

The effects of *Erysiphe quercicola* inoculation on the phyllosphere microbiome of *Hevea brasiliensis* in Hainan Province, China

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Abstract

Rubber tree powdery mildew, a major fungal leaf disease of *Hevea brasiliensis* trees caused by obligate parasitic fungi (*Erysiphe quercicola*), is among the most important diseases affecting rubber planting areas worldwide. Phyllosphere microbiomes are currently a new research hotspot in molecular ecology, and their biological community is extremely rich. This study used high-throughput sequencing and other molecular techniques to analyze the structure and diversity of dominant fungi and bacteria in the communities of rubber tree leaves (N=16) in four representative rubber-producing regions in Hainan Province, China, after the occurrence of white powder disease. The sampled area had high species richness and stable biodiversity. This study mainly focused on (i) the dominant bacterial and fungal phyla and genera of the sampled regions; (ii) differences in the phyllosphere microbial community structure and relative abundance between healthy leaves and powdery mildew Level 3-diseased leaves within the same region; and (iii) differences in the phyllosphere microbial community structure and relative abundance between healthy leaves and diseased leaves, as well as their correlations with environmental factors, in different regions. This work is innovative in three respects: (i) distinguishing the dominant populations of phyllosphere microbes for rubber trees with powdery mildew via high-throughput detection technology to determine the impact of powdery mildew on these microbes; (ii) exploring the effects of pathogenic bacteria on phyllosphere microbes and hosts from a relatively macro perspective; and (iii) investigating the effects on phyllosphere microbial communities before and after the infection of obligatory parasite leaf pathogenic bacteria. This project is among the first conducted in China's representative rubber-producing areas to study *Erysiphe*'s influence on rubber tree leaf microorganisms. These findings provide data support to better understand the biological evolution of powdery mildew diseases and establish new strategies for their ecological control in rubber tree plantations.

INTRODUCTION

Hevea brasiliensis, or the rubber tree, is a tall perennial native to the Amazon basin. The rubber tree has been introduced to more than 40 countries and regions in tropical and subtropical areas, where it plays a pivotal role in the economic development of plantations (Lieberei, 2007). As a major producer and consumer of natural rubber, China began to plant rubber trees within its territory in 1906. In 1954, Chinese researchers made a breakthrough in the large-scale cultivation of rubber trees in the subtropics (latitude 18°N and above), leading China to become the fourth largest producer of rubber globally, despite previously having no rubber plantations at all. Currently, there are five natural rubber plantation bases in China established in Hainan, Yunnan, Guangdong, Guangxi, and Fujian provinces. The experiment reported in the present study was conducted at plantations in Hainan.

Rubber tree powdery mildew is one of the most damaging and widespread foliar diseases of rubber trees. This disease affects the profit of the natural rubber industry by causing defoliation and reductions in latex yield. In severe cases, rubber production can be reduced by 45% (Liyanage et al., 2016). As the most serious epidemic disease affecting China's rubber plantations, powdery mildew occurs in various rubber planting areas every year. For example, the Xishuangbanna land reclamation area suffered major damage due to

powdery mildew in 2017, delaying rubber tapping by nearly 2 months and reducing rubber production by tens of thousands of tons, resulting in losses totaling hundreds of millions of yuan (Liu et al., 2018). It is known that phenological and meteorological factors can jointly influence powdery mildew disease. Under optimum conditions, the conidia can repeatedly infect the host tree throughout the year, leading to greatly reduced photosynthesis, which results in slower growth and substantially decreased latex yield (Liyanage et al., 2017). At present, plantation managers rely on chlorothalil and sulfur powder for mildew prevention and control. Although the application of these agents is effective to some extent, chlorothalil and sulfur powder cannot fundamentally prevent disease outbreaks, and their use causes environmental pollution.

The pathogen responsible for rubber tree powdery mildew is *Erysiphe quercicola*. *E. quercicola* is an obligate parasitic fungal pathogen that cannot be cultured in vitro without host tissue. The pathogen spreads easily through the air and causes serious damage to rubber trees, notably their leaves, as well their inflorescences, buds, and other tissues. The conidia and hyphae of powdery mildew form a white powder that appears on infected leaves, buds, and shoots. Later on, light yellow spots appear on infected leaves, causing the leaves to twist, buckle, or otherwise become distorted in shape and fall off.

Phyllosphere microorganisms are intimately related to plant growth and the occurrence of plant diseases (Liu et al., 2022). The phyllosphere microbial community is determined by various factors such as leaf age, canopy position, season, and chemical spraying (Jager et al., 2001). Two important indicators for inferring the phyllosphere's microecology are its community structure and metabolic functions (Liu et al., 2022). The regulation of phyllosphere microbial communities has opened a new and alternative approach to the biological control of foliar diseases. In this respect, the structural dynamics of microorganisms on leaf surfaces have great significance for shaping the near-surface environment and ecology (Li et al. 2021). Although the phyllosphere provides relatively stable conditions for the growth and reproduction of its microorganisms, the phyllosphere living environment is harsh. To better adapt to this habitat adversity, the phyllosphere microbial community relies on its diversity and complexity as evinced by its viable but non-culturable (VBNC) features. Although the phyllosphere environment is hostile, its microbial communities are still highly diverse, consisting of bacteria, filamentous fungi, yeasts, algae, and protozoans. However, bacteria are the main microorganisms constituting the phyllosphere. The community's diversity, dispersal, and development on the leaf surface are mainly associated with physiological and biochemical changes in the host leaf, its living environment, and its level of immunity (Liu et al., 2022). Many phyllosphere microorganisms have co-evolved with their hosts or epiphytes, and thus play key roles in plant productivity and health by affecting leaf function and lifespan, seed mass, apical growth, flowering, and fruit development, as well as by assisting in the removal and degradation of environmental pollutants (Stone et al., 2018).

The community structure and diversity of phyllosphere microorganisms influence the characteristics and strength of the ecological functions of leaves. The phyllosphere's microecological dynamic equilibrium is central to the composition of its microbial community. The impacts of phyllosphere microbes upon their host plants, surrounding atmospheric conditions, and microbial interactions have been studied. The results suggest that global climate change has altered phyllosphere microbial communities, with potential cascading effects on plant function and evolution and ecosystem functioning (Vacher et al., 2016). In contrast, little research has been conducted on smaller scales, especially in regard to the relationship between phyllosphere microbes and their hosts and pathogens following powdery mildew infection in a cash crop such as rubber trees. To address this knowledge gap, the present study employed MiSeq high-throughput sequencing (i) to investigate the similarities and differences in the composition of the phyllosphere microbial community of rubber trees in Hainan between regions where powdery mildew occurs and where it does not; and (ii) to understand the effects of powdery mildew on the phyllosphere microbial community of rubber tree leaves and the changes in each microbial community. The overarching aim of this study was to provide an empirical basis for the biological control of powdery mildew and further elucidate the evolution of powdery mildew in rubber trees.

MATERIALS AND METHODS

Leaf Samples

To study the structure and diverse differences of the phyllosphere microbial communities of rubber trees with powdery mildew in different regions of Hainan, four representative rubber plantation farms were selected as sampling sites, namely, Baisha (BS), Danzhou (DZ), Wanning (WN), and Wuzhishan (WZS). For each plantation, leaf samples were collected in April 2021 (during the phenological stage when leaves appeared pale green) from infected rubber trees (diameter at breast height: 25–35 cm) diagnosed with a Level 3 powdery mildew infection (i.e., white spots covering more than 33% of the leaf area of the tree) (Qin et al., 2015).

The five-point sampling method—i.e., the diagonal line’s midpoint was first set as the central sampling point, then four equi-spaced additional points along it were set—was applied to collect samples from each rubber farm, using three trees per point, with four leaves from each tree placed in one sterile bag as a sample. Three replications were set and marked as _1, _2 and _3. Uninfected leaves of the same size (at Level 0) were collected from non-infected healthy trees using the same method to serve as a blank control (denoted as _CK).

The sample number, time, and place of collection, in addition to the latitude, longitude, altitude, and ambient temperature and humidity on the day of leaf sampling were recorded (Table S1).

Extraction of DNA from Phyllosphere Microbiota

1. First, 6.0 g of rubber leaves was transferred into a sterile conical flask containing 100 mL 0.1 mol/L phosphate buffer solution (pH=7.0), and then 100 μ L Tween 80 was added.
2. Each sample was shaken with ultrasonic oscillation for 8 minutes, and then shaken at a speed of 200 rpm for 30 minutes at 25°C.
3. After 2 minutes of ultrasonic oscillation, sterilized forceps were used to remove the leaves on the super-clean worktable.
4. The microorganisms in the conical flask were collected using suction filtration with a 0.45- μ m microporous membrane.
5. DNA was extracted from each sample using the MP Biomedicals FastDNA® Spin Kit for Soil (CA, USA).
6. The concentration and purity of DNA were verified using the NanoDrop 2000C (Thermo Scientific, USA) to $A_{260}/A_{280} = 1.8\text{--}2.0$, and stored at -20degC until use.

Amplification and Sequencing of 16S and Internal Transcribed Spacer (ITS) DNA of Phyllosphere Microbiota

Using the DNA of the sample as a template, the bacterial 16S rDNA gene and fungal ITS genes were polymerase chain reaction (PCR)-amplified with universal primers (Lee et al., 2012).

Primer sequences:

Primers	Sequences (5'-3')
16S-338F	ACTCCTACGGGAGGCAGCA
16S-806R	GGACTACHVGGGTWTCTAAT
ITS-1F	CTTGGTCATTTAGAGGAAGTAA
ITS-2043R	GCTGCGTTCTTCATCGATGC

PCR-amplified products were detected using 1.0% agarose gel electrophoresis. The ensuing products were purified and sent to Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) for MiSeq high-throughput sequencing.

Sequence Data Processing and Statistical Analysis

(1) The DNA matrix was calculated. The UPARSE program (Edgar, 2013) was employed to divide the operational taxonomic units (OTUs) and cluster all the sequences considered very similar (i.e., with a minimum 97% identity threshold). The RDP-Classifer software v2.11 (<https://sourceforge.net/projects/rdp->

classifier/) was used to classify and annotate the sequences of each leaf sample, with the confidence parameter set to 50%. Microorganisms corresponding to sequences with a genetic distance of less than 3% are generally considered to belong to the same microbiota (Wang et al., 2007).

(2) The data were homogenized, and the number of OTUs at different taxonomic levels (genus and phylum) were calculated and their rarefaction curves plotted. To assess the alpha diversity of sequences in each sample, the Chao1, Ace, Shannon, and Simpson indexes were used.

(3) Phyllosphere microbiota in leaf samples from different regions were subjected to principal component analysis (PCA) (Gewersand et al., 2021) and nonmetric multidimensional scaling (NMDS) multivariate analyses (the correlations used in the PCA were linear).

(4) A Kruskal–Wallis (KW) sum-rank test was performed using the linear discriminant analysis effect size (LEfSe) algorithm to compare the distribution of OTUs between the leaves of rubber trees in different regions.

RESULTS Basic Composition and Structure of the Phyllosphere Bacteria of Rubber Trees with Powdery Mildew

Four samples from each site region (BS, DZ, WZS, and WN), for a total of 16 tree-level leaf samples, were subjected to MiSeq high-throughput sequencing of the phyllosphere microbial DNA of hosts with powdery mildew. A total of 567,903 high-quality sequence fragments were read after processing the raw data, with the number of sequences in a single sample ranging from 30,261 to 44,775, with an average of 35,494. A total of 520 OTUs were detected in the four regions after implementing the quality control protocol. Across all regions, there were 36 overlapping OTUs, constituting 6.92% of the total. This indicated that, although all the samples were collected from a rubber powdery mildew phyllosphere environment, there were significant differences in OTU levels among the four regions (Figure 1).

The rarefaction curves showed differences in the diversity of the different sample phyla. The flattening of the rarefaction curves for each sample indicated that the amount of sequenced data was reasonably sufficient, with a robust sequencing depth encompassing nearly all phyla in the samples (Figure S1).

The community composition of each sample was tallied at various taxonomic levels, and a total of 24 phyla, 49 classes, 100 orders, 172 families, and 256 genera of bacteria were identified. Apart from WZS_3, the other samples were dominated at the phylum level by *Cyanobacteria*, *Poteobacteria*, and *Actinobacteria*. The dominant phyla in individual regions also included *Firmicutes* and *Bacteroidetes*, which had different relative abundances across regions (Figure 2-1, 2-2). Excluding WZS_3, in the 15 samples the relative abundance of *Cyanobacteria* ranged from 71.24% to 98.29%, that of *Proteobacteria* from 1.55% to 23.13%, and that of *Actinobacteria* from 0.82% to 1.23%. The relative abundances of *Cyanobacteria*, *Proteobacteria*, and *Actinobacteria* in WZS_3 were 0.04%, 28.92%, and 10.59%, respectively.

The dominant genera in BS were *Cyanobacteria_norank* (“norank” is used when there is no scientific name for this level in the taxonomic genealogy), *Mitochondria_norank*, and *Rhodococcus*; in DZ, *Cyanobacteria_norank*, *Mitochondria_norank*, and *Curtobacterium* dominated; in WN, *Cyanobacteria_norank*, *Pseudomonas*, and *Pantoea* dominated; and in WZS, *Cyanobacteria_norank*, *Rhodococcus*, and *Pantoea* dominated (Figure 2-1, 2-2). In these regions, the most dominant communities in healthy rubber tree leaves (Level 0) consisted of *Cyanobacteria_norank*, and likewise for the dominant communities of infected leaves (Level 3). The relative abundances (mean values) are shown in Table S2. Among subdominant genera, the subdominant genera of both uninfected and Level 3-infected leaves in BS were *Mitochondria_norank* and *Rhodococcus*; the subdominant genera in DZ were *Mitochondria_norank* (uninfected leaves) and *Curtobacterium* (Level 3-infected leaves); the subdominant genera in WN were *Mitochondria_norank*, *Bacillus* (uninfected leaves), *Pseudomonas*, and *Pantoea* (Level 3-infected leaves); and the subdominant genera in WZS were *Rhodococcus* (uninfected leaves) and *Acidobacteria_norank* (Level 3-infected leaves). The relative abundances of subdominant genera in healthy leaves and Level 3 leaves from the four regions are listed in Table S2.

In summary, the composition of the dominant genera varied little between healthy leaves and Level 3-infected leaves. Although other dominant genera were different among regions, their relative abundances were not

high. Therefore, the composition of genus-level communities of the three levels of infected and healthy leaves of rubber trees at the same growing site was similar, except for some differences in members' relative abundances, while community composition at the genus level differed markedly among regions.

Diversity Index Analysis of Phyllosphere Bacteria of Rubber Trees with Powdery Mildew

The member abundance and diversity of the phyllosphere microbiome community were studied by analyzing the diversity of individual samples (alpha diversity) using various statistical indexes (Table S3).

The OTU statistics were analyzed at a 97% similarity level, and the OTU numbers for the 16 samples ranged from 24 to 324. Using the Ace index and Chao index, the total number of species in the phyllosphere microbiome was estimated to be 34–329 and 29–329, respectively. The Shannon index and Simpson index estimated samples' microbial diversity at 0.14–5.09 and 0.01–0.96, respectively. The coverage of the 16 samples exceeded 99%, confirming that the sequencing results accurately represented the microorganisms in rubber tree leaves. Despite their distinct algorithms, the Ace index and Chao index generally yielded consistent results and did not differ considerably in terms of measured OTU numbers. Both the Shannon index and Simpson index revealed a ranking of WZS > WN > DZ > BS for the alpha diversity in the microbiota of the four regions.

PCA and NMDS Multivariate Analyses of Phyllosphere Bacteria in Leaf Samples from Different Regions

PCA indicated that the microorganism composition of DZ and BS samples was similar, likely due to their geographical proximity to each other. The samples from WN and WZS were clearly distinguishable from each other, except for the WZS_3 sample, which was more dispersed but still close to clustering (Figure 3). These results indicated that bacterial communities differed significantly between the four regions, which suggests that different geographical locations are influential in accounting for the variation in community composition and structure of phyllosphere bacteria in rubber tree leaves.

NMDS showed that, except for the WZS_3 sample, samples that had close geographical proximity or similar climates shared more similarities in composition, given the clustering found (Figure 3). For example, the DZ and BS sites are a short distance apart, and both have a dry climate, whereas the WN and WZS sites are nearer to each other and have a wet climate. These results further suggest that different geographical locations or climate types can lead to the separation of distinct microbial communities, and that samples with geographical proximity or similar climate types can generate prominent clustering results.

Analysis of Phyllosphere Bacteria Taxa LEfSe in Samples from Different Regions

A KW sum-rank test was performed using the LEfSe algorithm to compare the distribution of OTUs between the leaves of rubber trees in different regions. These results showed that five OTUs were distributed in BS, three in DZ, five in WN, and 24 in WZS. *Cyanobacteria* were the most abundant taxon in BS, Oxalobacteraceae in DZ, Pseudomonadales in WN, and both *Proteobacteria* and *Acidobacteria* in WZS (Figure 4).

The plotted cladogram analysis also revealed which community taxa had significantly different impacts on distinguishing samples by region (Figure 5). These taxa were *Cyanobacteria* in BS, *Methylobacterium* in DZ, *Firmicutes* in WN, and *Proteobacteria* and *Acidobacteria* in WZS.

Correlation Analysis Between Clustering of Phyllosphere Bacterial OTUs and Environmental Factors

Correlations between the environmental factors (temperature, humidity, and altitude) at each sampling location and their OTUs were calculated and tested. The OTUs and environmental factors clustered separately (Figure S2). These results confirmed that OTU280, OTU257, OTU146, OTU27, OTU437, OTU196, OTU385, OTU30, and OTU510 were significantly correlated with temperature and clustered into one large branch; OTU430 was significantly correlated with temperature and humidity and was in this large branch as well; and OTU144 was significantly correlated with temperature and humidity, but was in a separate

branch. Altitude had no effect on the OTUs. Notably, OTU144 was highly correlated with temperature and humidity, OTU196 and OTU30 were highly correlated with temperature alone, while OTU 430 was moderately correlated with humidity alone.

Basic Composition and Structure of the Phyllosphere Fungal Community of Rubber Trees with Powdery Mildew

A total of 593,237 high-quality sequence fragments were obtained from the 16 samples of rubber tree powdery mildew fungi via MiSeq high-throughput sequencing. The number of sequences in a single sample ranged from 30,129 to 44,619, with an average of 37,077, and a total of 1406 OTUs were detected. Their average sequence length was 293.01 bp, with 57.38% of the sequences being 301–320 bp in size and 26.99% being 241–260 bp. The samples' dilution curves showed differences in their fungal diversity. The general flattening of these dilution curves indicated that the amount of sequenced data was robust, and that the depth of sequencing largely covered all species in the rubber tree leaf samples (Figure S1).

A total of 1398 OTUs were detected in the four regions after quality control, with a ranking of WZS > WN > BS > DZ (Figure 1). There were 90 overlapping OTUs across the four regions, accounting for 6.44% of the total number of OTUs in all pooled samples. This indicated that although the samples were all sourced from the rubber powdery mildew phyllosphere, there were stark differences in OTU levels among the four rubber tree-growing regions.

The community composition of each sample was calculated at different taxonomic levels, for which a total of six phyla, 26 classes, 69 orders, 157 families, and 351 genera of fungi were identified. At the phylum level, the community composition of samples from all four regions was similar, with *Ascomycota* and *Basidiomycota* dominating, though their relative abundances varied across regions (Figure 2-1). The relative abundance of *Ascomycota* ranged from 43.76% to 99.80%, and that of *Basidiomycota* ranged from 0.17% to 10.91% in the 16 samples (Figure 2-2).

The dominant genera in all four regions consisted of *Erysiphe* and *Cladosporium*. In addition, the dominant fungal taxa of the regions' healthy leaves differed (Figure 2-2). The dominant genera in the Level 3 leaves of the samples from the four regions are listed in Table S4. In addition, the healthy leaves (Level 0) and Level 3 leaves in the four regions also included a number of less abundant community members. The relative abundance of fungi (others) whose taxonomic status was unclear or not yet identified ranged from 1.04% to 10.58% (Table S4).

In summary, apart from WZS, the dominant fungal genera in the other three regions for both Level 3-infected leaves and healthy leaves consisted of *Erysiphe* and *Cladosporium*, with one to three different subdominant genera per region, albeit all at low relative abundances. Therefore, the two levels of infected and healthy leaves of rubber trees growing at the same site differed in the relative abundance of phyllosphere microbiome but not in the genus-level community composition, whereas the latter varied across the four regions. Although powdery mildew had little effect on the structure of the phyllosphere fungal community, it did affect the relative abundance of each community.

Diversity Index Analysis of the Phyllosphere Fungal Flora of Rubber Trees with Powdery Mildew

The fungal OTU statistics were analyzed at the 97% similarity level, and the OTU numbers for the 16 samples ranged from 71–692. As shown in Table S5, the Ace and Chao indexes estimated the total number of species to be 121–723 and 120–713, respectively. Both the Shannon and Simpson indexes indicated that the alpha diversity of fungal communities in the leaves of the four regions was ranked as follows: WZS > BS > DZ > WN.

LEfSe Analysis of Phyllosphere Fungal Taxa in Leaf Samples from Different Regions

A KW sum-rank test was applied using the LEfSe algorithm to compare the distribution of OTUs between the leaves of rubber trees in different regions. These results revealed 11 OTUs that were exclusively distributed

in BS, three OTUs that were exclusively distributed in DZ, 22 OTUs that were exclusively distributed in WN, and three OTUs that were exclusively distributed in WZS. *Trichosphaeriales* was the most abundant taxon in BS, *Sporidiobolaceae* was the most abundant taxon in DZ, both *Dothideales* and *Dothioraceae* were the most abundant taxa in WN, and *Chaetomiaceae* was the most abundant taxon in WZS (Figure 4). The plot cladogram results also revealed the community members that significantly influenced the delimitation of samples in different regions (Figure 5).

Hierarchical Clustering Tree of Phyllosphere Fungal Samples from Different Regions

A hierarchical clustering tree was constructed based on a beta diversity distance matrix to compare similarities and differences among multiple samples (Figure 6). The results showed that the community composition of the WN_1, WN_2, and WN_3 samples was similar, and clustered into a single branch. The similarity between WZS_1 and WZS_2 was also high, and both DZ_1 and DZ_2 clustered together. Clearly, the similarity of the community composition of the WZS-CK, WN-CK, BS-CK, and DZ-CK samples was not high, and the similarity with the other three samples in the region was low, with significant differences.

Correlation Analysis of OTU Clustering of Leaf Fungi with Environmental Factors

The environmental factors (temperature, humidity, and altitude) sampled at each location were tested to determine their correlation with OTUs, and the OTUs and environmental factors clustered separately (Figure S2). OTU1391, OTU202, OTU953, OTU985, OTU1073, and OTU769 were significantly correlated with temperature and humidity and clustered into one group; OTU420, OTU419, OTU173, and OTU856 were significantly correlated with temperature and clustered into one branch; OTU652, OTU758, OTU1289, and OTU12 were significantly correlated with altitude and clustered into one group; OTU223, OTU76, OTU1066, and OTU96 were significantly correlated with altitude, forming one cluster; and OTU919 was significantly correlated with temperature. Notably, among them, OTU76 and OTU1066 were strongly correlated with altitude, OTU1391 was strongly correlated with humidity, and OTU420, OTU856, and OTU1073 were strongly correlated with temperature.

DISCUSSION

Much research has focused on the relative macroscopic behavior of phyllosphere microorganisms, and on the behavior of phyllosphere microorganisms when non-specific parasitic bacteria infest plants. However, there are no reports on obligate parasitic bacteria. In work by Liu et al. (2022) on the community composition of phyllosphere microorganisms of female and male ash trees (*Fraxinus*), the core phyllosphere microbiota was consistent, with the dominant phyla being *Cyanobacteria* and *Proteobacteria*; however, some differences were identified at the genus level, with the genus *Arsenophonus* present in female ash only. As in the present study, Guo (2009) collected samples from several sites and found that the phyllosphere microorganisms of *Populus tremula* mainly consisted of bacteria, followed by actinomycetes and fungi; however, the populations and numbers of phyllosphere bacteria differed between sites. Recently, by analyzing the structure of the phyllosphere fungal communities of dominant tree species in broadleaf red pine forests in Changbai Mountains, Wang et al. (2022) found that the dominant phyla were *Ascomycota* and *Basidiomycota* and the dominant classes were *Dothideomycetes* and *Taphrinomycetes*, and that the canopy phyllosphere fungal communities of the six dominant tree species differed significantly in composition. Some researchers have also explored the complexity in host-microbe interactions and suggested that the rational design and use of microbial tools, such as phyllosphere probes, have broad potential for applications in economic production activities, such as agriculture, viticulture, forestry, and biofuels, and even for improving human health (Whitaker et al., 2019).

Sampling was based on the fact that geographical location and its relative environment are decisive factors in the community composition of interstitial microorganisms. Therefore, samples were collected from four representative areas in Hainan Province, China: WN in eastern Hainan, WZS and BS in central Hainan, and DZ in western Hainan. Sample WZS_3 from Wuzhishan was markedly different when compared with sample WZS_1 or WZS_2, and the sequencing results of its bacterial community composition showed that a portion of the microorganisms came from the soil, which might be related to the changeable climate in the field. It

was difficult to distinguish whether the samples were affected by the nearby environment before sampling.

Only Level 3-infected leaves were collected because under field conditions, Level 3 infections are the most common, Level 4 (entire leaf covered with spots) infections mostly occur in greenhouse conditions, while Level 2 (scattered spots on the surface) infections normally occur on older leaves, or when the powdery mildew spots have faded, and only form at the beginning or at the end of the powdery mildew period, when the leaves age faster under higher temperatures. In addition, there were differences in the relative abundances of the communities of Level 3-infected leaves in each region, which might be associated with the number of environmental influences in the field. As a result, the mean values were taken for comparison with uninfected healthy leaves (Level 0).

In this study, state-of-the-art molecular biotechnology was used to investigate the phyllosphere microorganisms of rubber trees with powdery mildew, to identify their dominant populations at the phylum and genus levels, and to determine whether the incidence of powdery mildew disease affected the phyllosphere microbial community structure and the relative abundances of their taxa members. Because one plantation site was sampled per region, the following inferences about regional differences were made with caution.

Structure and Diversity of the Phyllosphere Bacterial Community of Rubber Tree Powdery Mildew

The dominant community at the phylum level was *Cyanobacteria*, with a relative abundance ranging from 71.24% to 98.29%. This dominance can presumably be explained by the strong viability of *Cyanobacteria* and their strong symbiosis with some eukaryotes. Because of its widespread presence on the leaf surface of rubber trees, this paper speculates that genera of this bacterial phylum may have colonized the leaf surface via wind dispersal and then multiplied rapidly, thereby fixing nitrogen to help replenish the leaf with nitrogen. In the long term, *Cyanobacteria* has evolved in a mutually beneficial symbiosis with host leaves, enabling it to occupy an overwhelmingly dominant position in the phyllosphere bacterial community. The increased nitrogen supplies more nutrition for the leaf, which then provides a better parasitic environment for the causal agents of powdery mildew and allows the three actors—the rubber tree leaf, *Cyanobacteria*, and *Erysiphe*—to coexist peacefully.

The genus-level community composition and relative abundance of healthy leaves and Level 3-infected leaves at the same site were not significantly different, while community composition and relative abundance at the genus level were significantly different among the four representative regions. This could suggest that *Erysiphe* had little effect on the overall phyllosphere bacterial community structure, though they may affect the relative abundances of the members of each community. The correlation between OTUs and environmental factors in the four regions demonstrated that certain OTUs were significantly correlated with the ambient temperature and humidity, while the altitude had a negligible influence on OTUs.

Structure and Diversity of Phyllosphere Fungal Communities of Rubber Tree Powdery Mildew

The dominant phyla in all four regions were *Ascomycota* and *Basidiomycota*. At the genus level, *Erysiphe* and *Cladosporium* were dominant, with differing subdominant genera between regions.

Erysiphe was still present in healthy leaves, and so it is speculated that they persist in healthy leaves which do not develop the disease. Compared with these healthy leaves, the relative abundance of *Erysiphe* on Level 3-infected leaves was significantly higher in all four sampled regions. The relative abundance of *Cladosporium* increased significantly in DZ and decreased significantly in the other three regions. In both BS and WN, the abundance of most fungal taxa declined as *Erysiphe* became more abundant, which suggested competitive interactions between them.

The fungal community composition and relative abundance varied significantly at the genus level among regions; however, it was similar between Level 3-infected and healthy leaves within the same region. This suggested that powdery mildew did not affect the structure of fungal communities but only the relative abundance of taxa members in each community.

The taxonomic status of many bacteria and fungi in this study has not been identified, so further analysis of the specific relationships between powdery mildew and these taxa is challenging. Advances in biotechnology tools will eventually lead to the development and utilization of these difficult-to-cultivate microorganisms, enabling further research.

Innovation

1. Studies on rubber tree powdery mildew infections and the causal pathogenic bacteria are common in the literature. However, research using high-throughput detection techniques to determine the dominant taxa in the community of phyllosphere microorganisms on rubber trees with powdery mildew, and the effect of powdery mildew on these phyllosphere microorganisms, have not yet been reported. This study did so for the first time in order to provide data support for more effective new ideas for the biocontrol of powdery mildew in rubber tree plantations.
2. Most previous reports investigated the relationship between powdery mildew and hosts from a molecular perspective, while this work assessed the influence of pathogenic bacteria on the phyllosphere microorganisms of host leaves from a relatively macroscopic perspective. The results confirm that there is little, if any, difference between the phyllosphere microorganism populations of diseased leaves (Level 3) and healthy leaves at the same plantation site. Based on this finding, it can be inferred that phyllosphere microorganisms may exist in a satisfactory symbiotic relationship with the host plant as well as its pathogenic bacteria. The findings of this study can also confirm, from a fresh perspective, that *Erysiphe* is an obligate parasitic fungi. In the hostile phyllosphere environment, phyllosphere microorganisms need to maintain robust symbiotic associations with hosts and pathogens to survive and evolve, thus leading to a relatively stable evolutionary relationship between rubber trees, *Erysiphe*, and their attached phyllosphere microorganisms in tropical and subtropical regions. In the future, this research team will further investigate the interactions between the host, host pathogens, and phyllosphere microorganisms.
3. Few studies have evaluated the effects of foliar pathogens on the phyllosphere microbial community before and after plant infestation, and even fewer studies have examined the impact of obligate parasitic fungi on phyllosphere microorganisms. For example, Dai (2022) investigated the microbial diversity of tobacco leaves infested with *Streptomyces* and concluded that the relative abundance of *Alternaria* in healthy leaves increased from the first to the third collection, while the relative abundances of *Pseudomonas*, *Sphingomonas*, and *Methylobacterium* from the same location increased as leaves aged. Huang et al. (2021) reported on the phyllosphere microbial composition and diversity of tobacco leaves infested with subseptate strains of *Phytophthora* spp. and found that the relative abundances of *Didymella* and *Boeremia* (*Ascomycota*) fungi and *Pseudomonas* and *Pantoea* (*Ascomycota*) bacteria were higher in diseased leaves than in healthy leaves, with healthy tissue harboring a richer and more diverse fungal community. The above-mentioned studies uncovered consistent changes in the abundance and species of microorganisms in the phyllosphere after the pathogenic infection of their hosts. The present study is the first to gauge the effect of the powdery mildew's obligate parasitic fungi, *Erysiphe*, on the phyllosphere microorganisms of rubber trees in representative plantation areas in China.

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Figure legends

Fig. 1. Venn diagram illustrating the number of unique and shared OTUs (operational taxonomic units) from the bacterial (a) and fungal (b) community in the phyllosphere of rubber

trees

a. A total of 520 OTUs were detected in the four regions: 102 OTUs in BS, 113 OTUs in DZ, 81 OTUs in WN, and 479 OTUs in WZS. The proportions of unique OTUs in the four regions were as follows: 3.27% in BS, 1.54% in DZ, 0.77% in WN, and 67.69% in WZS. Across all regions, there were 36 overlapping OTUs, constituting 6.92% of the total.

b. A total of 1398 OTUs were detected in the four regions: 420 OTUs in BS, 295 OTUs in DZ, 500 OTUs in WN, and 875 OTUs in WZS. The proportions of unique OTUs in the four regions were as follows: 11.09% in BS, 1.93% in DZ, 13.23% in WN, and 44.49% in WZS. There were 90 overlapping OTUs across the four regions, accounting for 6.44% of the total number of OTUs in all pooled samples.

This indicated that although the samples were all sourced from the rubber powdery mildew phyllosphere, there were stark differences in the OTU levels among the four regions.

Fig. 2-1. Heat maps of the relative abundances of bacterial and fungal community members in the rubber phyllosphere at the phylum and genus level

Blue blocks indicate high correlation, green blocks indicate medium correlation, and the lighter-colored blocks indicate low correlation.

a. Heat map of the relative abundance of bacterial community members in rubber phyllosphere at the phylum level.

b. Heat map of the relative abundance of bacterial community members in rubber phyllosphere at the genus level.

c. Heat map of the relative abundance of fungal community members in rubber phyllosphere at the phylum level.

d. Heat map of the relative abundance of fungal community members in rubber phyllosphere at the genus level.

Fig. 2-2. Relative abundances of bacterial and fungal community members in the rubber phyllosphere at the phylum and genus level

a. The relative abundance of bacterial community members in rubber phyllosphere at the phylum level.

b. The relative abundance of bacterial community members in rubber phyllosphere at the genus level.

c. The relative abundance of fungal community members in rubber phyllosphere at the phylum level.

d. The relative abundance of fungal community members in rubber phyllosphere at the genus level.

Fig. 3. Principal component analysis (PCA) and non-metric multi-dimensional scaling (NMDS) plots of the bacterial community in the rubber phyllosphere

a. PCA plot of the bacterial community in the rubber phyllosphere. PC1 contributed the most significantly (91.93%), whereas PC2 explained just 4% of the variation.

The DZ and BS samples (closer geographically close) are clustered together in the PCA, while the WN and WZS samples (farther geographically) can be clearly distinguished from them. There are significant differences in bacterial communities among the different regions.

b. NMDS of the bacterial community in the rubber phyllosphere. Except for WZS_3 samples, samples with a closer geographical distance or similar climate type had higher composition similarity and tended to cluster together.

Fig. 4. Linear discriminant analysis effect size (LEfSe) of differences in bacterial and fungal OTUs in the rubber tree phyllosphere in different regions

- a. LEfSe of differences in bacterial OTUs in the rubber tree phyllosphere in the four sampled regions.
- b. LEfSe of differences in fungal OTUs in the rubber tree phyllosphere in the four sampled regions.

The threshold on the logarithmic LDA score for discriminative features was set to 2.0. BS = Baisha, DZ = Danzhou, WN = Wanning, WZS = Wuzhishan.

Fig. 5. Analysis of bacteria (a) and fungi (b) with significant effects in different regions

The plotted cladogram results revealed those community members which significantly influenced the delimitation of samples in the four regions.

Different circles represent different classification levels. Levels from the inside to the outside represent phylum, class, order, and family in turn. Yellow nodes are groups that have no important role, and other color nodes are groups that play an important role in the corresponding region.

Fig. 6. Hierarchical clustering tree of the fungal community in the rubber phyllosphere

The branch length represents the phylogenetic distance between samples; the more similar the samples, the closer they are positioned to each other. Branches with the same color represent the same location of sampled rubber leaf tissue.

Competing Interests Statement

The authors declare no conflict of interest.

Author Contributions

Weiguo Miao: Conceptualization (lead); writing – review and editing; Resources. **Jiaxin Shan:** Writing – original draft (lead); Writing – review and editing (equal); Investigation (equal); Data Curation (equal). **Hua Wu:** Writing – original draft (equal); Investigation (equal); Data Curation (equal). **JinYao Yin:** Writing – review and editing (equal); Methodology (equal). **Xiao Li:** Formal Analysis (equal); Methodology (equal). **Chunhua Lin:** Resources (equal).

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Data Availability Statement

This manuscript has no associated data.

Hosted file

Tables.docx available at <https://authorea.com/users/754611/articles/724482-the-effects-of-erysiphe-quercicola-inoculation-on-the-phyllosphere-microbiome-of-heveae-brasiliensis-in-hainan-province-china>









