

# The molecular and analytical methods reveal aflatoxin B1-producing *Aspergillus flavus* isolated from ready-to-eat peanut samples are resistant to the antifungal agent methyl-thiophanate

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Research

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## ABSTRACT

The peanut is a product of high nutritive value and an economically important crop. However, peanuts can be contaminated during storage with *Aspergillus flavus*, which produces aflatoxins. In humans, aflatoxins can cause hepatocellular carcinoma. Exposure of animals to aflatoxins can result in weight loss and generate residues in meat and milk. The objective of this study was to isolate and identify aflatoxigenic fungi, to quantify the production of aflatoxin B1, and to correlate its production with resistance to the antifungal agent methyl-thiophanate (MT) in ready-to-eat peanuts samples. Nine of the 50 samples selected were found to be contaminated with *A. flavus* by PCR amplification of the ITS region. In addition, three genes of the aflatoxin B1 biosynthesis pathway were amplified. HPLC analysis revealed that the isolates produced high levels of aflatoxins ranging from 4 to 285 µg/mL. Furthermore, the isolate producing the highest concentration of aflatoxin (285 µg/mL) exhibited the highest resistance to antifungal MT (MIC ≥ 250 µg/mL), factors that might be related. The present results confirm the presence of aflatoxin-producing fungi resistant to MT in peanut samples, which may pose health risks to consumers.

**Keywords:** HPLC, VER, APA, OMT, mycotoxins, methyl-thiophanate, resistance, antifungal

## 1. INTRODUCTION

The peanut (*Arachis hypogaea* L.) is a legume native to South America and the most widely grown crop around the world because of its pleasant taste and high content of proteins with high biological value, a fact that renders it a product for raw consumption (TOOMER, 2017). According to the Observatory of Economic Complexity (OEC), the top importers of peanuts are China, The Netherlands, Vietnam and Indonesia, with a market worth about 1.1 billion dollars per year. Leading exporters are the United States, India, Argentina, China and Brazil, with peanut exports worth about 2 billion dollars per year.

In Brazil, the export of peanuts has increased from 9% in 2016 to 61% in 2017, demonstrating that the development of this crop is extremely important for the Brazilian economy, as well as for the world economy Sampaio; Mogiana (2018). However, peanuts are susceptible to contamination with fungi such as *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. In addition to peanut, these fungi infect other foods such as corn, peppercorn, pistachio, chestnut, walnut and products derived from these grains, which are high-value crops in the international market (KRTZMAN et al., 1987; IDRIS et al., 2010; ALDARS-GARCIA et al., 2018).

*Aspergillus flavus* produces a carcinogenic metabolite called aflatoxin (OHKURA et al., 2018). About 20 different types of aflatoxins have been identified and the most common are aflatoxins B1, B2, G1 and G2. Studies have shown that *A. flavus* produces aflatoxins B1 and B2, with the former being the most toxic (LEE et al. 1986; JAIMEZ et al. 2000). Peanuts can be contaminated with these mycotoxins during harvest, storage, processing, and distribution (KADER; HUSSEIN, 2009).

Aflatoxin is converted to aflatoxin-8,9-epoxid in the liver of humans or animals. In humans, the effects caused by ingestion of this mycotoxin can be chronic or acute, including hepatotoxic and immunotoxic effects, an increase in reactive oxygen species, and modifications in DNA strands and protein structure (EATON; GALLAGHER, 1994).

Crops such as corn, soybean, peanut and wheat are commonly used in animal feed and have become vehicles of aflatoxin-producing fungi (RUSTOM, 1997). In general, animals that feed on contaminated grains store the mycotoxin in the liver, with consequent health risks such as weight loss, liver damage and kidney problems (QUEZADA et al., 2000). A strong relationship exists between the presence of aflatoxin in dairy cattle ration and the presence of this mycotoxin in milk used for human consumption (OMEIZA et al., 2018).

The objective of this study was to isolate and identify aflatoxigenic fungi in ready-to-eat peanut samples collected in the State of São Paulo, Brazil, as well as to quantify the production of aflatoxin B1 and to correlate this production with resistance to the antifungal agent methyl-thiophanate (MT).

## 2. MATERIAL AND METHODS

### 2.1 Isolation, identification and antifungal activity of aflatoxin-producing fungi

*Aspergillus flavus* ATCC 9643, obtained from Fundação André Tosello and used as control, was cultured on Sabouraud-dextrose agar (Oxoid, Hampshire, England) for 4 days at 35°C according to CHAI et al. (2009). The isolation of the aflatoxin-producing fungi and the determination of antifungal activity were previously described by AREVABINI et al. (2014). *Aspergillus flavus* was isolated from peanut samples collected at different retail markets in the region of Ribeirão Preto, São Paulo, Brazil, according to SHAPIRA et al. (1996), with some modifications described below: the peanut kernels were washed in sterile water and incubated on Czapeck medium (Merck, Germany) for 7 day at 35°C. The *A. flavus* isolates were then streaked on Czapeck medium to obtain pure cultures.

### 2.2 DNA extraction

Once pure cultures were obtained, DNA was extracted with TRIzol Reagent® following manufacturer's recommendations and agarose gels were prepared according to of SHAPIRA et al. (1996), with modifications described below: mycelia were collected by scraping the surface of the colonies with a sterile

spatula and inoculated into 100 mL liquid Sabouraud medium (Oxoid, Hampshire, England). The cultures were incubated for 24 h at 35°C under shaking at 150 rpm. After incubation, the mycelia were filtered through a Buchner funnel and pressed between sheets of filter paper to remove excess water. The mycelia were immersed in liquid nitrogen and ground in a sterile mortar and pestle. About 100-150 mg of mycelium was homogenized in 1 mL extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS).

### 2.3 Detection of aflatoxigenic *Aspergillus flavus* by the PCR technique

After the extraction of genomic DNA from the isolates, PCR was performed using the primers ITS18S and ITS28S, as well as primers targeting the *omt-1*, *ver-1* and *apa-2* genes (SHAPIRA et al., 1996; ZACHOVA et al., 2003; LEVIN, 2012). The PCR assays were conducted as described by SHAPIRA et al. (1996). The primers used are shown in Table 1.

**Table 1.** Sequence of the primers used for the detection of aflatoxin-producing fungi

Primer	Sequence	Reference
VER-496	5'-ATGTCGGATAAATCACCGTTTAGATGGC-3' (F)	Shapira et al., 1996
VER-1391	5'-CGAAAAGCGCCACCATCCACCCCAATG-3' (R)	
APA-450	5'-TATCTCCCCCGGCATCTCCCGG-3' (F)	Shapira et al., 1996
APA-1482	5'-CCGTCAGACAGCCACTGGACACGG-3' (R)	
OMT-208	5'-GGCCCGTTCTTGGCTCCTAAGC-3' (F)	Shapira et al., 1996
OMT-1232	5'-CGCCCCAGTGAGACCCTTCCTCG-3' (R)	
ITS 1 (18S)	5'-TCCGTAGGTGAACCTGCGG-3' (F)	White et al., 1990
ITS 4 (28S)	5'-TCCTCCGCTTATTGATATG-3' (R)	

### 2.4 Purification and sequencing of the PCR products

The PCR-positive samples were purified using the GFX Purification Kit (GE Healthcare, USA) following manufacturer's recommendations. The PCR products were directly sequenced using the ITS 1 and ITS 4 primers and DNA sequencing was performed on a Perkin-Elmer/ABI model 373 DNA Sequencer.

### 2.5 Fermentation conditions for aflatoxin B1 production

In 250-ml Erlenmeyer flasks,  $1 \times 10^6$  conidia/mL were added to 50 mL YES fermentation medium and incubated for 10 days under moderate shaking at 27°C in the dark, according to CVETNIC and PEPEL-JNJAK (2007).

### 2.6 High-performance liquid chromatography (HPLC/UV-DAD)

For the quantification of aflatoxin production by each isolate, a standard curve was constructed by HPLC using aflatoxin B1 (Sigma). HPLC analysis was carried out on a Shimadzu chromatograph (LC-10AD *vp*) coupled to a diode array detector using a Phenomenex

C<sub>18</sub> column (25 cm x 4.6 mm, id 5 µm). The mobile phase consisted of water:methanol:acetonitrile (50:40:10), eluted a flow rate of 0.80 mL.min<sup>-1</sup>. Twenty microliter of each sample was injected and the analyses were monitored at a wavelength of 350 nm.

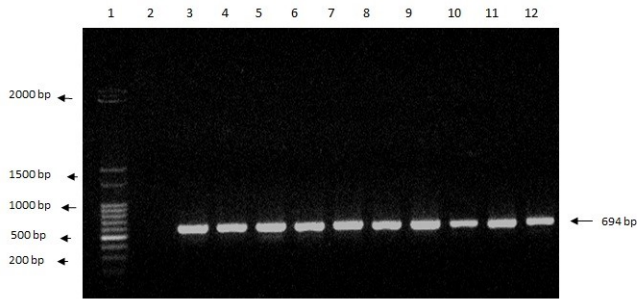
## 3. RESULTS

### 3.1 Isolation of aflatoxin-producing *Aspergillus flavus* strains

Fifty peanut samples collected at different retail markets in the region of Ribeirão Preto from June 2011 to October 2012 were analyzed. Nine of the 50 samples exhibited the growth of *Aspergillus flavus* strains.

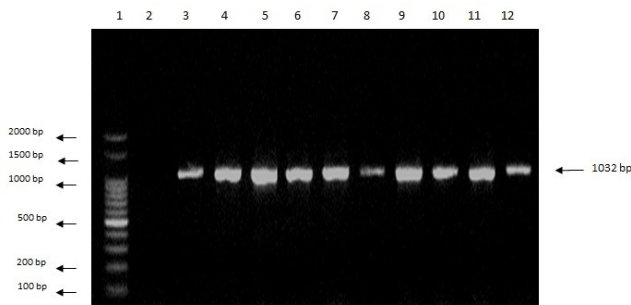
### 3.2 Identification of *Aspergillus flavus* isolates and of the genes involved in aflatoxin biosynthesis by PCR

PCR permitted the detection of a band corresponding to a 694-bp fragment of the ITS region in the selected isolates. This band is characteristic of *A. flavus* (HENRY et al., 2000). In addition, the amplification pattern observed for the ATCC strain was similar to that of the isolates (Figure 1).

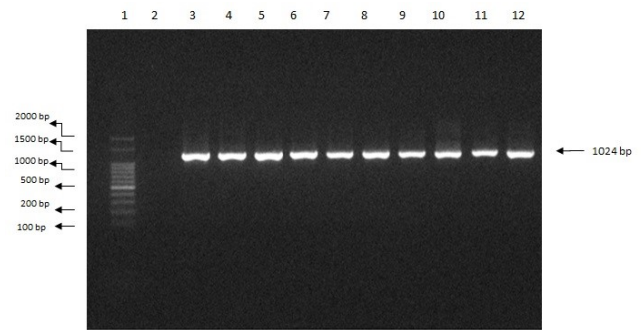


**Figure 1.** Agarose gel (1.2%) electrophoresis showing amplification of the 694-bp fragment of the ITS gene that characterizes *Aspergillus flavus*. Lanes: 1: 1-kb marker; 2: blank; 3: isolate Afl1; 4: isolate Afl2; 5: isolate Afl3; 6: isolate Afl5; 7: isolate Afl6; 8: isolate Afl7; 9: isolate Afl8; 10: isolate Afl9; 11: isolate Afl10; 12: *A. flavus* ATCC 9643.

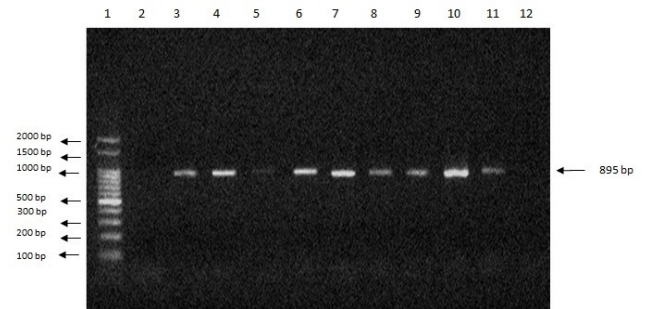
We also observed amplification (expression) of the *apa-2* (a regulator of aflatoxin biosynthesis, which controls the expression of the *ver-1* and *nor-1* genes), *omt-1* (the gene encoding sterigmatocystin-o-methyltransferase), and *ver-1* genes (versicolorin A dehydrogenase) (Figures 2, 3 and 4) (SHAPIRA et al., 1996).



**Figure 2.** Agarose gel electrophoresis showing amplification of a 1032-bp fragment of the *apa-2* gene. Lanes: 1-kb marker; 2: blank; 3: isolate Afl1; 4: isolate Afl2; 5: isolate Afl3; 6: isolate Afl5; 7: isolate Afl6; 8: isolate Afl7; 9: isolate Afl8; 10: isolate Afl9; 11: isolate Afl10; 12: *A. flavus* ATCC 9643.



**Figure 3.** Agarose gel electrophoresis showing amplification of a 1024-bp fragment of the *omt-1* gene. Lanes: 1: 1-kb marker; 2: blank; 3: isolate Afl1; 4: isolate Afl2; 5: isolate Afl3; 6: isolate Afl5; 7: isolate Afl6; 8: isolate Afl7; 9: isolate Afl8; 10: isolate Afl9; 11: isolate Afl10; 12: *A. flavus* ATCC 9643.



**Figure 4.** Agarose gel electrophoresis showing amplification of an 895-bp fragment of the *ver-1* gene. Lanes: 1: 1-kb marker; 2: blank; 3: isolate Afl1; 4: isolate Afl2; 5: isolate Afl3; 6: isolate Afl5; 7: isolate Afl6; 8: isolate Afl7; 9: isolate Afl8; 10: isolate Afl9; 11: isolate Afl10; 12: *A. flavus* ATCC 9643.

All *A. flavus* isolates obtained from peanuts as well as the *A. flavus* ATCC 9643, showed similarity to strains deposited in the database of the National Center for Biotechnology Information (NCBI). Amplification of the ITS gene revealed identity with ITS regions of the ribosomal 18S and 28S gene of *A. flavus* (accession number JF951750.1). Likewise, analysis of samples for the *omt-1* gene revealed identity with the O-methyltransferase gene of an *A. flavus* isolate (accession number L25836.1) and the *ver-1* gene showed identity with the reported gene of an *A. flavus* strain (accession number AB007805).

**Table 2.** Concentration of aflatoxin B1 and determination of Minimal Inhibitory Concentration (MIC) in ( $\mu\text{g}/\text{mL}$ ) of *Aspergillus flavus* strains

Strain	Concentration of aflatoxin B1	MIC of methyl-thiophanate (MT)
ATCC	ND	3.9
Afl1	18 $\pm$ 1	7.8
Afl2	135 $\pm$ 5	7.8
Afl3	47 $\pm$ 1	3.9
Afl5	4 $\pm$ 1	3.9
Afl6	ND	15
Afl7	285 $\pm$ 11	$\geq$ 250
Afl8	ND	1.9
Afl9	168 $\pm$ 6	1.9
Afl10	193 $\pm$ 7	1.9

ND: not detectable.

### 3.3 Quantification of aflatoxin B1 by HPLC/UV-DAD and antifungal activity

The detection and quantification of aflatoxin B1 by HPLC/UV-DAD demonstrated that 7 fungi samples were aflatoxin producers. Afl7 was the isolate with the most expressive production of the mycotoxin, followed by Afl10, Afl9 and Afl2. No aflatoxin production was also observed for the ATCC strain. In this study, 4 of the *A. flavus* isolates were resistant to MT compared to the MIC of 3.9  $\mu\text{g}/\text{mL}$  of the *A. flavus* ATCC strain (Table 2).

### 4. DISCUSSION

Despite hygiene-sanitary control of grains destined for human consumption, nine of the 50 peanut samples collected in the present study were contaminated with *A. flavus*, the main producer of aflatoxin B1.

The present study confirmed the presence of *A. flavus* in 18% of the peanut samples by the amplification of bands corresponding to the *ITS18S* and *IT S28S* genes. Mahmoud (2015) identified *A. flavus* in 53.6% (n=28) of peanut samples collected in Saudi Arabia by PCR amplification of the *FL1* gene.

Comparison of the molecular and analytical (HPLC/UV-DAD) methods showed that both were adequate for detecting aflatoxin in *A. flavus* isolates. Aflatoxin B1 was quantified by HPLC in fungal isolates with

positive PCR results for aflatoxin biosynthesis genes (*omt-1*, *ver-1* and *apa-2*). Aflatoxin B1 levels ranging from 4 to 285  $\mu\text{g}/\text{mL}$ . Mahmoud (2015) detected aflatoxin B levels ranging from 1.64 to 109.18  $\mu\text{g}/\text{mL}$  by HPLC in 70.37% of *A. flavus* strains isolated from peanuts. Mupunga et al. (2014) also observed contamination with *A. flavus* and *A. parasiticus* in peanut and peanut butter samples collected in Zimbabwe. Oliveira et al. (2009) selected 240 peanut samples in Brazil and found positive results for aflatoxins (B1, B2, G1, and G2) in 44.2%.

The isolate Afl7 produced the highest level of aflatoxin B1 (285  $\mu\text{g}/\text{mL}$ ) and was resistant to MT (MIC  $\geq$  250  $\mu\text{g}/\text{mL}$ ) as shown by Arevabini et al. (2014). Moreover, the isolate Afl2 produced high levels of aflatoxin B1 (135  $\mu\text{g}/\text{mL}$ ) and was also resistant to MT (MIC of 7.8  $\mu\text{g}/\text{mL}$ ). These fungal isolates may have been selected by the indiscriminate use of this commercial antifungal agent, which persisted in the field because of their resistance.

Methyl-thiophanate is a fungicide widely used in global agriculture for the control of many fungal diseases in vegetable, fruit and field crop plantations IBTISSEM et al., (2017).

The problem of the abusive use of pesticides in agriculture is linked to the agricultural production poli-



cies in Brazil, which are based on quantity rather than quality of the food and whose focus is unprecedented productivity gain (PIGNATI et al., 2017).

To guarantee the food and nutritional safety of the population, control parameters needs to be established for the effective monitoring of pesticide use, as well as incentives for the development of natural antifungal agents that pose no public health risks. AREVABINI et al. (2014) demonstrated that marine natural products were more effective against MT-resistant *A. flavus* isolates.

Resistance to MT could be related to aflatoxin production. These data makes us question the efficiency of the antifungal agents used in peanuts for elimination of aflatoxigenic fungi in the field t.

Isolates Afl6 and Alf8 were found to be resistant to MT, with MIC of 15 µg/mL. However, no aflatoxin B1 production was observed in these isolates. This finding might be due to the detection limit of the analytical technique used, which is on average 2 µg/mL (ESPINOSA-CALDERON et al., 2011). In this case, PCR is a more sensitive technique since it can detect very low amounts of DNA such as nanograms and femtograms (AREVABINI et al., 2014). In addition, *A. flavus* isolate Afl6 and Af18 may produce a mycotoxin other than aflatoxin B1, such as B2, G1 and G2 (GHAFI et al., 2018)

High levels of aflatoxins in grains are harmful to human and animal health, with a negative impact on the world economy. JAGER et al. (2016) quantified high levels of aflatoxin M1 in urine samples of patients, confirming the consumption of contaminated foods. AMIRKHIZI et al. (2015) observed aflatoxins at relatively high levels in the liver and eggs of chickens. KANG'ETHE AND LANG'A (2009) found contamination of dairy cattle feed with aflatoxins and a consequent expressive increase of aflatoxin B1 and M1 levels in the milk consumed by the population.

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