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(126) Bacterial flora and its association with the pine wood nematode (*Bursaphelenchus xylophilus*)

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ABSTRACT

In recent years a hypothesis was proposed that not pine wood nematode, but its accompanying bacteria is responsible for the symptom development in pine wilt disease. To ascertain this, we investigated the bacterial flora associated with the nematode and its possible roles in the disease by means of molecular biological techniques. As a result, the dominant bacterial species were different from those in past researches and none of them showed a significant pathogenicity against susceptible pine seedlings. On the other hand, one of the dominant species was frequently detected in seedlings inoculated with bacteria-free nematodes and in vector beetle-associated samples, indicating its possible involvement in the disease.

1. INTRODUCTION

Pine wilt disease is caused by the pathogen, pine wood nematode (PWN; *Bursaphelenchus xylophilus*). Recently, it is suggested that PWN needs bacteria inhibiting its body surface in host infection to cause wilt symptoms (Han *et al.* 2003), although this hypothesis still remains a matter of debate. At this time we have no promising method for effectively controlling pine wilt disease, partly because of lack of definitive causal therapy, and it is necessary to identify the pathogenic factor(s) precisely. In this study, in order to clarify the significance of PWN-associated bacteria in this disease, we described the bacterial flora on the PWN body and determined the potential pathogenicity of isolated bacteria to the host pine trees.

2. MATERIALS AND METHODS

2.1. Bacteria Accompanied by PWN in Naturally Infected Pine Stands

We investigated the bacterial flora on the body surface of PWN isolated from naturally-infected pine trees. Woody tissues were taken from dead pine trees during the infection season in 2011 at two pine stands; one of Japanese black pine, *Pinus thunbergii*, located at Arid Land Research Center, Tottori University, Tottori, Japan, and the other of Japanese red pine, *Pinus densiflora*, on Mt. Ogura, Ukyo, Kyoto, Japan. Samples were used for nematode extraction with Baermann funnels to obtain *B. xylophilus*, from which accompanying bacteria were isolated on a plate of R2A medium. All bacterial isolates served DNA extraction for species identification based on the nucleotide sequence of 16S rRNA.

2.2. Pathogenicity of Accompanying Bacteria

To examine the interaction between host plant (pine) and PWN-accompanying bacteria, we test the pathogenicity of two bacteria which were frequently isolated in experiment 2.1, namely, *Serratia proteamaculans* and *Erwinia mallotivora*. Seedlings of 4-month old *P. thunbergii* which were grown under axenic condition were challenged with 1) bacteria-free PWN (virulent isolate, Ka4), 2) 1) mixed with *S. proteamaculans*, 3) 1) mixed with *E. mallotivora*, 4) *S. proteamaculans* alone, 5) *E. mallotivora* alone, and 6) sterilized water as control and the symptom development was monitored.

2.3. Succession of Bacterial Flora on the Body Surface of PWN during Symptom Development

Potted seedlings of *P. thunbergii* were artificially inoculated with bacteria-free cultured PWN (virulent isolate, Ka4) in the open air to monitor the transition of bacterial flora accompanied by PWN inside host plant. One, 2, 4, and 6 weeks from inoculation, woody tissues of the seedlings were sampled to serve Baermann funnels extraction, nematode thus obtained were used as bacterial source, and the cultured bacterial mixture served molecular characterization by t-RFLP techniques with a set of universal primers specific to bacteria.

2.4. Interaction of Accompanying Bacteria and the Vector Beetle

The result of experiment 2.3 showed that *S. proteamaculans* was frequently accompanied by PWN. Considering the past report that *S. proteamaculans* was symbiotic to a kind of beetles (Morales-Jimenez *et al.* 2009), we examined the interaction between this bacteria and the vector beetle of PWN. Sampling of dead pine log that harbored the beetle larvae were conducted at Mt. Ogura in January 2013. By chopping the log with an ax, larvae of Japanese pine sawyer beetle, *Monochamus alternatus* and its pupal chambers were taken. DNA samples extracted from the larvae gut and from bacterial bodies scraped from the chamber were used for molecular characterization by t-RFLP in a same manner as experiment 2.3.

3. RESULTUS AND DISCUSSION

3.1. Bacteria Accompanied by PWN in Naturally Infected Pine Stands

In the analysis of bacteria accompanied by PWN isolated from pine forests naturally infected with pine wilt, neither *Pseudomonas* nor *Bacillus* were frequently detected and in especially *Pseudomonas fluorescens* was never detected (Figure 1), all which were suggested to be associated with pathogenicity of pine wilt (Kawazu & Kaneko 1997; Zhao *et al.* 2003). Dominant species in both pine stands were *Serratia proteamaculans* and *Erwinia mallotivora*, which is consistent with the previous study in Portugal (Vicente *et al.* 2011).

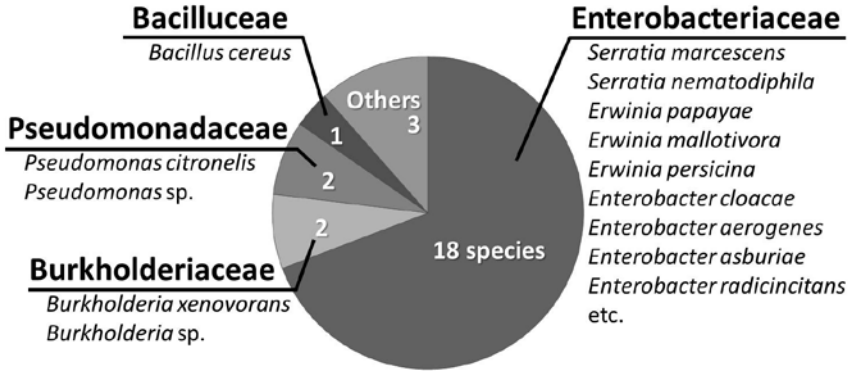

Figure 1. Bacterial flora on the body surface of pine wood nematode isolated from naturally infected pine stands in Japan

3.2. Pathogenicity of Accompanying Bacteria

In pathogenicity test using axenic pine seedlings, PWN inoculation caused a significant mortality regardless of whether and which bacteria was inoculated (Figure 2). Also, no bacteria was detected in the seedlings killed by PWN inoculation. All these suggest that PWN-accompanying bacteria is not necessary for the pathogenicity of pine wilt disease.

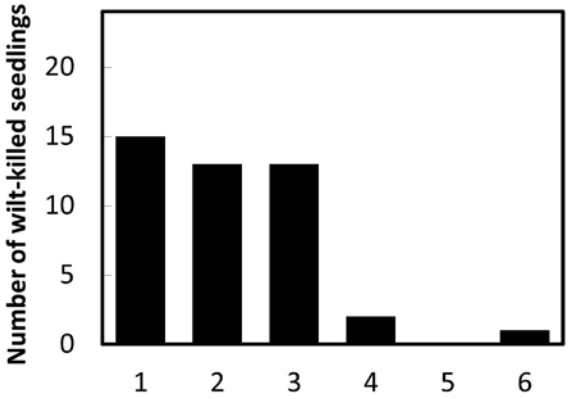

Figure 2. Mortality of pine seedlings after inoculation with (1) bacteria-free PWN, (2) PWN mixed with *S. proteamaculans*, (3) PWN mixed with of *E. mallotivora*, (4) *S. proteamaculans* alone, (5) *E. mallotivora* alone, and (6) sterilized water is shown. Values are averaged number of dead seedlings in two experiments (n=24 for each).

3.3. Succession of Bacterial Flora on the Body Surface of PWN during Symptom Development

Bacterial flora accompanied by PWN after inoculation into host pine was monitored by means of t-RFLP. In all samples a clear peak of 366 bp was detected, which was identical to that of *S. proteamaculans*. It suggests that *S. proteamaculans* get infected into pine plant by any way to establish a specific relation with PWN.

3.4. Interaction of Accompanying Bacteria and the Vector Beetle

Bacterial flora in the pupal chamber and the gut of vector beetle of PWN was characterized by means of t-RFLP. In the pupal chamber, more species of bacteria were detected and the dominance of *S. proteamaculans* (detected as a peak at 366 bp) was lower than in the gut. Thus *S. proteamaculans* may be accompanied by the body surface of PWN which is infecting host plant, propagate inside the plant (on the surface of pupal chamber), and then preferentially colonize the beetle gut to encounter PWN.

4. CONCLUSION

Our results strongly suggest a lack of pathogenicity of PWN-accompanying bacteria, although it is highly possible that certain species of bacteria inhabit on the body surface of PWN. Further studies need to determine the ecological significance of such bacterial species in association with pine wilt disease.

5. ACKNOWLEDGEMENTS

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