Do Ectomycorrhizal Mutualists Influence Douglas-fir Resistance to Defoliation by the Western Spruce Budworm?

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Abstract - Douglas-fir seedlings derived from parent trees that are resistant versus susceptible to western spruce budworm defoliation were inoculated with *Laccaria bicolor* ectomycorrhizal fungi or untreated. Inoculated resistant seedlings had more infested root tips than susceptible seedlings. *L. bicolor* increased foliar concentrations of phosphorus and magnesium in resistant seedlings, and it increased the growth rate of susceptible seedlings. These divergent responses to *L. bicolor* treatment suggest that ecotmycorrhizae might play a role in Douglas-fir resistance to damage from the budworm.

I. Introduction

Mycorrhizae are symbiotic, mutually beneficial associations between the roots of trees and specialized soil fungi [1]. All forest trees form mycorrhizae in natural soils. These fungus-root organs provide many benefits to the seedling and adult tree, especially enhancing water and nutrient uptake [1]. Thus, mycorrhizal associations may affect plant resistance to herbivory by way of their influence on mineral nutrients, host plant vigor, etc. [2, 3]. There are >2,000 mycorrhizal species associated with Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) trees [4, 5].

Because mycorrhizae have important influences on plant growth and nutrient levels in plant tissues, they might affect plant resistance to herbivores such as the western spruce budworm (*Choristoneura occidentalis* Freeman). For example, interior Douglas-fir (*P. menziesii* var. *glauca* [Beissn.] Franco) trees that are phenotypically resistant to damage from *C. occidentalis* had higher levels of nitrogen (N) and sugars, plus lower mineral/N ratios for phosphorus/N, magnesium/N, potassium/N, and zinc/N, in their foliage than susceptible trees [6]. Resistant trees also had greater radial growth rates compared to susceptible trees in two of three populations studied [7].

Four mechanisms of interior Douglas-fir resistance to damage from the western spruce budworm have been documented [7, 8]; three of the mechanisms could be linked with potential mycorrhizal mediation of herbivore resistance. First, phenological asynchronony [7, 9-11] resulting from delayed bud burst enables resistant trees to reduce the exposure of vulnerable developing tissues to western spruce budworm larvae when they emerge to feed on swollen buds in the spring; this seems unlikely to be influenced by mycorrhizae. Second, foliar nutritive quality influences budworm larval performance, with intermediate levels of sugars and key elements including P, Mg, and K (plus mineral/N ratios) being optimal and lower and higher tissue concentrations reducing larval performance [6, 7, 12-14]. Variation among trees in foliar nutrients could be associated with mycorrhizae. Third, shoot vigor, measured as growth rate, is positively related to western spruce budworm resistance [9, 10, 15], and tree growth rates could also be affected by mycorrhizae. The fourth mechanism is induced susceptibility, whereby defoliation alters foliar nutrients to make trees more favorable for insect feeding [8]. Differences in mycorrhizae might help explain why susceptible trees appear to be more prone to changes in foliar nutritional chemistry in response to western spruce budworm defoliation than resistant trees.

The objective of this study was to investigate the potential role of ectomycorrhizal fungi in determining Douglas-fir resistance to defoliation by the western spruce budworm [3]. We used half-sib seedlings grown from open-pollinated cones collected from resistant and susceptible Douglas-firs [10] to address the following questions: 1) Do ectomycorrhizal fungi change the chemical composition of Douglas-fir foliage, or the growth rate of seedlings? 2) Could ectomycorrhizae help determine Douglas-fir resistance to defoliation by the budworm?

II. Materials and Methods

We used 30–90 cm, 3-year-old Douglas-fir half-sib seedlings. The seedlings were grown from open-pollinated seeds collected from eight pairs of trees that are phenotypically susceptible (showing obvious signs of a history of defoliation) or resistant (healthy-looking) to the budworm on the Pike National Forest near Deckers, CO and from three pairs of trees on the San Isabel National Forest near Buena Vista, CO [6, 9, 16]. The resistant and susceptible parent trees were paired in the field (within 60 m of one another) based on similarities in age, height, and microsite. Seeds were collected from the 11 pairs of mature Douglas-fir trees (11 resistant trees plus 11 susceptible trees, for a total of 22 parent tree genotypes) to produce the half-sib seedlings, which were grown in the Rocky Mountain Research Station greenhouses in Flagstaff, AZ [10]. We matched half-sib seedlings derived from the

original pairs of resistant and susceptible parent trees throughout the experiments in order to control for variation among the 11 pairs. The seedlings were raised in a nutrient-poor peat moss-vermiculite growing medium and had not been fertilized for the previous 2 years.

The seedlings were subjected to one of two treatments: 36 were inoculated with ectomycorrhizal fungi, and 54 served as untreated controls. Seedlings from both groups were removed from their small plastic pots (15 cm diameter × 20 cm in height) and transplanted into larger pots (30 cm in diameter × 27 cm in height). All of the seedlings were irrigated with pH-adjusted water to promote fungal growth. Soil pH was checked regularly for all treatments to ensure that it remained between 5.0 and 6.0. Most species of mycorrhizal fungi prefer slightly acidic soils; pH levels that are too high or too low can kill the fungus and inhibit the uptake of nutrients [4, 17].

At the time of transplant, 36 seedlings (18 resistant and 18 susceptible) were artificially inoculated with *Laccaria bicolor*, an ectomycorrhizal fungus associated with Douglas-fir trees and commonly used in nurseries [18]. Root samples (20 cm in length) were cut from 10 randomly selected trees (five resistant and five susceptible) and inspected under a microscope to calculate the percentage of short roots infected with the fungus prior to inoculation (number of infected root tips ÷ the total number of root tips). This is the same procedure used by Gehring and Whitham [19] to compare the proportion of ectomycorrhizal colonization on resistant versus susceptible pinyon pine (*Pinus edulis* Engelmann) trees. Pre-treatment inspection of the roots revealed the presence of little or no mycorrhizae (0 – 6%), most likely because of the highly alkaline water used for irrigation. The proportion of short roots that were infected with the fungus averaged $2 \pm 0.6\%$ (± SE, here and throughout) on both resistant and susceptible genotypes. The seedlings were root-dipped into a hydrogel containing *L. bicolor* inoculant (vegetative mycelium) grown in sterile culture on a vermiculite carrier. The inoculant (MycorTree[TM] *Laccaria*) was purchased from Plant Health Care, Inc., in Pittsburgh, PA. An absorptive compound (Terra-Sorb[TM] Fine, Plant Health Care, Inc., Pittsburgh, PA) was added to the mixture to ensure adhesion to the roots.

The remaining 108 seedlings (54 resistant and 54 susceptible) were untreated. The large number of controls was necessary to ensure enough were available for three sets of budworm-feeding comparisons.

All seedlings were maintained in a greenhouse where temperatures were controlled by a computer to simulate early spring conditions in a high elevation Douglas-fir habitat (12 h at 4^oC and 12 h at 10^oC). The cooler temperatures were necessary to delay flushing and provide sufficient time for the inoculation treatment to have an effect. Once individual trees began to flush, greenhouse temperatures were increased (12 h at 10^oC and 12 h at 25^oC) to promote flushing of the remaining trees.

We used three paired treatment comparisons (i.e., three paired *t*-tests) to evaluate the effects of parent tree category (resistant versus susceptible) and ectomycorrhizae

(inoculated versus not inoculated) on foliar nutritional chemistry and seedling growth. Resistant versus susceptible half-sib seedlings were matched according to physical similarities (i.e., height, foliage density, and general architecture) to minimize potential budworm feeding preferences based on differences in host plant morphology. Originally, there were 18 pairs of resistant versus susceptible half-sib seedlings in each of the three paired *t*-tests, totaling 54 pairs (108 seedlings). However, 9 pairs had to be eliminated because one or both seedlings within the pair died during the experiment, leaving a total of 45 pairs (90 seedlings).

We compared: 1) Twelve pairs of resistant versus susceptible control seedlings to find out if there were inherent differences in responses (i.e., foliar nutritional chemistry and seedling growth) between seedlings from the two parent tree categories; 2) Sixteen pairs of resistant control versus inoculated susceptible seedlings to determine if ecotmycorrhizae changed responses for susceptible seedlings; and 3) Seventeen pairs of resistant inoculated versus susceptible control seedlings to decide if ecotmycorrhizae changed responses for resistant seedlings. Pairs of resistant versus susceptible seedlings from the 11 different maternal tree pairs were distributed in approximately even proportions among the three treatment comparisons to avoid any bias.

Three or four newly flushed shoots were sampled from each seedling. The Analytical Services Laboratory at Northern Arizona University analyzed foliage samples for total Kjeldahl N and P (colorimetrically), plus Mg and Zn (by flame atomic absorption spectroscopy). These four key nutrients are known to affect budworm performance [6, 7, 12-14]. Only the current-year shoots were sampled because nutrients accumulate in the youngest needles and because they are the preferred food source of the budworm [20]. Results from the analyses were used to compare foliar nutritional quality among inoculated and untreated seedlings. Trees were sampled as soon as they began to flush to preserve the chemical composition of the expanding needles. The samples were temporarily stored in zip-lock bags and frozen at 0^oC until all of the trees had flushed.

We measured seedling height and basal diameter when the trees were transplanted into larger pots and again when the foliage was sampled for chemical analysis. Stem height and basal diameter were combined to calculate an overall growth ratio ([post-treatment height • (diameter)^2] ÷ [pre-treatment height • (diameter)^2]) to determine seedling growth due to treatment.

We used paired *t*-tests to contrast the seedling (foliar concentrations of N, P, Mg, and Zn, and growth) responses between the paired resistant and susceptible seedlings used in each of the three treatment comparisons. Because we used the same experimental units to measure multiple dependent variables, we could expect to detect differences for about 0.3 of the five response variables from random chance ($P = 0.05$).

III. Results

A. Inoculation with Ectomycorrhizal Fungi

Post-treatment inspection of 10 randomly selected seedlings that were inoculated (five resistant and five susceptible) revealed higher levels of ectomycorrhizae (8 − 14%) compared to the pre-treatment inspection (0 − 6%). Prior to inoculation with *L. bicolor*, both resistant and susceptible seedlings averaged 2 % fungal colonization on their roots.   After treatment, the average proportion of infected roots on inoculated resistant seedlings was significantly higher than the average proportion of infected roots on susceptible seedlings that were inoculated (Fig. 1).

B. Effects of Mycorrhizal Fungi on the Chemical Composition of Douglas-fir foliage

There were no significant differences in foliar concentrations of N (data not shown), P (Fig. 2A), Mg (Fig. 2B), or Zn (data not shown) between the resistant versus susceptible control seedlings ($P \geq 0.453$ [3]).   This suggested that there were no inherent differences in foliar chemistry between the resistant versus susceptible seedlings.

The ectomycorrhizal inoculation did not change the foliar chemistry of the susceptible seedlings (Figs. 2A, B; [3]). However, it significantly increased foliar concentrations of P and Mg in the resistant seedlings (Figs. 2A, B), although there was no effect on levels of N or Zn   [3].

C. Effect of Mycorrhizal Fungi on Seedling Growth

Seedling growth rates were equivalent for the resistant versus susceptible control seedlings (Fig. 2C), indicating there were no inherent differences in growth rates between the resistant and susceptible seedlings.   The ectomycorrhizal inoculation caused a slight but significant increase in the growth rate of the susceptible seedlings, but it had no detectable effect on growth of the resistant seedlings (Fig. 2C).
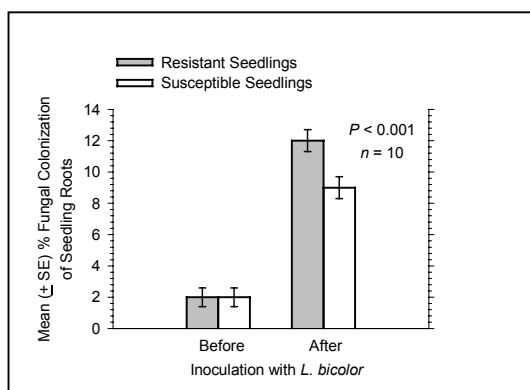

Fig. 1. Douglas-fir seedlings from resistant parent trees had more root tips infested with ectomycorrhizal fungi after inoculation with *L. bicolor* than susceptible seedlings did ($t = -17.36$, $P < 0.001$, $n = 10$).
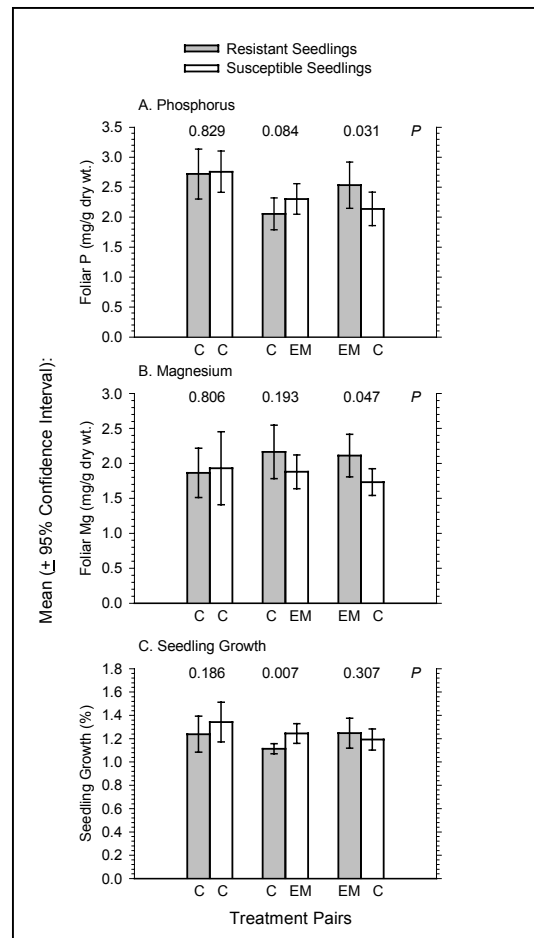

Fig. 2. Dry weight concentrations of P (A) and Mg (B) in the new (i.e., current-year) foliage, and seedling growth rates (C) of half-sib seedlings grown from open-pollinated seeds collected from interior Douglas-fir trees that are resistant versus susceptible to defoliation by the western spruce budworm. Results are shown for three treatment pairs, from left to right: 1) C C, resistant control (i.e., untreated) versus susceptible control seedlings; 2) C EM, resistant control versus susceptible seedlings treated with *Laccaria bicolor* ectomycorrhizal fungi; and 3) EM C, resistant ectomycorrhizal-treated versus susceptible control seedlings. The *P*-values from paired *t*-tests ($n = 12$-17) used to compare the seedlings' responses to the two treatments in each pair are shown in the first row at the top of the figure.

IV. Summary and Conclusions

L. bicolor increased foliar concentrations of P (Fig. 2A) and Mg (Fig. 2B) in resistant seedlings, and increased the growth of susceptible seedlings (Fig. 2C), despite the short treatment period.   The effects of mycorrhizal fungi on half-sib Douglas-fir seedlings in this experiment were limited to just 48 days, on average.   Yet, post-treatment inspection of roots from randomly selected seedlings revealed higher levels of fungal colonization (8 − 14%) compared to the pre-treatment inspection (0 − 6%) (Fig. 1). Unusually sunny weather during the experiment caused

greenhouse conditions to fluctuate, oftentimes exceeding the intended maximum temperature. As a result, the trees flushed earlier than we expected and collection of foliage samples for chemical analysis had to be conducted earlier than planned. Nonetheless, there were detectable differences from the ectomycorrhizal inoculations on seedling growth rates and on foliar P and Mg. The fungus had no detectable effect on N or Zn [3].

The inoculated half-sib seedlings from resistant parent trees had significantly more infected root tips than the inoculated seedlings from susceptible parent trees did (Fig. 1). The ectomycorrhizal inoculation also had different effects on the resistant versus susceptible seedlings for levels of P and Mg in the foliage (Figs. 2A, B) and for seedling growth rates (Fig. 2C). Overall, these results suggest that ectomycorrhizae might play a role in Douglas-fir resistance to damage from the western spruce budworm.

Muzika and Liebhold [21] stated that despite considerable research, the effect of foliage quality on population dynamics of defoliators is not well understood. Nonetheless, previous research has shown that budworm defoliation may alter foliar nutrients [8, 22], and reduce ectomycorrhizal colonization [23]. There may also be genetically based differences in the way resistant and susceptible trees respond to mycorrhizal fungi. We must consider the results presented here as preliminary, given the short treatment period and the relatively small sample sizes. Nonetheless, they suggest that mycorrhizal fungi may affect the differences in growth and foliar chemistry observed between the resistant versus susceptible parent trees in the field.

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