ETIOLOGIC STUDIES OF *Verticicladiella procera* Kendr. IN PINE CHRISTMAS TREES

by

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(ABSTRACT)

Colonization of Pine Christmas trees by *Verticicladiella procera* Kendr. causes Procera root disease. Little is presently known regarding the pattern and effects of fungal development within colonized trees. The present studies were undertaken to elucidate the developmental pattern of the fungus in colonized trees, to gather information on possible mechanisms and physiological effects of disease development, and to explore the relationship between *V. procera* and other, well documented bluestain fungi. The presence of cellulose was demonstrated in the cell walls of *V. procera*, indicating the probable genetic relatedness of this fungus with *Ophiostoma* (*Ceratocystis*) bluestain fungi. Inoculation studies revealed that the fungus could penetrate wounded sapwood, and that colonized seedlings had lower water potentials than uncolonized seedlings. In addition, it was found that the fungus could persist in resinous stem lesions for 22 months without foliar symptoms, and resinous stem

lesions with the fungus were significantly longer and deeper than wound lesions. An intensive isolation study revealed that the initial point of colonization in a tree is apparently at the root collar, progressing acropetally in both directions. Analysis of radial growth from increment cores showed that colonized trees had grown more slowly for the preceding three years than uncolonized trees. The sapwood moisture content of these cores was also significantly reduced in the colonized trees, indicating that the stem was drying out as symptoms developed. Histological examination of colonized sapwood showed that fungal colonization of tissues progressed along rays and resin ducts, in a fashion similar to that of bluestain fungi. Permeability measurements demonstrated that symptomatic sapwood, either resin-soaked or black-stained, had significantly reduced water movement relative to asymptomatic sapwood.

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Chapter I

Introduction

The earliest Christian use of evergreens at the time of the winter solstice is attributed to Martin Luther (1483-1546). Sowder (1949a) relates that Luther attempted to reproduce the glory of snowflakes sparkling on the evergreens by attaching candles to a conifer. The custom of trimming trees at Christmas apparently remained in the Rhineland for two centuries. It was beginning to spread in central Europe though, when Hessian mercenaries were unsuccessfully taming rebellious English colonies. Homesick Hessians, trying to liven up their holidays, are thus credited with introducing Christmas trees to North America.

Christmas tree demand long ago outstripped the number of available trees of a size appropriate for a front parlor or living room. Early in the 19th century, the tradition of a Christmas tree had taken hold in much of the United States. By the mid-20th century, the demand for Christmas trees had generated an industry with an estimated U.S. production in 1949 of 21.4 million trees (Sowder, 1949b). Current production in Virginia alone is estimated at 2.2 million trees in 1985 valued at 30 million dollars. The Christmas tree has thus become a center of economic as well as cultural attention.

With the advent of a market for Christmas trees,

commercial interest and production techniques soon developed. Growing large numbers of trees in one place, i.e. cultivation, is the most commercially feasible, and therefore profitable, means of tree production. Since cultivation also favors the pests of trees, it is not surprising that cultivation of trees for use as Christmas trees exacerbated problems with pests. One pest problem of recent notice in Virginia (Anderson and Alexander, 1979; Lackner and Alexander, 1982; 1984) and other states (Towers, 1978) is Procera root disease (PRD) caused by Verticicladiella procera Kendr. (Lackner and Alexander, 1982; 1983). Lackner and Alexander (1982, 1984) reported considerable losses of Christmas trees by producers in Virginia due to this disease. Since PRD is poorly understood, and of economic importance, an understanding of the disease is needed so that disease management strategies can be developed.

Literature concerning Procera root disease is scant. Etiologic studies of the disease are needed. The central purpose of this research was to explore the etiology of the disease, particularly the symptom development phase. Symptoms used in the diagnosis of PRD are suggestive of drought stress, e.g. reduced shoot elongation, uniformly discolored foliage and wilted needles, but the water relations of infected trees have not been described. The manner in which the fungus progresses in the tree, within

and among tissues, also remains to be described. Knowledge of the etiology of PRD might lead to developing control measures or provide clues to the mechanism(s) of pathogenesis.

The objectives of these investigations were to describe events associated with disease development, and search for possible mechanisms of pathogenesis. Specifically, the course of colonization within trees and within tissues was sought to provide insights regarding initial points of penetration and routes of colonization. An examination was made for interactions between V. procera colonization and radial growth since shoot growth is known to be reduced in colonized trees. In addition, the cell walls of V. procera were assayed for the presence of cellulose. Cellulose is very rare among the fungi, occurring only in Ophiostoma H. & P. Sydow (Ceratocystis Ellis and Halsted) of the Eumycota (Bartnicki-Garcia, 1968; von Arx, 1974). Determining the presence of cellulose in V. procera could indicate a common phylogeny between V. procera and Ophiostoma.

Chapter 2

Literature Review

Introduction.

The first report of lethal infections of Eastern white pine (*Pinus strobus* L.) by *Verticicladiella procera* appeared in 1967 (Dochinger, 1967). The fungus was reported to be killing E. white pine in six eastern states from Maryland and Pennsylvania to Indiana. The trees had delayed bud break, exhibited wilt symptoms and were girdled by a basal canker. Since this early report, *V. procera* has been found in a wide range of habitats including Christmas tree plantations (Anderson and Alexander, 1979; Lackner and Alexander, 1982, 1984), seed orchards (Webb and Alexander, 1982), landscape plantings and thinned forest plantations (S. A. Alexander, pers. commu.) and natural forest stands (Lackner and Alexander, 1983; Livingston and Wingfield, 1982; Bertagnole and Partridge, 1982). These occurrences were found from the mountains (Lackner and Alexander, 1982) to the coastal plain (Webb and Alexander, 1982). Towers (1977) reported that tree mortality had been associated with this fungus throughout Pennsylvania with 20% of the trees in one stand dead or dying. In a survey of eight Christmas tree plantations in Virginia, losses exceeded 700 saleable trees (6-10 yr old) valued at $5-$15 each in 1982 (Lackner and Alexander, 1982).

The broad range of habitats in which V. procera has been found generated concern about its potential impact on silvicultural activities in the East. A thorough understanding of this disease is central to developing effective control measures. In particular, knowledge of the mechanism(s) involved in symptom development and the means of fungal spread within and among trees is needed.

Pathogenicity reports.

Reports of successful inoculations of E. white pine with V. procera appeared in early abstracts concerning the association of V. procera with diseases of E. white pine. The fungus was found associated with basal cankers of E. white pine in New York (Houston and Eno, 1965), but inoculations showed it to cause annual cankers rather than the lethal cankers prevalent on the study site (Houston, 1969). Houston (1969) did note less callus around cankers formed by V. procera than around other annual cankers in his study. This suggests that the fungus was preventing the production of callus. Dochinger (1967) reported that "pathogenicity was established" but included no details of method other than the plants were root dip inoculated. Halambek (1976) in Yugoslavia also reported fulfilling Koch's postulates by killing E. white pine seedlings with V. procera. This report also indicated that root dip inoculations were used. Shaw and Dick (1980) effected infection and colonization of wounded root tissue of

pole-sized E. white pine in New Zealand with V. procera. Their inoculations resulted in formation of lesions proximal to the inoculation point although crown symptoms were not evident one year later.

This body of information was viewed as inconclusive by many plant pathologists. Therefore, additional pathogenicity studies were undertaken and reported by Lackner and Alexander (1982, 1983). Inoculations of two-year-old E. white pine seedlings by root-dipping in a conidial suspension and by insertion of colonized blocks in taproot wounds resulted in the death of 50% and 25% of the trees, respectively (Lackner and Alexander, 1982). In a second study, E. white pine and loblolly (Pinus taeda L.) pine were each inoculated with one of three isolates. Each of the six treatments resulted in 85%-100% of the seedlings becoming symptomatic or dead (Lackner and Alexander, 1983). Reisolations yielded V. procera thus demonstrating the pathogenicity of V. procera. They also found that one isolate killed significantly fewer loblolly than E. white pine seedlings.

Four other inoculation trials yielded less definite results. Swai (1980) and Bertagnole (1981) each included inoculation trials in unpublished thesis work. Swai was generally unsuccessful in obtaining infection of E. white pine seedlings by V. procera using agar plugs inserted in wounds on roots or stems. Bertagnole filled increment borer

holes in the secondary xylem of lodgepole pine (P. contorta Dougl.) roots with match wood colonized by one of five Verticicladiella Hughes species, including V. procera. Although crown symptoms typical of black stain root disease (generally considered to be caused by V. wageneri Kendr.) were not seen after 14 months, V. procera did colonize tissue and cause lesions around the inoculation points. In another study on the various species of Verticicladiella found in association with black-stained conifer roots in the western U.S., Harrington and Cobb (1983) inoculated seedlings with several species, including V. procera. V. procera was readily reisolated from wound-inoculated taproots of seedlings. The absence of lethal infections by V. procera led these workers to conclude that V. procera is less virulent than V. wageneri, which they regarded as the single causal agent of "black stain root disease". Wingfield (1983b) reported that point inoculations of V. procera produced local lesions after 5 months in E. white pine in the field.

Although some discrepency exists concerning the results of pathogenicity tests of V. procera, it should be noted that evidence for differences in virulence among isolates has been reported (Lackner and Alexander, 1983). Several of the inoculations did not kill trees (Bertagnole, 1981; Harrington and Cobb, 1983; Wingfield, 1983b) but did produce root lesions. These attempts were deemed unsuccessful since

trees did not die, but were ended before colonization encircled the root collar. This is significant since reports based on field observations state that trees are killed when lesions coalesce at the root collar (Sinclair and Hudler, 1980). The limited duration of reported 'unsuccessful' inoculations, the indication of virulence differences among isolates and recent repeated fulfillment of Koch's postulates (Lackner and Alexander, 1982; 1983) should establish the pathogenicity of these Virginia isolates.

Symptoms, control measures and disease names.

The symptoms associated with V. procera root infections of E. white pine are a delayed bud break and reduction of shoot elongation followed by chlorosis, wilting and a uniform browning of the needles which may remain attached to the tree for a year or more (Towers, 1977; Anderson and Alexander, 1979; Alexander, 1980). A dark brown cambial discoloration may appear at the base of the stem of infected trees, where the bark is frequently marked by resin exudation (Lackner and Alexander, 1982). The wood of colonized roots is resin soaked, and stem cross sections frequently have prominent wedge-shaped black stain evident across the sapwood zone (Shaw and Dick, 1980).

The symptoms (reduced terminal growth, chlorosis and wilting of needles) suggest that water relations within the

plant are affected. The fungus colonizes an appropriate tissue of the plant to cause vascular dysfunction, as indicated by the black staining in the xylem of the butt and roots.

Control measures are necessarily general since so few data on the biology and means of spread of the fungus are available. Current suggestions for control are: 1) avoid planting E. white pine on wet sites, 2) remove diseased trees, including root systems, and 3) do not replant E. white pine in infested areas (Anderson and Alexander, 1979; Alexander, 1980).

Some confusion exists concerning the common name(s) of this disease. The most frequently encountered name is "white pine root decline". Lackner and Alexander (1982) stated that in Virginia the disease appears more as a wilt than a decline, and suggested the name "white pine wilt". The isolation of V. procera from other pine species makes either of these names confusing when applied to diseases of these other species. Alexander (1980) included the name "Verticicladiella wilt" in his pest management guide, but predictably this polysyllabic name has not gained acceptance. The applicable, descriptive and easily-remembered name, "black stain root disease" is already in use for a lethal conifer disease in the western U. S. caused by V. wageneri. Procera root disease has recently been proposed (S. A. Alexander, personal

communication) for conifer root diseases caused by V. procera. This name has the advantages of indicating the particular fungus involved, yet remaining general about host species.

Geographical and host ranges.

Verticicladiella procera has a worldwide distribution. It has been reported from Finland and Yugoslavia (Hallaksela, 1977; Halambek, 1976), New Zealand (Shaw and Dick, 1980; Wingfield and Marasas, 1983) and Ontario, Canada (Kendrick, 1962). In the U.S. V. procera has been reported from states in the southeast, mid-Atlantic, midwest, lake states and inland northwest. Particular states include Idaho (Harrington and Cobb, 1983), Minnesota, Wisconsin, Michigan (Wingfield, 1983), New York (Sinclair and Hudler, 1980) and from South Carolina to Pennsylvania and Indiana (Anderson and Alexander, 1979). In addition, V. procera has been isolated in Florida, Alabama, Tennessee and Mississippi (S. A. Alexander, pers. commu.).

Pine species are the most frequently listed hosts of V. procera. Reported pine hosts are E. white, Scots (P. sylvestris L.), Austrian (P. nigra Arn.) (Lackner and Alexander, 1982), jack (P. banksiana Lamb.), red (P. resinosa Ait.) ponderosa (P. ponderosa Laws.) (Wingfield, 1983), loblolly, Virginia (P. virginiana Mill.), shortleaf (P. echinata Mill.), slash (P. elliottii Engelm.) (Horner

and Alexander, 1983), sand (P. clausa (Chapm.) Vasey.) (Barnard, et al., 1982) and lodgepole (Bertagnole, et al., 1982). Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) is also a host (Harrington and Cobb, 1983) and the fungus has been isolated from Norway spruce (Picea abies (L.) Karst.) stumps (Hallakskela, 1977). In addition to those published reports, V. procera has been isolated from Fraser fir (Abies fraseri (Pursh) Poir.) (S. A. Alexander, personal communication).

Site aspects.

Several reports of the occurrence of V. procera root disease have included observations on site specificity. In reference to V. procera, Towers (1977) stated that "In Pennsylvania, diseased trees are usually associated with shallow, heavy clay, poorly-drained soils". The general acceptance of this association is indicated by its inclusion in extension publications from both federal and state agencies (Anderson and Alexander, 1979; Alexander, 1980). However, no quantitative association has been established between any site parameter and the incidence of PRD.

A recent report of V. procera from two areas in Minnesota stated that when E. white pines with V. procera-infected roots were on good sites, trees were not affected. At another site though, V. procera-infected E. white pines were dying (Livingston and Wingfield, 1982).

The only site description was that the trees at the latter location were on a hillside. Sinclair and Hudler (1980) reported that V. procera caused girdling cankers and death of E. white pine up to 20-yr-old in landscapes or forest situations in six counties in New York. They stated that affected trees were "always on soils with poor internal drainage". These authors also reported an association between V. procera colonization and root lesions on declining red pine on poorly-drained sites. Root samples were dug from different portions of a transect running between well and poorly drained portions of the plantation. Percent dead root length increased from 0% in the well-drained portion of the transect to 7.1% in an intermediate zone and 29.6% in the poorly-drained area (Sinclair and Hudler, 1980).

The association between trees colonized by V. procera and wet sites is not absolute, however. Alexander noted that wet sites had been associated with the presence of the disease but "this is not always the case" (Alexander, 1980). In a comparison of root diseases and management practices, V. procera was the primary pathogen found in declining/subsoiled loblolly pine seed orchard trees located on droughty soils (86-88% sand) (Webb and Alexander, 1982). Subsoiling was used to intentionally stress the trees.

Other site conditions noted in association with V. procera infections are root disturbances along temporary

access roads in New Zealand plantations (Shaw and Dick, 1980), and exposure to oxidant air pollution (Lackner and Alexander, 1983).

Clearly most of these reports concern the association with wet sites. The findings of V. procera in 3 of 10 declining/subsoiled seed orchard trees on droughty soils raises questions about the effects of soil moisture on V. procera root infections. The common component may be stress, either air pollution, unfavorable soil moisture levels which are either too high or too low, or disturbance due to construction.

Growth patterns in wood.

A frequently-cited symptom of trees infected with V. procera is the presence of black or resinous streaks extending from roots to the stem base (Lackner and Alexander, 1982; Alexander, 1980). This discoloration, when viewed in cross-section, is reported to extend across the sapwood zone (Shaw and Dick, 1980). Although not described in the literature, sap-staining by V. procera is often prominent as wedge-shaped areas in cross-sectional view, as reported for infections of conifers by other species of sapstain or blueing fungi (Himelick, 1982; Mathre, 1964; Rumbold, 1931).

At present, four reports mention growth patterns of V. procera in wood tissue. Halambek (1976) mentioned that

hyphae were in tracheids and bordered pit pairs. Shaw and Dick (1980) observed hyphae concentrated in axial tracheids, but also present in rays. It was subsequently noted (Wingfield and Marasas, 1983) that V. procera was one of three fungal species involved in this second study. These two studies were conducted with E. white pine. Two papers are available from the western United States which contain histological observations of V. procera in other conifers. Lodgepole pine roots were inoculated in one study which resulted in colonization of axial tracheids and ray parenchyma (Bertagnole, et al., 1982). Harrington and Cobb (1983) inoculated Ponderosa pine and Douglas-fir seedlings with several species of the Leptographium Lagerberg and Melin complex, including V. procera. Their histological observations on "these other species" mentioned pigmented hyphae in both tracheids and ray parenchyma, but emphasized the radial extent of colonization. There is a discrepency among these reports regarding relative colonization of axial tracheids and rays in pine sapwood by V. procera. One author (Halambek, 1976) emphasizes tracheids, two (Shaw and Dick, 1980; Bertagnole, et al., 1982) report hyphae in tracheids and rays with no mention of relative abundance or extent, and the most recent (Harrington and Cobb, 1983) mentions tracheids and rays, with the emphasis on radial extent of colonization.

Ballard et al. (1984) described the development of

bluestain in lodgepole pine attacked by mountain pine beetle. No attempt was made to identify the fungi in that report, but the fungi staining lodgepole pine in other reports are Ceratocystis (including Europhium Parker), Verticicladiella and Leptographium species (Robinson, 1962; Robinson-Jeffrey and Grinchenko, 1963; Robinson-Jeffrey and Davidson, 1968). Ballard, et al. (1984) report that growth of the bluestain fungi in their system was confined initially to rays, with only 5-18% of tracheids occupied by hyphae at the inner margin of visible stain. Later stages of bluestain development had "considerably more" colonized tracheids. This is in agreement with previous histological studies of bluestain development (Lagerberg, et al., 1927; Bakshi, 1951).

The wedge-shaped stain pattern of V. procera, usually confined to the sapwood, is similar to macroscopic patterns of bluestain. Assuming that colonization of sapwood by V. procera at the cellular level also follows the bluestain pattern, the discrepencies noted above may represent different degrees of colonization.

A study using transmission electron microscopy of beech (Fagus sylvatica L.) wood colonized by V. procera in an agar block test revealed cell wall erosion, suggesting a cellulolytic capability for V. procera (Kilbertus et al., 1980). Although V. procera has not been reported on beech in nature, it is interesting to note that a lignified

substrate was degraded.

The stain pattern, evident in cross-section, is similar to that noted by both Mathre (1964) and Basham (1970) for Ceratocystis spp. Both found in dye conductance studies that sap was not conducted through stained areas, nor in adjacent unstained areas. This was interpreted by those authors as evidence that bluestained sapwood did not adequately supply the crown of bluestain infected trees. Sapwood dysfunction due to bluestain colonization was considered to be the mechanism underlying stem drying and crown death in bluestain infected trees. Determining the ability of V. procera stained xylem to conduct sap would be useful in establishing the etiology of the wilt symptoms caused in pines by V. procera. One way in which this may be estimated is to determine the permeability to water of affected sapwood (Kelso, et al., 1963; Comstock, 1965).

No mention is evident in the literature of the presence of gels, gums or insoluble carbohydrates reported as general blocking agents in dicot vascular wilt diseases (Subramanian, 1983).

Insect associations.

The suggested association between V. procera and insects remains to be fully elucidated. There is however, a large body of information concerning associations between insects and Ophiostoma (Ceratocysis) bluestain fungi

(Mathieson-Kaarik, 1960; Hunt, 1956; Verrall, 1941; Mathre, 1964; Findlay, 1959; Bakshi, 1950). Associations between insects and members of the Leptographium complex have also been established. There is substantial evidence linking the long distance dispersal of V. wageneri to the activity of root-feeding bark beetles (Harrington, et al., 1985; Goheen and Cobb, 1978). Livingston, et al. (1983) examined pockets of dying and recently dead Ponderosa pine, which were colonized by stem bark beetles, for root bark beetles and diseases. There was a significantly greater proportion of diseased primary roots among trees with stem bark beetles than trees without stem bark beetles. Assuming that root disease requires more time than bark beetle infestations to attain detectable levels, it is implied that the root infections preceded the bark beetles. Root bark beetles were also always present in a tree if stem bark beetles were present and present in adjacent trees uncolonized by stem bark beetles. This implies that the root bark beetles and pathogenic fungi together were preceding the stem bark beetles. Three Verticicladiella species were among the fungi isolated from diseased roots with root bark beetles.

Dowding (1969) demonstrated that fungi with sticky spores, i.e. gloiospores, such as V. procera, are poorly dispersed by air currents. Included in that study were species of Ceratocystis, Leptographium, three dry-spored species (Penicillium sp., Trichoderma viride and

Cladosporium herbarum) and a non-blueing, sticky-spored species (Gliocladium sp.). Dispersal of these spores was examined in a wind tunnel using dry air, dry air with particulate matter, mists of water, glycerol and paraffin. Dowding found that spore dispersal of the gloiosporous species was favored only in hydrophilic mists or by splashing drops of water. Dowding concluded from this study that the likely means of dispersal of gloiosporous-type species such as V. procera was not by air currents but by splash-drops or by insects. Since splash drops will not function for long range dispersal, insect involvement is suggested.

A specific association between V. procera and insects, is strongly suggested by several lines of evidence. Lackner and Alexander (1982), noted the presence of bark beetles and weevils in dying trees that were colonized by V. procera. They later isolated V. procera from roots infested by weevils (Lackner and Alexander, 1983) and subsequently from adult bark beetle (Lackner and Alexander, 1984). In a report of V. procera from Minnesota, Livingston and Wingfield (1982) noted Pissodes weevils in the roots of five of six trees from which the fungus was isolated. Wingfield (1983a) subsequently isolated V. procera from weevil (Hylobius spp.) infested pine roots in Wisconsin and Michigan. Lewis (1985) obtained convincing evidence of an association between V. procera and weevils by isolating the

fungus from a high proportion of weevils trapped in a survey of insects in Christmas tree plantations. Bark beetles recovered in this survey also carried the fungus, but in a lower proportion.

The most incriminating evidence for the involvement of insects as vectors of V. procera is the recovery of the fungus from Lewis' trap bolts. Tissue was cultured from these split pine stem sections, to verify the absence of the fungus, prior to use of the sections as trap bolts. After exposure in the field, V. procera was recovered from these bolts. A subsample of the weevils recovered were caged with a second set of freshly split pine bolts. The second set was also verified, by tissue plating, to be free of V. procera prior to use. After exposure to insects with V. procera, in muslin-covered cages, the fungus was also recovered from the second set of stem sections.

The existence of a specific vector association between V. procera and (an) insect(s) remains to be proven although strong suggestive evidence is available. The work of Cobb, et al. (1973) and Alexander, et al. (1981) has shown that bark beetles preferentially infest trees with root disease, which would potentially expose the insects to spores. Lackner and Alexander have shown that trees colonized by V. procera are often infested with bark beetles from which the fungus can be isolated. Wingfield (1983a) and Livingston and Wingfield (1982) determined that V. procera and weevils

are frequently present in the same roots. Lewis (1985) has established that naturally infested weevils can inoculate V. procera into host tissue (freshly cut, split pine bolts). It is reasonable to assume from this evidence that insects are associated with V. procera in diseased pine roots, and that some of these insects may be vectors of the fungus. It only remains to be demonstrated under controlled conditions whether any of these insects can actually effect a successful inoculation and kill a tree.

Verticicladiella wageneri

A salient difference between the root disease caused by V. procera and black stain root disease (BSRD), caused by V. wageneri, is that BSRD has received more attention, and is therefore, better understood. The disease was first reported in 1938 and pioneering work on BSRD started in the early 1960s (Wagner and Mielke, 1961). Unlike V. procera, the pathogenicity of V. wageneri has never been seriously questioned. Early workers established the pathogenicity of V. wageneri (Wagner and Mielke, 1961), which has been subsequently corroborated (Cobb and Platt, 1967; Smith, 1967).

The symptoms of BSRD are a reduction in the growth of the terminal shoots, reduced needle retention, thinning and yellowing of the crown. These symptoms are similar to those of root diseases common in the western U.S. caused by

Inonotus weirii (Murrill) Kotl. & Pouz. and Armillaria mellea (Vahl. ex Fr.) Kummer (Hunt and Morrison, 1979; Smith and Graham, 1975). Internal symptoms include a dark, usually black staining in the main roots and butt of infected trees. This stain, when seen in cross-section, is evident as narrow, concentric discolored bands which are restricted to the outermost growth rings (Hunt and Morrison, 1979). This is in marked contrast to the stain pattern found in stems and roots colonized by V. procera which is wedge-shaped and deeply penetrating.

As of 1979, control measures specifically for BSRD had not been developed. Suggested practices to reduce the severity of BSRD include: minimizing tree disturbance during road-building operations, harvesting heavily infected stands and regenerating with non-host species, and limiting the expansion of extant disease centers by felling all trees in a buffer strip around the center (Hunt and Morrison, 1979; Smith and Graham, 1975). The existence of disease centers of BSRD is in contrast to the irregular distribution pattern of trees with PRD within a stand of trees. The name 'black stain root disease' is prevalent and generally accepted in the current literature for the conifer root disease caused by V. wageneri.

The geographical range of BSRD includes Canada and the U.S. west of the continental divide. It has been reported in states from Arizona (Walters and Walters, 1977) to

Washington (Goheen and Hansen, 1978) and Colorado (Landis and Helburg, 1976), and in British Columbia (Hunt and Morrison, 1979).

The major host species reported for BSRD include pinyon pines (P. monophylla Torr. and Frem., and P. edulis Engelm.) in the southwest as well as Ponderosa pine and Douglas-fir in the Pacific northwest, California and British Columbia. Other coniferous host species have been reported (Smith and Graham, 1975).

The disease is affected by site. Goheen et al. (1978) established a positive correlation between disease severity and soil moisture. In Oregon, the disease is more prevalent in roadside strips, than in forest away from roads. This suggests root disturbances incurred during road construction are favorable to disease development (Hansen, 1978).

The local spread of BSRD has been shown by excavation of trees to occur via root grafts and close root contacts (Landis and Helburg, 1976). Goheen (1971) also found local spread through root contacts. He found however that local spread was most frequent through small rootlets not particularly close to another root. He concluded that the exact mode of local spread was still uncertain. No such means of spread has been noted for V. procera.

Several aspects of BSRD were elucidated by the discovery and description of perithecia of Ceratocystis wageneri Goheen and Cobb in insect galleries (primarily Hylastes

macer Lec.) in the roots of trees with BSRD. The discovery of the teleomorph of the BSRD fungus established that the fungus was in fact a species of Ceratocystis (Goheen and Cobb, 1978). The presence of the fungus sporulating in insect galleries was cited as concrete evidence to support the hypothesis of an insect vector. In addition, there were more perithecia in the galleries of H. macer than in the galleries of three other insects where perithecia were found. Cobb et al. (1973) found that bark beetles preferentially infest trees with root disease. In the case of fungi (such as species of Verticicladiella with sticky spores which may adhere to insects), this attraction to root-diseased trees would potentially bring the insects in contact with inoculum sources. The long-range spread of V. procera may involve insects as shown by Goheen for V. wageneri.

The question of host specificity of V. wageneri strains from different regions and hosts has received attention. The consistent morphological differences between isolates from pinyon in the southwest and Ponderosa pine in California have been recognized (Smith, 1967). Pathogenicity comparisons between these isolates on various hosts have yielded different results. Smith (1967) inoculated and killed both pinyon and Ponderosa pine with isolates from the other species. Harrington and Cobb (1982) included a third morphological group of isolates, that from

Douglas-fir, in a group of inoculations on Ponderosa pine and Douglas-fir. Their results indicated a significant difference between percent infection of the two host species indicating some variation in pathogenicity of these isolates.

The physiology of artificially-inoculated Ponderosa pine seedlings has been studied by Helms et al. (1971). They examined foliar water stress, transpiration rates, net photosynthesis and stomatal closure. One month after inoculation, prior to symptom development, net photosynthesis had decreased substantially (but not significantly at P=0.05), transpiration had decreased, and foliar water stress had increased significantly. At the two month sampling period, net photosynthesis had become negative. These authors attributed this to an inhibition of photosynthesis resulting from increased foliar water stress and stomatal closure. An additional conclusion was that the measurement of foliar water stress was the best single technique for establishing the relative health and vigor of seedlings.

The concentric growth pattern of V. wageneri in wood is unlike the patterns known for other species of Verticicladiella. Further, the stain does not traverse the sapwood zone but rather colonizes only the outermost annual rings of sapwood (Hunt and Morrison, 1979; Smith and Graham, 1975). Microscopically, the fungus colonizes only axial

tracheids, traversing the cell walls solely via bordered pit pairs (Smith, 1967; Wagener and Mielke, 1961). Smith mentions that hyphae were observed to abut half-bordered pits, but ray parenchyma cells were never seen to be penetrated.

Other species of Verticicladiella.

There are several reports of root diseases of pines associated with other species of the genus Verticicladiella. These come from the Pacific northwest, Italy, South Africa and New Zealand.

Four reports from the University of Idaho found Verticicladiella species involved in a 'root stain disease complex'. Kulhavy et al. (1978) reported isolating four species of Verticicladiella from western white pine (P. monticola Dougl.). One of these was V. wageneri which is widely accepted as the single causal agent of black stain root disease. The other three species, V. antibiotica Kendr., V. penicillata (Grosm.) Kendr., and V. abietina (Pk.) Hughes, were isolated but not tested for pathogenicity. Mielke (1981) later presented results of inoculation studies with V. penicillata which showing that this fungus could infect and colonize conifer roots. Although no trees were killed in the 160-day test period, root lesions were produced. Histological analysis revealed that hyphae were concentrated in the axial tracheids, but

also occurred in the ray parenchyma and that cell walls were traversed via bordered pit pairs.

Bertagnole and Partridge (1980) presented results of a three-year study concluding that the 'root stain disease complex' of conifers in the inland northwest was the result of a concert of stain fungi. This included five species of Verticicladiella in addition to species of Leptographium, Graphium, Ceratocystis and other stain fungi. Bertagnole (1981) later inoculated the roots of lodgepole pine (P. contorta Dougl.) with five Verticicladiella species: V. serpens (Goid.) Kendr., V. procera, V. penicillata, V. abietina and V. antibiotica. All except V. antibiotica produced lesions from which the particular fungus was recovered. As in other studies, trees were neither killed nor crown symptoms reproduced at the time of reisolation and measurement of lesion sizes.

Harrington and Cobb (1983) took exception to the concept of a 'root stain disease complex' and present results which they believed refuted it. They were able to reproduce the symptoms of black stain root disease only with V. wageneri of seven species of Verticicladiella and Leptographium used in their inoculation studies.

Wingfield and Marasas (1983) described a new species, V. truncata Wingfield and Marasas, from E. white pine in New Zealand and loblolly pine in South Africa. The pathogenicity of this species was established by inoculation

studies with slash pine.

Wingfield and Knox-Davies (1980) were able to produce lesions on roots of Monterey pine (P. radiata D. Don) and French maritime pine (P. pinaster Ait.) with an isolate of V. serpens (as V. alacris Wingfield and Marasas) from diseased pines in South Africa. The incidence of this disease was also common along roads, suggesting an association between root disturbances and disease incidence. Lorenzini and Gambogi (1976) also reported a root disease of Italian stone pine (P. pinea L.) caused by an isolate of Verticicladiella which they later determined to be V. serpens (Gambogi and Lorenzini, 1977). In both of these cases, symptoms included reduced growth of terminals, yellowing and wilting of needles and staining in the roots and/or butt of infected trees. Dark hyphae were noted in the tracheids of stained wood by Wingfield and Knox-Davies (1980).

History and characteristics of the genus Verticicladiella Hughes

The taxonomic value of conidiogeny was recognized early by Vuillemin (1910) and Langeron (1945). Hughes (1953) extended the use of conidiogeny to deuteromycete systematics and argued for the use of conidiogeny to establish major groupings among the deuteromycetes. Hughes, (1953) established several new genera and segregated those members

of Leptographium with sympodular conidiogeny into Verticicladiella.

A brief description of sympodular conidiogeny (= 'Section II' of Hughes, 1953) and the morphology of a Leptographium-type conidiophore suffices to characterize Verticicladiella. Hughes (1953, p. 581) defined the sympodula conidiogeny of Verticicladiella as:

"conidia arising as blown-out ends of apex of simple or branched conidiophores and the ends of successively produced new growing points developing to one side of the previous conidium".

Leptographium-type conidiophores are distinguishable from the vegetataive hyphae (macronematous), have a stipe consisting of a single hypha (mononematous), which becomes repeatedly divided into branches (metulae) at several levels near the apex and bear brush-like clusters of conidiogenous cells (sympodulae). The conidiophores are in part darkly pigmented (dematiaceous) and produce conidia in a drop of mucilage (gloiosporous).

The genus Verticicladiella was later reviewed (Kendrick, 1962) at which time V. procera, V. wageneri (as V. wagenerii) and other new species were described. Subsequently, several species of Europhium Parker (=Ceratocystis sensu Upadhyay) and Ceratocystis have been

described with Verticicladiella anamorphs (Robinson-Jeffrey and Davidson, 1968; Kendrick and Molnar, 1965; Davidson, 1971; Griffin, 1968). New species of Verticicladiella, without teleomorphs, have also been described (Wingfield and Marasas, 1980, 1983). which apparently have modes of conidiogeny intermediate between sympodular and annellidic. This has also been found in Leptographium and has resulted in the recent proposal to discard the distinction between these two anamorph genera (Wingfield, 1985a).

The Ceratocystis Connection

Two independent lines of evidence suggest that V. procera is an anamorph of a Ceratocystis species. All known anamorph-telemorph connections of Verticicladiella species are with species of Ceratocystis or Europhium (=Ceratocystis sensu Upadhyay) (Kendrick, 1962; Robinson-Jeffrey and Davidson, 1968; Kendrick and Molnar, 1965; Subramaniam, 1983). This criterion alone is not definitive. The absence of exceptions does strengthen its suggestion of a link though. Tolerance of the antibiotic cycloheximide, rare among eukaroytes, is common in certain sections of the genus Ceratocystis and is also a characteristic of V. procera (Harrington, 1981). This rare tolerance provides the basis of selective media for species of Ceratocystis (Hicks et al., 1980; Vaartaja, 1968) and for V. procera (McCall and Merrill, 1980; Swai and Hindal, 1981).

Harrington (1981) surveyed 53 species of fungi, known or thought to be related to Ceratocystis, for tolerance to cycloheximide. The distribution of cycloheximide tolerance among these fungi correlated well with earlier work by Jewell (1974) concerning the distribution of species within Ceratocystis with cellulosic cell walls. The near perfect correlation of these two traits provides an opportunity to predict that V. procera, which has a known tolerance of cycloheximide, also has cellulosic cell walls, if it is related to Ceratocystis.

The significance of cellulose within the realm of 'higher fungi', beyond Ceratocystis, lies in the rarity of its occurrence. Among the major groups of 'fungi' sensu lato, only the Acrasids, oomycetes and hyphochytridiomycetes regularly possess cellulosic cell walls. Cellulose is considered to be virtually absent from ascomycetous cell walls (Upadhyay, 1981; Bartnicki-Garcia, 1968) but anomalously occurs with regularity among species of Ceratocystis. This includes several species with Verticiladiella anamorphs (Jewell, 1974; Rosinski and Campana, 1964). Considering the anamalous nature among the 'higher' fungi, of cellulosic cell walls in Ceratocystis, a positive determination for cellulose in the cell walls of V. procera would be a strong indication of affinity with Ceratocystis.

The importance of a possible connection between V.

procera and the genus Ceratocystis lies in the wealth of information available concerning pathogenesis by Ceratocystis species on conifers. The general symptoms of uniform discoloration, rapidity of foliar symptom development, and prominent sapwood staining in a wedge pattern, are similar to of sapwood diseases associated with bark beetle infestations. These similarities justify an attempt to look for any such link between V. procera and Ceratocystis. If there is a link, then research approaches might be suggested from prior work on these other sapwood diseases.

Nelson (1934) established that infections of Ceratocystis blueing fungi, which almost invariably accompany bark beetle attacks, resulted in a reversal of the normal gradient of increasing sapwood moisture content from the base to the top of trees. He further found that the moisture content of stained wood was reduced and that stained wood would not conduct sap as indicated by dye transmission studies. Similar results were obtained by Caird (1935) who correlated Ceratocystis infections with a drying of sapwood and concommitant failure to conduct dye solutions. Additional reports of Ceratocystis infections of conifers, associated with bark beetles and vascular dysfunction, in the older literature include those of Bramble and Holst (1940) and Craighead and St. George (1940).

More recently, Molnar (1965) demonstrated the pathogenic nature of C. dryocoetidis Kendrick and Molnar associated with Dryocoetes confusus Sw., the western balsam bark beetle, on true firs. Crown symptoms were seen only after lesions caused by C. dryocoetidis had coalesced around the stem. Effects on stem moisture content were not determined in this study however. Mathre (1964) and Basham (1970) also demonstrated that the pathogenic nature of blueing species of Ceratocystis was associated with stem wood drying and concomitant failure to conduct dye solutions. Himelick (1982) has reproduced the wilting and drying symptoms of wilt, caused by the pine wood nematode (Bursaphelenchus xylophilus) with inoculations of several pine species with C. ips.

The drying of sapwood commonly associated with infections of blueing fungi is thought by Newbanks et al. (1983) to result from embolisms introduced by the degradation of host cell walls by fungi. Experimental evidence for this theory was derived from studies on Dutch elm disease, caused by C. ulmi. These studies show evidence of embolisms in the absence of hyphae or occluding gums (Newbanks et al., 1983). Nelson (1934) suggested that the mechanism for sapwood drying by blueing fungi involves pit aspiration. Bramble and Holst (1940), however, suggest that tracheid plugging by resin globules, rather than pit aspiration, is the mechanism. One of these proposed

mechanisms may be applicable to symptom development in E. white pine colonized by V. procera.

Summary.

Economic losses have resulted across the eastern U. S. from the death of marketable Christmas trees that are colonized by V. procera, for which Procera root disease was recently proposed as the common name. The fungus has been found in diseased conifer roots in seed orchards, plantations and natural forest stands. The impact of the fungus is not fully known, but is potentially serious. The fungus is known to occur in Europe, across North America and in two areas of the southern hemisphere where pines were planted. Although pathologists have sometimes obtained varying pathogenicity results, Lackner and Alexander (1982, 1983) successfully fulfilled Koch's postulates. The symptoms associated with colonization by V. procera are reduced shoot elongation, chlorosis, wilting, sapwood resinosus and the development of wedges of black stain in the sapwood. These symptoms are reminiscent of the bluestain infections associated with the dying of beetle-infested conifers.

Incidence of the disease was first related to poorly drained or low areas. The disease is now known to occur also on droughty soils and along forest roads where roots likely were damaged during construction. The common

component of these situations may be abiotic root stresses. Development of V. procera in host tissues has not been described in detail. It has been reported to colonize ray parenchyma, and the wedge-shaped pattern of stain suggests that ray colonization is prevalent. This also is similar to bluestain fungi. The fungus has been reported in association with subcortical feeding insects in several situations. Although no vector relationship has been proven, weevils have been shown to carry the fungus in high proportions and are capable of inoculating fresh pine bolts with V. procera.

The genus Verticicladiella was erected as a segregate of Leptographium. Leptographium contains bluestain fungi which are asexual states of Ceratocystis species, where known. All three genera contain species which share the rare traits of cellulosic cell walls and cycloheximide tolerance. All three genera also have species which colonize conifer roots, often in association with insects. These facts strongly suggest that V. procera may be related to Ceratocystis. If this is true, then the damage to conifer roots caused by V. procera may be due to one or more of the mechanisms proposed for the killing of trees by bluestain fungi.

Chapter 3

Qualitative Determination of Cellulose in the cell walls of *Verticicladiella procera*

Introduction.

The presence of cellulose-containing cell walls has been proposed as a taxonomic character in the genus *Ceratocystis* Ellis and Halsted (Jewell, 1974; Upadhyay, 1981; De Hoog, 1974; Harrington, 1981; von Arx, 1974). Cellulose is rare in cell walls of fungi. Among the Eumycota *sensu* von Arx (1974), cellulose is known to occur only in certain species of *Ceratocystis sensu lato* (Bartnicki-Garcia, 1968; Barr, 1983; Upadhyay, 1981). Olchowecki and Reid (1974) established four subgeneric taxa within the genus based on ascospore morphology. Species in three of these groups typically exhibit holoblastic conidiogeny, have cellulose and rhamnose in their cell walls, and are tolerant of cycloheximide (Spencer and Gorin, 1971; Harrington, 1981; Jewell, 1974). The fourth group splits rather cleanly into two sets of species. One set is similar to the other three groups, except in ascospore morphology. The second set does not have cellulose or rhamnose, is intolerant of cycloheximide, and has *Chalara* anamorphs (enteroblastic) (Harrington, 1981). Harrington (1981) compared data from several sources and discussed exceptions concerning these taxonomic criteria. This suite of characters -biochemical,

developmental/morphological, and metabolic- argue strongly for the segregation of Ceratocystis sensu lato into Ceratocystis sensu stricto (enteroblastic) and Ophiostoma (holoblastic), as accepted by recent treatments (De Hoog, 1974; De Hoog and Scheffer, 1974, von Arx, 1974), supported by Harrington (1981) and used herein.

The anamorph Verticicladiella procera Kendrick is associated with dying Christmas trees in Virginia, and is pathogenic on seedlings of loblolly and E. white pine (Lackner and Alexander 1982, 1983). This fungus is considered to be an anamorph of an unknown species of Ophiostoma for the following reasons. (1)All connected teleomorphs for Verticicladiella and the closely related genera Leptographium and Phialocephela, are in Ophiostoma (as Ceratocystis) (Subramanian, 1983; Carmichael et al., 1980). (2)The colonization of host sapwood by V. procera and several species of Ophiostoma involves the production of deep "wedges" of discolored wood, commonly referred to as "bluestain" (Findlay, 1959). (3)Tolerance to cycloheximide is an unusual metabolic trait among eukaryotes (Sisler and Siegel, 1967). The trait is shared, however, by V. procera and numerous species of Ophiostoma (Harrington, 1981; McCall & Merrill, 1980; Fergus, 1956).

The cell walls of V. procera were examined for cellulose. Since no teleomorph of V. procera is known, the presence of cellulose would greatly strengthen the evidence

for a genetic link between V. procera and Ophiostoma, because of the rarity of cellulose among the Eumycota.

Materials and Methods.

The isolate of V. procera used in this study was recovered from a dying E. white pine Christmas tree in Greenbrier Co., WV., and an isolate of Dr. O. ulmi (Buism.) Nannf. was acquired from the laboratory of Dr. R. J. Stipes. Isolates were maintained on 2% (w/v) malt extract agar (MEA) slants at 4 C and were prepared for use by center-inoculating MEA plates, which were then incubated at 20 C for ca. 14 da. The plates were flooded with 10 ml sterile distilled water and the resulting conidial suspensions decanted into sterile glass vials. Mycelium of each species was produced by aseptically transferring a loopful of conidial suspension into 10 sterile 125 ml flasks. Each flask contained 50 ml cellulose-free culture medium composed of dextrose, 10 g; Difco peptone, 5 g; $MgSO_4$, 1 g; KH_2PO_4, 5 g; $FeSO_4.7H_2O$, 1 mg; $ZnCl_2$, 0.5 mg; $MnSO_4.H_2O$, 0.3 mg; and distilled water to 1 liter. The pH was adjusted to 5.3 with KOH or H_2SO_4. After inoculation, cultures were incubated at room temperature on a rotary shaker at 100 rpm for ca. 6 wk. Except for plastic centrifuge tubes and petrographic slides, all glassware was washed in 50% (v:v) hydrochloric acid and rinsed with distilled water prior to use.

Before harvest a sample of each flask was examined microscopically for contamination, and a loopful of broth from each flask was streaked onto MEA plates to verify the identity and purity of the fungus. Contents of flasks were pooled by species and rinsed three times with distilled water followed by centrifugation for 3 min at 900 x g to remove the culture medium. The mycelial pellets were covered with 60% (w/v) KOH in glass beakers and autoclaved at 121 C for 3 h. This procedure de-acetylates chitin to chitosan, which is then soluble in hot dilute acetic acid (Fuller, 1960). After being autoclaved, the KOH was removed by repeated centrifugation with distilled water until the supernatant had a pH of 7. The residues were boiled in 5% (v/v) acetic acid for 5-10 min. Residues were then washed seven times with hot distilled water followed by centrifugation to remove the acetic acid. The washed residues were transferred to beakers and covered with freshly prepared Schweizer's reagent (Fuller and Barshad, 1960) to extract cellulose; the beakers were capped with aluminum foil.

Schweizer's reagent was prepared by covering excess technical grade, stabilized copper (II) hydroxide (Alfa/Thiokol, Danvers, MA) with ammonium hydroxide. The residues were extracted with Schweizer's reagent at 7 C for three successive 24-hr periods. After each extraction period, the Schweizer's reagent was filtered through a

medium porosity, fritted glass funnel, and the residues covered again with fresh reagent. The pooled filtrate was titrated to pH 4-4.3 by the slowly adding hydrochloric acid. Precipitates from the titrations were transferred to fritted glass filters and washed three times in 5% acetic acid and five times in hot distilled water. Washes were repeated, if necessary, until no blue color remained in the precipitate.

The washed precipitates were allowed to dry and then were ground to a powder in acetone with an agate mortar and pestle. The powders were transfered to acetone-rinsed glass petrographic slides, and allowed to dry in glass Petri dishes. Powder specimens were examined by x-ray diffractometry with a Picker Nuclear (Cleveland, OH) model 3668A recording diffractometer using Cu K X-rays monochromated by graphite and operated at 16 ma and 35 kV.

Cleaned shrimp exoskeletons and non-absorbent cotton were treated in a similar fashion, from the point of KOH treatment onward, as negative and positive controls, respectively, for cellulose.

Results.

The x-ray diffraction patterns indicated that cellulose was present in O. ulmi, V. procera and cotton (Table 3.1). The x-ray scans were run between 8° and 30° two theta. One run of the powder from O. ulmi was extended to 60° two theta. No additional peaks were seen in that region. The

Table 3.1. Comparison of reflection position and intensity for the three samples, Verticicladiella procera, Ophiostoma ulmi, and regenerated cellulose from cotton. The two theta measurements were converted to d-spacings by Bragg's formula $n\lambda = 2d \sin\theta$ where $\lambda = 1.5418\ K_{\alpha}$, and n = 1.

	Miller Indices (hkl)		
Sample	101	$10\bar{1}$	200
Ophiostoma ulmi	12.0°[1] (1.8°)[2] 7.38A[3] 22%[4]	20.6° (1.6°) 4.31A 100%	22.5° (1.8°) 3.95A 65%
Verticicladiella procera	12.4° (2.0°) 7.13A 17%	20.4° (2.0°) 4.35A 100%	22.1° (2.2°) 4.02A 90%
Regenerated cellulose (cotton)	12.6° (1.4°) 7.02A 8.4%	20.6° (2.3°) 4.31A 100%	22.1° (2.4°) 4.02A 79%

1. Two theta value in degrees for peak centers.
2. The angular spread from the peak maximun to 1/2 peak maximum (1/2 peak width).
3. d-spacing.
4. Per cent height of peak $10\bar{1}$.

precipitate obtained from shrimp exoskeleton gave no x-ray reflections indicative of crystalline material. Likewise, a glass petrographic slide alone gave no discernible x-ray reflections. A precipitate obtained from titrating Schweizer's reagent alone yielded three peaks, one below 10°, one at 13.4°, and one above 28° two theta. Upon sufficient washing, these peaks disappeared from x-ray diffractograms of cellulose samples, along with blue coloration in the powders. In each of the samples indicating crystalline material, O. ulmi, V. procera, and cellulose regenerated from cotton, three reflections were seen. The positions and relative intensities of these three reflections agreed in each of the three samples, O. ulmi, V. procera, and cellulose regenerated from cotton. These values are given in Table 3.1. The positions agree with the monoclinic cell for cellulose II, (i.e., regenerated cellulose) with dimensions a=8.0A, b=10.3A, c=9.1A, B=63° (Ellefsen and Tonnesen, 1971). The relative intensities also agree with published results for regenerated plant cellulose (Ellefsen and Tonnesen, 1971) and for regenerated fungal cellulose (Bartnicki-Garcia, 1966; Jewell, 1974; Fuller and Barshad, 1960).

Discussion.

One of the values of a phylogenetic classification scheme is to enhance the ability to infer relationships from

the scheme where observations are missing or otherwise unavailable. Based upon cycloheximide tolerance and the teleomorph connections of Leptographium and Verticicladiella anamorphs presently known, I hypothesized that V. procera is closely related to Ophiostoma and inferred the presence of cellulose in V. procera. The present work has confirmed the hypothesis that V. procera has cellulose and therefore strengthens the possibility that V. procera is either an anamorph of an Ophiostoma species, or shares a common phylogeny with Ophiostoma.

The confused history of Ceratocystis sensu lato derives partly from the original misinterpretation of the ascigerous state as a pycnidium, and partly from the taxonomic debate concerning an appropriate generic circumscription. Elliott (1923, 1925) established the presence of asci in C. fimbriata, thus settling the question of Ceratocystis being an ascomycete genus. However, the question of an appropriate circumscription for the genus (genera) remains. The segregation of Ceratocystis sensu lato into two genera, Ceratocystis sensu stricto and Ophiostoma, has been proposed in various forms since Munch, in 1907 (see Upadhyay, 1981) erected Endoconidiophora as a segregate of Ceratostomella, based strictly on the morphology of conidia. There now exist lines of evidence in addition to morphology that support this segregation. There is an excellent correlation among conidiogeny, conidial morphology, cell wall

biochemistry and cycloheximide tolerance supporting this segregation. In the event that a teleomorph is discovered for V. procera, it is predicted that it will fit readily into Ophiostoma.

Chapter 4

Inoculation Studies

Introduction.

An original objective of this research project was to elucidate possible mechanisms of PRD symptom development. This requires plants in which disease development has progressed to the point of recognizable symptom development. Standard textbooks of plant pathology (Agrios, 1978; Manion, 1981) outline generalized disease cycles for biotic pathogens. These sources establish the point that symptom development is one of the later stages of disease development, and follows after infection. In woody plant species, symptom development frequently occurs only after the plant is well colonized. The period of time after infection and prior to symptom development is referred to as the latent or incubation period of disease development. In order to study symptom development, it is necessary to obtain plants in which disease has developed into the colonization phase and beyond the latent period.

The preferred method to produce experimental material for studies of symptom development is to artificially inoculate suitable plant material. This approach has several advantages. It provides diseased plants where host and pathogen are of known origin. Individual plants can be assigned randomly to treatment groups to prevent bias from

the plant group. The time of inoculation is known, and therefore parameters of interest can be quantified accurately with regard to time. In order to apply the benefits cited above to studies of PRD symptom development, various artificial inoculation procedures were attempted with Verticicladiella procera.

Lackner and Alexander (1982, 1983) successfully used a root dip technique to artificially inoculate pines with V. procera. This technique was used to satisfy Koch's postulates for proof of pathogenicity on E. white pine (Lackner and Alexander, 1982). Colonized wood blocks were inserted into stem slits as a second inoculation procedure in their study. The wood block procedure killed fewer trees (25% vs. 50% with the root dip), but nonetheless also satisfied Koch's postulates (Lackner and Alexander, 1982).

These authors later compared the pathogenicity and virulence of three isolates of V. procera on loblolly and E. white pine by inoculating a group of each pine species with each isolate (Lackner and Alexander, 1983). In this study, also using the root dip technique, members of all six groups of pines became diseased and died. One of the isolates killed significantly fewer loblolly than E. white pines. This indicates that some variability exists among isolates of V. procera in terms of virulence. The experiments in these two reports comprise the most solid body of evidence establishing the pathogenicity of V. procera. Reports of

other inoculations are reviewed in Chapter 2.

The purpose of the artificial inoculations in the present study was to obtain plants of known origin expressing PRD symptoms. These PRD symptomatic plants were to be compared to healthy, asymptomatic plants in a study of possible mechanisms of symptom development. This comparison therefore required material in which disease development had progressed to the visual level of PRD symptom expression, in order to address the specific objectives listed below. It was hypothesized that colonization by V. procera affected plant water relations in some manner. The first objective was to determine whether such an effect existed. A second objective was to determine where a dysfunction in water transport occurred and whether it could be related to the area or areas colonized by V. procera. The procedure proposed for this effort was to quantify the water potentials of PRD symptomatic and asymptomatic plants.

Materials and Methods.

A. General Procedures.

Pine seedlings used for root dip inoculations were potted and maintained under standard greenhouse conditions until used. Loblolly pine seedlings were obtained from the Virginia Division of Forestry (VDF) nursery at New Kent, Va, except as noted below. Eastern white pine seedlings were from the VDF nursery at Waynesboro, Va. Seedlings from VDF

were transplanted into Pro-Mix^{R} in 2.8 l plastic pots of 17 cm dia. Loblolly pine seedlings used in three of the temperature tank studies (experiments 8, 9 and 10) were grown from seed in the Forest Pathology greenhouse at VPI&SU. These seedlings were germinated in moist vermiculite and transplanted into Pro-Mix^{R} in plastic pots as above.

Fungus cultures were maintained on 2% (w:v) malt extract agar (MEA) slants. Isolates in pure culture were allowed to grow one to two weeks at 20 C upon transfer to slants and then were stored at 4 C. To prepare inoculum for root dip inoculations, 9 cm petri plates of MEA were center inoculated with V. procera and incubated at 20 C. Colonies were allowed to develop for approximately 10 to 14 days, at which time the plates were covered with abundantly sporulating mycelium. Conidia were harvested by flooding each 10-14 da old colonies with 10 ml each sterile distilled water. Concentrated conidial suspensions taken directly from the plates were diluted with additional sterile distilled water to the desired volume and conidial density. From three to six plates were usually required to provide adequate numbers of conidia. The density of the conidial suspensions was adjusted after counting the number of conidia ml^{-1} with a hemacytometer.

Germinability of conidial suspensions used for inoculum was determined by dilution plating onto MEA plates and

direct observation of conidia under a compound microscope at 100x. Conidial suspensions were diluted to 10^5, 5 x 10^4, and 10^4 conidia ml^{-1} for counting. Each dilution was plated onto five MEA plates at the rate of 1 ml per plate. Plates with diluted inoculum suspension were incubated at 20 C for approximately 24 hr before counting. The 5 x 10^4 dilution was usually the optimal density for counting. The number of germinated and ungerminated conidia were counted in each of five 100x microscope fields from each of the five plates. The germinability of the conidial suspension was taken as the mean percentage of the 25 observations.

Seedlings to be used in the inoculation experiments were examined for any symptoms of chlorosis and/or wilting prior to selection. Only seedlings free of these symptoms were used. The seedlings selected for use in an experiment were then randomly assigned to treatment groups. Random selection was conducted in one of two ways. In the first method, plants were assigned numbers and the numbers were assigned to treatments with the use of a random number table. The second method assigned treatment designations to pot labels which were then thoroughly mixed and pulled blindly from a container and placed in the plant pots. The plant was then treated according to treatment designations on the pot label. The purpose of random assignment of plants to treatment groups was to assure that no bias would accumulate in any group due to unnoticed but detrimental

plant conditions.

To prepare for root dip inoculations, seedlings were unpotted and the root systems gently washed free of the potting mix under running tap water. Root systems were immediately wrapped in moistened paper towels to prevent desiccation. Unless immediately dipped and repotted, unpotted seedlings were kept at 4 C until inoculated, usually less than 1 hr. Seedlings were inoculated by submerging their root systems in the conidial suspensions for 15 min. The root systems of control seedlings were mock-inoculated for 15 min in sterile distilled water. Dipped seedlings were immediately potted in clean plastic pots. Pasteurized Spasoff's potting mixture containing weblite, vermiculite and peat (2:2:1, v:v:v) was used to repot treated seedlings.

In one series of experiments, treated and repotted seedlings were placed in soil temperature tanks. These tanks were set to maintain constant soil temperatures of 20 C. This temperature is the optimum temperature for *in vitro* growth of *V. procera*. Seedlings in temperature tanks had supplementary light from fluorescent lamps to maintain a 14 hr photoperiod.

Water potential readings were made with a Scholander pressure bomb (Soilmoisture Equipment Corp., Santa Barbara, CA). Needle fascicles were detached manually by pulling the fascicle downward such that stem tissue was removed with the

fascicle. Fascicles were placed in the pressure bomb and the expression of sap noted with a 10x hand lens. The balancing pressure required for sap expression was taken as the needle water potential. Readings were taken either at the diurnal maximum, i.e. pre-dawn, or in some cases at the diurnal minimum, i.e. early afternoon. Afternoon readings are not comparable between diurnal cycles since vapor pressure deficits, and hence transpirational demands, would seldom be comparable. Afternoon readings therefore were only used to compare the mean water potentials among groups of plants at the same sampling time.

The rationale for afternoon readings was to compare the seedling water potentials in the presence of transpirational demand. Predawn readings (diurnal maximum) only allow comparisons between treatments after a nighttime equilibration period. If fungal infection lowered the efficiency of a root system, foliar water potentials in infected plants might be reduced relative to uninfected plants, in the presence of transpirational demand. The lower efficiency of the infected root system still might allow infected plants to attain equilibrium water potentials, i.e. pre-dawn, comparable to healthy plants, although not as rapidly. In this case, daytime water stress due to infected roots might be detected earlier with afternoon measurements than with pre-dawn measurements.

The mean water potentials or lesion lengths of groups

were compared by student's t test or analysis of variance using the Statistical Analysis System (SAS) on an IBM 360/370 computer. The numbers of symptomatic seedlings in different treatment groups were compared by a Chi-square or Kolgomorov-Smirnov goodness of fit test (Ostle and Mensing 1975).

Seedlings in the root dip experiments were scored for symptoms either at the end of the experiment or at predetermined times during the experiment. Symptoms of particular interest were those associated with PRD, i.e. a uniform chlorosis and/or wilting of foliage, and black staining in the stem.

Isolations for pathogenic fungi were made from inoculated and control seedlings at the end of each experiment, at predetermined harvest points, or when seedlings died. Seedlings to be isolated from were unpotted and the soil washed from their roots under running tap water. Small diameter roots (<2mm dia) were surface disinfested by submerging in aqueous 0.5% (v:v) NaClO (10% commercial bleach) for two minutes, before aseptically plating onto agar media. The tap roots of seedlings in the root dip experiments were surface disinfested by swabbing with 70% (v:v) EtOH and flaming briefly. Wood chips were then aseptically excised with a flamed knife and plated onto agar medium. All isolations were performed in duplicate. Two tissue samples were taken from each sample point, and

one plated onto a general agar medium, MEA, and one plated onto an agar medium selective for V. procera (McCall and Merrill, 1980). Tissue platings were incubated at 20 C for at least two weeks before scoring for the presence of V. procera.

Identification of fungal cultures recovered from diseased plants were identified as V. procera based on the conidial state. The salient characters used were the presence of a Leptographium type conidiophore, sympodular conidiogenous cells, and ovoid, uncurved conidia. The first branch of the penicillus immediately becomes parallel to the main axis of the conidiophore stipe in many V. procera conidiophores (Kendrick, 1962). Although not universal, this feature is usually present in some conidiophores of a colony, and taken with the uncurved conidia, which are unusual among Verticicladiella spp. (Kendrick, 1962), serve well to distinguish this species.

Materials and Methods.

B. Specific Experimental Procedures.

Experiment 1. A long term study was conducted to determine the plant water potential status and extent of fungal colonization prior to and at the time of visual symptom development. Specific hypotheses were: plant water potentials are not affected at the time of visual symptom development, and there is no difference between the levels

of colonization of the taproot and the secondary roots when symptoms become visible.

A root dip inoculation was used to inoculate 50 2-yr-old E. white pine seedlings (Table 4.1). Another 50 seedlings were mock inoculated with sterile distilled water as controls. The pre-dawn water potential of each seedling was measured the day prior to inoculation. At 2 wks after inoculation, 10 inoculated and 10 control seedlings were harvested. Ten seedlings from each group were subsequently harvested at 4, 6, 8, and 10 wks after inoculation. At each harvest period, the pre-dawn water potential was recorded for all 20 seedlings to be harvested. The presence of visible foliar symptoms of PRD, uniform chlorosis or wilting, was recorded for each seedling. Root tissue samples were then surface disinfested and plated onto agar media.

Experiment 2. A comparison was made between the effects of two artificial potting mixes on the development of symptom expression in inoculated seedlings. Superimposed on this was a comparison between immediate and delayed (3 da) post-inoculation watering. The hypothesis tested in each comparison was that no difference existed.

A population of 40 2-yr-old loblolly pine seedlings was randomly divided into eight groups of five seedlings each. One group was assigned to each of eight treatment combinations in a 2x2x2 factorial design (Table 4.1). The

Table 4.1. Materials and methods summary for five root dip inoculations with *Verticicladiella procera* (*V. p.*). All inoculations were conducted by soaking seedling roots for 15 minutes in conidial suspensions adjusted to approximately 10^6 conidia ml^{-1}. Controls were soaked in sterile distilled water.

Experiment no. and date.	Number, age and species of tree.	Fungal isolate[1] (and % germination)	Potting mix	Treatments.
1. Jan.-March, 1983	100 2+ yr old white pine	125 (ND[2])	Pro-mix[R]	With and without *V. p.*
2. April-July, 1983	40 2+ yr old loblolly pine	125 (TNTC[3])	Pro-mix[R] and Spasoff's	Two potting mixes, watered just after and 3 days after inoculation, and with and without *V. p.*
4. June-July, 1983	15 loblolly and 15 white pine	152 (TNTC)	Spasoff's	Taproots wounded, with and without *V. p.*

1. Sources of *V. p.* isolates used were: 125-white pine (Va.), 152-white pine (W. Va.), 154-white pine (W. Va.), 254-white pine (Va.).
2. Not determined.
3. Too numerous to count at dilution used.

Table 4.1. (continued)

Experiment no. and date.	Number, age and species of tree.	Fungal isolate[1] (and % germination)	Potting mix	Treatments.
6. July-August, 1983	30 2 yr old loblolly pine	153 (84%)	Spasoff's	With and without *V. p.*, root dip and taproot in vial
14. April-July, 1985	80 2 yr old white pine	254 (56%)	Spasoff's	Ozone fumigated, potted, in aerated water, and directly from cold storage, each with and without *V. p.*

treatments were inoculated or non-inoculated, repotted in either Spasoff's or Pro-Mix^{R} and watered either immediately after inoculation or three days after inoculation. Three months after inoculation, a single seedling from each group was harvested. Isolations were made from the root tissues of each of these eight seedlings to determine whether the roots of the inoculated seedlings had become colonized by V. procera.

Experiment 3. Field grown saplings were inoculated in the field. The purpose of these inoculations was to obtain colonized sapwood for histologic examination. Several techniques were employed to assure that some colonized sapwood would be available for study. The purpose of the histologic study was to prepare a description of the penetration of sapwood by V. procera.

Blocks of E. white pine sapwood were colonized with V. procera using the agar wood-block technique (Behr, 1973). Three incisions were made aseptically through the bark at the stem base of trees to be inoculated such that a flap of bark still attached at the bottom could be peeled back from the underlying sapwood (Table 4.2). Colonized blocks were inserted beneath this flap and sealed in place with autoclaved, molten paraffin. Sterile sapwood blocks were placed beneath bark flaps on the opposite side of the trees as control inoculations. This procedure was repeated on four trees each of loblolly, Scots and E. white pine in

Table 4.2. Materials and methods summary for three wound inoculation experiments with *Verticicladiella procera* (*V. p*).

Experiment no. and date.	Number, age and species of tree.	Fungal isolate[1]	Treatments
3. December, 1982	4 each, 10-15 yr old Scots, white and loblolly pine	122 145 130	Colonized blocks inserted beneath bark wounds
April, 1983	as above	as above	as above
May, 1983	as above	146 (6.6×10^5 ml^{-1})	Three holes bored in the base of each tree and filled with a conidial suspension.
June, 1983-May, 1984	8 each 10-15 yr old Scots and white pine	151	Poultice of sterile sawdust and *V. p.* broth culture packed around debarked band of sapwood at base of stem.
5. July, 1983-May, 1985	20 3 yr old white pine	151	Colonized white pine twig segments secured to wounded or unwounded taproot.
15. April-July, 1985	60 2 yr old white pine	254	Seedlings divided among three watering regimes, half of each group inoculated, half mock-inoculated.

1. Isolate sources were: 122-white pine (Blue Ridge Parkway, VA), 145-loblolly pine (SC), 130-Scots pine, 146-Scots pine (VA), 151-E. white pine (WV), 254-E. white pine (VA).

December, 1982. The entire procedure was repeated on another set of 12 trees in April, 1983.

In May, 1983, the trees inoculated in December were re-inoculated with a conidial suspension. Three holes were bored into the base of each tree. These holes were approximately 5 mm dia, spaced evenly around the stem circumference and angled downward towards the pith. Ten ml of conidial suspension was then distributed among the three holes in each tree. The concentration of conidia in the suspension was approximately 6.6×10^5 ml^{-1}. After filling with conidial suspension, the holes were plugged with moistened cotton which was held in place with masking tape.

In June, 1983 16 field grown saplings were used in inoculations using a poultice of V. procera culture in malt extract broth and sterile sawdust (Basham, 1970; Mathre, 1964). Eight Scots and eight E. white pine (10-15 cm basal dia) were used. Four trees of each species were inoculated with V. procera and four were treated similarly but with sterile malt broth mixed with the sawdust as controls. A 15 cm wide strip of bark (vascular cambium and periderm, Sutton and Tinus, 1983) was removed from approximately 75% of the circumference of the trees at ground level. This debarked strip was covered with the poultice which in turn was wrapped with clear plastic film to physically secure the inoculum in place and retard drying.

The poultice inoculated E. white pine were harvested in

May, 1984. The trees were excavated and returned to the laboratory where they were dissected. Upon dissection, the presence of sapwood symptoms, i.e. resin-soaking and/or black staining, was noted. Isolations were made from resinous and black-stained sapwood onto both MEA and AMA.

Experiment 4. A previously untried inoculation procedure was tested for the ability to generate PRD symptoms in seedlings. The effect of a taproot wound at the time of inoculation was examined in a root dip inoculation experiment. The hypothesis tested was that no difference would develop between the degree of symptom expression in inoculated and uninoculated seedlings.

In this procedure a wound was made in the taproot of each unpotted, washed seedling immediately prior to the root dip (Table 4.1). The wounds were made by excising a chip of bark and xylem with a flamed knife. The chip removed was approximately 1 cm long and one third of the diameter of the taproot in width. The previously described root dip protocol was followed except for the wounding. Fifteen E. white and 15 loblolly pine potted seedlings were used. Ten seedlings of each species were inoculated and five mock-inoculated with sterile distilled water as controls.

Experiment 5. An inoculation technique commonly used with V. wageneri was attempted with V. procera. The hypothesis tested was that the development of symptom expression would be different between inoculated and

uninoculated seedlings. The procedure used was to attach colonized twig segments to either wounded or unwounded taproots of seedlings. This technique had been used successfully to compare the pathogenicity of several members of the Leptographium complex, including V. procera (Harrington and Cobb, 1983).

Twig segments of E. white pine were trimmed to 1 cm, boiled in 10% malt extract for 2 hr and autoclaved. Sterile, malt-impregnated twig segments were then aseptically placed in French square bottles and inoculated with 2 ml conidial suspension of V. procera (Table 4.2). This was enough to thoroughly cover the bottom side of the French square with a thin film of moisture. French squares with inoculated twig segments were incubated for six weeks at 20 C. Seedlings were unpotted and their roots rinsed free of soil with running tap water. The surface of the taproot 3 cm below the root collar was swabbed with ethanol. A 1 cm long cut was made at this point about one third way through the root diameter. Colonized blocks were placed on this cut surface and physically attached with masking tape. Seedlings were then repotted, the pots watered to field capacity and placed on a bench under an open shed. Ten seedlings were wound inoculated, five were wounded and sterile twig segments attached to the wound, and five were inoculated with colonized wood blocks on unwounded taproots.

Experiment 6. The root dip procedure was slightly

modified in an additional inoculation trial and a previously untried procedure was attempted as an inoculation procedure. In each case, the hypothesis tested was that differences in the amount of symptom expression would develop between inoculated and uninoculated seedlings. The modification of the root dip was to prune the root systems of the seedlings just prior to inoculation. The untried procedure was to sever the lower portion of the taproot and repot the seedling with the freshly cut taproot end in the buried vial of conidial suspension.

Ten seedlings were unpotted, their root systems washed free of soil and trimmed with pruning shears to 50-75% of their original length (Table 4.1). Pruning was done immediately prior to seedlings being placed in the conidial suspension. No untrimmed seedlings were included. Seedlings were then repotted, watered to field capacity and placed on a bench under an open shed. Five seedlings that were trimmed but treated with sterile distilled water served as controls.

Another 10 seedlings were unpotted, their root systems cleaned and trimmed as above. These seedlings were inoculated by repotting the seedling with the trimmed taproot end in a vial of conidial suspension. Five seedlings were treated similarly, but with taproots placed in vials of sterile distilled water as controls.

Experiment 7. Experiment 4 was repeated under

controlled soil temperature conditions. The hypothesis tested was again that differences would develop between the level of PRD symptom expression in inoculated and uninoculated seedlings. Seedlings with wounded taproots were root-dip inoculated and subsequently held in soil temperature tanks.

Twenty seedlings were unpotted, washed and wounded by aseptically removing a chip of bark and xylem from the taproot (Table 4.3). The chip removed was approximately 1 cm long and one third the taproot diameter in width. All seedlings in this study were wounded. Fifteen seedlings were inoculated and five were mock inoculated with sterile distilled water as controls. After repotting, these seedlings were held in a soil temperature tank maintained at 20 C for three weeks. Isolations were made from root tissue of all seedlings 20 da after inoculation.

Experiment 8. Unwounded loblolly pine seedlings were root dip inoculated and maintained in soil temperature tanks after inoculation. The hypothesis tested was that PRD symptom development would differ between inoculated and uninoculated seedlings.

Twenty, 6-8 month old loblolly pine seedlings which were grown from seed in a greenhouse were used in this experiment. Fifteen of these seedlings were root dip inoculated with V. procera and the remaining five were treated similarly, but with sterile distilled water as

Table 4.3. Materials and methods summary for seven root dip inoculation experiments with Verticicladiella procera (V. p). Seedlings in all experiments were incubated in soil temperature tanks maintained at 20 C with 14 hr photoperiods under fluorescent lamps. Conidial suspensions of approximately 10^6 conidia ml^{-1} were used for all inoculations; sterile distilled water was used for controls. All seedlings were repotted in Spasoff's rooting mix[1] except where noted.

Experiment no. and date.	Number, age and species of trees.	Fungal isolate (and % germination).	Treatments.
7. August 1983	20 white pine	154 (88%)	Taproots of all seedlings wounded, with and without V. p.
8. September 1983	20 6-8 month old, loblolly pine with white root tips.	153 (81%)	With and without V. p.
9. October-December, 1983	35 10-12 month old, loblolly pine.	153 (85%)	With and without V. p.
10. February-April, 1984.	18 2(?) yr old loblolly pine with white root tips.	153 (59%)	With and without V. p.

1. Spasoff's rooting mix consists of weblite, vermiculite and peat (2:2:1, v:v:v).

Table 4.3. (continued)

Experiment no. and date.	Number, age and species of trees.	Fungal isolate (and % germination).	Treatments.
11. March-May, 1984.	28 2(?) yr old loblolly pine with white root tips.	153 (54%)	With and without V. p.
12. April-July, 1984.	54 2 yr old loblolly pine with white root tips.	7 isolates[2] (48-67%, 57% for #153)	With differnt isolates of V. p., and controls with sterile distilled water.
13. July-October, 1984.	32 2(?) yr old loblolly pine with white root tips.	153 (60%)	Planted in 2:1 soil:sand, irrigated to establish high and low soil moisture regimes, with and without V. p.

2. V. p. isolates used and their sources were: 153-white pine (W. Va.), 122-white pine (Blue Bidge Parkway, Va.), 121-Scots pine (Va.), 125-white pine (Va.), 134-red pine (W. Va.), 133-Austrian pine (Va.), 127-white pine (Va.).

controls (Table 4.3). Immediately after inoculation, all seedlings were repotted, and the pots watered to field capacity and placed in a soil temperature tank. Pre-dawn fascicle water potentials were recorded at 16 da after inoculation. Isolations were made from root tissue of all seedlings at 16 da after inoculation.

Experiment 9. Loblolly pine seedlings were root dip inoculated, maintained in soil temperature tanks and seedling water potentials monitored over the course of the experiment. The hypothesis tested was that PRD symptom development would be accompanied by the development of a difference between the water potentials of inoculated and control seedlings.

Potted loblolly pine seedlings were root-dip inoculated and maintained in a 20 C temperature tank (Table 4.3). Control and inoculated groups contained seven and 28 seedlings, respectively. Pre-dawn water potential readings were taken at three da intervals throughout the course of the experiment. Isolations were made from the root tissue of all seedlings at 60 da after inoculation.

Experiment 10. Root dip inoculated loblolly pine seedlings were maintained in a soil temperature tank after inoculation and monitored for water potential effects. The hypothesis tested was that PRD symptom development would be accompanied by the development of a difference between the water potentials of inoculated and control seedlings.

Eighteen 2-yr-old loblolly pine seedlings with numerous white growing tips were selected for this inoculation experiment. These seedlings were held in aerated water tanks to promote root growth prior to use in this experiment. One half of the seedlings were inoculated with V. procera and the other seedlings treated in a similar manner, but with sterile distilled water as controls (Table 4.3). Pre-dawn and afternoon fascicle water potentials were recorded weekly from the first through the fourth week after inoculation. Seedling pots were watered to field capacity the afternoon before each set of pre-dawn readings was taken. One nighttime equilibration period was allowed between pot waterings and afternoon fascicle water potential readings. Isolations were made from root tissues of all seedlings at four weeks after inoculation.

Experiment 11. Experiment 10 was repeated. Root dip inoculated seedlings maintained in a soil temperature tank were monitored for fascicle water potential differences. The hypothesis was that water potential effects would accompany symptom development in inoculated seedlings.

Twenty-eight seedlings with white root tips were treated, the pots watered to field capacity and placed in a soil temperature tank at 20 C (Table 4.3). These seedlings had been held in aerated water tanks to promote root growth prior to use in this experiment. One half of the seedlings were inoculated with V. procera and the other seedlings

treated with sterile distilled water as controls. Pre-dawn and afternoon water potential readings were taken at one, two, three and four weeks after inoculation, as in the previous experiment. Seedling pots were watered to field capacity the afternoon before each set of pre-dawn readings was taken. One nighttime equilibration period was allowed between pot waterings and afternoon fascicle water potential readings. Isolations were made from root tissues of all seedlings at four weeks after inoculation.

Experiment 12. The virulence of seven isolates of V. procera were compared in a root dip inoculation. The hypothesis tested was that no differences regarding virulence existed among the isolates tested.

Groups of six 2 yr old loblolly pine seedlings were inoculated with one of seven isolates of V. procera from various host species and geographical areas (Table 4.3). These seedlings had been held in aerated water tanks to promote root growth prior to use in this experiment. Seedlings from this treatment with white root tips were selected for the inoculation study. A group of six control seedlings were dipped in sterile distilled water. Afternoon fascicle water potentials were recorded at weekly intervals from one week to ten weeks after inoculation. Isolations were made from root tissue of all seedlings at ten weeks after inoculation, or upon death of the seedling.

Experiment 13. Root dip inoculated seedlings were

allowed to extract soil moisture to predetermined levels before being watered. The hypothesis tested was that soil moisture levels did not affect the degree of PRD symptom development in inoculated seedlings.

Loblolly pine seedlings with numerous white root tips were root dip inoculated and repotted in a soil-sand mix (Table 4.3). Seedlings were watered to field capacity upon repotting and placed in a soil temperature tank. The moisture contents of the soil sand mixture corresponding to -0.05 mPa and -1.5 mPa were 11.8% and 4.4% as determined on a pressure plate (Table 4.4). Pots were again watered to field capacity when soil moisture levels fell to the prescribed levels, as determined gravimetrically. Fascicle water potentials were measured the afternoon after inoculation. Fifteen weeks after inoculation all pots were watered to field capacity and fascicle water potentials measured the following afternoon. After final water potential measurements were taken, isolations were made from the roots of all seedlings.

Experiment 14. Seedlings which had been had been previously treated in four different ways were inoculated and the degree of PRD symptom expression compared. The hypothesis tested was that previous treatment of the seedlings would have no effect on the degree of PRD symptom expression among the inoculated seedlings.

The four sources of seedlings for this experiment were:

Table 4.4. Soil moisture retention values for the soil sand mixture used in Experiment 13. Soil moisture contents are gravimetrically determined values corresponding to the various equilibrium pressures.

Equilibrium Pressure[1]	Moisture Content[2]
0.05	11.82
0.1	9.53
0.3	7.19
0.5	6.97
1.0	5.57
1.5	4.39

1. Pressure differential in -mPa that wetted soil was equilibrated with.
2. Moisture content %, on an ovendry basis, of equilibrated soil.

potted seedlings taken directly from the greenhouse bench, ozone fumigated potted seedlings, seedlings held in a tank of aerated water and seedlings taken directly from cold storage (Table 4.1). The ozone treated and the potted seedlings had been potted for approximately one month before inoculation. These two groups had received routine greenhouse maintenance of weekly watering and protection from freezing, but no supplemental lighting. The ozone treatment consisted of a single fumigation of 0.3 ppm ozone for 6 hr, two da prior to inoculation. The seedlings from the aerated water tank were held there for three weeks prior to inoculation and all seedlings used had abundant white root tips. The seedlings from cold storage were stored at 4 C in open plastic bags to prevent desiccation. These seedlings were still in bundles as delivered from the nursery when removed for inoculation.

Twenty seedlings were selected from each source for a total of 80 seedlings in the experiment. Each group of 20 seedlings was randomly divided into inoculated and control groups with 10 seedlings each. Thus the experiment contained 40 inoculated and 40 control seedlings. All plants in each inoculated group were simultaneously treated in the root dip, immediately potted or repotted, and the pots watered to field capacity and placed on a greenhouse bench. The control groups were treated similarly except dipped in sterile distilled water. Arrangement of seedlings

on the bench was randomized. Afternoon fascicle water potentials were recorded for all seedlings the day before inoculation. Potted seedlings had all been watered to field capacity the day before. The entire group of seedlings was again watered to field capacity two days after inoculation, and fascicle water potentials recorded on the third afternoon following inoculation. Thereafter weekly water potential readings were made, always in the afternoon one day after watering to field capacity. No isolations of root tissue were conducted.

Experiment 15. An inoculation technique which did not require uprooting seedlings was used on three groups of potted seedlings. Each group was exposed to one of three watering regimes. The hypothesis tested was that symptom expression would not differ among inoculated seedlings from the three watering regimes.

Potted seedlings were randomly assigned to one of three watering cycles (Table 4.2). These cycles were: watered to field capacity every third day, watered to field capacity every day, and flooded constantly. The first two watering cycles were established two wk before inoculation and seedlings in the third watering cycle were flooded three da before inoculation.

Within each watering cycle group, seedlings were randomly assigned to either inoculated or control treatments. The inoculation technique used in this

experiment placed a MEA agar plug cut with a #3 cork borer beneath a small wound in the bark of the seedling. Inoculated seedlings received a plug from the actively growing margin of a colony of V. procera, and control seedlings received a sterile plug of MEA. The lower stem of the seedling was first swabbed in 70% ethanol. A flamed scapel was then used to aseptically cut through the bark. This cut was made in the shape of an upward pointing semicircle such that a flap of bark could be peeled back from the underlying sapwood. The agar plug was placed in the wound beneath this bark flap. Wounds with agar plugs were then wrapped with cheesecloth moistened with sterile distilled water and covered with Parafilm^{R}.

Afternoon fascicle water potentials were taken the day of inoculation and every six or nine days thereafter. The sampling period for water potentials varied from six to nine da since afternoon water potential readings require that all seedlings be watered the day before reading. Isolations were made from stem tissue in the vicinity of the stem inoculation point of all seedlings at three months after inoculation, or when seedlings died.

Results.

Experiment 1. All 50 inoculated seedlings yielded V. procera upon isolation (Table 4.5). This included seedlings harvested at all five time periods from 2 wk to 10 wk after

Table 4.5. Results summary for five root dip experiments with *Verticicladiella procera* (*V. p.*).

Experiment no.	foliar symptom development[1]	Reisolation of *V. p.*	Water potential (WP) data	Remarks
1.	None	50/50 inoc. 0/50 cntrl.	WP not different, initial or final	2
2.	None	4/4 inoc.[3] 0/4 cntrl.	Not measured	2
4.	16/20 inoc. 7/10 cntrl.	8/20 inoc. 0/10 cntrl.	Not measured	Technique execution failure[4]
6.	3/10 and 1/5 and 3/10 and 1/5 and	3/3 root dip inoc.[5] 0/1 root dip cntrl. 3/3 vial inoc. 0/1 vial inoc.	Not Measured	4

1. Uniform chlorosis, wilt.
2. Level of disease development not extensive enough to allow comparisons between healthy and symptomatic plants.
3. Isolations made from one randomly selected seedling from each group.
4. Too many controls died to allow comparison between healthy and symptomatic plants.

Table 4.5. (continued).

Experiment no.	foliar symptom development[1]	Reisolation of *V. p.*	Water potential (WP) data	Remarks
14.	1/40 inoc. 1/40 cntrl.	None attempted	WP significantly different the day before inoculation (cold storage seedlings) WP not different among treatments 3 days or 13 weeks after inoculation	2

2. Level of disease development not extensive enough to allow comparisons between healthy and symptomatic plants.

inoculation. Disease development did not progress enough however for visible PRD symptom development to occur, even after 10 wk. The mean (± standard deviation) fascicle water potentials of the control and inoculated seedlings one day prior to inoculation were -1.15 (± 0.27) and -1.22 (± 0.25) mPa, respectively. The mean water potentials of control and inoculated seedlings at harvest were -0.80 (± 0.21) and -0.75 (± 0.24) mPa, respectively. Analysis of variance did not indicate a significant difference between groups either before inoculation or at harvest.

Experiment 2. Three months after inoculation there was no noticeable PRD symptom development among the inoculated seedlings (Table 4.5). All four of the randomly selected, inoculated seedlings (one from each inoculated treatment group) yielded V. procera upon isolation. The fungus was not recovered from any of the four uninoculated, control seedlings (one from each of the uninoculated treatment groups).

Experiment 3. The poultice-inoculated E. white pine were harvested after one year. At this time, resinous lesions had developed beneath the inoculation bands of all eight trees, four each inoculated and control (Table 4.6). Some areas of black stain had developed along parts of the inoculation band circumference. V. procera was readily isolated from these black stained tissues and from some of the resinous areas. Particular care was taken to examine

Table 4.6 Results summary for three wound inoculation experiments.

Experiment no.	Foliar symptom development[1]	Reisolation of *V. p.*	Water potential (WP) data	Remarks
3.	None	Not attempted	Not measured	Only white pine with poultice harvested.
	None	Not attempted	Not measured	Only white pine with poultice harvested.
	None	Not attempted	Not measured	Only white pine with poultice harvested.
	None (resinosus and black stained sapwood)	4/4 inoc. white pine 4/4 cntrl. white pine	No comparison possible (all trees with *V. p.*)	Colonized sapwood used for histology study.
5.	*V. p.* & wound: 1/10 wound only: 1/5 *V. p.* only: 0/5	9/10 5/5 2/5	Not measured	Technique Execution failure (controls contaminated).

1. Uniform chlorosis and wilt.

Table 4.6. (continued).

Experiment no.	Foliar symptom development[1]	Reisolation of *V. p.*	Water potential (WP) data	Remarks
15.	Flooded with *V. p.*: 9/10	10/10	WP differnces among watering regimes, but not between inoc./cntrl.[2]	Lesions with *V. p.* significantly longer and deeper than those without *V. p.* (flooded seedlings excluded).
	w/o *V. p.*: 10/10	6/10		
	One day cycle with *V. p.*: 0/10	10/10		
	w/o *V. p.*: 0/10	6/10		
	Three day cycle with *V. p.*: 0/10	10/10		
	w/o *V. p.*: 2/10	4/10		

1. Uniform chlorosis, wilt.
2. Analysis of variance of WP data revealed significant differences among mean values of the different watering regimes at the time of inoculation. The means of the flooded groups were much lower than the other two. Differences between inoculated and control groups were not significant.

the isolation plates for any darkly pigmented fungi. V. procera was the only dematiaceous fungus recovered on either MEA or AMA from isolations made from these trees. The fungus was isolated from the sawdust surrounding the inoculation band on each tree. Weevils were also noted in the sawdust of some trees. One weevil (source tree not noted) was aseptically placed on an AMA plate and yielded V. procera upon incubation. Symptomatic sapwood, i.e. resinous and/or black-stained, was collected, fixed in FAA (formalin: acetic acid: ethanol, 13:5:200) and stored for later histologic examination.

Experiment 4. At the end of one month, numerous seedlings in both the control (70%) and the inoculated (80%) groups were exhibiting symptoms of chlorosis and wilt (Table 4.5). Upon isolation, V. procera was recovered from 40% of the inoculated group and from none of the control seedlings.

Experiment 5. The trees in this experiment were harvested at 22 months after inoculation. At that time chlorosis and wilt had developed in only two seedlings, both of which had died (Table 4.6). One seedling was a wound inoculated seedling, and the other was an uninoculated check plant. None of the unwounded, inoculated seedlings were chlorotic or wilted. Upon isolation the wound inoculated, wound uninoculated, and unwounded inoculated groups yielded V. procera from 9/10, 5/5 and 2/5 of the seedlings, respectively.

Experiment 6. Eight seedlings of the total 30 in the experiment had developed chlorotic foliage and/or wilted within one month (Table 4.5). In the root dip inoculation, there were 3/10 inoculated and 1/5 controls with chlorosis and/or wilt. In the inoculation in which seedlings were repotted with the taproots in vials of a conidial suspension, there were also 3/10 inoculated and 1/5 control seedlings with chlorosis and/or wilt. V. procera was isolated from all six inoculated seedlings, but from neither of the two uninoculated seedlings with chlorosis and/or wilt.

Experiment 7. Three weeks after inoculation, many of the seedlings root dipped with wounded taproots and held in soil temperature tanks had developed chlorosis and/or wilt (Table 4.7). Among the inoculated seedlings, 9/15 were chlorotic and/or wilted as were 3/5 uninoculated seedlings. None of the control seedlings (uninoculated) yielded V. procera however, whereas 6/15 of the inoculated seedlings yielded the fungus.

Experiment 8. Chlorosis and/or wilt had developed in 11/15 inoculated seedlings after 16 da, at which time all of the control seedlings remained asymptomatic (Table 4.7). All 15 of the inoculated seedlings and none of the controls yielded V. procera upon isolation. Mean (± standard deviation) pre-dawn fascicle water potentials were -3.18 (± 0.14) and -0.86 (± 0.13) mPa for the inoculated and control

Table 4.7. Results summary for seven root dip experiments with *Verticicladiella procera* (*V. p*). Inoculated seedlings were incubated in soil temperature tanks maintained at 20 C.

Experiment no.	Foliar symptom development[1]	Recovery of *V. p.* upon reisolation	Water potential (WP) data	Remarks
7.	9/15 inoc. 3/5 cntrl.	6/15 inoc. 0/5 cntrl.	None taken.	2
8.	11/15 inoc. 0/5 cntrl.	15/15 inoc. 0/5 cntrl.	WP significantly different at end of exp't.	Successful inoculation.
9.	3/28 inoc. 0/7 cntrl.	28/28 inoc. 0/7 cntrl.	WP not different 6 wk after inoculation.	3
10.	7/9 inoc. 1/9 cntrl.	8/9 inoc. 0/9 cntrl.	WP different (only measured after inoculation).	Unusually low WP values.

1. Uniform chlorosis, wilt.
2. Too many controls died to allow comparison between healthy and symptomatic plants.
3. Level of disease development not extensive enough to allow comparisons between healthy and symptomatic plants.

Table 4.7. (continued)

Experiment no.	foliar symptom development[1]	Recovery of *V.p.* upon reisolation	Water potential (WP) data	Remarks
11.	9/14 inoc. 2/14 cntrl.	11/14 inoc. 0/14 cntrl.	WP different (only measured after inoculation).	Unusually low WP values
12.	16/42 inoc. (9/26 in less than 1 wk) 0/6 cntrl.	34/42 inoc. 0/6 cntrl.	WP not different among treatments at 1 wk or 10 wk.	7/42 inoc.[4] 0/6 cntrl.
13.	1/16 inoc. 1/16 cntrl.[5]	16/16 inoc. 1/16 cntrl.[5]	WP not different.	3

1. Uniform chlorosis, wilt.
3. Level of disease development not extensive enough to allow comparisons between healthy and symptomatic plants.
4. The number of symptomatic seedlings among treatments (isolates and control) was not significantly different. The group of 7 inoculated plants which became symptomatic does not include the plants which had very low WP values (-3.5 mPa) at the first week and were considered not to have recovered from transplanting.
5. Not the same tree.

groups, respectively at the end of the experiment. Analysis of variance indicated that these means were significantly different (P=0.05)

Experiment 9. Three of 28 inoculated seedlings died in the first 9 days of the experiment. No other seedlings had either developed symptoms or died when the experiment was terminated at 60 da (Table 4.7). Upon isolation, V. procera was recovered from all 28 inoculated seedlings but from none of the seven uninoculated seedlings. The mean (± standard deviation) pre-dawn fascicle water potentials for the inoculated and control groups were -1.48 (± 0.97) and -0.93 (± 0.20) mPa , respectively, one da prior to inoculation. One da after inoculation, inoculated and control means were -0.66 (± 0.10) and -0.63 (± 0.11) mPa, respectively. At six wk after inoculation, inoculated and control means were -0.73 (± 0.21) and -0.79 (± 0.12) mPa, respectively. Analysis of variance did not reveal a difference between the inoculated and control mean water potentials at any of these sampling times. Fascicle water potentials were not available for the last 18 da of the experiment due to a limited number of fascicles remaining on some seedlings.

Experiment 10. Within four weeks of inoculation, 7/9 inoculated seedlings had developed chlorosis and/or wilt (Table 4.7). A single control seedling had wilted, but remained green. Significant differences developed between the mean water potentials of the inoculated and control

seedlings both at pre-dawn and afternoon readings. The mean pre-dawn readings were significantly different at 2, 3 and 4 wk after inoculation (Table 4.8). The mean afternoon readings were significantly different at all four weekly readings, from 1-4 wk after inoculation. Both pre-dawn and afternoon measurements of fascicle water potential were quite low in this experiment. Isolations made at 4 wk after inoculation yielded V. procera from 8/9 inoculated and 0/9 control seedlings.

Experiment 11. Nine of 14 inoculated seedlings developed chlorosis and/or wilt within 4 wk after inoculation (Table 4.7). In this time 2/14 control seedlings had wilted and died. A chi-square test indicated that these frequencies were significantly different (P=0.05). The mean water potentials of these two groups of seedlings (inoculated and control) became significantly different through the course of the experiment. The afternoon means were different at 2 wk after inoculation and remained different to the end of the experiment (Table 4.8). The means of the pre-dawn measurements were different only 3 and 4 wk after inoculation. Isolations for V. procera recovered the fungus from 11/14 inoculated and 0/14 control seedlings.

Experiment 12. The comparison of isolates did not reveal differences among isolates, although 7/42 of the inoculated group became symptomatic while 0/6 controls

Table 4.8. Mean pre-dawn and afternoon needle water potentials for control seedlings and inoculated seedlings with PRD symptoms in Experiments 10 and 11.

Experiment 10	Week 1	Week 2	Week 3	Week 4
AM				
Inoculated	1.96±0.81[1]	2.52±1.09 *[2]	2.21±1.31 **[3]	2.50±1.39 **
Control	1.82±1.09	1.40±0.83	0.74±0.18	0.73±0.24
PM				
Inoculated	3.15±0.45 **	3.20±0.41 **	3.01±0.65 **	2.79±0.96 **
Control	2.41±0.51	2.24±0.72	1.76±0.33	1.49±0.54
Experiment 11				
AM				
Inoculated	2.09±1.17	2.28±1.27	2.32±1.38 *	2.28±1.40 *
Control	1.33±0.71	1.45±0.92	1.15±0.99	1.24±0.98
PM				
Inoculated	2.79±0.87	2.80±0.88 *	2.75±1.03 *	2.80±0.99 **
Control	2.51±0.73	2.00±0.85	2.01±0.66	1.62±0.85

1. Values in -mPa, means of 9 control and 8 inoculated trees in Exp. 10 and 14 control and 11 inoculated trees in Exp. 11.
2. Significantly different by student's t test at P=0.05.
3. Significantly different by student's t test at P=0.01.

developed symptoms (Table 4.9). At 1 wk after inoculation, the water potentials of nine inoculated seedlings were at or below -3.5 mPa. These seedlings were not considered to have recovered from the transplanting associated with inoculation. Disregarding this mortality, 17% (7/42) of the inoculated seedlings developed uniform chlorosis and/or wilted, compared to 0% of the controls. Analysis of variance did not reveal any differences among the mean water potentials of seedlings in the different treatments, either at 1 wk or 10 wk after inoculation (Table 4.9). Goodness of fit tests did not reject the hypothesis of a uniform distribution of symptomatic seedlings among treatments (Table 4.9). Isolations made at 10 wk after inoculation recovered the fungus from 34/42 inoculated seedlings and from 0/6 controls.

Experiment 13. Two seedlings developed chlorosis and/or wilt in the 15 wk of the experiment (Table 4.7). One seedling in the inoculated, high soil moisture treatment group developed chlorosis and wilted. The other seedling, in the low soil moisture, uninoculated treatment group, became chlorotic and died without wilting. Both of these seedlings had very low fascicle water potentials, -3.5 mPa, at 2 wk after inoculation. Mean afternoon fascicle water potentials are presented in Table 4.10 for each treatment group at one da after and 12 wk after inoculation. Analysis of variance of fascicle water potentials did not indicate

Table 4.9. Distribution of symptomatic seedlings and mean water potentials among treatment groups in Experiment 12. Each treatment group was inoculated with a different isolate of *V. procera*; controls were not inoculated

	Number of symptomatic seedlings			Water potentials[1]	
Isolate[2]	wk 1	wk 4-10	total	wk 1	wk 10
122	1	1	2	2.45±1.20	2.07±1.50
134	1	2	3	2.33±0.72	2.22±1.67
127	2	2	4	2.95±0.60	2.94±1.65
153	1	1	2	2.51±1.10	2.18±1.47
125	2	0	2	2.60±1.04	2.13±1.47
133	1	1	2	2.31±0.78	2.08±1.76
121	1	0	1	2.56±0.78	1.41±1.30
Cntrl.	0	0	0	2.13±0.76	1.07±0.31
Total:	9	7	16		
D value[3]:	0.125	0.250	0.125	NS[4]	NS[4]
P(D):	0.2	0.2	0.2		

1. Values are the mean ± standard deviation in -mPa for the six seedlings in each treatment group.
2. The sources of isolates used were: 122-E. white pine (Blue Ridge Parkway, Va.), 121-Scots pine (Va.), 125-E. white pine (Va.), 134-red pine (W.Va.), 133-Austrian pine (Va.), 127-E. white pine (Va.), 153-E. white pine (W. Va.).
3. Calculated D value for Kolgomorov-Smirnov goodness of fit test comparing the observed frequencies to an assumed uniform distribution of the total frequency within a column.
4. Means within a column not significantly different by analysis of variance.

Table 4.10. Mean afternoon fascicle water potentials of seedlings in Experiment 13. Values are the mean ± standard deviation of eight seedlings per group, expressed in -mPa.

Fungus Treatment[1]	Soil Moisture Regime[2]	Day 1[3]	Week 12[4]
Inoculated	11.8%	1.63±0.32	1.64±0.98
Inoculated	4.4%	1.69±0.44	1.23±0.10
Control	11.8%	1.54±0.55	1.17±0.34
Control	4.4%	1.51±0.37	1.64±0.98
		NS[5]	NS[5]

1. Either inoculated with Verticicladiella procera or sterile distilled water as controls.
2. Potted seedlings were allowed to extract soil moisture to the indicated levels before being irrigated again. Percent values were calculated on an oven dry basis and correspond to -0.05 mPa (11.8%) and -1.5 mPa (4.4%).
3. Afternoon fascicle water potentials measured the day after inoculation.
4. Afternoon fascicle water potentials measured at 12 wk after inoculation. All seedlings were watered to field capacity one day prior.
5. Mean values within a column not significantly different by analysis of variance.

significant differences among treatment group means at either date. Isolations made at 15 wk after inoculation yielded V. procera from 16/16 inoculated and 1/16 control seedlings. The dead control seedling did not yield the fungus.

Experiment 14. Two of 80 seedlings developed chlorosis and wilted in the seven months of this experiment (Table 4.5). One was a control (uninoculated) seedling from the cold storage treatment and the other was an inoculated seedling from the potted, non-fumigated treatment. A two way analysis of variance was conducted on fascicle water potentials by seedling source (ozone, cold storage, potted and from aerated water) and by fungal treatment (inoculated or control). This analysis was conducted for water potential values for 1 da prior to inoculation, 3 da after inoculation and 13 wk after inoculation. Significant differences were found only among seedling source the day before inoculation (Table 4.11). The mean value for seedlings still in cold storage was less negative than the means of the other treatments. There were no significant differences between means of inoculated and control seedlings at any of the three times. Isolations from root tissues of these seedlings were not attempted.

Experiment 15. Four months after inoculation, 21/60 seedlings had developed chlorosis, wilted and died (Table 4.6). Ten of these 21 were flooded controls (uninoculated),

Table 4.11. Mean afternoon fascicle water potentials for treatment groups in Experiment 14. The values are the mean ± standard deviation of 10 seedlings in each treatment group, expressed in -mPa.

Seedling History[1]	Inoculation Treatment[2]	Sampling Time[3]		
		Day -1	Day 3	Week 13
Cold Storage	Inoculated	0.27±0.15	1.30±0.19	0.83±0.19
	Control	0.35±0.16	1.47±0.63	0.86±0.28
Ozone	Inoculated	1.14±0.26	1.36±0.24	0.75±0.17
	Control	1.28±0.42	1.49±0.56	0.95±0.45
Potted	Inoculated	1.13±0.17	1.33±0.14	1.21±1.23
	Control	1.15±2.60	1.39±0.20	0.77±0.07
Aerated Water	Inoculated	1.36±0.23	1.58±0.25	0.91±0.15
	Control	1.44±0.30	1.60±0.34	0.79±0.13
		**[4]	NS[5]	NS

1. Cold storage, seedlings taken directly from cold storage; Ozone, potted seedlings fumigated with ozone two da prior to inoculation; Potted, seedlings taken directly from greenhouse benches; Aerated water, seedlings taken from cold storage and held in aerated water tanks for three wk prior to inoculation.
2. Seedlings either inoculated with Verticicladiella procera, or dipped in sterile distilled water as controls.
3. The three sampling times were the day prior to inoculation (da -1), the third day after inoculation (da 3) and 13 wk after inoculation.
4. Mean values within a column significantly different (P=0.01) by analysis of variance.
5. Mean values within a column not significantly different by analysis of variance.

nine were flooded, inoculated and two were uninoculated seedlings from the three day watering cycle treatment. Thus 19/21 dead seedlings were in the flooded treatment. The extent of resinous lesion development was not discernible due to the dark color of the sapwood throughout these stems. The extent of resinous lesion development in unflooded seedlings was determined, and comparisons of lesion extension made between seedlings which did and did not yield V. procera upon isolation. The analysis of variance for these comparisons indicated significant differences between both the mean length and the mean depth of the resinous lesions in seedlings with and without V. procera. In both cases, the greater lesion sizes were from the group with the fungus (Table 4.12).

Analysis of variance indicated that mean afternoon fascicle water potentials were different among watering regime groups, but not inoculation treatments the day before inoculation (Table 4.12). The values for the flooded seedlings was approximately twice the magnitude of values for the other two watering regime groups.

Discussion.

Based on prior work (Lackner and Alexander, 1982, 1983), it was considered feasible at the beginning of this project to reliably induce PRD with root-dip inoculations. In the earlier work, Lackner and Alexander (1982, 1983) were

Table 4.12. Mean fascicle water potentials and mean lesion dimensions of seedlings wound inoculated with agar plugs of *Verticicladiella procera* (*V. p.*) in Experiment 15.

Watering Regime[1]	Inoculation Treatment[2]	Initial WP[3]	*V. p.* Reisolation Frequency[4]		Lesion dimension[5] Length	Width
3 day:	Inoc.	0.99±0.14	+	14	15.0±8.1	2.7±1.5
	Cntrl.	1.19±0.69	-	6	4.4±4.4	0.8±0.8
1 day:	Inoc.	0.92±0.14	+	16	15.5±9.5	1.9±1.5
	Cntrl.	0.86±0.12	-	4	7.5±5.0	1.0±0.8
Flooded:	Inoc.	2.38±0.69	+	16	-------	-------
	Cntrl.	2.34±0.66	-	4	-------	-------
	Inoculation Treatment:	NS[6]			**[7]	**
	Watering Regime:	**			NS	NS

1. Watering regimes were: pots kept flooded, watered daily, or every third day.
2. An agar plug with or without (sterile) *V. p.* placed beneath a bark wound.
3. Water potentials one day prior to inoculation. Values are the mean ± standard deviation of 10 seedlings per group expressed in -mPa.
4. Number of seedlings within a watering regime from which *V. p.* was (+) or was not (-) reisolated at the end of the experiment. Lesion dimensions were compared between seedlings with and without *V. p* at the end of the experiment.
5. Linear dimensions in mm (mean±standard deviation) of resinous sapwood lesions beneath inoculation wound of seedlings with or without *V. p.*
6. Means within a column not significantly different by analysis of variance.
7. Means within a column significantly different (P=0.01) by analysis of variance.

consistently able to get successful inoculations with the root dip technique. These authors reported 50% mortality in one study (Lackner and Alexander, 1982) and 85-100% symptomatic or dead seedlings among each of six inoculated groups in the second study (Alexander and Lackner, 1983). All of the above values were from experiments using root dip inoculations. These workers were able to consistently induce disease with visual symptoms and mortality in pine seedlings with root dip inoculations of V. procera.

Two other reports in the literature supported the feasibility of using the root dip inoculation technique to induce symptoms of PRD. Although neither included data in their report, Dochinger (1967) and Halambek (1976) each stated that the root dip technique successfully induced disease and killed pine seedlings in their artificial inoculations with V. procera. Based primarily on the work of Lackner and Alexander (1983, 1983), and supported by these other reports (Dochinger, 1967; Halambek, 1976), the present research was initiated with the root dip inoculation technique. The purpose of using this technique was to obtain seedlings with PRD symptoms for further experimentation regarding PRD development.

Inoculations results in the present study fall into three categories: colonization with PRD symptom development, colonization without PRD symptom development, and confounded controls. Colonization with accompanying foliar PRD

symptoms was the result needed to allow further experimentation. This did occur in four root dip experiments. Anticipating when PRD symptomatic seedlings would be available for comparisons was prevented however by the five experiments where colonization was not accompanied by development of PRD symptoms. This compromised my reliance on this procedure to obtain symptomatic seedlings for further experimentation. The experiments with problematic controls suggest the activity of insects in some cases, and too harsh an inoculation treatment in others. The experiments are discussed below within the context of the three categories listed above.

When colonization accompanied the development of foliar symptoms, the inoculation was successful. This occurred in four of the root dip experiments, all in the soil temperature tanks (Exp'ts 8, 10, 11, 12). The object of my inoculations was to obtain PRD symptomatic plants for comparison, and these successes did not occur reliably enough to fulfill that purpose. They do corroborate, in part, the earlier work regarding the pathogenicity of V. procera. The water potential readings from both inoculated and control seedlings in two of these experiments were consistent (Exp'ts. 10 & 11) but abnormally low. This could indicate either that the entire group of seedlings was stressed or a systematic source of error was introduced into the readings. The seedlings used in these experiments all

were selected because they possessed white root tips. This would suggest that the root systems of these seedlings were healthy, and that the very low water potential readings may be erroneous.

The presence of V. procera in control (uninoculated) seedlings confounded inoculation and contamination effects in the results in one set of experiments. In two experiments (Exp'ts. 3 & 5) with contaminated controls, the control seedlings were wounded as part of the mock-inoculation procedure and then maintained outside for extended periods of time (11 and 22 months, respectively). In Exp't 3 weevils, from which V. procera was cultured, were noted in association with the trees at harvest time. It is possible that insects were involved in the contamination of controls, at least in Exp't 3.

In Exp't 15, a greenhouse experiment of shorter duration (3 months), many controls also yielded the fungus at the end of the experiment. Three of the contaminated seedlings in this experiment which yielded V. procera also yielded Ceratocystis pilifera, a fungus associated sometimes with bark beetles and frequently on mechanically injured stems (Mathiesen-Kaarik, 1960). Mathiesen-Kaarik (1960) mentions that this species may be windborne as well as associated with insects. Reports concerning the occurrence of Ceratocystis species in the apparent absence of insect activity rarely have included efforts to prevent insect

visitation (Dowding, 1960). Dowding (1969) reports of windborne Ceratocystis inoculum which may instead be due to contamination by casual visits of insects rather than by successful establishment of insects. The likelihood of windborne inoculum reaching the wounds in the trees in Exp't 15 are particularly unlikely since the wounds were wrapped in cheesecloth and plastic. The entry of C. pilifera into wounded, wrapped seedlings in this experiment may have also resulted from insect involvement. If insects introduced C. pilifera, then V. procera might also have been carried between inoculated and control seedlings.

Another type of confounded result was the death of control seedlings without the isolation of V. procera at the end of the experiment. The undetermined cause of control seedling death was confounded with inoculation when this happened in three instances (Exp'ts. 4, 6 & 7). In all three cases, the inoculation procedure involved wounding the taproot or pruning the root system. The wounding or pruning associated with the inoculations could have been too severe, and compromised the vigor of the seedlings.

Five of the inoculation experiments reported here did not result in symptom development although isolation results indicated that infection and colonization occurred. Isolate variability, different physiological states of the seedlings used, different proportions of the root systems becoming colonized or unrecognized environmental factors affecting

the latent period are all potential sources of variability. These sources, either singly or in combination, could be involved in the lack of symptom development.

Lackner and Alexander (1983) found that among three isolates of V. procera, one had reduced virulence on one host species. Isolates from several sources were used in various parts of my experiments to reduce the likelihood of using a less virulent strain. However, the possibility of isolate virulence as a source of variability cannot be eliminated.

Seedling condition is another possible source of variability regarding symptom development. In three of the experiments with colonization but no symptom development, the condition of the root systems was not noted. The root tips could have been actively growing or suberized at the time of inoculation, which could affect the proportion of the root system becoming colonized. Without specific information regarding whether root tips were growing or suberized at the time of inoculation, the possible effect of root dormancy cannot be eliminated.

The purpose of the isolations from inoculated root tissues at the end of these experiments was to determine if V. procera had colonized the roots. This was inferred from positive isolations assuming that disinfestation eliminated V. procera spores on the surface of the root. Since the information desired was qualitative (whether the fungus had

or had not penetrated), precise determinations of the percent of each root system colonized were not attempted. The onset of foliar symptom development might occur only after a certain percent of the root system is colonized. The possibility of colonization at levels readily detected by isolation, but too low to induce foliar symptom development cannot be eliminated in these experiments.

The expression of symptoms is regarded as the end of the latent period of disease development (Agrios, 1978). The length of a latent period is known to vary with environmental parameters in many diseases. There is the possibility therefore that an unrecognized environmental factor was affecting the length of the latent period in these experiments.

The absence of foliar symptoms in the presence of root system colonization could result from any of the four factors discussed above. There is evidence for variability in virulence among isolates of V. procera which could account for the variability of my results (Lackner and Alexander, 1983).

The possible sources of variability cited above were considered in the present work in attempts to match the conditions of the earlier inoculations (Lackner and Alexander, 1982, 1983). The root dip inoculations in the present set of experiments were not as reliable as the earlier inoculations however, which indicates that the

conditions were not duplicated. A particular combination of conditions may be needed for successful root dip inoculation, or an unrecognized factor which was present for the earlier work may be required. Additional experiments which focus on the infection phase of disease development, the rate at which root systems become colonized, and the proportion of root systems colonized at the onset of symptom development would prove useful in elucidating additional parts of the disease cycle.

Chapter 5

Colonization Patterns of *Verticicladiella procera* In Scots and Eastern White Pine, Associated Resin-Soaking, and Reduced Sapwood Moisture Content and Needle Water Potential.

Introduction.

Significant mortality of Christmas trees in Virginia has been associated in recent years with stem and root colonization by *Verticicladiella procera* Kendrick (Lackner and Alexander, 1982), referred to as Procera Root Disease (PRD)(S.A. Alexander, pers. comm.). Lackner and Alexander (1982, 1983) fulfilled Koch's postulates for *V. procera* on loblolly and E. white pine. The method of spread of *V. procera* remains unclear however. Early reports (Alexander, 1980; Anderson and Alexander, 1979; Dochinger, 1967; Towers, 1977) noted higher incidences of PRD in low and wet areas of plantings. Lackner and Alexander (1984), however, reported the incidence of PRD in plantings to be scattered rather than prodominantly in low or wet areas. There is not an accepted distribution pattern of PRD in plantings which might suggest the means of fungal spread.

Lackner and Alexander (1984) planted seedlings in naturally infested soil. These seedlings became diseased, died and yielded *V. procera* upon isolation. The absence of visual evidence of insect activity on these seedlings led

these authors to conclude that infection occurred from soil-borne inoculum.

Lewis subsequently gathered evidence that soil-borne inoculum is probably not the major means of spread of this fungus (Lewis and Alexander, 1985; Lewis, 1985). In her studies, germinability of propagules in artificially infested soil declined rapidly both under controlled conditions and in the field. Propagule germinability decreased most sharply when soil dried beyond -1.5 mPa at moderate to high temperatures (20 C or above) under controlled conditions. Propagules in the soil were found only in the vicinity of colonized host tissue, with populations decreasing rapidly with distance from the host stem. In those studies, seedlings planted in naturally infested soil (24-170 cfu/g) did not become infected (Lewis, 1985). This argues against the importance of soil-borne inoculum, except possibly as colonized root segments, in this disease.

The discrepancies among these lines of evidence regarding infection from soil-borne inoculum precludes a clear understanding of the disease cycle. This, in turn impedes the development of effective control strategies for PRD. The present study was undertaken to more fully describe the distribution of V. procera within naturally infected Christmas trees. Knowledge of the distribution was needed to determine the probable point of initial

colonization in a tree. Additional data concerning the status of symptomatic trees were collected as etiologic observations.

Materials and Methods.

Ten trees each of E. white and Scots pine, showing symptoms of PRD were selected from commercial Christmas tree plantings where PRD had been previously confirmed. Both plantings were established on former pastureland, and managed for retail sales on a 'choose and cut' basis. Trees had been harvested for at least the preceding five years in both plantings. Scots pines were collected from Warren Co. and E. white pines from Montgomery Co., VA. After selection, trees were cut at ca. 0.5 m above the ground and the root system carefully excavated by hand with a mattock. Care was taken to keep root systems as intact as possible and to recover all woody roots, i.e. primary and low-order lateral roots (Sutton and Tinus, 1983), from within 0.5 m of the stem base and more distal portions if possible. Excavated root systems were returned to the laboratory for dissection and isolation. Collecting began in October, 1984 and ended in July, 1985.

At each point from which isolations were made, bark (phloem and periderm) and sapwood were separated and each tissue type plated separately onto acidified 2% (w:v) malt extract agar (MEA) and a medium selective for V. procera

(AMA) (McCall and Merrill, 1980). All tissue plated was surface disinfested by swabbing lightly with 70% ethanol and flaming briefly. Plates were incubated at 20 C for at least 14 da. The presence of resin-soaked and/or black-stained tissue was noted at each sampling point, as well as any indications of insect activity.

Individual roots were cut from the root collar with pruning shears. Tissue platings were then made from tissue immediately adjacent to the root collar, 5 and 10 cm from the root collar, and every 10 cm thereafter to the root end. Isolations were made from equidistant positions around the root collar. The majority were sampled at eight points; one E. white was sampled at four and one at six points. Two Scots were sampled at 12, one at 10 and one at seven points. Two sides of the stem 180° apart were sampled at 5, 10, 20 and 30 cm above the root collar in the Scots pines. Subsequent sampling of the E. white pines (the latter six) included samples along four sides of the stem, 90° apart at the same distances above the root collar. In total, 1522 points were sampled from the 20 trees.

Observations from each set of 10 trees were pooled within species and analyzed by categories corresponding to the tree parts sampled (stem, root collar and roots). Recovery patterns within each of the six categories were analyzed first by comparing a hypothetical uniform distribution with the observed frequencies for each category

with a Kolgomorov-Smirnov goodness of fit test (Ostle and Mensing, 1975). Fewer roots were present at greater distances from the root collar. This caused the uniform distribution to predict reduced frequencies at those distances, as seen in Fig.1.

Further analysis of the recovery patterns from stems consisted of regressing recoveries on position, then testing with student's t test the hypothesis that the regression line slope was different than zero. Further analysis of the root recovery patterns of both species consisted of deriving negative exponential equations fitting the observed frequencies and comparing values predicted from this equation to the observed values, again with the Kolgomorov-Smirnov test. The number of isolation points that yielded the fungus from the bark only was compared to the number yielding the fungus from the wood only by a sign test (Ostle and Mensing, 1975).

The sapwood moisture content (MC%) based on an ovendry (105 C) weight basis (Panshin and DeZeuuw, 1970) was determined for the basal stem section of 10 Scots and eight of the E. white pine trees sampled. A visual estimate was made of the proportion of the stem circumference at groundline with resin-soaked sapwood for these same trees. Needle samples for water potential determinations were collected from 15 pairs of trees (eight E. white and seven Scots). Each pair consisted of a sample tree (to be

excavated) and an asymptomatic neighbor tree of a similar age class. Branches with needles were collected with pruning shears, bagged separately in clean plastic bags with a moist paper towel and returned to the laboratory. A minimum of two water potential readings were made from each branch within 24 hr after collection. Needle water potentials were taken as replicate balancing pressure determinations from needle fascicles in a Scholander pressure bomb. Comparisons between mean water potentials for sample and neighbor trees were made with a paired t-test.

Results.

The recovery pattern was similar on Scots and on E. white pine. In both cases, relative recovery frequencies were root collars > roots > stems. Recovery frequencies were higher overall in Scots pine than in E. white pine.

The fungus was recovered from 97% of Scots pine root collar isolations, with a range of 85-100% from individual sampling points. In E. white pine, 42% of root collar isolations yielded the fungus, with a range of 33-44% for individual points. In both cases, the goodness of fit test did not reject (P=0.05) an assumption of uniform distribution around the root collar (D=.02 for both species; n=31, E. white; n=86, Scots).

The proximal parts of roots also yielded the fungus in high frequencies. A rapid decline in recovery frequency

occurred acropetally along roots in both pine species (Figures 5.1 and 5.2). In E. white pine, 35% of the roots yielded the fungus from isolations at the the root collar. Recovery declined to 0% at and beyond 40 cm from the root collar. Scots pine roots showed a similar pattern declining from 84% at the root collar to 0% at and beyond 70 cm from the root collar. Goodness of fit testing between observed and expected frequencies in roots showed that a uniform distribution did not match (P=0.05) the observed frequencies for Scots (D=0.3, n=223) or E. white (D=0.38, n=73) pine. When expected values derived from negative exponential distributions were compared to the observed frequencies from roots of both species, goodness of fit testing did not reject (P=0.05) the fit (D=.02).

Recoveries along stems declined acropetally, although not as rapidly as in roots. V. procera was recovered from 45, 35, 35, and 25% of the stem isolations at 5, 10, 20, and 30 cm respectively, above the root collar in Scots pine. E. white pine stems yielded the fungus from 9, 6, 3 and 0% of the isolations from these positions. Goodness of fit tests did not reject (P=0.05) a uniform distribution for Scots (D=.07, n=28) or E. white pine (D=0.32, n=6). Subsequent regression of frequency on stem position however, in both species yielded slopes significantly different from zero (P=0.05).

The frequency of 'bark only' recoveries was greater than

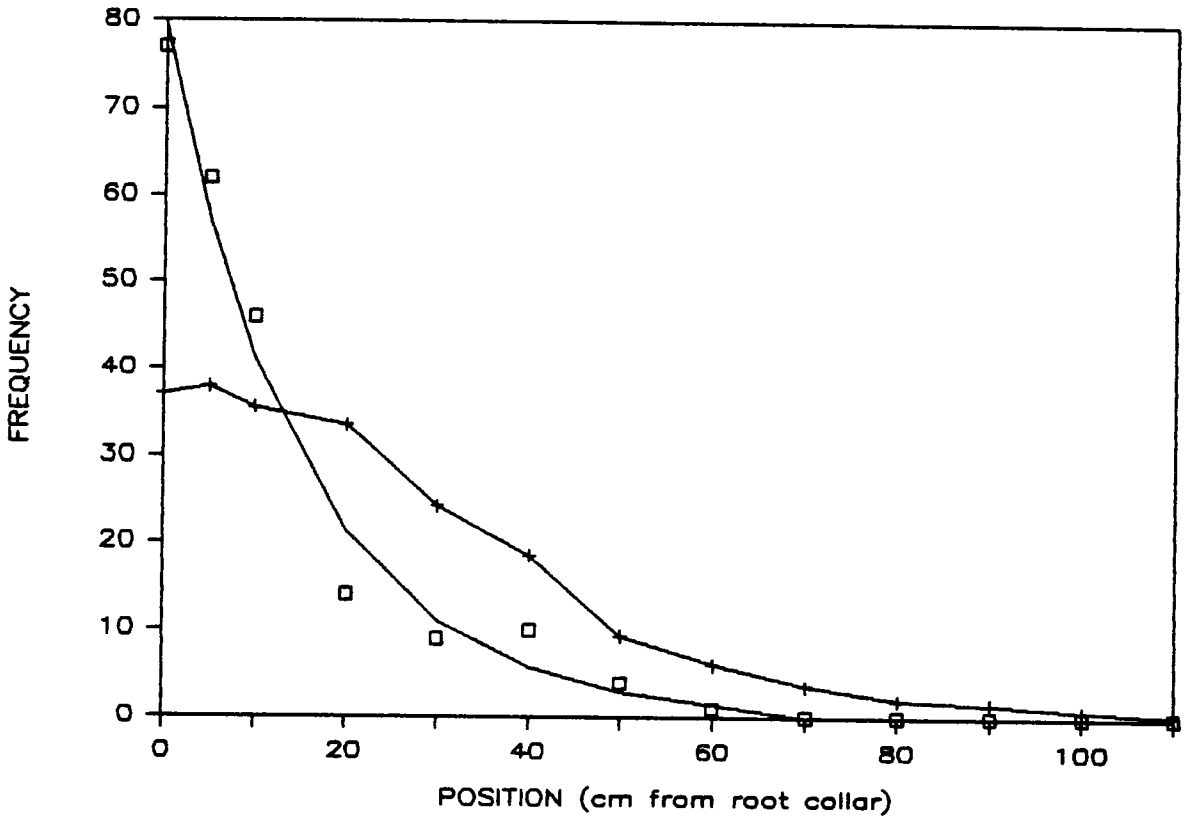

Figure 5.1. Observed recovery pattern of V. procera along roots of Scots pine. Included are the hypothetical uniform (+++++) and negative exponential (——————) distributions to which the observed distributions (□) were compared. The negative exponential function was of the form $Y=79.423e^{-0.0659x}$.

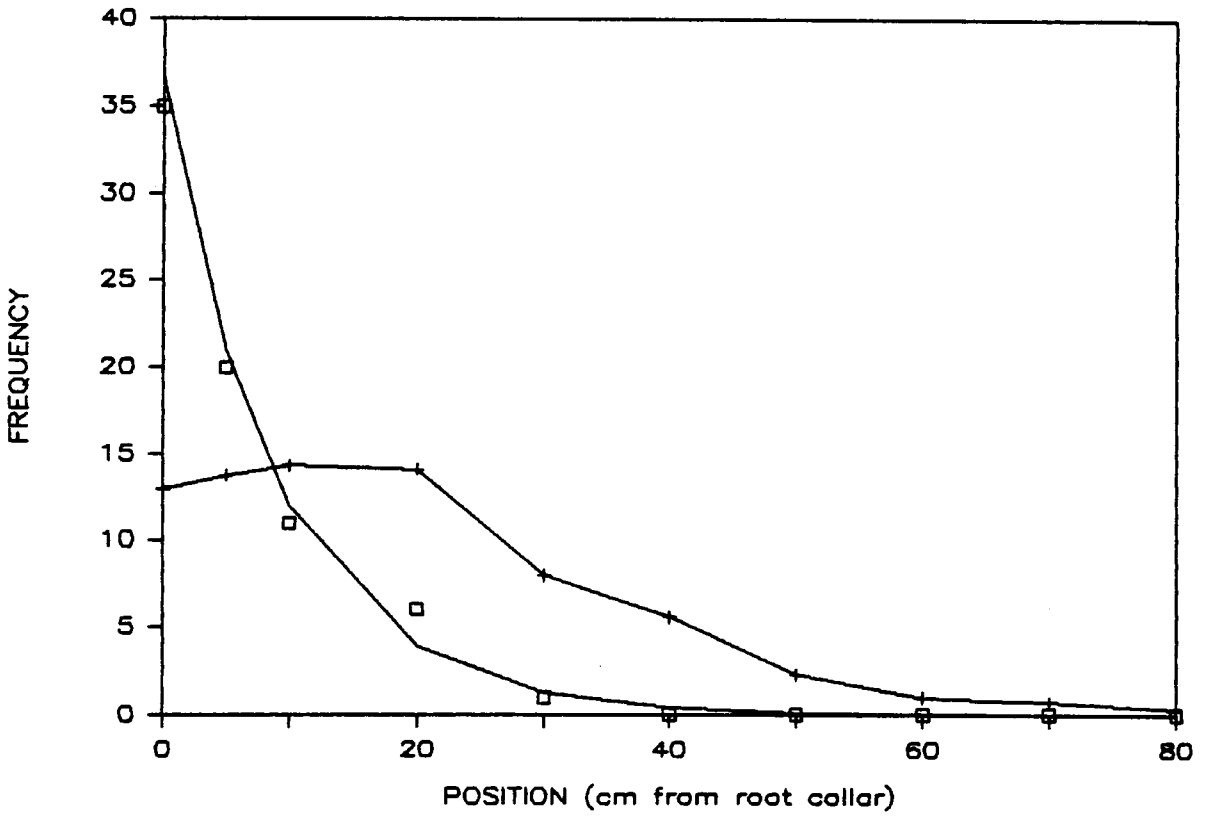

Figure 5.2. Observed recovery pattern of V. procera along roots of E. white pine. Included are the hypothetical uniform (+ + + +) and negative exponential (————) distributions to which the observed distributions (□) were compared. The negative exponential function was of the form $Y=36.624e^{-0.1118x}$.

'wood only' recoveries in both species for each category sampled, except for the white pine stems (Table 5.1). These differences were significant in Scots pine roots and collar (P=0.01), and in white pine roots (P=0.05). These trends were accentuated when categories were pooled.

Means ± S.D. (and ranges) of sapwood MC% for 10 Scots and eight E. white pine in this study were 86 ± 6% (34-123%) and 50 ± 5% (32-93%) respectively. The needle water potentials of Scots pine symptomatic sample and asymptomatic neighbor trees averaged -2.66 and -1.51 mPa, respectively. Sample and neighbor E. white pine needle water potentials averaged -3.15 and -1.29 mPa, respectively. A paired t-test showed that the mean difference was significant for both Scots (P=0.05) and E. white pine (P=0.01). The estimated amount of stem cross-sectional area at groundline that was resin-soaked and/or black-stained averaged 53 ± 6% (5-95%) and 73 ± 5% (30-100%) for Scots and E. white pine.

Discussion.

In both Scots and E. white pine the greatest recovery frequency of *V. procera* was from the root collars. Recovery frequency declined acropetally both up the stem and out the roots in both species. Regression analysis indicated an acropetal decline in frequency along the stem in both species; although this decline was not significantly different from a uniform distribution by goodness of fit

Table 5.1. Ratios of isolations from bark only and wood only from individual sampling points by species and tree part.

	Tree Species		
Tree Part	E. White	Scots	Combined
stem	2:3[1] (TF)[2]	8:3 (NS)	10:6 (NS)
collar	5:3 (NS)	12:0 (**)	17:3 (**)
roots	29:12 (*)	56:28 (**)	85:40 (**)
total	36:18 (*)	76:31 (**)	112:49 (**)

1. The first and second values of the ratio are respectively the number of sampling points which yielded Verticicladiella procera from the bark only and from the wood only.

2. Indicators of significance levels are: TF, too few observations to test; NS, not significant; *, significantly different at P=0.05; **, significantly different at P=0.01.

testing. This lack of significance is probably related to low values overall for stem frequencies. The acropetal decline along roots of both species did not fit a uniform distribution, but was described adequately by a negative exponential function. These data strongly suggest that the fungus occurs more frequently in proximal than in distal portions of roots. This agrees with data from stem and root collar isolations indicating that in both species, the root collar is the most frequently colonized part of the tree and that stem and root parts adjacent to the root collar are more frequently colonized than those away from the root collar. This pattern strongly suggests that initial colonization develops in the vicinity of the root collar and moves into root and stem tissues from the root collar.

Root tip infection, followed by basipetal root colonization, could also lead to root collar colonization. Subsequent acropetal colonization of other roots could then yield a pattern similar to that observed in this study. If this were true, the presence of some roots (at least one per tree) colonized at the distal end but not at the root collar would be expected. When colonization data were examined by individual root, however, only rare instances of this were seen. Three of 20 trees examined had a single root colonized at portions distal from but not proximal to the root collar. These three roots are less than 2% of the total 219 roots in the study population. This does not seem

sufficient to support the assumption of root tip colonization preceding root collar colonization.

The results of the sign tests (Table 5.1) indicate that V. procera occurred more frequently in the bark than in the wood, except in the sole case of the stems of E. white pine. Interpretation of results from E. white pine stems is complicated by low numbers precluding statistical testing. These results imply that initial colonization is in the bark or at least adjacent to bark tissues.

Lewis (1985) has obtained data that implicate weevils (Coleoptera) as vectors of V. procera in E. white pine Christmas tree plantings in Virginia. This evidence includes recovery of V. procera from a high proportion of weevils trapped in Christmas tree plantings and the demonstration of transmission of V. procera to freshly cut E. white pine bolts both in the field and in cloth-covered cages. Pertinent features of the ecology of these weevils (Hylobius pales and Pissodes sp., probably P. approximatus) are: 1) they typically infest the root collars and roots of trees, and 2) they feed in the phloem and oviposit in the cambium (Baker, 1972). The colonization patterns seen among tree parts and between bark and wood tissues in this study implicate an infection mechanism operating in the bark at the root collar. Lewis' suggestion that weevils are vectors of V. procera in E. white pine implies that initial colonization by V. procera, at least in that species, would

be at the root collar, which agrees with the findings of this study.

Horner and Alexander (1985) presented evidence demonstrating that resin-soaked or black-stained sapwood was much less permeable to water flow than was clear sapwood. The basal stem sections examined in the present study all were resin-soaked. Although the amount varied widely, average values for the cross-sectional area that were resin-soaked were 53% and 73% for Scots and E. white pine, respectively. This suggests that some symptomatic trees are functioning with less than half of their cross-sectional area at the stem base. This is probably a conservative estimate since some resin-soaking likely occurs and probably decreases permeability at levels too low to detect visually.

Mean sapwood MC% of E. white pine samples in this study was 50 $\pm$ 5%. Reported literature values for the MC% of E. white pine sapwood are 120% (Harrar, 1957) and 175% (Cech and Pfaff, 1977). The Scots pines (a diploxylon species) in this study had a mean basal sapwood MC% of 86 $\pm$ 6%. Koch (1972) presents a range of values for diploxylon pine species (southern yellow pines) of 115%-135% for trees less than 25 yr old in the southeastern United States. Scots pine in Siberia has sapwood MC% values of 102-164% (Isaeva, 1970). The trees in this study were less than 12 yr old and therefore contained mostly juvenile wood (Senft, et al., 1985). Since juvenile wood has a higher MC% than mature

wood (Koch, 1972, p. 269), values expected from this study would be as high, or higher than the cited MC% values, which are for stems containing mature wood. Contrary to expectation however, values from this study vary from the low end of the range to well below the cited range.

Evidence gathered during the course of this study suggests that colonization by V. procera in pine Christmas trees in Virginia originates in the vicinity of the root collar, most likely in the bark. Additional evidence gathered in this study suggests that foliar PRD symptoms are due to xylem dysfunction leading to crown desiccation. Lower than expected sapwood moisture contents support this contention. Resin-soaked sapwood, which has a drastically reduced permeability to water (Horner and Alexander, 1985) is prevalent at the root collar in trees with PRD. This permeability reduction could be responsible for symptom development in PRD.

Chapter 6

Radial growth and Sapwood Moisture Content Analysis from Increment Cores of Eastern White Pine.

Introduction.

One of the more noticeable symptoms of pines colonized by V. procera is the reduced springtime elongation of terminal and lateral shoots. The growth reduction of shoots is cited by several reports concerning the diagnosis and description of Procera root disease (PRD) (Alexander, 1980; Anderson and Alexander, 1979; Dochinger, 1967; Halambek, 1976; Towers, 1977). Sinclair and Hudler (1980) state that E. white pines infected by V. procera exhibit growth reduction one to two years prior to death, but do not specify radial or shoot growth. Stone, et al. (1954) also reported terminal growth reductions as part of a disease syndrome of red pine. Subsequent reports implicated V. procera in this syndrome (Kendrick, 1962; Sinclair and Hudler, 1980). Although colonization of pines by V. procera has been shown in these reports to be associated with reduced shoot growth, the radial growth rates of colonized trees have not been examined.

Reduced sapwood moisture content (MC%) is a well documented aspect of bluestain colonized trees. Early workers (Caird, 1935; Nelson, 1934) described the MC%

reductions associated with bluestain colonization and the optimum MC% levels for growth of these fungi in wood (Lagerberg, et al., 1927). More recent workers corroborated these results with artificial inoculations of bluestain fungi in pines (Mathre, 1964; Basham, 1970). The association between bluestain colonization and stem drying has also been modeled in relation to bark beetle attacks (Fares, et al., 1980). This synthesis states that water content reductions in loblolly pine can be induced by increasing water stress or by the introduction of the blue (stain) fungus via southern pine beetle. Since histologic, symptomologic and taxonomic evidence suggest similarities between V. procera and bluestain colonizations of pine sapwood, it is reasonable to postulate that sapwood colonized by V. procera also has reduced moisture contents.

The following study was undertaken to explore the possibility that host radial growth is affected by V. procera colonization. The second purpose of this study was to determine whether sapwood moisture content in colonized trees is lower than in uncolonized trees.

Materials and Methods.

An E. white pine Christmas tree planting with a known history of PRD was selected for the study. The planting was in Carroll Co. VA, planted on former pasture land and managed for sale to wholesale dealers. Trees sampled were generally 10-18 years old.

Twenty-two candidate symptomatic trees were selected on the basis of foliar discoloration and basal resinosus. Candidates were sampled for V. procera by aseptically plating three bark and sapwood plugs from the root collar onto an agar medium selective for V. procera (AMA) (McCall and Merrill, 1980). Previous work (Chapter 5) showed that the root collar was the most frequently colonized part of the tree. It was assumed from this work (Chapter 5) that a tree was uncolonized if V. procera was not detected from the root collar isolations. Plugs were removed from points equidistant around the root collar with a #5 cork borer after swabbing the bark with 70% ethanol. Plated tissue was incubated at 20 C for at least 10 da and scored for the presence of the fungus.

Symptomatic trees with V. procera were subsequently paired with an asymptomatic neighbor tree. Neighbor trees were selected on the basis of healthy appearance, i.e. asymptomatic, being in a size class similar to and in proximity to the symptomatic tree. Candidate neighbor trees were then sampled for the presence of V. procera in the same manner as the symptomatic trees. Trees were used as neighbors only if V. procera was not detected from the isolations. The fungus was recovered from three of 18 candidate neighbor trees. Sample pairs of trees consisted therefore, of a symptomatic tree which yielded the fungus, and its nearest asymptomatic, neighbor of a similar size class from which no V.

procera was detected. Ten sample pairs comprised the test population.

Increment cores were extracted from six points along the stem of each of the 20 trees. Each core was taken through the entire tree diameter such that pith and two opposite radii were included. The six points were spaced at 15 cm intervals from 15-90 cm above ground. Extraction of cores took place within an eight da period in the first half of October, 1985. Cores were sealed in plastic straws and returned to the laboratory where they were immediately shaved to facilitate ring width reading, and weighed.

Widths of the outer five growth rings on both sides of each core were measured with a dendrochronograph (Techtron Systems, Little Ferry, New Jersey). Widths were recorded to the nearest 0.01 mm. The cores were then oven dried at 105 C for at least one da, allowed to cool in a desiccator and weighed. Moisture contents were calculated on an ovendry basis for each core (Panshin and DeZeeuw, 1970). The two ring width readings for a particular year from opposite core sides were averaged and used as the width value for that year from that core.

Moisture content and ring width values were analyzed by a split plot experimental design with plots split by colonized trees and trees with no detected V. procera. Each set of ring widths from the current year (1985) to four years prior (1981) were analyzed separately. Values for the

last three years were summed and compared, as was the five year sum.

Regression analysis was subsequently used to examine the data for trends in growth rates over the last five years and in moisture content with stem position.

Results.

The analysis for MC% of cores revealed that trees colonized by V. procera had significantly lower moisture contents than trees with no detected V. procera (Table 6.1). The expected trend of increasing MC% with height (Koch, 1972) was observed in both sets of trees. The correlation of moisture content and height was essentially parallel for both classes of trees although with lower values in the colonized trees. Graphic representation of the mean values for disease class and core position, as well as analysis of variance indicated that no interaction occurred between core position and presence of the fungus.

Core width analysis also revealed significant differences in each of the last three years (1983-85) between trees with V. procera and trees from which V. procera was not detected (Table 6.2). Comparison of radial growth rates from five and four years prior to sampling (1981-82) did not reveal significant differences. When the growth of the last three years was summed and compared, the magnitude of the growth reduction of colonized trees was

Table 6.1. Moisture contents of sapwood cores from trees with and without detected Verticicladiella procera colonization.

Symptom class	Significance Level	Core position[1]					
		1	2	3	4	5	6
A[2]	** [4]	48.3[3]	56.1	61.2	65.2	78.3	70.0
B		119.2	129.8	149.1	144.6	154.3	137.9

1. Position 1 was 15 cm above ground level, with subsequent positions at 15 cm intervals upward on the stem.
2. A = trees with V. procera, B = trees from which V. procera was not detected.
3. Values are the mean moisture content of 10 cores.
4. Significant at P=0.01.

Table 6.2. Average ring widths for trees with and without detected Verticicladiella procera colonization, ordered by stem position and by year.

Year	Symptom Class	Significance Level	Stem Position[1] 1	2	3	4	5	6
1985	A[2]	*[4]	190[3]	215	222	202	201	220
	B		239	262	231	293	251	224
1984	A	*	224	269	244	237	255	241
	B		291	319	304	310	266	250
1983	A	*	254	317	306	273	272	277
	B		314	308	276	281	309	276
1982	A	NS	278	334	287	318	273	282
	B		327	285	304	314	316	267
1981	A	NS	286	311	298	327	275	291
	B		292	298	314	297	303	312

1. Position 1 was 15 cm above ground level, with subsequent positions at 15 cm intervals upward along the stem.
2. A = trees with V. procera, B = trees without detected V. procera.
3. Values are the mean ring width in mm/100 of 10 trees.
4. Significant at P = 0.1 (*), or not significant (NS).

accentuated and the difference was significant. The five year sums were also significantly different, but the magnitude of difference was less than with the three year sum, as expected with two years included that did not differ.

Regression analysis of moisture content and stem position indicated that the moisture content increase with stem height was significant (P=0.05) in trees without V. procera. In trees with V. procera, the gradient was less defined (P=0.09). The growth rates of trees without detected V. procera infections was decreasing significantly (P=0.05) over the prior five years, as indicated by regression analysis.

Discussion.

Radial growth and sapwood MC% were both significantly lower in colonized trees than in trees with no detected V. procera. The presence of drier sapwood in colonized trees agrees with previously reported conditions of trees colonized by bluestain fungi (Basham, 1970; Caird, 1935; Mathre, 1964; Nelson, 1934). The radial growth reduction is similar to previous reports of conifers infected by root diseases (Alexander, et al.,1975; Alexander, et al., 1981; Bradford, et al., 1978).

There was a general increase of MC% with stem height

among all trees in this study, both with and without detected V. procera colonization. Although MC% values were lower among trees with the fungus, the patter of increasing MC% with height still was obvious. This pattern agrees with published results for standing pine timber (Koch,1972). The pattern observed in the present study contrasts, however with earlier reports concerning lethal bluestain infections. Mathre (1964) was able to kill ponderosa pines with artificial inoculations of Ceratocystis ips and C. minor. Basham (1970) was able to kill loblolly pines by inoculating with each of the above two species and with C. montia and C. pilifera. In both of these reports, sapwood moisture content at and above the point of inoculation was lower than the moisture content below the point of inoculation. That is, a reversal in the typical height, MC% correlation (Koch, 1972) was observed.

Caird (1935) and Nelson (1934) determined moisture contents throughout bark beetle infested pines in which bluestain was developing. Caird, by correlating dye conductance with MC% was able to establish that sapwood below 75% MC did not conduct. In both studies, moisture contents decreased at and above the point of bluestain development but remained unchanged, or increased below the point of bluestain development. In the present study however, the moisture content of stems colonized by V. procera still had generally higher MC% at greater stem

heights, thereby retaining the normal correlation of height and MC% (Koch, 1972).

The four reports cited above were able to sample MC% below the point of fungal colonization, where MC% developed to higher than normal values. This contributed to the 'reversal' of the normal correlation of higher MC% with stem height (Koch, 1972). In the present study, bole samples were not available from below the presumed point of colonization since V. procera colonizes primarily the root collar. This may explain the lack of congruency between previous reports and the present report. The observation of uniformly reduced MC% in colonized trees is consistent with the supposition that water availability to the entire stem is reduced in trees colonized by V. procera.

The association of reduced radial growth and V. procera colonization documented in this study is consistent with at least two equally plausible explanations. Reduced radial growth could reflect reduced vigor of the trees that existed prior to fungal colonization. This possibility would suggest that stressed trees are predisposed to colonization by V. procera. Alternatively, fungal colonization may have occurred several years prior to the development of foliar symptoms and caused the reduced radial growth. This possibility would suggest that V. procera colonization may affect radial growth for several years prior to the development of foliar symptoms.

The work of Alexander, et al. (1975, 1981) and Bradford, et al. (1978a) has demonstrated that pines colonized by Heterobasidion annosum (Fr.) Bref. exhibit reduced radial growth for several years prior to the development of foliar symptoms. The extent of root decay was determined by excavations of entire root systems in the later two of these studies (Bradford, et al., 1979b; Alexander, et al., 1981). These workers report total colonization levels of 14% and 54% of tree root systems. These values indicate that colonization probably occurred several years prior to growth rate sampling.

These studies (Bradford, et al., 1979b; Alexander, et al., 1981) did not address possible mechanisms underlying the growth reduction associated with H. annosum. Since annosus root rot does destroy the root system, and therefore the water supply of the tree, it is reasonable to assume that water deficits are involved in this growth reduction. Colonization of pine stems by V. procera involves the proximal parts of roots and the root collar. Severe resinosus of sapwood is associated with V. procera colonization and renders sapwood essentially impermeable to water (Horner and Alexander, 1985). Thus a likely mechanism exists for V. procera colonization to reduce radial growth.

Alexander, et al. (1981) further related the radial growth reductions associated with H. annosum colonization with increased probability of bark beetle attack. This work

shows that stressed trees, exhibiting reduced growth, were more likely than vigorous trees to be attacked by bark beetles, which vector bluestain fungi. There is now substantial evidence that V. procera is associated with root and root collar inhabiting insects, and may be vectored by these insects (Lackner and Alexander, 1983; Lewis, 1985; Wingfield, 1983a). The weevil species found associated with V. procera in Virginia (H. pales and Pissodes sp., probably P. nemorensis) are attracted to weakened or stressed trees (Baker, 1972; Wingfield, 1983a; Lewis, 1985). Although radial growth rates were greater in trees without detected V. procera, these growth rates were decreasing over the previous five years. The decreasing growth rates of trees without detected V. procera and the ecology of the insects associated with the fungus support the alternative that infected trees were stressed prior to infection.

The presence of dry sapwood lends credence to the contention that trees colonized by V. procera die in response to an interrupted water supply. Reduced radial growth could be explained by reduced water flow through the stem, or indicate that slow growing trees are more frequently colonized by V. procera, possibly via insect vectors. Since it is not known when the trees in the present study became colonized, discrimination between these explanations is not possible without additional data.

Chapter 7

Histologic and Functional Studies of Pine Sapwood Colonized by *Verticicladiella procera*.

Introduction.

Procera root disease (PRD) was previously shown to be caused by *Verticicladiella procera* and responsible for important economic losses in Christmas tree production in Virginia (Lackner and Alexander, 1982, 1983, 1984). Symptoms associated with PRD are a prominent drooping of needles in E. white pine, reduced shoot elongation in the spring, pronounced resin-soaking of sapwood at the stem base, and frequently a prominent wedge-shaped pattern of black staining in the sapwood of both E. white and Scots pine (Anderson and Alexander, 1979). The anatomical aspects of *V. procera* colonization in PRD are incompletely described however.

Reports associating *V. procera* with diseased E. white pine in New Zealand (Shaw and Dick, 1980; identity in Wingfield and Marasas, 1983) and Yugoslavia (Halambek, 1976) have included some histological observations of colonized sapwood. Halambek (1976) reported the presence of hyphae in tracheids and bordered pit pairs. There is no mention in that report of whether hyphae were also present in ray cells. Shaw and Dick (1980) observed hyphae concentrated in

axial tracheids but also present in the rays. Interpretation of the role of V. procera in this study is difficult however, as a subsequent report (Wingfield and Marasas, 1983) determined that the Verticicladiella sp. from New Zealand (Shaw and Dick, 1980) included V. procera, V. truncata Wingfield and Marasas and a third, undescribed species. The lack of agreement and uncertainty of the fungal identity compromise the conclusiveness of these reports.

Reports of artificial inoculation studies with V. procera on other conifer species have briefly noted colonization patterns (Harrington and Cobb, 1983; Bertagnole and Partridge, 1983). Bertagnole and Partridge (1983) observed hyphae of V. procera in both axial tracheids and ray parenchyma of lodgepole pine. These authors did not report relative amounts of hyphae present in these different cells. Harrington and Cobb (1983) compared colonization by V. wageneri with several other staining fungi, one of which was V. procera, in Douglas-fir and ponderosa pine. They discussed fungi other than V. wageneri as a group, noting that these fungi were generally reisolated from their inoculated pine, but not from Douglas-fir. They also noted that these other fungi exhibited more radial and less longitudinal colonization of pines than V. wageneri. Hyphae of these other fungi were present in both tracheids and ray parenchyma whereas V. wageneri hyphae were present only in

axial tracheids in their study. These reports (Bertagnole and Partridge, 1983; Harrington and Cobb, 1983) agree that V. procera colonization involves both axial tracheids and rays. Each addresses a range of fungal species however and neither is a definitive account of V. procera colonization of sapwood.

Available observations of colonization patterns in E. white pine indicate that the salient feature is the presence of hyphae in axial tracheids. The staining pattern in colonized pine sapwood however is wedge-shaped when viewed in transverse section (Anderson and Alexander, 1979). Anatomical considerations suggest that a wedge pattern of staining would likely involve colonization primarily of ray tissues, at least initially. The stain pattern of bluestain fungi is also wedge-shaped (Rumbold, 1931; Manion, 1981) and is known to proceed initially along rays with subsequent development into axial tracheids (Ballard, et al., 1984; Lagerberg, et al., 1927). To determine what tissues are initially penetrated would contribute to an understanding of the colonization of pine sapwood by V. procera.

The symptoms of PRD are similar to the desiccation symptoms of trees infected with bluestain fungi, either by artificial inoculation (Basham, 1970; Mathre, 1964) or bark beetle infestations (Caird, 1935). The prominent wedge-shaped sapstain associated with V. procera is macroscopically similar to bluestain. The available reports

do not agree though on the host cell types involved in V. procera colonization. It is not known therefore what degree of similarity exists between V. procera colonization and colonization of pine sapwood by bluestain fungi. The absence of a definitive account of sapwood colonization by V. procera precludes reconciling these discrepencies and precludes an assessment of the degree of similarity between bluestain and V. procera colonization of pine sapwood. The objective of the present study was to prepare a detailed description of the colonization of pine sapwood by V. procera.

Materials and Methods.

Sapwood of naturally infected Scots and E. white pine was taken from trees with PRD collected from commercial Christmas tree plantings in VA. Scots pine were collected from Warren Co. and E. white from Montgomery Co. The presence of V. procera was confirmed by culturing the fungus from collected trees. All isolations were conducted in duplicate on a general media (2% malt extract agar), and a medium selective for V. procera (McCall and Merrill, 1980). Particular care was taken to examine all isolation plates for dematiaceous or other darkly pigmented fungi. V. procera was the only dark fungus recovered on either medium from tissue samples used in the present study. Sapwood that was resin soaked or black stained was considered symptomatic

of PRD. Symptomatic sapwood was excised, fixed in FAA (formalin: acetic acid: ethanol, 13:5:200) and stored until sectioned in FAA. Sections 15-20 u thick were cut with a sliding microtome. Sections were stained using a modified version of Cartwright's (1929) safranin, steaming picro-aniline blue method. Sections were immersed for one minute in picro-aniline blue which was already heated to steaming. Two separate dehydration schedules were employed after staining and rinsing. A graded ethanol series followed by clearing in xylene and mounting in a synthetic mounting resin (Permount^{R}) was used initially. Sections prepared for examination of pine resin inclusions were dehydrated in glycerol and mounted in corn syrup. Prepared slides were examined and photographed with a Leitz microscope.

Samples of resin soaked and black stained tissue for permeability comparisons were taken from naturally infected E. white pine Christmas trees with crown symptoms. Clear sapwood was taken from an asymptomatic E. white pine approximately 20 yr old in an unmanaged woodlot. After felling, the lower bole section of the trees were removed to the laboratory, and each end trimmed under water to minimize introduction of embolisms. Bole sections ca. 10 cm long were secured with clamps to prevent splitting by the increment borer. Longitudinally oriented cores of 5 mm dia were removed from submerged bole sections, and cores trimmed

under water to 5 cm with a razor blade. Excised and trimmed cores were placed in a Scholander pressure bomb with one end in degassed, filtered water and exposed to a 250 kPa pressure differential. Permeability was determined by measuring the amount of water passing through the cores in 5 min.

Results.

The observed colonization pattern of V. procera within pine sapwood generally agreed with descriptions of bluestain fungi. The greatest concentrations of hyphae were seen in the ray tissues and in the resin-ducts. No differences were noted in the colonization patterns between the two pine species examined.

Asymptomatic sapwood was devoid of hyphae or resinous inclusions. The ray parenchyma cells were intact with nuclei visible in radial section (Fig. 7.1). When viewed in tangential section (Fig. 7.2), the ray parenchyma were also seen to be intact, although nuclei were not usually visible, probably due to the small cell volume visible in this orientation. Resin duct epithelial cells from asymptomatic tissue appeared intact, and the resin duct was readily distinguishable (Figs. 7.3 & 7.4). No hyphae or resin globules were seen in asymptomatic tissue dehydrated in either ethanol or glycerol.

The ray parenchyma cells in resin soaked sapwood

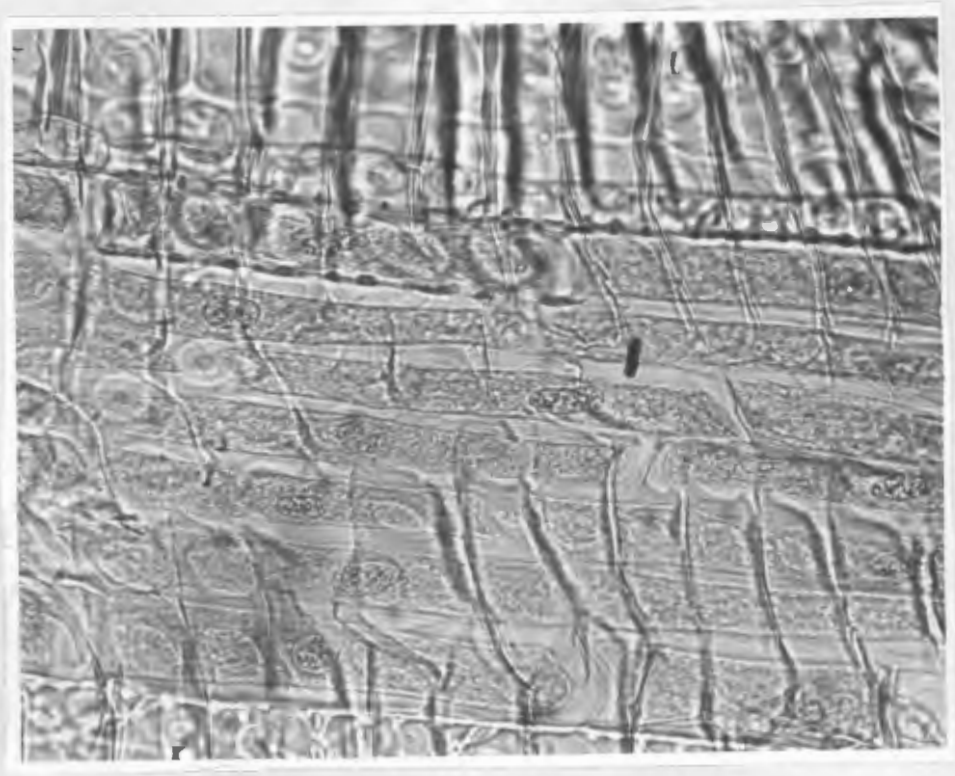

Figure 7.1. Radial section of asymptomatic Scots pine sapwood (250x, ethanol dehydration). Note intact nuclei.

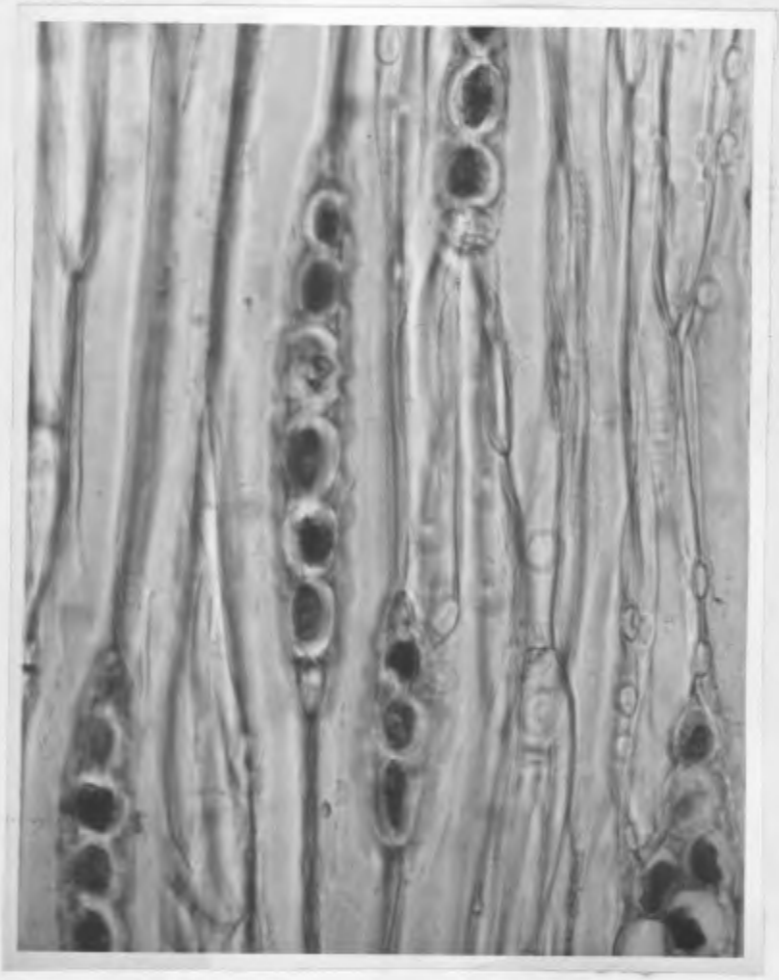

Figure 7.2. Tangential section of asymptomatic Scots pine sapwood (250x, ethanol dehydration. Note intact cell walls of ray parenchyma.

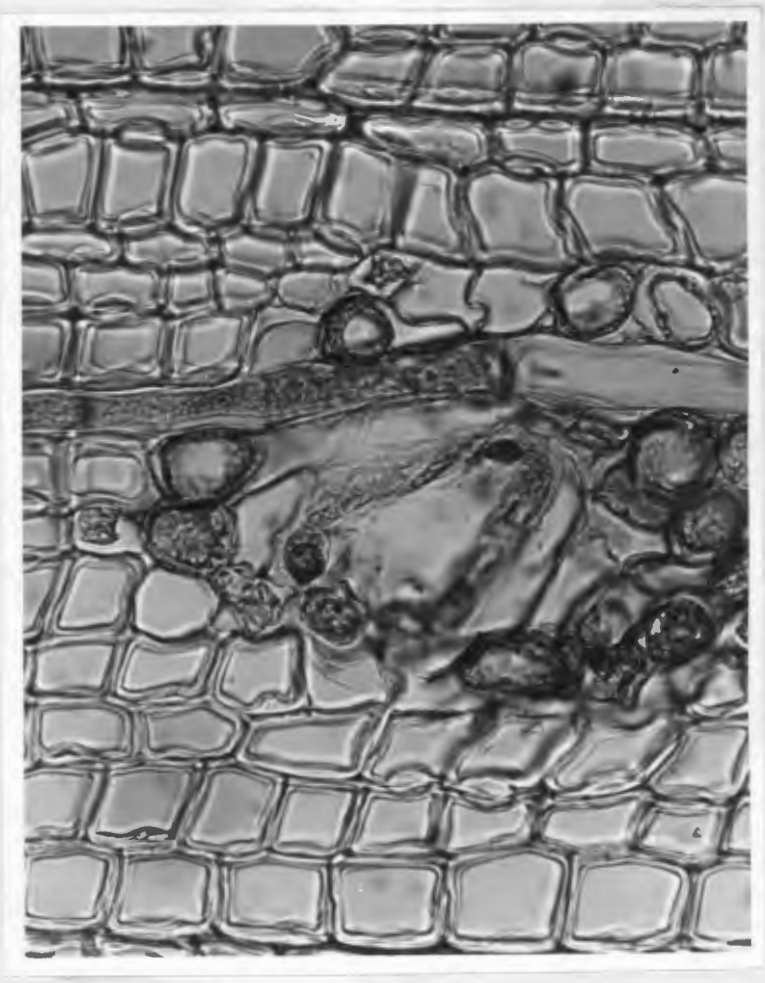

Figure 7.3. Transverse section of asymptomatic Scots pine sapwood (250x, ethanol dehydration). Note open resin duct and intact parenchymatous cells.

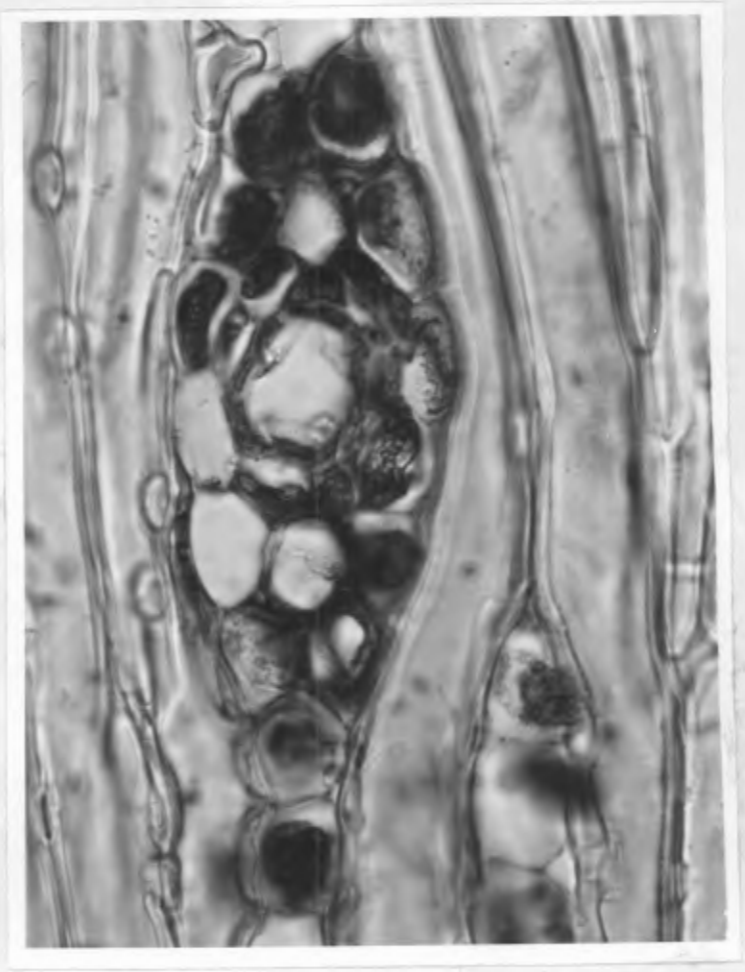

Figure 7.4. Tangential section of asymptomatic Scots pine sapwood (400x, ethanol dehydration). Note open resin duct.

typically did not have discernable nuclei (Fig. 7.5). When viewed in tangential sections, ray parenchyma frequently had cell walls which appeared disintegrated or deformed (Fig. 7.6). These effects were seen occasionally in the absence of resinous inclusions, whereas resinous inclusions were always accompanied by degenerated parenchyma cell walls. The sometimes copious amounts of resinous material visible in sections dehydrated in glycerol (Fig. 7.5) were sparse or not present at all in sections dehydrated in ethanol. The material, never seen in any sections of asymptomatic sapwood, was readily visible in glycerol-dehydrated sections of resin soaked sapwood, but not visible in ethanol-dehydrated sections of resin soaked sapwood.

Resinous inclusions were frequently observed spread across the entire width of cell lumena (Figs. 7.5, 7.7, 7.8). When viewed in transverse or tangential section, tracheids in closer proximity to ray cells more frequently contained resinous inclusions than did tracheids more distant from the ray, at that point (Figs. 7.7 & 7.8). When resinous inclusions were numerous, or viewed in radial section (Fig. 7.5), association with rays was not discernable.

Black stained sapwood invariably contained darkly pigmented hyphae (Figs 7.9-7.15). When present in a section, hyphae were always in ray cells (Fig. 7.9). In sections where hyphae were also present in longitudinal

Figure 7.5. Radial section of resin soaked white pine sapwood (250x, glycerol dehydration). Note prevalent resinous inclusions in longitudinal tracheids and absence of hyphae.

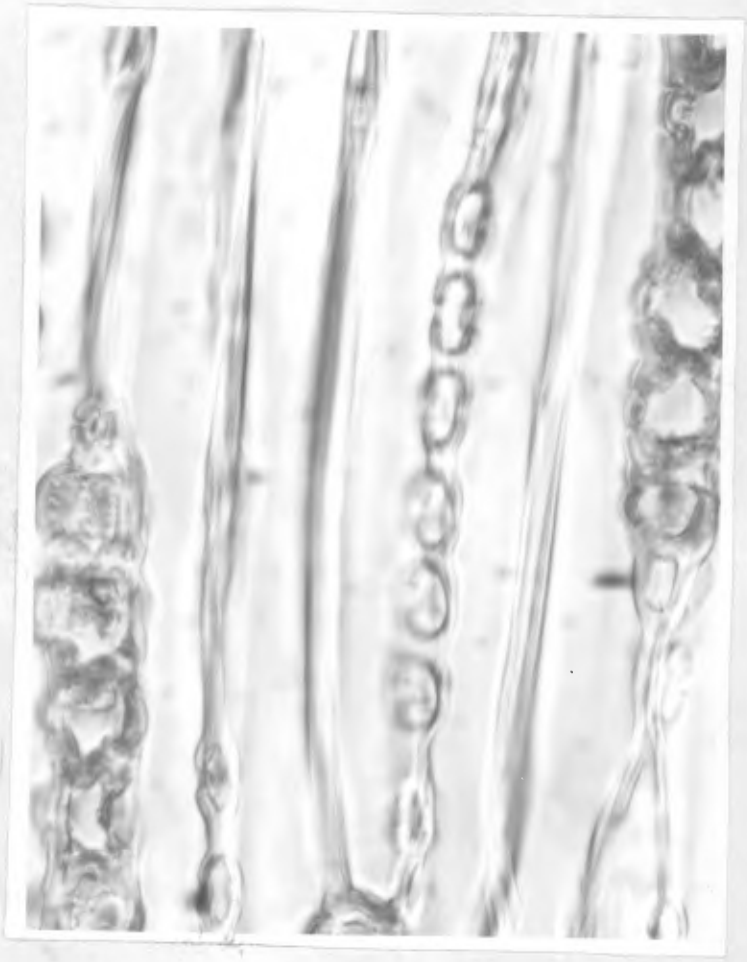

Figure 7.6. Tangential section of white pine sapwood with degenerating ray parenchyma, but no resinous inclusions in longitudinal tracheids (400x, glycerol dehydration).

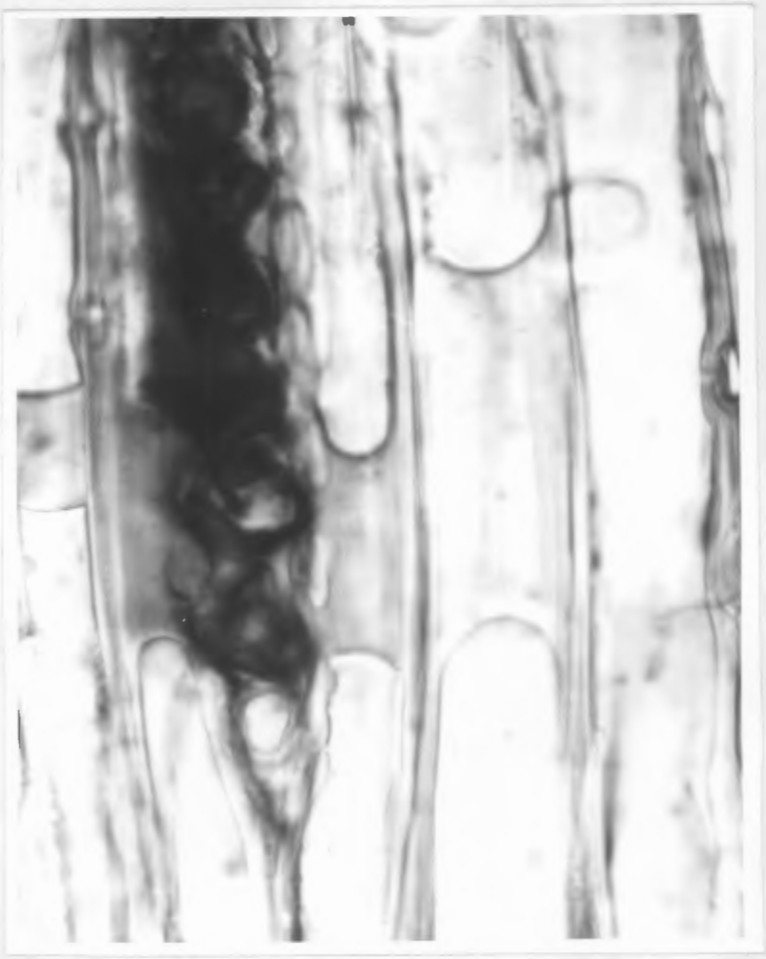

Figure 7.7. Resin soaked Scots pine sapwood (400x, glycerol dehydration). Resinous inclusions are obvious in longitudinal tracheids.

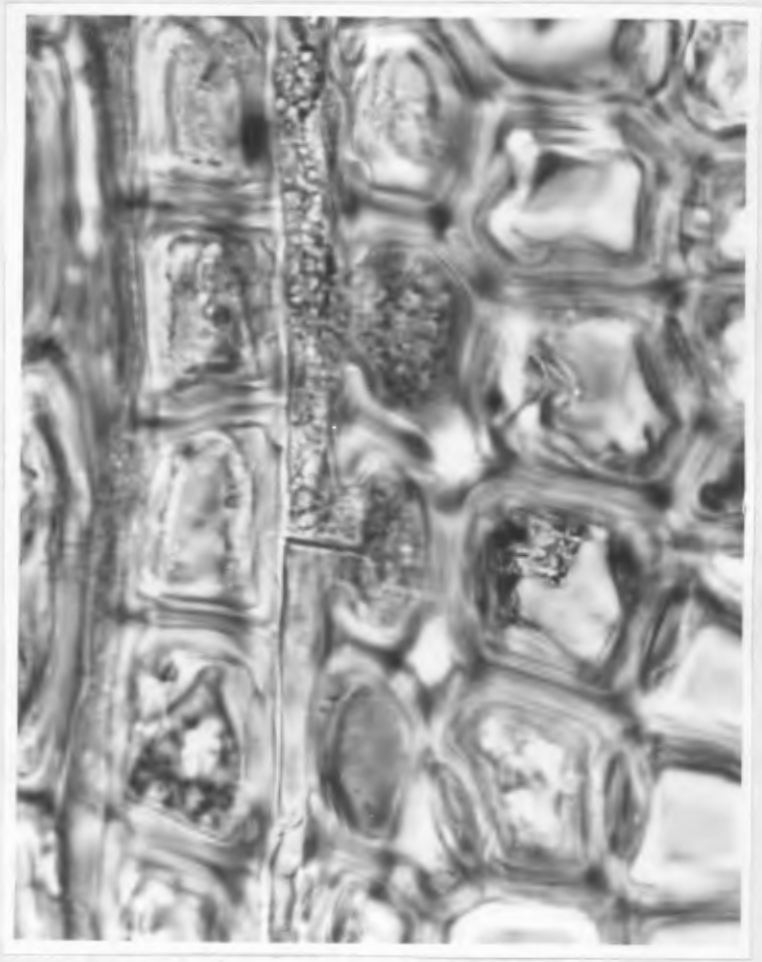

Figure 7.8. Resinous inclusions in Scots pine, transverse section (400x, glycerol dehydration). Resinous inclusion apparently is continuous between ray parenchyma and adjacent longitudinal tracheid.

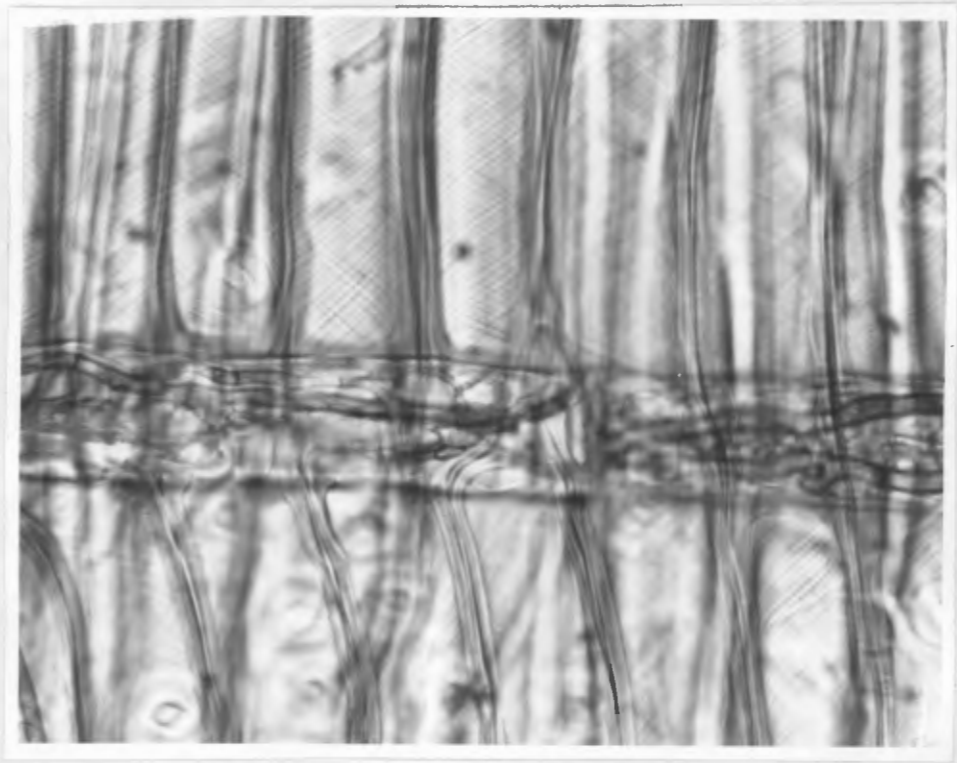

Figure 7.9. Pigmented hyphae in ray parenchyma, visible in radial section of Scots pine (250x, ethanol dehydration).

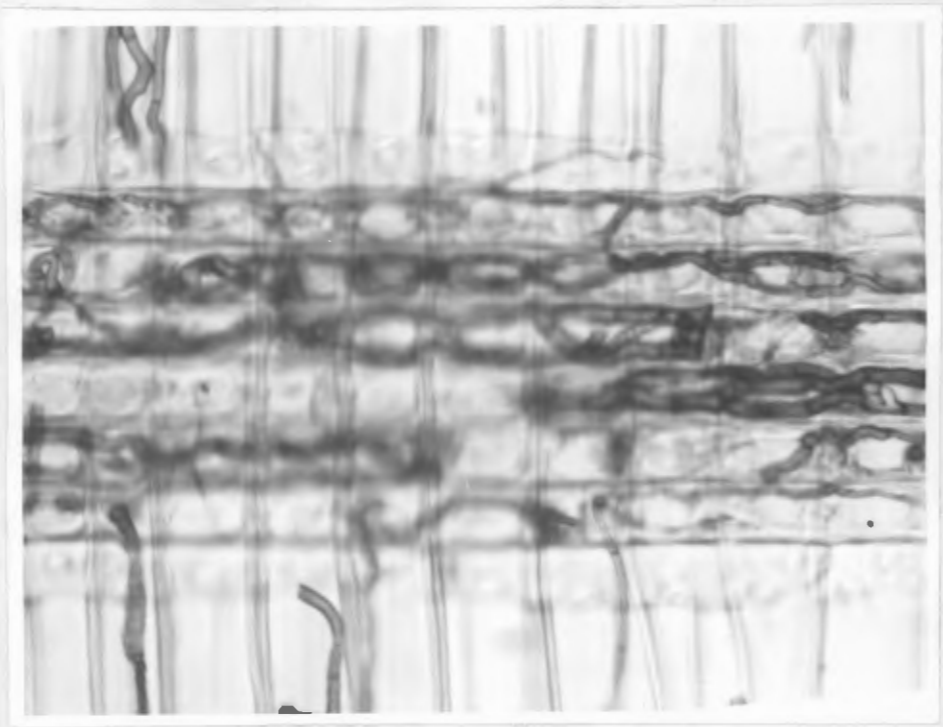

Figure 7.10. Numerous pigmented hyphae in ray of white pine, seen in radial section (250x, ethanol dehydration).

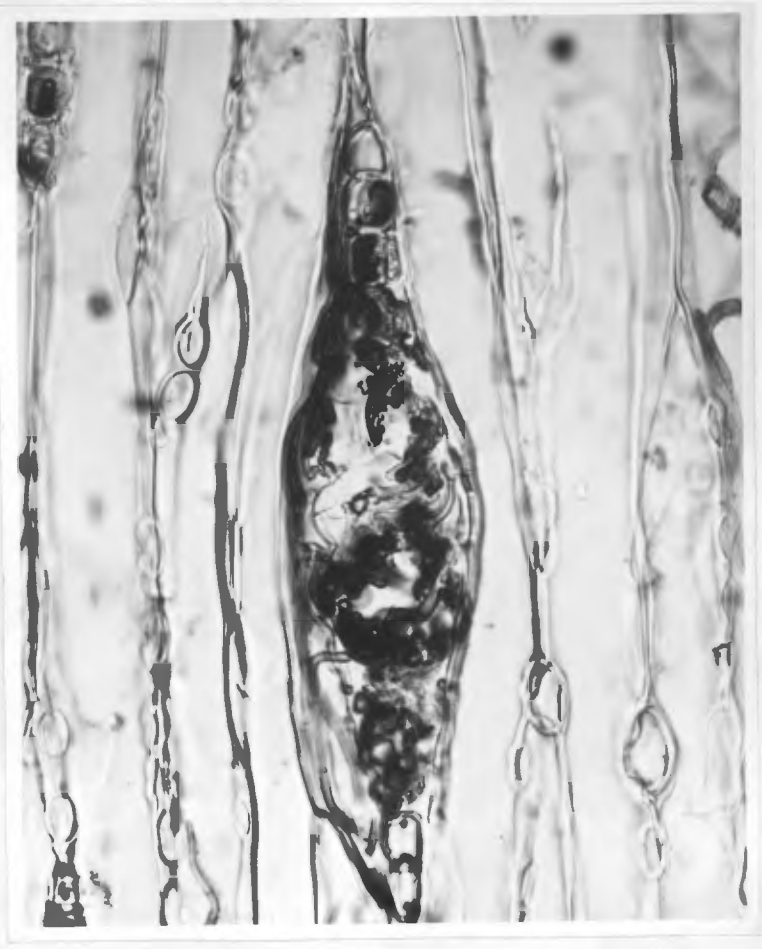

Figure 7.11. Horizontal resin duct of white pine, seen in tangential section filled with pigmented hyphae (250x, ethanol dehydration).

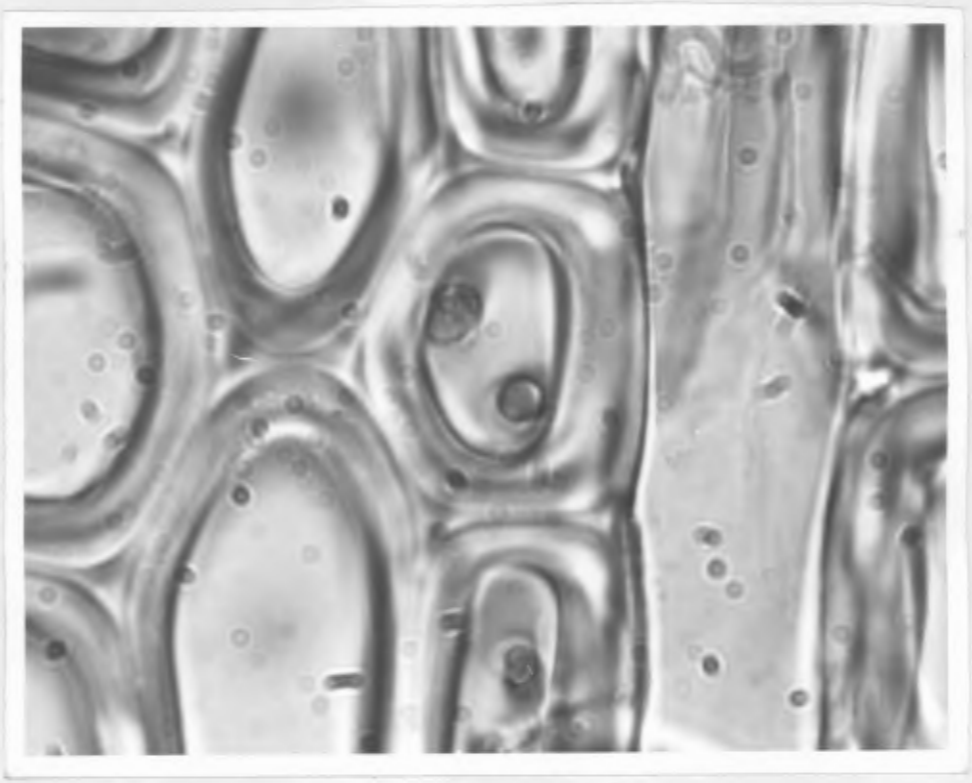

Figure 7.12. Transverse section of Scots pine with pigmented hyphae in the lumen of a longitudinal tracheid (900x, ethanol dehydration). Note the amount of lumen cross sectional area not occluded by hyphae.

Figure 7.13. Pigmented hypha lying in a bordered pit pair of Scots pine, seen in transverse section (900x, ethanol dehydration).

Figure 7.14. Pigmented hypha traversing the cell wall between a longitudinal tracheid and ray via a half bordered pit pair (900x, ethanol dehydration).

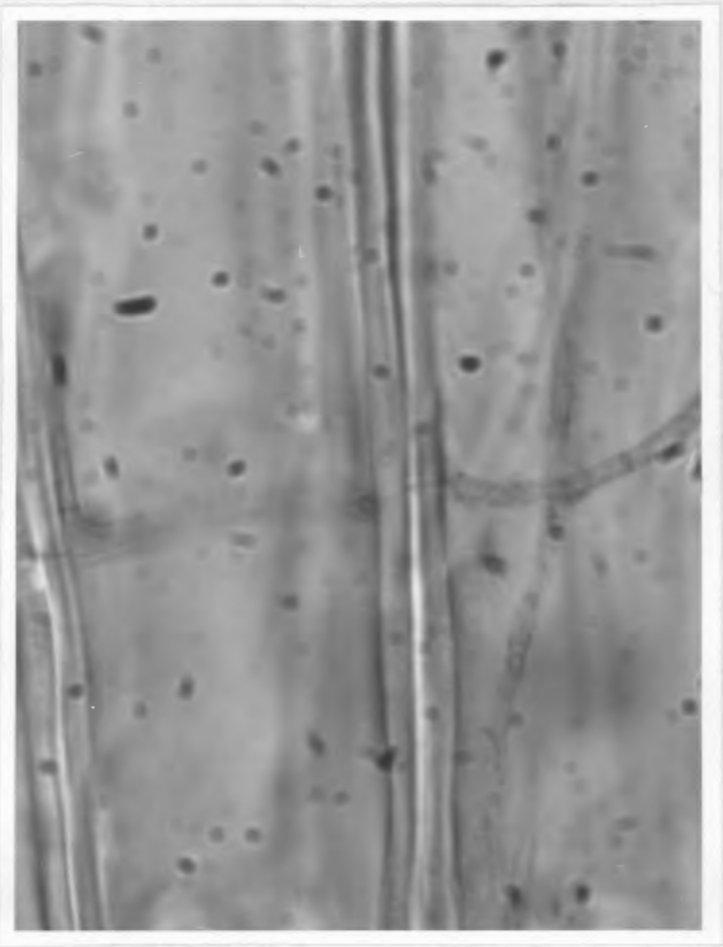

Figure 7.15. Hypha traversing cell walls via a narrow diameter bore hole in white pine (900x, ethanol dehydration).

tracheids, hyphae were more abundant in ray parenchyma (Fig. 7.10). Resin duct epithelial cells in black stained sapwood contained numerous pigmented hyphae as well (Fig. 7.11). In resin ducts with hyphae the epithelial cell walls were difficult to discern or not visible (Fig. 7.11). When viewed in transverse section, colonized sapwood was seen to have hyphae in tracheids. Typically, there was but a single hypha per tracheid, with occasional tracheids having two or rarely three hyphae (Fig. 7.12). Considerable cross sectional area of the tracheid lumen was always free of hyphae, even when more than one hypha was present (Fig. 7.12). Hyphae traversed cell walls in two ways. The most frequently observed manner was by pit penetration (Figs. 7.13, 7.14,). Hyphae were commonly observed lying in bordered pit pairs in heavily colonized sapwood (Fig. 7.13). Hyphae were also seen lying in half-bordered pit pairs (Fig. 7.14). An infrequent mode of traversing cell walls was through bore holes (Fig. 7.15). Wood inhabiting fungi produce bore holes by enzymatic penetration of secondary cell walls (Manion, 1981). The bore holes observed in this study were of a restricted or narrow diameter, and were only seen in heavily colonized tissue.

Water flow through clear sapwood cores averaged 3.41 ml whereas flows through resin soaked and black stained cores averaged 0.04 and 0.45 ml, respectively (Fig. 7.16). The flow through clear cores was significantly different

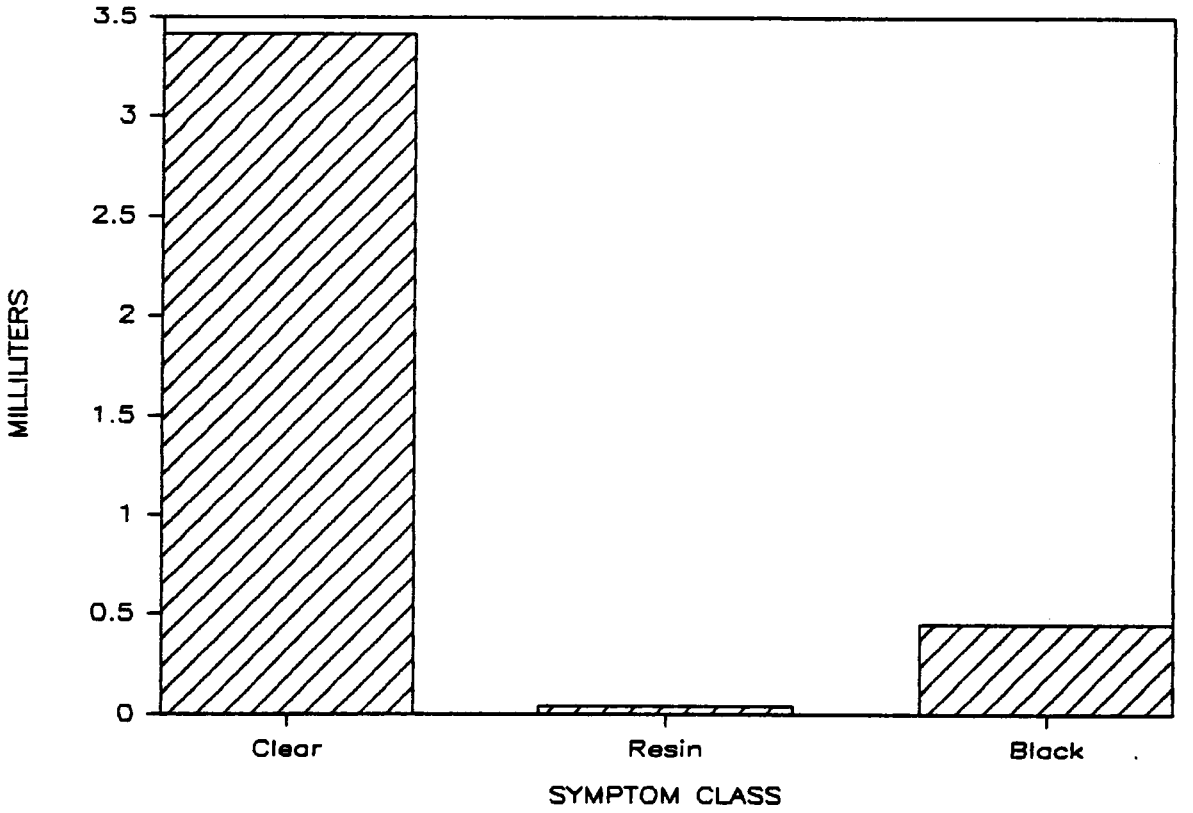

Figure 7.16. Permeability of sapwood cores of different symptom classes. Water was forced against tissue samples from the three symptom classes with a 250 kPa pressure differential. Clear sapwood allowed a significantly greater (P=0.05) amount of water to pass than either of the other classes.

(P=0.05) from the flow through both resin soaked and black stained cores by student's t test. The flows through resin soaked and black stained cores were not significantly different from one another. Black stained sapwood occasionally yielded cores that allowed passage of the pressurizing gas, causing a vigorous bubbling and preventing a measurement of liquid flow.

Discussion.

Parenchymatous cells were always degraded in symptomatic tissue if resinous inclusions or hyphae were present. Likewise, if hyphae were present, resinous inclusions were always present in sections dehydrated in glycerol. Macroscopically, resin soaked sapwood is usually present between clear sapwood and black stained sapwood. Ray cells always contained hyphae, if a tissue sample was black stained. When longitudinal tracheids did contain hyphae, the hyphae were invariably present in fewer numbers than in nearby ray parenchyma and/or epithelial cells. The inference is made from these observations that the sequence of events in sapwood colonization by V. procera begins with the death of parenchymatous cells, followed by resin impregnation and ultimately the development of the pigmented mycelium of V. procera. The cellular route of penetration appears to be through rays and resin ducts since hyphae were always present in these cell types in colonized tissue and

longitudinal tracheids were only colonized when hyphae were abundant in ray and resin duct tissues.

This sequence agrees with published accounts of bluestain development (Ballard, et al., 1984; Lagerberg, et al., 1927; Rumbold, 1931; Nelson, 1934; Bakshi, 1951; Boyce, 1948; Manion, 1981). The walls of longitudinal tracheids were penetrated by very narrow diameter bore holes, as described for bluestain fungi by Panshin and DeZeuuw (1970). The predominant means of hyphae traversing tracheid cell walls was via bordered pit pairs, as illustrated for bluestain fungi by Bakshi (1951). Macroscopically, the prominent wedge pattern of staining seen in stem cross sections agrees with the descriptions of bluestain development given by Rumbold (1931) and Nelson (1934).

The degradation of parenchyma cells apparently prior to physical contact with fungal hyphae raises the possibility of the involvement in pathogenesis of toxic metabolites. No attempt was made in the present study though to determine the mechanism of parenchyma cell death.

The significantly diminished flow of water through symptomatic sapwood in these permeability tests indicates that resin soaked or black stained sapwood is much less permeable to water than clear sapwood. Field observations have shown that much of the cross sectional area of the root collars of trees with crown symptoms is resin soaked and/or black stained. This suggests that water conduction through

the root collars of symptomatic trees is significantly diminished at comparable levels of transpirational tension. Diminished water conduction is consistent with the presence of dry sapwood in colonized trees and symptoms of PRD: wilting of needles (E. white pine), and reduced candle elongation.

These histological data indicate that sapwood colonized by V. procera is similar to sapwood colonized by bluestain fungi. The prominent resin soaking that accompanies V. procera colonization is not emphasized with bluestain fungi in the literature, however. This appears therefore to be a salient distinction between V. procera and other bluestain fungi. The functional implications for the physiology of colonized trees are reduced xylem flow, which would account for the foliar symptoms of PRD. The flow of resin which is commonly associated with wounds or infected tissue (Boyce, 1948) is generally inhibitory to fungal pathogens (Cobb, et al., 1968). Verticicladiella procera was seen in the present study however, to apparently grow into resin impregnated sapwood. This apparent ability to colonize resinous sapwood would circumvent the defensive capabilities of the tree in sealing off an infection, thus requiring impregnation of additional sapwood and consequently reducing xylem conducting capacity.

Chapter 8

Discussion, Conclusions and Recommendations for Future Work.

The presence of cellulose in the cell walls of V. procera is strong evidence of a phylogenetic link between this fungus and Ophiostoma (Ceratocystis). This provides an additional line of evidence suggesting that V. procera has certain features similar to bluestain fungi since most of the conifer bluestain fungi in the temperate zones are Ophiostoma species.

Inoculation studies revealed information on several aspects of the biology of V. procera. The fungus was shown capable of colonizing wounded sapwood in two trials, and persisted in one case for 22 months. In both of these trials, V. procera readily colonized resinous areas beneath wounds without producing foliar symptoms. Resinous lesions beneath inoculations were significantly larger on the colonized trees. These results indicate that V. procera persists and can expand within resinous tissues.

The root dip technique was found to have limitations for examining soil moisture effects on disease development. Root dip inoculations did show that afternoon water potentials detected early stages of stress in disease development, relative to predawn measurements.

The colonization pattern observed on naturally infected trees suggests that the root collar area is the initial point of colonization. Results from separate isolations of bark and wood from common sampling points further suggest specifically the bark at the root collar. Other workers have shown that V. procera is associated with, and may be vectored by root collar infesting insects. The colonization patterns observed in the present study corroborates this hypothesis. Symptomatic trees in this study were also shown to have copious amounts of resin soaked tissue at the root collar along with the fungus, and abnormally low sapwood moisture contents. This reinforces the association of V. procera and resinous sapwood with low sapwood moisture contents in symptomatic trees.

Radial growth analysis revealed that colonized trees had experienced reduced radial growth rates for the preceding three years. More data are needed to determine whether the fungus caused the growth reduction, or slow growing trees are more likely to become colonized. The moisture contents of sapwood cores from colonized trees was significantly lower than that of uncolonized trees. This is additional evidence that the crowns of symptomatic trees are desiccating, and in turn, is another similarity between V. procera colonized trees and trees dying with bluestain. Koch (1972) and Isaeva (1972) present data showing increased MC% with greater stem height in pines. Trees with V.

procera in the present study, did not exhibit reversal of the moisture content gradient as observed in trees with bluestain, however. If a source of interference to xylem conduction were present in the stem, then such a gradient reversal could be explained by the fact that the lower stem would still be well watered while the upper still transpiring crown was desiccating. Trees with V. procera however, show a consistent reduction in sapwood moisture content throughout the stem such that the normal trend, of MC% and height, in colonized trees is retained, but at lower moisture contents. This pattern suggests that the reduction in stem moisture does not originate in the stem, but could originate in the roots or root collar.

Histological examination of sapwood colonized by V. procera reveals similarities to bluestain colonized sapwood. The copious amounts of resin present in V. procera colonized sapwood however is not frequently cited as typical for bluestain infections. Successful bark beetle attacks, with subsequent inoculation of bluestain fungi has in fact been correlated with reduced oleoresin exudation pressure, rather than the copious production of resin typical of PRD (Vite, 1961). The persistence of V. procera in resinous lesions and the ability of the fungus to grow in these situations underscores this difference between bluestain fungi and V. procera. The permeability measurements in this study demostrate that resinous sapwood is essentially impermeable

to water. V. procera apparently is capable of persisting in and enlarging resinous areas of sapwood. Ultimately, resinous areas containing V. procera and impervious to water may coalesce and completely occlude the stem. This would account for the symptom development typical of PRD.

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