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Development of *Bursaphelenchus xylophilus* Populations in Wood Chips with Different Moisture Contents[1]

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Abstract: Bags of *Pinus strobus* wood chips with moisture contents of 38, 92, 164, and 217% (oven dry weight) were inoculated with *Bursaphelenchus xylophilus* and incubated at 30 C in order to determine the effect of wood moisture on nematode population development. Nematodes were extracted after 2, 4, 8, and 12 weeks. Population levels were greatest in wood chips with a moisture content of 38% and decreased successively with each higher moisture content. In chips with the three lower moisture contents, populations peaked at 2 weeks, but at 217% moisture, they peaked at 8 weeks. By 12 weeks, nematode populations had declined in wood chips with 92 and 164% moisture contents. The fungi most frequently isolated from the wood chips were *Alternaria, Fusarium, Gliocladium, Graphium, Penicillium, Trichoderma,* and Mucorales.

Key words: Bursaphelenchus xylophilus, moisture content, pinewood nematode, *Pinus strobus,* wood chip.

Since the early 1900s, the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970, has caused extensive mortality of native pines (*Pinus densiflora* Sieb. & Zucc. and *P. thunbergii* Parl.) in Japan, especially in warm coastal areas. Today this nematode is considered the most serious pest of conifers in Japan (19). Although it causes mortality of exotic pines (*P. nigra* Arnold and *P. sylvestris* L.) in the United States, it is more commonly found associated with conifers dying from other factors (3,17,33,34). The PWN was discovered in the United States in 1929 (31) but it has not caused a serious pine wilt epidemic in this country. Researchers hypothesize that *B. xylophilus* is native to North America and was introduced to Japan during the late 1800s (17,20,26,34).

Due to the impact of the PWN on their forests, the Japanese have extensively studied the disease cycle of *B. xylophilus* (18,19). The nematode is vectored primarily by pine sawyer beetles (*Monochamus* spp.) and is transmitted to healthy conifers during maturation feeding by the beetle. The nematode invades bark and xylem tissues where it is believed to feed on living epithelial cells lining the resin canals and on parenchymal cells and ray and fusiform initials in the cambium (24). Female beetles may also transmit the PWN to dead and dying trees during oviposition (32). In these trees, the nematode is believed to feed on fungal hyphae colonizing the wood and is then able to survive in cut logs and other wood products. Consequently, the recent spread of the PWN to cooler northern and inland areas of Japan is thought to have resulted from the transport of PWN-infested and beetle-infested logs from coastal areas (19,21).

In 1984, Finnish plant quarantine authorities discovered the PWN in wood chips imported from North America. In view of the impact of the PWN in Japan and its proven pathogenicity to *P. sylvestris,* Finland, Sweden, and Norway initiated embargoes against all coniferous wood from known infested regions of the world (1,25,28). The embargoes have had a substantial economic impact on forest industries in North America (2). By 1987, Canadian wood industries had lost about $140 million worth of wood export trade (4).

The biology of *B. xylophilus* in wood products, especially chips, had not been studied before 1984, but the embargoes stimulated interest in PWN-wood product research which has focused on temperature requirements of the PWN in wood chips. Kinn (11) determined the temperatures and short term time periods required

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to kill nematodes in southern pine wood chips using dry heat, moist heat, and a water bath. Dwinell (5) observed nematode population development in inoculated southern pine wood chips at a range of temperatures for up to 3 weeks and found 35 C to be optimum for growth of populations. In a subsequent study, he noted that nematode population levels decreased as wood moisture was lost from naturally infested wood chips, but specific moisture levels were not tested (5). Moisture contents ranging from 25 to 300% have been recorded from stored wood chip piles (8,29), but how this range of moisture contents affects PWN population levels is not known.

In a previous study (7), we observed that inoculated PWN populations increased 30-fold at 30 C after 8 weeks in eastern white pine (*P. strobus* L.) wood chips with a moisture content of 140%, oven dry weight (ODW). How population levels change after 8 weeks is not known but could be an important consideration because time in transit may be as long as 12–13 weeks from harvest to delivery at European ports (Peeples, pers. comm.). The purpose of the present study was to determine the effect of wood moisture content on population development of the PWN in wood chips maintained at 30 C for up to 12 weeks.

Materials and Methods

Apparently healthy 20-cm-d eastern white pines were harvested from the University of Vermont Research Forest in Jericho in October 1986. The logs were debarked and stored at ambient conditions for 12 days before chipping. The wood chips were mixed and a 600-g sample was removed for nematode extraction to determine if natural populations of the PWN were present. A modification of the Baermann technique (9), consisting of a 33-cm-d plastic funnel, was used to extract the nematodes.

To determine initial wood moisture content, 10 30-g samples of wood chips were oven dried at 98 C for 24 hours. The remainder of the wood chips was maintained in frozen storage for 7 days. The following four wood moisture treatments were chosen for this study: 40, 100, 160, and 220%. Sets of wood chips from frozen storage were either air dried or moistened with sterile distilled water in an attempt to achieve the four target moisture levels. For each of the four moisture treatments, the wet weight of wood chips placed into each of 20 sealable plastic bags (38 cm × 30 cm) was determined such that its ODW would be 200 g.

An isolate of *B. xylophilus* from white pine in Vermont was cultured on a nonsporulating strain of *Botrytis cinerea* Pers. growing on a 1:1 mixture of autoclaved oats and barley in a 250-ml flask. After incubation at 30 C for 9 days, the nematodes were extracted by filling the flask with sterile distilled water, capping it with a milk filter lid, and placing it upside-down in a beaker of sterile distilled water. After 24 hours, the nematodes in the beaker were diluted with sterile distilled water to 333/ml. Three milliliters of this dilution were pipetted into each of the bags of wood chips (four nematodes/gram ODW wood chips). The bags were then shaken, sealed, and placed in an environmental growth chamber at 30 C, a temperature commonly attained in stored wood chip piles (10,27,29) and one that is favorable for *B. xylophilus* development (7). Five replicates (bags) per moisture treatment were incubated for either 2, 4, 8, or 12 weeks. At the end of each incubation period, the 5 bags per moisture treatment were removed from the growth chamber and a sample of the wood chips (one-sixth by weight) was removed from each bag for fungal isolation and moisture content determination. The mean moisture content for the 20 bags in each moisture treatment was determined after 12 weeks.

For fungal isolation, at the time of each extraction, approximately 14 wood chips per bag were cut into small sections, surface sterilized in a 2% sodium hypochlorite solution, rinsed in sterile distilled water, and plated on 1.5% malt extract agar and 1.5% malt extract agar with lactic acid and cycloheximide (22). The latter medium was used to suppress growth of *Trichoderma* spp. All plates were incubated for 2 weeks at 20

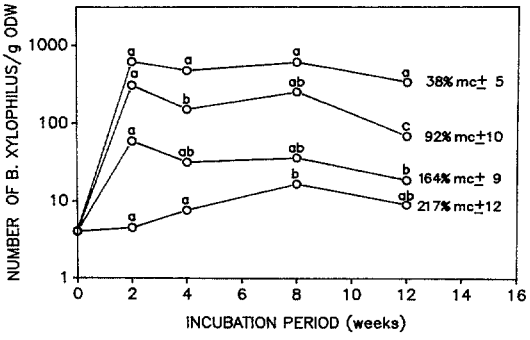

FIG. 1. Population levels of *Bursaphelenchus xylophilus* inoculated onto white pine wood chips with four moisture contents (mc) and incubated for four time periods. Number of *B. xylophilus* per gram oven dry weight (/g ODW) of wood chips represented logarithmically. Each mc is the mean of 20 bags of wood chips ± the standard deviation. Within each mc, population means labeled with the same letter are not significantly different using Duncan's multiple-range test ($P < 0.05$).

C and the fungi that grew out were identified to genus. Frequencies of isolation for each genus were recorded as percentages of total fungal colonies isolated on the two media combined.

Nematodes were extracted from the remaining chips using the modified Baermann funnel already described. After 48 hours, nematodes were counted using a dissecting microscope at 25× and the population in each bag was recorded per gram ODW of the wood. Two-way analysis of variance and Duncan's multiple-range test were used to test for treatment differences. Data were transformed prior to analysis using a $\log_{10}$ transformation. The contribution (in percent) of each moisture treatment to the interaction between moisture and time was calculated using the method described by Shelbourne (30).

RESULTS

Nematodes were not extracted from the sample of freshly chipped wood, which had an initial moisture content of 190%. The mean moisture contents for the four treatments were 38, 92, 164, and 217%, corresponding to the initial target moisture groups of 40, 100, 160, and 220%, respectively.

After inoculation, PWN population levels

TABLE 1. Fungi most frequently† isolated from wood chips of *Pinus strobus* inoculated with *Bursaphelenchus xylophilus*.

Fungus	Isolation frequency‡
Alternaria	3
Fusarium	3
Gliocladium	1
Graphium	3
Mucorales	3
Penicillium	14
Trichoderma	67
All other fungi	6

† Frequency of isolation ≥ 1%.
‡ Percentage of 639 total fungal colonies isolated.

varied significantly ($P < 0.01$) with wood moisture content and time of incubation, and a significant ($P < 0.01$) interaction existed between moisture and time (Fig. 1). Approximately 62% of this interaction was due to the effects of the 217% moisture treatment, at which nematode numbers increased gradually for 8 weeks. At all other moistures, population levels peaked within 2 weeks.

The highest nematode population levels were recovered from wood chips at 38% moisture, and nematode numbers decreased successively with each higher moisture treatment ($P < 0.05$). At 2 weeks, nematode population levels in the 38 and 92% moisture treatments did not differ from each other but were significantly greater than numbers in the two higher moisture content treatments, which were also significantly ($P < 0.05$) different from each other. At all other time periods, the nematode population levels at the four moisture treatments differed significantly ($P < 0.05$).

In the 217% moisture treatment, nematode numbers at 8 weeks were significantly ($P < 0.05$) greater than populations at 2 and 4 weeks. In chips at 92 and 164% moisture contents, nematode levels at 12 weeks were less than those at 2 weeks ($P < 0.05$). Population levels at 38% moisture, however, showed no significant time differences ($P > 0.05$).

The most common fungi isolated from the wood chips were *Alternaria, Fusarium,*

Gliocladium, Graphium, Penicillium, and *Trichoderma* (Table 1). Members of the order Mucorales were also isolated but were not identified to genus.

Discussion

Ray parenchyma cells in unpeeled logs of fresh aspen (*Populus tremuloides* Michx.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) have been shown to remain active for 2–3 months at ambient summer conditions (6). In fresh wood chips of the same two species, parenchyma cells may function for 2 weeks at 21 C. At the time the white pine chips in the present study were inoculated with nematodes, the trees had been cut for 12 days, chipped, and then maintained in frozen storage for 7 days. Thus, the epithelial cells may have served as food sources when the nematodes were introduced, contributing to the initial increases in populations. However, after several days, fungi probably became the major food source. The fungi isolated from wood chips in this study also have been isolated from wood chip piles by others (10,14,29). *Fusarium, Gliocladium,* and *Graphium* are known to support growth of *B. xylophilus* (12,23). *Alternaria* and *Trichoderma* are not believed to be preferred food sources (12); however, we (unpubl.) have successfully reared the PWN on cultures of *Trichoderma.*

Most wood colonizing fungi require a moisture content of at least 22%, whereas a moisture content nearing 100% begins to inhibit fungal growth (14). In this study, conditions were probably most favorable for fungal development in the wood chips with a moisture content of 38% and were progressively less favorable with each higher moisture treatment. Pinewood nematode population levels in the wood chips followed this pattern. Population differences among wood moisture treatments probably resulted from differences in the abundance of the fungal food supply for the nematodes.

Fungal development was most likely inhibited in the wood chips with 217% moisture; therefore, nematode populations increased more slowly than in the three lower moisture treatments, peaking at 8 weeks instead of at 2 weeks. The high water content present in the bags of the 217% moisture treatment may have limited available oxygen, resulting in lower population levels of aerobic organisms. Dwinell (5) placed PWN-infested wood chips in an anaerobic environment and found that nematode population levels decreased after 3 days. Nematode population levels in both the 92 and 164% moisture treatments dropped by about 70% from 2 to 12 weeks, suggesting that as fungal populations became exhausted, nematode development slowed. In wood chips with 38 and 217% moisture contents, similar trends probably occurred but were not significant in this study. Dwinell's (5) PWN inoculation studies in southern pine wood chips show a similar decline in populations over a 3-week period, but he did not speculate as to the cause.

According to our present study, PWN populations could remain at high levels in wood chip piles after 12 weeks, which is the approximate delivery time to European ports. The fungi isolated from the wood chips in our study also have been found in 5-year-old chip piles intended for use as mulch (10); thus, as long as temperature and moisture conditions remain favorable, PWN populations should stay at high levels well beyond 12 weeks.

Wood chips may be a biological "dead end" for the nematode, as pine sawyer beetles are not known to survive the chipping process, nor are they known to visit chip piles (5,13). There are, however, other potential beetle vectors, such as *Hylobius* sp. and *Pissodes* sp., in northern Europe that may frequent chip piles (15,28). The possible survival and movement of the PWN in soils has not been adequately researched, but many of the fungi that colonize wood chips and are used as food sources by the PWN are also known to inhabit soil (10). Transport of the pinewood nematode from a wood chip pile to living or dead pines by potential beetle vectors has not been demonstrated nor has transmission through the soil been shown. How-

ever, until more is known about whether *B. xylophilus* can be spread from wood chip piles to highly susceptible *P. sylvestris*, northern Europeans believe it is absolutely necessary to maintain wood import restrictions (16,28).

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