1 **Changes in Microorganisms in the Rhizosphere of Mulberry**

2 **Genotypes with Differing Resistance to Bacterial Wilt**

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10 **Abstract**

11 A close relationship between soil-borne diseases, soil microbial community structure, and
12 functional diversity has been described in the mulberry plant. In the present study, microbial
13 abundance, community structure, and functional diversity in the soil rhizosphere were compared
14 in resistant (Kangqing10) and susceptible (Guisang12) mulberry genotypes using the dilution plate
15 method, micro-ecology technology, and polymerase chain reaction-denaturing gradient gel
16 electrophoresis (PCR-DGGE). The goal of this study was to develop better management methods
17 for mulberry cultivation and preventing and controlling bacterial wilt. Rhizosphere soil
18 microorganisms were more abundant in the resistant normal mulberry genotype than in the
19 susceptible normal mulberry genotype. Carbon source utilization was better in the susceptible
20 normal mulberry genotype. These properties were lower in the resistant sickly mulberry genotype
21 than in the susceptible sickly mulberry genotype. PCR-DGGE indicated that the bacterial and
22 fungal community structures of the resistant genotypes were more stable than those of the

susceptible genotypes. Correlation regression analysis implicated mulberry bacterial wilt in the loss of soil nutrients, particularly organic matter and nitrogen, which can disrupt the balance of the soil microbial community. Loss of soil organic matter and nitrogen had a lower impact on resistant genotype plants than on susceptible genotype plants. Therefore, resistant genotype plants displayed some resistance to bacterial wilt. Further insights into the soil rhizosphere microbial diversities of resistant and susceptible genotypes will help in the control and prevention of mulberry bacterial wilt.

Keywords

Mulberry; Bacteria Wilt; Genotypes; Microorganisms

Introduction

Bacterial wilt is a devastating soil-borne disease caused by *Ralstonia solanacearum* and intestinal bacteria. It is widely distributed in tropical, subtropical, and temperate regions [1] and is common in commercial crops including Solanaceae [2], Leguminosae [3], tobacco [4], Cucurbitaceae [5], and Sanko [6-8]. One of the areas that has been affected is Guangxi, a key silkworm area in China. Guangxi accounts for more than 30% of the national mulberry area. However, the extension of mulberry planting years and improper cultivation management have led to a continuous annual decrease in the yield of mulberry leaves. Feeding of silkworms requires the collection of mulberry leaves every year to ensure that the mulberry supply exceeds demand. Thus, farmers tend to apply inorganic fertilizer, particularly nitrogenous fertilizer, over long periods to increase yield. Although this practice can increase production, in the long-term it will alter the physical and chemical properties of the soil [9], reduce soil microbial diversity [10], and promote the development of severe soil-borne diseases such as bacterial wilt [11, 12]. As a result, the stability

and sustainable development of the silk industry may be seriously threatened by the considerable loss of mulberry plants or yield of mulberry leaves.

Several recent studies have suggested that primary self-regulation of the soil micro-ecological environment by adding organic fertilizer can help prevent soil-borne diseases. Application of organic fertilizer can improve soil microbial community composition, enrich soil microbial community structures, and increase the quantities of root exudates, thus significantly reducing the incidence of bacterial wilt [13-18]. Organic fertilizer is applied to prevent and control soil-borne diseases of various vegetables [16] and strawberries in greenhouse environments [13]. The occurrence of tomato bacterial wilt can be controlled and tomato yield increased by adding silicon fertilizer and bagasse [18].

Another approach involves the use of a balanced soil bacterial community structure using different cropping systems to control bacterial wilt. The incidence of tobacco bacterial wilt can be significantly reduced by rotating tobacco with corn, lily, and carrot [19]. A third approach involves biological control to reduce the rate of bacterial wilt. For example, an endophytic *Bacillus subtilis* strain can prevent mulberry bacterial wilt [20]. Despite these approaches, further improvements have been limited by gaps in knowledge. The microbial community structure in the rhizosphere surrounding the plant roots, as well as the changes in the microbial community that promote bacterial wilt, is not well-understood. To explore these issues, we carried out a study in Guangxi province, the site of the largest sericulture production in China. Our results revealed the diversity of the rhizosphere microbial community structure between resistant and susceptible genotypes and their association with bacterial wilt, increasing the understanding of the micro-ecological mechanisms of bacterial wilt in the surrounding rhizosphere and conditions that

improve resistance to bacterial wilt. These results will help improve the prevention and control of mulberry bacterial wilt.

Material and Methods

Collection and preparation of soil samples

Eight soil samples were collected from a mulberry breeding site in Xinsheng Village, Gula town, Binyang County, Guangxi province (23°06'N, 108°59'E) in September 2017. All mulberry trees were 7Y cultivations. The rhizosphere soil of the resistant and susceptible mulberry genotypes was both sandy loam. Urea-based fertilizer was applied twice per year (1100 kg/hm² each time), with 40% applied in the spring (March) and 60% after the summer harvest in June. Soil rhizosphere samples were collected using the five-point sampling method [21] and shaking root method [22]. The control group, which was designated as CK, comprised rhizosphere soil from the base of normal (including resistant and susceptible genotypes) mulberry plants. Rhizosphere soil was also acquired from the base of sickly (including resistant and susceptible genotypes) mulberry plant genotypes (T1, T2, and T3). Additional information is presented in Table 1. Each obtained rhizosphere soil was placed in three labeled and sealed bags. One sample was partially dried for physical and chemical analyses. The other two samples were stored at 4°C or -20°C for microbial analysis.

Table 1

Designation of soil samples

Resistant normal mulberry genotype is denoted as RN (resistance normality), susceptible normal mulberry genotype is denoted as SN (susceptibility normality), resistant sickly mulberry genotype is denoted as RS (resistance sickness), and susceptible sickly mulberry genotype is

89 denoted as SS (susceptibility sickness).

Soil sample numbers and variety names		
Normality	CK	RN (Kangqing10, normal and control group)
		SN (Guisang12, normal and control group)
Sickness	T1	RS1 (Kangqing10-1 group)
		SS1 (Guisang 12-1 group)
	T2	RS2 (Kangqing 10-2 group)
		SS2 (Guisang 12-2 group)
	T3	RS3 (Kangqing 10-3 group)
		SS3 (Guisang 12-3 group)

90 **Physicochemical properties of rhizosphere soil**

91 The physicochemical properties of the rhizosphere soil, including alkaline nitrogen, available
 92 phosphorus, available potassium, and soil organic matter, can reflect the basic fertility status of the
 93 tested soil. The physicochemical properties of the collected rhizosphere soil are shown in Table 2.
 94 Alkaline N was extracted with 1.07M NaOH by the alkali-hydrolysis and diffusion method [23].
 95 Available P was extracted with 0.5 M NaHCO₃ as previously described [23, 24]. Available K was
 96 extracted with 1M NH₄OAc (1:10 soil:solution ratio) for 1h and analyzed by atomic absorption
 97 spectrophotometry [23, 25]. Soil organic matter was extracted with 0.8M K₂Cr₂O₇ using the
 98 standard Walkley-Black potassium dichromate oxidation method [23, 26].

99 **Table 2**

100 *Comparison of physicochemical properties in soil rhizosphere with different resistant mulberry*
 101 *genotypes*

102 The genotypes included Kangqing10, normal and control group (RN), Guisang12, normal and
103 control group (SN), Kangqing10-1 group (RS1), Guisang 12-1 group (SS1), Kangqing 10-2 group
104 (RS2), Guisang 12-2 group (SS2), Kangqing 10-3 group (RS3), and Guisang 12-3group (SS3). [†]

		Alkaline N	Available P	Available K	SOM [‡] (%)
		(mg kg ⁻¹)	(mg kg ⁻¹)	(mg kg ⁻¹)	
CK	RN	82.25±0.35 ^a	44.59±15.09 ^b	35.00±0.00 ^e	4.26±0.05 ^a
	SN	53.55±0.35 ^d	96.36±19.94 ^a	102.50±2.50 ^b	2.69±0.00 ^d
T1	RS1	71.75±3.15 ^b	49.77±3.97 ^b	52.50±2.50 ^c	3.83±0.05 ^b
	SS1	65.80±0.70 ^c	27.52±0.44 ^c	40.00±2.50 ^d	3.23±0.05 ^c
T2	RS2	11.55±1.05 ^g	1.41±0.11 ^e	10.00±0.00 ^g	0.48±0.00 ^g
	SS2	8.40±0.70 ^h	5.93±1.54 ^e	41.25±1.25 ^d	0.45±0.05 ^g
T3	RS3	30.80±0.00 ^e	21.57±1.54 ^{cd}	122.50±2.50 ^a	0.69±0.03 ^f
	SS3	26.60±0.70 ^f	7.91±0.88 ^{de}	15.00±0.00 ^f	0.79±0.01 ^e

105 [†]Data are expressed as the mean ± SD (n = 3). The superscript letters that differ within a column
106 indicate significant differences between treatments (P < 0.05).

107 [‡]Soil organic matter (SOM).

108 Viability of cultured bacteria and actinomycetes

109 The total numbers of viable cultured bacteria and actinomycetes were both measured as colony
110 forming units (CFUs) on agar plates using the dilution plate method [27]. The media used to
111 separately culture bacteria and actinomycetes were beef extract peptone medium and Gause's No.1
112 synthetic medium, respectively [28]. No antibiotics were present. The sample dilution used for
113 culture was 5g of each fresh soil sample in 45mL sterilized 0.85% NaCl. Each dilution was shaken
114 for 15 min at 150 rpm and then left standing for 30 min. The individual bacterial and actinomycete

strains from the 1×10^{-4} and 1×10^{-3} dilution plate, respectively, were isolated and purified. The purified strains were cultured on Biolog recommended agar media (BUG agar; Biolog, Inc., Hayward, CA, USA) and incubated at 25°C in the dark. Pure cultures from the BUG agar were then grown on GEN III MicroPlates (Biolog) for 24–72h and analyzed using a Biolog MicroStation equipped with Microlog microbial identification software (Ver. 5.2.01). The identification results were accepted if the similarity index value was ≥ 0.5 . Three replicates were analyzed for each sample.

Microbial community structure diversity analysis and microbial identification

Soil microbial community diversity

Ninety-six-well microplates (Ecoplates; Biolog, Inc.) were used to analyze soil microbial community diversity based on the utilization of 31 carbon sources as previously described [29]. The culture dilution was obtained as described above. The suspension was obtained from 1×10^{-2} dilutions after 30 min. Each suspension was dispensed into the wells of Ecoplates (125 mL per well) with a multi-channel repetitive-dispensing pipette. The plates were incubated at 25°C in the dark and absorbance at 590 and 750 nm was recorded every 24h for 7 days using the reader incorporated into the GEN III microStation. Three replicates per treatment and sampling time were analyzed.

Each well of an Ecoplate was loaded with a single carbon source. Thirty-one single carbon sources belonging to six classes (carbohydrates, amino acids, carboxylic acids, amines, polymers, and miscellaneous) were used in the analysis. The average well color development, which reflects the overall utilization of soil microorganisms on a single carbon source, was calculated as described by Garland JL [30]. Average well color development data from 144h were used to

calculate the functional diversities using the Shannon index and Shannon evenness [31, 32]. The Shannon index formula was calculated as $H = -\sum P_i \ln P_i$, P_i by subtracting the control from each substrate's absorbance and then dividing this value by the total color change recorded for all 31 substrates. Evenness was calculated as $E = H/\ln(\text{richness})$, where richness is the number of substrates utilized[31].

DNA extraction and PCR amplification of 16s rRNA and ITS

Total DNA was extracted using 0.3g of each fresh soil sample. DNA was extracted using an E.Z.N.A.® Soil DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions. The remaining samples were kept at -20°C for subsequent use. Specific gene fragments of bacteria and fungi were specifically amplified using the extracted DNA sample as a template with appropriate primers. Primers GC(CGCCCCGCCGCGCCCCGCGCCCGGCCGCGCCCCCGCCCC) + 341F (5'-CCTACGGGAGGCAGCAG-3') and R518 (5'-ATTACCGCGGCTGCTGG-3') were used to directly amplify the V3 hypervariable regions of 16s Rrna bacterial sequences from the microbial genome DNA [33, 34]. Amplification of the internal transcribed spacer (ITS) regions of fungal sequences from the microbial genome DNA used primers GC (CGCCCCGCCGCGCCCCGCGCCCGGCCGCGCCCCCGCCCC) + Fung (5'-ATTCCCCGTTACCCGTTG-3') and NS1 (5'- GTAGTCATATGCTTGTCTC-3') [35, 36]. The reaction mixtures (30 µL) contained 15 µL of 2 × Es Taq MasterMix, 1.2 µL of 10µM forward primer, 1.2 µL of 10µM reverse primer, 9.6 µL of RNase-free water, and 3 µL of template DNA. PCR conditions of bacteria were 36 cycles of 94°C for 5 min, 94°C for 1min, 64°C for 1min, and 72°C for 1min; and a final elongation at 72°C for 7 min. PCR conditions of the fungus were 35

cycles of 94°C for 5 min, 94°C for 30s, 47°C for 45s, and 72°C for 1min; and a final elongation at 72°C for 10 min. PCR products were excised from a 1.0% agarose gel and quantified in a Qubit1 Fluorometer (Invitrogen, Carlsbad, CA, USA) using a Quant-iT™ dsDNA HS assay kit (Invitrogen).

Denaturing gradient gel electrophoresis (DGGE) of 16s rRNA and ITS fragments

The amplified bacterial and fungal gene fragments (both 75 ng of DNA) obtained using the V3 hypervariable regions of 16s rRNA and ITS regions, respectively, were separated by DGGE using a polyacrylamide gel containing 8% acrylamide. The D-code system (Bio-Rad, Hercules, CA, USA) was used; this system can separate DNA fragments that differ in sequence but have the same length. Gradients of 50–80% and 20–50% were used for bacteria and fungi, respectively. The cross-linking agent was ammonium persulfate (APS) and N,N,N,N-tetramethyl-ethylene diamine (TEMED) (gradient gel:APS:TEMED = 1000:10:1). Electrophoresis was conducted in 1×TAE buffer at 60°C at a constant voltage of 80 V for 18 h. The DGGE gels were stained with SYBR1 Green (Molecular Probes, Eugene, OR, USA) and analyzed using the Quantity One v4.6.2 gel analysis software (Bio-Rad). Bands with intensities <0.05 were excluded from analysis. Two replicates were analyzed for each sample. The Shannon index and Shannon evenness were both calculated as described for the Ecoplate analysis.

Statistical analyses

The data were mainly analyzed using one-way analysis of variance (Levene's test was used to assess the equality of variances before performing analysis of variance), and significant differences between the means were determined by the least significant difference test. The differences were considered as significant when $P < 0.05$. DPS v7.05 software was used to analyze

the diversity index and evenness of Ecoplates and DGGE, the differences in the main six types of carbon sources utilization, and their correlations. The absolute value of the correlation coefficient (R) revealed no correlation at $R=0-0.09$, weak correlation at $R=0.1-0.3$, moderate correlation at $R=0.3-0.5$, and strong correlation at $R=0.5-1.0$. Quantity One v4.6.2 software (Bio-Rad) was used to quantitatively analyze DGGE bands. Excel 2016 software (Microsoft, Redmond, WA, USA) was used to analyze and map the data. The NCBI website was used for BLAST nucleic acid alignment for partial post-recovery sequencing results.

Results

Viability of cultured bacteria and actinomycetes in soil rhizosphere of mulberry with different resistant genotypes to bacterial wilt

The abundance of cultured microorganisms differed in the soil rhizosphere of mulberry genotypes that were resistant and susceptible to bacterial wilt (Fig 1). The numbers of viable bacteria were greater than those of viable actinomycetes in resistant mulberry genotypes (RN, RS1, RS2, RS3) and susceptible mulberry genotypes (SN, SS2, SS2, SS3). The numbers of cultured rhizosphere bacteria and actinomycetes of the resistant normal mulberry genotypes (RN) were both significantly higher ($P<0.05$) than those of the susceptible normal mulberry genotypes (SN). The number of rhizosphere actinomycetes of the resistant sickly mulberry genotypes (RS1, RS2, RS3) was also higher than that of the susceptible sickly mulberry genotypes (SS1, SS2, SS3), except for T1. However, the number in the rhizosphere of the resistant sickly mulberry genotypes (RS1, RS2, RS3) was significantly lower ($P<0.05$) than those of the susceptible sickly mulberry genotypes (SS1, SS2, SS3), except for T3 bacteria. The numbers of cultured rhizosphere bacteria and actinomycetes of the resistant normal mulberry genotypes (RN) were all

significantly higher ($P < 0.05$) than those of the resistant sickly mulberry genotypes (RS1, RS2, RS3), but the number of cultured rhizosphere bacteria and actinomycetes of the susceptible normal mulberry genotypes (SN) were significantly higher ($P < 0.05$) than those of the susceptible sickly mulberry genotypes (SS1, SS2, SS3), except for T3 bacteria. These results indicate that the abundance of soil microorganisms of the RN genotypes in the rhizosphere was higher than that of the SN genotypes, while the number of soil microorganisms of the RS genotypes in the rhizosphere was lower than that of the SS genotypes.

Fig 1. Comparison of abundance of (A) bacteria and (B) actinomycetes in the soil rhizosphere with different resistant mulberry genotypes treated with CK: Kangqing10, normal and control group (RN), Guisang12, normal and control group (SN); T1: Kangqing10-1 group (RS1), Guisang 12-1 group (SS1); T2: Kangqing 10-2 group (RS2), Guisang 12-2 group (SS2); T3: Kangqing 10-3 group (RS3), Guisang 12-3 group (SS3). The bars indicate the mean $\pm$ SD ($n = 3$). Different letters indicate significant differences between treatments ($P < 0.05$).

Average well color development in soil rhizosphere of mulberry with different resistant genotypes to bacterial wilt

The average well color development can reflect the overall utilization of soil microorganisms on a single carbon source. Microbial activity in the soil samples was examined as previously described [29]. During 168h incubation of the soil, the average well color development values of all soil samples increased rapidly starting at 24h and reached a steady state at approximately 144h. In this experiment, 144h was selected for statistical analysis (Fig 2). Soil microbes in the different resistant mulberry genotypes displayed differing utilization of the single carbon source. The equilibrium values of the steady state of single carbon source utilization were larger in susceptible

genotypes than in resistant genotypes, suggesting that the rate of carbon source utilization of resistant mulberry genotypes was higher than that of susceptible mulberry genotypes. For soil microbes of resistant mulberry genotypes, RN and RS2 sickly plants showed the highest utilization of the carbon source, followed by RS3 sickly plants, with RS1 sickly plants displaying the weakest utilization of the single carbon source. For soil microbes of the susceptible mulberry genotypes, the SS1 sickly plants displayed the greatest utilization of the single carbon source, followed by SS3 sickly plants, with SN normal plants displaying the weakest utilization of the single carbon source. Additionally, the utilization of carbon sources by RN in the rhizosphere was higher than that of SN. In contrast, utilization of the carbon sources by RS in the rhizosphere was lower than that of susceptible sickly mulberry genotypes in the order of $RS1 < SS1$; $RS2 < SS2$; and $RS3 < SS3$. These results suggest that the microbial function in the resistant normal genotypes in the surrounding rhizosphere was much greater than that of the susceptible normal genotypes, while the microbial function in the resistant sickly rhizosphere genotypes was lower than in the susceptible sickly genotypes.

The Shannon function index reflecting the rhizosphere soil microbe carbon source utilization by RN was greater than that of SN, while the Shannon function index of resistant sickly mulberry rhizosphere genotypes (RS1, RS2, RS3) was less than that of susceptible sickly mulberry genotypes (SS1, SS2, SS3) (Fig 3). The results may reflect the larger numbers of RN genotypes of cultured bacteria and actinomycetes than those of SN genotypes, while the numbers of RS1, RS2, and RS3 rhizosphere bacteria genotypes (RS1, RS2, RS3) were lower than those of the SS1, SS2, and SS3 genotypes, which differed based on the cultivation and fertilization systems in the mulberry garden.

In general, RN genotypes displayed the greatest utilization of the single carbon source, followed by SS genotypes, with SN genotypes most weakly utilizing the single carbon source. The data indicate that the utilization of a single carbon source in rhizosphere soil was greater in resistant mulberry genotypes and lower in susceptible mulberry genotypes.

Fig 2. Changes in the average well color development of 31 carbon sources in the soil rhizosphere with different resistant mulberry genotypes treated with Kangqing10, normal and control group (RN); Guisang12, normal and control group (SN); Kangqing10-1 group (RS1); Guisang 12-1 group (SS1); Kangqing 10-2 group (RS2); Guisang 12-2 group (SS2); Kangqing 10-3 group (RS3); and Guisang 12-3 group (SS3).

Fig 3. Comparison of the Shannon index (H) of carbon source in soil rhizosphere with different resistant mulberry genotypes treated with CK: Kangqing10, normal and control group (RN), Guisang12, normal and control group (SN); T1: Kangqing10-1 group (RS1), Guisang 12-1 group (SS1); T2: Kangqing 10-2 group (RS2), Guisang 12-2 group (SS2); T3: Kangqing 10-3 group (RS3), Guisang 12-3 group (SS3). The bars indicate the mean $\pm$ SD (n = 3). Different letters indicate significant differences between treatments (P < 0.05).

Classes of carbon sources in soil rhizosphere of mulberry with different resistant genotypes to bacterial wilt

The six classes of carbon sources in soil rhizosphere showed a sequence of utilization for resistant and susceptible mulberry genotypes. The order of utilization was carbohydrates > carboxylic acids > amino acids > polymers > amines > miscellaneous (Fig 4). Amino acids, amines, polymers, and miscellaneous utilization of the RN rhizosphere genotypes were all higher than that of the SN genotypes, while utilization of carbohydrates and carboxylic acids was lower

than that of the SN genotypes. Utilization of carbohydrates, carboxylic acids, amino acids, and miscellaneous molecules by the RS rhizosphere genotypes was all lower than those of the SS genotypes, while utilization of amines and polymers utilization was higher than that of the SS genotypes. Furthermore, utilization of carboxylic acids, amino acids, polymers, and miscellaneous molecules by the RN rhizosphere genotypes was higher than that of the RS1, RS2, and RS3 genotypes, with the exceptions of T2 amino acids, polymers, and carboxylic acids. Moreover, they were all significantly different from RS1 ($P < 0.05$), and utilization of carboxylic acids and polymers differed significantly in RS3 ($P < 0.05$). However, the utilization of carbohydrates and amines of the RN rhizosphere genotypes was lower than that of the RS1, RS2, and RS3 genotypes, except for T1 amines and T2 carbohydrates; both displayed a significant difference from RS3 ($P < 0.05$). In comparison, the utilization of all six classes of carbon sources by the SN rhizosphere genotypes was lower than the utilization by the SS1, SS2, and SS3 genotypes, except for T2 amines and carbohydrates; additionally, utilization of the polymers was significantly different from SS1, SS2, and SS3 ($P < 0.05$). The utilization of carbohydrates, carboxylic acids, and miscellaneous molecules displayed significant differences from SS1 and SS3 ($P < 0.05$), and utilization.

Fig 4. Comparison of the six classes of carbon sources in soil rhizosphere with different resistant mulberry genotypes treated with CK: Kangqing10, normal and control group (RN), Guisang12, normal and control group (SN); T1: Kangqing10-1 group (RS1), Guisang 12-1 group (SS1); T2: Kangqing 10-2 group (RS2), Guisang 12-2 group (SS2); T3: Kangqing 10-3 group (RS3), Guisang 12-3 group (SS3). Substrates: (A) amino acids, (B) amines, (C) polymers, (D) miscellaneous, (E) carboxylic acids, and (F) carbohydrates. The bars indicate the mean $\pm$ SD ($n = 3$). Different letters

indicate significant differences between treatments ($P < 0.05$).

PCR and recycling initial inspection

PCR electrophoresis bands of bacteria and fungi were pronounced, indicating the high accuracy of the DGGE profiles (Fig 5). The recycling electrophoresis bands of bacteria and fungi were also quite pronounced, also indicating the high accuracy of partial sequencing and NCBI alignment.

Fig 5. Comparison of the PCR and recycling initial inspection of bacteria and fungi in soil rhizosphere with different resistant mulberry genotypes treated with Kangqing10 normal and control group (RN); Guisang12, normal and control group (SN); Kangqing10-1 group (RS1); Guisang 12-1 group (SS1); Kangqing 10-2 group (RS2); Guisang 12-2 group (SS2); Kangqing 10-3 group (RS3); Guisang 12-3 group (SS3). (A) Bacterial productions of PCR amplification. (B) Bacterial productions of recycling initial inspection. (C) Fungal productions of PCR amplification. (D) Fungal productions of recycling initial inspection.

DGGE profiles analysis

Differences in DGGE profiles were evident between bacteria and fungi in the eight soil samples (Fig 6). Bands appearing in the bacteria DGGE profiles were significantly different from those in the fungi DGGE profiles; all were higher than those in the fungi DGGE profiles. The bands of bacteria and fungi of the RN rhizosphere genotypes were all more prominent than those of the RS1, RS2, and RS3 genotypes. The bands for bacteria and fungi of the SN rhizosphere genotypes were more prominent than those for the SS1, SS2, and SS3 genotypes. These results indicate that the microorganism community structures of normal genotype plants were richer than those of the sickly genotype plants. However, there was no obvious regularity between the bands of resistant and susceptible genotypes, which may be associated with the soil nutrients or cropping systems.

used.

Fig 6. DGGE profiles and results of the analysis of bacteria and fungi in soil rhizosphere with different resistant mulberry genotypes treated with 1-SS1 (Guisang 12-1 group) 2-RN (Kangqing10, normal and control group); 3-SN (Guisang12, normal and control group); 4-SS3 (Guisang 12-3 group); 5-RS3 (Kangqing 10-3 group); 6-RS2 (Kangqing 10-2 group); 7-RS1 (Kangqing10-1 group); 8-SS2 (Guisang 12-2 group).

Microbial community structure diversity index and evenness analysis in DGGE technology

The Shannon indices of microbial community structure are presented in Fig 7. The Shannon index of bacteria community structure of the RN rhizosphere genotypes was lower than that of the SN genotypes (SN). The Shannon index of bacterial community structure of the RS1, RS2, and RS3 rhizosphere genotypes was larger than those of the SS1, SS2, and SS3 genotypes, which was opposite to the Shannon index of soil microbial carbon source utilization. The Shannon index of the bacterial community structure of the RN rhizosphere genotypes was larger than those of the RS1, RS2, and RS3 genotypes, which is consistent with the Shannon index of soil microbial carbon source utilization. However, the Shannon index of bacterial community structure of the SN rhizosphere genotypes was larger than that of the (SS1, SS2, SS3 genotypes, with a significant difference in SS1 and SS2 ($P < 0.05$). These results contrasted those of the Shannon index of the soil microbial carbon source utilization. In contrast, the Shannon index of the fungal community structure of the RS1, RS2, and RS3 rhizosphere genotypes was lower than those of the SS1, SS2, and SS3 genotypes, which is consistent with the Shannon index of soil microbial carbon source utilization. The results for the other compared groups (RN with SN, RN with RS, and SN with SS)

were also consistent with the Shannon index of bacterial community structure, which was consistent with the Shannon index of soil microbial carbon source utilization. Furthermore, for all Shannon index values of bacterial and fungal community structure, the Shannon index of the SN genotypes was largest, followed by the Shannon index of the RN genotype, with the Shannon index of the SS genotypes showing the lowest value. These results contrast those of the Shannon index of soil microbial carbon source utilization. For susceptible genotypes, although the microbial community structure in normal genotype plants is richer than that in sickly genotype plants, the ability to utilize carbon sources is poor. The microbial community structure, particularly bacteria, is easily altered. In contrast, the microbial community structure in resistant genotypes, which better utilize the carbon, is more stable than that in susceptible genotypes. Thus, the resistant genotypes exhibit some resistance to bacterial wilt.

Data concerning the Shannon evenness of microbial community structure are shown in Fig 8. The Shannon evenness of bacterial community structure was larger than that of the fungal community structure. The Shannon evenness of bacterial community structure in the resistant and susceptible genotypes was consistent with that of the fungal community structure in resistant and susceptible genotypes. The Shannon evenness values of the bacterial and fungal community structure of the RN rhizosphere genotypes were both lower than those of the SN genotypes, but the Shannon evenness values of bacterial and fungal community structures of the RS1, RS2, and RS3 rhizosphere genotypes were larger than those of the SS1 and SS2 genotypes and significantly differed in SS1 ($P < 0.05$). The Shannon evenness values of the bacterial and fungal community structure of RN rhizosphere genotypes were both smaller than those of the RS1, RS2, and RS3 genotypes, but the Shannon evenness values of bacterial and fungal community structure of SN

rhizosphere genotypes were both larger than those of the SS1, SS2, and SS3 genotypes, except for the SS3 fungal genotype. Additionally, the Shannon evenness of the bacterial community structure of the SN genotype was significantly different in SS1 and SS2, and the Shannon evenness values of the fungal community structure of the SN genotypes both displayed a significant difference in SS1 ($P < 0.05$). The Shannon evenness values of the bacterial and fungal community structure were both opposite to the Shannon index of soil microbial carbon source utilization, further indicating that the microbial community structure in susceptible genotypes is not as stable as that in resistant genotypes which show some resistance to bacterial wilt.

Fig 7. Comparison of the Shannon index (H) of bacterial and fungal community structure in soil rhizosphere with different resistant mulberry genotypes treated with CK: Kangqing10, normal and control group (RN), Guisang12, normal and control group (SN); T1: Kangqing10-1 group (RS1), Guisang 12-1 group (SS1); T2: Kangqing 10-2 group (RS2), Guisang 12-2 group (SS2); T3: Kangqing 10-3 group (RS3), Guisang 12-3 group (SS3). Substrates: (A) amino acids, (B) amines, (C) polymers, (D) miscellaneous, (E) carboxylic acids, and (F) carbohydrates. The bars indicate the mean $\pm$ SD ($n = 3$). Different letters indicate significant differences between treatments ($P < 0.05$).

Fig 8. Comparison of the Shannon evenness (E) of bacterial and fungal community structure in soil rhizosphere with different resistant mulberry genotypes treated with CK Kangqing10, normal and control group (RN), Guisang12, normal and control group (SN); T1: Kangqing10-1 group (RS1), Guisang 12-1 group (SS1); T2: Kangqing 10-2 group (RS2), Guisang 12-2 group (SS2); T3: Kangqing 10-3 group (RS3), Guisang 12-3 group (SS3). Substrates: (A) amino acids, (B) amines, (C) polymers, (D) miscellaneous, (E) carboxylic acids, and (F) carbohydrates. The bars indicate the mean $\pm$ SD ($n = 3$). Different letters indicate significant differences between

379 treatments ($P < 0.05$).

380 **Partial sequencing and NCBI alignment**

381 NCBI alignments of bacteria and fungi are shown in Table 3 and 4, respectively. There were more
382 bacterial species than fungal species in the soil rhizosphere with different resistant mulberry
383 genotypes. *Ralstonia solanacearum* was found in the rhizosphere of resistant and susceptible
384 genotypes, indicating that bacterial wilt likely occurred because of the destruction of the bacterial
385 community structure in mulberry rhizosphere soil by *R. solanacearum*.

386 **Table 3**

387 *NCBI alignment of bacteriarandomly recovered from the bacterial DGGE profiles*

Treatments	Community structure of bacteria
A1-1(SS1-1)	Staphylococcus(98%)
A5-2(RS3-2)	Flavobacteria(93%)Sphingobacteria(93%)
A8-3(SS2-3)	Chryseobacterium(99%)
A5-5(RS3-5)	proteobacterium(99%)
A1-7(SS1-7)	Pseudomonas(94%)
A1-8(SS1-8)	Pseudomonas(96%)
A8-9(SS2-9)	Acidobacteria(97%)
A1-10(SS1-10)	Ralstonia solanacearum(99%)
A6-10(RS2-10)	Ralstonia solanacearum(99%)
A8-11(SS2-11)	Rhizobium(100%)Agrobacterium(100%)
A8-12(SS2-12)	Rhizobium(99%)Agrobacterium(99%)
A8-13(SS2-13)	Rhizobium(88%)proteobacterium(88%)

A5-14(RS3-14)	Candidatus Glomeribacter gigasporarum(92%)
B8-1(SS2-1)	Sphingomonadaceae bacterium(97%)
	Bacteroidetes bacterium(97%)
B1-2(SS1-2)	Ralstonia solanacearum(100%)
B6-2(RS2-2)	Ralstonia solanacearum(100%)
B8-3(SS2-3)	Rhizobium(99%)
B8-4(SS2-4)	Rhizobium(99%)
B8-5(SS2-4)	Rhizobium(95%)
B5-6(RS2-6)	Chujaibacter soli strain(99%)
C5-8(RS2-8)	proteobacterium(95%)Pelagibacter(95%)

388 **Table 4**

389 *NCBI alignment of fungal randomly recovered from the fungal DGGE profiles*

Treatments	Community structure of fung
A1-1(SS1-1)	Fusarium(99%)
A1-2(SS1-2)	Diplodia mutila,Guignardia,Phyllosticta,Lasiodiplodia,Melanops,Botryosphaeria laricina(99%)
A1-3(SS1-3)	Entomobryomorpha(98%)
A2-1(RN-1)	Tectocephus(99%)
A2-2(RN-2)	Tectocephus(98%)
A2-3(RN-3)	Enchytraeus(94%)
A3-1(SN-1)	Ramicandelaber(99%)
A3-2(SN-2)	Benoinyssus(92%)
A3-4(SN-4)	Benoinyssus(91%)

A3-5(SN-5)	Benoinyssus(91%)
A3-6(SN-6)	Benoinyssus(90%)
A3-7(SN-7)	Enchytraeidae(99%)
A7-1(RS1-1)	Enchytraeus(99%)
B1-1(SS-1)	Fusarium(99%)
B1-2(SS-2)	Cryptopygus sverdrupi(97%)
B1-3(SS-3)	Nematoda(98%)
B1-4(SS-4)	Enchytraeus(94%)
B1-5(SS-5)	Enchytraeus(99%)
B2-2(RN-1)	Enchytraeus(97%)
B5-2(RS3-2)	Enchytraeus(99%)

Correlation regression analysis between soil microbial community structure and soil nutrients

Correlation regression analysis data between bacterial and fungal community structure and soil nutrients are presented in Table 5 and 6, respectively. The bacterial and fungal community structures of mulberry resistant rhizosphere genotypes were significantly correlated with soil organic matter ($R=0.9697$ and 0.9666 , respectively) and alkaline nitrogen ($R=0.9519$ and 0.9759 , respectively), but displayed a weak correlation with available soil phosphorus and potassium. The bacterial and fungal community structures of the mulberry susceptible genotypes in rhizosphere were also significantly correlated with soil organic matter and alkaline nitrogen, except for bacterial alkaline nitrogen. This may be because of the high humidity, temperature, and rainfall in Southern China. More frequent precipitation and higher humidity accelerate soil decomposition

and mineralization. As a result, the occurrence of mulberry bacterial wilt may be related to the loss of soil nutrients, particularly soil organic matter and nitrogen. These losses may cause an imbalance in the soil microbial community structure. In contrast, the correlations between the bacterial and fungal community structures of mulberry resistant genotypes in the rhizosphere and soil organic matter ($R=0.9697$ and 0.9666 , respectively) and alkaline nitrogen ($R=0.9519$ and 0.9759 , respectively) were greater than those between the bacterial and fungal community structures of mulberry susceptible genotypes in the rhizosphere and soil organic matter ($R=0.8808$ and 0.8993 , respectively) and alkaline nitrogen ($R=0.0745$ and 0.6899 , respectively). These results indicate that the loss of soil organic matter and nitrogen has a lower impact on resistant genotypes than on susceptible genotypes. Thus, the bacterial and fungal community structures in resistant genotypes are more stable than in susceptible genotypes.

Table 5

Correlation regression analysis of that between soil nutrients and soil bacterial community structure in rhizosphere treated with resistant genotypes (Kangqing10) and susceptible genotypes (Guisang12), including alkaline N, alkaline P, alkaline K, and soil organic matter (SOM).

			Available			
Bacteria			Alkaline N	P	Available K	SOM
Resistant	genotypes	$R^{\dagger}$	0.9519	0.7734	0.7715	0.9697
	(Kangqing10)	$P^{\ddagger}$	0.0126	0.0712	0.0723	0.0003
Susceptible	genotypes	R	0.0745	0.4516	0.4962	0.8808
	(Guisang12)	P	0.0830	0.4452	0.3168	0.0485

$\dagger$ Correlation coefficient in correlation regression analysis (R).

417 ‡Value for the rejection of the null hypothesis (P).

418 Table 6

419 Correlation regression analysis of that between soil nutrients and soil fungal community structure
420 in rhizosphere treated with resistant genotypes (Kangqing10) and susceptible genotypes
421 (Guisang12), both including alkaline N,alkaline P, alkaline K and soil organic matter (SOM)

Fungi			Alkaline N	Available P	Available K	SOM
Resistant	genotypes	R†	0.9759	0.7521	0.7472	0.9666
	(Kangqing10)	P‡	0.0009	0.0846	0.0878	0.0017
Susceptible	genotypes	R	0.6899	0.5845	0.4470	0.8993
	(Guisang12)	P	0.1974	0.1682	0.3741	0.0378

422 †Correlation coefficient in correlation regression analysis (R).

423 ‡Value for the rejection of the null hypothesis (P).

424 Discussion

425 The health of plants is affected by interactions among plants, the soil environment, and soil
426 microorganisms. Changes in one or more oftheelements can lead to plant diseases. As a dynamic
427 part in the plant-soil system,the homeostasis of soil microorganisms is extremely important for
428 controlling bacterial wilt and other soil-borne diseases [37-42]. Resistant mulberry genotypes are
429 more stable than susceptible mulberry genotypes and exhibit better protection against bacterial
430 wilt. Thus, the incidence of bacteria wilt is relatively low. Recent studies have reported that
431 eggplant, tobacco, and pepper plants with a resistant genotypehave a significantly reduced
432 incidence of bacterial wilt [43-47]. The present study was conducted to explorethe relationship
433 between the occurrence of bacterial wilt and abundance and community structure diversity of soil
434 microorganisms with plants of different genotypes. The genotypes included resistant normal

435 mulberry genotype plants(RN), susceptible normal mulberry genotype plants (SN), resistant sickly
436 mulberry genotype plants(RS), and susceptible sickly mulberry genotype plants (SS).This is the
437 first study to show similar changes in the abundance and carbon source utilization of soil
438 microorganisms in mulberry genotypes resistant or susceptible to bacterial wilt. Additionally,
439 changes in microbial community structures were significantly correlated with soil organic
440 matterand alkaline nitrogen. For normal plants, the abundance of bacteria in resistant mulberry
441 genotypes was higher than that in susceptible mulberry genotypes. For sickly plants, the
442 abundance of bacteria in resistant mulberry genotypes was lower than that in susceptible mulberry
443 genotypes. Average well color development values and the Shannon index of carbon source
444 utilization revealed significantly better utilization of carbon sources by resistant normal mulberry
445 rhizosphere genotypes than by susceptible normal mulberry genotypes. In contrast, carbon sources
446 utilization in the RS rhizosphere genotypeswas poorer than that in the SS genotypes. These
447 observations are related to the abundance of soil microorganisms. Irikiin Y [48] has suggested that
448 the occurrence of tomato bacterial wilt is related to the pattern of carbon source utilization by
449 rhizosphere soil bacteria. The present PCR-DGGE examination revealed that the microbial
450 community structures of RN rhizosphere genotypes were not as rich as those of SN genotypes.
451 However, the microbial community structure of the RS rhizosphere genotypes was richer than that
452 ofthe SS genotypes. Finally, regression correlation analysis of the soil microbial community
453 structure and soil nutrients revealed that loss of soil organic matter and nitrogen may have a
454 greater negative impact on susceptible mulberry genotype plants than on resistant mulberry
455 genotype plants, and the microbial community structures in resistant mulberry genotype plants are
456 more stable than those in susceptible mulberry genotype plants.

The collective results of previous [49, 50] and the present study suggest that the occurrence of mulberry bacterial wilt is related to the loss of soil nutrients, particularly organic matter and nitrogen, which can disrupt the soil microbial community structure. However, these losses have a lower impact on resistant genotype plants than on susceptible genotype plants. Therefore, resistant genotype plants have some resistance to bacterial wilt. A previous study [51] indicated that the rate of utilization of soil nutrients is greater in resistant genotype plants than in susceptible genotype plants. In the current study, carbon utilization in resistant genotype plants was better than that in susceptible genotype plants. Our empirical data indicate that susceptible genotype plants are more dependent on soil nutrients than are resistant genotype plants.

Our results will be useful for developing methods to prevent and control bacterial wilt involving the application of organic fertilizer and nitrogen-containing fertilizer. These fertilizers can revitalize nutritionally depleted soil. Mulberry trees display growth vitality that can naturally prevent and control bacterial wilt naturally. Further studies in our lab will focus on the micro-ecological mechanisms of mulberry bacterial wilt, emphasizing the microbes in the surrounding rhizosphere by choosing resistant genotype varieties and appropriately applying organic fertilizer or nitrogen fertilizer.

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Carbon source

CK(RN,SN) T1(RS1,SS1)
T2(RS2,SS2) T3(RS3,SS3)

Bacteria

Bacteria

Fungi

Fungi

Bacteria

CK(RN,SN) T1(RS1,SS1)
T2(RS2,SS2) T3(RS3,SS3)

Fungi

CK(RN,SN) T1(RS1,SS1)
T2(RS2,SS2) T3(RS3,SS3)

Bacteria

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T2(RS2,SS2) T3(RS3,SS3)

Fungi

CK(RN,SN) T1(RS1,SS1)
T2(RS2,SS2) T3(RS3,SS3)

M RN RS1 RS2 RS3 SN SS1 SS2 SS3

A. Bacterial productions of PCR amplification

M RN RS1 RS2 RS3 SN SS1 SS2 SS3

B. Bacterial productions of recycling initial inspection

M RN RS1 RS2 RS3 SN SS1 SS2 SS3

C. Fungal productions of PCR amplification

M RN RS1 RS2 RS3 SN SS1 SS2 SS3

D. Fungal productions of recycling initial inspection

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M RN RS1 RS2 RS3 SN SS1 SS2 SS3

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■ T1(RS1,SS1)

□ T2(RS2,SS2)

▨ T3(RS3,SS3)

C**CK(RN,SN)****T1(RS1,SS1)****T2(RS2,SS2)****T3(RS3,SS3)**

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