Journal of Nematology 32(1):110–116. 2000.

Temperature Effects on the Transmission of *Bursaphelenchus xylophilus* (Nemata: Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae)[1]

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Abstract: The object of this research was to investigate the effects of ambient temperature on the transmission of the pinewood nematode, *Bursaphelenchus xylophilus,* by its vector, *Monochamus alternatus.* Ninety *M. alternatus* were reared individually at one of three constant temperatures (16, 20, and 25 °C). As the ambient temperature decreased from 25 to 16 °C, longevity of vectors decreased, nematode transmission efficiency decreased, and the peak period of nematode transmission was delayed and its peak height decreased. Low temperature may inhibit the transmission process, and this inhibition could be partially responsible for preventing pine wilt disease from devastating pine forests in cool regions.

Key words: Bursaphelenchus xylophilus, Monochamus alternatus, nematode, pine wilt, pinewood nematode, temperature, transmission.

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, is the causative agent of pine wilt disease (PWD) (Kiyohara and Tokushige, 1971). The nematode is believed to be native to North America (Tares et al., 1992) and was first introduced into Japan early in the 1900s (Mamiya, 1984). Since then, the disease has been devastating forests of Japanese red pine, *Pinus densiflora* Sieb. & Zucc., and Japanese black pine, *P. thunbergii* Parl., in Japan (Mamiya, 1984). Epidemics of PWD have recently occurred in China, Taiwan, and South Korea (Kishi, 1995). Europe is threatened by *B. xylophilus* invasion because extensive European forests of *P. sylvestris* L. are susceptible to this nematode (Fielding and Evans, 1996). Pinewood nematode has been found in pinewood chips exported from the United States and Canada to Finland (Magnusson, 1986; Rautapää, 1986). In 1999, the nematode was found in dead *Pinus pinaster* trees in Portugal (Mota et al., 1999). Many *Pinus* spp. have been introduced into the southern hemisphere (Richardson and Higgins, 1998), including species susceptible to *B. xylophilus* such as *P. radiata, P. pinaster,* and *P. nigra* (Futai and Furuno, 1979). This host range suggests that *B. xylophilus* is one of the potentially most serious pathogens of pine forests in the world.

The nematode is transported from diseased to healthy trees by adult insects, especially in the genus *Monochamus* (Coleoptera: Cerambycidae), through their feeding wounds on pine twigs (e.g., Linit et al., 1983; Morimoto and Iwasaki, 1972; Sato et al., 1987). *Monochamus alternatus* Hope is the primary vector in Japan (e.g., Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972) and in other areas of Asia (Enda, 1988).

Pine wilt disease is widespread along coastal areas and at low-elevation inland areas in warmer regions of Japan (Mamiya, 1984). The disease is becoming more common in the cooler regions of Japan, but damage to the forest is less severe and disease spread is slower (Rutherford et al., 1990). The difference in the epidemic of PWD between warm and cool regions is associated with different temperature-dependency profiles for this disease (Rutherford et al., 1990). Several studies have been conducted on the effect of low temperature on the biology of *B. xylophilus* and its vector in association with PWD. At lower temperatures, the reproductive rates of both *B. xylophilus* and its vector are inhibited (Kiyohara, 1973; Kobayashi et al., 1984), and asynchronous development occurs between the period of tree death and the oviposition

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Received for publication 12 May 1999.

[1] This work was partly supported by a Grant-in-Aid for Scientific Research (C) (#10660148) from the Ministry of Education, Science, Sports and Culture of Japan and the JAFTA Forest Research Scholarship.

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The authors acknowledge Dale R. Bergdahl and Marc J. Linit for reviewing the manuscript.

This paper was edited by E. C. Bernard.

period of the vector (Zinno et al., 1987). However, the effect of temperature on *B. xylophilus* transmission from its vector has not been studied.

The objective of this research was to determine the effect of temperature on transmission of *B. xylophilus* through *M. alternatus* feeding wounds. This study was directed at the longevity of vectors, the exit pattern from vectors, transmission efficiency, and the pattern of nematode transmission from vectors to pine twigs.

Materials and Methods

Dead *P. densiflora* and *P. thunbergii* were cut into logs in Tokuyama City, Yamaguchi Prefecture, and in Higashi-Hiroshima City, Hiroshima Prefecture, on 17–18 March 1994 and 13 May 1994, respectively. The logs were transported to the Higashi-Hiroshima (previously called Saijo) Campus of Hiroshima University, Hiroshima Prefecture. They were placed in field cages (65 cm × 45 cm × 80 cm) covered with a fine wire mesh. A total of 90 *M. alternatus* adults, which had emerged from 1 June to 7 July 1994, were used in this experiment. Their average body weight at emergence was 370 mg (SD = 136 mg) for females and 322 mg (SD = 140 mg) for males.

Adult beetles were individually placed in transparent rearing containers (Jikumaru and Togashi, 1995) within 1 day of emergence and maintained at one of three constant temperatures (16, 20, and 25 °C) and a fixed 12 hours light-12 hours dark photoperiod until death. Each beetle was supplied with a 1-year-old, 11-cm-long fresh twig of *P. densiflora,* and the insect was transferred to a new container with a new pine twig every 5 days. Nematodes that had entered the pine twig through feeding wounds and those failing to enter the twig were extracted separately with the Baermann funnel technique described by Jikumaru and Togashi (1995). Daily observations were made, and dates of vector deaths were recorded. Dead vectors were crushed individually with mortar and pestle, and *B. xylophilus* remaining in the vector's body were extracted with the Baermann funnel technique.

Funnels for extracting *B. xylophilus* were placed in a room maintained at 25 °C. On the second or third day after the start of extraction, the number of *B. xylophilus* in 20 ml of water at the bottom of the funnel was determined. A count also was made of *B. xylophilus* in the remaining water (ca. 80 ml) for 8 to 10 funnels, and a regression equation was developed to estimate the total number of nematodes extracted. The number of nematodes leaving the vector was estimated by a summation of *B. xylophilus* succeeding or failing to invade the pine twig. The total number of *B. xylophilus* carried by a vector at emergence (the initial nematode load) was estimated as being the summation of those leaving the living vector and those remaining in the dead vector body. Pine twigs not exposed to insect feeding were controls, to confirm that twigs were not previously infected with *B. xylophilus.*

Insects used in this study were collected from two sites (Tokuyama and Higashi-Hiroshima Cities); but since there was no difference in the vector-nematode interaction between the two populations, these data were combined and analyzed statistically. Analysis of variance (ANOVA) was used to test the differences in the mean longevity and body weight among the three different temperature treatments. The Tukey-Kramer multiple comparison test was used to compare means following one-way ANOVA. The Kruskal-Wallis test was used to determine a difference in transmission efficiency among the three temperature treatments. Multiple regression was used to investigate the contribution of vector body weight and treatment temperature on the longevity of vectors.

Results

Frequency distributions in initial nematode *load per vector:* A total of 74 *M. alternatus* (82.8%) were infested with *B. xylophilus,* and no vectors were found to carry more than 10,000 nematodes each (Table 1). The mean initial nematodes load per vector was 353 (SD = 924), 157 (SD = 339), and 142 (SD = 399) at 16, 20, and 25 °C, respectively.

TABLE 1. Frequency distribution of the initial number of *Bursaphelenchus xylophilus* carried by *Monochamus alternatus* adults maintained at three temperature regimes.

Temperature (°C)	Number of vectors	Initial nematode load					
		0	1–9	10–99	100–999	1,000–9,999	Mean ± SD[b]
16	30	0[a]	8	11	8	3	353 ± 924
20	30	5	8	7	9	1	157 ± 339
25	30	11	7	5	6	1	142 ± 399

[a] Number of insects that contained a specified load of *B. xylophilus.*

[b] Mean number of *B. xylophilus* per insect vector at a specified temperature.

Temperature effect on vector longevity: Mean vector longevity differed significantly among the three temperatures but not between sexes (two-way ANOVA, $P > 0.05$). Temperature had a significant effect on vector longevity, but initial *B. xylophilus* load did not (two-way ANOVA, $P > 0.05$). Lack of a deleterious effect of initial nematode load on vector longevity was probably due to the low nematode load values in all treatment groups. Mean longevity of vectors decreased as the temperature decreased (Fig. 1): 65.9 (SD = 37.9), 87.9 (SD = 59.1), and 143.6 (SD = 55.9) days at 16, 20, and 25 °C, respectively. Mean longevity of vectors was greater at 25 °C than at 16 and 20 °C (Tukey-Kramer multiple comparison test, $P < 0.05$).

Mean body weight of vectors was greater at 16 °C than at 25 °C (Tukey-Kramer multiple comparison test, $P < 0.05$). This difference in body weight may have been related to the difference in mean longevity. However, multiple linear regression indicated that the difference in vector longevity was associated with differences in temperature ($P < 0.001$) but not body weight ($P > 0.05$).

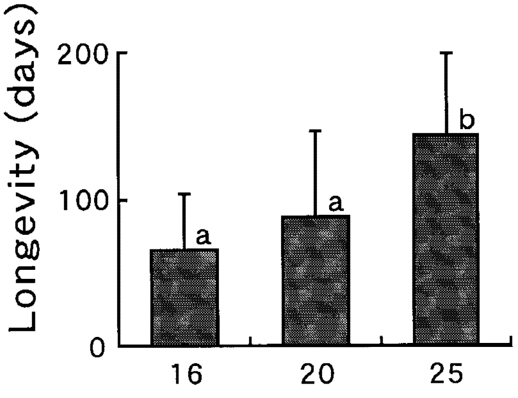

FIG. 1. Mean longevity of *Monochamus alternatus* adults reared at three constant temperatures. Vertical lines are standard deviations. Means with same letter are not significantly different according to the Tukey-Kramer multiple comparison test ($P > 0.05$).

Temperature effect on age-specific changes in number of B. xylophilus *contained in vector body:* The number of *B. xylophilus* carried by a vector remained constant for the first 5 days and then decreased as the age of vector increased. Numbers declined rapidly from 10 to 45 days after vector emergence at all three temperatures for vectors that carried 100 to 999 nematodes at emergence (Fig. 2). The time needed for 50% of total *B. xylophilus* to exit the vectors increased as temperature decreased (Table 2). The time needed for 50% *B. xylophilus* departure from vectors carrying 1,000 or more nematodes at emergence was shortest at 20 °C and longest at 16 °C, although only a few vectors were monitored (Table 2). A rapid increase in number of nematodes leaving the vectors occurred at temperatures of 16 and 20 °C from 10 to 40 days after vector emergence, when the vector carried 10 to 99 nematodes at emergence, and the 50% departure time was sooner at 20 °C than at 16 °C (Tukey-Kramer multiple comparison test, $P < 0.05$). There was no difference in *B. xylophilus* departure time from the vector at different temperatures when the initial nematode load was less than 10.

Temperature effect on transmission efficiency of B. xylophilus: Transmission efficiency is defined as the ratio of the total number of nematodes transmitted successfully into pine twigs to the total number of *B. xylophi-*

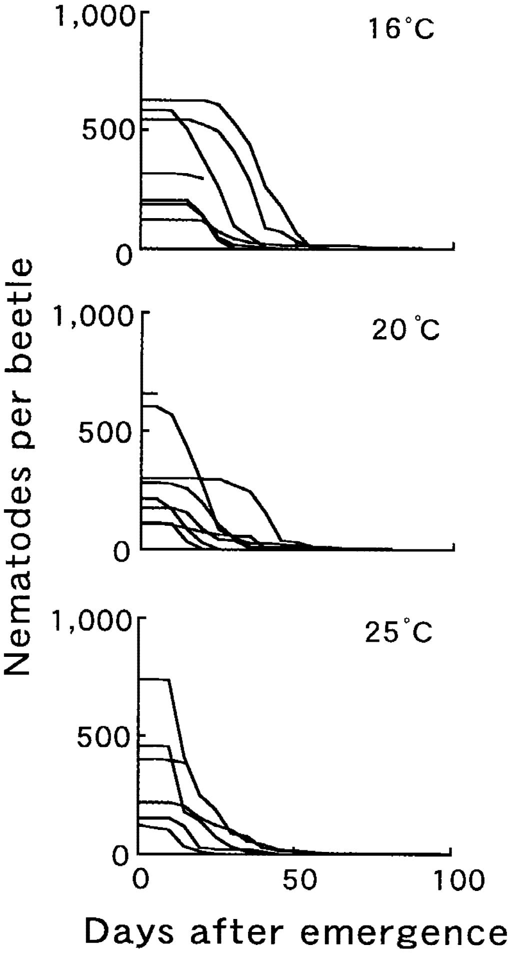

FIG. 2. Age-specific changes in the number of *Bursaphelenchus xylophilus* in *Monochamus alternatus* adults reared at three constant temperatures. Data are presented for adults with an initial nematode load of 100-999. Each line represents one beetle.

lus leaving the vector. Mean transmission efficiency decreased as ambient temperature decreased when vectors carried 100 to 999 *B. xylophilus* at the emergence ($P < 0.05$) (Table 2). Mean transmission efficiency was not significantly different among the three different treatment temperatures when the initial nematode load was 1 to 9 or 10 to 99 (Table 2).

Temperature effect on B. xylophilus transmission curve: The *B. xylophilus* transmission curve for a vector shows the age-specific change in the number of *B. xylophilus* successfully transmitted to pine twigs. In our study, the number of nematodes transmitted by each vector was counted at intervals of 5 days. The average *B. xylophilus* transmission curve for vectors with an initial nematode load of 100 to 999 was unimodal and peaked between days 10 and 30 after vector emergence at all temperatures (Fig. 3). This peak was delayed and its magnitude decreased as the temperature decreased (53, 19, and 13 nematodes per 5 days at 25, 20, and 16 °C, respectively) (Fig. 3). Vectors with an initial nematode load of 1,000 to 9,999 had a unimodal transmission curve with a peak between days 10 and 25 after vector emergence at all ambient temperatures. The height of the peak decreased as temperature decreased (276, 245, and 23 nematodes per 5 days at 25, 20, and 16 °C, respectively). When vectors carried 10 to 99 *B. xylophilus* at emergence, the transmission curve had a peak of 4 nematodes per 5 days between days 20 and 25 at 16 °C, and a peak of 5 nematodes per 5 days between days 15 and 20 at 20 °C. No peak occurred at 25 °C. Transmission of nematodes was rare when vectors carried only 1 to 9 *B. xylophilus* at emergence.

DISCUSSION

These experiments demonstrated the inhibitory effect of cool temperature on the transmission of *B. xylophilus* from its insect vector to healthy pine twigs via feeding wounds. This effect must be regarded as an important component of the temperature dependency of PWD. The inhibitory effect was composed of three processes: (i) reduced vector longevity, (ii) reduced transmission efficiency, and (iii) delayed nematode transmission at lower ambient temperatures. Delayed and decreased nematode transmission, resultant of these three processes, are highly important phenomena because PWD incidence is higher and disease development is accelerated when more *B. xylophilus* are inoculated into pine trees earlier in a nematode-transmission season (Hashimoto and Sanui, 1974; Kiyohara and Tokushige, 1971).

The rate of nematode movement decreases with decreasing ambient tempera-

TABLE 2. Time required for 50% of *Bursaphelenchus xylophilus* to depart from *Monochamus alternatus* adults and transmission efficiency of *B. xylophilus* at three temperatures.

Initial nematode load per vector	Temperature			
	16 °C	20 °C	25 °C	P^c
		Days to 50% departure[a]		
1–9	25.8 ± 16.5 a(4)	27.7 ± 16.1 a(8)	27.1 ± 11.4 a(6)	>0.05
10–99	26.1 ± 4.5 a(8)	15.6 ± 4.2 b(5)	27.4 ± 10.0 a(3)	<0.05
100–999	28.2 ± 7.1 a(6)	23.4 ± 10.5 a(7)	17.1 ± 6.6 a(5)	>0.05
1,000–9,999	30.1 (1)	20.8 (1)	28.3 (1)	
		Transmission efficiency[b]		
1–9	0.39 ± 0.44 a(6)	0.44 ± 0.40 a(8)	0.53 ± 0.46 a(7)	>0.05
10–99	0.27 ± 0.19 a(9)	0.25 ± 0.12 a(6)	0.23 ± 0.21 a(5)	>0.05
100–999	0.13 ± 0.07 a(7)	0.25 ± 0.16 ab(9)	0.40 ± 0.11 b(6)	<0.05
1,000–9,999	0.03 ± 0.04 (2)	0.28 (1)	0.30 (1)	

[a] Numbers are means ± SD (number of vectors used is in parentheses). Means in a row followed by a common letter are not significantly different ($P > 0.05$; one-way ANOVA and Tukey-Kramer multiple comparison test).

[b] Transmission efficiency was calculated as the ratio of total number of *B. xylophilus* transmitted successfully into pine twigs over the total number of *B. xylophilus* leaving the vector. Numbers are means ± SD (number of vectors used is in parentheses). Means in a row followed by a common letter are not significantly different ($P > 0.05$; Kruskal-Wallis test).

[c] Probabilities determined for each row.

ture between 25 and 5 °C (Iwahori and Futai, 1995), which might explain the delayed departure of *B. xylophilus* from the insect vectors. In addition to the temperature-dependent movement of *B. xylophilus,* there is a decrease in the amount of vector feeding with decreasing temperature (Morimoto, 1974), which might lead to lower transmission efficiency. Therefore, delayed departure and low transmission efficiency of *B. xylophilus* may induce a delayed, lower peak of transmission at lower temperatures (Fig. 3).

Temperature affects the severity of PWD through the biology of *B. xylophilus* and its vectors, and the interactions between *B. xylophilus,* its vectors, and host pine trees. Cool temperatures decrease the adult life span of *M. alternatus* (this study) and the lifetime fecundity of adult *M. saltuarius* (Gebler), another vector of *B. xylophilus* in Japan (Nakayama et al., 1998). *Monochamus alternatus* requires 1 or 2 years to complete development. The proportion of individuals requiring 2-year development is higher in cool districts than in warm districts (Zinno et al., 1987). Thus, cool temperatures cause a reduced potential reproductive rate per year of insect vectors. The pinewood nematode has lower reproduction rates and dispersal within trees at cool temperatures (Kiyohara, 1973; Mamiya, 1983; Shoji et al., 1986), which may result in reduced nematode load for insect vectors. The incidence of PWD on pine trees artificially infected with equal number of *B. xylophilus* is decreased and delayed with decreasing temperature (Kiyohara, 1973). In addition, the shorter life span of the adult vector, lower transmission efficiency, and delayed transmission due to cool temperatures result in the infection of host trees with fewer nematodes, leading to lower and delayed incidence of PWD (Hashimoto and Sanui, 1974). Because pine trees killed by *B. xylophilus* are one of the important oviposition resources for insect vectors, low incidence of PWD might lead to reduced reproductive rate of insect vectors. Delayed incidence of PWD results in asynchronous resource availability relative to the ovipositional period of the insect vectors, leading to low realized reproductive rate of vectors (Zinno et al., 1987). Cool temperatures decrease the transmission rate and virulence of *B. xylophilus,* which is consistent with the temperature dependency of PWD incidence reported by Rutherford et al. (1990).

Our results demonstrate the inhibitory effects of cool temperatures on the *B. xylophilus* transmission process. These findings help explain the interactions that prevent

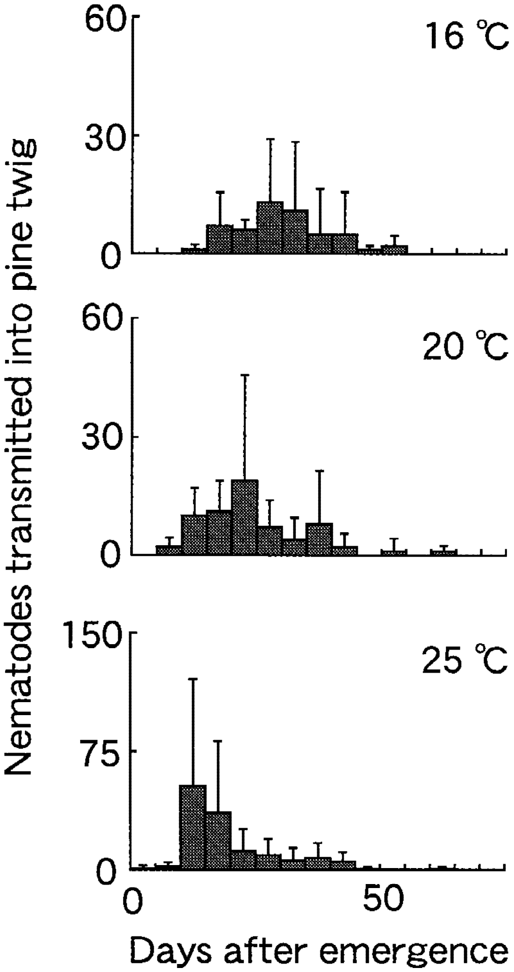

FIG. 3. Transmission of *Bursaphelenchus xylophilus* into pine twigs by *Monochamus alternatus* reared at three constant temperatures. Data are presented for beetles with an initial nematode load of 100-999. Heights of columns and error bars indicate the mean numbers and standard deviations of nematodes transmitted by beetles. No nematode transmission occurred after 75 days.

PWD from devastating pine forests in cooler areas. This inference may be reinforced by clarifying the effects of cool temperatures on nematode transmission by vectors with heavy initial loads of more than 10,000 *B. xylophilus.*

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