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Inhibitory Effect of *Bursaphelenchus mucronatus* (Nematoda: Aphelenchoididae) on *B. xylophilus* Boarding Adult *Monochamus alternatus* (Coleoptera: Cerambycidae)

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Abstract: Inhibitory effects of *Bursaphelenchus mucronatus* on the number of *B. xylophilus* carried by an adult *Monochamus alternatus* were investigated using artificial pupal chambers. When pupal chambers were infested with either *B. xylophilus* or *B. mucronatus,* the load of *B. xylophilus* onto the beetle was greater ($P < 0.001$) than that of *B. mucronatus.* However, within the pupal chamber there was no difference in the abundance of the third-stage dispersal juveniles, which would molt to the fourth-stage dispersal juveniles to board beetles. The nematode load on beetles that emerged from pupal chambers infested with both *Bursaphelenchus* species was smaller ($P = 0.015$) than that of beetles with *B. xylophilus* alone but greater ($P < 0.001$) than that of beetles with *B. mucronatus* alone, suggesting an inhibitory effect of *B. mucronatus.* As a result of this study, the rate of inhibition of *B. mucronatus* on molting of third-stage dispersal juveniles of *B. xylophilus* to fourth-stage dispersal juveniles was 0.65, which resulted in great inhibition on boarding beetles at a rate of 0.7.

Key words: Bursaphelenchus mucronatus, Bursaphelenchus xylophilus, interspecific competition, *Monochamus alternatus,* nematode load, phoresy, pine wilt disease, species replacement.

Replacement of native species by alien species has sometimes caused the disturbance of ecosystems worldwide (Elton, 1958; Liebhold et al., 1995). Increasing inter-continental trade increases the chance of various organisms moving to new ecosystems. Recently there have been many reports about species replacement—for example, the gecko (Case et al., 1994), mosquito (Juliano, 1998), ant (Holway, 1999), snail (Byers, 2000), and bullfrog (Kiesecker et al., 2001).

Bursaphelenchus xylophilus, the causative agent of pine wilt disease, is considered to be native to North America and believed to have been introduced in Japan in the early 1900s (Mamiya, 1988; Rutherford and Webster, 1987). In contrast, *B. mucronatus,* a species closely related to *B. xylophilus,* is native to Japan and considered to be avirulent in native *Pinus* species in this country (Mamiya and Enda, 1979). The biology of the two nematode species is similar. They have propagative and dispersal phases within their life cycle. Under favorable environment, they undergo a propagative phase but if the environment becomes unfavorable, development switches to the dispersal phase (Mamiya, 1984). The dispersal phase includes two juvenile stages, third-stage dispersal juveniles (JIII), and fourth-stage dispersal juveniles (JIV), the latter being a special stage for travel between host trees. The JIII molt to the JIV when they are near pupae or callow adults of vector beetles, particularly in the genus *Monochamus.* The JIV board beetles and enter new host trees through feeding and oviposition wounds made by the beetle (Arakawa and Togashi, 2002; Linit, 1988; Mamiya and Enda, 1979; Mamiya and Kiyohara, 1972). The two nematode species share resources such as food and vector species; thus, one might expect a rigorous interspecific interaction when they co-exist in a pine stand. Evidence indicates that *B. mucronatus* may be replaced in the pine forests during the infestation of *B. xylophilus,* causing pine wilt disease (Kishi, 1995).

Differences in the number of JIV of each species that a beetle carries at emergence (initial nematode load) is important to understand the replacement of *B. mucronatus* by *B. xylophilus* because the initial nematode load determines the number of nematodes transmitted to new host trees (Jikumaru and Togashi, 2001; Togashi, 1985). Only one of the nematode species is usually found in a dead tree, although Nagashima et al. (1975) and Kishi (1995) reported that both species were recovered from identical dead pine trees. In the laboratory, initial load of *B. mucronatus* on *Monochamus alternatus* is smaller than that of *B. xylophilus* (Jikumaru and Togashi, unpubl. data). However, there has been no information about initial nematode load of beetles that emerged from pine trees infested with both nematode species. The goal of this study was to determine the interaction of *B. mucronatus* and *B. xylophilus* relative to boarding an insect vector and to discuss a role of the inhibitory effect with respect to replacement of *B. mucronatus* by *B. xylophilus* in Japan.

Materials and Methods

Nematodes: Bursaphelenchus mucronatus and *B. xylophilus* were collected at Takano Town, Hiroshima Prefecture, and subcultured monoxenically on *Botrytis cinerea* Pers. at 25 °C. After 2 to 3 weeks on *B. cinerea,* the nematodes were recovered at 25 °C for 24 hours using the Baermann funnel technique and adjusted to a suspension of 3,000 nematodes (mixed stages)/ml of distilled water for each nematode species. A suspension of the two nematode species was obtained by mixing equal quantities of the two nematode suspensions. The mixed

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suspension contained 1,500 *B. mucronatus* and 1,500 *B. xylophilus*/ml.

Insects: An *M. alternatus* population originating from Miyoshi City, Hiroshima Prefecture, was used for the experiment. To obtain post-diapause, final instar larvae, small depressions were made on the exposed wood of healthy *P. densiflora* logs after removing small rectangular pieces (1.5 cm × 1.5 cm) of bark, and newly hatched larvae were singly placed in the depressions and covered with the bark pieces fastened with adhesive cloth tape. The logs were kept at 25 °C for ca. 4 months and then stored at 10 °C for ca. 4 months to terminate the larval diapause. The post-diapause, final instar larvae were collected from the logs in June 2000. The mean weight of the larvae was 593 mg (SD = 155 mg, range = 300 to 1,071 mg) just prior to the beginning the loading experiment.

Loading beetles with nematodes: The method developed by Aikawa et al. (1997) was used for loading *M. alternatus* adults with nematodes. A hole (5 cm deep and 1-cm diam.) was drilled in the center of a cut end of each of *P. densiflora* bolts (7 cm long and 4-cm mean diam.) and was used as an artificial pupal chamber. The bolts were set upright individually in polycarbonate containers (11 cm tall and 6.7-cm inside diam.) with quartz sand spread on the bottom. The bolts were autoclaved at 121 °C for 15 minutes, inoculated with the blue-stain fungus, *Ophiostoma minus* (Hedgcock) H. et P. Sydow, which had been cultured on PDA medium at 25 °C for 2 weeks, and kept at 25 °C in the dark for 4 weeks. The bolts then were inoculated with a 1-ml suspension containing 3,000 nematodes of *B. mucronatus* alone (Bm treatment), *B. xylophilus* alone (Bx treatment), or a mixed population of *B. mucronatus* and *B. xylophilus* (Bm+Bx treatment). Immediately after nematode inoculation, a post-diapause *M. alternatus* larva was placed in the hole and the hole was plugged with rounded aluminum foil. The number of artificial pupal chambers, each with a single beetle larva, was 25, 25, and 26 for Bm, Bx, and Bm+Bx treatments, respectively. The bolts were kept at 25 °C in the dark and checked daily for the adult beetle emergence. After the newly emerged adults were sexed and weighed, they were crushed individually using a mortar and pestle, and the nematodes they carried were recovered with the Baermann funnel technique at 25 °C for 2 to 3 days to determine the initial nematode load.

Nematode census: To determine the number and developmental stages of nematodes present at pupal chamber, the bolts were chopped into four pieces with a small hatchet and the wood surrounding the wall of the artificial pupal chamber was collected to a depth of 0.5 cm just after the emergence of adults. Most nematodes concentrated at pupal chamber were present in xylem within 0.5 cm of the surface of pupal chamber wall (Mamiya, 1972). Nematodes were extracted from the chipped wood of the wall using the Baermann funnel technique at 25 °C for 2 to 3 days.

An average of 54 nematodes (SD = 8.9) were randomly selected from the respective populations that had been recovered from the wood surrounding the artificial pupal chambers. These were classified by microscopic examination into three groups of the JIII, JIV, and others including propagative juveniles and adults. The *B. xylophilus* JIII were identified by the round tail and the dark body coloration due to the presence of lipid granules (Mamiya, 1984), whereas *B. mucronatus* JIII were done by its mucro on the body terminal and the dark body coloration. The JIV were determined by doomed head, lack of stylet, and dark body. The total number of nematodes (T) concentrated at the pupal chamber was calculated as the sum of initial nematode load on the emerging beetle (N_i) and the number of nematodes remaining in the pupal chamber (N_p), or $T = N_i + N_p$. N_p included JIII, JIV, propagative juveniles, and adults recovered from the wood surrounding the artificial pupal chamber. The numbers of JIII (R_{JIII}) and JIV (R_{JIV}) in N_p were estimated as the product of the number of nematodes (N_p) in wood wall and the ratios of JIII and JIV in the sample. The number of JIII concentrated in the pupal chamber so far (T_{JIII}) was calculated as the sum of the initial nematode load and total number of JIII and JIV remaining in the pupal chamber ($T_{JIII} = N_i + R_{JIII} + R_{JIV}$). The number of JIV produced (T_{JIV}) was calculated as summation of initial nematode load and number of JIV remaining in the pupal chamber ($T_{JIV} = N_i + R_{JIV}$). The proportion of JIII concentrated in the pupal chamber was defined as the ratio of the number of so-far concentrated JIII to the total number of nematodes concentrated in the pupal chamber (T_{JIII}/T). The proportion of JIII molting to JIV was defined as the ratio of the number of JIV produced to the number of JIII concentrated at pupal chamber (T_{JIV}/T_{JIII}). The proportion of JIV boarding the beetle was defined as the ratio of the initial nematode load to the number of JIV produced in the pupal chamber (N_i/T_{JIV}).

Interspecific inhibition coefficient: The effect of one nematode species on another species in the boarding of an insect when both are present in the pupal chamber was evaluated. The number of nematodes concentrated in the pupal chamber, the number of JIII concentrated in the pupal chamber, the number of JIV produced, the initial nematode load, the proportion of JIII concentrated in the pupal chamber, the proportion of JIII molting to JIV, and the proportion of JIV boarding beetle were considered. The total number of nematodes from a single *B. xylophilus* population, single *B. mucronatus* population, and a mixed population at a given step were scored as N_x, N_m, and N_{m+x}, respectively. The proportions for a single *B. xylophilus* population, single *B. mucronatus* population, and a mixed population at a given step were P_x, P_m, and P_{m+x}, respectively.

When h_m and h_x represent the inhibitory coefficients by *B. mucronatus* and *B. xylophilus* against each other, respectively, the nematode number and proportion for a mixed population can be expressed as follows,

$$N_{m+x} = N_x(1 - h_m) + N_m(1 - h_x) \quad (1)$$

and

$$P_{m+x} = P_x(1 - h_m) + P_m(1 - h_x). \quad (2)$$

The ranges of h_m and h_x values were estimated by using the equations on the conditions that *B. xylophilus* does not promote the molting and behavior of *B. mucronatus* and vice versa ($0 \leq h_m \leq 1$ and $0 \leq h_x \leq 1$).

Statistical analysis: One-way analysis of variance (ANOVA) was conducted to test the differences in the number of nematodes at respective steps in the boarding process, the proportion of JIII concentrated at pupal chamber, the proportion of JIII molting to JIV, and the proportion of JIV boarding beetle among three treatments. Following one-way ANOVA, the Tukey-Kramer multiple comparison test was used to compare pairwise means. Before the analyses, the numbers of nematodes were logarithmically transformed to make the variance independent of the mean. The proportions were arcsine-transformed for the same purpose.

Results

Nineteen, 16, and 19 *M. alternatus* adults emerged from pine bolts infested with *B. mucronatus, B. xylophilus,* and a mixed population, respectively (Table 1). There was no difference in the days from *M. alternatus* larval inoculation to adult emergence among Bm (mean ± SE = 35.9 ± 1.0), Bx (mean ± SE = 39.8 ± 1.9), and Bm+Bx treatments (mean ± SE = 35.8 ± 1.1), respectively.

There was no difference in the number of nematodes concentrated inside the pupal chamber prior to beetle emergence among *B. mucronatus* population, *B. xylophilus* population, and the mixed population, respectively (Table 1). The proportion of JIII concentrated at pupal chamber was the smallest for *B. mucronatus* and the greatest for *B. xylophilus* (one-way ANOVA, $P < 0.001$; Tukey-Kramer multiple comparison test, $P < 0.05$) (Table 1). The total number of JIII concentrated at pupal chamber did not differ among the three treatments (Table 1).

The proportion of JIII molting to JIV was smaller for the *B. mucronatus* population than for *B. xylophilus* population or the mixed population (one-way ANOVA, $P < 0.001$; Tukey-Kramer multiple comparison test, $P < 0.05$) (Table 1). Consequently, the number of JIV produced was smaller for the *B. mucronatus* population than for the *B. xylophilus* population and the mixed population (one-way ANOVA, $P < 0.05$; Tukey-Kramer multiple comparison test, $P < 0.05$) (Table 1).

The proportion of JIV boarding beetles was smaller for the *B. mucronatus* population than for the *B. xylophilus* population and the mixed population (one-way ANOVA, $P < 0.001$; Tukey-Kramer multiple comparison test, $P < 0.05$) (Table 1). Consequently, the initial nematode load was significantly smaller for the *B. mucronatus* population than for the *B. xylophilus* population and the mixed population (one-way ANOVA, $P < 0.05$; Tukey-Kramer multiple comparison test, $P < 0.05$) (Table 1).

Range estimation of h_m and h_x values by using the data in Table 1 showed that *B. mucronatus* inhibited *B. xylophilus* JIII from molting to JIV at the rate of 0.65, irrespective of the degree of inhibition by *B. xylophilus* against *B. mucronatus* (Table 2). It also indicated that *B. mucronatus* made both the number of *B. xylophilus* JIV produced and the initial load decrease at a large rate of 0.7 (Table 2). In addition, positive h_m values suggested that *B. mucronatus* inhibited *B. xylophilus* at other steps up to boarding. On the other hand, the degree of inhibition by *B. xylophilus* against *B. mucronatus* could not be estimated (Table 2).

Discussion

Our study indicated that the presence of *B. mucronatus* had an inhibitory effect on the number of *B. xylophi-*

Table 1. Comparison in boarding process of *Bursaphelenchus mucronatus* and *B. xylophilus* on *Monochamus alternatus* adults. The two nematode species were inoculated on *Pinus densiflora* bolts alone or together. A single beetle larva was placed in the hole of bolt as an artificial pupal chamber.

Nematode species inoculated	*B. mucronatus*	*B. xylophilus*	Both species
No. of beetles emerging	19	16	19
No. of nematodes concentrated in the pupal chamber[ab]	30,106 ± 3,711a	34,491 ± 5,776a	27,521 ± 4,030a
Proportion of JIII in nematodes concentrated in the pupal chamber[abc]	0.871 ± 0.016a	0.974 ± 0.006b	0.914 ± 0.007c
No. of JIII concentrated in the pupal chamber[abc]	26,481 ± 3,395a	33,707 ± 5,710a	25,222 ± 3,651a
Proportion of JIII molting to JIV[abc]	0.022 ± 0.007a	0.613 ± 0.054b	0.222 ± 0.042b
No. of JIV produced in the pupal chamber[abc]	649 ± 204a	23,918 ± 4,860b	7,236 ± 2,126b
Proportion of JIV boarding beetle[abcd]	0.698 ± 0.104a	0.977 ± 0.011b	0.925 ± 0.038b
Initial nematode load[ab]	316 ± 128a	23,669 ± 4,820b	6,982 ± 2,076b

[a] Mean ± SE.
[b] Means followed by the same letter are not significantly different at the 5% level (one-way ANOVA and Tukey-Kramer multiple comparison test).
[c] JIII and JIV indicate the third- and fourth-stage dispersal juveniles, respectively.
[d] Mean and SE in Bm treatment was calculated for 15 beetles excluding 4 beetles that emerged from pupal chambers harboring no JIV.

TABLE 2. Estimated inhibitory coefficients (h_m) of *Bursaphelenchus mucronatus* against *B. xylophilus* boarding on *Monochamus alternatus* and those (h_x) of *B. xylophilus* against *B. mucronatus* boarding.

Steps up to boarding	h_m Value	h_x Value
Number of nematodes concentrated in the pupal chamber	0.202–1.000	0.086–1.000
Number of JIII concentrated in the pupal chamber[a]	0.252–1.000	0.048–1.000
Number of JIV produced[a]	0.697–0.725	0.000–1.000
Initial nematode load	0.705–0.718	0.000–1.000
Proportion of JIII concentrated in the pupal chamber[a]	0.062–0.956	0.000–1.000
Proportion of JIII molting to JIV[a]	0.638–0.674	0.000–1.000
Proportion of JIV boarding beetle[a]	0.053–0.768	0.000–1.000

[a] JIII and JIV indicate the third- and fourth-stage dispersal juveniles, respectively.

lus that boarded *M. alternatus* adults. This conclusion was based on the result that the initial nematode load of a mixed population of *B. mucronatus* and *B. xylophilus* was extremely small as compared to that of *B. xylophilus* alone (Table 1). The decreased initial nematode load by a mixed population of *B. mucronatus* and *B. xylophilus* was unrelated to the reduced inoculum of each nematode species. Aikawa and Togashi (1997) reported that the inoculum density of *B. xylophilus* ranging from 1,000 to 50,000 did not affect the initial nematode load on *M. alternatus* or the number of nematodes remaining in the pupal chambers immediately following beetle emergence by using the same rearing system as this study. Their results indicated that the *B. xylophilus* population reached the saturation level during the time required for beetle emergence, averaging 32.9 days. Thus, our results showed that *B. mucronatus* must have an inhibitory effect on *B. xylophilus* load of *M. alternatus*. The inhibitory effect might be one of biotic resistance by native species against invading species (Elton, 1958; Pimm, 1989). However, the pine wilt disease epidemic in Japan suggests that the resistance by *B. mucronatus* was not strong enough to prevent the spread of *B. xylophilus*.

As morphology cannot discriminate between JIV of both species, we could not determine the numbers of *B. mucronatus* and *B. xylophilus* harbored in beetles that emerged from pine bolts containing a mixed species inoculum. Thus, assuming an inhibitory effect of each nematode species on the other, *B. mucronatus* was shown to inhibit *B. xylophilus* at all steps of the boarding process. In addition, *B. mucronatus* inhibited *B. xylophilus* JIII from molting to JIV at a high rate of 0.65. This resulted in a rate of inhibition of 0.7 for the production of JIV and initial nematode load of *B. xylophilus* (Table 2). An inhibition by *B. xylophilus* on *B. mucronatus* remains to be determined.

Some environmental factors are known to affect the initial *B. xylophilus* load on *M. alternatus*. Extremely dry or wet wood at the pupal chambers lowers the initial nematode load (e.g., Kobayashi et al., 1976; Morimoto and Iwasaki, 1973; Togashi, 1989). Experimentally it has been shown that fungi unsuitable for *B. xylophilus* propagation also reduce the initial nematode load (Maehara and Futai, 1996). Our study adds *B. mucronatus* to the list of factors that decrease the initial *B. xylophilus* load on *M. alternatus*. However, how such factors suppress the initial nematode load remains to be understood.

Interspecific matings between *B. xylophilus* and Japanese *B. mucronatus* in the laboratory produce hybrids that are sterile and fail to produce an F_2 generation (Mamiya, 1986). However, the crossing between *B. xylophilus* and European *B. mucronatus* produces fertile progeny for at least one generation (Bolla and Boschert, 1993; de Guiran and Bruguier, 1989), although it is unlikely that both nematodes mate in nature because they lack pheromone attraction (Riga and Webster, 1992). Morphology of the hybrid from such matings is similar to that of *B. mucronatus* (Mamiya, 1986). Thus we were unable to determine the occurrence of the hybrid in Bm+Bx treatment. The two nematode species are discriminated from each other by polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) analysis (Iwahori et al., 1998); thus, such analysis may be used to separate the hybrid from *B. mucronatus*.

Outbreaks of *M. alternatus* occur during the propagation of pine wilt disease in Japan. Based on the results of this study, when *M. alternatus* adults emerge from trees infested with one of the two nematode species, their initial *B. xylophilus* load is much greater than that of *B. mucronatus*. In addition, when the beetles emerge from trees infested with both species, the number of *B. xylophilus* carried by each of the beetles is much greater than that of *B. mucronatus*. This means that a smaller number of *B. mucronatus* are transmitted to pine trees than *B. xylophilus*. Therefore such difference in the number of nematodes carried by beetles between both species results in the nematode species replacement in several years.

It has been observed that *M. alternatus* adults sometimes carry more than 10,000 *B. mucronatus* at emergence prior to the invasion of pine forests by *B. xylophilus* (Mamiya and Enda, 1979). The conditions under which a great initial *B. mucronatus* load is produced and the effect of interspecific competition under such conditions are needed to further understand nematode species replacement via competition.

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