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Occurrence of *Aspergillus* section *Flavi* and aflatoxins in Brazilian rice: From field to market

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A B S T R A C T

The guarantee of the high quality of rice is of utmost importance because any toxic contaminant may affect consumer health, especially in countries such as Brazil where rice is part of the daily diet. A total of 187 rice samples, from field, processing and market from two different production systems, wetland from the state of Rio Grande do Sul, dryland, from the state of Maranhão and market samples from the state of São Paulo, were analyzed for fungi belonging to *Aspergillus* section *Flavi* and the presence of aflatoxins. Twenty-three soil samples from wetland and dryland were also analyzed. A total of 383 *Aspergillus* section *Flavi* strains were isolated from rice and soil samples. Using a polyphasic approach, with phenotypic (morphology and extrolite profiles) and molecular data (beta-tubulin gene sequences), five species were identified: *A. flavus*, *A. caelatus*, *A. novoparasiticus*, *A. arachidicola* and *A. pseudocaelatus*. This is the first report of these last three species from rice and rice plantation soil. Only seven (17%) of the *A. flavus* isolates produced type B aflatoxins, but 95% produced kojic acid and 69% cyclopiazonic acid. Less than 14% of the rice samples were contaminated with aflatoxins, but two of the market samples were well above the maximum tolerable limit (5 μg/kg), established by the Brazilian National Health Surveillance Agency.

1. Introduction

Rice is a major component of the diet in Brazil and many other parts of the world. Brazil is the world's ninth largest producer and the largest outside Asia (FAO. (Food and Agriculture Organization of the United Nations), 2016), so rice quality and safety are of great importance. Two production systems exist in Brazil, the wetland system, prevalent in the South region, and the dryland system, prevalent in the Northeast region. In the wetland system, the rice is irrigated by controlled flooding. Two processes are in use in the dryland system: one that depends totally on rainfall and the other where supplementary irrigation decreases drought stress and provides higher grain quality.

The occurrence of species from *Aspergillus* section *Flavi* in the rice production chain is a concern due to the production of aflatoxins mainly by *Aspergillus flavus*, but perhaps by less common species also. Aflatoxin B_1 is classified by the International Agency of Research on Cancer (IARC (International Agency for Research on Cancer), 1993) as a Group 1 carcinogen. Indeed aflatoxin B_1 is the most toxic known liver carcinogen.

Rice is an excellent substrate for fungal growth and mycotoxin production (Shotwell et al., 1966). *Aspergillus* section *Flavi* species and aflatoxins in rice have been studied in several countries including Brazil (Almeida et al., 2012; Beber-Rodrigues and Scussel, 2013; Carvalho et al., 2010; Dors et al., 2011; Silva et al., 2008), South Korea (Ok et al., 2014; Park et al., 2005), India (Jayaraman and Kalyanasundaram, 2009; Reddy et al., 2009; Toteja et al., 2006), Uganda (Taligoola et al., 2011), Philippines (Sales and Yoshizawa, 2005), Nigeria (Makun et al., 2011) and Turkey (Aydin et al., 2011). Some studies have used inadequate fungal isolation methods, such as dilution plating or direct plating without surface disinfection, resulting only in data on the surface contamination of rice grains (Aydin et al., 2011; Fredlund et al., 2009). Many identifications of these species from rice have been based on identification by morphology and aflatoxin production (Beber-Rodrigues and Scussel, 2013; Carvalho et al., 2010; Park et al., 2005; Reddy et al., 2009), and have reported that *A. flavus* and *A. parasiticus* are the main species found in rice. A review of the occurrence of aflatoxigenic fungi and aflatoxins in rice from different parts of the world

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has been published elsewhere (Katsurayama and Taniwaki, 2017).

The aim of this study was to analyze the presence of aflatoxin producing fungi using modern methodology for isolation and polyphasic approaches for identification; to investigate the presence of aflatoxins in field, drying, storage, processing and market rice samples from the two different production systems mentioned previously, together with some market samples from São Paulo. Soil samples from wetland and dryland were also analyzed for the presence of aflatoxin producing fungi.

2. Materials and methods

2.1. Samples

A total of 187 rice samples each of approximately 1 kg were collected from wetland farms in Rio Grande do Sul (latitude 30° 51′ 04″ S, longitude 51° 48′ 44″ W) and dryland farms in Maranhão (latitude 05° 31′ 35″ S, longitude 47° 29′ 30″ W), and during processing stages with paddy rice, husk, husked rice, bran, broken, brown, polished, parboiled and red rice. Included were samples from markets in these two regions along with São Paulo, because this state does not grow rice but consumption is high. Samples from São Paulo were of polished, brown, red, and parboiled rice, plus flake and, flour. Only two samples from the drying yard were collected in Maranhão because most of the rice is dried in mechanical dryers in processing plants. Table 1 shows the number of rice samples collected at each stage. Soil samples (approximately 1 kg) were also collected from the rice plantation surface, 12 samples from Rio Grande do Sul and 11 from Maranhão. Rice and soil samples were enclosed in plastic bags during transport from the collecting place to the laboratory, were all samples were refrigerated until analyzed.

2.2. Water activity of rice samples

The water activity (a_W) of all rice samples was determined in triplicate using an Aqualab Series 3TE instrument (Decagon, Pullman, WA, USA) at 25 °C ± 1.

2.3. Fungal isolation

From each sample, approximately 100 g was taken randomly and surface disinfected with sodium hypochlorite solution (0.4%) for 1 min. Fifty grains were then distributed evenly in five Petri dishes containing Dichloran 18% Glycerol Agar (DG18) and incubated at 25 °C for 5 to 7 d, according to Pitt and Hocking (2009). Non-particulate samples, i.e. bran, flour and soil, were analyzed by dilution plating. Samples (25 g) were diluted in peptone water (0.1%, 225 mL). Aliquots (0.1 mL) were inoculated onto DG18 plates and incubated at 25 °C for 5 to 7 d (Pitt and Hocking, 2009).

2.4. Fungal identification

Isolates that had the appearance of belonging to *Aspergillus* section *Flavi* were transferred to Czapek Yeast Extract Agar (CYA; Pitt and Hocking, 2009) and incubated at 25 °C for 7 d. Isolates were then examined on standard identification media for *Aspergillus* species: CYA, at 25 °C and 37 °C, Malt Extract Agar (MEA) and *Aspergillus flavus* and parasiticus agar (AFPA), at 25 °C (Pitt and Hocking, 2009). The incubation time for all media and conditions was 7 d. Representatives of each species, initially distinguished by morphological and physiological characteristics, were further analyzed by molecular methods and the production of extrolites.

2.5. Molecular analysis

Forty isolates from *Aspergillus* section *Flavi*, representing different groups according to morphological and physiological characteristics, were chosen. Genomic DNA was extracted using the Biopur mini spin genomic DNA extraction kit® (Biometrix, Curitiba, PR, Brazil) following the manufacturer's recommended protocol. For molecular phylogeny, part of the β-tubulin (*BenA*) gene sequence was determined as described in Taniwaki et al. (2012). The *BenA* sequences were aligned with those from type or neotype strains of all recognized species in *Aspergillus* section *Flavi* using Clustal W (Thompson et al., 1994). A maximum-likelihood tree was inferred using the Kimura-2-parameter model (Kimura, 1980) and to determine the support for each clade, a bootstrap analysis was performed with 1000 replicates in the MEGA 7.0 software package (Kumar et al., 2016).

2.6. Potential for aflatoxin production by Aspergillus section Flavi isolates

Fungi identified as potential producers of aflatoxins (n = 383) were inoculated onto yeast extract sucrose agar (YES agar, Filtenborg et al., 1983) for 7 d at 25 °C and then the agar plug technique (Filtenborg et al., 1983) was used to evaluate the capability of isolates to produce aflatoxins. Fungal extracts taken as plugs with a cork borer were placed on thin layer chromatography (TLC) plates, developed in a toluene: ethyl acetate: formic acid 90%: chloroform (7:5:2:5, v/v/v/v) mobile phase, and visualized under UV light at 365 nm. A mixture of standard preparations of aflatoxins B_1, B_2, G_1 and G_2 (Sigma Aldrich, St. Louis, MO, USA) was used for comparison.

2.7. Extrolite analyses

Forty-seven representative isolates were analyzed for extrolite formation according to Frisvad and Thrane (1987) as modified by Houbraken et al. (2012). Isolates were grown on both CYA and YES agar for 7 and 14 d at 37 °C. Five plugs were taken from each medium and extracted with ethyl acetate/dichloromethane/methanol (3:2:1) (v/v/v; 0.75 mL) with formic acid (1%; v/v) using 50 min ultrasonication. The solvents were evaporated and the dry extract re-dissolved in methanol (0.4 mL). After filtration, the extracts were analyzed using HPLC with diode array detection (Agilent series 1100 system, Santa Clara, CA, USA) with a Phenomenex Luna C18 column (Agilent, Santa Clara, CA, USA), according to Nielsen et al. (2011). Standards of the secondary metabolites were used to confirm identity.

2.8. Aflatoxin determination in raw rice

Aflatoxin analyses were carried out on rice and rice products as follows, based on the method of Stroka et al. (2000).

2.8.1. Clean-up

Rice (approximately 200 g) was finely ground using a laboratory mill (IKA A11 basic, Campinas, SP, Brazil), and passed through a sieve (18 mesh, 1.0 mm). An aliquot (25 g) was extracted with methanol: water (8: 2, v/v; 100 mL) with NaCl (2.5 g) and homogenised in a shaker for 30 min. The solution was first filtered using a quantitative filter (Nalgon, Itupeva, SP, Brazil) and a glass microfiber filter (VICAM, Milford, MA, USA). The filtrate (10 mL) was diluted in phosphate buffered saline (PBS, 60 mL) and applied to an immunoaffinity column for aflatoxins (R-Biopharm Rhône Ltd., Darmstadt, Germany) at a flow rate of 2–3 mL/min. The column was then washed with distilled water (30 mL) and aflatoxins eluted with methanol (1250 μL) and diluted with milli Q water (1750 μL). This method was used for polished rice, paddy rice, rice flakes, black rice, brown rice and parboiled rice samples. For red rice, rice flour and bran rice samples, the PBS was replaced by PBS tween (0.01%) and the elution was carried out with methanol (2 mL) and milli Q water (1 mL). For the red and brown rice mix and rice husks, methanol: water (8:2, 150 mL) was used for extraction, with PBS tween (0.01%) instead of PBS, and eluted with methanol (2 mL), dried in a nitrogen flow and resuspended in methanol: water (2:3, v:v, 3 mL).

Table 1
Infection of rice samples by *Aspergillus* section *Flavi*, and water activity (a_W) at different stages (fields, processing and markets) in three Brazilian regions: wetland (RS), dryland (MA) and markets of São Paulo.

Processing stages	Regions									
	Wetland (RS)			Dryland (MA)			São Paulo (SP)			
	N° positive samples/N° samples	Range of infection (%)	Mean a_W (range)	N° positive samples/N° samples	Range of infection (%)	Mean a_W (range)	N° positive samples/N° samples	Range of infection (%)	Mean a_W (range)
Field	7/12	24–100	0.932 (0.894–0.973)	1/12	0–2	0.825 (0.770–0.953)	–	–	–
Drying	–	–	–	0/2	0	0.708 (0.596–0.821)	–	–	–
Processing									
Paddy rice	7/13	0–38	0.645 (0.516–0.818)	1/2	0–22	0.738 (0.677–0.799)	–	–	–
Husk	3/4	0–58	0.600 (0.562–0.646)	1/1	0–28	0.740	–	–	–
Husked rice	1/2	0–2	0.611 (0.595–0.627)	0/2	0	0.685 (0.680–0.690)	–	–	–
Broken rice	–	–	–	0/4	0	0.633 (0.609–0.649)	–	–	–
Brown rice	1/4	0–12	0.590 (0.575–0.599)	–	–	–	–	–	–
Polished rice	1/4	0–2	0.532 (0.516–0.557)	0/3	0	0.639 (0.635–0.647)	–	–	–
Parboiled rice	1/5	0–2	0.591 (0.542–0.668)	–	–	–	–	–	–
Red rice	1/2	0–2	0.579 (0.571–0.586)	–	–	–	–	–	–
Markets									
Polished rice	0/6	0	0.565 (0.544–0.582)	1/19	0–6	0.591 (0.509–0.673)	0/10	0	0.597 (0.474–0.694)
Brown rice	0/12	0	0.618 (0.527–0.719)	0/2	0	0.592 (0.526–0.658)	2/19	0–2	0.581 (0.530–0.624)
Parboiled rice	0/1	0	0.593	0/6	0	0.661 (0.619–0.694)	2/4	0–4	0.620 (0.595–0.643)
Red rice	0/1	0	0.621	–	–	–	3/10	0–18	0.607 (0.544–0.678)
Mix Brown + red	0/3	0	0.622 (0.604–0.640)	–	–	–	0/3	0	0.584 (0.560–0.598)
Black rice	–	–	–	–	–	–	4/8	0–10	0.632 (0.580–0.696)

2.8.2. Chromatographic conditions

The HPLC system used was an Agilent 1260 Infinity model system (Santa Clara, CA, USA) with a fluorescence detector set at 362 nm excitation and 455 nm emission for aflatoxins G_1 and G_2 and 425 nm emission for aflatoxins B_1 and B_2. An ODS (1.8 µm, 40 × 15 mm; Agilent, Santa Clara, CA, USA) guard column and a Zorbax Eclipse Plus C18 column (5 µm, 4.6 × 150 mm; Agilent, Santa Clara, CA, USA) were employed. The mobile phase was water:acetonitrile:methanol (6:2:3, v/v/v), containing KBr (119 mg) and nitric acid (4 M, 350 µL/L) at a flow rate of 1 mL/min with injection volume of 20 µL. A post-column derivatization of aflatoxins B_1 and G_1 was performed with bromine using a KobraCell (R-Biopharm Rhône Ltd., Darmstadt, Germany).

Aflatoxin B_1, B_2, G_1 and G_2 standards (Sigma, St Louis, MO, USA) were used for quantification. The concentration of aflatoxins in the sample was determined by interpolation of the resulting peak area of each standard curve.

2.8.3. Methodology optimization

Detection and quantification limits were determined according to Eurachem Guides (Magnusson et al., 2015). To calculate the LOD, s_0' value was multiplied by 3 and for LOQ by 10. Spiked samples (0.5 µg/kg of total aflatoxins) in a polished rice sample free of aflatoxins were added. Eight parallel extractions were performed to calculate the recovery and the standard deviation.

The detection and quantification limit for aflatoxin B_1, B_2, G_1 and G_2 were 0.016, 0.012, 0.011 and 0.004 µg/kg and 0.054, 0.039, 0.038 and 0.012 µg/kg, respectively. The recovery percentage of total aflatoxins for polished rice at the level of 0.5, 5 and 25 µg/kg was 81.6%, 88.5 and 95.8%, respectively. The positive control analyses carried out in parallel for each rice sample had recovery values similar to those observed for polished rice. The Directives 98/53/EC of the European Union (Commission Directive 1998/53/EC, 1998) state that analytical methods for control of aflatoxins in food should provide recovery between 70 and 110% at levels between 1 and 10 µg/kg and 50 and 120% at levels < 1 µg/kg.

The mean values of contamination were shown as lower bound (Lb), where the values below the limit of detection (LOD) were replaced by zero and upper bound (Ub) where the values were replaced by LOD divided by two.

3. Results

3.1. Molecular identification

A phylogenetic tree of *Aspergillus* section *Flavi* based on *BenA* is shown in Fig. 1. This is constructed with sequences obtained in the present study and those retrieved from GenBank for each species type recognized in *Aspergillus* section *Flavi* (Samson et al., 2014). *A. flavus* was the predominant species (n = 34), in addition to this species these species from *Aspergillus* section *Flavi* were also found: *A. novoparasiticus* (n = 1), *A. arachidicola* (n = 2), *A. caelatus* (n = 1) and *A. pseudocaelatus* (n = 2), which mostly confirmed the extrolite identification which helped identify the cultures as: *A. flavus* (n = 42), *A. novoparasiticus* (n = 1), *A. arachidicola* (n = 1), *A. caelatus* (n = 1) and *A. pseudocaelatus* (n = 2).

3.2. Incidence of Aspergillus section Flavi species and water activity of rice, rice products and soil

Infection of rice samples by species from *Aspergillus* section *Flavi*, and water activity of samples from different stages (fields, processing and markets) in wetland (RS), dryland (MA) and also São Paulo (SP) markets is shown in Table 1. As expected, field samples from wetland areas had an average a_W of 0.932, a maximum of 0.973 a_W, and a minimum of 0.894 a_W, higher than dryland samples (average 0.825, maximum 0.953, minimum 0.770). At processing, water activity

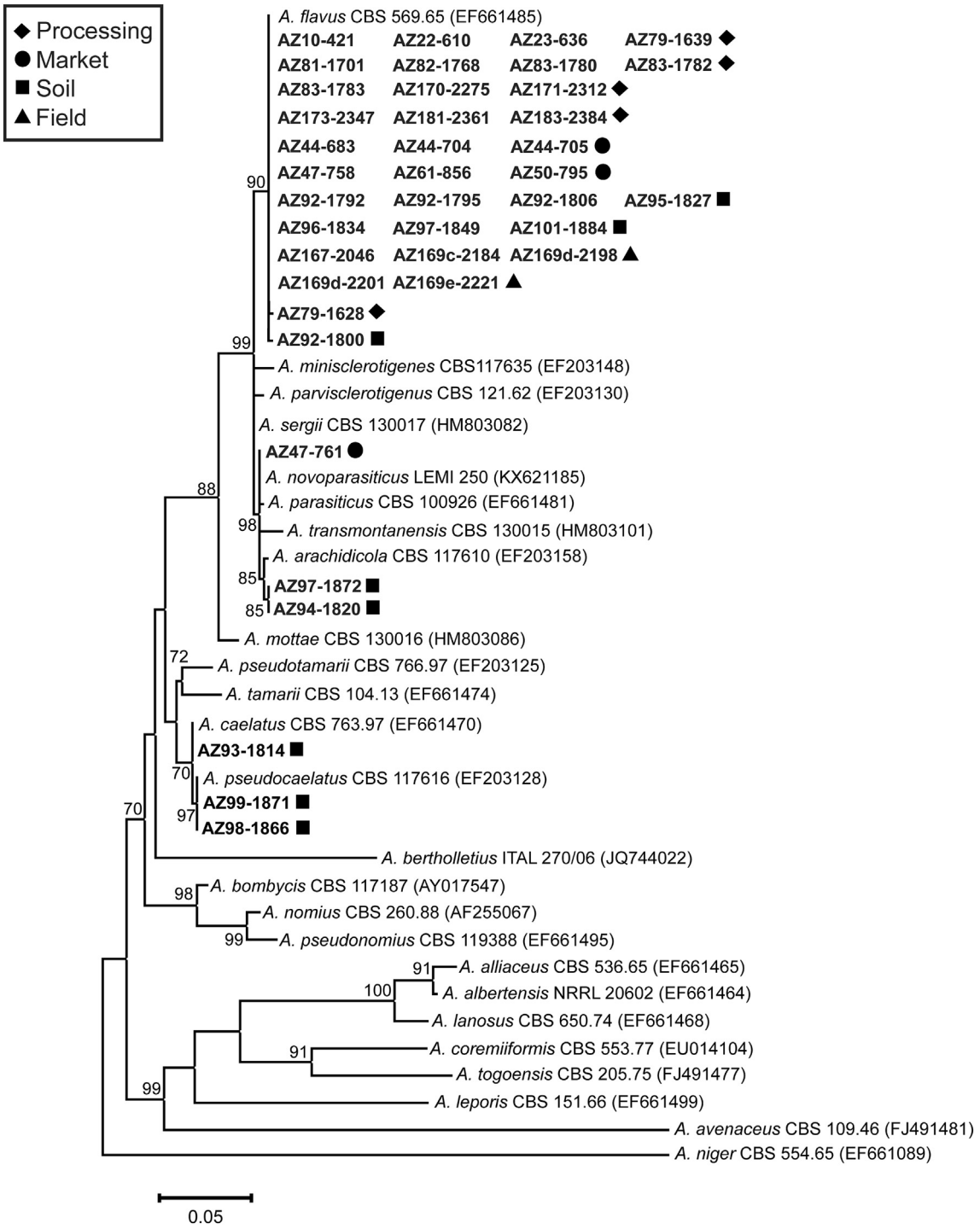

Fig. 1. Maximum likelihood tree based on *BenA* sequences data of *Aspergillus* section *Flavi* type strains and *Aspergillus* isolates from this study (in bold). Nodes supported by bootstrap values ≥ 70% are indicated by numeric values.

decreased from 0.818 and 0.799, for paddy rice samples to 0.516 and 0.635 for polished rice samples in wetland and dryland, respectively. Samples from markets showed a range of 0.474 to 0.719 in different types of rice sold in the markets of the three regions.

Field samples from the wetland showed a higher rate of infection with *Aspergillus* section *Flavi* species (7/12 samples) than samples from dryland (1/12; Table 1). The range of infection of individual grains was 24–100% and 0–2% from wetland and dryland samples, respectively. On the other hand, dryland soil showed a higher incidence of samples contaminated with *Aspergillus* section *Flavi* than wetland soil (Table 2). Infection in rice samples decreased during drying and processing stages in both regions, coinciding with a decrease in water content and also processing effects such as de-husking, polishing and/or parboiling. In the markets, some red and black rice samples showed higher infection than other samples (polished, brown and parboiled) coinciding with higher a_W. *Aspergillus* section *Flavi* species were not found in rice flake and flour samples (Table 2).

3.3. Potential production of aflatoxins and extrolite analyses

Of the 383 isolates from *Aspergillus* section *Flavi*, 44 produced aflatoxins in culture: 39 producing B aflatoxins and five producing both

Table 2
Colony Forming Units per gram (CFU/g) of *Aspergillus* section *Flavi* and water activity (a_W) of rice products (bran, flake and flour) and rice soil samples from wetland (RS) and dryland (MA).

Stage	Region					
	Wetland (RS)			Dryland (MA)		
	N° positive samples/N° samples	Range of CFU/g	Mean a_W (range)	N° positive samples/N° samples	Range of CFU/g	Mean a_W (range)
Processing						
Bran	2/4	< 100–10^3	0.609 (0.465–0.749)	2/2	< 100–2 × 10^3	0.636 (0.607–0.664)
Markets						
Flake	0/1	< 100	0.515	0/2	< 100	0.612 (0.602–0.621)
Flour	0/1	< 100	0.503	0/1	< 100	0.512
Rice soil	3/12	< 100–2 × 10^3	1.00	9/11	< 100–2 × 10^3	1.00

Table 3
Production of aflatoxins by *Aspergillus* section *Flavi* isolated from rice and soil samples from wetland (RS) and dryland (MA) and São Paulo.

Stage	State (number of isolates)	AFB +	AFB + G
Soil	Wetland (3)	0	1
	Dryland (47)	34	3
Field	Wetland (182)	0	0
	Dryland (1)	0	0
Processing	Wetland (70)	0	0
	Dryland (45)	1	0
Markets	Dryland (3)	0	0
	SP (32)	4	1

B and G (Table 3). *Aspergillus* section *Flavi* species were common in wetland rice samples (182 isolates): none of these isolates produced aflatoxins, while only one isolate from soil produced B and G aflatoxins. From dryland samples, the isolate from field rice did not produce aflatoxins, but 34 of 47 isolates from soil did so, including three that produced both B and G aflatoxins.

At processing, only one isolate from the dryland system produced aflatoxins. Five of 30 isolates from markets in São Paulo were positive for aflatoxin production; four producing only B and one producing both B and G aflatoxins.

Table 4 shows the extrolites produced by the 48 isolates of *Aspergillus* section *Flavi*. Most *A. flavus* isolates did not produce aflatoxins, only seven (17%) produced B aflatoxins, but 95% produced kojic acid and 69% cyclopiazonic acid as well. Other common metabolites found in *A. flavus* isolates were: aflavinines, flavimin, paspaline and paspalinine; three produced versicolorin and two 3-*O*-methysterigmatocystin. Isolates identified as *A. arachidicola*, *A. novoparasiticus* and *A. pseudocaelatus* produced aflatoxins B and G. All of them produced kojic acid and, except for *A. pseudocaelatus*, all produced cyclopiazonic acid as well. *A. flavus* AZ83-1783, isolated from a bran sample at the processing stage in the wetland, was unusual, as it produced only kojic acid.

3.4. Aflatoxin analysis

The levels of aflatoxins from field to processing and from markets are shown in Tables 5 and 6, respectively.

The occurrence of total aflatoxins in samples was low as only 25 from 187 were positive (13.4%). The average of total aflatoxin level found in wetland and dryland field samples was 0.35 μg/kg and < LOD (0.14 μg/kg), respectively. At drying, the average of the two samples was 0.23 μg/kg. In wetland and dryland processing samples the average was 0.04 μg/kg and < 0.14 μg/kg, respectively. In the markets of wetland, dryland and São Paulo the following situation was found for the presence of total aflatoxins: 2 samples (< LOD – 0.28 μg/kg), 6 samples (< LOD – 1.6 μg/kg) and 9 samples (< LOD – 70.9 μg/kg), respectively.

Two samples of red rice from São Paulo markets showed high concentrations of total aflatoxins, 70.9 μg/kg and 23.4 μg/kg, well above the maximum permitted level of aflatoxins in cereals, 5 μg/kg, established by resolution RDC 07/2011 of the National Health Surveillance Agency (ANVISA. Agência Nacional de Vigilância Sanitária, 2011). Most rice products were found to have no detectable aflatoxin levels including paddy rice, rice husks, husked rice, polished rice, brown, red and parboiled rice from wetland processing; brown

Table 4
Extrolites produced by the *Aspergillus* section *Flavi* isolated from rice (number of positive strains/total number of tested strains).

Extrolites	*Aspergillus flavus*	*A. arachidicola*	*A. novoparasiticus*	*A. pseudocaelatus*	*A. caelatus*
Aflatoxin type B	7/42	1/1	1/1	2/2	0/1
Aflatoxin type G	0/42	1/1	1/1	2/2	0/1
Aflatrem	6/42	0/1	0/1	0/2	0/1
Aflavazol	6/42	0/1	0/1	0/2	0/1
Aflavinines	18/42	1/1	0/1	1/2	0/1
Asperfuran	1/42	0/1	0/1	0/2	0/1
Chrysogine	0/42	1/1	0/1	0/2	0/1
Cyclopiazonic acid	29/42	1/1	1/1	0/2	0/1
Flavimin	12/42	0/1	0/1	0/2	0/1
Kojic acid	40/42	1/1	1/1	2/2	1/1
Miyakamide	0/42	0/1	0/1	1/2	0/1
Parasiticolide	0/42	0/1	0/1	1/2	0/1
Paspaline	14/42	1/1	0/1	1/2	0/1
Paspalinine	10/42	1/1	0/1	1/2	0/1
Pseurotin	0/42	0/1	0/1	0/2	0/1
Tenuazonic acid	0/42	0/1	0/1	0/2	1/1
Versicolorin	3/42	1/1	0/1	2/2	0/1
3-*O*-methylsterigmatocystin	2/42	0/1	0/1	0/2	0/1

Table 5
Incidence of aflatoxins (μg/kg) in rice samples from field to processing in wetland (MA) and dryland (RS).

State	Wetland					Dryland				
Stage (number of samples)	Field (12)					Field (12)				
	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Total aflatoxins	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Total aflatoxins
Mean (Lb)	0.337	0.011	< LOD	< LOD	0.349	< LOD	< LOD	< LOD	< LOD	< LOD
Mean (Ub)	0.343	0.016	0.006	0.002	0.374	0.008	0.006	0.006	0.002	0.02
Median	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Range	< LOD - 2.826	< LOD - 0.085	< LOD	< LOD	< LOD - 2.95	< LOD	< LOD	< LOD	< LOD	< LOD
No of positive samples	3 (25%)	2 (16.7%)	0	0	3 (25%)	0	0	0	0	0
Stage (number of samples)	–					Drying (2)				
						Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Total aflatoxins
Mean (Lb)						0.224	< LOD	< LOD	< LOD	0.225
Mean (Ub)						0.228	0.006	0.006	0.002	0.235
Median						0.224	< LOD	< LOD	< LOD	0.225
Range						< LOD - 0.448	< LOD	< LOD	< LOD	< LOD - 0.45
No of positive samples						1 (50%)	0	0	0	1 (50%)
Stage (number of samples)	Processing - Paddy Rice (13)					Processing - Paddy Rice (2)				
	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Total aflatoxins	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Total aflatoxins
Mean (Lb)	0.042	< LOD	< LOD	< LOD	0.046	< LOD	< LOD	< LOD	< LOD	< LOD
Mean (Ub)	0.050	0.006	0.006	0.002	0.065	0.008	0.006	0.006	0.002	0.02
Median	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Range	< LOD - 0.551	< LOD	< LOD	< LOD	< LOD - 0.60	< LOD	< LOD	< LOD	< LOD	< LOD
No of positive samples	1 (7.69%)	0	0	0	1 (7.69%)	0	0	0	0	0
Stage (number of samples)	Processing - Bran (4)					Processing - Bran (2)				
	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Total aflatoxins	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Total aflatoxins
Mean (Lb)	0.164	0.012	< LOD	< LOD	0.175	0.167	< LOD	0.007	< LOD	0.175
Mean (Ub)	0.17	0.122	0.006	0.002	0.19	0.167	0.006	0.007	0.002	0.175
Median	< LOD	< LOD	< LOD	< LOD	< LOD	0.167	< LOD	0.007	< LOD	0.175
Range	< LOD - 0.656	< LOD - 0.047	< LOD	< LOD	< LOD - 0.70	0.101–0.232	< LOD	< LOD - 0.014	< LOD	0.10–0.25
No of positive samples	1 (25%)	1 (25%)	0	0	1 (25%)	2 (100%)	0	1 (50%)	0	2 (100%)
Stage (number of samples)	–					Processing - Broken rice (4)				
						Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Total aflatoxins
Mean (Lb)						0.084	< LOD	< LOD	< LOD	0.084
Mean (Ub)						0.088	0.006	0.006	0.002	0.094
Median						0.121	< LOD	< LOD	< LOD	0.12
Range						< LOD – 0.23	< LOD	< LOD	< LOD	< LOD – 0.23
No of positive samples						2 (50%)	0	0	0	2 (50%)

LOD: Limit of detection.

rice, rice flour, red and flake rice from wetland markets, polished rice from wetland and São Paulo markets and parboiled rice from both dryland and São Paulo markets.

4. Discussion

In the present study, the wetland rice samples had a high infection with *Aspergillus* section *Flavi* species, while soil had only low levels, but aflatoxin concentrations in rice were low. According to Pitt et al. (2013), rice cultivated with an irrigation system in the first stages of development shows low levels of *A. flavus* in soil, with low infection in mature grains and in the final product, but if the grains are collected wet and then dried, the infection by *A. flavus* becomes more probable (Pitt et al., 2013). This was the case in the wetland region, where the production system used irrigation.

In the dryland production system, the seeds are cultivated when the rain becomes more regular, so the soil is not flooded as in the wetland system and fungi can develop. The dryland soil samples showed high contamination by *Aspergillus* section *Flavi* species, but the rice samples from the field had low infection rates and low levels of aflatoxins (below the limit of detection). As rice is not known to be infected with *A. flavus* before harvest (Pitt et al., 1994), these data indicate that rice from the wetland fields were harvested wet and then dried, while in the dryland field, the samples were drier when harvested. In South Korea, where the wetland system predominates, only one non-toxigenic *A. flavus* was found in 80 fresh harvest samples (Ok et al., 2014), but in India, where the dryland system predominates, the occurrence of *A. flavus* was reported in 83.5% of rice samples analyzed (Reddy et al., 2009).

Drying and storage are stages that require attention because if the drying is slow or the storage is poor, toxigenic fungi can develop and produce mycotoxins (Pitt et al., 2013). At processing, 7 of 13 samples of paddy rice from the wetland showed the presence of *Aspergillus* section *Flavi* species but none was an aflatoxin producer and only one sample

Table 6
Incidence of aflatoxins (μg/kg) in rice from market samples in wetland (RS), dryland (MA) and São Paulo (SP).

State	Wetland					Dryland					SP				
Type of rice (number of samples)	Polished (6)					Polished (19)					Polished (10)				
	AFB_1	AFB_2	AFG_1	AFG_2	AFT	AFB_1	AFB_2	AFG_1	AFG_2	AFT	AFB_1	AFB_2	AFG_1	AFG_2	AFT
Mean (Lb)	< LOD	< LOD	< LOD	< LOD	< LOD	0.136	0.007	< LOD	< LOD	0.143	< LOD	< LOD	< LOD	< LOD	< LOD
Mean (Ub)	0.008	0.006	0.006	0.002	0.02	0.142	0.012	0.006	0.002	0.157	0.008	0.006	0.006	0.002	0.02
Median	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Range	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD - 1.463	< LOD - 0.128	< LOD	< LOD	< LOD - 1.59	< LOD	< LOD	< LOD	< LOD	< LOD
No of positive samples	0	0	0	0	0	5 (26.32%)	1 (5.26%)	0	0	5 (26.32%)	0	0	0	0	0
Type of rice (number of samples)	Brown (2)					Brown (2)					Brown (19)				
	AFB_1	AFB_2	AFG_1	AFG_2	AFT	AFB_1	AFB_2	AFG_1	AFG_2	AFT	AFB_1	AFB_2	AFG_1	AFG_2	AFT
Mean (Lb)	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.004	< LOD	< LOD	< LOD	0.004
Mean (Ub)	0.008	0.006	0.006	0.002	0.02	0.008	0.006	0.006	0.002	0.02	0.011	0.006	0.006	0.002	0.023
Median	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Range	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD - 0.069	< LOD	< LOD	< LOD	< LOD - 0.069
No of positive samples	0	0	0	0	0	0	0	0	0	0	1 (5.26%)	0	0	0	1 (5.26%)
Type of rice (number of samples)	Parboiled (1)					Parboiled (6)					Parboiled (4)				
	AFB_1	AFB_2	AFG_1	AFG_2	AFT	AFB_1	AFB_2	AFG_1	AFG_2	AFT	AFB_1	AFB_2	AFG_1	AFG_2	AFT
Mean (Lb)	0.102	< LOD	< LOD	< LOD	0.102	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Mean (Ub)	0.102	0.012	0.11	0.004	0.102	0.008	0.006	0.006	0.002	0.02	0.008	0.006	0.006	0.002	0.02
Median	0.051	< LOD	< LOD	< LOD	0.051	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Range	0.102	< LOD	< LOD	< LOD	0.102	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
No of positive samples	1 (100%)	0	0	0	1 (100%)	0	0	0	0	0	0	0	0	0	0
Type of rice (number of samples)	Flake (1)					Flake (2)					–				
	AFB_1	AFB_2	AFG_1	AFG_2	AFT	AFB_1	AFB_2	AFG_1	AFG_2	AFT					
Mean (Lb)	< LOD	< LOD	< LOD	< LOD	< LOD	0.108	< LOD	< LOD	< LOD	0.108					
Mean (Ub)	0.008	0.006	0.006	0.002	0.02	0.058	0.006	0.006	0.002	0.118					
Median	< LOD	< LOD	< LOD	< LOD	< LOD	0.108	< LOD	< LOD	< LOD	0.108					
Range	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD - 0.215	< LOD	< LOD	< LOD	< LOD - 0.215					
No of positive samples	0	0	0	0	0	1 (50%)	0	0	0	1 (50%)					
Type of rice (number of samples)	Red Rice (1)					–					Red Rice (10)				
	AFB_1	AFB_2	AFG_1	AFG_2	AFT						AFB_1	AFB_2	AFG_1	AFG_2	AFT
Mean (Lb)	< LOD	< LOD	< LOD	< LOD	< LOD						8.440	1.078	< LOD	< LOD	9.428
Mean (Ub)	0.008	0.006	0.006	0.002	0.02						8.446	1.093	0.006	0.002	9.444
Median	< LOD	< LOD	< LOD	< LOD	< LOD						< LOD	< LOD	< LOD	< LOD	< LOD
Range	< LOD	< LOD	< LOD	< LOD	< LOD						< LOD - 63.32	< LOD - 8.591	< LOD	< LOD	< LOD - 70.91
No of positive samples	0	0	0	0	0						2 (20%)	2 (20%)	0	0	2 (20%)
Type of rice (number of samples)	Brown w/ red (3)					–					Brown w/ red (3)				
	AFB_1	AFB_2	AFG_1	AFG_2	AFT						AFB_1	AFB_2	AFG_1	AFG_2	AFT
Mean (Lb)	0.098	< LOD	< LOD	< LOD	0.098						2.111	0.358	< LOD	< LOD	2.467
Mean (Ub)	0.100	0.006	0.006	0.002	0.101						2.114	0.360	0.006	0.002	2.473
Median	< LOD	< LOD	< LOD	< LOD	< LOD						2.243	0.23	< LOD	< LOD	2.47
Range	< LOD - 0.283	< LOD	< LOD	< LOD	< LOD - 0.283						< LOD - 4.09	< LOD - 0.845	< LOD	< LOD	< LOD - 4.93
No of positive samples	1 (33.3%)	0	0	0	1 (33.3%)						2 (66.67%)	2 (66.67%)	0	0	2 (66.67%)

(*continued on next page*)

Table 6 (*continued*)

State	Wetland	Dryland	SP					
Type of rice (number of samples)	–	–	Black Rice (8)					
			AFB_1	AFB_2	AFG_1	AFG_2	AFT	
Mean (Lb)			0.011	< LOD	0.047	< LOD	0.059	
Mean (Ub)			0.018	0.006	0.050	0.002	0.069	
Median			< LOD	< LOD	0.067	< LOD	0.067	
Range			< LOD - 0.09	< LOD	< LOD - 0.133	< LOD	< LOD - 0.133	
No of positive samples			1 (12.5%)	0	4 (50%)	0	4 (50%)	

LOD: Limit of detection.

had a low level of aflatoxins. These data therefore indicate that if drying and storage are carried out in a safe way, aflatoxin production is minimal.

The milling process decreases the level of aflatoxins in cereals, including rice. Sales and Yoshizawa (2005) observed a reduction of 78% of aflatoxins from brown rice to polished rice and a reduction of 38% of aflatoxins from milled rice to well milled rice. The milling process removes the bran from rice and in our study bran samples from both regions showed the presence of low levels of aflatoxins. In a study on aflatoxin reduction during shelling and milling, Trucksess et al. (2011) found that bran was the fraction with the highest contamination (357 μg/kg), followed by brown rice (158 μg/kg), paddy rice (114 μg/kg), polished rice (56 μg/kg) and husk (39 μg/kg). In our study, the presence of *Aspergillus* section *Flavi* species at the processing stage was low, and the samples with highest contamination were paddy rice, rice husk and rice bran.

Polished, brown and parboiled rice are the market types of rice most consumed in Brazil. Previous studies have shown low infection or absence of *Aspergillus* section *Flavi* species (Carvalho et al., 2010; Ok et al., 2014; Taligoola et al., 2011). In our study, red rice showed the highest contamination by *Aspergillus* section *Flavi* species and aflatoxins. This type of rice is not commonly consumed in Brazil. Aflatoxin contamination in polished rice in Brazil has been reported to be high, including 176 μg/kg in rice stored under inappropriate conditions (Almeida et al., 2012). Other reports show lower levels such as 2.04 μg/kg (Silva et al., 2008) and 1.2 μg/kg (Carvalho et al., 2010). Reports on brown rice have shown levels of 2.7 μg/kg (Ok et al., 2014) and parboiled rice high levels such as 30 μg/kg (Toteja et al., 2006) and 74 μg/kg (Dors et al., 2011). In the present study, polished, parboiled, brown, brown with red, flake, flour, and black rice had levels lower than 5 μg/kg.

The presence of *Aspergillus* section *Flavi* species, especially *A. flavus*, has been reported in rice in several countries (Carvalho et al., 2010; Ok et al., 2014; Park et al., 2005; Reddy et al., 2009; Sales and Yoshizawa, 2005). After taxonomic revision of *Aspergillus* section *Flavi* using polyphasic approaches (Samson et al., 2014), a few strains previously identified as *A. flavus* or *A. parasiticus*, have been reclassified. In the present study, five species have been distinguished: *A. flavus*, *A. caelatus*, *A. novoparasiticus*, *A. arachidicola* and *A. pseudocaelatus*. This is the first report of these last three species from rice and rice plantation soil. *A. novoparasiticus* was first isolated in Brazil from clinical environments (Gonçalves et al., 2012), *A. arachidicola* from peanuts in Argentina (Pildain et al., 2008) and *A. pseudocaelatus* from *Arachis burkartii* leaf in Argentina (Varga et al., 2011). *A. novoparasiticus*, *A. arachidicola* and *A. pseudocaelatus* have also been found in maize kernels (Viaro et al., 2017), showing that with molecular data these species can be distinguished from other *Aspergillus* section *Flavi* species in foods.

In the present study, few isolates of *A. flavus* were able to produce aflatoxins in culture and most rice samples were not contaminated with aflatoxins. Using the agar plug technique, Carvalho et al. (2010) found that 26% of *A. flavus* isolates from Brazilian rice collected from markets produced aflatoxin B_1 and B_2 These results differ from many results with other crops where a higher proportion of *A. flavus* producing aflatoxins have been reported, including peanuts 50% (Martins et al., 2017), brazil nuts 46% (Calderari et al., 2013), and maize 70% (Giorni et al., 2007). Although most of the *A. flavus* isolated in this study did not produce aflatoxins, 69% produced cyclopiazonic acid.

The present research has shown that species from *Aspergillus* section *Flavi* can occur in rice and in soils from rice plantations, from both wetland and dryland agricultural systems. However, most *A. flavus* strains encountered here did not produce aflatoxins and consequently the risk of aflatoxin production in rice was low at pre-harvest. However, if the rice is dried slowly or poorly stored, aflatoxin production may occur. In our study, red rice showed the highest contamination by *Aspergillus* section *Flavi* species and the highest aflatoxin levels. This type of rice is not commonly consumed in Brazil.

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