AN ABSTRACT OF THE THESIS OF

Katarina Sweeney for the degree of Master of Science in Botany and Plant Pathology presented on August 8, 2013.

Title: Histological Comparisons of Needle Tissues of Four Species of White Pine infected by *Cronartium ribicola.*

Abstract approved:

_____

Jeffrey K. Stone

Hypersensitive response-like (HR-like) needle reactions to infection by the white pine blister rust pathogen, *Cronartium ribicola,* have been reported for several species of five needle pines native to western North America. The best-studied examples are in *Pinus monticola* and *P. lambertiana*. In these species a "needle spot" phenotype has been identified in which HR-like needle reactions are related to disease resistance that is conditioned by a major gene. Conventionally it is believed that the HR-like needle reactions in resistant pines prevent spread of the pathogen to vascular stem tissue by HR mechanisms commonly seen in other plant-pathogen incompatibility interactions, i.e. a rapidly induced plant cell death and subsequent localized tissue necrosis. The dead cells present a barrier to colonization by biotrophic pathogens and cause degeneration of

fungal hyphae preventing further pathogen colonization. Structural analyses of early *C. ribicola* needle colonization in resistant *Pinus* spp. have shown, however, that these symptoms and their underlying physiology are fundamentally different from the clearly defined HR described in other host-pathogen systems. Contrary to the pattern of HR responses seen in most incompatible host reactions, onset of needle lesions was first seen several weeks after initial entry of *C. ribicola.* We observed extensive proliferation of fungal hyphae in the host and penetration of the needle endodermis and vascular tissue by the pathogen prior to the onset of a discernable HR or cell necrosis. The amount of fungal tissue present and progress of needle colonization was similar for both resistant and susceptible *Pinus* spp. Therefore, typical HR does not appear to function in needles as the mechanism of disease resistance in the "needle reaction" phenotype.

Histological Comparisons of Needle Tissues of Four Species of White Pine Infected with *Cronartium ribicola.*

By
Katarina Sweeney

A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented on August 8, 2013
Commencement June 2014

Master of Science thesis of Katarina Sweeney presented on August 8, 2013.

APPROVED:

_____

Major Professor, representing Botany and Plant Pathology

_____

Head of the Department of Botany and Plant Pathology

_____

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

_____

Katarina Sweeney, Author

ACKNOWLEDGEMENTS

I would like to thank Jeff Stone and the department of botany and plant pathology for giving me the opportunity to study at Oregon State University, Ken Johnson and Tom Wolpert for valuable discussions about my research and Barb Taylor for support and practical guidance in the planning stages of my thesis. Especially, I would like to thank Kathy Cook who trained me in microtomy and histo-chemical techniques and became a true mentor during my grad school career. She always provided insightful advice whenever it was of need during the last three years. Moreover, I would like to thank Richard Sniezko, Angelia Kegley and the Dorena staff in Cottage Grove, OR for their help with my project. Finally, I would like to thank my friends for their ongoing support and everyone else who open-mindedly showed encouragement of my decision to change careers.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

Page

LIST OF FIGURES

Page

Figure

LIST OF FIGURES (continued)

Page

LIST OF TABLES

Page

Table

Dedicated to Kathy Cook

1. INTRODUCTION

1.1 Biology of the White Pine Blister Rust Pathosystem

White pine blister rust (WPBR) is caused by the Pucciniomycete pathogen *Cronartium ribicola* (Cronartiaceae) J.C. Fischer (Rabenhorst, 1872). The obligate biotroph exhibits five spore stages (TABLE 1) and requires two unrelated species as host plants to complete its life cycle. Only five-needle pines (Pinaceae, subsection *Strobus*) are susceptible to infection by *C. ribicola* and serve as the spermatial (via pycniospores) and the aecial host for the pathogen; *Ribes* spp. (Grossulariaceae) are the main uredinial and telial host for the pathogen. In North America, *Pedicularis* and *Castilleja* species are also known to serve as uredinial and telial hosts in this pathosystem (McDonald et al. 2006; Mulvey, 2011) **.**

Table 1: Spore stages of *Cronartium ribicola* and their characteristics.

	Ploidy	Spore stage	Host	Season
Spore Stage 0	monokaryotic	pycnia	on pine	Spring
Spore Stage I	dikaryotic	aecia	on pine	Spring
Spore Stage II	dikaryotic	uredinia	on ribes	Summer
Spore Stage III	dikaryotic	telia	on ribes	summer/fall
Spore Stage IV	monokaryotic	basidia	on ribes	Fall

The disease is initiated when haploid basidiospores, which are produced from germinating dikaryotic telia in the fall, infect foliage of susceptible white pine hosts. Basidiospores deposited on pine needles germinate to produce a germ tube capable of entering needles via the stomata. Following initial penetration of the needle, the fungus proliferates, but does not begin to form reproductive structures until colonization reaches

the stem tissue, usually nine months or longer following initial needle infection (Bingham, 1983). In needles the fungus obtains nutrients from host tissues via haustoria, which penetrate the host mesophyll cells. Fungal hyphae eventually breach the endodermis, which surrounds the central vascular bundle and enter the vascular cylinder, colonizing xylem and phloem to the base of the needle and into stems where reproductive spore structures, pycnia and aecia, are produced (Maloy, 1997). Pycnia form in the first growing season following infection, aecia are formed during the second growing season. Disruption of host cambium and phloem as a result of aecial sporulation causes the stem cankers that are a distinctive symptom of this disease (Maloy, 1997). New pycnia can develop annually at the margins of aecial cankers (Spaulding, 1911) (FIG.1).

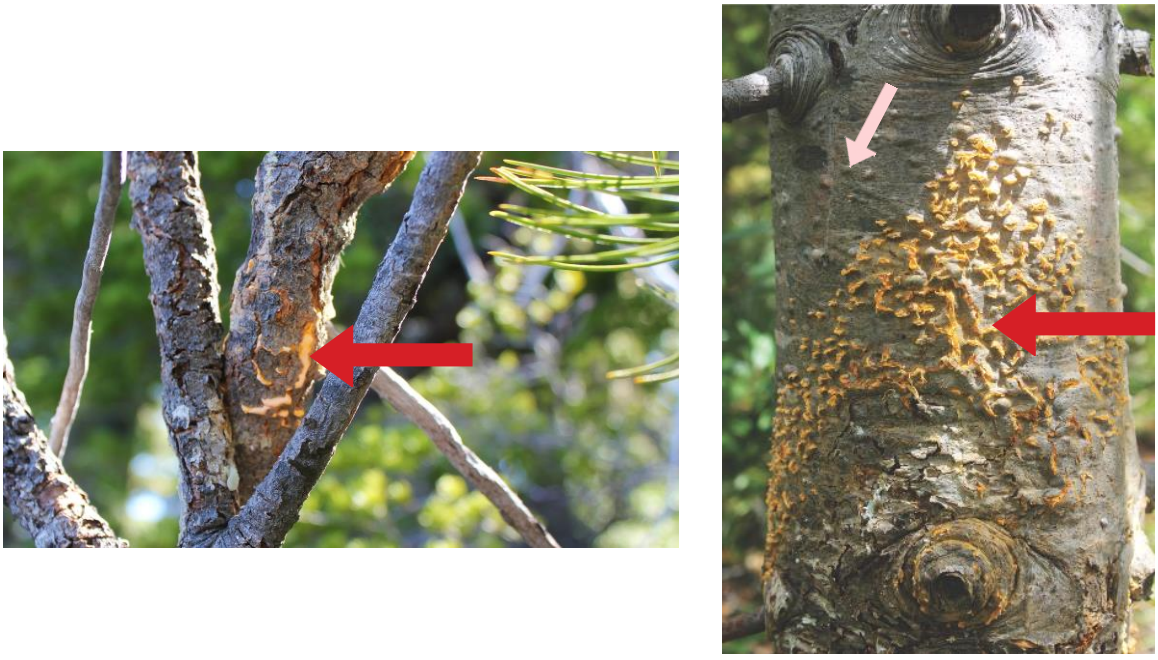


Figure 1: Aecial stage of *C. ribicola* on *P. monticola*. Aecia make up the prominent orange masses inside blisters, which burst in the spring to release orange spores. Red arrows point to aecia. White arrow points to pycnia at the margin of the aecial canker.

Pycnia, consisting of pycniospores (which function as spermatia) and receptive hyphae, formed from the monokaryotic, primary mycelia are produced in the spring and initially form on the pine stem below the bark, which ruptures as the pycnia enlarge (Maloy, 1997). Pycnial exudate oozes through affected bark and persists as a sticky sap, which attracts insects that aid in the dispersal of pycniospores to pycnia of compatible mating types (FIG.2) (Maloy, 1997; Geils et al., 2010).

Figure 2: Pycnial Stage of *C. ribicola* on *P. monticola*. Arrows point to pycnial droplets oozing from swollen areas on the stem in the spring. Photo: J. Stone

Cronartium ribicola is a heterothallic fungus; fusion of two individuals having compatible mating types controlled by nuclear *MAT* loci results in formation of a stable binucleate mycelium (dikaryon) that is the dominant vegetative nuclear condition (Geils

et al., 2010). The two nuclei in the dikaryon will eventually fuse and undergo meiosis, completing the sexual cycle. Fusion of pycniospores and receptive hyphae, i.e. mating of two different compatible mating types, gives rise to the dikaryotic mycelium (Hiratsuka and Sato, 1982). This dikaryotization event leads to maturation of aecia and localized swellings and cankers appear which are often diamond- or spindle- shaped. Dikaryotization during the pycnial stage can be regarded as genetic outcrossing through fusion of different mating types, which is believed to influence evolutionary potential of this pathosystem (Geils et al., 2010).

Aecia are pustules or blisters, filled with masses of initially yellow or bright orange, dikaryotic aeciospores that erupt through the pine stem and disperse on air currents to *Ribes* spp. hosts, on which they germinate and enter the host foliage and form reproductive structures called uredinia (Hiratsuka and Sato, 1982). Dikaryotic urediniospores are able to re-infect the *Ribes* spp. hosts throughout the summer, thereby intensifying the disease via secondary infection, but cannot infect pines (Maloy, 1997). In autumn, telial columns develop from the uredinia. Karyogamy and meiosis occur within the telia resulting in haploid, monokaryotic basidiospores, which are able to infect white pines and in so doing completing the fungal life cycle (Maloy, 1997). The entire cycle of infection will take a minimum of two years to complete (Spaulding, 1911) (FIG. 3).

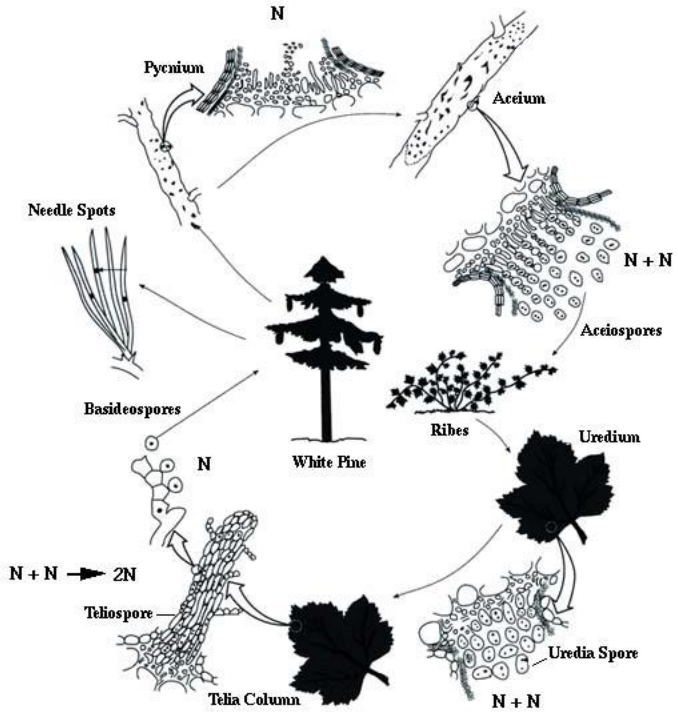


Figure 3: The life cycle of white pine blister rust (diagram: US Forest Service). N=haploid monokaryotic mycelium (primary mycelium); N+N = haploid dikaryotic mycelium (secondary mycelium); 2N = diploid nuclei.

1.2 White pine blister rust symptoms and damage

The first noticeable symptom of WPBR on pines is yellow needle spots, which appear within two to three months after basidiospore infection (Kinloch and Littlefield, 1977) (FIGS. 4, 5). These spots are initially pinpoints and gradually enlarge to encircle the needle and produce yellow patches on needle foliage**.**

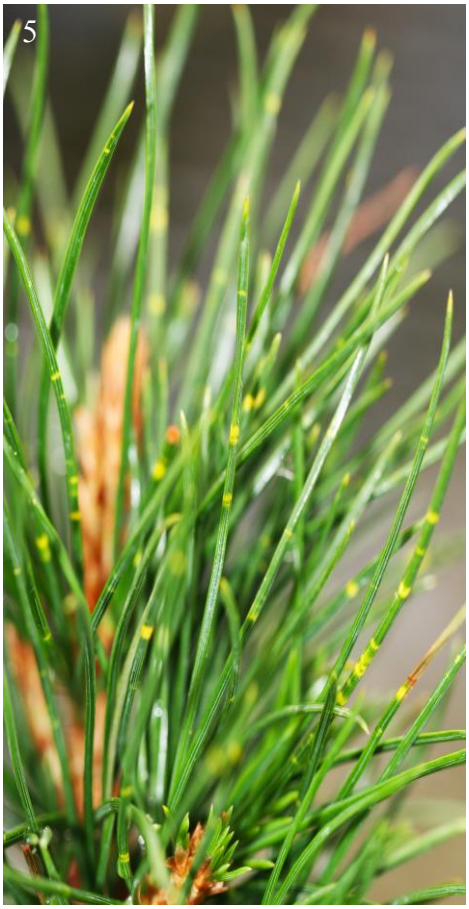


Figure 4: Foliage of susceptible P. *lambertiana* 97 days after initial inoculation.
Figure 5: Foliage of susceptible *P. albicaulis* 97 days after initial inoculation.

As the *C. ribicola* colonization reaches twigs and branches, infected branches become yellow and wilt. On diseased mature trees, branch flagging and top kill are common (Maloy, 1997). Orange spindle-like areas of the pine stem are early symptom, which precedes formation of aecial cankers that are visible symptoms of WPBR on pines (FIG.6). Infection by *C. ribicola* leads to tree mortality through girdling stem cankers that result from aecial sporulation. Rust cankers can girdle the tree on both bole and branches, produce resinosis and necrosis and account for killing of all parts of the tree

above the point of infection (Geils et al., 2010). Often opportunistic fungi begin to colonize cankered tree tissues and accelerate death of the tree (Wicker, 1970).

Figure 6: Early bark discoloration symptom of *C. ribicola* stem infection on *P. monticola*. Photo: J. Stone.

1.3 The white pines of North America

All *Pinus* spp. are forest trees of the Northern Hemisphere and are members of Pinaceae, Coniferae. The genus *Pinus* is divided into two subgenera, *Pinus* and *Strobus*, each of which is subdivided into sections and subsections. *Cronartium ribicola* only infects members of the subgenus *Strobus*, commonly called "white pines" or "five-needle pines" due to the presence of five needles per fascicle (Price et al. 1998). Pine species in the subgenus *Strobus*, sect. *Strobus*, subsection *Strobi* include: *P. amamiana, P. armandii, P. ayacahuite, P. bhutanica, P. chiapensis, P. dabeshanensis, P. dalatensis, P. fenzeliana, P. flexilis, P. lambertiana, P. monticola, P. morrisonicola, P. parviflora, P. peuce, P. strobus, P. wallichiana, P. wangii.*

Other North American pine species that are noteworthy in regards to blister rust are in the subgenus *Strobus*, sect. *Strobus*, subsection *Balfourianae*: *P. aristata*, *P. balfouriana*, and *P. longaeva* ; as well as one of the pine species in the subgenus *Strobus*, sect. *Strobus*, subsection *Cembrae*: *P. albicaulis* (TABLE 2).

Table 2: Taxonomy and nomenclature of the genus *Pinus* Lemm. Classification of species follows Price et al. (1998)

***Pinus* L. Subgenus *Strobus* Lemm.**
Section ***Strobus*,** Subsection ***Strobi*** Loudon: *P. amamiana, P. armandii, P. ayacahuite, P. bhutanica, P. chiapensis, P. dabeshanensis, P. dalatensis, P. fenzeliana, P. flexilis, P. lambertiana, P. monticola, P. morrisonicola, P. parviflora, P. peuce, P. strobus, P. wallichiana, P. wangii*

Section ***Strobus***, Subsection ***Balfouriana*** Loudon *P.aristata, P. balfouriana , P. longaeva*
Section ***Strobus***, Subsection ***Cembrae*** Loudon *P. albicaulis, P. cembra, P. koraiensis, P.pumila, P. sibirica*

The genus *Pinus* was previously categorized based on needle anatomy. Shaw (1914) was the first to recognize two distinct groups in the genus, *Haploxylon*, and *Diploxylon*, based on the presence of one or two needle vascular bundles. Systematists have subsequently rearranged and subdivided the two groups, which were elevated to the rank of subgenus, but the two groups are still recognized as major lineages within *Pinus* (Mirov, 1967; Little and Critchfield, 1969; Price et al., 1998; Gernandt et al., 2005). *Haploxylon*, which is a category equivalent to the current subgenus *Strobus*, is described as a group of species that all share the characteristic single, central vascular bundle in needles (Mirov, 1967). Nine species of subg. *Strobus* native to North America are susceptible to *C. ribicola*: *P. monticola*, *P. strobus*, *P. lambertiana*, *P. albicaulis*, *P. flexilis*, *P. strobiformis*, *P.aristata*, *P. longaeva*, and *P. balfouriana*. The pathogen and the disease is currently present in native populations of each species, except Pinus longaeva D.K. Bailey (Schwandt et al., 2010; Sniezko et al., 2011).

The North American white pines, in particular eastern white pine (*P. strobus*), were recognized by Europeans early on as high-value species useful for many commercial applications. The Euro-American settlers processed lumber of eastern white pines up until the 1880s and its wood was one of the most commonly used building

materials in the USA (Howard, 1986). The demand for white pine lumber was immense and natural stands of *P. strobus* in the USA were depleted at the turn of the previous century (Howard, 1986). *Pinus monticola*, western white pine, which grew extensively in the interior Pacific Northwest prior to the introduction of *C. ribicola* also became a timber species of high economic value. As the Pacific railroad expanded its range out west in 1880, access to *P. monticola* stands and a market for the straight-grained wood grew (Harvey et al., 2008). Together eastern and western white pines were the backbone of the lumber industry in the USA but most native stands of these pine species were depleted rapidly by the timber industry (Maloy, 1997).

1.4 Introduction of white pines into Europe

Swiss stone pine *(P. cembra)* and Macedonian pine (*P. peuce)* are the only two members of the subg. *Strobus* native to Europe (Radu et al., 2008). It was through introduction from North America that seeds and seedlings of *P. strobus* arrived in Europe and a greater variety of pine species was subsequently established in the new locality (Radu et al., 2008). The first imports of *P. strobus* into Europe can be traced back to 1553 but plantations did not become established until the mid 18th century (van Arsdel, 2011). In Britain, introduced *P. strobus* became known subsequently as Weymouth pine after one English traveler who imported them. The wood of *P. strobus* was highly sought after for the construction of ships for the British navy and the species became economically important throughout Europe, similar to its status in North America (Maloy, 1997). Other

pine species were introduced and established but a timber industry began to focus on the creation of white pine plantations in many European countries (Spaulding, 1929).

Meanwhile, white pines were in high demand to fuel an expanding forestry industry in the USA (Maloy, 1997). North American nursery owners concentrated their trade efforts on the succesful white pine plantations in Europe and subsequently millions of white pine seedlings were imported from Europe to the USA (Maloy, 1997) in an attempt to supply the demands of Americans and also to take advantage of lower plant stock prices of European seedlings (Spaulding, 1911).

1.5 *Cronartium ribicola* and its invasion of North America

In 1856 , H.A. Dietrich first identified *C. ribicola* infections on *Ribes nigrum, R. rubrum, R. palmatum* and *P. strobus* in Russia (Spaulding, 1911). Yet, the *C. ribicola* life cyle and its heteroecious nature were not described until 1889 by Klebahn. The pathogen began to spread outside of the Russian Baltic regions into other European countries with centers of infection in areas where *P. strobus* plantations had been established (Maloy, 1997). Concurrently with the spread of the pathogen across Europe, white pine seedling export to North America had begun. A decade after Klebahn's initial species description in 1898, *C. ribicola* was first introduced to eastern North America (Spaulding, 1911). Spaulding (1922) reported that it is likely that *C.ribicola* was present before the turn of the century, however, no infected *Ribes* collection could be found in any of the east coast herbaria to provide evidence of an earlier introduction of the pathogen in the USA. It was not until 1906 that that the pathogen was first discovered and documented in a *Ribes*

planation in Geneva, NY and 1909 that infection on pines was first discovered in the US (Spaulding, 1909).

The earliest documentation of *C. ribicola* in western North America occurred in 1910 (Spaulding, 1922). However, the rust was not detected until 1921 when inoculum was found on *Ribes* spp. near Vancouver, British Columbia (Hunt, 2009). Although only one introduction of the pathogen has been documented; because WPBR spread very quickly along the Pacific Northwest in the following years, multiple introductions of infected European plant stock to Canadian and American west coast ports that lacked trade documentation are suspected to have occurred (Hunt, 2009).

1.6 Review of Resistance Research

Silvicultural approches, host eradication and sanitation efforts have been unsuccessful to control spread of WPBR in North America (Maloy, 1997). To date, it appears that the most successful and lasting control efforts are achieved by utilizing techniques to breed trees for genetic resistance to *C. ribicola*. Planting resistant trees into natural stands can reduce the amount of disease incidence. Resistance research efforts to combat WPBR were initiated in the USA by A.J. Riker's work on *P. strobus* in Wisconsin (Kriebel, 1972). By 1938, scouting in the field allowed Riker to find over 100 trees in native stands that appeared to have a resistance phenotype evidenced by absence of stem cankers even though these trees were growing near infected ribes plants (Riker and Kouba, 1940; Riker et al., 1943). Initially, Riker collected cones from these putatively resistant trees to use their seeds to grow seedlings, which would be used in artificial inoculations in a nursery

setting in the following year. Later, Riker used grafting techniques to make use of cuttings from the resistant parent plantstock (Riker et al., 1943). Also, rooted tree cuttings taken from resistant trees were included in artificial inoculation trials (Patton and Riker, 1966). It was found that there were differences in the tree responses to these controlled inoculations with *C. ribicola* in all of the plantstock under study (Riker et al., 1943). Riker was able to deduce that certain phenotypes of infected trees failed to develop further symptoms after needle spots appeared, and never showed further incidence of disease (Riker et al., 1943). Riker's research was a first indication that resistance existed in natural populations of *P. strobus*, that resistant trees had the ability to transmit this resistance to their progeny, and that resistance comprised more than one phenotype. Riker's work also provided motivation to continue to test for complete resistance to WPBR in future studies.

While small scale resistance research combined with resistance screening continued throughout the USA (Kriebel, 2004), a federally supported resistance breeding program began for *P. monticola* in 1946 (Bingham, 1983). The "Inland Empire", a region in the northwestern USA between the Cascade Range and the Rocky Mountains became the focus for large scale tree breeding experiments.The resistance trials conducted at Moscow Idaho, also called the Rocky Mountain program, were overseen by R.T. Bingham (McDonald et al., 2004). Canker-free trees called "candidate" trees were located in natural forest stands and their seed was obtained from cone collections. In nursery settings, seedlings were grown from the collected seed and subsequently exposed to *C. ribicola* inoculum. Bingham and his staff expanded on the existing resistance

screening methodology and incorporated positive and negative test trees into the breeding program (Bingham, 1983). Moreover, tree progeny were classified under different resistance categories according to the amount of stem cankering that was visible after inoculaton with the pathogen and in this way quantitative measures of resistance were devised (Bingham, 1983). If the level of resistance promised a successful tree, able to withstand disease, its seed was planted into seed orchards at various sites in order to generate germplasm that could be used for further tests and more mating crosses with previously selected successfull planting stock (Bingham et al., 1969).

The controlled pollination technique to produce progeny trees that receive genes from each of the two known parent trees was widely used during Bingham's time at the Inland Empire (Bingham et al., 1969; McDonald et al., 2004). Another important feature of Bingham's work included large-scale setups for controlled inoculation with *C. ribicola* (McDonald et al., 2004). In contrast to natural infection by the pathogen in forest stands, artificial inoculations allow both the amount of inoculum and seedling exposure time to be varied in the nursery setting. For artificial inoculations, leaves of *Ribes* spp. were commonly collected in late summer, when telial columns were visible on the underside of leaves and *C. ribicola* basidiospores were actively shed. These infected leaves were placed above young white pine seedlings in greenhouses to ensure spore dissemination onto pine foliage where new needle infection would occur (Bingham, 1983).

In the following years USDA Forest Service provided funding for the expansion of the existing research in the Moscow, Idaho breeding program and additional locations were included in the WPBR research program. In 1956, Oregon and Washington (Forest

Service Pacific Northwest, Region 6) began resistance research on *P. monticola* and *P. lambertiana* (McDonald et al., 2004), currently the Dorena Genetic Resource Center near Cottage Grove, Oregon. Lastly, an addition to the program in California was begun in 1957, intended to focus on resistance research on *P. lambertiana* (McDonald et al., 2004).

The resistance breeding program in Moscow, Idaho switched to open-pollinated progeny trials in 1965 (King et al., 2010), while USFS Region 6 began to employ open-pollination (OP) research in 1970 (Sniezko, 1996). Little or nothing is known about the pollen source in OP trials, which takes place in the form of wind pollination; however, the phenotypic characteristic of the maternal trees are usually known. In general , OP breeding allows for a wider genetic diversity in the tree progenies than controlled pollination (Sniezko, 1996).

1.7 Three types of white pine resistance

Three different general categories of resistance to WPBR in five-needle pine species are recognized based on accumulated reasearch findings:
1) Ontogenic resistance, 2) partial resistance, and 3) major gene resistance (MGR) (King et al., 2010). Ontogenic resistance is described as an increasing disease tolerance to WPBR as trees mature (Patton, 1961). Accordingly, it appears that the incidence of mortality due to infection by *C. ribicola* is less in older , larger diameter trees than in young trees with small diameter stems. Partial resistance, which is also referred to as "slow-rusting resistance" (SRR) is characterized by symptoms of lesser severity

compared to the phenotype seen in most susceptible trees. SRR does not prevent or delay the infection process of *C. ribicola*; however, SRR can delay the death of an infected tree and often tree survival is possible (King et al., 2010). This type of resistance is believed to be under the control of several different genes and has also been termed multigenic resistance (Hoff and McDonald, 1980; King et al., 2010; Zambino and McDonald, 2003).

SRR in WPBR is often expressed in the form of bark reactions, which take place in stem tissues as a means to stop *C. ribicola* from becoming established in the stem. Bark reactions involve the formation of necrotic tissues in the stem to hinder *C. ribicola* from colonizing healthy stem regions, which is often evidenced by sunken cankers in the bark of the tree (Kinloch et al., 2007). Also, "short shoot fungicidal reactions" that are believed to eliminate *C. ribicola* at the junction of the base of the needle fascicle and the stem of the seedling, are regarded as partial resistance mechanisms that can arrest spread of *C. ribicola* to other tissues (Hoff and McDonald, 1971). MGR resistance is a monogenic resistance that is controlled by a dominant R-gene and conforms to the gene-for-gene model after Flor (1942) in which the resistant host exhibits a hypersensitivity response (HR) that is able to curtail pathogen colonization . This type of resistance is capable of producing a host which is highly resistant to the pathogen. MGR to *C. ribicola* was first described by Kinloch and Littlefield to exist in sugar pine (Kinloch and Littlefield, 1977). All of the resistance mechanisms of white pines in response to infection by *C. ribicola* have been difficult to interpret and dynamics of the pathosystem are only marginaly understood.

1.8 Major gene resistance (MGR)

Major gene resistance (MGR), also known as R-gene resistance, qualitative, mono- or oligogenic resistance, or vertical resistance, can be characterized by a distinct specificity of a host species and a pathogen, meaning that a single host individual may be highly resistant to one pathogen genotype but not to others. Fundamental research aimed to describe MGR was completed by Flor in his work with the flax rust pathogen *Melampsora lini* (1942). Flor hypothesized that the presence of two genes is necessary for MGR in plant-pathogen interactions to occur: one resistance gene (R-gene) in the plant host and one complimentary avirulence gene (*avr*-gene) in the pathogen. This model became known as the gene-for-gene relationship of plant resistance. It implies that plant host R-gene products recognize the products of the pathogen's *avr*-genes. Following the host's recognition of the pathogenic *avr*-proteins also known as effectors, plant host defense mechanisms are triggered and host cellular reactions take place aimed to ward off the pathogen. Conversely, in the absence of a corresponding R-gene in the host, effectors will enable the pathogen to cause disease (Dangle and Jones, 2001).

MGR is believed to be conferred by a single gene locus, in which resistance is dominant over the susceptibility allele. Accordingly, in tree breeding crosses that include one homozygous parent for the resistance gene, all offspring will be resistant. Major genes are inherited in a Mendelian pattern. If the resistant parent plants are both heterozygous for the major gene, the predicted 3:1 Mendelian ratio of

resistant: susceptible phenotypes would be present in the F1 generation. One can regard Mendelian inheritance patterns as evidence of a single locus of resistance (Kinloch and Dupper, 2002).

1.9 The hypersensitivity response (HR)

The hypersensitivity response (HR) or localized cell death is one of the major components of defense responses in plants against pathogen attack (Dangl and Jones, 2001). It is generally recognized by the rapid onset of cell death surrounding the infected tissues via vacuolar lysis, degrading of cellular machinery and complete cellular collapse. Generally, HR phenotypes are visible as yellow or brown necrotic lesions in host tissues at the site of pathogen entry (Heath, 2000; Morel and Dangl, 1997). HR is triggered by the interaction of disease resistance (R) genes of host plants and corresponding avirulence (*avr*) genes in pathogens (Hammond-Kosack and Jones, 1997); *avr* genes in the pathogen code for elicitor or effector proteins that interact with R genes in the host. Hence, host and pathogen recognize each other by their gene products and activate signal transduction cascades, which orchestrate defense responses (Gilchrist, 1998; Innes, 1998).

This system is highly specific, as only pathogens with *avr* genes are detected by the host and subsequently induce the HR response in host tissues. HR constitutes coordinated plant responses to pathogen colonization, which involve oxidative burst with accumulation of reactive oxygen species (ROS), calcium ion fluxes, changes to the host cell wall, expression of pathogenesis-related PR proteins and localized host cell death (van Doorn, 2011; Innes, 1998; Liu and Ekramoddoullah, 2004). These are key

components of classic HR, which usually occur within hours of attack by pathogens (Lamb and Dixon 1997). This autolytic cell death is a means to eliminate infected cells, which will simultaneously restrict flow of nutrients to the invading pathogenic hyphae and delimit fungal spread.

1.10 Major gene resistance (MGR) in five needle pines

MGR resistance to *C. ribicola* has been described for four species of pine, namely sugar pine (*P. lambertiana),* western white pine (*P. monticola),* southwestern white pine (*P. strobiformis),* and limber pine (*P. flexilis)* (Schoettle et al., 2013, in press). The MGR classification was assigned to these species via segregation analysis, which is generally considered an adequate method of identifying major genes. Major genes produce distinct phenotypes and show Mendelian segregation, which suggests a single locus pattern of inheritance. These major R-genes are presumed to operate on a gene-for-gene basis with *avr* genes in the pathogen. During infection the product of the *avr* gene is recognized by the product of the corresponding R-gene that in turn induce a cascading series of events leading to resistance, namely the Hypersensitivity reaction (HR) (Kinloch and Dupper, 2002). The MGR phenotype has been assumed to be coded by an R-gene (King et al., 2010; Kinloch and Littlefield, 1977; Kinloch et al., 1999).

The first R-gene described in the WPBR pathosystem, called *Cr1*, was identified in sugar pines (Kinloch et al., 1970). It was reported that *Cr1* confers resistance of sugar pines to WPBR by promoting a HR in needle tissues, which is evidenced by a necrotic needle spot on the needles of resistant seedlings (Kinloch and Littlefield, 1977). A

similar R-gene was recognized in western white pine in the western Cascade Range of Oregon and became known as *Cr2* (McDonald et al., 1984; Kinloch et al., 1999). *Cr2* produces HR lesions on western white pine foliage that are similar in appearance to *Cr1* needle lesions. *Cr3* is a third R-gene that has been reported to confer HR-mediated resistance in southwestern white pine (Kinloch and Dupper, 2002). On limber pine, HR lesions on needles have been noted and a *Cr4* locus for HR mediated resistance is currently discussed (Schoettle et al. 2013, in press).

1.11 The hypersensitivity response (HR) in five-needle pines

One type of resistance to *C. ribicola* has been attributed to major gene resistance (MGR) and has been described as a classical hypersensitivity response (HR) in infected needles believed to be conferred through a dominant resistant allele at loci designated *Cr1, Cr2, Cr3 and Cr4* in *P. lambertiana*, *P. monticola*, *P. strobiformis*, and *P. flexilis* respectively (Kinloch and Dupper, 2002; Kinloch and Littlefield, 1977; Kinloch et al., 1970, 1999; Schoettle et al., 2013, in press). In *Cr1*, *Cr2*, *Cr3* and *Cr4* genotypes, it has been presumed that HR-mediated cell death near the point of *C. ribicola* entry confines fungal growth and prevents colonization of the mesophyll and ultimately the vascular cylinder. In these species a “needle spot” phenotype has been identified. The needle spot phenotypes on needles of resistant pines differed distinctly from the susceptible spots (Kinloch and Littlefield, 1977; Kinloch et al., 2003). The resistant needle spots appear localized, small, yellow and brown, often with a necrotic band (Kinloch, 1982). The

needle spots on susceptible needles are larger and more diffuse in spread and have yellow or red coloration (Kinloch, 1982).

1.12 Virulence genes in the blister rust pathogen

R-gene resistance has been described as an effective mechanism to prevent infection in four species of white pines (Kegley and Sniezko, 2004; King et al., 2010; Kinloch and Byler, 1981). Research has shown however, that MGR in specific populations of *P. lambertiana* carrying the *Cr1* resistance genes, and *P. monticola* carrying the *Cr2* resistance genes, was overcome, apparently due to evolution of virulent races or populations of the pathogen (Kinloch and Dupper, 2002; Kinloch et al., 2004, 2007). In both cases it is assumed that the R-genes prevalent in the pine populations under study were matched by a pathogen mutation that enabled the pathogens to become virulent to the resistant host phenotypes. In this scenario, virulence can be regarded as a pathogen's means to overcome vertical resistance (Priyamvada and Tiwari, 2011). Virulence in the pathogen is usually recessive to avirulence (Priyamvada and Tiwari, 2011) and the appearance of virulent genotypes is commonly associated with mutations in *avr* gene loci (TABLE 3).

Table 3: Gene-for-gene interaction for plant host and pathogen after Flor (1942).

GENOTYPE OF HOST

GENOTYPE OF PATHOGEN	R R[a]	R r	r r
AVR AVR[b]	incompatibility reaction	incompatibility reaction	INFECTION
AVR avr	incompatibility reaction	incompatibility reaction	INFECTION
avr avr	INFECTION	INFECTION	INFECTION

[a]R/r denote resistance alleles in the host.
[b]AVR/avr denote avirulence alleles in the pathogen. Virulence (avr avr) is recessive to avirulence in the pathogen.

The resistance conferred by *Cr1*, the resistance gene in *P. lambertiana,* was overcome by a virulent race of *C. ribicola,* which was found at a site in the USDA Forest Service Region 5, near Happy Camp, California (Kinloch and Dupper, 2002). The Happy Camp site has been used as a research site to test resistance in *P. lambertiana* since 1957 (McDonald et al., 2004). The virulent race of *C. ribicola* was first encountered at Happy Camp in 1976 and was named *vcr1* after the R-genes that it neutralizes (Kinloch et al., 2007). *P. lambertiana* seedlings that were bred on site to have the *Cr1* gene were subsequently infected by the *vcr1* rust and showed very high levels of infection and mortality (Kinloch et al., 2007).

Similarly, resistant *P. monticola,* which carry the resistance gene *Cr2,* were found to show an erosion of resistance to *C. ribicola* if a virulent race of the pathogen, *vcr2,* was present. The virulent form of *C. ribicola* able to neutralize *Cr2* was first found at the Champion Mine site in Oregon in the Umqua National Forest, Forest Service Region 6

(Kinloch et al., 2003). This site comprises a naturally regenerating forest stand. *vcr2* is believed to have nullified the effects of *Cr2* for a number of years (Kinloch et al., 1999) but it was not confirmed to be present at the Champion mine stand until 1996 (McDonald et al., 1984; Kinloch et al., 2007). It was reported that virulence of *vcr2* showed a lesser destructive potential than virulence of *vcr1*, which was evidenced by the infection rate curves produced by Kinloch et al. (2007) from research trials previously completed. Also, it was shown that virulence in *vcr2* is specific to host genotype, as *vcr2* is only virulent to *P. monticola* and does not neutralize *Cr1* in *P. lambertiana* (Kinloch et al., 1999, 2007). Specifically, *P. monticola* carrying the *Cr2* allele are highly susceptible to *vcr2* while *P. monticola* that are not carriers of this gene do not show loss of resistance mechanisms (Kinloch et al., 1999).

1.13 Research hypothesis and objectives

The principal objective of this study is to compare the process of colonization of needles of susceptible and resistant phenotypes and to reevaluate mechanisms of resistance to *C. ribicola* in North American species of five-needle pines. It has been suggested that colonization of needle tissues by *C. ribicola* is delimited by HR-mediated resistance reactions in pine needles that are similar to the incompatibility reactions seen in agricultural host-pathogen interactions. However, preliminary observations of *C. ribicola* colonization in resistant pine phenotypes suggests that this may not be the case. Contrary to the classical HR paradigm, in which colonization of a biotrophic pathogen is prevented by rapid localized cell death, *C. ribicola* colonizes needles of resistant

seedlings extensively for months without any discernable restriction of growth of the pathogen.

This study will compare the colonization of needle tissues of resistant and susceptible pine seedlings (*P.lambertiana, P.monticola, P. flexilis, P. albicaulis*) by *C. ribicola* and follow the colonization process over a period of nine months. Specifically, this study aims to determine if and when fungal hyphae enter host vascular tissues to allow the pathogen to reach the stem tissue in resistant and susceptible pines. Histological analysis of needles of resistant and susceptible host seedlings will comprise embedding of needle tissues with methacrylate resin, thin sectioning of embedded specimens with a rotary microtome, staining of sections and light microscopy. Morphological comparisons of infected needle tissues with uninfected samples will create a baseline of data that will supply relevant information regarding seedling responses to infection by *C. ribicola.* In this way, a timeline of infection for both resistant and susceptible pine seedlings can be created. Since genotypes of seedling families are known, the seedling phenotypes can now be assessed and characterized for each genotype. In particular, the nature of needle reactions in response to infection can be described.

2. METHODS

2.1 White pine seedlings and inoculations

In 2008, 2009, 2011 and spring of 2012, needles were harvested from two-year-old seedlings, *P. lambertiana*, *P. monticola*, *P. flexilis,* and *P. albicaulis*, grown at the Dorena Genetic Resource Center (DGRC) in Cottage Grove, OR. DGRC is the USDA

Forest Service regional service center for genetics in the Pacific Northwest region (Oregon and Washington). *P. monticola* , *P. lambertiana, P. flexilis and P. albicaulis* families with known resistance and susceptibility genotypes are tested annually in resistance trials at DGRC. 112 seedlings were included in this study (TABLE 4).

Table 4: Seedlings used for needle samples in 2008, 2009, 2011 and spring of 2012. Dorena Genetic Resource Center (DGRC) in Cottage Grove, OR provided the seedlings.

	Resistant seedlings	Susceptible seedlings
Pinus flexilis	32	25
Pinus monticola	12	12
Pinus lambertiana	3	2
Pinus albicaulis	8	16

In late September to early October, wild collections of *Ribes* leaves with telia were collected throughout Oregon by DGRC technicians. Planted *Ribes* gardens were established to provide a known source of *C. ribicola* inoculum from which infected *Ribes* leaves were also collected to supplement the wild collections. Artificial inoculation of pine seedlings took place in a temperature and humidity controlled inoculation chamber at DGRC. Inside the inoculation chamber a relative humidity of 100% and air temperature of 18°C are maintained for the duration of the infection period. Infected *Ribes* spp. leaves bearing *C. ribicola* telia were suspended above the seedlings in the inoculation chamber to facilitate basidiospore drop onto pine foliage (FIGS. 6 -9). The inoculum remained in place until a basidiospore density, which was monitored by spore

deposition on glass microscope slides in each inoculation chamber, of >5000/cm^2 was reached. The inoculated pine seedlings remained in the inoculation chamber until 48 hrs after inoculation (*Ribes* leaves removed) for basidiospores to germinate. During this time the chamber temperature was raised to 20°C and humidity maintained at 100%.

Figure 7: The underside of a *Ribes nigrum* leaf infected with *C. ribicola*.
Figures 8, 9, 10: The inoculation chamber at Dorena Genetic Resource Center near Cottage Grove, OR, during an inoculation trial in October 2012. *Ribes* spp. leaves with mature *C. ribicola* telia are suspended on wire mesh frames above pine seedlings.

2.2 Pine needle sample processing

Samples of infected secondary needles were collected from seedlings beginning two wks after inoculations. Three to four secondary needles were plucked off the seedling in a random fashion allowing foliage from different parts of the seedling to be included. Care was taken to not injure the seedling. The overall state of the seedling was examined at time of sample collection and presence or absence of macroscopic needle spots was noted. The needles were brought to the lab to initiate the embedding process within two to three hrs after collecting. In the lab, samples were cross-sectioned into 2mm pieces with the aid of a sterile razor blade and immediately transferred into 4% paraformaldehyde fixative (4% PFA) in fresh 0.1 M sodium phosphate buffer inside 20mL screw top glass jars. Subsequently, needles were fixed under vacuum (17-23 mmHg) in 4% PFA for 72 hrs prior to beginning the embedding procedure.

In preparation for tissue embedding with Tecnovit 7100 glycol methacrylate plastic (Heraeus Kulzer), samples (2mm needle sections) were dehydrated in a graded alcohol series (50%, 70%, 95% EtOH) for 24 hrs each. Afterwards, sample infiltration under vacuum took place in four infiltration steps with decreasing ratios of 95% EtOH to Tecnovit 7100 infiltration solution. For the first infiltration step, needle sections were held in a vacuum for three hrs suspended in a 2:1 ratio of infiltration solution (EtOH: Tecnovit 7100 infiltration solution) and then were refrigerated for 24 hrs.

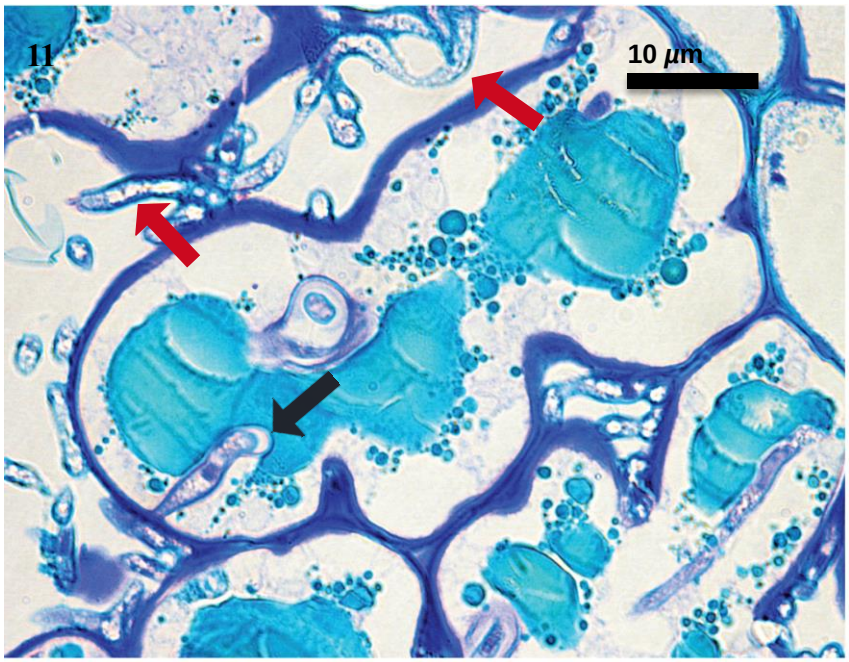
Next, needle sections were held under a vacuum for three hrs suspended in a 1:1 ratio of infiltration solution (EtOH: Tecnovit 7100 infiltration solution) after which samples were refrigerated for 48 hrs. The infiltration solution was replaced with 1:2 ratio of infiltration

solution (EtOH: Tecnovit 7100 infiltration solution) and the samples were held in a vacuum for three hrs and then refrigerated for 24 hrs. The infiltration solution was then replaced with undiluted Tecnovit and Hardener I and samples were placed under vacuum for three hrs following which the samples were kept suspended in this solution under refrigeration for 72hrs. The embedding process of samples was completed by placing two needle cross sections into each embedding mold and filling it with the polymerization solution (Tecnovit 7100 and Hardener II) prepared according to manufacturer's directions. The embedded samples were left untouched inside the mold for 24 hrs to cure and to completely harden. The polymerized blocks were affixed to 2 cm long segments of an acrylic rod with cyanoacrylate adhesive to allow for easy loading in a rotary microtome. Prior to microtomy the polymerized blocks were trimmed and faced to expose the area of interest. A rotary microtome with a steel blade was used to vertically section the embedded pine needles perpendicular to the surface of the tissue. The resulting pine needle cross sections, which were 4-6 µm thick, were placed onto water droplets on glass microscope slides and heat fixed in place. Subsequent, tissue staining was completed by submerging the slide in 0.4% Toluidine Blue 0 in pH 4.4 citric acid buffer for four minutes, followed by rinsing the slides for four minutes in sterile water.

3. RESULTS

3.1 Structural analysis of host tissues

In this study, histological observations of embedded needle tissues from resistant and susceptible seedlings of four pine species sampled at different post-infection time intervals were obtained. Morphological variation in pine needle tissues was noted as well as variation and patterns of fungal colonization inside needle tissues. Intercellular hyphae and haustoria were observed in both resistant and susceptible needles of all examined pine species. Occurrence of haustoria in needle cross sections was common and haustoria were observed inside both mesophyll and phloem cells. Only one haustorium per cell was observed, however, inside host cells haustoria can be branched. Hyphae and haustoria of *C. ribicola* appeared purple when stained with toluidine blue and were found to be 3-5µm in diameter (FIGS. 11, 12).



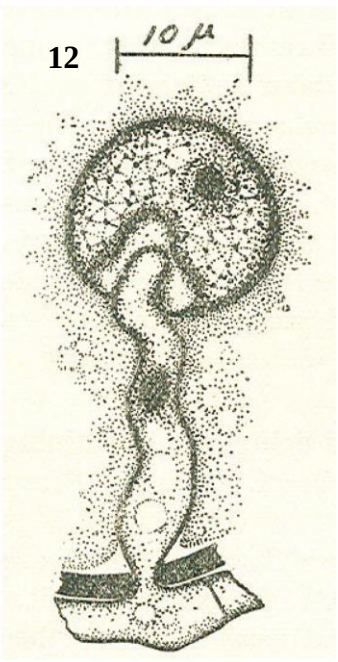


Figure 11: Microtome cross-section of *P. albicaulis* needle stained with TBO. Black arrow points to a haustorium of *C. ribicola* invading a *P. albicaulis* mesophyll cell. Red arrows point to intercellular, branching hyphae of *C. ribicola* in the mesophyll tissue.
Figure 12: *Cronartium ribicola* haustorium invading a host cell nucleus. Drawing by Reginald H. Colley, 1917, Journal of Agricultural Research, 11, Fig.1a.

The progression of *C. ribicola* colonization in needles obtained from the same seedling was monitored first biweekly, then monthly for some of the samples. Other sample collections were obtained haphazardly throughout the year depending on external symptom appearance. The following character key was employed to visually score and to categorize observations of host tissues:

0 = no hyphae present (FIG.13)

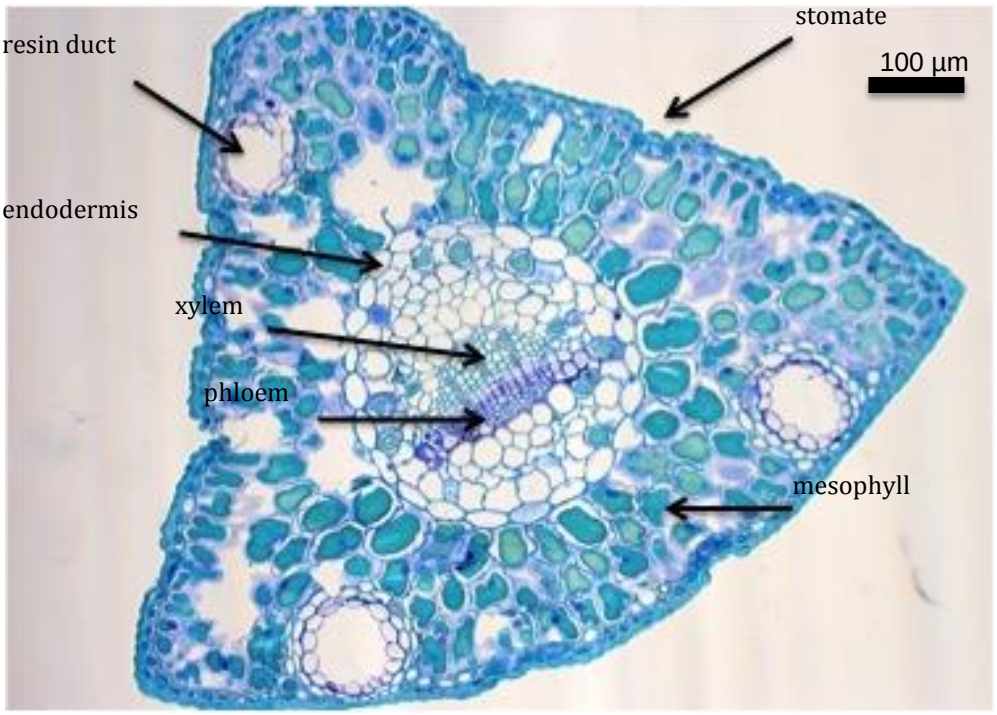
1 = intercellular hyphae present in the mesophyll (FIGS. 14, 15, 16, 17)

2 = hyphae present in mesophyll and hyphal mass appressed to endodermis (EN)(FIGS. 15, 16)

3 = EN deformed (FIG.16)

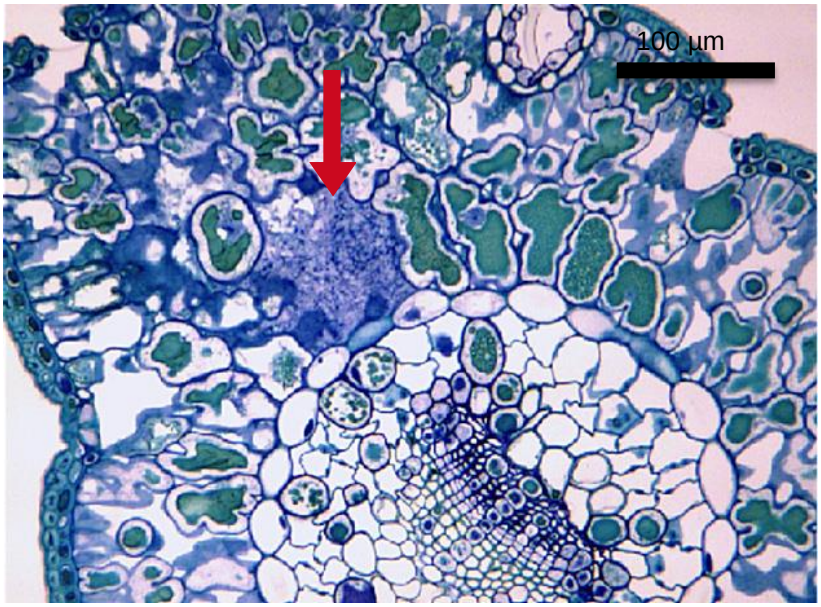
4 = intracellular fungal hyphae inside EN (FIGS.15, 16, 17)

5 = fungal hyphae inside the vascular cylinder (FIGS. 16, 17, 18)



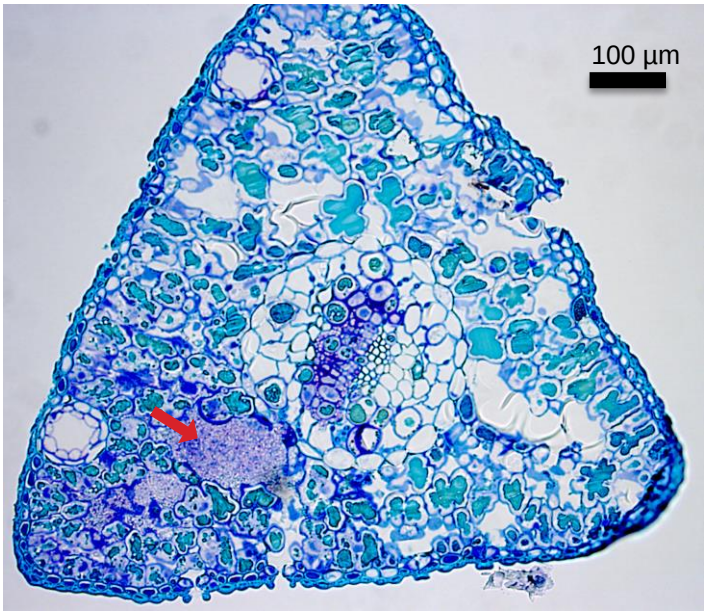
Visual score "0"

Figure 13: A cross section of an uninoculated secondary needle taken from a two-year old *Pinus monticola* seedling. The visual score rating of this cross section is "0", meaning no hyphae are present. The section was stained with TBO.



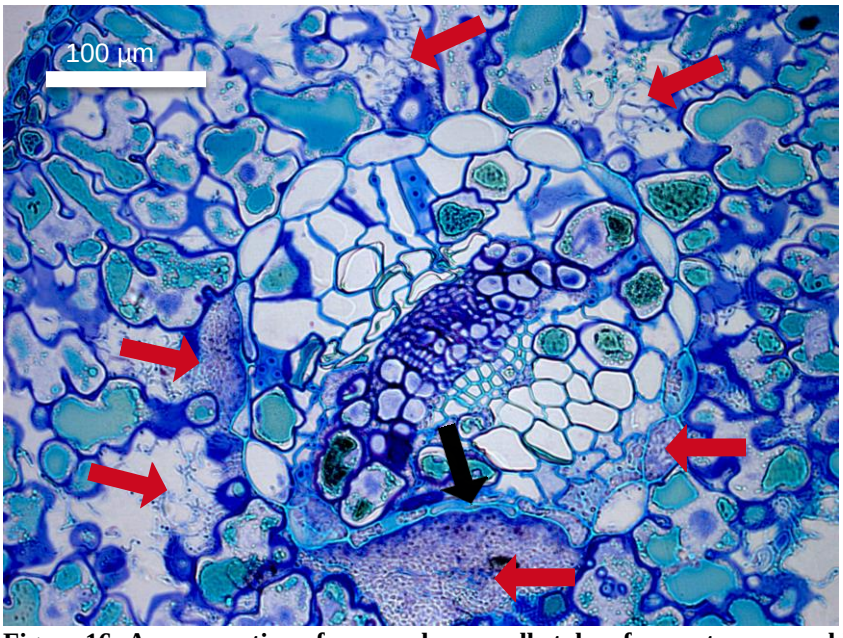
Visual score "1"

Figure 14: Cross section of secondary needle taken from a two-year old *Pinus flexilis* seedling. The rating of this cross section is "1", intercellular hyphae are present in the mesophyll and denoted by red arrow. The section was stained with TBO.



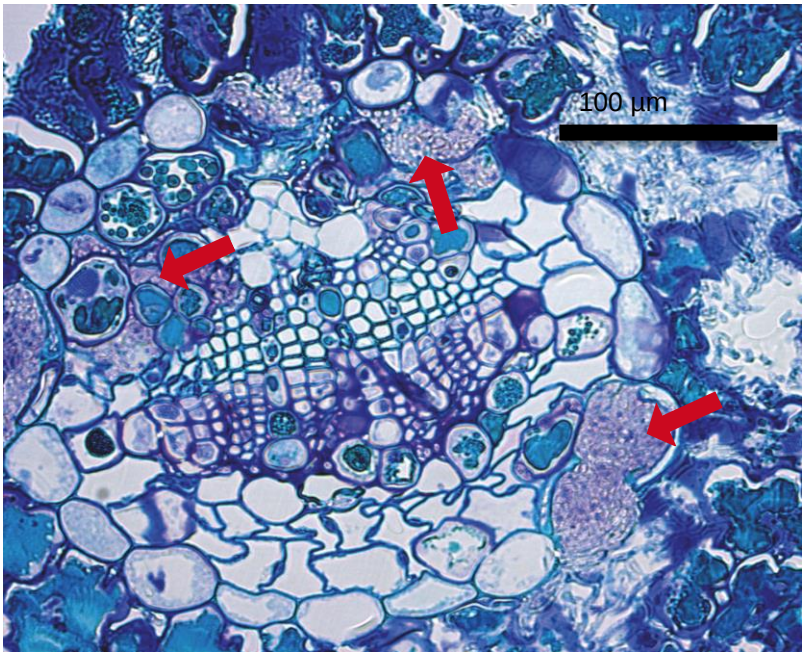
Visual score
"1,2,4"

Figure 15: A cross section of a secondary needle taken from a two-year old *Pinus albicaulis* seedling. The rating of this cross section is "1,2,4", intercellular hyphae is present in the mesophyll and a hyphal mass is appressed to the endodermis. The section was stained with TBO.



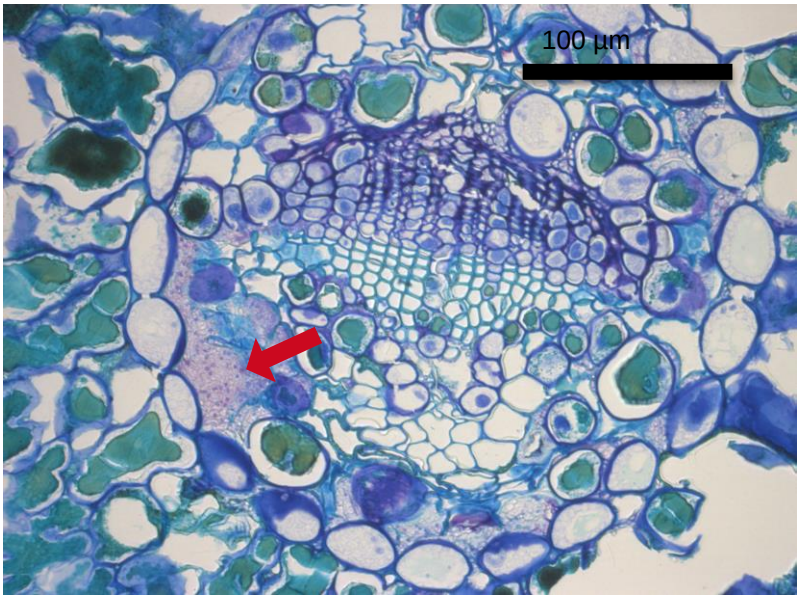
Visual score
"1, 2, 3, 4, 5"

Figure 16: A cross section of a secondary needle taken from a two-year old *Pinus albicaulis* seedling. The rating of this cross section is "1, 2, 3, 4, 5", intercellular hyphae are present in the mesophyll and a hyphal mass is appressed to the endodermis, the endodermis is deformed and there are hyphae in some of the endodermal cells; hyphae can also be seen within the vascular cylinder .The section was stained with TBO. Black arrow points to the deformed endodermis, red arrows point to inter-and and intracellular hyphae.



Visual score
"1, 4, 5"

Figure 17: A cross section of a secondary needle taken from a two-year old *Pinus lambertiana* seedling. The rating of this cross section is "1, 4, 5". Intercellular hyphae are present in the mesophyll, intracellular fungal hyphae inside endodermis (EN), and hyphae are visible inside the vascular cylinder. Arrows point to fungal hyphae inside the EN and inside the vascular cylinder.



Visual score
"5"

Figure 18: A cross section of a secondary needle taken from a two-year old *Pinus flexilis* seedling. The rating of this cross section is "5"; hyphae are visible inside the vascular cylinder. Arrows point to hyphae inside the endodermis and inside the vascular cylinder. Arrow points to hyphae inside the vascular cylinder.

3.2 Structural observations of *Pinus flexilis*

A total of 57 *P. flexilis* samples were examined of which 31 samples were taken from the resistant-phenotype seedlings; i.e. seedlings that developed the R-type needle spots and did not develop stem cankers up to 15 months following inoculation, and 26 samples were taken from susceptible-phenotype seedlings; i.e. seedlings that developed S-type needle spots and stem cankers. Both resistant and susceptible seedling families, i.e. seed parent trees that consistently yield progeny that have a low incidence (R families) or high incidence (S families) were known. Collections were obtained as early as 47 days after inoculation and continued until 223 days post-inoculation being the latest needle collection completed for this species of pine. Large masses of hyphae were visible in the needle mesophyll of both resistant and susceptible *P. flexilis* seedlings at 47 days post-inoculation. Hyphae invading host tissues in the needle vascular cylinder were observed in 18 of 31 needle samples from resistant seedlings and in 14 out of 26 needle samples from susceptible *P. flexilis* seedlings (TABLE 5) (FIGS.19, 20,21).

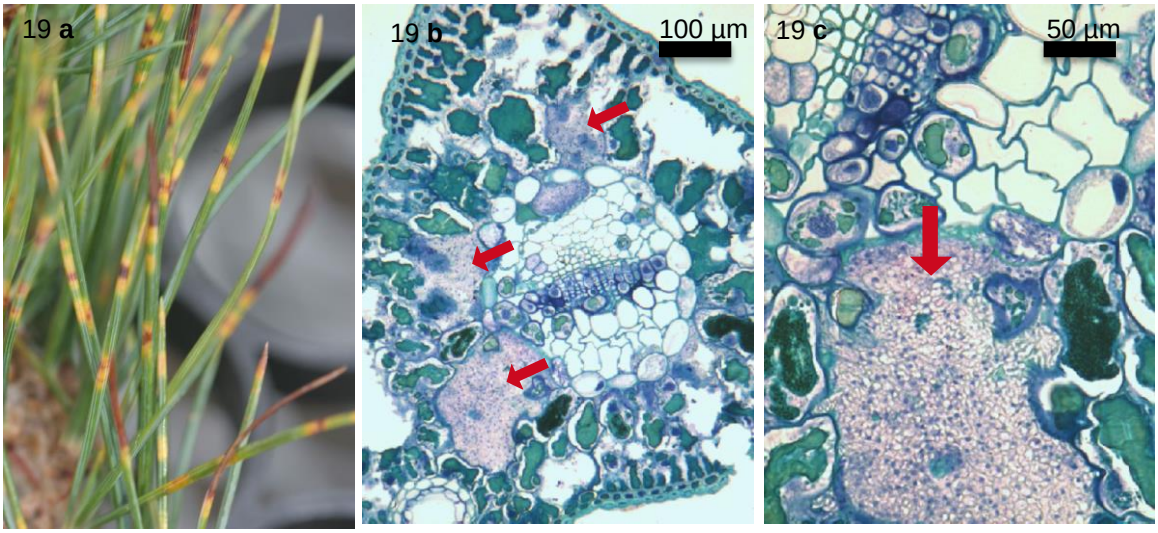


Figure 19a-c: *Pinus flexilis* resistant
19 a: *P. flexilis* foliage on two-year old seedling from a resistant family in the spring following fall inoculation. 19 b and c: *P. flexilis* secondary needle in cross sections 47 days after inoculation. Arrows point to inter- and intracellular hyphae.

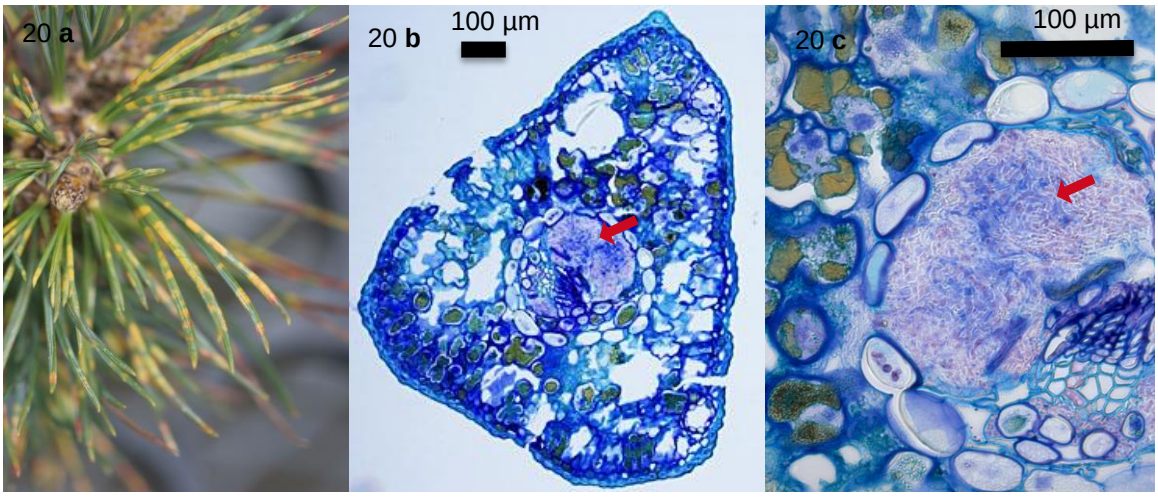


Figure 20a-c: *Pinus flexilis* susceptible
20a: *P. flexilis* foliage on two-year old seedling from a susceptible family in the spring following fall inoculation. 20 b and c: *P. flexilis* secondary needle in cross sections 47 days after inoculation. Arrows point to inter- and intracellular hyphae.

Table 5: *Pinus flexilis* needle samples.

Species	Inoculation	ID #	phenotype	sampled	days post-inoc.	observations [a]
P. flexilis	9/17/08	2115 R	resistant family	2/6/09	142.00	0
P. flexilis	9/17/08	2139 S	resistant family	2/6/09	142.00	0
P. flexilis	9/17/08	2133 S	x	2/6/09	142.00	1,4,5
P. flexilis	9/17/08	2127 S	x	2/6/09	142.00	4,5
P. flexilis	9/17/08	F=2-50 S	resistant family	4/2/09	197.00	4,5
P. flexilis	9/17/08	F=2-47 S	susceptible family	4/2/09	197.00	1,2,3
P. flexilis	9/17/08	F=2-49 S	x	4/2/09	197.00	1,4
P. flexilis	9/17/08	5116 R	resistant family	4/28/09	223.00	0
P. flexilis	9/23/10	44 S	susceptible family	11/9/10	47.00	1,5
P. flexilis	9/23/10	44 S	susceptible family	11/30/10	68.00	0
P. flexilis	9/23/10	44 S	susceptible family	2/8/11	138.00	4,5
P. flexilis	9/23/10	44 S	susceptible family	3/8/11	166.00	1,5
P. flexilis	9/23/10	45 S	susceptible family	11/9/10	47.00	1
P. flexilis	9/23/10	45 S	susceptible family	2/8/11	138.00	1
P. flexilis	9/23/10	45 S	susceptible family	3/8/11	166.00	1,5
P. flexilis	9/23/10	45 S	susceptible family	4/26/11	215.00	1,4,5
P. flexilis	9/23/10	15 R	susceptible family	11/9/10	47.00	1
P. flexilis	9/23/10	15 R	susceptible family	3/8/11	166.00	1,5
P. flexilis	9/23/10	15 R	susceptible family	4/26/11	215.00	1,3,5
P. flexilis	9/23/10	73 S	resistant family	11/9/10	47.00	1
P. flexilis	9/23/10	73 S	resistant family	11/30/10	68.00	1,5
P. flexilis	9/23/10	75 S	resistant family	11/9/10	47.00	1,5
P. flexilis	9/23/10	4 R	resistant family	11/30/10	68.00	1
P. flexilis	9/23/10	4 R	resistant family	2/8/11	138.00	1

Species	Inoculation	ID #	phenotype	sampled	days post-inoc.	observations[a]
P. flexilis	9/23/10	4 R	resistant family	3/8/11	166.00	1,5
P. flexilis	9/23/10	4 R	resistant family	4/26/11	215.00	1,3,5
P. flexilis	9/23/10	72 R	resistant family	11/9/10	47.00	1
P. flexilis	9/23/10	72 R	resistant family	11/30/10	68.00	1,4,5
P. flexilis	9/23/10	72 R	resistant family	2/8/11	138.00	1
P. flexilis	9/23/10	72 R	resistant family	3/8/11	166.00	1
P. flexilis	9/23/10	72 R	resistant family	4/26/11	215.00	1,5
P. flexilis	9/23/10	14 R	resistant family	11/30/10	68.00	1,4,5
P. flexilis	9/23/10	14 R	resistant family	2/8/11	138.00	1,4,5
P. flexilis	9/23/10	21 R	resistant family	11/9/10	47.00	1,2
P. flexilis	9/23/10	21 R	resistant family	11/30/10	68.00	1
P. flexilis	9/23/10	21 R	resistant family	2/8/11	138.00	1
P. flexilis	9/23/10	21 R	resistant family	3/8/11	166.00	1
P. flexilis	9/23/10	21 R	resistant family	4/26/11	215.00	1,2
P. flexilis	9/23/10	46 R	resistant family	11/9/10	47.00	1,2,5
P. flexilis	9/23/10	46 R	resistant family	11/30/10	68.00	1,2,5
P. flexilis	9/23/10	46 R	resistant family	2/8/11	138.00	1,5
P. flexilis	9/23/10	46 R	resistant family	3/8/11	166.00	1,5
P. flexilis	9/23/10	46 R	resistant family	4/26/11	215.00	1,5
P. flexilis	9/23/10	51 S	resistant family	11/30/10	68.00	1
P. flexilis	9/23/10	52 R	resistant family	11/9/10	47.00	1,4,5
P. flexilis	9/23/10	52 R	resistant family	11/30/10	68.00	1,4
P. flexilis	9/23/10	52 R	resistant family	2/8/11	138.00	1,5
P. flexilis	9/23/10	52 R	resistant family	4/26/11	215.00	1,5
P. flexilis	9/23/10	57 R	resistant family	4/26/11	215.00	1
P. flexilis	9/23/10	70 S	susceptible family	11/9/10	47.00	1,5

Species	Inoculation	ID #	phenotype	sampled	days post-inoc.	observations[as]
P. flexilis	9/23/10	70 S	susceptible family	3/8/11	166.00	1,5
P. flexilis	9/23/10	71 S	susceptible family	11/9/10	47.00	1
P. flexilis	9/23/10	71 S	susceptible family	11/30/10	68.00	1,5
P. flexilis	9/23/10	71 S	susceptible family	3/8/11	166.00	1,4,5
P. flexilis	9/23/10	71 S	susceptible family	4/26/11	215.00	1,4,5
P. flexilis	9/23/10	27 S	susceptible family	4/26/11	215.00	1,3,5

[a]**Observations legend:**
0 = no fungal hyphae present.
1 = fungal hyphae present in the mesophyll.
2 = fungal hyphae present in mesophyll and hyphal mass appressed to endodermis (EN).
3 = EN deformed.
4 = fungal hyphae inside EN.
5 = fungal hyphae inside the vascular cylinder.

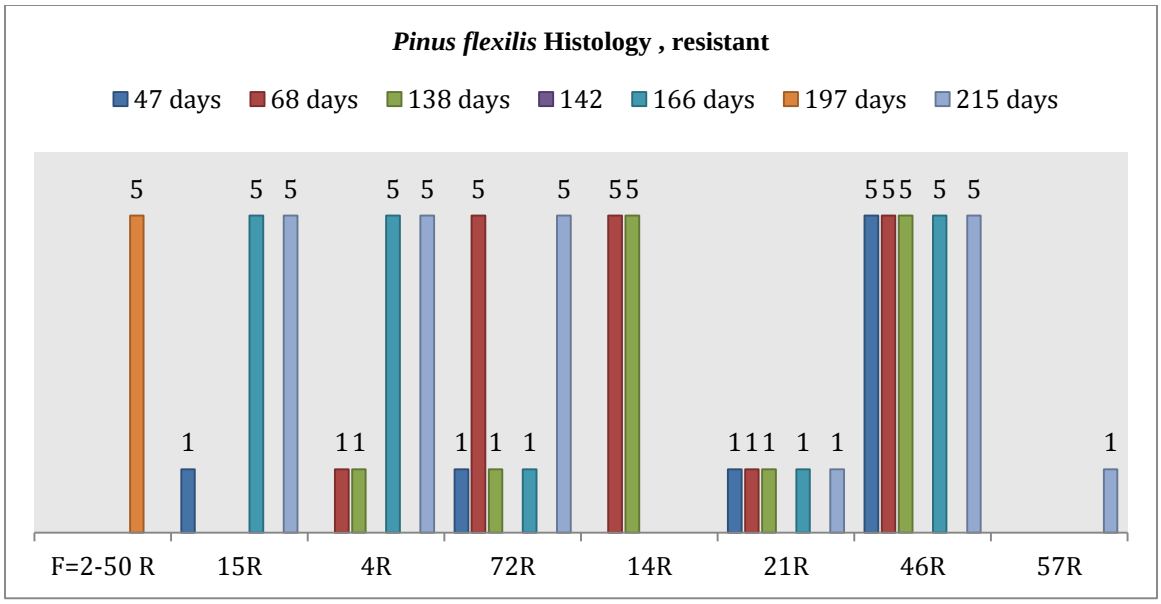


Figure 21a: *Pinus flexilis* needle samples, resistant.
1 = fungal hyphae present in the mesophyll
5 = fungal hyphae inside the vascular cylinder

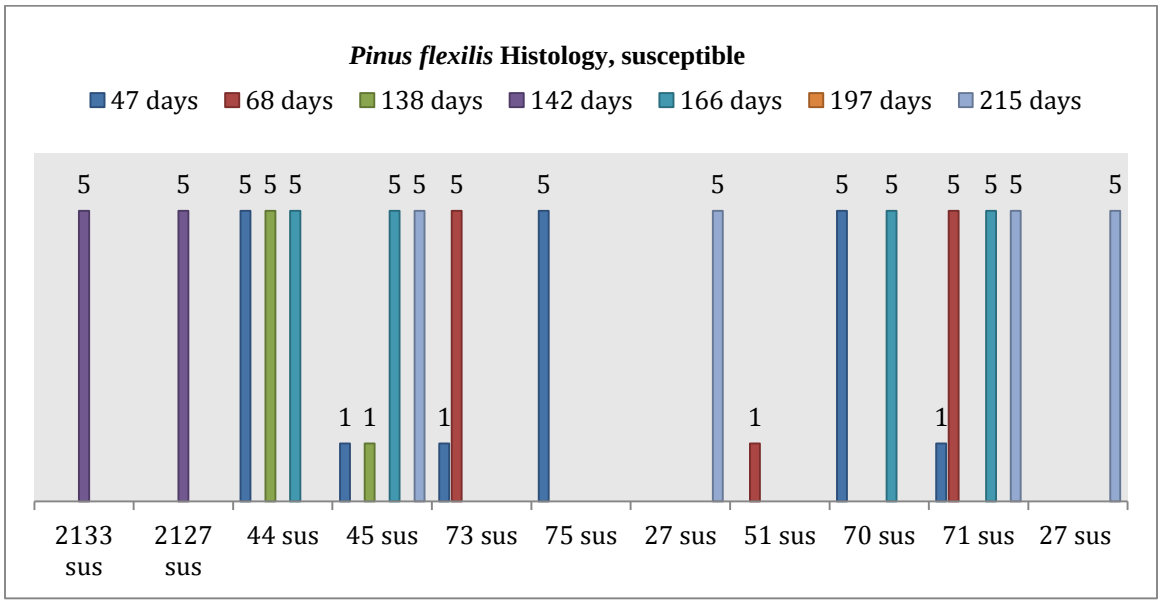
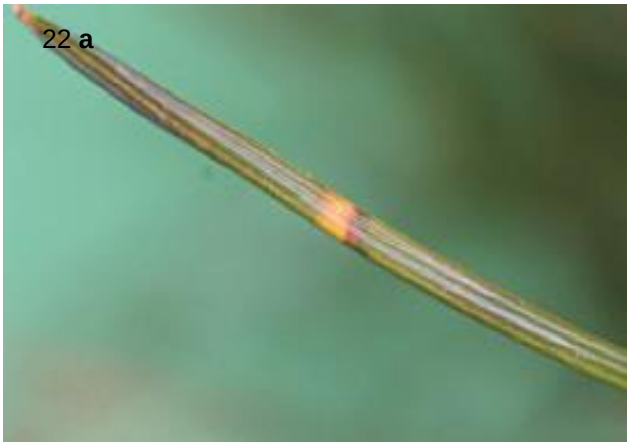


Figure 21b: *Pinus flexilis* needle samples, susceptible.
1 = fungal hyphae present in the mesophyll
5 = fungal hyphae inside the vascular cylinder

3.3 Structural observations of *Pinus monticola*

A total of 25 *P. monticola* needle samples were examined comprising 12 samples from resistant seedlings, i.e seedlings that developed R-type needle spots and remained free of stem cankers up to 15 months following inoculation, and 12 samples from susceptible seedlings, i.e seedlings that had S-type needle spots and developed stem cankers. The resistance phenotype of one additional *P. monticola* sample is unknown. Collections were obtained as early as 27 days after inoculation and continued until 168 days post-inoculation. Large condensed hyphal masses were visible in the needle mesophyll of both resistant and susceptible *P. monticola* seedlings at 27 days post-inoculation. Hyphae invading host tissues in the needle vascular cylinder were seen in all but three samples from resistant individuals. In none of the needle cross sections examined was the fungal infection limited to the mesophyll and hence a standalone observation rating “1” was never given. The amount of the fungal colonization inside host needle tissues in *P. monticola* always extended past the needle mesophyll into other tissue types (TABLE 6) (FIGS.22-24).



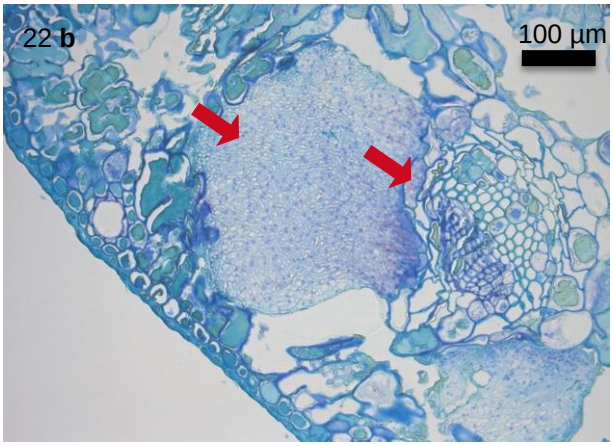


Figure 22a-b: *Pinus monticola*, resistant
22a: *P. monticola* foliage on two-year old seedling from a resistant family in the spring following fall inoculation. 22b: *P. monticola* secondary needle in cross sections 27 days after inoculation. Arrows point to inter- and intracellular hyphae.

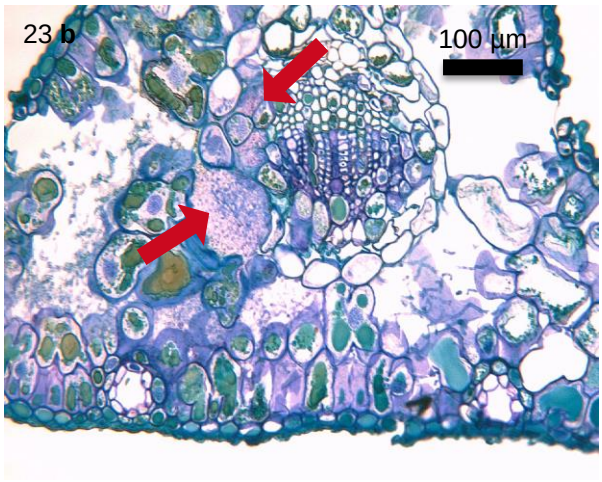


Figure 23a-b: *Pinus monticola*, susceptible
23a: *P. monticola* foliage on two-year old seedling from a resistant family in the spring following fall inoculation. 23b: *P. monticola* secondary needle in cross sections 27 days after inoculation. Arrows point to inter- and intracellular hyphae, and hyphae inside the vascular cylinder.

Table 6: *Pinus monticola* needle samples.

Species	Inoculation	ID #	phenotype	sampled	days post-inoc.	observations.[a]
P. monticola	9/17/08	5617 S	susceptible family	3/4/09	168.00	1,4,5
P. monticola	9/9/09	5619 S	susceptible family	10/20/09	41.00	1,4,5
P. monticola	9/9/09	5619 S	susceptible family	10/27/09	48.00	1,4,5
P. monticola	9/9/09	5619 S	susceptible family	11/3/09	55.00	1,5
P. monticola	9/9/09	5619 S	susceptible family	11/10/09	62.00	1,5
P. monticola	9/9/09	5619 S	susceptible family	11/17/09	69.00	1,5
P. monticola	9/9/09	9001 R	resistant family	10/13/09	34.00	0
P. monticola	9/9/09	9001 R	resistant family	10/20/09	41.00	1,5
P. monticola	9/9/09	9001 R	resistant family	10/27/09	48.00	1,4
P. monticola	9/9/09	9001 R	resistant family	11/3/09	55.00	1,2,5
P. monticola	9/9/09	9001 R	resistant family	11/10/09	62.00	1,5
P. monticola	9/9/09	9001 R	resistant family	11/17/09	69.00	1,5
P. monticola	9/28/11	601 S	susceptible family	10/25/11	27.00	1,2,5
P. monticola	9/28/11	615 S	susceptible family	11/1/11	34.00	1,4,5
P. monticola	9/28/11	631 S	susceptible family	11/8/11	41.00	1,4,5
P. monticola	9/28/11	655 S	susceptible family	11/15/11	48.00	1,2,5
P. monticola	9/28/11	681 S	susceptible family	11/29/11	62.00	1,4,5
P. monticola	9/28/11	706 S	susceptible family	1/2/12	96.00	1,4,5
P. monticola	9/28/11	599 R	resistant family	10/25/11	27.00	1,3,5
P. monticola	9/28/11	613 R	resistant family	11/1/11	34.00	1,3,5
P. monticola	9/28/11	627 R	resistant family	11/8/11	41.00	1,4,5
P. monticola	9/28/11	653 R	resistant family	11/15/11	48.00	1,5

Species	Inoculation	ID #	phenotype	sampled	days post-inoc.	observations[a]
P. monticola	9/28/11	679 R	resistant family	11/29/11	62.00	1,4,5
P. monticola	9/28/11	704 R	resistant family	1/2/12	96.00	0

[a]**Observations legend:**
0 = no fungal hyphae present.
1 = fungal hyphae present in the mesophyll.
2 = fungal hyphae present in mesophyll and hyphal mass appressed to endodermis (EN).
3 = EN deformed.
4 = fungal hyphae inside EN.
5 = fungal hyphae inside the vascular cylinder.

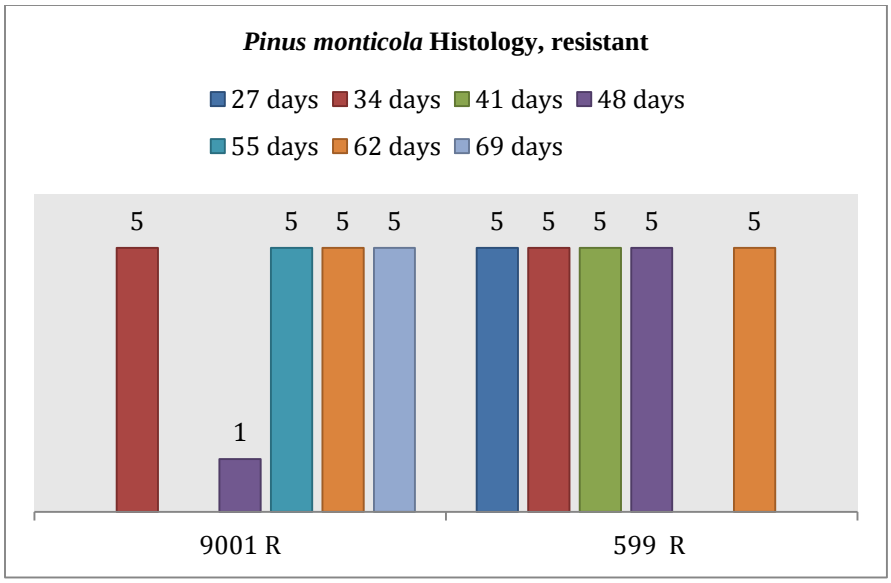


Figure 24a: *Pinus monticola* needle samples, resistant.
1 = fungal hyphae present in the mesophyll
5 = fungal hyphae inside the vascular cylinder

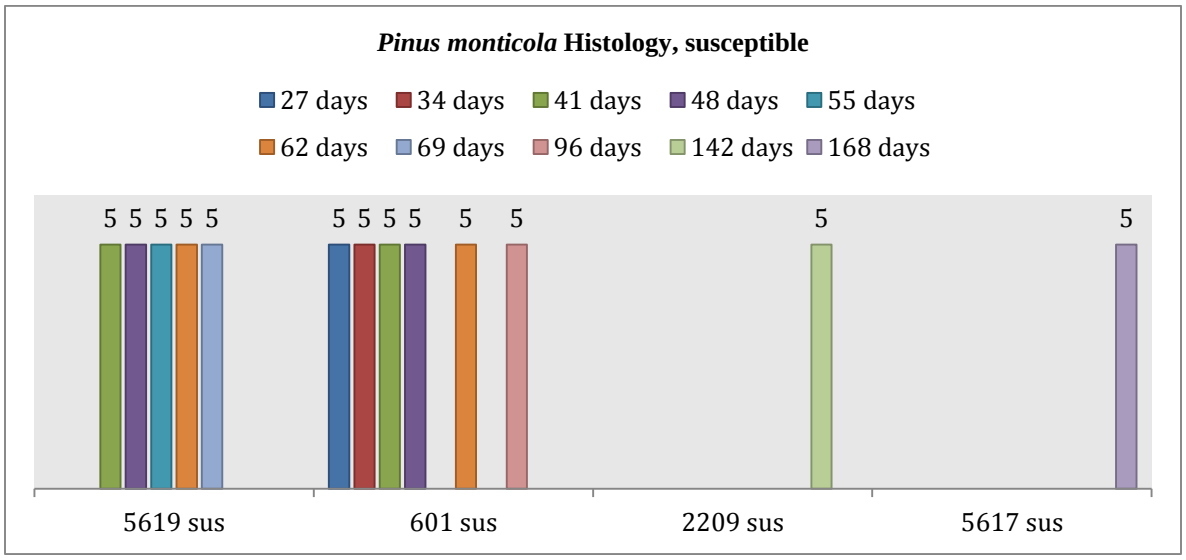


Figure 24b: *Pinus monticola* needle samples, susceptible.
1 = fungal hyphae present in the mesophyll
5 = fungal hyphae inside the vascular cylinder

3.4 Structural observations of *Pinus lambertiana*

A total of five *P. lambertiana* samples were examined, of which three samples were taken from resistant seedlings, which developed R-needle spots and remained free of stem cankers for 15 months, and two samples were taken from susceptible seedlings, which developed stem cankers. Collections were obtained as early as 22 days after inoculation and continued until 286 days post-inoculation. Large masses of fungal hyphae were visible in the needle mesophyll of resistant *P. lambertiana* seedlings only at 183 days post-inoculation. Hyphae invading the mesophyll of susceptible samples were observed at 172 and 286 days post- inoculation. Fungal hyphae invading host tissues in the needle vascular cylinder were seen in one resistant sample and in both susceptible samples (TABLE 7) (FIGS. 25-27).

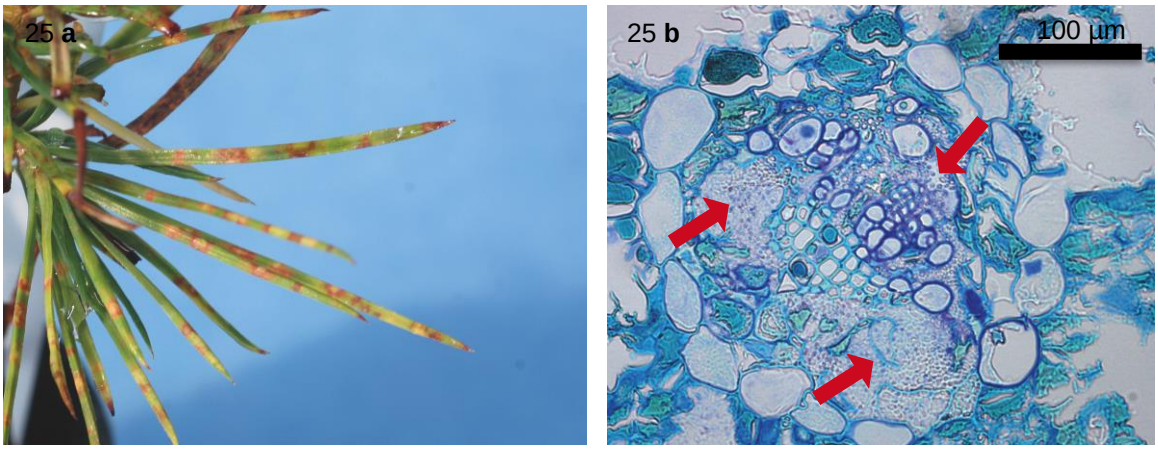


Figure 25a-b: *Pinus lambertiana*, resistant
25a: *P. lambertiana* foliage on two-year old seedling from a resistant family in the spring following fall inoculation. 25b: *P. lambertiana* secondary needle in cross sections 183 days after inoculation. Arrows point to hyphae inside the vascular cylinder.

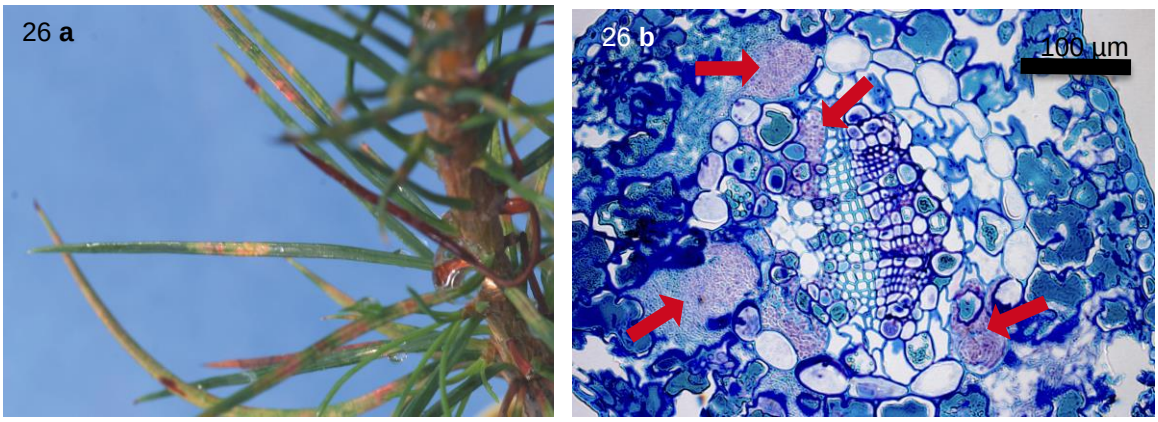


Figure 26a-b: *Pinus lambertiana*, resistant
26a: *P. lambertiana* foliage on two-year old seedling from a resistant family in the spring following fall inoculation. 26b: *P. lambertiana* secondary needle in cross sections 173 days after inoculation. Arrows point to hyphae appressed to the endodermis and inside the vascular cylinder.

Table 7: *Pinus lambertiana* needle samples.

Species	inoculation	ID#	phenotype	sampled	days post-inoc.	observations[a]
P.lambertiana	9/17/08	5673 R	resistant family	3/19/09	183.00	1,5
P.lambertiana	9/17/08	5793 S	susceptible family	6/30/09	286.00	1,2,5
P.lambertiana	9/9/09	5629 R	resistant family	10/1/09	22.00	0
P.lambertiana	9/9/09	5629 R	resistant family	10/8/09	29.00	0
P.lambertiana	9/9/09	5652 S	susceptible family	3/1/10	173.00	2,4,5

[a]Observations legend:
0 = no fungal hyphae present.
1 = fungal hyphae present in the mesophyll.
2 = fungal hyphae present in mesophyll and hyphal mass appressed to endodermis (EN).
3 = EN deformed.
4 = fungal hyphae inside EN.
5 = fungal hyphae inside the vascular cylinder.

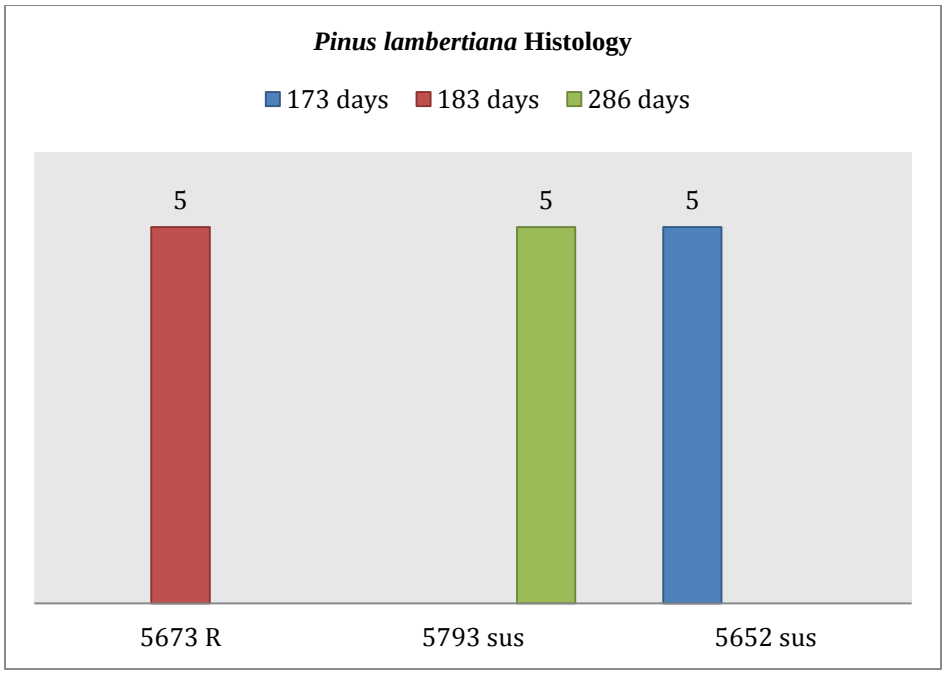


Figure 27: *Pinus lambertiana* needle samples, resistant and susceptible.
1 = fungal hyphae present in the mesophyll
5 = fungal hyphae inside the vascular cylinder

3.5 Structural observations of *Pinus albicaulis*

A total of 24 *P. albicaulis* samples were examined of which eight samples were taken from resistant seedlings that remained stem canker free (from resistant families) and eight samples were taken from susceptible seedlings that developed stem cankers (from susceptible families) 15 months after initial inoculation. Collections from eight additional susceptible seedlings of unknown families were also examined. The collections were obtained as early as six days after inoculation and continued until 132 days post-inoculation for this species of pine. Large masses of *C. ribicola* hyphae were visible in the needle mesophyll of both resistant and susceptible *P. albicaulis* seedlings. Hyphae invading host tissues in the needle vascular cylinder were seen at 132 days post-inoculation in one of the susceptible seedlings of unknown seedling family. Moreover, one resistant sample collected 96 days post-inoculation showed colonization of needle vascular tissues. Hyphae in vascular tissues were also seen in two collections from susceptible *P. albicaulis* seedlings from susceptible families from which needles were sampled at 62 and 96 days post inoculation (TABLE 8) (FIGS. 28-30).

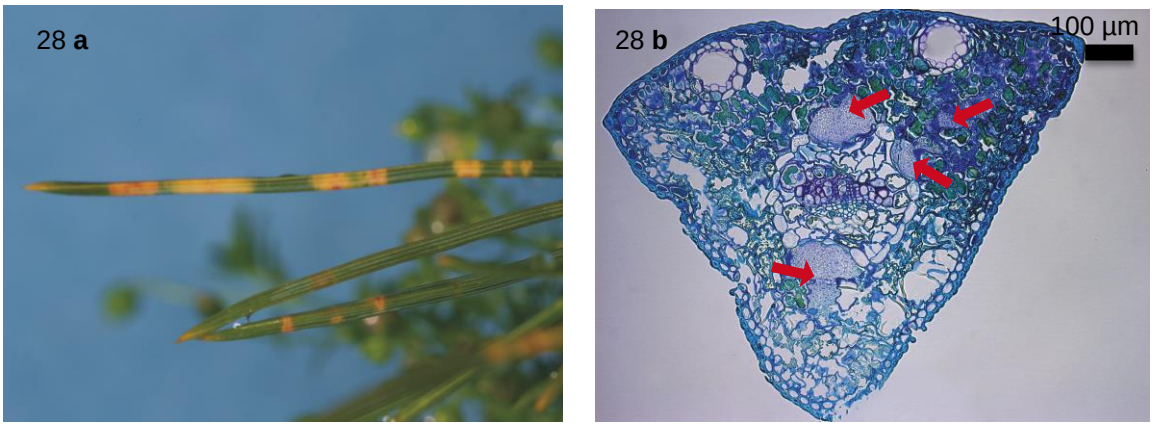


Figure 28a-b: *Pinus albicaulis*, resistant
28a: *P. albicaulis* foliage on two-year old seedling from a resistant family in the spring following fall inoculation. 28b: *P. albicaulis* secondary needle in cross sections 96 days after inoculation. Arrows point to inter- and intracellular hyphae.

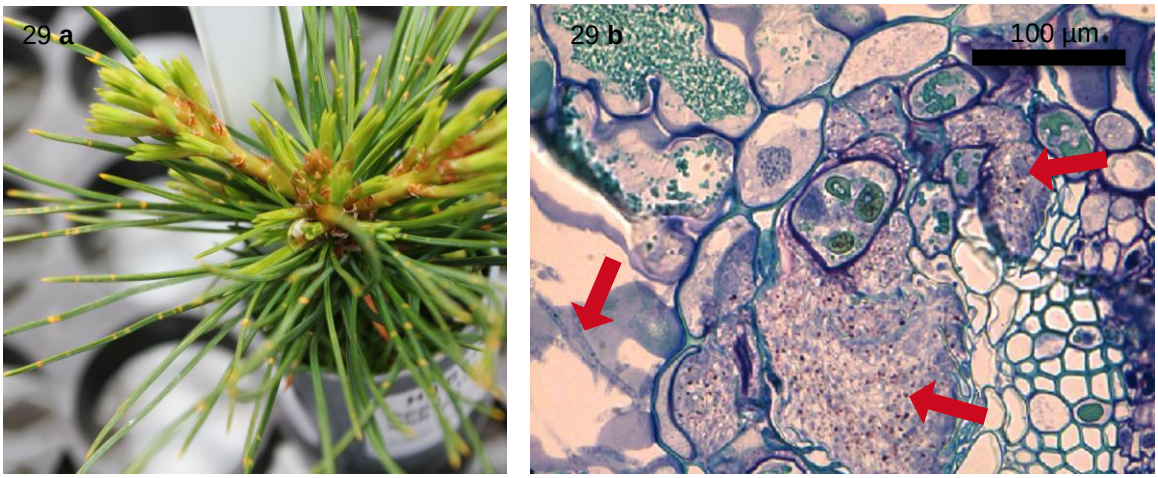


Figure 29a-b: *Pinus albicaulis*, susceptible
29a: *P. albicaulis* foliage on two-year old seedling from a resistant family in the spring following fall inoculation. 29b: *P. albicaulis* secondary needle in cross sections 96 days after inoculation. Arrows point to inter- and intracellular hyphae and hyphae inside the vascular cylinder.

Table 8: *Pinus albicaulis* needle samples.

Species	Inoculation	ID #	phenotype	sampled	days post-inoc.	observations
P. albicaulis	9/9/09	5290 S	x	10/8/09	29.00	1,3
P. albicaulis	9/9/09	5290 S	x	10/20/09	41.00	1,2,3
P. albicaulis	9/9/09	5290 S	x	11/3/09	55.00	1,2
P. albicaulis	9/9/09	5290 S	x	11/17/09	69.00	1,2,3
P. albicaulis	9/9/09	5290 S	x	12/15/09	97.00	1,2,4
P. albicaulis	9/9/09	5290 S	x	1/5/10	118.00	1,4
P. albicaulis	9/9/09	5290 S	x	1/19/10	132.00	1,4,5
P. albicaulis	9/9/09	5315 S	x	10/20/09	41.00	1,3
P. albicaulis	9/28/11	579 R	resistant family	10/4/11	6.00	0
P. albicaulis	9/28/11	593 R	resistant family	10/18/11	20.00	0
P. albicaulis	9/28/11	607 R	resistant family	10/25/11	27.00	0
P. albicaulis	9/28/11	621 R	resistant family	11/1/11	34.00	0
P. albicaulis	9/28/11	643 R	resistant family	11/8/11	41.00	0
P. albicaulis	9/28/11	660 R	resistant family	11/15/11	48.00	0
P. albicaulis	9/28/11	686 R	resistant family	11/29/11	62.00	1
P. albicaulis	9/28/11	711 R	resistant family	1/2/12	96.00	1,5
P. albicaulis	9/28/11	577 S	susceptible family	10/4/11	6.00	0
P. albicaulis	9/28/11	591 S	susceptible family	10/18/11	20.00	0
P. albicaulis	9/28/11	605 S	susceptible family	10/25/11	27.00	0
P. albicaulis	9/28/11	619 S	susceptible family	11/1/11	34.00	0
P. albicaulis	9/28/11	639 S	susceptible family	11/8/11	41.00	0
P. albicaulis	9/28/11	659 S	susceptible family	11/15/11	48.00	0
P. albicaulis	9/28/11	685 S	susceptible family	11/29/11	62.00	1,5
P. albicaulis	9/28/11	710 S	susceptible family	1/2/12	96.00	1,5

[a]**Observations legend:**
0 = no fungal hyphae present.
1 = fungal hyphae present in the mesophyll.
2 = fungal hyphae present in mesophyll and hyphal mass appressed to endodermis (EN).
3 = EN deformed.
4 = fungal hyphae inside EN.
5 = fungal hyphae inside the vascular cylinder

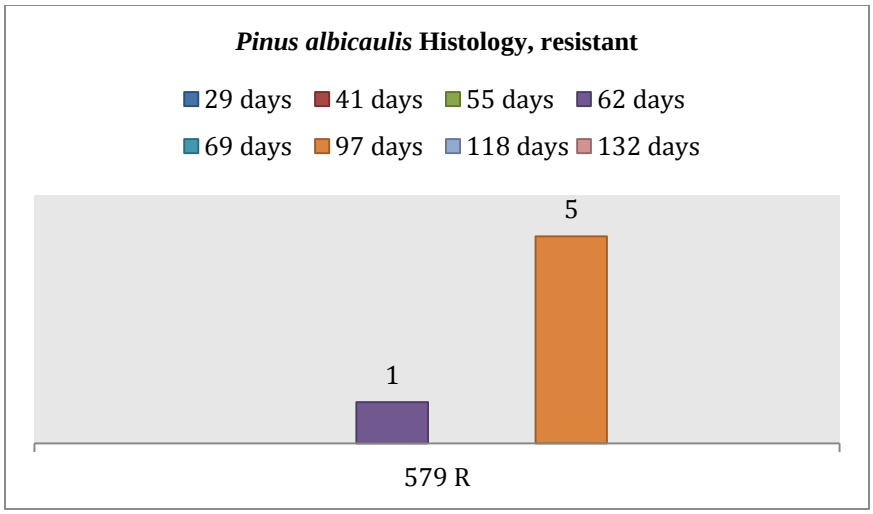


Figure 30a: *Pinus albicaulis* needle samples, resistant.
1 = fungal hyphae present in the mesophyll
5 = fungal hyphae inside the vascular cylinder

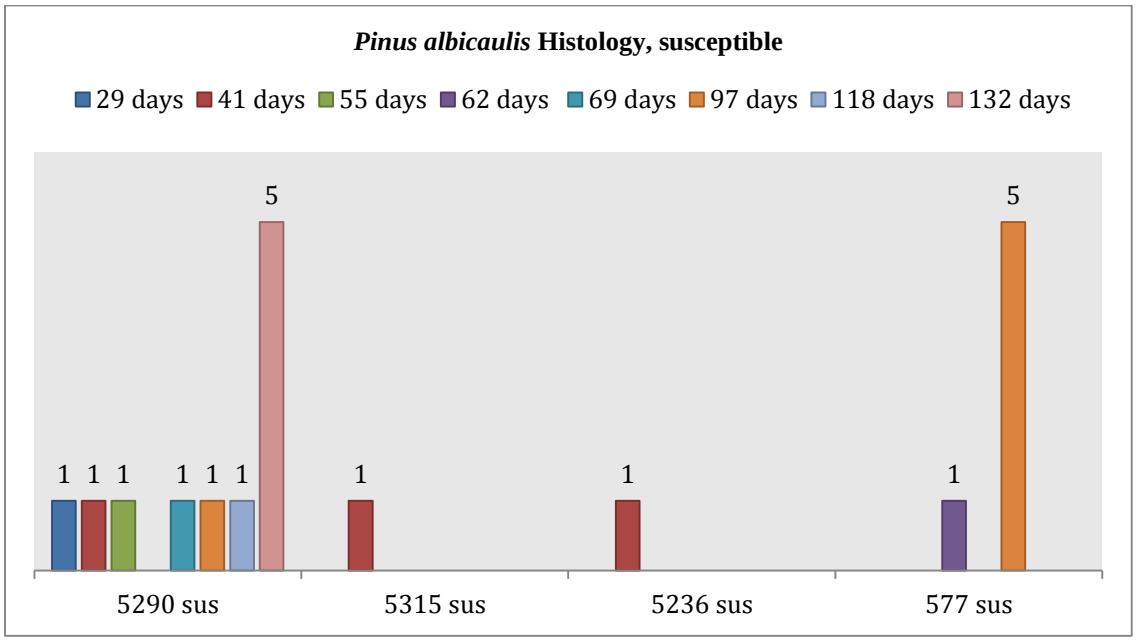


Figure 30b: *Pinus albicaulis* needle samples, resistant.
1 = fungal hyphae present in the mesophyll
5 = fungal hyphae inside the vascular cylinder

4. DISCUSSION

4.1 Histology and structural analyses

This study provides insights into the process of needle colonization by *C. ribicola* in selected five-needle pines. HR-mediated resistance does not appear to function in halting needle colonization, as has been suggested for one type of resistance to WPBR (Kinloch and Littlefield, 1977; Kinloch et al., 2003). No difference in pattern of needle colonization by *C. ribicola* was seen between resistant and susceptible phenotypes of four species of white pines. *C. ribicola* extensively colonizes needles of the MGR phenotypes of *P. flexilis, P. lambertiana,* and *P. monticola,* which are thought to restrict needle colonization by a hypersensitivity response mechanism without associated localized death of needle mesophyll cells, or any discernable restriction of the growth of the pathogen in needle tissues. The onset of auto-induced HR-like needle lesions in the resistant host does not begin until five to nine months after initial colonization. In comparison to the non-HR resistance believed to take place in *P. albicaulis* seedlings (Sniezko et al., 2012), no differences in the onset and progression of fungal growth was observed.

4.1.1 *Pinus flexilis*

Overall, no differences in the progress of colonization by *C. ribicola* were observed in needle samples from susceptible and resistant seedlings. For the majority of the *P. flexilis* seedlings under study information on heritability of major gene based resistance to *C. ribicola* among progeny of identified seed parent trees (i.e. ‘families’) was known. Seed

parents that uniformly yielded rust resistant seedlings (‘resistant families’) are operationally considered homozygous dominant at the MGR resistance locus. Those that have progeny segregating for the resistance phenotypes are presumed to be heterozygous or the resistance phenotype are presumed to be heterozygous at the MGR locus (Sniezko, 2006). The *Cr4* gene locus has been associated with MGR in *P. flexilis* (Schoettle et al, in press). The HR-like needle spots become visible approximately eight months after initial inoculation in the resistant phenotype; in the susceptible phenotype the yellow needle spots continue to enlarge along the length of the needle.

4.1.2 *Pinus monticola*

No differences in the needle colonization pattern by *C. ribicola* were observed between the resistant and susceptible seedlings of *P. monticola* examined. In none of the sampled seedlings was the extent of fungal colonization limited to tissues outside of the needle vascular cylinder. In comparison to the other pine seedlings in this study, this may be an indication of an accelerated rate of fungal proliferation in *P. monticola* seedlings. Yet, a wider scope of seedling genotypes should be examined to provide confirmation. MGR-type resistance to *C. ribicola* in *P. monticola* is thought to confer an HR-mediated resistance through the *Cr2* locus (Kinloch et al., 1999). HR-like needle spots (necrotic) become visible approximately eight months after initial inoculation. Populations of *C. ribicola* that are virulent to *P. monticola* carrying the MGR *Cr2* phenotype have been designated as the *vcr2* pathovar race which is known to exist in Oregon (Kinloch et al., 2004). Few studies have been completed to estimate the genetic diversity of natural

populations of *C. ribicola* in western North America and to assess the variation in virulence among different *C. ribicola* races (pers. communication R. Hamelin). Genetic variation in rust races operationally termed 'wild-type' and *vcr1*, *vcr2* etc. has not been investigated to date. It is therefore possible that accelerated rates of fungal infection or colonization are attributes of unique races of the pathogen. Further seedling testing with genotyped fungal races is necessary to investigate this claim. Such studies are underway (Sweeney, unpublished; Hamelin et al., unpublished) to relate molecular analyses of the fungal race to the variation seen in pine host reaction.

4.1.3 *Pinus lambertiana*

No differences in the pattern of needle colonization by *C. ribicola* were observed between the resistant and susceptible seedlings of *P. lambertiana* examined. Only a few seedlings were included in the study and samples examined included both primary and secondary needles. Although fewer *P. lambertiana* samples were examined, patterns of colonization were similar between resistant seedlings expressing the MGR phenotype and susceptible seedlings. MGR-type resistance in *P. lambertiana* is thought to confer HR-mediated resistance through the *Cr1* locus (Kinloch and Comstock, 1980). The HR-like needle spots become visible approximately nine month after initial inoculation; needle spots in the susceptible phenotype continue to expand along the needle length. The virulent *vcr1* race of *C. ribicola*, which is able to neutralize the effects of *Cr1*, is not known to exist in Oregon but has only been found in California (Kinloch et al., 2004). It appears that the occurrence of *vcr1* is coincidental with the presence of forest stands

showing the *CR1* phenotype. At the WPBR testing site 'Happy Camp', located in the Siskiyou Mountains in California, a population of *P. lambertiana* with high levels of *CR1* co-occurs with the *vcr1* race of *C. ribicola* (Kinloch and Dupper, 2002; Kinloch et al., 2004). It was hypothesized that a strong selection pressure at this site may have contributed to the development of the *vcr1* race, and accelerated its proliferation in the population (Richardson et al., 2008). The *vcr1* rust race was not used (to our knowledge) for any of the inoculations in the present study.

4.1.4 *Pinus albicaulis*

No differences in the process of colonization of needles by *C. ribicola* were observed between susceptible and resistant seedlings of *P. albicaulis*. However, the interval between inoculation and first visible hyphae in needle tissues differed between seedlings in the two inoculation trials studied here. The earliest hyphae detected in mesophyll tissue from the inoculation trial completed in 2009 was at 29 days after initial inoculation. Hyphae were not observed in needle sections until 96 and 62 days after inoculation in resistant and susceptible needle collections, respectively, in the 2011 trial. Only susceptible seedling phenotypes were used for the collections completed in 2009. In the 2011 inoculation trial, seedlings of known phenotypes but unknown seedling families were available. The reason for the late onset of fungal growth in the 2011 samples may be due to differences in the seedling families included in the study. Other factors that influenced the host colonization may include environmental conditions as well as the inoculum density employed. It is possible that basidiospore germination on seedling

foliage inside the growth chamber failed to produce host infection rates that were achieved in the 2009 inoculation. There appears to be considerable variation in the first appearance of *C. ribicola* hyphae in needles and it will be necessary to collect additional data to have more conclusive indications of what environmental and biological factors might have been responsible for it. MGR/HR- mediated resistance has not been observed in *P. albicaulis*. Needle spots become visible approximately nine months after initial inoculation on average. The needle lesions lack a necrotic center and resemble susceptible spots, which in general have a more diffuse appearance.

4.2 Integration of histological findings

In the classical gene-for-gene model of plant disease resistance, plant disease resistance gene (R gene) products have the ability to detect pathogen elicitor molecules, the products of *avr* genes, and initiate a host response. Numerous plant resistance genes that function successfully in pathogen suppression have been identified in many agricultural crops (Jones and Dangl, 2006). This type of plant disease resistance includes a rapid host cell death in the infected plant region. The cell death response known as the HR is a central feature of gene-for-gene resistance in plants (Heath, 2000). Classification of HR in plants is largely based on the morphological features of the resulting cell death lesion at the pathogen site of infection and the subsequent suppression of growth of the pathogen (Heath, 2000; Morel and Dangl, 1997). Initiation of HR leads to localized necrotic tissue spots in the host plant, usually at the point of pathogen entry (Heath, 2000). The dead plant cells accumulated during an HR present a barrier to colonization

by the biotrophic pathogen, causing degeneration of fungal hyphae and stopping further pathogenic invasion (Goodman and Novacky, 1994). Hence, accumulation of necrotic host cells deprives the biotrophic fungus of nutrients and eventually leads to a macroscopically visible necrotic HR lesion. The timing of the onset of this cell death is crucial, as immediate arrest of pathogen colonization is necessary to prevent the pathogen from reaching vascular tissues from which it could spread systemically through the host (Heath, 2000). One can summarize the cardinal features of the classical HR paradigm as follows: A promptly induced plant cell death with subsequent localized tissue necrosis to limit pathogenic invasion of tissues.

Findings of this study show that typical HR as described above does not appear to function in needles of *Pinus spp*. as the mechanism of disease resistance. Host colonization by *C. ribicola* revealed that there is no apparent difference in the needle colonization by *C. ribicola* in resistant phenotypes (individuals that developed typical MGR-type needle spots and remained free of stem cankers) and susceptible phenotypes (individuals that developed typical S-type needle spots and stem cankers), nor any limitation of *C. ribicola* to specific tissues in resistant host phenotypes. Colonization of both resistant and susceptible phenotypes entails extensive intercellular colonization of the mesophyll, intracellular haustoria in mesophyll cells, the formation of a large condensed mass of *C. ribicola* hyphae closely appressed to the endodermis, penetration of the needle endodermis and needle vascular tissues by *C. ribicola*. Histological examination showed that *C. ribicola* hyphae are already present in the needle vascular cylinder when necrotic lesions associated with HR-type interactions were

macroscopically visible on needles of resistant seedlings, and hyphae progressively continued to grow for months after. Thus the mechanism of resistance to *C. ribicola* in seedlings of pine species that express the MGR phenotype, i.e. absence of stem cankers and fungal reproductive structures such as pycnia and aecia in individuals that display the 'necrotic spot' needle reaction, does not seem to be due to any barrier to pathogen colonization of needles as has been suggested (Kinloch and Littlefield, 1977; Kinloch et al., 2003).

Macroscopic needle lesions resembling HR-type tissue spots were first visible as early as five months following initial inoculation in *P. monticola*. The delayed onset of such needle spots displaying a necrotic region was first recognized by Kinloch and Littlefield (1977) on foliage of resistant *P. lambertiana*. The authors termed HR-like spots 'fleck'-spots, as these differed from the coalescing and more extensive yellowed tissues seen on infected needles of susceptible seedlings. The 'fleck spot' phenotype was attributed to a classical HR even though its onset took much longer than typical HR in other host-parasite systems. Kinloch and Littlefield (1977) concluded that the 'fleck spot' phenotype seedlings were indicators of resistance to *C. ribicola* as none of the tested 'fleck spot' trees continued to produce stem symptoms as was seen in susceptible trees. Kinloch et al. (1999) later used the same spot phenotype categories to describe the foliage of infected *P. monticola* seedlings. HR-like spots on *P. monticola* were recognized and a comparative histological analysis of infected resistant and susceptible tissues followed. The analysis revealed that large fungal masses were present in the susceptible needle tissues, while tissue sections of resistant needles showed necrotic tissues and "cellular

disorganization" which would result in fungal death (Kinloch et al. 1999). Kinloch et al. (1999) extrapolated from these data that an HR must have been taking place, resulting in host deterioration, which would deprive the fungus of nutrients and create a barrier of degenerative host cells to arrest further fungal spread. While the histological observations of colonization of needles of resistant and susceptible phenotypes in this study agree with the observations by Kinloch and Littlefield (1977) that HR-like lesions are present on foliage of resistant seedlings several months after infection, the results generated here indicate that the delayed tissue necrosis is unrelated to preventing host colonization by *C. ribicola* or disease development in seedlings.

The colonization of needles by *C. ribicola*, including invasion of the vascular tissues, seen in this study appear more similar to observation by Hoff and McDonald (1971), connecting the needle spot appearance in resistant seedlings to a fungicidal reaction that is believed to take place in the "short shoot" or brachyblast of needles. Hoff and McDonald (1971) described how *C. ribicola* invading *P. monticola* secondary needles produces a large fungal mass below the point of infection, which continues to grow inside the needle vascular cylinder until it reaches the basal portion of the infected needle fascicle and the adjacent short shoot. Upon entering the brachyblast a resistance mechanism is triggered causing the host cell and the invading fungus to die. This "toxic" reaction was suggested as the mechanism preventing the spread of fungal hyphae into stem tissues of the seedling and stem canker development is avoided (Hoff and McDonald, 1971).

This hypothesized toxic or fungicidal reaction in the short shoot is mechanistically similar to the 'fleck spot' reaction described by Kinloch and Littlefield (1977) in that progress of *C. ribicola* is prevented by necrotic host tissues, which forms a barrier to fungal growth and simultaneously compartmentalizes the attacked area. Also, both theories have characterized the resistant phenotypes by grouping seedlings into needle spot categories differentiating the diffuse yellowing of needles of susceptible seedlings from the distinct and confined needle spots seen on the foliage of resistant trees. It was further hypothesized that the short shoot fungicidal reaction underlies the control of recessive genes (Hoff and McDonald, 1971) unlike the HR-like reactions observed in *P. lambertiana* and *P. monticola* , which are thought to be under the control of unique major R-genes. Regardless of the nature of the genes controlling the resistant phenotypes in this pathosystem, it remains unclear why there is a necrotic tissue reaction in the foliage of resistant seedlings even though its onset appears unrelated to the mechanism of arresting fungal growth in resistant seedlings.

Needle histology of infected foliage of *P. strobus* was examined at a much larger scale by Jurgens et al. (2003) and Jacobs et al. (2009); however, both studies focused on foliage of *P. strobus* infected with *C. ribicola*. Resistant seedlings of *P. strobus* do not display a macroscopically visible necrotic band; yet in this host species two spot categories were also recognized, with susceptible needle spots being more diffuse than the localized yellowing observed on resistant *P. strobus* foliage. Jurgens et al. (2003) observed an increased deposition of polyphenolic compounds in the areas surrounding infection in needles of the resistant phenotype. Furthermore, disturbed host cells were

present in the resistant seedlings, which were absent in the examined susceptible tissues. Since polyphenols and tissue abnormalities were absent in susceptible seedlings, Jurgens et al. (2003) interpreted the presence of phenolic compounds as a fungicidal reaction. It was not clear however, which factors contributed to the host cell disturbance. Jacobs et al. (2009) examined exclusively primary needles of *P. strobus* and generated observations consistent with Jurgens polyphenol-reaction hypothesis. It was observed that collapsed cells adjacent to infection sites, as well as heavy deposition of phenolic compounds occurred more frequently in resistant seedlings. Nonetheless, smaller amounts of polyphenols were also found in needle tissues of some susceptible seedlings, in what appeared to contribute to a weaker fungicidal reaction unable to delimit the fungal growth (Jacobs et al., 2009).

This study did not specifically test for presence of polyphenolic compounds in needle tissues; however, the nonspecific stain employed, toluidine blue, stains polyphenols green and therefore we were able to recognize equivalent distributions of polyphenol containing cells in all of the sectioned needles of the five-needled pines studied, including the uninoculated control needles. Kinloch et al. (1977) also reported presence of phenolic compounds in sectioned tissues adjacent to necrotic spots in resistant *P. lambertiana* and considered this a result of plant defense responses, which seemed to only be present in resistant seedlings showing the ‘fleck-spot’ phenotype. In regards to presence of phenolic compounds in needles of resistant seedlings, this study does not support findings by Jurgens et al. (2003), Jacobs et al. (2009) or Kinloch et al.

(1977), as no increased occurrence of polyphenolic cells were observed in any of the needle cross sections examined, based on staining by toluidine blue.

Previous studies describing the histological patterns of colonization by *C. ribicola* are also not supported by the findings of this study. Rather than being a manifestation of programmed cell death or a fungicidal reaction, necrotic needle spots associated with MGR resistance phenotypes of pine species may instead be the product of an ongoing wound response that is initiated after fungal haustoria are established in the host mesophyll cells or due to disruption of needle tissues due to the enlarging hyphal mass.

Plant responses to wounding such as wound plug formation and papillae formation, as well as an increased accumulation of phenolics, tannins, and reactive oxygen species (ROS) are inducible plant responses that are initiated through pathogen invasion as well as mechanical tissue damage (Aist, 1976). Specifically, wound plugs are aimed at reducing cytoplasmic losses after plant cells are injured, while papillae are structural barriers to block pathogenic entry into plant tissues. However, a wound plug at the site of injury may function as a means to block the invasion of pathogens (Aist, 1976). Wound responses are slower than HR and can be regarded as cell wall reinforcement, which can either consist of structural or biochemical fortification. Structural cell wall modifications (papillae, wound plugs) in response to pathogen attack include callose (1,3-beta-D-glucan) depositions into the host cell wall to slow the invading pathogens (Brown et al., 1998). The callose depositions (papillary callose) are formed at the site of attempted microbial penetration. Biochemical responses associated with the plant wound response include release of ROS as a signaling molecule for

activation of defense genes (Thordal-Christensen et al., 1997), and induction of the synthesis of enzymes of the phenylpropanoid pathway, with the subsequent synthesis of phenolic compounds (Matern et al., 1995). Phenolics are a group of carbon based secondary compounds that can be found in conifers and other plants (Wink, 2003). When oxidized, phenolics may become ROS and are able to damage tissues and pathogens (Matern et al., 1995; Vargas et al., 2012). These reactions may describe the events that potentially lead to the localized needle spot with necrotic tissues that were observed in needles of resistant seedlings in this study.

C. ribicola, an obligate biotroph, initially colonizes the host foliage after it enters through stomata. The fungal life strategy keeps the host cell alive while minimizing cell damage. This describes a special kind of plant-pathogen compatibility allowing *C. ribicola* to parasitize the pine host while evading the host plant defense responses. The activities required by the biotroph to maintain this interaction are not well understood. Hahn and Mendgen (2001) hypothesized that a successful fungal biotroph secretes suppressor molecules that interfere with the plant's recognition of pathogen invasion; yet, it was also stated that no fungal suppressor molecule of the plant defense response to biotrophic fungi has been identified. Research with *Magnaporthe oryzae* has shown that this fungal pathogen is capable to mask its biochemical make-up by secreting a surface alpha-1, 3-glucan that enables the pathogen to evade detection by the plant host and allows for pathogenic destruction of the host cell wall (Fujikawa et al., 2012). This finding describes an elegant way of the fungus to parasitize its host without being attacked by the plant defense responses, such as wound responses. Yet, research with

other fungal pathogens is unlikely to describe the events leading to successful biotrophy in the *C. ribicola* pathosystem. Further research is needed to identify mechanisms that enable *C. ribicola* to successfully invade the host in susceptible seedlings while escaping recognition by the host.

Initially, hyphae grow in the intercellular spaces of the mesophyll. Intercellular hyphae give rise to intracellular haustoria, which provide a means for the fungus to obtain carbohydrates from the plant host. No breach of the host plant cell wall takes place until haustorium formation is initiated. The haustorium is an intracellular structure that connects to the intercellular hyphae via a haustorial mother cell adjacent to the host cell, from which the haustorium invades the inside of the host cell. Research in the *Uromyces viciae-fabae* pathosystem has shown that small amounts of cellulases are secreted, which underlies strict developmental control and are only to be found after the haustorial mother cell was formed (Deising et al., 1995). This suggests that a highly localized and limited secretion of cell wall degrading enzymes that may also be present in the *C. ribicola* haustoria takes place inside needle tissues. Since the fungus does not provoke immediate cell death and only minimal damage to the host cells is caused, an ongoing low-grade wound response inside the needle tissues of resistant seedlings may be the cause of the HR-like needle spot morphology. It appears that haustoria in the susceptible host go unrecognized and formation of a wound tissue with a necrotic center is absent.

Another possible trigger of wound response initiation in needle cells may be the mechanical stress exerted on host cells as large, condensed masses of hyphae build up in the mesophyll and within and around the vascular bundle of infected cells. The extensive

proliferation of hyphae in needles, which was observed in collections of all of the five-needle pine species under study, appears to compress and deform the host tissue. While this mechanical cellular stress caused by the expanding fungal hyphae in susceptible seedlings continues apparently uninhibited by host responses, an ongoing plant response to tissue injury may manifest itself in needle tissues of resistant seedlings, eventually leading to a needle spot displaying a necrotic center.

It remains unclear why the necrotic needle spot appears on foliage of resistant seedlings, in light of the fact that its presence is likely unrelated to the arrest of fungal growth in plant tissues. It is possible that the resistant seedlings have a transcriptionally activated pathway in response to mechanical damage that is absent in the susceptible seedlings. Genes activated in response to wounding may trigger signals that enhance the wound response in the resistant seedlings. Hydrogen peroxide and other ROS, which play a role in tissue necrosis seen in HRs (Lamb and Dixon, 1997) are also known to be a wound inducing signal, regulating gene expression following stresses to the plant cell (Pellinen, 2002). It is possible that the lower levels of ROS released for signaling act to enhance and maintain systemic wound response-signaling, while inadvertently causing a delayed tissue necrosis in the resistant seedlings. Pellinen et al. (2002) studied this phenomenon in birch trees, which were artificially wounded, by using molecular probes to detect the up-regulation of certain defense genes in response to ROS release into tissues after wounding occured. Future studies with *Pinus* spp. infected with *C. ribicola* might be useful to find out if low levels of ROS released in resistant seedlings may function as regulatory signal molecules of defense gene expression.

While host colonization patterns of *C. ribicola* in needle tissues of resistant and susceptible *P. lambertiana, P. monticola, P. flexilis,* and *P. albicaulis* are very similar, no phenotypes of *P. albicaulis* have been identified as having an HR-like needle lesion displaying a band of necrotic tissues. Nevertheless, heritable variation in development of stem cankers of *C. ribicola* has been observed for *P. albicaulis*. While the needle resistance in *P. monticola* appears to be conferred as an MGR, resistance to *C. ribicola* in *P. albicaulis* appears to be based on polygenic traits (Sniezko, 2012).

C. ribicola is considered native to eastern Asia where its hosts included *P. sibirica, P. armandii, P. koraiensis, P. wallichiana* and *P. pumila* (Kinloch and Dupper, 2002). Interestingly, the North American white pines all seem to possess a small proportion of individuals naturally resistant to infection by *C. ribicola* (Sniezko et al., 2008). To decipher the resistance mechanisms in *P. albicaulis* one could relate phylogenetic research to this study.

P. albicaulis is classified in the subsection Cembrae, a distinct group from other North American white pines, most of which are members of subsection Strobi (Price et al. 1998). The closest relative of *P. albicaulis, P. cembra*, is known to be highly resistant to *C. ribicola* (Hoff et al., 1980; Sniezko, 2008) and its native distribution coincides with that of the pathogen. With molecular analyses it may be possible to identify ancestral genes that are shared between *P. albicaulis* and *P.cembrae* and that would contribute to resistance of *P. albicaulis* to *C. ribicola*. Selection of such genes might become available to improve *P. albicaulis* in future tree breeding efforts.

5. Conclusions

Needle reactions in response to *C. ribicola* do not appear to present an HR similar to the host incompatibility reaction seen in other plant-pathogen interactions. The needle spot phenotype seen in resistant P. *lambertiana, P. monticola, P. flexilis* seedlings interpreted as carrying the MGR phenotype displays a necrotic band, which may be due to an ongoing plant response to tissue injury taking place in the host. This necrotic lesion on resistant foliage can be useful in identification of resistant individuals; however, histological analyses presented here revealed that necrotic spots are not directly involved in resistance to needle colonization by *C. ribicola;* the pathogen appears to grow unimpeded in the needles of both susceptible and resistant phenotypes.

BIBLIOGRAPHY:

Bingham, RT. 1983. *Blister rust resistant western white pine for the Inland Empire: the story of the first 25 years of the research and development program*. USDA For. Serv. Intermt. Res. Stn. Gen. Tech. Rep. Int-146 Ogden Utah.

Bingham, RT, Olson, RJ, Becker, WA, and Marsden, MA. 1969. Breeding Blister Rust Resistant Western White Pine. V. Estimate of heritability, combining ability, and genetic advance based on tester matings. *Silvae Genet*.**18**:23–38.

Brown, I, Trethowan, J, Kerry, M, Mansfield, J, and Bolwell, PG. 1998. Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of Xanthomonas campestris and French bean mesophyll cells. *Plant J*.**15**:333–343.

Dangl, JL and Jones, JD. 2001. Plant pathogens and integrated defence responses to infection. *Nature***411**:826-833.

Deising, H, Rauscher, M, Haug, M, and Heiler, S. 1995. Differentiation and cell wall degrading enzymes in the obligately biotrophic rust fungus Uromyces viciae-fabae. *Can. J. Bot.*73:624–631.

Flor, HH. 1942. Inheritance of pathogenicity in Melmapsora lini. *Phytopathology***32**: 653-669.

Fujikawa, T, Sakaguchi, A, Nishizawa, Y, Kouzai, Y, Minami, E, Yano, S, Koga, H, Meshi, T, and Nishimura, M. 2012. Surface α-1,3-Glucan Facilitates Fungal Stealth Infection by Interfering with Innate Immunity in Plants. *PLoS Pathog*.**8**,

Geils, BW, Hummer, KE, and Hunt, RS. 2010. White pines, Ribes, and blister rust: a review and synthesis. *For. Pathol*.**40:**147–185.

Gernandt, D. S., Lopez, G. G., Ortiz Garcia, S., and Liston, A. 2005. Phylogeny and classificaton of Pinus. Taxon 54:29-42.

Gilchrist, DG. 1998. Programmed cell death in plant disease: the purpose and promise of cellular suicide. *Annu. Rev. Phytopathol*.**36**:393–414.

Goodman RN, Novacky AJ.1994. The hypersensitive reaction in plants to pathogens. St Paul, MN: APS Press.

Hahn, M, and Mendgen, K. Signal and nutrient exchange at biotrophic plant-fungus interfaces. Curr. Opin. *Plant Biol*.**4**:322–327.

Hammond-Kosack, KE, and Jones, JD. 1997. Plant disease resistance genes. *Annu. Rev. Plant Biol.***48**:575–607.

Harvey, AE, Byler, JW, McDonald, GI, Neuenschwander, LF, and Tonn, JR. 2008. *Death of an Ecosystem: Perspectives on Western White Pine Ecosystems of North America at the End of the Twentieth Century.* USDA For. Serv. Rocky Mt. Res. Stn. Gen. Tech. Rep. Rmrs-Gtr-208.

Heath, MC. 2000. Hypersensitive response-related death. *Plant Mol. Biol.***44**:321–334.

Hiratsuka, Y and Sato, S. 1982. Morphology and taxonomy of rust fungi. 1-36 in: The Rust Fungi. Scott, KJ and Chakravorty, AK, eds. Academic Press, London.

Hoff, RJ, and McDonald, GI. 1980. Resistance to Cronartium ribicola in Pinus monticola: reduced needle-spot frequency. *Can. J. Bot.***58**: 574–577.

Howard, TE. 1986. The Lore and Lure of Eastern White Pine. General Technical Report – WO*, US Department of Agriculture Forest Service***51**:10-15.

Hummer, KE. 2000. History of the origin and dispersal of white pine blister rust. *HortTechnology***10**:515–517.

Hunt, RS. 2009. History of western white pine and blister rust in British Columbia. *For. Chron.***85**:516–520.

Innes, RW. 1998. Genetic dissection of R gene signal transduction pathways. *Curr. Opin. Plant Biol.*1:299–304.

Jacobs, JJ, Burnes, TA, David, AJ, and Blanchette, RA. 2009. Histopathology of primary needles and mortality associated with white pine blister rust in resistant and susceptible *Pinus strobus*. *For. Pathol*.**39**:361–376.

Jones, JDG and Dangl, JL. 2006. The plant immune system. *Nature***444**:323–329.

King, JN, David, A, Noshad, D, and Smith, J. 2010. A review of genetic approaches to the management of blister rust in white pines. *For. Pathol*.**40**:292–313.

Kinloch, BB. 2003. White pine blister rust in North America: past and prognosis. *Phytopathology***93**:1044–1047.

Kinloch, BB, and Comstock, M. 1980. Cotyledon test for major gene resistance to white pine blister rust in sugar pine. *Can. J. Bot.***58**:1912–1914.

Kinloch, BB, Davis, DA, and Burton, D. 2007. Resistance and virulence interactions between two white pine species and blister rust in a 30-year field trial. *Tree Genet. Genomes***4**:65–74.

Kinloch, BB, and Dupper, GE. 2002. Genetic specificity in the white pine-blister rust pathosystem. *Phytopathology***92**:278–280.

Kinloch, BB, and Littlefield, JL. 1977. White pine blister rust: hypersensitive resistance in sugar pine. *Can. J. Bot*.**55**:1148–1155.

Kinloch, BB, Parks, GK, and Fowler, CW. 1970. White Pine Blister Rust: Simply Inherited Resistance in Sugar Pine. *Science***167**:193–195.

Kinloch, BB, Sniezko, RA, Barnes, GD, and Greathouse, TE. 1999. A major gene for resistance to white pine blister rust in western white pine from the western Cascade Range. *Phytopathology***89**:861–867.

Kinloch, BB, Sniezko, RA, and Dupper, GE. 2003. Origin and distribution of Cr2, a gene for resistance to white pine blister rust in natural populations of western white pine. *Phytopathology***93**:691–694.

Klebahn, H. Bemerkung uber den Weymouthkieferrost. *Abhandlungen herausgegeben von Naturwissenschaftlichen Vereine zu Bremen*. **10**:427-428.

Kriebel, HB. 1972. White pines in North and Central America: *Pinus Strobus* and introduced Asian and European species. In Biology of rust resistance in forest trees: Proceedings NATA-IUFRO Advanced Study Institute, 17-24 August 1969, Moscow, ID. Misc. Publ. **1221**, USDA Forest Service. 201-214.

Kriebel, HB. 2004. Genetics and breeding of five-needled pines in the eastern United States. In:*Breeding and genetic resources of five-needle pines : Growth, adaptability and pest resistance*, IUFRP Working Party 2.02.15, Medford, OR, USA; 23-27 July 2001. Ed. by Sniezko, Richard A.; Samman, Safiya; Schlarbaum, Scott E.; Kriebel, Howard B. eds. 2004. *Breeding and genetic resources of five-needle pines: growth, adaptability, and pest resistance*; 2001 July 23-27; Medford, OR, USA. IUFRO Working Party 2.02.15. Proceedings RMRS-P-32. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 259 p.

Lamb, C and Dixon, RA. 1997. The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol***4**8:251–275.

Little, E. L. Jr. and Critchfield, W. B. 1969. Subdivisions of the Genus *Pinus* (Pines). U.S.D.A. Forest Service Miscellaneous Publication 1144, Washington, D.C.

Liu, JJ, and Ekramoddoullah, AKM. 2004. Isolation, genetic variation and expression of TIR-NBS-LRR resistance gene analogs from western white pine (Pinus monticola Dougl. ex. D. Don.). *Mol. Genet. Genomics***270**: 432–441.

Maloy, OC. 1997. White pine blister rust control in North America: a case history. *Annu. Rev. Phytopathol*.**35**:87–109.

Matern, U, Grimmig, B, and Kneusel, RE. 1995. Plant cell wall reinforcement in the disease-resistance response: molecular composition and regulation. *Can. J. Bot*.**73**:511–517.

McDonald, GI, Hansen, EM, Osterhaus, CA, and Saman, S. 1984. Initial characterization of a new strain of Cronartium ribicola from the Cascade Mountains of Oregon. Plant Dis. **68**(9):800-804.

McDonald, G, Zambino, P, and Sniezko, R. 2004. Breeding rust-resistant five-needle pines in the western United States: lessons from the past and a look to the future. *Breed. Genet. Resour. Five-Needle Pines Growth Adapt. Pest Resist. Iufro Work. Party***2**:23–27.

McDonald, GI, Richardson, BA, Zambino, PJ, Klopfenstein, NB, and Kim, MS. 2006. Pedicularis and Castilleja are natural hosts of Cronartium ribicola in North America: a first report. *For. Pathol*.**36**:73–82.

Mirov, NT. 1967. *The genus Pinus* (Ronald Press Co.).

Moerschbacher, BM, Mierau, M, Graeßner, B, Noll, U, and Mort, AJ. 1999. Small oligomers of galacturonic acid are endogenous suppressors of disease resistance reactions in wheat leaves. *J. Exp. Bot*.**50**:605–612.

Morel, JB and Dangl, JL. 1997. The hypersensitive response and the induction of cell death in plants. *Cell Death Differ*.**4**:671–683.

Mulvey RL, Hansen EM. 2011. Castilleja and Pedicularis confirmed as telial hosts for *Cronartium ribicola* in whitebark pine ecosystems of Oregon and Washington. *Forest Pathology*. **41**(6):453-463.

Patton, RF. 1961. The effect of age upon susceptibility of Eastern White Pine to infection by Cronartium ribicola. *Phytopathology***51**(7):429-34.

Patton, RF and Riker, AJ 1966. Lessons from nursery and field testing of eastern white pine selections and progenies for resistance to blister rust, p.403-414. In H.D. Gerhold, et al., *Breeding pest-resistant trees*. Pergamon Press, Oxford. 505p.

Pellinen, RI. 2002. Hydrogen Peroxide Activates Cell Death and Defense Gene Expression in Birch. *Plant Physiol*.**130**:549–560.

Price, RA, Liston, A and Strauss, SH. 1998. Phylogeny and systematics of *Pinus*. P.49-68 in Richardson, D.M. (ed.), *Ecology and Biogeography of Pinus*. Cambridge University Press.

Priyamvada, MS and Tiwari, R. 2011. Priyamvada et al.pdf. *Int. J. Genet. Mol. Biol*.**3**: 108–114.

Rabenhorst, GL. 1872. *Fungi Europaei Exsiccati.Cent. XIV*.

Radu, S, Blada, I, King, J, and Sniezko, R. 2008. Pinus strobus: past and future in Europe. A page of silvicultural history and international scientific cooperation. *Ann. For. Res.***51:**133–140.

Richardson, BA, Klopfenstein, NB, Zambino, PJ, McDonald, GI, Geils, BW, and Carris, LM. 2008. Influence of host resistance on the genetic structure of the white pine blister rust fungus in the western United States. *Phytopathology***98**:413–420.

Riker, AJ, Kouba, TF. 1940. White pine selected in blister rust areas. *Phytopathology* **30**(1): 20.

Riker, AJ, Kouba, TF, Brener, WH, Byam, LE. 1943. White pine selections tested for resistance to blister rust. *Journal of Forestry***41**(10):753–760.

Schoettle, AW, Sniezko, R, Kegley, A, Burns, KS. 2013. White pine blister rust in limber pine: evidence for a major gene. *Phytopathology*. In press.

Schwandt, JW, Lockman, IB, Kliejunas, JT, and Muir, JA. 2010. Current health issues and management strategies for white pines in the western United States and Canada. *For. Pathol*.**40**:226–250.

Shaw, G. R. 1914. The Genus *Pinus*. Publications of the Arnold Arboretum No. 5. The Riverside Press, Cambridge, Massachussets.

Sniezko, RA. 1996. Developing resistance to white pine lister rust in sugar pine in Oregon. In:*Sugar pine: Status, values, and roles in ecosystems*, Davis, CA March 30-April 1. Ed. by Kinloch, B.B., JR;Marosy, M.; Huddleston, M.E. 171–178.

Sniezko, RA. 2006. Resistance breeding against nonnative pathogens in forest trees—current successes in North America. *Can. J. Plant Pathol*.**28**:S270–S279.

Sniezko, RA, Mahalovich, MF, Schoettle, AW, and Vogler, DR. 2011. Past and current investigations of the genetic resistance to Cronartium ribicola in high-elevation five-needle pines. Keane Robert E Tomback Diana F Murray Michael P Smith Cindy M Eds Future High-Elev. Five-Needle White Pines West. North Am. Proc. High Five Symp. Proc Rmrs-P-63 Ft Collins Co Us Dep. Agric. For. Serv. Rocky Mt. Res. Stn. 246–264.

Sniezko, RA, Kegley, AJ, and Danchok, RS. White Pine Blister Rust Resistance in Pinus monticola and P. albicaulis in the Pacific Northwest U.S. –A Tale of Two Species.

Sniezko, RA, Yanchuk, AD, Kliejunas, JT, Palmieri, KM, Alexander, JM, Frankel, SJ. tech. coords. 2012. Proceedings of the fourth international workshop on the genetics of host-parasite interactions in forestry: Disease and insect resistance in forest trees. Gen. Tech. Rep. PSW-GTR-240. Albany, CA: Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture. 372 p.

Spaulding, PC. 1911. The blister rust of white pine. US Bur. Plant Indus. Bul. 206.

Spaulding, PC. 1929. White pine blister rust: A comparison of European with North American conditions. USDA Tech. Bul. 87:58.

Thordal-Christensen, H, Zhang, Z, Wei, Y, and Collinge, DB. 1997. Subcellular localization of H202 in plants. H202 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. *Plant J*.**11**:1187–1194.

Van Arsdel, EP. 2011. Blister Rust in North America: What we have not learned in the Past 100 Years. Fairweather Mj Palacios Pproceedings 58th Annu. West. Int. For. Dis. Work Conf. 2010 Oct. 4-8 Val. Bc Flagstaff Az Us Dep. Agric. For. Serv. Az Zone For. Heal. 61–69.

Van Doorn, WG. 2011. Classes of programmed cell death in plants, compared to those in animals. *J. Exp. Bot.***62**:4749–4761.

Vargas, WA, Martin, JMS, Rech, GE, Rivera, LP, Benito, EP, Diaz-Minguez, JM, Thon, MR, and Sukno, SA. 2012. Plant Defense Mechanisms Are Activated during Biotrophic and Necrotrophic Development of Colletotricum graminicola in Maize. *PLANT Physiol*. **158**:1342–1358.

Vogler, DR, Delfino-Mix, A, and Schoettle, AW. 2005. *White pine blister rust in high-elevation white pines: screening for simply-inherited, hypersensitive resistance*. In 53rd Annual Western International Forest Disease Work Conference, Jackson, WY, 26–30 p.

Zambino, PJ, and McDonald, GI. 2003. *Resistance to white pine blister rust in North American fiveneedle pines and Ribes and its implications*. In 51st Western International Forest Disease Work Conference, 18–22 p.