PROCERUM ROOT DISEASE PHYSIOLOGY AND DISEASE INTERACTIONS WITH OZONE

by

Jodi Ann Carlson

Dissertation submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Plant Pathology, Physiology and Weed Science

APPROVED:

Samuel A. Alexander
S. A. Alexander, Chairman

Boris Chevone
B. I. Chevone, Co-chairman

Laurence D. Moore
L. D. Moore

G. J. Griffin
G. J. Griffin

John R Seiler
J. R. Seiler

February 1994
Blacksburg, Virginia

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(ABSTRACT)

Procerum root disease of eastern white pine (*Pinus strobus* L.), caused by *Leptographium procerum* (Kendr.) Wingf., has been epidemic in Virginia Christmas tree plantations since 1990. Symptoms of chlorosis, wilt, and decreased apical growth resemble those of water stress. Resin infiltration of the xylem at the stem base may be responsible for vascular occlusion leading to severe water deficits and mortality. The pathogen has been isolated from the roots of ozone-sensitive eastern white pines in the field, although not from nearby tolerant trees, and it may be that ozone sensitivity predisposes the trees to infection. The objectives of my studies were to investigate the physiology of diseased white pines, and to determine the effects of ozone fumigation on disease development. Impacts of vascular occlusion upon host water relations and gas exchange were investigated in 8-yr-old, plantation-grown, white pine Christmas trees. Disease severity was estimated as the proportion of resin-soaked cross-sectional area at the base of the stem. The linear response of a suite of six physiological variables to disease severity was highly significant. Individually, the variables pre-dawn water potential, daily change in pre-dawn to mid-day water potential, stomatal conductance, and photosynthetic and transpiration rates all decreased significantly with increasing disease severity. Fumigation studies were conducted on white and loblolly (*P. taeda* L.) pine seedlings to determine if ozone exposure increased the incidence of

root disease or the amount of stem tissue colonized by *L. procerum*. Roots were inoculated by soil drenching with conidial suspension, and stems were wounded at the base and inoculated with mycelium. Beginning 24 h post-inoculation, and for 14 consecutive days, seedlings were fumigated in closed chambers with charcoal-filtered air or 200 ppb ozone for 5 h/day, then removed to a charcoal-filtered greenhouse. Six weeks post-inoculation, root and stem tissue were plated on a medium selective for *L. procerum*. Ozone treatment did not significantly affect the proportion of diseased roots per seedling or the vertical colonization of stem tissue in seedlings of either species.

ACKNOWLEDGEMENTS

I thank my advisor, Dr. S. A. Alexander, for giving me the opportunity to come to Virginia to work on a challenging plant disease, and for his financial support through the years of my studies. I am particularly grateful to my co-advisor, Dr. Boris Chevone, for countless hours of stimulating discussion and for his critical approach to science.

To the members of my advisory committee; Drs. L. D. Moore and G. J. Griffin of the Department of Plant Pathology, Physiology and Weed Science, and Dr. J. R. Seiler of the Department of Forestry, I give my thanks. Through discussion of my research, and formal coursework, they have provided me a strong foundation for future endeavors.

I extend a special thank you to Ms. Moss Baldwin for her technical assistance, many hours of which occurred before sunrise and were above and beyond the call of duty, and for her friendship and moral support.

Dr. David Gray; my most constructive critic, occasional technical support person, and partner, has contributed greatly to my accomplishment of this work. In addition to his sharing valuable insight and advice regarding my research, he has provided welcome relief from it.

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Chapter 1

Introduction and Literature Review

PROCERUM ROOT DISEASE

The Pathogen

Leptographium procerum (Kendr.) Wingf. is an imperfect fungus (Hyphomycetes, Dematiaceae) that was originally described by Kendrick (1962) as *Verticicladiella procera.* Wingfield (1985) subsequently reclassified it to the genus *Leptographium* Lagerb. & Melin based upon its annelidic conidiogenesis, in contrast to the sympodial type found in *Verticicladiella* Hughes species. *Leptographium* now includes species with both types of conidiogenesis and has replaced the genus *Verticicladiella* (Zambino and Harrington 1992). While the teleomorph has yet to be found, Horner and Alexander (1986) predicted that it would be in the genus *Ophiostoma* H.& P. Sydow based on their finding of cellulose in the cell wall. In addition, *L. procerum* shares other definitive characteristics with anamorphs of *Ophiostoma* spp., including cycloheximide tolerance (Harrington 1981) and a typical "bluestain" colonization pattern in the xylem of affected trees which leads to wedge-shaped blue or black areas (Horner and Alexander 1986). *L. procerum* is cosmopolitan in distribution (Alexander *et al.* 1988) and is often isolated from host tissue together with related species. In the Pacific Northwest, several *Leptographium* spp. including *L. procerum* have been isolated, two or three together, from lodgepole pine (*P. contorta* Dougl.) exhibiting staining root disease (Bertagnole *et al.* 1983). *L. procerum*, *L. serpens* and *L. wageneri* were isolated together from a diseased Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Bertagnole 1981). The authors stated that these fungi rarely occur alone, and that they may work interactively, or perhaps successionally, within

the host. Studies have shown that bark beetles frequently carry more than one fungus, however, it is thought that they may work antagonistically once inoculated into the host (Owen *et al 1987, Parmeter et al.* 1989). Extensive isolations from procerum root disease (PRD) symptomatic eastern white pine Christmas trees in Virginia revealed that *L. procerum* was isolated together with various *Ceratocystis* and *Ophiostoma* spp. in 2.0% of sampled trees in 1988, 12.0% in 1989 and 9.0% in 1990 (Nevill 1990). In the case of PRD, *L. procerum* occurring alone appears to be the common condition.

Host Range

In the United States, *L. procerum* has been most frequently isolated from pines, including ten native species (Alexander *et al.* 1988). In addition, Douglas-fir (Harrington and Cobb 1983) and Fraser fir (*Abies fraseri* (Pursh) Poir.) (Alexander *et al.* 1988) are recorded hosts. While landscape and Christmas tree plantings of eastern white pine have yielded the majority of infected trees, the pathogen has been isolated from 60 to 80-yr-old loblolly pines (*P. taeda* L.) in the piedmont region of North Carolina, South Carolina and Georgia (Alexander *et al.* 1992). Lackner and Alexander (1983) isolated it from roots in a 40-yr-old natural stand of eastern white pine in the Blue Ridge Mountains of Virginia. It has been found associated with wounded roots in subsoiled loblolly pine seed orchards in the southern U.S. (Webb and Alexander 1982). Given the geographic range of the pathogen, and an apparent lack of host specificity, it is likely present in many forest systems.

PRD Symptoms

A characteristic set of disease symptoms has not been described for most species of trees from which *L. procerum* has been isolated. However, the fungus has recently been associated with a decline disease of red pine (*P. resinosa* Aiton), first reported in Wisconsin in 1975 in 20 to 40-yr-old plantation-grown trees (Klepzig *et al.* 1991). PRD has been described on eastern white and Scots (*P. sylvestris* L.) pines. The first unambiguous record is from 1967 (Dochinger), where *L. procerum* was associated with eastern white pine exhibiting the symptoms of 'white pine root decline' (=PRD). Similarly, the fungus was identified as the agent causing this disease in Christmas tree plantings in Virginia in 1980 (Alexander 1980).

Koch's Rules for proof of pathogenicity have been fulfilled for *L. procerum* and PRD of eastern white pine. Chlorosis and wilt of foliage, reduced root growth, black staining of xylem, and mortality were observed beginning 2 to 4 weeks following inoculation of seedlings (Lackner and Alexander 1982, Smith 1991). Many other workers, however, have not been able to obtain PRD upon inoculation of *L. procerum* into eastern white pine using similar, as well as different, techniques (Swai 1980, Wingfield 1986, Horner 1985, Nevill and Alexander 1992a, Carlson unpublished). In all cases, the pathogen had successfully infected and colonized the host, and could be re-isolated from it. Symptoms of PRD did not occur, even several months following inoculation, and mortality was very rare. Horner (1985) cited variability in virulence of isolates as a cause for discrepancies between studies. This is not supported by the few data available for *L. procerum* and eastern white pine. In studies with loblolly pine seedlings, significantly less mortality was found 7 wk following inoculation with a Scots pine isolate than with two eastern white pine isolates (Lackner and Alexander 1983). However, within

this study, there were no differences between isolates when inoculated into eastern white pine. Similarly, no differences were detected in eastern white pine inoculated with eight *L. procerum* isolates of diverse geographic and host origin (Wingfield 1986). Although there was no PRD development or mortality 5 months following inoculation, the length and width of wound-associated lesions were not significantly different between any of the isolates tested. The author concluded that variability in virulence among isolates has not been responsible for conflicting results in reported pathogenicity studies. The intervening years have not yielded a resolution regarding the presence or absence of procerum root disease following *L. procerum* colonization of eastern white pine.

PRD is a disease of the root collar and adjacent areas of the roots and lower stem. It should be noted that PRD is not a root rot, and there is no decay associated with the colonization of wood by *L. procerum.* Rather, the disease symptoms, and the behavior of the pathogen in the tree, make it more similar to diseases caused by vascular wilt fungi (Beckman 1987, Horner 1985). Horner (1985) most frequently isolated the pathogen from the root collar area, with isolation success decreasing as samples were taken more distally in either direction. These findings led him to suggest that *L. procerum* was introduced into the tree at the root collar, rather than via the roots. The colonization pattern of *L. procerum* in host vascular tissue is similar to that of bluestain fungi. The fungus proliferates in ray parenchyma and tracheids, and to a lesser extent in axial tracheids (Horner 1985). Horner showed that tangential travel is commonly via bordered pit pairs, occasionally through narrow diameter bore holes, and is rather limited. Radial colonization is therefore much greater than tangential, resulting in the characteristic wedge-shaped stains associated with these fungi. Bertagnole *et al.* (1983) noted that under certain conditions, *L. procerum* could assume a yeast-like

form in lodgepole pine, thus facilitating its movement within the host. It is not known if this occurs in eastern white pine.

The basic symptoms of PRD in eastern white pine have been described (Alexander *et al.* 1988). I have observed that the timing, sequence and presence of some or all PRD symptoms are highly variable in young eastern white pine. Often the first indication of disease will be an overall change in foliar color. A diseased tree may be only slightly less green than its healthy neighbors for a year or longer, or the color may deteriorate to a marked chlorosis, then yellow and finally red within a few weeks to months. An overall wilting of foliage may occur before, or after, a color change is observed. During the spring, it is often possible to detect diseased trees by the occurrence of delayed bud break relative to their neighbors. In addition, subsequent shoot and needle elongation are frequently reduced. Examination of the base of the stem may reveal resin being forced from the bark in the absence of any wounding. Observation of the circumference at the base of the tree may show a flat side due to death of the cambium in a particular area. Beneath the bark, varying degrees of brown cambial discoloration and resin-soaking are visible in the roots, root collar and lower stem. This may infrequently be accompanied by black streaking. A transverse section at the base of the stem shows wedge-shaped areas of resin-soaking extending from the outer xylem to the pith. Penetrating black stain is rarely observed in diseased trees of the age (6 to 10 yr) found in Christmas tree plantations. Histological studies of the tissues of naturally occurring PRD symptomatic eastern white pine tissues showed the presence of dark-pigmented hyphae in colonized wood (Horner 1985). In contrast, Bertagnole *et al.* (1983) primarily observed hyaline hyphae in the xylem of 10 to 40-yr-old lodgepole pine 14 months post-inoculation. The reasons for these differences are presently unknown.

Water Relations and PRD

Trees infected with bluestain and related fungi exhibit altered water relations. Horner (1985) found evidence of reduced water potential in *L. procerum* inoculated white pine seedlings, as well as reduced moisture content in sapwood throughout the stems of diseased trees. *L. wageneri* infection of ponderosa (*P. ponderosa* Dougl. ex Laws.) pine resulted in foliar water stress within one month of inoculation (Helms *et al.* 1971). External symptoms of disease, including wilt, caused by these fungi are frequently identical to those achieved by withholding water (Basham 1970, Paine 1982). As with vascular wilt diseases, the means by which water uptake is critically impaired are not understood (Beckman 1987, Parmeter *et al.* 1992).

The physical presence of the fungus in tracheids appears to have little or no effect on water conduction. Histological studies of colonized ponderosa pine sapwood indicated that direct blockage of the xylem by hyphae of *Ceratocystis ips* or *C. minor* was unlikely (Mathre 1964). Studies of *L. wageneri* in Douglas-fir showed that much of the black-stained, non-conducting xylem did not contain fungal hyphae (Hessburg 1984), although when present, hyphae were thought to be responsible for 10 to 80% of the observed occlusion. In the case of *L. procerum*, the proportion of internal tracheal volume occupied by mycelium was found to be too small to impede water conduction (Horner 1985).

Restricted water flow in colonized hosts may be due to plugging of tracheid pits by compounds formed by the host and/or the pathogen. Substances such as decomposition products of fungus and ray cells (Nelson 1934), emboli caused by introduction of air to the vascular system (Mathre 1964), and gums (Hessburg 1984), have been postulated to be the occluding materials. Considerable research has been conducted in the area of resin responses in conifers, particularly pines,

attacked by bark beetles and their associated bluestain fungi. Pines have well developed, independent resin systems in their needles, stems and roots, and breakage of a duct due to insect activity or other wounding results in a substantial flow of resin out of the wound (Shrimpton 1978). There appear to be two major mechanisms with which a tree defends itself when attacked by beetles carrying fungi (Lieutier and Berryman 1988). The first is constitutive, a passive flow of resin preformed by epithelial cells lining resin ducts. It is followed by the production of secondary resins from parenchyma cells, a response induced by the presence of the fungi. The secondary response is thought to be the major defense against invasion, and results in a resinous barrier being formed in advance of the fungal mycelium (Basham 1970, Paine *et al.* 1987). In studies of lodgepole pine and *Europhium* (=*Ophiostoma*) *clavigerum*, Raffa and Berryman (1982) stated that defense responses were localized, with neither lesion formation nor resinosis occurring away from the inoculation site in the phloem. Examination of inoculation sites of *C. minor* on loblolly pine showed that lesions were confined to the phloem and only rarely extended into the sapwood (Paine *et al.* 1987). Resin globules were noted in tracheids of shortleaf (*P. echinata* Mill.) pine colonized by the beetle-vectored *Ceratostomella pini* (=*Ophiostoma minus*) (Bramble and Holst 1940). The authors postulated that this resin may have impaired water transport by blocking the pit passages. Other workers have not considered the presence of resin to be responsible for serious vascular dysfunction (Mathre 1964, Hessburg 1984). Mathre (1964) cited studies in which bluestained wood dried faster than clear wood, and in which bluestained wood was more absorptive of preservatives than uninfected wood. These findings seem unlikely if the blue staining had been accompanied by high resin infiltration.

The copious production of resin in the conducting elements of trees with PRD appears to be unique, and may play an important role in PRD development in eastern white pine. A cross-sectional view at the base of the stem of a diseased tree reveals wedge-shaped resin-soaked areas, frequently extending from the phloem all the way through the sapwood to the pith. Microscopically, resinous inclusions were often found spanning the entire width of tracheids in samples taken from resin-soaked sapwood of trees with PRD (Horner 1985). Water flow through this sapwood was reduced by 99% in relation to clear wood.

A reduced capacity for water conduction in diseased eastern white pine trees likely makes them more susceptible than healthy trees to water deficits during periods of water shortage or high transpirational demand. These deficits may impact upon the host in a number of ways. The sensitivity of the processes of cell division and growth to water stress are well documented, and Kramer (1983) stated that even relatively moderate water stress may severely inhibit vegetative growth and particularly, leaf expansion. Decreased apical growth and needle elongation are characteristic of PRD-affected trees. This decreased foliar production may affect photosynthesis by reducing the photosynthetic area of the plant (Kramer 1983). In addition, photosynthesis is reduced under water stress by stomatal closure and direct effects upon the photosynthetic machinery (Bradford and Hsiao 1982).

Epidemiology of PRD

Most conifer root diseases cause expanding centers of dead and dying trees, and therefore show a clumped pattern in the field. This typically occurs in the cases of soilborne pathogens, or those that move from one tree to another below ground by rhizomorphs or naturally occurring root grafts. In contrast, trees with

PRD are scattered within plantations (Alexander *et al.* 1988). Although *L. procerum* propagules can be detected in the rhizosphere of diseased trees (Lewis *et al.* 1987, Swai and Hindal 1981), their poor survival in soil makes it unlikely that the pathogen is soilborne (Lewis 1985). The distribution of PRD in the field, and Horner's findings (1985) that the fungus was not isolated from the roots further than 40 cm from the root collar, make tree to tree spread via root grafts unlikely. The above factors, plus the known association of these types of fungi with subcortical arthropods (Harrington 1988), suggest that *L. procerum* is vectored by insects.

Recent studies have shown that in Virginia, *L. procerum* is routinely carried by two weevil species, *Hylobius pales* (Herbst.) and *Pissodes nemorensis* Germ. (Nevill and Alexander 1992b). Nevill and Alexander (1992a) demonstrated in controlled studies that both naturally and artificially contaminated weevils efficiently transmit the pathogen to eastern white pine seedlings and saplings. Adult weevils breed in dead and dying trees (Anderson 1980) and it appears likely that the brood becomes contaminated with *L. procerum* when the breeding site is in a diseased tree or stump. Openings within wood, such as those made by larval feeding, have been shown to be the sites of conidial production by *L. procerum* (Lackner and Alexander 1984) and related species (Goheen and Cobb 1978). Conidia are borne in a mucilaginous droplet atop the conidiophore (Kendrick 1962). The sticky conidia readily adhere to the insects (Harrington 1988) and are presumably transported to healthy trees during feeding activities of the newly emerged adults. Practices within Christmas tree plantations exacerbate problems which would normally be associated with high-density monoculture plantings. Culling diseased trees throughout the year, and harvesting healthy and diseased trees for sale in November and December, provides large numbers of

contaminated stumps within which the weevils may breed (Anderson 1980).

Various reports have indicated that PRD is associated with the lower, more poorly drained sites in plantations (Sinclair and Hudler 1980, Smith 1991), however, the disease is often found in areas with good drainage (Swai and Hindal 1981, Nevill 1990). Nevill (1990) suggested that the correlation with wet sites may be related to behavior of the insect vectors and that mortality may occur more quickly, but not exclusively, on trees in these poorer sites.

OZONE

Ozone in the Plant Environment

Ozone in the stratosphere, >10 km above the earth's surface, is of critical importance in protecting life on earth from ultraviolet radiation (Krupa and Manning 1988). In the planetary boundary layer, the lower 2000 m of the troposphere, it is generally recognized as the regional scale air pollutant most harmful to plant life in the United States (Manning and Krupa 1991). Formed through photolysis of NO_2 and subsequent reaction of the atomic oxygen with O_2, ozone occurs naturally in the troposphere in fairly low concentrations of 30-50 ppb (Skelly 1980). With anthropogenic NO_2 inputs due to plumes from urban areas, fossil fuel power plants, and petroleum refineries (Altshuller 1988) concentrations of >300 ppb have been recorded (Wolff and Lioy 1980).

Ozone events are episodic and can be very widespread. Since a photochemical reaction is involved, ozone reaches high levels annually during the summer months, and diurnally, peaks mid-day (Altshuller 1988). Depending upon meteorological conditions, ozone episodes may last a few to many days, and may occur several times within an area during the summer months (Skelly *et al.* 1982, Garner *et al.* 1989). During the period July 12-21, 1977, a mass of ozone reached

from the Texas-Louisiana Gulf Coast east to the northeastern Atlantic Coast. Pollutant concentrations ranged from 120-130 ppb, with peak levels of 328 ppb recorded in Connecticut (Wolff and Lioy 1980). This report noted that a prolongation of the episode due to cold front activity in the north resulted in a simultaneous ozone exposure of nearly two-thirds of the United States. Garner *et al.* (1989) stated that a major portion of the United States east of the Mississippi River has been frequently exposed to phytotoxic ozone concentrations for many years.

In the west, the San Bernardino Mountains of California are subject to high oxidant levels due to their location relative to the Los Angeles Basin. Peak daily 1 h maxima have been recorded in the range of 200-400 ppb (Skelly 1980), and concentrations up to 330 ppb are common (Fenn *et al.* 1990).

Hayes and Skelly (1977) demonstrated the occurrence of high oxidant levels in rural southwest Virginia and the Shenandoah Valley, recording peak hourly concentrations in excess of 60 ppb from June through August 1975. These levels were associated with North and Northeast winds, which brought the oxidant south from urban centers. In the Shenandoah National Park in Virginia, Duchelle *et al.* (1983) observed several ozone episodes of 1-3 days duration each year from 1979 through 1981. Peak hourly concentrations ranged from 80-100 ppb. From mid-April through September of 1979-1981, Skelly *et al.* (1982) continuously monitored ozone levels at three sites in the Blue Ridge Mountains. They characterized mid-April through early October as the oxidant season, with monthly hourly average concentrations of 35-60 ppb and peak hourly concentrations of 80-120 ppb. One-hour average values of $<$20-200 ppb ozone have been reported for near-urban and rural forests in the southeastern United States (Garner *et al.* 1989).

Ozone as a Plant Disease Agent

Weather fleck of tobacco (Heggestad and Middleton 1959) and grape stipple (Richards *et al.* 1958) were two of the first plant diseases for which acute ozone injury was determined to be the cause. Skelly (1980) defines acute injury as that involving death, whether of cells, entire plants, or plant communities. Chronic injury is manifested by non-lethal symptoms, such as reduced chlorophyll production and reduced growth rates. Acute foliar symptoms on dicotyledonous plants are initially a water-soaking of leaf tissue in the vicinity of stomates, followed by localized necrosis of the mesophyll which is visible as a stippling on the adaxial surface (Lacasse and Treshow 1976). On conifers, acute injury results in a tipburn of the current year's growth and frequently a chlorotic mottling (Lacasse and Treshow 1976). Chronic injury on dicots may not be evident as foliar symptoms, but plants may sustain premature defoliation, and reduced growth and yield (Reich *et al.* 1984, Miller 1988). Likewise on conifers, chronic injury may or may not result in foliar symptoms similar to those of acute injury, but does lead to poor needle retention, reduced growth, and decline (Kress and Skelly 1982, Shafer and Heagle 1989).

Biochemical Effects of Ozone on Plants

Ozone gains entry to the interior of a plant via stomata (Rich *et al.* 1970), and once inside, the primary site of deposition is the mesophyll (Taylor *et al.* 1982). Following deposition, it is unclear when, and with what, ozone or its decomposition products react. Heath (1988) describes possible means by which entry could be gained through the cell wall, and states that once inside the cell "Interactions of O_3, or its decomposition products, may be immediately external to, within, or inside the membrane depending upon how far O_3, and its products, can move before reaching

reacting biochemicals." These products include the superoxide anion radical (O_2^-), hydroxide radical (·OH), and hydrogen peroxide (H_2O_2) (Grimes *et al.* 1983), although in aqueous solutions of ozone in physiological conditions, the authors were able to detect only ·OH. Runeckles and Chevone (1992) point out that while the production of oxyradicals presents an attractive hypothesis for the observed effects of ozone, it is not based on verification *in vivo*, but rather theoretical plausibility. These highly reactive materials can be expected to interact with many exposed biological materials, including sufhydryl groups and unsaturated carbon bonds (Heath 1988). This has been amply demonstrated *in vitro*, although a perusal of the review of Runeckles and Chevone (1992) shows that these studies have generally been conducted with levels of ozone far higher than those normally encountered by plants during ozone episodes.

Effects of Ozone on Photosynthesis

Ozone exposure can negatively impact upon a plant's photosynthetic capacity in a number of ways. Under conditions of acute injury, resulting tissue damage reduces the photosynthetic area of the plant. Even in the absence of visible, acute injury, typical ambient ozone levels may cause a reduction in photosynthetic rate (Miller 1988). Comparing several studies, Miller (1988) and Runeckles and Chevone (1992) observed marked similarities in the photosynthetic response of higher plants to ozone, regardless of the species used or the exposure conditions. Reductions may occur indirectly through changes in stomatal aperture, as well as directly, through the action of ozone on the photosynthetic machinery. Stomatal resistance increases in many plants in the presence of ozone (Winner *et al.* 1988), and the consequent reduction in gas exchange has been implicated as a cause of photosynthetic depression (Hill and Littlefield 1969). However, Sen

Gupta *et al.* (1991) observed decreased photosynthetic rates in fumigated hybrid poplar (*Populus deltoides* Bartr. x *P.* cv. caudina) during and after exposure, with no attendant change in stomatal aperture. Runeckles and Chevone (1992) postulated that increased stomatal resistance in the presence of ozone may be a consequence, rather than a cause, of alterations in photosynthetic rate and internal CO_2 concentrations. While the mechanisms by which ozone, or its products, directly affect photosynthesis have not been defined, exposure results in a number of events which clearly may have an impact upon the process. Oxidants cause alterations in the permeability of membranes, and in the case of the plasmalemma, render it 'leaky' (Heath 1988). Changes in chloroplast ultrastructure are rapidly seen following the onset of fumigation, and destruction of chlorophyll is a common consequence of exposure (Guderian *et al.* 1985). Decreases in both the activity and quantity of ribulose bisphosphate carboxylase/oxygenase, accompanied by reductions in photosynthetic rate, have been demonstrated (Dann and Pell 1989, Farage *et al.* 1991).

Effects of Ozone on Carbon Allocation

Whether plants are subject to short-term, high level exposures to ozone resulting in permanent damage of the photosynthetic apparatus, or long-term, low level exposures leading to frequent, transient photosynthetic depression, the result will be a decrease in carbon assimilation. This decrease likely leads, in part, to the reduced growth which has been documented in ozone exposed plants (Krupa and Manning 1988, Heagle 1989). In addition, carbon allocation within plants is frequently altered by ozone exposure, with shoot growth typically being favored over root growth (McLaughlin *et al.* 1982, Cooley and Manning 1987). The loss of resources at the roots may impact plant health in several ways, including depleted

carbohydrate reserves for overwintering and growth in the spring, decreased root growth, enhanced root senescence, and alterations in associations with mycorrhizal fungi (Shafer and Heagle 1989, Manning *et al.* 1971, McCool *et al.* 1979). Appropriate allocation of resources to reproductive structures may be prevented due to ozone exposure, resulting in decreases in fruit size or seed weight (Chevone *et al.* 1990).

Sensitivity of Plants to Ozone

The impact which ozone exposure may have upon a plant is dependent upon a number of variables. Considerable evidence supports the importance of genetically determined tolerance (Gillespie and Winner 1989, Houston 1973, Kress *et al.* 1982, Nicholson 1977). The success with which a plant scavenges ozone, or the free radicals formed upon ozone uptake, likely influences the amount of damage it sustains when exposed. Since it is unclear with what systems ozone or its products react once having entered stomata, any or all of a number of oxidant scavenging systems may be utilized. Scavenging may occur outside the plasmalemma, and/or in the cytosol and/or organelles (Runeckles and Chevone 1992). The antioxidants ascorbate (Barnes 1972), glutathione (Sen Gupta *et al.* 1991), α-tocopherol (Mehlhorn *et al.* 1986) and the enzyme superoxide dismutase (Lee and Bennett 1982), have been shown to increase as a result of oxidant exposure. In addition to scavenging, the ability of a plant to repair oxidant damage undoubtedly influences the outcome of exposure.

Oxidant flux to the inside of a leaf is a major factor in determining the effect of any particular concentration x duration of ozone (Hogsett *et al.* 1988, Runeckles 1992). Among species, or cultivars within a species, plants with fewer stomates and/or increased stomatal resistance have been shown to sustain less damage due

to ozone exposure than those which do not share these characteristics (Evans and Miller 1972, Butler and Tibbitts 1979, Reich 1987).

The physiological and environmental conditions a plant experiences prior to, during, and following exposure will modify its inherent tolerance. The developmental stage of a plant at the time of exposure is important. Fumigation of bean plants showed that they were most susceptible to damage when the leaves were 7-14 days old (Dugger *et al.* 1963). Eastern white pine foliage is most sensitive to ozone when it is semimature, and thereafter sensitivity decreases (Costonis and Sinclair 1969a). Plant water status, light intensity and relative humidity exert control over stomatal aperture (Kramer 1983) and will therefore regulate ozone flux into the leaf. High temperatures in combination with high humidity were found to increase ozone damage in eastern white pine (Costonis and Sinclair 1969a). High humidities during exposure likewise led to increased ozone damage in Virginia pine (Davis and Wood 1973). Chloroplastic damage during exposure is thought to be photooxidative and therefore exacerbated by direct illumination (Anderson 1990). As a consequence, the degree of damage will be influenced by the relative position of a plant within a canopy, or of leaves within a single plant. Nutrient status appears to have variable, and poorly understood, effects on the severity of ozone damage to a plant (Runeckles and Chevone 1992, Chappelka and Chevone 1992).

Ozone as a disease agent of conifers

Due to their long-lived nature and retention of foliage for more than one growing season, evergreen conifers are in a rather unique position regarding their potential for long-term, cumulative injury from ozone exposure. Many forested areas in the United States are subject to annually and seasonally repeated ozone

episodes of sufficient concentration and duration to cause injury (Garner *et al.* 1989, Bartuska 1990). The role of ozone as a forest disease agent has been investigated in three major areas of the country.

Red spruce (*Picea rubens* Sarg.) forests in the high elevation Appalachians of the northeastern United States have been declining for approximately three decades (Chevone and Linzon 1988). Ozone has been implicated as a contributing factor to the decline and researchers found that the symptoms of needle chlorosis and reduced retention, thin crowns, and mortality resembled those of ozone-injured conifers in other areas of the United States (Chevone and Linzon 1988). While one-hour average values as high as 90 ppb have been reported for northeastern high elevation forests (Garner *et al.* 1989), studies have not shown red spruce to be particularly sensitive to ozone (Laurence *et al.* 1989). A recent review (Johnson 1992) does not include a role for ozone in what the author states is a consensus view that winter injury and acid deposition are preeminent contributors to the decline.

Photochemical air pollutants have been documented to cause a chlorotic decline of ponderosa and Jeffrey (*P. jeffreyi* Grev. and Balf.) pines in the San Bernardino Mountains of southern California (Parmeter and Miller 1968). Symptoms of chlorosis, poor foliage retention and death were first observed in the early 1950's and were related to ozone about ten years later (Miller *et al.* 1963, Cobb and Stark 1970).

Eastern white pine is a major component of four forest types and is found in fourteen others throughout its range in the eastern United States (USDA FS 1973). In his treatise on forest tree disease, Hepting (1971) stated of the species, "It is in a class by itself in its sensitivity to atmospheric insults." A broad range of sensitivity to ozone is exhibited in eastern white pine (Gerhold 1977) and has been

demonstrated frequently in controlled studies (Houston 1974, Nicholson 1977). Houston and Stairs (1973) found that ozone-induced needle injury and the effect of exposure on needle elongation were under strong genetic control.

As long ago as 1907, a common needle blight was described on eastern white pine with the symptoms being variously attributed to frost, drought, winter injury, foliar fungi and air pollutants (Costonis and Sinclair 1969a). It was found that the acute symptoms of this blight could be induced in controlled fumigation studies with ozone, and that necrotic flecking occurred with doses as low as 30 ppb for 48 h (Costonis and Sinclair 1969b), while tip burn was observed following exposure to 65-70 ppb for 4 h (Berry and Ripperton 1963). The severity of injury in these genetically sensitive trees was clearly related to the maturity of the needle tissue, being reduced as the growing season advanced.

Chlorotic dwarf of eastern white pine was first observed in 1936 in Ohio, and was thereafter reported from many states in the northern and eastern United States (Swingle 1944). Swingle described it as a chronic disease characterized by trees which exhibited stunted roots and tops, chlorotic foliage, premature foliar abscission and tipburn. While early work investigated the possibility of biotic causal agents, including viruses, Dochinger *et al.* (1965) showed that trees in chambers receiving charcoal-filtered air recovered from the condition, but that those in chambers with fine filters to exclude microbes did not. They stated that the disease was caused by '... the harmful action of gaseous dispersoids on the foliage of susceptible individuals.' In controlled chamber studies with a pollutant-sensitive clone, Dochinger *et al.* (1970) were able to duplicate the foliar symptoms of chlorotic dwarf through simultaneous exposure to ozone and sulphur dioxide. They found that the pollutants interacted synergistically and that either compound acting alone could induce the symptoms, but to a lesser extent than in

combination.

Chronic ozone exposure has been well documented to cause growth losses in eastern white pine. A survey in the southern Appalachian Mountains of Virginia, North Carolina, South Carolina, Georgia, Tennessee and Kentucky detected ozone injury in 23% of 201 stands examined (Anderson *et al.* 1988). The authors pointed out that while other agents can cause symptoms which mimic ozone-induced tip burn, chlorotic mottling is a symptom unique to ozone injury. Therefore, only trees showing both needle tip necrosis and chlorotic mottling were considered to have ozone injury, making this study somewhat more stringent than most. Ozone-injured trees had dramatically different growth than did non-symptomatic trees; they were found to be shorter, smaller in diameter, and had 49% less volume. Substantial impacts of ozone on sensitive eastern white pine trees were also found along the length of the Blue Ridge Mountains in Virginia (Benoit *et al.* 1982). In order to evaluate the effects of ozone on long-term growth, trees were divided into three ozone sensitivity classes based on the needle characteristics of retention, injury symptoms and length. The mean annual radial increment of ozone-sensitive trees over the period 1955-1978 was nearly 29% lower than in ozone-tolerant trees. The authors suggested that the growth reductions were due to the cumulative stress of photosynthetic suppression in the year of injury, plus a reduced photosynthetic capacity in future seasons as a consequence of premature needle senescence and loss. A long-term study on eastern white pine in the Shenandoah National Park showed a decline in crown condition, attributed to ozone exposure, over the period 1980-1992 (Skelly 1979, 1992). It should be noted, however, that much of this decrease was due to heavy weighting of acute symptoms on the current year's foliage. While mottling and banding are definitive ozone characteristics, they do not reflect the long-term condition of a tree in as

meaningful a way as the more lightly weighted characteristics in this study, such as needle retention and needle length. Eastern white pine has been reported to sustain growth loss in the absence of visible foliar damage (Gerhold 1977). Significant reductions in height growth of loblolly pine seedlings were measured following exposure to ozone for 6h/d x 28d at concentrations as low as 50-100 ppb (Kress and Skelly 1982). No foliar damage was observed.

INTERACTIONS BETWEEN OZONE AND FUNGAL DISEASE AGENTS

Ozone and Foliar Disease

Studies of the interactions between ozone and plant disease or its agents have been extensively reviewed (Treshow 1975, Rist and Lorbeer 1981, Manning and Keene 1988). Two major types of effects have been found. In the first case, there may be a direct effect of ozone on the pathogen. Conidia of *Botrytis cinerea* Pers. which developed on geranium leaves during exposure to low levels of ozone had reduced germination and germ tube growth when placed on water agar (Krause and Wiedensaul 1978a). Low ambient levels of ozone delivered to geraniums inoculated with *B. cinerea* resulted in abnormal growth of the fungus, reduced germination and an apparent inability to penetrate (Krause and Weidensaul 1978b).

Secondly, the host pathogen relationship may be influenced by direct effects on the host. Generally, if a pathogen is biotrophic and requires living mesophyll tissue for infection and colonization, it will be negatively affected. Such was the case with uredospores of the rust fungus *Puccinia graminis* f. sp. *tritici* Eriks. & E. Henn. which exhibited poor penetration capabilities when inoculated onto ozone-damaged wheat (Heagle and Key 1973). In contrast, Hibben and Taylor (1975) found that the biotrophic powdery mildew, *Microsphaera alni* (Wallr.) G.

Wint., was not affected by host ozone damage, presumably since it penetrates only the epidermal tissues.

Ozone damage to host tissue may be expected to facilitate infection and colonization by non-biotrophic fungi. This was shown to be the case in a study of *B. cinerea*, in which ozone-induced injury of potato leaves apparently provided increased numbers of infection courts for the pathogen (Manning *et al.* 1969). Rist and Lorbeer (1981) cite a study in which fumigation of *B. cinerea* inoculated broad beans resulted in increased infection, both in the presence and absence of visible foliar injury.

A more common occurrence between ozone and necrotrophic fungi appears to be a lack of interaction, or sometimes a decrease in disease due to host damage. Scots pine seedlings were inoculated with the needle blight pathogen, *Scirrhia acicola* (Dearn.) Siggers, prior to, or following, exposure to high levels of ozone (Weidensaul and Darling 1979). Infection 8 wks post-inoculation was not significantly greater in fumigated trees despite significant foliar damage. Likewise, Costonis and Sinclair (1971) found no interaction between ozone fumigation and infection of eastern white pine by *Lophodermium pinastri* (Schrad.) Chev.

Ozone and Root Disease

Few studies have been conducted to explore the interactions between ozone and root diseases or their causal agents. Unlike the case with foliar pathogens, most organisms causing root disease are not exposed directly to ozone. Significant interaction would therefore likely be due to the impact of ozone on the health, and resultant susceptibility, of the host. Although mycorrhizal fungi are not generally considered to have a pathogenic relationship with their host, the overall processes involved in establishment and maintenance of the relationship are similar to those

of disease-causing fungi. Interactions between these symbionts, their hosts, and ozone have been investigated and show the same types of variability demonstrated in ozone exposure studies of fungal pathogens and their hosts.

Naturally occurring ectomycorrhizae on northern red oak (*Quercus rubra* L.) seedlings were shown to increase significantly in laboratory and field studies following ozone treatment (Reich *et al.* 1985). Laboratory exposures were conducted at 20, 70 and 120 ppb ozone for approximately 7h/d for 52d. Field exposures had daily 7h means of 23 (charcoal-filtered), 44 (ambient) and 68 (1.5x ambient) ppb ozone over the 56d fumigation period. Ozone treatment had a significant effect on the amount of infection, and depending upon soil type, resulted in 20-50% increases in the number of infected short roots in laboratory and field studies. A similar study was conducted to determine the effects of ozone on the interaction of the ectomycorrhizal fungus *Pisolithus tinctorius* (Pers.) Coker and Couch and its host, loblolly pine. Seedlings were exposed to 70 ppb ozone for 6h/d for 35 days. In this case, fumigation had no effect on the incidence of infection by the fungus (Mahoney *et al.* 1985).

An often-cited study was conducted with the vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatus* (Thaxter) Gerd. & Trappe, on seedlings of 'Troyer' citrange (*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck) (McCool *et al.* 1979). Plants were treated with extremely high doses of ozone for 19 weeks; 900 ppb x 6h/d x 1d/wk or 450 ppb x 3h/d x 2d/wk. Fungal infection of the roots was reduced by 22% at 450 ppb ozone and there was no decrease in chlamydospore production. Infection decreased by only 15% at 900 ppb, but chlamydospore production was reduced by 39%. The very high ozone levels used in this study may not accurately reproduce anything that would happen in nature. The outcome of a similar study, using long-term exposure with a moderated ozone

concentration, was quite different. Root colonization of soybean by the vesicular-arbuscular mycorrhizal fungus *Glomus geosporum* (Nichol. and Gerd.) Walker was not affected by moderate ozone levels of 79 ppb administered for 9h/d x 139 d (Brewer and Heagle 1983). This exposure did, however, interfere with normal growth processes of the fungus. Total chlamydospore production per gram of root tissue was reduced by 40%. Brewer and Heagle suggested that sporulation was inhibited due to ozone depression of photosynthesis, resulting in reduced translocation of carbohydrates to the soybean roots. This would have the effect of reducing nutrient availability to the fungus. Working with a pathogenic fungus, Moore *et al.* (1984) investigated the effects of ozone exposure on development of phytophthora root rot in 11 rhododendron and azalea cultivars. The plants were inoculated with *Phytophthora cinnamomi* Rands and fourteen days later fumigated with 200 ppb ozone for 6 h/day on 3 consecutive days. Little ozone injury was detected on the plants 8 wk following inoculation, while most had symptoms of root rot. Only one cultivar showed a significant increase in root rot severity. The authors felt that the ozone tolerance of the plants, and their high susceptibility to the pathogen, may have contributed strongly to the observed lack of interactions. Manning and Keane (1988) reviewed several fumigation studies of *Fusarium* spp., vascular wilt and root decay fungi, on various host plants. While significant differences were detected in plants due to ozone injury, there were no interactions with disease.

The cycle of annosus root disease is one in which the pathogen, *H. annosum*, may directly interact with ozone. The basidiocarp is somewhat exposed to the atmosphere and produces spores which are airborne to susceptible host material, particularly freshly cut stumps (Alexander and Anderson 1985). The basidiospores are therefore exposed during dissemination, deposition, germination and

penetration. In culture, the fungus produces asexual conidia. James *et al.* (1982) investigated the effects of ozone on growth rate, conidial production, and germination of this pathogen. Linear growth rate of mycelium and conidial production decreased significantly at concentrations ≥ 100 ppb. Germination was not substantially reduced except at very high dosages (450 ppb for 8 h), and was stimulated at lower, more realistic dosages. Colonization of wood disks by the pathogen during exposure to ozone was only slightly affected. Although significant decreases in colonization were found at concentrations > 100 ppb, even extremely high dosages resulted in reductions of only 20-35%. It should be noted that conidia are not the spore form which is responsible for infecting stumps with *H. annosum*, and it is not clear whether the effect of ozone on basidiospores would be similar to that observed for conidia. The authors concluded that direct effects of ozone on *H. annosum* would not be of a magnitude to influence the epidemiology of annosus root disease.

Further studies were conducted to determine whether ozone had an indirect effect on annosus root disease, via injury of the host resulting in increased susceptibility to *H. annosum* infection and colonization (James *et al.* 1980a). Injury was expressed as the amount of needle retention in the upper crown of ponderosa and Jeffrey pines. Trees were then cut and the percentage of cross-sectional stump area colonized was measured. Horizontal colonization increased with increasing host injury in ponderosa, but not Jeffrey, pine. Vertical colonization of stumps increased with injury in Jeffrey pine, and in some of the ponderosa pine studied.

In a similar study, James *et al.* (1980b) examined the effects of severe oxidant injury on the susceptibility of ponderosa and Jeffrey pine roots to *H. annosum.* They found that both infection and colonization were increased with increasing ozone injury. They attributed this, in part, to visible decreases in resin

production in injured trees.

Interactions between ozone and *L. wageneri* var. *ponderosum* (Harrington & Cobb) Harrington & Cobb, were investigated in controlled studies on ponderosa pine (Fenn *et al.* 1990). Seedlings were inoculated by placing *L. wageneri* infected pine twigs adjacent to the taproot. The presence and length of black staining in the taproot were used as measures of infection and colonization. Fumigations were conducted for 11 weeks in open-top chambers. Ozone concentrations increased from early morning to a late afternoon peak of 100, 200 or 300 ppb, then decreased until sundown. Long-term exposure of ponderosa pine seedlings to ozone in this study resulted in increased incidence of infection and increased colonization. There was a dose response evident with colonization, which increased with increasing ozone levels.

Lackner and Alexander (1983) assessed the fungal and insect populations in the root systems of ozone-sensitive and tolerant eastern white pine in natural stands along the Blue Ridge Parkway in Virginia. Two lateral roots were sampled from each of 25 trees exhibiting symptoms of ozone sensitivity, and from 18 nearby asymptomatic trees, in order to compare their populations of insects and fungi. The authors isolated *L. procerum* from six of the sensitive trees and from none of the tolerant trees. Two weevils, *Pissodes approximatus* Hopk., and an unidentified *Hylobius* sp., were found in five of these six sensitive trees. *Heterobasidion annosum* (Fr.:Fr.) Bref., the causal agent of annosus root disease, was isolated from one of the trees which had *L. procerum* and weevils, plus another tree in which it occurred alone. Neither it nor any of the bluestain fungi found in the sensitive trees were recovered from ozone-tolerant trees.

A survey conducted in the Coweeta Basin of North Carolina failed to show a relationship between ozone symptomatology and root disease agents (Leininger *et al.* 1990). Two 25-yr-old eastern white pine stands, located within the same drainage basin and with similar soil chemistry and stand characteristics, were evaluated for the presence of root disease agents. One stand exhibited foliar ozone injury, while the other did not. Only 2 of 344 roots sampled in the injury-free stand yielded pathogenic fungi, and none were isolated from 330 roots in the injured stand. The apparent absence of root disease agents make this study neither support, nor refute, the potential for ozone and root disease interactions.

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Chapter 2

Water Relations and Gas Exchange in Eastern White Pine With Procerum Root Disease

INTRODUCTION

Procerum root disease (PRD) of eastern white pine (*Pinus strobus* L.) has been epidemic in Virginia Christmas tree plantations since 1990. The disease is caused by the imperfect fungus *Leptographium procerum* (Kendr.) Wingf. The pathogen, related to the bluestain fungi vectored by numerous species of beetles, is likely introduced into the lower stem or upper portion of a host's roots by one or more species of pine reproduction weevils (Nevill and Alexander 1992). Once established in a feeding or oviposition wound, *L. procerum* grows radially along the rays and vertically through the tracheids, with limited tangential spread (Horner *et al.* 1987). Death of the host frequently occurs following colonization by *L. procerum* and is most common in trees 7 to 10 years of age (Alexander *et al.* 1988). A prominent, and possibly unique, feature of the *L. procerum*-eastern white pine interaction is abundant resin production in the xylem of the roots and lower stem, and often several dm up the stem (Horner 1985). Resin is also exuded forcibly to the outer surface of stems and roots through what appears to be previously intact bark.

Symptoms of PRD resemble those of water-stressed trees and include chlorosis, wilt and reduced apical growth (Alexander *et al.* 1988). The processes of cell division and elongation are especially sensitive to water deficits, and Kramer (1983) stated that even relatively moderate stress may severely inhibit vegetative growth, particularly leaf expansion. Reductions in both shoot and needle elongation are common in PRD affected trees. The drying evident in the foliage and xylem as the diseased trees approach death strongly suggests that the cause of

mortality is water stress.

Recent studies indicate that the appearance of visible symptoms in PRD affected eastern white pine trees may not occur until some years following inoculation and that these trees may exhibit mild symptoms for a few years before death (Carlson, unpublished). Before we can implement control measures to prevent this disease or minimize its impact, we must have a basic understanding of the disease cycle. A portion of the knowledge required relates to the host-pathogen interaction. The objective of these studies on eastern white pine was to investigate the effects of *L. procerum*-induced vascular occlusion on selected water-stress-sensitive physiological processes. The use of well established, mature trees in the field will increase our understanding of the physiology of PRD over that which may be obtained in controlled studies with seedlings.

MATERIALS AND METHODS - Study I

The study was conducted in an 8-yr-old planting of eastern white pine Christmas trees. Twenty-one trees were selected which showed some or all of the following PRD symptoms: mild to moderate chlorosis, reduced needle length, foliar wilt, and external basal resinosis. Ten trees with no visible PRD symptoms were chosen for comparison with diseased trees. Because the investigations focused on water stress, the study was undertaken in August when soil conditions often become dry in southwest Virginia. In addition, all new growth on the trees was fully mature at that time.

Pre-dawn and mid-day shoot xylem water potential were measured with a pressure chamber (Model 3005 Plant Water Status Console, Soilmoisture Equip. Corp., Santa Barbara, CA) (Scholander *et al.* 1965). Values were averaged from two shoots on opposite sides of the exposed upper crown of each tree. The daily

change in water potential was calculated by subtracting the pre-dawn from the mid-day water potential values.

Gas exchange measurements were conducted mid-day (≈12:00 to 1:30 EST) using a LI-COR 6000 portable photosynthesis system to estimate photosynthetic and transpiration rates, and stomatal conductance (LI-COR Inc., Lincoln, NE). Three to five fascicles were removed from the upper crown on the side of the tree exposed to direct sunlight and placed in a 0.25-l cuvette. Air flow rate through the cuvette was set to allow stabilized relative humidity over the 30-second duration of water and gas exchange measurements. The cuvette was placed in the shade with the cover open between measurements to prevent heating of the next sample. The CO_2 analyzer and flow rate meter were zeroed approximately every 20 min. Within the cuvette, light intensity during the measurements was 1060 - 1250 μmol m^{-2} sec^{-1}, RH ranged from 57 - 71% and the temperature was 33 - 38 C. Following measurement, each sample was placed in an envelope and oven dried 24 h at 55 C. All rates were expressed using foliar dry weight.

Disease severity was estimated upon completion of gas exchange and water potential measurements. Stems were severed directly above the root collar and the branches removed. The 1 m basal portion of each stem was placed upright in 10 cm of a 0.75 g/l Fast Green FCF solution (U.S. Biochemical Corporation, Cleveland, OH) for 24 h in a technique adapted from Parmeter *et al.* (1989). The lower end was trimmed with a band saw and a 1 cm section was cut for examination. The disease severity variable was the proportion of occluded cross-sectional xylem area at the stem base and was estimated using a mylar grid with 2 mm divisions.

Due to their proportional nature, disease severity estimates were transformed (arcsin (disease severity$^{0.5}$)) prior to analysis. Linear trends in the response to

disease severity of the suite of six variables; pre-dawn water potential, mid-day water potential, daily change in water potential, stomatal conductance, photosynthetic rate and transpiration rate, were evaluated using multivariate analysis of variance (PROC GLM, MANOVA) (SAS Institute 1988). In addition, the analysis allowed examination of the response to disease severity of each of the variables individually. Data for healthy trees in these analyses were the means of the 8 estimates of each variable. Non-linear trends in the responses were investigated through visual examination of the residuals calculated in the GLM procedure. Relationships among the variables were further examined on the residuals by conducting a principal component analysis (PROC PRINCOMP) (SAS Institute 1988). The relationship between selected dependent variables was examined by analysis of correlation (Zar 1984).

MATERIALS AND METHODS - STUDY II

The study was performed in a planting of 8-yr-old eastern white pine Christmas trees. As in Study I, work was initiated in August. Thirty trees were selected which expressed mild to moderate PRD symptoms, including varying levels of chlorosis, reduced needle length and basal resinosis. An additional six trees with no visible PRD symptoms were chosen to provide baseline data for comparison with diseased trees on each measurement date. Using the techniques of Study I, water potential and gas exchange measurements were performed weekly, or as weather conditions allowed, for a maximum of 8 measurement dates. Cuvette conditions for each date are listed in Table 1. Trees were sacrificed following mid-day water potential and gas exchange measurements if their pre-dawn water potential was $\leq$-1.5 MPa, indicating moderate to severe water stress.

Table 1: Range of light intensity, relative humidity and temperature within the LI-COR cuvette during measurement periods in Study II.

Date	Light intensity (μmol m-2 sec-1)	RH (%)	Temp. (° C)
8/15	1870-2160	47-69	31-37
8/28	2950-3501	58-70	32-36
9/04	1230-2050	57-66	34-40
9/10	1280-1630	44-63	29-36
9/16	1560-1855	28-32	30-34
9/26	950-1280	36-51	27-31
10/4	1350-2560	27-33	34-40
10/18	1250-1550	15-28	26-36

Disease severity was estimated and data were analyzed as in Study I. Where data are compared between diseased and healthy trees over the entire study period, those presented for diseased trees represent each tree on the day it reached ≤-1.5 MPa PDWP.

RESULTS - STUDY I

Disease severity in the studied trees ranged from 0.01 to 0.94 (Figure 1). With the exception of a small pith, healthy trees conducted water over the entire stem cross-sectional area. The pith was included in the estimate of non-conducting xylem, resulting in a disease severity rating of 0.01-0.02 for healthy trees. One of the ten trees selected as healthy on the basis of no external symptoms was found to have 0.07 disease severity.

The linear response of the suite of six variables to disease severity was highly significant ($P = 0.0008$). Visual examination of the residuals did not suggest any existing non-linear trends. The reduction in dimensionality provided through principal component analysis showed that virtually all the variability remaining after that accounted for by the linear model was contained in the first principal component. This component was constructed of a very high positive weight (0.998) on photosynthetic rate.

Significant linear trends in the response of the variables pre-dawn water potential (PDWP), mid-day water potential (MDWP) and daily change in water potential (ΔWP) indicated that disease severity affected host water relations. PDWP became more negative as disease severity increased ($P = 0.0069$, $R^2 = 0.23$) (Figure 2). The mean (± S.E.) PDWP of healthy trees ($n = 10$) was -0.66 ± 0.11 MPa versus -1.71 ± 0.98 MPa for diseased ($n = 21$). Four trees with disease severities of 0.25, 0.42, 0.42 and 0.73 were visibly wilted. Highly negative PDWP

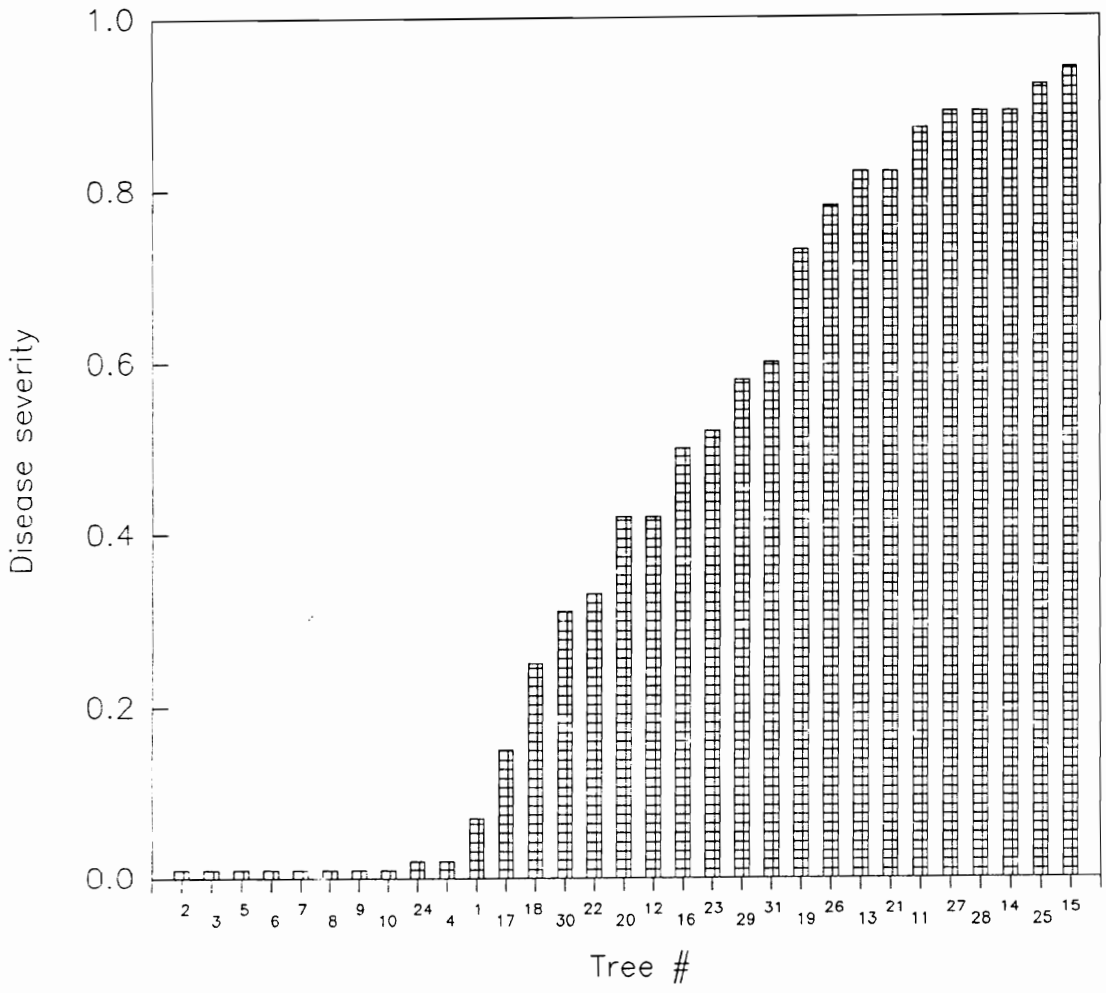

Figure 1: Disease severity estimates of eastern white pine Christmas trees with procerum root disease. Disease severity = proportion of cross-sectional stem area occluded at the base of the trees.

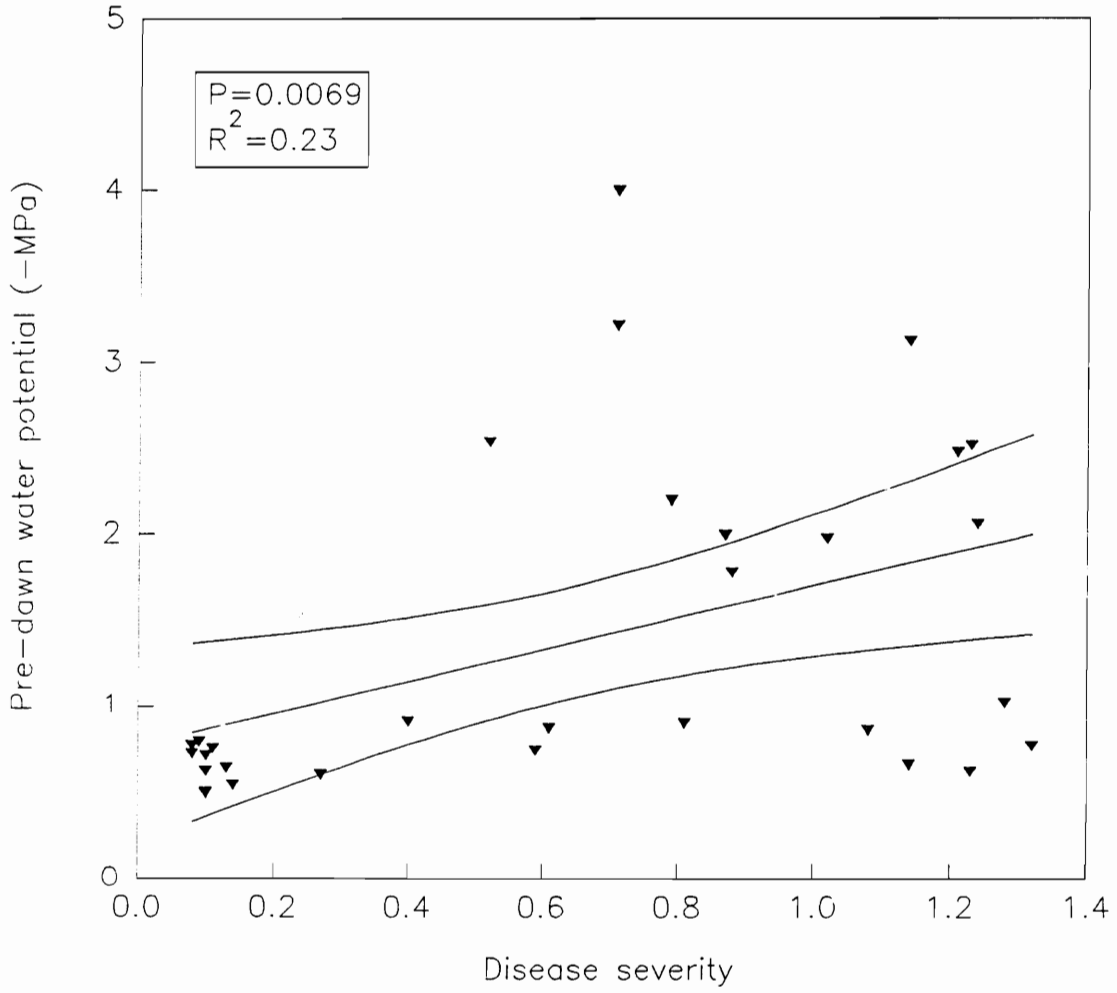

Figure 2: Effect of disease severity on the pre-dawn water potential (±95% C.I.) of eastern white pine with procerum root disease. Disease severity = (arcsin (proportion vascular occlusion at the stem base$^{0.5}$)).

estimates ranging from -2.0 to -4.0 MPa were associated with these wilted trees, however, a further 6 trees with similar PDWP (-2.1 to -3.2 MPa) did not exhibit wilt. Mid-day water potential decreased with increased disease severity ($P = 0.0316$, $R^2 = 0.15$) (Figure 3). The greatest effect of vascular occlusion was on the ΔWP ($P = 0.0030$, $R^2 = 0.27$) (Figure 4). Vascular water loss in the hours between pre-dawn and mid-day was substantially reduced in diseased trees, with a mean (± S.E.) ΔWP of 0.62 ± 0.34 MPa, versus healthy trees at 1.06 ± 0.18 MPa. At disease severities $> \approx 0.40$, both PDWP and ΔWP were highly variable among trees. Ten trees exhibited a greatly reduced ΔWP of ≤ 0.5 MPa, and all had > 0.40 disease severity ratings.

Stomatal conductance was significantly, and inversely, affected by disease severity ($P = 0.0001$, $R^2 = 0.45$) (Figure 5). Transpiration rate was likewise reduced ($P = 0.0001$, $R^2 = 0.44$) with increasing vascular occlusion. The reduction in transpiration rate was highly positively correlated with the reduced stomatal conductance ($R = 0.97$). Vascular occlusion resulted in a significant depression of the photosynthetic rate ($P = 0.0060$, $R^2 = 0.24$) (Figure 6). The mean photosynthetic rate (± S.E.) of healthy trees was 48.1 ± 15.6 μmol CO_2 $g^{-1}sec^{-1}$ while that of diseased trees was 24.9 ± 24.8 μmol CO_2 $g^{-1}sec^{-1}$. There was a high A high correlation between stomatal conductance and photosynthetic rate ($R = 0.94$) suggested that most of the reduction in photosynthesis was due to decreased gas exchange.

RESULTS - Study II

Disease severities ranged from 0.26 - 0.92 in the 14 trees which had reached a PDWP of $\leq$-1.5 MPa at the termination of the study (Figure 7).

As in Study I, the linear response of the suite of six variables to disease

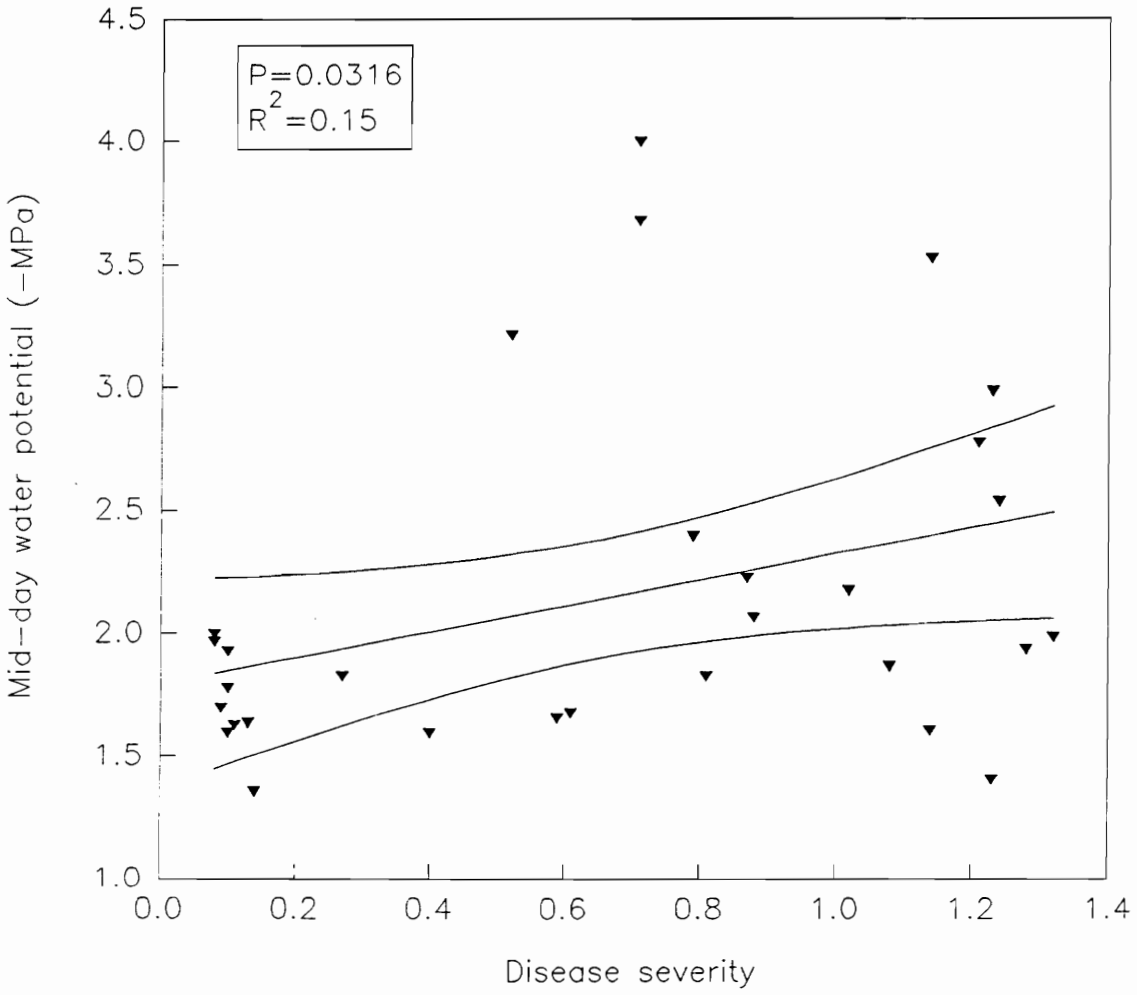

Figure 3: Effect of disease severity on the mid-day water potential (±95% C.I.) of eastern white pine with procerum root disease. Disease severity = (arcsin (proportion vascular occlusion at the stem base$^{0.5}$)).

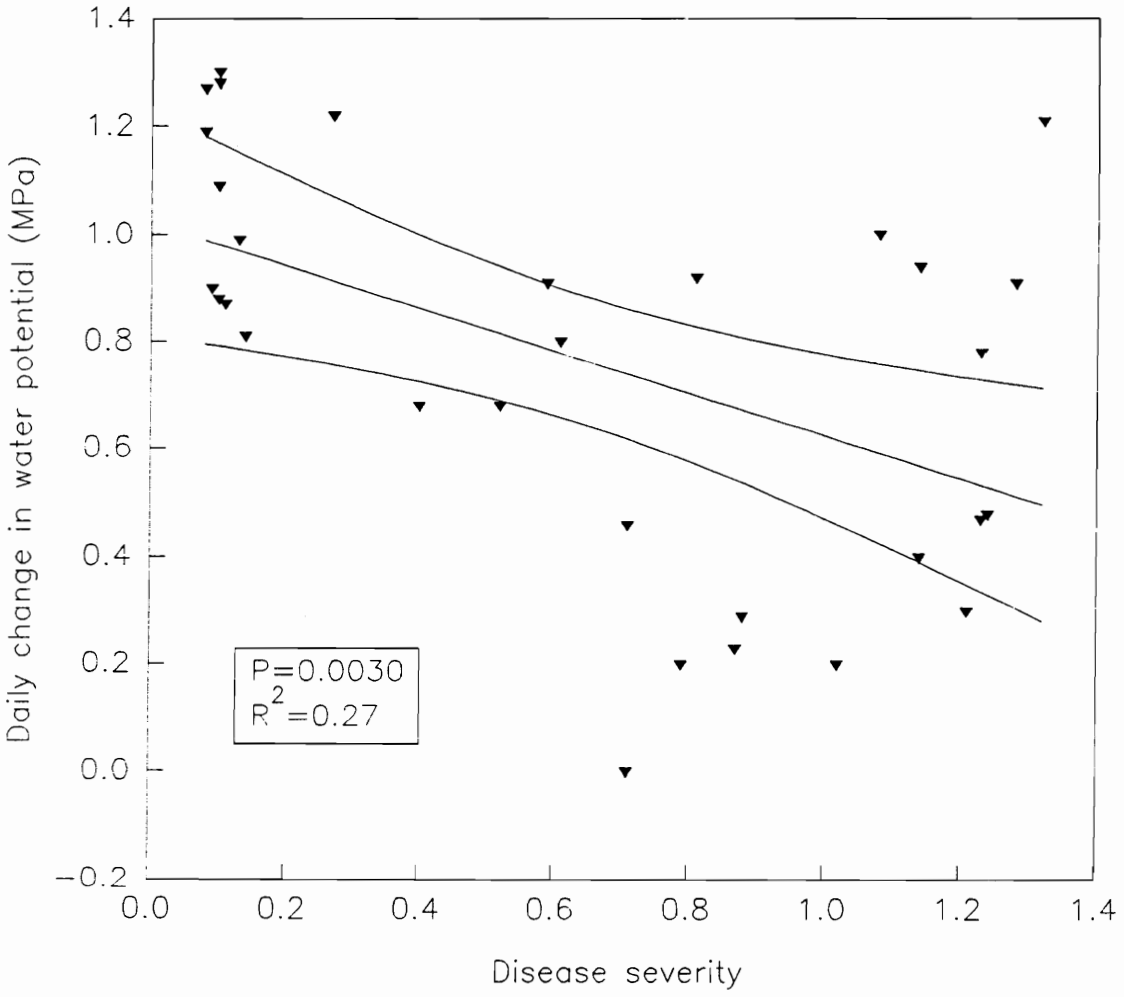

Figure 4: Effect of disease severity on the daily change in water potential (±95% C.I.) of eastern white pine with procerum root disease. Disease severity = (arcsin (proportion vascular occlusion at the stem base$^{0.5}$)).

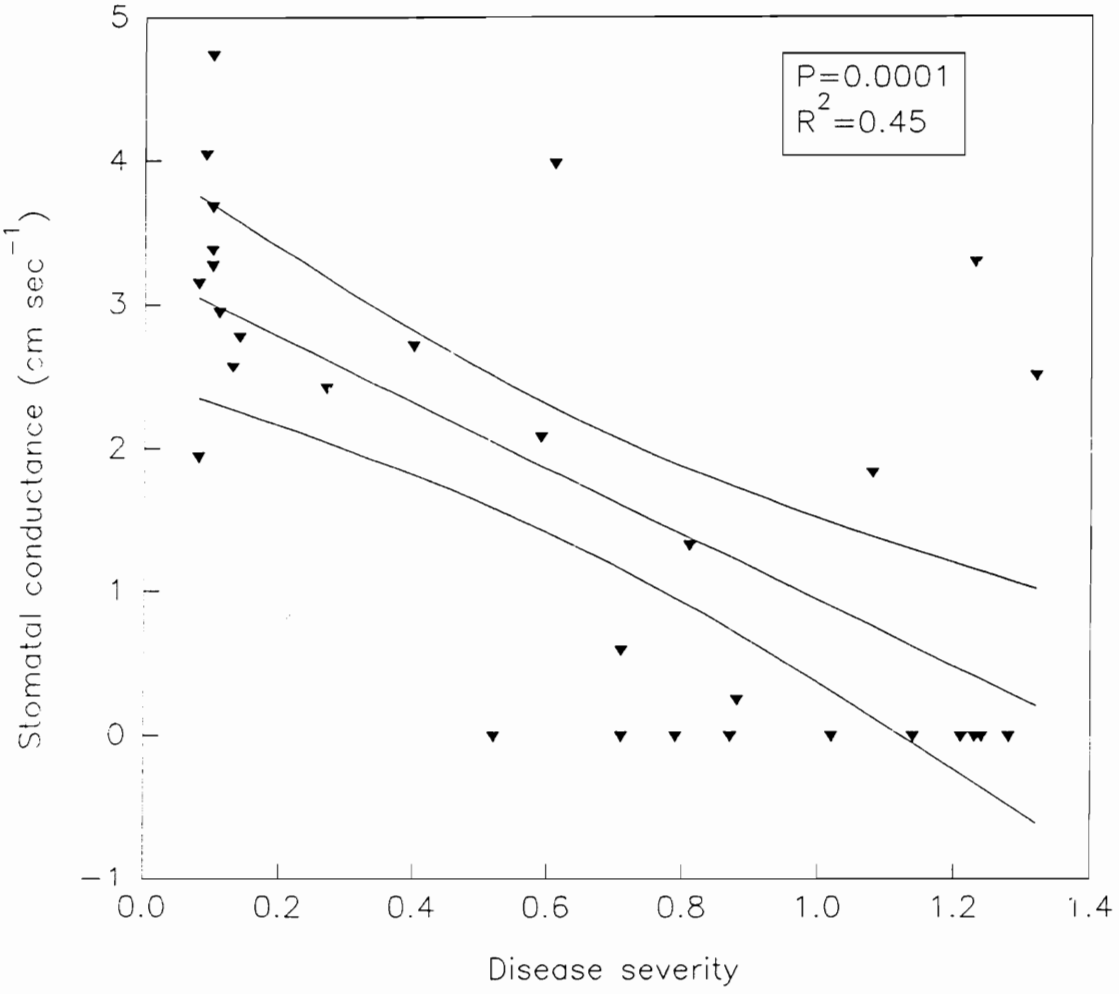

Figure 5: Effect of disease severity on the stomatal conductance (±95% C.I.) of eastern white pine with procerum root disease. Disease severity = (arcsin (proportion vascular occlusion at the stem base$^{0.5}$)).

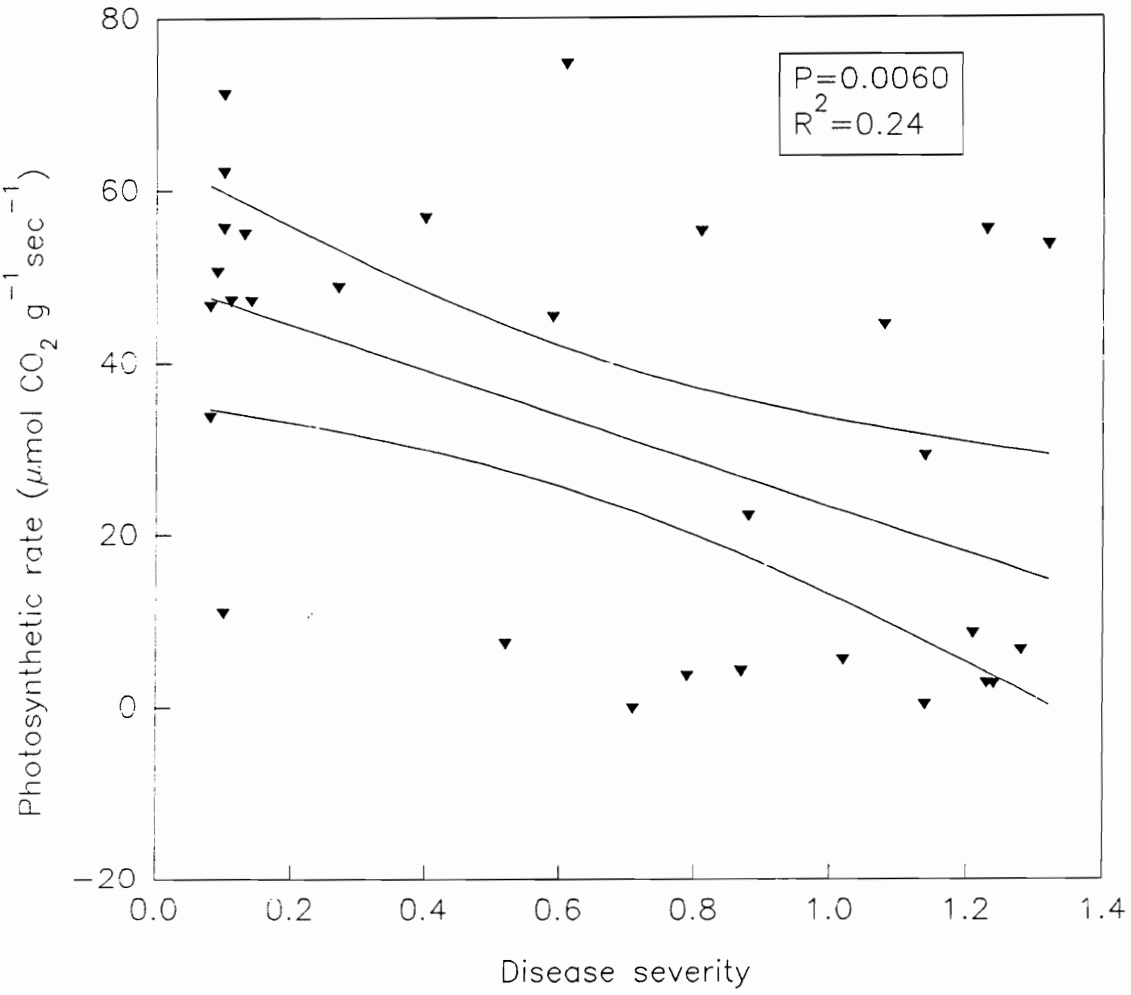

Figure 6: Effect of disease severity on the photosynthetic rate (±95% C.I.) of eastern white pine with procerum root disease. Disease severity = (arcsin (proportion vascular occlusion at the stem base$^{0.5}$)).

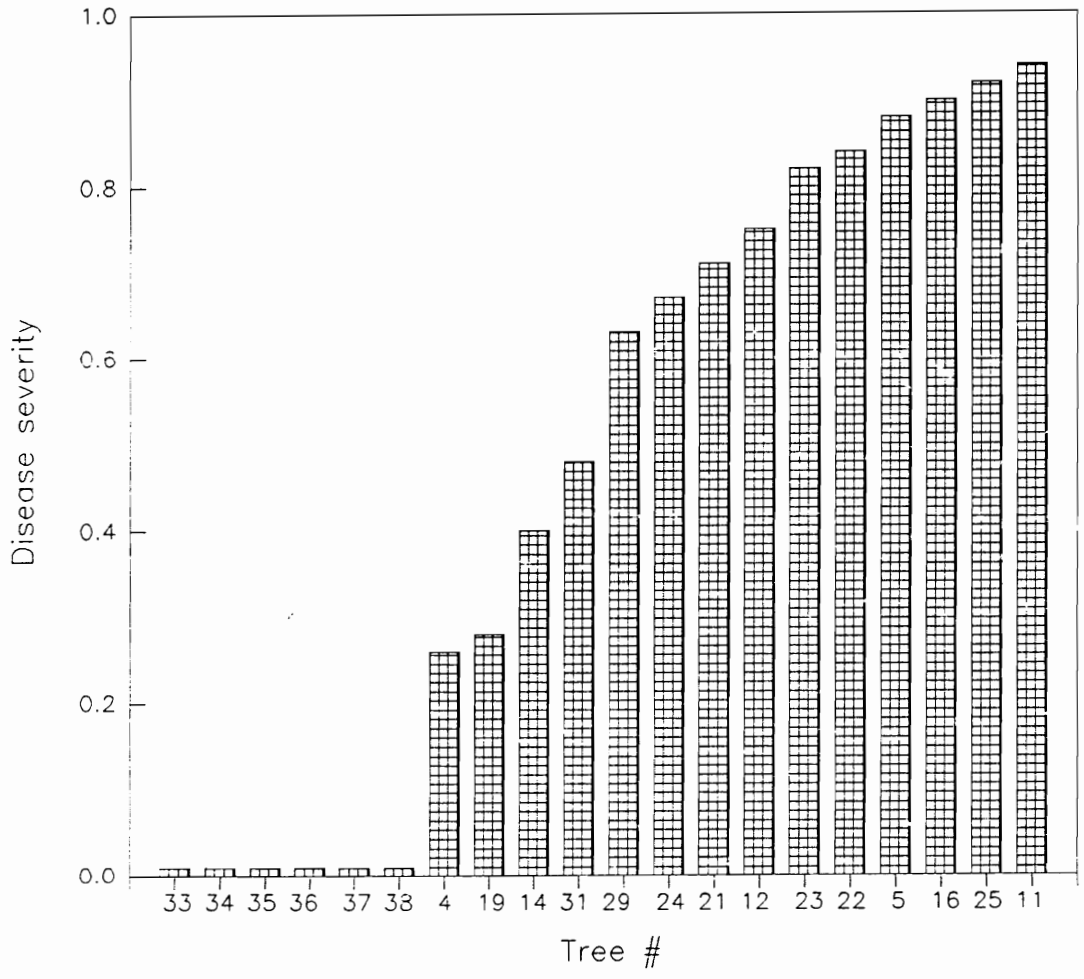

Figure 7: Disease severity estimates of eastern white pine Christmas trees with procerum root disease. Disease severity = proportion of cross-sectional stem area occluded at the base of the trees.

severity was highly significant ($P = 0.0002$). No non-linear trends were seen in the residuals of the model. Evaluation of the residuals through principal component analysis again showed that the first principal component accounted for all the variability not explained by the linear model. This component was comprised almost solely of a very high positive weight on photosynthesis (0.999).

The PDWP of all trees decreased over the course of the study (Figure 8), likely reflecting reductions in soil moisture which commonly occur in the late summer in southwest Virginia. The PDWP was more negative, and the ΔWP (Figure 9) consistently smaller, in diseased than healthy trees. The stomatal conductance of the trees showed an overall decrease with time and was generally much lower in diseased than healthy trees (Figure 10). Photosynthetic rate followed a similar pattern (Figure 11). The close association between these variables can be seen in a comparison of the preceding two figures. For example, in the healthy trees at week 6 the stomatal conductance was increased 6.5-fold over week 5, and was accompanied by a 5-fold increase in photosynthetic rate.

Individual examination of the variables showed a significant effect of disease severity on the PDWP of the trees ($P = 0.0001$, $R^2 = 0.70$) (Figure 12). MDWP was not significantly affected ($P = 0.4130$, $R^2 = 0.04$) and several trees with high disease severity ratings had MDWP in the same range as healthy trees. The impact of disease severity was reflected most strongly in the ΔWP ($P = 0.0001$, $R^2 = 0.43$) (Figure 13). There was a high correlation between ΔWP and PDWP ($R = 0.89$).

Stomatal conductance decreased significantly with increasing disease severity ($P = 0.0004$, $R^2 = 0.51$) (Figure 14). Transpiration ($P = 0.0001$, $R^2 = 0.57$) and photosynthetic rate ($P = 0.0006$, $R^2 = 0.49$) likewise decreased. The high correlation between stomatal conductance and photosynthetic rate suggested that photosynthesis was limited primarily by reduced stomatal conductance ($R = 0.95$).

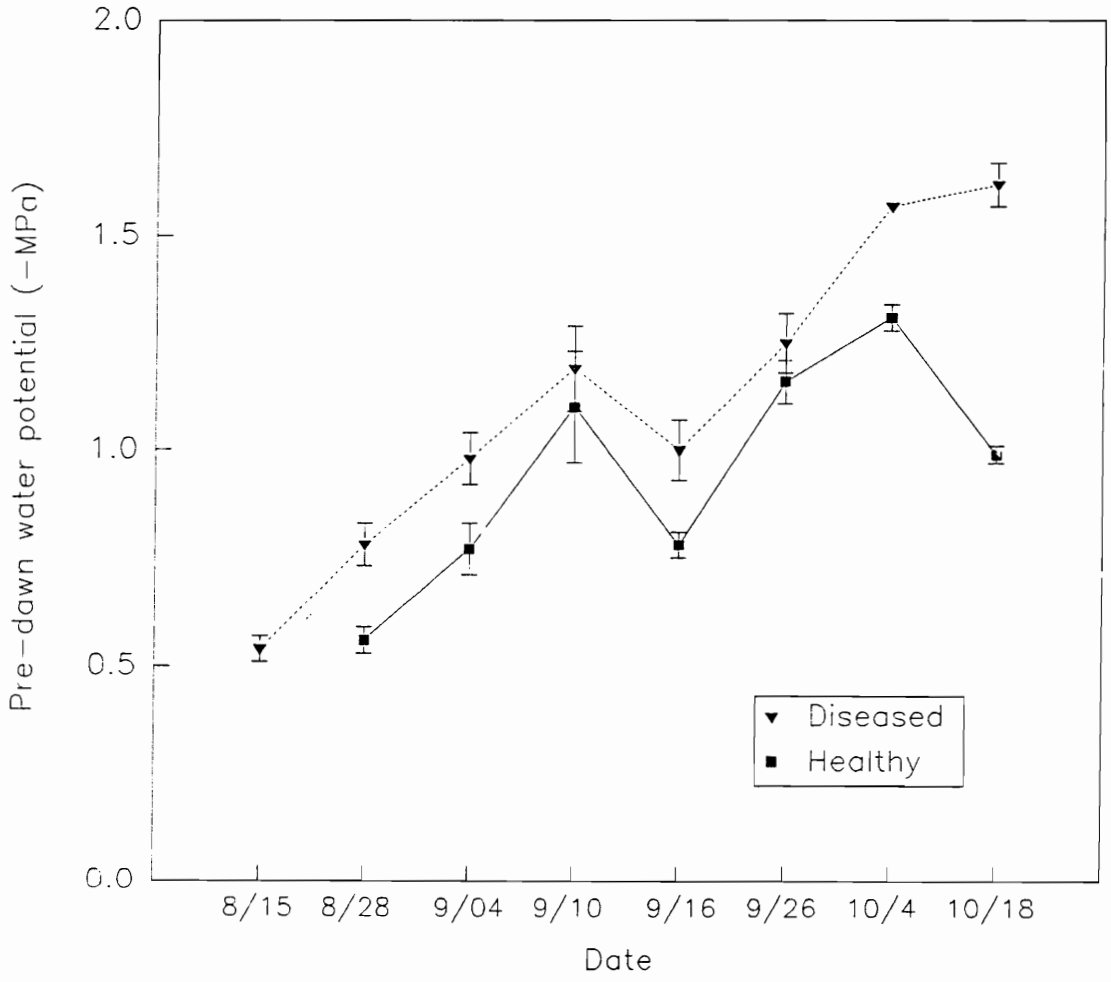

Figure 8: Pre-dawn water potential of eastern white pine trees with and without procerum root disease. Values for diseased trees are the mean (±S.E.) of 14 (wk.1, 2, 3), 13 (wk.4), 11 (wk.5), 10 (wk. 6, 7), and 4 (wk.8) trees. Values for healthy trees are the mean (±S.E.) of 6 trees.

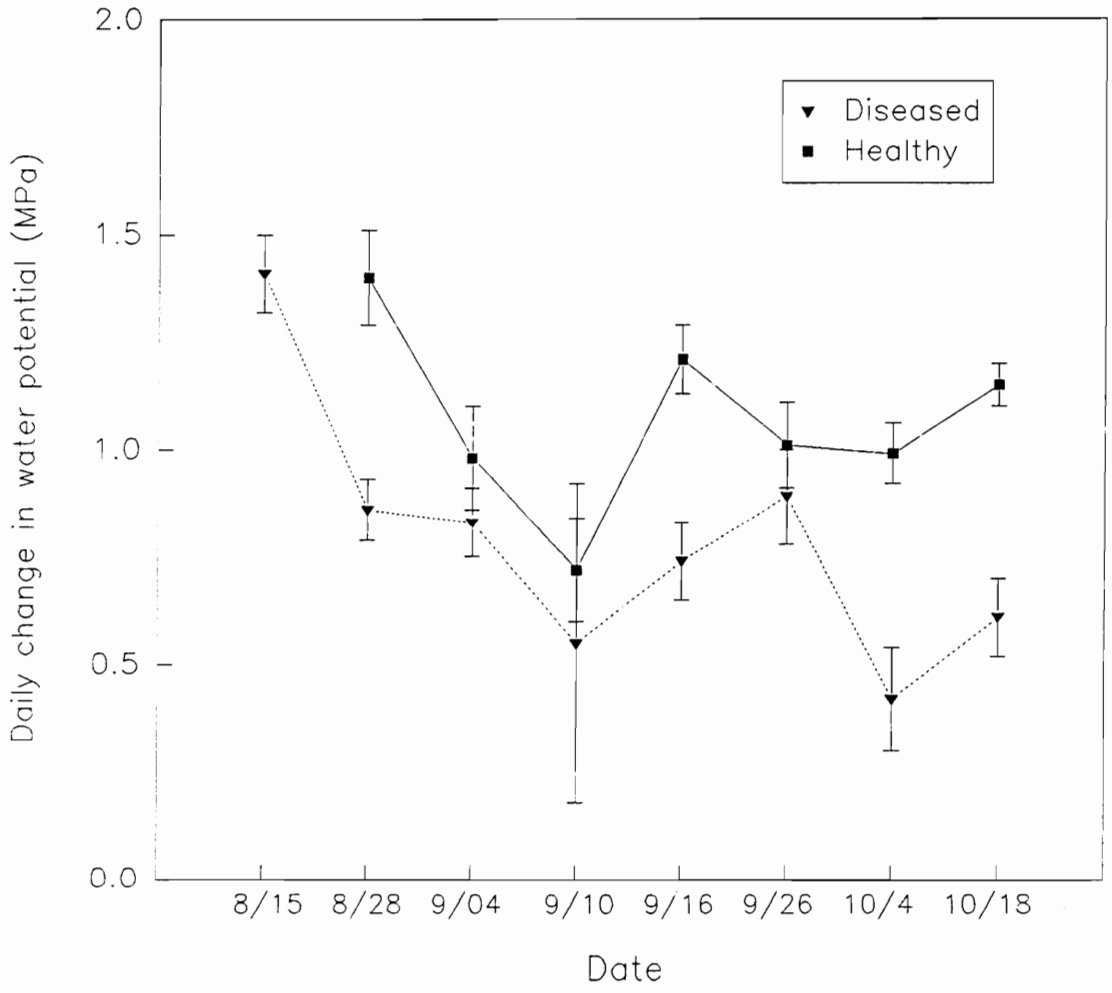

Figure 9: Daily change in water potential of eastern white pine trees with and without procerum root disease. Values for diseased trees are the mean (±S.E.) of 14 (wk.1, 2, 3), 13 (wk.4), 11 (wk.5), 10 (wk. 6, 7), and 4 (wk.8) trees. Values for healthy trees are the mean (±S.E.) of 6 trees.

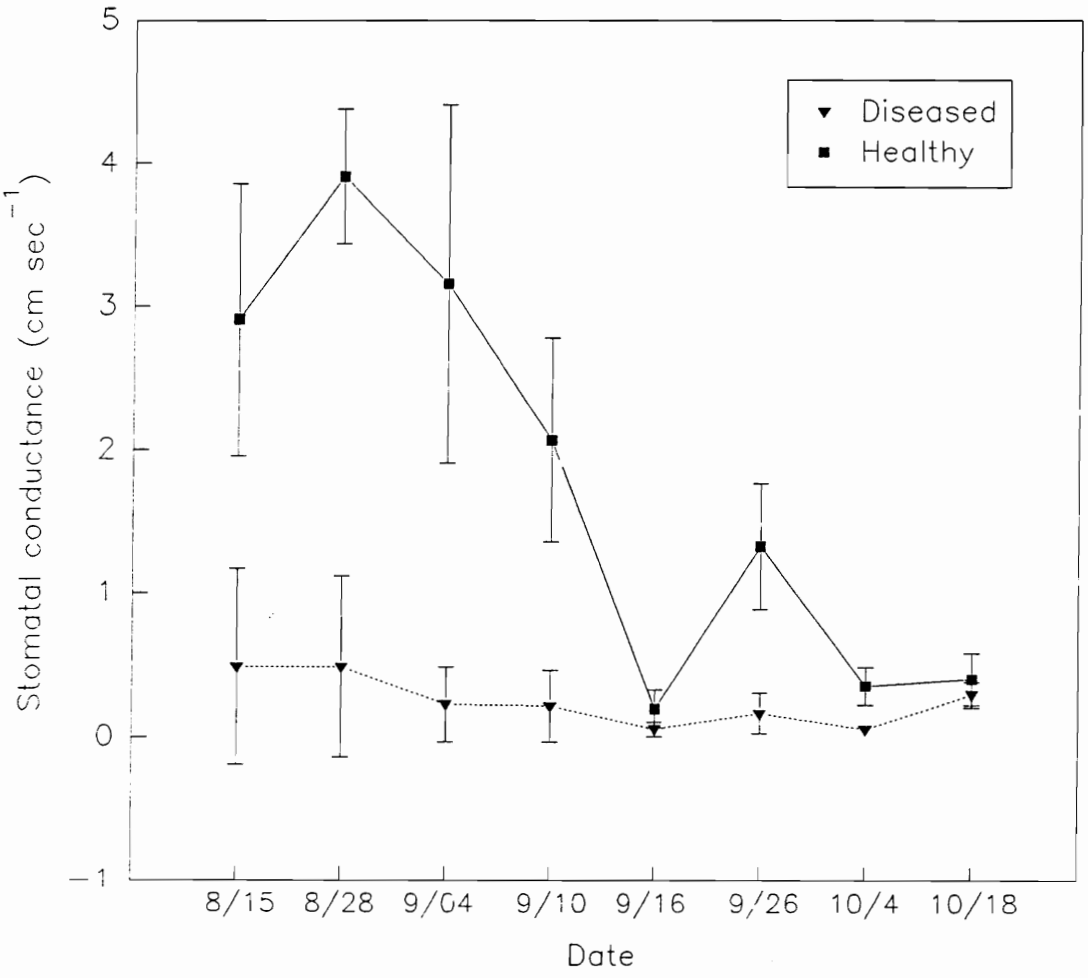

Figure 10: Stomatal conductance of eastern white pine trees with and without procerum root disease. Values for diseased trees are the mean (±S.E.) of 14 (wk.1, 2, 3), 13 (wk.4), 11 (wk.5), 10 (wk. 6, 7), and 4 (wk.8) trees. Values for healthy trees are the mean (±S.E.) of 6 trees.

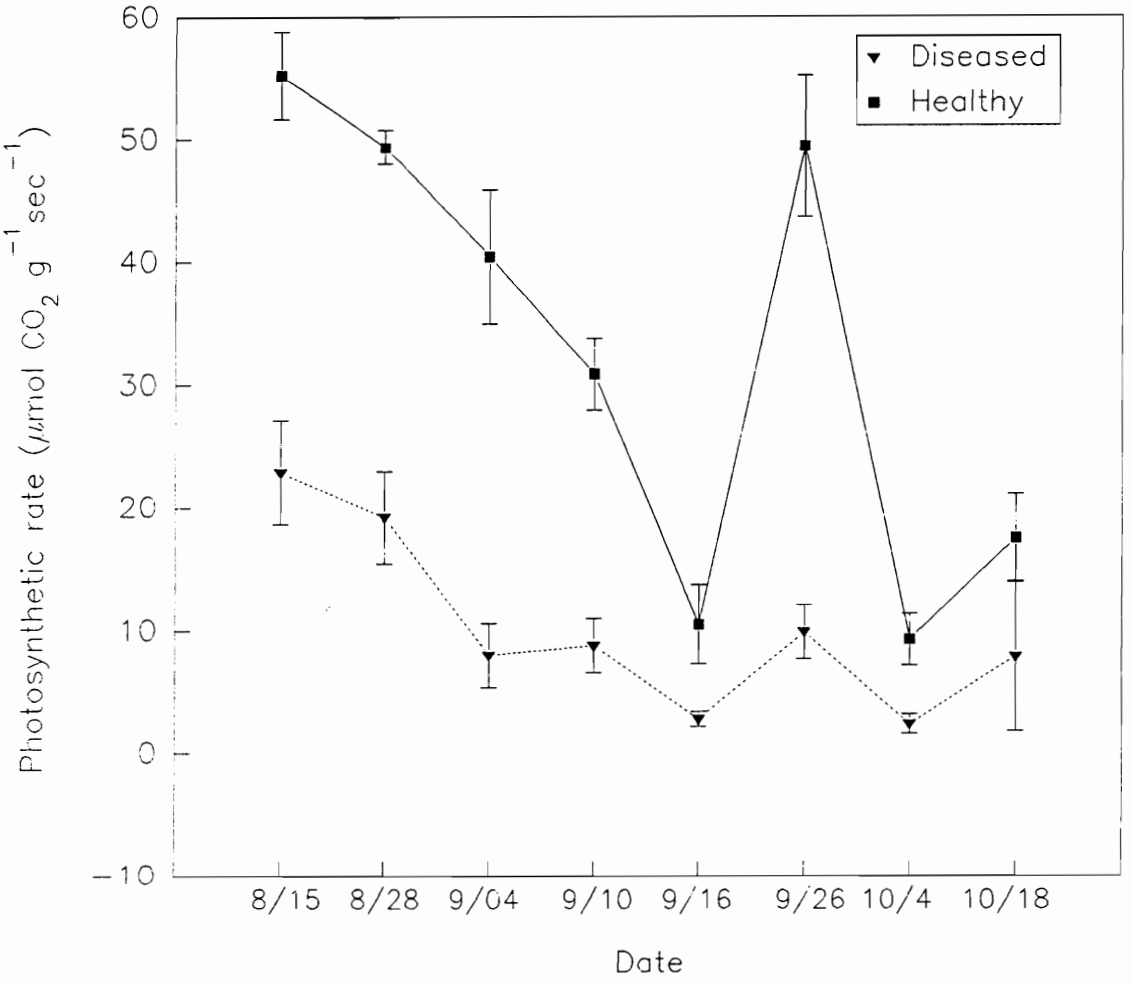

Figure 11: Photosynthetic rate of eastern white pine trees with and without procerum root disease. Values for diseased trees are the mean (±S.E.) of 14 (wk.1, 2, 3), 13 (wk.4), 11 (wk.5), 10 (wk. 6, 7), and 4 (wk.8) trees. Values for healthy trees are the mean (±S.E.) of 6 trees.

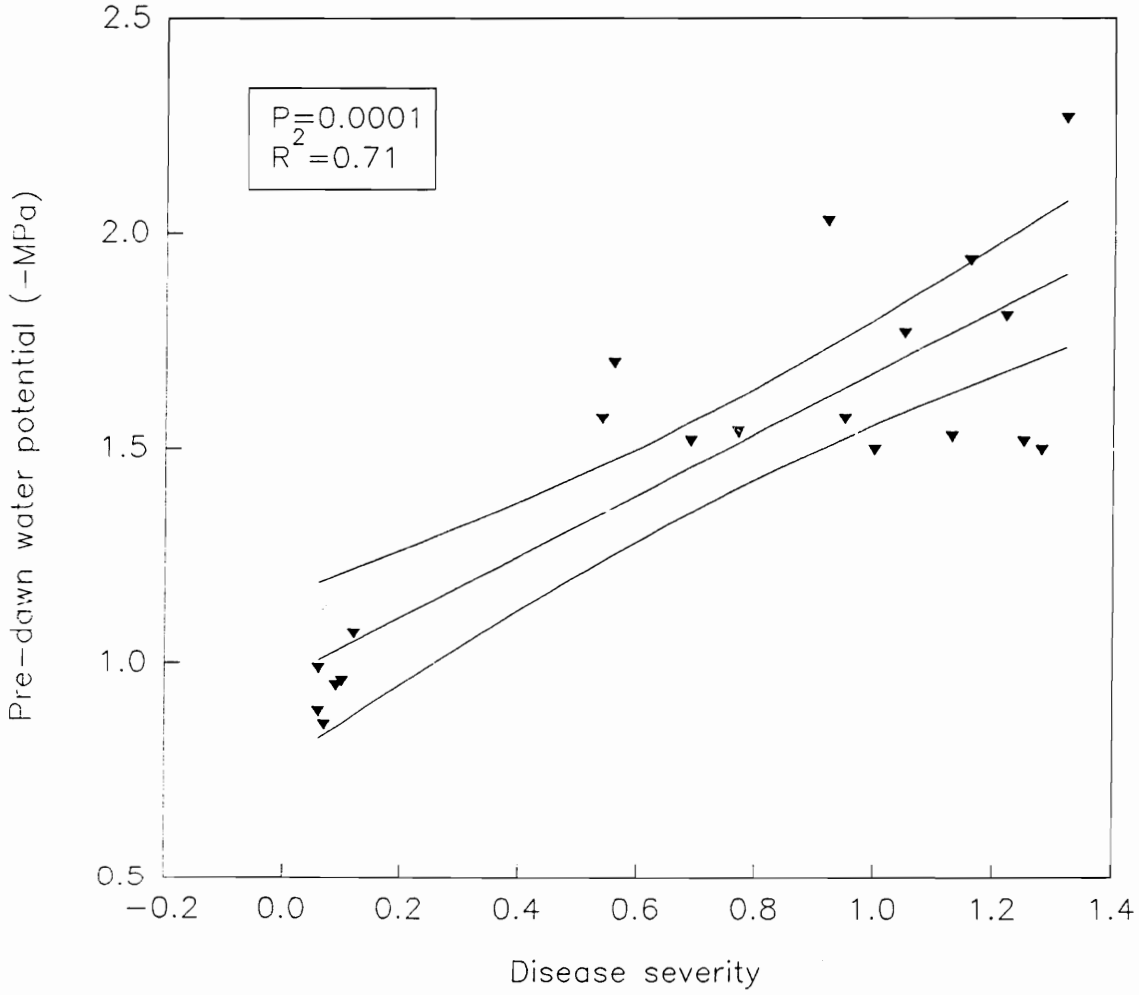

Figure 12: Effect of disease severity on the pre-dawn water potential (±95% C.I.) of eastern white pine with procerum root disease. Disease severity = (arcsin (proportion vascular occlusion at the stem base$^{0.5}$)).

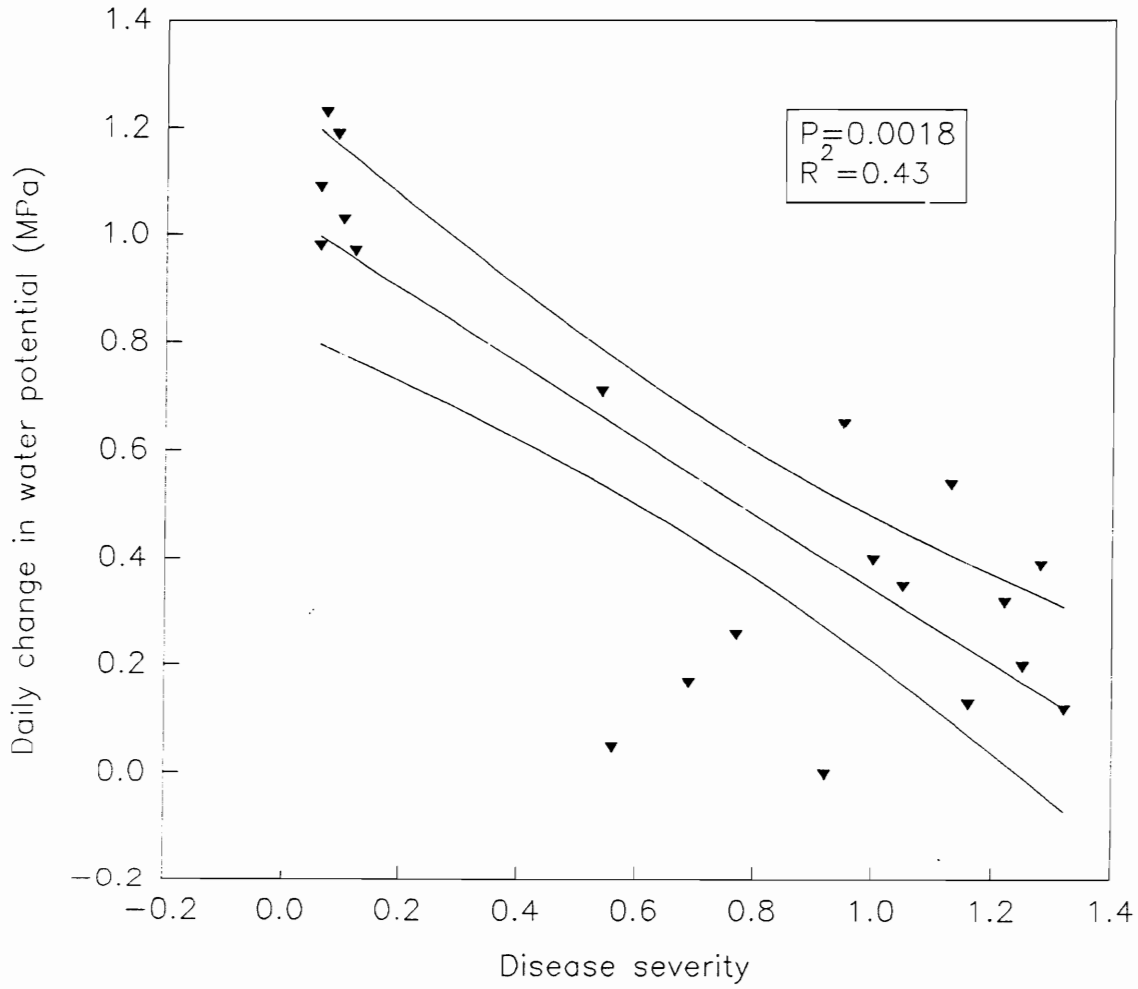

Figure 13: Effect of disease severity on daily change in water potential (±95% C.I.) of eastern white pine with procerum root disease. Disease severity = (arcsin (proportion vascular occlusion at the stem base$^{0.5}$)).

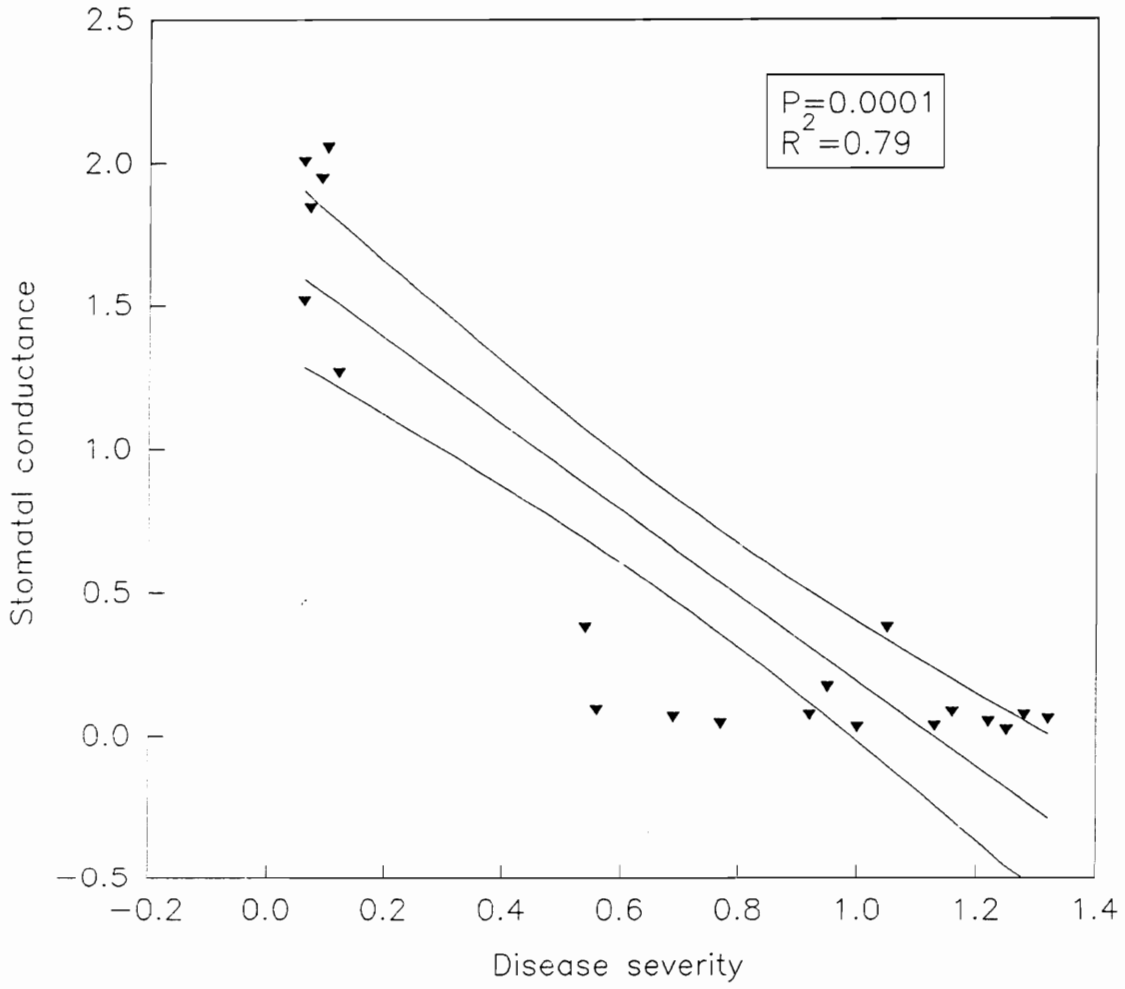

Figure 14: Effect of disease severity on the stomatal conductance (±95% C.I.) of eastern white pine with procerum root disease. Disease severity = (arcsin (proportion vascular occlusion at the stem base$^{0.5}$)).

DISCUSSION

Water relations were significantly altered in trees with *L. procerum*-induced occlusion of the xylem. Prominent among the effects was a reduced capacity to rehydrate overnight, resulting in low PDWP. This may not have been due solely to vascular occlusion. In Study 2, trees with PDWP of ≤-1.5 MPa had disease severities ranging from 0.26 to 0.92. Furthermore, the remaining 16 PRD-symptomatic trees which were not sacrificed in the study likely had disease severities within the above range, yet their PDWP did not decrease to -1.5 MPa. Of particular interest are those trees experiencing low PDWP in the absence of high vascular occlusion, for example PDWP = -2.5 MPa, severity = 0.25 or PDWP = -4.0, severity = 0.42 in Study I. Even a small amount of occlusion in the xylem will result in increased internal resistance to water movement, however, the low water potentials in these trees appear out of proportion to the disease severity. This may be due to microsite differences in the availability of soil moisture. The combination of increased resistance at the soil-root interface, and increased internal resistance, may be enough to substantially dehydrate the crown in plants which have only a minimal amount of vascular occlusion. Several trees with moderate to high disease severity retained PDWP similar to those observed in healthy trees. Two trees in Study I with disease severities of 0.82 and 0.52 had PDWP of -0.7 and -0.9 MPa, respectively. As suggested for trees with very low PDWP, the maintenance of high PDWP may have resulted, in part, from microsite variation in available soil water. Trees with severely reduced water potential on sites with low moisture availability likely die before those which maintain hydration, in spite of vascular occlusion, on wetter sites. Visual examination of the plot layout versus the gross topography did not suggest an effect of location in trees exhibiting unusually high or low water potential for their degree of vascular

occlusion.

The above hypothesis would appear to conflict with anecdotal information in the literature which states that PRD of eastern white pine is associated with low, wet sites (Smith 1991, Sinclair and Hudler 1980). Although initial PRD mortality in a plantation may be observed in a low area, subsequent mortality is scattered throughout the plantation regardless of slope, aspect or elevation (Nevill 1990, Swai and Hindal 1981). The suggestion that microsite differences are the cause of some variability in tree response is based on the assumption that they are planted in suitable areas. In its natural range, eastern white pine is most closely associated with well-drained sandy soils and is infrequent on poorly-drained soils (USDA FS 1965). The stress incurred by trees planted on low, wet sites may cause more rapid mortality due to disease than would occur in a favorable location. In addition, it is probable that the stressed trees would be highly attractive to the insect vectors of *L. procerum* (Rieske and Raffa 1993), and would be attacked first. There is no evidence to suggest that stressed trees are more susceptible to infection by the pathogen.

Another factor which may contribute to the apparent anomalies in PDWP is that a simple estimate of vascular occlusion at the base of the stem may not accurately reflect the degree of impairment of the root system. Depending upon the point of origin of roots at the root collar, and the location of occlusion, a disease severity of 0.50 could include the vascular tissue directly associated with one, two, or several, primary roots. This could lead to high variability among trees in their ability to take up adequate amounts of water from the soil.

These studies indicate that even a small amount of vascular occlusion at the base of the stem may have a substantial impact upon water relations and gas exchange in PRD-affected eastern white pine. Following occlusion, the key

regulatory point appears to be at the stomata. Their conductance may be decreased directly by guard cell response to reduced shoot water potential (Kramer 1983). It is also possible that the stomata close independently of shoot water potential in response to a "signal" received from the lower part of the tree as internal resistance to water movement increases (Zieger *et al.* 1987). Whatever the cause, the decreased conductance in trees with high levels of vascular occlusion limited transpirational water loss, resulting in reduced ΔWP. The reduced xylem water potential experienced by a plant following the onset of transpiration is the driving force for water uptake. Therefore, stomatal closure in the diseased trees may have compounded the water stress resulting from vascular occlusion. Coincident with reduced water loss was limitation of gas exchange at the stomates. This resulted in significantly decreased photosynthetic rates. In addition, virtually all the unexplained variability in the linear model was due to photosynthetic rate. This variability may have been caused by non-stomatal effects of water stress on the photosynthetic process.

Loss of foliar turgor leading to wilt generally occurs when a plant sustains a moderate to severe water deficit. Depending upon environmental and physiological conditions at the time, the plant may be unable to rehydrate and the wilt becomes permanent (Kramer 1983). Wilt in PRD-affected eastern white pine is, at present, poorly understood. Although usually occurring in trees which exhibit chlorosis and reduced needle length, it is often observed in diseased trees that have few or no other foliar symptoms. The wilted trees in Study I had foliage which was otherwise healthy in appearance. Decreased water potential was associated with these trees, but the absence of wilt in trees exhibiting similar amounts of water stress suggests the existence of a contributing factor(s). As discussed above, this may be the availability of soil moisture. Another contributing factor may be

genetic differences between the trees in their ability to remain turgid while experiencing water deficits. Osmotic adjustment, the active accumulation of solutes to maintain turgor at lower water potentials, has been observed in many species of woody and herbaceous plants (Kramer 1983). A third possibility may be the speed with which symptoms occur in the host tree. In trees with a slow rate of occlusion, one or more years of water stress may precede wilt. This would account for the presence of chlorosis and reduced needle length. If vascular occlusion occurs very quickly, the tree may wilt without having experienced long-term water stress.

Basham (1970) stated that symptoms in loblolly pine seedlings inoculated with several species of bluestain fungi were indistinguishable from those killed by withholding water. The results of these studies in eastern white pine Christmas trees suggest that not only are the symptoms of *L. procerum* infection and colonization indistinguishable from those of water stress, but that water stress is directly responsible for the symptoms. The cause(s) of critical impairment of water conduction in trees infected by wilt-inducing fungi has not been determined (Beckman 1987, Parmeter *et al.* 1992). Vascular occlusion due to fungal infection in conifers has been attributed to air emboli or physical plugging of tracheids by compounds formed by the host or pathogen, but not to flooding of the majority of the functional xylem by resin (Nelson 1934, Mathre 1964, Hessburg 1984). The abundant resin visible in the xylem of *L. procerum*-colonized eastern white pine is a probable cause of the water stress they experience. Horner *et al.* (1987) found that the resinous wood from *L. procerum*-infected pines had a reduced moisture content and was virtually impervious to water. Resin infiltration occurs prior to the onset of serious water deficits, as indicated by the high water potential observed in many trees with vascular occlusion in these studies. The elicitation of a resin response

following inoculation of conifers with *L. procerum* and related fungi, or even wounding, has been well documented. In pines, the initial reaction is a passive release of preformed resins, followed by reactivation of the epithelial cells in the resin ducts and secondary resin secretion (Lieutier and Berryman 1988). Descriptions of hosts colonized by other *Leptographium* spp., and fungi in related genera, indicate that resin generally accumulates in a somewhat restricted area surrounding the inoculation site (Raffa and Berryman 1982, Paine *et al.* 1987). Of interest in the *L. procerum*-eastern white pine interaction is the magnitude of the response. Inoculation studies of maritime pine (*P. pinaster* Ait.) with a *Verticicladiella* (=*Leptographium*) sp. (Cheniclet 1987) have provided a possible explanation of the origin of the resin throughout the xylem in PRD affected trees. The author's work showed that the reactivated secretory ducts quickly lyse and are not functional during the time when terpenes (resin = terpenes + resin acids) are accumulating quickly. Cheniclet found that terpene formation becomes delocalized in "traumatic cavities" (Wong and Berryman 1977) created through cell degradation, and hypothesized that enzymes and substrates involved in terpene synthesis randomly meet following their release from lysed cells. While this may account for the copious amounts of resin in the diseased eastern white pine, an appropriate stimulus would be required. *L. procerum* frequently cannot be isolated from the majority of the resin-soaked xylem. Horner (1985) did not isolate the pathogen further than about 20 cm from the root collar, even though resin may have extended further. This has also been found in other conifer disease interactions where resin is produced (Wong and Berryman 1977, Cheniclet 1987). Cheniclet suggested that diffusible substances from the mycelium may stimulate resin enrichment by acting directly upon terpene metabolism or indirectly on host cells. Diffusion of these substances throughout the xylem could account for the

widespread occurrence of resin in trees with PRD.

In summary, the relationship demonstrated between disease severity and the suite of physiological variables indicates that vascular occlusion at the base of the stem causes water stress in *L. procerum* infected eastern white pine. It is hypothesized that soil moisture availability may have an effect, either positive or negative, upon the physiological consequences of a given amount of vascular occlusion. Further studies will be required to determine the impact, if any, of site factors on disease development. Following onset of vascular occlusion, water stress is detected by the stomatal guard cells and stomatal conductance is decreased. This reduces transpirational water loss but also limits gas exchange, resulting in reduced photosynthetic rate. If resin infiltration is responsible for the vascular occlusion observed in PRD-affected trees, it would be unique among related host-pathogen systems.

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Chapter 3

Interactions Among *Leptographium procerum*, Ozone, and Two Pine Species

INTRODUCTION

Leptographium procerum (Kendr.) Wingf. is the causal agent of procerum root disease (PRD) of eastern white (*Pinus strobus* L.) and Scots (*P. sylvestris* L.) pines. While the fungus has been isolated from other pine species, particularly loblolly (*P. taeda* L.) (Webb and Alexander 1982), a suite of characteristic disease symptoms has not been described for them (Klepzig *et al.* 1991, Bertagnole *et al.* 1983). Most frequently observed in eastern white pine Christmas tree plantations, PRD is characterized by foliar chlorosis and wilt, basal resin exudation, and mortality (Alexander *et al.* 1988). Internally, xylem of the roots, root collar and lower stem is infiltrated with resin and may be stained. In studies with eastern white pine seedlings, Lackner and Alexander (1982) described the occurrence of PRD symptoms including chlorosis, wilt and mortality as early as two weeks following root inoculations with colonized wood blocks or conidial suspensions.

Trees colonized by bluestain fungi, which include *Leptographium* spp., exhibit altered water relations. The causes are unknown, but have been variously attributed to formation of emboli (Mathre 1964), plugging of tracheids by gums (Hessburg 1984) and disruption of phloem and cambium (Rane and Tattar 1987). Water transport in trees with PRD is further impeded by the copious production of resin in the vascular tissue of roots and lower stems (Horner *et al.* 1987).

Ozone episodes of sufficient concentration and duration to injure ozone-sensitive forest trees routinely occur during the summer months over large areas of the eastern United States (Garner *et al.* 1989). Anderson *et al.* (1988) found air pollution (ozone) symptoms in 23% of the white pine stands sampled in

the southern Appalachian Mountains in five states. Little is known of the impact which ozone may have upon forest disease, and specifically, upon the ability of individual trees to defend themselves against root pathogens. Overall reductions in carbohydrate reserves of pines following ozone exposure (Miller *et al.* 1968), and alterations in biomass partitioning to favor shoot growth over root growth (McLaughlin *et al.* 1982, Adams *et al.* 1990), may decrease the ability of roots to resist invasion by pathogenic fungi. Resin acids and terpenes play a role in defense against fungi and insects (Lieutier *et al.* 1989, Shain 1967), and ozone-induced alterations in host physiology may affect their production. The yield, exudation pressure and rate of flow of resins were found to be decreased in ozone-injured ponderosa pine (*P. ponderosa* Dougl. ex Laws.) (Cobb *et al.* 1968).

Correlations between foliar ozone injury and *L. procerum* infection have been observed in the field. Isolations from the roots of dead eastern white pine trees which had sustained repeated, injurious ozone exposures in the Shenandoah National Park frequently yielded *L. procerum* (Skelly 1980). Eastern white pine exhibiting injury typical of ozone-sensitivity in the Blue Ridge Mountains of Virginia were found to be infected with *L. procerum* and other *Leptographium* spp., while nearby uninjured trees were not (Lackner and Alexander 1983).

The objectives of this study were to determine if ozone exposure of *L. procerum* inoculated eastern white or loblolly pine seedlings affects disease development, or the amount of fungal infection or colonization. The effects of ozone exposure and *L. procerum* colonization on host water relations were also investigated.

MATERIALS AND METHODS

Seedlings

Bareroot, improved, 2 + 0 eastern white and 1 + 0 loblolly pine seedlings were obtained from the Virginia Department of Forestry Augusta Nursery at Crimora, Virginia. Seedlings were planted in 3.5 L, 17 cm tall x 15 cm diameter plastic pots in a mix of 2:1:1 peat:vermiculite:perlite and maintained in a greenhouse under natural light for 6-12 months. Approximately two weeks preceding the initiation of each study, seedlings were transferred to a charcoal-filtered greenhouse.

Root Infection Studies

Root inoculations of eastern white and loblolly pine seedlings were performed through infestation of the growing medium with a conidial suspension of *L. procerum*. Preliminary studies allowed selection of an inoculum concentration and volume which resulted in infection of 25-50% of the roots of eastern white pine seedlings 6 weeks following application of inoculum. Four *L. procerum* isolates were used together in the event of differences in infectivity among them. Isolate #143 was collected in 1982 from an eastern white pine in West Virginia and maintained in the VPI&SU Forest Pathology Laboratory culture collection. Prior to use in this study, it was inoculated into an *L. procerum*-free eastern white pine seedling and re-isolated (isolate #4-1) from stem tissue 8 wk later. Isolate #283A was obtained just prior to the study from the root collar of an eastern white pine Christmas tree in Floyd Co., VA. Isolates #207A and #239A were obtained 1 yr previously from the roots of two ≈40-yr-old asymptomatic loblolly pines in Fairfield Co., S. Carolina. Initial isolations were made onto an actidione-amended malt agar selective medium (AMA) (McCall and Merrill 1980)

and subcultures were grown on malt extract agar (MEA) for 14 d at 20C. Conidia were dislodged from plates with a bent glass rod and distilled water. Inoculum was prepared by combining equal concentrations of conidia of each isolate to achieve a final concentration of $5x10^4$ conidia/ml of distilled water. One hundred sixty seedlings of each species were watered thoroughly and a 20 mm diameter cork borer was used to make four equally spaced holes through the entire depth of the potting mix around the margin of each pot. Twenty-five ml of inoculum was pipetted into each hole and the potting mix replaced.

Immediately following infestation, plants were moved to 1.3 m^3 continuous-stirred tank reactor chambers with 10 seedlings of a species placed in each of 16 chambers. After a 24-h equilibration period, seedlings in half the chambers received ozone delivered in a step function profile which peaked at 200 ppb within 1 h and was maintained for 5 h/d. Seedlings in the remaining chambers received charcoal-filtered air containing ≤20 ppb ozone. Ozone was generated through ultraviolet irradiation of O_2 with a Welsbach Laboratory Ozonator Model T-408 (Welsbach Ozone Systems Corporation, Philadelphia, PA) and monitored with a Teco UV O_3 analyzer (Model 49s, Thermo Electron Corporation, Environmental Instruments Division, Hopkinton, MA) calibrated with a Photocal 3000 Automated O_3 calibrator (Columbia Scientific Industries, Austin, TX). Light was provided from 1 h pre- to 1 h post-fumigation by 1000 W metal halide lamps producing a photosynthetic photon flux density of 575 ± 25 μmol m^{-2} s^{-1}. Temperatures were maintained at 27 ± 2 C, and relative humidity varied from 45 - 75%. Fumigations continued for 14 consecutive days, from approximately 0800 - 1500 daily. The cumulative ozone exposure was 14 ppm·hr. Following the fumigation period, seedlings were placed in a charcoal-filtered greenhouse.

Four weeks after the conclusion of fumigation, seedlings were removed

from pots and all loose growing medium shaken from the roots. Seedling tops were removed ≈5 cm above the root collar and the root systems and lower stems washed 5 min in running tap water, then surface sterilized 2 min in 0.5% NaOCl. The tap root, and each primary root plus associated lower order roots, were removed from each plant and aseptically cut into 1.5 - 2.0 cm segments with a razor blade. Five samples from each root were plated onto AMA and incubated 14 d at 20 C. Plates were observed microscopically for the presence of *L. procerum*. The observation of *L. procerum* on any sample of a single root resulted in that root being recorded as infected. The proportion of infected roots was recorded for each seedling. For each species, a mean proportion of infected roots was calculated for each replicate of 10 seedlings per chamber, and the means were transformed (arcsin (mean proportion)$^{0.5}$) prior to analysis. The effect of ozone on root infection was evaluated for each species using a t-test (PROC TTEST, SAS Institute 1988).

Stem Colonization Studies

In a subsequent test, the effect of ozone exposure on the extent of fungal colonization was determined through measurement of the upward vertical growth of the pathogen in the stem of inoculated seedlings. A 5-mm diameter piece of bark from the base of the stem on each of 160 seedlings per species was removed with a cork borer. A single eastern white pine isolate of *L. procerum* (isolate #283A) was grown on MEA for 14 d at 20 C. A 5 mm diameter piece of mycelium from a single eastern white pine isolate, grown on MEA for 14 d at 20 C, was placed on the wound and the inoculation site wrapped with Parafilm®. Immediately following inoculation, 10 seedlings were fumigated per chamber, as described in the root inoculation study.

Seedlings were sacrificed six weeks after inoculation. The stem was severed

at a point approximately 1 cm below the inoculation site and again 15-20 cm above the site. Plastic film covering the inoculation site was removed, the entire stem surface was sterilized 2 min in 0.5% NaOCl solution, rinsed in distilled water and air-dried on paper towelling. Ten contiguous 1 cm pieces were cut from the stem immediately above the mid-point of the inoculation site. Pieces were plated sequentially on AMA and incubated 10-14 d at 20C. The presence or absence of *L. procerum* growth on each piece was recorded and a mean colonization distance calculated for the 10 seedlings per chamber. The effect of ozone on colonization distance was evaluated for each species with a t-test (PROC TTEST, SAS Institute 1988).

Evaluation of Water Relations

Water potential and transpiration were estimated for 240 seedlings per inoculation technique, per species, to evaluate the impacts of fungal infection, ozone fumigation, and their interactions, on water relations. To determine the effects of fungal inoculation, an additional five seedlings were included with the ten *L. procerum* inoculated seedlings per chamber described in the previous studies. These additional seedlings received 25 ml of distilled water at each infestation site in root infection studies, while those in colonization studies were inoculated with a 5 mm piece of sterile MEA and wrapped with Parafilm®.

Transpirational water loss was determined gravimetrically as follows. Three weeks after fumigation (5 wk post-inoculation), seedlings were watered to dripping. Each pot was then enclosed in a plastic bag sealed at the base of the seedling stem, and weighed. Plants were re-weighed 7 d later. The change in weight equalled the water lost through transpiration. Mid-day xylem water potential was then measured on the terminal shoot of each seedling with a pressure chamber (Model

3005 Plant Water Status Console, Soilmoisture Equip. Corp., Santa Barbara, CA) (Scholander *et al.* 1965). Foliage was removed from each seedling and oven-dried 48 h at 50 C. Transpirational water loss for each plant was calculated as the 7-day change in weight divided by the foliar dry dry weight. Transpiration and water potential means were calculated for the 10 inoculated, and the 5 uninoculated, seedlings per chamber. Data were analyzed by ANOVA (PROC GLM, SAS Institute 1988).

RESULTS AND DISCUSSION

Effects of inoculations on seedlings

Infestation of the growing medium with a conidial suspension resulted in root infection of 95% of the eastern white and 87% of the loblolly pine seedlings. The mean proportions of infected roots on the two species were 0.47 ± 0.03, and 0.36 ± 0.02, respectively (Table 1). Staining of root tissue, which may result from *L. procerum* colonization, was minimal. No disease symptoms were observed over the 6 wk study period. The single eastern white pine seedling which died did not appear to have PRD.

Stem inoculations resulted in infection and colonization of 99% of the eastern white and 100% of the loblolly pine seedlings. The mean vertical upward spread of the pathogen from the inoculation site was 2.22 ± 0.10 cm in eastern white pine and 2.20 ± 0.11 cm in loblolly pine (Table 2). Brown lesions were visible originating at the inoculation sites, but the PRD-associated resin-soaking observed in older trees was not evident. Staining was not seen in the xylem of any seedling and there were no external symptoms of PRD. The two eastern white pine and four loblolly pines that died did not exhibit PRD symptoms.

Table 1: Proportion of roots of eastern white and loblolly pine seedlings infected 6 wk following soil infestation with *Leptographium procerum* and ozone fumigation.

Host	Treatment	Root infection (±S.E.)[1]	P(T)
eastern white pine	c. f. air[2]	0.47 (0.03)	0.82
	200 ppb ozone	0.48 (0.02)	
loblolly pine	c. f. air	0.36 (0.02)	0.18
	200 ppb ozone	0.29 (0.02)	

[1] Values are the mean of 8 replicates of 10 seedlings per chamber.
[2] Charcoal-filtered air < 20 ppb ozone.

Table 2: Vertical growth of *Leptographium procerum* in eastern white and loblolly pine seedling stems 6 wk following inoculation and ozone fumigation.

Host	Treatment	Growth (cm) (±S.E.)[1]	P(T)
eastern white pine	c. f. air[2]	2.22 (0.10)	0.40
	200 ppb ozone	2.09 (0.10)	
loblolly pine	c. f. air	2.20 (0.11)	0.85
	200 ppb ozone	2.16 (0.10)	

[1] Values are the mean of 8 replicates of 10 seedlings per chamber.
[2] Charcoal-filtered air < 20 ppb ozone.

The six week duration of these studies should have been adequate for symptom expression to occur. Two weeks after root inoculation of 2-yr-old eastern white and loblolly pine, Lackner and Alexander (1982) observed the PRD symptoms of chlorosis and wilt of foliage, reduced root growth, black staining of xylem, and mortality. Lackner and Alexander (1982) did not quantify *L. procerum* growth in the root systems of symptomatic and dying seedlings. The possibility exists that their root dipping technique resulted in a higher incidence of infection than was achieved in the current studies.

In the case of eastern white pine, this implies that greater than the 47% root infection achieved in this study is required before symptom expression occurs. In preliminary tests, we found that seedlings inoculated with concentrations of conidia 10X greater than those used in the fumigation studies exhibited no symptoms 8 wk following inoculation, although a mean of 82% (range 62.5 - 100%) of their roots were infected. It does not then appear that the proportion of infected roots determines the disease outcome. Lackner (1981) observed seedling mortality following single, point inoculations with colonized wood placed on a slit on the taproot. This suggests that the location of fungal growth within the root system may influence the outcome. Impairment of taproot function can be expected to have a considerably greater effect on a plant than would dysfunction in some other portion of the root system. In the fumigation studies, *L. procerum* was isolated from 54% and 36% of the taproots of eastern white and loblolly pines, respectively. As previously noted, these trees exhibited neither disease symptoms nor mortality.

Although no visible disease resulted from stem inoculations, fungal growth in both species extended 4-5X further than the diameter of the inoculation wound. Wingfield (1986) suggested that his failure to obtain disease in 2-yr-old eastern white pine seedlings 5 mo following inoculation with *L. procerum* may have been

due to inoculating at the base of the stem rather than the roots. While this could be the case in the current work, colonization patterns of the fungus in diseased trees strongly suggest that under natural conditions, inoculation occurs at the root collar rather than on the roots (Horner 1985). The proximity of the lower stem to the root collar, coupled with the known movement of *L. procerum* in both directions from the point of inoculation (Horner 1985) make the base of the stem a likely inoculation point. Furthermore, Smith (1991) inoculated 1-yr-old eastern white pine by placing mycelium in a slit on the stem and reisolated the pathogen from seedlings exhibiting chlorosis, wilt and mortality approximately 4 wk later (Smith, pers. comm.).

An hypothesis that the lack of disease development in inoculated eastern white pine is due to differences in virulence (aggressiveness) among isolates of the pathogen has not been supported in studies (Lackner 1981, Wingfield 1985). There may, however, be variability among host species in their susceptibility to various isolates. Host specialization does occur in *L. wageneri*, the causal agent of black stain root disease of western conifers (Harrington and Cobb 1984). Lackner (1981) found reduced mortality in loblolly pine inoculated with a Scots pine (*P. sylvestris* L.) isolate when compared to two isolates from eastern white pine. Fewer loblolly than eastern white pine seedlings became infected in the current root inoculation studies with mixed loblolly and eastern white pine-derived inoculum. Furthermore, of those loblolly in which infection occurred, the proportion of infected roots was 29% to 46% lower than that in the eastern white pine. The similar growth of the pathogen in the stems of both host species suggests that the mechanism responsible for the decreased infection in the roots of loblolly pine is avoided in stem inoculations.

Effects of inoculations and fumigation

Fumigation with the relatively high ozone concentration of 200 ppb did not result in visible foliar injury of either eastern white or loblolly pine seedlings. This was not unexpected as needles were fully expanded and therefore past their most ozone-sensitive stage (Costonis and Sinclair 1969, Kress *et al.* 1982). In addition, the seedlings used in these studies were not selected based upon their ozone sensitivity. Visible injury is not required for the occurrence of ozone-induced alterations in metabolism and physiology of these pine species; exposure to ambient or near-ambient levels has been shown to decrease photosynthesis (Reich *et al.* 1987, Sasek and Richardson 1989), alter carbon allocation (McLaughlin *et al.* 1982), and reduce growth (Gerhold 1977, Kress 1978). Seedlings of an ozone-tolerant full-sib family of loblolly pine sustained 15% decreases in total and shoot dry weights after receiving an ozone dosage of only 7ppm·hr at a concentration of 80 ppb (Chevone and Yang, pers. comm.). Although the fumigation period in the current studies was short, a dosage of 14 ppm·hr was achieved due to the high ozone concentration.

The proportion of infected roots per seedling was not significantly affected by exposure to 200 ppb ozone in either of the species tested (Table 1). The increased infection incidence observed by Lackner and Alexander (1983) may have been due to a reduced capacity for defense following long-term exposure. Ponderosa pine trees exhibiting severe foliar ozone injury were significantly more susceptible than healthy trees to infection following inoculation with *H. annosum* (James *et al.* 1980a).

The possibility also exists that a greater incidence of *L. procerum* infection was observed in trees with visible ozone injury (Lackner and Alexander 1983) because the trees had been preferentially inoculated with the pathogen.

Stark *et al.* (1968) reported that two bark beetles, the western pine beetle (*Dendroctonus brevicomis* LeConte) and the mountain pine beetle (*D. ponderosae* (Hopkins)), more frequently attacked oxidant-damaged ponderosa pine than healthy trees, and that attack frequency increased with increasing severity of damage. Certain lower stem-and root-infesting insects, such as the pales weevil (*Hylobius pales* (Herbst)) are likewise more attracted to stressed than healthy trees (Rieske and Raffa 1993, Nevill and Alexander 1992a). This insect, the putative primary vector of *L. procerum* in Virginia Christmas tree plantations, routinely carries the pathogen and efficiently transmits it to eastern white pine seedlings and saplings in controlled studies (Nevill and Alexander 1992b). If ozone injury increases primary attraction of the vector and thereby, the probability of inoculation, fumigation following artificial inoculation is unlikely to alter the incidence of infection.

Ozone fumigation did not have a significant effect on the extent of stem colonization of either host species by *L. procerum* (Table 2). Increased colonization, presumably arising from decreased host defense mechanisms, has been observed in fumigation studies with *L. wageneri* and ponderosa pine seedlings (Fenn *et al.* 1990). Ozone-treated ponderosa and Jeffrey (*P. jeffreyi* Grev. and Balf.) pine saplings had significantly greater *H. annosum* colonization than did saplings receiving no ozone (James *et al.* 1980a). Common to studies in which significant interactions were found was moderate to severe foliar injury, whether hosts were naturally or experimentally ozonated. James *et al.* (1980a,b) found that the extent of *H. annosum* colonization in their studies was directly related to the amount of foliar injury. It is possible that visible injury to the foliage, whether of an acute or chronic nature, must be present in order for there to be sufficient impact upon the host to alter its capacity for defense against root pathogens.

Water relations

Neither fungal inoculation and growth, nor ozone fumigation, significantly affected the shoot water potential of eastern white or loblolly pine seedlings (Tables 3, 4), and no seedlings exhibited symptoms of water stress. Lackner and Alexander (1982) observed wilt 2 wk following *L. procerum* inoculation of eastern white pine seedlings, although water potentials were not measured. Rane and Tattar (1987) found increasingly negative water potentials in saplings of *P. thunbergiana* Franco and *P. sylvestris* L. beginning approximately 10 days after inoculation with *L. terebrantis* Barras & Perry.

There was no effect of fungal inoculation or ozone fumigation on the transpirational water loss root-inoculated ($p = 0.96$) (Table 5) and stem-inoculated ($p = 0.98$) (Table 6) loblolly pine seedlings or in stem-inoculated eastern white pine seedlings ($p = 0.99$) (Table 6). Transpirational water loss in root-inoculated eastern white pine did differ significantly between treatments ($p = 0.05$) (Table 5); seedlings which had received ozone, with or without accompanying inoculation, experienced increased transpirational water loss ($p = 0.02$). Reductions in stomatal conductance have been observed during ozone fumigations (Chevone *et al.* 1990). Maintenance of higher water potentials in red spruce (*Picea rubens* Sarg.) exposed to 150 ppb O_3 for 8 wk versus those exposed to charcoal-filtered air was thought to be due to reduction in transpirational water loss following ozone-induced stomatal closure (Roberts and Cannon 1989). In the current study, stomatal closure during fumigation would not account for the reduced water loss in the fourth week following exposure. The cause of the observed reduction is not known.

Table 3: Water potential of eastern white (EWP) and loblolly (LP) pine seedlings 6 wk following soil infestation with *Leptographium procerum* and ozone fumigation.

Treatment	Water potential (-MPa) (±S.E.)[1]	
	EWP	LP
c. f. air[2]	0.64 (0.02)	0.57 (0.03)
200 ppb ozone	0.60 (0.02)	0.60 (0.03)
c. f. air/*L. procerum*	0.61 (0.01)	0.57 (0.02)
200 ppb ozone/*L. procerum*	0.64 (0.02)	0.60 (0.02)
	P = 0.28	P = 0.97

[1] Each value is the mean of 8 replicates of 10 (*L. procerum*-inoculated) or 5 (uninoculated) seedlings per chamber.
[2] Charcoal-filtered air < 20 ppb ozone.

Table 4: Water potential of eastern white (EWP) and loblolly (LP) pine seedlings 6 wk following stem inoculation with *Leptographium procerum* and ozone fumigation.

Treatment	Water potential (-MPa) (±S.E.)[1]	
	EWP	LP
c. f. air[2]	0.67 (0.02)	0.86 (0.07)
200 ppb ozone	0.65 (0.02)	0.81 (0.06)
c. f. air/*L. procerum*	0.66 (0.01)	0.86 (0.04)
200 ppb ozone/*L. procerum*	0.67 (0.01)	0.91 (0.04)
	P = 0.84	P = 0.87

[1] Each value is the mean of 8 replicates of 10 (*L. procerum*-inoculated) or 5 (uninoculated) seedlings per chamber.
[2] Charcoal-filtered air < 20 ppb ozone.

Table 5: Transpiration (g H_2O/g foliage/7 d) of eastern white (EWP) and loblolly (LP) pine seedlings 6 wk following soil infestation with *Leptographium procerum* and ozone fumigation.

Treatment	Transpiration (±S.E.)[1]	
	EWP	LP
c. f. air[2]	40.2 (2.0)	29.3 (1.3)
200 ppb ozone	50.5 (3.4)	28.5 (1.1)
c. f. air/*L. procerum*	47.0 (3.7)	29.1 (1.1)
200 ppb ozone/*L. procerum*	55.5 (2.7)	30.0 (1.6)
	P = 0.05	P = 0.96

[1] Each value is the mean of 8 replicates of 10 (*L. procerum*-inoculated) or 5 (uninoculated) seedlings per chamber.
[2] Charcoal-filtered air < 20 ppb ozone.

Table 6: Transpiration (g H_2O/g foliage/7 d) of eastern white (EWP) and loblolly (LP) pine seedlings 6 wk following stem inoculation with *Leptographium procerum* and ozone fumigation.

Treatment	Transpiration (±S.E.)[1]	
	EWP	LP
c. f. air[2]	29.9 (1.9)	43.6 (3.3)
200 ppb ozone	29.9 (1.8)	40.9 (2.6)
c. f. air/*L. procerum*	29.6 (1.2)	41.7 (2.0)
200 ppb ozone/*L. procerum*	30.5 (1.2)	43.0 (1.8)
	P = 0.99	P = 0.98

[1] Each value is the mean of 8 replicates of 10 (*L. procerum*-inoculated) or 5 (uninoculated) seedlings per chamber.
[2] Charcoal-filtered air < 20 ppb ozone.

Ozone fumigation did not result in an increased incidence of root infection or increased colonization of stem tissue in these studies with eastern white and loblolly pine seedlings. Since the seedlings used in the study were standard nursery-grade stock, and not selected based upon their response to ozone, it is likely that they encompassed the normal range of genetic variability in ozone-sensitivity. Benoit et al (1982) estimated that 4% of the eastern white pine population in the Blue Ridge Mountains of Virginia was ozone-sensitive, with varying degrees of tolerance exhibited by the remainder. If 4% of the seedlings in the current studies were ozone-sensitive, and they did have an increased susceptibility to *L. procerum*, the effect would have been hidden amongst the responses of the ozone-tolerant trees.

The amount of *L. procerum* colonization in the root systems of seedlings was not evaluated in the studies. However, colonization may have been extensive given the high incidence of infection. The lack of effect on water potential or total transpiration following inoculation by either technique was therefore surprising. If occlusion of the vascular system did occur in the roots or stem, it was not of sufficient magnitude to induce a measurable effect on host water relations.

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Chapter 4

Discussion and Directions for Future Study

Little is known of the development or epidemiology of procerum root disease. The long-held hypothesis that inoculation or infection and death occurred within a single season or year was supported by several lines of evidence: mortality occurred very quickly following inoculations of seedlings in early greenhouse studies, death may occur within a few weeks to months following the appearance of symptoms in older trees in Christmas tree plantations, and weevil larvae or pupae are often found in the base of the stem in PRD affected trees. An alternative hypothesis, that *L. procerum* is acquired during the seedling stage and that symptom expression and death may not be apparent for several years, I base upon the following observations:

1. As described earlier, neither symptom expression nor mortality occurred in seedlings in these fumigation studies, or in numerous inoculation studies by other workers. Isolations from seedlings up to 2 yrs following inoculation indicated that the pathogen had successfully infected and colonized the host in all cases; in the current work there was abundant infection of roots and colonization of stem tissue. This suggests that the lethal consequences of *L. procerum* colonization are not generally manifested in seedlings.
2. Although older Christmas trees may die quickly following the onset of visible symptoms, none of the trees observed in the current physiology studies died over this period in spite of dry conditions and high levels of vascular occlusion. The symptomatic phase of the disease may persist for several years; trees with low water potential and low vascular occlusion may have survived one or more seasons following these studies.

3. Rather than the presence of weevil brood within a symptomatic tree indicating a causal relationship, it may be due to adult weevils being attracted to already diseased trees for oviposition activities. The adults which emerge from these trees are likely contaminated with *L. procerum* and may then transmit it to healthy trees during feeding, rather than breeding, activities. A preferred food of adult pales weevils is seedling stem tissue. In recent studies in Christmas tree plantations we isolated *L. procerum* from 100% of the feeding areas on stems and roots of seedlings which had been attacked by wild weevils. This is the first evidence of naturally occurring transmission of *L. procerum* to eastern white pine.

If it does take several years for trees to manifest symptoms, seedlings are a poor model for investigating procerum root disease. Long-term studies have been initiated to annually evaluate the disease process in inoculated seedlings and trees. The above hypothesis has serious implications for disease control measures, which to date have been ineffective in reducing PRD in mature Christmas trees. If inoculation occurs at the seedling stage, protection with an appropriate insecticide early in the rotation may result in long-term disease control.

Our fumigation studies showed that exposure to high concentrations of ozone post-inoculation did not increase *L. procerum* susceptibility in seedlings which were likely principally ozone-tolerant. It is possible that exposure injury to older, ozone-sensitive trees over many years in the field may result in a predisposition to the pathogen. As mentioned in Chapter III, ozone injury may serve to increase the host attractiveness to the vector. In this case, weevils may be attracted to the stressed trees for oviposition and simultaneously introduce the fungus. I would suggest that *L. procerum* acts as a contributing factor (sensu Manion), at most, in an overall decline of these trees.

The physiology studies of diseased eastern white pine demonstrated a strong linear dependence of host water relations on vascular occlusion. Of particular interest are those trees whose response to vascular occlusion was much lesser or greater than expected. As discussed in Chapter II, this may have been due to site factors or to inaccuracy in the estimation of vascular occlusion. It should be noted that inaccuracies of this sort would have served to underestimate the amount of occlusion, and would not explain those trees with both high occlusion and high pre-dawn water potential. The contribution of site factors to the development of PRD is currently being investigated. This work is designed to elucidate the role of soil moisture in the disease process, and will perhaps provide information on the strength of the relationship between vascular occlusion and development of wilt in trees with PRD.

It is not evident why the resin response in the *L. procerum*-eastern white pine interaction should be so great in comparison with that in similar host-pathogen systems. The speed with which the various diseases progress may be of importance. Most staining fungi are introduced into trees through mass attacks of the bole by vector beetles, and death follows very quickly. If death of eastern white pine does not occur for several years following *L. procerum* infection, ample time would be available for resin to accumulate. The means by which death occurs may also differ between *L. procerum* and these other fungi. Although drying of sapwood occurs after inoculation with other staining fungi, mortality is thought to be due to girdling of the phloem and cambium. My casual observations have been that even in trees nearing death from PRD not more than ≈50% of the circumference of the cambium has been killed. If this is the case, the difference in disease progression between PRD and disease caused by other staining fungi may be due to the vastly different inoculation intensities experienced by the hosts.

Vita

Jodi Ann Carlson was born in Penticton, British Columbia on November 4, 1954. She graduated from Centennial Senior Secondary School in Coquitlam, British Columbia in June, 1972. Studies commenced in the Biology Department of Simon Fraser University, British Columbia, in 1975 following a year of travel in Europe. The B. Sc. was awarded in 1980 and a Master of Pest Management degree was begun at the same institution in 1981 under the supervision of Dr. J. Rahe. In August of 1982 Ms. Carlson began work as a research scientist at a private R&D company in Victoria, British Columbia and completed her degree in 1985. She left work in August of 1987 to begin doctoral studies at Virginia Polytechnic Institute and State University under the direction of Dr. S. A. Alexander. The dissertation was defended on February 8, 1994.