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Exploring the diversity and potential interactions of bacterial and fungal endophytes associated with different cultivars of olive (*Olea europaea*) in Brazil

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ABSTRACT

The olive crop has expanded in the southeastern region of South America, particularly in Brazil. Thus, the objectives of this study were to identify the diversity of endophytic microorganisms associated with olive leaves with culture-dependent and culture-independent methods, to explore which factors influence the composition and abundance of this microbial community, to identify the trophic mode of these fungi by FunGuild and, to verify type associations between bacterial and fungal communities. Leaf samples were collected from 93 plants in nine locations in the Brazilian states of São Paulo and Minas Gerais. Leaves were first superficially disinfected before fungal isolation and next-generation metabarcoding sequencing was completed targeting the 16S rRNA regions for bacteria and ITS1 for fungi. In total, 800 isolates were obtained, which were grouped into 191 morphotypes and molecularly identified, resulting in 38 genera, 32 of which were recorded for the first time in cultivated olive trees in Brazil. For the isolated fungi, the most abundant trophic level was pathotrophic and for the culture-independent method was unidentified followed by symbiotrophic. The metabarcoding results revealed that factors such as plant age, altitudinal gradient, and geographic location can influence the microbial community of commercial olive plants, while the specific cultivar did not.

1. Introduction

Endophytes are microorganisms that live in plant tissue for at least part of their life cycle without causing harm to the host (Le Cocq et al., 2017; van Overbeek and Saikkonen, 2016), however, there is a disagreement in the literature about the definition. Some authors suggest that endophyte should refer only to its habitat and not its function (Hardoim et al., 2015). Theses microorganisms, mainly fungi and bacteria, have attracted increasing attention, as they have important properties, such as providing their hosts resistance under conditions of water stress and can influence the production of plant hormones and other compounds, such as enzymes and potential medical compounds (Araújo et al., 2002). Next generation sequencing (NGS) technologies have accelerated research by enabling the production of large volumes of sequence data at a drastically lower price per base, compared to traditional sequencing methods. These developments permit addressing research questions in plant-microbe biology that were not conceivable just a few years ago (Knief, 2014). The combination of cultivation-independent and improved cultivation technologies has allowed the exploration of uncultured groups living in association with plants (de Melo Pereira et al., 2012; Dissanayake et al., 2018). In addition surveys that infer the trophic level and guilds for fungi have been conducted to explore the functional diversity of microorganisms in the community in habitats such as soil and leaves (Pang et al., 2019; Skaltsas

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et al., 2019; Tedersoo et al., 2014). Currently a tool that can be used for this purpose is the FunGuild, this database estimates trophic levels, guilds and fungal growth mode (Nguyen et al., 2016). This permits the inference of trophic mode such as (1) pathotroph = individuals receiving nutrients through the destruction of host cells (including phagotrophs); (2) symbiotroph = individuals receiving nutrients through the exchange of resources with host cells; and (3) saprotroph = individuals receiving nutrients through the destruction of dead host cells (Nguyen et al., 2016).

The endophytic microbial community is influenced by numerous factors such as plant age, geography, altitude, soil, cultivar strain, climate and even different parts of the host such as roots and leaves (Bergelson et al., 2019; Carper et al., 2018; Nascimento et al., 2015; Siles and Margesin, 2017; Wemheuer et al., 2019). One plant that has increased in crop area in South America, but little is known about the diversity of the endophytic community, is the olive tree (*Olea europaea*). In Brazil, the southeast region is rapidly increasing olive cultivation, leading to the creation of the state government project OLIVA SP (http://www.apta.sp.gov.br/olivasp). This project aims to understand the factors that may influence the successful growing of olive trees in this Neotropical region including specific climate zones, the endophytic microorganisms, potential diseases, and the pests that attack the culture in agricultural settings.

Overall, numerous studies have been done investigating the microbial communities in plants (Carper et al., 2018; Wemheuer et al., 2019; Yao et al., 2019). Some studies with other perennial plants such as apple and pear have shown that the age of the plant and tissue can alter the endophytic microbial community (Arrigoni et al., 2018). In *Boechera stricta* leaves, age was an important factor for explaining endophytic bacterial composition. The endophytic community of the phyllosphere of *Populus deltoides* varied with leaf senescence (Redford and Fierer, 2009). Previous studies with olive have demonstrated that the endophytic and epiphytic community in different cultivars were distinct from each other and that diversity changes among the cultivars studied (Mina et al., 2020). Using phyllosphere-dependent methods on cultivars (Cobrançosa vs. Galega vulgar vs. Azeiteira) demonstrated distinct microorganism profiles among the olive cultivars (Materatski et al., 2019). Research on the richness of endophytic fungi along altitudinal gradients, is still much more poorly explored compared to other variables (Rojas-Jimenez et al., 2016). Olive crop production has high economic potential in Brazil, with new cultivation areas in the southern region reaching 11,000 ha in 2020 (IBRAOLIVA - Instituto Brasileiro de Olivicultura). Because olive cultivation is recent in Brazil, compared to its place of origin in the Mediterranean region, little is known about the community of endophytes associated with olive trees in Neotropical regions, particularly in Brazil. Therefore, this work will contribute to a better understanding of the endophytic microbial community of this perennial plant in the Neotropics. To expand our knowledge of the microbial diversity of this crop in Brazil we used cultivation-dependent and cultivation-independent methods, to characterize the endophytic microbial community of olive. The goals of our study were: 1) to identify the microbial communities of fungi (ITS) and bacteria (16S rRNA) in olive trees through metabarcoding amplicon sequencing; 2) to explore factors that can be influential on the composition and abundance of these communities such as age of the plant, altitude of the crop, cultivar identify, and geographic location; 3) to identify correlations between fungi and bacteria; and 4) to identify the fungal communities using culture-dependent methods to predict the trophic modes based on the FunGuild database in olive trees planted in Brazilian farms in the states of São Paulo and Minas Gerais.

2. Materials and methods

2.1. Study site and sampling of olive tree leaves

The study was carried out on farms located in the southeastern region of Brazil. In the state of São Paulo six properties were sampled and for the state of Minas Gerais three properties. In the state São Paulo, properties were located in the following municipalities: Cunha (CUN) - 23° 7′ 51’’S 44° 54’ 41’’W, Pinhalzinho (PIN) - 22° 43’ 40’’ S 46° 32’ 1’’ W, São Pedro (SPD) - 22° 31’ 42"S 47° 54’ 4"W, Pilar do Sul (PDS) - São Bento do Sapucaí (SBS) - 22° 37’ 25’’ S 45° 41’ 26’’ W and São Paulo (SP) - 23° 35’ 10’’ S 46° 38’ 58’’ W. In the state of Minas Gerais, the following properties were sampled: Extrema (EXT) - 22° 52’ 17’’ S 46° 19’ 29’’ W, Andradas (AND) - 21° 54’ 45’’ S 46° 22’ 48’’ W and Caldas (CAL) - 21° 54’ 45’’ S 46° 22’ 48’’ W (Figure S1).

The cultivars sampled in this study are of the same species *Olea europaea* and all seedlings were purchased from orchards in Brazil. However, the origin of each cultivar is distinct (Supplementary Table 1). Although, it is not known how each cultivar adapts to the different regions of Brazil, so planting with different cultivars shows high variance of fruit production across locations.

A total of 93 trees were sampled from nine farms (Supplementary Table 1). Subsequently, eight branches with healthy leaves were collected from each tree, thus make one pool per sample. Each cultivar was sampled in triplicate and were placed in sterile plastic bags, labeled, and transported in a refrigerated Styrofoam box until processing in the lab.

2.2. DNA extraction

Fresh leaves were first sterilized by immersion in 70% ethanol for 5 min, 2 % sodium hypochloride for 5 min, 70 % ethanol for 30 s and three washes in autoclaved water, according to (Araújo et al., 2002), after sterilized the leaves were freeze-dried using liquid nitrogen and homogenized using a sterilized mortar and pestle before then being transferred to a 1.5 mL tube with 600 μL cetyltrimethylammonium bromide extraction buffer (CTAB, 100 mM Tris–HCl, 1.4 M NaCl, 20 mM EDTA, 2-mercaptoethanol, pH 8.0) and incubated to 65 °C for 60 min. We added 600 μL of chloroform: isoamyl alcohol (24:1 ratio) into each tube and mixed thoroughly to form an emulsion. The mixture was centrifuged at 12.000 rpm for 15 min at 4 °C in a microcentrifuge and 500 μL of the aqueous phase was removed into a clean 1.5 mL tube. The supernatant was discarding and the pellet containing DNA was re-extracted with 70% ethanol twice and dried using an Eppendorf Vacufuge (Concentrator 5301; Hamburg, Germany) for 10 min or until dry. The DNA pellet was then resuspended in 70 μL sterile ultrapure water. The DNA concentration was measured using the Quantifluor (Promega, Lyon, France) staining kit, according to the manufacturer’s instructions.

2.3. Library construction for Illumina MiSeq 16S rRNA and ITS sequencing

The 16S rRNA /ITS1 library construction and Illumina MiSeq sequencing was a service provided by Genomics Facility Center (ESALQ, Piracicaba, SP, Brazil). Amplification of the V4 region of 16S rRNA (515 F 5′- GTGCCAGCMGCCGCGGTAA −3′/ 806 R 5′- GGACTACHVGGGTWTCTAAT −3′ primers) was performed in triplicate following Earth Microbiome Project (EMP) protocols (http://www.earthmicrobiome.org/emp-standard-protocols/) from (Thompson et al., 2017), also described in (Caporaso et al., 2011) and for the fungi we amplified the ITS region (ITS1f 5′- CTTGGTCATTTAGAGGAAGTAA −3′/ITS2 5′– CTTGGTCATTTAGAGGAAGTAA −3′) (Caporaso et al., 2012) (Gardes and Bruns, 1993; White et al., 1990). Three negative controls as blank samples (ultrapure water instead of DNA in the PCR reaction) were used to remove contaminants that may have been introduced in the process of obtaining the data. PCR reactions followed recommendations from the Taq Platinum Kit (Invitrogen, Schwerte, Germany) with 2.5 μL Buffer 10X, 1.0 μL $MgCl^2$ 50 mM, 1.0 μL dNTPs 10 mM, 1.0 μL BSA (Bovine Serum Albumin) 1 mg/mL, 0.5 μL each primer 10 μM, 0.5 μL Taq Platinum 5 U/μL, 5 μL template DNA (10 ng/μL), and

13.0 μL PCR Water (Certified DNA-free). The reaction was amplified on a BIO-RAD T-100 thermal cycler with the following conditions: 95 ºC for 3 min, 30 cycles at 95 ºC for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 10 min and, 4 °C hold. Purification was performed with AMPure XP beads (Beckman Coulter) and adapter ligations (NEXTERA XT, Illumina) were performed following the manufacturer's recommendations. Samples were quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Pooling and normalization were performed so that samples had the same concentration. The DNA pool was diluted to 2 nM, denatured, and then diluted to a final concentration of 16 pM with 20% PhiX for 2 × 250 pb sequencing on an Illumina MiSeq machine.

2.4. Bioinformatic analysis

Demultiplexed sequence data were analyzed using Qiime2–2019.7 (Bolyen et al., 2019) with plugin demux (Bolyen et al., 2019). Sequence quality control and feature table construction were performed using the Dada2 plugin (Callahan et al., 2016; McDonald et al., 2012). For the 16S rRNA libraries, the forward and reverse reads were input in to the pipeline, which started with the "dada2 denoise-paired" command, this method requires two parameters that are used in quality filtering (Callahan et al., 2016). In this step, there was a truncation by read length with commands "–p-trim-left-r = 21 \–p-trim-left-f = 20 \ –p-trunc-len-r = 239 \ –p-trunc-len-f = 244″ and for the ITS1, there was a truncation by read length "–p-trim-left-r = 13 \ –p-trim-left-f = 19 \ –p-trunc-len-r = 171 \ –p-trunc-len-f = 230″. Bacterial taxonomic assignment of contigs was carried out using a Naïve Bayes classifier trained on SILVA version 132 (Quast et al., 2013). The classifier was set to include V4 regions of 16S rRNA genes at 99% sequence similarity (Quast et al., 2013; Yilmaz et al., 2014). The sequence reads from chloroplasts and mitochondria were removed from the final set of Amplicon Sequence Variants (ASVs). For fungal communities, primers ITS1f/ITS2 were employed and taxonomic assignment was conducted with the UNITE database (Nilsson et al., 2019). Thereby, a classifier was created using the "feature-classifier fit-classifier-naive-bayes" command. Once the classifier was obtained, reads (rep-seqs) were classified by taxon using the "feature-classifier classify-sklearn" command (Bokulich et al., 2018) and finally a feature table were obtained. Following the recommendations of Łukasik et al. (2017), three negative controls were included, and contaminants were removed through the Decontam package (Davis et al., 2017) using R software (R Core Team, 2019). The prevalence method removed the largest number of contaminants and therefore was applied to our samples. These decontaminated data were then brought back into Qiime2 where subsequent filtering also removed mitochondria and chloroplast from the feature-tables. The feature-table summarize command created a visual summary of the 16S rRNA and ITS data. For the 16S rRNA dataset, inference of the phylogenetic tree was performed using fragment-insertion SEPP (Mirarab et al., 2011) and for the ITS dataset, inference was performed using the align-to-tree-mafft-fasttree command (Katoh and Standley, 2013), all in Qiime2. Raw sequence reads are available on the NCBI Short Read Archive under BioProject PRJNA790928.

2.5. Statistical analysis

The statistical analysis was made using variables such as plant age, altitudinal gradient, cultivars of olive and geographic location and were tested under these alpha diversity metrics. Alpha diversity metrics were computed to measure the richness of the communities within samples: Shannon index and Observed ASV, these metrics were all computed in QIIME2 version 2019.7 (Bolyen et al., 2019) using the Kruskal-Wallis command "p- pairwise" for pairwise comparison test to determine which specific pairs of groups differ from each other. We considered composition and abundance of all variables tested such as plant age, altitudinal gradient, geographic location and cultivars of the olives tree. In the beta diversity analyses in QIIME2 implemented using the command "beta-group-significance", the beta diversity analysis between samples were measured with a permutational multivariate analysis of variance (PERMANOVA) on weighted and unweighted Unifrac distance matrices as well as a Bray-Curtis distance metric with 999 permutation with Bonferroni correction (McArdle and Anderson, 2001). Principal coordinate analysis ordination (PCoA) was calculated based on the Bray-Curtis dissimilarity matrix using implemented statistics in the PAST3 software (Clarke, 1993; Hammer et al., 2001; McMurdie and Holmes, 2013). In addition, through the Simper analysis we explored the contribution of the main ASVs in the present study. The SIMPER analysis calculates the contribution of each species (%) to the dissimilarity between the two groups. It is calculated from the Bray-Curtis dissimilarity matrix. This analysis shows the contributions of each ASV in descending order and cumulative, this analysis was also implemented in the software PAST3 (Hammer et al., 2001). To test if there are any differentially abundant taxa in our sample groups (i.e. bacteria from hosts of different plant ages and localities) we used a statistical power analysis ANCOM plugin (analysis of composition of microbiomes) implemented in QIIME2 (Mandal et al., 2015). FunGuild was used to compare and identify functional guilds (Nguyen et al., 2016).

2.6. Correlation analysis between bacterial and fungal communities using culture-independent methods

We searched for specific correlations between bacterial ASVs and fungal ASVs by performing a Regularized Canonical Correlation Analysis (rCCA). We used the matrix with taxon and occurrence of both groups and multiple data set integration was done using rCCA and network functions from the mixOmics package (Gonzalez et al., 2008; (Rohart et al., 2017) implemented in the R software (R Core Team, 2019). The correlation analysis was done with threshold 0.7 and only the significant correlations were used. The network was edited and visualized in the software Cytoscape (Shannon et al., 2003).

After the results of the correlation analyses, we separated the bacterial and fungal ASVs involved in the positive and negative correlations. Next, we obtained taxonomic information of the sequences from these correlations (Table S3) using Blast-n (Genbank), as well as additional information when available such as collection environment, microorganism occurrence site and host. For the sets containing bacterial and fungal sequences from each type of correlation we preformed phylogenetic analysis using maximum likehood. The dataset of sequences that was recovered in the correlation analyses (negative and positive) between bacteria and fungi were submitted to the ModelFinder plugin (Kalyaanamoorthy et al., 2017) which determines the best fit model for the type of data, this plugin is available on the IQTree website (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos et al., 2016). Subsequently phylogenetic analyses were carried out with different models. For the sequences involved in the negative correlation the model for the bacterial ASVs was TIM3e+G4 and for the fungal ASVs was K2P+I. For the sequences involved in the positive correlation the model used for the bacterial ASVs was TIM2e+I+G4 and for the fungal ASVs was K2P+I. The trees were visualized and edited in iTOL (Letunic and Bork, 2019).

2.7. Culture-dependent method

Leaf surfaces were washed with mild detergent and running tap water, and then air-dried. Leaves were surface sterilized by successively immersion in 70% ethanol for 5 min, 2 % sodium hypochloride for 5 min, 70 % ethanol for 30 s and three washes in autoclaved water, according to Araújo et al. (2002). The surface-disinfected fragments were placed on potato dextrose agar (PDA) (supplemented with 500 mg/L pentabiotic to suppress bacterial growth) and incubated at 25 °C until the growth of endophytic fungi was discerned. Hyphal tips originating from plant segments were transferred to PDA plates without antibiotics. Each fungal isolate was checked for purity and transferred to new

medium by the hyphal tip method. For each olive tree, five plates with 10 sterilized leaf fragments were prepared. Isolated fungi were grouped according to colony morphology and submitted to molecular identification.

2.8. Obtaining DNA sequences from mycelia

Genomic DNA was extracted from fresh cultures using a modified protocol of (Doyle and Doyle, 1987). Fresh fungal mycelia were scraped from the surface of the Potato Dextrose and Agar (PDA) plate and transferred into a 1.5 mL microcentrifuge tube with 600 μL of preheated (65 °C) CTAB extraction buffer (2% w\v CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0). Fungal mycelia were ground using plastic pestles and then incubated in a heat block at 65 °C for 60 min with occasional mixing. An equal volume of chloroform: isoamyl alcohol (24:1) was added to each tube and mixed thoroughly in a vortex. The mixture was centrifuged at 12,000 g for 15 min at room temperature and 500 μL of the aqueous phase was transferred into a fresh 1.5 mL tube. After adding 300 μL of 100% isopropanol, microtubes were mixed by inversion and centrifuged at 12,000 g for 10 min. Supernatant was discarded, the DNA pellet washed twice with 70% ethanol and dried in a vacuum centrifuge for 10 min. The DNA pellet was then resuspended in 70 μL sterile water.

Polymerase chain reaction (PCR) was performed to amplify the ITS (internal transcribed spacer) region using universal primers ITS1-F (5′-TCCGTAGGTGAACCTGCGG −3′) and ITS-4R (5′- TCCTCCGCTTATT-GATATGC −3′) and the following thermocycler conditions: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min; and final extension at 72 °C for 5 min (White et al., 1990). The sizes of the amplified products were confirmed by 1 % agarose gel electrophoresis. PCR products were purified using Illustra™ PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequencing was performed with Big Dye Terminator Cycle Sequencing kit 3.1 (Applied Biosystems), according to manufacturer's recommendation and ran in an ABI 3500 xL Genetic Analyzer (Applied Biosystems).

ITS sequences were aligned using Clustal-X software and manually edited using Bioedit (Hall, 1999). Nucleotide sequence BLAST searches were performed using the National Center for Biotechnology Information (NCBI) GenBank database to identify the closest species for each fungal strain.

3. Results

3.1. Bacterial community analysis through culture-independent methods

Overall, 6,378,200 reads were obtained and after removing low-quality sequences, mitochondrial, chloroplast, and singletons, 134 Amplicon sequence variants (ASVs) were recovered. The bacterial community recovered *Bacteroides* as the predominant bacterial genus (15 %), followed by *Lactobacillus* (13%), *Stenotrophomonas* (10 %), *Phascolarctobacterium* (10 %), *Listeria* (5%), *Achromobacter* (4 %) and others in smaller quantities. These six genera corresponded to 57 % of the entire community. Fig. 1 shows the 10 ASVs of bacteria endophytes most commonly recovered in our samples of olive tree leaves. We included the most specific taxonomic level possible for the identification of this group, which for these samples was kingdom, order, and genus levels.

3.2. Alpha diversity of the bacterial communities in olive tree leaves

To test for differences in alpha diversity we used Observed_ASV as it is considered a qualitative measure of community richness. No significant differences were found when plant age was tested Observed_ASV (Kruskal-Wallis H = 5.533, p-value = 0.236). The same was observed for altitude (H = 0.109, p-value = 0.946), geographic location (H = 6.443, p-value = 0.597) and for the effect of cultivars (H = 3.643, p-value = 0.456).

3.3. Beta diversity of the bacterial communities in olive tree leaves

Beta diversity results showed that age influences the bacterial community in olive tree leaves (PERMANOVA Bray-Curtis: all groups Pseudo-F = 2.058, p-value = 0.005, Fig. 2A). Each age group was also compared in pairs, and significant results were obtained when bacterial communities associated with plants of ages four, five and seven years were compared to eight-year-old plants. When age eight was removed from the analysis, differences were no longer significant (Bray-Curtis Pseudo-F = 1.037, p-value = 0.385).

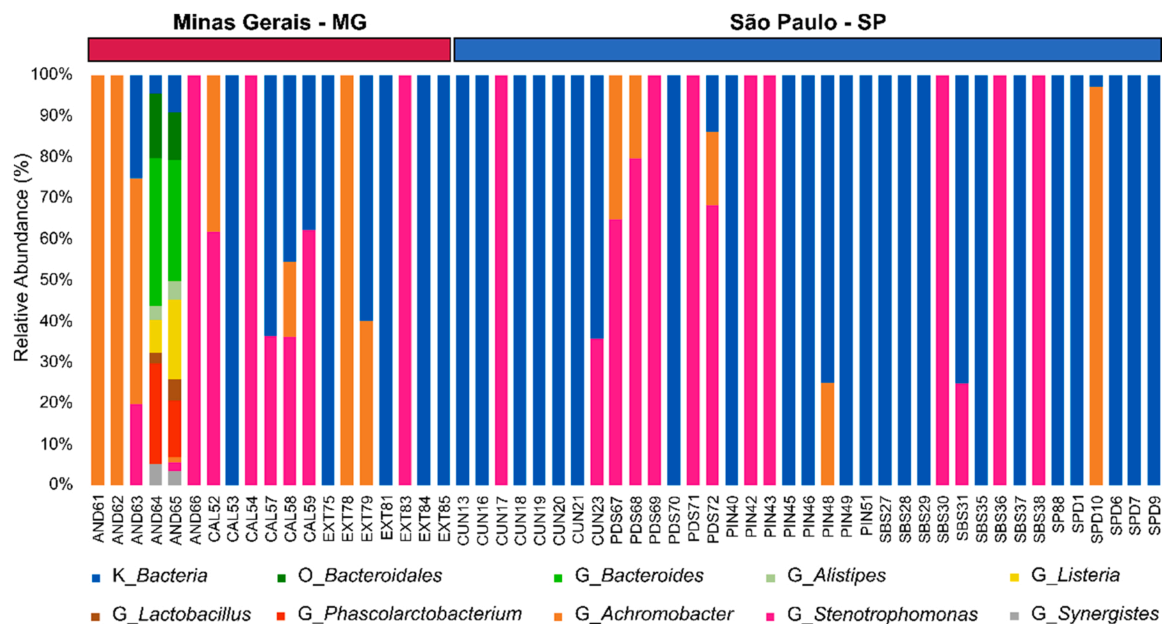

Fig. 1. Relative abundance of main bacterial ASVs recovered from each sample of olive tree leaves. Each sample represents a different tree. Bar graphs (one column = community from a sample). Each color represents a distinct bacterium at the most specific taxonomic level (K=Kingdom; O=Order and G= Genera). The horizontal bars on the top indicate farms in the states of Minas Gerais (pink) and São Paulo (blue).

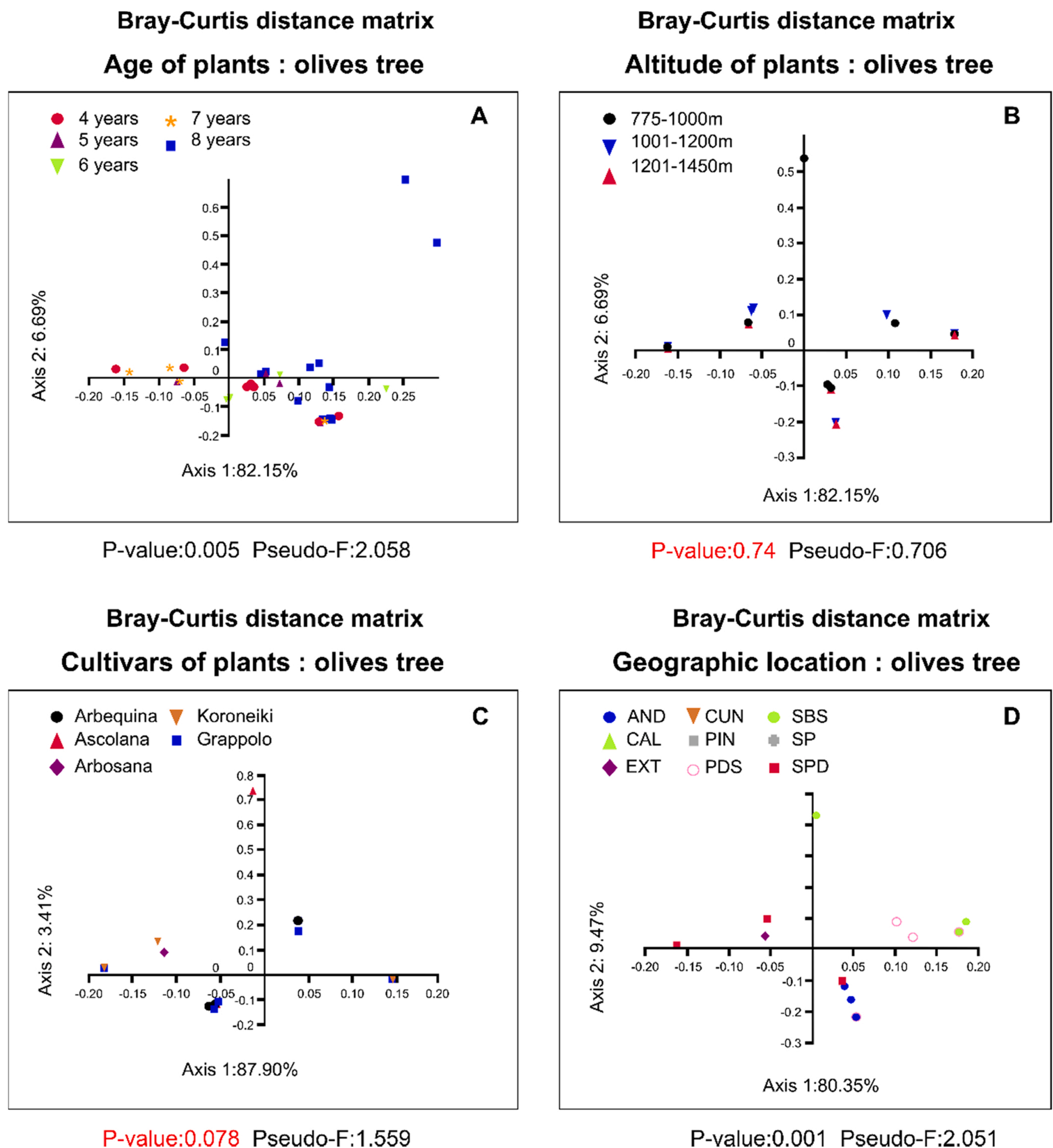

Fig. 2. Beta diversity results with Principal coordinates analysis (PCoA) with Bray-Curtis distance illustrating bacterial community structure among leaves of the olive tree. **A.** PCoA for age of trees, each color represents a different age group. **B.** PCoA for altitude of farms where plants were collected, each color represents a different altitude. **C.** PCoA for cultivar, each color represents a different cultivar of olive tree. **D.** PCoA for geographic location influencing bacterial communities, each color represents a different farm, the abbreviations are of the municipalities of São Paulo states: Cunha (CUN), Pinhalzinho (PIN), São Pedro (SPD), Pilar do Sul (PDS), São Bento do Sapucaí (SBS) and São Paulo (SP). In the state of Minas Gerais, properties were located in: Extrema (EXT), Andradas (AND) and Caldas (CAL). The statistics highlighted in red show p-values greater than 0.05.

To explore this result more deeply, through SIMPER analysis, the main bacteria (ASVs) associated with age eight-year-old plants, contributing to the differences observed, were *Stenotrophomonas*, followed by *Achromobacter* (Table 1). In the ANCOM analyses the genus *Stenotrophomonas* (W = 43) was also identified as the main contributor to differences observed.

Differences in beta diversity were not found when altitude was tested (PERMANOVA Bray-Curtis distance: Pseudo F = 0.706, p-value = 0.740). The same was observed when the cultivars were tested (PERMANOVA Bray-Curtis distance: Pseudo F = 1.559, p-value = 0.078). However, the beta diversity analysis demonstrated the influence of the geographic location on the composition of the bacterial communities

Table 1
SIMPER analysis of the main bacteria with the ASVs more frequents. This test indicates the contribution of specific ASVs to the observed differences in community structure among different ages of olive trees.

Age of olive trees	p-value	Pseudo-F	Overall Average Dissimilarity	Most Influential ASVs	Contribuition to Difference (%)	Cumulative (%)
Four vs. Eigth (N = 28)	0.001	4.921	89.10 %	*Stenotrophomonas*	47.14	47.14
				Achromobacter	29.16	76.30
Five vs. Six (N = 23)	0.05	2.308	69.78 %	*Stenotrophomonas*	55.29	79.23
				Achromobacter	14.49	20.77
Five vs. Eigth (N = 28)	0.005	5.060	86.17 %	*Stenotrophomonas*	49.73	49.73
				Achromobacter	31.37	81.09
Seven vs. Eigth (N = 21)	0.02	2.714	82.39 %	*Stenotrophomonas*	50.45	50.45

(PERMANOVA Bray-Curtis distance: Pseudo F = 2.051, p-value = 0.001). The SIMPER analysis showed that the most frequent bacteria were *Stenotrophomonas* and *Achromobacter*. The cumulative contribution of both account for more than 50% of the differences between counties (Table 2).

The ANCOM analyses showed that the taxa responsible for the differences in beta diversity between counties was the genus *Stenotrophomonas* (W = 73).

The principal coordinates analysis (PCoA) with the Bray-Curtis distance matrix of the bacterial communities revealed no significant influence of altitude and cultivar, but the effect of plant age and geographic locality were important for influencing the abundance and composition of bacterial communities in leaves olive trees (Fig. 2).

3.4. Fungal community analyses through culture-independent methods

Overall, 4,430,548 reads were obtained and after removing low-quality, chimeric and singletons ITS sequences, 2475 taxa were recovered and grouped into 661 amplicon sequence variants (ASVs) of fungi associated with leaves of olive trees. The main taxa recovered revealed Dothideomycetes as the predominant fungi class (56%), followed by Leotiomycetes (9 %), Tremellomycetes (8 %), Cystobasidiomycetes (8 %) and others with lower percentages. These four classes together corresponded to 81 % of all ASVs. At the Kingdom level 31 % were unidentified Fungi, at the Order level, the most frequent ASVs were Venturiales (12 %) and Capnodiales (7 %), and at the Genus level were *Pseudocercospora* (7 %), *Hyphozyma* (6%), *Symmetrospora* (3 %), *Genolevuria* (2%), *Nigrospora* (1 %) and *Catenulostroma* (1%) corresponded to 74 % of all ASVs. Fig. 3 shows the 10 ASVs of fungi endophytic more common that we recovered in samples on olive tree leaves. We consider the highest taxonomic level possible for the identification of this group. This way, we had a kingdom, orders, and genera levels.

3.5. Alpha diversity of the fungal communities in olive tree leaves

We found that alpha diversity for abundance and composition were influenced by plants age according to Observed_ASV (H = 14.813, p-value = 0.005). Altitude did not show significant differences according to Observed_ASV (H = 0.503, p-value = 0.777). The same was observed for cultivar as a factor according to Observed_ASV (H = 1.480, p-value = 0.830). Results for geographic location were significant according to Observed_ASV (H = 39.691, p-value = 0.001) (Figure S2).

Table 2
SIMPER analysis of the ASVs contributing to differences between geographic locations of groups of olive trees. All the geographic locations in this table had significant results in the Bray-Curtis distance matrix.

Geographic Location *	p-value	Pseudo-F	Overall Average Dissimilarity	Most Influential ASVs	Contribution to Difference (%)	Cumulative (%)
AND vs. CAL (N = 12)	0.022	3.630	77.53 %	*Achromobacter*	29.84	29.84
				Stenotrophomonas	28.66	58.5
AND vs. CUN (N = 14)	0.015	2.700	94.2 %	*Achromobacter*	42.18	42.18
				Stenotrophomonas	23.23	65.41
AND vs. EXT (N = 14)	0.043	2.556	89.32 %	*Achromobacter*	41.19	41.19
				Stenotrophomonas	22.32	63.5
AND vs. PIN (N = 14)	0.008	3.240	92.22 %	*Achromobacter*	41.06	41.06
				Stenotrophomonas	23.61	64.67
AND vs. SBS (N = 15)	0.021	2.826	91.09 %	*Achromobacter*	40.48	40.48
				Stenotrophomonas	23.77	64.25
AND vs. SPD (N = 13)	0.005	4.148	99.79 %	*Achromobacter*	39.47	39.47
				Stenotrophomonas	18.57	58.04
CAL vs. CUN (N = 14)	0.038	2.757	71.04 %	*Stenotrophomonas*	84.47	84.47
CAL vs. EXT (N = 14)	0.034	3.476	81.36 %	*Stenotrophomonas*	77.12	77.12
CAL vs. SPD (N = 13)	0.005	5.030	85.71 %	*Stenotrophomonas*	71.66	71.66
CUN vs. PDS (N = 14)	0.019	3.032	77.11 %	*Stenotrophomonas*	84.06	84.06
EXT vs. PDS (N = 14)	0.031	3.303	84.08 %	*Stenotrophomonas*	77.04	77.04
PDS vs. PIN (N = 15)	0.028	3.196	80.31 %	*Stenotrophomonas*	81.15	81.15
PDS vs. SPD (N = 13)	0.003	5.293	85.71 %	*Stenotrophomonas*	71.99	71.99

*Label: AND (Andradas), CAL (Caldas), EXT (Extrema) for Minas Gerais state; CUN (Cunha), PDS (Pilar do Sul), PIN (Pinhalzinho), SBS (São Bento do Sapucaí) and SPD (São Pedro) for São Paulo state.

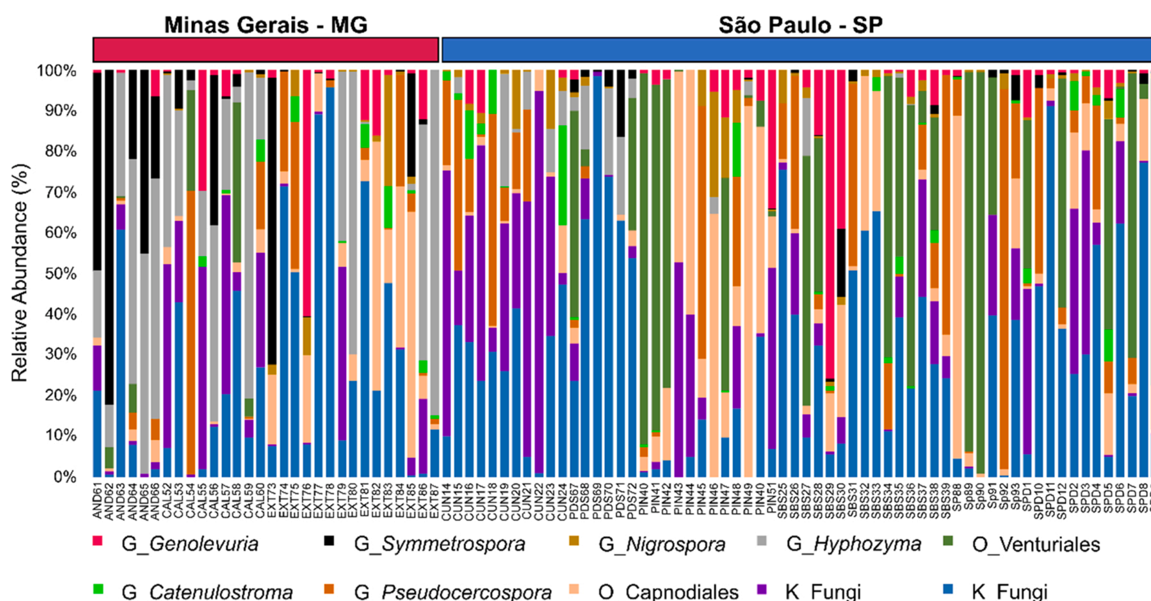

Fig. 3. Relative abundance of main fungal ASVs recovered from each sample of olive tree leaves. Each sample represents a different tree. Bar graphs (one column = community from a sample). Each color represents a distinct fungal at the most specific taxonomic level (K=Kingdom; O=Order and G= Genera). The horizontal bars on the top indicate farms in the states of Minas Gerais (pink) and São Paulo (blue).

3.6. *Beta diversity of fungal communities in olive tree leaves*

To explore if any factors influenced the beta diversity of fungi in olive tree leaves, we found age as a factor influencing the fungal community (PERMANOVA Bray-Curtis distance: Pseudo-F = 2.481, p-value = 0.001). Subsequently, SIMPER analysis showed *Pseudocercospora*, *Hyphozyma* and *Symmetrospora* as the most influential genera contributing to differences between fungal communities of plants with different ages (Table S1).

ANCOM analysis showed that the main fungi responsible for differences between plants of different age groups were in the order Venturiales (W = 221); family Mycosphaerellaceae (W = 224); and the following genera: *Hyphozyma* (W = 225); *Cyphellophora* (W = 223); *Erythrobasidium* (W = 222); *Glomerella* (W = 207).

Altitude also influenced the composition and abundance of fungal communities in olive tree leaves (Bray-Curtis Pseudo-F = 2.503 p-value = 0.001). Through SIMPER analysis, the main fungi (ASVs) associated with altitudes, contributing to the differences observed, were *Hyphozyma* and *Pseudocercospora* (Table 3).

Cultivars do not influence to beta diversity abundance and composition of fungi in leaves of olive trees (PERMANOVA Bray-Curtis distance: Pseudo-F = 0.812, p-value = 0.928).

Geographic location was found to influence the diversity of fungal communities (PERMANOVA Bray-Curtis distance: Pseudo-F = 2.364, p-value = 0.001). SIMPER analysis showed *Hyphozyma*, *Pseudocercospora*, *Symmetrospora*, and *Genolevuria* as the most influential genera contributing to dissimilarity between fungal communities between counties (Table S2). ANCOM analysis for beta-diversity, the main fungal taxa responsible for the differences found between counties were the family Mycosphaerellaceae (W = 222) and the genera *Hyphozyma* (W = 225); *Cyphellophora* (W = 225); *Symmetrospora* (W = 224); *Erythrobasidium* (W = 223); *Glomerella* (W = 223), *Sporobolomyces* (W = 216); *Didymella* (W = 205) and unidentified fungi (W = 211).

The principal coordinates analysis (PCoA) with the Bray-Curtis distance matrix revealed cultivar as not significant, but plant age, altitude and geographic location were important in influencing the abundance and composition of fungi in leaves of olive trees (Fig. 4).

3.7. *Interactions between bacterial and fungal endophytes communities*

In addition to the analyses of community diversity and composition, a network analysis was constructed using only statistically significant correlations ($p \geq 0.05$). Positive correlations were found between bacteria and fungi more frequently than negative ones (Fig. 5).

Microorganisms can correlate positively, neutrally, or negatively for numerous reasons, such as occurrence in the same place or competition

Table 3
SIMPER analysis of main fungal genera contributing to differences between altitude groups of olive plants.

Altitude	p-value	Pseudo-F	Overall Average Dissimilarity	Most Influential ASVs	Contribution to Difference (%)	Cumulative (%)
A: 774–1000 m vs. B: 1001–1200 m (N = 75)	0.008	2.121	88.46 %	*Hyphozyma*	24.9	24.9
				Pseudocercospora	17.72	42.61
				Cyphellophora	12.53	55.14
A: 774–1000 m vs. C: 1201–1450 m (N = 63)	0.001	2.921	86.42 %	*Pseudocercospora*	18.83	18.83
				Hyphozyma	12.97	31.8
				Genolevuria	10.31	42.11
				Symmetrospora	8.493	50.6
B: 1001–1200 m vs. C: 1201–1450 m (N = 48)	0.001	2.540	84.11 %	*Hyphozyma*	23.31	23.31
				Pseudocercospora	16.69	40
				Cyphellophora	10.48	50.48

*Label: Altitude A = 774 – 1000 m, Altitude B = 1001 − 1200 m and Altitude C = 1201–1450 m.

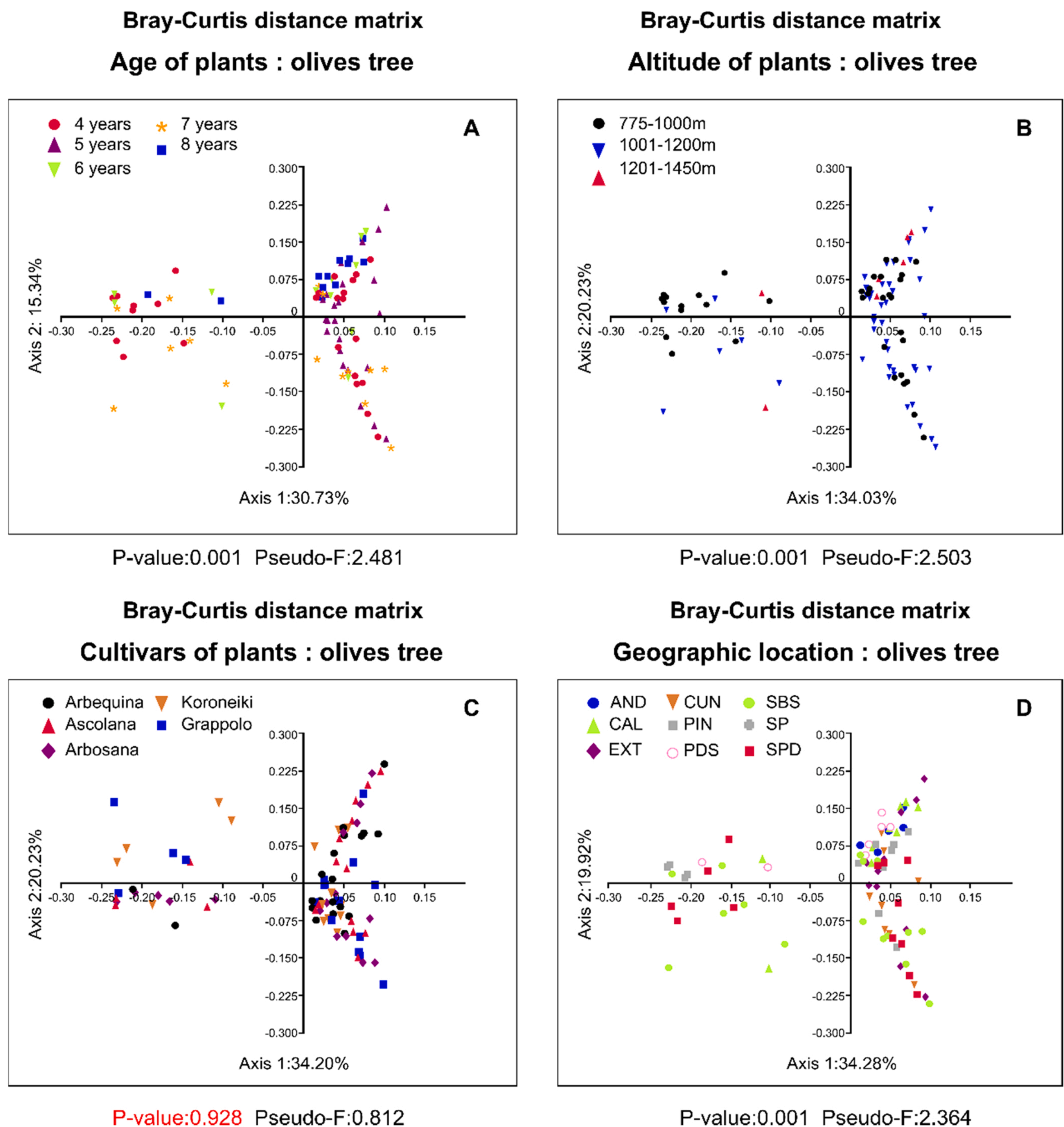

Fig. 4. Beta diversity data with Principal coordinates analysis (PCoA) with Bray-Curtis distance illustrating fungal community structure among leaves of the olive tree. A. PCoA for age of trees, each color represents different ages. B. PCoA for altitude of farms where leaves were collected, each color represents a different altitude. C. PCoA for cultivar, each color represents different cultivars of olive trees. D. PCoA for geographic location influencing fungal communities, each color represents different farms, the abbreviations are of the municipalities of São Paulo states: Cunha (CUN), Pinhalzinho (PIN), São Pedro (SPD), Pilar do Sul (PDS), São Bento do Sapucaí (SBS) and São Paulo (SP). In the state of Minas Gerais, properties were located in: Extrema (EXT), Andradas (AND) and Caldas (CAL). The statistics highlighted in red show p-values greater than 0.05.

for niche and nutrients. The types of associations between fungi and bacteria can be classified as harmonic (symbiosis) or disharmonic (dysbiosis). Of the bacterial and fungal ASVs involved in the negative correlation in Fig. 6 only 16 bacterial ASVs could be identified. For the fungal community involved in the negative correlation all 5 ASVs were reported to be isolated from plant tissues and 3 ASVs were taxonomically assigned to the order Venturiales and identified as Mycosphaerellaceae are foliar pathogens commonly found in olive crops.

The bacteria involved in negative correlations (N = 16), such as *Conexibacter*, *Burkholderia*, Betaproteobacteriales_2, Elsterales, and *Methylobacterium* were similar to bacteria from leaves, rhizosphere and soil. Also, bacteria such as *Planctomycetia*, *Edaphobacter*, *Gemmata*, Bacteria_15 were related to bacteria from soil and rhizosphere. Fungi

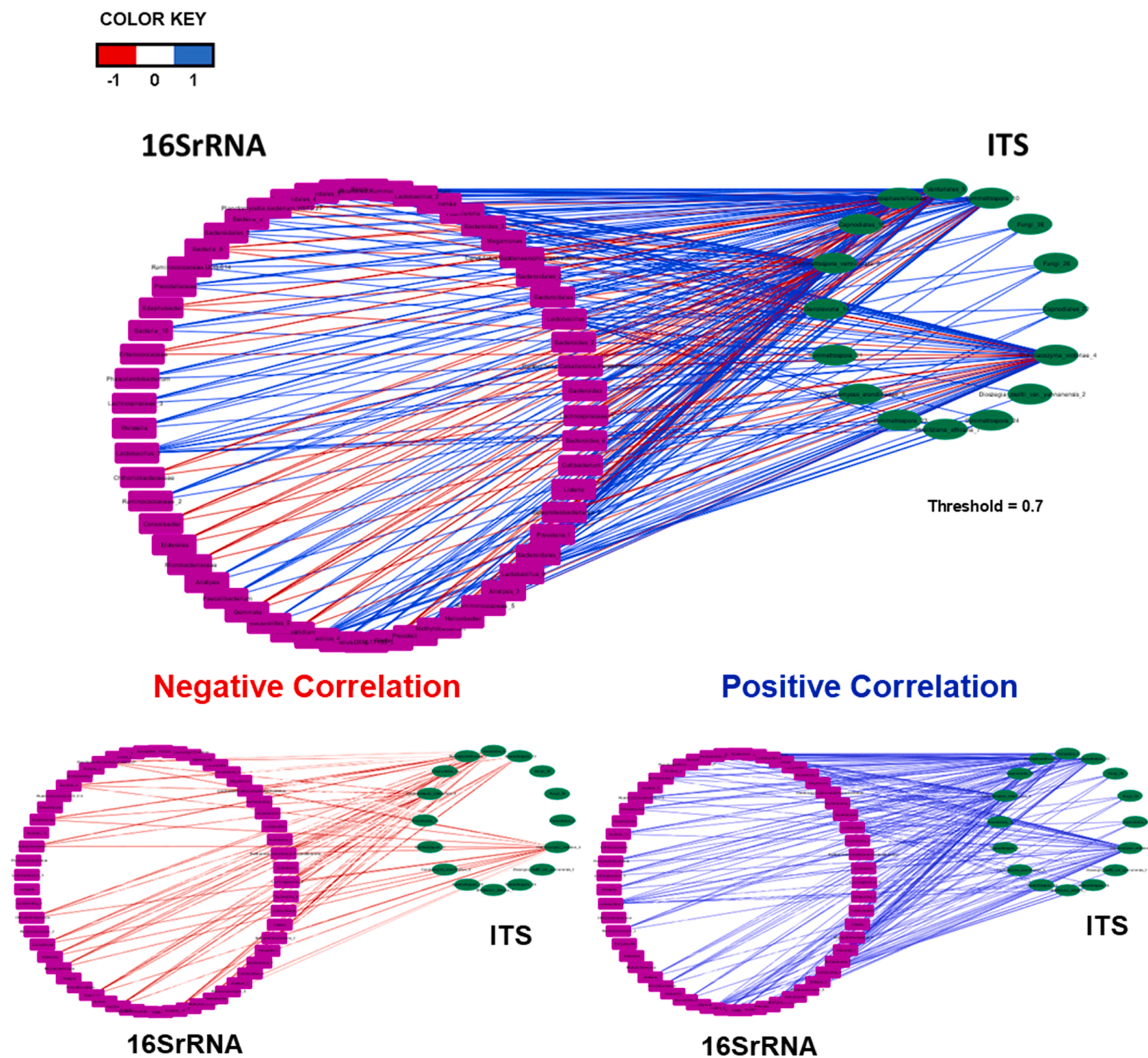

Fig. 5. A. Analysis of correlation between bacterial (violet rectangles) and fungal (green ovals) communities, B. Negative correlations are highlighted, and C. Positive correlations are highlighted.

involved in negative correlations such as Venturiales_3 and Mycosphaerellaceae_7 were similar to fungi found in olive trees and other plants. Other fungi such as *Symmetrospora*_10, *Symmetrospora vermiculata*_8 and *Vishniacozyma victoriae*_4 were similar to fungi found in maize, sugarcane and rice.

Thirty-nine bacterial ASVs were identified in the positive correlations (Fig. 7) and were similar to strains commonly found in the gut, intestine and feces of vertebrates such as cow, chicken, mouse and *Homo sapiens*, not showing specificity to a host species. Among the 16 fungal ASVs involved in positive correlations, most were similar to species associated with plants (Table S2).

Fungi of the Order Tremellales highlighted in the phylogeny (*Carcinomyces arundinariae*_8, *Genolevuria*, *Dioszegia, Vishniacozyma victoriae*_4) are found in the phyllosphere of maize and olive trees and three ASVs of the genus *Symmetrospora* (Symmetrospora_21, *Symmetrospora*_24 and *Symmetrospora vermiculata*_8) are also found in the phyllosphere of maize and olive trees and in mangrove areas.

The relation among fungi that are plant pathogens in the Order Venturiales (*Venturia oleaginea*, syn. *Fusicladium oleagineum*) that are considered important leaf pathogen of olive trees and the order Capnodiales are abundant in the plant phyllosphere. Members of the family Mycosphaellaceae are leaf pathogens of crop plants such as *Eucalyptus globulus* and also of olive trees.

3.8. Diversity of endophytic fungi recovered through culture-dependent methods

Approximately 800 fungal isolates were obtained and morphologically separated into 191 groups. One isolate of each group was submitted to ITS amplicon sequencing. The endophytic fungi were assigned to seven classes of which Sordariomycetes (59 %) and Dothideomycetes (33 %), were the most frequent. Thirty-eight genera were recovered among which *Diaporthe* (26 %), *Colletotrichum* (11 %), *Xylaria* (9 %), *Alternaria* (5 %) and *Neofusicoccum* (5 %) were the most frequent. These five genera accounted for 56 % of the diversity of the whole fungal community (Table 4).

3.9. Trophic mode of endophytic fungi through culture-independent methods

According to the FunGuild database, the endophytic fungi were assigned to four trophic modes: pathotroph, saprotroph, symbiotroph and unidentified for isolated fungi. The most common trophic mode was unidentified (51 %) followed by symbiotrophic (21 %), pathotrophic (15

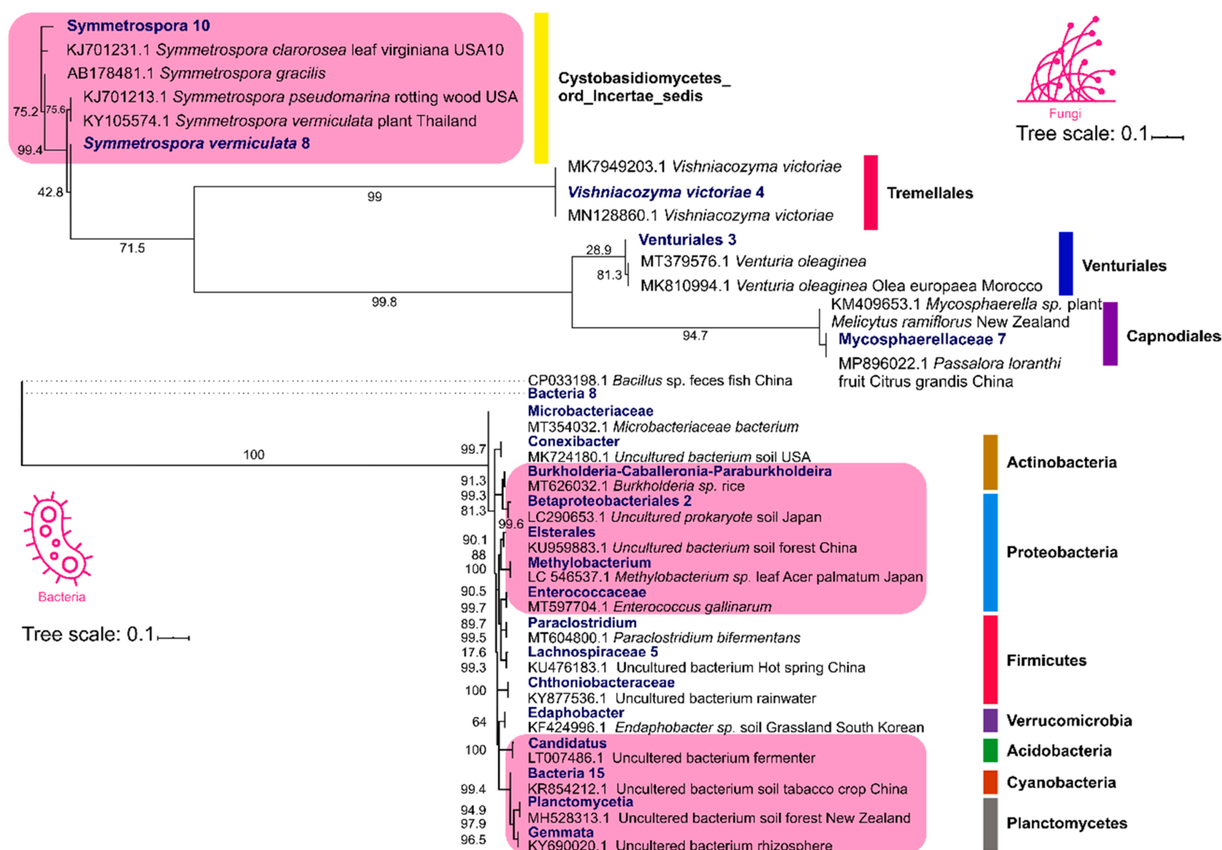

Fig. 6. Phylogenetic trees of bacterial and fungal ASVs that showed negative correlation in the network analysis. ASVs with names in colored letters are from this study and in black letters are from the GenBank. Fungal ASVs (substitution model K2P + I). Bacterial ASVs (substitution model TIM3e+G4). The pink boxes highlighted in the images, refer to the GenBank sequences that had information about the host or isolated location and that were correlated with plant materials e.g., wood, leaf, fruit, roots, etc.

%) and saprotroph with 13 %, (Fig. 8 B). In all counties, the pathotrophs were predominant with frequencies between 45 % and 80 % (Fig. 8A).

3.10. Trophic mode of endophytic fungi through culture-dependent methods

According to the FunGuild database, the endophytic fungi were assigned to four trophic modes: pathotroph, saprotroph, symbiotroph and unidentified for isolated fungi. The most common trophic mode was pathotrophic (85 %) followed by saprotrophic (8 %), unidentified (6 %) and symbiotroph with 1 %, (Fig. 9B). In all counties, the pathotrophs were predominant with frequencies between 65 % and 100 % (Fig. 9A).

4. Discussion

4.1. Culture-independent bacterial and fungal community analyses

Many studies using bacterial 16S rRNA and fungal ITS sequences have improved our understanding of the relationship between microorganisms that live in association with plants. This study contributes to our understanding of the diversity (composition and abundance) of endophytic bacterial and fungal communities in olive tree leaves. We investigated factors that may influence this microbial endophytic diversity. Plant age and geographic location affected both groups of microorganisms while altitude only impacted the fungal community.

Alpha diversity analysis demonstrated that the phylum Proteobacteria was the most abundant in olive tree leaves. This finding corroborates the results obtained in other studies of the endophytic bacterial diversity in olive leaves in cultivated and wild plants (Müller et al., 2015) and in conventional and sustainable orchards (Fausto et al., 2019).

4.2. Age influences the diversity of bacterial and fungal communities

Age was important for both composition and abundance of bacterial and fungal communities. Previous studies revealed that age was also an important factor affecting diversity of bacterial communities in other plants such as *Pinus flexilis* (Carper et al., 2018) and *Panax ginseng* (Hong et al., 2019). In *Panax ginseng*, an increase in endophytic bacterial diversity was observed in older plants, corroborating our results with olive trees. Eight- year- old plants have a bacterial community that is distinct from those observed in younger plants, with *Stenotrophomonas* and *Achromobacter* being the most influential bacteria contributing to dissimilarity. The genus *Stenotrophomonas* is commonly found in the phyllosphere of *Arabidopsis thaliana*, clover, and soybean (Delmotte et al., 2009) and roots of rice (Zhu et al., 2012). Whereas the bacteria *Achromobacter* and *Stenotrophomonas* have been reported to be present on the leaf surface of *Hedera helix* (Schreiber et al., 2005). *Achromobacter* species are frequently found in screenings for endophytic plant growth promoting bacteria and phytoremediation (Ho et al., 2012; Jha and Kumar, 2009).

The age of eight years is reported as an important period for olive trees. This can be explained by the physiology of the plant in the Southeast region of Brazil, where plants at this age and onwards are considered in the adult phase and show stability in production (Oliveira, 2012).

The increase in richness of fungal endophytes in leaves along with the plant's age has also been reported for *Coccoloba cereifera*

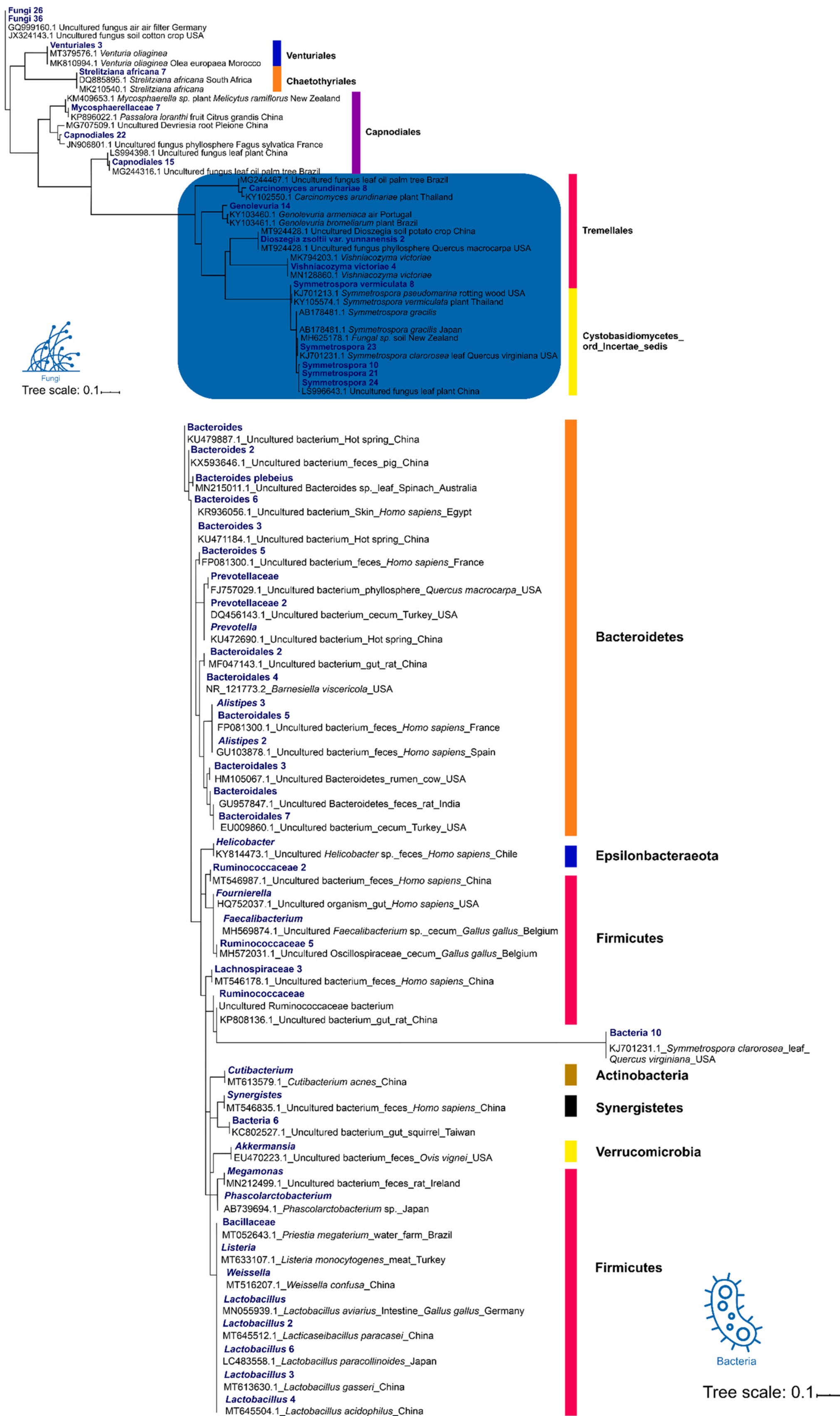

Fig. 7. Phylogenetic trees of bacterial and fungal ASVs that showed positive correlation in network analyses. ASVs with names in colored letters are from this study and in black letters are from GenBank. Fungal ASVs (substitution model TIM3e+G4). Bacterial ASVs (substitution model TIM2e+I+G4). The blue box highlighted in the image, refers to the GenBank sequences that had information about the host or isolated location and that were correlated with plant materials e.g., soil, leaf, fruit, roots, parts of plants etc.

Table 4
Diversity of endophytic fungi genera isolated from leaves of olive trees cultivated in the state of São Paulo [Cunha (CUN), Pinhalzinho (PIN), São Pedro (SPD), Pilar of Sul (PDS), São Bento do Sapucaí (SBS) and São Paulo (SP)]; and in the state of Minas Gerais [Extrema (EXT), Andradas (AND) and Caldas (CAL)].

Genus	Nº of	Geographic location								
	isolated	AND	CAL	EXT	CUN	PDS	PIN	SBS	SP	SPD
Alternaria	10						6		4	
Aspergillus	2							2		
Aureobasidium	6							1	5	
Bipolaris	2						1	1		
Botryosphaeria	2							2		
Cercospora	2					2				
Cladosporium	6								4	2
Colletotrichum	21		6			3	3	6	3	
Diaporthe	49	1	7	11	5	3	7	10	5	
Didymela	3								3	
Epicoccum	1						1			
Fusarium	1								1	
Lectera	1						1			
Leptosphaerulina	1								1	
Muscodor	1								1	
Mycosphaerella	3							1	2	
Nemania	2		1					1		
Neocucurbitaria	1	1								
Neofusicoccum	10		2			3		2	3	
Nigrograna	1								1	
Nigrospora	3					2	1			
Penicillium	1							1		
Pestalotia	1						1			
Pestalotiopsis	1						1			
Peziza	1							1		
Phlebia	1							1		
Phoma	2								2	
Phomopsis	7		3	1				1	2	
Phyllosticta	6			1		3		1	1	
Preussia	1					1				
Pseudocercospora	4					1	1	2		
Rhizopus	1						1			
Sarocladium	3				3					
Tremateia	2					3				
Trichoderma	3						3			
Trichophaea	2							2		
Xepicula	3								3	
Xylaria	18	3	1		3		4	7		
Unidentified	6				3			2	1	
Total	**191**	**3**	**6**	**3**	**4**	**9**	**13**	**18**	**17**	**1**

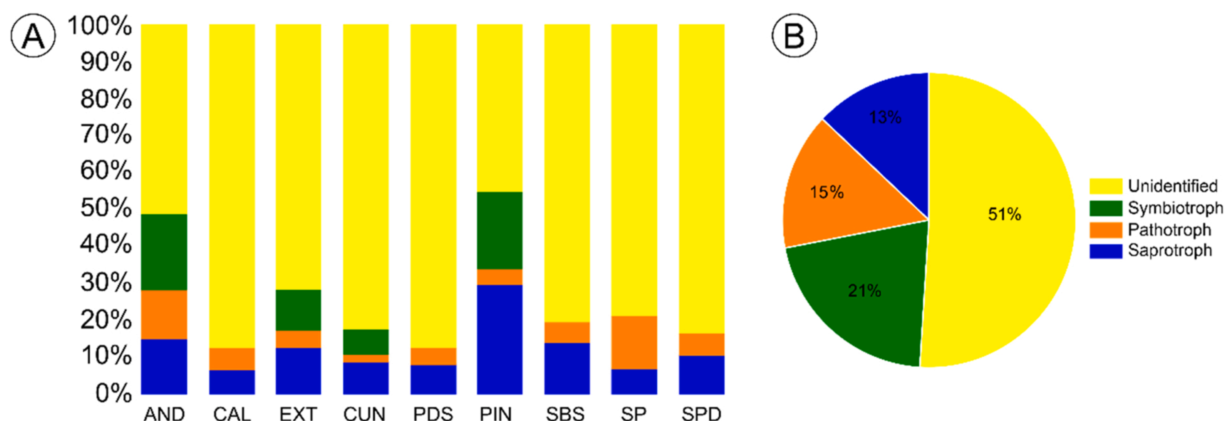

Fig. 8. Trophic mode of endophytic fungi from leaves of olive trees according to the FunGuild database. A. Relative frequencies of the trophic modes by counties, the color blue is Pathotroph, the color orange is Saprotroph, the color green is Symbiotroph and, color yellow is unidentified. B. Overall relative frequencies of trophic modes, the color blue is Pathotroph, the color orange is Saprotroph, the color green is Symbiotroph and, color yellow is unidentified. The abbreviations are of the municipalities of the state of São Paulo were: Cunha (CUN), Pinhalzinho (PIN), São Pedro (SPD), Pilar do Sul (PDS), São Bento do Sapucaí (SBS) and São Paulo (SP). In the state of Minas Gerais, properties were located in: Extrema (EXT), Andradas (AND) and Caldas (CAL).

(Sanchez-Azofeifa et al., 2012). The community of endophytic fungi also changed at different ages and localities in *Calotropis procera* leaves, and most of the endophytes were found in senescent leaves (Nascimento et al., 2015). According to Skaltsas et al. (2019), host developmental stage is a determinant factor of endophytes community of tropical plants and differences such as composition of nutrients in the phloem may

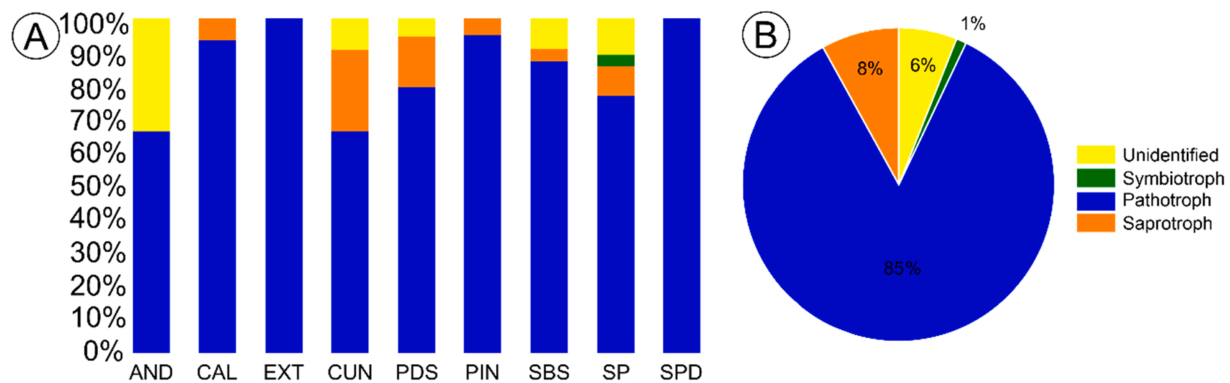

Fig. 9. Trophic mode of endophytic fungi from leaves of olive trees according to the FunGuild database. A. Relative frequencies of the trophic modes by counties, the color blue is Pathotroph, the color orange is Saprotroph, the color green is Symbiotroph and, color yellow is unidentified. B. Overall relative frequencies of trophic modes, the color blue is Pathotroph, the color orange is Saprotroph, the color green is Symbiotroph and, color yellow is unidentified. The abbreviations are of the municipalities of the state of São Paulo were: Cunha (CUN), Pinhalzinho (PIN), São Pedro (SPD), Pilar do Sul (PDS), São Bento do Sapucaí (SBS) and São Paulo (SP). In the state of Minas Gerais, properties were located in: Extrema (EXT), Andradas (AND) and Caldas (CAL).

cause adaptations of the endophytic community to the developmental stage.

4.3. Altitude did not influence the diversity of bacterial and fungal communities

In this study, altitude was not a significant factor influencing the bacterial community in olive trees, contrasting to the study of Carper et al. (2018) that has shown significantly different endophytic bacterial communities in the leaves of mature trees across an elevation gradient. In Rojas-Jimenez et al. (2016) plants sampled in different stratum showed that the population structure of endophytes in each altitudinal gradient differed from the structure of the other strata. The same was observed for bacterial and fungal communities in vineyards (Corneo et al., 2013).

In coffee plants, altitude, soil parameters, or season were of minor importance in the diversity of septate black fungi isolated from roots (Bonfim et al., 2016). In another study of coffee plants, richness, evenness, and diversity of the mycobiome and bacteriome were higher in soil than in fruits, independently of altitude (Veloso et al., 2020). Although we have not found differences in bacterial diversity/richness along with the altitudinal range, however, this information supports other studies that suggest that bacteria may not follow the patterns of plants and animals along with the altitude (Fierer et al., 2011; Shen et al., 2013; Siles and Margesin, 2017).

4.4. Cultivars did not influence the diversity of bacterial and fungal communities

Cultivar has been reported as an important factor in differentiating the fungal and bacterial endophytic community in olive trees (Costa et al., 2021; Mina et al., 2020). However, the five cultivars (cv. Arbequina, cv. Arbosana, cv. Ascolana, cv. Koroneike and cv. Grappolo) evaluated in this work did not influence the abundance and composition of leaf endophytic fungi and bacteria, and therefore we consider that geographic locality has a greater weight in modulating diversity.

According to Costa et al., (2021) who assessed five olive cultivars, there was no difference in the microbial community when the cultivars were from the same farm, but the opposite was truth when the cultivars were from different farms. The authors mention that geographic location may have an influence on fungal assemblages, because cultivars sampled at the same location (cv. Cobrançosa, cv. Madural and cv. Verdeal Transmontana) did not differ in endophytic diversity.

In work conducted by Mina et al. (2020) that explored endophytic bacterial community in two different olive cultivars (cv. Verdeal Transmontana and cv. Cobrançosa) with and without symptoms of the olive knot (OK) disease, found that the factor cultivar explained only 3.6 % of the total community variation, and that the relative contribution associated with the presence/absence of symptoms of OK would be 7.3 % and 7.1 % of the total contribution related to the sampled habitat (epiphytic or endophytic). According to the work of Fernández-González et al. (2019), the endophytic microbial communities of the rhizosphere (bacteria and fungi) are mainly shaped by the genotype of the olive tree; however, they mention that the cultivar shapes the subterranean microbial communities being this factor more determinant for the rhizosphere than for the endosphere, and more crucial for the community of bacterial than for the community fungal.

4.5. Geographic location influences the diversity of bacterial and fungal communities

The geographical location factor influenced the diversity in the endophytic microbial community in olive leaves in this work. Similar data were found in research conducted in Italy, Spain, Greece, Portugal, Tunisia, Syria and Morocco with olive trees (Müller et al., 2015), and on other perennial plants such as *Hevea* and *Micrandra* in three reserves in the Peruvian Amazon region (Skaltsas et al., 2019).

The locality also influenced the bacterial and fungal community of two woody plant species of Maple Tree in Germany (Wemheuer et al., 2019). In the research samples from nine olive orchards in Portugal revealed that the endophytic fungal communities showed significant differences between their geographical locations (Martins et al., 2016). These data corroborate our results that also showed differences in the microbial community between plants from different geographic locations in Brazil.

The possible reasons for this difference in endophyte diversity could be associated with soil characteristics, such as physicochemical properties, soil type or even conductivity and pH (AlSharari et al., 2022; de Souza Rocha et al., 2020; Szymańska et al., 2018), in this work we did not consider such data; however, in future studies these metrics should be considered for a better understanding of the community of endophytic microorganisms.

4.6. Olive trees hosts a high diversity of endophytic fungi – culture-dependent methods

We recovered thirty-eight genera of fungi in leaves of olives tree from our cultured isolates, among these the most frequent was *Diaporthe*, of which some species are known as pathogens of plants, including olive trees (Materatski et al., 2018), while second most frequent was

Colletotrichum, an important genus of phytopathogens with many species reported as pathogenic for olive trees in numerous producing regions such as Portugal and Spain (Materatski et al., 2018; Moral et al., 2014). *Colletotrichum* species such as *C. gloeosporioides*, *C. aenigma*, *C. kahawae*, *C. siamense*, *C. acutatum*, *C. godeatiae*, *C. nymphaeae*, and *C. rhombiforme* can cause the anthracnose disease that attacks trees and mainly fruits, causing severe losses and impacting on the quality of the oil causing reddish color, high acidity and reduction of polyphenols (Kolainis et al., 2020; Moral et al., 2014). These results were interesting because the leaves collected were apparently healthy suggesting that the pathogen could be quiescent and given the proper conditions it may proliferate and induce disease symptoms. The third most frequent genus was *Xylaria* which is a known decomposer of wood and frequently found in surveys for endophytes (Davis et al., 2003; Thomas et al., 2016). The genus *Xylaria* has already been found in olive trees in Brazil (Sia et al., 2013).

The two most frequent genera in this study were also recovered at frequencies of 61 % for *Diaporthe* and 12 % for *Colletotrichum* in *Hevea* and *Micrandra* seedlings (Skaltsas et al., 2019). Interestingly, the three most frequent genera in our study, *Diaporthe*, *Colletotrichum* and *Xylaria* were also the most commonly found, 75 % of all isolates, in several plant species along an altitudinal gradient in a study conducted in wet forests of Costa Rica (Rojas-Jimenez et al., 2016). In general, these fungi show rapid growth in a Potato Dextrose Agar (PDA) medium that is composed of a simple sugar source of Dextrose, maybe this may have based the growth of these genera.

Of the seven isolated fungal genera from the leaves of olive trees in Brazil, two of them, *Daldinnia* sp. and *Guignardia* sp. were not found in our study but five genera, *Xylaria*, *Nigrospora*, *Diaporthe*, *Colletotrichum*, and *Aspergillus* were common to both studies (Sia et al., 2013). In the current study thirty-eight fungal genera were recovered, of which thirty-two are recovered for the first time from olive trees in Brazil. Nevertheless, our list of fungal genera is strikingly congruent with that presented by (Nicoletti et al., 2020), in their review article on endophytic fungi of olive trees, despite that most of the data came from studies conducted in European countries such as Portugal, Italy and Spain. The olive trees encountered and recruited a very similar set of fungal endophytes in such distinct and distant environments is noteworthy.

4.7. Interactions between fungi and bacteria

Interactions between microorganisms such as fungi and bacteria are complex and infrequently studied. In this study we aimed to explore correlations between leaf endophytic microorganisms.

Correlation analyses showed that positive correlations between fungi and bacteria were more frequent than negative ones. Bacteria involved in negative correlations were mostly from Proteobacteria and Actinobacteria that are also predominant in roots of *Panax ginseng* (Hong et al., 2019). The genus *Methylobacterium* were recovered, thus we suggest this group of bacteria may be in disharmony with the related fungi. This genus also can be found on the surface of rice leaves (Sanjenbam et al., 2020), however this bacterium has also been reported to act against pathogens in potatoes (Ardanov et al., 2012).

Some species of this genus, such as *M. mesophilicum* can inhibit growth in vitro of *Xylella fastidiosa* an important citrus pathogen (Lacava et al., 2004) and also of olive trees in Brazil (Coletta-Filho et al., 2016). The genera *Burkholderia* and *Conexibacter* have also been found in the rhizosphere of sweet potato (Marques et al., 2014). Fungi in the Order Venturiales and family Mycosphaerellaceae were also frequently found among those involved in negative correlations, as well as the genus *Symmetrospora* (Into et al., 2020; Nicoletti et al., 2020; Srisuk et al., 2019).

In contrast the bacteria involved in positive correlations were mostly found in Bacteroidetes and Firmicutes, and showed similarity to species found in the gut, intestine and feces of vertebrates, as part of animal microbiota (Chen et al., 2019; Fouts et al., 2012; Qin et al., 2010). The 22 ASVs recovered are known to be associated with vertebrates mainly in the gut, cecum, and feces, suggesting that these bacteria were potentially taken up by the roots (Frank et al., 2017). These bacteria were also the most frequently found in roots of dwarf wheat (Kavamura et al., 2020) and the phyllosphere of olive trees (Müller et al., 2015). Exploratory studies are required for a better insight into the types of associations among the endophytic community investigated, but we cannot rule out fertilizer as a potential source of some of these bacteria.

4.8. Trophic mode of endophytic fungi

In recent years, studies of the putative functional ecology of fungal communities using the FunGuild database has been employed in order to better understand the niches and behavior of these organisms (Martínez-Diz et al., 2019; Pang et al., 2019; Skaltsas et al., 2019). Our results on the trophic mode for culture-independent methods showed that the most abundant trophic mode was unidentified followed by symbiotrophic, pathotrophic and saprotrophic. The unidentified trophic mode was the most prevalent demonstrates that FunGuild database is still largely incomplete. As an example, *Symmetrospora* was very frequent in our samples but it is classified as unknown. Nevertheless, the culture-independent method detected many symbiotrophic fungi that were not recovered by isolation in the culture medium in this study. According to Nguyen et al. (2016), symbiotroph fungi are those that receive nutrients by exchanging resources with host cells. Compared to other plants such as a wetland grass the most frequent trophic modes were pathotrophs, saprotrophs and symbiotrophs among fungi associated with roots of *Phragmites australis* (Bickford et al., 2018). The most frequent modes among endophytic fungi found in *Hevea* and *Micrandra* were saprotrophs and symbiotrophs (Skaltsas et al., 2019).

In endorhizosphere of grapevines mostly symbiotrophs, followed by pathotrophs, unidentified and saprotrophs, while in bulk soil the order was saprotroph, unidentified, symbiotroph and pathotroph (Martínez-Diz et al., 2019). These authors suggested that root tissues may act as a barrier for colonization by fungi (Martínez-Diz et al., 2019). Their results differed from what has been found in the current study, in which unidentified and symbiotroph were more frequent, possibly because leaf endophytes were sampled.

Our results for isolated fungi, showed that the most abundant trophic mode was pathotrophic followed by saprotrophic, unidentified and symbiotrophic. Pathotrophic fungi, according to Nguyen et al. (2016), receive nutrients from host cells but also harms host cells. Some of the recovered genera such as *Diaporthe*, *Colletotrichum*, *Pseudocercospora*, and *Phoma* harbor many phytopathogenic species.

In a study carried out in eight localities with native and non-native lineages of the plant species *Phragmites australis*, it has been hypothesized that beneficial associations with bacteria, fungi and oomycetes could promote non-native species to invade new sites (Bickford et al., 2018).

Research carried out in vineyards with the aim of understanding the relationships between soil-plant compartments (bulk soil, rhizosphere and endorhizosphere) and fungal microbiome diversity showed that diversity was lower in the endorhizosphere, suggesting that the root tissue may act as a physical barrier for fungi. Trophic modes also differed between bulk soil and rhizosphere compartments compared to the endorhizosphere, with a higher frequency of saprotrophs and symbiotrophs in the latter (Martínez-Diz et al., 2019). These results were different from what we have found in our study, where the majority of fungi were pathotrophic and saprotrophic, possibly because we sampled leaf endophytes.

5. Conclusions

In summary, our results support similar findings regarding the diversity of endophytic microorganisms in olive leaves. For the richness of

the bacterial community the most frequent genera found were *Stenotrophomonas* and *Achromobacter*, for the endophytic fungal community the genera were *Pseudocercospora, Hyphozyma* and *Symmetrospora*. Our investigation highlights that plant age and geographical location of olive trees influence bacterial and fungal communities, in contrast to olive tree cultivar, which was not an important variable for differences in microbial diversity. However, the altitudinal gradient was important for fungal composition, but not for bacteria. When investigating the potential interactions between these two microbial groups more positive correlations were identified than negative correlations, which were less frequent. Fungal communities were categorized by guilds, with unidentified and symbiotrophic being predominant for culture independent methods, and pathotrophic for isolated fungi. In total, 38 genera of isolated fungi were found, with 32 reported for the first time in association with olive trees in Brazil.

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CRediT authorship contribution statement

Amanda Aparecida de Oliveira: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Manuela de Oliveira Ramalho:** Software, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Corrie Saux Moreau**: Validation, Formal analysis, Investigation, Resources, Writing – review & editing, Supervision, Funding acquisition. **Ana Eugênia de Carvalho Campos:** Conceptualization, Methodology, Resources, Funding acquisition. **Ricardo Harakava:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision, Funding acquisition. **Odair Correa Bueno:** Methodology, Investigation, Resources, Writing – review & editing, Supervision, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2022.127128.

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