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Responses of Endophytic Microbial Community in Masson Pine to the Early Stage of Pine Wilt Disease

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Research Article

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ABSTRACT

The pinewood nematode (PWN), *Bursaphelenchus xylophilus (B. xylophilus)*, is one of the causal agents of the pine wilt disease (PWD). Early diagnosis of PWD is of a great significance, as effective treatment at the late course of this disease does not exist. At present, microbial flora has been suggested to play a role in PWD. However, whether microbial flora can be the diagnosis index of early PWD is unknown. In this study, we applied 10 healthy wild adult *Pinus massoniana* (*P. massoniana*) to analyzed the role of endophytic microbial communities in the early infection of *B. xylophilus*By using PCR-DGGE, we found the bacterial structure in *P. massoniana* was significantly different after inoculation with *B. xylophilus*. The bacterial bands of No.8, 16, 11 and 12 bacteria were correlated with the time of nematode infection, which indicates that these four bacteria may have potential in the prevention or treatment of PWD. In addition, the alterations of bacterial flora structure in individual *P. massoniana* were similar by analyzing NMDS. With the multidimensional calibration of the microbial community, we found that the population structures of endophytic bacteria are markedly different between the early 30 days and the 40th day. Therefore, by identifying the endophytic bacteria structure in *P. massoniana*, we are able to diagnose the PWD in the first 40 days before the onset of symptoms. Early diagnosis of PWD would provide valuable time for the treatment of the disease. Our study showed significant data to prove that endophytic bacteria are a novel index for the early diagnosis of PWD.

INTRODUCTION

Pine wilt disease (PWD) is referred to the “cancer” of pine trees, induced by the infection of *Bursaphelenchus xylophilu* (*B. xylophilus*), which also called pine wood nematode (PWN), constitutes one of the most serious worldwide conifer diseases [1]. This disease represents a significant economic and environmental damage to the affected countries, with huge annual losses of timber, increased costs in disease control and irreversible changes to the native forest ecosystems including loss of biodiversity, wild life habitat destruction, soil and water conservation and tree species conversions [2,3].

The pine wood nematode has strong pathogenicity. Once the pine sawyer beetle, the insect vector, introduces the nematode, infected tree typically dies within a few weeks or months. There are no cures for PWD once a susceptible tree becomes infested with the pinewood nematode and removing and chipping infested trees to prevent the spread to nearby susceptible trees is the limited way of defense. Rapid and accurate detection of PWN in pine trees is critical in preventing the introduction of *B. xylophilus.* However, no obvious symptoms would be showed in early infestation of PWN, which increases the difficulty for the early diagnosis of this disease [4].

In recent years, with the development of microecology theory, the relationship among the microbial environment, host and microbial population has been further elaborated [5]. It was report that plant endophytes could enhance the nutritional physiology function and resistance activity of plants in diverse ways and induce the defense enzymes in plants. These results indicate that endophytic bacteria and plant defense system are closely related [6]. Le Chatelier found that people with a low bacterial richness are characterized by more marked overall metabolic diseases and a more pronounced inflammatory phenotype when compared with high bacterial richness individuals [7]. Although PWN is considered the primary pathogenic agent of PWD, growing evidence

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indicates that bacteria may play an active role in PWD development. Diogo found the microbial community associated with *B.* xylophilus present in *pinus pinaster* with PWD, and the majority of the strains isolated belonged to phylogenetic groups usually isolated as endophytic bacteria in the *Pinus pinaster* Several PWN-associated bacteria could increase PWN virulence and induce PWD symptoms in various pine hosts [1, 8-10]. What's more, the bacterial communities within the insect vector have been reported to influence insect lifestyle and development as well as the interactions with PWN (PMID: 23927049).

Although many studies have suggested a role for PWN-associated and insect vector-associated bacteria in PWD, but not many have paid attention to the endophytic bacteria in pine trees, and how the response of *P. massoniana* endophytic bacteria applied to *B. xylophilus* infection, whether endophytic bacteria can be an index for the early diagnosis of PWD. In this study, we analyzed the microbial community structure and dynamic changes of endophytic bacteria of *P. massoniana* after infection with *B. xylophilus*, and constructed the theoretical basis for early diagnosis technology of *B. xylophilus* based on microorganism fingerprint map [11].

MATERIALS AND METHODS

Plant Material

P. massoniana forestland (119˚55'E, 30˚59'N) located in 2 kilo meters west of Taiyangshan Road Xiaofeng Town, Anji County, Huzhou City of Zhejiang Province, China. The elevation of the forestland is 81 meters. Ten healthy wild *P. massoniana* with diameter at breast height (DBH) of about 50 cm from the forest were selected as the experiment objects, and the time for inoculation was July 2015.

Preparation for Pine Wood Nematode Suspension

Dead *P. massoniana* which were infected by PWN were collected, and the sick wood was split into small pieces of 5 cm long, 5 mm diameter. Gauze was used to wrap 300 g woodchips, placed them in the glass funnel whose bottom was covered by rubber tube with flat jaw pinchcock. 100 ml deionized water at 25˚C was poured into the funnel with the flat jaw pinchcock closed. After 24 hours, 15 ml of liquid at the bottom of funnel was taken to inspect the presence of PWN under a microscope [12].

The nematodes were collected by centrifugation (4000×g, 2 min), and transferred to a PDA medium which filled with *Botrytis cinerea*. Cultivation was carried out under constant temperature of 25˚C after the inoculation of pine wood nematodes. After the nematodes ate up the mycelium (8 to 12 days later), Baermann Funnel Method was used to extract nematodes from the medium. The isolated nematodes were centrifugal disinfected, and concentrated to a suspension of 6000 nematodes/ml.

Pine Wood Nematode Inoculation

Growth cone (5.15 mm aperture) was used to drill in the middle of trunk (height of about 1.3 m to 1.5 m), and the prepared wet cotton balls with a good adsorption of PWN suspension were put into the drilled holes. Topical erythromycin antibiotics was coated in the orifice with sterile gauzes covered outside, and after wrapped with sterile plastic film, the inoculation spots were tape sealed [13].

Ten strains of healthy *P. massoniana* were prepared to be inoculated. Three trees were received water, three trees were inoculated with *B. xylophilus*, four trees had non-inoculation but only the inoculate opetation. The tested *P. massoniana* were covered with a mesh screen made of fine-mesh screens to prevent natural spread of beetles and other insects [14]. Labeled ten healthy *P. massoniana* without any operation as J1-J10, trees with *B. xylophilus inoculation are* J3, J6, J8, water-inoculated *P. massoniana* are J1, J2, J4, and non-inoculated trees are J5, J7, J9, J10. The samples collected from tree J3 at 10, 20, 30 and 40 days were labeled as J310, J320, J330 and J340 respectively, and this method of labeling is also applicable in other samples.

Sample Collection

Wood samples were collected at 3-5 cm away from the inoculation holes by four directions (up, down, left, right). Samples were collected before the inoculation and after the inoculation for 10, 20, 30 and 40 days, each collation was in the clockwise rotation 22.5 ˚ points from the previous sampling positions. Collected samples were placed in sterile sample bags and kept in a liquid nitrogen tank until back to the laboratory. The samples were keeping in -80°C for long-term storage. The specific division of sampling tree's state of an illness was according to the color of trees and needlepoint leaf, and the wilting condition of *P. massoniana* [14].

Extraction of Endophytic Bacteria Genome from P. Massoniana

The specific operation procedure was as follows: weighted and took 3 g of *P. massoniana.* After surface disinfection, samples were put in sterile centrifuge tubes, sealed with plastic wrap which has holes in it. Pre-frozen in -80˚C for more than 2 h and transferred into the 2.5 L desktop freeze-drying instrument (U.S. Labconco Free zone) for freeze-drying. Weighed 1 g freeze-dried samples and grinded rapidly by U.S. MP FastPrep-24 sample preparation system with parameter of 6 m/s, in 45 s for two cycles, and the cycle interval was 3 min. After passing through a 40 mesh sieve, 0.2 g of grinded *P. massoniana* was weighed for the extraction of endophytic bacteria genome by using Omega Bacterial DNA Kit D3350. The extraction method was followed with the manufactorial instructions, and the DNA samples were purified and stored at -20˚C.

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PCR Amplification

The purified DNA was used as templates, 16S rDNA gene at V3 region in bacteria and archaea were amplified by PCR, and the amplified fragments were about 230 bp. The specific primers are GC-341F (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CGCGGGGGGACT-CCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3').

PCR was performed in 0.2 ml tubes using a Hybaid Px2 thermal cycler. For direct PCR, the 50µl reaction mixture consisted of 25 µl of Premix taq (Takara Taq, version 2.0 plus dyc), 21.5 µl of double distilled water, 0.5 µl of upstream and downstream primers (10 µM of working concentration) and 2.5 µl of DNA template. PCR amplification conditions of PCR amplification conditions of 94˚C for 5 min, 94˚C for 30s, 55˚C for 30 s, 72˚C for 1 min, 30 cycles, with a final extension at 72˚C for 10 min. These optimized PCR conditions were then used for all the results for primer sets described in this study. Agarose gel electrophoresis was applied to detect PCR products.

Denaturing Gel Gradient Electrophoresis (DGGE) Performance

PCR products were analyzed by DGGE with 8% polyacrylamide gel, and the denaturation gradient was 40% to 55%. The gels were prepared and run with 1×TAE electrophoresis buffer at constant voltage of 80V at 60˚C for 13 h. The PCR products (10 µl) were mixed with 6 µl 10×gel loading dye. After electrophoresis, the gels were stained at room temperature for 30 min in GelRed ™ Nucleic Acid Gel Stain and visualized by UV transillumination [15].

Sequence Analysis of DGGE Bands

The interested clear bands shown in the DGGE map of the infected *P. massoniana* samples were cut off with a sterile scalpel and were transferred to a sterile tube containing 20 µl sterile DNase/RNasefree water and incubated overnight at 4˚C. Before PCR, the gel block was placed at -20˚C for 1 h. Then melt it, 1-2 µl of the supernatant was used as a template for PCR amplification. The eluted DNA was amplified with the respective GC-341F and 518R primers set and the application condition is as previous descripted. The PCR products were purified by Takara minibest DNA Fragment purification kit ver.4.0, followed with T Vector of Takara pMD ™ 19-T Vector Cloning Kit. The DH5a competent cells were transformed and the positive clones were picked out and sent to Shanghai Sangon Biotech Co., Ltd. for sequencing. The results were compared with available 16S rRNA gene sequences from GenBank using the BLAST program (http://www.ncbi. nlm.nih.gov/blast/). For phylogenetic analysis, the 16S rRNA gene sequences of the isolate and closely related strains were aligned using CLUSTAL X software [16]. Mega6 software was used to construct a phylogenetic tree by the Neighbor-Joining method, with 1000 times boostrap in the confidence of each branch [17].

Data Analysis

Data analysis of PCR-DGGE was performed using software including Image lab 5.2.1 (gel labography), Quantity One v4.6.2 (quantitative analysis), PAleontological Statistics Version 2.03Data Analysis (data analysis), Matlab 2016 and Mega6. DGGE banding patterns were assessed by cluster analysis with a Dice similarity coefficient constructed using UPGMA, Schema graph, Shannon-Weaver Index, species evenness index, and richness index (Margalef), NMDS non-metric multidimensional scale, value of similarity index P and R [18,19].

RESULTS AND DISCUSSION

P. massoniana Inoculated with B. xylophilus Performed Pine Wilt Disease

Since the wild adult pines was valuable, and the potential danger of spreading *B. xylophilus* in the forest, we picked 10 healthy *P. massoniana* for this study, and all of which are grow in the forest for 50 years with the trunk diameter is more than 50cm. Subtropics monsoon's burning hot, humid summer conditions may place pine trees under stress, making them more prone to attack by the PWN and provide the warm conditions needed by the nematode for rapid reproduction, so the inoculation time was in July. The dosage of nematode we use in this study was followed with Liqun Xie's study [20].

We observed that all the ten trees had no obvious symptoms during the forty days experiment. After 50 days, the trees with nematodes inoculation started to show 10% brown leaves, 40–50% brown leaves at the day 80, and finally dead without leaf. However, the 3 trees inoculated with water and 4 trees without inoculation anything but the inoculate operation has no symptoms of illness. To confirm the infection of *B. xylophilus*, we also isolated nematodes from the ten trees by using modified Baermann funnels. The results show that nematodes can be extracted from the trees with nematodes inoculation, but not from the other seven trees without nematodes inoculation.

The Species and Abundance of Endophytic Bacteria were Similar in Healthy P. massoniana.

The structure of endophytic bacteria population in the sample J1-J10 from healthy *P. massoniana* was analyzed by PCR-DGGE. DNA extracted from all wood samples gave the expected around 230 bp PCR fragment with the bacterial primers GC-341F and 518R (Data see PCR amplification). Separation of these fragments by DGGE produced complex banding patterns reflecting the microbial diversity present in these samples **(Figure 1)**. It was observed that, in general, the DGGE samples from the untreated

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trees **(Figure 1A)** has similar predominant bands, which means the species and abundance of endophytic bacteria from the ten healthy trees were close to each other.

The Structure of Endophytic Bacteria in P. massoniana has Slight Variations after Inoculation with Water for 40 Days

In order to eliminate the interference of the pile holes and the inoculated water on the endophytic bacteria of *P. massoniana,* we compared the bacterial structure of endophytic bacteria in *P. massoniana* before inoculation and after the inoculate operation or inoculation water for 40 days. Water-inoculated *P. massoniana* at day 40 are labeled as J140, J240 and J440, and non-inoculated samples at 40 days are labeled as J540, J740, J940 and J1040. By PCR-DGGE technique, we found that the species and abundance of endophytic bacteria did not change much after the water inoculation, and the band richness was comparable to the band of the samples from healthy trees **(Figure 1B)**. The shannon-weaver coefficient, evenness and Margalef of these fourteen *P. massoniana* samples were analyzed by Quantity One software. From **Table 1**, we observed that the shannon index of J1 and J140 is 1.84 and 1.83 respectively, the shannon index of J2 and J240 is 1.8 and 1.81, and J540 is 1.89, close to the shannon index of J5, 1.87. Thus, the pile hole and water-inoculation had little effect on the structure of endophytic bacteria in *P. massoniana.*

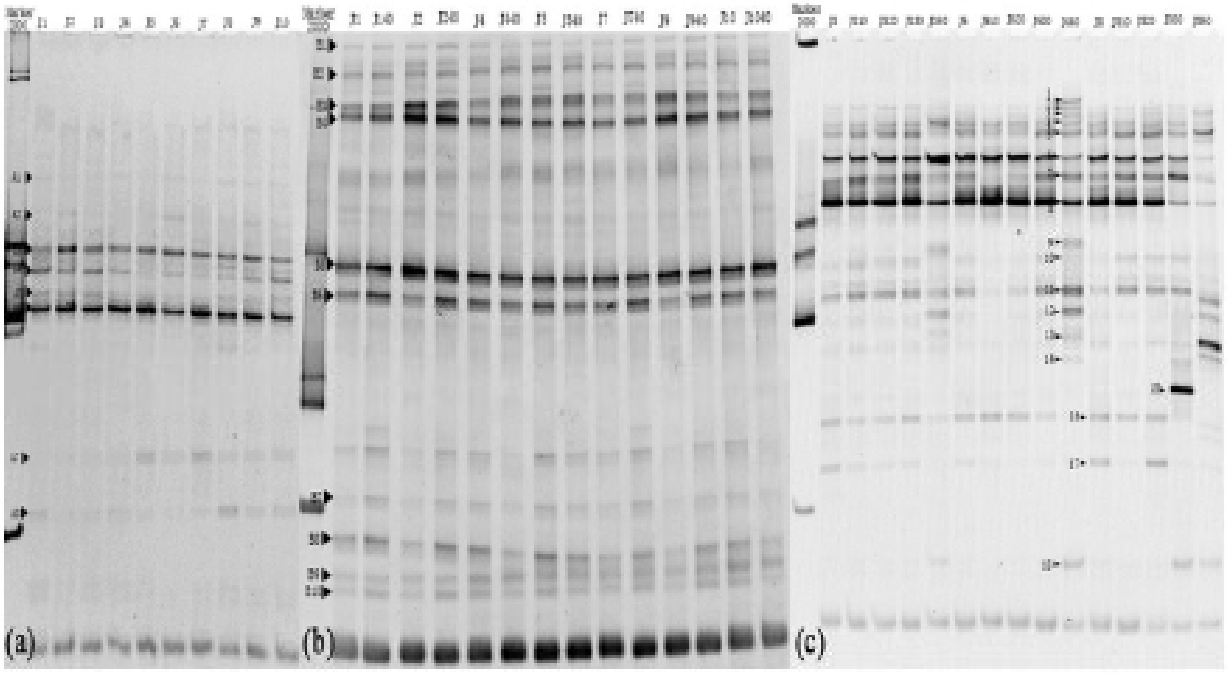

Figure 1. (A) DGGE analysis of endophytic bacteria population structure of healthy *P. massoniana* J1-J10 before any operations. (B) DGGE analysis of endophytic bacteria population structure of water-inoculated and non-inoculated *P. massoniana* samples before and after the operation for 40 days. (C) DGGE analysis of endophytic bacteria population structure of *P. massoniana* samples with *B. xylophilus* infection for 10, 20, 30 and 40 days.

Table 1. The variety, evenness and abundant analysis of the endophytic bacteria from water-inoculated and non-inoculated *P. massoniana* before the operation (J1, J2, J4, J5, J7, J9 and J10) and after the operation for 40 days (J140, J240, J440, J540, J740, J940 and J1040).

Lane	Shannon-H	Evenness	Margalef
J1	1.84	0.93	0.67
J140	1.83	1.91	0.65
J2	1.82	0.86	0.64
J240	1.81	0.85	0.64
J4	1.93	0.88	0.77
J440	1.94	0.88	0.76
J5	1.87	0.81	0.65
J540	1.89	0.82	0.64
J7	1.76	0.80	0.55
J740	1.73	0.80	0.54
J9	1.93	0.85	0.76
J940	1.91	0.87	0.77
J10	1.84	0.90	0.66
J1040	1.82	0.90	0.66

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The Structure of Endophytic Bacteria has Significant Changes after the Inoculation with Nematodes.

The population structure of endophytic bacteria in diseased *P. massonian* was analyzed by PCR-DGGE. We found that the structure of endophytic bacteria obviously changed, and the species and abundance of endophytic bacteria were increased over time **(Figure 1C)**. The shannon-weaver coefficient, evenness and Margalef of the 15 *P. massoniana* samples were further analyzed by Quantity One software. From **Table 2**, we obvious that the shannon index of J340 was 2.12, which is larger than that of J3. Compare to the shannon index of J6 and J8, the J640 and J840 shannon index is also markedly increased. Moreover, the mean evenness index of these three trees changed from 0.62 to 0.84 in the 40 days of infection, while the mean richness index has no significant changes. Therefore, after infection of nematodes for 40 days, the diversity of endophytic bacteria is markedly increased, which is mostly depends on the increase in the abundance of species rather than the richness in species. It can be speculated that with the infection of *B. xylophilus*, the bacteria carried by the trees break the balance, resulting in the propagation of pathogens in *P. massoniana* and the increased diversity of endophytic flora.

Table 2. The variety, evenness and abundant analysis of the endophytic bacteria from the P. massoniana (J3, J6 and J8) with B. xylophilus infection for 10, 20, 30 and 40 days.

Lane	Shannon-H	Evenness	Margalef
J3	1.93	0.53	1.31
J310	2.02	0.63	1.22
J320	1.99	0.74	1.05
J330	2.16	0.79	1.15
J340	2.12	0.80	1.22
J6	1.99	0.73	0.95
J610	1.62	0.56	0.89
J620	1.73	0.63	0.94
J630	2.02	0.83	0.96
J640	2.03	0.85	0.95
J8	1.79	0.61	1.02
J810	1.71	0.62	0.91
J820	2.03	0.69	1.13
J830	2.02	0.84	0.97
J840	2.05	0.87	1.03

Treatment-Dependent Bacterial Responders

Bands in **Figure 1C** that were clearly separated from neighboring bands were isolated for sequencing. Similarity searches with BLAST were conducted on 16S rRNA gene consensus sequences. The results showed that the dominant species of endogenous bacterial in healthy and diseased *P. massoniana* are *proteobacteria* and *bacteroides*, whose corresponding species are *sphingomonas, asticcacaulis, polaribacter, klebsiella, pseudomonas,* and etc. Some of them are uncultured bacteria. Interestingly, we found that No.8 and 16 bacteria bands showed a significant decline or disappear with the inoculationtime increased, while No.11 and 12 bands density is increased. We applied the matlab2016 software to draw the fitted curve of No. 8, 11, 12 and 16 bacterium with the time variation of inoculation. The results show that the strip brightness of the dominant bacteria No.8 and 16 was negatively correlated with the time of nematode infection, while the strip brightness of the bacteria NO.11 and 12 was up-regulated in a time-dependent manner **(Figure 2)**. After the phylogenetic tree of all endophytic strains were clustered and analyzed **(Figure 2)**, we found that among the four dominant bacteria No.8, 11, 12 and 16, the No.8 and 16 bacteria were closely related (93%), and the No.11 and 12 bacteria were related (48.6%).

A variety of phytopathogenic bacteria causing harm and diseases to plant hosts have been reclassified because of advances in taxonomy such as molecular polyphasic, hierarchical approaches for improved phylogeny [21]. However, in pine trees, there are no known diseases associated with the presence of phytopathogenic bacteria. In this study, we identified The NO.11 bacterium in diseased *P. massoniana* as *Mucilaginibacter sp*., and this bacterium was originally from the trees, as samples from healthy trees have strong NO. 11 bands in the DGGE map too. It also reported that *Mucilaginibacter pineti* sp. was isolated from the endophytic microbial community of a *Pinus pinaster* tree branch from a mixed grove of pines **(Figure 3)**. Phylogenetic analysis of 16S rRNA gene sequences showed that this organism represented one distinct branch within the family *Sphingobacteriaceae* (PMID:24711588). *Mucilaginibacter sp* belongs to bacterial strains, which tend to parasites in the rotten wood, and takes rotting materials as food. We postulate that with the PWN infection, the infected trees start to decay inside without showing any symptoms in the early of PWD, meanwhile, the *Mucilaginibacter sp* multiples vigorously. In addition, it may have nematode activity which has great potential in prevent and treatment of this disease. Therefore, *Mucilaginibacter sp* could be used as the response index for early infected *P. massoniana* by PWD. The No. 16 bacterium was identified as *Klebsiella*. Some strains of *Klebsiella* showed strong nematocidal activity *in vitro*, it was also reported to have growth-promoting function for trees. *Klebsiella* sct5 had the highest nematocidal activity for 4 days, with PWN mortality rate reaching 100% [22,23]. A study, conducted in Portugal, found

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Klebsiella is associated with PWN, which may from the pine trees. The abundance of *Klebsiella* is reduced with the inoculation time increases [24]. We suspect that this bacterium is of pine tree origin, and it community balance got interrupted by the infection. A downregulation response of *Klebsiella*. is a typical symptom for the early PWD.

With the comparison of NO.8 and 12 sequences with available 16S rDNA gene sequences in GenBank, we found these two sequences are two unculturable bacteria. Despite the abundance of bacterial species in the trees, more than 99% of these species cannot be cultured by traditional techniques. In addition, the less than 1% of bacteria that can be cultured are not representative of the total phylogenetic diversity [25]. However, identifying species and their new functions of NO.8 and 12 bacteria is still an important task for this study, and has a great potential in prevention and treatment of PWD.

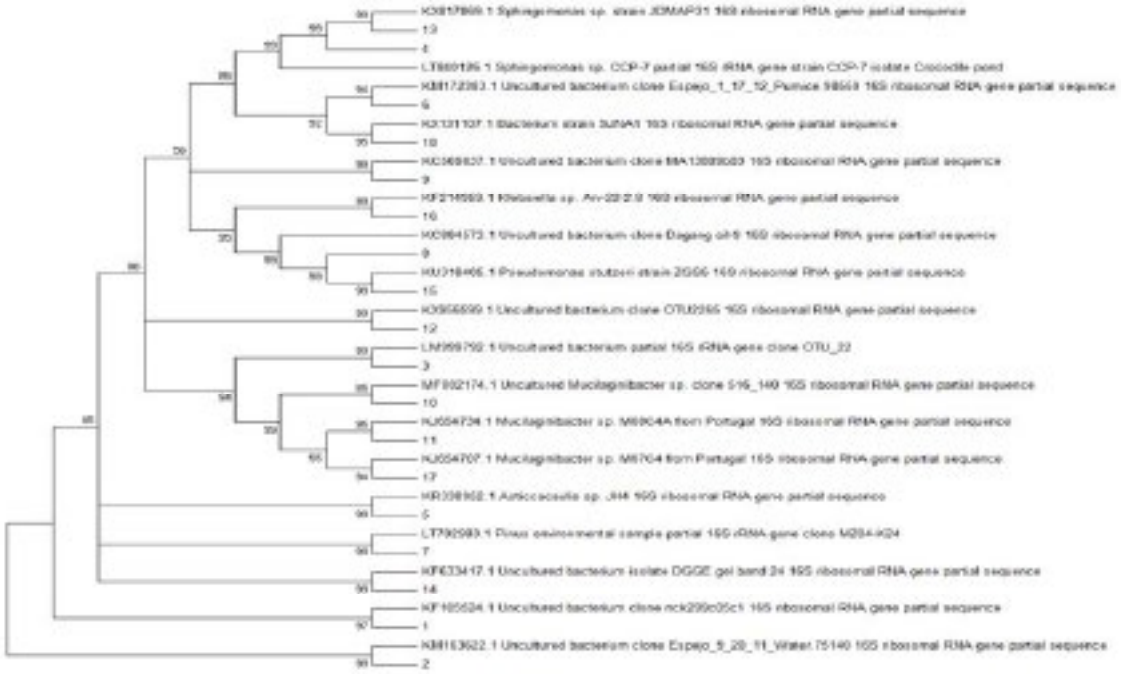

Figure 2. Cluster analysis of interested endophytic bacteria 16S rRNA gene sequence from diseased *P. massoniana* and blast the similar sequence in GenBank. Phylogenetic tree of 16S rRNA of bacteria was constructed with Mega6.0.6 version.

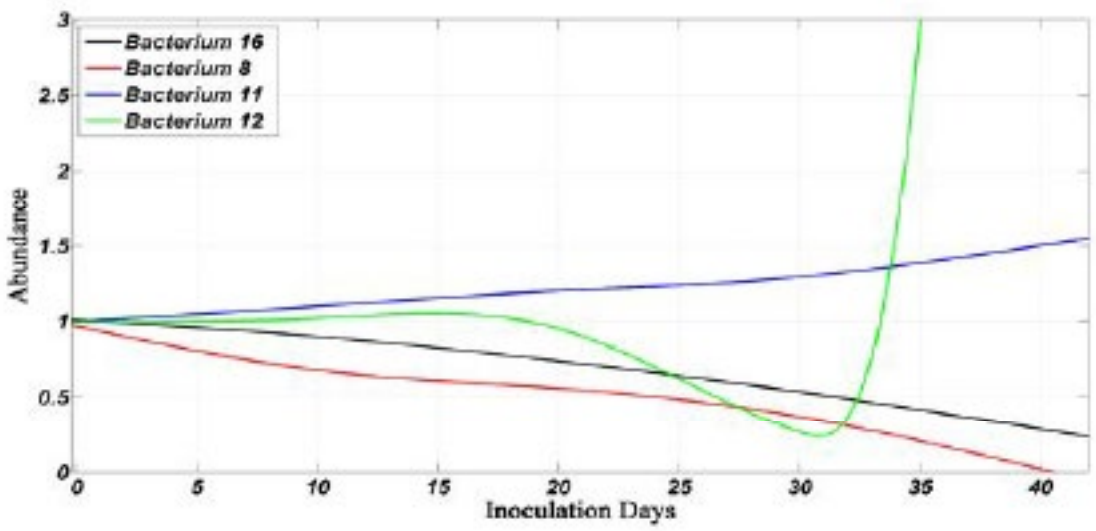

Figure 3. The fitted curve of four species abundance (No. 8, 11, 12 and 16) with time gradients. The abundance of bacterium 8 and 16 were positive correlated with the infection time, while the abundance of bacterium 11 and 12 were decreased in a time-dependent manner.

Endophytic Bacteria Can be a Potential Index for Early Diagnosis of Pine Wilt Disease

In order to explore the alteration rule of endophytic bacteria community to nematode infection, we carried out NMDS in different *P. massoniana* with same infection time gradient (10, 20, 30, and 40 days). We observed that the *P. massoniana* received inoculation of nematodes had a very high coincidence degree in in the same time and space **(Figure 4)**. The alteration of

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population structure of endophytic bacteria had similarity and synchronism characteristic among different *P. massoniana* infected by *B.* xylophilus.

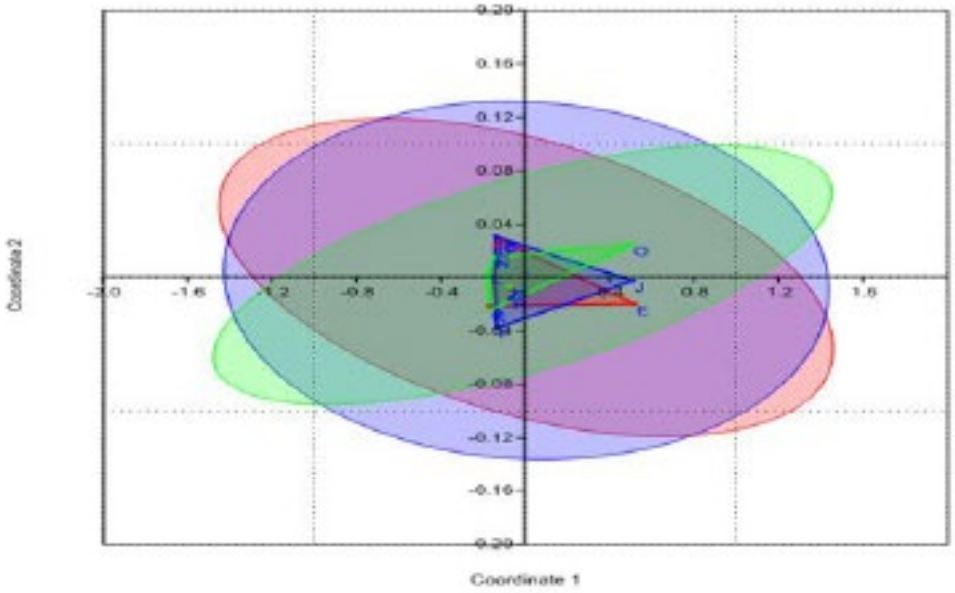

Figure 4. NMDS of endophytic bacteria population variety in different *P. massoniana* with the same treatment. The distribution of endophytic bacteria in inoculated pathogenic bacteria was expressed in the form of point. Sample J3 is in blue color, J6 is in red, and J8 is in green. The population structure of endophytic bacteria had similarity and synchronism characteristic among different *P. massoniana* infected by *B.* xylophilus.

To compare the differences of endophytic flora structure of *P. massoniana* after the inoculation, we applied NMDS to analyze the data of endophytic flora from *P. massoniana* in the first 30 days and in the 40th day of inoculation. With the multidimensional calibration of the microbial community, it is shown that the two layers are obviously separated, and the population structures of endophytic bacteria are markedly different **(Figure 5A)**. Using PAST software analysis, we obtained the value P is 0.0005999, and the correlation of value R was 0.5418 **(Figure 5B)** [26]. Therefore, the structures of endophytic bacteria have significant differences and moderate correlation after the inoculation at 40 days. These results suggest that by detecting the changes in the endophytic bacteria of *P. massoniana,* we are able to diagnose whether the trees are sick in the first 40 days of the disease.

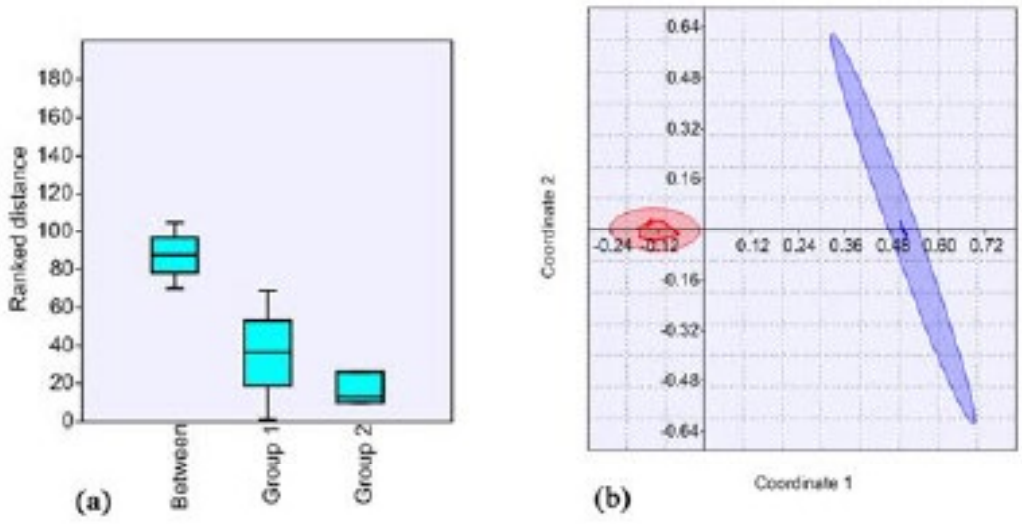

Figure 5. (A) Box plot of value P and value R difference in endophytic bacteria population structure of *P. massoniana* on the 30th day and the 40th day of inoculation.Gourp1 represents the dispersion of bacterial flora in the first 30 days, group 2 represents the dispersion of bacterial flora in 40th day.The value P is 0.0005999, and the correlation of value R was 0.5418. (B) NMDS of endophytic bacteria population structure of *P.massoniana* in the first 30 days (red) and in the 40th day (blue) of inoculation. Coordinate 1 represents the difference of observation; coordinate 2 represents sort distance.

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Pine wilt disease constitutes a major threat to forest ecosystems worldwide, both from the economic point of view as well as from the environmental perspective. Early diagnosis is particularly important for prevention and treatment of this disease. According to different principles, methods of external symptoms, pathogens nematode microscopy, physical spectrum and electrical, molecular biology, biochemistry and other identification methods have been used to confirm the diagnosis [27-31]. Traditional detection of PWD is time consuming and basically depend on the observation of the trees, which usually too late to stop the spreading of the disease. Though the newest molecular detection techniques like LAMP (loop-mediated isothermal amplification) or PCR amplification in SCAR characteristic sequence specificity showed rapid and direct detection methods, without extraction of nematodes or the need of using expensive and specialized equipment as well as taxonomic expertise [32]. However, none of them perform the experiments in wild forest with a longtime period, and none of the studies could provide the specific time of diagnosis. Our study indicated that the structure changes of endophytic bacteria in *P. massoniana* can diagnosis the pine wilt disease as early as infection time at 40 days. In addition, the bacterium we found in diseased *P. massoniana* which was correlated with the time of nematode infection any have great potential in prevent and treatment of this disease.

CONCLUSION

In this study, we found the bacterial structure of *P. massoniana* was significantly different after inoculation with *B. xylophilus*The bacterial bands of No.8, 16, 11 and 12 bacteria were correlated with the time of nematode infection, which suggests a great potential of the bacterial in the prevention or treatment of PWD. The structure changes of endophytic bacteria in *P. massoniana* can diagnosis the PWD as early as infection time at 40 days. Our study provides a new role of endophytic bacteria in *P. massoniana*on the prevention of PWD. The early diagnosis of PWD would provide valuable time for the treatment of this disease. The experiments were performed in ten wild *P. massoniana,* the relatively small size is a limitation. In addition, further studies are needed to identify the specific bacteria that correlated with nematodes inoculation. However, our study provides significant data to prove that endophytic bacteria is a new research field for early diagnosis of PWD, and our ongoing experiments in a larger sample size will provide more evidence for this new theory.

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