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ORIGINAL ARTICLE

Genetic diversity of endophytic fungi from *Coffea arabica* cv. IAPAR-59 in organic crops

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Abstract Endophytes are microorganisms that live inside plant tissues throughout its life cycle and during certain phases of development. The endophyte–host relationships can provide benefits to the host, protecting it against attack by insects and diseases. Several studies have demonstrated the diversity of endophytes from *Coffea arabica*, but few studies in varieties of organic crops. Thus, the objective in this study was to corroborate these reports with knowledge of the endophytic fungi communities in an organic variety of *C. arabica* L. cultivar IAPAR-59. We identified the endophytic fungi by molecular methods using the ITS1-5.8S-ITS2 region of rDNA and phylogenetic analyses. In the antagonist activity tests, the endophytes were tested against phytopathogens with the evaluation of the kind of interactions between them. Analyses demonstrated a diversity of genera, including: *Colletotrichum*, *Trichoderma*, *Schizophyllum*, *Mycosphaerella*, *Cladosporium*, and *Cercospora*, as well as the first record of the genus *Ophiognomonia* in *C. arabica*. The antagonist activity showed reduced growth of the phytopathogenic fungi *Glomerella* sp., *Colletotrichum* sp. and *Sclerotinia sclerotiorum*. The results obtained in this work, with the identification for the first time, of a highly diverse isolated endophytic of the species *Ophiognomonia* sp. in the coffee plants, is the second report of the detection of these fungi in Brazil. Also, the detection of different isolates with the ability to antagonise pathogens, emphasises the importance of further research, involving the isolation, identification and exploration of endophytes of other genetic varieties of coffee, obtained by breeding programs of this important crop in Brazil.

Keywords Coffee · Endophytes · Antagonism · *Ophiognomonia* · Biodiversity · Phytosanitary

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Introduction

Endophytic microorganisms or endophytes are defined as fungi or bacteria that colonise the interior of tissues or plant organs of host plants, without causing symptoms of disease or injury (Petrini 1991; Azevedo et al. 2000; Bernardi-Wenzel et al. 2010; Garcia et al. 2012; Rhoden et al. 2012; Almeida et al. 2015); they have properties potentially useful in agriculture, biological control, and the development of bioactive compounds. Studies have indicated that their presence in large quantities can reduce attacks by insects (Kogel et al. 2006; Koulman et al. 2007). Previous studies have shown that the plant genus *Coffea* sp. has a wide variety of endophytic fungi (Santamaría and Bayman 2005; Sette et al. 2006; Vega et al. 2008; Vega et al. 2010).

Coffee is a strategic agricultural product in Brazil and research into the plant is essential for the increased production and quality of the drink. However, the number of publications

relating to endophytes extracted from coffee plants is still low. The species *Coffea arabica* presents several varieties due to programs of genetic improvement. IAPAR-59 coffee is one such product, and was developed especially for high planting densities, while maintaining rust resistance. IAPAR-59 has complete resistance to rust and is derived from the cross between *Coffea arabica* cv. Villa Sarchi 971/10 and the cv. Timor Hybrid 832/2 held at the Coffee Rust Research Centre (Centro de Investigação das Ferrugens do Cafeeiro - CIFC) in Portugal. Beyond rust resistance, this cultivar is resistant to *Meloidogyne exigua*, which is highly disseminated in Brazil (Salgado et al. 2005; Sera et al. 2010).

Organic crops from this cultivar appear to be an alternative to the use of pesticides, especially due to their low impact on the environment and human health. According to Carvalho et al. (2011), differentiation between organic and conventional coffee has increased due to the growing demand and high consumption of healthy foods that contain compounds with antioxidant potential, which have been associated with the reduction of chronic diseases.

Oliveira et al. (2014) performed comparisons between endophytic fungal communities from leaves of *Coffea arabica* in organic and conventional crop systems in the Northeast Brazil, observing 50.61 % similarity, with 6 species occurring uniquely in organic coffee and five in conventional coffee. *Colletotrichum gloeosporioides* complex and *Phyllosticta capitalensis* were the most common fungi in conventional and organic crop systems, respectively. Xia et al. (2015) study the diversity and specificity of culturable bacterial endophytes in four vegetable crops, corn, tomato, melon, and pepper, grown under organic or conventional practices. Endophytic bacteria were isolated from surface-sterilized shoots, roots, and seed tissues, and the sequences were identified. Importantly, endophytic microbial species abundance and diversity was significantly higher in the organically grown plants compared to those grown using conventional practices, potentially indicating that organic management practices may increase endophyte presence and diversity.

Camatti-Sartori et al. (2005) isolated endophytic fungi from leaves, flowers and fruit of healthy apple trees (*Malus domestica*, BORKH) growing in southern Brazilian orchards under three different cultivation systems (conventional, integrated and organic), during two vegetative cycles. The greatest total numbers of endophytic isolates were obtained from the orchards under organic cultivation when compared to integrated and conventional cultivation systems. Filamentous fungi from the genera *Colletotrichum*, *Xylaria* and *Botryosphaeria* were the most frequent ones, and the most representative yeast genera were *Sporobolomyces*, *Rhodotorula*, *Debaryomyces* and *Cryptococcus*. It is suggested that some isolates may be used as indicators of the different management systems.

Thus, studying the diversity of endophytic fungi in organic crops, as in the case of *Coffea arabica* L. cv. IAPAR-59, which has complete resistance to all known physiological races of rust, is a source in the search for new endophytes to act as biological controllers, because, in this crop, endophytes do not suffer interference from fungicides. Therefore, the aim of this study is to determine the community of filamentous endophytic fungi inside the leaf tissues of *Coffea arabica* L. variety IAPAR-59, in an organic coffee plantation, and evaluate the antagonistic activity of these isolates against pathogenic fungi of coffee and other plants of agricultural interest.

Materials and methods

Biological material

The collection of *Coffea arabica* L. cv. IAPAR-59 leaves was undertaken in an organic coffee plantation, certified by the Biodynamic Institute (IBD), in an oxisol soil type in the city of Jesuítas (latitude: 24° 23′06″ South, longitude: 53° 23′15″ W; elevation: 489 m above sea level), west of State Paraná - Brazil. The season was spring and the collection occurred at the end of October. The coffee crop was 10 years old at time of collection and they were not fruiting or flowering. The number of leaves sampled was about 125.

Endophytic fungi isolation from leaf tissues

Endophytic fungi of *Coffea arabica* L. cv. IAPAR-59 were isolated. For isolation, branches with apparently healthy leaves without smearing or any kind of injury were collected. In order to make sure that only endophytic fungi and not epiphytic fungi were obtained from the isolation process, surface disinfection of the leaves was performed.

For the isolation of fungi, a fragmentation technique was used. The leaves were collected randomly and washed in running water and "Tween 80" detergent solution. They were then rinsed in sterile distilled water and superficially disinfected with 70 % ethanol (1 min), 3 % sodium hypochlorite (4 min), 70 % ethanol (30 s) and rinsed again in sterile distilled water. Fragments of leaves were cut into approximately 3 mm^2 and placed in Petri dishes containing potato dextrose agar (PDA) medium supplemented with tetracycline (50 $ug.ml^{-1}$) and incubated for 7 days at 28 °C. Aliquots of final rinse water of 100 μL were similarly incubated in plates with PDA medium to assess the efficiency of the disinfection process. To determine the colonisation frequency (CF%), the number of fragments that showed fungal growth compared to the total number of sampled fragments was evaluated according to the following formula:

$$CF\% = (Fg/Ft) \times 100$$

where Fg is the number of leaf fragments with fungal growth and Ft is the number of total leaf fragments.

The morphogroups were obtained by observing morphological characteristics, considering colour, mycelial growth of the colonies, and the presence and development of reproductive structures (Garcia et al. 2012).

Extraction, amplification, purification and sequencing of DNA

The fungi were grown in a dialysis membrane which stayed on PDA medium within a Petri dish for 7 days at 28 °C. The method for DNA extraction has been described by Pamphile and Azevedo (2002). The DNA concentration was estimated by agarose gel electrophoresis on 1.0 % using a standard molecular weight *High DNA Mass Ladder* (Invitrogen).

The polymerase chain reaction (PCR) amplification of ITS1-5.8-UTS2 (ITS = internal transcribed spacer) regions was performed using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCCCGCTTATTGATATGC-3′) as described by Rhoden et al. (2012). The amplified regions were purified using the *GFX PCR DNA* and *Gel kit Band Purification* (Amersham Biosciences) according to the manufacturer's specifications. The purified DNA from endophytes was subjected to quantification agarose gel 1 % and photodocumentation.

The sequencing reaction was performed according to Almeida et al. (2015), using the *MegaBACE TM 1000 sequencer* (Amersham Biosciences).

After sequencing, samples were analysed. For the identification of fungi, the nucleotide sequences found were compared with those deposited in the National Center for Biotechnology Information database (NCBI website). For the research of the species, the BLAST tool limited to sequences from type material, was used (McGinnis and Madden 2004). Species identification was determined based on the best value obtained for the similarity and the sequences were deposited in the NCBI database.

Genetic distance of isolates

The determined sequences were aligned using the program MEGA version 6.05 with grouping using the "*neighbour-joining*" (Saitou and Nei 1987), "*p-distance*" to nucleotides with "*the gap pairwise deletion*" and bootstrap with 10,000 repetitions to build the phylogenetic tree.

Antagonism in vitro of endophytes against phytopathogenic fungi

The phytopathogenic fungi *Glomerella* sp. (CNPUV 378), responsible for ripe rot of grape, provided by EMBRAPA Grape and Vine of Bento Gonçalves – RS., *Colletotrichum* sp. and *Sclerotinia sclerotiorum* [IGSALQ collection of the Laboratory João Lucio de Azevedo ESALQ, University of São Paulo, Brazil (www.cria.org.br/cgee/junho/docs/cadastro_colecoes.xls)] were used. The endophytic strains were selected randomly from endophytes previously isolated. The paired culture technique was modified from Campanile et al. (2007).

On opposite sides of Petri dishes (9 cm diameter) containing PDA, discs with 6 mm diameter of cultures of endophytes and phytopathogenic fungi, previously grown for 7 days at 28 °C, were inoculated at 4 cm distance. The tests were performed in triplicate and all plates were incubated at 28 °C for 7 days. Antagonism rates were calculated according Campanile et al. (2007) and the competitive interactions between endophytic and phytopathogenic fungi were determined based on 3 types of interactions, according to the scale of Badalyan et al. (2002): A, B, and C, where C is divided into 4 sub-categories (CA1, CA2, CB1, and CB2), with A= blocking mycelial growth with contact, B=blocking distance, C=endophytic growth on the initial pathogen without blocking; CA1 and CA2=partial and complete endophyte growth on the pathogen after initial blocking with mycelial contact, and CB1 and CB2=partial and complete endophyte growth on the pathogen after initial distance blocking. Mean antagonisms rates were statistically evaluated using analysis of variance (ANOVA) and the means were compared with the Skott-Knott test ($p>0.05$) in order to avoiding ambiguity in the multiple comparisons. The test was performed using the Sisvar v.5.3 statistical software (Ferreira 2011).

Results

Isolation of endophytes

The endophyte colonisation frequency was 48.6 %, totalling 203 isolates. The absence of microorganisms indicated in the control, shows the efficiency in surface disinfection of the plant leaves process.

Molecular identification of fungi isolated

The molecular identification using ITS regions can provide the identification of 32 isolates. These isolates were distributed in seven genera including: *Colletotrichum*, *Ophiognomonia*, *Trichoderma*, *Cladosporium*, *Mycosphaerella*, *Cercospora*, and *Schizophyllum* (Fig. 1, Table 1).

Phylogenetic analysis

Of the 32 isolates that were very similar when compared to the NCBI databases, these were grouped into three

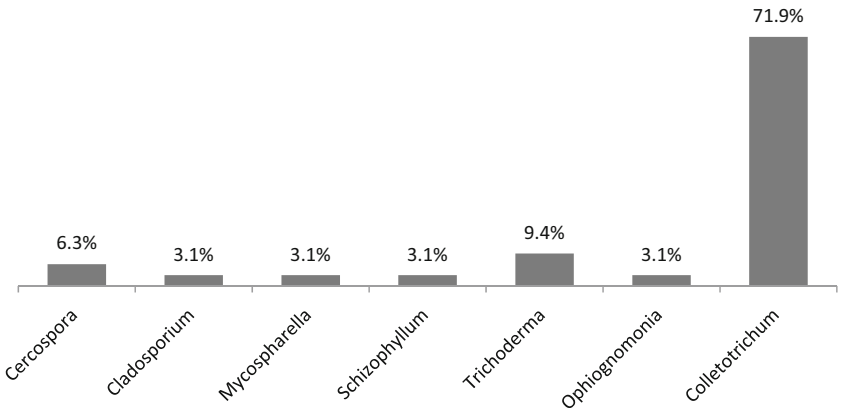

Fig. 1 Frequency of isolation of each taxa of endophytes from *Coffea arabica* L. cv. IAPAR-59

Table 1 Endophytic fungi isolated from *Coffea arabica* with greater identity to other sequences found in the NCBI (National Center for Biotechnology Information website)

Strain	Class	Order	% Identity	Fungi with greater similarity in the NCBI	N° access in NCBI
185	Dothideomycetes	Capnodiales	93 %	*Cercospora iranica*	KJ886513.1
175			99 %	*Cercospora tezpurensis*	KC351743.1
179			97 %	*Cladosporium pini-ponderosae*	NR_119730.1
183			96 %	*Mycosphaerella pseudovespa*	DQ530216.1
115	Agaricocomycetes	Agaricales	98 %	*Schizophyllum commune*	AF280759.1
164b	Sordariomycetes	Hypocreales	92 %	*Trichoderma neokoningii*	DQ841734.1
82			98 %	*Trichoderma neokoningii*	DQ841734.1
36b			87 %	*Trichoderma neokoningii*	DQ841734.1
187		Diaporthales	98 %	*Ophiognomonia* sp.	JQ936328.1
153		Glomerellales	97 %	*Colletotrichum* sp.	KJ955201.1
137a			97 %	*Colletotrichum* sp.	KJ955201.1
137b			97 %	*Colletotrichum ti*	NR_120143.1
174			95 %	*Colletotrichum ti*	NR_120143.1
60			88 %	*Colletotrichum karstii*	HM585409.1
27			93 %	*Colletotrichum gloeosporioides*	JX010152.1
64			97 %	*Colletotrichum* sp.	KJ955201.1
65			94 %	*Colletotrichum endophytica*	KC633854.1
163b			97 %	*Colletotrichum endophytica*	KC633854.1
29b			93 %	*Colletotrichum* sp.	KJ955201.1
173b1			97 %	*Colletotrichum ti*	NR_120143.1
176			95 %	*Colletotrichum ti*	NR_120143.1
155			97 %	*Colletotrichum ti*	NR_120143.1
149			92 %	*Colletotrichum endophytica*	KC633854.1
167			96 %	*Colletotrichum endophytica*	KC633854.1
118			97 %	*Colletotrichum queenslandicum*	JX010186.1
136a			98 %	*Colletotrichum ti*	NR_120143.1
189			96 %	*Colletotrichum aeschynomenes*	NR_120133.1
11			99 %	*Colletotrichum karstii*	HM585409.1
47			98 %	*Colletotrichum* sp.	KJ955201.1
165b3			98 %	*Colletotrichum endophytica*	KC633854.1
61			95 %	*Colletotrichum* sp.	KF242094.1
202a			92 %	*Colletotrichum* sp.	KJ955201.1

clades identified by classes of fungi Agaricomycetes, Dothideomycetes and Sordariomycetes (Fig. 2). Among the Sordariomycetes, 23 isolates presented them selves as fungi genus *Colletotrichum*, while isolates 36b, 82 and 164b clustered on the *Trichoderma* genus and the isolated 187 to a clade with *Ophiognomonia* and Ophioceras leptosporum (CBS 894, 70) genus of fungi. In according to The New Zealand Collections of Fungi and Plant Pathogenic Bacteria (http://nzfungi2.landcareresearch.co.nz/), the *Ophioceras leptosporum* genus is the preferred name of *Ophiognomia*. The isolate 115 stayed grouped concisely in clade *Schizophyllum* genus. The clade represented by class Dothideomycetes was divided into three smaller clades. One of these clades represented the genus *Cladosporium*, with isolate 179, the clade *Mycosphaerella* had isolate 183 and the *Cercospora* clade had isolates 175 and 185.

Fig. 2 Phylogenetic tree of leaf from *Coffea arabica* endophytic fungi with other fungi obtained from the international database GenBank, built by clustering method "*Neighbor-joining*" using "*p-distance*" to nucleotides with "*the pairwise gap deletion*" option and 10,000 repetitions

In vitro antagonism of endophytic isolates against pathogenic fungi

With the objective of looking for endophytic with phytosanitary activity of a broad spectrum against *Glomerella* sp., *Colletotrichum* sp. and *Sclerotinia sclerotiorum*, pathogens of several crops, including coffee, soybeans, grape and others, we obtained different levels of inhibition of mycelial growth of these different pathogens from the assessed endophytes (Fig. 3, Table 2).

The analysis of variance (ANOVA) showed that when compared to *S. sclerotiorum*, the antagonism rates varied from 0 to 39.04 %, and generated 6 different groups. Isolate 36b (*Trichoderma* sp.) was the endophyte that showed the highest inhibition index (AI=39.04 %).

With respect to *Glomerella* sp., the antagonism indices varied between 6.66 and 60.86 %, and generated 2 distinct groups. The isolate 165b2 was the endophyte that showed the highest inhibition index (AI=60.86 %).

In relation to *Colletotrichum* sp., endophytes 147a (IA= 49.71 %) and 144 (IA=48.90 %) were the most efficient, presenting the greatest inhibition rates, ranging from 17.52 to 49.71 %. Statistical analysis generated two distinct groups.

According to the Badalyan scale, the competitive interactions between endophytes and *Colletotrichum* sp. were: A= deadlock (mutual inhibition, in which neither organism was able to overgrow the other) at mycelia contact (60.6 %), B= deadlock at distance (26.3 %), CA1=partial replacement after initial deadlock with mycelia contact (7.9 %), CB1=partial replacement after initial deadlock at a distance (2.6 %) and CB2=complete replacement after initial deadlock at a distance (2.6 %). Between endophytes and *Glomerella* sp. were: A=(85.7 %), B=(2.4 %), CA1=(4.8 %) and CA2=total overlay after antagonist inhibition by mycelial contact (7.1 %). In the case of endophytes coffee against *Sclerotinia sclerotiorum*, the competitive interaction was 100 % A.

Discussion

Isolation and phylogenetic analysis

The phylogenetic analysis of the endophytic fungi from organic coffee, genetic variety IAPAR-59, was successful in confirming the taxonomic identification of the isolates, including the determination of *Ophiognomia* genus (isolated-19),

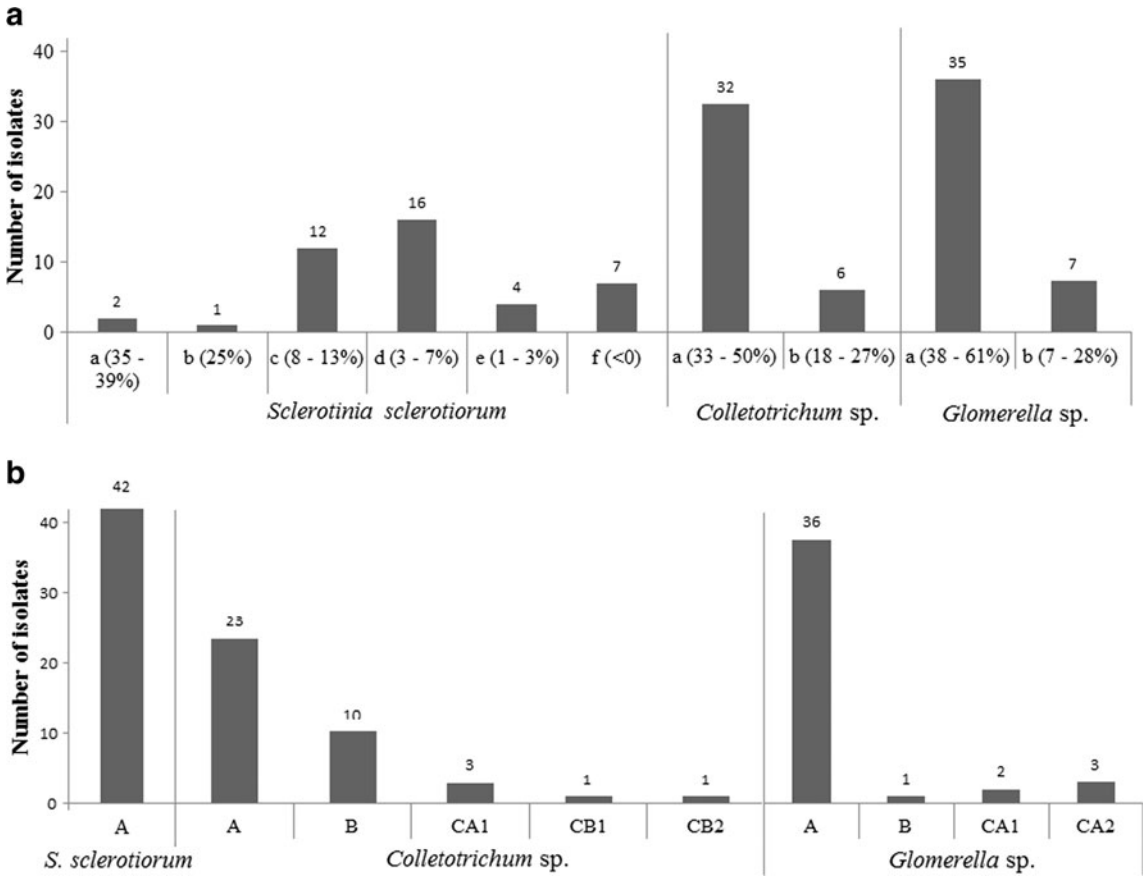

Fig. 3 Summary of antagonism test. **a** Group distribution of endophytes isolates according to statistical analysis and the antagonism rate (%); **b** Interactions types according to the scale of Badalyan et al. (2002)

Table 2 Inhibition rate of mycelial growth of pathogens against endophytic fungal strains of *Coffea arabica*, followed by the statistical group and the interaction between mycelial fungi

Endophytic strain	Antagonism rates (%)* (competitive interactions)		
	Sclerotinia sclerotiorum	*Glomerella sp.*	*Colletotrichum sp.*
3a	2.85^{e} (A)	40.94^{a} (A)	33.15^{a} (A)
3b	0.00^{f} (A)	39.18^{a} (A)	–
4a	8.57^{c} (A)	43.92^{a} (A)	38.26^{a} (A)
4b	10.47^{c} (A)	48.60^{a} (A)	27.30^{b} (A)
5	1.90^{e} (A)	19.41^{b} (A)	18.09^{b} (B)
11 - *Colletotrichum* sp.	7.61^{d} (A)	44.68^{a} (A)	45.19^{a} (A)
25a	6.66^{d} (A)	47.61^{a} (A)	33.98^{a} (A)
25b	8.56^{c} (A)	42.92^{a} (A)	38.46^{a} (A)
29a	3.80^{d} (A)	47.56^{a} (A)	37.98^{a} (A)
36a	0.00^{f} (A)	44.92^{a} (CA1)	42.48^{a} (CA1)
36b - *Trichoderma* sp.	39.04^{a} (A)	21.83^{b} (CA2)	21.97^{b} (CB2)
47 - *Colletotrichum* sp.	11.42^{c} (A)	49.03^{a} (A)	44.47^{a} (CB1)
51a1	6.66^{d} (A)	47.75^{a} (A)	34.61^{a} (A)
54	5.71^{d} (A)	45.71^{a} (A)	40.95^{a} (A)
59c	0.00^{f} (A)	14.28^{b} (A)	–
60 - *Colletotrichum* sp.	8.56^{c} (A)	48.65^{a} (A)	41.90^{a} (A)
65 - *Colletotrichum* sp.	7.61^{d} (A)	56.69^{a} (A)	45.53^{a} (B)
82 - *Trichoderma* sp.	0.95^{e} (A)	44.28^{a} (A)	24.33^{b} (A)
86	0.00^{f} (A)	40.23^{a} (A)	24.76^{b} (B)
115 - *Schizophyllum* sp.	3.80^{d} (A)	54.37^{a} (A)	38.09^{a} (B)
129a2	11.42^{c} (A)	46.57^{a} (A)	–
136a - *Colletotrichum* sp.	11.42^{c} (A)	38.41^{a} (A)	45.21^{a} (B)
137a - *Colletotrichum* sp.	0.95^{e} (A)	45.10^{a} (A)	36.18^{a} (A)
137b - *Colletotrichum* sp.	5.71^{d} (A)	47.69^{a} (A)	41.15^{a} (A)
141b	4.75^{d} (A)	42.33^{a} (A)	37.04^{a} (A)
144	7.61^{d} (A)	51.17^{a} (A)	48.90^{a} (A)
147a	35.23^{a} (A)	18.09^{b} (CA2)	49.71^{a} (CA1)
147b	11.42^{c} (A)	55.50^{a} (A)	37.98^{a} (A)
149 - *Colletotrichum* sp.	12.37^{c} (A)	54.42^{a} (A)	41.73^{a} (B)
153 - *Colletotrichum* sp.	4.75^{d} (A)	50.00^{a} (A)	33.41^{a} (A)
155 - *Colletotrichum* sp.	6.66^{d} (A)	45.11^{a} (A)	37.32^{a} (A)
164b - *Trichoderma* sp.	24.76^{b} (A)	27.77^{b} (CA2)	37.20^{a} (CA1)
165b2	5.71^{d} (A)	60.86^{a} (A)	46.21^{a} (B)
168b2	8.57^{c} (A)	42.11^{a} (A)	43.06^{a} (B)
173b1 - *Colletotrichum* sp.	12.37^{c} (A)	50.02^{a} (CA1)	38.25^{a} (A)
176 - *Colletotrichum* sp.	10.47^{c} (A)	42.08^{a} (A)	35.49^{a} (B)
179 - *Cladosporium* sp.	0.00^{f} (A)	22.85^{b} (B)	17.52^{b} (B)
181a	0.00^{f} (A)	49.82^{a} (A)	33.88^{a} (A)
181b	5.71^{d} (A)	43.18^{a} (A)	38.57^{a} (A)
183 – *Mycospharella* sp.	0.00^{f} (A)	6.66^{b} (A)	–
187 - *Ophiognomonia* sp.	3.81^{d} (A)	45.41^{a} (A)	43.47^{a} (A)
202a – *Colletotrichum* sp.	5.71^{d} (A)	45.17^{a} (A)	36.52^{a} (A)

* Means followed by the same letter superscript in columns do not differ each other by Skott-Knott test at a 5 % significance level

first found in endophytic conditions in *C. arabica*. According to Walker et al. (2012), *Ophiognomonia* species are hosted in leaves, colonising as endophytes, pathogens and saprobes that infect the plants of families Betulaceae, Fagaceae, Juglandaceae, Lauraceae, Malvaceae, Platanaceae, Rosaceae, Salicaceae and Sapindaceae. Considering the relationship of

host plants of *Ophiognomonia* presented by Walker et al. (2012), it is observed that these had not been isolated from coffee. This genus has worldwide distribution and is especially present in the temperate zones, with some species being also found in subtropical regions. Regarding the geographical distribution of this genus, Walker et al. (2012) cited Argentina, Canada, China, Europe, Iran, Japan, Panama, Russia and the United States. In Brazil, this genus had a unique isolate identified by Leite et al. (2013), using molecular methods, endophytically colonising soybean leaves.

Among the different genera of fungi isolated in this work, *Colletotrichum*, *Trichoderma*, *Schizophyllum*, *Mycosphaerella*, *Cladosporium* and *Cercospora* had also been found in other coffee plants as endophytes (Santamaría and Bayman 2005; Vega et al. 2008; Vega et al. 2010; Oliveira et al. 2014). Similar to that shown by Vega et al. (2010) and Oliveira et al. (2014), the present study also obtained a high frequency of *Colletotrichum* sp. The genus *Colletotrichum* causes anthracnose and other diseases on the leaves, and also on many other important crops (Cai et al. 2009), but *Colletotrichum gloeosporioides* was found as an endophyte in *Justicia gendaruss* as a producer of taxol (Gangadevi and Muthumary 2008).

Lima et al. (2012) evaluated the genetic diversity of thirty-nine endophytic fungi identified as *Colletotrichum* spp. associated with *Aroeira brazilian* plant (*Schinus terebinthifolius* Raddi. Anacardiaceae). These endophytes were identified by morphological and molecular methods. The molecular analysis of isolates was based on PCR using primers specific for taxonomy CaInt/ITS4, CgInt/ITS4 and Col/1/ITS4 that amplify specific bands in, respectively, *Colletotrichum acutatum*, *C. gloeosporioides* and *C. boninensis*, and by analysis of the DNA sequence of the rDNA - ITS1-5.8S-ITS2. Combining the morphological and molecular data, these authors were able to identify the species *C. gloeosporioides*, *C. boninense* and *C. simmondsii*. They also found a high intraspecific variability in *C. gloeosporioides*, suggesting the existence of several other species.

The ITS regions of ribosomal DNA are conserved regions of DNA that are capable of assisting in the establishment of phylogenetic relationships and species distinction (Chen et al. 2004; Zervakis et al. 2004; Rhoden et al. 2013). Rhoden et al. (2013), performed in silico phylogenetic analysis of fungi isolated from various plant families in Brazil. For this study, they chose twelve articles published between 2005 and 2012 that examined endophytes isolated in Brazil. They analyzed sequences deposited in the NCBI GenBank database and carried out alignment to determine the genetic distance of strains using the MEGA v. 5 program. The articles yielded 73 plant species belonging to 13 families found in the Brazilian states of Amazonas, Bahia, Minas Gerais, Paraná, and São Paulo. The use of GenBank and the MEGA program for phylogenetic observation revealed that several endophytes had been incorrectly identified because inconsistencies were apparent in their location in the phylogenetic tree. However, approximately 98 % of the sequences deposited in GenBank were consistent with the identification of related genera, indicating that the database is sufficiently robust to support studies in which molecular identification of endophytes is made via analysis of ribosomal DNA sequences.

The high rate of the genus *Colletotrichum* isolated in endophytic condition on coffee cultivar IAPAR-59 suggests a resistance relationship from virulence of the fungus and possible antagonism balanced between plant–fungus, as proposed by Schulz and Boyle (2005). The genus *Trichoderma*, according to Harman (2000), is typically considered saprobes soil fungi and can also have a more intimate relationship with plants that can be characterised as an opportunistic avirulent symbiont. Orlandelli et al. (2012), with endophytic isolates of *Piper hispidum*, showed that genus *Schizophyllum* also presents on leaves of this plant as a endophyte. Moreno et al. (2011), isolating an endophyte of *Psychotria horizontalis* (Rubiaceae), also found the fungus *Mycosphaerella* sp., with the fungus producer cercorporinas.

The genus *Cladosporium* is one of the largest genera of hyphomycetes, being one of the most common isolates in any environment in the world. Many species are plant pathogens, while others are regularly found as contaminants and deteriorating agents in food or industrial products and frequently associated with asthma grievances as well as endophytic fungi (Schubert and Braun 2004).

Antagonism

Biological control of pests and diseases is an alternative for reducing or eliminating the use of chemicals in agriculture, and the selection of effective antagonistic organisms is an initial and important step for biological control (Kamalakannan et al. 2004). According to Silva et al. (2014), production of the Arabica coffee cultivar in Brazil during 2011 generated 50.83 million bags, with Brazil standing out as the world's largest producer and exporter of the product. Given that the use of pesticides for plant disease control has promoted several environmental problems via contamination of natural resources (food, soil, water, animals) and the poisoning of farmers, alternative farming methods, such as organic crops, are perceived as being more environmentally and economically viable. Considering this, Silva et al. (2014), evaluated the efficiency of aqueous extracts from medicinal plants against the major etiological agents of coffee trees: *Cercospora coffeicola*, *Colletotrichum* sp., *Fusarium oxysporum*, *Phoma tarda*, *Rhizoctonia solani* and *Hemileia vastatrix*. Similar to the present article, the screening for harmful extracts was done based on mycelial growth on PDA medium.

According to Shiomi et al. (2008), the evaluation of antagonism in vitro (in tubes or petri dishes) is a fast and efficient method of selecting biocontrol agents of plant diseases to reduce time and cost expended in the field tests (in vivo or in plant assays).

In the present study, the endophyte 165b2, from *C. arabica*, was the best isolate obtained for inhibition of pathogens, with inhibition rates of 46.22 % to *Colletotrichum* sp. and 60.86 % to *Glomerella* sp. (anamorph=*Colletotrichum* sp.). This isolate belongs to the same morphogroup of endophytes identified molecularly as *Trichoderma*, showing the same taxonomic characteristics observed in light microscopy (highly branched characteristic conidiophore). Waller et al. (1993) studied representative isolates of *Colletotrichum*, including the coffee berry disease (CBD) pathogen, obtained from across the range of its distribution. These isolates can be used to distinguish it from other *Colletotrichum* strains occurring on coffee and some other tropical crops. The authors reinforce the conclusions made on the differentiation of the pathogen in Kenya which stipulated the CBD pathogen exists as a distinct and quite separate population in the coffee mycobiota. The CBD pathogen appears to be closely related to *C. gloeosporioides* and possibly evolved from it fairly recently; it has been sufficiently distinct in its pathogenicity and related ecology, colony morphology and biochemical characteristics to warrant recognition as a distinct species.

According to Teles et al. (2013), one of the main problems of the soybean (*Glycine max* L. Merrill) crop has been white mold, which is caused by *Sclerotinia sclerotiorum* (Lib) de Bary. This disease is causing significant losses because, in addition to attacking more than 400 species, the pathogen may survive in soil for many years without hosts. The soybean is currently one of the most important crops in the world. In Brazil, 40 % of the total production area was grown with this legume during the 2011/2012 crop season. The main producers were the states of Mato Grosso, Rio Grande do Sul, Goiás and also Paraná (where coffee cultivar IAPAR-59 was collected for endophytic studies in the present work). So, the endophytic isolate 36b (*Trichoderma* sp.) showed 39.04 % inhibition of that phytopathogen, with mycelial contact.

Campanile et al. (2007), evaluating the endophyte *Trichoderma viride*, observed 28.5 % inhibition of *Diplodia corticola*. These authors reported a predominance of competitive interaction type A, inhibition of mycelial contact, similar to our study. In contrast, the majority of fungi tested by Badalyan et al. (2002) against pathogens of cereals (*Bipolaris sorokiniana*, *Fusarium culmorum*, *Gaeumannomyces graminis* var. Tritici and *Rhizoctonia cerealis*) had total or partial overlapping of their mycelium over the pathogens. Similarly, Mónaco et al. (2004) found that *Trichoderma* strains inhibited the growth of *A. alternata* between 41 and 61 % and the growth of *Bipolaris sorokiniana* between 36 and 71 %; given that, one complete overlap of endophytes over pathogens was observed by these authors.

The microbiota in organic crops can function as natural sources for the control of pathogens and the metabolic products generated starting this complex system. As shown by Badri et al. (2013) the microbiome composition of *Arabidopsis thaliana* leaves and the physical–chemical composition of the soil can affect the metabolome and, consequently, the plant microbiome. The effect of agricultural chemical controllers are discussed as factors that directly influence the diversity and survival of the natural microflora of plants, especially in studies related to mycorrhizae (Tommerup and Briggs 1981; Gosling et al. 2006).

One of the major issues related to intrinsic plant–endophyte relations is the connection between the diversity of plants, the microbial community in the soil and the plant, and the connection between these ecosystems (Zak et al. 2003; Gottel et al. 2011). The use of chemical controllers in agricultural crops (e.g., fungicides and bactericides, among others) may compromise access to some natural microbial strains of these cultures, affecting the biological balance of an ecosystem and, thus, making it difficult to access specific and susceptible groups of fungi and bacteria. Isolation of the genus *Ophiognomonia* in this work, in organic coffee farming, can be related to this factor, not affecting the natural microflora of the soil-plant complex. In addition to this, other genera with pathogen antagonist activity were observed.

According to Souza et al. (2004), generally, the knowledge generated by studies on plant–microorganism associations, has been of enormous value in understanding the role that these microorganism play in their hosts. Studies on the specificity and establishment of the phenomena of host infection, on the regulatory functions among endophytes and hosts, as well as on the control of this association, during the endophytic phase or not, could be useful to the use of endophytes as a biotechnological material to increase or improve plant production. In that point of view, working with endophytes of leaves of *Coffea arabica* L. cv. IAPAR-59 cultivated in organic system management we were able to show biotechnological potential to control diverse phytopathogens. Further experiments in vivo will be useful to identify the effective biotechnological application of endophytes in a phytosanitary area.

Conclusion

The results obtained in this work, with the identification for the first time of a highly diverse isolated endophytic of the species *Ophiognomonia* sp. in the coffee plants, is the second report of the detection of these fungi in Brazil.

The detection of different endophytic isolates with the ability to antagonise pathogens, such as *Colletotrichum* sp., speaks to future application of these microorganisms as

possible pathogenic fungi controllers in crops grown in the Parana state of Brazil, as they presented great potential, and since they were isolated in this specific habitat. They may remain on the crops (e.g., in coffee), reducing the need of successive applications of these controllers, emphasising the importance of others research involving the isolation, identification and exploration of endophytes of other genetic varieties of coffee obtained by breeding programs in Brazil.

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