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The root disease fungus Inonotus tomentosus, common in the old growth boreal forests of British Columbia, poses a threat to the health of second growth forests established on sites with a previous history of root disease. Colonized stumps occur in groups of 1 to 6; the groups are clumped within a clearcut. Therefore, surveys for disease incidence need to employ wide (10 m) transects and cover at least 10% of the stand area.

The fungus remains viable in stumps for at least 30 years and can infect roots of regeneration trees in contact with stump roots. Spruce stump roots cause twice as many infections as pine stump roots because more spruce roots are colonized, they are longer, and they are horizontally oriented. Trees growing within 200 cm of diseased spruce stumps have a 25% chance of infection. At 350 cm - 400 cm the chance of infection is 10%. Trees growing within 50 cm and 250 cm - 300 cm of diseased pine stumps have a 25% and 10% chance of infection respectively.

Within a stand, disease centres are usually small (less than 5 trees) and composed of a single genotype. Larger centres consist of

several genotypes. Electrophoretic protein profiles and vegetative compatibility tests were co-supportive methods used to determine genotype similarities. The clumped distribution of disease centres and the frequency of unique genotypes suggest that spores may have an important role in disease spread.

Infection of roots by I. tomentosus occurs through direct penetration of the bark of small roots, or by infection of a feeder root. In roots approximately 5 cm diameter or less, the fungus grew in or on the bark and often preceded decay in the wood. In larger roots fungal growth is in the heartwood; radial growth to the bark is limited until root death.

Inoculations of mature spruce and pine trees suggest that spruce is more readily infected than pine, due in part to greater resin production and phenol accumulation by challenged pine roots. Infection of root wood by I. tomentosus caused host cell death at the hyphal front, increased phenolic deposition, peroxidase activity, and starch depletion beyond the hyphal front.

Inonotus circinatus (a pathogen of pine in eastern North America) and I. tomentosus show consistent differences at the protein level as indicated by protein electrophoresis and southern hybridization of a homologous probe (random genomic clone) to total genomic DNA. Protein patterns of I. tomentosus isolates are more variable than patterns of I. circinatus which may be due to greater diversity of I. tomentosus hosts and climate.

Epidemiology and Population Biology of *Inonotus tomentosus* as a Pathogen of Forests of British Columbia

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Chairman of the Department of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

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To my parents, Alan and Carolyn Lewis,

and to "Wimp Tours"

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter VI

LIST OF TABLES

EPIDEMIOLOGY AND POPULATION BIOLOGY OF *INONOTUS TOMENTOSUS* AS A PATHOGEN OF YOUNG FORESTS IN BRITISH COLUMBIA

INTRODUCTION

Fungi causing root diseases operate below ground and in the interior of trees; therefore, their biology is often poorly understood and diagnosis, detection surveys and loss measurements are much more difficult than with agents whose effects are expressed above ground (Garrett, 1956). The study of root diseases in the past progressed relatively slowly. At the time when old growth forests dominated the timber supply, root disease studies were focused on the old growth forests. Now, the emphasis is changing and root diseases are recognized as threats to plantations and young forests as well.

Inonotus tomentosus (Fr.)Teng is a basidiomycete which causes a root disease of conifers in the boreal forest (Whitney, 1962). Most reports of the disease are from old growth spruce and pine forests (Merler et al., 1988; VanGroenewoud, 1955; Whitney, 1973). The vastness of the old growth, the general acceptance of rots and decays in virgin timber and the relative infrequency of *I. tomentosus* in the more productive and better studied coastal Douglas-fir (*Pseudotsuga menziesii* (Mirb.)Franco forests, partly explain why tomentosus root disease has not been intensively studied.

In north central British Columbia, timber supply has been based on old growth spruce (*Picea glauca* (Moench)Voss and *P. glauca* x *engelmannii* Engelm.) and pine (*Pinus contorta* Dougl. var. *latifolia*

Engelm.) forests. Reduction in the availability of old growth timber due to cutting and alternative uses has initiated a major change in emphasis from old growth management to second growth management. The goal of second growth management is to efficiently produce healthy, productive stands of trees.

The possibility of damage to the second growth stands by fungi such as I. tomentosus has generated many questions regarding damage potential and disease management. However there have been no previous studies which addressed the survival of I. tomentosus in stumps and subsequent spread to young trees. This thesis addresses the likelihood and means of infection and damage to second growth stands established in areas with a root disease history. This information is important for the design of disease management strategies. The problem was approached in a series of studies and the results are presented in separate chapters. The first study (Chapter II) documents the spread of I. tomentosus from old infected stumps to young trees. The distribution of diseased stumps in clearcuts, the development of decay in stumps and the likelihood of root contacts and subsequent infection between old growth stumps (spruce or pine) and regeneration trees were measured.

Chapter III addressed the question of the role of basidiospores in disease spread. A population genetics approach was used, examining the distribution of unique fungal genotypes within stands. Pairing reactions and protein electrophoresis were used to define the genotypes.

Vegetative spread of I. tomentosus was descriptively examined in Chapter IV. Roots of trees at the edge of disease centres where the

fungus was advancing were excavated and examined for potential infection courts and to describe the progression of the disease within the roots.

Artificial inoculation experiments are reported in chapter V. Differences between spruce and pine in reaction to inoculation with I. tomentosus were observed. The reaction of roots to wounding and inoculation was measured by the length of resin soaking response, the length of colonization by the fungus and by staining thin sections for phenols, starch and several enzymes. The effect of root size and wounding on the likelihood of infection was determined and the virulence of I. tomentosus and I. circinatus was compared. The latter is a fungus closely related to I. tomentosus that causes significant damage to pine in the southeastern United States.

The final study (Chapter VI) was undertaken to determine the relatedness of I. tomentosus and I. circinatus by comparison of electrophoresed protein profiles and restriction fragment length polymorphisms. The last chapter summarizes the conclusions from all of the studies and discusses their implications for control of tomentosus root disease.

Chapter I. Literature review

I. THE PATHOGEN

Taxonomy

The taxonomic history of Inonotus tomentosus is varied and confusing due in part to the lack of type specimens and the fact that the first description and classifications by Fries are based on the nature of the context and pore layer depth, both of which are highly variable (Haddow, 1941). Much of the confusion is a result of Fries' original description of Polyporus tomentosus (1821) and P. circinatus (1848), with the former having an homogenous context and the latter a duplex context, whereas they both have a duplex context according to Gosselin (1944). In 1869, Peck described another species, Polyporus dualis from New York which had a duplex context.

In 1882, Karsten transferred P. tomentosus to the genus Polystictus (cited in Haddow, 1941 and Gosselin, 1944), which was supported by Cooke in 1886 who declared Polystictus dualis and P. circinatus to be synonymous, and by Saccardo (1888) who described Polystictus tomentosus and P. circinatus, but adhered to Fries' distinction of the species based on the nature of the context.

Karsten in 1889 erected the genus Onnia which emphasized the hymenial setae and distinguished Onnia tomentosa from O. circinata by the context. In the same year, upon examining some species of Polyporus, Ellis and Everhart (1889) discovered several species with reddish brown spines along the pore inner surface. They erected the new genus Mucronoporus to distinguish the spined species from Polystictus. They described M. circinatus with curved spines and

equated M. dualis with M. circinatus. At first M. tomentosus specimens from Prince Edward Island were thought to be a new species, but the authors examined Karsten's specimens of Polystictus tomentosa and determined they were one and the same. Mucronoporus tomentosus is described as having lanceolate (no curve) spines.

In 1900 Patouillard erected the genus Xanthochrous to include Trametes Fr., Pelloporus Quelet, Polystictus (Fr.), Onnia Karst. and Mucronoporus Ellis. Both Xanthochrous circinatus and X. tomentosus are described as having "cystidia", one figure shows X. circinatus with straight "cystidia".

In Murrill's (1904) extensive review of this group of fungi, he placed P. tomentosus into the genus Coltricia which was erected in 1821 (by S.F. Gray, in Murrill, 1904) based on species later described as Polystictus and Polyporus. Examination of specimens of Mucronoporus tomentosus and of Onnia tomentosa resulted in their incorporation into Coltricia. Murrill examined some of Fries' specimens of Polyporus tomentosus and found them to be the same as Coltricia tomentosus. Murrill makes no distinction between P. tomentosus and P. circinatus. Lloyd (1908) described Polyporus circinatus with straight setae, then in 1912 was the first to state that P. tomentosus was a European species not found in North America. Overholts (1933), Shope (1931) and Lowe (1934) also recognized only P. circinatus in North America, which was described in all three references as having straight setae.

Sartory and Maire (1922) were the first to place P. circinatus as a variety of P. tomentosus which was later supported by Jorstad and Juul (1938) working on Norwegian specimens and by Haddow (1941)

working on specimens labelled by Fries. Haddow separated the varieties based on setal morphology but did not believe this characteristic to be substantial enough to denote separate species.

The genus Inonotus was erected by Karsten in 1879 to include polypores with "Fruiting body dimidiate-sessile, at first sponge soft, becoming firm and elastic, anoderm, setos-hispid with colour. The heterogenous hymenium and spores have colour." (Pegler, 1964). The genus Inonotus as described by Gilbertson (1976) comprised lignicolous Hymenomycetes in the order Aphyllophorales with annual basidiocarps, united pores, and brown context that gives a darkened xanthocroic reaction with KOH. Hyphae are septate but unclamped, and the spores are pigmented or hyaline. Setae in the hymenium or setal hyphae are sometimes present. Inonotus species give a positive oxidase reaction and cause a white rot. Gilbertson placed both P. tomentosus and P. circinatus as separate species in the genus Inonotus in 1974. However, in his 1986 "North American Polypores", Gilbertson recognizes Teng (1964) for his earlier transfer of Polyporus tomentosus to Inonotus tomentosus.

According to Gilbertson (1986) I. tomentosus and I. circinatus differ in the following morphological characteristics:

I. tomentosus	I. circinatus
-central or lateral stipe	-usually lateral stipe
-yellowish brown pileus	-buff to reddish brown
-tube layer 3 mm thick	-tube layer 10 mm thick
-context up to 4 mm thick	-context up to 1 cm thick
-setae abundant, subulate, straight	-setae scattered, common, hooked

-basidia 13-15x5-6 um	-basidia 18-27x6-7 um
-particularily in spruce	-particularily in pine

The gross morphological features of these two species are quite similar which suggests that mis-diagnoses are possible in reports of the occurrence and pathogenicity of these species. There are several conflicting reports of pathogenic differences between the two species (Whitney, 1977, 1976; Hubert, 1931).

Host and Geographic Ranges

Inonotus tomentosus and I. circinatus are found throughout much of North America. Most reports of I. tomentosus are from the northern Boreal forests of Canada, Wisconsin and the Pacific Northwest (Basham and Morowski, 1964; Davidson and Redmond, 1957; Hobbs and Partridge, 1979; Patton and Myren, 1968; Whitney, 1962). Inonotus circinatus has been found in the southeastern United States (Boyce, 1963; Ross, 1966) and eastern Canada (Whitney, 1962). Outside of North America, these fungi have been reported from the following countries:

India	I. circinatus	(Bakshi, 1976)
USSR	I. tomentosus, I. circinatus	(Bondartsev, 1953)
Sweden	I. tomentosus, I. circinatus?	(Fries, 1821, 1863; Murrill, 1904)
Japan	I. circinatus	(Shirai, 1927)
China	I. tomentosus	(Teng, 1932)
Germany	I. tomentosus	(Murrill, 1904)
Austria	I. tomentosus	(Lloyd, 1920)

Gossellin (1944) gave a detailed review of Polystictus circinatus and tomentosus and cited a possible report of P. tomentosus in Africa (Lloyd, 1920) but cautioned that the diagnosis may be incorrect. No other occurrences in the southern hemisphere have been documented, although Gosselin believed that I. tomentosus or I. circinatus may be found at higher elevation in lower latitudes due to temperature limitations.

There are many coniferous tree species susceptible to tomentosus root disease. No reports of infection of hardwoods are known except for successful artificial inoculations of white birch (Betula papyrifera Marsh.) and trembling aspen (Populus tremulloides Michx.) by Whitney (1964).

In Canada, most native pine species and all native spruce species are attacked (Whitney, 1977). Table I.1 lists the species reported to be hosts for I. tomentosus.

Measurements of susceptibility have been made by inoculation tests on a limited number of species. Pathogenicity was greatest on white spruce, intermediate on blue spruce and lowest on Norway spruce in an inoculation experiment on germlings in nutrient agar (Whitney and Bohaychuk, 1977). In a second experiment Whitney and Bohaychuck (1976) inoculated germlings of 11 tree species and by infection ratings, determined that ponderosa pine was most susceptible, followed by lodgepole pine, white spruce, black spruce and tamarack. However, in 1964 Whitney stated that in general spruce is more susceptible to tomentosus root disease than is pine, and tamarack (Larix laricina (DuRoi)K.Koch) is very susceptible. This latter experiment was on trees, not germlings which may explain the

Table I.1. Hosts of *Inonotus tomentosus*.

Host			Reference
Canadian			
Picea engelmanni Parry	engelmann spruce		1, 2
P. sitchensis (Bong.)Carr.	sitka spruce		1, 2
P. glauca (Moench)Voss	white spruce		1, 2
P. abies Karst.	Norway spruce		1, 2
P. engelmanni x glauca	interior spruce		1, 2
P. mariana (Mill.)B.S.P.	black spruce		1, 2
P. rubens Sarg.	red spruce		1, 2
Pinus contorta Dougl.	lodgepole pine		1, 2
P. banksiana Lamb.	jack pine		1, 2
P. monticola Dougl.	western white pine		1, 2
P. strobus L.	eastern white pine		1, 2
P. sylvestris L.	Scots pine		3, 2
P. ponderosa Laws.	ponderosa pine		2
P. resinosa Ait.	red pine		2
Pseudotsuga menzeisii (Mirb.)Franco	Douglas-fir		2
Tsuga canadensis (L.)Carr.	eastern hemlock		2
T. heterophylla (Raf.)Sarg.	western hemlock		2
Abies amabilis (Dougl.) Forbes	amabalis fir		2
A. lasiocarpa (Hook.)Nutt.	subalpine fir		2
Larix laricina (DuRoi) K.Koch	tamarack		2
L. occidentalis Nutt.	western larch		2
Thuja plicata Donn.	western red cedar		2
elsewhere			
Abies procera Rehd.	Noble fir	U.S.	4
Picea pungens Engelm.	Blue spruce	U.S.	4
P. abies (L.)Karst.	Norway spruce	Europe	1
P. smythiana Boiss.	Indian spruce	India	5
Pinus elliottii Engelm.	slash pine	U.S.	4
P. radiata D.Don.	Monterey pine	U.S.	4
P. rigida Mill	pitch pine	U.S.	4
P. taeda L.	loblolly pine	U.S	4
P. nigra Arnold	Austrian pine	Europe	1
P. sylvestris L.	Scots pine	Europe	1
P. griffithii McClell.	Bhutan (blue)	India	3
P. clausa (Chapm.)[1]	sand pine	U.S.	6
Cedrus deodara Roxb.	deodar cedar	India	5
Abies grandis	grand fir	U.S.	7

1. *I. circinatus*

References: 1=Whitney, 1977; 2=Whitney, 1980; 3=Buchanan, 1964; 4=Hepting, 1971; 5=Bakshi, 1976; 6=Barnard and Blakeslee, 1985; 7=Miller and Partridge, 1973.

conflicting results. Susceptibility of a tree species when artificially inoculated does not imply that the species is substantially damaged in nature. Likewise, isolation of I. tomentosus from particular tree species does not necessarily imply significant damages occur in that species. Some of these reports are incidental isolations. Most reports of damage caused by I. tomentosus are from field studies and surveys in white and black spruce (VanGroenewoud and Whitney, 1969; Whitney, 1962), Engelmann and interior spruce (Merler et al., 1988; Garbutt and Turnquist, 1986) and in a variety of pine species: red pine and jack pine (Myren and Patton, 1971; Whitney, 1980), slash pine (Boyce, 1963) and lodgepole pine (Merler et al., 1988). There are many ecological factors that may affect how damaging a particular pathogen is to a particular host species. It may be that spruces and certain pines are most heavily attacked by I. tomentosus because the host and pathogen ranges overlap. However, balsam fir (Abies balsamea (L.)Mill.) and amabalis fir (A. amabilis (Doug.)Forbes) are both susceptible to the fungus and are found within the range of the fungus but decay and mortality losses in both species are much less than those in spruce (Morrison, 1987; Whitney et al., 1974). Therefore, host resistance mechanims are likely an important factor in determining susceptibility and damage.

Cultural Characteristics

In culture I. tomentosus is variable, particularily in cultures maintained for over a year on artificial media (Whitney and Bohaychuck, 1977). In general, the colony is brown or whitish brown,

somewhat cottony and slow-growing (2mm/day on malt extract agar-MEA). No clamp connections are formed (Whitney, 1977). These characteristics are similar to those of Phellinus pini (Thore.:Fr)A. Ames in culture; however, I. tomentosus has a darker brown mat with the media taking up some pigmentation and the hyphae have chlamydospore-like swellings (Christensen, 1940; Hubert, 1924; Whitney, 1977). Perhaps one reason why I. tomentosus and I. circinatus have not been separated by some authors is because they are quite indistinguishable in culture (Whitney and Bohaychuck, 1977). Using several cultural characteristics (mat colour, growth rates and chlamydospore production) Whitney and Bohaychuck (1977) were not able to distinguish isolates with straight setae from isolates with curved setae (the basis for separation of I. tomentosus and I. circinatus). However, tests of pathogenicity to germlings in nutrient media did distinguish the two species (or the two varieties of P. tomentosus) which the authors concluded supported separation of the varieties.

Infection and Colonization Biology

The means by which I. tomentosus infects and colonizes trees is not well understood. Whitney (1962) observed a progression of infection from small lateral roots 0.5 cm - 1.5 cm diameter which were often encrusted with resin-soaked soil. Inonotus tomentosus was isolated from stained wood and bark in these roots. Roots less than 0.25 cm diameter were not stained. The infection progressed into larger lateral roots at which point the fungus was located primarily in the heartwood and not commonly found in the sapwood or bark. In

the larger roots, stain in the heartwood was observed up to 1 m ahead of the infected bark and sapwood. From the roots, the decay progressed into the base of the trunk, and from there extended up the stem 1 m - 2 m and down the heartwood of other main roots. Decay moved into the sapwood near the root collar, but radial expansion in both roots and stem was much slower than longitudinal growth.

In some cases, attack by I. tomentosus was primarily in the bark and sapwood, and invasion of these tissues was often in advance of heartwood colonization, indicating that I. tomentosus is capable of colonizing living bark. Schulting (1987) noted that infected pine roots in pine/spruce disease centres had necrotic phloem and that colonization of the bark was commonly found without accompanying colonization of the heartwood. In spruce however, he observed typical heartwood decay with little colonization of the sapwood and bark. In white spruce resin production in response to wounding inhibited growth of some fungi but not I. tomentosus (Whitney and Denyer, 1969).

Whitney (1962) determined that initial infection of trees by I. tomentosus was through the roots. Observations of decay patterns and the location of advanced decay suggested that infection occurred in branching lateral roots 1 cm in diameter or smaller. Several sources of infection and actual entrance points were investigated. Many of the infected lateral roots were observed to be wounded by Hylobius spp. In a separate study, Whitney (1962) found a high correlation between Hylobius wounds and root rot. However, I. tomentosus did not enter the root directly through the wound. Whitney (1962) postulated that the Hylobius wounds predisposed roots to infection but were not

infection courts. Dead rootlets 1 mm - 3 mm were also ruled out as infection courts. Whitney concluded that the infection occurred at root contacts with diseased roots although in his excavations he found very few completely convincing examples of spread from one root to another.

Merler (1984) proposed the following route of infection and colonization by I. tomentosus in spruce trees. Initial infection occurs via root contacts between a colonized root and a healthy root which is usually less than 2 cm in diameter. The fungus grows ectotrophically on the small root for a short distance before penetration occurs. Multiple points of infection and cambial necrosis occur as the fungus grows proximally along the bark. On the small roots these multiple infection points can coalesce and girdle the root. As the root becomes larger (approx. 5 cm), the fungus moves into the xylem and may no longer be able to advance ectotrophically. It advances in the centre of the root to the tree stem where it colonizes the heartwood of the stem. Merler also stated that the fungus could grow from the stem down the xylem of uninfected roots. Once the root diameter decreases to a few centimetres, the fungus re-emerges and grows ectotrophically. These statements were based on observations of small white pustules from under the root bark distal to more advanced decay which appeared to originate from older decay at the root collar.

From observatons of radial spread of the fungus in disease centres (Myren and Patton, 1971; Whitney, 1962) it is apparent that root contacts are a very important means of spread. The role of basidiospores in disease spread is not known. Whitney (1963)

observed germinated basidiospores and subsequent mycelium beneath sporophores, and in inoculation experiments he successfully obtained colonization using basidiospore suspensions. However, successfull inoculations occurred only with very severe wounding - holes drilled half way through the root and puncture wounds 0.5 cm deep. Bark or shallow wood wounds yielded no infections from basidiospores.

In a second study Whitney (1966b) concluded that viability of basidiospores was greatest from young sporophores. In addition, germination rate increased on water agar ammended with spruce bark extracts, but decreased on water agar ammended with humic and soil extracts, suggesting that spores landing on roots are more likely to germinate than spores in soil or humus. Furthermore, viability improved after storage for several months at -18C, and spores germinated after several freezing and thawing cycles during storage. This suggests that spores released in the fall when the fruiting bodies develop are more likely to initiate infections in the spring after having a chilling requirement fulfilled. The natural range of this fungus further supports at least a chilling tolerance, if not a chilling requirement.

Once again, in Whitney's (1966) inoculation tests, holes drilled 5 cm into root heartwood and inoculated with basidiospore suspensions were more conducive to infection than holes 1 cm deep in the root sapwood. Whitney suggested that exposed, broken root ends on windthrown trees could provide infection courts for the establishment of new infection centres; further development of the centre would be through root contacts.

Basidiospores play a variable role in spread of root disease

caused by other pathogens. Tree to tree spread by Phellinus weirii (Murr.)Gibn. occurs by root to root contact (Wallis, 1957). Basidiospores have not been shown to be involved in spread. Buckland et al. (1949) and Wright and Isaac (1956) extensively surveyed stump surfaces and wounds looking for evidence of basidiospore infection but they were unsuccessful. Attempts to inoculate stump surfaces with P. weirii basidiospores were unsuccessful, but vegetative mycelium preparations were successful (Nelson, 1971).

With Heterobasidion annosum, basidiospores have an important role in disease spread. Rishbeth (1951b) successfully inoculated Scots pine stumps with spore suspensions and found evidence that stumps are naturally infected by spores. Basal scars also provide an infection court to H. annosum (Buckland et al., 1949).

To date, the only clearly demonstrated mode of entry for I. tomentosus mycelium is through the roots, not through stump tops, branch stubs or stem scars (Aho, 1971; Myren and Patton, 1971; Whitney, 1962), and as discussed earlier, infection most likely takes place in the small branching lateral roots. There are many questions remaining with respect to infection biology. Where does infection occur? Is a wound required and if so, how deep? Does the fungus enter a root through small branchlets or does it attack larger roots? Whitney (1962) was not able to bait I. tomentosus from soil using two different techniques, but the limitations of these techniques preclude conclusive statements regarding spread of the fungus through the soil or duff layer.

Ecology

Ecological studies concerning I. tomentosus are limited. In an early study of root disease (attributed to I. circinatus but was more than likely I. tomentosus) in Saskatchewan, Van Groenewoud (1956) compared soil types in diseased and nondiseased areas. In the stand openings (disease centres), trees were more shallow-rooted than in healthy stands due to an impenetrable layer near the surface or to sandy soils where the subsoil is often below permanent wilting point.

Stand opening disease was never found by VanGroenewoud in soils with a topsoil pH greater than 7.0. The pH of the soil in diseased areas was lowest, down to 4.5 in severely diseased areas. VanGroenewoud attributed the pH effect to the greater buffering capacity of higher pH soils. Inonotus tomentosus grew more favorably in media which were slightly acidic and would alter the pH of the media (Whitney, 1962). Soil buffering inhibits pH changes. Therefore VanGroenewoud (1956) suggested I. tomentosus did not grow where soils were well buffered and could not be made more acidic. VanGroenewoud and Whitney (1969) further described three soil conditions associated with diseased stands in the same area.

1. Shallow soil type: This type was characterized by a sharp transition between stand opening and surrounding stands, coinciding with abrupt changes in soil profile and properties. Opening profiles were shallow, wet, beta-gley with B horizon at 10 in. The organic layer was highly acid (pH 4.5) changing rapidly to alkaline at the B horizon (pH 9.3). Root penetration was only 4-5 in. from the top of the organic layer and the air space and nutrient content of rooting layer were low. Feather mosses (Hylocomium splendens and Pleurozium

schreberi) made up the entire ground cover.

2. Dry type: This type also had sharp transition between openings and healthy coinciding with change in soil type. The openings occupied pockets of sandy soil wedged between silty clay soils. Root penetration was fair but soil was very low in nutrients and moisture holding capacity. The organic layer pH was acid (4.5). Ground cover of openings was needles with sporadic mosses and herbaceous plants.

3. Variable soil type: In this type, diseased trees were scattered throughout the stands. The soil was very heterogenous. Where the disease was present, the pH of the organic layer was always acidic (pH 4-5) and the nutrient content varied from low to extremely low. Texture varied widely from silty clay to fine gravelly sand. Soil moisture and profiles were also highly variable.

Common factors between all diseased areas were a low pH in the organic horizon, low nutrient content in the rooting zone, and high stand density (and therefore high root competition). Just as Van Groenewoud (1956) named the white spruce - Hylocomium - Calliergonella plant association as being the most conducive to disease development, VanGroenewoud and Whitney (1969) found a strong relationship between increasing white spruce and feather mosses with decreasing pH in the organic layer. Therefore, as white spruce density increases, a more favorable pH is created for disease development, and less favorable conditions for the host are produced.

In B.C. Merler (1984) described four ecological subassociations where tomentosus root disease was prevalent.

1. Sub boreal spruce (SBS) - mesic oak, fern, thimbleberry

-humoferric podzol, submesic-submesotrophic.

2. SBS - mesic oak, fern, black huckleberry

-leached brunisol, sandy loam, mesic-submesotrophic.

3. SBS - mesic oak

-silty clay lacustrine luvisol with moder humus.

4. SBS - sub mesic queen's cup

-ferrohumic podzol, submesic-submesotrophic.

The submesotrophic classification suggests that I. tomentosus may be more prevalent in soils that are nutrient-poor. Gosselin (1944) also indicated that infection is less frequent in rich soils. The sample sizes in these two studies are too small to make conclusive statements or to attempt to explain the possible relationships between root disease and soil types. Gosselin (1944) found a wide variation in root disease incidence in southern Quebec but did not examine soil or ecological factors. In Idaho however, a specific pattern of incidence was observed in a transect line study along an elevation gradient. Frequency increased from a low in stands of type C (see Table I.2), to a high in stands of type A. Inonotus tomentosus was not evenly distributed along the elevation gradient but was found most frequently above 2,000 m (Hobbs and Partridge, 1979). This study is supported by Gosselin's (1944) observation that incidence of infection (measured by stump section butt rot) on the north shore of the St. Lawrence (S. Quebec) was about 20% in the valley and 80-90% up the mountain slope. Inonotus tomentosus has a competitive advantage over other microorganisms at low temperature (Whitney, 1962), therefore it is likely that temperature along an elevation gradient is a limiting factor. This

Table I.2. Stand types clustered by species and elevation (Hobbs and Partridge, 1979)

Stand group	Major Species	Elevation
A	Engelmann spruce, amabalis fir lodgepole pine	1082-2134 m
B	grand fir, western red cedar Douglas-fir, white pine	587-1402 m
C	Douglas-fir, western larch, grand fir, white pine, engelmann spruce	762-1585 m

factor may be in part responsible for disease distribution rather than stand composition alone.

Fire history is another factor which has been suggested to effect disease incidence (Hubert, 1931) but has not been conclusively demonstrated. In India, I. tomentosus attacks deodar, spruce and blue pine primarily through fire scars (Bakshi, 1976).

Sporophore Production

Sporophore production by I. tomentosus is very dependent upon weather conditions (Whitney, 1977). Some studies have examined the factors that influence sporophore production because sporophores are sometimes used to identify and delineate disease centres (Myren and Patton, 1971). Furthermore, fruit bodies may provide spores which could be responsible for establishing new disease centres in previously uninfected areas. In studying I. circinatus fruit body production, Gosselin (1944) measured maximum and minimum temperature and relative humidity (RH) before and during fruit body production. He found fruit body production occurred when average maximum temperature decreased below 60 F and RH was approximately 85%. Inonotus circinatus sporophores grew better in diffused light than in darkness or direct light, although this factor was not deemed important in sporocarp initiation. Spore discharge in I. tomentosus was also found to be greatest during periods of high RH (greater than 85%), moderate temperatures (16C - 22C) and low light (Bohaychuck, and Whitney, 1973). Under controlled conditions of darkness and high RH, spore discharge increased with increasing temperature from 5 to 23C, but this relationship was disrupted by light. In nature, discharge rates were controlled by an interaction of RH, temperature

and light; no periodicity was observed with any single factor.

Mycorrhizal Trials

Evidence for the formation of a symbiotic relation between I. circinatus and its host before parasitism was provided by a growth rate comparison between healthy and diseased trees on mountain slopes (nutrients limiting) and valley bottoms (nutrients plentiful) (Gosselin, 1944). Gosselin states that the symbiotic relationship is only effective when there is some mineral deficiency. He further supports this statement by observations of fruit bodies associated with tree rootlets and by foliage analysis. Percent dry weight of potassium, ammonium and phosphate increased in needles from trees with increasing decay.

Whitney (1965) viewed Gosselin's conclusions with some skeptisism and initiated experiments to determine whether or not I. tomentosus or I. circinatus formed mycorrhizae with red pine or white spruce. Germlings grown aseptically in nutrient -sphagnum - sand medium were inoculated with one of the two fungi by placing inoculum plugs onto a layer of shredded spruce bark in the flasks. None of the I. tomentosus or I. circinatus germlings developed mycorrhizae and a few died. Germlings inoculated with forest mycorrhizal tips did develop mycorrhizae.

II. THE DISEASE

Symptoms

Inonotus tomentosus was not considered an economically

significant pathogen in Canada until tree disease surveys in Saskatchewan and Manitoba detected a forest condition characterized by openings in a stand in which trees were dead or dying or were windthrown and butt rotted (Whitney, 1962). Whitney began his extensive studies of stand opening disease in 1952 in an area where trees were 40-180 years old, and where up to 87% of the spruce trees were dead or butt rotted. In these spruce stands, the disease openings had distinct margins and were variable in size. Dead or windfallen trees occupied the centre of the opening with unhealthy-appearing trees around the perimeter. Crown symptoms in declining trees were reduced internode length, short needles and a tendency for the ends of the branches to curl upward. Later, the crown thinned and became chlorotic; liquid and solidified resins often acculumlated at the tree base and in duff around the major roots. Extensive root decay was observed in trees with visible crown symptoms. Infected trees with small amounts of decay usually did not exhibit crown symptoms. Crown symptoms were evident when more than 40% of the roots were dead and where decay had extended into the root collar. According to Whitney's classification of stages of disease (1962), the tree died when more than 80% of its roots were girdled. Merler et al. (1988) maintained that in spruce, crown symptoms were not consistent and could not be related to the extent of colonization. They found a low mortality rate compared to the number of diseased trees.

In lodgepole pine, disease develoment and symptomology were found to be different from those in spruce (Schulting, 1987). At the point of infection the outer sapwood and cambium were dead and the

root tissues were highly resin-soaked and brittle. Progressing away from the infection, a faint stain was observed in the xylem. Much less decay occurred in the pine roots. Crown symptoms were much more prominent in the pines due to the much greater extent of cambial necrosis. The mortality rate was also higher. However, in several study sites, pine displayed the symptoms and butt rot typical of spruce. These pines still displayed greater cambial necrosis than the spruce (Schulting, 1987). The reason for variation in disease development between pine and spruce and within pine is unknown, but similar differences in disease development between spruce and pine infected with Heterobasidion annosum have been observed. In spruce and hemlock, H. annosum causes a butt and root rot, whereas in resinous pine species it causes cambial necrosis and mortality (Hadfield et al., 1986).

Damage

Root diseases caused by decay fungi result in four types of losses: mortality (standing), windthrow, butt cull and growth reduction (Whitney, 1976). Windthrow and mortality result in complete tree loss, and are continuous processes, not sporadic such as losses to fire or insects. The magnitude of losses measured can vary widely from stand to stand.

Whitney and VanGroenewoud (1964) measured tree diameter, crown class and disease condition in two young (51 and 38 years) stands in 1952 and again in 1962. The number of dead trees increased by 21 and 16% respectively. In the 51-year-old stand, the basal area was reduced by 24% over the ten years but on the second plot (38 years)

the reduction due to mortality was offset by the growth of remaining trees.

To estimate losses on a larger scale, Whitney (1973) conducted a survey of black and white spruce in 23 stands. In 14 black spruce stands (73-111 years) there was an average of 30% dead trees with 14% more diseased. In 5 black spruce stands averaging 71-85 years old, 35% of the trees were dead and an additional 20% were diseased. Finally, four white spruce/aspen stands (70-88 years) showed 41% average mortality with 13% living diseased trees.

Increment loss is the most difficult to measure because of the slow development of crown symptoms and the technical difficulties and lack of suitable controls associated with measuring volume reduction. VanGroenewoud and Whitney (1969) stated that growth increment is reduced 15-20 years before mortality, and that increment losses in diseased trees are probably as great as losses from outright mortality. By comparing radial increment over 5 years between healthy and diseased trees, Merler (1984) estimated that there is an average 20% decrease in basal area increment in diseased spruce trees. Whitney and MacDonald (1985) recorded a slightly smaller growth reduction in balsam fir ranging from 20 to 156 years. The average height decrease was 13.5% and the average radial decrease was 10.9%. However their comparison was between trees with and without ground level decay; hence, trees with extensive root rot and growth reduction, but without butt rot would reduce the measured difference. Tree age did not appear to effect the growth reduction.

There have been several studies measuring defect or decay losses in merchantable timber; some of these include data for I. tomentosus

(or I. circinatus) losses (Aho, 1971; Basham and Morawski, 1964; Davidson and Redmond, 1957; Loman and Paul, 1963; Thomas and Thomas, 1954). In 1964 in Ontario 6.3% of the annual cut was culled. Phellinus pini was responsible for 1/3 of this loss and several other fungi, including I. tomentosus, were responsible for about 2/3 of the loss (Basham and Morawski, 1964).

Spread Rates

In root wood, I. tomentosus grows quite slowly. Whitney's (1962) study of inoculated roots determined a growth rate of 1-10 cm/year, and in a second study, the average annual rate of growth was 3.8 cm (Whitney and VanGroenewoud, 1964). Ectotrophic growth of the fungus has been reported (Merler, 1984) but the rate of ectotrophic growth has not been determined. The limiting factors in tomentosus root disease spread are the slow rate of growth in roots and the frequency of root contacts.

In a 45 year old white spruce (unthinned) plantation, disease centres were identified and measured to their outside perimeter by the presence of sporophores (Myren and Patton, 1970). Excavations of some centres provided examples of I. tomentosus spread through root contacts. Over the four year study period the total area occupied by 10 of the root rot pockets increased from 99.4 m^2 to 157.4 m^2, an annual increase of 14.5%. In addition, over the whole study area (1.9 ha) 18 new disease centres were detected; the total area encompassed by disease centres at the end of the study equalled 263 m^2 or 1.4% of the area.

Reports of severe damage in spruce stands of central and eastern Canada prompted the intensive and extensive study of tomentosus root

disease by Whitney (1962). In one of the first papers to describe the disease as more serious than a cull-causing pathogen, VanGroenewoud (1956) states that "Management aimed at producing saw timber on these (referring to heavily diseased sites) is doomed to failure." After summarizing many of his studies to estimate losses, Whitney (1980) stated that losses in central and eastern Canada were considerable in natural stands but it was the recently documented losses in spruce plantations which were of most concern. Concern over the impact of I. tomentosus discovered in some Wisconsin plantations led to the 1970 study by Myren and Patton. They concluded that disease impact would increase with time and that the disease would intensify. Merler et al. (1988) determined that in B.C., I. tomentosus was the dominant root pathogen of spruce. They too felt that plantation management may be severely impacted by this fungus.

Early work on I. tomentosus was concerned with decay studies and losses in old growth stands. Discovery of the pathogen's existence in second growth stands, and the decreasing availability of old growth (and therefore an increase in second growth management) have led to greater concern about this pathogen (Cozens, 1986; Geisler, 1988) although few studies have been conducted.

In a Wisconsin white spruce plantation during a four year period, the reduction in the number of living dominant trees was 2.1%, codominants 2.4%, intermediates 15.4% and suppressed trees 48.5% in diseased plots compared to a 29.3% decrease in suppressed living trees only in control plots. Several reports of significant losses to pine plantations due to I. circinatus have surfaced from

the southeastern U.S. Boyce (1963) identified I. circinatus in a Georgia slash pine plantation and noted a relationship between the presence of basal rust cankers (Cronartium fusiforme Hedge. & Hunt) and the presence of I. circinatus sporophores. He later (1967) found that 29% of trees with basal cankers were also infected with I. circinatus. Similarly Ross (1966) in a survey of an 18 year old slash pine plantation which identified infected trees by sporophores only, found an infection rate of 1.6%. Of the cankered trees, 8% were infected with I. circinatus. It is suspected that the percentage of trees infected was actually much greater due to trees which were infected that were not yet supporting sporophores. There are many unknowns regarding the infection biology of I. circinatus and I. tomentosus, their virulence and host specificity. However, the occurrence of I. circinatus in young pine plantations (Boyce, 1963; Ross, 1966) suggests that plantations established in I. tomentosus disease areas may also become infected at a significant rate.

III. VEGETATIVE INCOMPATIBILITY

As mentioned previously, the role of basidiospores in disease spread is not understood. Basidiospores of I. tomentosus are small and hyaline and therefore very difficult to observe in nature. Furthermore, the lifecycle of the Basidiomycetes and their growth habits makes it difficult to define an individual. One well-accepted means of determining individuals is by vegetative incompatibility reactions between isolates of the same species within diseased areas (Rayner and Todd, 1977; Todd and Rayner, 1980). This technique has

been used on many Basidiomycetes to determine "clones" which are genetically identical at those loci which determine vegetative compatibility (Adams and Roth, 1967; Barrett and Uscuplic, 1971; Brodie, 1936,; Childs, 1963). Pairing of two vegetative isolates on artificial media which results in the two isolates growing together indicates that the isolates are of the same vegetative compatibility group (Vc Group). The formation of a "Barrage" line or a "zone of demarcation" indicates that the isolates are of different Vc groups, are genetically different and are therefore probably derived from different basidiospore infections. Childs (1970) used this technique to identify clones (or identical Vc groups) of Phellinus weirii. He found that the large disease centres caused by P. weirii were usually of one Vc group and that the size was due to persistence of the fungus in stumps and dead trees from one generation to the next allowing for continuous vegetative spread. The lack of many different Vc groups led to Childs' conclusion that basidiospores were not important in disease spread of P. weirii. With Heterobasidion annosum however, disease centres are generally smaller,and Vc studies showed a much greater frequency of different clones (Stenlid, 1985) suggesting that basidiospores are involved in disease spread. Disease centres of I. tomentosus are typically smaller (a few metres in diameter) than those of P. weirii which suggests that different Vc groups may be more frequent.

Chapter II. Survival of *Inonotus tomentosus* in stumps and subsequent infection of young stands

INTRODUCTION

The old growth boreal forests of British Columbia (B.C.) consist primarily of white spruce (*Picea glauca* (Moench) Voss), interior spruce (*P. glauca* x *engelmannii*), and lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) which are hosts to an indigenous and ubiquitous root disease caused by *Inonotus tomentosus* (Fr.) Teng. The potential for damage by this disease in stands established after harvest of diseased old growth stands is of considerable concern to forest managers because second growth management will be increasingly important for future timber supply.

The pattern of disease development and excavations of root systems in disease centres indicate that root contacts are an important means of disease spread (Merler, 1984; Patton and Myren, 1970; Schulting, 1987; Whitney, 1962). The fungus infects tree roots by penetration of smaller lateral roots, killing cambium and sapwood before reaching the heartwood where it moves up and down the root (Merler, 1984; Lewis chapter VI). Eventually the fungus progresses to the lower stem. Infected roots become structurally weakened and disfunctional, leading to windthrow or growth reductions eventually followed by mortality.

In plantations and naturally regenerated stands, roots of young trees which contact stumps of the previous stand that are colonized by *I. tomentosus* may become infected at a very early age compared to infection of trees in an old growth forest system, resulting in

greater losses and spread of the disease. Most plantations in B.C. are less than 25 years old and too young to observe the effects of I. tomentosus on yield. One survey in the Prince Rupert Forest Region of B.C. found that 3.1% of white spruce trees in eight, 20-year-old and younger plantations were infected or had been killed by I. tomentosus (Unger and Humphreys, 1984). Other studies of disease spread and damage were reported from Wisconsin. Mortality in one white spruce and one red pine (Pinus resinosa Ait.) plantation (both 55 years old and thinned once) was followed over 4 years (Myren and Patton, 1971). Excavations showed that new infection occurred at root contacts with colonized stumps and infected suppressed trees. Mortality in disease plots was greatest in suppressed and least in dominant trees (suppressed 48.5%, intermediate 15.4%, codominant 2.4%, dominant 2.1%). In root rot free control plots mortality was confined to suppressed trees (29.3%) (Myren and Patton, 1971).

Many basic questions need to be answered before conclusive statements can be made about the effects of I. tomentosus on second growth stands. There are several factors that affect the likelihood of disease spread into future stands from past rotation stumps, including the distribution of colonized stumps in a clearcut, the viability of the mycelium in stumps, the location of the mycelium in the root system (stump core or peripheral roots) and the likelihood of root contact with a susceptible regeneration species. These are addressed in the study outlined below, the objectives of which were to determine: A. The incidence of disease in clearcuts in two Forest Regions of B.C.; B. The distribution of colonized stumps in clearcuts; C. The longevity of I. tomentosus mycelium in old growth

stumps; D. The location of mycelium within stumps (colonization patterns); and E. The frequency of fungus transfer to surrounding regeneration.

MATERIALS AND METHODS

Stand selection and survey of incidence of disease in clearcuts

The selection of clearcuts for stump excavation was determined by several criteria. Stands should be in either the Prince George Forest Region (interior forest, sub-boreal spruce zone) (Krajina, 1965) or the Prince Rupert Forest Region (coastal forest, sub-boreal spruce and interior cedar-hemlock zones) of central British Columbia. They were to be of three age classes: 0-2 years, 10-15 years, and 24-30 years since harvest. Finally they were to have white or interior spruce or lodgepole pine regeneration (except the 1-to 2-year-old sites which were not yet planted).

Major areas (road systems, drainages) were selected on maps and several clearcuts meeting the selection criteria within each major area were examined by walking through the clearcut along several compass bearings and sampling stump roots. The presence or absence of I. tomentosus in the clearcut was recorded on area maps. Brief observations of the moisture regime (Krajina, 1969) and some shrub species were recorded for each major area in an effort to relate root disease incidence with habitat. The final selection of stands for stump excavation was made from the list of clearcuts with I. tomentosus according to the age and regeneration species criteria above. In addition sites were to have at least 15 stumps infected with I. tomentosus.

Distribution of I. tomentosus in stands selected for stump excavation

A transect survey was run through all selected clearcuts with

the purpose of determining the incidence and distribution of I. tomentosus in the clearcuts and of selecting 15 or 20 infected stumps per site for excavation. Transect lines were 10 m wide and at least 500 m long, and were run from a landing or a road on a randomly-chosen bearing. All stumps within the transect were examined by cutting into three primary roots approximately 1 m from the root collar with a chainsaw or pulaski. Stumps with I. tomentosus in the roots were marked for later excavation, and presence or absence of decay on the stump surface was noted. Decay on the stump surface was visible as areas of advanced pocket rot, usually in the centre. The number of colonized stumps per hectare and the relationship between root infection and butt rot visible at the stump surface was determined. A map was drawn of the transect line from which the number of disease centres (disease centres being defined here as a group of adjacent colonized stumps surrounded by uncolonized stumps) per hectare was determined.

The pattern of distribution of infected stumps was determined by analyzing the frequency distributions of the number of sampling units (25 m sections of transect line) with 0, 1, 2, 3, 4, 5, and 6 or more colonized stumps. Variance to mean ratios were calculated from these distributions for each stand. Variance to mean ratios greater than one suggest a clumped pattern (Ludwig and Reynolds, 1988). Further analysis of the pattern of colonized stumps was carried out with Pielou's (1965) method of run lengths of colonized stumps. Run length is the number of adjacent colonized stumps along the transect not separated by a healthy stump. The frequency of observed run lengths was compared to expected run lengths by calculating the Chi-

square statistic. Frequencies expected, should the colonized and uncolonized stumps be randomly mingled, were calculated from a geometric distribution (Pielou, 1965).

Longevity of viable mycelium, location of mycelium in stumps and frequency of fungus transfer to surrounding regeneration

All roots on selected stumps were excavated by pulaski to their end or to a depth of 50 cm below the soil surface. The length of each root was measured and a map of each stump and its roots was drawn to scale.

Longevity and location of viable mycelium: Decay was estimated in three categories: 1) stain (incipient decay); 2) advanced decay with apparently viable and vigorous mycelium; and 3) old decay lacking mycelium or with wet, yellowish mycelium. The length along the root in which I. tomentosus was found and the root diameter at the end of each stage of decay were recorded in the field. Visual estimation of the vigour of the mycelium was tested by laboratory isolation on 3% malt extract agar (MEA). Samples were removed from areas of transition from one decay category to another and where viability of the mycelium was questionable. These samples were plated on MEA and incubated at room temperature.

To determine whether the fungus was in a position to transfer to regeneration tree roots should contacts occur, the radial distance from the outer margin of the decay column to the surface of the root (rdm) was measured. Mean measurements of rdm were calculated for each site.

Frequency of fungus transfer: The five regeneration trees closest to each stump were located on the stump map by distance and bearing. The total height and leader growth increment of each tree were measured; age was estimated by counting internodes, and disease symptoms were recorded. The occurrence of root contacts between stump and tree were recorded and notes were made on the condition of the roots at the point of contact. Trees that exhibited symptoms or that had root contacts with infected stump roots were closely examined at the root collar and along the root bark. In some cases regeneration tree roots or whole trees were removed for observation and subsamples were cultured on MEA for attempted isolation of the fungus. Root contacts with regeneration trees and infection of those roots were analyzed statistically by calculating the proportion of trees with root contacts and infections. Such proportions represented a binomial distribution and were compared using the method of normal approximation of a binomial distribution (Ostle and Mensing, 1982).

RESULTS

Stand selection and survey of incidence of disease in clearcuts

A total of 49 stands were examined in 10 major areas. Stumps with I. tomentosus were found in 19 stands in six of these areas. Infected stumps were usually located in moist habitats (subhygric, Krajina, 1969) with pure spruce or spruce-pine mixes. These sites were characterized by the presence of the mosses Rhytidiadelphus triquetrus(Hedw.)Warnst., Pleurozium schreberi (Brid.)Mitt., and Polytrichum commune Hedw., as well as false sarsparilla (Aralia naudicaulis L.), blue huckleberry (Vaccinium ovalifolium Sm. in Rees), and twin flower (Linnea borealis L.). In the Prince George Region, I. tomentosus was found in 17 of 42 stands (40%); in the Prince Rupert Region two stands of seven examined (29%) had I. tomentosus. Table II.1 lists those stands selected for stump excavations, and their general locations are shown in Figure II.1.

Distribution of I. tomentosus in stands selected for stump excavation

The number of stumps with I. tomentosus ranged from 8-71 per hectare (2.1% to 27.5%) (Table II.1). Frequently, stumps colonized by I. tomentosus were found in small groups of two to three stumps. The 30-year-old stumps were very decayed and ant-infested. Signs of past colonization by I. tomentosus were eroded by unidentified brown cubical rots, by ant infestation, and by destruction of the stump tops by bears feeding on ants. Therefore the estimate of 20 colonized stumps/ha in these old clearcuts is conservative. Individual disease centres, or groups of colonized stumps, ranged in

Table II.1. Clearcuts selected for stump excavation and the incidence of stumps colonized by Inonotus tomentosus.

Forest Region	BGC[1] zone	Site name(no.)	Stump age	Stump species	Colonized stumps/ha (total/ha)	Disease centres/ha[2]	Regeneration description
PG[3]	SBS[4]	Wansa (1)	1	spruce	32 (476)	14	No treatment
PG	SBS	Km 162 (2)	2	spruce	40 (196)	24	Burned, not yet planted
PG	SBS	Site 21 (3)	13	spruce	71 (258)	41	Planted - Sx[5]
PG	SBS	Pinney Ck. (4)	26	spruce	27 (165)	16	Selective logged natural regen.
PG	SBS dry	Bob 102 (5)	2	pine	8 (383)	5	Burned, not planted
PG	SBS dry	Km 12 Pel. (6)	24	pine	21 (238)	9	Natural regen.
PR	ICH[6]	Kispiox (7)	15	spruce			
			15	pine	40 (203)	17	Burned, planted Pl[5], Sw[5]
PR	SBS	Jonas Ck. (8)	30	spruce			
			30	pine	20 (261)	8	Burned, natural Pl and planted Sw

[1] Biogeoclimatic zone (Krajina, 1965)
[2] Disease centres defined as a group of colonized stumps surrounded by healthy stumps.
[3] PG = Prince George; PR = Prince Rupert
[4] SBS = Sub-boreal spruce zone
[5] Sx = interior spruce (Picea glauca x engelmanni)
Sw = white spruce (P. glauca)
Pl = lodgepole pine (Pinus contorta)
[6] ICH = Interior cedar hemlock zone

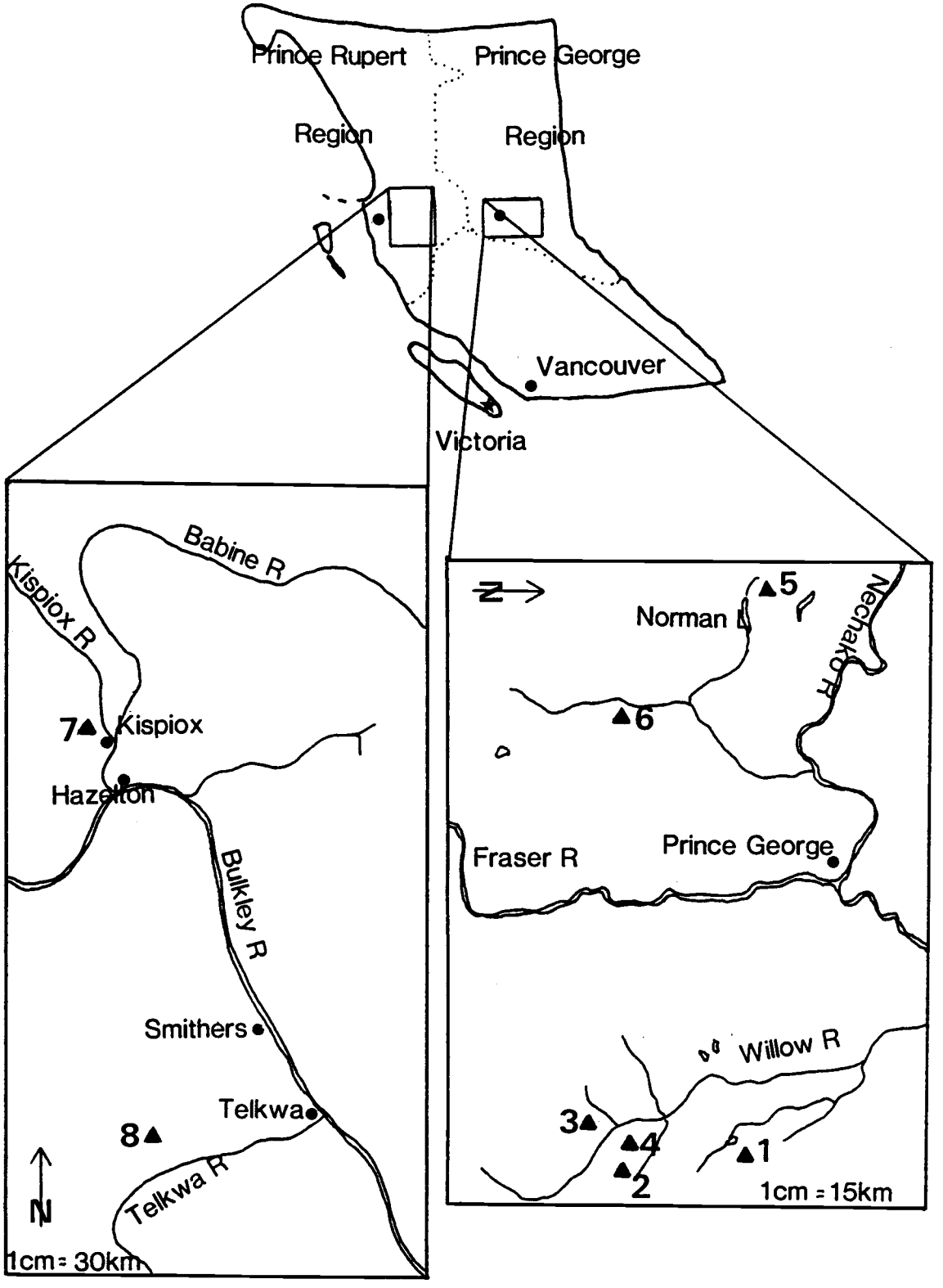


Figure II.1. The location of stump excavation sites in the Prince George and Prince Rupert Forest Regions in British Columbia.

size from one stump to six stumps encompassing an area averaging 20 m in diameter.

Variance to mean ratios calculated from frequency distributions (see appendix A) of the number of sampling units along transects with 0, 1, 2, 3, 4, 5, and 6+ diseased stumps were all greater than one, ranging from 5.04 to 23.26 across the eight sites (Table II.2). These ratios and the field maps of transect lines and sampled stumps suggest that diseased stumps occur in clumps. In further support of a clumped pattern, analysis of colonized stump run length (Pielou, 1965) in all but one stand rejected the null hypothesis that diseased and healthy trees were randomly mingled within "disease patches" (infected areas of the stand) (Table II.2). Therefore, the probability of a stump being diseased is not independent of the condition of its neighbour.

For both pine and spruce, all stumps that displayed I. tomentosus decay on the surface also had root decay. The existence of root rot in spruce stumps, but not in pine stumps was reliably indicated by decay at the stump surface. The percentage of stumps with infected roots that also exhibited surface decay ranged among sites from 67% to 93% for spruce, and 0% to 75% for pine (Fig. II.2). Butt rot was much less common in pine than spruce.

Longevity of viable mycelium, location of mycelium in stumps and frequency of fungus transfer to surrounding regeneration

Isolation success from all samples including those visually identified as non-viable was 50% from advanced decay, 18.7% from stain, and 21.1% from old decay. Isolation success from only those

Table II.2. Variance to mean ratios of the number of sampling units (25m sections of transect) with 0, 1, 2, 3, 4, 5, and 6+ colonized stumps, and p values from X^2 tests comparing observed to expected distributions of colonized stumps in clearcuts.

Stand no.	Species	Age	Var./mean	P values Runs Analysis[1]
1	spruce	1	8.16	0.012
2	spruce	2	12.28	0.024
3	spruce	13	5.04	0.006
4	spruce	26	8.28	0.060
5	pine	2	16.63	idf[2]
6	pine	24	13.27	0.033
7	both	15	7.49	0.006
8	both	30	23.26	0.016

1 Pielou, 1965

2 idf = insufficient degrees of freedom

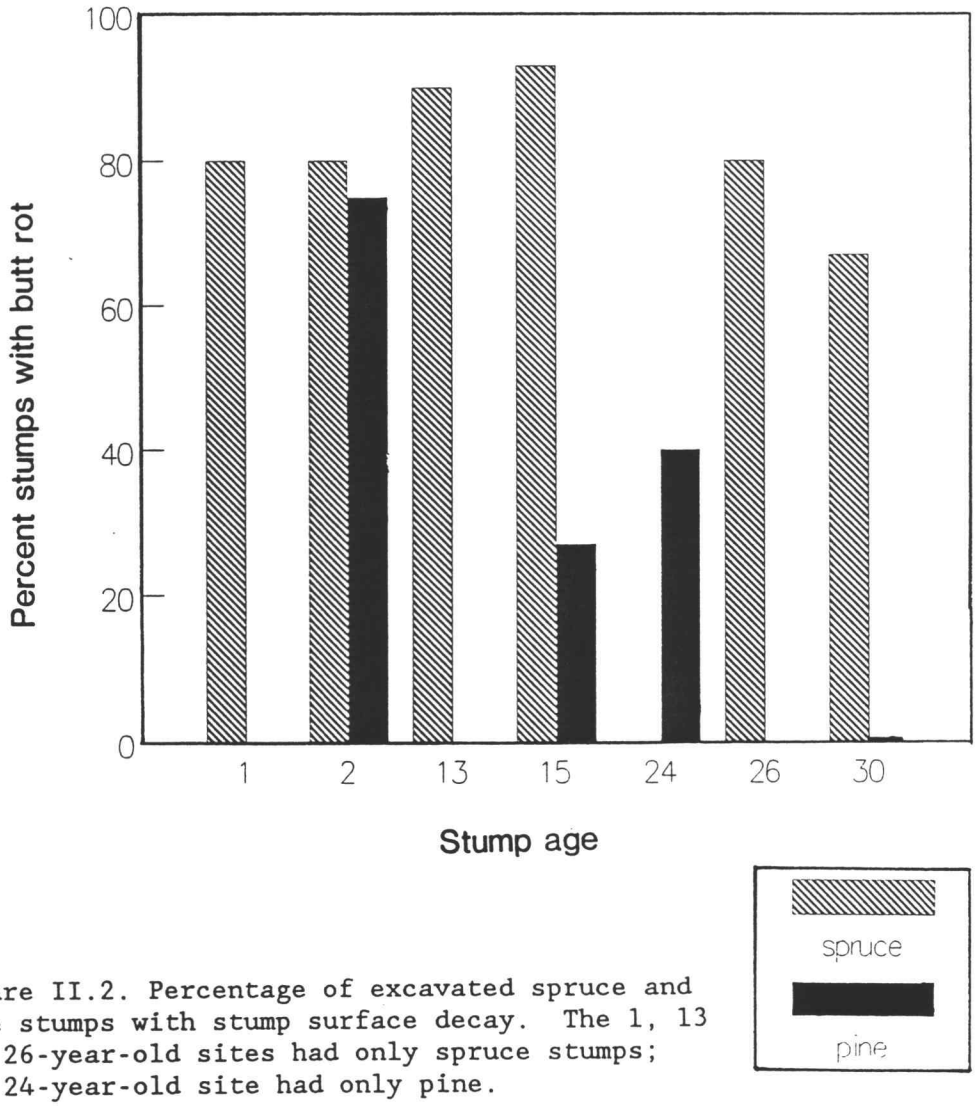


Figure II.2. Percentage of excavated spruce and pine stumps with stump surface decay. The 1, 13 and 26-year-old sites had only spruce stumps; the 24-year-old site had only pine.

samples visually identified as viable was slightly improved for advanced decay and much improved for stain and old decay (60%, 50% and 50% respectively). Some of the advanced decay samples which did not yield I. tomentosus in culture were transitional to old decay. Stain samples which were a very dark red-brown, in close proximity to advanced decay, were much more likely to yield I. tomentosus in culture than were lightly stained samples. Very old decay without visible mycelium in the decay pockets never produced mycelium in culture.

Longevity of viable mycelium: Mean excavated root length per stump (excavated to the root end or to 50 cm below the soil surface) for pine ranged from 638 cm to 1250 cm and for spruce from 1019 cm to 3042 cm (Table II.3). Viable mycelium remained in 80% of the oldest spruce stumps examined (30 years) as determined by visual observations of decay with verification by isolation on MEA. Viable mycelium was found in 100 percent of infected spruce stumps 2 - 26 years after harvest. However, the percentage of infected pine stumps with viable mycelium decreased from 95% after 2 years to 53% after 30 years.

Location of mycelium in stumps: For both species, roots less than 2 cm in diameter and up to 4.5 m from the stump harboured viable I. tomentosus mycelium. In roots where most of the cross section was colonized, mycelium in or near the bark appeared younger and more robust than mycelium in the root centre.

Spruce stumps: Of all the spruce roots examined, 69.6% were at least

Table II.3. Percent of stumps with viable mycelium of *I. tomentosus* and average excavated stump root length.

Site	Species	Age	% Stumps with viable mycelium	Avg. combined root length/stump[1]
Wansa	spruce	1	100	2658 cm
Km 162	spruce	2	100	3042 cm
Site 2	spruce	13	100	1434 cm
Kispiox	spruce	15	100	1675 cm
Pinney Ck	spruce	26	100	1039 cm
Jonas Ck.	spruce	30	80	1615 cm
Bob 102	pine	2	95	1250 cm
Kispiox	pine	15	80	657 cm
Km 12 Pel.	pine	24	87	770 cm
Jonas Ck.	pine	30	53	638 cm

1. Standards for excavation: all roots excavated to the end or to 50 cm below the soil surface.

partially colonized by I. tomentosus. Mean root length for colonized and healthy roots was 188 cm. Decay columns in the colonized roots averaged 162 cm. Average excavated root length and average colonized root length per stump both decreased with stump age at very similar rates (Fig. II.3). Both data sets fit a power law equation ($y = ax^b$) with exponents of -0.25 and -0.28 respectively (Table II.4). Therefore, as seen in Fig. II.3, the percent of root length colonized by recognizable I. tomentosus remained constant for all stump ages.

The advanced decay category predominated in spruce stump roots (see Appendix B for an example of stump maps). The stain category was very high in the 1- and 2- year-old stumps and decreased abruptly in the older stumps. Old decay was greatest in the oldest stumps (Fig. II.4).

In roots of newly cut spruce stumps, stain or decay columns were generally surrounded by uncolonized wood. In older stumps, the total root cross-section was often occupied by advanced decay which extended to just beneath the bark, partially through the bark, or to the root surface in areas where bark was missing (Fig. II.5a). Apparently, colonization of the spruce stump roots continued after harvest, however, development of decay progressed radially, with little advance longitudinally. The radial distance from the decay column to the root surface decreased in stump roots from harvest to age 15 (Table II.5), then remained constant.

Pine stumps: Of the pine stump roots examined, 54.3% were colonized. Average root length in pine was 119 cm and the mean decay column length in colonized roots was 104 cm. Average excavated root length of pine stumps also decreased with stump age, but unlike spruce, the

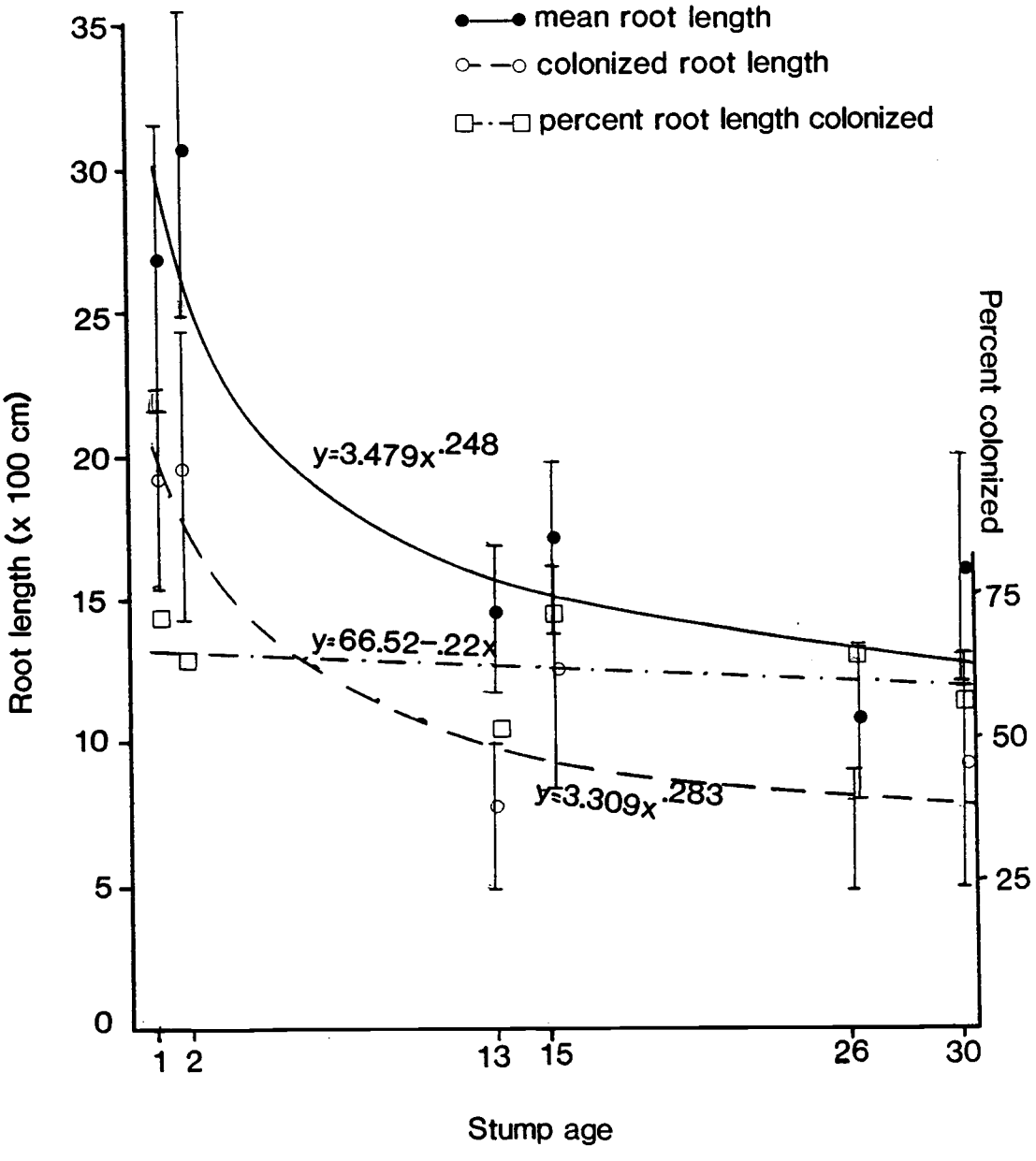


Figure II.3. Mean root length, root length colonized by I. tomentosus and percent root length colonized in spruce stumps by stump age.

Table II.4. Regression equations and analysis of variance of spruce stump root length and colonized root length on stump age.

Source	Sum Squares	Df	Mean Square	F-ratio	p
log (Total Root length) =3.54 - 0.248 log(stump age)					
model	.616	1	.616	13.594	0.211
error	.181	4	.045		
total	.797	5		R^2 = 77.26%	
log (colonized length) = 3.309 - 0.283 log(stump age).					
model	.803	1	.803	14.656	0.019
error	.219	4	.055		
total	1.022	5		R^2 = 78.56%	
Percent root length colonized =66.524 - 0.220(stump age)					
model	34.547	1	34.547	0.510	0.514
error	270.787	4	67.697		
total	305.334	5		R^2 = 11.31%	

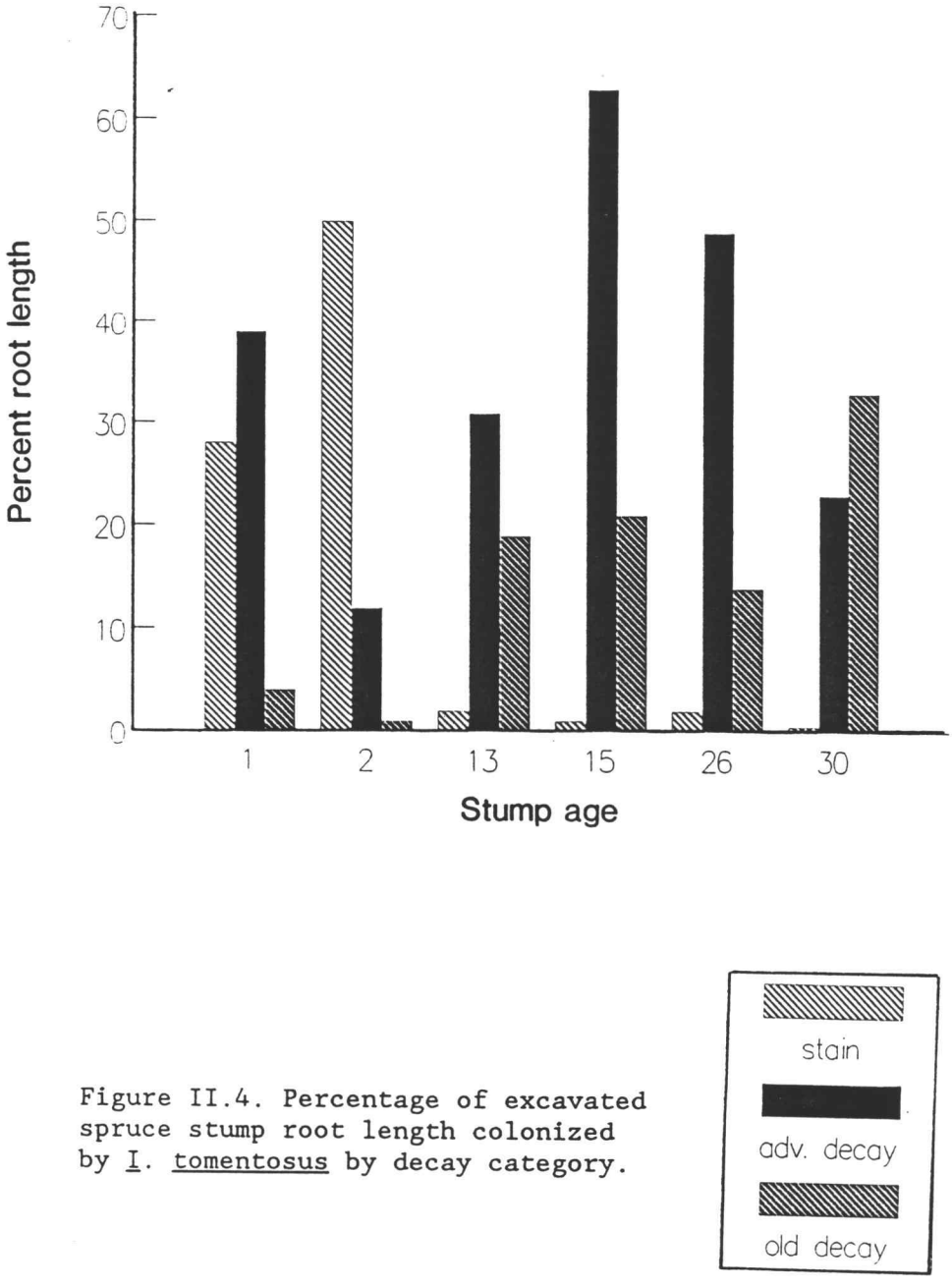


Figure II.4. Percentage of excavated spruce stump root length colonized by I. tomentosus by decay category.

Figure II.5a. Spruce stump root colonized by I. tomentosus advanced decay.

Figure II.5b. Pine stump root colonized by I. tomentosus advanced decay. Arrows indicate bands of decay.

Table II.5. The average radial distance from the decay column to the root surface in spruce and pine stump roots.

Species	Stump age	Average radial distance (rdm)
spruce	1	0.78 cm
spruce	2	0.90
spruce	13	0.52
spruce	15	0.24
spruce	26	0.54
spruce	30	0.54
pine	2	0.54
pine	15	0.62
pine	24	0.32
pine	30	0.66

length of colonized root did not decrease. Therefore the percentage of root length colonized increased with stump age (Fig. II.6). Equations fit to the excavated length and colonized length are in Table II.6.

Old decay was as prevalent as advanced decay in the pine stumps, both categories were greater in the 15- to 30-year stumps than in the 2-year-old stumps. The stain category decreased abruptly after 2 years (Fig. II.7).

In the pine stump roots, the fungus was also in a position to cause infection of contacting tree roots. Radial development of decay was highly variable as indicated by the rdm values in Table II.5. However, pine roots from stumps of all ages were frequently observed with mycelium in the bark, which sometimes preceeded decay in the root wood. Discrete decay columns were often lacking and were replaced by apparently discontinuous bands of decay (Fig. II.5b). Total root decay did occur but was less common than in spruce.

Frequency of fungus transfer to surrounding regeneration: Root contacts between stump roots and regeneration tree roots were common. The percentage of total trees contacted, infected, and symptomatic were calculated because the number of trees examined were not equal for all sites (Table II.7). With one exception (spruce, 26 years old) the percentage of young trees contacted, infected, and symptomatic increased with stump age. The 26-year-old spruce site was naturally regenerated and had a lower stand density than some other sites, which may explain the lack of conformation to the described trend.

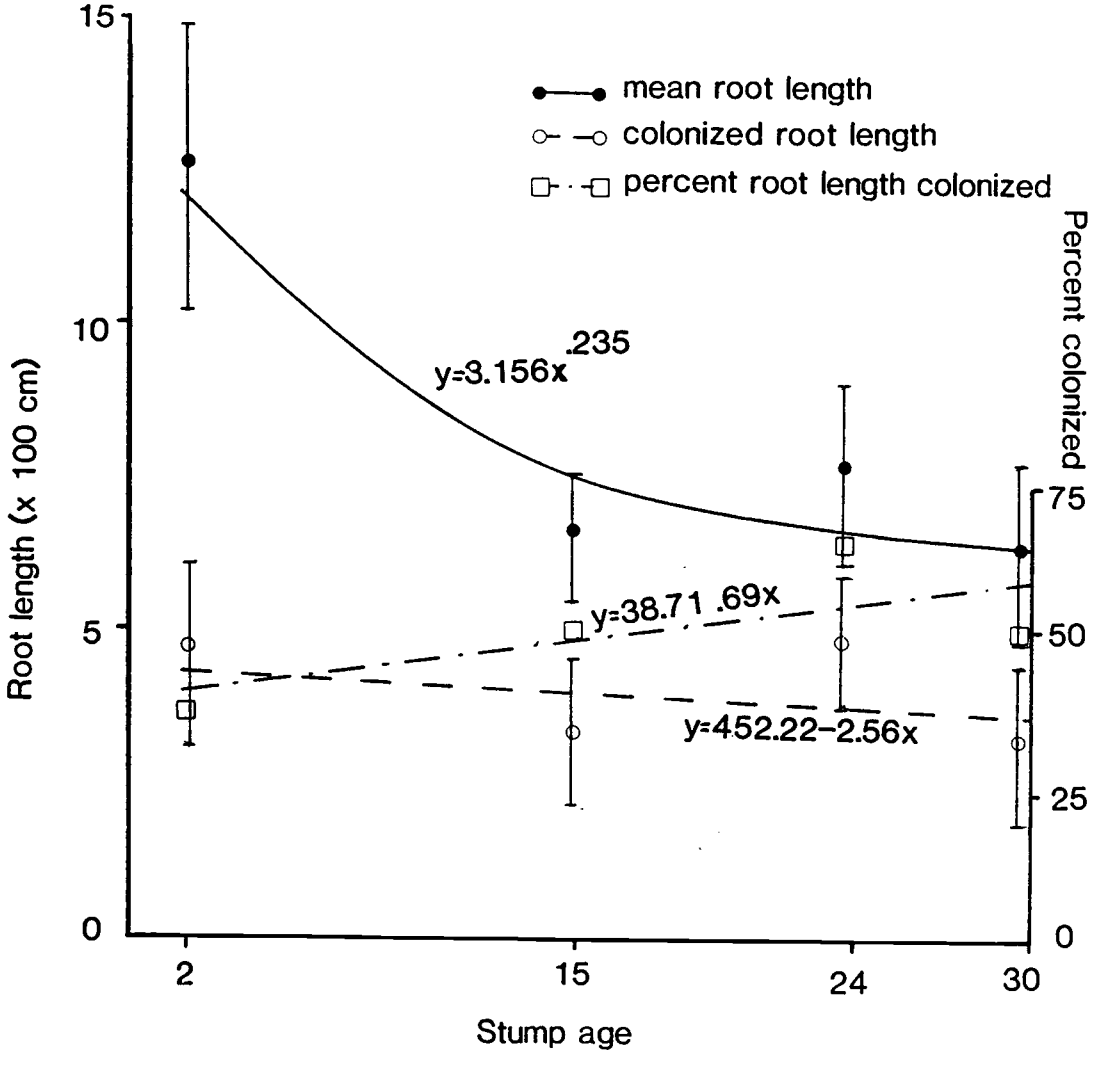


Figure II.6. Mean root length, root length colonized by I. tomentosus and percent root length colonized in pine stumps by stump age.

Table II.6. Regression equations and analysis of variance of total pine stump root length and colonized root length on pine stump age.

Source	Sum Squares	Df	Mean Squares	F-ratio	p
log (total root length) = 3.156 - 0.235 log(stump age)					
model	.253	1	.253	13.635	.066
error	.037	2	.018		
total	.291	3		R^2 = 87.21	
colonized root length: = 452.218 - 2.56(pine stump age)					
model	2918.25	1	2918.25	.293	.643
error	19944.50	2	9972.25		
total	22862.75	3		R^2 = 12.76%	
Percent root length colonized =38.708 + 0.693(stump age)					
model	213.297	1	213.297	2.387	.262
error	178.703	2	89.351		
total	392.000	3		R^2 = 54.4%	

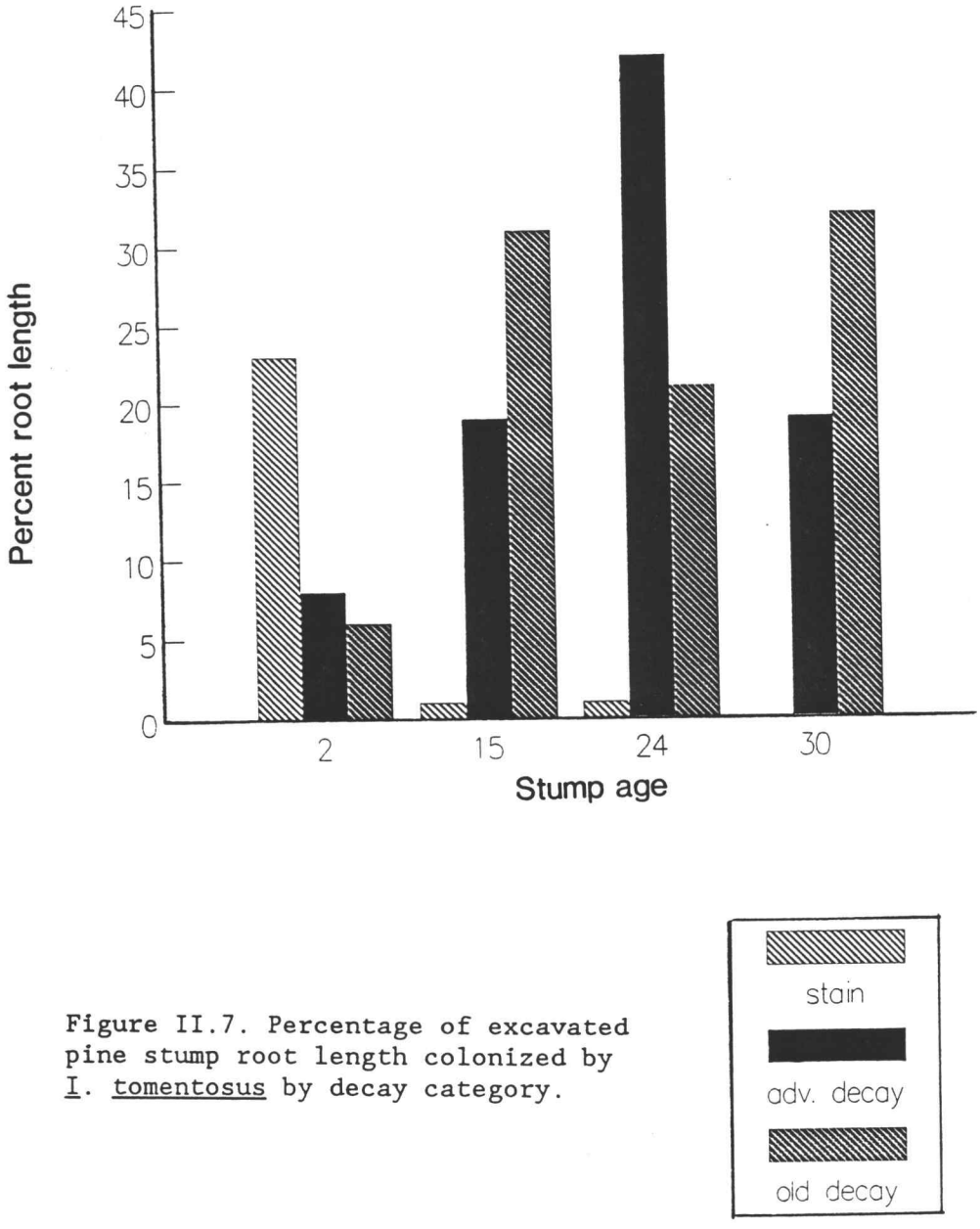


Figure II.7. Percentage of excavated pine stump root length colonized by I. tomentosus by decay category.

Table II.7. Frequency of root contact, root infection, and crown symptom development by the 5 susceptible[1] regeneration trees growing closest to each old spruce and pine stumps colonized by *I. tomentosus*.

Stump Species	Site age	Total trees	Number (%) Contacted	Infected	Symptomatic	Infect:[2] contact
spruce	13	100	20 (20)	14 (14)	5 (5)	0.70
spruce	15	72	21 (29)	8 (11)	6 (8)	0.30
spruce	26	100	20 (20)	10 (10)	4 (4)	0.42
spruce	30	75	32 (43)	16 (21)	7 (9)	0.38
Total		347	93 (27)	48 (14)	22 (6)	0.41
pine	15	56	6 (11)	1 (2)	1 (2)	0.17
pine	24	75	11 (15)	3 (4)	1 (1)	0.21
pine	30	75	21 (28)	7 (9)	5 (7)	0.27
Total		206	38 (18)	11 (5)	7 (3)	0.24

1. *Picea*, *Pinus*, *Abies*

2. Ratio of infected roots to contacted roots.

Not only were the number of root contacts greater with spruce stumps but the proportion of contacts resulting in infections was greater for root contacts with spruce stumps than for root contacts with pine stumps (Table II.7). Comparisons were made between the percentage of trees in root contact and infected by a) spruce stump roots and b) pine stump roots using data from the two sites where pine and spruce stumps were sampled together (Kispiox - 15 years and Jonas - 30 years). Comparisons were by normal approximation of a binomial distribution. Spruce stump roots contacted more regeneration tree roots than did pine stump roots ($p=.01$ and $p=.17$ for 15- and 30-year-old sites respectively, refer to Table II.7) and more infections resulted ($p=.05$ and $p=.09$ respectively, Table II.7).

Symptoms on infected trees included foliage chlorosis, reduced leader growth, abundant and premature cone crop and less commonly, basal resinosus. Symptoms were expressed more frequently in trees which were in contact with spruce stump roots than in trees in contact with infected pine stump roots. An average of 23% (22 of 93) of young trees in root contact with spruce stumps and 18% (7 of 38) in contact with pine stumps showed symptoms (Table II.7). Symptom expression was related to the degree of infection in individual trees.

Young spruce trees were more common than pine trees on most sites. A total of 364 spruce trees and 101 pine trees were examined. However, the percent of trees which were in contact with stump roots was not significantly different between spruce and pine regeneration (25% and 19% respectively).

The distance between colonized stumps and regeneration trees was

an important factor affecting the number of root contacts and regeneration tree infections. The probabilities of root contacts and infections were calculated by stump-to-tree distances grouped in 50 cm distance classes and regression curves were fit. For both spruce and pine stumps, the number of root contacts and infections were inversely proportional to distance from the stump to the tree. Regressions were fit for both individual stands and for the combined data from each stump species. Table II.8 shows the regression equations and Analysis of Variance tables for the data from all sites combined. There was a significant (p ranged from <.0001 to 0.013) relationship between root contacts or infection of regeneration trees and distance from the stump for both pine and spruce stumps. The R^2 values ranged from 51% to 87%.

Table II.8. Regression equations of 1) the number of regeneration trees in contact with spruce and pine stump roots regressed on distance; and 2) the number of infected trees regressed on distance.

1: spruce stumps: Y = 0.678 - 0.00131(distance)

Source	SS	Df	MS	F	p
model	.522	1	.522	58.88	<0.0001
error	.089	9	.009		
total	.602	10			

R^2 = 86.74%

pine stumps: Y = 0.741 - .00164 (distance)

Source	SS	Df	MS	F	p
model	.809	1	.809	18.085	0.002
error	.403	9	.045		
total	1.212	10			

R^2 = 66.77%

2: spruce stumps Y = 0.406 - 0.000823 (distance)

Source	SS	Df	MS	F	p
model	0.204	1	0.204	12.93	.00579
error	0.142	9	0.015		
total	0.347	10			

R^2 = 58.96%

pine stumps Y = 0.281 - 0.000636 (distance)

Source	SS	Df	MS	F	p
model	.122	1	.122	9.63	.01265
error	.114	9	.013		
total	.237	10			

R^2 = 51.69%

DISCUSSION

Stand selection and survey of incidence of disease in clearcuts

Root disease occurrence on a Forest District-wide basis is patchy. The present study did not include an extensive survey on a Forest District-wide basis; such a survey would be necessary for incidence and loss estimates and for correlation of root disease incidence with ecosystem associations. Three inter-related factors affecting disease distribution became quite apparent during the survey. One was that the disease can be quite extensive within one area and absent from another. This may be related to the second factor, that moist but not wet habitats are much more likely to have root disease present than dry habitats. The third observation is that tomentosus root disease was very uncommon in pure pine stands. Inonotus tomentosus was quite common and easy to find in mixed stands of spruce and pine which generally grow on moister sites than where pure pine is found.

Relationships between I. tomentosus and habitat type have not been studied in B.C., but observations made during stand selection for this study strongly suggest that areas with moist but well drained soils support more extensive disease development than areas with dry soils. These observations agree with Merler's (1984) ecological classification of his five study sites which were chosen for the presence of I. tomentosus. Elsewhere in Canada, Loman and Paul (1963) found I. tomentosus decay in Alberta lodgepole pine stands in which white spruce was also a major component. In Saskatchewan and Manitoba VanGroenewoud and Whitney (1969) described

moist, low pH soil types that were most conducive to disease development in white spruce.

Distribution of I. tomentosus in stands selected for stump excavation

Within individual clearcuts, incidence of tomentosus root disease is also patchy with some large (up to six stumps) clumps of colonized stumps occupying up to .03 ha and some isolated individual colonized stumps. Other studies found opening sizes in mature forests ranging from .001 ha to .04 ha (occasionally as high as .4 ha) (VanGroenewoud and Whitney, 1969; Myren and Patton, 1971).The patchy occurrence of I. tomentosus, the occurrence of single, colonized stumps, and the relatively small size of groups of colonized stumps indicates that the fungus is not only spread by means of root contacts. The observed pattern could be explained several ways. For example, vegetative spread may predominate, with disease centres made discontinuous by trees which escape infection. Alternatively, repeated spore infections may be important with each infection expanding outwards to produce several disease centres. Phellinus weirii, which is spread primarily by root contacts, produces relatively large, continuous root disease centres (Childs, 1960). Heterobasidion annosum on the other hand, infects stump surfaces and fresh wounds by spores in addition to spreading by root contact (Rishbeth, 1951). Disease centres are usually quite small; Stenlid (1985) identified nine clones of H. annosum within a 60 x 60 m area. Unlike H. annosum, there is no evidence to suggest that I. tomentosus spores can infect stumps through the cut surfaces.

The population genetics of I. tomentosus have been investigated

using vegetative compatability and protein profiles as measures of genetic similarity (see chapter IV). Both the vegetative compatability and protein profile analysis showed that while some disease centres consist of a single genotype, others are composed of two or more genotypes. The high numbers of vegetative compatability groups or unique genotypes observed within a single stand of two to three hectares suggests that disease centres are established from separate spore infections, with subsequent root to root colonization of adjacent trees. The runs analysis (Pielou, 1965) performed on the stump survey data showed that the presence of a diseased stump in one location positively affects the probability of finding the fungus in neighbouring stumps. Such a result could arise from root contact spread of the fungus from tree to tree prior to harvest. One could also speculate that sporophores were produced near the established disease centres in the old growth stand and that limited transport of the spores gave rise to new centres near old ones, resulting in the grouped pattern of disease centres. Pielou's method of analysis of run lengths of diseased trees (stumps), also includes an analysis of run length of healthy trees which is used to test the hypothesis that the patches of diseased trees are located randomly throughout the stand. One of the shortcomings of the present study is that the healthy stump run lengths were not recorded. However, observations made during the preliminary surveys for site selection, and the fact that some stump survey transect lines yielded no or very few diseased stumps while other lines had many, support the suggestion of grouped disease centres.

Such a pattern of grouped clumps has serious implications with

regard to design of surveys for I. tomentosus incidence. Surveys either in clearcuts or stands need to be extensive and systematic, giving thorough coverage of the area of interest, to avoid missing grouped disease centres entirely. The frequency of colonized stumps per site generally was low relative to the total number of stumps, therefore the sampling method would need to be quite intensive as well. Bloomberg's (1980) survey method for Phellinus weirii using transect lines in grids should give appropriate coverage for I. tomentosus surveys. P. weirii centres are often larger than I. tomentosus centres, therefore transect line width should be greater for I. tomentosus surveys. In this study, ten metre wide transects efficiently located colonized stumps. From the data in this study, the average proportion of infected stumps from pure spruce, pure pine and mixed spruce-pine sites is P= 0.151, 0.057 and 0.106 respectively. These estimates are used to calculate sample size where $n = (t_{a/2}/\text{error})^2(p)(1-p)$. To obtain an estimate of P with 0.02 error with 90% confidence, 870 spruce, 245 pine and 640 stumps in a spruce-pine mixed stand must be examined. Using the average number of stumps per 25 m section of 10 m wide transect, these figures translate to 3175 m, 685 m and 1500 m of transect line respectively. If the allowable error is increased to 0.03, the transect line lengths are halved. It is suggested that these figures be used for stands approximately 30 ha or less, sample size should be adjusted accordingly for larger stands. Phellinus weirii surveys by Bloomberg's method use only above ground symptoms for identification of root disease centres. Inonotus tomentosus centres consist of few trees and symptoms are often difficult to distinguish, therefore all

suspect trees should be root sampled. Inonotus tomentosus in B.C. occurs most frequently in moist habitats. Further detailed work on disease incidence and distribution relative to habitat types would be extremely valuable for hazard ratings, stratification of surveys and as a basis for establishment of operational control trials.

The relationship between stump surface decay and root colonization is very good for spruce stumps (average =82%) and poor for pine stumps (average =35%). Stump top surveys would be suitable for identification of the presence or absence of root disease in clearcuts with spruce stumps or spruce with a minor pine component, but not for sites with pine as the major species. The presence of decay at the stump surface is dependent upon the degree of colonization of the roots and bole prior to harvest and the height of stumps; winter logging leaves high stumps therefore the root rot is less likely to be evident at the stump surface. There was no apparent increase in stump surface decay with an increase in clearcut age suggesting that following harvest, saprophytic colonization of the stump surface by decay present in the roots is limited. This limitation may be due to colonization of the stump by cubical brown rot fungi which eventually replace I. tomentosus in the stump, or dessication of the stump surface.

Longevity of viable mycelium, location of mycelium and frequency of fungal transfer to surrounding regeneration

The fungus remains viable in spruce and pine stump roots for at least 30 years. In many cases, the mycelium in the 24-to 30-year-old stumps appeared vigourous and was easily isolated from wood samples. It is quite probable that mycelium viability, and therefore

infectivity, extends significantly beyond 30 years. Furthermore, the location of I. tomentosus in both spruce and pine roots in stumps of all ages studied is such that the fungus is in a position to cause infection should a tree root contact the stump root. It is interesting to contrast these results with those for P. weirii. In Douglas-fir, P. weirii remains viable in stumps for about 50 years (Hansen, 1979; Wallis and Reynolds, 1965). However, P. weirii is often confined to the area it occupied prior to harvest and may not extend through the root wood to the surface. Ectotrophic mycelium on stump roots is often patchy and confined to those roots with bark (Hansen, 1976). In the case of P. weirii, transfer to surrounding regeneration is therefore likely to be less frequent than that observed with I. tomentosus.

Schulting's (1987) work on I. tomentosus in spruce and pine trees revealed a basic difference in the behaviour of the fungus in the two tree species. In spruce, I. tomentosus caused a butt rot and weakening of the structural root system. In pine, the fungus caused a necrosis of the cambium and phloem. The differences observed in the present work in location of the fungus in spruce and pine stumps support the different behaviours in spruce and pine trees noted by Schulting. A general description of the pattern of colonization in stumps in each of the two species follows.

Spruce stumps

Whitney (1962) observed that in large living spruce roots, stain and decay in the heartwood may precede the infection in the sapwood and bark by up to a metre. Radial spread to the bark was much slower than longitudinal growth in the heartwood. Observations in the

present study suggest that incipient decay is common in roots of two-year-old spruce stumps but is rare in older stump roots which are often completely colonized by I. tomentosus. After tree death, decay spreads radially from the centre of the root suggesting that host responses such as the mobilization of phenolic compounds (Johansson and Stenlid, 1985; Shain, 1967; Stenlid and Johansson, 1986) encumber radial spread in living tree roots. After the tree is cut, the central core of decay or stain expands radially and the fungus penetrates the bark so that the root cross section becomes completely colonized as observed in stumps older than 13 years. Total root length and colonized root length decrease similarily and logarithmically such that as age increases the rate of decrease in root length and colonized length decreases. Therefore, the percent of root length colonized does not change over time. This and the lack of incipient decay in stumps greater than 13 years old suggests longitudinal expansion halts shortly after harvest and roots are gradually colonized from their distal ends by other decay fungi.

Maximum colonization of the root system is reached between 15 and 20 years after harvest. Brown cubical rots caused by other fungi that invade the stump from its surface grow down the roots and eventually replace the I. tomentosus decay. Such replacement may explain the decrease in colonized root length over time.

Pine stumps

Upon harvest of infected pine trees, I. tomentosus, which is often already in the cambial region of dead or dying roots, continues to survive in the cambium. In pine stumps therefore, the fungus is available at the root surface to cause infection soon after harvest

and can remain in the cambium and bark for at least 30 years. Unlike spruce, colonized root length did not decrease significantly over time, perhaps because I. tomentosus in pine roots is seldom replaced from the stump end by brown rot fungi. Pine stump roots and the stump itself rarely become fully colonized by I. tomentosus, perhaps because of the resinous nature of pine. The lack of change of colonized length over time also suggests that post-harvest longitudinal spread of the fungus is lacking.

In both spruce and pine, partially colonized roots are often more degraded by saprophytes than fully colonized roots. Once it occupies the root, I. tomentosus colonization appears to delay colonization and breakdown of the root by other saprophytes. The fungus colonizes root surfaces and organic matter surrounding colonized roots, which are areas where many other soil microorganisms may be found. This suggests that I. tomentosus has the ability to slightly extend from the decayed root centre, which it dominates, to a more competitive environment.

Root contacts or very close associations between stump and tree roots are necessary for infection to occur. The closer to the stump a tree is planted, the greater the chance for a root contact to occur. Decay in spruce stump roots occupies an area of 8.3 m^2 around the stump (assuming roots grow straight out from stumps). Furthermore, a greater percentage of spruce roots are colonized compared to pine stump roots. Trees planted within approximately 200 cm have a much better chance of becoming infected (25%) than do trees planted outside a 200 cm radius (10% chance of infection at 375cm) (calculated by regression equations, Table II.8). Decay in pine

stump roots occupies an average of 3.4 m^2 around pine stumps. Therefore, pine stumps with shorter, more vertically oriented roots do not pose a signicant threat until regeneration is established within approximately 100 cm (25% chance of infection at 50 cm; 10% at 275). The percentage of pine compared to spruce regeneration which come into contact with stump roots is not statistically different. However, it is possible that as the stand develops, infected spruce trees with a horizontal rooting habit would lead to larger disease centres than pine trees with a vertical rooting habit. Mycelium of I. tomentosus does not grow through mineral soil but can grow on woody organic matter in the duff (personal observations). Therefore roots may be in close association and still become infected across a short bridge of organic matter. Superficial mycelium is often observed on root surfaces (both the inoculum source root and the regeneration tree root). Although intensive observations on infection biology were not made in this study, lesions were observed in the cambium and wood of tree roots in contact with stump roots. The fungus appeared to penetrate at areas of thin bark on small diameter roots (less than 2 cm) and at disruptions in the bark (such as branch points) in larger roots (greater than 2.5 cm).

The results of this study can be used to estimate the rate of infection of new trees planted on a site with a history of root disease.

For spruce stumps: From the oldest clearcuts (26 and 30 years) the average number of regeneration trees in contact with stump roots is 1.5 trees/colonized stump. The infection to contact ratio is .50,

therefore 0.75 trees/colonized stump (1.5 trees/stump x .50 infections/contact) become infected. Estimates of the number of colonized stumps/ha are obtained by transect surveys. For a site with 20 colonized spruce stumps/ha it is estimated that 15 regeneration trees will become infected (20 stumps x .75 trees/stump).

For pine stumps: roots of each colonized stump contact an average of 1.1 trees (calculated from the 24 and 30-year-old sites). The proportion of trees that become infected per stump is 0.32. For a pine site with 20 colonized pine stumps/ha, an estimated 7 trees will become infected.

Sources of error in these estimates lean generally towards underestimates. Root contacts which once existed may not be observed during sampling, especially of pine stumps older than 15 years, because of decay of the stump roots by I. tomentosus and other fungi. The number of root contacts increased with stump age but the figure used in these calculations was an average over two ages and therefore is less than that which could occur over a complete rotation. The proportion of root contacts leading to infection may be underestimated because of the time required for the fungus to expand radially to the stump root surface (in spruce), and to grow across the contact and along the tree root to a suitable point for infection. It is also possible that the infection rate of less than one is not an underestimate and that root contacts play a smaller role in disease spread in these young second growth stands than that assumed earlier. The additional information on clone size (Lewis chapter IV) and the observations of small but numerous and clumped

disease centres suggest that spores are potentially very important in spread of I. tomentosus. Assuming that the calculated infection rates are not underestimated, one would expect the number of infected trees to decrease from one rotation to the next. However, there is enough evidence of spread by spores to suggest that once the fungus in disease centres established by root contacts begins to reproduce, further infections will result from spores.

There are several characteristics of plantation management and root disease biology that make even the conservative estimates of infection rates cited above of significant concern. One of these is that prompt crop re-establishment on root disease sites will allow more time for initial stump root-tree root contacts to develop, and therefore more time for development of the fungus in the individual trees leading to increased losses. Furthermore, early establishment of root disease allows more time for tree to tree spread and greater opportunity for sporophore production and possible infection of trees by spores, such that larger areas will be occupied by a greater number of root disease centres. In British Columbia, plantations are usually established within 3 years of harvest, are re-entered several times until the trees are declared "free to grow" (free of brush competition), and then are often not entered again unless the stand is to be spaced. The process of infection may take several years, and symptoms are not expressed until at least one root is well colonized. Therefore, even on sites with a history of heavy root disease where second growth trees become infected soon after planting, infection, or potential infection of these trees will go unnoticed for many years. Even with shortened rotations of 80 years,

damage can be considerable when trees have been infected for 60 of those years. Losses will be directly affected by the number and size of disease centres and the number of trees with severe root colonization in the previous stand, and the density and species of the regeneration.

This study has furthered our understanding of what happens to I. tomentosus in tree stumps following harvest, the number of root contacts and regeneration tree infections to expect within a 30 year period, the relationship between distance from stump to tree and regeneration infection, and some differences in disease spread between spruce and pine stumps. In order to understand the degree of damage to be expected, further studies are necessary to collect basic information on the effects of a regeneration lag, stand density, natural compared to artificial regeneration, alternative harvesting systems and rotation length. Such information could be used directly for harvesting and regeneration prescriptions or it could be incorporated into a model and used as a tool to examine stand development over time and the effects of different management strategies. Current studies are addressing the regeneration species effect, and the role of spores in disease spread.

This work provides several general conclusions with respect to the potential for infection of second growth stands from old growth stumps in north-central and north-western British Columbia.

1. The disease is common in second growth stands in the Prince Rupert and Prince George Forest Regions although it is patchily-distributed among stands. In moist habitats I. tomentosus can be expected in 30 to 40% of spruce and mixed spruce-pine stands.

2. Stump surface decay reliably predicts root decay in spruce stumps (67 to 93% of stumps with root disease also had surface decay), but less reliably in pine stumps (0 to 75% prediction).

3. Within a stand, stumps colonized by I. tomentosus are distributed in a clumped pattern, with portions of the stand containing up to 40 clumps/ha of colonized stumps and other portions with none.

4. I. tomentosus can remain viable in stumps for at least 30 years. The often healthy appearance of the mycelium in decay columns suggests mycelium viability extends well beyond 30 years.

5. The fungus can be found in roots at some distance from the stump (4-5 m) and is often in a position (within 1 cm of the root surface) to cause an infection should a root contact occur with a regeneration tree.

6. The number of stump to tree root contacts and the number of resultant infections increases as the regeneration becomes older. Forty percent of the regeneration trees growing closest to spruce stumps and 30% of those closest to pine stumps are expected to be in root contact with the stumps. Of these 20% and 10% respectively, may become infected.

7. The number of root contacts with regeneration trees and the number of infections decreased linearly with increasing distance from the stump. Trees within 200 cm of spruce stumps have a 25% chance of infection; at 350 cm - 400 cm the chance of infection drops to 10%. With pine stumps, trees within 50 cm have a 25% chance of infection and trees within 250 cm - 300 cm have a 10% chance of infection.

8. Spruce stumps are likely to cause more infections of regeneration than are pine stumps. As stated above, spruce stump roots cover more area than pine stumps (8 m^2 and 3 m^2 respectively), and the ratio of root infections to contacts is greater in spruce stumps because more of the roots contain inoculum.

Chapter III. Vegetative compatibility and electrophoresis groups of Inonotus tomentosus and the role of spores in disease spread

INTRODUCTION

Inonotus tomentosus causes a white pocket rot root disease of conifers in the boreal forests of North America (Whitney, 1962). Most reports of this disease are from natural old growth forests of spruce and pine species (Merler et al., 1988; Van Groenewoud, 1956; Whitney, 1973).

Tomentosus root disease has also been reported to cause losses in young stands in eastern Canada and the northern U.S.A. (Myren and Patton, 1971; Whitney, 1980). In British Columbia, excavations of spruce (Picea glauca x engelmannii) and pine (Pinus contorta) stumps and planted second growth trees show that the fungus infects the young trees from inoculum in stumps (chapter II). Basidiospores may provide another means of disease spread. The purpose of this study is to determine the role of basidiospores in disease spread.

Inonotus tomentosus sporulates prolifically in the late summer and fall of wet years, but the importance of spores in disease spread had not been assessed. Germinating basidiospores were observed by Whitney (1962) beneath sporophores, but inoculations performed with basidiospore suspensions were successful only in deep root wounds (Whitney, 1966).

In general, spores of wood decaying basidiomycetes germinate to produce homokaryotic, haploid mycelia which grow until another mycelium carrying an unlike mating allele is met. Anastamosis and nuclear migration occur and the ensuing dikaryon grows vegetatively

and is responsible for most of the decay. In contrast, when unrelated dikaryons or heterokaryons meet they produce a reaction line between them (Vegetative incompatibility (Vc) reaction) which prohibits nuclear exchange. In a woody substrate, the reaction is called a zone line (Rayner and Todd, 1977); the reaction is readily demonstrated on synthetic media (Adams and Roth, 1967; Todd and Rayner, 1980). Such vegetative incompatibility prevents somatic integration between genetically distinct individuals (Esser and Blaich, 1973; Todd and Rayner, 1980) and is a useful indicator of unique genotypes or vegetative compatibility (Vc) groups.

Ascomycetes such as Cryphonectria and Ophiostoma also show vegetative incompatibilty. The reaction is heterogenic and controlled by several loci (Anagnostakis, 1977 and Brasier, 1986 respectively). Less is known about genetic control of Vc in basidiomycetes, but it has been shown that heterogenic incompatabilty also governs Vc reactions and is thought to be polygenic (Adams and Roth, 1967; Barrett and Uscuplic, 1971; Hansen, 1979; Stenlid,1985).

Root disease-causing Basidiomycetes spread from one tree to the next by root contacts or by spores. Spread by root contacts results in outward expansion of a single genotype. On the other hand, basidiospores are products of recombination, therefore each spore may have different allele combinations at the Vc loci. In a stand of trees infected by a fungus spread only by root contacts, all isolates are of the same genotype. In a stand of trees infected by multiple spore infections, isolates of the fungus represent different genotypes, their individuality preserved by vegetative incompatibility.

Childs (1963, 1970) cross-plated isolates of Phellinus weirii collected from disease centres in a Douglas-fir stand. He found large (up to 50m in diameter) "clones" (vegetative compatibility groups) which encompassed multiple disease centres. Vc reactions have also been used to document the continuity of P. weirii infections from one rotation to the next (Childs, 1970; Tkacz and Hansen, 1982). These studies and evidence from root excavations of infected trees (Wallis and Reynolds, 1965) suggest that P. weirii spreads primarily by root contacts.

In contrast to P. weirii, each tree colonized by the root pathogen Phaeolus schweinitzii has a unique genotype of the fungus (Barrett and Uscuplic, 1971; Childs, 1937). Subsequently Barrett (1985) determined that basidiospores and mycelium of P. schweinitzii were common members of the soil microflora. He suggested that soil infestation from basidiospores can explain why each tree contained a different genotype of the fungus.

Stenlid (1985) working with Heterobasidion annosum, identified nine Vc groups within a 60m x 60m area and the number of trees in one group ranged from 1 to 13. Furthermore, each Vc group had different mating type alleles although several Vc groups shared one common allele. This work indicates a role for both basidiospores and root contacts in Annosus root disease spread.

Isozyme analysis has also been used to define populations within a species, for example the biological species of Armillaria (Morrison et al., 1985), the host variants of Leptographium wageneri (Ostrosina and Cobb, 1987), or several closely related species of Polyporus (Shannon et al., 1973). Total protein electrophoresis is a

more sensitive indicator of variation than isozyme analysis. Hansen et al. (1986) found protein profiles sensitive enough to distinguish groups within the Phytophthora megasperma complex. The use of protein profiles to identify different genotypes of the same fungal species depends on the degree of genetic variation between the genotypes and the degree of resolution of individual bands on the gel.

The objective of this study is to assess the importance of spore infection in tomentosus root disease spread using 1) vegetative compatibility analysis, and 2) protein profiles.

MATERIALS AND METHODS

Collection of Isolates

To observe the upper limits of genetic diversity by Vc and protein profile analysis, geographically diverse isolates of *I. tomentosus* were obtained in British Columbia (Fig. III.1), eastern Canada, Alaska and the north central U.S.A. (Table III.1). Four isolates collected in B.C., including two from the same disease centre (AV-1 and AV-2) were included to provide continuity between the comparison of geographically diverse isolates and the comparison of isolates collected in close proximity to each other described below, and as a check to establish the lower limits of genetic diversity.

To compare diversity within disease centres (groups of symptomatic or dead trees) and between disease centres at various distances apart, five sites each with several disease centres were selected for intensive isolate collection in the Prince George and Prince Rupert Forest Regions of north-central B.C. All stands were virgin timber, less than 120 years old, probably established after fires. In the Prince George Forest Region, collections were made at Averil Lake (AV), Jerry Creek (JC), Pelican Forest Rd. (Pln), and Bobtail Forest Rd (Bob). In the Prince Rupert Forest Region, a collection was made near Smithers (SM) (Fig. III.1).

At the Averil and Smithers locations a 50 m x 50 m square plot, which included several disease centres, was marked in the field and mapped to scale. Ten disease centres outside each plot were also located and referenced to the plot by distance and bearing. These outlying centres were at various distances from the square. At the

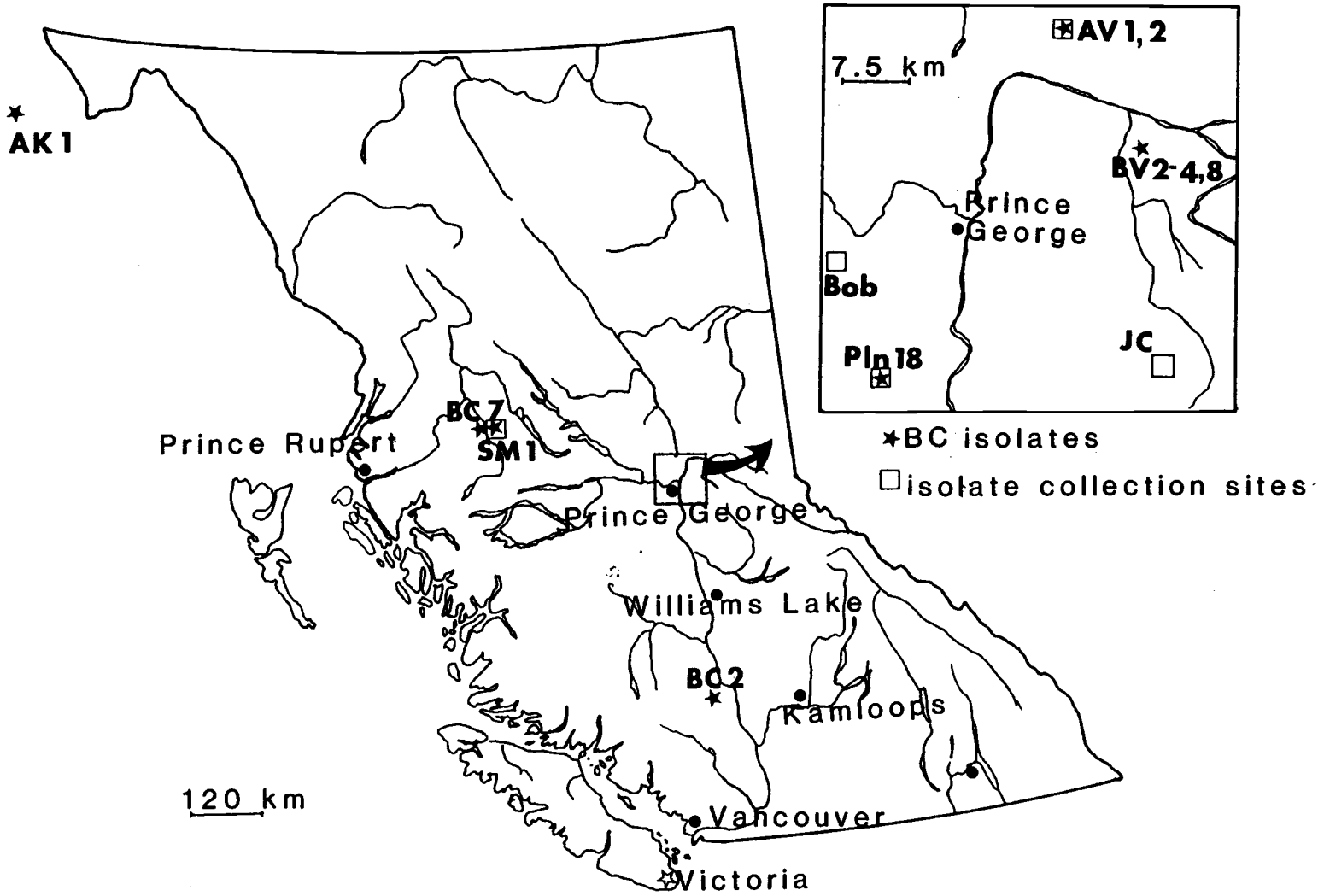


Figure III.1. Location of isolate collection sites in British Columbia

Table III.1. Origin of Inonotus tomentosus isolates collected from geographically diverse locations.

Isolate	Region	Host	Sample	Donor
Ak-1	Alaska, Kenai	P. glauca	decay	PH[1]
BC-2	Lillooet, B.C.	Pseudotsuga menziessi	decay	DM[2]
BC-7	Smithers, B.C.	Abies lasiocarpa	decay	DM
WH-85	Grand-mere, Que.	P. glauca	decay	RW[3]
WH-110	Tobin Rapids, Sask.	P. glauca	decay	RW
WH-140	Candle Lake, Sask.	P. glauca	sporophore	RW
WH-91	Finland	Picea exelsa x fennica	decay	RW
BV 2-4	Beaver R. B.C.		single spore	KL[4]
BV 2-8	Beaver R. B.C.		single spore	KL
AV-1	Averil lake, B.C.	P. glauca x engelmannii	decay	KL
AV-2	Averil Lake, B.C.	P. glauca x engelmannii	decay	KL
Bob-11	Bobtail Rd, B.C.	P. glauca x engelmannii	decay	KL
Pln-8	Pelican Rd, B.C.	P. glauca x engelmannii	decay	KL
KL-2	Missoula Montana	Pinus monticola	decay	USFS[5]
Mad-2	State col. Penn.	Pinus rigida	decay	LO[6]
Mad-3	Arlington, VA		soil, small roots	RD[7]
Wal-1	Wallowas, Idaho	P. engelmannii	decay	KL

1. Paul Hennon, USDA Forest Service
2. Duncan Morrison, Forestry Canada, Victoria, B.C.
3. Roy Whitney, Forestry Canada, Sault Ste. Marie, Ont.
4. Kathy Lewis, Oregon State University.
5. U.S. Forest Service, Missoula Montana
6. L. Overholts, from the Forest Products Lab. Madison, Wisconsin
7. R. Davidson, from the Forest Products Lab. Madison, Wisconsin

Averil location, all trees (dead, symptomatic and healthy) in the square area were mapped. In the Smithers collection, only symptomatic trees were mapped. At the other three locations (Jerry creek, Bobtail and Pelican), ten disease centres were identified in the field and located on a map (no 50 m x 50 m plot). For the Averil and Smithers sites, root samples from all dead and symptomatic trees within the plots and from 2-3 trees in each outlying centre were removed. For the remaining sites, one to three trees in each disease centre were sampled. Small subsamples from the root pieces were plated onto 3% malt extract agar (MEA) for isolation of I. tomentosus.

Vegetative compatibility analysis

From each of the five British Columbia collections, 14-18 isolates were selected to allow comparisons both within and between disease centres. Isolates from each collection were paired in all possible combinations on 3% MEA. In addition, the collection of geographically diverse isolates was paired in all possible combinations. Isolates were paired using plugs of colonized agar (0.8 cm diameter) placed 1 cm from each other, hyphae down. Following incubation for 4, 6 and 8 weeks, the plates were examined for the production of lines between the isolates. At each examination of the plates, line reactions and changes in morphology were drawn and rated as 0 (no reaction, compatible); 1/2 (no intermingling of hyphae but no line formed, reaction uncertain); 1 (slight line, no heavy pigmentation, incompatible); 2 (light pigmentation or raised line, incompatible); and 3 (heavy pigmentation and/or raised line, incompatible). Notes were made on the colour and

morphology of each of the paired isolates and on changes that occurred in the reaction line over time.

Protein electrophoresis

Protein extracts were prepared from each isolate used in the pairing study. The isolates were started in 30 mls liquid glucose-yeast-peptone (GYP) medium (Gill and Zentmeyer, 1978) in a 100 x 15 mm petri dish. After one week the cultures were macerated, then pieces used to inoculate 4 additional plates of GYP. Cultures were incubated at room temperature (20C) for 3 weeks, then harvested by vacuum filtration through Whatman No. 1 filter paper with 3 washes of distilled water. Mycelium was loaded into a frozen steel vessel with acid washed sand and 1 ml of 0.1M phosphate buffer pH 7.0. An electric drill with a grinding head attachment was used to grind the mycelium, then the mixture was centrifuged at 13000 g for 20 min. The clear supernatent was removed to 1.5 ml eppendorf tubes. A 0.3 ml subsample of supernatent was placed in a second tube with 0.3 ml sample buffer (2% sodium dodecyl sulphate (SDS), 10% mercaptoethanol, 0.1M TRIS pH 6.8, 20% glycerol). A second subsample (0.1 ml) was placed in a glass tube with 5 mls of BioRad protein assay buffer to analyze the concentration of proteins from each sample. The assay ensured that equal amounts of protein were loaded into each lane of the gel. SDS polyacrylamide gels were prepared and electrophoresis was carried out according to the methods of Thomas and Kornberg (1975). Gels were run at room temperature at 105v until the tracking dye ran off the gel. Following electrophoresis, the gels were removed, placed in 1% Coomassie brilliant blue R-250 (BioRad) in acetic acid, methanol, and water (1:5:5) and gently shaken overnight.

The gels were destained with several changes of ethanol, acetic acid and water (20:7:73) prior to photography.

Analysis

Protein patterns from the geographically diverse isolates were compared and similarity coefficients calculated for all possible combinations according to the formula used by Hansen et al. (1986):

similarity coefficient:

$$sc = \left(\frac{2 \times \text{number of bands in common}}{\text{bands in isolate \#1 and \#2}}\right) \times 100$$

The mean sc was calculated by dividing the sum of the sc's for all comparisons with one isolate and by the number of comparisons. This was done for each isolate.

For the isolates collected at the 5 intensively sampled sites within B.C., the pairing reactions (compatible and incompatible) and differences in protein profiles were related to the isolate maps. Isolates belonging to the same Vc group (all compatible pairing reactions) or electrophoresis group (all with identical banding patterns) were identified on the maps and the boundaries were compared to disease centre boundaries. Similarity coefficients were calculated for all possible isolate-pair combinations in each gel.

All sc values from pairings which also showed compatible Vc reactions were grouped together; likewise, all sc values from pairings which showed incompatible Vc reactions were placed in a second group. The mean sc of each group was calculated and compared using a Student's t-test.

RESULTS

Vegetative compatibility

All pairings between geographically diverse isolates of I. tomentosus showed incompatible reactions. Incompatible reactions generally began as a gap between the two isolates (3-4 weeks), followed by the formation of either a thick, raised white line (4-5 weeks) or a pigmented line (six or more weeks) (Fig. III.2). Reaction intensity often increased from one examination date to the next.

Two basic colony types were observed over the length of the study. The yellow-brown appressed, slowly-growing type was prevalent after isolation from decayed wood tissue. The second morphological type was apparently induced with age of the culture, from oxygen deprivation, or upon pairing with an unlike isolate. The hyphae of this second group were white and aerial, usually faster-growing and often produced dark brown or black pseudosclerotial plates, especially at the edge of the petri dish. Individual isolates were frequently observed to switch from the brown type to the white fluffy type, and pairings of white fluffy isolates sometimes resulted in one of them converting to the brown morphology. Dark brown to black pseudosclerotial plates sometimes formed between paired isolates of different morphological types. These reactions were in addition to the line reactions produced between different Vc groups and were noted but not included in the reaction rating.

Pairings among isolates from each of the 5 intensively sampled sites gave comparable results, therefore detailed results and examples are given only for the Averil and Smithers sites. Isolate

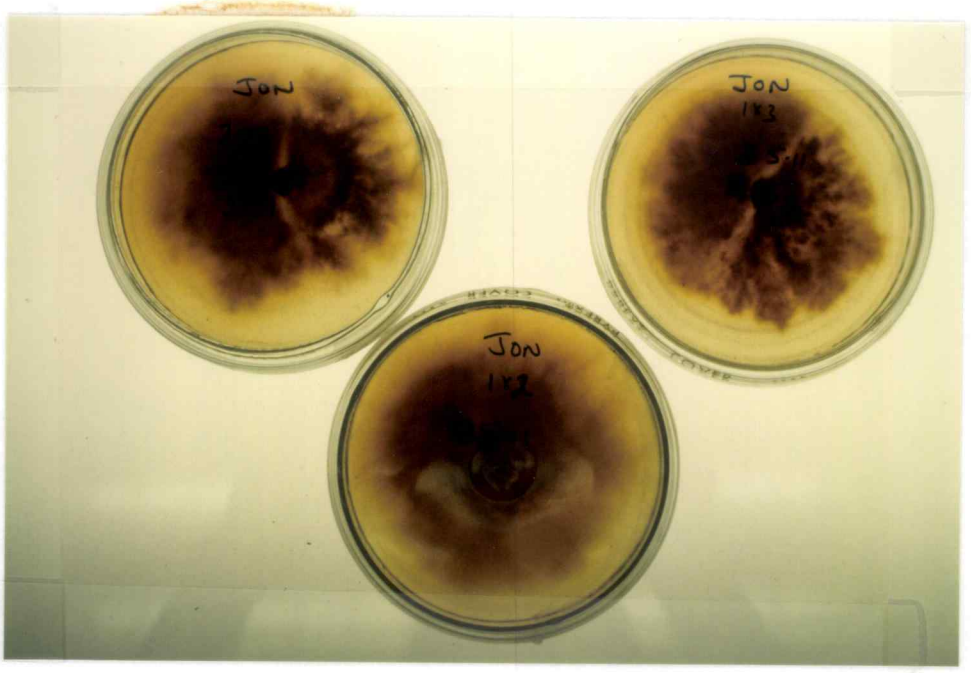


Figure III.2a. Vc reactions between paired I. tomentosus isolates. Top plates are incompatible reactions seen as gaps of sparse mycelium, lower plate is a compatible reaction where the hyphae have grown together.

Figure III.2b. Incompatible reaction between paired I. tomentosus isolates showing the two morphological states. Top left plate shows an incompatible reaction between 2 isolates of the brown, appressed morphology. Other plates show incompatible reactions between the white, fluffy morphological state.

maps, pairing and protein profile results from the remaining sites are in Appendix C and D, and are included as general results and in the discussion.

Among the Averil Lake and Smithers isolates, compatible reactions occurred only between isolates collected from the same disease centre. Five disease centres (I to V) well removed from each other in the Smithers site (Fig. III.3) were all unique Vc groups. Two Vc groups were defined from the large, patchy disease centre (VI) in the 50 m x 50 m plot at Smithers. Isolates 1, 2, 11 and 12 were compatible with each other but incompatible with isolates 4, 5, 6, and 7. Therefore, from 6 apparent disease centres identified in the Smithers plot, 7 Vc groups were identified. The number of Vc groups in the Averil area (Fig. III.4) was greater because several of the disease centres apparent in the field were composed of two or more Vc groups. Twelve Vc groups were identified in the seven disease centres that were sampled. Compatible pairings occurred between isolates 1, 2 and 3 of centre II, isolates 13 and 29 of centre VI, isolates 18 and 19 of centre VII, and between 20 and 21 of centre I. All other isolates were incompatible in every pairing. Pairings of isolates from JC, Pln and Bob also showed incompatibility between centres and compatibility within centres. There were three cases where two Vc groups were found in one isolated disease centre.

Distance between isolates had no effect on the intensity of the line reaction. For example, Averil isolate 10 was paired with isolates 11, 12 and 20, collected from an adjacent symptomatic tree, a tree 12 m away and a tree 400 m away respectively. The reaction intensities were 3, 1 and 3 respectively.

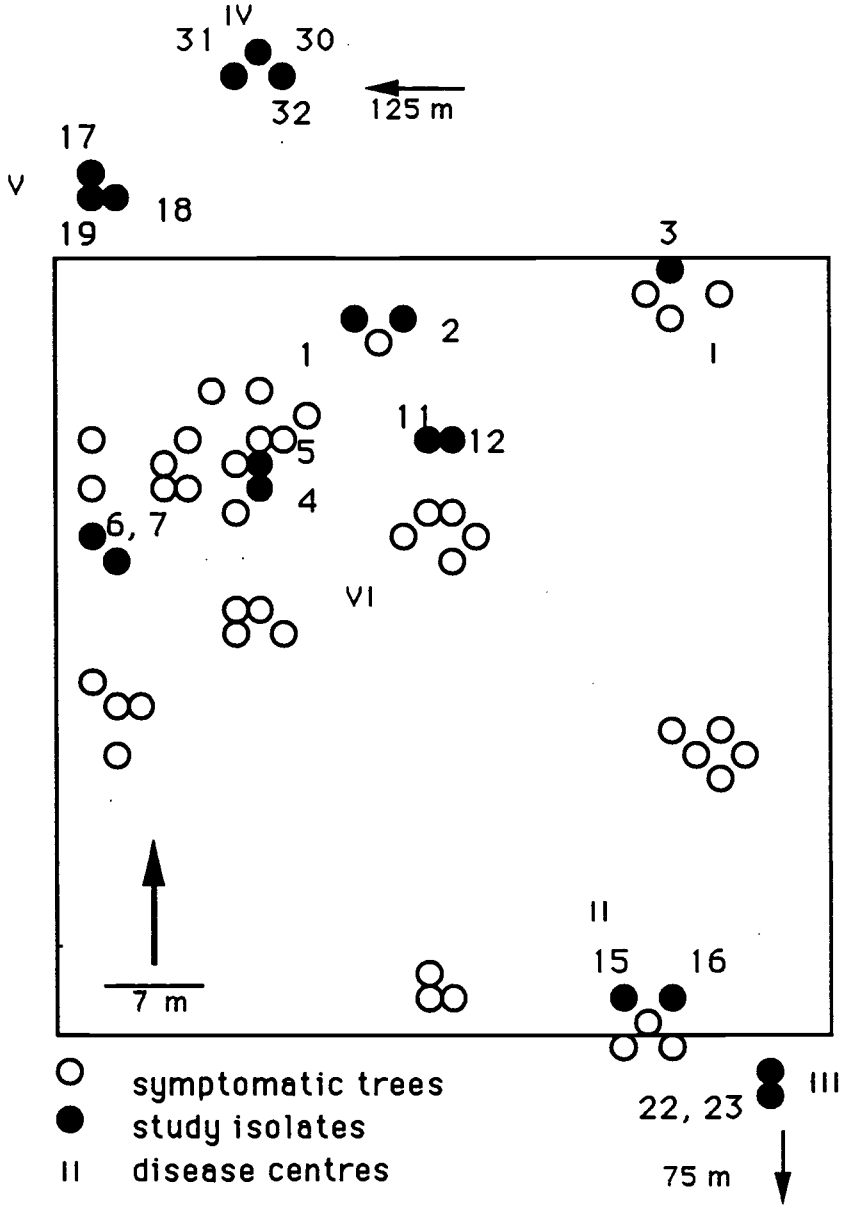


Figure III.3. Map of the Smithers collection site.

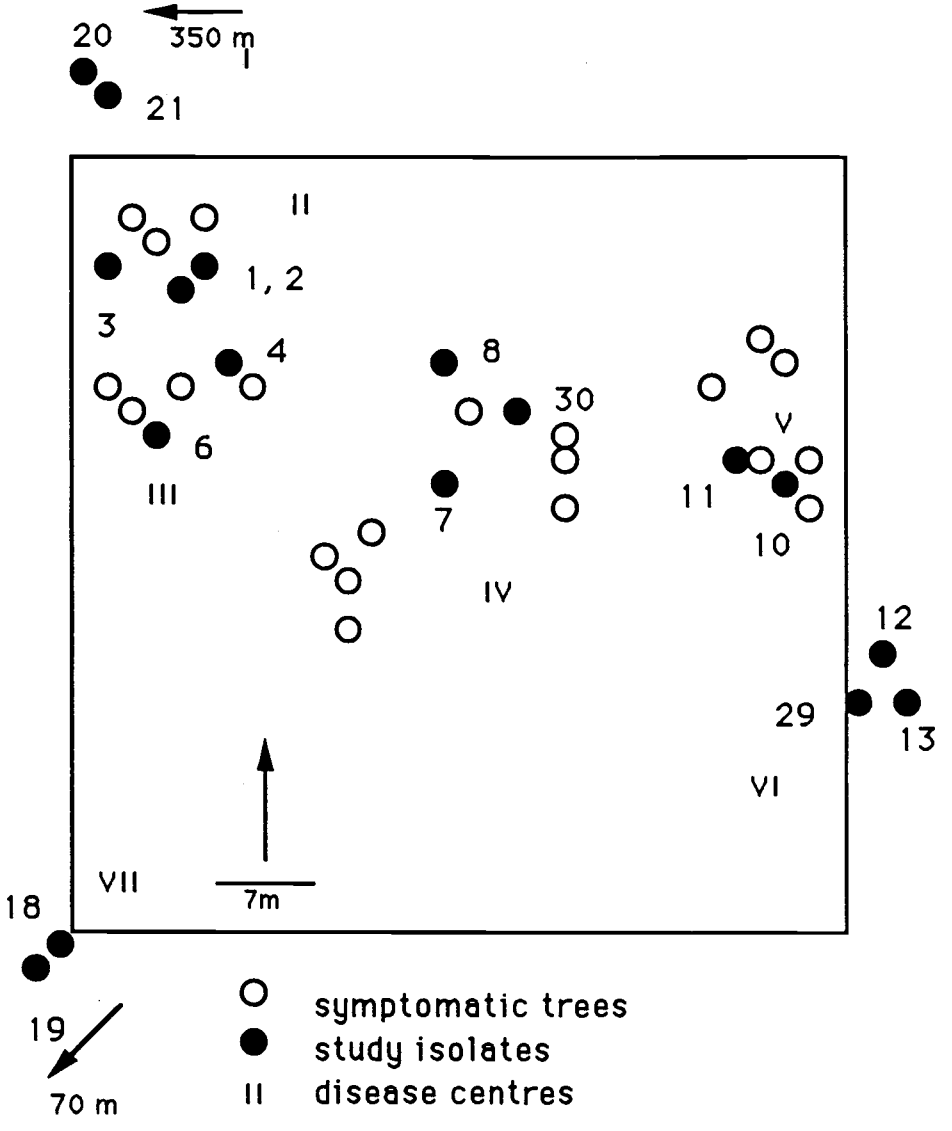


Figure III.4. Map of the Averil collection site.

Protein electrophoresis

For all analyses of protein profiles, differences in band intensity were not used; only differences in the band pattern (presence or absence of bands) were counted. The number of bands counted and compared ranged from 25 to 40. Bands at each end of the profiles were not counted because they were often faint or blurred.

All isolates from various geographic locations compared to define the upper limits of diversity, showed similar protein profiles, but none were identical except for the two Averil (AV) isolates. These were from the same disease centre and were included to define the lower limits of diversity. Similarity coefficients for the isolate comparisons ranged from 75.5% to 98.0% (Table III.2). The average estimate of similarity in banding patterns between the geographically diverse isolates was 91.4%. The only isolate not from the North American continent was WH-91, from Finland, which showed the least similarity to the other isolates (average sc = 85.9%). Two single spore isolates from one sporophore (BV 2-4, BV 2-8) had two protein polymorphisms (Fig. III.5). Host species did not relate to protein profile patterns: several isolates from the same host had different patterns (e.g. Ak-1, WH-85, and Sm-1, all isolated from Picea glauca).

Protein profile comparisons of isolates collected from trees within and between disease centres at the five sites in B.C., showed identical patterns in most cases for isolates within Vc groups, and different patterns for isolates from different Vc groups. Similarity coefficients for all Averil and Smithers isolate comparisons are presented in Table III.3. Comparisons which also showed compatible

Table III.2. Similarity coefficients calculated from total protein profiles of geographically diverse isolates of I. tomentosus.

	BC-2	BC-7	WH-85	WH-140	WH-91	BV2-4	BV2-8	AV-1	AV-2	SM-1	KL-2	WAL-1	MAD-3	PLN-8	BOB-11
Ak-1	94.9	93.1	85.7	94.1	76.0	91.7	88.0	89.4	89.4	89.8	90.2	88.0	92.3	89.8	91.7
BC-2		92.3	75.5	90.9	81.5	88.5	90.6	90.2	90.2	90.6	94.5	96.3	83.0	90.6	94.1
BC-7			82.3	85.2	79.2	92.0	94.3	90.2	90.2	94.3	96.3	88.9	85.7	94.3	92.3
WH-85				92.0	90.2	89.8	93.9	87.5	87.5	85.7	90.2	92.0	82.3	81.6	87.5
WH-140					83.0	92.0	88.5	87.5	87.5	92.3	88.9	96.2	94.5	88.5	97.9
WH-91						84.0	94.1	85.7	85.7	82.4	86.3	85.7	94.3	88.0	92.0
BV2-4							93.9	95.6	95.6	91.7	92.0	89.8	96.0	95.8	91.7
BV2-8								95.8	95.8	95.8	96.0	90.2	86.8	92.0	89.8
AV-1									100	91.7	92.0	89.8	90.2	89.4	97.9
AV-2										91.7	92.0	89.8	90.2	89.4	97.9
SM-1											92.3	94.1	90.6	96.0	93.9
KL-2												94.3	92.6	94.1	94.1
WAL-1													92.6	92.0	92.0
MAD-3														94.3	92.3
PLN-8															98.0
BOB-11															

Mean similarity coefficients for each isolate compared to the rest:

AK-1	BC-2	BC-7	WH-85	WH-140	WH-91	BV2-4	BV2-8	AV-1	AV-2	SM-1	KL-2	WAL-1	MAD-3	PLN-8	BOB-11
89.6	89.6	90.0	86.9	90.6	85.9	92.0	92.4	91.5	91.5	91.5	92.4	91.4	90.5	91.6	93.5

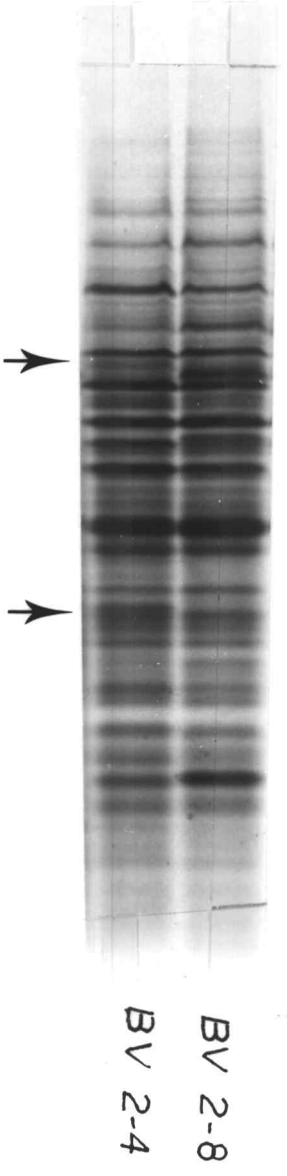


Figure III.5. Single spore isolates BV2-4 and BV2-8 from the same sporophore showing protein polymorphisms.

Table III.3a. Similarity coefficients calculated from protein profiles of I. tomentosus isolates collected from the Smithers location in British Columbia.

Smithers

	2	3	4	5	6	7	11	12	15	17	19	22	30	31
2	100	96.7	93.5	93.5	93.5	93.5	[100]	[100]	96.9	98.4	98.4	98.4	93.3	93.3
3		100	93.5	93.5	93.5	93.5	96.7	96.7	93.8	95.2	95.2	95.2	93.3	93.3
4			100	[100]	[100]	[100]	93.5	93.5	90.6	92.1	92.1	92.1	90.0	90.0
5				100	[100]	[100]	93.5	93.5	90.6	92.1	92.1	92.1	90.0	90.0
6					100	[100]	93.5	93.5	90.6	92.1	92.1	92.1	90.0	90.0
7						100	93.5	93.5	90.6	92.1	92.1	92.1	90.0	90.0
11							100	[100]	96.9	98.4	98.4	98.4	93.3	93.3
12								100	96.9	98.4	98.4	98.4	93.3	93.3
15									100	95.4	95.4	95.4	90.3	90.3
17										100	[100]	100	91.8	91.8
19											100	100	91.8	91.8
22												100	91.8	91.8
30													100	[100]
31														100

[ ] pairing also Vc compatible

Table III.3b. Similarity coefficients calculated from protein profiles of I. tomentosus isolates collected from the Averil location in British Columbia.

Averil

	3	6	7	8	10	11	12	13	18	19	20	29	30
3	100	88.9	96.3	96.3	94.7	94.7	93.1	92.9	87.7	87.7	92.6	92.6	96.4
6		100	96.3	85.2	94.7	94.7	93.1	89.3	91.2	91.2	92.6	92.6	92.9
7			100	88.9	94.7	94.7	93.1	92.9	91.2	91.2	96.3	88.9	92.9
8				100	91.2	91.2	86.2	96.4	91.2	91.2	88.9	96.3	92.9
10					100	96.7	95.1	88.1	90.0	90.0	91.2	91.2	94.9
11						100	95.1	91.5	93.3	93.3	94.7	94.7	94.9
12							100	94.9	95.1	95.1	93.1	93.1	90.0
13								100	98.3	98.3	92.9	92.9	89.7
18									100	100	91.2	91.2	91.5
19										100	91.2	91.2	91.5
20											100	96.3	89.3
29												100	89.3
30													100

☐ pairing also Vc compatible

Vc reactions are boxed. A total of 377 isolate - pairs were compared by protein and Vc groups. Vc and protein groups were the same for 356 of the comparisons (94.4%), including 73.7% of Vc compatible reactions and 96.7% of Vc incompatible reactions. Band differences were usually limited to one or two bands for each comparison. From the large, patchy disease centre VI at the Smithers location, two electrophoretic groups were identified (Fig. III.6) which corresponded to the Vc groups defined earlier. Isolates 4, 5, 6 and 7 had a 2-band difference with isolates 2, 11, and 12 (sc = 93.5%, isolate 1 was not on the gel), but within the Vc groups the band patterns were identical (sc=100%). All sc values between Smithers isolates which showed compatible reactions were 100%. Similarity coefficients between isolates which showed incompatible reactions ranged from 90.0% to 98.4%.

Averil isolates had lower sc values, ranging from 85.2% to 96.4% between isolates with incompatible Vc reactions (Table III.3). From disease centre VI (Fig. III.4) isolates 13 and 29 were Vc compatible but had a one band difference (Fig. III.7). Isolate 12 had a one band difference and was incompatible with both 13 and 29. There was no relation between reaction intensity and sc for any of the sites. Figure 8 shows plate reactions and protein profiles of Averil isolates 8 and 30 from centre IV, and isolates 10 and 11 from centre V. When paired, 8 and 30 produced a Vc reaction rated as 3 (pigmented line) and the sc was 92.9%. Av 10 and 11 produced a Vc reaction rated as 2 (light pigmentation or raised line) with an sc of 96.7%.

For each B.C. collection sc values for each isolate comparison

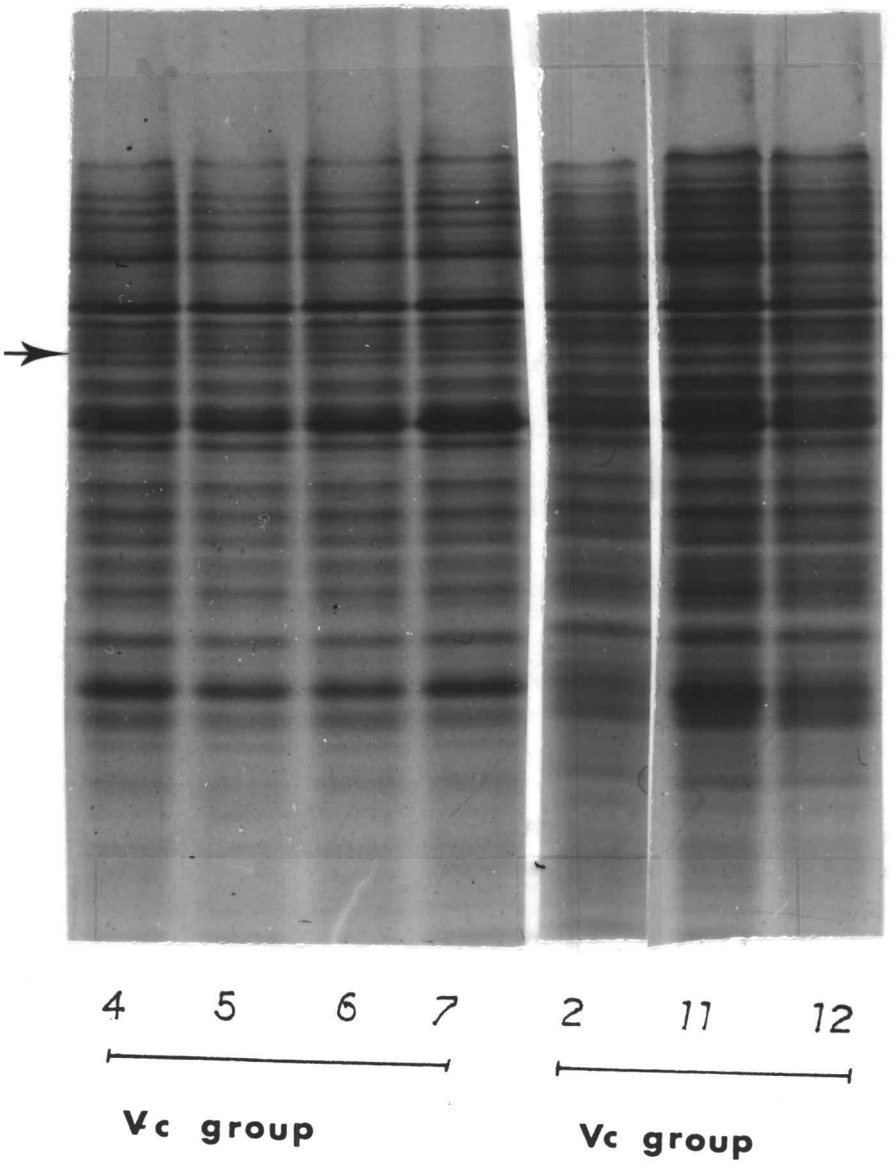


Figure III.6. Protein profiles showing polymorphisms between Smithers isolates 4, 5, 6, 7, and 2, 11, 12.

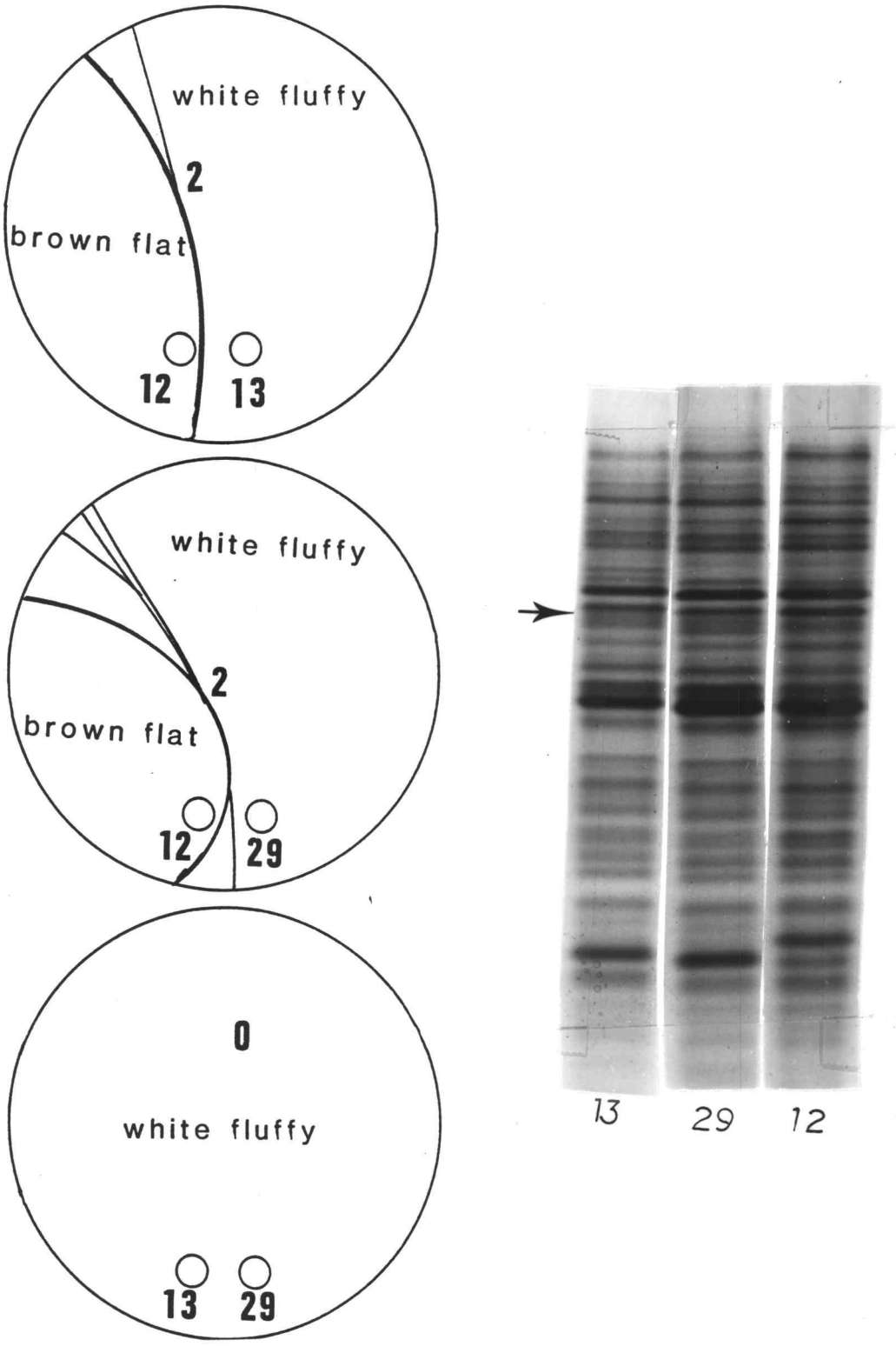


Figure III.7. Protein profiles and Vc reactions of Averil isolates 12, 13, and 29.

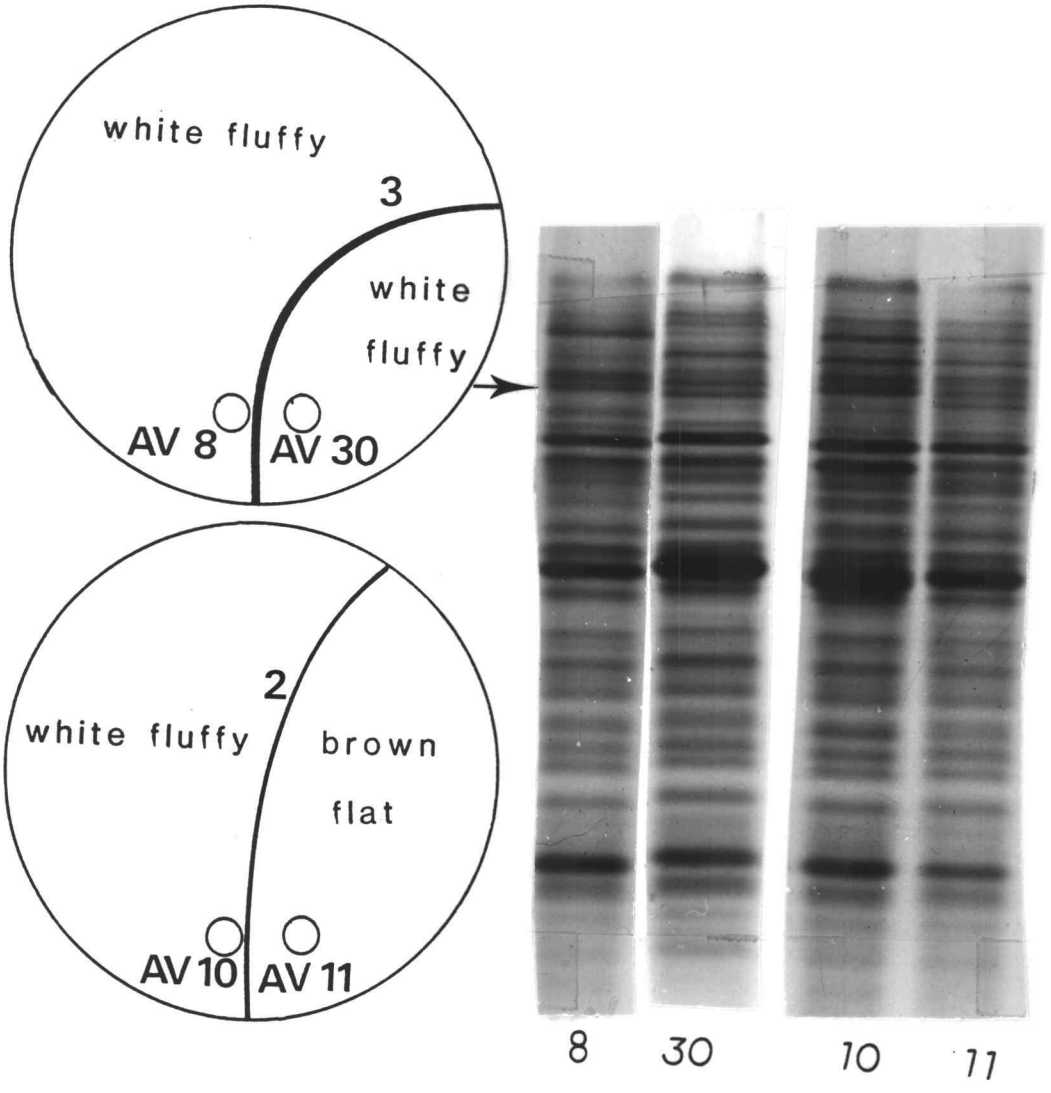


Figure III.8. Protein profiles and Vc reactions of Averil isolates 8 and 30, 10 and 11.

were grouped by Vc reaction (compatible or incompatible). Mean similarity coefficients from the compatible Vc reaction group compared to the incompatible Vc reaction group were significantly different (Table III.4).

Table III.4. Mean similarity coefficients calculated from similarity coefficients grouped by compatible Vc reaction and incompatible Vc reaction. Comparisons of mean sc's by student t-tests.

Site		Compatible Vc	Incompatible Vc	Significance
Smithers	n	11	78	
	mean sc	100	93.5	**[1]
	std. dev.	0	6.4	
Averil Lake				
	n	2	76	
	mean sc	96.5	88.8	**
	std. dev.	16.8	4.7	
Jerry Creek				
	n	8	70	
	mean sc	99.6	95.2	**
	std. dev.	1.6	10.5	
Pelican				
	n	8	58	
	mean sc	99.0	91.8	**
	std. dev.	2.0	6.6	
Bobtail				
	n	9	57	
	mean sc	96.6	95.2	NS
	std. dev.	16.7	12.4	

1. ** = p less than 0.0001
   NS = p greater than 0.25

DISCUSSION

Pairing of isolates on MEA plates and total protein electrophoresis were both consistent and co-supportive means of determining unique genotypes of I. tomentosus. The numbers of unique genotypes that existed within one stand suggested that the genetic diversity was due to separate infections by spores.

Both methods have some limitations in interpretation of results, but these were minimized by their coordinate use. Hansen (1979) cautioned that incompatible Vc reactions indicated isolates differed genetically, but compatible reactions indicated only that the isolates had identical alleles at the Vc loci; they may differ at other loci. Therefore interpretations of genotypes based on Vc reactions have to be made with caution. Protein electrophoresis produces protein banding patterns that are a product of the genotype of the fungus as it was functioning prior to protein extraction. Differences in patterns may be due to differences in expression rather than actual allelic differences. All isolates were grown under the same conditions and for the same length of time which should have minimized differences in gene expression. Interpretation of band patterns in this study was conservative and avoided differences in protein production by not including band intensity as a distinguishing characteristic.

The switching in colony morphology typical of this fungus did not alter Vc reactions or protein profiles. Such morphological switching is not uncommon among basidiomycetes, particularly in association with dikaryotization or diploidization following fusion of homokaryotic hyphae. However, examples of vegetative

pleomorphisms, such as described here, are less frequent. Sharland et al (1986) described a very similar pleomorphism in Hymenochaete corrugata, and juxtapostion of the two colony types of this fungus (from a single Vc group) produced a dark brown pseudosclerotial plate (psp). With pairings of I. tomentosus, psp formation was often not directly between the colonies, and did not seem to be precipitated solely by hyphal interaction. Pseudosclerotial plates did form on compatible pairings, but were usually at the colony margin, not between the two isolates. An understanding of this phenomenon may be essential for a thorough understanding of Vc reactions in I. tomentosus but was beyond the scope of this study.

The amount of genetic variation in I. tomentosus as measured by Vc and protein groups was substantial. Protein polymorphisms existed even between single spore isolates from one sporophore. The level of diversity evident from comparisons of geographically diverse isolates established the basis for comparison of isolates from the same and from different disease centres.

Both the pairing study and protein electrophoresis profiles gave similar results. Protein profiles were identical between two isolates from different Vc groups in 11 of 339 (3.2%) cases. Of 30 apparently Vc compatible reactions between paired isolates, 10 (33.3%) (usually between separate and isolated disease centres) were not supported by identical protein profiles. Overgrowth of one isolate by the other was suspected in a few of these cases leading to a "compatible" classification. It was also possible that the two isolates were sib-composed heterokaryons which were known to show reduced interactions in H. annosum (Stenlid, 1985), P. weirii

(Hansen, 1979) and P. schweinitzii (Barrett and Uscuplic, 1971). If vegetative compatibility was under polygenic control, as suggested by Stenlid (1985), weak interactions may be due to common alleles at several of the loci. With I. tomentosus, the reaction intensity between juxtaposed sib-composed isolates has not been tested. It is suspected that the anomalous compatible pairings were primarily due to overgrowth of one isolate by the other.

Total protein profiles served to support the Vc group results and helped to clarify anomalous results such as stated above, but electrophoresis groups were more difficult and time consuming to determine than were Vc groups. Protein preparations of some of the isolates were degraded and had to be re-extracted from new cultures, a time-consuming process for a slow-growing fungus. Interpretation of banding patterns was difficult at times due to slight differences in the concentration of protein loaded and differences in individual band intensity. Band intensity was not used to distinguish genotypes, and highly intense bands sometimes could not be distinguished from close double bands. Only easily distinguishable band differences were used in the analysis, therefore the similarity coefficients may underestimate differences between isolates.

Disease centres observed in the field, defined by a group of dead or symptomatic trees, ranged in size from 2 to 3 trees (2m diameter) to 25-30 trees (25 to 30m diameter). In most cases, the small, isolated disease centres represented one genotype as defined by Vc and electrophoresis. The larger, more patchy centres and some of the small centres (2-3 trees) were composed of two or more genotypes. Stenlid (1985) states that "the more important airborne

inocula are in relation to vegetative spread, the smaller the clones will be in their spatial distribution." Both H. annosum and P. schweinitzii have small clone sizes and evidence of their spread by spores is convincing (Barrett, 1985; Rishbeth, 1951). The small clone size observed in this study suggested that airborne inocula of I. tomentosus were important relative to vegetative spread. In contrast, the large clone size of P. weirii suggested the opposite: that airborne inocula are not important for spread. Evidence is lacking for spread by spores and extensive for spread by root contacts with P. weirii (Childs, 1970; Tkacz and Hansen, 1985; Wallis and Reynolds, 1965).

Another explanation for the large numbers of genotypes found was that the disease centres were very old, having carried over from one natural stand rotation (fire caused) to the next. Isolated mutations or parasexual recombination occurring over time could explain the high number of genotypes. To argue against mutations, one could expect to find some discontinuous areas within the large disease centres that belonged to the same Vc group. This was not the case. Furthermore, to produce the large numbers of observed genotypes, many of the mutations would have to occur at the Vc loci, a less likely event. A mutation may occur originally in one cell. For that mutation to manifest itself, the new genotype must be successful in establishing itself in an environment already occupied by a successful individual. While mutation cannot be ruled out as cause of the genotypic variation observed, spore infections were probably the major contributor. Parasexual recombination was a possible factor in determining genetic diversity, but little is known of the

nuclear cycle of this fungus and the likelihood of parasexual recombination is unknown.

In trees or stumps decayed by H. annosum or P. schweinitzii, there exists only one clone of the fungus (Barrett and Uscuplic, 1971; Stenlid, 1985). With P. schweinitzii, Barrett and Uscuplic (1971) state that infection occurs in a single root and that multiple genotypes may exist during early stages of colonization. They suggest that the strain which first colonizes the stem base has a selective advantage and will monopolize further colonization leading to a single genotype per tree. Stenlid (1985) described similar diversity limiting mechanisms in H. annosum: low numbers of initial infections, the need for two homokaryotic mycelia to mate to cause extensive decay and the decreasing numbers of genotypes due to competition in H. annosum colonized trees. In the current study, multiple isolations were not made from single trees and some of the disease centres were represented by only one isolate of I. tomentosus. Therefore, while it can be concluded that multiple genotypes in one disease centre were common, the existence of multiple genotypes within one tree has not been tested.

Many of the collection sites showed a grouped distribution of the disease centres themselves, an observation supported by transect surveys through clearcuts which identified groups of colonized stumps (previous chapter). The number of genotypes of I. tomentosus found within a small area suggests that spores had an important role in spread of the disease. The prevalence of disease centres in one area of a stand and total lack of diseased trees in another area was also consistent with limited dispersal of the basidiospores from their

point of production. Limited outward progression of the fungus may also be due to the need for fusion between homokaryotic strains to support disease development. Chances of fusion would be greater where many spores were deposited. Such information about the life history of I. tomentosus is lacking. Whitney (1966) successfully produced infections in roots using basidiospore suspensions, but whether or not fusion occurred before or after the infection progressed very far was unknown. The vast majority of vegetative isolates consisted of mycelia with two nuclei per cell. Mycelia from single spores had a much higher proportion of cells with multiple nuclei (Lewis, unpublished data). Whether or not homokaryotic hyphal fusion is necessary for extensive decay and when this takes place are important questions yet to be addressed.

Other important questions include the processes which occur from sporulation to the production of a new, genotypically unique disease centre. Germinated basidiospores and subsequent mycelium were observed beneath sporophores (Whitney, 1962). Whitney (1966) also found that spores germinated more readily on agar amended with spruce bark extracts and were less likely to germinate on agar with humic extracts. Both Whitney (1962) and Merler (1985) determined that infection occurs in lateral roots less than 2 cm in diameter, although Whitney (1962) ruled out rootlets (1-3mm diameter) as potential infection courts. In 1966, Whitney suggested that exposed roots of windthrown trees could act as a potential infection court for spores. In a study discussed in a later chapter, small rootlets and disruptions in the bark at root junctions were suggested as infection courts (Lewis, Chapter IV). The increase in spore

germination on spruce bark amended agar, and observations of the ability of I. tomentosus to grow in the bark suggests (Lewis, Chapter IV) that it is likely that spores are washed by rain or snowmelt through soil and deposited on a small root. Germination would follow, with limited growth on the bark before penetration into the bark and wood. However, direct evidence to support spore infections is lacking.

This study demonstrated that multiple genotypes, determined by Vc and protein groups, within a single disease centre were common and suggested that spore infections were an important means of disease spread. Determinations of the potential for loss to tomentosus root disease in second growth stands established in sites with a history of root disease should consider spread by spores. Previous work (Lewis, chapter II) has demonstrated the potential for vegetative spread of I. tomentosus from stumps to regeneration trees. The current study implied that as a second growth stand developed, mycelium in trees infected from I. tomentosus in stump roots colonize the root system until there is enough for the production of a sporophore. From this point, two means of disease spread will be active in the stand, one by vegetative means across root contacts and a second by spore dispersal causing new infection centres.

Chapter IV. Vegetative spread of Inonotus tomentosus (Fr.)Teng and disease development in spruce (Picea glauca x engelmannii Engelm.)

INTRODUCTION

Stand-opening disease as described by Whitney (1962), is characterized by variable-sized patches of dead, dying or fallen trees, often with extensive butt rot. The causal agent is the Basidiomycete Inonotus tomentosus which has a broad host range but most of the damage in Canada is reported from Picea spp (Whitney, 1977).

The openings range in size from a few trees (most common) to several acres (Whitney, 1962). Many trees not in the stand opening may be infected but may not show crown symptoms (reduced leader growth, chlorotic and thin foliage). Merler et al. (1988) maintained that crown symptoms were inconsistent and were not related to the degree of root decay. Whitney (1962) observed crown symptoms only when more than 40% of the roots were dead. Trees usually died when root mortality reached 80%. Therefore the disease may extend further into the stand than the margin of the opening indicates.

Few studies have examined closely the means of spread by I. tomentosus. Root contacts were implicated as the primary means of spread by this fungus in mature stands (Whitney, 1962) and in plantations (Myren and Patton, 1971). From observations of excavated roots, Whitney (1962) reported a progression of infection from small (0.5 cm to 1.5 cm) lateral roots into larger roots where the fungus was primarily found in the heartwood. A similar progression was described by Merler (1984). Initial infection occurred at a root

contact between a small healthy root (less than 2cm diameter) and a colonized root. Limited ectotrophic growth by I. tomentosus lead to multiple points of penetration through the bark. In roots greater than 5 cm, the fungus was unable to grow ectotrophically and resided in the xylem.

While several infection courts have been suggested, such as trunk scars (Gosselin, 1944; Hubert, 1931), basal rust cankers (Boyce, 1963), and small healthy or injured roots (Gosselin, 1944; Merler, 1984; Whitney, 1962), no conclusive evidence exists to support any of these. Whitney (1962) ruled out weevil feeding sites as infection courts although the fungus and weevils were associated. He also determined that small feeder roots were probably not involved because I. tomentosus was not isolated from these roots (Whitney, 1962).

Although root contacts were implicated as the major means of spread of I. tomentosus by Whitney (1962), he could find only two convincing examples of spread from one root to another by root contact. Some evidence exists of spores causing new infections. Whitney (1962) observed germinated spores and subsequent mycelium beneath sporophores. Furthermore, disease centres are often small and represent unique genotypes which suggests that separate spore infections are involved (Lewis, chapter III). The infection court for potential spore infections has not been identified.

The objectives of this study were to determine the point of entry into healthy roots of spruce (Picea glauca x engelmannii Engelm.) by I. tomentosus, to closely examine disease development in roots and to relate crown symptoms and tree mortality to root

colonization and mortality.

MATERIALS AND METHODS

Five rectangular plots were established at the edge of disease centres in the Prince George Forest Region. The plots were between 250 cm and 400 cm on a side, and were situated to include obviously symptomatic and asymptomatic spruce trees. Moss and soil were excavated with hand trowels to expose all roots to a depth of 30 cm. Very few roots went below this level in the soil. Once the plot was fully excavated, a string grid was laid out over the plot at 20 cm x 20 cm spacing. The grid was used to accurately map the location and approximate sizes of roots. Each tree and all its roots were labelled with corresponding numbers (Fig. IV.1). Root contacts were noted on the map and each root contact was examined carefully for signs of infection of one root by inoculum from another. Recently infected roots, determined by a thin line of light red-brown stain (Fig. IV.2), were examined closely for potential infection courts. The surface of each root was examined for mycelium, and the outer bark was scraped away to look for mycelium in the bark and cambium. Each root was cut with an axe every 5 cm to 10 cm to sample for stain and decay. To verify the identification of I. tomentosus, roots with stain, decay or intrabark mycelium were taken to the lab where chips from colonized tissue were plated on 3% malt extract agar. The observations were noted on the maps and these were used to compile common observations between plots, and to assess the development of the fungus within the disease centres.

Figure IV.1. Excavation, grid layout and root labelling of excavated plots.

Figure IV.2. Cambial lesion at pencil point (a) and stain line (b) caused by I. tomentosus infection through a small feeder root.

RESULTS

All plots were in the sub-boreal spruce biogeoclimatic zone (Krajina, 1969), with spruce the major stand component. Rooting was shallow beneath a moss layer and a thin layer of soil and duff.

Plots 1 and 2 were in a 60-year-old spruce stand which was the most densely stocked of the 3 stands studied. Plots 3 and 4 were in a spruce stand with pine as a major component. This 60- to 80-years-old stand was on a slightly drier site than 1 and 2, and had been burnt during development of the present stand. Plot 5 was in a pure spruce stand with trees 100- to 140-years-old. The moss layer in this stand was deep and the soils were more moist than in the other stands. Plot 5 was 5 km from plots 1 and 2; plots 3 and 4 were approximately 45 km from 1, 2 and 5.

General observations are summarized below, followed by brief descriptions of relevant observations from each site.

In all 5 plots, 134 primary roots were excavated (many of these had branch roots also) from 28 trees. Approximately 75% of the roots were colonized by I. tomentosus. For most of the plots, the direction of the spreading fungus was quite evident. Very old, heavily decayed and dead roots were found on one side of the plot, with stained, still living roots on the other side. The fungus moved in roots in one of two ways. The most commonly observed was a progression from a thin band of light red-brown stain in the heartwood of recently infected wood, to a darker red and broader stain, followed by decay which eventually fully occupied the root cross section. This decay progression was observed moving from root ends towards the tree (oldest decay at root end) and also was

observed moving from butt decay down a root. The second means of fungal growth in a root was in the bark and cambium. This was usually observed in small roots (2 cm or less), particularily in roots in contact with another root, or in larger roots which were fully colonized. In small roots, mycelium in the bark often preceded decay and sometimes stain in the heartwood. Ectotrophic mycelium was much less common than intra-bark mycelium. The outer bark was colonized first followed by the cambium. At this point the root was dead or dying. Roots with stain were often still alive unless they were extensively colonized in the cambium. Roots with advanced decay were usually dead.

Intra-bark mycelium was common in roots with stain and in decayed roots. Ectotrophic or intrabark mycelium was found in 39 roots which ranged from 1 to 21 roots for each plot. The mycelium was yellowish to white and inhabited the bark and cambial regions (Fig. IV.3). In roots with very old decay, it was evident from the presence of decay pockets in the inner bark (sometimes with traces of mycelium), that the intra-bark mycelium had been present at one time but was now gone. Isolations from bark with yellowish white mycelium present produced I. tomentosus in culture; isolations from the bark of these old decay roots were unsuccessful. The probable point of infection of a previously uninfected root was identified in 13 of 59 root contacts examined (22%). Actual penetration was through unwounded bark (5 of 13) or small rootlets (7 of 13). The infection had progressed too far in many of the roots to determine the actual point of infection although the general source was readily identifiable in 90% of the primary roots. The source of infection

Figure IV.3. Intrabark mycelium (right arrow) and I. tomentosus stain in one half of a contacting root (left arrow).

for the remaining 10% could not be identified.

Root contacts between a healthy root and a diseased root without intra-bark or ectotrophic mycelium did not lead to infection of the healthy root. On most of the contacts which did appear to result in infections there was an abundance of ectotrophic mycelium and intra-bark mycelium.

Roots which did become infected by root contacts with diseased roots were 1.5 cm to 4 cm in diameter. There was no difference in size of roots which were infected by direct bark penetration and access through a feeder root.

All dead trees (except for the windthrown tree in plot 4),and all trees rated as very symptomatic (almost dead) had no living excavated roots. Root mortality of trees rated as symptomatic ranged from 0 to 80% (mean mortality was 44%). Root mortality of asymptomatic trees ranged from 0 to 33% (mean mortality was 12%).

Plot 1 (Fig. IV.4)

Trees 2 and 4 were notably symptomatic (thin crown and reduced leader growth) and trees 1 and 3 were dead. Tree 3 was cut at 0.5 m above ground to determine the extent of butt decay. Butt decay was apparent only in the half of the stump closest to tree 2. The fungus was moving into tree 5 through root 5c and 5a. A small branch rootlet (0.8 cm diameter) from root 5a in contact with Y1 was the point of entry to 5a. There was no colonization of the bark; I. tomentosus penetrated through the xylem of the rootlet directly into the heartwood of 5a. Inonotus tomentosus was isolated from the junction of the stained rootlet and the main root (5a). Penetration

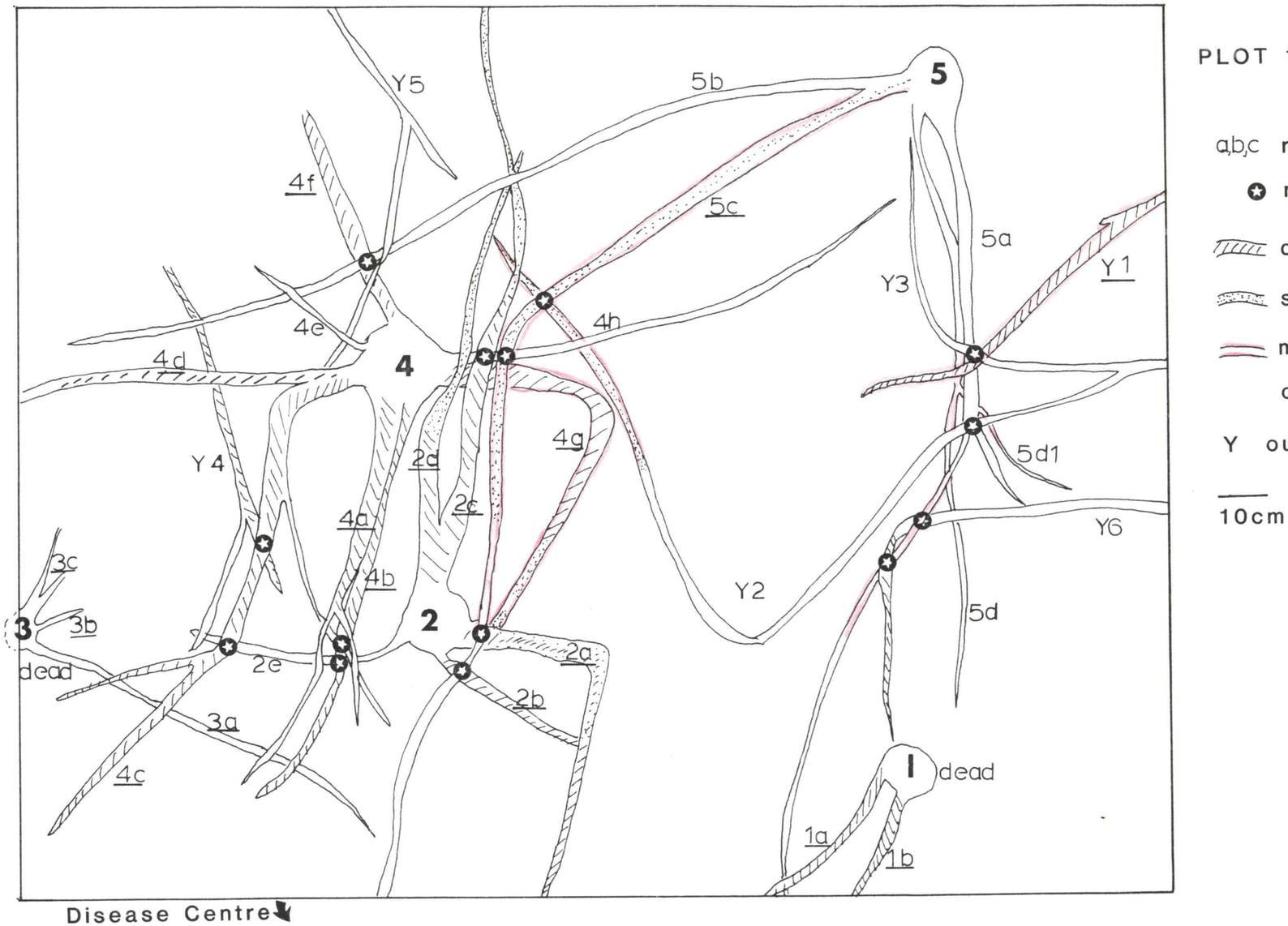


Figure IV.4. Plot 1 root map.

of root 5c by I. tomentosus in root 2a was directly through the bark. The root tissue at the contact was resin soaked and the cambium was dying. The stain originated from the resin soaked area. It is possible that 5c was also infected by the contact with 4g, but the decay had progressed too far to discern an infection point.

Inonotus tomentosus was cultured from ectotrophic mycelium on root 4h. A lesion was observed in the cambium beneath the ectotrophic mycelium and the wood below was stained. Inonotus tomentosus was not successfully isolated from the resin soaking or the stain. However, in root Y3 samples of resin soaked wood in lesions distal to the root contact between Y3 and Y1 produced I. tomentosus in culture.

Plot 2 (Fig. IV.5)

Tree 1 showed slight crown symptoms; trees 2 and 3 were very symptomatic. The source of infection in this plot was uncertain. Tree 3 was the most diseased and probably lead to the infection of tree 2 but tree 1 was apparently infected from a source opposite the disease centre from tree 3. Root 1b had patches of encrusted resin and soil moulded around the root. Mycelium was abundant in the cambium of encrusted sections. The infection of root 1d appeared to occur through a rootlet in contact with Y5; stain and beginning decay in the heartwood (successfully isolated) were surrounded by healthy sapwood (unsuccessful isolation). However, infection of root 2a was by direct penetration of the bark. Ectotrophic mycelium on root 2b was abundant and apparently lead to the infection of root 2c through a small root at the junction with 2c1. The cambium was dead in this region and the roots were encrusted with resin and soil. Stain in

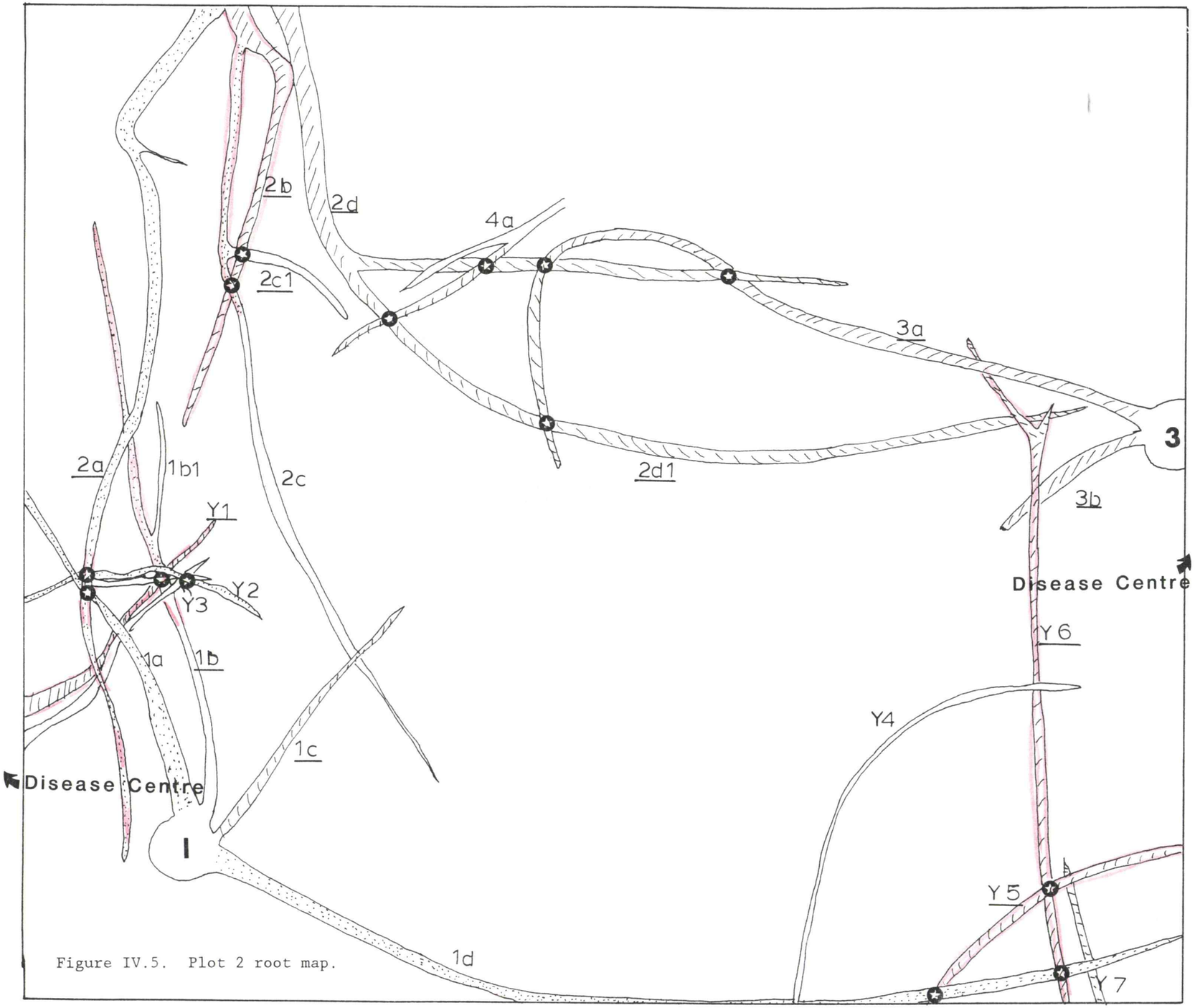


Figure IV.5. Plot 2 root map.

root 2c1 proximal to the junction may also have come up the heartwood from root 2b.

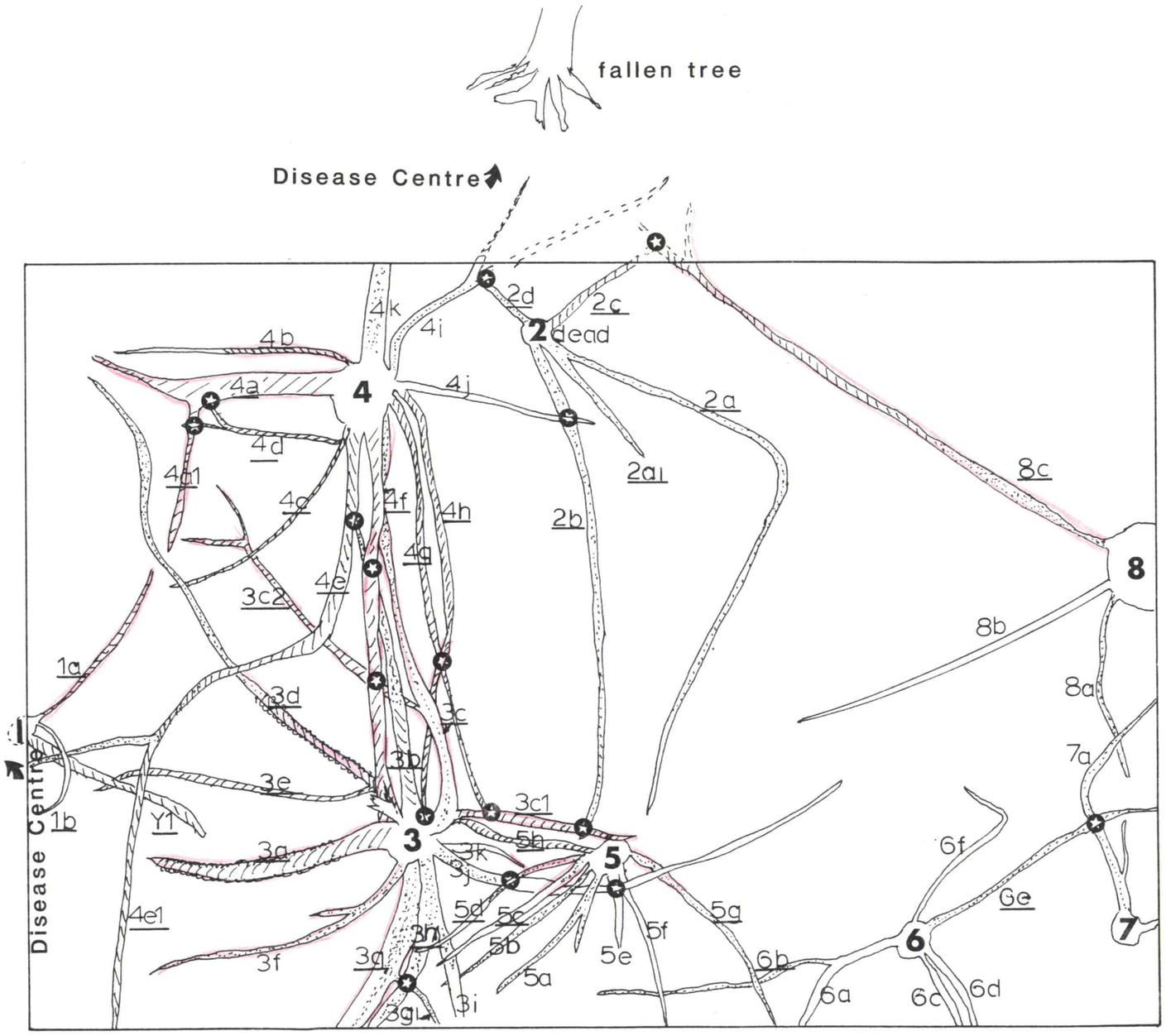
Root collar weevil (most likely Hylobius warreni Wood) feeding was evident on root Y2 at the contact point with 2a, but evidence of infection of one root by the other was lacking.

Plot 3 (Fig. IV.6)

Tree 2 was dead, trees 7 and 8 were asymptomatic and the remainder were clearly symptomatic. The disease moved into this plot from a very rotten root near tree 1, and/or from a very decayed, fallen tree outside the plot. Where root 5d contacted 3j, ectotrophic mycelium was abundant and the fungus had penetrated only the cambium of 3j. Root 5d was stained in the half of the root closest to the contact (Fig. IV.3). The stain originated at a small (0.4 cm diameter) feeder root. Likewise, at root 5c on the side closest to 5d, ectotrophic mycelium and cambial mycelium were present and had penetrated the cambium to cause a lesion (Fig. IV.2a). Further penetration to the wood occurred at a feeder root (Fig. IV.2b). Root 5h was root grafted with tree 3 and was severely decayed. The fungus apparently came into 3 on the opposite side, traversed the butt and grew down 5h into tree 5.

Plot 4 (Fig. IV.7)

Tree 8 was dead and fallen, tree 5 was dead, trees 1, 3, 4 and 6 showed crown symptoms, and trees 2 and 7 were asymptomatic. Inonotus tomentosus had not progressed far into this plot; the source was through tree 8 which had fairly extensive butt rot. Root 3b was severely decayed and where it contacted root 4c (not shown, it was beneath 3b), there was heavy resin exudation and cambial death but no



PLOT 3

a,b,c roots; a,b,c dead roots

root contact

stain

decay

mycelium in or on bark

Y outside plot

20cm

Figure IV.6. Plot 3 root map.

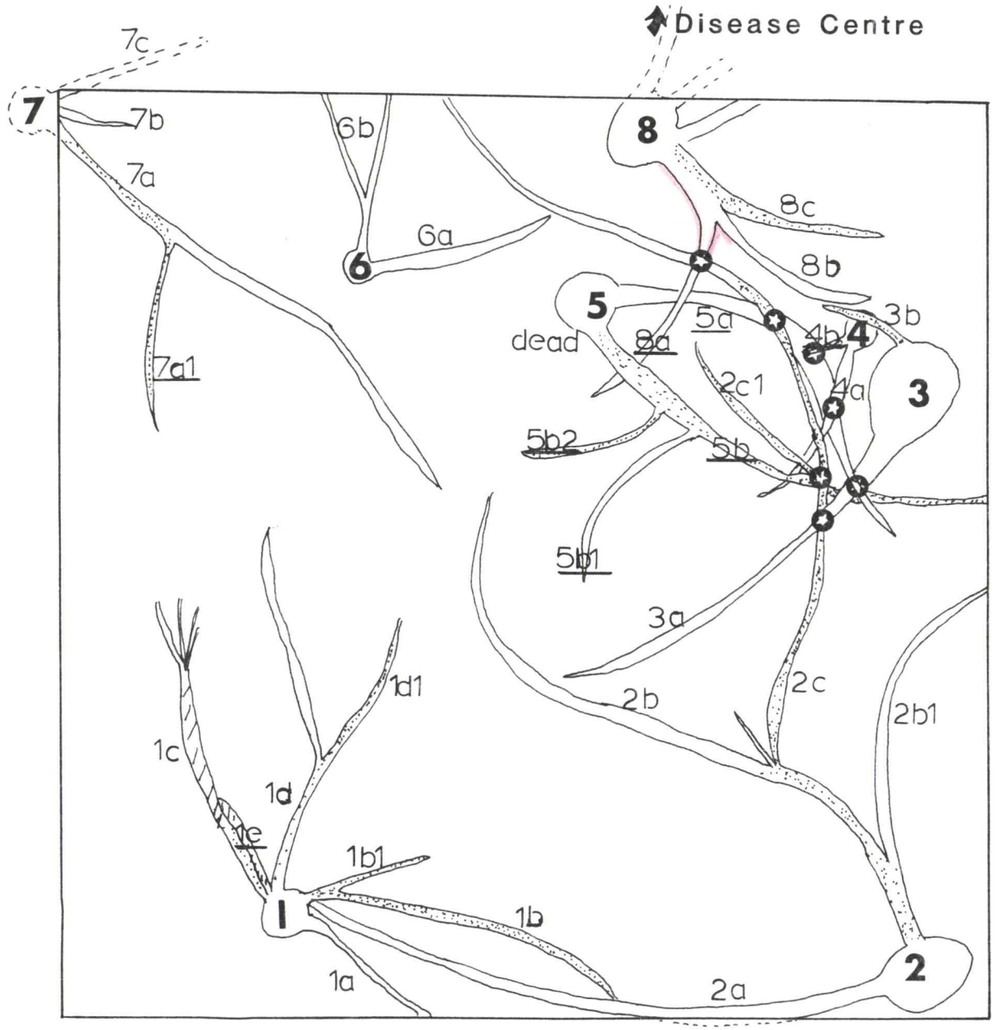


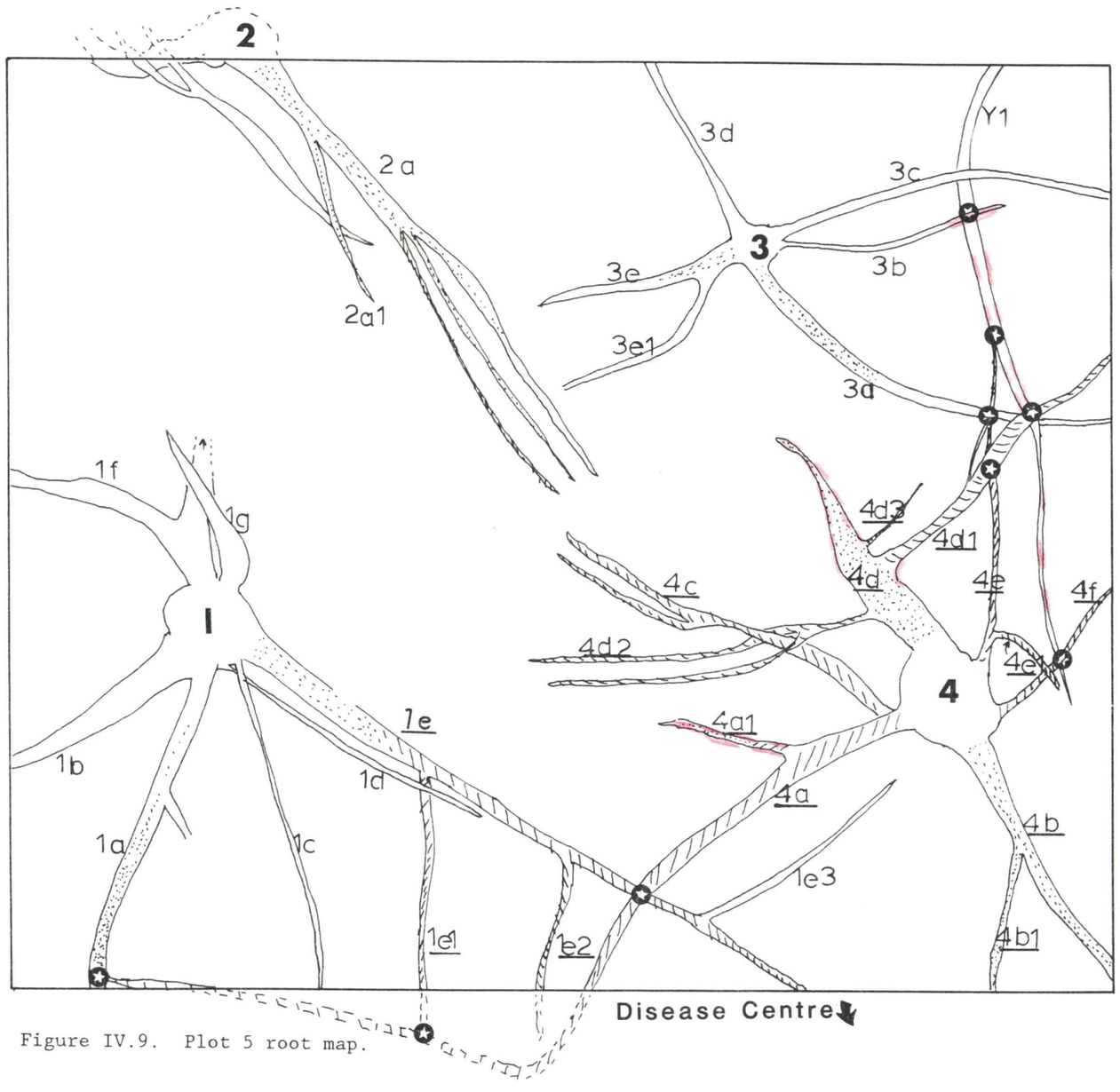
Figure IV.7. Plot 4 root map.

ectotrophic mycelium or bark mycelium were evident. Penetration of 4c occurred at a root branch point (Fig. IV.8). The sources of infection for trees 5 and 7 were not discovered, although root 1c was very rotten and may have rotted back from a previous contact with 7a1.

Plot 5 (Fig. IV.9)

All trees were living; tree 4 was severely symptomatic, and the remaining trees were moderately symptomatic. The fungus was moving up root 1e from the tip towards the stem, the infection source may have been the contact with 4a. Root 3b (1 cm diameter) in contact with Y1 had ectotrophic mycelium which directly penetrated the bark and cambium, then grew down a nearby small feeder root into the wood. It is most likely that the infection of roots 2a occurred by contact with 4c which had rotted back.

Figure IV.8 Cambial lesion from penetration by I. tomentosus at a root junction.



PLOT 5

a,b,c roots; a,b,c dead roots

root contact

decay

stain

mycelium in or on bark

Y outside plot

20 cm

Figure IV.9. Plot 5 root map.

DISCUSSION

The relationship between crown symptoms and percentage of roots decayed was similar to that found by Whitney (1962). Trees with 1 or 2 roots containing stain with little advanced decay did not show crown symptoms. Crown symptoms were obvious when 40% to 50% of the root system was decayed or stained. In this study, trees with 100% of their roots colonized were still alive, as long as the fungus had not girdled the cambium in all the roots. Some roots contained a band of decayed or stained heartwood surrounded by healthy sapwood; these roots were still alive. This may be why Merler et al. (1988) found little relation between root decay and crown symptoms; the important factor is the degree of cambial death, not root colonization.

In roots approximately 5 cm or less in diameter, intra-bark mycelium often preceded decay by 20 cm. In larger roots however, the stain in the heartwood generally preceded colonization of the bark. The bark of the larger roots was colonized only when the decay core expanded radially to reach the surface. Radial expansion by the fungus and colonization of the bark in larger roots was also observed in stumps of harvested old growth trees and was shown to lead to infection of regeneration trees in root contact with the stumps (Lewis and Hansen, 1988). Unlike stump roots however, stain columns in the living trees were often very long (2.5 m or more). In dead trees, as in the stumps, the stain (incipient decay) had progressed to advanced decay which expanded radially. Therefore the living cambium and sapwood may have some resistance to radial invasion which prior to tree death makes heartwood the path of least resistance for

I. tomentosus colonization.

Two infection courts were identified in this study. The first was direct penetration of the bark on small (less than 4 cm diameter) roots, particularily at root junctions where bark was disrupted. This usually resulted in colonization of the bark and cambium, but little penetration into the wood. Merler (1984) also noted direct penetration of small roots. The second infection court, small (less than 1 cm) feeder roots, provided a direct path to the root wood by the rootlet's vascular system. In some cases this route of infection was the only one taken. In others, the fungus penetrated and colonized a large patch of bark and cambium before colonizing a feeder root and gaining access to the heartwood. This supports Merler's (1984) contention that hyphae could not grow on or through larger roots, perhaps because of thicker bark. Whitney (1962) was unsuccessful at isolating I. tomentosus from the small feeder roots probably because they were the first tissue colonized and the fungus would have died by the time the isolation was made. An isolation from a rootlet of a very recently infected root was successful in this study.

There were only two wounded roots observed which were in contact with colonized roots. However in neither case was infection by I. tomentosus related to root wounds. One was a weevil feeding wound and the other was fire-related. However in other studies, fire wounds have been implicated as enhancing infection (Hubert, 1929; Whitney, 1962) and a relationship between fire history and disease incidence either by providing infection courts or by predisposing the roots to infection, is suspected.

In two of the plots, the sources of infection of the trees were not clear. The fungus appeared to have originated from 2 or more directions. This could be due to missed roots and contacts, or contacts that once existed which were completely degraded. However the plots were chosen at the edge of disease centres to observe the advancing front of the fungus. This should have minimized the number of severely decayed roots. A second explanation is that the stand became established after a fire and the plot trees were situated over previous disease centres. Therefore infection could come from several sources within the old disease centre. This is supported by observations of some old decayed, sometimes partially burnt logs and stump stubs in the area. A third explanation is that multiple infection sources are due in part to spore infections. Little evidence exists to support this except for the fact that several fungal genotypes may occupy one disease centre which indirectly supports infections by spores (Lewis, chapter III).

Phellinus weirii is thought to spread primarily by root contacts with little contribution by spores (Childs, 1970). Ectotrophic growth of P. weirii can be extensive (Buckland et al., 1954) and therefore can readily infect roots in contact with diseased roots. Inonotus tomentosus does not have extensive ectotrophic mycelium and root contacts without ectotrophic mycelium or intra-bark mycelium do not result in infection.

On the other hand, there is no evidence that Phaeolus schweinitzii infects trees by root contacts between healthy and diseased roots but basidiospores were shown to persist in soil (Barrett, 1985). Furthermore, each tree was colonized by a unique

genotype suggesting that spore infections were involved to the exclusion of root contacts (Barrett and Uscuplic, 1971). Infections by P. schweinitzii sp. were associated with Armillaria sp. infections such that Armillaria may provide an infection court for P. schweinitzii.

In between the two extremes of ectotrophic growth and vegetative spread by Phellinus weirii and spread by spores by Phaeolus schweinitzii, is Heterobasidion annosum which grows ectotrophically under appropriate soil conditions (Gibbs, 1967), infects healthy roots at root contacts with diseased roots, and causes new infection centres by spore infections (Rishbeth, 1951a, b). It is suggested that I. tomentosus is most like H. annosum in disease spread and population genetics, although infection by spores has not been directly proven.

In conclusion, ectotrophic and intrabark mycelium have an integral role in disease spread. Infection of the bark occurs by direct penetration of the bark in small (less than 4 cm diameter) roots, and xylem infections occur through feeder roots or at root junctions. Progression of decay in small roots often follows behind hyphal growth in the bark, but in larger roots (5 or more cm diameter) the fungus does not grow readily through the bark and the hyphal advance is through the heartwood. The fungus advances through the heartwood until tree death at which time the stained wood is decayed and decay progresses radially. Mycelium colonizes the bark until the food source is depleted, then it dies out of the bark and cambium.

Chapter V. Inoculation of *Picea glauca* x *engelmannii* Engelm. and *Pinus contorta* Dougl. with *Inonotus tomentosus* (Fr.)Teng and *I. circinatus* (Fr.)Gilbn.: infection and host response

INTRODUCTION

In the development of control or prevention strategies for diseases of forest trees, host response to infection and resistance mechanisms are important considerations. *Inonotus tomentosus* (Fr.)Teng, a root pathogen of spruce and pine species in Canada and the northern U.S.A., and *I. circinatus* (Fr.)Gilbn., a pathogen of pine in eastern North America, have the potential to cause significant damage in second growth forests (Boyce, 1963; Lewis and Hansen, 1988; Myren and Patton, 1971; Ross, 1966; Whitney, 1980).

Taxonomically, *I. tomentosus* and *I. circinatus* differ slightly in sporophore morphology, especially the hymenial setae. Host and geographic ranges, and pathogenicity differences between the species are not clear. In the southeastern U.S.A. the *Inonotus* species most frequently reported to cause significant damage in pine plantations is *I. circinatus* (Boyce, 1963; Ross, 1966). In eastern Canada the apparent ranges of the two species overlap (Whitney, 1962), while in western Canada there have been no reports of *I. circinatus*. Inoculation tests of germlings of 11 species including lodgepole and ponderosa pines (*Pinus contorta* Dougl. var *latifolia* Engelm. and *P. ponderosa* Laws.) and white and black spruce (*Picea glauca* (Moench)Voss and *P. mariana* (Mill.)B.S.P.) in nutrient agar, and of 40 to 120-year-old trees (including white and black spruce and jack pine (*P. banksiana* Lamb.)in Saskatchewan suggested that *I. tomentosus*

was more virulent than I. circinatus on all species tested (Whitney, 1967, 1964; Whitney and Bohaychuck, 1976).

While the damage to pines from I. circinatus in the southeastern U.S. is well documented (Boyce, 1963; Ross, 1966), there are conflicting reports on the effects of I. tomentosus on pines in Canada. It has been reported from many pine species (Buchanan, 1964; Hepting, 1971; Whitney, 1977, 1980) but the potential for infection and the degree of damage to pine compared to spruce in a forest situation is not clear. This has lead to the suggestion (Merler et al. 1988) that I. circinatus is a pathogen of pine and I. tomentosus a pathogen of spruce. One inoculation study (in eastern Canada) indicated that white spruce, black spruce and tamarack (Larix laricina (DuRoi)K. Koch) were significantly more susceptible than was jack pine (Whitney, 1964). In British Columbia, Merler (1984) found that lodgepole pine trees in root contact with infected interior spruce (Picea glauca x engelmannii Engelm.) did not become infected. However a different study in the same Forest Region involved plots established in diseased spruce and pine stands where both species were infected (Schulting, 1987). Schulting found that the frequency of infection of pine and spruce were equal, but the manifestation of the disease varied with host. In spruce I. tomentosus caused a root and butt rot and crown symptoms were slow to develop. In pine, cambial necrosis was common with less butt decay and earlier crown symptoms (Schulting, 1987).

In north-central British Columbia, pine and spruce are often equally suitable for some sites. Pines are often recommended as alternative species for establishment in sites infested with root

diseases such as Phellinus weirii (Hadfield, 1985). If the chance of infection or damage by I. tomentosus on pine were less than spruce, it could serve as an attractive alternative species for establishment in root disease areas.

Root diameter and the sapwood to heartwood ratio may affect host response to infection. Observations of infected roots in situ suggest that small roots (less than 1cm - 2cm) are the initial infection site for I. tomentosus spreading across root contacts (Lewis, Chapter IV; Merler, 1984; Whitney, 1962). However, inoculation studies involving inner bark wounds showed that roots 7 to 8.5 cm in diameter were more susceptible to infection than were small roots 1.0-1.5 cm diameter (Whitney, 1967), and that fungal growth in the wood was positively correlated with root size. The latter study raises the question of the role of wounding in infection. Whitney (1962) determined that infection by I. tomentosus does not naturally occur through wounds, but under artificial inoculation conditions infection was most successful through deep root wounds (Whitney, 1963).

The success of deep (compared to shallow) wound inoculations suggests that the inner bark and outer xylem produce a defensive response to wounding or infection. The histochemical response of excised Norway spruce (Picea abies (L.)Karst.) roots to infection by Heterobasidion annosum (Fr.)Bref. was described by Johansson and Stenlid (1985). They found an increase in phenols and a decrease in starch in advance of the mycelium with a corresponding increase in fungal laccase slightly behind the hyphal front. The region of highest enzymatic activity and phenolic deposition corresponded with

the "reaction zone" described by Shain (1967). Death of ray and epithelial parenchyma, and synthesis and deposition of phenols occurred in the reaction zone, ahead of the hyphae. Such processes are similar to the formation of heartwood. Shain (1967) reported that the formation of a reaction zone significantly increased the resistance of sapwood to infection and decay by H. annosum. Biochemical changes induced by wounding or infection are well documented in other plants (Bostock and Stermer, 1989; Kuc, 1972) and have been studied in search of resistance mechanisms which may be exploited for disease management purposes.

The purpose of this study was to compare the frequency of infection and biochemical response of pine and spruce to I. tomentosus and I. circinatus. The objectives were: 1) develop inoculation techniques for future studies; 2) compare infectivity of I. tomentosus and I. circinatus; 3) determine root sizes most susceptible to infection; and 4) compare responses of spruce and pine to inoculation.

MATERIALS AND METHODS

Two separate inoculation studies were established. The first used natural inoculum, and the second used artificial inoculum.

Natural Inoculum test: Fully colonized spruce roots (7 to 20 cm diameter) of diseased stumps and trees were excavated from a 500 m^2 area in an infested stand in north-central British Columbia. The roots were cut into 15 to 20 cm lengths; only sections with intact bark were used. Some of the larger pieces were split in half lengthwise to an approximate mean diameter of 10 cm.

Two areas were selected (Fig. V.1), each with mature trees (>80 yrs old) and young trees (<25 yrs old). Two age groups were used to assess the effect of age on infection. One root of each of ten trees in the two age groups was carefully excavated to the desired size for inoculation (2-3 cm diameter). The inoculum was placed bark side against the root and adjusted to obtain maximum contact between root and inoculum. The block was tied to the root at the two ends with flagging tape. The hole was filled with soil with one end of the flagging tape protruding from the hole to mark the inoculation.

Artificial Inoculum Test: Alder (Alnus rubra Bong.) branches were cut into pieces approximately 7 cm long x 2.5 cm diameter. These were loaded into quart jars with malt broth (3%) added to a depth of 2 cm, and autoclaved for one hour. Each jar of alder pieces was inoculated with one isolate of I. tomentosus or I. circinatus growing on 3% malt agar (Table V.1). Controls consisted of autoclaved alder pieces. Inoculum was incubated for 3.5 months at room temperature.

Ten pine and ten spruce stands (>80 yrs) were selected in the

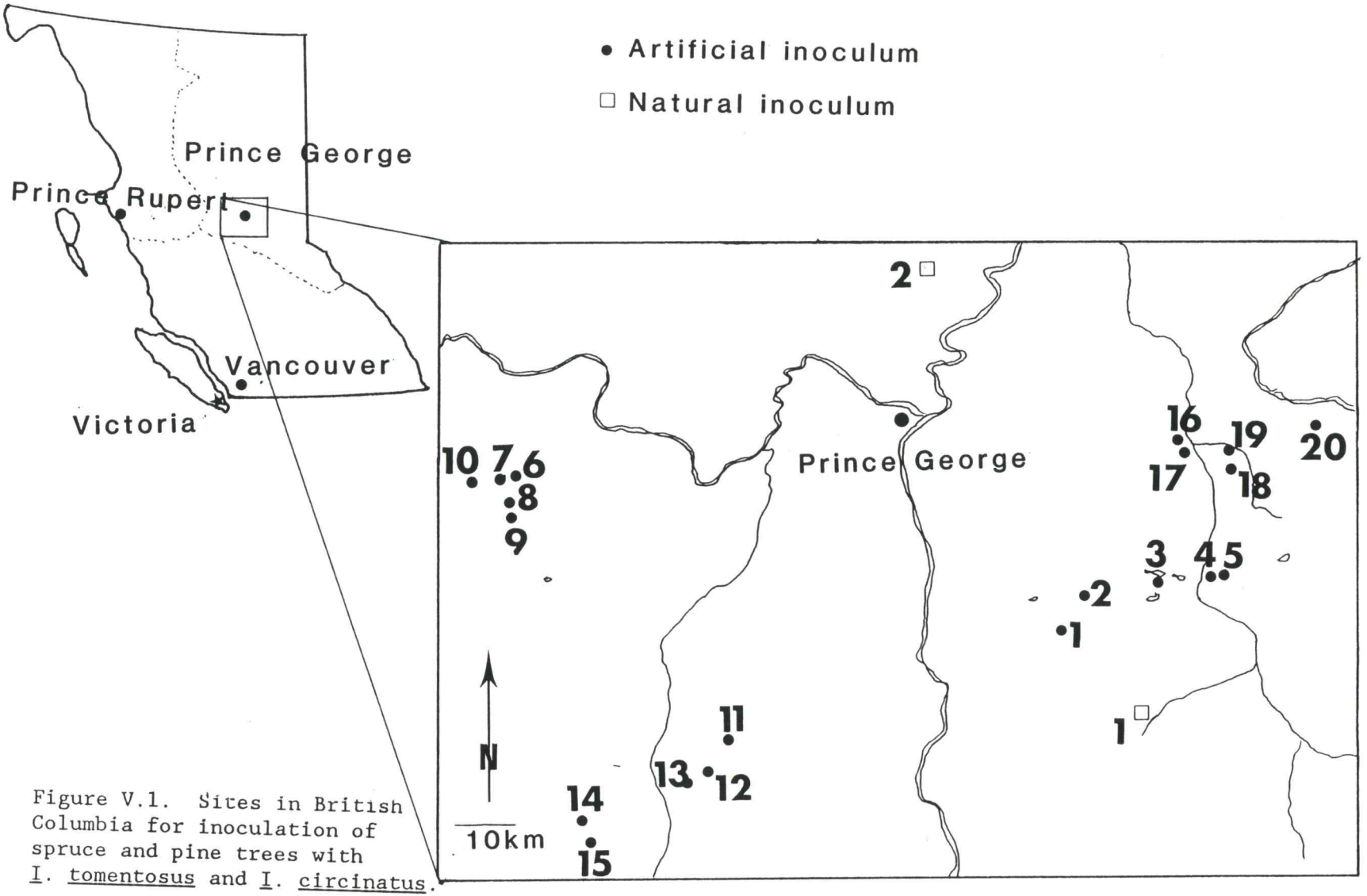


Figure V.1. Sites in British Columbia for inoculation of spruce and pine trees with I. tomentosus and I. circinatus.

Table V.1. Isolates of Inonotus used for inoculation of pine and spruce trees.

Isolate	Species	Location	Host
BC-1	It[1]	Kispiox, B.C	Pinus contorta
BC-7	It	Smithers, B.C.	Abies lasiocarpa
AV-3	It	Averil Lake, Prince George B.C.	Picea glauca x engelmannii
Sm-5	It	Smithers, B.C.	Picea glauca
WH-11	Ic[2]	Candle Lake, Saskatchewan	Picea glauca
Control		Sterilized alder	

1. Inonotus tomentosus
2. Inonotus circinatus

Table V.2. Mean length (cm) from the inoculum margin of resin-soaking in roots inoculated with Inonotus tomentosus and I. circinatus by host species and inoculation treatment. Sample size is in parentheses.

	Spruce				Pine			
	wounded		unwounded		wounded		unwounded	
	+[1]	-	+	-	+	-	+	-
Inoculated	3.1(8)	2.2(91)	4(2)	0.3(44)	1.2(6)	2.3(95)	0(2)	0.2(45)
Control		1.3(17)		0 (7)		1.6(17)		0(7)

1. + = infected roots; - = uninfected roots (as determined by isolation).

Prince George Forest Region for inoculations. These were in accessible areas not in current harvesting plans (Fig. V.1). At each site three roots from each of six trees were excavated. A record of root diameters was kept to ensure that roots from 0.5 cm to 7 cm diameter were inoculated with each isolate. Roots were lightly rinsed with sterile distilled water, then two of the three roots were wounded with a sterilized knife. The wound was shallow, barely into the outer sapwood, and was as long as the inoculum. All three roots of one tree were inoculated with the same isolate (or a control). Each tree, of the six trees on a site, was inoculated with a different isolate. Inoculum blocks were tied first with gauze strips then wrapped with flagging. The excavation hole was filled with one piece of tape protruding as a marker (Fig. V.2).

All roots from both studies were harvested one year following inoculation. Roots were re-excavated and notes were made on root appearance and signs of infection, such as mycelium on or in the root bark, and on the condition of the inoculum. A 30 cm section including the inoculum block was excised. Roots were labelled by site, isolate and treatment (wounded or unwounded) then transported back to the lab on ice.

Each root was bisected longitudinally with a bandsaw and the length and depth of resin soaking and stain were measured and the presence of mycelium in the bark was noted. Isolations were made from one of the root halves. Chips from the inoculation margin (the wound end or where the inoculum block ended on unwounded roots), and from 1 cm and 2 cm beyond those points were transferred to 3% malt extract agar. Further isolations were made at 1 cm intervals from

Figure V.2. Inoculation procedure, counterclockwise from top: excavation of root, wounding and inoculum attachment.

roots with signs of infection.

Cubes 1 cm - 1.5 cm square were cut by hand saw and chisel from the second half of the root at the same locations as the isolations (Fig. V.3). Thin sections (20-30 microns) were cut with a sliding microtome and stained immediately. Phenols were stained with diazotized tolidine (Shain, 1967) and Hoepfner-Vorsatz reagent (Reeve, 1951), and starch was stained with iodine potassium iodide (IKI) (Jensen, 1962). Protocols for dehygrogenase, β-glucosidase, acid phosphatase, peroxidase and laccase were those modified and described by Johansson and Stenlid (1985). A brief description of each method follows. The staining reactions were rated as low, moderate or high and their location in the wood described. Differences in activity within one section, especially in relation to the hyphal front, were noted.

Phenols - (i) Diazotized tolidine: a stock solution of 5 g o-tolidine in 15.1 ml HCl and 970 ml distilled H_2O was prepared. The solution was diazotized by mixing 1:1 with 10% (w/v) $NaNO_2$ and used within 15 min.

(ii) Hoepfner-Vorsatz: Sections were placed in 2 ml of 10% $NaNO_2$ (w/v) and 2 ml acetate (10%). After 3 min. this solution was replaced with 4 ml 2N NaOH.

For both protocols, phenols stained an orange-red.

Starch - sections were incubated in a solution of 0.2% iodine and 2% KI in water for approximately 3 min., then were rinsed in dH_2O before examination. Starch granules were seen as black deposits.

Dehydrogenase (Tetrazolium reductase) - a stock solution was

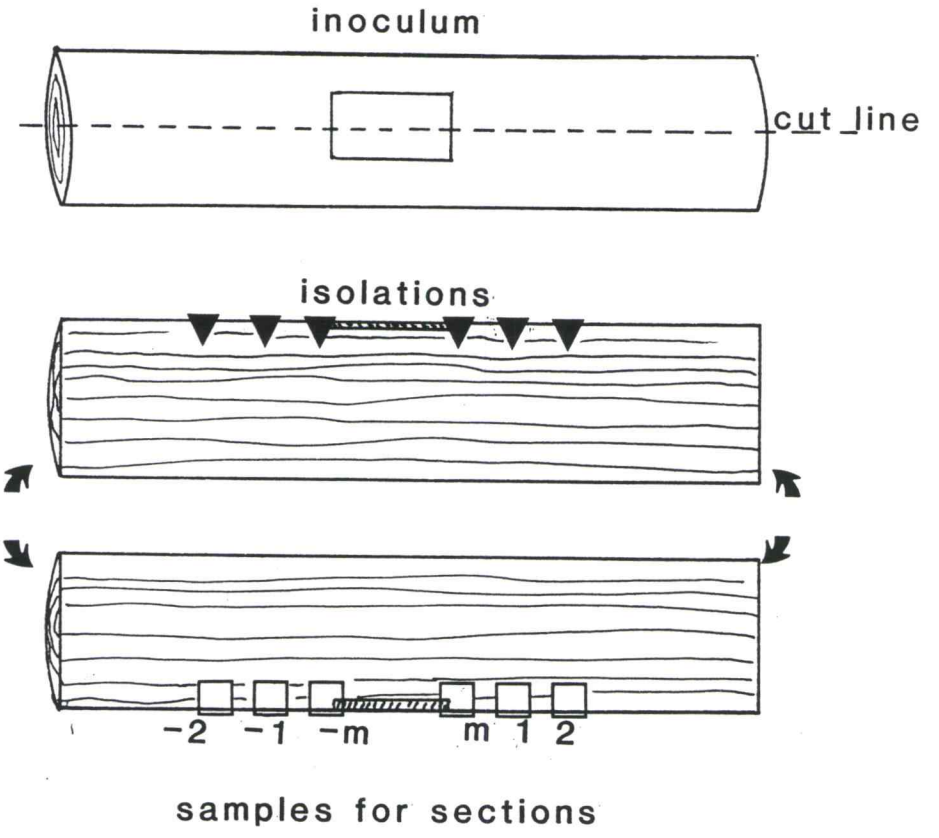


Figure V.3. Location of samples from inoculated roots for isolation and sectioning.

prepared consisting of 10 ml 0.1M PO_4 buffer pH 7.2 and 40mg nitroblue tetrazolium (Sigma) in 0.5ml N,N dimethylformamide, 17.5 ml dH_2O, and 4ml 1% $MgCl_2$ $6H_2O$. Prior to use, 4 mg of the substrate NADH (Sigma) was added to 2 ml stock solution and the sections were incubated for 30 min. at 30C. A blue reaction product indicated dehydrogenase activity.

Glucosidase - 20 ml phosphate buffer pH 6.5 was added with 1 ml of 0.3% substrate (6 bromo-2-naphthyl-β-D glucopyranoside) (Sigma) in N,N-dimethylformamide and 20 mg fast garnet GBC salt (Sigma). Sections were incubated for 30 min. at 30 C. A positive reaction was indicated by the red-violet colour.

Acid Phosphatase - 10mg Na α-napthyl phosphate (Sigma-substrate) and 20mg fast garnet GBC salt were added to 10ml 0.2M acetate buffer pH 4.8. A purple colour indicated activity.

Peroxidase - sections were immersed in a 1.5% water solution of guaicol, then transferred to 0.3% peroxide and washed in dH_2O after 30 sec. Peroxidase activity was indicated by a brown-red colour.

Laccase - sections were immersed in a 1.5% water solution of guaicol for 30 min. at room temp. Brown-red reaction products suggested laccase activity.

RESULTS

Very few infections resulted from the inoculations. Only one root of 40 inoculated with natural inoculum showed signs of infection, and that one was questionable. One small feeder root (2 mm diameter) located between the inoculum and the main root was dead and appeared to have been colonized by I. tomentosus although the fungus did not go far into the main root. Inonotus tomentosus was not recovered from any isolations. No living mycelium remained in any of the inoculum blocks. This set of inoculations was not studied further.

A total of 360 roots were inoculated using colonized alder pieces; all but two were successfully recovered after one year. At the time of inoculation, I. tomentosus had not caused any visible decay in wood of the alder blocks, but a thick mat of mycelium covered the surface. At harvest, no decay was observed in the alder, and the superficial mycelium was reduced to a crust, or to small mounds of highly pigmented mycelium on the block ends (Fig. V.4).

During root harvest, I. tomentosus was observed growing on the bark surface of ten spruce roots and seven pine roots. Nine of the 17 roots with mycelium on the bark had been inoculated with isolate AV-3. However, successful isolations were made from only two roots with ectotrophic mycelium.

It was noted that pine roots produced a much greater surface resin flow in response to wounding than did spruce roots. No difference between unwounded roots of the two species was observed.

Two methods were used to determine successful infection by I. tomentosus or I. circinatus in the roots. One was by isolation and

Figure V.4. Inoculum blocks one year after inoculation showing small white mounds of pigmented mycelium on the block ends.

the second by observing hyphae in sections examined microscopically. These two methods gave different results. Inonotus tomentosus and I. circinatus were detected most frequently by the isolation method. Nine percent of the inoculated spruce roots were infected, according to the isolation results, and the proportion of infected wounded and unwounded roots was the same. Five percent of inoculated pine roots were infected; the percentage of infected wounded roots was 5 times that of the unwounded.

The greatest resin soaking response was from infected, wounded spruce roots (3 cm beyond the wound margin) which was significantly different ($p=.1$) from wounded, infected pine roots (average for pine was 1.2 cm). There was no significant species effect on resin-soaking in uninfected roots, wounded or unwounded. Unwounded infected roots were too few for a comparison (Table V.2). The length of resin soaking in the wood was not significantly different for failed inoculations and for uninoculated controls (Table V.2). Resin soaking penetrated only 2 to 3 growth rings. The typical pink-brown stain produced by incipient I. tomentosus was not observed.

Inonotus tomentosus was recovered from roots ranging in diameter from 1.2 cm to 5.2 cm; the larger (6-7 cm) and smaller (less than 1 cm) roots did not become infected (Table V.3). Mean diameters of infected roots were quite uniform: 2.5, 2.7, 3.6, 2.9 and 2.8 cm for AV-3, BC-1, BC-7, Sm-5 and WH-11 respectively.

Ten of 20 successful isolations were from the wound margin and in only 4 roots was the fungus recovered further than 1 cm from the inoculation margin (Table V.3). The mean distances from the margin that the fungus was recovered were 0.88 cm and 0.77 cm for pine and

Table V.3. Inoculated roots from which I. tomentosus was isolated by isolate and site. Inoculation treatment and distance from the margin that I. tomentosus was recoverable.

Site (sp)	Isolate	Root Diam. (cm)	Treatment	Distance from margin (cm)	Field obs.
12 (Sx)[1]	AV-3	2.4	wounded	margin	
14 (Sx)	AV-3	1.4	wounded	1	
16 (Sx)	AV-3	3.6	wounded	3	patchy dead cambium
2 (Sx)	BC-1	4.1	unwounded	margin	
12 (Sx)	BC-1	1.2	wounded	margin	
2 (Sx)	BC-7	2.3	unwounded	margin	
4 (Sx)	BC-7	5.2	wounded	1	
9 (Sx)	BC-7	4.7	wounded	1	
14 (Sx)	BC-7	3.0	unwounded	margin	
20 (Sx)	BC-7	2.7	unwounded	margin	
12 (Sx)	Sm-5	1.4	unwounded	3	mycelium on and in bark
14 (Sx)	Sm-5	3.0	wounded	margin	
13 (Pl)	Sm-5	2.6	wounded	1	
15 (Pl)	Sm-5	4.5	wounded	1	
17 (Pl)	Sm-5	4.9	wounded	margin	dead cambium
19 (Pl)	Sm-5	2.0	wounded	margin	
15 (Pl)	Sm-5	2.2	unwound	2	
6 (Pl)	WH-11[2]	1.9	wounded	margin	
12 (Sx)	WH-11	3.1	wounded	1	mycelium on bark
15 (Pl)	WH-11	3.4	wounded	2	

1. Sx = interior spruce (Picea glauca x engelmannii Engelm.)
   Pl = lodgepole pine (Pinus contorta Dougl.)
2. WH-11 = I. circinatus (Fr.)Gilbn.

spruce respectively; these were not significantly different (Students t-test).

A subsample of inoculated roots was selected for histochemical examination. This subsample included a) all roots with external signs of infection; and b) all unwounded roots and half of the wounded roots inoculated with isolates BC-7, WH-11 and Sm-5 recovered from 10 of the 20 sites. These isolates were selected because they yielded the highest proportions of positive isolations.

Of 360 roots inoculated with colonized alder blocks, 68 were examined histochemically. Hyphae were observed in the wood of only seven roots (five spruce and two pine). The I. circinatus isolate WH-11 was the most successful in causing infection of the wood (four of seven roots), but the small sample size precluded further comparisons between isolates. The range of diameters of these roots (average 4.0 cm) was consistent with those stated above. Hyphae were not observed beyond 2 cm from the margin of the inoculation (Table V.4). Resin soaking in these roots extended an average of 2.2 cm for spruce and 0.5 cm for pine (only 2 samples), which is similar to the length of resin soaking for aseptic wounds (Table V.2).

Of the 7 chemical reactions used, only phenols and dehydrogenase varied with species. A description of each reaction follows. Roots described below as infected refer only to those 7 roots where hyphae were observed in the wood. Reactions in uninfected, inoculated roots were the same as those observed in uninoculated control roots, therefore roots described as uninfected refer to both of these.

Hyphae were sparse in pine roots in contrast to more densely colonized spruce roots. Hyphae occupied the tracheids and rays in

Table V.4. Roots infected in the wood (as determined from histochemical examination) from inoculation with isolates of I. tomentosus and I. circinatus.

Isolate	Site	Species	Distance from margin[1]
AV-3	7	Interior spruce	2 cm
BC-7[2]	4	Interior spruce	0 - at margin
Sm-5	2	Interior spruce	0 - at margin
WH-11	14	Interior spruce	0 - at margin
WH-11	16	Interior spruce	0 - at margin
WH-11	6	Lodgepole pine	0 - at margin
WH-11	13	Lodgepole pine	0 - at margin

1. Distance from the inoculum margin that hyphae were found in the root.
2. I. tomentosus was also recovered from reisolation from this root.

the oldest part of the infection, but near the hyphal front, hyphae were observed only in the tracheids. Penetration by the hyphae was limited to 2 to 3 growth rings from the cambium.

Phenol

Uninfected: Phenols were observed in the ray cells and resin canal parenchyma only. They were infrequently observed in unwounded roots and common on the wound side of wounded roots (Fig. V.5). Phenols were more evident in wounded pine roots than in wounded spruce roots.

Infected: Accumulation in tracheids was observed in infected sections and phenol content of rays was slightly higher than in aseptic wounds. Phenol concentrations decreased more rapidly in aseptic wounds compared to infected sections where phenol concentration was greatest 1 to 2 cm ahead of the hyphal front. In heavily colonized cells with the oldest hyphae, phenols were less obvious. Phenol accumulation in infected pine was approximately twice that of spruce.

Dehydrogenase

Uninfected: Moderate background levels in rays and outer tracheids were observed. Wounding increased the reaction at the wound margin where callose was being formed. The staining reaction decreased abruptly beyond the wound margin. Pine produced more of a reaction than did spruce in wounded roots.

Infected: Dehydrogenase was secreted by the hyphae but was very low or absent in rays near hyphae (Fig. V.6). Moderate background levels were restored in rays 2 cm ahead of the hyphae. Therefore ray cells were apparently killed (no colour reaction was observed) at the

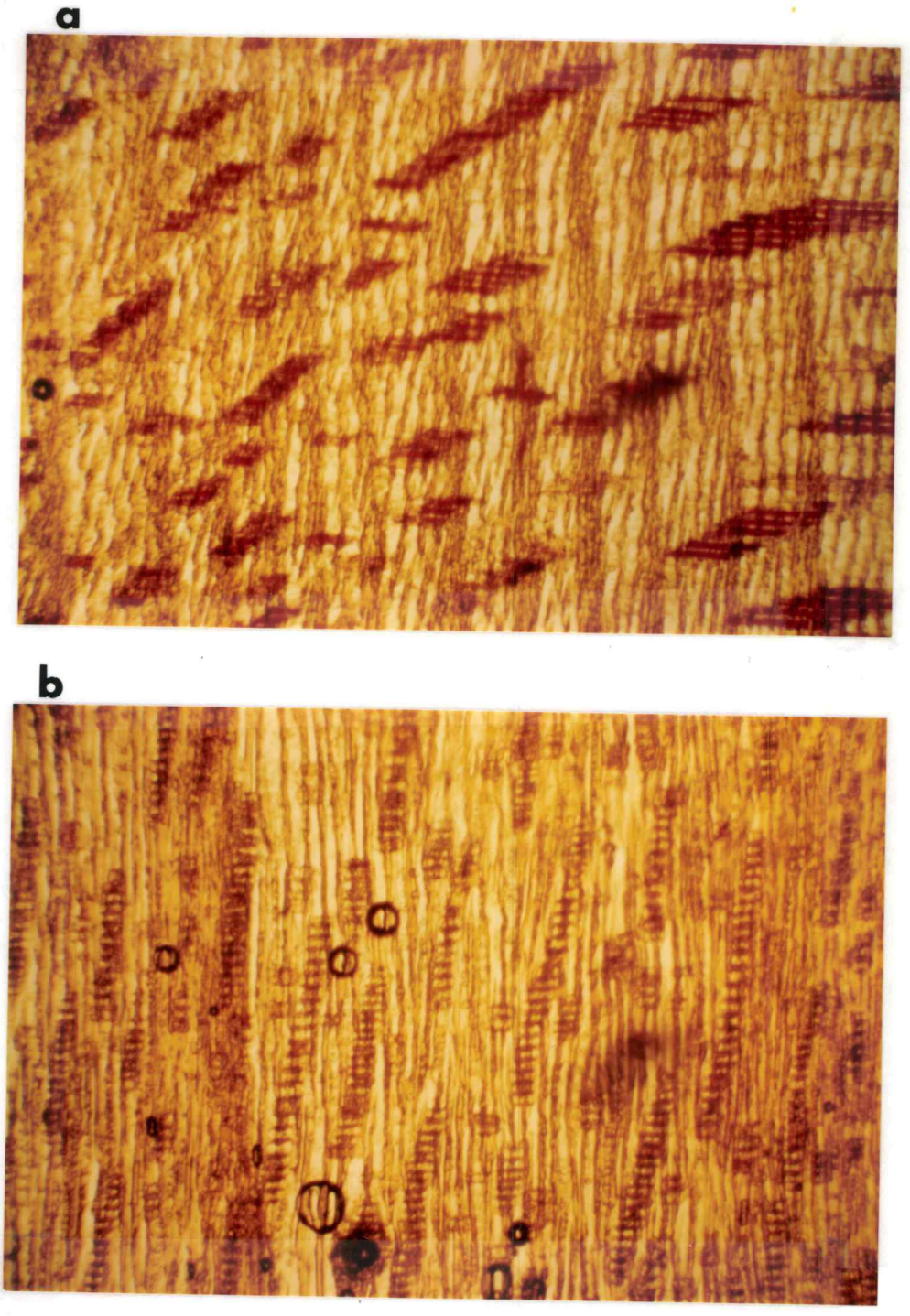


Figure V.5. Accumulation of phenols (stained orange-red) in rays near a wound (a); and lack of phenol accumulation from a section opposite the wound (b). x100.

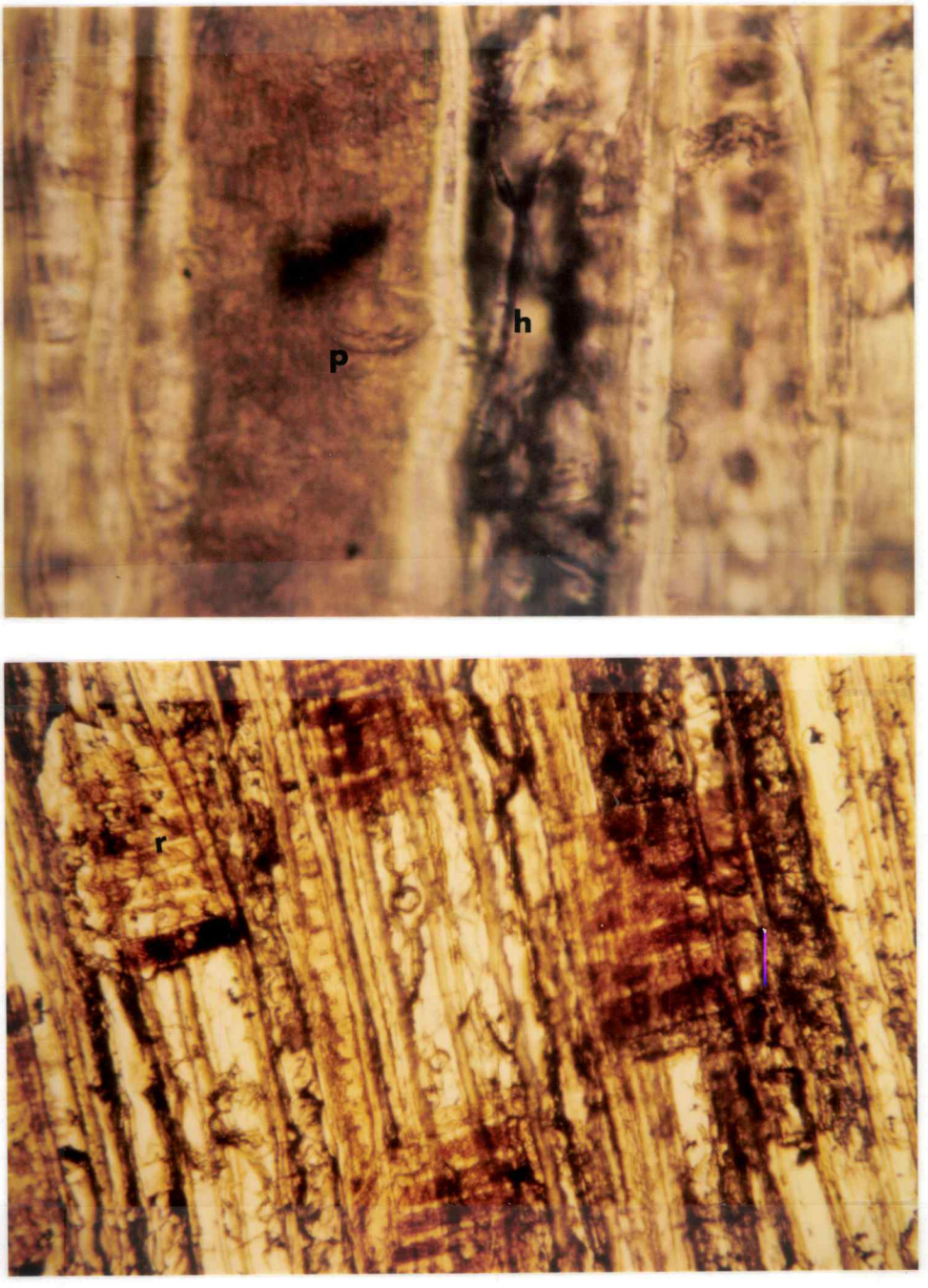


Figure V.6. Dehydrogenase extruded from hyphae (h), phenols (p) in neighbouring tracheid (x400), and lack of dehydrogenase in ray (r) (x100) in the colonized sections.

hyphal margin but killing decreased at 2 cm from the hyphal front.

Glucosidase

Uninfected: The glucosidase reaction was very low or absent in unwounded roots but low levels were observed in wounded roots. Spruce and pine showed no difference in glucosidase reaction.

Infected: Glucosidase was indicated in hyphae of infected roots, but there was little difference in host response from the uninfected roots (Fig. V.7).

Phosphatase

Uninfected: Not observed in uninfected (wounded or unwounded) roots.

Infected: Phosphatase reaction was observed in hyphae and in rays near the hyphae. The reaction decreased rapidly ahead of the hyphal front.

Starch

Uninfected: Starch content was variable, but in general the highest concentrations were observed in unwounded roots and on the unwounded side of wounded roots (Fig. V.8). Near wounds, starch levels were rated as low, which reverted to moderate levels 1 to 2 cm ahead of the wound. No difference was found in starch deposits between spruce and pine.

Infected: Starch at the front of the hyphae was depleted below the levels observed in aseptic wounds. At 4 cm from the hyphal front, starch levels remained depleted.

Peroxidase

Uninfected: Compared to very low levels in unwounded roots, peroxidase activity increased in rays close to the wound margin.

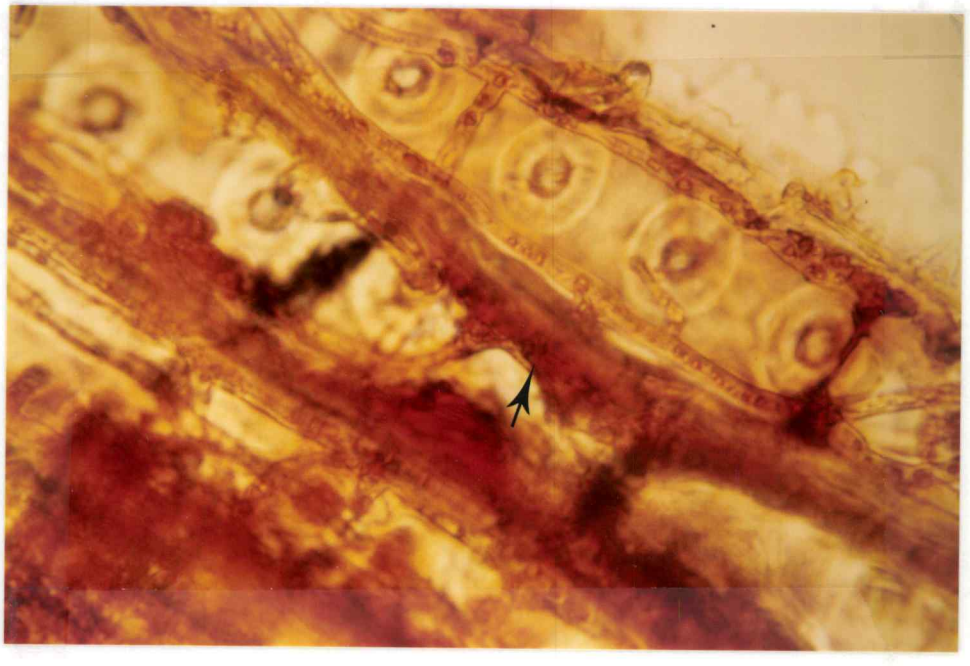


Figure V.7. Glucosidase in hyphae (arrow) x400.

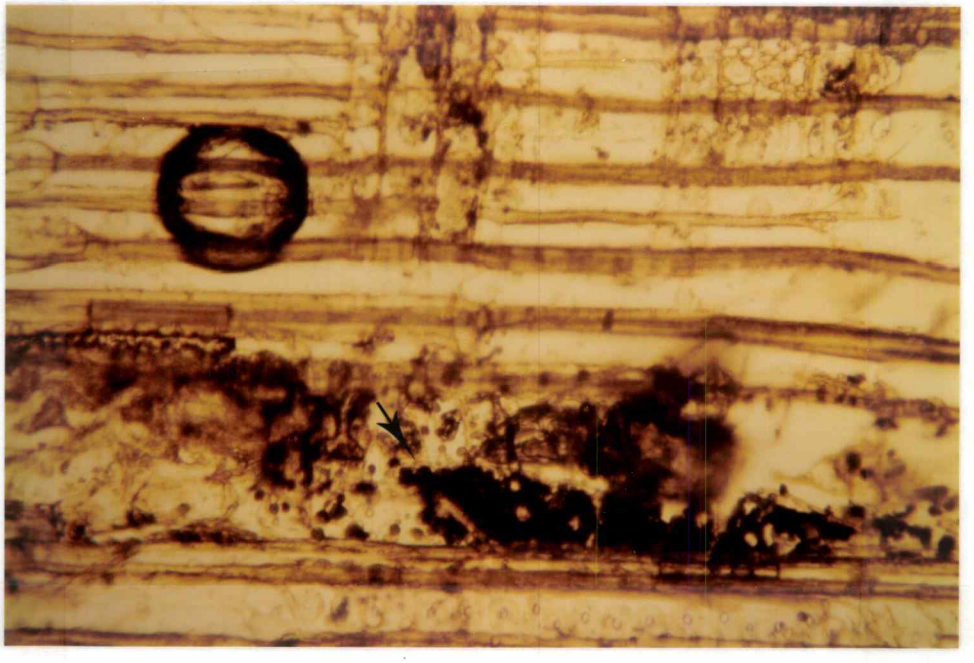


Figure V.8. Accumulation of starch (arrow) in unwounded, uninoculated roots. x400.

Infected: Activity at the hyphal front increased to a high intensity then slowly decreased to elimination at 4 cm in front of the hyphae. Peroxidase was very high in rays at the hyphal margin.

Laccase

Uninfected: The laccase reaction was not observed in unwounded or wounded controls.

Infected: Laccase was exuded from the hyphae and was very active at the hyphal front, decreasing in the older hyphae. Laccase activity positively corresponded with phenolic content in tracheids (Fig. V.9).

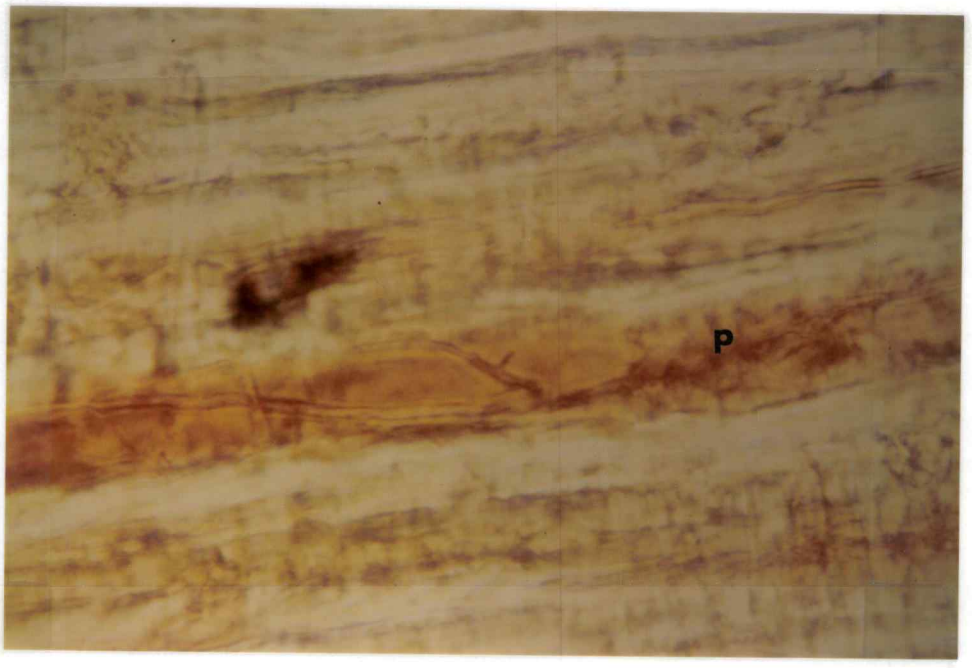


Figure V.9 Phenol deposition (p) caused by fungal laccase activity. x400.

DISCUSSION

By the inoculation and infection-determination methods used in this study, lodgepole pine was less frequently infected by I. tomentosus than was interior spruce. More pine than spruce roots were infected by I. circinatus, but the sample size was too small to draw many conclusions. Resin flow upon wounding, phenolic production and dehydrogenase activity were greater in pine than in spruce and may account for the lower infection rates.

Interpretation and extrapolation of the results in this study were constrained by the low infection rate of inoculated roots. Inoculations were left for one year before harvest; a longer period of time may have increased the infection rate. There was little evidence of mycelium on the bark of roots inoculated with naturally colonized root pieces. During the year the inoculum changed from having reddish-brown decay pockets full of mycelium, to brown apparently empty pockets. This suggests that the fungus was declining in fully colonized roots and may not have had a large enough food base remaining in the root piece to grow across and penetrate the tree root. Furthermore, exposure of the decay columns in the root by cutting may have contributed to the decline.

Artificial inoculation was more successful. Inonotus tomentosus was isolated from 20 roots, but hyphae were observed in the wood of only 7 roots. Samples removed for isolation were wedges containing both bark and wood, whereas the tissue sectioned and stained to detect hyphae was primarily wood tissue. Even though isolation of I. tomentosus is difficult (especially from bark) due to the slow growth of the fungus, isolation plates apparently revealed bark infections

that had not yet grown into the wood. Both I. tomentosus and I. circinatus grow very slowly in culture (Nobles, 1965). Growth of mycelium already established in roots was measured at 3.8 cm/year (Whitney and VanGroenewoud, 1964). One year may not have provided enough time for the fungus to become established in the wood. Whitney and Denyer (1969) obtained an 86% success rate from inoculations left for three years. However the inoculation holes were two inches or more into the heartwood, therefore the high success rate could be due to increased incubation time or to bypassing the defense response of the tree by inoculating directly into the heartwood.

In contrast Heterobasidion annosum is a faster-growing fungus (Nobles, 1965). Johansson and Stenlid (1985) measured growth rates of H. annosum in wood as 2-3 mm per day (compared to 38 mm per year for I. tomentosus). They found hyphae in root wood 3 days following inoculation and observed more dramatic biochemical changes than those seen in the present study. They were also able to measure response over time which was not done in this study.

Although the percentages of infections which occurred in unwounded and in wounded roots were similar, the observations of ectotrophic mycelium and mycelium in the bark (in this study and under natural conditions - Lewis Chapter II and IV) suggests that the preferred mode of entry is through the bark. Resin production and phenolic deposition at the wound site may actually inhibit direct entry through wounds although Whitney and Denyer (1969) found that resin impregnation of inoculum holes inhibited I. circinatus but not I. tomentosus. Inhibition of I. circinatus by resin production

suggests that I. circinatus would be more virulent on spruce than on pine based on observations of an abundant resin flow from pine. From the limited sample in this study, the opposite was true. Only the I. circinatus isolate, WH-11, infected pine to the wood, although, as determined by the results from isolations, several I. tomentosus isolates caused bark infections of pine. Even though the alder blocks were inoculated at the same time under the same conditions, several isolates produced more luxuriant mycelium on the blocks. The I. circinatus isolate grew more rapidly relative to the others and thickly colonized the surface of the alder blocks which may explain its apparent virulence. Isolates BC-7 and AV-3 also grew rapidly, but did not infect pine in this study.

The relationship between I. tomentosus and I. circinatus is of practical concern because of their morphological similarity and possible difference in virulence on pine. The primary distinguishing character between the two species is the morphology of the setae. For many years I. circinatus was considered a variety of Polyporus tomentosus. In 1974 Gilbertson placed them as separate species in the genus Inonotus, based on morphological factors.

Different geographical distributions and habitats of I. tomentosus and I. circinatus, with limited range overlap could indicate that the two host-pathogen interactions evolved separately from a common ancestor. One interaction was between I. circinatus and pine in the southeastern pine regions, and the second was between I. tomentosus and spruce in the northern boreal forests. Perhaps I. circinatus has evolved a capacity to withstand fungitoxic phenolic compounds such as pinosylvin and pinosylvin monomethyl ether, found

in pine heartwood. These phenolics were also found in sapwood of Pinus resinosa Ait. near wounds and Heterobasidion annosum-infected tissue (Jorgensen, 1961). This would explain the infection of both spruce and pine roots with I. circinatus and the lack of infection of pine by I. tomentosus observed in this study. However, in mixed stands of pine and spruce, pine was as frequently infected as spruce by I. tomentosus, although the fungus was not as prevalent in the heartwood of pine as in spruce (Schulting, 1987). Further work on host specificity and virulence needs to be done before conclusions can be made regarding their host ranges and pathogenic adaptations.

The potential for infection of pine compared to spruce is an important consideration for management of areas in the boreal forest with tomentosus root disease. Inoculations tests with germlings ranked ponderosa pine and lodgepole pine as more susceptible than white spruce (Whitney and Bohaychuck, 1976), although other tests with living trees showed the opposite (Whitney, 1964). The present study found spruce to be more commonly infected than pine which may be due in part to the greater phenolic response by pine. In addition, Lewis and Hansen (1988) found that colonized pine stump roots covered less area than spruce and fewer roots were colonized so that the likelihood of root contacts between regeneration trees and pine stump roots was less. The vertically oriented roots of pine, compared to horizontally oriented spruce roots, and the lower infection rate of inoculated pine determined in this study makes pine a good alternative to spruce for establishment in sites with a history of root disease.

Histochemical responses of spruce and pine roots to wounding

and/or to infection of the wood tissue by I. tomentosus or I. circinatus primarily occurred in the ray parenchyma. Wounding alone of both spruce and pine roots caused an accumulation of phenols in cells adjacent to the wound, and death of cells in the immediate vicinity of the wound as indicated by a decrease in dehydrogenase activity. Dehydrogenase activity was greater in the cells involved in callose formation near the wound. Starch content was greater in unwounded roots than in cells near wound-damaged cells. Bark infections of roots were not different from aseptic wounds in host response. However, the presence of hyphae in tracheids intensified some of the reactions compared to aseptic wounds. One of seven roots with hyphae in the wood also produced I. tomentosus in culture. The appearance of the fungus in wood tissue and the responses observed were similar between this root and the other five roots. Therefore, hyphae observed colonizing the rays and tracheids were assumed to be I. tomentosus or I. circinatus even though the reisolations did not substantiate all of them.

Initial colonization appeared to occur primarily in the tracheids. As colonization developed, the rays also became colonized. Ray cells ahead of the hyphal front appeared to increase dehydrogenase activity, which decreased abruptly at and behind the hyphal front where ray parenchyma were killed, probably by invading hyphae and phenol deposition. The killing of ray cells observed by Johansson and Stenlid (1985) occurred prior to invasion of the dead cells by H. annosum through tori. They postulated that chemical signals induced by wounding and infection moved between rays via the tracheids. In the present study, response in wood infected by I.

tomentosus was more subdued compared to what Johansson and Stenlid (1985) observed with H. annosum. It is possible that the paucity of ray parenchyma infection by I. tomentosus and I. circinatus resulted in a less dynamic response. The accumulation of phenols extended 1 to 2 cm beyond the hyphal front. In the one case where hyphae were well established in the rays and tracheids, phenols in the immediate vicinity were depleted and had a grainy appearance in the tracheids. Starch depletion coincided with dehydrogenase activity and phenol accumulation perhaps signifying increased host activity aimed at the biosynthesis of phenolics from starch.

According to Hammerschmidt and Kuc (1982) host peroxidase activity is important in the production of lignin-like materials (phenolics) by polymerizing alcohols during phenol biosynthesis. Johannson and Stenlid (1985) found that peroxidase activity was associated with the dysfunction of the tori during lignification and dry zone formation. In addition, peroxidase may be involved in hydrolytic reactions to produce phytoalexin-like compounds in ray cells in advance of the hyphae (Hammerschmidt and Kuc, 1982; Johannson and Stenlid, 1985). In this study peroxidase activity increased at the hyphal front and in the zone of phenolic deposition, and remained elevated up to 4 cm beyond the hyphal front. This suggests that a signal is involved which induces peroxidase activity. One such signal may be ethylene which is known to increase peroxidase activity (Day, 1974), and Johannson and Stenlid (1985) have observed increases in ethylene production in infected Norway spruce roots.

These processes are similar to those found during the production of heartwood in which peroxidase plays an important role (Shigo and

Hillis, 1973). Lignification of host cell walls changes permeability of the cells, and therefore reduces food availability to the fungus. Pine roots produced more phenolics and had a greater surge of activity in advance of the hyphae than did spruce, although no differences in peroxidase activity between spruce and pine were noted. This may be due to sparse infection of pine and the small sample size. Fungal laccase was observed in four of seven of the infected roots, all of which were spruce. Laccase activity was negatively correlated with age of the hyphae, and positively correlated with the degree of hyphal establishment in the wood and the phenol content of the cells. Laccase is thought to be induced by the presence of toxic host phenolics (Johansson and Stenlid, 1985). There may be some variation among isolates in the quantity and lag time between induction and production of laccase, which may explain observed differences in infectivity. Further experimentation with more isolates needs to be done to address this.

In conclusion, further inoculation studies to examine host susceptibility and response to infection, and pathogen virulence should involve unwounded roots, and long incubation times. Using the methods described above, pine was less susceptible than spruce to infection, which was correlated with greater resin flow and phenolic production by pine. The I. circinatus isolate was the only isolate to cause infection in the wood of pine roots although both Inonotus species caused bark infections.

A reaction zone, more subtle than that described in H. annosum by Johansson and Stenlid (1985) did form in spruce and pine. Phenolic deposition, dehydrogenase reduction, peroxidase increase and

starch depletion characterized the host responses. The reaction zone of pine appeared to be more active than that of spruce, and may be more effective at deterring infection.

Chapter VI. Comparison of Inonotus tomentosus (Fr.) Teng and I. circinatus (Fr.) Gilbn. by total protein electrophoresis and restriction fragment length polymorphisms.

INTRODUCTION

Inonotus tomentosus and I. circinatus both cause root rot of conifers in North America. Inonotus tomentosus has a centrally stipitate sporophore and straight hymenial setae, whereas I. circinatus has a sessile shelving sporophore and curved hymenial setae. There are some confusing similarities and differences in their morphology, mode of action, host and geographic range.

The taxonomic history of these two fungi begins with their description by Fries as two separate species: Polyporus tomentosus and P. circinatus (Fries, 1821, 1848) based on the pileus context. Both species underwent several changes of genus (eg. Cooke, 1886; Karsten, 1882; Patouillard, 1900). Some authors made no distinction between the two species (eg. Murrill, 1904) and in 1922 Sartory and Maire placed P. circinatus as a variety of P. tomentosus. Haddow (1941) separated the two primarily because of the differences in the hymenial setae, but determined they were too similar to be different species. It was Teng (1964) who transferred P. tomentosus to Inonotus and Gilbertson (1974) who transferred P. circinatus to the genus Inonotus. Gilbertson separated primarily on morphological characteristics which sometimes overlap, and host preference which does overlap.

In the southeastern United States, I. circinatus predominates and is reported (as Polyporus tomentosus var. circinatus) to cause significant damage to pine plantations (Boyce, 1963; Ross, 1966). In

western Canada, the only species found is I. tomentosus which primarily causes a root and butt rot of spruce (Merler, 1984), but to a lesser extent causes cambial necrosis of pine (Schulting, 1987). In central and eastern Canada, Whitney (1962) reports both species on spruce and pine, although I. tomentosus is more common. Inoculation tests with I. tomentosus and I. circinatus suggest that I. tomentosus is more virulent than I. circinatus (Whitney, 1967, 1964; Whitney and Bohaychuck, 1976) but interpretation of the results is limited because of the artificial wounding technique used for inoculation.

One hypothesis which explains the overlapping of geographic and host ranges and morphological characteristics is that the two species have evolved from a common ancestor. Inonotus circinatus evolved in a warm dry environment with pine as the predominant host, while I. tomentosus evolved in a boreal climate with spruce as the predominant host and pine as a minor host.

Several techniques are available to examine genetic similarities between the two species. One such technique is total protein electrophoresis which was found by Hansen et al. (1986) to be a sensitive indicator of subgroups of Phytophthora megasperma. Another technique is analysis of restriction fragment length polymorphisms (RFLP). RFLPs have been widely used as markers in plant genetics (Michlemore and Hulbert, 1987) and are becoming a common tool in fungal taxonomy (Armstrong et al. 1989; Raeder and Broda, 1986; Specht et al. 1984).

Genetic analysis using molecular techniques has been slow to develop with filamentous fungi, particularly Basidiomycetes. This is due in part to slow growth rates and infrequent or lack of sporulation

in culture. No molecular work has been done with I. tomentosus or I. circinatus. The objectives of this study were to: 1) find an efficient DNA extraction protocol; 2) construct homologous probes using I. tomentosus DNA; 3) to use molecular markers (protein profiles and RFLP's) to compare several isolates each of I. tomentosus and I. circinatus in order to relate genetic differences to geographical separation and species separation.

MATERIALS AND METHODS

Isolates

Isolates of I. tomentosus were collected in British Columbia and from other workers in the United States and Canada; isolates of I. circinatus were all donated (Table VI.1). Geographically widespread isolates were chosen to compare genetic differences within a species to differences between species. For both protein and DNA extraction, isolates were grown in glucose-yeast-peptone (GYP) broth (Gill and Zentmeyer, 1978) for 3 weeks at room temperature (20C). Cultures were harvested by vacuum filtration through Whatman No. 1 filter paper with 3 washes of distilled water.

Protein electrophoresis

Harvested mycelium was loaded into a frozen steel vessel with 1 ml of phosphate buffer pH 7.0, and acid washed sand. The mycelium was ground with a drill and grinding bit, then the mix was centrifuged at 13000 g for 20 min. A 0.3 ml sample of the yellow, clear supernatant was placed with 0.3 ml of sample buffer (2% sodium dodecyl sulphate (SDS), 10% mercaptoethanol, 0.1M TRIS pH 6.8, 20% glycerol) in an eppendorf tube, heat fixed at 100 C for 1 min. and frozen at -20C. A 0.1 ml sample was placed in a tube with 5 mls diluted BioRad protein assay buffer to analyze protein concentrations such that equal amounts of protein were loaded into each lane of the gel. The remainder of the supernatant was stored at -20C in eppendorf tubes.

SDS polyacrylamide gels were prepared and electrophoresis carried out according to the methods of Thomas and Kornberg (1975). Samples were electrophoresed at room temperature at 105 v, then placed in 1%

Table VI.1. The location, host and donor of I. tomentosus and I. circinatus isolates compared by protein electrophoresis and restriction fragment length polymorphisms.

Isolate	Sp.	Location	Host[1]	Donor
BV2-4	It[2]	Beaver Rd, Prince George, B.C.	Sx	KL[3]
BV2-8	It	Beaver Rd, Prince George, B.C.	Sx	KL
Sm-1	It	Smithers, B.C.	Sw	KL
Av-2	It	Averil Lk., Prince George, B.C.	Sx	KL
KL-2	It	Missoula, Montana	Pm	USFS[4]
Ak-1	It	Kenai Peninsula, Alaska	Sw	PH[5]
WH-85	It	Grand-mere, Quebec	Sw	RW[6]
WH-110	It	Tobin Rapids, Saskatchewan	Sw	RW
WH-91	It	Finland	Pef	RW
Mad-2	It	Pennsylvania	Pr	MD[7]
Mad-3	It	Arlington, Virginia	P	MD
WH-140	It	Candle Lake, Saskatchewan	Sw	RW
BC-1	It	Kispiox, B.C.	Sw	DM[8]
Mad-5	Ic[9]	Wedgefield, S. Carolina	Ps	MD
Mad-6	Ic	Maryland	Pv	MD
Mad-7	Ic	Virginia	P	MD
Mad-8	Ic	Maryland	P	MD
Mad-9	Ic	Arkansas	Pe	MD
Mad-10	Ic	Sand Hills, S. Carolina	P	MD
WH-11	Ic	Candle Lake, Saskatchewan	Sw	RW
WH-32	Ic	Candle Lake, Saskatchewan	Sw	RW
WH-96	Ic	Candle Lake, Saskatchewan	Sw	RW

1. Hosts: Sx = interior spruce, Picea glauca x engelmanni Engelm.
   Sw = white spruce, P. glauca (Moench)Voss
   Se = engelmann spruce, P. engelmannii Parry
   Pef = P. excelsa x fennica
   Pm = western white pine, Pinus monticola Dougl.
   Pr = pitch pine, P. rigida Mill
   Ps = slash pine, P. elliottii Engelm.
   Pv = Virginia pine, P. virginiana
   Pe = shortleaf pine, P. echinata Mill
2. It = Inonotus tomentosus
3. KL = Kathy Lewis, Oregon State University
4. USFS = U.S. Forest Service, Missoula Montana
5. PH = Paul Hennon, U.S. Forest Service, Alaska
6. RW = Roy Whitney, Forestry Canada, Ont.
7. MD = Forest Products Lab, Madison Wisconsin
8. DM = Duncan Morrison, Forestry Canada, Victoria, B.C.
9. Ic = I. circinatus

coomassie brilliant blue R-250 in acetic acid, methanol and water (1:5:5) and gently shaken overnight. The gels were then destained with ethanol, acetic acid and water (20:7:73) prior to photography.

Band differences were compared between all isolates and similarity coefficients were calculated for each pair of isolates.

similarity coefficient =

$$\left(\frac{\text{number of bands in common}}{\text{total number in both isolates}}\right) \times 100$$

DNA extraction, electrophoresis and Southern hybridizations

Hyphae were harvested by vacuum filtration through Whatman No. 1 filters, lyophilized then ground with a mortar and pestle and stored at -20C. Total genomic DNA was extracted from approximately 100 mg lyophilized, ground mycelium by incubation in lysis buffer (50mM Tris HCl; 50mM EDTA; 3% SDS and 1% 2-mercaptoethanol) at 65C for one hour. Lysate was extracted with chloroform:phenol (1:1) and chloroform, then DNA was precipitated in isopropanol. The pellet was resuspended in 300 ul TE (10mM Tris, 1mM EDTA, pH 8.0) and precipitated in ethanol. Procedures outlined by Maniatis et al. (1982) were followed except where noted. One ug of DNA was digested overnight with EcoRl (BRL)(according to the manufacturers instructions) at 37C. Electrophoresis was carried out in 0.8% agarose gels in 0.5x TBE (0.089M Tris, 0.089M Boric acid, 0.002M EDTA, pH 8.0) buffer with RNase A (lul/50ml agarose) and ethidium bromide (2.5ul/50ml agarose) incorporated. Digested DNA was transferred to Genetran nylon membranes and stored at 5C (Southern, 1975).

Random genomic clones were prepared from DNA of isolate BC-1 by

restriction with EcoR1, ligation to pUC 19, which carries a β-galactosidase gene and ampicillin resistance, and transformed into competent E. coli cells (DH5α). Plasmids containing insert DNA were identified by digestion with EcoR1 and gel electrophoresis. Plasmid mini-preps were performed on transformants, and probe DNA was isolated from low melting temperature agarose (FMC BioProducts, 5 Maple st., Rockland, ME) mini-gels and eluted from the gel using mini ion-exchange columns (Elutip-d, Schleicher and Schuell, Keene, NH). Probe DNA was labelled with ^{32}P-dCTP by random priming (Feinberg and Vogelstein, 1983).

The blot was prehybridized overnight at 42C (in 3x SSPE, 0.5% SDS, 0.5% Denhardts, 50% formamide and 200ul salmon sperm DNA in 20 ml final volume), then hybridized with labelled probe in 10-15 ml prehybridization buffer overnight at 42C. The blot was washed once with 250 ml of 50% formamide, 5x SSPE and 0.1% SDS in water (60C), then 1 or 2 times in SSPE and SDS buffers of increasing stringency. Kodak X-ray film was exposed at -70C using intensifying screens.

RESULTS

Protein electrophoresis

Each isolate had a unique protein banding pattern. The range of similarity coefficients (sc) was from 75.4 to 93.5, based on approximately 30 bands that were counted. Except for two I. circinatus isolates (WH-32 and WH-96), two bands were found consistently in the I. circinatus isolates which were not in the I. tomentosus isolates (Fig. VI.1). WH-32 and WH-96 were from basidiospores from sessile sporocarps with curved setae. Mean sc values from comparisons between WH-96 and all of the I. tomentosus all of the I. circinatus isolates were not different (mean sc values were 84.3 and 85.1 respectively). The setal morphology of WH-91 from Finland was unknown. However the protein pattern of WH-91 was more similar to I. circinatus patterns than to I. tomentosus patterns (mean sc's were 90.3 and 85.3 respectively). WH-32 was not included in the sc analysis because the protein preparation was degraded.

Mean sc values were calculated for the comparison of patterns within each species and between species. The isolates WH-96 and WH-32 were not included in this calculation. The protein patterns of I. circinatus isolates were significantly more similar to each other (sc = 92.1) than to the I. tomentosus isolates (85.3). But patterns of I. tomentosus isolates were not more similar to each other than to I. circinatus isolates (85.5 for within I. tomentosus; 85.3 for between I. tomentosus and I. circinatus).

DNA restriction fragments

The method used for DNA extraction rapidly provided an adequate amount of DNA especially when several phenol:chloroform extractions

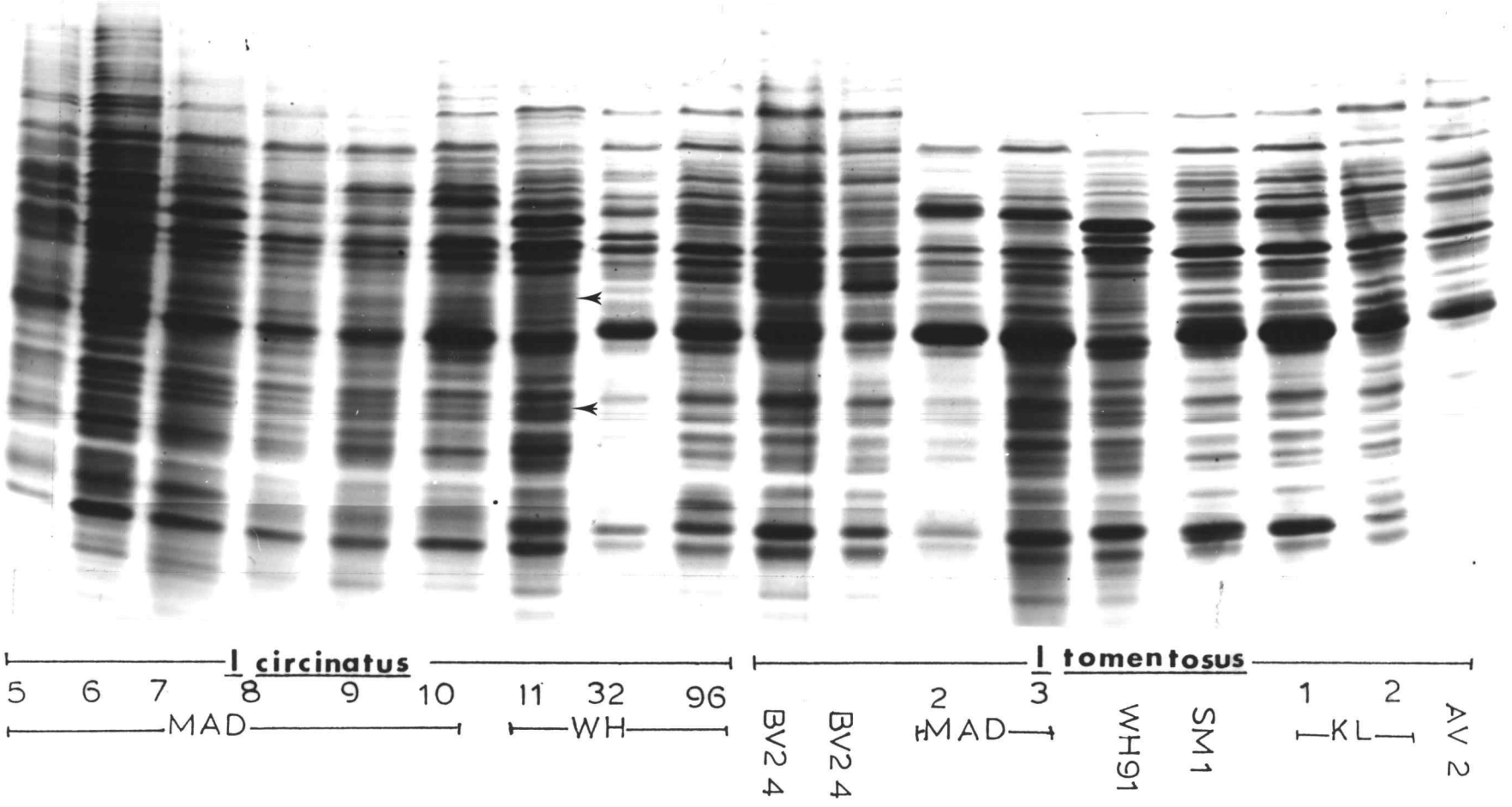


Figure VI.1. Electrophoretic protein patterns of isolates of I. tomentosus and I. circinatus. Arrows denote protein polymorphisms.

were carried out. Yield was approximately 50 ug DNA from 60-100mg lyophilized mycelium. DNA extracted by this procedure was generally susceptible to digestion by EcoR1 although several isolates produced a dark precipitate. Several random genomic clones were obtained, but following EcoR1 digestion and electrophoresis of DNA mini-preps of each clone, only one showed a second band not attributed to uncut, supercoiled or linear plasmid. This clone (clone 10), was developed into a probe. The insert was approximately 500 base-pairs in size. Hybridization of the insert to EcoR1 digested total genomic DNA of I. tomentosus and I. circinatus was successful for 9 of 12 isolates (8 of 9 I. tomentosus and 1 of 3 I. circinatus). Two DNA fragments were identified in the total genomic DNA preparations which had homologous sequences with the probe. However restriction fragment length polymorphisms were not revealed in any isolates.

DISCUSSION

Isolates of Inonotus were variable at the protein level, but the single restriction enzyme and DNA probe used to measure variation at the DNA level showed no variation between isolates. Variation in protein banding patterns was greater between the isolates in this study than between isolates collected from within one infested stand (Chapter III). Therefore geographical separation positively affected diversity in protein profiles. Protein patterns also appeared to correspond to species because most of the isolates could be identified as I. tomentosus or I. circinatus based on their protein band pattern. The isolates that did not fit within the suggested protein pattern-species group may have been mis-identified. The protein profile and doubt about the morphology of hymenial setae of the Finnish isolate (WH-91), suggest that it may belong in the I. circinatus group, not within I. tomentosus as it was labelled.

The overlap in morphological characteristics sometimes makes it difficult to conclusively identify species. This may have been the case for the isolates WH-32 and WH-96 labelled as I. circinatus, which more closely resemble the I. tomentosus isolates based on the 2 bands marked in fig. VI.1. This result should be further supported by mating compatibility studies or further molecular genetic work before conclusions can be made.

Inonotus circinatus isolates were more similar within their taxonomic species than were the I. tomentosus isolates. This may suggest greater variation and more rapid change occurring in I. tomentosus. It is possible that I. circinatus is derived from a population that split from the I. tomentosus group. Perhaps the

limitation of hosts to Pinus spp. in the eastern U.S., and the more uniform climate and selection pressures on I. circinatus have reduced genetic divergence compared to I. tomentosus on several host genera in the boreal forest.

Electrophoretic protein patterns were found by Angwin (1989) to consistently separate biological species groups of Phellinus weirii. The biological species (Douglas-fir type and cedar type) first defined by mating compatibility, were then shown to correspond to electrophoretic patterns which differed by only a few major bands. The cedar type was determined to have a narrow host range whereas the Douglas-fir type had a wider host range. This is similar to the depth of host range evident in I. circinatus and I. tomentosus. Furthermore, as with Inonotus, the geographical ranges of the P. weirii biological species overlapped. Angwin (1989) concluded that speciation was sympatric, rather than allopatric. Morphological differences between Douglas-fir and cedar types were more subtle than those between I. tomentosus and I. circinatus. These latter two species may have diverged further as suggested by morphological differences, than the Douglas-fir and cedar biological species of P. weirii.

Sympatric speciation is also suspected in Heterobasidion annosum, where host preferences are found in the spruce and pine types (Korhonen, 1978). In the case of H. annosum, the mode of pathogenicity in the host is different between spruce and pine. Like I. tomentosus, H. annosum generally causes a butt rot in spruce and cambial necrosis in pine. Such differences may be due more to the host biochemistry than to the fungal species, but different

biochemical responses to infection may place different selection pressures on the biological species that attacks pine compared to spruce.

Results from the Southern hybridizations of total genomic DNA have provided a basis for further molecular work. Although no restriction fragment length polymorphisms were revealed, a homologous probe was constructed and shown to hybridize in two locations to total genomic DNA. The use of additional probes with different enzymes is needed to identify RFLPs which can be used as markers to study the genetic relatedness of populations of I. tomentosus and I. circinatus.

In conclusion, there are notable genetic differences at the protein level between I. tomentosus and I. circinatus. Electrophoretic protein patterns may not be mutually exclusive but they do allow identification of the two species. More variation is evident in I. tomentosus isolates than in I. circinatus isolates which may be due to uniformity of the habitat and host range of I. circinatus, or perhaps other mechanisms are involved such as more common spread by spores of I. tomentosus than of I. circinatus. Evidence of the role of spores in disease spread exists for I. tomentosus (Chapter III) but has not been studied with I. circinatus.

SUMMARY

Inonotus tomentosus causes a root rot of spruce and pine species in the boreal forest of Canada. In British Columbia the fungus attacks old growth Picea glauca (Moench.)Voss., P. glauca x engelmannii Engelm. and Pinus contorta Dougl. and causes significant damage by windthrow, mortality, butt cull and growth reductions.

Pine species are thought by some to be less susceptible to infection by I. tomentosus (Merler, 1984; Whitney, 1962) but other evidence suggests that pine are equally susceptible to infection (Schulting, 1987). In spruce the fungus causes a root and butt rot; in pine, cambial necrosis and more rapid mortality are common effects of I. tomentosus infection (Schulting, 1987). The relative susceptibility and damage of pine compared to spruce is an important question for tomentosus root disease management. This is particularly important with regard to carry-over of the disease from old growth stands to second growth forests. Second growth management is of increasing importance in British Columbia, and the potential for damage to the young stands by pathogens from the previous rotation is a major concern.

Many components of the tomentosus root disease cycle in old growth stands are different in second growth stands. The lag time between tree death or harvest and replacement by a susceptible species, the proximity of regeneration, and the species which are established are examples of these differences. The likelihood of carry-over from past-rotation stumps to new trees and the rate of spread in new stands is influenced at many stages of the disease

cycle, some of which were examined for this dissertation.

The first study (chapter II) examined the distribution of diseased stumps on a regional basis (such as a watershed, or main road system), distribution within clearcuts, and distribution of the fungus within a stump. The likelihood of root contacts with old growth trees and the subsequent infection of regeneration was studied by excavations of stumps ranging in age from one year to 30 years.

The disease was commonly found in pure spruce or in mixed spruce-pine stands in moist habitats. It was rare in drier pure pine stands. In clearcuts, disease incidence was patchy with 1 to 6 diseased stumps in a patch; most patches consisted of 2 to 3 stumps. The patches (defined as disease centres) themselves were clumped within a clearcut, with up to 40 disease centres per hectare in some areas of the stand, and other areas with none. Such a patchy distribution suggests that disease surveys should employ wide transects (10m was satisfactory) which are long enough to cover 10% of the stand area. This coverage gives an allowable error of 0.02 with 90% confidence in spruce stands. For mixed pine-spruce, the area to be covered was less (6%). Stump surface decay reliably predicted root decay in spruce therefore stump top surveys in clearcuts will give accurate results. This is not the case with pine stumps.

Inonotus tomentosus in spruce and pine stumps was still viable at 30 years. Furthermore, in both species it was found near the root surface where it was in a position to cause infection of a contacting root. In young spruce stumps (1 and 2 years) the fungus existed primarily in the interior of the root. Post-harvest longitudinal

growth was limited, but radial expansion from the root centre to the surface was common. Colonization of spruce stump roots peaked at age 15-20; in older stumps brown rot fungi, which apparently entered through the stump surface replaced I. tomentosus from the stump down the roots. This caused a decrease in colonized root length over time. Total root length also decreased over time as roots were decayed by other fungi from the distal end, particularly in roots not colonized by I. tomentosus. In pine stumps, the fungus commonly colonized the cambium and bark as well as isolated bands in the root wood in both young and old stumps. Total root length in pine decreased over time but colonized length remained the same; pine roots were not invaded by brown rot fungi as were spruce roots.

The number of root contacts between stumps and regeneration trees increased with regeneration age. In this study 20% of trees growing closest to spruce stumps and 10% of trees closest to pine stumps were infected. Likewise, the chance of infection increased with decreasing distance between the stump and the tree. Spruce stumps were more likely to contribute to carry-over of I. tomentosus from one rotation to the next because the roots covered more area and more of the roots were colonized. Trees growing within 200cm of spruce stumps had a 25% chance of infection. At 350-400cm the chance of infection is 10%. Trees within 50cm and 250-300cm of pine stumps had a 25% and 10% chance of infection respectively.

The percent of pine and spruce regeneration in root contact with old growth stumps was not significantly different, but it is suspected that in sites 30-years-old or more, the vertical rooting habit of pine will decrease its chances of becoming infected relative

to spruce. Furthermore, infected pine stumps cause fewer regeneration tree infections. Therefore the establishment of pine in root disease areas will contribute to disease control even though it is a susceptible species.

Calculations of the number of regeneration trees infected from past rotation spruce and pine stumps suggest that the frequency of disease may decrease from one rotation to the next even without control measures. These calculations most likely produce underestimates of the chance of infection because the stands were not followed over a complete rotation, and contacts that did not show infection when examined may cause infection in the future once the fungus grows out from the centre of the root. Considering the small size and clumped distribution of disease centres found in the transect survey, and the evidence for spore infections discussed below, spread by root contacts may not be the only means of disease spread and may be less important than assumed earlier.

The second study (Chapter III) employed two techniques to determine the genotype composition of disease centres observed in the field. Isolates collected from different disease centres were almost always vegetatively incompatible in pairings on malt extract agar, and they also frequently showed repeatable, unique protein profiles. Isolates collected in British Columbia and from several locations in Canada, the U.S. and Finland were all vegetatively incompatible and showed very different banding patterns. Banding pattern differences between isolates collected from disease centres within one stand were usually limited to one or two bands. Protein profiles of isolates from the same vegetative compatibility (Vc) group were identical.

Small (less than 5 trees), isolated disease centres consisted of one genotype; larger centres usually contain several genotypes of I. tomentosus. The role of spores in disease spread is well established for several other root disease fungi for which there is also information on Vc groups. Fungi that have large, single Vc group centres (such as Phellinus weirii) have an efficient means of spread by root contacts and spores have a much less important role in disease spread. Heterobasidion annosum has smaller disease centres composed of several genotypes, and the role of spores in spreading disease with this fungus is well documented. Therefore, the many genotypes and small centres found in this study suggests that spores of I. tomentosus do play an important role in disease spread although direct evidence is lacking. Once the young stands develop to pole-sized stands (30-50 years) it is anticipated that infections inherited from the old growth stumps will have developed into disease centres which will produce fruiting bodies thereby increasing the number of disease centres by spore infections.

The third study (Chapter IV) closely examined disease development and spread within an infection centre. Excavations of tree roots at the edge of disease centres showed that the fungus moved either in the heartwood of roots (usually larger than 5 cm in diameter) or in the bark of smaller roots. Ectotrophic or intrabark mycelium often preceded wood decay in the small roots. Conversely in large roots, stain (incipient decay) in the heartwood preceded radial expansion of the decay to the root surface where colonization of the bark occurred. The infection point at root contacts was in small roots, either at a small feeder root, or at a disruption in the bark

such as a root junction. Direct penetration of the bark in small roots occurred, but penetration to the wood was facilitated by feeder roots. The direction of fungal spread was generally from the centre of the infection centre outward into the stand, but several infected roots were excavated which did not fit the overall pattern of radial spread. These anomalies may be explained by coalescence of two disease centres spreading vegetatively, or the stray diseased roots may be the result of more recent spore infections.

The fourth study (Chapter V) compared the response of pine and spruce to inoculation with I. tomentosus. One isolate of I. circinatus was also used because reports of I. circinatus damage to pine in the southeastern United States and eastern Canada indicated that I. circinatus may be more a pine pathogen than I. tomentosus. Pine was infected by inoculations with I. tomentosus less frequently than spruce. Greater resin soaking in response to the wound treatment, and greater phenol production in pine may account for the lower infection rate of pine. Inonotus tomentosus and I. circinatus caused infections of the bark in spruce and pine but penetration to the pine root wood was accomplished only by the I. circinatus isolate, although the sample size of roots with I. tomentosus or I. circinatus in the wood was too small to draw conclusions.

The observed response to infection of spruce and pine occurred primarily in the ray parenchyma cells. Wounding alone caused an accumulation of phenols adjacent to the wood and death of cells near the wound in the zone of heavy phenol deposition. Starch was depleted from ray parenchyma near the wound. The presence of hyphae in the wood enhanced phenol deposition and the effect was seen

further from the hyphal front than from the wound margin alone. Dehydrogenase activity increased just ahead of the hyphal front, then was nonexistent in colonized cells and adjacent ray parenchyma. Host peroxidase activity increased at the hyphal front and remained elevated up to 4 cm from the hyphae. The accumulation of phenolic substances was thought to induce activity of fungal laccase which was depleted in older hyphae.

Finally the last chapter (VI) made preliminary comparisons of the genetic relatedness of I. tomentosus and I. circinatus. Protein profiles showed dramatic differences between most of the isolates and most isolates could be identified to species by their pattern. Several random genomic clones of I. tomentosus were prepared; one was used in conjunction with EcoR1 restriction of total genomic DNA of I. tomentosus and I. circinatus. Successful hybridization of this one probe and enzyme system showed no restriction fragment length polymorphisms. However, this study was only preliminary and further work with additional probes and restriction enzymes may yield better results. The apparent difference in host preference of the two species, the consistent difference at the protein level and morphological variation suggest significant genetic divergence, although the mechanism of speciation (allopatric or sympatric) is not clear.

This dissertation has produced some management suggestions with respect to survey strategies, species to establish on infected sites, planting distance from infected stumps, and the longevity of I. tomentosus in stumps. This work has also supported the contribution to disease spread by spores and has proposed infection courts for

inoculation by root contacts which may also apply to spore infections. Disease development from a centre outward into a stand and within a single root was described. Preliminary investigations into spruce and pine host response to infections by I. tomentosus and I. circinatus have provided a basis upon which to recommend pine as an alternative species in root disease sites and to support further studies of host response to infection.

Suggestions for future research which stem from observations made during these studies are:

1) To relate I. tomentosus incidence to habitat at the ecosystem association level (ecosystems similar physically and biologically which have similar vegetation at climax), or ecosystem association phase (recognizes contrasting soil or landform characteristics). Inonotus tomentosus was most frequently found in moist, but not wet sites and its distribution in the boreal forest suggests there may be temperature constraints on distribution. Such information would be useful for site prescriptions and root disease hazard rating.

2) To determine whether a relationship exists between the incidence and/or severity of tomentosus root disease and fire history. During site selection for the studies discussed in this dissertation, evidence of fairly recent (less than 100 years ago) fires were observed in a high proportion of heavily diseased stands. Fire is a common component of forest growth in the north and may be coincidental. On the other hand, it may lead to greater damage to root rot infected trees by decreasing host response. Fire could also provide infection courts by damaging roots.

3) Determine the relationship between root colonization, root

mortality, symptom expression and volume losses. Symptom expression relates to root mortality, but there is a lack of information on losses to I. tomentosus relative to the degree of root mortality. Such information, especially when combined with knowledge of disease development over time, is necessary for calculation of expected losses and for development of disease models.

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APPENDICES

Appendix A

1. Frequency distribution of the number of sampling units (25 m sections of transect line) with 0. 1, 2, 3, 4, 5, and 6+ diseased stumps.

2. The number of run lengths of 1, 2, 3, 4, 5, and 6+ diseased stumps by site.

Site	0	1	2	3	4	5	6
1. Number of diseased stumps by 25 m section of transect line							
Km 162	15	8	9	1	1	0	0
Site 21	8	6	8	3	3	1	1
Pinney Ck	13	4	4	1	0	0	0
Km 12 Pel	27	6	4	3	0	0	0
Kispiox	14	2	5	1	0	2	0
Jonas	40	6	8	2	0	0	0
Bob 102	35	5	4	2	2	0	0
Wansa	21	5	4	2	2	0	0
2. Number of diseased stumps by run							
Km 162		11	7	0	1	1	0
Site 21		19	5	4	2	1	0
Pinney Ck.		4	4	1	0	0	0
Km 12 Pel		6	4	3	0	0	0
Kispiox		3	7	1	0	1	0
Jonas		8	7	2	0	0	0
Bob 102		5	1	1	0	0	0
Wansa		4	3	3	2	0	0

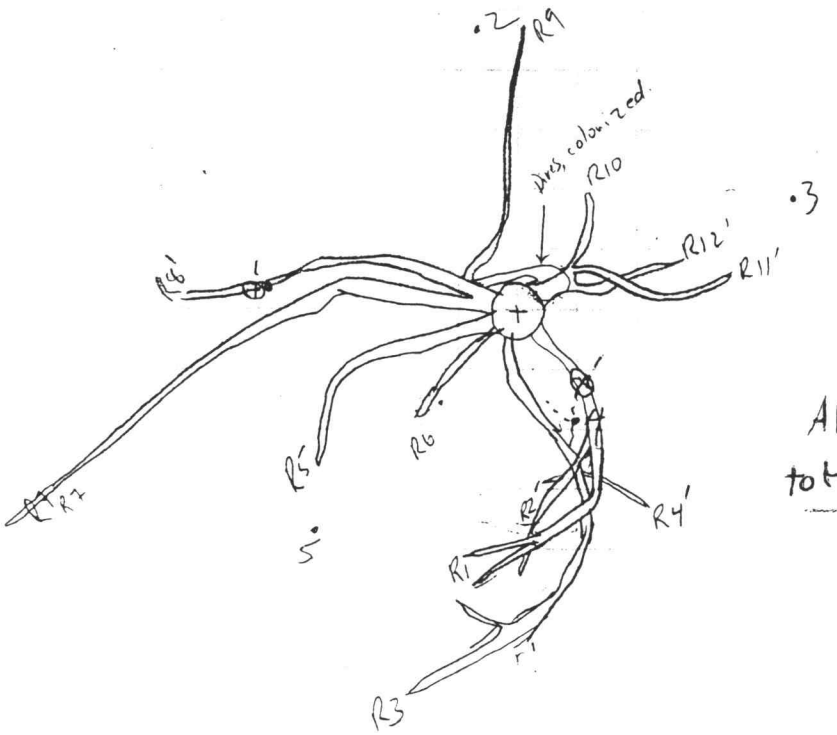
Appendix B. Example of stump maps and field notes

Site # Kapoy Transect # 1 Stump # 1 Diam. 40

Tree #	Height	Age	Leader	Distance	Brg.	Symptoms	Contact
1 Sx	120		2	~~250~~ 210	N8W	chlor., shut backs	Root 8' contact at sum RS injected
2 Ba	400		50	240	N8W		
3 Sx	170		40	255	N66E		
4 Sx	220		25	100	~~S78E~~	chlorotic, sum on root at cont.	R1 @ 70 cm
5 Ba	625		60	250	S43W		

Spruce
Red Rot

N 1:50



ROOT #	LENGTH RECOVERED	LENGTH ALONG ROOT (DIAMETER)			CM FROM EDGE	SAMPLES
		EMPTY POCKETS (Ø)	MYCELIUM (Ø)	STAIN (Ø)		
R-1	200		0-200(3)		100 = 0 200 = 0	
R-2'	80		0-80(2)		80 = 0	
R-3	360		0-360(2)		80 = 1 360 = 0 120 = 0	
R-4'	70		0-70(1.5)		70 = .3	
R-5	195	180-195 hollow	0-180(5.8)		100 = 0 180 = 0	
R-6	100	0-100 hollow (2.3)				
R-7	490	300-490 hollow (6)	0-300(6.4)		100 = .1 300 = .2	
R-8'	170	110-170 (	0-110 (3.5)			
R-9	180		0-160(1.2)	160-180(1.3)	160 = 0	
R-10	120		0-120(2.1)		120 = 0	
R-11'	80		0-80(1.3)		70 = 0	
R-12'	80		0-80(1.3)		80 = 0	

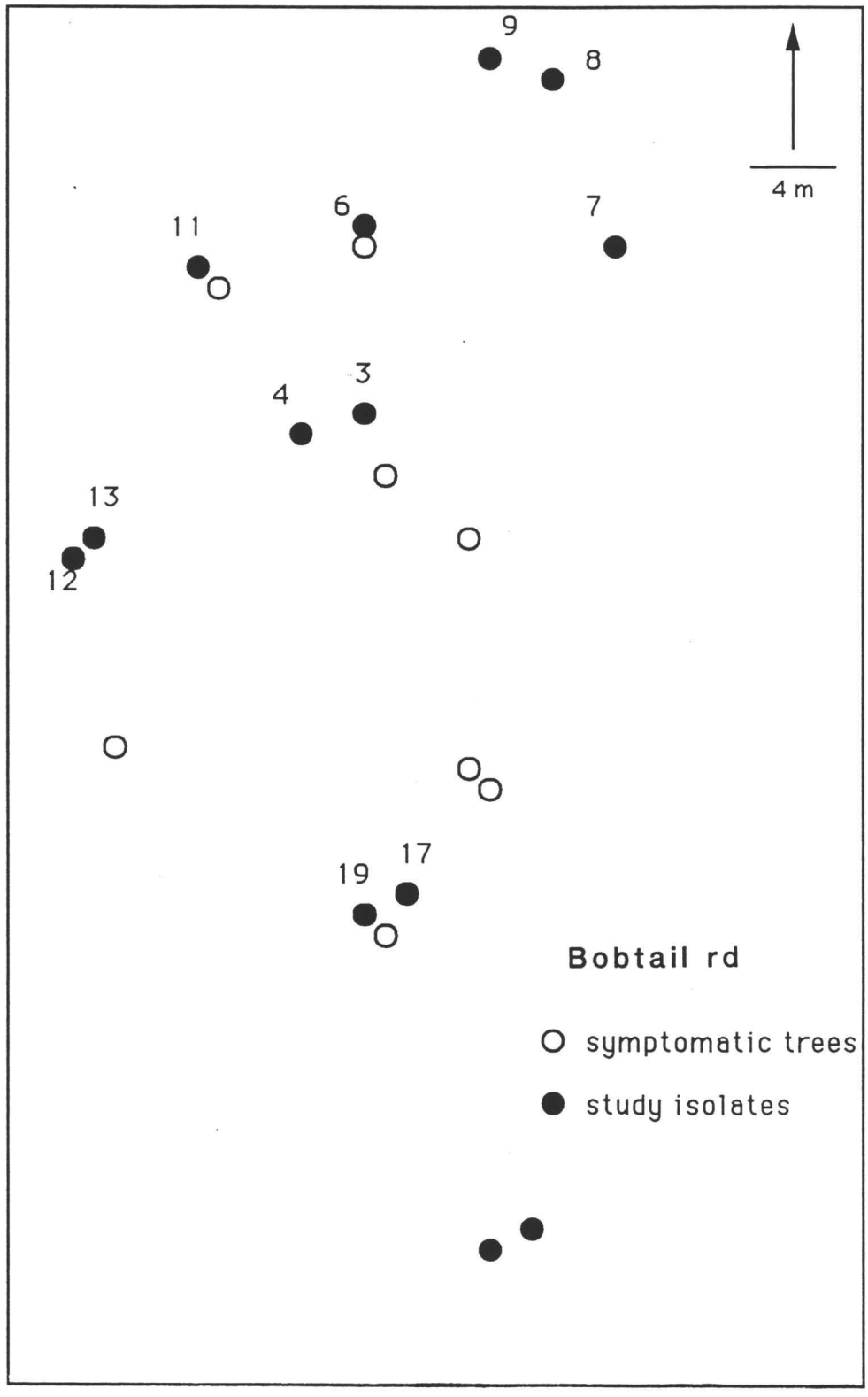
COMMENTS:

T-1(sample) whole root collar

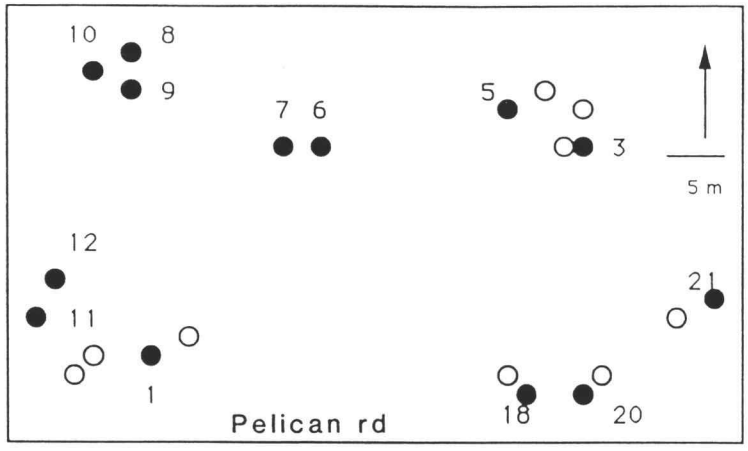
T4 two roots with S.U.M samples

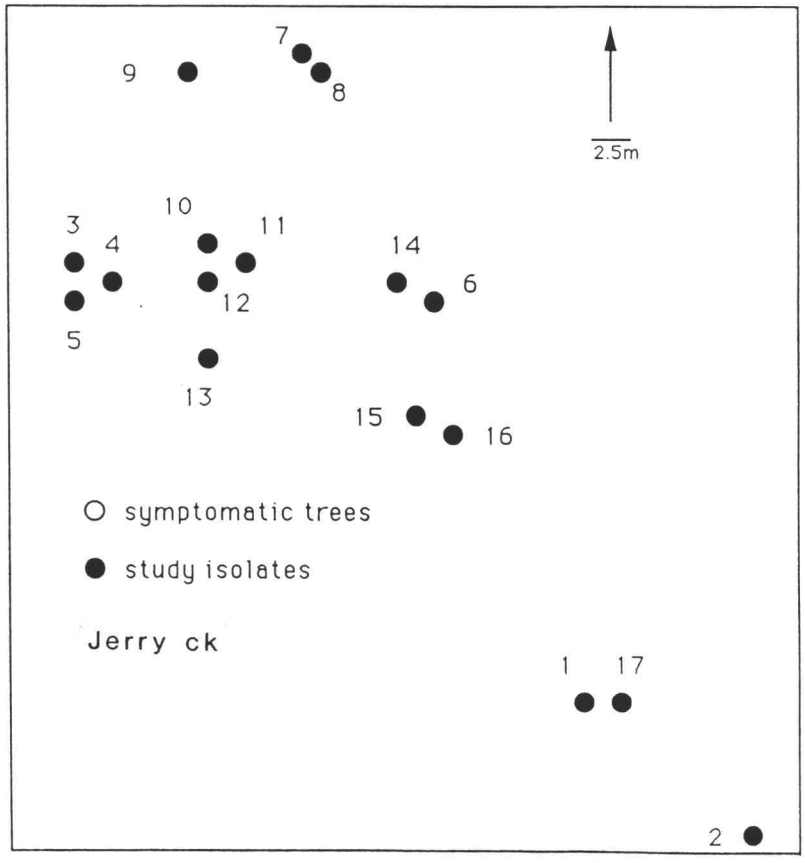
No other tree root contacts obvious . other trees etc

Appendix C. Isolate collection sites, Bobtail rd., Pelican rd., Jerry ck.



Appendix C. cont'd.





Appendix D

Similarity coefficients of protein profiles between I. tomentosus isolates from Jerry Creek (JC), Pelican Rd. (Pln), and Bobtail Rd. (Bob). Shaded numbers are isolate pairs which showed vegetative compatibility.

Jerry Ck.

	2	3	4	5	10	11	12	13	14	15	16	17
1	98.3	98.3	98.3	98.3	98.3	98.3	98.3	94.9	96.4	94.9	94.9	98.2
2		100	98.3	98.3	100	100	100	96.7	87.7	96.7	96.7	91.5
3			100	100	100	100	100	96.7	87.7	96.7	96.7	91.5
4				100	98.3	98.3	98.3	98.4	94.7	98.3	98.3	98.3
5					98.3	98.3	98.3	98.4	94.7	98.3	98.3	98.3
10						100	100	96.7	87.7	96.7	96.7	91.5
11							100	96.7	87.7	96.7	96.7	91.5
12								96.7	87.7	96.7	96.7	91.5
13									94.7	96.7	96.7	98.3
14										96.4	96.4	91.5
15											100	98.3
16												98.3

Pelican Rd.

	3	5	6	9	10	11	12	15	18	20	21
2	100	96.9	90.3	92.1	92.1	90.0	90.0	90.0	90.0	90.0	91.8
3		96.9	90.3	92.1	92.1	90.0	90.0	90.0	90.0	90.0	91.8
5			90.3	92.1	92.1	90.0	90.0	90.0	86.7	90.0	88.5
6				95.1	95.1	93.3	93.3	93.3	90.0	90.0	85.2
9					100	94.9	94.4	94.9	98.3	91.5	88.5
10						94.9	94.9	91.5	98.3	91.5	88.5
11							100	100	96.4	92.9	91.2
12								100	96.4	92.9	91.2
15									96.4	92.9	91.2
18										94.7	94.7
20											94.7

Bobtail Rd.

	4	6	7	8	9	11	12	13	17	18	19
3	93.6	100	97.8	91.3	91.3	87.5	87.5	87.5	97.8	97.8	97.8
4		91.7	95.6	91.3	91.3	93.3	93.3	93.3	95.6	95.6	93.6
6			97.8	91.3	91.3	87.5	87.5	87.5	97.8	97.8	100
7				97.8	97.8	95.6	95.6	95.6	100	100	97.8
8					100	97.7	97.7	97.7	97.8	97.8	93.6
9						97.7	97.7	97.7	97.8	97.8	93.6
11							100	100	95.6	95.6	93.6
12								100	95.6	95.6	93.6
13									95.6	95.6	93.6
17										100	97.8
18											97.8