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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.
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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.

2.8.4. Aspergillic acid

All isolates were cultured on AFPA at 30 °C for 48 h in the dark to confirm the production of aspergillic acid by analyzing the color on the reverse side of the colony (Pitt et al., 1983).

2.9. Statistical analysis

Statistical analysis was performed with the R 2.9 GAMLSS package and SAS 9.1 program. Exploratory analysis, Spearman's correlation coefficient, and GAMLSS models were used.

3. Results and discussion

3.1. Frequency of fungal isolation from kernel and pod samples

The moisture content of the stored peanut samples was 10.7% (Caiapó) and 10.4% (886). These values agree with those recommended by Fonseca (2012) who suggested the storage of shelled grains with a moisture content of less than 11% to prevent fungal contamination and the production of mycotoxins.

The fungi isolated from pods and kernels during the different sampling times are showed in Table 1. With respect to freshly harvested peanuts, an elevated frequency of isolation of *Fusarium* spp. was observed, probably due to high A_w and competition with other fungi present in pods and kernels. The significance of *Fusarium* as a competitor of *A. flavus* has been reported by other authors (Rheeder, Marasas, & Van Wyk, 1990; Wicklow, Horn, Shotwell, Hesseltine, & Caldwell, 1988).

A decline in the frequency of isolation of *Fusarium* spp. was observed in peanut samples of the two cultivars after storage. This finding might be associated with the decrease of A_w in the samples as a result of peanut drying before storage. The lower A_w, in turn, may have favored an increase in the isolation of *Aspergillus* section *Flavi* and *Aspergillus* section *Nigri* strains. Similar results have been reported by Nakai et al. (2008), who observed a high frequency of isolation of *Fusarium* spp. from peanut samples, with a decrease in the frequency of this fungus during storage and a gradual increase in the frequency of *Aspergillus* spp.

During storage, pod and kernel samples of 886 had higher frequency of isolation of *Aspergillus* (mainly *Aspergillus* section *Flavi* and *Aspergillus* section *Nigri* isolates) when compared with the Caiapó. Spearman's correlation test between fungal presence variables in peanut pods showed a weak negative association between the presence of *A. flavus* and *Aspergillus* section *Nigri* strains for the Caiapó ($r = -0.389$ and $p = 0.009$), suggesting some antagonism between these fungi. However, no association between these fungi was observed for the 886 ($p = 0.362$). *Aspergillus* section *Nigri* species are considered an important competitor of *A. flavus* (Ashworth, Schroeder, & Langley, 1965; Hill, Blankenship, Cole, & Sanders, 1983), which have similar nutritional requirements for growth and conidia germination and are often isolated in the same habitat (Griffin & Garren, 1974; Jackson & Bell, 1969). In addition, the relative abundance of these two species is dependent on climatic and agronomic factors that are poorly understood (Paterson & Lima, 2010; Pitt & Hocking, 2009). In our research, the high frequency of isolation of *Aspergillus* section *Nigri* in the 4th collection may explain the low frequency of isolation of *A. flavus*. Other fungi commonly found in peanuts are *A. parasiticus*, *Fusarium* spp., *Rhizopus* spp. and *Penicillium* spp.

In samples of 886 negative correlations between *A. parasiticus* and *Fusarium* spp. (correlation $= -0.497$, $p = 0.005$) and between *A. parasiticus* and *Penicillium* spp. (correlation $= -0.409$, $p = 0.025$) were observed, suggesting a possible competition between these fungi. A significant correlation was observed between the presence of *Aspergillus* section *Nigri* strains and *Fusarium* spp. ($r = -0.378$ and $p = 0.047$) for the Caiapó and between the presence of *Fusarium* spp. and *Penicillium* spp. ($r = -0.438$ and $p = 0.016$) for the 886. *Fusarium* and *Penicillium* are less xerophilic fungi than *A. parasiticus* and this negative correlation may therefore be a natural response to environmental changes. Environmental conditions, such as temperature,

Table 1
Mycobiota contamination of kernels and pods of peanut cultivars Runner IAC Caiapó and 886 stored in the period April to September 2006.

Peanut samples	Collection	Runner IAC cultivar	Mycobiota									
			Fusarium spp.	*A. flavus*	*A. parasiticus*	*Aspergillus* section *Nigri*	*Penicillium* spp.	*A. terreus*	*Cladosporium* spp.	*Mucor* spp.	*Rhizopus* spp.	*Alternaria* spp.
			Fungal frequencies (%)									
Kernel samples	Freshly harvested	Caiapó	77.6	1.8	0	0	0	0	0	1.8	0	1.2
		886	7.3	3.6	0	0	1.8	0	0	0	0	0
	1st	Caiapó	1.5	12.1	0	6.1	4.5	0	0	6.1	0	0
		886	7.9	2.4	0	4.2	4.8	0	1.2	0	0	0
	2nd	Caiapó	0.6	6.1	0	3.6	14.5	4.2	0.6	0	0	0
		886	1.2	1.8	0	5.4	6.7	0	1.2	0	0	2.4
	3rd	Caiapó	8.5	6.1	0.6	1.8	6.7	10.9	0	0	0	0
		886	4.8	25.4	15.7	7.9	1.2	0	0	0	0	0
	4th	Caiapó	0	13.9	0	12.1	3.6	0	1.8	0	68.7	0
		886	0.6	30.3	0	7.3	6.7	0.6	0	0	6.7	0
	5th	Caiapó	0	11.5	0	0.6	2.5	4.2	0.6	0	0	0
		886	0	0	0	1.8	0	0	0	0	12.1	0
	6th	Caiapó	2.4	5.4	0	2.4	4.8	16.4	0	13.3	6.7	0
		886	7.3	5.5	1.2	9.7	4.8	0	0.6	0	13.3	0
Pod samples	Freshly harvested	Caiapó	96.2	0	0	0	0	0	0	0	0	7.5
		886	60.0	41.2	0	0	1.2	0	0	0	0	2.5
	1st	Caiapó	8.3	91.7	0	0	0	0	0	0	0	2.8
		886	43.7	72.5	0	52.5	18.7	0	1.2	8.7	10.0	0
	2nd	Caiapó	11.2	41.2	0	11.2	6.2	0	1.2	1.2	20.0	40.0
		886	8.7	42.5	0	23.0	22.5	0	1.2	2.5	15.0	0
	3rd	Caiapó	28.7	56.2	8.7	5.0	3.7	0	0	1.2	0	16.2
		886	0	35.0	41.2	20.0	0	0	0	1.2	0	1.2
	4th	Caiapó	12.2	67.5	0	16.2	0	0	1.2	1.2	33.7	22.5
		886	1.2	78.7	5.0	40.0	0	0	0	0	30.0	0
	5th	Caiapó	2.5	57.5	0	20.0	2.5	0	0	2.5	25.0	8.7
		886	0	38.7	1.2	32.5	0	0	0.6	0	50.0	1.2
	6th	Caiapó	11.2	18.7	0	18.0	11.2	21.2	1.2	7.5	30.0	0
		886	5.0	8.7	0	27.5	3.7	0	1.2	7.5	55.0	0

humidity and sunlight can affect the survival of pathogens, including mycotoxigenic fungi. Thermotolerant species are adapted to warmer climates that nonthermotolerant fungi, for example, *A. flavus* and *A. parasiticus* are more adapted to tropical areas than *Penicillium* and *Fusarium* (Paterson & Lima, 2010, 2011). In a previous study analyzing peanuts in the field, Zorzete et al. (2011) also observed a possible antagonism between *Fusarium* and *Penicillium* in kernels and pods of Runner cultivars. Furthermore, the larger the number of *A. flavus*, the greater was on average the presence of *A. parasiticus* ($r = 0.592$ and $p = 0.001$).

Descriptive statistical analysis indicated that *A. flavus* was predominant in both cultivars during the period of storage. In addition, *Alternaria* spp. and *Aspergillus terreus* were frequently isolated from the Caiapó, whereas a high frequency of *A. parasiticus* was observed in the 886. According to Paterson and Lima (2010), the mycobiota is influenced significantly by temperature, water availability and nutrient status. In the present study, factors that may explain the differences in mycobiota between cultivars were the chemical composition of kernels and the development cycle of the peanut plant. In the Caiapó, the oleic/linoleic acid ratio is higher (approximately 2) than in other cultivars (almost 1). This feature results in the lower oxidation of Caiapó grains compared to the 886 and prolongs shelf life. Moreover, the development cycle of Caiapó plants is longer, a fact that prolongs kernel dormancy and prevents budding, with a consequent reduction in infection with *A. flavus* and *A. parasiticus* (CONAB, 2007). The high frequency of *Rhizopus* spp. in the two cultivars is in accordance with the results reported by other investigators (Fernandez, Rosolem, Maringoni, & Oliveira, 1997; Nakai et al., 2008).

3.2. Fungi isolated from air

The following airborne fungal species were isolated inside the warehouse where the Caiapó and 886, respectively, were stored: *Cladosporium* spp. (306.0 and 300.1 CFU/m^3), *Aspergillus* section *Nigri* strains (24.9 and 23.3 CFU/m^3), *Fusarium* spp. (4.6 and 5.6 CFU/m^3), and *Rhizopus* spp. (1.7 and 2.5 CFU/m^3). *A. flavus* was only isolated from air in the warehouse where the 886 was stored (0.8 CFU/m^3). The detection of *Cladosporium* spp., and *Rhizopus* spp. in air samples agrees with others Brazilian studies on peanuts (Gonçalez et al., 2008; Zorzete et al., 2011), and corn (Almeida et al., 2002). In the present investigation, although both peanut cultivars and air samples were contaminated with *Aspergillus* section *Nigri* strains, statistical analysis showed no correlation.

3.3. A_w and abiotic factors

The decrease in average A_w in kernel samples from 0.77 to 0.51 (Caiapó) and from 0.62 to 0.45 (886) may explain the higher frequency of isolation of *A. flavus* and *A. parasiticus* at the beginning of storage. The decline in the frequency of *Fusarium* spp. during the storage period indicates the absence of adequate conditions for the growth of this fungus, including a low A_w (Tables 1, 2 and 3). The A_w observed in the present study was below the ideal value for the growth of *Aspergillus*. According to Lacey, Ramakrishina, Hamer, Magan, and Marfleet (1991), the ideal A_w for growth ranges from 0.78 to 0.80 for *A. flavus* and from 0.78 to 0.82 for *A. parasiticus* at a temperature range of 6 to 45 °C.

The highest mean monthly temperature (24.1 °C) and RH (82.2 mm) and lowest rainfall index (45.8 mm) were recorded in the region of the study (1st sampling). The frequency of isolation of *A. flavus* and *A. parasiticus* increased during the 3rd and 4th samplings presumably by temperature (mean monthly = 21.2 °C) and RH (mean monthly = 80.2 to 75.2); and low rainfall index (7.6 mm) observed only on the 3rd collection. According to the Codex Alimentarius Commission (1979, 2004), temperatures higher than 25 °C and relative humidity higher than 70% favor fungus growth, and a temperature of 0 and 10 °C and relative humidity of less than 70% are therefore recommended for warehouses.

The relative humidity was lower inside the warehouse than outside, with an average monthly humidity of 43.8 to 31.6% and 64.5 to 87.1%, respectively. The highest temperature measured inside the warehouse was observed in September (40.2 °C). Although the temperature was adequate for the growth of *A. flavus* and *A. parasiticus*, the A_w of the samples and RH measured inside the warehouse did not permit fungal growth. According to Paterson and Lima (2010, 2011), climate changes (temperature and rainfall) can influence host–pathogen dynamics.

Statistical analysis showed that the presence of *A. flavus* in kernel samples in the warehouse varied according to the presence of *A. flavus* in pods ($p = 0.0046$) and pluvial precipitation ($p = 0.0213$). Descriptive statistics indicated low variability in the A_w of pods ($p = 0.0463$) and kernels ($p = 0.0001$) in the same month of storage.

3.4. Occurrence of mycotoxins

3.4.1. Performance of analytical methods for the detection of AFs and CPA

The detection limits were 0.03 ng/mL for AFB_1, 0.03 ng/mL for AFB_2, 0.03 ng/mL for AFG_1, and 0.01 ng/mL for AFG_2. The quantification limits were 0.18 ng/mL for AFB_1, 0.15 ng/mL for AFB_2, 0.15 ng/mL for AFG_1, and 0.07 ng/mL for AFG_2 in kernels and pods determined in triplicate.

The following concentrations were used for construction of the calibration curves: 1.5, 3.1, 6.2, 12.4, and 24.8 ng/mL for AFB_1; 1.5, 2.9, 5.9, 11.8, and 23.6 ng/mL for AFB_2, 1.8, 3.6, 7.1, 14.2, and 28.5 ng/mL for AFG_1, and 1.5, 2.9, 5.9, 11.8, and 23.6 ng/mL for AFG_2. The coefficient of the calibration curves was $r^2 = 0.999$ for all AFs.

Recovery rates of AFs from kernels were 87% for AFB_1, 79% for AFB_2, 76% for AFG_1, and 81% for AFG_2; recovery rates from pods were 75% for AFB_1, 83% for AFB_2, 78% for AFG_1, and 73% for AFG_2.

The detection limits and quantification limits for CPA in kernels analyzed in triplicate were 80 and 156 μg/kg, respectively. Concentrations of 0.625, 1.25, 2.50, 5.00 and 10.00 μg/mL were used for construction of the calibration curve, with an r^2 of 0.998. Recovery from kernels was 72%.

3.4.2. Aflatoxins and CPA

Four (11.4%) of the 35 kernel samples of the 886 were contaminated with AFs, with mean levels ranging from 0.03 to 16.6 μg/kg. Aflatoxins were also detected in 3 (8.6%) of the 35 pod samples of this cultivar, with mean levels ranging from 1.3 to 3.3 μg/kg. In the Caiapó, 4 (11.4%) of the 35 kernel samples were contaminated with AFs, with mean levels ranging from 0.3 to 2.7 μg/kg, and 2 (5.7%) of the 35 pod samples, with mean levels ranging from 0.5 to 2.3 μg/kg (Tables 2 and 3). According to Ding, Li, Bai, and Zhou (2012), low AF contamination is found in peanuts after harvest, but AF levels might be higher during storage and processing. It is therefore, necessary to monitor the AF contamination status of peanuts during growth, storage, and processing. Moreover, high levels of contamination with AFs were detected in commercial peanut samples as a consequence of insufficient postharvest handling practices (Mutegi et al., 2013). Nakai et al. (2008), studying peanut samples collected in the Tupã region which were stored for 1 year, detected AFs in 33% of samples.

According to Rodrigues, Venâncio, and Lima (2012), aflatoxigenic isolates are able to persist or even grow in almonds, but may not produce AFs. Another factor that can influence the amount of AFs in samples is simultaneous infection with other fungi, such as *Aspergillus* section *Nigri*, *Rhizopus* spp., *Trichoderma*, and *Penicillium* spp. (Aziz & Shahin, 1997; European Union, 2006; Wicklow, Hesseltine, Shotwell, & Adams, 1980). Paterson and Lima (2011) showed that conditions such as heat and drought can help maintain the culture in a dry condition that is inadequate for mold growth and mycotoxin production.

Table 2
Frequency of *A. flavus*, *A. parasiticus* and *Aspergillus* section *Nigri*, abiotic factors (temperature, relative humidity, rainfall), water activity, levels of total aflatoxins and CPA, measured in the 886 cultivar.

Collection	Samples	Frequency (%)			Temperature (°C)[a]			Temperature (°C)[b]			RH (%)[a]	RH (%)[b]	Rainfall (mm)	A_w	Total aflatoxins (μg/kg)[c]	CPA (μg/kg)[c]
		A. flavus	*A. parasiticus*	*Aspergillus* section *Nigri*	Max	Min	Mean	Max	Min	Mean	Mean	Mean	Mean	Mean		
Freshly harvested	Kernels	3.6	0	0	nr	nr	nr	nr	nr	nr	nr	nr	nr	0.62	0.3/2	2606.0/2
	Pods	41.2	0	0										0.60	nd	na
1st (April)	Kernels	2.4	0	0	30.3	19.3	24.1	nr	nr	nr	82.3	nr	45.8	0.51	3.4/1	859.4/5
	Pods	72.5	0	52.5										0.52	nd	na
2nd (May)	Kernels	2.4	0	4.2	26.4	11.2	18.8	27.0	13.9	20.4	87.1	43.8	40.2	0.56	nd	288.0/4
	Pods	42.5	0	23.0										0.55	nd	na
3rd (June)	Kernels	25.4	15.7	5.4	28.7	13.8	21.2	25.2	13.2	19.2	80.2	39.6	7.6	0.51	nd	4918.1/4
	Pods	35.0	41.2	20.0										0.51	nd	na
4th (July)	Kernels	30.3	0	7.3	28.3	13.2	21.1	24.0	12.5	18.2	75.2	35.5	30.6	0.52	16.6/1	833.8/3
	Pods	78.7	5.0	40.0										0.49	nd	na
5th (August)	Kernels	0	0	0	30.9	15.0	22.9	29.2	15.3	22.2	64.5	34.9	2.5	0.45	nd	2141.0/5
	Pods	38.7	1.2	32.5										0.42	3.3/1	na
6th (September)	Kernels	5.5	1.2	5.5	29.2	14.3	21.7	27.9	14.0	21.0	68.1	31.6	71	0.53	nd	2491.3/3
	Pods	8.7	0	27.5										0.50	1.3/2	na

nr = not recorded; nd = not detected; na = not analyzed; RH = relative humidity.
[a] Plantation region.
[b] Inside the warehouse.
[c] Mean levels/no. of positive samples.

Cyclopiazonic acid was detected in a large number of kernel samples. The frequency of detection was 60% for the Caiapó, with mean levels ranging from 304.1 to 2583.7 μg/kg, and 74.3% for the 886, with levels ranging from 288.0 to 4918.1 μg/kg (Tables 2 and 3). Other studies investigating the production of CPA in peanuts also reported high rates of 89% (Blaney, Kelly, Tyler, & Connole, 1989), 93% (Horn, Greene, Sobolev, Dorner, & Powell, 1996), and 97% (Resnik et al., 1996).

Aflatoxins and CPA were also detected simultaneously in kernel samples (11.4% for Caiapó and 886). The co-occurrence of CPA and AFs has been reported by several investigators (Fernández-Pinto et al., 2001; Gonçalez et al., 2008; Heperkan, Somuncuoglu, Karbancioglu-Güler, & Mecik, 2012; Vaamonde, Patriarca, & Pinto, 2006). In addition, Smith et al. (1992) demonstrated possible synergistic and cumulative effects of the two mycotoxins.

In the 886, the level of detection of CPA and the frequency of isolation of *A. flavus* and *A. parasiticus* increased in the 3rd collection. The frequency of these aflatoxigenic fungi may have caused an increase in the level of AF detection in subsequent collections. The competition between *A. flavus* and *A. parasiticus* strains observed on the 3rd collection may have caused a decrease in the production of CPA, found in subsequent collections of grains (Table 2).

Analysis of kernel samples of the Caiapó showed a predominance of *A. flavus* strains in the early collection (1st and 2nd). The highest levels of AFs and CPA were detected in the 3rd collection when *A. parasiticus* strains were isolated for the first time. A similar frequency of *A. flavus* (13%) and *Aspergillus* section *Nigri* isolates (12.1%) was observed in the 4th collection. Competition between these fungi may explain the decrease in the level of CPA (1/5 positive samples) and the lack of detection of AFs in subsequent samplings (Table 3).

In pod samples of the 886, the high frequency of isolation of *A. flavus* and *A. parasiticus* in the 4th collection may explain the detection of AFs in the subsequent sampling (Table 2). In pod samples of Caiapó, the occurrence of AFs coincided with the highest frequency of isolation of *A. flavus* (1st and 4th collection) and *A. parasiticus* (4th collection) (Table 3). Cole (1986) suggested that even after peanut was infected

Table 3
Frequency of *A. flavus*, *A. parasiticus* and *Aspergillus* section *Nigri*, abiotic factors (temperature, relative humidity, rainfall), water activity, levels of aflatoxins B_1, B_2, G_1, G_2 and CPA, measured in the Caiapó cultivar.

Collection	Samples	Frequency (%)			Temperature (°C)[a]			Temperature (°C)[b]			RH (%)[a]	RH (%)[b]	Rainfall (mm)	A_w	Aflatoxins (μg/kg)[c]	CPA (μg/kg)[c]
		A. flavus	*A. parasiticus*	*Aspergillus* section *Nigri*	Max	Min	Mean	Max	Min	Mean	Mean	Mean	Mean	Mean		
Freshly harvested	Kernels	1.8	0	0	nr	nr	nr	nr	nr	nr	nr	nr	nr	0.77	1.8/1	nd
	Pods	0	0	0										0.65	nd	na
1st (April)	Kernels	12.1	0	6.1	30.3	19.3	24.1	nr	nr	nr	82.3	nr	45.8	0.45	nd	420.8/1
	Pods	91.7	0	0										0.49	0.5/1	na
2nd (May)	Kernels	6.1	0	3.6	26.4	11.2	18.8	27.0	13.9	20.4	87.1	43.8	40.2	0.57	nd	402.7/5
	Pods	41.2	0	11.2										0.56	nd	na
3rd (June)	Kernels	6.1	0.6	1.8	28.7	13.8	21.2	25.2	13.2	19.2	80.2	39.6	7.6	0.51	2.7/2	2583.7/4
	Pods	56.2	8.7	5.0										0.51	nd	na
4th (July)	Kernels	13.0	0	12.1	28.3	13.2	21.1	24.0	12.5	18.2	75.2	35.5	30.6	0.57	nd	20635/5
	Pods	67.5	0	16.2										0.53	2.3/1	na
5th (August)	Kernels	11.5	0	0.6	30.9	15.0	22.9	29.2	15.3	22.2	64.5	34.9	2.5	0.40	nd	394.1/1
	Pods	57.5	0	20.0										0.40	nd	na
6th (September)	Kernels	5.4	0	2.4	29.2	14.3	21.7	27.9	14.0	21.0	68.1	31.6	71	0.53	0.3/1	304.1/5
	Pods	18.7	0	18.0										0.50	nd	na

nr = not recorded; nd = not detected; na = not analyzed; RH = relative humidity.
[a] Plantation region.
[b] Inside the warehouse.
[c] Mean levels (μg/kg)/no. of positive samples.

with *A. flavus* and *A. parasiticus*, aflatoxin production does not occur in the kernel until the natural resistance mechanism of the plant breaks down as a result of environmental stress (water deficiency and elevated temperature).

3.4.3. Aspergillic acid production

All strains identified as *A. flavus* and *A. parasiticus* were confirmed by a bright orange color on the reverse side of the colony on AFPA. This orange color is due to the reaction of ferric citrate with aspergillic acid, forming a colored complex (Pitt et al., 1983). Similar results have been reported by other investigations (Baquião, Oliveira, Reis, Zorzete, & Corrêa, 2013; Doster, Cotty, & Michailides, 2009).

4. Conclusions

The presence of fungi can damage grains. Furthermore, if the strains were toxigenic, mycotoxin production is an imminent risk. In this study, despite the high frequency of isolation of *A. flavus* and *A. parasiticus*, good agricultural practices such as the control of grain moisture content (<11%) before and during storage may have prevented the production of aflatoxins. The large number of samples contaminated with CPA, and the simultaneous detection of AFs and CPA highlights the need to investigate factors related to control and co-occurrence of these toxins in peanuts.

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