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Mycobiota, aflatoxins and cyclopiazonic acid in stored peanut cultivars

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ABSTRACT

This study evaluated the presence of fungi and mycotoxins [aflatoxins (AFs), cyclopiazonic acid (CPA), and aspergillic acid] in stored samples of peanut cultivar Runner IAC Caiapó and cultivar Runner IAC 886 during 6 months. A total of 70 pod and 70 kernel samples were directly seeded onto *Aspergillus flavus* and *Aspergillus parasiticus* agar for fungi isolation and aspergillic acid detection, and AFs and CPA were analyzed by high-performance liquid chromatography. The results showed the predominance of *Aspergillus* section *Flavi* strains, *Aspergillus* section *Nigri* strains, *Fusarium* spp., *Penicillium* spp. and *Rhizopus* spp. from both peanut cultivars. AFs were detected in 11.4% of kernel samples of the two cultivars and in 5.7% and 8.6% of pod samples of the Caiapó and 886 cultivars, respectively. CPA was detected in 60.0% and 74.3% of kernel samples of the Caiapó and 886 cultivars, respectively. Co-occurrence of both mycotoxins was observed in 11.4% of kernel samples of the two cultivars. These results indicate a potential risk of aflatoxin production if good storage practices are not applied. In addition, the large number of samples contaminated with CPA and the simultaneous detection of AFs and CPA highlight the need to investigate factors related to the control and co-occurrence of these toxins in peanuts.

1. Introduction

Peanuts are considered to be a high-risk product for contamination with aflatoxins (AFs) since they are frequently contaminated with fungi, particularly *Aspergillus flavus* and *Aspergillus parasiticus*, and because of long peanut drying times and occurrence of rainy periods after uprooting (Fonseca, 2012). *A. flavus* strains usually produce aflatoxin B1 (AFB_1) and aflatoxin B_2 (AFB_2), whereas *A. parasiticus* strains produce AFB_1, AFB_2, aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) (Pitt & Hocking, 2009). Contamination of peanuts with aflatoxins in the field is difficult to control because of the influence of climatic conditions, mainly relative humidity and temperature. In addition, factors such as soil moisture content, damage caused by insects, mineral deficiency, and stress play an important role in fungal contamination. However, high concentrations of AFs are related to the growth of *A. flavus* and *A. parasiticus* after harvest when storage conditions are propitious (Moss, 1991). One important factor that contributes to the contamination of stored peanuts is the high moisture content of peanut grains during postharvest drying and the inability to maintain adequate moisture during storage (Davison, Whitaker, & Dickens, 1982).

Aflatoxins are associated with toxicity and carcinogenicity in humans and animals (IARC, 1993). Acute aflatoxicosis can be fatal, whereas chronic toxicosis can lead to cancer and immunosuppression (Hsieh, 1988). Another mycotoxin produced by *A. flavus*, cyclopiazonic acid (CPA), causes necrotic foci in internal organs such as the liver and exerts neurotoxic effects (Frisvad, Thrane, Samson, & Pitt, 2006). CPA occurs naturally in peanuts (Fernández-Pinto, Patriarca, Locani, & Vaamonde, 2001; Lansden & Davidson, 1983) as a co-contaminant with AFs and may have contributed to the "Turkey X" syndrome in England in 1960 (Cole, 1986).

The Runner is the most important peanut cultivar in the State of São Paulo, Brazil, which has gained acceptance on the international market because it is similar to cultivars of other peanut-exporting countries such as the United States and China (USDA, 2012).

The objectives of the present study were to determine the presence of fungi, mainly *Aspergillus* section *Flavi* and mycotoxins (AFs, CPA, and aspergillic acid) in stored peanut samples of two Runner IAC cultivars (886 and Caiapó) and to correlate these results with water activity (A_w) and abiotic factors (temperature, rainfall, and relative humidity).

2. Materials and methods

2.1. Study place and peanut planting

The study area was located in the municipality of Tupã, State of São Paulo, Brazil. Peanuts of Runner IAC 886 cultivar (886) and Runner IAC Caiapó cultivar (Caiapó) were planted in October 2005. A conventional tillage method was used and the experiment was laid

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out in a randomized block design. The peanut tillage system was installed using a row spacing of 90 cm (Atayde et al., 2012).

2.2. Evaluation of climatic conditions

Temperature (°C), relative humidity (RH) (%) and rainfall index (mm) data were supplied throughout the period of storage (6 months) by the climatologic station of Instituto Agronômico, municipality of Adamantina, State of São Paulo. The temperature and relative humidity inside the warehouse were measured with a Sphere digital thermo-hygrometer (Oregon, Tualatin, Oregon, USA).

2.3. Samples and storage conditions

The plants were windrowed in the field during the natural drying process until a moisture content of less than 11% was obtained. Ten peanut samples (5 samples per cultivar, each weighing 400 g) were collected before storage (freshly harvested peanuts) and analyzed for A_w, mycobiota and mycotoxins. The remaining samples were transferred to 25 kg sacks, stacked on wooden pallets (10 sacks per pallet), and stored at the agricultural cooperative Cooperativa Agrícola Mista da Alta Paulista (CAMAP) under proper ventilation conditions. Each 400 g sample was obtained from five different sites within each sack. A_w, mycobiota, and mycotoxins were analyzed monthly over a period of 6 months. A total of 70 peanut samples were analyzed.

2.4. Determination of A_w

The A_w of the peanut samples (separately for kernels and pods) was determined using an Aqualab CX-2 apparatus (Decagon Devices, Inc., Pullman, WA, USA).

2.5. Isolation of fungi from peanut samples

Fungi were isolated and identified in accordance with good laboratory practice. Direct inoculation was used for fungal isolation from kernel and pod samples (Berjak, 1984). A 30 g aliquot of each sample was disinfected with 0.4% sodium hypochlorite solution for 3 min, followed by three washes with sterile distilled water in order to eliminate external contaminants. After disinfection, the pods were separated aseptically from the kernels and inoculated directly into Petri dishes containing *A. flavus* and *A. parasiticus* agar (AFPA) (Pitt, Hocking, & Glenn, 1983). Three plates containing 11 kernels were prepared for each sample, corresponding to a total of 33 kernels per sample. For pods, four plates containing four pairs of pods each were used, corresponding to a total of 16 pods per sample. The plates were incubated at 25 °C for 5 days, and the results are expressed as total percentage of kernels and pods infected with fungi.

2.6. Isolation of airborne fungi from the warehouse

For isolation of airborne fungi from the warehouse, five plates were exposed monthly around the sacks of each cultivar for 6 months, totaling 60 air samples. Air sampling of fungi was carried out using the programmable M Air T air monitoring system (Millipore, Marlborough, MA, USA). This method is based on the quantification of airborne fungal spores (1000 L or m^3 of air) seeded onto Petri dishes containing Sabouraud dextrose agar (Gambale, 1998). After exposure in the air monitoring device, the Petri dishes were incubated for 5 days at 25 °C and the isolated colonies were subcultured on Sabouraud dextrose agar and potato dextrose agar.

2.7. Identification of isolated fungi

The fungi were morphologically identified to genus level and those belonging to *Aspergillus* section *Flavi* were identified to the species level according to Raper and Fennel (1965), and Pitt and Hocking (2009), and confirmed by evaluation of the production of AFs, CPA (Pitt & Hocking, 2009), and aspergillic acid (Pitt et al., 1983).

2.8. Determination of mycotoxins

All solvents used were of liquid chromatography grade and were obtained from Merck (Darmstadt, Germany). Milli-Q water was produced in our laboratory using an Academic System (Millipore, Marlborough, MA, USA). AFB_1, AFB_2, AFG_1, AFG_2 and CPA standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.8.1. Performance of analytical methods of AFs and CPA

The performance of the analytical methods for the detection of AFs and CPA was determined according to IUPAC and Currie (1999).

2.8.2. Aflatoxins

For extraction of AFB_1, AFB_2, AFG_1 and AFG_2 from peanut kernels and pods, 25 g of each previously ground peanut sample was added to 5 g NaCl and 125 mL methanol–water (7:3, v/v) and the mixture was stirred for 30 min in a horizontal shaker. After filtration, 15 mL of the sample was collected, diluted in 30 mL distilled water, and filtered through a microfiber filter. Fifteen milliliters of the extract was loaded onto an immunoaffinity column (Aflatest, Vicam, Watertown, MA, USA) at a flow rate of 1–2 drops/s until air passed through the column. The column was washed with 10 mL distilled water, aflatoxins were eluted with 1.0 mL methanol (Trucksess et al., 1991), and the eluent was evaporated to residue. The residues were derivatizated with 50 μL trifluoroacetic acid and 200 μL n-hexane heated to 40 °C in an oven (Tarin, Rosell, & Guardino, 2004). The solution was evaporated and resuspended in 200 μL acetonitrile–water (3:7, v/v) and 20 μL was injected into the Shimadzu HPLC system (Kyoto, Japan) equipped with an RF 10AXL fluorescence detector (Shimadzu) at excitation and emission wavelengths of 360 and 440 nm, respectively. A Luna C18 analytical column (4.6 × 250 nm, 5-μm particle size; Phenomenex, Torrance, CA, USA) was used for analysis. The isocratic mobile phase consisted of water–acetonitrile–methanol (60:25:15, v/v/v) eluted at a flow rate of 1 mL/min.

2.8.3. Cyclopiazonic acid

Cyclopiazonic acid was analyzed in the 70 kernel samples. An aliquot (25 g) of each sample was crushed and transferred to an Erlenmeyer flask, and 100 mL methanol–2% sodium bicarbonate in water (7:3, v/v) was added. After stirring in a horizontal mechanical shaker for 30 min, the content was filtered through filter paper, 50 mL of the extract was transferred to a separation funnel, and 100 mL hexane was added. This solution was shaken, the hexane fraction was discarded, and 50 mL of an aqueous 10% KCl solution was added. The mixture was acidified with 2.0 mL HCl (6 N). Cyclopiazonic acid was extracted twice with 50 mL chloroform. The chloroform phases were collected, filtered through anhydrous sodium sulfate, and evaporated under vacuum. Micotox® MS2300 solid phase extraction column (Micotox Ltda., Bogota, Colombia) was conditioned with 5 mL chloroform before use at a rate of 1–2 drops/s until air passed through the column. Next, the sample was resuspended in 10 mL chloroform and transferred to a cartridge. The column was washed with 10 mL ethyl ether, 10 mL chloroform–acetone (1:1, v/v), and 10 mL chloroform–methanol (95:5, v/v), and CPA was eluted with 10 mL chloroform–methanol (75:25, v/v) at a flow rate of 2.0 mL/min. The extract was evaporated to residue under nitrogen, resuspended in 1 mL methanol (Urano, Trucksess, Matusik, & Dorner, 1992), and stored at −20 °C until the time for chromatography. CPA was quantified by HPLC using a diode array detector (SPD-M10Avp, Shimadzu) at 284 nm and a Luna C8 column (4.6 × 250 mm, 5-μm particle size; Phenomenex). The isocratic mobile phase consisted of acetonitrile–0.05 M ammonium acetate buffer, pH 5 (8:2, v/v), and was eluted at a flow rate of 0.6 mL/min.