Endogenous bacterial community structure of *Pinus massoniana* with differing resistance to pine wilt disease

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Summary – Pine wilt disease (PWD) is a devastating pine disease caused by *Bursaphelenchus xylophilus* and its main host in China is *Pinus massoniana*. The relationship between endophytic bacteria and disease resistance in *P. massoniana* remains unclear. In this paper, the leaves, roots, stems and treetops of different disease-resistant *P. massoniana* were studied as the research objective and Illumina MiSeq sequencing was used to analyse whether there were significant differences in the composition and diversity of endophytic bacterial communities between different disease-resistant *P. massoniana*. The results showed that at the genus level there were no obvious differences in the composition of the endophytic bacterial community of different disease-resistant *P. massoniana* in the leaves, but there were obvious differences in the roots, stems and treetops. The richness and diversity of endophytic bacteria in *P. massoniana* had no significant impact on its disease resistance, whilst the structure of endophytic bacterial community in stems and treetops may be related to its disease resistance.

Keywords – *Bursaphelenchus xylophilus*, China, endophytic bacteria diversity, Illumina MiSeq sequence, pine wood nematode.

Pine wilt disease (PWD) is one of the most destructive diseases in trees of the genus *Pinus* and is responsible for environmental and economic losses around the world. The only known causal agent of the disease is the pine wood nematode (*PWN*) *Bursaphelenchus xylophilus* (Vicente et al., 2012; Proença et al., 2017a). The tree-to-tree natural transmission of *B. xylophilus* is only possible through *Monochamus* beetles. The *B. xylophilus* enters in its dispersal form into *Monochamus* when the beetle larva molts to the late pupa (Alves et al., 2016). Previous studies showed that PWD symptoms and histological changes in the pine host begin to appear before the *B. xylophilus* population increases, which led to the hypothesis of a role for *B. xylophilus*-related bacteria in the development of PWD (Oku et al., 1980; Kawazu et al., 1996). Studies using metagenomics methods found that *B. xylophilus*-related bacteria played a role in detoxification of xenobiotics inside conifers, indicating that there is a mutualistic symbiotic relationship between *B. xylophilus* and its microbiome (Alves et al., 2016). Bacteria associated with *B. xylophilus* may also help the *B. xylophilus* tolerate the tree’s defence metabolites (Cheng et al., 2013). In addition, bacterial lipases may mediate the production of systemic signalling molecules that trigger plant resistance (Proença et al., 2010). *Serratia quinivorans* BXF1 was found to be widely associated with *B. xylophilus* and its insect vectors, possibly obtained from the endophytic bacterial community of the host pine; the strain can also be used as a pine endophyte that has fungal and bacterial antagonistic activity and promotes plant growth (Nascimento et al., 2016, 2018). In short, the tree microbiota has been shown to have the potential to help the tree’s defence, but it may also change its role as the disease progresses, eventually leading to tree rot (Alves et al., 2018); the pine endophytic bacteria and the bacteria carried by the *B. xylophilus* may participate in the pathogenesis of the disease.

Plants constitute a very complex micro-ecosystem, including different tissues or intercellular spaces from roots to buds and leaves, all of which may be occupied by microorganisms. The microbial communities associated with organisms have long attracted the attention of researchers, especially the interaction between bacteria and hosts. The plant microbiome has been shown to play an important role in modulating plant growth and development, as well as stress response and resistance. Endo-
Phytophthora parasitica has been shown to provide different services to the plants; besides acting as plant growth promoters, they can exhibit strong anti-fungal activity, antagonise bacterial pathogens and control plant-parasitic nematodes (Hardoin et al., 2015; Proença et al., 2017b). According to reports, infected trees may exert a selective pressure on the plant microbiome, modulating the bacterial community structure and selecting for resistant microorganisms (Huot et al., 2013; Alves et al., 2018). Therefore, the health of the organism is considered to be the result of the interaction between the microorganisms and its host (Turner et al., 2013).

The resistance of *P. massoniana* from different provinces and different pine species to *B. xylophilus* and the course of disease are very different (Wang et al., 1997). Different pine species from different places have physiological differences that will affect their microbiota (Geib et al., 2009). In terms of other diseases, studies have also pointed out that the richness and diversity of endophytic microbial communities are very different between resistant and sensitive varieties of some plants (Li et al., 2019). Based on this, we hypothesised that the resistance of pine trees to *B. xylophilus* may be related to their endophytic bacteria, and tried to look at the relationship between PWD and endophytic bacteria from the perspective of the host. To find out whether there are differences in the community structure of endophytic bacteria in different disease-resistant *P. massoniana*, the V5-V7 region amplicon sequencing of the 16S rRNA gene was used to study endophytic bacteria community structure in different disease-resistant *P. massoniana* to understand further the relationship between endophytic bacteria in *P. massoniana* and PWD.

**Materials and methods**

**PREPARATION OF Bursaphelenchus xylophilus INOCULUM**

*B. cinerea* was inoculated on potato dextrose agar (PDA) medium in a Petri dish (diam. 90 mm) and placed in a 25°C incubator until the hyphae filled the entire medium, then *B. xylophilus* were inoculated on *B. cinerea* and placed in the 25°C incubator for 1 week. Cultured *B. xylophilus* were separated by the Baermann funnel method. Liquid containing *B. xylophilus* was collected in a 15 ml centrifuge tube, washed once with sterile distilled water, and the suspension was adjusted to the required concentration. This nematode strain AMA3 was isolated from the infected *P. elliottii* of Ma’an mountain in Anhui Province, P.R. China.

**DETERMINATION OF DISEASE RESISTANCE OF P. massoniana FROM DIFFERENT PROVINCES**

In April 2019, 2-year-old *P. massoniana* potted seedlings were purchased from seedling bases in five different provinces (Suzhou Jiangsu, Suqian Jiangsu, Hechi Guangxi, Liuzhou Zhejiang, Wenzhou Zhejiang) and planted in a glasshouse at Nanjing Forestry University, China. In June of the same year, *B. xylophilus* was inoculated. A sterile blade was used to cut a small wound deep into the xylem of *P. massoniana* stems approximately 15 cm near the ground, and a sterile cotton ball was inserted. Then, the incision was covered with a cotton ball and funnel-shaped Parafilm. The inoculation amount of *B. xylophilus* was 5000 nematodes plant\(^{-1}\) with 10 plants treatment\(^{-1}\) and sterile water was inoculated as a control. Seedling growth was regularly observed every day after inoculation and a disease index was calculated. Disease severity was classified as follows: 0, all leaves were green; I, a few leaves had turned yellow; II, approximately half of the leaves had turned yellow or brown; III, most of the leaves had turned brown; and IV, the entire seedling was withered. The formula for calculating the disease index was as follows: \[\sum (\text{number of disease plants} \times \text{symptom stage}) \times 100/(\text{total number of plants} \times \text{highest symptom stage})\] (Fu et al., 2020).

**COLLECTION AND PRETREATMENT OF P. massoniana SAMPLES**

*Pinus massoniana* with obvious differences in disease resistance were selected from three provinces and 1 g samples were taken from tissues of the leaves, roots, stems and treetops (the tip of a seedling) of healthy seedlings, three seedlings from each province were used as three biological replicates. The collected samples were washed with sterile water for 30 s, soaked in 70% ethanol for 2 min, soaked with 2.5% NaClO (containing 0.1% Tween-80) for 5 min, transferred to 70% ethanol for 30 s, and finally washed with sterile water three times. Surface-sterilised pine seedling tissues were frozen in liquid nitrogen and then stored in a refrigerator at \(\sim 80°C\) for future use (Bodenhausen et al., 2013; Beckers et al., 2016).
**DNA EXTRACTION AND PCR AMPLIFICATION OF SAMPLES**

Microbial DNA was extracted from *P. massoniana* samples using the FastDNA SPIN Kit for Soil (MP Biomedicals) according to the manufacturer’s protocols. The final DNA concentration and purification were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific) and DNA quality was checked by 1% agarose gel electrophoresis. The first round of PCR amplification was performed with 799F (5′-AACMGATATACCGKGG-3′) and 1392R (5′-ACGATTAGATACCCGAC-3′) primers. The first round of the amplification program was pre-denaturation at 95°C for 2 min, 27 cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s) and finally an extension at 72°C for 10 min. The V5-V7 variable region was amplified in a second round of PCR with 799F (5′-AACMGATATACCGKGG-3′) and 1193R (5′-ACGGGCGGTGTGTRC-3′) primers. The second round of the amplification program was: pre-denaturation at 95°C for 3 min, 13 cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s) and an extension at 72°C for 10 min. The PCR amplification volume was 4 μl 5 × FastPfu buffer, 2 μl 2.5 mM dNTPs, 0.8 μl forward primer (5 μM), 0.8 μl reverse primer (5 μM), 0.4 μl FastPfu polymerase, 0.2 μl BSA, and 10 ng template DNA; ddH2O was supplemented to 20 μl.

**ILLUMINA MISEQ SEQUENCING AND SEQUENCE DATA PROCESSING**

The resulting PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences) and quantified using QuantiFluor™-ST (Promega) according to the manufacturer’s protocol. Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina) according to the standard protocols by Majorbio Bio-Pharm Technology. The raw fastq files were quality-filtered by Trimmomatic and merged by FLASH and the effective data were finally obtained through the above steps. After obtaining effective data, Operational Taxonomic Units (OTU) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) with a novel greedy algorithm that performs chimera filtering and OTU clustering simultaneously. The taxonomy of each 16S rDNA sequence was analysed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the silva132/16s_bacterial database using a confidence threshold of 70% and subsampled by the minimum number of sample sequences. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (accession number PRJNA642684). Sequencing was completed by Majorbio Bio-Pharm Technology.

The disease index diagram was made using Excel. The sample data were analysed by mothur (version v.1.30.1), and Alpha diversity indices such as Chao, Shannon, Sobs were obtained, and SPSS 20.0 software was used for one-way ANOVA. The dilution curve diagram was made by R Programming Language. Based on the data table in the tax_summary_a folder, Community Bar diagrams were produced by R Programming Language. Statistics and production of Venn diagrams were obtained through R Programming Language. Principal co-ordinates analysis (PCoA) and Anosim analysis were performed through R Programming Language. Linear effect size (LEfSe) discriminant analysis was performed through software LEfSe (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload).

**Results**

**DISEASE RESISTANCE OF P. MASSONIANA FROM DIFFERENT PROVINCES**

*Pinus massoniana* from all five provinces had no symptoms after inoculation with sterile water. The disease indexes from 17-30 days after inoculation (DAI) with *B. xylophilus* are shown in Figure 1. The disease indexes of the *P. massoniana* from the five provinces of Suqian Jiangsu, Hechi Guangxi, Suzhou Jiangsu, Wenzhou Zhejiang and Liuzhou Guangxi were 20, 42.5, 60, 67.5 and 75 on the 30th DAI. To understand the relationship between endophytic bacteria of *P. massoniana* and its disease resistance more clearly, *P. massoniana* from three of the provinces, Suqian Jiangsu, Hechi Guangxi and Liuzhou Guangxi were selected as follow-up research materials. The disease resistance of *P. massoniana* from Suqian Jiangsu, Hechi Guangxi and Liuzhou Guangxi was high, moderate and low, respectively.

**NUMBER OF ENDOPHYTIC BACTERIA SEQUENCES OF P. MASSONIANA**

A total of 36 samples was obtained by sampling different tissues of *P. massoniana*. A total of 784 592 sequences was obtained from all samples and the average length
Fig. 1. Disease indexes of *Pinus massoniana* from the different provinces 17-30 days after inoculation with *Bursaphelenchus xylophilus*.

Fig. 2. Individual rarefaction curves for different disease-resistant *Pinus massoniana* samples. In the key, 1 is high resistance, 2 is moderate resistance and 3 is low resistance. Three lines of the same colour represent three repetitions.

of sequences was 375 bp (Supplementary Table S1). All sequences were assigned to 27 phyla, 52 classes, 160 orders, 323 families, 635 genera, 1071 species and 1736 OTUs in bacterial taxonomy. By drawing the rarefaction curves at the OTU level, the sequencing depth of the samples could be obtained. With the increase of sequencing quantity, all sample curves tended to level, indicating that the sequencing of each sample basically reached the saturation state with satisfactory confidence (Fig. 2).

**ENDOPHYTIC BACTERIAL RICHNESS AND DIVERSITY IN DIFFERENT DISEASE-RESISTANT *P. MASSONIANA***

The community richness of three disease-resistant *P. massoniana* leaves was moderate resistance > high resistance > low resistance; of the roots it was high resistance > low resistance > moderate resistance; of the stems it was low resistance > high resistance > moderate resistance; and of the treetops it was high resistance > low resistance > moderate resistance. The endophytic bacte-
Bacterial community structure of Pinus massoniana

Fig. 3. Histograms of Chao test for endophytic bacterial richness of different disease-resistant Pinus massoniana; A: Leaf; B: Root; C: Stem; D: Treetop. OTU = Operational Taxonomic Units. In the key, 1 is high resistance, 2 is moderate resistance and 3 is low resistance. Bars = standard errors. Means are not significantly different, $P > 0.05$.

Endophytic bacterial community structure of different disease-resistant Pinus massoniana at the phylum and genus level

Species with less than 1% abundance in all samples were merged into others at the phylum level, species with less than 5% abundance in all samples were merged into others at the genus level, and the community bar map was generated after calculating the mean value of repeated samples within the group (Fan, 2015; Zachow et al., 2016; Cheng, 2018). There were six main phyla with a relative abundance of more than 1%, which included Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Chloroflexi and Armatimonadetes (Fig. 5A). The abundance of the main phyla in the leaves, roots and treetops of three disease-resistant Pinus massoniana were all similar. The Proteobacteria in the stems of low resistant Pinus massoniana was relatively low and Actinomycota was relatively high. The abundance of the remaining phyla in the stems of three disease-resistant Pinus massoniana were similar.

There were 17 genera with a relative abundance of more than 5% (Fig. 5B). The dominant genera were Burkholderia, Ralstonia, Methylobacterium, Sphingomonas, Kosakonia and Brevundimonas, among others. The compositions of endophytic bacterial species in the leaves of the three disease-resistant Pinus massoniana were similar and their abundance was also similar. In stems and treetops of the three disease-resistant Pinus massoniana, the composition of endophytic bacteria species was similar but there were obvious differences in abundance. There were obvious differences in the composition of species and abundance...
of endophytic bacteria in the roots of the three disease-resistant *P. massoniana*.

**Endophytic bacterial community structure of different disease-resistant *P. massoniana* at the OTU level.**

A Venn diagram was constructed at the OTU level to analyse the common and unique species of three disease-resistant *P. massoniana* (Zhou et al., 2018; Wang et al., 2019). A total of 825 OTUs was detected in the three disease-resistant *P. massoniana* leaves, and by analysing the ratio of the unique OTUs to the total OTUs, the endophytic bacteria from leaves had the highest specificity in the moderately resistant *P. massoniana* (27.2%). A total of 1025 OTUs was detected in the roots, and the endophytic bacteria from roots had the highest specificity in the low resistant *P. massoniana* (18.2%). A total of 1020 OTUs was detected in the stems, and the endophytic bacteria from stems had the highest specificity in the low resistant *P. massoniana* (17.4%). A total of 817 OTUs was detected in the treetops, and the endophytic bacteria from treetops had the highest specificity in the highly resistant *P. massoniana* (23.0%) (Fig. 6).

Principal co-ordinates analysis (PCoA) is a non-constrained data dimensionality reduction analysis method that can be used to study similarities or differences in sample community composition (Wang et al., 2012). We used PCoA analysis at the OTU level and the distance algorithm Abund_Jaccard (Fig. 7). The results showed that only the roots of low resistant *P. massoniana* were completely distinguished from other roots, while the leaves, stems and treetops of the three disease-resistant *P. massoniana* were not completely distinguished. Anosim analysis was a nonparametric test used to test whether the differences between groups were significantly greater than the differences within groups to judge whether grouping was meaningful. We used Anosim analysis at the OTU level and the distance algorithm Abund_Jaccard (Fig. 8). The results showed that there were more intra-group differences than inter-group differences in the leaves and roots of three disease-resistant *P. massoniana* and group-
Bacterial community structure of Pinus massoniana

Fig. 5. Relative abundance of endophytic bacteria at the phylum (A) and genus (B) level in different disease-resistant Pinus massoniana. 1, 2, 3 designations are as in Figure 3.

ing was not meaningful. There were significant differences in the stems of three disease-resistant P. massoniana ($P = 0.023$), and significant differences in the treetops of three disease-resistant P. massoniana ($P = 0.016$) (Supplementary Table S3).

DIFFERENT SPECIES OF ENDOPHYTIC BACTERIA IN DIFFERENT DISEASE-RESISTANT P. MASSONIANA

LEfSe can be used to discover significant differences in the species composition of two or more groups of
samples and to analyse the magnitude of the impact of these different species on group discrimination (Jin et al., 2019). In the LEfSe analysis of endophytic bacteria in stems and treetops of the three disease-resistant *Pinus massoniana*, the LDA threshold was 2.0, classification level was from phylum to species, and an ‘all-against-all’ strategy was adopted for comparison (Fig. 9). The results showed that *Burkholderia*, *Rothia*, *Brachybacterium* and *Micrococcus* were more abundant in the stems of highly resistant *P. massoniana*. *Paracraurococcus* and *Adhaeribacter* were more abundant in the stems of moderately resistant *P. massoniana*. *Jatrophihabitans*, *Candidatus*, *Alsiosphaera* and *Bacillus* were more abundant in the stems of low resistant *P. massoniana*. *Azospirillum*, *Ruegeria*, *Paenibacillus*, *Cobetia*, *Novosphingobium*, *Halocynthiibacter* and *Haliangium* were more abundant in the treetops of highly resistant *P. massoniana*. *Methylorosula* was more abundant in the treetops of moderately resistant *P.
massoniana. *Pantoea* and *Romboutsia* were more abundant in the treetops of low resistant *P. massoniana*.

**Discussion**

Endophytic microbiota are shaped by both the host plant and environmental stimuli and, in turn, may enhance the biotic and abiotic tolerance of their host plants as a multispecies functional unit (Podolich *et al*., 2015). It has been proposed that endophytes can activate the plant immune system, inhibit pathogens through competition, produce antibacterial substances, and improve resistance to adverse environments and pests (Estrada *et al*., 2013; Mejia *et al*., 2014; Pieterse *et al*., 2014). Pine endophytic bacteria were suggested to be part of the plant-nematode interaction by growth promotion or induction of resistance in pine trees (Nascimento *et al*., 2015). The rich microbial community of the pine tree can represent a wide untapped resource of functional characters with potential uses for environmentally safe management of this devastating...
Fig. 8. Box plot of distance between treatment groups of different disease-resistant *Pinus massoniana* samples; A: Leaf; B: Root; C: Stem; D: Treetop. Between box refers to the difference between groups and the others represent the difference within each group. In the key, 1 is high resistance, 2 is moderate resistance and 3 is low resistance.
Fig. 9. Linear Effect Size (LEfSe) discriminant analysis of endophytic bacteria in different disease-resistant Pinus massoniana; A: Stem; B: Treetop. In the key, 1 is high resistance, 2 is moderate resistance and 3 is low resistance.

disease (Kim et al., 2019). However, there are few studies on the relationship between endophytic bacteria and disease resistance in P. massoniana. Therefore, in this study, we focused on whether there are differences in the community structure of endophytic bacteria in different tissues of P. massoniana with different disease resistance. The main purpose is to understand whether the community structure of endophytic bacteria in P. massoniana affects the resistance to B. xylophilus.

The abundance and diversity of endophytic microbial communities vary substantially between resistant and susceptible cultivars of some plants (Tan et al., 2006), a diverse and balanced microbial system would be more conducive to disease resistance (van Elsas et al., 2012).

A study on tomato showed that the diversity of endophytic bacteria in seedling roots of resistant cultivars was higher and more antagonistic bacteria were found, which may play a role in natural defence against pathogens (Upreti & Thomas, 2015). The abundance of endophytic bacteria in apple leaves had no significant effect on the resistance of apple cultivars to Alternaria leaf spot, but the abundance of endophytic fungi had a significant effect on it (Hirakue & Sugiyama, 2018). The difference in resistance of rape varieties to pathogens may be related to the quality and quantity of endophytic microbial population (Graner et al., 2003). However, this study showed that the abundance and diversity of endophytic bacteria had no significant effect on the disease resistance of P. massoniana.
Endophytic bacteria that are beneficial to plant growth and development are found across many phyla, including the Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Kandel et al., 2017). The endophytic bacteria composition of *P. massoniana* also includes these phyla, and the abundance of Proteobacteria is the highest in all tissues. Proteobacteria is the most prevalent phylum of endophytic bacteria in plants (Rangjaroen et al., 2014), and this result is consistent with most research results in China and abroad. In this study, there were no obvious differences in the species composition and abundance of endophytic bacteria between the four tissues of three disease-resistant *P. massoniana* at the phylum level. The endophytic bacterial communities are similar within a plant species and even between contemporary cultivars and ancestors, a systemic study of endophytic microbiome of *Arabidopsis thaliana* plants clearly demonstrating that the core microbiome is linked with plant host genotype and is consistent across different soil types and developmental stages (Podolich et al., 2015). In European *P. sylvestris*, the major genera of endophytic bacteria found were *Methylobacterium*, *Pseudomonas*, *Bacillus* and *Paenibacillus*. In China, the main bacteria associated with *B. xylophilus* isolated from infected trees were *Burkholderia*, *Enterobacter*, *Pantoea*, *Peptostreptococcus* and *Pseudomonas* (Proença et al., 2017a, b). In this study, the community structure of endophytic bacteria of three disease-resistant *P. massoniana* differed in different tissues, but there were no differences in leaves. Studies have shown that geographical distribution has little effect on
the microbial community of *P. ponderosa* leaves (Redford et al., 2010). This study found a higher abundance of *Azospirillum* in treetops of highly resistant *P. massoniana*, and endophytic bacteria of the *Azospirillum* as a major component confer resistance to diseases and pests in gramineous plants (Mei & Flinn, 2010). *Azospirillum* may play an important role in the resistance of *P. massoniana* to PWD. In addition, endophytic bacteria of stems had the highest specificity in low resistant *P. massoniana*, which may indicate that endophytic bacteria in the stems of low resistant *P. massoniana* play a role in promoting the development of PWD.

PWD-infected trees exhibit reduced photosynthesis, resin canal formation disruption, and malformations in the xylem, cambium, and cortex tissues (Fukuda, 1997). The results of artificial inoculation experiments showed that *B. xylophilus* entered pines first through the cortical resin channels, destroyed the cells around the cortical resin channels into the cortical tissues, and then entered the xylem resin channels. However, if the wound at the inoculation site reaches deep into the xylem, *B. xylophilus* can directly enter the axial and radial resin channels of the xylem (Zhao et al., 2003). It has been reported that in pine infected by *B. xylophilus*, the content of *B. xylophilus* in the crown branch is more than that in other tissues within 1 month after the initial symptoms appear, and the distribution of *B. xylophilus* in pine tends to be more uniform later (Jing, 2007). The distribution of *B. xylophilus* in the tree body is tree-wide and can exist in almost all of the resin channel system, except for pine needles, pine buds, and cones in the current year where *B. xylophilus* has not been isolated (Long, 2007). Compared with the more sensitive pine trees, the migration of *B. xylophilus* in resistant pine trees was slowed down in the cortex and restricted in the xylem axial resin channels, which could partly explain the resistance of some pine trees to *B. xylophilus*. In addition to morphological differences between susceptible and resistant pine trees, other key factors may also be involved (Son et al., 2015). The results of the present study showed that there were significant differences in endophytic bacterial community structure in stems and treetops of three disease-resistant *P. massoniana*. In the experiment, *B. xylophilus* was inoculated in the stems where *B. xylophilus* mainly propagates and spreads, eventually migrating to the treetops. Therefore, it is speculated that the difference in disease resistance of *P. massoniana* is related to the endophytic bacterial community structure of stems and treetops. This study revealed the composition of endophytic bacteria community structure of *P. massoniana* with different disease resistance, and further research is needed to clarify how endophytic bacteria affect the pathogenesis of PWD.

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**References**

Alves, M., Pereira, A., Matos, P., Henriques, J., Vicente, C., Aikawa, T., Hasegawa, K., Nascimento, F., Mota, M., Correia, A. et al. (2016). Bacterial community associated to the pine wilt disease insect vectors Monochamus galloprovincialis and Monochamus alternatus. *Scientific Reports* 6, 23908. DOI: 10.1038/srep23908

Alves, M., Pereira, A., Vicente, C., Matos, P., Henriques, J., Lopes, H., Nascimento, F., Mota, M., Correia, A. & Henriques, I. (2018). The role of bacteria in pine wilt disease: insights from microbiome analysis. *FEMS Microbiology Ecology* 94, fiy077. DOI: 10.1093/femsec/fiy077

Beckers, B., De Beeck, M.O., Thijs, S., Truyens, S., Weyens, N., Boerjan, W. & Vangronsveld, J. (2016). Performance of 16s rDNA primer pairs in the study of rhizosphere and endosphere bacterial microbiomes in metabarcoding studies. *Frontiers in Microbiology* 7, 650. DOI: 10.3389/fmicb.2016.00650

Bodenhausen, N., Horton, M.W. & Bergelson, J. (2013). Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. *PLoS ONE* 8, e56329. DOI: 10.1371/journal.pone.0056329

Cheng, X.-Y., Tian, X.-L., Wang, Y.-S., Lin, R.-M., Mao, Z.-C., Chen, N. & Xie, B.-Y. (2013). Metagenomic analysis of the pine wood nematode microbiome reveals a symbiotic relationship critical for xenobiotics degradation. *Scientific Reports* 3, 1869. DOI: 10.1038/srep01869

Cheng, Z.Q. (2018). [Community structure, diversity, succession and functional and function of the tomato rhizobacteria and endophytes.] M.D. Dissertation, Fujian Normal University, Fujian, P.R. China.

Estrada, C., Wcislo, W.T. & Van Bael, S.A. (2013). Symbiotic fungi alter plant chemistry that discourages leaf-cutting ants. *New Phytologist* 198, 241-251. DOI: 10.1111/nph.12140

Fan, X.X. (2015). [Study on spatiotemporal structure and diversity of endophytic community of Aconitum carmichaeli](#)
Debx.] M.D. Dissertation, Shanxi Normal University, Shanxi, P.R. China.

Fu, Y.-M., Liu, H.-B. & Wu, X.-Q. (2020). Diversity and function of endo-bacteria in Bursaphelenchus xylophilus from Pinus massoniana Lamb. in different regions. *Forests* 11, 487. DOI: 10.3390/f11050487

Fukuda, K. (1997). Physiological process of the symptom development and resistance mechanism in pine wilt disease. *Journal of Forest Research* 2, 171-181. DOI: 10.1007/BF02348216

Geib, S.M., Jimenez-Gasco, M.D., Carslon, J.E., Tie, M. & Hoover, K. (2009). Effect of host tree species on cellulase activity and bacterial community composition in the gut of larval Asian longhorned beetle. *Environmental Entomology* 38, 686-699. DOI: 10.1603/022.038.0320

Grané, G., Persson, P., Meijer, J. & Alström, S. (2003). A study on microbial diversity in different cultivars of *Brassica napus* in relation to its wilt pathogen, *Verticillium longisporum*. *FEMS Microbiology Letters* 224, 269-276. DOI: 10.1016/S0378-1097(03)00449-X

Hardoim, P.R., van Overbeek, L.S., Berg, G., Pirtilă, A.M., Compart, S., Campisano, A., Döring, M. & Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary considerations for defining microbial endophytes. *Microbiology and Molecular Biology Reviews* 79, 293-320. DOI: 10.1128/MMBR.00050-14

Hirakue, A. & Sugiyama, S. (2018). Relationship between foliar endophytes and apple cultivar disease resistance in an organic orchard. *Biological Control* 127, 139-144. DOI: 10.1016/j.biocontrol.2018.09.007

Huot, O.B., Nachappa, P. & Tamborindeugy, C. (2013). The evolutionary strategies of plant defenses have a dynamic impact on the adaptations and interactions of vectors and pathogens. *Insect Science* 20, 297-306. DOI: 10.1111/1744-7917.12010

Jin, Z.Y., Li, W. & Sun, B.L. (2019). [Taxonomic and metabolic analysis of gut microbiota from male athletes based on metagenomics.] *Journal of Biology* 36, 7-13. DOI: 10.3969/j.issn.2095-1736.2019.04.007

Jing, G. (2007). [Study on programmed cell death during the interaction between Pinus thunbergii and pine wood nematode.] Ph.D. Dissertation, Nanjing Forestry University, Nanjing, P.R. China.

Kandel, S.L., Joubert, P.M. & Doty, S.L. (2017). Bacterial endophyte colonization and distribution within plants. *Microorganisms* 5, 77. DOI: 10.3390/microorganisms5040077

Kawazu, K., Zhang, H. & Kanzaki, H. (1996). Accumulation of benzoic acid in suspension cultured cells of *Pinus thunbergii* Parl. in response to phenylacetic acid administration. *Bioscience, Biotechnology, & Biochemistry* 60, 1410-1412. DOI: 10.1271/bbb.60.1410

Kim, N., Jeon, H.W., Mannaa, M., Jeong, S.-I., Kim, J., Lee, C., Park, A.R., Kim, J.-C. & Seo, Y.-S. (2019). Induction of resistance against pine wilt disease caused by *Bursaphelenchus xylophilus* using selected pine endophytic bacteria. *Plant Pathology* 68, 434-444. DOI: 10.1111/ppa.12960

Li, Q., Guo, R.J., Li, Y.J., Hartman, W.H., Li, S.F., Zhang, Z.X., Trings, S.E. & Wang, H.Q. (2019). Insight into the bacterial endophytic communities of peach cultivars related to crown gall disease resistance. *Applied and Environmental Microbiology* 85, e02931-18. DOI: 10.1128/AEM.02931-18

Long, R.M. (2007). [Study on the migration of pine wood nematode and extraction of active substances from Botryis cinerea liquid and their attraction to pine wood nematode.] M.D. Dissertation, Xiamen University, Xiamen, P.R. China.

Mei, S.C. & Flinn, B.S. (2010). The use of beneficial microbial endophytes for plant biomass and stress tolerance improvement. *Recent Patents on Biotechnology* 4, 81-95.

Mejía, L.C., Herre, E.A., Sparks, J.P., Winter, K., García, M.N., Van Bael, S.A., Stitt, J., Shi, Z., Zhang, Y., Zhang, Y.F. et al. (2014). Pervasive effects of a dominant foliar endophytic fungus on host genetic and phenotypic expression in a tropical tree. *Frontiers in Microbiology* 5, 16. DOI: 10.3389/fmicb.2014.00479

Nascimento, F.X., Hasegawa, K., Mota, M. & Vicente, C.S.L. (2015). Bacterial role in pine wilt disease development – review and future perspectives. *Environmental Microbiology Reports* 7, S1-63. DOI: 10.1111/1758-2229.12202

Nascimento, F.X., Espada, M., Barbosa, P., Rossi, M.J., Vicente, C.S.L. & Mota, M. (2016). Non-specific transient mutualism between the plant parasitic nematode, *Bursaphelenchus xylophilus*, and the opportunistic bacterium *Serratia quinivorans* BXF1, a plant-growth promoting pine endophyte with antagonistic effects. *Environmental Microbiology* 18, 5265-5276. DOI: 10.1111/1462-9290.13568

Nascimento, F.X., Vicente, C., Cock, P., Tavares, M., Rossi, M., Hasegawa, K. & Mota, M. (2018). From plants to nematodes: *Serratia grimesii* BXF1 genome reveals an adaptation to the modulation of multi-species interactions. *Microbial Genomics* 4, 000178. DOI: 10.1099/mgen.0.000178

Oku, H., Shiraishi, T., Ouchi, S., Kurozumi, S. & Ohta, H. (1980). Pine wilt toxin, the metabolite of a bacterium associated with a nematode. *Naturwissenschaften* 67, 198-199. DOI: 10.1007/BF01086307

Pieterse, C.M.J., Zamiodis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C.M. & Bakker, P.A.H.M. (2014). Induced systemic resistance by beneficial microbes. *Annual Review of Phytopathology* 52, 347-375. DOI: 10.1146/annurev-phyto-082712-102340

Podolich, O., Ardanov, P., Zaets, I., Pirtilă, A.M. & Kozyrovska, N. (2015). Reviving of the endophytic bacterial community as a putative mechanism of plant resistance. *Plant and Soil* 388, 367-377. DOI: 10.1007/s11104-014-2235-1

Proença, D.N., Francisco, R., Santos, C.V., Lopes, A., Fonseca, L., Abrantes, I.M.O. & Morais, P.V. (2010). Diversity of bacteria associated with *Bursaphelenchus xylophilus* and other nematodes isolated from *Pinus pinaster* trees with pine
Bacterial community structure of Pinus massoniana

Proença, D.N., Grass, G. & Morais, P.V. (2017a). Understanding pine wilt disease: roles of the pine endophytic bacteria and of the bacteria carried by the disease-causing pine wood nematode. *Microbiology Open* 6, e415. DOI: 10.1002/mbo3.415

Proença, D.N., Francisco, R., Kublik, S., Schöler, A., Vestergaard, G., Schloter, M. & Morais, P.V. (2017b). The microbiome of endophytic, wood colonizing bacteria from pine trees as affected by pine wilt disease. *Scientific Reports* 7, 4205. DOI: 10.1038/s41598-017-04141-6

Rangjaroen, C., Rerkasem, B., Teaumroong, N., Sungthong, R. & Lumyong, S. (2014). Comparative study of endophytic and endophytic diazotrophic bacterial communities across rice landraces grown in the highlands of northern Thailand. *Archives of Microbiology* 196, 35-49. DOI: 10.1007/s00203-013-0940-4

Redford, A.J., Bowers, R.M., Knight, R., Linhart, Y. & Fierer, N. (2010). The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. *Environmental Microbiology* 12, 2885-2893. DOI: 10.1111/j.1462-2920.2010.02258.x

Son, J.A., Matsushita, N. & Hogetsu, T. (2015). Migration of *Bursaphelenchus xylophilus* in cortical and xylem axial resin canals of resistant pines. *Forest Pathology* 45, 246-253. DOI: 10.1111/efp.12164

Tan, H.M., Cao, L.X., He, Z.F., Su, G.J., Lin, B. & Zhou, S.N. (2006). Isolation of endophytic actinomycetes from different cultivars of tomato and their activities against *Ralstonia solanacearum* in vitro. *World Journal of Microbiology and Biotechnology* 22, 1275-1280. DOI: 10.1007/s11274-006-9172-y

Turner, T.R., James, E.K. & Poole, P.S. (2013). The plant microbiome. *Genome Biology* 14, 249-253. DOI: 10.1186/gb-2013-14-6-209

van Elsas, J.D., Chiurazzi, M., Mallon, C.A., Ellhottová, D., Krištufek, V. & Salles, J.F. (2012). Microbial diversity determines the invasion of soil by a bacterial pathogen. *Proceedings of the National Academy of Sciences of the United States of America* 109, 1159-1164. DOI: 10.1073/pnas.1109326109

Wang, Q.M., Xu, F.Y., Ge, M.H., Wang, Z.R. & Cheng, T.H. (1997). A preliminary study on the variation of resistance in 39 Masson pine provenances to *Bursaphelenchus xylophilus*. *Journal of Zhejiang Forestry College* 14, 31-36.

Wang, X., Zenda, T.S., Liu, S.T., Liu, G., Jin, H.Y., Dai, L., Dong, A.Y., Yang, Y.T. & Duan, H.J. (2019). Comparative proteomics and physiological analyses reveal important maize filling-kernel drought-responsive genes and metabolic pathways. *International Journal of Molecular Sciences* 20, 3743. DOI: 10.3390/ijms20153743

Zhao, B.G., Wang, H.L., Han, S.F. & Han, Z.M. (2003). Distribution and pathogenicity of bacteria species carried by *Bursaphelenchus xylophilus* in China. *Nematology* 5, 899-906. DOI: 10.1163/15685410377304817

Zhou, Z.F., Ling, G.H., Ding, N., Xun, Z., Zhu, C., Hua, H. & Chen, X.C. (2018). Molecular analysis of oral microflora in patients with primary Sjögren’s syndrome by using high-throughput sequencing. *PeerJ* 6, e5649. DOI: 10.7717/peerj.5649
## Supplementary Table S1. Sequence data statistics of samples of *Pinus massoniana*.

| Sample       | Sequence number | Base number | Mean length | Min. length | Max. length |
|--------------|-----------------|-------------|-------------|-------------|-------------|
| Leaf 1(1)    | 10 608          | 3 975 984   | 374.81      | 210         | 499         |
| Leaf 1(2)    | 22 475          | 8 396 167   | 373.57      | 259         | 499         |
| Leaf 1(3)    | 20 528          | 7 657 268   | 373.01      | 206         | 483         |
| Root 1(1)    | 23 437          | 8 824 757   | 376.53      | 202         | 499         |
| Root 1(2)    | 22 984          | 8 675 716   | 377.46      | 203         | 500         |
| Root 1(3)    | 23 158          | 8 735 982   | 377.23      | 212         | 499         |
| Stem 1(1)    | 21 602          | 8 090 454   | 374.52      | 207         | 499         |
| Stem 1(2)    | 24 925          | 9 360 119   | 375.53      | 203         | 506         |
| Stem 1(3)    | 23 172          | 8 707 591   | 375.78      | 208         | 499         |
| Treetop 1(1) | 24 767          | 9 309 388   | 375.87      | 212         | 499         |
| Treetop 1(2) | 23 156          | 8 705 880   | 375.96      | 212         | 509         |
| Treetop 1(3) | 16 402          | 6 173 498   | 376.38      | 203         | 502         |
| Leaf 2(1)    | 19 780          | 7 391 135   | 373.66      | 362         | 499         |
| Leaf 2(2)    | 19 406          | 7 268 257   | 374.53      | 360         | 499         |
| Leaf 2(3)    | 19 577          | 7 325 073   | 374.16      | 331         | 499         |
| Root 2(1)    | 23 478          | 8 807 503   | 375.13      | 214         | 499         |
| Root 2(2)    | 21 022          | 7 930 604   | 377.25      | 201         | 499         |
| Root 2(3)    | 18 930          | 7 136 083   | 376.97      | 203         | 488         |
| Stem 2(1)    | 24 855          | 9 342 463   | 375.87      | 204         | 499         |
| Stem 2(2)    | 24 965          | 9 378 195   | 375.65      | 201         | 499         |
| Stem 2(3)    | 24 069          | 9 066 532   | 376.68      | 208         | 499         |
| Treetop 2(1) | 20 271          | 7 589 001   | 374.37      | 214         | 499         |
| Treetop 2(2) | 21 244          | 7 976 947   | 375.49      | 207         | 499         |
| Treetop 2(3) | 21 842          | 8 203 661   | 375.59      | 215         | 499         |
| Leaf 3(1)    | 12 911          | 4 821 958   | 373.47      | 214         | 391         |
| Leaf 3(2)    | 23 916          | 8 955 169   | 374.44      | 212         | 499         |
| Leaf 3(3)    | 24 069          | 8 990 099   | 373.51      | 360         | 499         |
| Root 3(1)    | 24 265          | 9 162 299   | 377.59      | 201         | 502         |
| Root 3(2)    | 24 769          | 9 362 417   | 377.98      | 209         | 499         |
| Root 3(3)    | 23 557          | 8 871 762   | 376.60      | 212         | 397         |
| Stem 3(1)    | 23 250          | 8 756 485   | 376.62      | 202         | 499         |
| Stem 3(2)    | 20 614          | 7 768 305   | 376.84      | 201         | 499         |
| Stem 3(3)    | 23 424          | 8 835 328   | 377.19      | 211         | 499         |
| Treetop 3(1) | 24 742          | 9 266 131   | 374.51      | 212         | 499         |
| Treetop 3(2) | 23 176          | 8 706 800   | 375.68      | 212         | 499         |
| Treetop 3(3) | 19 246          | 7 225 002   | 375.40      | 212         | 499         |

The first number in the sample name is disease resistance, 1 is high resistance, 2 is moderate resistance, 3 is low resistance, and the number in parentheses represents number of biological replicates.
**Supplementary Table S2.** Alpha diversity index of samples of *Pinus massoniana.*

| Sample   | Sobs   | Shannon  | Chao    |
|----------|--------|----------|---------|
| Leaf 1   | 199.3 ± 91.74 | 3.06 ± 0.8906 | 228.4 ± 77.47 |
| Leaf 2   | 238.0 ± 85.16  | 3.32 ± 0.3477  | 315.9 ± 144.0  |
| Leaf 3   | 168.3 ± 52.27  | 3.103 ± 0.2458 | 212.8 ± 74.86  |
| Root 1   | 427.3 ± 77.53  | 4.023 ± 0.6686 | 599.2 ± 84.84  |
| Root 2   | 327.7 ± 37.54  | 3.983 ± 0.2857 | 449.4 ± 52.12  |
| Root 3   | 372.7 ± 163.4  | 3.62 ± 1.5707  | 493.1 ± 172.2  |
| Stem 1   | 386.3 ± 60.12  | 4.19 ± 0.3863  | 454.3 ± 98.09  |
| Stem 2   | 351.3 ± 84.6   | 4.467 ± 0.1531 | 411.9 ± 102.0  |
| Stem 3   | 389.3 ± 123.8  | 4.543 ± 0.3625 | 492.7 ± 154.1  |
| Treetop 1| 276.7 ± 49.0   | 3.157 ± 0.4954 | 359.2 ± 59.9   |
| Treetop 2| 221.3 ± 32.13  | 3.027 ± 0.2781 | 273.1 ± 61.32  |
| Treetop 3| 242.0 ± 30.41  | 3.247 ± 0.3164 | 300.2 ± 2.836  |

The values in the table are mean ± standard deviation (n = 3). There are no significant differences between the three values for leaves, roots, stems or treetops, *P* < 0.05. The number after the sample name is disease resistance, 1 is high resistance, 2 is moderate resistance, and 3 is low resistance.

**Supplementary Table S3.** Anosim analysis of different disease-resistant *Pinus massoniana* samples.

| Group   | Statistic (R) | *P* value |
|---------|---------------|-----------|
| Leaf    | 0.168         | 0.035     |
| Root    | 0.209         | 0.185     |
| Stem    | 0.341         | 0.023     |
| Treetop | 0.358         | 0.016     |

The statistic in Anosim is *R* and the theoretical range is −1 to +1; the closer the *R* value to 1, the greater the differences between groups than within groups. The smaller the *R* value indicates no significant differences between and within groups. The reliability of this statistical analysis is expressed by the *P* value. *P* < 0.05 indicates statistical significance.