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Resistance and tolerance of Douglas-fir seedlings to artificial inoculation with the fungus *Ophiostoma pseudotsugae*

M. G. Cruickshank[1] 🆔 | K. P. Bleiker[2] | R. N. Sturrock[2] | E. Becker[2] | I. Leal[2]

[1]Natural Resources Canada, Canadian Forest Service, Canadian Wood Fibre Centre, Victoria, BC, Canada

[2]Natural Resources Canada, Canadian Forest Service, Victoria, BC, Canada

Correspondence
Mike G. Cruickshank, Natural Resources Canada, Canadian Forest Service, Canadian Wood Fibre Centre, Victoria, BC, Canada.
Email: mike.cruickshank@canada.ca

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Summary

We tested the resistance and tolerance of 79 Douglas-fir halfsibling seedling families to artificial inoculation with *Ophiostoma pseudotsugae* in a shade house experiment. The Douglas-fir beetle vectors the fungus where it colonizes the phloem and sapwood, often leading to tree mortality. The 79 halfsibling seedling families originated from four seed planning zones in BC that span ecological gradients ranging from moist-warm to cool-wet. We tested resistance to the fungus by measuring lesion size and tolerance by measuring seedling height. We found variation in both resistance and tolerance within seed zones and halfsibling hierarchies. Trees from zones and families that were shortest before inoculation appeared to have the most tolerance after inoculation suggesting a cost for carrying tolerance traits. There was a cost in height growth for resistance after inoculation versus wounding alone. There was no trade-off between family resistance and tolerance defence strategies indicating that both developed independently in the population. Higher resistance and lower tolerance to the fungus were the least commonly occurring trait combination in families. Douglas-fir trees are moderately shade intolerant at the sapling stage, and as height increment is crucial for light capture, they probably avoid costly strategies such as resistance alone. This defence strategy may change in older stands.

1 | INTRODUCTION

The sessile nature of plants means that they cope with a large array of biotic and abiotic stressors that cannot be simply avoided. Repeated or continuous exposure to stress usually leads to a physiological expression that manifests in specific symptoms, such as reduced growth. Disease is defined as a malfunctioning of host cells or tissues upon continuous irritation by a pathogenic agent or environmental factor leading to symptomology (Agrios, 2005). After exposure to continuous or repeated stress, plants that die are considered susceptible, or they survive and are considered resistant or tolerant. Resistant plants minimize damage to their tissues. In contrast, tolerant plants endure damage and display superior growth relative to other plants (Schafer, 1971). The pathological definition of plant resistance and tolerance can also apply to insects (e.g., Shen & Bach, 1997), or in general, where there is population variation in response to an environmental stress gradient (biotic or abiotic).

The native range of Douglas-fir (*Pseudotsuga menziesii*) extends from central British Columbia (BC) into northern Mexico (Burns & Honkala, 1990), but it has been introduced to other temperate forest regions due to its desirable wood quality and rapid growth. Douglas-fir is one of the tallest conifer species in the world, an early colonizer and moderately shade tolerant in succession. Douglas-fir copes with a wide range of temperature and precipitation climes as well as biotic stress agents, such as insects and pathogens, over its range. The species plasticity to multiple co-occurring stress agents facilitates its survival over its range.

One of the stress agents affecting Douglas-fir is the Douglas-fir bark beetle and the associated fungi it vectors. Douglas-fir has co-evolved with the beetle, *Dendroctonus pseudotsugae*, which is an eruptive herbivore capable of killing mature trees during outbreaks. Beetles introduce a variety of microorganisms to trees as they construct galleries in the inner bark. The most common blue-stain fungal associate of the Douglas-fir beetle is *Ophiostoma pseudotsugae*

(Rumbold) von Arx. This fungus has been described as moderately virulent, and it elicits a defence response when inoculated into trees (Ross & Solheim, 1997; Solheim & Krokene, 1998).

Douglas-fir's great height growth and high wood strength combine to produce low-cost high-value wood products, hence, the need to understand factors affecting survival and growth of this important commercial species. There were 79 Douglas-fir maternal halfsiblings (same mother) from four seed planning zones originating in the interior of BC challenged with *O. pseudotsugae*. We examined resistance and tolerance of Douglas-fir seedlings to determine patterns in population variation at the zone and family levels using mixed hierarchical models.

2 | MATERIALS AND METHODS

2.1 | Seedling preparation

Wind-pollinated maternal halfsibling seed from four tree-breeding zones in the British Columbia Interior Douglas-fir tree breeding program was selected for study: Shuswap Adams, SA; West Kootenay

low elevation (< 1,000 m, WKL); West Kootenay high elevation (>1,000 m, WKH); and Mica (M). These breeding zones largely make up the Interior Cedar Hemlock (ICH) biogeoclimatic zone in British Columbia (Lloyd, Angrove, Hope, & Thompson, 1990). The ICH is isolated from the maritime influence by a large range of coastal mountains that maintain a strong continental climate. The ICH has moist summers and cold wet winters with a snow pack that reduces summer moisture deficits, although considerable extremes in environmental conditions occur in subzones of this ecosystem.

In the BC interior, seed transfer is based on seed zones that act as surrogates for the biological and physical environment (Ying & Yanchuk, 2006). The four seed planning zones partition the ICH ecosystem mainly along clines of temperature and moisture but also on latitude. Zones M and WKH are the coolest and wettest zones; SA and M occur adjacent to, but north of WKL and WKH in southern BC. All of the trees were included in wind-pollinated progeny tests within their respective zone, and individual tree breeding values for volume growth varied from low, medium and high.

Douglas-fir [*P. menziesii* var. *glauca* (Beissn.) Franco] consisting of 79 families with 11 in M, 23 in SA, 22 in WKH and 23 in WKL

TABLE 1 Maximum likelihood analysis of variance table for the effects of days since wounding zone, stem girdling, and family after *Ophiostoma pseudotsugae* inoculation on Douglas-fir tree height

Effect	Estimate	Standard Error	*p*-value
Fixed main effects			
Intercept	59.7639	2.0449	<.0001
Time (days)	0.1676	0.01449	<.0001
Zone[a]			
M	−3.1638	1.5055	.0389
SA	−0.07567	1.2071	.9502
WKH	−3.1874	1.2217	.0109
WKL	0	–	–
Stem gird[b]	−10.5793	3.0082	.0004
Zone[a]*gird[b]			
M	1.0183	1.2875	.4314
SA	−0.9620	1.1095	.3484
WKH	2.4063	1.0338	.0266
WKL	0	–	–
	Variance		***p*-value**
Random effects			
Family within zone[a] intercept	10.0153		.0001
Family within zone[a] girdling[b]	4.8548		.0157
Covariance a*b (intercept*girdling[b])	4.0123		.0070
Bench	13.7775		.1161
Repeated measures covariance	0.8582		<.0001
Residual	133.64		<.0001
Akaike`s information criteria	22077.1		

[a]See the methods section for zone details.
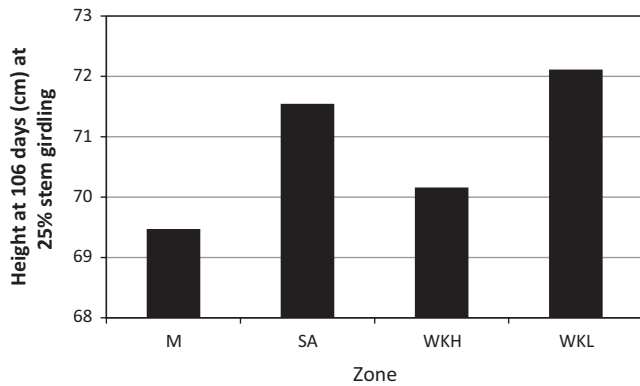[b]Stem girdling is transformed. See stats methods for details.

FIGURE 1 Least squares means for tree height from each zone at 106 days and average Douglas-fir stem girdling after inoculation with *Ophiostoma pseudotsugae*. See the methods for descriptions of the zones

zones were used for the experiment. In spring 2014, seed was stratified and sown in 130-ml cell styroblocks at the Canadian Forest Service 506 W. Burnside Rd. Victoria, BC, and grown for one season. In spring 2015, a total of 3127 trees seedlings were transferred to 4.5-L plastic containers containing a soil mixture of 40% sand, 30% coarse forestry grade peat and 30% forest loam mineral soil. The seedlings were then grown for one complete growing season in a plastic-covered shade house. The layout of the shade house consisted of four benches with each family randomly assigned to each bench.

2.2 | Inoculation of seedlings

An isolate of *O. pseudotsugae* (isolate OM5 listed in Table 1 of Gorton, Kim, Henricot, Webber, & Breuil, 2004; obtained from the Culture Collection of Forintek Canada Corporation, Vancouver, Canada) was subcultured on 1.5% malt extract agar in 10-cm diameter Petri dishes at approximately 22°C for 10 days prior to inoculation. Trees were inoculated on 23–25 March 2015. The stem was sprayed with 70% ethanol approximately 18 cm above the soil line. A 4-mm sterilized cork borer was used to remove the outer bark and phloem, and a 4-mm diameter plug of fungus-colonized MEA or sterile MEA (control) was placed in the resultant wound using sterilized forceps. A small piece of sterilized cotton was placed over the hole and secured with a strip of Parafilm. Tree height and lesion size were assessed in July 2015, 103 to 106 days following inoculation.

2.3 | Seedling measures

Trees were measured for total height and diameter at the beginning in March before wounding and at the end of the experiment after 103–106 days post-inoculation. The Parafilm was removed at the end of the experiment, and lesion width, length and the presence of callus (none, partial and full) were noted. Resistance to an agent is usually measured by the amount of tissue affected by the agent. Lesion width was used to study resistance. Tolerance is

usually measured as a measure of host growth for a given level of host damage, so the per cent stem circumferential girdling versus height growth was used to study tolerance. Tree height can be used to measure fitness because survival and growth depend on light capture especially in shade-intolerant species (Younginger, Sirová, Cruzan, & Ballhorn, 2017).

2.4 | Statistics

2.4.1 | Test for fungal tolerance

Tolerance at the family level was assessed at two time periods by analysing tree height before wounding and then after inoculation at the end of the experiment. Three hierarchical effects were identified as follows: zone, family and bench levels. The MIXED procedure within the SAS statistical program was used to model this according to:

$$\begin{aligned} Y_{ijklm} = &\beta_0 + \beta_1 \text{days}_{ij} + \beta_2 \text{zone}_k + \beta_3 \text{stem girdling}_{ij} \\ &+ \alpha_1 \text{family}_l \text{intercept} + \alpha_2 \text{family girdling}_{il} + \alpha_3 \text{bench}_m + \varepsilon_{ijklm} \end{aligned} \quad (1)$$

where Y_{ijk} are the individual tree heights (cm) within the fixed effects, zone, per cent stem girdling and time since wounding, and for the random effects of family intercept and girdling and bench location; β_0 is the intercept; β_1 is the effect for continuous time in days i since wounding tree j; β_2 are the categorical effect for the four seed zones k; β_3 is the continuous effect for per cent stem girdling transformed by an arcsine square root girdling function for tree j at time i; α_1 is the random intercept for family l; α_2 is the random intercept for stem girdling in family l at time i; α_3 is the random intercept for the bench where the seeding was located within the shade house; ε_{ijklm} is the residual error at time i for tree j within zone k, family l, and bench m. Inclusion of the categorical treatment in the model (wounding or wounding plus Ophiostoma) did not significantly improve the model fit because the continuous effect per cent girdling of the tree stem was a better predictor of tree height. All variables were tested for inclusion into the model using a likelihood ratio test at $p \leq .05$ if they were not highly correlated. Multicollinearity was assessed using the CORRB option within the MIXED procedure. Two-way interactions between the fixed effects were also considered for inclusion with a likelihood ratio test.

Covariance was accommodated between the random family intercept and family stem girdling which indicated a relationship between tree heights before treatment (i.e., healthy and unwounded) and subsequent growth increment for the year after treatment. Repeated measures of tree height were accommodated with a first-order autoregressive structure, which assumes constant variance of the residuals in time. The covariance is governed by $\sigma^2 \rho^{i-j}$ where σ^2 is the variance, ρ is the correlation between i and j observations in time constrained to $-1 < \rho < 1$.

2.4.2 | Test for resistance

The model used to test resistance was based on lesion width. Lesion width was tested in an allometric fashion by including tree diameter

in the model to account for differences in seedling size. Resistance was assessed at the end of the experiment at the zone, bench and family levels using the following model:

$$Y_{ijk} = \beta_0 + \beta_1 \text{diameter}_i + \beta_2 \text{zone}_j + \beta_3 \text{treatment}_i \\ + \alpha_1 \text{family}_k \text{intercept} + \alpha_2 \text{bench}_l + \varepsilon_{ijkl}$$

(2)

where Y_{ijk} are the individual lesion widths (cm) within the fixed effects of stem diameter (cm), zone and inoculation treatment, and the random effects of bench location and family; β_0 is the intercept; β_1 is the continuous effect for diameter of tree i; β_2 are the categorical effects for the four seed zones j; β_3 is the categorical effect for treatment (wounded=0 or wounded plus fungus=1) in tree i; α_1 is the random intercept for family k; α_2 is the random intercept for bench location l within the shade house; ε_{ijkl} is the residual error for tree i for zone j, family k and bench l.

2.5 | Best linear unbiased predictors (BLUPs)

BLUPs were output for each model. BLUPs are an estimate of the random effect for an individual subject (family) which described how that subject differed from the population average of all trees (fixed-effect model). A positive BLUP meant that family had a response that was larger than the average response of all observations (i.e., the fixed-effect model), and vice versa for negative BLUPs. Family BLUPs were graphed on scatter plots, and the distance from the origin was calculated based on the hypotenuse of the "x" and "y" coordinates. The origin of this graph (x = 0, y = 0) represented a BLUP that has zero value for that family trait lesion width (resistance) and height growth (tolerance); in other words, it was described by the fixed-effect model only. The sign of the "x" coordinates (tolerance) and "y" coordinates (resistance) BLUP determined which quadrant each family BLUP was assigned. Each quadrant on the scatter graph represents a combination of these traits into four types: low tolerance and resistance response, or low tolerance and high-resistance response and vice versa, or high response for both traits.

Correlations between tolerance and resistance responses predicted by family BLUPs were assessed. BLUPs from resistant trees described lesion size; therefore, large BLUPS equated to larger lesions or the opposite of resistance. The increases in tolerance BLUPs show increasing tolerance. To adjust for the differences in how BLUPs measure resistance or tolerance, 1-R BLUPs controlling lesion width were used in correlations so that increases in resistance response occurred in parallel with increasing tolerance response.

3 | RESULTS

3.1 | Height growth

3.1.1 | Tolerance at the population level

The tallest trees originated from zones WKL and SA, with trees from zones M and WKH approximately 1.3 cm shorter on average over the duration of the study (Table 1, Figure 1). Increasing stem girdling resulted in reduced height over all trees (p = .0004, Table 1) indicating that the level of damage affected growth. Stem girdling also interacted with zone (Table 1 zone by gird interaction, Figure 2), indicating the greatest tolerance to damage occurred in trees from zone WKH, followed by zone M, WKL and SA (at average girdling of 25%, Figure 2). Tree height differences within zones were approximately 11% of the average height increment (12.2 cm) for that year.

3.1.2 | Tolerance at the family and bench level

Family initial tree heights at the beginning of the season differed from their zone average heights (p = .0001, family intercept, Table 1). Family height growth as a function of stem girdling damage differed significantly (p = .0157, family girdling, Table 1) meaning that there was a difference in family tolerance to damage. For example, in zone WKL, family 9146 (one of the tallest families) had the greatest effect of stem girdling on height growth (lowest slope) and family 9143 (one of the shortest) the least difference (Figure 3). The average height growth increment for this zone was about 12 cm so that a 1.3-cm difference in heights between the two families was an 11% reduction in increment at average stem girdling (25% girdling) or 27% reduction at the maximum stem girdling (40% girdling).

On average, the families with greatest initial height before wounding showed higher growth impact for a given level of stem girdling (p = .0070, Table 1—pooled covariance a*b, Figure 3) after wounding and inoculation. The effect of stem damage on height growth at the family level paralleled that at the zone level closely. Taller than average trees in zones and families had more height reduction for a given level of stem girdling than those trees in zones or families that were smaller before wounding (compare Figure 1 with 2). Families with shorter than average trees before inoculation were more tolerant on average to height growth reduction following inoculation.

The effect of bench location where the trees grew explained some variation (Table 1, bench) of ±7% from the average height of all trees at most. Trees in the middle benches away from the shade house walls were tallest.

3.2 | Lesion width

3.2.1 | Resistance at the population level

Stem lesion width perpendicular to the stem was used to test for host resistance at the zone and family levels. Stem lesions can easily girdle the whole stem circumferentially leading to death; therefore, smaller lesion widths are critical for survival. Stem diameter had a significant effect on lesion width (p < .0001, Table 2) indicating that larger trees also had larger lesion widths on average. The zone where the trees originated also affected lesion width with trees from zone M having the smallest widths, and increasing in order in zone WKL, with WKH and SA tied (Table 2). Lesions within zones ranged at most ±3% of the average lesion width of all trees. The fungus treatment
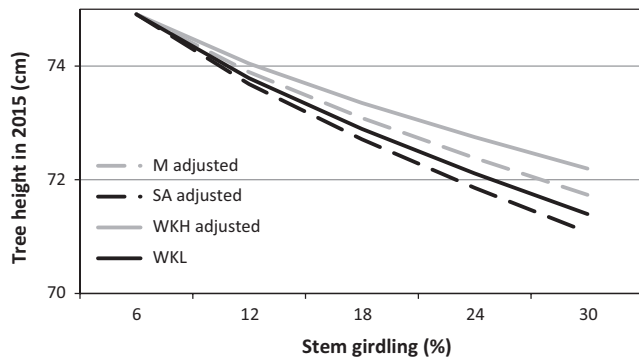
FIGURE 2 Tree height growth at the end of the experiment as a function of Douglas-fir stem girdling after inoculation with *Ophiostoma pseudotsugae*. The WKH zone had the most tolerant trees to stem girdling and SA the least. Intercepts were adjusted at low girdling (6%) to match WKL intercept (the tallest seedlings) for comparison of slopes. See the methods for descriptions of the zones

increased the width of lesions by 1 mm ($p < .0001$, Table 2—inoculated) over wounding alone, which was 11% larger than the average lesion width from wounded but not inoculated trees (7 mm vs. 8 mm). There was no interaction between tree diameter and fungal inoculation.

3.2.2 | Resistance at the family and bench level

The effect of family also explained significant variation ($p = .0008$, Table 2); in fact, average lesion widths in one family ranged ±5% from the average lesion width of all fungal inoculated trees. We could not accommodate an additional random effect of family diameter in the model (Equation 2), as the size effect was best fit as a fixed effects term. The correlation between family BLUPs for resistance (lesion width) and family height before inoculation (family intercepts) was not significantly different from zero when pooled among zones (Spearman's $r_s = -0.03$, $p = .78$) or by zone ($p > .16$). No cost of family resistance before inoculation was apparent. On the other hand, families with height increment reduction after inoculation were associated (Spearman's $r_s = 0.16$, $p < .0001$) with smaller lesions (higher resistance) indicating a cost for induced resistance.

The location of the bench in the shade house where the trees grew also explained some variation in lesion width at most about ± 4% of the average for all trees (Table 2, bench).

3.3 | Relation between tolerance and resistance

Family BLUPs for resistance (lesion width) and tolerance (height growth) from the two models (Equations 1 and 2) were plotted (Figure 4). Each quadrant on the graph represented a different combination of tolerance and resistance. The origin represented the average of all trees, so the distance from the origin indicated the strength of that family trait combination. This meant that families with greater effects of resistance or tolerance would reside further from the origin. For example, zone WKL had one family with high tolerance and resistance (lower right quadrant, Figure 4). Figure 5 shows the cumulative proportion of observations falling into each quadrant and zone in Figure 4. Across all zones, the most frequent trait combinations were low tolerance with low resistance, high tolerance with high resistance or high tolerance with low resistance. The least frequently occurring trait combination across all zones

TABLE 2 Maximum likelihood analysis of variance table for the effects of *Ophiostoma pseudotsugae* inoculation, tree diameter, zone and family on Douglas-fir lesion width

Effect	Estimate	Std. Error	*p*-value
Fixed main effects			
Intercept	5.1618	0.2627	<.0001
Diameter	0.1991	0.02041	<.0001
Zone[a]			
M	−0.2727	0.1254	.0329
SA	0.1935	0.1006	.0583
WKH	0.1882	0.1019	.0687
WKL	0	–	–
Inoculated	0.7618	0.05406	<.0001

	Variance	*p*-value
Random effects		
Family within zone[a] intercept	0.06050	.0008
Bench	0.08541	.1185
Residual	1.0999	<.0001
Akaike`s information criteria	4637.2	

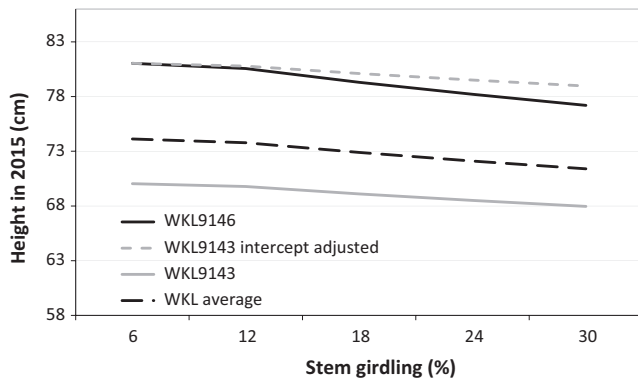[a]See the methods section for zone details.

FIGURE 3 Douglas-fir height for families within zone WKL, representing the range of differences in tree sizes (intercept) and tolerance to stem girdling (line slope) within that zone, after inoculation with *Ophiostoma pseudotsugae*. The zone average is shown, and the intercept of family 9143 was adjusted at the intercept (6% girdling) to match family 9146 for comparison of slopes. See the methods for descriptions of the zones

was low tolerance and high resistance (Figure 5). Both high tolerance and low resistance and low tolerance with high resistance had the strongest overall effect on height and lesion size (Figure 5). The

taller families that occurred in both the drier and warmer zones (WKL and SA) showed higher frequency of the high-high or low-low resistance tolerance trait combinations compared to the other two wetter and cooler zones (Figure 5).

4 | DISCUSSION

The study results of artificial inoculation of Douglas-fir with *O. pseudotsugae* indicated that there were different defence responses at the population level (all trees), but also within seed zones and half-sibling families. At the highest level on average, Douglas-fir trees showed a reduction in height growth or an increase in lesion width after inoculation with *O. pseudotsugae*. Next, trees from the wetter and cooler seed zones (M and WKH) had greater tolerance in maintaining height increment following stem damage. Finally, resistance and tolerance to *O. pseudotsugae* were better explained at the family level, and the two strategies were not positively or negatively correlated among families. A lack of trade-off (negative correlation) between resistance and tolerance strategies is known to be common in other studies for a range of crop plants (Leimu & Koricheva, 2006a) and in poplar trees (Stevens, Waller, & Lindroth,
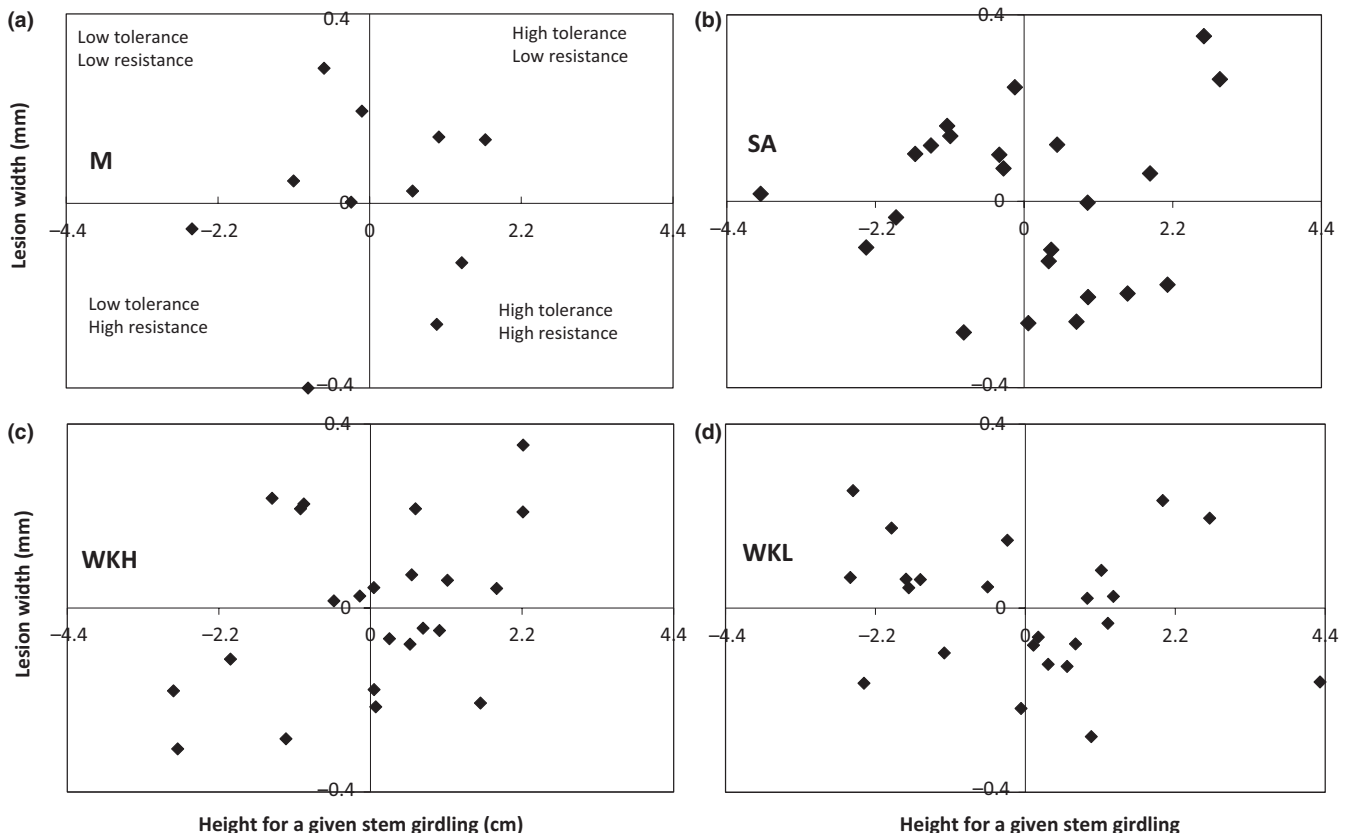

FIGURE 4 Family BLUPs controlling Douglas-fir lesion width (from Equation 2) versus family BLUPs controlling growth for a given Douglas-fir stem girdling (from Equation 1) for zones. Each observation represents one family which falls into one of four quadrants: low resistance-low tolerance (upper left), low resistance-high tolerance (upper right), high resistance-high tolerance (lower right) and high resistance-low tolerance (lower left). The distance from the origin in each case indicates the strength of the tolerance or resistance trait combination for that family after inoculation with *Ophiostoma pseudotsugae*. See the methods for descriptions of the zones
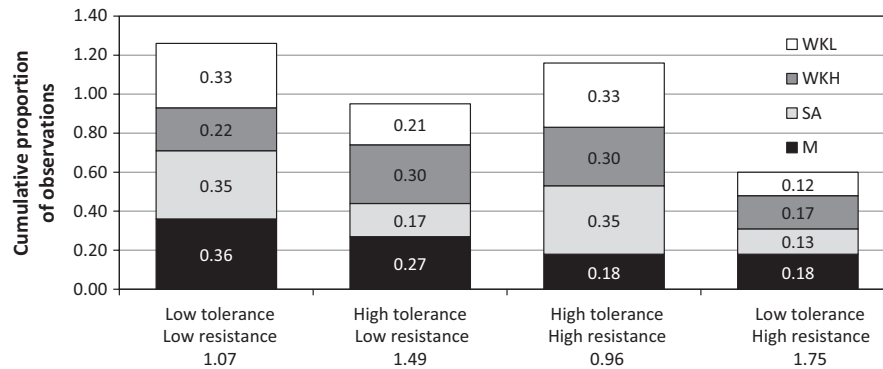
FIGURE 5 Halfsibling family BLUPs describing the level of tolerance and resistance in Douglas-fir seedlings from each zone. Low tolerance combined with high resistance is the least frequent trait combination, but it had higher impact when it occurred. Proportions within columns sum across the columns within zones. The number below each bar is the average distance from the origin in Figure 4 for the trait combination and indicates its strength. See the methods for descriptions of the zones

2007), meaning that either strategy appears to be free to develop independently.

The resistance and tolerance rankings to *O. pseudotsugae* in the families we tested were not similar to the rankings of five of the same families inoculated with Armillaria root disease (Cruickshank & Jaquish, 2014); however, that was a small sample of families for comparison. Evidence from elsewhere suggests that plant resistance and tolerance rankings to multiple pests may not align completely (Leimu & Koricheva, 2006b) or that it is uncommon for families to be top performers in all traits. The family-level variation in tolerance and resistance response to fungal infection demonstrated that there were examples of all combinations of strategies. Variation in defence strategy occurs when costs and benefits vary in time and space (Strauss & Agrawal, 1999), most likely due to alternating environmental conditions (Lazzaro & Little, 2009). Hamilton, Zangerl, DeLucia, and Berenbaum (2001) suggested that defence benefits must outweigh costs from an evolutionary perspective if they are to remain in the population. The current study suggested that family tolerance to a fungus was a cost to trees before inoculation because the initially shorter trees on average were the most tolerant after inoculation. In another study, older infected 20-35-year-old Douglas-fir trees had small volume before infection that allowed trees to become almost as large as their healthy counterparts did over time (Cruickshank, Morrison, & Lalumière, 2011), probably demonstrating disease tolerance. Combined, these results suggest that the initial cost of tolerance could pay back over time in trees exposed to an environmental stress. In this study, family resistance came at no detectable cost to trees before induction, but there was a cost after inoculation with *O. pseudotsugae* that may last for some time. Cost of resistance to Armillaria root disease in five infected 22-year-old Douglas-fir families accumulated long after fungal infection (Cruickshank & Jaquish, 2014). For seedlings, the implication of defence costs described above might indicate that having some tolerance to damage would be preferable when height growth is critical.

In the current study, elevated resistance combined with lowered tolerance to *O. pseudotsugae* was the only defence strategy that clearly appeared less frequently than other combinations

of the two defence strategies. Considering that Douglas-fir is a relatively shade-intolerant species (Burns & Honkala, 1990), any reduction in height growth might cause lethal overtopping in the long term. At sapling stages when growth is maximized, height increment reduction would have greater consequences than later on in the rotation when growth is restricted. Our data support the competition hypothesis as seedlings in families from the most productive zones (SA and WKL) tended to have higher tolerance combined with higher resistance or lower tolerance with lower resistance as the most frequent strategies. Higher tolerance within a family in conjunction with higher resistance might negate some of the cost of inducing resistance. Families possessing lower response of both resistance and tolerance strategies would also have lower cost to growth.

In plants, the frequency of resistance and tolerance may be associated with neighbourhood and temporal stand dynamics. Strategy switching with age could also be common in plants and is possibly even more likely in long-lived trees where fitness costs accumulate over time (Boege, Dirzo, Siemens, & Brown, 2007; Muola, Mutikainen, Laukkanen, Lilley, & Leimu, 2010). Strong resistance traits expressed in response to a stress agent may have less growth impact at older stand ages when trees are already growing slowly. Resistance also might be more effective in taller trees when height also causes constraints to water balance (Ryan, Phillips, & Bond, 2006) and damage exacerbates this constraint. In other studies of Douglas-fir concerning radial wood properties (Cruickshank & Filipescu, 2017) and survival (Cruickshank, 2016), it was suggested that water balance was connected to fungal root disease. Understanding the frequency of each strategy based on costs and benefits in an ecological framework is not straightforward (Stamp, 2003), especially in longer-lived plants where conditions also change with time. More work needs to be carried out to understand these connections.

ORCID

M. G. Cruickshank http://orcid.org/0000-0003-4687-1242

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