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Brief Report

A Brief Snapshot of *Aspergillus* Section *Nigri* Isolated from Brazilian Peanuts and Soil

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Abstract: The occurrence of mycotoxigenic species in peanuts is a major concern, and has been investigated in depth for many years. However, most studies focus on the occurrence of aflatoxigenic fungi, such as *Aspergillus* section *Flavi*. The present study aimed to clarify the occurrence of *Aspergillus* section *Nigri*, a group that harbors species capable of producing ochratoxin A (OTA), which has scarcely been investigated in peanuts. A total of 52 peanut samples, collected in the field and from storage, were analyzed. *Aspergillus* section *Nigri* was isolated from 64% and 100% of field and storage samples, respectively, and 218 strains were obtained. Based on the multiloci phylogeny of the *CaM* and *BenA* loci, six species of *Aspergillus* section *Nigri* were identified: *A. brasiliensis*, *A. niger*, *A. neoniger*, *A. welwitschiae*, *A. costaricaensis*, and *A. japonicus*. The incidence of ochratoxigenic strains was 5.0% (11/218), and only *A. niger* and *A. welwitschiae* were able to produce OTA. The presence of OTA in peanuts was found in 6 field and 8 storage samples, with levels ranging from 0.106 to 0.387 and 0.090 to 0.160 µg/kg, respectively.

Keywords: ochratoxin A; *Aspergillus* section *Nigri*; peanuts

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1. Introduction

Brazil is the third-largest peanut producer in the Americas, behind the United States and Argentina [1]. The state of São Paulo stands out as the biggest national producer; in the last few years, it has shown great changes in the planted cultivar and, consequently, an increase in productivity. Peanuts grow in tropical and subtropical climate areas and are mainly composed of lipids (~48%), carbohydrates (~20%), and protein (~25%), as well as bioactive compounds such as flavonoids, phytosterols, stilbenes, and vitamins (mainly tocopherol, folate, and niacin) [2].

The characteristics of peanut production, such as development inside the soil, uneven maturation, and rainfall during the drying stage, make the peanut grain susceptible to invasion by microorganisms, especially fungi, which may lead to kernel deterioration and contamination by fungal secondary metabolites such as mycotoxins. Most studies related to peanut contamination by fungi have focused on *Aspergillus* section *Flavi*, a fungal group that harbors most aflatoxigenic species, since the occurrence of aflatoxins in peanuts is of great concern due to its carcinogenic aspect [3–5]. In peanuts, studies on the occurrence of *Aspergillus* species of the section *Nigri* and their related toxigenic ability are scarce.

Aspergillus section *Nigri* are food spoilage fungi which are widely distributed in nature. Although the main source is soil, some members of this section have been reported in diverse habitats, including decaying organic matter and on the surface of living plants [6], and have caused food spoilage and disease in maize [7], onions [8], grapes [9,10] and peanuts [11,12].

Among the members of *Aspergillus* section *Nigri*, *Aspergillus niger* stands out, often found in food; in addition, it is widely used in industry for the production of organic acids and extracellular enzymes. Other applications include the biotransformation of xenobiotics,

bioremediation and waste pre-treatment, and cell protein for feed [13]. It has usually been regarded as a benign fungus [13], and it holds GRAS (generally regarded as safe) status from the US Food and Drug Administration [14].

The significance of *Aspergillus* section *Nigri* regarding its occurrence and its ability to produce mycotoxins has been investigated worldwide. Two mycotoxins commonly associated with *A. niger* aggregate are ochratoxin A (OTA) and fumonisin B2 (FB2). However, only fungal strains belonging to *A. niger* and *A. welwitschiae* have been reported to be able to produce these mycotoxins in culture media or on natural substrates [15–17]. OTA is a nephrotoxic and carcinogenic mycotoxin [18], and FB2 has shown cytotoxic activities to animals [19].

Considering the risk inherent to the occurrence of *Aspergillus* section *Nigri* in peanuts, the objective of this study is to investigate the biodiversity of *Aspergillus* section *Nigri* based on a polyphasic approach (using phenotypic and molecular data). In addition, we evaluate the ochratoxygenic potential of *Aspergillus* section *Nigri* isolates and the presence of OTA in peanuts.

2. Materials and Methods

2.1. Peanut Samples

A total of 52 peanut samples (variety IAC OL3) were examined, of which 42 were obtained in the field at an experimental farm in the city of Araraquara (São Paulo state) and 10 samples from storage were provided by the Cooperative of Sugarcane Planters in the West of São Paulo State (Coopercana), in the city of Sertãozinho (São Paulo state). Around 1 kg of each sample was collected and used for the analyses.

2.2. Water Activity of Peanut Samples

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

2.3. Fungal Isolation and Counting

The mycological analysis of the peanut kernel samples was performed according to Pitt and Hocking [6]. Briefly, fifty kernels from each sample were surface-disinfected in a 0.4% sodium hypochlorite solution for 2 min. Then, direct plating of 50 kernels was carried out so that they were distributed in 10 plates (replicates, each containing 5 kernels) containing 18% glycerol dichloran agar (DG18), according to Pitt and Hocking [6]. The plates were incubated in the dark at 25 °C for 5 days, and the results are expressed as percentages of infected peanuts. A total of 2600 peanut kernels were analyzed.

2.4. Morphological Characterization

Isolates that appeared to belong to the *Aspergillus* section *Nigri* (colonies of brown to black color, with great sporulation) were purified by monosporic culture and transferred to Czapek Yeast Extract Agar (CYA, [6]), then incubated at 25 °C for 7 days. For micromorphological observations (optical microscopy AX10, Carl Zeiss™, White Plains, NY, USA), microscopic mounts were made in lactic acid. The microstructures, including vesicles, conidia, metulae and phialides, were analyzed and/or measured using the software AxioVision Release 4.8.2 (Carl Zeiss™, White Plains, NY, USA).

2.5. Molecular Identification

Based on the morphological analysis, 78 isolates (78/218; about 35%) were selected for molecular identification at the species level; the choice of these representatives was based on the morphogroups characterized as described in Section 2.4. The characterization of the morphogroups and the distribution of the isolates is provided in the Supplementary Materials (Table S1).

The purified strains were grown in liquid YES (yeast extract sucrose) medium at 25 °C for 3 days until the formation of a mycelial skin, which was then manually macerated

with the aid of liquid nitrogen. The macerated material was used to obtain genomic DNA through the DNA purification kit (Mebep Bioscience, Shenzhen, China) according to the protocol recommended by the manufacturer.

To identify the selected strains, a multilocus analysis was performed based on the calmodulin (*CaM*) and beta-tubulin (*BenA*) loci; the primers used and the amplification conditions were the same as those described in Silva et al. [20]. Amplification products were purified using ExoSAP-IT (Thermo Fisher Scientific, Swindon, UK), as per the manufacturer's protocol. The fragments were submitted to direct sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA), and then the SeqStudio Genetic Analyzer device was used (Applied Biosystems, Waltham, MA, USA).

The obtained sequences were compared by local alignment using the BLAST tool against the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 25 April 2023); moreover, a maximum likelihood phylogenetic species tree was inferred based on the evolutionary general time-reversible model [21]. To determine the support for each clade, a bootstrap analysis was performed with 1000 replicates in the MEGA 11 program [22].

Representatives of all intra- and interspecific variability found in this study have been deposited in GenBank under accession numbers OQ923274-OQ923282 (*CaM* locus) and OQ923283-OQ923291 (*BenA* locus).

2.6. Potential for OTA Production by Aspergillus Section Nigri Isolates

All *Aspergillus* section *Nigri* isolates (n = 218) were tested for potential ochratoxin A production according to Filtenborg et al. [23]. The *Aspergillus* section *Nigri* isolates were inoculated on 15% sucrose yeast extract agar medium (YESA) and incubated at 25 °C for 7 days [23]. Then, the agar plug of each isolate was taken with a cork borer and applied on a Thin Layer Chromatography (TLC) plate, with an OTA standard (Sigma Aldrich, St. Louis, MO, USA). The mobile phase used was toluene/ethyl acetate/formic acid 90%/chloroform (7:5:2:5). OTA detection was performed using UV light (365 nm) on the Chromatovisor (Cole-Parmer, Vernon Hills, IL, USA). Isolates that showed a retention factor and a fluorescence spot similar to the OTA standard pattern were considered producers of this toxin.

2.7. OTA Analysis in Peanut Samples

Ten grams of ground peanut kernels were extracted with 200 mL of 3% sodium bicarbonate methanol solution (1:1). The suspension was shaken for 30 min on a shaker. This homogenized mixture was filtered using a quantitative filter (Nalgon, Itupeva, SP, Brazil) and a glass microfiber filter (Vicam, Milford, MA, USA). Then, 20 mL of the filtrate was diluted in 20 mL of phosphate-buffered saline (PBS) with 0.01% Tween 20. This mixture was passed through an immunoaffinity column of the brand R-Biopharm (R-(Biopharm Rhône Ltd., Darmstadt, Germany), specifically for ochratoxin A, at a flow of 2–3 mL/min, followed by washing with 20 mL of distilled water. In the next step, 4 mL was eluted with HPLC methanol in an amber flask. The solution was evaporated under a flow of nitrogen at 40 °C, and the dry extract was resuspended in 300 µL of mobile phase. The OTA analysis was performed in duplicate for each peanut sample.

HPLC equipment with a fluorescence detector (Agilent 1260 Infinity model system, Santa Clara, CA, USA) was used. A C18 chromatography column was used, with dimensions of 4.6 mm × 150 mm and particle size of 5 µm, as well as a mobile phase containing acetonitrile/water/acetic acid (51:47:02 $v/v/v$) filtered through a membrane of 0.45 µm. The mobile phase programmed flow was 1.0 mL/min, and the oven temperature was 40 °C. An OTA standard at a concentration of 1 mg/mL was used to construct the standard curve, with peak area versus toxin mass (ng). The concentration of OTA in the sample extract was determined by interpolating the resulting peak area on the calibration graph. The injection volume was 20 µL.

The limits of detection (LOD) and quantification (LOQ) were obtained by analyzing 8 peanut samples of a 0.5 µg/kg contamination level, according to EURACHEM [24]. Recovery was calculated by inoculating peanuts with OTA, in triplicate, with standard OTA at levels of 0.40 µg/kg, 5.0 µg/kg, and 12.0 µg/kg.

3. Results and Discussion

3.1. Aspergillus Section Nigri in Peanut Samples

Aspergillus section *Nigri* was found in 64% (27/42) of the field samples and 100% (10/10) of the storage samples (Table 1). This frequency was corroborated by other authors, who found 9.4–52.6% of *Aspergillus* section *Nigri* in peanuts after harvest [11] and 100% in stored peanut seeds [12]. One of the main sources of contamination by black aspergilli is the soil [25], so it is not surprising that this fungal group is present in peanuts, a creeping herbaceous plant where the food is in direct contact with the soil. The water activity values ranged from 0.521–0.724, with an average of 0.623, for the field samples after drying, and 0.541–0.609, with an average of 0.584, for the storage samples (Table 1).

Table 1. Occurrence and range of infection of *Aspergillus* section *Nigri* in peanut samples from the field and from storage.

Determinations	Field (42 Samples)	Storage (10 Samples)
Range of water activity	0.521–0.724	0.541–0.609
Number of positive samples (%)	27 (64%)	10 (100%)
Range of *Aspergillus* section *Nigri* infection (%)	0–42	6–34

A total of 218 strains of *Aspergillus* section *Nigri* were isolated, 121 from the field and 97 from storage samples. Out of 78 isolates selected for molecular identification at the species level, based on the multilocus analysis, six species of *Aspergillus* section *Nigri* were identified: *Aspergillus brasiliensis* (*n* = 29), *Aspergillus welwitschiae* (*n* = 27), *Aspergillus niger* (*n* = 12), *Aspergillus costaricaensis* (*n* = 8), *Aspergillus japonicus* (*n* = 1), and *Aspergillus neoniger* (*n* = 1) (Figure 1).

Aspergillus niger aggregate species have been reported to be frequent in peanuts. The *A. niger* aggregate is an informal taxonomic group that harbors morphologically similar and phylogenetically close species, namely: *A. niger*, *A. welwitschiae*, *A. piperis*, *A. costaricaensis*, *A. eucalypticola*, *A. tubingensis*, *A. vadensis*, *A. luchuensis*, *A. brasiliensis*, and *A. neoniger* [17,25,26]; recently, *Aspergillus vinaceus* was described and included in this group [20].

Magnoli et al. [12] investigated peanut samples stored in Argentina, and *A. niger* aggregate species were found to be isolated in all samples. In addition, the authors reported the occurrence (at low frequency) of *A. carbonarius* and *A. aculeatus*, but neither species was found in our study. Magnoli et al. [12] was based only on phenotypic data for species identification; therefore, the authors did not discriminate the species of the *A. niger* aggregate.

Chein et al. [27] used molecular data to identify *Aspergillus* and *Penicillium* isolates in peanut samples in Myanmar; according to the authors, *A. flavus* and *A. niger* were the species that occurred most frequently, although *Aspergillus terreus* and *Penicillium citrinum* were also found. Palencia et al. [28] investigated peanut samples from the USA and reported an isolation frequency of 67% for *A. niger* and 20% for *Aspergillus foetidus*. It is noteworthy that *A. foetidus* is currently considered to be synonymous with *A. niger sensu stricto* [25].

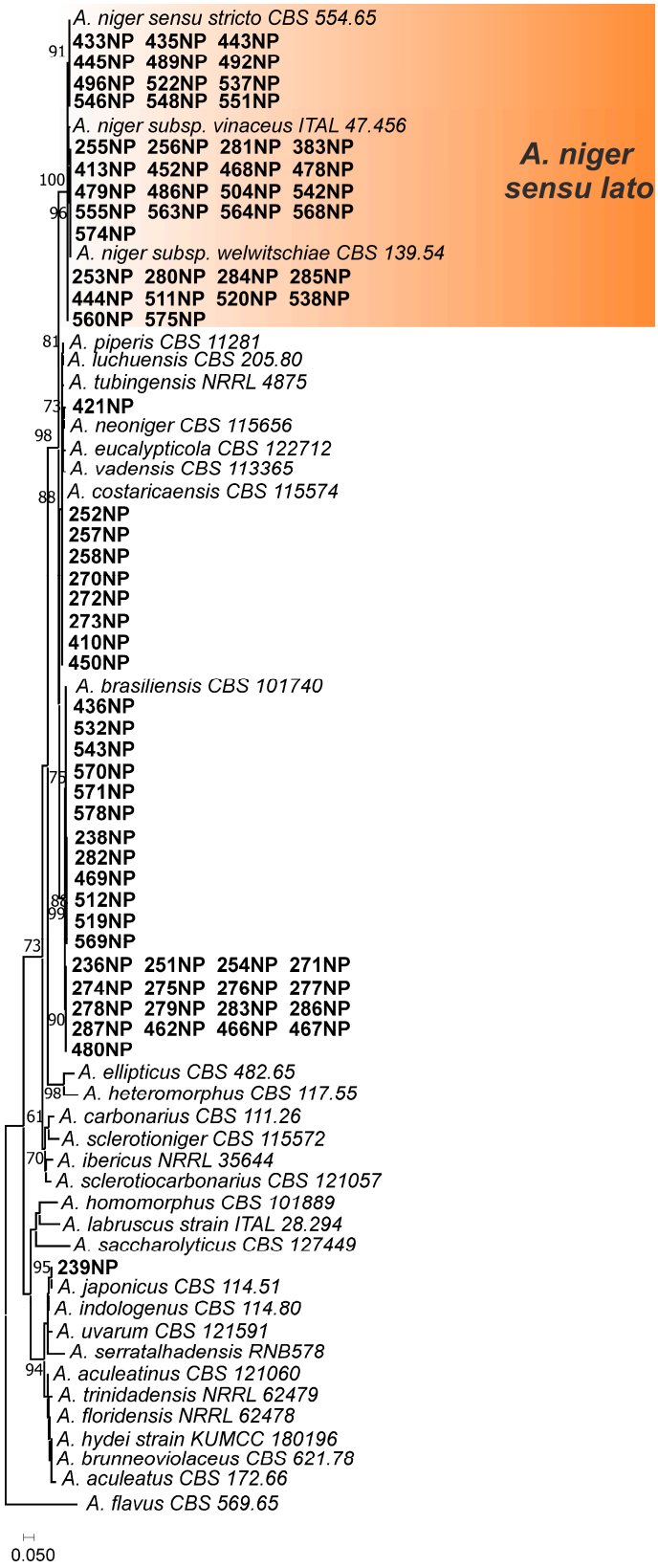

Figure 1. Maximum likelihood species tree, based on calmodulin (*CaM*) and beta-tubulin (*BenA*) sequences, showing the relationships between *Aspergillus* section *Nigri* and isolates from peanut samples. Bootstrap values (BS) higher than 60% are shown. Isolates from this study are in bold. *A. flavus* is the outgroup.

In general, *A. brasiliensis*, *A. welwitschiae,* and *A. niger* were the most frequently found species in our study. *A. brasiliensis* was described in 2007 [29], having been reported in onions, grapes, soils, and clinical sources [8,30–32]. *A. niger* and *A. welwitschiae* are species widely distributed in nature, having been found in several foods, including fruits, meats, coffee, dairy products, vegetables, nuts, and cereals [33–37]. *A. niger* and *A. welwitschiae* are potentially OTA- and FB2-producing species, so their high frequency (Table 1) in peanuts may be of concern.

A. japonicus was the only uniseriate species of *Aspergillus* section *Nigri* found in our study; this species had already been reported in peanuts by other authors [12,28]. *A. neoniger* and *A. costaricaensis* are infrequent species when compared to other species of the *A. niger* aggregate; *A. neoniger* has been found in grapes, yerba mate, clinical sources, mangrove water, and desert sand [25,38–40], while *A. costaricaensis* is rare, with few studies reporting its occurrence [41].

To the best of our knowledge, the present study is the first report of the presence of *A. brasiliensis*, *A. welwitschiae*, *A. neoniger,* and *A. costaricaensis* in peanuts.

3.2. Ocratoxigenic Potential of Aspergillus Section Nigri Isolates

All isolates identified as belonging to the *Aspergillus* section *Nigri* were tested for OTA production. The incidence of ochratoxigenic strains was 5.0% (11/218). The phenotype of OTA production within these species is variable; Massi et al. [36] investigated the ochratoxigenic profile of strains isolated from coffee, Brazil nuts, grapes, onions, dried fruits, and cocoa, and reported the incidence of strains producing OTA to be 32% and 1% of *A. niger* and *A. welwitschiae*, respectively. Out of 78 species identified molecularly, as expected, *A. brasiliensis*, *A. costaricaensis*, *A. japonicus,* and *A. neoniger* were not able to produce OTA, confirming the findings reported in the literature [42].

3.3. Occurrence of Ochratoxin A in Peanuts

The limits of detection (LOD) and quantification (LOQ) of the method were 0.08 µg/kg and 0.27 µg/kg, respectively. Recovery for contamination levels of 0.4 µg/kg, 5.0 µg/kg, and 12 µg/kg was 87.60%, 91.69%, and 89.80%, respectively. These values are within the range of 50 to 120% for the low level and 70 to 110% for the medium level established by European Community Directive EC 401/2006 [43].

Out of 52 samples analyzed, 6 samples from the field and 9 from storage showed OTA contamination of 0.106–0.387 µg/kg and 0.090–0.16 µg/kg, respectively. Most of these samples had levels lower than the LOQ (<0.27 µg/kg) of the method. Freshly harvested peanuts had a lower incidence of OTA than stored ones, showing that OTA was formed at the post-harvest stage, during drying and storage. This may be because *A. niger* and *A. welwitschiae* are xerophilic species, and are favored when peanuts have lower a_w.

Magnoli et al. [12] studied the formation of OTA in peanuts kept in a stored plant in Córdoba, Argentina. The percentages of contaminated samples were 50% during the first month and 66.7% at the second and third month of storage. The mean value of OTA obtained in the first month (30 µg/kg) was significantly higher than from those obtained in the second (6.5 µg/kg) and third (13 µg/kg) months. These authors found *A. carbonarius* as the main OTA producer, which may explain the difference in the data compared to our study. In our study, this species was not found, and the levels of OTA in peanuts were very low. *A carbonarius* is considered a strong OTA producer and has been recognized as the primary source of OTA contamination in grapes and grape products [9,44,45], coffee [46], cocoa [47], and others. In another study carried out in peanut samples from local markets of Tripoli, Libya, out of 15 samples, 5 were positive for OTA, ranging from 4.0 to 6.5 µg/kg [48]. These authors also found aflatoxins in co-occurrence.

In the present study, *A. niger* and *A welwitschiae* were found to produce OTA. Although the OTA values found were lower than those found in other reported studies, our data demonstrated that there is potential for peanuts to be a substrate for OTA production. This is a short study on the occurrence and diversity of *Aspergillus* section *Nigri*, its potential to

produce OTA in culture media, and the presence of this toxin in peanuts; more studies are required in this area. The present study allowed us to reach some conclusions.

4. Conclusions

The *Aspergillus* section *Nigri* was frequent in field and stored peanut samples. Six species were found, mostly belonging to the *A. niger* aggregate: *A. niger sensu stricto*, *A. welwitschiae*, *A. neoniger*, *A. costaricaensis,* and *A. brasiliensis*; in addition, *A. japonicus* was also present.

A. niger clade species were among the most prevalent, which would denote risks due to the ochratoxygenic potential of these species; however, the incidence of strains with a positive phenotype for OTA production was very low. In line with these results, OTA contamination was also low in peanut samples. The present study reported, for the first time, the occurrence of *A. welwitschiae*, *A. neoniger*, *A. costaricaensis,* and *A. brasiliensis* in peanut samples.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol3020033/s1. Table S1: Morphological characterization of groups.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding authors.

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