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RESEARCH ARTICLE

Identification and characterization of aflatoxigenic *Aspergillus* Section *Flavi* by polyphase approach from Brazil nuts produced in agroforesty production system

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ABSTRACT

Brazil nut is an Amazonian raw material with a fundamental role in the socioeconomic organization of the extractive areas of the Legal Amazon Brazil's largest socio-geographical division, composed of nine states are part of the Amazon basin. However, due to the precarious conditions of storage and processing, the nut is frequently subjected to contamination by aflatoxigenic fungi. These fungal species have high similarity, which makes it difficult to identify them only by traditional methods. This research had as objective the identification of a species of *Aspergillus Flavi* isolated from Brazil nut samples, through the polyphase approach. To identify the fungal isolate, macromorphological and micromorphological characterization techniques by microcultive in a slide were used; The molecular identification followed by sequencing compared the nucleotide sequences with the GenBank database, the aflatoxigenic potential of the strain was evaluated by HPLC. According to the results, the macromorphological and micromorphological analysis showed color characteristics and reproductive structures typical of the genus *Aspergillus* and the *Flavi* section. However, only from the results of molecular identification in which the strain showed 100% similarity with *Aspergillus nomius* and the metabolic production profile in which the species was producing the 4 types of aflatoxins (AFG2 = 1177,23, AFG1 = 2458, 90, AFB2 = 860,23, AFB1 = 2370,06) it was possible to carry out the correct identification of the fungus as *Aspergillus nomius.* The combination of different techniques for identification of the strain *Aspergillus* section *Flavi* allowed a more accurate characterization. The identification of strain as *Aspergillus nomius* with aflatoxigenic potential in Brazil nuts confirms the affinity of these microorganisms for this substrat

Keywords: Brazil nuts; Contamination; Identification; Aflatoxins; Fungi

INTRODUCTION

Brazil nut (*Bertholletia excelsa,* H.B.K) plays a fundamental role in the socioeconomic organization of large extractive areas in the states of Legal Amazon, with its importance in the support of low income families as part of their food base (Freitas-Silva et al., 2011). The differenced and unique flavor of this nut combined with its nutritional benefits promoted the recognition of this product in the international market. However, because of the precarious conditions of storage and processing, the nut is subject to contamination (Baquião et al., 2013).

The fungal species *Aspergillus flavus, Aspergillus nomius* and *Aspergillus parasiticus*, which belong to the *Flavi* section, stand out as potential aflatoxin producers and brazil nut contaminants (Midorikawa et al., 2014). Aflatoxins are extremely toxic secondary metabolites, they arecarcinogens that can contaminate food in the field, in storage or after processing (Arrus et al., 2005; Baquião et al., 2012; Taniwaki et al., 2016).

The species belonging to the *Flavi* section have high morphological similarity, which makes their identification impossible based only on microscopic and cultural characteristics. Hence the need to use polyphasic taxonomy. This approach uses several combined methods (microscopic characterization, molecular biology and aflatoxin production potential) for correct taxon designation (Varga et al., 2011; Moore et al., 2015). Phylogenetically,

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section *Flavi* is split into eight clades and currently contains 33 species (Frisvad et al., 2019).

For many years *A. flavus* has been described as the main contaminant of Brazil nuts, but with advances in molecular biology, several studies have highlighted *A. nomius*, a producer of the four types of aflatoxins, as an important contaminant of this substrate (Olsen et al., 2008; Freitas-Silva and Venâncio, 2011; G. Moore et al., 2015). These species with identical morphological characteristics may have been confused, since their identification was quite subjective (Moore et al., 2015).

The correct identification of the species belonging to the *Flavi* section is important for the knowledge of the mycobiota present in Brazil nuts, and so, it is possible, to define methods to reduce its contamination, since micro-organisms have distinct characteristics of resistance (Fraise et al., 2004).

Thus, this work aimed to identify a species of *Aspergillus* section *Flavi* isolated from Brazil nuts, through morphological characterization, biomolecular identification and secondary metabolite profile production potential.

Fig 1. (A) Brazil nut samples: Brazil nut pods, Brazil nuts, In-shell Brazil nuts, Shelled Brazil nuts (B) The strain of *Aspergillus* section *Flavi* isolated from Brazil nut samples. Source: MAY, Thomas.

MATERIALS AND METHODS

Strain of *Aspergillus* section *Flavi*

The strain of *Aspergillus* section *Flavi* was isolated from Brazil nut samples (Fig. 1) and provided by the Laboratory of Residues and Contaminants of Embrapa Food Agroindustry (Laboratório de Resíduos e Contaminantes da Embrapa Agroindústria de Alimentos – RJ).

Identification of the strain of *Aspergillus* section *Flavi*

Characterization macro and micromorphological

For the characterization of the fungal isolate by means of the micromorphological aspects, the technique of microculture on a slide using Lactrimel Agar was carried out according to the technique of Riddell (1950) with modifications for observation of asexual reproduction structures. Blocks of 1 cm^3 of Lactrimel agar were maintained on the culture medium in Petri dishes. The samples were then seeded on the side of the agar blocks and covered with coverslip (Fig. 2). After inoculation the plate was incubated at 30 °C for 5 days. After this period, the coverslips containing the fungal growth were placed on slides containing blue cotton Lactophenol solution and/or Lactophenol from Amman. Slides were analyzed under an optical microscope with 40X and 100X Objectives. Other phenotypic features based on colony texture, degree of sporulation, obverse and reverse colony colors, were determined and recorded after 7 d of incubation at 27 °C. The assays were performed in duplicate.

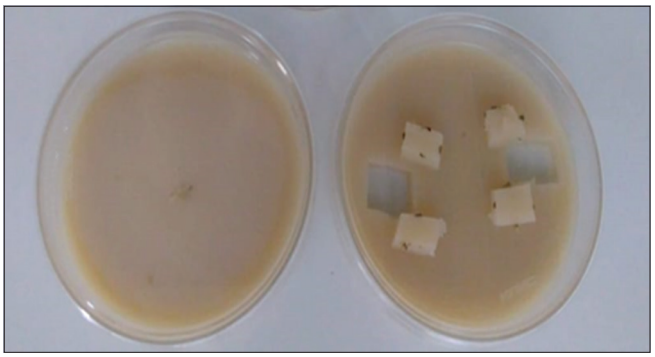

Fig 2. Microculture in Petri dish containing Lactrimel agar from the fungal isolate of *Aspergillus* section *Flavi*. Source: author.

Molecular identification of fungi

Genomic DNA was extracted from pure mycelial cultures of the fungal isolate grown on Lactrimel agar and using the method proposed by (Najafzadeh et al., 2010).

Initial identification using molecular techniques was based on sequencing of the ITS rDNA region (ITS 1, 5.8S e ITS 2). Reaction mixtures with primer sets ITS1(F)TCCGTAGGTGAACCTGCGG and ITS4(R) TCCTCCGCTTATTGATATGC were thermally cycled (PX2 Thermo Hybaid - Kingdom United) once at 95°C for 5 min, 35 times at 94°C for 60 s, 55,5°C for 120 s and 72°C for 120 s and then once at 72°C for 10 min (Chen et al., 2001; Fugita et al., 2001).

The amplification reaction was performed with 4 mM MgCl2, 0.4 mM of each dNTP (deoxynucleotide triphosphate), 1 mM of the primers, 0.1 µL of Taq DNA polymerase (Platinum® Taq, Invitrogen, Brazil) and 2 µl DNA, in a total volume of 25µL. PCR was performed on Thermo Hybaid PX2 thermocycler (Kingdom United). The amplification product was developed by 2% agarose gel electrophoresis to observe the test positivity. Subsequently, purification of the amplicons was performed using HiYield Gel/PCR DNA mini kit (Royal Biotech Corporation, China) according to the manufacturer's recommendations.

The ITS region was amplified by PCR using the sense primer and the antisense primer as mentioned above for the PCR. Amplicons were purified using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit, according to the manufacturer's instructions and then bidirectionally sequenced using an ABI Prism 3130⁄3130- Genetic Analyser (Applied Biosystems, Foster City, CA, USA) with the same primers used for PCR amplification. The nucleotide sequences obtained were compared with those available in the GenBank database using the BLASTn database.

Assessment of aflatoxin production potential B1, B2, G1, G2 and M1 by Aspergillus

The aflatoxigenic potential of the strain was carried out at the Embrapa Laboratory. The *Aspergillus* strain was grown in YES-Yeast Extract Sucrose medium at 25 ° C for 10 days, according Midorikawa et al 2014 (Fig. 3).

Samples were analyzed by HPLC (high performance liquid chromatography), with a "pool" of aflatoxins B1, B2, G1 and G2 as standard. The chromatographic conditions for the analysis of aflatoxins B1, B2, G1 and G2 in the fungal extract were as follows: Mobile phase: Acetonitrile: Methanol: Water: KBr:HNO34M (290:390:1320:390:1,17mL; Flow: 0,8mL/min.; Column: XBridge™ C18 (250 x 4,6 mm, 5. µm); Detector: Fluorescence; Excitation: 360, emission 440; Injection volume: 40 µL; flowing: 100 µA (Fig. 4).

For the calculation of post-column derivatization (with Kobra-cell®) the following expression was used:

$$\text{Amount of aflatoxins } (\mu g/kg): \frac{Ca \times V1 \times V3}{m \times V2}$$

Ca = concentration of the sample obtained through the calibration curve (µg/mL).
V1 = volume of sample extraction (250 mL).
V2= volume of the filtrate added to the immunoaffinity column (10 mL).
V3= volume of recovery of the purified extract (3 mL).
m= mass of the sample in kg.
Elution order: G2, G1, B2, B1

RESULTS AND DISCUSSION

Identification of the *Aspergillus* section *Flavi* isolate: Morphological characterization and molecular identification

The *Aspergillus* section *Flavi* strain was evaluated according to its macro morphological and microscopic characteristics after 5 days of incubation at 30 °C (Fig. 5).

The macrocolony (Fig. 5 A) presented radial growth, of cottony texture with dotted yellow-green on the colony. Microscopically, it was possible to observe reproductive structures after the fifth day of culture, which may be

Fig 3. Strain of *Aspergillus* section *Flavi* isolated from Brazil nut samples grown in YES-Yeast Extract Sucrose medium at 25 ° C for 10 days. Source: MAY, Thomas

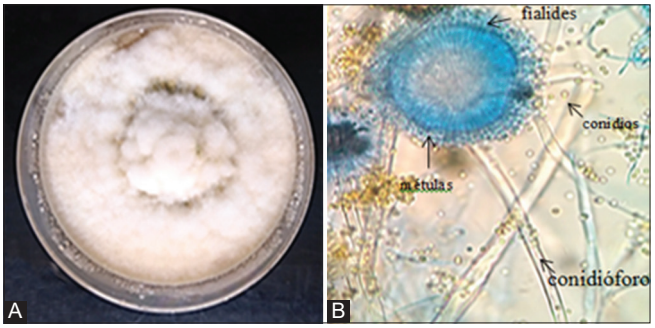

Fig 4. (A) Aflatoxins Extracts (B). Samples analyzed by HPLC. Source: MAY, Thomas

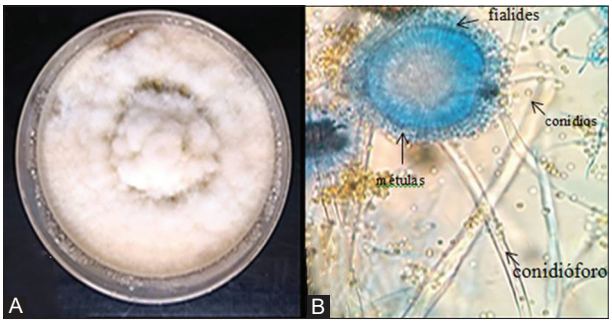

Fig 5. A. Colonial macroscopic aspect of the fungal isolate of *Aspergillus* section *Flavi* seeded in Lactrimel Agar. B. Microscopic Aspects. Source: author.

Table 1: Aflatoxigenic potential of *Aspergillus* section *Flavi*

Aflatoxins (ng/mL)				
AFG2	**AFG1**	**AFB2**	**AFB1**	**AFM1**
1177,23	2458,90	860,23	2370,06	0

AFG2: Afatoxin G2, AFG1: Aflatoxin G1, AFB2: Aflatoxin B2, AFB1:Aflatoxin B1, AFM1:Aflatoxin M1

related to the culture medium used, Lactrimel agar, which has the characteristic to favor the rapid sporulation of filamentous fungi (Jorgensen et al., 2015). In other culture media, such as Malt Extract Agar, Sabouraund Agar and Czapeck Agar, sporulation occurs after 7 days of incubation at 25 °C (Doster et al., 2009).

Initially, the formation of a large white endovelate structure, aerial mycelium with little sporulation was observed, and in the center of the colony (Fig. 5 A) the formation of greenish yellow conidia (spores). With the passage of time and, consequently, maturing of the colony, the green center became darker, in addition, the reverse of the plates remained colorless. The coloration of the colonies is one of the macroscopic characteristics that allows the differentiation between the sections of *Aspergillus*. As can be seen in Fig. 5 A, the greenish conidia of the culture confirm that the strain belongs to section *Flavi* (Varga et al., 2009).

In microscopic observation (Fig. 5 B) after coloring with cotton blue lactophenol, it is possible to identify the typical reproductive structures of the genus *Aspergillus*, composed of conidiophore, presenting at its extremity the formation of a bisserial globose vesicle, composed of phialides and metulae from which the conidia emerge.

This last aspect, referring to vesicular bisection, excludes the possibility of the strain being *A. parasiticus,* leaving the possibility of the strain being characterized as *A. nomius* or *A. flavus*. Only through morphological analysis, it is not possible to differentiate between these two species, as they have very similar phenotypic characteristics (Midorikawa et al., 2014; Samson et al., 2014, Frisvad et al., 2019).

Several works reported the difficulty of identifying members belonging to the *Flavi* section, either by the similarity between species or by the fact that, constantly, new species have been discovered and grouped in the section. Nowadays, in order to correctly identify a species, a polyphase approach is required, in which the macro and micromorphological characteristics, the molecular identification and the production profile of metabolites are evaluated (Varga et al., 2011; Samsom et al., 2014; Garcia et al., 2018).

In this sense, for the correct identification of the fungal isolate used in this study, the molecular identification of the species was performed after the comparison of the sequence obtained with the *GenBank* database. With the use of *Blast* tool, the strain was identified as *Aspergillus nomius* (accession number MK397788) presenting 100% similarity.

Potential for the production of aflatoxins B1, B2, G1, G2 and M1 of the fungal isolate

It was detected that the evaluated strain produces the four types of aflatoxins (Table 1), this information corroborates with the molecular analysis that identifies the strain as *Aspergillus nomius*, besides discarding the hypothesis in the morphological characterization that the isolate could be *Aspergillus flavus*, since, due to a genetic mutation, this species only produces aflatoxins B (P-K. Chang, 2010).

G. Moore et al. (2015) points out that many paths still need to be explored in relation to the aflatoxigenic fungus *A. nomius*, which may be indicated as an ancestral aflatoxigenic species from genomic comparisons with other section *Flavi* members. Emphasizing that future genomic comparisons should include more species in this section and elucidate the reasons for the emergence of other aflatoxigenic species, as well as the purpose of aflatoxin production.

Historically, aflatoxin profiles have been used to distinguish between *A. flavus* and *A. nomius*, since their macro and micro-morphologies are highly similar. If an isolate produced only B-aflatoxins, it would be classified as *A. flavus* and, if aflatoxins B and G were produced, it would be considered *A. nomius* (G. Moore et al., 2015).

Nielsen et al. (2011) and Kildgaard et al. (2014) emphasized that the production of a given secondary metabolite consists of an efficient mode for the allocation of a strain in a section, as well as the identification of several secondary metabolites can effectively ensure the identification of a species.

According to Frisvad et al. 2019, some of the most efficient producers of aflatoxins have not been described yet. Using a polyphasic approach combining phenotype, physiology, sequence and extrolite data, we can describe the species into section Flavi. There is also another factor that is the presence of atoxigenic strains, and detection methods shall to be combined for correct taxon identification.

CONCLUSION

The combination of different techniques for identification of the strain *Aspergillus* section *Flavi* allowed a more accurate identification of the species. In this study, the identification of the species such as *Aspergillus nomius* with its aflatoxigenic potential in Brazil nuts confirms the affinity of these microorganisms for this substrate.

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Author's contributions

All authors participated effectively in the elaboration of this manuscript. Maria do Socorro Souza Ribeiro (M. Ribeiro), was responsible for performing the experiments and wrote the article. Silvia Helena Marques da Silva (S.H. M. Silva) contributed the correction of the article and molecular identification. Otniel Freitas-Silva (O. Freitas-Silva) contributed with aflatoxin analysis, review & editing. Laura Figueiredo Abreu (L.F. Abreu) elaborated the research project and review. Consuelo Lucia Sousa de Lima supervised the whole study and reviews of the article.

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