

Diversity of Endo and Exo-Bacteria Associated With Fungi Isolated From Plant Rhizospheres: A Pilot Study

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Abstract

Background: Over the past several years, the scientific community has described the diversity of microbial communities in a variety of soils associated with plants, but at present, little is known about the specific diversity of the soil fungal microbiome involving bacteria colonizing the surface of fungi (i.e., exo-bacteria) or existing within fungal hypha (i.e., endobacteria). This study aimed to collect, identify, and characterize several fungi and their associated (endo- and exo-) microbiome obtained from the rhizosphere of six different plants. Microcosm devices called fungal highway columns, containing one of four plant-based media as attractants, were placed in the rhizosphere of six different plants. The isolated fungi and their associated endo- and exo- bacteria were identified by sequencing of the ITS (fungi) or 16S (bacteria) rRNA regions, followed by Scanning Electron Microscope (SEM), and fluorescence *in situ* hybridization (FISH) imaging.

Results: Most of the fungi recovered are known plant pathogens, such as *Fusarium*, *Pleosporales*, and *Cladosporium* together with species associated with the soil, e.g. *Kalmusia*. The exo-bacteria recovered were previously described as plant promoters, such as *Bacillus*, *Rhizobium*, *Acinetobacter* or *Ensifer*. The interactions between fungi and exo-bacteria recovered from fungal highway columns were further investigated via confrontation assays. From the reconstruction of the potential co-occurring bacterial-fungal associations in the rhizosphere, we discovered that the most promiscuous exo-bacterium group (associated with diverse fungi) was *Bacillus*. From the study of the endobacterial community, emerged a core of shared endosymbionts with a potential implication in the nitrogen cycle.

Conclusions: The present study demonstrated the importance of selecting and studying cultivable fungi and bacteria from the rhizosphere. Our findings demonstrated that at the rhizosphere level, the range of interactions between fungi and bacteria, both internal and external to the fungal hypha, could vary even among closely related species.

1. Background

The effects of plant symbiotic relationships with fungi or bacteria have been studied and reported in countless ecosystems [1–10]. Symbiotic relationships between plants and microorganisms have been shown to be able to reduce the use of chemical fertilizers [11, 12], increase plant resistance to abiotic stress, such as salinity and drought [13–15], and increase plant defenses against harmful microorganisms [16]. Furthermore, the establishment of a positive microbial community in the rhizosphere could also increase plant growth due to the reduction of N_2 to ammonia (NH_3) [11, 17, 18] and enhance plant absorption of phosphate since organic acids produced by microorganisms can solubilize inorganic phosphate [19–22]. However, most of these studies analyzed individual plant-fungi or plant-bacteria interactions [23]. Considering the complexity of soil ecosystems, these studies provide only a partial understanding of the role of microbes in the soil and plants. The microbial interactions should, in fact, be viewed in a more integrated manner and take into consideration the complex microbial networks in the soil. For instance, the rhizosphere is a highly diverse ecosystem, where complex interactions between different species, e.g. plant, fungi, and bacteria, create unique networks and niches in soils [24].

The experimental proof of the intimate relationships of bacterial-fungal interactions inside or outside the fungal hyphae originated in the early '70s [25–29]. However, a quarter century later, Garbaye was the first to postulate the importance of bacterial-fungal interactions with plants in soil ecosystems [30]. Following this investigation, other studies found that the presence of fungal-bacterial associations can increase the bioavailability of nutrients, such as nitrogen [31, 32] and phosphorus [33]. In fact, the symbiotic relationship between plant, fungi and bacteria has been demonstrated, not only to positively affect nutrient uptake, but also improve plant fitness, either by conferring increased host resistance to pathogens [34–37], or by preventing establishment of antagonistic microbial communities [7, 34, 37, 38].

The study of interactions among bacteria-fungi-plant is, however, still in its infancy. To understand this three-way interaction, it is necessary to first gain a broader understanding of the diversity of the bacterial-fungal interactions, both phylogenetically and functionally. Most studies investigating bacterial-fungal interactions have been with exo-bacteria (bacteria growing on the surface of the hyphae) and obligatory endobacteria (bacteria growing inside the fungal hyphae), such as *Gigaspora margarita* and the bacterium *Burkholderia* [39] or *Mortierella elongata* and *Mycoavidus* [40]. Studies on these model pairs of organisms demonstrate the importance of these interactions. For instance, curing the fungus from having the endo-symbiont can cause a significant alteration in the fungal metabolism [40], negatively affect the fungal growth and phenotype, and therefore the ecological fitness of the fungus [41]. The studies with these model organisms begin demonstrating the ecological importance of these interactions and the large knowledge gap related to the extent that fungal-bacterial partners can influence each other in the environment. [42].

Hence, the goal of this study was to gain a broader understanding of the phylogenetic diversity of bacterial-fungal interactions. To do this, we collected fungi and their associated bacteria, including endo- and exo-bacteria, in six different plant rhizospheres, and characterized their diversity, abundance, and interactions with fungal isolates using four different plant-based media.

2. Materials And Methods

2.1. Media

Potato dextrose broth (PDB), Luria Bertani broth (LB), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ were purchased from Sigma Aldrich. Oatmeal, cornmeal, and sorghum grain agar plates were prepared following the ATTC medium 551 modified protocol [43]. In summary, oat, corn, and sorghum were purchased as whole grains and grounded using a commercial grinder (National Panasonic Co.) to obtain a fine powder (size smaller than 3 mm). Twenty grams of ground grains were boiled in DI water on a hot plate (temperature set at 180 °C) for 30 minutes, simmered, then sieved (Nominal Sieve Size Opening 0.09 mm). After adding 18 grams of agar and bringing the volume to 1 L, the solution was then autoclaved for 20 minutes, and the trace salts solution from the ATTC medium 551 was added. The oatmeal (oat) (100% whole grain), cornmeal (corn) (100% dried corn), sorghum grain (Bob's Red Mill sorghum grain), or potato-carrot (PC) (HiMedia, VWR) media were used in the isolation process of the fungi and their associated endo- and exo-bacteria in the soil. All media prepared for the fungal isolation were supplemented with 150 mg/L final concentration of chloramphenicol as the antibiotic (Alfa Aesar). The bacterial isolation media were prepared with 50 mg/L cycloheximide as a fungicide (Sigma Aldrich). Briefly, the sterile media for fungal and bacterial isolations were allowed to cool under the biosafety cabinet to around 50–60 °C before adding the antibiotic/fungicide and plating.

2.2. Isolation of fungi and their associated bacteria from soil using modified microcosm fungal highway columns

The method of collection of the fungi and associated bacteria used was previously described as the "fungal-highway column." It has been established as a novel method for fungal isolation from soil together with its associated exo-bacteria [44]. We used the same system with a few modifications. Briefly, the column microcosm shell was prepared using two screw caps and two round-bottom polypropylene tubes. The round bottom tubes were cut at the bottom and glued with superglue to make a narrow passage between the tubes. The microcosm was then sterilized by boiling with deionized water for 15 minutes and then soaked in 70% ethanol overnight. After sterilization, it was air-dried inside the biological hood. The other parts of the microcosm, such as caps, mesh, and 1 mm glass beads, were autoclaved for 20 min at 121 °C and 15 psi. The assembly of the microcosm parts under sterile conditions was performed under a biological hood with sterilized tweezers and a spatula. The glass beads were used to fill the inside of the entire column.

The selected attracting medium, (either potato-carrot, oatmeal, cornmeal, and sorghum grain agar) was placed on top and at the bottom of the sterile caps of the column (Fig. 1). At the bottom, a perforated cap was used to allow unhindered contact with the rhizosphere. The medium in the bottom cap of the column was crushed to serve as an attractant to the fungi/bacteria, and at the same time, to allow them to grow throughout the column to reach the top cap of the column. At the entrance of the column, in contact with the soil, a nylon mesh (150 µm) was added to prevent the ingress of mites from the soil in the column. These modifications to the originally published column design [44] were made to reduce the overall size, to be cost-effective, easier to manufacture, and to minimize the possible contamination of the column with mites and their associated bacteria. The full microcosm setup is represented in Fig. 1.

The microcosm fungal highway columns (Fig. 1) were set up at the surface of the rhizosphere of six different plants, which were selected based on their tree or bush growth forms, and named in this study as Tree sites and Bush sites. The Tree sites (T) were composed of two angiosperms, *Citrus sinensis* (Orange tree) (T1), and *Diospyros kaki* (Persimmon tree) (T2), and a gymnosperm, *Cycas revoluta* (Cycad) (T3). The plants included in the Bush sites (B) had three evergreen angiosperm shrubs, *Ilex vomitoria* (Yaupon) (B1), *Myrica cerifera* (Wax myrtle) (B2), and *Buxus sempervirens* (Boxwood) (B3). In each site, triplicate fungal highway columns for each media were set up in the soil under the corresponding plants selected for this study. After collection, the isolates of each of the triplicate fungal highway columns of each media in each site were plated in their respective media, but finally analyzed as pooled samples to maximize the number of isolates per site in each media.

After one week of contact with the soil, the microcosms were removed and then taken to the laboratory in sterile whirl Pak bags for immediate microbial isolation. Under the biological hood, the column's top cap with the media was spread onto sterile plates containing the respective media used as attractant: Oatmeal Agar, Cornmeal Agar, Sorghum grain Agar or Potato-carrot Agar. The initial media was prepared without antibiotic or fungicide to obtain the whole community growing on the plate. All the plates were incubated at 28 °C for two weeks. After the incubation, the fungal colonies grown on the plates were isolated into their corresponding growth media. These media contained either antibiotic to purify the fungi from exo-bacteria or fungicide to purify the bacteria from the fungi, as described earlier. Most fungal isolates were transferred to fresh plates every seven days until the culture was pure. For the few slow-growing fungal isolates, the transfer to new plates was made every 14 days. In the case of bacterial isolates, most grew well after 16 to 24 hours and were transferred to new plates every 24 hours until purified. The isolation process continued until each plate had only colonies with a single morphology of fungi or bacteria. Then, the purified isolates were used for total DNA extraction.

The distilled water stasis technique was applied to store the fungal isolates [45]. Up to five pieces of media containing fresh cultures of fungus were transferred to a sterile 15 ml tube containing 10 ml of sterile DI water. The cap was tightly closed and sealed with parafilm. After the bacterial isolates were purified, they were grown in the corresponding liquid media under optimal growth conditions (Additional file 1 – Supporting Information) and stored with 25 % sterile glycerol at – 80 °C.

2.3. DNA extraction and quality control

Sterilized cellophane paper was used on top of the agar plates for DNA extraction of the fungal isolates. Cellophane (Gel Company cellophane sheet 35x45cm PK100, Fisher Scientific) was cut into smaller pieces to match the size and shape of the Petri dishes used for the media. The cut cellophane sheets were wrapped individually with aluminum foil for autoclaving at 121 °C, 15 psi for 20 minutes. After autoclaving, these sheets were dried in the biohood. Under the biosafety hood, sterile tweezers were used to place the cellophane sheets on top of the agar media. Then, a disposable

spreader was used to flatten and place the cellophane evenly over the surface of the media. On top of the cellophane paper, an agar piece containing the fungal isolate was placed in the middle of the plate to grow for DNA extraction. After the growth diameter reached approximately 1/2 to 2/3 of the plate, the biomass was removed, under sterile conditions, using disposable inoculation loops and transferred into the bead beating tube of the extraction kit (Zymo Quick-DNA Fungal/Bacterial Kit, D6005). The tube was weighed before and after adding the fungal biomass to estimate the amount of biomass to be extracted. The kit lysing solution was added inside the biosafety hood. To attain optimal DNA extraction yields, beta-mercaptoethanol was added in the lysing step to a final dilution of 0.5 % (v/v), as per the kit manufacturer's suggestion.

For the DNA extraction of bacterial isolates, each isolate was grown in LB or PDB liquid media depending on their growth ability in either of these two media (Additional file 1 - Supporting information). For the ones that could not grow in liquid media (Additional file 1 - Supporting information), they were inoculated on plates, and then the biomass was scraped off for extraction. Bacterial isolates were also extracted using the same Zymo kit as the fungal isolates. For DNA quality and quantity control, the microplate reader (Take3, BioTek Instruments, U.S.A) was used to evaluate DNA concentration and degree of purity (260/280 ratio).

2.4. Sequencing and bioinformatics

2.4.1. Identification of Fungal and Exo-bacterial Isolates

The exo-bacterial isolates were identified using amplicon sequencing of the V3/V4 region of 16S rRNA, that was amplified using the F341/R806 primer pair (Table 1 – Supporting Information)[46]. The sequencing was conducted using a 300-cycle paired-end Illumina MiSeq protocol. All sequence analysis steps were performed using the QIIME2 application within the EDGE Bioinformatics environment [47, 48]. The PCR primer sequences were removed from the forward and reverse reads. The sequences were then quality screened using maximum expected error parameters before being denoised with DADA2 [30]. The denoised sequences were merged before the taxonomic assignment. For the 16S amplicon sequences, the taxonomy assignment was done with a custom taxonomy classifier built using the portion of the SILVA database sequences that matched the amplicon used here.

The fungal isolates were sequenced using the Sanger sequencing method with primers for the internal transcribed spacer (ITS) (ITS1F and ITS4) [49]. The conditions of the PCR are presented in Table 1 in the Supporting Information. First, the fungal DNA was amplified with regular PCR, and the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, USA). All the purified products were eluted with sterile nuclease-free water and then quantified with the Take 3 plate reader. These purified PCR products were sent for Sanger sequencing with BigDye™ Terminator Version 3.1 sequencing kit (Lone Star Labs Genetic Sequencing, Houston, TX). The sequencing was done with both reverse and forward primers. The assembly, trimming, and editing were performed with MEGA version X [50]. The sequences were analyzed using NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to find the most related sequences and identify the fungal isolates. For those isolates that could not be identified definitely with the ITS, additional Sanger sequencing results for the large 25-28S RNA subunit (LSU) were obtained using the primer pair LROR and LR5 (Table 1 – Supporting Information)[49].

2.4.2. Quantification and identification of the fungal endobacterial community

To determine the presence of endo-bacteria in the fungal isolates, we performed a PCR on the fungal DNA using the 16S rRNA primers (EUB9-27F and 907r), as described in Table 2. The successful amplification was determined visually on a 1% gel electrophoresis.

To further quantify the number of endobacteria in the fungal isolates, quantitative real-time PCR (qPCR) was carried out. The qPCR was performed using primers E8-F and E533-R, as previously described [51]. The thermal cycling conditions and primers are included in Table 1 – Supporting information. The gene copy per DNA sample was normalized by nanograms of DNA then per gram of fungal biomass used to extract the DNA (Additional file 2 - Supporting information).

The endobacteria identification was performed with nested PCR for V1/V5 region with the primer pair EUB9-27F/907r (Table 1 – Supporting Information) and then V3/V4 region with F341/R806. The identification was obtained following the same protocol as described above for the identification of the exo-bacterial isolates. All the sequencing data were deposited in NCBI (ITS sequences: accession numbers MT771292-MT771337; LSU sequences: accession number MT625979-MT626024; 16s rRNA gene sequences: BioProject PRJNA644907).

2.5. Confrontation assays to determine fungal-bacteria interactions

For each location-media combination, a set of fungi and bacteria were isolated (Table 1, Additional file 3 – Supporting information). Following the isolation, each fungal strain was tested against the co-isolated bacteria obtained from the same location-media combination following a modified protocol [49]. The confrontation assay consisted of inoculating the fungi on the corresponding isolation medium plate. A cylinder of the agar media (collected with the wider part of a 200 μ L pipette tip) containing the fungal hyphae was transferred from the edge of a 5-day-old culture (pre-inoculum) to a fresh plate and incubated at 28°C.

These fungi were grown to reach about 5 cm of diameter, which, on average, took seven days. After allowing the fungus to grow for six days, in a parallel experiment, the exo-bacterium was inoculated in sterile liquid media. Then, on the day the fungus reached the expected diameter of approximately 5 cm, the bacterial culture was centrifuged and adjusted to a 600 nm optical density (OD_{600}) of 1 with phosphate buffer solution (PBS). Then, 25 μ L of the bacterial suspension was placed on three equidistant sides of the plate at approximately 3 cm from the center of the fungus inoculation (Fig. 1 – Supporting Information). After the bacterial drop dried, the plates were incubated for another five days until the fungal diameter reached the plates' edges. The interactions between the fungi and bacteria were recorded (Fig. 6). Control plates were prepared in the same manner but without adding the bacterial suspension to the plate (Fig. 1 – Supporting Information); the same volume of sterile PBS was added instead. The time of growth for each fungus and bacteria growth were optimized and recorded (Additional file 1 - Supporting information). All experiments were performed in triplicates and the results, based on the visual screening, were reported as neutral, when fungal growth was not affected by the bacterial inoculum, or negative, when the fungal growth was inhibited by the bacterium (Fig. 2 – Supporting information, Additional file 4 - Supporting information).

2.6. Scanning Electron Microscopy (SEM)

The fungi and bacteria that displayed a neutral interaction from the confrontation assay were further analyzed with SEM. The experiment involved growing the fungus and bacteria on the same plate for ten days until the fungus grew over the bacteria. A 5 mm portion of the media where fungus and bacterium overlapped was collected and then immediately fixed with 2.5% glutaraldehyde solution at 4°C for 24 h in the dark. Post-fixation and dehydration procedures followed our previous publication [52]. Briefly, following the washing steps with phosphate buffer solution (PBS), the sample was incubated for 2 h with 1 % osmium tetroxide (Sigma Aldrich, USA). Dehydrations were done with ethanol/water (v/v %) at 50 %, 70 %, 95 % and 100 %. The final rinses were with acetone/ethanol at a ratio of 1:2, 1:1, and 2:0. The SEM samples were dried on glass slides [52] and then sputter-coated with gold (Denton V, USA) for 40 s at 45 mAmps. The images were obtained with SEM JSM 6010LA (Jeol, USA) with an accelerating voltage of 10 kV and a spot size of 26.

2.7. Fluorescence *in situ* Hybridization (FISH)

For the fluorescence *in situ* hybridization (FISH) analysis, *Didymella* (F41c) and *Fusarium* (F54) were selected to confirm the presence of endobacteria in the fungal hyphae. FISH involved preparing growth media pads by casting 4 % phytigel (Sigma Aldrich) supplemented with potato dextrose broth (Sigma) between two standard microscope slides spaced by #1.5 coverslips. Growth pads were shaped using a sterile 15 mL conical tube as a mold cutter and were transferred to the center of microscope slides equipped with a 25 mL Gene Frame (Thermo Fisher Scientific). The slides were placed in a 100 mm disposable petri dish (Thermo Fisher Scientific) alongside a cap from a 15 mL conical tube filled with DI-water to control humidity. Mycelia from fungal tissue was transferred to the media pads and incubated for three days at 21°C and then fixed with 4 % paraformaldehyde (Thermo) in PBS buffer at 4°C overnight. Samples were washed three times with PBS, and cell walls were lysed with a cocktail of 5 mg mL⁻¹ lysozyme (Sigma Aldrich) and chitinase (Sigma Aldrich) for 1 hour at 37°C. The samples were washed three times with PBS and subsequently dehydrated with a series of ethanol treatment at 50 %/75 %/100 %/75 %/50 % ethanol in PBS for 3 minutes each at room temperature then resuspended in a solution of PBS.

Hybridization chain reaction FISH (HCR-FISH) was adapted from Choi et al [53]. Stellaris RNA Fish buffers (Biosearch Technologies) were used for subsequent hybridization and washing steps. Samples were preconditioned with Stellaris Wash Buffer A supplemented with 10 % formamide for 5 minutes and replaced by Stellaris Hybridization Buffer supplemented with 10 % formamide and containing a final 125 nM probe concentration. Probes used in this study are a variant of the Eub338 extended with the initiator sequence for HCR (5'-GCTGCCTCCCGTAGGAGTATTTACATTTACAGACCTCAACCTACCTCCAACCTCTCAC-3') and the universal eukaryote probe, Euk516 (5'-ACCAGACTTGCCCTCC-3'). Probes were hybridized for 4 hours at 37°C and then washed three times with Wash Buffer A for 20 minutes each at 37°C. For hybridization chain reaction FISH, samples were preconditioned with amplification buffer 2X saline sodium citrate buffer (SSC, Thermo Fisher Scientific), 0.1 % Tween 20 (Thermo Fisher Scientific), and 10 % low MW dextran sulfate (Sigma Aldrich) for 20 minutes at room temperature and exchanged with HCR amplification probes (Molecular Instruments): B4-Alexa Fluor 647 in amplification buffer. HCR amplification was performed overnight at room temperature and subsequently washed with Stellaris Wash Buffer A without formamide three times for 20 minutes each at room temperature. DAPI staining was performed with the addition of 1 µM DAPI in Stellaris Wash Buffer A and incubated for 10 minutes at room temperature. Excess dye was washed with Stellaris Wash Buffer B for 5 minutes at room temperature and rinsed with 2X SSC buffer three times prior to imaging. Images were collected with a Zeiss Axio Observer microscope and were processed using FIJI.

3. Results And Discussion

Bacteria have been described to colonize and use the fungal hyphae as highways to translocate in unsaturated soil matrices [51, 52]. Recently, a microcosm fungal highway column was developed as a unique isolation technique that allows bacteria to be transported using the fungus hyphae and to experimentally obtain insights on the bacterial-fungal interactions occurring in the soil [44, 54]. In the present study, this device was placed in the soil in proximity to the roots of six different plants using four different plant-based media as attractant for the isolation of cultivatable fungi and their associated bacteria from plant rhizospheres. The setup of the microcosm requires that the fungus grow upward in the unsaturated microcosm column to colonize the agar on top of the column. In this setup, the bacteria would also have to interact directly with the fungi to get transported to the top of the column. Therefore, we hypothesized that in these highway columns the bacteria should only have a positive or neutral interaction with the fungus to travel to the top of the column.

3.1. Fungal isolation from highway column samplings: relationship with types of plants and media

The fungal highway columns yielded a total of 46 fungal isolates from six different plant rhizospheres using four different plant-based media (Table 1). Most of the fungal isolates were identified as belonging to known plant pathogen species. The most represented fungal genera found in the collection were *Fusarium* and *Cladosporium*, both common soil-borne pathogens largely associated with plants and common inhabitants of the soil microbial community [55, 56]. Other fungal isolates, also associated with plant diseases, were *Alternaria* [57, 58], *Diaporthe*, and its asexual state *Pomopsis* [59, 60]. Moreover, we were able to isolate *Aspergillus niger* and *Aspergillus fumigatus*, which are often present in soils [61, 62], and *Didymella*, known for its pectolytic activities against plant cell walls [63]. We also isolated fungi often described as endophytes, such as *Pestalotiopsis* [64–69] and *Plectosphaerella* [70], and non-pathogenic slow-growing mold, such as *Stachybotrys* [71] and *Kalmusia*. The latter is a fungus commonly associated with the soil crust [72]. In summary, all the fungal isolates have been previously described to be part of the soil microbial community.

Additional investigation involved the identification of possible relationships between plant type and the fungal isolates. For this purpose, we grouped the plants based on their tree or bush aspect and described them as tree sites and bush sites. In the case of the tree sites, they were divided into T1, T2, and T3, which corresponded to *Citrus sinensis* (Orange tree), *Diospyros kaki* (Persimmon tree), and *Cycas revoluta* (Cycad), respectively (Table 1). The *Citrus sinensis* (T1) was characterized by the fewest recovered fungal isolates (Fig. 2), i.e. one from Sorghum grain agar and one from Potato-carrot agar. Similar results and low fungal yield was previously observed in another study with the same type of tree [73]. These authors described that the principal taxa of fungi associated to *Citrus sinensis* was *Basidiomycota*, a generally slow growing phylum of fungi, and consequently difficult to isolate with conventional techniques of soil mycology [74]. This location was the only site where the genus *Plectosphaerella* was isolated (Additional file 1 - Supporting information). *Plectosphaerella* is a plant pathogen previously isolated from nine different plant genera [75–77] but never from *Citrus sinensis*. *Kalmusia* was another plant pathogen isolated that, as with *Plectosphaerella*, had never been described to be associated with orange trees. Among all the locations studied, the largest number of fungal species were obtained in the rhizosphere of *Diospyros kaki* (T2) (Fig. 2). This result may be explained by the natural decay of fallen persimmon fruits, which increased the nutrient content in the soil [78, 79]. Such a finding is also corroborated by the fact that the majority of the fungi obtained in this location were saprophytes [80–82].

In the case of the bush sites, they were divided into B1, B2, and B3, which corresponded to *Ilex vomitoria* (Yaupon), *Myrica cerifera* (Wax myrtle), and *Buxus sempervirens* (Boxwood), respectively (Table 1). Among all the bush locations, the number of fungi retrieved from these locations ranged from five, coming from the *Buxus sempervirens* (B3), to nine isolates from *Ilex vomitoria* (B1) (Fig. 2). Also, *Ilex vomitoria* (B1) was the only location with the presence of the fungus *Diaporthales* (Fig. 3). This fungus is a known plant pathogen commonly associated with various plants [59, 83]. The overall comparison of the bush and tree sites showed that the bush sites presented a higher diversity of fungal isolates (Fig. 3 – Supporting Information). In general, both sites seemed to share a core of four fungi commonly known as plant pathogens and soil inhabitants, namely, *Pleosporales*, *Fusarium*, *Aspergillus* and *Cladosporium* [55, 56, 61] (Fig. 3).

In addition to the types of plants, we determined the effect of different plant-based media on the diversity of fungi collected for each plant type. The results showed a noticeable difference in the culture collections, confirming previous findings on the effect of different media on the isolation of fungi [84–86]. Cornmeal agar recovered the highest number of fungal isolates, while oatmeal agar recovered the lowest (Fig. 2). Furthermore, we found at least one common taxon i.e. *Fusarium* and *Pleosporales*, in both tree and bush locations, as well as in all growth media, except for Potato-carrot. This finding suggests that these two taxa are ubiquitous in different plant rhizospheres.

Due to the intrinsic specificity of the nutrient requirement of the different microorganisms [87] and the fact that plant-based media do not have a defined chemical composition, it is difficult to draw a conclusion related to the effects of

the media effects. However, we could conclude that none of the media used in this experiment can be defined as "optimal" considering the different number of fungi and bacteria obtained in the different media and different sites. Still, the community of culturable fungi was influenced by a combination of plant coverage types and the type of media used as a fungal attractant.

Table 1

Number of fungal and bacterial isolates obtained with the different media (Oatmeal Agar, Cornmeal Agar, Sorghum grain Agar, and Potato-carrot Agar) in six different types of rhizospheres. The area code in the table corresponds to the type of plant and was assigned as tree (T) and bush (B).

Area code	Description	Oatmeal Agar		Cornmeal Agar		Sorghum grain Agar		Potato-carrot Agar	
		Fungal Isolates	Bacterial Isolates	Fungal Isolates	Bacterial Isolates	Fungal Isolates	Bacterial Isolates	Fungal Isolates	Bacterial Isolates
T1	<i>Citrus sinensis</i> (Orange tree)					1	1	1	
T2	<i>Diospyros kaki</i> (Persimmon tree)	2		6	5	5	5	3	12
T3	<i>Cycas revoluta</i> (Cycad)	2				4	1		2
B1	<i>Ilex vomitoria</i> (Yaupon)	3		6					4
B2	<i>Myrica cerifera</i> (Wax myrtle)	3		3	5	2	3		8
B3	<i>Buxus sempervirens</i> (Boxwood)	2		2				1	5

3.2. Linking fungal and bacterial isolates from the same columns

The isolated fungi and associated exo-bacteria carried along the columns with the fungal hyphae were further investigated. A total of 51 exo-bacterial isolates were successfully collected with serial transfers on plant-based media with fungicide (Fig. 4, Fig. 5). The most predominant phyla isolated were *Firmicutes* (57%), followed by *Proteobacteria* (29%) and *Actinobacteria* (16%). These phyla are the most prominent endophytic representatives described to be associated with diverse plants, such as *Arabidopsis thaliana* [89], *Setaria italica* (Foxtail millet) [90], *Glycine max* (soybean), [91] and *Panicum virgatum* (switchgrass) [92]. These results confirm the capacity of this isolation technique to obtain a similar rhizosphere microbiome to other techniques.

Among all locations, *Bacillus* was the most common isolate, followed by other well-known soil bacteria: *Acinetobacter*, *Ensifer*, *Pseudomonas*, *Microbacterium*, *Paenibacillus*, *Rhizobium*, and *Stenotrophomonas*. Most of these bacterial isolates are known to either act as plant growth promoters [93–104] or as biocontrol agents against plant pathogens [105–107].

Similar to the finding from the fungal isolates, *Diospyros kaki* (T2) presented the most exo-bacterial isolates, while *Citrus sinensis* (T1) presented the least. Among the tree rhizospheres, the total number of different bacterial isolates retrieved ranged from 22 associated with *Diospyros kaki*, to 1 with *Citrus sinensis* (Fig. 4). Interestingly, from the latter location, we were able to retrieve *Pseudorhodoferax*, a bacterium, that from the best of our knowledge, has never been described as being associated with the fungus *Kalmusia*.

In the bush sites, *Myrica cerifera* (B2) was characterized by the highest percentage of isolates ascribed to the genus *Bacillus* (62.5%). This genus was retrieved from almost all locations, except from *Citrus sinensis* (T1) and *Cycas revoluta* (T3) (Fig. 5).

Despite the small number of locations and isolates, a positive correlation was observed between the number of fungal and bacterial isolated in the different sites (Pearson's $r^2 = 0.84$) (Fig. 7 – Supporting Information). Additionally, a positive correlation between the diversity indices of fungal and bacterial isolates were obtained from the different locations (Pearson's $r^2 = 0.76$) (Fig. 8 – Supporting Information). From this data, we hypothesized that a species-specific partnership between fungus and bacterium could be occurring. However, the possibility of non-specific interactions cannot be excluded considering the co-occurrence of multiple bacteria and fungi retrieved in the columns. These results suggest that the abundance and diversity of the retrieved fungi should be able to directly affect the capacity to represent and recover the associated bacterial community. He et al. reported a similar result where a positive correlation was described when they studied the possible presence of a linkage between diversities of plants, fungi, and bacteria [108].

3.3. Bacterial-fungal interactions: reconstruction of the microbial associations in the soil

The positive linear correlation between fungal diversity and associated bacteria observed earlier suggested the need for a deeper understanding of the types of interactions between fungi and their associated bacteria. As mentioned earlier, we hypothesized that only positive or neutral interactions with fungi would have allowed the translocation of exo-bacteria via the fungal hyphae. Therefore, co-isolated bacteria and fungi in the same locations and media were further investigated via the confrontation assay (Fig. 6) in order to reconstruct the type of partnerships that were occurring in the soil.

As hypothesized, some of the bacteria isolated from the same location and growth medium displayed species-specific associations and neutral/positive interactions with the co-isolated fungus. This was the case for *Agrococcus*, *Brevibacillus*, and *Exiguobacterium*, which displayed positive or neutral interactions only in the presence of *Didymella*. A similar pattern was observed with the bacteria *Cellulomonas* associated with the fungus *Staphylotrichum coccosporum* and *Stenotrophomonas* with *Aspergillus fumigatus*. However, contrary to our initial hypothesis, in some cases different bacterial isolates belonging to the same species displayed different types of interactions with the same fungal isolate, suggesting that the bacterial-fungal interactions are not necessarily species-specific but rather strain-specific. (Additional file 4 - Supporting information). For instance, in the present study, we observed that some *Bacillus* isolates presented both neutral and antagonistic relationships with different fungal isolates of the same genus. One example was the association of *Fusarium* and *Bacillus*, where we recorded both negative and neutral interactions (Fig. 6). The negative interactions between *Fusarium* and *Bacillus* have also been observed and explained by others [109, 110].

The negative effects of bacteria toward fungi is typically a result of the bacterial production of antifungal secondary metabolites. For instance, as described by Mnif et al [111] lipopeptides produced by *Bacillus* display antifungal activity against *F. solani*. Another antifungal compound produced by different bacteria, including *Pseudomonas*, is 2,4-

diacetylphloroglucinol (DAPG) [112]. DAPG is an active compound against various plant-pathogenic fungi, including *Fusarium* [113–116]. Besides the negative interactions observed between *Fusarium* and *Bacillus*, other studies also reported antagonistic interactions between *Ensifer* and the fungus *Pleosporales*, due to the production of alliinase, as a secondary metabolite for bacterial defense [113, 114, 117–119]. The present study also corroborates these previous findings that *Bacillus*, *Ensifer* and *Pseudomonas* present antagonistic patterns towards these fungal genera/species.

In addition to the negative interactions between *Fusarium* and *Bacillus*, this fungus also presented a neutral interaction with *Rhodococcus*. This interaction, however, was not species-specific since this bacterium also presented neutral interactions with other fungi. To further understand the nature of the neutral interactions observed in the present study, scanning electron microscopies were obtained. A representative image of the neutral interaction between *Fusarium* and the bacterium *Rhodococcus* is presented in Fig. 7. The results showed that the bacteria displayed the capacity to grow on top of the hyphae (Fig. 7). This type of finding was also reported by others [44]. However, it is important to point out that except for a few documented associations, such as the co-existence of *A. fumigatus* and *Stenotrophomonas* as a hyphae biofilm, [120] there is a lack of literature about those types of interactions in natural environments. This study shows that bacteria are commonly associated with fungi in plant rhizospheres. Furthermore, this isolation technique using highway columns promises to shed light on the specificity and types of relationships between bacteria and fungi in plant rhizospheres.

3.4. Endo-hyphal microbiome and bacterial-fungal specificity

Like other taxonomic groups of eukaryotic organisms, fungi have also been reported to have established associations with bacteria as endosymbionts [28, 29, 92, 121–124]. To determine if any of our fungal isolates harbored bacterial endosymbionts within their fungal hyphae, we performed both qualitative and quantitative investigations of our fungal isolates. Initial qualitative determination of bacterial presence inside the fungal hyphae was determined via 16S rRNA gene signal using fluorescence *in situ* hybridization (FISH) imaging (Fig. 8) [53]. The FISH results confirmed the presence of bacteria inside the hyphae of selected isolates. Figure 8 shows an example of bacterial signatures observed inside the *Didymella* mycelium. At low magnification, the presence of the signal (Cyan) can be observed ubiquitously across the fungal hyphae, while at higher magnifications bacteria can be observed within the hyphae. Innate fluorescence of the fungi was investigated to determine whether the fungi exhibited any fluorescent artifacts. *Fusarium* (F54) was also observed to exhibit internalized bacterial signals, which was to be expected since this fungus exhibited one of the highest bacterial loads determined by quantitative real time PCR (Additional file 1 - Supporting Information).

After confirming the presence of endosymbionts in the fungal isolates, 16S rRNA gene sequencing of these endosymbionts were done. The taxa of endobacteria present in each fungal isolated are listed in the Supporting information (Fig. 11, 12, 13, 14, 15, 16 – Supporting Information). On average, the fungal genera characterized by the largest numbers of endobacterial taxa were *Pleosporales*, followed by *Fusarium* (Fig. 13–14 – Supporting Information). This is the first study reporting the presence of endobacteria in *Fusarium*. Most of the endosymbiotic bacteria found in the genus *Fusarium* and in the order *Pleosporales* were composed of bacterial phyla commonly associated with the soil microbial community, such as *Proteobacteria*, *Firmicutes*, and *Actinobacteria* [125], suggesting that these bacteria probably have a mechanism to enter the fungal hyphae. Comparing exo-bacterial and endo-bacterial communities, we found that in most cases there was no intersection between these two microbial communities. Among the different exo-bacteria co-isolated with the fungi, only *Bacillus*, *Microbacterium*, *Pseudomonas*, and *Stenotrophomonas* were also found as endosymbionts. One of the most compelling examples is *Bacillus spp.* These species, despite having been associated as exo-bacteria with various fungal isolates (almost 70% of our collection), were present as endobacteria in only five fungi. More specifically, this genus was present in conjunction with the fungal taxa of *Pleosporales*, *Didymella*, and *Neopestalotiopsis*. This result validates the

hypothesis that the endobacterial microbial community present in the fungi is not necessarily affected or linked to the exogenous microbial community. On the other hand, *Pseudomonas* and *Stenotrophomonas* were found to be present as both endo- and exo-bacteria, respectively, in 85% and 71% of their co-isolated fungi, suggesting that these genera could be horizontally transmitted from the fungus to the external environment and vice-versa [126].

The analysis conducted on the bacterial endosymbionts present in fungal isolates resulted in the observation that different genera of fungi present both a unique subset of bacteria and a shared core of five endosymbionts (Fig. 9) composed of isolates from *Stenotrophomonas*, *Pseudomonas*, *Achromobacter*, *Candidatus Finniella*, and *Methylobacterium*. This core of endosymbionts was previously documented as fungal endobacteria [121–123, 127, 128]. Among the core of endosymbionts, *Stenotrophomonas* [129, 130], *Pseudomonas* [131, 132] and, *Achromobacter* [133], they have also been described to be involved in nitrogen fixation in soil. Other endosymbionts, such as *Bacillus* [134], *Bradyrhizobium* [135], *Novosphingobium* [136], and *Herbaspirillum* [137], were also previously described to be participating in nitrogen fixation in soil [131–142]. Previous studies proposed that the bacteria could exploit the nitrogen resources of the fungus. The proposed mechanism involved the production of peptidases by the endophytic bacteria and consequent absorption of the resulting oligopeptides produced by their biodegradation [143, 144]. However, the identification of microorganisms, possibly capable of carrying out nitrogen fixation, present as fungal endosymbionts, indicate that there could be a more complex nutrient exchange pattern between the endobacteria and its host.

4. Conclusions

In the present study, fungal highway columns allowed us to glimpse the complexity of the network of bacterial-fungal interactions in plant rhizospheres by successfully selecting a group of cultivatable fungi and their intimately associated bacteria. The diversity of fungi and bacteria obtained was clearly influenced by the types of plants and media used. The bush sites presented a larger diversity of isolates than the sites with trees. However, both sites shared a core of four fungi, namely *Pleosporales*, *Fusarium*, *Aspergillus* and *Cladosporium*. In general, most of the isolated fungi were previously described as plant pathogens [55, 56, 59, 61, 80-83]. Among the media used in this study, we can conclude that cornmeal could be considered as an excellent compromise between the number and diversity of isolates. These results suggest that a careful selection of the growth medium for different types of plant rhizospheres to be used in the fungal highway column is essential to achieve isolation of larger subsets of the fungal and bacterial communities associated with the rhizospheres. This approach could also be used with selective media to select and enrich a specific subset of the fungal-bacterial community associated with the rhizosphere for more detailed studies of model soil microorganisms.

Detailed investigation of the exo-bacterial and endobacterial communities associated to this isolated subset of the fungal community showed that both species-specific and generalistic associations can occur. Although, generalistic associations were more frequently observed in the fungal isolates. Furthermore, the exo-bacteria and endobacteria communities showed little or no intersection between these two microbial communities. Among the different exo-bacteria co-isolated with the fungi, only *Bacillus*, *Microbacterium*, *Pseudomonas*, and *Stenotrophomonas* were also found as endosymbionts. *Bacillus* was, however, associated as an exo-bacteria with the vast majority of the fungal isolates and was present as an endosymbiont in only 15% of the fungal isolates. *Pseudomonas* and *Stenotrophomonas*, on the other hand, were found in both endo- and exo-bacterial communities. This result suggested that the horizontal transmission of bacteria from the fungus to the environment and vice-versa is not always host specific.

The most notable findings of this study are the identification of a core of endobacteria shared among the principal fungal taxa in soil, and some species-specific interactions observed with some pairs of exo-bacteria and fungi. On the other hand, the cosmopolitan distribution of the *Bacillus* genus among the exo-bacteria associated with fungi has not been previously described and could provide an excellent model organism to gain a better understanding of exo-bacterial-fungal interactions in future investigations.

Abbreviations

PDB: potato dextrose broth; LB: Luria Bertani broth ; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; qPCR: quantitative polymerase chain reaction; ITS: internal transcribed spacer; LSU: large subunit; SEM: scanning electron microscopy; FISH: fluorescence in situ hybridization.

Declarations

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Ethical approval and consent to participate

Not applicable.

Authors' contributions

DFR and PSGC conceived the study. HNN and SP performed the collection and isolation of the samples. SL, HNN, and SP performed DNA extraction of the samples. SL and HNN performed the identification of the isolates, data analysis and visualization. SL performed the confrontation assays and qPCR analysis. GLH performed the identification of the endobacterial community. HNN performed the SEM imaging. DM-III performed the fluorescent *in situ* analysis and microscopic imaging. SL, DFR, HNN, GLH, DM-III, and PSGC wrote the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

All data derived from this work is publicly available in the NCBI-GenBank database under the following accession numbers: ITS sequences: accession numbers MT771292-MT771337; LSU sequences: accession number MT625979-MT626024; 16s rRNA gene sequences: BioProject PRJNA644907.

Consent for publication

All authors have read and participated in the preparation of the manuscript. All authors consent to the publication.

Competing interests

The authors declare no competing interests.

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Figures

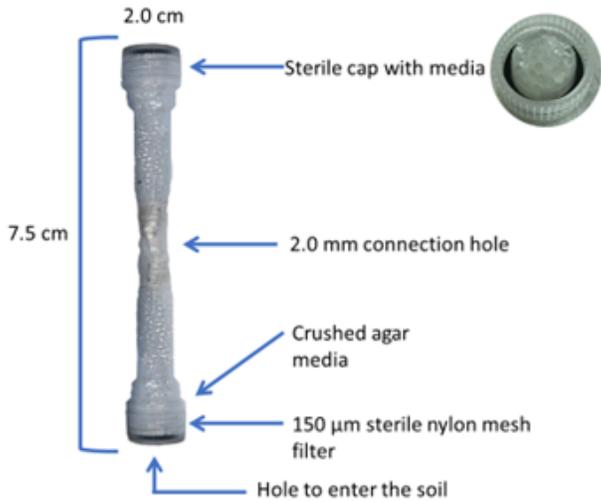


Figure 1

Microcosm column for isolation of fungi and bacteria from soil

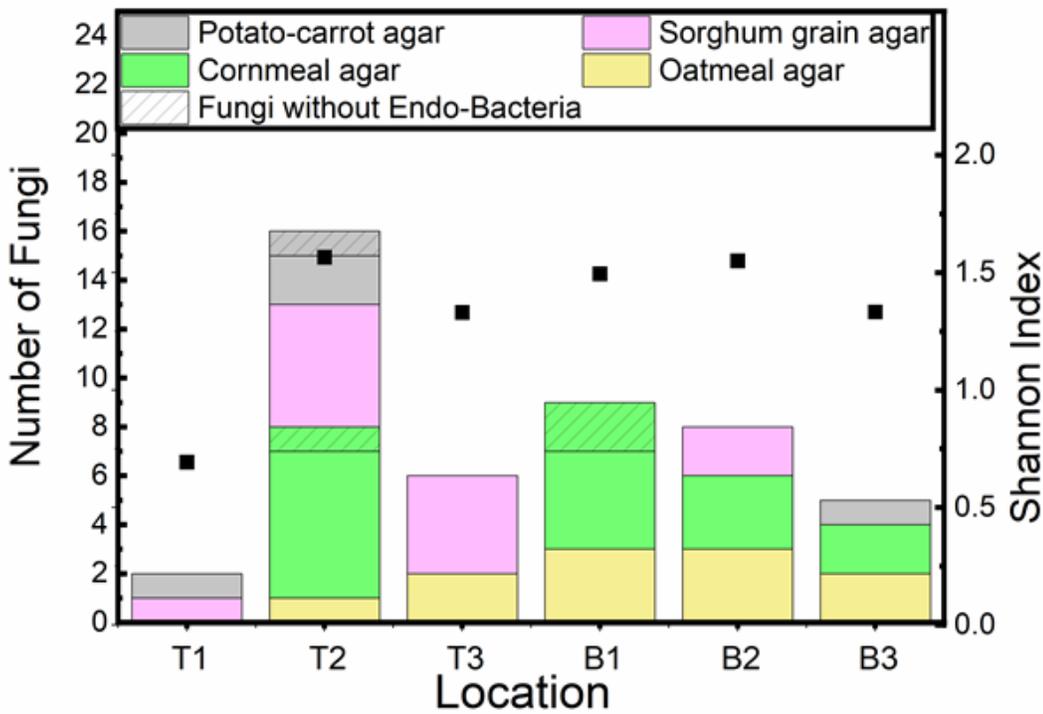


Figure 2

The bar graph represents the number of fungal isolates obtained from different locations (T1=Citrus sinensis, T2= Diospyros kaki, T3= Cycas revoluta, B1= Ilex vomitoria, B2= Myrica cerifera, B3= Buxus sempervirens) using different plant-based media (Oatmeal agar, Cornmeal agar, Sorghum grain agar, and Potato-carrot agar) grown at 28°C. The pattern in the bar graphs corresponds to fungi isolated in the corresponding media that did not contain any endobacteria. The diversity within each fungal collection was calculated using the Shannon Index [88] and is represented by black squares in the graph.

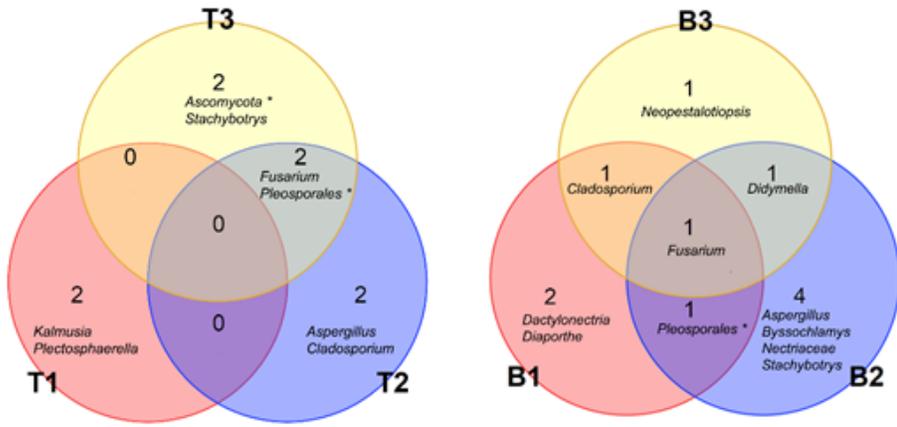


Figure 3

Venn diagram of the fungal genera in the different locations based on the relative plant coverage: Tree sites (T1=Citrus sinensis, T2= Diospyros kaki, T3= Cycas revoluta, B1= Ilex vomitoria, B2= Myrica cerifera, B3= Buxus sempervirens). *: isolates not identified to the genus level.

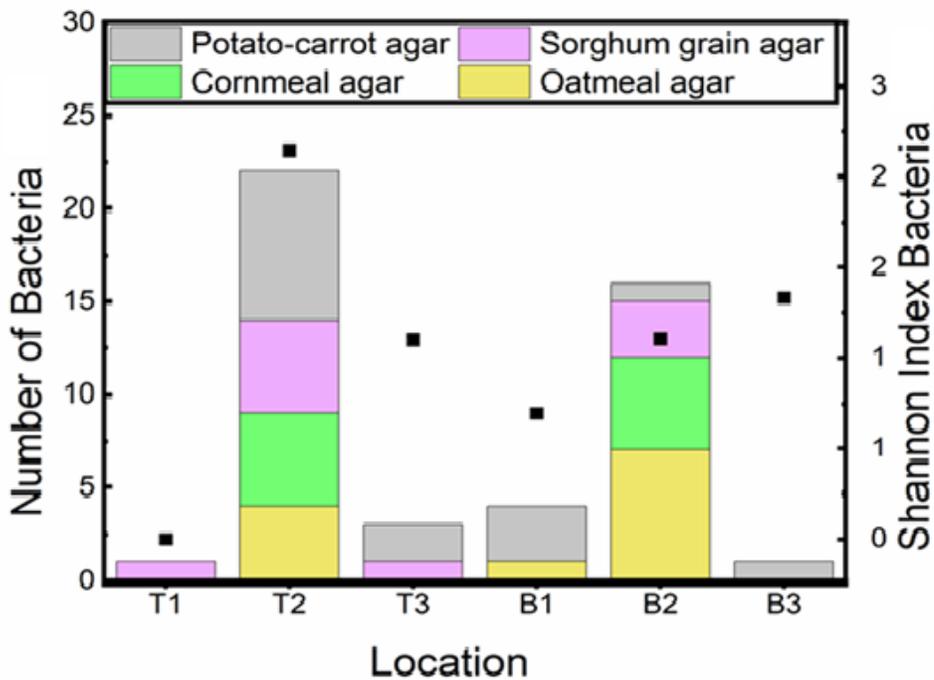


Figure 4

The bar graph represents the number of exo-bacterial isolates obtained in the different locations (T1=Citrus sinensis, T2= Diospyros kaki, T3= Cycas revoluta, B1= Ilex vomitoria, B2= Myrica cerifera, B3= Buxus sempervirens) using different plant-based media (Oatmeal agar, Cornmeal agar, Sorghum grain agar, and Potato-carrot agar) grown at 28°C. The diversity within each bacterial collection was calculated using the Shannon Index and represented by black squares.

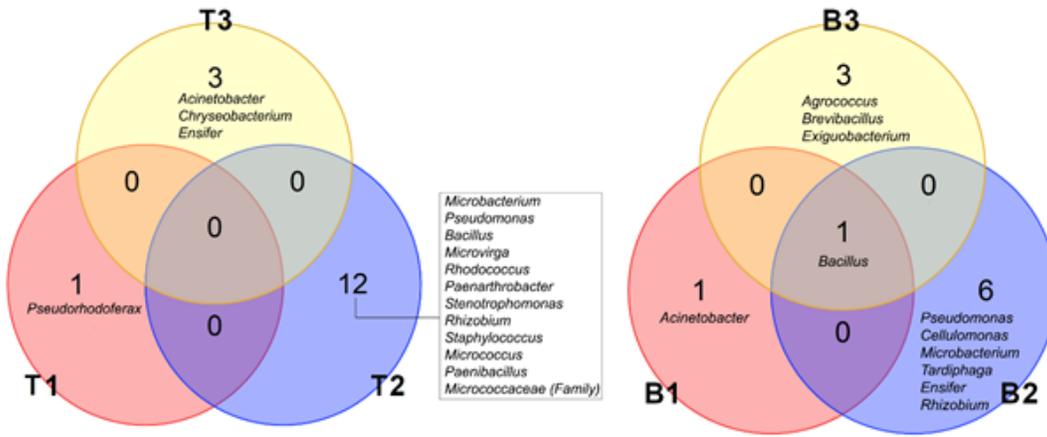


Figure 5

Venn diagram of the bacterial genera in the different locations based on the relative plant coverage: Tree (T1=Citrus sinensis, T2= Diospyros kaki, T3= Cycas revoluta) and Bush B1= Ilex vomitoria, B2= Myrica cerifera, B3= Buxus sempervirens) sites.

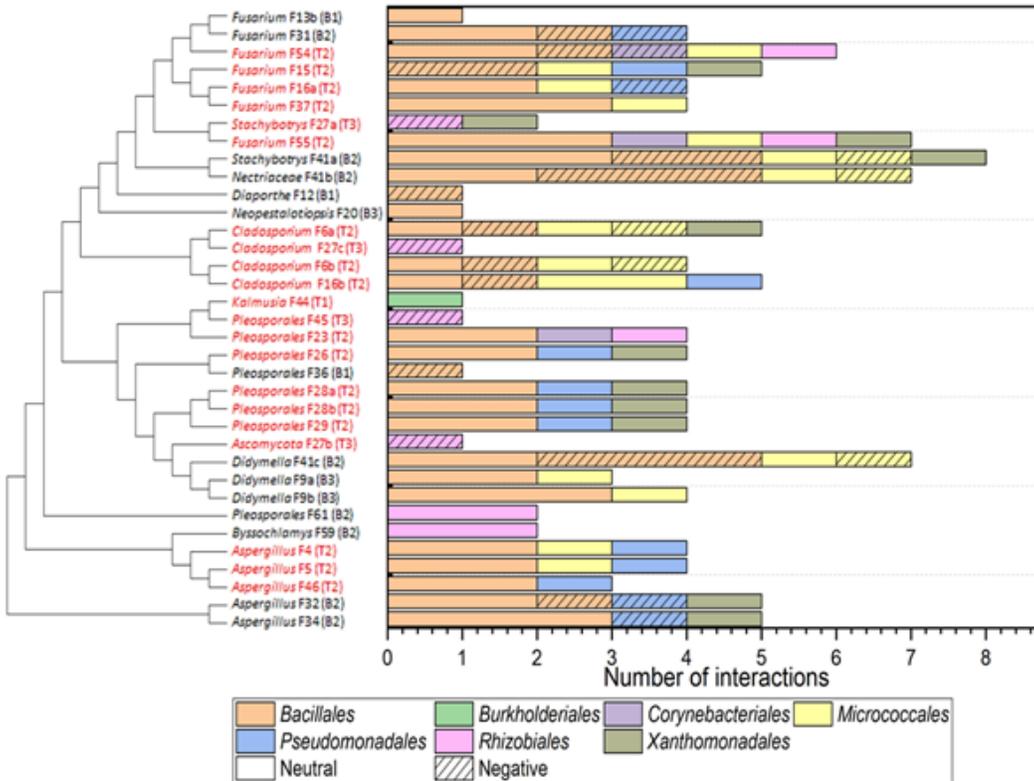


Figure 6

Phylogenetic tree of the bacterial-fungal interaction in different locations. The phylogenetic distance is based on the ITS sequences. In the phylogenetic tree, the fungal taxa in red is the fungi isolated in the tree sites (T1=Citrus sinensis, T2= Diospyros kaki, T3= Cycas revoluta), and in black is the fungi isolated in bush sites (B1= Ilex vomitoria, B2= Myrica cerifera, B3= Buxus sempervirens). The confrontation between bacteria and fungi was performed on a plate containing partially growing fungi and 25 μ L of the bacterium inoculum in three equidistant corners of the plate. The confrontation was carried at 28°C for 7 days and then evaluated. The results of the confrontation assay for each isolate are

presented in the bar graph with or without patterns for negative or neutral interactions, respectively. The colors in the bar graphs correspond to the bacterial taxonomic level of Order interacting with their respective fungi.

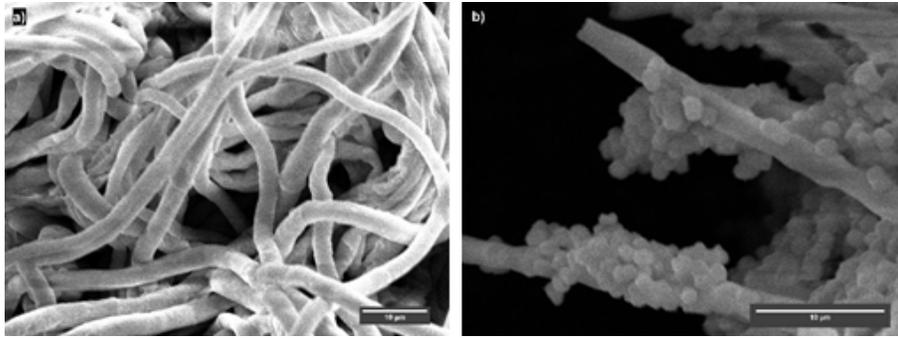


Figure 7

SEM images of bacteria attached to the fungal hyphae: a) *Fusarium* (F54) at 3.3 k magnification; b) *Rhodococcus* (B23a) on hyphae of *Fusarium* (F54) at 7 k magnification. The scale bar in both images corresponds to 10 µm.

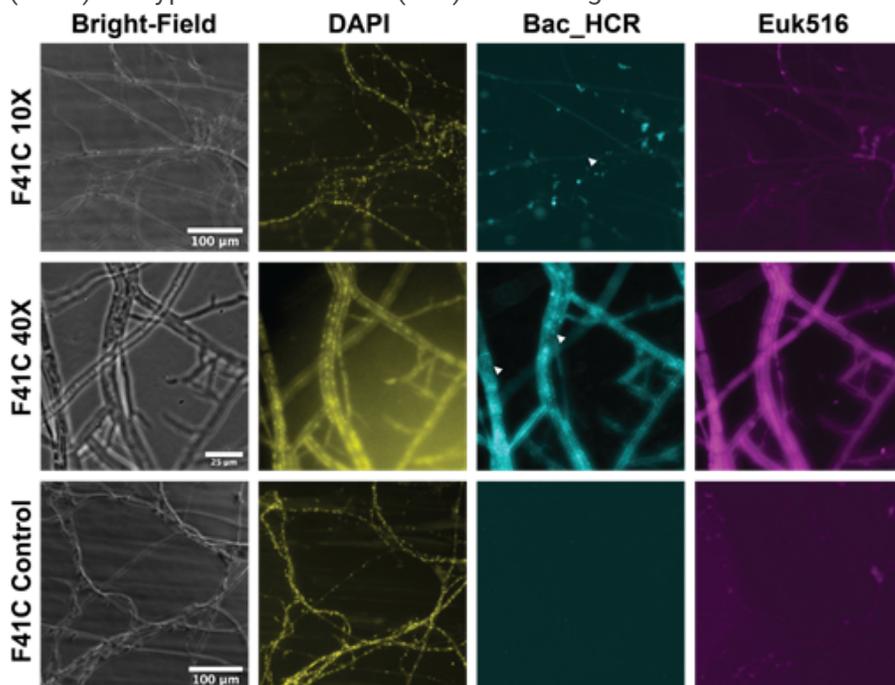


Figure 8

Fluorescence light microscopy of the fungal hyphae of the fungus *Didymella* (F41c), the bacteria were stained with the HCR FISH probe for Bacterial 16S rRNA (Cyan) while the fungi was stained with a standard FISH probe Euk516 region of 18S rRNA (Magenta), nuclei of fungi were stained with DAPI (Yellow). Example spherical bacteria-like structures observed within fungi are pointed out by white arrows. Control autofluorescence of Bacterial Channel (Cyan) determined background fluorescence of fungi.

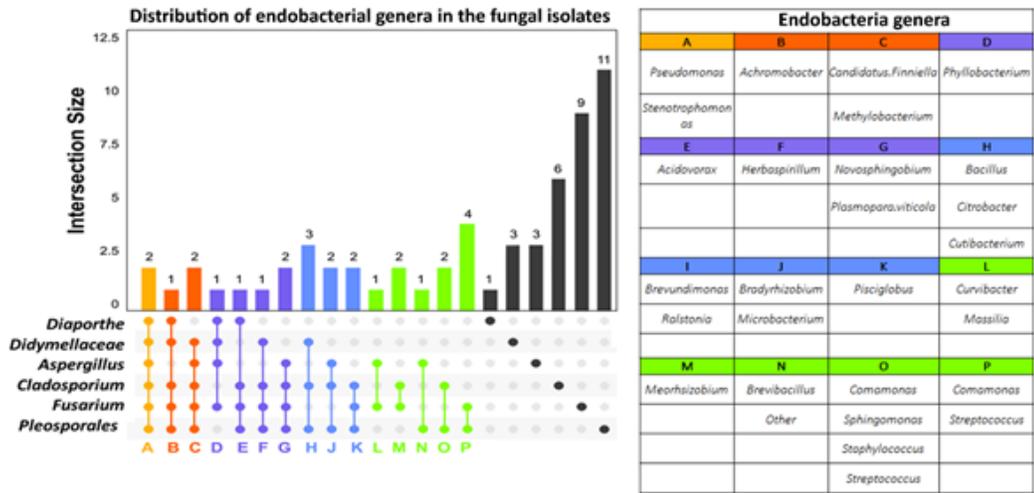


Figure 9

UpSet plot of the shared taxa of endobacteria identified in the principal fungal taxa analyzed. The analysis was performed clustering all the isolates obtained in the rhizosphere of *Citrus sinensis*, *Diospyros kaki*, *Cycas revoluta*, *Ilex vomitoria*, *Myrica cerifera*, and *Buxus sempervirens* and the plant based media (Oatmeal agar, Cornmeal agar, Sorghum grain agar and Potato-carrot agar). The table on the right visualizes the alphabetized and color-coded endobacteria genera, the number of endobacteria genera found in the fungi, and the taxonomic composition of each specific aggregates of endobacteria.

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