# Influence of Seedling Physiology on Expression of Blister Rust Resistance in Needles of Western White Pine

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**Abstract**—Growth conditions for nursery-grown western white pine seedlings have been shown to affect levels of blister rust infection (from *Cronartium ribicola).* In an experiment initially designed to test the influence of environmental conditions at two nurseries in northern Idaho on the blister rust pathosystem, western white pine seedlings of a single resistant seedlot were unintentionally held in cold storage for 6 months longer at one nursery than at the other. Inoculation of these long-stored seedlings with blister rust spores occurred at 1 month after growth resumed under nursery conditions, versus 7 months for those with shorter storage. Infection percent was nearly double and infection efficiency (infections per unit area of stomata) was 70 times greater on the seedlings with only 1 month of growth than on the seedlings with the more mature foliage. Since the seedlings had originated from the same genetic source, the overwhelming difference suggests that phenology and/or nursery regimes can strongly influence infectability of seedling needles in western white pine using artificial inoculations. If phenology is the key factor, it may help explain why infection levels have been relatively high on northern Idaho resistant selections when grown at milder locations. Furthermore, if resistance genes can be selectively activated by manipulating phenology, molecular tools that examine gene expression might be employed to enhance our understanding of environmental regulation of genes for blister rust resistance.

**Key words**: *Cronartium ribicola*, blister rust pathosystem, infection efficiency, phenology.

## Introduction

Western white pine's (*Pinus monticola* Dougl.) susceptibility to the blister rust fungus (*Cronartium ribicola* J.C. Fisch. in Rabenh.) has been shown to vary with tree age, type, and age of needles, and age of the shoot (Lachmund 1933; Pierson and Buchanan 1938; Bingham 1972; Hunt 1991). But the environment in which seedlings are reared may also influence their susceptibility to infection. This possibility became apparent when, at three years postinoculation with *C. ribicola* spores, 47.6 percent of the seedlings grown in one nursery in northern Idaho were dead, compared to only 29.8 percent mortality of the seedlings grown in a second Idaho nursery. The result was the reverse of expectations because the seedlings grown in the second nursery were from a previously untested seed orchard, which was later determined to have low rust resistance compared to those grown in the first nursery. It appeared that, in addition to genetic variation, factors associated with nursery location and/or growing regimes had influenced "susceptibility," or perhaps "infectability" of the seedlings to *C. ribicola* (Eramian and Foushee, personal communication).

If rust resistance levels do vary as a function of growth environment, then estimates of resistance levels of the same genetic stock may vary widely depending on the nursery regime in which the stock is grown, or between stocks placed in field environments and their nursery-grown counterparts. Such variation in estimated resistance would imply a need to refine nursery rearing and testing protocols such that they produce comparable test results that are reliable predictors of long-term resistance levels under a variety of field conditions.

The original objective of the study reported here was to address this issue and test the hypothesis that blister rust infection levels in western white pine seedlings are indeed influenced by differences in nursery growing environment. We began by growing seedlings of the same genetic stock in two nurseries in northern Idaho, with the intent of following their routine protocols and exposing the seedlings to blister rust spores at the end of their second growing season after germination. However, an unexpected physiological difference was induced between the stocks when the seedlings at one of the nurseries were accidentally kept in cold storage six months longer than their counterparts at the second nursery. As environmental conditions during inoculation and/or disease development can influence the expression of blister rust-resistance genes in white pines, we

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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recognized the possibility that the physiological differences induced by the accidental extension of cold storage might also have important and potentially profound effects on expression of blister rust resistance in the seedlings (Yokata 1983; Bower 1987; Hunt and Meagher 1989; McDonald and others, this volume). As a measure of the relative infectability and early expression of resistance in the two stocks by *C. ribicola*, we compared their levels of needle infection at 5 months postinoculation. Subsequent mortality from rust was not evaluated.

# Materials and Methods

## Plant Material and Artificial Inoculation

Western white pine seedlings from a single genetic source (blister rust-resistant $F_2$ from the R.T. Bingham White Pine Seed Orchard in Moscow, ID, as described in Hoff and others 1973) were grown for two growing seasons at two nurseries in northern Idaho: Potlatch Corporation's Nursery in Lewiston, and the University of Idaho Forest Research Nursery in Moscow, hereafter referred to as the Lewiston and Moscow nurseries. The seedlings from both nurseries were inoculated with spores of *C. ribicola* at the USDA Forest Service Nursery in Coeur d'Alene, ID, in September 1999 using the routine procedures described in Mahalovich and Eramian (1995). The rust inoculum was collected from an established *Ribes* garden that had been inoculated with aeciospores collected from blister rust cankers in locations in Idaho, western Montana, and eastern Washington. Spore cast was monitored on slides that were placed at regular intervals among the seedlings.

At the time they were inoculated, the seedlings from the Moscow Nursery were about 36 cm tall and had calipers of 0.6 to 0.8 cm. Seedlings from the Lewiston Nursery, which had inadvertently been left in cold storage from December 1998 to early August 1999, were physiologically immature and substantially smaller, measuring about 22 cm in height, with a caliper of 0.3 to 0.5 cm, and had short (but expanding) secondary needles. We entered 200 seedlings into this study (25 per nursery in each of four replications). Nursery groups were randomized within replications, with one plot per nursery per replication.

In the months following inoculation, the seedlings were irrigated but no nutrients were supplied. At 5 months postinoculation, the seedlings were inspected for needle spots as a measure of successful penetration by basidiospore germ tubes. Overall seedling survival was 98.5 percent (197/200.) Needle spots were counted on all needles. Infection efficiency (IE) was calculated as:

[1] IE = spots per seedling / infective target area per seedling / spore cast

Infection efficiency was determined for each seedling and averaged for each nursery (McDonald and others 1981, 1991). The number used for spore cast in this equation was determined the by spore count on a monitoring slide that was closest to the seedlings during the inoculation process. Infective target area (ITA) ($cm^2$/seedling) was estimated as:

[2] ITA = SR x N x L (cm) x 0.01 (cm)

where SR= average number of stomatal rows on two adaxial sides per needle, N= number of needles per seedling, and L= average length of stomatal row (estimated by needle length) (McDonald and others 1981). Average number of stomatal rows and needle length were based on samples of six needles per seedling (one from each of six fascicles, two each from the top, middle, and bottom of each seedling). Lesion frequency was calculated as the number of needle spots/infective target area. Subsequent mortality as a function of blister rust infection was not evaluated.

## Statistical Analyses

Data were analyzed using the SAS-PROC GLM (general linear model) statistical package and Type III Sums of Squares (SAS Institute Inc. 1989). Data were transformed by arcsine when the exploratory PROC UNIVARIATE test showed they were not normally distributed.

# Results

Seedlings from the Moscow Nursery averaged 3.25 spots per seedling (range: 0 to 50) whereas seedlings from the Lewiston Nursery averaged 87.83 (range: 0 to 710) (table 1). Infection efficiency was significantly higher on seedlings

**Table 1**—Infection efficiency of *Cronartium ribicola* on western white pine seedlings from two nurseries in northern Idaho[a].

| Nurseries | Percent infection | Number of spots/seedling | Infective target area ($cm^2$/seedling) | Spore concentration (spores/$cm^2$) | Germination (%) | Lesion freq. | Infection efficiency |
|---|---|---|---|---|---|---|---|
| Lewiston | 95 | 87.83 (11.04) | 94.15 (4.30) | 5521.20 (232.04) | 44.77 (1.42) | 1.050 (0.138) | $2.1x10^{-4}$ ($2.9x10^{-5}$) |
| Moscow | 51 | 3.25 (0.73) | 322.36 (11.31) | 4465.75 (116.58) | 61.39 (1.08) | 0.011 (0.002) | $3x10^{-6}$ ($6x10^{-7}$) |

[a]Values are means with SE given in parentheses. P-values (shown below) are based on comparisons between samples of seedlings from the Lewiston versus the Moscow nurseries when P<0.05. Needle spots were counted on all needles per seedling (P=0.0009); Infective target area was estimated by average number of stomatal rows on two adaxial sides per needle x number of needles per seedling x average length of stomatal row (estimated by needle length) x 0.01cm (P=0.0037); Spore concentration = total number of spores in ten fields per slide x 60.2 (P=0.6574); Germination % (P=0.003); Lesion frequency = # spots/infective target area (P=0.0025); Infection efficiency = spots per seedling/infective target area per seedling/spore cast (P=0.0105).

from the Lewiston Nursery (mean=$2.1x10^{-4}$) compared to those grown in the Moscow Nursery (mean=$3x10^{-6}$) (P=0.0105). Also statistically significant were differences in the number of spots per seedling (P=0.0009), infective target area (P=0.0037), spore germination percentage (P=0.003), and lesion frequency (P=0.0025). Only five of 98 seedlings from the Lewiston Nursery had no spots, compared to 49 of 99 for the Moscow seedlings. No statistical difference was found between nurseries in spore concentration (P=0.6574).

Compared to the seedlings grown in the Moscow Nursery, seedlings grown in the Lewiston Nursery had a relatively small infective target area (94 versus 322 $cm^2$/seedling) and, although spore germination percentage was lower on nearby monitoring slides (45 vs 61 percent), the Lewiston Nursery seedlings developed more rust spots per seedling than did those from the Moscow Nursery.

The mean spore concentration (spores/$cm^2$) for the two stocks at the end of the inoculation period was 4,994 spores/$cm^2$, ranging from 2,047 to 11,920 spores/$cm^2$. Mean spore germination was 53 percent (range 25 to 82 percent).

## Discussion

Compared to the Moscow Nursery seedlings, the Lewiston Nursery seedlings had 27 times the number of spots and 70 times the infection efficiency (table 1). As the seedlings from the two nurseries had originated from the same genetic source (open pollinated seed from the same seed orchard), and the seedlings were inoculated at the same time and under the same inoculation conditions, observed differences in infection were not likely to be a function of genetic differences or differences in inoculation conditions. The most likely explanation of the observed differences in infection is either nursery cultural practices and/or a difference in developmental and physiological state of the seedlings at the time of inoculation.

As previous attempts to infect white pine seedlings grown in the Lewiston Nursery had resulted in relatively low infection percentages (Foushee, personal communication), the standard nursery practice used at the Lewiston Nursery is not a likely explanation for the observed, relatively high infection levels in this study. However, the Lewiston Nursery seedlings were kept in cold storage for six months longer than the seedlings from the Moscow Nursery and were removed from cold storage only one month prior to inoculation with rust. The seedlings had small needles (that were probably still expanding) and succulent tissues when they were inoculated. These observations suggest that some needle resistance mechanisms may not be fully operational in needles that have not reached their full development within a current growing season.

In addition, the very low frequency of needle spotting on the seedlings from the Moscow Nursery (compared to target levels of greater than 90 percent for routine rust screenings) may indicate a nursery regime that, at least temporarily, protects seedlings from infection. If so, rust screening that includes only artificial inoculations of their seedlings will not reflect actual long-term rust resistance levels under field conditions.

Data on needle spots were not recorded by needle type or location on the seedlings. However, it was clear that most of the observed rust spots appeared on current-year needles that were at the tops of the western white pine seedlings from both nurseries. Although it is possible that this reflects a purely spatial phenomenon, with uppermost younger needles having greater spore deposition than those lower on the stem, our results with *P. monticola* are consistent with previous findings for *P. monticola* and *P. strobus* that current needles are more susceptible to blister rust than older needles (Snell 1936; Van Arsdel 1968; Hunt 1991). Alternatively, physio-mechanical attributes may explain the differences, since stomata in older needles are less active than those of the current-year needles (Hirt 1938).

Bingham (1973) reported that seedling height was significantly related to the frequency of needle spots on nonresistant western white pine seedlings, with taller seedlings more highly cankered than shorter ones. However, this relationship did not hold true for three types of resistant stocks, one of which consisted of a bulk lot of $F_2$ seedlings similar to those used in our study. In either case, our result differs from Bingham's in that the relatively small seedlings from Lewiston Nursery had, by a large margin, more needle spots than the seedlings grown in the Moscow Nursery.

The average spore germination for our study was 53 percent, considerably lower than the overall average of 73 percent for the 1999 routine inoculations at the Coeur d'Alene Nursery (Eramian, personal communication; data on file at the USDA Forest Service Coeur d'Alene Nursery). Also, spore germination percentages on the slides near the Lewiston seedlings were consistently lower than those near the Moscow seedlings (45 percent versus 61 percent respectively). The reasons for the overall lower spore germination and the differences between samples near the two stock types are not apparent but may be related to variation in microsite associated with seedling size and foliage density.

Target spore deposition at the Coeur d'Alene Nursery is 6,000 to 7,000 spores per square centimeter and 95 percent or higher infection percent (Eramian, personal communication). Thus the mean spore deposition of 5,258 spores per square centimeter was lower than desired, but the 95 percent infection of the Lewiston Nursery seedlings indicates the deposition of spores and the environmental conditions in the inoculation tent were sufficient to achieve a high level of infection in at least one of the groups. The reason for the low infection percentage for the Moscow nursery seedlings is not known.

We found no relationship between needle spot development and either spore concentration or percent spore germination on slide traps (table 1), suggesting that at relatively high spore concentrations, seedling physiology may have more influence on infection efficiency than either spore concentration or percent germination.

An investigation of actual physiological differences between the groups was beyond the scope of this study. However, in other studies, a western white pine protein, *Pin m* III, was found to increase both during winter months and in tissue infected with blister rust (Ekramoddoullah and others 1995; Ekramoddoullah and others 1998). The normal winter increase in the protein was suppressed in rust-infected trees with the slow canker growth resistance mechanism, further suggesting a relationship between *Pin m* III and rust resistance. If the abundance of *Pin m* III is related to rust resistance, and if it is relatively easy to manipulate

by subjecting trees to differing lengths of cold storage, the protein may be a useful indicator of desirable or undesirable genotypes for selection in tree breeding programs. This hypothesis is easily tested by subjecting groups of resistant and susceptible seedlings to long versus short cold storage treatments. If there is a relationship only the resistant stocks exposed to normal cold storage period are predicted to show low levels of the protein.

Our results are also consistent with the infection of Japanese stone pine shoots by *Endocronartium sahoanum* (Kaneko and Harada 1995) after cold storage synchronization. Increased susceptibility in these related situations argues for the existence of a physiological cause associated with cold-storage treatment and/or immature tissue.

In our study, seedlings established under different nursery environments displayed different infection levels, but the likely causal factors were confounded and could not be isolated to explain the differences. Physiological and developmental conditions, growing regimes and nursery environment may all have influenced infectability of the study seedlings.

Needle infection was higher on current-year needles than on older needles (magnitude not quantified in this study) but older needles may have been sheltered from inoculum by the newer foliage, or they may have been less physiologically active. The highest infection levels occurred on physiologically immature needles. It appears that some needle resistance mechanisms may not be fully developed in actively growing, nonmature, succulent needles. If true, movement of stock to other climatic conditions, or differences in annual weather patterns under field conditions may influence the effectiveness of some resistance mechanisms, and may, at least in part, account for suspected "wave year" phenomena. However, it is also possible that, in this study, the effect of needle maturation state was confounded by physiological changes associated with the prolonged period of cold storage and/or the nursery growing regime. The "needle immaturity" hypothesis could be tested by inoculating second year seedlings of the same genetic stock at monthly intervals from June through September, or by inoculating only in September using groups of seedlings (of the same genetic stock) that had been subjected to different periods of cold storage.

Our results suggest that expression of genes related to rust resistance in northern Idaho white pine seedlings is sensitive to physiological state as related to phenology and/or to growing regimes that alter needle infectability in the nursery. If phenology is a critical factor, new molecular tools, such as cDNA PCR detection assays and micro arrays may facilitate experiments designed to explore environmental regulation of resistance genes and their association with phenologic traits. If variation in growing regimes is found to critically affect the infectability of seedling needles, then either protocols for evaluating resistance must be fine-tuned to address this variation, or growth regimes and testing protocols should be standardized for test seedlings across nurseries.

Any program that relies on artificial inoculation for evaluating seedlings for rust resistance must also include long-term field tests under a variety of conditions to fully assess resistance and validate the predictions of early screenings. If studies determine that rust infection under field conditions generally tends to be substantially higher than is indicated by current rust screening procedures for tree improvement programs, it may be useful to alter testing protocols to better mimic field conditions, such as including multiple exposures to rust spores, and exposing seedlings to spores earlier, potentially beginning when teliospores are first produced on the *Ribes* leaves. Further study is needed to test these hypotheses and to refine rust screening procedures (and perhaps nursery rearing procedures) that, together, will provide accurate long-term predictions of rust resistance.

## Acknowledgments

The authors thank Paul Zambino and Ned Klopfenstein for their thoughtful reviews of the manuscript, Aram Eramian for providing critical baseline information on routine rust screenings at the Coeur d'Alene Nursery, and the Inland Empire Tree Improvement Cooperative for its support of the original study on which the manuscript is based.

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