



The Analysis of Microbial Community Characteristics Revealed that the Pathogens of Leaf Spot of *Rosa roxburghii* Originated from the Phyllosphere

Yuxing Liu¹ · Wei Ge¹ · Chunbo Dong¹ · Qiuyu Shao¹ · Zhiyuan Zhang¹ · Xiao Zou¹ · Haiyan Hu² · Yanfeng Han¹

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Abstract Members of the plant mycobiota are all associated to varying degrees with the development of plant diseases. Although many reports on the plant mycobiota are well documented, the relationships between mycobiota of *Rosa roxburghii* and plant diseases are poorly understood. Mutual interactions and extent of the roles of microbial communities associated with *R. roxburghii* and the source of pathogens are still unclear, and more research is needed on the health benefits of this ecologically important population. Using high-throughput sequencing, we analyzed the mycobiota composition and ecological guilds of the rhizosphere, root, and phyllosphere of healthy and diseased *R. roxburghii* from the Tianfu *R. roxburghii* Industrial Park in Panzhou city, Guizhou province. Analysis of community composition showed that the relative abundance of pathogens of leaf spot, including *Alternaria*, *Pestalotiopsis* and *Neofusicoccum* in the phyllosphere of diseased plant (LD), were 1.15%, 0.15% and 0.06%, and the relative abundance of *Alternaria* and *Pestalotiopsis* were 0.96% and 0.58% in healthy plant (LH). The alpha diversity indices indicated that fungal diversity was higher in healthy plants compared to diseased plants in each compartment. The alpha diversity index of fungi in the phyllosphere (LH) of healthy *R. roxburghii*, including Shannon, Chao-1, and Faith-pd indices, was 1.02, 81.50 and 10.42 higher than that of the diseased (LD), respectively. The fungi in the rhizosphere of healthy

was 1.03, 59.00 and 5.56 higher than the diseased, respectively. The Shannon index of fungi in the root of healthy was 0.29 higher than that of diseased. Principal Coordinate analysis and ANOSIM results showed that there were significant differences in mycobiota composition between healthy and diseased phyllospheres ($P < 0.05$), as well as rhizosphere fungal community, while there was no significant difference between healthy and diseased roots ($P > 0.05$). Linear discriminant analysis effect size revealed that, at different taxonomic levels, there were significantly different taxa between the healthy and diseased plants in each compartment. The ecological guilds differed between healthy and diseased plants according to the FUNGuild analysis. For example, of healthy compared to diseased plants, the percentages of “lichenized-undefined saprotroph” were increased by 2.34%, 0.44%, and 1.54% in the phyllosphere, root, and rhizosphere, respectively. In addition, the plant pathogens existed in each compartment of *R. roxburghii*, but the percentages of “plant pathogen” were increased by 1.16% in the phyllosphere of diseased compared to healthy plants. Together, the ecological guild and co-occurrence network indicated that the potential pathogens of leaf spot were mainly found in the phyllosphere. This study explained one of pathogen origin of leaf spots of *R. roxburghii* by the microbial community ecology, which will provide the new insights for identification of plant pathogens.

✉ Yanfeng Han
swallow1128@126.com

¹ Institute of Fungus Resources, Department of Ecology, College of Life Sciences, Guizhou University, Guiyang 550025, Guizhou, China

² State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang 550081, Guizhou, China

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Introduction

Rosa roxburghii is a perennial deciduous shrub of the family *Rosaceae*. In China, it is mainly distributed in the

southwest, with the most extensive cultivation and highest production in Guizhou Province [1]. With the intensive development of the *R. roxburghii* industry in various planting areas of Guizhou, diseases in this population of plants are becoming more and more serious. Leaf spot is one of the common diseases and can lead to serious premature leaf shedding and reductions in plant growth and fruiting, resulting in large areas of yield reduction or even no harvest [1]. Pathogenic fungi, such as *Pestalotiopsis microspora*, *Neofusicoccum parvum* and *Alternaria alternata* can cause leaf spot disease in *R. roxburghii* [2]. The current leaf spot control strategies are mainly based on the selection of resistant varieties and the use of chemicals [3–6]. In recent years, biological control has paid more and more attention due to the harm of chemical pesticides to human health and the ecological environment. However, at present, the high-efficiency biocontrol microorganisms screened in the laboratory may be largely affected by the environment, and the application effect in the field environment is not stable [1, 7]. Actually, plants under natural environment facing various pathogens, tend to produce defense to maintain their fitness and minimize pathogenic damage [8]. We should maximize the efficiency of disease control based on the biological regulatory mechanisms of plants. However, biological control of leaf spot of *R. roxburghii* has not been widely implemented. The identification of the causal pathogens of leaf spot and the details of colonization, infection, and disease development is key to develop effective biological control strategies.

The tissues and rhizosphere soil of plants are colonized by a variety of microorganisms, which altogether form the host–microbiome holobionts as a result of long-term co-evolution [9]. The plant microbiome has been shown to have an important impact on the physiology and health of the host, and the evolution and ecology of the plant host can only be understood in the context of symbiotic functions [10, 11]. For example, plant-associated microorganisms may determine plant fate by affecting plant adaptation and growth, protecting plants from herbivores, or by driving the evolution of disease resistance [12–14]. The impact of the plant microbiome on host health and fitness depends not only on the microorganisms present in the community, but also on how they interact with each other. The plant microbiome is not equal to the sum of all the individual microbial components. They tend to be structured with microbes interacting strongly and frequently with each other and form interconnected microbial networks [15]. In microbial networks, certain microbes often co-occur with other taxa, and they may interact with many others, playing a key role in the microbial community. These interactions may potentially affect the adaptability of various components, and even directly affect soil fertility and plant health. It is critical to fully exploit the potential of beneficial plant-microbial interactions [16, 17].

Normally, microorganisms in different compartments of the plant exchange material and energy with the host plant, reaching a state of equilibrium through interactions, which may lead to disease when the steady state is disrupted, such as ecological dysregulation due to the proliferation of pathogens or the loss of microbial diversity [18, 19]. A recent study showed that a quadruple *Arabidopsis* mutant (*min7 fls2 efr cerk1*, abbreviated as *mfec*) in the leaves of *Arabidopsis thaliana* has defects in certain pattern-triggered immune and cell surface component structuring genes. It assembles a more numerous but less diverse bacterial community in the phyllosphere, which in turn induces disease symptoms (leaf yellowness and necrosis) [20].

The plant microbiome comes from a wide range of sources. Microbes can be transmitted vertically between plant generations or within different development stages via seeds [21, 22]. Microbes can also be transmitted horizontally within plant tissues (e.g. rhizosphere microbes can enter roots, via emerging root sites or injury sites generated by soil herbivores or other pathogens, and migrate to the above-ground parts of the plant via xylem and phloem) or be obtained from external sources such as the atmosphere, water, soil, animals, and other plants [23–27]. Microbes in the soil and leaves/roots are known to overlap, that is, the same microbial taxa appear repeatedly in different compartments of the plant [28, 29]. Plant pathogens may also overlap in different compartments of the plant, leading to the occurrence of plant diseases. It is important to pay attention to the dynamic changes of plant pathogens [30]. Therefore, this paper studied leaf spot disease from the perspective of fungi.

In this study, healthy and leaf spot-diseased *R. roxburghii* cv. Guinong No. 2 were selected from the Tianfu *R. roxburghii* Industrial Park in Panzhou City, Guizhou Province. High-throughput MiSeq sequencing based on the ITS1 locus was used to sequence the mycobiota inhabiting in the phyllosphere, root, and rhizosphere of *R. roxburghii*. We then used FUNGuild and a co-occurrence network analysis to reveal the mycobiota composition and its relationship with leaf spot of *R. roxburghii*.

Materials & Methods

Sample Collection

In August 2020, eight 10 × 10 m² plots were randomly set up in Tianfu *Rosa roxburghii* base in Panzhou City, Guizhou Province. The healthy and diseased plants (leaves and roots) and soil (rhizosphere soil) in each sample were collected and mixed by five-point method. A total of 24 samples were put into aseptic plastic bag, and the sampling time and number were recorded and brought back to the laboratory for timely processing. All the treated samples were stored in – 80 °C

cryopreservation for DNA extraction. The sample codes were shown in Table 1. The sampling method was according to our previous culturable experiment, and the current work is a follow-up study to explore the response of the phyllosphere, root and rhizosphere mycobiota of *R. roxburghii* to the leaf spot and the source of potential pathogens [31]. The process for obtaining the rhizosphere soil is as follows: first removing the topsoil and finding the plant roots, then cutting off the plant roots with a sterilized shovel and gently shaking off the soil on the roots, that is, the rhizosphere soil [32, 33].

DNA Extraction, PCR Amplification, and Sequencing

Tissue samples (roots and leaves) of *R. roxburghii* were ground under liquid nitrogen using a mortar and pestle to accelerate the grinding process. Phyllosphere, root and rhizosphere microbial DNA were extracted from 0.2 g of leaf powder, root powder and soil, respectively, using a total DNA extraction kit (FastDNA[®] SPIN Kit for Soil) according to the manufacturer's instructions. The ITS rDNA was amplified with primer pairs ITS1F (5'-CTTGGTCATTTA GAGGAAGTAA-3'), ITS2R (5'-GCTGCGTCTTCATCG ATGC-3'), by an ABI GeneAmp[®] 9700 PCR thermocycler (ABI, CA, USA). The conditions and mixtures for PCR amplification referred to our previous method [34]. PCR products were subjected to high-throughput sequencing using an Illumina MiSeq platform by Shanghai Meiji Biomedical Technology Co. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRR15096517).

Raw Data Processing

The raw ITS sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 and merged by FLASH version 1.2.7 with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of < 20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio

of overlap region is 0.2. Reads that could not be assembled were discarded; (iii) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching. The operational taxonomic units (OTUs) were then determined based on 97% similarity using UPARSE 7.1. To obtain species annotation information for each OTU, representative OTU sequences were compared to the sequences in the Unite 7.0 fungal database (<http://unite.ut.ee/index.php>) and subjected to taxonomic assignment using the Ribosomal Database Project (RDP) Bayesian classifier 2.2 (<http://rdp.cme.msu.edu/>) with a confidence threshold of 0.7 [35–37].

Diversity Analysis

The alpha diversity indices (Shannon's index, Faith's phylogenetic diversity) were calculated and compared between samples using one-way analysis of variance (ANOVA) [38]. The statistical analyses were conducted in Excel 2010 (Microsoft Corporation., Redmond, Washington) and SPSS 26.0 (SPSS Inc., Chicago, IL).

The beta diversity (Bray Curtis distance) for all samples were calculated using QIIME2. For constrained PCoA analysis from the vegan package in R was used [39]. The ANOSIM (analysis of similarity) was performed with 999 permutations using the function vegan package in R [40].

Analysis of Differences in Community Composition

To comprehensively evaluate the *R. roxburghii* mycobiota composition and relative abundances of fungal orders and genera, the abundant, intermediate, and rare taxa in each sample were determined based on the relative abundance thresholds of > 1%, 0.01–1%, and < 0.01%, respectively [41–43]. Bar graphs and Wayne plots were plotted using Excel 2010 and Lianchuan Bioplatform (<https://www.omics.tuduo.cn/index>), respectively.

According to the OTU abundance matrix, the taxa with significantly differences were determined by the online analysis software LEfSe (<http://huttenhower.sph.harvard.edu/galaxy/>) [44]. The linear discriminant analysis (LDA) score was more than 4, and the Kruskal-Wallis test value was less than 0.05.

Analysis of Ecological Guilds

The FUNGuild database (<https://github.com/UMNFun/FUNGuild>) was used to determine the ecological guilds in each sample [45]. The relative abundance of each guild is determined based on the number of sequences in each taxon as a percentage of the total number of sequences in each sample and then plotted using Excel.

Table 1 Definition of the sample code

Code	Definition
LD	The phyllosphere fungi of infected <i>R. roxburghii</i>
LH	The phyllosphere fungi of healthy <i>R. roxburghii</i>
RD	The root fungi of infected <i>R. roxburghii</i>
RH	The root fungi of healthy <i>R. roxburghii</i>
RSD	The rhizosphere fungi of infected <i>R. roxburghii</i>
RSH	The rhizosphere fungi of healthy <i>R. roxburghii</i>

Co-occurrence Network Analysis

A co-occurrence network of the mycobiota in each sample was constructed using Gephi 0.9.2 [46]. Based on the relative abundance information, six samples of LH, LD, RSH, RSD, RH and RD were analyzed by co-occurrence network. The nodes represent samples and taxa, the edges between the nodes represent the relationships between the samples and taxa, and the thickness of the edges represent the abundance of the taxa in the samples.

Results

Fungal Community Composition and Diversity in Various Compartments of Healthy and Diseased *R. roxburghii*

Based on the Illumina Miseq sequencing for each sample, approximately 27,009–46,459 high-quality sequences were detected from each sample, and a total of 1184 OTUs were obtained from all the samples. These OTUs belonged to 11 phyla, 30 classes, 83 orders, 188 families, 283 genera, and 296 species. The alpha diversity indices (Shannon, Chao-1, and Faith-pd) of various compartments of healthy and diseased plants are shown in Fig. 1. The results showed that the Shannon, Chao-1, and Faith-pd indices of fungi in the phyllosphere (LH), root (RH), and rhizosphere (RSH) of

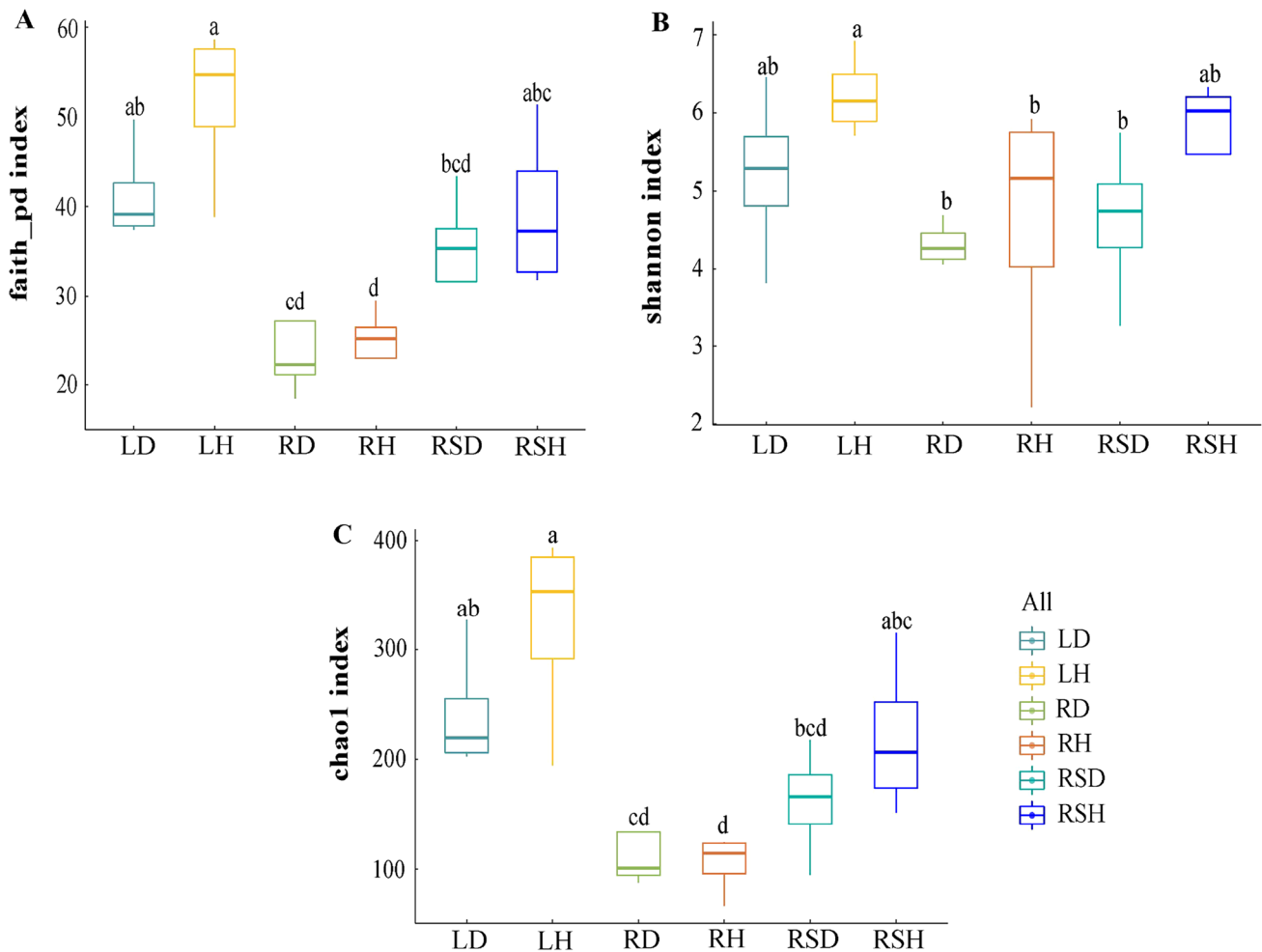


Fig. 1 Comparison of alpha fungal diversity in various components of healthy and diseased *Rosa roxburghii* (the degree of replication n=4). **A** Comparison of Faith-pd index in various components of healthy and diseased *R. roxburghii*. **B** Comparison of Shannon index in various components of healthy and diseased *R. roxburghii*; **C** Comparison of Chao1 in various components of healthy and dis-

eased *R. roxburghii*. *Note:* LD: The phyllosphere fungi of infected *R. roxburghii*; LH: The phyllosphere fungi of healthy *R. roxburghii*; RD: The root fungi of infected *R. roxburghii*; RH: The root fungi of healthy *R. roxburghii*; RSD: The rhizosphere fungi of infected *R. roxburghii*; RSH: The rhizosphere fungi of healthy *R. roxburghii*

healthy *R. roxburghii* were generally higher than the corresponding indices of diseased plants in the phyllosphere (LD), root (RD) and rhizosphere (RSD), respectively, with higher indices corresponding to more diverse fungal communities. However, none of the differences was significant. Also, the Chao-1 and Faith-pd indices were lower in RH than RD. On the compartments of *R. roxburghii*, the whole, healthy plants showed higher fungal community diversity than diseased plants.

In addition, the alpha diversity indices were higher in the phyllosphere (LH, LD) than in the rhizosphere (RSH, RSD), which were higher than in the root (RH, RD), except that the Shannon index was higher in RSH than LD. In other words, the alpha diversity of fungal communities in different varied greatly.

Bray Curtis distance was calculated based on OTU abundance information, and principal coordinate analysis was conducted for each sample to compare the composition of

different fungal communities in different compartments of *R. roxburghii* (Fig. 2). The results showed that in phyllosphere (Fig. 2A), the principal coordinate explained 41% of the community variation, with the abscissa explaining 22% of the relationship value and the ordinate explaining 19%. There was no similarity between the two groups. The results of ANOSIM analysis (Table 2) further indicated that there were statistically significant differences between healthy and diseased phyllospheres ($R=0.65$, $P=0.03$). In root (Fig. 2B), the total explanatory degree was 30%, in which 15.4% of the relationship value was explained by the

Table 2 ANOSIM analysis among each samples

Group	R-value	P-value
LD-LH	0.65	0.03
RD-RH	0.05	0.25
RSD-RSH	0.77	0.03

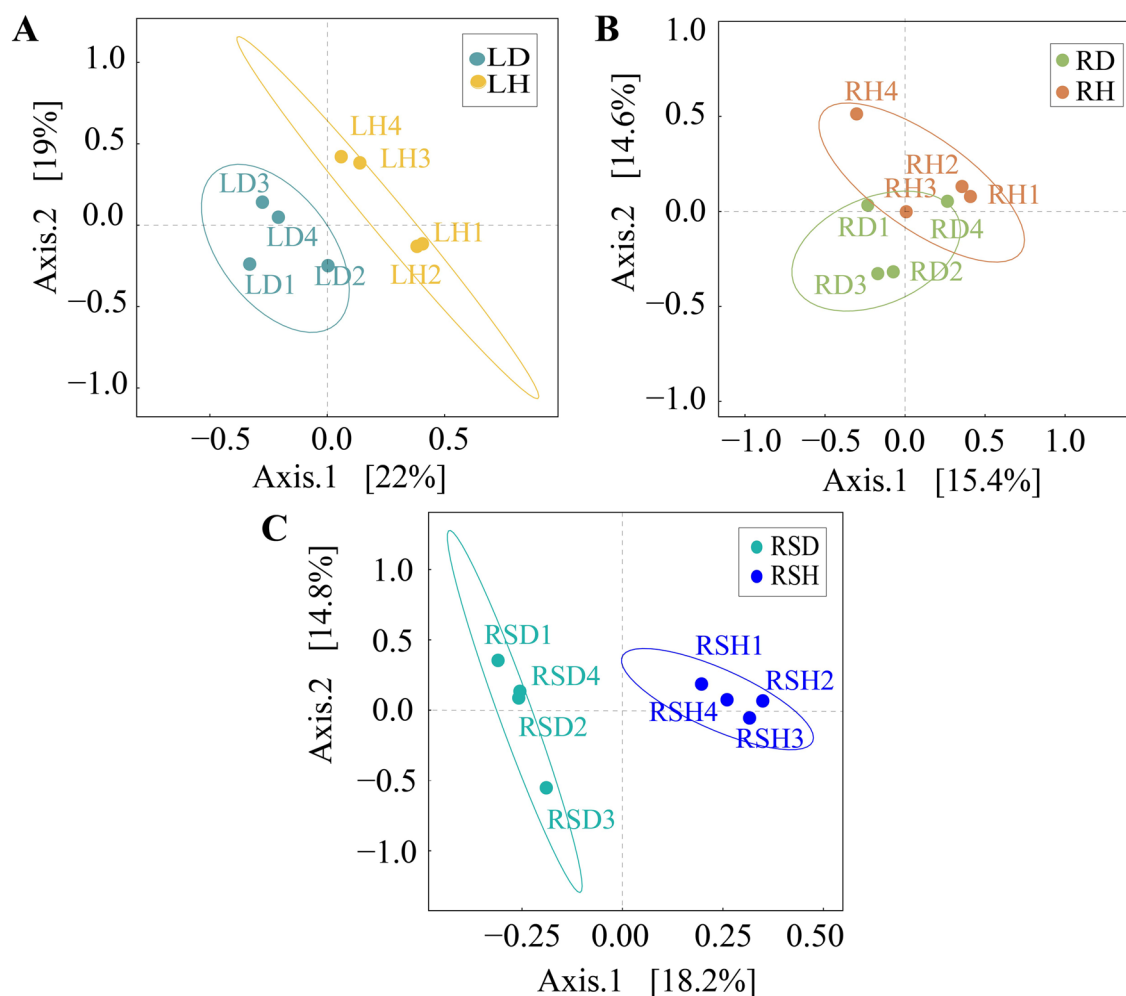


Fig. 2 Comparison of beta fungal diversity in various components of healthy and diseased *Rosa roxburghii*. **A** PCoA analysis in phyllosphere of healthy and diseased *R. roxburghii*. **B** PCoA analysis in

root of healthy and diseased *R. roxburghii*; **C** PCoA analysis in rhizosphere of healthy and diseased *R. roxburghii*

abscissa and 14.6% by the ordinate. There was no significant difference between healthy and diseased roots ($R=0.05$, $P=0.25$). In rhizosphere (Fig. 2C), abscissa explained 18.2% of the relationship value, ordinate explained 14.8% of the relationship value, and the total explanation was 33%. There was no similarity between the two groups, and ANOSIM analysis between healthy and diseased rhizospheres reached a significant level ($R=0.77$, $P=0.03$).

Fungal Community Composition in Various Compartments of Healthy and Diseased *R. roxburghii*

At the genus level, there were differences in abundant taxa in various compartments of healthy and diseased *R. roxburghii* (Fig. 3A). The dominant genera in LD were, in order, Unspecified_Pleosporales (14.83%), *Taphrina* (12.25%), and Unspecified_Mycosphaerellaceae (12%); those in LH were Unspecified_Pleosporales (15.91%), Unspecified_Chaetothyriales (11.86%), and Unspecified_Mycosphaerellaceae (8.98%); those in RD were Unspecified_Helotiales (17.85%), Unspecified_Sebacinales (10.01%), and Unspecified_Xylariales (9.94%); those in RH were Unspecified_Helotiales (45.47%), *Cladosporium* (12.42%), and Unspecified_Pleosporales (7.23%); those in RSD were *Mortierella* (33.34%), Unspecified_Trechisporales (25.18%), and Unspecified_Nectriaceae (5.07%); and those in RSH were *Mortierella* (32.36%), *Fusarium* (11.66%), and Unspecified_Sordariomycetes (5.73%).

There were also great differences in rare genera in various compartments of healthy and diseased *R. roxburghii* (Fig. 3B). The proportions of rare taxa were 0.18% in LD (36 genera, including *Bensingtonia*, *Neofusicoccum* and *Steccherinum*), 0.46% in LH (51 genera, including Unspecified_Corticiales, *Hypoxylo* and *Bensingtonia*), 0.06% in RD (5 genera, including Unspecified_Ramalinaceae, *Arthrinium* and Unspecified_Agaricostilbales), 0.03% in RH (5 genera, including Unspecified_Tricholomataceae and *Ampelomyces*), 0.16% in RSD (17 genera, including *Arthrinium*, *Rugosomyces* and Unspecified_Physalacriaceae), and 0.14% in RSH (17 genera, including *Microascus*, *Hydnocystis* and *Steccherinum*).

Furthermore, there were also great differences in intermediate genera in various compartments of healthy and diseased *R. roxburghii* (Fig. 3C). The proportions of intermediate taxa were 19.76% in LD (69 genera, including *Pestalotiopsis*, Unspecified_Elsinoaceae and *Pseudocercospora*), 21.39% in LH (81 genera, including *Pestalotiopsis*, Unspecified_Dothideomycetes and *Kondoa*), 23.98% in RD (79 genera, including *Lophiotrema*, *Hymenoscyphus* and *Pestalotiopsis*), 17.70% in RH (74 genera, including *Pestalotiopsis*, *Mycena* and *Plectosphaerella*), 17.27% in RSD (73 genera, including *Solicoccozyma*, *Exophiala* and

Pestalotiopsis), and 22.81% in RSH (93 genera, including Unspecified_Clavariaceae, *Talaromyces* and *Pestalotiopsis*).

Linear discriminant analysis effect size (Fig. 4) was used to conduct preliminary analysis on the biomarkers with statistical differences in different compartments of healthy and diseased *R. roxburghii*. The results showed that there were 58 taxa at different taxonomic levels with significant differences among the samples ($\log LDA > 4$, $p < 0.05$). Among them, 21 taxa were significantly different in LD, including Ascomycota, *Taphrina* (from class to genus) and Erysiphales, and 9 taxa in LH, including Chaetothyriales, *Strelitziana* and Agaricostilbales (from class to order). Four taxa played an important role in RD, including Helotiales, Eurotiales and Aspergillaceae; and 5 taxa in RH, including Dothideomycetes, Capnodiales and *Alternaria*. Thirteen taxa were significantly different in RSD, including *Mortierella* (from order to genus), *Trichoderma* and Hypocreaceae, and 6 taxa in RSH, including Hypocreales, Sordariomycetes and Nectriaceae. All of the above taxa contributed significantly to the differences in fungal community composition between healthy and diseased *R. roxburghii*.

Fungal Ecological Guilds in Various Compartments of Healthy and Diseased *R. roxburghii*

According to the results of FUNGuild annotation, after disregarding the Unspecified guilds, the fungi in various compartments of healthy and diseased *R. roxburghii* involved 57 ecological guilds such as “plant pathogen”, “plant pathogen-wood saprotroph”, “endophyte-plant pathogen”, “dung saprotroph-plant pathogen”, “dung saprotroph-plant pathogen”, “endophyte-plant pathogen-wood saprotroph”, “endophyte-plant pathogen-undefined saprotroph” and “endomycorrhizal-plant pathogen-undefined saprotroph”. Among them, the plant pathogens (red boxes in Fig. 5) were mainly *Pestalotiopsis*, *Pseudocercospora*, *Neofusicoccum*, *Thanatephorus*, *Taphrina*, and *Alternaria*. The plant pathogens were present in all compartments of healthy and diseased plants, exhibiting the following order: LD (1.32%), LH (0.16%), RD (0.19%), RH (0.35%), RSD (0.12%), and RSH (0.14%).

In LD compared to LH, “plant pathogen”, “plant pathogen-undefined saprotroph” and “leaf saprotroph-plant pathogen-undefined saprotroph-wood saprotroph” were increased by 1.16%, 1.79%, and 0.69%, respectively. In RD compared to RH, “animal pathogen-endophyte-lichen parasite-plant pathogen-soil saprotroph-wood saprotroph”, “lichen parasite-plant pathogen-wood saprotroph” and “endophyte-lichen parasite-plant pathogen-undefined saprotroph” were increased by 3.42%, 3.12%, and 1.40%, respectively. In RSD compared to RSH, “animal pathogen-endophyte-lichen parasite-plant pathogen-soil saprotroph-wood saprotroph”, “epiphyte-leaf saprotroph-lichen parasite-lichenized-plant pathogen-wood saprotroph” and “animal

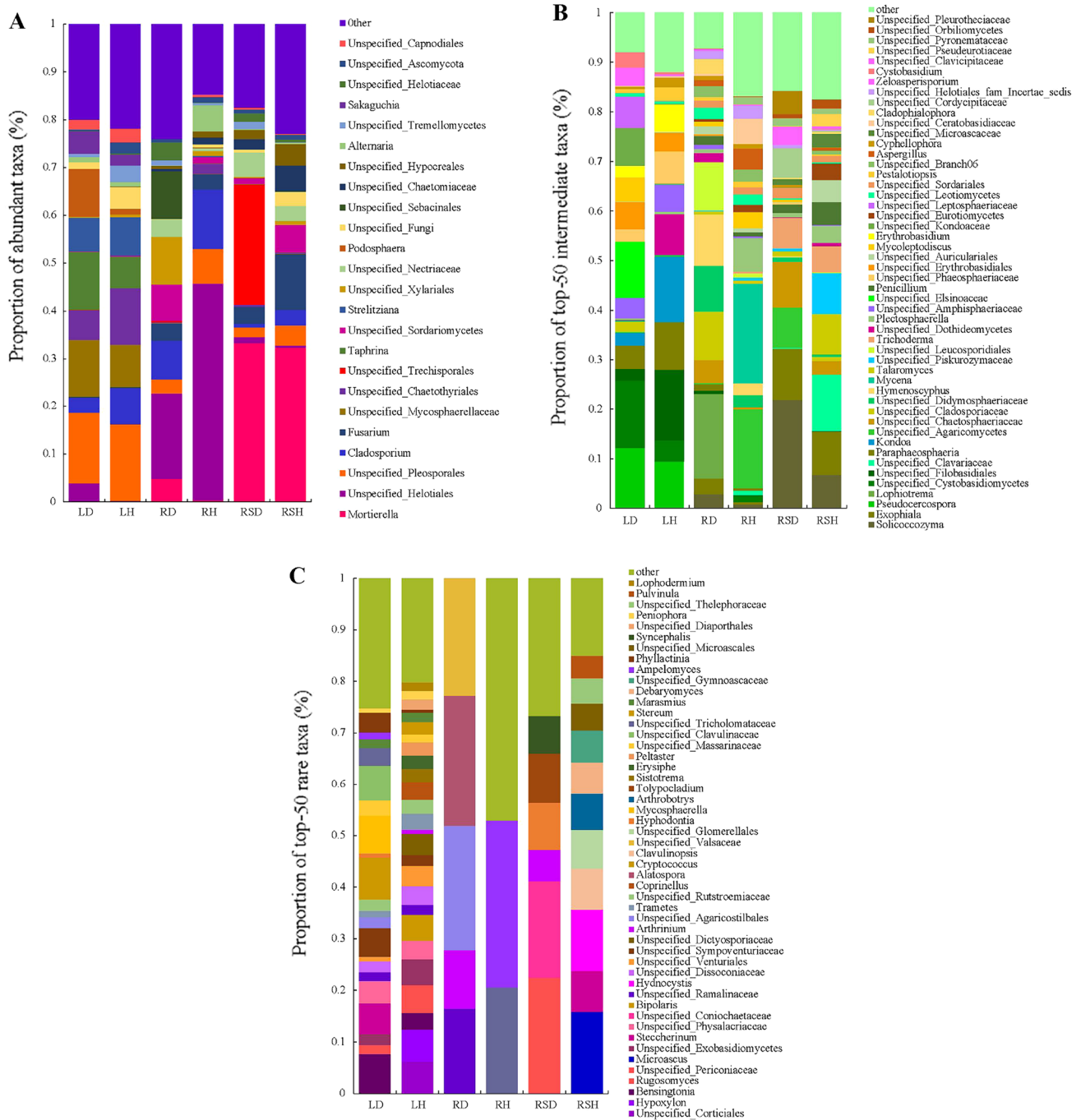


Fig. 3 Community Composition and relative abundance of fungal community at genus level in each sample. **A** Community composition and proportion of abundant taxa at the genus level; **B** Community composition and proportion of the top-50 intermediate taxa at the genus level; **C**, Community composition and proportion of the top-50 rare taxa at the genus level

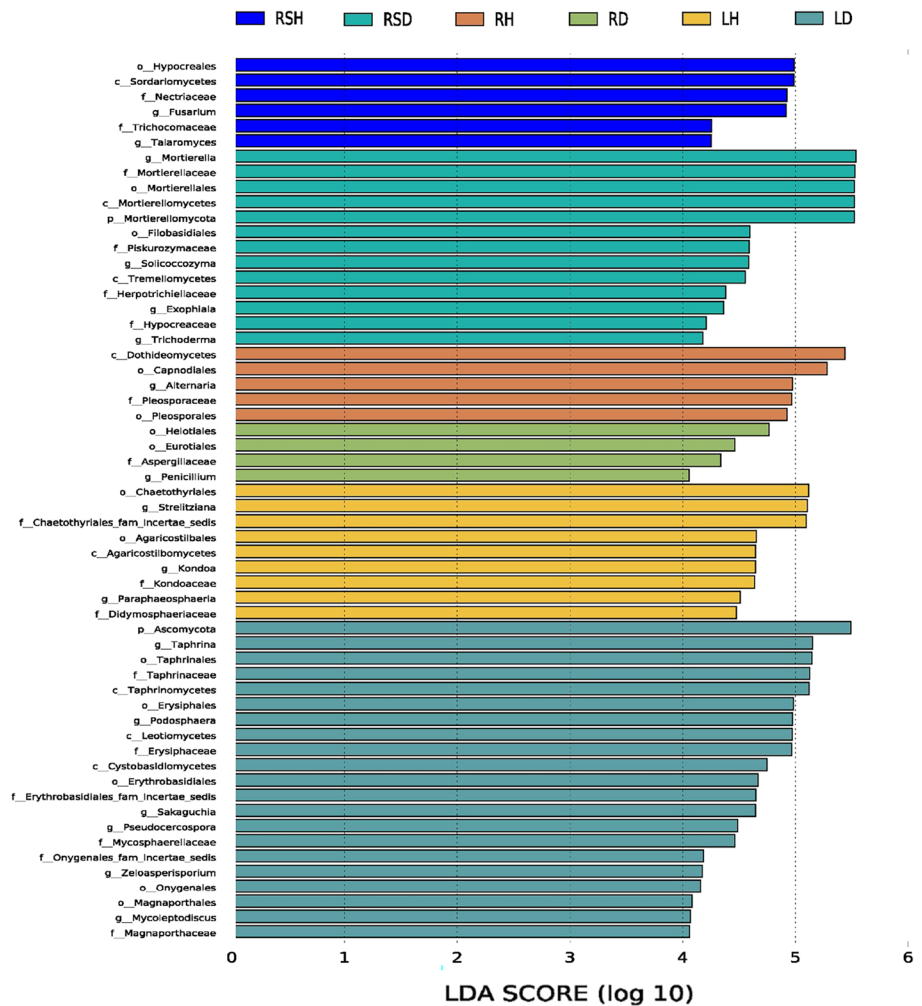
pathogen-endophyte-plant pathogen-undefined saprotroph” were increased by 0.96%, 1.05%, and 0.66%, respectively. In summary, the proportion of plant pathogen-related taxa was higher in diseased plants compared to healthy plants in each compartment.

Additionally, “lichenized-undefined saprotroph” was higher in LH, RH, and RSH compared to LD, RD, and

nity composition and proportion of the top-50 intermediate taxa at the genus level; **C**, Community composition and proportion of the top-50 rare taxa at the genus level

RSD, respectively, by 2.34%, 0.44%, and 1.54%, respectively. In contrast, saprotrophs such as “dung saprotroph-plant saprotroph” and “undefined saprotroph” accounted for relatively high proportions in LD compared to LH, increasing by 2.16% and 4.40%, respectively.

Fig. 4 LEfSe analysis on the significance of fungal community difference between healthy and diseased *R. roxburghii*



Co-occurrence Network Analysis

To further determine the potential source of the pathogens of leaf spot of *R. roxburghii*, this study combined the three leaf spot pathogens (*Alternaria*, *Pestalotiopsis*, *Neofusicoccum*) identified by our previous research with the potential plant pathogens (*Taphrina*, *Thanatephorus*, *Pseudocercospora*) annotated in the ecological guilds, and performed a co-occurrence network analysis of the mycobiota using Gephi software [2]. The co-occurrence network diagram showed that there were 5 potential pathogens in LH (Fig. 6a), including *Taphrina* (6.55%), *Pseudocercospora* (2.02%), *Alternaria* (0.96%), *Pestalotiopsis* (0.58%) and *Thanatephorus* (<0.01%), and 5 potential pathogens in LD (Fig. 6b), including *Taphrina* (12.25%), *Pseudocercospora* (2.40%), *Alternaria* (1.15%), *Pestalotiopsis* (0.15%) and *Neofusicoccum* (<0.01%). Potential pathogens were also found in root and rhizosphere. There were 3 potential pathogens in RH (Fig. 6c), including *Taphrina* (0.03%), *Alternaria* (5.55%) and *Pestalotiopsis* (0.17%), and 4 in RD (Fig. 6d), including *Taphrina* (0.02%), *Pseudocercospora* (<0.01%), *Alternaria*

(0.13%) and *Pestalotiopsis* (0.16%). RSH included 3 potential pathogens (Fig. 6e), that were *Taphrina* (0.03%), *Alternaria* (0.28%) and *Pestalotiopsis* (0.08%), and RSD included 3 potential pathogens (Fig. 6f), which were *Taphrina* (0.02%), *Alternaria* (0.12%) and *Pestalotiopsis* (<0.01%). That is to say, there were differences in the distribution of pathogens in different compartments of *R. roxburghii*, and the types of pathogens and potential pathogens distributed in the phyllosphere were the most, accounting for the highest proportion. From this, it can be inferred that the potential pathogens in our sampling area mainly came from phyllosphere and existed in all compartments of *R. roxburghii*.

Discussion

Generally speaking, leaf spot of *R. roxburghii* is postulated to be caused by a variety of pathogenic fungi [1, 47, 48]. Plant pathogens, including *Pestalotiopsis*, *Neofusicoccum* and *Alternaria*, have been reported to cause leaf spot of *R. roxburghii* [2]. *Pseudocercospora*, *Thanatephorus*

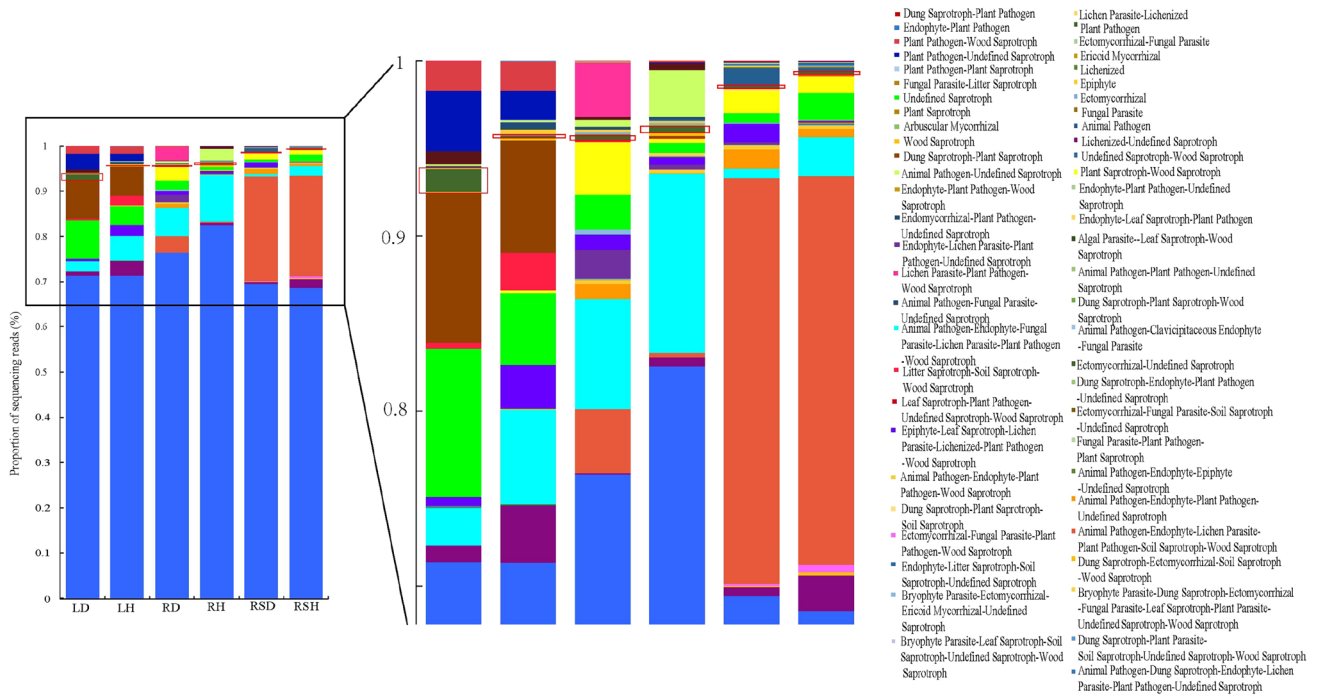
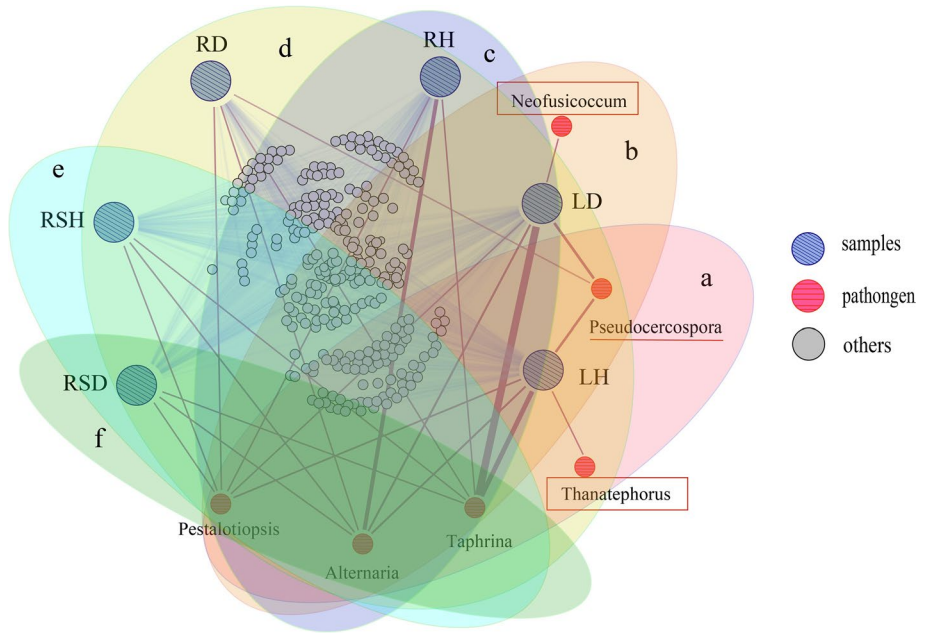


Fig. 5 Fungal ecological guilds based on FUNGuild analysis. The boxes indicate plant pathogen

Fig. 6 Co-occurrence network diagram of mycobiota in the root, rhizosphere, and phyllosphere of *R. roxburghii*. Nodes with oblique lines represent samples, nodes with transverse lines represent pathogens, and nodes with no lines represent other taxa. The thickness of the edges represent the abundance of the taxa in the samples. **a** pathogenic fungi present in LH. **b** pathogenic fungi present in LD. **c** pathogenic fungi present in RH. **d** pathogenic fungi present in RD. **e** pathogenic fungi present in RSH. **f** pathogenic fungi present in RSD



and *Taphrina*, as pathogens of leaf spot with a wide host range, may also be the potential pathogens of leaf spot disease of *R. roxburghii* [49–51]. In the study, the results of ecological guilds and co-occurrence network showed that the above potential leaf spot pathogens mainly existed in the phyllosphere of *R. roxburghii*, but they were found in all compartments of the plant. This is similar to our

previous results based on culturable experiments, that is, the phyllosphere, including the diseased and healthy phyllosphere, is a major potential reservoir for the pathogens that cause leaf spot of *R. roxburghii* due to air pollution and insect-mediated transmission [31]. In addition, the co-occurrence network also found that there were multiple pathogenic fungi in the phyllosphere of *R. roxburghii*, such

as *Taphrina*, *Alternaria* and *Pestalotiopsis*, which often caused leaf spot disease [21, 50, 52, 53]. Whether this may suggest that the leaf spot of *R. roxburghii* may also be the result of mixed infections by multiple pathogens. Of course, this hypothesis still needs to be verified by further experiments.

Plant-associated microbiome assembly is regulated by host factors, and plant health is often closely related to its microbial community composition [54–56]. The microbial community of diseased plants may be altered compared to healthy plants. This alteration is similar to the dysbiosis in the gastrointestinal tract of patients with inflammatory bowel disease, and it leads to the development of associated plant diseases which may account for the significant difference in fungal community composition between healthy and diseased *R. roxburghii* in our study. In general, plant microbial communities often have a small number of high-abundance dominant species co-existing with a large number of low-abundance rare species [57, 58]. The functions of rare microorganisms have often been overlooked and underestimated in previous studies because changes in the abundance of dominant microorganisms tend to mask the population dynamics of low-abundance taxa [59, 60]. In this study, not only the dominant taxa were considered, but the rare taxa were also given extra attention.

For example, *Cladosporium* was a abundant genus in all samples, and its abundance in various compartments was higher in healthy compared to diseased plants ($P > 0.05$). This is consistent with the results of our previous culturable study, that is, *Cladosporium* only exist in the root epiphytic microbiota of healthy *R. roxburghii* [2]. These fungi are able to synthesize a lot of different bioactive compounds, and some of their metabolites show antagonistic activity against certain pathogens. For example, four compounds produced by *Cladosporium cladosporioides* (isocladosporin, cladosporin, 5-hydroxyasperentin, and cladosporin-8-methyl ether) have large inhibitory effects on seven pathogens of strawberry anthracnose, with isocladosporin significantly inhibiting the growth of the pathogens *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides* [61]. The higher abundance of *Cladosporium* in healthy plants compared to diseased plants may be related to the antagonistic activity of its secondary metabolites against leaf spot pathogens. *Hypoxylon* was a rare genus, which exists only in the phyllosphere of healthy plants, but not in diseased plants. This is consistent with the culturable results that the proportion of *Hypoxylon* in the phyllosphere epiphytic microbiota of healthy *R. roxburghii* is higher than that of diseased *R. roxburghii*. *Hypoxylon* can produce bioactive products and have great potential in resistance to human diseases and plant diseases [62]. For example, the ethyl acetate extract of *Hypoxylon* had high inhibitory activity against *Sphaeropsis sapinea* [63].

Another interesting point is that *Exophiala* was mainly distributed in the rhizosphere (RSD, RSH), followed by the root (RD, RH) and phyllosphere of healthy plants (LH), but not in the phyllosphere of diseased plants (LD). *Exophiala* is a common type of dark septate endophyte (DSE), which is a kind of endophytic soil fungi that colonize plant roots without causing obvious negative effects [64]. *Exophiala* have strong biological adaptability, promote plant growth by improving plant nutrition, and exhibit strong stress tolerance [65, 66]. As *Exophiala* has a similar niche to the potential leaf spot pathogens, it inhibits the overgrowth of the pathogens through competition for resources such as nutrients and space. This hinders disease development, which may explain why this genus is present in LH but not LD.

The generalized definition of ecological guild refers to a group of related or unrelated species that utilize the same type of ecological resources in a similar way [67]. The focus of the classification on trophic preferences, rather than on species abundance and taxonomic homogeneity, provides a different perspective on community composition [68, 69]. In this study, based on the FUNGuild analysis, it was found that there were differences in the guilds in the various compartments of the mycobiota of healthy and diseased *R. roxburghii*. The proportions of pathogens were higher in diseased compared to healthy plants in all compartments. This may mean that the health status of *R. roxburghii* is directly related to the number of pathogens. “Lichenized-undefined saprotroph” accounted for a high proportion of fungi in various compartments of healthy *R. roxburghii*. These symbiotrophs are the result of long-term plant–environment co-evolution, and the symbiotic relationships may promote plant growth and resistance. In addition, the proportions of plant pathogens and saprotrophs in the phyllosphere were higher in diseased compared to healthy plants. There was evidence of a functional shift between healthy and diseased plants, from colonization by symbiotrophs (e.g., “lichenized-undefined saprotroph”) to colonization by pathotrophs (e.g., “plant pathogen-undefined saprotroph”) and saprotrophs (e.g., “undefined saprotroph”), which may be an important reason for disease in *R. roxburghii*.

Plant health and pathogen resistance are often closely related to microbial diversity. More complex networks in the plant environment can inhibit pathogen colonization by competing for resources and occupying niches, thereby reducing the risk of pathogen outbreaks [70]. In general, microbial communities with high diversity and abundance levels may enhance the functional redundancy of the inhabited environment, which may help to maintain the health of the host plant [70, 71]. In this study, we found that the alpha diversity indices of the six samples were basically consistent (except that the Chao-1 and Faith-pd indices were lower in RH than RD), that is, the diversity of fungi in various compartments was generally higher in

healthy compared to diseased plants. This implies that the degree of plant-associated fungal diversity is positively correlated with the health of *R. roxburghii*.

Different compartments of the plant act as different microhabitats with diverse morphological characteristics, metabolic activities, and corresponding functions. These distinct microhabitats selectively recruit different microorganisms to colonize, thus leading to differences in microbial diversity in different compartments of the plant [26, 72]. Typically, microbial diversity is more abundant in rhizosphere soils than in non-rhizosphere compartments [73]. However, our results showed that the compartment with the highest fungal diversity was the phyllosphere, followed by the rhizosphere and then the root, which may be due to the fact that the phyllosphere, as a microhabitat with great variability, is susceptible to strong disturbances of fungal communities by environmental factors, so the phyllosphere was more diverse than other compartments [23].

Conclusions

This study revealed that there were differences in microbial composition and ecological functional groups between healthy and diseased *R. roxburghii*, which provided a theoretical basis for the construction of antagonistic synthetic communities. As for what is a healthy plant microbiome, researchers still do not have a definite conclusion, which is a problem that needs further research in the future. In addition, this study also clarified that *R. roxburghii* leaf spot is caused by mixed pathogens, which mainly come from phyllosphere. As a reservoir of pathogens of leaf spot disease, phyllosphere can spread to other compartments of the plant through plant tissue or external force, resulting in the recurrence of pathogens in different parts of the plant, so cutting off the spread pathway of pathogens may provide a new idea for disease control.

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Author Contributions YH: conceptualization and Funding acquisition; YL, WG, ZZ, CD, and QS: data acquisition; YL, and CD: formal analysis; YL: writing the first draft; YH: writing, review, and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRR15096517). The datasets generated during and/or analyzed during the current study are available from the corresponding authors upon request.

Declarations

Conflict of interest The authors declare Conflict of interest.

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