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The biology and ecology of the pinewood nematode, Bursaphelenchus xyophilus (Steiner and Buhrer) Nickle, in Massachusetts /

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THE BIOLOGY AND ECOLOGY OF THE PINEWOOD NEMATODE *BURSAPHELENCHUS XYLOPHILUS* (STEINER AND BUHRER) NICKLE, IN MASSACHUSETTS

A Thesis Presented

By

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To Mom, Dad, Laurie and Jean

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TABLE OF CONTENTS

Chapter

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934) Nickle 1970, formerly *B. lignicolus* Mamiya and Kiyohara 1969, was first recovered in Massachusetts from the sapwood of a dying red pine (*Pinus resinosa* Ait.) on Cape Cod (Rohde 1983).

This plant parasitic nematode had been reported to cause a rapid wilt disease of most *Pinus* spp. trees in Japan known as pine wilt (Mamiya 1972a). The first noticeable symptom of disease is a change in needle coloration. Infected trees change from dark green to grey-green, then to yellow green, followed by the bright reddish brown of dead trees (Figure 1). In warmer climates of Japan this change may occur in only six to eight weeks, while in cooler climates, trees which become infected in the summer may not die until the following spring (Mamiya 1983). Another important symptom of pine wilt is a decrease in the oleoresin flow from bark wounds or from broken branches (Mamiya 1972). Whole trees may be affected or pine wilt disease may be limited to one or several diseased branches of a single tree (Dropkin *et al*. 1981, Malek and McClary 1981, Malek *et al*. 1982 and Malek and Appleby 1984).

At various times pine wilt was reported to be caused by many different agents, such as bark engravers, drought, off-site planting, fungal infection, adverse soil conditions, rodent injury, lightning,

Figure 1. Red pine (*Pinus resinosa* Ait.) at the University of Massachusetts at Amherst exhibiting reddish brown needle coloration similar to pine wilt disease.

and other forms of stress (Kobayashi 1984, Malek et al. 1982 and Robbins 1979). In 1969, Kiyohara and Tokishige first indicated the nematode, B. xylophilus, as the primary pathogen of pine wilt disease in Japan. There was at the time of discovery only one other known instance of a migratory endoparasitic nematode causing a vascular disease. Rhadinaphelenchus cocophilus, which causes red ring of coconut, is an economically important disease of coconut and oil palms in the West Indies and South America (Blair and Darling 1968). The only other known wilt disease of pine had been reported by Basham (1970) on loblolly pine and was caused by a blue-stain fungus Ceratocystis (Mamiya 1983).

Pine wilt disease first occurred in 1905 in Japan and has since developed into a serious epidemic there. It has been estimated that twenty-five percent of Japan's 2.6 million ha of pine forest has been affected. Losses of pine 1.5 million m^3 was lost. In 1980, the Japanese government spent 35 million dollars on control of pine wilt (Nickle et al. 1981, Mamiya 1980, 1983a,b).

Since the pinewood nematode's discovery in the United States in 1979 by Dropkin and Foudin, much interest, research, and controversy has surrounded pine wilt disease. There have been several symposia, a joint meeting between U.S. and Japanese scientists and approximately 50 papers published by American researchers. In some areas of the United States the pinewood nematode has proven to be of economic importance (Adams and Morehart 1981, 1982, Bergdahl 1982, Birchfield et al. 1981, Dropkin 1982, Dropkin et al. 1981, Nickle 1982, Robbins 1982, Wingfield 1982, and Wingfield & Blanchette 1984). In Illinois, losses of trees in Christmas tree plantations and in urban settings due to pine wilt disease have been reported (Malek and Appleby 1984 and Malek *et al*. 1981). In Massachusetts, *B. xylophilus* has been isolated from dying Japanese black (*Pinus thunbergiana* Franco), red (*P. resinosa* Ait.) and Scots pine (*P. sylvestris* L.) on Cape Cod (Dorrance and Rohde, unpublished).

Japanese black pines originally were planted in 1895 in Wauwinet, Nantucket, because of their salt tolerance. They are commonly used to help prevent soil erosion and to stabilize beaches (Jones 1930). These trees have also been used as landscape trees for many homes. Since 1983, losses of Japanese black pines due to pine wilt disease have been reported along the eastern shore line from Cape Cod to N. Carolina (Nickle 1984).

Death of Japanese black pines on Cape Cod has also been associated with blue-stain fungi and black turpentine beetles (Highley 1983). In some instances populations of *B. xylophilus* and *Monochamus* spp. have also been found in declining trees (Dorrance and Rohde, unpublished). This study was undertaken to determine what role the pinewood nematode plays in pine tree decline and death on Cape Cod as well as the rest of the state of Massachusetts.

CHAPTER II

LITERATURE REVIEW

Disease Cycle

Two extension bulletins, Battenfield and Bird (1983) and Malek et al. (1984), give a detailed description of the pine wilt disease cycle. *Bursaphelenchus xylophilus* is introduced into healthy trees in the early summer during maturation feeding by adult Long-horn beetles (Coleoptera: Cerambycidae) following beetle emergence from dead trees. The nematodes are carried in the respiratory organs of these beetles and as the beetle feeds on the new expanding growth of the pine trees, the nematodes enter through the feeding wound (Dropkin and Linit 1982, Kobayashi et al. 1984, Luzzi et al. 1984, Mamiya and Enda 1972, Wingfield 1982). They move through the resin canals where they multiply and feed on the epithelial cells as well as parenchyma cells in the cortex (Dropkin and Linit 1982, Myers 1982, Mamiya 1972 and Mamiya 1980a,b). Tree response is a reduction in oleoresin flow and disruption in transpiration with eventual wilt and tree death. This may take as little as six to eight weeks from the time that nematodes are initially introduced into the tree (Dropkin and Linit 1982 and Mamiya 1972).

After maturation feeding on healthy trees, adult beetles oviposit in stressed or dying trees (Baker 1972, and Kobayashi et al. 1984). Eggs hatch in six to nine days and the larvae bore into the wood and overwinter. The nematodes also overwinter in wood, as third-stage juveniles which are high in lipid content, food reserves and possess a thick cuticle. In the spring, the insect pupates and molts to an adult where it remains in the tree for four to six days (Appleby 1982, Kobayashi et al. 1984, Yamane 1975 and Wingfield 1982). Nematodes begin to accumulate in the tracheids and resin canals surrounding the pupae. At the callow adult stage, nematode dauer larvae, or dispersal stage, enter the beetle through its spiracles. Cerabycid beetles then emerge from the dead tree to begin maturation feeding and continue the cycle carrying the nematodes and releasing them into healthy trees (Dropkin and Linit 1982, Kobayashi et al. 1984, Luzzi et al. 1984, Mamiya and Enda 1972, Mamiya 1976, 1983, Wingfield and Blanchette 1983a,b, and Wingfield et al. 1982a,b). Another means of transmitting B. xylophilus is through oviposition. Female beetles carve niches into dying or dead trees and lay their eggs. At this time nematodes also enter the tree and immediately colonize the area beneath the wound (Wingfield 1983, Wingfield and Blanchette 1983a,b, and Wingfield et al. 1982).

The Nematode

Discovery of the pinewood nematode in Japan by Kiyohara and Tokushige (1969) led to the search for the nematode's origin. Mamiya and Kiyohara (1972) originally named the pinewood nematode, Bursaphelenchus lignicolus, believing it to be a new species. After discovery of the pinewood nematode in the U.S. (Dropkin and Foudin

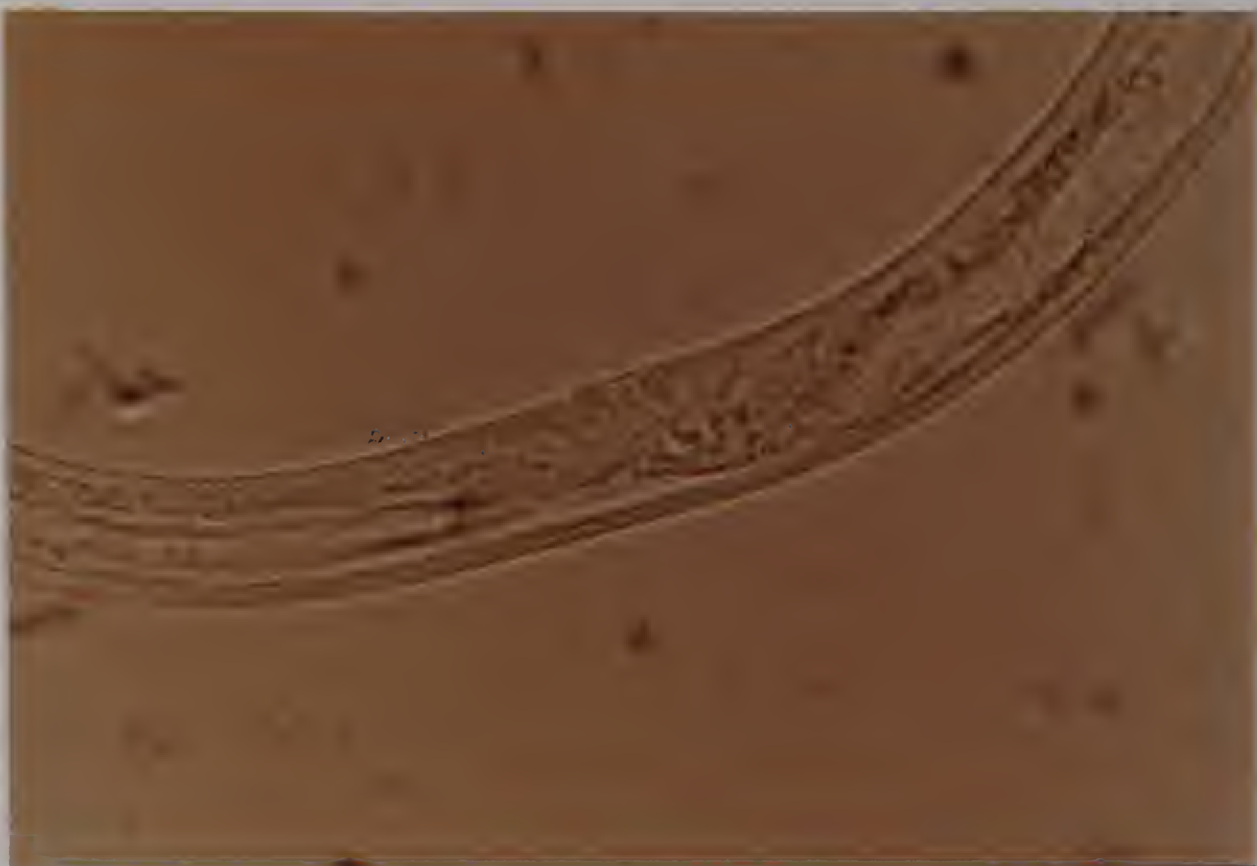
1979) taxonimists searched the U.S. collection to determine how long the nematode had been present in the U.S. Steiner and Buhrer (1934) had previously described, Aphelenchoides xylophilus, recovered from a blue stained log of Pinus palustris Mill. in Louisiana (Nickle and Golden 1980). The original nematode specimens were found in the collection and comparisons between the U.S. and Japanese nematodes determined them to be the same (Nickle et al. 1981). The earliest record of the pinewood nematode in the United States is from Texas, October 15, 1929, recovered from Pinus palustris found on a preserved slide in the USDA collection (Nickle et al. 1981). Nickle et al. (1981) redescribed the species as Bursaphelenchus xylophilus after successful mating studies were completed between the U.S. and Japanese populations. B. xylophilus is now believed to have originated in the United States and to have been transported to Japan, where the different pine species are more susceptible to pine wilt disease (Nickle et al. 1981, Holdeman 1980, and Mamiya 1983).

Unique characteristics of B. xylophilus include large rosethorn shaped spicules with an expanded tip and strongly arcuate tails in the males. The females have a characteristic overlapping vulval flap (Figures 2 and 3). B. xylophilus can be distinguished from B. mucronatus, a closely related species, by the presence of a digitate tail tip (Nickle 1970, Nickle et al. 1981 and Tarjan and Aragon 1982).

Mamiya (1975) first described the life history of B. xylophilus. Eggs took 26 to 32 hours to hatch in water at 25°C. Four molts occur as in other nematodes and the first occurs in the egg. Feeding is

Figure 2. *Bursaphelenchus xylophilus* female with overlapping vulval flap. (100 X)

Figure 3. *Bursaphelenchus xylophilus* male with rosethorn shaped spicules with an expanded tip. (100 X)



necessary for further development to proceed normally. At 25^{o}C, nematodes reached the adult stage in four to five days. Mamiya (1975) also noted that copulation is necessary for reproduction. Female adults lived an average of 15 days up to a maximum of 32. Females need to be inseminated periodically for continued egg production. Eggs were laid at an average of 79 over a 28-day period with a maximum of 216 eggs over the same period of time (Mamiya and Furukawa 1977).

B. xylophilus also has two juvenile stages besides the propagative stages. Survival third stage and dispersal fourth stage or dauer larvae are the dispersal forms (Ishibashi and Kondo 1977). The survival stage ($L_{III's}$) are better adapted to survive under starvation conditions. After three deaths, there is an overall drop in total nematode population followed by an increase in the ratio of $L_{III's}$ present (Ishibashi and Kondo 1977 and Mamiya 1976). An increase of $L_{III's}$ has also been observed in laboratory cultures that have been stored for long periods of time without subculturing. The exact stimulus for the formation of the L_{III} stage is unknown.

Molting of $L_{III's}$ to the dauer larvae (L_{IV}) stage cannot be induced without the presence of a stimulus from a pupating insect (Ishibashi and Kondo 1977). The authors suggest that a possible insect hormone may be involved, for the rate of L_{IV} increased as pupation of beetle continued. When the insect was removed $L_{III's}$ left the insect gallery and twenty days later had still failed to molt to $L_{IV's}$.

The $L_{III's}$, the overwintering stage, have stored food reserves and contain high levels of lipids to aid in their survival. In the dauer larvae internal structures are absent or poorly developed; the median bulb, stylet, and intestinal tract undergo histolysis. Presumably larvae in this stage are better adapted to survive dry conditions. Dauer larvae have been found still viable after six months in dried insect cadavers (Kondo and Ishibashi 1978).

Ishibashi et al. (1978) described suppressed gonad development in dispersal forms when compared to propagative juveniles. Dispersal forms had little gonad development after the second molt. According to the authors this ability serves to conserve energy for the long survival time. They also reported on the increase in time needed for fourth stage juveniles to molt to adults. At 25°C, five days were needed for $L_{IV's}$ to molt to adults. Propagative juveniles complete an entire life cycle in the same amount of time.

Botrytis cinerea is used routinely to maintain nematode populations in culture (Dozono and Yoshida 1974, Dropkin et al. 1981, Kondo et al. 1982, Mamiya 1978, and Wingfield 1983). Several other fungi have also been reported to be favorable for nematode multiplication; Pestalotia spp., Rhizosphaera sp., Ceratocystis spp., Diplodia, and Fusarium sp. (Dropkin et al., 1981, Kobayashi et al. 1974, Kobayashi et al. 1975, and Mamiya 1976). Kiyohara (1976) reported that continued subculturing of B. xylophilus on a fungal mat resulted in a decrease in pathogenicity as well as decreases in propagation rate and survival in adverse conditions. The nematode population was not able to molt to $L_{III's}$ as readily under starvation conditions with repeated subculturing. *B. xylophilus* is also able to maintain populations on pine callus tissue (Tamura and Mamiya 1979).

Insect Associates

Pinewood nematode lives in association with several different insects (Table 1). The majority of nematodes are found in the tracheae of long-horn beetles in the Genus *Monochamus* (Coleoptera: Cerambycidae). There are also a few reports from the U.S. of associations between these nematodes and members of the Buprestidae and Curculionidae (Kondo *et al*. 1982, Linit 1982, Linit *et al*. 1983, Wingfield 1983 and Wingfield and Blanchette 1983a,b). *Monochamus alternatus* (Hope) is the principle vector of *B. xylophilus* in Japan. Insects may carry an average of 10,000 nematodes per beetle with reports of up to 230,000 nematodes in one beetle. In forests with severe infestations, 70-100% of the beetles may carry the nematode (Kobayashi 1978). In the U.S. *Monochamus carolinensis* (Oliver) has been reported to carry similar numbers of nematodes per beetle. The large numbers of pinewood nematodes found in association with *M. carolinensis* is the reason it is thought to be the main vector in this country (Kondo *et al*. 1982, Linit 1982, Linit *et al*. 1983).

The life cycles of these two longhorn beetles are similar. *M. alternatus* and *M. carolinensis* are attracted to recently dead trees or to freshly cut logs to oviposit (Malek 1982). On late summer nights the beetles fly to these dead or dying trees. Copulation occurs and females carve funnel shaped niches in the bark with their mandibles

Table 1. Insects associated with Bursaphelenchus xylophilus in Japan and the United States.

Insects	References
Japan	Coleoptera: Cerambycidae
Acalolepta fraudatrix Bates	Kobayashi *et al*. 1984 Mamiya 1976
Acanthocinus griseus Frabricus	Kobayashi *et al*. 1984 Mamiya 1976
Aropalus rusticus Linne	Kobayashi *et al*. 1984 Mamiya 1976
Corymbia succedanca Lewis	Kobayashi *et al*. 1984
Monochamus alternatus Hops.	Kobayashi 1978 Kobayashi *et al*. 1984 Mamiya 1976 Mamiya and Enda 1972 Morimoto and Iwaski 1972
Monochamus nitens Bates	Kobayashi 1978 Kobayashi *et al*. 1984 Mamiya 1976 Mamiya and Enda 1972 Morimoto and Iwaski 1972
Corymbia succendanca Lewis	Kobayashi *et al*. 1984 Mamiya 1976
Spondylis buprestoides Linne	Kobayashi *et al*. 1984 Mamiya 1976
Uraecha bimaculata Thompson	Kobayashi *et al*. 1984 Mamiya 1976
United States	Coleoptera: Cerambycidae
Amniscus sexguttatus (Say)	Kondo *et al*. 1984 Linit 1982 Linit *et al*. 1983 Malek *et al*. 1982 Wingfield 1983
Arhopalus rusticus obsoletus (Rand)	Dropkin et al. 1981 Kondo *et al*. 1982 Linit *et al*. 1983 Wingfield 1983
Assum seriatum L.	Linit 1982 Linit *et al*. 1983

Table 1, cont'd.

Neocanthocinus pusilus (Kirby)	Wingfield 1983 Wingfield *et al*. 1983
Monochamus carolinensis (Oliv.)	Appleby 1982 Dropkin *et al*. 1981 Kondo *et al*. 1982 Linit 1982 Linit *et al*. 1983 Malek *et al*. 1982 Malek and Appleby 1984 Wingfield 1983 Wingfield and Blanchette 1983
Monochamus marmorator Kirby	Wingfield 1983 Wingfield and Blanchette 1983
M. mutator Lec.	Wingfield 1983 Wingfield and Blanchette 1983
M. obtusus (Casey)	Dropkin *et al*. 1981
M. scutellatus (Say)	Dropkin *et al*. 1981 Nickle *et al*. 1980 Wingfield and Blanchette 1983
M. titillator (Fabricus)	Dropkin *et al*. 1981 Luzzi and Tarjan 1982 Luzzi *et al*. 1984
Xylotrechus saggitatus	Wingfield and Blanchette 1983
	Coleoptera: Buprestidae
Chrysobothris sp.	Kondo *et al*. 1982 Linit 1982 Linit *et al*. 1983 Wingfield and Blanchette 1983
	Coleoptera: Curculionidae
Hylobius pales (Herbst.)	Kondo *et al*. 1982 Linit *et al*. 1983 Wingfield and Blanchette 1983
Pissodes approximates Hopkins	Kondo *et al*. 1982 Linit *et al*. 1983 Wingfield and Blanchette 1983

and deposit an average of one egg per niche (Appleby 1982, Kobayashi 1978, Kobayashi et al. 1984, Linit et al. 1983, Malek et al. 1982 and Yamane 1975). The eggs hatch within six to nine days and the first and second instar larvae feed on the inner bark (Appleby 1982, Kobayashi et al. 1984, Malek et al. 1982 and Yamane 1975). Third instar larvae tunnel into the sapwood and overwinter there as fourth instars. Pupation occurs in the spring (April and May for both Japan and Illinois) and lasts for seventeen to nineteen days for M. alternatus (Kobayashi et al. 1984). The pupae transforms to a callow adult and remains stationary for four to eight days (Kobayashi et al. 1984 and Yamane 1975). Adult M. alternatus emerge mostly at night from June to August and begin maturation feeding on succulent new growth of pines for three to four weeks (Kondo et al. 1982, Malek et al. 1982, Wingfield 1983 and Yamane 1975). The life cycle of M. alternatus occurs within one year but occasionally may carry over into two years (Kobayashi et al. 1984 and Yamane 1975). Monochamus spp. in the U.S. may take two or more years in northern regions to complete a life cycle while in more southern areas less than a year is needed (Dillon and Dillon 1941 and Rose 1957).

Monochamus alternatus has been reported from many tree species. Eighteen species of Pinus, three of Picea and one species of Abies, Cedrus and Larix. The preferred hosts are Pinus spp., Cedrus deodora and Larix leptolepsis (Kobayashi et al. 1984). In the U.S. laboratory tests Monochamus carolinensis fed readily on a variety of pine species including; P. sylvestris, P. echinata, P. strobus, P. banksiana and

P. nigra (Kondo et al. 1982). The pine sawyers have been reported to reproduce on Pinus, Picea, Abies, Pseudotsuga and Larix (Dillon and Dillon 1941).

Mamiya and Enda in 1972 first reported transmission of B. xylophilus by M. alternatus. M. carolinensis has been reported to transmit nematodes during feeding on Scots (Linit 1982) and red pine seedlings as well as during oviposition (Malek and Appleby 1984, Wingfield and Blanchette 1983a,b). Beetles infested with dauer larvae were allowed to feed on excised branches, seedlings, or on branches of ten to twenty-year-old trees (Holdeman 1980, Linit et al. 1983, Luzzi et al. 1984, Mamiya and Enda 1972, Wingfield and Blanchette 1983a,b). M. carolinensis did not transmit B. xylophilus after feeding on P. echinata, P. strobus, P. banksiana or P. nigra (Linit 1982). Pieces of wood taken from the feeding site did not contain nematodes.

M. titillator has also been reported to transmit B. xylophilus during feeding and oviposition on slash pine in Florida (Luzzi et al. 1984 and Luzzi and Tarjan 1982) and in Iowa during feeding on red pine (Holdeman 1980). Malek and Appleby (1984) reported transmission of B. xylophilus to Scots pine during feeding but with a less than 30% frequency. Wingfield and Blanchette (1983b) reported that transmission did not occur on seedlings until after beetles had fed for two weeks. They also reported that transmission was more common during oviposition.

Cerambycid beetles have been dissected to determine where the nematodes are transported within insect bodies. The thoracic region contains most of the nematodes with only a few located in the head and abdominal regions. Nematodes were isolated from metathoracic spiracles as well as tracheae of the metathoracic, abdominal, antennae and leg regions (Kobayashi et al. 1984, Kondo et al. 1982, Linit et al. 1983, and Mamiya and Enda 1972).

Histopathology of Infected Pines

Several studies by Bergdahl and Smeltzer (1981), Mamiya (1972a), Mamiya (1980), Myers (1982), and Sasaki et al. (1984) discuss the histopathology of infected seedlings. Nematodes enter the tree through insect feeding wounds and move into the resin canals, cambial zone and cortex from this exposed xylem (Mamiya 1980). Mamiya (1972) reported that the axial and radial resin canals were the major sites used by the nematode for infestation and movement throughout the tree while epithelial cells served as feeding sites. Cross sections of inoculated seedlings showed damaged epithelial cells and parenchyma tissue of the cambium phloem and cortex (Mamiya 1980). Myers (1982) reported that young seedlings were girdled by the destruction of epithelial and parenchyma cell resulting in formation of large spaces which surrounded the cambium. These large spaces or excessive traumatic resin ducts were sites for nematode reproduction and contained large numbers of eggs. Sasaki et al. (1984) using water soluble stains determined that death of seedlings was a result of complete damage to the cambium. Comparisons between infected larch and red pine seedlings were made by Bergdahl (1982). He reported that

B. xylophilus was limited to the phloem, cortex and resin canals of the bark in larch seedlings while in red pine nematodes were found in the longitudinal and radial resin canals of xylem as well as the bark tissues.

Death of ray and axial parenchyma cells occurs within 24 hours of inoculation (Mamiya 1980a,b). Reduction in oleoresin flow did not occur until six to nine days after inoculation. Nematode populations increased prior to the reduction in transpiration which occurs 20-35 days after inoculation in seedlings (Koyohara and Suzuki 1977 and Mamiya and Tamura 1977). Nematodes are evenly distributed within seedlings at every stage of symptom development (Kiyohara and Suzuki 1978).

Other Factors Associated With Pine Wilt Disease

Oku *et al*. (1979) first reported the presence of toxic metabolites in diseased pines. Since then, several researchers have investigated the possible involvement of toxins in pathogenesis of *B. xylophilus* (Bolla *et al*. 1982, 1984, Oku 1984, Oku *et al*. 1980 and Shaheen *et al*. 1984). Internal symptoms of pine wilt, a decrease in oleoresin flow in three to five days, followed by a decrease in water transpiration begin long before nematode populations have increased to levels at which external symptom development occurs. Extracts from diseased pines, when added to healthy seedlings, reproduce the symptoms of nematode infected trees. Extracts from healthy and girdled trees give no such response (Oku 1984 and Shaheen *et al*. 1984).

Several different compounds have been identified with wilt producing ability. Oku (1984) isolated benzoic acid and 8-hydroxycarvotanacetone from naturally infected P. thunbergiana, which produced wilt symptoms when added to susceptible seedlings. Bolla et al. (1982) isolated toxic compounds from naturally infected P. sylvestris. These compounds, oxygenated monoterpenes, are thought to be derived from natural resin components of the tree and also produce wilt symptoms when added to seedlings.

Shaheen et al. (1984) reported on the specificity of the toxin extracted from nematode infected P. sylvestris. When the toxin was added to various pine seedlings, a longer time was necessary for symptom development in white pine than Scots; no symptoms were produced in Jeffery pine seedlings. Symptom development from toxin injection are similar to those in nematode inoculated seedlings. The authors suggested that phytotoxin production was linked to restriction of water flow in inoculated seedlings. An increase in stomatal closure occurred in infected seedlings which corresponded with increased phytotoxin production. As more stomates closed, more phytotoxin was extracted followed by eventual wilt of the seedling.

Researchers have also studied these compounds to determine if they may be phytoalexins. Toxic compounds are synthesized shortly after infection but do not make the host resistant to infection of B. xylophilus (Bolla et al. 1984). Oku (1984) reported at low concentrations, 10-30ug/ml of benzoic acid and 3ug/ml of 8-hydroxycarvotanacetone stimulated pinewood nematode multiplication. At higher concentrations, 8-hydroxycarvotanacetone (30ug/ml) inhibited nematode multiplication. The oxygenated monterpenes, compounds originally isolated from Scots pine, have shown limited affects against Ceratocystis ips and B. xylophilus in vitro (Bolla et al. 1984).

Higher temperatures and water stress are reported to favor disease development. Suzuki and Kiyohara (1978) found that pines failed to develop pine wilt disease in the absence of some form of water stress. They described a decline in transpiration that occurs naturally in pines as a means of drought tolerance. During this phase of decreased transpiration, pinewood nematodes increased rapidly within trees and wilt soon followed (Suzuki 1984). Hunt et al. (1984) reported similar results with greenhouse seedling inoculations. In other seedling studies, high temperatures, 25-30°C, resulted in wilting while inoculated seedlings maintained at lower temperatures (20-15°C) failed to develop symptoms (Kiyohara 1977 and Mamiya 1983).

In the U.S. several reports show differences in pathogenicity to native pine species (Dropkin et al. 1981, Dropkin and Linit 1982, Kobayashi 1978, Kondo et al. 1982, Mamiya 1972, 1983, and Wingfield et al. 1982, 1984). Mamiya (1983) reported the three most susceptible species to pinewood nematode in Japan are: P. densiflora, P. thunbergiana, and P. luchiensis, while others are resistant. Highly resistant pines have included P. echinata, P. banksiana, P. strobus, P. pungens, P. palustrus, and P. taeda (Kobayashi 1978). Smith (1981) reported P. echinata, P. banksiana, P. radiata, P. lambertiana and P. sylvestris as more susceptible than P. jefferyi and P. strobus.

Differences in pathogenicity have been reported among isolates of *B. xylophilus* from various areas in the U.S. and in Japan. Kondo *et al*. (1982) found *B. xylophilus* from Japan was more pathogenic to Scots pines than an isolate from Missouri. Wingfield *et al*. (1983) reported on differences between *B. xylophilus* recovered from balsam fir (*Abies balsamea* (L.) Mill.) and from pine species. Although slightly different in morphology, the two isolates were concluded to be the same species after mating experiments were completed. The balsam fir isolate was only pathogenic on balsam fir seedlings and not to pine when compared in inoculation experiments.

Several control strategies for pine wilt disease aim at various parts of the disease cycle. Most management strategies are aimed at the insect to prevent further spread of the disease. The major control has been sanitation. Infected trees are removed and burned or chipped. Such practices have been utilized with some success in Delaware and Illinois as well as Japan (Green 1982, Holdeman 1980, Kobayashi *et al*. 1984 and Nickle 1984).

Due to massive tree death in Japan, logs have been sprayed with insecticides which resulted in 95-100% mortality of beetles depending on the material used. Trees have also been sprayed to prevent maturation feeding of insects (Holdeman 1980, Kobayashi *et al*. 1984 and Mamiya 1976). Matsuura reported in 1981 and 1984 on the use of nematicides in trees. He found that systemics higher in water solubility are able to reach higher concentrations in pines.

Japanese scientists are now looking for alternative tree species and resistant varieties to plant in place of *P. densiflora* and *P. thunbergiana* (Kobayashi *et al*. 1984 and Holdeman 1980). *Beauvaria bassiana*, a fungus pathogenic on beetle larvae and adults, and *Steinernema feltiae* an entomogenous nematode, are being studied for use as potential biocontrol agents (Kobayashi 1978 and Kobayashi *et al*. 1984). Beetle attractants have also been studied. The combination of a-pinene and ethanol are used as attractants as well as paraquat treated trees. These traps are only effective for ovipositing females. This control measure is aimed at future populations of Cerambycid beetles; it will not deter any increase in disease incidence in the current year (Ikeda *et al*. 1980, Kobayashi *et al*. 1984 and Yamaski *et al*. 1980).

CHAPTER III

MATERIALS AND METHODS

Inoculum Preparation

To determine the pathogenicity of the Massachusetts isolate of B. xylophilus several greenhouse and field inoculations were implemented. B. xylophilus to be used for inoculum was collected from the main stem of recently dead red pine trees displaying symptoms of pine wilt, i.e. bright reddish-brown foliage. Inoculum was started with small sections of wood incubated on two percent Potato-dextrose agar (Difco) with Botrytis cinerea to allow nematodes to multiply. Nematodes were extracted in a modified Baermann funnel (Southey 1970) for twelve to sixteen hours and surface sterilized with 2.5% solution of 1:1000 merthiolate for two hours followed by three washings of sterile distilled water. B. xylophilus males and females were placed on sterile cover glass slips, transferred to PDA plates and incubated at 25^{o}C on B. cinerea. A new isolate was started from the same location in Barnstable County, Massachusetts, each spring to avoid changes in nematode pathogenicity from prolonged culturing (Kiyohara 1976). Nematode isolate Bx-1 was used in 1983 inoculation studies and FTF-84 was used in 1984. B. cinerea was also placed in a Baermann funnel to produce a spore suspension for control treatments.

Seedling Inoculations

Inoculation studies were carried out in the greenhouse during the summers of 1983 and 1984. Two-year-old seedlings of Scots, Japanese black and red pine growing in a sand, soil and peat mix in four-inch plastic pots, were inoculated with 2,000 nematodes per seedling with 15 replicates of each treatment in 1983. In 1984, ten seedlings each of Japanese black, Austrian and white pine were inoculated with 2,000 nematodes each with three Botrytis inoculated control seedlings for white and Austrian and two controls for red pine. An incision was made in the stem with a razor blade exposing the sapwood. The bark was folded back, a folded paper tissue was placed over the wound and then wrapped with parafilm to form a funnel. One ml of either nematode suspension or Botrytis spores was added to each of the seedlings. The following were treatments for 1983: (i) unsterilized Bx-1; (ii) Bx-1 on B. cinerea; (iii) B. xylophilus isolate from V. H. Dropkin; and (iv) B. cinerea. In 1984 only FTF-84 on B. cinerea was used for seedling studies.

Thirty-five three-year-old seedlings of Austrian and Japanese black and 30 of white pine were inoculated with 2,000 nematodes and maintained outdoors in pots. There were five controls of each pine species. Every 14 days three to five nematode inoculated seedlings of each tree species were removed from their pots, the roots were washed, seedlings were weighed, cut into small sections and placed in a Baermann funnel. Nematodes were recovered after 24 hours and the numbers of B. xylophilus per gram of wood recorded. Comparisons were made among the three groups of the number of nematodes per gram of wood and the nematode population fluctuations over time.

Two field experiments used 80 two-year-old larch, 70cm in height and 21 four-year-old, one meter in height Japanese black pine seedlings at Montague fields, Amherst, Massachusetts. Japanese black pine were planted in February of 1983, and inoculated in July, 1984. Larch seedlings were planted in November 1983, and inoculated in July, 1984.

Ten of the Japanese black pine seedlings were inoculated with one ml suspension of 2,000 nematodes of FTF-84 and 11 controls inoculated by the procedure described above. The larch seedlings consisted of four treatments: (i) LG-1, an unknown species of Aphelenchoides, recovered from a dying larch, Gardner, Massachusetts; (ii) Bx-1; (iii) FTF-84; and (iv) B. cinerea spore suspension. Each treatment was replicated on 20 trees and another 12 seedlings were left untreated. In October, seedlings were harvested and approximately 20cm of the main stem, including the inoculation site, was sampled for B. xylophilus.

Plantation Inoculations

Six plots were established on Cape Cod and at Mt. Toby Reservation in Massachusetts in the summers of 1983 and 1984. Table 2 gives a brief summary of all these plots, their locations, age and height of trees, amount of inoculum and dates of inoculation and harvest. Plots were selected because they were scheduled to be thinned or clearcut in the following two years.

Table 2. Summary of field inoculations of Red (1,3-4), Japanese black (2) and Pitch (5) with *B. xylophilus* in Massachusetts.

Study	Location	Age/height	Inoculum n/tree	Number inoculated	Date inoculated	Date harvested
1	Mt. Toby Franklin County	30/13.7m	30,000	20n[1] 20c	7/15/83	9/18/84
2	Rebello's Nursery Falmouth	2/0.6m	2,000	1n 1c	6/9/83	6/7/84
	Barnstable County	18/5.5m	4,000	1n 1c	6/9/83	3/22/84 6/7/84
		6/2m	2,000	1n 1c	6/8/83	3/22/84 6/7/84
		--/4.5m	6,000	1n	6/9/83	9/9/83
3	Mt. Toby Franklin County	40/19.5m	15,000	15n 5c	7/6/84	10/27/84
4	Mt. Toby Franklin County	24/7.6m	15,000	15n 5c	7/6/84	10/24/84
5	Falmouth Town Forest Barnstable County	28/11.3m	2,000	10n 5c	7/12/84	9/13/84
6	Falmouth Town Forest Barnstable County	50/9.1m	3,000	4n 1c	7/12/84	9/13/84

[1] n = *B. xylophilus*, c = *Botrytis* spore suspension

Six Japanese black pines, at Rebello's Nursery, Falmouth, Massachusetts, ranging in age from two to 22 years, were inoculated with a suspension of 2,000 to 6,000 nematodes. The inoculation procedure was similar to previous inoculations except that incisions were made on branches of the tree vs. the main stem of the seedling. Trees were cut and sampled after symptom appearance or on June 7, 1984.

Eighteen red pine trees with branches six feet from the ground were selected at Mt. Toby Reservation Forest in Sunderland, Massachusetts. One to three branches of each tree were inoculated with a suspension of 30,000 nematodes per tree. Nine trees were treated with B. xylophilus and nine with a Botrytis spore suspension. Two trees, a treated and control, were cut and dissected to determine the presence of nematodes within the tree in September, 1983. The remaining 16 trees were cut in August and September of 1984. Samples were taken from the original inoculated branches and at 1.5 meter intervals along the main stem and samples were processed as before.

Red and Pitch pines at Mt. Toby Reservation and the Falmouth Town Forest were also inoculated with B. xylophilus. An increment core was taken from the main stem of each tree approximately one meter from the ground. A suspension of B. xylophilus or Botrytis spores was introduced into the wound which then sealed with parafilm. Symptom development was monitored periodically until harvest. At harvest the trees were felled, and samples were taken from the inoculation site, as well as 50 cm above and below the inoculation wound.

State Survey

A partial state survey was conducted during the course of this study. Letters were sent to private consulting foresters and to county foresters throughout the state of Massachusetts. Articles about the pinewood nematode were also sent for publication to the New England Branch of the Professional Grounds Management Society newsletter and Tree News published by Massachusetts Cooperative Extension Service. Surveys of known declining pine and larch plantings in Berkshire, Northern Worcester, Middlesex, Barnstable counties were conducted in August and September, 1983, with the help of the State of Massachusetts Department of Environmental Affairs, Bureau of Insect and Pest Control. Approximately thirty samples were collected from suspect trees via increment cores from the main stem, branch sections and/or wood chips from logs on the ground. Subsamples were taken of 30 to 80 grams fresh weight depending on the amount of wood collected and placed in Baermann funnels. Increment cores were sampled directly and placed in test tubes with water or Syracuse dishes. These were checked at 24 to 48 hours for the presence of *B. xylophilus*.

Histopathology

Sections of wood from the inoculation site of two red pine trees, a control and inoculated, from the 1983 stem inoculation study were sampled in September, 1984. Wood blocks were stained in Goodey's formula of acid fuchsin in lactophenol (McBeth *et al*. 1941) for one minute, rinsed in running tap water and stored in glycerin. Sections, 40 to 60 microns thick, were cut with a sliding microtome and observed with a microscope.

Vector Studies

Sections from the main stem of trees displaying symptoms of pine wilt were collected from Falmouth Town Forest, Rebellos Nursery and Granby in January, 1983; March and April, 1984. Samples were processed to determine the presence of B. xylophilus. The bark was also peeled from a small portion of these logs to determine the presence of Monochamus spp. The logs were then placed in barrels originally used for collecting elm bark beetles. These barrels consisted of 55-gallon drums which had three holes cut along one side where mesh funnels were attached. Glass bottles were placed over the end of the funnels and were checked periodically for emergence of insects.

After emergence, beetles were collected and processed to establish the presence of nematode dauer larvae. Beetles were dissected while still alive, wrapped in a paper tissue and placed in a Syracuse watch glass or petri dish. If the intact beetle was needed for identification purposes, the insect was allowed to "swim" in a petri dish with distilled water. Nematode dauer juveniles recovered from insects were placed on cultures of B. cinerea. After ten days, cultures were examined for the presence of adult B. xylophilus.

In 1983, five Monochamus carolinensis beetles were allowed to feed on two-year-old red pine seedlings in cages and maintained outdoors. After feeding for four days, the trees were placed in a greenhouse to determine if pine wilt would develop.

To determine if nematodes could be transmitted through oviposition, several logs from a healthy red pine were placed on the ground at the Falmouth Town Forest, Barnstable County, on July 12, 1984. Logs were taken from a recently felled red pine and cut into sections averaging one meter in length. In September, 1984, the logs were collected, sampled for B. xylophilus and the numbers of larvae and Scolytids were determined.

CHAPTER IV

RESULTS AND DISCUSSION

Seedling Studies

Three-year-old pine seedlings, when inoculated with the Massachusetts isolate of *B. xylophilus*, readily developed pine wilt disease in both 1983 and 1984 greenhouse trials. Typical symptoms of pine wilt disease were reproduced and infected seedlings changed from green to bright reddish brown, whereas control trees remained green (Figure 4).

In the 1983 greenhouse study, symptom development occurred as early as 24 days after inoculation (Table 3) in Japanese black, red and Scots pines. Nematodes were recovered from 33 out of the total 104 seedlings of all three species inoculated. Verification of nematodes from these seedlings was determined over several months after symptom development and seedling death. The nematodes may have been unable to survive in the seedlings under greenhouse conditions over this extended period of time.

In 1984, three-year-old seedlings of Japanese black, white and Austrian pine were inoculated with *B. xylophilus*. All trees inoculated with the pinewood nematode developed symptoms of pine wilt and died. Japanese black pine seedlings contained significantly greater numbers of nematodes than Austrian but not white pine (Table 4).

Japanese black, Austrian and white pine seedlings were used to

Figure 4. Scots pine (*Pinus sylvestris* L.) inoculated with (left to right) 2,000 *B. xylophilus* from Missouri, 2,000 *B. xylophilus* from Massachusetts and *Botrytis* spores. 1983 greenhouse study.

Table 3. Number of three-year-old pine seedlings with Pine wilt foliar symptoms after inoculation with three different populations of B. xylophilus and Botrytis spores, 24 days after inoculation in the greenhouse 1983[a].

Treatment	Seedlings[b] Japanese black	Red	Scots
Unsterile, Bx-1	5	12	...
Bx, Dropkin	10	11	1
Bx-1, Botrytis	10	11	0
Botrytis	0	0	0

[a]Symptom expression included seedlings with reddish-brown needles and wilted to yellowing needles.

[b]Japanese black and red pine seedlings, 15 trees/treatment Scots pine, 7 trees/treatment.

Table 4. Comparison of Japanese black, white and Austrian pine three-year-old seedlings in greenhouse studies at 60, 54 and 59 days, respectively, after inoculation.[1]

Seedling	No. of nematodes/gram of wood[2]
Japanese black	153.752 ± 16.469 a[3]
White	100.076 ± 16.575 ab
Austrian	16.738 ± 18.224 b

[1]Seedlings were inoculated with 1500 Bursaphelenchus xylophilus, adults and juveniles from cultures of Botrytis cinerea and maintained in the greenhouse.

[2]Average of ten seedlings.

[3]Means not followed by the same letter differ significantly (P 0.05) according to Duncan's multiple range test.

monitor population fluctuations of *B. xylophilus* over time (Figure 1). These potted seedlings were maintained outdoors to decrease some of the large temperature fluctuations which occur in the greenhouse. As mentioned earlier, white pine is described as resistant, Austrian--moderate, and Japanese black highly susceptible (Kobayashi 1978, Kondo *et al*. 1982, Mamiya 1983 and Smith 1981). When comparisons were made at two-week intervals, significant differences in the numbers of *B. xylophilus* present in the seedlings of the three pine species occurred only at six and eight weeks after inoculation. Japanese black and Austrian pine had significantly greater numbers of nematodes than white at six weeks after inoculation. At eight weeks after inoculation Japanese black pine had significantly greater numbers of nematodes than Austrian but not white. In previous inoculation studies, differences in susceptibility of pine species to the pine-wood nematode have been monitored by the number of seedlings dying and the nematode populations within diseased seedlings (Kondo *et al*. 1982). As depicted in Figure 5 these results can be misleading. To determine actual resistance to infection of the pinewood nematode other methods should be tested to give more reliable results, such as field inoculations of the different species at different sites under natural conditions.

Potted seedlings studies have received much criticism because they do not approximate field conditions (Holdeman 1980, Mamiya 1983 and Wingfield *et al*. 1984). Relatively fewer Japanese black pine seed-lings, planted at Montague fields, developed pine wilt disease when

Figure 5. Depicts the number of nematodes/gram of fresh weight of wood over time in three-year-old white (---), Japanese black (——) and Austrian (........) pine seedlings. Japanese black and Austrian pine had significantly greater numbers of nematodes than at six weeks. At eight weeks after inoculation Japanese black pine had significantly greater numbers of nematodes than Austrian but not white. There were no significant differences among the three species at 2, 4, 10, 12 and 14 weeks.

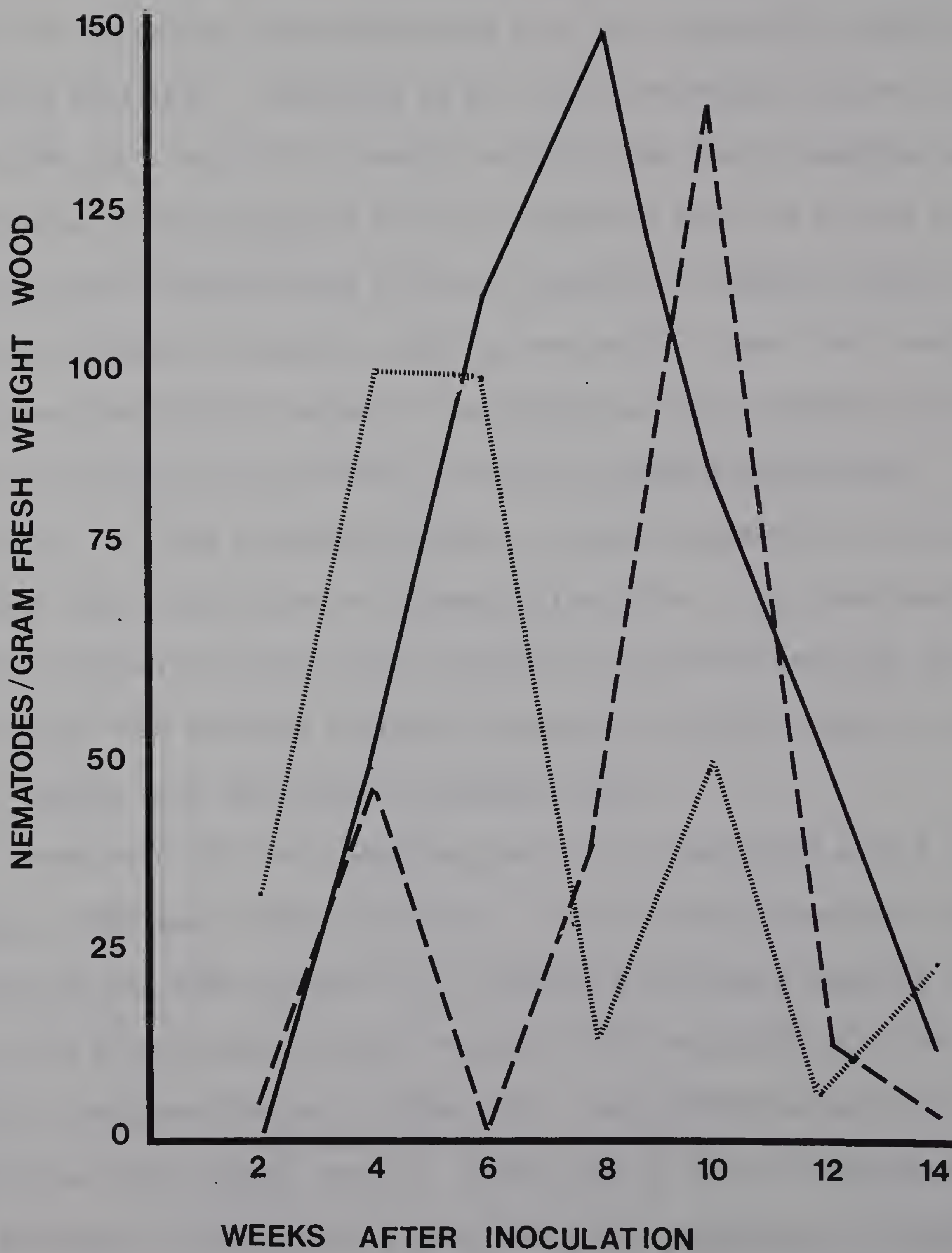

NEMATODES/GRAM FRESH WEIGHT WOOD
150
125
100
75
50
25
0
2
4
6
8
10
12
14
WEEKS AFTER INOCULATION

Figure 5

inoculated with _B_. _xylophilus_ than greenhouse studies (Table 5). Only one of ten inoculated seedlings developed pine wilt and died over the fifteen-week period. All the inoculations were determined to be successful, nematodes were recovered from all inoculated seedlings and there was no callus tissue formation over the inoculation wound in nematode seedlings. Wingfield _et al_. (1984) reported failure of callus formation over inoculation wounds in Scots pine where nematodes were introduced. They suggested this as a possible mode for branch death. Two seedlings had developed yellowing needles at sampling time and the top branches of another seedling were dead. These four seedlings contained the highest numbers of nematodes per gram compared with the other six inoculated seedlings in which no symptom development occurred. In 1983 greenhouse studies, symptoms appeared as early as 24 days after inoculation of Japanese black pine. The slow development of pine wilt disease that occurred in the field has been reported previously from northern regions in Japan and in Scots pines in Illinois (Mamiya 1983 and Malek and Appleby 1984).

Three-year-old larch seedlings were also inoculated with _B_. _xylophilus_ at Montague fields (Table 6). Five of the 20 seedlings inoculated with the 1984 isolate of _B_. _xylophilus_ developed symptoms of pine wilt disease whereas only one out of 20 inoculated with the 1983 isolate developed pine wilt (Figure 6). The difference was not significant (chi square test, p 0.05), but it is an indication of the decrease in pathogenicity over time of _B_. _xylophilus_ maintained in culture as reported by Kiyohara, 1976. This nematode was recovered

Table 5. Recovery of B. xylophilus (FTF-84) from Japanese black pine (Pinus thunbergiana), 105 days after inoculation.[1]

Treatment	trees treated	trees with callus formation	Trees: Foliar symptoms	n/g[2]	no symptoms	n/g
B. xylophilus	10	0	4	265.982	6	0.4745
Botrytis cinerea	11	11	0	0.000	11	0.0000

[1]One ml. suspension of B. xylophilus was added to each seedling at a rate of 2,000 nematodes per seedling. Controls were treated with a one ml. suspension of Botrytis spores.

[2]Mean number of nematodes per gram of fresh weight.

Table 6. Field inoculations of Larch (*Larix laricina*) with *B. xylophilus* and *Aphelenchoides* at Montague farms, Amherst, Massachusetts, 1984.

Inoculum	no. of trees	No. of trees Dead	Symptoms[2]	No. of trees yielding nematodes	n/g[3]
FTF-84	20	5	7	11	22.7470
Bx-1	19	1	2	2	4.3075
LG-1	20	0	7	8	.7630
Botrytis	20	0	0	0	0.0000

[1]FTF-84 (isolated 1984) and Bx-1 (isolated 1983) = *B. xylophilus*, LG-1 = *Aphelenchoides*.

[2]Symptoms were determined as a general yellowing of needles.

[3]Mean number of nematodes per gram of fresh wilt in infected trees.

from 13 of the total 40 seedlings inoculated with *B. xylophilus* including both the 1983 and 1984 isolates. From these field results larch has been shown to be a host of the Massachusetts isolate of *B. xylophilus*. The nematode can produce wilt symptoms and reproduce in larch seedlings.

Bergdahl (1982) described a wilt disease of larch in Vermont which was often associated with *B. xylophilus*. Following inoculation studies, he reported that *B. xylophilus* was pathogenic to young seedlings. Berhdahl *et al*. (1984) also reported on the association of another nematode: *Aphelenchoides* sp., associated with this wilt disease complex. He reported inoculations with *Aphelenchoides* sp. on seedlings that resulted in "some mortality."

Figure 6. Larch (*Larix laricina*) seedling developing symptoms of pine wilt following inoculation with *B. xylophilus* (FTF-84).

An Aphelenchid nematode, Aphelenchoides sp., originally isolated from a dying larch tree in Gardner, Massachusetts, proved to be non-pathogenic to three-year-old larch seedlings under field conditions (Table 6). No comparisons were made between the Massachusetts and Vermont Aphelenchoides sp. isolate to determine if they were the same. Aphelenchoides nematodes are often found in association with bark beetles (Massey 1974). There was an extensive amount of beetle activity, mainly Ips sp. in the declining larch tree which was the original source of inoculum.

Pine wilt does not occur in nature on seedling trees and aside from proving pathogenicity, experiments with small trees are of limited value.

Field Studies

Few workers report successful field inoculations of mature trees with B. xylophilus in the United States. There is much speculation about native trees being resistant as mentioned earlier, but no studies have been undertaken to determine this. In this study, pinewood nematodes were inoculated into eight to 18 meters in height Japanese black, pitch, and red pine trees under natural conditions on Cape Cod and in western Massachusetts.

Two Japanese black pines at Rebellos nursery, Falmouth, developed pine wilt disease after inoculation. Smaller Japanese black pines inoculated with B. xylophilus did not develop pine wilt nor was the pinewood nematode recovered from the inoculation site. There were bluestain fungi and evidence of black turpentine beetle activity in the two trees which developed pine wilt disease as well as in controls where no symptom development occurred. One of the younger Japanese black pine seedlings appeared to be developing pine wilt disease in March, 1984. The buds were smaller when compared to controls and there was a general yellowing of needles (Figure 7). Mamiya (1983) described a recovery phenomenon in Japanese black pine which occurred under cool, wet conditions. The spring of 1984 was very wet and this may have favored recovery.

At Mt. Toby research plot, Leverett, Massachusetts, no red pine trees developed pine wilt disease following branch inoculation. A significantly greater number of nematode inoculated branches did develop wilt and died compared to control inoculations (Table 7). One of the control trees did die from unknown causes. An additional three of the nematode inoculated branches produced symptoms from the inoculation point to the end of the branch. In two of these branches high concentrations of nematodes (45.5 and 189.5n/g) were recovered from areas below the inoculation point bordering onto healthy tissue. Similar results were recently reported by Wingfield et al. (1984) with Scots pine branch inoculations. They also reported the failure of callus formation over the inoculation wound and speculated that this may have led to partial branch death. Lack of callus formation is not a symptom of pine wilt disease but has been associated with artificial inoculations with pinewood nematodes. Nematodes also failed to move from the branches into the main stem of the inoculated trees and were

Figure 7. Japanese black pine (Pinus thunbergiana) in March, 1984, with stunted buds and slight yellow discoloration of needles (branch on right) compared with control (left).

Table 7. Pathogenicity of *Bursaphelenchus xylophilus* to branches of red pine trees.

Treatment	No. of trees	No. of branches	No. of branches that died			Total dead	% of total
			2 mo.	9 mo.	14 mo.		
Nematodes	9	23	12	6.5	0.5	19*[2]	82.61
Controls	9	20	5	1.0	0.0	6	30.00

[1]One to three branches of 25-40' trees were inoculated with 30,000 nematodes per tree on July 18, 1983.

[2]Number followed by an asterisk is significantly different (P = 0.01) according to Chi Square test.

only recovered from the B. xylophilus inoculated branches. In previously reported inoculation studies, nematodes did not move from the original site of introduction until symptom development had readily progressed (Kiyohara and Suzuki 1978 and Mamiya 1980).

One of the B. xylophilus stem inoculated red pine trees at Mt. Toby in 1983 developed symptoms of pine wilt in March, 1984. Branches began "wilting" from the lowest branch and progressed upward. The top meter of the tree was still green and healthy when the tree was cut in March, 1984. Nematodes were recovered from the roots and xylem two meters above the inoculation point. B. xylophilus was also recovered from the other nineteen nematode inoculated trees, but only from the area around the original inoculation wound. The inoculation site was always heavily soaked with resin in both control and nematode treated trees. Nematodes were only isolated from the resin soaked area (Figures 8 and 9). The one tree that did develop symptoms of pine wilt was an understory tree, only nine meters in height compared to approximately 12 meters of other trees.

Nematodes were found in the tracheids and resin canals in stained wood sections from the sapwood below the inoculation site (Figure 10). Extensive destruction of the epithelial cells in the resin canals could be seen with comparisons between control and nematode inoculated trees (Figures 11 and 12).

In the understory plot, in 1984 inoculation studies, one red pine of 15 inoculated developed pine wilt disease eight weeks after inoculation with B. xylophilus. Nematodes were recovered from all

Figure 8. Resin soaking in B. *cinerea* (4c) and B. *xylophilus* (2) inoculation sites in red pines from Mt. Toby, September, 1984.

Figure 9. Inoculation wound from B. *xylophilus* inoculated red pine at Mt. Toby, September, 1984.

Figure 10. *Bursaphelenchus xylophilus* stained with Goodey's formula from inoculation site of a red pine. Tree was inoculated with 30,000 nematodes in July, 1983, and harvested September, 1984. (63 x)

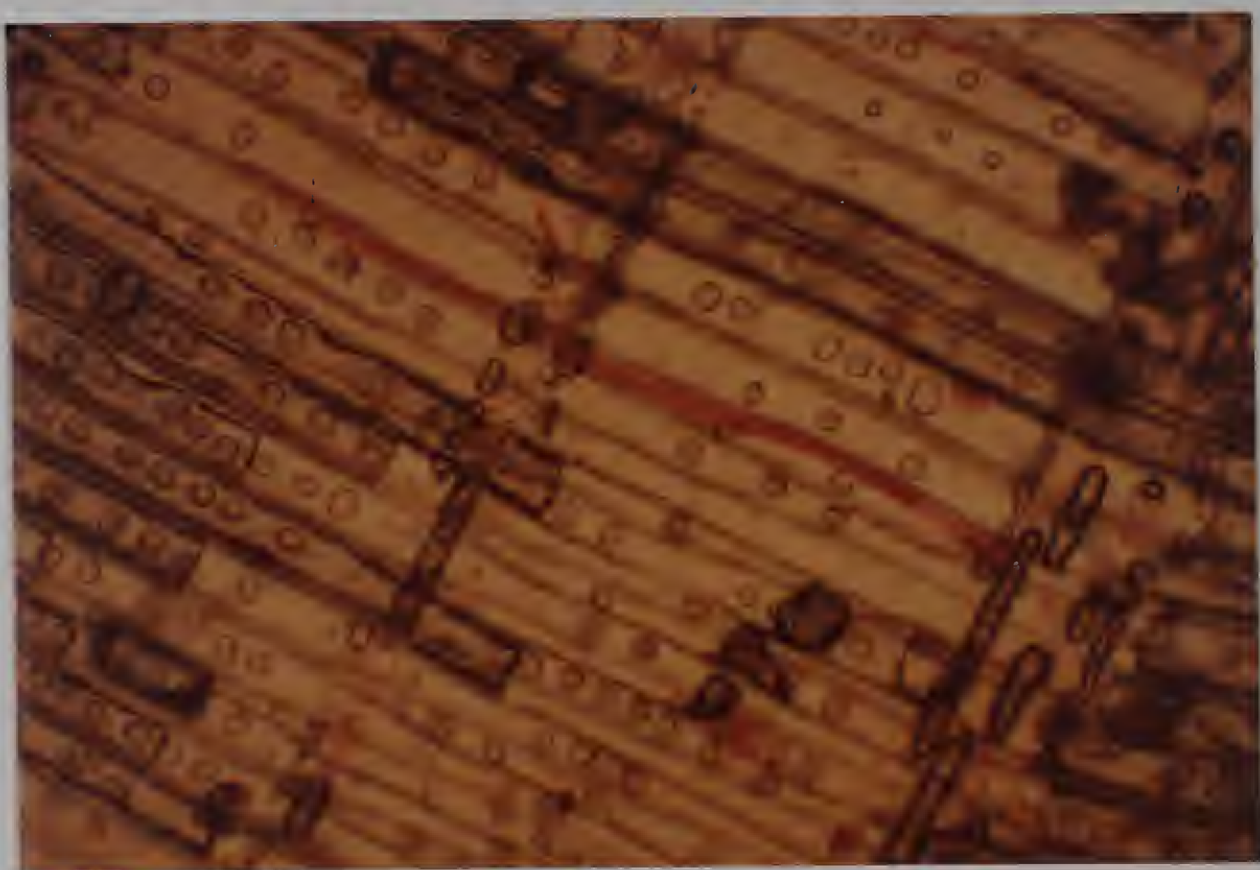


Figure 11. Tangential section of red pine following inoculation with *B. xylophilus*. Fusiform ray with destroyed epithelial cells. (100 x)

Figure 12. Tangential section of red pine with healthy fusiform ray following inoculation with *Botrytis*. (100 x)

parts of the tree in relatively high numbers (258.87n/g). Nematodes were also recovered from the inoculation site from all nematode inoculated trees and 50 cm above the inoculation site in three out of six that were sampled. No nematodes were recovered from 50 cm below the inoculation site of the eight trees that were sampled (Table 8).

Fifteen red pines at Mt. Toby, 18 meters in height, were also inoculated with B. xylophilus. None of the inoculated trees developed symptoms of pine wilt. Nematodes were recovered from all inoculation sites as well as 50 cm above and below the inoculation site in 12 and nine out of the 15 nematode inoculated trees, respectively. More nematodes were recovered from above the inoculation site with an average of 9.161 n/g than below the inoculation site (1.074 n/g) (Table 8).

Nematodes were recovered from the inoculation point in red and pitch pines at Falmouth Town Forest, Barnstable County. Nematodes migrated below the inoculation point in two of the red pines. Samples from below the inoculation point were processed one month after they were collected from the field which could explain the large number, 16.333 n/g (Table 8). Nematodes were recovered from two of the three inoculated pitch pines only from the inoculation site and at very low population levels (.112n/g).

Red and Japanese black pine in natural forest situations, inoculated with B. xylophilus, may develop pine wilt disease. Some form of stress was involved in each of the four trees that died. The two red pines were understory trees and the two Japanese black pines had

Table 8. *B. xylophilus* populations in stem inoculated red pine (*Pinus resinosa*) trees in July, 1984, at various locations in Massachusetts.[1]

Location	Inoculum per tree	no. Inoculated	inoculation point		50 cm above		50 cm below	
			+	n/g[2]	+	n/g	+	n/g
Mt. Toby 62', 43 years	15,000	15	15	36.231	12	9.161	9	1.074
Mt. Toby understory	15,000	15	15	34.000	3	43.560	0	0.0
Falmouth Town Forest	2,000	10	9	5.289	0	0.0	2	16.333

[1]Increment cores were taken from the main stem approximately three feet from the ground. The nematode suspension was then pipetted in.

[2]Nematodes per gram of fresh weight of wood.

been previously attacked by other agents. None of the red pines at Mt. Toby displayed any differences when an additional stress of partial girdling was added (Table 9). Differences might have appeared the spring of 1985 if the trees were not cut.

Table 9. Comparison between girdled and nongirdled B. xylophilus stem inoculated red pine at Mt. Toby, Massachusetts, 1984.

Treatment	no. of trees	Inoculation point	50 cm above	50 cm below
Girdled[1]	5	44.2n/g[2]	3.478	1.110
Nongirdled	10	41.52	9.250	1.056
Botrytis	5	0.0	0.0	0.0

[1]Selected trees were girdled half around 30 cm above the ground line on September 22, 1984. Trees were harvested October 27, 1984.

[2]All values are an average from treated trees; nematodes per gram fresh weight.

The pinewood nematode is able to survive within the trees for an extended length of time, shown by recovery of B. xylophilus from healthy red pine inoculated the previous year. High incidences of pine wilt disease could occur under unfavorable growing conditions, such as drought, which has actually occurred in the midwest. In the field study where Japanese black pine seedlings were inoculated, pine wilt disease developed more slowly than in other reported studies. Temperatures in Massachusetts are similar to those in northern Japan where disease development is slower or the disease is not present.

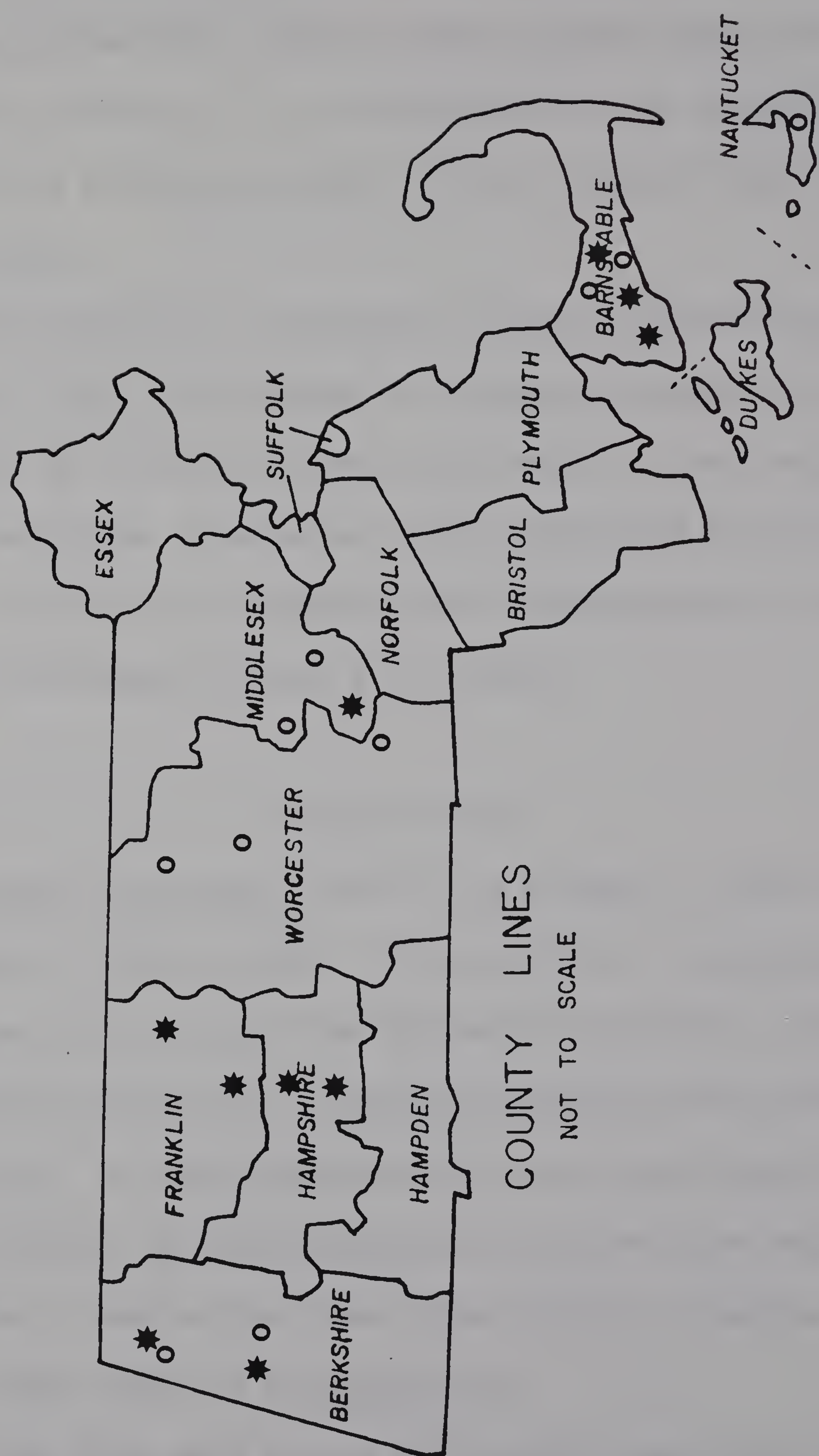
State Survey

An independent forester, Mr. Charles Walker, told us of a plantation of dying red pines in Granby, Massachusetts, that was in the process of undergoing a clearcut. The stand was overstocked, with evidence of Cerambycid activity. B. xylophilus was isolated from logs that were lying on the ground.

B. xylophilus was also isolated from logs that had been on the ground from Scots pine at Mt. Toby, red pine at Wendell and Hopkinton, and white pine in northern Berkshire County. In all five cases, there was evidence of Cerambycid activity either in the form of larvae, or entrance or exit holes in the sapwood.

Thirty-two trees were sampled which were symptomatic (rapid browning of needles with characteristic reddish-brown discoloration) from Barnstable, Berkshire, Hampshire, Middlesex and northern Worcester County (Figure 13). Two red pines on the University of Massachusetts campus at Amherst, a white pine at Berkshire County Community College and the red pines at the Falmouth Town Forest in Barnstable County were the only samples positive for B. xylophilus. With the exception of the University of Massachusetts campus, B. xylophilus appears to be associated with trees that have died or are in a very late stage of decline in western Massachusetts. Similar situations have been reported from Minnesota, Iowa, and Wisconsin (Wingfield et al. 1982b). On Cape Cod, the nematode shows a stronger association with trees in a state of decline. There is Armelliaria mellea (Vahl ex Fries) Krammer, black turpentine beetle activity, and Cerambycid

Figure 13. Locations of pinewood nematode, *Bursaphelenchus xylophilus* from declining pines in Massachusetts (✸), sites of declining pines or larches where no pinewood nematodes were recovered (O).


ESSEX
SUFFOLK
MIDDLESEX
NORFOLK
PLYMOUTH
BRISTOL
BARNSTABLE
DUKES
NANTUCKET
WORCESTER
FRANKLIN
HAMPSHIRE
HAMPDEN
BERKSHIRE
COUNTY LINES
NOT TO SCALE

beetle activity in these stands of declining pines. The pinewood nematode may be the final blow to these weakened trees which results in their terminal decline. It is also possible that pinewood nematode was present and weakened the host defenses against these other pathogens and insects.

Pinewood nematode is widespread throughout Massachusetts in low populations. Higher populations of pinewood nematodes exist on Cape Cod, possibly as a result of the large numbers of declining pines. The pine plantations surveyed are widely separated and well managed. From these findings it is doubtful that Massachusetts is likely to experience an epidemic of pine wilt disease.

Vector Studies

Monochamus carolinensis (Oliv.) was trapped in 1983 and *M. scuttelatus* (Say) in 1983 and 1984. Three of the *M. carolinensis* from *B. xylophilus* infested logs from Falmouth Town Forest contained pinewood nematodes (Table 10). No pinewood nematodes were recovered from *M. scuttelatus*. *M. carolinensis* beetles were only found in logs from Barnstable County. *M. scuttelatus* was recovered from logs from Granby and Hopkinton. None of the other three species of beetles recovered from these logs contained *B. xylophilus*.

Trap logs which were placed at Falmouth Town Forest to determine if nematodes were transmitted through oviposition did not become infested. One log contained 65 Cerambycid borers, and eight entrance holes were present in another. None of the seven logs contained

Table 10. Cerambycids collected from various sources of *B. xylophilus* infested logs, 1983 and 1984.

Genus	No. of Beetles	No. with *B. xylophilus*
Beetles collected, 1983		
Monochamus carolinensis	10	3
Monochamus scutellatus	7	0
Graphisurus obsoletus	2	0
Xylotrechus sp.	1	0
Beetles collected, 1984		
Monochamus scutellatus	9	1
Stenocorus sp.	1	0

B. xylophilus.

Few beetles from pinewood nematode infested logs contained nematodes. No studies were undertaken to determine what other organisms were present in these logs, to determine if there were any that were predatory on *B. xylophilus*. The logs that were placed out at Falmouth Town Forest were attacked by Cerambycid beetles but did not contain nematodes. This area has the highest populations of nematodes in dying red pine trees. Transmission through oviposition has been reported to be the main form of transmission in Minnesota, Iowa and Wisconsin (Wingfield *et al*. 1983a,b). The failure of transmission in Falmouth could mean that conditions are similar to Japan, where transmission occurs through maturation feeding. From the beetle traps, few beetles carried the pinewood nematode which may also explain why there was no transmission. A more thorough study is needed to determine why there is such a low association of the pinewood nematode with reported vectors in Massachusetts. The traps used in this study may have affected the results. Logs which were collected in the spring of 1984 were water soaked from the heavy spring rains. They were placed in the barrels before they were allowed to dry, resulting in an extensive amount of fungal growth. No beetles or nematodes were recovered from these logs when they were sampled in November, 1984.

CHAPTER V

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