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Cambium Destruction in Conifers Caused by Pinewood Nematodes[1]

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Abstract: Percentage and rate of mortality in 2–4-year-old conifers depended upon the numbers of pinewood nematodes *Bursaphelenchus xylophilus* inoculated into their stems. In addition, percentage of conifer mortality was greater for spring inoculations when cambial activity was greater than for late summer and fall inoculations. Gross and histological examination of stems revealed destruction of the cambial layer, including fusiform and ray intitials and their derivatives. These data suggest that cambial and ray destruction causes tree death through blockage of tracheids by gas, oleoresin, or metabolites from dying ray tissues.

Key words: histopathology, *Bursaphelenchus xylophilus,* pinewood nematode, pinewilt disease, conifer death, mortality, *Pinus* spp.

Bursaphelenchus xylophilus (Steiner & Buhrer, 1934) Nickle, 1970, the pinewood nematode (PWN), kills many different conifers (2,3,6,9). Causes of tree death were suggested to be toxic compounds synthesized or released during the nematode–conifer interaction (1,2) or metabolic leakage into tracheids from rays resulting in blockage of water transport to the foliage (12). Tissue destruction in Japanese red pine was previously described (7). Histopathological damage and its relationship to mortality in conifers has not been thoroughly documented.

My objective was to study the progressive pathology in conifers after inoculation with PWN. Data are presented to support the hypothesis that death of trees results from mechanical destruction of cambium and rays by PWN. Preliminary data on the relationship of PWN to tissue pathology was previously reported (8,10,11).

Materials and Methods

Trees 2–4 years old of the following species were used over a 3-year period: *Pinus densiflora* Sieb. & Zucc. (Japanese red pine), *P. nigra* Arn. (Austrian pine), *P. resinosa* Sol. (red pine), *P. rigida* Mill. (pitch pine), *P. strobiformis* Engelm. (southwestern whie pine), *P. strobus* L. (eastern white pine), *P. sylvestris* L. (Scots pine), *P. thunbergii* Parl. (Japanese black pine), *Picea pungens* Engelm. (Colorado blue spruce), and *Pseudotsuga menzezii* (Mirb.) Franco (Douglas fir).

A New Jersey isolate of PWN was extracted from a dead Japanese black pine by soaking wood chips in water on a Baermann funnel. Nematodes were surface sterilized using the Aretan-agar migration method (4). Nematodes were picked from the surface of the agar containing 100 μg/ml Aretan and stored in a solution containing 1,000 units/ml each of streptomycin sulfate and penicillin G. The Aretan contained 3% methoxyethyl mecurous chloride (Bayer Agricultural Ltd.). Ten nematodes were transferred to *Pyrenochaeta terrestris* deNot cultures on potato dextrose agar and reared on this fungus or on *Botrytis cinerea* Pers. PWN were collected on a 45-μm-pore sieve from water after they migrated through filter paper from the fungal cultures. After a second migration through filter paper, the nematodes were centrifuged twice in sterile, deionized water before the suspension was adjusted to the desired nematode concentration. Trees were stem inoculated with 0, 100, 500, or 5,000 PWN as follows: A shallow slice, 1–2 cm long, was cut through the bark to expose the xylem. The wound was then covered with cotton. Parafilm was wrapped around the stem to build a cavity, ca. 1.5 cm^3, that was open at the top and sealed at the bottom and sides. Nematodes suspended in water were then pipeted onto the cotton. Control inoculations utilized water from which PWN had been removed. Parafilm and cotton were removed 1 week after inoculation.

Ten trees each of Austrian, eastern

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white, pitch, Scots, and Japanese black pines were inoculated with 5,000 PWN every month from May to September. Dead and dying trees were recorded each month. This experiment was designed to determine if date of inoculation influenced the rate of sympton expression and tree mortality.

Stem pathology was examined on trees harvested at 4, 8, 15, and 28 days after inoculation. Six control trees were stem inoculated with water from which nematodes had been removed, and 36 trees were each inoculated with 5,000 nematodes. Cross and longitudinal sections of stems floating in water were examined at 15–40 × magnification. Discs of stem tissues cut from just above the inoculation wounds, in the middle of the new candle growth, and just above the soil line were fixed in TAF. Tissues were dehydrated in a *tert*-butyl alcohol series and embedded in paraffin (5). Exposed faces of the woody tissue were softened in water and sectioned on a rotary microtome. Longitudinal, tangential, and cross sections of stems were cut 12 μm thick. Celloidin was needed to keep sections on slides during staining. Sections were stained with safranin-fast green (8,11) or Delafield's hematoxylin followed by counterstaining with 3.5% aqueous phloxine-B before dehydrating and mounting in Permount (Fisher Scientific). The hematoxylin–phloxine stain provided greater contrast between animal and plant tissues.

Results and Discussion

Fewer conifers died when 500 rather than 5,000 nematodes were inoculated. Inoculation with 500 nematodes during early spring resulted in the death of trees as follows: 80% Austrian, 80% eastern white, 60% Scots, 75% southwestern white, and 25% Japanese black pines. All these pines were killed when inoculated with 5,000 PWN. No pitch pines, Douglas fir, or Colorado blue spruce were killed after inoculation with 500 PWN although 20–80% of these trees succumbed to 5,000 PWN. No mortality was observed 120 days after inoculation of Austrian, eastern white, Scots, or Japanese black pines with 100 PWN. The more PWN inoculated, the more rapid the expression of symptoms. Trees that survived for several months were usually alive the following year, and no PWN were extracted from them. Even nonhosts such as pitch pine, Colorado blue spruce, Douglas fir, and red pine were killed if sufficient numbers of PWN were inoculated into their stems (11). These findings indicated that percentage of tree death was dependent on the number of PWN in the inoculum and suggested that the amount of tissue damage was directly related to tree mortality.

Date of PWN inoculation was the most important factor in producing tree mortality (Table 1). Data recorded on Austrian pine for May and June followed a pattern similar to that for eastern white, Scots, Japanese black, and pitch pines. The period from bud break during late April or early May to candle maturation in July was when trees died rapidly from PWN, whereas trees inoculated during August and September were resistant. These data correspond with the pattern of cambial activity occurring in New Jersey. The cambium reactivates after 12–16 weeks of winter dormancy. Stimulated by bud growth during the middle of April to early May, cell divisions move downward through the stem to the roots over a period of several weeks. Before this reactivation, the cambium is represented by a single layer of cells lying between differentiated xylem and phloem. Oblique cell division is followed by sliding intrusive growth between thin-walled cambial cells. This stage of cambial development would be extremely vulnerable to PWN damage. Maturation lags during this period of rapid cell divisions, so that a layer of cell derivatives may be present in various stages of differentiation up to mature phloem, tracheids, and ray tissues. The period of active cambial growth continues until late July or candle maturation when only individual, scattered mitoses occur. Because the greatest cambial activity, between May and July in New Jersey, coincides with the period of highest mortality for trees inoculated with PWN, my experiments point to damage to the cambium as the cause of tree death.

Gross examination of stem tissues was made by cutting slices at various places and examining them in water. Nematodes were observed emerging from various stem tissues. A browning reaction of cortex, phloem, and cambium was observed only

TABLE 1. Percentage of *Pinus* spp. trees dying 2–4 months after inoculation with 5,000 *Bursaphelenchus xylophilus* per tree.

Species	Date of inoculation	Elapsed time (months) 1	2	3	4
Pinus strobus	20 May	90	100	100	
Eastern white	18 June	70	80	80	80
pine	15 July	90	100	100	100
	18 Aug.	10	30	60	
	17 Sept.	0	20		
Pinus sylvestris	20 May	0	70	70	
Scots pine	18 June	30	60	60	60
	15 July	0	70	70	80
	18 Aug.	0	0	40	
	17 Sept.	0	0		
Pinus thunbergii	20 May	0	50	70	
Japanese black	18 June	0	60	80	80
pine	15 July	0	20	20	40
	18 Aug.	0	0	20	
	17 Sept.	0	20		
Pinus rigida	20 May	0	0	0	
Pitch pine	18 June	10	10	10	10
	15 July	30	60	60	60
	18 Aug.	0	0	0	
	17 Sept.	0	0		

in inoculated stems. Circumferential damage progressed with elapsed time as nematodes moved around the stem. While this circumferential damage was in progress, other nematodes migrated up and down the stems. Up to 100% of the stems of Austrian, eastern white, Scots, and Japanese black pines were girdled (browned) within 8 days after inoculation. This browning appeared similar to the reaction of stem tissues to cutting or mechanical damage after exposure to atmosphere, and probably resulted from the action of polyphenol oxidase. The reaction was especially severe in eastern white pine where widespread discoloration occurred rapidly wherever nematodes moved through plant tissues. The faster the tissue browning rates in trees, the quicker the trees died. In trees that were dying, a band of brown, dead tissue several centimeters long encircled the stems, often leaving the tops and bottoms of the trees still alive. If PWN damage was not fatal to the trees, the trees produced secondary growth to repair and wall off the damaged tissues. Wilting of elongating candles was especially notable in Scots pine. Death of the total tree appeared to result from dehydration. Nematode population densities declined rapidly as trees desiccated after dying, and living PWN could not be extracted from dried trees.

Stem tissues, especially those related to the cambial layer, were examined histologically. Sections cut with a microtome from trees 4, 8, 15, and 28 days after PWN inoculation showed severe progressive tissue destruction when compared with sections from noninoculated control trees. PWN migrated from the cortex through the radial rays or directly across the sieve cells of the phloem to the cambium, moving both vertically and circumferentially and destroying the fusiform and ray initials and their derivatives (Fig. 1A–C). Circumferential trails of destruction were observed. Occasionally cambial gaps developed in which nematodes of all sizes were found (Fig. 1D–G). Nematodes occurred between the xylem and phloem (Fig. 1H, I). The anterior ends of nematodes, apparently feeding, were often found buried in the dense cytoplasm of fusiform initials, their derivatives, or in parenchymatous cells (Fig. 1J, K). Females in cambial gaps often contained eggs in utero, and an occasional deposited egg was observed. If the cambium was only partially destroyed by PWN, it regenerated and callus grew over the wounded tissues.

Destruction of the cambial layer by PWN ultimately resulted in tree death. During

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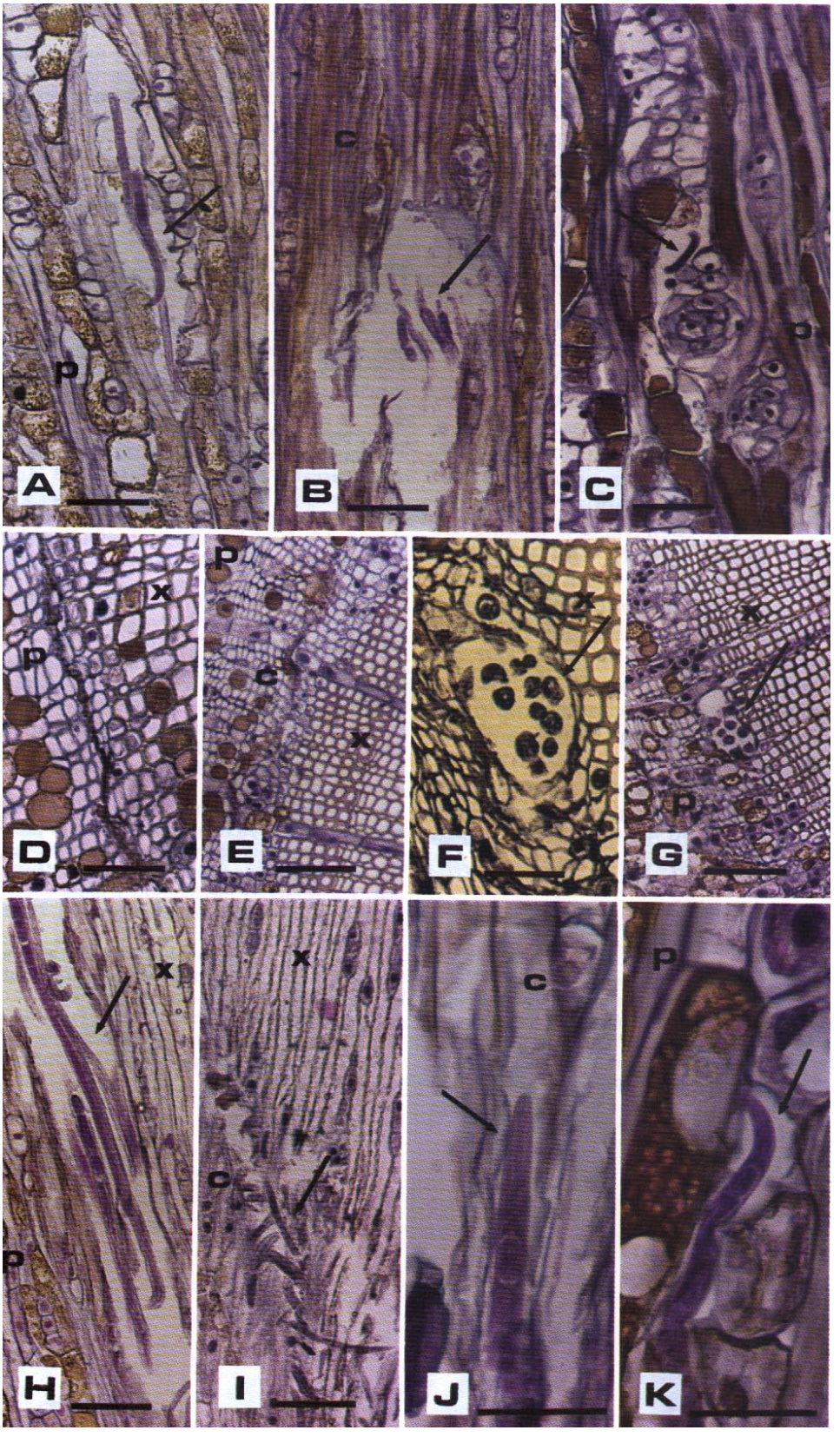
FIG. 1. Histopathology at or near the stem cambium of pine trees 2–4 years old inoculated with *Bursaphelenchus xylophilus*. A–C) Tangential sections showing nematodes in phloem cavity (p) of red pine (A), among cambial cells (c) and their derivatives in Austrian pine (B), and near a bundle of cells that is a precursor to a radial resin canal in Japanese black pine (C). D, E) Cross sections showing nematode-caused stem injury in the cambial area due to circumferential migration of nematodes along the cambial–xylem (x) interface in eastern white (D) and Scots pines (E). F, G) Cross sections showing nematodes in cambial cavities formed from cells that normally would have become axial resin ducts in Scots (F) and red pines (G). H, I) Longitudinal sections showing nematodes in cambial area of red (H) and eastern white pines (I). J) Nematode among cell derivative of stem cambium in Austrian pine. K) Nematode that is possibly feeding upon a phloem parenchyma cell containing tanniferous bodies in eastern white pine. Bars represent 100 μm on A–C, E, G, H, I; 50 μm on D, F; and 20 μm on J, K.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the most active period of tree growth, the cambial layer is fragile and easily penetrated by PWN. Since new tracheids and sieve cells generally retain functionality for less than 2 years, damage to the cambium, even if trees were not killed, ultimately interfered with water translocation and food conduction. Because mortality in young trees was related to numbers of PWN inoculated and the time of year or stage of development of cambial layers, no prediction of degree of resistance or susceptibility of young trees was attempted. Such predictions have been reported on the basis of percentage of tree mortality (2,3,7).

A possible mechanism for wilting of pines is as follows: Tracheids measure up to 4 mm long, so destruction of cambium, both fusiform and ray initials, in a band less than 6 mm in depth would create a solid disk of wood with each tracheid lacking the contact of living ray cells. All tracheids in pines contact living ray cells. Water conduction would be prevented by blockage of tracheids through aerification (10) or by leakage and diffusion of oleoresin and metabolites from dying rays (13). Wilting of needles and developing candles would follow. The obvious damage to the cambium and the association of increased rate of mortality with the period of greatest cambial activity indicate that mechanical damage interacting with normal plant processes is responsible for tree mortality.

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