GMR

The Original by FUNPEC-RP

Genetic variability of *Aspergillus flavus* isolated from commercial peanut and bulgur wheat in Southern Brazil and antifungal activity of essential oils against some of the isolates

K.L. dos Reis and I.P. Barbosa-Tessmann

Universidade Estadual de Maringá, Departamento de Bioquímica, Maringá, PR, Brasil

Corresponding author: I.P. Barbosa-Tessmann
E-mail: ipbtessmann@uem.br

Genet. Mol. Res. 20 (2): gmr18787
Received January 06, 2021
Accepted May 11, 2021
Published June 30, 2021
DOI http://dx.doi.org/10.4238/gmr18787

ABSTRACT. *Aspergillus flavus* produces carcinogenic aflatoxins. This species is divided based on the type of sclerotia produced, designated as "S" for numerous small and "L" for fewer and large. "L" strains are concerning since they are prevalent in corn, peanut, rice, and soil samples. Nearly all "S" strains and approximately 70% of "L" strains produce aflatoxins. Host specificity within isolates is not well understood and needs to be better characterized. We isolated *A. flavus* from commercial peanut samples from Southern Brazil and investigated their genetic variability and their genetic correlation with four *A. flavus* isolates previously obtained from commercial bulgur wheat in the same region. We also evaluated the antifungal activity of essential oils on some of the isolates. Eleven "L" type *A. flavus* strains were isolated from peanut kernels. Eight of these isolates were considered aflatoxigenic, based on thin-layer chromatography and culture techniques. The genetic biodiversity of the *A. flavus* isolates was analyzed using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) analyses, which indicated differences according to host, however, did not regarding aflatoxin production. Essential oils of oregano, thyme, cinnamon, lavender, and fennel inhibited the isolate's growth. In conclusion, *A. flavus* isolates differ according to host and essential oils have potential to control this fungus.

Key words: Phylogenetics; RAPD and ISSR; Host; Fungi; Aflatoxin

INTRODUCTION

Aspergillus flavus is a filamentous cosmopolitan pathogenic fungus that can occur in both field and post-harvest conditions. It occurs in soils, living plants, and plant products, such as oil-rich seeds, which is concerning because this fungus can produce aflatoxins. Aflatoxins are hepatotoxic, immunosuppressive, teratogenic, and mutagenic. The four main aflatoxins, named B1, B2, G1, and G2, are distinguished based on their fluorescence under UV light (blue or green) and relative chromatographic mobility. Aflatoxin B1 is the most well-known potent natural carcinogen and is classified as a group I carcinogen by the International Agency for Research on Cancer (IARC, 2009; Kumar et al., 2017).

A. flavus isolates are divided into "S" and "L" types, based on whether they produce numerous small (S) or fewer and large (L) sclerotia (Cotty, 1989). Some of the S type isolates secrete both B- and G-type aflatoxins, but the L type isolates secrete only B aflatoxins (Saito et al., 1986; Saito et al., 1989). Nearly all S strains and approximately 70% of L strains produce aflatoxins (Horn et al., 1999). L strains exhibit much higher virulence since they are far more prevalent in corn, peanut, rice, and soil samples than S strains (Abbas et al., 2005). By sequence analysis of coding gene regions, *A. flavus* strains were also divided into two distinct phylogenetic groups, termed group I and group II (Geiser et al., 1998). Group I consists of both S- and L-strains that produce aflatoxin B1 and G-aflatoxins, while group II is comprised of S-strains that produce aflatoxin B1 and/or G-aflatoxins (Geiser et al., 2000; Abbas et al., 2005).

Currently, there are concerns about synthetic preservatives to handle grain and food fungal contamination due to their toxic side effects and, because of this, there is an increasing consideration on natural antimicrobials such as essential oils, which have been generally recognized as safe (GRAS) (Nazzaro et al., 2017; Pandey et al., 2017; D'agostino et al., 2019). The major constituents of essential oils, which may be involved in the antifungal activity are terpenes (e.g., pinene and limonene), terpenoids (e.g., geraniol), aromatic phenols (e.g., carvacrol, thymol, and eugenol), and cinnamaldehyde (Montes-Belmont and Carvajal, 1998; Viuda-Martos et al., 2007; Silva et al., 2012). There are several reports in the literature about *A. flavus* growth inhibition by thyme, oregano, cinnamon, peppermint, basil, clove, ginger, and fennel essential oils (Montes-Belmont and Carvajal, 1998; Viuda-Martos et al., 2007; Silva et al., 2012).

Peanut (*Arachis hypogaea*) is widely cultured in tropical and subtropical regions. The world annual production of peanuts was estimated to be 47.3 million metric tons in 2020-21, led by China and India, with 17.5 and 6.5 million metric tons, respectively (USDA, 2021). The primary center of peanut origin is South America (Grabiele et al., 2018). Brazilian production in 2020-21 was estimated to be 0.5 million metric tons (USDA, 2021). Peanut cultures are widely sensitive to infection by toxin-producing fungi, mainly during the harvest and post-harvest periods (Norlia et al., 2019). Peanut is appreciated and used in Brazilian cuisine for centuries to prepare several traditional dishes, especially in sweet desserts.

Previous to this work, 41 strains of *A. flavus* were obtained from commercial bulgur wheat, an ingredient from Arabic cuisine, also much appreciated in Brazil (Faria et al.,

2017). Our objectives were to obtain isolates of *A. flavus* from peanut kernels and investigate their genetic variability and their genetic relation with four (one aflatoxigenic and three non-aflatoxigenic) *A. flavus* isolates obtained previously from another host, the commercial bulgur wheat. In addition, we evaluated the efficiency of several essential oils as growth inhibitors of some of the isolates.

MATERIAL AND METHODS

Sample collection

Six different peanut samples were bought in stores of the city of Maringá (latitude 23° 25' 30"; longitude S 51° 56' 20" W; altitude 530 m) located in the Southern region of Brazil, from September to October 2017 (Table 1). Samples consisted of individual packs of raw peanut kernels with 500 g each, from different brands. Three of the samples were from small-sized and red-skinned varieties, and three were medium-sized and white-skinned varieties. All samples were conserved at room temperature and were analyzed before the expiration dates.

Table 1. Samples, number of peanut kernels contaminated with *Aspergillus* spp. on AFPA medium, isolates obtained, and aflatoxin production.

Samples	Peanut variety	Nº of kernels presenting *Aspergillus* spp. on AFPA*	Isolates of *Aspergillus* obtained	Species (DNA barcoding)&	GenBank Accession number (5.8S-ITS)	Fluorescence in CMA	Ammonium hydroxide analysis	TLC
1	Red	4	UEM 1-1	*Aspergillus flavus*	MN661377	-	-	-
			UEM 1-2	*A. flavus*	MN661378	+	-	+
2	White	20	UEM 2-1	*A. flavus*	MN661379	-	-	+
			UEM 2-2	*A. flavus*	MN661380	+	+	+
3	White	4	UEM 3-1	*A. flavus*	MN661381	+	+	+
			UEM 3-2	*A. flavus*	MN661382	-	-	-
4	Red	4	UEM 4-1	*Aspergillus caelatus*	MN661383	-	-	-
			UEM 4-2	*A. flavus*	MN661384	+	+	+
5	Red	12	UEM 5-1	*A. flavus*	MN661385	-	-	+
			UEM 5-2	*A. flavus*	MN661386	+	+	+
6	White	3	UEM 6-1	*A. flavus*	MN661387	-	-	+
			UEM 6-2	*A. flavus*	MN661388	-	-	-

*Nº of kernels presenting *Aspergillus* spp. on AFPA from a total of 24 kernels analyzed. &The indicated species was identified using DNA barcoding. AFPA – *Aspergillus flavus* and *parasiticus* Agar. 5.8S-ITS – 5.8S rDNA and Internal Transcribed Sequence. CMA – Coconut Milk Agar. TLC – Thin Layer Chromatography. (+) – Positive result in the test. (-) – Negative result in the test.

Aspergillus spp. isolation and morphologic characterization

For surface disinfection, six peanut kernels from each sample were incubated with 50 mL of 0.4% active chlorine solution for 1 min at room temperature, with agitation, and rinsed once with 50 mL of sterile distilled water. The six kernels from each sample were placed on 10 cm Petri dishes containing *Aspergillus flavus* and *parasiticus* Agar (AFPA): 2 g/% of

yeast extract, 1 g/% of peptone, 0.05 g/% of ferric ammonium citrate, and 1.5 g/% of agar (Pitt and Hocking, 2009). Fast-growing fungi, such as *Rhizopus* and *Mucor*, were prevented by adding 2.5 µg/mL malachite green to the culture medium prior to autoclaving. Bacterial growth inhibition was performed by aseptically adding 641 U/mL penicillin and 256.4 µg/mL streptomycin to the medium after sterilization by autoclaving and cooling until 60°C. Four Petri dishes were inoculated for each peanut sample; therefore, 24 kernels from each sample were analyzed in the AFPA medium. The Petri dishes were incubated at 25°C for five days with a 12 h photoperiod in an incubator. In this medium, the potentially aflatoxigenic *Aspergillus* spp. strains colonies reverse side have an orange color. The kernels showing reverse side orange color colonies were counted.

A fragment of a colony on the AFPA medium with a reverse side orange color was transferred to a tube containing a slanted potato-dextrose agar (PDA) medium. This tube was incubated at 25°C in a 12 h photoperiod incubator for five to seven days for spore formation. Cultures with *A. flavus* and *A. parasiticus* characteristic green color were chosen for monosporic isolation. For that, a fragment of approximately 1 cm^3 of the PDA culture was stirred in 20 mL of sterile distilled water, and an aliquot of 100 µL of the obtained spore suspension was spread on Petri dishes containing 2.5% agar-water medium. After incubation at 25°C for approximately 24 h, a single germinating spore was transferred to new slanted PDA tubes (Nelson et al., 1983). After growth at 25°C for 5 days, the isolates were stored at room temperature, with passages every three months.

For the morphological identification, the obtained monosporic isolates were inoculated, in duplicate, in the center of dishes containing Czapeck Dox Agar (Pitt and Hocking, 2009). The inoculum consisted of a touch of a sterile wooden skewer stick tip covered with spores collected from the monosporic culture in slanted PDA. The dishes were incubated at 25°C for 7 days in a 12 h photoperiod incubator before analysis. The conidiophores were observed under a stereoscopic microscope. Macroscopic aspects, such as coloration and colony surface, were observed. In this culture medium, isolates of *A. parasiticus* are dark olive green colored, and isolates of *A. flavus* are yellowish-green colored. The isolate of *A. parasiticus* UEM 443, previously isolated from peanut and maintained for several years in our laboratory, and the isolate of *A. flavus* UEM 2-1, isolated from commercial bulgur wheat, were used as standards (Faria et al., 2017).

Molecular identification of the isolates

For DNA extraction, a fragment of approximately 1 cm^3 of a monosporic culture in PDA was cut into small pieces and shaken in 10 mL of sterile distilled water. One hundred microliters of the spore suspension (4.25×10^6 spores) were inoculated in a 125 mL Erlenmeyer flask containing 25 mL of liquid AFP medium (without malachite green and antibiotics). The inoculated flasks were incubated for five days at 25°C, with a photoperiod of 12 h. This culture medium was used to obtain mycelium without spores. The isolate *A. flavus* NRRL 5940 also had its DNA extracted to be used as a reference.

The mycelium was collected by filtration with sterile gauze and used for genomic DNA extraction by the method described by Koenig et al. (1997) and modified by Faria et al. (2017). The DNA was quantified in a spectrophotometer at 260 nm, and the final concentration was adjusted to 100 ng/µL.

The isolates were identified by DNA *barcoding* through PCR amplification of a 5.8S-ITS region DNA fragment with the primers ITS4 and ITS5 (Table 2) (White et al., 1990). The amplification reactions contained: 50 mM KCl; 10 mM Tris, pH 7.5; 1.5 mM $MgCl_2$; 1.5 U of *Taq* DNA polymerase; 0.2 mM of each dNTP; 25 pmol of each primer; and 400 ng of the DNA

sample, in a final volume of 25 µL. The cycling conditions were 25 cycles of 1 min and 30 s at 94°C, 1 min and 30 s at 50°C, and 2 min at 72°C, which were run in a Techne TC-312 thermocycler (England). Samples were heated for 5 min at 94°C, prior to the cycles, and 10 min at 72°C after the cycles. The PCR products were kept frozen at -20°C until use. The amplification of a DNA fragment was confirmed by electrophoresing 10 µL of the PCR reaction in a 1.5% agarose gel containing ethidium bromide (0.25 µg/mL) and visualizing it under UV light. The PCR products were purified with the Illustra ExoProStar™ (GE Healthcare Life Sciences, USA), following the manufacturer's protocol, and sequenced at the Center for Human Genome Studies (CEGH) from the University of São Paulo (USP). All obtained sequences had 5´and 3´ends trimmed and were deposited in GenBank (numbers available in Table 1 and Figure 1). The sequences were compared with others deposited in databanks for species definition.

Table 2. Primers used for the phylogenetic, RAPD and ISSR analysis of *Aspergillus flavus*.

	ITS primers	
Primer	**Sequence**	**Reference**
ITS4	5´-TCCTCCGCTTATTGATATGC	White et al., 1990
ITS5	5´-GGAAGTAAAAGTCGTAACAAGG	White et al., 1990
β-tubulin primers		
Bt2a	5´-GGTAACCAAATCGGTGCTGCTTTC	Glass and Donaldson, 1995
Bt2b	5´-ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson, 1995
RAPD primers		
Primer 1	5´-GGTGCGGGAA	Mahmoud et al., 2012
Primer 3	5´-GTTTCGCTCC	Mahmoud et al., 2012
Primer 5	5´-AACGCGCAAC	Mahmoud et al., 2012
OPW 04	5´-CAGAAGCGGA	Batista et al., 2008
OPW 05	5´-GGCGGATAAG	Batista et al., 2008
OPA 10	5´-GTGATCGCAG	Batista et al., 2008
OPA 14	5´-TCTGTGCTGG	Batista et al., 2008
OPA 17	5´-GACCGCTTGT	Batista et al., 2008
OPA 20	5´-GTTGCGATCC	Batista et al., 2008
$(GACA)_4$	5´-GACAGACAGACAGACA	Batista et al., 2008
$(AGAG)_4G$	5´-AGAGAGAGAGAGAGAGG	Batista et al., 2008
$(GTG)_5$	5´-GTGGTGGTGGTGGTG	Hatti et al., 2010

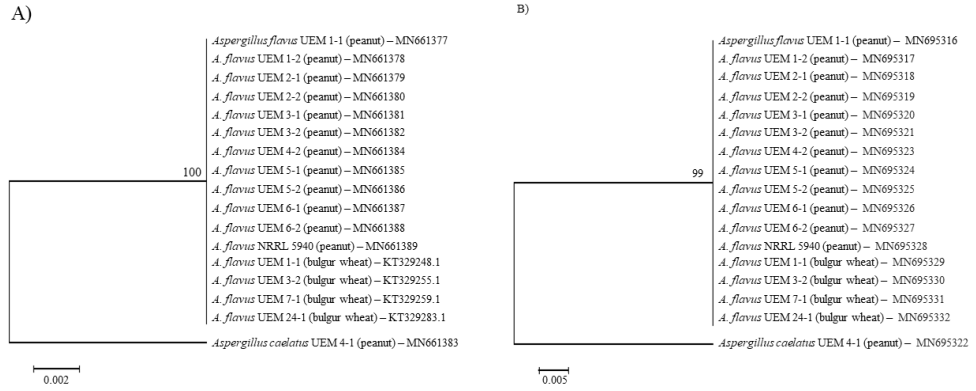

Figure 1. Phylogenetic analysis of the 5.8S-ITS and β-tubulin gene sequences from the *Aspergillus flavus* isolates. A) Phylogenetic analysis with the partial sequence from the 5.8S-ITS region. The analysis involved 17 nucleotide sequences. There was a total of 527 positions in the final dataset. B) Phylogenetic analysis with the partial sequence from the β-tubulin gene. The analysis involved 17 nucleotide sequences. There was a total of 490 positions in the final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches. The isolate *A. flavus* NRRL 5940 was used as a reference.

Aflatoxin production analyses in specific culture medium

The isolates were cultured in coconut milk agar (CMA) medium, which was prepared with 200 mL of coconut milk, 600 mL of distilled water, pH 6.9, and 16 g of agar (Lin and Dianese, 1976). Ten cm diameter Petri dishes containing approximately 20 mL of CMA medium were inoculated in duplicate. The inoculum was made by a touch of a sterile wooden skewer stick tip covered with spores collected from a monosporic culture in a slanted PDA medium. The dishes were incubated at 25°C for seven days in a 12 h photoperiod incubator, and fluorescence was observed in a UV transilluminator with emission at 312 nm. The appearance of fluorescence around the colonies indicated aflatoxin production.

For ammonium hydroxide vapor analysis, CMA dishes with seven-day colonies were inverted, and drops of 28-30% ammonium hydroxide were added on the inside of the lid. The dishes were incubated like this for 10 min at room temperature. The appearance of a pink color around the colonies indicated aflatoxin production (Saito and Machida, 1999). The *A. parasiticus* isolate UEM 443 was used as a positive control in both analyses.

Aflatoxin production analyses by thin-layer chromatography (TLC)

A fragment of approximately 1 cm^3 of the monosporic cultures in PDA was cut into smaller pieces and shaken by hand in 10 mL of sterile distilled water. One hundred microliters of the spore suspension (4.25×10^6 spores) were inoculated in Erlenmeyer flasks with 25 mL of YES medium (2% yeast extract and 20% sucrose) (Davis et al., 1966). These flasks were incubated for 15 days, without shaking, at 25°C in a 12 h photoperiod incubator. The isolate of *A. parasiticus* UEM 443 was also inoculated as a positive control.

The obtained cultures were filtered through filter paper. A volume of 10 mL of hexane was added to the filtrates, with shaking for 1 min in a vortex. Chloroform (10 mL) was then added to the aqueous fraction with shaking for 3 min in a vortex. After phase separation, the chloroform fraction was collected and filtrated in filter paper containing approximately 3 g of anhydrous sodium sulfate. The filtrate was entirely evaporated with incubation at 50°C for 18 h, and the obtained extracts were resuspended in 200 µL of chloroform. Approximately 40 µL of each extract in chloroform was applied with a capillary tube on a thin-layer chromatography plate (TLC) (Silica gel, Sigma-Aldrich, Germany). After application, the samples were dried at room temperature, and the chromatogram was developed with the solvent system toluene/ethyl acetate/chloroform/formic acid in the proportion of 7:5:5:2 (v/v). The aflatoxin standards B1 and B2 (Sigma-Aldrich, Germany) were dissolved in toluene at 0.125 µg/µL, and the G1 and G2 standards were dissolved in methanol:water (9:1, v/v) in the same concentration. Approximately 20 µL of each standard (2.5 µg) were applied on the chromatographic plate.

All the analyses, beginning from the culture, were repeated at least twice for all of the isolates. The isolates with negative results were repeated three times. Isolates that produced aflatoxins in at least one of the extractions were considered aflatoxigenic.

Phylogenetic analyses

The phylogenetic analyses of the *A. flavus* isolates were conducted in the MEGA7 program (Kumar et al., 2016), using the Neighbor-Joining method (Saitou and Nei, 1987). The confidence limits of the branching were assessed using Bootstrap analyses with 1000

heuristic replicates (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004), in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair.

A phylogenetic tree was constructed using the partial amplified sequences of the 5.8S-ITS region (525 bp), as described in the Molecular identification of the isolates section of the Materials and Methods together with 5.8S-ITS region sequences (GenBank: KT329248.1, KT329255.1, KT329259.1, and KT329283.1) of four *A. flavus* isolates previously obtained from bulgur wheat (Faria et al., 2017). Those isolates corresponded to three non-aflatoxigenic isolates (UEM 1-1, UEM 3-2, UEM 7-1) and one aflatoxigenic isolate (UEM 24-1).

Another phylogenetic tree was built using partial sequences of the β-tubulin gene. First, a portion (485 bp) of the β-tubulin gene was amplified from the peanut isolate's DNA and the four bulgur-wheat isolate's DNA (Faria et al., 2017) with the primers Bt2a and Bt2b (Table 2) (Glass and Donaldson, 1995). The PCR conditions were the same as those described for the 5.8-ITS sequences amplification, but using an annealing temperature of 60°C and 32 cycles. The PCR products were purified with the Illustra ExoProStar™ (GE Healthcare Life Sciences, USA) and sequenced at CEGH from USP, and the obtained sequences had the 5´and 3´ends trimmed and were deposited in GenBank (Numbers available in Figure 1).

Determination of *A. flavus* morphotypes

To determine sclerotium type formation, cultures were performed in PDA and Czapeck Yeast Agar (CYA) in slant tubes (Pitt and Hocking, 2009). The eleven *A. flavus* isolates obtained from peanut and the four chosen isolates of *A. flavus* obtained previously from bulgur wheat were analyzed. The inoculum was made with a sterile wooden skewer stick tip covered with spores collected from a monosporic culture in a slanted PDA medium. The tubes were incubated at 25°C and 30°C for seven days in the dark (Gilbert et al., 2018). The formation of sclerotia was observed after that.

RAPD and ISSR analyses

Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) analyses were used to evaluate the genetic variability among the *A. flavus* isolated from peanuts in this work, and the four *A. flavus* isolated from bulgur wheat (Faria et al., 2017) mentioned in the phylogenetic analyses.

The RAPD PCR mixtures were made to a final volume of 25 µL, containing reaction buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4), 3.0 mM $MgCl_2$, 0.20 mM dNTP, 1 µM primer (Table 2), 1.5 U *Taq* DNA polymerase (Thermo Fisher Scientific, USA), and 400 ng of genomic DNA (Williams et al., 1990). Amplification consisted of an initial denaturation step at 95°C for 10 min (one cycle) followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min, and amplification at 72°C for 2 min, with a final extension at 72°C for 5 min.

For the ISSR analyses, the PCR mixtures were made to a final volume of 25 µL, containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.20

mM dNTP, 1 μM primer (Table 2), 1.5 U *Taq* DNA polymerase (Thermo Fisher Scientific, USA), and 400 ng genomic DNA (Williams et al., 1990). Amplification consisted of an initial denaturation step at 93°C for 10 min, followed by 35 cycles of denaturation at 93°C for 20 s, annealing at 55°C for 45 s, and amplification at 72°C for 90 s, with a final extension at 72°C for 6 min.

As a control of the banding pattern consistency, each primer's PCR was repeated twice on different days. Negative controls (no DNA template) were made for each RAPD and ISSR primer reaction to test for DNA contamination of reagents and reaction mixtures. Fragments that were also amplified in the negative controls were removed from the analysis.

For all samples, the PCR reactions (15 μL) were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.25 μg/mL) in 1X TAE buffer (Tris-acetate EDTA, pH 8.0), at 110 V for 3h, using the 100-bp ladder DNA marker (Thermo Fischer Scientific, USA). The DNA was visualized and photographed under UV light in a transilluminator. The agarose gel pictures were analyzed for the DNA banding pattern in PyElph 1.4 software (Pavel and Vasile, 2012), which generated the phylogenetic trees; the method used for clustering was the Unweighted Pair Group Method with Arithmetic mean (UPGMA). The running distance of each DNA fragment was considered in the analyses.

Effect of essential oils on fungal growth

The essential oils were purchased in stores in the city of Maringá. Essential oils of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), fennel (*Foeniculum vulgare*), lavender (*Lavandula angustifolia*), and cinnamon (*Cinnamomum cassia*) were tested non-diluted (100%) on some of the *A. flavus* isolates for their potential as growth inhibitors. The *A. flavus* isolates were chosen at random, two aflatoxigenic peanut isolates (UEM 3-1 and UEM 4-2) and two non-aflatoxigenic (UEM 3-2 and UEM 6-2), as well as two bulgur wheat isolates, one non-aflatoxigenic (UEM 1-1), and one aflatoxigenic (UEM 24-1) (Faria et al., 2017). A fragment of approximately 1 cm^3 of a monosporic culture in PDA was cut into small pieces and shaken in 10 mL of sterile distilled water. One hundred microliters of the obtained spore suspension (4.25×10^6 spores) were inoculated in triplicate in 10 cm diameter Petri dishes containing approximately 20 mL of PDA medium. After inoculation, a sterile filter paper disc with 1.5 cm diameter was covered with 10 μL of the sterile essential oil and placed on the center of the inoculated plate. The dishes were incubated at 25°C for five days in a 12 h photoperiod incubator. A halo around the paper disc indicated growth inhibition. The inhibition halo was measured from the paper disc's edge to the halo's end, where the mycelial growth was present. The negative control (0%) had 10 μL of sterile mineral oil (Vaseline) in the disc paper. The essential oils that showed the best inhibitory effect were also tested at concentrations of 25, 50, 75, and 100%, with dilutions (v/v) made with sterile mineral oil (liquid Vaseline).

Statistical analyses

Averages and standard deviations of the obtained data were submitted to ANOVA and compared using the Tukey test in the SASM–Agri program (Canteri et al., 2001). Identical letters indicate no difference among averages ($\alpha = 0.01$).

RESULTS

Aspergillus spp. isolation, identification, and characterization

Table 1 presents the number of contaminated kernels with *Aspergillus* spp. on the AFPA medium of each peanut sample. Every sample was contaminated with potentially aflatoxigenic *Aspergillus* spp. Peanut samples 2 and 5 were heavily contaminated (83% and 50%), while the other samples were less contaminated (12.5 to 16.7%). The total amount of contaminated kernels was 32.6%, considering 47 contaminated kernels out of 144 analyzed. Twelve *Aspergillus* spp. monosporic isolates were obtained, two from each peanut sample. All isolates presented culture and microscopic characteristics (not shown) of *Aspergillus* from the Flavi group in the Czapeck Dox Agar (Table 1). The molecular identification confirmed all isolates as *A. flavus*, except for isolate UEM 4-1, identified as *Aspergillus caelatus* (Table 1).

The eleven *A. flavus* isolates obtained in this work from peanut and the other four isolates obtained previously from bulgur wheat (Faria et al., 2017) were determined to be from the "L" morphotype. They produced large or no sclerotia in PDA or CYA media (not shown).

Aflatoxin production

Five isolates presented fluorescence in CMA, and four of those isolates pigmented the CMA medium with pink color in the ammonium hydroxide vapor analysis (Table 1). In the TLC analysis, eight isolates produced aflatoxins B1 and B2 in the YES medium, while the control isolate *A. parasiticus* UEM 443 produced the four types of aflatoxins (Table 1).

Phylogenetic analyses

In the phylogenetic analyses with the 5.8S-ITS rDNA and β-tubulin partial gene (Figure 1), only the UEM 4-1 isolate, identified as *A. caelatus*, did not group with the *A. flavus* isolate's sequences.

RAPD and ISSR analyses

From the nine primers tested for RAPD analysis and the three primers tested for ISSR analysis (Table 2), only the RAPD Primer 1 resulted in no DNA amplification. All the other primers could generate amplified DNA products and separation between the isolates (Figures 2, 3, and 4).

The best results for RAPD and ISSR analyses are shown in Figure 2. In the obtained dendrograms with RAPD Primers 3 and OPA 17 (Figure 2A and B), most peanut isolates do not group in the same clade as the bulgur wheat isolates. The same pattern was seen in the dendrograms obtained with the ISSR Primers $(GACA)_4$ and $(AGAG)_4G$ (Figure 2C and D). There was little differentiation among all tested primers between aflatoxigenic and non-aflatoxigenic *A. flavus* isolates from peanuts (Figures 2, 3, and 4).

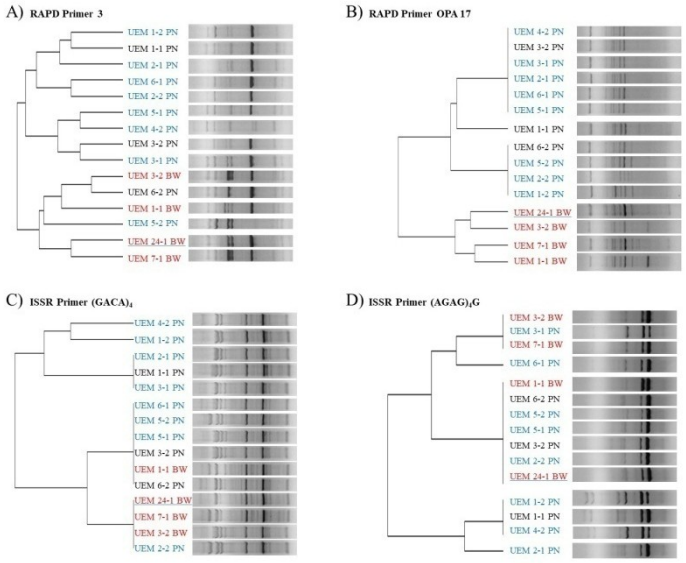

Figure 2. Dendrograms derived from PCR-amplification banding of RAPDs with primer 3 (A) and primer OPA 17 (B). Dendrograms derived from PCR-amplification banding of ISSR with primer $(GACA)_4$ (C) and primer $(AGAG)_4G$ (D). The dendrograms were obtained using the UPGMA method, with the results obtained from eleven peanut *Aspergillus flavus* isolates (aflatoxigenic in blue and non-aflatoxigenic in plain black) and four bulgur wheat isolates (in red; underlined in blue if aflatoxigenic).

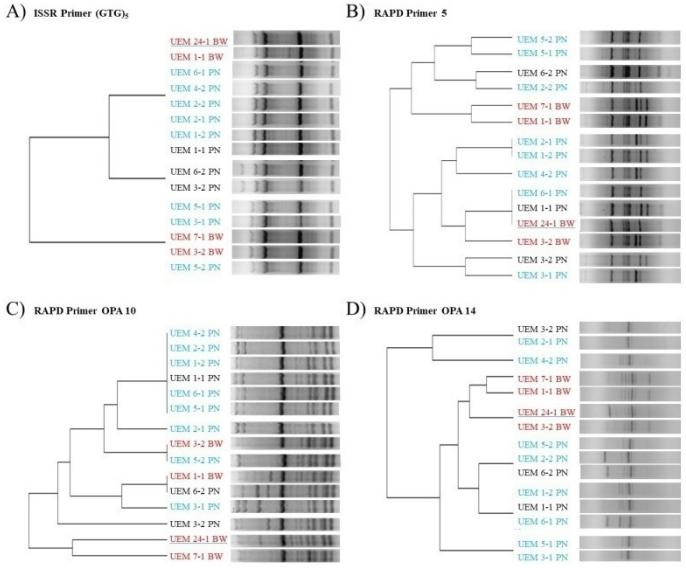

Figure 3. Dendrogram derived from PCR-amplification banding of ISSR with primer $(GTG)_5$ (A). Dendrograms derived from PCR-amplification banding of RAPD with Primer 5 (B), Primer OPA 10 (C), and Primer OPA 14 (D). The dendrograms were obtained using the UPGMA method, with the results obtained from eleven peanut *Aspergillus flavus* isolates (aflatoxigenic in blue and non-aflatoxigenic in plain black) and four bulgur wheat isolates (in red; underlined in blue if aflatoxigenic).

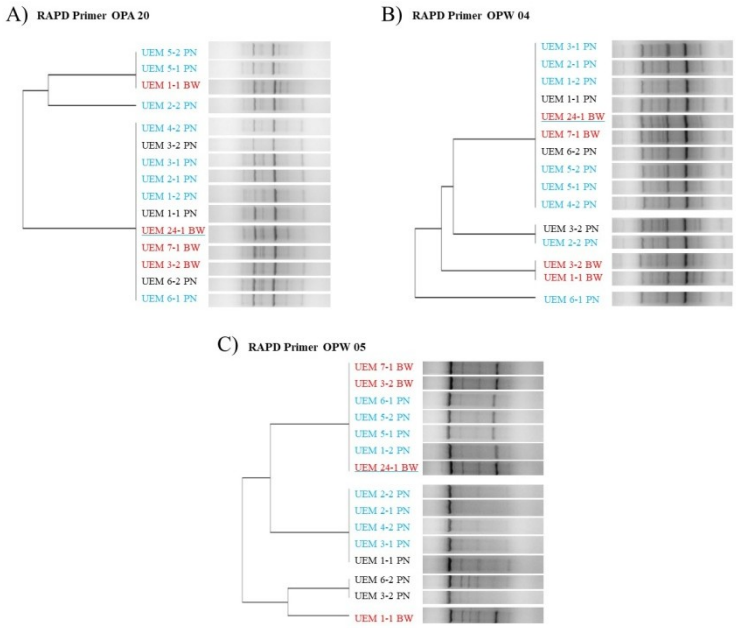

Figure 4. Dendrograms derived from PCR-amplification banding of RAPD with primer OPA 20 (A), Primer OPW 04 (B), and Primer OPW 05 (C). The dendrograms were obtained using the UPGMA method, with the results obtained from eleven peanut *Aspergillus flavus* isolates (aflatoxigenic in blue and non-aflatoxigenic in plain black) and four bulgur wheat isolates (in red; underlined in blue if aflatoxigenic).

Effect of essential oils on fungal growth

Although not statistically significant at $\alpha = 0.01$, oregano and thyme had the highest averages of inhibition halos on mycelial growth among the tested essential oils (Table 3 and Figure 5). However, no statistically significant ($\alpha = 0.01$) mycelial growth inhibition halos were observed between host or aflatoxigenic and non-aflatoxigenic *A. flavus* isolates. Fennel and cinnamon essential oils also showed considerable growth inhibition activity, depending on the isolate (Table 3 and Figure 5). The lavender essential oil was the least effective growth inhibitor, although it inhibited pigmentation in some isolates, which may indicate an effect on sporulation inhibition (Table 3 and Figure 5).

Since oregano and thyme had the highest averages of growth inhibition halos at 100%, smaller dilutions of those oils were also tested on the growth inhibition of the same *A. flavus* isolates (Table 3 and Figure 6). The oregano oil could inhibit the isolates' growth equally at 100% or 75%, but the thyme oil was most effective at 100% (Table 3). The minimal concentration of oregano oil that caused growth inhibition in our isolates was 25%, and for thyme oil was 50% in most of the isolates (Table 3 and Figure 6).

Table 3. Diameter of mycelial growth inhibition halos of *Aspergillus flavus* isolates by essential oils.

Isolates	Essential oil (100%)				
	Oregano*	**Thyme***	**Cinnamon***	**Fennel***	**Lavender***
UEM 3-1 (peanut)▲	^{a} 3.54 ± 0.28 ^{A}	^{ab} 2.45 ± 0.78 ^{A}	^{ab} 2.43 ± 0.08 ^{B}	^{ab} 1.40 ± 1.45 ^{A}	^{b} 0.00 ± 0.00
UEM 3-2 (peanut)	^{a} 2.05 ± 0.09 ^{C}	^{a} 3.35 ± 0.13 ^{A}	^{a} 2.03 ± 0.49 ^{B}	^{a} 2.98 ± 0.73 ^{A}	^{b} 0.00 ± 0.00
UEM 4-2 (peanut)▲	^{a} 3.70 ± 0.00 ^{A}	^{a} 2.98 ± 0.63 ^{A}	^{a} 3.70 ± 0.00 ^{A}	^{a} 3.58 ± 0.20 ^{A}	^{b} 0.00 ± 0.00
UEM 6-2 (peanut)	^{a} 3.47 ± 0.40 ^{AB}	^{a} 3.43 ± 0.24 ^{A}	^{ab} 2.69 ± 0.23 ^{AB}	^{b} 2.17 ± 0.38 ^{A}	^{c} 0.00 ± 0.00
UEM 1-1 (bulgur wheat)	^{a} 2.40 ± 0.23 ^{BC}	^{a} 1.95 ± 0.51 ^{A}	^{a} 1.87 ± 0.32 ^{B}	^{a} 1.73 ± 1.72 ^{A}	^{b} 0.00 ± 0.00
UEM 24-1(bulgur wheat)▲	^{a} 2.78 ± 0.49 ^{ABC}	^{ab} 2.70 ± 0.68 ^{A}	^{ab} 2.00 ± 0.43 ^{B}	^{ab} 1.32 ± 1.03 ^{A}	^{b} 0.00 ± 0.00
Average#	2.99 ± 0.58 ^{A}	2.81 ± 0.44 ^{A}	2.45 ± 0.49 ^{A}	2.2 ± 0.72 ^{A}	0.00 ± 0.00 ^{B}
	Oregano				
	100%*	**75%***	**50%***	**25%***	**0%***
UEM 3-1 (peanut)▲	^{a} 3.54 ± 0.28 ^{A}	^{ab} 2.37 ± 0.15 ^{A}	^{bc} 1.18 ± 0.71 ^{A}	^{bc} 1.08 ± 0.5 ^{A}	^{c} 0.00 ± 0.00
UEM 3-2 (peanut)	^{a} 2.05 ± 0.09 ^{C}	^{a} 2.07 ± 0.26 ^{A}	^{b} 0.78 ± 0.41 ^{A}	^{b} 0.53 ± 0.35 ^{A}	^{b} 0.00 ± 0.00
UEM 4-2 (peanut)▲	^{a} 3.70 ± 0.00 ^{A}	^{ab} 2.43 ± 0.26 ^{A}	^{ab} 1.6 ± 1.05 ^{A}	^{b} 1.02 ± 0.91 ^{A}	^{b} 0.00 ± 0.00
UEM 6-2 (peanut)	^{a} 3.47 ± 0.40 ^{AB}	^{b} 1.92 ± 0.58 ^{A}	^{bc} 0.82 ± 0.45 ^{A}	^{bc} 1.10 ± 0.2 ^{A}	^{c} 0.00 ± 0.00
UEM 1-1 (bulgur wheat)	^{a} 2.40 ± 0.23 ^{BC}	^{a} 2.05 ± 0.43 ^{A}	^{ab} 1.68 ± 0.03 ^{A}	^{ab} 1.37 ± 0.98 ^{A}	^{b} 0.00 ± 0.00
UEM 24-1(bulgur wheat)▲	^{a} 2.78 ± 0.49 ^{ABC}	^{a} 2.63 ± 0.18 ^{A}	^{ab} 1.77 ± 0.42 ^{A}	^{bc} 0.88 ± 0.45 ^{A}	^{c} 0.00 ± 0.00
Average#	2.99 ± 0.58 ^{A}	2.25 ± 0.23 ^{A}	1.3 ± 0.38 ^{B}	0.99 ± 0.19 ^{B}	0.00 ± 0.00 ^{C}
	Thyme				
	100%*	**75%***	**50%***	**25%***	**0%***
UEM 3-1 (peanut)▲	^{a} 2.45 ± 0.78 ^{A}	^{b} 0.80 ± 0.28 ^{A}	^{b} 0.28 ± 0.19 ^{B}	^{b} 0.08 ± 0.03 ^{B}	^{b} 0.00 ±0.00
UEM 3-2 (peanut)	^{a} 3.35 ± 0.13 ^{A}	^{b} 1.23 ± 0.50 ^{A}	^{bc} 0.50 ± 0.36 ^{AB}	^{c} 0.12 ± 0.16 ^{AB}	^{c} 0.00 ±0.00
UEM 4-2 (peanut)▲	^{a} 2.98 ± 0.63 ^{A}	^{b} 0.98 ± 0.39 ^{A}	^{b} 0.50 ± 0.20 ^{AB}	^{b} 0.12 ± 0.03 ^{AB}	^{b} 0.00 ±0.00
UEM 6-2 (peanut)	^{a} 3.43 ± 0.24 ^{A}	^{b} 1.17 ± 0.51 ^{A}	^{bc} 0.80 ± 0.22 ^{AB}	^{c} 0.03 ± 0.06 ^{B}	^{c} 0.00 ±0.00
UEM 1-1 (bulgur wheat)	^{a} 1.95 ± 0.51^{A}	^{ab} 1.47 ± 0.26 ^{A}	^{ab} 1.42 ± 0.31 ^{A}	^{bc} 0.42 ± 0.03 ^{A}	^{c} 0.00 ±0.00
UEM 24-1(bulgur wheat)▲	^{a} 2.7 ± 0.68 ^{A}	^{ab} 1.33 ± 0.43 ^{A}	^{b} 0.28 ± 0.41 ^{B}	^{b} 0.08 ± 0.08 ^{B}	^{b} 0.00 ±0.00
Average#	2.81 ± 0.44 ^{A}	1.16 ± 0.18 ^{B}	0.63 ± 0.32 ^{BC}	0.14 ± 0.09 ^{C}	0.00 ± 0.00 ^{C}

Values are the mean ± standard error (n = 3). *Means with the same superscript uppercase letter on the right and lowercase letter on the left do not differ by Tukey's Test at α = 0.01, in the treatment (column) and in the isolate (line), respectively. # Averages with the same superscript uppercase letter on the right do not differ by Tukey's Test at α = 0.01 ▲Aflatoxigenic.

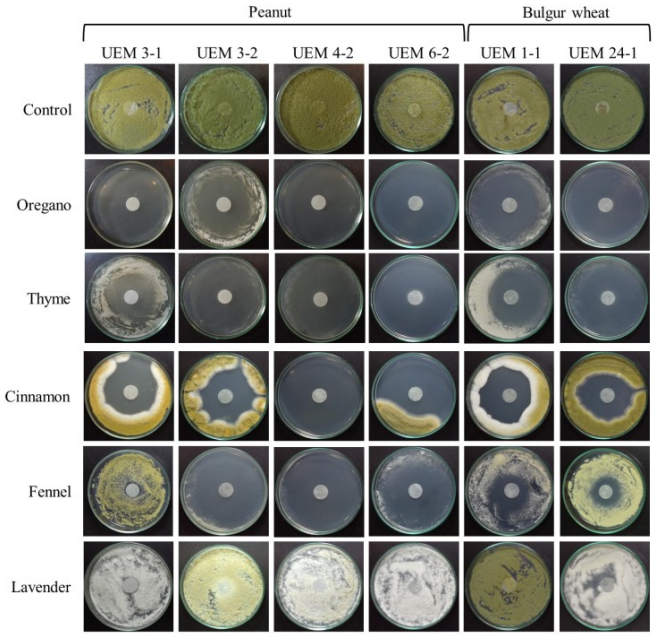

Figure 5. Effect of essential oils on fungal growth. The *Aspergillus flavus* isolates selected for these analyses are identified and separated in isolates from peanut and bulgur wheat. The essential oils tested were oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), fennel (*Foeniculum vulgare*), lavender (*Lavandula angustifolia*) and cinnamon (*Cinnamomum cassia*), all at 100%.

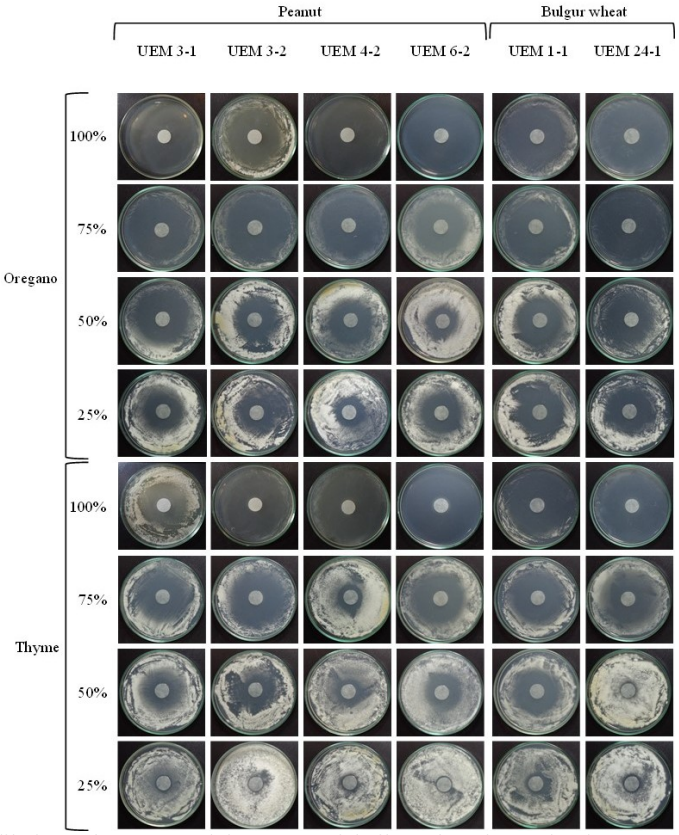

Figure 6. Effect of dilutions of oregano and thyme essential oils on fungal growth. The *Aspergillus flavus* isolates are identified and separated between isolates from peanut and bulgur wheat. The concentrations tested were 25, 50, 75, and 100% for each essential oil.

DISCUSSION

Moreira et al. (2016) also found a 33.3% contamination of aflatoxigenic *Aspergillus* sp. in commercial peanuts in the State of Ceará in Brazil, from the Northeastern region. Regarding other countries, Segunya and Yortee (1990) found 36% of potentially aflatoxigenic *Aspergillus* spp. contamination in commercial peanut samples from Uganda markets.

Although four species of the Flavi group (*A. flavus*, *A. parasiticus*, *A. caelatus*, and *A. tamarii*) are reported to occur in the early stages of the peanut chain in the State of São Paulo, Brazil, only the species of *A. flavus* and *A tamarii* are reported to occur when the kernels are ready to eat (Martins et al., 2017). This change in *Aspergillus* occurrence was also reported in the peanut food chain of several other countries (Norlia et al., 2019). It is possible that this occurrence correlates with the reduced amount of water in the dried peanut-based products, which reduces the levels of viable aflatoxigenic fungi. Those results and correlations agree with the results obtained by Variane et al. (2018) in corn silage, which is a more humid substrate, where they have obtained *A. parasiticus* isolates.

Regarding the occurrence of *A. caelatus*, this species was also isolated in Algeria from commercial peanut kernels imported from China (Guezlane-Tebibel et al., 2013).

All analyzed isolates in this work, from peanut and bulgur wheat, were determined to be from the "L" morphotype. The prevalence of L-type strains in peanut and other cereals has been reported before (Abbas et al., 2005).

The obtained aflatoxin production results agree with the literature, which states that only 70% of the *A. flavus* "L" type isolates can produce aflatoxins (Kumar et al., 2017). However, in a previous study performed in our laboratory, 41 strains of *A. flavus* were obtained from commercial bulgur wheat, and only five (12%) were aflatoxigenic (Faria et al., 2017). This may reflect host colonization by genetically diverse isolates. The culture in YES medium and TLC analysis identified eight of the eleven *A. flavus* isolates obtained in this work as aflatoxigenic, while the culture in CMA identified only five isolates as aflatoxigenic. This indicates either that the TLC analysis may be more sensitive to detect aflatoxigenic isolates or that the YES medium may provide better conditions for aflatoxins production by the isolates under the employed culture conditions, considering that the optimal aflatoxins production depends on the temperature, pH, moisture, medium composition, time, and radiation (Norlia et al., 2019).

Phylogenetic studies of *A. flavus* have shown that it consists of two subgroups, called groups I and II, and morphological studies indicated that it consists of two morphological groups based on sclerotium size, called "S" and "L" (Geiser et al., 2000). Although there are reports in the literature about the use of ITS and β-tubulin sequences, which were able to separate *A. flavus* isolates in several different clades, these markers were not capable of discriminating among our isolates (Okoth et al., 2018). It could be assumed that our isolates were not genetically different, but there were differences among them, such as the ability to produce aflatoxin.

Excepting one, all RAPD and ISSR primers could amplify DNA fragments from the tested *A. flavus* isolates' genomic DNA. The best primers were those that produced more amplified DNA fragments and fragments that could differentiate among isolates, producing more clades in the phylogenetic analysis. Some of the used primers could differentiate among the host's isolates. These primers were also successfully used by other authors to separate among *A. flavus* isolates (Batista et al., 2008; Midorikawa et al., 2008; Hatti et al., 2010; Mahmoud et al., 2014). According with our results, Midorikawa et al. (2008) obtained the separation of *A. flavus* strains from Brazil nuts and cashew by RAPD DNA analysis. It is possible to infer that different strains of *A. flavus* evolved with different types of hosts. The used primers, however, could not differentiate among aflatoxigenic and non-aflatoxigenic isolates. Mahmoud et al. (2014) were also not able to differentiate among aflatoxigenic and non-aflatoxigenic *A. flavus* isolates from corn, using RAPD and ISSR.

Besides RAPD and ISSR to differentiate among aflatoxigenic and non-aflatoxigenic *A. flavus* strains, PCR and RT-qPCR have also been used to amplify portions of aflatoxin biosynthesis pathway genes (Criseo et al., 2001; Faria et al., 2017; Variane et al., 2018; Rao et al., 2020). However, the reported results do not always reflect the metabolic profile indicating possible mutations or upstream regulation of the involved genes expression. Aflatoxins are produced by a polyketide pathway with at least 27 enzymatic reactions, with 30 genes potentially involved (Caceres et al., 2020). In *Aspergilli*, the aflatoxin pathway genes are clustered within a 75 Kb region of the fungal genome on chromosome III, approximately 80 kb away from telomere (Yu and Ehrlich, 2011).

In agreement with our results of the essential oil analyses, oregano, thyme, cinnamon, and fennel essential oils were also reported to inhibit *A. flavus*' mycelial growth (Montes-Belmont and Carvajal, 1998; Viuda-Martos et al., 2007; Silva et al., 2012). The lavender essential oil was the least effective growth inhibitor for our isolates. However, Rashidi et al. (2011) have demonstrated that lavender essential oil was able to inhibit *A. flavus* growth. The main antifungal properties of essential oils reside in their effect on cell membrane disruption, alteration and inhibition of cell wall formation, dysfunction of the fungal mitochondria, and inhibition of efflux pumps (Nazzaro et al., 2017; D'agostino et al., 2019). It is possible that all these processes were equally affected in all isolates regarding their host or ability to produce aflatoxins.

Oregano and thyme oils in smaller concentrations could also not differentiate among the isolates, regarding hosts and aflatoxin production. Regarding growth, the minimal concentration of thyme oil that caused growth inhibition in our isolates of 50% was also found by Silva et al. (2012) in the growth inhibition of a strain of *A. flavus* isolated from peanut.

The use of essential oils is common since ancient times in several world regions (Nazzaro et al., 2017; Pandey et al., 2017). Their use has been proposed for microbial spoilage control, food quality preservation, and shelf-life prolongation (Nazzaro et al., 2017). To develop techniques for food commodities protection with GRAS compounds is a prerogative for health safety. Considering that essential oils are GRAS, host specific, biodegradable, and pose no harm for non-target organisms (Pandey et al., 2017), their use for *A. flavus* control in peanut and bulgur wheat could be studied in the field of food technology.

CONCLUSIONS

Twelve strains of *Aspergillus* spp. were isolated from peanut kernels. Eleven of the isolates were identified as *A. flavus* and one as *A. caelatus*. Among the eleven *A. flavus* obtained isolates, there were eight aflatoxigenic strains. Although there were no phylogenetic differences among these isolates regarding the ITS region and β-tubulin gene partial sequence, these isolates could be separated from *A. flavus* isolated from bulgur wheat based on ISSR and RAPD analysis, indicating host specificity of the isolates. The essential oils of oregano, thyme, cinnamon, and fennel inhibited the mycelial growth of some *A. flavus* isolates but did not differentiate among them.

ACKNOWLEDGMENTS

The authors are thankful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, for the Project funding (grant 001) and the scholarship given to K. L. dos Reis, respectively.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abbas HK, Weaver MA, Zablotowicz RM, Horn BW, et al. (2005). Relationships between aflatoxin production and sclerotia formation among isolates of *Aspergillus* section Flavi from the Mississippi Delta. *Eur. J. Plant Pathol.* 112(3): 283-287.

Batista PP, Santos JF, Oliveira NT, Pires APD, et al. (2008). Genetic characterization of Brazilian strains of *Aspergillus flavus* using DNA markers. *Genet. Mol. Res.* 7: 706-717.

Caceres I, Al Khoury A, El Khoury R, Lorber S, et al. (2020). Aflatoxin Biosynthesis and Genetic Regulation: A Review. *Toxins*. 12: article 150.

Canteri MG, Althaus RA, Virgens Filho JS and Giglioti EA (2001). SASM-AGRI - system for analysis and mean separation in agricultural assays using Scott-Knott, Tukey and Duncan methods. *Braz. J. Agrocomp.* 1: 18-24.

Cotty PJ (1989). Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. *Phytopathology*. 79: 808-814.

Criseo G, Bagnara A and Bisignano G (2001). Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group. *Lett. Appl. Microbiol.* 33(4): 291-295.

D'agostino M, Tesse N, Frippiat JP, Machouart M, et al. (2019). Essential oils and their natural active compounds presenting antifungal properties. *Molecules*. 24: 3713.

Davis ND, Diener UL and Elridge DW (1966). Production of aflatoxin B1 and G1 by *Aspergillus flavus* in semisynthetic medium. *Appl. Microbiol.* 29: 378-380.

Faria CB, Santos FC, Castro FF, Sutil AR, et al. (2017). Occurrence of toxigenic *Aspergillus flavus* in commercial Bulgur wheat. *Food Sci. Technol.* 37(1): 103-111.

Felsenstein J (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*. 39: 783-791.

Geiser DM, Dorner JW, Horn BW and Taylor JW (2000). The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. *Fungal Gen. Biol.* 31(3): 169-79.

Geiser DM, Pitt JI and Taylor JW (1998). Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *PNAS USA* 95(1): 388-393.

Gilbert MK, Mack BM, Moore GG, Downey DL, et al. (2018). Whole genome comparison of *Aspergillus flavus* L-morphotype strain NRRL 3357 (type) and S-morphotype strain AF70. *PLoS one.* 13(7): e0199169.

Glass NL and Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61: 1323-1330.

Grabiele M, Chalup L, Robledo G and Seijo G (2012). Genetic and geographic origin of domesticated peanut as evidenced by 5S rDNA and chloroplast DNA sequences. *Plant Syst. Evol.* 298: 1151-1165.

Guezlane-Tebibe N, Bouras N, Mokrane S, Benaya T, et al. (2013). Aflatoxigenic strains of *Aspergillus* section Flavi isolated from marketed peanuts (*Arachis hypogaea*) in Algiers (Algeria). *Ann. Microbiol.* 63(1): 295-305.

Hatti AD, Taware SD, Taware AS, Pangrikar PP, et al. (2010). Genetic diversity of toxigenic and non-toxigenic *Aspergillus flavus* strains using ISSR markers. *Int. J. Curr. Res.* 5: 061-066.

Horn B and Dorner J (1999). Regional differences in production of aflatoxin B1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. *Appl. Environ. Microbiol.* 65(4): 1444-1449.

IARC – International Agency for Research on Cancer (2009). Chemical agents and related occupations. A review of human carcinogens. *IARC – WHO.* 100F: 225-248.

Koenig RL, Ploetz RC and Kistler HC (1997). *Fusarium oxysporum* f. sp. *cubense* consists of a small number of divergent and globally distributed clonal lineages. *Phytopathology*. 87(9): 915-923.

Kumar P, Mahato DK, Kamle M, Mohanta TK, et al. (2017). Aflatoxins: a global concern for food safety, human health and their management. *Front. Microbiol.* 7: 2170.

Kumar S, Stecher G and Tamura K (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33: 1870-1874.

Lin MT and Dianese JCA (1976). Coconut-agar medium rapid detection of aflatoxin production by *Aspergillus* spp. *Phytopathology*. 66(12): 1466-1469.

Mahmoud MA, Ali HM, El-Aziz ARM, Al-Othman MR, et al. (2014). Molecular characterization of aflatoxigenic and non-aflatoxigenic *Aspergillus flavus* isolates collected from corn grains. *Genet. Mol. Res.* 13 (4): 9352-9370.

Mahmoud MA, Al-Sohaibani SA, Abdelbacki AM, Al-Othman MR, et al. (2012). Molecular characterization of the pathogenic plant fungus *Rhizoctonia solani* (Ceratobasidiaceae) isolated from Egypt based on protein and PCR-RAPD profiles. *Genet. Mol. Res.* 11: 3585-3600.

Martins LM, Sant'Ana AS, Fungaro MHP, Silva JJ, et al. (2017). The biodiversity of *Aspergillus* section Flavi and aflatoxins in the Brazilian peanut production chain. *Food Res. Int.* 94: 101-107.

Midorikawa GEO, Pinheiro MRR, Vidigal BS, Arruda MC, et al. (2008). Characterization of *Aspergillus flavus* strains from Brazilian Brazil nuts and cashew by RAPD and ribosomal DNA analysis. *Lett. Appl. Microbiol.* 47: 12-18.

Montes-Belmont R and Carvajal M (1998). Control of *Aspergillus flavus* in maize with plant essential oils and their components. *J. Food Prot.* 61(5): 616-619.

Moreira MF, Oliveira TR, Vieira IGP, Freire FCO, et al., (2016). Occurrence of fungi and aflatoxins B in nuts and products marketed the Brazilian northeastern regions. *Rev. Inst. Adolfo Lutz* 75: 1-6.

Nazzaro F, Fratianni F, Coppola R and De Feo V (2017). Essential oils and antifungal activity. *Pharmaceuticals*. 10(4): 86.

Nelson PE, Tousson TA and Marasas WFO (1983). *Fusarium* species: an illustrated manual for identification. Pennsylvania State University Press, Philadelphia, USA.

Norlia M, Jinap S, Nor-Khaizura MAR, Radu S, et al. (2019). *Aspergillus* section Flavi and aflatoxins: occurrence, detection, and identification in raw peanuts and peanut-based products along the supply chain. *Front. Microbiol.* 10: 2602.

Okoth S, De Boevre M, Vidal A, Di Mavungu JD, et al. (2018). Genetic and toxigenic variability within *Aspergillus flavus* population isolated from maize in two diverse environments in Kenya. *Front Microbiol*. 9: 57.

Pandey AK, Kumar P, Singh P, Tripathi NN, et al. (2017). Essential oils: sources of antimicrobials and food preservatives. *Front. Microbiol.* 7: 2161.

Pavel AB and Vasile CI (2012). PyElph - a software tool for gel images analysis and phylogenetics. *BMC Bioinform.* 13: 9.

Rao KR, Vipin AV and Venkateswaran G (2020). Molecular profile of non-aflatoxigenic phenotype in native strains of *Aspergillus* flavus. *Arch. Microbiol.* 202: 1143-1155.

Pitt JI and Hocking AD (2009). Fungi and food spoilage, 3rd ed. Springer, New York, USA.

Rashidi A, Mousavi B, Rahmani MR, Rezaee MA, et al. (2011). Evaluation of antifungal effect of *Lavandula officinalis*, *Salvia officinalis* L., Sumac, *Glycyrrhiza glabra*, and *Althaea officinalis* extracts on *Aspergillus niger*, *Aspergillus fumigatus*, and *Aspergillus flavus* species. *J. Med. Plant Res*. 6(2): 309-313.

Saito M and Machida S (1999). A rapid identification method for aflatoxin producing strains of *A. flavus* and *A. parasiticus* by ammonia vapour. *Mycoscience*. 40(2): 205-208.

Saito M, Tsuruta O, Siriacha P, Kawasugi S, et al. (1989). Atypical strains of *Aspergillus flavus* isolated in maize fields. *Jpn. Agric. Res. Q.* 23: 151-154.

Saito M, Tsuruta O, Siriacha P, Kawasugi S, et al. (1986). Distribution and aflatoxin productivity of the atypical strains of *Aspergillus flavus* isolated from soils in Thailand. *Proc. Jpn. Assoc. Mycotoxicol*. 24: 41-46.

Saitou N and Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.

Sebunya TK and Yourtee DM (1990). Aflatoxigenic *Aspergilli* in foods and feeds in Uganda. *J. Food Qual.* 13: 97-107.

Silva FC, Chalfoun SM, Siqueira VM, Botelho DMS, et al. (2012). Evaluation of antifungal activity of essential oils against potentially mycotoxigenic *Aspergillus flavus* and *Aspergillus parasiticus*. *Rev. Bras. Farmacogn.* 22(5): 1002-1010.

Tamura K, Nei M and Kumar S (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *PNAS USA*. 101: 11030-11035.

USDA – United States Department of Agriculture (2021). World agricultural production. Circular Series WAP 1-21, January 2021. Available at [https://apps.fas.usda.gov/PSDOnline/Circulars/2021/01/production.pdf]. Accessed May 10, 2021.

Variane ACF, Santos FC, Castro FF, Barbosa-Tessmann IP, et al. (2018). The occurrence of aflatoxigenic *Aspergillus* spp. in dairy cattle feed in Southern *Brazil. Braz. J. Microbiol.* 49: 919-928.

Viuda-Martos M, Ruiz-Navajas Y, Fernández-López J and Pérez-Álvarez JA (2007). Antifungal activities of thyme, clove and oregano essential oils. *J. Food Saf.* 27(1): 91-101.

White TJ, Bruns T, Lee S and Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols, a Guide to Methods and Applications (Innis MA, Gelfand H, Sninsky JJ, White TJ, eds.). Academic Press Inc, San Diego, USA. 315-322.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, et al. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*. 18(22): 6531-6535.

Yu J and Ehrlich KC (2011). Aflatoxin biosynthetic pathway and pathway genes. In: Aflatoxins – Biochemistry and molecular biology (Guevara-González RG, ed.). IntechOpen, London, UK, 41-66.