New Forests **24:** 113–129, 2002.
© 2002 *Kluwer Academic Publishers. Printed in the Netherlands.*

Effects of nursery environment on needle morphology of *Pinus monticola* Dougl. and implications for tree improvement programs

KWAN-SOO WOO[1], LAUREN FINS[1,*], GERAL I. MCDONALD[2], DAVID L. WENNY[1] and ARAM ERAMIAN[3]

[1]Department of Forest Resources, University of Idaho, Moscow, Idaho 83844-1133, USA; [2]USDA Forest Service, Rocky Mountain Research Station, 1221 South Main Street, Moscow, Idaho 83843, USA; [3]USDA Forest Service, Coeur d'Alene Nursery, 3600 Nursery Road, Coeur d'Alene, Idaho 83814, USA; [*]Author for correspondence (e-mail: woo9431@uidaho.edu; phone: (208) 885-9158; fax: (208) 885-6226); (e-mail: lfins@uidaho.edu; phone: (208) 885-7920; fax: (208) 885-6226); (e-mail: gimcdonald@fs.fed.us; phone: (208) 883-2343; fax: (208) 883-2318); (e-mail: dwenny@uidaho.edu; phone: (208) 885-7023; fax: (208) 885-6226); (e-mail: aeramian@fs.fed.us; phone: (208) 765-7372; fax: (208) 765-7474)

Received 24 April 2001; accepted in revised form 17 December 2001

Key words: *Cronartium ribicola* J. C. Fisch. ex Rabenh, *Pinus monticola* Dougl., Stomatal occlusion, Wettability, White pine blister rust

Abstract. Statistically significant differences were found in 14 needle traits of western white pine (*Pinus monticola* Dougl.) seedlings grown from the same seed orchard source in the three nurseries in northern Idaho. Traits with significant variation included 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 placed on adaxial needle surfaces. Wax crystallites on needle surfaces were hollow and tubular and the amount of surface wax appeared to be associated with surface wettability. Our results may have important implications for tree improvement programs that require successful inoculation of nursery-grown seedlings with spores of *Cronartium ribicola* J. C. Fisch. ex Rabenh. to reliably screen white pines for resistance to blister rust.

Introduction

Blister rust is a devastating disease of western white pine (*Pinus monticola* Dougl.) that is caused by the fungus *Cronartium ribicola* J. C. Fisch. ex Rabenh., which requires alternating hosts of pines and *Ribes* species to complete its life cycle. Infection occurs when basidiospores of the rust germinate on needle surfaces and germ tubes enter the needles through their stomata. White pine blister rust was inadvertently introduced into eastern North America from Europe on imported nursery stock about 1898 and into western North America in 1910 (Spaulding 1922; Benedict 1981; Fins et al. 2001; McDonald and Hoff 2001). The disease appears to have reached Idaho's forests in 1923 (Mielke 1943). Of the original five million acres of white pine cover type in the Inland Northwest (northern Idaho, northwestern Montana, northeastern Washington, northeastern Oregon, and southeastern British

Columbia), only 5 to 10% still carries a major component of white pine, largely as a result of decimation by blister rust (Fins et al. 2001). Efforts to restore white pine to inland northwestern ecosystems rely on seeds produced by a genetic improvement program designed to increase resistance to blister rust.

Part of the genetic improvement program entails routine evaluations of white pine seedlings for rust resistance after they have been inoculated with *C. ribicola* basidiospores. The seedlings are usually grown in the nursery where the inoculation takes place, but on several occasions, seedlings grown in other nurseries were also subjected to the inoculation and screening procedure. Casual observations of morphological differences between these stocks suggested possible links to differences in infectability. For example, water beaded up and accumulated on needles of the relatively ''infectable'' seedlings from one nursery, but little water collected on the thick-needled, relatively difficult-to-infect seedlings grown in another. Since rust infection occurs through stomata in the needles and rust spore germination depends largely on moisture availability (Huttunen 1984), the physical differences suggested the possibility that in addition to the genetic constitution of the seedlings, differences in nursery environments and growing regimes may affect needle surface structure and needle chemistry in ways that limit or promote inoculation success. If so, the findings would have crucial implications for the white pine blister rust screening program, which hinges on detecting genetic differences among stocks.

Water collection on needle surfaces is essential for fungal infections (Huttunen 1984), and is determined by wax chemistry, surface roughness, and the amount of air between the water droplet and the needle surface (Van Overbeek 1956; Leyton and Juniper 1963; Leyton and Armitage 1968; BukoVac et al. 1981; Cape and Fowler 1981; Cape 1983; Haines et al. 1985; Schreiber 1996). Contact angle of water droplets, a measure of needle wettability (high contact angles = low wettability), can be used to indicate the amount of water that collects on needle surfaces. Variation in wax deposits and other needle surface traits, and the number, size and distribution of stomata, are also thought to be related to variation in infectability of white pine by blister rust (Patton and Johnson 1970; Patton and Spear 1980).

The quantity, quality, and distribution of needle waxes vary between species, within species, and between needles on individual trees (Hanover and Reicosky 1971; Sauter and Voss 1986). These wax deposits are related to age and physiological condition of the trees. They protect needle tissues against ultraviolet light and other damaging agents, and minimize cuticular transpiration (Cape et al. 1989; Gordon and Percy 1999). Degraded waxes on needle surfaces are associated with higher probabilities of infection by parasites (Huttunen 1984; Elstner et al. 1985).

The objective of our study was to describe and assess differences in needle surface traits of western white pine seedlings that may be related to differences in infectability by white pine blister rust and which may be influenced by differences in nursery environment. Our hypothesis was that important needle surface traits would vary significantly on seedlings of the same genetic stock grown in different nurseries.

Table 1. Nurseries, treatments and sample collection information.

	Nurseries		
	Coeur d'Alene	Lewiston	Moscow
Latitude	47° 37′ N	46° 23′ N	46° 44′ N
Longitude	116° 45′ W	117° 01′ W	117° 00′ W
Elevation (m)	675	426	789
Container size	3.8×20 cm; 164 cm^3	3.8×20 cm; 164 cm^3	6×15 cm; 336 cm^3
Media	Peat moss:Sawdust (70:30)	Peat moss:Vermiculite (50:50)	Peat moss:Vermiculite (50:50)
Fertilization[1] (N-P-K)	9-45-15 20-7-19 4-25-35	7-40-17 20-7-19 27-15-12 4-25-35	7-40-17 20-7-19 27-15-12 4-25-35
Acid Rinse	No	No	Yes
Sowing date	June 1997	late March 1997	early March 1997
Surfactant	No	No	Yes[2]
Min/max temp. (°C)[3]	6.2 – 31.4	13.2 – 33.6	11.8 – 26
Sample collection date and time	September 21, 1998 (9~10 a.m.)	September 22, 1998 (9~10 a.m.)	September 23, 1998 (9~10 a.m.)

[1]/ Fertilization regimes are changed during the growing season as appropriate to the seedlings' physiological state.[2]/ R-11 Spreader Surfactant applied twice weekly from July through September.[3]/ Mean minimum and maximum temperatures in greenhouse during May to September of a year

Materials and methods

Western white pine seedlings were grown for two years (1997–1998) in 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. The Coeur d'Alene and Lewiston nurseries are separated (north to south) by about 200km, with the Moscow nursery between them at about 55km north of the nursery site in Lewiston. Of the three, the Lewiston nursery is lowest in elevation, with higher mean minimum and maximum temperatures in the greenhouses between May and September than at either of the other two nurseries. Specific locations and differences among nurseries in container size, media and fertilizer treatments and temperature regimes are shown in Table 1.

The nurseries used different fertilizers, rates and application frequencies to suit their particular environment and objectives. The seedlings used in the study were grown with regular nursery production stock and the exact per-seedling amounts of nutrients were not monitored. Seedlings were watered and fertilized with either fixed overhead systems or traveling booms. Only one of the nurseries used a surfactant (surface active agent) (Moscow); one used sawdust in the medium (Coeur d'Alene), the other two used vermiculite. The sowing dates at the Coeur d'Alene nursery were about 2 and 3 months later than at Lewiston and Moscow, respectively. The Moscow nursery used an acid rinse H_3PO_4(Table 1).

All seedlings originated from bulked collections of open-pollinated seeds collected from the R.T. Bingham White Pine Seed Orchard in Moscow, Idaho. This

stock has been reported as approximately 66% resistant to blister rust at 2.5 years after inoculation (Hoff et al. 1973).

Morphometric traits and stomatal wax

Samples for measurement of morphometric traits and surface waxes were collected in September 1998. For most of the morphometric traits, samples consisted of three secondary needles per seedling, one needle from each of three directions on the current stem of each of 30 seedlings per nursery. Additional samples, consisting of two secondary needles per seedling (one from each of two fascicles) from 25 seedlings per nursery, were used to assess stomatal size (major and minor axes), area and stomatal wax.

Stomatal characteristics were assessed using two 1 mm square sample plots located at random distances from the center of each of the two adaxial surfaces (total of four 1-mm plots per needle). Eight needle characteristics were evaluated using a stereo microscope at 62.5X. Needle length was measured with a ruler to the nearest 1.0 mm. A calibrated grid (eyepiece) was used to measure the width of the center portion of each adaxial side of the needles to the nearest 0.05 mm and to count the number of stomata per mm. Measurements of needle length and width were used to estimate adaxial surface area. The number of stomatal rows in each plot was used to determine average number of stomatal rows per plot on each needle. In each plot, the number of stomata per row in two randomly selected rows was used to calculate the average number of stomata per row. The total number of stomata per needle was estimated using the assumption that needles were rectangular with a constant number of stomatal rows. Some needles had a small number of stomata on the abaxial side, but only stomata on the adaxial sides were used to determine stomatal density.

Length and width (major and minor axes) of stomata were measured to the nearest 1 mm directly through the monitor of a scanning electron microscope (Hitachi S-570 SEM at 10–15 kV, 500X, 15 mm working distance). The center portion of each needle was prepared for SEM by cryofixation in a precooled cryovessel and lyophilized overnight at −55 °C for 12 h (Larsen and Green 1992). Dried needle specimens were coated with 1-to 2-nm gold (Au) in a Polaron sputter coater. Stomatal areas were calculated according to Mitton et al. (1998):

$$\text{Stomatal Area} = 3.14 \times (\text{length of major axis}/2) \times (\text{length of minor axis}/2) \quad (1)$$

The average values of the major and minor axes of each seedling's stomata were based on 20 measurements (five randomly selected stomata on each of two randomly selected stomatal rows on one adaxial side of each of two needles per seedling). Mean stomatal areas, the ratios of the major to minor axes, and stomatal wax characteristics were calculated from measurements of 500 stomata (20 stomata on each of 25 seedlings) per nursery.

The general morphology, structure and distribution of surface waxes were

observed using a stereomicroscope and SEM with a range of magnifications. Stomata were classified as either occluded (blockage of the stomatal opening by wax) or partially open. The overall condition of the waxes was observed using an SEM at 2000X; higher magnifications (5000X – 7000X) were used for detail. Epistomatal waxes in the stomatal area were classified by percentage of fine crystallites vs. fused or platy (degraded) wax using the 5-category classification method suggested by Trimbacher and Eckmullner (1997).

Surface waxes were extracted and quantified from two sets of samples. Samples consisted of one current year needle fascicle from each of five randomly selected seedlings in each nursery. Needles were immersed in 40 ml chloroform ($CHCL_3$) for five seconds. SEM observations indicated that a five-second dip was sufficient to remove the wax from most needles, but additional immersion time was required for large needles. The $CHCL_3$-dissolved wax was filtered through a Whatman No. 1 filter paper into 20 ml glass scintillation pre-washed $CHCL_3$ vials, and evaporated to reduce volume (Percy et al. 1992; Gordon et al. 1998). The samples were dried overnight on a heater (40 °C) within a fume hood.

Needles were oven-dried for 24 h at 70 °C and cooled in a desiccator for 40 min, then weighed to the nearest 0.01 mg to assess needle dry weight. Wax quantity per dry weight of needle was calculated as:

$$\text{Extracted wax weight}(\mu\text{g})/\text{Dry weight of needle(mg)} \quad (2)$$

Needle wettability

Wettability of current-year needles was evaluated as a function of the contact angles of water droplets placed on their two adaxial surfaces (xylem side). Samples consisted of one needle from each of three fascicles from each of 30 seedlings per nursery (total 90 needles per nursery). Samples were collected at approximately the same time of day in each nursery (one-day interval between nurseries) and stored in plastic bags at 4 °C prior to use.

Needles were brought to room temperature and surfaces allowed to dry. Using a micropipette (or ml syringe) and a dissecting microscope (50X), two droplets of distilled water (2 ml) at room temperature (22 °C) were placed at the centers of both adaxial surfaces of each needle, or at other locations of the adaxial needle surfaces if droplets did not adhere at the center. Contact angles were measured with a protractor on a television monitor (enlarged X75) that was connected to a dissecting microscope fitted with a light source and camera. Both the left and right angles of each water droplet were measured.

To evaluate the effect of surfactant on needle wettability, contact angles of water droplets were measured on 30 needles each (three from each of 10 seedlings) from the Coeur d'Alene nursery (no surfactant used during seedling production) and the Moscow nursery (surfactant used) before and after rinsing the needles with cool distilled water for 15 to 25 s. Surface water was evaporated from the needles at room temperature before water droplets were applied and measured. Contact angles were

also measured on wax-extracted needles to determine how waxes and cuticle differentially affected needle surface wettability.

Statistical analyses

Data were analyzed using multivariate analysis of variance (MANOVA), canonical variate analysis, pairwise comparisons (contrast), and LSMEAN (Least Squares Means; multiple comparisons) procedures using the SAS-PROC GLM (general linear model) statistical package (SAS Institute Inc. 1989). All MANOVAs used Roy's Greatest Root statistics (λ; λ_{max}). The exploratory PROC UNIVARIATE test was done on all dependent variables to determine whether they satisfied the assumption of a normal distribution. When distributions of the variables were not normal, residual ($r_{ijk} = y_{ijk} - \hat{y}_{ijk}$) values were used as the new data for the univariate test.

MANOVA with completely randomized hierarchical design was used to determine whether differences were significant among nurseries in needle characteristics using the following experimental models:

$$\underset{(8x1)}{\underline{Y}_{ijk}} = \underset{(8x1)}{\underline{\mu}} + \underset{(8x1)}{\underline{\alpha}_i} + \underset{(8x1)}{\underline{\beta}_{j(i)}} + \underset{(8x1)}{\underline{\varepsilon}_{k(ij)}} \quad (3)$$

where $\underline{Y}_{ijk}$ is the observation of the k^{th} needle of the j^{th} seedling in the i^{th} nursery, $\underline{\mu}$ is the overall sample mean, $\underline{\alpha}_i$ is the effect of i^{th}nursery, $\underline{\beta}_{j(i)}$ is the effect of the j^{th} seedling nested in the i^{th} nursery, and $\underline{\varepsilon}_{k(ij)}$ is the residual representing variability within seedlings. The null and alternative hypotheses of the MANOVA are the following:

$$H_o: \underline{\alpha}_1 = \underline{\alpha}_2 = \underline{\alpha}_3$$
$$H_1: \text{at least one } \underline{\alpha}_i \neq \underline{\alpha}_{i'} (i = 1, 2, 3)$$

Canonical variate analysis (canonical correlation analysis) was used to determine the combinations of canonical variables that affect the significance of the MANOVA test. From the SAS output, 'Within Canonical Structure' and 'Standardized Canonical Coefficients' were used to determine the important variables. Univariate analysis using GLM procedures was used to determine differences among nurseries and seedlings within nurseries for each trait.

Data on stomatal occlusion and wax structure (degradation) were analyzed using the SAS-PROC GLM statistical package. Data on the proportion of occluded stomata were transformed by arcsine to normalize the distribution as:

$$Y = \arcsin \sqrt{P} \quad (4)$$

where P = proportion of occluded stomata per seedling and Y is a new variable (SAS Institute Inc. 1989; Ott 1993).

Data on the condition of epistomatal waxes were analyzed by analysis of variance (ANOVA) using the SAS-PROC GLM statistical package and type III sums of squares (SAS Institute Inc. 1989). The GLM procedure was also used to evaluate

Table 2. Means and standard errors of western white pine needle traits

Variables[1]	No. of needles	Nurseries			P-value[2]
		Coeur d'Alene (mean ± SE)	Lewiston (mean ± SE)	Moscow (mean ± SE)	
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 axes (μm)	150	59.5 ± 0.61	54.8 ± 0.55	57.9 ± 0.66	0.0001
Minor axes (μm)	150	33.2 ± 0.27	33.1 ± 0.31	33.0 ± 0.28	0.9369
Major/Minor	150	1.80 ± 0.03	1.66 ± 0.02	1.76 ± 0.02	0.0001
Mean stomatal area (μm^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 (μg/mg)	75	6.26	8.48	4.38	0.0002
Wax per surface area ($\mu g/mm^2$)	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

[1]/ Stomata/needle: (stomatal rows/plot x stomata/row x needle length x 2); Adaxial surface area: needle length x (needle width 1 + needle width 2); Stomatal density: (stomata/needle)/(adaxial surface area); Stomatal area (πab)= 3.14 × (major axis/2) × (minor axis/2); Wax per dry weight (weight of wax per dry weight of needle); Wax per area: total leaf area= $(2\pi RL/n) + 2RL$ where, L = the length of the needle (mm); R= half of the diameter of the fascicle (mm); n= the number of needles per fascicle; π= 3.14. [2/]P-values are tests of significance between nurseries.

differences in contact angles of water droplets among nurseries and among seedlings within nurseries. In all tests, when differences among nurseries were significant at $P < 0.05$, least squares means procedures were used to test differences among means of nursery samples for specific variables.

Results

Morphometric traits

Nursery environment clearly affected most of the needle and stomatal traits evaluated (Table 2). The exceptions were minor axes of stomata ($P=0.94$) and the amount of wax per surface area ($P=0.07$). Nearly all of the samples from the Moscow nursery had stomata on their abaxial sides (97%), which was more than two and three times the percentages for the Lewiston and Coeur d'Alene samples (42 and 27% respectively). The numbers of abaxial stomata were small (10 – 50) compared to adaxial needle surfaces, with most on distal ends of the needles. Mean length of the major axes of stomata differed significantly among nursery samples (longest from Coeur d'Alene; shortest from Lewiston), but mean stomatal width was

nearly identical. Thus, the greater mean stomatal area and higher ratio of major/minor axes of stomata on needles from the Coeur d'Alene nursery were primarily a function of longer primary axes of their stomata (Table 2).

Wax characteristics

Needles from the Moscow nursery had the smallest amount of wax when compared on a basis of either needle dry weight (P=0.0002) or needle surface area (P=0.07) (Table 2). Within a needle, it was common to find larger deposits of waxes along the stomatal rows and over epistomatal chambers. Generally, more wax was visible on the distal third of the adaxial sides than in the middle or at the proximal end. Wax crystallites appeared either tubular with hollow centers or fused and platy (Woo 2000; Woo et al. 2001).

Needles from the Lewiston nursery had the smallest percentage of occluded stomata (56%), compared to 86% and 90% on samples from Coeur d'Alene (P=0.0001) or Moscow (P=0.0001), respectively (Figure 1). The latter two did not differ significantly from each other (P=0.47). The mean length of the openings of partially opened stomata from the Moscow nursery (24.42 mm) was greater than that from the Coeur d'Alene nursery (22.69 mm) and the Lewiston nursery (22.84 mm).

Epistomatal wax degradation on current-year needles varied from less than 10% to more than 75% (Figure 2). Average levels of epistomatal wax degradation differed significantly among nurseries (P=0.0003) and among seedlings within a

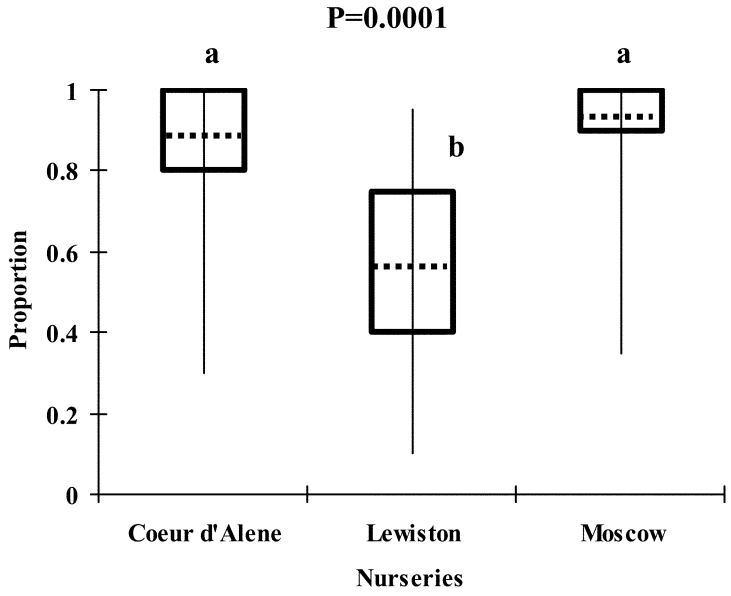


Figure 1. The proportions of stomatal occlusion on needles of western white pine from three nurseries in northern Idaho.

nursery (P=0.02). Samples from the Moscow nursery had statistically higher levels of degraded wax than those from either the Coeur d'Alene nursery or the Lewiston nursery (P=0.0002 and P=0.001, respectively), which were not statistically different from each other (P=0.55). Relatively little degraded wax appeared on most

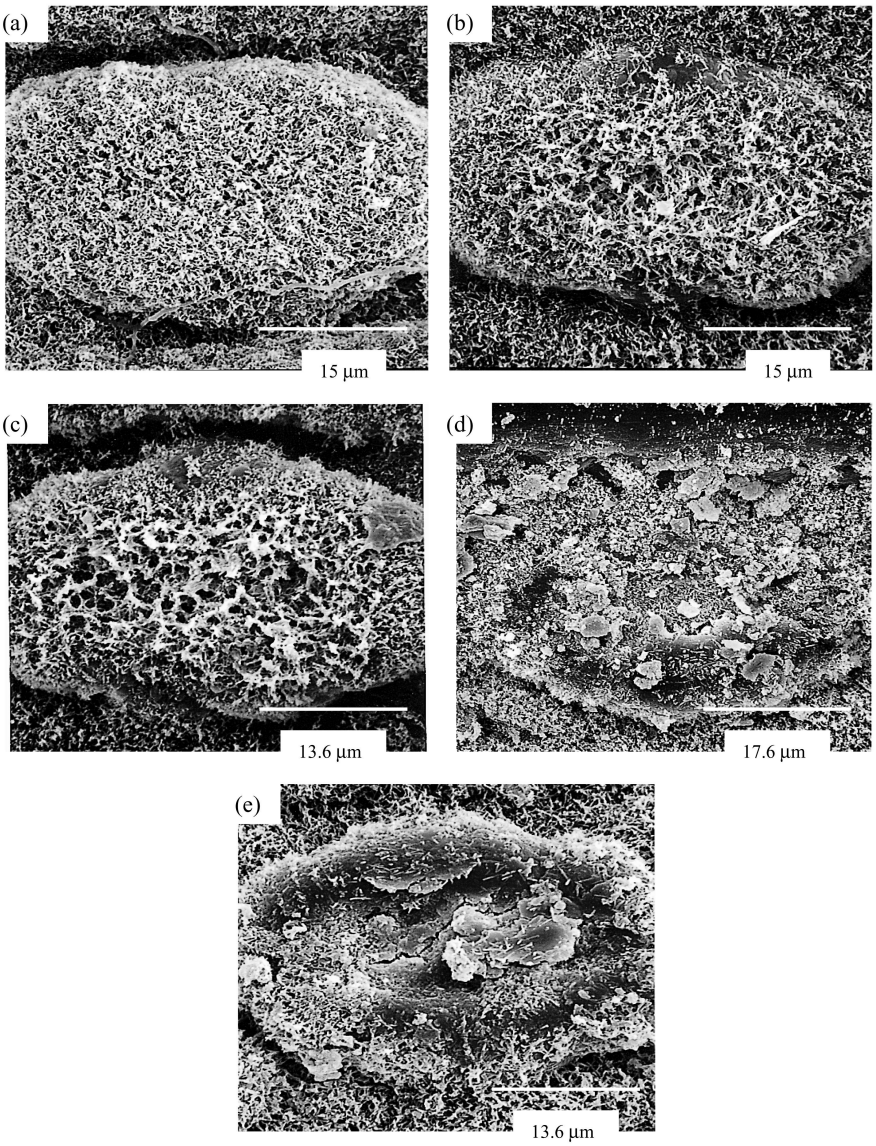


Figure 2. Percent of degraded epistomatal wax of current-year needles of western white pine: (a) Less than 10%; (b) 10 to 25%; (c) 25 to 50%; (d) 50 to 75%; (e) greater than 75%.

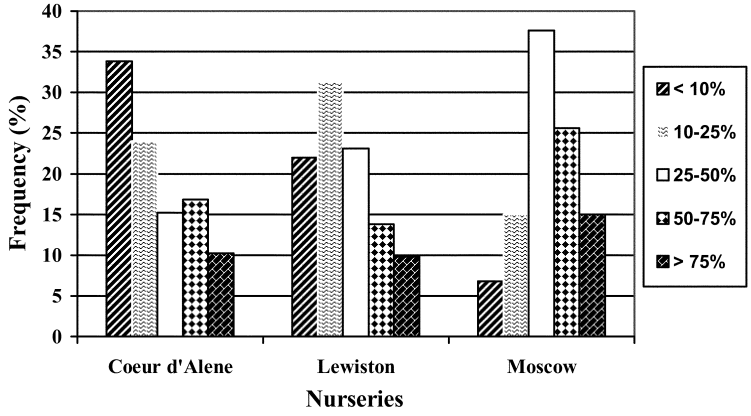


Figure 3. Epistomatal wax degradation on western white pine needles of seedlings grown in three nurseries in northern Idaho. Levels of degradation of epistomatal waxes: 1- less than 10%; 2–10 to 25%; 3–25 to 50%; 4–50 to 75%; 5- greater than 75%.

samples from the Coeur d'Alene nursery whereas most samples from the Moscow nursery had moderate to high levels of wax degradation (Figure 3).

Needle wettability

Mean contact angles of water droplets on needle samples differed significantly among the three nurseries both before ($P=0.0001$) and after ($P=0.005$) removing surface waxes with chloroform with the smallest angles on the Moscow samples (Table 2). The shapes of water droplets reflect the observed differences in contact angles (Figure 4). Contact angles on needle samples with wax also differed among individual seedlings within nurseries ($P=0.0001$), and between rinsed and unrinsed needles from the Moscow nursery ($P=0.0001$), but not between rinsed and unrinsed samples from the Coeur d'Alene nursery ($P=0.56$) (Figure 5). Contact angles were higher on samples from both the Lewiston and Moscow nurseries when their needles had surface waxes, but were higher on samples without wax from the Coeur d'Alene nursery (Table 2).

Discussion and conclusions

The observed differences in white pine needle traits were clearly associated with differences in nursery environments and/or growth regimes since the genetic source of the seedlots was the same in all three nurseries.

The larger needles of the Moscow nursery seedlings likely reflect the larger containers in which they were grown and differences in other environmental factors such as fertilization and temperature regimes. Although container size is an unlikely factor in variation in infection intensity (Kinloch 1980), differences in fertilization

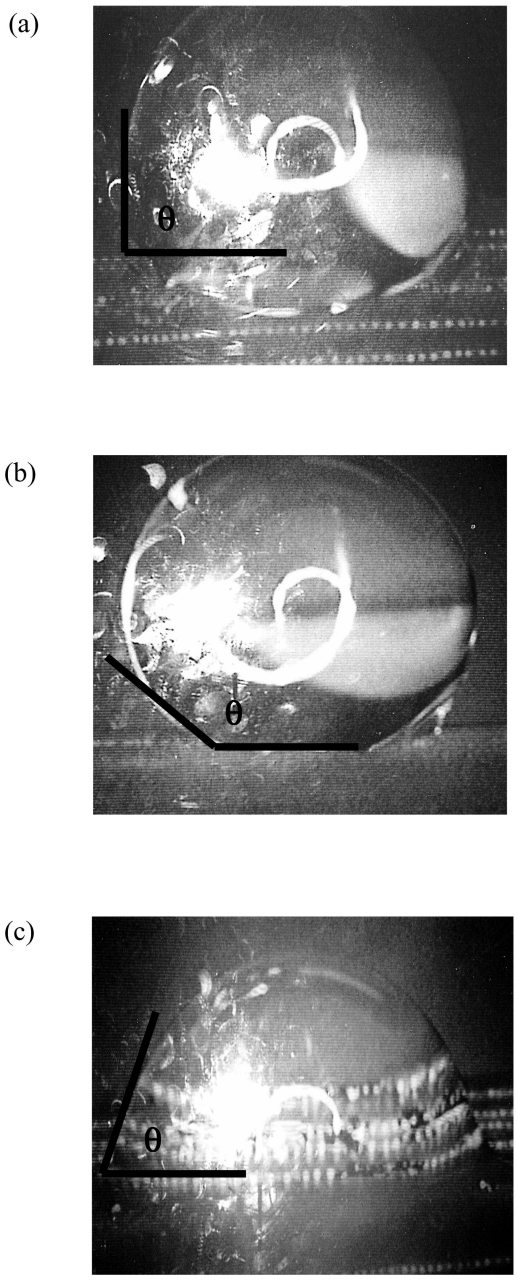


Figure 4. Contact angles of water droplets on needles of western white pine seedlings grown in three nurseries in northern Idaho. (a) Coeur d'Alene nursery (92° left; 94° right); (b) Lewiston nursery (137° left; 112° right); (c) Moscow nursery (67° left; 71° right;).

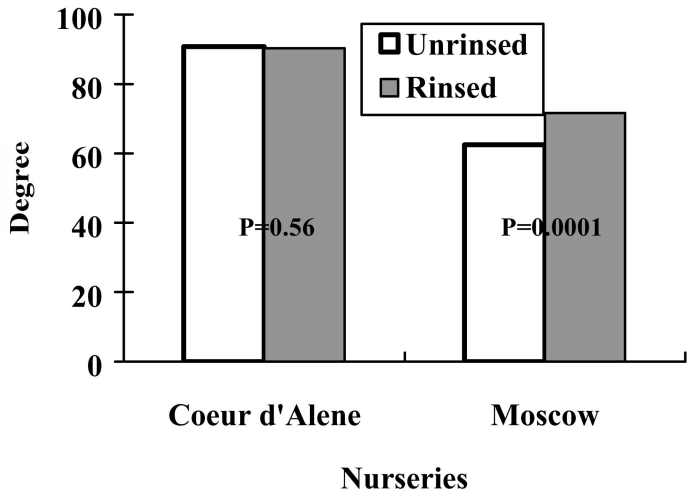


Figure 5. Mean contact angles of water droplets on rinsed and unrinsed needles of western white pine seedlings grown in two nurseries in northern Idaho.

regimes may well be related to initial infectability of white pine with blister rust, as has been reported for loblolly and slash pines with fusiform rust (Schmidt et al. 1972; Blair and Cowling 1974; Rowan and Steinbeck 1977).

Since the blister rust fungus enters white pine needles through stomata and subsequently develops substomatal vesicles and infection hyphae (Patton and Johnson 1970), differences in stomatal numbers and traits (particularly size and shape) may be the most critical factors in initial infection. Although the numbers of stomata per row varied significantly among nurseries, the actual difference between the largest and smallest means was relatively small (about 5%). The influence of nursery on number of stomatal rows per plot was much greater, however, with 37.5% and 50% more stomatal rows per plot on the Lewiston and Moscow seedlings than on the Coeur d'Alene seedlings. The samples from the Moscow nursery had the largest number of stomata per needle, which appeared to be mostly a function of needle size; the samples from the Lewiston nursery had the highest stomatal density. The latter was associated with a relatively high number of rows of stomata coupled with relatively small needle width (the narrowest of the three nurseries). Such differences in numbers of stomata and stomatal density are potentially important to the infection process as they may change the probability that a germinating rust spore will find an entry point to the needle.

A study comparing resistant and susceptible families of western white pine showed the stomata of susceptible families to be larger than those of resistant families (Woo et al. 2001). If relative stomatal size proves important, we would expect seedlings from the Coeur d'Alene nursery, with their larger stomata, to have higher levels of initial infection compared to the Moscow and Lewiston nurseries. However, stomatal area of the Coeur d'Alene samples in this study was still considerably smaller (by about 8%) than mean stomatal area of susceptible families

in the genetics study. Although the stomata of samples from Coeur d'Alene in the current study were slightly longer than those of susceptible families, the latter were considerably wider. These differences in stomatal size are interesting in that all the seedlings in the genetics study were grown at the Coeur d'Alene nursery. One possible explanation lies in the difference in age of the sampled seedlings: samples for the genetics study were collected from 5-year-old seedlings, whereas those for the nursery study were from 2-year-olds.

Differences in stomatal behavior may also play a significant role in levels of blister rust infection. For example, a seedling may be 'functionally resistant' to blister rust if its stomata remain closed or close relatively quickly during periods when blister rust germ tubes normally enter and grow into stomatal openings (Hart 1929; Hirt 1938). If size and shape of stomata are related to the timing and efficiency with which they open and close, then it is easy to see how initial infection levels in seedlings would vary with the nursery in which the seedlings were grown.

Waxes on conifer needles serve as a defense mechanism, reflecting UV-B radiation and intense light in the visible range, reducing transpiration, shedding water that might otherwise promote the growth of fungi and reducing the time pollutants remain on needle surfaces (Gordon and Percy 1999). But the amounts, chemical composition and structure of epicuticular waxes are not fixed; they are influenced by the environmental conditions under which conifer seedlings are grown and to which they are subsequently exposed (Gordon and Percy 1999; Chiu et al. 1992). For example, exposure to high temperatures increased the amounts of epicuticular needle waxes on 4 species of *Picea* (Cape and Percy 1993); fertilization with N and K altered the morphological structure of epicuticular waxes on *Pseudotsuga menziesii* (Mirb.) Franco seedlings (Chiu et al. 1992); and exposure to wind increased wettability of spruce needles, possibly due to removal of wax crystallites from the needle surfaces and/or deposition of hydrophilic particles (Cape and Percy 1993). Changes in the properties of epicuticular waxes, such as those described here, potentially alter the ability of fungal spores to adhere to and germinate on needle surfaces, thus altering the apparent and immediate ability of seedlings to avoid infection by fungal pathogens such as blister rust.

The nurseries differed not only in the nutrient regimes that were applied but also in other features that may have affected surface traits, such as the media in which they grew the seedlings (peat moss:sawdust at Coeur d'Alene, peat moss:vermiculite at the other two), pH of the media (Moscow used H_3PO_4 in all irrigation and rinse applications), the twice weekly application of a surfactant (only at the Moscow nursery, from July through September), and the temperatures to which the seedlings were exposed during the growing season (highest minimum and maximum in Lewiston). The influences of these factors were not assessed in this study, but it is likely that the larger amounts of surface wax in Lewiston were associated with higher mean temperatures (Cape and Percy 1993) while the lower mean contact angle of surface water on the Moscow seedlings was associated with the application of surfactants.

Some of our findings, such as the presence of structural (fibrillar, netted, or thread-like), amorphous (platy, ridged, or encrusted) and tubular wax crystallites

with hollow centers have previously been observed on other conifer needles (Leyton and Juniper 1963; Leyton and Armitage 1968; Hanover and Reicosky 1971; Trimble et al. 1982; Crossley and Fowler 1986; Günthardt-Goerg et al. 1994). However, the greater abundance of wax on the distal third of the adaxial sides of the needles compared to the central and proximal portions has not been previously reported.

Both the amount of surface wax per dry weight of needle and the contact angles of water droplets on needle surfaces followed the same order as the recorded high temperatures of the three nurseries (Lewiston > Coeur d'Alene > Moscow) (Table 1). However, the amount of wax per unit of surface area followed a different order (Coeur d'Alene > Lewiston > Moscow). The latter results are consistent with studies of broad-leaf species in which the amount of wax on leaves was not critical to the hydrophobic properties of a leaf surface (Silva Fernandes 1965; Holloway 1969a, 1969b). In either case, the Moscow seedlings, which were exposed to the lowest maximum temperature, had the largest needles and the smallest relative amount of wax. If the amount of surface wax is inversely related to infectability with blister rust, we would expect the Moscow seedlings to be more easily infected than those from the other nurseries.

The higher proportions of degraded (amorphous) wax on needles from the Moscow nursery may be related to the application of surfactants or possibly the regular acid rinses or other environmental factors such as dust or wind. Occlusion of stomatal antechambers on white pine needles may reduce the chance of blister rust infection (Patton and Johnson 1970; Patton and Spear 1980); if so, we would predict higher infection levels on seedlings from the Lewiston nursery than on those from the other two nurseries (Figure 1).

Our comparisons of rinsed and unrinsed white pine needles from the Coeur d'Alene and Moscow nurseries (Figure 5) support previous findings that surfactants can significantly alter contact angles of water droplets (Ford et al. 1965). Huttunen (1984) suggested that water collection on the needle surface is an essential factor for initial fungal infections. If infectability by blister rust is a function of amount of surface water, and if the amount of surface water is inversely related to the contact angle of water droplets, then seedlings from the Moscow and Lewiston nurseries are predicted to have the lowest and highest infectability, respectively.

Whether the differences we observed in white pine needle surface properties are associated with differences in infectability by *C. ribicola* spores has yet to be determined. If differences in infectability exist, they are not likely to be associated with stomatal occlusion by waxes, which did not vary significantly among nurseries in our study. However, infectability may be related to differences in the amounts, chemistry and degradation of surface waxes, wettability and roughness of needle surfaces, and density, area, and behavior of stomata. Most, if not all, of these traits appear to be influenced by nursery environment.

Our results have important implications for tree improvement programs in which reliable screening of nursery-grown seedlings for rust resistance is a critical and costly component of the selection process. This screening process requires the successful infection of both test seedlings and ''standard lots'' (whose levels of susceptibility are well characterized, and which are used as a gauge of the success of

the inoculation itself). Ultimately, the ability to differentiate seedlings that have blister rust resistance (other than immunity) from seedlings that are susceptible to rust is based on the expression of resistance traits in a known sequence at specific intervals of time. Thus, successful initial infection of the seedlings is fundamental to this process and any nursery (or other) regime that temporarily inhibits, prevents, or otherwise changes the ''infectability'' of the seedlings by blister would result in unreliable data and thus impair the genetic selection process.

Acknowledgements

The authors thank the Inland Empire Tree Improvement Cooperative for supporting this study. We also thank Sue Morrison and Abbie Acuff for their assistance in growing the seedlings and Dr. Kas Dumroese for his thoughtful review and insightful comments on a previous version of the manuscript.

References

Benedict W.V. 1981. History of white pine blister rust control: a personal account. USDA Forest Service, FS-355, Washington, D.C., 47 p.

Blair R.L. and Cowling E.B. 1974. Effect of fertilization, site, and vertical position on the susceptibility of loblolly pine seedlings to fusiform rust. Phytopathology 64: 761–762.

BukoVac M.J., Rasmussen H.P. and Shull V.E. 1981. The cuticle: Structure and function. Scanning Electron Microsc III: 213–233.

Cape J.N. 1983. Contact angles of water droplets on needles of Scots pine (*Pinus sylvestris*) growing in polluted atmospheres. New Phytologist 93: 293–299.

Cape J.N. and Fowler D. 1981. Changes in epicuticular wax of *Pinus sylvestris* exposed to polluted air. Silva Fennica 15: 457–458.

Cape J.N. and Percy K.E. 1993. Environmental influences on the development of spruce needle cuticles. New Phytologist 125: 787–799.

Cape J.N., Paterson I.S. and Wolfenden J. 1989. Regional variation in surface properties of Norway spruce and Scots pine needles in relation to forest decline. Environmental Pollution 58: 325–342.

Chiu S.T., Anton L.H., Ewers F.W., Hammerschmidt R. and Pregitzer K.S. 1992. Effects of fertilization on epicuticular wax morphology of needle leaves of Douglas-fir, *Pseudotsuga menziesii* (Pinaceae). Am. J. Botany 79: 149–154.

Crossley A. and Fowler D. 1986. The weathering of Scots pine epicuticular wax in polluted and clean air. New Phytologist 103: 207–218.

Elstner E.F., Osswald W. and Youngman R.J. 1985. Basic mechanisms of pigment bleaching and loss of structural resistance in spruce (*Picea abies*) needles: Advances in phytomedical diagnostics. Experientia 41: 591–596.

Fins L., Byler J., Ferguson D., Harvey A., Mahalovich M.F., McDonald G. et al. 2001. Return of the Giants. Idaho Forest, Wildlife, and Range Experiment Station Bulletin 72. University of Idaho, Moscow, Idaho, 20 p.

Ford R.E., Furmidge G.G.L. and Montagne J.T.W. 1965. The role of surface-active agents in the performance of foliar sprays. In: Surface Activity and the Microbial Cell. Society of Chemical Industry, London SCI Monographs No. 19, p. 214.

Gordon D.C. and Percy K.E. 1999. Effect of UV-B dose on biosynthesis of epicuticular waxes in blue spruce (*Picea pungens* Engelmann.) primary needles: preliminary investigation. Water, Air and Soil Pollution 116: 429–436.

Gordon D.C., Percy K.E. and Riding R.T. 1998. Effects of U.V.-B radiation on epicuticular wax production and chemical composition of four *Picea* species. New Phytologist 138: 441–449.

Günthardt-Goerg M.S., Keller T., Matyssek R. and Scheidegger C. 1994. Environmental effects on Norway spruce needle wax. Eur. J. For. Path. 24: 92–111.

Haines B.L., Jernstedt J.A. and Neufeld H.S. 1985. Direct foliar effects of simulated acid rain. II. Leaf surface characteristics. New Phytologist 99: 407–416.

Hanover J.W. and Reicosky D.A. 1971. Surface wax deposits on foliage of *Picea pungens* and other conifers. Am. J. Bot. 58: 681–687.

Hart H. 1929. Relation of stomatal behavior to stem-rust resistance in wheat. J. Agr. Res. 39: 929–948.

Hirt R.R. 1938. Relation of stomata to infection of *Pinus strobus* by *Cronartium ribicola*.. Phytopathology 28: 180–190.

Hoff R.J., McDonald G.I. and Bingham R.T. 1973. Resistance to *Cronartium ribicola* in *Pinus monticola*: structure and gain of resistance in the second generation. USDA Forest Service Research Note INT-178. Intermountain For. and Range Exp. Sta., Ogden, Utah, 8 p.

Holloway P.J. 1969a. The effect of superficial wax on leaf wettability. Annals of Applied Biology 63: 145–153.

Holloway P.J. 1969b. Chemistry of leaf waxes in relation to wetting. J. Sci. Food and Ag. 20: 124–128.

Huttunen S. 1984. Interactions of disease and other stress factors with atmospheric pollution. In: Treshow M. (ed.), Air pollution and plant life. John Wiley and Sons, New York, pp. 321–356.

Kinloch B.B. 1980. Effect of photoperiod and container size on sugar pine seedling growth and infection by white pine blister rust. USDA Forest Service Res. Note PSW-343. Pacific Southwest For. and Range Exp. Sta, Berkeley, Calif, 4 p.

Larsen M.J. and Green F. III 1992. Mycofibrillar cell wall extensions in the hyphal sheath of *Postia placenta*. Can. J. Microbiol. 38: 905–911.

Leyton L. and Armitage I.P. 1968. Cuticle structure and water relations of the needles of *Pinus radiata* (D. Don). New Phytologist 67: 31–38.

Leyton L. and Juniper B.E. 1963. Cuticle structure and water relations of pine needles. Nature 198: 770–771.

McDonald G.I. and Hoff R.J. 2001. Blister rust: An introduced plague. In: Tomback D.F., Arno S.F. and Keane R.E. (eds), Ch. 10 *in* ''Whitebark pine communities: ecology and restoration''. Island Press, Washington, D.C., USA, pp. 440.

Mielke J.L., Yale University School of Forestry 1943. White pine blister rust in western North America. Bull. 52: 155.

Mitton J.B., Grant M.C. and Yoshino A.M. 1998. Variation in allozymes and stomatal size in pinyon (*Pinus edulis*, Pinaceae), associated with soil moisture. Am. J. Bot. 85: 1262–1265.

Ott L. 1993. An introduction to statistical methods and data analysis. 4th edn. Wadsworth, Inc., Belmont, California, 1051 p.

Patton R.F. and Johnson D.W. 1970. Mode of penetration of needles of eastern white pine by *Cronartium ribicola*. Phytopathology 60: 977–982.

Patton R.F. and Spear R.N. 1980. Stomatal influences on white pine blister rust infection. Phytopathologia Mediterranea 19: 1–7.

Percy K.E., Jensen K.F. and McQuattie C.J. 1992. Effects of ozone and acidic fog on red spruce needle epicuticular wax production, chemical composition, cuticular membrane ultrastructure and needle wettability. New Phytologist 122: 71–80.

Rowan S.J. and Steinbeck K. 1977. Seedling age and fertilization affect susceptibility of loblolly pine to fusiform rust. Phytopathology 67: 242–246.

SAS Institute Inc. 1989. SAS/STAT user's guide version 6.4. SAS Institute Inc., Cary, N.C.

Sauter J.J. and Voss J.U. 1986. SEM observations on the structural degradation of epistomatal waxes in *Picea abies* (L.) Karst. - and its possible role in the 'Fichtensterben'. Eur. J. For. Path. 16: 408–423.

Schmidt R.A., Foxe M.J., Hollis C.A. and Smith W.H. 1972. Effect of N, P, and K on the incidence of fusiform rust galls on greenhouse-grown seedlings of slash pine. Phytopathology 62 (Abstr.).: 788.

Schreiber L. 1996. Wetting of the upper needle surface of *Abies grandis*: influence of pH, wax chemistry and epiphyllic microflora on contact angles. Plant, Cell and Env. 19: 455–463.

Silva Fernandes A.M.S. 1965. Studies on plant cuticle. VIII. Surface waxes in relation to water-repellency. Ann. Appl. Biol. 56: 297–304.

Spaulding P.C. 1922. Investigations of the white pine blister rust. USDA Bull. 957: 100.

Trimbacher C. and Eckmullner O. 1997. A method for quantifying changes in the epicuticular wax structure of Norway spruce needles. Eur. J. For. Path. 27: 83–93.

Trimble J.L., Kelly J.M., Tolin S.A. and Orcutt D.M. 1982. Chemical and structural characterization of the needle epicuticular wax of two clones of *Pinus strobus*differing in sensitivity to ozone. Phytopathology 12: 652–656.

Van Overbeek J. 1956. Absorption and translocation of plant regulators. Ann. Rev. Pl. Phys. 7: 355.

Woo K.-S. 2000. Variation in needle morphology of blister rust susceptible and resistant western white pine, PhD, University of Idaho, Moscow 161 p.

Woo K.-S., Fins L., McDonald G.I. and Wiese M.V. 2001. Differences in needle morphology between blister rust restant and susceptible western white pine stocks. Can. J. For. Res. 31: 1880–1886.