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Mycobiota and mycotoxins in Brazil nut samples from different states of the Brazilian Amazon region

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ABSTRACT

The objective of this study was to evaluate the presence of fungi and mycotoxins (aflatoxins and cyclopiazonic acid) in Brazil nut samples collected in different states of the Brazilian Amazon region: Acre, Amazonas, Amapá, and Pará. A total of 200 husk samples and 200 almond samples were inoculated onto *Aspergillus flavus–parasiticus* agar for the detection of fungi. Mycotoxins were analyzed by high-performance liquid chromatography. The mycobiota comprised the following fungi, in decreasing order of frequency: almonds — *Phialemonium* spp. (54%), *Penicillium* spp. (16%), *Fusarium* spp. (13%), *Phaeoacremonium* spp. (11%), and *Aspergillus* spp. (4%), husks — *Phialemonium* spp. (62%), *Phaeoacremonium* spp. (11%), *Penicillium* spp. (10%), *Fusarium* spp. (9%), and *Aspergillus* spp. A polyphasic approach was used for identification of *Aspergillus* species. Aflatoxins were detected in 22 (11%) of the 200 almond samples, with 21 samples presenting aflatoxin B_1 levels above 8 μg/kg, the limit established by the European Commission for Brazil nuts for further processing. Nineteen (9.5%) of the 200 husk samples contained aflatoxins, but at levels lower than those seen in almonds. Cyclopiazonic acid (CPA) was detected in 44 (22%) almond samples, with levels ranging from 98.65 to 161.2 μg/kg. *Aspergillus nomius* and *A. flavus* were the most frequent *Aspergillus* species. The presence of fungi does not necessarily imply mycotoxin contamination, but almonds of the Brazil nut seem to be a good substrate for fungal growth.

1. Introduction

The Brazil nut tree (*Bertholletia excelsa*) is a plant native to the Amazon region. Nuts, especially Brazil nuts, are among the most nutritious human foods because of their high content of proteins, carbohydrates, unsaturated lipids, vitamins, and essential minerals (USDA, 2010). The production and extraction of the Brazil nut occurs only in the Amazonian biome (Freitas-Silva and Venancio, 2011).

The Brazilian Amazon region is divided into Western and Eastern regions. The Western Amazon region comprises part of Amazonas (starting from Manaus city to the West), and the Eastern Amazon region comprises the states of Para, Amapa, the Northern part of Maranhao, and the Eastern part of Amazonas (starting from Manaus city to the East) (Brazil, 1968).

Fungal infection of Brazil nuts has been studied since the beginning of the last century (Spencer, 1921; Castrillón and Purchio, 1988). Contamination with aflatoxigenic *Aspergillus* species is directly related to the climatic conditions in the Amazon rainforest (hot and humid climate) during the period of harvest (Pacheco and Scussel, 2006; Olsen et al., 2008). *Aspergillus* section *Flavi* comprises a closely related group of fungi, which are widely distributed in soil, air, organic matter, and plant parts all over the world (Raper and Fennell, 1965; Cotty and Cardwell, 1999). Three species of this group (*Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*) have received considerable attention because of their ability to produce aflatoxins.

Aflatoxins are the most potent natural carcinogens affecting animals and humans (JECFA, 1997). Contamination with aflatoxins is a matter of serious concern for exporters of Brazil nuts as the Commission Regulation of European Union no. 165/2010 of 26 February 2010 has established maximum aflatoxin levels for Brazil nuts. In nuts for further processing and ready to eat nuts, respectively, the limits are 8.0 μg/kg and 5.0 μg/kg for AFB_1 and 15.0 μg/kg and 10.0 μg/kg for total aflatoxins $AFB_1 + AFB_2 + AFG_1 + AFG_2$ (EC, 2010). Cyclopiazonic acid (CPA) is an indole tetramic acid that is toxic to animals and has been implicated in human poisoning (Rao and Husain, 1985). This toxin is produced by a variety of fungi, including some *Penicillium* species (Le Bars, 1979), and some *A. flavus* strains (Yokota et al., 1981).

The taxonomy of *Aspergillus* is in a transition phase and there is currently no consensus regarding the definition of taxa within groups. The discussion continues about which molecular tools are most appropriate for the characterization of taxa, since the identification of some species is difficult due to variations and overlapping morphological and biochemical characteristics of the isolates. Sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) is recommended

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for the identification of species of the genus *Aspergillus* (Samson et al., 2006). Lately there has been a trend in the sequencing of more than one gene for the purpose of increasing the reliability of results. Besides the use of ITS region, the β-tubulin (Glass and Donaldson, 1995) and calmodulin (Carbone and Kohn, 1999) genes have been used for sequencing of fungi. In this respect, the study of DNA sequences may provide important information for the definition of species and their appropriate identification (Hinrikson et al., 2005).

The objective of the present investigation was to identify the mycobiota and occurrence of aflatoxins and CPA in Brazil nut samples (husks and almonds) collected in different states of the Brazilian Amazon region (Amazonas, Acre, Amapá, and Pará).

2. Materials and methods

2.1. Study area — geographic and climatic characterization

Brazil nuts from storage were collected in different states of the Brazilian Amazon region: Amazonia, at latitude 5° 48′S, longitude: 61° 18′O, and altitude 45 m, Acre at latitude: 9° 58′S, longitude: 67° 48′O, altitude: 153 m, Amapá, at latitude: 1° 07′S, at longitude: 52° 00′O, altitude: 20 m and Pará at latitude: 1° 57′S, at longitude: 48° 11′O, altitude: 10 m (Fig. 1).

2.2. Samples

The samples were collected in March 2009 from cooperatives in northern Brazil: Amazonas, Acre, Amapá, and Pará. Fifty samples of 400 g each were collected per state, corresponding to 20 kg Brazil nuts per cooperative warehouse. The husks of each nut were carefully and aseptically removed by cracking it with a breaker nut. After the nut samples were dehusked, they were divided into 200 husk and 200 almond samples. These samples were subsequently analyzed for moisture content, presence of fungi, aflatoxins, and CPA.

2.3. Moisture content and a_w

The moisture content was performed in duplicate by the classical method, according to ISO 712. The husks and almonds of Brazil nuts were triturated and 5 g ± 1 mg of each were placed on a metal plate in a ventilated oven at constant temperature (130–133 °C) for 3 h and the samples were weighed again. The percentage of difference between the initial and final weight of the samples was moisture content.

A few pieces of the samples (almonds and husks) were placed in a disposable cup and the water activity (a_w) was determined in duplicate by the dew point method with an Aqualab CX-2 apparatus (Decagon Devices, Inc., Pullman, WA, USA).

2.4. Isolation and identification of mycobiota

Ten grams of each husk and almond sample was mechanically triturated and diluted in 90 mL sterile distilled water. Successive decimal dilutions were prepared to 10^{-4}, and 0.1 mL of each dilution was inoculated into *A. flavus–parasiticus* agar supplemented with chloramphenicol (Pitt et al., 1983). The plates were incubated at 25 °C for until 7 days in the dark. The results are reported as colony-forming units per gram of substrate (CFU/g).

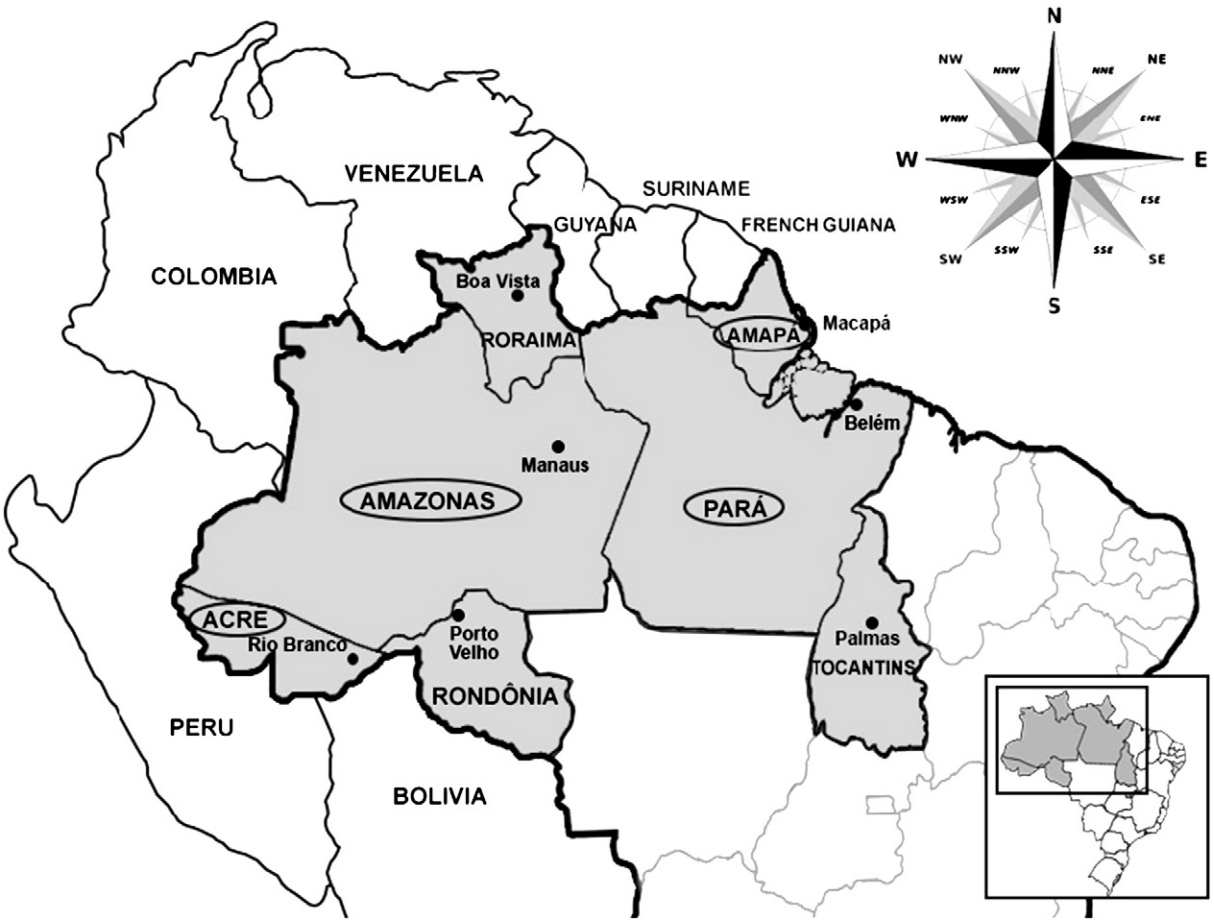

Fig. 1. Geographical location of the study area (Acre, Amazonas, Amapá, and Pará).

All colonies were subcultured on potato dextrose agar (Oxoid, Basingstoke, UK) at 25 °C for 7 days. Colonies were identified to genus level according to Barron (1972) and Pitt and Hocking (2009). A polyphasic approach consisted of the following steps for the identification of *Aspergillus* strains: investigation of morphological features according to the textbooks of Pitt and Hocking (2009) and Raper and Fennell (1965), growth and examination of cultures in Czapek agar and Blakeslee's malt agar at 25 °C and 42 °C for 7 days (Kurtzman et al., 1987; Peterson et al., 2001), observation of colony reverse color in AFPA at 25 °C for 3 days (Pitt et al., 1983), aflatoxins production (Lin and Dianese, 1976; Pitt and Hocking, 2009) and sequencing of rDNA regions: (i) internal transition spacer (ITS) as described by White et al. (1990), (ii) β-tubulin as described by Glass and Donaldson (1995) and (iii) calmodulin according to Carbone and Kohn (1999).

2.5. Chemicals and reagents

AFB_1, AFB_2, AFG_1, AFG_2 and CPA standards were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Stock solutions were prepared in methanol and kept at −20 °C. All solvents used were of liquid chromatography grade and were purchased from Merck (Darmstadt, Germany). Milli-Q water was produced in our laboratory using the Millipore Academic System.

2.6. Determination of aflatoxins

For extraction, 25 g of each sample was triturated and homogenized. Next, 12.5 mL of an acetonitrile:water (85:15, v/v) solution was added to 2.5 g of almonds and to 5 g of husks. The mixture was shaken for 60 min. After centrifugation, 5 mL of the supernatant containing almonds was mixed with 45 mL acidified water (0.5% glacial acetic acid), and 2.5 mL of the supernatant containing husks was mixed with 47.5 mL acidified water. The solutions were cleaned on a Strata C18-E cartridge (500 mg/3 mL) (Phenomenex, Santa Clara, CA, USA) at a flow rate of 1 drop/s. The cartridges were washed with 12 mL acidified water. Aflatoxins were eluted with 1 mL methanol and the eluent was evaporated to residue (Tanaka et al., 2002; Lebret et al., 2004). The residues were derivatized with trifluoroacetic acid (TFA) and hexane, again evaporated, and resuspended in 400 μL of a methanol:water (1:1, v/v) solution. The mixture (40 μL — almonds and 20 μL — husks extracts) was injected into the Shimadzu Prominence HPLC system (Kyoto, Japan) equipped with an RF 10AXL fluorescence detector (excitation: 365 nm, emission: 450 nm) and an autosampler system. The analytical column (Shimadzu, Shim-Pack VP ODS, 150×4.6 mm) was coupled to a pre-column cartridge (Shim-Pack GVP-ODS, 10 mm×4.6 mm) maintained at 40 °C in an oven. The isocratic mobile phase consisted of acetonitrile:methanol:water (1.5:1.5:8, v/v/v) + 0.1% TFA and was eluted at a flow rate of 1 mL/min.

2.7. Determination of CPA

CPA was analyzed according to the method of Urano et al. (1992), with modifications. Twenty-five grams of each previously triturated and homogenized sample was divided into aliquots of 2.5 g and mixed with 20 mL methanol: 2% sodium bicarbonate in water (7:3, v/v). After shaking for 45 min, the samples were centrifuged and 8 mL of the supernatant was transferred to a separating funnel. Then, 10 mL hexane was added and the mixture was gently shaken. After separation of the phases, the hexane fraction was discarded and 8 mL 10% KCl was added to the aqueous fraction. The latter was acidified with 6 N HCl until pH 3.0 was obtained.

CPA was extracted with two partitions of 10 mL chloroform. The chloroform phase was collected and evaporated to residue. The residue was then resuspended in 1000 μL methanol and injected 10 μL into the Shimadzu Prominence HPLC system equipped with a Shimadzu SPD-10A UV–vis detector set at 282 nm, an autosampler system, and a Luna C8 column (4.6×250 mm, 5-μm particle size, Phenomenex, Torrance, CA, USA) maintained at 40 °C in an oven. The isocratic mobile phase consisted of 4 mM $ZnSO_4$ in methanol: water (6:4, v/v) and was eluted at a flow rate of 1 mL/min.

2.8. DNA extraction, amplification and sequencing

The strains were confirmed by sequencing of the ITS (White et al., 1990), β-tubulina (Glass and Donaldson, 1995) and calmodulin (Carbone and Kohn, 1999) gene regions of rDNA. DNA was extracted and purified directly from fungal colonies grown on yeast extract sucrose agar at 25 °C in the dark for 3 days using the PrepMan Ultra® kit (Applied Biosystems, Carlsbad, CA, USA). DNA was quantified with the GeneQuant pro Calculator (Amersham Pharmacia Biotech, Cambridge, UK). A fragment of ITS region was amplified with the ITS1 (5′ TCCGTAGGTGAACCTGCG 3′) and ITS4 (5′ TCCTCCGCTTATTGATAT 3′) primer pairs. Part of the gene β-tubulin was amplified using primers T22 (5′ TCTGGATGTTGTTGGGAATCC 3′) and TUB-F (5′ CTGTCCAACCCCTCTTAGGGCGACT 3′). Part of the calmodulin gene was amplified using primers CMD 42 (5′ GGCCTTCTCCCTATTCGTAA 3′) and CMD 637 (5′ CTCGCGGATCATCTCATC 3′). The PCR mixture contained 12.5 μL 2× PCR Master Mix (Promega, San Luis Obispo, CA, USA), 6.5 μL Milli-Q water, 2 μL DNA (40 ng), and 2 μL (20 pmol) of each primer (Prodimol Biotecnologia, Minas Gerais, Brazil) in a final volume of 25 μL. The amplification program included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C (ITS), 49 °C (β-tubulin), or 54 °C (camodulin) for 1 min, and extension at 72 °C for 1 min. A final extension step at 72 °C for 5 min was included at the end of the amplification.

After PCR, the products were purified with the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and stored at −20 °C until the time of sequencing. The PCR products were sequenced using the same primers as those employed for amplification. The Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) was used. The reactions were run on a 3100 DNA sequencer (Applied Biosystems). Consensus sequences were obtained using the AutoAssembler program (Perkin Elmer-Applied Biosystems) and SeqMan software (Lasergene, Madison, WI, USA). The sequences were used in BLASTn searches (www.ncbi.nlm.nih.gov) in order to confirm preliminary identifications.

2.9. Aflatoxigenic potential of Aspergillus section Flavi strains

A fragment of each colony was inoculated into a plate containing coconut agar (Lin and Dianese, 1976) and incubated at 25 °C for 14 days. After growth, the whole content of each plate was removed and chloroform (30 mL per 10 g of culture) was added. After shaking, the sample was filtered through Whatman No. 1 filter paper. The content was evaporated to residue and the extracts were resuspended in 2 mL chloroform. Next, 100 μL of this solution was evaporated and resuspended in 3 mL acetonitrile:water (85:15, v/v), and diluted in 27 mL acidified water. The cleanup steps and injection into the HPLC system were the same as those described in item 2.6.

3. Results

3.1. Moisture content, a_w, and mycobiota in almonds and husks

The mean moisture content of almond samples was 4.37, with the highest value (10.30) being observed in sample from the State of Amapá and the lowest value (2.50) in sample from Pará. The mean moisture content of husks samples was 14.96, with the highest value (22.00) being observed in sample from the State of Amapá and the lowest activity (12.2) in the State of Pará (Table 1).

The mean a_w of almond samples was 0.76, with the highest value (0.96) being observed in sample from the State of Acre and the lowest

Table 1
Water activity and moisture content (mean, maximum and minimum levels, and standard deviation) in Brazil nut samples (200 almonds and 200 husks) in four states of Brazil.

State	Sample	Water activity				Moisture content			
		Mean[a]	SD[b]	Max.[c]	Min.[d]	Mean	SD	Max.	Min.
Acre	Almond	0.85	0.08	0.96	0.69	3.70	0.36	5.30	3.20
	Husk	0.77	0.10	0.94	0.51	14.30	0.50	15.30	13.40
Amazonas	Almond	0.81	0.05	0.93	0.68	3.83	0.25	4.50	3.40
	Husk	0.75	0.06	0.87	0.58	14.1	0.66	16.30	13.10
Amapá	Almond	0.76	0.11	0.95	0.55	5.82	1.59	10.30	3.80
	Husk	0.68	0.08	0.85	0.56	16.73	2.44	22.00	12.90
Pará	Almond	0.64	0.06	0.79	0.56	4.15	0.85	8.30	2.50
	Husk	0.62	0.03	0.71	0.55	14.51	1.36	17.10	12.20

[a] Mean level.
[b] Standard deviation.
[c] Maximum level.
[d] Minimun level.

value (0.55) in sample from Amapá. The mean water activity of husk samples was 0.70, with the highest value (0.94) being observed in sample from the State of Acre and the lowest activity (0.51) in the same State (Table 1).

Identification of mycobiota from Brazil nut samples (200 husk and 200 almond samples) revealed the presence of the following fungi, in decreasing order of frequency: almonds — *Phialemonium* spp. (54%), *Penicillium* spp. (16%), *Fusarium* spp. (13%), *Phaeoacremonium* spp. (11%), and *Aspergillus* spp. (4%), husks — *Phialemonium* spp. (62%), *Phaeoacremonium* spp. (11%), *Penicillium* spp. (10%), *Fusarium* spp. (9%), and *Aspergillus* spp. (5%) (Table 2).

Phialemonium dimorphosporum (=*P. curvatum*) was the most frequent species of this genus in the samples analyzed and could only be identified by sequencing of the ITS region. Species of the genus *Aspergillus*, which are important from the mycotoxicological point of view, were detected in husks (5%) and almonds (4%). The level of contamination (CFU/g) with *Aspergillus* was 307 (Amazonas), 910 (Acre), 377 (Amapá) and 91 CFU/g (Pará) in almonds, and 1948 (Amazonas), 9000 (Acre), 1521 (Amapá) and 687 CFU/g (Pará) in husks. *A. nomius* and *A. flavus* were the most frequent *Aspergillus* species (Table 3).

3.2. Limits of detection, limits of quantification, and recoveries tests for mycotoxins

Limit of detection (LOD) was the lowest concentration that could be determined to be different from a blank. The estimated values of the LOD were based on the relationship of five times of baseline noise and determined by analysis of standard solutions of each analyte, decreasing the concentrations until the lowest detectable level (IUPAC and Currie, 1999).

Limit of quantitation (LOQ) was the level above which quantitative results could be obtained with a specified degree of confidence (IUPAC and Currie, 1999). The LOQ was determined by analysis of standard solutions of each mycotoxin, decreasing the concentrations until the lowest quantified level with acceptable precision and accuracy.

To test for recoveries of mycotoxins, each 5 g Brazil nut flour samples (almonds and husks) was spiked with a mixture of four aflatoxins or CPA standards. The samples were allowed to stand overnight and then extracted by using the described procedures, according to Sections 2.6. and 2.7.

3.3. Determination of aflatoxins

The calibration curves were constructed using five concentrations of each aflatoxin standards: 0.625, 1.25, 2.5, 5.0, and 10 ng/mL corresponding to 0.25, 0.5, 1.0, 2.0, and 4 μg/kg, respectively. Quantification of aflatoxins was performed by measuring peak area at each aflatoxin retention time and comparing it with the relevant standard curve. If test solution area response was outside (higher than) the standard range, the test extract was diluted with methanol:water (1:1, v/v) solution and reinjected into the LC column (Bao et al., 2010). The coefficients of the calibration curves were 0.999 for all aflatoxins. The detection and quantification limits in almonds and husks were 0.75 and 0.5 μg/kg for each aflatoxin, respectively.

Almond and husk samples were spiked with a mixture of four aflatoxin standards to obtain a level of 10 μg/kg. The samples were allowed to stand overnight and then extracted using the procedure described in Section 2.5. Average recoveries of aflatoxins from samples spiked (determined in quintuplicate) were 80.4% for AFB_1, 81.1% for AFB_2, 83.3% for AFG_1, and 84.3% for AFG_2, and the coefficients of variation of recovery in quintuplicate samples were: 6.4%, 3.5%, 4.7%, and 6.7% for AFB_1, AFB_2, AFG_1, and AFG_2, respectively. Retention times were about 5.0, 7.5, 11.0, 17.0 min (±5%) for AFG_1, AFB_1, AFG_2, and AFB_2, respectively (Fig. 2).

Among the 200 almond samples analyzed, aflatoxins were detected in 4 (8%) samples from Acre, in 9 (18%) from Amazonas, in 5 (10%) from Amapá, and in 4 (8%) from Pará. The aflatoxin levels in samples from the State of Acre exceeded 15 μg/kg. According to the Brazilian Health Surveillance Agency (ANVISA, Resolution RDC No. 7 of February 18, 2011, published February 22, 2011) and Commission Regulation of European Union (EC, 2010), the maximum tolerance limit of aflatoxins in shelled Brazil nuts destined for subsequent processing is 15 μg/kg. The highest concentrations of aflatoxins were detected in a sample from the Acre region (1058.00 μg/kg AFB_1 and 1655.04 μg/kg total aflatoxins). Eight of the 9 positive samples from Amazonas presented total aflatoxin levels higher than 15 μg/kg. In contrast, only one of the contaminated samples from Amapá had levels higher than 15 μg/kg (46.41 μg/kg total aflatoxins). The maximum level detected in positive samples from Pará was 7.12 μg/kg total aflatoxins (Table 4).

Table 2
Mean relative frequency of the main fungi isolated from 400 Brazil nut samples (200 almonds and 200 husks) in four states of Brazil.

State	Sample	Frequency of the main isolated fungi (%)					
		Aspergillus spp.	*Phialemonium* spp.	*Phaeoacremonium* spp.	*Penicillium* spp.	*Fusarium* spp.	Others[a]
Acre	Almond	10	61	10	11	5	3
	Husk	16	57	5	16	2	4
Amazonas	Almond	1	50	12	14	22	1
	Husk	1	68	9	11	10	1
Amapá	Almond	3	54	12	6	23	2
	Husk	3	55	12	6	24	0
Pará	Almond	2	50	12	34	2	0
	Husk	2	68	19	9	2	0
Mean	Almond	4	54	12	16	13	1
	Husk	6	62	11	11	10	0

[a] Other isolated fungi: *Alternaria* spp., *Cladosporium* spp., *Curvularia* spp., *Dreschlera* spp., *Rhizopus* spp., *Thrichoderma* spp., and *Verticillium* spp.

Table 3
Relative frequency of 254 *Aspergillus* strains isolated from Brazil nut samples.

State	Sample	Frequency of *Aspergillus* species (%)									
		A. nomius	*A. flavus*	*A. parasiticus*	*A. pseudotamarii*	*A. caelatus*	*A. tamarii*	*A. sclerotium*	*A. sydowii*	*A. persii*	*A. versicolor*
Acre	Almond	52.5	17.5	2.5	0	0	15.0	2.5	2.5	0	7.5
	Husk	50.0	39.6	1.7	3.5	0	0	0	1.7	0	3.5
Amazonas	Almond	80.0	13.3	0	0	0	0	0	0	0	6.7
	Husk	75.0	12.5	0	0	6.25	6.25	0	0	0	0
Amapá	Almond	67.7	19.4	6.5	0	0	0	0	0	3.2	3.2
	Husk	66.0	34.0	0	0	0	0	0	0	0	0
Pará	Almond	7.7	69.2	0	0	0	0	0	15.4	0	7.7
	Husk	77.4	16.2	3.2	0	3.2	0	0	0	0	0
Mean	Almond	56.0	24.5	2.85	0	0	6.1	0.8	2.85	0.8	6.1
	Husk	63.8	30.5	1.3	1.3	1.3	0.5	0	0	0	1.3

The frequency of aflatoxins among the 200 husk samples was lower than that seen among almond samples: Acre (10%), Amazonas (14%), Amapá (10%), and Pará (4%) (Table 2).

3.4. Determination of CPA

The calibration curve was constructed using five concentrations of CPA standards: 66.6, 100, 133.4, 166.7, and 333.2 μg/mL corresponding to 66.6, 100, 133.4, 166.7, and 333.2 μg/kg, respectively. Quantification of CPA was performed by measuring peak area at CPA retention time and comparing it with relevant standard curve. The coefficient of the calibration curve was 0.997 for CPA. The detection and quantification limits of CPA in both husks and almonds samples were 50 and 100 μg/kg, respectively.

Almond and husk samples were spiked with a CPA standard to obtain a level of 500 μg/kg. The samples were allowed to stand overnight and then extracted using the procedure described in Section 2.6. Average recovery of CPA from samples spiked (determined in quintuplicate) was 76.4%. The coefficient of variation of recovery in quintuplicate samples was 7.3%. Retention time was about 10.0 min (±5%) for CPA (Fig. 3). Among the 200 almond samples analyzed, CPA was detected in 6% of samples from Acre, in 2% from Amazonas, in 4% from Amapá, and in 10% from Pará. In almond samples, the levels of CPA ranged from 124.67 to 151.33 μg/kg in the three positive samples from the State of Acre, and from 98.65 to 123.31 μg/kg in the five positive samples from Pará. The concentration of CPA was 132.86 μg/kg in the positive sample from the State of Amazonas, and 143.26 and 161.2 μg/kg in the two contaminated samples from Amapá.

In husk samples, the levels of CPA ranged from 101.30 to 133.5 μg/kg, being detected in 10% of husk samples from Acre, in 10% of samples from Amapá, and in 10% of samples from Pará. However, this mycotoxin was not detected in samples from Amazonas.

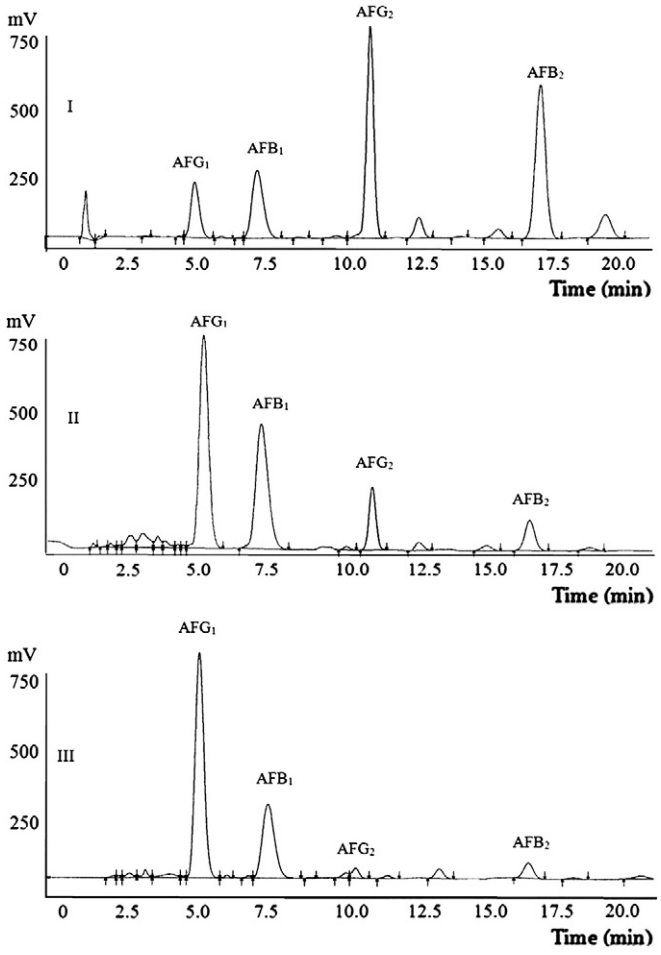

Fig. 2. Chromatographic representations of aflatoxins: (I) standards, (II) almond sample, and (III) husk sample.

3.5. Identification of Aspergillus section Flavi

Among the 254 *Aspergillus* strains isolated from almonds and husks, 98 strains were from Acre, 31 from Amazonas, 81 from Amapá, and 44 from Pará.

Aspergillus section *Flavi* strains isolated were identified by polyphasic approach as *A. nomius* (Acre: 56%, Amazonas: 81%, Amapá: 68%, and Pará: 62%). *A. flavus* was isolated at a lower frequency in all regions (Acre: 33%, Amazonas: 13%, Amapá: 29%, and Pará: 35%). Other species isolated at low frequency were *A. parasiticus* (Acre: 2%, Amapá and Pará: 3%), *Aspergillus tamarii* (Acre: 7% and Amazonas: 3%), *Aspergillus pseudotamarii* (isolated only in Acre: 2%), and *Aspergillus caelatus* (Amazonas: 3% and Pará: 2%). Other *Aspergillus* species were also identified: one *Aspergillus sclerotium* strain in a sample from Acre and five *Aspergillus versicolor* strains in samples from Acre and one strain each in samples from Amazonas, Amapá and Pará. Two *Aspergillus sydowii* strains (Acre and Pará) and one *Aspergillus persii* (Amapá) were also isolated.

Analysis of aflatoxigenic potential of the *A. flavus* revealed aflatoxin production (AFB_1 and AFB_2) by 52% of the strains, with levels ranging from 67.83 to 187,098.86 μg/kg (AFB_1). However, all of the *A. nomius*, *A. parasiticus*, and *A. pseudotamarii* strains produced AFB_1, AFB_2, AFG_1, and AFG_2, with levels ranging from 2313.68 to 285,901.89; 2102.64 to 44,722,00, and 1137.83 to 23,538.80 μg/kg for AFB_1, respectively. The maximum level of AFB_1 was observed in the strain of *A. nomius* isolated from state of Amazonas.

4. Discussion

Contamination by fungi and aflatoxins is a problem for Brazil nuts. Industry and producers have made considerable efforts over the last 15 years to minimize fungal growth and aflatoxin production, particularly in the case of Brazil nuts. Due to climatic conditions in the Amazon region, with an average temperature of 26 °C and relative humidity of 80–95%, favor the production of mycotoxins. Because Brazil nuts are harvested from the wild, it is hard to control these factors (Freitas-Silva and Venancio, 2011).

The main stages in Brazil nut production are: collection in the forest (cleaning paths between trees, gathering the fruit, opening the fruit and transporting them to the camp), processing (cleaning, drying and soaking, peeling the nuts, drying the peeled nuts) and packaging in the packing house (Freitas-Silva and Venancio, 2011). In the Amazon forest, after the fruit pods fall, they stay in direct contact with the soil for several days prior to harvest and during this time, the fruit pods can be contaminated with fungi species (Arrus et al., 2005). Baquião et al. (2012) nominated soil as the main route of fungal contamination in Brazil nuts. Fungal infection in Brazil nuts may occur by the porosity of the husks (Spencer, 1921), through the action of insects, birds and other mechanical damage, and these factors, in association with the climatic conditions of the region, may favor the penetration and propagation of fungi from fruit pod to husks and from husks to almonds (Castrillón and Purchio, 1988; Arrus et al., 2005).

In our study, the frequency of fungal isolation was higher in husks than almonds of Brazil nuts. Husks of Brazil nuts are mainly composed of lignin and cellulose (Bonelli et al., 2001). Cellulose endows the husk with hygroscopic characteristics, that linked to climatic conditions of the Amazon region, influence on their moisture content. There is a tendency for the moisture content of the husk to balance with air relative humidity. However, the moisture content of the husks is not fully transferred to almonds due to their high lipid content (hydrophobic characteristic). This would explain the different values of moisture content in husk (mean: 14.96) and almond (mean: 4.37) samples shown in our study (Table 1). While the moisture content refers to total amount of water determined in a substrate, the water activity measures the amount of free water. In the present study, the means of water activity were 0.70 and 0.76 for husks and almonds, respectively (Table 1), which were below the minimum range of 0.78–0.80 established for the growth of *Aspergillus* (Lacey et al., 1991) and mycotoxin production (higher than 0.85) (Schmidt-Heydt et al., 2009). Moreover, the husks of Brazil nuts can be a protective barrier against oxidizing agents in almonds, and also maintain their quality (Ribeiro et al., 1993).

In the present study *Phialemonium* spp. was the main isolated fungal species. According to Guarro et al. (1999a, 1999b), *Phialemonium* spp. is characterized by its morphological similarity with the genera *Fusarium* and *Acremonium*. *Phialemonium* spp., a dematiaceous fungus, has no evident dark pigments. These species were initially described as an intermediate genus between *Acremonium* spp. and *Phialophora* spp. (Gams and McGinnis, 1983). The genus *Phialemonium* includes the species *Phialemonium curvatum*, *Phialemonium dimorphosporum* and *Phialemonium obovatum*. However, *P. dimorphosporum*, which is considered a synonym of *P. curvatum*, is the most frequent species (Guarro et al., 1999a, 1999b). These fungi are widely distributed in

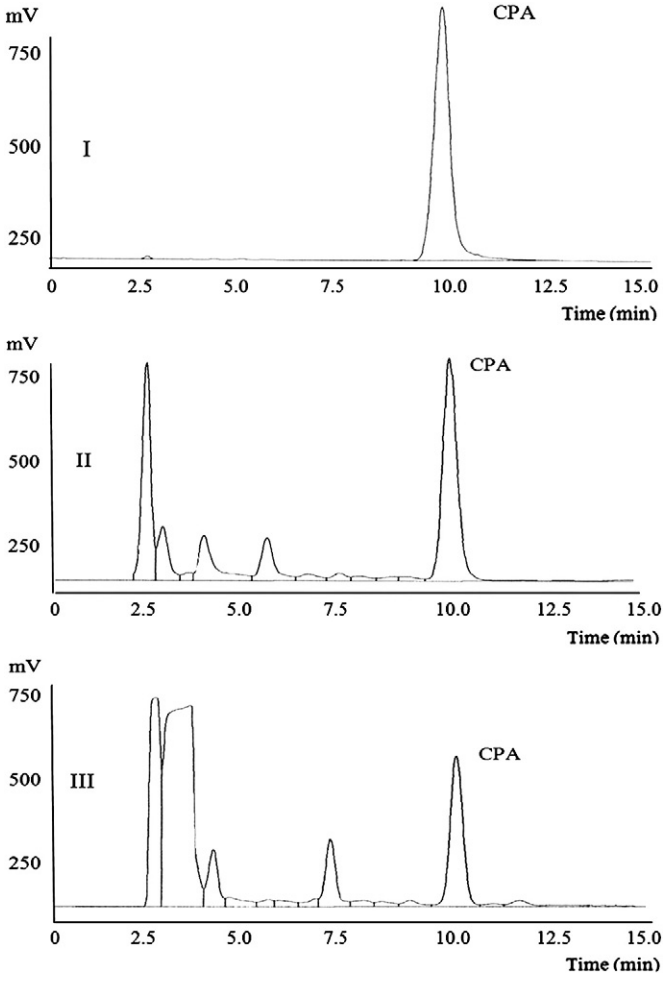

Fig. 3. Chromatographic representations of CPA: (I) standard, (II) almond sample, and (III) husk sample.

Table 4
Mycotoxin (aflatoxins and cyclopiazonic acid) contamination of Brazil nut samples.

State	Sample	Aflatoxins								Cyclopiazonic acid	
		AFB_1		AFB_2		AFG_1		AFG_2			
		Positive samples (%)	Range (μg/kg)	Positive samples (%)	Range (μg/kg)	Positive samples (%)	Range (μg/kg)	Positive samples (%)	Range (μg/kg)	Positive samples (%)	Range (μg/kg)
Acre	Almond	8	11.9–1058.0	6	13.7–130.4	6	17.9–243.0	4	14.7–223.6	6	124.7–151.3
	Husk	12	6.8–61.8	4	1.1–3.1	8	4.4–46.3	2	6.0	10	101.3–133.5
Amazonas	Almond	18	6.8–36.6	4	1.1–1.6	14	2.7–12.6	2	1.8	2	132.8
	Husk	14	2.9–9.5	ND[a]	–	14	1.6–3.2	ND	–	ND	ND
Amapá	Almond	10	2.5–38.7	4	0.5–7.7	2	2.9	2	0.8	4	143.3–161.2
	Husk	10	3.6–15.1	ND	–	2	1.9	ND	–	10	105.8–133.5
Pará	Almond	8	1.7–6.9	ND	–	ND	–	ND	–	10	98.65–123.31
	Husk	4	2.8–3.3	ND	–	ND	–	ND	–	10	101.9–151.1

Limits of detection were 0.5 μg/kg for each aflatoxin and 50 μg/kg for cyclopiazonic acid. Limits of quantification were 0.75 μg/kg for each aflatoxin and 100 μg/kg for cyclopiazonic acid.

[a] Not detected.

nature and can be isolated from air, soil, industrial effluent, and sewage (Gams and McGinnis, 1983; Perdomo et al., 2011).

In a previous study, Castrillón and Purchio (1988) identified *Aspergillus* spp., *Penicillium* spp. and *Acremonium* spp. as the predominant genera in samples of Brazil nuts from Amazonia. Freire et al. (2000) detected *Aspergillus* spp. as the predominant species in husks of the Brazil nut. *Penicillium*, *Cunninghamella*, *Rhizopus*, *Fusarium* and *Acremonium*, among others, were also isolated in that study.

Morphological differentiation is insufficient to distinguish many *Aspergillus* species, due to interspecific similarities, and intraspecific variability (Rodrigues et al., 2009). Samson and Varga (2009) showed that no single method is flawless in recognizing species and recommend a polyphasic approach, where morphological examination, extrolite analysis and DNA sequence data are considered together. In the present study, *Aspergillus* isolates were identified by a polyphasic approach. Distinguishing between *A. flavus* and *A. nomius* has been difficult, because these species share many characteristics (Kurtzman et al., 1987). In addition to their toxigenic profile, these species are distinct with many nucleotide substitutions present in comparisons of their genes. In addition, growth rate at 42 °C, stipe roughness and DNA sequences differ between *A. nomius* and *A. bombycis* (Peterson et al., 2001).

The isolation of *A. nomius* from Brazil nuts agrees with the findings of Olsen et al. (2008) who studied two batches of Brazil nuts (60 and 20 kg). Twenty-two of the fungal isolates belonged to the *Aspergillus* section *Flavi*. Three isolates were producers of aflatoxins B and G and were identified as *A. nomius* according to Kurtzman et al. (1987). Two of these isolates were sent to the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands) for confirmation. Nineteen isolates were identified as *A. flavus*, including 10 producers of aflatoxins B and nine non-producers. *A. parasiticus* was not isolated in that study and *A. nomius* was found to produce large amounts of aflatoxins.

A. nomius has been isolated from Brazil nuts (Olsen et al., 2008; Baquião et al., 2012; Gonçalves et al., 2012), pistachio (Feibelman et al., 1998), wheat (Kurtzman et al., 1987), corn, and peanut (Pitt et al., 1993), as well as from agricultural soil in the United States (Kurtzman et al., 1987; Egel et al., 1994), Iran (Razzaghi-Abyaneh et al., 2006), and Thailand (Ehrlich et al., 2007). The last authors concluded that *A. nomius* may be more widespread than commonly thought. This species is a major aflatoxin producer in Brazil nuts (STDF, 2009). In addition, *A. nomius* might be particularly important because of its ability to produce larger amounts of aflatoxins than *A. flavus* isolates.

It should be highlighted that the presence of aflatoxins is a matter of serious concern for exporters of Brazil nuts since the maximum tolerance limit established by the European Commission to Brazil nuts for further processing is 15 μg/kg for the sum of aflatoxins $AFB_1 + AFB_2 + AFG_1 + AFG_2$, and 8 μg/kg for AFB_1 (EC, 2010). In our study, Brazilian states that had the most samples with aflatoxins levels higher than the maximum tolerance limit were Amazonas (8/9) and Acre (4/4). On the other hand, only one sample from Amapá (1/5) and none from Pará (0/4) had levels higher than 15 μg/kg (Table 4). Regarding to CPA, 5.5% of the almonds samples were positives. The minimum and the maximum levels were 98.65 μg/kg and 161.2 μg/kg, respectively (Table 4). Baquião et al. (2012) did not detect CPA in Brazil nuts.

The production chain of Brazil nuts has many peculiarities, which require a holistic approach to prevent decaying of these nuts. Their production begins in the forest, when the fruits containing the seeds fall to the ground; they are collected and stored by gatherers who are responsible for the initial handling and processing still in the forest. This system does not allow pests and diseases to reach damaging levels characteristically observed in monocultures, because of the forest ecosystem equilibrium (Freitas-Silva and Venancio, 2011). In fact, the most influential parameters that affect fungal growth and mycotoxin production are storage conditions, particularly as high moisture and temperatures directly affect the a_w. Drying should take place soon after harvest and as rapidly as feasible, followed by controlled atmospheres in terms of relative humidity, temperature, ozone treated, and oxygen reduction in warehouses. High levels of temperature and a_w create conditions favorable for the growth of fungi, particularly aflatoxigenic strains. In the present study, the presence of aflatoxigenic strains also indicates the need for good storage practices to prevent the occurrence of aflatoxins in Brazil nuts.

5. Conclusion

The frequency of fungal isolation was higher for husks than almonds of Brazil nuts, showing that contamination occurred from the husk to the almond. *Phialemonium* spp. was the main fungal genus isolated. Aflatoxin production was higher in the almonds than in the husks. The presence of the fungus did not necessarily imply aflatoxin contamination, but the high frequency of aflatoxigenic *Aspergillus* strains showed that the almond of Brazil nuts was a potential substrate for aflatoxin production. Therefore, increased attention is necessary since the climatic conditions (high temperature and humidity) of the Amazon region favor the growth of fungi, particularly aflatoxin-producing *Aspergillus* species. Multiple approaches will be needed to prevent the negative economic impact of the aflatoxins and their harmful effects on human and animal health.

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