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Full Length Research Paper

The Relationship Between *Bursaphelenchus xylophilus* and the Endoparasitic Fungus *Esteya vermicola*: Attraction Dynamics

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The investigations on attraction of nematodes to nematophagous fungi have mostly dealt with the nematode-trapping species and just were limited to the assays on plate. In present study, attraction of pinewood nematodes to the living mycelia of endoparasitic fungus, *Esteya vermicola*, was investigated. It was confirmed that the living mycelia of *E. vermicola* were attractive not only to pinewood nematodes on plate, but also to that in the discs of infected pine seedling, dead blocks of infected pine tree, 15 and 30 days infected pine seedlings. Moreover, the volatiles released by *E. vermicola* also were attractive to pinewood nematodes in 15 and 30 days infected pine seedlings. This study may provide information for the application of *E. vermicola* as biological control agent of pinewood nematode.

Key words: *Esteya vermicola*, pinewood nematode, *Bursaphelenchus xylophilus*, *Pinus densiflora*, living mycelia, attraction, volatiles.

INTRODUCTION

The attraction of nematodes to fungi has been investigated primarily to show the host-finding mecha-nisms of fungus-feeding nematodes (Townshend, 1964; Klink et al., 1970). Subsequently, researches further demonstrated the attraction of nematodes to culture filtrates and living mycelia of several nematophagous fungi. It was suggested that certain exudative and volatile compounds, which were continuously produced by living fungal mycelia, might be responsible for the attraction (Balan et al., 1976; Field and Webster, 1977; Jansson, 1982; Jansson and Nordbring-Hertz, 1979, 1980; Monoson et al., 1973). Mamiya (2006) reported that the living mycelia and exudative substances of nemato-phagous wood-decay fungi showed a very strong ability to attract pinewood nematode (PWN), *Bursaphelenchus xylophilus*, which is released by pine tree and larval of *Monochamus alternatus* (Zhao et al., 2007). The causal agent of the pine wilts disease. PWN fed on living pine tissue and strongly were attracted by volatiles.

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Esteya vermicola is the first recorded endoparasitic fungus of PWN and exhibits high infection activity in plate (Liou et al., 1999; Wang et al., 2008; 2009). On the other hand, PWN is mycophagous (Kobayashi et al., 1974). Throughout experiments in previous studies, it was commonly observed that PWN congregated around colonies of *E. vermicola* on agar plates soon after introduction of nematodes to the plate. Accordingly, the purposes of present study were to investigate the attraction of PWN to living mycelia of *E. vermicola*, and to compare its attraction ability with pine tree. Since the pine wilt disease is a complex interrelationship among pine tree, fungi, bacteria and nematode, this study may have significant practical implication, provide information for the development of *E. vermicola* as biological control agent to combat the devastating pine wilt disease.

MATERIALS AND METHODS

Culture of *E. vermicola*

E. vermicola CNU 120806 was obtained from the Agriculture Bioscience Biotech Centre, Chungnam National University, Korea. Fungus was cultured on the potato dextrose agar (PDA) plate at

26°C. The conidia suspension was prepared by using sterilized 0.05% tween 80 solution to dislodge the conidia from 7-day-old colony. Conidia concentration was determined by employing a haemocytometer and adjusted to 10^6 ml^{-1}. 0.3 ml conidial suspension was spread onto the surface of 2% water agar (WA) plate in a 9 cm diameter Petri dish. The plate was uncovered for 30 min in a laminar flow hood to evaporate excess water, and then cultured at 26°C for 10 days. Discs (1 cm diameter) were cut with a sterilized cork borer for the next works.

Monoxenic culture of *B. xylophilus*

Monoxenic PWN was cultured and prepared as aqueous suspension according to the reported method (Wang et al., 2008, 2009). Briefly, *Botrytis cinerea* was cultured on PDA plate at 26°C, and then inoculated with PWN while the fungus grew fully. Subsequently, the plate was cultured until fungal mycelia had been completely consumed. The cultured nematodes were separated from culture medium using the Baerman funnel technique. The harvested nematodes were suffered from the surface disinfection with 0.1% NaOCl solution for 1 min, rinsed three times with sterilized distilled water, and then prepared as an aqueous suspension for next work.

Culture of 4-year pine seedlings of *Pinus densiflora*

Four years old pine seedlings of *P. densiflora* were potted in pots (15 cm in diameter, 18 cm in height) with mixture of sand soil and organic fertilizer and cultured in greenhouse at 27°C. Axenic discs (0.4 cm in height; 1 cm in diameter) were cut from the surface disinfected (0.1% NaOCl, 1 min) stem of pine seedling with a sterilized scissors, and prepared for next work.

Attraction assay of PWN to living mycelia of *E. vermicola* and pine seedling discs

Attraction assay was carried out on the 2% WA plate in a 9 cm diameter Petri dish. On a fresh WA plate, two *E. vermicola* mycelia discs or pine seedling discs were placed opposite to each other and 5 cm apart, 1 cm from the edge of plate. Two WA control discs without fungal mycelia also were placed opposite to each other on a line which is at right angle to that of fungal discs or pine seedling discs. The plate was incubated in the dark at 26°C for 24 h to establish a concentration gradient of exudative substances which diffuse from the fungal or pine seedling discs into the WA plate. 20 l of nematode suspension (about 100 individuals) was applied to the centre point of the plate and sealed well with parafilm. After another 24 h, the total numbers of nematodes accumulating in, on or under the two fungal discs and control discs were counted, respectively, under light microscope in a 40 - 100× magnification. Since it was difficult to directly count the nematodes in or on the pine seeding discs by using light microscope, pine discs together with agar blocks under them were cut with a knife and then immersed in the water in 6 cm diameter Petri dish. 6 h later, the number of nematodes released into water was counted and recorded under light microscope.

In a parallel experiment, the attraction ability of PWN to *E. vermicola* mycelia was directly compare with the pine seedling discs by placing them on the same WA plate. The assay was carried out by following the same experimental procedure mentioned above. When both *E. vermicola* and pine seedling discs have a strong ability to attract nematodes, nematodes were attracted by both of them. Accordingly, there was no statistically significant difference between the number of nenatodes attracted to the *E. vermicola* mycelia discs and that attracted to the pine seedling discs. Otherwise, more nematodes were attracted to the one which had stronger attraction ability to nematodes.

The test plates had to be prepared for at least 2 days before use so that water of the nematode suspension disappeared quickly. WA agar provided a very thin mycelia mat, thus facilitating microscope observation of both mycelia and nematodes.

Attraction assay of PWN to *E. vermicola* from infected pine seedling discs

In this experiment, pine discs (0.4 cm in height; 1 cm in diameter) were cut from the infected pine seedlings, which were inoculated with PWN suspension (200 l, about 3000 individuals) 10 days ago, but no wilting symptoms. On a fresh WA plate, two *E. vermicola* mycelia discs were placed opposite to each other 5 cm apart, 1 cm from the edge of plate. The plate was incubated in the dark at 26°C for 24 h to establish a concentration gradient of fungal exudative substances which diffuse from the discs into the WA plate. One infected pine seedling disc was applied to the centre point of the plate and well sealed with parafilm. After another 24 h, the number of nematodes aggregating in, on or under the mycelia discs of *E. vermicola* was counted under light microscope. After removing *E. vermicola* discs together with agar blocks under them, remanent agar plate and pine disc were prepared for nematode extraction by the Baermann funnel technique. The number of released nematodes was recorded after 12 h. Total number of nematodes per plate was tallied. The number of nematodes attracted to the mycelia discs of *E. vermicola* was expressed as a percentage of the total number per plate. On the control plate, two WA discs without fungal mycelia were placed and treated exactly the same way with the fungal discs.

Attraction assay of PWN to *E. vermicola* from dead block of infected pine tree

Infected pine blocks (0.4 × 0.4 × 0.4 cm^3) were randomly cut from the logs of about 25 years old *P. densiflora*, which was killed by PWN in nature environment. Assay was conducted according to the method mentioned above, just instead of infected pine seedling discs with dead infected pine blocks.

Attraction assay of PWN to *E. vermicola* from infected pine seedlings

Pine seedlings were inoculated with PWN suspension (200 l, about 3000 individuals) at the middle part 7, 15 and 30 days before the assay, and showed no wilting, partial wilting and dying symptom, respectively. From the infection point, barks were removed at intervals of 5 cm and replaced with mycelia discs of *E. vermicola*, which were fixed and sealed with parafilm. WA discs were used as control and fixed at the opposite side against the discs of *E. vermicola* totally in the same way. 12 h later, the discs were taken out and inspected under light microscope.

In a parallel experiment, the attraction of PWN by volatile substance discharged by living mycelia of *E. vermicola* was determined by using hollow tubes. 2 ml sterile Eppendorf tube was cut off the lid and round bottom, appressed with a hollow, thin layer of 2% WA around the wall for the movement of nematode. A fungal disc of *E. vermicola* was gently pressed into the tube at one end to make mycelia connect with the WA layer. Another end of the tube was contacted with the stem of infected pine seedling to fix and seal the tube well by the same way mentioned above. As control, tubes with WA discs were fixed at the opposite side against the tubes of *E. vermicola* by following the same procedure. 12 h later, the number of nematodes accumulating around the fungal or mycelia

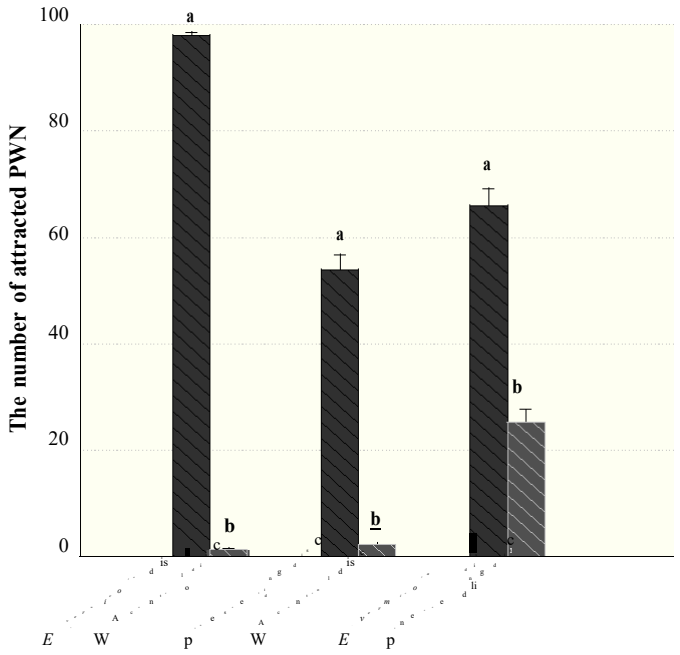

Figure 1. Attraction of PWN to the living mycelia discs of *E*. *vermicola* and pine seedling discs. Each bar represents means ± SE of 3 replicates. Bars with the different letter above are highly significantly different (P < 0.01).

control disc was recorded. If the volatile attractive substance was discharged by living mycelia of *E*. *vermicola*, nematodes could be attracted to *E*. *vermicola* discs though the WA lay in the hollow tube. Otherwise, none or few of nematode could be observed around *E*. *vermicola* discs.

Data analysis

Each assay consisted of three replicates. The data were analyzed using SPSS 17.0 version for Windows.

RESULTS

Attraction assay of PWN to living mycelia of *E. vermicola* and pine seedling discs

The numbers of nematodes accumulating on, in or under *E*. *vermicola* discs and pine seedling discs were statistically significantly greater (P < 0.01) than that of the WA control discs (Figure 1), suggesting they were attractive to PWN. Almost all of the tested nematodes (98%) were attracted to the living mycelia discs of *E*. *vermicola*, while only about 50% of the tested nematodes were attracted to the pine seedling discs. When discs of *E*. *vermicola* and pine seedling were inoculated into the same plate, their attraction abilities to PWN were compared directly. According to the results, the number of nematodes aggregating in, on or under the mycelia discs of *E*. *vermicola* was significantly higher (P<0.01) than that

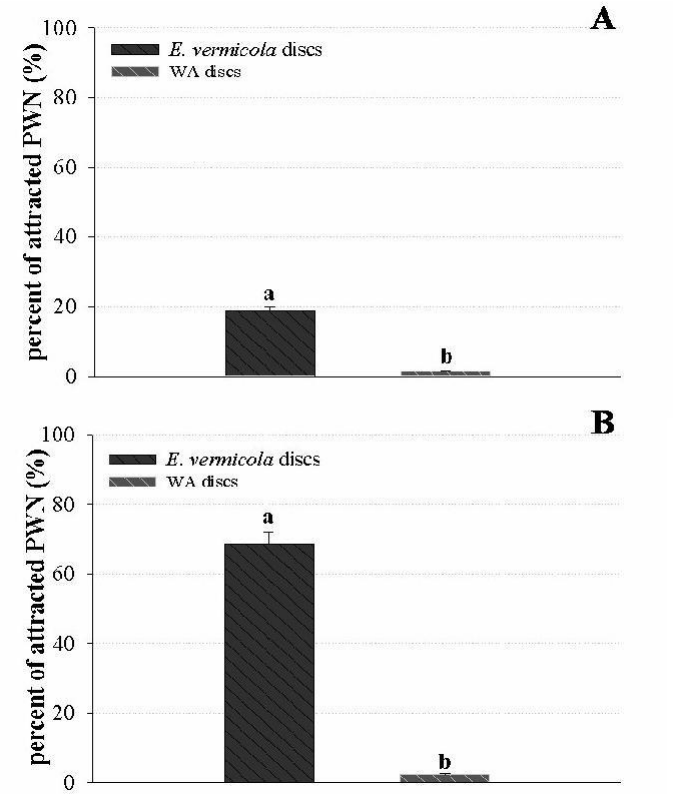

Figure 2. Attraction of PWN to the living mycelia discs of *E*. *vermicola* from the 10 days infected pine seedling discs and the dead infected pine wood blocks. **(A)** Attraction of PWN to the living discs of *E*. *vermicola* from the 10-day-infected pine seedling discs. **(B)** Attraction of PWN to the living mycelia discs of *E*. *vermicola* from the dead infected pine wood blocks. Each bar represents means ± SE of 3 replicates. Bars with the different letter above are highly significantly different (P < 0.01).

that of pine seedling discs (Figure 1). It was suggested that the attraction ability of *E*. *vermicola* discs was significantly stronger (P < 0.01) than that of pine seedling discs in this experiment.

Attraction assay of PWN to *E. vermicola* from infected pine seedling discs

Ten days after PWN inoculation, the infected pine seedlings didn't yet show any wilting symptom. Although the secretion of resin slightly decreased, they looked like healthy, fresh and green. There were about 50 - 70 nematodes inside of per pine disc, while only 9 - 14 nematodes were observed in, on or under the mycelia discs of *E*. *vermicola*. However, the number of nematodes accumulated around *E*. *vermicola* discs was significant higher (P < 0.01) than that around WA control discs (Figure 2; a) . It was therefore confirmed that the mycelia discs of *E*. *vermicola* were attractive to nematodes inside of infected pine seedling discs, though the attraction ability was somewhat low.

Table 1. The number of PWN attracted to the mycelia disc of *E*. *vermicola* or WA control disc, which was directly fixed to the stem of infected pine seedling.

Pine tree	Disc	-15 cm	-10 cm	-5 cm	+5 cm	+10 cm	+15 cm
7 d after infection	*E. vermicola*	0	0	0	0	0	0
	WA control	0	0	0	0	0	0
15 d after infection	*E. vermicola*	0	0	7-12	1 - 3	0	0
	WA control	0	0	0	0	0	0
30 d after infection	*E. vermicola*	20-34	40-55	140 - 220	10 - 24	2 - 7	0
	WA control	1 - 3	2 - 4	4 - 7	2 - 3	0	0

+ means above infection point; - means below infection point.

Table 2. The number of PWN attracted to the *E*. *vermicola* tube or WA control tube, which was fixed to the stem of infected pine seedling.

Pine tree	Tube	-15 cm	-10 cm	-5 cm	+5 cm	+10 cm	+15 cm
7 d after infection	*E. vermicola*	0	0	0	0	0	0
	WA control	0	0	0	0	0	0
15 d after infection	*E. vermicola*	0	0	4 - 6	1 - 2	0	0
	WA control	0	0	0	0	0	0
30 d after infection	*E. vermicola*	10-20	40-50	80 - 120	6-13	1 - 5	0
	WA control	0	1 - 3	3 - 7	1 - 2	0	0

+ means above infection point; - means below infection point.

Attraction assay of PWN to *E. vermicola* from dead block of infected pine tree

After being killed by PWN in the nature environment, the pine tree no longer secreted resin, lost most of water and was colonized by many blue-stain fungi, which provided food and nutrition for nematodes. Accordingly, the number of PWN inside of pine wood sharply increased, reached 120 - 160 nematodes per infected pine block. According to the result, about 70% of them were observed on, in or under the mycelia discs of *E*. *vermicola*, while only 2.3% nematodes were checked around the WA discs on the control plate (Figure 2; b). It seemed that PWN inside of dead infected pine blocks were stronly attracted by the mycelia discs of *E*. *vermicola*, though there were many blue-stain fungi within pine blocks.

Attraction assay of PWN to *E. vermicola* from infected pine seedlings

Seven days after infection, the number of PWN inside of pine seedlings was very low and no any wilting symptom could be observed. Pine seedlings still had so strong attraction ability to PWN that nematode was found from neither the mycelia discs nor the tubes of *E*. *vermicola* (Tables 1 and 2). 15 days after infection, the number of PWN was sharply increased, causing the loss of water, stop of resin secretion and partial wilting of pine seedlings. A few of nematodes were observed from both the tubes and discs of *E*. *vermicola* only at the points that 5 cm below or above the infection point, while no nematode was found from the WA control discs and tubes (Table 1 and 2). 30 days after infection, there was a high number of PWN inside of infected pine seedlings, which were dying, no resin secretion, and getting dried on the top part. Except for the point 15 cm above the infection point, nematodes were observed from both the tubes and discs of *E*. *vermicola* at all of the tested points. Moreover, the numbers of nematodes were obviously higher ($P < 0.05$) than those from 15 day infected pine seedlings. With regard to the WA control discs and tubes, however, nematodes only were seen at several points and the numbers of nematodes were significantly lower ($P < 0.05$) than those of *E*. *vermicola* (Table 1 and 2).

According to the above analysis, it seemed that certain attractive volatile substances were discharged by the living mycelia of *E*. *vermicola* and PWN could be attracted from the 15 and 30 day infected pine seedlings. Moreover, the attraction ability was related to the number of PWN inside of infected pine seedlings and the condition of pine seedlings.

DISCUSSIONS

Although many investigations on attraction of nematodes to nematophagous fungi have been conducted, they were mainly dealt with the nematode-trapping fungi and just limited to the assays on plate. The attraction of nematodes from plant tissues to living mycelia of fungi has never been investigated and reported. In present study, it was investigated and confirmed that the attraction of PWN to the living mycelia of an endoparasitic fungus *E*. *vermicola* not only from plate, discs of infected pine seedlings and dead blocks of infected pine tree, but also from the infected pine seedlings. According to the results, in the earlier stage of pine wilt disease, the number of PWN was low and nematodes were mainly attracted by the substances released by the pine tissues. With the increase of PWN number, pine wilt disease stepped into the second stage and the production of attractive substances was decreased. Accordingly, the attraction ability of *E*. *vermicola* to PWN was relatively increased. Finally, the infected pine seedling was dying and lost most of attraction ability to PWN. On the contrary, *E*. *vermicola* showed the high attraction ability to PWN.

By employing the hollow tube in this study, in addition, it was proved that the living mycelia of *E*. *vermicola* produced some attractive volatile substances, which could attract PWN from the 15 and 30 days infected pine seedlings. Moreover, reproducible results were obtained in this case. This study may provide the reference and instruction for the development and application of *E*. *vermicola* as biological control agent of PWN.

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