*, with values of 160−570 CFU/g after 9 months (252 days) of storage (Table 2).

The low levels of aflatoxin B_1 and fumonisin B_1 observed can be attributed to moisture contents below 14.5% in most of the grains, levels recommended by the Brazilian Ministry of Agriculture for the trading of cereal grains (Bastos, 1987). Despite the large number of CFU of *Aspergillus* isolated, high levels of secreted mycotoxins were absent. This could be due to the influence of various abiotic factors, especially low water activity of the grains: it is known that the minimal water activity necessary for fungal growth is below that required for the production of aflatoxins. In this respect, Lacey et al. (1991) mentioned 0.78 and 0.83 as the minimum water activity values that favor proliferation of *A. flavus* and production of aflatoxins, respectively; the minimum value needed for fumonisin secretion is 0.87. In our study, the values recorded were closer to required fungal growth of the genera analyzed.

Because we detected an association between two independent variables (relative humidity and mean rainfall) and aflatoxin contamination, the data were submitted to polynomial linear regression analysis. The results indicated a statistically significant correlation ($P < 0.009$ and $r^2 = 0.99$) between relative humidity and the presence of aflatoxins (Table 2). The simple linear regression analysis also pointed to a statistically significant influence of mean rainfall on aflatoxin contamination ($P < 0.001$ and $r^2 = 0.98$).

The statistical analysis of our results indicated that mean rainfall, relative humidity, and time of storage had a significant influence on contamination with fumonisins. Mean rainfall correlated negatively with funomisin production, as also reported by Pozzi et al (1995) in his study on maize grains.

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