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# Ochratoxin A and related fungi in Brazilian black pepper (*Piper nigrum* L.)

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, genotoxic, teratogenic and carcinogenic properties, produced by several species of *Aspergillus*, mainly those belonging to the *A*. section *Circumdati* and *A*. section *Nigri*. Although this toxin has been detected in spices and condiments, in black pepper (*Piper nigrum* L.) few studies have investigated the mycobiota (based on a molecular approach) and the presence of OTA in this food. The aim of this study was to investigate the presence of potentially ochratoxigenic species and ochratoxin A in black pepper marketed in Brazil, one of the largest producers in the world. A total of 60 samples of black pepper (29 in powder and 31 in grain) were collected in markets. The presence of OTA was investigated in black pepper samples using High-Performance Liquid Chromatography (HPLC), OTA was detected in 55% of the samples, with levels ranging from 0.05 to 13.15 μg/kg, all of which were below the Brazilian legal tolerances. *A*. section *Nigri* and *A*. section *Circumdati* were found in 80% of the samples, but the species of *A*. section *Nigri* were significantly more frequent than those of *A*. section *Circumdati*. The potential for OTA production by fungal isolates was tested using the agar plug technique and confirmed by HPLC. Among the isolates belonging to *A*. section *Nigri* (n = 1,083) and *A*. section *Circumdati* (n = 129), 3.7% and 3.8%, respectively, were able to produce OTA in Yeast Extract Sucrose Agar (YESA). A total of 25 strains from *A*. section *Circumdati* and 64 from *A*. section *Nigri* were identified using molecular data. The following potentially ochratoxigenic species were found in black pepper: *A. niger, A. welwitschiae, A. carbonarius, A. westerdijkiae* and *A. ochraceus*. The occurrence of these species denotes the need for continuous monitoring of black pepper by regulatory bodies in order to safeguard consumers' health.

## 1. Introduction

Black pepper (*Piper nigrum* L.) is one of the most consumed spices worldwide. It is mainly cultivated in countries with tropical and/or semi-tropical climates. Brazil is the fourth-largest producer in the world (FAOSTAT, 2020) with production standing at around 40,000 tons per year, 85% of which is exported (http://agrospice.com.br/product). The crop needs a hot and humid climate for its development, from 23 to 27 °C and total annual rainfall above 1,500 mm and water availability during the flowering and fruiting period. High temperatures and humidity in the growing areas can provide ideal conditions for fungal proliferation and mycotoxin production (Yogendrarajah et al., 2016). In 2015, the Codex Committee on Contaminants in Foods (CCCF) included black pepper in Group 1, assigning it a high priority for evaluation and quantification of mycotoxins.

Ochratoxin A (OTA) is a mycotoxin considered nephrotoxic, teratogenic, immunotoxic, and hepatotoxic to domestic and laboratory animals (O'Brien & Dietrich, 2005). Based on ample evidence of carcinogenicity in animal studies, the International Agency for Research on Cancer (IARC) categorized OTA as a possible human carcinogen (category 2B) (IARC, 1993). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has confirmed a Provisional Tolerable Weekly Intake (PTWI) of 100 ng/kg bodyweight for this mycotoxin, which corresponds to 14 ng/kg body weight per day (CAC, 1998; JECFA, 1995).

Several studies have been conducted on the occurrence of OTA in spices, but most of the research is conducted in Asian countries such as Malaysia, Turkey, India, Iran (Jalili, 2016; Jalili, Jinap, & Radu, 2010; Ozbey & Kabak, 2012; Ramesh & Jayagoudar, 2014), and in Hungary (Fazekas, Tar, & Kovácz, 2005) and Cameroon (Nguegwouo et al.,

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2018). Brazilian legislation establishes the Maximum Tolerated Limits (MTL) for OTA in spices of 30 μg/kg (ANVISA, 2011), but only a limited amount of data is available on the presence of this mycotoxin in black pepper (Freire, Kozakiewicz, & Paterson, 2000; Gatti, Fraga, Magnoli, Dalcero, & Rosa, 2003; Garcia, Mallmann, & Copetti, 2018).

*Aspergillus* section *Nigri* and *A.* section *Circumdati* have been highlighted as the main source of OTA contamination in black pepper (Garcia, Mallmann, & Copetti, 2018; Jeswal & Kumar, 2015). Although the use of DNA sequencing has become routine for identifying *A.* section *Nigri* and *A.* section *Circumdati* at species level, none of the studies designed to evaluate the occurrence of ochratoxigenic *Aspergillus* in black pepper has used this approach to complement morphological findings and accurately identifies the species.

The aim of this research was to investigate the occurrence of OTA in samples of powdered black pepper and grains, as well as the presence and identification of OTA-producing species.

## 2. Materials and methods

### 2.1. Samples

Sixty samples (200–300 g) of black pepper were analyzed, comprising 29 black pepper powder and 31 black pepper grain samples. Fifty-one samples were obtained in bulk from different spice shops and nine samples of commercial brands were bought in supermarkets in the Brazilian states of São Paulo (Southeast), Maranhão (Northeast), and Rio Grande do Sul (South).

### 2.2. Water activity determination

Water activity in the pepper samples was measured in triplicate at 25 °C (±1 °C) using AquaLab – Decagon (WA, USA) equipment.

### 2.3. Direct plating for grain samples

The direct plating method was used, both with (Pitt & Hocking, 2009) and without sample surface disinfection. Fifty grains per sample were surface disinfected with 0.4% sodium hypochlorite for 2 min. Grains from each sample disinfected with sodium hypochlorite were plated onto Dichloran 18% Glycerol Agar (DG18) with 5 grains per plate. The same procedure was performed for the non-disinfected sample. The plates were incubated at 25 °C for 5–7 days, and fungal contamination was expressed as a percentage (%).

### 2.4. Dilution method for powder samples

The dilution method was used for powder black pepper samples. Twenty-five grams of the sample were homogenized with 225 mL of 0.1% peptone water for 1 min. Serial dilutions were performed, and aliquots of 0.1 mL of each dilution plated onto the DG18 medium. Plates were incubated at 25 °C for 5–7 days. Plates containing 15 to 150 CFU (Colony Forming Unit) were then selected and the results expressed in CFU/g (Pitt & Hocking, 2009).

### 2.5. Morphological evaluation

Potentially ochratoxigenic *Aspergillus* species were purified and inoculated at three equidistant points on Czapek Yeast Extract Agar (CYA), and then incubated at 25 °C for 5–7 days, according to Pitt & Hocking (2009). Colonies were then inoculated on CYA and Malt Extract Agar (MEA), incubated at different temperatures, and separated into *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* according to Klich (2002).

The fungi were evaluated according to their microscopic and macroscopic characteristics (diameter, texture, pigmentation, surface and reverse appearance, shape, and size of the structures and conidia) using the taxonomic keys described.

### 2.6. Molecular identification

A total of 64 *Aspergillus* section *Nigri* and 25 *Aspergillus* section *Circumdati* isolates were grown in 7 mL of Yeast Extract Sucrose medium (YES) at 25 °C for 24 h. The mycelia were collected, frozen in liquid nitrogen and ground to a fine powder. Genomic DNA was extracted using the Biopur Mini Spin Planta (Biometrix, Brazil) commercial extraction kit according to the manufacturer's instructions. Part of the calmodulin gene (*CaM*) sequence was amplified using CMD5 and CMD6 primer pairs (Hong, Cho, Shin, Frisvad, & Samson, 2006). PCR products were purified using ExoProStarTM 1-Step (GE Healthcare Life Sciences, UK) according to the manufacturer's recommendations and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in an ABI 3500XL Genetic Analyzer (Applied Biosystems, USA). The sequences obtained were aligned with sequences from all *A.* section *Nigri* or *A.* section *Circumdati* type strains available in the NCBI database (http://www.ncbi.nlm.nih.gov) using Clustal W (Thompson, Higgins, & Gibson, 1994). The maximum-likelihood trees were inferred using the Kimura-2-parameter model (Kimura, 1980) with a gamma distribution (+G). The shape of the gamma distribution was 0.61 for phylogenetic tree of *Aspergillus niger* aggregate and 0.43 for phylogenetic tree of *Aspergillus* section *Circumdati.* In order to determine each clade support, a bootstrap analysis was performed with 1,000 replicates using MEGA 7.0 software (Kumar, Stecher, & Tamura, 2016).

The *CaM* sequences representing the diversity found in this study were deposited in GenBank, under access numbers MW362100 to MW362117.

### 2.7. Evaluation of ochratoxin A production by strains

*Aspergillus* section *Circumdati* and *A.* section *Nigri* isolates were tested for OTA production. They were inoculated onto Yeast Extract Sucrose Agar (YESA) and incubated at 25 °C for seven days, and the agar plug technique combined with Thin Layer Chromatography (TLC) applied according to Filtenborg, Frisvad, & Svendensen (1983). Pieces of agar (5–7 $mm^2$) were removed from the YESA plate, and the toxins extracted with a chloroform/methanol mixture (1:1). These pieces were applied to 20 cm × 10 cm silica gel plates (Merck, Darmstadt, Germany) and the OTA standard added on the same plate. The mobile phase was toluene/ethyl acetate/formic acid/chloroform (7:5:2:5, v/v/v/v). After elution, the plate was dried, and the results visualized in a cabin with ultraviolet light at wavelengths of 365 nm and 254 nm. A qualitative determination was performed by comparing the fluorescence and retention factor (Rf) of spot strains to the OTA standard.

The results were confirmed using High Performance Liquid Chromatography (HPLC). Three pieces of agar were cut and ochratoxin A was extracted with methanol, diluted when necessary, filtered and injected into the equipment, using the same parameters described in 2.8.3 item.

### 2.8. Determination of ochratoxin A in black pepper by HPLC

#### 2.8.1. Sample preparation

Approximately 100 g of each grain pepper sample were finely ground using a mill (IKA, Mod A11, Brazil) and sifted through an 18 mesh (1 mm) sieve (Bronzinox, Brazil).

#### 2.8.2. Extraction and cleaning

Five grams of the sample were extracted with 100 mL of 3% methanol: bicarbonate (1:1) solution and homogenized in a shaker for 30 min. Filtration was carried out using a qualitative filter followed by a glass microfiber filter (Vicam, Sweden). The filtrate (4 mL) was diluted in 20 mL of PBS with 0.01% Tween 80, passed through an OchraTest immunoaffinity column (IAC) (Vicam, USA) at a flow rate of 2–3 mL/min, and washed with 20 mL of ultra-pure water (MilliQ, France). The

mycotoxin was eluted eight times with 500 μL of methanol: acetic acid (98:2) in an amber vial. The extract was dried using nitrogen and resuspended with 300 μL of acetonitrile: water: acetic acid (51:47:02) solution. Before injection into the HPLC system, the extract was filtered through a 0.45 μm PVDF filter (Analítica, China). Twenty microliters were injected into the HPLC system with a fluorescence detector (Shimadzu, Japan).

#### 2.8.3. HPLC parameters

A Shimadzu LC-10VP HPLC system (Shimadzu, Japan) was used with manual injection and fluorescence detector wavelength settings of 333 nm (excitation) and 477 nm (emission). A C18 Shimadzu Shim-pack (4.6 × 250 mm) column was used at an oven temperature of 40 °C. The mobile phase was methanol: acetonitrile: water: acetic acid (35:30:34:01), with an isocratic flow rate of 0.8 mL/min. The methodology was based on Copetti, Pereira, Iamanaka, Pitt, & Taniwaki (2010). Extraction solution (1% bicarbonate), volume of the filtrate (20 mL) and mobile phase (51:47:2 acetonitrile:water:acetic acid) were changed from the previous method.

An OTA standard was used for the construction of a six-point calibration curve of peak areas versus OTA mass (ng). Recovery was calculated spiking OTA standard at 3 levels: low (0.7 μg/kg), medium (11.5 μg/kg) and high (57.4 μg/kg), on a blank black pepper sample, in triplicate. Limits of detection (LOD) and quantification (LOQ) were calculated according to Eurachem Guides (Magnusson, Ellison, & Örnemark, 2015).

## 3. Results and discussion

### 3.1. Fungi species: occurrence, identification and OTA producers

Levels of water activity ($a_w$) in the black pepper samples herein analyzed were low; for grain samples, the average was 0.599 and for powder samples 0.561. Of the sixty samples analyzed (29 powder and 31 grain), a total of 1,212 *A.* section *Nigri* and *A.* section *Circumdati* isolates were obtained. The study focused on *A.* section *Nigri* and *A.* section *Circumdati* because some species in these groups have been highlighted as the main sources of contamination with OTA in black pepper (Garcia, Mallmann, & Copetti, 2018; Jeswal & Kumar, 2015).

Table 1 gives the frequency of occurrence, average, range of contamination by *Aspergillus*, in the grain and powder black pepper. Grains were also plated without external disinfection to evaluate the presence of *Aspergillus* on the surface. As black pepper can be used in powder and as an ingredient in various products, it is important to know the external contamination of the grains, since these contaminants can become part of the mycoflora of the final product. The contamination rate for samples plated without surface disinfection was higher than the infection rate of disinfected samples, indicating that the *A.* section *Nigri* and *A.* section *Circumdati* isolates were also on the surface of the grains.

As mentioned in the Material and Methods section, 51 bulk samples and 9 branded packaged samples were obtained. Fungal contamination in bulk samples was higher (frequency of occurrence 73% in powder samples, and 76% for disinfected and 90% for non-disinfected grain samples) than in packaged samples (frequency of occurrence 43% in powder samples, 0% for disinfected and 50% for non-disinfected grain samples.). The lower fungal contamination in branded samples could be a result of some companies' using well-selected raw material, or gamma irradiation, which has been used by some spice companies to reduce fungal and mycotoxin contamination (Jalili, Jinap, Noranizan, 2010).

*A.* section *Nigri* and *A.* section *Circumdati* were found in 80% of samples investigated herein, but *A.* section *Nigri* isolates were significantly more frequent than those of *A.* section *Circumdati*. As shown in Table 2, the isolates were grouped according to morphology and physiology.

Currently, 27 species are included in *A.* section *Nigri,* and four (*A. carbonarius, A. sclerotioniger*, *A. niger,* and *A. welwitschiae*) have the potential to produce OTA (Fungaro et al., 2017; Samson et al., 2014). Based on morphology and physiology, the strains of *A.* section *Nigri* obtained were split into three Groups: *Aspergillus niger* aggregate, *A. carbonarius* group, and Uniseriate group.

*Aspergillus niger* aggregate is a biseriate species that are morphologically very similar. *A. costaricaensis, A. luchuensis, A. neoniger, A. piperis, A. tubingensis, A. eucalypticola, A. vadensis*, *A. niger sensu stricto*, *A. welwitschiae,* and *A. brasiliensis* have been generally classified as *Aspergillus niger* aggregate. Only *A. niger sensu stricto* and *A. welwitschiae* are recognized as OTA producers, but this is limited to a smaller proportion of strains. *A. niger* aggregate was the most prevalent in *A.* section *Nigri,* corresponding to 87% of the isolates ($n = 1{,}063$). All these isolates had biseriate heads, black or slightly brown colonies (70 mm) on CYA at 25 °C after 7 days, and smooth or slightly rough globose conidia (4–5 μm). The frequency of occurrence for this group was similar in both powder and disinfected grains (65–67%), and higher in non-disinfected grains, rising to 84%. As shown in Table 3, only 3.7% of the *A.* section *Nigri* strains were OTA producers. These findings corroborate those of Garcia, Mallmann, & Copetti (2018), Jeswal & Kumar (2015), and Yogendrarajah et al. (2014), who reported *A. niger* aggregate as more frequent in pepper. Garcia, Mallmann, & Copetti (2018) examined 15 black peppers and 15 white peppers, among other spices marketed in Brazil (rosemary, cinnamon, clove, fennel, pepperoni, oregano), totaling 112 samples. For black pepper, *A. niger* aggregate was the most frequent group (80%). Jeswal & Kumar (2015) examined the occurrence of toxigenic fungi and OTA in different Indian spices (red chili, black pepper, turmeric, coriander, cumin, fennel, caraway, fenugreek, and dry ginger). *A. niger* was one of the predominant species isolated in all the spices studied, with average contamination ranging from 2.6% to 19.6% (the highest contamination was found in black pepper). Yogendrarajah et al. (2014) also found *A. niger* to be one of the predominant species in *Piper nigrum* from Sri Lanka. According to the authors, *A. niger* was present in 62% of the samples, with contamination levels higher than $1.3 \times 10^3$ CFU/g. However, it is important to highlight that none of the authors above referenced (Garcia, Mallmann, & Copetti, 2018; Jeswal & Kumar, 2015; Yogendrarajah et al., 2014) used molecular data for identifying *A.* section *Nigri* at species level, which is essential for discriminating evolutionary close species.

It is known that OTA production is not widespread in *A. niger* aggregate. Of the 1,063 isolates analyzed in this study, only 2.5% tested positive. This frequency is similar to that found by Ferranti et al. (2018), who found that 4.3% of *A. niger* aggregate strains were OTA producers.

**Table 1**
Contamination of black pepper by *Aspergillus* section *Nigri* and *A.* section *Circumdati*.

| Group | Black pepper grains (n = 31) | | | | | | Powdered black pepper (n = 29) | | |
|---|---|---|---|---|---|---|---|---|---|
| | With disinfection | | | Without disinfection | | | | | |
| | FO (%) | Average (%) | Range (%) | FO (%) | Average (%) | Range (%) | FO (%) | Average (CFU/g) | Range (CFU/g) |
| **Section *Nigri*** | 67.7 | 15.4 | 0–100 | 84.0 | 40.8 | 0–100 | 65.5 | $3.12 \times 10^3$ | $<10$–$5.0 \times 10^4$ |
| **Section *Circumdati*** | 19.4 | 1.0 | 0–10 | 58.1 | 6.0 | 0–38 | 13.8 | $7.65 \times 10^2$ | $<10$–$1.7 \times 10^4$ |

FO = Frequency of occurrence (n° contaminated samples/n° total samples).
Average = Sum of contamination value of total sample/n° total samples).

**Table 2**
Main groups of *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati*.

| Species/group | | Black pepper grains (n = 31) | | | | | | Powdered black pepper (n = 29) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | With disinfection | | | Without disinfection | | | | | |
| | | FO (%) | Average (%) | Range (%) | FO (%) | Average (%) | Range (%) | FO (%) | Average (CFU/g) | Range (CFU/g) |
| **Section *Nigri*** | ***A. niger* "aggregate"** | 67.7 | 15.0 | 0–100 | 84.0 | 40.0 | 0–100 | 65.5 | $3.08 \times 810^3$ | $<10–5.0 \times 10^4$ |
| | ***A. carbonarius* Group** | 16.1 | 0.5 | 0–6 | 9.7 | 0.4 | 0–6 | 3.4 | 34 | $<10–1.0 \times 10^3$ |
| | **Uniseriate Group** | 0 | 0 | 0 | 9.7 | 0.4 | 0–8 | 0 | 0 | <10 |
| **Section *Circumdati*** | **Group 1** | 16.1 | 0.9 | 0–10 | 54.8 | 5.0 | 0–32 | 13.8 | $6.27 \times 10^2$ | $<10–1.3 \times 10^4$ |
| | **Group 2** | 3.2 | 0.1 | 0–2 | 16.1 | 0.5 | 0–6 | 3.4 | $1.37 \times 10^2$ | $<10–4.0 \times 10^3$ |
| | **Group 3** | 0 | 0 | 0 | 12.9 | 0.5 | 0–6 | 0 | 0 | <10 |

FO = Frequency of occurrence (n° contaminated samples/n° total samples).
Average = Sum of contamination values of total samples/n° total samples).

**Table 3**
Ochratoxin A producing fungi in black pepper samples.

| Species/group | | Grain | | | | Powder | | Total | |
|---|---|---|---|---|---|---|---|---|---|
| | | With disinfection | | Without disinfection | | | | | |
| | | N | OTA + (%) | N | OTA+ (%) | N | OTA+ (%) | N | OTA+ (%) |
| **Section *Nigri*** | *A. niger* "aggregate" | 231 | 0.0 | 620 | 3.5 | 212 | 2.4 | 1063 | 2.5 |
| | *A. carbonarius* Group | 7 | 100 | 6 | 83.3 | 1 | 100 | 14 | 92.8 |
| | Uniseriate Group | 0 | 0 | 6 | 0 | 0 | 0 | 6 | 0 |
| | Total | 238 | 2.9 | 632 | 4.3 | 213 | 2.8 | 1083 | 3.7 |
| **Section *Circumdati*** | Group 1 | 14 | 0 | 76 | 0 | 20 | 0 | 110 | 0 |
| | Group 2 | 1 | 100 | 7 | 14.3 | 4 | 0 | 12 | 16.6 |
| | Group 3 | 0 | 0 | 7 | 42.9 | 0 | 0 | 7 | 42.9 |
| | Total | 15 | 6.7 | 90 | 4.4 | 24 | 0 | 129 | 3.8 |

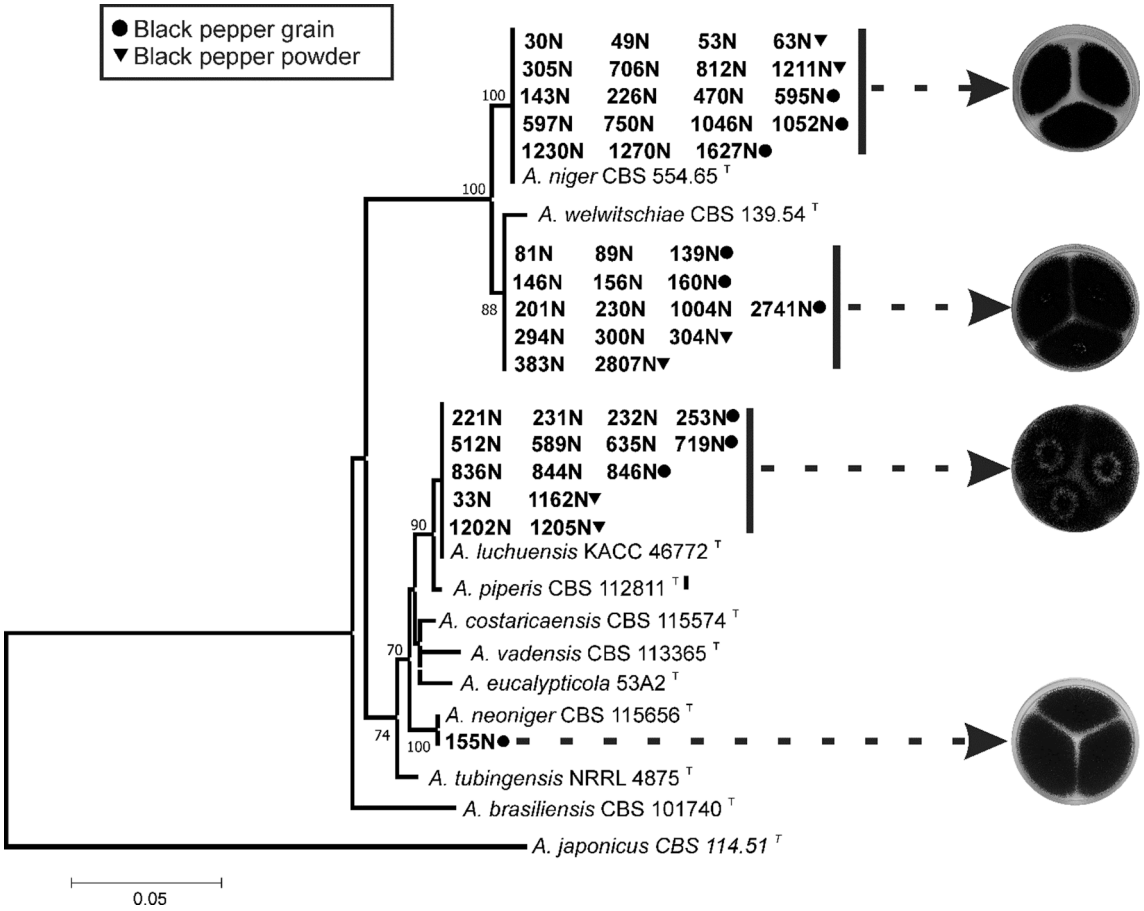


**Fig. 1.** Maximum Likelihood (ML) phylogenetic tree reconstructed from the partial calmodulin gene sequence aligned with the corresponding sequence of type strains (T) of *Aspergillus niger* aggregate deposited in the NCBI database. Bootstrap values ≥ 70% are shown.

Based on calmodulin gene sequence (*CaM)* which has been determined for some representative isolates of *A. niger* aggregate (n = 50), we identified *A. niger* (38%), *A. welwitschiae* (30%), *A. luchuensis* (30%) and *A. neoniger* (2%) (Fig. 1). Among these species, *A. niger* and *A. welwitschiae* were proven to be OTA producers, but this ability is limited to a small number of strains (Massi et al., 2016, Susca et al., 2016, Ferranti et al., 2018, Gil-Serna et al., 2019).

Within *A.* section *Nigri*, the *A. carbonarius* group was the second most frequent, corresponding to 1.3% of the isolates. This group differs from *A. niger* in terms of conidia size (diameter ranging from 7 to 10 μm), and spiny conidia ornamentation. *A. carbonarius* group was found respectively in 16%, 9.7%, and 3.4% of the disinfected, non-disinfected, and powder samples, and respective average contamination was low, at 0.5%, 0.4%, and 34 CFU/g. According to the literature, the species comprising the *A. carbonarius* group are *A. carbonarius*, *A. sclerotioniger*, *A. ibericus*, and *A. sclerotiicarbonarius* (Varga et al., 2011). Of these species, *A. carbonarius* and *A. sclerotioniger* are very effective OTA producers. The *A. carbonarius* group showed the highest percentage of OTA-producer strains (92.8%, 13 out of 14). Based on sequence data, all strains of the *A. carbonarius* group were identified as *A. carbonarius* (identity > 99% in NCBI BLASTn ref). Although *A. carbonarius* non-ochratoxigenic isolates are very rarely found in nature (Castellá, Bragulat, Puig, Sanseverino, & Cabañes, 2018), one from the 14 strains analyzed tested negative for OTA production, according to the agar plug technique and Thin Layer Chromatography (TLC) (Table 3). The inability of OTA production by this strain was confirmed on High Performance Chromatography (HPLC). The chromatogram is shown in the supplementary Fig. 1. *A. carbonarius* was also found in Brazilian black pepper by Garcia, Mallmann & Copetti (2018) and Gatti, Fraga, Magnoli, Dalcero & Rosa (2003).

In addition to the groups described previously, the *A.* section Nigri Uniseriate was isolated only from non-disinfected black pepper grains and was found in 9.7% of these samples at a low average contamination level of 0.4%. The isolates were characterized by dark brown and gray colonies (70 mm) on CYA at 25 °C after seven days, with globose and spiny conidia (4–5 μm). Although *A.* section Nigri Uniseriate has been frequently found in some crops cultivated in tropical and subtropical zones (Chiotta, Ponsone, Sosa, Combina, & Chulze, 2013; Ferranti et al., 2018), it has not been previously reported in black pepper. As expected, no strains produced OTA (Table 3).

*Aspergillus* section *Circumdati*, which includes many OTA-producing species, was found at lower frequencies compared to *A.* setion *Nigri*; however, they were found at high frequency in non-disinfected grains. It was found respectively in 19.4%, 58.1%, and 13.8% of disinfected and non-disinfected grains and powder samples (Table 1). Based on morphology, *A.* section *Circumdati* isolates were classified in three groups: Group 1, was the most prevalent, with 16.1% (disinfected), 51.6% (non-disinfected) and 13.8% (powder), followed by Group 2, with 3.2% (disinfected), 13% (non-disinfected) and 3.4% (powder). Group 3 of *Aspergillus* section *Circumdati* was found only in non-disinfected samples (13%) (Table 2).

Of the 27 species currently accepted in section Circumdati, 13 produced large amounts of OTA, and seven produced OTA inconsistently and/or in trace amounts (Visagie et al., 2014). In terms of potential OTA contamination of agricultural produce, the most important species are *A. ochraceus*, *A. steynii*, and *A. westerdijkiae*. Jeswal & Kumar (2015) analyzed different spices with no external disinfection and also reported black pepper contamination with *A. ochraceus,* 45% of which produced OTA and Garcia, Mallmann, & Copetti (2018) examined 15 samples of black pepper available on the Brazilian market and detected *A. ochraceus* in 20% of samples. In our study, only 129 *Aspergillus* section *Circumdati* isolates were recovered from the total samples analyzed, but only 3.8% of strains were OTA producers (Table 3).

The main characteristics of the *Aspergillus* section *Circumdati* Group 1 were small vesicles (25.7 μm) and the ability to grow at 37 °C (15 mm). The isolates were not able to produce OTA. Isolates in this Group exhibited yellow to pinkish sclerotia on CYA at 25 °C and a brown reverse. Colony sizes ranged from 39 to 60 mm, with conidia of 3.8 μm, metulae of 8.0 μm and phialides of 9.0 μm, on CYA at 25 °C. Group 2 exhibited microcolonies up to 9 mm and, in some cases, did not grow at 37 °C. Sclerotia were pinkish to brown; the reverse exhibited brown areas but was otherwise grayish and yellow, and 16.6% were OTA producers. Colony sizes ranged from 40 to 53 mm, conidia of 2.96 μm, vesicles of 40.57 μm, metulae of 11.76 μm and phialides of 12.3 μm. Group 3 did not grow to 5 mm at 37 °C; sclerotia were white to yellow with a brown reverse and sometimes olive-brown near the margin. The isolates exhibited colony sizes ranging from 35 to 52 mm, conidia of 2.92 μm, vesicles of 39.5 μm, metulae of 12.36 μm and phialides of 9.6 μm on CYA at 25 °C. The OTA-producing strains belonged to Groups 2 and 3.

Twenty-five *A.* section *Circumdati* isolates, representing the three fungal groups morphologically identified, were subjected to molecular identification. As shown in Fig. 2, three species were found. These results are well-correlated with the morphological/physiological analyses performed in this study and the information in the literature for *A.* section *Circumdati*. The strains from Groups 1, 2, and 3 were identified as *A. pallidofulvus*, *A. ochraceus,* and *A. westerdijkiae,* respectively. *A. pallidofulvus* was reported by Visagie et al. (2014) as unable to produce OTA. Silva et al. (2019) confirmed and expanded these findings by analyzing 10 *A. pallidofulvus* strains on YES, CYA and MEA media using two detection approaches (DAD and HRMS) and there was no trace of OTA in any of them. However, *A. ochraceus* and *A. westerdijkiae* are acknowledged as OTA producers (Frisvad, Frank, Houbraken, Kuijpers, & Samson, 2004).

In this study higher production of OTA by strains of *Aspergillus ochraceus* and *A. carbonarius* was confirmed on TLC plate, showing stronger fluorescence intensity of the spots when compared to *A. niger*. Results of OTA production by three different groups found, positive and negative strains, which are shown in the Supplementary Fig. 2.

*Aspergillus* have been cited as fungal post-harvested contaminants in different crops, including coffee, cocoa and spices (Garcia, Mallmann, & Copetti, 2018; Taniwaki, Pitt, Teixeita & Iamanaka, 2003; Copetti, Pereira, Iamanaka, Pitt & Taniwaki, 2010). These species occurs when low quality practices in the post-harvesting processing have been applied and their growth is favored with water activity decreasing. *Aspergillus* section *Nigri* and *Circumdati* are xerophilic fungi and are able to survive and grow at lower water activity (<0.85) (Pitt & Hocking, 2009). In this study water activity of the samples collected from the market was lower than 0.599 which is safe for fungal growth. However, there is no information about previous processing. The presence of potential toxigenic species in black pepper analyzed indicates contamination along the process.

### 3.2. Occurrence of OTA in black pepper samples

The recovery at the three defined levels were: 97% (low level of 0.76 μg/kg), 96% (medium level of 11.50 μg/kg) and 88.5% (high level of 57.45 μg/kg), being satisfactory and following the requirements of European Community Directive EC 401/2006 (EC, 2006) that establishes recovery values between 50 and 120% for OTA levels lower than 1.0 μg/kg and between 70 and 110% for 1.0 to 10 μg/kg. The limits of detection (LOD) and quantification (LOQ) for OTA were 0.05 μg/kg and 0.16 μg/kg, respectively, with a retention time of around 10.5 min (Fig. 3).

Studies have shown that the average for OTA contamination of black pepper samples ranges from 43.5% to 78.57% and the level of OTA contamination varies from ND to 154.1 μg/kg (Jalili, 2016; Jalili, Jinap, & Radu, 2010; Jeswal & Kumar, 2015; Ramesh & Jayagoudar, 2014). The average of OTA contamination herein for the total samples analyzed (n = 60) was 1.0 μg/kg. Twenty-seven samples (45%) exhibited no OTA contamination (<LOD). All contaminated samples (55%) were compliant with the Brazilian legislation (maximum of 30 μg/kg) and also satisfied European regulatory requirements (15 μg/kg). Twenty-one

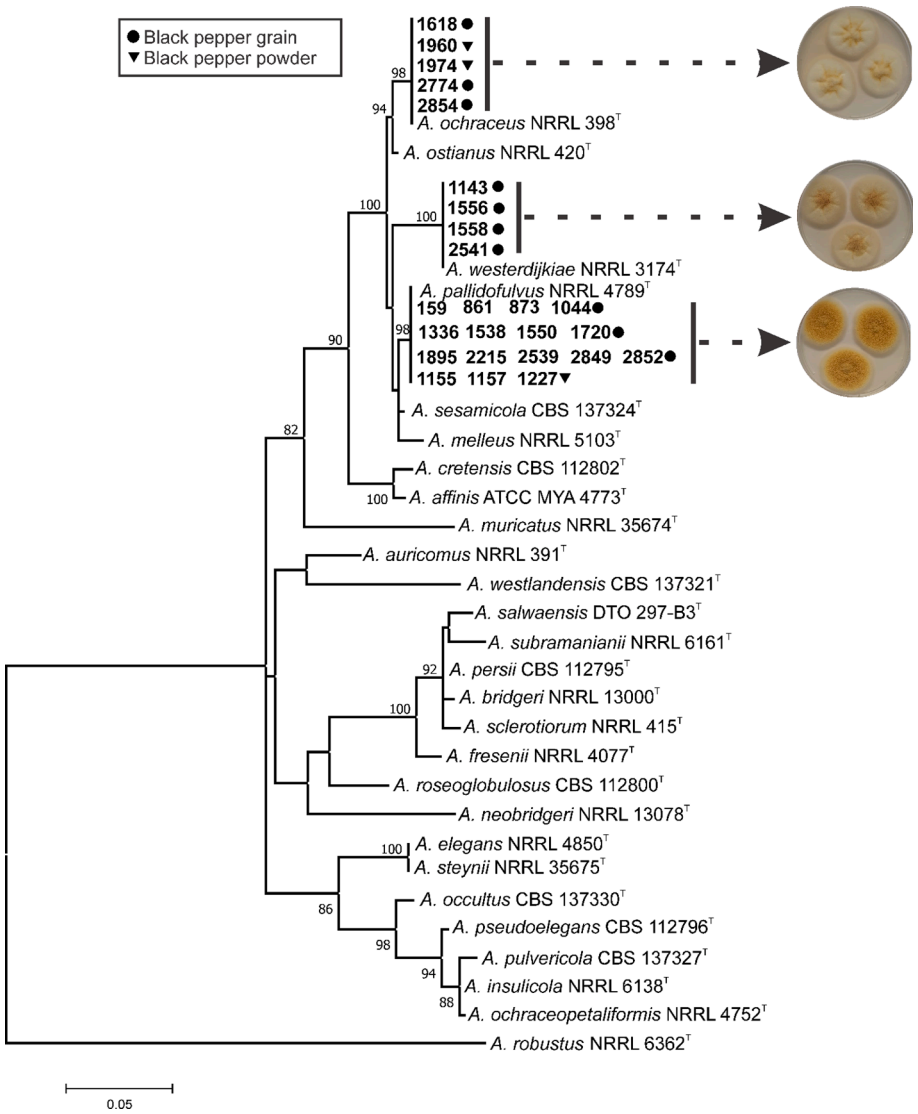


**Fig. 2.** Maximum Likelihood (ML) phylogenetic tree reconstructed from the partial calmodulin gene sequence aligned with the corresponding sequence of type strains (T) of *Aspergillus* section *Circundati* deposited in the NCBI database. Bootstrap values ≥ 70% are shown.

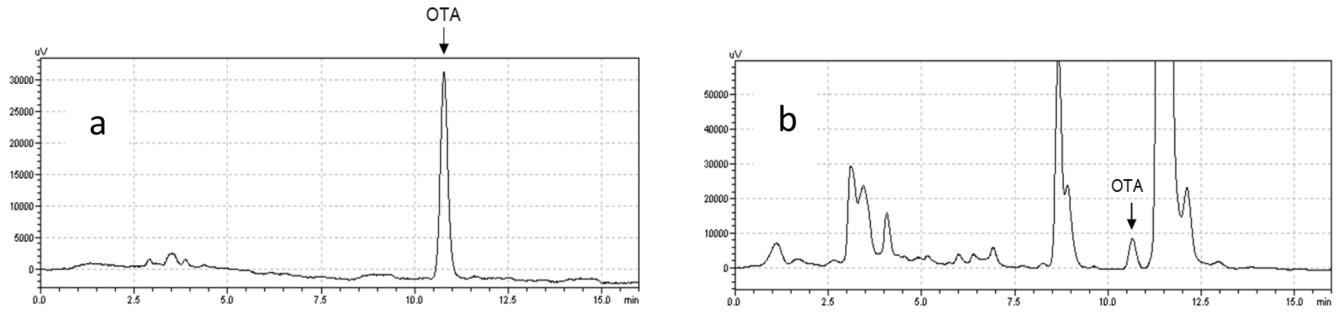


**Fig. 3.** Chromatogram of ochratoxin A standard (a) and contaminated black pepper sample (13.2 μg/kg) (b).

samples (35%) exhibited OTA contamination between the detection limit (0.05 μg/kg) and 1.0 μg/kg, and 12 samples (20%) showed contamination higher than 1.0 μg/kg. Only two samples (3.3%) exceeded 10 μg/kg (Table 4).

In terms of sample types, bulk samples showed higher OTA contamination, with an average of 1.11 μg/kg, whereas packaged samples bought in the supermarket showed average contamination of 0.48 μg/kg. Eleven of the twelve samples contaminated with OTA (higher than 1 μg/kg) were bulk samples. Five were powder samples, and the average contamination of OTA-producing fungi was $1.3 \times 10^4$ CFU/g. Six grain samples exhibited average infection and contamination with OTA-producing fungi of 39.3% and 64.3%, respectively. Interestingly, no OTA-producing fungi were found in 7 samples (11.6%) contaminated with this mycotoxin. In these cases, OTA may have been produced at some point along the black pepper production chain before commercialization, and the pepper remained contaminated with OTA even after

**Table 4**
Ochratoxin A contamination in the black pepper samples.

| Ochratoxin A (μg/kg) | N° of samples | |
|---|---|---|
| | Grain | Powder |
| <0.05 | 16 | 11 |
| ≥0.05 – <1.0 | 9 | 12 |
| ≥1.0 – <5.0 | 3 | 5 |
| ≥5.0 – <10.0 | 2 | 0 |
| ≥10.0 – <15.0 | 1 | 1 |
| ≥15.0 | 0 | 0 |

the fungi had died.

The four highest contaminated samples (13.2; 10.6; 7.9 and 7.8 μg/kg) were bulk samples purchased in spice shops The sample with the highest level of OTA contamination (13.2 μg/kg, (Fig. 3b) also exhibited the highest incidence of *A.* section *Circumdati* (10% disinfected and 38% non-disinfected). However, the other three samples with high levels of OTA (10.6, 7.9 and 7.8 μg/kg) were contaminated by *A.* section *Nigri* (above 42% and above $10^3$ CFU/g). It is important to emphasize that among the 15 grain samples contaminated with OTA, 13 (86.6%) were contaminated by *A.* section *Nigri* and 11 (73,3%) by *A.* section *Circumdati*, representing a total of 14 (93,3%) grain samples contaminated with both sections. Of the 18 powder samples contaminated with OTA, 12 (66.6%) were contaminated by *A.* section *Nigri* and 3 (16.6%) by *A.* section *Circumdati,* indicating that *A.* section *Nigri* species are predominant and represent a risk factor.

## 4. Conclusions

The percentage of Brazilian black pepper samples contaminated with OTA was similar to that found in other countries, but OTA contamination levels in all samples were compliant with Brazilian law and European regulatory requirements. Although the levels of OTA were generally low compared to other food products, the presence of ochratoxigenic species, such as *A. carbonarius, A. niger*, *A. welwitchiae*, *A. westerdijkiae,* and *A. ochraceus,* indicates that black pepper needs to be continuously monitored by the regulatory agencies. Grain moisture content must be controlled along the entire black pepper production chain in order to prevent contamination with OTA and subsequent consumer exposure.

## Author contributions

Beatriz Thie Iamanaka and Marta Hiromi Taniwaki contributed to conceiving and designing the study. Material preparation, data collection, and analysis were performed by Adriana Raquel Persson da Silva, Ligia Manoel Martins, Josué José Silva, and Beatriz Thie Iamanaka. The first draft of the manuscript was written by Beatriz Thie Iamanaka, Adriana Raquel Persson da Silva and Maria Helena Pelegrinelli Fungaro. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110207.

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