Journal of Nematology 23(4):477–484. 1991.

Incubation Temperature and Time Effects on Life Stages of *Bursaphelenchus xylophilus* in Wood Chips[1]

J. Tomminen,[2] S. Halik,[3] and D. R. Bergdahl[3]

Abstract: Wood chips of *Pinus strobus* inoculated with *Bursaphelenchus xylophilus* were incubated at 3, 12, 30, or 40 C during intervals of 47, 82, and 130 days to determine the effects of incubation temperature and time on total number of nematodes and occurrence of each life stage. Nematodes did not survive at 40 C; the greatest number of nematodes was maintained at 3 C. The number and percentage of juveniles in the propagative cycle were greatest at 3 C after 47 days, but the percentage was greatest at 30 C after 130 days. More third-stage dispersal larvae, with percentages as high as 85%, were extracted at 3 and 12 C than at 30 C by the end of the study. Dauer larvae were extracted from the chips, but percentages never exceeded 5%. The percentage of adults was greater at 30 C than at 3 and 12 C after 82 and 130 days. When a 1-week heat treatment at 30 C was applied to samples at 3 and 12 C, numbers and percentages of adults increased. Percentages of dauer larvae increased very slightly when the heat treatment was applied after 47 days, but numbers and percentages of juveniles and dispersals were affected erratically.

Key words: Bursaphelenchus xylophilus, dauer larva, dispersal larva, nematode, pinewood nematode, temperature, wood chip.

In 1984, Finland placed an embargo against the importation of certain coniferous wood products from areas of the world where the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer 1934) Nickle 1970, is known to occur (17). Included in such materials is pulpwood in the form of chips and particles (Rautapää, pers. comm.). The embargo was initiated after the Finnish Plant Quarantine Service discovered *B. xylophilus* in wood chips imported from the United States and Canada (17). Later, this action was followed by Sweden and Norway.

In northern Europe, the only native pine species of economic and aesthetic importance is Scots pine (*Pinus sylvestris* L.). The PWN has been found to be very pathogenic to this exotic pine in the midwestern United States (1,9,10). Because of the possible threat to native stands, Nordic countries are concerned about transmission of the PWN from imported wood chip piles to surrounding forests.

In the natural forest ecosystem, *B. xylophilus* is most commonly vectored as a specialized dauer larval stage by species of cerambycid beetles of the genus *Monochamus* (6–8,13,15). Nematodes are vectored to recently dead or dying trees during beetle oviposition or to healthy pine branches during beetle maturation feeding (23,24). Dauer larvae move onto the wounds and invade bark and xylem tissues where they molt to adults (13) and feed on fungi in dead and dying trees (24) and on host cells in living trees (11,13,19,20).

While conditions remain favorable, PWN populations persist in a propagative cycle of four larval stages and adults. As ambient temperatures and host tree moisture decline, second-stage larvae molt to specialized third-stage dispersal larvae adapted to adverse conditions (5,11,12). The majority of the nematode population exists in this form from winter until spring, when dispersals are attracted to beetle pupal chambers where they molt to dauer larvae before transmission to another host (7,13).

Dauer larval formation in wood chips may be crucial for nematode dispersal.

Received for publication 20 July 1990.

[1] This research was supported in part by the Academy of Finland, the Research Foundation of Finnish Natural Resources, the USDA Cooperative State Research Service, McIntire-Stennis Research Program, and the University of Vermont.

[2] Visiting post-graduate student, School of Natural Resources, University of Vermont, Burlington, VT 05405. Current address: Department of Agricultural and Forest Zoology, University of Helsinki, SF-00710 Helsinki, Finland.

[3] Technician and Professor, School of Natural Resources, University of Vermont, Burlington, VT 05405.

We thank Drs. Erkki Annila from the Finnish Forest Research Institute and Matti Nuorteva from the University of Helsinki for reviewing the manuscript. We also thank Dr. Juha Helenius from the University of Helsinki for assistance with statistical analyses.

Dwinell (3) studied population development of *B. xylophilus* in wood chips maintained at 25–40 C and recovered only adults and juveniles of the reproductive cycle. Tomminen et al. (22), however, reported the presence of dauer larvae in wood chips. Thus, a more thorough investigation of the factors involved in regulating formation of various life stages of the PWN is necessary. The main objective of this study was to determine the effects of incubation temperature and time on occurrence of different life stages of the PWN in wood chips.

Materials and Methods

Two hundred grams (fresh weight) of eastern white pine (*P. strobus* L.) wood chips were placed in each of 125 sealable plastic bags (23 × 15 cm). The bags were each inoculated with an aqueous suspension containing 1,000 nematodes of isolate 16E of the PWN from white pine in Vermont. All bags were incubated at 27 C for 25 days, at which time five bags were used for nematode extractions in modified Baermann funnels (18) for 48 hours. Samples of extracted nematodes were counted and the total populations per bag were estimated. Subsamples of counted nematodes were evaluated for the percentage of each of four life stages: juveniles, dispersals, dauer larvae, and adults as previously described (7,14,16).

The remaining 120 bags were randomly divided into 24 treatments with five replicates. Treatments consisted of four temperatures (3, 12, 30, or 40 C) and three incubation times (47, 82, or 130 days). For treatments at 12 and 3 C, temperatures were gradually reduced to 20 C for 7 days, then to 12 C either for the remainder of the incubation time or for 15 days followed by incubation at 3 C for the remainder of the treatment. A 1-week heat treatment at 30 C was applied to half the bags at the end of each incubation time.

At the conclusion of each treatment, nematodes were extracted from the chips and counted, and the number and percentage of each life stage were estimated. Wood chips were weighed and oven dried at 98 C, and moisture content was calculated on the basis of oven dry weight.

Two-way analyses of variance and pairwise *t*-tests were used to determine the extent to which temperature and incubation time influenced total number of nematodes and numbers and percentages of each life stage, using only data from chips not receiving the extra 1-week incubation at 30 C. Before analysis, total number of nematodes and numbers of each life stage were $\log_{10}$-transformed, following addition of a constant 0.001 to each observation. Percentages of each life stage were modified with an arcsin transformation. When variances between treatment groups under comparison were not equal, Brown-Forsythe analysis of variance was used. One-way analysis of variance was used to determine the impact of the extra 1-week heat treatment on total nematode numbers and numbers and percentages of each life stage. The Spearman rank correlation was used to test the correlations of wood moisture content to total number of nematodes and percentage of each life stage (2).

Results

The initial five bags used for extraction contained 48% (± SD 3) juveniles, 12% (± SD 4) dispersals, 1% (± SD 1) dauer larvae, and 39% (± SD 8) adults. Mean nematode population level per bag was 3,650 (± SD 1,175). Because all nematodes in the 40-C treatment died during the first 47 days, this treatment was excluded from analysis.

Numbers of juveniles were affected ($P < 0.001$) by temperature and incubation time (Fig. 1A–C). More ($P < 0.001$) juveniles were extracted from wood chips incubated at 3 C than at 30 C after 47 and 82 days. At 30 C, there were more ($P < 0.001$) juveniles after 130 days than after 82 days.

Variation in percentages of juveniles was affected ($P < 0.001$) by time and an interaction between temperature and time (Fig. 2A–C). There was a greater ($P < 0.001$) percentage of juveniles at 3 and 12 C after 47 (40 and 44%) days than after both 82 (14 and 21%) and 130 days (13 and 12%). After 47 days, the percentages of juveniles

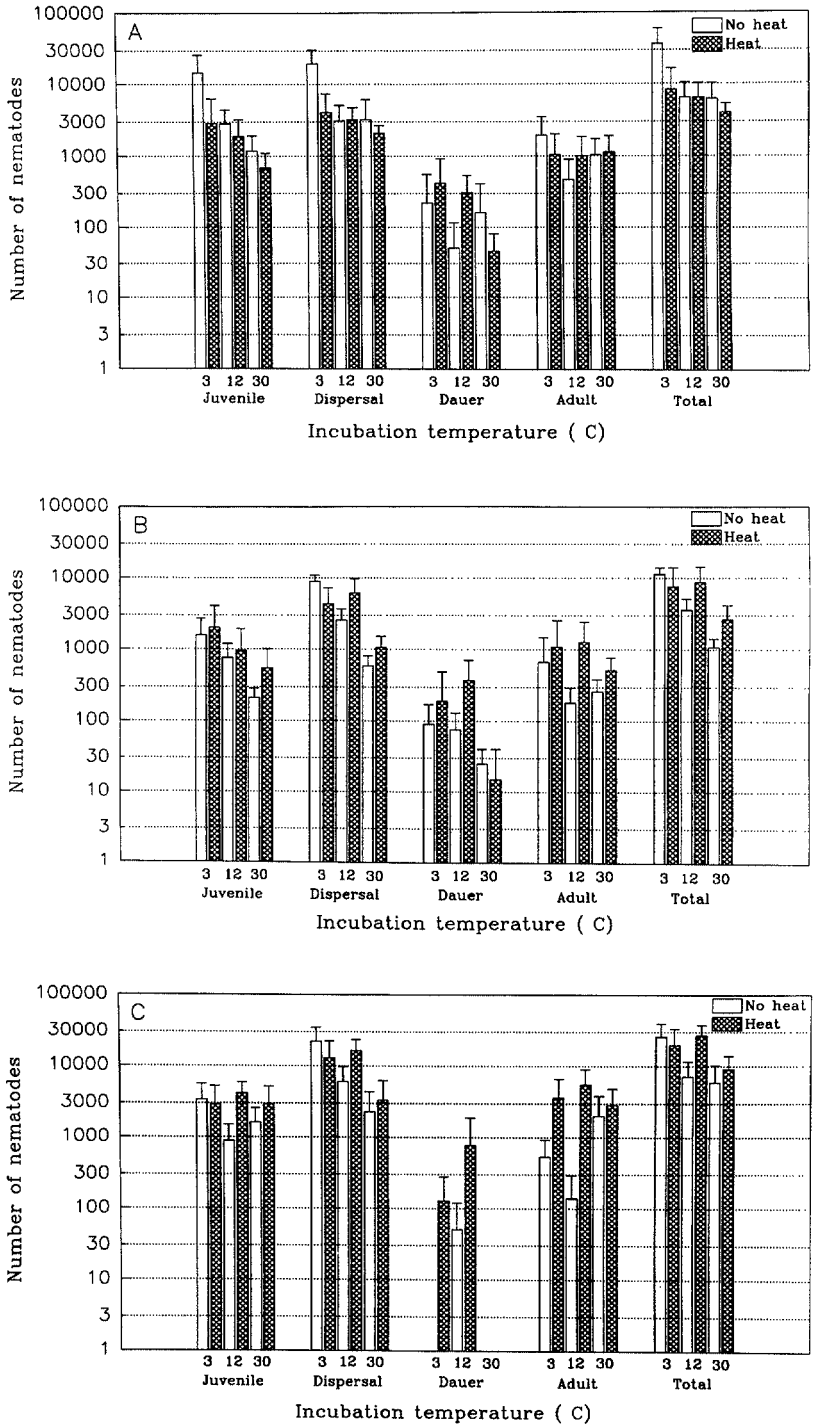

FIG. 1. Effects of incubation temperature (3, 12, and 30 C), time, and a final 1-week heat treatment at 30 C on the total number of nematodes and the numbers of each life stage of *Bursaphelenchus xylophilus* in 200 g of inoculated wood chips of *Pinus strobus*. A) 47 days. B) 82 days. C) 130 days. Number of nematodes represented logarithmically. Error bars indicate SD.

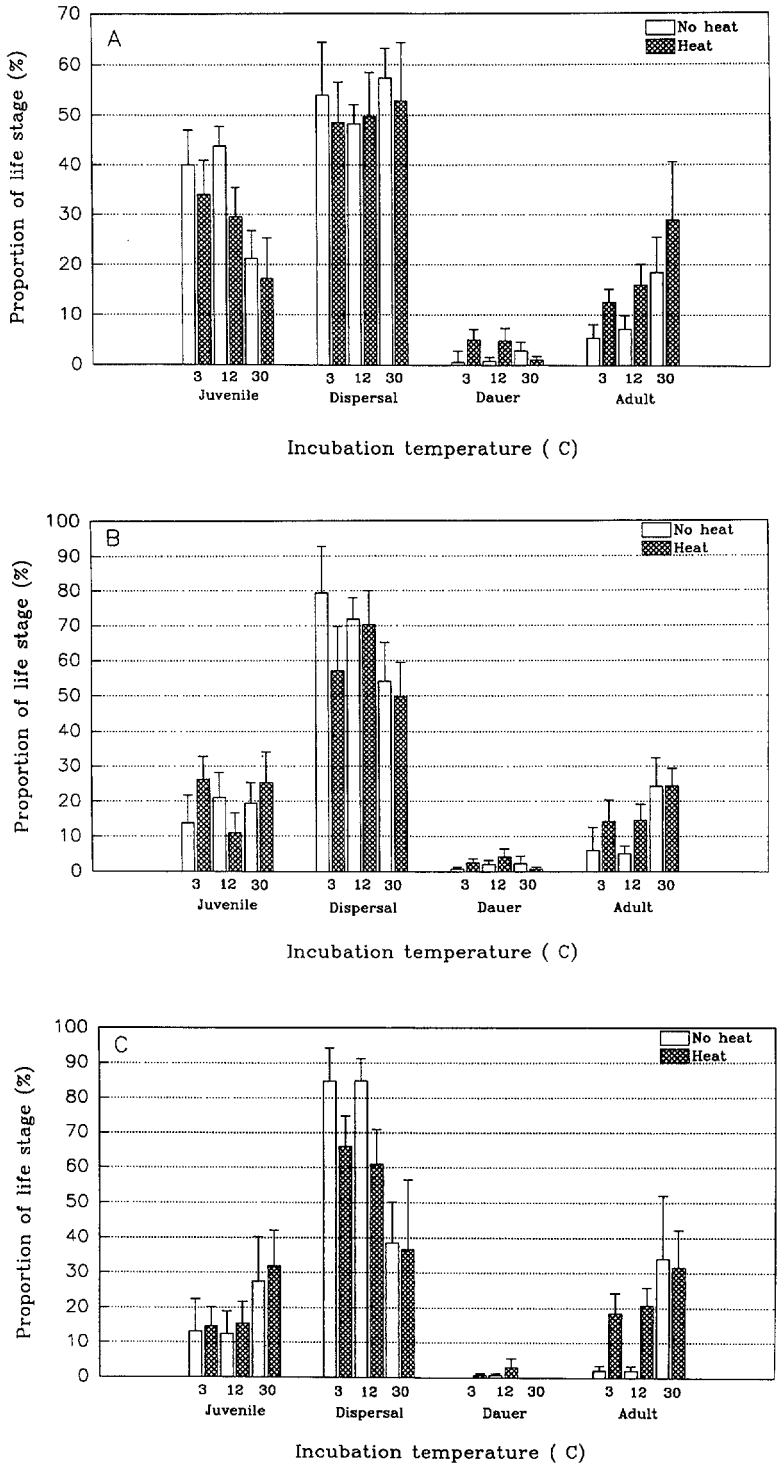

FIG. 2. Effects of incubation temperature (3, 12, and 30 C), time, and a final 1-week heat treatment at 30 C on the percentage of each life stage of *Bursaphelenchus xylophilus* in 200 g of inoculated wood chips of *Pinus strobus*. A) 47 days. B) 82 days. C) 130 days. Error bars indicate SD.

were greater ($P < 0.001$) at 3 and 12 C than at 30 C; but after 130 days, the percentage was greater ($P < 0.001$) at 30 C than at the lower temperatures.

The extra 1-week heat treatment following 47 days at the 3-C treatment resulted in a fivefold decrease ($P < 0.05$) in number of juveniles (Fig. 1A). Following 130 days at the 12-C treatment, the extra 1-week incubation resulted in a fivefold increase ($P < 0.05$) in number of juveniles (Fig. 1C). The percentages of juveniles decreased ($P < 0.05$) after 47 and 82 days at 12 C followed by the heat treatment (Fig. 2A, B).

Numbers and percentages of dispersals were affected ($P < 0.05$) by temperature, time, and an interaction of the two variables (Figs. 1, 2). Greater ($P < 0.001$) numbers and percentages of dispersals were extracted from chips incubated at 3 and 12 C than from chips maintained at 30 C by the end of the study. There were greater ($P = 0.001$) percentages of dispersals at 3 and 12 C after 130 days (85%) than after 47 days (50%).

The 1-week heat treatment applied to chips maintained at 3 C for 47 and 82 days resulted in a decrease ($P < 0.05$) in numbers of dispersals (Fig. 1A, B). When chips at 12 C received the heat treatment after 130 days, the number of dispersals increased ($P < 0.05$) (Fig. 1C). As a result of the heat treatment, percentages of dispersals decreased ($P < 0.05$) at the two lower temperatures after 130 days (Fig. 2C).

Dauer larvae were present in almost all samples that did not receive the extra heat treatment but remained less than 3% of the population (Fig. 2). When chips at 3 and 12 C received the extra heat treatment after 47 days, there was an increase ($P < 0.05$) in percentages of dauer larvae to 5% (Fig. 2A).

There were no differences in numbers of adults among treatments, but percentages varied ($P < 0.001$) among the three temperatures at 82 and 130 days (Fig. 2B, C). A greater ($P < 0.001$) percentage of adults was extracted from chips at 30 C than from chips at 3 and 12 C.

The heat treatment applied after 82 and 130 days resulted in an increase ($P < 0.05$) in numbers of adults in the 12-C treatment (Fig. 1B, C). Percentages of adults increased ($P < 0.01$) in the 12-C treatments when heat was applied after each incubation time and in the 3-C treatments after 47 and 130 days (Fig. 2).

TABLE 1. Mean moisture content (based on oven dry weights) of *Pinus strobus* wood chips inoculated with *Bursaphelenchus xylophilus* and maintained at three temperatures for three incubation times.

Temperature treatment (C)	Incubation time (days)	Mean moisture content (%)	(SD)
3	47	126	(1)
	82	126	(1)
	130	125	(1)
12	47	125	(1)
	82	122	(1)
	130	120	(1)
30	47	119	(1)
	82	110	(1)
	130	100	(2)

Total number of nematodes was affected ($P < 0.01$) by temperature (Fig. 1). Over all incubation times, there was a greater ($P < 0.05$) number of nematodes at 3 C (24,460 ± SD 18,030) than at 12 (5,730 ± SD 3,630) or 30 C (4,390 ± SD 4,010). As a result of the heat treatment, the total number of nematodes decreased ($P < 0.05$) in the 3-C treatment after 47 days and increased ($P < 0.01$) in the 12-C treatment after 130 days (Fig. 1A, C).

A loss of wood moisture content was observed throughout the duration of the study; at 30 C, mean wood moisture content was 119% after 47 days and 100% after 130 days (Table 1). The percentage of dispersals was negatively correlated ($r = -0.53$) with wood moisture content. The proportion of adults ($r = 0.45$) and total number of nematodes ($r = 0.61$) were positively correlated with moisture. There was also a strong negative correlation ($r = -0.90$) between temperature and moisture content of the wood.

DISCUSSION

The PWN in our study did not survive in wood chips incubated at 40 C. This is in

accordance with the observation (12) that PWN reproduction on *Botrytis cinerea* Pers. was inhibited at temperatures above 33 C. Dwinell (3) found 40 C supported reproduction by a southern pine isolate of *B. xylophilus* in southern pine wood chips; he also isolated from the chips thermotolerant fungi that may have served as food sources for the nematode. In our study, however, fungal composition was not evaluated. Halik and Bergdahl (4) did not isolate thermotolerant fungi from white pine chips incubated at 30 C; however, this temperature may not be conducive to development of these fungi (3). Also, Dwinell (3) observed that *B. xylophilus* did not survive in an anaerobic environment at 38 C. Thus, an alternative explanation for nematode mortality in our study may have been existence of near anaerobic conditions within the plastic bags at 40 C.

The high percentages of juveniles and adults and the low percentages of dispersals present in the wood chips before the start of the study indicate the environment was favorable for reproduction. Although the percentages of juveniles remained high after 47 days at the lower temperatures, nematode reproduction was limited because of nematode passivity and the low percentages of adults. Mamiya (12) reported that reproduction of the Japanese isolate of *Bursaphelenchus xylophilus* did not occur at temperatures below 9.5 C on *Botrytis cinerea*; but at 15 and 30 C, the nematode completed its life cycle in 12 and 3 days, respectively. Although temperature requirements may differ for the Vermont isolate of the PWN, nematode reproduction probably did not occur at 3 C but it did occur at a greatly reduced rate at 12 C in our study, based on Mamiya's findings (12). Because total number of nematodes was greatest in the 3-C treatment throughout the study, nematode mortality was also limited at low temperatures. However, the total numbers were remarkably high, relative to the total before the start of the study. This may be explained, in part, by the gradual temperature decrease to 3 and 12 C, which apparently allowed reproduction to continue as long as the temperature was above 9.5 C.

Because there were more dispersals at the lower temperatures by the end of the study, the extended cool period probably stimulated further development of dispersals, which survive unfavorable conditions (5,11,12). Thus, even though reproduction may have ceased in the chips at 3 C, nematodes were still physiologically active and juveniles continued molting to dispersals.

The lower number and percentage of dispersals at 30 C than at 3 C by the end of the study, coinciding with an increase in percentages of juveniles and adults, suggests the abundance of fungi in the wood at 30 C may have increased enough to cause a significant shift in nematode population life cycle from dispersal to reproductive. The PWN was much more active in the wood chips at 30 C than at the other temperatures.

Because the extra 1-week heat treatment at 30 C was applied abruptly to the chips at 3 C, the decline in total numbers of nematodes after 47 days may have resulted from severe thermal shock. Conversely, the 1-week heat treatment applied to the wood chips incubated at 12 C appeared to result in an increase in nematode numbers by the end of the study. This rise in numbers suggests that the nematode populations were at least partly reverting back to the reproductive cycle with resurgence of favorable conditions. Similarly, the numbers of juveniles and adults increased at 12 C, especially when the heat treatment was applied after 130 days, indicating escalation of reproduction. Although the numbers of dauer larvae were not affected by the heat treatment, the percentage was slightly increased when the treatment was applied after 47 days of low temperatures. This result is probably related to the decrease in number of juveniles and dispersals, some of which may have molted to dauer larvae but not enough to affect dauer larval numbers. Previous observations (21,22) show heat-induced increases in the proportion of dauer larvae to 30 and 74%, although

dauer larvae were 5% or less of the population in the present study. If the wood chips are incubated at high temperatures for a longer period of time (several months, for instance) before lowering the temperature, the proportion of dispersals increases significantly, possibly as a result of gradually diminishing nutrient sources (unpubl.). This further enhances the probability of obtaining high percentages of dauer larvae, especially if the warm period following the cold treatment is considerably extended. In our study, 25 days at 27 C before the cool period and 1 week at 30 C after it may not have been long enough to stimulate much dauer larval development. Similar, though more gradual, temperature changes exist in the natural environment. After the low temperatures of winter, dauer larval formation begins in spring at the time vector beetles start to emerge. Ishibashi and Kondo (5) proposed chemical stimuli from pupating vectors as inducers of dauer larval formation, although they also found dauer larvae in wood without beetles.

Decreases in wood moisture content were small, especially at 3 and 12 C, and probably did not have a substantial impact on nematode populations. The gradual loss of wood moisture at 30 C probably contributed significantly to correlations with changes in numbers and percentages of nematodes. The increase in percentage of dispersals associated with the decline in wood moisture agrees with observations in the literature that PWN populations revert to the dispersal cycle with the onset of drier conditions (5,14). Overall, there was a tendency for nematode numbers to decrease with declining moisture. However, moisture and temperature were also highly correlated, so it is difficult to isolate the causal factors. At warmer temperatures, moisture declines more rapidly and should have a greater effect on *B. xylophilus* populations.

Dwinell (3) also found PWN population levels to decrease with decreasing moisture content of southern pine wood chips. Halik and Bergdahl (4) found nematode population levels tended to decline over time within wood chip moisture treatments, but greatest population levels were maintained at 38% moisture, the lowest moisture tested.

Our experimental conditions did not induce increased formation of PWN dauer larvae in wood chips. However, observations of both dispersals and dauer larvae in wood chips in the absence of beetle vectors can now be taken into consideration when evaluating the threat of transmission of *B. xylophilus* to conifer forests of northern Europe.

Literature Cited

1. 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.

2. Dixon, W. J., ed. 1985. BMDP statistical software manual. Berkeley: University of California Press.

3. Dwinell, L. D. 1986. Ecology of the pinewood nematode in southern pine chip piles. Research Paper SE-258, USDA Forest Service Southeastern Forest Experiment Station, Asheville, NC.

4. Halik, S., and D. R. Bergdahl. 1990. Development of *Bursaphelenchus xylophilus* populations in wood chips with different moisture contents. Journal of Nematology 22:113–118.

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

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

7. Kondo, E., and N. Ishibashi. 1978. Ultrastructural differences between the propagative and dispersal forms in pine wood nematode, *Bursaphelenchus xylophilus*, with reference to the survival. Applied Entomology and Zoology 13:1–11.

8. 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.

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

10. Malek, R. B., and J. B. Appleby. 1984. Epidemiology of pine wilt in Illinois. Plant Disease 68: 180–186.

11. Mamiya, Y. 1972. Pine wood nematode, *Bursaphelenchus lignicolus* Mamiya and Kiyohara, as a causal agent of pine wilting disease. Review of Plant Protection Research 5:46–60.

12. Mamiya, Y. 1975. The life history of the pine wood nematode, *Bursaphelenchus lignicolus*. Japanese Journal of Nematology 5:16–25.

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. The pine wood nematode. Pp. 589–626 *in* W. R. Nickle, ed. Plant and insect nematodes. New York: Marcel Dekker.

15. Mamiya, Y., and N. Enda. 1972. Transmission of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae). Nematologica 18:159–162.

16. Nickle, W. R., A. M. Golden, Y. Mamiya, and W. P. Wergin. 1981. On the taxonomy and morphology of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934) Nickle 1970. Journal of Nematology 13:385–392.

17. Rautapää, J. 1986. Experiences with *Bursaphelenchus xylophilus* in Finland. European Plant Protection Organization Bulletin 16:453–456.

18. Southey, J. F., ed. 1986. Laboratory methods for work with plant and soil nematodes. London: Her Majesty's Stationery Office.

19. Tamura, H., and Y. Mamiya. 1975. Reproduction of *Bursaphelenchus lignicolus* on alfalfa callus tissues. Nematologica 21:449–454.

20. Tamura, H., and Y. Mamiya. 1979. Reproduction of *Bursaphelenchus lignicolus* on pine callus tissues. Nematologica 25:149–151.

21. Tomminen, J., and H. Akar. 1990. Infestation of four coleopteran species by the pine wood nematode *Bursaphelenchus xylophilus* (Nematoda, Aphelenchoididae) living in wood chips. Entomologica Fennica 1:171–174.

22. Tomminen, J., S. Halik, and D. R. Bergdahl. 1988. Dauerlarvae of *Bursaphelenchus xylophilus* formed in wood chips of *Pinus strobus.* Nematologica 34:298 (Abstr.).

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

24. Wingfield, M. J. 1987. A comparison of the mycophagous and the phytophagous phases of the pine wood nematode. Pp. 81–90 *in* M. J. Wingfield, ed. Pathogenicity of the pine wood nematode. St. Paul, MN: American Phytopathological Society Press.