Physiological Change Alters Endophytic Bacteria Community in Clubroot of Tumorous Stem Mustard Infected by Plasmodiophora Brassicae

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Abstract

Background: Endophytic bacteria are considered as symbionts living within plants and are influenced by abiotic and biotic environmemts. Here, we reveal how endophytic bacteria in tumorous stem mustard (*Brassica juncea* var. tumida) are affected by clubroot disease using 16S rRNA high-throughput sequencing.

Results: The results showed that Proteobacteria was the dominant group in both healthy roots and clubroots, but their abundance differed. At the genus level, *Pseudomonas* was dominant in clubroots, whereas *Rhodanobacter* was the dominant in healthy roots. Hierarchical clustering, UniFrac-weighted principal component analysis (PCA), non-metric multidimensional scaling (NMDS) and analysis of similarities (ANOSIM) indicated significant differences between the endophytic bacteria communities in healthy roots and clubroots. The physiologcial properties including soluble sugar, soluble protein, methanol, POD and SOD significantly differed between healthy roots and clubroots. The distance-based redundancy analysis (db-RDA) and two-factor correlation network showed that soluble sugar, soluble protein and methanol were strongly related to the endophytic bacteria community in clubroots, wheares POD and SOD correlated with the endophytic bacteria community in healthy roots.

Conclusions: Our results illustrate that physiologcial change caused by *P. brassicae* infection may alter the endophytic bacteria community in clubroots of tumorous stem mustard.

Background

Endophytic bacteria are symbionts living within plants for the majority of their life cycle without any negative effects on a host plant [1, 2]. It is well known that endophytic bacteria are beneficial to plant growth and development because they synthesize plant hormones (indole-3-acetic acid), solubilize phosphate and promote plant tolerance to biotic and abiotic stresses [3–5] by producing siderophores, competing with pathogens for space and nutrients, and modulating the plant resistance response [6, 7]. Moreover, some endophytic bacteria provide biological nitrogen fixation for host plants [8, 9].

Endophytic bacteria often live in plant intercellular spaces, where they easily absorb carbohydrates, amino acids, and inorganic nutrients [8, 10, 11]. When endophytic bacteria survive in the intracellular environment, they must adapt to the environment and be compatible with a host. This specific niche within host plants results in endophytic bacteria competing with fewer competitors. However, pathogens in infected plants would compete with endophytic bacteria for space and nutrients. In diseased plants, pathogens become the dominant microorganisms and fight with endophytic bacteria as well as plant. For example, the endophytic bacteria community in grapevine and apple infected by phytoplasmas [12, 13] and in tomato infected by root knot nematode [14] changed compared with healthy plants.

Clubroot is a serious disease of cruciferous crops caused by biotrophic *P. brassicae* Woronin [15], significantly change the morphology and physiology of the diseased plant, finally forming galls [16]. *P. brassicae* survive and absorb carbohydrates in galls [17, 18], thus they occupy most space in root cells

and probably suppress endophytic bacteria. However, how clubroot disease influences endophytic bacteria communities in tumorous stem mustard is unclear. The objectives of our study were (1) to reveal the diversity and composition of the endophytic bacteria community associated with tumorous stem mustard, and (2) to uncover how pathogen shape the endophytic bacteria community in clubroots of tumorous stem mustard caused by *P. brassicae* compared to healthy roots.

Results

α-diversity analysis

High quality sequences of partial 16S rRNA were produced by the Miseq PE3000 platform. The raw sequencing data have been deposited at the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) under accession numbers SUB7398877. According to the taxonomy of the sequences and abundance (Additional file 1:Table S1), we analyzed the composition of the endophytic bacteria community. Rarefaction curves analysis confirmed that the number of observed OTUs increased asymptotically with an increase in reads (Fig. 1A).The sobs index and Shannon index of the endophytic bacteria community in healthy roots were higher than that in clubroots, showing that healthy roots possessed higher diversity community (Fig. 1A, B). However, the Simpson index showed no significant differences between healthy roots and clubroots (Fig. 1C).

At the phylum level, Proteobacteria was the dominant group in healthy roots (relative abundance ranging from 57.8 to 63.8%) and in clubroots (relative abundance ranging from 80.4 to 89.0%) (Fig. 2A). Actinobacteria in healthy roots were the second abundant bacterial group with relative abundance from 21.6 to 31.8%. However, the second abundant bacterial group in clubroots was Bacteroidetes (relative abundance ranging from 8.0 to 18.2%). At the genus level, *Rhodanobacter* (relative abundance ranging from 10.7 to 17.8%) dominant in the endophytic bacteria community within healthy roots, followed by *Rhizobium*. However, *Pseudomonas* (relative abundance ranging from 24.7 to 30.9%) in clubroots was the dominant group, followed by *Rhizobium* and *Acidovorax* (Fig. 2B).

β-diversity analysis

The endophytic bacteria community in the healthy roots and clubroots clustered in two branches on the Hierarchical clustering tree (Fig. 3A). UniFrac-weighted PCA showed variations between the healthy roots and the clubroots with the first two axes explaining 57.5% and 7.2% of the total variation (Fig. 3B). The endophytic bacteria community in healthy roots was clustered on the right side of PCA, whereas the communities in the clubroots was clustered on the left side, indicating a clear separation between the community in R and C samples. Likewise, NMDS results with stress 0.038 also showed the same trends between the community in R and C (Fig. 3C), although some samples exhibited differences among three fields in one group such as healthy roots or clubroots. The results of ANOSIM with R 0.997 demonstrated the communities in R and C significantly differered (Fig. 3D). The network analysis reflected that healty

roots had a more complex endophytic bacteria community (Degree 3140 and Clustering 66.53) than clubroots (Degree 2632 and Clustering 58.77) (Additional file 2: Figure S1).

Significantly different taxa were found between the two communities from results of LEfSe (Fig. 4A). At the genus level, *Methylobacterium*, *Bradyrhizobium*, *Sphingomonas*, and *Bordetella* enriched in healthy roots and *Duganella*, *Rhizobium*, *Hydrogenophaga* and *Sphingopyxis* were biomarker species (Fig. 4A). Furthermore, the 15 most abundant genera of the two communities were compared by the Student's t-test (Fig. 4B). *Pseudomonas* and *Rhizobium* were significantly abundant in the clubroots, whereas *Rhodanobacter* were markedly more abundant in the healthy roots.

Relationship between physiological properties and endophytic bacteria community in healthy roots and clubroots

The physiological properties, such as SS, SP, M, POD and SOD in healthy roots and clubroots were markedly different, except for MDA (Additional file 3: Figure S2). Futhermore, we analyze the relationship between physiological properties and the endophytic bacteria community. The results of d-b RDA showed that SS, SP and M were strongly related to the community in clubroots, wheares POD and SOD correlated with the community in healthy roots (Fig. 5A). Moreover, we conducted two-factor correlation network and found that physiological properties correlated with some endophytic bacteria (Fig. 5B). For example, SS, SP and M were related to endophytic bacteria with number of 76, 74 and 71, respevtively, suggesting that they play important role in shaping the endophytic bacteria community in clubroots.

Discussion

In our study, we found that the endophytic bacteria community in healthy roots and clubroots were markedly different in alpha diversity and beta diversity. The domiant bacteria in healthy roots and clubroots were Proteobacteria at phylum level, while the relative abundance of proteobacteria were different. These results were in line with previous studies that many kinds of bacteria live in plant roots, including the phyla Proteobacteria, Actinobacteria and Bacteroidetes [19, 20]. In most studies, Proteobacteria are the predominant group of endophytic bacteria in various plant hosts [21, 22], suggesting they are suited to the ecological niche of plant tissue. Zhao also reported Proteobacteria as the dominant group of endophytic bacteria in the roots of oilseed rape (*Brassica napus*) [23]. Actinobacteria was the second dominant groups in healty roots and had high relative abundance, which is in line with Zhao's results [23]. Some previous studies found that endophytic Actinomycetes had biocontrol capacity to inhibit some pathogens and also showed plant-growth-promotion traits [24–26]. In this study, Actinobacteria in healty roots maybe also play benefitial roles.

At the genus level, *Pseudomonas* dominanted in clubroots, suggesting that this bacteria play an important role in the ecological niche. They probably had advantage to tumorous stem mustard and

maybe compete with *P. brassicae* for space and nutrition. Many previous studies verified that *Pseudomonas* possessed plant growth-promoting characteristics such as nitrogen-fixing [27], production of plant hormone or antimicrobial substances, or inducing systemic plant defense responses [28]. The main bacteria in healthy roots was *Rhodanobacter*, which is also isolated from the roots of Spathiphyllum plants and has biocontrol activity against root rot fungal pathogen *Fusarium solani* [29, 30]. *Rhizobium* is widely distributed in plant root tissues and play a role in nitrogen fixation for plant hosts [31–33]. In healthy roots and clubroots, we observed abundant *Rhizobium*, indicating that the bacteria probably fix nitrogen for tumorous stem mustard.

It was reported that endophytic bacteria community was altered by pathogen infection in many plants species such as grapevine [34], apple [12] and tomato [14]. Similarly, the differences in the endophytic bacteria community in healthy roots and clubroots were revealed by Hierarchical clustering analysis, PCA, NMDS and ANOSIM, suggesting that *P. brassicae* can restructure the endophytic bacteria community. To reveal how *P. brassicae* altered the community, we compared the physiological properties between healthy roots and clubroots and found that marked differences exist in SS, SP, M, SOD and POD, showing that *P. brassicae* infection significantly change the physiological characteristic. This is accordance with previous studies that soluble sugar distinctly increase under *P. brassicae* infection [35, 36]. In our study, soluble sugar had the most strongest correlation with the endophytic bacteria community in clubroot, suggesting that high concentration of soluble sugar change the community. In addition, soluble protein also increase in clubroots and correlated with the endophytic bacteria community. The high nutritional substances in clubroot induced by *P. brassicae* infection promote some endphytic bacteria proliferation, such as *Pseudomonas*, which possessed strong adaptation and ability of quick growth [37].

The methanol was also related to the endophytic bacteria community in clubroots. Previous studies showed that methanol production increased when plant cell wall endured mechanical wounding or other stresses such as pathogens or unsuitable temperature [38]. *P. brassicae* infection leads to root cell swellling and damages cell walls, which may promote root cells releasing more methanol. The content of methanol in clubroots were markedly higher than healthy roots, which probably imparct on the endophytic bacteria community and promoted or inhibited some bacteria. For example, *Duganella* was the biomarker species in the community in clubroots, can utilize methanol as a carbon source [39]. Abundant *Duganella* in clubroots may stimulated by methanol. The two-factor correlation network revealed that SS, SP and M were related to more endophytic bacteria, confirming SS, SP and M resturctured the endophytic bacteria community in clubroot.

SOD and POD are the antioxidase in plants and enhance plants stress tolerance to environment and pathogen. In general, POD and SOD increased when pathogen infected [40, 41]. However, SOD and POD in clubroots were lower than in healthy roots, reflecting that the normal physiological function maybe destroyed by *P. brassicae* infection. Moreover, the db-RDA demonstrated that SOD and POD positively and negatively correlated with the endophytic bacteria community in healthy roots and clubroots, verifing the fact that *P. brassicae* infection inhibited the activity of SOD and POD.

Conclusion

The discrimination in the endophytic bacteria community within the clubroots and healthy roots were revealed by high throughput sequencing method. *P. brassicae* infection caused marked changes in physiological properties within clubroots. Theses physiological alterations inhibited or promoted some bacteria, and regualted compositon of the endophytic bacteria community.

Methods

Samples

The clubroots of tumorous stem mustard were obtained at the harvest-stage (February 2, 2019) from three fields with distances 5 km in Fuling (29.21° N, 106.56° E) where clubroot disease had been found 20 years ago. The roots were classified as healthy roots (named R) and clubroots (named C). From one field, 30 plants for 15 R samples and 15 C samples were randomly selected and formed two groups, thus 6 groups containing 90 plants from 3 fields were named R1, C1, R2, C2, R3 and C3. Soil particles attached to roots were removed by washing with tap water. The healthy roots with 0.5 cm diameter from healthy plants and clubroot galls with 1 cm diameter from diseased plants were cut off, surface sterilized by 70% (v/v) ethanol for 40 s, followed by 4% (w/v) sodium hypochlorite for 60 s and were finally rinsed three times in sterile distilled water. The surface-sterilized healthy roots and galls cut with a sterilized razor and separated into two parts. One part was used for genomic DNA extraction, one part for phsiologial properties determination.

Determination of phsiologial properties of healthy roots and clubroots

The content of soluble sugar (SS), soluble protein (SP), peroxidase (POD), superoxide dismutase (SOD), malonaldehyde (MDA) and methanol (M) in healthy roots and clubroots were detected accaording standard method in Nanjing cavenex testing technology co. LTD. SS, SP and MDA was determined by anthrone-sulfuric acid colorimetric method, coomassie brilliant blue method and thiobarbituric acid method, respectively. SOD and POD were assessed by NBT-illumination method and guaiacol method. The methanol were measured by gas chromatography (GC-17A, Shimadzu, Kyoto, Japan).

PCR amplification and 16S rDNA sequencing

Genomic DNA of the health roots and cluroots were extracted using cetyltrimethylammonium bromide (CTAB). DNA concentration and purity were monitored on 1% w/v agarose gel. The bacteria V3 + V4 region of 16S ribosomal RNA gene was amplified by PCR for barcoded pyrosequencing using the primers (338F: 5'-ACTCCTACGGGAGGC AGCAG – 3' and 806R : 5'-GGACTACHVGGGTWTCT AAT-3') [42]. Forward primer 338F was linked to A-adaptor, a specific 8-bp multiplex identifier (MID) barcode, while the reverse

primer 806R carried the B-adapter. The PCR conditions were: 95 °C for 2 min (one cycle), 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s (25 cycles), 72 °C for 5 min (one cycle). The sequencing was performed using an Illumina MiSeq sequencer (Asbios Technology Co.,Ltd, China). PCR reactions were performed in triplicate of 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. PCR products were confirmed by electrophoresis in agarose gel (2%) and resulted in amplified fragments of 500 bp. and further purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol. Purified amplicons were pooled and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Bioinformatics processing and data analysis

The bioinformatics analysis was conducted on the free online Majorbio I–Sanger Cloud Platform (http://www.i-sanger.com/). Firstly, the raw sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME) package (v1.8) [43]. The low-quality sequences, such as primer and barcode sequence mismatches, sequences shorter than 50 bp, sequences containing ambiguous characters, PCR-based or sequencing errors and chimeras, were removed. The quality-filtered sequences were used to carry out identification of taxonomy of each operational taxonomic unit (OTU) representative sequence by Unite (Release 7.2) software under the threshold of 97% identity [44]. Taxonomic assignment of representative sequences for each operational taxonomic unit (OTU) was carried out on the basis of Silva (Release123 http://www.arb-silva.de) and the Ribosomal Database Project RDP (Release 11.3 http://rdp.cme.msu.edu/). The rarefaction curves, Shannon and Simpson index were used to indicate the community richness. Relative abundance of endophytic bacteria were assessed at the phylum, class, order, family, genus, species and OTU levels.

For β-diversity, the hierarchical cluster dendrograms (Bray-Curtis distance dissimilarities) were constructed according to OTU composition [45], UniFrac-weighted PCA, NMDS and ANOSIM were performed to reveal the discrimination in the endophytic bacteria communities between healthy roots and clubroots using R 3.1.1 statistical software [46, 47]. Discriminant Analysis Effect Size (LEfSe) software was used to screen for the markedly different genera between healthy roots and clubroots for biomarker discovery [48]. Network analysis was performed to reveal the relationship among the top 50 OTUs within the endophytic bacteria communities by Networkx software based on Pearson's rank correlation coefficients [49]. The distance-based redundancy analysis (db-RDA) and Two-factor correlation network were used to investigate relationships between the endophytic bacteria communities and physiological properties by Canoco statistical software (Version 5.0) with default parameter settings.

Abbreviations

PCA: UniFrac-weighted principal component analysis; NMDS: non-metric multidimensional scaling; ANOSIM: analysis of similarities; M:methanol; MDA: malonaldehyde; POD: peroxidase; SOD: superoxide dismutase.

Declarations

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Consent to publication

Not applicable.

Authors' contributions

DDW and TTS conceived and designed the study and wrote the manuscript.. SYZ collected the samples. LMP helped draft and revised the manuscript. XJT modified the manuscript to prepare its final version. HFL helped with statistics. All authors read and approved the final manuscript.

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Availability of data and materials

The raw reads of 16S MiSeq data were deposited into the NCBI Sequence Read Archive database (SUB7398877).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

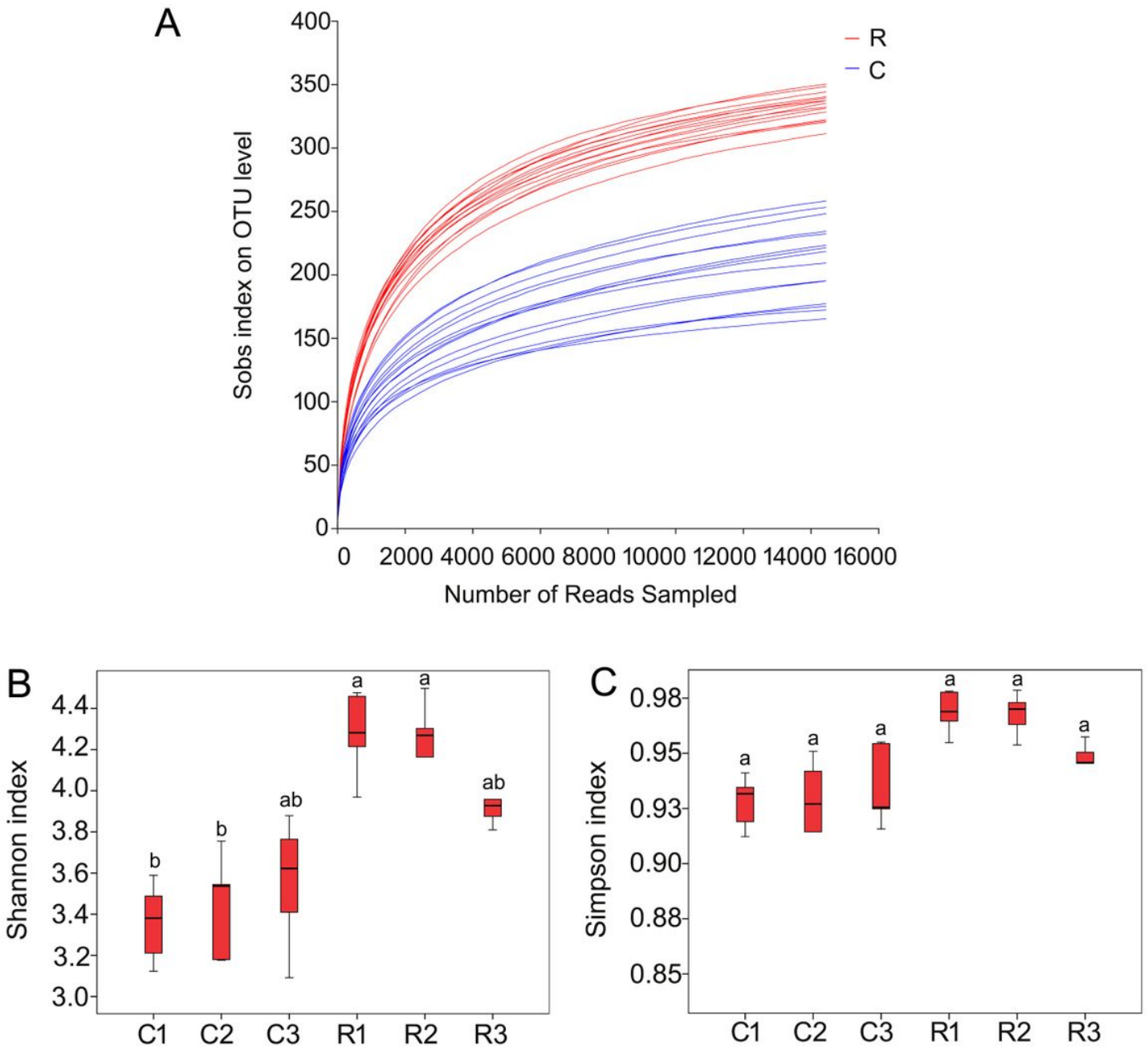

Figure 1

Alpha diversity of the endophytic bacteria communities in healthy roots and clubroots. a Rarefaction curves. b Shannon index. c Simpson index. R, healthy roots. C, clubroots. Various letters on the column showed the marked difference between healthy roots and clubroots.

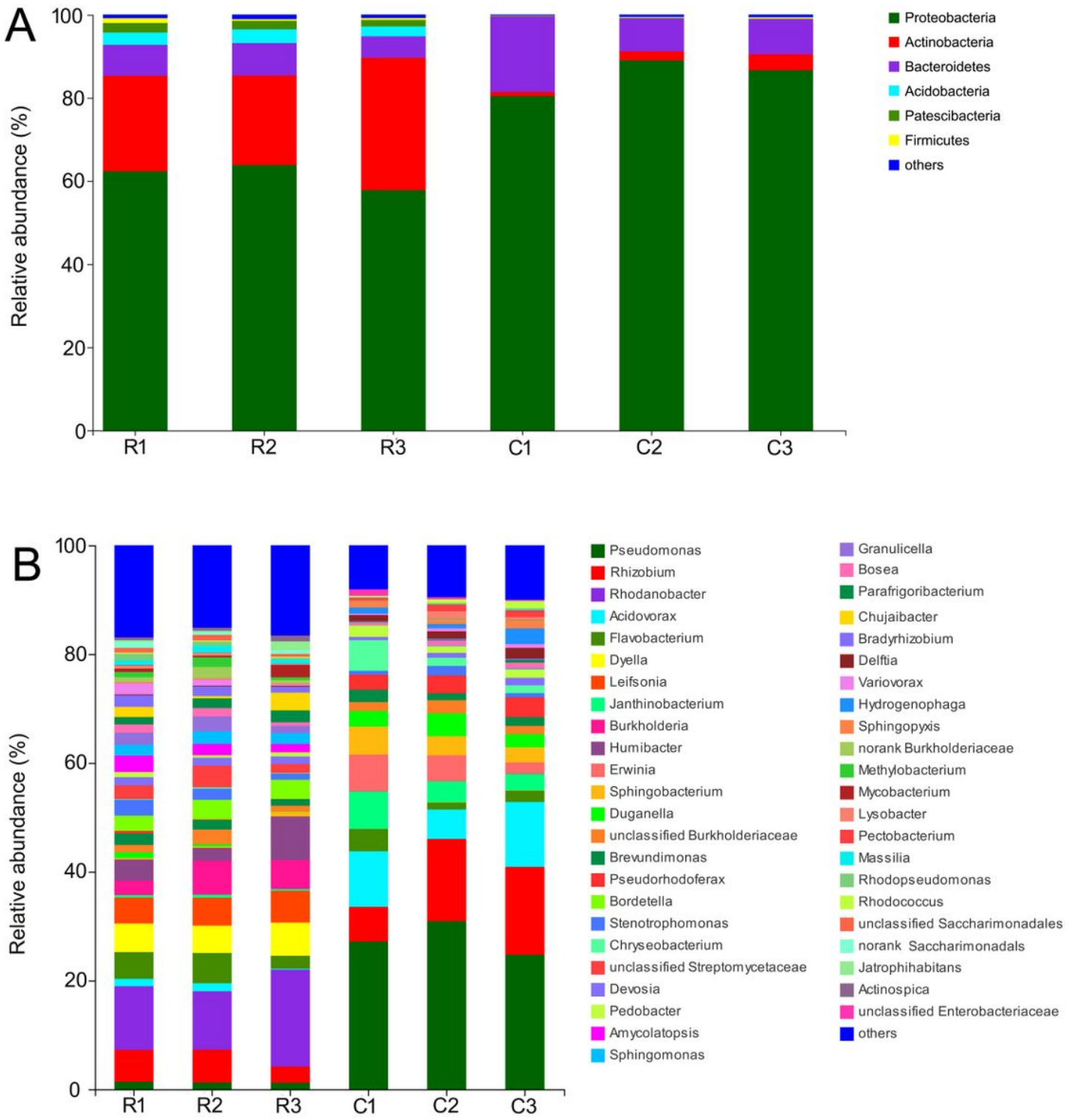

Figure 2

Distribution of endophytic bacteria at phylum (a) and genus (b) level. R, healthy roots. C, clubroots.

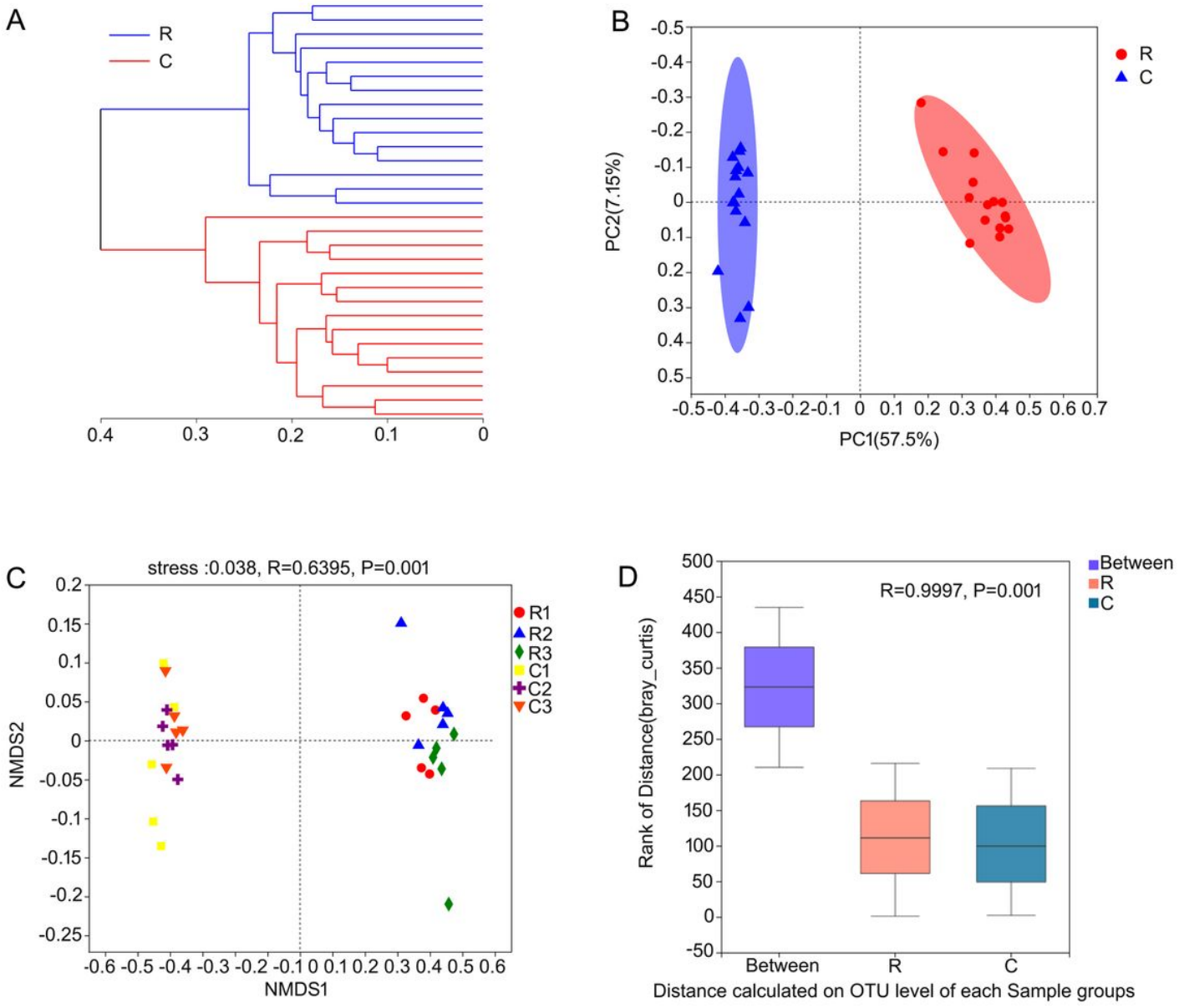

Figure 3

Beta diversity analysis of the endophytic bacteria communities in the healthy roots and clubroots. a Hierarchical clustering analysis. b UniFrac-weighted PCA. c NMDS. d ANOSIM. R, healthy roots. C, clubroots.

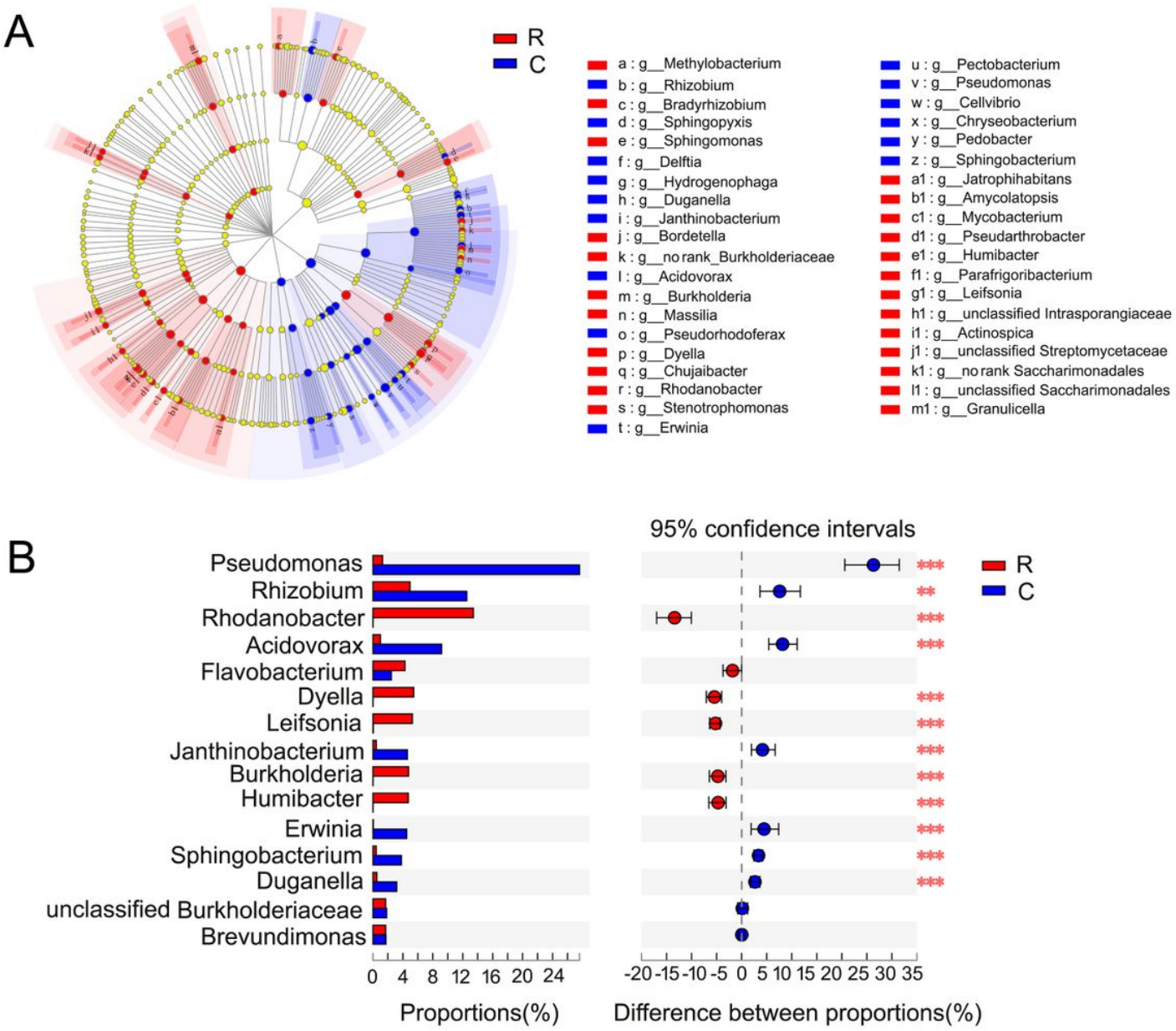

Figure 4

The markedly different bacteria in the endophytic bacteria communities between healthy roots and clubroots. a LefSe analysis. The cladogram diagram shows the taxas with marked differences in the two endophytic bacteria communities. Red and blue indicate different groups, with the classification of taxas at the level of class, order, family, and genus shown from inside to the outside. The red and blue nodes in the phylogenetic tree represent taxas that play an important role in the two endophyte communities, respectively. Yellow nodes represent taxas with no significant difference. b Student's t-test bar plot of the endophytic bacteria communities at the genus level in the healthy roots and clubroots. $p<0.05$*, $p<0.01$**, $p<0.001$***. R, healthy roots. C, clubroots.

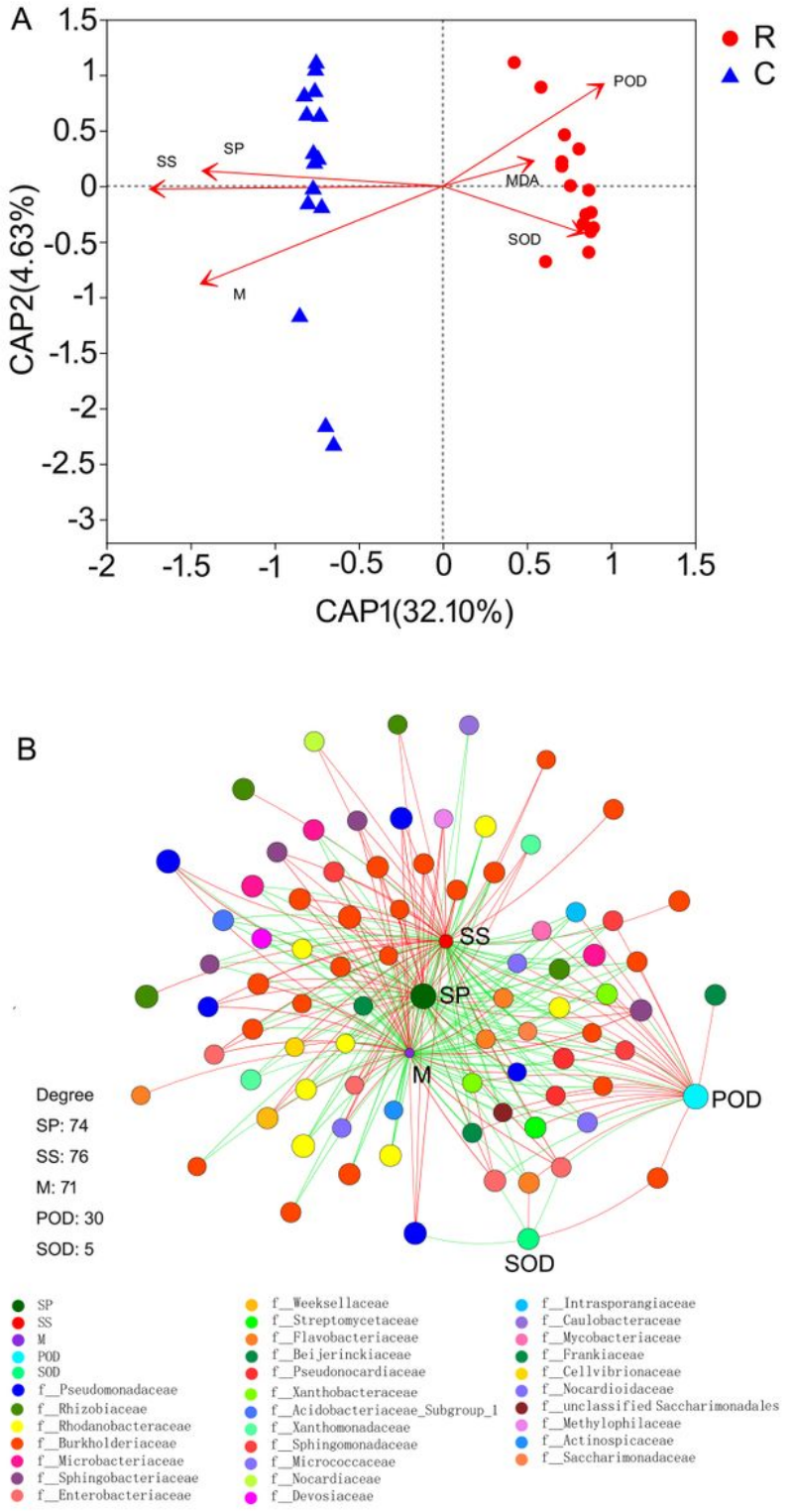

Figure 5

Relationship between physiological properties and the endophytic bacteria communities in healthy roots and clubroots. a db-RDA. SS, soluble sugars. SP, soluble protein. M, methanol. MDA, malonaldehyde. POD, peroxidase. SOD, superoxide dismutase. b Two-factor correlation network. The number represented the quantities of bacteria markedly correlated with physiological properties. A red line indicates a positive correlation. whereas a green line indicates a negative correlation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS2.tif
- FigS1.tif
- TableS1.xls