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INCIDENCE AND DISTRIBUTION OF FILAMENTOUS FUNGI DURING FERMENTATION, DRYING AND STORAGE OF COFFEE (*COFFEA ARABICA* L.) BEANS

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ABSTRACT

The objective of this work was to isolate and characterize filamentous fungi present in different stages of harvest, fermentation, drying and storage of coffee beans processed by natural method. The cherries were hand-picked and then placed on a cement drying platform where they remained until reached 11% of humidity. Microbial counts were found in all samples during fermentation and drying of the coffee beans. Counts of fungi in the coffee cherries collected from the tree (time 0) were around 1.5 x 10^3 CFU/g. This number increased slowly during the fermentation and drying reaching values of 2 x 10^5 CFU/g within 22 days of processing. Two hundred and sixty three isolates of filamentous fungi were identified. The distribution of species during fermentation and drying was very varied while there was a predominance of *Aspergillus* species during storage period. The genera found were *Pestalotia* (4), *Paecelomyces* (4), *Cladosporium* (26), *Fusarium* (34), *Penicillium* (81) and *Aspergillus* (112) and comprised 38 different species.

Key-words: Fermentation, filamentous fungi, coffee, toxigenic fungi

INTRODUCTION

Coffee quality is evaluated by key factors including the selection of the *Coffea arabica* variety, climatic conditions during growth, processing method and storage conditions. The main aim of coffee processing is removal of the pulp, mucilage, parchment and silver skin surrounding the coffee beans, which leaves the so called ´green‘ coffee beans. In Brazil and Ethiopia and for robusta coffees generally, the dry or natural method of fermentation is usually used. During the natural processing, coffee fruits are spread on the ground (earth, concrete or tarmac) in layers approximately 10 cm thick, heaped at night and respread each morning. During the course of 10-25 days of sun drying, the natural microbial fermentation that occurs can influence the final quality of the product (32). Microbial contamination can occur in the cherries and during harvesting, fermentation, drying and storage coffee beans (32). Bacteria, yeasts and filamentous fungi have been already reported in the pulp and beans of coffee processed in Brazil, India, Hawaii, Congo, Argentina, Colombia, Costa Rica, Ethiopia and Mexico (2,12,30,32). Filamentous fungi predominate at the end of the processing and during storage, and may affect the quality and safety of the final product due to production of mycotoxins (4,6,34,35). Several studies have reported the occurrence of toxin-producing fungi and ochratoxin in green coffee beans (4,18,20,26, 28). A survey on stored green coffee beans from various origins has shown that coffee samples from African origin have significantly higher levels of OTA than those from America and Asia (21). The aim of this work was to detect the occurrence of filamentous fungi present in the different stages of coffee production by natural method including harvest, fermentation, drying and storage of beans.

MATERIALS AND METHODS

Sampling

One hundred and eight kg of beans from *Coffea arabica* var. Acaiá were collected from a farm located 750 to 800 m above

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sea level in Lavras, State of Minas Gerais, Brazil. The hand picked red cherries were fermented and dried on a concrete platform for about 25 days until reaching humidity of 11-12%. The beans were then packed in either polystyrene bags or jute sacks and stored in a cold chamber at 3ºC for 136 days, when the relative humidity reached 90%.

Isolation and identification of filamentous fungi

Every 48 h, three samples of two hundred beans were aseptically removed from the stored packages and placed in sterile flasks and transferred to the laboratory in an ice box. The beans were transferred to a bottle containing 1800 ml of saline peptone diluent (0.1% of peptone, 0.5% of NaCl, 0.03% $Na_2H_2PO_4$). After 15 min mixing, ten-fold dilutions were prepared. Appropriate dilutions were spread on triplicate plates of DG18 (Dicloran Glycerol 18%) agar (11) containing 100 mg/L chloramphenicol and 50 mg/L chlortetracycline to inhibit bacterial growth. Plates were incubated at 28ºC for 5 days, and the colonies were counted and expressed as CFU/g.

For identification, the isolates were purified by streaking in Malt Extract agar (Merck) according to Christensen (8) for species of the *Circumdati* section, Christensen (9) for species of the *Flavi* section, Klich and Pitt (14) for species of the *Nigri* and *Aspergillus* section, Pitt (24) for species of the *Penicillium genus* and Klich (13) for species of the *Versicolores* section. Further support for fungi identification was found in Raper and Fennell (27), Booth (5), Nelson *et al.* (19), Samson *et al.* (29) and Pitt and Hocking (25).

RESULTS AND DISCUSSION

Coffee cherries and beans are subjected to contamination and consequent colonization by microorganisms during different phases of development, harvesting, preparation, transport and storage. Microbial counts were found in all samples during fermentation and drying of the coffee beans. Counts of fungi in the coffee cherries collected from the tree (time 0) were around 1.5 x 10^3 CFU/g (Table 1). Most papers on coffee contamination reported microbial counts from a mixture of green (immature), red (mature), over mature, dark brown and shriveled cherries (3,4,31,32,35). In this work, the fungi population in hand-picked mature cherries increased slowly during fermentation and drying, reaching values of 2 x 10^5 CFU/g within 22 days of processing, when the beans contained about 11% humidity. The highest count of filamentous fungi was found at the 20th day of fermentation and drying. This growth of fungi was probably due to reduction of water activity which inhibits the growth of other microbial groups e.g. bacteria and yeasts (Table 1). On the twentieth day of drying, the humidity level and the water activity were 12.90% and 0.63, respectively. Two days later, the humidity in the grains decreased and reached the ideal 11%, which corresponded to a water activity of 0.52. According to Pitt and Hocking (25) water activity below 0.60 does not allow the growth of known microorganisms. The fungi population during of 136 days of storage was also monitored. During this period, the green coffee packed in polystyrene bags presented a decrease in fungi population whereas the coffee stored in jute sacks presented a small increase (Table 2). Coffee beans stored in jute sacks presented a much higher water activity after 136 days of storage, making possible that the counts reached values of 9 x 10^5 CFU/g (Table 2).

Table 1. Total counts of filamentous fungi, relative humidity and water activity of the coffee grains during fermentation and drying.

Time (days)	Counts (CFU/g)	Relative humidity (%)	Water activity
0	1.5 x 10^3	67.45	>0.85
2	2.8 x 10^3	60.83	>0.85
4	5.9 x 10^3	38.85	>0.85
6	7.6 x 10^3	29.35	>0.85
8	2.0 x 10^4	28.56	>0.85
12	4.0 x 10^4	19.72	0.82
14	6.8 x 10^4	19.30	0.82
16	9.0 x 10^4	19.70	0.82
18	1.7 x 10^5	15.78	0.71
20	2.0 x 10^5	12.90	0.63
22	1.6 x 10^5	11	0.52

Two hundred and sixty three filamentous fungi isolates were identified to species level (Table 3). The distribution of species during fermentation was very varied while there was a predominance of *Aspergillus* species during storage (Table 3). Isolates belonged to 6 genera: *Pestalotia* (4 isolates), *Paecelomyces* (4 isolates), *Cladosporium* (26 isolates), *Fusarium* (34 isolates), *Penicillium* (81 isolates) and *Aspergillus* (112 isolates) (Table 3). These isolates comprised 38 different

Table 2. Fungi population, relative humidity and water activity in green coffee grains stored in polystyrene and jute sacks.

Time (days)	Type of packing	Counts (CFU/g)	Relative humidity (%)	Water activity
40	polystyrene	3.7 x 10^3	13.05	0.63
	jute	6.0 x 10^4	17.90	0.82
84	polystyrene	1.2 x 10^3	12.90	0.63
	jute	9.7 x 10^4	19.00	0.82
136	polystyrene	0.7 x 10^3	12.50	0.63
	jute	1.8 x 10^5	19.0	0.82

fungi species. These differences found in the number and diversity of fungi in the coffee beans could be related with the long period of fermentation and drying (15-25 days). This high frequency and diversity of fungi in green coffee samples analyzed at different stages of maturation and processing was also reported by Urbano *et al.* (37) and Pardo *et al.* (21).

A high diversity of fungi species was observed in cherries collected from trees. Fourteen different species belonging to genera *Cladosporium*, *Fusarium*, *Pestalotia*, *Paecelomyces* and *Penicillium* were detected (time 0 - Table 3). After the second day of fermentation, the total number of fungi increased slightly but there was a decrease in the number of identified species (Tables 1 and 3).

The microbial diversity on the surface of coffee cherries and beans in the South region of Minas Gerais State was also reported by Silva *et al*. (32). *Aspergillus*, *Penicillium*, *Fusarium* and *Cladosporium* are known as natural coffee contaminants, and are present from the field to the warehouse (18,22,32). The humidity and chemical composition of the coffee beans, environmental conditions and crop and product management can influence development of microorganisms and their metabolic activity.

Table 3. Species of filamentous fungi detected in cherries and green coffee during different phases of processing: fermentation, drying and storage in polystyrene and jute sacks.

Time (days)		Species of filamentous fungi
Fermentation and drying	0	*Cladosporium cladosporioides* (seven), *Fusarium lateritium* (four), *F. solani* (four), *F. illudens* (two), *F. moniliforme* (sin. *verticilioides*) (two), *F. nivale* (one), *Pestalotia* sp. (three), *Paecelomyces* sp. (one), *Penicillium minioluteum* (three), *P. roqueforti* (one), *P. solitum* (one), *P. funiculosum* (two), *P. brevicompactum* (two), *P. chrysogenum* (one)
	2	*C. cladosporioides* (three), *Paecelomyces* sp. (one), *P. minioluteum*(one), *P. crustosum* (one)
	4	*C. cladosporioides* (two), *F. solani* (two)
	6	*C. cladosporioides* (one), *F. solani* (two), *P. purpurogenum* (two)
	8	*C.cladosporioides* (five), *Aspergillus flavus* (one), *F. illudens* (one), *Pestalotia* sp. (one)
	12	*C. cladosporioides* (one), *A. flavus* (one), *Paecelomyces* (two), *P. fellutanum* (one), *P. corylophilum* (two), *P. solitum* (one)
	14	*A. flavus* (one), *P. roqueforti* (one), *P. expansum* (one), *P. citrinum* (one), *P. janthinelum* (one), *P. fellutanum* (one), *P . brevicompactum* (nine), *P. chrysogenum* (two)
	16	*F. solani* (three), *F. illudens* (two), *F. xylarioides* (two), *F. stilboides* (one), *F. concolor* (one), *F. equiseti* (one), *P. solitum* (one)
	18	*C. cladosporioides* (two), *A. flavus* (one), *P. roqueforti* (six), *P. citrinum* (one), *P. brevicompactum* (one), *P.crustosum* (one)
	20	*A. ochraceus* (twelve), *A. flavus* (three), *F. xylarioides* (one), *F. trincictum* (one), *P. brevicompactum* (twelve), *P. roqueforti* (two), *P. aurantiogriseum* (one), *P. waksmanii* (one), *P. citrinum* (one), *P. minioluteum* (one), *P. solitum* (one)
	22	*A. flavus* (five), *A. niger* (twenty), *A. tamari* (one), *A. sydowii* (one), *F. lateritium* (one), *P. aurantiogriseum* (one)
Storage	40 days (polystyrene bags)	*A. flavus* (six), *P. citrinum* (two), *P. corylophilum* (one), *P. chrysogenum* (one), *P. roqueforti* (one)
	40 days (jute sacks)	*A. flavus* (one)
	84 days (polystyrene bags)	*A. flavus* (two), *P. brevicompactum* (one), *P. viridicatum* (one), *P. citrinum* (one)
	84 days (jute sacks)	*C. cladosporioides* (five), *F. concolor* (one), *P. roqueforti* (one), *P. citrinum* (one), *P. solitum* (one)
	136 days (polystyrene bags)	*A. flavus* (eleven), *A. niger* (nine), *F. lateritium* (one), *P. citrinum* (two)
	136 days (jute sacks)	*C. cladosporioides* (two), *A. flavus* (twenty one), *A. niger* (thirteen), *A. foetidius* (one), *A. dimorphicus* (two), *F. lateritium* (one), *P. citrinum* (one), *P. implicatum* (one), *P. crustosum* (one), *P. waksmanii* (one)

Cladosporium cladosporioides was the most frequent specie found on cherries in the tree (time 0), on the grains during the fermentation until 12th day and in grains stored in jute sacks for 84 and 136 days (Table 3). Magan and Lacey (15) studied the colonization in coffee grains with *Cladosporium cladosporioides, Fusarium culmorum*, species of *Aspergillus* and *Penicillium brevicompactum* and *P. roqueforti,* and observed that the two first species were present in the field and the two last species were colonizers of dried coffee grains during storage. The authors observed that the growth of *Cladosporium cladosporioides* was inhibited by species of *Fusarium, Penicillium* and *Aspergillus*. Comparing the species of fungi present in the coffee beans, the highest incidence of *C. cladosporioides* was detected when competitors were absent or present in a reduced number (Table 3). These results are in agreement with those reported by Pereira *et al.* (22), who observed that this *C. cladosporioides* specie corresponded to 100% of the identified isolates in the cherries and grains of coffee. There is evidence that this fungus is common in coffees of good quality (22).

The *Fusarium* species was detected in cherries on the tree, in the beans during the process of fermentation and drying and in the beans stored both in jute sacks (84 and 136 days) and in polystyrene packs (136 days) (Table 3). *Fusarium lateritium* and *F. solani* were more frequent than other *Fusarium* species. *F. lateritium* also was isolated in cherries on the 22nd day of drying and at the end of storage in both types of packages (Table 3). *F. illudens* was observed in cherries in the tree (time 0), in grains on the 8th and 16th days of fermentation and drying. Although the majority of the toxigenic fungi reported in coffee are *Aspergillus* and *Penicillium* (4,17,31,35,38), *Fusarium* species, involved in the coffee processing, were also reported by Silva *et al.* (32) and Pimenta and Chalfoun (23), possibly as mycotoxin producers.

Isolates belonging to the genus *Penicillium* were observed in all samples, during fermentation, drying and also during the storage in the two types of packages (Table 3). This genus presented the highest diversity of species as 17 different species were identified (Table 3). *P. brevicompactum* was identified in cherries in the tree, in grains on the 14th, 18th and 20th days of drying and in grains after 84 days of storage in polystyrene packs. Among species of *Penicillium* studied by Magan and Lacey (15), *P. brevicompactum* was the most prevalent due to release of metabolites in the substratum that had inhibited or limited the growth of other species of fungi. In the present study, the second specie most frequent belonging to the genus *Penicillium* was *P. roqueforti*, representing 15.2% of the isolates. This specie was identified in coffee beans on the14th, 18th and 20th days of fermentation and drying of the beans after 40 days of storage in polystyrene bags and after 84 days of storage in jute sacks. Magan and Lacey (15) reported that this specie inhibits the growth of other *Penicillium* species. *P. citrinum* was not identified in cherries in the tree but identified in the grains containing humidity between 19.49% and 13.74% (14, 18 and 20 days of fermentation). During the storage, this specie was detected in jute sacks (84th day) and in polystyrene packing. Species of *Penicillium* were identified in coffee beans during processing, especially in the last two days of drying (a_w of 0.71 and 0.63, respectively), being therefore xerophilic fungi. *P. citrinum* was found in coffee cherry is in agreement with its requirement for high water activity (a_w) for growth. Our results indicated that *P. citrinum* occurred only when the water activity was about 0.84. Besides being xerophilic, *P. roqueforti* is a psychrophilic fungus (25), so its presence can be explained by the low temperature of storage chamber. Some species of *Penicillium* found in the coffee grains, such as *P. roqueforti*, *P. citrinum*, *P. chrysogenum*, *P. crustosum, P. aurantiogriseum, P. funiculosum*, *P. janthinellum*, *P. expansum* can produce mycotoxins, and their presence in the grains is important because they compromise the quality and safety of the product. However the presence of toxigenic fungi does not indicate that mycotoxins were also present, as evidenced by Batista *et al.* (3) and Silva *et al.* (31).

The presence of *P. brevicompactum, P. citrinum, P. aurantiogriseum* and *P. expansum* found in this study agree with results reported by Batista et al. (4) and Mislivec et al. (17), who cited these species as the principal members of *Penicillium* genus found in coffee beans. The high frequency of *Aspergillus* (96%) and *Penicillium* (50%) species confirms the widespread natural contamination of coffee with these fungi (1,4,31,35).

One hundred and twelve isolates of *Aspergillus* had been identified: *A. flavus* (53 isolates), *A. niger* (42 isolates), *A. ochraceus* (12 isolates), *A. tamarii* (1 isolate), *A. sydowii* (1 isolate), *A.foetidius* (1 isolate), *A. dimorphicus* (2 isolates). These species were detected at the 8th day of drying of the beans and during storage, representing 59.6% of the total isolates. *A. flavus*, the most frequent specie, was presented in the 8th day of drying and also in the12th, 14th, 18th, 20th and 22nd days of fermentation, and at the end of storage in the two kinds of packing. *Aspergillus niger* represented 37.8% of the isolates of the genus *Aspergillus*, being only found in the last day of fermentation/drying of the beans (22nd day), and in beans stored in jute and polystyrene sacks (Table 3). *A. ochraceus* was found only at the 20th day of fermentation, not being detected during storage. *Aspergillus* species have been frequently reported in beans and stored grains of coffee (3,4,6,7,17,32,33,35,37). Besides compromising the quality of the product, the presence of *Aspergillus* may affect their safety due to production of secondary metabolites toxic to man and animals (mycotoxins). *Aspergillus ochraceus*, *A. niger* and *A. carbonarius* had been also found in samples of grains of coffee by Taniwaki and collaborators (35). Although *A. carbonarius* is a common contaminant of wines (10), its presence in stored coffee beans is rare in Brazil (35).

Biotic and abiotic parameters such as water activity (aw) and interactions among fungi determine the extent of

colonization and the microbiota found in coffee beans (16). The ability of fungus to germinate, grow and sporulate in coffee grains is also dependent of a_w, temperature and intergranular composition of gases (36). The biotic and abiotic factors that influence the establishment of groups of microorganisms (bacteria, yeasts and filamentous fungi) in coffee during natural processing are not known. However, it is known that maize with high humidity can become contaminated by microorganisms that interact during pos-harvesting and storage. Species of filamentous fungi can interact in different ways, including the production of metabolites that can influence the settlement of specific strains (15). The interaction among filamentous fungi observed in maize could be extended for those observed in coffee beans containing 67.45% humidity. According to Pitt and Hocking (25), *Aspergillus* competes for substrate with *Fusarium* and *Penicillium*, and its incidence increases only in environments with high temperature and low water activity, which are the ideal conditions found in the final stages of processing and drying during storage of coffee. Many species of fungi identified in this work had been already detected in cherries and grains of coffee (3,17,31,32).

In this work, the number of isolated fungi in coffee grains increased during storage, where *Aspergillus flavus* and *A. niger* predominated. During storage, the number of species isolated in samples from jute sacks was higher than in samples stored in polysterene bags. The polystyrene bags are less permeable and re-absorption of water occurs in lesser extent that in the jute sacks. Despite the high humidity of the green coffee grains, humidity and temperature were not favorable for the growth of potentially toxigenic species such as *Aspergillus flavus* and *A. ochraceus*. The minimum water activity for production of aflatoxin by *A. flavus* is 0.82, which corresponds to approximately 18.4% of humidity. For *A. ochraceus,* the minimum water activity to produce ochratoxin is 0.85 corresponding to approximately 20% of humidity in coffee grains (34). The minimum and maximum temperature of growth for *A. flavus* range between 6 and 10ºC and between 25 and 37ºC, respectively, however for aflatoxin B_1 and B_2 production the ideal temperature is between 16 and 31ºC. For *A. ochraceus* the minimum and maximum temperature of growth is between 8 and 12ºC and between 24 and 31ºC, respectively, while for ochratoxin production, the temperature should be between 25 and 31ºC (34). It is interesting to observe that despite the isolation of these fungi in stored coffee grains, ideal temperature for development of the species and for production of toxins is much higher than the temperature in the cold storage chamber (3ºC). Although several species of toxin producing fungi were found during coffee processing neither ochratoxin nor aflotoxin were detected in any sample (data not shown).

The species identified in this study are among the most common species of fungi present in storage environments. They can tolerate growth in different substrates and environmental conditions, and their complete elimination is difficult. However, the use of good hygiene practices and good management of the beans during processing can minimize the production of the mycotoxins in coffee.

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RESUMO

Incidência e distribuição de fungos filamentosos durante a fermentação, secagem e armazenamento de frutos e grãos de café (*Coffea arabica* L.)

O objetivo deste estudo foi isolar e caracterizar fungos filamentosos presentes em diferentes estágios de beneficiamento de café processado pelo método natural, incluindo: colheita, fermentação, secagem e armazenamento. O café cereja foi colhido manualmente e então colocado em uma plataforma de cimento, onde permaneceu até atingir 11% de umidade. A contagem microbiana foi realizada em todas as amostras durante a fermentação e secagem do café. A população de fungos filamentosos no café cereja ainda nos pés (tempo 0) foi em torno de 1,5 x 10^3 UFC/g. Este número aumentou vagarosamente durante a fermentação e secagem, alcançando valores de 2 x 10^5 UFC/g em 22 dias do processamento. Duzentos e sessenta e três isolados de fungos filamentosos foram identificados. A distribuição das espécies durante fermentação e secagem foi bastante variada, mas no armazenamento dos grãos ocorreu o predomínio de espécies de *Aspergillus*. Foram encontradas 38 espécies de fungos distribuídas nos seguintes gêneros: *Pestalotia* (4), *Paecelomyces* (4), *Cladosporium* (26), *Fusarium* (34), *Penicillium* (81) e *Aspergillus* (112).

Palavras-chave: Fermentação, fungos filamentosos, café, fungos toxigênicos

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