

Frequency of Hypersensitive-Like Reaction and Stem Infections in a Large Full-Sib Family of *Pinus monticola*

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Introduction

Western white pine (WWP) (*Pinus monticola* Douglas ex D. Don) is a long-lived forest tree species with a large native range in western North America. The tree species is highly susceptible to the non-native fungal pathogen, *Cronartium ribicola*, the causative agent of white pine blister rust (WPBR).

Several types of genetic resistance to WPBR are present in WWP, of which the best documented is a hypersensitive-like reaction (HR) in the needles that conveys complete resistance (generally no stem infection) and is conditioned by a single dominant gene (*Cr2*). The HR resistance is rare, and its occurrence is geographically limited (Kinloch et al. 1999, 2003). Virulence to *Cr2* in the rust (*vcr2*) is known (Kinloch et al. 2004). Most screening trials examine relatively few seedlings for any one family for HR. We report on the frequency of HR (using needle phenotypes) in a large full-sib family (3,592 individuals) in a cross between two putative *Cr2* heterozygotes (this is part of a larger genetic study to map *Cr2*); the frequency of stem symptoms and mortality in HR and non-HR seedlings; and the number of stem symptoms per seedling for HR and non-HR phenotypes.

Materials and Methods

Western white pine (WWP) seed of a single full-sib family (15045-862 x 15045-837) was stratified for 120 days and then sown in June 2010. The 3,592 seedlings from this 1991 seed collection were grown in a greenhouse at Dorena Genetic Resource Center (DGRC), Cottage Grove, Oregon in the summer of 2010 (fig. 1). Both parents are heterozygotes (*Cr2cr2*) for HR resistance and originated from the Champion Mine area on the Cottage Grove Ranger District, Umpqua National Forest in Oregon. This stand had previously been noted for having the highest incidence of parents with *Cr2* of any areas tested (Kinloch et al. 1999, 2003). Seedlings had both primary needles and cotyledons present at inoculation (only a few seedlings displayed any secondary needles). Seedlings were inoculated with *C. ribicola* in September 2010 using standard DGRC protocols (Danchok et al. 2004). For artificial inoculation, infected *Ribes* spp. leaves (the alternate host of *C. ribicola*) from geographical areas outside of the areas of known occurrence of *vcr2* were collected and suspended above the pines under optimal conditions for spore drop and germination. Inoculum density for the trial averaged 6,805 basidiospores/cm², and spore germination averaged 89 percent. After inoculation, seedlings were placed in an unheated greenhouse for nursery culture and subsequent development of rust symptoms.

Seedlings were assessed at periodic intervals when needle spots and stem symptoms were evident. The assessments for spot type included rating each seedling for whether all spots were susceptible (code 1) or whether all spots were HR type (code 4). Seedlings with a combination of HR and susceptible spots (or some of the seedlings with HR-like spots, but stem symptoms at the early assessments) were initially assigned a separate category (code 2), as were seedlings where the spot phenotype was ambiguous (code

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3). The presence of stem symptoms was also recorded, and in March and April 2011, a count of stem symptoms was made. Mortality was also recorded. In June 2011, the spot type was re-evaluated on the 33 seedlings coded as '3' to classify into type 1, 2, or 4. A subset of 150 seedlings was examined for type of stem symptoms in July 2010.

Results

The artificial inoculation was very successful, with most seedlings having dozens to hundreds of needle spots (fig. 1). All seedlings were ultimately classified as having type 1, 2, or 4 spots. All 3,592 seedlings showed needle spots by April 2011, and 36 percent had only susceptible (S), non-HR spots (fig. 1). The 64:36 ratio (HR spots: S spots) differed significantly from the expected 3:1 ratio (Chi-square p-value < 0.0001) and also from a 1:1 ratio (p-value < 0.0001).

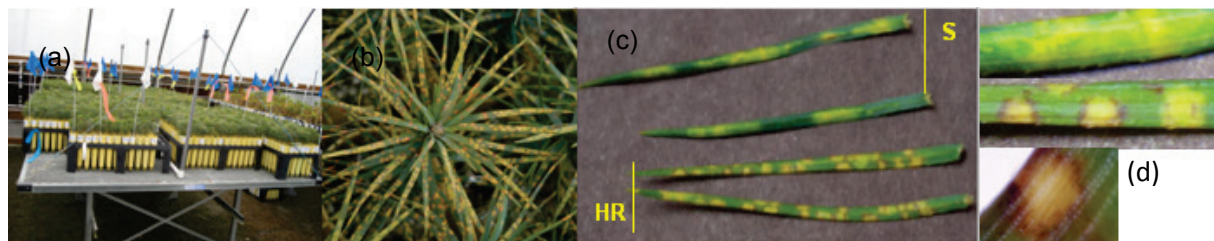


Figure 1—March 2011 photos (6 months after inoculation): (a) overview of *P. monticola* trial; (b) hundreds of needle spots (HR) on inoculated *P. monticola* seedling; (c) resistant (HR) and susceptible (S) spots on *P. monticola*; note many infections per needle, (d) needles with S and HR spots (note incomplete development at this point of HR spots on one needle).

By April 2011, 94.4 percent of the category 1 (S) seedlings had stem symptoms, versus 29.5 percent for the HR seedlings (groups 2 and 4) (57.4 percent and 13.8 percent of the category 2 and 4 (HR) seedlings, respectively) (figs. 1 to 3). By November 2011, stem symptoms had increased to 97.5 and 53.1 percent for S and HR seedlings, respectively. The non-HR (S) seedlings averaged 4.6 stem symptoms per infected tree in April, while the HR seedlings averaged 1.6 (type 2 and 4 seedlings averaged 1.7 and 1.4 stem symptoms per infected tree, respectively (fig. 4)). This difference was significant (two-sided p-value < 0.0001 from a likelihood ratio test); the mean number of stem symptoms per infected tree is estimated to be 2.9 times greater for S seedlings than HR seedlings (95 percent confidence interval from 2.7 to 3.1 times greater) (figs. 2 and 4). Stem symptoms generally appeared earlier on non-HR than on the HR seedlings: 77.9 percent of non-HR had SS in March, while only 14.1 percent and 3.1 percent of the HR seedlings in categories 2 and 4, respectively, had SS (fig. 3).

Overall mortality (seedlings with and without stem symptoms) by November 2011 was 92.2 and 72.6 percent for S and HR seedlings, respectively; the odds of mortality for S seedlings were estimated to be 3.6 times to 5.6 times as large as the odds of mortality for HR seedlings (approximate 95 percent confidence interval). The mortality for the subset of seedlings with stem symptoms was 92.2 and 77.0 percent for S and HR seedlings, respectively. For seedlings with no visible stem symptoms, mortality was also high: 90.9 and 67.5 percent for the S and HR groups.

Discussion

Based on the needle phenotypes following artificial inoculation, the ratio of HR:non-HR seedlings (64:36) was less than the 75:25 ratio anticipated in a cross of two *Cr2* heterozygotes. In a previous trial, progenies of some of the parents from the Champion Mine stand have showed reduced penetrance of *Cr2*— expressed by altered Mendelian ratios (mostly less-than-expected resistant phenotypes) in specific combinations of certain parents, indicating the potential presence of modifier genes with effects that ranged from mild to almost complete suppression of *Cr2* (Kinloch et al. 1999). This may explain the

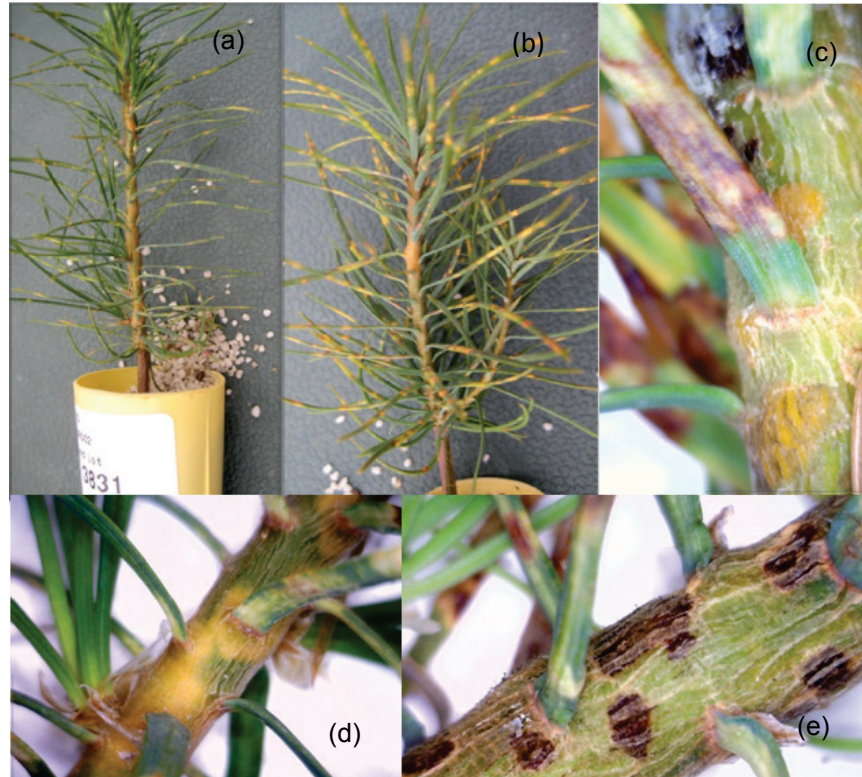


Figure 2—Stem infections observed on seedlings with S spots (a, c, and e) and on seedlings with HR spots (b and d).

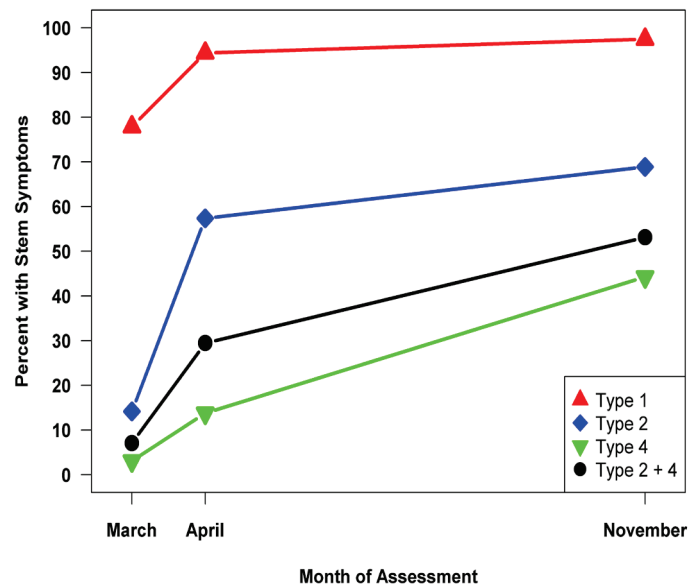


Figure 3—Percentage of *Pinus monticola* seedlings with stem symptoms by assessment date for the three needle spot types: 1: all susceptible (S) spots, 2: at least one resistant (HR) spot, 4: all (HR) spots.

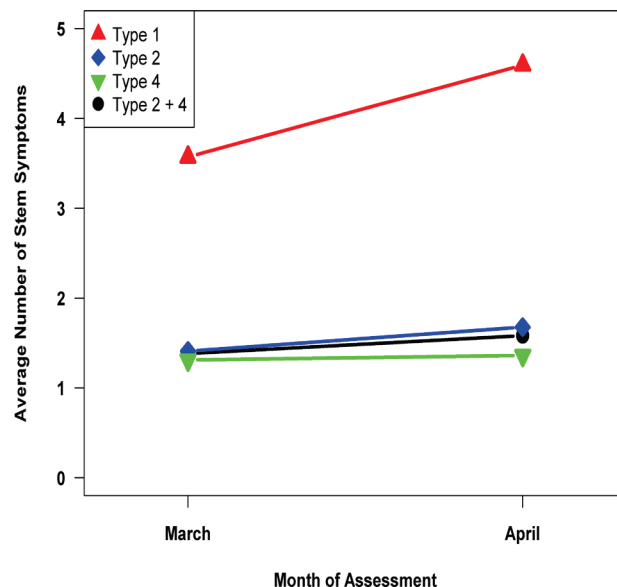


Figure 4—Mean number of stem symptoms (per infected tree) by assessment date for the three needle phenotype category types: 1: all susceptible (S) spots, 2: at least one HR spot, 4: all HR spots.

slightly lower than expected frequency of HR phenotypes in this full-sib family. Previous tests in 2002 and 2008 using this full-sib family from five different seed collection years (1974, 1980, 1988, 1989, 1991) showed similar results, with ratios in all tests exhibiting <75 percent HR type spots (unpublished data). There was no evidence of a difference in the ratio of HR:non-HR seedlings for any of the five different seed collection years (p -value = 0.14 from a likelihood ratio test). This suggests that inadvertent contamination of this full-sib seedlot by other pollens or seedlots is unlikely, but further investigation is needed to discern whether this is a possibility. Other possible explanations are discussed below.

The number of HR seedlings with stem symptoms was unexpected. In previous trials, including the 2002 and 2008 trials with this same family, few or no seedlings of WWP with HR had stem symptoms in the absence of known *vcr2* sources of rust. The reason for the high incidence of stem infection of HR seedlings in this trial is unknown, but it is possible that a very low frequency of *vcr2* was present in the inoculum and coupled with a very large number of needle infections per seedling resulted in stem infections on some seedlings. Another possible explanation is that there could be ‘leakage’ in the HR, at least under this high-effective infection level and some environments. Many seedlings had several hundred needle spots with some individual needles having more than 15 needle spots (fig. 1). It was observed that the timing of development of the necrotic bands around the yellow spots could vary even within a seedling and that on several seedlings the necrotic band did not fully encircle the spot, suggesting that HR was sometimes only partially effective. Some of these seedlings showed stem symptoms at the base of such needles. The HR in some plant pathosystems is a temperature-dependent trait (Goodman and Novacky 1994, Wang et al. 2009) and that may have had some influence here, but investigations of the temperature sensitivity of HR in WWP (and other white pines) have not been done. However, relatively high levels of stem symptoms of HR seedlings are not unprecedented. Kinloch and Comstock (1980) reported that about 32 percent of sugar pine (*P. lambertiana* Dougl.) seedlings with HR showed evidence of stem symptoms, and that this infection was generally only apparent on seedlings with primary needles (versus cotyledons or secondary needles). In sugar pine HR seedlings, the stem infections were abnormal and never sporulated.

The latency in stem symptom development observed on HR seedlings in this WWP trial is similar to that reported on HR seedlings in sugar pine by Kinloch and Littlefield (1977). They reported that 27 percent of HR (‘fleck’) phenotypes developed atypical bark symptoms from primary needle infection. The

bark symptoms resulting from primary needle infection on fleck phenotypes of sugar pine developed at a slower rate and were always incompatible and healed by the second year after inoculation. It was hypothesized that primary needles may react differently or have a somewhat lower resistance than secondary needles (Kinloch and Comstock 1980). Primary needles were the predominant needles present on the WWP in this study. In this study, in contrast to S seedlings, the HR seedlings with stem symptoms tended to show SSs later, had fewer SSs, and had lower mortality of both seedlings with SSs and those showing no SSs. This trial had a very high number of needle spots on the primary needles and a high level of stem symptoms on HR seedlings, as well as an unexpected and relatively high mortality of seedlings with no stem symptoms (reason unknown). In the earlier 2002 and 2008 trials of this full-sib family, very few of the HR seedlings developed stem symptoms and there was little mortality of HR seedlings. Thus, if very young WWP seedlings are used, needle spot type would generally be a more reliable indicator of presence or absence of HR in WWP than relying on whether stem symptoms are present.

The nature of the resistance for seedlings showing an HR-like needle spot is still unknown. Some recent histological investigations indicate that it is not the classic, very rapid, hypersensitive response that often occurs within hours of infection; further histological work is underway to further characterize the nature of this needle resistance in WWP (Sweeney et al., Are needle reactions in resistance to *Cronartium ribicola* a hypersensitivity response?, these proceedings). Tissue from the seedlings in this WWP study will be used by Dr. Liu's group for molecular characterization by RNA-seq for discovery of single nucleotide polymorphisms (SNPs) between phenotypic groups, with some future work using high throughput SNP genotyping technique to find functional genes tightly linked to *Cr2* in this full-sib family for positional characterization of *Cr2*.

Acknowledgments

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