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Transmission of Pinewood Nematode Through Feeding Wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae)[1]

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Abstract: Transmission of pinewood nematode, *Bursaphelenchus xylophilus,* to mature, field grown Scots pines through feeding wounds of *Monochamus carolinensis* was investigated by caging nematode-infested beetles on pine branches for 24 hours. Nematodes were transmitted to 31 of 64 branches. Frequency of successful transmission was independent of the sex of the beetle but dependent upon beetle age. Transmission frequencies were highest for beetles 2, 3, and 4 weeks after emergence as adults. The number of nematodes transmitted per branch was low and did not differ between beetle sexes or among beetle age categories. The number of nematodes extracted per branch was correlated with the number of nematodes carried per beetle but was not correlated with the feeding area on the branch.

Key words: *Bursaphelenchus xylophilus, Monochamus carolinensis,* pine sawyer, pine wilt disease, pinewood nematode.

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970, is the causal agent of pine wilt disease (7). The nematode is transported from infested to uninfested trees by insect vectors. Worldwide, 24 insect taxa have been identified as associates of the pinewood nematode (4). Of these, species in the genus *Monochamus* (Coleoptera: Cerambycidae) are thought to be the most important as vectors of the nematode. Transmission of *B. xylophilus* can occur in two ways. Nematodes can be introduced into a healthy tree through feeding wounds of *Monochamus* spp. Should this occur on a susceptible tree species, the tree may die as a consequence of nematode infection. Alternately, nematodes can enter a dying tree, or recently cut log, through *Monochamus* oviposition sites.

Reported here are results of studies conducted to determine the ability of *Monochamus carolinensis* (Olivier), a common associate of pinewood nematode in the midwestern United States (5), to introduce *B. xylophilus* to new host trees through feeding wounds. The goal of this study was to document successful transmission of *B. xylophilus* to healthy, field grown Scots pines (*Pinus sylvestris* L.). The objectives were to determine 1) the frequency of successful transmission and the mean number of nematodes transmitted by *M. carolinensis* during feeding and 2) the influence of beetle age and sex and the number of nematodes carried per beetle on transmission.

Materials and Methods

Beetles were laboratory reared according to procedures developed for previous studies on *M. carolinensis* (3). The procedure was modified as follows in order to obtain nematode-infested beetles. Each pine log used for beetle rearing was inoculated with ca. 25,000 *B. xylophilus* (all life stages) before being subjected to *M. carolinensis* oviposition. Nematodes colonized the logs and were available to enter newly formed adult beetles prior to beetle emergence (4). Upon emergence, beetles were placed in wooden boxes and maintained under room conditions until needed. Water and pine twigs on which they fed were replenished regularly.

Feeding trials were conducted on ca. 20-year-old Scots pine at the Thomas A. Baskett Wildlife Research and Education Center (Ashland Wildlife Research Area), Boone County, Missouri. Xylem samples were taken from prospective study trees to determine the presence of pinewood

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nematodes. Only nematode-free trees were selected for inclusion in the study. Beetles in their first, second, third, fourth, or fifth week of adult life were individually caged on a 2–3-year-old section of branch. The cage was constructed of 0.3-cm wire mesh designed to enclose a 20-cm section of the branch. All beetles were placed on the branches during the morning hours.

After 24 hours the beetles were collected and the branch sections within the cages were removed from the tree. Each branch was divided into two 10-cm sections. One randomly selected section of each branch was processed for nematode extraction immediately upon return to the laboratory. These branch sections contained only nematodes transmitted during beetle feeding. The remaining section of each branch was held at 25 C and 75% RH for 2 weeks to allow the nematodes introduced during beetle feeding to reproduce, thus increasing the probability of extracting nematodes from the branch. These branch sections provided a comparison of nematode infestation frequencies with the branch sections that were processed immediately. The amount of beetle feeding was estimated by measuring the length of all feeding wounds on each section of all branches; total length of all feeding wounds was the feeding area on that branch. The weight and diameter of each branch section was recorded, and the amount of foliage was categorized as none, moderate, or full.

The modified Baermann technique (13) was used to extract nematodes from branch sections and beetles. Nematodes were extracted from the beetles immediately upon return to the laboratory. The cortex and xylem of each branch section were separated and nematodes were extracted independently.

During the 1985 field season, 116 beetles were caged. Approximately one-half of the beetles were caged during the last week of June and the first week of July. The remaining beetles were caged during the period of 20 August through 10 September. Estimating within-beetle nematode load by the Baermann technique necessitates destruction of the beetle; therefore, the procedure was done after each beetle's 24-hour feeding period was complete. Nematode infestation of beetles could not be determined before caging and some nematode-free beetles were caged. The nematode-free beetles were used as a control group to verify that nematodes extracted from branches fed upon by beetles were introduced during the feeding process.

Chi-square analysis was used to determine if frequency of successful transmission was independent of branch section type. Transmission was considered successful if at least one pinewood nematode was extracted from the bark or wood of the branch sample. Based upon these results, subsequent statistical analyses were conducted only on data from the branch sections that were dissected immediately. An additional chi-square analysis was used to determine if frequency of successful transmission was independent of beetle age and sex.

Analysis of variance was used to investigate mean differences in the number of nematodes transmitted between seasons of caging, among branch foliage categories, among beetle age categories, and between sexes. Analysis of the latter two treatments was hampered because of lack of variation in two of the treatment combinations. A one-way ANOVA was conducted to test for differences among the age–sex treatment combinations with variances greater than zero ($n = 8$). A two-way ANOVA was then conducted on age and sex. The two age categories with no associated variance (age classes 1 and 5) were eliminated from this analysis. The ANOVA table was constructed using the error mean square from the one-way analysis for computation of the age, sex, and interaction F-values.

Regression analyses were conducted to investigate relationships between the number of nematodes transmitted per branch, the number of nematodes carried per beetle, and the feeding area per branch; Pearson correlations were run between the number of nematodes extracted per branch and the feeding area, weight, and diameter of the branches. The Spearman rank correlation procedure was used to determine whether the number of nematodes transmitted was related to either the number of nematodes carried per beetle or the feeding area per branch. Square root transformations were used to stabilize variances associated with nematode transmission values and nematode load values. All statistical analyses were conducted using the Statistical Analysis System (11).

Results

Fifty-two caged beetles were found to be nematode free, leaving 64 nematode-carrying beetles for analysis of nematode transmission success frequencies and for calculating the mean number of nematodes transmitted during feeding. The infested beetles averaged 9,597 (± SE 1,670) nematode dauer juveniles per beetle. Individual nematode loads ranged from 42 to 63,333 nematodes per beetle. Female and male beetles carried the same mean number of nematodes ($t = 0.01$; df = 60.2; $P = 0.991$). No nematodes were extracted from the wood and bark of branches fed upon by the 52 nematode-free beetles. All nematodes found in branches fed upon by nematode-carrying beetles were therefore assumed to have been introduced during beetle feeding.

Comparison of branch section types: A mean of 27.7 ± 8.18 nematodes were extracted from the branch sections that were dissected immediately. The majority (94%) of these were extracted from the cortex. Branch sections that were stored before extraction contained 4,900 ± 932 nematodes. The majority (80%) of nematodes in these branches were collected from the woody tissue. The frequency of successful transmission was independent of branch section treatment ($\chi^2 = 0.125$; df = 1; $P = 0.72$). Nematodes were extracted from 31 of the 64 branch sections that were processed immediately and from 33 of the stored branch sections.

Frequency of successful transmission: Transmission was successful in 31 of 64 branches (Table 1). About two-thirds of the branches fed upon by female beetles contained nematodes, whereas nematodes were recovered from less than half of those fed upon by males. The frequency of successful transmission, however, was independent of beetle sex ($\chi^2 = 3.01$; df = 1; $P = 0.083$), the season of caging ($\chi^2 = 2.34$; df = 1; $P = 0.126$), and foliage category ($\chi^2 = 3.04$; df = 2; $P = 0.219$). The frequency of successful transmission was dependent upon the age of the beetle ($\chi^2 = 13.62$; df = 4; $P = 0.009$). Only 2 of 12 branch sections fed upon by 1-week-old beetles contained nematodes. The frequency of successful transmission was highest for branches fed upon by 2-week-old beetles, high for branches fed upon by beetles in age classes three and four, and lower for beetle age class five (Table 1).

Analyses of successful transmission frequencies for the stored branch sections were similar for beetle sex ($\chi^2 = 3.35$; df = 1; $P = 0.067$), season of caging ($\chi^2 = 0.23$; df = 1; $P = 0.632$), and beetle age ($\chi^2 = 17.65$; df = 4; $P = 0.001$).

Number of nematodes transmitted: Numbers of nematodes extracted from branch

TABLE 1. Frequency of successful pinewood nematode transmission to branches fed upon by *Monochamus carolinensis* beetles of differing sex and age (weeks post adult emergence) during two seasonal feeding periods.

	Frequency of transmission†
Sex	
Female	16/26
Male	15/38
Age	
1	2/12
2	7/9
3	7/11
4	11/17
5	4/15
Period	
June–July	20/35
August–September	11/29

† Frequencies (number of branch sections from which nematodes were extracted/total number of branch sections) were calculated from branch sections processed immediately after beetle feeding.

TABLE 2. Analysis of variance of nematode transmission data.

Source of variation	df	SS	F	$P > F$
Treatment	7	259.6	1.92	0.09
Sex	1	61.9	3.20	0.76
Age	2	10.8	0.28	0.08
Sex × Age	2	50.6	1.31	0.28
Error	43	832.5		

sections fed upon by beetles ranged from 0 to 314. No nematodes were extracted from 33 branch sections; 2–10 from 11 sections; 11–100 from 15 sections; and 100 or more nematodes from five branch sections. Two-thirds (66.3%) of the nematodes recovered from all 64 branch sections were isolated from the five branches containing more than 100 nematodes; thus, the introduction of nematode dauer juveniles through feeding wounds was aggregated.

There was no difference in the mean number of nematodes extracted from branches fed upon in June–July compared with those fed upon in August ($F = 0.52$; df = 1, 109; $P = 0.471$) and no difference among the foliage categories ($F = 1.52$; df = 2, 61; $P = 0.227$). Data were pooled across month of caging and foliage categories for the remaining analyses.

The number of nematodes extracted per branch was positively correlated with the number of nematodes carried per beetle ($r = 0.31$, $P = 0.01$). There was no correlation between the number of nematodes extracted per branch and the feeding area per branch ($r = 0.07$; $P = 0.59$), or the diameter ($r = 0.21$; $P = 0.09$) or weight ($r = 0.12$; $P = 0.32$) of the branch.

Analysis of sex and age treatments indicated that differences among sex–age treatment combinations were not significant, nor were differences between sexes or among ages (Table 2). Nematode transmission rates were low at both ends of the beetle age distribution and most efficient for 3-week-old and 4-week-old beetles (Table 3). A mean of 52.8 nematodes were extracted from branches fed upon by female beetles, whereas 10.6 nematodes per branch were recovered from branches fed upon by males; however, this difference was not significant (Table 4).

TABLE 3. Mean number of pinewood nematodes extracted from branches fed upon by *Monochamus carolinensis* beetles of differing sex and age.

Beetles	n	Nematodes extracted	SE
Sex			
Female	26	52.81	17.45
Male	38	10.58	5.61
Age (weeks)			
1	12	1.00	0.72
2	9	12.89	5.13
3	11	49.18	20.42
4	17	42.71	20.29
5	15	25.33	20.76

Data are from branch sections processed immediately after beetle feeding.

TABLE 4. Mean number of pinewood nematodes extracted from branches fed upon by *Monochamus carolinensis* beetles by sex-age treatment combination.

Beetles				
Age	Sex	n	Nematodes extracted	Variance
1	Female	4	0.00	0
	Male	8	1.50	9
2	Female	3	5.00	338
	Male	7	6.57	50
3	Female	5	45.60*	3,677
	Male	6	52.17*	6,708
4	Female	9	77.22*	11,097
	Male	8	3.88	56
5	Female	6	63.33*	15,212
	Male	9	0.00	0

Data are from branch sections processed immediately after beetle feeding.

Means followed by an asterisk (*) differed significantly from 0 ($P = 0.05$) according to a *t*-test. Raw means reported above, statistical differences based on square root transformed data.

DISCUSSION

Transmission of pinewood nematode dauer juveniles through insect feeding wounds is an essential component in the epidemiology of pine wilt disease. Most pinewood nematode transmission studies have used potential insect vectors placed on pine seedlings or excised pine branches in the laboratory or greenhouse. Transmission studies that use mature, healthy pines under field conditions are few. Transmission of pinewood nematode dauer juveniles through feeding wounds under such conditions has been documented for two species of insect vectors in the genus *Monochamus*: *M. alternatus* Hope on Japanese black pine (*P. thunbergii* Parl.) in Japan and *M. titillator* (Fabricius) on slash pine (*P. elliottii* Engelm.) in Florida (4). The present study is the first to document the frequency of successful transmission and number of nematodes transmitted through *M. carolinensis* feeding wounds on mature, field-grown pines. Transmission through *M. carolinensis* feeding wounds had been documented previously on Scots pine seedlings (2,12) and five other species of pine seedlings (12). Results of previous transmission studies were summarized recently (4).

Forty-nine percent of the branches fed upon by nematode-carrying beetles contained nematodes. Transmission success tended to be higher for female beetles than for males, although the difference was not significant. No differences in transmission frequencies between the sexes of *Monochamus* spp. has been reported previously. Enda (1) reported 100% successful transmission to 9-year-old and 20-year-old Japanese black pines fed upon by *M. alternatus*; however, the feeding period was 40 days compared with 24 hours in the present study. Luzzi et al. (6) reported successful transmission to four of seven slash pine after *M. titillator* feeding for 4 weeks.

Successful transmission was 16.7, 77.8, 63.6, 64.7, and 26.7% for age classes one through five, respectively. Mineo and Kontani (10) found the frequency of successful transmission was highest for 2–3-week-old *M. alternatus* on Japanese black pine. Numbers of nematodes recovered from branches fed upon by nematode-carrying *M. carolinensis* and processed immediately ranged from 0 to 314. This was the number of nematodes extracted from one-half of each branch that was fed upon in the study. The range of nematodes transmitted during the 24-hour feeding period may have been greater had both branch sections been processed immediately. In studies on transmission by *M. alternatus*, a maximum of 374 nematodes was transmitted during a 24-hour feeding period (8) and 654 during 48 hours (9).

Relationships between the numbers of nematodes transmitted and the independent variables for which data were collected were difficult to establish because of the high variance associated with the dependent variable. No relationship was found between the transmission values and the feeding area on each branch. Similar results have been reported by Mineo (9). Transmission rates were correlated with the number of nematodes carried by the beetles during feeding. This relationship is similar to that found for *M. alternatus* (15). Transmission rates were also found to be independent of branch characteristics such as amount of foliage, branch section weight, and branch section diameter.

Most nematodes extracted from the branch section dissected immediately after the feeding trial were located in the cortex. Nematodes extracted from the stored branch sections were found primarily in the xylem. Tamura (14) reported that most dauer juveniles inoculated on artificial wounds on Japanese black pine remained in the resin on the wound surface for 2–4 days, during which time molting to the adult stage occurred, and then moved into the cortex.

The results of the present study illustrate patterns of pinewood nematode transmission through feeding wounds of *M. carolinensis*. The rates of successful transmission and the mean number of nematodes transmitted provide an insight into the role of this beetle in the epidemiology of pine wilt disease. Caution must be used, however, in relating these results to transmission rates under normal field conditions. In the present study, beetles were caged on Scots pine branches for 24 hours. This resulted in feeding wound densities higher than those observed on trees within the same plantation which were fed upon by the local *M. carolinensis* population. The feeding behavior of *M. carolinensis* is poorly understood. Neither the area of a typical feeding wound nor the time involved in its construction has been determined. The transmission rates reported here must be interpreted as a 24-hour feeding event. Additional research is necessary to relate these results to actual transmission rates that occur in nature.

Ten or fewer nematodes were extracted from 44 of the 64 branch sections analyzed in this study. Only five branch sections had more than 100 nematodes. Hashimoto and Sanui (15) demonstrated that as few as 300 pinewood nematode dauer juveniles could kill a young Japanese black pine. No data exist for Scots pine grown in North America. Results of this study suggest that pinewood nematode infection of healthy pines by *M. carolinensis* is the result of the introduction of a small number of nematodes through a feeding wound or series of feeding wounds distributed throughout the crown of the tree, rather than a single inoculation of many nematodes. This should be considered in future studies on host tree susceptibility.

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