Diversity of Fungal microbiome obtained from plant rhizoplanes

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Highlights

- Reconstruction of potential associations between bacteria and fungi through the combination of highway column isolation and network analysis.
- The microbiome inside the fungal hyphae is different from the microbiome colonizing the surface of the hyphae.
- There is a core of bacteria potentially associated with the nitrogen cycle identified in the isolated fungal collection.
- Metabolic pathways identified with PICRUSt2 analysis of the putative endobacterial community are associated with their survival within the fungal host.

Abstract

Microbial communities, and their ecological importance, have been investigated in several habitats. However, so far, most studies could not describe the closest microbial interactions and their functionalities. This study investigates the co-occurring interactions between fungi and bacteria in plant rhizoplanes and their potential functions. The partnerships were obtained using fungal-highway columns with four plant-based media. The fungi and associated microbiomes isolated from the columns were identified by sequencing the ITS (fungi) and 16S rRNA genes (bacteria).

Statistical analyses including Exploratory Graph and Network Analysis were used to visualize the presence of underlying clusters in the microbial communities and evaluate the metabolic functions associated with the fungal microbiome (PICRUSt2). Our findings characterize the presence of both unique and complex bacterial communities associated with different fungi. The results showed that *Bacillus* was associated as exo-bacteria in 80% of the fungi but occurred as putative endo-bacteria in 15%. A shared core of putative endo-bacterial genera, potentially involved in the nitrogen cycle was found in 80% of the isolated fungi. The comparison of potential metabolic functions of the putative endo- and exo-communities highlighted the potential essential factors to establish an endosymbiotic relationship, such as the loss of pathways associated with metabolites obtained from the host while maintaining pathways responsible for bacterial survival within the hypha.

Keywords: fungal highways; exo-bacteria; fungal microbiome; bacteria–fungi interactions; Network Analysis.

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, NMDS: Non-metric multidimensional scaling; PERMANOVA: Permutational Multivariate Analysis of Variance; EGA: Exploratory Graph Analysis; PICRUSt2: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

1. Introduction

The importance of plant symbiotic relationships with fungi and bacteria have been reported in many ecosystems (Rodriguez and Redman 2008, Behie and Bidochka 2014). Symbiotic relationships between plants and their associated microbiomes have been shown to reduce the need for chemical fertilizers (Franche, Lindström et al. 2009, Hungria, Nogueira et al. 2013, Sharma, Sayyed et al. 2013), improve plant fitness, increase resistance to abiotic (*i.e.* salinity and drought) and biotic stresses (Aroca, Ruiz-Lozano et al. 2013, Gómez-Bellot, Nortes et al. 2015), either by conferring increased host resistance to pathogens (Linderman 2000), or by preventing the establishment of antagonistic microbial communities (Azcón-Aguilar and Barea 1997, Linderman 2000, Bordiec, Paquis et al. 2011). However, most of these studies analyzed individual plant-fungi or plant-bacteria interactions (Broeckling, Broz et al. 2008), which correspond to only a fragment of the complex network of interkingdom associations occurring in the rhizosphere (Weston 2003).

The intimate bacterial-fungal interactions (BFI) inside or outside the fungal hyphae, neglected until the early '70s (Mosse 1970), have demonstrated how the presence of the endobacterium can play a key role in the fitness of the fungal host (Lumini, Bianciotto et al. 2007, Uehling, Gryganskyi et al. 2017, Lupini, Peña-Bahamonde et al. 2022). Despite previous efforts, current literature on BFI is either focused on in-depth studies of complex ecosystem by sequencing whole soil microbial communities or centered on pairwise interactions (de Boer 2017). Broad studies of soil microbial communities can be misleading, as they may underestimate the co-occurrence of specific fungal-bacterial partnerships. On the other hand, studies of model associations may not be representative of the overall diversity of interactions in the soil (Robinson, House et al. 2021).

78 Therefore, a different approach is needed to gain both a broad perspective on the diversity of the
79 co-occurring fungal-bacterial partnerships in rhizoplanes, determining whether there is a
80 presence of core endo- and exo- microbiomes associated with the soil fungi, as well as a more in-
81 depth insight on the potential functions associated with these BFI. To fill this knowledge gap, we
82 centered our investigation on obtaining a subset of the fungal community and their intimately
83 associated bacterial microbiome found in close proximity to the root system of six different
84 plants via the fungal highway columns followed by traditional isolation techniques of bacteria
85 and fungi, taxonomic profiling, and functional prediction analyses.
86 This work, albeit, based on an isolation method involving the microcosm fungal-highway
87 column, which is characterized by a reduced fungus recovery yield, allowed us to identify both
88 unique and recurrent patterns of BFI in fungi commonly found in soils. The diversity of BFI
89 obtained also allowed us to determine the potential metabolic functions associated with the
90 whole fungal microbiome, which includes both putative endo- and exo-bacteria.
91

92 # 2. MATERIALS AND METHODS

93 ## 2.1 Media

94 Potato dextrose broth (PDB), Luria Bertani broth (LB), $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, and
95 $ZnSO_4.7H_2O$ were purchased from Sigma Aldrich. Potato carrot (PC) was purchased from
96 HiMedia (VWR). Oatmeal (100% whole grain), cornmeal (100% dried corn), and sorghum grain
97 (Bob's Red Mill sorghum grain) agar plates were prepared following the ATCC medium 551
98 modified protocol (Atlas 2010). All media prepared for the fungal isolation were supplemented
99 with 150 mg/L final concentration of chloramphenicol as the antibiotic (Alfa Aesar) (Abass

2017). The bacterial isolation media were prepared with 50 mg/L cycloheximide, as a fungicide (Sigma Aldrich) (Ha, Ricke et al. 1994).

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

The method used for the collection of fungi and associated bacteria was previously described as the "fungal-highway column". This approach was established as a novel method for fungal isolation from soil together with its associated exo-bacteria (Simon, Bindschedler et al. 2015). We used the same system with a few modifications. Briefly, the modified 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 sterilized by boiling it with deionized water for 15 minutes and soaked in 70% ethanol overnight. After sterilization, it was air-dried aseptically. The other parts of the microcosm, such as caps, mesh, and 1 mm glass beads, were autoclaved for 20 min at 121 ^{o}C and 15 psi. The assembly of the microcosm parts was performed using aseptic techniques. The glass beads were used to fill the inside of the entire column. The selected attracting medium, (either potato-carrot, oatmeal, cornmeal, or sorghum grain agar) was placed on top and at the bottom of the sterile caps of the column (Figure A1_Appendix A). At the bottom, a perforated cap was used to allow unhindered contact with the rhizoplane. 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 (Simon, Bindschedler et al. 2015) were made to reduce the overall microcosm size, be cost-

123 effective, easier to manufacture and minimize the possible contamination of the column with
124 mites and their associated bacteria.
125 The microcosm fungal highway columns (Figure A1_Appendix A) were set up in the rhizoplane
126 of six different plants, which were selected based on their tree or bush growth forms and named
127 in this study as Tree type and Bush type. The Tree type (T) was composed of two angiosperms,
128 *Citrus sinensis* (Orange tree) (T1), and *Diospyros kaki* (Persimmon tree) (T2), and a
129 gymnosperm, *Cycas revoluta* (Cycad) (T3). The plants included in the Bush type (B) had three
130 evergreen angiosperm shrubs, *Ilex vomitoria* (Yaupon) (B1), *Myrica cerifera* (Wax myrtle) (B2),
131 and *Buxus sempervirens* (Boxwood) (B3). At each site, triplicate fungal highway columns for
132 each media were set up in the soil under the corresponding plants selected for this study. After
133 one week of direct contact with the plant roots in the soil, the microcosms were removed and
134 transported to the laboratory in sterile Whirl-Pak® bags for immediate microbial isolation. Under
135 sterile conditions, the bait on top of the column cap was spread onto sterile plates containing the
136 same media used as an attractant for the isolation of culturable microorganisms.
137 All the plates were incubated at 28 ^{o}C for two weeks. After the incubation, the fungal colonies
138 grown on the plates were isolated into their corresponding growth media. These media contained
139 either antibiotic to purify the fungi from exo-bacteria (Li, Sato et al. 2010, Medrano, Grauke et
140 al. 2017, Heydari, Siavoshi et al. 2021) or fungicide to purify the bacteria from the fungi, as
141 described earlier (Ha, Ricke et al. 1994). The isolation of fungi or bacteria continued until a
142 colony with single morphology was distinguishable. Here we clarify the boundaries between
143 endo- and exo-bacteria, defining “putative endo-bacteria" as the community of bacteria present
144 in the form of endosymbionts within the hypha, and " exo-bacteria", as the bacteria capable of
145 colonizing and surviving on the surface of the hypha. The curation of the exo-bacteria from the

surface of the hypha for the analysis of the endobacteria was confirmed via scanning electron microscopy (SEM) of several fungal isolates before the endobacteria community sequencing (Figure A2-A10_Appendix A) (Cruz and Ishii 2011, Medrano, Grauke et al. 2017, Heydari, Siavoshi et al. 2021). Due to the reduced number of microorganisms obtained following the isolation process, the different isolates from the same locations were pooled together at the expense of the number of replicates.

In this present study, the cultivable bacteria obtained from the first step of isolation, as associated with the surface of the fungus, will be referred to as exo-bacteria. The presence of the endobacterium inside the host has been confirmed for the fungus *Didymella* (F41c) using the Fluorescent In Situ Hybridization (FISH) technique (Figure A11_Appendix A). Although this preliminary validation procedure has confirmed the presence of the bacteria inside the hyphae, due to the number and diversity of the fungal hosts and specific optimization requirements for the FISH technique for each type of fungi, it is not possible to do for every single fungal isolate in the scope of this study. Hence, not having confirmed the presence of the bacterial community inside all fungi, we have referred to the endobacteria, as putative endo-bacteria in this study (Izumi, Anderson et al. 2006).

The distilled water stasis technique was applied to store the fungal isolates (Humber 1997). The bacterial isolates were stored in 25 % sterile glycerol at – 80 ^{o}C. Detailed information about the corresponding liquid media under optimal growth conditions for the bacteria and fungi can be found in Appendix B.

2.3 DNA extraction and quality control

Sterile disks of cellophane paper (Gel Company cellophane sheet 35x45cm PK100, Fisher Scientific) were placed on top of the agar plates to facilitate the fungal mycelium collection for

DNA extraction. An agar plug, containing the fungal mycelium, was transferred to the center of a Petri dish containing cellophane and incubated until the fungus reached approximately 1/2 to 2/3 of the plate diameter. As a control for the DNA extraction, a Petri dish containing sterile cellophane paper was also incubated. After the incubation, the biomass was processed, and the DNA was extracted using the Zymo 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. To obtain 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 its growth ability in either of these two media (Appendix B). The ones that could not grow in liquid media (Appendix B) were inoculated on plates, and 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 Isolates

The fungal isolates were sequenced using the Sanger sequencing method with primers for the internal transcribed spacer (ITS) (ITS1F and ITS4) (White, Bruns et al. 1990). The conditions of the PCR are presented in Table A1 in Appendix A. First, the fungal DNA was amplified, 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 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 sequences were grouped into OTU (cluster cutoff=0.03) using Mothur (Schloss, Westcott et al. 2009) (Appendix B) and processed with *vegan* package (Dixon 2003) in R to calculate Shannon diversity indices (Shannon 1948). The sequences were aligned using MUSCLE (Edgar 2004) and manually trimmed with MEGA version X (Kumar, Stecher et al. 2018). The fungal identification was assigned using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the most closely related taxa in the reference databases. 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 A1_Appendix A)(White, Bruns et al. 1990). The phylogenetic analysis was inferred using the Neighbor-Joining method (Saitou and Nei 1987) build in MEGA version X (Kumar, Stecher et al. 2018) (Figure A12_Appendix A).

2.4.2 Identification of the exo- and putative endo-bacterial community associated with the fungal hyphae

To reduce the technical biases and therefore being able to better compare the two communities of bacteria (exo- and putative endo-bacteria) associated with the fungal isolates we decided to use the same sequencing technology (Illumina MiSeq). The exo-bacterial isolates were identified by sequencing the V3/V4 region of 16S rRNA, which was amplified using the F341/R806 primer pair (Table A1_Appendix A) (Nguyen, Castro-Wallace et al. 2017). The sequencing was conducted using a 300-cycle paired-end Illumina MiSeq protocol. Controls of the sequencing reagents used in this study were performed with the same PCR master mix used to prepare the

libraries for the MiSeq sequencing of the isolates to ensure lack of contamination. All sequence analysis steps were performed using the QIIME2 application within the EDGE Bioinformatics environment (Callahan, McMurdie et al. 2016, Bolyen, Rideout et al. 2019). The PCR primer sequences were removed from the forward and reverse reads. The sequences were quality screened using maximum expected error parameters before being denoised with DADA2 (Callahan, McMurdie et al. 2016). The denoised sequences were merged before the taxonomic assignment. For the 16S amplicon sequences, the taxonomy assignment was done with a Naive Bayes Classifier that was trained using the portion of the SILVA database sequences that matched the amplicon used here (Robinson, House et al. 2021) (Appendix C). To better compare the exo-bacterial results with the fungi, the sequences of the 16s rRNA genes were also grouped into OTU (cluster cutoff=0.03) using Mothur (Schloss, Westcott et al. 2009) (Appendix B).

To determine the presence of putative endo-bacteria associated with the fungal isolates, the DNA obtained from the fungal mycelium, after removal of exo-bacteria and confirmation by SEM (as described above), was used as template for the amplification of the bacterial 16S rRNA genes (primers EUB9-27F and 907r), as described in Table A1_Appendix A. The amplification was visually determined on 1% gel electrophoresis. After that, the putative endo-bacteria identification was performed with nested PCR (Lumini, Bianciotto et al. 2007, Salvioli, Lumini et al. 2008) targeting the V1/V5 region and theV3/V4 region with the primers F341/R806 (Table A1_Appendix A). The identification was obtained following the same protocol as described above for the identification of the exo-bacterial isolates (Robinson, House et al. 2021). The DNA sequences originated from the nested 16S rRNA sequencing of the fungus were analyzed using the QIIME2 application to exclude the presence of reads corresponding to the mitochondria in the 16S rRNA V3-V4 hyper-variable region (Thomas, Sekhar et al. 2017).

All sequencing data were deposited in the NCBI database (ITS sequences: accession numbers MT771292-MT771337; LSU sequences: accession number MT625979-MT626024; 16S rRNA gene sequences: accession number MZ374450-MZ374743; BioProject PRJNA644907).

2.5 Statistical analysis and Data Processing

2.5.1 Non-metric multidimensional scaling (NMDS) Analysis and Permutational Multivariate Analysis of Variance (PERMANOVA)

To assess similarities between the putative endo- and exo-bacterial communities, we performed a non-metric multidimensional scaling (NMDS) analysis (Kruskal 1964, Faith, Minchin et al. 1987).

To remove the possible biases due to the difference in library preparation between putative endo- and exo-bacterial communities, the data sets were aggregated based on the identified bacterial genus and transformed into presence/absence matrix. The distance matrix, calculated based on the Jaccard dissimilarities, was performed using the *vegan* package (Dixon 2003) using 2 dimensions (k). The obtained results were displayed using the ggplot2 package (Wickham 2016). The points were color-coded based on the fungal taxa and shape-coded based on the type of isolation media.

To test the statistical significance of the results, the NMDS was complemented with a Permutational Multivariate Analysis of Variance (PERMANOVA) analysis using the *vegan* function adonis2. (Anderson 2001)

2.5.2 Diversity index and Indicator Species Analysis

The OTUs obtained for both bacterial and fungal isolates were processed with *vegan* package (Dixon 2003) in R to calculate Shannon diversity indices (Shannon 1948) and displayed using OriginPro (OriginPro, Version 2021b. OriginLab Corporation, Northampton, MA, USA) and the R package *ggplot2*. (Wickham 2016)

To determine the characteristic of the specific functions associated with the putative endo- and exo-bacteria communities in the different locations, the multipattern analysis, the build-in function of the *indicspecies* package in R studio (Cáceres and Legendre 2009, De Cáceres 2013), was used. This was implemented using the IndVal.g function and 99 permutations. Only species with a p-value <0.05 were considered.

2.5.3 Exploratory Graph Analysis

To define the presence of clusters of similarities across the putative endo-bacteria, the Exploratory Graph Analysis (EGA) (Golino and Epskamp 2017) was performed using the R studio package EGAnet (Christensen and Golino 2019, Golino, Christensen et al. 2020). The values of the different nodes and the different clusters were calculated using the Triangulated Maximally Filtered Graph (TMFG) model, the walktrap algorithm, and 50 bootstraps iterations (descriptive statistics reported in Table A2-A3_Supplementary information). A similar analysis was performed for the identified exo-bacteria but due to the reduced number of isolates, no significant result was obtained.

2.5.4 UpSet Plot and Community Characterization

To determine the presence of a specific pattern of putative endo-bacteria and exo-bacteria associated with the fungal host, the UpSet plot (Conway, Lex et al. 2017) analysis was performed using the R studio package UpSetR (Jake Conway 2019). This type of graph, unlike an ordinary Venn Diagram, allows you to visually compare a greater number of sets. The

visualization was based on the most abundant fungal taxa. The counts of specific fungal taxa within each intersection were color-coded based on the fungal taxa.

2.5.5 Network Analysis

The network analysis was performed using the program igraph in R-studio. The vertexes corresponding to the identified genus of fungi and relatively co-isolated bacteria were color-coded and labeled accordingly. The edges were constructed based on the nature of co-occurrence, exo- or putative endo-symbiont, and the number of each specific association. The size of the vertex was calculated and plotted as coreness (K-core decomposition) (Bader and Hogue 2003). The layout of the network was obtained using the Kamada Kawai algorithm (Kamada and Kawai 1989). The Kamada and Kawai algorithm was chosen for the visualization of the network due to the capacity to cluster nodes based on their similarity, centering those microorganisms characterized by greater interconnectivity (Kobourov 2004).

2.5.6 Prediction of bacterial community functions

To metabolic functions of the bacteria obtained in this study were inferred based on their identification with the 16S rRNA genes sequences using the pipeline Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) (Langille, Zaneveld et al. 2013). The potential metabolic functionalities were assigned using the MetaMyc (Caspi, Foerster et al. 2006, Caspi, Altman et al. 2014) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto 2000) (Appendix D). The obtained results were analyzed to determine the presence of specific metabolic patterns associated with microbial communities using the R package *indicspecies* (Cáceres and Legendre 2009, De Cáceres 2013).

3. RESULTS AND DISCUSSION

306 Bacteria have been described to colonize and use the fungal hyphae as highways to translocate in
307 unsaturated soil matrices (Simon, Bindschedler et al. 2015, Simon, Herve et al. 2017, Pilar
308 Junier, Guillaume Cailleau et al. 2021). Recently, a microcosm fungal highway column was
309 developed as a unique isolation technique that allows bacteria to be transported using fungal
310 hyphae and to experimentally obtain insights into the bacterial-fungal interactions occurring in
311 the soil (Simon, Bindschedler et al. 2015, Simon, Herve et al. 2017, Pilar Junier, Guillaume
312 Cailleau et al. 2021). In the present study, this device was placed in the rhizoplane of six
313 different plants using four different plant-based media as attractants for the isolation of
314 cultivatable fungi and their associated bacteria.

315 ## 3.1 Fungal isolation from highway columns: relationship with types
316 of plants and media

317 The fungal highway columns yielded a total of 46 morphologically distinct fungi from six
318 different plant rhizoplanes using four different plant-based media (Figure 1, Appendix A). The
319 OTU classification of these isolates via ITS (ITS1F and ITS4) sequencing determined the
320 isolation of 32 fungal OTUs, which were used for the subsequent analyses (Appendix B). The
321 fungi obtained in this study belong to taxa ubiquitously found in soil (Nesci, Barros et al. 2006).
322 Although many of the identified genera possess species known to be plant pathogens, the
323 pathogenicity of our isolates was not determined. The most represented fungal genera found in
324 the collection were *Fusarium* and *Cladosporium,* both common soil-borne fungi largely
325 associated with plants and common inhabitants of the soil microbial community (Steinkellner,
326 Lendzemo et al. 2007). Other fungi isolated in this study, which possess species that are plant
327 pathogens, were *Alternaria* (Yoder 1980), *Diaporthe*, and its asexual state *Phomopsis* (Masirevic
328 and Gulya 1992, Gomes, Glienke et al. 2013). Moreover, we were able to isolate *Aspergillus*,

329 which is often present in soils (Bossche, Cauwenbergh et al. 2013), and *Didymella*, known for its
330 pectolytic activity against plant cell walls (Chilosi and Magro 1998). We also isolated fungi
331 often described as endophytes, such as *Pestalotiopsis* (Hu, Jeewon et al. 2007) and
332 *Plectospaerella* (Carlucci, Raimondo et al. 2012), and non-pathogenic slow-growing molds, such
333 as *Stachybotrys* (Wu and Zhang 2009) and *Kalmusia.* The latter is a fungus commonly associated
334 with the soil crust (Christensen and Kinter 2001).
335 The additional investigation involved the identification of possible relationships between plant
336 type and the fungal taxa. For this purpose, we grouped the plants based on their morphological
337 characteristics in "tree" and "bush". In the group "tree" were included *Citrus sinensis* (Orange
338 tree), *Diospyros kaki* (Persimmon tree), and *Cycas revoluta* (Cycad), respectively labeled T1, T2,
339 and T3. The *Citrus sinensis* (T1) was characterized by the fewest recovered fungal OTUs (Figure
340 1), *i.e.* one from Sorghum grain agar and one from Potato-carrot agar. Similar results and low
341 fungal yield was previously observed in another study with the same type of tree (Sun, Zhang et
342 al. 2017). These authors described that the principal taxa of fungi associated with *Citrus sinensis*
343 were Basidiomycota, a phylum generally characterized by slow-growing fungi, and consequently
344 difficult to isolate with conventional culturing techniques (Warcup 1959). This was the only site
345 where *Kalmusia* and *Plectosphaerella* were isolated. Neither *Kalmusia* nor *Plectosphaerella*
346 were previously described to be associated with orange trees (Alderman and Polglase 1985,
347 Palm, Gams et al. 1995, Zhang, Chen et al. 2019). Among all the sites studied, the largest
348 numbers of fungal OTUs were obtained in the rhizoplane of *Diospyros kaki* (T2) (Figure 1). This
349 result may be explained by the natural decay of fallen persimmon fruits, which increased the
350 nutrient content in the soil (Palou, Montesinos-Herrero et al. 2009). Such a finding is also

corroborated by the fact that the majority of the fungi obtained at this site were saprophytes (Dickinson and J.P 1981, Fracchia, Garcia-Romera et al. 2000).

In the case of the bush-type plants, the number of fungi retrieved ranged from five, coming from the *Buxus sempervirens* (B3) to nine from *Ilex vomitoria* (B1) (Figure 1). Also, *Ilex vomitoria* (B1) was the only plant with the presence of the fungus Diaporthales (Figure 1). This order comprises many known plant pathogens commonly associated with various plants (Udayanga, Liu et al. 2012, Gomes, Glienke et al. 2013), which confirmed the association of the fungus with the sampling site. The results related to the microbial diversity associated with the plant type showed that there is no statistically significant difference between the "Trees" and "Bush (Figure A13, Table A4_Appendix A). Although not statistically significant, the overall comparison of the bush and tree sites showed that the bush sites were characterized by a fungal collection more diverse (average bush Shannon index=1.64, average tree Shannon index=1.54) (Figure 1). In general, both sites seemed to share a core of four fungi that had several species commonly found in soils, namely, *Fusarium, Aspergillus*, *Cladosporium,* and, albeit not identified at the genus level, Pleosporales (Steinkellner, Lendzemo et al. 2007, Bossche, Cauwenbergh et al. 2013) (Figure 1, Figure A14_Appendix A). However, a greater number of replicates is necessary to determine taxa-specific associations between the selected plants and the identified fungi.

In addition to the types of plants, we determined the effect of different plant-based media by analyzing the Shannon diversity index (referred to as diversity in the subsequent text) of fungi collected for each plant type. Sorghum grain agar recovered the more diverse fungal community (Shannon index 2.21), while potato-carrot agar recovered the less diverse (Shannon index 1.33) (Figure 1). Furthermore, we found at least one common taxon, *i.e*. *Fusarium* and Pleosporales, in both tree and bush plant types, as well as in all growth media, except for potato-carrot. This

finding suggests that these two taxa, possibly due to the ability to utilize different nutrients, are ubiquitous in different plant rhizoplanes.

Considering the different number of fungal and bacterial OTUs obtained in the different sites, we were not able to define an optimal plant-based media for the isolation of fungi and their associated bacteria. This is due to diverse nutrient requirements for different microorganisms (Muggia, Kopun et al. 2017) and the complexity of substrates provided by plant-based media (Rees, Bashir et al. 2021). In summary, we cannot exclude that the combination of selected media and microcosm column may have favored the selection of some types of fungi over others (*i.e* Basidiomycetes and Glomeromycota).

3.2 Linking fungal and exo-bacterial isolates from the same columns

As previously found in the literature, the coexistence of fungi and bacteria in soils has led to the formation of strong associations in which the partners have mutually adapted (Garbaye 1994). Among the examples of this adaptation is the bacterial ability to be transported along the fungal hypha to reach niches underground that would otherwise be impossible to reach (Kohlmeier, Smits et al. 2005) and facilitate the translocation of other bacteria through the production of biofilms (Warmink, Nazir et al. 2011). In our study, the exo-bacteria associated with the fungal hyphae were also investigated. A total of 51 exo-bacteria were successfully collected with serial transfers on plant-based media with fungicide (Figure 1). Based on OTU classification, 37 bacterial OTU emerged from our collection (Appendix B).

As expected, a method of isolation linked to the selective translocation of fungi and associated bacteria has influenced the number and diversity of isolates obtained. However, the aim of this study was to select the fraction of bacteria intimately associated with fungi to try to understand

397 their interactions and not determine the whole soil microbial community. Despite the limited
398 number of sites and isolates, there was a positive linear correlation between the fungal taxa and
399 their associated exo-bacteria. This correlation holds true for both the number of isolates and the
400 diversity index, suggesting that a wider range of fungi have the ability to transport a more
401 diverse array of bacteria. (Pearson's r^2 respectively 0.90 and 0.92) (Figure A15-A16, Table A5-
402 A6_Appendix A). He et al. reported a similar result where a positive correlation was described
403 when they studied the possible presence of a linkage between diversities of plants, fungi, and
404 bacteria (He, Wang et al. 2008). However, in the study of He and collaborators, they determined
405 the bacterial diversity using the PCR-DGGE fingerprinting technique. In our study, we
406 demonstrate that the use of the microcosm columns could obtain similar results, corroborating
407 the effectiveness of this new approach. Based on the literature and our findings, we can infer
408 there might be species-specific partnerships between fungus and certain exo-bacteria occurring.
409 Moreover, considering the co-occurrence of multiple bacteria and fungi retrieved in the columns,
410 the possibility of non-specific interactions cannot be excluded. For these reasons, future studies,
411 and a greater number of isolates, will be necessary to confirm this specific hypothesis.
412 A more detailed analysis of the exo-bacterial community obtained showed that the most
413 predominant exo-bacterial phyla were Firmicutes (57%), followed by Proteobacteria (29%) and
414 Actinobacteria (16%). These phyla have been described as the most prominent in microbial
415 communities associated with the rhizoplane of diverse plants, such as *Arabidopsis thaliana*
416 (Lundberg, Lebeis et al. 2012), *Setaria italica* (Foxtail millet) (Jin, Wang et al. 2017), *Glycine*
417 *max* (soybean), (Mendes, Kuramae et al. 2014) and *Panicum virgatum* (switchgrass) (Xia,
418 DeBolt et al. 2015). In our study, based on the plant type, we noticed a wider range of isolates
419 from the tree-rhizoplanes, with a difference between the minimum and the maximum number of

unique isolates almost 3 times higher than the bush-rhizoplanes (Figure 1). Among all sites, except *Citrus sinensis* (T1) and *Cycas revoluta* (T3), *Bacillus* was the most common genera, followed by other well-known soil bacteria: *Acinetobacter*, *Ensifer*, *Pseudomonas*, *Microbacterium*, *Paenibacillus*, *Rhizobium,* and *Stenotrophomonas*.

Species belonging to the aforementioned bacterial genera have previously been characterized by their ability to act as plant growth promoters (Rogel, Hernández-Lucas et al. 2001, Patten and Glick 2002, Dardanelli, Manyani et al. 2010, Rokhbakhsh-Zamin, Sachdev et al. 2011, Cordovez, Schop et al. 2018) or as biocontrol agents against plant pathogens (Shafi, Tian et al. 2017). In the case of fungi, most of the identified fungal isolates belong to genera that contain plant pathogens. However, further investigations on the presented fungi and their associated exo-bacteria will be necessary to confirm what was previously defined as a dynamic equilibrium between the populations of pathogenic fungi and biocontrol bacterial agents at the level of the rhizoplane (Raaijmakers, Paulitz et al. 2009). Among the various associations found, it should be noted the one between *Pseudorhodoferax* and *Kalmusia*, to the best of our knowledge, has never been described.

The study of these isolates, both those found in association with different fungi (such as *Bacillus*, *Pseudomonas,* and *Ensifer*) and those found only in association with a type of fungus (such as *Pseudorhodoferax*) could define potential fungiphile bacteria, both specific and universal (Warmink, Nazir et al. 2011). These findings further confirm the crucial role of the "fungal-highway column" technique to reveal new potential intimate associations between fungi and bacteria.

3.3 Putative endo-hyphal microbiome and bacterial-fungal specificity

443 Like other eukaryotic organisms, fungi have also been reported to have established associations
444 with bacteria as endosymbionts (Bonfante 2003, Lumini, Ghignone et al. 2006, Xia, DeBolt et al.
445 2015). To determine if any of our fungal isolates harbored bacteria within their hyphae, after
446 confirming via SEM that the exo-bacteria were successfully removed (Figure A2-A10_Appendix
447 A), we performed an initial quantitative determination of the 16S rRNA gene signal using the
448 qPCR method (Table A1_Appendix A).
449 After confirming the presence of putative endo-symbionts in the fungal isolates, sequencing of
450 the 16S rRNA gene was performed (taxa of putative endo-bacteria present in each fungal isolate
451 are listed in the Appendix C, Figure A17_Appendix A). Most of the putative endo-symbiotic
452 bacteria found in this study were composed of phyla commonly associated with the soil
453 microbial community, such as Proteobacteria, Firmicutes, and Actinobacteria (Schloss and
454 Handelsman 2006).
455 To test the hypothesis that there is a distinct separation between the exo- and putative endo-
456 bacterial communities associated with the fungi, we performed a Permutational multivariate
457 analysis of variance (PERMANOVA) (Anderson 2001). We found that there is a statistically
458 significant difference between the two communities (P-value 0.001, Figure 2 and Table
459 A7_Appendix A). Among the identified putative endo-symbionts, only *Bacillus*,
460 *Microbacterium*, *Pseudomonas,* and *Stenotrophomonas* were also co-isolated as exo-bacteria.
461 *Pseudomonas* and *Stenotrophomonas* were found to be present as both putative endo- and exo-
462 bacteria, respectively, in 85% and 71% of their co-isolated fungi. *Bacillus*, on the other hand,
463 was associated as exo-bacteria in almost 80% of our collection but present in 15% of the fungal
464 isolates as putative endo-bacteria, suggesting that these genera could be horizontally transmitted
465 from the fungus to the external environment and vice versa. Further studies are required to

466 confirm the potential horizontal transfer between the microbial community present outside the
467 fungus and its endosymbionts. However, these preliminary results suggest a potential link
468 between putative endo- and exo-bacterial communities(Bianciotto, Genre et al. 2004), although
469 not all bacterial taxa possess equal capability to cross the fungal hypha barrier.
470 The non-metric multidimensional scaling (NMDS) (Kruskal 1964, Faith, Minchin et al. 1987,
471 Dixon 2003), followed by Permutational multivariate analysis of variance (PERMANOVA)
472 (Anderson 2001) was performed on the community of putative endo-bacteria to determine
473 similarity in the microbiota within the same fungal taxa (Figure A18_Appendix A) but, possibly
474 due to the reduced number of isolates, no evident clusters were found (Table A8_Appendix A).
475 Therefore, to cluster the different bacteria identified, we used Exploratory Graph Analysis, an
476 analysis proved to accurately measure and visualize underlying correlations among nodes in
477 complex datasets (Golino, Shi et al. 2020). This approach presents the clusters of bacterial taxa
478 by nodes, depicting underlying similarities within the putative endo-bacteria community. Three
479 different clusters, characterized by a descending correlation between the nodes, emerged from
480 this analysis (Figure 3_A). The first cluster contained five different bacteria (*Pseudomonas*,
481 *Methylobacerium*, *Phyllobacterium,* and *Brevundimonas* and Microbacterium), the second,
482 contained six different bacteria (*Stenotrophomonas*, *Citrobacter*, *Herbaspirillum*,
483 *Achromobacter*, *Candidatus Finniella,* and *Acidovorax*) and finally, the third consisted of four
484 bacteria (*Bradyrhizobium*, *Bacillus*, *Cutibacterium,* and *Novosphingobium*). Most of these
485 bacterial taxa appear to be part of the phylum Proteobacteria, which as previously described, is
486 among the most representative phyla of fungal endosymbionts (Hoffman and Arnold 2010,
487 Robinson, House et al. 2021).

The presence of these 3 bacterial clusters was then characterized at the level of the single fungal host through the UpSet plot analysis. From the analysis, emerged that the genera belonging to the three bacterial cores were associated as putative endosymbionts in most fungal hosts (Figure 3_B-C-D) confirming the presence of a core of putative endobacteria.

It is important to mention that, even if more than 90% of our isolates harbored a diverse putative endobacteria community, it is possible that, if the antibiotic used for the removal of the exo-bacteria from the hyphae was able to penetrate inside the hyphae, some changes in the putative endobacteria microbial community could have occurred (Partida-Martinez, Monajembashi et al. 2007, Castro-Seriche, Jerez-Morales et al. 2021). This effect of cultivation with antibiotics on the putative endobacterial microbial community should be investigated in future studies.

3.4 Fungal microbiome: reconstruction of the microbial networks between the fungi and bacteria in the soil

To determine potential ecological niches underlying the microbial associations found in this study, a network analysis was carried out. In this analysis, the two bacterial populations, putative endo- and exo-bacteria, were visualized separately (Figure 4).

In the center of the network associated with the exo-bacteria, emerged a cluster of bacterial nodes, characterized by a high number of interconnections (vectors) with their related fungal nodes. As we can observe, four main bacterial nodes corresponding to *Bacillus*, *Stenotrophomonas*, *Pseudomonas,* and *Herbaspirillum,* were grouped in the center of the graph. These genera are frequently encountered in studies of microbial communities associated with rhizospheres (Caesar-TonThat, Caesar et al. 2007, Aravind, Kumar et al. 2009, Çakmakçı, Dönmez et al. 2010). The observed co-occurrence of those bacteria with different fungi suggests a cosmopolitan association of these bacteria compared to others. This result is in line with what

511 was previously found in other studies, where bacteria, such as *Pseudomonas* and
512 *Stenotrophomonas,* were interacting closely with saprophytic fungi in the soil. (Verrecchia and
513 Braissant 2006, Yoon, Kang et al. 2006, Gahan and Schmalenberger 2014, Brabcová, Nováková
514 et al. 2016)
515 In addition to the cosmopolitan associations observed, a more varied array of co-occurring
516 microorganisms emerged. Although our study was characterized by a reduced number of
517 isolates, we determined the presence of new fungal-bacterial associations potentially important
518 from an ecological point of view (de Menezes, Richardson et al. 2017). For instance, we
519 determined that bacterial genera usually associated with root nodules, such as *Ensifer* (Shiro,
520 Matsuura et al. 2013, Pastorino, Martínez Alcántara et al. 2015, Andrews and Andrews 2017),
521 also interact with fungi.
522 As previously mentioned, the isolation typology investigated in this study entailed the sacrifice
523 of the number of isolates in order to focus on biological associations. We cannot therefore
524 exclude how this may have played a role in the identification and structuring of the network.
525 Due to the reduced number of bacterial isolates, no specific groups of associations within the
526 exo-bacteria community were found by the EGA. A similar result to that of the network analysis
527 can be also visualized from the Upset plot (Figure A19 _Appendix A).

528 3.5 Fungal microbiome: reconstruction of the bacterial functional
529 profiles

530 To determine the potential metabolic capacities associated with the fungal microbiome, the
531 Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2)
532 analysis was used. Through this approach, 336 unique pathways (MetaCyc database) have been
533 inferred based on the 16S rRNA gene sequences (Appendix D).

534 The pathways were subsequently analyzed to determine characteristic functions
535 associated with the community of bacteria inside and outside the fungal hypha using the
536 *indicspecies* analysis. This analysis allowed us to identify 26 unique functions of the putative
537 endobacterial community and 35 associated only with the exo-bacterial community (Table 1).
538 Most of the pathways identified in the putative endobacterial community were connected to the
539 production of lipids (Superpathway of fatty acid biosynthesis initiation, stearate, oleate, cis-
540 vaccenate, and cis-dodecenoate biosynthesis), followed by pathways related to amino acid
541 degradation (L-leucine degradation I, L-histidine degradation II) (Figure 5, Table A8_Appendix
542 A). In the case of the exo-bacterial community, most pathways were associated with the
543 synthesis of cofactors and carriers (superpathway of tetrahydrofolate biosynthesis, superpathway
544 of tetrahydrofolate biosynthesis, 2-carboxy-1,4-naphthoquinol biosynthesis, biotin biosynthesis
545 II) (Figure 5, Table A8_Appendix A), followed by amino acid and nucleotide salvage and
546 biosynthesis (adenine and adenosine salvage III, superpathway of pyrimidine
547 deoxyribonucleotides de novo biosynthesis, pyrimidine deoxyribonucleotides de novo
548 biosynthesis I, superpathway of pyrimidine deoxyribonucleoside salvage, pyrimidine
549 deoxyribonucleosides salvage and superpathway of pyrimidine ribonucleosides salvage) (Figure
550 5, Table A9_Appendix A). Notably, we also found two functions related to the degradation of
551 phenylpropanoids, compounds produced from breakdown of plant materials such as lignin and
552 resins (Dagley 1971), along with different functions responsible for the recycling of
553 biomolecules such as Nicotinamide adenine dinucleotide (NAD) and DNA products (Figure 5,
554 Table A8_Appendix A). These biomolecules are frequently present in soils as
555 deoxyribonucleosides, ribonucleosides, and pyrimidine. In fact, as previously demonstrated, the
556 salvage and reassimilation of partial DNA breakdowns products such as bases, ribose, and

557 nucleosides into nucleic acids, drastically reduce the energy consumption associated with DNA
558 synthesis (Levy-Booth, Campbell et al. 2007). Besides the reduction of the metabolic cost, the
559 capacity to uptake intact genetic information from the environment can also act as a driving
560 factor in microbial adaptation in soils and rhizospheres (Whipps 2001, Berg, Eberl et al. 2005).
561 From the exo- and putative endo-specific functionalities, we determine the potential metabolic
562 pathways possibly involved in the process of adaptation to bacterial endosymbiotic life. For
563 instance, among the putative endobacterial-specific functionalities, we did not find pathways
564 associated with tricarboxylic acid (TCA), biosynthesis of amino acids, and nucleic acids
565 functions, instead they were present among the exo-bacterial specific pathways (Figure 5, Table
566 A8-A9_Appendix A). These metabolites, being essential for the survival and reproduction of any
567 cell must be inevitably assimilated from the fungal host by the endosymbiont. This result
568 supports previously found results in Mycoplasma-related endobacteria (MRE), where the
569 endosymbiont obtains metabolites linked to these pathways from the host (Naito, Desirò et al.
570 2017). Furthermore, among the functions associated with the putative endobacteria but absent in
571 exo-bacteria, we found the lipid IV_A biosynthesis, a necessary precursor for the production of
572 lipopolysaccharides (LPS) (Figure 5, Table A8_Appendix A) (Sleytr, Egelseer et al. 2010). The
573 biosynthesis of LPS, as previously demonstrated in the symbiotic system *Rhizopus* –
574 *Burkholderia*, appears to be part of an adaptive mechanism, which shields the bacterium from the
575 fungus's defense mechanisms. In fact, lower production of lipopolysaccharides by the
576 endobacterium has been shown to result in a drastic reduction of the bacterial survival capacity
577 inside the host (Leone, Lackner et al. 2010). The combination of gene losses (Uehling,
578 Gryganskyi et al. 2017) and maintenance of defense mechanisms against the host defenses are
579 crucial for the establishment of a parasitic or mutualistic association between the bacterium and

580 the fungus. Despite the small number of isolates obtained, this approach was able to identify
581 potential metabolic pathways previously associated with the survival of the endobacterium
582 within the fungal host.
583 Although not identified by the *indicspecies* analysis, we found the presence of pathways related
584 to the nitrogen metabolism in the soil associated with the taxa previously defined as belonging to
585 putative endobacterial core 1 (*Brevundimonas, Methylobacterium, Phyllobacterium,*
586 *Pseudomonas*), 2 (*Acidovorax, Achromobacter, Citrobacter, Herbaspirillum*) and 3
587 (*Stenotrophomonas, Bradyrizobium, Cutibacterium, Novosphingobium).*
588 Among those pathways, we found nitrate reduction (Yin, Chen et al. 2002, Torres, Simon et al.
589 2016) as well as pathways related to the transport and assimilation of ammonium such as L-
590 glutamate and L-glutamine biosynthesis (Figure 5, Table A8_Appendix A) (Merrick and
591 Edwards 1995, Cabello, Roldan et al. 2004, Stadtman 2004). Moreover, we determined that
592 86.8% of the fungi with putative endobacteria had at least one of these bacteria, 66.8% at least
593 two, and 15.8% at least three. Therefore, having found metabolic pathways potentially linked to
594 nitrogen metabolism, within what we defined as the core fungal putative endosymbiont could
595 indicate that this form of nutrient exchange with the host is potentially a common characteristic
596 of this type of fungal-bacterial association. In fact, a previous study on the relationship between
597 *Mortierella elongata* and *Mycoavidus* sp. has demonstrated that, when growing under nitrogen-
598 limiting conditions, despite the reduced growth of the fungus, the endobacterial growth was not
599 affected. This was explained by the capacity of the bacterium to uptake different types of
600 nitrogen-containing compounds from the host (Li, Yao et al. 2017).

It is important to reiterate that the results obtained from the PICRUSt2 analysis are inferred based on the 16s rRNA gene sequences, and they will therefore have to be confirmed using other methods (*i.e* metatranscriptomics).

4. Conclusions

In the present study, by complementing the fungal-highway column with taxonomic profiling of culturable fungi and their intimately associated bacteria, we were able to gain a broad understanding of the diversity of the potential co-occurring fungal-bacterial partnerships in close proximity to the root system of six different plants. Despite the reduced number of isolates, we confirmed the hypothesis that there is a clear distinction between the fungal microbiome potentially present inside and outside the hypha. From the observations of the potential fungal-bacterial associations emerged both a complex and common pattern, with a core of putative endobacteria, potentially related to the nitrogen cycle, shared among the principal fungal taxa. The comparison of the metabolic functionalities inferred using the PICRUSt2 analysis, highlighted the functions potentially involved in the establishment of an endosymbiotic relationship between bacteria and the fungus, such as the loss of pathways associated with metabolites obtained from the host, alongside the maintenance of pathways responsible for survival within the fungal hypha. While the study of the functionality associated with exo-bacteria emphasized how these bacteria are characterized by the ability to degrade organic compounds, deriving from the decomposition of plant tissues, and uptake of nitrogenous compounds present in the soil. Our results show how complementing the fungal highway column with function and network analysis allows us to better understand the ecological importance of the BFI.

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Ethical approval and consent to participate

Not applicable.

Authors' contributions

DFR and PSGC conceived the study and develop the experimental design. 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, SL performed data analysis and visualization. SL performed qPCR analysis. GLH performed the identification of the putative endobacterial community. HNN performed the SEM 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: accession number MZ374450-MZ374743; BioProject PRJNA644907.

Consent for publication

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

Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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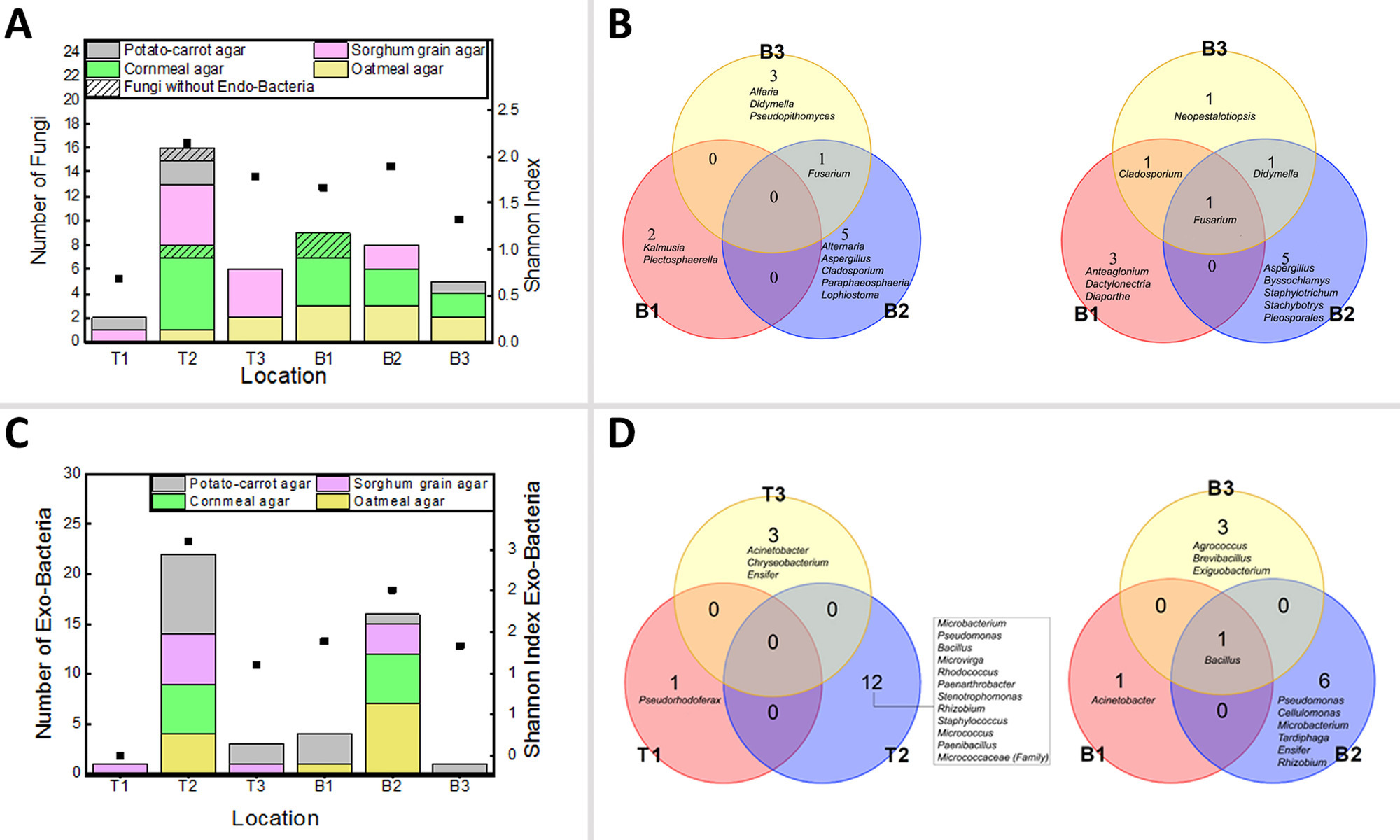
Figure 1: Bar graph represents A) the number of fungal isolates, C) the number of exo-bacterial isolates obtained from different plant types (T1=*Citrus sinensis*, T2= *Diospyros kaki*, T3= *Cycas revoluta*, B1= *Ilex vomitoria*, B2= *Myrica cerifera*, B3= *Buxus sempervirens*) using different plant-based media (Oatmeal agar, Cornmeal agar, Sorghum grain agar, and Potato-carrot agar) grown at 28°C. The pattern in the graph A corresponds to the number of isolated fungi that, based on the qPCR analysis, did not contain any endo-bacteria. The diversity within each collection was calculated using the Shannon Index (Shannon, 1948) and is represented by black squares in the graphs. B) Venn diagram of the B) fungal, D) bacterial genera in the different sites 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.

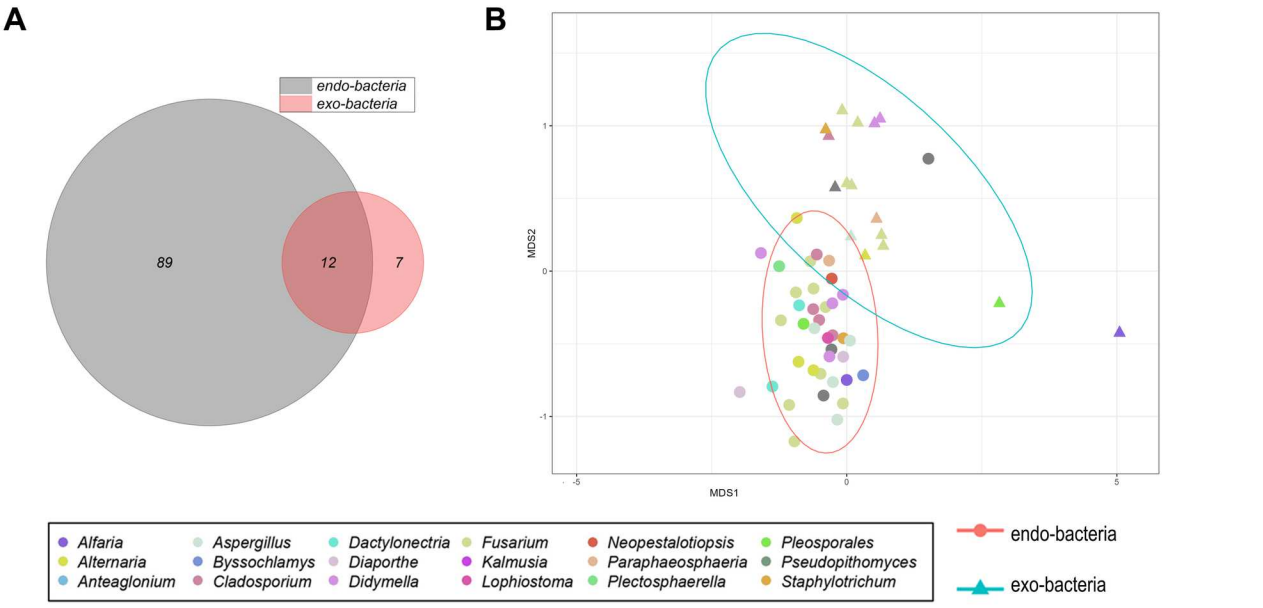
Figure 2: Comparison between the endo and the exo-bacterial community associated with different fungal hosts. **A)** Venn diagram of the different genera present (detailed list in Additional_file _2.xlsx) **B)** Non-metric Multi-dimensional Scaling (NMDS) plot showing the comparison between endo-bacterial and exo-bacterial microbiome communities (shape-coded) associated to each fungal isolate (color-coded) subdivided by putative endo- and exo-bacteria. The dissimilarities indices between putative endo-bacterial and exo-bacterial communities were calculated using the Jaccard distance. (NMDS Stress: 0.096).

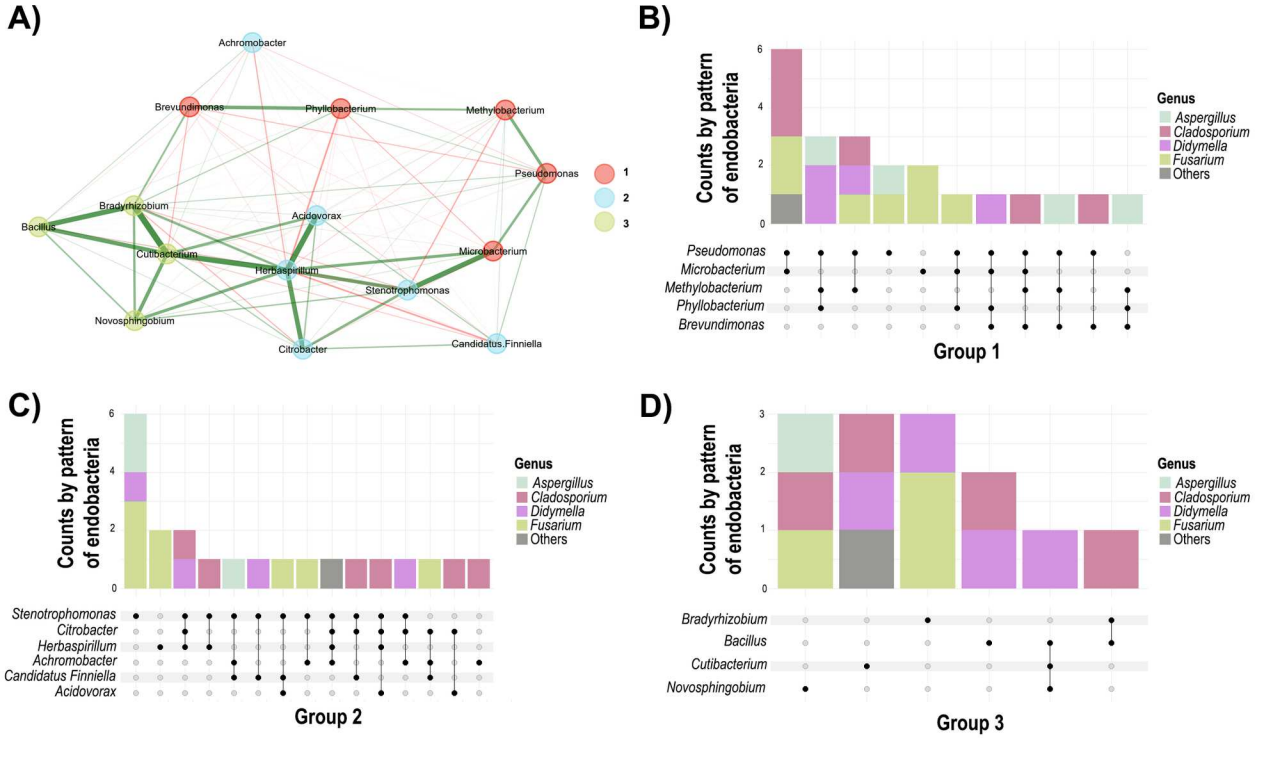
Figure 3: A) Exploratory Graph Analysis (EGA) of the endo-bacteria identified. The different taxa were clustered in three different groups based on their similarities using the Triangulated Maximally Filtered Graph model, walktrap algorithm and 50 bootstraps iterations. UpSet plot of the shared taxa of endo-bacteria identified in the core groups determined by the EGA analysis: **B)** *Pseudomonas*, *Methylobacerium*, *Phyllobacterium Brevundimonas* and Microbacterium), **C)** *Stenotrophomonas*, *Citrobacter*, *Herbaspirillum*, *Achromobacter*, *Candidatus Finniella* and *Acidovorax*) and **D)** *Bradyrhizobium*, *Bacillus*, *Cutibacterium* and *Novosphingobium*. 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 counts per pattern, displayed on the y-axe, were color-coded based on the fungal taxa.

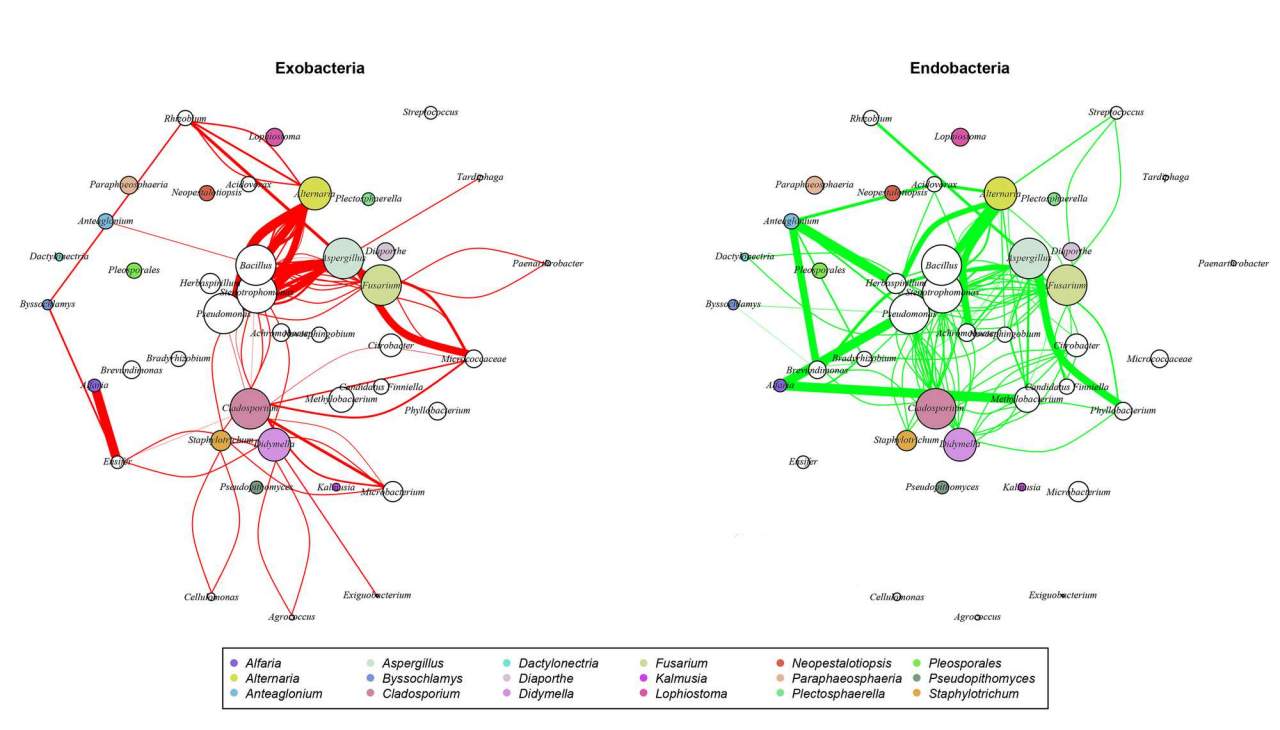
Figure 4: Reconstruction of the possible association occurring in the soil based on the network analysis. The layout was calculated based on the Kamada Kawai algorithm. Edges were constructed based on the number of each specific association. The size of the vertex, calculated and plotted as coreness (K-core decomposition) (Bader and Hogue 2003) and color-coded based on the taxa. The network obtained was divided based on the origin of the bacteria: Exo-bacteria (red) and Endo-bacteria (green).

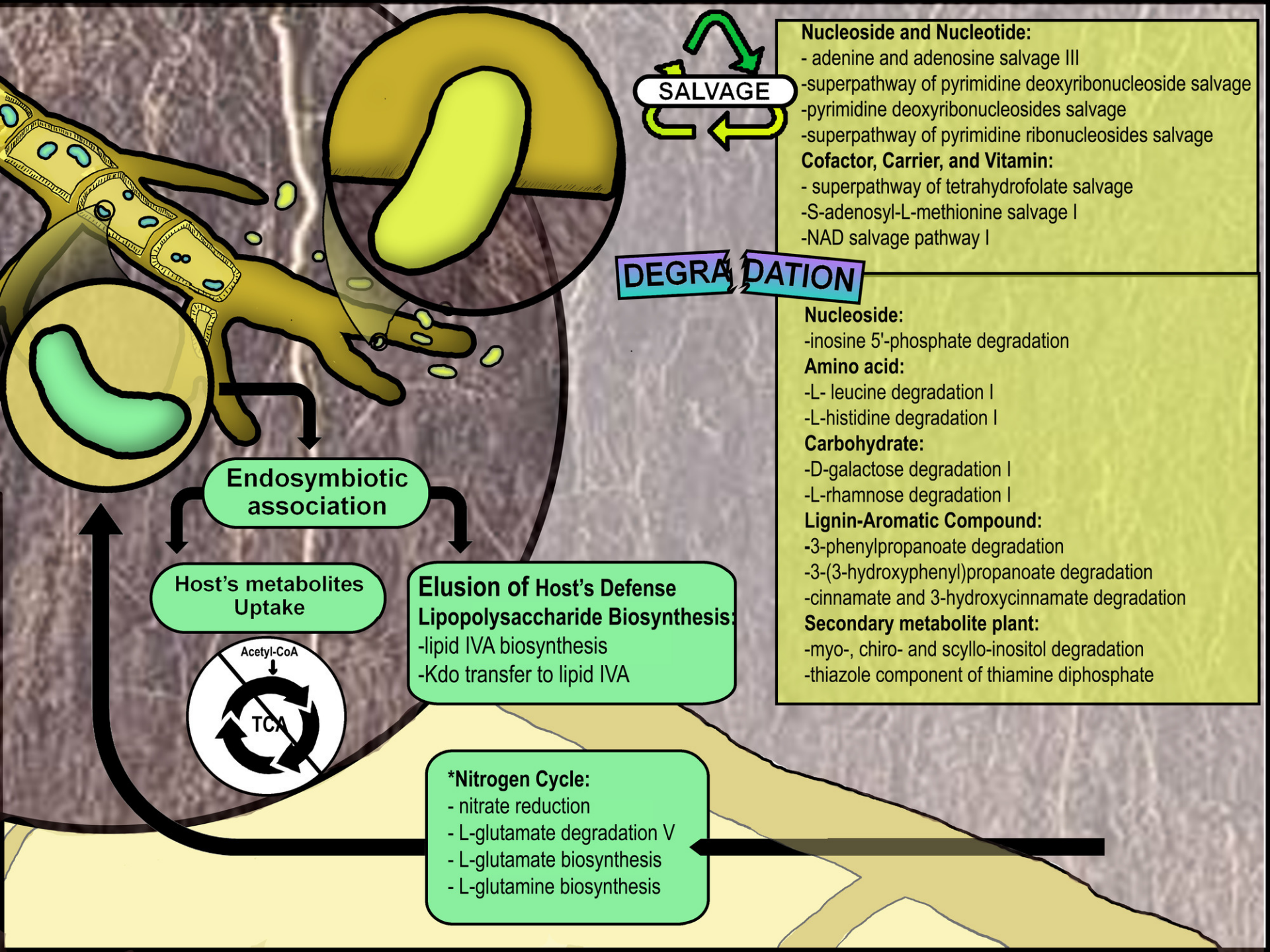
Figure 5: Illustration of the unique metabolic functions (MetaCyc pathways inferred by the PICRUSt2 analysis) associated with the endo-bacterial community (highlighted in green) and exo-bacterial community (highlighted in yellow) determined from the indicspecies analysis (number of permutations=99, p value = 0.001). Functions with "*" were found with a p value lower than 0.01.

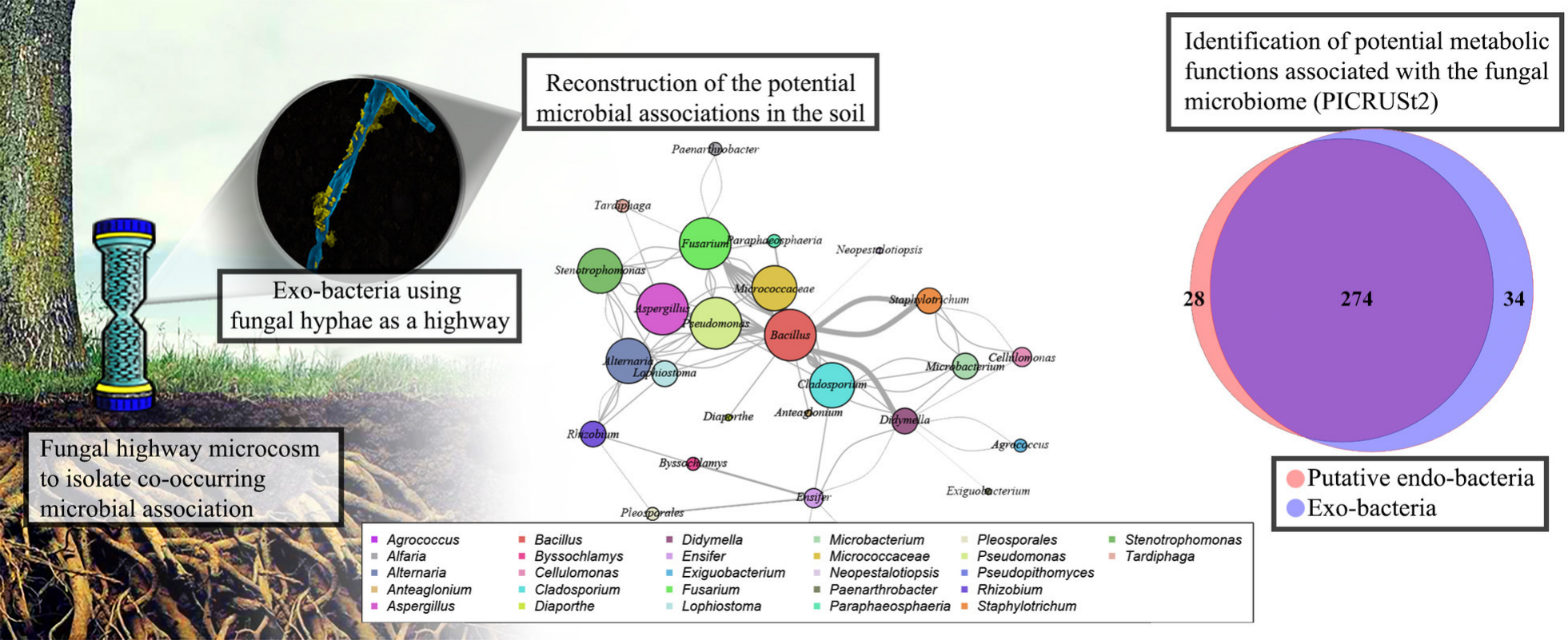

A
Potato-carrot agar
Sorghum grain agar
Cornmeal agar
Oatmeal agar
Fungi without Endo-Bacteria
Number of Fungi
Shannon Index
Location
T1
T2
T3
B1
B2
B3
B
B3
3
Alfaria
Didymella
Pseudopithomyces
0
1
Fusarium
0
2
Kalmusia
Plectosphaerella
0
5
Alternaria
Aspergillus
Cladosporium
Paraphaeosphaeria
Lophiostoma
B1
B2
B3
1
Neopestalotiopsis
1
Cladosporium
1
Didymella
1
Fusarium
3
Anteaglonium
Dactylonectria
Diaporthe
0
5
Aspergillus
Byssochlamys
Staphylotrichum
Stachybotrys
Pleosporales
B1
B2
C
Potato-carrot agar
Sorghum grain agar
Cornmeal agar
Oatmeal agar
Number of Exo-Bacteria
Shannon Index Exo-Bacteria
Location
T1
T2
T3
B1
B2
B3
D
T3
3
Acinetobacter
Chryseobacterium
Ensifer
0
0
0
1
Pseudorhodoferax
12
0
T1
T2
Microbacterium
Pseudomonas
Bacillus
Microvirga
Rhodococcus
Paenarthrobacter
Stenotrophomonas
Rhizobium
Staphylococcus
Micrococcus
Paenibacillus
Micrococcaceae (Family)
B3
3
Agrococcus
Brevibacillus
Exiguobacterium
0
0
1
Bacillus
1
Acinetobacter
0
6
Pseudomonas
Cellulomonas
Microbacterium
Tardiphaga
Ensifer
Rhizobium
B1
B2


A
endo-bacteria
exo-bacteria
89
12
7
B
MDS2
MDS1
1
0
-1
-5
0
5
Alfaria
Alternaria
Anteaglonium
Aspergillus
Byssochlamys
Cladosporium
Dactylonectria
Diaporthe
Didymella
Fusarium
Kalmusia
Lophiostoma
Neopestalotiopsis
Paraphaeosphaeria
Plectosphaerella
Pleosporales
Pseudopithomyces
Staphylotrichum
endo-bacteria
exo-bacteria


A)
Achromobacter
Brevundimonas
Phyllobacterium
Methylobacterium
Pseudomonas
Bradyrhizobium
Acidovorax
Bacillus
Cutibacterium
Herbaspirillum
Microbacterium
Stenotrophomonas
Novosphingobium
Citrobacter
Candidatus Finniella
1
2
3
B)
Counts by pattern of endobacteria
Genus
Aspergillus
Cladosporium
Didymella
Fusarium
Others
Pseudomonas
Microbacterium
Methylobacterium
Phyllobacterium
Brevundimonas
Group 1
C)
Counts by pattern of endobacteria
Genus
Aspergillus
Cladosporium
Didymella
Fusarium
Others
Stenotrophomonas
Citrobacter
Herbaspirillum
Achromobacter
Candidatus Finniella
Acidovorax
Group 2
D)
Counts by pattern of endobacteria
Genus
Aspergillus
Cladosporium
Didymella
Fusarium
Others
Bradyrhizobium
Bacillus
Cutibacterium
Novosphingobium
Group 3


Exobacteria
Endobacteria
Rhizobium
Lophiostoma
Streptococcus
Paraphaeosphaeria
Neopestalotiopsis
Acinetobacter
Alternaria
Plectosphaerella
Tardiphaga
Anteaglonium
Dactylonectria
Pleosporales
Bacillus
Aspergillus
Diaporthe
Paenarthrobacter
Herbaspirillum
Stenotrophomonas
Fusarium
Byssochlamys
Pseudomonas
Actinomycetospora
Bradyrhizobium
Cirrobacter
Micrococcaceae
Brevundimonas
Alfaria
Candidatus Finniella
Methylobacterium
Cladosporium
Phyllobacterium
Staphylotrichum
Didymella
Exiguobacterium
Pseudopithomyces
Kalmusia
Micrococcus
Cellulomonas
Agrococcus
Alfaria
Alternaria
Anteaglonium
Aspergillus
Byssochlamys
Cladosporium
Dactylonectria
Diaporthe
Didymella
Fusarium
Kalmusia
Lophiostoma
Neopestalotiopsis
Paraphaeosphaeria
Plectosphaerella
Pleosporales
Pseudopithomyces
Staphylotrichum


SALVAGE
Nucleoside and Nucleotide:
- adenine and adenosine salvage III
-superpathway of pyrimidine deoxyribonucleoside salvage
-pyrimidine deoxyribonucleosides salvage
-superpathway of pyrimidine ribonucleosides salvage
Cofactor, Carrier, and Vitamin:
- superpathway of tetrahydrofolate salvage
-S-adenosyl-L-methionine salvage I
-NAD salvage pathway I
DEGRADATION
Nucleoside:
-inosine 5'-phosphate degradation
Amino acid:
-L- leucine degradation I
-L-histidine degradation I
Carbohydrate:
-D-galactose degradation I
-L-rhamnose degradation I
Lignin-Aromatic Compound:
-3-phenylpropanoate degradation
-3-(3-hydroxyphenyl)propanoate degradation
-cinnamate and 3-hydroxycinnamate degradation
Secondary metabolite plant:
-myo-, chiro- and scyllo-inositol degradation
-thiazole component of thiamine diphosphate
Endosymbiotic association
Host's metabolites Uptake
Acetyl-CoA
TCA
Elusion of Host's Defense
Lipopolysaccharide Biosynthesis:
-lipid IVA biosynthesis
-Kdo transfer to lipid IVA
*Nitrogen Cycle:
- nitrate reduction
- L-glutamate degradation V
- L-glutamate biosynthesis
- L-glutamine biosynthesis


Exo-bacteria using fungal hyphae as a highway
Fungal highway microcosm to isolate co-occurring microbial association
Reconstruction of the potential microbial associations in the soil
Paenarthrobacter
Tardiphaga
Fusarium
Paraphaeosphaeria
Neopestalotiopsis
Stenotrophomonas
Micrococcaceae
Staphylotrichum
Aspergillus
Pseudomonas
Bacillus
Alternaria
Lophiostoma
Cellulomonas
Microbacterium
Cladosporium
Diaporthe
Anteaglonium
Didymella
Rhizobium
Agrococcus
Byssochlamys
Ensifer
Exiguobacterium
Pleosporales
Agrococcus
Alfaria
Alternaria
Anteaglonium
Aspergillus
Bacillus
Byssochlamys
Cellulomonas
Cladosporium
Diaporthe
Didymella
Ensifer
Exiguobacterium
Fusarium
Lophiostoma
Microbacterium
Micrococcaceae
Neopestalotiopsis
Paenarthrobacter
Paraphaeosphaeria
Pleosporales
Pseudomonas
Pseudopithomyces
Rhizobium
Staphylotrichum
Stenotrophomonas
Tardiphaga
Identification of potential metabolic functions associated with the fungal microbiome (PICRUSt2)
28
274
34
Putative endo-bacteria
Exo-bacteria