Mycotoxin Res (2017) 33:49–58
DOI 10.1007/s12550-016-0262-x

ORIGINAL ARTICLE

Occurrence and fumonisin B_2 producing potential of *Aspergillus* section *Nigri* in Brazil nuts

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Received: 12 July 2016 / Revised: 24 October 2016 / Accepted: 26 October 2016 / Published online: 5 November 2016

Abstract *Bertholletia excelsa* is the tree that produces Brazil nuts which have vast economic importance in the Amazon region and as an export commodity. The aim of this study was to assess the presence of *Aspergillus* section *Nigri* in Brazil nut samples at different stages of its production chain and to verify the toxigenic potential for fumonisin B_2 (FB_2) production of these isolates along with the presence of this mycotoxin in Brazil nut samples. The fungal infection ranged from 0 to 80% at the different stages of the harvest and processing chain and the water activity of the nuts from 0.273 to 0.994. A total of 1052 *A.* section *Nigri* strains were isolated from Brazil nuts and 200 strains were tested for their ability to produce FB_2: 41 strains (20.5%) were FB_2 producers with concentrations ranging from 0.09 to 37.25 mg/kg; 2 strains (1%) showed traces of FB_2, less than the detection limit (0.08 mg/kg); and 157 (78.5%) were not FB_2 producers. Although several samples showed high contamination by *A.* section *Nigri*, no sample was contaminated by FB_2.

Highlights
- This is the first report of *A.* section *Nigri* strains isolated from Brazil nuts producing FB_2.
- The PCR assay revealed 100 and 33% of *A. niger* and *A. welwitschiae* isolates, respectively, harboring the *fum8* gene in their genomes.

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Keywords *Bertholletia excelsa* · Black *Aspergilli* · Fumonisins

Introduction

The Brazil nut is of great importance for the Brazilian economy. It is the main non-timber product extracted from the Amazonian rainforest and is connected to communities where trees are used as a source of food and for income (Wadt et al. 2005). Brazil supplies 75% of the world market and produces about 38,300 t of Brazil nuts annually which are predominantly exported (Conab 2015). Brazil nuts are considered a food rich in proteins, lipids, and vitamins in addition to being an excellent source of selenium. However, the characteristic low technological level of the production chain as well as inadequate management and handling of the raw material favors the establishment of points of contamination with consequent risk to consumer health as well as economic losses at all stages (Souza and Menezes 2008).

Aspergillus section *Nigri*, known as black *Aspergilli*, are commonly found in foods such as grapes and grape products (Cabañes et al. 2002; Abarca et al. 2004; Leong et al. 2004), coffee (Joosten et al. 2001; Urbano et al. 2001; Taniwaki et al. 2003; Leong et al. 2007), cocoa (Mounjouenpou et al. 2008; Copetti et al. 2010), dried fruits (Doster et al. 1996; Lombaert et al. 2004; Iamanaka et al. 2005), and among others. It is considered a common fungus in nuts, especially peanuts and tree nuts (Magnoli et al. 2007; Pitt and Hocking 2009). The taxonomy of *A.* section *Nigri* has been reviewed several times using the polyphasic approach, and new species have been described from different sources each time (Samson et al. 2004; Varga et al. 2011; Hong et al. 2013; Samson et al. 2014). Nowadays, *A.* section *Nigri* comprises a set of 27 species, but only *A. niger* and *Aspergillus welwitschiae* have been

sufficiently proven to produce the mycotoxin FB_2. *A. niger* and *A. welwitschiae* belong to an informal taxonomic rank, *A. niger* "aggregate," that represents a group of morphologically very similar species of *A.* section *Nigri* demanding DNA sequence analysis of calmodulin (*CaM*) for the identification of their members.

A. niger is the most important filamentous fungus used in biotechnology where it is used extensively for organic acid production and for the production of extracellular enzymes. Other applications include the following: biotransformation of xenobiotics, bioremediation and waste pre-treatment, and cell protein for feed (Frisvad et al. 2011). It has usually been regarded as a benign fungus (Frisvad et al. 2011) and it holds GRAS (generally regarded as safe) status from the US Food and Drug Administration (FDA 2001). Usually, only a low percentage of *A. niger* isolates are able to produce ochratoxin A (OTA). Although *A. niger* is very frequently isolated, it is not a significant source of OTA as a rule (Taniwaki and Pitt 2013). *A. welwitschiae* (=*A. awamori* sensu, Perrone et al. 2011) is a new species, which was recently dismembered from the *A. niger* taxon (Varga et al. 2011).

Frisvad et al. (2007) reported for the first time the production of fumonisin B_2 (FB_2) in cultures of three full genome sequenced strains of *A. niger*, including the ex-type culture *A. niger*. Until that time, production of fumonisin had been reported only in species of *Fusarium* especially *Fusarium verticillioides* and *Fusarium proliferatum* which are common in maize. Isolates of *A. niger* were able to produce FB_2 and FB_4 in grapes and raisins (Mogensen et al. 2010), and FB_2 was produced by fungi isolated from coffee (Noonim et al. 2009). Fumonisins have been reported to cause a fatal disease in horses called leuko-encephalomalacia (Marasas et al. 1988), pulmonary edema in pigs (Haschek et al. 2001) and possibly esophageal cancer in humans (Yoshizawa et al. 1994). Even though fumonisins are less acutely toxic than aflatoxins, they are found in high concentrations in maize compared to aflatoxins (Yoshizawa et al. 1994).

Contamination with aflatoxins and aflatoxigenic fungi in Brazil nuts have been the focus of studies in Brazil and in several other countries (Arrus et al. 2005; Pacheco and Scussel 2007; Olsen et al. 2008; Baquião et al. 2012; Calderari et al. 2013). This is due to the requirement established primarily by the European Union (EU 2010). Fumonisins are also regulated in foods and feeds in the EU and Brazil, with the maximum levels ranging from 200 to 5000 μg/kg (EU 2010; ANVISA 2011). Positive findings of FB_2 in Brazil nuts would therefore have potential implications on trade. Few studies have investigated the presence of *A.* section *Nigri* and production of FB_2 in Brazil nuts. Therefore, the aim of this work was to verify the incidence of *A.* section *Nigri* in Brazil nuts collected from different stages of its production chain and their potential for FB_2 production.

Materials and methods

Isolation and identification of fungi from Brazil nuts

A total of 100 samples of in shell and shelled Brazil nuts, (approximately 2 kg each) were collected from the Amazon region (rainforest, street markets, and processing plants) and in São Paulo state (supermarkets). One hundred grams of each sample was immersed in a solution of sodium hypochlorite 0.4% for 1 min. Fifty pieces of kernel (which were cut using a scalpel) of each sample were plated onto agar Dichloran Glycerol 18% (DG18). Each plate had 5 pieces of kernels (10 plates × 5 pieces = 50). The plates were incubated for 5 days at 25 °C. The isolates apparently identified as *A.* section *Nigri* were isolated and purified in Czapek Yeast Extract Agar (CYA). The pure cultures were grown in CYA and incubated at 25 °C for 7 days (Pitt and Hocking 2009; Klich and Pitt 1988).

Test for fumonisin B_2 production by *A.* section *Nigri* by HPLC

From 1052 *A.* section *Nigri* isolates, 200 strains from different samples that were morphologically biseriates were tested for the ability to produce FB_2. *A.* section *Nigri* were inoculated onto agar Czapek Yeast Extract 20% Sucrose (CY20S) and incubated at 25 °C for 7 days, following the methodology of Frisvad et al. (2007) with modifications. Five small pieces of mycelium were removed (plugs) from the central portion and transferred into a pre-weighed vial (Frisvad et al. 2007). The bottles were weighed again, thereby obtaining the weights of mycelia for later calculation of the concentration of FB_2. One milliliter of methanol was added and stirred by vortex for 3 min. After that, two filtrations were carried out with membrane of Millex 0.45 and 0.22 μm. Then, 55 μl of the extract was transferred to a vial for HPLC, adding 55 μl of ortho-phthaldialdehyde reagent (OPA), according to the method of Shephard et al. (1990). The vial was stirred in a vortex for 30 s. The detection of FB_2 by high-performance liquid chromatography (HPLC) was performed according to the method of Visconti et al. (2001). Chromatography from Shimadzu LC-10VP (Shimadzu, Japan), with a fluorescence detector at 335 nm excitation and 440 nm emission was used. A YMC column—Pack ODS-A (5 μm, 4.6 × 150 mm)—was employed and the mobile phase used acetonitrile/water/acetic acid (51:47:02 *v*/*v*/*v*) and was filtered through a 0.45-μm

membrane. The flow of the mobile phase was set to 1.0 ml/1 min. The oven temperature was 40 °C.

Test for fumonisin B_2 production by *A.* section *Nigri* by UHPLC-HRMS

To confirm the efficiency of the test in the "Test for fumonisin B_2 production by *A.* section *Nigri* by HPLC" section, an analysis with nine isolates was performed in ultra-high-performance liquid chromatography high-resolution mass spectrometry (1290 UHPLC-HRMS system, Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector (Frisvad et al. 2007). Separation was obtained on an Agilent Poroshell 120 phenyl-hexyl column (2.1 × 250 mm, 2.7 μm) with a linear gradient consisting of water (A) and acetonitrile (B) both buffered with 20 mM formic acid, starting at 10% B and increased to 100% B in 15 min where it was held for 2 min, returned to 10% B in 0.1 min and remaining at 10% B for 3 min (0.35 ml/min, 60 °C). An injection volume of 1 μl was used. MS detection was performed on an Agilent 6540 QTOF MS equipped with Agilent Dual Jet Stream electrospray ion source with a drying gas temperature of 250 °C, gas flow of 8 l/min, sheath gas temperature of 300 °C, and flow of 12 l/min. Capillary voltage was set to 4000 V and nozzle voltage to 500 V. Mass spectra were recorded at 10, 20, and 40 eV as centroid data for *m*/*z* 85–1700 in MS mode and *m*/*z* 30–1700 in MS/MS mode, with an acquisition rate of 10 spectra. Lock mass solution in 70:30 methanol/water was infused in the second sprayer using an extra LC pump at a flow of 15 μl/min using a 1:100 splitter. The solution contained 1 μM tributylamine (Sigma-Aldrich, USA) and 10 μM Hexakis (2,2,3,3-tetrafluoropropoxy) phosphazene (Apollo Scientific Ltd., Cheshire, UK) as lock masses. The $[M + H]^+$ ions (*m*/*z* 186.2216 and 922.0098, respectively) of both compounds were used. Standards of fumonisin B_2 and B_4 were obtained from Biopure (Tulln, Austria), FB_6 was obtained from a previous study (Mansson et al. 2010), and verification of identity was performed via exact mass confirmation, spectra similarity and retention times.

Molecular analysis

Out of 200 isolates, a total of 37 (13 producing FB_2) were randomly subjected to *CaM* gene sequence analysis, representing different origins. After growing in liquid complete medium (Pontecorvo et al. 1953), the mycelia were collected, frozen in liquid nitrogen, and ground to a fine powder. Nucleic acids were extracted using the BioPurMini Spin Extraction Kit (Biometrix, Brazil), according to the manufacturer's instructions. Amplification of a *CaM* gene region was performed using the primers cmd5 (5′ CCG AGT ACA AGG AGG CCT TC 3′) and cmd6 (5′ CCG ATA GAG GTC ATA ACG TGG 3′) described in Hong et al. (2006). The PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI 3500XL Genetic Analyser (Applied Biosystems, USA). The partial *CaM* sequences were subsequently aligned with those from *A.* section *Nigri* available in the GenBank database, and a phylogeny reconstruction was performed using a distance-based Neighbor-Joining method (Saitou and Nei 1987). The tree was drawn using MEGA 6.05 (Tamura et al. 2013).

The isolates were also screened by PCR to assess the presence of the *fum8* gene, which is essential for fumonisin production. A primer-pair to detect the gene *fum8* (fum8vn-F TCG TTT GAG TGG TGG CAG AAT and fum8-R GTT GGG CAC AGA TAC CAT TTG), which encodes α-oxoamine synthase, an essential enzyme for FB_2 biosynthesis was used. The forward (fum8vn-F) consisted of the same sequence designed by Susca et al. (2010) denoted vnF1, but with five additional nucleotide residues. The reverse primer (fum8-R) was designed after the selection of a conserved region, 128 nucleotides away from the target sequence of the forward primer (Massi et al. 2016). PCRs were performed in a final volume of 10 μl containing 1× PCR buffer, 5 ng of DNA template, 0.3 pMoL of each primer, 0.2 mM of dNTP set (Invitrogen, Life Technologies, USA), 2.0 mM of $MgCl_2$ and 1.0 U of Platinum Taq DNA polymerase (Invitrogen, Life Technologies, USA). The DNA was amplified in a Veriti® Thermal Cycler (Applied Biosystems, USA) under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 40 s, annealing at 58 °C for 35 s, and extension at 72 °C for 40 s followed by final extension of 72 °C for 30 min. The amplified products were analyzed by standard agarose gel (1.5%) electrophoresis.

Analysis of fumonisin B_2 in Brazil nut samples

All the samples were analyzed for FB_2 and were shelled, approximately 100 g was finely ground using a grinder (IKA, Mod A11, Brazil) and placed in a plastic bag. Twenty grams was taken and extracted with 50 ml of methanol/acetonitrile/water (25:25:50, *v*/*v*/*v*). The suspension was stirred for 3 min in an Ultra-Turrax homogenizer (Polytron, Switzerland) at a speed of 1118 g. This mixture was centrifuged (International Company Equipment-Mod: B20A, USA) for 20 min at 3578 g. The supernatant was removed by pipette and stored in a 100-ml flask. The pellet was re-suspended

Table 1 Water activity, frequency of occurrence, mean and variation for the level of infection of brazil nut kernel by *Aspergillus* section *Nigri* in rainforests, processing plants, street markets and supermarkets.

Data	Rainforest	Street market	Processing	Supermarket
N samples	23	35	29	13
Water activity	0.920 (0.796-0.993)	0.814 (0.444-0.994)	0.695 (0.316- 0.984)	0.482 (0.273-0.597)
Frequency of occurrence (%)	100	66	90	92
Average of infection (%)	11	3	8	5
Range of infection (%)	2 - 80	0 - 54	0 - 72	0 - 58
FB_2 (mg/kg)	ND	ND	ND	ND

Frequency of occurrence = number of samples that contained a fungal species/total of samples evaluated; Average of infection = sum of infection level/ total number of samples; Range of infection = range of infected kernels in a sample; and ND = not detected (Limit of detection of FB_2 = 0.032 mg/kg; Limit of quantification = 0.054 mg/kg).

with 50-ml solution of methanol/acetonitrile/water (25:25:50, *v*/*v*/*v*), stirred again for 3 min in the Ultra-Turrax homogenizer (Polytron, Switzerland) and centrifuged for 20 min at 4000 rpm. The two supernatants were joined and then filtered through filter paper Whatman n 4. The filtrate was withdrawn at a rate of 10 ml and transferred to a 50-ml volumetric flask. The volume was completed with phosphate buffered saline (PBS). This solution was filtered through a Whatman glass fiber filter. Ten milliliters of the filtrate was passed through an immunoaffinity column FumoniTestTM^{WB} (Vicam®, USA) at a flow rate of 2–3 ml/min followed by washing with 10 ml PBS. FB_2 was eluted with 1.5 ml of methanol, dried under a stream of nitrogen at 40 °C and the dried extract was re-suspended in a solution of 0.2 ml of acetonitrile/water (1:1, *v*/*v*).

A Shimadzu LC-10VP HPLC (Shimadzu, Japan) was used with a fluorescence detector wavelength at 335-nm excitation and 440-nm emission and automatic injector. A YMC column—Pack ODS-A (5 μm, 4.6 × 150 mm)—was employed and a mobile phase consisting of acetonitrile/water/acetic acid (51:47:0.2. *v*/*v*/*v*) with a flow rate of 1 ml/min was utilized. The oven temperature was 40 °C.

The recovery, detection and quantification limits of this methodology were estimated spiking the Brazil nut kernels with FB_2 standard (Sigma-Aldrich, USA) at the level of 0.07 and 1 mg/kg. Tests were carried out in triplicate.

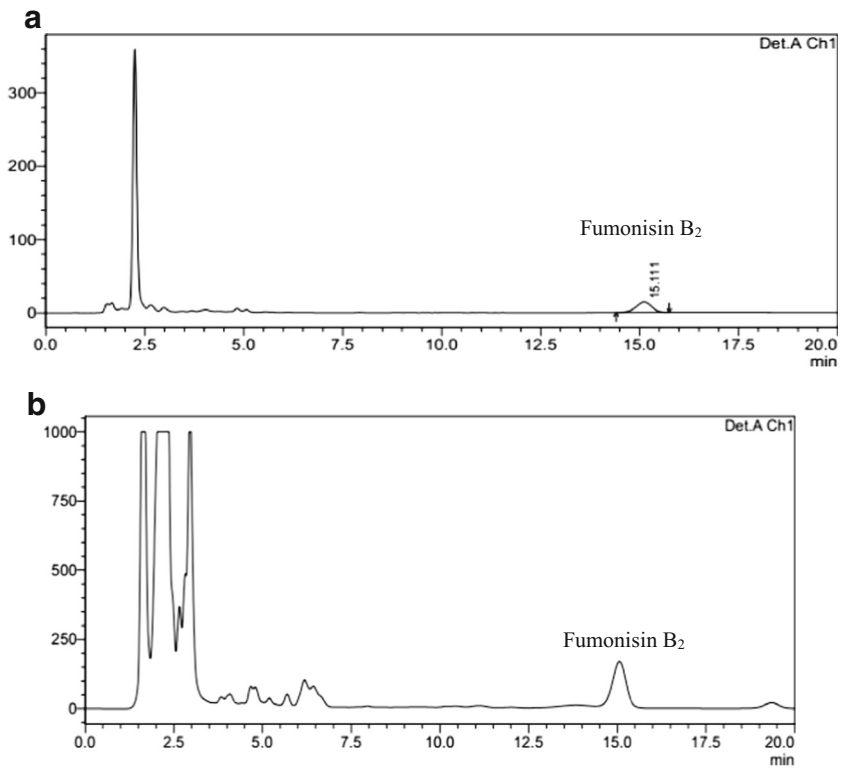

Fig. 1 Chromatogram of FB_2 **a** standard solution and **b** produced by *A. niger*

Test for fumonisin B_2 production by *A. niger* in Brazil nuts

One strain of *A. niger* (ITAL 1206) isolated in the present study produced a high amount of FB_2 (14 mg/kg) which was used to test the production of FB_2 in Brazil nuts. *A. niger* (ITAL 1206) was inoculated on Czapek Yeast Extract Agar (CYA) and incubated at 25 °C for 7 days. For inoculation in Brazil nut kernels, the shells were used as an inoculum vector of *A. niger* so as to not change the water activity of the Brazil nut kernel. The shells were sterilized using an autoclave at 121 °C for 15 min and dried for 20 min before being removed from the autoclave. The shells were ground in a grinder (IKA, Mod A11, Brazil) at 425-μm mesh and turned into a powder. Spores of *A. niger* were inoculated in a flask containing 100-ml peptone water (0.1%), tween 80 (0.1%), and glass bead. The flash was stirred and filtrated with cheese cloth. Ten milliliter of this suspension were added aseptically to 60 g of Brazil nut shell powder and mixed. Two batches of 200 g of crushed Brazil nut kernels with different water activity, 0.705 (A) and 0.965 (B) were prepared previously (before introducing the fungal inoculum). They were placed in sterile bags and 30 g of the contaminated powder were added and mixed. Each contaminated sample was divided into 10 portions of 20 g. A control of Brazil nuts without *A. niger* was also carried out. The samples were incubated at 30 °C and analyzed in duplicate for FB_2 production as described above, every 7 days for a period of 28 days.

Results

Aspergillus section *Nigri* infection in Brazil nuts

A total of 1052 *A.* section *Nigri* strains were isolated from the Brazil nuts. They were found in several samples of Brazil nuts collected in the rainforest, processing plants, street markets, and supermarkets. The frequency of occurrence, the average infection rate, the range of infection, and the water activity and presence of fumonisin B_2 of each stage of Brazil nut production are given in Table 1.

Samples collected from Amazonian rainforests had water activity between 0.796 and 0.993 and fungal infection from 2 to 80%. One sample showed 80% of infection by *A.* section *Nigri*, showing that the growth conditions of Brazil nuts in rainforests are favorable to their development. Samples collected in street markets had a_w between 0.444 and 0.994 and fungal infection from 0 to 54%. Samples collected from processing plants had water activity between 0.316 and 0.984, due to the different stages of processing and infection ranging from 0 to 72%. Samples collected in supermarkets in São Paulo were all processed and had low a_w between 0.273 and 0.597 and infection by these species was between 0 and 58%.

Table 2 Production of FB_2 (mg/kg) by *Aspergillus* section *Nigri* isolated from brazil nuts.

Isolates	# ITAL	Origin (Local of collection/States)	FB_2
1	ITAL161	Street Market/ Amazon	0.15
2	ITAL 162	Street Market/ Amazon	0.09
3	ITAL 361	Rainforest/ Pará	1.80
4	ITAL 429	Supermarket/ São Paulo	2.53
5	ITAL 578	Street Market/ Amazon	33.43
6	ITAL 671	Street Market/Amazon	26.63
7	ITAL 676	Street Market/ Amazon	1.26
8	ITAL 678	Street Market/ Amazon	1.64
9	ITAL 683	Street Market/ Amazon	0.10
10	ITAL 713	Street Market/ Amazon	0.09
11	ITAL 749	Street Market/ Amazon	0.27
12	ITAL 798	Street Market/ Amazon	2.74
13	ITAL 903	Processing/ Pará	4.63
14	ITAL 920	Processing/ Pará	0.97
15	ITAL 927	Processing/ Pará	<LD
16	ITAL 933	Processing/ Pará	0.13
17	ITAL 934	Processing/ Pará	0.82
18	ITAL 1001	Street Market/ Amazon	0.50
19	ITAL 1157	Street Market/ Pará	0.61
20	ITAL 1206	Street Market/ Pará	14.00
21	ITAL 1207	Street Market/ Pará	<LD
22	ITAL 1214	Street Market/ Pará	4.94
23	ITAL 1340	Processing/ Pará	0.43
24	ITAL 1344	Processing/ Pará	1.36
25	ITAL 1546	Processing/ Pará	2.04
26	ITAL 1547	Processing/Pará	2.22
27	ITAL 1642	Processing/ Pará	15.63
28	ITAL 2208	Street Market/ Amazon	0.18
29	ITAL2221	Street Market/ Amazon	3.63
30	ITAL 2636	Rainforest/ Pará	2.90
31	ITAL 3779	Rainforest/ Pará	12.82
32	ITAL 4117	Processing/ Amazon	0.85
33	ITAL 4138	Street Market/ Amazon	1.17
34	ITAL 4195	Processing/ Amazon	3.77
35	ITAL 4262	Street Market/ Amazon	29.57
36	ITAL 4956	Processing/ Pará	1.83
37	ITAL 5446	Processing/ Pará	2.19
38	ITAL 5470	Processing/ Pará	9.43
39	ITAL 6504	Supermarket/ São Paulo	2.44
40	ITAL 6573	Supermarket/ São Paulo	24.55
41	ITAL 6626	Supermarket/ São Paulo	2.19
42	ITAL 6951	Street market/ Amazon	37.25
43	ITAL 7061	Street market/ Amazon	0.98

ITAL: refers to the number of isolates in the culture collection of Instituto de Tecnologia de Alimentos; LD: Limit of detection = 0.08 mg/kg.

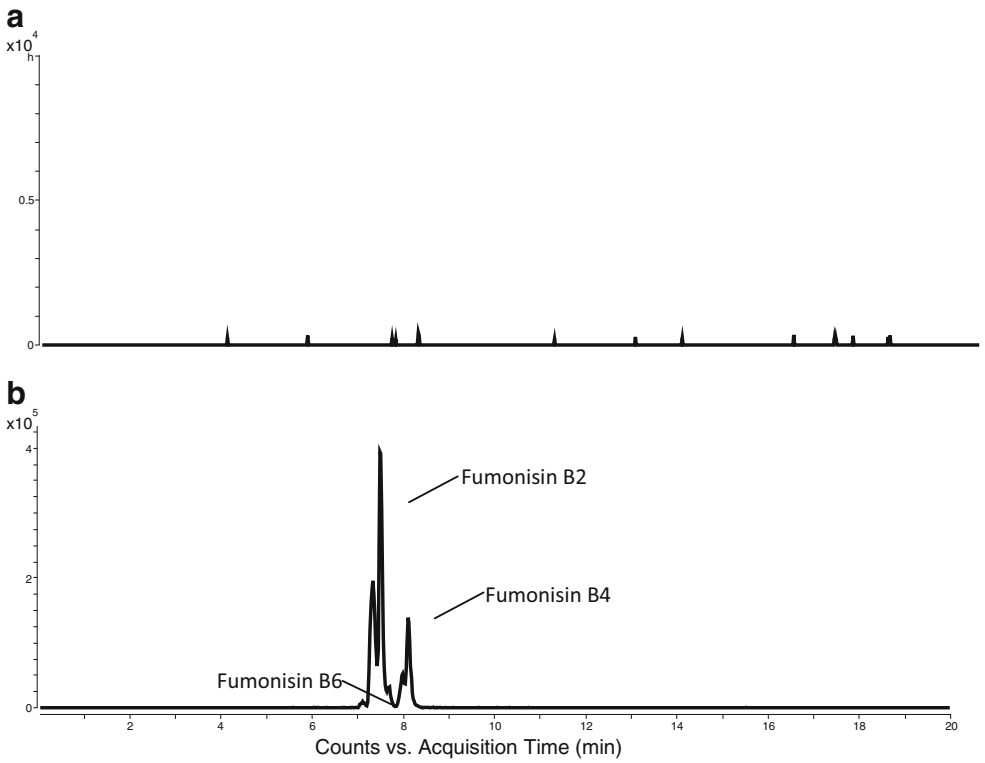

Fig. 2 Chromatogram from HRMS-UHPLC of FB_2, FB_4, and FB_6. **a** Negative strains. **b** Positive strains

Fumonisin B_2 production from *A.* section *Nigri* by HPLC

The methodology for fumonisin B_2 production and extraction had a good performance. The detection limit of this method was 0.08 mg/kg. Figure 1 shows the chromatogram peak (HPLC) of FB_2 standard and the fungal extract with retention time of 15.1 min.

Table 2 shows the isolates of *A.* section *Nigri* that were positive for FB_2 production. A total of 41 strains (20.5%) produced FB_2 in concentrations which ranged from 0.09 to 37.25 mg/kg, 2 strains (1%) had values less than the detection limit (0.08 mg/kg), and 157 (78.5%) did not produce FB_2.

Fumonisin B_2 production by *A.* section *Nigri* by UHPLC-HRMS

Figure 2 shows the chromatogram peak of (a) one isolate that did not produce FB_2, FB_4, and FB_6 and b) one isolate that produced FB_2, FB_4, and FB_6. Table 3 shows the nine isolates tested for FB_2, FB_4, and FB_6 production using UHPLC-HRMS, which confirmed the results obtained in HPLC.

Occurrence of fumonisin B_2 in Brazil nut samples

The methodology of FB_2 in Brazil nuts showed a good performance with recovery of 73 and 109% for spiking levels of 0.07 and 1 mg/kg, respectively. The detection and quantification limit was 0.032 and 0.054 mg/kg, respectively. Although several samples showed high contamination by *A.* section *Nigri*, none of the 100 samples analyzed was contaminated with FB_2. This is the first report investigating the incidence of FB_2 in Brazil nut samples at different stages of its production chain.

Molecular analysis

A portion of the *CaM* gene was sequenced using a sample of 37 isolates which were previously identified as *A. niger* "aggregate." The phylogenetic tree showed that the isolates spread into two distinct clades: one grouping with *A. niger*

Table 3 Comparison of fumonisin production by *Aspergillus* section *Nigri* using HRMS - UHPLC and HPLC detectors

# ITAL	UHPLC-HRMS	HPLC
ITAL 154	ND	ND
ITAL 181	ND	ND
ITAL 671	FB_2, FB_4 and FB_6	FB_2
ITAL 1206	FB_2, FB_4 and FB_6	FB_2
ITAL 1530	ND	ND
ITAL 3779	FB_2, FB_4 and FB_6	FB_2
ITAL 4262	FB_2, FB_4 and FB_6	FB_2
ITAL 6573	FB_2, FB_4 and FB_6	FB_2
ITAL 6951	FB_2, FB_4 and FB_6	FB_2

ITAL: refers to the number of isolate at the culture collection of Instituto de Tecnologia de Alimentos.

ND: Not Detected.

CBS 554.65 type strain and the other with its sibling species, *A. welwitschiae* CBS 139.54 type strain (Fig. 3). Twenty-one isolates (57%) were identified as *A. welwitschiae* and 16 isolates (43%) were identified as *A. niger* sensu stricto. No other species belonging to the *A. niger* "aggregate" were found.

Taking into account that not all *A. niger* and *A. welwitschiae* strains produce FB_2, all *A. welwitschiae* and *A. niger* sensu stricto here identified were analyzed for the presence of the gene *fum8* into the genoma. The PCR assay revealed 100 and 33% of *A. niger* and *A. welwitschiae* isolates, respectively, harboring the *fum8* gene in their genomes (Fig. 4).

Inoculation of fungi in Brazil nuts

FB_2 was not produced in Brazil nut kernels at a water activity of 0.705 and 0.965. The result obtained in this study showed

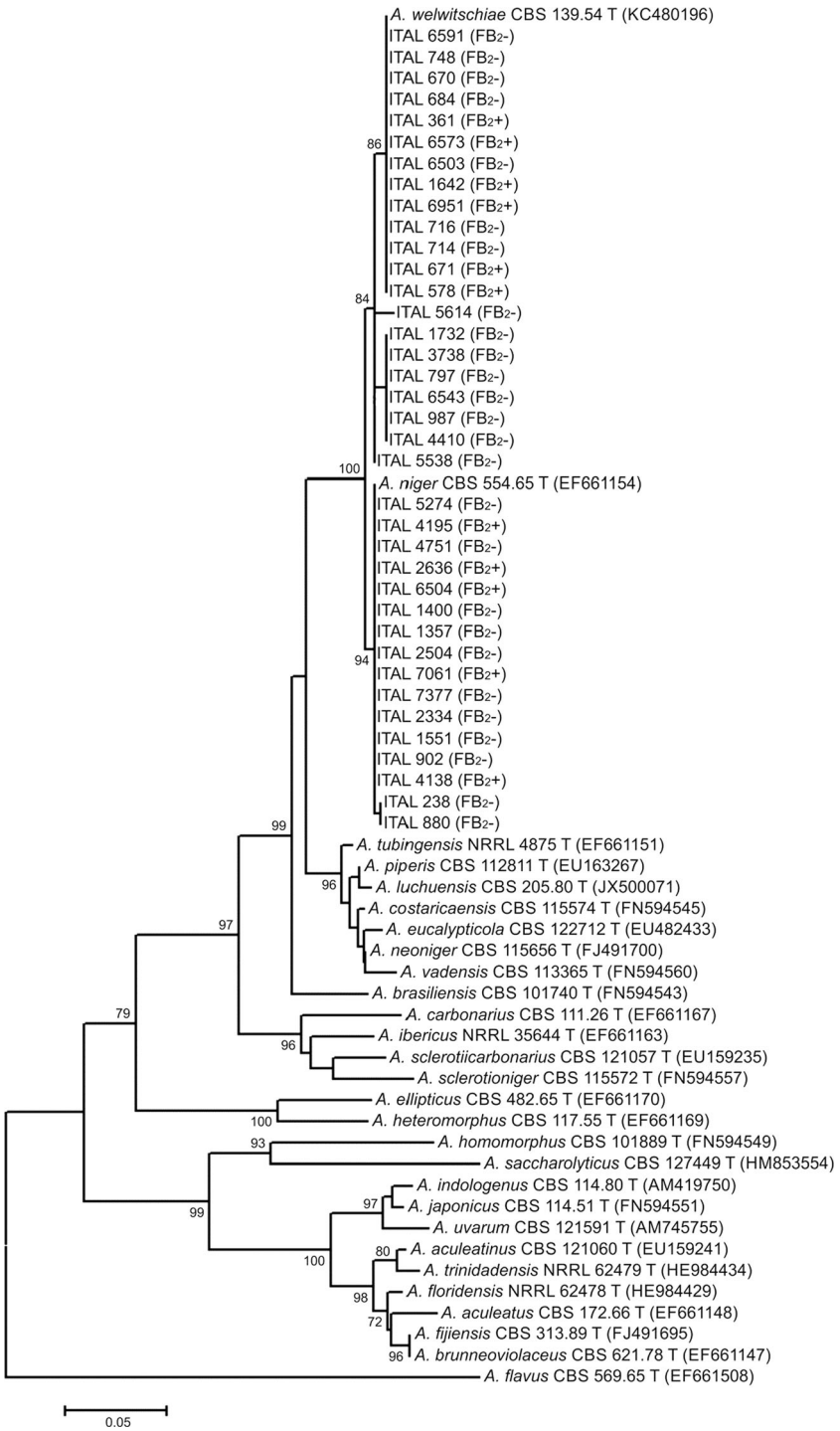

Fig. 3 Phylogenetic tree based on partial calmodulin gene sequences, using strains isolated from Brazil nuts (*ITAL*) and those sequences of type strains, obtained from GenBank database, suggested by Varga et al. (2011) and Samson et al. (2014). Numbers at the notes are percentage-bootstrapping values on 1000 replicates, and only values of >70% are shown

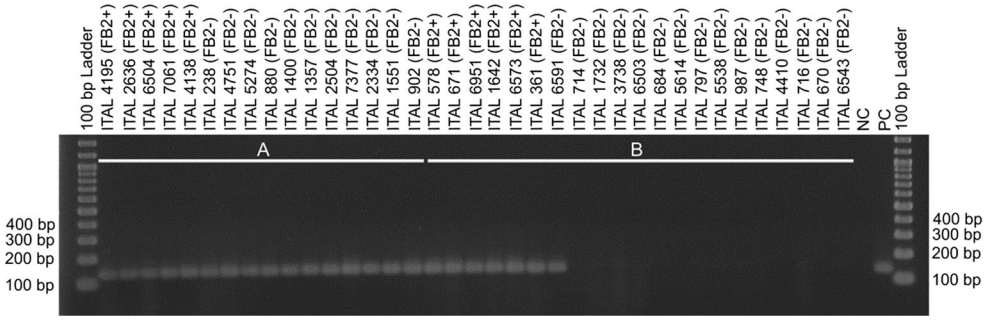

Fig. 4 Agarose gel of PCR products showing the presence or the absence of the amplicon for the gene *fum8* from *A. niger* strains (*A* group) and *A. welwitschiae* strains (*B* group). Lane NC (negative control) is the result of the PCR reaction without any DNA template. Lane PC (positive control) is a PCR product which had its identity confirmed by sequencing, as a portion of the *fum8* gene

that Brazil nuts may not be a good substrate for FB_2 production by *A. niger* or FB_2 reacted with Brazil nut components. In the sample with high water activity (0.965), there was a significant growth of the inoculated fungus but not at low a_w (0.705).

Discussion

Out of 200 isolates of *A.* section *Nigri* from Brazil nut samples tested for FB_2 production, 43 (21.5%) isolates were able to produce this toxin. This frequency is somewhat lower than found for other commodities. For example, in grapes, Abrunhosa et al. (2011) found 176 (29%) out of 597 samples to be FB_2 producers. Susca et al. (2010) found 7 (23%) out of a total of 30 in grape samples. Noonim et al. (2009) found 13 (76%) out of 17 isolates of *A. niger* from coffee in Thailand, producing FB_2 and FB_4. For industrial strains, Frisvad et al. (2011) found 145 (81%) out of 180 isolates producing FB_2. This discrepancy could be explained due to the differences in testing *A.* section *Nigri* isolates or *A. niger* sensu stricto and *A. welwitschiae*, the food source, the culture media, and/or the number of tested strains. In this study, FB_2 concentrations ranged from 0.09 to 37.25 mg/kg, while Abrunhosa et al. (2011) found a range from 0.003 to 6.0 mg/kg, which was lower than the levels reported by Susca et al. (2010) 0.1 to 293.0 mg/kg, and those reported by Varga et al. (2010) who found levels between 0.017 and 19.6 mg/kg. These data show that FB_2 production may vary among the isolates, origin, and substrates.

In the present study, FB_2 production was not detected in Brazil nuts with the method used in this work. Some reports show that due to fumonisin chemical structures, they can react with food components causing extractability problems in some matrices such as maize (Humpf and Voss 2004; Scott and Lawrence 1994). Shier et al. (2000) proposed that fumonisins can link covalently to starch and proteins present in food; this fact was later confirmed by Seefelder et al. (2003). *A. niger* grew well when inoculated in Brazil nuts at water activity of 0.965, but FB_2 was not produced. This mycotoxin was also not found in Brazil nut samples in the study carried out by Freitas-Silva et al. (2011) in which 235 different metabolites were analyzed. In other studies, *A. niger* was reported to produce FB_2 and FB_4 in grapes and raisins (Mogensen et al. 2010), and small amounts (1 to 9.7 ng/g) of FB_2 were detected naturally in coffee (Noonim et al. 2009). Studies by Mogensen et al. (2010) comparing the growth and FB_2 production in *A. niger* and *Fusarium* spp. showed that *A. niger* can grow at a_w values from 0.92 to 1.0 but FB_2 production only occurred at a_w 0.99 and 0.98. On the other hand, Frisvad et al. (2007) reported that *A. niger* was able to produce high amounts of FB_2 in culture media with a_w of 0.955. The food matrix composition may play a role in FB_2 production in agricultural products and more studies are needed to clarify the importance and interaction of *A. niger* and food matrix on food safety.

Aspergillus welwitschiae (=*A. awamori* sensu, Perrone et al. 2011) is a new species, which was dismembered from the *A. niger* taxon (Samson et al. 2014). It was revealed that the morphological characteristics of *A. niger* and *A. welwitschiae* (=*A. awamori* sensu, Perrone et al. 2011) overlap and extrolite profiles of *A. niger* and *A. welwitschiae* were similar. OTA and FB_2 and FB_4 were produced by some isolates of both species (Frisvad et al. 2011). Therefore, these two species can only be distinguished using molecular approaches. *A. welwitschiae* has been found in dried fruits, grapes, coffee beans, and cocoa (Massi et al. 2016), although its proportion with regard to *A. niger* sensu stricto is still unknown. Gherbawy et al. (2015) reported *A. welwitschiae* as the prevalent species in onion samples. Based on *CaM* sequences, we found that *A. welwitschiae* and *A. niger* occur with similar frequency in Brazil nuts, e.g., 57 and 43%, respectively. PCR results revealed that the frequency of strains harboring the gene *fum8*, which encodes an essential enzyme for fumonisin biosynthesis, was clearly different from *A. welwitschiae* and *A. niger* sensu stricto. Interestingly, the *fum8* gene was detected in all *A. niger* isolates, regardless of their ability to produce fumonisin. Differently, *fum8* was not

detected in most of the *A. welwitschiae* fumonisin non-producing isolates. Our results are in accordance with those recently found by Susca et al. (2014) when studying the mycobiota recovered from grapes and by Massi et al. (2016) that investigated *A.* section *Nigri* from different sources such as dried fruits, grapes, Brazil nuts, onions, coffee beans, and cocoa.

In conclusion, the results obtained from this study showed that Brazil nuts can be infected by *A.* section *Nigri* that produce FB_2 such as *A. welwitschiae* and *A. niger.* Although the Brazil nut samples had FB_2 producing fungi, the toxin was not detected in any sample.

Acknowledgements This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (Process 2009/07693-6), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) together with Ministério da Agricultura, Pecuária e Abastecimento, (MAPA, Process 578485/2008-7).

Conflict of interest The authors declare that they have no conflict of interest.

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