Potential resistance mechanisms in *Pinus strobus* to infection by *Cronartium ribicola*

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Abstract

Although putative blister rust-resistant Pinus strobus were selected nearly 40 years ago, seedlings with increased resistance to blister rust are still unavailable. In the studies reported here, seedlings from open pollination and controlled crosses (factorial mating design) were subjected to artificial inoculation with Cronartium ribicola in an effort to facilitate selection of seed sources with resistance to blister rust. Disease incidence and severity data were collected and histology was used to study the infection process in needles of inoculated seedlings. Inoculation of seedlings from open pollination and controlled crosses indicate that seedlings with P. strobus selections P 30, P 312 or P 327 as a parent are more resistant than others tested. Specifically, seedlings with P 327 as a parent have a lower incidence of infection (approximately 10 – 20 % reduction in incidence compared to susceptible controls) and consistently longer survival following inoculations (393 days longer than susceptible controls). In addition,

seedlings originating from P 327 had the lowest values for needle infection based on spot index (a rating used to evaluate disease severity). Histological studies indicate that different mechanisms of resistance to infection exist in the needles. Seedlings of P 327 respond to infection by producing a hypersensitive-like reaction where cells near the infection site die rapidly and inhibit the fungus from penetrating the tissue further. Seedlings from open-pollinated P 30 exhibit a concentration of phenolic compounds near the infection site that appears to restrict hyphal growth. Needles from infected and non-infected seedlings of P 327 (resistant) X H109 (susceptible) were also compared using scanning electron microscopy. Preliminary results suggest that non-infected seedlings have a greater proportion of stomata plugged with wax than infected seedlings. Grafts of the parent trees used in this study were made in 2002 and will be compared to seedlings and used to further elucidate the mechanisms of resistance. These results support previous findings by Patton done in the 1960's (University of Wisconsin) that selection P 327 shows promise for the development of blister-rust resistant P. strobus seedlings. In addition to selection P 327, selections P 30 and P 312 exhibit degrees of resistance that may result from different mechanisms. These results provide a basis for future selection and breeding programs aimed at developing blister rust- resistant P. strobus.

Introduction

Resistance to *Cronartium ribicola* has been found in several North American species in section *Strobus* of *Pinus*, including *P. monticola* and *P. lambertiana* (2,3). Major-gene resistance in these species has facilitated the development of blister-rust resistant seedlings for planting (3). The mechanisms of blister-rust resistance in these species are well characterized (2,3) and

breeding efforts are underway to combine resistance traits and increase the durability of the resistance.

Despite the discovery of several rust-free *P. strobus* in high hazard areas nearly forty years ago (5), blister-rust resistant *P. strobus* seedlings are still unavailable for planting. Limited knowledge about the genetics of blister-rust resistance in *P. strobus* is further compounded by a lack of knowledge about what mechanisms are important for blister-rust resistance in this species. As a result, there has been little progress towards breeding blister-rust resistant *P. strobus*.

Grafts of putative resistant *P. strobus* selections are planted at the U.S.F.S Oconto River Seed Orchard in White Lake, Wisconsin (**Fig. 1**). These grafts are now being used for breeding and genetic improvement projects and seed from superior trees (and crosses) are being grown and tested for resistance to *C. ribicola*. In the studies reported here, seedlings from open pollination and controlled crosses (factorial mating) have been subjected to artificial inoculation with *C. ribicola* to further our understanding of the resistance behavior and possible mechanisms of resistance in these *P. strobus* selections.

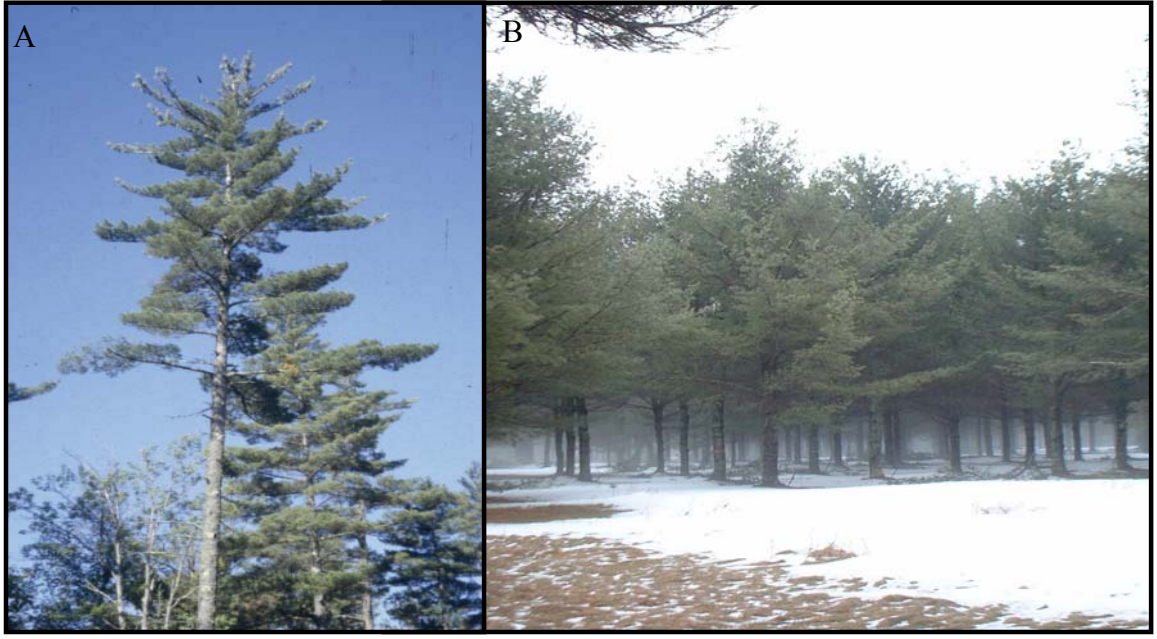

Fig. 1 - (A) Blister-rust free *P. strobus* in a high hazard area of Minnesota and (B) grafts of putative blister-rust resistant selections of *Pinus strobus* planted at Oconto River Seed Orchard in White Lake, Wisconsin, USA.

Methodology

Resistance screening

Seedling production - Seed from controlled crosses (factorial mating design) were collected and stratified for 12 weeks. After stratification, the seed were planted and grown in the greenhouse under supplemental lighting for 12 weeks.

Experimental design - Sixty 12-week-old seedlings per seed lot were randomly selected and placed in a randomized block design. For each experiment, 60 seedlings of the most susceptible selection (H 111) were included for comparison. Control seedlings were not inoculated.

Inoculations - Seedlings were inoculated by suspending telia-bearing leaves (from a monouredial isolate) over the seedlings for approximately 24 hours at 17 ^{O}C and 100% humidity. After 7 days, the seedlings were removed from the growth chamber and placed in the greenhouse and monitored for disease development. The inoculated seedlings were inspected visually for symptoms at 4 and 8 weeks post-inoculation. A rating scale called spot index (**Table 1**), was

used to indicate the number of spots observed on each seedling. In addition data were taken on the number of seedlings per seed lot with symptoms at each rating. For open pollinated seedlings, mortality rates from two experiments were determined and based on whether a plant was alive or dead on the date of data collection, which was taken every two weeks after inoculation. Mortality data were also taken weekly for seedlings (from open pollination) from replicated inoculations (2 experiments) used in histology studies

Host-parasite Interactions

Needle histology - Secondary needles were collected from open-pollinated seedlings 7 weeks after inoculation and prepared for histological examination. Needle tissues were fixed in formalin-acetic acid-ethyl alcohol (FAA), dehydrated in a tertiary-butyl alcohol series and embedded in paraffin. Serial sections were cut at 12-14 mm and stained with the periodic acid-Schiff technique and phoroglucinol-HCL.

Wax on needle surfaces - Primary needles from infected and non-infected inoculated seedlings of P 327 X H 109 were collected for SEM evaluation of wax occlusion of stomata. After coating with gold using a sputter-coater, the needles were evaluated under low vacuum, at room temperature using a Hitachi variable pressure S3500 scanning electron microscope. Images were collected at three pre-determined locations (using the x-axis). Stomata were considered occluded with wax when the wax covering the stomatal opening contained no gaps larger than 5 microns.

Results

Resistance screening -Inoculations of seedlings from controlled crosses indicates that seedlings with P 327 as a parent are most resistant. Seedlings with this selection as a parent have smaller, fewer spots than susceptible controls (**Fig. 2**) and survive longer following infection

(**Fig. 5**). The lowest average spot index (at 4 weeks) (**Table 1**) for all inoculations was recorded for P 327 X H109 (spot index = 1.2) followed by P 312 X P327 (spot index = 1.5). Open pollinated P 327 had an average spot index of 1.9. In comparison, the susceptible seed source, H 111 had an average spot index of 3.0. Seedlings with P 327 as a parent also had lowest percentage of seedlings infected after 4 weeks. Only 66% of seedlings of P 327 X H109 and 74 % of P 312 X P327 were infected after 4 weeks, whereas 85% of seedlings of H 111 were infected. Survival of open-pollinated seedlings used in histology studies was greatest for selections P 327 and P 30 and worst for H 111. The average number of days required to reach 75% mortality was 212 for H111, whereas 605 days were required for P 327 (although in one study P 327 did not reach 75% mortality) and 597 days for P 30.

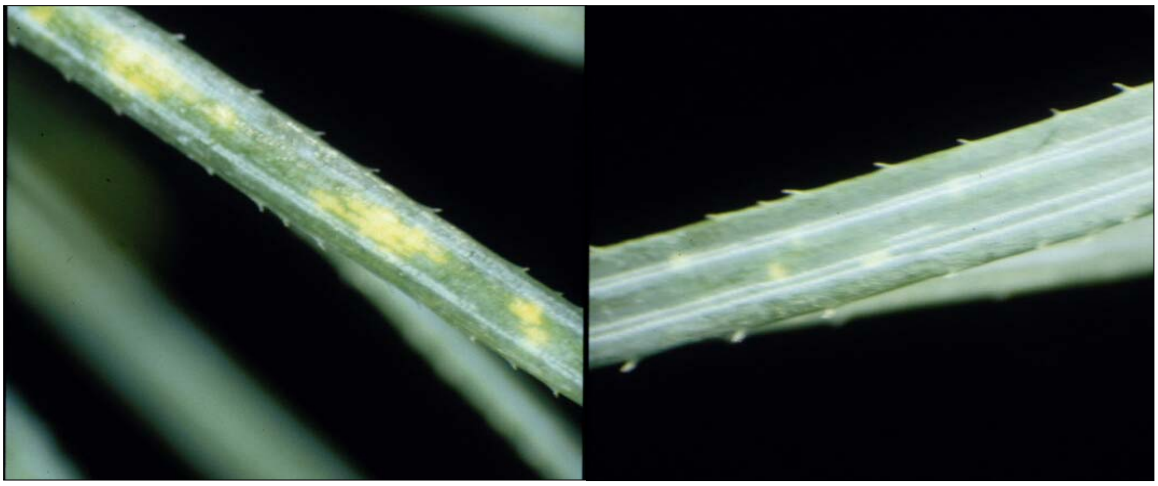

Fig. 2 – Needle lesions from inoculated seedlings of *P. strobus* selections H 111 (A) and P 327 X P 312 (B) at 4 weeks after inoculation. Note the reduced lesion frequency and size on P 327 X P312; whereas H 111 has larger, more numerous lesions.

Seed Source	Average Spot Index	Percentage of seedlings infected
P 327 X H109	1.2	66
P 312 X P 327	1.5	74
P 327 (open pollinated)	1.9	83
P 30 X P 343	2.7	84
H 111	3.0	85

Table 1 – Data from artificial inoculations of *P. strobus* selections (controlled crosses and open-pollination) 4 weeks after inoculation. Seedlings with P 327 as a parent are much less susceptible than other selections. The spot index values are as follows: 1 = 1-3 spots per seedling, 2 = 4-10 spots, 3 = 11-32 spots, 4 = 33-100 spots, 5 = 101-316 spots, 6 = 317-1000 spots and 7 = 1001-3162 spots.

Host-parasite Interactions

Needle histology - Histological studies show that needle symptoms of seedlings from open pollinated P 327 are characterized visually by a small, bright yellow spot and microscopically exhibit a collapse of mesophyll cells and an absence of phenolic compounds (**Fig. 4**). The cells and transfusion tissue in close proximity to the infection were necrotic and vascular elements remained unaltered. Phenolic compounds were found deposited near the infection site in P 30 and P 312 and the hyphae were distorted and shrunken indicating inhibition of fungal development. Susceptible selections (H 111 and WI 352) had densely packed mycelium penetrating the vascular bundle and presumably not restricted by host responses.

Wax on needle surface - Of the 371 stomata observed on secondary needles of non-infected seedlings of P 327 X H109, 244 (66%) were occluded (**Fig. 3**) with wax (gaps were less than 5 microns). Of the 338 stomata observed on the secondary needles of infected seedlings of P 327 X H109, 67 (20%) were occluded with wax.

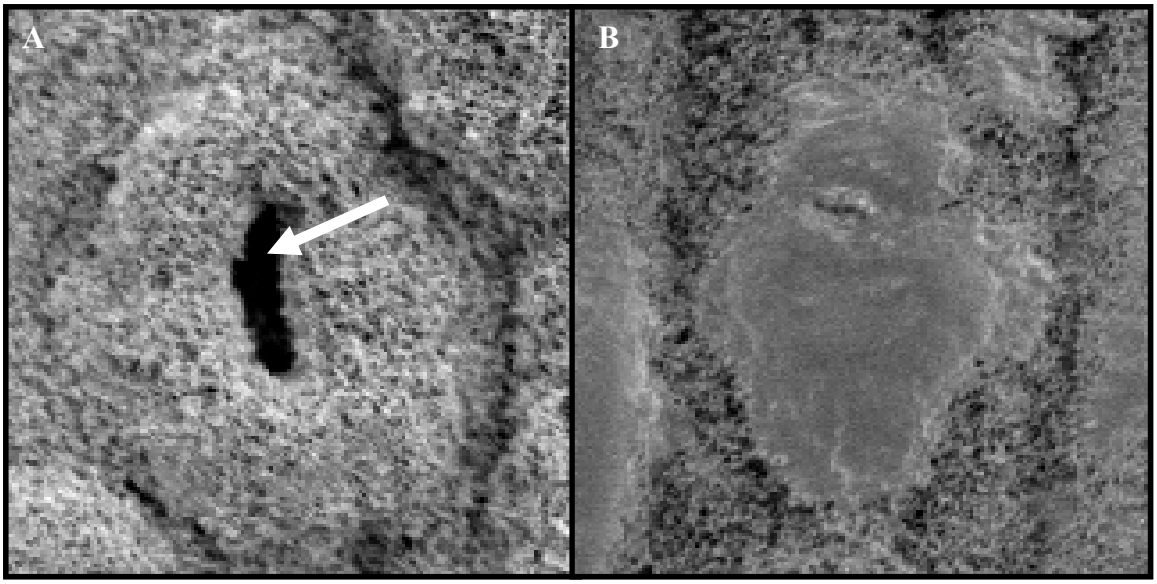

Fig. 3 – ESEM micrographs (8000x magnification) of stomata from infected (A) and non-infected (B) P 327 X H109 seedlings. Note uniform covering of epistomatal wax in B and large gap in wax at stomatal opening (white arrow) in A.

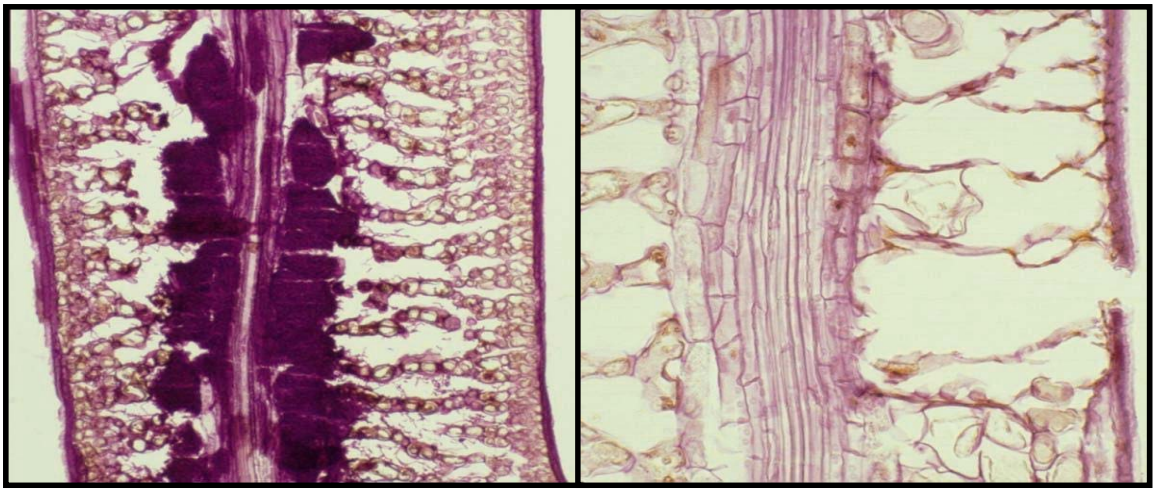

Fig. 4 –Micrographs of needles from susceptible (A – WI 352) and resistant (B – P 327) seedlings 7 weeks after inoculation. Note the collapsed mesophyll cells and lack of penetration of vascular bundles in B. In contrast, mesophyll cells remain intact in A and the mycelium has penetrated the vascular bundle.

Fig. 5 – Inoculated seedlings P 327 x P 312 and H 111 12 weeks after inoculation. Mortality is greater in H 111 (red arrows) than P 327 (black arrows).

Discussion

The preliminary results from these studies indicate that there are different mechanisms responsible for resistance to *C. ribicola* in *P. strobus*. Inoculations have shown that selection P 327 exhibits smaller and fewer needle lesions. The fewer lesions may result from more stomatal wax that prevents entry of the fungus into the needle tissue. More experiments are underway to determine what role epistomatal wax plays in resistance in P 327.

The slower progression and smaller size of needle spots in P 327 may result from a hypersensitive-like response where the host cells die rapidly and prevent the fungus from penetrating the tissue. The accumulation of phenolic compounds and inhibition of fungal growth in cells of seedlings of P 30 and P 312 indicates that other mechanisms of resistance are present in these selections.

These studies have supported earlier findings by Patton et al. (5,7) and Hunt et al. (2) that selections P 327, P 312 and P 30 show promise for developing resistant *P. strobus*. Further

studies are underway to further elucidate the mechanisms responsible for resistance to blister-rust in *P. strobus*. More seed sources are being tested and grafts will be used in inoculation studies during the fall of 2002.

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Acknowledgements

The authors wish to thank Paul Zambino for providing assistance and inoculated seedlings for histological studies. We also thank Bill Sery and the staff at Oconto River Seed Orchard for providing seed and scions used for this project.