

Esteya vermicola Controls the Pinewood Nematode, *Bursaphelenchus xylophilus*, in Pine Seedlings

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Abstract: *Esteya vermicola* (Ophiostomataceae) is an endoparasitic fungus that has great potential as a biological control agent against the pinewood nematode *Bursaphelenchus xylophilus* which causes pine wilt disease. We tested *E. vermicola* for control of pine wilt disease by spraying *E. vermicola* conidia on artificial wounds on pine seedlings, and the optimum *E. vermicola* treatment density and application time were also investigated in the greenhouse. The wounds were similar to those made by sawyer beetles (Coleoptera: Cerambycidae). *Esteya vermicola* treatments significantly increased the survival rate of pine seedlings that were infected by pinewood nematodes. Wounded plants sprayed with 10^7 CFU/ml *E. vermicola* had a 73.0% greater survival rate than nonwounded pine seedlings treated similarly. The treatment of pine seedlings with 10^7 CFU/ml *E. vermicola* 14 d before nematode infection increased their survival rate by 90.0%. The number of pinewood nematodes isolated from dead pine seedlings sprayed with *E. vermicola* was 76% less than the number of pinewood nematodes in the controls. Moreover, infected nematodes and the hyphae of *E. vermicola* were detected in the dead or wilting pine seedlings. Therefore, spraying *E. vermicola* on the wounds of pine seedlings made by sawyer beetles provides good control of the pine wilt disease that is caused by pinewood nematodes.

Key words: biological control, *Esteya vermicola*, pinewood nematode, survival rate, wound.

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle, is the causal agent of pine wilt disease (PWD) (Kiyohara and Tokushige, 1971). The spread of this nematode occurs via pine sawyer beetles (*Monochamus* spp., Cerambycidae), which are attracted to pine trees for feeding or oviposition (Mamiya and Enda, 1972). The PWN is native to North America, where it is not considered to be a primary pathogen of native pines but is the causal agent of pine wilt in some nonnative pines (Dwinell, 1997). So far, PWD has been reported in Japan, China, Korea, Portugal, and Spain (Wang et al., 2010; Abelleira et al., 2011). The PWN is a worldwide threat to pine forests and forest ecosystems and causes significant economic losses.

Protection of pine forests against the PWN is an important problem in China. Several methods are currently used to control this disease. These include fumigation, burning, clear-cutting, breeding, aerial insecticide spraying, and trunk injection. The fumigation of *Pinus pinaster* (Pinaceae) boards with sulfuryl fluoride at a certain temperature is effective in controlling the PWN (Luís et al., 2013). Preventative clear-cutting of neighboring asymptomatic pine trees and the removal of fallen logs or branches can curb the spread of PWD (Kwon et al., 2011). Pesticides are also used to kill sawyer beetles but this is only a temporary solution and may cause unwanted damage to the ecosystem (Kwon et al., 2005). A breeding program for pine trees resistant to PWD has been initiated in Japan, and selected clones of *Pinus densiflora* and *Pinus thunbergii* have been

identified as being resistant to PWD (Nose and Shiraishi, 2008). Injecting a formulation of emamectin benzoate prevents the wilting of trees infested by the PWN, and this treatment lasts for up to 3 yr (Kazuya et al., 2003). Inoculating logs (*P. densiflora*) with *Trichoderma* sp. to decrease the number of nematodes carried by the emerging Japanese pine sawyer adults also helps to reduce the prevalence of PWD in Japan (Maehara, 2008). Saiki et al. (1984) tried to control PWNs in pine seedlings by spraying a spore suspension of the nematode-trapping fungus, *Arthrobotrys* sp. Because the growing region of pine trees is very large and the variety of pine habitats, such as cliffs and steep hills, is complex, operations such as cutting, burning, fumigation, and trunk injection are unfeasible. Thus, aerial spraying with selective environmentally friendly fungal formulations appears to be one of the best practicable means to control PWD.

Esteya vermicola is the first endoparasitic fungus shown to exhibit high infectivity against the PWN. *Esteya vermicola* produces two kinds of conidia, lunate and bacilloid. Lunate conidia can adhere to and infect the nematode by cuticle penetration, immobilization, and digestion of the internal contents of PWNs (Liou et al., 1999; Wang et al., 2011a). *Esteya vermicola* produce α -pinene, β -pinene, and camphor, which are the major volatile organic compounds that attract PWNs (Lin et al., 2013). *Esteya vermicola* exhibits strong nematocidal effects in vitro (Wang et al., 2011b); however, its field efficacy remains undetermined.

Adult beetles feed on the bark of young branches, and the nematodes present in the insect spiracles enter the tree through the feeding wounds caused by the beetle (Donald et al., 2003). During this process, fungi are introduced into the tree by the beetle from the initial insect wound (Reid, 1967). Therefore, we artificially inflicted wounds that were similar to those caused by the sawyer beetle on healthy pine seedlings that were uninfected with PWNs and sprayed *E. vermicola* on these wounds to control PWD. We also investigated the

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optimum treatment density of *E. vermicola* and spraying time to control PWD in the greenhouse.

MATERIALS AND METHODS

Sources of fungal strains and nematodes: *Esteya vermicola* CNU 120806 and *Botrytis cinerea* KACC 40573 were obtained from the Agriculture Bioscience Biotechnology Center, Chungnam National University, Korea. PWNs feed on fungi such as *B. cinerea* (Kikuchi et al., 2005), which was treated as a negative control. *Esteya vermicola* and *B. cinerea* were cultured on PDA (Acumedia, U.S.A.) plates at 26°C. A conidial suspension was prepared by using a 0.05% Tween 80 solution to dislodge conidia from 7-d-old colonies. The conidia density was determined using a hemocytometer, and the density of *E. vermicola* was adjusted to 10⁷, 10⁵, and 10³ CFU/ml, whereas the density of *B. cinerea* was adjusted to 10⁵ CFU/ml.

Bursaphelenchus xylophilus (the PWN) was isolated from wilted pine tree logs using the Baermann funnel technique (Ogura and Nakashima, 2002). *Botrytis cinerea* was cultured on PDA culture medium at 26°C for 5 to 7 d until the fungal colony covered the whole petri dish (9 cm diam.), and then PWNs were transferred onto the fungi. The plates containing *B. cinerea* and PWNs were maintained at 20°C for 28 d (Maehara and Futai, 2000). The living PWNs (mixture of adults and propagative generation juveniles) were isolated from the culture medium using the Baermann funnel technique. The collected PWNs were rinsed three times with sterile water with the assistance of centrifugation (650×g, 3 min) and finally adjusted to the desired concentration by the addition of sterile water or by decantation (Kawazu, 1996).

Spraying the conidial suspension onto the wounds of pine seedlings in 2012: One hundred eighty-three-year-old pine seedlings (*P. densiflora* Sieb. et Zucc, 1.5 cm trunk diam., 50 cm high) were purchased from Songoun Garden in Daejeon, Korea. The seedlings were randomly classified into three groups (*n* = 60), and each group was planted in a separate location covered with a small greenhouse (3 m long × 1.5 m wide × 1.8 m high). In the first group of 60 pine seedlings, four pieces (0.5–0.8 cm diam.) of bark were removed from areas near the shoot on each young branch of the seedling using a scalpel, imitating the beetles' biting habit. The beetles' habit of mining initially at an oblique angle before mining upward results in a broader streak or embolism (Reid, 1967). No bark samples were removed from the other two groups. The wounded and unwounded pine seedlings in the first and second groups were sprayed with 10⁵ CFU/ml *E. vermicola*, and the unwounded pine seedlings in the third group were sprayed with water as a control. Ten days after spraying, all pine seedlings in each group were injected with 100 µl of nematode suspension (approximately 3,000

nematodes) and then sealed with film paper (Parafilm, Bemis, U.S.A.). The injection point was near the roots of the pine seedlings.

Spraying the conidial suspension onto the wounds of pine seedlings in 2013 and 2014: Three hundred four-year-old pine seedlings (*P. densiflora* Sieb. et Zucc, 1.9 cm trunk diam., 60 cm high) were purchased from Songyuan Garden in Linyi, China. The experiments were replicated two times in April 2013 and March 2014. The seedlings were randomly classified into five groups, and each group was planted in a separate location covered with a small greenhouse (3 m long × 1.5 m wide × 1.8 m high). Ten pine seedlings were arranged in one row, and six rows were maintained in each greenhouse (Table 1).

The 60 pine seedlings (*n* = 60) were divided into two groups (wounded [*n* = 30] and unwounded seedlings [*n* = 30]) in each small greenhouse. In the first group of 30 pine seedlings (*n* = 30), four pieces (0.5–0.8 cm diam.) of bark were removed as described above. No bark samples were removed from the other 30 pine seedlings (*n* = 30). The 60 pine seedlings in the first group, which were sprayed with 10⁷ CFU/ml *E. vermicola*, were labelled as E1, and the second and third groups which were sprayed with 10⁵ or 10³ CFU/ml *E. vermicola*, were labelled as E2 or E3, respectively. Water was sprayed onto the 60 pine seedlings in the fourth group, which served as a control. The pine seedlings in the fifth group, which were sprayed with 10⁵ CFU/ml *B. cinerea*, were labelled B and served as a negative control. After spraying, each pine seedling was covered with a plastic bag for 1 d to maintain moisture. Ten days after spraying, 20 pine seedlings (wounded seedlings *n* = 10, unwounded seedlings *n* = 10) in each group (Control, E1, E2, E3, and B) were injected with 100 µl of nematode suspension (approximately 3,000

TABLE 1. Arrangement of pine seedlings undergoing different treatments in five greenhouses in 2013 and 2014 (E1, E2, E3, control, and B). Sixty pine trees were split into two groups (wound group and no wound group) in each greenhouse.

Treatment	Wound group	No wound group
E1	Infected PWN after 10 d	Infected PWN after 10 d
	Infected PWN after 20 d	Infected PWN after 20 d
	Infected PWN after 30 d	Infected PWN after 30 d
E2	Infected PWN after 10 d	Infected PWN after 10 d
	Infected PWN after 20 d	Infected PWN after 20 d
	Infected PWN after 30 d	Infected PWN after 30 d
E3	Infected PWN after 10 d	Infected PWN after 10 d
	Infected PWN after 20 d	Infected PWN after 20 d
	Infected PWN after 30 d	Infected PWN after 30 d
Control	Infected PWN after 10 d	Infected PWN after 10 d
	Infected PWN after 20 d	Infected PWN after 20 d
	Infected PWN after 30 d	Infected PWN after 30 d
B	Infected PWN after 10 d	Infected PWN after 10 d
	Infected PWN after 20 d	Infected PWN after 20 d
	Infected PWN after 30 d	Infected PWN after 30 d

PWN = pinewood nematode; E1 = 10⁷ CFU/ml *Esteya vermicola*; E2 = 10⁵ CFU/ml *E. vermicola*; E3 = 10³ CFU/ml *E. vermicola*; B = 10⁵ CFU/ml *Botrytis cinerea*.

nematodes) and then sealed with film paper. The injection point was near the roots of the pine seedlings. After 20 and 30 d, another 10 wounded seedlings and 10 unwounded seedlings in each group were injected with 100 µl of nematode suspension (approximately 3,000 nematodes) and then sealed with film paper during each period. The time intervals between spraying the pine seedlings with *E. vermicola* and infection with PWNs were 10, 20, and 30 d. All the PWNs that were injected into the pine seedlings at the different time points were cultured in petri dishes with *B. xylophilus*, and isolated using the Baermann funnel technique 1 d before injection into the seedlings.

All the pine seedlings were continuously grown in the greenhouse, and the test was evaluated in December. The seedlings were visually inspected for discoloration and wilting every 14 d for 7 mon. Dead pine seedlings were cut into small segments and prepared for the isolation of PWNs.

RESULTS

Survival rate of pine seedlings in 2012: From March 1 to December 26, 2012, the survival rates of wounded and unwounded pine tree seedlings that were sprayed with *E. vermicola* and infected with PWNs were investigated (Table 2). The survival rate of seedlings that were wounded and sprayed with *E. vermicola* was higher than that of unwounded seedlings ($P < 0.05$). However, the survival rate of seedlings that were not sprayed with *E. vermicola* was the lowest ($P < 0.05$), only $13.3\% \pm 1.9\%$.

Survival rates of pine seedlings in 2013 and 2014: The survival rates of pine seedlings sprayed with *E. vermicola* on the wound ($n = 30$) and infected with PWNs are shown in Fig. 1. The survival rates of pine seedlings increased to $73.0\% \pm 3.3\%$, $59.0\% \pm 4.3\%$, and $50.0\% \pm 3.2\%$ after 6 mon in response to spraying with 10^7 , 10^5 , and 10^3 CFU/ml *E. vermicola* on the wound, respectively. Spraying *E. vermicola* on the wound at a higher density led to a higher survival rate against PWNs compared to spraying the wound at a lower density. Spraying 10^7 CFU/ml *E. vermicola* on the wound produced the highest survival rate, which was 4.8-fold that of the control. Moreover, the survival rates of pine seedlings sprayed with 10^7 CFU/ml *E. vermicola* on the wound were significantly different

TABLE 2. Survival rates of wounded and unwounded pine seedlings sprayed with *Esteya vermicola* and infected with PWNs in 2012. The control was unwounded pine seedlings.

Treatment	Total number of seedlings	Number of living seedlings	Number of dead seedlings	Survival rate of seedlings (%)
Wounded	60	35 ± 5	25 ± 5	58.3 ± 4.9 ^c
Unwounded	60	29 ± 3	31 ± 3	48.3 ± 3.4 ^b
Control	60	8 ± 1	52 ± 1	13.3 ± 1.9 ^a

PWN = pinewood nematode.

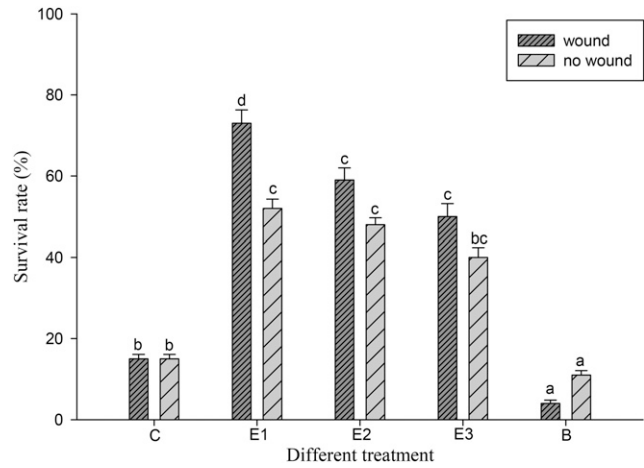


FIG. 1. Survival rates of wounded or unwounded pine seedlings that were sprayed with *Esteya vermicola* and infected with pinewood nematodes. C = control; E1 = 10^7 CFU/ml *E. vermicola*; E2 = 10^5 CFU/ml *E. vermicola*; E3 = 10^3 CFU/ml *E. vermicola*; B = 10^5 CFU/ml *Botrytis cinerea*. Error bars represent the standard deviations of triplicate determinations. Different letters above the bars indicate significant differences (Tukey's test, $P < 0.05$).

($P < 0.05$) than those of nonwounded seedlings treated with *E. vermicola*.

Survival rates of pine seedlings treated at different time intervals: The time intervals between spraying the pine seedlings with *E. vermicola* and infection with PWNs produced different effects on the survival rate (Fig. 2). The 20-d interval between spraying the pine seedlings with *E. vermicola* and infection with PWNs significantly differed ($P < 0.05$) from those of the 10- and 30-d interval times ($P < 0.05$). In addition, there was no significance in survival rates between the time intervals of 10 and 30 d. However, infection with PWNs 20 d after spraying *B. cinerea* on the pine seedlings decreased the survival rate to 10.0%.

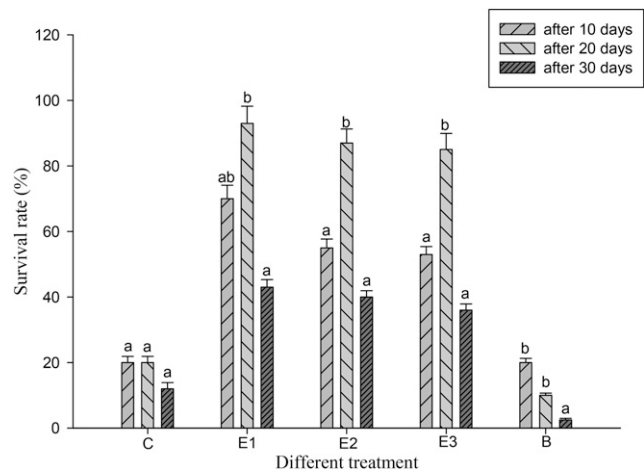


FIG. 2. Survival rates of pine seedlings sprayed with *Esteya vermicola* conidia suspensions before being infected with pinewood nematodes at different intervals. C = control; E1 = 10^7 CFU/ml *E. vermicola*; E2 = 10^5 CFU/ml *E. vermicola*; E3 = 10^3 CFU/ml *E. vermicola*; B = 10^5 CFU/ml *Botrytis cinerea*. Error bars represent the standard deviations of triplicate determinations. Different letters above the bars indicate significant differences (Tukey's test, $P < 0.05$).

The PWN populations in living and dead pine seedlings: We determined the number of PWNs in living or dead pine seedlings sprayed with *E. vermicola* and infected with PWNs (Fig. 3). The numbers were determined as $6,880 \pm 1,000$, $12,000 \pm 855$, and $12,200 \pm 1,310$ after spraying with 10^7 , 10^5 , and 10^3 CFU/ml *E. vermicola*, respectively, whereas the number of PWNs in the control (dead pine seedlings not sprayed with *E. vermicola*) was $27,300 \pm 3,000$. The number of PWNs in dead pine seedlings sprayed with the *B. cinerea* conidia suspension was $51,300 \pm 3,500$ (Fig. 3A). We also determined the number of PWNs in living pine seedlings under different treatments. The number of PWNs in these seedlings decreased significantly (Fig. 3B). The number of PWNs in living pine seedlings was 78 ± 7.8 , 141 ± 8.8 , and 156 ± 9.1 after spraying with 10^7 , 10^5 , and 10^3 CFU/ml *E. vermicola*, respectively, whereas the number of PWNs in the control (living pine seedlings not sprayed with *E. vermicola*) was 432 ± 21.5 . The number of PWNs in the living pine seedlings was much

lower ($P < 0.05$) than the original number of PWNs injected into the pine seedlings. The *E. vermicola* conidia suspension sprays decreased the number of nematodes to a number lower ($P < 0.05$) than that in the treatment control and the negative control. The living seedlings showed some wilt symptoms; however, the partially wilted seedlings survived in the following year and no more wilt symptoms happened in partially wilted seedlings.

Detection of *E. vermicola* in treated pine seedlings: *Esteya vermicola* was detected in the wilting or dead pine seedlings after spraying with *E. vermicola*. This species has uniquely shaped lunate conidia and conidiophores, so it was easily distinguished from other fungi. By using scanning electron microscope, infected nematodes with hyphae and lunate conidia or conidiophores of *E. vermicola* were clearly observed (Fig. 4A,B). Because of external forces when slices were made, some lunate conidia had fallen down from the conidiophores.

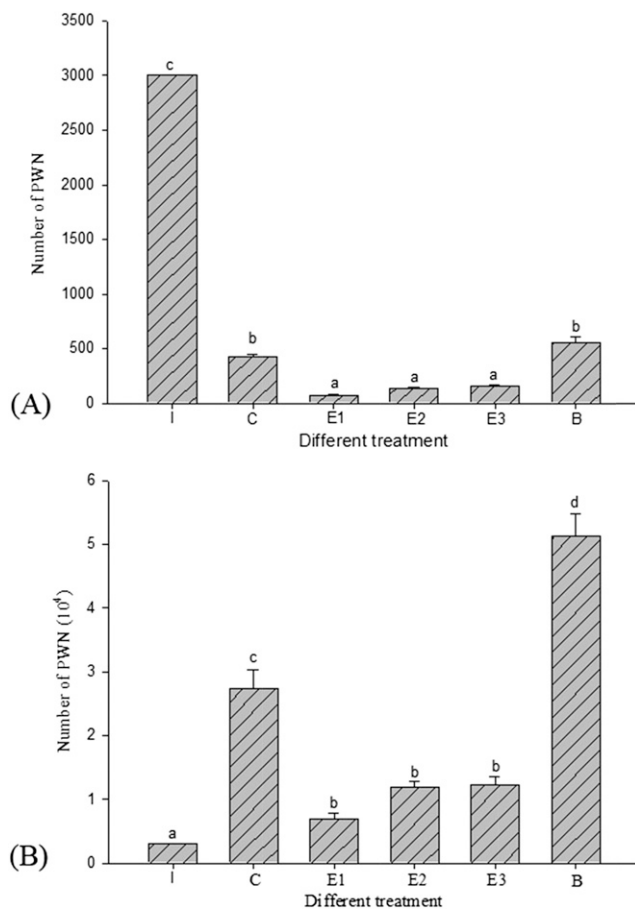


FIG. 3. Number of pinewood nematodes (PWNs) in dead and living pine seedlings sprayed with *Esteya vermicola* conidia suspensions. A. Number of PWNs in dead seedlings. B. Number of PWNs in living seedlings. I = the number of PWNs initially infecting pine seedlings; C = control; E1 = 10^7 CFU/ml *E. vermicola*; E2 = 10^5 CFU/ml *E. vermicola*; E3 = 10^3 CFU/ml *E. vermicola*; B = 10^5 CFU/ml *Botrytis cinerea*. Error bars represent the standard deviations of triplicate determinations. Different letters above the bars indicate significant differences (Tukey's test, $P < 0.05$).

DISCUSSION

Kobayashi et al. (1974) and Maehara and Futai (2000) compared nematode propagation on various fungi and showed that some fungi were unsuitable for nematode propagation. *Trichoderma* are unsuitable for propagation and the occurrence of the third-stage dispersal juveniles of the PWN (Fukushige, 1991; Maehara, 2008). Meyer et al. (2002) demonstrated that *Trichoderma virens* (Miller, Giddens & Foster) von Arx filtrate inhibited egg hatching and second-stage juvenile mobility of another root-knot nematode, *Meloidogyne incognita* (Kofid & White) Chitwood. *Esteya vermicola* also has a similar effect on *B. xylophilus*.

The nematodes present in the insect spiracles enter the tree through feeding wounds caused by the beetle, and the fungi could be introduced into the tree by the beetle from the initial insect wound (Donald et al., 2003). The blue-staining fungi introduced into pine trees by the beetle played an important part in aggravating and extending the necrotic resinous reaction

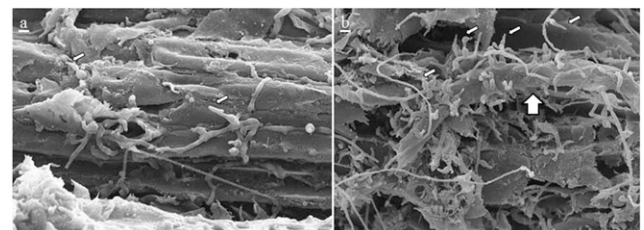


FIG. 4. Scanning electron micrographs of pinewood nematodes (PWNs) infected by *Esteya vermicola* and hyphae of *E. vermicola* in wood sections of pine seedlings. A. Lunate conidia of *E. vermicola* in wood section and conidiophores where lunate conidia had fallen down (small arrow). B. Infected PWN (large arrow) in tracheid with hyphae and lunate conidia. Bars: A and B = 50 μ m.

originating from the initial insect wound (Reid, 1967). Sawyer beetles wound new branches of pine trees and produce channels for fungi to enter. The blue-staining fungi are likely transmitted by *Monochamus* beetles when they feed on the young shoots of healthy pine trees in early summer (Kobayashi et al., 1974). Therefore, we can infer that *E. vermicola*, which is an endoparasitic fungus in trees, could enter the pine trees through wounds.

The efficacy of *E. vermicola* in reducing the number of PWNs and extending the survival of pine seedlings was clearly demonstrated in the greenhouse experiments. Spraying *E. vermicola* on the wounds ensures a higher survival rate than that of “no wound seedlings.” After *E. vermicola* enters a pine tree through a wound, the PWNs are attracted by volatile organic compounds produced by *E. vermicola*, and the fungus eventually kills the nematodes (Wang et al., 2008). Similarly, the conidia of *B. cinerea* sprayed onto the wounds of pine seedlings caused more damage compared to “no wound seedlings.” The emergence period of sawyer beetles begins in the middle of April, and the peak emergence period is from late May to the middle of June in the Fujian Province of China (Wang, 2004). Therefore, the optimal time for spraying *E. vermicola* for the biocontrol of PWNs is during the feeding period of the sawyer beetle on the branches of pine trees.

Saiki et al. (1984) reported that *Arthrobotrys* sp. could not be reisolated from surviving seedlings 5 months after spraying with the fungus. Bouchier (1961) reported that hyphae were difficult or impossible to find in stems of lodgepole pine known to be naturally infected with fungi. Reid (1961) reported that the blue-staining fungi involved in the resistant reaction may occur mainly in the form of microspores. Infected PWNs and *E. vermicola* were observed in wilting and dead pine seedlings, but never in healthy seedlings (Wang, 2011). After the PWNs were infected by *E. vermicola*, the fungus consumed the contents of the infected nematode’s body, grew out from its cadaver, and then produced new lunate conidia for the next infection cycle. The behavior of the conidia after spraying and the mechanism of *E. vermicola* in killing PWNs in healthy pine seedlings are not clear. Therefore, further experiments should be designed to identify the conidia in living seedlings and the mechanism of *E. vermicola* in controlling PWNs in healthy pine seedlings.

Trunk injection of nematicides and aerial spraying of chemical pesticides cannot be done on a large scale for PWD control because of high chemical and labor costs, environmental pollution, and nontarget effects (Lee et al., 2003; Kong et al., 2007). *Esteya vermicola* is a biocontrol agent isolated from wood and soil that does not add to environmental pollution. It has a high potential to be a useful biological control agent of the PWN.

However, real-world validation of this potential will require a very large amount of *E. vermicola* and several years of field testing.

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