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Use of glycerol coating to control aflatoxin production by *Aspergillus parasiticus* in peanut grains

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ABSTRACT

Peanut grains are very susceptible to aflatoxin contamination. Aflatoxins are toxic metabolites produced by *Aspergillus flavus*, *A. nomius* and *A. parasiticus*. The aflatoxin B_1 is most frequently found in peanuts, posing a high toxicological risk due to its carcinogenic, teratogenic and mutagenic properties. Alternative methods to fungicides can be used to protect grains. GRAS (Generally Regarded As Safe) substances can be an interesting option to avoid contamination, specially glycerol. In the present work, the ability of glycerol films to prevent aflatoxin production by *A. parasiticus* in peanuts was evaluated. Glycerol was established in two different ways: by immersion and aspersion. Aspersion was more efficient in reducing aflatoxin production (86.3%) than was the immersion process (66.9%) ($P < 0.05$). At the same time, a progressive reduction in *A. parasiticus* colony diameters was observed (from 38.6 ± 0.9 to 34.4 ± 1.7 mm) when the fungus was grown on GYEP medium supplemented with glycerol (0 to 5%). However, varying concentrations did not influence the production of spores, colonies, conidiophores or spore condition. Peanuts coated with 5% glycerol (by immersion or aspersion) had improved characteristics, with a cleaner and more shiny appearance, which can make the resulting product more acceptable to the population. In conclusion, the reduction of aflatoxin production in peanut grains with glycerol, particularly by aspersion, was satisfactory, and this GRAS substance shows promising potential to be used to prevent mycotoxin contamination in grains.

Key words: GRAS substances. Mycotoxin. Grain storage. Food preservation.

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INTRODUCTION

Due to their high protein and lipid content, peanut grains are greatly appreciated by consumers but are also very susceptible to mycotoxin contamination, particularly by aflatoxins. This contamination can occur during all the steps of peanut processing and storage (Santos et al., 2012; Viegas & Rossetto, 2006).

Mycotoxins are toxic metabolites produced by some filamentous fungi, mainly species related to the *Aspergillus* genus. Among the mycotoxins, aflatoxins represent a major concern for public health and agriculture. Aflatoxins are mainly produced by the fungal species *A. flavus*, *A. nomius* and *A. parasiticus* Klich (2007), and aflatoxin B_1 (AFB_1) is the most frequently found in peanuts, posing a high toxicological risk due to its carcinogenic, teratogenic and mutagenic properties (WHO, 1979).

In cases of aflatoxin contamination, peanuts are normally discarded due to the high cost of detoxification, such that prevention of aflatoxin production is the only strategy to maintain product safety. Consequently, governmental agencies recommend intensifying the search for alternative methods (i.e., without fungicides) to preserve seeds (Boxstael et al., 2013).

GRAS (Generally Regarded As Safe) substances are a good option to prevent food degradation and, among these, glycerol and its variations have been studied as growth inhibitors of a wide range of detrimental microorganisms (Arruda et al., 2007; Campana-Filho et al., 2007).

Glycerol is a colorless, odorless, non-toxic liquid with many applications as an additive in the food and pharmaceutical industries due to its antioxidant, emulsification and humectant proprieties (Arruda et al., 2007).

With the development of biofuel in the last few years, high amounts of glycerol have been produced as a residue of biodiesel production. When used as a food additive, glycerol is absorbed and metabolized, and studies show that ingestion of concentrations up to 5% is not toxic to experimental animals (Aider, 2009; Mali et al., 2010; Mali & Grossmann, 2003).

Additionally, other studies have demonstrated that glycerol is an efficient antimicrobial agent against many pathogenic microorganisms (fungi and bacteria) and also can act as a barrier to colonization and degradation by deleterious microorganisms, improving the shelf life of foods when used as a biofilm (Gioso et al.,2002; Mali et al., 2010; Rabelo et al., 2004).

In the present work, we evaluated the efficacy of two glycerol films to prevent aflatoxin production by *A. parasiticus* in peanut grains.

MATERIALS AND METHODS

Peanut grains

Autoclaved peanut grains, cultivar IAC Caiapó (Campinas Agronomical Institute, São Paulo, Brazil), were used. The grains, harvested in 2009/2010, had a medium size, brownish color and high oil concentration and were free of mycotoxins.

Phytopathogen

Aspergillus parasiticus strain IMI 242695, a producer of aflatoxins B_1, B_2, G_1 and G_2, was obtained from the Mycology Laboratory of the Ezequiel Dias Foundation (FUNED), Belo Horizonte, Brazil. The strain was stored in potato broth supplemented with 20% glycerol at -80°C.

Glycerol solution

A solution of glycerol (Synth – PA®, PM = 92.09) at 5% (v/v) in distilled sterile water was used to produce biofilms on peanut grains by the immersion or aspersion methods.

Immersion method

After immersion in glycerol solution (5% v/v) for 30 minutes, peanut grains were dried under air flow at 35 ± 5°C until completely dry. Grains immersed in sterile distilled water were used as a negative control.

Aspersion method

Peanut grains were coated with the glycerol biofilm using a manual spray device operated with compressed air. After spraying the glycerol solution (5% v/v), peanut grains were dried under air flow at 35 ± 5°C. The cycle of spraying/drying was repeated two hundred times. Peanut grains sprayed with sterile distilled water were used as a negative control.

Reagents

All solvents and reagents were purchased from Merck (Darmstadt, Germany) or EM Science (Gibbstown, NJ, USA) and were of analytical grade. Water used in the analytical process was obtained through a Milli-Q purification and filtration system with an 18 MΩ cm-1 resistivity (Millipore, Bedford, MA, USA). All glassware used in evaluating aflatoxin production was decontaminated by immersion in 5% Alcaline Extran MA 01, 7555 (Merck) for 24 hours and then washed exhaustively with distilled water.

Aflatoxin determination

Aflatoxin standards were purchased from Sigma Chemicals (St. Louis, MO, USA), dissolved to 10 µg/ml in high performance liquid chromatography-grade benzene-acetonitrile (98:2), and stored at – 20°C. From the stock solutions (aflatoxins (B_1 10.92, B_2 10.02, G_1 12.17, and G_2 8.36 µg/mL-1), a calibration curve ranging from 0.1 to 9.8 ng/ml was constructed, and sample concentrations were determined using a Shimadzu UV-1601PC spectrophotometer (Shimadzu Scientific Instruments, Japan) as described by (Horwitz & Latimer-Junior, 2011). Intermediate standard solutions were prepared from the stock solutions in benzene:acetonitrile (98:2).

Aspergillus inoculums

Aspergillus parasiticus was inoculated onto Petri dishes containing Czapek agar medium (4% sodium nitrate, 1% potassium chloride, 1% magnesium sulfate 7H2O, 0.02% ferrous sulfate 7H2O, 2% dibasic potassium phosphate, 3% sucrose, 0.1 ml of 1% zinc sulfate 7H2O, 0.1 ml of 0.5% copper sulfate and 2% agar) and incubated for 7 days at 25°C. After sporulation, the spore suspension was transferred from the medium surface to an assay tube in 1.5 ml of 0.1% Tween 80 and homogenized by shaking at 300 rpm for 30 seconds. Then, 200 µl was transferred to a 250-ml Erlenmeyer flask containing 25 ml of GYEP agar medium (2% glucose, 0.3% yeast extract, 1% peptone and 2% agar) and incubated at 25°C for 7 days to induce new sporulation. After incubation, 20 ml of 0.1% Tween 80 and 10 glass beads were introduced into the Erlenmeyer flask, which was hand-agitated, and the supernatant was transferred to a new Erlenmeyer flask. Spore concentration was determined using a hemocytometer and then adjusted to 1 x 10^6 spores/ml in 0.1% Tween 80 (Prado et al., 2011).

Aflatoxin production control assays

Autoclaved peanut grains were separated for two experiments (1 and 2, with three groups, each assay used 15 g approximately of peanuts) with immersed grains and aspersed grains. Experimental conditions for each group were as follows: Group 1-1 (positive control 1) had grains previously immersed in distilled sterile water that were inoculated with 2.5 ml of a suspension containing 1.0 x 10^6 spores/ml of *A. parasiticus*; Group 1-2 had grains previously immersed in 5% glycerol that were inoculated with 2.5 ml of a suspension containing 1.0 x 10^6 spores/ml of *A. parasiticus*; Group 1-3 (negative control 1) had grains previously immersed in 5% glycerol that were inoculated with 2.5 ml of Tween 0.1% 80. The same experimental design was made to evaluate the aspersion method. After inoculation, all groups from both experiments were incubated in sterile 250 ml Erlenmeyer flasks at 25 °C for seven days (Prado et al., 2011).

Aflatoxin quantification

For aflatoxin quantification, 75 ml of a 4% methanol-KCl (270:30) solution were added to each treatment sample, shaken for 30 minutes, and blended at high speed for 1 minute. After filtration with Whatman filter N°1, 30 ml of the extract were mixed with 30 ml of 10% cupric sulfate and filtered again. An aliquot of 30 ml of this filtrate was mixed with 30 ml of water in a separation funnel and extracted twice with 25 ml of chloroform. Then, the chloroform phase was evaporated to dryness under a gentle stream of nitrogen. The dry extract was dissolved (100 to 2000 µl) in benzene-acetonitrile (98:2), and 2 to 10 µl was spotted onto thin layer aluminum chromatography plates (20 by 20 cm) pre-coated with silica gel 60 (Merck).

The plates were developed with a mixture of toluene-ethyl acetate-chloroform-formic acid (70:50:50:20, v/v/v/v), and bands with appropriate retention factor values were quantified by fluorodensitometry at 365 nm with a fluorodensitometer (model CS9301, Shimadzu Corp. Kyoto, Japan). Aflatoxin concentrations were determined by comparing the fluorescent intensity of sample spots with known amounts of standards spotted on the same plate (Soares & Rodriguez-Amaya, 1989; Prado et al., 2011).

Influence of glycerol on *A. parasiticus* growth

Three aliquots of 10 µl containing 1.0 x 10^6 spores/ml of *A. parasiticus* were spotted onto Petri dishes containing GYEP medium. Medium without glycerol was used as a control, and experimental tests were performed using the same medium supplemented with 1.5%, 2.5% and 5% glycerol. After incubation for seven days at 25 °C, colony diameter was measured with a digital pachymeter (Jomarca®) (Pimenta et al., 2012).

Influence of glycerol on A. parasiticus morphology

Spores and hyphae from the experiments described above were observed by scanning electronic microscopy (JEOL JSM-6360LV) according to protocols used by the Microscopy Center of the Federal University of Minas Gerais, MG, Brazil.

Statistical analysis

The experimental design used to evaluate the influence of glycerol on aflatoxin production and *A. parasiticus* growth was entirely randomized with five repetitions, two treatments and two types of coatings (immersion and aspersion) for four treatments. Results were analyzed with F tests, and averages were analyzed with Tukey's test. Data were subjected to the *Kolmogorov-Smirnov* test to evaluate instability among averages of groups. Results were considered significant at $P < 0.05$. Tests were performed using the Sisvar program (version 5.3 UFLA).

RESULTS AND DISCUSSION

Table 1 shows that the two methods of coating (immersion and aspersion) reduced aflatoxin production of *A. parasiticus* on peanut grains ($P < 0.05$). However, the aspersion method was more efficient (86.3%) than the immersion process (66.9%) ($P < 0.05$). This fact could be explained by the higher number of applications performed with the spray coating (200 cycles) when compared to the single immersion in glycerol solution. Another interesting observation is the higher aflatoxin concentration in immersion control in comparison with aspersion control, probably this occur due a high humidity in the grains treated with immersion (Garcia et al., 2012) As aflatoxins are carcinogenic and teratogenic substances that act in a cumulative manner, any process leading to a significant reduction in their concentration in foods can be considered important for agroindustry.

Table 1 - Aflatoxin concentrations (µg/kg) and reduction (%) in peanut grains previously treated by immersion or aspersion with 5% glycerol and inoculated with *A. parasiticus* IMI 242695.

	Aflatoxin concentration (µg/Kg)		Aflatoxin reduction (%)		P value
	Immersion	Aspersion	Immersion	Aspersion	
Control	45,170.20 Aa	15,405.20 Ab	-	-	0.0009
Glycerol (5%)	14,964.80 Ba	2,111.00 Bb	66.9	86.3	

A, B, a, b Different letters (lowercase letters for rows and uppercase letters for columns) indicate significant differences according to Tukey's test ($P < 0.05$).

A progressive reduction of *A. parasiticus* colony diameters was observed (from 38.63 ± 0.98 to 34.42 ± 1.79 mm) when the fungus was grown on GYEP medium supplemented with increasing concentrations of glycerol (from 0 to 5%), but this phenomenon was not statistically significant ($P > 0.05$). This result must be confirmed with more experiments to evaluate whether glycerol acts only on aflatoxin production or also on the growth of *A. parasiticus*, as described in other studies examining the antimicrobial properties of glycerol (fungicidal or fungistatic) (Melo-Filho et al., 2011; Rabelo et al., 2004).

Figures 1 and 2 show that increasing concentrations of glycerol (0% to 5%) supplemented in GYEP medium did not influence the production of *A. parasiticus* spores, colonies, conidiophores and spore appearance. Glycerin can to promote a dehydration of cells (Vulcani et al., 2008). But despite this, the colonies remained homogeneous, with well-developed conidiophores and a large amount of spore production. Vesicles exhibited a regular form (ampulliphorm) with the presence of phialides, and a normal appearance was observed for spores (globose, hyalines and with spicules). These results are interesting since they show the glycerol effect in aflatoxin biosynthesis but not in mycelial structure.

Glycerol is frequently used in the food and pharmaceutical industries as a humectant, solvent, and sweetener (Monosik et al., 2012), and no cases of damage or negative symptoms have been observed with its daily

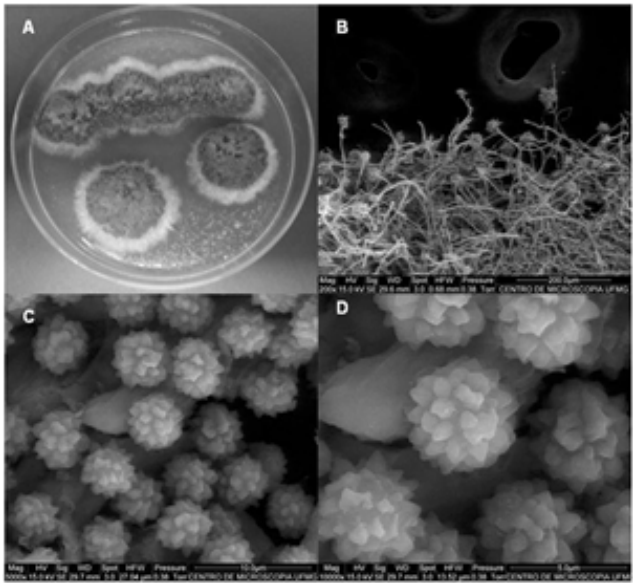

Figure 1 - *Aspergillus parasiticus* IMI 242695 grown on GYEP medium for seven days at 25°C (positive control): (A) macroscopic appearance of colonies; (B) electron micrography of colonies (mag. 200 x); (C) electron micrography of spores (mag. 5,000 x); (D) electron micrography of spores (mag. 10,000 x).

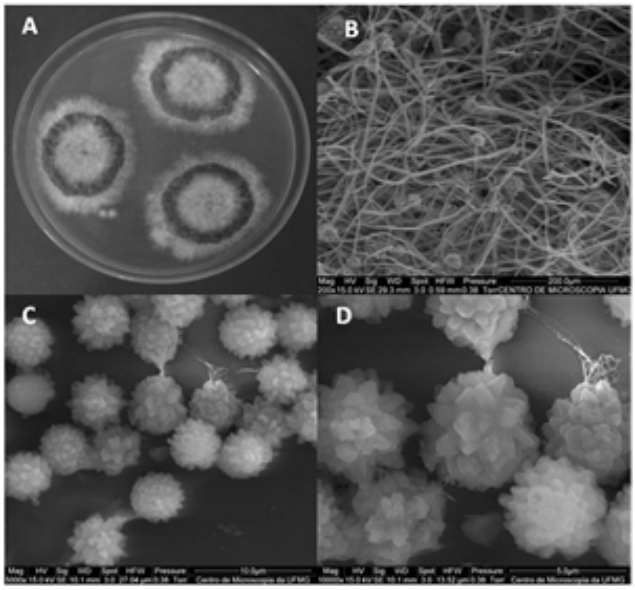

Figure 2 - *Aspergillus parasiticus* IMI 242695 grown on GYEP medium supplemented with 5% glycerol for seven days at 25°C (positive control): (A) macroscopic appearance of colonies; (B) electron micrography of colonies (mag. 200 x); (C) electron micrography of spores (mag. 5,000 x); (D) electron micrography of spores (mag. 10,000 x).

ingestion by consumers. Additionally, glycerol has recently experienced overproduction as a secondary product of the growing biodiesel industry in Brazil. Therefore, use of glycerol as food additive could decrease the problems involved in discarding this residue of biofuel production (Fountoulakis & Manios, 2009; Gu & Jérôme, 2010). In addition to its protective effects against mycotoxin, peanuts coated with 5% glycerol (both by immersion and aspersion) had an improved aspect, with a clean and more shiny appearance, which can make the resulting product more acceptable to the population.

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RESUMO

Utilização de um filme de glicerol para o controle da produção de aflatoxina por Aspergillus parasiticus em amendoim

O amendoim é muito suscetível à contaminação por aflatoxinas, que são metabólitos tóxicos produzidos por *Aspergillus flavus*, *A. parasiticus* **e** *A. nomius*. **A aflatoxina B_1 é a mais frequentemente encontrada nos amendoins e apresenta risco toxicológico devido às suas propriedades carcinogênicas, teratogênicas e mutagênicas. Entre os métodos de prevenção da contaminação, o uso de substâncias GRAS (substâncias geralmente consideradas seguras) pode apresentar grande potencial de exploração, especialmente o glicerol. No presente trabalho, foi avaliada a capacidade de filmes de glicerol para o controle da produção de aflatoxinas em amendoins. O glicerol foi inoculado por imersão ou aspersão, sendo a aspersão mais eficiente na redução da produção de aflatoxina (86,3%) que a imersão (66,9%) ($P < 0,05$). Ao mesmo tempo, foi observada uma redução nos diâmetros das colônias de** *A. parasiticus* **(38,6 ± 0,9 para 34,4 ± 1,7 mm) quando cultivado em meio GYEP suplementado com glicerol (0 a 5%). Apesar disto, diferentes concentrações não influenciaram a produção ou morfologia dos esporos e conidióforos. Amendoins revestidos com 5% de glicerol apresentaram características interessantes, tais com: maior brilho e coloração mais intensa, o que pode tornar o produto mais atraente para o consumidor. Em conclusão, a redução da produção de aflatoxinas em amendoim pelo glicerol, principalmente por aspersão foi satisfatória. Sendo assim, esta substância apresenta um potencial promissor para utilização para a prevenção da contaminação do amendoim por aflatoxinas.**

Palavras-chave: Substâncias GRAS. Micotoxinas. Armazenagem de grãos. Conservação de alimentos.

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