STUDIES ON THE SPREAD OF VERTICICLADIELLA PROCERA BY SOIL-BORNE AND INSECT-BORNE PROPAGULES

by

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(ABSTRACT)

Studies were undertaken to determine the dispersal mechanisms of _Verticicladiella procera_ Kendrick, the causal agent of Procera Root Disease (PRD).

Propagule germinability in artificially infested soil decrease rapidly under natural and controlled conditions. Colonization of seedlings in artificially infested soil was rare and symptoms were not displayed by colonized seedlings.

Natural populations of _V. procera_ were closely associated with colonized root tissue. Colonization of field planted seedlings was related to proximity to root collars of diseased trees and insect activity on the seedlings.

Insects (Coleoptera) contaminated with _V. procera_ were found in plantations both with and without PRD. The percent of weevils and bark beetles contaminated with _V. procera_ was 64 and 0.76 respectively. _Verticicladiella procera_ was transmitted to white pine bolts in the field and under controlled conditions following visitation by contaminated insects. _Verticicladiella procera_ was

associated with larval galleries and frass in trap bolts and was observed fruiting in insect galleries in root systems of diseased trees.

This evidence suggests that transmission by insects, especially weevils, is the more important mechanism for dispersal and that soil-borne propagules have a minor role in pathogen spread.

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Chapter I

Literature Review

HISTORY

Verticicladiella procera Kendrick is the causal organism of Procera Root Disease (PRD) in the eastern United States. Although *V. procera* was first described in 1962 by Kendrick, it was not until later that *V. procera* was recognized as a pathogen of pines in the eastern U.S. Earlier reports described *Leptographium* and *Verticicladiella* species that were associated with basal cankers on eastern and western white pine (*Pinus strobus* L. and *P. monticola* Dougl.) (Hubert, 1953; Leaphart and Gill, 1959) and with a black staining root disease of white pine (Gill and Andrews, 1949; Leaphart, 1960). Confusion regarding the identification of these two genera was due in part to similarities with respect to staining patterns in wood and fungal morphology on agar medium.

Lackner and Alexander (1982), with their work in Christmas tree plantations, were the first to document the species *V. procera* as causing significant economic losses of trees due to mortality. Proof of pathogenicity by the fulfillment of Koch's postulates was also established. Earlier, several workers reported lethal "cankers"

incited by *V. procera* but these reports were not considered conclusive evidence of pathogenicity. Dochinger (1967) described the symptoms of a disease associated with a *Leptographium* sp. (later recognized as *V. procera* (Towers, 1978) and performed a single pathogenicity test on several pine species. While conducting a study to determine the causal agent of a basal canker on white pine, Houston (1969) infrequently isolated *V. procera* from cankers. Inoculations of a few healthy white pine with *V. procera* did result in annual cankers, however *Pragmopara pithya* ([Fr.] Fr.) Groves was identified as the primary cause of the basal canker disease.

TAXONOMY

Verticicladiella procera is a member of the Leptographium complex, a group of wood-staining fungi (Bluestain fungi). As defined by Kendrick (1962), members of the complex are "hyphomycetes with darkly pigmented mononematous conidiophores which bear at the apex a complex sporogenous apparatus consisting of one to six multiplicative series of metulae, the ultimate series of which bears numerous sporogenous cells." The conidia are amerospores borne in a mucilaginous drop. Hughes (1953) separated the genera *Leptographium* and *Verticicladiella* based on conidiogeny. *Verticicladiella* spp. form sympodulospores whereas *Leptographium* spp. form annellospores. The genus *Verticicladiella* was originally

erected by Hughes in 1953 to accomodate the single species *V. abietina* (Peck) Hughes. In 1962 Kendrick described the species *V. procera*. The teleomorph of *V. procera* is unknown although there are several lines of evidence which indicate that if a sexual state exists, it would belong to the genus *Ophiostoma* (or *Ceratocystis* sensu lato) (Horner *et al.*, in press).

SYMPTOMS

Above ground symptoms of Procera Root Disease are a delay in bud break and reduction in shoot growth followed by uniform chlorosis. Wilting occurs in pine species with long, pliable needles such as white pine. Tree growth ceases and the needles turn brown but remain on the tree. These symptoms usually first appear in spring as higher temperatures result in increased transpiration rate. A canker may form in the root collar region accompanied by resin accumulation (Alexander, 1980; Dochinger, 1967; Swai and Hindal, 1981). The roots are often resin soaked and may show irregular, brownish-black stain lines. The resin-soaking and staining may extend up the lower bole beneath the bark (Dochinger, 1967; Sinclair and Hudler, 1980; Swai and Hindal, 1981).

HOST RANGE AND GEOGRAPHICAL DISTRIBUTION

At present *V. procera* has been isolated from trees

in Florida, Indiana, Kentucky, Maryland, North Carolina, Ohio, Pennsylvania, South Carolina, Virginia, West Virginia (Alexander, 1980), Idaho (Bertagnole *et al*., 1983), Minnesota, Michigan, Wisconsin (Wingfield, 1982), New York (Sinclair and Hudler, 1980), Alabama, Tennessee and Mississippi (S.A. Alexander, personal communication). The fungus has also been reported in Ontario (Kendrick, 1962), New Zealand (Wingfield and Marassas, 1983), Finland (Hallakskela, 1977) and Yugoslavia (Halambek, 1981).

Most reports of trees colonized by *V. procera* are from studies with pines in plantations less than 20 years old (Houston, 1969; Towers, 1977) and many of these are Christmas tree plantations (Anderson and Alexander, 1979; Lackner and Alexander, 1982). Anderson (1980) recovered the fungus from eastern white pine in plantations that were up to 25 - years - old. One report of *V. procera* is from a 40 - year - old natural stand (Lackner and Alexander, 1983). Webb and Alexander (1982) successfully isolated *V. procera* from trees in seed orchards. In an extensive study of root pathogens associated with cultural practices in seed orchards, Alexander (1984) examined trees in 18 southern pine seed orchards. *Verticicladiella procera* was found in 14 seed orchards in association with root wounds. It was isolated from 47.8 % of the wounded roots.

Verticicladiella procera has been reported most frequently from eastern white pine (Alexander, 1980).

Species from which the fungus has been isolated are listed in Table 1.1

DISEASE NAME

Prior to the identification of V. procera the basal canker symptom was used to describe the disease (Dochinger, 1967; Towers, 1977) and Houston (1969) gave it the name Basal Canker Disease. Dochinger (1967) introduced the name Leptographium root decline. Following the determination of V. procera as the causal organism, early investigators referred to the disease as "white pine root decline" (Alexander, 1980; Anderson, 1980; McCall and Merrill, 1980). However, as noted by Lackner and Alexander (1982), on white pine the disease symptoms resemble those of a sudden wilt rather than a decline which usually involves several factors operating in concert to cause a prolonged loss of vigour. Therefore the disease name "white pine wilt" was recommended. The wilt symptom so obvious in white pine does not occur in pine species with short, stiff needles such as Scots pine. Thus, the disease was also commonly called "white pine root disease" (S.A. Alexander, personal communication).

The common name for a disease should not imply limitations of host range. The disease caused by V. procera occurs on many pine species and several conifer genera. A new name for the disease has been proposed (S.A. Alexander,

Table 1.1. Host Range of *Verticicladiella procera*

Common name	Scientific name	citation
eastern white pine	*Pinus strobus* L.	a,b
Austrian pine	*P. nigra* Arnold	a,b
Scots pine	*P. sylvestris* L.	a,b
red pine	*P. resinosa* Ait.	a,b
Virginia pine	*P. virginiana* Mill.	a
loblolly pine	*P. taeda* L.	a
shortleaf pine	*P. echinata* Mill.	a
jack pine	*P. banksiana* Lamb.	b
ponderosa pine	*P. ponderosa* Laws.	b
sand pine	*P. clausa* (Chapm.)Vasey	c
slash pine	*P. elliottii* Engelm. var. *elliottii*	g
Douglas-fir	*Pseudotsuga menziessi* (Mirb.) Franco	d
Fraser fir	*Abies fraseri* (Pursh)Poir.	e
Norway spruce	*Picea abies* (L.)Karst.	f

a) Alexander, 1980
b) Wingfield, 1982
c) Barnard et *al*, 1982
d) Bertagnole, 1981
e) Alexander, personal communication
f) Hallekskela, 1977
g) Alexander, 1984

personal communication). The name "Procera Root Disease" is general with respect to host species, names the species of *Verticicladiella* involved which avoids confusion with the Black Stain disease on the west coast incited by *Verticicladiella wageneri* Kendrick (Cobb and Platt, 1967), and defines the type of disease.

LOSSES CAUSED BY PROCERA ROOT DISEASE

Most of the documented evidence of losses due to *V. procera* has been from work with Christmas tree plantations.

Towers (1977), one of the first to survey losses due to PRD, reported that in some young eastern white pine plantations in Pennsylvania a mortality of 20% in seven years could be expected. Anderson and Alexander (1979) observed annual mortality rates of 1-3% in Christmas tree plantations with a total stand mortality of 20%. Some very wet sites reached 50% mortality. Losses from eight Christmas tree plantations totaled about 700 marketable trees of three species (Lackner and Alexander, 1982).

Losses from PRD are not limited to Christmas trees although mortality is most frequently observed for trees less than 20 years old. *Verticicladiella procera* was observed causing damage to white pine (planted and naturally seeded trees up to 20 years old in landscapes and forests) in six counties in New York and the fungus was isolated

from red pine in four counties (Sinclair and Hudler, 1980).

A study in Croatia (Halambek, 1976) determined V. procera to be the cause of white pine dieback in many localities. Mortality was very rapid following the onset of symptoms which first appeared in plantations five to eight years after establishment.

HISTOLOGY

Verticicladiella procera inhabits the axial and ray tracheids and the ray parenchyma of the vascular system (Halambek, 1976; Horner and Alexander, 1985a; Shaw and Dick, 1980). The hyphae extend further into the axial tracheids than into the rays, and move tangentially through bordered pit pairs (Halambek, 1976). Growth of the fungus in wood is greatest in longitudinal and radial directions. Tangentially growth is limited, resulting in a wedge-shaped stain in cross section typical for bluestain fungi.

Microscopic examination of thin sections of wood colonized by V. procera showed that the actual physical blockage of tracheids by hyphae is insufficient to cause vascular dysfunction (Horner and Alexander, 1985a). Resin droplets were very common along the walls of tracheids in black stained or resin-soaked tissue. A study on the permeability of wood to water showed that the accumulation of resin in the stained and resin-soaked areas effectively

blocked water movement. Sapwood without an accumulation of resin was very permeable to water (Horner and Alexander, 1985b). Colonization of the vascular system by V. procera may mediate transpiration dysfunction, resulting in decreased water potential (Horner unpublished data) and the wilting symptom.

PATHOGENICITY

Lackner and Alexander (1983) established pathogenicity of V. procera on eastern white pine (P. strobus) and loblolly pine (P. taeda) seedlings by dipping the roots in V. procera spore suspensions followed by potting the seedlings in an artificial medium (vermiculite, weblite and peat 2:2:1, v:v:v). A high percentage of treated white and loblolly pines developed PRD symptoms and died. The fungus was successfully reisolated from the symptomatic and dead seedlings.

Several other reports exist which document the establishment of local lesions following inoculation of healthy trees by V. procera. Houston (1969) observed the formation of local lesions on white pine inoculated with V. procera. However, only five trees were inoculated; three formed cankers and two of these were found to be contaminated with a second fungus which was more virulent. Using a root wound inoculation technique on lodgepole pine (Pinus contorta Dougl.) with five species of

Verticicladiella, Bertagnole et al. (1983) observed the formation of resin-soaked root lesions in response to all species, with V. procera and V. penicillata (Grosm.)-Kendrick producing the longest lesions. Verticicladiella procera was found colonizing axial tracheids and ray parenchyma, resulting in cytoplasm degradation. An inoculation technique involving the insertion of colonized toothpicks into the root collar of white pine was attempted by Wingfield (1983). Small local lesions at the point of inoculation were formed from which the fungus was re-isolated.

Wood blocks colonized by an isolate of V. procera from Idaho were secured to wounded or unwounded tap roots of Douglas-fir and ponderosa pine. The inoculated trees failed to develop symptoms although the fungus was recovered from the wound-inoculated trees (Harrington and Cobb, 1983).

The results of these pathogenicity tests range from development of small local lesions to mortality. These studies involve a wide variety of isolates, inoculation techniques and host species which may account for the differences in inoculation trial results.

SITE AND STRESS FACTORS

Procera Root Disease incidence and severity may be increased by stress factors such as excessive soil moisture. Such factors may weaken the host thereby aiding the

pathogenic process.

The relationship between trees colonized by _V_. _procera_ and soil moisture was observed by several workers. Towers (1977) related diseased trees to poorly drained soils which led to the general acceptance of this idea. However, this phenomenon has not been thoroughly studied. One report states that all trees displaying symptoms similar to those caused by _V_. _procera_ were on poorly drained soils throughout several New York counties. Furthermore, red pine roots excavated along a transect running from well-drained to poorly drained soil were measured for proportion of total dead length and were 29.6, 7.1 and 0% for poorly, intermediately and well-drained soils respectively. _Verticicladiella_ _procera_ was the only organism associated exclusively with active lesions (non-crystallized resin in the lesion) (Sinclair and Hudler, 1980). Shaw and Dick (1980) observed greatest damage in a poorly drained portion of a stand in New Zealand. Likewise, a survey of a wilting disease of _Pinus_ _strobus_ in Yugoslavia demonstrated consistent isolation of _V_. _procera_ from symptomatic trees. The diseased trees were primarily located on poorly-drained sites with an unsuitable water-air regime (Halambek, 1981). Webb and Alexander (1982) however, observed that _V_. _procera_ was the primary organism isolated from resin-soaked, subsoiled roots in a seed orchard with very sandy

soil. Swai and Hindal (1981) state that disease occurrence and the ability to recover the pathogen were not always associated with poor drainage. They found symptomatic trees on steep hillsides where soils were well-drained.

The feeding and breeding activities of insects may also have an effect on host vigour. The majority of bark beetle (Scolytidae) species are secondary invaders, they attack only those trees which are already under stress. However, there are some species of beetle which, with appropriate population densities, will attack healthy trees. Some members of the Curculionidae, namely the pine reproduction weevils, preferentially feed on young pine trees (Knight and Heikkenen, 1980). Bark beetles and weevils have been found colonizing trees showing symptoms of PRD from which V. procera has been isolated (Lackner and Alexander, 1984; Livingston and Wingfield, 1982; Wingfield, 1983; Wingfield, 1982). The activity of these insects may contribute to the reported losses.

In one white pine stand from which V. procera was isolated, numerous additional pathogenic agents were identified including Armillaria mellea (Vahl ex Fr.) Kummer, white pine blister rust (Cronartium ribicola Fisher ex Rabenh), root weevils (Pissodes spp.), round headed borers (Cerambicydae), and Ips spp. It was concluded that biotic agents had predisposed the trees to disease incited by A. mellea and V. procera (Livingston and

Wingfield, 1982).

A strong association between root wounds from cultural practices in a seed orchard and infection by _V_. _procera_ was documented by Alexander (1984). Root excavations of trees in 18 orchards revealed 14 orchards with trees colonized by _V_. _procera_. The fungus was associated only with roots wounded during cultural practices. Of the roots wounded by a subsoiler or a power till seeder, _V_. _procera_ was recovered from 20.4 % and 27.4 % respectively.

A survey of 25 air pollution sensitive and 18 air pollution tolerant eastern white pine in the Blue Ridge mountains revealed that trees expressing air pollution injury are more susceptible to root disease and insect infestations (Lackner and Alexander, 1983). _Verticicladiella procera_ was isolated from 24% of the trees showing pollution sensitivity, and from none of the tolerant trees. Two species of weevil (_Pissodes_ _approximatus_ Hopkins and _Hylobius_ sp.) were found in the roots of 20 % of the sensitive trees and in none of the tolerant trees.

SOIL-BORNE PROPAGULES

To understand the Procera Root Disease cycle, the question of the source of _V_. _procera_ inoculum needs to be answered for both local (between adjacent trees) and long distance spread of the pathogen. The primary sources of inoculum are thought to be either soil-borne propagules

and/or insect vectors. Dowding (1969) in a study on the dispersal and survival of bluestain spores concluded that dry air dispersal may carry spores short distances. These spores are highly susceptible to dessication and therefore have a low chance for survival. Dowding suggested that spores carried in run-off water and deposited in a moist environment are more likely to survive. The nature of the spore and its formation suggest that air-borne dispersal is not likely (Dowding, 1969).

Swai and Hindal (1981) developed a selective medium for the isolation of _V. procera_ from soil. Using this medium, the authors were able to successfully isolate the fungus from 72% of all soil samples collected from the rhizosphere of symptomatic white pines and from 4% of the soil samples taken from the rhizosphere of asymptomatic trees.

In a study on the development of _V. procera_ in Christmas tree plantations, Lackner and Alexander (1984) determined propagule densities in soil using the selective medium of Swai and Hindal (1981). Soil was sampled from plots where symptomatic trees had been excavated and from plots around symptomless trees. Sampling was conducted at two month intervals for 10 months to study the duration of _V. procera_ propagules over time. _Verticicladiella procera_ was not isolated from soil samples collected from the base of healthy trees. Initial propagule recoveries

from the excavated sites were 3.4 x 10^4/g of soil from one plantation and 1.7 x 10^4/g of soil from a second plantation. Levels detected of soil-borne propagules decreased rapidly from August (when the symptomatic trees were excavated) to December at which time no propagules were detected. The fungus overwintered in soil at only one of 10 sites sampled. Detectable spore survival decreased rapidly once the colonized plant material was removed. These results indicate propagules exist in soil near symptomatic trees, that propagule density in soil decreases rapidly and that a small proportion of propagules are capable of surviving in soil for short time periods.

One of the characteristics of a pathogen which infects its host through the root system by soil-borne propagules is the ability to survive in soil between colonization of successive hosts. Garrett (1970) lists five means by which root disease fungi survive:

(1) as competitive saprophytes on dead organic substrates;

(2) saprophytic survival on dead tissues of a host crop or of weeds infected during the parasitic phase;

(3) dormant survival as 'resting' propagules, e.g. chlamydospores and microsclerotia;

(4) parasitic survival on living roots and other underground parts of weed hosts and 'volunteer' susceptible crop plants;

(5) parasitic survival on living root systems of plants that show no disease symptoms above ground.

There has been little research demonstrating mechanisms of *V. procera* survival between successive hosts which relates to the source of inoculum for new infections. Houston's work (1969) determined that *V. procera* does survive by method (2), saprophytic survival on the host. He determined that *V. procera* remains viable in dead white pine bark tissue for at least two years. Lackner and Alexander (1984) documented propagule survival in soil for four months near excavated trees. However, colonization of small pieces of organic matter in the soil may also enhance survival. No attempts have been made to isolate *V. procera* from, or to inoculate *V. procera* into, non-coniferous plants; therefore it is not known whether method (4), colonization of roots of weed and volunteer hosts, is a means of survival. The fungus has been isolated from asymptomatic trees, particularily Scots pine (Horner and Alexander, 1983) but the duration of parasitic survival without causing symptoms is unknown. The majority of soil-borne plant pathogenic fungi don't survive as mycelium but produce a resting structure or dormant propagule (Coley-Smith, 1979). For example, sclerotia produced by organisms such as *Sclerotium cepivorum* Berk. and *Rhizoctonia tuliparum* Whetzel and Arthur can survive for several years in soil (Coley-Smith, 1979).

The formation of chlamydospores within hyphal and conidial cells also enhance survival of fungi in soil. The possibility exists that a resistant form of resting spore is formed by *V. procera*. Bertagnole (1981) observed chlamydospores produced by *V. serpens* (Goid.) Kendrick and *V. penicillata* but did not observe any produced by *V. procera*. Conidia may also have some survival value. Coley-Smith (1979) showed that conidia of *Botrytis tulipae* Lind. were able to survive for at least six weeks in field soil at temperatures from 0 to 10 C.

INSECT VECTORS

The fruiting bodies of bluestain fungi and certain wood- inhabiting arthropods have common habitats (Dowding, 1984). Due to the mucilaginous drop encompassing the conidia, Dowding (1969) proposed that during feeding and breeding activities the insects may become contaminated with spores. When the insects seek fresh habitats, insect-borne spores are a potential source of inoculum for infection of new hosts.

The connection between bark beetle infestations and blue stain fungi has been well established. According to Rumbold (1931), von Shrenk in 1903 was the first to establish this connection in North America. von Shrenk isolated *Ceratostomella pilifera* (=*Ceratocystis pilifera* (Fr.) C. Moreau) from ponderosa pine infested with

Dendroctonus frontalis Zimmerman. Furthermore, he stated that "The spread of the blue fungus within wood, through the agency of wood-boring beetles, is an occurrence frequently found in many coniferous woods." Craighead (1928) noted the association between bluestain and Dendroctonus spp.. He proposed that the fungi were introduced under the bark by bark beetles which played an important role in the death of the tree. Additionally, fungi may be important in conditioning the tree for bark beetle breeding and brood rearing. In a study on the effect of bluestain fungi on southern pines attacked by bark beetles, Nelson (1934) observed that bluestain in living trees not attacked by bark beetles was extremely rare. However, bluestain appeared in the vicinity of beetle tunnels in less than a week following infestation by the southern pine beetle. Nelson was able to make some conclusions about the means by which the fungus entered wood. The two possible infection courts with respect to bark beetles are entrance tunnels and ventilation tunnels. Of the 218 stained areas in shortleaf pine attacked by D. frontalis, 97% originated at the entrance tunnels. If the inoculum were wind-blown or splash dispersed, one would expect the fungal infestations to also originate at the ventilation tunnels. In support of the vector hypothesis, he consistently isolated blue stain fungi from bark beetles plated on agar medium

(Nelson, 1934).

Since these early works, transmission of fungi by insects has been well established especially with members of the Ceratocystis group (Dowding, 1984).

Verticicladiella procera has been found in association with bark beetle and weevil infestations. Wingfield (1982) isolated V. procera from several species of pine infested with the root collar weevil (Hylobius radicis (Buchanan)) and with pine root tip weevil (H. rhizophagus Millers, Benjamin, and Warner). In a survey of the occurrence of V. procera in Christmas tree plantations, the pales weevil (Hylobius pales (Herbst)) and Pissodes approximatus were found in stems of dead pines. Bark beetles were observed in both symptomatic and asymptomatic trees (Lackner and Alexander, 1982). Lackner and Alexander (1984) isolated V. procera from 22 of 320 bark beetles (species of Pityokteines Fusch., Pityogenes Bedel., and Pityophthorus Eich.) recovered from heavily infested white pine Christmas trees. Several weevils (Pissodes approximatus) were collected from symptomatic trees but V. procera was not recovered from these insects. Pupal chambers of the weevils were most common at the stem base and were observed to be associated with stained wood. Wingfield (1983) studied the association of the fungi V. procera and Leptographium terebrantis Barras and Perry with insects in pines. Of 37 pines damaged by Hylobius

radicis, 36 yielded *V. procera* from tissue pieces plated on agar. In addition, *V. procera* was isolated from roots recently infested by weevils but not from decayed roots. *Verticicladiella procera* was also isolated from roots damaged by *Pachylobius picivorus* (Germar) in all of the Scots pines sampled. *Pachylobius picivorus* produces puncture holes in living roots which become surrounded by necrotic bark and cambium. It was from these lesions that *V. procera* was isolated, not from maturation feeding damage on the lower green branches. *Verticicladiella procera* was also isolated from the galleries of *Pissodes approximatus* and *H. pales* in tree stumps. Furthermore, *V. procera* was commonly isolated from surface-sterilized *H. radicis*, *H. pales*, *Pa. picivorus*, and less commonly from the bark beetle *Dendroctonus valens* LeConte (Wingfield, 1983).

Wingfield (1983) felt that the presence of *V. procera* was secondary to the activities of the insects and that PRD may be confused with root collar weevil damage because the symptoms are similar. Furthermore, because there was a low level of *V. procera* in the soil and a high percentage of insects were carrying *V. procera*, Wingfield suggested that the fungus was probably introduced into the trees by the insects.

There is a lack of conclusive evidence concerning the mechanism for infection of pine trees in the field by

V. procera and the subsequent spread of the pathogen. Insect transmission of V. wageneri which causes black stain root disease of Douglas-fir and pines in the Pacific northwest has been more clearly documented. There are obvious differences between these two pathogens but some parallels may be drawn.

Hunt and Morrison (1979), state that long distance spread is likely via root feeding insects. This is supported by the work of Goheen and Cobb, (1978). Conidiophores of V. wageneri were found in the galleries of several bark beetle species, primarily those of Hylastes macer LeConte. It was during this study that the perfect state of V. wageneri, Ceratocystis wageneri (Goheen and Cobb, 1978) was discovered fruiting in 19 H. macer galleries in seven roots on five trees examined. James and Goheen (1981) consistently isolated a Verticicladiella sp. from beetle (Dendroctonus pseudotsugae Hopkins) infested Douglas- fir in mortality centres. However, the species was not identified, nor was its role in pathogenicity determined. In a study to determine the range of black stain root disease in Colorado, Landis and Helburg (1976) excavated root systems of symptomatic Pinyon pines (Pinus edulis Engelm.). Black stain could be traced from one tree to the next through the root contacts and grafts and there was a definite pattern of disease development from extensive colonization in one tree to less developed

colonization in adjacent trees. Several species of Scolytids were found associated with diseased trees and beetle galleries were found in the vicinity of stained wood. *V. wageneri* has been recovered from a bark beetle of the species *Pseudohylesinus grandis* Swaine (=*P. sericeus* (Mannerheim)) which is one of the species suspected of transmitting *V. wageneri* in California (Jackman and Hunt, 1975).

Conclusive evidence of an insect-disease relationship was obtained by Witcosky and Hansen (1985). Diseased trees from three stands were divided into symptom classes prior to root excavation and insect collection. Numbers of root and root collar inhabiting insects were found to correspond directly to the degree of symptom expression and the extent of fungal colonization. Heavily infected roots were found to be suitable for oviposition. The authors state the sequential infestation of the root system by *Hylastes nigrinus* (Mannerheim) (Scolytidae), *Steremnius carinatus* (Mannerheim) (Curculionidae) and *Pissodes fasciatus* LeConte (Curculionidae), increases the opportunity for contact between emerging insects and propagules of *V. wageneri*. Furthermore, the fungus was recovered from a low percentage of the insects collected, and artificially infested insects of the same species transmitted the fungus to seedlings (which subsequently developed symptoms) under laboratory conditions (unpub-

lished data cited in the above publication).

Transmission of *V. wageneri* was demonstrated by Harrington *et al.* (1985). Three of 22 seedlings caged with a single artificially contaminated bark beetle (*Hylastes nigrinus*) became diseased. Of 47 seedlings caged with pairs of *H. nigrinus* collected from the forest (not artificially contaminated), one became diseased.

RESEARCH OBJECTIVES

The importance of PRD incited by the fungus *Verticicladiella procera*, has only recently been recognized. During the last decade research has been centred on determining pathogenicity, describing the host and geographical ranges and describing some of the site and biotic factors associated with the disease. Many questions regarding the ecology and epidemiology of the disease still remain. Some of these questions need to be answered before recommendations for control may be made.

One important question is the means by which the fungus spreads. Is it by propagules in soil which germinate on and infect roots, is it transmitted by insect vectors during feeding and breeding activities, is it transmitted by root grafts, or is it a combination of several methods? In relation to the question of epidemiology, what environmental factors enhance or inhibit development and spread of the pathogen?

The focus of this research was to determine the primary source of inoculum for infection of the host. Specific objectives were to determine the importance of soil-borne propagules in disease development and spread, and to initiate studies of insect transmission of the fungus.

Chapter II

Tests on the Efficiency of the Medium Selective for Soil-borne Propagules of Verticicladiella procera

Much of the research described in the following chapters required an efficient method for consistent and accurate detection of soil-borne propagule densities.

Serial dilution and plating on a selective medium is an accepted technique for determining the level of microbial populations in soil (Clark, 1965). A medium (VPIM) selective for propagules of V. procera in soil was developed by Swai and Hindal (1981) and has been used successfully since then (Lackner and Alexander, 1984). However, the accuracy of propagule recovery by this medium has not been reported.

The objective of the following study was to test the selective medium, using several different methods, in order to quantify the accuracy of propagule recovery from soil.

MATERIALS AND METHODS

Three different techniques were used to test the performance of the selective medium for germination and growth of V. procera, and recovery of propagules from artificially infested soil.

Germination of V. procera conidia. A conidial suspension was prepared by pouring 10 ml of sterile distilled water

onto a plate culture of V. procera (isolate #153, from a white pine Christmas tree in West Virginia), then gently rubbing the culture with a flame sterilized bent glass rod which dislodged the conidia. The suspension was collected with a Pasteur pipet and the number of conidia per ml were determined with a hemacytometer. The suspension containing 1.4 x10^4 spores/ml was diluted three times (1:10) for an approximate concentration of 14 spores/ml. One ml of this concentration was spread plated onto 10 plates each of MEA and VPIM. Five plates of each medium were incubated at 15 C, and five at 20 C for nine days. The number of colonies per plate were counted and the average colony diameter measured.

Growth rate on VPIM and VPIM-antibiotics. VPIM was prepared with and without the antibiotics (chlorotetracycline HCL, cycloheximide and streptomycin sulphate). Ten plates of each medium and were inoculated in the centre with a plug (0.5 cm diameter) of V. procera culture (isolate # 153). The plates were incubated at 20 C. Every two days for 28 days, the colonies were measured in two directions perpendicular to each other. The average colony size was calculated for each medium at each sampling time and plotted.

Percent recovery from artificially infested soil. The efficiency of recovery of V. procera from soil infested with a known number of spores was tested. Soil obtained

locally was sifted through a one cm screen then artificially infested with a spore suspension. The conidial suspension was prepared as described previously then was serially diluted to the desired concentration for each test. Five ml was reserved to spread plate on five MEA plates. These were incubated for 20 to 24 hours at which time germ tubes were present. The number of germinated conidia were counted to obtain the percent germinable which was used to correct the expected number of propagules recovered.

A measured weight of soil was placed in a plastic bag to which the suspension was added. After thorough mixing, subsamples were removed, serially diluted using the method of Clark (1965) and plated on the selective medium. Several dilution levels were plated to obtain the optimum number of colonies/plate for counting. A soil sample was reserved and oven dried at 105 C for 24 hours to determine soil moisture content.

Following incubation of the soil dilution plates for 14 days at 20 C, the average number of colonies/plate was determined, multiplied by the dilution factor then corrected for oven dry soil weight. The number of spores recovered per gram soil (oven dry weight) was compared to the expected recovery using a Chi- square test. The experiment was repeated three times.

RESULTS

Germination of Verticicladiella procera conidia. The number of colonies/plate and the average size of the colonies on the two media (MEA and VPIM) are compared in Table 2.1.

At both temperatures, there was no significant difference between germination of conidia on MEA and VPIM as determined by a "Students" t-test with P=.05. The average colony diameter on MEA was significantly greater than on VPIM ("Students" t-test, P=.05) for both temperatures.

Growth rate on VPIM and VPIM-antibiotics. The mean growth rate of *V. procera* on the two media are plotted in Fig. 2.1.

The growth rate slopes were 0.2229 and 0.25557 for VPIM and VPIM-antibiotics respectively. Simultaneous regression with dummy variables (Draper and Smith, 1981) determined no significant difference in growth rate of *V. procera* between the two media. Furthermore, mean colony diameters on the two media measured at 14 days (which was the incubation period used for isolations from soil) were found to be not significantly different by "Students" t-test at the .01 level.

Percent recovery from artificially infested soil. For each repetition, an average percent recovery was calculated from the dilution plates of several subsamples. The results from each repetition are shown in Table 2.2.

Table 2.1. Number of conidia germinated on the all-purpose medium MEA and the selective medium VPIM and the average colony diameters.

	MEA		VPIM	
Temp. (C)	#/plate	avg.diam (cm)	#/plate	avg. size (cm)
20	8	2.2	13	1.5
	21	2.0	9	1.5
	11	2.2	18	1.8
	13	2.5	16	1.5
	19	-	13	1.5
mean	14.4[a]	2.2[b]	13.8[a]	1.6[c]
15	18	1.2	21	1.0
	15	1.2	16	0.8
	13	1.0	12	0.9
	14	1.1	16	0.8
	15	1.2	18	0.8
mean	15.0a	1.1[b]	16.6[a]	.9[c]

Numbers followed by the same letter at each temperature level are not significantly different. "Students" t test at P=.05.

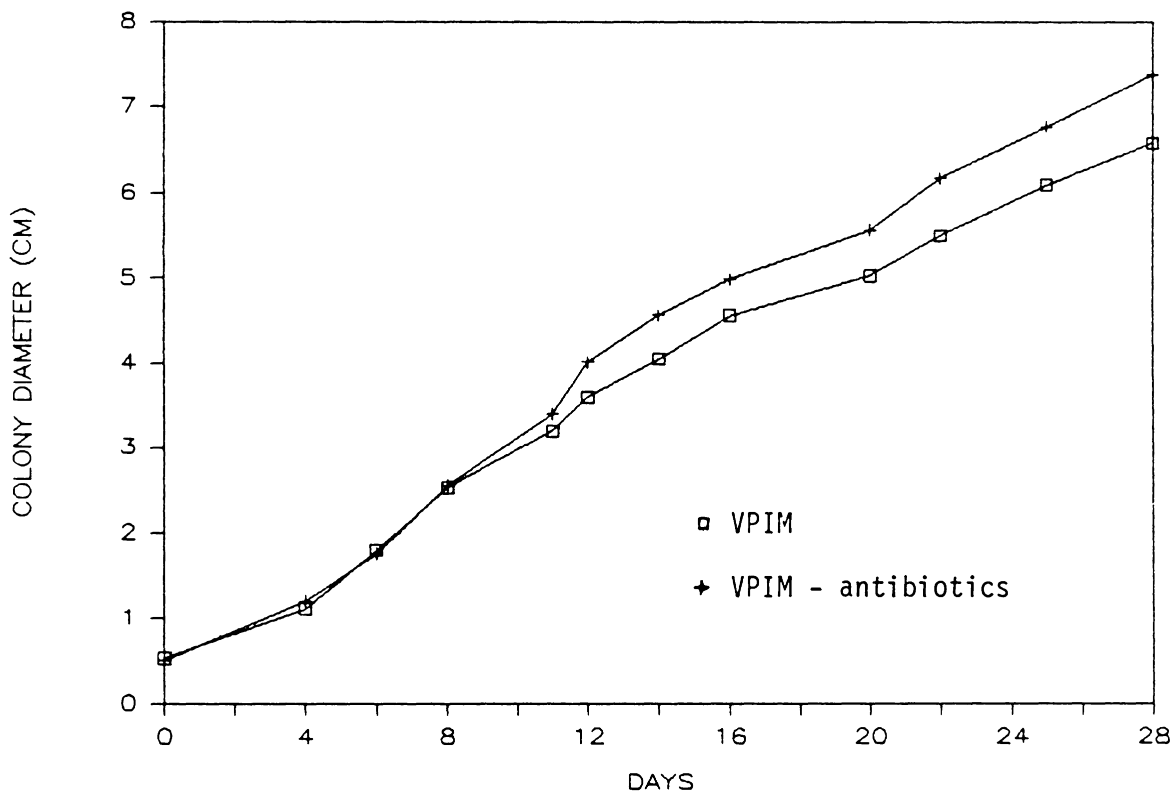

Figure 2.1. A comparison of the growth rate of Verticicladiella procera on two media: V. procera soil isolation medium (VPIM) and VPIM- antibiotics. Growth rates on the two media are not significantly different (P=.05).

Table 2.2. Recovery of *V. procera* propagules from artificially infested soil using the selective medium VPIM.

Test	Expected number/g	Subsample	Dilution level	Avg. recovery (oven dry wt.)	Avg. % recovery	Chi-Square
1	14400	1	10^{-3}	14011.54		
			10^{-2}	11799.27		
		2	10^{-3}	16777.09	92.6%[a]	S[c]
			10^{-2}	10250.62		
		3	10^{-3}	9218.18		
				8351.67		
2	24600	1	10^{-4}	17200.0		
			10^{-3}	25628.0	100%[a]	S
		2	10^{-3}	27864.0		
			10^{-2}	TNTC		
3	24700	1	10^{-3}	13645.45		
		2	10^{-3}	17272.73	62.6%	NS
4	50000	1	10^{-2}	TNTC		
			10^{-3}	32800.0		
			10^{-4}	34000.0	90.0%	S
		2	10^{-2}	TNTC		
			10^{-3}	36200.0		
			10^{-4}	56000.0		

[a] Average of 10^{-3} dilution levels

[b] Average of 10^{-4} dilution levels

[c] Chi Square test results comparing observed recovery to expected. S=observed = expected; NS= observed = expected.

The range of percent recovery was between 63 and 100%. There appeared to be some inhibition of colony formation at higher population levels. Recovery was greatest at dilution levels yielding 10-30 colonies per plate. With the exception of one repetition, the efficiency of propagule recovery on the selective medium was very high. Chi-Square tests comparing the observed recovery to the expected showed no significant difference between expected and observed for test numbers one, two and four and a significant difference for test number three (P=.05).

DISCUSSION

Verticicladiella procera isolation medium, as developed by Swai and Hindal (1982), was found suitable for quantitative analysis of soil-borne propagule density. The antimicrobial compounds in the medium did not inhibit germination and did not cause a significant decrease in growth rate of *V. procera*. Furthermore, mean colony diameters on the two media at the end of the normal incubation time for isolations from soil were not significantly different. Recovery from artificially infested soil was quite high and relatively consistent. The one repetition with only 63 % recovery suggests that the techniques used during sampling and serial dilution processes are as important as the efficiency of the medium. Measures taken to create a

random distribution of propagules from the clumped distribution found naturally may be especially important depending upon the objectives of the study.

Chapter III

Propagules of _Verticicladiella procera_ in Artificially Infested Soil: the Duration of Germinability Under Controlled and Natural Conditions and Their Inability to Cause Disease

Soil-borne propagules that retain the ability to germinate and infect roots for a longer period of time have a greater chance of causing infection. Lackner and Alexander (1984), recovered _V. procera_ propagules from soil where diseased trees had been excavated using a selective medium for soil-borne propagules of _V. procera_ (Swai and Hindal, 1981). Propagule numbers were observed to decline rapidly within two months. The fungus appeared to overwinter in the soil at only one of ten sites. _V. procera_ was not detected at the base of symptomless trees.

The ability of _V. procera_ to survive in soil under various environmental conditions in both the presence and the absence of the host is important with respect to disease development and spread. This study was undertaken to determine the duration of propagule germinability in the absence of a host, and the ability of propagules in artificially infested soil to infect seedlings and cause disease.

MATERIALS AND METHODS

Propagule germinability over time. Artificially infested soils were subjected to various treatments and

sampled periodically. The samples were serially diluted by the method of Clark (1965). One ml of each desired dilution was spread onto a medium selective for soil-borne propagules of _V. procera_ (VPIM) (Swai and Hindal, 1981). Following incubation for 14 days at 20 C, the number of colonies per plate were counted. The number of colony-forming units (cfus) was averaged for each treatment at each sampling, multiplied by the dilution factor and corrected for soil oven dry (OD) weight. The number of colony forming units per gram OD weight soil for each sampling was termed propagule germinability.

The first experiments involved infested soil placed in a controlled environment at various temperatures. Two types of inoculum were prepared for soil infestation. A conidial suspension was prepared by gently washing a culture of the fungus (isolate #153) growing on 2% malt extract agar (MEA) with 10 ml sterile distilled water then collecting the suspension with a pasteur pipet. Mycelial fragments in suspension were prepared as follows. _Verticicladiella procera_ isolate #153 was inoculated into 125 ml erlenmeyer flasks containing 2% malt broth, and placed on a rotary shaker for 14 days. The mycelium was harvested by pouring off the broth and centrifuging the pellet at 900 x g in sterile distilled water with three changes. The mycelium was suspended in water and macerated in a blender for 2 min. Most of the conidia were removed

by this process.

Soil (Aquic hapludult, fine-loamy, mixed, mesic; see appendix for soil properties) was collected from a mature white pine stand, sifted through a 1 cm screen and mixed with sand (2 parts soil:1 part sand). A moisture retention curve for this soil:sand mix was determined using a pressure membrane and tension plate (see appendix for soil moisture retention curve). A plastic bag was used to thoroughly mix 2880 grams of soil to which 100 mls of a conidial suspension containing 2.52 x 10^5 spores/ml was added. One ml of the suspension was reserved and diluted to 25 spores/ml. One ml of this dilution was spread onto each of five MEA plates. After incubation at 20C for 24 hrs, the number of germinating conidia were counted to obtain percent germination. This was used to correct for the number of germinable propagules mixed with the soil. Infested soil:sand mix was shaken thoroughly for 10 min then divided equally into seven containers. Eleven grams were reserved to calculate soil OD weight. Four of the containers were plastic 11 cm diameter x 4.5 cm high (Dowpac). These were fitted with cheesecloth over the opening so the soil inside could air dry. The remaining three containers were glass jars with loosely fitting lids which decreased the rate of evaporation. Soil:sand mix in these containers was kept moist by the addition of sterile distilled water every two weeks. The water

potential for "air-dry" and "moist" soil was determined by measurements of moisture content and the soil moisture retention curve. One each of the plastic containers and glass containers was placed in incubators at 10, 20 and 30 C. The remaining plastic container was placed in a freezer at -5C. Likewise, 1350 grams of the soil:sand mix was infested with a 150 ml suspension of mycelial fragments, thoroughly mixed and divided equally into three jars with lids. These were incubated at 10, 20 and 30 C. The soil was kept moist as described above. Treatments are summarized in Table 3.1. The treatments were sampled periodically by emptying the soil from each container into a separate plastic bag and thoroughly mixing the soil. Two 11 gram subsamples were removed and the remaining soil placed back in the container. One sample from each treatment was dilution plated as outlined above. The remaining sample was oven-dried at 105 C for 24 hours to obtain soil moisture content.

Artificially infested soil was buried in an attempt to simulate more natural conditions. Five local sites were chosen to represent different cover types and moisture regimes. At each site two pits were dug 30 cm square by 20 cm deep. Serial dilution and plating was performed with an 11 gram subsample of soil (Typic paleudult, clayey, mixed, mesic; see appendix for properties) from each pit to ensure that there were no detectable natural

TABLE 3.1. Treatments used to test the germinability over time of *Verticicladiella procera* propagules under controlled temperature conditions in a soil:sand mix.

	Temperatures			
Soil Moisture	-5	10	20	30
"air dry"	C[a]	C	C	C
"moist"	-	C/M[b]	C/M	C/M

[a]C=soil infested with conidia.
[b]M=soil infested with mycelial fragments.

populations of *V. procera*. At each site, a portion of the soil (1020 g) from one pit was infested with 100 mls of a conidial suspension with 2.5×10^8 spores using the same technique described previously. Soil from the other pit was infested with a suspension of mycelial fragments. The 1020 g of infested soil was divided equally into three fiberglass mesh tubes (15 cm long and 8 cm diameter with a pore size of 1 mm). These were sewn shut then buried at the original site of the soil sample (Fig. 3.1). Once a month for ten months the tubes were excavated, two subsamples removed and the tubes reburied. One sample was serially diluted as described previously and the other was oven-dried to determine soil moisture content. Data collected were the number of propagules/g oven-dry soil remaining germinable at each sampling period.

Seedlings planted in artificially infested soil. There were five experiments in this part of the study. Each one involved seedlings planted in soil, a soil:sand mix, or a potting medium ("Pro-mix", a peat mix) artificially infested with either conidia or mycelial fragments (Table 3.2 summarizes the treatments and the duration of each experiment).

Conidial suspensions were serially diluted to the appropriate concentrations for the desired number of spores/gram soil (Table 3.2). The soil and inoculum for each treatment were thoroughly mixed in a plastic bag.

Figure 3.1. Mesh tubes filled with artificially infested soil and placed in the pit prior to burial.

TABLE 3.2. Summary of treatments for seedlings planted in artificially infested soil mixes.

Experiment	Weeks	Inoculum	Soil	Seedlings	Treatments
1	11	conidial susp. isolate #153	white pine stand (Aquic hapludult)	loblolly pine 5 mo. old	-conidial susp.: 0, 10, 10^2, 10^3, 10^4, 10^5, 10^6 spores/gm soil. 10 seedlings each.
2[a]	9.5	conidial susp. mycelial frag. (#153)	soil:sand 2:1	white pine 2 yrs old	-conidial suspension - as above -mycelial fragments[b] 10 seedlings each.
3[a]	40	"	"	"	-conidial susp.: 0, 10, 10^2, 10^3, 10^4, 10^5 spores/gm soil -mycelial fragments -both propagule types infested pasteurized and nonpasteurized[c] soil. 5 seedlings each
4	12	conidial susp. (#254)	"	"	-conidial susp.: 0 and 3.6 x10^4 spores/gm. -water every day -water every third day 10 seedlings each
5	12	"	promix and clay loam soil (1:1)	"	-conidial susp.: 0 and 3.2 x10^6 spores in 100 ml poured at base of seedlings. 10 seedlings each.

[a]Greenhouse overheated, many treated and control seedlings showed heat and drought stress. Results modified.

[b]Mycelial fragments. 3 replications with 4 flasks of 75 mls of 14 da. old culture for one replication

[c]Pasteurized soil allowed to recolonize with saprophytes prior to infestation.

Five or ten seedlings per treatment were planted in the infested soil in plastic pots (15 cm diameter) with 2.5 cm of gravel in the bottom. Potted seedlings were placed randomly on benches in the greenhouse and watered as needed or according to the treatment. Observations were made on symptom development: chlorosis or wilting of the foliage, reduced candle extension and resin exudation on the stem. When a seedling died or at the termination of the experiment, the root system was rinsed in tap water and the following observations were made. Root colour, presence of new, white growing tips, health of the cortex and the presence of any areas of resin-soaking or excess resin exudation. All lateral roots were removed; one of the larger roots was surface sterilized in 2% chlorox for two minutes. The tap root was dipped in alcohol and flamed briefly. From the tap root and lateral root two segments were removed at each of three positions (Fig. 3.2) and plated onto MEA and actidione amended, malt extract agar (AMA) (McCall and Merrill, 1980). After incubation for ten days at 20 C, the plates were observed for colonies of _V_. _procera_.

RESULTS

Water potential of the artificially infested sand:soil mix initially was above field capacity (> -3.3 kPa). The water potential for each treatment calculated from moisture content of the latest sample for each treatment are in

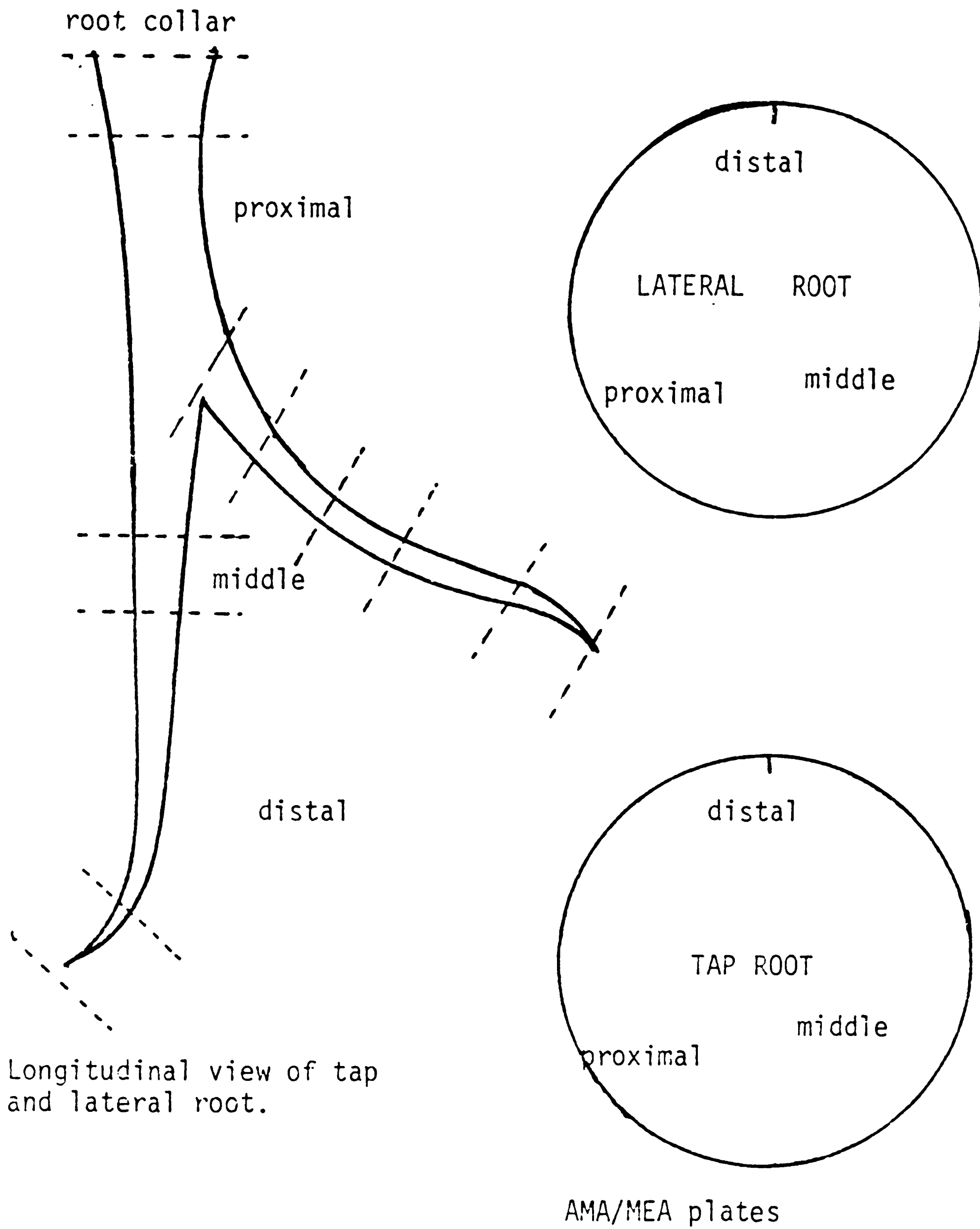

Figure 3.2. Isolations made from the tap and lateral roots of seedlings. Two segments from each isolation plated at a specific location, one on MEA and the other on AMA.

Table 3.3. The "moist" soil treatments were characterized as soil with a water potential from "field capacity" (-3.3 kPa) to -100 kPa. The "air-dry" soil treatments were characterized by soils with water potentials of less than -160 kPa (or much less than "permanent wilting point", -150 kPa).

Propagule germinability over time. Under controlled conditions, both conidia and mycelial fragments in soil retained the ability to form a colony on agar medium for a longer period of time at lower temperatures. The proportion of the original concentration of conidia that remained germinable at each sampling time in air dried soil:sand is plotted in Fig. 3.3. Conidia in soil maintained at 20 C and 30 C were not detected after 139 and 15 days respectively. However, at -5 and 10 C, approximately 20% of the conidia remained germinable after nine months. The number of colony-forming-units was regressed on time for each temperature using quadratic transformations. The slope for each curve was significant which indicates that time has an effect on germinability. A simultaneous regression with dummy variables (Draper and Smith, 1981) was performed on the data at -5, 10 and 20 C. The regression lines for -5 and 10 C were different from 20 C but not from each other (P=.01).

Figure 3.4 compares germinability/time under moist and dry (see Table 3.3) conditions at 10, 20 and 30 C.

TABLE 3.3. Moisture contents and water potentials of soils artificially infested with *V. procera* conidia and mycelial fragments and subjected to several temperatures.

Treatment	Day sampled[a]	% Moisture Content	Water Potential(kPa)
Start of exp.	0	approx. 16	>-3.3[b]
10 C "moist"	280	8.0	- 30
20 C "moist"	140	5.3	-100
30 C "moist"	18	12.0	- 10
10 C "air-dry"	280	.8	<< -150
20 C "air-dry"	105	3.6	< -150
30 C "air-dry"	18	4.0	-160
-5 C "air-dry"	140	3.0	< -150

[a] Day sampled= last day the treatment was sampled due to absence of recovery of *V. procera*.

[b]< > means moisture contents beyond measured water retentions.

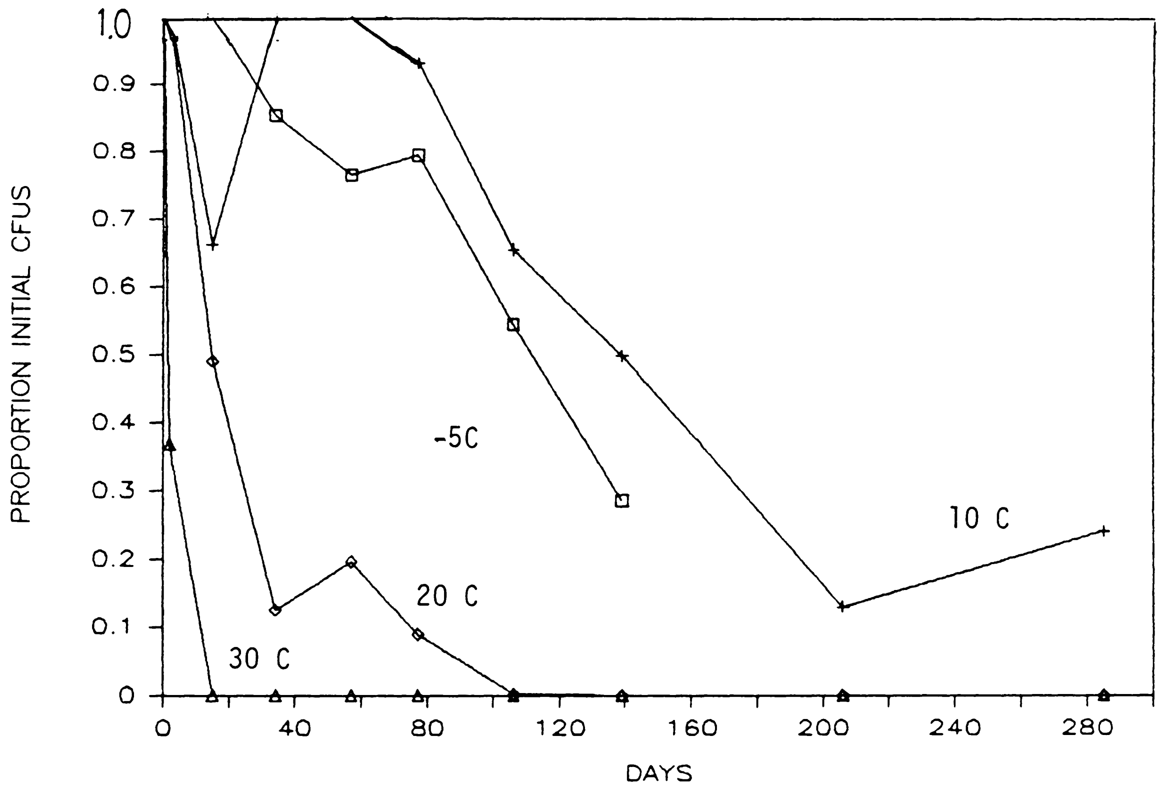

Figure 3.3. The duration of V. procera spore germinability over time in soil allowed to air dry at four different temperatures.

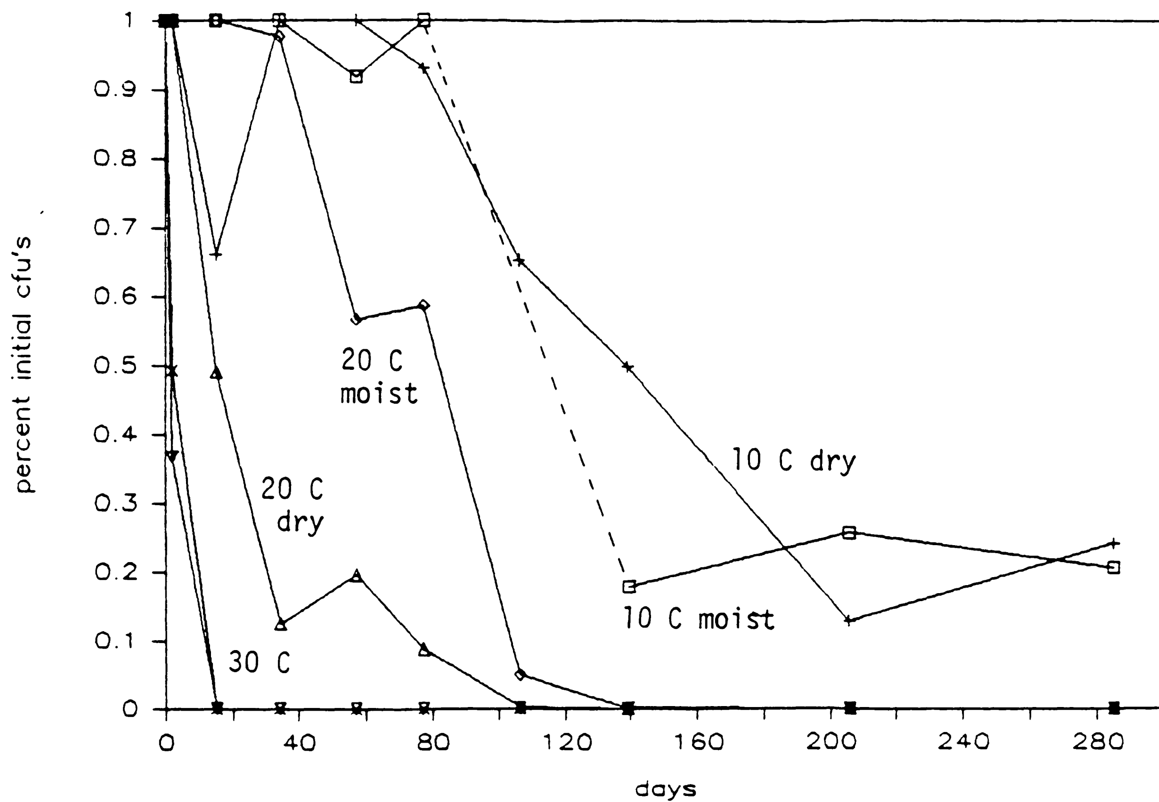

Figure 3.4. A comparison of V. procera spore germinability over time between moist and dry soil conditions at 10, 20, 30 C.

Simultaneous regressions (Draper and Smith, 1981) were performed on the 10 and 20 C data for moist and dry soil. At 10 C moist soil:sand, in comparison with air-dry soil:sand, did not have a significant effect on germinability, however this effect was significant (P=.05) at 20 C.

Mycelial fragments in moist soil at 30 C were not detected after 20 days incubation. At 10 and 20 C, colony forming units were still detected after 316 da (Table 3.4).

Results from dilution platings of buried soil also showed a rapid decline in the proportion of cfus recovered over the sampling period. Results were similar for all five sites. The average proportion of cfus at each sampling time for both conidia and mycelial fragments is plotted in Fig. 3.5. At the end of the experiment, an average of five percent of the conidia and eight percent of the mycelial fragments remained germinable.

Seedlings planted in artificially infested soil. None of the experiments showed significantly greater mortality of seedlings in infested soil than in uninfested soil. There was no observed development of disease symptoms and root and crown condition were similar for treated and control seedlings. In only a few cases was *V. procera* isolated from seedling root systems.

The observations made in experiments two and three were modified due to overheating in the greenhouse, resulting

TABLE 3.4. The number of colony forming units recovered at each sampling time from soil artificially infested with mycelial fragments

	colony forming units/gram soil (OD weight)		
Days	10 C	20 C	30C
0[a]	$3.0x10^9$	$3.0x10^9$	$3.0x10^9$
20	TNTC[b]	TNTC	0
106	$5.0x10^5$	$3.2x10^5$	0
170	$4.2x10^5$	$1.6x10^5$	0
237	$2.7x10^5$	$4.0x10^4$	0
316	$4.0x10^5$	$5.8x10^3$	0

[a]=estimate of the number of cfus/gram soil originally infested.

[b]TNTC=too numerous to count.

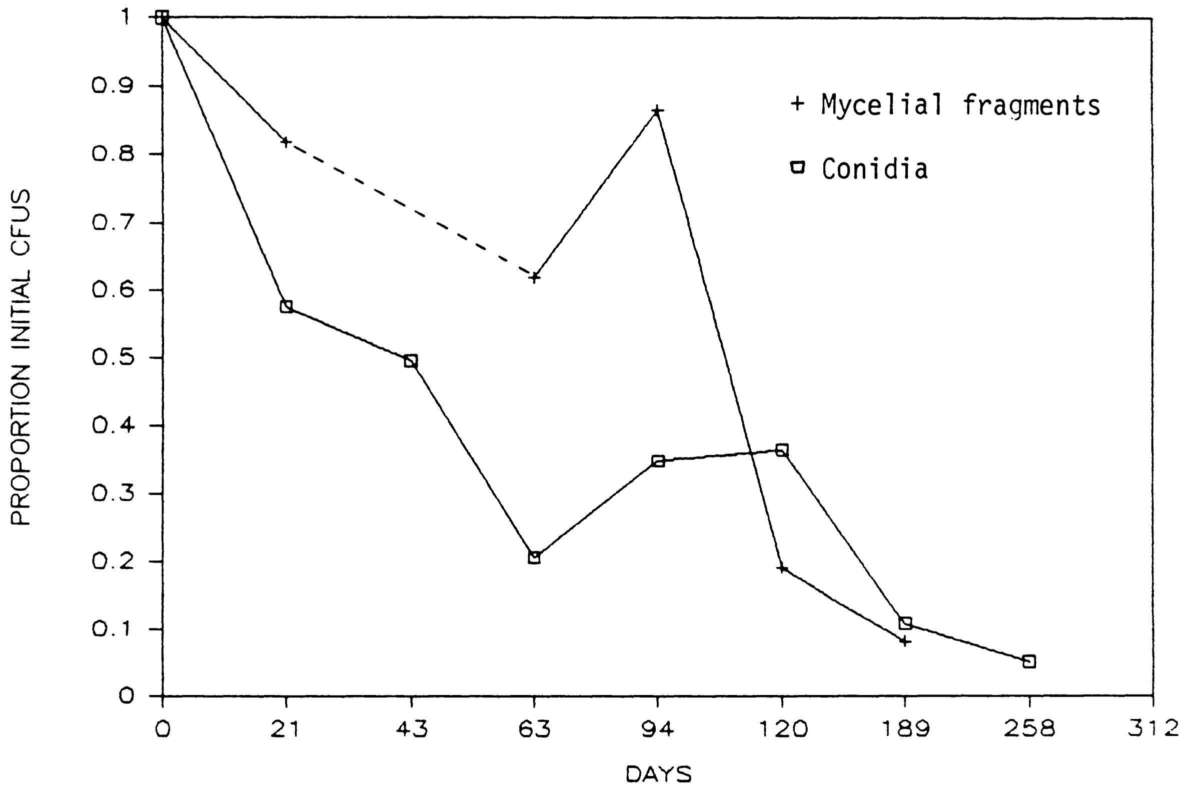

Figure 3.5. The average proportion of colony-forming-units of *V. procera* recovered from buried soil (Blacksburg, VA) over the study period.

in heat-stressed seedlings. The observations were limited to the number of seedlings whose root systems were penetrated and subsequently colonized by _V_. _procera_. Colonized trees were divided into two groups according to their condition (dead or alive) at the time the isolations were made. A high mortality rate of seedlings in all treatments, including controls, disallows observations on symptom development or comparisons of seedling condition between treatments. Furthermore, the percent of colonized seedlings is expected to be greater because of the stress, therefore comparison of percent colonized cannot be made between seedlings in experiments two and three and the remaining experiments. For experiments one, four and five, observations included symptom development.

Of the loblolly pines in experiment one, only one died during the 11 week period and no _V_. _procera_ was recovered from the root system. At the termination of the experiment. _Verticicladiella procera_ was not recovered from any of the seedlings and no seedlings showed symptoms of Procera Root Disease.

Verticicladiella _procera_ was recovered from one seedling in experiment two. This seedling was in soil artificially infested with mycelial fragments. The fungus was recovered from the distal portion of a lateral root.

In experiment three, of the seedlings planted in

soil artificially infested with conidia, penetration and colonization by V. procera occurred only in the 100,000 spores/ gram soil treatment. Verticicladiella procera was recovered from a total of five out of ten seedlings, one of five in pasteurized soil and four of five in nonpasteurized soil (Table 3.5). A total of seven out of ten of the seedlings planted in soil artificially infested with mycelial fragments became colonized, four in pasteurized soil and three in nonpasteurized soil. Soil artificially infested with mycelial fragments had a greater success rate for infection and colonization of seedlings compared to soil artificially infested with conidia. Twenty percent more seedlings in soil infested with mycelial fragments became colonized than seedlings in conidia-infested soil at 100,000 conidia/g soil.

The positions in the lateral and tap roots from which V. procera was recovered are seen in Fig. 3.6. The total number of successful isolations from the lateral roots was 25 out of 36 possible (three positions at each of 12 trees) compared to 16 out of 36 for the tap root. These are significantly different by a "Students" t-test ($P=.05$).

In experiment four, there was no symptom development in seedlings planted in infested soil. The fungus was recovered from one seedling in the watered daily treatment and from three seedlings in the remaining treatment

TABLE 3.5. Recovery of *Verticicladiella procera* propagules from seedlings planted in artificially infested soil at various treatment levels

Treatment	Number of trees colonized by *V. procera*: Live	Dead
Control - pas[a]	0	0
- unpas[b]	0	0
1x10 spores/gram - pas	0	0
- unpas	0	0
$1x10^2$ - pas	0	0
- unpas	0	0
$1x10^3$ - pas	0	0
- unpas	0	0
$1x10^4$ - pas	0	0
- unpas	0	0
$1x10^5$ - pas	1	0
- unpas	0	4
Mycelial fragments - pas	3	1
- unpas	2	1
total:	6	6

[a]pas = pasteurized soil
[b]unpas = unpasteurized soil

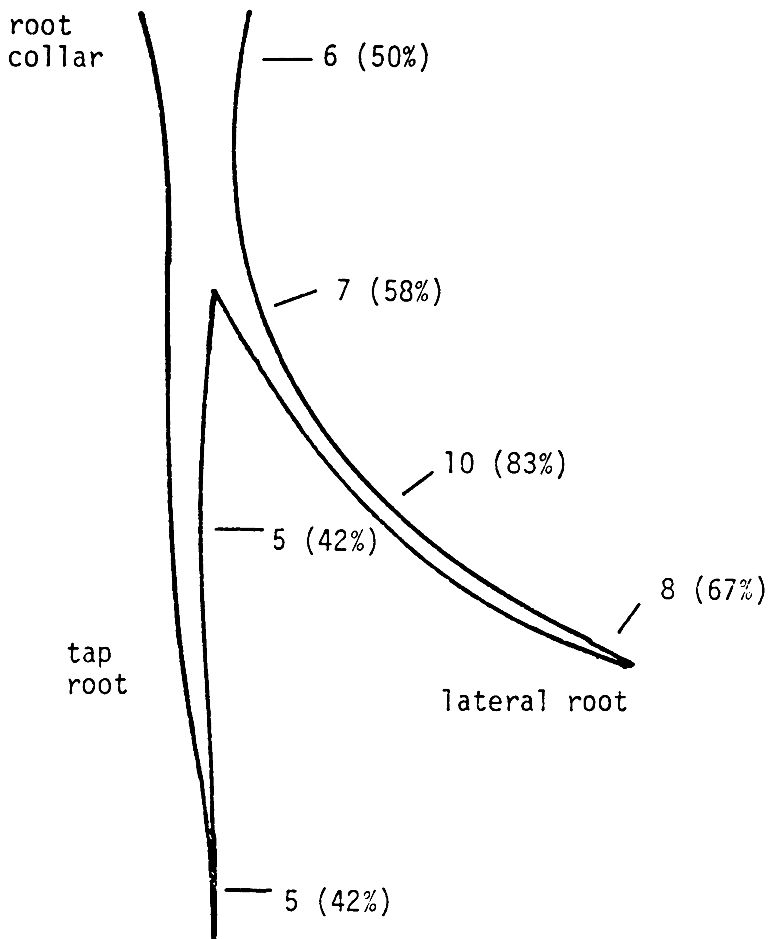

Figure 3.6. Incidence of recovery of Verticicladiella procera at each isolation position from the 12 colonized seedlings. Tap and lateral root isolations at positions distal, middle, and proximal to the root collar. Numbers in brackets are percent recovery at that postion for all the colonized seedlings.

(three day watering cycle). No control seedlings yielded _V. procera_ upon isolation. A Chi-square test for binomial distributions was performed on the data. The number of infected seedlings from the infested soil was not significantly different from the control seedlings (P=.05). The fungus was recovered an equal number of times from the tap and lateral roots of infected seedlings.

In experiment five, _V. procera_ was recovered from one seedling in infested soil. The proportion of colonized, treated seedlings was not significantly different from the controls (P=.05), none of which were colonized. There was no difference in crown and root condition between inoculated and control seedlings. Most of the trees had dark green foliage, good shoot extension and new growing tips on the roots.

DISCUSSION

Conidia of _V. procera_ in soil are quite ephemeral relative to many soil-borne plant pathogens which commonly produce resting structures (Coley-Smith, 1979). Loss of germinabilty occurs most rapidly at temperatures greater than 10 C with "air-dry" soil conditions. At -5 C, the decrease in germinability over time was similar to that at 10 C which may be due to dessication of the soil:sand mix. Mycelial fragments lose the ability to produce colonies most rapidly at temperatures greater than 10 C.

Compared to the hyaline conidia, the melanin pigments in the hyphae do not give additional protection while exposed to the soil environment.

Although the soil temperature is more stable than ambient air temperature, soil temperatures near the surface during the soil may go much higher than 10 C (Pritchett, 1979). During periods of drought, low soil moisture content could have a detrimental effect on soil-borne propagules. This information can be of use in Christmas tree management. The rapid loss of propagule germinability may decrease the likelihood of infection; therefore, following the removal of a diseased tree and its root system, the chances of seedling infection at that site may be decreased by leaving the site fallow for one year.

Under greenhouse conditions, seedling infection by soil-borne propagules is an uncommon occurrence. When seedlings do become infected, disease development is very slow or non-existant. One explanation may be that the number of infections and subsequent colonization occurring on any one seedling is not high enough to result in disruption of the vascular system. This is supported by the isolations made from the tap and lateral roots of planted seedlings. In one experiment, _V_. _procera_ was recovered more frequently from the lateral root than the tap root. Recovery of _V_. _procera_ from both the lateral and the tap root of a single seedling was uncommon. To

be most efficient in disrupting water uptake, the pathogen must be detrimental to the vascular system of many lateral roots and/or be concentrated in the root collar area. Colonization of infected seedlings in these experiments was not extensive and not concentrated in the root collar area.

It has been shown that *V. procera* can easily penetrate and colonized dead wood tissue in the field and in the laboratory. Houston (1969) reported that *V. procera* can remain viable in dead stump tissue for at least two years. In addition, pieces of colonized tissue have been used in the laboratory as sources of inoculum during study of this pathogen (Harrington and Cobb, 1983; Lackner and Alexander, 1983; Wingfield, 1983). Therefore *V. procera* can easily penetrate and colonize dead wood tissue. The frequency of recovery of *V. procera* from the seedlings planted in artificially infested soil in experiments two and three (in which many seedlings died due to heat stress) was greater than in the remaining experiments. However, in spite of the ease with which *V. procera* colonizes dead wood tissue, the frequency of infection and colonization was still low. This suggests that the low rate of infection seen in all experiments may be partly due to infrequent penetration and infection of the root with little subsequent colonization, rather than due to a host response alone.

Pasteurization of the soil prior to infestation did not increase the incidence of infection. This suggests that with soils used in this study, competing microorgansims were not important in the inhibition of root infection by soil-borne propagules.

Germinability of soil-borne propagules declined rapidly. With this in mind, there are several possible scenarios which could explain infection through roots by soil-borne propagules. There could be a resevoir of inoculum such as in a colonized root which supplies inoculum for infection of a new host, or the new host must be available soon after the inoculum source is available, or very few propagules are required to cause infection and disease development. The latter two possibilities are in conflict with the conclusions drawn from the low frequency of infection of seedlings planted in artificially infested soil. The results suggest therefore, that soil-borne propagules are not important in the spread of _V_. _procera_.

Chapter IV

STUDIES ON THE DISTRIBUTION OF SOIL-BORNE VERTICICLADIELLA PROCERA PROPAGULES AND THE INFECTION OF SEEDLINGS PLANTED IN NATURALLY INFESTED SOIL

There are several lines of evidence which indicate that infection of trees may occur through the roots by soil-borne propagules. Firstly, *Verticicladiella procera* is most frequently recovered from the roots and root collar area (Dochinger, 1967; Horner *et al*., 1985; Sinclair and Hudler, 1980), the parts in contact with soil. Secondly, the fungus has been recovered from soil sampled at symptomatic trees (Lackner and Alexander, 1984; Swai and Hindal, 1981). Thirdly, seedlings planted in naturally infested soil (at the site of an excavated, diseased tree) developed symptoms and *V. procera* was recovered from the root systems (Lackner and Alexander, 1984). Many questions remain with regard to the mechanism of entry into the root system, the location of the fungus in soil, the quantity of propagules available for infection (which relates to the inoculum potential), and the number of propagules required for infection and sufficient colonization to result in vascular dysfunction. Also to be considered is the association of root, stem and soil-inhabiting insects with diseased trees (Lackner and Alexander, 1982; Wingfield, 1982, 1983). Insects may influence propagule distribution and penetration into roots.

To further understand the role of soil-borne propagules of _V. procera_ in the development and spread of PRD, studies involving naturally infested soil were undertaken to meet the following objectives: (1) to determine the large-scale density and distribution of soil-borne propagules in plots encompassing symptomatic and asymptomatic trees; (2) to focus on the density and distribution of propagules around individual diseased trees; (3) to relate this distribution to fungal colonization patterns in root and root collar tissue; (4) to examine the ability of soil-borne propagules to infect seedlings planted in naturally infested soil.

MATERIALS AND METHODS

Each of the soil samples collected during this study was processed by soil dilution, using the method of Clark (1965), and plated on a medium selective for _V. procera_ (VPISM) (Swai and Hindal, 1981). Following incubation at room temperature (18-22 C) for 14 days, the number of colonies per plate were counted, the mean was calculated for each sample, and multiplied by the dilution factor. The number of propagules/gram of soil was corrected for oven-dry soil weight.

Density and distribution of soil-borne propagules in a large plot. Four plots were established in a white pine Christmas tree plantation in Montgomery County, Virginia. Primarily one hillside of the plantation was

affected by the disease. The plots were established in this area at the top, mid slope (two plots) and bottom of the hill. Symptomatic trees surrounded by asymptomatic trees served as plot centres. Ten soil sampling locations per plot were located on a 2 m by 2 m grid. These locations were at least 50 cm from the base of the centre symptomatic tree. A soil auger (one litre capacity) was used to remove a sample from each location once a month (starting in May, 1984) for three months. A sub sample was removed after thoroughly mixing the soil in a plastic bag. Each subsample was serially diluted using the technique described by Clark (1965) and plated on VPIM. The centre tree in each plot was sampled by removing wood chips from the root collar and plating them on actidione amended malt extract agar (AMA).

Propagule density and distribution around individual trees. Ten symptomatic trees from each of two plantations on sites typical for Christmas tree plantations in Virginia were sampled. Plantation A was planted with Scots pine (Warren County, VA) and plantation B with white pine (Montgomery County, VA). Two roots approximately 180° apart were selected and carefully excavated by brushing soil away from the top of the root to minimize soil disturbance. Using a teaspoon dipped in alcohol and flamed, small soil samples (approximately 30 grams) were removed from precise locations along the two roots (figure

4.1) and placed in plastic bags labelled by tree, root and sample location. Samples a, b, c and f were next to the root at 0, 10, 20 and 30 cm, respectively, from the root collar. Samples pairs d, g and e, h were taken at 5 and 10 cm respectively, perpendicular to the root surface at positions c and f. All samples were from the same depth as the root. Following dilution plating of the samples, the mean number of germinating propagules at each position was determined for each root then averaged for the two tree species.

Association of soil-borne propagules with colonization patterns in roots. Remaining roots of trees sampled in the previous section were carefully excavated and the lower bole and root system removed to the laboratory. A drawing was made of the stump and the major roots were labelled with particular attention paid to roots I and II (Fig. 4.1). Tissue samples were taken along each major root at 0, 10, 20 and 30 cm from the root collar. The samples taken from roots I and II corresponded with the soil samples from locations a, b, c and f (Fig. 4.1). Therefore, from each of roots I and II there were four isolation pairs (root isolation and adjacent soil isolation) at 0, 10, 20 and 30 cm from the root collar. The tissue samples were removed with a flame sterilized cork borer (0.8 cm diameter) and cut in half. The bark and wood were separated and plated individually. One half was plated onto malt extract agar

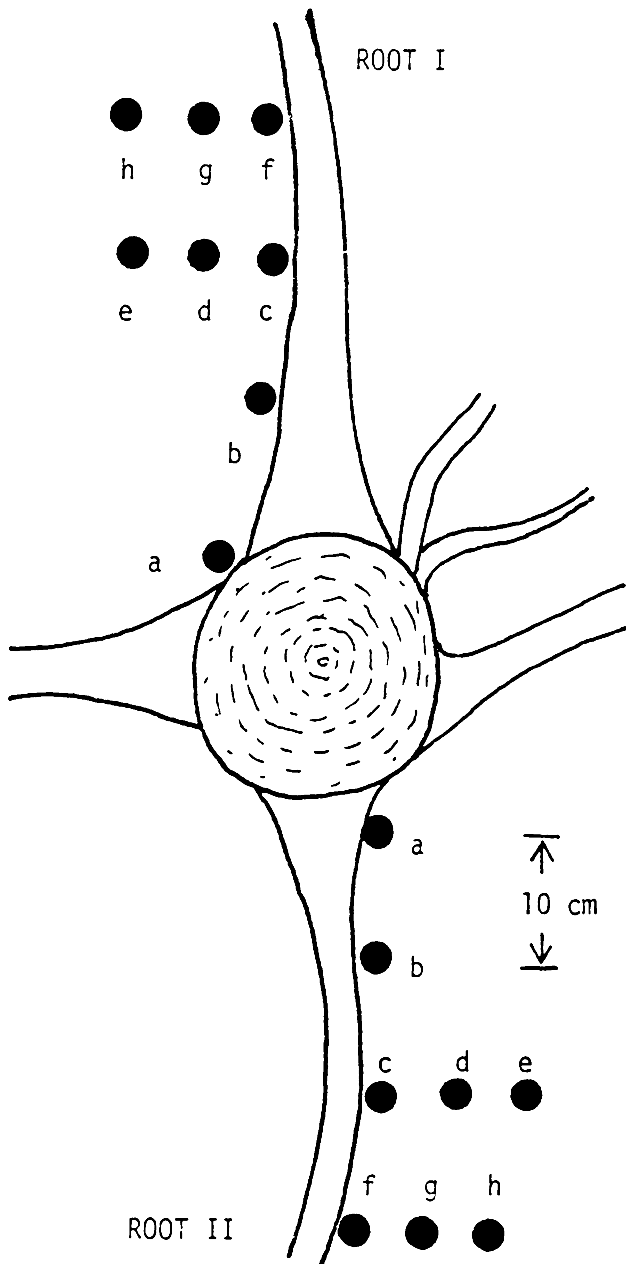

Figure 4.1. Soil sampling scheme. Two roots from each of 10 white pine and 10 Scots pine. Eight soil samples per root.

(MEA) and the other half onto AMA[1]. After incubation at 20 C for 14 days, each piece of tissue was scored for the presence or absence of V. procera. The positions from which V. procera was recovered from tissue and/or soil isolations were marked on the drawing. Observations were made on the colonization patterns in wood and the corresponding distribution of propagules in the soil adjacent to the wood tissue. Each root tissue and soil isolation pair was placed in a category according to the criteria in Table 4.1. The frequency of occurrence of each category for the four positions along the roots were determined. The frequencies were plotted over sampling position for the two tree species separately.

Infection of seedlings by soil-borne propagules. Seedlings were planted in a large plot to bait for propagules which may be generally distributed over a large area. An 8 x 10 m plot was established in plantation B in an area encompassing several symptomatic trees and the sites of excavated trees from which V. procera had been isolated. Twenty- two-year-old white pine seedlings were planted at 2m x 2m intervals. To test for methods of infection other than by propagules in soil, one seedling in a pot with a weblite, vermiculite and peat mix (2:2:1, v:v:v)

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[1] Tissue sample data was collected by W.E. Horner in a co-study on colonization patterns by V. procera in diseased trees (Horner et al., 1985).

TABLE 4.1. Categories of isolation results from root tissue - soil isolation pairs.

Soil	Tissue	Category
–[a]	–	0
+[b]	–	1
–	+	2
+	+	3

[a] - *V. procera* not recovered.
[b] + *V. procera* recovered.

was placed next to each of the planted seedlings (Fig. 4.2). Prior to outplanting seedlings, five from the same source were randomly selected for root isolations as described in chapter III. This was to ensure no pre-treatment infection by V. procera. A soil sample (approx. 60 g) was removed from the location of each planted seedling and dilution-plated onto VPIM. The plot was established in August 1984 and maintained for 10 mo. As planted and potted seedlings died, they were replaced with a healthy seedling. The dead seedlings were labelled by their position in the grid and taken back to the laboratory. Isolations were made from the tap root and one lateral root of each seedling as described in chapter III. At the termination of the experiment (June 1985), all of the seedlings were lifted and labelled according to their position in the grid. Potted seedlings were inspected for roots growing into the soil through holes in the bottom of the pot. Observations of the crown and stem (color, length of new growth, presence of resin-soaked areas or lesions) and root condition (presence of new roots, health of cortex) were made. Following an inspection for insect activity (such as puncture holes or feeding marks), isolations were made from the seedlings as previously described. The number of seedlings showing symptoms of PRD and/or from which V. procera was recovered was recorded. Seedlings were also planted directly adjacent to individual symptomatic

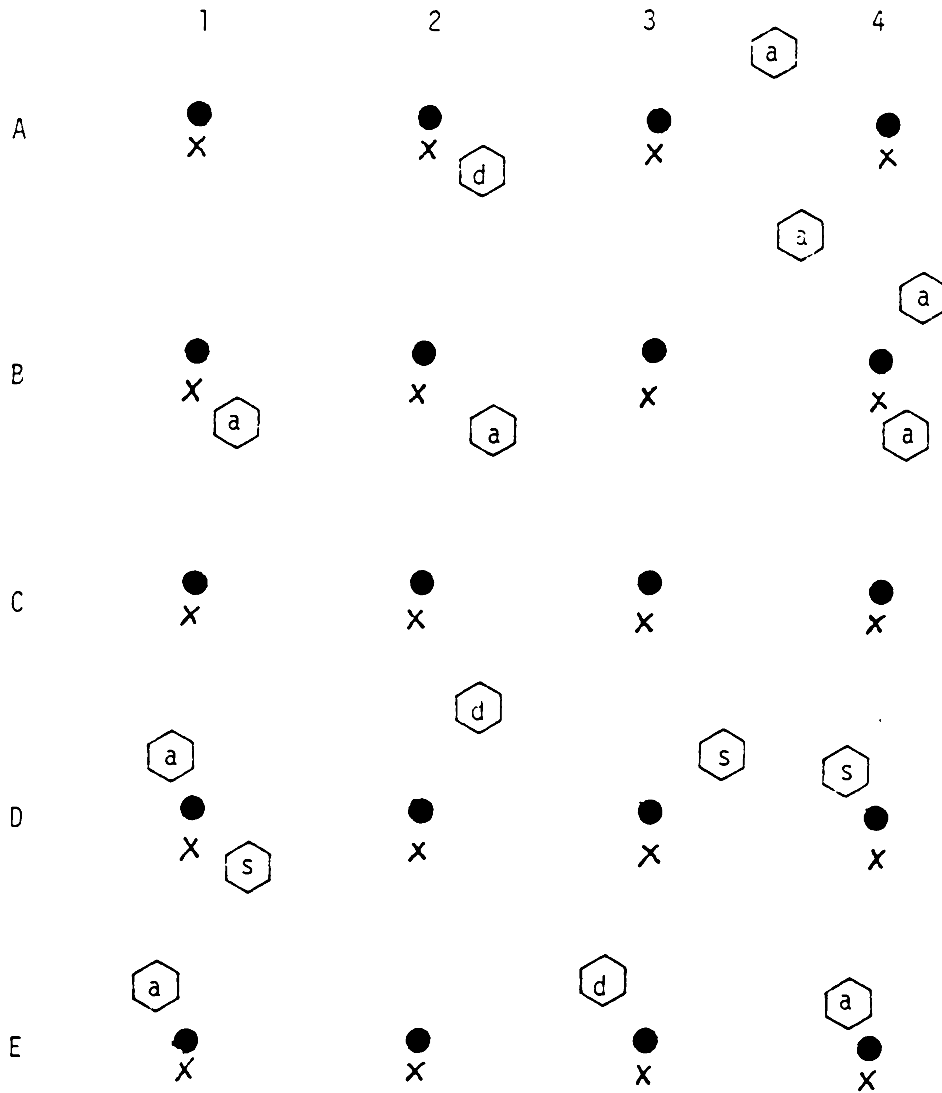


Fig. 4.2. Location of planted and potted seedling pairs relative to crop trees. ● =potted, x=planted, a=asymptomatic, s=symptomatic, d=dead, ⬡ =crop trees.

trees in order to examine the effect of propagule density and distribution on infection of seedlings. Three seedlings in each of four perpendicular directions were planted at 10, 25 and 40 cm from the root collar of five symptomatic trees and one asymptomatic tree in plantation B. Seedlings were labelled by bearing (N, S, E, W) and distance (10, 25 and 40 cm). Soil samples (approximately 60 g each) were taken at each planting location and processed by soil dilution as before. These plots were maintained for 14 wks by replacing and isolating from dead seedlings. At the termination of the experiment, the seedlings were lifted and the centre trees excavated except for the asymptomatic tree. The root collar of this tree was swabbed with 70 % ethanol and two cork borer plugs (size #3) of bark and wood tissue were removed. These were plated on AMA. The seedlings were treated as described previously with observations of possible symptoms followed by isolations from one lateral root and the tap root. From the centre trees, three or more roots closest to the planted seedlings were selected. Tissue samples at 0, 15, and 30 cm (from the root collar) and from around the root collar were removed aseptically and plated onto AMA.

RESULTS

Density and distribution of soil-borne propagules in a large plot. *Verticicladiella procera* was not recovered

from any of the soil samples. However, V. procera was recovered from the tissue samples of the four centre symptomatic trees.

Propagule density and distribution around individual trees. The mean propagule densities at each sampling location for both species are plotted in Figure 4.3.

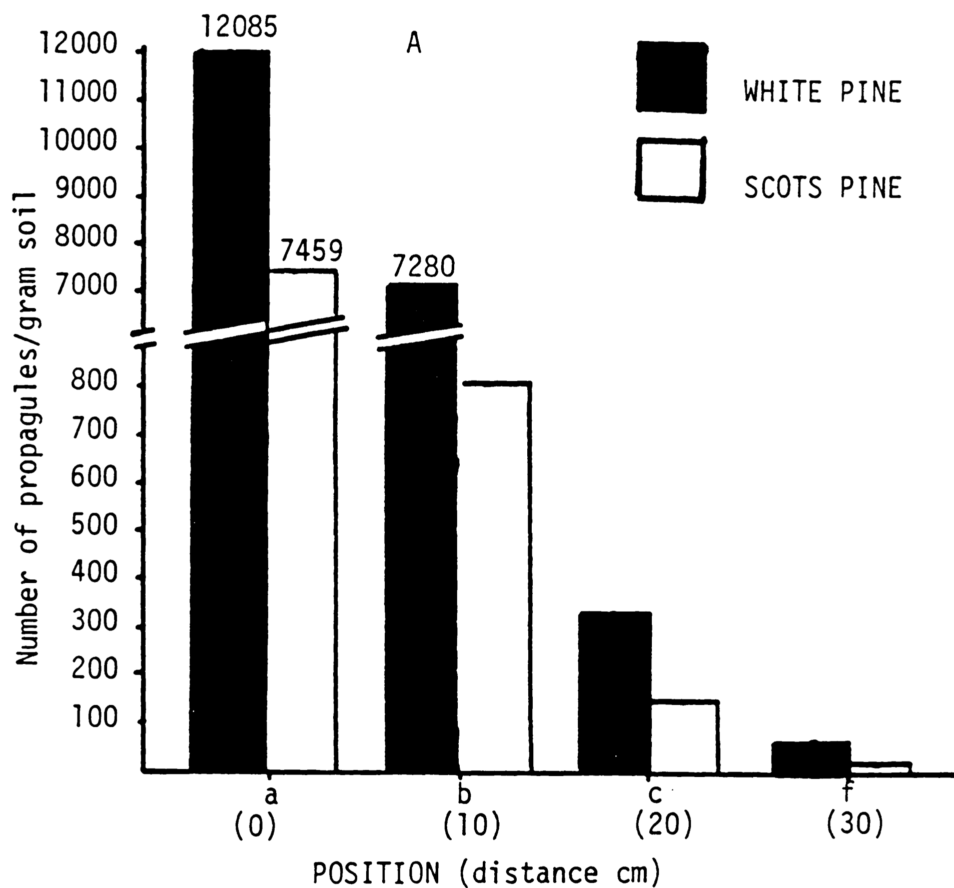
The data for positions a, b, c and f (those immediately adjacent to the root surface) for both species were fit to a negative exponential curve which describes a very high number of propagules at position 'a', decreasing rapidly towards the root tip. In both cases, the slope of the regression line of the transformed data was significant (P=.05) indicating a significant difference in propagule density between sample locations. The equations are as follows:

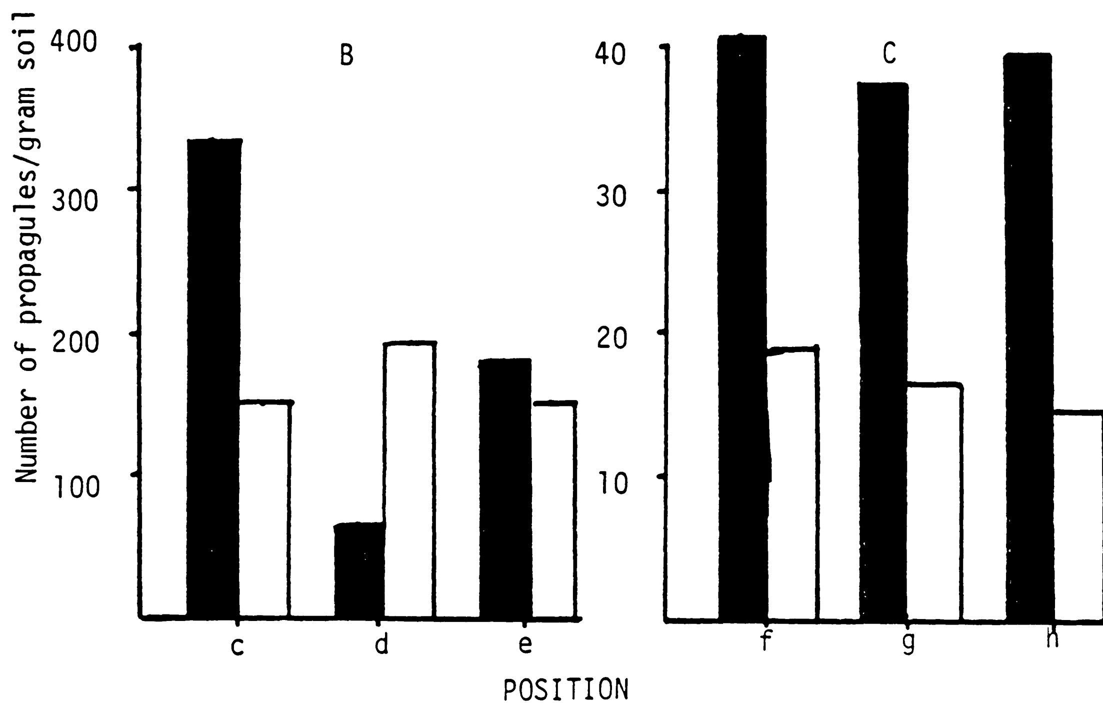
Scots pine: $Y=51021e^{-1.7x}$

white pine: $Y=158419e^{-2.003x}$

An analysis of variance on each set of lateral samples (positions c, d, e and f, g, h) for both species showed an insignificant position effect. The number of propagules recovered from the root surface and at five and 10 cm from the root surface were not significantly different for either species. Propagule density was greatest at the root collar and decreased significantly towards the root tip. Lateral samples showed a trend of decreasing propagule density with increasing distance from the root surface but

Figure 4.3. Recovery of *Verticicladiella procera* from soil sampled around symptomatic Scots and white pine trees. A= samples adjacent to the root surface, B= samples perpendicular to the root surface from position 'c', C= samples perpendicular to the root surface from position 'f' (see Fig. 4.1).


12085
A
WHITE PINE
SCOTS PINE
7459
7280
12000
11000
10000
9000
8000
7000
800
700
600
500
400
300
200
100
Number of propagules/gram soil
a
(0)
b
(10)
c
(20)
f
(30)
POSITION (distance cm)


B
C
400
300
200
100
Number of propagules/gram soil
40
30
20
10
c
d
e
f
g
h
POSITION

this was not significant.

Association of soil-borne propagules with colonization patterns in roots. The pattern of propagule distribution in soil was similar for both Scots and white pine. However, the numbers of propagules recovered were different. Neither root nor soil isolation of V. procera remained constant for the samples adjacent to the roots. Successful isolation of V. procera from both the root and soil (category 3, Table 4.1) was greatest at the root collar and decreased distally. The unsuccessful isolation of V. procera from both the tissue and the soil was least at the root collar and increased distally (Fig. 4.4). This would not have been the case had successful isolation of V. procera from either the root or the soil been constant (or with insignificant change) for samples adjacent to the root. Isolation of V. procera from only one component of the tissue-soil sample pair was constant from the root collar toward the root tip (Fig. 4.4). Category 0 occurred much more frequently with the white pine samples than with the Scots pine samples. Likewise, category 3 occurred much more frequently with Scots pine than with white pine. However, when propagules were recovered from soil samples at white pines, the numbers were generally higher than those from Scots pine.

Infection of seedlings by soil-borne propagules. None of the 20 soil samples from sites of the planted seedlings in the 8x10 m plot yielded V. procera upon

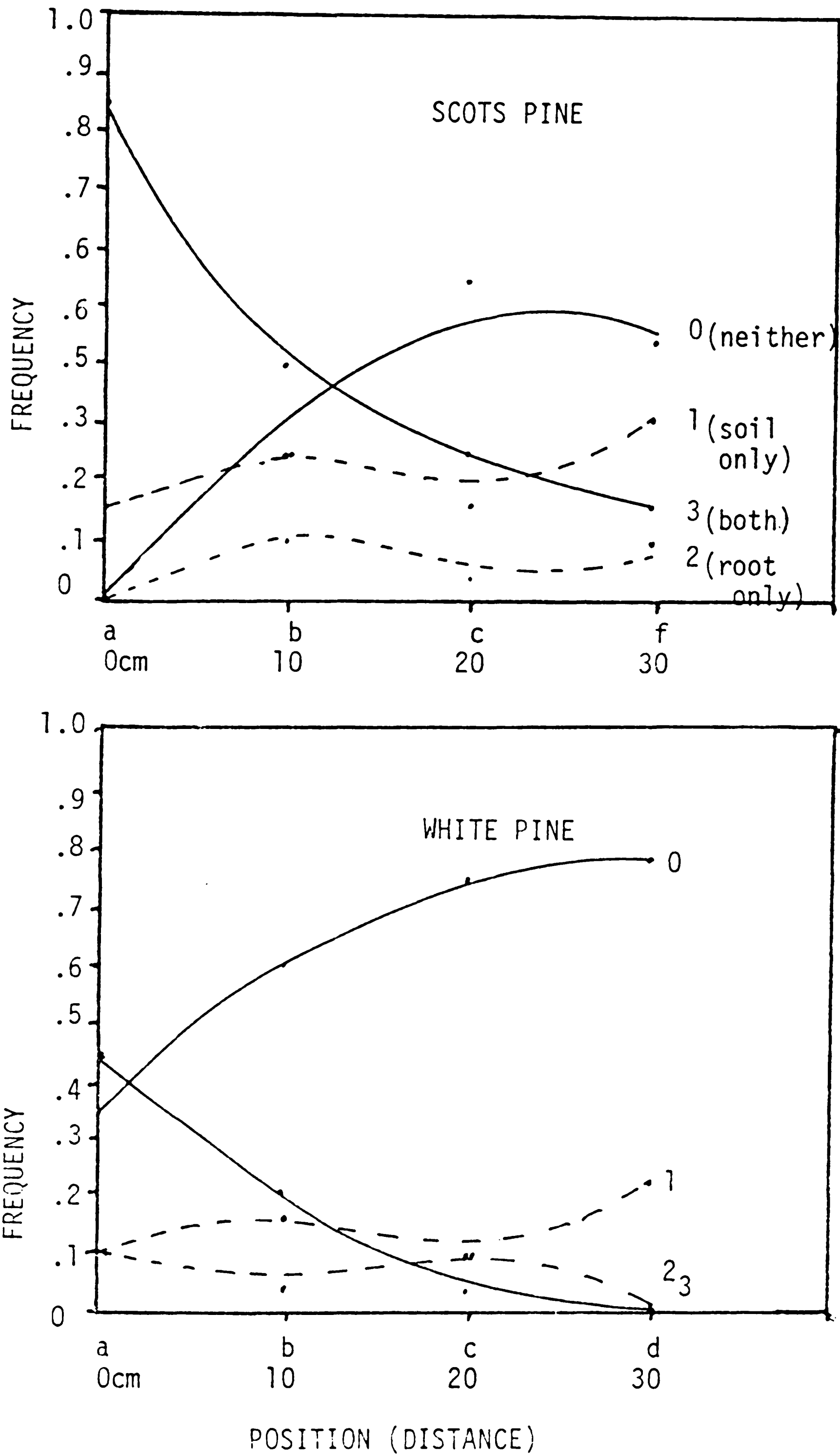


Fig. 4.4. Soil/Root isolation pairs. Frequency of occurrence of each category (see Table 4.1 for description of categories).

dilution plating.

All of the planted and potted seedlings removed at experiment termination had healthy green tops and new, white root tips. During the course of the experiment a few seedlings died and were replaced by healthy seedlings but _V_. _procera_ was not recovered from the dead seedlings.

Isolations made from all the seedlings at the termination of the experiment showed one potted seedling, at position D4, which was colonized by _V_. _procera_; however no symptoms of disease were observed. The fungus was isolated from the proximal section of a lateral root. No planted seedlings yielded _V_. _procera_. Lower stem and root wounds (cause not determined) were not uncommon.

Of the five symptomatic trees around which seedlings were planted, four were colonized by _V_. _procera_. Conidiophores and spore masses were observed in insect galleries in roots of two trees (Table 4.2). The fungus was recovered from at least one seedling out of the 12 planted around each of the four colonized centre trees (Table 4.2). Seedlings planted around tree E (no _V_. _procera_ recovered) and the symptomless tree (G) did not yield the fungus. _Verticicladiella_ _procera_ was not recovered from cork borer plugs of bark and wood tissue removed from the root collar of tree G. Seven of the 48 seedlings planted around trees colonized by _V_. _procera_ were infected. Of these, three had been planted at 10 cm from the root collar and

TABLE 4.2. Recovery of *Verticicladiella procera* from the centre trees and the planted seedlings.

Tree	Recovery from centre tree	Recovery from seedlings: seedling	location of recovery	Comments on centre trees
A	Root II: 0, 15 cm	N-25	tap root-distal	-*V. procera* fruiting in root II insect gallery (see Fig. 4.5).
	III: 0, 15			-most roots resin-soaked and black stained.
	collar: 2/3 isolations[a]			
C	Root I: 0 cm	E-25	lateral root- middle and proximal	-roots resin-soaked and black stained.
	Root III: insect gallery			-*V. procera* fruiting in root III insect gallery
D	Root III: 0 cm	E-25	tap root-proximal	
	collar: 1/3 isolations	S-10	lateral root- distal	
F	collar: 3/3 isolations	N-10	tap root-middle and proximal	-roots not excavated
		E-10	tap root-all 3 positions	
		W-25	lateral root- middle	
E	None	None		-most roots resin-soaked, several with insect feeding sites.
G	None	None		-healthy roots.

[a] Root collar isolations: successful recovery/attempts

four had been planted at 25 cm from the root collar. The appendix contains drawings of the location of V. procera recovery from the centre trees and the seedlings. The fungus was recovered from the tap and lateral roots an equal number of times.

Verticicladiella procera was infrequently recovered from the soil samples taken at each of the 12 planting sites/tree (Table 4.3). Recovery of V. procera from seedlings did not correspond to the locations from which V. procera was recovered from the soil. Seedlings planted directly adjacent to colonized roots did not become infected. Infection of seedlings at 10 cm from colonized root collar was observed six times.

There was no difference in root and crown condition between infected and uninfected seedlings. Verticicladiella procera was recovered from one seedling with obvious signs of weevil feeding (Fig. 4.5).

DISCUSSION

Verticicladiella procera was found in association with diseased trees. Soil samples taken in the general area of, but not adjacent to, diseased trees did not yield the fungus. Verticicladiella procera was not recovered from soil sampled around asymptomatic trees. These results concur with those of Lackner and Alexander (1984) who recovered V. procera from soil at symptomatic trees only. However, Swai and

TABLE 4.3. Recovery of *Verticicladiella procera* from soil samples removed from the location of a planted seedling.

Tree	Position of sample	V.p[a] in seedling at sample site	Propagules/gram OD wt.
A	E-10	-	24.5
	S-10	-	147.2
	S-25	-	24.5
C	---		0
D	---		0
E	---		0
F	W-10	-	88.8
G	---		0

[a] V.p.=*V. procera*

Figure 4.5. Weevil feeding marks at arrow. Also some mechanical damage to the stem. Verticicladiella procera was recovered from this wound.

Hindal (1981) recovered _V. procera_ from soil at four percent of the asymptomatic trees. Possible explanations are that the asymptomatic trees were colonized by _V. procera_ but not showing symptoms, or propagules may have been brought to the site by insects. A much higher percentage of soil from symptomatic trees yielded _V. procera_, therefore it is safe to conclude that _V. procera_ propagules in soil are associated with diseased trees. Therefore, a possible source of propagules in soil is from the colonized roots and root collar of diseased trees.

Focusing more closely on the soil immediately surrounding diseased trees revealed a definite pattern of propagule distribution. Propagule density around individual diseased trees was greatest at the root collar and decreased toward the root tip. This distribution reflected the pattern of colonization in the root system and root collar. Dense colonization at the root collar decreasing up the stem and towards the root tips indicated that colonization originated at the root collar (Horner _et al._, 1985). Large numbers of propagules in soil corresponded with recovery of _V. procera_ from the adjacent tissue sample, another line of evidence suggesting that soil-borne propagules originate from colonized root systems.

There were differences in the density of propagules in soil around Scots and white pine. How much of this difference may be attributed to host species is unknown.

In the two plantations studied, the incidence of *V. procera* in soil at the root collar and along the roots was greatest for the Scots pine plantation, but the total numbers of propagules recovered was greatest in the white pine plantation. The patterns of distribution were similar for both host species. Soil around the Scots pine yielded *V. procera* more consistently but in fewer numbers. If insects do play a role in propagule distribution, the difference in habits of insects associated with the two tree species may account for the difference in propagule numbers.

It is possible that the density of propagules in soil, in a direction perpendicular to the root surface, would also decrease significantly at distances greater than 10 cm from the root surface. However, numbers of propagules recovered from samples at greater distances would have an increased chance of being influenced by another colonized root.

It is evident that the fungus is not uniformly or randomly distributed in soil, its presence in soil may be dependent upon the presence of a diseased root and/or upon the activities of insects.

Seedling pairs (one planted, one in a pot) were placed in the vicinity of symptomatic trees in order to (i) compare *V. procera* recovery between soil samples and planted seedlings, (ii) to bait for soil-borne propagules

not recovered by the selective medium, and (iii) to determine whether an alternative means of seedling infection exists. Both planted and potted seedlings should be susceptible to inoculation by a fungus-bearing insect. Only planted seedlings are susceptible to infection from propagules in soil, because none of the potted seedling roots were in contact with soil. The infection of a potted seedling but not of planted seedlings indicates that infection can occur by means other than propagules in soil. The most likely alternative is insect vectors. This hypothesis is supported by the recovery of _V_. _procera_ from insect galleries in roots of the colonized centre trees around which seedlings were planted. A low percentage of the planted seedlings (around individual colonized trees) became infected by _V_. _procera_. There are two possible explanatations for the infection of these seedlings and of those in the 1984 study by Lackner and Alexander. One is that infection occurred through small wounds in the roots with propagules in soil as the source of inoculum. The second is that insects carrying propagules of _V_. _procera_ transmitted the fungus to the seedling while feeding on the roots or root collar. The low incidence of recovery of _V_. _procera_ from soil sampled at the sites where seedlings were planted and subsequently infected, and the lack of infection of seedlings planted in soil from which the fungus was recovered supports the

latter possibility. Additionally, studies with artificially infested soil discussed in the previous chapter suggests that infection and colonization requires very high numbers of propagules which are not found in nature except for those closely associated with colonized roots. The presence of the fungus in soil may be due in part to the movement of insects in soil around colonized roots which provide the source of inoculum. In the study by Lackner and Alexander (1984), 46% of the seedlings planted at the sites of excavated diseased trees became infected by V. procera, a higher rate of infection than the 14.5% observed in this study. Insects feeding on roots may have contributed to the incidence of infection in Lackner and Alexander's (1984) study. In addition it is possible that small pieces of colonized wood tissue left from the tree excavations may have provided concentrations of inoculum for infection.

Propagules may have been present in soil below the level of detection by the dilution-plating technique, and these propagules could have caused the infection of seedlings. However, the lack of infection of seedlings planted in soil from which the fungus was detected does not support this. From these results, it is doubtful that infection of the seedlings in Lackner and Alexander's (1984) study and in this study in particular, is due solely to propagules in soil.

This study indicates that the role of soil-borne propagules in disease spread is unimportant. Furthermore, insect transmission was observed as an alternative method of infection. Results from this study suggest that insect transmission is more important than infection via soil-borne inoculum.

Chapter V

PRELIMINARY STUDIES ON THE TRANSMISSION OF VERTICICLADIELLA PROCERA BY INSECT VECTORS

One of the proposed mechanisms for the spread of *V. procera* is transmission of the fungus by insects of the order Coleoptera (Lackner and Alexander, 1984; Wingfield, 1983). Spores of *V. procera* are borne in sticky, mucilaginous droplets which adhere to insect bodies. Many bluestain fungi are known to be transmitted by insects (Craighead, 1928; Dowding, 1984).

The insect transmission aspect of Procera Root Disease epidemiology has not been closely examined beyond the association of colonized trees with insect infestations (Lackner and Alexander, 1982; Wingfield, 1983, 1982) and the recovery of *V. procera* from insects (Lackner and Alexander, 1984; Wingfield, 1983).

The objectives of this study were based on examining more closely the potential role of insects as vectors of *V. procera*. The first objective was to determine which insect groups carry the fungus and therefore are potential vectors. Secondly, to determine the proportion of *V. procera*-contaminated insects in Christmas tree plantations with and without diseased trees. Thirdly, to carry out preliminary studies on the ability of contaminated insects to transmit *V. procera*.

MATERIALS AND METHODS

Insect survey. An insect survey in 10 white pine Christmas tree plantations was conducted in southwestern Virginia. Plantations with and without Procera root disease were selected for the survey. Selection criteria were based on symptoms and the recovery of *V. procera* from symptomatic trees. The plantations selected are listed in Table 5.1.

Twenty trees per plantation were sampled to obtain a reliable estimate of the presence or absence of *V. procera*. Trees were selected with a bias towards those displaying symptoms. Every tree was assigned to one of the following symptom categories: asymptomatic, symptomatic and dead. The duff around the base of each tree was removed and the root collar swabbed with 70 % EtOH. Two cork borer (0.8 cm diameter) samples of wood and bark tissue were removed from opposite sides of the root collar and plated onto AMA. After incubation for 14 days at 20 C, the plates were observed for colonies of *V. procera*.

One insect trap per plantation was established starting the first week in June 1985. The traps were modified split-bolts (Taylor and Franklin, 1970). White pine trees were cut into bolts 30 cm long with diameters of 10 to 20 cm. Tissue platings onto AMA certified that no *V. procera* was present in trees from which bolts were cut. Tissue samples for plating were taken from the basal portion of

TABLE 5.1. Plantations surveyed for insects contaminated with *Verticicladiella procera*.

Plantation Name	Location	Tree Condition - No. sampled (No. with *V. procera*)		
		Asymptomatic	Symptomatic	Dead
EB	Floyd County, VA	7 (0)	13 (0)	0 (0)
LC	Montgomery County, VA	8 (0)	10 (0)	2 (0)
OMR	Montgomery County, VA	19 (0)	1 (0)	0 (0)
RK	Bedford County, VA	5 (0)	12 (0)	3 (0)
LS #3	Floyd County, VA	19 (0)	1 (0)	0 (0)
LS #4	Floyd County, VA	20 (0)	0 (0)	0 (0)
DL	Floyd County, VA	6 (0)	11 (0)	3 (1)
RS	Floyd County, VA	9 (0)	10 (0)	1 (0)
BL[a]	Montgomery County, VA		10 (10)	
SC	Wythe County, VA	9 (2)	8 (2)	3 (2)

[a] = presence of *V. procera* predetermined in this plantation.

the stem since that is the most frequently colonized. The bolts and freshly cut pine boughs were immediately transported to the plantations where the bolts were split longitudinally. The trap sites were located arbitrarily either within an area of symptomatic trees, or if no trees displayed symptoms, at least 30 m from roads or standing timber. Each trap consisted of a small pit 30 cm square and 20 cm deep into which six split bolt halves were placed bark side up. Fresh pine boughs were placed over the pit to completely shade the bolts. Bolts were exposed for one week, then collected in clean plastic bags and removed to the laboratory with attached insects. When bolts were collected, the pit was also examined carefully for weevils not attached to the bolts which were also collected. Fresh split bolts were placed in the pits and covered with boughs. This sampling procedure was repeated four times at weekly intervals in all 10 plantations.

In the laboratory, each bolt was carefully examined for evidence of insects. Weevils were picked off the bolts with flamed forceps and placed onto AMA plates. Most weevils were identified to genus. Selected weevils were aseptically removed from the plates after 24 hours (at which time they were still alive) and fixed in Kahles solution (90 mls 95% EtOH, 5 mls acetone, 5 mls formalin) for verification of identification.

Bark beetles in trap bolts were located by the presence of frass around entry holes. A one cm square portion of bark around the entry holes was aseptically removed to expose the beetles. Bark beetles removed from the bolts with sterilized forceps and spatula were pushed into the agar in an AMA plate. Approximately one half of the beetles from plantations with PRD were reserved for a caging study (see below) or identification. Insects reserved for identification were fixed in Kahles solution[1]. Insect isolation plates were observed for colonies of V. procera after incubation at 20 C for 14 da. The number of insects trapped at each collection from each plantation and the number carrying V. procera was recorded.

Transmission studies. An experiment with two stages was designed to test the ability of the trapped insects to transmit the fungus. In the first stage, the trap bolts from selected collections (previous section) were placed in loose plastic bags labelled with the date and plantation from which they were collected. These were incubated at room temperature (ca. 18-20 C) for 21 days. Isolations from trap bolts were made at the end of the incubation period. Areas of insect activity (as indicated by frass, discolored and flattened areas of bark or weevil feeding

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[1] Identification of insects was by comparison with specimens previously identified by the Smithsonian Institute, Washington D.C. and by the Entomology Clinic, VPI & SU, Blacksburg, VA.

holes) were swabbed with 70% EtOH and flamed briefly. A cork borer (0.8 cm diameter) was used to remove bark and wood tissue which were plated individually onto MEA and AMA. The number of isolations made from each set of bolts (one collection from each plantation) varied from six to 12 depending upon the amount of insect activity. When present, beetle and weevil larvae and frass from larval galleries were plated on AMA. After incubation at 20 C for 14 days, the plates were observed for presence of *V. procera*.

For the second stage of the transmission study, weevils were placed in separate muslin-covered frames (1m x 1m x .6m) (Fig. 5.1) along with two uninfested split bolts and a white pine seedling. The caged weevils were from certain collections that had been plated on AMA in the previous section. Three white pine seedlings from the same source as those placed in cages but not exposed to insects were isolated from as described in chapter III as controls to verify no colonization by *V. procera* prior to caging. Bark beetles collected from the trap bolts and reserved (section above) were also caged with bolts and a seedling. Detailed notes were made on the source of the insects (date and plantation) so that the proportion of caged insects carrying *V. procera* could be estimated from the isolation plates. After two weeks in the cage, the bolts and seedlings were removed. The caged bolts were incubated

Figure 5.1. Insect cage. Muslin-covered frame with a removable lid. Bark beetles and weevils were placed in the cages with split white pine bolts and white pine seedlings.

for three weeks and isolated from as previously described. The seedlings were examined for insects then placed on a bench in a lath house. After one month the seedlings were observed for root and crown symptoms, and isolations made. Segments of the tap root and two lateral roots were plated onto MEA and AMA as described in chapter III.

RESULTS

Insect survey. The number of trees in each symptom category, and the number colonized by *V. procera* for each plantation are in Table 5.1. Only three of the 10 plantations were confirmed to have trees colonized by *V. procera*. In one of these (DL), *V. procera* was recovered from only one dead tree of the three sampled; it was not recovered from any of the 11 symptomatic trees sampled. In plantation RS there were many trees showing symptoms of PRD and *V. procera* was recovered from the trap bolts. This plantation probably contained trees colonized by *V. procera*, but they were missed in the 20-tree sample.

Weevils were trapped in all 10 plantations and bark beetles were trapped in nine out of 10 plantations (Table 5.2).

Weevils contaminated with *V. procera* were trapped in seven out of 10 plantations. Five of these seven were plantations from which *V. procera* was not isolated from crop trees. Bark beetles carrying *V. procera* were collected from

TABLE 5.2. Insect survey in Christmas tree plantations: insects trapped and proportion carrying Verticicladiella procera.

Plantation	No. trees/20 with V.p.[a]	Total bark beetles	Proportion with V.p.	Total Weevils H.p.[b]		Total Weevils P.sp.[c]	Proportion with V.p.
EB	0	137	0	6		11	0.412
LC	0	3	0	3		2	0.200
OMR	0	0	0	1		0	0
RK	0	65	0	1		3	0
LS # 3	0	129	0	1	+5[d]		0.830
LS # 4	0	98	0.01	3	+5	6	0.357
DL	1	19	0		1		0
RS	0	29	0.33	8		3	0.909
BL	10[e]	76	0.021	16	+7	7	0.933
SC	6	58	0	19		1	0.800
		overall proportion:	0.00756			overall proportion:	0.642

[a] V.p. = Verticicladiella procera
[b] H.p. = Hylobius pales
[c] P.sp. = Pissodes spp.
[d] = no identification attempted
[e] = presence of V. procera determined in previous studies.

three plantations, only one of which was confirmed to have trees colonized by _V_. _procera_. Bark beetles reserved for identification were in the following genera: _Pityogenes_ Bedel, _Orthotomicus_ Ferrari, _Xyleborus_ Eichhoff, _Hylastes_ Eichhoff. Weevils collected and identified were _Hylobius pales_ and _Pissodes_ spp.

The proportion of weevils contaminated with _V_. _procera_ was 0.642 compared to 0.00756 for the proportion of bark beetles contaminated with _V_. _procera_. These are significantly different at the .01 level by a Z-test for two binomial populations.

Transmission studies. Seventeen sets of trap bolts (stage one of the transmission study) were incubated. Four of these sets had trapped insects free of _V_. _procera_ (set numbers 4, 11, 12 and 14, Table 5.3). Isolations from areas of insect activity (entrance holes, bark beetle and weevil galleries and feeding puncture holes) on these four bolts did not yield _V_. _procera_ except for one set from plantation RK. Thirteen of the 17 sets of trap bolts which were incubated trapped insects carrying _V_. _procera_. All but one of the 13 sets of trap bolts yielded _V_. _procera_ from wood and bark tissue isolations.

Many of the bolts were completely colonized by insect larvae and their galleries (Fig. 5.2). A large majority of the larvae observed were of the family Curculionidae, larvae of the Scolytidae family were infrequently observed.

TABLE 5.3. Recovery of *V. procera* from trapped insects and from the trap bolts.

Bolt set[d]	Plantation	Collection number	V.p. from insects	V.p. from bolt
1	LS#3	1	+ (w)[a]	+
2	LS#3	3	+ (w)	+
3	LS#4	1	+ (w)	+
4	DL	1	0	0
5	RS	1	+ (w & b)[b]	+[c]
6	RS	3	+ (w)	+
7	BL	1	+ (w & b)	+
8	BL	2	+ (w)	+
9	BL	3	+ (w)	+
10	BL	4	+ (w)	0
11	RK	1	0	0
12	RK	3	0	+
13	EB	1	+ (w)	+[c]
14	EB	3	0	0
15	SC	1	+ (w)	+
16	SC	2	+ (w)	+[c]
17	SC	3	+ (w)	+[c]

[a] (w) = *V. procera* isolated from weevils
[b] (b) = *V. procera* isolated from bark beetles
[c] = *V. procera* isolated from larvae and frass also
[d] = bolts certified free of *V. procera* initially as described in text chapter V materials and methods by platin tissue from basal most bolt of tree on a medium selective for *V. procera* (AMA).

Figure 5.2. White pine trap bolt colonized by weevil larvae following three weeks incubation at ca. 18-22 C.

Even in bolts without larvae, areas of insect activity were obvious due to the presence of frass, discoloured bark and sunken areas.

Transmission of _V. procera_ into white pine bolts by caged insects (stage two of the transmission study) occurred in four of 11 cages, three with weevils and one with bark beetles (Table 5.4). The proportion of caged bark beetles carrying _V. procera_ was estimated by the proportion of plated beetles (a subsample of beetles collected) from which _V. procera_ was recovered. Transmission of _V. procera_ to a bolt occurred in a cage with only seven bark beetles, a very small percentage of which were carrying _V. procera_ (Table 5.4).

Actual proportions of weevils contaminated with _V. procera_ was determined since the caged weevils were actually plated on AMA for 24 hours prior to caging. At least 74% of the weevils in each cage were contaminated with _V. procera_. Transmission of the fungus to the bolts occurred three of seven times (Table 5.4) when fresh bolts were exposed to infested insects in cages.

Signs of insect activity were not as common on the caged bolts as on the trap bolts. Entry of bark beetles to the bolts was evidenced by frass at small holes. Small puncture holes and patches of chewed bark were signs of weevil feeding.

Verticicladiella procera was not recovered from any

TABLE 5.4. Insects caged with split bolts and seedlings: recovery of *Verticicladiella procera* from bolts and seedlings.

Insect family	number in cage	estimated prop. of insects with V.p.	bolts[a] with V.p.	seedlings[a] with V.p.
bark beetles	17	0.09	0	0
bark beetles	104	0	0	0
bark beetles	7	0	+	0
bark beetles	58	0	0	0
weevils	8	0.75	+	0
weevils	11	0.74	+	0
weevils	4	1.00	0	0
weevils	4	0.80	0	0
weevils	4	1.00	+	0
weevils	5	0.92	0	0
weevils	5	0.92	0	0

[a] Isolations were made from a sample of the seedling and bolt population prior to placement in cages to ensure that *V. procera* was not initially present.

of the caged seedlings. No signs of insect activity were seen on any part of the seedlings.

DISCUSSION

The association of insects of the order Coleoptera with blue stain fungi has been well documented (Craighead, 1928; Dowding, 1984; Nelson, 1934; Rumbold, 1931; Wingfield, 1982). To contribute to the development of a strong argument for insect transmission of V. procera, insects from two families of the order Coleoptera were examined for their potential as vectors of V. procera: Scolytidae (bark beetles) and Curculionidae (weevils). Most of the evidence collected strongly supports weevils as the primary vectors for the following reasons. The majority of the weevils trapped carried the fungus on their bodies whereas very few bark beetles were contaminated with V. procera. Weevils inhabit the lower stem, root collar and roots of trees (Baker, 1972; Finnegan, 1959; Speers, 1958) especially during reproductive stages. The root collar and proximal portions of roots are generally the most densely colonized portion of the tree (Horner et al., 1985) hence ample opportunity exists for inoculum acquisition. Weevils are attracted to young trees for feeding (Baker, 1972; Speers, 1958) where inoculation may occur. Bark beetles however are generally secondary invaders (Knight and Heikkenen, 1980).

Weevils and bark beetles contaminated with V. procera

were trapped in plantations from which the fungus was not found in crop trees. The 20 tree sample may have missed trees colonized by V. procera. However, the preferential sampling of symptomatic trees and the lack of symptoms in several plantations suggests that V. procera was present at low levels, if at all, in these plantations. From the isolation data, it is concluded that where the disease occurs, there are fungus-bearing insects. However, there are also fungus-bearing insects where the disease is not detected. This association is supported by Lackner and Alexander (1984) and Wingfield (1983, 1982) who isolated V. procera from insect-infested trees. However, the absence of diseased trees in a plantation does not imply the absence of V. procera on insects from that plantation. There are two possible reasons to explain the presence of fungus-bearing insects in plantations without diseased trees. The 20 tree sample may have missed trees that were colonized by V. procera, or insects carrying the fungus were immigrants which had picked up the fungus elsewhere. If the latter possibility is true, there are important implications for the dispersal of V. procera by insects.

Transmission of the fungus into white pine bolts was established in this study. Under field conditions, previously uncolonized bolts became colonized following a week of exposure to Coleopteran insects contaminated with V. procera. A likely explanation for this is that insects

attracted to the freshly-split bolts inoculated the bolts with *V. procera* during feeding and oviposition. This hypothesis was tested by placing insects in a more controlled environment. Bolts in cages with contaminated insects also became colonized with *V. procera* although to a lesser extent than the trap bolts. The isolation of *V. procera* from areas of insect feeding and from larvae, frass and galleries further supports this hypothesis.

The lack of infection of caged seedlings may be explained by the probable absence of insect activitiy as suggested by an absence of signs of insect activity on the seedlings. The seedlings apparently were undesirable for either feeding or oviposition, possibly because of their small size.

Similar to Koch's postulates for proof of pathogenicity, there are rules of proof for insect transmission of plant pathogens (Leach, 1940). These are:

> "1. A close, although not necessarily a constant, association of the insect with diseased plants must be demonstrated.
>
> 2. It must be demonstrated that the insect also regularily visits healthy plants under conditions suitable for the transmission of the disease.
>
> 3. The presence of the pathogen or virus in or on the insect in nature or following visitation to a diseased plant must be demonstrated.
>
> 4. The disease must be produced experimentally by insect visitation under controlled conditions with adequate checks."

The first three of these four rules have been demonstrated. Insects (both Curculionidae and Scolytidae) have been associated with diseased trees (Lackner and Alexander, 1982; Wingfield, 1983, 1982). Weevils and bark beetles identified in this study are usually found in association with their host trees during all stages of their life cycle (Baker, 1972; Knight and Heikkenen, 1980). Therefore there are many chances for visits to healthy plants under conditions suitable for transmission which fulfills rule number two. This study (and studies by Lackner and Alexander, 1984 and Wingfield, 1983) satisfied rule number three, the occurrence of the fungus on insects in nature. The last rule has not been fulfilled. Transmission of the fungus from insect to wood has been shown to be possible. Further work is necessary to prove transmission to healthy seedlings and subsequent disease development.

Chapter VI

CONCLUSIONS

Soil-borne propagules appear to have a minor role in the spread of _V. procera_ in Christmas tree plantations. Infection of trees by soil-borne propagules is important only with those trees planted in the immediate area of a concentrated source of inoculum. It is suggested that spread of the pathogen both within and between plantations is primarily due to insect vectors.

Soil-borne propagules rapidly lose the ability to germinate, especially at temperatures greater than 10 C with low soil moisture. Therefore, the opportunity to cause infection may also decline rapidly. Nevertheless, a low percentage of propagules retain germinability for several months. This is important if low numbers of propagules can cause sufficient infection and colonization to result in vascular dysfunction. However, the lack of infection of seedlings planted in soil artificially infested with several propagule concentrations is not in support of infection by low numbers of propagules. Lackner and Alexander (1984) observed infection of seedlings planted in naturally infested soil at the site of excavated trees. Under greenhouse conditions, infection of seedlings in artificially infested soil was rare and only occurred with high concentrations of propagules (30,000 spores/gram

soil). When infection did occur, symptom development was negligible and colonization was limited, suggesting that the number of infections present did not result in colonization sufficient to induce vascular dysfunction. Successful infection and symptom development in seedlings in the field may be due to factors not operative in the greenhouse. Colonized root wood remaining in the soil after tree excavations (Lackner and Alexander, 1984) may have supplied concentrations of inoculum sufficient for infection through root wounds. The process of infection may have been mediated by the activities of insects which inhabit the roots and root collar of trees.

Verticicladiella procera was not recovered from soil sampled over a large area in the vicinity of, but not directly adjacent to, diseased trees. Propagules in soil were closely associated with colonized roots and root collars of diseased trees. Distribution of soil-borne propagules reflects the pattern of colonization in the root system, therefore movement of propagules through the soil is limited.

Seedlings planted around colonized trees in soil from which *V. procera* was recovered did not become colonized. However, seedlings planted in soil from which *V. procera* was not detected did become colonized. Colonization did occur in seedlings closest to colonized root collars (10 and 25 cm from the root collar).

This information suggests that an alternative means of infection exists, such as transmission of the fungus by insects. Insect feeding was observed on several seedlings planted around colonized trees, one of which yielded *V. procera* upon isolation. Furthermore, *V. procera* was observed fruiting in insect galleries in roots of several centre trees.

There are strong indications of an intimate association between *V. procera* and insects. *Verticicladiella procera* was consistently isolated from species of weevils (Curculionidae) which are frequent pests of Christmas trees. Bark beetles (Scolytidae) were found to carry the fungus less frequently. The collection of contaminated insects from plantations not showing symptoms of PRD (assuming sufficient time for symptom development) and from which *V. procera* was not recovered from sample trees suggests that insects migrating from one area to another may provide the source of inoculum for new infections.

Following visitation by insects, transmission of the fungus to uncolonized white pine bolts frequently occurred in the field and under controlled conditions. Larval galleries and frass were common substrata for the fungus in both a vegetative and a reproductive state. The ability of weevils and bark beetles to carry the fungus on their bodies and to transmit it to bolts of wood was proven. Three out of four of Leach's rules of proof for

insect transmission of a pathogen have been demonstrated.

The information gathered during these studies contributes towards a description of the PRD cycle in Christmas tree plantations. Colonized stumps and trees may provide a concentrated source of inoculum. Insects inhabiting colonized stumps and trees create galleries which are protected microenvironments that promote fruiting of *V. procera*. Inoculum may be acquired by the insects during egg-laying, hatching, pupation and maturation activities.

When the insects emerge and search for new hosts for maturation feeding and/or breeding activities, the inoculum is carried along on their bodies. Feeding and oviposition creates small wounds which may serve as infection courts.

An important component of the disease cycle may be the apparent requirement for many points of infection in the root system. Single point inoculations with *V. procera* have resulted in local lesion formation but no symptom development (Bertagnole *et al.*, 1983; Harrington and Cobb, 1983; Houston, 1969; Wingfield, 1983). Symptom development was observed in seedlings inoculated by root-dip into conidial suspensions (Lackner and Alexander, 1982) which allows multiple inoculations. Numerous infection points may cause colonization sufficient for disruption of the vascular system.

It is suggested that infection through the roots by soil-borne propagules occurs only when new hosts are located adjacent to a concentrated source of inoculum, such as a colonized root. The activities of root-inhabiting insects may enhance the incidence of infection by creating root wounds and dispersing inoculum.

The remainder of the proposed disease cycle consists of continued colonization in the phloem and the xylem, followed by symptom development in colonized trees due to blockage of the tracheids by excessive amounts of resin. Mortality occurs in one to two years. Insects are attracted to the stressed or dead trees for breeding purposes which starts the cycle over again.

Further study of the insect-fungus association is required to: 1) prove transmission of the fungus and subsequent symptom development in trees; 2) determine the efficiency of insect transmission of the fungus; 3) determine the number of infections required for symptom development; 4) describe inoculum potential with respect to insect transmission 5) examine the relationships between the life cycles of the insect, the fungus and the host; 6) propose avoidance and control measures for Procera Root Disease.

In Christmas tree plantations, stumps may serve as inoculum resevoirs. Initial colonization may occur in a crop tree which is either sold before symptoms develop,

or culled. Or initial colonization may occur in the stump after the tree is cut. The stump attracts insects for breeding purposes at which time inoculum may be acquired.

Control measures for PRD have been tentatively proposed. These are primarily concerned with reducing the amount of inoculum available for infection through roots by soil-borne propagules and for insect transmission. In areas where PRD is a concern, sites should be left fallow for one year, which will decrease the amount of inoculum in the soil. Seedlings should not be planted adjacent to colonized stumps, and a non-susceptible species should be considered. In severe cases, stump removal is recommended to decrease both the available inoculum and the vector populations. Any control measures aimed at reducing the population of the vector, may also limit spread of *V. procera*.

LITERATURE CITED

Alexander, S.A. 1984. Evaluate the association between root-disturbing cultural practices and root pathogens, and related losses in forest tree seed orchards. Final Report. USDA Forest Service. Forest Pest Management, Region 8. 44pp.

Alexander, S.A. 1980. White pine root decline (*Verticicladiella* wilt). Virginia Polytechnic Inst. and State Univ. Pest Management Guide 503. 4pp.

Anderson, Robert L. 1980. Method for recovering *Verticicladiella procera* from basal cankers on eastern white pine. Plant Disease 64:680.

Anderson, Robert L. and S.A. Alexander. 1979. How to identify and control white pine root decline. USFS Southeastern area, state and private forestry. Forestry Bulletin SA-FR/P6 July, 1979. 4pp.

Baker, W.L. 1972. Eastern Forest Insects. USDA For. Serv. Misc. Pub. No. 1175. 642pp.

Barnard, E.L., R.L. Anderson, J.T. English and G.M. Blakeslee. 1982. Sand pine root disease survey. Florida - 1980. U.S. Forest Service Forest Pest Management, Asheville field office, Report #82-1-30.

Bertagnole, Catherine L. 1981. Pathogenicity and pleomorphism among five *Verticicladiella* species in lodgepole pine and laboratory cultures. Masters Thesis, Univ. of Idaho.

Bertagnole, Catherine L., John Y. Woo and Art D. Partridge. 1983. Pathogenicity of five *Verticicladiella* species to lodgepole pine. Can J. Bot. 61:1861-1867.

Clark, F.E. 1965. Agar-plate method for total microbial count. Pages 1460-1466 in: C.A. Blake, ed. Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties. American Society of Agronomy, Inc. Madison WI. 1572 pp.

Cobb, Jr., Fields W. and W.D. Platt. 1967. Pathogenicity of *Verticicladella wageneri* to Douglas-fir. Phytopathology 57:998-999.

Coley-Smith, J.R. 1979. Survival in the absence of host

plants. In: Soil-borne Plant Pathogens. B. Schippers and W. Gams (eds). Academic Press. p.39-57.

Craighead, R.C. 1928. Interrelation of tree-killing barkbeetles (Dendroctonus) and blue stains. J. of Forestry 26:886-887.

Dochinger, L.S. 1967. (abstr.). Leptographium root decline of eastern white pine. Phytopathology 57:809.

Dowding, P. 1984. The evolution of insect-fungus relationships in the primary invasion of forest timber. In: Invertebrate- Microbial Interactions. J.M. Anderson, A.D.M. Rayner and D.W.H. Walton (eds). Brit. Mycol. Soc. Symposium 6. Cambridge University Press. 349 pp.

Dowding, P. 1969. The dispersal and survival of spores causing bluestain in pine. Trans. Br. Mycol. Soc. 52:125-137.

Draper, N. and H. Smith. 1981. Applied Regression Analysis 2nd Ed. John Wiley & Sons. 709 pp.

Finnegan, R.J. 1959. The pales weevil, Hylobius pales (Hbst.) in Southern Ontario. Can. Entomol. 61: 664-670.

Garret, S.D. 1970. Pathogenic Root-Infecting Fungi. Cambridge University Press. 294 pp.

Gill, Lake S. and Stuart R. Andrews. 1949. Note on a Scopularia attacking western white pine. Plant disease Reptr. 33:227.

Goheen, Donald J. and Fields W. Cobb Jr. 1978. Occurrence of Verticicladiella wageneri and its perfect state, Ceratocystis wageneri sp. nov. in insect galleries. Phytopathology 68:1192- 1195.

Halambek, Marija. 1981. Verticicladiella procera Kendrick causal organism of eastern white pine wilting in conifer cultures. Zastita bilja 32:313-323.

Halambek, Marija. 1976. Dieback of eastern white pine (Pinus strobus L.) in cultures. Agriculturae Conspectus Scientificas 39:495-498.

Hallaksela, A.M. 1977. Microbial flora isolated from Norway Spruce stumps. Acta Forest. Fenn. 158:1-42.

Harrington, T.C. and F.W. Cobb Jr. 1983. Pathogenicity of Leptographium and Verticicladiella spp. isolated from roots of western North American conifers. Phyto-

pathology 73:596- 599.

Harrington, T.C., F.W. Cobb, Jr., and J.W. Lownsbery. 1985. Activity of Hylastes nigrinus, a vector of Vertcicladiella wageneri, in thinned stands of Douglas-fir. Can. J. For. Res. 15:519-523.

Horner, W. Elliott, and S.A. Alexander. 1985a. Histopathology of Verticicladiella procera in Scots and eastern white pine (Abst.). Phytopathology, in press.

Horner, W. Elliott, and S.A. Alexander. 1985b. Permeability of asymptomatic, resin-soaked and Verticicladiella procera- black-stained pine sapwood (Abst.). Phytopathology, in press.

Horner, W. Elliott, K.J. Lewis and S.A. Alexander. 1985. Colonization patterns of Verticicladiella procera in Scots and eastern white pine (abstr.). Phytopathology 75:625.

Horner, W. Elliott, and S.A. Alexander. 1983. Verticicladiella procera on Pinus sylvestris Christmas trees (Abst.). Phytopathology 73:966.

Horner, W. Elliott, S.A. Alexander and M.M. Julian. Qualitative determination of cellulose in the cell walls of Verticicladiella procera. Mycologia, in press.

Houston, David R. 1969. Basal canker of white pine. Forest Science 15:66-83.

Hubert, Ernest E. 1953. Studies of Leptographium isolated from western white pine. Phytopathology 43:637-641.

Hughes, S.J. 1953. Conidiophores, conidia and classification. Can. J. Bot. 31:577-659.

Hunt, Richard S. and D.J. Morrison. 1979. Black stain root disease. Canadian Forestry Service pest leaflet #67. 4pp.

Jackman, Raymond E. and Richard Hunt. 1975. Black stain root disease in Douglas-fir on Jackson State Forest. Cal. Div. of Forestry. State Forest Note #58. 4pp.

James, R.L. and D.J. Goheen. 1981. Conifer mortality associated with root disease and insects in Colorado. Plant Disease 55:506-507.

Kendrick, W. Bryce. 1962. The Leptographium complex.

Verticicladiella Hughes. Can. J. Bot. 40:771-797.

Knight, F.B. and H.J. Heikkenen. 1980. Principles of Forest Entomology. McGraw-Hill Book Co. 461 pp.

Lackner, A.L. and S.A. Alexander. 1984. Incidence and development of Verticicladiella procera in Virginia Christmas tree plantations. Plant Disease 68:210-212.

Lackner, A.L. and S.A. Alexander. 1983. Root disease and insect infestations on air-pollution sensitive Pinus strobus and studies of pathogenicity of Verticicladiella procera. Plant Disease 67:679-681.

Lackner, A.L. and S.A. Alexander. 1982. Occurrence and pathogenicity of Verticicladiella procera in Christmas tree plantations in Virginia. Plant Disease 66:211-212.

Landis, Thomas D. and Lawrence B. Helburg. 1976. Black stain root disease of pinyon pine in Colorado. Plant Disease Reptr. 60:713- 717.

Leaphart, Charles D. 1960. A root stain disease of eastern white pine. Plant Disease Reptr. 44:704-706.

Leaphart, Charles D. and Lake S. Gill. 1959. Effects of inoculations with Leptographium spp. on western white pine. Phytopathol. 49:350-353.

Livingston, W.H. and M.J. Wingfield. 1982. First report of Verticicladiella procera on pines in Minnesota. Plant Disease 66:260-261.

McCall, K.A. and W. Merrill. 1980. Selective medium for Verticicladiella procera. Plant Disease 64:277-278.

Nelson, R.M. 1934. Effect of bluestain fungi on southern pines attacked by bark beetles. Phytopath. Z. 7(4): 327-353.

Rumbold, C.T. 1931. Two blue-staining fungi associated with bark beetle infestation of pines. J. of Agric. Res. 43:847-973.

Shaw, C.G.III and M. Dick. 1980. Verticicladiella root disease of Pinus strobus in New Zealand. Plant Disease 64:86-98.

Sinclair, W.A. and G.W. Hudler. 1980. Tree and shrub pathogens new or noteworthy in New York State. Plant Disease 64:590-592.

Speers, C.F. 1958. Pales weevil rapidly becoming serious pest of pine reproduction in the south. J. Forestry 56: 723-726.

Swai, I.S. and D.F. Hindal. 1981. Selective medium for recovering Verticicladiella procera from soils and symptomatic white pines. Plant Disease 65:963-965.

Taylor, John W. and Rudolph T. Franklin. 1970. Biology of Hylobius pales (Coleoptera:Curculionidae) in the Georgia Piedmont. Can. Entomol. 102: 729-735.

Towers, Barry. 1977. The occurrence of Verticicladiella procera in Pennsylvania: 1976. Plant Disease Reptr. 61:477.

Towers, Barry. 1978. Summarization: work of Leon Dochinger. Pathology section, Div. of Forest Pest Management. Bureau of Forestry. Middletown, Pennsylvania.

Webb, Roger S. and S.A. Alexander. 1982. Incidence of resin-soaked roots in subsoiled loblolly pine seed orchards on sandy soils. Southern J. of Applied Forestry 6:104-107.

Wingfield, M.J. 1983. Association of Verticicladiella procera and Leptographium terrebrantis with insects in the Lake States. Can. J. For. Res. 13:1238-1245.

Wingfield, M.J. 1982 (abstr.) Verticicladiella procera associated with root weevil damage. Phytopathology 72:1141.

Wingfield, M.J. and W.F.O. Marasas. 1983. Some Verticicladiella species, including V. truncata sp. nov., associated with root disease of fpine in New Zealand and South Africa. Trans. Brit. Mycol. Soc. 80:231-236.

Witcosky, J. J. and E. M. Hansen. 1985. Root-colonizing insects recovered from Douglas-fir in various stages of decline due to Blackstain Root Disease. Phytopathology 75:399-402.

APPENDIX

SOIL PROPERTIES[1]

1) <u>Aquic hapludult, fine - loamy, mixed, mesic</u>
Cotaco Series

Ap horizon: dark grayish-brown, loam.
Bt horizon: yellowish-brown, strongly acid, sandy, clay loam. Friable to firm.

- solum thickness 70 - 100 cm.
- moderately well drained with medium runoff and moderate permeability.
- see following page for moisture retention curve.

2) <u>Typic paleudult, clayey, mixed, mesic.</u>
Frederick Series

Ap horizon: brown, silt loam, moderate-fine granular structure, friable.
B1 horizon: yellowish-red, silty clay, moderate to medium subangular blocky, firm.
B22, 23 and 24t horizon: yellowish-red, clay, mod to medium subangular blocky, firm, very strong acid reaction.

- solum thickness 150 - 250 cm.
- well drained, moderate permeability, runoff medium.
- see following page for moisture retention curve.

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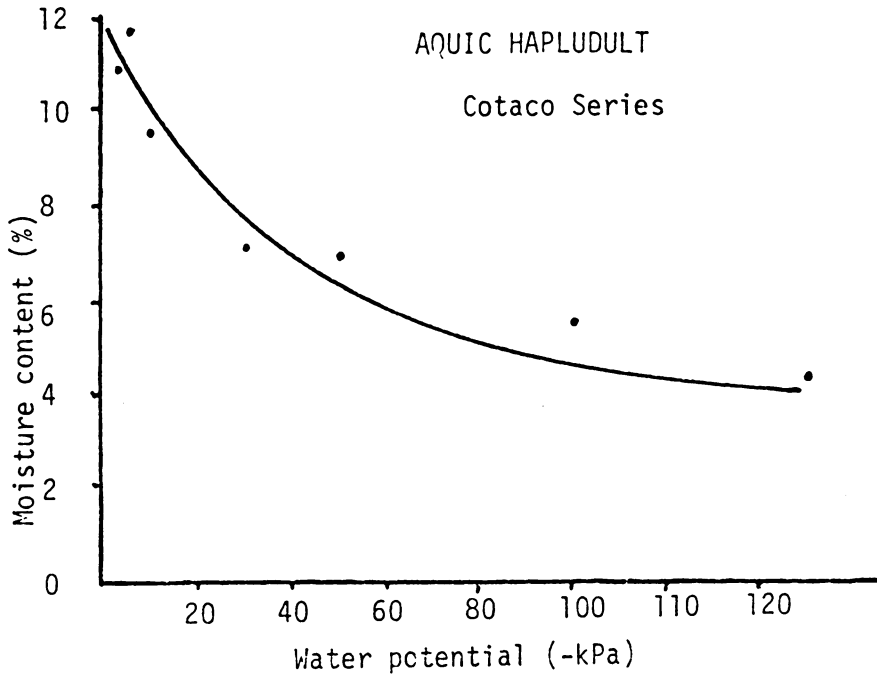
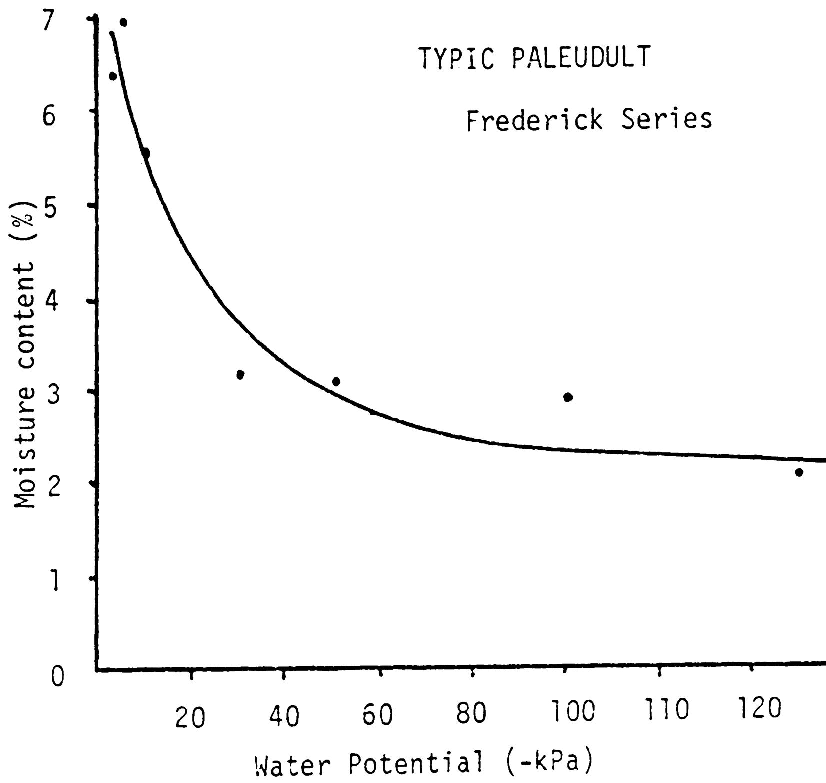
[1] Soil Survey field sheets of Montgomery County. VPI & SU. Agronomy department. January 1973. Supplemental to "Soils of Montgomery Count, VA". VPI & SU Extension report # 6.


AQUIC HAPLUDULT
Cotaco Series
Moisture content (%)
Water potential (-kPa)
0
2
4
6
8
10
12
20
40
60
80
100
110
120


TYPIC PALEUDULT
Frederick Series
Moisture content (%)
Water Potential (-kPa)
0
1
2
3
4
5
6
7
20
40
60
80
100
110
120

MAPS OF SEEDLINGS PLANTED AROUND INDIVIDUAL TREES

The following pages are maps of the tree root systems and the location of the seedlings planted around them. Also shown are the results of isolations from the centre trees, the seedlings and the soil.

LEGEND

○ tissue sample, no _V_. _procera_

● tissue sample, _V_. _procera_ recovered

X planted seedling, no _V_. _procera_

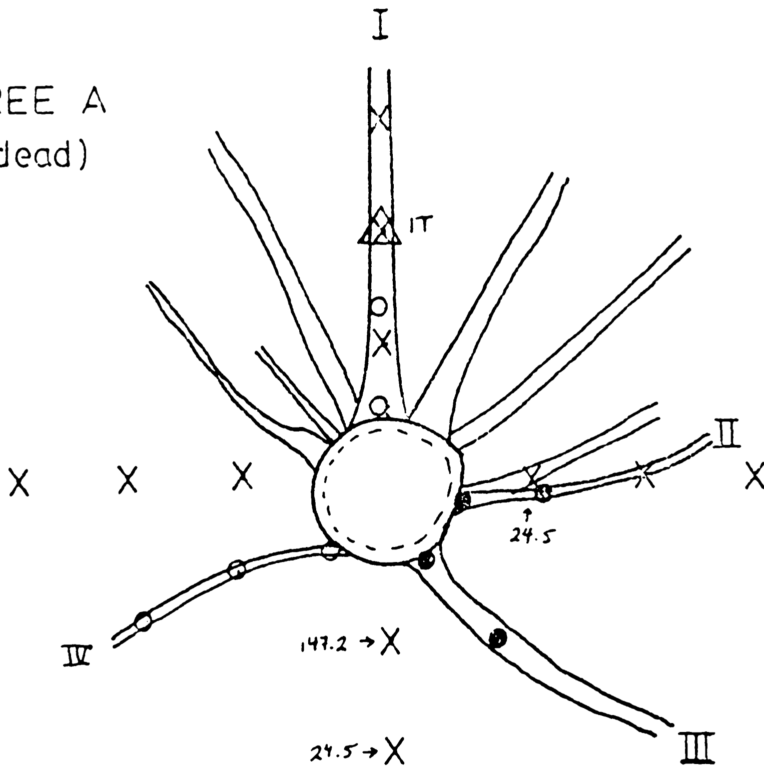
△ planted seedling, _V_. _procera_ recovered

5L number = position of recovery: 1=distal, 2=middle, 3=proximal, 4=1&2, 5=1&3, 6=2&3, 7=1,2&3.
L=lateral root, T= tap root

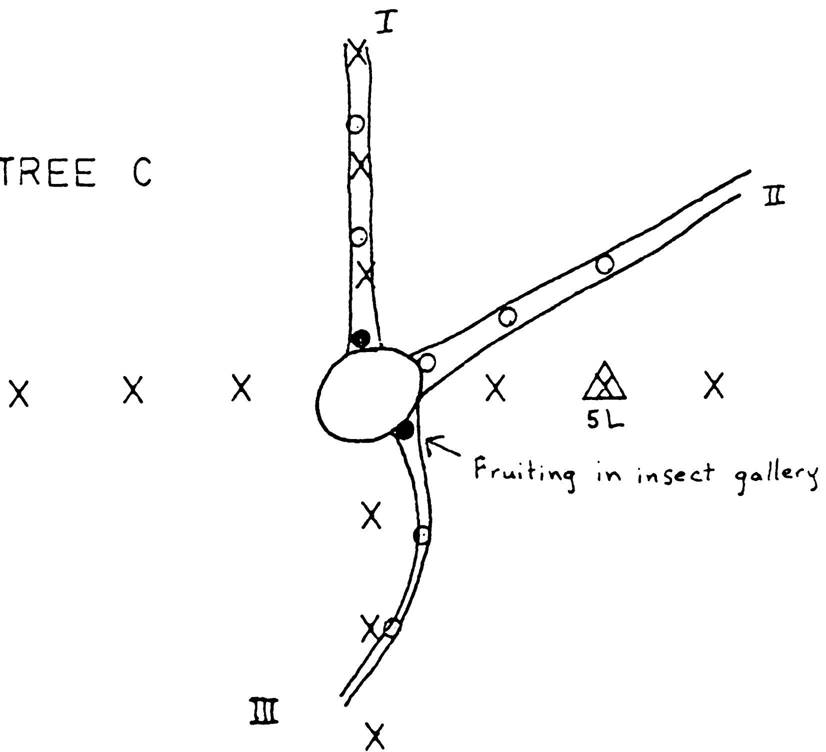
24 propagules/gram soil recovered from sample at each planted seedling

) _V_. _procera_ recovered from root collar of centre tree

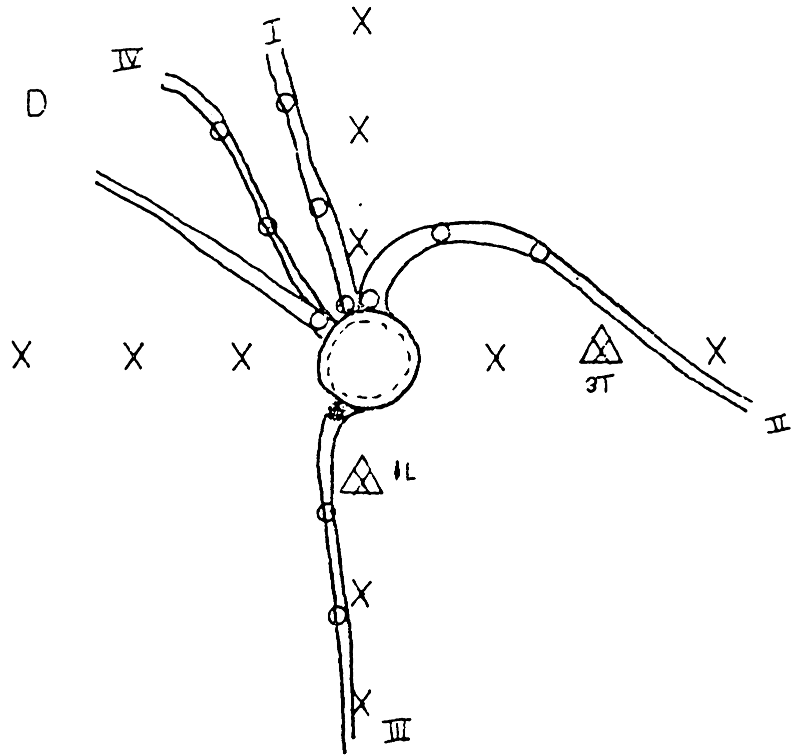
) _V_. _procera_ not recovered from root collar

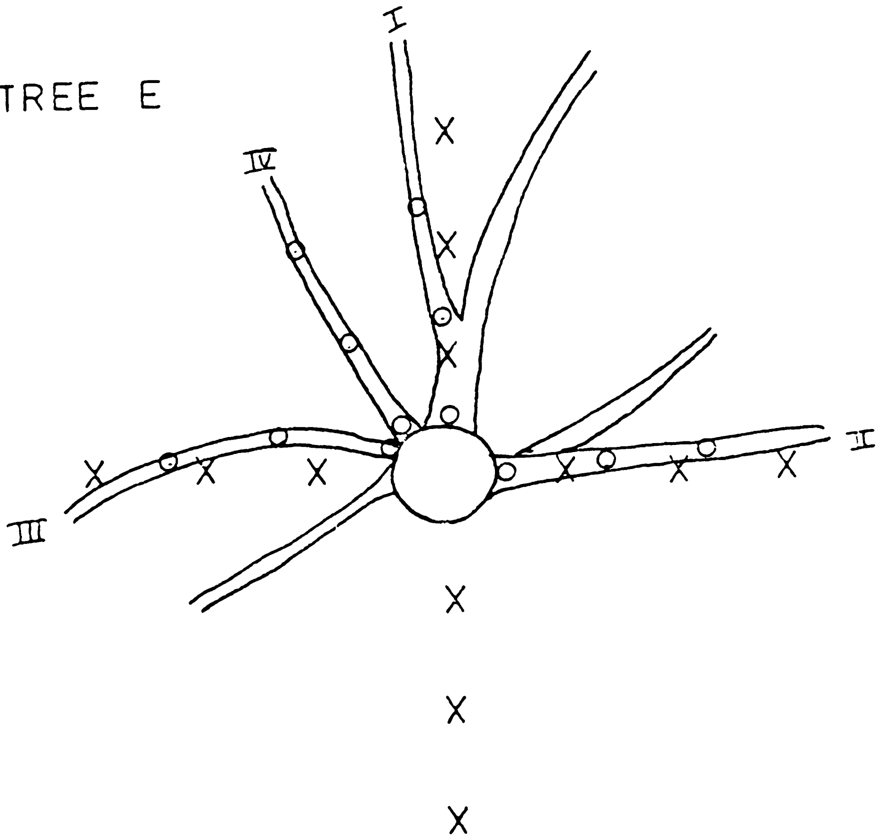


I
TREE A
(dead)
1T
II
24.5
IV
147.2 →
24.5 →
III

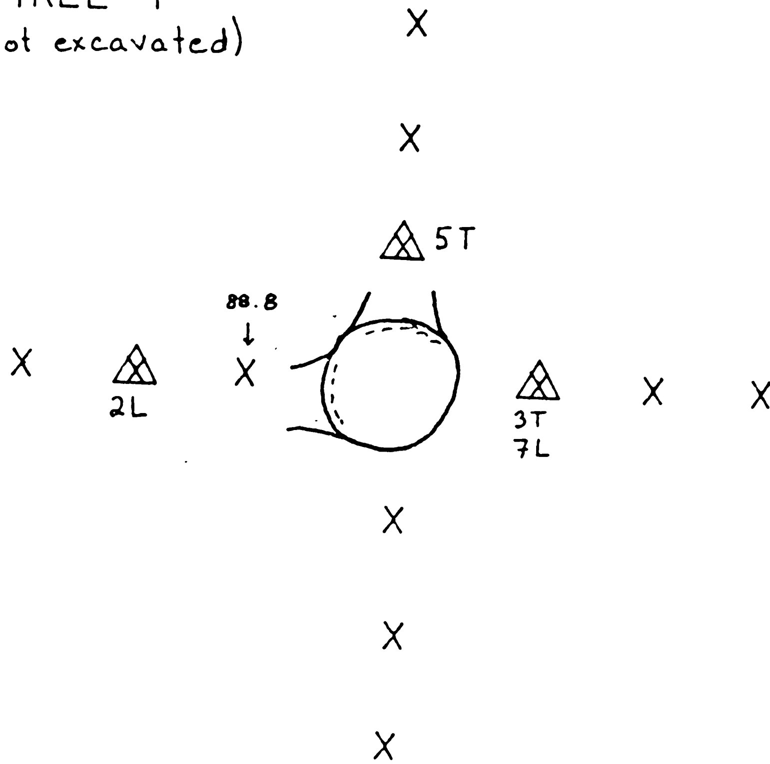


I
TREE C
II
5L
Fruiting in insect gallery
III


TREE D
I
IV
II
III
3T
1L


TREE E
I
IV
II
III

TREE F
(Not excavated)



TREE G - asymptomatic

No *V. procera* from
- seedling
- soil samples
- cork borer tissue samples

The vita has been removed from the scanned document