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Analysis of the difference in endophytic bacterial community structure between infected and non-infected rice plants with rice bakanae disease

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Abstract. *Yu Y, Liu H, Xu Z, Yan P, Wu H, Wu L. 2024. Analysis of the difference in endophytic bacterial community structure between infected and non-infected rice plants with rice bakanae disease. Biodiversitas 25: 197-204.* Rice bakanae disease, caused by the main pathogen *Fusarium fujikuroi*, is an important fungal disease that seriously threatens rice production. This study analyzed the effect of *Fusarium fujikuroi* on the community structure and diversity of endophytic bacteria in different rice tissues. The total DNA of different tissues, such as root, stem, and leaf, was extracted after artificial inoculation, and the bacterial 16SrRNA gene library was constructed. The high-throughput sequencing was used to analyze the biological information of bacterial community composition, diversity and function. The results showed that a total of 467841 effective sequences were obtained. Alpha diversity analysis showed that the diversity and richness of the endophytic bacterial community in infected plants were higher than in non-infected plants. At the phylum level, *Cyanobacteria*, *Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Actinobacteria* were the dominant phyla. The infection of rice seedling bacteria causes changes in the abundance of endophytic bacteria in rice. Compared with non-infected plants, the abundance of *Streptophyta* and *Acidovorax* in the root decreased by 1.29 and 0.74%, respectively. The abundance of *Geobacter*, *Devosia*, *Pleomorphomonas*, and *Herbaspirillum* increased by 1.05, 1.55, 1.28, and 1.76%, while the abundance of *Streptophyta* in stem decreased by 7.83%. The abundance of *Xanthomonas*, *Acidovorax*, *Pseudomonas* and *Sphingomonas* increased by 1.55, 1.49, 1.11 and 1.15%, whereas the abundance of *Streptophyta* in the leaf decreased by 12.03%, *Actinomycetales*, *Pseudomonas*, *Sphingomonas* are significant biological differences between different tissues of infected and non-infected plants. The study provides a theoretical basis for the biological control of rice bakanae disease.

Keywords: Community structure, endophytic bacteria, *Fusarium fujikuroi*, rice bakanae disease

INTRODUCTION

Rice bakanae disease is a fungal disease caused by the main pathogenic pathogen *Fusarium fujikuroi*, also known as chronic disease or male rice, and it is one of the most common diseases of rice (Matić et al. 2021). The disease symptoms at the seedling stage are elongated seedlings, slender stems, yellow-green leaves, and poor root development. Seeds with serious bakanae disease do not germinate or die after germination (Raghu et al. 2023). The symptoms of the plant growth stage are similar to those of seedling stage: the rice becomes thin and tall, leaf color becomes light, leaf angle becomes larger, tillers reduce, or no-tillers, stem or internodes elongate, white to yellow adventitious root grows at the stem section (Krishnan et al. 2019), stem stalk gradually rots, leaves dry from top to bottom, ears become smaller, and the seed setting rate decreases and even dies. Rice bakanae disease generally leads to 10-20% or more than 50% yield loss (Sharma et al. 2022; Bashyal 2018). In addition, mycotoxins, such as fumarin and fususin produced by pathogenic bacteria are toxic to both humans and animals (Elamawi et al. 2020). Heilongjiang province is an important high-quality rice-producing area in China. In recent years, the incidence of rice bakanae disease is increasingly serious in Heilongjiang due to the lack of disease-resistant variety and long-term use of a single agent control. The disease causes huge losses to rice production, and the incidence of disease frequency is rising, easy to cause a large area of spread, it becomes the limiting factor for good quality and high yield of rice in Heilongjiang province, so it's urgent to study rice bakanae disease (Yadav et al. 2020).

Endophytes are microorganisms that both compete with the host and coexist harmoniously (Bertani et al. 2016; Ansari and Ahmad 2019). They reproduce by absorbing nutrients provided by the host to produce secondary active substances, such as antibiotics and hormones to promote host growth and increase host immunity (Banik et al. 2019; Hu et al. 2018). Endophytic bacteria have complex community structures and dynamic change characteristics; plant endophytic bacterial community structure and relative abundance affect the severity of disease (Sarwar et al. 2018). It was found that community structure and diversity of endophytic bacteria in bananas were closely related to plant health. The bacterial community structure and diversity of different parts of healthy tobacco plants and tobacco

plants infected with *Ralstonia solanacearum* was analyzed, compared with healthy tobacco plants, the bacterial community abundance and diversity of rhizosphere soil and stem samples of tobacco plants infected with *Ralstonia solanacearum* increased to varying degrees (Walitang 2023). In recent years, rice bakanae disease has occurred seriously in Heilongjiang Province, so breeding disease-resistant varieties and promoting green prevention and control technology is urgent. The biological control of this disease mainly focuses on screening biological bacteria; the effect of rice bakanae disease infection on the community structure and diversity of endophytic bacteria in rice has not been reported. The aim of this study was to used high-throughput sequencing to analyze the community structure and diversity of endophytic bacteria in different tissues of non-infected rice and infected rice and to explore the regulation of endophytic bacteria on disease resistance of rice. This study can provide a theoretical basis for green prevention and control of rice bakanae disease.

MATERIALS AND METHODS

Study area

The experiment was carried out in the long-term field test site of FSR in Minle Country, Wuchang City, Heilongjiang Province (E127°03′, N45°03′, 450 m above sea level) in 2021. The soil was sandy loam, with organic matter content of 81.6 $g.kg^{-1}$, hydrolyzable nitrogen of 228.0 $mg.kg^{-1}$, available phosphorus of 43.5 $mg.kg^{-1}$, available potassium of 106 $mg.kg^{-1}$, and pH of 7.28.

Experimental materials

The local main variety “Wuyoudao 4” was used in the experiment. It was found to be suitable for the first accumulated temperate zone in Heilongjiang Province.

Procedures

Preparation of conidium suspension

Fusarium fujikuroi was provided by the Crop and Livestock Molecular Breeding Laboratory of Heilongjiang and cultured on potato sucrose medium at 28°C for 5 days. The spores were washed with sterile water and filtered by sterile gauze to prepare a spore suspension (200 spores at 8×15 times the microscopic field of view).

Artificial inoculation

Rice seeds were soaked in 50% carbendazin 500 times solution for 3d to sterilize, washed with water 3 times, then germinated at 32°C for 24 hours to reach grain length following the method of Li et al. (1994) with slightly modification. The neat bud valleys were selected and inoculated for 3 h by soaking in spore suspension and were seeded in the seedling bed after inoculation. When the rice seedlings reached the 3.5 leaf stage, the seedlings were transplanted to the field where rice bakanae disease occurs throughout the year. The row length of the planting area was 8 m, planted four lines were planted; the transplanting specification was 30.0×13.3 cm, single plant implantation, 3 repeats. No fungicide was applied during the whole growth period. Other field management was the same as the local general field management, with sterilization without inoculating pathogenic bacteria as a non-susceptible control.

Sample collection

Healthy and infected rice plants were taken respectively according to the five-point sampling method on 13 July 2023. After three repetitions, sample were brought to the laboratory to gently wash off the root mud, then sterilized with 75% ethanol, and washed with sterile water. The roots, stems, leaves, and panicles were separated with sterilized scissors. The treatment settings were HR: healthy rice root; HS: healthy rice stem; HL: healthy rice leaf; DR: infected rice root; DS: infected rice stem; DL: infected rice leaf. Samples were stored at -80°C for endophytic diversity analysis.

Surface sterilization

Referring to the method of Liu et al. (2018), different tissues of rice roots, stems, leaves, and panicles were rinsed with running water for 10 min. Then, they were dried with sterile paper and treated with 75% absolute ethanol for 4 min. After washing with sterile water, they were soaked with 3% H_2O_2 for 3 min, then rinsed with sterile water 3-4 times. Finally, the endophytic bacterial DNA was extracted under sterile conditions after the last sterile water-washing solution was tested to be sterile.

Genomic DNA extraction, amplification and sequencing

Total DNA was extracted using OMEGA E.Z.N. ATMMag-Bind Soil DNA Kit, DNA integrity was detected by 1% agarose gel electrophoresis, concentration and purity were determined by Thermofisher Qubit3.0 DNA detection kit. PCR amplification was performed using bacterial V3-V4 region universal primers 341F (5'-CCTACGGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGGTATCTAATCC-3'). PCR reaction system consisted of (30μL): 15 μL 2×PCR Master Mix, 1 μL Bar-PCR primer F, 1 μL Primer R, 1 μL DNA template, ddH_2O add up to 30 μL. Steps of amplification procedure were: 94°C 3 min, 94°C 30 s, 45°C 20 s, 65°C 30 s, 5 cycles, 94°C 20 s, 55°C 20 s, 72°C 30 s, 20 cycles, finally 72°C extension for 5 min. The amplification products were detected by electrophoresis on a 2% agarose gel and recovered using the catSK8131 Agarose Recovery Kit. The PCR recovered products were sent to Shanghai Sangong Bioengineering Co. Ltd. for high-throughput sequencing by Illumina MiSeq2x300bp platform.

Data analysis

The effective sequence was obtained by data splitting; PE Reads splicing, Tags filtering, and Tags removing chimera sequences of the original sequence. Then, OTUs (operational taxonomic units) clustering and species classification analysis were performed based on the valid data, and OTU and species annotation were combined. Finally, the abundance and diversity indexes of endophytic bacteria OTUs at various taxonomic levels were analyzed using Mothur software.

RESULTS AND DISCUSSION

Quality analysis of sequencing

A total of 477080 raw sequences were measured from six rice plant samples, and the bases with mass values below 20 in the tail of the read were removed. After splicing and filtering, 467841 valid sequences were obtained. The effective sequence length was concentrated in the range of 406-413 bp, with an average length of 408 bp (Table 1).

Alpha diversity analysis

The coverage index of each treatment was more than 99%, indicating that the sequencing data can represent the real situation of the sample and adequately reflect the diversity of endophytic bacterial community. Diversity indexes "Shannon and Simpson" showed that the diversity of endophytic bacterial community in infected plants was higher than that in non-infected plants. The abundance indexes "Chao and ACE" showed that the abundance of endophytic bacterial community in infected plants was higher than that in non-infected plants. The Shannon evenness index showed that the evenness of the endophytic bacterial community in infected plants was higher than in non-infected plants (Table 2).

Classification and analysis of different bacterial populations

The population classification shows that the OUT obtained from different tissues of infected and non-infected plants belonged to 9 taxonomic bacterial phyla, 13 taxonomic bacterial classes, 15 taxonomic bacterial orders, 14 taxonomic bacterial families, and 9 taxonomic bacterial genera. The total number of bacteria at all levels in different tissues was also significantly different. The total number of endophytic bacteria in infected and non-infected roots was higher, followed by infected and non-infected stems, and the least in leaves (Table 3).

OUT distribution of endophytic bacteria

Venn diagram was used to count the common and unique OUT numbers of samples and intuitively show the similarity and overlap of OUT number composition of environmental samples. There were 178 OUTs from 6 different infected and non-infected rice tissues, accounting for 60.96% of the total OTUs in the library. The number of unique species in the root of infected and non-infected rice was 98 and 13, and 2 and 1 stem, respectively. The results showed differences in endophytic bacteria between infected and non-infected rice plant tissues (Figure 1).

Analysis of the structural composition of the community

Phylum level composition

There were 7 phyla (Figure 2) at the phylum level, including *Cyanobacteria*, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Acidobacteria*. There were differences in the dominant phyla of different tissues. *Cyanobacteria*, *Proteobacteria*, and *Actinobacteria* in non-infected root were dominant phyla with an abundance of 51.76, 14.18, and 2.18%, respectively. The dominant bacteria in the stems and leaves of the non-infected plant were *Cyanobacteria*, with an abundance of 72.10 and 90.93%, respectively. For the infected plant, *Cyanobacteria*, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* were dominant phyla with an abundance of 45.09, 23.95, 4.06, and 2.45%. *Cyanobacteria* and *Proteobacteria* in the infected stem were dominant phyla with an abundance of 62.13 and 10.32%, while the abundance of *Cyanobacteria* was 78.99% in the leaf as the dominant phyla (Figure 2).

Genus level composition

There were 14 genera with relative abundance ≥0.1% in the panicle, of which 7 can be accurately classified. In infected and non-infected plants, the abundance of *Streptophyta* was 44.87 and 51.73%, respectively, which was the dominant genus, the abundance of *Acidovorax* was 0.44 and 1.12% respectively, the abundance of *Geobacter* was 1.49 and 0.79% respectively, and the abundance of *Devosia* was 1.53 and 0.42% respectively, the abundance of *Pseudomonas* was 0.36 and 0.31% respectively, the abundance of *Sphingomonas* was 0.18 and 0.17%, respectively, the abundance of *Pleomorphomonas* was 1.14 and 0.20%, respectively, and the abundance of unclassified and other genera in infected and non-infected plants were 48.67 and 45.25% respectively.

Table 1. Statistics of sample sequence length

Samples	Number of raw sequences (strip)	Number of valid sequences (strip)	Average length of sequence (bp)
HR	71633	71309	411
HS	77506	76502	406
HL	84052	78223	406
DR	73104	72505	413
DS	77284	76974	408
DL	93501	92328	406
Total	477080	467841	408

Table 2. Alpha diversity index

Samples	Shannon	Chao	Ace	Simpson	Shannon Evenness	Coverage（%）
HR	2.366±0.595	1042.112±91.029	1049.886±78.840	0.324±0.085	0.348±0.085	99.6
HS	0.682±0.025	373.821±50.292	480.694±93.828	0.592±0.017	0.127±0.004	99.8
HL	0.360±0.163	324.398±20.243	352.652±48.796	0.840±0.101	0.068±0.031	99.9
DR	3.112±0.631	1044.234±63.105	1055.095±56.080	0.236±0.088	0.454±0.091	99.7
DS	1.244±0.869	399.112±60.762	419.255±48.769	0.452±0.185	0.220±0.145	99.8
DL	0.612±0.064	374.702±17.820	411.107±75.671	0.663±0.038	0.111±0.011	99.9

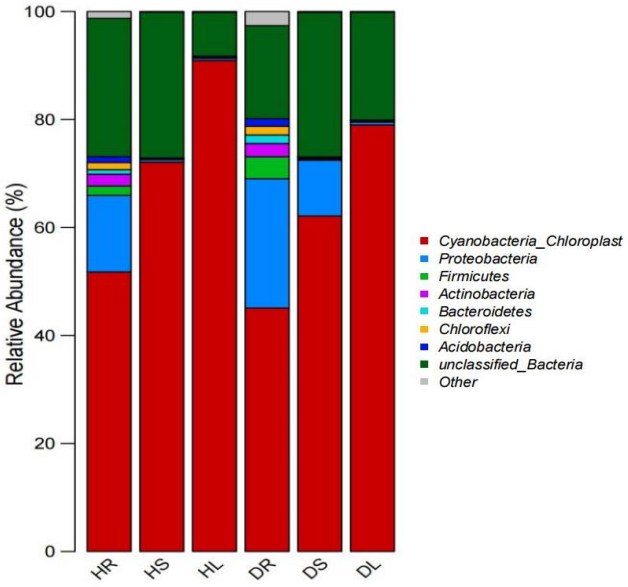

Figure 2. Composition of dominant bacterial phyla of endophytic bacterial communities in different tissues of infected and non-infected rice plants

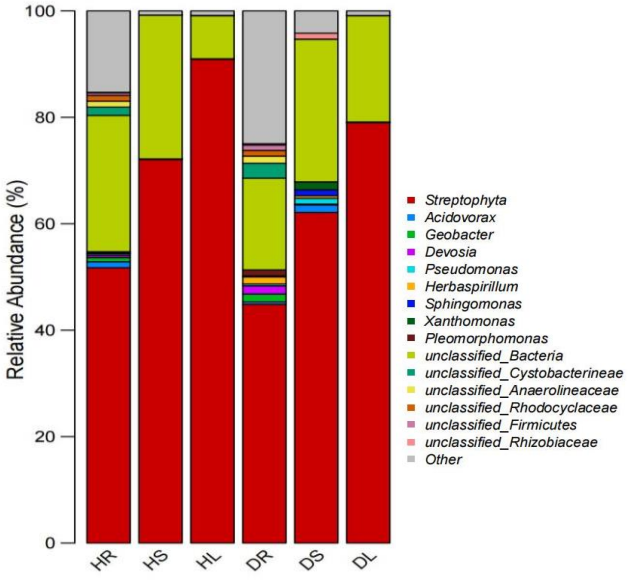

Figure 3. Composition of dominant bacterial genera in endophytic bacterial communities in different tissues of infected and non-infected rice plants

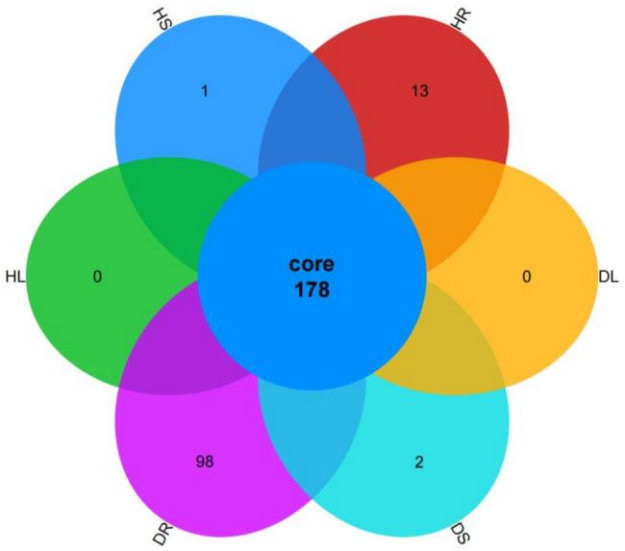

Figure 1. Venn diagram of OUT distribution of endophytic bacterial communities in different tissues of infected and non-infected rice plants

Table 3. Total number of endophytic bacterial communities in different tissues of infected and non-infected rice

Samples	Phylum	Class	Order	Family	Genus
HR	7	8	10	9	5
HS	1	1	1	1	1
HL	1	1	1	1	1
DR	9	13	16	15	9
DS	2	4	7	10	9
DL	1	1	1	1	1
Total	9	13	15	14	9

There were 10 genera with relative abundance ≥0.1% of endophytic bacteria in the stem, of which 7 genera can be accurately classified. *Streptophyta* was the dominant genus shared by infected and non-infected plants, with an abundance of 62.12 and 72.10%, respectively. In infected plant stems, there were *Acidovorax*, *Devosia*, *Pseudomonas*, *Herbaspirillum*, *Sphingomonas*, and *Xanthomonas*; the abundance was 1.49, 1.43, 0.12, 1.08, 0.49, and 1.12%. The abundance of these 6 genera in the stems of non-infected plants was very low, only 0.002-0.043%. In addition, the abundance of unclassified and other genera in infected and non-infected plants was 32.13 and 27.81%, respectively.

At the genus level, only *Streptophyta* with relative abundance ≥0.1% of endophytic bacteria in the leaves, with an abundance of 78.99 and 90.93% in infected and non-infected plants, respectively. The abundance of unclassified and other genera in infected and non-infected plants was 20.88 and 9.00% (Figure 3).

Relative abundance of dominant genera

Moreover, 29 bacterial genera with high abundance in rice samples were selected to draw the abundance heatmap. The abundance of the top 29 genera in each sample was visually reflected by color. The sample level cluster tree at the top shows that the three samples from different rice tissues are separately clustered into one branch, indicating that the community structure and composition of the three treatments in the same tissues of infected and non-infected rice plants are the most similar. The endophytic bacteria in the stem have common characteristics with those in the root. The species-level cluster tree on the left side of the abundance heatmap shows that bacteria genera with similar abundance are clustered together and divided into 4 branches. The abundance of bacteria genera in infected and non-infected plant roots differed from that of the other

treatments. The genus with the most significant difference in abundance was *Strephophyta*, followed by *Pantoea* (Figure 4).

Genus level species distribution bubble map

The infection of pathogenic bacteria caused changes in the abundance of endophytic bacteria in rice. Compared with non-infected plants the abundance of *Streptophyta* and *Acidovorax* in the root of infected plants decreased by 1.29 and 0.74% respectively, the abundance of *Geobacter*, *Devosia*, *Pleomorphomonas*, *Herbaspirillum*, *Pseudomonas* and *Sphingomonas* increased by 1.05, 1.55, 1.28, 1.76, 0.10, and 0.05%, respectively. The abundance of *Streptophyta* in the stem decreased by 7.83%, the abundance of *Xanthomonas*, *Acidovorax*, *Pseudomonas*, *Sphingomonas*, *Devosia*, and *Herbaspirillum* in the stem increased by 1.55, 1.49, 1.11, 1.15, 0.08, and 0.51%, respectively. The abundance of *Streptophyta* and *Devosia* in the leaves decreased by 12.03 and 0.01%, and *Sphingomonas* increased by 0.06%. The remaining bacterial genera did not change. Infection of the main pathogenic fungus, *Fusarium fujikuroi*, led to an imbalance of the endophyte community, and the original microbial balance of rice endophytes was affected, which might be an important reason for the incidence of rice (Figure 5).

Discriminant analysis of LEfSe differential species

LEfSe conducted the difference analysis of all taxonomic levels, and different species were found between groups. LEfSe species analysis of endophytic bacteria in different parts of infected plants and non-infected plants with rice bakanae disease was shown in Figure 6; there were relatively many different biological markers within the group, including 7 phyla, 12 classes, 18 orders, 25 families, and 28 genera. Among the differential species, *Actinomycetales* played an important role in the root of non-infected plants, while *Pseudomonas* and *Sphingomonas* played an important role in the stem of infected plants (Figure 6).

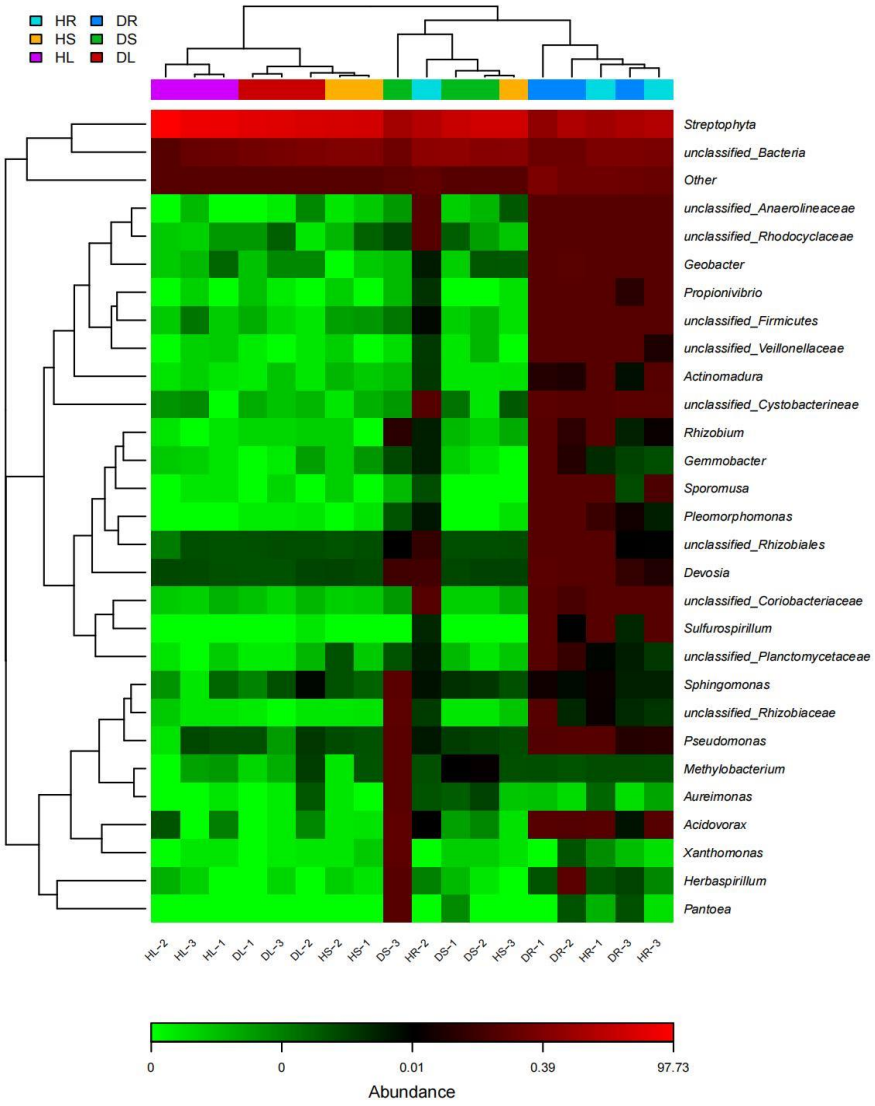

Figure 4. Heatmap of the relative abundance at the endophytic bacterial genus level in different tissues of infected and non-infected rice plants

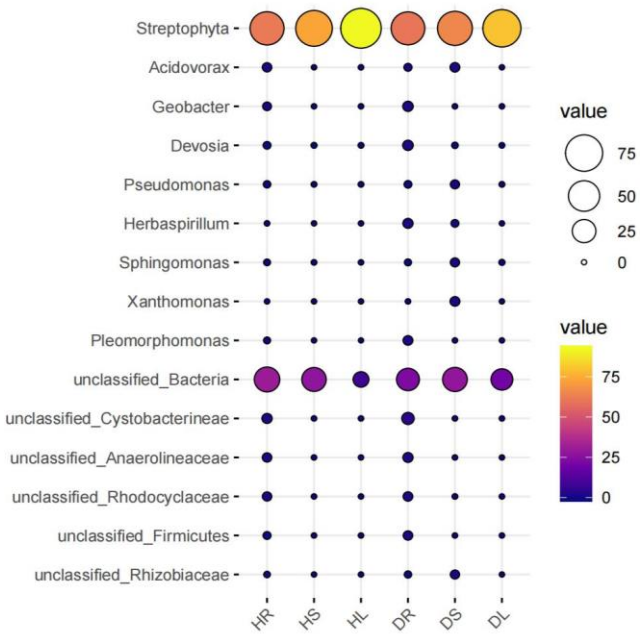

Figure 5. Bubble plots of endophytic bacterial genus level in different tissues of infected and non-infected rice plants

Discussion

The results showed that the number of endophytic bacteria was different in different parts of rice plants, with the largest number in the root, and the number of endophytic bacteria gradually decreased from the bottom to the top. The health level of the plant was closely related to the pathogen, and the infection of the pathogen had an impact on the endophytic bacteria (He and Li 2013), so the health status was the main factor determining the distribution of endophytic bacteria in various parts of the plant (An et al. 2023; Martin and Dombrowski 2015). This study analyzed the differences of endophytic bacterial communities in different tissues, such as roots, stems, leaves, and panicles of infected and healthy rice.

Result showed that there were significant differences in the structure and composition of endophytic bacterial communities in different tissues, such as roots, stems, leaves and panicles of rice, indicating that different parts of rice were selective for the bacterial communities, which was consistent with the research results of Takahashi (2011). There were also differences in community structures of endophytic bacteria in different tissues between infected and healthy rice. Furthermore, the infection of *Ustilaginoidea virens* impacted the composition and diversity of endophytic bacterial communities. Alpha diversity analysis showed that the abundance of endophytic bacterial communities in infected plants was higher than that in healthy plants, indicating that the invasion of *Ustilaginoidea virens* changed the community structure of endophytic bacteria in rice. In addition, a similar finding was also made by Sarwar (2018), who reported that there was a higher bacterial diversity in roots than in other parts of the plant in both infected and healthy plants. The endophytic bacteria of rice can come from the plant surface, rhizosphere soil and plant. Due to the differences in the sources of endophytic bacteria in various parts, there were differences in the structure composition and diversity of the bacterial communities (Rawat et al. 2022; Nawaz et al. 2021).

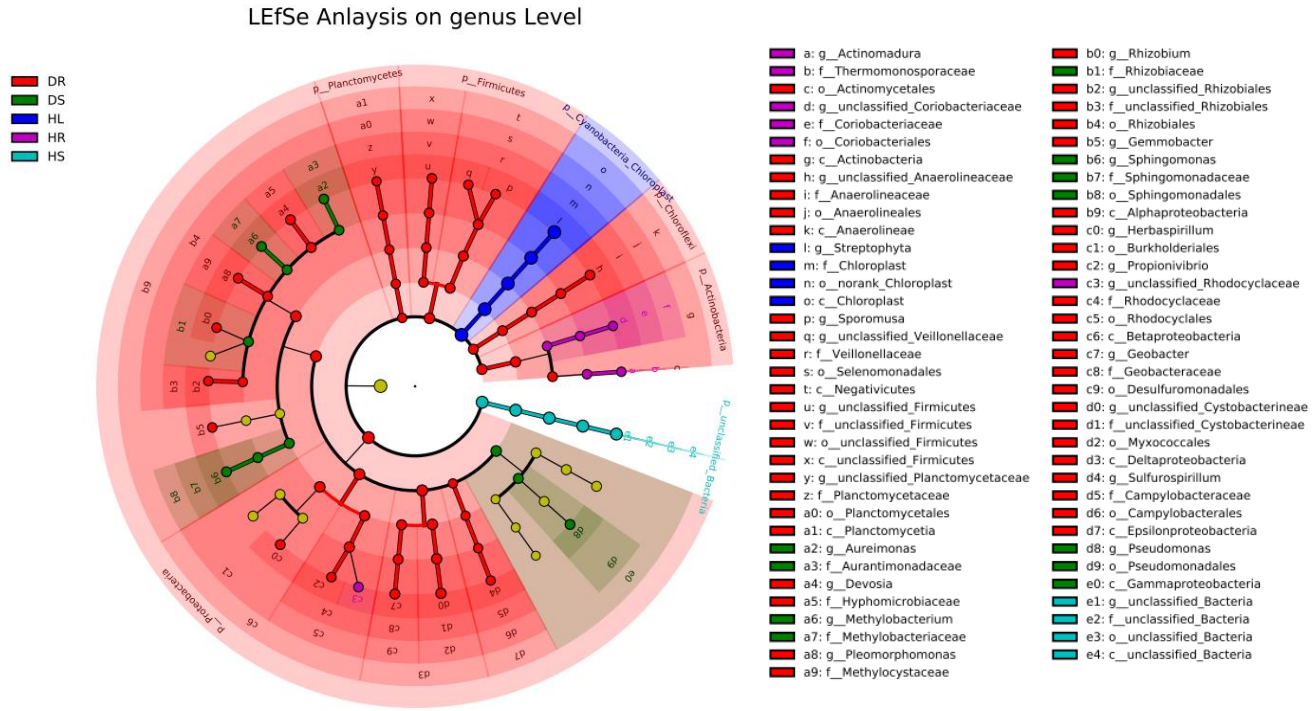

Figure 6. Circular dendrogram of the LEfSe difference in endophytic bacteria in different tissues of infected and non-infected rice plants

In this study, *Streptophyta* and *Acidovorax* were found as the dominant genus in the root and stem, which played an important role in maintaining the balance of endophytic microbial populations in rice and promoting the health of rice. The analysis of bacterial community structures showed that the phyla composition of bacteria in different parts of infected plants and healthy plants' corresponding parts were similar. After infection of rice malignant pathogens, the abundance of *Proteobacteria* increased by 9.77%, *Actinobacteria* increased by 0.27%, and the abundance of *Proteobacteria* in the stem increased by 10.32%. LEfSe analysis showed that *Actinomycete* was an important microbial species in the roots of non-infected rice plants. *Actinomycete* played an important role in promoting disease resistance in rice plants, which may be related to the fact that *Actinomycete* could produce different types of antibiotics. *Pseudomonas* and *Sphingomonas* are important species in infected rice stems. Compared with non-infected rice tissues, the abundance of *Pseudomonas* in infected roots, stems, and leaves increased by 0.10, 1.11, and 0.06%, respectively. The abundance of *Sphingomonas* in infected roots, stems, and leaves increased by 0.05, 1.12, and 0.06%, respectively. *Devosia* in infected roots and stems increased by 1.05 and 0.08%. It could be seen that the infection of rice bakanae disease bacteria destroyed the original microbial balance of rice endophytes, which may be an important reason for the incidence of this disease. Studies show that *Pseudomonas* can inhibit the pathogens of rice blast and rice leaf blight (Chakraborty et al. 2021). *Devosia* has the function of biodegradation of toxins and their derivatives (Shakeel et al. 2023); *Sphingomonas* can secrete active peptides and other substances to promote the growth of banana (Hossain et al. 2016). Whether *Pseudomonas*, *Sphingomonas*, and *Devosia* can antagonize rice bakanae disease bacteria and whether they play a role in the process of rice endophytes in response to pathogen infection needs further study.

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