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Title: PATHOGENESIS AND INTERTREE TRANSMISSION OF VERTICICLADIELLA WAGENERI IN DOUGLAS-FIR (PSEUDOTSUGA MENZIESII)

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Dr. Lewis F. Roth for Dr. Everett M. Hansen

Verticicladiella wageneri Kendr. is a vascular wilt pathogen of Douglas-fir in the Pacific Northwest. The disease is characterized by black staining of colonized sapwood; crown symptoms are those typical of a wilting syndrome. Histopathological studies revealed that the pathogen is limited to the xylem but causes vascular dysfunction in both the xylem and phloem. In xylem, hyphae grew in lumens of mature tracheids increasing resistance to the flow of xylem sap; bordered pit penetration facilitated intertracheal growth. Living host cells were never invaded by hyphae and evidence for the primary involvement of translocateable phytotoxins was lacking. Increased vertical and circumferential extension of the fungus, systematically reduced the capacity of vascular tissue to conduct water. Phloem vascular dysfunction occurred with no evidence of mycelial invasion of phloem tissues. The appearance of engorged sieve cells and flattened albuminous cells, adjacent only to regions of heavily ramified xylem, suggested that

this xylem colonization indirectly impeded centripetal transport of photosynthate through rays.

Xylem pressure potential and transpiration water uptake were periodically measured on V. wageneri inoculated and control seedling groups to indicate the earliest significant consequence of vascular tissue colonization. Circumferential colonization of inoculated seedling roots consistently exceeded 80% when significant differences in pressure potential and water uptake were first apparent; radial colonization was proportionally less (35-61%). This pattern, supported by the histopathological evidence, suggested that foliage wilting was related to vascular occlusion.

Root infections of dip-inoculated Douglas-fir seedlings were initiated through artificial wounds and natural openings to exposed xylem, and living bark and cambial tissues were never directly penetrated by hyphae.

Root graft transmission of V. wageneri in Douglas-fir was verified from field excavations in natural infection centers. In potted seedling experiments, healthy seedlings regularly became infected whether intertree root contact was allowed or completely restricted.

In growth chamber experiments, cool soil temperatures favored infection and establishment of V. wageneri in inoculated Douglas-fir seedlings; warm temperatures decreased the likelihood of infection. Vertical growth rate varied predictably with soil temperature fluctuations in the greenhouse; soil temperatures within or above the growth optimum range favored faster growth of V. wageneri in xylem. Growth rates in roots of older trees compared favorably with estimates of the annual rate of radial increase of infection centers.

Pathogenesis and Intertree Transmission of *Verticicladiella wageneri* in Douglas-fir (*Pseudotsuga menziesii*)

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To my wife, Johnette Marie Hessburg,

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter III

Chapter IV

Chapter V

Chapter VI

LIST OF TABLES

PATHOGENESIS AND INTERTREE TRANSMISSION OF *VERTICICLADIELLA WAGENERI* IN DOUGLAS-FIR (*PSEUDOTSUGA MENZIESII*)

INTRODUCTION

Black-stain root disease, caused by *Verticicladiella wageneri* Kendrick, was first discovered causing tree mortality in western pine forests in the 1930's. Discovery of the disease in Douglas-fir in the Pacific Northwest occurred much later (Cobb and Platt, 1967; Goheen and Hansen, 1978). Little is known of the host-pathogen relationship in the Douglas-fir ecosystem; in particular, information is needed on disease ontogeny and infection center epidemiology.

This dissertation is comprised of two major areas of study: 1) the pathogenesis, and 2) intertree transmission of *V. wageneri* in Douglas-fir. Studies addressing the first area are described in Chapters II through IV, those addressing the second, in Chapters V and VI. Chapter II is an in-depth study of the pathological anatomy of the disease. In Chapter III vascular tissue colonization and occlusion by *V. wageneri* are related to changes in seedling water status. In Chapter IV, the process of root infection in Douglas-fir seedlings is examined and the infection courts described. Three intertree transmission pathways are described in Chapter V, and the effect of soil temperature on infection and growth of *V. wageneri* in roots is reported in Chapter VI.

Results from both areas of study should contribute to our understanding of the root disease syndrome and the epidemiology within infection centers.

LITERATURE REVIEW

Chapter I

In 1961, Wagener and Mielke published a report on a new fungal root disease of ponderosa (Pinus ponderosa Laws.), Jeffrey (P. jeffreyi Grev. et Balf.), and single leaf pinyon pines (P. monophylla Torr. et Frem.). Long vertical columns of black-stained sapwood were characteristic of the disease. Microscopic examination showed that darkly pigmented hyphae were present within tracheids of black-stained roots. Infected trees became chlorotic, were attacked by bark beetles and eventually succumbed. Initial isolates were provisionally identified as Leptographium lundbergii Lag. et Melin, but further study determined that the fungus more closely resembled the anamorphic state of Ceratocystis (Grosmannia) serpens (Goid.) Moreau. In 1962, Kendrick placed the fungus in the genus Verticicladiella Hughes, because of its sympodial rather than anellic conidiogenous apparatus, and suggested the specific epithet wageneri in honor of W.W. Wagener who conducted pioneering research on the disease. Soon afterwards, there were numerous new reports of the disease in new hosts and new locations. The geographic range of V. wageneri was extended from California and Arizona to include Colorado (Landis and Helburg, 1976), Oregon and Washington (Goheen and Hansen, 1978), Idaho, Utah, Nevada, and British Columbia (Smith and Graham, 1975; Hunt and Morrison, 1979; Goheen, 1976). The host range was expanded to include Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) (Cobb and Platt, 1967), lodgepole, knobcone, pinyon, sugar, and western white pines (Pinus contorta

Dougl., P. attenuata Lemm., P. edulis Engelm., P. lambertiana Dougl., and P. monticola Dougl.) (Goheen, 1976; Landis and Helburg, 1976), and western and mountain hemlocks (Tsuga heterophylla (Raf.) Sarg., and T. mertensiana (Bong.) Carr.) (Goheen and Hansen, 1978).

In 1978, Goheen and Hansen surveyed Douglas-fir plantations in Oregon and Washington and found black-stain root disease centers widely distributed throughout the west-side Cascade and coastal mountain ranges. Tree mortality typically occurred in pockets which appeared as areas of standing dead trees surrounded by infected trees in various stages of decline. Asymptomatic infected trees were located on the perimeter of infection centers and most centers were associated with some sort of major site disturbance including: road cuts, clear-cut margins, stream drainages, and plantation thinnings. Hansen (1978) later corroborated these findings by demonstrating that road-side trees were more frequently infected by V. wageneri than trees located at a distance from roads.

Goheen (1976) studied V. wageneri infection center epidemiology in ponderosa pine and found that intertree transmission occurred through root grafts and perhaps through contacts between major roots. Most new infections, however, occurred in small roots not in contact with, but growing within 15 cm of infected roots. Goheen hypothesized that small roots acted as infection courts and that the fungus was capable of limited growth through soil. Hicks and others (Hicks, 1978; Hicks et al, 1980) experimentally demonstrated fungal growth through untreated soil from artificial inoculum-blocks and later isolated V. wageneri from soil that was up to 6 cm away from infected roots of ponderosa pine. The pattern of mortality within

pine infection centers further supported these hypotheses. Clearly, two patterns of disease distribution began to emerge; one by which new infection centers were initiated, and a second by which the pathogen spread to healthy trees at the margins of expanding infection centers. Goheen (1976) found that ponderosa pines weakened by V. wageneri were attacked by various root and stem inhabiting insects and he hypothesized that certain subcortical insects, particularly the root breeding scolytid Hylastes macer LeC. (Coleoptera/Scolytidae), might act as vectors. In 1975, V. wageneri conidiophores, and perithecia of a Ceratocystis spp. were found in galleries of root inhabiting insects, most commonly those of H. macer (Goheen, 1976; Goheen and Cobb, 1978). Goheen determined that these perithecia were the teleomorphic stage of V. wageneri and the new species Ceratocystis wageneri Goheen et Cobb was described. In a number of cases, the pattern of black staining in roots associated with galleries of H. macer strongly suggested that infection originated from the galleries. They suggested that H. macer was a vector in ponderosa pine/black-stain root disease.

In Douglas-fir black-stain root disease, the same two patterns of disease distribution are apparent. Witcosky (1981) hypothesized that certain individuals, within the guild of subcortical, root-inhabiting insects in dead and dying Douglas-fir, might also act as vectors of the disease. In 1981, he reported that the weevils Steremnius carinatus Boh. and Pissodes fasciatus LeC. (Coleoptera/Curculionidae) and the bark beetle Hylastes nigrinus Mann. (Coleoptera/Scolytidae) were consistently associated with infected roots and root collars of diseased trees. Insects sequentially colonized roots of diseased

trees over a 2-4 year period, as each root succumbed to infection. Conidiophores of V. wageneri were observed in galleries and pupal cells of all three insect species. Soon afterward, Witcosky and Hansen (1984) reported that some field-collected individuals of H. nigrinus, S. carinatus, and P. fasciatus carried viable V. wageneri inoculum, providing further support for the vector hypothesis. To date, it has been shown that potential vectors are consistently associated with diseased trees and that propagules of the fungus are regularly on these insects in nature. In order to confirm an insect vector-pathogen relationship it must yet be shown that insects carrying viable inoculum, regularly visit healthy trees under conditions suitable for disease transmission, and produce the disease syndrome in the host as a result of such visitation.

Several investigators have reported observations on the pathological anatomy of the disease in Pinus spp. (Wagener and Mielke, 1961; Smith, 1967; Landis and Helburg, 1976). V. wageneri mycelia were confined to the lumens of mature xylem tracheids, elongating from tracheid to tracheid via bordered pit-pairs; living parenchyma cells were never attacked; and radial movement through ray tracheids was lacking. Smith concluded that except for initial infection and egress, the pathogen was confined to the xylem of its conifer hosts, and he compared V. wageneri to other wilt pathogens of trees. Harrington (1983) examined a large collection of V. wageneri isolates representative of the great diversity of geographic and host ranges, and found that there were three distinct variants, one from hard pines, one from pinyon pines, the third from Douglas-fir. Variants were classified on the basis of cultural morphology and growth

response to temperature. In a later study (Harrington and Cobb, 1984), host preferences were demonstrated for each of the three variants.

Helms et al (1971) studied the effect of V. wageneri infection on net photosynthesis, transpiration, foliar water stress, dark respiration and stomatal aperture of ponderosa pine seedlings. One month after inoculation, net photosynthesis and transpiration decreased dramatically, concurrent with large increases in foliar water stress and stomatal closure despite the absence of visual symptoms. Dark respiration remained roughly constant. The authors speculated that such changes in host physiology might increase the susceptibility or attractiveness of ponderosa pines to the bark beetles, Dendroctonus brevicomis LeC. and D. ponderosae LeC.. Goheen and Cobb (1980) later confirmed that black-stain root diseased pines were much more likely to become bark beetle infested than healthy ones. They speculated that diseased trees were more susceptible due to decreased oleoresin exudation pressure and rate of flow, and more attractive due to disease induced production of attractive compounds.

Recent investigations have explored the relationship of various edaphic factors to infection and colonization of Douglas-fir and ponderosa pine by V. wageneri (Goheen et al, 1978; Smith, 1967; Wilks et al, 1983; Harrington and Cobb, 1984). High soil moisture, intermediate soil redox and aeration conditions, and cool to moderate soil temperatures favored infection and growth in xylem. Hansen and Goheen (Hansen et al, 1983; Hansen and Goheen, unpublished data) monitored disease increase, in natural V. wageneri infection centers in Douglas-fir plantations in Oregon and Washington over a 5-year period and

found that infection centers expanded radially at an average rate of 1.5 m $\cdot$ yr^{-1}. Cobb et al (1982) reported comparable rates of infection center enlargement in ponderosa pine in the California Sierra Nevada.

Although only discovered in 1938 (Wagener and Mielke, 1961), black-stain root disease is probably indigenous to western North American forests. It is likely that prior to 1938, the disease escaped detection and tree mortality was attributed to other causes (i.e., bark beetles and known root-rot pathogens). Better diagnostic and survey methods have allowed forest pathologists and forest managers to detect the presence of V. wageneri in stands and begin to evaluate the potential impact on forests. Natural stands are not seriously threatened by the disease, but managed stands show an increased potential for serious damage and loss. The shift from predominantly natural forests to managed forests, brought about by increased harvesting and intensified forest management practices, has established the need for a better understanding of the basic biology of this host-pathogen relationship.

PATHOLOGICAL ANATOMY OF BLACK-STAIN ROOT DISEASE OF DOUGLAS-FIR

Chapter II

ABSTRACT

Twenty black-stain root diseased Douglas-fir trees from the Oregon Cascade and Coast Ranges were dissected and the macro- and microscopic patterns of fungal colonization were described. Verticicladiella wageneri colonized secondary xylem, disrupting xylem transport. Histopathological studies revealed that xylem colonization by this fungus is unique among Ceratocystis wilt pathogens. Axial colonization by hyphae was confined to xylem tracheids and radial development of mycelia in ray tracheids was limited. Parenchyma cell and cambial necroses did not appear to involve phytotoxic metabolites. Alternative mechanisms for parenchyma cell necroses not involving toxins are proposed. Living host cells, although never penetrated, responded variously to the hyphal attack of adjacent functional xylem. Secondary phloem was never invaded by hyphae, but some dysfunction was evident. Host responses to infection were numerous, but unsuccessful in curtailing spread of the pathogen. These findings contribute to our understanding of the wilting mechanism and provide clues to the actual infection and egression processes.

INTRODUCTION

Verticicladiella wageneri Kendrick (Ceratocystis wageneri Goheen and Cobb) was first reported causing a fatal root disease of ponderosa (Pinus ponderosa), Jeffrey (P. jeffreyi), and pinyon pines (P. monophylla and P. edulis) in the southwestern United States in 1961 (Wagener and Mielke, 1961). In 1965, Cobb and Platt (1967) isolated V. wageneri from a young Douglas-fir in northern California and subsequently demonstrated its pathogenicity. Since then, it has been reported throughout the range of Douglas-fir in Oregon, Washington and northern California (Goheen and Hansen, 1978; Goheen, 1976).

Goheen and Cobb (1978) reported on the occurrence of the Ceratocystis teleomorphic state in galleries of Hylastes macer in roots of ponderosa pine. Their discovery of both anamorphic and teleomorphic spore stages of Ceratocystis (Verticicladiella) wageneri in insect galleries provided strong empirical support for insect vectors in that conifer ecosystem. To date, the Ceratocystis teleomorphic state of V. wageneri has not been reported in Douglas-fir, therefore, I refer to it using the Verticicladiella anamorph.

Several investigators have reported histological observations about the disease in pines (Wagener and Mielke, 1961; Smith, 1967; Landis and Helburg, 1976). Smith (1967) reported that hyphae of V. wageneri were confined to xylem tracheids, and living parenchyma cells were never attacked, and concluded that except for initial infection or egression, the pathogen was confined to the xylem and compared it to other vascular wilt pathogens of trees.

Wagener and Mielke (1961) initially reported that intertree

spread of the disease in Pinus spp. occurred through contact between diseased and healthy roots, although no infection pathway was suggested. This assertion has been perpetuated in subsequent writings on the host-parasite relationship. Recent investigations have newly illuminated the question of intertree transmission. Goheen (1976), working with ponderosa pine, documented evidence for root graft transmission and spread of the disease through a few major root contacts and presented strong circumstantial evidence for intertree transmission involving rootlets. Excavation studies revealed that 92% of diseased trees showed evidence of infection unexplained by root grafting or contact between healthy and diseased roots. Goheen proposed that fine rootlets were functioning as infection courts, although the infection process was not demonstrated. Other evidence has been presented for pinyon pine black-stain root disease in Colorado where root grafts and contacts were again implicated (Landis and Helburg, 1976). It has been assumed of root contact transmission that hyphae of V. wageneri penetrate directly through bark and cambial tissues to exit from infected roots and infect healthy roots of adjacent trees (Smith, 1967; Landis and Helburg, 1976). This assumption lacks supporting evidence and conflicts with observations of the pathological anatomy of this disease in Pinus spp.. Hessburg (Chapter IV) recently demonstrated that small roots were the site of infection on Douglas-fir seedlings and openings to exposed xylem were requisite for infection. Studies reported here characterize the pattern of colonization of Douglas-fir by V. wageneri and clarify the evidence for infection and intertree transmission.

To characterize the pattern of vascular colonization, it is

necessary to: 1) delineate the tissues attacked by hyphae; and 2) describe the mode of attack in each effected tissue type (i.e., enzymatic vs. mechanical).

MATERIALS AND METHODS

Root systems of 20 naturally infected, 10-15 year old Douglas-fir trees were hand excavated from five widely separated disease centers; three from the Oregon Coast Range, two from the Central Oregon Cascades. Tissue samples, ca. 3 x 4 x 4 mm, taken from roots, root collar and lower stem, were fixed in FAA (Johansen, 1940) or Karnovsky's fixative (Karnovsky, 1965) and dehydrated under vacuum (20-25 mm Hg) in a graduated tertiary-butanol sequence (Johansen, 1940). Additional specimens were held for free-hand sectioning without fixation. Fixative and alcohol solutions were slowly infiltrated to minimize bordered pit aspiration; final vacuum was achieved within 20-30 minutes. Residence time for each step in the process leading to embedding was 24 hr. Specimens were embedded in paraffin (Paraplast®) and serially thick-sectioned (20-38 μm) in warm, moist air using a Spencer rotary microtome. Permanent mounts were made following complete dissolution of the paraffin from the sections with two 5 minute changes of 100% xylene. Unstained sections were examined microscopically using bright-field and phase-contrast methods.

Primary isolations were made from each infected tree onto a 10% Potato Dextrose Agar (PDA) selective medium containing 200 μg/L each of cycloheximide and streptomycin sulfate (Hicks, 1978; Hicks *et al*, 1980). From these isolations, single conidiophore subcultures were

taken and plated onto standard PDA. Isolates were also obtained in pure culture using another technique used by Anderson (1980) with Verticicladiella procera. Small chips of infected xylem were aseptically removed from roots and placed in petri dishes on sterile, wet filter paper. After 10 days incubation at 17C, conidiophores of V. wageneri were transferred to standard PDA. In most cases, pure cultures were obtained in the first transfer. The identity of the 20 V. wageneri isolates was confirmed microscopically using Kendrick's (1962) taxonomic criteria. To verify pathogenicity, 10 2-year-old Douglas-fir seedlings were wound inoculated with each isolate (Chapter III). Colonized root and stemwood specimens were taken from each isolate-group of seedlings. Seedling specimens were fixed, dehydrated, paraffin embedded and sectioned as described above.

RESULTS

Macroscopic Characteristics of Disease Development. Macroscopic examination of infected trees revealed more or less vertical columns of purplish- to chocolate-black stain tapering to a point at their advancing margins (Figure II.1). Within annual rings, columns of stained tissue often divided near their apex, producing several vertically advancing margins of varying lengths. In earlywood, stain columns were brown and often diffuse, while those in latewood tended to be more concentrated and black. Stain columns adjacent to necrotic cambia were often resin-soaked or covered by a partially hardened layer of oleoresin. External resinosus was common on the surface of heavily infected roots.

The height of stain columns in stems was variable among the excavated trees; several tree stems showed no visible stain, one stem was colonized 1.5 m above the root collar. The length of stain columns in stems appeared to be related to the number, distribution and location of infections within root systems. Infections originating from a single source produced stain columns with a long vertical ascent into the stem. Trees having multiple infections, widely distributed throughout the root system, succumbed quickly, with little vertical development of stain in the stem.

Extension of the fungus to new roots in the root system occurred when stain columns reached the root collar. Branches of stain columns moved vertically into the stem and down again into new roots. Rapid spread of the fungus throughout the entire root system proceeded once the fungus was established in the root collar.

The pattern of stain column development was the same in roots and stems. Vertical development of stain columns was more rapid than radial or circumferential development (Hessburg and Hansen, 1982; Hessburg, unpublished). In cross-section, stain columns were characteristically arc- or crescent-shaped in contrast to blue-staining Ceratocystis spp., which produce a wedge-shaped staining pattern in cross-section (Figure II.2). Stain columns often divided radially into several initially smaller columns, colonizing the earlywood of successive annual rings and appearing as concentric arcs in cross-section. Hyphae of V. wageneri developed radially between successive annual rings, via bordered pits on the tangential walls of late-season latewood tracheids (Panshin and deZeeuw, 1964). As many as 15 successive annual rings were traversed by a single stain column.

Stain columns moved upward through lateral root junctions following the path of translocation in the earlywood. When several annual rings of a lateral root were colonized, stain columns developed through the branching point in the same annual rings (Figure II.3). Stain columns were observed in roots and rootlets of all sizes, however, in some instances the fine fibrous portion of lateral roots was not colonized prior to root death.

Microscopic Characteristics of Disease Development.

A. Colonization of Longitudinal Elements. In the axial plane, hyphae of V. wageneri were always confined to sapwood xylem tracheids. Hyphal growth in tracheid lumens was serpentine and helicoid (Figure II.4). In older trees, tracheid diameters ranged from 20-60 μm, with an average of 45 μm. Hyphal diameters ranged from 3-12 μm, while most were between 5 and 7 μm. Hyphal walls were often thick (0.5-2.0 μm), averaging at least 1.0 μm. In tracheids, advancing hyphal tips were hyaline. After a residence time in the lumen, they slowly melanized to an amber brown. Mature hyphae were frequently encrusted with a coarse, granular, dark amber sheath, which at times more than doubled the overall diameter of the hyphae. Hyphae grew adjacent to the lumenal wall and were commonly anchored to it by hyphal wall or sheath material that filled in the depressions between the spiral thickenings (Figure II.5). Hyphal branching was common and most frequently observed directly adjacent to bordered pit-pairs on the radial walls (Figure II.6). Average inside diameter of bordered pit apertures was 8.0 μm. The combined pattern of serpentine and helical growth in tracheid lumens allowed hyphae to encounter the majority of bordered pits in radial walls. Intertracheal colonization

occurred through bordered pit-pairs on common radial walls. Bordered pits tended to be more concentrated towards the ends of the tracheids and less frequent throughout the middle portions. Hyphal penetrations of bordered pits appeared to be purely mechanical (Figure II.7). Narrow hyphal tips entered the pit cavity and lodged themselves in one of the many openings in the margo, which eventually ruptured as the hyphae increased in diameter. The torus was left intact, still attached to the margo, but pushed aside, clear of the exit pit border (Figure II.8). Bordered pits were completely occluded since both pit apertures were blocked with hyphae. The margo, torus and pit borders were frequently amber-stained adjacent to hyphae passing through bordered pit-pairs.

Sequential longitudinal colonization of tracheids was through bordered pits on their tapered ends; however, since the vascular cambium of Douglas-fir is nonstoried, longitudinal movement of hyphae to the next tracheid could occur along the entire length of any tracheid (Figures II.4 and II.9). Nearly every longitudinal tracheid had a parenchyma cell associated with it. Parenchyma cells were never necrotic in advance of hyphal colonization of their adjacent tracheids. Ray cells associated with colonized axial tracheids ultimately died, but far behind the advancing margin of colonization (Figure II.10). Ray parenchyma cell cytoplasm darkened in stages as the cells died. Cell walls were ultimately amber colored.

Longitudinal strand tracheids, although much less common than axial tracheids, were observed in several occasions. When a strand was composed completely of strand tracheids, they were commonly colonized by hyphae. Hyphae were never observed penetrating the

simple pit membrane in mixed strands where longitudinal strand parenchyma were interspersed, or where longitudinal epithelial cells were terminal in the strands. Hyphal tips were frequently observed entering the pit aperture of half-bordered pits from the tracheid side, and either stopping or exiting through the same aperture (Figures II.4 and II.6). Neither longitudinal strand nor epithelial parenchyma cells were invaded by hyphae of V. wageneri.

Within the sapwood, earlywood was often colonized before latewood (Figure II.11). Tracheid lumens were smaller in latewood than in earlywood and elliptical in cross-section. When colonized, hyphae effectively occluded both the lumens and bordered pits of latewood tracheids (Figure II.12). The last formed latewood axial tracheids had bordered pits in both radial and tangential walls (Figure II.12). Tangential wall pitting was only found in late-season latewood tracheids. Hyphae of V. wageneri ramified intensely in this region, often imparting a darker, black coloring to the colonized wood (Figures II.1 and II.2). Hyphae colonized latewood tracheids immediately adjacent to the vascular cambium without ever invading cambial initials or disturbing their meristematic function (Figure II.13). New earlywood tracheids were commonly produced directly over regions of heavily ramified latewood xylem (Figure II.14). By contrast, hyphal movement to the vascular cambium in earlywood was restricted. Separating the cambium from dead, mature xylem tracheids was a band of living, often mitotically dividing, xylem mother cells, which, like parenchyma cells, hyphae were unable to penetrate. Trabeculae were absent in the xylem region adjacent to the cambium, indicating that fusiform initials were not directly exposed to fungal infection prior

to cytokinesis (Panshin and deZeeuw, 1964).

B. Radial and Circumferential Colonization. Movement of the stain column tangentially was accomplished via bordered pit-pairs on radial walls of axial tracheids (Figure II.15). The interspersing of fusiform and uniseriate rays with longitudinal xylem elements had the effect of increasing or decreasing the circumferential development of the stain column by forcing tracheids to pass out of the vertical plane on either side of a ray.

Radial development of the stain column occurred principally in three ways:

1. Through growth in ray tracheids.

2. Through bordered pit-pairs on tangential walls of the last formed latewood longitudinal tracheids.

3. Via the deviation from true vertical declination by longitudinal tracheids. The vertical axis of any radial file of longitudinal tracheids is roughly constant and dependent upon the axis of the fusiform initial that produced them. The axes of laterally adjacent files of tracheids are often quite different, allowing tracheids in one file to diagonally traverse the radial walls of as many as 3-5 tracheids (Figure II.16). This difference in alignment accounted for most of the radial movement of hyphae. Hyphal growth in ray tracheids was limited by comparison; pit aperture and lumen size were less than half of that observed in earlywood tracheids (Figure II.17). Uniseriate rays were mostly parenchymatous; when present, tracheids were most often terminal in the ray. Fusiform rays were composed of ray parenchyma cells and epithelial cells lining the resin canals, none of which were penetrated by hyphae. Centrifugal

elongation of rays occurs via the production of cells by ray initials in the cambium. Rays are therefore continuous up to the cambium and beyond to the phelloderm. Hyphae that were observed colonizing ray tracheids centrifugally, approached but did not penetrate the cambium.

C. Host Response to Infection. Hyphae of V. wageneri elicited numerous responses from living host cells without directly attacking or penetrating them. Black-stain columns were frequently resin-soaked, and localized patches of resinosus were common on the exterior of roots or stems where stain occurred adjacent to the cambium in latewood (Figure II.18). In several cases, localized necrotic lesions were observed in the cambium adjacent to colonized latewood. Lesions were often surrounded by developing, uncolonized folds of callus tissue (Figure II.19). Small lesions were covered over with new wood within one season. Microscopically, the new xylem in the region of callus tissue formation had developed more wound parenchyma tissue, resin canals, and associated epithelial tissue. Tracheids followed a circuitous path through these regions. Most localized resinous lesions were not effective in limiting spread of the fungus; hyphae invariably colonized under or around the lesions in adjacent xylem. Widespread cambial necrosis occurred late in disease development and was associated only with advanced stages of chlorosis and necrosis of permanently wilted foliage. Even when necrotic, cambial tissues were not invaded.

Tyloses were frequently formed in tracheids invaded by hyphae, but not in uncolonized cells. Tyloses were small and never coalesced to block hyphal passage through tracheid lumens (Figure II.20). The tylose-forming layer protruding through the pit cavities from

parenchyma cells was heavily stained brown. In more advanced stages of infection, parenchyma cells adjacent to colonized tracheids were darkened and showed signs of breakdown. The browning of the living tylose cells preceded that of the adjacent parenchyma cells.

The most striking localized response to infection was the plugging of tracheids by gums. Except for the advancing margin of stain, tracheids that were previously colonized became all or partially occluded by the gums. Eventually the gums filled adjacent uncolonized vascular elements. Close inspection of cross-sections of stain columns frequently revealed that only a portion of the "black-stain" column was actually colonized by hyphae; hyphae accounted for 10-80% of the xylem occlusion, the remainder of the tracheids were without hyphae and were occluded only by gums (Figure II.21). Gums thoroughly plugged affected tracheids, filling in the depressions between the spiral thickenings of the inner wall surrounding the lumen. Pit apertures and chambers were likewise filled with the viscous material. In older, well established infections, both the gum and the tracheid walls were deeply amber stained. Gums appeared to be homogeneous in composition since they stained amber evenly. In a few cases gums appeared as plugs, encrusting end or side walls of tracheids near bordered pits.

Mortality in fine rootlets was often not directly associated with mycelial colonization. Fine roots of inoculated seedlings and older trees were at times colonized down to the point where secondary xylem graded into late primary metaxylem and perhaps beyond. More often nonmycorrhizal rootlets and mycorrhizal short roots died distal to the furthest advance of mycelia. I was not able to determine whether

these dead roots would eventually be colonized.

Another acute expression of vascular dysfunction was observed in the secondary phloem of heavily infected roots. In several instances, the secondary phloem surrounding these roots was swollen and the outer surface convoluted. Occasionally small fissures were present in rhytidomes, exposing portions of the secondary phloem (Figure II.22). Microscopically, sieve cells appeared engorged, presumably with sugars from photosynthesis or storage. In extreme cases, albuminous cells (gymnospermous counterpart to the companion cell) were crushed or flattened by swollen sieve cells. Death of xylem ray parenchyma cells preceded this condition in the secondary phloem.

DISCUSSION

V. wageneri was found exclusively in mature xylem tracheids and more frequently in earlywood than latewood. Translocation occurs only in sapwood xylem, and with the exception of the current year's xylem, it is confined to earlywood. Colonization of the current year's latewood axial tracheids is therefore considered significant, since this is the only time when latewood tracheids are involved in water transport. As new earlywood is formed in the spring, latewood bordered pits are naturally aspirated and the tracheids become embolized. Extensive colonization of earlywood in outer annual rings should significantly reduce water transport to transpiring foliage.

Living host cells in the xylem and phloem responded variously to the attack on xylem by V. wageneri. Witcosky (1981) first reported on resinous lesions on roots of Douglas-fir, citing that hyphae readily

escaped from "xylem tracheids into the subcortical environment created when the cambium was killed and the bark had been lifted away from the xylem surface by growth of adjacent, living inner bark". Witcosky isolated viable V. wageneri from the lesions and suggested that hyphae grow out of the roots through cracks and fissures in the outer bark. In my investigations, I incubated several roots with lesions in moisture chambers at 17C. Within one week's time, extensive conidiophore production of V. wageneri was observed. I suggest that these lesions are ready sources of external inoculum for additional intratree or intertree spread of the disease.

Compared to other Ceratocystis wilt and sapstain fungi, the colonization behavior of Ceratocystis (Verticicladiella) wageneri is singularly different. Absence of parenchyma cell colonization in the wilt pathogenesis of Douglas-fir and other coniferous hosts is trophically significant (Landis and Helburg, 1976; Smith, 1967; Wagener and Mielke, 1961; Hessburg and Hansen, 1982). Several well studied pathogens in the genus Ceratocystis, including C. ulmi (Wilson, 1965; Pomerleau, 1970; Ouellette, 1962), C. fagacearum (Sachs et al, 1970; Wilson, 1961), C. fimbriata (Zalasky, 1965; DeVay et al, 1968), and C. piceae (Wilson, 1959), demonstrate a highly specialized attack on living parenchyma cells. Less pathogenic sapstain fungi, C. ips, C. minor, C. montia, C. pilifera also attack parenchyma and epithelial tissues in addition to secondary xylem tracheids (Rumbold, 1931; Mathre, 1964; Nelson, 1934; and Basham, 1970). Ceratocystis spp. other than C. wageneri exhibit a colonization behavior where colonization of xylem conduits (tracheids or vessels) allows access to parenchyma cells. When penetrating parenchyma cell walls or simple

pit membranes, C. piceae and C. fagacearum produce appressoria and penetration pegs. Others produce haustoria or haustoria-like appendages once inside parenchyma cell walls.

Several species hydrolyse cellulose or pectic substances and are reported to bore through cell walls and ramify within the compound middle lamella (Sachs et al, 1970). Under certain conditions, some Ceratocystis species cause typical "Type 1" soft rot cavities in wood (Corbett, 1965; Nilsson, 1973; Nilsson, 1974). Colonization of Douglas-fir by V. wageneri reveals none of these specialized activities. Histological studies reveal conclusively that V. wageneri is confined to mature xylem tracheids of all types in each conifer host it invades and differs from all other pathogenic Verticicladiella spp. in this respect (Wingfield, 1983). Smith (1967, 1969) looked for evidence of cell wall penetration and degradation in Pinus ponderosa, and tested for cellulolytic and pectolytic activity in a series of cultural assays. He found no evidence of either activity. I duplicated this work using isolates from Douglas-fir, western hemlock, and ponderosa and lodgepole pines, and confirmed Smith's results (Hessburg, unpublished). V. wageneri is confined to tracheids and while in tracheids does not appear to rely on cellulose or pectic substances as a major source of nutrition. Examination of several thousand roots revealed not a single penetration of bark or cambial tissues by hyphae. Pathological anatomy and cultural assays support the observation that openings to the xylem are required for fungal infection and egress (Hessburg and Hansen, 1982; Hansen et al, 1983).

In the absence of parenchyma cell colonization, sapwood xylem tracheids are apparently nutritionally adequate to sustain rapid and

extensive fungal growth. Mycelia, developing preferentially in earlywood longitudinal xylem and often in the current year's growth, have access to storage sugars, salts and photosynthate that are routed to elongating and expanding tracheids. Mycelia also have access to cytoplasmic constituents and encrusted cytoplasm of maturing tracheids in various stages of cytoplasmic autolysis. Mycelia in recent earlywood, the region of greatest translocation, have access to solutes of xylem sap.

Vascular dysfunction is apparent in both the xylem and phloem. In the xylem, hyphae in tracheid lumens increase resistance to the flow of xylem sap. Sequential invasion of bordered pits laterally and terminally eliminates tracheid involvement in translocation. Adjacent tracheids are adversely affected, pits aspirate, and tracheids are embolized. With increasing tracheidal colonization and occlusion, larger regions of xylem are circumnavigated by the transpirational flow. Once xylem colonization and gum plugging of tracheids is extensive, I suggest that trees lose their ability to translocate sufficient quantities of xylem sap to meet the daily demands established by evapotranspiration. The result is a gradual increase in the level of net nonrecovery from diurnal transpiration stress, ultimately to some lethal level (Chapter III).

Phloem transport dysfunction occurred in the absence of mycelial colonization of the secondary phloem. Disruption of normal phloem transport was only apparent adjacent to large regions of heavily colonized rootwood, including the current year's xylem. The appearance of distended sieve cells and flattened albuminous cells indicates that photosynthate was not emptying rapidly enough from rays

into the xylem. Sieve cells are the conduit in phloem translocation; albuminous cells empty and fill sieve cells at sinks where sugars and other carbohydrates can be utilized (Cronshaw, 1981; Evert, 1977; Esau, 1977). Organic and inorganic molecules are loaded into the phloem from leaf mesophyll and are transmitted to sieve cells by contiguous albuminous cells (Giaguinta, 1983). Albuminous cells transfer these materials ultimately into phloem ray parenchyma cells which they border terminally in rays. Loss of normal function of ray cells adjacent to colonized tracheids might reduce or eliminate the transference of photosynthate from rays.

ACKNOWLEDGEMENTS

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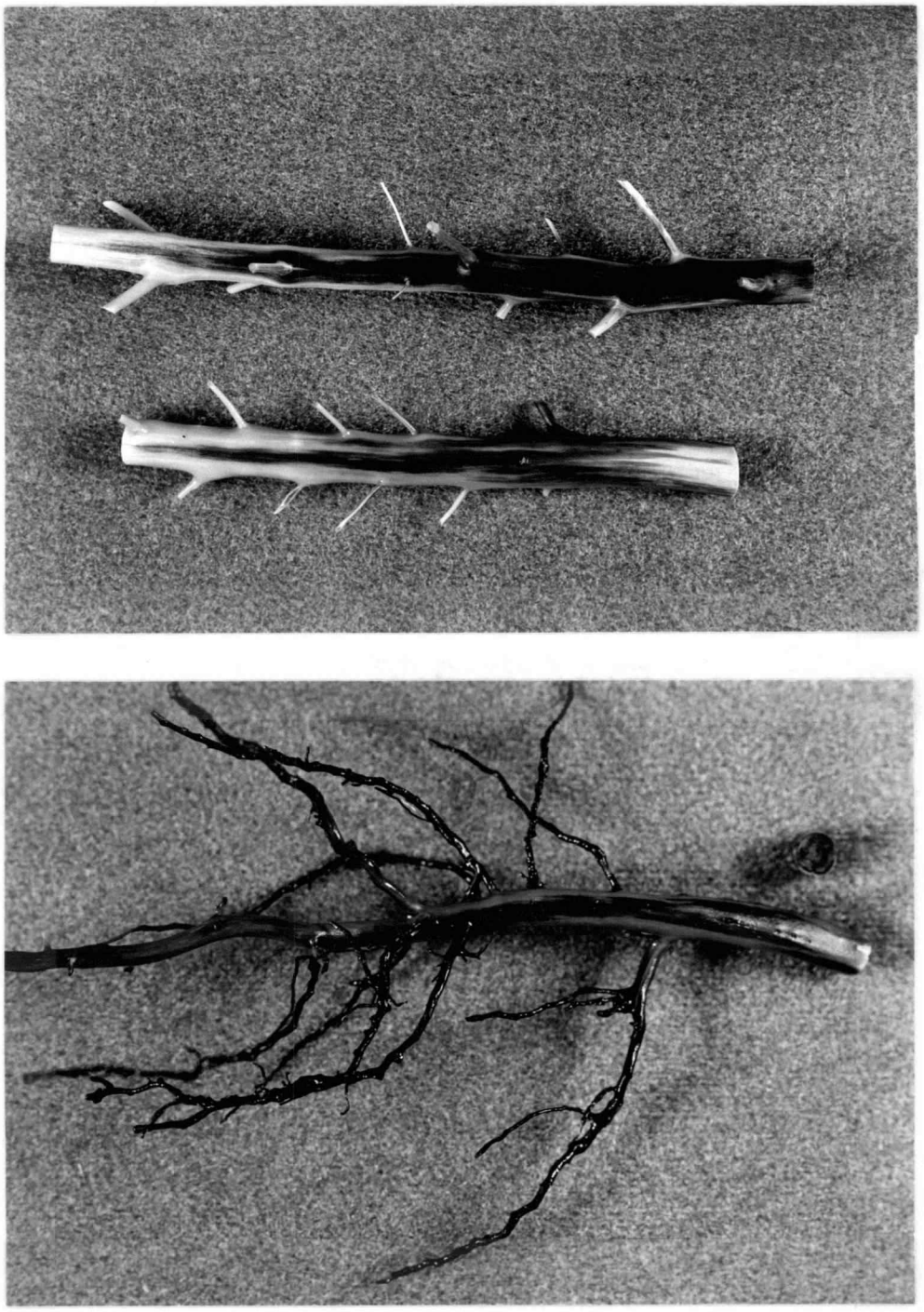

Figure II.1. Typical black-stain columns in Douglas-fir seedling root and stemwood. Note vertical extension of stain versus circumferential. (actual size).

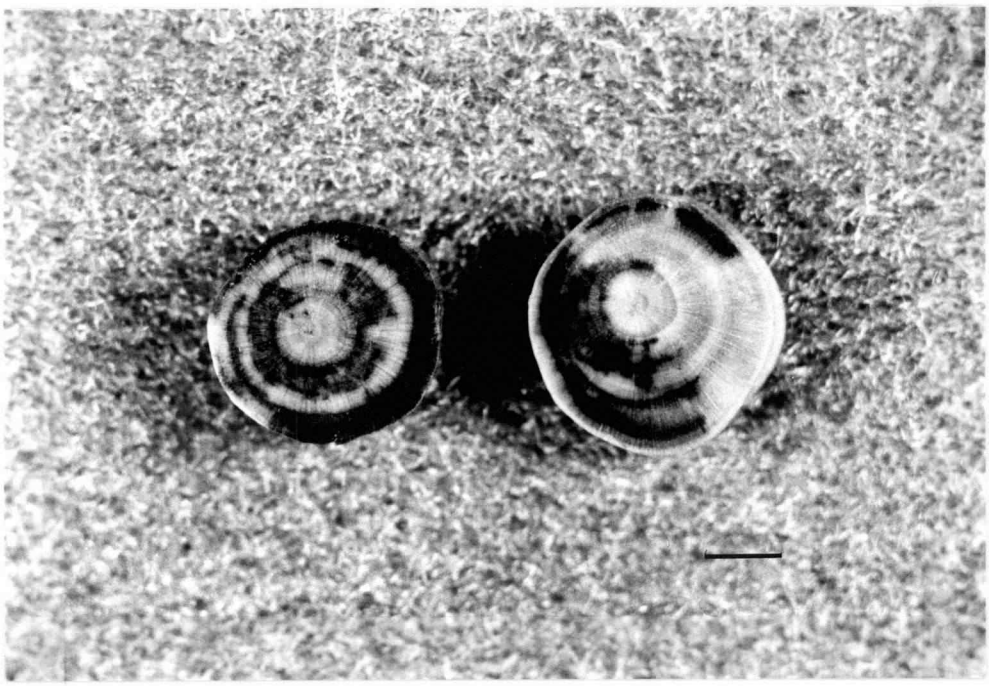

Figure II.2. Cross-sectional view of black-stain columns showing characteristic concentric arcs of stain in the earlywood. (bar = 5mm).

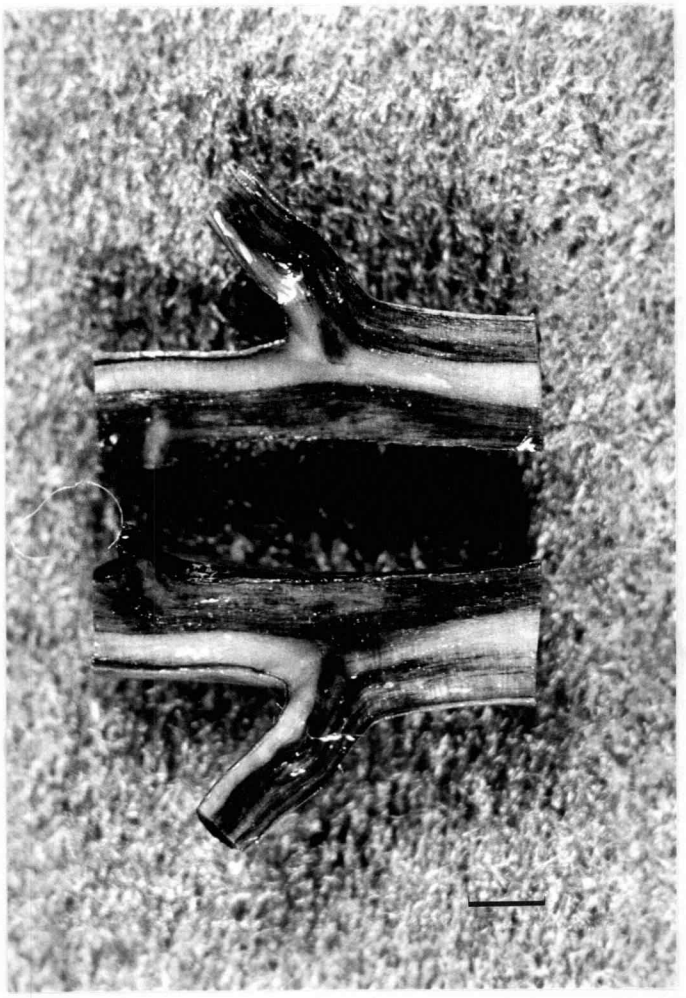

Figure II.3. Longitudinal view of stain column development through a lateral root junction. *V. wageneri* mycelia follow the path of translocation in earlywood. (bar = 5mm).

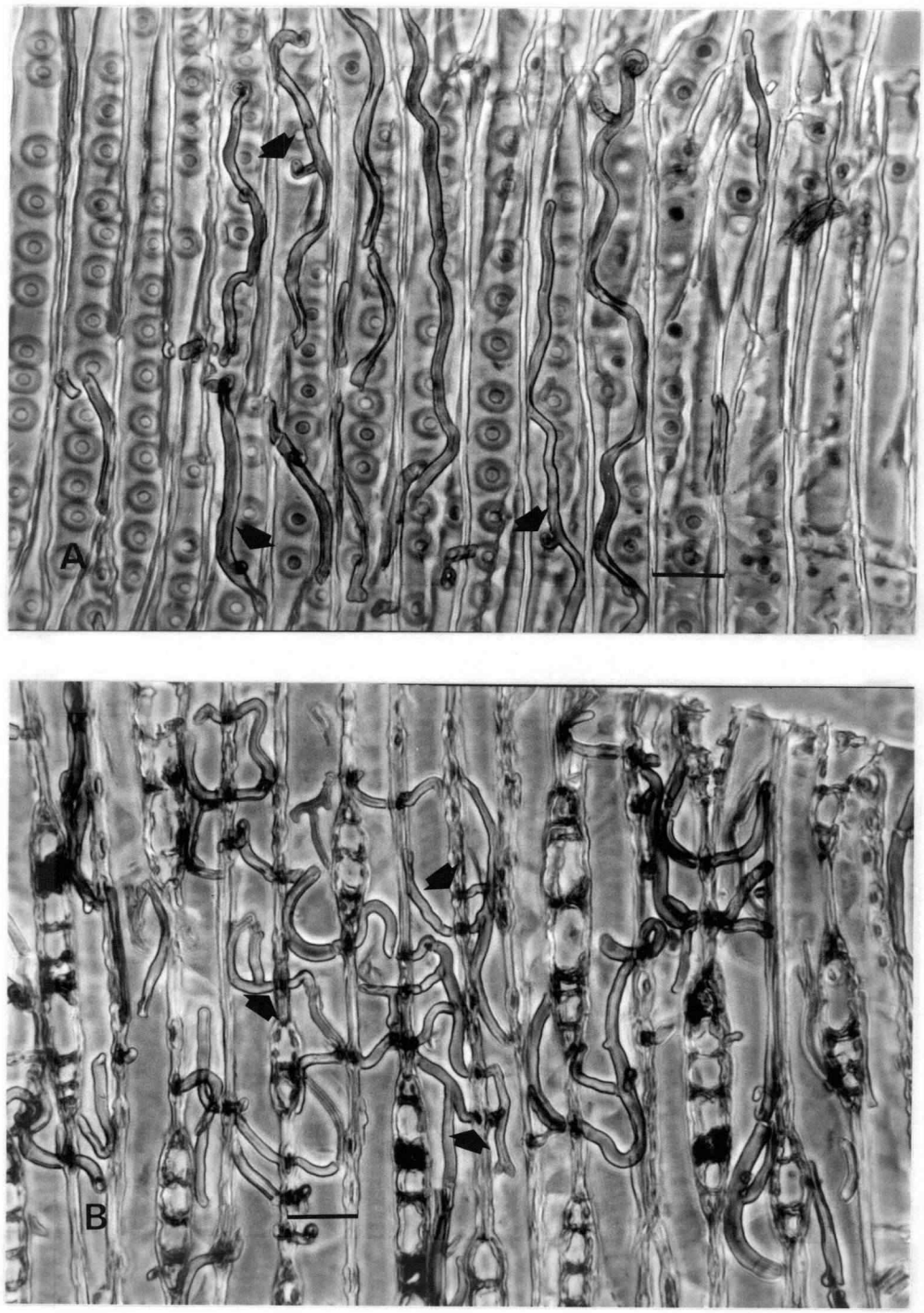

Figure II.4. Serpentine and helical growth of V. wageneri hyphae in tracheid lumens. A, Radial section of infected xylem; hyphae entering bordered pits on radial walls. (arrows). Bordered pit tori darkening in infected tracheids (X250) (bar = 40 μm). B, Tangential section showing growth pattern and confinement of hyphae to tracheids. Hyphae passing through bordered pit-pairs (arrows). (X250) (bar = 40 μm).

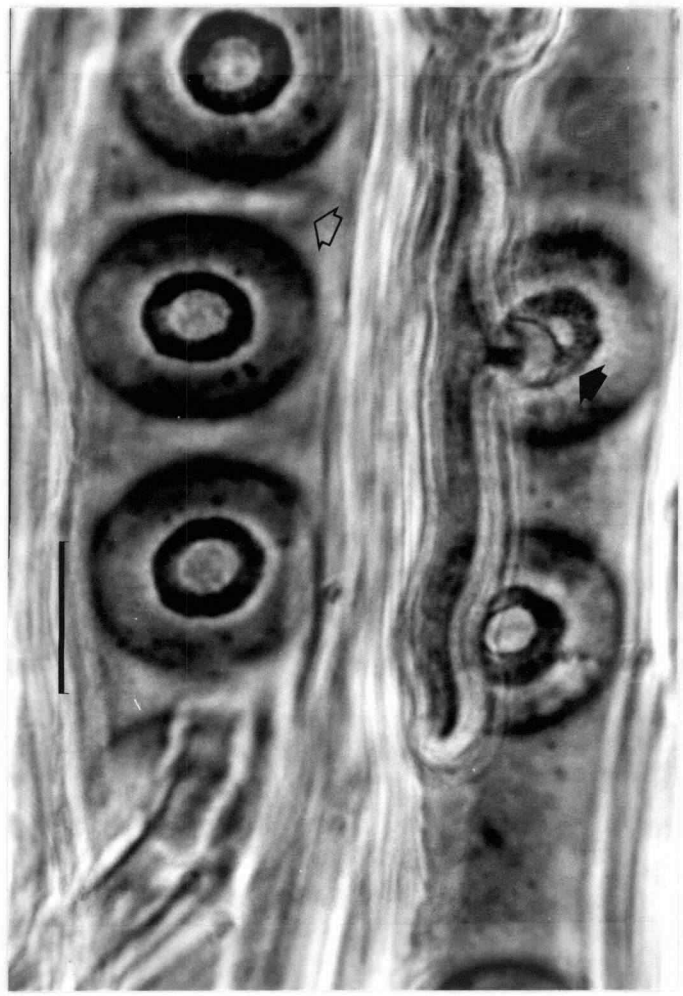

Figure II.5. V. wageneri hypha anchored to tracheid lumenal wall. Branch hypha beginning to enter bordered pit (solid arrow). Note crassula between bordered pits of the adjacent tracheid (X1600) (bar = 12.5 µm).

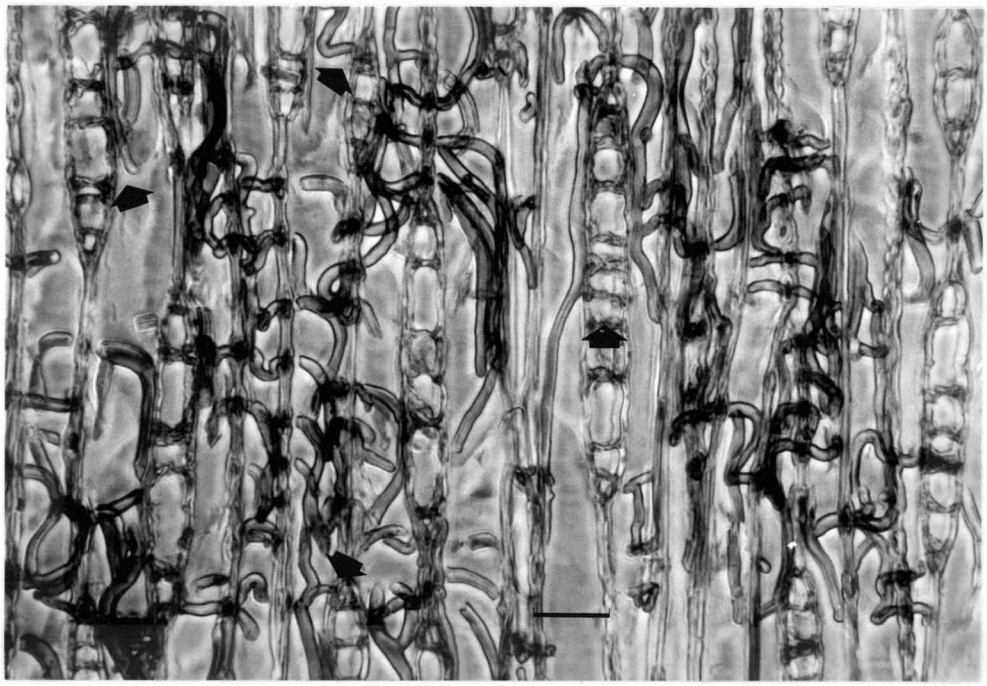

Figure II.6. Tangential view of hyphal branching adjacent to bordered pit-pairs in the radial walls of earlywood tracheids. Absence of ray parenchyma cell invasion (arrows) and systematic plugging of bordered pits by hyphae (X200) (bar = 50 μm).

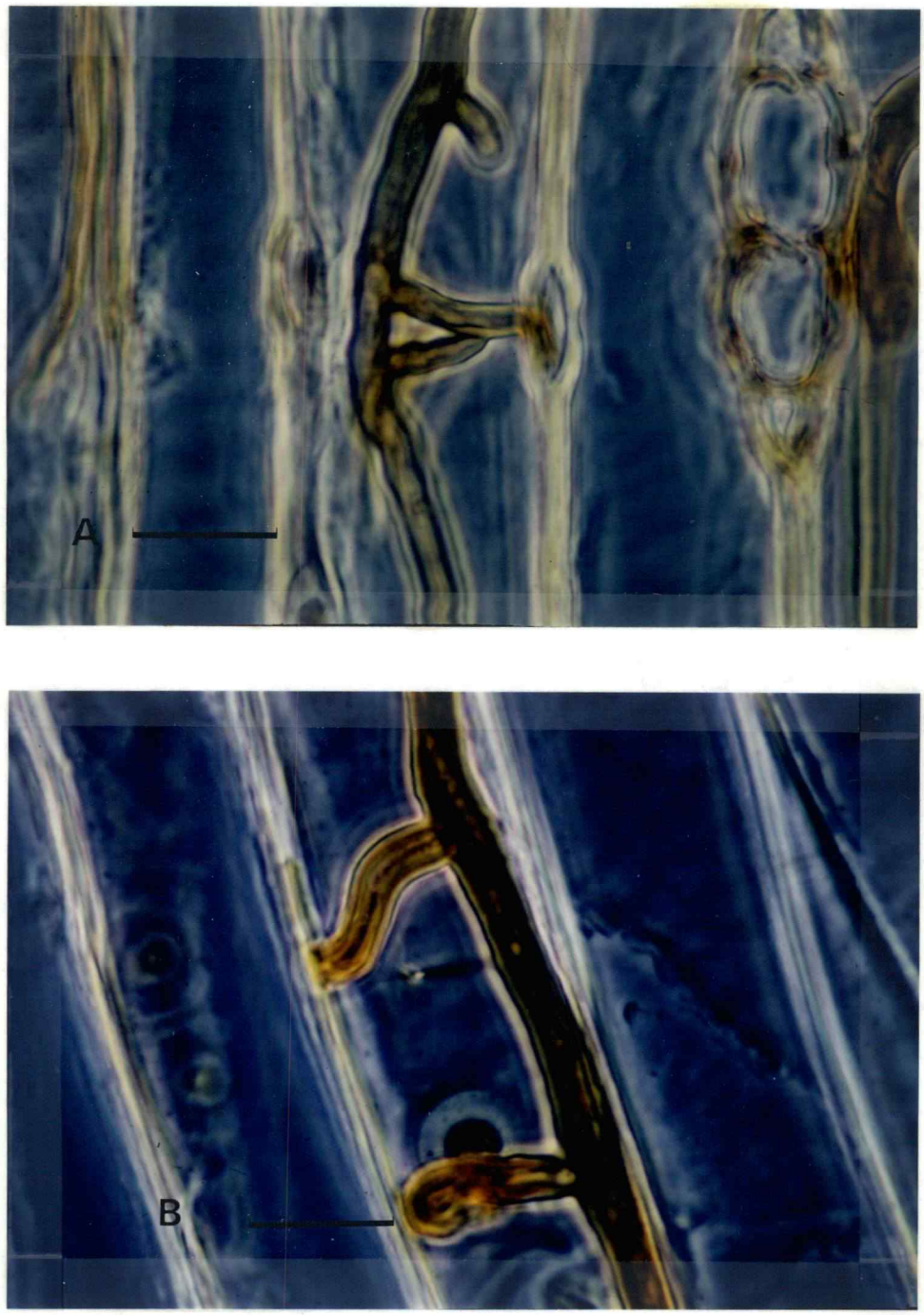

Figure II.7. Hyphal penetration of a bordered pit-pair. A, new hyphal branch entering bordered pit aperture. B, new hyphal branch orienting towards a bordered pit on the radial wall of the lumen (X1000) (bar = 20 μm).

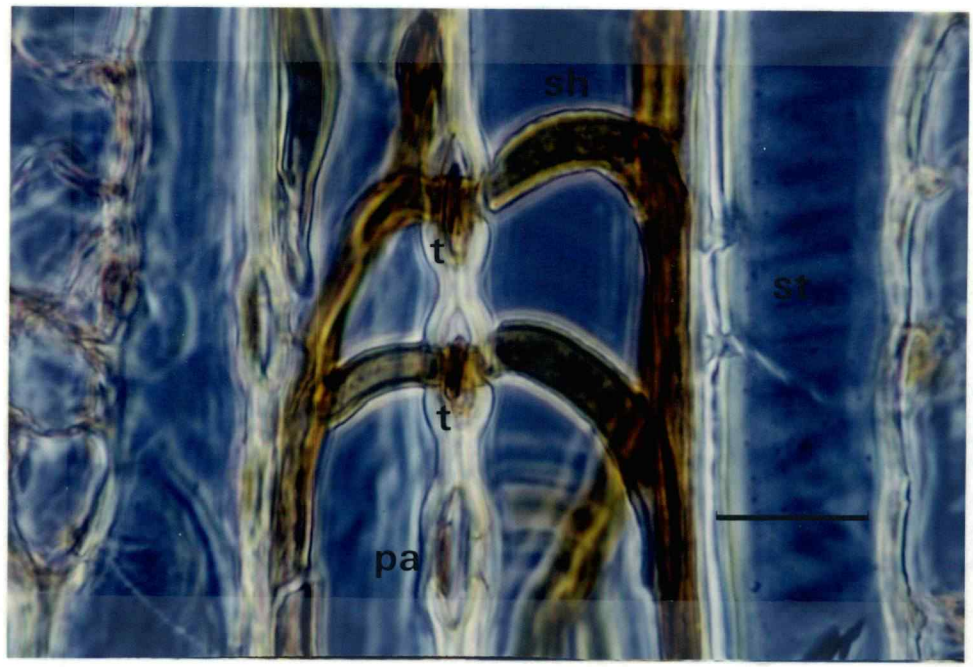

Figure II.8. Complete blockage of bordered pit apertures by hyphae. Pit apertures (pa) of adjoining tracheids are occluded and tori (t) are pushed to the foreground. Note granular sheath (sh) encrusting the hyphal wall and increasing the overall dimensions of hyphae. Spiral thickenings of the secondary cell wall (st) of the adjacent uncolonized tracheid (X1000) (bar = 20 μm).

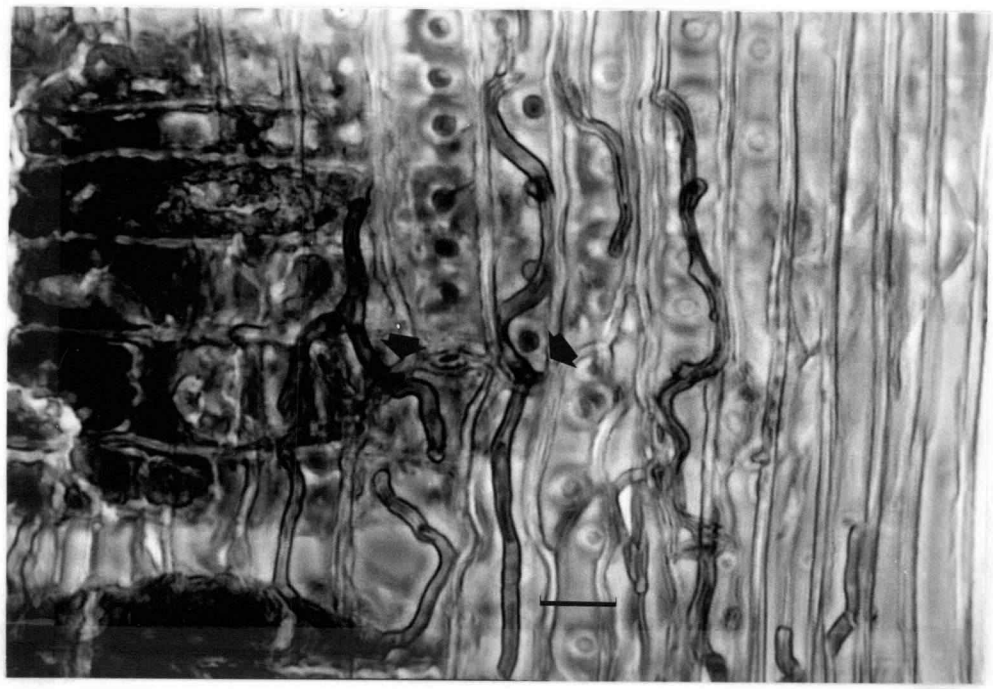

Figure II.9. Sequential colonization of axial tracheids by hyphae through bordered pits on tracheid ends (arrows). (X250) (bar = 40 μm).

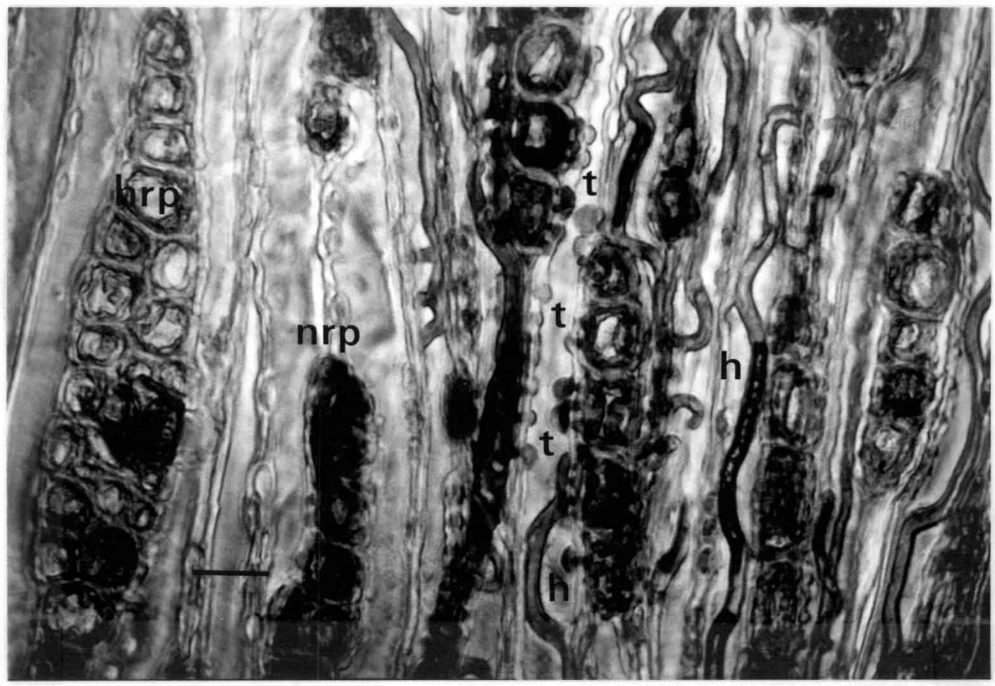

Figure II.10. Gradual necrosis of xylem ray parenchyma cells adjacent to colonized axial tracheids. Hypha (h) growing around tyloses (t) produced by adjacent ray parenchyma. Note the contrast between healthy (hrp) and necrotic ray parenchyma (nrp). (X250) (bar = 40μm).

Figure II.11. Prefential colonization of earlywood before latewood in successive annual rings. (Photograph courtesy of J.J. Witcosky).

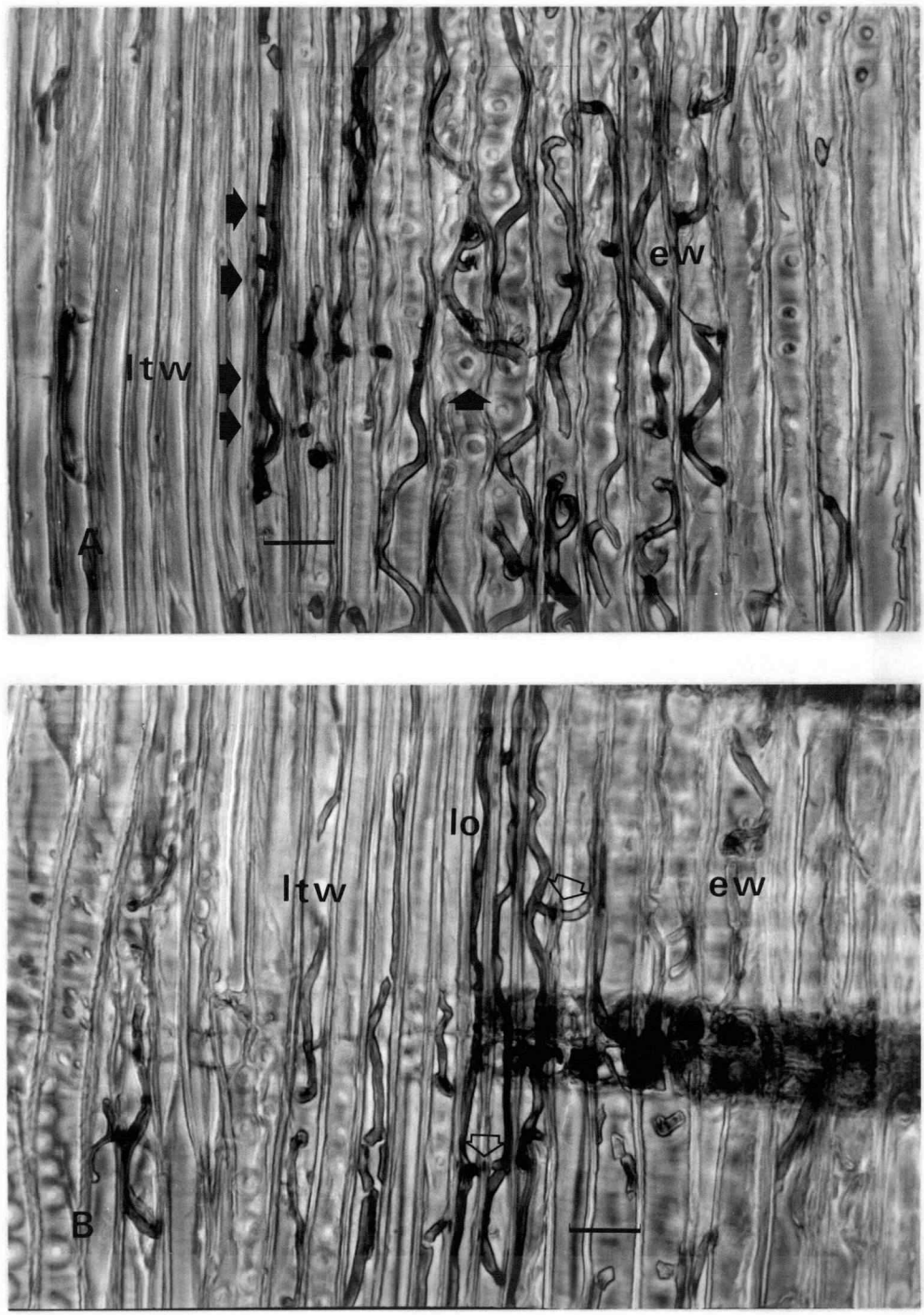

Figure II.12. Hyphal occlusion of latewood tracheid lumens and bordered pits. A. Bordered pit-pairs on tangential walls in latewood (ltw) (solid arrows) (radial section). Larger lumens in earlywood (ew). B, bordered pit penetration by hyphae (open arrows) and lumenal occlusion (lo) in latewood (X200) (bar = 50 µm).

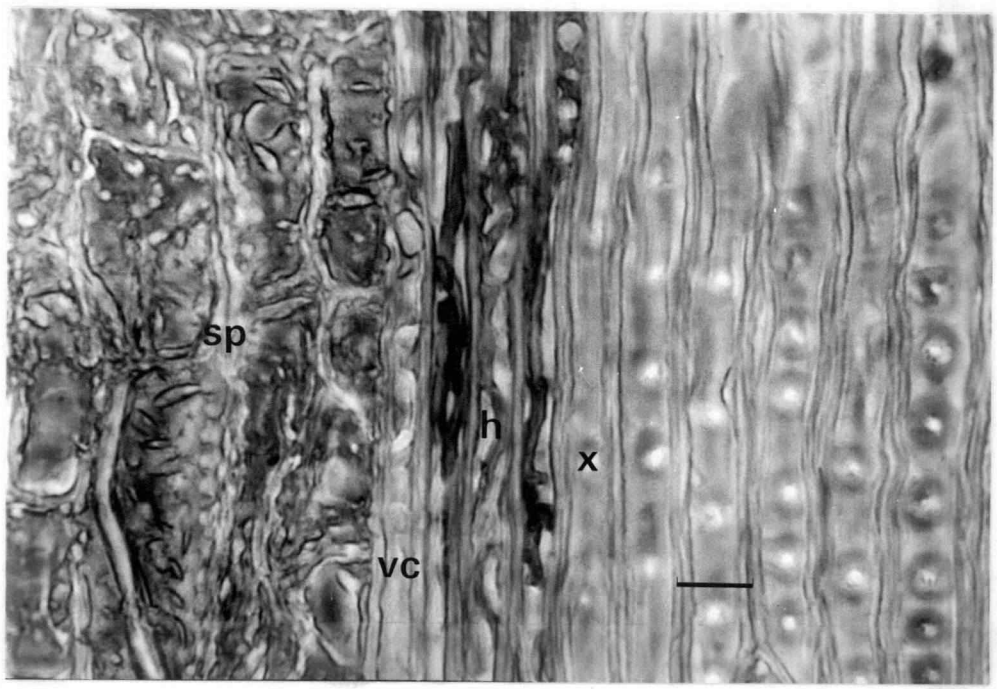

Figure II.13. Hyphal colonization of latewood tracheids immediately adjacent to the vascular cambium. Hyphae (h) in xylem (x) tracheids, absent in vascular cambium (vc) and secondary phloem (sp) (X250) (bar = 40 µm).

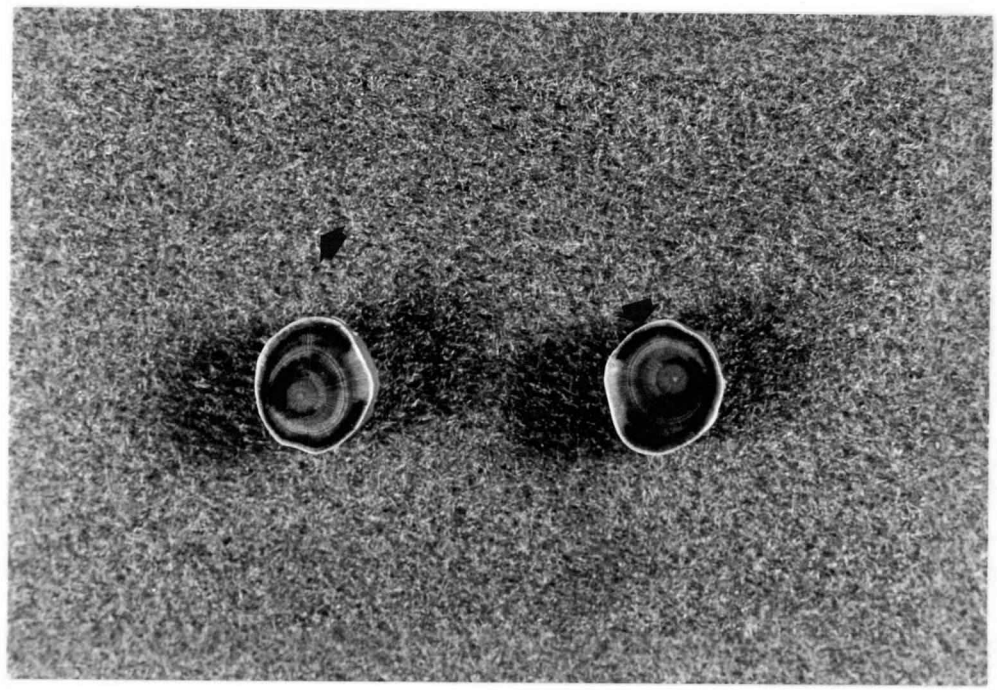

Figure II.14. Production of new earlywood tracheids over regions of heavily ramified latewood xylem. Narrow rind of new uncolonized earlywood (solid arrows); darkly stained wood is hyphal colonized, in lighter stained wood hyphal colonization is sparse and tracheids are occluded by gums. (actual size)

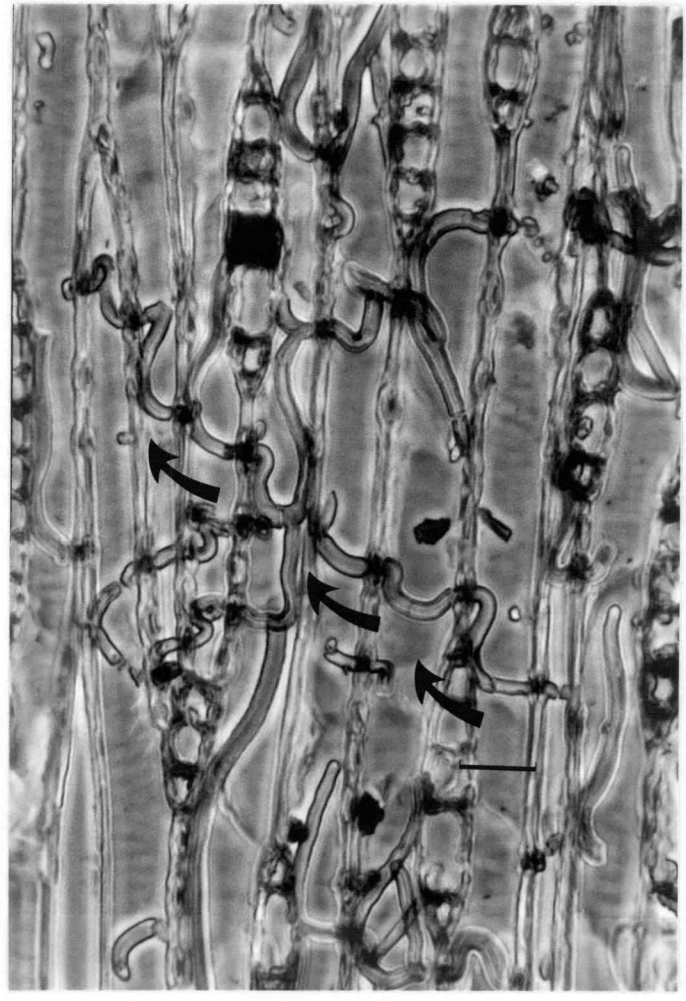

Figure II.15. Circumferential colonization via bordered pit-pairs on radial walls of axial tracheids. (X250) (bar = 40 μm).

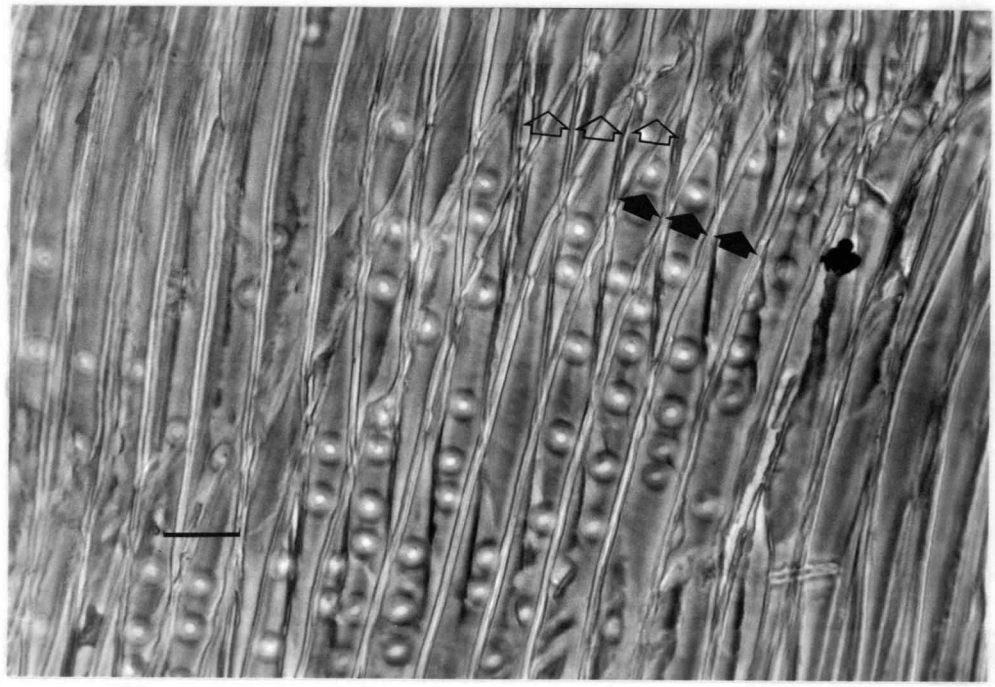

Figure II.16. Longitudinal section showing the difference in vertical declination of adjacent files of axial tracheids (X200) (bar = 50 µm).

Figure II.17. Radial growth of hyphae through xylem ray tracheids. Hyphae enter ray tracheids (rt) from longitudinal tracheids (lt) through bordered pit-pairs (open arrows). Ray tracheids are generally terminally positioned in rays (r) and ray parenchyma (rp) cells are never attacked. (X200) (bar = 50 μm).

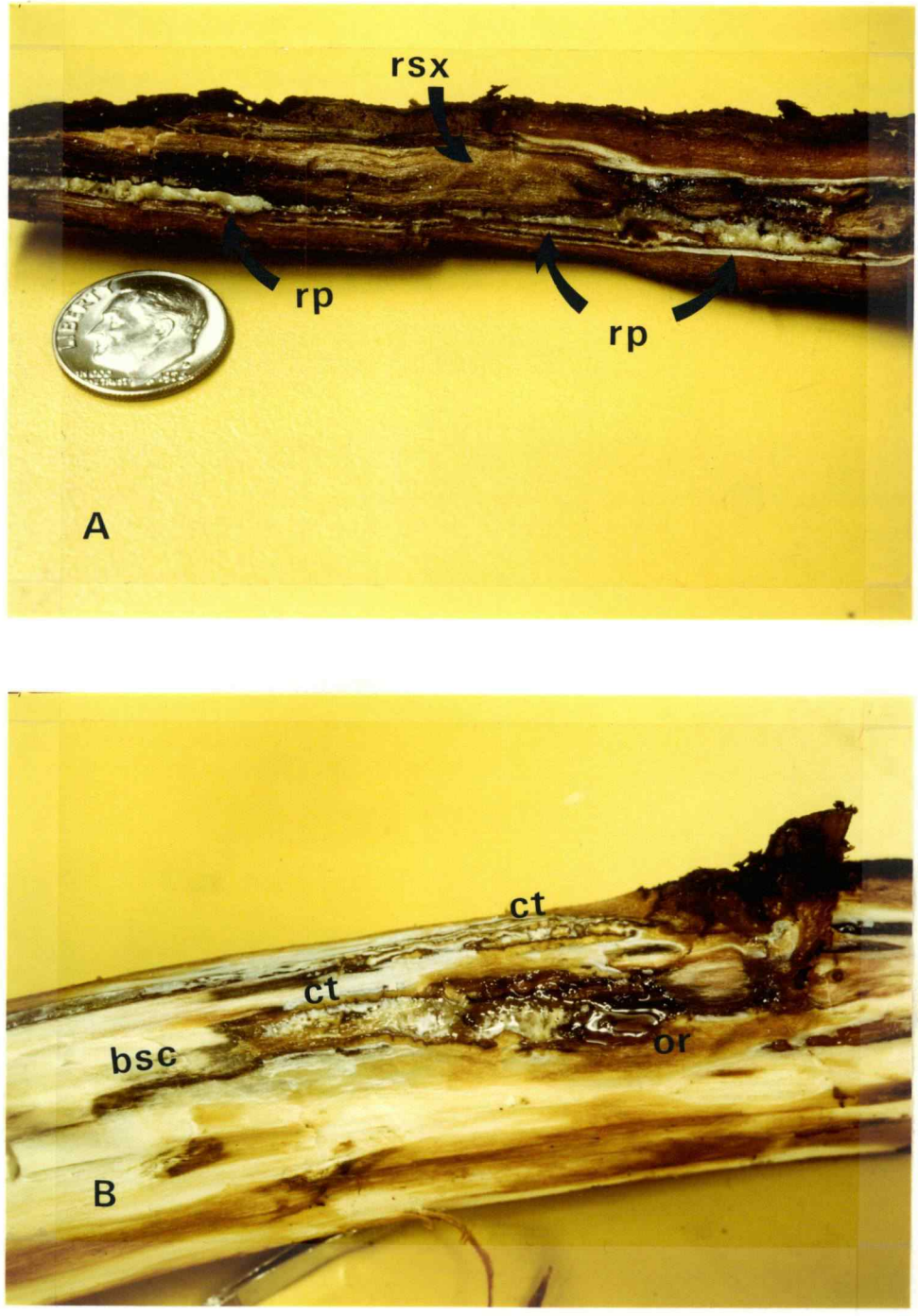

Figure II.18. External resinosus and resin-soaking associated with heavily colonized latewood in roots. A, pockets of crystalized oleoresin (rp) adjacent to deeply stained latewood, and resin soaking of latewood xylem (rsx). B, oleoresin (or) collecting in a lesion, active callus tissue (ct) growth over lesion. Black stain columns (bsc) associated with healing root lesion.

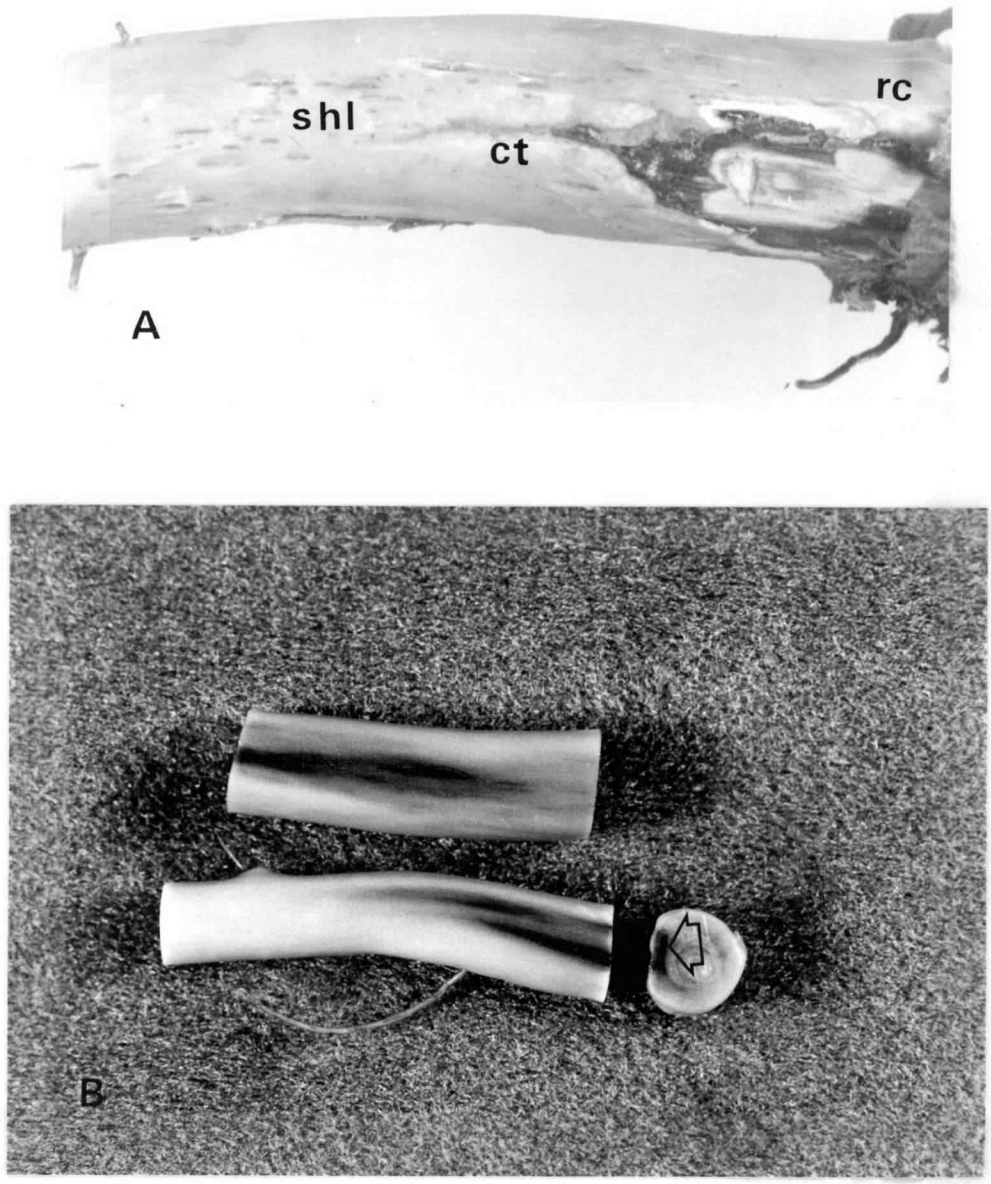

Figure II.19. Localized necrotic lesions in the cambium adjacent to V. wageneri colonized latewood. A, callus tissue (ct) associated with a large basal lesion in the stem eminating from the root collar (rc). Stain column is in latewood, new wood is earlywood. Small lesions (shl) are quickly healed (actual size). B, lesion developing in seedling stem, new earlywood production already beginning. (Note flattening of stem caused by non-production of wood by cambium) (actual size).

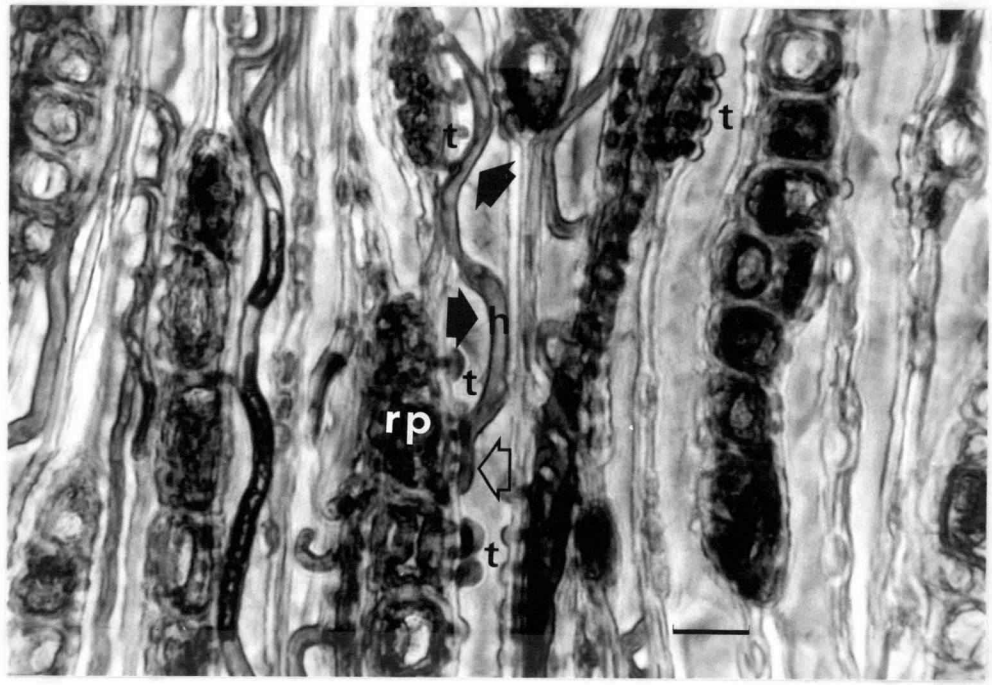

Figure II.20. Tyloses forming in colonized axial tracheids. Hypha (h) growing around tyloses (t) in earlywood tracheid lumen (solid arrows). Hyphal tip unable to penetrate half-bordered pit-pairs of adjacent ray parenchyma cell (rp) (open arrow) X250. (bar = 40μ m).

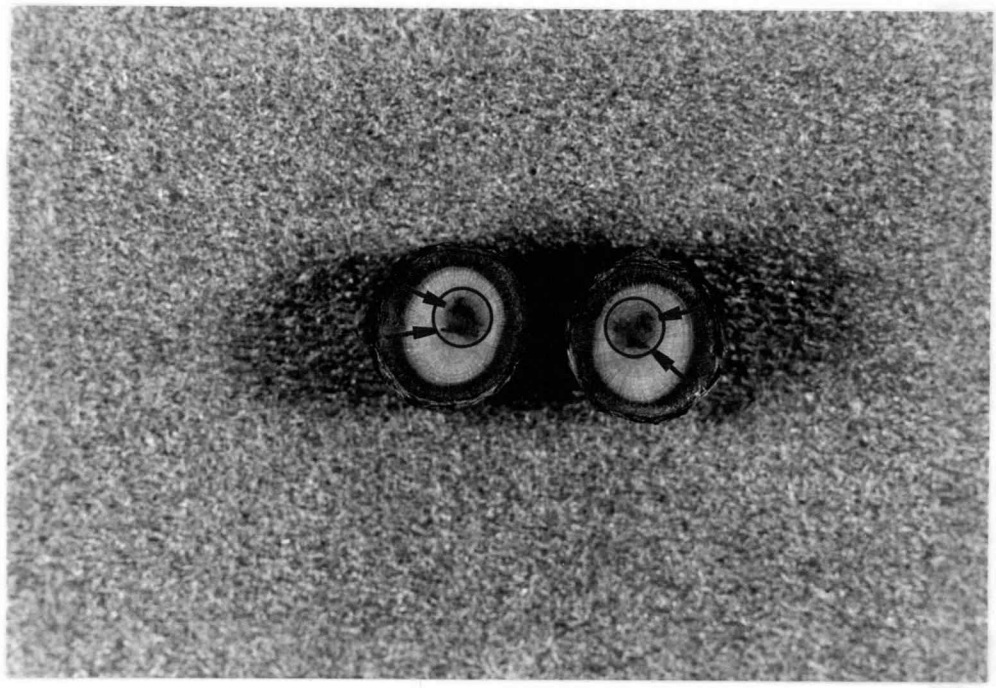

Figure II.21. Gum plugged tracheids surrounding immersed black stain columns. Arrows point to hyphal stain, circles delimit extent of gum plugging. (actual size).

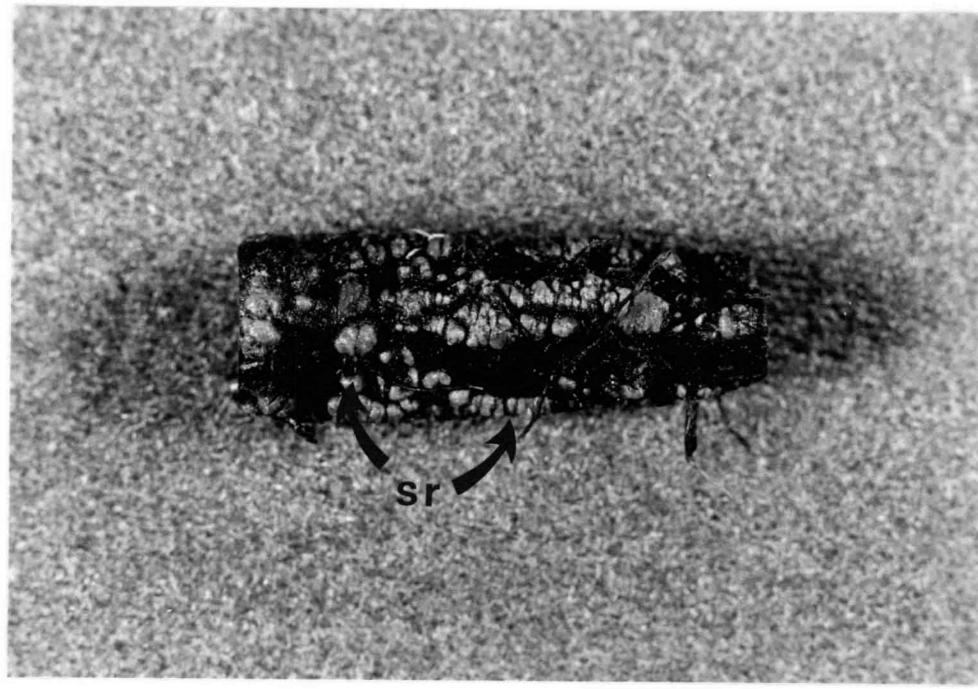

Figure II.22. Engorgement of the secondary phloem of a root over a region of heavily ramified xylem. Swollen rhytidomes (sr) have ruptured through cortical tissues (actual size).

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VASCULAR OCCLUSION IN THE WILT PATHOGENESIS OF *VERTICICLADIELLA WAGENERI* IN DOUGLAS-FIR

Chapter III

ABSTRACT

Xylem pressure potential and water uptake were periodically measured for *V. wageneri* inoculated and uninoculated control seedlings in two paired experiments. Inoculated seedlings were destructively sampled when pressure potential and water uptake values differed from control values at a statistically significant level. Root systems of inoculated seedlings were systematically dissected and the proportion of the root system colonized by *V. wageneri* to the total volume was estimated by measuring the portions of lateral and tap-root cross-sections visibly colonized by the fungus. T-tests confirmed that differences between inoculated and control seedlings in both experiments were significant after one month. At the time of final sampling, circumferential colonization was extensive (80-92%) in inoculated seedlings in both experiments at the root collar and for the entire root system, while radial colonization, measured on area cross-sections, was proportionally less (35-74%). Results of these experiments and empirical evidence from studies of the pathological anatomy of this disease suggest that wilting is primarily the result of vascular occlusion. Wilt mechanisms of phyllogenetically related wilt pathogens are discussed and compared.

INTRODUCTION

Field observations and preliminary experiments indicate that extensive colonization of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) by Verticicladiella wageneri Kendrick (1962) (teleomorph: Ceratocystis wageneri Goheen et Cobb) (1978) can occur before foliage wilts or becomes chlorotic. This stands in direct contrast to wilt diseases caused by Ceratocystis ulmi and C. fagacearum, which exhibit a restricted pattern of vascular colonization and reportedly produce translocateable phytotoxins. Experiments were designed to determine the relationship of wilting of Douglas-fir to vascular tissue colonization and occlusion by V. wageneri.

Black-stain root disease, caused by Verticicladiella wageneri was first reported killing young-growth Douglas-fir in 1967 (Cobb and Platt, 1967). Smith (1967), "awaiting further evidence on the mode of pathogenesis", tentatively classified the disease as a vascular wilt on the basis of several characteristic features of xylem colonization. Goheen et al (1978) similarly adopt the classification, calling it a "wilt-type" root disease of numerous western coniferous species. The mechanism of wilt pathogenesis of V. wageneri has not been demonstrated to date.

Symptom development is typically slow in infected trees. Tree size, number and distribution of infections, and season of first symptom development determine the rate of foliage symptom expression. Trees less than 10-years-old die quickly once they are symptomatic, with crowns fading from yellow to rusty-red in a single

growing season. Older trees, once chlorotic, may take 2 to 5 years to die completely. Witcosky (1981) determined the relationship between crown symptom expression and levels of root and stem wood colonization for trees 14, 15 and 24 years old. Trees were categorized according to foliage color, needle retention, and reduction in terminal growth. V. wageneri colonized sapwood over 64% of the stem circumference at ground level before chlorosis was evident, although terminal growth and needle retention were measurably reduced. At least 70% of the xylem circumference was colonized by the time trees were visibly chlorotic. Terminal growth at this stage was less than 10% of that preceding infection (Witcosky, 1981). Helms et al (1977) report similarly that 60-90% of the stem circumference is colonized for ponderosa pine (Pinus ponderosa) seedlings that are newly chlorotic. Witcosky (1981) showed that for all symptomatic trees, hyphae of V. wageneri were always associated with the three outermost annual rings, colonizing earlywood rather than latewood tracheids.

Sapwood colonized by V. wageneri stands out in contrast to healthy sapwood. Areas of colonized xylem are deeply stained, amber to chocolate brown and are easily delimited along any axis in the wood. Sapwood discoloration is the result of a combination of features: amber hyphal wall and sheath pigmentation, acquired pigmentation of tracheid walls and lumenal deposits, darkening of cytoplasm in xylem parenchyma cells, and host resin and phenolic discoloration in colonized areas (Hessburg and Hansen, 1982; Chapter II).

Wagener and Mielke (1961) made the first histological observations concerning the disease in ponderosa, Jeffrey (Pinus jeffreyi) and pinyon pines (P. edulis and P. monophylla, stating that

colonization was "almost entirely" confined to tracheids. Smith (1967) confirmed that observation adding that xylem longitudinal and ray parenchyma and cambial tissues were never invaded in pines, and cell walls were not penetrated or disrupted. Hessburg (1982, Chapter II) demonstrated similar pathological anatomy in Douglas-fir. Axial colonization by hyphae was confined to mature sapwood xylem longitudinal tracheids, predominantly in recent earlywood, and radial development of mycelia in ray tracheids was limited. Longitudinal and ray parenchyma, and epithelial tissues were never directly attacked by hyphae although they eventually died when adjacent to colonized conductive tissue. Several host responses were reportedly associated with these tissues adjacent to colonized conductive tissue. Evidence of toxic necroses of parenchyma and cambial tissues was lacking and alternative mechanisms were proposed for the necroses. Vascular occlusion of xylem tracheids by hyphae and gums was extensive in the outer annual rings. The authors suggested that continuous reduction of the amount of functional conductive tissue by such occlusion would eventually lead to critical water shortages in trees.

Helms et al (1977) examined the effect of black-stain root disease on rates of net photosynthesis, dark respiration, transpiration, foliar water stress and stomatal aperture of ponderosa pine seedlings transplanted to a forest environment. Dramatic changes in net photosynthesis, transpiration and foliar stress (xylem pressure potential) were observed one month after inoculation.

Experiments were designed to determine the effect of colonization by V. wageneri on water uptake and xylem pressure potential in Douglas-fir seedlings. The location, pattern and extent of vascular

tissue colonization was recorded and quantified when significant changes in seedling water status first occurred.

MATERIALS AND METHODS

Seedling Inoculations. Two separate paired experiments were conducted in which either xylem pressure potential or transpiration water uptake was periodically measured for inoculated and control groups of seedlings. The isolate of *V. wageneri* (VW-45) used in this study was obtained from a diseased Douglas-fir in the Oregon Cascade mountain range. Two-year-old Douglas-fir seedlings were used in the experiments. To inoculate seedlings, they were first wounded by removing a wedge of bark and wood with a sterile scalpel 3 to 10 cm below the root collar. Wound length was approximately equal to the diameter of the root at the wound site and their width never extended over more than 25% of the root circumference. Sporulating *V. wageneri* colonies growing on potato dextrose agar were cut into 1 cm^2 blocks. A block was placed on each wound. The inoculum was wrapped in 3 x 10 cm pieces of moistened cheesecloth and then enclosed in 5 x 10 cm pieces of 0.50 mil poly-ethylene plastic. Ends of the inoculum bandage were loosely tied with "twistems"; the seam and bandage ends were sealed with melted paraffin. This procedure was used to insure that infections and developing stain columns originated from a single source. Control seedlings were similarly treated except that no inoculum was applied to the wound.

Seedlings were individually transplanted to plastic seedling

transplant tubes (7 cm diameter x 25 cm length) which were watered from a reservoir of 20% Hoaglund's solution by cotton wicks. Seedlings were transplanted into the tubes with the wick running alongside the taproot to within 1 cm of the soil surface. The potting mix was 65% washed silica sand (EI-20), 35% a mix of equal volumes of 1/4" mesh washed river sand, clay loam, and peat.

Xylem Pressure Potential. Nine inoculated and nine control seedlings were randomized in a tube rack. The rack was placed in a plastic wash tub with the 20% Hoaglund's solution so that the bottom of the tubes touched the nutrient solution and the wicks were immersed. Seedlings were incubated at 17C in a controlled temperature growth chamber. Seedling xylem pressure potential was measured using a Scholander pressure chamber (Scholander et al, 1965), which measures pressure potentials in bars (1 bar = 100 k Pa) (Ritchie and Hinckley, 1975; Waring and Cleary, 1967). Pressure potentials were measured on all seedlings 22, 28, 34, 39 and 43 days after inoculation. Lateral branch tips, each having at least 15 mature needles, were used in the measurements (Cleary, 1968).

Inoculated seedlings were unpotted and the extent of V. wageneri colonization was measured when xylem pressure potentials at two consecutive readings fell outside of the range of potentials observed for control seedlings.

Transpiration Water Uptake. Twelve control and 12 inoculated seedlings were transplanted to tubes with wicks. Each tube was nested in a second empty tube then placed upright in a narrow-mouth quart jar with the wick extending into 400 ml of the nutrient solution in the bottom of the jar. A reference line for refilling was drawn on the

jar at the bottom of the meniscus. The top of each tube was covered with a double layer of 4 mil poly-ethylene fitted snuggly around the stem and secured just below the top lip of the tube with strapping tape. The juncture of the tube and jar was wrapped in a double thickness of Parafilm®. Inoculated and control trees were randomized and incubated at 17C in a controlled temperature growth chamber. Seedlings were monitored and jars refilled 10, 22, 30, 36, 41, 45 and 49 days after inoculation. Uptake volumes were recorded in $ml \cdot day^{-1}$. Refilling accuracy was ± 2 ml.

Inoculated seedlings were destructively sampled when water uptake values fell outside of the range observed for the control seedlings for two consecutive readings. If the trend was to increased uptake, destructive sampling was deferred until the trend reversed.

Measuring the Extent of Root System Colonization. The extent of colonization was determined similarly in both experiments. For each seedling, roots were washed and cleanly cut from the stem at the root collar. All lateral roots were removed at the taproot and the cut ends were decorticated for easier measuring. The remaining taproot was cut into thirds, and the large ends were also decorticated. Radius and circumference of the wood of each lateral and taproot cross-section were measured with a template made as follows: Circles of measured radius, ranging from 2-12 mm, in 1/2 mm increments were drawn with a drafting compass on tracing velum and photocopied onto transparent film. From this, rapid and precise measurements were made. Roots 1.5 mm in radius and smaller were measured under a compound microscope at 125X magnification.

The proportion of the root system colonized (stained) was approximated by measuring the visibly colonized portions of each lateral and

taproot cross-section. The Relative Stain Proportion (RSP) was computed for each section (Figure III.1).

$$RSP = PC\ (R^2_{SO} - R^2_{SI})\ x\ \ 100, \text{ where:}$$

PC = the proportion of the circumference that is stained, which is equal to the length of the outside arc of V. wageneri stain divided by the outside circumference of the cross-section; R_{SO} = the radius to the outside edge of the arc of stain; R_{SI} = the radius to the inward edge of the stain arc. The radius measurements, R_{SO} and R_{SI} were not measurements with units, but proportions of the full radius of a given cross-section measured to the nearest tenth radius off a template.

A third template was constructed to measure (PC), the length of the outside arc of stain, as a proportion of the circumference of each cross-section. Arcs, increasing in increments of $(\pi/8)r$, were drawn for circles of radius 0.5-6.0 mm and photocopied onto transparent film.

RSP's computed for each lateral and taproot piece cross-section expressed the percentage of that root volume that was colonized. RSP and PC were also computed for each seedling, both for the cross-section at the root collar and for the sum of all lateral root and taproot cross-sections (RSP_T, PC_T).

$$RSP_T = \frac{\sum_{i=1}^{n} [RSP_i\ (\pi r^2_i)]}{\sum_{i=1}^{n} (\pi r^2_i)}\ x\ 100 \text{ where;}$$

RSP_T = the proportion of the sum of all cross-sections that is stained by fungus, expressed as a percent; πr^2_i = cross-sectional area of the ith root, with radius measured in mm;

RSP_i = Relative Stain Proportion of the ith root;

n = the total number of lateral and taproot cross-sections.

$$\text{Similarly, } PC_T = \frac{\sum_{i=1}^{n} [PC_i(\pi d_i)]}{\sum_{i=1}^{n} (\pi d_i)} \times 100, \text{ where;}$$

PC_T = the proportion of the sum of all circumferences that is stained, expressed as a percent;

PC_i = the proportional circumference of the ith root;

πd_i = the circumference of the ith root; and

n = the total number of lateral and taproot cross-sections.

RESULTS

Xylem Pressure Potential. At the time of the first reading, all seedlings had broken dormancy and were in varying stages of bud burst and new shoot elongation. The mean pressure potential, after 22 days, was -4.9 bars for healthy control seedlings. Xylem pressure varied from -4.7 to -5.4 bars over the 43 day sampling period, with a mean of -5.0 bars. The 99% Confidence Interval about the mean of control seedling means was -5.0 ± -0.7 bars (Figure III.2), and variation among the sample period means was not significant (range of SE's -0.15

to -0.30 bars).

The mean xylem pressure potential for inoculated seedlings at 22 days was -5.3 bars, fully within the range observed for the controls. By the 28th day, the mean decreased to -6.2 bars, and by the 34th and 39th days, means were -11.9 and -16.5 bars, respectively (Figure III.2). Standard Errors (SE) increased with larger size of sample mean values, however, the magnitude of the variation (CV) about each sample mean did not increase. Even though the seedlings represented a range of varying heights, diameters, and root-to-shoot ratios, their response to infection and colonization by V. wageneri as measured by the pressure chamber was consistent.

Inoculated seedlings #2 and #9 died early in the study of what appeared to be transplant failure. Following transplanting, these seedlings failed to break dormancy and there was no evidence of any new root growth. The existing foliage was shrivelled and dried after 34 days, when all other inoculated seedlings were asymptomatic. Although both seedlings were infected, the extent of fungal colonization was less than half of that observed for the other inoculated seedlings. Both seedlings were eliminated from further computations.

T-tests were computed for both inoculated and control seedlings to compare pressure potentials at each sampling time. At 34 and 39 days, but not at 22 and 28 days, pressure potentials of inoculated seedlings were significantly ($p = 0.01$) lower than those of the controls. Inoculated seedlings were destructively sampled and dissected at either 34 or 39 days when their pressure chamber readings differed significantly from the controls.

Circumferential colonization of the xylem was extensive in each inoculated seedling by the time pressure potentials differed significantly from control seedlings, both at the root collar (PC = 87%) and for the total root system (PC_T = 80%). Colonization measured on area cross-sections was much less, at the root collar (RSP = 35%), and for the total root system (RSP_T = 39%) (Table III.1, Figure III.4).

Transpiration Water Uptake. All seedlings had broken dormancy in the first three weeks of the study. Water uptake in control seedlings was greatest during the period after initial potting. Ten days after establishment, mean water uptake for the controls was 21.9 ml $\cdot$ day^{-1}. Uptake decreased to a rate of 10.6 ml $\cdot$ day^{-1} at 22 days and to 7.9 ml $\cdot$ day^{-1} at 30 days (Figure III.3). Four to five weeks following establishment, all control seedlings were growing vigorously with rapid shoot development; the pattern of decreasing water uptake reversed. Water uptake increased to 10 ml $\cdot$ day^{-1} after 36 days and was at 21.5 ml $\cdot$ day^{-1} by day 45 (Figure III.3).

Seedling number 2 of the inoculated group died early in the study of what appeared to be transplant shock. Dissection of its root system revealed successful infection but limited colonization. Since early dying could not be attributed to colonization, it was dropped from further computations.

T-tests were computed for inoculated and control seedlings for each periodic sampling. Water uptake for inoculated seedlings was significantly ($p = 0.01$) less than that of the control seedlings at days 30 and 36, and at all subsequent sampling periods, but not at 10 and 22 days. The trend of increased water uptake observed for control seedlings between 30 and 36 days was less pronounced for the inocu-

lated seedlings. A trend of increased uptake was observed for several of the larger inoculated seedlings between 36 and 41 days, but they were subsequently overcome by the fungus and water uptake declined. The first seedling in this experiment was sampled after 30 days and the rest were sampled on or before 45 days following inoculation. The smallest seedlings never entered into a pattern of increasing uptake. Figure III.3 plots the periodic sample means for control and inoculated seedlings and shows the 99% Confidence Interval.

Seedling root systems were dissected and evaluated, following procedures used in the previous experiment. Again, circumferential colonization at the root collar and for the total root system was high at the time of final sampling, PC = 92%, PC_T = 85%, respectively (Table III.1). The proportion of the cross-sectional areas colonized for the root collar (RSP) and total root system (RSP_T) were 74% and 61%, respectively (Table III.3, Figure III.4).

T-tests were computed to compare PC's and RSP's of inoculated seedlings from the xylem pressure potential (XPP) and transpiration water uptake (TWU) groups (Table III.1). RSP and RSP_T were significantly ($p = 0.01$) higher for the TWU group. PC and PC_T were not significantly different. Inoculated seedlings from the TWU group were sampled over a period of 15 days ending 45 days after inoculation. Seedlings from the XPP group were sampled over a period of 5 days ending at 39 days. Mean sampling times for inoculated seedlings of XPP and TWU groups were 36.8 and 38.8 days from inoculation, respectively.

DISCUSSION

Since pathogen behavior is histologically identical irrespective of host age or size (Chapter II), seedlings were used in these experiments, providing a convenient and representative model system for elucidating mechanisms of V. wageneri pathogenesis in Douglas-fir. With seedlings, whole root systems were easily dissected and the extent of colonization could be assessed fairly rapidly. Seedlings could also be cultivated under controlled conditions in sufficient quantity to allow treatment replication and statistical analysis. It is understood that the rate and extent of colonization needed to cause wilting of foliage may vary somewhat with increased host age and size and other environmental conditions.

The parameters, xylem pressure potential and transpiration water uptake, were measured to indicate the earliest significant consequence of vascular colonization by mycelia of V. wageneri. Both measurement techniques indicated that critical shortages occurred in inoculated seedlings 30 to 40 days after inoculation. On the average, pressure potential readings gave an earlier warning of increased vascular distress and were therefore a more sensitive indicator. The high PC and PC_T values observed in both experiments at the time of final sampling indicated that a high level of circumferential colonization in the outer annual rings occurred early in wilt pathogenesis, and was responsible for early expression of vascular dysfunction. This expression was probably keyed to a threshold level of colonization in excess of ca 75%.

Transpiration water uptake (TWU) seedlings were sampled an

average of 2 days and up to 6 days later than the xylem pressure potential (XPP) seedlings. RSP and RSP_T were significantly greater for the TWU seedlings. This suggests that centripetal colonization proceeds more slowly and is of secondary importance.

All sapwood annual rings conduct water and solutes (Harris, 1961; Kozlowski et al, 1967; Swanson, 1975; Whitehead and Jarvis, 1981), but water conductivity decreases with increasing age of the sapwood (Markstrom and Hann, 1972; Puritch, 1971) due to higher levels of pit aspiration and cavitation in older tracheids (Gregory and Petty, 1973; Hart and Thomas, 1967; Milburn, 1966; Whitehead and Jarvis, 1981). Since recent annual rings conduct most of the xylem sap, their elimination from the water column via embolism, aspiration and fungal occlusion should significantly alter the water economy of trees.

Colonization of outer sapwood xylem is perhaps nutritionally important to the pathogen. Cowling and Merrill (1966) demonstrated that the outer ring possesses the greatest quantity of soluble nitrogen, reserve carbohydrates, and recently derived photosynthate. Xylem mother cells derived from cambial initials ultimately autolyse. Cytoplasmic constituents are disintegrated and solubilized in the transpiration flow of functional conductive tissue. Large sources of free soluble N and amino acids are available in the outer annual ring (Merrill and Cowling, 1966). The outer ring always has a zone of elution where cells are autolysing and becoming functional in water conduction.

Fungi lumped under the generic heading of "vascular wilt pathogen" are taxonomically and trophically a diverse group. Representatives, including Fusarium, Verticillium and Ceratocystis

spp. and respective anamorphs or teleomorphs, have been studied in great detail. Toxins have been demonstrated to be of primary importance in the wilting syndrome caused by: Fusarium vasinfectum, F. oxysporum, F. heterosporum, F. moniliforme, Verticillium dahliae, V. albo-atrum, Ceratocystis ulmi and C. fagacearum (Dimond, 1955; McWain and Gregory, 1972; Pegg, 1981; Sadasivan, 1961). The toxins are usually liberated in the host by the pathogens, translocated to living cells where they impair normal host physiology, culminating in wilting of foliage. A toxin theory of wilt pathogenesis is attractive because it offers an explanation for the development of wilt symptoms in advance of the pathogen. A number of different toxins have been isolated, purified, chemically characterized and re-introduced into healthy plants, reproducing all or part of the wilting syndrome (Dimond, 1955; Zentmeyer, 1942; Stevenson and Slater, 1970; Pegg, 1981; Salemink et al, 1965; VanAlfen and Turner, 1975; Takai, 1974, 1980; Turner, 1975). Toxins seem to be an essential element in the wilt pathogenesis of this group of pathogens. Most investigators now accept that wilt pathogenesis is caused by the combination of vascular plugging and the action of a translocateable toxin on xylem parenchyma cells and foliage.

Involvement of a toxin in primary wilt pathogenesis of Douglas-fir by V. wageneri is unlikely, although toxins may be involved secondarily. Three lines of evidence support this.

1) Axial and circumferential colonization are extensive when significant reductions in xylem pressure potential and water uptake are evident. The earliest indication of wilting appears to be related to extensive vascular tissue colonization and occlusion rather than an

alteration of leaf cell permeabilities by a translocateable toxin. The opposite is true in wilt diseases caused by Ceratocystis ulmi and C. fagacearum, where vascular tissue colonization is restricted and phytotoxins cause foliage wilting.

2) Parenchyma cells adjacent to tracheids do not die in advance of hyphal colonization of those tracheids, indicating that a diffuseable toxin is lacking (Chapter II). Neither xylem parenchyma cells nor cambial initials are attacked by hyphae of V. wageneri in adjacent tracheids. C. ulmi and C. fagacearum demonstrate a highly developed ability to colonize living cells.

3) Cambial necrosis does not occur as a result of intimate association between cambial initials and recently colonized, newly functional latewood axial tracheids (Chapter II).

The wilt pathology of V. wageneri in Douglas-fir differs from that of Ceratocystis wilt fungi in angiosperms in other ways as well.

For example, Ceratocystis ulmi, the cause of Dutch Elm disease, and C. fagacearum, the oak wilt pathogen, display a pathological anatomy much different from that of Verticicladiella (Ceratocystis) wageneri. Hyphae of the oak wilt fungus are rarely observed in the xylem vessel elements at the time of initial leaf wilt symptoms; however, after most leaves have browned or dropped, mycelia are found in vessel elements (Struckmeyer et al, 1958). Conidiophores of the Chalara anamorph, originating from hyphae in parenchyma cells, penetrate half-bordered pit-pairs leaving the conidiogenous end in the lumen of the adjacent vessel element (Wilson, 1961). Conidia are produced, and move vertically, in the ascent of xylem sap. Hyphae of C. fagacearum are commonly found growing near the plasma membrane of

pith and uniseriate ray parenchyma cells, and in other xylem parenchyma (Wilson, 1961). Hyphae produce cellulolytic and pectolytic enzymes (Geary and Kuntz, 1962) and can penetrate intercellularly, decomposing regions of the compound middle lamella and intracellularly, penetrating cell walls directly especially through the plasmodesmata of primary pit fields (Sachs et al, 1970). Cell wall penetration is both mechanical and enzymatic via an appressorium and penetration peg (Wilson, 1961). Hyphae also corrode the walls of fiber tracheids and other parenchyma cells (Sachs et al, 1970). Colonization of vessel elements by hyphae facilitates rapid and direct movement of hyphae to adjacent parenchyma cells, and allows systemic distribution of conidia in the xylem sap. Since parenchyma cells are reservoirs of nutritious compounds, their colonization by hyphae is central to this host-parasite relationship in the absence of extensive vascular colonization.

Investigators working on the pathological anatomy of C. ulmi in elm wood found a similar colonization strategy (Ouellette, 1962a, 1962b; Wilson, 1965). Banfield (1944) and Ouellette (1962a) demonstrated that spores from the Cephalosporium anamorph spread rapidly in the tree similar to C. fagacearum. Wilson (1965) and Ouellette (1962b) reported that parenchyma cells adjacent to colonized vessels were invaded by hyphae either through half-bordered pits or by direct cell wall penetration.

Wilson (1965), sampled bole and branchwood of naturally infected elm trees (Ulmus americana L.) showing early foliar symptoms of Dutch Elm Disease and found hyphae and spores lacking in vessels and fiber tracheids. Hyphae found in vessels entered adjacent parenchyma

cells. Movement from cell to cell was through pits only in early wilting stages and later also by direct cell wall penetration. Beckman (1956) has shown that the fungus produces pectin and cellulose hydrolysing enzymes which are implicated in cell wall and pit membrane degradation (Ouellette, 1962b). As in the case of *C. fagacearum*, gums and tyloses are frequently encountered in the colonization of elms by *C. ulmi*.

While Ouellette (1962b) emphasized that plugging of the smaller vessels by spores, mycelium and by-products of cell wall and cytoplasm degradation could result in the acute wilt expression, this is probably not the case. Wilson (1965) has shown that only a fraction of the total conductive tissue is plugged in the early stages of wilt expression. Numerous others have since confirmed this observation (MacDonald and Hindal, 1981).

Wilson (1963) proposed a wilt pathogenesis for the wilt disease of persimmon (*Diospyros virginiana*), caused by *Cephalosporium diospyri*, that is nearly identical to that of *C. ulmi* and *C. fagacearum*.

On the basis of these studies it is concluded that the principle mechanism in the wilt pathogenesis of *V. wageneri* in Douglas-fir is vascular occlusion and evidence for the primary involvement of a toxin is lacking.

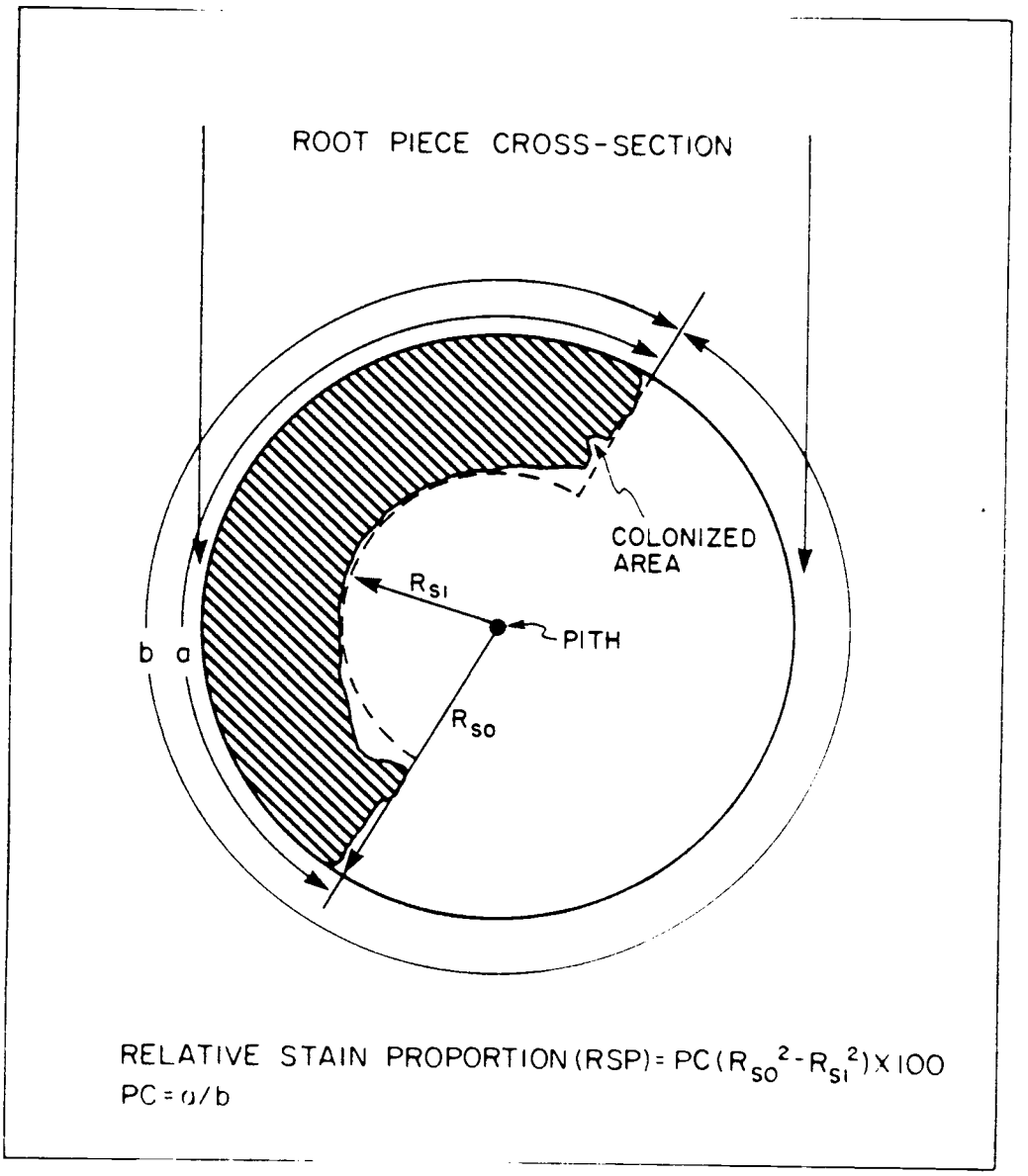

Figure III.1. Schematic drawing for the computation of the Relative Stain Proportion (RSP) for individual root piece cross-sections.

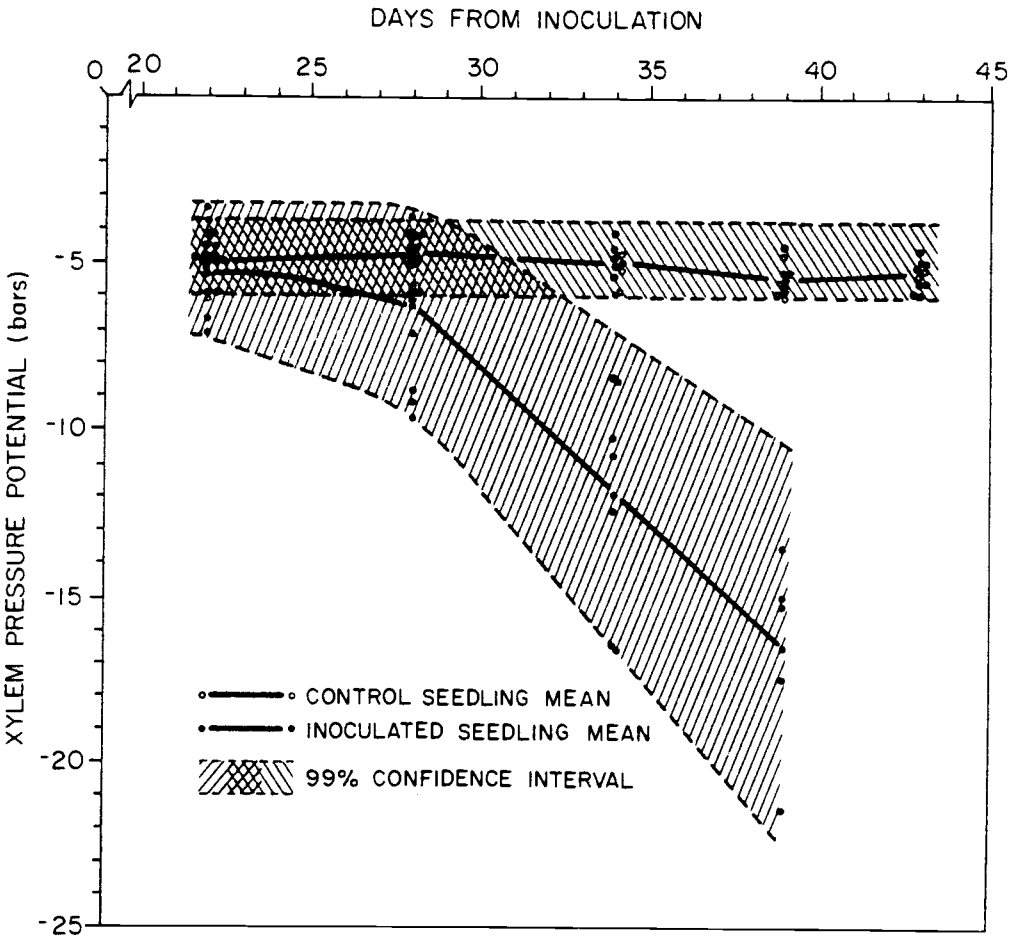

Figure III.2. Comparison of xylem pressure potentials for healthy control seedlings and seedlings inoculated with V. wageneri. Treatment means are significantly different at 34 and 43 days (P = 0.01).

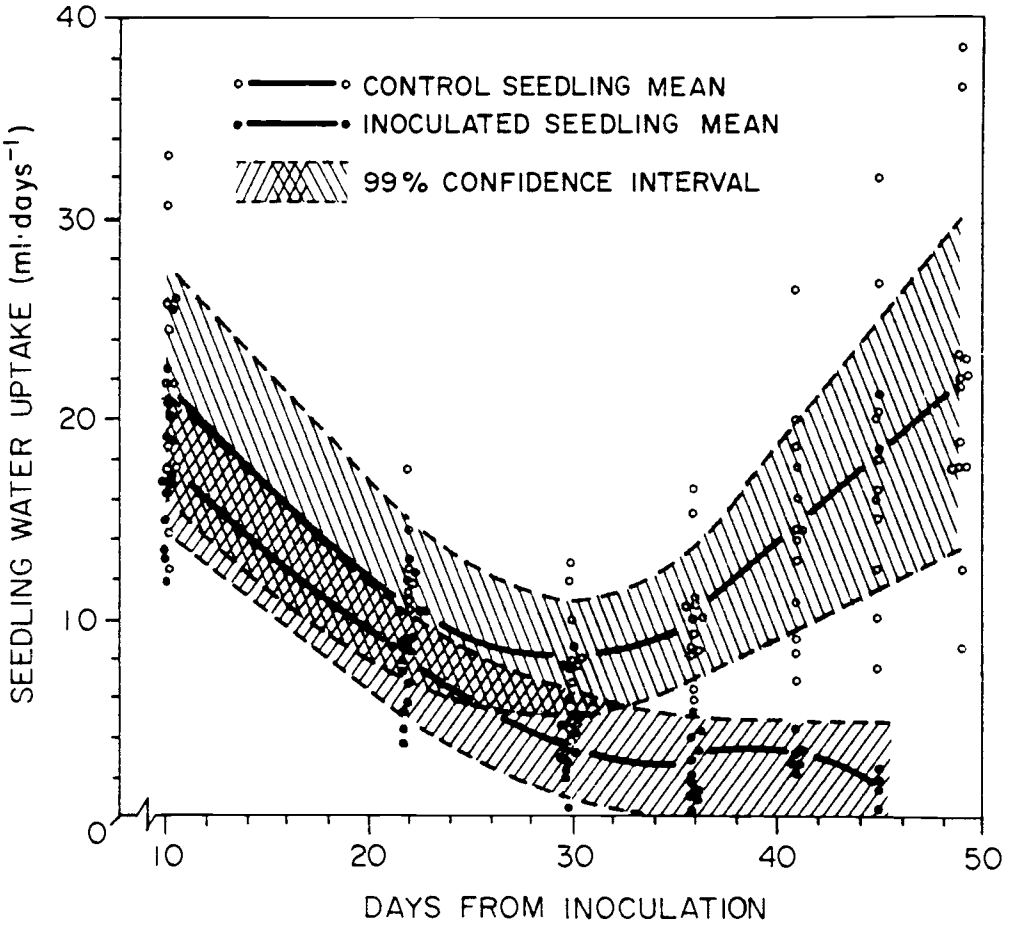

Figure III.3. Comparison of water uptake for healthy control seedlings and seedlings inoculated with V. wageneri. Treatment means are significantly different at 30, 36, 41, and 45 days (P = 0.01).

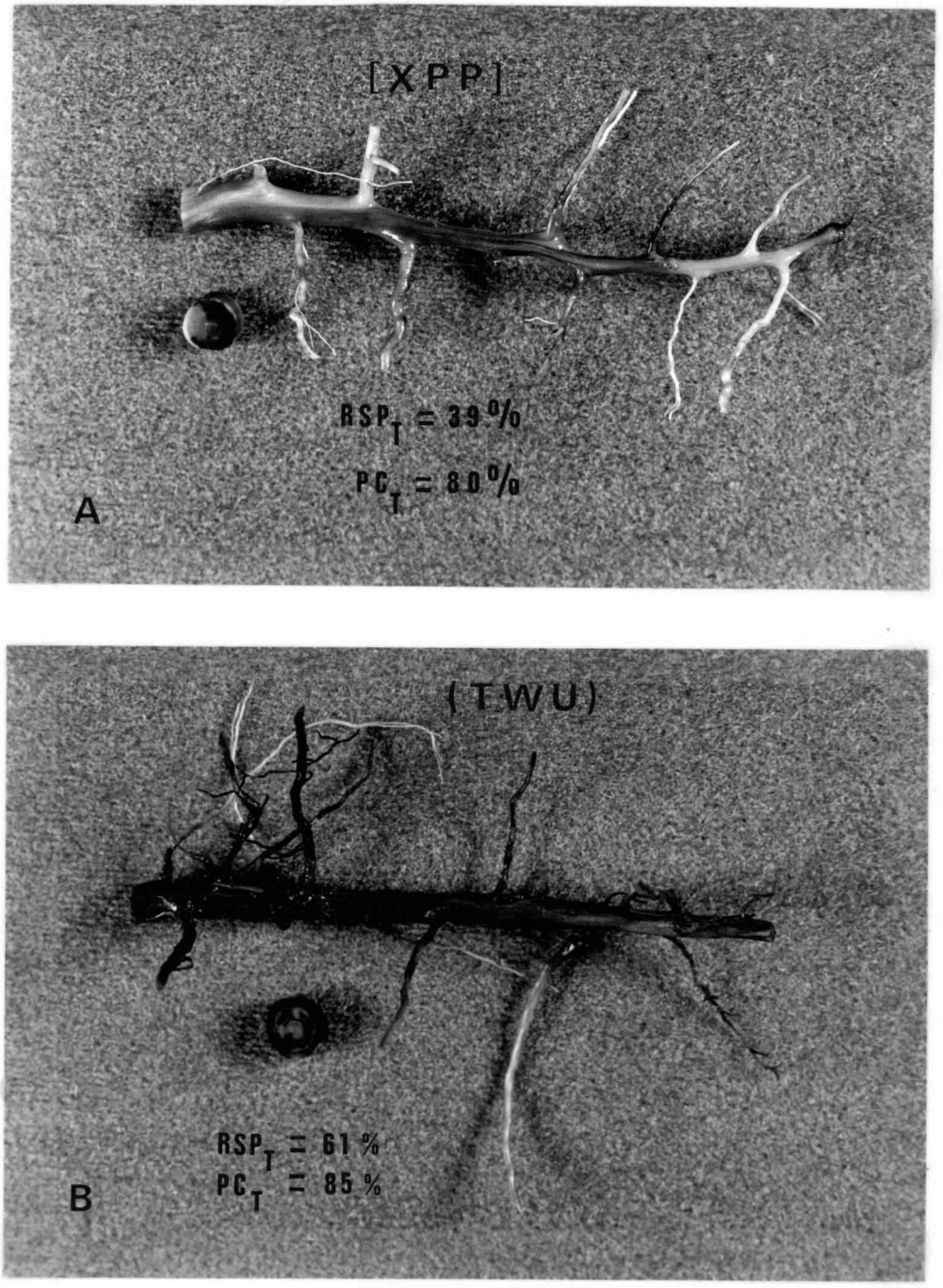

Figure III.4. Extent of root system colonization at the time of final sampling for seedlings inoculated in the xylem pressure potential (XPP) and water uptake studies (TWU). A, xylem pressure readings gave an earlier warning of increased vascular distress due to xylem colonization than did B, water uptake readings. Radial colonization was significantly greater in TWU sampled seedlings.

TABLE III.1. Measurement of the extent of root system colonization for Douglas-fir seedlings inoculated with *V. wageneri* and sampled according to their record of xylem pressure potential or water uptake relative to uninoculated controls.

		ROOT COLLAR CROSS-SECTIONS		SUM OF ALL ROOT SYSTEM CROSS-SECTIONS	
TREATMENT	SEEDLING NUMBER	PROPORTIONAL CIRCUMFERENCE (PC) %	RELATIVE STAIN PROPORTION (RSP) %	PROPORTIONAL CIRCUMFERENCE (PC_T) %	RELATIVE STAIN PROPORTION (RSP_T) %
Xylem Pressure Potential (XPP)	1	87	44	81	65
	2	38[x]	7[x]	33[x]	8[x]
	3	75	14	82	23
	4	87	31	73	26
	5	100	36	84	38
	6	87	31	76	31
	7	88	17	75	38
	8	87	73	87	49
	9	28[x]	10[x]	48[x]	19[x]
MEAN (SE)		87.3(2.7)a[y]	35.1(13.3)a	79.7(2.0)a	38.6(5.5)a
Transpiration Water Uptake (TWU)	1	100	100	99	79
	2	63[x]	23[x]	45[x]	22[x]
	3	75	75	83	58
	4	100	84	94	86
	5	88	80	84	46
	6	84	71	84	75
	7	94	18	83	29
	8	100	96	79	64
	9	88	32	82	35
	10	94	93	93	88
	11	87	79	85	67
	12	88	87	74	47
MEAN (SE)		91.6(2.3)a	74.1(7.8)b	85.5(2.2)a	61.3(6.1)b

[x]Seedling dead from transplant failure; not used in computing means.

[y]Mean percentages followed by the same letter are not significantly different (P = 0.01). Analysis by T-test.

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INFECTION OF DOUGLAS-FIR ROOTS BY *VERTICICLADIELLA WAGENERI* THROUGH WOUNDS AND NATURAL OPENINGS

Chapter IV

ABSTRACT

Root systems of 2-year-old Douglas-fir seedlings were dip-inoculated in a mycelium-spore suspension of *Verticicladiella wageneri*. Infection frequency was scored for dormant inoculated seedlings and seedlings inoculated 4 and 8 weeks from dormancy. Dissections of whole seedling root systems revealed that roots were consistently infected through wounds and natural openings to exposed xylem; direct penetration of root bark and cambial tissues by hyphae was never observed. Most dormant inoculated seedling infections (63%) occurred through wounds incurred in lifting and handling; wound infection frequency decreased to zero in 8-week inoculated seedlings. Four and 8-week inoculated seedlings were most frequently infected through natural openings occurring at sites of new lateral root initiation; dead fine root stubs were also infected. The infection of seedling roots exclusively through openings to exposed xylem demonstrates that openings are required for infection by *V. wageneri*. The demonstrated infection susceptibility of dead fine root stubs suggests that during periods of high fine root mortality, these sites may be important for new fungal infection of healthy trees, and fungal egress from already diseased trees.

INTRODUCTION

Verticicladiella wageneri Kendrick (teleomorph: Ceratocystis wageneri Goheen et Cobb) (Goheen and Cobb, 1978), the causal agent in black-stain root disease of conifers, is a primary root pathogen of several economically important timber species in the western United States and Canada. In the Northwest, mortality occurs in Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco), and ponderosa and lodgepole pines (Pinus ponderosa and P. contorta) (Goheen and Hansen, 1978; Goheen, 1976; Hunt and Morrison, 1979; Smith and Graham, 1975). In the Southwest, pinyon pines (Pinus edulis and P. monophylla) are the principal hosts (Wagener and Mielke, 1961; Landis and Helburg, 1976; Goheen, 1976).

Host colonization by V. wageneri is completely restricted to mature xylem tracheids and tree mortality is the result of extensive vascular tissue colonization and occlusion concurrent with insect attack of stem and roots (Chapter II; Chapter III; Harrington, 1983; Goheen, 1976; Goheen and Cobb, 1980; Witcosky, 1981; Witcosky and Hansen, 1984).

Long distance spread of the disease is likely via insect vectors that feed and breed in roots of V. wageneri infected trees. New infection centers are thought to arise from vector attacks on stressed, injured, or otherwise weakened trees (Goheen and Cobb, 1978, 1980; Goheen 1976; Witcosky, 1981; Harrington, 1983; Witcosky and Hansen, 1984). Intertree spread of the disease has been demonstrated through root grafts, and between rootlets of diseased and healthy trees intimately associated or in actual contact with each other

(Goheen, 1976; Landis and Helburg, 1976; Wagener and Mielke, 1961; Chapter V). In ponderosa pine, most root infections occurred through small roots (≤ 5 mm diam.) growing within 15 cm of diseased roots (Goheen, 1976). Hicks and others (Hicks, 1978; Hicks et al, 1980) have demonstrated that the fungus can grow through soil from infected roots for distances of 15 cm or more. Still lacking is a clear elucidation of the infection process and adequate description of the infection court(s). Since the fungus is confined to xylem tracheids, is unable to support primary growth on cellulosic or pectic substances, and appears to be unable to penetrate walls of living cells (Smith, 1969; Chapter V), infection of healthy roots may occur by means other than direct hyphal penetration. The objective of this study was to examine the process of root infection in Douglas-fir seedlings and describe the infection courts that were observed.

MATERIALS AND METHODS

In two replicated experiments, whole seedling root systems were dip-inoculated with an isolate of V. wageneri. In the initial experiment (A), dormant seedlings were inoculated and potted. In the weeks following inoculation, seedlings were periodically sampled and carefully dissected to observe the initial points of entry and progress of new infections, and the number of infection sites was counted. In a second experiment, (B) seedlings were grown in pots for 4 or 8 weeks to allow healing of obvious wounds on roots damaged during lifting, sorting and handling at the nursery, then dip inoculated. Again, seedlings were periodically

sampled and systematically dissected, and infection courts were tallied.

All seedlings used in these experiments had been grown for two years in a nursery using seed from a low elevation Oregon Cascades seed zone. All seedlings were from a single seedlot, lifted from a single seedbed, and root-pruned 3 months prior to lifting.

All seedling root systems were dipped to the root collar in a mycelium-spore suspension prepared as follows: 4-week-old cultures of V. wageneri (isolate VW-45) grown on standard Potato Dextrose Agar were macerated at low speed in a sterilized blender for 45 seconds. The mixture of spores and mycelial fragments was diluted with an equal volume of sterile, distilled H_2O and remixed. Haemocytometer readings for the various batches of inoculum were in the range of 10^4-10^6 spores/ml of suspension and hyphal fragments typically included several septa.

Following inoculation, seedlings were potted in a pasteurized medium composed of equal volumes of washed silica sand (EI-20) and peat (Baker, 1957).

Seedlings were destructively sampled 1 to 2 months after inoculation. At the time of sampling, seedlings were unpotted, washed, wrapped with a wet paper towel, and stored in a plastic bag at 4C awaiting dissection. Seedling dissections, viewed through a binocular dissecting microscope, were made by systematically removing the bark from the entire root system beginning at the root collar and proceeding carefully towards the root ends. The inner surface of the bark was examined for evidence of direct hyphal penetration which would appear as rust colored necrotic flecks in the secondary

phloem. Once the bark was removed, rootwood was kept continually wet to discourage the rapid phenolic discoloration of wood. The number of infections and types of infection courts were tallied for each seedling. When necessary, infection courts were free-hand sectioned and examined under the compound microscope to verify the origin of an infection.

In Experiment A, 102 dormant seedlings were inoculated and transplanted, three seedlings to a pot, in 34, 2.5 L plastic planting containers. Seedlings were randomized in the greenhouse and watered from above three times a week for 9 weeks (61 days) with a 20% Hoaglunds solution. Seedlings were destructively sampled 34, 44, 50, 57 and 61 days after inoculation. Six pots were selected at each sampling, including pots with trees showing early wilt symptoms. Preliminary experiments indicated that once foliage symptoms were expressed, colonization of roots by V. wageneri was too extensive to reliably determine the infection origins.

Experiment B consisted of three treatments. In the first two treatments, seedlings were grown in pots 4 weeks before inoculation. Prior to inoculation, seedlings were carefully removed from their pots and gently washed with cool tap water. Forty-five seedlings were dip-inoculated in the first treatment and planted 2-3/tube in 18 transplant tubes (7 cm diam. x 25 cm length). In nine cases, it was feasible to put only two seedlings in a tube because root systems of these seedlings were very large. Treatment 2 served as a control treatment; root systems were purposely clipped prior to inoculation to provide wounds for infection. Fifty-four seedlings were inoculated and transplanted 3/tube in 18 tubes. Seedlings in treatment 3 were

grown in pots for 8 weeks prior to inoculation. After careful washing, 18 seedlings were dip-inoculated and transplanted individually to tubes; two seedlings were wounded in the transplanting and eliminated from the experiment. The remaining 16 seedlings were randomized in racks with the seedlings from treatments 1 and 2 and incubated at 17C in a growth chamber. Seedlings in the three treatments were individually wick watered on demand with 20% Hoaglund's solution (Chapter III), and destructively sampled after 40, 35, and 27 days, respectively.

RESULTS

Of the 102 dormant seedlings inoculated in Experiment A, 48 (47%) were infected with V. wageneri; the remaining seedlings showed no evidence of infection. Infection origins were identifiable on all but three seedlings (Table IV.1). No seedlings were infected more than three times; an average of 1.3 infections were tallied for all infected seedlings. At 34 days, only 1 of 18 sampled seedlings was infected, at 50 days 12 of 18 seedlings (67%) were infected, and by 57 and 61 days 13 of 21 (62%) and 9 of 18 (50%) seedlings were infected, respectively (Figure IV.1). All infections originated at wounds or natural openings to exposed xylem. Direct penetration of roots through bark and cambial tissues was never observed. Stain columns representative of infection ranged from 1 to 10 cm in length and were easily distinguished by their chocolate-black coloration against the light color of uncolonized xylem. In all cases, both ends of a stain column were clearly identifiable. An infection court was tallied as

the point where typical hyphae and stain of V. wageneri reached to the exterior of a root. This occurred only at sites where roots were broken, cut, cracked, or abraded, where new lateral roots had emerged by penetrating and rupturing the bark, or where small roots had died back to the point of attachment. Figure IV.2 depicts the types of infection courts encountered in the initial experiment; six types were observed. Type 1, "spider-root" stubs, were produced by root pruning in the nursery. Cut root ends were surrounded by new roots formed since pruning. V. wageneri infected through the cut ends which were not healed over at the time of inoculation (Figure IV.3) Type 2, broken roots, were detached during lifting or sorting at the nursery; V. wageneri entered the xylem through the broken ends (Figure IV.4). Type 3, bark abrasions, were also the result of lifting injury at the nursery. A small section of bark (usu $<1cm^2$) was torn or abraded from roots, exposing the outer surface of the xylem. V. wageneri penetrated into the xylem only on the perimeter of these wounds where tracheids were at least partially covered by bark (Figure IV.5). Type 4 (lateral root) infection courts occurred at sites of new lateral root development. Hyphae of V. wageneri invaded through openings in the bark created by recently emerged lateral roots. Stain columns developed in the parent root around the point of lateral root attachment; lateral roots were not initially infected (Figure IV.6). Type 5 infection courts occurred on roots that were partially fractured or at acute-angled root junctures that were partially split. Type 5 differed from Type 2 in that damaged roots were alive and still attached. V. wageneri penetrated through the wound to exposed xylem and in many cases colonized both proximal and distal

portions of wounded roots (Figure IV.7). Type 6 infection courts occurred at points of partial or complete detachment of dead fine roots from parent roots. Dead roots were not penetrated by hyphae, and stain columns originated in the parent root around the point of detachment or through small stubs that remained attached (Figure IV.8). Of the total number of infected seedlings in Experiment A, 63% of the infections were through artifically induced wounds (Types 1, 2, 3 and 5) and 31% through natural openings to exposed xylem (Types 4 and 6).

In Treatment 1 of Experiment B, all 45 seedlings were infected by inoculation; exact infection courts were not ascertained on seven seedlings. An average of 3.4 infections occurred on seedlings in Treatment 1 and no seedling had more than seven infections. Seedlings were inoculated 4 weeks after initial planting and sampled 40 days after inoculation, at which time new root and shoot development was evident on all seedlings. Of the total infections (Table IV.1), 57% of the seedlings were infected through natural openings (Types 4 and 6) and 28% through wounds (Types 1 and 2). Infection by direct hyphal penetration of bark and cambium was never observed.

Treatment 2 seedlings were sampled 35 days after inoculation. Eighty-nine percent of the inoculated seedlings were infected, 94% of which were repeatedly infected through clipping wounds. Clipping removed nearly all of the previous wounds and most infected seedlings had multiple (>15) infections. Many clipped roots were infected on each seedling and stain columns coalesced while they were still quite short. It was impossible, therefore, to get an accurate total count of the number of infections per infected seedling. Again, no root

infection by direct hyphal penetration was observed.

In Treatment 3, seedlings were grown for 8 weeks prior to inoculation. Eight of 16 seedlings (50%) were infected after 27 days, all through Type 4 infection courts. Numerous old wounds were apparent on the roots of these seedlings but none were successfully colonized. In many cases, adequate wound healing was apparent; regions of localized resinosus and callus tissue formation were observed. At the time of final sampling (i.e., 3 months after the initial planting), the growth of new foliage was nearly completed, although new needles were still somewhat succulent in appearance and extensive new root growth was evident. Infected seedlings were multiply infected, each having five or more separate infections.

Differences in observation frequency of infection Types 1-6 were compared for Experiment A and Experiment B, Treatments 1 and 2 using the Least Significant Difference Test (LSD) (Steel and Torrie, 1980). Treatment 3 was not included in the analysis because of its small sample size. Analyses confirmed that differences in the frequency of Type 1, 2 and 4 infections were significant ($P = 0.01$) for all treatments and no other differences were significant (Table IV.2).

DISCUSSION

In all seedlings where infection courts were identified, root infections occurred only through wounds or openings to exposed xylem. Results from the two experiments indicated that a sufficient number of openings occurred on roots of Douglas-fir seedlings to allow infection by *V. wageneri* via natural means. Results from Experiment

B, Treatment 3, indicated that after 8 weeks of vigorous growth, old lifting or pruning wounds on seedling roots were adequately healed and infection at these sites was discouraged.

Significant differences in the frequency of Type 1, 2 and 4 infections were apparent in Experiment A and Treatments 1 and 2 of Experiment B, and all infections in Treatments 3 were Type 4. The frequency of seedling infection through Type 1 and 2 infection courts decreased with increasing period of growth prior to inoculation; wound healing discouraged some infection after 4 weeks and no wounds were infected after 8 weeks. In Treatment 2, roots were clipped and most spider root stubs (Type 1) were removed. The frequency of Type 1 infections was therefore lowest in Treatment 2, and the frequency of Type 2 infections (broken/cut root) was highest. Twice as many Type 4 infections occurred in Treatment 1 of Experiment B as occurred in Experiment A. Type 4 infections occurred at the site of new lateral root initiation, and increases in seedling infection frequency were related to longer periods of new lateral root initiation (Table IV.1, Treatments 1 and 3).

Wound infection courts (Types 1-3, 5) were common on all seedlings used in the experiments despite the special care afforded them in lifting and handling. Spidering in seedling root systems was related to fall root pruning at the nursery. Roots were cut and replacement roots regenerated at or proximal to the point of damage. Replacement roots were long (usu $\geq$ 10 cm) with well developed secondary tissues, indicating that the new roots had developed in the 3 months after root pruning before the seedlings were lifted from the nursery. The cut root stubs, however, did not heal over during the 3

winter months prior to lifting, and they were later infected by inoculation. For Type 1 infections to occur in Treatment 1, cut root stubs had to remain open to infection for at least 4 months. Type 1 infections did not occur in Treatment 3; pruning wounds healed adequately in the 2 month growing period prior to inoculation. Roots are frequently damaged on trees in field conditions and multiplicative root replacement often results. Root stubs such as these may be important sites for infection where roots of healthy and diseased trees are in close proximity.

Infection through bark abrasions (Type 3) occurred only in dormant inoculated seedlings (Experiment A). Although these sites were easily invaded by hyphae, infection took place only where tracheids were at least partially covered by bark. The remainder of the exposed xylem appeared to be embolized and was not colonized.

In Type 4 infections (lateral root), hyphae of V. wageneri appeared to invade through openings in the bark created by recently emerged lateral roots. Lateral roots arise from the pericycle, the outermost layer of the primary vascular stele. Cells of the pericycle become meristematic, a growing point forms with a definite root cap and the developing lateral root forces its way through the endodermis, cortex and epidermal tissues by enzymatic digestion and mechanical pressure (Esau, 1977; Kramer and Kozlowski, 1960; Kozlowski, 1971). The lack of 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 infection court for V. wageneri. Dissections of newly infected roots showing the Type 4 pattern of infection revealed that typical V. wageneri hyphae had

colonized the xylem of the parent root opposite these openings, and stain columns elongated axially away from these sites. In Experiment A, dormant seedlings were inoculated, and most infections occurred through wounds. Once seedlings had broken dormancy, new roots developed at an increasing rate and new shoot growth was evident. Type 4 infection courts were observed on 25% of the seedlings. In Experiment B (Treatments 1 and 3), seedlings were grown 4 and 8 weeks before they were inoculated. In Treatment 1, 55% of the observed infections were through Type 4 infection courts; in Treatment 3 all infections were Type 4. The evidence from seedling inoculations indicates that seedling infection through Type 4 infection courts increased with the increased production of lateral roots. I suggest that these sites are infected on roots of large Douglas-fir trees as they are on seedlings, and are important in intertree transmission.

Infection of openings left by dead roots (Type 6) occurred in both dormant inoculated seedlings (Experiment A) and seedlings grown 4 weeks prior to inoculation (Experiment B, Treatment 1). Dead root infection courts were absent on seedlings grown 8 weeks prior to inoculation (Treatment 3). I would anticipate low observation frequency of Type 6 infection courts on seedlings that are rapidly increasing their fine root systems. Santantonio (1982), studying the annual production and turnover of fine roots of mature Douglas-fir trees, found that peak fine root (<1 mm diam.) growth, occurred in the spring and fall of each year, root mortality and decomposition followed in the late summer and winter months. My observations in seedling Douglas-fir seem consistent with this pattern. Type 6 infections occurred either through short stubs (<2 mm) immediately

adjacent to the branching point, or on the parent root itself eminating from the original branching point.

Dead roots may be important sites for both new infection establishment and fungal egress. Fine roots are generally considered to be short lived, living from 1 to 4 years. Cold weather, excessive soil moisture, poor soil drainage and aeration, droughty soil conditions, attacks by insects, fungi, and other organisms, advancing tree age and foliage losses have been associated with fine root mortality (Kramer and Kozlowski, 1979; Zimmerman and Brown, 1971; Esau, 1977; Kozlowski, 1971; Torrey and Clarkson, 1975; Kramer and Kozlowski, 1960). Estimates of the annual turnover (mortality and decomposition) of fine roots were given by Santantonio (1982) for mature Douglas-fir (70-170 yrs.) growing on dry, moderate and wet sites. Estimates of mean annual fine root mortality were 5.5 MT/ha/yr on wet sites and 7.2 MT/ha/yr on dry and moderate sites. Annual turnover estimates in other hardwood and softwood tree species range from 10-90% (Kramer and Kozlowski, 1979; Kozlowski, 1971). It appears that during the summer and winter months there is an abundance of dead roots on healthy trees. Douglas-fir trees infected with V. wageneri show a marked reduction in foliage retention in comparison with healthy trees; two or more years of older needles are lost on most symptomatic trees (Witcosky, 1981). This may have the effect of further increasing the amount of fine root mortality on black-stain root diseased trees.

I have shown, using seedlings, that dead fine roots provide infection courts for hyphae of V. wageneri and I suggest that infection at these sites occurs in large Douglas-fir trees as it does

in seedlings. Since it has been shown that the amount of annual turnover of fine roots of Douglas-fir trees is considerable, I further speculate that infection by this means may be important in intertree transmission of this disease. In the field, root systems of symptomatic, black-stain root diseased Douglas-fir trees are widely colonized. When trees succumb to the disease and the associated insect attack, most major roots have been colonized to some degree. With the occurrence of seasonal fine root mortality, it is likely that living hyphae of V. wageneri would be in roots where fine roots were dying. Since infection occurs at these sites, fungal egress may also occur by a simple reversal of the same process.

In natural V. wageneri infection centers, wounds such as those involved in Type 2, 3 and 5 infections may occur less frequently than do the openings in Type 4 and 6 infection courts. Small root breakage, and splitting or fracturing of roots may occur as results of wind action on tree crowns. In subalpine environments, frost-heaving and alternating freezing and thawing of soils may also contribute to root wounding. Bark abrasions can occur on radially enlarging roots in contact with other roots (as in the case prior to grafting), or with permanent obstructions in the soil (i.e., stones or larger concretions).

The evidence for infection of Douglas-fir roots through wounds and natural openings may be summarized as follows:

1) intact roots of seedlings were never directly penetrated by hyphae of V. wageneri.

2) obvious wounds were invaded by hyphae and infected seedlings succumbed to the disease.

3) natural openings in the bark were invaded by hyphae and infected seedlings succumbed to the disease.

4) the number of infections per infected seedling was low and many seedlings escaped infection, even though entire root systems were dipped in a concentrated inoculum suspension (10^4-10^6 spores/ml). I would expect to see a higher infection frequency at that inoculum density if infection by direct penetration was possible.

5) the pathological anatomy of this disease has been critically examined in pines and in Douglas-fir. Hyphae of V. wageneri never invaded living parenchyma cells, xylem mother cells or cambial initials and were instead confined to xylem tracheids. Fungal egress through living bark and cambium was never observed.

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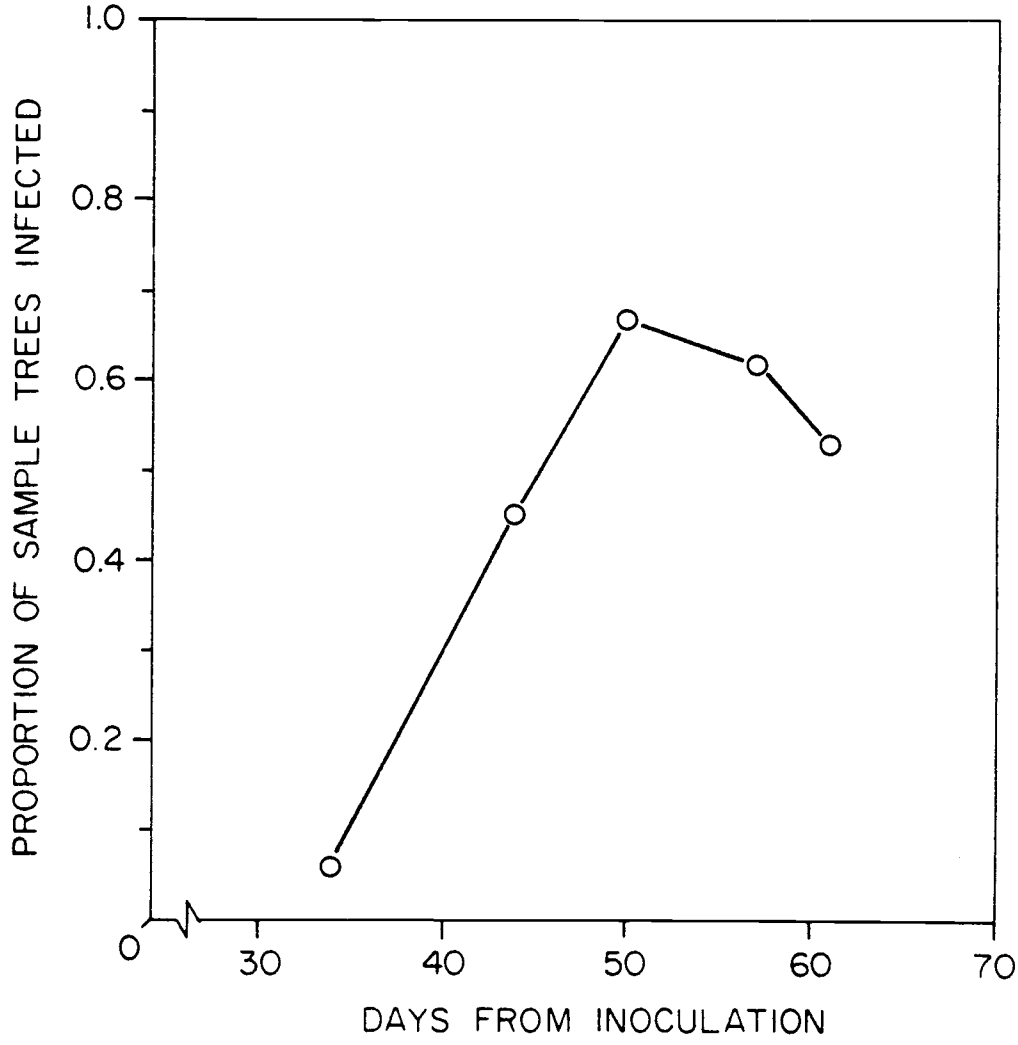

Figure IV.1. Time to infection for Douglas-fir seedlings root dip-inoculated with V. wageneri and incubated two months in a greenhouse. Data are from seedlings sampled in Experiment A.

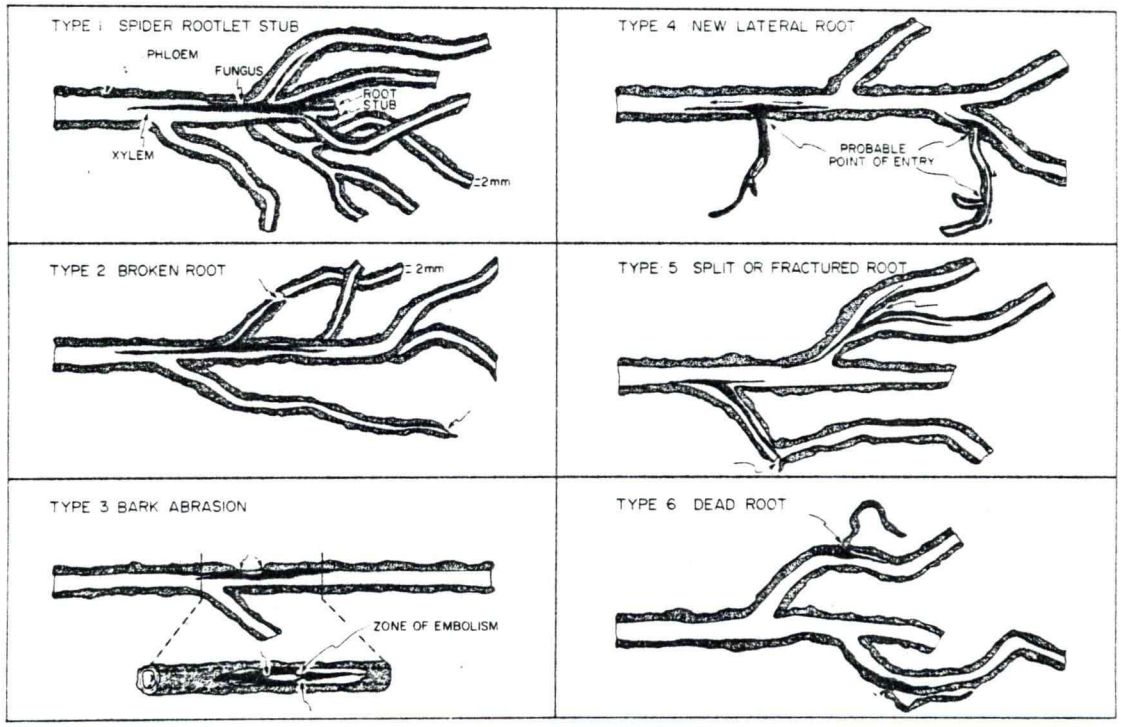

Figure IV.2. Schematic simplifications of six types of infection courts observed from dormant inoculated, newly infected Douglas-fir seedlings in Experiment A.

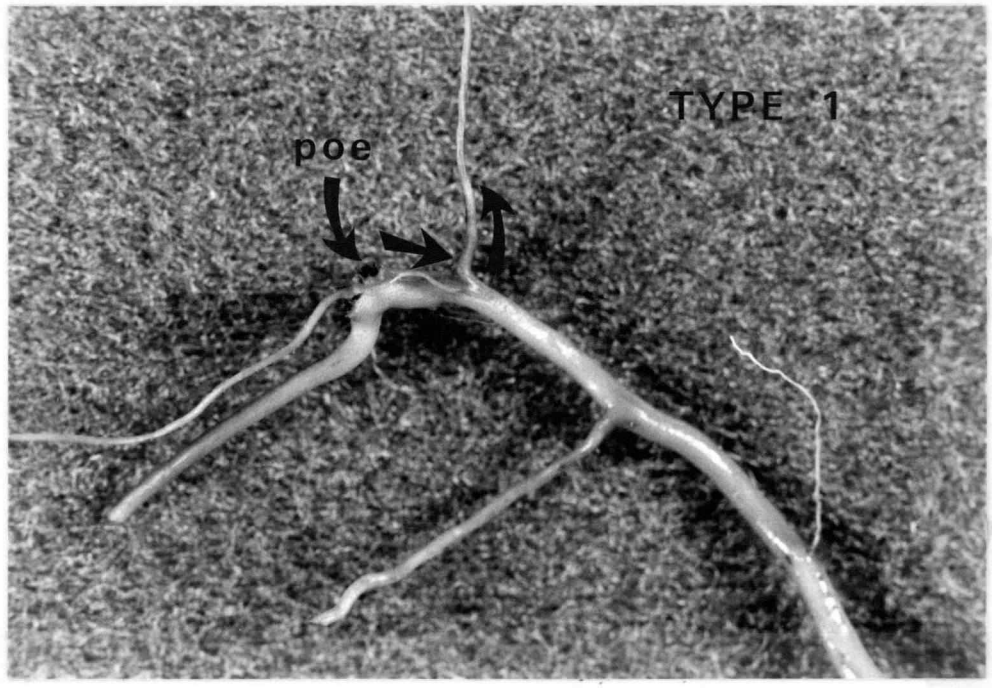

Figure IV.3. Root infection through a spider-root stub (Type-1). Point of entry (poe) and extension of the fungus shown with arrows. (actual size).

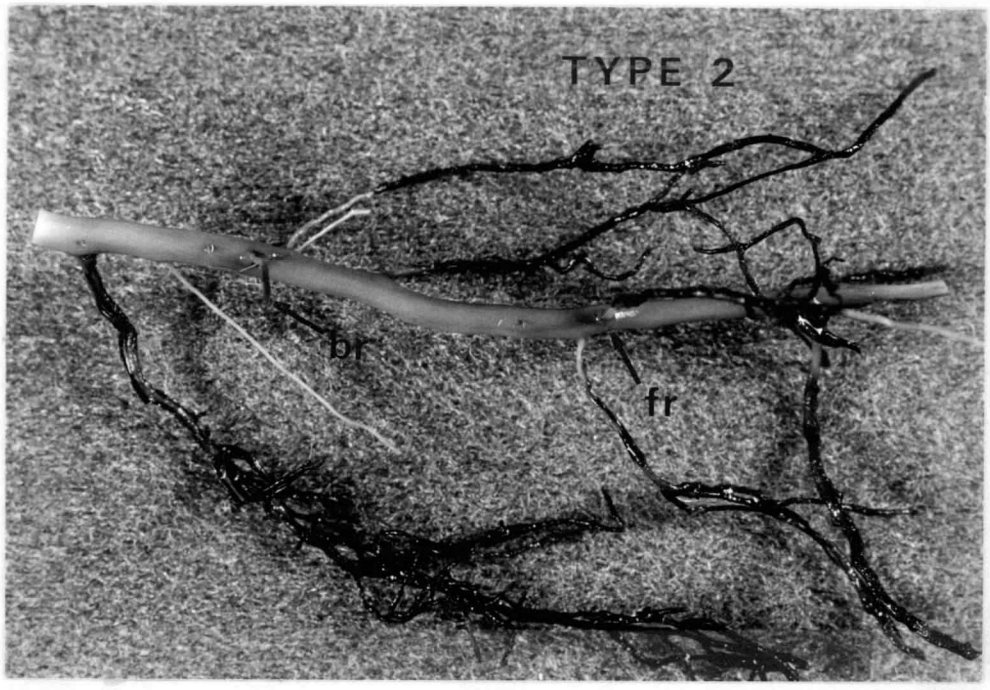

Figure IV.4. Root infection through broken root ends (Type-2). Two infections are shown, one through a small broken root (br) the other through a fracture at the base of a lateral root (fr). (actual size).

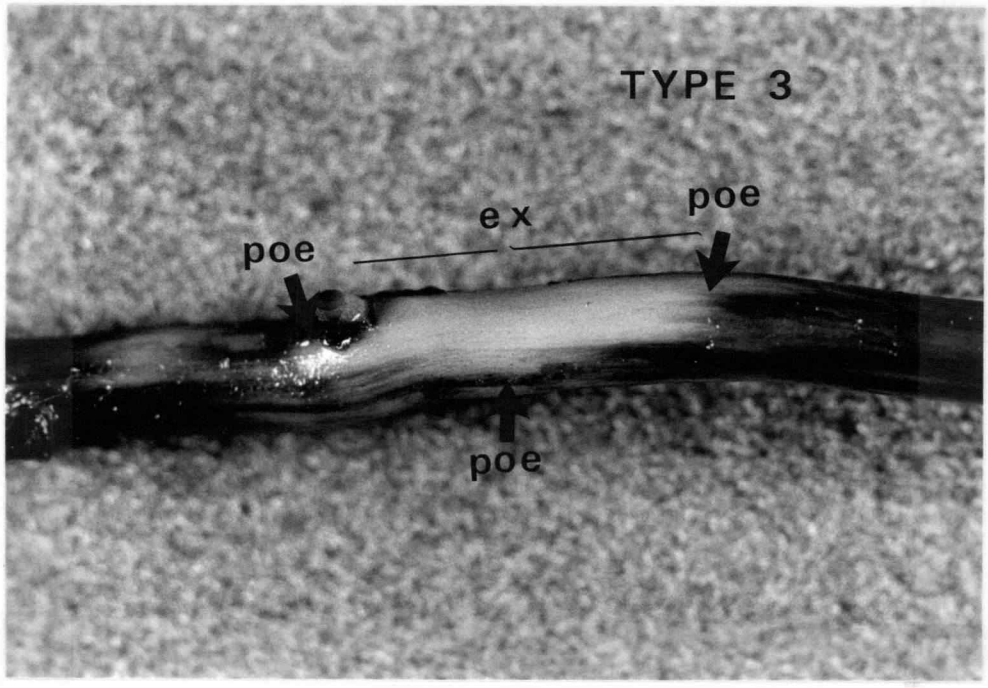

Figure IV.5. Root infection through bark abrasion (Type-3). Large exposed regions of xylem are quickly embolized (ex) and are not colonized by hyphae of V. wageneri. Points of entry (poe) (twice actual size).

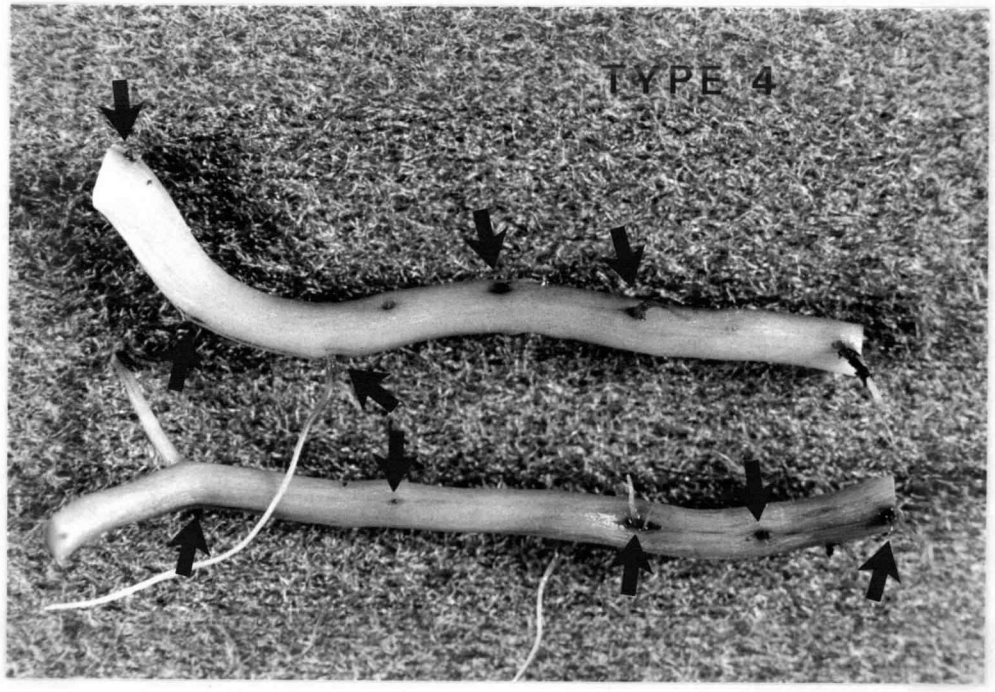

Figure IV.6. Root infection through openings in the bark created by recently emerged lateral roots (Type-4). Arrows indicate multiple points of entry. (actual size).

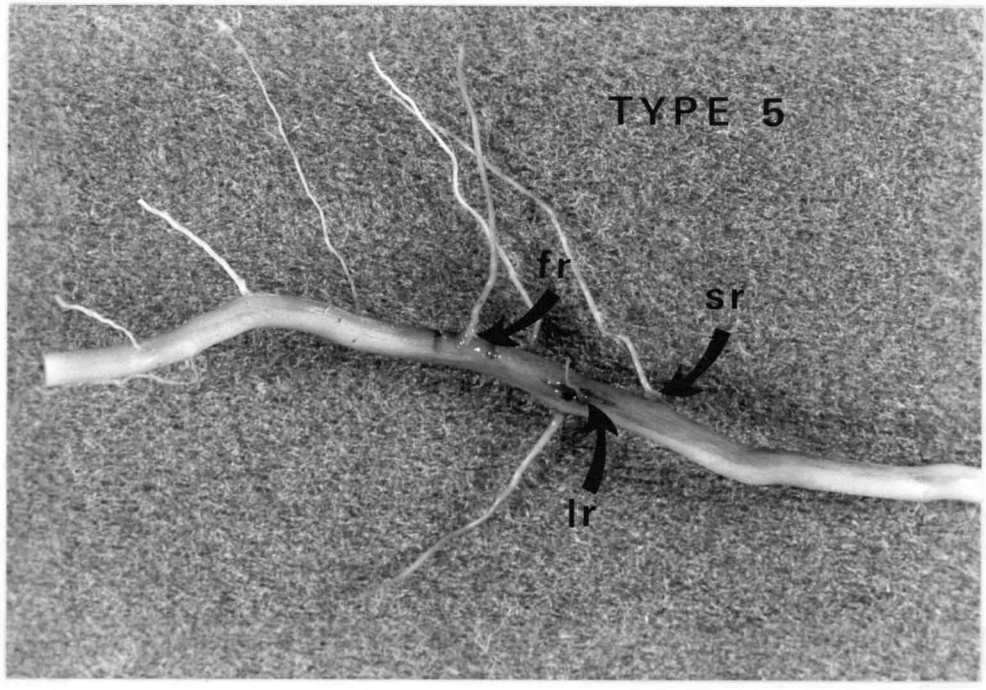

Figure IV.7. Root infection through split or fractured roots (Type-5). Lateral root infection (lr) also present. Split root (sr) and fractured root (fr) still attached; new lateral root (lr) is infected (actual size).

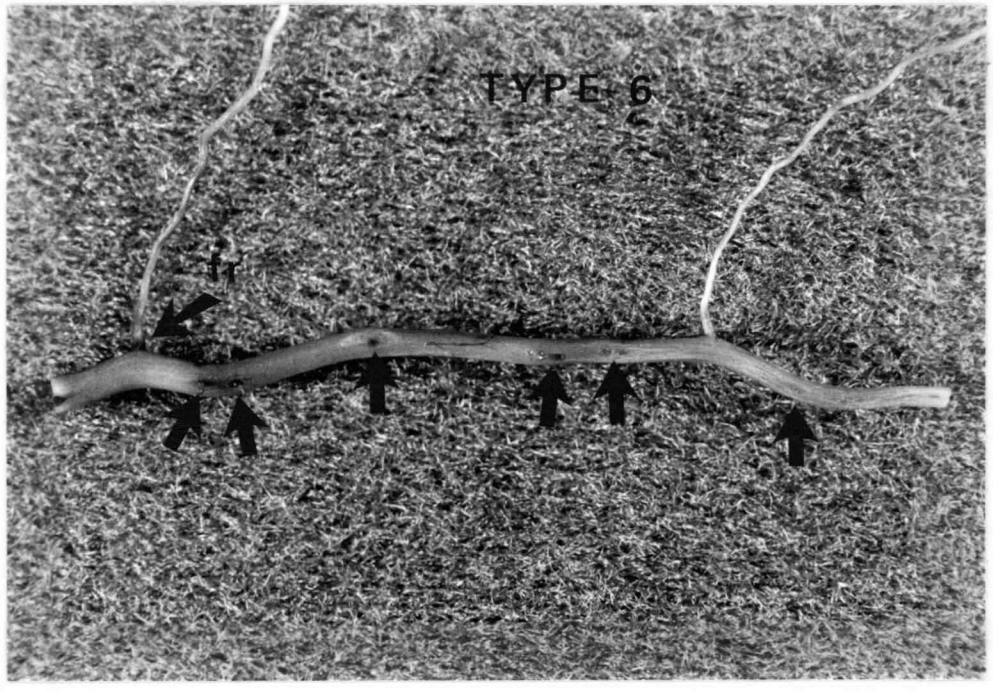

Figure IV.8. Infection through openings in the bark left by dead fine roots (Type-6). A fractured root (fr) is also infected (actual size).

Table IV.1 Infection of Douglas-fir seedlings root dip-inoculated with Verticicladiella wageneri 0, 4 and 8 weeks after planting.

Treatment	Number of Seedlings	Percent Infected	Infection Court Observation Frequency[v] Type[w]	Number of Seedlings	Percent of Observations
Experiment A				20	38
Seedlings			(1) s-rt stub	8	15
dormant planted[x]	102	47	(2) broken/cut rt	4	8
(roots intact)	(34 pots)		(3) bk abrasion	13	25
			(4) lateral rt	3	6
			(5) split/fract rt	3	6
			(6) dead rt	3	6
			(7) unknown	3	6
Experiment B					
(Treatment 1)			(1) s-rt stub	12	26
Seedlings growing	45	100	(2) broken/cut rt	1	2
4 weeks[y]	(18 pots)		(3) bk abrasion	0	0
(roots intact)			(4) lateral rt	26	55
			(5) split/fract rt	0	0
			(6) dead rt	1	2
			(7) unknown	7	15
(Treatment 2)			(1) s-rt stub	1	2
Seedlings growing	54	89	(2) broken/cut rt	44	94
4 weeks[y]	(18 pots)		(3) bk abrasion	0	0
(roots clipped)			(4) lateral rt	0	0
			(5) split/fract rt	2	4
			(6) dead rt	0	0
			(7) unknown	0	0
(Treatment 3)			(1) s-rt stub	0	0
Seedlings growing			(2) broken/cut rt	0	0
8 weeks[z]	16	50	(3) bk abrasion	0	0
(roots intact)	(16 pots)		(4) lateral rt	8	100
			(5) split/fract rt	0	0
			(6) dead rt	0	0
			(7) unknown	0	0

[v]Number of seedlings in which each infection court type was observed.

[w]See Figure 1 for diagrams of infection court types.

[x]Seedlings inoculated while fully dormant.

[y,z]Seedlings inoculated 4 and 8 weeks after initial transplanting, respectively.

Table IV.2 Comparison of infection court frequency between treatments for seedlings inoculated with Verticicladiella wageneri.

Treatments	Infection Court Observation Frequency		
	Type[x]		Percent of Observations
Experiment A Seedlings dormant planted (roots intact)	(1)	Spider-rt stub	38 a[y]
Experiment B, Treatment 1 Seedlings growing 4 weeks (roots intact)			26 b
Experiment B, Treatment 2 Seedlings growing 4 weeks (roots clipped)			2 c
A	(2)	Broken/cut root	15 a
B-1			2 b
B-2			94 c
A	(3)	Bark abrasion	8 a
B-1			0 a
B-2			0 a
A	(4)	Lateral root	25 a
B-1			55 b
B-2			0 c
A	(5)	Split or fractured root	2 a
B-1			0 a
B-2			4 a
A	(6)	Dead root	6 a
B-1			2 a
B-2			0 a

[x]See Figure IV.2

[y]Percentages followed by the same letter are not significantly different at P = 0.01 level based on the Least Significant Test of the arcsin of the square root of the percentages. LSD at (P = 0.01) is 9%.

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MECHANISMS OF INTERTREE TRANSMISSION OF *VERTICICLADIELLA WAGENERI* IN YOUNG DOUGLAS-FIR

Chapter V

ABSTRACT

Black-stain root disease caused by *Verticicladiella wageneri*, is typically found in foci or infection centers in young Douglas-fir plantations. Intertree transmission of the disease is reportedly via root grafts and major root contacts in *Pinus* spp., although only root graft transmissions have been verified. *V. wageneri* is readily isolated from soil adjacent to infected roots and intertree spread by mycelial growth through soil has been proposed. To test this hypothesis, potted seedling experiments were conducted where contact between roots of inoculated and healthy seedlings was either allowed or restricted. Transmission occurred in both treatments but was significantly more frequent ($P = 0.01$) when intertree root contact was allowed. In the root contact treatment, 100% of the inoculated transmitters contracted the disease; 67% of the receptor seedlings died as a result of successful transmission. In the no-contact treatment, the figures were 100% and 35%, respectively. Evidence points to fine roots as principal infection courts. Root graft transmission of *V. wageneri* in Douglas-fir was verified through field excavation and microscopic examination of 23 root grafts from three widely separated sites.

INTRODUCTION

Verticicladiella wageneri Kendrick (Ceratocystis wageneri Goheen et Cobb) (1978) causes a lethal root disease of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco), western and mountain hemlock (Tsuga heterophylla and T. mertensiana) and numerous Pinus spp. throughout western North America (Cobb and Platt, 1967; Goheen, 1976; Goheen and Hansen, 1978; Hansen, 1978; Wagener and Mielke, 1961; Hunt and Morrison, 1979; Smith and Graham, 1975). Tree mortality results from a systemic colonization, by fungal hyphae, of the functional vascular tissue of root systems, which limits uptake and translocation of water to foliage (Chapter III). Crown symptoms are those typical of a wilting syndrome. In pure stands, trees are killed in irregularly expanding infection centers which enlarge as the pathogen is transmitted to adjacent trees on the margin. Mortality in V. wageneri infection centers follows a pattern typical of many root rot pathogens (e.g., Phellinus weirii, Fomes annosus, and Armillaria mellea, that spread from tree to tree through root contacts or by rhizomorph infection of healthy roots in proximity (Smith, 1978).

Disease weakened trees are attacked by various root breeding bark beetles (Coleoptera/Scolytidae) and weevils (Coleoptera/Curculionidae) (Cobb et al, 1974; Goheen and Cobb, 1978; Witcosky, 1981; Witcosky and Hansen, 1984; Harrington, 1983), some of which have been implicated as vectors of the disease (Witcosky et al, 1981; Witcosky, 1981; Hansen et al, 1983; Witcosky and Hansen, 1984a, Harrington, 1983; Goheen and Cobb, 1978; Goheen, 1976). The hypothesis that insect vectors of V. wageneri initiate new foci of infection has expanding empirical

support. Insects are also undoubtedly involved to some degree in the local intertree spread of *V. wageneri*.

Hansen (1978) documented a clear association of the root disease with roads and proposed that trees on disturbed sites are more likely to be infected. Goheen and Hansen (1978) reported that 80% of infection centers were associated with some sort of obvious site disturbance, including road edges, clearcut margins, and plantation thinnings. Soil compaction and altered soil water drainage patterns are disturbances common to tractor logged sites with a high incidence of black-stain root disease. These disturbances may be of principal importance in the initiation of new infection centers by creating foci of stressed trees which are attractive to insect vectors of *V. wageneri*.

Intertree transmission of the disease in *Pinus* spp. can occur through root grafts and perhaps through major root contacts (Goheen, 1976; Landis and Helburg, 1976). In ponderosa pine, however, most new infections occur in small roots not in contact with, but within 15 cm of infected roots, and successful transmission through root grafts and major root contacts is infrequent (6%) (Goheen, 1976). Since hyphae of *V. wageneri* are confined to xylem tracheids (Hessburg and Hansen, 1982; Chapter II; Smith, 1967; Wagener and Mielke, 1961; Landis and Helburg, 1976), transmission through major root contacts is unlikely, unless there are regions of exposed xylem on both roots in the contact. Hicks and others (1978; Hicks *et al*, 1980) demonstrated limited fungal growth through soil and isolated the fungus from soil adjacent to infected roots of ponderosa pine. In pines, mycelial growth through soil and fine root infections appear to be two

important elements of intertree transmission.

In Douglas-fir black-stain root disease, infection center epidemiology and pathways of intertree transmission have not been adequately studied. In this study, I excavated root-grafted Douglas-fir trees in three locations and looked for evidence of graft transmission of V. wageneri. Two seedling experiments were conducted to demonstrate the importance of rootlets in intertree transmission and determine if root contact is required for successful transmission.

MATERIALS AND METHODS

Root-grafted, V. wageneri infected, Douglas-fir trees were excavated over a two year period from 1978-80, from three widely separate young-growth stands: two in the Oregon Coast Range (Ball Bearing Hill-Yamhill County, and Alsea-Benton County), and one in the westside Central Oregon Cascades (Balm Creek Drainage-South Fork McKenzie River). Trees ranged in age from 10-15 years for the three stands. Diseased trees were identified by crown symptoms (i.e., chlorotic foliage and reduced needle length and terminal shoot growth) (Figure V.1), and by the characteristic black-stain columns in the xylem of roots and stems. Infection centers in naturally seeded overstocked stands (>6000 trees/ha) were targeted for the excavations to increase the frequency of root graft observation. Groups of 2-4 trees, including apparently healthy trees on infection center margins, were excavated with hand implements and examined for the presence of root grafting. All grafts were removed to the laboratory for dissection and microscopic examination.

During the initial examination, isolates were taken from each pair of grafted roots from stained wood on the advancing margin. Wood chips were placed on sterile, wet filter paper in petri dishes and incubated 10 days at 17C (Anderson, 1980; Chapter II). Sympodioconidia were taken from conidiophores fruiting on the wood chips and plated onto standard PDA plates. Sixteen isolates of V. wageneri were obtained by isolation; each was examined microscopically in wood and checked culturally against Kendrick's (1962) taxonomic criteria. To verify pathogenicity in Douglas-fir, three seedlings were inoculated with each isolate.

Root grafts were washed and decorticated. When possible, the movement of stain columns through grafts was observed on the wood surface, otherwise grafts were completely dissected. Free-hand sections were made of colonized xylem throughout each graft to verify xylem continuity and observe the path of hyphal colonization. Sections were viewed under bright-field and phase-contrast microscopy. In cases of successful graft transmission, evidence of colonization other than intratracheal was sought.

Intertree transmission experiments were conducted in the greenhouse and growth chamber using V. wageneri inoculated and healthy seedlings. In the growth chamber, intertree root contact was either completely allowed or restricted in two experiments; in the greenhouse, root contact transmission was tested in a single experiment. Douglas-fir seedlings grown two years in the nursery were inoculated by removing a small wedge of bark and wood from the taproot with a sterile scalpel, and placing a 1 cm^2 block of V. wageneri (isolate VW-25) colonized agar inoculum on each wound. The inoculum was wrapped

first with a small (3 x 10 cm) piece of moistened cheesecloth and then wrapped with a piece (5 x 10 cm) of 0.50 mil clear polyethylene. The ends of the bandage were loosely secured with twist ties and melted paraffin (Chapter III). Control seedlings were handled identically, except that no inoculum was applied to the wounds.

In the greenhouse experiment, three or four healthy "receptor" seedlings were transplanted around an inoculated "transmitter" seedling in a pasteurized clay-loam Douglas-fir forest soil in 2.5 L planting pots. Seedlings were watered three times per week. Sixty-six inoculated treatment pots and 25 control pots were established in the experiment. The study was initiated in February of 1981 and taken down in May of 1983.

In the growth chamber each transmitter seedling was planted with two to four healthy receptor seedlings in a plastic seedling transplant tube (7 cm diam. x 25 cm length). Seedlings were planted in a pasteurized potting mix composed of 65% washed silica sand (EI-20) and 35% a mix of equal volumes of 1/4" mesh washed river sand, clay loam and peat, and were watered by a wick from a reservoir of 20% Hoaglund's solution (Chapter III). Thirty-six inoculated treatment tubes and 18 controls were incubated in a constant 17C growth chamber for 4 months.

Intertree transmission of V. wageneri without root contact was demonstrated by confining transmitter seedling roots in 45 µm mesh nylon bags sewn from Nitex® No. HD 3-45; Tetko® Inc., Los Angeles, CA 91754. Inoculated transmitter seedlings were transplanted into the nylon bags using the previously defined potting mix. Two to four uninoculated receptor seedlings placed around a transmitter seedling

in its nylon bag were transplanted into a plastic transplant tube. Potting mix was used to fill in around the roots of the receptor seedlings and seedlings were watered on demand by wicks from a reservoir of 20% Hoaglund's solution. Transplant tubes containing the seedlings were randomized in racks and incubated for 4 months at 17°C in a growth chamber. Thirty-six treatment tubes and 18 controls were used.

RESULTS

Transmission of V. wageneri through root grafts occurred at all three sites. Excavations at the three sites yielded relatively few root grafts with the exception of the Alsea 2 infection center; stocking density for this center exceeded 10,000 trees/ha. Excavations yielded 5, 11, and 7 grafts at the Ball Bearing, Alsea and Balm Creek sites, respectively (Table V.1). Microscopic examination of freehand sections revealed V. wageneri hyphae in the vicinity of 16 of the 23 grafts; the fungus was traced through 10 grafts. Root graft transmission always followed lines of functional axial xylem continuity and hyphae were confined solely to tracheids (Figure V.2). Three cases of within-tree or self-grafting were observed, one of which successfully transmitted the disease to another root of the same tree. Of the six infected grafts not transmitting the disease, four were due to a lack of functional xylem continuity between roots. In the fifth, the xylem was continuous through the graft in the two outer annual rings, however, hyphae of V. wageneri were colonizing an inner annual ring that was not continuous. In the remaining instance,

hyphae had not reached the point of xylem continuity, yet successful transmission appeared possible. All isolates from root grafts were pathogenic on seedlings and each isolate fit Kendrick's (1962) description of Verticicladiella wageneri.

In both the greenhouse and growth chamber potted seedling studies, transmission of V. wageneri occurred when roots of inoculated and healthy seedlings were allowed free and intimate contact. In the greenhouse, 76% of the inoculated transmitters became infected and 14% of the receptors (Table V.2). Infection of new receptors occurred in several pots throughout the 2 year period of the study. Since infected seedlings usually died within 2-3 months following inoculation, it was assumed that mortality in some receptor seedlings was the result of infection from adjacent receptor seedlings. In all cases but one, successfully inoculated transmitter seedlings died in the first 6 months after inoculation. Of the 66 seedlings inoculated, 50 became infected, and 20 (40%) of these transmitted the disease to receptor seedlings. An average of nearly two receptor seedlings ($\bar{x}$ = 1.8) were infected per pot, with a maximum of five receptors killed in a pot. The cause of death was confirmed by seedling dissection and microscopic examination of freehand sections for all transmitter and receptor seedlings that succumbed to the disease. Dissections of the 30 infected transmitter seedlings that did not transmit the disease revealed that they succumbed too quickly to allow transmission to the receptor seedlings. All control seedlings were healthy and growing vigorously when the greenhouse experiment was taken down.

The same experiment repeated under controlled conditions in a

growth chamber produced similar results. One hundred percent of the inoculated transmitters contracted the disease; 67% of the receptors died as a result of transmission. Root to root transmission occurred in all but one pot, with an average of two seedlings ($\bar{x}$ = 1.8) killed per pot. Of the 94 receptor seedlings in the study, 63 contracted the disease and died within 4 months. All control seedlings were healthy at the time of final sampling.

In the no contact test, root contact between transmitters and receptors was completely prevented by the use of the fine mesh nylon bags. All 36 transmitter seedlings were successfully inoculated with *V. wageneri* and 27 of 77 receptor seedlings (35%) succumbed to the disease within 4 months (Table V.2). An average of less than one ($\bar{x}$ = .8) receptor seedling was killed per pot. All control seedlings were healthy at the time of final sampling. The difference in infection of receptor seedlings between root contact and no contact was significant (T-test, $p = 0.01$).

DISCUSSION

Root graft transmission of *Ceratocystis* vascular wilt pathogens is well documented in the forest pathology literature (Boyce, 1961; Jones and Phelps, 1972; Agrios, 1969; Himelick and Nelly, 1965). In hardwood tree species, *Ceratocystis fagacearum* and *C. ulmi* are the notable examples. Landis and Helburg (1976) excavated roots of pinyon pine (*Pinus edulis*) and observed the spread of *V. wageneri* from tree to tree through root grafts. Goheen (1976) observed similar graft transmission between ponderosa pine (*Pinus ponderosa*) trees in

California. Evidence reported here indicates that root grafts between Douglas-fir trees also reliably transmit the disease in young-growth plantations when grafted xylem is continuous.

Twenty-three grafts were observed from the excavations, 16 of which were infected. Of the infected grafts, 63% (10) of them successfully transmitted the disease. Root grafts can provide an efficient means of intertree spread of the disease; the importance of graft transmission in infection centers will depend on the frequency of grafting and the frequency of intertree spread by other means. In young, densely stocked stands, grafting frequency may be high. The incidence of black-stain root disease is also often high in such stands.

Reynolds (1981) and Reynolds and Bloomberg (1982) have shown that the probability of intertree root contact (and grafting) increased with increasing DBH (i.e., increasing size and age of trees), and decreased with increasing intertree slope distance. It follows that increasing tree age and stand density not only increase the incidence but also the importance of root grafting as a potential pathway of intertree transmission of contagious root diseases. These excavations were concentrated in areas of abnormally high stocking within V. wageneri infection centers, so extrapolation to normal spacings is tenuous. Root graft transmission of V. wageneri, although potentially significant, is probably not the primary means of intertree spread in managed, young, second-growth Douglas-fir plantations.

Goheen (1976) excavated 126 V. wageneri infected and 139 healthy ponderosa pines and found 69 apparent root grafts and 79 major root contacts. Evidence of intertree transmission of V. wageneri occurred

in only three and five instances, respectively. Of the 126 diseased trees examined, 116 (92%) showed evidence of infection unrelated to grafts or major root contacts. Goheen proposed that fine rootlets were the principle infection court in pines, however, the process of infection was not described. Hicks et al (1980) were able to isolate the fungus from forest soil up to 6 cm from V. wageneri infected ponderosa pine roots, indicating that the fungus is capable of some growth and survival in the soil.

Results reported here show that intertree root contact between diseased and healthy seedlings increases the frequency of successful intertree transmission, but it is not required. In the growth chamber experiment, where intertree root contact was allowed, transmission of V. wageneri to healthy receptor seedlings occurred in most of the pots. Hyphae of V. wageneri apparently exited from infected roots and easily located sites to establish new infections either on roots in contact or in close proximity. The interpositioning of a thin microporous nylon bag between roots of inoculated and healthy seedlings in the no-contact treatment decreased the number of successful transmissions to half of that observed in the contact treatment; roots were only separated by a distance of 1-2 mm. The loss of intertree root contact apparently decreased the opportunity for the fungus to find a suitable infection court within an adequate time frame outside of the host. Nevertheless, results from the no-contact treatment clearly demonstrate that V. wageneri can exit from roots, grow for a limited distance outside the host, find an infection court and successfully establish itself in a new host plant. This corroborates evidence from pines that V. wageneri can survive and grow in the soil.

V. wageneri is a pathogen confined to tracheids throughout the disease cycle in the host, and is apparently incapable of direct penetration of walls of living cells. The evidence suggests that hyphal infection of roots can only occur at points where hyphae can enter xylem tracheids directly through bordered-pits. Recent evidence from infection tests with Douglas-fir seedlings indicates that natural openings or wounds to exposed xylem are necessary for infection of healthy roots (Chapter IV). In seedling tests, fine roots of Douglas-fir provided ample opportunity for infection; all infections occurred through wounds or natural openings to xylem.

Local spread of *V. wageneri* within infection centers probably consists of several components:

1) root grafts transmit the disease in each conifer ecosystem where the disease occurs. Root grafts are potentially an efficient means of spread. The importance of graft transmission of *V. wageneri* depends on the frequency of root grafting which is regulated by specific stand and site factors (i.e., species mix, stocking level and tree spacing, tree age and size, slope, etc.);

2) spread may occur between rootlets in contact or in close proximity to each other. Root infection in either case is by direct growth of hyphae to open infection courts;

3) insects may be directly or indirectly responsible for further spread of the pathogen to new healthy trees (Goheen and Cobb, 1978; Witcosky and Hansen, 1984; Harrington, 1983).

Witcosky and others (1981; Witcosky and Hansen, 1984a; Witcosky *et al*, 1981; Harrington, 1983) have presented strong empirical evidence implicating two curculionids and a root breeding scolytid

beetle as potential vectors of V. wageneri in Douglas-fir. Emergence holes created by teneral adults of each species may link infected xylem with the surrounding rhizosphere. Insects may be responsible for carrying inoculum into the soil as they emerge from infected roots or carrying it directly to roots of adjacent healthy trees in the process of maturation feeding or prior to breeding.

Least plausible of all of the previously proposed modes of intertree transmission is that occurring through major root contacts between large roots. Since healthy, intact bark is not colonized or penetrated by hyphae of V. wageneri (Chapter II), mere contact between roots is not sufficient for transmission to occur.

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Figure V.1. Chlorotic foliage and reduced shoot growth of black-stain root diseased Douglas-fir trees. Note stunting of needles, reduction in the retention of older needles; and distress cone crop.

Figure V.2. Root graft transmission of *V. wageneri*. Note how the stain column follows the path of translocation.

Table V.1. Incidence of root grafting and efficiency of graft transmission in black-stain root diseased Douglas-fir at three widely separated excavation sites.

	Root Grafts			
Excavation Site	Total Grafts	Infected Grafts	Transmitting	Nontransmitting
Yamhill County				
Ball Bearing 1	2	1	0	1[a]
Ball Bearing 2	3	2	2	0
Benton County				
Alsea 1	2	2	1	1[c]
Alsea 2[b]	8[e]	7	5[d]	2[a]
Alsea 3	1	1	1	0
Lane County				
Balm Creek 1	3	1	0	1[a]
Balm Creek 2	4[e]	2	1	1[a]
Total	23	16	10	6

[a]Nontransmission in infected grafts due to lack of functional xylem continuity.

[b]Stocking level in this infection center exceeded 10,000 trees/ha.

[c]Furthest advance of hyphae of *V. wageneri* had not reached graft union.

[d]Transmission occurred through one self-grafted specimen.

[e]One root graft is self-grafting.

Table V.2. Transmission of Verticicladiella wageneri from inoculated transmitter seedling to adjacent receptor seedlings in pots.

Treatment	Infection Success			
	Transmitters		Receptors	
Roots in contact[a] (1982-1983)	36/36	100%	63/94	67%[c]
Controls	0/18	0%	0/18	0%
Roots separated[a] (1982-1983)	36/36	100%	27/77	35%[c]
Controls	0/18	0%	0/18	0%
Roots in contact[b] (1981-1983)	50/66	76%	25/250	14%
Controls	0/25	0%	0/125	0%

[a]Results from growth chamber experiments.

[b]Results from greenhouse experiment.

[c]Percentages are significantly different at the P = 0.01 level, based on the T-test comparing all replicates from each treatment with two receptor seedlings per pot.

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EFFECT OF SOIL TEMPERATURE ON THE GROWTH OF *VERTICICLADIELLA WAGENERI* IN DOUGLAS-FIR

Chapter VI

ABSTRACT

Growth chamber, greenhouse and field experiments were conducted to determine infection success and growth rate of *V. wageneri* in Douglas-fir as affected by soil temperature. In growth chambers at 10 and 17C, 92 and 97% of the seedlings became infected; at 28C only 19% of the seedlings were infected. Vertical growth rate of *V. wageneri* in seedling xylem was 2-3 times faster at 17C than at 10C; growth rate at 28C was intermediate.

Growth rates varied predictably with soil temperature fluctuations in the greenhouse; increases in the proportion of days where soil temperatures were between 15 and 18C produced increases in fungal growth rate in xylem; conversely increases in the proportion of days >18C or <15C depressed growth rate. Soil temperatures above 15C generally favored faster growth of *V. wageneri* in xylem.

Growth rate of *V. wageneri* in roots of 20-year-old trees averaged 2.2 m $\cdot$ yr^{-1}, with a maximum of 3.6 m $\cdot$ yr^{-1}, and an average of eight successive annual rings were colonized within three months. Recent estimates of the annual rate of radial enlargement of infection centers range from 1 to 2.5 m $\cdot$ yr^{-1}. Results from these experiments indicate that radial spread of the disease in infection centers can be explained by fungal growth in roots without insect vectors.

INTRODUCTION

Verticicladiella wageneri Kendrick (teleomorph: Ceratocystis wageneri Goheen et Cobb), the causal agent in black-stain root disease, is a vascular wilt pathogen of Douglas-fir (Pseudotsuga menziesii Mirb. Franco), ponderosa pine (Pinus ponderosa) and several other species in the Pinaceae of western North America (Smith and Graham, 1975; Hunt and Morrison, 1979; Goheen, 1976; Landis and Helburg, 1976; Wagener and Mielke, 1961; Goheen and Hansen, 1978). In ponderosa pine, most new infections are initiated in small roots ($\leq$5 mm dia.) growing within 15 cm of diseased roots (Goheen, 1976). Root infections in Douglas-fir seedlings are initiated through wounds and natural openings to exposed xylem, and living bark and cambial tissues are never directly penetrated by hyphae (Chapter IV).

In each conifer host, V. wageneri mycelia are confined to mature sapwood xylem tracheids and radial colonization in ray tracheids is limited (Smith, 1967; Landis and Helburg, 1976; Hessburg and Hansen, 1984c). During host colonization, earlywood is colonized more often than latewood, and recent sapwood xylem is colonized before older xylem. Colonization is widespread in root systems of symptomatic trees; the root collar and most major roots are colonized to some degree prior to overall tree decline. Tree mortality results from extensive vascular tissue colonization of the lower stem and root system which limits water uptake and impedes translocation of xylem sap to transpiring foliage, causing critical water shortages in trees (Chapter III; Smith and Graham, 1975). Infected trees are attacked by

various root-feeding and root-breeding bark beetles (Coleoptera/Scolytidae) and weevils (Coleoptera/Curculionidae) which contribute to rapid tree decline; some insects have been implicated as vectors of the disease (Goheen, 1976; Goheen and Cobb, 1978, 1980; Witcosky *et al*, 1981; Witcosky, 1981; Harrington, 1983; Witcosky and Hansen, 1984).

Intertree spread of the disease can occur without insects, through root grafts, and between small roots of healthy and diseased trees that are in intimate contact or close proximity to each other (Goheen, 1976; Landis and Helburg, 1976; Chapter V). Healthy seedlings planted in the same pot with inoculated seedlings regularly become infected whether intertree root contact is allowed or is completely restricted (Chapter V). Wagener and Mielke (1961) reported that pinyon pine isolates of *V. wageneri* had an *in vitro* growth optimum temperature of 15C; others (Goheen, 1976; Smith, 1967) have confirmed this observation reporting an *in vitro* growth optimum range of 15-18C.

Several researchers have evaluated the relationship of edaphic factors to infection and colonization of Douglas-fir and ponderosa pine by *V. wageneri* (Harrington and Cobb, 1984; Smith, 1967; Wilks *et al*, 1983; Goheen *et al*, 1978). Goheen *et al* (1978) examined the relationship of soil moisture to infection and colonization of ponderosa pine by *V. wageneri*. In greenhouse and lathhouse studies, mean values for vertical colonization of pine seedlings under different soil moisture treatments ranged from 40-90 mm after 80 days, or 0.50-1.1 mm $\cdot$ day^{-1}; high soil moisture (1/3 bar tension) favored seedling infection. Wilks *et al* (1983) examined the relationship of

soil moisture and redox potential to infection and colonization of ponderosa pine seedlings by V. wageneri. Mean values for vertical colonization of pine seedlings placed in soils of varying redox potential ranged from approximately 6.5 to 14.5 cm after 52-56 days, or approximately 1.2 - 2.7 mm $\cdot$ day^{-1} of vertical growth in xylem. Intermediate redox and aeration conditions (300-700 mV potential) favored seedling infection; maximum vertical colonization occurred under slightly higher redox conditions (550-750 mV). Soil temperature was not a controlled variable in either study, and maximum vertical growth was measured in only one direction from the point of inoculation.

Smith was first to evaluate the role of temperature in infection of pine seedlings by V. wageneri. Ponderosa pine seedlings were inoculated with a hardpine isolate of V. wageneri and incubated at four different constant temperatures (16, 21, 27 and 32C) for 2 months. At 16C, 90% of the seedlings were infected; at 21C, 30% became infected; no seedlings were infected at either 27 or 32C. Harrington and Cobb (1984) showed host preferences of three morphological variants of V. wageneri in inoculations of seedling and mature ponderosa pine and Douglas fir. In the seedling inoculations, percentage infection and extent of vertical colonization were evaluated at the same four soil temperatures (16, 21, 27 and 32C). Neither Douglas-fir nor ponderosa pine seedlings were infected at 27 or 32C by the hard pine, pinyon pine, or Douglas-fir variants. For all three variants, percent infection success and maximum vertical extension were greatest in Douglas-fir seedlings incubated at 21C; infection and growth in pine seedlings was greatest at 16C. Vertical colonization

of pine seedlings by hard pine variants, measured in both directions from the point of inoculation, ranged from 22-28 cm after 14 weeks, or 2.2 - 2.9 mm $\cdot$ day^{-1}. Vertical colonization of Douglas-fir seedlings by Douglas-fir variants ranged from 14-15 cm after 14 weeks or 1.4 - 1.5 mm $\cdot$ day^{-1}. In inoculations of mature trees of both species, vertical colonization of pine roots by hard pine variants ranged from 2.3 - 6.4 mm $\cdot$ day^{-1}; vertical colonization of Douglas-fir roots by Douglas-fir variants ranged from 0.3 - 2.3 mm $\cdot$ day^{-1}.

Harrington and Cobb confirmed Smith's results showing that infection success in pine seedlings was greatest at 16C; however, they reported that percent infection and maximum vertical extension in Douglas-fir were greatest at 21C. Our preliminary experiments indicate that soil temperatures $\leq$ 18C favor infection of Douglas-fir, and maximum vertical extension in xylem occurs when soil temperatures are within the *in vitro* growth optimum range. Preliminary observations further indicate that previous estimates of maximum growth rate under optimum soil temperature conditions are conservative. In these experiments, we studied the effects of three constant soil temperatures (10, 17 and 28C) on infection and growth of *V. wageneri* in Douglas-fir; the lowest temperature was chosen to reflect soil temperature conditions in the Pacific Northwest. Infection success and maximum fungal growth in xylem were compared at each temperature. *In vivo* growth response to fluctuating soil temperature conditions was demonstrated in greenhouse experiments. Fungal growth measurements from roots of field inoculated trees gave reliable estimates of *in vivo* growth rate under typical field conditions.

MATERIALS AND METHODS

Experiments were conducted with inoculated trees in the growth chambers, greenhouse, and field to evaluate the effects of optimal and suboptimal soil temperatures on the growth rate of *V. wageneri* in Douglas-fir xylem.

Inoculation Procedures. Two-year-old seedlings used in greenhouse and growth chamber studies were wounded with a sterile scalpel at a right angle to the root axis, 3-10 cm below the root collar. A wedge of bark and wood was removed from each seedling. Wound length was approximately equal to the diameter of the root, and wound width never exceeded 25% of the root circumference. A 1 cm square of *V. wageneri* colonized agar (Douglas-fir isolate VW-45 on standard PDA) was placed on the wound and wrapped with a 3 x 10 cm piece of sterile, moistened cheesecloth. The cheesecloth was covered with a 5 x 10 cm piece of 0.50 mil polyethylene plastic and the ends were loosely wrapped with "twist ties". The seam and ends of the inoculum bandage were sealed with melted paraffin. Roots of large Douglas-fir trees in the field were similarly inoculated, but wound, inoculum, and bandage size were increased to accommodate the larger roots.

Growth Chamber Studies. Inoculated seedlings were placed in 10, 17 or 28C constant temperature growth chambers to maintain soil temperatures at their respective levels.

Seedlings were individually transplanted with a cheesecloth wick (Chapter III) to plastic seedling transplant tubes (7 cm diam. x 25 cm

length) in a pasteurized potting medium composed of equal volumes of washed silica sand (EI-20) and peat (Baker, 1957). Potted seedlings were randomly sorted into three blocks of 36 seedlings (108 inoculated seedlings); one block was placed in each of the three growth chambers. Seedlings were wick-watered from a reservoir of 20% Hoaglund's solution. Air temperature was continuously recorded and soil temperatures were periodically monitored in each chamber. Lighting conditions were the same for the three growth chambers; light and dark periods alternated at 12 hr intervals.

Six randomly selected seedlings were destructively sampled from each growth chamber at each sample time. Sampling was done 28, 43, 49, 57, 64 and 72 days after inoculation for the 10C treatment, and 26, 41, 47, 55 and 62 days after inoculation for the 17 and 28C treatments. Seedling root systems were washed and decorticated, and maximum extension of the fungus vertically, radially and circumferentially was measured (Chapter III). Maximum vertical extension was measured with a clear metric ruler; maximum radial and circumferential development were measured using prefabricated templates. Mean growth rates were computed for each sampling.

Greenhouse Studies. Growth rate of V. wageneri in seedling xylem was evaluated in the greenhouse where soil temperature was allowed to fluctuate diurnally and seasonally. The greenhouse study, first conducted in 1981, was repeated in 1982. In 1981, 220 Douglas-fir seedlings were inoculated and transplanted to 2.5 L plastic planting containers in a pasteurized clay-loam forest soil. Soil temperature in pots was measured continuously by soil thermograph. Seedlings were sampled at weekly intervals beginning 2 days and ending 151 days after

inoculation. Ten seedlings were randomly selected at each sampling as well as other seedlings showing advanced stages of wilting; preliminary evidence indicated that fungal growth rate declined rapidly with overall tree decline. Maximum fungal growth was determined as for the growth chamber study. To test the hypothesis that soil temperatures above and below the *in vitro* growth optimum range depressed the growth rate of *V. wageneri* in xylem, daily soil temperatures were partitioned as follows: proportion of the day when soil temperatures were <15C, proportion between 15-18C, proportion >18C. Growth rates for each sample period were compared with the daily soil temperature profile for the same period.

In 1982, 100 seedlings were inoculated, transplanted, and measured as in 1981. Soil temperature was measured continuously by soil thermograph, and seedlings were sampled 24, 38, 48, 54, 65, 75 and 82 days after inoculation. Twelve seedlings were randomly selected at each sampling as well as other seedlings showing advanced wilting symptoms.

Field Study. Roots of 20-year-old Douglas-fir trees were wound inoculated with the same isolate of *V. wageneri* used in the other studies, at a soil depth of 20-40 cm, and 1-2 m slope distance from the bole. A total of 101 roots on 50 trees were inoculated. The test plantation was located in the Oregon Coast Range at an elevation of 440m in the Mary's Peak Watershed along the south fork of Rock Creek, T. 12 S., R. 7W., NE 1/4 SE 1/4 Sec. 22 Willamette Meridian. The stand was fully stocked on a southeastern exposure, and all inoculated roots were shaded by the fully closed canopy. Roots were inoculated in May 1982 and removed in August 1982 and February 1983. Soil

temperature was measured continuously on the site by soil thermograph at a depth of 30 cm. Fungal growth was measured in infected roots at both sample times and periodic growth rates were computed.

RESULTS

Growth Chamber Studies. Cool soil temperatures favored seedling infection success. Percent infection was similar at 10 and 17C (92 and 97%, respectively), but was significantly ($P = 0.05$) reduced at 28C (19% infection) (Table VI.1). Time to infection was shortest at 17C, slightly increased at 10C, and substantially increased at 28C (Figure VI.1, Table VI.1). Vertical growth rate of *V. wageneri* in seedling xylem was 2-3 times more rapid at 17C than at 10C; differences in growth rate were significant ($P = 0.05$) for each sample period (Table VI.1). Circumferential growth rate was 1.5 to 3 times faster at 17C than at 10C, and differences were significant for most sample periods. Radial growth rates at 17C were $\geq$ those observed at 10C, in two cases rate differences were significant. Growth rate at 28C was intermediate to that observed at 10 and 17C, lack of infection made it impossible to estimate growth rate in later samples. Peak growth rates were observed at 64 days for the 10C treatment, 47-55 days at 17C, and 41 days at 28C (Figure VI.2). All infected seedlings in the 28C treatment were sampled 47 days after inoculation.

Greenhouse Studies. In 1981, soil temperatures in the greenhouse were frequently above 18C. The coolest soil temperatures occurred in the first month after inoculation; percent infection increased steadily over this period (Figures VI.1 and VI.3). In the second

month, soil temperatures increased drastically (Figure VI.3) and percent infection increased at a reduced rate. Vertical growth rate of V. wageneri peaked 51 days after inoculation at approximately 2.5 mm • day^{-1} and declined steadily with higher soil temperatures. Vertical and circumferential growth rates responded predictably to fluctuations in soil temperatures. Increases in the proportion of days where soil temperatures were between 15 and 18C produced increases in growth rate, increases in the proportion of days >18C or <15C depressed growth rate (Figure VI.3). Radial growth was slow and fluctuations in growth rate were imperceptible with changes in soil temperature.

In 1982, greenhouse soil temperatures were generally <18C. Percent infection of seedlings and time to infection establishment were similar to that observed in the greenhouse in 1981 (Figure VI.1). Vertical growth rate of V. wageneri peaked 65 days after inoculation at approximately 1.9 mm • day^{-1} (Figure VI.4). The delay in days to peak growth rate appeared to be related to the drastic cooling of soil temperatures between days 42 and 54, producing a 10-13 day delay (Figure VI.4). Vertical growth rate responded to temperature fluctuations above and below the growth optimum range, circumferential and radial growth rates were slow and unresponsive by comparison.

Field Study. Soil temperatures were cool from May 1982 to May 1983 at the Rock Creek field plot (Figure VI.5). Except for 20 days in late August and early September, soil temperatures were continously below 15C; during 7.5 months temperatures were less than 10C. The highest soil temperature (16.5C) was recorded in late August, the

lowest (2.5C) was recorded in early December. Of the 101 roots that were inoculated, 25% (25 roots) were infected at the time of sampling. Resinosus and resin-soaked xylem were apparent around inoculation wounds of all 76 unsuccessfully inoculated roots; in one root, infection had occurred and 46 mm of vertical xylem were colonized after 270 days. Host resin response had apparently curtailed further spread of the fungus early in infection and it could not be reisolated. V. wageneri was reisolated and identified from each of the other 25 infected roots.

Low infection success allowed only two sampling times: once in August during the period of highest soil temperatures and once in February during the low soil temperature period. Of the roots sampled, 95 days after inoculation, 17 percent were infected and average vertical growth rate was nearly 6 mm $\cdot$ day^{-1} (Table VI.2). In roots sampled 270 days from inoculation, 26% were infected and average vertical growth rate was 4.3 mm $\cdot$ day^{-1}; the difference was not statistically significant. The three-fold decrease in radial growth between the two samples was significant ($P = 0.05$) (Table VI.2). Circumferential growth rate likewise decreased but differences were not significant. An average of eight annual rings were colonized at either sample time (Table VI.2).

DISCUSSION

In the greenhouse and growth chambers, soil temperatures $\leq$18C increased infection and establishment of *V. wageneri* in seedling xylem; warm temperatures >18C decreased the likelihood of infection and establishment. Results from the growth chamber experiments indicated that the *in vivo* growth optimum temperature for this Douglas-fir isolate of *V. wageneri* was the same as that which has been reported for *in vitro* growth.

Greenhouse soil temperatures in 1981 were most often above the growth optimum range. Soil temperatures in the 1982 study were mostly within or below the optimum range. Vertical and circumferential growth rates in 1981 were greater than those observed in 1982. Temperature effects on radial growth rate were imperceptible in either study. In comparing results from greenhouse and growth chamber studies, growth rates observed in the greenhouse in 1981 were comparable with those of the 28C treatment, growth rates in the cooler 1982 study were comparable with those of the 10C treatment. It appears that temperatures $\leq$18C favor infection and establishment, and temperatures $\geq$ 15C favor faster growth in xylem.

Peak vertical growth rate of *V. wageneri* in seedling xylem at a constant 17C soil temperature averaged 4.6 mm $\cdot$ day^{-1} or 1.7 m $\cdot$ yr^{-1}, with a maximum of 7.2 mm $\cdot$ day^{-1} or 2.6 m $\cdot$ yr^{-1}. In the field, peak vertical growth rate in roots of 20-year-old trees averaged 5.9 mm $\cdot$ day^{-1} or 2.2 m $\cdot$ yr^{-1}, with a maximum of 1 cm $\cdot$ day^{-1} or 3.6 m $\cdot$ yr^{-1}. Since soil temperatures at the field plot were below the growth optimum range for all but 20 days of the study year, growth rates in

the field under optimum conditions might be greater than any that we observed.

Soil moisture also appears to be very important in the dynamics of infection and colonization (Goheen, et al, 1978; Wilks, et al, 1983), but it is difficult to separate the effects of soil moisture and soil temperature since the two are inversely related in nature. Summer rains are infrequent during periods of near optimum soil temperature in the Pacific Northwest. In winter, soil moisture conditions may approach field capacity yet soil temperatures are cool. Such conditions may be optimum for root infection by V. wageneri and suboptimum for growth. Concurrence of optimum conditions of both edaphic factors may be infrequent throughout the northern distribution of this disease.

In field inoculated roots, an average of eight annual rings (beginning with the current year's xylem) were colonized after 95 days; after 270 days, there was no significant increase in the number of annual rings colonized. While radial growth rate of V. wageneri in xylem is considerably slower than vertical growth rate, radial colonization of successive annual rings occurred early in root infections. It would therefore be of marginal value to measure the number of successive annual rings colonized in roots or stems of infected trees in the field to estimate the number of years of infection.

Hansen and Goheen (Hansen et al, 1983; Hansen and Goheen, unpublished data) monitored disease increase in natural V. wageneri infection centers in 28 Douglas-fir plantations in Oregon and Washington over a 5-year period and found that infection centers enlarged radially at an average rate of 1.5 m $\cdot$ yr^{-1}. Cobb and others

(Cobb et al, 1982) determined the rate of spread of V. wageneri in ponderosa pine in the Central Sierra Nevada from aerial photographs taken at 2-year intervals during an 11-15 year period. Data from 52 infection centers showed that the average rate of radial spread was 1.0 m • yr^{-1}, but radial spread rates varied from 0 to 7 m • yr^{-1}. Results of these two field studies are comparable with the results obtained in our growth chamber, greenhouse and field studies. In seedlings, the growth rate of V. wageneri in roots ranged from 0.5 - 1.7 m • yr^{-1}. In 20-year-old Douglas-fir trees, growth rates ranged from 0.5 - 2.2 m • yr^{-1}. While insects are undoubtedly involved in some intertree spread of the disease, the observed rates of infection center enlargement can be explained in terms of fungal growth rates in roots.

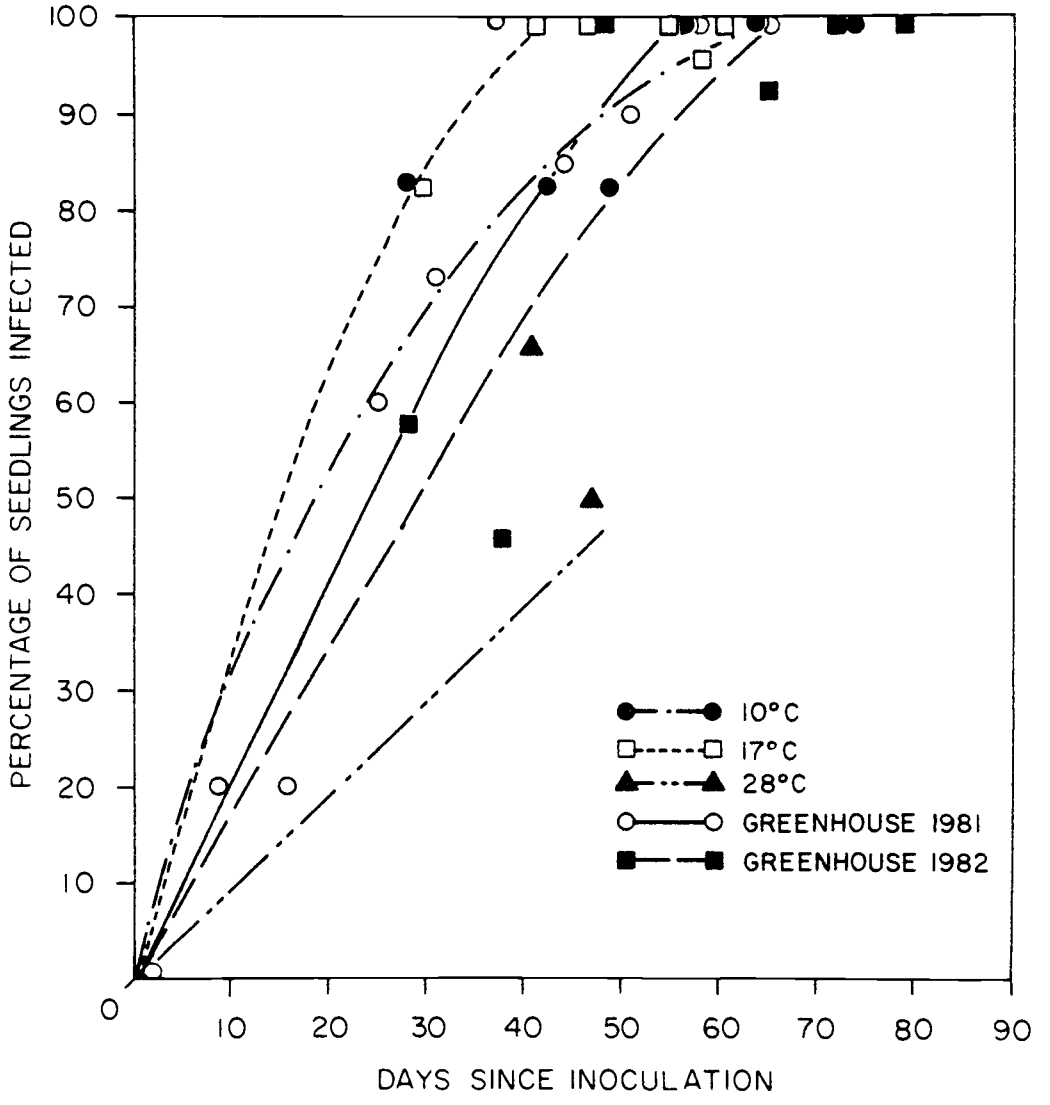

Figure VI.1. Percentage infection and time to infection for V. wageneri inoculated seedlings grown in five different soil temperature environments. Percentages computed from periodic samplings of six or more seedlings.

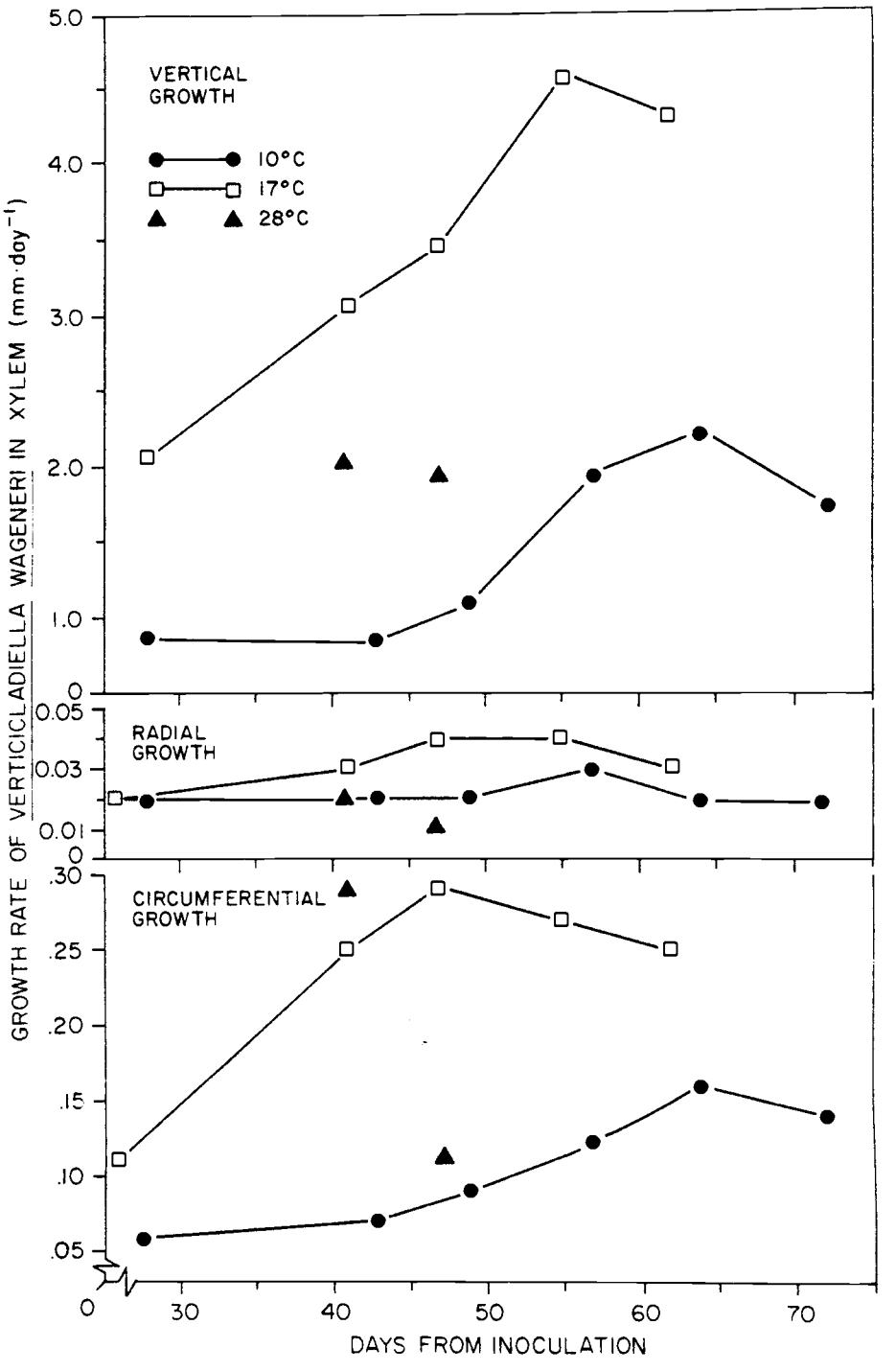

Figure VI.2. Effect of three constant soil temperatures on vertical, radial, and circumferential growth of V. wageneri in seedling Douglas-fir xylem. Data points are periodic sample means.

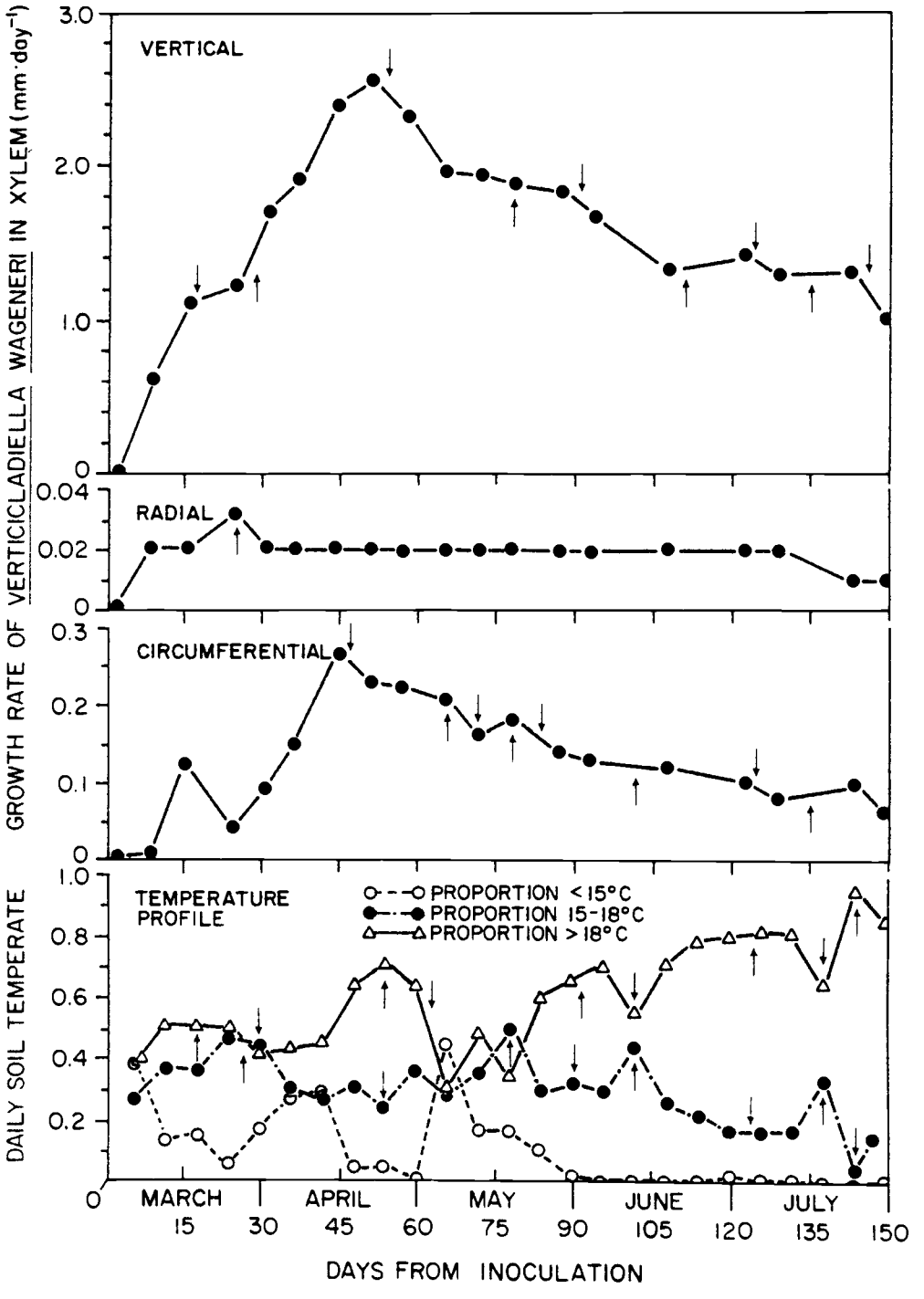

Figure VI.3. Relationship of growth rate of V. wageneri in seedling Douglas-fir xylem to soil temperature fluctuations in the greenhouse (1981). Soil temperature data points are six day periodic means.

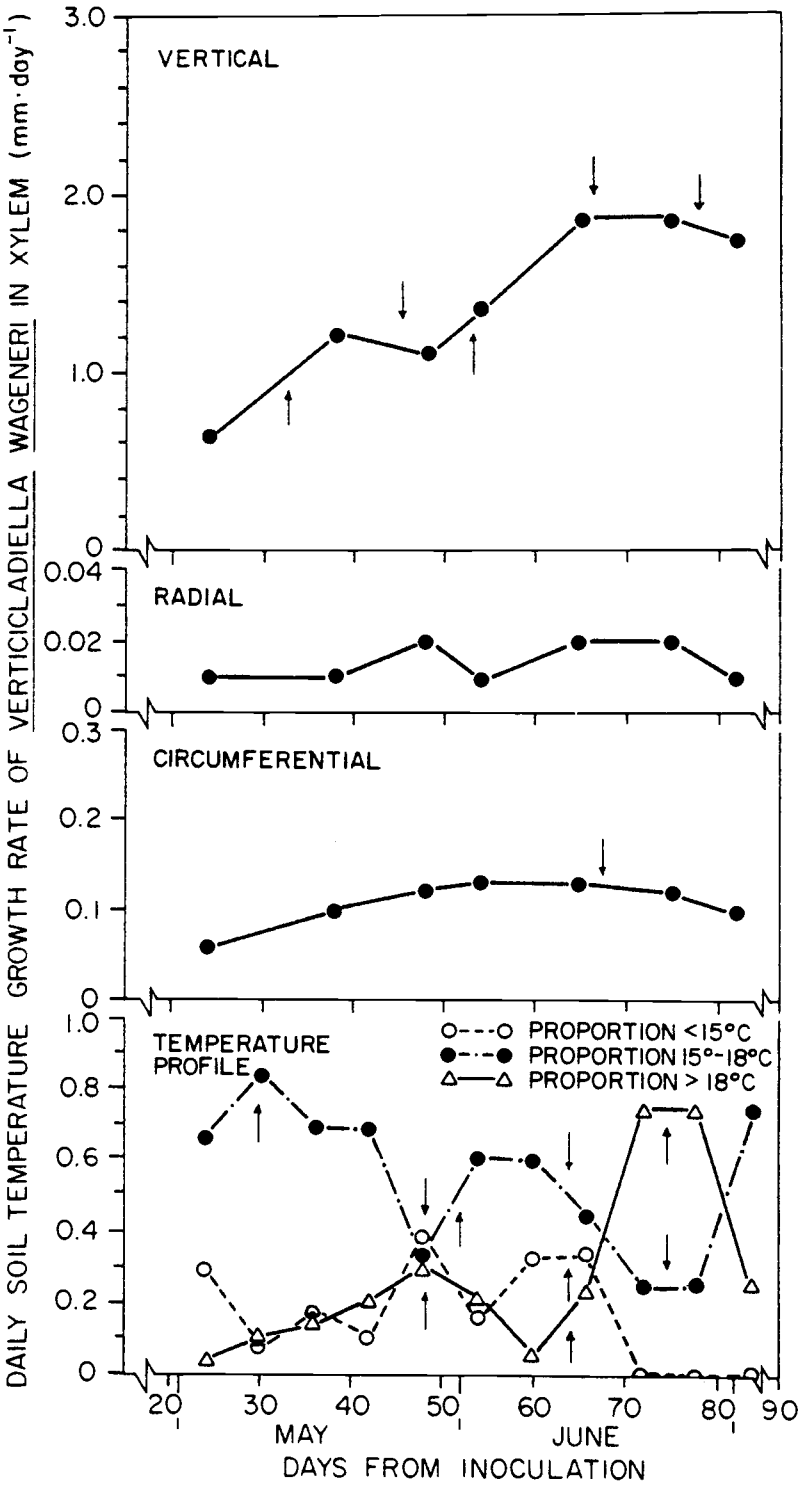

Figure VI.4. Relationship of growth rate of V. wageneri in seedling Douglas-fir xylem to soil temperature fluctuations in the greenhouse (1982). Soil temperature data points are six day periodic means.

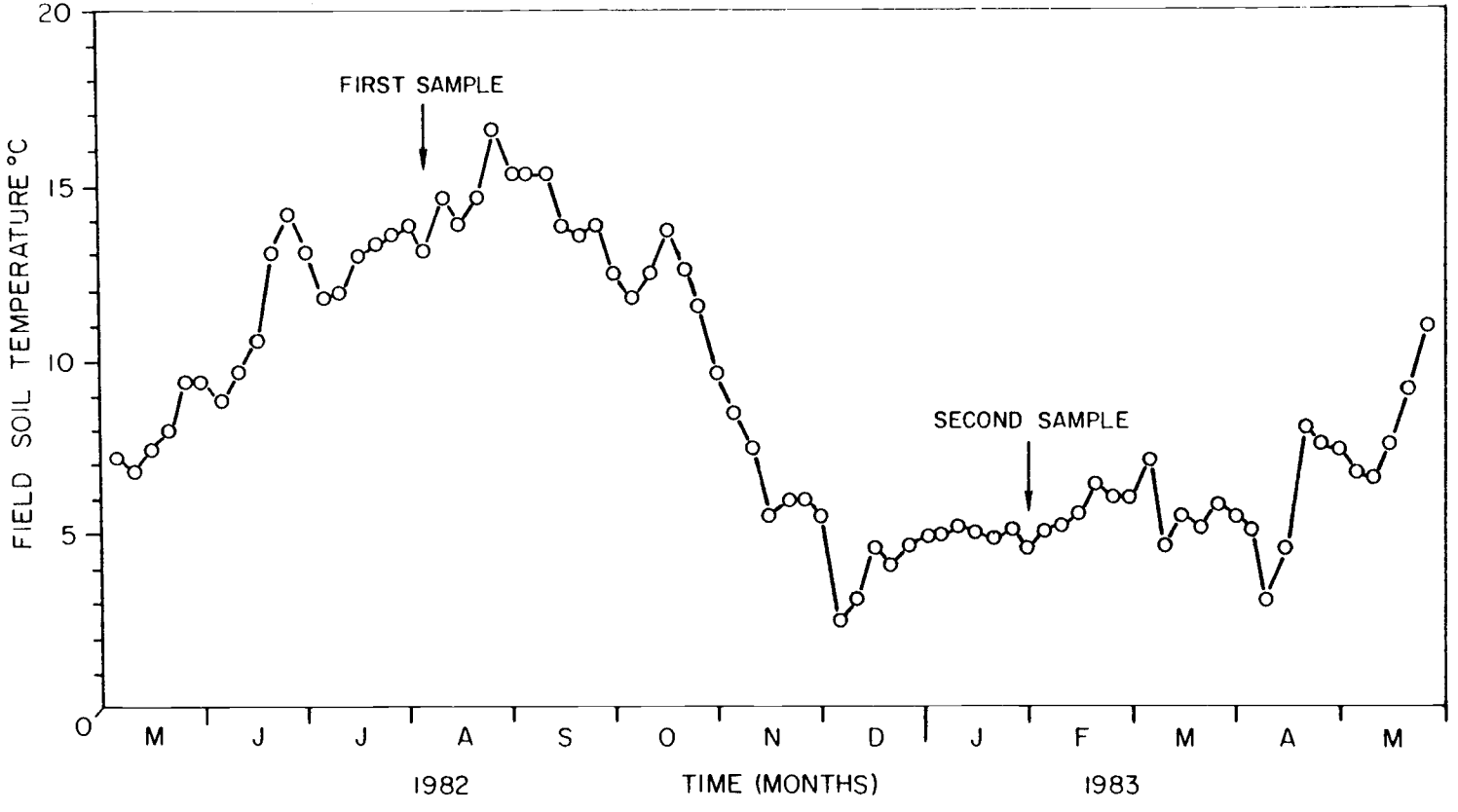

Figure VI.5. Soil temperature record for the Rock Creek field plot from May 1982 to May 1983. Soil temperature data points are five day periodic means.

Table VI.1. Effect of three soil temperatures on percent infection and growth rate of *Verticicladiella wageneri* in inoculated Douglas-fir seedlings.

Treatment	Days From Inoculation	Seedlings Sampled	Percent Infected	Mean Fungal Growth Rate ($mm \cdot day^{-1}$)		
				Vertical	Radial	Circumferential
(1) 10°C	28	6	83	0.86a[z]	0.02a	0.06a
(2) 17°C		6	83	2.05b	0.02a	0.10a
(3) 28°C		6	0	-	-	-
(1)	41-43	6	83	0.84a	0.02a	0.07a
(2)		9	100	3.06b	0.03a	0.25b
(3)		6	67	2.03b	0.02a	0.29b
(1)	47-49	6	83	1.1a	0.02a	0.09a
(2)		7	100	3.46b	0.04b	0.29b
(3)		6	50	1.93a	0.01a	0.11a
(1)	55-57	6	100	1.93a	0.03a	0.12a
(2)		9	100	4.56b	0.04b	0.27b
(3)		6	0	-	-	-
(1)	62-64	6	100	2.22a	0.02a	0.16a
(2)		5	100	4.3b	0.03a	0.25b
(3)		6	0	-	-	-
(1)	70-72	6	100	1.7	0.02	0.14
(2)		-	-	-	-	-
(3)		6	0	-	-	-
MEANS ($\bar{X}$)				PEAK GROWTH RATE ($MM \cdot DAY^{-1}$)		
10C		36	92a[y]	2.22[u]	0.02[u]	0.16[u]
17C		36	97a	4.56[v]	0.04[w]	0.29[w]
28C		36	19b	2.03[x]	0.02[x]	0.29[x]

[u,v,w,x]peak growth rate observed 64, 55, 47, and 41 days, respectively, after inoculation.

[y]mean percentages followed by the same letter are not significantly different (P=0.05), analysis by T-test.

[z]mean growth rates followed by the same letter are not significantly different (P=0.05), analysis by T-test.

Table VI.2. Growth of V. wageneri in roots of artificially inoculated 20-year-old Douglas-fir trees.

Root Sample	Days From Inoculation[W]	Number of Roots Sampled	Percent Infected	Growth Rate of V. wageneri in Xylem[X]												Annual Rings Colonized	
				Vertical				Radial				Circumferential					
				mm/day		m/yr		mm/day		cm/yr		mm/day		cm/yr			
				(Mean)	Max.	(Mean)	Max.	(Mean)	Max.	(Mean)	Max.	(Mean)	Max.	(Mean)	Max.	(Mean)	Max.
August 1982	95	23	17	5.9a[Y]	6.6	2.2	2.4	0.06a	0.09	2.2	3.3	0.15a	0.26	5.5	9.5	8.3a	10
February 1983	270	78	26	4.3a	10.0	1.6	3.7	0.02b	0.05	0.7	1.8	0.10a	0.36	3.7	13.1	7.5a	15

[W]Roots inoculated May 1982.

[X]Maximum extension in xylem measured in one direction from the point of inoculation.

[Y]Values in columns followed by the same letter not significantly different at P=0.05. Analysis by T-test.

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SUMMARY

Verticicladiella wageneri Kendr. is a xylem-limited vascular wilt pathogen and the causal agent in a fatal root disease of Douglas-fir. Long vertical columns of black-stained sapwood and stunted, chlorotic foliage are diagnostic evidence of the disease. In Douglas-fir stands, tree mortality typically occurs in pockets which appear as areas of standing dead trees surrounded by infected trees in various stages of decline. From observations of infection centers it is clear that there are two distinct patterns of disease distribution: one by which new infection centers are initiated, involving long distance spread of the pathogen by insects; a second whereby the pathogen is locally transmitted to healthy trees at the margins of expanding infection centers, where insect involvement is probably of secondary importance. This dissertation explores the epidemiology of local intertree transmission and disease development in Douglas-fir. In the first section of the thesis (ChapterII), I studied the pathological anatomy of the disease in Douglas-fir to characterize pathogen behavior in the host and host response to infection. *V. wageneri* mycelia only colonized mature xylem tracheids and earlywood was preferentially colonized before latewood. Intertracheal movement of hyphae occurred exclusively through bordered pit-pairs with limited radial movement in ray tracheids. Bordered pits were completely occluded when penetrated by hyphae. Living cells were never directly penetrated by hyphae and hyphal passage through simple pit membranes of half-bordered pits was never observed. Circumferential colonization occurred by hyphal penetration of bordered pits on radial

walls of axial tracheids but was impeded by uniseriate and fusiform rays that were uniformly distributed throughout the xylem. Observations of the pathogen in newly produced xylem tracheids consistently revealed that the vascular cambium and adjacent living xylem mother cells provided a barrier to fungal egress through the secondary phloem. Lack of evidence for fungal invasion of living bark, cambial and xylem tissues, and the absence of direct cell wall penetration, allowed me to hypothesize that wounds or openings in the bark that exposed the xylem were requisite for fungal infection of roots and egress from roots. Furthermore, histological evidence of primary involvement of a translocateable phytotoxin was lacking. I hypothesized that foliage wilting in Douglas-fir may result from occlusion of the most important xylem conductive tissue by hyphae and host-produced gums.

Since the pathological anatomy and physiology of black-stain root disease were apparently identical in seedling and mature Douglas-fir trees, I chose to use seedlings as a model system in subsequent experiments. This allowed complete host dissection and larger sample sizes for statistical analysis of experimental results.

In the second section of the thesis (Chapter III) I tested the vascular occlusion hypothesis experimentally. Xylem pressure potential and transpiration water uptake were periodically measured on V. wageneri inoculated and control seedling groups to indicate the earliest significant consequence of vascular tissue colonization. Circumferential colonization of inoculated seedling roots consistently exceeded 80% when significant differences in pressure potential and water uptake, relative to control seedlings, were first apparent. In

the pressure potential experiment, average xylem pressure for inoculated seedlings was significantly reduced after 34 days (-12 bars vs. -5 bars) and an average of 80% of the circumference of root systems was colonized by hyphae or occluded by gums. Radial colonization of root systems, measured from area cross-sections, averaged 39%. In the water uptake experiment, average water uptake for inoculated seedlings had decreased significantly 30 days after inoculation (3.6 vs. 7.9 ml $\cdot$ day^{-1}), and an average of 85% of the circumference of root systems was colonized by hyphae or occluded by gums; radial colonization of root systems averaged 61%. These results indicated that a high level of circumferential colonization in the outer annual rings was responsile for the early expression of vascular dysfunction. The earliest measureable change in seedling water status was related to significantly reduced water uptake and translocation, reflected by a reduced xylem pressure potential. I proposed that the occlusion of vital conductive tissue was the principal cause of foliage wilting and diffuseable toxins, if present, operated secondarily.

In the third section of the thesis (Chapter IV) I tested the wound infection court hypothesis. Root systems of 2-year-old Douglas-fir seedlings were dip-inoculated to the root collar in a mycelium-spore suspension of V. wageneri. Infection frequency was scored for dormant inoculated seedlings and seedlings inoculated 4 and 8 weeks from dormancy. Root system dissections revealed that seedling roots were consistently infected through small wounds and natural openings to exposed xylem. Direct penetration of bark and cambial tissues by hyphae was never observed. Most infections of dormant inoculated

seedlings (63%) occurred through wounds incurred in lifting and handling at the nursery; wound infection frequency decreased to zero in 8-week inoculated trees. Four and 8-week inoculated trees were most frequently infected at sites of new lateral root initiation or through dead fine root stubs. Infection of seedling roots exclusively through openings to exposed xylem demonstrated that such openings were necessary for infection by V. wageneri. The demonstrated infection susceptibility of roots initiating new lateral roots or bearing dead fine roots suggested that these sites may be seasonally important for new fungal infection of healthy trees, and fungal egress from already diseased trees.

In section four of the thesis (Chapter V) I continued to develop this line of reasoning and looked for evidence of intertree transmission of V. wageneri without insect mediation. Root graft transmission of V. wageneri was verified from field excavations in natural infection centers in Douglas-fir stands. In functioning root grafts, hyphae followed the path of translocation through the graft in the outer xylem. Root grafts can provide an efficient means of intertree spread of the disease; the importance of graft transmission in infection centers will depend on the frequency of grafting and the frequency of intertree spread by other means. In young, densely stocked stands, grafting frequency may be high; the incidence of black-stain root disease is also often high in such stands.

V. wageneri is readily isolated from soil adjacent to infected roots; intertree spread by mycelial growth through soil has been proposed. To test this hypothesis, I conducted potted seedling experiments where contact between roots of inoculated and healthy

seedlings was either allowed or completely restricted. Transmission of the disease occurred in both treatments. In the root contact treatment, 100% of the inoculated seedlings contracted the disease and 67% of the healthy seedlings died as a result of successful transmission. In the no-contact treatment, all inoculated seedlings were again infected but 35% of the healthy seedlings became infected. Intertree root contact between diseased and healthy trees increased the probability of successful tranmission, but it was not required. These results clearly demonstrated that V. wageneri can exit from roots, grow for a limited distance outside the host, find a suitable infection court and successfully establish itself in a new host plant.

In the final section of the thesis (Chapter VI) I conducted experiments in the growth chambers, greenhouse and field to determine the growth rate of V. wageneri in Douglas-fir xylem as influenced by soil temperature. The current literature indicates an in vitro growth optimum temperature range of 15-18C. In growth chamber experiments, 92 and 97% of inoculated seedlings were infected in the 10 and 17C soil temperature treatments respectively; but vertical growth rate of V. wageneri in seedling xylem was 2-3 times faster at 17C than at 10C. In the 28C treatment, only 19% of the inoculated seedlings were infected, yet vertical growth rate in xylem was intermediate to that observed in the 10 and 17C treatments. In greenhouse tests, soil temperatures were allowed to fluctuate diurnally and seasonally; growth rates responded predictably to fluctuations in soil temperatures. Increases in the proportion of days where soil temperatures were between 15 and 18C produced increases in fungal growth rate; conversely, increases in the proportion of days > 18C or < 15C

depressed growth rate. In an extended field test, the growth rate of V. wageneri in roots of 20-year-old trees averaged 2.2 m $\cdot$ yr^{-1}, with a maximum of 3.6 m $\cdot$ yr^{-1}, and soil temperatures rarely exceeded 15C. Current estimates of the annual rate of enlargement of infection centers range from 1 to 2.5 m $\cdot$ yr^{-1}. While insects may be involved in some intertree spread of the disease, the observed rates of infection center enlargement can be explained by fungal growth rates in roots. From these results I speculate that the seasonal optimum conditions for infection and growth are as follows: optimum conditions for infection may occur both in the fall and spring. In the spring, soil temperatures are cool but rising, and soil moisture levels are still high. In the fall soil temperatures are cooling and fall rains begin to elevate soil moisture. Both periods may be conducive to infection. Optimum conditions for in vivo growth invariably occur during the summer months, yet the fungus is apparently capable of sustaining growth at nearly any temperature.

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