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Diversity and association of filamentous fungi in coffee beans under organic and conventional cultivation

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Brazil is a country with great biodiversity; however, knowledge of this microbiological wealth is currently insufficient for its utilization in a sustainable manner. Agricultural expansion represents one of the largest current dangers to biodiversity and threatens to cause the extinction of a variety of species. This study therefore aimed to isolate and identify the species of fungi present in coffee beans cultivated in organic and conventional systems. Eighteen (18) samples of coffee beans from southern Minas Gerais were analyzed, and 346 fungal isolates were obtained from the analyzed coffee beans. These isolates belonged to 32 species in the following 14 genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Mucor*, *Rhizopus*, *Trichoderma*, *Epicoccum*, *Phoma*, *Bipolaris*, *Glomerella*, *Colletotrichum*, *Alternaria* and *Gliocladium*. Organic coffee bean samples exhibited the highest indices of fungal diversity. Two species identified in this study, *Aspergillus flavus* and *Aspergillus ochraceus*, are extremely important for their toxigenic characteristics. We utilized simple correspondence analysis to evaluate the interaction of the identified fungi with the toxigenic species. An association of toxigenic fungi with other fungi is important because some microorganisms can degrade mycotoxins. In the organic coffee beans, *A. flavus* was associated with *Cladosporium cladosporioides*, *A. ochraceus*, and *Penicillium brevicompactum*. In contrast, in the conventional coffee beans, *A. ochraceus* was only associated with *C. cladosporioides*. These results demonstrate that greater fungal diversity exists in organic coffee beans.

Key words: Interactions, microorganisms, mycotoxins, *Aspergillus.*

INTRODUCTION

Fungi exhibit greater species richness than most other organisms and, thus, are of significant environmental and economic importance (Varga et al., 2011; Blackwell, 2011). Recent predictions based on molecular methods

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have suggested that there are 5.1 million fungal species (O'Brien et al., 2005); however, only approximately 5% of the predicted filamentous fungal species have been described (Hawksworth, 1991).

The biodiversity of Brazil is among the greatest in the world (Agostinho et al., 2005), and studies to estimate the risk of mycotoxins, biological control, or fungal diversity in agricultural systems are therefore critical for the utilization of these microorganisms in biotechnological processes. However, global warming and the need to expand agriculture significantly threaten microbial diversity (Hole et al., 2005). Pesticides that are intensively used in conventional cultivation can lead to a diminished ecosystem, reducing biodiversity while allowing for pathogen increase (Lima and Vianello, 2011).

In agricultural products, such as coffee, interactions between different fungal species represent a natural phenomenon that affects the development of these microorganisms and their subsequent production of mycotoxins. Several studies have been undertaken to assess the growth of mycotoxigenic fungi and mycotoxin degradation (Noonim et al., 2008; Batista et al., 2009, Abrunhosa et al., 2010). These interactions are influenced by environmental conditions, microorganism diversity (Logrieco et al., 2007), agricultural products (Perrone et al., 2007), the type of processing (Batista et al., 2003), and the cultivation system used (Schneider et al., 2010). Interactions between mycotoxigenic and nontoxic species can lead to the appearance of diseases, the production of different types of mycotoxins, or their degradation. The results of these interactions are influenced by environmental conditions (Logrieco et al., 2007).

Ochratoxin A (OTA) has been detected in various agricultural products, including coffee (Clouvel et al., 2008; Batista et al., 2009; Duarte et al., 2010). According to Petzinger and Weidenbach (2002), coffee drinks significantly contribute to the ingestion of OTA in the human diet. OTA is primarily produced by *Aspergillus* section *Circumdati* (*A. westerdijkiae* and *A. ochraceus*) and section *Nigri* (*A. carbonarius* and *A. niger*) species (Batista et al., 2009; Gil- Serna et al., 2011).

In Brazil, conventional farming is dependent on the use of herbicides, pesticides, and inorganic nutrient applications, whichare less beneficial to the environment than organic methods (Bettiol, et al., 2002).In contrast, organic coffee is produced without the use of pesticides and soluble fertilizers, which are replaced by recycling organic by-products such as animal manure, biofer-tilizers, pulp and coffee husk compounds, and earthworm castings (Theodoro and Guimarães, 2003). For the production of coffee to be considered organic, the crop must be free from the use of pesticides and chemical fertilizers for at least three years (Bakutis et al., 2006).

The objectives of this study were therefore to evaluate the diversity of filamentous fungi in coffee beans cultivated in organic and conventional systems and to assess the ecological interactions between species of fungi that produce toxins and those that do not.

MATERIALS AND METHODS

Samples

Samples of stored coffee beans (500g per sample) were obtained from acooperative in southern Minas Gerais. The samples consisted of both conventional and organic coffee beans (*Coffea arabica* L.), which were harvested from three districts in Southern Minas Gerais. Two types of coffee were analyzed: coffee harvested onto cloth and coffee swept from the ground. The harvested fractions were as follows: organic coffee (two samples) from a district at latitude 20° 56' and longitude 44° 55', with a mean annual temperature of 20°C, a mean annual rainfall of 1597.6 mm, and an altitude of 1013 m (IBGE, 2013); organic coffee (eight samples) and conventional coffee (seven samples) from a second district located at latitude 21° 46' and longitude 45° 57', with a mean annual temperature of 20°C, a mean annual rainfall of 1592.7 mm and an altitude of 836 m (IBGE, 2013); and conventional coffee (one sample) from a district located at latitude 21° 42' and longitude 46° 14', with a mean annual temperature of 18.2°C, a mean annual rainfall of 1605 mm, and an altitude of 1076 m (IBGE, 2013). These 18 samples were sent to the Laboratory for Food Mycotoxins and Mycology of the Department of Food Science of the Federal University of Lavras in Minas Gerais for analysis.

Isolation of filamentous fungi

The fungi associated with green coffee beans were isolated by direct plating on Dichloran Rose Bengal Chloramphenicol HiMedia medium (10 g of glucose; 5 g of bacteriological peptone; 1 g of KH_2PO_4; 0.5 g of $MgSO_4 \cdot 7H_2O$; 15 g of agar; 1 L of distilled water; 25 mg of Rose Bengal and 2 mg of dichloran) as described by Samson et al. (2000). The isolates were selected according to morphological differences in the color and appearance of their colonies.

From each sample, 200 beans were randomly collected; 100 beans were plated following surface disinfection, and 100 beans were plated without surface disinfection. For disinfection, the samples were immersed in a solution of 70% alcohol followed by a solution of 1% sodium hypochlorite for 30 s. The samples were then washed with distilled water. After seven days of culture, the cultures were purified using malt agar (MA) medium.

Identification of filamentous fungi

Samples of the fungal species were removed from the pure cultures and cultured in specific media. Standard identification manuals were used for each genus. *Aspergillus* and *Penicillium* isolates were cultured in Czapek yeast agar (CYA: 1 g of K_2HPO_4; 10 mL of Czapek concentrate; 5 g of fungal extract; 15 g of agar and 1 L of distilled water), Czapek concentrate (30 g of $NaNO_3$; 5 g of KCl; 5 g of $MgSO_4 \cdot 7H_2O$; 0.1 g of $FeSO_4 \cdot 7H_2O$; 0.1 g of $ZnSO_4 \cdot 7H_2O$; 0.05 g of $CuSO_4 \cdot 5H_2O$ and 100 mL of distilled water) and MEA (20 g of malt extract; 1 g of peptone; 30 g of glucose; 20 g of agar and 1 L of distilled water) at 25 and 37°C, respectively. After seven days of incubation, macroscopic and microscopic characteristics were observed. *Aspergillus* isolates were identified according to Klich (2002), and *Penicillium* species were identified according to Pitt (2000), as these identification processes have been supported by Pitt and Hocking (1997) and Samson et al. (2000). Synthetic nutrient-poor agar medium (SNA: 1 g of KH_2PO_4; 1 g of KNO_3; 1 g

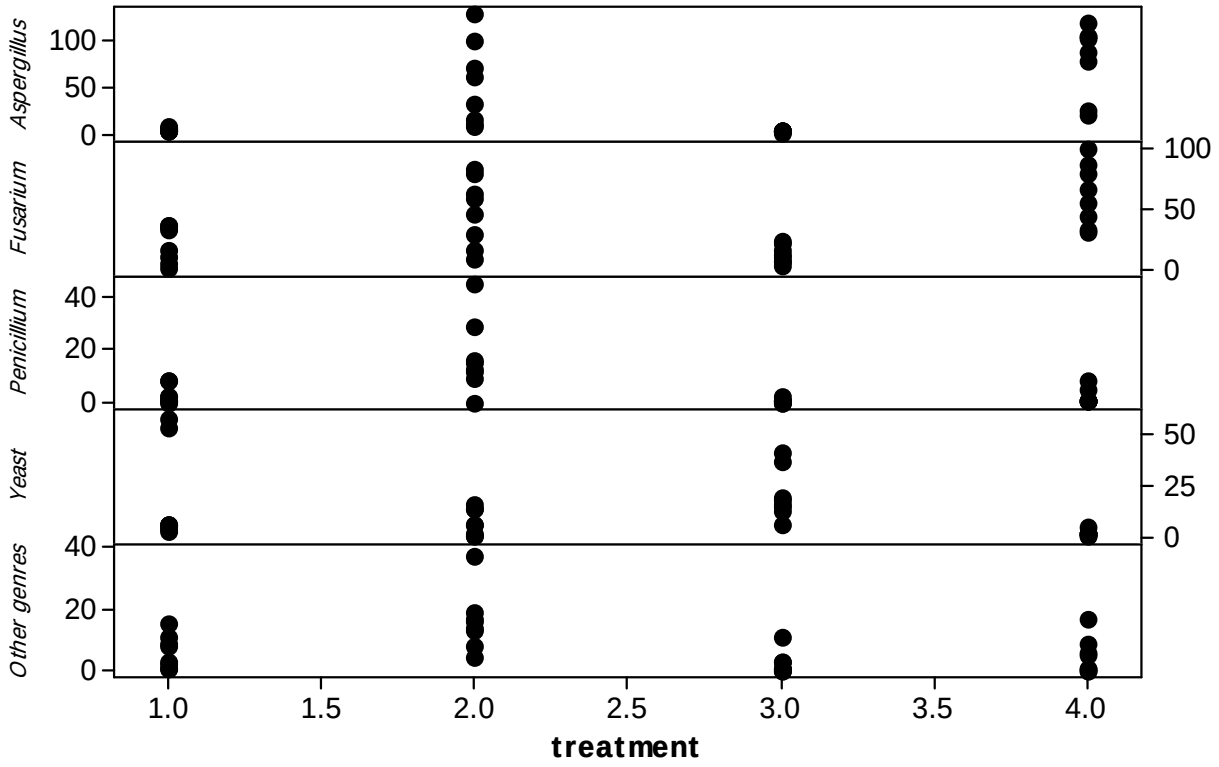

Figure 1. 1) Organic coffee with surface disinfection; 2) organic coffee without surface disinfection; 3) conventional coffee with surface disinfection; and 4) conventional coffee without surface disinfection.

of $MgSO_4 \cdot 7H_2O$; 0.5 g of KCl; 0.2 g of glucose; 0.2 g of saccharose;20 g of agar and 1 L of distilled water) was used for isolates of the *Fusarium* genus, and MA (malt extract) was used for the analysis of microscopic characteristics. Potato dextrose agar (PDA) was used to observe the colony color. The isolates were maintained in a photoperiod for 10 days at 21°C. Isolates belonging to the genera *Mucor*, *Rhizopus*, *Cladosporium* and *Trichoderma* were cultured in malt extract (MEA) at 25°C for seven days, and isolate identification was accomplished according to Samson et al. (2000). *Colletotrichum*, *Glomerella*, *Bipolaris* and *Epicoccum* were cultured in MA, whereas *Phoma* and *Alternaria* were cultured in OA medium (oatmeal agar: 30 g of oats, 1 L of distilled water and 15 g of agar) at 25°C for seven days. Fungal identification was performed as previously described by Ellis (1971).

Statistical analyses

The statistical methodology used in this study involved the construction of a 95% confidence interval for the standard deviation, aiming to infer the dispersion of the occurrence of filamentous fungi in organic and conventional coffee samples. To identify the relationship between toxigenic and nontoxigenic fungi through grouping, a simple correspondence analysis was utilized according to the methodology described by Hair et al. (1998).

Biodiversity indices

Margalef's richness index (Rm) was used to determine the diversity and richness of the filamentous fungalspecies isolated from organic and conventional coffee beans. In this index, $Rm = (S - 1)/(\ln n)$; where, S is the number of species and n is the number of identified individuals. The Shannon-Weiner diversity index (H'), $H' = -\Sigma (pi \ln pi)$, was also used, as described by Magurran (1988); where, pi is the proportion of individuals in each species with respect to the total number of individuals.

RESULTS AND DISCUSSION

Coffee sample contamination

Filamentous fungi were detected in all coffee bean samples from both the conventional and the organic cultivation systems. The highest contamination indices were found in the samples that were not disinfected with 1% sodium hypochlorite.

Aspergillus, *Fusarium* and *Penicillium* (Figure 1) represent the major identified fungal genera and are primarily responsible for mycotoxin production in agricultural products (Cast, 2003). These genera were also identified in coffee bean samples by Batista et al. (2009), Batista and Chaulfoun (2007), and Rezende et al. (2013). The presence of these genera in coffee is of concern given their potential production of mycotoxins, such as aflatoxin B1, aflatoxin B2 and OTA (Batista et al. 2003; Amézqueta et al., 2009).

Organic and conventional coffee bean samples that were not disinfected with 1% sodium hypochlorite were

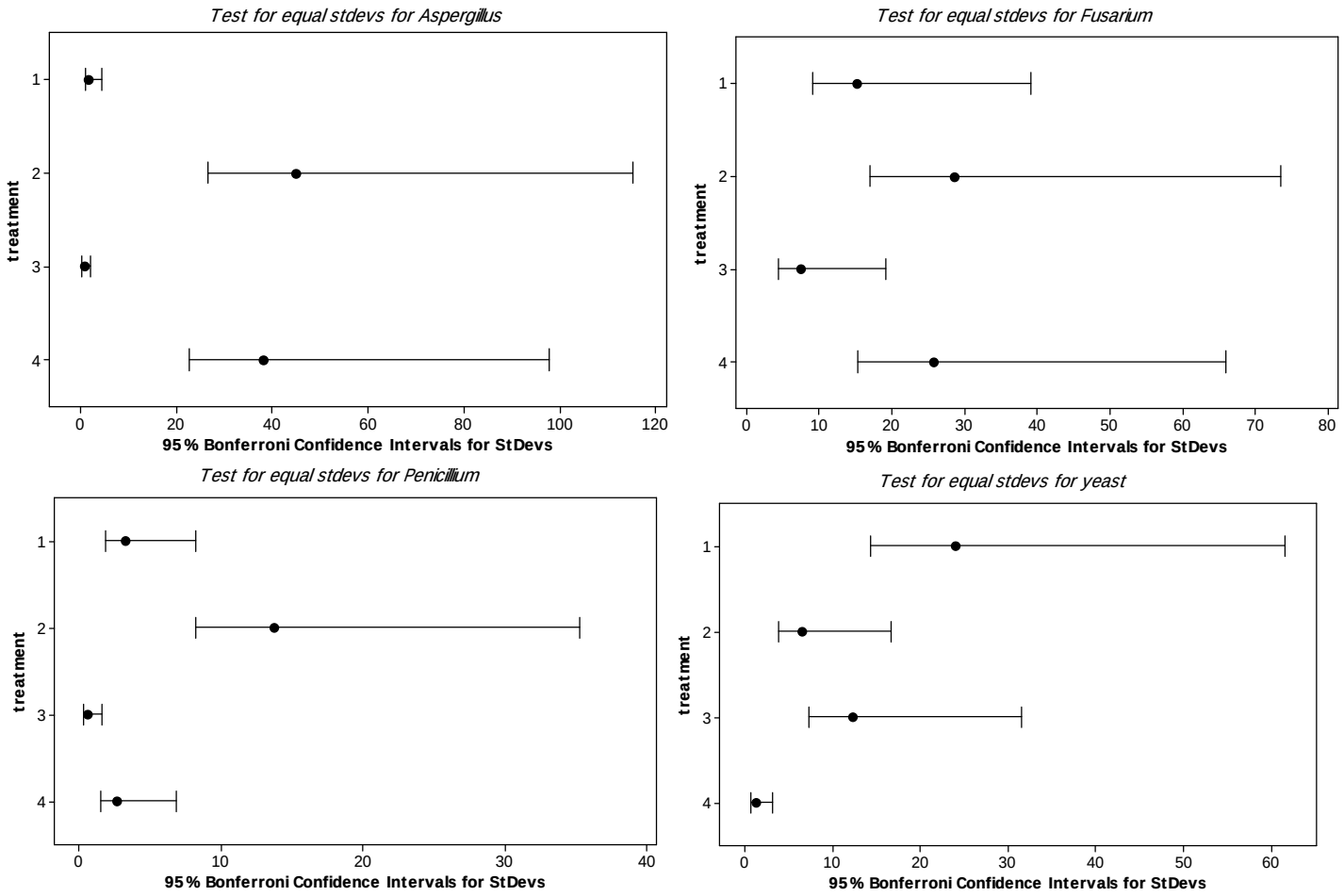

Figure 2. The frequency of filamentous fungi in processed coffee beans cultivated under organic and conventional systems, with and without surface disinfection. Treatment 1: organic coffee with surface disinfection. Treatment 2: organic coffee without surface disinfection. Treatment 3: conventional coffee with surface disinfection. Treatment 4: conventional coffee without surface disinfection.

found to exhibit a higher frequency of filamentous fungi (Figure 1). The analysis of coffee beans with and without surface disinfection accounts for fungal spores present inside and outside of the beans, respectively. In Figure 1, the vertical axis represents the fungal counts (in arbitrary units according to the data distribution) for each treatment. The distribution of the fungal counts for each treatment indicates the scatter between observations; a shorter distance between the fungal counts indicates a greater homogeneity between the scores of each fungus for each treatment.

Based on the fact that the variability within the samples cannot be directly observed (Figure 1), the homogeneity of the coffee bean samples was analyzed (Figure 2). A high diversity of *Aspergillus*, *Fusarium* and *Penicillium* was identified in the coffee bean samples with and without disinfection (Figures 2A, 2B, and 2C). Organic coffee samples exhibited a larger species variability,which was indicated by a higher contamination index for filamentous fungi.

The presence of the fungal genus *Aspergillus* in coffee is not desirable because the species *A. ochraceus*, *Aspergillus sclerotiorum*, *Aspergillus sulphureus*, *Aspergillus steynii*, and *Aspergillus westerdijkiae* have been associated with OTA production in coffee. The presence of the *Fusarium* genus was confirmed in coffee samples that had not been disinfected. Similar results were also reported by Pasin et al. (2009), who observed that the *Fusarium* genus was found with high incidence on the external portion of the beans under different coffee cultivation conditions.

Identification of filamentous fungi

Three hundred and forty-six (346) fungal isolates were obtained from all analyzed coffee beans, with 32 species belonging to the following 14 genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Mucor*, *Rhizopus*, *Trichoderma*, *Epicoccum*, *Phoma*, *Bipolaris*, *Glomerella*,

Table 1. Number of isolates of filamentous fungi present in coffee beans from organic and conventional farming

Species	Conventional				Organic			
	Cloth*		Sweeping**		Cloth*		Sweeping**	
	With disinfection	Without disinfection	With disinfection	Without disinfection	With disinfection	Without disinfection	With disinfection	Without disinfection
Alternaria alternata	6	-	-	-	-	-	-	-
Aspergillus flavus	-	-	-	-	7	6	4	2
Aspergillus foetidus	-	-	-	-	-	9	-	3
Aspergillus niger	3	-	-	-	-	-	-	-
Aspergillus ochraceus	-	-	-	-	5	14	-	4
Aspergillus oryzae	-	-	-	-	-	-	-	2
Aspergillus sulphureus	-	-	-	-	2	-	-	-
Aspergillus tubingensis	-	5	-	-	-	-	-	-
Aspergillus versicolor	-	-	-	-	-	3	-	-
Cladosporium cladosporioides	4	-	-	3	5	5	-	-
Colletotrichum gloeosporioides	-	-	-	-	3	4	-	-
Epicoccum purpurascens	6	-	-	-	-	-	-	-
Fusarium oxysporum	1	-	-	6	-	-	-	2
Fusarium semitectum	-	5	-	8	-	-	-	8
Fusarium solani		6						9
Gliocladium sp.	-	-	-	-	-	-	1	-
Mucorhiemalis	-	-	-	-	4	-	-	7
Penicillium brevicompactum	8	11	-	-	1	11	-	-
Penicillium citrinum	2	-	-	-	-	-	-	-
Penicillium hirsutum	-	-	-	-	-	-	3	-
Penicillium solitum	-	-	-	-	-	-	2	-
Rhizopus stolonifer	8	1	-	-	-	-	-	-
Trichoderma harzianum	-	-	-	-	-	-	-	4

*: Coffee harvested onto cloth; **: harvested coffee swept from the ground.

Colletotrichum, *Alternaria* and *Gliocladium* (Table 1). Various fungal genera have also been reported in studies on conventional coffee beans, notably species of *Aspergillus*, *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium*, *Rhizopus* and *Trichoderma*, among others (Chalfoun and Batista, 2003; Joosten et al., 2001; Prado et al., 2004; Leong et al., 2007; Rezende et al., 2013).

Fungi of the genus *Aspergillus* were detected in all samples that were not subjected to disinfection. The section *Circumdati* accounted for 49.35% of the observed contamination, followed by section *Nigri* (47.26%) and section *Flavi* (3.37%).*A. ochraceus* of section *Circumdati* was the predominant speciesand accounted for 73.17% of the total contamination. This fungal species has been identified in other studies on coffee beans (Batista et al., 2003; Silva et al., 2008;

Suarez-Quiroz et al., 2004; Batista and Chaulfoun, 2007). Additional species were also identified in the coffee samples, including *A. sulphureus* (17.7%) (Batista et al., 2003) and *A. ostianus* (9.75%). Studies using coffee beans harvested in Minas Gerais reported that 80% of the identified isolates belonged to the genus *Aspergillus* section *Circumdati* at all harvesting and processing stages (Batista et al., 2009). In comparison to the coffee swept from the ground, the coffee harvested onto cloth exhibited a high level of mold contamination. The coffee swept from the ground was found to contain the highest level of microorganisms, which is undesirable in a high-quality coffee (Batista et al., 2007). However, the quality of coffee swept from the ground is influenced by geographical location and climatic conditions. In high altitude regions without rain during the harvest, the level of microorganism contamination for coffee swept from the ground would be equivalent to that for coffee harvested onto cloth.

Fungal richness and diversity

Twelve (12) of the thirty-two species identified in organic and conventional coffee beans were present only in organic coffee beans and include *Fusarium solani*, *Colletotrichum gloeosporioides*, *Aspergillus tamarii*, *A. oryzae*, *A. parasiticus*, *A. versicolor*, *Penicillium hirsutum*, *Trichoderma harzianum*, *Phoma* sp., *Bipolaris* sp., *Glomerella cingulata* and *Mucor hiemalis*. The highest richness index was observed in the organic coffee bean samples (Rm = 4.18) in comparison to the conventional coffee bean samples (Rm = 3.24). This result indicates that the species richness is greater in the organic coffee than in the coffee from conventional cultivation. Several studies have indicated that organic farming allows for greater species richness than conventional farming (Mader et al., 2002; Stokstad, 2002; Delate and Cambardella, 2004; Bengtsson et al., 2005).

Regarding species diversity, the Shannon index for the organic coffee was 5.18, whereas the Shannon index for the conventionally cultivated coffee was 4.60. Thus, consistent with both the Margalef and the Shannon indices, the species richness and diversity of filamentous fungi are greater in the organic system. In organic agriculture, it is possible to observe an increase in soil biodiversity and biological activity. The greater organism diversity in this system maintains the biological equilibrium, promoting a reduction in disease- and pest-related problems (Hyde, 2001; Lima and Vianello, 2011).

The genera with the greatest number of represented species were *Aspergillus* and *Penicillium*. Twelve (12) *Aspergillus* species were divided into four groups (sections *Nigri*, *Circumdati*, *Flavi* and *Versicolores*) according to Klich (2002), whereas five species of *Penicillium* were identified (*Penicillium brevicompactum*, *Penicillium hirsutum*, *Penicillium crustosum*, *Penicillium citrinu* and *Penicillium solitum*).

Association of fungi with toxigenic species

The existence of different fungal species and their ability to produce different classes of secondary metabolites has enabled these microorganisms to become strong competitors within several ecosystems (Logrieco et al., 2007). Although the functions of mycotoxins have not been entirely elucidated, they are believed to participate in the elimination of other competing microorganisms from the environment (Brase et al., 2009). Therefore, the investigation of the association between different species of filamentous fungi is necessary to understand their complex ecological relationship and potential interactions that occur to promote the synthesis or degradation of mycotoxins in the environment.

The filamentous fungi that are associated with toxigenic species are shown in Figure 3. For these analyses, samples contaminated with *A. ochraceus*, which is one of the major OTA-producing species in coffee beans and *A. flavus*, which is known to produce aflatoxin (Chalfoun and Parizzi, 2008), were used for each cultivation system.

The association between toxigenic fungi and filamentous fungi in both the organic and the conventionally cultivated coffee beans was determined using a correspondence analysis technique (Hair et al., 1998), in which the homogeneity criteria included the location of profiles next to the center and their contribution to other components. Accordingly, in Figure 3A, an association between the toxigenic fungus *A. flavus* and species of filamentous fungi in the organic coffee bean samples can be observed. These *A. flavus*-associated species include *Cladosporium cladosporioides*, *Aspergillus ochraceus* and *Penicillium brevicompactum*. However, according to the established criterion for grouping, an association with *A. flavus* was not observed for *Colletotrichum gloeosporioides*, *Fusarium equiseti* and *Aspergillus foetidus* for either coffee cultivation system.

Using the coordinates and contributions obtained from the correspondence analysis, the organic coffee samples S1, S2, S3, and S4 did not confirm a clear association between toxigenic fungal species and *Aspergillus foetidus*, *Aspergillus flavus*, *Penicillium brevicompactum*, and *Cladosporium* (Figure 3B). Regarding group formation among the species *A. flavus*, *A. ochraceus* and *C. cladosporioides* in conventional coffee, the results illustrated in Figure 3C demonstrate that these fungi were associated with sample S2.

Microorganisms such as protozoa, bacteria, yeast, and filamentous fungi are capable of degrading mycotoxins through the production of proteolytic enzymes (Abrunhosa et al., 2006; Abrunhosa et al., 2010). Although a report from Shantha (1999) revealed that the fungus *Cladosporium* sp. represented the least efficient species to degrade aflatoxin, producing an inhibition of less than 10%, Figure 3 D indicates that only *C. cladosporioides* was associated with *A. ochraceus* in

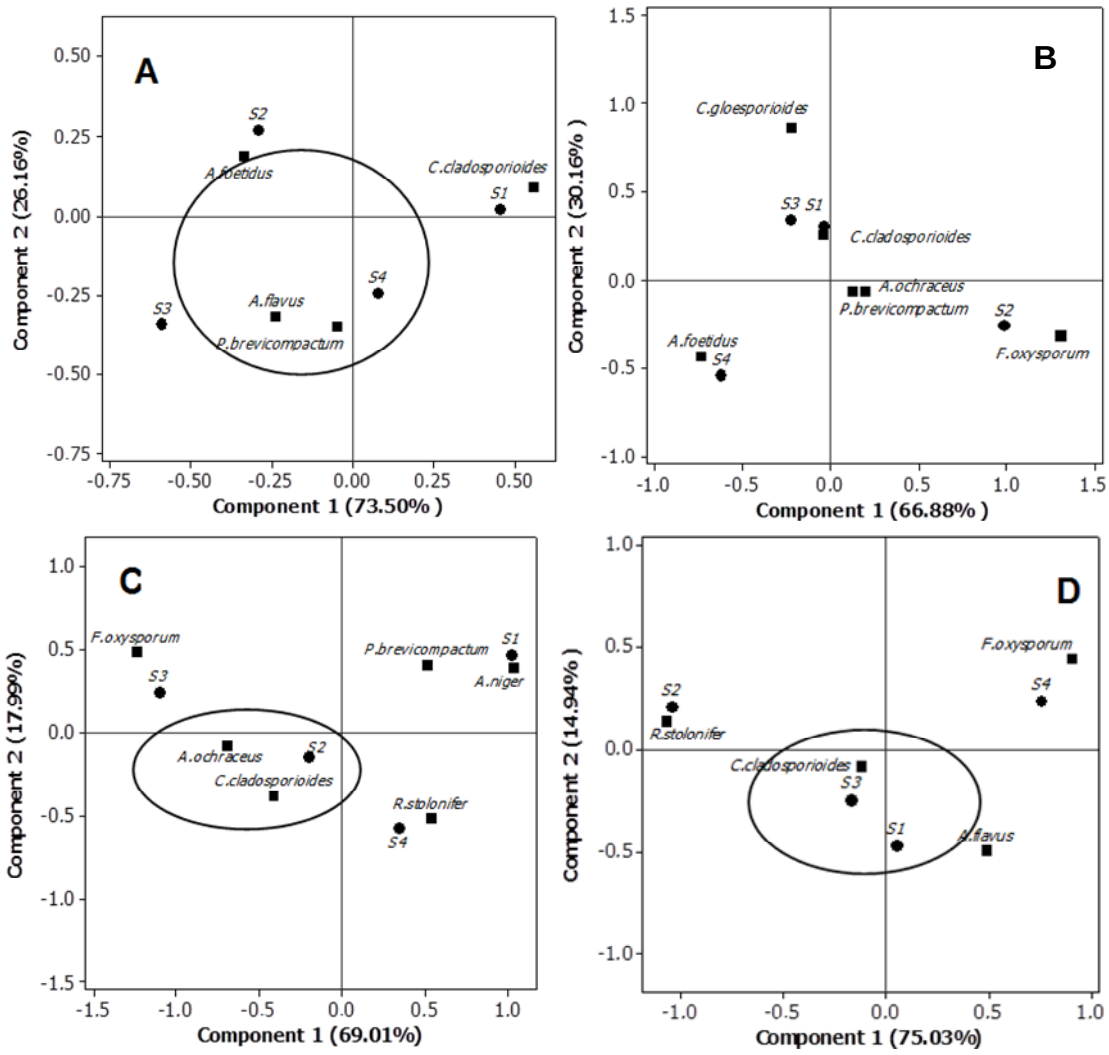

Figure 3. The association profile of toxigenic fungi in organic and conventionally cultivated coffee beans. A: *A. flavus* in organic coffee x fungi. **B:** *A. ochraceus* in organic coffee x fungi. **C:** *A. flavus* in conventional coffee x fungi. **D:** *A. ochraceus* in conventional coffee x fungi.

samples 1 and 3. However, *Rhizopus stolonifer*, *Fusarium oxysporum* and *Aspergillu sflavus* were not associated with this toxigenic fungus. Hence, a comparison of these associations with the results of Abrunhosa et al. (2002) suggests that *C. cladosporioides* exhibits a significant ability to degrade OTA. The occurrence of potentially toxigenic species in coffee represents a significant threat to the economy of this agricultural production addition to human and animal health.

The results of the association of fungal species with toxigenic species indicate that in both conventional and organic coffee, *A. ochraceus* and *A. flavus* are associated with *C. cladosporioides*. In the presence of *A. ochraceus*, *C. cladosporioides* can serve as a bioprotectorin organic and conventional coffee due to its ability to degrade mycotoxins. According to Martins et al. (2001), *Cladosporium* growth functions as a barrier to the growth of other fungi that are considered detrimental to coffee quality. Therefore, the interaction between *A. ochraceus* and *C. cladosporioides*is considered to demonstrate a positive effect on the safety and quality of the produced coffee.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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