Population changes of the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), on fungi growing in pine-branch segments

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Abstract

Temporal changes in pinewood nematode (PWN) populations and the percentage of third-stage dispersal juveniles (J_{III}s) of the PWN were examined on nine species of fungi growing in pine-branch segments. On *Ophiostoma minus*, *Macrophoma* sp., and *Trichoderma* sp. 1, the total PWN populations increased quickly for 4 weeks after nematode inoculation and then increased slightly or remained high. The number and percentage of J_{III}s also increased with time, i.e., with increasing numbers of PWNs. In contrast, the total population of PWNs decreased and the number and percentage of J_{III}s were small on *Verticillium* sp. and *Trichoderma* sp. 3.

Key words: *Ophiostoma minus*, pinewood nematode, third-stage dispersal juvenile, *Trichoderma* spp., *Verticillium* sp.

INTRODUCTION

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, the causal agent of pine wilt disease (Kiyohara and Tokushige, 1971), is vectored from wilt-killed to healthy pines by beetles in the genus *Monochamus* (Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972; Linit, 1988). PWNs invade healthy trees through maturation feeding wounds caused by vector beetles. Kobayashi et al. (1974, 1975), Fukushima (1991), and Kuroda and Ito (1992) considered the PWNs to feed and reproduce on various wood-inhabiting fungi such as blue-stain fungi after the trees weakened and died. Kobayashi et al. (1974, 1975) and Fukushima (1991) also found fungi which were unsuitable for PWN propagation to inhabit killed pines. However, changes in PWN populations on fungi over time have been examined in only a few studies, i.e., on *Botrytis cinerea* Pers. by Ishibashi and Kondo (1977), on a species of blue-stain fungus *Ceratocystis* sp. by Fukushima (1991), and on a blue-stain fungus *Ophiostoma*

piceae (Munch) Syd. et P. Syd. by Forge and Sutherland (1996).

Formation of fourth-stage dispersal juveniles (J_{IV}s) via third-stage dispersal juveniles (J_{III}s) of the PWN is necessary for the nematode to be vectored from wilt-killed to healthy pines by *Monochamus* beetles. Fukushima (1991) examined the percentage of J_{III}s on 14 fungal isolates of 10 genera, 4 weeks after nematode inoculation and temporal changes in numbers of J_{III}s only on *Ceratocystis* sp. for 10 weeks following nematode inoculation.

In the present study, we investigated temporal changes of PWN populations and J_{III}s on several species of fungi, including some of which are unsuitable for PWN reproduction, to clarify the effects of wood-inhabiting fungi on PWN reproduction and the occurrence of J_{III}s.

MATERIALS AND METHODS

Inoculation with fungi. Two-year-old branches, 10.4 to 12.8 mm in diameter, of Japanese red pine, *Pinus densiflora* Sieb. et Zucc. were cut into 1 cm lengths and autoclaved at 121°C for 30 min. The segments were then

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Table 1. Origin of fungi used

Fungus	Origin and reference
<i>Arthrobotrys</i> sp.	PWN-inoculated <i>Pinus thunbergii</i> (Fukushige and Futai, 1987)
<i>Cryptoporus volvatus</i>	Donated by Forestry and Forest Products Research Institute
<i>Macrophoma</i> sp.	PWN-inoculated <i>P. thunbergii</i> (Fukushige and Futai, 1987)
<i>Mariannaea elegans</i> var. <i>elegans</i>	PWN-infected <i>Pinus densiflora</i>
<i>Ophiostoma minus</i>	PWN-infected <i>P. densiflora</i> (Fukushige, 1991)
<i>Trichoderma</i> sp. 1	Healthy <i>P. thunbergii</i> (Fukushige, 1991)
<i>Trichoderma</i> sp. 2	PWN-inoculated <i>P. thunbergii</i> (Fukushige and Futai, 1987)
<i>Trichoderma</i> sp. 3	PWN-infected <i>P. densiflora</i> (Fukushige, 1991)
<i>Verticillium</i> sp.	PWNs in PWN-infected <i>P. densiflora</i> (Fukushige, 1991)

placed on potato dextrose agar (PDA) in petri dishes, each containing one of the test fungi grown at 25°C in the dark. The plates were then incubated at 25°C in the dark for 1 month. Nine fungi were used: *Arthrobotrys* sp., *Cryptoporus volvatus* (Pk.) Hubb., *Macrophoma* sp., *Mariannaea elegans* (Corda) Samson var. *elegans* Samson, *Ophiostoma minus* (Hedgcock) H. et P. Sydow [called *Ceratocystis* sp. 2 by Fukushige (1991)], *Trichoderma* sp. 1, *Trichoderma* sp. 2, *Trichoderma* sp. 3, and *Verticillium* sp. (Table 1). As controls, branch segments were placed on PDA plates with no fungus.

Nematode inoculation. After the fungi had grown entirely over the branch segments, 30 segments of each fungus treatment were transferred individually into sterile, 105 × 18 mm test tubes and each segment was inoculated with ca. 350, axenic PWNs in 30 µl sterilized water. A pathogenic isolate (S10) of *B. xylophilus* (donated by the Kansai Branch of Forestry and Forest Products Research Institute) was propagated on the fungus *B. cinerea* growing on autoclaved barley grains. One week before inoculation, the PWN culture was transferred to new cultures of *B. cinerea* growing on PDA and incubated at 25°C. The nematodes used for inoculation were separated from the *B. cinerea* hyphae by the method of Iwahori and Futai (1993) where a suspension of nematodes is passed through four thin layers of Japanese typing paper in a Baermann funnel, then through Sephadex G-25 (coarse) and glass beads packed in a column tube. The resulting suspension was then centrifuged five times at 700 × g for 1 min to remove any remaining *B. cinerea* mycelium. After nematode inoculation, the

branch segments were incubated at 20°C in the dark.

Nematode extraction and data analysis. Two, 4, 6, 8, and 12 weeks after nematode inoculation, six shoot segments of each fungus treatment were harvested and cut into four pieces with a pair of pruning shears and placed in a Baermann funnel overnight to extract the PWNs. J_{III}S, J_{IV}S, and all other PWN developmental stages (J₂S–J₄S and adults) were counted separately for each sample. When numbers of nematodes were too great, the suspension was diluted to count the nematodes. A two-way repeated-measures ANOVA was used to determine the significance of mean differences among fungus treatments. For ANOVA, the numbers of nematodes were log₁₀-transformed.

RESULTS

There was a significant difference in the total numbers of PWNs of all stages among fungus-treatments ($p < 0.01$ for both the fungus species and time × the species interaction) (Table 2). For *O. minus*, *Macrophoma* sp., and *Trichoderma* sp. 1, the total population of PWNs increased quickly during 4 weeks after inoculation, then increased slightly or remained constantly high (Fig. 1). In contrast, the total number of PWNs decreased on *Verticillium* sp. and *Trichoderma* sp. 3. There were no differences in the total population of PWNs among controls and the other fungi, i.e., *Trichoderma* sp. 2, *Arthrobotrys* sp., *M. elegans*, and *C. volvatus*.

There was a significant difference in the number of J_{III}S among fungus-treatments ($p < 0.01$ for fungus species and $p < 0.01$ for

Table 2. Effect of fungi on the total population of pinewood nematodes (two-way repeated-measures ANOVA table)

Source of variation	df	SS	MS	F	
Fungus species	9	285.233	31.693	204.197	$p < 0.01$
Error	50	7.760	0.155		
Time	5	3.878	0.776	6.625	$p < 0.01$
Time $\times$ fungus species	45	74.919	1.665	14.219	$p < 0.01$
Time $\times$ error	250	29.273	0.117

Fig. 1. Changes in the total population of pinewood nematodes on nine fungi. Values are means of six observations.

time $\times$ the species interaction) (Table 3). On *O. minus*, *Macrophoma* sp., and *Trichoderma* sp. 1, the number of J_{III} s (Fig. 2) and the ratio of J_{III} s to the total number of PWNs (Fig. 3) steadily increased with time. Eight and 12 weeks after nematode inoculation the percentage of J_{III} s was also high on *Arthrobotrys* sp. J_{IV} s were not detected in any branch segments.

DISCUSSION

Kobayashi et al. (1974, 1975) and Fukushima (1991) compared PWN reproduction on various fungi from healthy and PWN-killed pines, and showed that while the PWNs fed and multiplied on some of the fungi, others were unsuitable for PWN reproduction. In these

studies, however, the PWN population was monitored only once. Ishibashi and Kondo (1977), Fukushima (1991), and Forge and Sutherland (1996) examined changes in PWN populations over time on a few fungi, but all these fungi were suitable for PWN reproduction and unsuitable fungi were not investigated. Thus, in the present study, we investigated temporal changes of PWN population on nine species of fungi, including some that are unsuitable for PWN reproduction. On *O. minus*, *Macrophoma* sp. and *Trichoderma* sp. 1, the total population of PWNs increased quickly for 4 weeks after nematode inoculation and then increased gradually or remained high. This shows that there are suitable fungi for PWN

Table 3. Effect of fungi on numbers of the third-stage dispersal juvenile of the pinewood nematode (two-way repeated-measures ANOVA table)

Source of variation	df	SS	MS	F	
Fungus species	9	252.414	28.046	149.453	$p < 0.01$
Error	50	9.383	0.188		
Time	5	124.643	24.929	142.085	$p < 0.01$
Time $\times$ fungus species	45	129.077	2.868	16.349	$p < 0.01$
Time $\times$ error	250	43.862	0.175

Fig. 2. Changes in numbers of third-stage dispersal juveniles of the pinewood nematode on nine fungi. Values are means of six observations.

Fig. 3. Changes in the percentage of third-stage dispersal juveniles of the pinewood nematode on nine fungi. Values are means of six observations.

reproduction in pine trees and they maintained the large population of PWNs at least until 12 weeks after the nematode inoculation. The PWN populations increased well on *Trichoderma* sp. 1, hardly at all on *Trichoderma* sp. 2, and decreased on *Trichoderma* sp. 3; i.e., there are suitable and unsuitable *Trichoderma* spp. for PWN reproduction. On *Verticillium* sp., the PWN population decreased drastically because this fungus is a nematode parasitic fungus and kills nematodes.

Mamiya et al. (1973) and Kiyohara and Suzuki (1975, 1977) showed that the percentage of J_{III}s in a PWN population in wilt-killed wood increased with time and reached 50 to 100%. However, Futai et al. (1986), Tamura (1986), and Fukushima and Futai (1987) reported that the percentage of J_{III}s did not always increase with time and overall averaged less than

50%. From a different point of view, all these studies stated that the percentage of J_{III}s reached a maximum when or after the number of PWNs was the highest. *In vitro* studies also indicate that the percentage of J_{III}s reached its maximum after PWN number peaked on *B. cinerea* (Ishibashi and Kondo, 1977), *Ceratocystis* sp. 1 (Fukushige, 1991), or *O. piceae* (Forge and Sutherland, 1996). In our study, the number and the percentage of J_{III}s increased with time on *O. minus*, *Macrophoma* sp., or *Trichoderma* sp. 1, and thus resulted from an increase in total numbers of PWNs. We conclude, from the above, that it is necessary to the occurrence of J_{III}s that PWNs propagate once. This is also supported by the results (Figs. 1, 3) that showed that the percentage of J_{III}s was low on fungi which were unsuitable for PWN. The accumulation of pheromones at high popula-

tion densities triggers formation of dauer larvae in *Caenorhabditis elegans* (Riddle et al., 1987) and a similar process could regulate formation of J_{III}s of *B. xylophilus* (Forge and Sutherland, 1996). Eight and 12 weeks after we made our nematode inoculations, the percentage of J_{III}s was also high on *Arthrobotrys* sp., a nematode-trapping fungus, but the total number of PWNs was small. The factors causing this are unknown.

J_{IV}s were not detected in any branch segments in this study, probably because the presence of the vector of PWNs is necessary for the formation of J_{IV}s (Maehara and Futai, 1996; Necibi and Linit, 1998).

In our *in vitro* study, some fungi were suitable and others unsuitable for PWN reproduction and the occurrence of J_{III}s. The relationship between these fungi and PWNs in pine wilt-killed trees should be further examined.

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