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CARACTERIZAÇÃO MOLECULAR DE ISOLADOS DE *Aspergillus flavus* DE AMENDOIM DO SUL DO BRASIL E ATIVIDADE ANTIFÚNGICA DE ÓLEOS ESSENCIAIS

Maringá
2020

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BIOGRAFIA

Karina Lima dos Reis nasceu em Paraíso do Norte, Paraná, em 10 de Novembro de 1996. Possui ensino médio pelo Colégio Nobel, Paranavaí, Paraná e Bacharelado em Bioquímica pela Universidade Estadual de Maringá (UEM) – Maringá, Paraná (2014-2017). Em Março de 2018 iniciou o curso de Mestrado em Ciências Biológicas – Biologia Celular e Molecular pelo programa de Pós-graduação em Ciências Biológicas (PBC-UEM). Tem experiência nas áreas de Bioquímica, Biologia Molecular, Microbiologia e Genética de Microrganismos.

DEDICATÓRIAS

Dedico este trabalho aos meus pais Fabiano Dias dos Reis e Elieni Fernandes Lima por todo o apoio e suporte em todas as etapas da minha vida até este momento e em todos que ainda estão por vir.

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A Deus.

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À Universidade Estadual de Maringá e ao Programa de Pós-graduação em Ciências Biológicas, ao seu corpo docente, à sua direção e administração.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo apoio financeiro ao Programa de Pós-graduação e pela bolsa de estudos concedida.

Finish each day and be done with it. You
have done what you could. Some
blunders and absurdities no doubt crept
in; forget them as soon as you can.
Tomorrow is a new day. You shall begin
it serenely and with too high a spirit to
be encumbered with your old nonsense.

Ralph Waldo Emerson.

APRESENTAÇÃO

Esta dissertação é composta de um artigo científico que descreve a obtenção de isolados de *Aspergillus flavus* de amendoim no sul do Brasil, a identificação molecular dos mesmos, a filogenética e o controle do crescimento de isolados aflatoxigênicos por óleos essenciais. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, esta dissertação foi redigida como um artigo científico que será enviado para análise quanto à publicação para a revista Journal of Food Science and Technology.

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LISTA DE ABREVIAÇÕES

°C – Graus Celsius

µL – Microlitro(s)

µmol – Micromol(s)

AN – Número de Acesso GenBank

bp – Base pairs

DNA – Ácido desoxirribonucleico

EDTA – Ácido etilenodiamino tetra-acético

g – Grama(s)

g/% - Grama(s) por porcentagem

g/L – Grama(s) por litro

h – Hora(s)

ISSR – Inter-simple sequence repeats

ITS – Internal transcribed sequence

L – Litro(s)

M – Molar

M.M. – Marcador Molecular

m/v – Massa por volume

mg – Miligrama(s)

mg/L – Miligrama(s) por litro

min – Minuto(s)

ml – Mililitro(s)

mM – Milimolar

ng – Nanograma(s)

ng/µl – Nanograma(s) por microlitro(s)

nm – Nanômetro(s)

PCR – Reação em cadeia da polimerase

pH – Potencial de hidrogênio

RAPD – Random amplified polymorphic DNA

U/ml – Unidade/mililitro

UV – Ultravioleta

v/v – Volume/volume

w/v – Weight per volume

RESUMO GERAL

Introdução: Micotoxinas são metabólitos secundários produzidos por fungos. Aflatoxinas são micotoxinas hepatotóxicas, mutagênicas, e carcinogênicas. Há quatro tipos principais aflatoxinas, chamadas B1, B2, G1 e G2. A espécie *Aspergillus flavus* é produtora de aflatoxinas, mas apenas 40-50% dos isolados desta espécie podem produzir estas micotoxinas, e somente as aflatoxinas B1 e B2. A razão de alguns isolados de *A. flavus* produzirem aflatoxinas e outros não ainda não está bem estabelecida. A espécie *A. flavus* pode contaminar grãos como o amendoim. O uso de conservantes naturais, como óleos essenciais, é uma opção para o controle da contaminação de alimentos. **Objetivos:** Avaliar a presença e obter isolados de *A. flavus* em grãos de amendoim cru comercializados na cidade de Maringá, Paraná, Brasil; identificar os isolados ao nível de espécie por DNA *barcoding*; avaliar o perfil de produção de aflatoxinas dos isolados por meio de técnicas em meio de cultivo e cromatografia; investigar a variabilidade genética dos isolados; e analisar o efeito antifúngico de cinco óleos essenciais em alguns dos isolados. **Materiais e métodos:** Inicialmente, amostras de amendoim foram compradas em mercados da cidade de Maringá. Grãos de cada amostra foram colocados em meio de cultivo sólido específico para *Aspergillus* spp. Foi feita a contagem dos grãos contaminados com *Aspergillus* spp. e foram feitos isolamentos monospóricos para obtenção de isolados. Os isolados monospóricos foram identificados por caracterização morfológica e DNA *barcoding*. A produção de aflatoxinas dos isolados foi testada por cultivo em meio sólido de leite de coco, exposição a vapor de hidróxido de amônio e cromatografia em camada delgada (TLC). Uma análise filogenética foi realizada com amplificação e sequenciamento da região ITS–5,8rRNA e de uma região do gene da β-tubulina. A variabilidade genética foi analisada com reações de PCR com oito iniciadores de RAPD (*Random Amplified Polymorphic* DNA) e três de ISSR (*Inter Simple Sequence Repeats*) com o DNA de 11 dos isolados obtidos neste trabalho e de quatro isolados de *A. flavus* obtidos de trigo para quibe em um estudo prévio. A atividade de inibição do crescimento micelial de cinco óleos essenciais foi testada com alguns dos isolados. **Resultados e discussão:** Onze isolados monospóricos de *A. flavus* e um de *Aspergillus caelatus* foram obtidos. Cinco isolados de *A. flavus* foram identificados como aflatoxigênicos em meio de cultivo e oito na análise de TLC, onde foi evidenciada a produção de aflatoxinas dos tipos B1 e B2. Na análise filogenética, somente o isolado de *A. caelatus* (UEM 4-1) não agrupou com os isolados de *A. flavus*, os quais eram filogeneticamente idênticos. As análises de RAPD e de ISSR mostraram separação entre os isolados de amendoim e de trigo para quibe, bem como uma pequena diferenciação entre isolados de *A. flavus* aflatoxigênicos e não aflatoxigênicos. Os óleos essenciais de orégano e tomilho a 100% tiveram as maiores médias dos halos de inibição do crescimento dos isolados. Os óleos de canela e erva-doce também foram muito eficientes, mas o óleo de lavanda foi pouco eficiente na inibição do crescimento dos isolados. O efeito do óleo de orégano a 100% ou a 75% não foi estatisticamente diferente, o óleo de tomilho foi mais eficiente a 100%. A concentração mínima que causou inibição do crescimento foi de 25% para o óleo de orégano e 50% para o óleo de tomilho. **Conclusões:** Foram obtidos 11 isolados de *A. flavus* e um de *A. caelatus* de grãos de amendoim do sul do Brasil. Oito dos isolados de *A. flavus* eram aflatoxigênicos. Não houve diferença filogenética entre os isolados de *A. flavus*. As análises de RAPD e ISSR mostraram que os isolados de *A. flavus* obtidos neste trabalho foram parcialmente separados de isolados de *A. flavus* obtidos previamente de trigo para quibe, indicando diferente colonização de *A. flavus* nestes hospedeiros. Por fim, os óleos essenciais de orégano, tomilho, canela e erva-doce inibiram o crescimento micelial dos isolados de *A. flavus* testados.

Palavras-chave: *Aspergillus flavus*, Aflatoxinas, RAPD e ISSR, Óleos essenciais.

GENERAL ABSTRACT

Introduction: Mycotoxins are secondary metabolites produced by fungi. Aflatoxins are mycotoxins that are hepatotoxic, mutagenic, and carcinogenic. There are four types of aflatoxins named B1, B2, G1, and G2. The species *Aspergillus flavus* produces aflatoxins, but only 40-50% of the isolates of this species can produce these mycotoxins, and they can only produce the aflatoxins B1 and B2. It is still not well known why some strains of *A. flavus* produce aflatoxins, and others do not. The species *A. flavus* can contaminate peanut grains. The use of natural preservatives, such as essential oils, is an option for the control of food contamination. **Objectives:** To evaluate the presence and to obtain isolates of *A. flavus* on raw peanut kernels commercialized in the city of Maringá, Paraná, Brazil; to identify the isolates at a species level by DNA barcoding; to evaluate the aflatoxin production profile of the obtained isolates by culture medium and chromatographic techniques; to investigate the genetic variability of the isolates, and; to analyze the antifungal effects of five essential oils on some of those isolates. **Materials and methods:** Initially, peanut samples were bought in stores of the city of Maringá, and kernels from each sample were placed on a solid culture medium, specific for the growth of *Aspergillus* spp. Peanut kernels contaminated with *Aspergillus* spp. were counted, and monosporic isolations were performed to obtain the isolates. The monosporic isolates were identified by morphological characterization and DNA barcoding. The ability of the isolates to produce aflatoxins was tested by culture in coconut milk agar, hydroxide vapor exposure, and thin-layer chromatography (TLC). A phylogenetic analysis was carried out with amplification and sequencing of the ITS–5.8rDNA region and a β-tubulin gene region. The genetic variability was studied with PCR reactions using eight primers of RAPD (*Random Amplified Polymorphic* DNA) and three of ISSR (*Inter Simple Sequence Repeats*) with the 11 isolates obtained in this work and with four *A. flavus* isolates obtained from bulgur wheat in a previous study. The growth inhibition activity of essential oils, pure and diluted, was tested on some of the isolates. **Results and discussion:** Eleven monosporic isolates of *A. flavus* and one of *Aspergillus caelatus* were obtained. Five isolates of *A. flavus* were identified as aflatoxigenic in culture medium and eight in the TLC analysis, in which the production of B1 and B2 aflatoxins was identified. In the phylogenetic analyses, only the *A. caelatus* isolate (UEM 4-1) did not group with the *A. flavus* isolates, which were phylogenetically identical. The RAPD and ISSR analysis showed separation between the peanut and bulgur wheat isolates, as well as a small separation between aflatoxigenic and non-aflatoxigenic *A. flavus* isolates. The oregano and thyme essential oils at 100% had the highest averages of growth inhibition halos. Fennel and cinnamon essential oils also showed considerable activity on growth inhibition, but the lavender essential oil was the least effective as a growth inhibitor. The effect of oregano oil at 100% or 75% was not statistically different, and thyme oil was most effective at 100%. The minimal concentration to cause growth inhibition was 25% for oregano oil, and 50% for thyme oil. **Conclusions:** Eleven isolates of *A. flavus* and one of *A. caelatus* were obtained from peanut kernels from the South of Brazil. Eight of the *A. flavus* isolates were aflatoxigenic. Analyses of RAPD and ISSR have shown that most of the *A. flavus* isolates obtained in this work were partially separated from the *A. flavus* isolates obtained previously from bulgur wheat, indicating different *A. flavus* colonization in these hosts. At last, the essential oils of oregano, thyme, cinnamon, and fennel could inhibit the mycelial growth of selected *A. flavus* isolates.

Keywords: *Aspergillus flavus,* Aflatoxins, RAPD and ISSR, Essential oils.

MOLECULAR CHARACTERIZATION OF *Aspergillus flavus* ISOLATES FROM SOUTHERN BRAZIL PEANUTS AND ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS

Reis, K.L.,[1] Barbosa-Tessmann, I.P.[2]

ABSTRACT

Aflatoxins are hepatotoxic, mutagenic, and carcinogenic mycotoxins. The objective of this work was to analyze the presence of aflatoxigenic *Aspergillus* spp. on commercial peanuts in the city of Maringá, Paraná, Brazil. Twelve strains of *Aspergillus* spp. were isolated from peanut kernels, and eleven were identified as *Aspergillus flavus* and one as *Aspergillus caelatus.* The potential for aflatoxin production of the isolates was studied by culture techniques and thin-layer chromatography (TLC). Eight of the *A. flavus* strains were aflatoxigenic. A phylogenetic analysis with partial sequences from the 5.8S-ITS and the β-tubulin gene showed no phylogenetic difference among the isolates of *A. flavus*. On a genetic variability analysis using PCR amplification of Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR), there was a separation between the isolates obtained in this work from isolates previously isolated from bulgur wheat, indicating differential host colonization. However, there was little differentiation between aflatoxigenic and non-aflatoxigenic *A. flavus*. Essential oils were tested for their antifungal activity; oregano, thyme, cinnamon, and fennel essential oils inhibited mycelial growth of selected *A. flavus* isolates, which could be useful on the control of this pathogen. The results showed that *A. flavus* contamination on peanuts is concerning.

Keywords: *Aspergillus flavus*, Aflatoxins, RAPD and ISSR, Essential oils.

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1 INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi. Aflatoxins are hepatotoxic, mutagenic, and carcinogenic mycotoxins, capable of causing disease and death in humans and other animals. These mycotoxins are produced by species of the genus *Aspergillus*, subgenus Circumdati, section Flavi (also known as the *Aspergillus flavus* group). The main aflatoxin producing species of the *Aspergillus* genus are *Aspergillus parasiticus, Aspergillus flavus*, and *Aspergillus nomius*, which can be found in several grains such as peanut, soybean, wheat, Brazil nut, among others (Bennett and Klich, 2003; Richard, 2007; Kumar et al., 2017). Aflatoxins also pose a significant economic burden, causing annual destruction of more than 25% in the world's food crops (WHO, 2018).

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 in thin-layer chromatography. The aflatoxin B1 is the most known potent natural carcinogen and is usually the main aflatoxin produced by toxigenic strains. Most isolates of *A. parasiticus* (90%) are aflatoxins producers, but only 40-50% of *A. flavus* isolates produce these mycotoxins (Konietzny and Greiner, 2003). It is still not well known why some isolates of *A. flavus* produce aflatoxins, and others do not. Isolates of *A. flavus* produce only aflatoxins B1 and B2, and isolates of *A. parasiticus* and *A. nomius* can produce all four aflatoxins (Richard, 2007; Kumar et al., 2017).

Aflatoxins are mainly hepatotoxic, immunosuppressive, teratogenic, and mutagenic (Richard, 2007) and were classified as group I carcinogens by the International Agency for Research on Cancer (IARC, 2009). The aflatoxins biosynthetic pathway involves approximately 25 genes, which are clustered in a 70 kb DNA region (Yu et al., 2004). The species *A. flavus* and *A. parasiticus*, as well as other *Aspergillus* species from the section

Flavi, share almost identical sequences and conserved order of genes in that cluster (Chang et al., 2005; Chang et al., 2006).

The peanut (*Arachis hypogaea*) is grown mainly for its edible seeds. It is widely cultured in tropical and subtropical regions, being essential to both small and large commercial producers. It is classified as both a grain legume and, due to its high oil content, as a crop. The world annual production of peanuts was estimated to be 46 million metric tons in 2019, led by China, with 37% of the world total (USDA, 2019). The primary center of peanut origin is South America (Grabiele et al., 2018), and the Brazilian production in 2019 was estimated to be 440 thousand metric tons (USDA, 2019). Peanut cultures are widely sensible to infection by toxin-producing fungi, which mainly occurs during the harvest and post-harvest periods (Souza et al., 2014).

Food contamination control is necessary to ensure that food products remain safe and uncontaminated throughout the supply chain (Pitt and Hocking, 2009). In general, the synthetic fungicides used in the management of food contamination by *Aspergillus* spp. are potentially carcinogenic, teratogenic, and have high and acute residual toxicity and other effects on humans. The use of natural preservatives, such as essential oils, is a safer alternative.

The aims of the present work were: 1) to evaluate the presence of *Aspergillus* in raw peanut kernels commercialized in the city of Maringá, Paraná, located in Southern Brazil; 2) to obtain isolates of *Aspergillus* sp. from peanut kernels and to identify them at a species level by DNA *barcoding*; 3) to evaluate the aflatoxin production profile of the obtained isolates by culture medium and chromatographic techniques; 4) to investigate the genetic variability of the isolates using PCR amplification of Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) molecular markers, and; 5) to analyze the antifungal effects of five essential oils on some of those isolates.

2 MATERIALS AND METHODS

2.1 Sample collection

Six different peanut samples were bought in stores of the city of Maringá, from September to October 2017. Samples consisted of individual packs of raw peanut kernels with 500 g each. Three of the samples corresponded to small-sized and red-skinned varieties, and three to medium-sized and white-skinned varieties. All samples were conserved at room temperature and were analyzed before the expiration dates. Samples belonged to six different brands and were from the following Brazilian cities: Pinhais (one sample), Maringá (two samples), Umuarama (one sample), and Cambará (one sample), all located in the Paraná State, and Pomerode (one sample), located in the Santa Catarina State. Both Paraná and Santa Catarina States are located in the Southern region of Brazil (Table 1).

2.2 *Aspergillus* spp. isolation and morphologic characterization

For asepsis, six peanut kernels from each sample were incubated with a 50 mL of 0.4% active chlorine solution for 1 min at room temperature, with eventual agitation, and rinsed once with 50 mL of sterile distilled water. Those 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 the addition of 2.5 µg/ml malachite green to the culture medium previous 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 to 60 °C. For each peanut sample, four Petri dishes were inoculated; 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 reverse of aflatoxigenic *Aspergillus* spp. colonies stains with an orange color. The kernels showing reverse orange colonies were counted.

A fragment of a colony on the AFPA medium with a reverse orange color was transferred to a tube containing a slanted potato-dextrose agar (PDA) medium. This tube was incubated at 25 °C in a photoperiod incubator for 12 h 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 100 µL of the obtained spore suspension were 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 medium (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 (Faria et al., 2017), isolated from commercial bulgur wheat, were used as standards.

2.3 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 media was used to obtain mycelium without spores. The isolate of *A. flavus* NRRL 5940 was 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 executed in a Techne TC-312 thermocycler (England). Samples were heated for 5 min at 94 °C, previous to the cycles, and for 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 the gel 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. The obtained sequences were compared with sequences deposited in databanks for species definition.

2.4 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). Petri dishes with 10 cm diameter 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 in 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 inverted in the lids and 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 isolate of *A. parasiticus* UEM 443 was used as a positive control in both analyses.

2.5 Aflatoxin production analyses by thin-layer chromatography (TLC)

A fragment of approximately $1cm^3$ 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 (2g/% yeast extract and 20g/% 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 solvents toluene/ethyl acetate/chloroform/formic acid in the proportion of 7:5:5:2 (v/v). The aflatoxin standards B1 and B2 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 chromatography plate. The aflatoxin standards were purchased from Sigma-Aldrich (Germany).

All the analyses, beginning with 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.

2.6 Phylogenetic analyses

Phylogenetic analyses of the *A. flavus* isolates were made by analyzing 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, in the MEGA 7 program (Kumar et al., 2016). The obtained sequences were used to build a phylogenetic tree together

with 5.8S-ITS region sequences (from GenBank) 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, and 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 from 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 the ones described for the 5.8-ITS sequences amplification, but using an annealing temperature of 60 °C and with 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 5´and 3´ends trimmed and were deposited in GenBank. The phylogenetic analysis was also conducted in the MEGA 7 program (Kumar et al., 2016).

2.7 RAPD and ISSR analyses

RAPD and 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, the PCR for each primer was repeated twice in different days. Negative controls (no DNA template) were made for each RAPD and ISSR primer reaction to test for the presence of 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 clustering method used was the Unweighted Pair Group Method with Arithmetic mean (UPGMA). The running distance of each DNA fragment was considered in the analyses.

2.8 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 peanut isolates that were aflatoxigenic (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 edge of the paper disc to the end of the halo, 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.

2.9 Statistical analyses

Statistical analyses were carried out by calculation of the means and standard deviations of the results and comparison with the Tukey test ($\alpha < 0.01$) using the program SAS (SAS Institute, Cary, NC, USA).

3 RESULTS AND DISCUSSION

3.1 *Aspergillus* spp. isolation and identification

Table 1 presents the number of contaminated kernels with *Aspergillus* spp. on AFPA medium of each peanut sample. All samples had kernel contamination with potentially aflatoxigenic *Aspergillus* spp. Peanut samples 2 and 5 had the highest contamination, with 83% and 50% of contamination, while other peanuts samples were less contaminated with 12.5% to 16.7% of contamination. The total amount of contaminated kernels was 32.6% if we consider that there were 47 contaminated kernels out of 144 analyzed. 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.

Twelve monosporic isolates were obtained, two from each peanut sample, all with culture features (Fig. 1) and microscopic characteristics (not shown) of *Aspergillus* of the group Flavi in the Czapeck dox agar medium (Table 1). The molecular identification confirmed all isolates as *A. flavus*, except for isolate UEM 4-1, which was identified as *Aspergillus caelatus* (Table 1). 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. The

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

3.2 Aflatoxin production

An example of the obtained results of fluorescence under ultraviolet light (UV) and ammonium hydroxide vapor analyses are presented in Fig. 2. Five isolates (41.2%) presented fluorescence in CMA, and four (33.3%) of those isolates pigmented the CMA medium with pink color in the ammonium hydroxide vapor analyses (Table 1).

In the TLC analysis, eight isolates (66.7%) were able to produce aflatoxins B1 and B2 in the YES medium, while the control isolate *A. parasiticus* UEM 443 produced the four types of aflatoxins (Fig. 3, Table 1). This result agrees with the literature, which states that only 50% of the *A. flavus* isolates can produce aflatoxins (Konietzny and Greiner, 2003). However, in a previous study by our group in which we isolated 41 strains of *A. flavus* from bulgur wheat obtained in stores, only five isolates (12%) were aflatoxigenic (Faria et al., 2017). From the eight aflatoxigenic isolates observed by culture in YES medium and TLC analysis, only five also presented characteristics of aflatoxin production in the CMA medium. This indicates that the TLC analysis was more sensitive to detect aflatoxigenic isolates. Besides, it is possible that the YES medium provided 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 (Hussein and Brasel, 2001; Klich, 2007; Ritter et al., 2011; Norlia et al., 2019).

3.3 Phylogenetic analyses

In the phylogenetic analyses with the 5.8S-ITS and β-tubulin partial genes (Fig. 4), only the isolate UEM 4-1, which was identified as *A. caelatus*, did not group with the *A.*

flavus isolate's sequences. Phylogenetic data in the literature support the division of *A. flavus* in two subgroups, called groups I and II (Gilbert et al., 2017), and morphological studies based on sclerotium size indicated that it consists of two groups, called "S" and "L" (Cotty, 1989). Although there are reports in the literature about the use of ITS and β-tubulin sequences, which were able to separate isolates of *A. flavus* in several different clades (Okoth et al., 2018; Oloo et al., 2019), these markers were not capable of discriminating among our isolates. It could be assumed that our isolates were not different, but there were differences among them, such as the ability to produce aflatoxin.

3.4 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 were able to generate amplified DNA products and separation among the isolates (Fig. 5 and Suppl. figs 1 and 2). 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., 2014a; Mahmoud et al., 2014b). 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.

The best results for RAPD and ISSR analyses are shown in Fig. 5. In the dendrograms obtained with RAPD primers 3 and OPA 17 (Fig. 5A and B), the majority of the peanut isolates do not group in the same clade of the bulgur wheat isolates. The dendograms obtained with ISSR primers $(GACA)_4$ and $(AGAG)_4G$ (Fig. 5C and D) follow the same standard, they cluster together, although there are more peanut isolates in their clade. According to 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.

With all tested primers, there was little differentiation among peanut *A. flavus* with relation to aflatoxigenic and non-aflatoxigenic isolates (Fig. 5, Suppl. figs. 1 and 2). Mahmoud et al. (2014a and 2014b) were also not able to differentiate among aflatoxigenic and non-aflatoxigenic isolates of *A. flavus* from corn and barley using RAPD and ISSR with the same primers, although they could get separation among isolates from different regions in Saudi Arabia. As our isolates are all from the same region in Brazil, this separation was not evident in our analysis

3.5 Effect of essential oils on fungal growth

Although not statistically significant ($\alpha = 0.01$), oregano and thyme had the highest averages of growth inhibition halos on mycelial growth (Table 3 and Fig. 6). Fennel and cinnamon essential oils also showed considerable activity on growth inhibition, depending on the isolate (Table 3 and Fig. 6). In agreement with our results, oregano, thyme, cinnamon, and fennel essential oils were also reported to inhibit *A. flavus*' mycelial growth (Paster et al., 1995; Montes-Belmont & Carvajal, 1998; Viuda-Martos et al., 2007; Silva et al., 2012).

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 Fig. 6). Differently, Thanaboripat et al. (2007) and Rashidi et al (2011) have demonstrated that lavender essential oil was able to inhibit *A. flavus* growth. The lack of inhibitory activity of lavender oil in our study could be related to its chemical composition, which may change depending on the plants' genetic variability, environmental conditions, harvest period, growth conditions, soil type and part of the plant (Hay and Sovoboda, 1993; Kokkini et al. 1997). Another explanation is that the *A. flavus* isolated from peanuts and

bulgur wheat analyzed in this study could be resistant to the inhibitory activity of lavender essential oil.

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). Even though all the essential oil components have their contribution to the oil´s biological activity, the antimicrobial activity is generally attributed to its main compounds. Carvacrol and thymol are reported to be the main components of oregano oil, thymol and cinnamaldehyde are the primary compounds of thyme oil, and linalool and linalyl acetate are the highest constituents of lavender oil. All those compounds were reported to inhibit fungal growth (Hili et al., 1997; Daferera et al., 2003; Nazzaro et al., 2017; D'agostino et al., 2019). *o*-Methoxycinnamaldehyde has been isolated from cinnamon and is reported to inhibit the growth of *A. flavus* (Morozumi, 1978; Nazzaro et al., 2017; D'agostino et al., 2019). A phenylpropanoid derivative, dillapional, and a coumarin derivative, scopoletin, were found to be the antimicrobial principles of fennel (Kwon et al., 2002; Nazzaro et al., 2017; D'agostino et al., 2019).

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 *A. flavus* isolates (Table 3 and Fig. 7). The oregano oil effect at 100% or 75% was not statistically different ($\alpha = 0.01$), but the thyme oil was most effective at 100%. The minimal concentration of oregano oil that caused growth inhibition in our isolates was 25%, and for thyme oil was 50%, with statistical significance in the majority of the isolates (Table 3 and Fig. 7). In agreement, Silva et al. (2012) also found that the minimal effective concentration for thyme essential oil in the growth inhibition of a strain of *A. flavus* isolated from peanut was 50% (essential oil/DMSO; v/v).

In this work, we have used the filter paper disc diffusion method in a solid medium to evaluate the effect of essential oils on our isolates (Medeiros et al., 2011). This method has some limitations, such as the diffusion may not be uniform, and the presence of volatile substances with an essential role in the antimicrobial activity could evaporate over time. In the literature, there are also several other methods described to test essential oils as inhibitors of fungal growth, mainly using the oil incorporation in the solid medium (Silva et al. (2012). However, these methods also have limitations because the hydrophobic oil incorporation in the medium may not be homogeneous, and the addition of an emulsifier is sometimes necessary (Lambert et al., 2001; Suhr and Nielsen, 2003). To better compare the results in the literature, a standardization of the methods would be necessary.

4 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* isolates, there were eight aflatoxigenic strains. Although there was no phylogenetic difference among these isolates regarding the most used ITS region and the β-tubulin partial sequence, these isolates could be separated from strains of *A. flavus* isolated from bulgur wheat, in ISSR and RAPD analysis, indicating host specificity of isolates. At last, the essential oils of oregano, thyme, cinnamon, and fennel could inhibit the mycelial growth of selected *A. flavus* isolates, which could be useful in the control of this pathogen.

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

Samples	**Geographical origin**	**Peanut variety**	**Nº of kernels presenting *Aspergillus* spp. on AFPA**	**Isolates of *Aspergillus* obtained**	**Species (DNA barcoding)**	**Fluorescence in CMA**	**Ammonium hydroxide analysis**	**TLC**
1	Pinhais, PR	Red	4	UEM 1-1	*A. flavus*	-	-	-
				UEM 1-2	*A. flavus*	+	-	+
2	Pomerode, SC	White	20	UEM 2-1	*A. flavus*	-	-	+
				UEM 2-2	*A. flavus*	+	+	+
3	Maringá, PR	White	4	UEM 3-1	*A. flavus*	+	+	+
				UEM 3-2	*A. flavus*	-	-	-
4	Maringá, PR	Red	4	UEM 4-1	*A. caelatus*	-	-	-
				UEM 4-2	*A. flavus*	+	+	+
5	Umuarama, PR	Red	12	UEM 5-1	*A. flavus*	-	-	+
				UEM 5-2	*A. flavus*	+	+	+
6	Cambará, PR	White	3	UEM 6-1	*A. flavus*	-	-	+
				UEM 6-2	*A. flavus*	-	-	-

Table 2. Primers used in this work.

ITS primers		
Primer	Sequence	Reference
ITS4	5´-TCCTCCGCTTATTGATATGC	White et al., 1990
ITS5	5´-GGAAGTAAAAGTCGTAACAAGG	White et al., 1990
β-tubulin primers		
Primer	Sequence	Reference
Bt2a	5´-GGTAACCAAATCGGTGCTGCTTTC	Glass and Donaldson, 1995
Bt2b	5´-ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson, 1995
RAPD primers		
Primer	Sequence	Reference
RAPD primer 1	5´-GGTGCGGGAA	Mahmoud et al., 2012
RAPD primer 3	5´-GTTTCGCTCC	Mahmoud et al., 2012
RAPD 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
ISSR primers		
Primer	Sequence	Reference
$(GACA)_4$	5´-GACAGACAGACAGACA	Batista et al., 2008
$(AGAG)_4G$	5´-AGAGAGAGAGAGAGAGG	Batista et al., 2008
$(GTG)_5$	5´-GTGGTGGTGGTGGTG	Batista et al., 2008

Table 3. Essential oils inhibition halos (cm) on the mycelial growth of the *A. flavus* isolates.

	Essential oil (100%)					
Isolates	**Oregano***	**Thyme***	**Cinnamon***	**Fennel***	**Lavender***	**Average#**
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	**1.96 ± 1.01 ^{A}**
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	**2.08 ± 0.87 ^{A}**
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	**2.79 ± 1.10 ^{A}**
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	**2.35 ± 1.01 ^{A}**
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	**1.59 ± 0.64 ^{A}**
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	**1.96 ± 1.01 ^{A}**
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					
Isolates	**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					
Isolates	**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 in the right and lowercase letter in the left do not differ by Tukey's Test at $\alpha = 0.01$, in the treatment (column) and in the isolate (line), respectively.

Averages with the same superscript uppercase letter in the right do not differ by Tukey's Test at $\alpha = 0.01$.

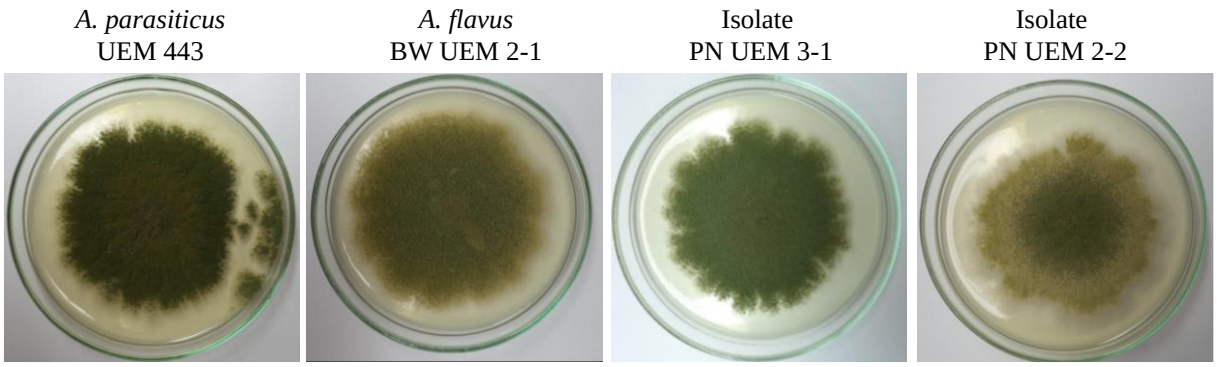


Figure 1. Cultural characteristics on the Czapeck dox agar medium. Cultures of *A. parasiticus* UEM 443 and *A. flavus* bulgur wheat isolate UEM 2-1 (Faria et al., 2017), used as controls. Cultural characteristics of representative peanut isolates UEM 3-1 and UEM 2-2, respectively, from this work. After inoculum, the dishes were incubated at 25 °C for 7 days in a 12 h photoperiod incubator.

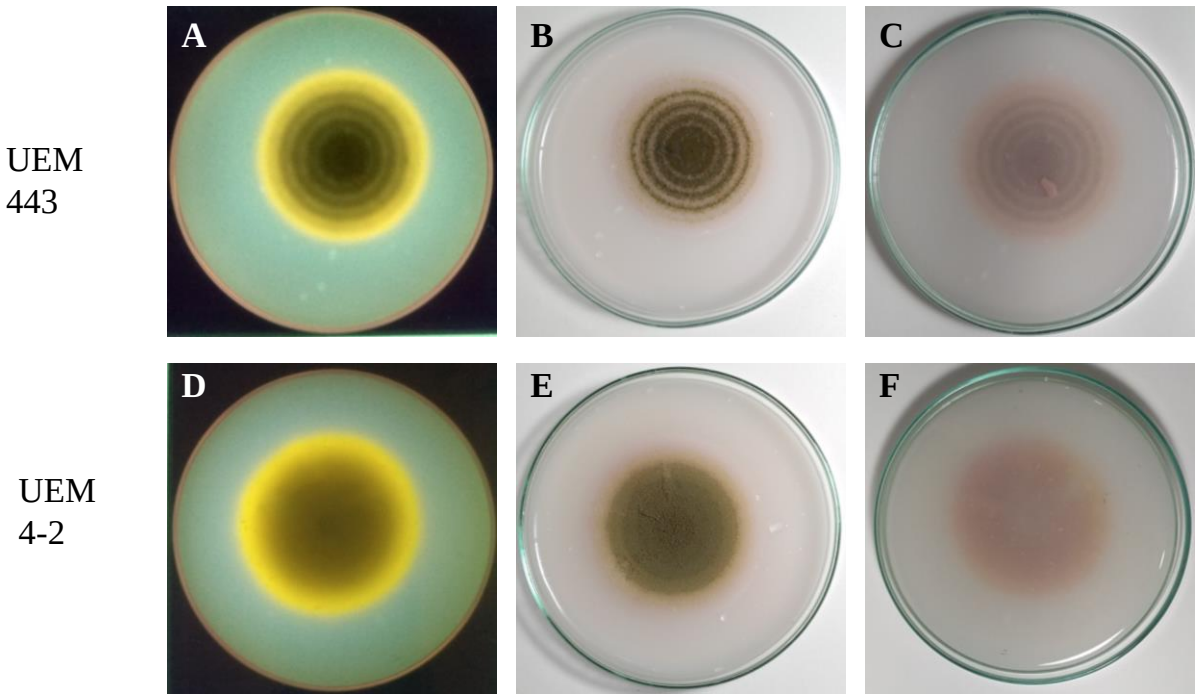


Figure 2. Fluorescence under UV light and color change after exposure to ammonium hydroxide vapor on CMA dishes. A) Back of a dish with a colony from the positive control, *A. parasiticus* UEM 443, under UV light at 312 nm. B) and C) Front and back, respectively, of the dish in A) after ammonium hydroxide vapor exposure. D) Back of a dish with a colony of *A. flavus* UEM 4-2 obtained from peanut in this study under UV light at 312 nm. E) and F) Front and back, respectively, of the dish in D) after ammonium hydroxide vapor exposure.

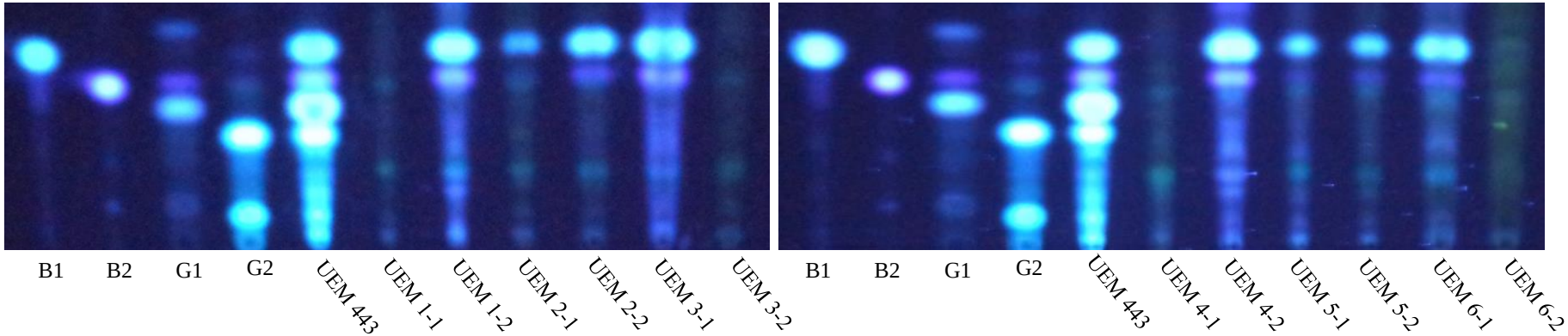


Figure 3. Results of the aflatoxin production analyses by TLC. The positive control used was the extract from *A. parasiticus* UEM 443. B1, B2, G1, and G2 are the aflatoxin standards. The sample numbers are identified.

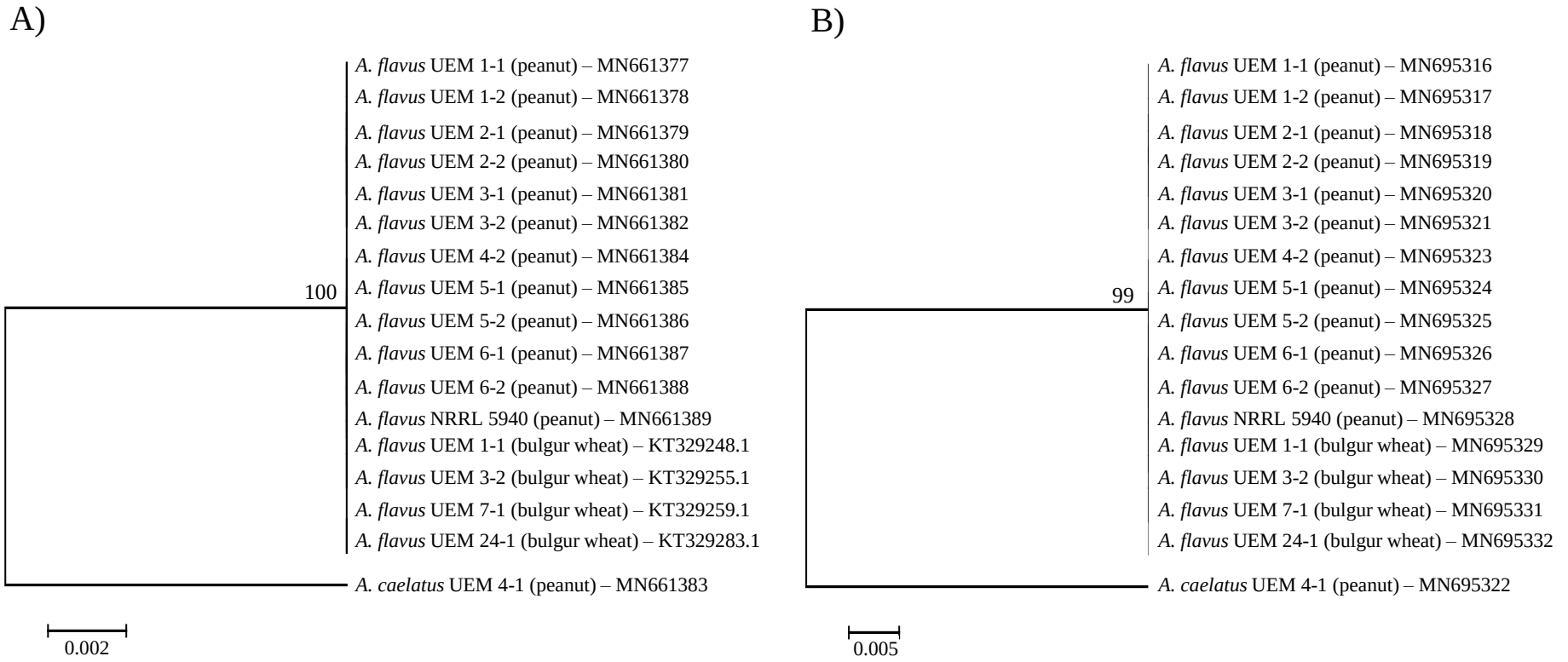


Figure 4. Phylogenetic analyses. A) Phylogenetic tree generated in MEGA software with the ITS gene fragments amplified with the ITS4 / ITS5 primers. B) Phylogenetic tree generated in MEGA software with the β-tubulin gene fragments amplified with the Bt2a / Bt2b primers.

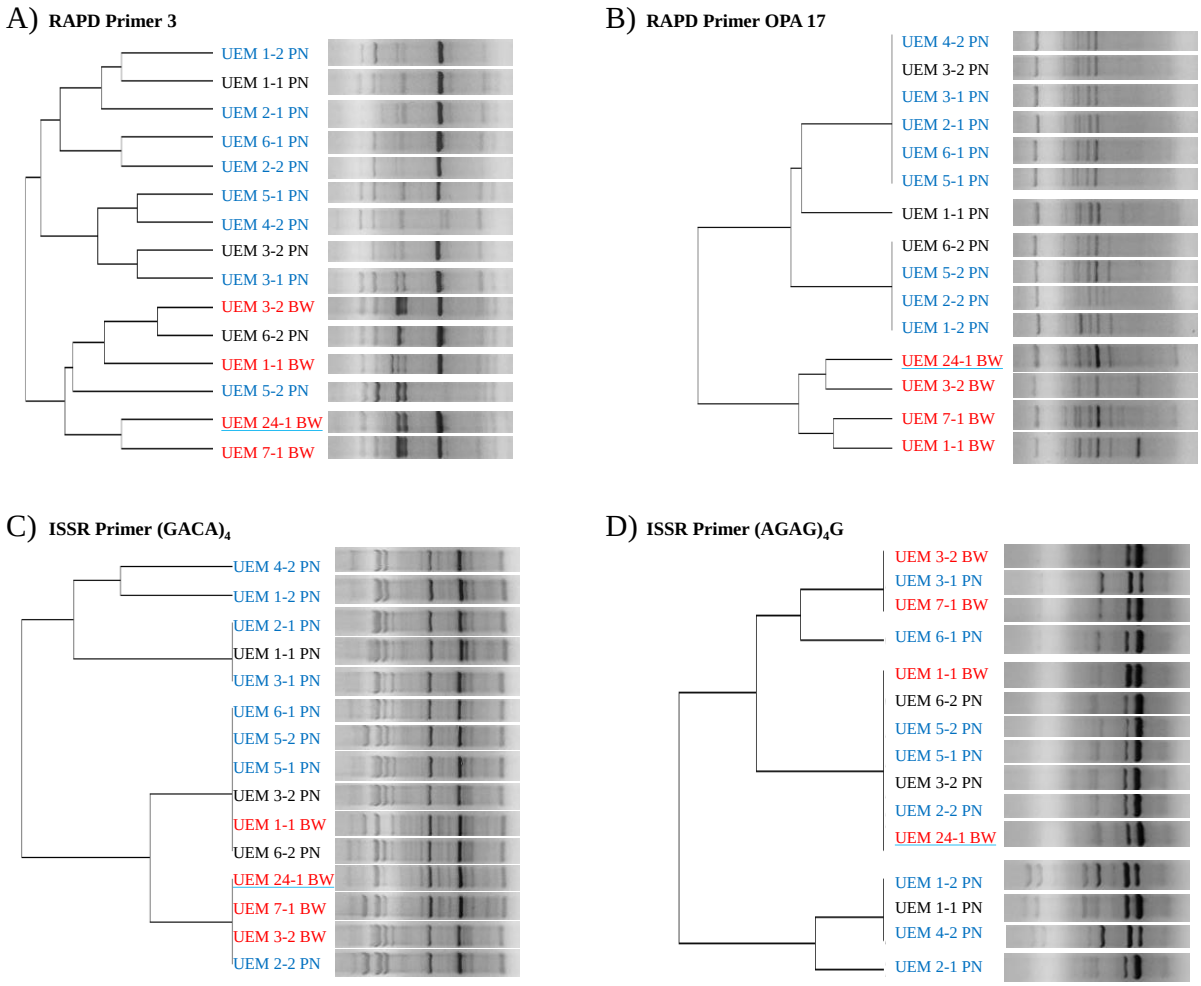


Figure 5. RAPD and ISSR analyses. A) Dendrogram derived from PCR-amplification banding of RAPD with primer 3. B) Dendrogram derived from PCR-amplification banding of RAPD with primer OPA 17. C) Dendrogram derived from PCR-amplification banding of ISSR with primer $(GACA)_4$. D) Dendrogram derived from PCR-amplification banding of ISSR with primer $(AGAG)_4G$. All dendrograms were obtained by the UPGMA method. The analysis used 11 isolates from peanut (aflatoxigenic in blue and non-aflatoxigenic in plain black) and four from bulgur wheat (in red; underlined in blue if aflatoxigenic).

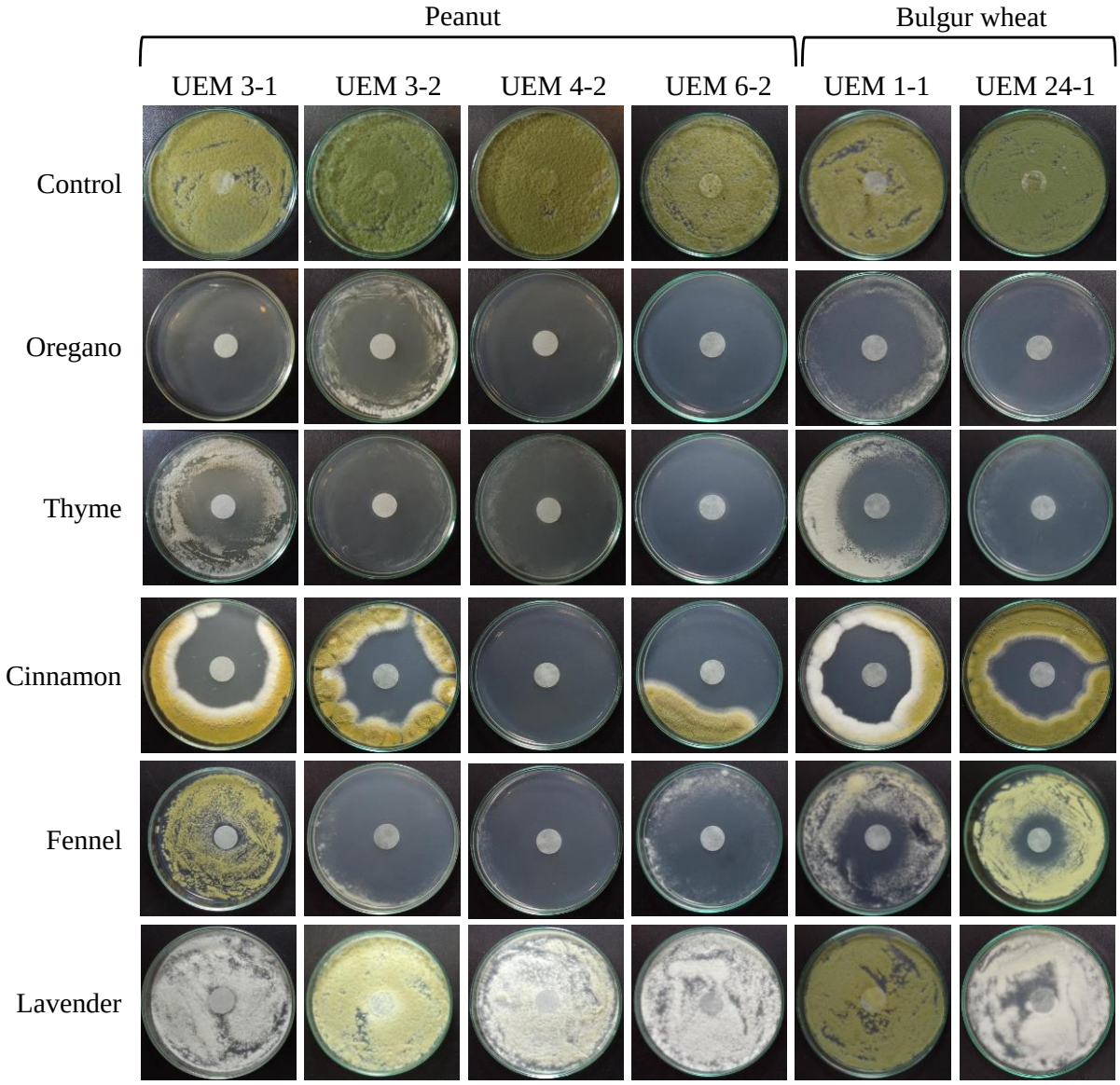


Figure 6. Effect of essential oils on fungal growth. The *A. flavus* isolates selected for these analyses are identified and separated in isolates from peanut and from bulgur wheat. The essential oils tested were oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), fennel (*Foeniculum vulgare*), lavender (*Lavandula angustifolia*) and cinnamon (*Cinnamomum cassia*), all 100% concentrated.

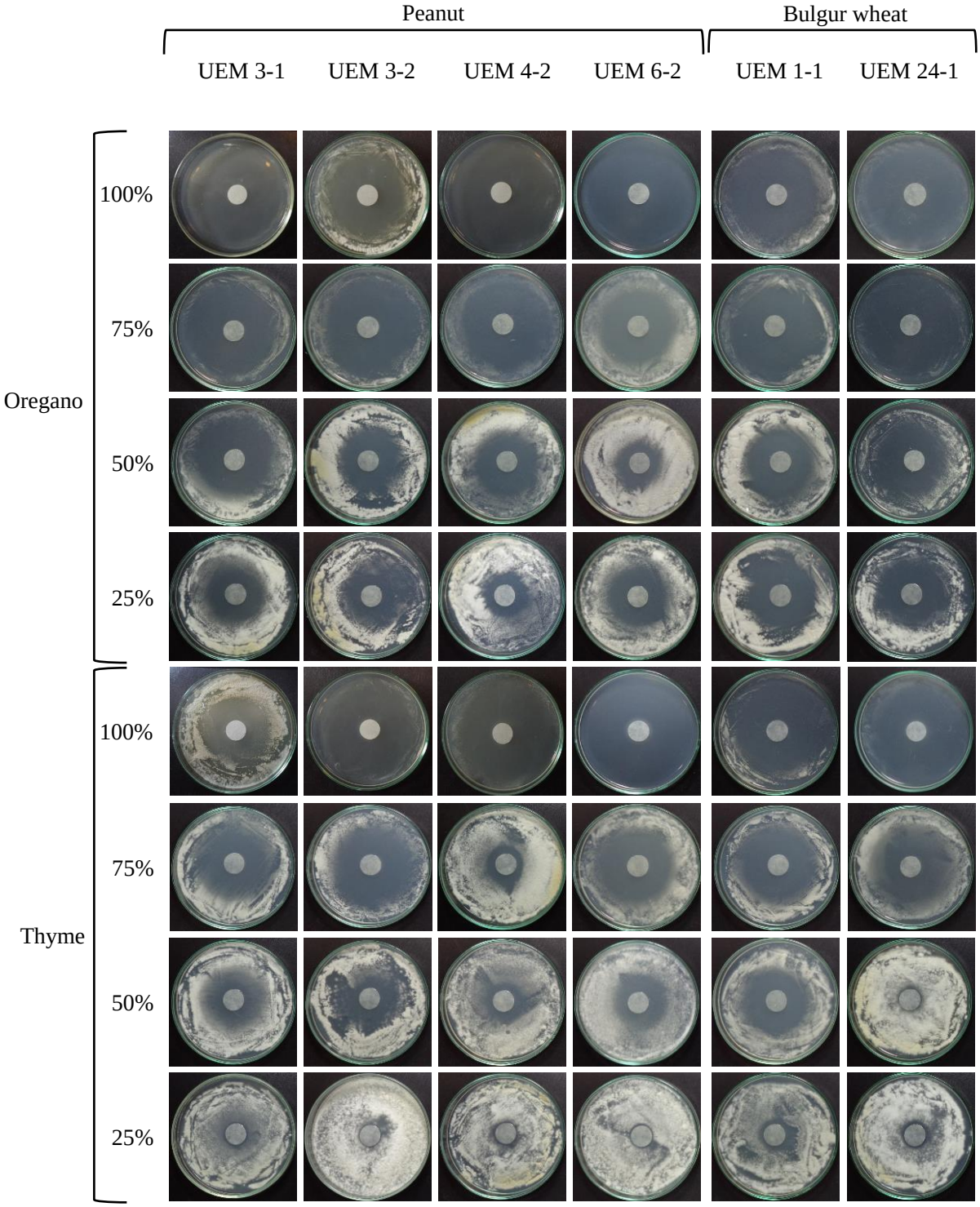
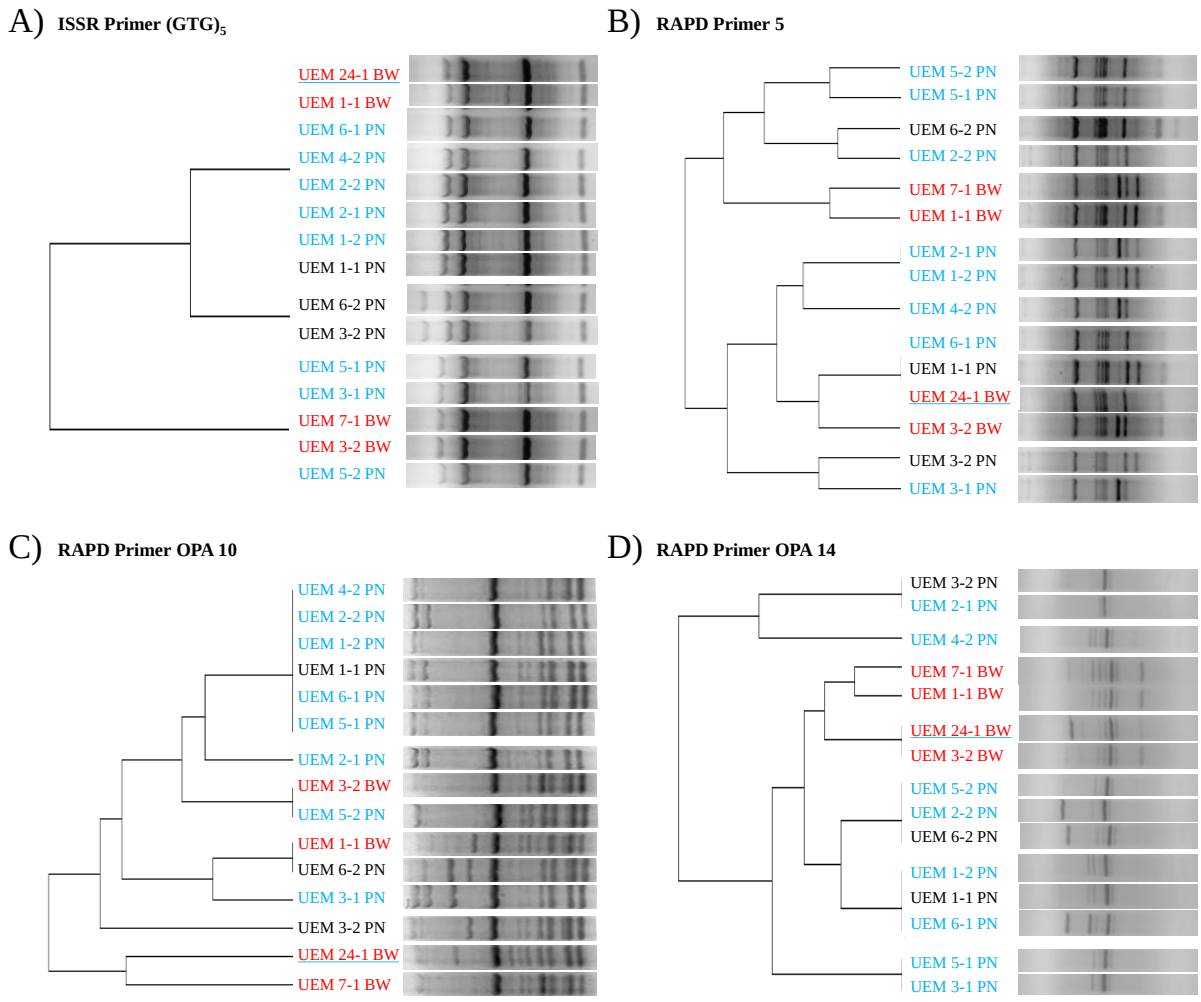
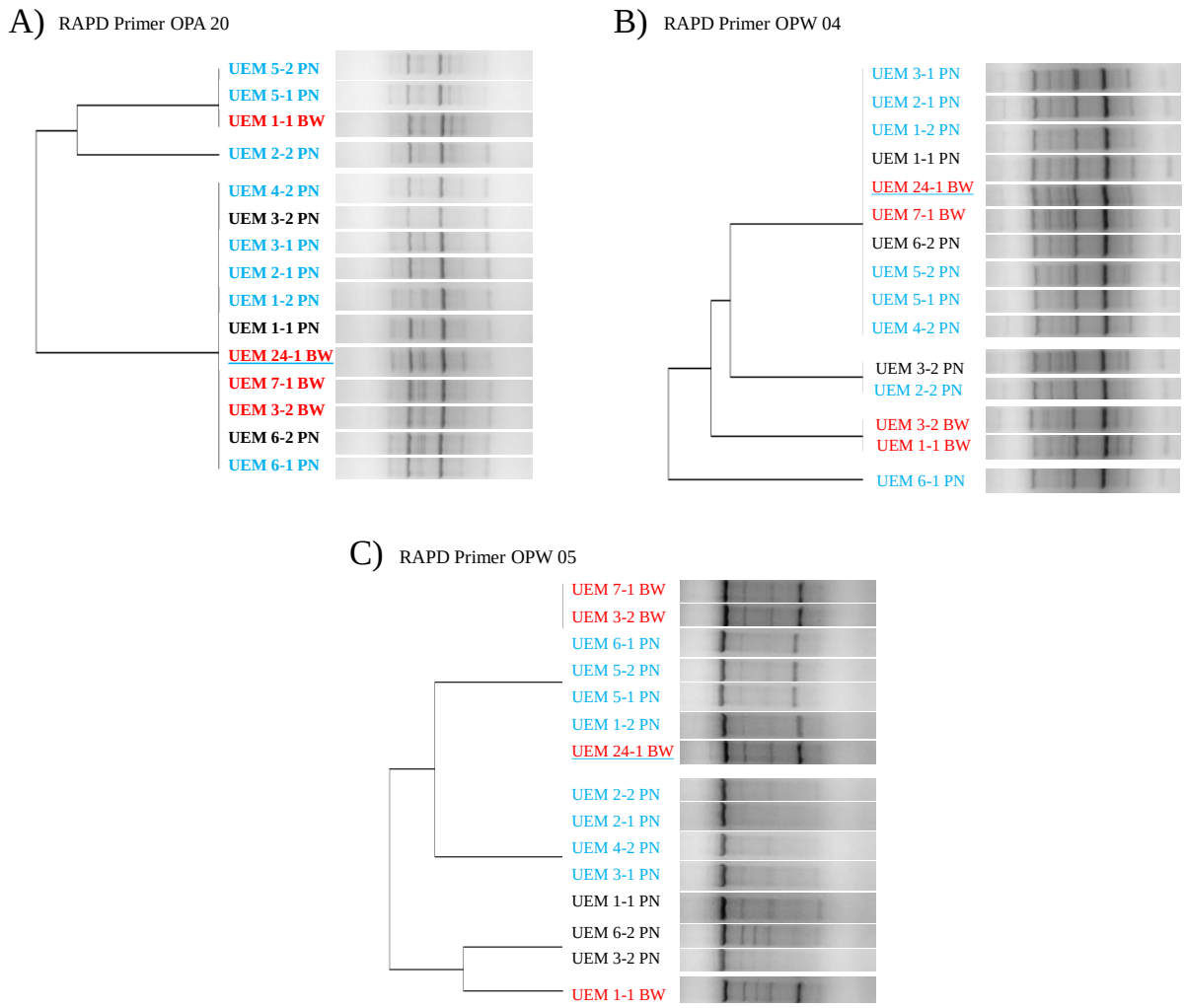


Figure 7. Effect of dilutions of oregano and thyme essential oils on fungal growth. The *A. flavus* isolates are identified and separated between isolates from peanut and from bulgur wheat. The concentrations tested were 25, 50, and 75% for each essential oil.



Supplementary figure 1. RAPD and ISSR analyses. A) Dendrogram derived from PCR-amplification banding of ISSR with primer $(GTG)_5$. B) Dendrogram derived from PCR-amplification banding of RAPD with primer 5. C) Dendrogram derived from PCR-amplification banding of RAPD with primer OPA 10. D) Dendrogram derived from PCR-amplification banding of RAPD with primer OPA 14. All dendrograms were obtained by the UPGMA method. The analysis used 11 isolates from peanut (aflatoxigenic in blue and non-aflatoxigenic in plain black) and four from bulgur wheat (in red; underlined in blue if aflatoxigenic).



Supplementary figure 2. RAPD analyses. A) Dendrogram derived from PCR-amplification banding of RAPD with primer OPA 20. B) Dendrogram derived from PCR-amplification banding of RAPD with primer OPW 04. C) Dendrogram derived from PCR-amplification banding of RAPD with primer OPW 05. All dendrograms were obtained by the UPGMA method. The analysis used 11 isolates from peanut (aflatoxigenic in blue and non-aflatoxigenic in plain black) and four from bulgur wheat (in red; underlined in blue if aflatoxigenic).