Relationship of *Bursaphelenchus xylophilus* Population Density to Mortality of *Pinus sylvestris*[1]

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Abstract: Seven-month-old Scots pine seedlings were inoculated with water or culture filtrate (controls), with 10,000, or 20,000 (experiment 1), and with 2,500 (experiment 2) *Bursaphelenchus xylophilus* B.C. isolate nematodes and maintained under defined experimental conditions. Controls did not develop pine wilt disease over a 2-month period. In experiment 1, less than 50% of the inoculum was recovered from the nematode-inoculated seedlings in the first 48 hours, after which the nematode population of both treatments increased exponentially resulting in pine death and approximately equal populations at 216 hours after inoculation. In the second experiment, plant mortality, which was always preceded by 2–3 days of chlorosis and associated stem vascular necrosis, first occurred 14 days after inoculation. The nematode population increased until about day 40 after inoculation and declined thereafter. Nematodes extracted from the roots 2 weeks after inoculation accounted for ca.15% of the total number of nematodes per pine. The study indicates that the rate of nematode reproduction is a factor in pine wilt disease. However, the lack of a linear correlation between the number of nematodes and the timing of pine mortality suggests that the timing of pine death may also depend on the location of nematode damage to the host tissue.
  Key words: *Bursaphelenchus xylophilus*, damage threshold, pinewood nematode, pine mortality, pine wilt disease, *Pinus sylvestris*, population dynamics, Scots pine.

Bursaphelenchus xylophilus (Steiner and Buhrer) Nickle, causal agent of pine wilt disease, is one of the few plant-parasitic nematodes that kill a mature host plant within a relatively short period after penetration (1). Under field conditions, bark beetles of the genus *Monochamus* are the primary vectors of *B. xylophilus* (14). Temperature affects nematode reproduction (7), and the number of nematodes from the initial inoculum that become established in host tissue influences disease expression. Although as many as 289,000 *B. xylophilus* per adult *M. alternatus* have been reported (14), the number of nematodes carried per beetle varies enormously with host plant and locality (19) and only 10–20% of the nematodes exit from the vectors (16). Under experimental conditions, inocula of as many as 200,000 (13) and as few as 100 (4) nematodes per plant have been used, but a common range seems to be 10,000 (9) to 40,000 (2) nematodes per plant.

When pines (*Pinus* spp.) are simultaneously inoculated with similar numbers of *B. xylophilus* under similar experimental conditions, pine death occurs over a period ranging from 2 weeks to several months after inoculation (2,6,12,15,17). Hence, the relationship between the number of nematodes in the plant and the timing of pine death is unclear. In order to better understand the mechanisms of nematode-induced pine wilt, the size of the nematode population at the time of pine death should be determined under a range of specified experimental conditions. The objectives of this paper are to show 1) the percentage of the nematode inoculum that becomes established in the host and 2) the time until death and nematode population level at pine mortality under specified experimental conditions.

MATERIALS AND METHODS

Seven-month-old Scots pine, *Pinus sylvestris* L., seedlings were grown in a 50:50 peat : vermiculite mixture in blocks of styrofoam seedling cavities (4 cm d × 15 cm deep). The experimental conditions were 30 C, 75% RH at 400 μE m^{-2} s^{-1} and 16-hour day and 25 C, 80% RH 8-hour night cycle in a Conviron growth chamber. Plants were watered daily with tap water.

A British Columbia isolate of *B. xylophi-*

lus was maintained under sterile conditions on potato dextrose agar on which a non-sporulating *Botrytis cinerea* was growing at 27 C. When required for experiments, nematodes were washed off the lids of 1-week-old culture plates under sterile conditions and aqueous nematode suspensions were concentrated to the desired volumes. Culture filtrates were collected similarly by washing the lids of the petri dish cultures and the suspensions were filtered under vacuum through 5-μm-pore nylon sieve. Scots pines, 21.8 ± 3.3 cm tall, were inoculated by pipetting the required number of nematodes in aqueous suspension into a 1-cm long vertical slit in the bark 11–12 cm from the base of the stem, and covering the wound with nonabsorbent poly filter wool (Rolf C. Hagen Inc., Canada) and parafilm immediately after inoculation. The inoculated seedlings were maintained under the prescribed experimental conditions; in order to obtain a uniform infection time, the poly filter wool and parafilm covers were removed 48 hours after inoculation.

Pines were divided into groups of 40 (experiment 1), 36 (experiment 2), and 20 (controls). In experiment 1, the 40 seedlings were divided into two equal groups and each seedling was inoculated with an estimated suspension of either 10,000 (low) or 20,000 (high) *B. xylophilus*. Four plants from each of the high and low nematode treatments were harvested at 6, 18, 48, 96, and 216 hours after inoculation, the total fresh plant weight was measured, and the numbers of nematodes in the shoot and root of each plant were determined after extracting nematodes separately from top and roots of pine seedlings on Baermann funnels for 3 days.

In experiment 2, each of the 36 seedlings was inoculated with an aqueous suspension of an estimated 2,500 *B. xylophilus*. The times of appearance of chlorosis and wilting, of seedling death, and of stem tissue necrosis were visually assessed and recorded. Four randomly selected pines were harvested and the shoot and root fresh weights and the number of nematodes in them were

recorded for each pine at 3, 7, and 11 days after inoculation. At 14, 18, 28, 53, and 68 days after inoculation, dead and dying pines were harvested and processed as described. A pine seedling was considered dead when stem wilt occurred together with either needle chlorosis or needle wilt.

Half of the controls received wound and water alone; the other half received wound and culture filtrate. They were kept under the same experimental conditions as in experiment 1 for 9 weeks after which all plants were weighed and processed on the Baermann funnel.

Data were analyzed using regression and (or) the following model:

$$y = b_1 + b_2X - b_3X^2$$

where y = the number of nematodes recovered per plant or per gram of plant weight, X = days after inoculation, and b_1, b_2, and b_3 are coefficients describing the shape of the curves (20).

RESULTS

In the first experiment, about 50% of the nematode inoculum was recovered from each plant 6 hours after inoculation, and the number of nematodes extracted from each plant decreased with time during the following 42 hours (Fig. 1). Thereafter, the population per plant increased exponentially up to approximately equal levels in the two treatments at 216 hours post infection, reaching the initial inoculum levels at 120 (Pi = 10,000) and 160 (Pi = 20,000) hours after inoculation. The population increased faster in seedlings inoculated with 10,000 than with 20,000 nematodes (Fig. 1). At 216 hours after inoculation, 10.2% and 9.6% of the total number of nematodes per plant in the low and high inoculum treatments, respectively, were recovered from the roots (Fig. 1). By that time all pines from both inoculum levels showed wilt and stem vascular destruction.

In the second experiment, the nematode population per plant increased to an estimated 60,000 at about day 40 and then declined by day 68 to ca. 12,500 (Fig. 2).
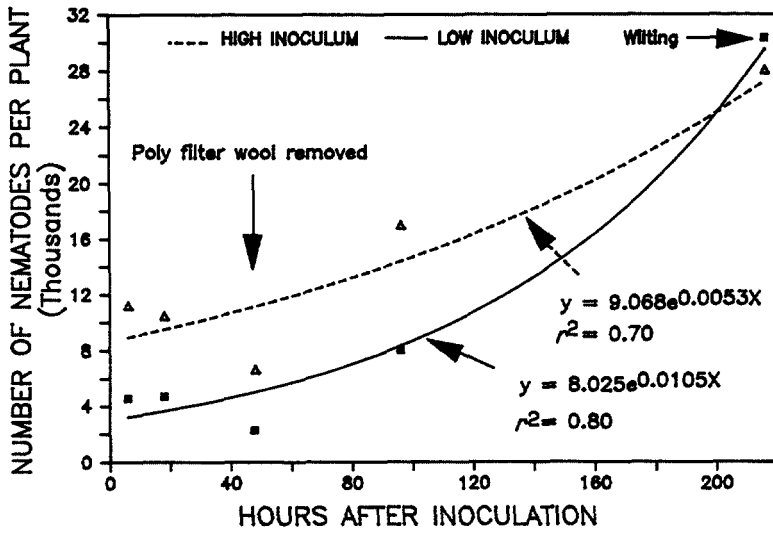
FIG. 1. Population density curves for *Bursaphelenchus xylophilus* in 7-month-old Scots pines inoculated with 10,000 (low) or 20,000 (high) nematodes per plant. Each data point represents a mean of four replicates. Removal of the poly filter wool cover from the point of inoculation and occurrence of wilt are shown.

The number of nematodes did not increase to a level significantly greater than the inoculum until 11 days after inoculation. The first disease symptom, chlorosis, occurred 2–3 days before pine death. After 14 days, all dead pines were examined on each sampling day; i.e., four pines at 14 days followed by four at 18, three at 28, four at 53, and two at 68 days after inoculation (Fig. 3).

The nematodes were mostly in the shoot tissue but from about 14 days after inoculation nematodes also were found in the roots (Fig. 4). Thereafter, ca. 15% of the
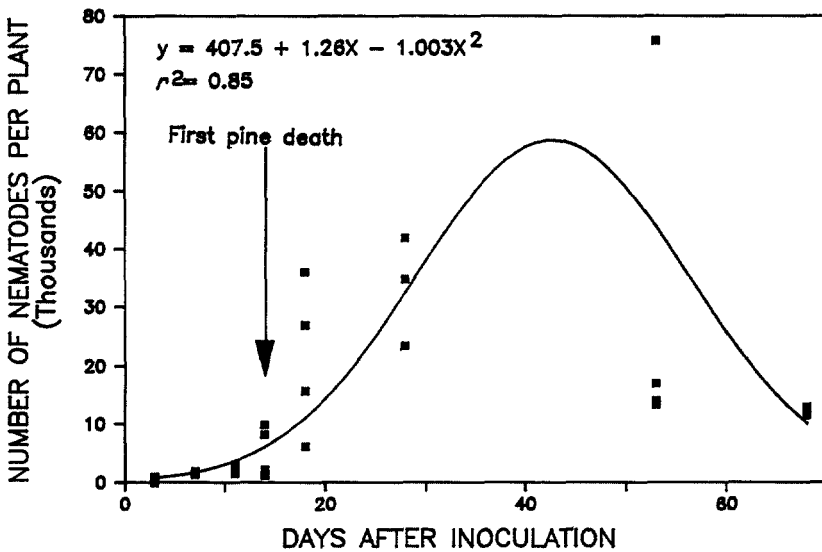

FIG. 2. Population density curves for *Bursaphelenchus xylophilus* in 7-month-old Scots pines inoculated with 2,500 nematodes. Date of first pine death is shown. Each sampling day has four data points except days 28 and 68 after inoculation, which have three and two data points, respectively.
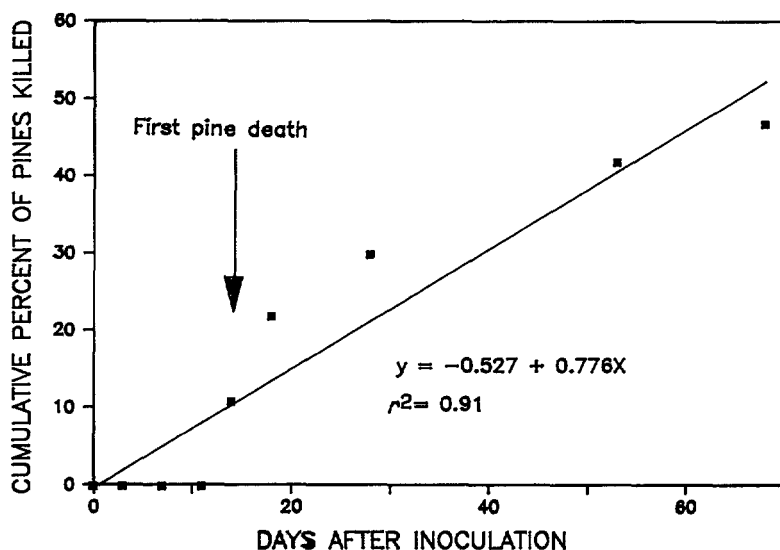
FIG. 3. Cumulative percentage mortality of 7-month-old Scots pines on each sampling date over a period of 68 days after inoculation with 2,500 *Bursaphelenchus xylophilus*. Each data point represents four pines, except days 28 and 68, which are three and two, respectively.

total number of nematodes per plant (Fig. 2) were extracted from the roots.

No nematodes were found in the water and culture filtrate control plants, and none of these plants showed disease symptoms after 2 months.

DISCUSSION

The population of *B. xylophilus* in pine seedlings declined during the first 48 hours, as less than 50% of the inoculum became established in the plant tissues, and then increased significantly as the nematodes reproduced (Fig. 1). Whereas the exponential increase in the population during the 9 days (Fig. 1) reflects the reproductive potential of *B. xylophilus*, the faster nematode population build up in plants with the lower inoculum suggests a possible density-dependent factor influencing the nematode reproduction rate. Despite the different initial infection levels of the first experiment, the nematode population in plants from both treatments reproduced to similar levels and caused pine wilt at about the same time (Fig. 1). Differences in reproductive rate of nematode populations arising from different sized inocula make prediction of host response difficult and may

explain the lack of relationship between inoculum level and host response reported by Bolla et al. (5).

Although infected pines died in both experiments (Figs. 1, 2), the pines differed in symptom expression. Stem tissue necrosis was evident in dead or wilting plants from both experiments. Chlorosis, which always preceded pine death by about 2–3 days, occurred in only the second experiment where pines received a much smaller inoculum than in the first experiment (Fig. 2). The number of nematodes per plant did not increase significantly above the level of inoculum until about the time chlorosis first appeared (Fig. 2). This suggests that when Pi = 2,500, the nematode population built up sufficiently slowly to allow the physiological effects on the host to be manifest in changes in needle cell structure resulting in chlorosis before permanent wilt occurred. When Pi = 10,000 to 20,000, the large nematode population per plant quickly destroyed the stem vascular tissue resulting in shoot wilt before needle chlorosis occurred.

Bursaphelenchus xylophilus migrates 40–50 cm per day within tree tissues (11,18) and the extent of tissue destruction prob-
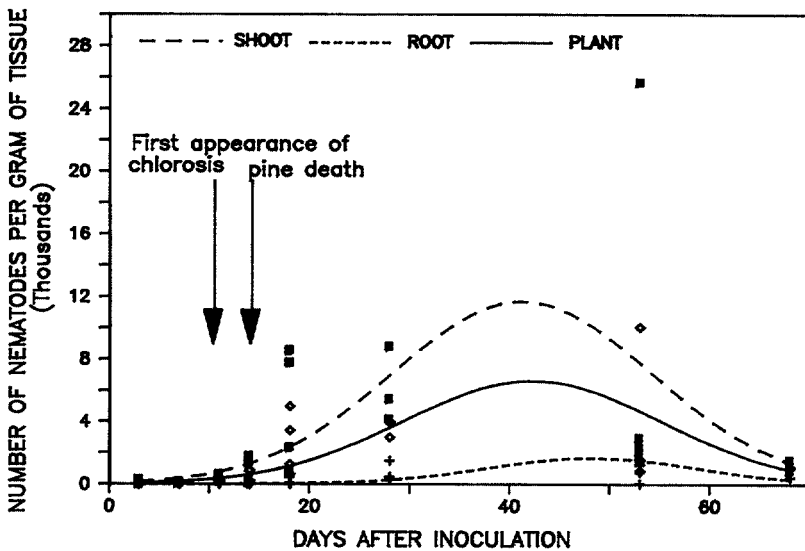
FIG. 4. Population density of *Bursaphelenchus xylophilus* per gram fresh weight of the shoot, root, and total plant over 68 days after the inoculation of 7-month-old Scots pine with 2,500 nematodes. Shoot = 76.7 + 1.28X − 1.003X², *r*² = 0.86. Root = −19.3 + 1.537X − 1.005X², *r*² = 0.85. Plant = 33.45 + 1.29X − 1.003X², *r*² = 0.90. Each sampling day has four data points, except days 28 and 68 after inoculation, which have three and two data points, respectively.

ably is proportional to spread of the nematode. Our study showed that a higher proportion of the nematodes was found in the shoot than in the root in both experiments and nematodes did not migrate to the roots until disease symptoms appeared in the stems and needles (Figs. 1, 4). This suggests that the nematodes migrate to the roots as shoot destruction intensifies, probably in search of nutrients or a less toxic environment.

The number of *B. xylophilus* that cause pine death varies with host type and experimental conditions. For example, Dwinel (8) reported a high correlation between the number of *B. xylophilus* and the mortality of five pine species 12 weeks after inoculation, whereas Bolla et al. (5) found no correlation between nematode population and the mortality of three pine species over a similar duration. There does not seem to be a correlation within a species between the number of nematodes, host age, and time of pine mortality. For example, Bedker and Blanchette (2) and Bedker et al. (3) showed death of 13-year-old and 11-year-old Scots pines with 224.5 and

13.8 nematodes per gram of wood at 50 and 58 weeks after inoculation, respectively, whereas in our study it took about 1,000–5,000 nematodes per gram of plant fresh weight to kill 7-month-old pines (Fig. 4). In addition to the difference in host age and differences in the experimental conditions, the uneven distribution of *B. xylophilus* within trees (10) may contribute to the difference in number required to cause pine mortality.

The present study suggests a possible correlation between the number of nematodes and pine mortality early in the infection period (Figs. 1, 2). However, the linear increase in percentage of pine mortality with time of infection (Fig. 3) without a corresponding linear increase in nematode population in pines (Fig. 2) suggests that there is some additional factor, such as the number and location of nematode-damaged host cells, contributing to the timing of pine death.

LITERATURE CITED

1. Bedker, P. J. 1987. Assessing pathogenicity of the pinewood nematode. Pp. 14–25 *in* M. J. Wing-

field, ed. Pathogenicity of the pinewood nematode. St. Paul, MN: The American Phytopathological Society Press.

2. Bedker, P. J., and R. A. Blanchette. 1988. Mortality of Scots pine following inoculation with the pinewood nematode, *Bursaphelenchus xylophilus*. Canadian Journal of Forest Research 18:574–580.

3. Bedker, P. J., M. J. Wingfield, and R. A. Blanchette. 1986. Pathogenicity of *Bursaphelenchus xylophilus* on three species of pine. Canadian Journal of Forest Research 17:51–57.

4. Bergdahl, D. R., and D. L. K. Smeltser. 1981. Histopathological observations of *Bursaphelenchus xylophilus* in symptomatic tissues of *Larix laricina* and *Pinus resinosa*. Phytolathology 72:257 (Abstr.).

5. Bolla, R. I., R. E. K. Winter, and M. J. Linit. 1986. Pathotypes of the pinewood nematode *Bursaphelenchus xylophilus*. Journal of Nematology 18:230–238.

6. Burnes, T. A., M. J. Wingfield, F. A. Baker, K. Knowles, and Y. Beaubien. 1985. The pine wood nematode in Jack pine infected with dwarf mistletoe. Forest Science 31:866–870.

7. Dwinel, L. D. 1986. Ecology of the pinewood nematode in southern pine chip piles. Research Paper SE-258, USDA Forest Service, Southern Forest Experimental Station, Asheville, NC.

8. Dwinel, L. D. 1985. Relative susceptibility of five pine species to three populations of the pinewood nematode. Plant Disease 69:440–442.

9. Futai, K. 1980. Population dynamics of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae) and *B. mucronatus* in pine seedlings. Applied Entomology and Zoology 15:458–464.

10. Harman, A. L., L. R. Krusberg, and W. R. Nickle. 1986. Pinewood nematode, *Bursaphelenchus xylophilus*, associated with red pine, *Pinus resinosa*, in western Maryland. Journal of Nematology 18:575–580.

11. Hashimoto, H., and T. Kiyohara. 1973. Migration of the pine wood nematode in a pine tree (III). 84th Transactions of Meeting of Japanese Forest Society. Pp. 330–332. (In Japanese.)

12. Ishibashi, N., and E. Kondo. 1977. Occurrence and survival of the dispersal forms of pine wood nematode, *Bursaphelenchus lignicolus* Mamiya and Kiyohara. Applied Entomology and Zoology 12:293–302.

13. Kondo, E., A. Foudin, M. J. Linit, R. Bolla, R. Winter, and V. Dropkin. 1982. Pine wilt disease—nematological, entomological and biochemical investigations. Bulletin SR 282, University of Missouri, Columbia.

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

15. Linit, M. J., and H. Tamura. 1987. Relative susceptibility of four pine species to infection by pinewood nematode. Journal of Nematology 19:44–50.

16. Mamiya, Y. 1984. The pinewood nematode. Pp. 589–626 *in* W. R. Nickle, ed. Plant and insect nematodes. New York: Marcel Dekker.

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

18. Terashita, T. 1977. Migration of the pine wood nematode immediately after entering pine wood tissues. Japanese Ministry of Agriculture, Forest and Fisheries Research Report 96:40–42. (In Japanese.)

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

20. Zar, J. H. 1974. Biostatistical analysis. Englewood Cliffs, NJ: Prentice-Hall.