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Exploring Microbial Rhizosphere Communities in Asymptomatic and Symptomatic Apple Trees Using Amplicon Sequencing and Shotgun Metagenomics

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Abstract: The rhizosphere is a dynamic and highly interactive habitat where diverse microbial communities are established, and it plays crucial roles in plant health and disease dynamics. In this study, microbial communities and functional profiles in the rhizosphere of both asymptomatic and symptomatic apple trees were investigated through amplicon sequencing and shotgun metagenomics. The research was conducted at a location in the municipality of Cuauhtemoc, Chihuahua State, Mexico, and a total of 22 samples were collected, comprising 12 for amplicon sequencing and 10 for shotgun metagenomic sequencing. Symptomatic trees were identified based on reddish branches and internal necrosis in the trunk and root, while asymptomatic trees exhibited a healthy physiology. The findings showed that the dominant bacterial phyla included Proteobacteria, Actinobacteria, and Bacteroidetes, with prevalent genera such as *Streptomyces*, *Pseudomonas*, and *Rhodanobacter*. The fungal communities featured Ascomycota, Mortierellomycota, and Basidiomycota, which were dominated by *Fusarium*, *Penicillium*, and *Mortierella*. In the fungal communities, Mortierellomycota, notably abundant in asymptomatic trees, holds potential as a biocontrol agent, as seen in other studies on the suppression of *Fusarium* wilt disease. The application of shotgun metagenomic sequencing revealed significant differences in alpha and beta diversities in bacterial communities, suggesting a health-dependent change in species composition and abundance. Functional profile analysis highlighted enzymatic activities associated with lipid synthesis/degradation, amino acid biosynthesis, carbohydrate metabolism, and nucleotide synthesis, which have been documented to participate in symbiotic relationships between plants. These insights not only contribute to understanding the dynamics of rhizosphere microbial activity but also provide valuable perspectives on the potential application of microbial communities for tree health and implications for the management of apple orchards.

Keywords: *Malus domestica*; rhizosphere; bacteria; fungi; microbiome; metagenomics

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1. Introduction

The rhizosphere, the soil region surrounding plant roots, is a dynamic and highly interactive habitat where diverse microbial communities are established. These communities play a fundamental role in numerous ecological and agricultural processes, including organic matter decomposition, nutrient cycling, and the promotion of plant growth [1–3]. Among the microorganisms present in the rhizosphere, bacteria and fungi stand out as

key components of the microbial community. These microorganisms not only exist in close proximity to plant roots but also interact intimately with them, influencing plant health and productivity [1].

The apple tree (*Malus domestica*), a fruit species of great economic and agricultural importance, heavily relies on the interaction between tree roots and the microbial communities present in the rhizosphere. This microbial diversity and its functions have been the subject of increasing interest in the rhizosphere of several agricultural crops [4–8]. For instance, certain bacterial species can fix atmospheric nitrogen, making it available to plants, while others produce phytohormones that stimulate root development and enhance nutrient uptake [9–11]. Additionally, microbial communities can protect plants from pathogens through resource competition or by producing antimicrobial compounds [12,13]. Understanding the composition and function of these microbial communities is crucial to improving tree health, increasing the quality and quantity of apple production, and developing sustainable agricultural practices [14,15].

Although numerous studies have been conducted on the microbiological aspects associated with apple trees, the generated data due to next-generation sequencing (NGS) techniques are crucial for the generation of knowledge benefiting this agriculturally important crop. The use of metagenomic analysis in research has been applied to solve existing gaps by identifying new genetic variants and trying to explain emerging diseases or diseases caused by various pathogens, as is the case in this study. In the same way, progress in these matters has revolutionized our understanding of microbial communities in the rhizosphere, providing insights into the taxonomic composition, functional potential, and ecological roles of microbial communities [16,17].

Apple crown and root rot diseases have scarcely been studied in apple orchards in the apple-growing region of Chihuahua, Mexico [18]. They induce symptoms such as necrosis in the feeder root system and in the trunk, and finally, they lead to the death of the tree. Around the world, fungi (e.g., *Cylindrocarpon* spp., *Rhizoctonia* spp., and *Fusarium proliferatum*) and oomycetes (e.g., *Phytophthora* spp. and *Pythium* spp.) have been reported as the pathogens causing apple root and crown rot diseases [19–21]. However, it has been observed that in many instances, it is not just an individual pathogen but rather the involvement of many other groups of microorganisms that affect the outcome of the infection in plants [22]. In fact, the diseases known as apple replant disorders are associated with various causal agents but differ among countries and remain unclear [23]. In Chihuahua, Mexico, the incidence of root diseases in apple trees was assessed, revealing a rate of 17%. Various isolates of fungi and oomycetes were identified, and their pathogenicity was subsequently determined. In in vitro antagonistic activity tests, *Trichoderma* and *Bacillus* emerged as promising alternatives for biological control against the evaluated phytopathogens [18]. It is crucial to study the microbial community in the rhizosphere of both asymptomatic and symptomatic trees affected by root and crown rot to gain a better understanding of the possible microbial interactions that exist in the rhizosphere and, consequently, to enhance our overall comprehension of the disease dynamics.

In light of the existing gaps in knowledge, our hypothesis suggests that changes in the rhizosphere microbial community composition of symptomatic apple trees are structured by the presence and activities of pathogens and saprophytes, as well as beneficial microorganisms exhibiting antagonistic properties against the pathogens. The aim of this study was to characterize and compare the composition and diversity of the microbial communities found in rhizosphere samples of both conditions at a location in the municipality of Cuauhtemoc, Chihuahua State, Mexico. In pursuit of this objective, metagenomic shotgun sequencing and amplicon sequencing targeting the 16S rRNA gene and internal transcribed spacer (ITS1) region were employed to generate new knowledge. Additionally, the functional profile was analyzed to elucidate the ecological functions of microbial communities in the rhizosphere. Thus, this study significantly contributes to understanding rhizosphere microbial dynamics, providing insights that could have potential implications for the effective management of apple orchards.

2. Materials and Methods

2.1. Site Description and Sample Collection

Soil samples from an apple orchard area containing asymptomatic and symptomatic crown and root rot of *Malus domestica* trees (golden glory apples grafted onto Emla7 rootstock) were collected at a location in the municipality of Cuauhtemoc, Chihuahua State, Mexico (28°42′54.9″ N, 106°55′06.6″ W; 2,018 masl). The first sampling took place in June 2020, during the early summer, and involved collecting three rhizospheric soil samples from both asymptomatic and symptomatic apple trees. These samples were used for amplicon sequencing. A subsequent sampling was conducted in February 2022, during late winter, where six asymptomatic and four symptomatic trees were sampled for metagenomic sequencing.

Rhizosphere soil samples from 7-year-old trees under each condition were sampled: first, rhizosphere soil samples from asymptomatic and symptomatic apple trees were selected, with a minimum distance of 40 m between samples, based on visual symptoms from the roots and foliage, such as dead areas at the base of the tree. These dead areas started in the bark between the soil line and the crown roots, comprising darkened and collapsed tissue. The foliage symptoms were reduced vegetative growth, small leaves, and little budding. The uppermost 20 cm of soil was removed using a shovel, and approximately 10 g of rhizosphere soil was collected per individual plant by employing a dry sterile toothbrush to brush off the soil around the surface of the apple root, which was then placed into sterile bags and stored on ice for transport to the laboratory, where the samples were then stored in an ultralow temperature freezer at −80 °C until processing. In addition, the physical and chemical properties of the soil at a depth of approximately 20 cm from asymptomatic and symptomatic trees were as follows: soil texture = loam and sandy loam; pH = 6.24 and 2.91; electrical conductivity (EC) = 2.71 and 3.75 mS/cm; organic matter = 1.42 and 1.50%.

2.2. Total DNA Extraction and Sequencing

Total DNA was extracted from each sample using a ZymoBIOMICS^{TM} DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The DNA quality and quantity were determined by using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) based on its A260/280 ratio, and the DNA was observed with a 1.0% agarose gel electrophoresis. The preparation and sequencing of amplicon libraries (16S rRNA gene and ITS1 region) and shotgun metagenomics, following the manufacturer's protocol, were carried out at Novogene (Beijing, China) and Illumina (San Diego, CA, USA), respectively. For amplicons, the NovaSeq sequencing platform was used alongside a paired-end 2 × 250 bp strategy carried out on an Illumina sequencer (Illumina Inc., San Diego, CA, USA). In the case of bacteria, a fragment of the 16S rRNA gene was amplified using the primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) flanking the V3 and V4 regions [24]. For fungi, the ITS1 region was amplified using the primer pairs ITS5-1737F (5′-GGAAGTAAAAGTCGTAACA AGG-3′) and ITS2-2043R (5′-GCTGCGTTCTTCATCGATGC-3′) [25]. For metagenomic library preparation and sequencing, the total DNA was shipped to Illumina (San Diego, CA, USA). Briefly, 100 ng of total DNA was processed following the instructions of the Illumina DNA prep (M) tagmentation kit (# Cat. 20018705). Because many samples were run in the same flow cell, specific indexes were added to each DNA library with IDT for Illumina DNA/RNA UD Indexes Set A Tagmentation (# Cat. 20027213). Library concentration was quantified with the Qubit dsDNA HS Assay kit (Thermo Fisher), and the integrity of the DNA libraries was assessed with the Bioanalyzer 2100 Agilent system (NGS 1-6000 kit). Libraries were sequenced in an S4 flow cell using a 2 × 150 bp strategy on an Illumina NovaSeq 6000 sequencer (Illumina Inc., San Diego, CA, USA).

2.3. Amplicon Data Analysis

Sequence data were obtained as fastq files in the CASAVA v1.8 paired-end demultiplexed format. Forward and reverse sequences were merged using FLASH v1.2.11 with

default settings [26]. Merged sequences were imported into Quantitative Insights Into Microbial Ecology 2 v2020.2 (QIIME 2) [27]. Firstly, quality filtering, trimming, dereplication, and denoising, as well as the removal of chimeric sequences from the merged data using DADA2 v1.16, were performed [28] to obtain representative amplicon sequence variants (ASVs). Each ASV was assigned a taxonomy with a trained naïve Bayesian classifier using the Greengenes v13.8 database [29] for bacteria and the UNITE v8.99 database [30] for fungi. Bar plots based on relative abundance were generated to show the taxonomic distribution.

A multiple sequence alignment and phylogenetic reconstruction were produced from the ASVs using MAFFT v7 [31] and FastTree v2 [32], respectively, to generate a rooted phylogenetic tree and conduct subsequent analyses. In order to analyze the α- and β-diversity of bacterial and fungal communities and conduct related statistical tests, the samples from the library were rarefied with the lowest number of reads, and the metrics were calculated using RStudio [33] with the packages vegan [34], tidyverse [35], and qiime2R [36]. To explore the α-diversity within these communities under both asymptomatic and symptomatic conditions, the Chao1, Shannon, Simpson, and evenness indexes were estimated, and the diversity was compared among groups using the Kruskal–Wallis test followed by the post hoc Dunn's test, considering the difference to be statistically significant at p-values < 0.05.

To assess differences in the bacterial and fungal communities' composition between the asymptomatic and symptomatic conditions, a principal coordinate analysis (PCoA) was performed based on Jaccard distances. Then, a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was used for testing, followed by the post hoc Benjamini–Hochberg FDR test to determine significant differences in bacterial and fungal communities. Additionally, a functional inference analysis was performed with PICRUSt2 v2.5.1 [37] to explore the possible functional profiles of the microbial communities through their enzymatic activities, and the accuracy of the analysis was assessed through the weighted Nearest Sequences Taxon Index (NSTI).

2.4. Metagenomic Data Analysis

Raw fastq reads of each sample were quality filtered with FASTP v0.20.0 [38]. The filtered quality scores were set to 25, and a trimming of the first and last five bases was carried out. The taxonomic assignment of trimmed reads was performed with Kraken2 v2.1.2 [39] using the –download-library option from Kraken2-build to download the complete bacterial and fungal genomes from the RefSeq database. In addition, fungal genomes from *Fusarium* spp. and *Pythium* spp. were included. The Kraken2 files from all samples were combined to generate a biom file with the Kraken-biom utility. Two main datasets were generated from the biom file through filtering: Kingdom == "Bacteria" and Kingdom == "Eukaryota". Analysis of the microbial diversity, including measurements of α- and β-diversity, was performed with the Phyloseq package v1.40.0 [40] for both datasets independently. In addition, Venn diagrams were made to visualize which bacterial and fungal genera were exclusive or shared between the symptomatic and asymptomatic conditions. All metrics presented in this work are based on relative abundances and calculated using RStudio [33] with the packages vegan [34], tidyverse [35], and qiime2R [36]. The statistical analysis of α-diversity was evaluated with the Kruskal–Wallis test, and β-diversity was determined using PERMANOVA with 999 permutations to test for significant differences in bacterial and fungal communities, considering the difference to be statistically significant at p-values < 0.05.

Finally, functional predictions for the shotgun metagenomic data were determined using the MG-RAST pipeline v4.0.3 [41]. The sequences in fastq format were uploaded to MG-RAST, and the default settings were used. MG-RAST classifies sequences into subsystems, which are grouped into hierarchical categories and can be used to construct a heatmap with the top 50 most abundant functions.

The datasets generated and analyzed during this current study are available in the NCBI Bioproject database under the accession numbers PRJNA1003089 (amplicon sequences) and PRJNA1003562 (metagenomic sequences).

3. Results

3.1. Composition and Abundance of Bacterial and Fungal Communities

A total of 837,294 (16S rRNA) and 1,074,605 (ITS1 region) high-quality sequences were obtained from all rhizosphere soil samples under both asymptomatic and symptomatic conditions (Table S1). The samples with the lowest number of sequences were rarefied, homogenizing all bacterial samples to a total of 126,057 sequences and fungal samples to a total of 166,349 sequences, which were the totals for subsequent analyses. In the case of shotgun metagenomics, a total of 826,840,106 raw sequences were generated. After a quality check analysis, 708,403,234 high-quality sequences were obtained and used for further analysis.

The community composition derived from both amplicon and metagenomic sequences exhibited robust similarity in relation to the most prevalent phyla. Nonetheless, metagenomic sequencing enabled a more comprehensive retrieval of information across all taxonomic levels, but especially that related to the phyla. The amplicon sequences allowed for the identification of a total of 51 phyla, 196 families, and 507 bacterial genera, as well as a total of 11 phyla, 143 families, and 268 fungal genera. The most abundant prokaryotic phyla in the rhizosphere of apple trees consisted of Proteobacteria (48.91%), Bacteroidetes (11.19%), Actinobacteria (10.46%), Acidobacteria (9.48%), Gemmatimonadetes (6.43%), Firmicutes (3.93%), Verrucomicrobia (3.25), and Chloroflexi (3.11%), followed by 43 genera with <1.00% relative abundance (Figure 1a). In the fungal communities, the phyla comprised Ascomycota (77.69%), Mortierellomycota (9.32%), Basidiomycota (9.19), Rozellomycota (1.59%), and Blastocladiomycota (1.47%), followed by eight genera with <1.0% relative abundance (Figure 2a). At the genus level, the only bacterial genus that presented a relative abundance >10% was *Rhodanobacter* (10.6%), followed by 25 genera (e.g., *Kaistobacter*, *Streptomyces*, *Rhodoplanes*, *Dechloromonas*, and *Pseudomonas*) that had a relative abundance ranging between 1 and 10% and 481 genera that had a relative abundance of <1.00% (Figure 1b). In fungi, at the genus level, *Penicillium* (28.62%) and *Mortierella* (11.14%) presented an abundance >10%, 18 genera (e.g., *Acremonium*, *Fusarium*, *Ilyonectria*, *Setophaeosphaeria*, and *Apiotrichum*) had a relative abundance ranging between 1 and 10%, and 248 genera had a relative abundance of <1.0%. (Figure 2b).

On the other hand, in the metagenomic analysis, 39 phyla, 451 families, and 1741 bacterial genera were recovered. The most abundant bacterial phyla were Proteobacteria (57.25%) and Actinobacteria (33.17%), followed by phyla with <5.00% relative abundance such as Bacteroidetes (2.22%), Planctomycetes (1.88%), Acidobacteria (1.63%), and Firmicutes (1.24%), and 33 phyla had <1.0% relative abundance (Figure 1c). At the genus level, *Streptomyces* was the most abundant, with a relative abundance of 6.81%, followed by *Bradyrhizobium* (3.87%), *Pseudomonas* (3.13%), *Nocardioides* (2.83%), *Sphingomonas* (2.67%), *Rhodanobacter* (2.63%), and nine other genera with relative abundances of up to 1.0%; the 1726 remaining genera showed relative abundances of <1.0% (Figure 1d). For fungi, the annotated sequences accounted for 0.10–0.30% of the total sequences, suggesting potential annotation biases that resulted in the underestimation of eukaryotic communities and led to the recovery of a total of 2 phyla, 32 families, and 55 genera. The phyla Ascomycota (91.87%) was the most dominant, followed by Basidiomycota (8.13%) (Figure 2c); *Fusarium* (30.00%) and *Trichoderma* (11.21%) were the most abundant genera, followed by *Metarhizium* (5.73%), *Beauveria* (4.48%), *Pseudozyma* (3.58%), *Pyricularia* (3.52%), *Clonostachys* (3.22%), *Penicillium* (3.11%), and 11 other genera with relative abundances of up to 1.00%, while the remaining 36 genera exhibited a relative abundance of <1.0% (Figure 2d). In addition, the comparison using Venn diagrams showed a core microbiome at the genus level. In the case of bacteria, 198 genera were shared between both health conditions analyzed, while 12 genera were prevalent for both conditions in the case of fungi (Figure S1).

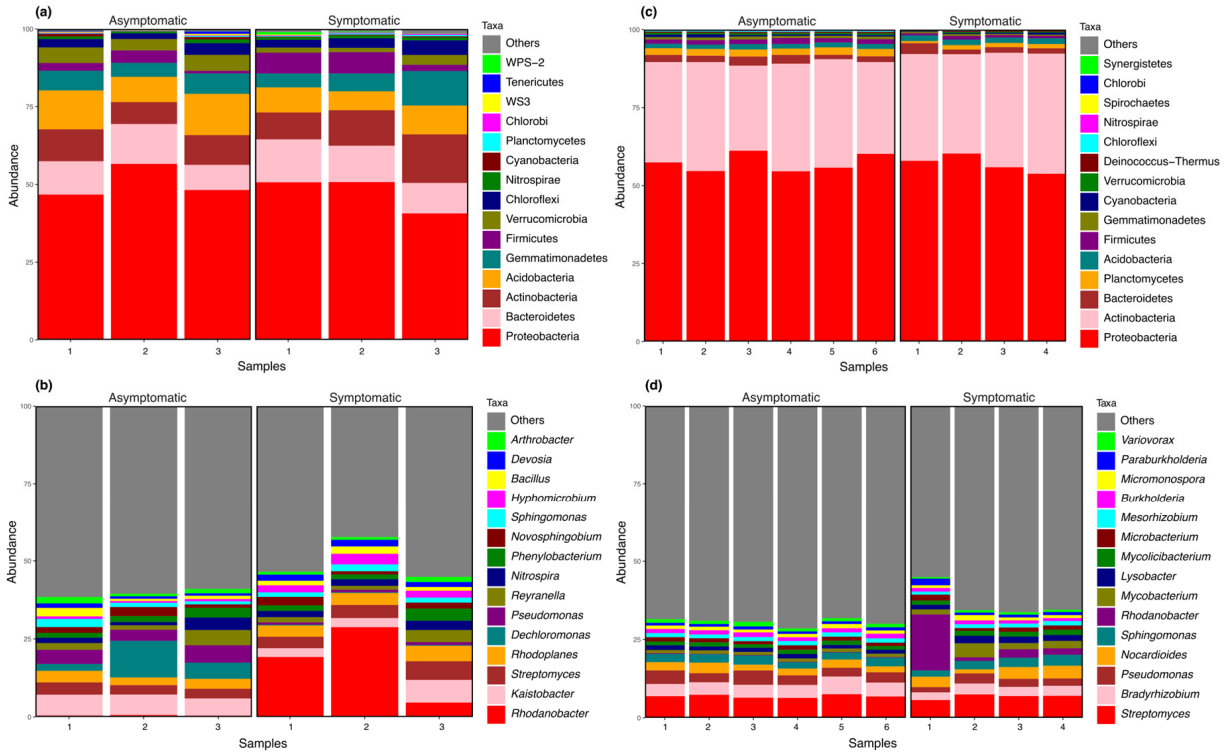

Figure 1. Bar plots depicting the relative abundance of bacterial communities at different taxonomic levels: (**a**) phyla using amplicon data, (**b**) phyla using metagenomic data, (**c**) genera using amplicon data, and (**d**) genera using metagenomic data.

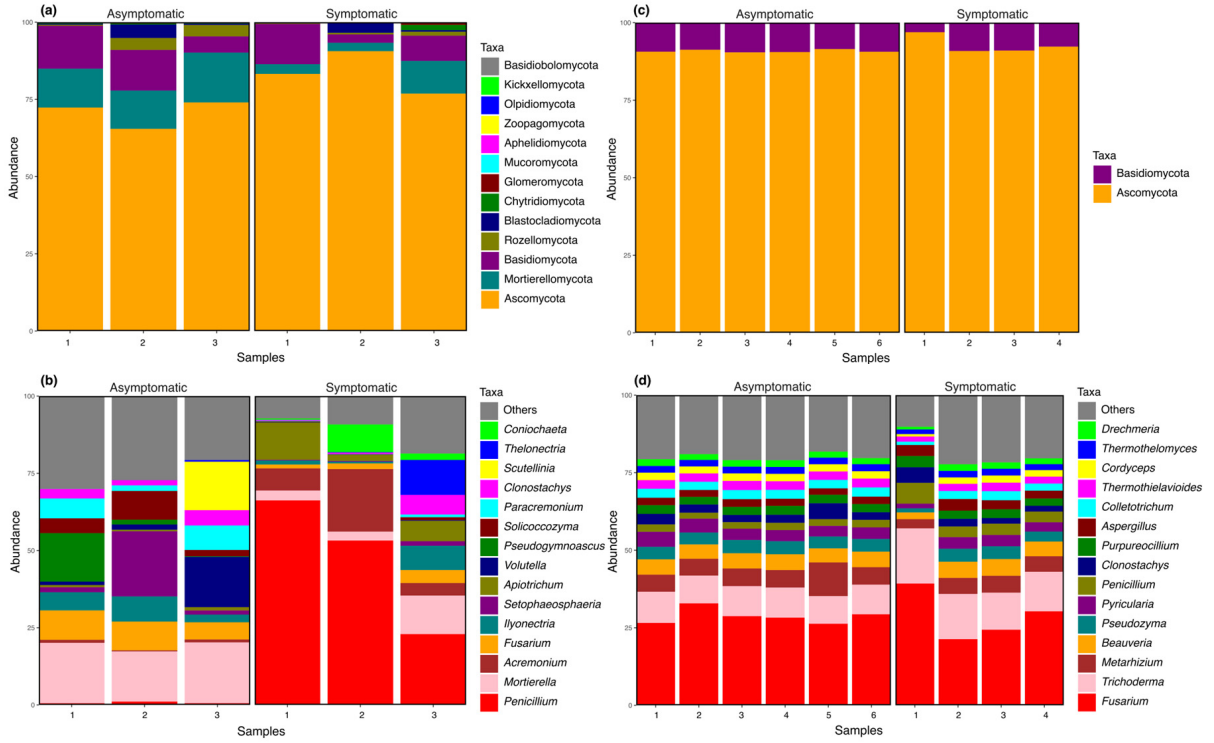

Figure 2. Bar plots depicting the relative abundance of fungal communities at different taxonomic levels: (**a**) phyla using amplicon data, (**b**) phyla using metagenomic data, (**c**) genera using amplicon data, and (**d**) genera using metagenomic data.

3.2. α- and β-Diversity of Asymptomatic and Symptomatic Apple Trees

The taxonomic distinctiveness of the rhizosphere in asymptomatic and symptomatic apple trees with regard to α-diversity was investigated. The analysis of amplicon sequences from bacteria and fungi revealed no significant differences ($p > 0.05$) in the Chao1, Shannon, Evenness, and Simpson indexes (Figures 3a and 4a). In the case of metagenomic data for bacteria, with the exception of the Simpson index ($p > 0.05$), the Chao1, Shannon, and Evenness indexes showed significant differences ($p < 0.05$) (Figure 3b). For fungi, no significant differences were observed in any index ($p > 0.05$) (Figure 4b).

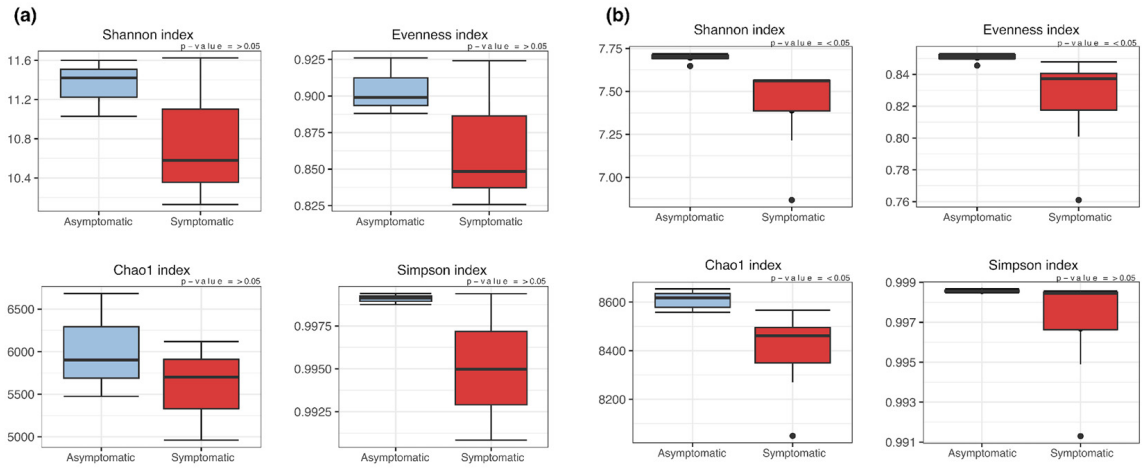

Figure 3. Box plots depicting the analysis of alpha diversity in the bacterial community using Shannon, Evenness, Chao1, and Simpson indexes, evaluated through (**a**) amplicon data and (**b**) metagenomic data.

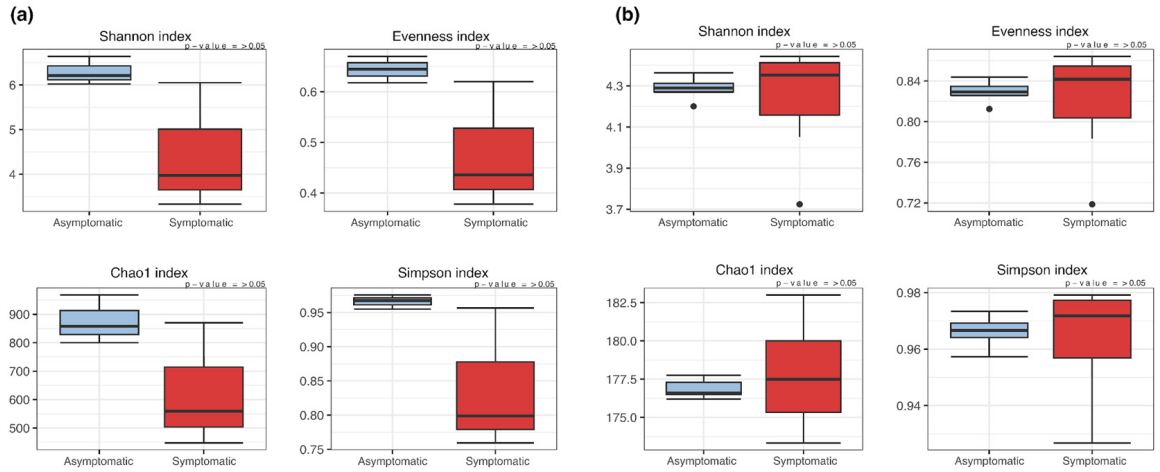

Figure 4. Box plots depicting the analysis of alpha diversity in the fungal community using Shannon, Evenness, Chao1, and Simpson indexes, evaluated through (**a**) amplicon data and (**b**) metagenomic data.

The β-diversity, as determined through the variation in microbial communities between asymptomatic and symptomatic conditions, was examined using PCoA analysis based on Jaccard distances. With bacterial and fungal amplicon data, the PCoAs showed 80.5% and 83.8% dissimilarity, respectively, and revealed that the microbial communities did not differ (PERMANOVA; $p > 0.05$) (Figure 5a,b). In contrast, the metagenomic data showed a dissimilarity of 81.11% and 90.18% in the PCoA analysis, unveiling significant differences in the bacterial communities (PERMANOVA; $p < 0.05$) (Figure 5c) but not in the fungal communities (PERMANOVA; $p > 0.05$) (Figure 5d).

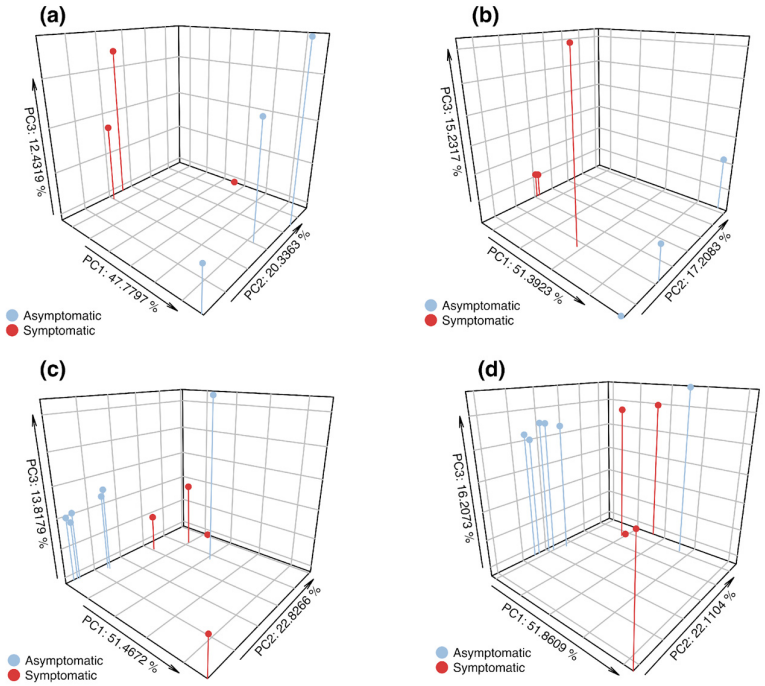

Figure 5. PCoA based on Jaccard distance shows the microbial community dissimilarity. (**a**) Bacterial 16S rRNA gene amplicon data; (**b**) fungal ITS region amplicon data; (**c**) bacterial metagenomic data; and (**d**) fungal metagenomic data.

3.3. Functional Profiles of Microbial Communities

PICRUSt2 analysis of the amplicon data was applied to identify the possible functional profiles of the microbial communities. A list of 2380 enzymatic functions was obtained, and the top 50 dominating enzymes were displayed in a heatmap (Figure 6a). The average NSTI value was 0.23, which is considered to be a low value; it indicates a satisfactory quality in the functional predictions made due to its proximity to the nearest reference genomes in the used database. In general, the top 50 enzymatic functions participate in essential processes for cellular functioning and the regulation of numerous metabolic pathways, such as DNA replication and transcription, protein synthesis and modification, lipid and carbohydrate metabolism, energy generation through cellular respiration, the synthesis and repair of nucleic acids, as well as the biosynthesis of secondary metabolites.

Based on the processed sequencing data from MG-RAST in 2019, genes/enzymes were identified using KEGG pathway annotation. Among these genes, a heatmap was generated for the top 50 most abundant enzymatic functions in the analyzed samples (Figure 6b). The analysis revealed a significant abundance of enzymes involved in various processes, including fatty acid, nucleotide, and protein synthesis, as well as amino acid biosynthesis, carbohydrate metabolism, and lipid metabolism. Additionally, enzymes related to energy generation and the degradation of toxic compounds were also highly prevalent. When comparing the top 50 enzymatic functions detected through PICRUSt2 and metagenomics, several enzymatic activities were found to be exactly the same. These activities are associated with various metabolic processes and biological functions related to lipid synthesis and degradation, amino acid biosynthesis, carbohydrate metabolism, and nucleotide synthesis. The enzymes involved in these processes were as follows: EC:1.1.1.100—3-oxoacyl-[acyl-carrier-protein] reductase; EC:1.8.1.9—thioredoxin-disulfide reductase; EC:2.2.1.6—acetolactate synthase; EC:2.3.1.9—acetyl-CoA C-acetyltransferase; EC:4.2.1.33—3-isopropylmalate dehydratase; EC:5.1.3.2—UDP-glucose 4-epimerase; EC:6.2.1.3—long-chain fatty acid CoA ligase; and EC:6.3.5.3—phosphoribosylformylglycinamidine synthase.

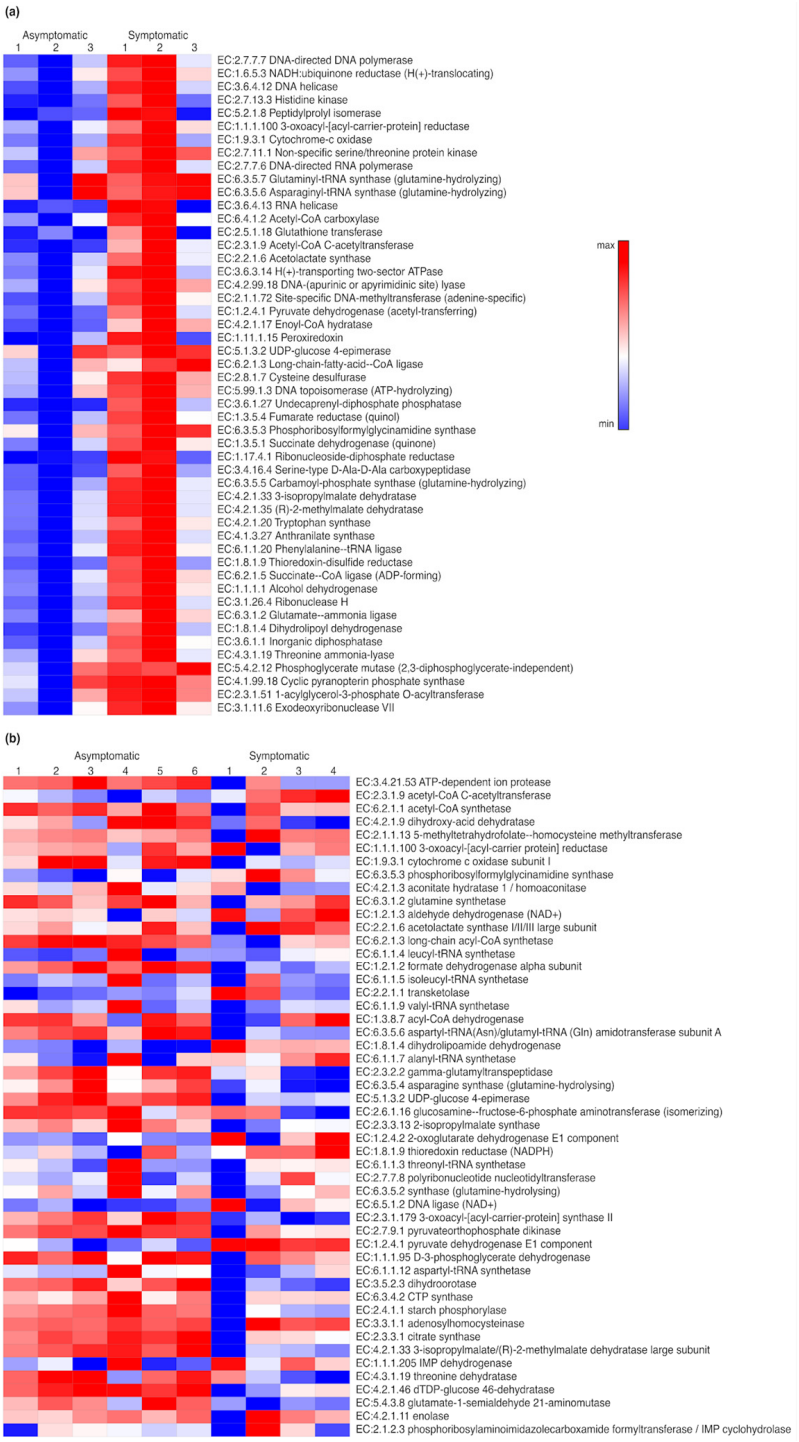

Figure 6. Heatmaps reveal the top 50 functional profiles predicted from (**a**) amplicon data using PICRUSt2 and (**b**) metagenomic shotgun data using MG-RAST.

4. Discussion

In this study, a thorough analysis of the taxonomic and functional traits of microbial communities in the rhizosphere of apple trees was conducted over two non-consecutive years. By using both shotgun metagenomic and amplicon sequencing, our aim was to enhance our understanding of the diversity and functional capabilities within these ecologically vital communities, which are linked to both asymptomatic and symptomatic trees. While previous studies on apple tree microbial communities have predominantly utilized

amplicon sequencing approaches [42–45], only a limited number of shotgun metagenomic studies have been conducted on rhizosphere microbiomes [46].

In the current study's orchard, the incidence of apple tree crown and root rot is less than 10% (Omar Quintana, personal communication); however, it remains a persistent and growing problem that has not been completely eradicated despite continuous monitoring and management. Some studies have reported on the associated microorganisms inhabiting the rhizospheric soil of apple trees affected by crown disease, mainly through cultivable characterization [19] and non-cultivable techniques such as DGGE [47]. Therefore, exploring this through NGS techniques such as amplicon and metagenomics represents a baseline for further investigation.

Metagenomic shotgun sequencing provided greater depth in the analyses performed (39 phyla, 451 families, and 1741 bacterial genera), whereas amplicon sequencing identified a total of 23 phyla, 196 families, and 507 bacterial genera. It is important to note that different factors can influence changes in the communities, such as sampling over two non-consecutive years, different seasons, and possible variations in climatic conditions. Studies on the soil rhizosphere have shown that, despite being different samplings, the composition of microbial communities remains largely consistent when using either amplicon-based or shotgun sequencing technologies [48]. However, it is important to emphasize that understanding microbial communities and their functional capabilities provides an advantage and is a reason for choosing metagenomic technology over amplicon sequencing. Furthermore, it is important to highlight that, despite using different technologies, the most abundant phyla were successfully recovered with both high-throughput sequencing approaches. These phyla, including Proteobacteria, Actinobacteria, and Bacteroidetes, have been previously reported as the most abundant in the rhizosphere, particularly in some studies on soil in which *M. domestica* is grown [49–51].

In fungi, as mentioned earlier, an underestimation in composition occurred when using shotgun sequencing; however, two out of the three most abundant phyla (Ascomycota and Basidiomycota) remained consistent in both analyses. A possible technical issue could have been related to the Kraken database used, which contained 36,246 bacterial species and 455 fungal species, resulting in a sub-estimation of fungal diversity. The phyla Ascomycota, Basidiomycota, and Mortierellomycota have been reported in other agriculturally important crops. Notably, the phylum Mortierellomycota was found to be more abundant in asymptomatic trees; previous studies have reported the presence of this phylum in vanilla orchards, highlighting its ability to produce antibiotics and act as a potential antagonist against various plant pathogens [52]. Additionally, Basidiomycota is another abundant phylum in the rhizosphere of both asymptomatic and symptomatic trees, as it has been reported as the most abundant in apple orchard soils in China [53].

At the genus level, microbial diversity was high and heterogeneous, regardless of the health conditions of the trees. The greater sequencing depth of the shotgun technology allowed for the detection of more genera at a ratio of 1:3 compared to amplicon sequencing. Genera such as *Streptomyces*, *Pseudomonas*, and *Rhodanobacter* were prevalent, regardless of the sequencing technology used, and maintained a similar proportion despite health status. In particular, *Rhodanobacter* exhibited a higher relative abundance in the rhizosphere of symptomatic trees. While the available literature on *Rhodanobacter* is limited, it has been reported that it has the capacity to act as a denitrifying bacterium, which, if it occurs excessively, can result in the loss of nitrogen, an essential nutrient for plant growth, and reduce nitrogen availability for crops in agricultural soils [54,55]. Furthermore, in the case of *Streptomyces* and *Pseudomonas*, these genera have been widely reported in agricultural soils due to their biocontrol properties and their ability to act as antagonists against plant pathogens [56,57], their capacity to degrade and metabolize a wide range of organic compounds [58–60], and their ability to produce substances that promote plant growth, such as phytohormones, enzymes, and siderophores, which can enhance nutrient absorption and stimulate plant growth [61–63]. In the case of less common genera, it is important to highlight some low-abundance genera that, despite their scarcity, may play significant roles

in the rhizosphere. One such is *Hydrogenobaculum*, a genus that participates in nitrate reduction and could be involved in modulating the distribution of microbial communities [64]. Another infrequent and low-abundance genus is *Metakosakonia*, which has been previously reported as a promoter of plant growth through in vitro assays, demonstrating increases in both the shoot and root growth of potatoes [65]. Therefore, it is of interest to investigate its interactions with other rhizosphere microorganisms and with the plant, which may lead to new discoveries for the development of biocontrol strategies and biostimulants for crop production.

In the case of fungi, *Fusarium* and *Penicillium* were the most abundant genera, followed by *Mortierella*, which was mainly abundant in samples from the rhizosphere of asymptomatic trees. Different *Fusarium* species have been documented as saprophytes [66], opportunists [67], and phytopathogens, meaning they are capable of causing diseases in plants such as vascular wilting, root rot, and stem decay, which results in a negative impact on the health and yield of crops [68–71]. Moreover, *Penicillium* is a common necrotrophic–saprophytic genus that might play an important role in diseased roots since it has been able to exhibit a variety of lifestyles, including mutualism, commensalism, and parasitism [72]. In fact, certain strains can also be phytopathogenic, leading to diseases and damage to crops [73,74]. On the other hand, in the rhizosphere of asymptomatic trees, the *Mortierella* genus presented the highest abundance, which was similar to the results reported by Xiong et al. [52], who found that suppressive soil was dominated by the fungal *Mortierella*, accounting for 37% of the total fungal sequences in a study of the suppression of vanilla *Fusarium* wilt disease; it seems that *Mortierella* produces fatty acid ethyl esters that contain arachidonic acid, which under greenhouse conditions reduced the development of tomato late blight and rhizoctonoise of potato tubers [75].

Significant differences in α-diversity were detected only in bacteria, exclusively through the shotgun metagenomic approach. Studies evaluating the α-diversity of rhizosphere communities in agricultural crops have reported variations in diversity due to different biotic factors (e.g., fungi, oomycetes, bacteria, and nematodes) and abiotic factors (e.g., temperature, tree age, sampling season, and physical and chemical soil properties) that influence the growth and distribution of microbes [76,77]. In fact, similar to the results from this study, greater microbial diversity has been reported in the rhizosphere of healthy plants, which decreases in diseased plants [78–80]. This was also demonstrated in sesame rhizosphere soil, where a positive correlation was found between the alpha diversity of the microbial community in the rhizosphere soil of crops and their health status. This correlation led to alterations in the dynamics of bacterial communities and their associated soil functions as part of a plant disease response mechanism [80].

The β-diversity results showed that the bacterial and fungal communities in the rhizosphere of asymptomatic trees were grouped separately from those of symptomatic trees. However, statistical analysis revealed no significant difference in the microbial community structure. In contrast, shotgun analysis showed a statistically significant difference, indicating a health-dependent shift in community structure. Regarding the amplicon-sequenced samples, several key observations were made when reviewing the β-diversity results, which may be interconnected. First, there was genuinely no significant difference in the analyzed samples, as observed in other studies evaluating microbial communities between asymptomatic and symptomatic avocado trees affected by root rot [81]. Secondly, the number of biological replicates used for amplicon sequencing is crucial. It is advised that at least three biological replicates be performed to obtain a more accurate representation of microbial diversity and reduce result variability. However, in some cases, additional replicates may be required for more robust and reliable results [82]. Thirdly, sampling for the first year was conducted in March 2020. Continuous monitoring was carried out in the orchard to detect trees with disease symptoms and apply appropriate management. It was found that out of the three rhizosphere samples collected from symptomatic trees, the apple tree, from which the third biological replication of bacteria and fungi was obtained, recovered, unlike the other two trees. This tree was treated with compost tea, which pos-

sibly promoted greater competition among soil microorganisms [83] due to the exudates produced by the roots and microorganisms and due to the self-regulation of microbial communities [84].

On the other hand, the observed variations in microbial communities between asymptomatic and symptomatic trees through the use of the two sequencing methodologies employed should be interpreted in the context of the seasonal differences during sample collection. Seasonal dynamics, such as temperature and moisture fluctuations, are known to influence microbial composition in soil ecosystems [85]. Recognizing the potential influence of seasonal variations on our results, it is crucial to emphasize that our data analysis utilized rigorous statistical methods to identify patterns related to health conditions. Indeed, similar to other studies such as those conducted by Bei et al. [86], despite the seasonal variations, the overall diversity of the rhizosphere microbiome remained relatively stable, as observed in the Venn diagrams. However, future studies with more frequent and extended sampling across seasons could offer a more comprehensive understanding of the interaction between seasonal dynamics and rhizosphere microbial communities.

Regarding the functional profiles of rhizosphere microbial communities, PICRUSt and MG-RAST have been used in several studies to identify enzymatic activities involved in the metabolic processes in the rhizosphere bacterial communities [87–89]. Considering the top 50 most abundant enzymatic functions from both approaches, several common metabolic functions were identified, including some directly associated with genes/enzymes involved in various metabolic processes and biological functions related to lipid synthesis and degradation, amino acid biosynthesis, carbohydrate metabolism, and nucleotide synthesis. For example, one of the important enzymes detected was 3-oxoacyl-[acyl-carrier-protein] reductase, which has been reported as an enzyme that participates in symbiotic relationships between plants and microorganisms, promoting the formation of nodules in plant roots [90]. In agricultural soil, the mentioned functions are of utmost importance as they perform activities that involve soil nutrient cycling, organic matter degradation, chemical compound transformation, secondary metabolite production, and interaction with plant roots [91,92]. Comprehending these metabolic profiles associated with the microbiome could serve as evidence of dependence on environmental factors and provide valuable insights into the interactions between the microbial community and the host plant [93].

Interestingly, shotgun sequencing allowed for the identification of the oomycetes *Phytophthora* and *Pythium*, which were found in the rhizosphere of both asymptomatic and symptomatic trees (Figure S2). It has been documented that this phytopathogen infects approximately 200 plant species, including economically important plants such as strawberries, pears, walnuts, and apples [94]. A higher relative abundance of *Phytophthora* and *Pythium* was observed in the rhizospheric soil samples from asymptomatic trees than those from symptomatic ones. *Phytophthora*, despite being known to cause diseases in apple trees, can also be present in healthy soil samples as part of its life cycle due to nutrient availability or the presence of related non-pathogenic species. It is also important to consider that the presence or abundance of a pathogen in soil does not always directly correlate with disease in plants. Other factors, such as plant defense mechanisms, the susceptibility of the host plants, or the interaction with other microorganisms present in the soil, can influence disease expression [95]. In fact, in studies conducted on the soils of various oak species showing nonspecific symptoms of branch death and canopy decline [96] and on those of asymptomatic *Eucalyptus coccifera* [97], several species of *Phytophthora* were recovered regardless of the health status of the trees, suggesting a possible ecological role as saprophytes. It is important to note that further analyses involving the isolation of these phytopathogens are required to understand the processes of pathogenicity or saprophytism. On the other hand, *Pythium* species are extensively spread as plant pathogens, including several apple pathogens [98]. However, other members of this genus are prevalent as soil saprophytes [99] and have been identified as exhibiting saprophytic behavior in soil samples, with distribution influenced by factors such as soil type, precipitation, and temperature. Hence, it is crucial to emphasize that not every Pythium species is pathogenic.

5. Conclusions

In summary, this study utilized amplicon sequencing and shotgun metagenomic sequencing to investigate the composition and diversity of bacterial and fungal communities in the rhizosphere of apple trees, revealing dominant taxa with similar relative abundances irrespective of the sequencing technology used. The findings showed significant variations in alpha and beta diversities within bacterial communities, indicating a shift in species composition and abundance influenced by both biotic and abiotic interactions in the dynamic ecological niche of rhizospheric soil, depending on tree health conditions. Additionally, microorganisms and their enzymes, which were previously identified in other studies as important agents of biological control due to their metabolic functions in the rhizosphere, particularly those involved in plant–microorganism interactions, collectively form the baseline for practical strategies in the development of sustainable agricultural practices.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14020357/s1, Table S1: Summary of Illumina Data. Figure S1: Venn diagrams depicting the shared and exclusive (a) bacterial and (b) fungal genera under both asymptomatic and symptomatic conditions. Figure S2: Barplots of oomycetes community composition at the genus level.

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