Genetic and Environmentally Related Variation in Needle Morphology of Blister Rust Resistant and Nonresistant *Pinus monticola*

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Abstract—This paper compares the results of two studies (differences related to genotype versus differences related to growing environment) that have been reported in previous publications (Woo and others 2001; Woo and others 2002) and highlight information that may be useful to tree breeders in refining rust resistance evaluation procedures. The objectives of these studies were to assess genetic and environmentally related variation in needle surface traits in rust–resistant western white pine (*Pinus monticola* Dougl.). Statistically significant differences were found in 14 needle traits (needle length and width, number of stomatal rows, number of stomata per row, total stomata per needle, adaxial surface area, stomatal density, major axes of stomata, stomatal shape, stomatal area, stomatal occlusion, epistomatal wax degradation, weight of wax per dry weight of needle, and the contact angles of water droplets) of western white pine seedlings grown from the same seed orchard source in three nurseries in northern Idaho. Waxes on needle surfaces were tubular in structure and the amount of surface wax appeared to be associated with surface wettability. In a separate study, stomata on needles from susceptible families were found to be significantly wider and larger than those from genetically resistant families and from genetically improved bulked lots from the seed orchard. Neither the percent of stomatal occlusion nor the amounts of degraded epistomatal wax were statistically different among the seed sources. Contact angles of water droplets on needles of the resistant families were significantly larger than those of the susceptible families and the seed orchard lots. Results of both the genetic study and the nursery study should be more broadly tested to determine their generality and applicability to refining rust screening procedures. Information from this study may be useful in refining protocols for selecting genotypes for tree improvement programs and/or for quantifying levels of rust resistance in selectively bred western white pine stocks. Rust screening protocols may be made more efficient if any of the traits prove to be reliable indicators of resistance.

Key words: Epistomatal wax, *Pinus monticola* Dougl., wettability, stomatal occlusion, blister rust, needle morphology, rust resistance

In: 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.

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Introduction

Routine tests of western white pine (*Pinus monticola* Dougl.) for blister rust resistance rely on successful inoculation of seedlings with spores of the fungus *Cronartium ribicola* J. C. Fisch. in Rabenh., and subsequent ocular evaluations for rust symptoms and expression of the resistance mechanisms. Presumably, phenotypic variation that has a genetic basis will play a critical role in infection success; for example, a specific resistance type may prevent entry of germinating rust spores into needle tissue. However, phenotypic variation caused by a particular nursery regime that temporarily prevents or diminishes successful inoculation of seedlings may hamper attempts to identify genetically resistant stocks for tree improvement programs or attempts to quantify realized levels of resistance in selectively bred stock. The studies reported here were designed to assess and quantify phenotypic variation in a variety of needle surface traits that may be associated with genetic differences in resistant versus susceptible genotypes and which may also be affected by differences associated with nursery growing regimes and or their environments. Results of the individual studies (differences related to genotype versus differences related to growing environment) have been reported in previous publications (Woo and others 2001; Woo and others 2002). The purpose of this paper is to compare the results of the studies and highlight information that may be useful to tree breeders in refining rust resistance evaluation procedures.

Background

White pine blister rust, a devastating disease caused by the fungus *Cronartium ribicola* J. C. Fisch. in Rabenh., was introduced into western North America in 1910 on eastern white pine seedlings imported from Ussy, France, to Point Grey, near Vancouver, British Columbia. The rust appears to have reached Idaho about 1923 (Mielke 1943). Blister rust is largely responsible for the drastic reduction in white pine cover type (greater than 90 percent) in the inland northwestern United States (eastern Washington, northeastern Oregon, northern Idaho, and northwestern Montana) since 1923 (Fins and others 2001). A breeding program designed to increase white pine's resistance to blister rust was initiated in the late 1940s as part of an effort to restore white pine to inland Northwestern ecosystems (Bingham 1983). The program produces genetically improved stocks that outperform unimproved (susceptible) stocks in operational plantings and field tests throughout the region (Fins and others 2002).

Rust Screening

As a key component of the ongoing breeding program, white pine seedlings are routinely evaluated for rust resistance after inoculation with *C. ribicola* basidiospores. Although most seedlings subjected to the screening process are grown at the USDA Forest Service Coeur d'Alene (Idaho) nursery, occasionally seedlings grown elsewhere have been included in the rust inoculations. At these times, differences in infection levels, mortality, variation in needle thickness, and the amount of water that collects on needle surfaces were observed between seedlings grown in different nurseries, suggesting that inoculation success may vary not only with the genetic backgrounds of the seedlings, but also with the nursery in which the seedlings are grown.

The relative number of needle spots that appear on white pine seedlings after exposure to blister rust has been shown to be under genetic control (Hoff and McDonald 1980; Meagher and Hunt 1996) and it appears to be a type of "rate reducing resistance" (Rossi and others 1999) that reduces infection efficiency by 10 times (Hoff and McDonald 1980).

At least two blister rust resistance types, "no spot" and "reduced needle lesion frequency," are recognized by the lack of or low numbers of needle spots that appear on needles after exposure to blister rust spores. "No spot," which occurs in high frequencies in Eurasian white pine species and occasionally in North American white pine species, is rare in western white pine, but the "reduced needle frequency" type of resistance can be found in higher frequencies (Hoff and others 1980). Although the exact mechanisms by which seedlings avoid or reduce infection are not known, it is known that *C. ribicola* germ tubes enter needles through their stomata (Patton and Johnson 1970), indicating that stomatal features are likely to be important to the infection process. Whether or how specific variations in stomatal features of western white pine needles are critical to infectability by blister rust is also not known.

Surface Wettability

"Wettability" refers to the tendency of a surface to retain water. Water beads up and remains on nonwettable surfaces longer than on wettable surfaces. Because water droplets take different shapes on wettable versus nonwettable surfaces, the angles they make with the surfaces on which they rest can be used as a measure of surface "wettability" (see Woo and others 2002 for images). A higher contact angle (produced by a more globular, rounder bead) indicates lower wettability of the surface (Cape 1983; Leyton and Juniper 1963). This phenomenon may be important in blister rust infection because water collection on needle surfaces is essential for fungal infections (Huttunen 1984), and the amount and distribution of free moisture have been reported to be the most important factors for germination of *C. ribicola* basidiospores and subsequent infection of eastern white pine needles (Spaulding and Rathbun-Gravatt 1926; Hansen and Patton 1977).

Study Objectives

The objectives of these studies were to describe and compare differences in needle surface traits, including surface wettability, stomatal size, frequency and distribution, and other needle traits that may be influenced by differences in nursery environment and/or genetic constitution. Our hypothesis was that both nursery environment and genetic differences affect needle surface traits that are related to infectability by *C. ribicola*.

Materials and Methods

Nursery Study

Seeds of western white pine were sown in 1997 and grown for 2 years (1997 and 1998) at three nurseries in northern Idaho, USA: the USDA Forest Service nursery in Coeur d'Alene, Potlatch Corporation's nursery in Lewiston, and the University of Idaho Forest Research nursery in Moscow. All seedlings used for the comparisons between nurseries originated from F_2 open pollinated seeds collected from the R.T. Bingham White Pine Seed Orchard in Moscow, ID. This stock has been reported as approximately 66 percent resistant to blister rust at 2.5 years after inoculation (Hoff and others 1973).

Morphometric traits were measured on three needles per seedling collected from each of 30 seedlings per nursery, one needle from each of three directions on the current stem of each seedling. Stomatal size (major and minor axes) and area were assessed on additional collections of one needle from each of two fascicles per seedling from 25 seedlings per nursery. Details on field, laboratory and statistical methods can be found in Woo (2000) and Woo and others (2002).

Genetics Study

For the genetics study, needle samples were collected from western white pine seedlings grown at the USDA Forest Service nursery in Coeur d'Alene as part of a routine rust screening operation of 271 entries that included open-pollinated families from phenotypic selections in natural stands, woods-run check lots, and open-pollinated bulk F_2 seed orchard lots from the R.T. Bingham White Pine Seed Orchard in Moscow. The seeds were sown in 164 cm^3 single super cells in spring 1993 and remained in a greenhouse for two growing seasons. They were inoculated in August 1994 by suspending blister rust-infected *Ribes* leaves over them in an inoculation chamber at 100 percent relative humidity.

In May 1995, the seedlings were transplanted to five outdoor nursery beds where they remained for the next 3 years (Mahalovich and Eramian 1995). Four families with low spot frequency (3062, 3653, 4437, and 4922), four families with high spot frequency (3162, 3233, 4110, and 4778), and two open-pollinated F_2 resistant bulked lots from the R.T. Bingham White Pine Seed Orchard (lots 4815 and 4816) were selected for this study (mean spot frequencies: 0.065, 3.32, and 0.20) (Woo and others 2001). One family in the "resistant" group (4437) also had 73 percent zero spot individuals 1 year after inoculation. Of the two seed orchard lots, lot 4815 was a general seed orchard collection, and lot 4816 was collected from parent trees that had been selected for the "short shoot" type of resistance (needle lesions appear after inoculation but rust infection is stopped between the needle and the branch), but seedlings from both lots exhibit a variety of rust resistance mechanisms (Rust 1998). Needle samples consisted of one needle from the current stem from each of three fascicles from each of 21 seedlings per source (seven seedlings per source per replication). Details on field, laboratory and statistical methods used can be found in Woo (2000) and Woo and others (2001).

Results

Nursery Study

Significant differences were found among the three nurseries (l=5.67; P<0.0001) and among seedlings within nurseries (l=4.17; P<0.0001) for the eight measured needle characteristics and three of the four stomatal measurements; that is, major axes, stomatal shape, and mean stomatal area (P<0.001) (table 1). Minor axes of stomata (stomatal width) were nearly identical among the three nurseries (P=0.94).

Relatively larger deposits of waxes were commonly distributed along the stomatal rows and over epistomatal chambers. Stomata with severely degraded epistomatal waxes were found side by side with stomata whose waxes were completely intact. Percent of occluded stomata was similar on needles from the Coeur d'Alene (86 percent) and Moscow nurseries (90 percent), and both had significantly more occluded stomata than needles from the Lewiston nursery (56 percent, P=0.0001). Needles from the Lewiston nursery produced more wax per dry weight of needle than those from either the Coeur d'Alene or the Moscow nursery, but those from the Moscow nursery had statistically higher levels of degraded wax than either of the other two (table 1).

Mean contact angles of water droplets on the needle surfaces differed significantly among the three nurseries and were highest on needles from the Lewiston nursery, both with and without the presence of surface waxes (table 1). Needles from the Moscow nursery had the smallest contact angles.

Genetics Study

In the genetics study, we found significant differences among the 10 seed sources ($P \leq 0.10$) for nearly all of the traits (Woo and others 2002). When grouped by resistance type (resistant, susceptible, and Moscow Seed Orchard), most of the comparisons were no longer statistically significant. Several exceptions stood out, however, including needles from the four resistant families were significantly shorter than those from the seed orchard lots (P=0.015), and the stomata of the susceptible families were significantly wider and greater in area than stomata on the seed orchard lots and the resistant families. The stomata of the susceptible families were also "rounder" in shape (smallest ratio of

Table 1—Means and standard errors of western white pine needle traits (from Woo and others 2002).

Variables	No. of needles	Nursery: Coeur d'Alene (mean ± SE)	Nursery: Lewiston (mean ± SE)	Nursery: Moscow (mean ± SE)	P-value
Needle length (mm)	270	54.3 ± 0.78	65.7 ± 1.08	81.2 ± 1.12	0.0001
Needle width 1 (mm)	270	0.8 ± 0.006	0.7 ± 0.008	0.9 ± 0.01	0.0001
Needle width 2 (mm)	270	0.8 ± 0.006	0.7 ± 0.008	0.9 ± 0.01	0.0001
Stomatal rows/plot	270	2.4 ± 0.05	3.3 ± 0.07	3.6 ± 0.07	0.0001
Stomata/row	270	13.1 ± 0.11	13.5 ± 0.13	13.8 ± 0.15	0.0228
Stomata/needle	270	3449 ± 76.9	5811 ± 158.7	8048 ± 246.7	0.0001
Adaxial surface area (mm^2)	270	82.8 ± 1.46	93.2 ± 1.92	146.2 ± 3.58	0.0001
Stomatal density	270	41.8 ± 0.63	62.2 ± 0.97	55.0 ± 0.92	0.0001
Major stomatal axes (mm)	150	59.5 ± 0.61	54.8 ± 0.55	57.9 ± 0.66	0.0001
Minor stomatal axes (mm)	150	33.2 ± 0.27	33.1 ± 0.31	33.0 ± 0.28	0.9369
Stomatal shape:					
Major axes/Minor axes	150	1.80 ± 0.03	1.66 ± 0.02	1.76 ± 0.02	0.0001
Mean stomatal area (mm^2)	150	1549 ± 19.41	1426 ± 24.41	1502 ± 23.73	0.001
Mean wax degradation	150	2.46 ± 0.06	2.58 ± 0.06	3.27 ± 0.05	0.0003
Wax per dry weight (mg/mg)	75	6.26	8.48	4.38	0.0002
Wax per surface area (mg/mm^2)	75	9.48	7.76	6.07	0.0719
Contact angles with wax	270	90.8 ± 0.79	105.4 ± 0.74	62.9 ± 1.68	0.0001
Contact angles, no wax	30	94.7 ± 1.31	101.0 ± 0.93	55.3 ± 2.3	0.0051

major to minor axes) than stomata on the seed orchard lots, and contact angle of water droplets on needles surfaces was significantly larger on resistant families than on susceptible families and Moscow Seed Orchard lots.

Discussion

Nursery Study

Growth Environment and Nursery Regimes—The differences we found in needle traits of the same seed source grown in three northern Idaho nurseries likely reflect differences in nursery growth regimes, temperature levels, container size, and/or sowing dates. Some of these nongenetic differences may be associated with variation in blister rust infection levels. For example, differences among nursery samples in stomatal traits are potential candidates as predictors of differences in initial infection levels because the blister rust fungus enters white pine needles through their stomata (Patton and Johnson 1970). We did not test this hypothesis in our current study. However, incidental evidence suggests a link between nursery growth regimes and rust infection following artificial inoculation, as on several occasions, attempts to inoculate seedlings grown in the Lewiston nursery had resulted in low infection levels compared to seedlings grown in Coeur d'Alene and inoculated at the same time.

Different growth regimes in each nursery (amounts and timing of water, temperature, light, growth medium and fertilization) likely contribute to differences in needle morphology and possible differences in infectability by rust infection. Fertilization, for example, may generally increase seedling growth and vigor but also increases susceptibility of southern pines to fusiform rust (Schmidt and others 1972; Blair and Cowling 1974; Rowan and Steinbeck 1977). Another potential association links differences in infection to a "functional resistance" associated with stomatal behavior (Hart 1929; Hirt 1938). If, for example, wider and larger stomata close slowly or incompletely compared to small narrow stomata, fungal germ tubes may more easily invade the larger ones.

Surface Waxes—Other, less obvious, surface traits may be implicated in differences in infection. For example, the high proportion of degraded waxes on seedlings from the Moscow nursery, which may be related to the use of surfactants and other treatments, such as the use of an acid rinse following fertilization, may be associated with rust infection. This relationship is suggested by a study showing that fungal hyphae penetrated Norway spruce needles more easily when surface or epistomatal waxes were degraded compared to when the wax structures were well-preserved (Huttunen 1984; Elstner and others 1985).

Stomatal Occlusion—Previous researchers have suggested a possible link between reduced blister rust infection and occlusion of the stomatal antechamber on needles of *Pinus strobus* L. (Patton and Johnson 1970; Patton and Spear 1980). However, seedlings from the Coeur d'Alene nursery (86 percent occluded) have historically been more easily infected than those from the Lewiston nursery (56 percent occluded). If previous infection patterns hold true, it would suggest that factors other than stomatal occlusion may be relevant to infectability, but this relationship was not tested in the current study.

Needle Wettability—The distribution and amount of water on a needle is important for basidiospore germination (Spaulding and Rathbun-Gravatt 1926; Hansen and Patton 1977). Thus, the wettability of a needle will likely affect infectability because "nonwettable" surfaces hold more water and for a longer time than "wettable" surfaces (Leyton and Juniper 1963; Cape 1983; Haines and others 1985). In routine rust screenings in 1989 and 1992, water tended to bead up more and remain longer on seedlings from the Coeur d'Alene nursery compared to seedlings from the Lewiston nursery; they also contracted higher infections (Eramian, personal communication). In our study, however, the contact angles on needles from the Lewiston nursery were significantly higher than those from the Coeur d'Alene nursery suggesting either that nursery regimes have changed since the mid 1990s or that is the contact angle of water droplets is not informative with regard to predicting levels of infection with blister rust fungus. We did not, however, test these hypotheses in this study.

We hypothesized that the amount of wax on the needle surface would be associated with needle wettability. Surface waxes on needles of Scots pine are more hydrophobic than the cuticle itself (Cape 1983), but the amount of wax is not critical to the hydrophobic properties of a leaf surface (Silva Fernandes 1965; Holloway 1969a and 1969b). In our study, the amount of extracted surface wax, expressed over needle dry weight, varied among nurseries and did appear to be associated with differences in needle wettability.

Genetics Study

Perhaps the most noticeable (and potentially the most important) finding came from the genetics study, in which the susceptible families had larger and rounder stomata and smaller contact angles of surface water droplets than either or both the resistant families and the seed orchard bulk lots.

Gansel (1956) investigated seven white pine needle traits based on the hypothesis of direct penetration of *C. ribicola* germ tubes through the epidermis but found no differences in the traits between four uninfected (but untested) and four susceptible individual trees growing in natural stands. Our results are generally consistent with Gansel's findings for needle traits other than stomatal size.

Stomatal Traits—Stomatal width and area may be important and potentially definitive traits that distinguish trees with "reduced needle lesion frequency" from those that are genetically susceptible. It has been suggested that the reduced needle lesion frequency may be related to occlusion of stomata by wax, which was hypothesized to reduce the chance of infection of *Pinus strobus* L. by *C. ribicola* (Patton and Johnson 1970; Patton and Spear 1980). However, we found no evidence that resistant families had different proportions of stomatal occlusion compared to susceptible families. Most of the observed stomatal chambers were occluded in all sampled families and lots.

Surface Waxes—At the time we sampled the seedlings, which was 3 years after inoculation, we found no differences among families and bulked lots in the proportions of degraded epistomatal wax on needles. It is possible that there were differences among families in the amounts of degraded wax and/or the proportions of occluded stomata at the time the seedlings were inoculated, possibly due to differences in wax chemistry or different melting temperatures. Alternatively, environmental factors may play a greater role than genetic differences in epistomatal wax degradation and stomatal occlusion (Cape 1983).

Needle Wettability—Previous research has indicated that the most important factor in basidiospore germination and needle infection of *Pinus strobus* L. with *C. ribicola* is the distribution and amount of free moisture on the needle surface during incubation (Spaulding and Rathbun-Gravatt 1926; Hansen and Patton 1977). However, our finding of larger contact angles of the water droplets on needles of the resistant families compared to both the susceptible families and the R.T. Bingham Seed Orchard bulked lots, whose contact angles were similar to each other, suggests relatively more moisture was retained on needles of the resistant families. Unless wettability changed differentially among the families from the time of inoculation to the time they were sampled, infectability does not appear to be a simple function of needle wettability. We note too that trees in the R.T. Bingham Seed Orchard were selected for resistance mechanisms other than low needle lesion frequency (although many of the trees exhibit this resistance trait as well) (Bingham 1983). Thus, if there were a relationship between needle wettability and specific resistance mechanisms, it would not be surprising to find differences in wettability between the Moscow bulks and the resistant families.

Summary and Conclusions

Most of the needle traits examined in these studies exhibited significant phenotypic plasticity, varying across samples of the same genetic stock grown in three nurseries. But only a few of the traits varied genetically (that is, across stocks of different rust resistance types grown in a single nursery). Although not tested in this study, our results suggest that variation in stomatal traits and/or the characteristics of surface water on pine needles may be critical features in the dynamics of blister rust infection. If some nursery regimes produce seedlings with needle surface characteristics similar to those of resistant genotypes, such seedlings may exhibit lower initial infection rates when planted under field conditions, even in “high rust hazard” areas. Nonetheless, such an effect would likely be short-lived as the needles produced in the nursery senesced and new ones were produced under field conditions. Furthermore, seedlings produced under these nursery regimes would be unsuitable for use in rust screening procedures, as initial infection is required for detection of most of the rust resistance traits.

Whether the variations in needle surface properties we observed are associated with differences in infectability by white pine blister rust has yet to be determined. Differences in infectability are not likely to be associated with stomatal occlusion, which did not vary among nurseries or between resistant and susceptible families. Larger wax deposits were associated with higher contact angles and lower wettability on western white pine needle surfaces. This apparent relationship may reflect a broader or more even distribution of wax as the quantities increased. Factors such as chemical composition of the waxes or surface roughness may play an important role in surface wettability and may be related to fungal spore attachment and germination. The physiological condition of seedlings may also be related to infectability because *C. ribicola* absorbs nutrients from its hosts.

Further exploration of nursery environments and growth regimes for effects on the morphology of seedling needles is warranted. Additional studies are needed to explore the relationships between the chemical and physical nature of needle surfaces (particularly at the time of inoculation) and their infectability by the blister rust fungus. Finally, the initial finding of differences in stomatal size and shape between resistant and susceptible families should be more fully explored and verified using a larger number of families.

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