

Histopathology of primary needles and mortality associated with white pine blister rust in resistant and susceptible *Pinus strobus*

By J. J. JACOBS^{1,*}, T. A. BURNES¹, A. J. DAVID² and R. A. BLANCHETTE^{1,3,*}

¹Department of Plant Pathology, University of Minnesota, St. Paul, MN, 55108-6030, USA; ²North Central Research and Outreach Center, University of Minnesota, Grand Rapids, MN, 55744, USA;

³E-mail: robertb@umn.edu (for correspondence)

Summary

White pine blister rust caused by *Cronartium ribicola* is a damaging non-native disease of five-needled pines in North America. Efforts to control the disease and mitigate damage to date have been only somewhat effective. Recent efforts to improve the health of eastern white pine and reestablish the tree as a dominant species in the North Central United States have focused on identification and propagation of disease-free eastern white pine (*Pinus strobus*) growing in areas with a high incidence of blister rust. Many of these selections have been shown to resist infection following artificial inoculation with *C. ribicola*. In this study, 13 eastern white pine families derived from controlled pollination of selections previously determined to possess putative resistance as well as susceptible selections were inoculated with *C. ribicola*. Mortality data from inoculation studies show superior survivability in three families with over 60% of seedlings able to survive the 52 week post-inoculation monitoring period compared to 0–10% survival of the most susceptible families. Primary needles were collected for histological analysis from all inoculated families 4 weeks after inoculation and from selected families 6.5 weeks and 38 weeks after inoculation. Histological observations of infection sites show distinct resistance reactions in the families more likely to survive infection based on mortality data. Analysis of the reactions in susceptible families revealed extensive hyphal colonization of the vascular bundle and adjacent mesophyll cells that appear uninhibited by tree responses. In resistant families, collapsed cells adjacent to infection sites, heavy deposition of phenolic compounds and abnormal cell growth were documented more frequently and appear to play an integral role in the ability of these eastern white pine families to impede growth of *C. ribicola* in primary needle tissue.

1 Introduction

White pine blister rust, an introduced fungal disease of 5-needled pines (*Pinus* subgenus *Strobus*) caused by *Cronartium ribicola* J.C. Fischer, has caused extensive damage throughout North America (PATTON 1967; KINLOCH and LITTLEFIELD 1977; MALOY 1997; KINLOCH 2003). The fungus was introduced to both coasts at the beginning of the 20th century and has since spread from these initial introduction sites to impact nearly the entire range of 5-needled pines in North America (STEWART 1906; MALOY 1997; KINLOCH 2003).

Efforts to effectively control white pine blister rust began with the initial eradication of wild and cultivated *Ribes* spp. and quarantine of cultivated varieties during the first half of the 20th century. Application of antibiotic compounds, various silvicultural techniques such as pruning the lower one-third of the tree and planting on low hazard sites, as well as selective breeding programs have been employed to mitigate damage caused by the disease with mixed results (KINLOCH 2003). Of these, silvicultural methods of control and breeding programmes have the most potential for success and are currently being utilized (MALOY 1997; KINLOCH 2003).

Received: 27.6.2008; accepted: 19.11.2008; editor: S. Woodward

*Corrections added after publication 28 April 2009. The authors' names have been corrected to J. J. Jacobs and R. A. Blanchette.

Breeding programmes focused on resistance to *C. ribicola* generally use collections made from apparently uninfected pine growing in areas of high disease incidence. For eastern white pine, many collections were made in the mid-20th century by Dr Robert Patton of the University of Wisconsin-Madison and others from locations in the upper Midwest and Canada. Many of these selections were grafted onto eastern white pine stock and subsequently planted at the U.S. Forest Service Oconto River Seed Orchard (ORSO) in White Lake, WI and other locations.

Previous work has been conducted to identify growth patterns of *C. ribicola* in pine tissues (COLLEY 1917, 1918; STRUCKMEYER and RIKER 1951; WATERMAN 1955; KREBILL 1968), resistance mechanisms in eastern white pine needles (BOYER and ISAAC 1964; BOYER 1966; JURGENS et al. 2003; SMITH et al. 2006a,b) and resistance mechanisms in stem tissues of *P. strobus* (STRUCKMEYER and RIKER 1951; BOYER 1966). Multiple mechanisms of resistance previously reported in white pine blister rust pathosystems suggest that the partial resistance observed in many of the 5-needled pines to *C. ribicola* is polygenic (KINLOCH 1972; SMITH et al. 2006a,b).

Histological techniques have been employed to elucidate the infection process in eastern white pine (COLLEY 1917, 1918; CLINTON and McCORMICK 1919; STRUCKMEYER and RIKER 1951; WATERMAN 1955; BOYER and ISAAC 1964; KREBILL 1968) and to characterize resistance mechanisms present in secondary needles of putative resistant trees (JURGENS et al. 2003), however, more work is needed to fully understand these processes.

In the most recent research conducted by JURGENS et al. (2003), four distinct interactions were observed in secondary needles of open-pollinated *P. strobus* seedlings. One type of needle reaction was characteristic of needle responses to infection by *C. ribicola* in susceptible open-pollinated selections and was similar to observations made by CLINTON and McCORMICK (1919). The three other needle reactions were classified as different resistance responses in selections able to survive for an extended period of time following greenhouse inoculation. Collapsed mesophyll cells bordering infection sites, hyperplastic and hypertrophic cell growth as well as increased deposition of polyphenolic compounds were documented and likely had a role in the increased survival rate of these 'resistant' families when compared with inoculated seedlings from more susceptible families (JURGENS et al. 2003).

Recently, seed from controlled pollinations of *P. strobus* selections established at ORSO became available and seedlings were used for greenhouse inoculation studies with *C. ribicola*. The investigations reported here were done to evaluate probable resistance mechanisms present in the primary needles of these seedling families and to compare results with previously documented infection reactions in needle tissues of open-pollinated *P. strobus* using a variety of histological techniques.

2 Materials and methods

2.1 Seed sources and seedling growth conditions

Eastern white pine seed produced from 13 controlled cross pollinations of previously selected clonally propagated putative resistant trees were obtained from ORSO. Selections carrying a 'P' prefix denote trees originally collected in Minnesota and Wisconsin by Dr Robert Patton of the University of Wisconsin during his early work on the interactions between *C. ribicola* and *P. strobus*. Selections with an 'H' prefix were collected in the 1960s by foresters from two counties in Wisconsin (JURGENS et al. 2003). Information on selection ON469 is limited and the origin is not known (C. Sweeney, personal communication). All prior collections of known origin (selections with either a 'P' or 'H' prefix) were made from apparently uninfected trees existing on sites with high incidences of white pine blister rust infection.

Seed was surface sterilized with a 10% bleach solution for 15 s, rinsed thoroughly and soaked in water for 24 h before being placed in Petri dishes lined with moist filter paper. The Petri dishes were sealed with parafilm, placed in a plastic bag and stratified for 30 days at 4°C. Following stratification, individual seeds were planted in 2.5 cm × 15 cm 66 ml cones using Sun Gro™ Sunshine SB500 (Vancouver, BC, Canada) high porosity growth mix. Seedlings were grown in the greenhouse (21–32°C) with additional light added to give a 12-h photoperiod. Fertilization was carried out monthly using a liquid drench of Peters Professional water soluble 14-14-14 fertilizer (Scotts, Marysville, OH, USA).

2.2 Inoculation methods

Approximately 35–45 seedlings from each family studied were randomly placed within cone racks before inoculation using a modified protocol similar to the procedure outlined in JURGENS et al. (2003). Briefly, the underside of *Ribes nigrum* leaves approximately 6–7 cm in diameter were inoculated with urediniospores of *C. ribicola* strain W14.1B suspended in water using a hand held sprayer (JURGENS et al. 2003). Following inoculation, whole *R. nigrum* plants were placed into plastic bags and incubated for 24 h at 20°C. After 24 h, the plants were removed from the plastic bags and left in the incubation chamber at 20°C. Approximately 1 week after inoculation, uredinial pustules were abundant and the temperature in the chamber was reduced to 17°C until telial columns developed approximately 4–5 weeks later. Following telia development, infected leaves were suspended above 13-week-old eastern white pine seedlings in sealed moisture chambers kept at 100% humidity and placed within temperature controlled incubation chambers at 18°C. Telia were allowed to germinate and a count of basidiospores cast over the seedlings was taken using 1 cm × 1 cm water agar plugs placed randomly throughout the moisture chambers. The plugs were retrieved and basidiospores counted using a compound microscope equipped with a calibrated grid. Once a predetermined basidiospore threshold was reached (approximately 6000 basidiospores/cm²), the *R. nigrum* leaves were removed and seedlings were kept in the incubation chambers at 21°C and 100% humidity for a further 72 h. Following removal from the moisture chambers, seedlings were grown under original greenhouse conditions. Approximately 10 non-inoculated control seedlings from each family were subjected to all inoculation conditions without suspended *R. nigrum* leaves.

2.3 Monitoring infection and mortality

Seedlings were monitored after inoculation for the appearance of chlorotic needle spots characteristic of infection with *C. ribicola* (CLINTON and McCORMICK 1919; HIRT 1939; JURGENS et al. 2003). Previous observations of inoculated *P. strobus* seedlings have shown that needle spotting can be observed as early as 2 weeks after inoculation but becomes more obvious macroscopically at approximately 4 weeks after inoculation. Following verification of inoculation success, groups of 10–15 seedlings from 12 of the 13 crosses were randomly separated from the remaining seedlings for use in histological analyses. Interactions in family H109 × H109 were not documented histologically because of an insufficient number of seedlings and the need to collect mortality data. The remaining seedlings from each of the crosses were used to assess differences in needle spot size, spot frequency and overall mortality for 52 weeks.

2.4 Statistical analyses

Mortality data indicating seedling family survivability were analysed using the Bonferroni-adjusted chi-square for multiple comparisons (JONES 1984). For all analyses, $\alpha = 0.05$.

Tissue colonization measurements were analysed using two-way ANOVA with time and family as the main effects.

2.5 Sample processing

Samples of infected primary needles were collected from seedlings with replacement from each family 4 weeks after inoculation followed by an additional collection from selected families 6.5 weeks after inoculation. At 38 weeks post-inoculation, additional collections were made from individual needle spots that were still discernible on families P327 × P327, P327 × ON469 and H111 × H111. All collected samples consisted of individual needle spots (based on macroscopic examination) which presumably encompassed an area of the needle where symptoms developed following infection by a single basidiospore. Excised needle samples were immediately placed in a solution of formalin–acetic acid–alcohol (FAA) to fix the tissues for further analysis. Samples were then processed through an ethyl alcohol/tert-butyl alcohol dehydration series and embedded in paraffin wax for processing using a protocol adapted from previously published work (RUZIN 1999; JURGENS et al. 2003).

Needle samples were cut longitudinally into serial sections using a manual rotary microtome set to excise sections at an 8–10 µm thickness. Following sectioning, individual sections were cut from ribbons and floated on a water bath containing de-ionized water heated to 48–50°C for approximately 10 min or until any noticeable wrinkles in the section were removed. Sections were presorted in the water bath to be laid out in a serial manner with 6–8 sections in each group and were then transferred from the water bath to Fischer Super Frost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA) before being placed on a slide warming plate to dry.

2.6 Staining and imaging

Prior to removal of paraffin and staining of sections, slides containing serial sections were placed in flat, covered slide holders and heated in an oven set just above the melting point of the paraffin wax used for embedding (58°C). Sections were kept in the oven for approximately 10 min or until the excess paraffin around the needle sections began to melt. This step significantly increased tissue adhesion to the slides without deterioration of needle structure. Two histological stains were utilized in this study. One stain, periodic acid-Schiff has been traditionally used as a specific stain for carbohydrates containing 1–2 glycols, giving them a reddish/purple colour when viewed with standard light microscopy (GAHAN 1984). The stain has been shown to be a specific for fungi in plant material and has recently been used to observe *C. ribicola* in eastern white pine secondary needle tissue (DRING 1955; JURGENS et al. 2003). The other stain, phloroglucinol-HCl, was used to differentiate tissues containing polyphenolic compounds, resulting in these compounds staining a brilliant red (GAHAN 1984; RUZIN 1999; JURGENS et al. 2003). Images were captured for further analysis using a Nikon DXM1200F digital camera mounted to a Nikon Eclipse E600 microscope and collected using Nikon ACT-1 imaging software (Nikon, Tokyo, Japan).

2.7 Measurements of tissue colonization

All infected needle samples collected 4 and 6.5 weeks after inoculation from families P327 × P327, H111 × H111 and H109 × H111 were measured to quantify any differences that may be present in the average size of the characteristic condensed mass of mycelium associated with needle infection by *C. ribicola*. Longitudinal sections were chosen for measurement by selecting the section containing the condensed mass at its largest point

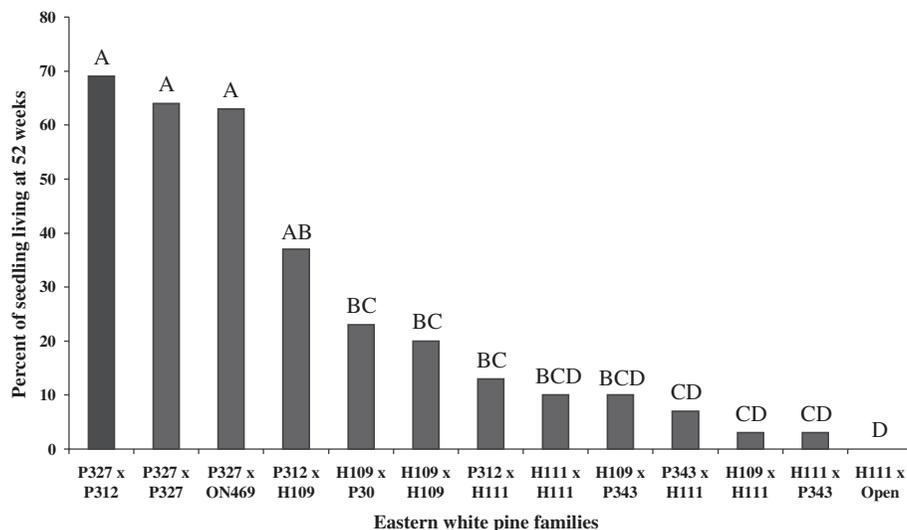


Fig. 1. Percent of eastern white pine seedling families surviving 52 weeks after inoculation with *Cronartium ribicola*. Bars with same letters are not significantly different (Bonferroni-adjusted chi-square test, $p > 0.05$).

closest to the centre of the needle (based on observed width of the vascular bundle). The condensed masses of hyphae were measured parallel and perpendicular to the vascular bundle at $100\times$ magnification using an ocular micrometer. When more than one infection was present in the section, the two condensed masses were measured and the average used in the comparisons.

3 Results

3.1 Seedling mortality

Mortality data, when compared as a percent of total seedlings living at the end of the 52-week monitoring period, clearly show families P327 \times P312, P327 \times P327 and P327 \times ON469 had superior resistance to infection by *C. ribicola* with more than 60% of the seedlings inoculated surviving (Fig. 1). Within the more susceptible families tested, only crosses containing selection H109 had $>20\%$ survival at the end of the study. The most susceptible families studied in this experiment were crosses made with selection H111. Prior inoculations involving this selection have shown it to be highly susceptible to infection by *C. ribicola* (HUNT and MEAGHER 1989; JURGENS et al. 2003). In the experiments reported here, an open-pollinated cross with selection H111 did not have any seedlings that survived the 52-week monitoring period (Fig. 1).

3.2 Histological observations in susceptible families

Reactions observed within needles from crosses which can be considered susceptible based on mortality data were varied (Table 1). Large condensed masses of hyphae were present within and around the vascular bundle (Fig. 2a,b) in many samples from these crosses. At 4 weeks post-inoculation, the condensed mass was smaller than those previously described in earlier reports of older needle infections (Fig. 2c,d) (CLINTON and MCCORMICK 1919;

Table 1. Comparison of relative abundance of mesophyll collapse and phenolic response in primary needles from different eastern white pine families after inoculation with *Cronartium ribicola*¹.

Seedling family	Mesophyll collapse	Phenolic response
P327 × P312 (R)	–	High
P327 × P327 (R)	+	High
P327 × ON469 ² (R)	–	Medium
H109 × H111 (S)	+	High
H111 × P343 (S)	–	Medium
H111 × H111 (S)	–	High
H111 × Open (S)	–	Low

¹Plus and minus designations signify the presence or absence of collapsed mesophyll cells after staining with periodic acid-Schiff and phloroglucinol-HCl. Gradients from high to low phenolic response signify relative intensity and abundance of phenolic reactions observed in response to infection after phloroglucinol-HCl staining. Seedling families followed by an R or S are classified as resistant or susceptible, respectively, based on mortality data.

²When samples from family P327 × ON469 were analysed 38 weeks after inoculation extensive cellular collapse was observed associated with macroscopically characteristic *C. ribicola* infection spots.

JURGENS et al. 2003). Reactions of this type often exhibited fungal hyphae and haustoria spreading within the vascular bundle outward from the established infection centre (Fig. 2b). Another type of reaction found frequently in the more susceptible crosses 4 weeks after inoculation was that of sparse hyphae spreading into the mesophyll. These hyphae often colonized a large area of the needle without the condensed mass of mycelia commonly associated with *C. ribicola* infection in susceptible tissues (CLINTON and McCORMICK 1919; JURGENS et al. 2003). Phenolic deposition was present in needle reactions from many of the susceptible families but the level of deposition varied considerably. Samples collected 4 weeks after inoculation from the most susceptible families often exhibited a weak phenolic reaction which did not appear to limit fungal growth (Fig. 2a,d).

Extensive colonization of infected susceptible needles was observed 6.5 weeks after inoculation (Fig. 3). In selection H111 × H111, hyphae were observed spreading within and along the edges of the vascular bundle apparently uninhibited by host responses (Fig. 3a). When areas of these needles not showing a condensed mass of hyphae were analysed, fungal structures were observed intermittently within the vascular bundle (Fig. 3b). The intense staining with phloroglucinol-HCl observed in many infections from this cross indicated deposition of phenolic compounds had occurred and completely surrounded the condensed mycelial mass (Fig. 3c). Although staining with phloroglucinol-HCl had apparently increased from 4 to 6.5 weeks post-inoculation, the phenolic compounds appeared unable to limit fungal growth (Figs 2a,d and 3c).

3.3 Histological observations in resistant families

Morphologically distinct reactions within needles were observed in crosses that were more likely to survive following inoculation, but varied between the resistant families (Table 1). In family P327 × P312, infection sites observed 4 weeks after inoculation typically had an elongated appearance extending outward from the infection centre along the edge of and within the vascular bundle (Fig. 4a,b). This elongated fungal mass was often bordered by a relatively intense reaction to phloroglucinol-HCl indicating that phenolic compounds had

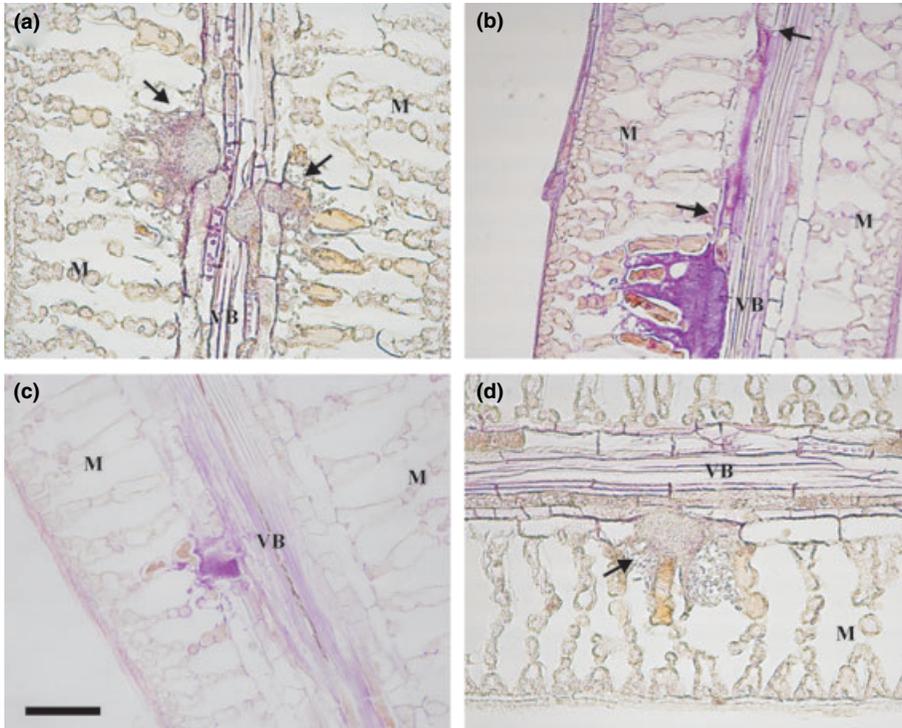


Fig. 2. Needle reactions observed in susceptible eastern white pine families 4 weeks after inoculation with *C. ribicola* showing unrestricted colonization and cellular disruption. Tissue labels: M, mesophyll; VB, vascular bundle. (a) Extensive colonization associated with highly susceptible families. Hyphae completely colonize the vascular bundle and have spread into adjacent mesophyll cells. Deposition of phenolic compounds (arrows) was observed surrounding the infection. (b) Established infections that are restricted in size from susceptible families often had condensed masses of hyphae within the vascular bundle (arrows showing extent of hyphal colonization). (c) Small condensed mycelial masses occasionally observed on the edge of the vascular bundle with minimal hyphae spreading into adjacent mesophyll. This type of reaction was often associated with observations of haustoria and hyphae within the vascular bundle spreading long distances from the established infection. (d) Weak phenolic deposition (arrow) surrounding an infection that is somewhat sparse along the edge of the vascular bundle with light pink color characteristic of a weak reaction. (a and d) Stained with phloroglucinol-HCl. (b and c) Stained with periodic acid-Schiff. (a and b) H111 \times P343. (c and d) H111 \times Open. Bar = 200 μ m for all micrographs.

been deposited along the periphery of the infection site (Fig. 4b). Needle samples collected 6.5 weeks after inoculation from family P327 \times P312 did not show appreciable differences in hyphal colonization when compared with needle reactions collected 4 weeks after inoculation.

Needle colonization in family P327 \times P327 appeared restricted 4 weeks after inoculation and varied from somewhat elongate and compact to a more rounded appearance with very little hyphae spreading into the mesophyll (Fig. 4c,d). Collapse of mesophyll cells near the infection site was evident in some of the needle samples collected from P327 \times P327 seedlings (Fig. 4e). When these collapsed cells were present in the needle they were often associated with the edges of the condensed mycelial mass, presumably serving as a barrier to hyphal growth. In addition, collapsed mesophyll cells were occasionally observed on the

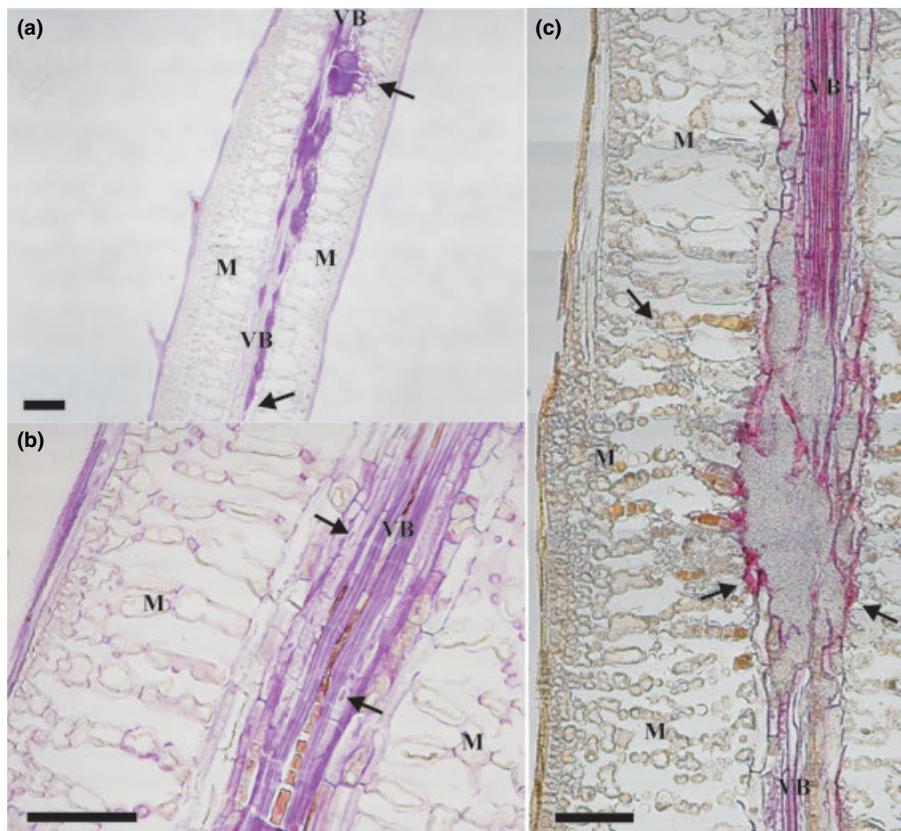


Fig. 3. Needle reactions observed in susceptible eastern white pine family H111 \times H111 6.5 weeks after inoculation with *C. ribicola*. Tissue labels: M, mesophyll; VB, vascular bundle. (a) A large area of fungal colonization within the vascular bundle. Lower arrow shows edge of condensed fungal growth. Upper arrow identifies likely site of initial infection. (b) Sparse haustoria and hyphal structures (arrows) near edge of collected sample away from the original site of infection. (c) Composite image of infection site showing apparently ineffective heavy deposition of phenolic compounds (arrows) surrounding the condensed mycelia almost exclusively colonizing the vascular bundle. (a and b) Stained with periodic acid-Schiff. (c) Stained with phloroglucinol-HCl. Bars = 200 μ m.

opposite side of the vascular bundle from the infection site (Fig. 4c). The collapsed mesophyll reaction was found in two of the eastern white pine families examined but occurred infrequently (Table 1). The collapse of cells observed within needles was not always associated with established infections. In this reaction, the collapsed cells were found just below the stomata and appeared to coincide with *C. ribicola* infection sites (Fig. 4f). However, upon histological analysis of the entire sample, no hyphae were observed in needle tissues. Analysis of sections taken from needles of family P327 \times P327 6.5 weeks after inoculation indicated the condensed mass of hyphae present along and within the vascular bundle was only slightly larger than in the same family 4 weeks after inoculation (Figs 4c and 5a). The main difference observed between reactions at 4 and 6.5 weeks was a very intense staining with phloroglucinol-HCl surrounding the condensed mycelial mass present in the 6.5-week samples. This staining was noticeably brighter than

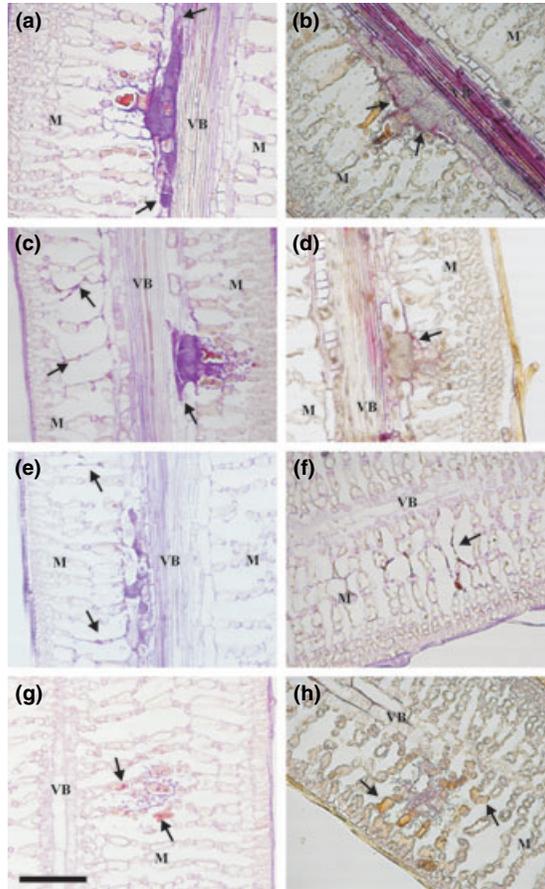


Fig. 4. Reactions observed in resistant eastern white pine families 4 weeks after inoculation with *C. ribicola* showing a localized cellular response. Tissue labels: M, mesophyll; VB, vascular bundle. (a) Localized infection with minimal spread of hyphae into mesophyll tissue. Arrows indicate extent of hyphal colonization of vascular bundle. (b) Section from the same infection as in 5A but stained with phloroglucinol-HCl, arrows indicate intense phenolic deposition. (c) Compact infection on edge of vascular bundle with limited spread of fungal hyphae, extensive mesophyll collapse indicated by arrows on opposite side of vascular bundle from infection. (d) Section from same infection as in 5C but stained with phloroglucinol-HCl, arrow indicates positive reaction for phenolic compounds associated with the zone immediately around the condensed fungal mass. (e) Collapsed cells (arrows) bordering infection area on the edge of the vascular bundle. (f) Collapse of mesophyll cells (arrow) related to stomatal openings but not associated with condensed hyphae. (g) Copious haustoria were observed in many reactions with spreading hyphae often bordered by cells filled with hyphae and haustorial structures, arrows designate cells with haustoria and hyphae. (h) Tan occluded cells (arrows) were observed surrounding infection sites and appear to delimit the edge of fungal growth. (a, c, e, f and g) Stained with periodic acid-Schiff. (b, d and h) Stained with phloroglucinol-HCl. (a and b) P327 × P312. (c–f) P327 × P327. (g and h) P327 × ON469. Bar = 200 μm for all micrographs.

the staining observed in the reactions from needles 4 weeks after inoculation and indicates that phenolic compound deposition had increased (Figs 4d and 5c). Some distortion of transfusion cells within the vascular bundle was also evident in many samples apparently as a result of fungal growth.

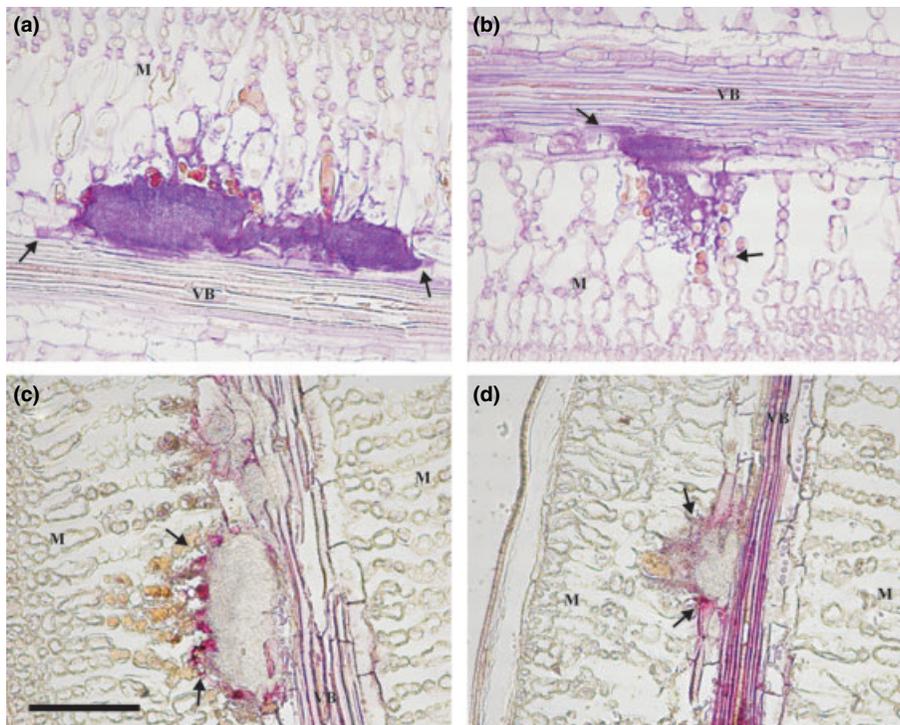


Fig. 5. Needle reactions observed in resistant eastern white pine families 6.5 weeks after inoculation with *C. ribicola*. Tissue labels: M, mesophyll; VB, vascular bundle. (a and b) Sections through approximate center of two needle infections showing the extent of the condensed mycelial mass (arrows). Only minimal hyphae were observed in the adjacent mesophyll cells. (c) Same infection as in (a) but stained with phloroglucinol-HCl indicating extensive deposition of phenolic compounds (arrows) around infection site. (d) Same infection as in (b) stained with phloroglucinol-HCl, indicating deposition of phenolic compounds (arrows) that appeared to effectively restrict growth of hyphae within needle. (a and b) Stained with periodic acid-Schiff. (b) P327 × P312. (a, c and d) P327 × P327. Bar = 200 μ m for all micrographs.

Although family P327 × ON469 was able to tolerate infection by *C. ribicola* and was more likely to survive than susceptible crosses, it did not exhibit a reaction similar to P327 × P312 or P327 × P327 (Table 1). Characteristic reactions observed in this family consisted of relatively small areas of infection along the vascular bundle with hyphae often spreading into adjacent mesophyll cells. Copious haustoria were also associated with this infection type (Fig. 4g) as well as tan occluded cells around the periphery of the infection bundle (Fig. 4h). A generalized phenolic reaction was observed in this family 4 weeks after inoculation, however, it did not appear to confine or inhibit growth of the fungus. No evidence of mesophyll cell collapse was found 4 or 6.5 weeks after infection.

3.4 Histological observations 38 weeks post-inoculation

Infection spots were present on three eastern white pine families 38 weeks after inoculation that were sufficiently separated from adjacent infections to allow collection of presumed individual infection sites. Histological observations of these infection sites collected from

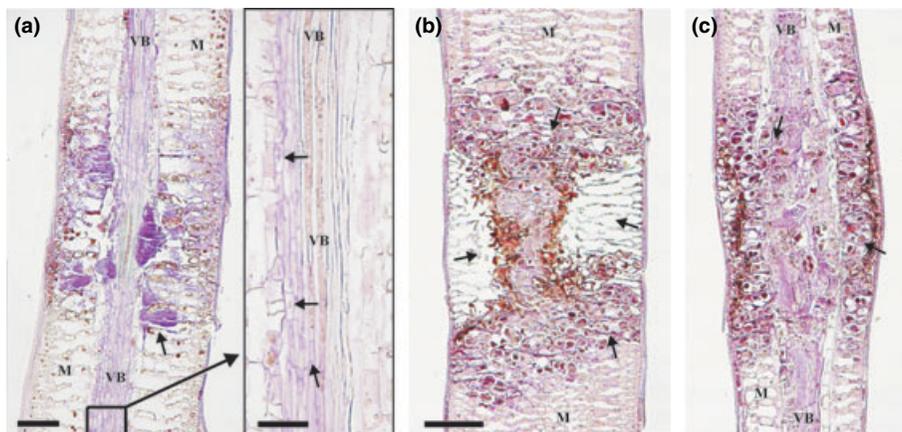


Fig. 6. Needle reactions in resistant eastern white pine families 38 weeks after inoculation with *C. ribicola*. Tissue labels: M, mesophyll; VB, vascular bundle. (a) Section showing extensive colonization of mesophyll cells by *C. ribicola* (arrow) along vascular bundle. Inset showing a magnified view of vascular bundle with many haustoria and hyphal structures (arrows) extending away from the condensed masses of hyphae. Inset is from an area outlined by the box as well as below this area not shown on micrograph. (b) Extensive mesophyll cell collapse (middle arrows) and copious hyperplastic and hypertrophic mesophyll cells (upper and lower arrows) throughout the area of infection. (c) Extensive hyperplasia and hypertrophy (arrows) in vascular and mesophyll cells in section from same sample as (b). (a-c) Stained with periodic acid-Schiff. (a) P327 \times P327. (b and c) P327 \times ON469. Bars = 100 μ m.

families P327 \times P327, P327 \times ON469 and H111 \times H111 revealed three distinct reaction types.

Reactions typical of cross P327 \times P327 exhibited extensive hyphal colonization of mesophyll and vascular cells (Fig. 6a). Heavy deposition of phenolic compounds was evident throughout the infection site as well as hyperplastic and hypertrophic growth of transfusion cells on the edge of the vascular bundle. Fungal hyphae and haustoria were observed in many cells far away from the main infection center. Hyphal and haustorial structures present in the vascular bundle not neighbouring the established infection centre may suggest that although characteristic resistance mechanisms were present some *C. ribicola* hyphae were able to move within the needle and into stem tissues.

Infection sites collected from family H111 \times H111 38 weeks after inoculation exhibited small masses of hyphae surrounded by dense deposits that stained dark purple with periodic acid-Schiff and a brilliant red with phloroglucinol-HCl, indicating that phenolic compound deposition was abundant. These dense deposits were only found in intercellular spaces. Intracellular spaces were coloured a light tan similar to reactions in samples collected 4 and 6.5 weeks after inoculation. The hyphae present in these infection bundles were noticeably deformed, appeared to be non-living and were not observed spreading outside of the dense deposits.

Samples from family P327 \times ON469 were not similar to needle spots from the previous two families and were dissimilar to samples collected from the same family 4 weeks and 6.5 weeks after inoculation. Microscopically, extensive hyperplasia, hypertrophy and collapsed cells were observed in mesophyll and vascular cells (Fig. 6b,c). Noticeable distortion of the vascular bundle was present, likely as a result of the excessive cell growth and not a result of hyphal growth. Hyphae were not detected in all samples examined exhibiting this reaction type. However, the reactions began just below stomatal openings

Table 2. Comparison of length and width measurements made from all condensed infection sites from one resistant and two susceptible eastern white pine families at two times following inoculation with *Cronartium ribicola*¹.

Seedling family	4 weeks post-inoculation			6.5 weeks post-inoculation		
	n	Mean infection length (μm)	Mean infection width (μm)	n	Mean infection length (μm)	Mean infection width (μm)
P327 \times P327 (R)	15	431 \pm 180	236 \pm 63	8	448 \pm 289	264 \pm 67
H109 \times H111 (S)	15	389 \pm 137	261 \pm 42	16	630 \pm 300	299 \pm 67
H111 \times H111 (S)	14	390 \pm 138	244 \pm 52	16	644 \pm 275	292 \pm 83

R, resistant; S, susceptible.
¹No statistically significant differences were found between measurements when compared using a two-way ANOVA, where n = number of samples from each family at each time point and $\alpha = 0.05$. All means reported with corresponding standard deviation.

and continued to the vascular bundle similar to the reaction observed in collected samples from family P327 \times P327 4 weeks after inoculation. Extensive bright red staining was observed in intercellular spaces and along the vascular bundle in these reactions after staining with phloroglucinol-HCl, indicating that copious phenolic compounds had been deposited throughout the infection site.

3.5 Measurement of tissue colonization

Relative size of condensed fungal masses present within needles were compared by measuring these masses at two time points from one resistant and two of the more susceptible eastern white pine families. Differences in measurements made of the condensed hyphae at 4 weeks and 6.5 weeks after inoculation in families P327 \times P327, H109 \times H111 and H111 \times H111 were not statistically significant when analysed using a two-way ANOVA (Table 2). However, a trend was observed indicating that the condensed mycelial masses found in resistant family P327 \times P327 may well be significantly smaller if more samples were available and the number of replicates increased.

4 Discussion

The results presented in this report clearly show that many observable differences in primary needle reactions between susceptible and resistant genotypes can be documented as early as 4 weeks after inoculation but become more pronounced 6.5 weeks after inoculation. In addition, these results show this type of study can be used in future investigations to assess newly collected and as yet untested eastern white pine selections for resistance responses using inoculated primary needle tissue. The myriad resistance and infection responses apparently present within the *C. ribicola*/*P. strobus* pathosystem indicate that many factors may be contributing to the level of resistance observed in this study.

Our collection of individual *C. ribicola* infection sites 4 weeks after inoculation provides a histological assessment of resistance mechanisms functioning within primary needle tissues early in the infection process. Although needle defence reactions are likely present much earlier, sampling these initial reactions becomes problematic as macroscopic disease symptoms seldom develop before 3 weeks after initial infection. Molecular techniques may be more suited to investigations of these initial stages of infection. Recently, a proteomic

study of primary needles of *P. strobus* selections inoculated with *C. ribicola* conducted by SMITH et al. (2006a) revealed up-regulation of several putative resistance proteins in primary needles of open-pollinated progeny of selection P327 when compared with open-pollinated susceptible selection H111 4 weeks after inoculation with *C. ribicola*. Although the specific role of these up-regulated proteins was not identified they are potentially intimately involved in the resistance responses observed in our study (SMITH et al. 2006a).

The differences in shape and extent of initial fungal colonization of primary needle tissues observed histologically may be indicative of chemical barriers produced by the resistant selections inhibiting mycelial growth (VANCE et al. 1980; SMITH et al. 2006a). Our results indicate that a general trend of decreased growth in the condensed mycelial mass characteristic of early infection by *C. ribicola* in eastern white pine needles may have occurred between the 4 and 6.5 week post-inoculation sampling of resistant family P327 × P327 when compared with more susceptible families. The condensed masses of mycelium present within primary needles of eastern white pine infected with *C. ribicola* likely serve as a nutrient reserve for the fungus as it moves through the vascular elements into the stem tissue as suggested in earlier work (CLINTON and McCORMICK 1919). Any differences present in relative size of this mass between resistant and susceptible eastern white pine families would give insight into the ability of resistant mechanisms present within the system to limit growth of the fungus and possibly prevent movement into stem tissue. The lack of statistically significant differences in the size of the condensed mycelial mass found in this study was likely due to the presence of a limited number of apparently uninhibited infections within resistant families and a relatively small sample size for each of the families tested. Further research and perhaps an increase in the number of samples dedicated to a quantitative comparison of the size of this mycelial aggregation may well show statistically significant differences in abundance of condensed hyphae present between resistant and susceptible *P. strobus*.

Extremely varied needle reactions were observed between eastern white pine families. The observed increase in phenolic deposition over time in resistant families and susceptible family H111 × H111, collapsed mesophyll cells in a susceptible and resistant family as well as other resistance responses observed in resistant families contribute further evidence for multiple mechanisms at work in the interaction between *C. ribicola* and *P. strobus*.

This research shows striking differences in survivability of eastern white pine families derived from controlled cross pollinations of *P. strobus* selections previously identified as resistant and susceptible in multiple artificial inoculations of their open-pollinated progeny (JURGENS et al. 2003). In addition, vast differences were noted histologically at three sampling points in needle reactions after inoculation with *C. ribicola*. These differences were evident when comparing the more resistant families with each other as well as to the susceptible families.

Large condensed masses of mycelium characteristic of infection by *C. ribicola* in pine needle tissues (COLLEY 1917; CLINTON and McCORMICK 1919) were observed in samples taken from the more susceptible families tested. Fungal growth within the vascular bundle of the needle as well as sparse hyphae spreading into adjacent mesophyll cells observed in these seedlings gives evidence for uninhibited progression of infection in the susceptible families. This is in contrast to the reactions observed in the three most resistant families tested. These three distinct reaction types indicate that multiple mechanisms may be effective in the inhibition of disease progression in primary needles of *P. strobus*. Collapsed mesophyll cells were previously proposed as a significant resistance mechanism in secondary needles of selection P327, however, no indication of frequency of occurrence was presented (JURGENS et al. 2003). Collapsed cells were documented in this study but were not ubiquitous within either *P. strobus* family where this trait was observed. When present in infections from family P327 × P327, they often delimited the extent of fungal expanse in that sample. However, collapsed cells were not limited to resistant families and

also appeared in the susceptible family H109 × H111. Although not as abundant in the susceptible family, when pooled with the observed heavy deposition of phenolic compounds in H109 × H111 and other crosses with selections H109 and H111, it may explain the previous results indicating that susceptible selections used in our inoculation studies were more resistant than general bulk eastern white pine seed often used for nursery production of *P. strobus* (JURGENS et al. 2003).

Several investigators have described growth of *C. ribicola* in needles following inoculation using one sampling time (COLLEY 1917; CLINTON and McCORMICK 1919; HIRT 1939; JURGENS et al. 2003). The most recent research directed at identifying resistance mechanisms in eastern white pine artificially inoculated with *C. ribicola* used only one collection time (7 weeks after inoculation) and successfully identified mechanisms present in secondary needles of seedlings more likely to survive infection (JURGENS et al. 2003). Our results using two collection times early in the infection process demonstrate that similar mechanisms of resistance are functioning throughout the early progression of the disease in primary needle tissue and they may significantly impact disease development between 4 and 6.5 weeks after infection.

Resistance mechanisms observed within primary needles of *P. strobus* were not omnipresent in the resistant families tested. Other studies have also documented this variability in some of the same eastern white pine selections used in this study (JURGENS et al. 2003; SMITH et al. 2006b). Some infections in highly resistant families are still apparently able to overcome resistance mechanisms present in the primary needles and then colonize main stem tissues. This is evident in the mortality data showing that a number of seedlings from the three most resistant crosses succumb to disease during the 52-week monitoring period. Evidence from this current research and past studies may suggest that although needle resistance in *P. strobus* selections is incomplete it is genetically controlled and heritable (JURGENS et al. 2003; SMITH et al. 2006a,b). Resistance in these selections appears to be an additive process with epicuticular wax serving as an initial physical and chemical barrier to infection on the surface of the needles and likely reducing the total number of needle infections (PATTON 1961; PATTON and SPEAR 1980; SMITH et al. 2006b). Collapsed mesophyll cells and increased deposition of phenolic compounds in both primary and secondary needles as well as an up-regulation of multiple putative resistance proteins in primary needles all further reduce the number of active infections that have the ability to reach stem tissues and cause mortality (JURGENS et al. 2003; SMITH et al. 2006a).

Prior observations of eastern white pine that have apparently recovered from *C. ribicola* stem infections have shown that resistance mechanisms are present in some *P. strobus* allowing them to form barriers against fungal growth in the stem (STRUCKMEYER and RIKER 1951; BOYER 1966). To date, no studies have investigated stem tissues of the selections and crosses discussed in this paper. However, previous research in other stem rust systems has shown that stem resistance is important in the overall resistance response. In the fusiform rust (*Cronartium quercuum* f. sp. *fusiforme*)/slash pine (*Pinus elliottii*) pathosystem multiple researchers have shown that stem resistance mechanisms exist in the genotypes able to survive following infection (JEWELL and SPEIRS 1976; MILLER et al. 1976; POWERS et al. 1981).

In order to understand all aspects of the additive process likely occurring in these *P. strobus* selections, further research is needed to elucidate resistance mechanisms almost certainly present in the stem tissues of these superior eastern white pine genotypes. Although stem resistance has yet to be studied, many of the selections used in this study have proven to survive infection and display considerable resistance in needles in multiple studies (JURGENS et al. 2003; SMITH et al. 2006a,b). This appears to be sufficient justification for utilization of these genotypes in urban tree planting and landscape reforestation activities to mitigate damage caused by *C. ribicola*.

Acknowledgements

Partial funding for this research was provided by the Minnesota Tree Improvement Cooperative, North Central Research and Outreach Center, University of Minnesota. The authors would like to thank Dr Jason Smith, University of Florida and Drs Les Szabo and Mike Ostry, USDA-ARS and U.S. Forest Service respectively, as well as Mr Joel Jurgens, University of Minnesota for reviewing early versions of the manuscript. The authors would also like to thank Dr Andrew Graves, University of California for assistance with the statistical analysis as well as Carrie Sweeney and the staff of the U.S. Forest Service Oconto River Seed Orchard for conducting crosses and obtaining the eastern white pine seed used in this study.

References

- BOYER, M. G., 1966: Auxin in relation to stem resistance in white pine blister rust. In: *Breeding Pest Resistant Trees*. Ed. by GERHOLD, H. D.; SCHREINER, E. J.; McDERMOTT, R. E.; WINIESKI, J. A. PA: Pergamon Press, University Park, pp. 179–184.
- BOYER, M. G.; ISAAC, P. K., 1964: Some observations on white pine blister rust as compared by light and electron microscopy. *Can. J. Bot.* **42**, 1305–1309.
- CLINTON, G. P.; McCORMICK, F. A., 1919: Infection experiments of *Pinus strobus* with *Cronartium ribicola*. In: *Report of the Botanist for Years 1917–1918*. Conn. Agric. Exp. Stn. Bull. **214**, 428–459.
- COLLEY, R. H., 1917: Diagnosing white pine blister rust from its mycelium. *J. Agr. Res.* **11**, 281–286.
- COLLEY, R. H., 1918: Parasitism, morphology, and cytology of *Cronartium ribicola*. *J. Agr. Res.* **15**, 619–659.
- DRING, D. M., 1955: A periodic acid-Schiff technique for staining fungi in higher plants. *New Phytol.* **54**, 277–279.
- GAHAN, P. B., 1984: *Plant Histochemistry and Cytochemistry. An Introduction*. London: Academic Press.
- HIRT, R. R., 1939: Canker development by *Cronartium ribicola* on young *Pinus strobus*. *Phytopathology* **29**, 1067–1076.
- HUNT, R. S.; MEAGHER, M. D., 1989: Incidence of blister rust on “resistant” white pine (*Pinus monticola* and *Pinus strobus*) in coastal British Columbia plantations. *Can. J. Plant Pathol.* **11**, 419–423.
- JEWELL, F. F.; SPEIRS, D. C., 1976: Histopathology of one and two year-old resisted infections by *Cronartium fusiforme* in slash pine. *Phytopathology* **66**, 741–748.
- JONES, D., 1984: Use, misuse, and role of multiple-comparison procedures in ecological and agricultural entomology. *Environ. Entomol.* **13**, 635–649.
- JURGENS, J. A.; BLANCHETTE, R. A.; ZAMBINO, P. J.; DAVID, A., 2003: Histology of white pine blister rust in needles of resistant and susceptible eastern white pine. *Plant Dis.* **87**, 1026–1030.
- KINLOCH B. B. JR, 1972: Mechanisms and inheritance of rust resistance in conifers. In: *Biology of Rust Resistance in Forest Trees*, Misc. Publ. 1221. Ed. by BINGHAM, R. F.; HOFF, R. J.; McDONALD, G. I. Washington, DC: U.S. Department of Agriculture, Forest Service, pp. 119–129.
- KINLOCH B. B. JR, 2003: White pine blister rust in North America: past and prognosis. *Phytopathology* **93**, 1044–1047.
- KINLOCH B. B. JR; LITTLEFIELD, J. L., 1977: White pine blister rust: hypersensitive resistance in sugar pine. *Can. J. Bot.* **55**, 1148–1155.
- KREBILL, R. G., 1968: Histology of canker rusts in pines. *Phytopathology* **58**, 155–164.
- MALOY, O. C., 1997: White pine blister rust control in North America: a case history. *Ann. Rev. Phytopathol.* **35**, 87–109.
- MILLER, T.; COWLING, E. B.; POWERS H. R. JR; BLALOCK, T. E., 1976: Types of resistance and compatibility in slash pine seedlings infected by *Cronartium fusiforme*. *Phytopathology* **66**, 1229–1235.
- PATTON, R. F., 1961: The effect of age upon susceptibility of eastern white pine to infection by *Cronartium ribicola*. *Phytopathology* **51**, 429–434.
- PATTON, R. F., 1967: Factors in white pine blister rust resistance. In: *Proceedings of the 14th IUFRO Congress, Munich, Germany*. US Forest Service Publication, pp. 876–890.
- PATTON, R. F.; SPEAR, R. N., 1980: Stomatal influence on white pine blister rust infection. *Phytopathol. Mediterr.* **19**, 1–7.
- POWERS, H. R.; SCHMIDT, R. A.; SNOW, G. A., 1981: Current status and management of fusiform rust in Southern pines. *Ann. Rev. Phytopathol.* **19**, 353–371.

- RUZIN, S. E., 1999: Plant Microtechnique and Microscopy. Oxford: Oxford University Press.
- SMITH, J. A.; BLANCHETTE, R. A.; BURNES, T. A.; JACOBS, J. J.; HIGGINS, L.; WITTHUHN, B. A.; DAVID, A. J.; GILLMAN, J. H., 2006a: Proteomic comparison of needles from blister rust-resistant and susceptible *Pinus strobus* seedlings reveals up-regulation of putative disease resistance proteins. *Mol. Plant Microbe Interact.* **19**, 150–160.
- SMITH, J. A.; BLANCHETTE, R. A.; BURNES, T. A.; GILLMAN, J. H.; DAVID, A. J., 2006b: Epicuticular wax and white pine blister rust resistance in resistant and susceptible selections of eastern white pine (*Pinus strobus*). *Phytopathology* **96**, 171–177.
- STEWART, F. C., 1906: An outbreak of the European currant rust (*Cronartium ribicola* Dietr.). NY Agric. Exp. Stn. Tech. Bull. **2**, 61–74.
- STRUCKMEYER, B. E.; RIKER, A. J., 1951: Wound periderm formation in white pine trees resistant to blister rust. *Phytopathology* **41**, 276–281.
- VANCE, C. P.; KIRK, T. K.; SHERWOOD, R. T., 1980: Lignification as a mechanism of disease resistance. *Ann. Rev. Phytopathol.* **18**, 259–288.
- WATERMAN, A. M., 1955: A stain technique for diagnosing blister rust in cankers on white pine. *For. Sci.* **1**, 219–221.