An effect of pine volatiles on departure of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) from *Monochamus alternatus* (Coleoptera: Cerambycidae)

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(Received 30 May 1997; Accepted 21 January 1998)

Abstract

To clarify the effect of the volatiles of *Pinus densiflora* on the temporal pattern of departure of *Bursaphelenchus xylophilus* from *Monochamus alternatus* adults (nematode departure curve), autoclaved or normal pine twigs were provided to the beetles. Monoterpenoids were not detected from the air of rearing containers with autoclaved pine twigs. A Kolmogorov-Smirnov test showed that there were significant differences in the averaged nematode departure curve between the 2 beetle groups supplied with different foods. The time of 50% nematode departure was shorter in the beetles supplied with autoclaved twigs. The proportion of nematodes which remained in the bodies of 51-day-old *M. alternatus* was significantly smaller in the beetles supplied with autoclaved pine twigs. Consequently, it is suggested that *B. xylophilus* had a trait of spontaneous departure from *M. alternatus* and that the volatiles from excised normal *P. densiflora* twigs repressed *B. xylophilus* departure from *M. alternatus*.

Key words: *Bursaphelenchus xylophilus*, *Monochamus alternatus*, nematode departure, pine volatiles

INTRODUCTION

The pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, causes a serious damage to the Japanese red and black pines, *Pinus densiflora* Sieb. et Zucc. and *P. thunbergii* Parl., in Japan (Kiyohara and Tokushige, 1971). The nematode is transmitted among host trees primarily by adult *Monochamus alternatus* Hope (Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972), feeds on the parenchymatous cells of the resin canal and fungi in trees, and then increases in number (Mamiya, 1983). Under unfavorable conditions, third-stage dispersal juveniles occur and aggregate around the pupal chambers of *M. alternatus* larvae during winter. They molt to the fourth-stage dispersal juveniles following the pupation of *M. alternatus* (Mamiya, 1983). The fourth-stage dispersal juveniles are attracted by CO₂, which is released in large amounts by respiration at the time of molting to the beetle adult, and enter the tracheal system of beetles (Miya-

zaki et al., 1978). After the sclerotization of the integument, *M. alternatus* adults carrying the nematodes emerge from the dead pine trees, feed on bark of fresh pine twigs and deposit eggs under the bark of declining or recently-killed host trees. The fourth-stage dispersal juveniles carried by *M. alternatus* adults invade healthy host trees via *M. alternatus* feeding wounds (Mamiya and Enda, 1972; Linit, 1988) and recently-killed trees via oviposition wounds (Edwards and Linit, 1992; Wingfield, 1983) made by *M. alternatus* females.

Elucidation of the transmission mechanism of *B. xylophilus* from *M. alternatus* adults to the host trees is necessary to fully understand and control the epidemic of pine wilt disease. The temporal pattern of nematode transmission has been described in relation with the age of *M. alternatus* adults and the number of nematodes carried initially (e.g. Kishi, 1978; Morimoto and Iwasaki, 1972; Togashi, 1985). But there have been only a few reports about the factors associated with the departure of nematodes from the

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insect vectors. Enda and Ikeda (1983) show that β -myrcene, which is one of the monoterpenes emitted from *B. xylophilus*-infected *P. densiflora* trees (Ikeda and Oda, 1980), has an accelerating effect on *B. xylophilus* departure from *M. alternatus*. However, they could not obtain an effect of β -myrcene on the temporal pattern of *B. xylophilus* departure in relation to the age of *M. alternatus* (nematode departure curve) because of the short lives of the beetles. The short lives were possibly attributed to the deteriorated food, *P. thunbergii* twigs which had been dried and then moistened. Edwards and Linit (1992) show that the volatiles emitting from wood chips of *Pinus banksiana* Lamb. promote the departure of *B. xylophilus* from adult *Monochamus carolinensis* (Olivier). As they examined the departure response of *B. xylophilus* during two consecutive 24 h periods using individual beetles, they could not show an effect of the volatiles on the nematode departure curve.

We noticed that *M. alternatus* adults supplied with autoclaved, excised *P. densiflora* twigs lived as long as those supplied with excised, normal twigs in the laboratory and that there was no smell of terpenoid emitting from the autoclaved twigs. Thus, to clarify the effect of the volatiles emitting from *P. densiflora* twigs on the nematode departure curve, *M. alternatus* adults contaminated with *B. xylophilus* were reared on autoclaved and normal pine twigs.

MATERIALS AND METHODS

Insects. Fourth-stadium larvae of *M. alternatus* which originated from Tokuyama City, Yamaguchi Prefecture, and Hiroshima City, Hiroshima Prefecture, were used for this experiment. The Tokuyama population was collected as larvae on November 14 and 16, 1994, and the next generation was reared on *P. densiflora* logs under constant conditions of 25°C and a photoregime of 12L–12D, and then placed at 10°C and 12L–12D to terminate the larval diapause. The Hiroshima population was obtained by cutting dead *P. densiflora* trees on October 27 and 28, 1995, and storing at 10°C and 12L–12D. The larvae were taken out of the pine logs in September 1996 and placed singly in Petri dishes 9 cm in diameter at 10°C in continuous dark-

ness until use on September 28, 1996.

Nematodes. *B. xylophilus* used was the S-10 isolate, which originated from Shimane Prefecture, and was one of the most virulent isolates. After the nematodes were cultured on *Botrytis cinerea* Pers. for 2 weeks at 25°C, they were extracted for 24 h using the Baermann funnel technique at 25°C and adjusted to a concentration of 10,000 nematodes per 1 ml of deionized, distilled water.

Method for loading *M. alternatus* with *B. xylophilus*. Stems of healthy *P. densiflora* trees were cut into 7.5 cm long bolts in September, 1996. The bolts averaged about 4 cm in diameter. A hole was drilled in the center of a cut end. The holes were 1.05 cm across and 5 cm deep. The bolts were individually set upright in polycarbonate containers (11 cm tall, 6.7 cm inside diameter, Agripot®, Kirin Co.) with quartz sand spread. They were autoclaved at 121°C for 30 min, and then each pine bolt was inoculated with a disk of bluestain fungus, *Ophiostoma minus* (Hedgc.) H. et P. Sydow, which had been kept on PDA medium at 25°C for 2 weeks. Two weeks later, a 0.5 ml suspension of nematodes was pipetted into each hole in the pine bolts to inoculate 5,000 nematodes. Immediately following inoculation, 59 fourth-stadium *M. alternatus* larvae were individually introduced into the pine bolt holes. Then the openings of holes were plugged with rounded aluminum foil (Aikawa et al., 1997). The incubation and rearing were conducted under constant conditions of 25°C, ca. 100% R.H. and continuous darkness. The pine bolts were monitored daily for the adult beetle emergence. The body weight and elytral length of beetles were determined just after emergence.

Insect foods. Two kinds of foods were used for *M. alternatus* adults to determine the effect of the pine volatiles on the temporal pattern of the departure of *B. xylophilus* from the vectors. One was fresh 1-year-old *P. densiflora* twigs which were excised to 11 cm long just after the collection, and another was 1-year-old *P. densiflora* twigs autoclaved for 40 min at 121°C after excising to 11 cm long. To confirm the lack of emission of *P. densiflora* volatiles from the autoclaved pine twigs, the autoclaved and normal pine twigs were individually placed in the

beetle rearing containers described later. The containers were placed at 25°C and 12L-12D. The air in the containers was analyzed by gas chromatography (Shimadzu GC-7 [H_2 : 0.7 kg/cm², Air: 0.5 kg/cm²], column: PEG-1000 10% on CW 60/80 Shimalite BT® [3 mm ϕ × 2 m]) 1 h and 3 days after the introduction of autoclaved twigs and 1 h after the introduction of normal twigs. Analysis conditions for GC were as follows: column temperature, programmed from 80 to 120°C; injection temperature, 90°C; carrier gas, N_2 40 ml/min; pressure, 1.1 kg/cm². Gas chromatogram showed that there were α -pinene, β -pinene, myrcene and β -phellandrene in the air of containers containing excised, normal *P. densiflora* twigs. In contrast, such volatiles were not detected from the air in containers with the autoclaved twigs.

Investigation of departure of *B. xylophilus* from *M. alternatus*. Newly-emerged *M. alternatus* adults were individually placed into a transparent rearing container (12 cm tall and 6.2 cm in inside diameter, Agripot®, Kirin Co.) with a floor made of stainless wire-netting which separated the adults from the deionized, distilled water in the container bottom. The con-

tainers were illustrated by Jikumaru and Togashi (1995). The adults were supplied with either a normal or autoclaved pine twig and kept at 25°C and under a photoregime of 12L-12D. The adults were moved to new containers with new food every 3 days. Soon after the removal of each adult, the surface of the old pine twig, the container wall and the stainless wire-netting were washed three times with deionized, distilled water, which was added to the water at the container bottom. The nematodes failing to enter the twigs were extracted from the water. The nematodes successfully entering the twigs were extracted after cutting the twigs into slices. When *M. alternatus* adults died or attained the age of 51 days, they were crushed using a mortar and pestle to extract the nematodes remaining in the bodies.

The Baermann funnel technique was used to extract the nematodes at 25°C. Two days after the start of extraction, a census was taken for the nematodes in the bottom 20 ml of water in the funnel. To estimate the total number of nematodes extracted, regression equations were made between the number of nematodes in the bottom 20 ml of water and that in the total

Fig. 1. Relationships between the number of *Bursaphelenchus xylophilus* in the bottom 20 ml of water and that in all the water of the extracting funnels. The nematodes were extracted from normal or autoclaved *Pinus densiflora* twigs provided to *Monochamus alternatus* adults in rearing containers. They were also extracted from water and dead or 51-day-old *M. alternatus* bodies. Water at the rearing container bottom was combined with water used to wash the container and twig surface.

water of the funnel for the respective samples (Fig. 1).

Analytical method. The initial number of nematodes (x) carried by *M. alternatus* (initial nematode load) was estimated as the sum of the numbers of nematodes departing from and remaining in the beetles, transformed into $\log(x+1)$ to make the mean independent of the variance and then analyzed by the one-way ANOVA. The proportion of nematodes which remained in insect bodies and the highest peak in the percentage temporal pattern in nematode departure (percentage nematode departure curve) were transformed to angle by arcsine transformation for ANOVA (Yonezawa et al., 1988). A Kolmogorov-Smirnov test was used for a comparison of the averaged nematode departure curves between *M. alternatus* adults supplied with normal and autoclaved pine twigs. The age-specific values of the averaged nematode departure curves were calculated as the mean numbers of nematodes departing during each 3-day period after the beetle emergence. Pearson's correlation coefficient was calculated to show the association between variables.

RESULTS

A total of 28 *M. alternatus* adults emerged from the pine bolts. The adult body weight ranged from 169 to 609 mg (mean=385 mg, SD=120 mg) and the elytral length from 1.11 to 1.73 cm (mean=1.50 cm, SD=0.16 cm). The initial nematode load ranged from 1 to 28,877 (mean=5,127, SD=6,831).

A group of 14 *M. alternatus* adults was supplied with normal pine twigs and the other 14 adults were supplied with autoclaved twigs. An adult fed with normal twigs died 4 days after the emergence whereas 3 adults with autoclaved twigs died 3, 6 and 14 days after the emergence. The 24 remaining adults lived for 51 days. There were no differences in the initial nematode load (one-way ANOVA, $F=0.491$, $df=1$, $p=0.489$) or in the adult body weight (one-way ANOVA, $F=1.141$, $df=1$, $p=0.295$) between the 2 adult groups. The initial nematode load was positively correlated with adult body weight ($r=0.554$, $p=0.002$, $n=28$).

The initial nematode load of adult beetles was

grouped into 5 classes; 1–9, 10–99, 100–999, 1,000–9,999, and 10,000–99,999 nematodes per adult beetle. The adults which carried less than 100 nematodes at emergence were eliminated from the analysis because of the lack of counterparts of nematode departure data in the 2 initial load classes of 1–9 and 10–99 nematodes. The nematode load, the number of nematodes carried, continued to decrease as the age of adult beetles increased except for the period comprising the first 3–6 days (Fig. 2). A rapid decrease was observed during the period from the 9th to the 30th day after adult emergence in both food conditions.

A Kolmogorov-Smirnov test showed that there were significant differences in the averaged nematode departure curves between the 2 adult groups supplied with the normal and autoclaved pine twigs at the 2 initial load classes of 1,000–9,999 and 10,000–99,999 nematodes ($p<0.001$

Fig. 2. Age-related changes in the number of *Buraphelenchus xylophilus* carried by *Monochamus alternatus* adults in relation to food conditions. The numerals connected by hyphen in each figure represent the class of initial nematode load of adult beetles. The numerals in parentheses indicate the number of adult beetles examined.

Fig. 3. Temporal pattern in the percentage (left scales) and number (right scales) of *Bursaphelenchus xylophilus* departing from *Monochamus alternatus* adults. The percentage represents the proportion of departing nematodes to the initial number of nematodes carried at beetle emergence. The numerals connected by hyphen in each figure represent the class of initial nematode load of adult beetles. The numerals in parentheses represent the number of adult beetles examined. The vertical bars indicate the standard deviation.

for the 2 classes of initial nematode load) (Fig. 3). A comparison of the averaged nematode departure curves could not be performed statistically for the beetles with an initial load of 100–999 nematodes because all adult beetles supplied with autoclaved pine twigs died before the end of the study.

Although the nematode departure curves tended to be unimodal irrespective of supplied foods and the initial nematode load classes, the kurtosis of the highest peaks seemed to be duller for the adults supplied with normal pine twigs (Fig. 3). Two-way ANOVA showed no effects of food conditions or initial nematode loads on the values of the highest peaks in the percentage nematode departure curves ($F=2.746$, $df=1$, $p=0.121$ for food conditions; $F=1.701$, $df=1$, $p=0.215$ for initial nematode loads), but there was a significant effect of food conditions on the times required for the half of initially carried nematodes to depart from individual adult beetles (Tables 1 and 2).

The proportion of nematodes which remained in the bodies of 51-day-old *M. alternatus* was significantly smaller in adult beetles supplied with autoclaved pine twigs than in those with normal pine twigs (one-way ANOVA, $F=8.856$, $df=1$, $p=0.008$) (Table 3).

DISCUSSION

The volatiles emitting from pine trees have great influences on the behavior of *B. xylophilus*. β -Myrcene placed on *P. densiflora* twigs, which were dried at 70°C and then moistened, has an accelerating effect on the *B. xylophilus*

Table 1. A comparison of the highest peaks of the percentage *Bursaphelenchus xylophilus* departure curve and the time required for a half of the initially carried nematodes to depart from *Monochamus alternatus* adults among 5 adult groups of the different foods and initial nematode load classes

Initial nematode load	Value of highest peak in age-specific nematode departure curve on (%)		Time of 50% nematode departure on (days after emergence)	
	Autoclaved twigs ^a	Normal twigs ^a	Autoclaved twigs ^a	Normal twigs ^a
100–999	—	22.9±1.4 [2]	12.3 [1]	38.6±17.5 [2] ^b
1,000–9,999	23.3±5.4 [8]	17.9±5.3 [4]	18.4±4.0 [8]	28.7±15.5 [4] ^b
10,000–99,999	28.3±9.7 [2]	22.3±8.5 [3]	14.3±3.6 [2]	17.2± 5.6 [3]

^a Mean±SD [No. of beetles examined].

^b Less than 50% of nematodes carried initially (34.5% and 49.0%) departed from two *M. alternatus* adults by the age of 51 days. So a value of 51 days was given as the time of 50% nematode departure for the respective adult beetles and the averages were calculated.

Table 2. The effect of *Pinus densiflora* twig conditions and initial load of *Bursaphelenchus xylophilus* on the time of 50% nematode departure from *Monochamus alternatus* adults (two-way ANOVA table)

Source of variation	df	SS	MS	F	p
Food condition (F)	1	575.475	575.475	6.618	0.022
Initial nematode load (N)	2	244.118	122.059	1.404	0.278
Interaction (F × N)	2	234.865	117.432	1.351	0.291
Error	14	1,217.320	86.951		

Table 3. The differences in the number and percentage of *Bursaphelenchus xylophilus* remaining in the bodies of 51-day-old *Monochamus alternatus* adults which had been supplied with normal or autoclaved twigs of *Pinus densiflora*

Twigs supplied	Number of beetles examined	Number of nematodes (%)	
		Departing from beetles ^{a b c}	Remaining in beetle bodies ^{b c}
Autoclaved	10	5,731 ± 4,069 a (84.2 ± 8.7 a)	1,037 ± 725 a (15.8 ± 8.7 a)
Normal	9	6,401 ± 8,121 a (67.0 ± 16.1 b)	2,016 ± 1,854 a (33.0 ± 16.1 b)

^a The percentages of nematodes departing from *M. alternatus* by the 51st day after beetle emergence are parenthesized.

^b Mean ± SD.

^c Means followed by different letters in each column are significantly different at the 1% level (one-way ANOVA). Number and percentage of nematodes departing from or remaining in *M. alternatus* adult were log and arcsine transformed before analysis, respectively.

departure from *M. alternatus* adults when compared with the excised normal twigs (Enda and Ikeda, 1983). The volatiles of *P. banksiana* wood chips also have an accelerating effect on nematode departure from *M. carolinensis* (Edwards and Linit, 1992). β -Myrcene attracts the fourth-stage dispersal juveniles of *B. xylophilus* placed on agar media (Ishikawa et al., 1986; Stamps and Linit, 1995).

The results of our study, however, showed that *B. xylophilus* departure from *M. alternatus* adults was suppressed by the presence of excised normal *P. densiflora* twigs compared with autoclaved twigs. The amount of volatiles from *P. densiflora* twigs was one of the greatest difference between normal and autoclaved twigs. As no volatiles emitting from autoclaved pine twigs were detected, our results suggested that *B. xylophilus* had a trait of spontaneous departure from *M. alternatus*. In addition, the volatiles from excised normal *P. densiflora* twigs were suggested to repress the *B. xylophilus* departure from *M. alternatus*. The aforementioned results of Enda and Ikeda (1983) may suggest the possibility that nematode-departure suppressing substances are included in the vola-

tiles emitting from the excised normal *P. densiflora* twigs. Our results can be understood if the suppressing effect of some substances on the nematode departure exceeds the accelerating effect of others. Another marked difference between the treatments of our study was probably the chemical constituents of pine twigs. The change of food quality may have induced the difference in physiological status of *M. alternatus* adults and consequently may have had an influence on the nematode departure. This question remains to be addressed.

B. xylophilus is native to North America, where it has never induced an epidemic in native pine forests in North America because of resistance or climatic conditions that are unsuitable for disease development (Rutherford and Webster, 1987). The nematode is transmitted via the oviposition wounds of insect vectors to recently-killed pine trees and other conifers and then reproduces in them (Wingfield, 1983). The facts suggest that it is more adaptive for *B. xylophilus* to respond more positively to the volatiles emitting from recently-killed trees compared with the volatiles from healthy trees. It is known that the volatiles from recently-

killed *P. densiflora* seedlings include ethanol in addition to volatiles such as monoterpenes emitting from healthy seedlings (Ikeda et al., 1980). The slightly suppressed departure response of *B. xylophilus* to the volatiles from excised normal *P. densiflora* twigs might suggest its original trait because monoterpenoids emitting from higher plants show protective actions against herbivores and pathogens (e.g., Langenheim, 1994).

ACKNOWLEDGEMENTS

The authors deeply thank the staffs of the Tokuyama Forest Experiment Station of Kyoto University and the Hiroshima Botanical Garden for assisting with the collection of dead pine trees.

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