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Transmission of *Bursaphelenchus xylophilus* through Oviposition Wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae)[1]

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Abstract: Transmission of pinewood nematode through *Monochamus carolinensis* oviposition wounds was documented. Nematode transmission was measured as the average number of nematodes isolated per oviposition wound excavated and also as the percentage of oviposition wounds from which nematodes were isolated. The influence of three factors that might affect nematode transmission was investigated: age of the beetle vector, number of nematodes carried per beetle, and egg deposition in the oviposition wound. Only the number of nematodes carried by the beetle was found to have a significant effect on transmission. Nematodes were transmitted more frequently and in slightly greater numbers by beetles carrying more nematodes. The influence of pinewood on nematode exit from beetles were investigated by comparing nematode exit from beetles placed over pine chips with those placed over distilled water. Nematodes exited in greater numbers and at a higher frequency from beetles over pine chips than from beetles over distilled water. Apparently, the nematodes are able to detect a factor from the pine chips that promotes their exit from the beetles.
Key words: Bursaphelenchus xylophilus, Monochamus carolinensis, nematode, pine sawyer, pinewood nematode.

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, is a mycophagous and phytophagous nematode associated with stressed pines in North America. The nematode is transported between trees by insect vectors. The principal vectors of the pinewood nematode throughout its distribution are cerambycid beetles in the genus *Monochamus* (9). These beetles lay their eggs under the bark of stressed or recently felled pine trees. The wounds created by these beetles during oviposition provides a means by which the nematodes can enter pine trees (20). Nematodes may also enter healthy pine trees through beetle feeding wounds (11). In susceptible pines, pinewood nematodes feed on the cells lining resin canals (13,14). These pine trees may show symptoms of pine wilt disease as a result of this type of feeding.

Historically, interest in the pinewood nematode has focused on its transmission to susceptible healthy pines. Recently, the detection of pinewood nematode in North American wood chips shipped to Finland resulted in an embargo on North American raw softwood products by that nation. Sweden and Norway have passed similar import restrictions (1). The economic loss associated with this embargo has increased interest in the transmission of the nematode into felled timber through beetle oviposition wounds.

The purpose of this study was to investigate pinewood nematode transmission through the oviposition wounds made by its principal vector in the midwestern United States, *Monochamus carolinensis* (Olivier) (12). The first objective of this study was to determine the frequency of successful transmission and the mean number of nematodes transmitted into *M. carolinensis* oviposition wounds. The influence of beetle age, number of nematodes carried per beetle (nematode load), and egg deposition on nematode transmission was examined. The second objective of this study was to investigate the potential role of pinewood volatiles in controlling nematode exit from the beetle.

Materials and Methods

M. carolinensis adults used in these experiments were from a laboratory colony

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infested with pinewood nematode. The beetles were reared in jack pine, *Pinus banksiana* Lamb., logs, as described previously (3). The wood bolts (12–18 cm in length, 5–8 cm in diameter) used in the transmission studies were from the main bole of recently felled jack pine trees from the Thomas A. Baskett Wildlife Research and Education Center in Boone County, Missouri. The bolts were taken from an area of the tree where the bark was thin (1–2 mm) in order to promote oviposition (7) and to minimize mandibular activity during oviposition (19). The ends of the wood bolts were coated with paraffin to retard desiccation and were held for at least 3 days before use to increase their attractiveness to ovipositing *M. carolinensis* (7). All statistical analyses were conducted using the Statistical Analysis System (15).

Transmission through oviposition wounds: Female beetles were removed from the colony at the beginning of the scotophase of the photoperiod. For each of 218 trials, one beetle was placed in a cylindrical cardboard container (19 cm high, 17.5 cm diameter) that contained a jack pine bolt. Bolts were exposed to beetles for 4 hours. Bolts that had oviposition wounds after 2 hours were removed to prevent nematode dispersal from the wood surrounding oviposition wounds prior to examination. These were replaced by fresh bolts for the remaining 2 hours. Bolts that contained no oviposition wounds after beetle exposure were discarded.

Each oviposition wound was examined to determine whether an egg had been deposited. To determine if transmission occurred, the bark, cambium, and heartwood around each oviposition wound were removed to a radius of 0.75 cm and a depth of at least 2 cm. Nematodes were extracted from the bark and wood together using a modified Baermann technique (16). One additional bark and wood sample from each bolt was taken from a region where oviposition did not occur. These samples were checked to ensure that no pinewood nematodes were present in the bolts prior to beetle oviposition. Beetles that made oviposition wounds were macerated immediately after the experiment, and the number of nematodes they carried was determined using the modified Baermann technique. All nematodes extracted from wood samples were individually examined to identify them positively as *B. xylophilus*.

Beetles from five adult age classes were tested: 1, 2, 3, 4, and 5 weeks post adult emergence. One day of leeway was used for the beetle ages, so that all 1-week-old beetles were 6–8 days old, 2-week-old beetles were 13–15 days old, and so forth. At least 50 oviposition wounds from nematode-infested beetles in each age class were tested. Because we could not determine without killing the beetles whether the beetles were nematode infested before carrying out the experiment, some beetles without nematodes were tested. The wounds made by these beetles were examined to ensure that the wood brought from the field was nematode-free.

The mean number of nematodes transmitted per oviposition wound was calculated for each age class. Percentage of successful transmission was calculated as the percentage of oviposition sites into which at least one nematode was transmitted. Analysis of variance was used to determine whether number transmitted was affected by beetle age; chi-square analysis was used to determine whether percentage of successful transmission was affected by age class.

Regression analysis was used to investigate the relationship between nematode load of beetles and number of nematodes transmitted. To analyze the effect of nematode load on percentage of successful transmission, the beetles were classed as follows: beetles carrying 1–100 nematodes, 101–1,000 nematodes, 1,001–10,000 nematodes, and more than 10,000 nematodes. Chi-square analysis was used to determine whether percentage of successful transmission was independent of nematode load.

Differences in mean nematode transmission and percentage of successful transmission were also examined between sites in which an egg had been laid and those without an egg by *t*-test and chi-square analysis, respectively.

Transmission by male beetles: An experiment to determine whether male beetles transmitted nematodes into oviposition wounds was performed concurrently with the videotaping of *M. carolinensis* oviposition behavior as described by Edwards and Linit (3). Nematode-free *M. carolinensis* females were placed in the videotaping arena with nematode-infested males. Nematode-free females were obtained by omitting nematode inoculation from the rearing procedure described previously (3). Videotaping provided a record of every oviposition event. The wood surrounding all oviposition wounds was excavated and checked for nematodes as described above. The number of nematodes transmitted during each oviposition event was recorded.

Pine volatiles and nematode exit: Beetles over 1 week of age were used to exclude those from which nematodes were exiting at very low rates (10). Beetles carrying fewer than 1,000 nematodes were not included in data analysis to eliminate the poor transmission associated with beetles carrying few nematodes.

Beetles were individually placed in an arena for two 24-hour assays of nematode exit. The arena consisted of a plastic Petri plate with an elevated floor and a lid, both constructed of wire screening. For one assay, only distilled water was beneath the elevated wire floor. For the other, 5 g of pine chips in distilled water was used. The pine chips consisted of bark, cambium, phloem, and xylem obtained by drilling 1.5-cm-deep holes into a freshly cut jack pine log with a 2.5-cm-diameter drill bit. The top portion of the chips extended above the water line, so that plant compounds could volatilize. Each beetle was assayed under both experimental conditions for two consecutive 24-hour assay periods; the assays were randomly assigned to the periods so that half the beetles were exposed to one set of conditions first, whereas the other half were exposed to the other set of conditions first. After each 24-hour test period, the arena was thoroughly washed with distilled water. The washing was placed with the distilled water and wood chips (if present) into funnels for nematode extraction using the modified Baermann technique. After the second 24-hour testing period, the beetles were macerated and their nematode loads were determined. All nematodes extracted were examined with a dissecting microscope for positive identification as dauer juveniles of *B. xylophilus*.

The data were analyzed as a crossover treatment design (2). Because there were only two treatments, residual effects could not be tested; any residual effects from the first to the second assay period were assumed to be the same for all the beetles. Analysis of variance was used to test for significant differences in nematode exit attributable to treatment, assay period, and beetle. A chi-square analysis was used to determine if the presence of wood chips affected frequency of nematode exit into each treatment arena.

Results

Transmission through oviposition wounds: During the testing period, 118 of 290 female beetles made oviposition wounds. A total of 437 oviposition wounds was made by these beetles during testing. Of these, 274 were made by 64 nematode-infested beetles. No nematodes were recovered from the wood surrounding wounds ($n = 163$) made by nematode-free beetles. All analyses were based on wounds made by nematode-infested beetles.

The mean (S.D.) nematode load for the beetles used in this experiment ($n = 64$) was 2,293 (5,019). The maximum number of nematodes recovered from one beetle was 29,500. The mean number of nematodes transmitted per oviposition wound was 1.48 (4.85), and nematodes were recovered from 27% of oviposition wounds. The mean number of nematodes transmitted per oviposition wound was not dependent on beetle age class ($F = 0.64$; df = 4,268; $P = 0.63$) (Table 1). Percentage of

TABLE 1. Number of nematodes transmitted and frequency of successful transmission of *Bursaphelenchus xylophilus* into oviposition wounds on jack pine bolts by *Monochamus carolinensis* beetles of differing age (weeks post adult emergence), nematode load, and when eggs were laid or not.

		Number of nematodes recovered			Number of oviposition wounds	
Factor	Condition	Mean	Minimum	Maximum	With nematodes	Without nematodes
Age†						
	1	0.74‡	0	11	16 (30%)§	37 (70%)
	2	2.09	0	39	16 (25%)	49 (75%)
	3	1.19	0	18	13 (25%)	40 (75%)
	4	1.63	0	30	16 (31%)	35 (69%)
	5	1.63	0	27	14 (27%)	38 (73%)
Nematode load						
	1–100	0.73‖	0	30	8 (9%)¶	77 (91%)
	101–1,000	1.67	0	39	16 (31%)	35 (69%)
	1,001–10,000	1.60	0	27	33 (36%)	59 (64%)
	10,000+	2.39	0	27	18 (39%)	28 (61%)
Eggs laid						
	Yes	1.61#	0	39	58 (29%)§	139 (71%)
	No	1.16	0	27	17 (22%)	60 (78%)

Means and frequencies were calculated by counting nematodes from the wood surrounding oviposition wounds after beetle oviposition.
† Weeks post adult emergence.
‡ Means did not differ significantly according to analysis of variance.
§ Counts did not differ significantly according to chi-square analysis.
‖ Significant linear relationship; data presented as class means in table.
¶ Counts significantly different according to chi-square analysis.
# Means did not differ significantly according to a *t*-test.

successful nematode transmission was also unaffected by beetle age class ($\chi^2 = 1.091$; df = 4; $P = 0.90$).

There was a significant ($F = 8.79$; df = 271; $P < 0.01$) linear relationship between nematode load and the number of nematodes transmitted, but the predictive value of the resulting relationship was low ($r^2 = 0.03$, slope = 0.00012 ± 0.00004). The mean number of nematodes transmitted ranged from 0.73 (4.12) in the lowest nematode load class to 2.39 (5.70) in the highest nematode load class (Table 1). Percentage of successful transmission increased with increased nematode load ($\chi^2 = 20.7$; df = 3; $P < 0.001$).

The deposition of an egg into the oviposition wound did not significantly affect the number of nematodes transmitted ($t = 0.48$; df = 271; $P = 0.49$). Percentage of successful transmission was also independent of the occurrence of egg deposition ($\chi^2 = 1.51$; df = 1, $P = 0.22$). Both values, however, were higher when egg deposition occurred (Table 1).

Transmission be male beetles: During the videotaping of *M. carolinensis* oviposition behavior, 21 oviposition events were observed that involved a nematode-infested male on top of an uninfested female. Pinewood nematodes were isolated from the wood surrounding two of these wounds. One wound contained one *B. xylophilus* dauer juvenile and one *B. xylophilus* adult. The other wound contained 221 *B. xylophilus* dauer juveniles.

Pine volatiles and nematode exit: Thirty nine beetles were tested, 15 of which were not included in the analysis because they carried less than 1,000 nematodes. The mean (S.D.) nematode load for the remaining beetles (n = 24) used in the analysis was 8,147 (11,006). There was no difference in the total number of nematodes recovered among beetles ($F = 1.02$; df = 23,22; $P = 0.49$) or between the assay periods ($F = 1.40$; df = 1,22; $P = 0.25$). There was a significant treatment effect on the number of nematodes collected ($F = 12.60$; df = 1,22; $P = 0.002$). The mean number of nematodes collected in distilled water alone was 0.92 (2.53), whereas the mean number collected in distilled water with pine chips was 11.79 (14.92) (Table 2). Nematodes dropped off the beetles more frequently in the trials with wood chips than in the trials without wood chips ($\chi^2 = 12.08$; df = 1; $P < 0.001$).

Discussion

Tranmission through oviposition wounds: Wingfield and Blanchette (21) were the first researchers to quantify nematode transmission through *M. carolinensis* oviposition wounds. Their values for percentage of successful transmission and for the mean number of nematodes transmitted per oviposition niche were much lower than the overall values obtained in this experiment. The beetles in their experiment did not carry a high number of nematodes, which explains why the values they obtained were very close to the values obtained in this experiment for the lowest nematode load class.

The absence of an age effect on transmission into oviposition wounds suggests that nematode transmission is not influenced by the lower rate of egg deposition into oviposition wounds that is found in 1- and 2-week-old beetles (3). The lack of a difference in nematode transmission between wounds with eggs and without eggs supports this conclusion. The pinewood nematode exit rate from *M. carolinensis* is low during the first week following adult beetle emergence (10). Low nematode exit rates in 1-week-old beetles did not influence transmission rates in the present study, perhaps due to the low numbers of nematodes transmitted per wound.

Female beetles spend less time with their ovipositors inserted under the bark when an egg is not laid than when an egg is laid (3). Nematode recovery from oviposition wounds was unrelated to the presence of an egg in the wound. This suggests that the amount of time in which the ovipositor is inserted under the bark does not influence the number of nematodes transmitted or the frequency of successful transmission.

The mean number of nematodes transmitted through oviposition wounds increased with increased beetle nematode load. The mean number transmitted, however, increased from 0.73 in the lowest to only 2.39 in the highest nematode load class. This weak relationship between nematode load and the number of nematodes transmitted is similar to that observed by other workers (11,18).

There was a dramatic effect of nematode load on percentage of successful transmission. This was especially apparent in beetles carrying less than 100 nematodes. Percentage of successful nematode transmission into oviposition wounds may be a more meaningful measurement than the mean number of nematodes transmitted. The only requisite for successful colonization of a dying tree is that enough nematodes are transmitted to ensure that mating can occur. The number of nematodes required to colonize a dying tree has not been documented.

Transmission by male beetles: Our observations indicate that nematodes can be intro-

Table 2. Number and frequency of *Bursaphelenchus xylophilus* drop-off from *Monochamus carolinensis* adults into distilled water and into jack pine (*Pinus banksiana*) chips in distilled water.

Treatment	No. beetles	Nematodes recovered		Number of trials	
		Mean	Maximum	With drop-off	Without drop-off
Distilled water	24	0.92†	9	5‡	19
Pine chips in distilled water	24	11.79†	53	17‡	7

Nematodes were collected after a 24-hour exposure of the beetles to the treatments.
† Means significantly different according to analysis of variance.
‡ Counts significantly different according to chi-square analysis.

duced into wood beneath oviposition wounds by infested *M. carolinensis* males. Therefore, it may not be biologically advantageous for pinewood nematodes to enter female beetles preferentially (5,12,17,21). Furthermore, the nematodes may not be able to distinguish between male and female beetles in the pupal chambers where beetle infestation occurs.

It is unknown if the nematodes entered the wounds directly from the male or were transferred to the females during mating. Additional research is required to determine if direct transmission from males does occur, and if it does, if nematodes are transmitted at the same frequency from males as from females.

Pine volatiles and nematode exit: Though much research has been conducted on pinewood nematode transmission into pine trees through *M. carolinensis* and *M. alternatus* Hope feeding and oviposition wounds, little is known about the factors that control nematode exit from beetles. Minor effects of nematode load (11,18), beetle age (8), and the size of the feeding wound (8) cannot account for all the variability associated with nematode transmission. Results of this study suggest that a factor within pinewood influences nematode transmission. Because no contact occurred between the beetles and the pine chips, we hypothesize that a volatile chemical in the pinewood was responsible for the movement of the nematodes. It must be noted, however, that nematodes could have responded to CO_2 from metabolic activity of the pinewood or microorganisms associated with the pinewood.

Pine volatiles alone are not sufficient to explain all the variability associated with pinewood nematode transmission. Control mechanisms within the nematode may explain some of the variability. Endogenous control has been suggested to explain erratic resumption of development following arrest in some parasitic nematodes (4). Resumption of development from the infective juvenile stage in many plant-parasitic and entomopathogenic nematodes is triggered by rehydration (22). Infective-stage pinewood nematode extracted from pinewood or adult beetles exhibit the same straight posture as inactive steinernematid nematodes (6). Activation of sinusoidal activity occurs after a few hours in distilled water (pers. obs.). Depletion of food reserves may be an endogenous control mechanism that enables the resumption of dauer juvenile activity within beetle tracheae in response to host volatiles, causing the nematodes to exit from the beetles into appropriate transmission sites. In order to test these hypotheses, the behavioral response to rehydration or to depleted food resources needs to be studied. Further investigations on the role of host tree chemistry on nematode exit are also needed. This information may help to explain the high degree of variability researchers must contend with when studying the nematode-vector complex.

Literature Cited

1. Bergdahl, D. R. 1988. Impact of pinewood nematode in North America: Present and future. Journal of Nematology 20:260–265.

2. Cochran, W. G., and G. M. Cox. 1957. Experimental designs. New York: Wiley.

3. Edwards, O. R., and M. J. Linit. 1991. Oviposition behavior of *Monochamus carolinensis* (Coleoptera: Cerambycidae) infested with the pinewood nematode. Annals of the Entomological Society of America 84:319–323.

4. Evans, A. A. F., and R. N. Perry. 1976. Survival strategies in nematodes. Pp. 383–424 *in* N. A. Croll, ed. The organization of nematodes. New York: Academic Press.

5. Hosoda, R., M. Okudo, A. Taketani, and K. Kobayashi. 1974. Number of pine wood nematode held in the pine sawyer adult emerging from dead pine trees in the late stage of heavy infestation stands. Transactions of the Annual Meeting of the Japanese Forestry Society 85:231–233. (in Japanese)

6. Ishibashi, N., and E. Kondo. 1990. Behavior of infective juveniles. Pp. 139–150 *in* R. Gaugler and H. Kaya, eds. Entomopathogenic nematodes in biological control. Boca Raton, FL: CRC Press.

7. Kobayashi, F., A. Yamane, and T. Ikeda. 1984. The Japanese pine sawyer beetle as a vector of pine wilt disease. Annual Review of Entomology 29:115–135.

8. Linit, M. J. 1987. The insect component of pine wilt disease in the United States. Pp. 66–73 *in* M. J. Wingfield, ed. Pathogenicity of the pine wood nema-

tode. St. Paul, MN: American Phytopathological Society Press.

9. Linit, M. J. 1988. Nematode–vector relationships in the pine wilt system. Journal of Nematology 20:227–235.

10. Linit, M. J. 1989. Temporal pattern of pinewood nematode exit from the insect vector *Monochamus carolinensis*. Journal of Nematology 21:105–107.

11. Linit, M. J. 1990. Transmission of pinewood nematode through feeding wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae). Journal of Nematology 22:231–236.

12. Linit, M. J., E. Kondo, and M. T. Smith. 1983. Insects associated with the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), in Missouri. Environmental Entomology 12:467–470.

13. Mamiya, Y. 1983. Pathology of the pine wilt disease caused by *Bursaphelenchus xylophilus*. Annual Review of Phytopathology 21:201–220.

14. Mamiya, Y. 1984. Behavior of the pine wood nematode, *Bursaphelenchus xylophilus*, associated with the disease development of pine wilt. Pp. 14–25 *in* Proceedings of the United States–Japan seminar, The resistance mechanisms of pines against pine wilt disease.

15. SAS Institute. 1985. SAS user's guide: Statistics 5th ed. SAS Institute, Cary, N.C.

16. Southey, J. F., editor. 1986. Laboratory methods for work with plant and soil nematodes. Ministry of Agriculture, Fisheries, and Food Reference Book 402. London: Her Majesty's Stationery Office.

17. Takizawa, Y. 1979. Number of nematodes carried by the pine sawyer and beetle feeding test on pine seedlings. Transactions of the Annual Meeting of the Yohoku Branch Japanese Forestry Society 31:153–155. (in Japanese)

18. Togashi, K. 1985. Transmission curves of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) from its vector, *Monochamus alternatus* (Coleoptera: Cerambycidae), to pine trees with reference to population performance. Applied Entomology and Zoology 20:246–251.

19. Walsh, K. D., and M. J. Linit. 1985. Oviposition biology of the pine sawyer, *Monochamus carolinensis* (Coleoptera: Cerambycidae). Annals of the Entomological Society of America 78:81–85.

20. Wingfield, M. J. 1983. Transmission of pine wood nematode to cut timber and girdled trees. Plant Disease 67:35–37.

21. Wingfield, M. J., and R. A. Blanchette. 1983. The pine wood nematode, *Bursaphelenchus zylophilus*, in Minnesota and Wisconsin: Insect associates and transmission studies. Canadian Journal of Forest Research 13:1068–1076.

22. Womersley, C. Z. 1990. Dehydration survival and anhydrobiotic potential. Pp. 119–137 *in* R. Gaugler and H. Kaya, eds. Entomopathogenic nematodes in biological control. Boca Raton, FL: CRC Press.