

Survival and Infectivity of *Bursaphelenchus xylophilus* in Wood Chip-Soil Mixtures¹

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Abstract: To determine the effect of soil environment on the life stages and total numbers of *Bursaphelenchus xylophilus*, nematode-infested wood chips alone and mixed with soil were incubated at 12 and 20 C. Nematodes were extracted at 2-week intervals for 12 weeks. Numbers of nematodes and percentage of third-stage dispersal larvae were greater at 12 C and in chips without soil. Percentage of juveniles of the propagative cycle was greater at 20 C and in chips with soil. Although *B. xylophilus* survived in chips with soil for 12 weeks, nematode numbers and life stage percentages changed little over time. To determine if *B. xylophilus* was capable of infecting wounded roots, infested and uninfested chips were mixed with soil in pots with white and Scots pine seedlings. Trees were maintained at 20 and 30 C and harvested at mortality or after 12 weeks. Only seedlings treated with infested chips contained nematodes. In field experiments, planted seedlings were mulched with infested chips to determine if nematodes would invade basal stem wounds. Among these trees, Scots pine was more susceptible than white or red pines to infection and mortality.

Key words: *Bursaphelenchus xylophilus*, nematode, pine, pinewood nematode, *Pinus strobus*, *P. sylvestris*, root, soil, temperature, wood chip.

Unlike the majority of plant-parasitic nematodes, the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, causal agent of pine wilt disease, is not known to inhabit soil (40). However, northern Europeans are concerned the nematode could be transmitted from imported wood chip piles through soil to infest roots of susceptible pines (*Pinus* spp.) (18,27). Finland, Norway, and Sweden have placed embargoes against importation of raw coniferous wood products from areas where *B. xylophilus* is known to occur, including the United States, Canada, and Japan (2). This nematode is not known to exist in Europe, but researchers there have reported the presence of a similar wood-inhabiting nematode, *B. mucronatus* (32). However, epidemics of pine wilt disease occur only in Japan and possibly China and Taiwan (23).

Because of large-scale mortality of native pines in Japan, much research has been conducted on the biology of *B. xylophilus*. It is vectored primarily by pine sawyer beetles (*Monochamus* spp.) that emerge

from dead trees in spring carrying nematode dauer larvae. Nematodes enter trees through wounds made during beetle maturation feeding or oviposition (14-16) and molt to reproductive adults, which feed on healthy host parenchyma tissue or on fungal hyphae in wood of dead trees (36,37, 39). While favorable conditions exist, the nematode persists in a propagative life cycle, with four juvenile stages. When ambient temperatures and wood moisture decline or food becomes scarce, second-stage larvae molt to specialized third-stage dispersal larvae, which are better adapted to survive adverse conditions (11,20,22). Dispersals are attracted to pupal chambers of the pine sawyer, where they molt to dauer larvae and infest beetles prior to emergence (15,16).

Reports on the ability of *B. xylophilus* to survive in soil are limited. Mamiya and Shoji (24) found that all nematodes died within 72 hours after inoculation of *B. xylophilus* into a soil environment. However, McGawley et al. (26) demonstrated population growth of *B. xylophilus* on several fungi commonly found in soil, and Blackwell and Gilbertson (3) successfully cultured the nematode on *Quasiconcha reticulata* isolated from roots of *Pinus halapensis*. Kiyohara and Tokushige (13,24) buried disks of pine wood contaminated with *B. xylophilus* around wounded roots of

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healthy pines, which became infected and wilted in 3 months. We (7) mixed *B. xylophilus*-infested wood chips with soil around wounded roots of potted eastern white pine (*P. strobus*), and 58% of the seedlings became infected with the nematode through the roots and wilted.

Because of both the concern that *B. xylophilus* may threaten conifer forests in Europe and the economic repercussions of continued trade restrictions, survival and transmission of the nematode in soil is of concern to countries involved in the embargoes. Thus, objectives of this study were to determine if *B. xylophilus* can survive in a soil environment and infect wounds on roots or stems of pine seedlings if wood chips are incorporated into soil or used as mulch.

MATERIALS AND METHODS

Healthy eastern white pines (20-cm-d) were harvested at the University of Vermont's Jericho Research Forest in northwestern Vermont in April 1988. Logs were debarked and chipped, and all wood chips were mixed. Ten 200-g samples of chips were randomly chosen and floated in 18-cm-d modified Baermann funnels (8,10). After 48 hours, extracts were examined for presence of nematodes using a dissecting microscope at 25 \times . This same procedure was used for all subsequent extractions.

Survival in wood chips with soil: Isolate 16E of *B. xylophilus* from a dead white pine in Vermont was cultured on *Botrytis cinerea* (8), and approximately 1,000 nematodes in 10 ml sterile distilled water were pipetted into each of 12 sealable plastic bags (38 cm \times 30 cm) containing 600 g (fresh weight) of chips, and the bags were incubated at 30 C. After 4 weeks, all bags were removed from the incubator and the chips were mixed in one large plastic bag. Nematodes were extracted from 10 randomly chosen 50-g samples and counted. Following extractions, chips were oven dried at 98 C for 24 hours, and numbers of nematodes were based on oven dry weight (ODW) of each

chip sample, as were all subsequent nematode counts.

Fifty grams (fresh weight) of the infested wood chips, containing about 700 nematodes/g ODW, were placed in each of 144 plastic bags (23 cm \times 15 cm), and 200 g of steam-sterilized greenhouse potting soil (1/3 sandy loam, 1/3 peat moss, 1/3 perlite) was mixed with chips in half of the bags. The bags were sealed, and each of the two chip treatments was randomly split between two incubators at either 12 or 20 C. Six replicates (bags) per treatment were randomly removed for nematode extraction at 2-week intervals for 12 weeks. Nematodes were counted and the first 40 nematodes counted per bag were identified as either adult, propagative juvenile, dispersal, or dauer larva.

Analyses of variance followed by Duncan's multiple-range tests were used to evaluate effects of incubation time, temperature, and chip treatment on total number of nematodes and on number and percentage of each life stage. Before analyses, total number of nematodes were log₁₀-transformed, percentages of juveniles were modified with an arcsine transformation, and remaining percentages of life stages were subjected to power transformations to satisfy variance assumptions (4,28).

Seedling infection: Wood chips were placed in plastic bags and half the bags were inoculated with *B. xylophilus* and incubated as previously described. The remaining chips were treated with sterile distilled water. Forty-eight 3-year-old white pine and 48 3-year-old Scots pine (*P. sylvestris*) nursery stock were potted in 15-cm-d plastic pots with sterile greenhouse potting soil. Seedlings were placed in environmental growth chambers with gradually increasing temperature and photoperiod. After budbreak and onset of shoot elongation, seedlings were removed from the pots and wounds 1 cm long were made at three locations on the roots of each seedling by scraping bark to expose xylem. For each of the two chip treatments, 150 g (fresh weight) of chips were mixed with

the soil in each of 48 of the pots. Twenty-four seedlings of each pine species were potted in the nematode-infested chip–soil mixture, containing about 2,500 nematodes/g ODW of chips, and 24 were potted in the uninfested chip–soil mixture. Each treatment was split between two growth chambers at 20 or 30 C, both with an 18-hour photoperiod. Seedlings were watered 2–3 times per week. At wilt or at 12 weeks, seedlings were removed from pots, and stems were cut from main roots at the soil line. For each seedling, stem and root were kept separate and were clipped into 1-cm sections, and nematodes were extracted.

For the field study at Jericho Research Forest in June 1988, roots of actively growing 3- to 5-year-old white pines, Scots pines, and red pines (*P. resinosa*) were wounded using the described method. Twelve seedlings of each species were potted in 15-cm-d pots with a mixture of field sandy loam and 150 g (fresh weight) of nematode-infested chips, containing about 560 nematodes/g ODW of chips. Twelve seedlings per species were potted in similar soil mixed with uninfested chips. Pots were then placed in soil to the depth of the pot, 0.5 m apart, in rows 1 m apart. Another set of the same pine species was planted on the same spacing and wounded at the base of the stem, and 12 seedlings of each species were surface mulched with 150 g of infested chips and 12 with uninfested chips. Twenty-four additional red pines were planted, left unwounded, and mulched as above. A layer of uninfested wood chips was placed on top of all mulch treatments to help prevent desiccation. Seedlings were watered every other day during droughty periods and air temperatures were recorded electronically. Seedlings were returned to the lab for nematode extraction when wilt symptoms appeared or after 18–25 weeks.

Effects of chamber temperature, host species, and chip treatment on frequencies of mortality and nematode infestation of seedlings were analyzed using log-linear modeling and chi-square tests of marginal and partial association (5).

RESULTS

Survival in wood chips with soil: No nematodes were extracted from wood chips prior to inoculation with *B. xylophilus*. There were fewer ($P < 0.001$) nematodes in wood chips with soil than in chips without soil during 12 weeks of incubation at both 12 and 20 C, and there were more ($P < 0.001$) nematodes in treatments at 12 than at 20 C (Fig. 1A). Time-related effects differed ($P < 0.003$) between chip treatments. Number of nematodes in chips with soil did not fluctuate ($P > 0.05$) with time, but in chips without soil, the number decreased ($P < 0.05$) at 6 weeks and then increased ($P < 0.05$) again at 8 weeks.

Number and percentage of adults in nematode populations were affected by a significant ($P < 0.003$) interaction between temperature and soil. There were greater ($P < 0.05$) number and percentage of adults at 12 C (18%) than at 20 C (11%) in chips without soil; but in chips with soil, these temperature-related differences did not occur ($P > 0.05$) (Figs. 1B,2). At 12 C, there were greater ($P < 0.05$) numbers and percentages of adults in chips without soil (18%) than in chips with soil (10%); but at 20 C, there were no differences ($P > 0.05$) in number or percentage of adults between chip treatments. Number of adults was affected ($P < 0.001$) by time. Effects of time on percentage of adults differed ($P < 0.001$) with chip treatment. In chips without soil, the percentage of adults dropped ($P < 0.05$) by 26% from 2 to 12 weeks but only by 9% in chips with soil.

After 6 weeks of incubation, mean number of juveniles in both chip treatments decreased ($P < 0.05$) but the mean percentage increased ($P < 0.05$) to 70% (Figs. 1C,2). The percentage began to decrease ($P < 0.05$) by 8 weeks and by 12 weeks was 50%. Effects of time on number of juveniles differed ($P < 0.001$) between chip treatments. In chips without soil, number of juveniles increased ($P < 0.05$) after 8 weeks and decreased ($P < 0.05$) again after 12 weeks, but number of juveniles in chips with soil remained stable after 6 weeks.

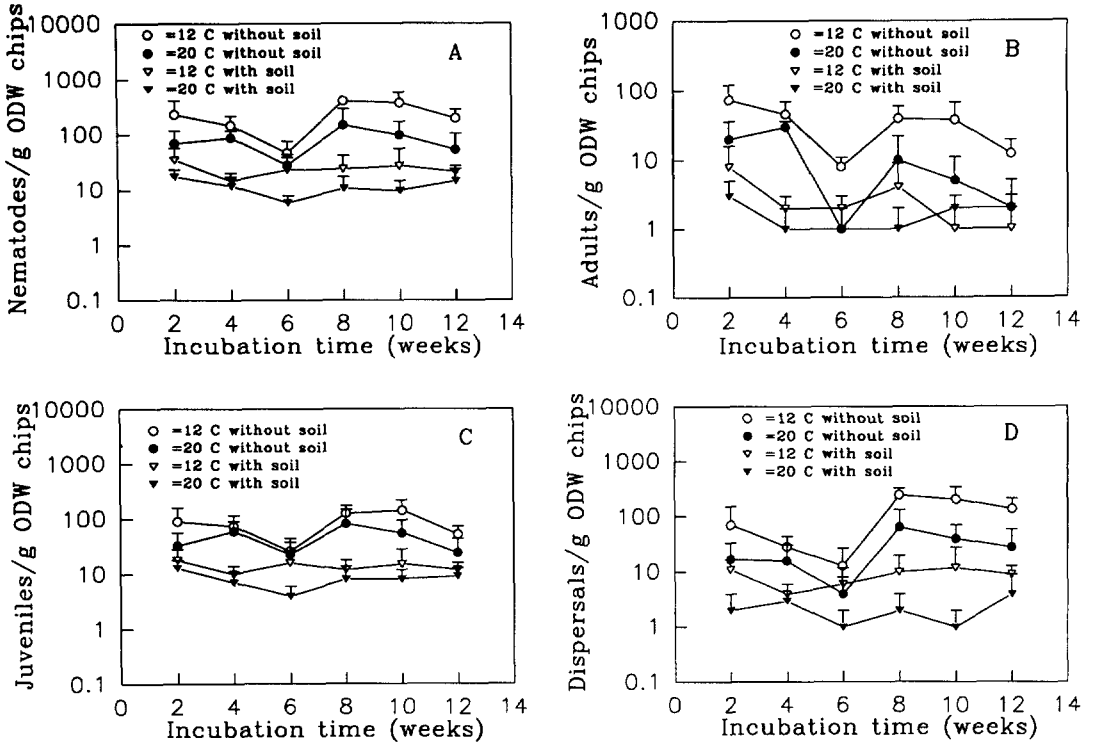


FIG. 1. Total number of nematodes and numbers within each of three life stages of *Bursaphelenchus xylophilus* in wood chips with soil and without soil and incubated at 12 and 20 C. A) Total numbers. B) Adults. C) Juveniles. D) Dispersals. Number of *B. xylophilus* per gram oven dry weight (ODW) of chips represented logarithmically. Error bars indicate SD. Missing error bars indicate too small a SD to be shown.

Number of juveniles was greater ($P < 0.001$) in chips without soil than in chips with soil, but percentage was greater ($P < 0.001$) in chips with soil than in chips without soil. At 12 C, there was a greater ($P < 0.001$) number but a lower ($P < 0.001$) percentage of juveniles than at 20 C.

Greater ($P < 0.001$) number and percentage of dispersal larvae were found at 12 than at 20 C in both chip treatments, and there were greater ($P < 0.001$) number and percentage of dispersals in chips without soil than in chips with soil (Figs. 1D, 2). Effects due to time differed ($P < 0.04$) between chip treatments. Number of dispersals decreased ($P < 0.05$) at 4–6 weeks and increased ($P < 0.05$) at 8 weeks in both chip treatments. However, in chips without soil, the number increased ($P < 0.05$) beyond the level found at 2 and 4 weeks, but in chips with soil, the number increased ($P < 0.05$) only to the level

found at 2 and 4 weeks. Percentage of dispersals in treatments without soil remained low until 6 weeks, at which time they constituted 19% of the population. At 8 weeks, percentage of dispersals began to increase ($P < 0.05$) and remained greater ($P < 0.05$) than that in chips with soil through 12 weeks, at which time dispersals averaged 55% of the population in chips without soil. However, percentage of dispersals in treatments containing soil remained stable ($P > 0.05$) over time until it increased ($P < 0.05$) to 31% at 12 weeks.

Dauer larvae were omitted from analyses. They were extracted from three bags of wood chips without soil but represented only 3% of the population in these bags.

Seedling infection: In the growth chambers, frequency of trees that became infected was associated ($P < 0.001$) with chip treatment. No control seedlings contained *B. xylophilus* at the time of extraction (Ta-

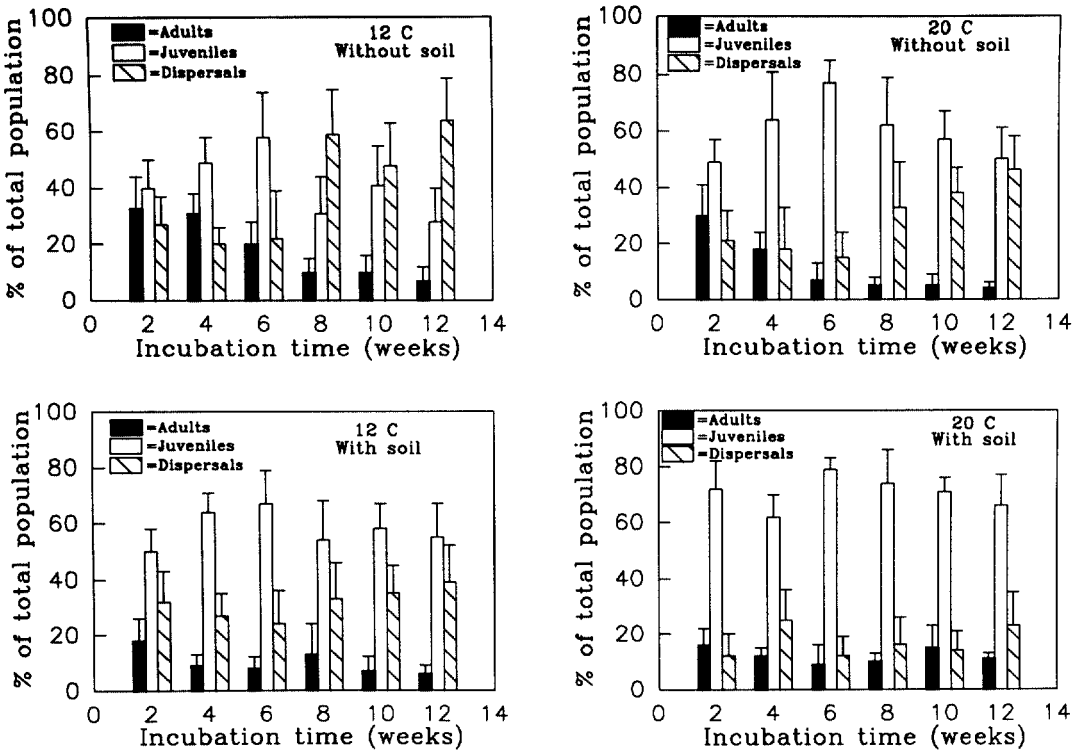


FIG. 2. Percentages of three life stages in populations of *Bursaphelenchus xylophilus* in wood chips with soil and without soil and incubated at 12 and 20 C. Error bars indicate SD.

ble 1), but nematodes were extracted from 92% of the Scots pines and 75% of the white pines treated with infested chips. Scots pine at 30 C showed no association ($P < 1.000$) between frequency of seedling mortality and chip treatment. Because of the extreme temperature, as many control Scots pines died as those treated with *B. xylophilus*. Within all other host-temperature combinations, more ($P < 0.02$) seedlings treated with nematode-infested chips died than did seedlings treated with uninfested chips. One of the white pines treated with infested chips at 20 C contained *B. xylophilus* but remained asymptomatic. For all treatments except Scots pine at 30 C, frequency of mortality was associated ($P < 0.007$) with frequency of infection by the nematode. Mortality and infection of seedlings were not associated ($P < 0.700$) with temperature treatment.

None of the seedlings in submerged pots in the field wilted, but 17% of the white

pines in these pots treated with infested wood chips contained *B. xylophilus* and remained asymptomatic. One of the non-wounded planted red pines treated with infested chips died and contained *B. xylophilus* (Table 2). Mortality and infection of planted Scots pines were associated ($P < 0.04$) with chip treatment; more Scots pines treated with nematode-infested chips (50%) died than did control Scots pines (8%). All trees that died and had been treated with infested chips contained *B. xylophilus*, but none of the control seedlings were infected. More ($P < 0.04$) Scots pines (50%) died and contained *B. xylophilus* than did white (8%) or red pines (8%).

DISCUSSION

Apparently, conditions in wood chip and wood chip–soil bags never became favorable for dauer larval development over the 12-week study period. Tomminen et

TABLE 1. Percentages of potted Scots pine (*Pinus sylvestris*) and eastern white pine (*P. strobus*) seedlings that died and were infected with *Bursaphelenchus xylophilus* following inoculation with wood chips and incubation at 20 or 30 C.

Temperature (C)	Chip treatment	Mortality (%)	Trees with nematodes (%)†
Scots pine			
20	Uninfested	42 b‡	0 b
	Infested	100 a	92§ a
30	Uninfested	92¶ a	0 b
	Infested	92 a	92 a
Eastern white pine			
20	Uninfested	17 b	0 b
	Infested	75 a	67¶ a
30	Uninfested	8 b	0 b
	Infested	92 a	83§ a

† Percentage of all seedlings in treatment.

‡ Percentages within a host-temperature treatment and within a column followed by a different letter are significantly different by a chi-square test ($P < 0.05$).

§ One seedling that died did not contain *B. xylophilus*.

¶ Mortality related to temperature stress.

¶ One seedling that did not die contained *B. xylophilus* at 12 weeks and two seedlings that died did not contain *B. xylophilus*.

al. (38) showed that a cool period followed by warming temperatures, as occurs naturally in spring when dispersal larvae molt to dauer larvae, may be necessary for dauer larval formation. Ishibashi and Kondo (11) suggested that formation of dauer larvae requires chemical stimuli from pupating vector beetles, although they have extracted dauer larvae from wood without beetles present. Neither warming temperatures nor beetle vectors were present in our study, and this may explain the very small percentage of dauer larvae in the bags containing wood chips alone.

Because the number of nematodes in each life stage was greater at 12 C than at 20 C and there was a larger percentage of juveniles at 20 C than at 12 C, at the higher temperature nematodes were more actively reproducing but also dying as food supplies became depleted. The larger number of nematodes at 12 C than at 20 C suggests that populations remained relatively stable, with slower reproduction and mortality after onset of the lower temper-

ature. However, more juveniles were molting to the dispersal form at 12 C than at 20 C, as occurs in natural populations of *B. xylophilus* when conditions in wood become less favorable (11,22). Tomminen et al. (38) also indicated that reproduction by *B. xylophilus* occurred in wood chips incubated at 12 C, but at a much reduced rate, and populations at this temperature were more likely to revert to the dispersal cycle.

In bags containing only wood chips, number of nematodes in each life stage fluctuated with time, possibly following the rise and fall of fungal food resources. However, the percentages of adults and juveniles declined over time, and because of the substantial increase in number of dispersals after 8 weeks, percentage of dispersals increased throughout the study. Tomminen et al. (38) observed the same transformation in nematode populations in wood chips at 12 C. We did not evaluate fungal populations in the bags, but believe that as food reserves diminished in the wood chips, nematode populations reverted to the dispersal form. By 8 weeks, there was a greater proportion of dispers-

TABLE 2. Percentages of Scots pine (*Pinus sylvestris*), eastern white pine (*P. strobus*), and red pine (*P. resinosa*) seedlings planted in the field and mulched with wood chips infested with *Bursaphelenchus xylophilus*.

Chip treatment	Mortality (%)	Trees with nematodes (%)†
Scots pine, wounded		
Uninfested	8 b‡	0 b
Infested	50 a	50 a
Eastern white pine, wounded		
Uninfested	0 a	0 a
Infested	8 a	8 a
Red pine, wounded		
Uninfested	0 a	0 a
Infested	8 a	8 a
Red pine, unwounded		
Uninfested	0 a	0 a
Infested	8 a	8 a

† Percentage of all seedlings in treatment.

‡ Percentages within a host treatment within a column followed by the same letter are not significantly different by a chi-square test ($P < 0.05$).

als in chips without soil than in chips with soil. Wood chips served as a more natural substrate and nematodes were more physiologically active than in the wood chip–soil mixture.

The fewer nematodes in chips mixed with soil and the fewer changes in each life stage over time suggest that nematode populations changed very little in the soil medium. Conditions in the wood chip–soil bags were less favorable for nematode survival and reproduction than in the wood-chip-only treatment. However, lower population levels may not be solely the result of nematode mortality caused by the unnatural soil medium. During the extraction process, nematodes may not have passed as freely through the layer of soil and chips as through wood chips alone. Soil mixed with the chips may have created anaerobic conditions that inhibited nematode movement. Whether reproduction occurred in soil is not as important as survival of nematodes for up to 12 weeks in the wood chip–soil medium. Up to 40% of the population (at 12 C) were in the dispersal stage at 12 weeks and therefore better able to survive such uncharacteristic conditions as a wood chip–soil environment. Although Mamiya and Shoji (24) demonstrated that *B. xylophilus* did not survive in soil after 72 hours at 25–28 C, the lower temperatures in our study may have been more conducive to nematode survival. Nematodes in our study also may have remained in association with wood chips in the soil.

Because soil nematodes move only a few centimeters to a meter per year at most (1,25), *B. xylophilus* is unlikely to travel great distances in soil. This study did not evaluate nematode migration through soil; wood chips were probably in contact with the wounded roots or stems of seedlings when infestation occurred. Our results are supported by those of Mamiya and Shoji (24), who found that wounding of the basal stem of a tree was necessary for successful nematode invasion. Because planted trees are likely to have some form of mechanical or insect wound (29), they

are subject to potential infection by *B. xylophilus*, especially if infested wood chips are used as mulch or soil near chip piles is used in transplanting.

Of the planted seedlings, Scots pine was clearly the species most susceptible to *B. xylophilus*. The apparently low frequency of infestation and disease development in the white and red pines may be related to variation in host species susceptibility. Linit and Tamura (17) obtained similar results when they inoculated mature Scots and white pines in the field. Researchers agree that in the United States, the nematode appears able to cause primary mortality of Scots pine, especially those planted on stressful sites (6,19,39).

In our growth chamber study, numbers of nematodes increased in the *B. xylophilus*-treated and -infected Scots pines, but because so many control seedlings died, it appears that heat stress resulted in tree mortality. Pathogenicity studies using potted seedlings, especially those disturbed during active growth, are not conclusive evidence of host susceptibility because of stressful conditions. However, our primary intention in conducting the growth chamber study was to confirm results of a previous study (7) that *B. xylophilus* is capable of infecting freshly wounded seedling roots from a wood chip–soil medium.

It is possible that *B. xylophilus* infested more of the planted white and red pines than indicated in Table 2 but did not reproduce to large enough populations for extraction or for host symptom expression. The extraction of pinewood nematodes from asymptomatic potted white pines both in the field and in the 20 C growth chamber suggests that symptom development can be inhibited by low temperature. Other researchers (12,21,34) have also reported low temperature inhibition of *B. xylophilus* reproduction and pine wilt symptom development. Average daily temperatures for the months of June, July, and August at the Jericho Research Forest ranged from 17–23 C. These temperatures may have been low enough to retard nematode reproduction and disease

expression in our native pine species. However, the summer also had some extremely hot, dry periods for the northeastern United States, with an average daily maximum temperature throughout June, July, and August of 27 C. The hot, dry weather may have rapidly lowered the moisture content of the chips around the seedlings and nematodes may have desiccated before they could infect the trees. Unfortunately, the moisture content of the field chips was not measured at any time during the study.

Rutherford and Webster (31) claimed that temperatures are too low in Nordic countries for pine wilt to develop and become widespread. They suggested that only in areas with several weeks' mean air temperature of greater than 20 C will trees be susceptible to *B. xylophilus*, and Scots pine does not naturally grow in these warmer areas of Europe. These authors probably based their hypotheses on the distribution of pine wilt disease in Japan, where currently the nematode only causes extensive damage in warm southern and coastal areas (22). However, Rutherford et al. (30) suggested that isolates of *B. xylophilus* from cooler areas of the world, such as the northern United States and Canada, and isolates of *B. mucronatus* from northern Europe may be more virulent at low temperatures than are isolates from warmer climates.

Sikora and Malek (34) showed that *B. xylophilus*-inoculated Scots pine seedlings incubated at ≤ 18 C for 8 weeks did not wilt, but did when subsequently incubated at 22 C. If Scots pines are stressed in ornamental situations or in managed stands, there is the chance that infection by *B. xylophilus* could occur in cooler regions. Infected pines may remain asymptomatic, but only a few weeks of high temperature and drought are needed for pine wilt to follow.

In his review of climatic change, Harrington (9) suggests global mean temperature may increase 2.5 C by 2050. Other researchers have estimated a 4 C increase (9,33). If these predictions prove correct

and cool areas become warm enough in the future for pine wilt disease manifestation, the possibility of *Bursaphelenchus* spp. being a threat to the world's conifer forests may be realized.

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