Effects of Ortet Genotype and Western Spruce Budworm Defoliation on Foliar Nutrients in Douglas-fir Clones

Karen M. Clancy
USDA Forest Service Research and Development, Rocky Mountain Research Station, Flagstaff, Arizona 86001-6381, USA

Zhong Chen, Thomas E. Kolb
School of Forestry, Northern Arizona University, Flagstaff, Arizona 86011-15018, USA

Abstract – Greenhouse experiments with Douglas-fir clones that are resistant versus susceptible to the western spruce budworm demonstrated that foliar concentrations of sugars and P had a genetic basis. Budworm defoliation changed levels of sugars, P, K, Mn, and Zn, and had divergent effects on concentrations of P, K and Zn in resistant compared to susceptible clones. Induced susceptibility, whereby defoliation alters foliar nutrients to make trees more favorable for insect feeding, appears to be an important determinant of Douglas-fir resistance to the western spruce budworm.

I. Introduction

Tree resistance plays an important role in the ecology of forest insects [1]. We have summarized the role of many potential mechanisms of resistance in trees to defoliators using western spruce budworm (Choristoneura occidentalis Freeman) and interior Douglas-fir (Pseudotsuga menziesii var. glauca [Beissn.] Franco) as a model system [2]. We studied this insect-plant system in detail because the western spruce budworm is the most important forest defoliator in western North America [3], and Douglas-fir is a commercially important host tree species [4-6].

We have evaluated mechanisms of resistance for the Douglas-fir/budworm model system using a combination of laboratory diet bioassays [7], field observations on pairs of mature Douglas-fir trees that are phenotypically resistant versus susceptible to damage from the western spruce budworm [8, 9], and greenhouse bioassays with grafted clones derived from the resistant and susceptible trees [10-12].

Three mechanisms appear to be important determinants of Douglas-fir resistance to the western spruce budworm: phenological asynchrony [8, 10, 12] (Fig. 1), vigor (i.e. growth rate; resistant trees had greater radial growth rates than susceptible trees in two of three populations studied [8, 10, 13]), and nutritive quality of foliage (resistant trees have higher levels of N and sugars and lower mineral/N ratios for P/N, Mg/N, K/N, and Zn/N in their current-year foliage than susceptible trees [2, 8, 9, 14-17]). On the other hand, the following five mechanisms have been excluded: compensatory photosynthesis [10], toughness of needles [2, 18], defensive compounds (i.e. monoterpenes) in foliage [8, 9, 11, 19, 20], induced defenses (i.e., induction of foliar monoterpenes [11]), and western spruce budworm feeding and oviposition behavior [21].

The overall objective of this study was to further understand the role of foliar nutrients as a resistance mechanism of Douglas-fir to western spruce budworm defoliation. We used data on concentrations of foliar nutrients from resistant and susceptible mature trees (i.e., ortets) and clones of these trees in the greenhouse to test three null hypotheses: (H0 1) Foliar nutritional chemistry does not have a genetic basis; there is no correlation between concentrations of foliar nutrients of the ortets and their corresponding clones. Alternatively, positive correlations between the ortets and clones would indicate that foliar nutrients are under genetic control to some degree. (H0 2) Foliar nutritional chemistry does not change in response to budworm defoliation, for either resistant or susceptible clones. On the other hand, changes in foliar chemistry in response to budworm defoliation that are not the same for resistant and susceptible clones would support induced susceptibility in host trees as an important mechanism [2]. (H0 3) Foliar nutritional chemistry is not different between resistant versus susceptible clones. Conversely, inherent differences in foliar chemistry between resistant versus susceptible clones would support nutritive quality as an important mechanism [2].

![Fig. 1. Mean (± 2 SE, or an ≈95% confidence interval) phenological scores (0 = overwintering bud stage, 7 = feather duster shoot growth stage [8, 22]) for paired Douglas-fir trees from three sites that were phenotypically resistant versus susceptible to western spruce budworm defoliation. The P-values from paired t-tests (Kaibab 90, San Isabel 95) or nested (i.e., paired) ANOVA (Kaibab 91 + Pike 91) used to compare resistant versus susceptible trees in each year are shown in the row at the top of the figure [2]. The resistant trees consistently had later budburst phenology than the susceptible trees.](image-url)
II. Materials and Methods

Our experimental plant material consisted of clones derived from mature Douglas-fir trees that differed in western spruce budworm defoliation under field conditions [8]. The mature Douglas-fir trees were from sites in the United States on the Pike National Forest near Deckers, CO and the Kaibab National Forest near Jacob Lake, AZ. At the time the trees were identified (1988 and 1989) most of the trees at the sites had sustained moderate to severe budworm defoliation for at least several years, as determined from their growth form and general condition. We selected seven phenotypically resistant trees at the Pike National Forest site and five phenotypically resistant trees at the Kaibab National Forest site by identifying trees with full crowns and little other evidence of budworm damage. These trees were visually distinct from other trees in the stand that were characterized as phenotypically susceptible based on their defoliated crowns. Each resistant tree was paired with a nearby (within 30 m) susceptible tree of similar size (height and DBH) and microsite (slope and aspect). In other words, the pairs of resistant and susceptible trees were “matched” as closely as possible to minimize any size-, age-, or microsite-related effects that could confound effects associated with different levels of herbivory. We deliberately chose pairs at each site that represented a range of size (i.e., age) classes. Age of the 24 trees ranged between 45 and 123 years (79.3 ± 4.1 years [mean ± SE, here and throughout]); height ranged between 6.4 and 14.9 m (10.4 ± 0.5 m); DBH ranged between 15 and 40 cm (25.3 ± 1.3 cm).

We cloned each of the 24 mature trees by whip-grafting branches collected from the lower third of the crown onto one-year seedling rootstocks in 1991 and 1992. This is a common and widespread technique for reproducing mature tree characteristics in a smaller plant [23, 24]. Such cloning resulted in the fixation of the genotype and tissue developmental stage of mature trees but not tree environment.

The experiment had a completely randomized block design composed of six blocks, each containing 48 clonally propagated trees (i.e. two treatments [budworm defoliation versus control] x two traits [resistant versus susceptible]/pair x 12 pairs). In total, 288 cloned trees were included in the experiment. However, 11 trees died before the experiment started, therefore, there were actually four to six replications of each treatment combination for each of the 12 pairs.

In order to test the role of budburst phenology as an influence on budworm performance, we conducted the budworm defoliation experiment differently in 1998 and 1999 [12]. In 1998, defoliation by budworm larvae was matched to the budbreak phenology of each individual clone. Because budworm larval feeding was purposely matched to the fourth budburst stage of each clone, the effect of genetic variation in budburst phenology among trees on budworm feeding was minimized. However, in 1999 the larvae were placed on all the trees at the same date when approximately 50% of all terminal buds in the population were in the second (i.e. yellow) budburst stage [22]. This schedule of larval introduction allowed genetic differences in budburst phenology among trees to influence the developmental stage of buds available for budworm feeding, as can occur in Douglas-fir forests [8]. All the trees were fertilized in 1998 and 1999. In 2000, none of the trees were defoliated and the clones were not fertilized so that we could determine if lower levels of soil nutrients had different effects on the foliar chemistry of resistant versus susceptible trees. The budworm larvae used in our study were from our laboratory cultures of diapausing and nondiapausing western spruce budworms, maintained in the Entomology Laboratory at the Rocky Mountain Research Station, Flagstaff, AZ, U. S. A. The nondiapausing colony has growth rates and feeding behavior similar to a wild population [26].

Current-year foliage from the clonal trees in the greenhouse was sampled in 1998 and 1999 when late instars of the budworm were actively feeding on the defoliated trees, and the foliage was at the seventh (or feather duster) developmental stage [22]. Seventh stage foliage was also sampled in 2000. Two clusters of current-year foliage were clipped at random from the upper third of the crown, sealed inside plastic bags, temporarily stored in a freezer, and later transferred to ultralow freezer and stored at −60 °C until analyzed. The foliage sampled from defoliated trees was not directly damaged by budworms.

The needles were pulled off the stems in preparation for the chemical analyses, and a composite subsample of all the current-year needles sampled in each year from the four to six trees from the same clone and treatment (i.e. budworm defoliation versus control) was analyzed (i.e., the samples taken from the four to six trees were pooled prior to chemical analyses). Therefore, no block effect was included in the analysis of the foliar nutrient data. A total of 48 pooled foliage samples were used for chemical analysis for each of the three sample years (i.e. two treatments [defoliated versus control] x two traits [resistant versus susceptible clones] x 12 pairs). We wore disposable gloves when handling the foliage to avoid contamination with minerals from our skin.

Foliage samples were analyzed by the Analytical Services Laboratory at Northern Arizona University for the following: total Kjeldahl nitrogen (N) and phosphorus (P) (colorimetrically); potassium (K), calcium (Ca), magnesium (Mg), manganese (Mn), copper (Cu), iron (Fe) and zinc (Zn) (by flame atomic absorption spectroscopy); and sugars – sucrose, fructose and glucose (by high-performance liquid chromatograph). Nutrient concentrations were based on the dry weight of the foliage.

We used Spearman’s rho (ρ) to examine correlations between the foliar chemistry of the Douglas-fir parent trees and the clones that were derived from them. We used the mean value of all observations for foliar nutrients for each of the 24 ortets (two or three samples/tree [9]), and the 1998, 1999, and 2000 corresponding grafted clone mean values (two samples/clone [defoliated and not defoliated treatments] for each sample year). Rho was determined for each of the 10 nutrients (dry weight foliar concentrations of N, sugars [sucrose + fructose + glucose], P, K, Mg, Ca, Mn, Cu, Fe and Zn); we could expect 1.5 of the 30 rho values to
be significant from random chance at $P = 0.05$.

Repeated measures analysis of variance (ANOVA) tests were used to investigate the effects of trait (resistant versus susceptible), treatment (defoliated versus not defoliated), and sample year (1998, 1999, and 2000), and their interactions on variations in concentrations of each of the 10 nutrients [25]. The Douglas-fir populations we sampled from the Pike (Colorado) and Kaibab (Arizona) National Forest sites were not genetically differentiated based on an isoenzyme study [27]; consequently, we treated the 12 pairs of trees from these two sites as one population for statistical analysis.

III. Results

A. $H_0$: Foliar Nutritional Chemistry Does Not Have a Genetic Basis

We calculated Spearman's rho ($\rho$) between the parent trees and their corresponding grafted clones ($n = 24$ ortet/clone pairs) to test this null hypothesis. We failed to reject the null hypothesis for foliar concentrations of N, K, Mg, Mn, Cu or Zn; 18 out of the 20 values for Rho were not significant ($P > 0.05$) [25].

However, significant and positive rank correlations led us to reject the null hypothesis for sugars in all three sample years (Fig. 2A), and for phosphorus in 1999 (Fig. 2B). There were also positive but non-significant rank correlations for P in 1998 ($\rho = 0.292, P = 0.166$) and 2000 ($\rho = 0.219, P = 0.302$).

We concluded that foliar concentrations of sugars and possibly P were under some degree of genetic control for Douglas-fir in our study, as evidenced by the robust significant positive correlations between the foliar chemistry of the ortets and their corresponding clones (Fig. 2). On the other hand, foliar concentrations of N, K, Mg, Ca, Mn, Cu, Fe and Zn did not appear to be under strong genetic control; positive correlations between foliar chemistry of the ortets and clones were weak or absent.

B. Effect of Sample Year

A noteworthy pattern in the data from the nutritional chemistry of the grafted clones is that the sample year main effect was very strong ($P < 0.001$) for all nutrients except Ca [25]. There were large increases in the foliar concentrations of N (Fig. 3A), P (Fig. 4A), K (Fig. 4B), Mg (data not shown), Mn (Fig. 3C), Fe (data not shown) and Zn (Fig. 4C) in all clones between 1998 and 1999. This increase is most likely because the grafted trees were transplanted from 1- to 5-gallon (15-liter) pots in 1997, prior to the start of the experiment, to give the roots more room to grow. The root mass area probably increased substantially between the 1998 and 1999 growing seasons, thus allowing the trees to absorb more nutrients from the soil in 1999 compared to 1998.

The trees were not fertilized in 2000 because we wanted to determine if lower levels of soil nutrients had different effects on the foliar chemistry of resistant versus susceptible trees. There were large decreases in levels of N (Fig. 3A), sugars (Fig. 3B), P (Fig. 4A), K (Fig. 4B), Mg (data not shown), Mn (Fig. 3C), Cu (data not shown), Fe (data not shown) and Zn (Fig. 4C) between 1999 and 2000 that apparently reflected the lower levels of these nutrients in the soil after we stopped fertilizing. Moreover, foliar concentrations of N, sugars, P, K, Cu and Zn were lowest in 2000 among all sample years.

Fig. 2. Mean values for dry weight concentrations of sugars in 1998, 1999, and 2000 (A) and phosphorus in 1999 (B) in current-year foliage from grafted Douglas-fir clones versus ortets (i.e., parent trees) that are resistant versus susceptible to defoliation by the western spruce budworm ($n = 24$ for each year). The lines on the graphs were fit with regression analysis. Spearman’s rho ($\rho$) between the ortet mean values and the corresponding grafted clone mean values are shown at the top of each graph.
We evaluated this null hypothesis by the significance of the treatment (defoliated versus not defoliated) main effect in the repeated measures ANOVAs, and by the two-way interactions that included the treatment effect. The only significant three-way interaction in the ANOVAs was for sugars [25].

We failed to reject the null hypothesis for N, Mg, Ca, Cu and Fe because there were no detectable effects of budworm defoliation on foliar concentrations of Mg, Ca, Cu and Fe [25], and inconsistent effects on N (Fig. 3A). There were variable effects of defoliation on N among sample years; foliar N was slightly lower in defoliated versus undefoliated trees in 1998, but was higher in the defoliated trees in 1999 for both resistant and susceptible clones. In 2000, previous defoliation had no effect on N levels in resistant clones, whereas past defoliation increased foliar N in susceptible clones.

Conversely, we rejected the null hypothesis for sugars (Fig. 3B), Mn (Fig. 3C), P (Fig. 4A), K (Fig. 4B), and Zn (Fig. 4C), based on values of $P \leq 0.042$ for the treatment main effect or interactions involving the treatment effect. Defoliation generally decreased levels of sugars in 1998 and 1999, but this trend was more consistent for the susceptible compared to the resistant clones (Fig. 3B). Sugars were equivalent in previously defoliated versus control trees in 2000 for both resistant and susceptible clones (Fig. 3B). Phosphorus, K and Zn increased in response to defoliation in susceptible clones in 1999 and 2000, whereas they were unaffected by defoliation in resistant clones (Fig. 4). Defoliation dramatically decreased levels of Mn in resistant clones in 1999, however it had little effect otherwise (Fig. 3C).

We concluded that budworm defoliation generally decreased levels of sugars in Douglas-fir foliage of both resistant and susceptible clones when sugar concentrations were highest (years 1998 and 1999), and it decreased levels of Mn in resistant clones in the year when overall Mn was highest (1999). Moreover, effects of budworm defoliation on concentrations of P, K and Zn differed between resistant versus susceptible clones. Effects of defoliation on other nutrients were non-significant or inconsistent.

D. $H_0$ 3: Foliar Nutritional Chemistry is Not Different between Resistant and Susceptible Clones

We tested this null hypothesis based on the significance of the trait (resistant versus susceptible) main effect in the repeated measures ANOVAs, and by interactions that included the trait effect. There were no clear differences between resistant and susceptible clones that were independent of the trait $\times$ year, trait $\times$ treatment, or trait $\times$ treatment $\times$ year interactions reported above [25]. Moreover, approximate 95% confidence intervals (i.e., mean values $\pm 2$ SE) for the three-year average values ($n = 36$) of nutrient concentrations for the control (i.e., not defoliated) trees did not differ significantly between the resistant and susceptible clones (Table 1). Consequently, we failed to reject this null hypothesis.

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C. $H_0$ 2: Foliar Nutritional Chemistry Does Not Change in Response to Budworm Defoliation

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Fig. 3. Mean (± SE, $n = 12$ clones per bar) dry weight concentrations of nitrogen (A), the sugars sucrose, glucose, and fructose (B), and manganese (C) in current-year Douglas-fir needles sampled at the feather duster (7th) bud development stage from clones derived from interior Douglas-fir trees (i.e., ortets) that are resistant (Res.) versus susceptible (Sus.) to defoliation by the western spruce budworm. Results are shown for three sample years (1998-2000). See text for details about how the experimental conditions differed among the three sample years.
A. Phosphorus
Res. or Sus. \( P = 0.081 \)
Defoliated or Not \( P = 0.006 \)
Interaction \( P = 0.023 \)

Mean (± SE, \( n = 12 \) clones per bar) dry weight concentrations of phosphorus (A), potassium (B), and zinc (C) in current-year Douglas-fir needles sampled at the feather duster (7th) bud development stage from clones derived from interior Douglas-fir trees (i.e., ortets) that are resistant (Res.) versus susceptible (Sus.) to defoliation by the western spruce budworm. Results are shown for three sample years (1998-2000). See text for details about how the experimental conditions differed among the three sample years. The dashed line on each graph and corresponding number on the right y-axis show the optimal dry weight concentrations of each nutrient for the budworm, established in diet bioassays [9].

### TABLE I

Approximate 95% Confidence Intervals for the Average Dry Weight Concentrations of Foliar Nutrients in Current-year Needles from Undefoliated Douglas-fir Clones that are Resistant Versus Susceptible to the Western Spruce Budworm

<table>
<thead>
<tr>
<th>Variable (units)</th>
<th>Resistant</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td>2.25-2.97</td>
<td>2.13-2.82</td>
</tr>
<tr>
<td>Sugars (%)</td>
<td>9.83-11.38</td>
<td>9.81-11.27</td>
</tr>
<tr>
<td>P (mg/g)</td>
<td>3.55-4.60</td>
<td>3.33-4.19</td>
</tr>
<tr>
<td>K (mg/g)</td>
<td>13.43-16.32</td>
<td>12.37-14.79</td>
</tr>
<tr>
<td>Mg (mg/g)</td>
<td>1.87-2.13</td>
<td>1.76-2.00</td>
</tr>
<tr>
<td>Ca (mg/g)</td>
<td>1.31-1.48</td>
<td>1.32-1.77</td>
</tr>
<tr>
<td>Mn (( \mu )g/g)</td>
<td>39.51-51.21</td>
<td>36.43-47.07</td>
</tr>
<tr>
<td>Cu (( \mu )g/g)</td>
<td>4.61-6.88</td>
<td>4.08-6.00</td>
</tr>
<tr>
<td>Fe (( \mu )g/g)</td>
<td>34.34-46.44</td>
<td>33.00-41.55</td>
</tr>
<tr>
<td>Zn (( \mu )g/g)</td>
<td>31.14-38.52</td>
<td>31.06-37.94</td>
</tr>
</tbody>
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IV. Discussion

**A. \( H_0 \) 1: Foliar Nutritional Chemistry Does Not Have a Genetic Basis**

There was convincing evidence to reject this null hypothesis for sugars (Fig. 2A) and P (Fig. 2B), indicating that foliar concentrations of sugars and P were under genetic control to some degree. Thus, these are heritable traits that could be altered by selection. However, we failed to reject the hypothesis for levels of N, K, Mg, Ca, Mn, Cu, Fe and Zn in the foliage [25]. The most parsimonious explanation for these divergent results relates to our method of clone propagation by grafting shoots of mature tree ortets onto generic seedling rootstocks that did not match the ortet genotype. This method of propagation produced clones that shared the same genotype of the ortet in above-ground tissues, but not roots. Evidence for genetic control of foliar nutrient levels was strongest for sugars produced directly by photosynthesis in the above-ground tissues, which were genetically identical to the ortet. In contrast, we found little evidence for genetic control of foliar concentrations of nutrients acquired by the root system, which was a different genotype than the ortet. Our experiment probably did not provide a very robust test of the null hypothesis for foliar nutrients that are heavily influenced by characteristics of the tree’s root system. Foliar concentrations of P, which were positively correlated between ortets and grafted clones in all years, are an exception to this explanation. Genetic control over foliar P and sugar concentrations might be linked given that P is critical to energy transfers in photosynthesis and respiration (e.g., ATP) that are required for sugar synthesis [28].
Palermo et al. [29] conducted another test of this hypothesis for nutrients absorbed by roots; they measured concentrations of N, P, Mg and Zn in the current-year foliage of 3-year-old half-sib seedlings grown from open-pollinated seeds collected from 11 pairs of resistant and susceptible Douglas-firs from our study sites. There was variation among the half-sib seedlings from the 11 resistant maternal genotypes in N ($P = 0.006$), and variation among the seedlings from the 11 susceptible maternal genotypes in N ($P = 0.002$) and P ($P = 0.004$). The existence of variation in levels of foliar nutrients among half-sib seedlings from different maternal trees suggests that foliar nutritional chemistry of Douglas-fir can be influenced by the genotype of the tree, although the relationship between this variation and resistance to western spruce budworm defoliation is presently unclear.

**B. $H_0$ 2: Foliar Nutritional Chemistry Does Not Change in Response to Budworm Defoliation**

We rejected this null hypothesis for sugars (Fig. 3B), P (Fig. 4A), K (Fig. 4B), Mn (Fig. 3C) and Zn (Fig. 4C). Defoliation by the western spruce budworm changed concentrations of these foliar nutrients, although it did not have detectable (Mg, Ca, Cu and Fe) [25] or consistent (N) (Fig. 3A) effects on the other nutrients we measured. Kolb et al. [30] also reported that budworm defoliation changed foliar nutrient levels in Douglas-fir; heavy defoliation increased concentrations of N, Ca and Mg in seedlings. Furthermore, Clancy et al. [31] documented several additional examples of how herbivory can change levels of foliar nutrients in coniferous trees.

More importantly, effects of budworm defoliation on foliar levels of P, K and Zn differed between resistant and susceptible clones, based on the significant trait × treatment interactions terms in the ANOVAs (Fig. 4). This result lends support for induced susceptibility as an important mechanism of resistance in the Douglas-fir/western spruce budworm system, as hypothesized by Clancy et al. [8] and Clancy [2].

On the whole, there was no convincing evidence from this experiment to support rejecting the null hypothesis that there are no inherent differences in foliar nutrients between the resistant and susceptible clones (Table 1). This was an unexpected result, given the differences in foliar nutrient concentrations observed between the resistant and susceptible mature tree ortets (plus 54 additional mature Douglas-fir trees sampled) in the forest [2, 8, 9, 14-17]. We believe that these previously reported differences in foliar nutrient levels between the resistant and susceptible mature trees in the forest after budworm outbreaks were caused by prior defoliation as discussed above. In other words, the differences in foliage nutrient levels are probably the result of different defoliation patterns rather than the cause.

**C. $H_0$ 3: Foliar Nutritional Chemistry is Not Different Between Resistant and Susceptible Clones**

We believe that the most important proximate mechanism causing lower levels of budworm defoliation in resistant Douglas-fir trees is mismatched phenology between budburst of the trees and the emergence of second instar budworm larvae in the spring; resistant trees have later budburst compared to susceptible trees [2, 8, 10, 12] (Fig. 1). Accordingly, the susceptible trees in a stand, which have earlier budburst, are defoliated more. However, the susceptible trees also have inherently slower growth rates than the resistant trees [2, 8, 10, 13], resulting in less capacity to tolerate or compensate for lost photosynthetic area (sensu McNaughton [32]). The results from this experiment demonstrate that the foliar nutrients of
susceptible trees appear to be more prone to change in response to budworm defoliation than resistant trees. Consequently, susceptible trees become a better source of food for the larvae with consecutive years of defoliation, but resistant trees do not.

Clancy et al. [8] suggested that budworm defoliation of susceptible Douglas-firs might have a positive feedback for subsequent generations, as in the “resource regulation hypothesis” proposed by Craig et al. [33]. This experiment provides empirical evidence that this is indeed the case for interior Douglas-fir trees. We conclude that induced susceptibility via changes in concentrations of foliar terpenoids is a fourth mechanism that appears to be an important determinant of Douglas-fir resistance to the western spruce budworm.

Acknowledgements

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