Comparison of Bacterial Communities in Soil Samples with and without Tomato Bacterial Wilt Caused by *Ralstonia solanacearum* Species Complex

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Abstract

Background

Ralstonia solanacearum is one of the most notorious soil-born phytopathogen that causes a severe wilt disease with deadly effects on many economically important crops. The microbial community structure and interactions are commonly changed between bacterial wilt susceptible soil and healthy soil. Here, the bacterial community structure, correlation analysis with soil chemical properties, interaction network of healthy soil (HS, nearly no disease happened at recent three years) and diseased soil (DS, suffered heavy bacterial wilt disease) were analyzed.

Results

Compared to DS, a higher bacterial community diversity index was found in HS, and the relative abundance of main genera *Bacillus*, *Gaiellales*, *Roseiflexus*, *Gemmatimonadaceae*, *Nocardioides* and *Anaerolineaceae* reached significant level. Redundancy analysis on genus level indicated that rapid available phosphate played key role on bacterial community distribution difference, and showed negative correlation with the other four chemical properties. Interaction network analysis further demonstrated that the higher genus community diversity and more extensive interactions were existed in HS network and formed stable network, and the genera *Mycobacterium*, *Cyanobacteria* and *Rhodobiaceae* should be the key components that sustain the network stably. Seven clusters of orthologous groups reached significant level difference between HS and DS. Moreover, 55 bacterial strains with distinct antagonistic activities to *R. solanacearum* were isolated and identified.

Conclusions

In summary, our findings indicate that the bacterial diversity and interaction network changed between the HS and DS samples, which are also provide a good research basis for future biological control to the bacterial wilt.

Background

Tomato (*Solanum lycopersicum*) is one of the most famous vegetables in the world, but the soil-borne disease caused by *Ralstonia solanacearum* species complex (RSSC) has been the serious threatening to tomato production. Not only the tomato, but also over 200s hosts in 54 botanical families can be infected by the members of RSSC[1], and caused severe economic and social impact worldwide[2, 3]. For the RSSC can exist in soil for many years, thus, except for the disease is difficult to control, the infected fields are always not adapt to cultivate the susceptible plants again[4].

Disease-suppressive soils are exceptional ecosystems, the known resident via as yet unknown microbiota confer the property that make crop plants suffer less from specific soil-borne pathogens[5, 6]. Increasing evidences have been reported for suppressing several soil-borne pathogens causing *Fusarium* wilt[7],

potato common scab[8], damping-off disease[9], sugar beet wilt[10], and bacterial wilt[11]. It is noteworthy that the diversity of soil microbial community is particularly crucial for keep the suppressing capacity, which can affect the colonization success of additional species[12, 13], and some introduced beneficial bacteria have been confirmed to increase the microbial community diversity or enhance the resistance to RSSC[14, 15]. Thus, the soil bacteria play key roles in suppressing plant disease and protecting plant health, for the bacteria wilt can be reduced significantly by the existing population of rhizosphere microorganisms[11].

Lingshui cherry tomato is one kind of tomato cultivar, and is honored as China's national geographical indication products. Taking a comprehensive investigation in Lingshui country, due to long-term continuous monoculture during the last ten years has led to severe bacterial disease in a large area of Lingshui country, Hainan province, China, however, though the tomato culturing and management patterns are same, we still found that the tomato growing well in some fields. What make the differences between healthy soil (HS, nearly no disease happened at recent three years) and diseased soil (DS, suffered heavy bacterial wilt disease)? Whether are the soil microorganism functioned to sustain the tomato health?

In this study, we used high-throughput sequencing technology to explore the bacterial diversities differences between HS and DS. Meanwhile, the soil chemical properties, bacterial community composition, network analysis and clusters of orthologous groups (COG) comparison were also analyzed. Importantly, we isolated and identified 55 bacteria strains with excellent antagonistic ability to *R. solanacearum* from the rhizosphere soil of the healthy tomato plants collected from DS, which supplies more biocontrol resources for the control of bacterial wilt.

Results

Bacterial diversity assessment of HS and DS samples

In order to detect whether the microbial functioned to sustain the tomato health, we established the difference in disease incidence of tomato plants cultivated with HS, DS, and DS with heat treatment (autoclaving, 121°C one hour and followed with dry heat sterilization, 180°C four hours). Results demonstrated that the tomato plants cultivated in DS showed typical wilt symptom and nearly 80% plants died, while the tomato plants growing in HS and heat treated soil with no plant wilt (Supplemental information figure 1). Bacterial diversity of soil samples collected from HS and DS were then assessed using phylotype taxonomy. Results revealed that there were 3,041 OTUs, the core OTU number was 2,488, and the HS and DS with 330 and 220 unique OTUs, respectively (Fig. 1A). Furthermore, the result of student's t-test indicated that the Sobs index of the OTU level of HS and DS samples reached the significant level (p value is 0.002216) (Fig. 1B).

Main bacterial composition of HS and DS

Result of HS samples demonstrated that genera *Bacillus* (relative abundance 7.18%), *Gaiellales* (5.20%), *Acidobacteria* (3.28%), *Nocardioides* (2.31%), *Nitrospira* (2.74%), norank_c_KD4-96 (1.82%), norank_f_Xanthobacteraceae (1.99%) were the main groups; the genera *Bacillus* (3.95%), *Gaiellales* (3.94%), *Acidobacteria* (3.76%), *Nocardioides* (3.77%), *Gaiella* (2.96%) norank_c_KD4-96 (2.47%), *Roseiflexus* (2.49%), and norank_f_Anaerolineaceae (2.41%) were the dominate groups of DS samples (Fig. 2). In addition, PCoA result revealed that the bacterial communities of HS and DS were separated distinctly, and the PC1 axis could show 56.74% bacteria community variations between HS and DS (Fig. 3).

Further to perform the relative abundance analysis, we found that the relative abundance of genera *Nocardioides* and norank_f_Anaerolineaceae between HS and DS reached significant level (95% CI; p value < 0.05). In addition, the relative abundance of genera *Bacillus*, *Gaiellales*, *Roseiflexus*, norank_o_Gemmatimonadaceae, and *Gaiella* reached very significant level (95% CI, p value < 0.01) (Fig. 4).

Chemical properties and RDA of HS and DS

No significant difference was observed for AHN content of HS and DS samples, the contents of pH, OM and RAK of HS were all significantly lower than DS sample (p < 0.05, Table 1), but the content RAP in HS was significantly higher than DS. Further to conduct the RDA at genus level, we found that RAP played key role on the bacterial community distribution difference between HS and DS, and had a negative correlation with the other four chemical contents (Fig. 5).

Network analysis of HS and DS

Network analysis of the 30 most abundant genera revealed the interaction relationships of HS and DS, respectively. Results indicated that there were extensive interactions among the identified genera. In the HS (Fig. 6A), the 30 most abundant genera were from ten phylum, including ten genera from Actinobacteria, six genera from Proteobacteria, four genera from Acidobacteria, three genera from Chloroflexi, two genera from Firmicutes, and one genus from Nitrospirae, Saccharibacteria, Planctomycetes, Gemmatimonadetes, respectively; obviously, the genera as *Mycobacterium*, *Rhodobiaceae* and *Cyanobacteria* shew interaction relationships with five, seven, and seven other genera, respectively. In the DS (Fig. 6B), these genera were only from seven phylum, such as the nine genera from Actinobacteria, six genera from Proteobacteria, six genera from Chloroflexi, four genera from Acidobacteria, one genus from Gemmatimonadetes, Nitrospirae, and Firmicutes, respectively.

Analysis of the COGs with significant differences

The COG function prediction was performed and compared between HS and DS samples. The COGs with significant differences were focused, our results demonstrated that there were seven COGs varied distinctly and reached significant level, such as the group S (Function unknown), H (Coenzyme transport and metabolism), A (RNA processing and modification), F (Nucleotide transport and metabolism), and D

(Cell cycle control, cell division, chromosome partitioning) reached very significant levels, the group C (Energy production and conversion) and Z (Cytoskeleton) (Fig. 7).

Isolation and identification of antagonistic strains

During the investigation, we found that there were few tomato plants in the DS field still growing well. We collected the rhizosphere soil of three healthy tomato plants and isolated the cultural bacteria. Followed by taking inhibition zone method, 55 bacterial strains with distinct antagonistic activities to *R. solanacearum* strain EP1 were found and identified by sequencing their 16S rDNA sequences (Table 2). Results of BLAST showed that these strains were belonging to the genera of *Bacillus* (17 strains), *Pseudomonas* (ten strains), *Sphingobacterium* (ten strains), *Chryseobacterium* (nine strains), *Serratia* (four strains), *Cellulosimicrobium* (one strain), *Staphylococcus* (one strain), *Fictibacillus* (one strain), *Microbacterium* (one strain), and *Paenibacillus* (one strain).

Discussion

Previous studies mainly focus on comparing the microbial species abundance and diversity of bacterial wilt disease outbreak soil samples[16-18]. Here, not only the microbial relative abundances and diversities of HS and DS, but also the associated soil chemical properties, microbial networks and COG groups with significant differences were also explored. Notably, we also isolated and identified 55 bacteria strains with excellent antagonistic activities to *R. solanacearum* strain EP1.

Results of Sobs index of the OTU level, main bacterial community distribution and PCoA indicated that the significant differences of bacterial community composition were existed between the HS and DS (Fig. 1, 2 and 3). Based on the result of Welch t test (95% CI) at genus level, we found that the relative abundances of following groups *Bacillus*, norank_c_*Gaiellales*, *Roseiflexus* and norank_f_*Gemmatimonadaceae* reached very significant level (Fig. 4), which demonstrate that relative abundances of these four genera changed distinctly as the bacterial wilt development. Numerous results have proved that the *Bacillus* species are often considered as beneficial microorganisms and microbial factories for producing a vast array of biological active molecules inhibitory for pathogens[19]. The cyclic lipopeptides antimicrobial compounds produced by *Bacillus* species, such as surfactin, iturin and fengycin, as well as other components have been well studied and applied for their antagonistic activities, which can efficiently reduce the diseases caused by *R. solanacearum*[20], *Rhizoctonia solani*[21], *Pythium aphanidermatum*[22] and *Podosphaera fusca*[23]. In our result, the relative abundances of genera *Bacillus* in HS samples were also higher than which in DS samples, which was consist with the reported results. Moreover, the result of antagonistic strain isolation and identification also support these data, for 17 *Bacillus* strains were found among the 55 antagonistic strains.

Network analysis can provide information regarding the co-existence relationships of species in environmental samples, as well as species interactions and mechanisms of formation of phenotypic differences between samples. Based on the network analysis results of HS and DS (Fig. 6), the genera *Cyanobacteria*, *Saccharibacteria* and *Planctomycetes* were not found in the interaction network of DS

sample, and several lonely interactions were found in DS network. Thus, the higher genus community diversities and more complex interaction were existed in the HS samples. The nodes in HS network analysis were connected with more interactions than DS network and formed stable network, especially the three genera (*Mycobacterium*, *Cyanobacteria* and *Rhodobiaceae*) with extensive interactions to other genera, which should be the key components that sustain the stable network.

It was noteworthy that among the main genera of HS and DS, there were ten genera belonging to phylum Actinobacteria in HS, and nine genera belonging to phylum Actinobacteria in DS. Obviously, the species of Actinobacteria with an important niches in HS and DS samples. Strains of Actinobacteria are also known as effective biocontrol microorganisms against many plant pathogens including the *R. solanacearum*[24, 25], however, these strains colonized on the plant surface also played potential inhibitory effects on beneficial microbes for their broad-spectrum antimicrobial compounds, for they can decrease the bacterial community components greatly. By comparing the network analysis between HS and DS, we found that several genera format negative correlation with the genera of *Actinobacteria* in HS network, such as the genera of *Chloroflexi*, *Cyanobacteria*, *Planctomycetes*, *Acidobacteria*, as well as some genera of Proteobacteria (*Bradyrhizobium*, *Rhodobiacear*), which can balance or limit the antagonistic effects of Actinobacteria to other microorganisms, then to construct a more stable bacterial community with higher diversities. Previous results have shown that the soil with higher bacterial community diversities will help microorganisms to participate in nutrient cycling, promote plant growth, adapt to environmental changes and suppress plant pathogens[26-28]. However, in the result of DS network, significant positive correlation were existed among Actinobacteria and other genera, thus, it is unfavorable to sustain the whole bacterial community diversity, especially once the plant-beneficial groups were inhibited, which may lead to DS fail to prevent the infection of RSSC, which is consist to another report on the bacterial wilt susceptible soil[29]. Thus, except for the accumulation of plant pathogen, the decreasing of plant-beneficial groups and bacterial diversity will be the main reason that resulted in the outbreak of bacterial wilt. While the stable and complex network existed in HS may help to prevent the infection of RSSC and sustain the tomato plant healthy.

Based on the associated analysis between soil chemical properties and bacterial diversity, it was found that pH, RAK, OM and RAP could affect their diversity significantly (Fig. 5). It has been demonstrated that pH is key driver of bacterial abundance[30], some related report predicted that the soil acidification could negatively affect soil bacterial community stability and easy to cause the bacterial wilt[29]. However, we found that the pH in HS was lower than which in DS, thus, except for the pH, some other soil chemical properties may contribute to the stability of soil bacterial community or the soil inhibition ability to RSSC. Interestingly, we found that RAP played vital role on the bacterial community distribution difference between HS and DS, meanwhile, a negative correlation with the other four chemical factors was also found, thus, the RAP may function differently from other chemical factors.

Soil is considered to be a highly complex and dynamic ecosystem[31], and result demonstrates that the formation of disease suppressive soils after a disease outbreak always due to the subsequent assembly of beneficial microbiota in the plants rhizosphere[32]. Our result was consist with the above reports, up to

55 strains from *Bacillus*, *Pseudomonas*, *Sphingobacterium*, *Chryseobacterium*, *Serratia*, *Cellulosimicrobium*, *Staphylococcus*, *Fictibacillus*, *Microbacterium*, and *Paenibacillus* with excellent antagonistic activities to *R. solanacearum* were isolated and identified. Especially the strains of *Sphingobacterium*, *Chryseobacterium*, *Serratia*, *Cellulosimicrobium*, *Staphylococcus*, *Fictibacillus*, *Microbacterium*, and *Paenibacillus* with antagonistic activity to *R. solanacearum* were not so extensive reported.

Conclusions

The study shows the microbial diversities differences between healthy soil and diseased soil caused by *R. solanacearum*. Meanwhile, the soil chemical properties and bacterial community composition were also analyzed. Importantly, we isolated and identified 55 bacteria strains with excellent antagonistic ability to *R. solanacearum* from the rhizosphere soil of the healthy tomato plants collected from DS, which will provide a good research basis for future biological control to the bacterial wilt.

Methods

Collection and DNA extraction of soil samples

Soil samples were collected from the Benhao town, Lingshui country, Hainan province, China, and the collection of all soil samples was permissible by Lingshui country government. The HS samples were located at N: 18°29'51", E: 109°56'54", the DS samples were located at N: 18°32'47", E: 110°3'1" (Table 1), at which cherry tomato has been continuously planted in the two sites more than five years. Firstly, the grass covered on the surface were cleared, within the 1m × 1m square, the soils from 10 cm depth of three different locus were mixed as one soil sample, five different mixed soil samples as repetitions were collected from healthy and diseased field, respectively. All samples were stored in sterile plastic bags and transported to the laboratory in an ice box immediately, where they were stored at -20°C until 16S rDNA were sequenced and analyzed.

Aliquots (0.25 g) of the soil samples were processed using a MOBIO PowerSoil® kit. The extracted DNA samples were then analyzed using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA), whereas the DNA quality was checked by 1% agarose gel electrophoresis. The extracted DNA samples were selected and used to conduct microbial community analysis by PCR using the following 16S rDNA primers: forward (5'-GTGCCAGCMGCCGCGG-3') and reverse (5'-CCGTCAATTCMTTTRAGTTT-3')[33]. The PCR reactions were as follows: 95°C, 3 min; followed by 27 cycles of 30 s at 95 °C, 30 s at 55 °C, and 72°C for 45 s and then final extension at 72°C for 10 min. The PCR reactions were performed in triplicate in 20 µL mixtures containing 4 µL 5× FastPfu Buffer, 2 µL 2.5 mM dNTPs, 1 µL primer mix (5 µL), 0.4 µL FastPfu Polymerase, and 5 ng extracted DNA as the template. The PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). The products were then quantified using QuantiFluor-ST (Promega, Madison, USA). Purified amplicons were then pooled in equimolar

concentrations and paired-end sequenced (2×300) using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to the standard protocols of Shanghai Majorbio Bio-pharm Technology Co., Ltd. Raw sequences were filtrated by using FASTX Toolkit 0.0.12 software to remove low quality reads with Q value < 20 and with less than 35 bp[29].

Diversity analyses of microbial communities

16S rDNA data were analyzed using the Majorbio I-Sanger cloud platform (<http://www.i-sanger.com/>). The similarity and differences between samples were compared using the shared and unique operational taxonomic unit (OTU) of Venn diagram, and student's t test was used to assess the level of significant difference. The bar and pie analysis were conducted at genus level. The principal coordinates analysis (PCoA) of β -diversity was calculated based on the Bray-Curtis algorithm. To compare significance testing of microbial community variance of HS and DS samples at genus level, Welch's t-test (two-tailed test) and false discovery rate (fdr) were used with Scheffe cutoff value of 0.95, the Welch's inverted was used to calculate confidence interval (CI) value. The network analysis was conducted at genus level to assess the correlation characterization of the HS and DS samples (the 30 most abundant OTUs were selected), for these analyses, the Spearman correlation coefficient model was used with a cutoff of 0.5. To conduct the 16S rDNA function prediction, firstly, the richness of OTU was standardized by PICRUSt, and then the COG family information corresponding to OUT and COG richness was obtained. The COGs with significant differences were analyzed by Stamp software (statistical test model is ANOVA, and post-hoc test model is Tukey-Kramer with a cutoff 0.95).

Correlation between soil chemical characteristics and bacterial diversities

Soil pH, contents of soil organic matter (OM), alkali-hydrolysable nitrogen (AHN), rapid available phosphate (RAP), and rapid available potassium (RAK) were determined as previously reported[29]. The redundancy analysis (RDA) was conducted to calculate the bacterial diversity distribution correlation with above soil chemical properties at genus level.

Isolation and identification of antagonistic bacteria

The rhizosphere soils from the healthy tomato plant roots planted in DS were collected, and then the root tissues were shredded and grinded fully with the collected rhizosphere soils, 50 ml sterile water was added to the above collecting pipe. Taking a series of gradient dilution, the suspension were spread on the CN agar medium (0.1% casamino acid, 0.1% nutrient broth, 1% agar), the dilution fold that each plate growing with 40-60 colonies was selected. The colonies with different phenotypes were separated and purified. The inhibition zone method was used to assess the inhibition ability of strain, the method is described briefly as below: each isolated strain was cultured in the CN liquid medium (about 72 h, 28 °C, 200 rpm), respectively; the *R. solanacearum* strain EP1[34] was selected as indicator strain, when was cultured in the CPG medium until the OD₆₀₀ up to 1.0, 1 ml EP1 suspension was added in the 100 ml CPG agar medium (melting but not burning) and was well mixed. Dispensing 4 μ L suspension of isolated rhizosphere strain on the CPG plate with added EP1 suspension, and cultured in the incubator at 28 °C,

the diameters of inhibition zone were recorded and analyzed after three days. All the strains with inhibition ability were identified with 16S rDNA sequencing (27F/1492R), the corresponding genus was conducted based on the BLAST results of 16S rRNA sequences database (Bacteria and Archaea).

Declarations

Author contributions

PL, WF collected the soil, YZ, AN contributed to isolate and identify the antagonistic strains, PL designed the experiment and wrote the paper, JN revised the manuscript.

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Data accessibility

The raw reads of 16S MiSeq data were deposited into the NCBI Sequence Read Archive database (accession No.: PRJNA564488). All the 16S rDNA sequences of 55 antagonistic strains have been upload to NCBI database (GenBank accession no. MN410647 - MN410701).

Ethics approval and consent to participate

All experiments were performed according to the experiment security regulations of Hainan Normal University, and approved by the biosafety committee in Hainan Normal University.

Consent to publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Healthy and diseased soil chemical properties

Properties	Healthy soils (n=5)	Diseased soils (n=5)
Location	N 18°29'51", E 109°56'54"	N 18°32'47", E 110°3'1"
pH	5.19 ± 0.16	6.64 ± 0.14 **
OM	0.85 ± 0.13	1.15 ± 0.082 *
RAP	152.02 ± 10.37	86.95 ± 5.58 **
RAK	102.34 ± 8.46	247.69 ± 54.68 **
AHN	35.77 ± 3.18	39.21 ± 2.82

Table 2 Antagonistic strains to *R. solanacearum* strain EP1

Genus	Strain number	GenBank accession No.
<i>Bacillus</i>	17	MN410647-48, 51-59, 61-62, 71, 74, 98, 70
<i>Pseudomonas</i>	10	MN410664-65, 68-69, 75-76, 79, 89, 92, 94
<i>Sphingobacterium</i>	10	MN410666-67, 72-73, 81-82, 85-86, 93, 96
<i>Chryseobacterium</i>	9	MN410677, 80, 83-84, 87-88, 90-91, 95
<i>Serratia</i>	4	MN410670, 78, 91, 99
<i>Staphylococcus</i>	1	MN410649
<i>Fictibacillus</i>	1	MN410650
<i>Microbacterium</i>	1	MN410660
<i>Cellulosimicrobium</i>	1	MN410663
<i>Paenibacillus</i>	1	MN410701

Figures

A

B

Figure 1

Venn analysis of shared and unique OUT between HS and DS (A). OTUs defined by 97% sequence similarity and Student's t test of OTU level between healthy and diseased soils (B). ** means $p \leq 0.01$

Community barplot analysis

Figure 2

Community barplot analysis of five healthy soils (H1, H2, H3, H4, H5) and five diseased soils (D1, D2, D3, D4, D5) at the genus level, others means the genera with relative abundance lower than 0.1%, x-axis of the column was the percent of community relative abundance at the genus level

PCoA on Genus level

Figure 3

PCoA analysis on genus level. The β -diversity was calculated based on the Bray-Curtis algorithm
Welch's t-test bar plot on Genus level

Figure 4

Welch's t-test and FDR comparison analysis of microbial communities on genus level between HS and DS samples, the Scheffe's value cutoff was 0.95, *** means $p \leq 0.001$, ** means $0.001 < p \leq 0.01$, and * means $0.01 < p \leq 0.05$

Figure 5

Correlations analysis between bacterial diversity (genus level) and soil chemical properties. The green arrows represent genus, red arrows represent environmental factors (EF): soil pH, organic matter (OM), alkali-hydrolysable nitrogen (AHN), rapid available phosphate (RAP), and rapid available potassium (RAK). The length of red arrows mean the impact degree affected by related EF, the angle between EF represent the positive and negative correlation, the acute angle represent positive correlation, the obtuse angle represent negative correlation, the right angle represent no correlation.

Figure 6

Interactions network analysis on genus level of HS (A) and DS (B) samples. Different node colors respective different bacteria genera. Blue lines represent negative interactions, red lines represent positive interactions among different nodes.

Figure 7

Significantly different COGs between HS and DS samples. S: Function unknown; H: Coenzyme transport and metabolism; A: RNA processing and modification; F: Nucleotide transport and metabolism; D: Cell cycle control, cell division, chromosome partitioning; C: Energy production and conversion; Z: Cytoskeleton.

Supplementary Files

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- [SupplementaryMaterial.tif](#)