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RESEARCH ARTICLE

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Mutual influences in growth and reproduction between pine wood nematode *Bursaphelenchus xylophilus* and bacteria it carries

Abstract The interactions between pine wood nematode and three bacterium strains isolated from the nematode, *Bursaphelenchus xylophilus,* which are two strong pathogenic bacterium strains, *Pseudomonas fluorescens* GcM5-1A and *Pseudomonas putida* ZpB1-2A and a weak-pathogenic bacterium strain, *Pantoea* sp. ZM2C, were studied. The result showed that the strong-pathogenic GcM5-1A strain and ZpB1-2A strain significantly increased fecundity, reproduction rate, and the body volume of the adult nematode. Meanwhile, pine wood nematodes significantly promoted reproduction of the two strong-pathogenic bacterium strains. However, the weak-pathogenic bacterium strain, ZM2C, completely inhibited reproduction of pine wood nematodes. Aseptic pine wood nematodes significantly inhibited reproduction of the strain ZM2C. The results indicated that mutualistic symbiosis exists between pine wood nematodes and the two pathogenic bacteria it carries. The phenomenon showed that the pathogenic bacteria carried by the nematode were not accidentally contaminated, but rather had existed as symbionts of the nematode with which it had coevoluted over a long period. The role of mutualistic symbiosis in the process of pine wilt disease was also discussed.

Keywords pine wood nematode (*Bursaphelenchus xylophilus*), pathogenic bacteria, mutualistic symbiosis

1 Introduction

Pine wilt, a devastating disease in Asia, is mainly transmitted by *Monochamus alternatus* Hope which causes serious damage to pines, such as *Pinus thunbergii* and *P. massoniana*. Since the mechanism of the disease has not been clearly elucidated, effective field control measures, to prevent its spreading, have not been found. Given that bacteria are associated with pine wood nematodes (PWN), the phenomenon of axenic PWN lost pathogenicity and the results of our inoculation experiments, we posit the hypothesis that pine wilt disease is a disease-complex induced by both PWN and the pathogenic bacteria it carries (Zhao et al., 2000a, 2003). It is of fundamental importance for the elucidation of the disease etiology and for finding a new effective control strategy.

2 Material and methods

2.1 Cultivation of PWN and callus of Japanese black pine used in the experiment and preparation of bacterial suspension

2.1.1 Source and culture of nematodes

Bursaphelenchus xylophilus was isolated with Baermann funnels (Zhao et al., 2003) from wilted Japanese black pine at the Liliu plantation in Nanjing (China). One adult male and one adult female were selected and identified as *B. xylophilus*, then cultured on a plate with the fungus *Botrytis cinerea* growing on potato dextrose agar (PDA). After propagation, the PWN were isolated and placed on the surface of 2% agar in a dish to obtain eggs in 6–24 h (Zhao et al., 2000b). The nematodes were washed off the plate with sterilized water and centrifuged for 5 min (1,500 r/min). The supernatant was discarded. The nematodes and eggs were treated with 30% hydrogen peroxide for 10 min. Then the treated nematodes and the eggs were washed and centrifuged three times in sterilized water. The sterilized eggs were collected in a microsyringe, kept under a microscope, and then cultured in an NB medium for 48 h to check if any bacterial colonies had appeared on the culture. If not, the axenic egg suspension was prepared with sterile water

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for further use (Guo et al., 2002). The remaining axenic eggs were put on callus of Japanese black pine to obtain aseptic nematodes.

2.1.2 Culturing Japanese black pine callus

Following the procedure of Zhang et al. (2002), calli were successfully induced, in the dark, on the tissue culture medium of 1/2 MS + 2,4-D 10.0 mg/L + KT 4.0 mg/L + 6-BA 4.0 mg/L, from mature embryos of *P. thunbergii* at 27±1°C. The callus was subcultured on a tissue culture medium of 1/2 MS + NAA 0.5 mg/L + IBA 0.1 mg/L + GA_3 1.0 mg/L + LH 100 mg/L + 2, 4-D 1.0 mg/L, under the same conditions as above.

2.1.3 Preparing bacterial suspension

Three bacterial strains, two strong pathogenic *Pseudomonas fluorescens,* GcM5–1A and Z *Pseudomonas putida*, ZpB1–2A strains and a weak strain of pathogenic bacterium *Pantoea* sp., ZM2C were isolated in relatively large numbers from PWN and cultured on a slant NB medium at 27°C for 24 h. Five loops (0.5 cm in diameter) of bacterial colonies were put in 10 mL of sterilized water in an aseptic glass tube to make a strain suspension.

2.2 Experiment on interactions between bacteria and PWN

2.2.1 Experiment on the effect of bacteria on fecundity of PWN

Two axenic PWN eggs, approximately 0.01 g callus of Japanese black pine, 0.05 mL of suspension of the bacterial strain and 2 mL of sterilized water were put into a small Petri dish (2 cm in diameter). Ten replicates were made for each strain. Four small Petri dishes were put into a bigger dish (8.5 cm in diameter). The dishes were then cultured at 25°C. The dishes were observed every day under sterile conditions from the fifth till the ninth day and the number of newly hatched juveniles was recorded and picked up with a sterilized hooked needle and discarded. The same preparation was made for the control but without a bacterial suspension. The mean number of juveniles produced per pair of nematodes in the ten replicates for each strain was calculated and compared with that of the control by Dunnett's test (SPSS 12.0).

2.2.2 Experiment on the effect of bacteria on egg hatch rates of PWN

One drop of axenic egg suspension, containing two eggs of the nematode, was put in a small Petri dish, which contained a 0.5 mL bacterial suspension of a particular strain in 2 mL sterilized water. The same preparation, but without any bacterium, was used as the control. Four replicates were made for each strain and the control. The dishes were then put into a bigger dish (8.5 cm in diameter). The dishes were cultured at 25°C for 32–40 h. Then the nematodes in each dish were counted and statistically compared with that of the control by Dunnett's test (SPSS 12.0).

2.2.3 Experiment on the effect of bacteria on development rate of PWN

Petri dishes were prepared in the same way as given in the section 2.2.1. The Petri dishes were observed with a microscope at 12 h intervals to determine the time required for one generation cycle of PWN, which was defined as the number of days from the time of the egg hatch, through the juvenile and adult stages till the appearance of the first new eggs.

2.2.4 Experiment of the effect of bacteria on growth and development of adult PWN

Petri dishes were prepared in the same way as given in section 2.2.1. Then 0.01 g calli was added into each small Petri dish and kept at 25°C for 4–8 days. The body length and thickest diameter of adults were measured for both the males and females. Their body volume was calculated using the formula: $2\pi \times (D/2)^2 \times (L/2)/3$, where D is body diameter and L the length of the body. The body volumes were analyzed by Duncan's new multiple range test (SPSS 12.0).

2.2.5 Experiment on the effect of PWN on reproduction of associated bacteria

Two axenic PWN eggs, approximately 0.01 g callus of Japanese black pine, 0.05 mL of suspension of a particular bacterial strain, and 2 mL of sterilized water were put into a small Petri dish (2 cm in diameter). Six replicates were made for each strain. The four small Petri dishes were put into a bigger dish (8.5 cm in diameter). The dishes were then cultured at 25°C. One dish was taken each day from the second till the seventh day from the treated and control groups. Then the same volume of the culturing liquid was put into the plates with the NB medium. The number of bacterial colonies that grew on the plates was compared and statistically evaluated with ANOVA (SPSS 12.0). Two curves of the number of bacterial colonies from the treated and corresponding control groups were drawn in order to compare the differences between each bacterial strain and its control.

Table 1 Effect of bacteria on the fecundity, egg hatch rate, development rate of pine wood nematode *Bursaphelenchus xylophilus*

Treatment	Fecundity	Rate of egg hatching /%	Days for a generation	Days to show wilting
Pseudomonas fluorescens	45.00±3.70a	100.00±0.00a	4.00±0.32a	4.2
Pseudomonas putida	43.20±0.35a	100.00±0.00a	4.22±0.34a	4.5
Pantoea sp.	0.00c	100.00±0.00a	–	9.7
Control	2.00±0.00b	100.00±0.00a	5.33±0.33b	>12

The data in the table are means±SE. Means in the same column followed by different letters differ significantly at $P<0.01$. "—"showing no data.

3 Results and analyses

3.1 Effect of bacteria on the fecundity, egg hatch rate, and development rate of PWN

The reproduction of PWN was significantly [$p(F) < 0.05$] promoted by *Pseudomonas fluorescens* and *P. putida* when compared to the control and other treatments (Table 1). PWN treated with *Pantoea* sp. showed a completely inhibitory effect on its reproduction and the second generation was entirely absent. The fecundity of PWN treated with the two strains, *P. fluorescens* GcM5-1A and *P. putida* ZpB1-2A, was significantly larger than that of the other treatments at $\alpha=0.01$ level (Table 1). It indicated that the two strains of *P. fluorescens* and *P. putida* did not affect egg hatching of PWN. However, the bacterium *Pantoea* sp. could inhibit reproduction of PWN.

The experiments of the bacteria on egg hatching of PWN showed that there was no obvious difference between that of the treatments with the bacteria and the controls at the $\alpha=0.01$ level of significance after the sterilization treatment with hydrogen peroxide (Table 1). It indicated that the egg hatch rate of PWN was not affected by the sterilization with a solution of hydrogen peroxide.

3.2 Effect of bacteria on the growth and development of female PWN

Table 2 shows that there were differences among the treatments with bacteria in the volume of female bodies. At $p(F)<0.05$ level, there were significant differences between the body volumes of adult females treated respectively with the strains of GcM5-1A and ZpB1-2A and that of the control, while there is no significant difference between female body volumes of PWN treated with *Pantoea* sp. and the control. However, the male body volume in the same treatment was significantly less than that of control. It indicated that *Pseudomonas fluorescens* and *P. putida* promoted the growth of female and male. At $p(F)<0.05$ bacteria of the *Pantoea* sp. had no noticeable effect on the growth of the body volume of female PNW, but showed significantly inhibitory effects on the growth of male bodies.

Pine wilt disease is a devastating disease, which, in Asia, is mainly transmitted by *Monochamus alternatus* Hope and causes serious damage to pine forests such as *Pinus thunbergii* and *P. massoniana*. Further discovery of the relationship between PWN and its carrying bacteria will be essential for verifying our hypothesis and clarifying etiology of the disease and the development of an efficient control strategy.

Table 2 Effect of bacteria on the growth and development of female pine wood nematode *Bursaphelenchus xylophilus*

Treatment	Adult female volume /mm^3	Adult male volume /mm^3
Pseudomonas fluorescens	6.756×10^{-4}±8.216E-05a	2.101×10^{-4}±2.173E-05a
Pseudomonas putida	6.387×10^{-4}±6.450E-05a	3.080×10^{-4}±3.192E-05a
Pantoea sp.	1.415×10^{-4}±4.755E-05b	0.654×10^{-4}±7.957E-06c
Control	2.125×10^{-4}±3.645E-05b	1.190×10^{-4}±2.276E-05b

The data in the table are means±SE. Means in the same column followed by different letters differ significantly at $P<0.01$. "—"showing no data.

3.3 Effect of PWN on reproduction of its associated bacteria

Figures 1–3 show the results of experiments on the effect of PWN on reproduction of the strains GcM5-1A, ZpB1-2A, and ZM2. Reproduction of the strains GcM5-1A and ZpB1-2A was significantly increased by the axenic PWN ($\alpha<0.01$) (Figs. 1 and 2). The amounts of the cells of treated bacteria were much more than that of the control. However, Fig. 3 indicates that reproduction of treated ZM2 was significantly inhibited by the axenic PWN ($\alpha<0.01$), i.e., the amount of the cells of ZM2 was significantly less than that of the control.

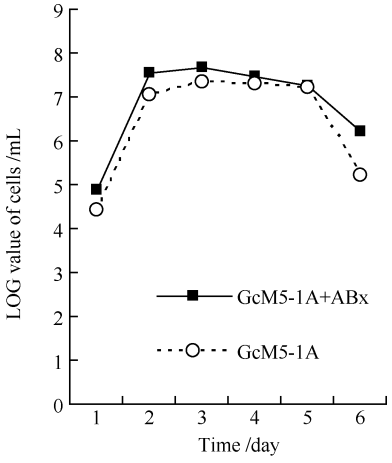

Fig. 1 Effect of PWN on reproduction of GcM5-1A strain

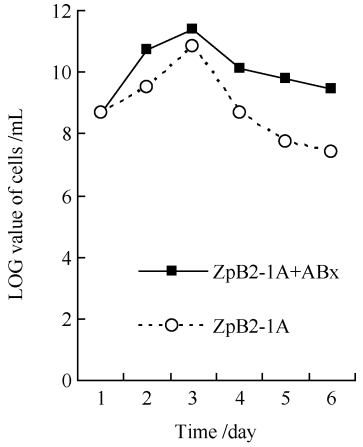

Fig. 2 Effect of PWN on reproduction of ZpB2-1A strain

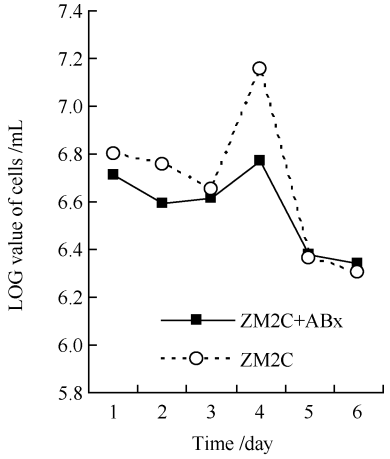

Fig. 3 Effect of PWN on reproduction of ZM2C strain

4 Discussion

Three bacterial strains, including two strong and one weak pathogenic bacterium strains isolated from PWN in relatively large numbers were used in the experiments. The strong pathogenic GcM5–1A and ZpB1–2A strains significantly increased fecundity, reproduction rate, and the body volume of adult nematodes. Meanwhile, PWN significantly promoted reproduction of the two strong pathogenic strains. In a mutualistic symbiosis both partners benefit from the relationship (Paracer et al., 2000). It was clear that mutualistic symbiosis existed in the complex of PWN and the pathogenic bacteria. This phenomenon showed that the pathogenic bacteria carried by the nematode might not be accidentally contaminated, but could be a symbiosis coevoluted with the nematode over long time periods. *Pantoea* sp. ZM2C not only completely inhibited reproduction of the nematode, but also decreased the body volume of the adult males. Meanwhile, aseptic PWN significantly inhibited reproduction of the strain ZM2C. It indicates that there is an interinhibitory tendency between PWN and the bacterium strain of *Pantoea* sp.

The phenomenon of the mutualistic symbiosis between PWN and certain of its associated bacteria implied that the mutualistic symbiosis of PWN and the bacteria might be the result of coevolution, rather than an accidental contamination. The mutualistic symbiosis between PWN and its carrying bacteria provided further evidence for our new theory that pine wilt disease is a complex disease caused by both the nematode and their pathogenic bacteria (Zhao et al., 2000a, 2003; Han et al., 2001).

The mutualistic symbiosis of PWN and the bacteria exhibited: 1) PWN (as vectors) carry mutualistic bacteria and invade the host through wounds; 2) PWN could promote bacteria to produce phytotoxins (Han et al., 2001); 3) Mutualistic bacteria may provide certain nutrients or favorable environmental factors to promote growth, fecundity, and reproduction of PWN. The mutualistic relationship accelerated toxin production from the symbiotic bacteria (Liang, 2004) and death of the host pine trees. This would favor colonization of the huge resources of the dead tree by PWN and its associated bacteria.

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