Infection of Douglas-fir by *Leptographium wageneri*

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Abstract: In three related experiments, root systems of 2-year-old Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings were dip-inoculated with a viscous blend of *Leptographium wageneri* var. *pseudotsugae* Harrington and Cobb spores and hyphal fragments and planted in a sterile potting medium. Infection frequency and points of entry were evaluated for dormant seedlings and seedlings that had been active for 4 and 8 weeks. All putative black stain infections and other areas of sapwood discoloration were free-hand sectioned and examined with a bright-field microscope and phase-contrast optics. This technique was shown to be 100% reliable in a prior experiment with 223 inoculated Douglas-fir seedlings that paired microscope examinations with pathogen isolation. In this study, all lesions were sectioned and examined at 250–1000 diameters magnification for the presence of *L. wageneri* var. *pseudotsugae* hyphae and characteristic pathological anatomy. Complete root system dissections revealed that *L. wageneri* var. *pseudotsugae* infected roots through wounds and natural openings where a direct path to the xylem was exposed and never penetrated living cortical or cambial tissues to infect its host. Among the dormant inoculated seedlings, 63% of infections occurred through wounds associated with nursery handling. Wound infection frequency decreased to zero in seedlings inoculated 8 weeks after coming out of dormancy. Seedlings inoculated 4 and 8 weeks after coming out of dormancy were most frequently infected through openings occurring at sites of new lateral root initiation. Infection of dead fine root stubs suggested that during periods of increased fine root mortality, these sites may be important for the new infection of healthy trees and egress from already diseased trees.

Key words: Black-stain root disease, *Leptographium wageneri*, *Verticicladiella wagnerii*, infection courts, wounding, vascular wilt.

Résumé : Les auteurs ont conduit trois expériences dans lesquelles les systèmes racinaires de sapins Douglas âgés de 2 ans ont été trempés dans un mélange visqueux du *Leptographium wageneri* var. *pseudotsuga* Harrington and Cobb comportant des spores et des fragments d'hyphes, avant d'être plantés dans un milieu d'empotage stérile. Ils ont évalué la fréquence d'infection et des points d'entrée chez des plantules dormantes, ainsi que des plantules remises en activité depuis 4 et 8 semaines. Ils ont sectionné à la main et examiné en microscopie sur fond clair et en contraste de phase, toutes les régions tachées de noir ou autres régions colorées de l'aubier, présumément infectées. Dans une expérience antécédente, on a démontré que cette technique est fiable à 100 % ; cette expérience portait sur 233 plantules de sapin Douglas où des observations microscopiques ont été couplées avec l'isolement du champignons pathogène. Dans cette étude, toutes les lésions ont été sectionnées et examinées à 250–1000 agrandissement en diamètre, afin de déceler la présence des hyphes du *L. wageneri* var. *pseudotsuga* et l'anatomie pathologique caractéristique. Les dissections de systèmes racinaires complets révèle que les hyphes du *L. wageneri* var. *pseudotsuga* pénètrent par des blessures ou des entrées naturelles donnant un accès direct au xylème; la pénétration ne s'effectue jamais à travers les tissus corticaux ou méristématiques pour infecter l'hôte. Parmi les plantules dormantes inoculées, 63 % des infections se font par des blessures provenant de manipulations en pépinière. La fréquence des infections par des blessures diminue jusqu'à zéro chez les plantules inoculées 8 semaines après le bris de la dormance. Les plantules inoculées 4 et 8 semaines après le bris de la dormance sont le plus souvent infectées par ouvertures localisées sur le site de l'initiation de nouvelles racines latérales. L'infection de bouts de racines fines suggère qu'au cours de périodes ou la mortalité des racines fines augmente, ces sites peuvent être importants pour de nouvelles infections de plants sains et peuvent sortir d'arbres déjà malades.

Mots clés : maladie racinaire à coloration noire, *Leptographium wageneri*, *Verticicladiella wagnerii*, sentiers d'infection, blessure, flétrissure vasculaire.

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Introduction

Leptographium wageneri var. *pseudotsugae* Harrington and Cobb (= *Verticicladiella wageneri* Kendrick, = *Verticicladiella wageneri* var. *pseudotsugae* Harrington and Cobb, = *Leptographium wageneri* (Kendr.) Wingf., Harrington and Cobb 1984, 1986, 1987; Kendrick 1962; Wingfield 1985), the agent of black-stain root disease, affects Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) throughout the western United States and British Columbia (Harrington 1988 and references therein). In the range of Douglas-fir, both the coastal (var. *menziesii)* and interior (var. *glauca)* varieties are affected, but the disease is most often reported in the coastal variety.

Host colonization by *L. wageneri* var. *pseudotsugae* is restricted to mature xylem tracheids. Living cortical, cambial, and parenchyma tissues are not invaded, and cell walls are not directly penetrated by hyphae (Hansen et al. 1988; Hessburg and Hansen 1987; Hessburg 1984). Tree mortality appears to be the result of rapid, systemic vascular tissue colonization, and tracheid occlusion by hyphae and host reaction compounds (Hessburg and Hansen 1987; Hessburg 1984) often concurrent with insect attack of the basal stem, root collar, and roots (Harrington 1983, 1988; Hansen et al. 1988; Witcosky 1985; Witcosky and Hansen 1985; Witcosky et al. 1986).

Primary disease spread over long distances is associated with insect vectors that feed and breed in roots of *L. wageneri* var. *pseudotsugae* infected trees. New disease centers arise when vectors attack stressed, injured, or weakened trees for breeding (Harrington 1983; Harrington et al. 1983, 1985; Witcosky and Hansen 1985; Witcosky et al. 1986). New infection centers also arise from vector maturation feeding on roots of healthy trees (Witcosky 1985; Witcosky et al. 1986). Secondary disease spread from established foci occurs through root grafts and via contact or intimate association of roots of diseased and healthy trees (Hessburg 1984; Hessburg and Hansen 1986). In ponderosa pine (*Pinus ponderosa* (Dougl.) Laws.), most new secondary spread of *L. wageneri* var. *ponderosum* (Harrington and Cobb) occurs in roots that are less than 5 mm in diameter growing within 15 cm of diseased roots (Goheen 1976; Harrington and Cobb 1984, 1986, 1987; Harrington 1988). Hicks (1978) and Hicks et al. (1980) demonstrated that *L. wageneri* var. *ponderosum* grows from infected roots through soil for 15 cm or more. Hessburg and Hansen (1986) demonstrated spread of *L. wageneri* var. *pseudotsugae* through soil when intertree root contact was prevented.

Still lacking is a clear demonstration of the infection process and adequate description of the most likely points of entry. Infection through unwounded roots, i.e., through cortical cells, fusiform cambial initials, and xylem mother cells, conflicts with our knowledge of the pathological anatomy of this disease, but this route is the most common mode of entry of other vascular wilt pathogens (Beckman 1987). *Leptographium wageneri* var. *pseudotsugae* has never been observed in any cell types other than mature xylem axial and ray tracheids (Hessburg 1984; Hansen et al. 1988; Hessburg and Hansen 1987; Smith 1967; Wagener and Mielke 1961), and in vitro assays indicate that the fungus lacks cellulolytic and pectolytic enzymes needed for direct penetration of primary and secondary cell walls and compound middle lamellae (Hessburg and Hansen 1987; P.F. Hessburg unpublished data; Smith 1969). Infection of healthy roots apparently occurs by means other than direct penetration by hyphae. The objectives of this study were to critically examine the process of root infection in Douglas-fir seedlings under controlled conditions, and describe possible points of entry.

Materials and methods

We learned in field and greenhouse experiments that preceded this work, that microscopic examination was a reliable technique for identifying new infections. For example, in a greenhouse experiment with 223 inoculated Douglas-fir seedlings, we paired microscopic examinations with pathogen isolations. Microscope examinations more reliably detected the presence of *L. wageneri* var. *pseudotsugae* than isolations in three important settings: (*i*) where fungal lesions were small (<1 cm) or minute (<3 mm), (*ii*) where mycelia were already vacuolized, or (*iii*) where mycelia were present with host-produced tylotic cells, resinosus, or gums in host reaction zones. Likewise, small and minute lesions were encountered in sapling and pole-sized Douglas-fir roots in association with insect maturation feeding wounds; pathogen isolation was seldom successful where similar host responses were apparent. Microscope evaluation of new infections was equivalent to isolation in cases where there was abundant fresh, infected plant material.

This study consisted of three experiments with Douglas-fir seedlings. All seedlings were bare-root, grown for 2 years in the nursery from a single local seedlot, and root pruned 3 months prior to lifting. In each experiment, we critically examined new infections in inoculated seedlings to identify probable points of entry. In one experiment, we inoculated dormant seedlings and looked for points of fungal entry. In the next two experiments, we inoculated seedlings that had been actively growing for 4 and 8 weeks. These latter experiments were designed to give seedlings time to callus over wounds incurred at the nursery so that we might observe fungal entrance at other locations.

In the first experiment, root systems of 103 dormant seedlings were dip-inoculated in a viscous blend of *L. wageneri* var. *pseudotsugae* spores and hyphal fragments (isolate Vw-45). Haemocytometer readings of potentially infective propagules ranged from 10^4 to 10^6 spores and hyphal fragments/mL, and hyphal fragments typically included several septa. Streakings of five 0.5-mL samples of the suspension on standard potato-dextrose agar (PDA) plates demonstrated retention of inoculum viability and a greater than required inoculum potential. Isolate Vw-45 was collected from the Balm Creek drainage, Blue River Ranger District, Willamette National Forest, T. 18 S., R. 5 E., Sec. 4, Willamette Meridian. Pathogenicity of Vw-45 was confirmed via inoculations to Douglas-fir seedlings in the greenhouse and to sapling Douglas-fir in the field.

After inoculation, the 103 dormant seedlings were transplanted, three to a pot, in 2.5-L containers. Seedlings were potted in a pasteurized planting medium composed of equal volumes of washed silica sand (EI-20) and peat (Baker 1957). Pots were randomized in the greenhouse and watered from above three times each week for 9 weeks (61 days) with 20% sterile Hoagland's complete mineral nutrient solution (Hoagland and Arnon 1950). Seedlings were destructively sampled 34, 44, 50, 57, and 61 days after inoculation, with six pots sampled each time, including pots with seedlings showing even the slightest indication of wilting. Early greenhouse trials with dip-inoculated seedlings showed that wilting was evident before chlorosis, and once seedlings were chlorotic, root colonization by *L. wageneri* var. *pseudotsugae* was too extensive to reliably determine points of entry. In essence, we needed to catch *L. wageneri* var. *pseudotsugae* in the act of initiating new infections.

At the time of destructive sampling, seedlings were removed from the pots, gently washed, wrapped in a wet paper towel, and cold stored in a plastic bag at 4°C for up to 24 h while awaiting dissection. Dissections were made under a binocular dissecting microscope by systematically removing the bark from the root collar to the root ends. The inside surface of the bark was examined for necrotic lesions or other visible evidence of direct hyphal penetration through secondary phloem and vascular cambium. Any discolored area, regardless of its size or shape was examined under the microscope. Once the bark was removed, the remaining tissue was repeatedly misted with a spray bottle containing a sterile dilute solution of distilled water and lemon juice to keep the wood moist and discourage phenolic discoloration. The number and types of infection were tallied for each seedling. To verify origins, all putative infections, including obvious black-stain columns ranging from minute (<3 mm in length) to several centimetres in length, resinous lesions in xylem, and sapwood or secondary phloem coloration abnormalities, were free-hand sectioned and examined under a microscope.

Free-hand sections ranged in thickness from 20 to 50 μm (estimated using the average tracheid width of 25–50 μm as a guide). Sections were observed under phase contrast microscopy (250–1000× magnification), with the incident light source in Nelson's critical illumination (Needham 1977, pp. 315–317). Sections were inspected for the presence of hyphae and microhyphae, and any conidiogenous apparatus. All observed hyphae were compared morphologically and histologically with those of *L. wageneri* var. *pseudotsugae* in Douglas-fir using the descriptions of Hessburg and Hansen (1987) as the standard. *Leptographium wageneri* var. *pseudotsugae* infection was tallied only when hyphae were identical to the published descriptions.

In the second experiment, 99 seedlings were grown for 4 weeks in a greenhouse prior to inoculation. The seedlings were carefully removed from the pots to avoid wounding, and the roots were gently rinsed free of the sand and peat potting medium with cool tap water. Forty-five seedlings were dip-inoculated as above and transplanted, three to a tube, into plastic transplant tubes (7 × 25 cm). In nine tubes, only two seedlings were transplanted because the root systems were large. Root systems of the remaining 54 seedlings were lightly and evenly clipped with a sterile scissors before dip-inoculation to create new wounds for possible infection. These seedlings were likewise dip-inoculated and transplanted three to a tube.

In a third experiment, 18 seedlings actively that had been growing for 8 weeks were dip-inoculated and transplanted individually to tubes. Two seedlings that were wounded during the transplanting were eliminated from the experiment. The remaining 16 seedlings were randomized in racks with seedlings that had been actively growing for 4 weeks, some of which were intact and some of which had clipped root seedlings, and incubated at 17°C in a controlled temperature growth chamber. Seedlings individually transplanted to tubes were wick-watered with 20% Hoagland's solution (Hessburg and Hansen 1986). Wick-watering minimized the development of anaerobic soil conditions that usually developed with top watering as diseased roots died.

Seedlings grown for 4 weeks from dormancy and transplanted with roots intact were destructively sampled 40 days after inoculation. Seedlings grown for 4 weeks and transplanted with roots clipped were destructively sampled 35 days after inoculation, and those grown for 8 weeks and transplanted were destructively sampled 27 days after inoculation.

Results

Dormant seedlings

Of the 103 dormant inoculated seedlings, 48 (47%) were infected with *L. wageneri* var. *pseudotsugae* (Table 1). All infections were dissected, and points of entry were identified under the compound microscope. The average number of *L. wageneri* var. *pseudotsugae* infections per seedling was 1.3, and no seedling was infected more than three times. At 34 days after inoculation, 1 of 18 sampled seedlings was infected (6%). At 44 days, 12 of 28 seedlings (43%) were infected, at 50 days, 12 of 18 seedlings (67%) were infected, at 57 days, 13 of 21 seedlings were infected (62%), and at 61 days, 9 of 18 seedlings (50%) were infected (Fig. 1). Complete root system dissections revealed that *L. wageneri* var. *pseudotsugae* infected roots through wounds and natural openings where a direct path to the xylem was exposed and never penetrated living cortical or cambial tissues to infect its host. Stain columns resulting from infection were from 1 to 10 cm long and were readily distinguished by their distinctive chocolate-black color against the unblemished light yellow-white of healthy xylem. In all cases, regardless of size, both ends of a fusiform stain lesion were clearly identifiable. A point of entry was tallied only when the characteristic hyphae (using the measures and descriptions of Hessburg and Hansen 1987) of *L. wageneri* var. *pseudotsugae* reached the outermost ring of xylem tracheids in a root as determined by microscopic exam. Fungal entrance occurred where roots were broken, cut, cracked, or abraded; where new lateral roots had emerged by penetrating and rupturing the cortex and epidermis; or where small roots had died back to the point of attachment.

Six different types of infection courts were identified (Fig. 2). Type 1 consisted of spider-root stubs that were produced by root pruning at the nursery. Pruned root ends were surrounded by a proliferation of newly formed roots; *L. wageneri* var. *pseudotsugae* infected the pruned ends not yet callused or overgrown with bark at the time of inoculation. Type 2 consisted of broken roots that were detached during lifting or sorting at the nursery; *L. wageneri* var. *pseudotsugae* entered the xylem through the broken ends. Type 3 consisted of bark abrasions, also the result of lifting injuries at the nursery, that exposed the outer rind of xylem tracheids; *L. wageneri* var. *pseudotsugae* penetrated the xylem on the perimeter of wounds where tracheids were partially covered by bark. Type 4 consisted of lateral root infection courts that occurred at sites of new root emergence. Hyphae of *L. wageneri* var. *pseudotsugae* invaded openings in the bark created by new lateral roots, which emerge by physically forcing their way through the living secondary phloem and cortex. Black-stain lesions developed in the parent root around the point of attachment of new lateral roots that were themselves not initially infected. Type 5 consisted of split or fractured root infection courts that occurred on partially fractured roots or at acute-angled root junctures that were partially split. Type 5 differed from type 2 in that damaged roots were still alive and attached. *Leptographium wageneri* var. *pseudotsugae* penetrated the wound to exposed xylem often colonizing proximal and distal portions of wounded roots. Type 6 consisted of dead root infection courts that occurred at points of partial or complete detachment of dead fine roots from parent roots. Dead roots that were partially detached were not penetrated by black-stain hyphae, and stain lesions originated in the parent root around the point of detachment or in small stubs that remained attached to the parent root. Of the infected seedlings,

Table 1. Infection frequency among Douglas-fir seedlings whose roots were dip-inoculated in a viscous blend of *Leptographium wageneri* var. *pseudostugae* spores and hyphal fragments 0, 4, and 8 weeks out of dormancy.

Treatments	No. of seedlings inoculated	Infected (%)[b]	Infection court type[a]						
			Type 1 (%)	Type 2 (%)	Type 3 (%)	Type 4 (%)	Type 5 (%)	Type 6 (%)	Type 7 (%)
Fully dormant (lifted/cold stored)	103	47	37*a*	15*a*	7*a*	24*a*	6*a*	6*a*	6
Nondormant 4 weeks (intact roots)	45	100	26*b*	2*b*	0*a*	55*b*	0*a*	2*a*	15
Nondormant 4 weeks (clipped roots)	54	87	2*c*	94*c*	0*a*	0*c*	4*a*	0*a*	0
Nondormant 8 weeks (intact roots)	16	50	0*c*	0*b*	0*a*	100*d*	0*a*	0*a*	0

Note: Percentages in the same column followed by the same letter are not significantly different according to tests of last significant difference (LSD; Steel and Torrie 1980) of the arcsine of the square root of the percentages. LSD at $p = 0.01$ was 9%. Unknown infection courts were not tested.

[a]See Fig. 2 for illustrations of infection court types. Values given are percentages of infected trees with that particular type of infection court. Type 1, spider root stub; type 2, broken or cut root; type 3, bark abrasion; type 4, new lateral root; type 5, split or fractured root; type 6, dead root; type 7, unknown.

[b]Percentage of inoculated trees that became infected. Infections were determined by microscopic examination.

Fig. 1. Time to infection for Douglas-fir seedling roots dip-inoculated with *Leptographium wageneri* var. *pseudotsugae* and incubated for 2 months in a greenhouse. Error bars are mean standard errors.

63% of the infections occurred through wounds incurred at the nursery (types 1–3 and 5, Fig. 2), and 31% occurred through natural openings to exposed xylem (types 4 and 6).

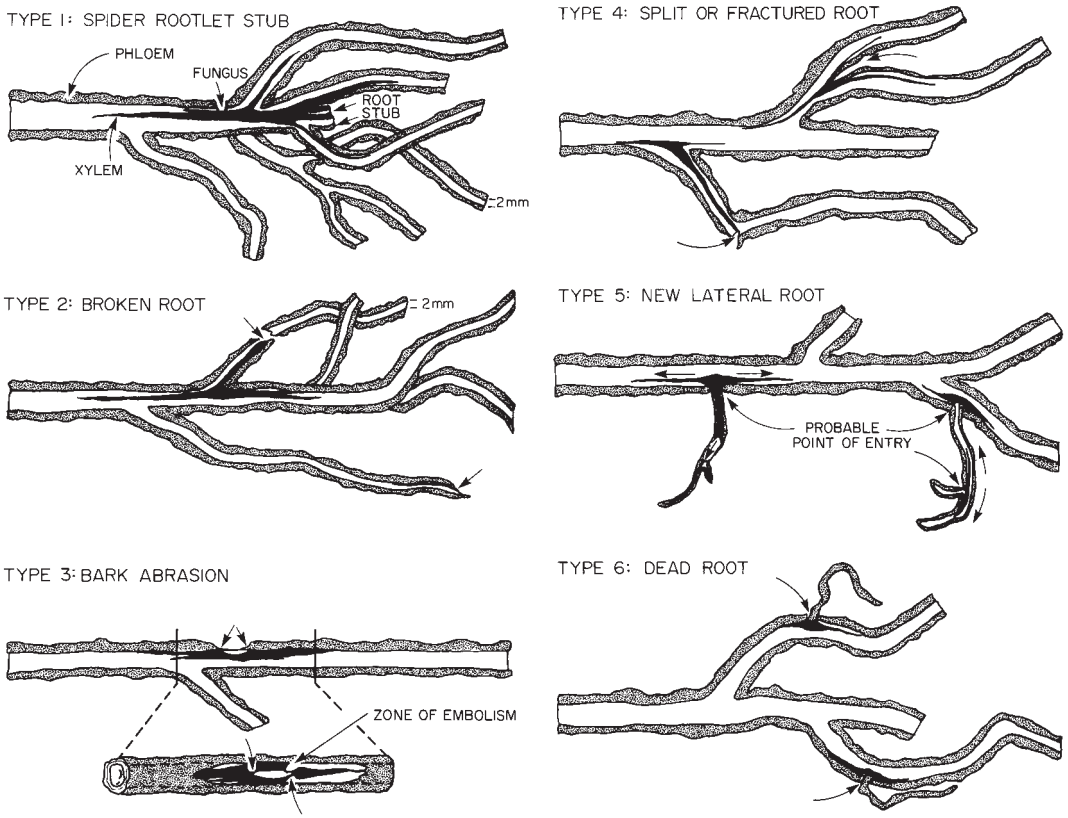
Nondormant (4 week) seedlings with intact roots

Among the 45 seedlings with intact roots (inoculated 4 weeks after transplanting and sampled 40 days after inoculation), all were infected with *L. wageneri* var. *pseudotsugae* (Table 1), but infection courts could not be ascertained with certainty on seven seedlings. The remaining 38 seedlings averaged 3.4 infections per seedling. New root and shoot development was evident on all seedlings. Infections occurred through natural openings in 26 of the 45 seedlings (55%), and through spider-root stubs and broken or cut roots in 28% (Table 1). No hyphal penetration of bark, cambium, or any parenchyma cell was observed.

Nondormant (4 week) seedlings with clipped roots

Among the 54 seedlings with clipped roots (inoculated 4 weeks after planting and sampled 35 days after inoculation), 47 were infected (87%), and of these, 94-percent were infected through clipping wounds (Table 1). Although clipping removed nearly all previous wounds, most infected seedlings had more than 15 infections, and stain columns were beginning to coalesce at the time of destructive sampling. It was therefore difficult to obtain an accurate count of the total number of infections in some seedlings, especially where infections were close together. We only tallied infections when the point of entrance could be clearly established. For this reason, our estimate of the number of infections per seedling is conservative. No infection by direct hyphal penetration of bark, cambium, or parenchyma was observed.

Fig. 2. Schematic drawings of the six types of infection courts observed on dip-inoculated Douglas-fir seedlings.



Nondormant (8 week) seedlings with intact roots

Among the 16 seedlings with intact roots (inoculated 8 weeks after planting and sampled 27 days after inoculation), 8 were infected, all through type 4 lateral root infection courts (Table 1). Many old wounds were apparent on roots of these seedlings, but none were colonized by *L. wageneri* var. *pseudotsugae* or any other fungus. In many cases, wounds were callused and exhibited localized resinosus. At final sampling nearly 3 months after the initial planting, new foliage growth was nearly completed, but new needles were still succulent, and new white root tips were present throughout most root systems. At least five points of entry were found on all infected seedlings.

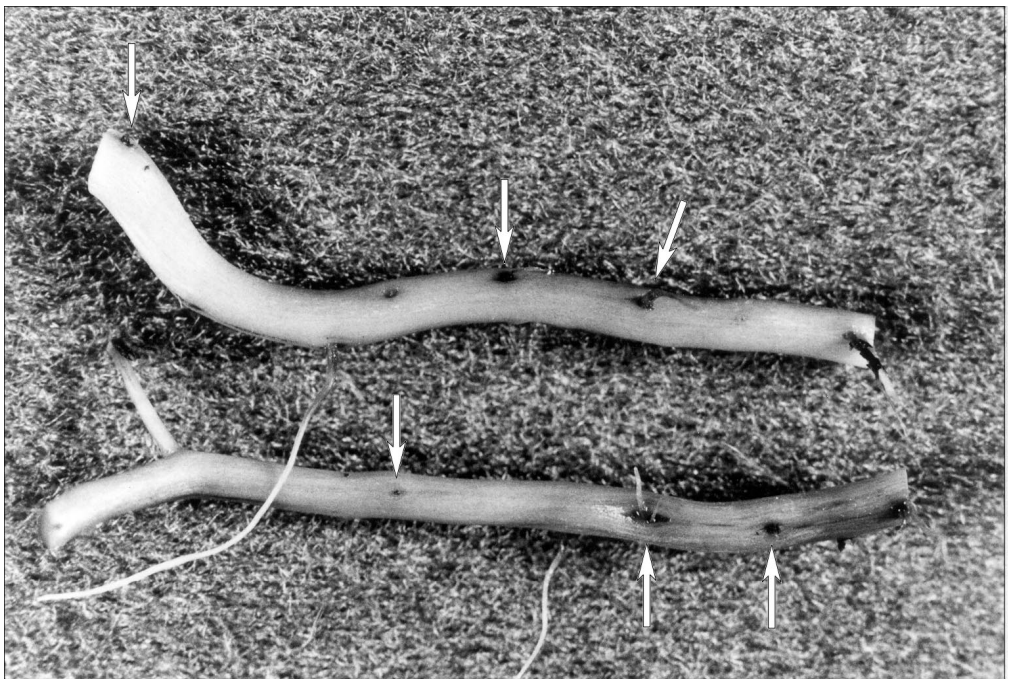
Once all root system dissections were completed, we compared the percentages of the six infection types for the three experiments (dormant, active 4 weeks, active 8 weeks) using the least significant difference test (Steel and Torrie 1980). Using the arcsine of the square root transformation for percentage data, we found a significant difference (p = 0.01) in the percentage of type 1, 2, and 4 infection courts among treatments (Table 1). Of special note, we observed a significant difference in the percentage of type 4 infection courts among seedlings across all treatments. As expected, most infection courts were type 2 among the seedlings with clipped roots that were inoculated 4 weeks after transplanting, and the percentages of type 2 infections were significantly different among most treatments. In general, we noted that after 1 month, most minor root injuries (<2 mm) were not infected by *L. wageneri* var. *pseudotsugae* because the site of injury was effectively cordoned off by callus tissue and resinosus. Larger injuries, such as those occurring by root pruning, could be infected even after 1 month, because callus development over the wound was incomplete.

Discussion

In the three experiments involving *L. wageneri* var. *pseudotsugae* inoculation of dormant and actively growing (4 and 8 week) seedlings, we identified six types of infection courts. In the seedlings scored *L. wageneri* var. *pseudotsugae* positive by microscopic examination, all infections occurred through wounds or natural openings, and in no case was there histological evidence of infection through bark or cambial tissues.

Our experiments yielded several important observations. First, dormant seedlings from the nursery are obviously replete with wounds incurred through root pruning, lifting, and handling. Wounds are readily infected by *L. wageneri* var. *pseudotsugae* in the first month after planting, but after 4 weeks of active growth, most wounds are closed, and by 8 weeks, wounds are no longer entrance courts for *L. wageneri* var. *pseudotsugae*. Second, seedlings active for 4 weeks with clipped roots displayed nearly five times the number of infections per seedling as seedlings active for 4 weeks with intact roots. Where wounds were abundant, infections were abundant. Third, new lateral roots emerge in the first month of active growth but they are more common in the second month. When old wounds were overgrown after 4 and 8 weeks of active growth, natural openings were

Fig. 3. Photograph of type 4 (new lateral root) infection courts on Douglas-fir seedling roots. Infections occured adjacent to new lateral roots that have recently forced their way to the outside through the endodermis, cortex, and epidermal tissues by enzymatic digestion and mechanical pressure. Several type 4 infection courts are indicated with arrows. Roots are actual size.



the only entrance courts for *L. wageneri* var. *pseudotsugae* (Table 1).

Infection frequency through spider root stubs decreased with increasing growth period before inoculation; wound closure discouraged infection after 4 weeks and appeared to prevent it after 8 weeks. In inoculation trials with seedlings active for 4 weeks with clipped roots, most spider root stubs were removed; the frequency of type 1 infection courts was reduced to its lowest level, and the frequency of type 2 infection courts (broken or cut roots) increased to its highest level.

Damaged roots are frequently observed on trees under field conditions. For example, we commonly observe wounds on roots adjacent to stones buried in the soil during field excavations. We suspect that wounds arise as roots grow and increase in girth, and when winds create broken or abraded roots under the force of high velocity gusts. Root wounds may be important sites for infection of healthy trees on the perimeter of infection foci, especially where root anchorage has been compromised by root disease.

Twice as many type 4 infection courts (lateral root) were apparent on seedlings inoculated 4 weeks out of dormancy than on dormant seedlings (Table 1). Because infections occurred at sites of new lateral roots, increases in infection frequency were related to longer periods of lateral root initiation. Lateral roots arise from cells of the pericycle, the outermost layer of the primary vascular stele, which becomes meristematic at sites of new lateral root initiation. A new lateral root forces its way through the endodermis, cortex, and epidermal tissues by enzymatic digestion and mechanical pressure (Esau 1977; Kramer and Kozlowski 1960; and Kozlowski 1971). The lack of an initial connection between the emerging lateral root, and the cortex and epidermis of the parent root leaves an opening to the xylem of the parent root that serves as an entrance court for *L. wageneri* var. *pseudotsugae* (Fig. 3). Dissections of newly infected roots showing this pattern of infection revealed that *L. wageneri* var. *pseudotsugae* hyphae had colonized the xylem of the parent root opposite these openings, and black-stain columns elongated axially away from these sites.

In the first experiment where dormant seedlings were inoculated and most infections occurred through wounds, new roots developed at an increasing rate, and new shoot growth was evident as dormancy was broken. Type 4 lateral root infection courts were observed 24% of the time on infected seedlings. In seedlings with intact roots grown for 4 or 8 weeks before inoculation, 55 and 100% of the infection courts were type 4, respectively. On larger Douglas-fir, new lateral roots are proliferated in the same manner. To understand the role and importance of new lateral roots in intertree transmission within established foci, it will be important to examine these possible entrance courts on larger Douglas-fir.

Infection of openings left by dead roots occurred in both dormant inoculated seedlings and those grown for 4 weeks before inoculation, but were absent on seedlings grown for 8 weeks before inoculation. We would expect a low frequency of type 6 dead root infection courts on seedlings that are rapidly increasing their fine root systems. Type 6 infections occurred either through stubs <2 mm long adjacent to a branching point or on the parent root emanating from the original branching point. Dead roots may be important sites of new infection and fungal egress. Fine roots generally live from 1 to 4 years, and mortality is associated with cold weather, excessive soil moisture, poor soil drainage and aeration, drought, attacks by insects, fungi, and other organisms, and foliage loss (Torrey and Clarkson 1975; Zimmerman and

Brown 1971). Estimates of the annual mortality and decomposition of fine roots for 70- to 170-year-old Douglas-fir range from 5.5 to 7.2 $MT \cdot ha^{-1} \cdot year^{-1}$ (Santantonio 1982). Estimates of the annual turnover in hardwood and softwood tree species range from 10 to 90% of the fine roots (Kramer and Kozlowski 1979). During the summer and winter months, dead roots are abundant on healthy trees and may be important entrance courts for *L. wageneri* var. *pseudotsugae* on large trees as on seedlings.

Douglas-fir trees infected with *L. wageneri* var. *pseudotsugae* retain much less foliage than healthy trees (Witcosky 1981). Such loss may further increase the amount of fine root mortality on black-stain root diseased trees. When trees succumb to disease and the associated insect attack, most major roots have been colonized. It is likely that *L. wageneri* var. *pseudotsugae* has colonized many of the lateral roots upon which fine roots are dying. Since infection occurs at these sites, fungal egress may also occur through the reverse route.

While we have observed seedling infections in the field on numerous occasions, and wounds and openings of the kinds we have described are readily found on seedling, sapling, and pole-sized Douglas-fir alike, knowledge of wounds and entrance courts for fungi on seedlings from greenhouse and growth chamber trials should be applied with a certain amount of caution. Microbial communities of the rhizosphere and rhizoplane, and edaphic factors will most certainly exert some measure of influence on infection ecology and frequency in relation to fungal entrance and egress.

The pathological anatomy of black-stain root disease has been critically examined in pines and Douglas-fir. Hyphae of *L. wageneri* var. *pseudotsugae* never invade living parenchyma cells, including those of the bark, cambium, and xylem. The black-stain fungus is unique among its pathogenic and saprophytic associates because it is unable to colonize any parenchyma cells, simple pits, or half -bordered pits (Hessburg and Hansen 1987). Neither fungal penetration nor egress through living bark and cambium has ever been observed. These features can be exploited in *L. wageneri* var. *pseudotsugae* detection and diagnosis. It is apparent that *L. wageneri* var. *pseudotsugae* can infect and ramify in its hosts via wounds and natural openings, and we have observed infection via these pathways in seedlings.

In one noteworthy instance in the field, we observed *L. wageneri* var. *pseudotsugae* entrance through a wound on a sapling. In the same stand where we were verifying pathogenicity of isolate Vw-45 with field inoculations to sapling Douglas-fir, we observed an occasion of natural *L. wageneri* var. *pseudotsugae* cross-over from a Douglas-fir sapling to an adjacent (approximately 0.5 m distant) western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) sapling. Cross-over was mediated by an aggressive *Armillaria ostoyae* (Romag.) Herink infection common to both saplings, which had created obvious open lesions on both the transmitting and receiving roots. The identity of the *L. wageneri* var. *pseudotsugae* pathovar was established in greenhouse pathogenicity trials using Douglas-fir and western hemlock seedlings. Seven of 10 Douglas-fir seedlings were infected by the isolate, and none of the 10 western hemlock seedlings were infected. From these results, we interpreted that *L. wageneri* var. *pseudotsugae* can infect western hemlock in the field, but frequency may be limited. Subsequent pathogenicity trials with western hemlock seedlings, confirmed that seedlings were only occasionally killed (<5%) by the *L. wageneri* var. *pseudotsugae* isolate from western hemlock.

Infection of wounds by vascular wilt pathogens is common. For example, Selman and Buckley (1959) studied factors that affect invasion of tomato roots by *Verticillium albo-atrum* and showed that transplanting injury and root cutting to expose the vascular system increased vascular infection frequency dramatically. Among hardwood vascular wilt pathogens, infection through wounds is also the norm. For example, the Dutch elm disease caused by *Ophiostoma ulmi* enters healthy elms when Scolytid bark beetles create feeding wounds in branch axils; the Oak wilt fungus (*Ceratocystis fagacearum*) is transmitted by sap-feeding Nitidulid beetles when they feed on sap emanating from fresh wounds; the verticillium wilt fungus (*Verticillium albo-atrum*) infects maples, elms, ashes, and other species through wounds; and the Persimmon wilt fungus (*Acromonium diospyri*) enters its hosts through wounds (Tainter and Baker 1996). But it is significant that *L. wageneri* var. *pseudotsugae* invades through natural openings because this feature is apparently rare or absent among vascular wilt pathogens (Beckman 1987, and references therein), especially among wilt pathogens of trees.

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