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# Distribution of fungi and aflatoxins in a stored peanut variety

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**Abstract**

The objective of the present study was to evaluate the mycoflora and occurrence of aflatoxins in stored peanut samples (hulls and kernels) from Tupã, State of São Paulo, Brazil. The samples were analyzed monthly over a period of one year. The results showed a predominance of *Fusarium* spp. (67.7% in hulls and 25.8% in kernels) and *Aspergillus* spp. (10.3% in hulls and 21.8% in kernels), and the presence of five other genera. The growth of *Aspergillus flavus* was mainly influenced by temperature and relative humidity. Analysis of hulls showed that 6.7% of the samples were contaminated with $AFB_1$ (mean levels = 15–23.9 μg/kg) and $AFB_2$ (mean levels = 3.3–5.6 μg/kg); in kernels, 33.3% of the samples were contaminated with $AFB_1$ (mean levels = 7.0–116 μg/kg) and 28.3% were contaminated with $AFB_2$ (mean levels = 3.3–45.5 μg/kg). Analysis of the toxigenic potential revealed that 93.8% of the *A. flavus* strains isolated were producers of $AFB_1$ and $AFB_2$.


*Keywords:* Peanut; Mycoflora; *Aspergillus flavus*; Abiotic factors; Aflatoxins; Toxigenic potential

## 1. Introduction

Peanuts are used in the fabrication of sweets, candies and pastes and mainly as a raw material in oil production. About 60% of the world production of peanut kernels are destined to the extraction of oil, with peanut oil being the fifth most consumed type of oil (Santos, 2000). The peanut seed possesses a high nutritional and commercial value due to the presence of proteins, fatty acids, carbohydrates, and fibers, in addition to vitamins, calcium and phosphorus (Câmara, 1998). Today peanuts are cultivated on a large scale, with China, India and Africa being the greatest producers. Brazil also possesses excellent soil and climate conditions for the cultivation of peanuts and peanut production was estimated to be 267.8 thousand tons during the 2005/2006 harvests, with the State of São Paulo being the greatest producer (CONAB, 2006).

Contamination of peanuts with aflatoxins is one of the main factors that compromise the quality of the product. Aflatoxins are secondary metabolites mainly produced by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*, fungi frequently isolated from peanut seeds especially in tropical and subtropical regions during the storage period (Smith & Ross, 1991).

Chemically, aflatoxins possess a polycyclic structure derived from a coumarin nucleus attached to a bifuran system on one side and either to a pentenone (series B aflatoxins) or a six-membered lactone (series G aflatoxins) on the other side. Eighteen different compounds are currently known; however, aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ are the most common in nature (Oga, 1996) and are named according to the fluorescence they emit when exposed to ultraviolet light (B = blue and G = green). Aflatoxins have a low molecular

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weight, are poorly soluble in water, and highly soluble in moderately polar solvents such as chloroform, methanol and dimethylsulfoxide. Aflatoxins are stable at high temperatures, with the fusion point of $AFB_1$ being 269 °C, and are deactivated by autoclaving in the presence of ammonium and by treatment with hypochlorite (World Health Organization, 1979). $AFB_1$ is the most toxic form for mammals and presents hepatotoxic, teratogenic and mutagenic properties, causing damage such as toxic hepatitis, hemorrhage, edema, immunosuppression and hepatic carcinoma (Santos, Lopes, & Kosseki, 2001). It has been classified as a class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 1993).

Aflatoxins, especially $AFB_1$, directly affect the quality of peanuts and their derivatives used for animal and human food consumption. After the ingestion, these toxins are absorbed in the gastrointestinal tract, and are biotransformed in the liver by microsomal enzymes of cytochrome P 450 system, creating the active form of $AFB_1$ ($AFB_1$ epoxide), which is able to affect the metabolism of nucleic acids, as DNA and RNA, and protein synthesis. Covalent binding of aflatoxin results in a decrease in both DNA and RNA synthesis rates in the liver. The inhibition of protein synthesis is not as rapid or extensive as that of nucleic acids. Polysomal disaggregation parallels this inhibition and is likely to represent the mode of inhibition of protein synthesis (Roebuck & Maxuitenko, 1994).

In view of these facts, together with the lack of studies regarding fungal contamination and mycotoxins in stored peanuts, the objective of the present investigation was to evaluate the mycoflora and the occurrence of aflatoxins in stored peanut samples (hulls and kernels), to determine the toxigenic potential of *A. flavus* strains isolated during the study, and to correlate the results obtained with abiotic factors (water activity, temperature and relative humidity).

## 2. Materials and methods

### 2.1. Samples

The study was conducted in the Tupã region, State of São Paulo. After harvest, peanuts of the Runner IAC 886 variety were placed in 25 kg bags (a total of five bags), piled up on a wooden frame, and stored for a period of 12 months in a warehouse under good conditions of ventilation. Samples were removed monthly from different points of each bag until completing a sample of 1 kg (Fonseca, 1991). Five samples were collected each month, for a total of 60 samples. Each sample was analyzed for the presence of fungi and aflatoxins in both hulls and kernels.

### 2.2. Water activity

The water activity of hulls and kernels was determined with the AquaLab CX-2 apparatus (Decagon Devices, Inc., Pullman, Washington, USA).

### 2.3. Isolation, enumeration and identification of the mycoflora

Approximately 30 g was removed from each of the 60 samples and disinfected with 0.4% sodium hypochlorite solution for 2 min, followed by washing with sterile distilled water for elimination of external contaminants (Berjak, 1984). After disinfection, some hulls and kernels were randomly separated and directly seeded onto Petri dishes containing *A. flavus–parasiticus* agar (Pitt, Glenn, & Hocking, 1983). Four plates containing four hulls and three plates containing 11 kernels were used for each sample. The plates were incubated at 25 °C for 5 days and the results are expressed as the percentage of total hulls and kernels infected with fungi. The colonies were identified to the genus level and those belonging to the genus *Aspergillus* were identified to the species level according to Pitt and Hocking (1997).

### 2.4. Determination of aflatoxins

Aflatoxins $B_1$ and $B_2$ were determined according to the method described by Soares and Rodrígiez–Amaya (1989). Briefly, 50 g of each sample of hulls and kernels was extracted with 270 ml methanol and 30 ml 4% potassium chloride. Samples were blended at moderate speed for 30 min and filtered, and 150 ml of the filtrate was collected into a graduated cylinder. Next, 150 ml 10% copper sulfate and 50 ml diatomaceous earth were added, followed by moderate stirring and filtration. The filtrate was again recovered up to 150 ml and transferred to a separation funnel, and toxins were extracted three times with 10 ml chloroform. The chloroform extracts were collected into a beaker and submitted to solvent evaporation in a water bath at 60 °C. Extracts were resuspended in 500 μl chloroform and immediately submitted to thin-layer chromatography (TLC).

Final identification and quantification of aflatoxins were performed by one-dimensional thin-layer chromatography on precoated silica gel plates (Merck). The plates were developed in a saturated chamber with chloroform/acetone (9:1, v/v). Aflatoxin spots were observed under long-wave ultraviolet light ($\lambda = 366$ nm) and determined by visual comparison with $AFB_1$ and $AFB_2$ standards prepared. Confirmatory tests for aflatoxins were carried out using trifluoroacetic acid (Scott, 1990). The detection limit of the method was 2 μg/kg, and the recovery rates of $AFB_1$ and $AFB_2$ were 87% and 86% for hulls and 92% and 96% for kernels, respectively.

### 2.5. Toxigenic potential of A. flavus strains

Each strain was initially maintained in a tube containing potato dextrose agar at 25 °C. A fragment of each colony was then inoculated into a plate containing coconut agar and incubated at 25 °C for 10 days. After growth, the whole content of each plate was removed and chloroform

(30 ml for each 10 g of culture) was added. After shaking, the sample was filtered through Whatman No. 1 filter paper with diatomaceous earth and sodium sulfate. The content was evaporated and the extracts were resuspended in chloroform and then submitted to thin-layer chromatography (Lin & Dianese, 1976). Confirmation and quantification of aflatoxins were done according to Scott (1990).

### 2.6. Climatic data

During the storage period, climatic data such as temperature (°C) and relative air humidity (%) were recorded monthly in the region of the experiment.

### 2.7. Statistical analysis

The data were analyzed with the SAS (statistical analysis software) program using analysis of variance (ANOVA) and linear regression models to determine the influence of storage time on the frequency of isolation of *A. flavus*. Regression analysis was also used to select the most representative explanatory variables (abiotic factors) that influenced the response variables (frequency of fungal isolation and aflatoxin production) (Neter, Wasserman, & Kutner, 1990).

## 3. Results and discussion

The results regarding the monitoring of the mycoflora in peanut samples (hulls and kernels) collected over 12 months of storage are shown in Table 1. Fungi were detected in all peanut samples analyzed. During monitoring, *Fusarium*, *Aspergillus*, nonsporulating fungi, *Rhizopus*, *Penicillium*, *Drechslera* were the genera commonly isolated from both substrates; but *Trichoderma* spp. was only detected in kernels. The isolation of *Fusarium* spp., nonsporulating fungi, *Rhizopus* spp. and *Drechslera* spp. was higher in hulls, because of the contact of this substrate with soil. The frequencies of *Aspergillus* spp. and *Penicillium* spp., on the other hand, were higher in kernels, because of the great adaptation of this fungus to this substrate, especially during storage (Rossetto, Silva, & Araújo, 2005). The isolation of *Aspergillus* spp., *Penicillium* spp. and *Rhizopus* spp. agrees with the findings of other investigators studying peanut kernels from Brazil (Fernandez, Rosolem, Maringoni, & Oliveira, 1997; Rossetto et al., 2005) and India (Bhattacharya & Raha, 2002). Particularly interesting were the high frequency of isolation of *Fusarium* spp. in the samples, the trend toward a decrease in the frequency of the fungus during storage, and the gradual increase in the frequency of *Aspergillus* spp. Since peanuts are groundnuts, their hulls are directly exposed to contamination with fungi present in soil, especially *Fusarium* spp. which is a field fungus but may survive several months in stored grains (Riley, Norred, & Bacon, 1993). On the other hand, the increase in the frequency of *Aspergillus* spp. can be explained by the fact that this fungus and *Penicillium* spp. are considered to be storage fungi. Among representatives of the genus *Aspergillus*, only *A. flavus* (10.3%) was isolated from hulls. In kernels, in addition to *A. flavus* (21.2%), *Aspergillus niger* was isolated but at a low frequency (0.6%). *A. parasiticus*, another important aflatoxin-producing species, was not isolated in the present experiment.

The mean water activities observed in the present study (0.38–0.65 in hulls and 0.44–0.65 in kernels) were below the minimum range of 0.78–0.80 established for the growth of *A. flavus* (Lacey, Ramakrishna, Hamer, Magan, &

Table 1
Relative frequency of fungi isolated from 120 peanut samples (60 hull and 60 kernel samples) over 12 months of storage (June 2005–May 2006)

| Sampling | Isolated fungi (%) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Aspergillus* spp. | | *Fusarium* spp. | | *Penicillium* spp. | | *Rhizopus* spp. | | *Drechslera* spp. | | *Trichoderma* spp. | | NSF[e] | |
| | *H*[b] | *K*[d] | *H* | *K* | *H* | *K* | *H* | *K* | *H* | *K* | *H* | *K* | *H* | *K* |
| S1[a] | ND[c] | 21.2 | 97.5 | 43.6 | ND | 1.8 | ND | 0.6 | ND | ND | ND | ND | 2.5 | 1.8 |
| S2 | ND | 3.0 | 95.0 | 53.3 | ND | 4.8 | 2.5 | 6.6 | ND | ND | ND | ND | 2.5 | 10.9 |
| S3 | 1.25 | 13.3 | 86.25 | 30.9 | ND | ND | ND | 17.0 | ND | ND | ND | ND | 12.5 | 3.0 |
| S4 | 2.5 | 13.9 | 82.5 | 35.2 | ND | 0.6 | 7.5 | 4.8 | ND | ND | ND | ND | 7.5 | 5.5 |
| S5 | 1.25 | 16.4 | 61.25 | 20.6 | ND | 1.2 | 5.0 | 10.3 | ND | ND | ND | 9.1 | 31.25 | 3.6 |
| S6 | 7.5 | 29.7 | 83.75 | 21.2 | 1.25 | 1.2 | 1.25 | 3.6 | ND | ND | ND | ND | 6.25 | 3.0 |
| S7 | 20.0 | 31.5 | 65.0 | 12.1 | ND | 1.2 | ND | ND | ND | ND | ND | ND | 15.0 | 10.9 |
| S8 | 13.75 | 27.3 | 55.0 | 24.2 | ND | 1.8 | 1.25 | 1.8 | ND | ND | ND | ND | 8.75 | ND |
| S9 | 13.75 | 24.8 | 53.75 | 20.0 | ND | 0.6 | 13.75 | 1.2 | ND | ND | ND | ND | 17.5 | 5.5 |
| S10 | 21.25 | 31.5 | 45.0 | 19.4 | ND | 0.6 | 11.25 | 4.2 | 2.5 | ND | ND | ND | 16.25 | 3.6 |
| S11 | 28.75 | 21.2 | 42.5 | 22.4 | 1.25 | 3.6 | 18.75 | 1.8 | 3.75 | 0.6 | ND | 1.2 | 5.0 | 1.2 |
| S12 | 13.75 | 27.9 | 45.0 | 6.1 | 1.25 | 1.2 | 18.75 | 5.5 | 1.25 | ND | ND | ND | 17.5 | ND |
| Total | 10.3 | 21.8 | 67.7 | 25.8 | 0.3 | 1.6 | 6.7 | 4.8 | 0.6 | 0.05 | | 0.9 | 11.9 | 4.1 |

[a] Stored peanut samples.
[b] Hulls.
[c] Not detected.
[d] Kernels.
[e] Nonsporulating fungi.

Marfleet, 1991). The low water activity levels detected in the stored samples are probably due to the previously used process of peanut drying. In the present experiment, the mean temperature ranged from 19.40 to 27.03 °C and the relative humidity ranged from 58.03% to 87.10%. Thus, the temperature was below 32–33 °C considered to be the optimum temperature for the growth of *A. flavus* by Hocking (1997). Regarding relative humidity, excepting the last month of storage (S12), values were lower than those observed by Christensen, Mirocha, and Meronuck (1977) who reported that a relative humidity of about 83–85% favors the growth of *A. flavus* (Table 2).

Statistical analysis of variance of the present results indicated a positive correlation ($p < 0.05$) between storage period and the prevalence of *A. flavus* in both hulls ($p = 0.0002$) and kernels ($p = 0.0068$).

The equation *A. flavus* $= -16.482 + 6.554A_w + 0.318$ temperature $+ 0.13$ relative humidity ($r = 0.575$), presented by the analysis of linear regression, indicated that water activity, temperature and relative humidity were the variables that most influenced contamination with *A. flavus* in hulls. With respect to kernels, a significant positive correlation between temperature, relative humidity and isolation of *A. flavus* was observed in the equation *A. flavus* $= -27.01 + 0.854$ temperature $+ 0.12$ relative humidity ($r = 0.449$).

Table 2 shows the mean levels of $AFB_1$ and $AFB_2$ detected in the 120 samples analyzed (hulls and kernels). Four (6.7%) of the 60 hull samples were contaminated with $AFB_1$ and $AFB_2$ at mean levels ranging from 15.0 to 23.9 μg/kg and from 3.3 to 5.6 μg/kg, respectively. Of the 60 kernel samples, 20 (33.3%) were contaminated with

Table 2
Relative frequency of *Aspergillus flavus* and mean aflatoxin $B_1$ and $B_2$ levels in the 120 peanut samples (60 hull and 60 kernel samples) analyzed over 12 months of storage (June 2005–May 2006), and mean values of the abiotic factors (water activity, temperature and relative humidity) recorded during the experiment

| Sampling | $A_w$ | | Temperature (°C) | Humidity (%) | *A. flavus* (%) | | $AFB_1$ (μg/kg) mean (positive) | | $AFB_2$ (μg/kg) mean (positive) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *H*[b] | *K*[c] | | | *H* | *K* | *H* | *K* | *H* | *K* |
| S1[a] | 0.43 | 0.46 | 22.00 | 71.93 | ND[d] | 19.4 | ND | 116.15 (2) | ND | 27.9 (2) |
| S2 | 0.42 | 0.44 | 19.40 | 69.94 | ND | 3.0 | ND | 95.65 (1) | ND | 45.5 (1) |
| S3 | 0.65 | 0.63 | 23.42 | 59.45 | 1.3 | 13.3 | 15.0 (1) | 34.2 (1) | 3.3 (1) | 5.6 (1) |
| S4 | 0.61 | 0.63 | 21.45 | 68.00 | 2.5 | 14.0 | ND | 68.33 (1) | ND | ND |
| S5 | 0.38 | 0.50 | 25.87 | 64.81 | 1.3 | 16.4 | 23.92 (1) | 68.33 (3) | 3.3 (1) | 28.3 (2) |
| S6 | 0.55 | 0.63 | 26.26 | 58.03 | 7.5 | 29.7 | 23.92 (1) | 59.8 (2) | 5.6 (1) | 4.45 (2) |
| S7 | 0.56 | 0.55 | 25.53 | 60.45 | 20.0 | 31.0 | ND | 69.7 (1) | ND | 3.3 (1) |
| S8 | 0.62 | 0.65 | 27.03 | 63.90 | 15.0 | 27.3 | ND | ND | ND | ND |
| S9 | 0.38 | 0.59 | 26.08 | 72.36 | 13.8 | 24.2 | ND | 18.4 (3) | ND | 3.3 (3) |
| S10 | 0.47 | 0.63 | 26.27 | 83.00 | 21.25 | 29.7 | 15.0 (1) | 21.0 (2) | 5.0 (1) | 3.4 (2) |
| S11 | 0.55 | 0.62 | 24.03 | 82.27 | 28.75 | 20.0 | ND | 7.0 (1) | ND | ND |
| S12 | 0.53 | 0.62 | 20.00 | 87.10 | 13.75 | 26.7 | ND | 9.7 (3) | ND | 22.7 (3) |

[a] Stored peanut samples.
[b] Hulls.
[c] Kernels.
[d] Not detected.

Table 3
Mean levels of aflatoxin $B_1$ ($AFB_1$) and $B_2$ ($AFB_2$) production by 81 *Aspergillus flavus* strains isolated during the period between June 2005 and May 2006

| Sampling | $AFB_1$ (μg/kg) mean (producer strains/strains analyzed) | | $AFB_2$ (μg/kg) mean (producer strains/strains analyzed) | |
|---|---|---|---|---|
| | *H*[b] | *K*[d] | *H* | *K* |
| S1[a] | ND[c] | 21,509 (5/5) | ND | 957 (5/5) |
| S2 | ND | 171 (1/1) | ND | 5.6 (1/1) |
| S3 | 27,888 (1/1) | 16,747 (5/5) | 1138 (1/1) | 338 (5/5) |
| S4 | 20,916 (2/2) | 13,481 (5/5) | 193 (2/2) | 525 (5/5) |
| S5 | 27,888 (1/1) | 19,528 (5/5) | 2275 (1/1) | 353 (5/5) |
| S6 | 8837 (3/3) | 23,519 (5/5) | 82.4 (3/3) | 331 (5/5) |
| S7 | 31,013 (3/3) | 13,465 (4/5) | 373 (3/3) | 604 (4/5) |
| S8 | ND | 18,944 (4/5) | ND | 599 (4/5) |
| S9 | 4898 (2/3) | 20,544 (5/5) | 20.3 (2/3) | 5764 (5/5) |
| S10 | 217,534 (5/5) | 8748 (5/5) | 190 (5/5) | 113 (5/5) |
| S11 | 6275 (4/4) | 24,419 (4/5) | 150 (4/4) | 199 (4/5) |
| S12 | 19,754 (2/3) | 7379 (5/5) | 187 (2/3) | 115 (5/5) |

[a] Stored peanut samples.
[b] Hulls.
[c] Not detected.
[d] Kernels.

$AFB_1$ at mean levels ranging from 7.0 to 116 μg/kg and 17 (28.3%) were contaminated with $AFB_2$ at levels ranging from 3.3 to 45.5 μg/kg. Seventeen (70.8%) of the 24 samples contaminated with aflatoxins exceeded the maximum limit of 20 μg/kg permitted for peanuts by the Brazilian Ministry of Health for the sum of aflatoxins $B_1 + B_2 + G_1 + G_2$ (Brasil, 2002).

Studies conducted in Brazil analyzing the occurrence of aflatoxins in peanuts and derived products generally reported higher contamination levels than those observed in the present investigation (Caldas, Silva, & Oliveira, 2002; Fonseca, Valarini, Calori-Domingues, Wettstein, & Silva, 1991; Sabino et al., 1999). Statistical analysis revealed the absence of significant correlations between the presence of $AFB_1$ and $AFB_2$ in hulls and kernels, storage time and abiotic factors.

Analysis of the toxigenic potential of the 81 *A. flavus* strains isolated from the peanut samples revealed the production of aflatoxins by 76 strains (93.8%), with mean levels ranging from 4898 to 31,013 μg/kg (hulls) and from 171 to 24,419 μg/kg (kernels) for $AFB_1$, and from 20.3 to 2275 μg/kg (hulls) and from 5.6 to 957 μg/kg (kernels) for $AFB_2$ (Table 3). In Brazil, a previous study conducted by Fonseca, Martinelli Filho, Nery, and Roncatto (1974) has shown that 32 (30%) of the *A. flavus* strains analyzed were producers of aflatoxins. Pildain, Vaamonde, and Cabral (2004), analyzing samples of peanut kernels from Formosa, Argentina, also observed high positivity rates, with 75% of the 40 *A. flavus* strains producing aflatoxins. The finding that *A. flavus* produced both group B aflatoxins ($AFB_1$ and $AFB_2$) has also been reported by Pier (1990) who observed that 90% of the strains produced both $AFB_1$ and $AFB_2$.

## 4. Conclusions

The present study demonstrated the susceptibility of peanuts to colonization with *A. flavus*, especially during storage. In addition, the results showed that storage period, water activity of the product and climatic factors (temperature and relative humidity) were important for the growth of the fungus and, consequently, presented a potential risk for aflatoxin production. The high percentage of aflatoxigenic strains also indicates the need of good storage practices in order to prevent the occurrence of aflatoxins in peanuts.

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