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Interactions among soil biology, nutrition, and performance of actinorhizal plant species in the H.J. Andrews Experimental Forest of Oregon☆

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Abstract

The study examined the effect of *Frankia*, macronutrients, micronutrients, mycorrhizal fungi, and plant-growth-promoting fluorescent *Pseudomonas* sp. on total biomass, nodule weight, and nitrogen fixation of red alder (*Alnus rubra*) and snowbrush (*Ceanothus velutinus*) under greenhouse conditions. The soil samples were collected from a 10-year-old clearcut on the H.J. Andrews Experimental Forest, Oregon. Within the clearcut, four sampling points were selected along a slope gradient. Red alder and snowbrush plants were greenhouse-grown in a mix of soil–vermiculite–perlite (2:1:1) for 6 and 12 months, respectively. Plants were inoculated with *Frankia* and a fluorescent *Pseudomonas* sp. Some of the red alder were also inoculated with ectomycorrhizal *Alpova diplophloeus*, and some snowbrush with endomycorrhizal *Glomus intraradix*. There was no interaction between treatment and slope location for either species. There were significant treatment effects for red alder, but not for snowbrush. Red alder seedlings given *Frankia* and macronutrients produced more biomass and had greater nitrogen fixation than seedlings grown without additions; adding *A. diplophloeus* increased nitrogen fixation by 33% over that obtained with *Frankia* plus macronutrients. *Frankia*, macronutrients, and the mycorrhizal fungus together increased nitrogen fixation by 136% over the control. Adding only micronutrients to *Frankia* and macronutrients, however, reduced nitrogen fixation by nearly one half; the presence of the mycorrhizal fungus appeared to buffer these negative effects. *Pseudomonas* inoculation did not affect any of the measured variables. Slope location of soil affected the two plant species differently. Red alder seedlings grown in upper slope soil had greater biomass and nitrogen fixation than those grown in soil from the lower slope. In contrast, snowbrush plants had greater biomass, nodule weight, and nitrogen fixation when grown in bottom slope soil rather than on soil from any of the other slope positions.

Keywords: *Frankia*; Red alder; Snowbrush; Actinorhizal plants; Nitrogen fixation

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1. Introduction

Actinorhizal plants (i.e. plants nodulated by actinomycetes in the genus *Frankia*) are the primary sources of biologically fixed nitrogen (N) in many temperate and boreal forest ecosystems; they may be responsible for most of the N inputs in northern forests that have low N inputs in precipitation (Perry, 1994). Predominant actinorhizal plants in the Pacific Northwest (North America) include four *Alnus* species, which occur in relatively mesic sites across a range of elevations (Hibbs et al., 1994), and eight species of *Ceanothus*, found in mesic to dry sites at mid-elevations (Rose and Youngberg, 1981; Conard et al., 1985). *Alnus rubra* Bong. (red alder) is by far the most abundant of the alders, covering 13% of the coastal commercial forest land in Oregon and Washington (Resch, 1988); *Ceanothus velutinus* Dougl. (snowbrush) is common on disturbed sites throughout the central Cascades and eastward into the northern Rocky Mountains. In legumes, nodule development and N fixation require the micronutrients Co, Cu, Fe, B, Mo, and Ni, as well as macronutrients; the first three can limit leguminous N fixation in the field (O'Hara et al., 1988a,b). Micronutrient limitations in actinorhizal plants are less well studied; however, as essential components of the nitrogenase enzyme, Fe and Mo are required by all diazotrophs. Mo limits asymbiotic N fixation in the Pacific Northwest (Silvester, 1989).

Past studies of Pacific Northwest clearcuts generally have found abundant nodulation on alders but variable nodulation on snowbrush (Zavitkovski and Newton, 1967, 1968; Wollum et al., 1968; Youngberg and Wollum, 1976; Youngberg et al., 1979; Binkley, 1981). Wollum et al. (1968) found that snowbrush nodulated better on sites formerly occupied by mid-aged conifer stands than on sites formerly occupied by old growth; they hypothesized that *Frankia* declined over time in the absence of host plants. However, that hypothesis was not supported by bioassays of an age sequence of stands that involved red alder and snowbrush, in which nodulation was highly variable within stands and varied between a recent clearcut (the site of the study reported here), a 20-year-old conifer plantation intermixed with snowbrush, and old growth (Rojas et al., 2001).

Soil microbes that are not diazotrophs may influence symbiotic N fixation either positively or negatively. Mycorrhizal fungi enhance symbiotic N fixation by improving host nutrition and perhaps water relations (Carling et al., 1978; Barea and Azcon-Aguilar, 1983). Rose and Youngberg (1981) found that mycorrhizal snowbrush plants had three times greater nodule biomass and fixed nearly twice as much N as non-mycorrhizal plants.

Nodulation of red alder is improved by *Pseudomonas fluorescens* Migula (Knowlton et al., 1980, Knowlton and Dawson, 1983), a common root-associated microbe that produces strong Fe^{3+} chelators, hydroxymate siderophores (HS) (Torres et al., 1986). Fe is known to enhance nodulation in legumes, at least for strains of *Rhizobium* that are inefficient at obtaining Fe on their own (O'Hara et al., 1988a).

The objective of this study was to determine whether the ability of red alder and snowbrush to nodulate and fix N in soils from the clearcut in the H.J. Andrews Experimental Forest was enhanced by amending soils with various combinations of *Frankia* plus macronutrients, micronutrients, mycorrhizal fungi, and fluorescent *Pseudomonas* sp. The ectomycorrhizal (EM) fungus *Alpova diplophleus* was for red alder, and the vesicular-arbuscular (VAM) *Glomus intraradix* for snowbrush. The research was stimulated by a widespread growth and survival failure of snowbrush seedlings germinating after clearcutting and burning. In addition, although red alder occurs in moist microsites near the study site, it has not colonized the clearcut. Previous work using soils from this site showed that nodulation of both red alder and snowbrush varied widely among sample points (Rojas et al., 2001).

We speculated that the failure of actinorhizal plants to establish in the clearcut was related to lack of *Frankia* inocula or other changes in soil biology stemming from site disturbance. We hypothesized that adding mycorrhizal fungi and fluorescent *Pseudomonas* sp. to clearcut soils would increase nodulation and N fixation of both plant species. Work elsewhere has shown that mycorrhizal fungi and HS may be lost in erosion from disturbed sites (Valdes, 1986; Amaranthus and Trappe, 1993; Perry et al., 1984). Mycorrhizal fungi function in nutrient gathering, and *Pseudomonas* functions in solubilizing Fe; we therefore expected the addition of nutrients to substitute, at least in part, for these two groups of microorganisms.

2. Materials and methods

2.1. Study area

Soils used for the greenhouse bioassays were collected from within a 10-year-old clearcut planted with Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) on the H.J. Andrews Experimental Forest in the Cascade Mountains, 80 km east of Eugene, Oregon, the only available site with a characteristic slope position suitable for this study. Shallow soils derived from ash flow, mudflow, and stream deposits are found at lower elevations; deeper soils derived from volcanic ash and andesite lava flows are found at middle and higher elevations (Swanson and James, 1975). On the Andrews Forest, soils derived from these parent materials are predominantly Inceptisols, with some Alfisols and Spodosols (Brown and Parson, 1973).

The clearcut is 890 m above sea level in the Western Hemlock Zone (Franklin and Dyrness, 1973). The site faces southeast on a slope averaging 22°. Formerly occupied by an old growth forest of Douglas-fir, western hemlock (*Tsuga heterophylla* (Raf.) Sarg.), and western redcedar (*Thuja plicata* Donn ex D. Don.), the site was clearcut in 1981, and logging slash was broadcast burned in 1982. Douglas-fir (900 seedlings/ha) was planted 6 months after slash burning. In surveys conducted in 1990, we found that most snowbrush seedlings in the field had not nodulated. The larger plants at slope bottoms had nodulated, but the small plants growing on mid- and upper slopes had not. Planted Douglas-fir has successfully established on the site.

2.2. Soil collection

Soils were collected along a transect originating at a randomly chosen point at the base of a 22° slope and extending 60 m up to the top of the slope at right angles to the contour. Four sampling points were selected along the transect, one each at the bottom of the slope, lower mid-slope, upper mid-slope, and top of the slope. At each sampling point, soils were collected at four locations (1 m from the sampling point in each of four compass directions) and pooled to make a single sample. This sampling scheme was chosen so that samples reflected a range of slope positions within the clearcut (the large number of planned treatments precluded replicating by slope position) All soil samples were obtained from the mineral soil to a depth of 15 cm.

2.3. Isolation and cultivation of Frankia

Frankia for use in inoculations of red alder was isolated from nodules of red alder collected near the study site. A modified Benson's (1982) filtration procedure (Rojas et al., 1992; Molina et al., 1994) was used to isolate *Frankia* in the N-free defined liquid basal medium with propionate (BAP) (Murry et al., 1984) incubated at 30 °C. *Frankia* was then transferred to BAP medium containing 5.0 mM of NH_4Cl and incubated for inoculum preparation. Isolation of *Frankia* from snowbrush nodules was not successful; therefore, we used freshly crushed nodules from snowbrush growing on the study site as *Frankia* inoculum for snowbrush.

2.4. Isolation of Pseudomonas

Three isolation sources (rhizosphere soils, nodule surfaces, and root surfaces of snowbrush plants) were chosen for the isolation of fluorescent *Pseudomonas* colonies. Several snowbrush plants were collected from the site with their nodules and root systems still attached. In order to obtain individual plants with their nodulated root systems as intact as possible, soil particles were shaken from each plant.

Fluorescent *Pseudomonas* in rhizosphere soil and on nodules and fine roots was isolated on modified King's medium following the procedure of Geels and Schippers (1983). Colonies that developed on the medium were examined under ultraviolet light at 366 nm, and colonies that strongly fluoresced under ultraviolet light were isolated. The bacterial cells grown in King's liquid medium were harvested and washed three times with 0.01 M phosphate buffer, pH 6.7, for inoculation.

2.5. Plant culture

Two greenhouse bioassays were conducted, one with red alder and the other with snowbrush. Red alder seeds (Brown Seed Company, Vancouver, WA) were from seed zone 042, elevation 2501–3000 ft, lot number B201-1987. Red alder seeds were surface sterilized with 30% hydrogen peroxide for 15 min and

then rinsed with sterilized distilled water. Snowbrush seeds were collected from the site and its vicinity. Snowbrush seeds contained in a mesh tea infuser were immersed in boiling water for 5 min, then soaked overnight in tap water The following morning, seeds were surface sterilized with 30% hydrogen peroxide for 15 min and then rinsed with sterilized distilled water. Seeds were partially immersed in potato dextrose agar in glass vials as described by Rose and Youngberg (1981) and left in a cold room (4 °C) for 3 months for stratification. Seeds began germinating after 3 months.

Five surface sterilized red alder seeds were planted in 150 ml Ray Leach tubes containing a mixture of soil–vermiculite–perlite (2:1:1); five germinating snowbrush seeds were planted in 590 ml D-cell tubes containing the same soil mixture. Both types of seeds were covered with a thin layer of sterilized silica. At 4 weeks, tubes were thinned to one plant each.

For the two greenhouse bioassays, red alder plants were grown for 6 months and snowbrush for a year. In the greenhouse, room temperature was kept at 21 °C during the day and at 16 °C at night; sodium-vapor lamps (11,000 μk) kept a 1 hour photoperiod. When necessary, plants were watered twice daily; otherwise, they were watered only once a day. To avoid cross-contamination from splash during irrigation, different treatments were separated by at least 20–30 cm. In order to minimize location effect, plants were rotated to different bench locations once or twice a week.

2.6. Treatments

Frankia, macronutrients, micronutrients, mycorrhizal fungi, and fluorescent *Pseudomonas*—all known to influence nodulation and nitrogen fixation—were added to soils. A complete factorial design was not possible because the size of the experiment would have been prohibitive; instead, we added treatment components sequentially. All seedlings except controls received *Frankia* and macronutrients. Depending on treatment, seedlings additionally received micronutrients, mycorrhizal fungi, micronutrients and mycorrhizal fungi, mycorrhizal fungi and fluorescent *Pseudomonas*, or micronutrients plus mycorrhizal fungi plus *Pseudomonas* (Table 1). Ten seedlings per treatment were used in the alder experiment and 12 in the snowbrush experiment.

Table 1
Treatments for red alder and snowbrush experiments

Treatment number	Treatment components
1	Control 1: pasteurized soil, no fertilizer
2	Control 2: non-pasteurized soil, no fertilizer
3	*Frankia* + macronutrients
4	*Frankia* + macronutrients + micronutrients
5	*Frankia* + macronutrients + mycorrhizae
6	*Frankia* + macronutrients + micronutrients + mycorrhizae
7	*Frankia* + macronutrients + mycorrhizae + *Pseudomonas*
8	*Frankia* + macronutrients + micronutrients + mycorrhizae +*Pseudomonas*

Control soils were pasteurized and non-pasteurized. The pasteurized soil was treated in the laboratory over a 3 day period. Each day, soils were brought to a temperature of 60–70 °C and the soil mixture was left in a steam bath for 0.5 h. After the soil had cooled overnight, the procedure was repeated until three successive pasteurizations were complete.

At 5 weeks, Pasteur pipettes were used to inoculate soil close to the roots of all red alder seedlings (except controls) with injections of *Frankia* suspension in sterile distilled water. Snowbrush seedlings (except controls) were inoculated with a crushed nodule suspension in sterile distilled water. There were 320 seedlings in the red alder experiment and 384 in the snowbrush experiment.

Beginning at 6 weeks after planting, 10 ml of a 1:4 dilution of full strength N-free mineral solution (Pregent and Camire, 1985) was used to fertilize each plant weekly until harvest. All treatments except controls (Treatments 1 and 2) received a macronutrient solution containing $NaH_2PO_4 \cdot H_2O$, K_2SO_4, $CaCl \cdot 2H_2O$, and $MgSO_4 \cdot 7H_2O$. Micronutrients were added to the appropriate treatments as NaFeEDTA, H_3BO_3, $MnCl_2 \cdot 4H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, and $ZnCl_2$.

For the mycorrhizal treatment, red alder seedlings were inoculated with 1 ml of an inoculum suspension containing 290.000 spores of the EM fungus *A. diplophleus*, which is host specific to alder (Molina, 1979, 1981; Molina et al., 1994), and snowbrush plants were inoculated with 1 g of soil containing

the VAM fungus *G. intraradix*. Seedlings receiving *Pseudomonas* were inoculated with 1 ml of bacterial suspension (1×10^6 CFU).

2.7. Measurement of variables

Two variables were measured in the alder experiment: total plant biomass and nitrogen fixation per plant. These variables plus nodule weight and nitrogen fixation/g of nodule were measured in the snowbrush experiment.

Alder plants were harvested after 6 months and snowbrush plants after a year. At harvest, tops (leaves and stems) and roots of both species were separated and stored in individual paper bags. Nodules were picked from snowbrush roots and transferred to test tubes. Tops, roots, and nodules were oven dried for 3 days at 80 °C prior to weighing.

We measured nitrogenase activity in intact root systems (with nodules still attached) using the acetylene reduction technique (Koo, 1989; Rojas et al., 1992). Each red alder plant was placed in a 525 ml plastic tube (PVC) in such a way that the root systems were sealed from the plant tops with a rubber stopper perforated to let the plant stem go through. A syringe was used to inject sealed plastic tubes containing the root systems with prepurified acetylene to 10% of the total gas volume of the tube. After 2 h of incubation at room temperature, a 0.1 ml gas sample was withdrawn from each tube and analyzed for acetylene and ethylene in a Hewlett-Packard Model HP5830A gas chromatograph (GC) fitted with a 2.0 m × 2.1 mm 80–100 mesh Porapak R filled column. Oven temperature was adjusted to 70 °C. Injection temperature and flame-ionization detector temperature were each adjusted to 100 °C. Flow rate of the nitrogen carrier gas was adjusted to 40 ml/min (Li and Castellano, 1987; Koo, 1989; Rojas et al., 1992).

Because snowbrush plants were smaller than red alder, each snowbrush plant, with soil adhering to roots and shoots, was inserted into a 900 ml Mason jar. Each jar with its whole plant was sealed with a lid into which a serum stopper had been inserted. Prepurified acetylene was injected into 10% of the total gas volume of the jar Following 2 h of incubation at room temperature, a 0.1 ml gas sample was withdrawn and analyzed for acetylene and ethylene in a GC as described above.

2.8. Data analysis

Statistical analyses were performed with SAS for Windows, version 6.10 (SAS Institute, Inc., 1996). The distribution of the residuals and the test for normality showed that transformations were not necessary for any of the variables.

Data were analyzed by using analysis of variance (ANOVA) and simple correlations among variables. For ANOVA, we used a modified split–plot analysis (Milliken and Johnson, 1989), with location (slope position) as the whole plot factor and treatments as subplots. In such cases, where whole plots are not replicated, Milliken and Johnson (1989) recommend using the Characteristic Root Test to test for interactions between whole plot and subplots (in our case, interaction between slope position and treatments). In our study, the multiplicative interaction model was

$$Y_{ijk} = \mu + \alpha_i + \beta_i + \lambda_{ji}\delta_j + \varepsilon_{ijk}$$

The following null (H_0) and alternative (H_A) hypotheses were tested by the Characteristic Root Test:

$$H_0 : \lambda = 0, \quad \text{and} \quad H_A : \lambda \neq 0$$

This procedure revealed no significant interaction between slope position and treatments for any variable. We then tested for a treatment effect using a standard F-test from the split–plot analysis.

Expected values of the mean squares, E(MS), and the expected value of the F statistic, $E(F)$, are as follows:

Source of variation	d.f.	$E(\text{MS})^{a,b}$	$E(F)^{a,b}$
Slope position	3	$\sigma_s^2 + \sigma_w^2 + \varphi_S$	
Treatment	6	$\sigma_s^2 + \varphi_T$	$(\sigma_s^2 + \varphi_T)/\sigma_s^2$
Error	18	σ_s^2	
Total	27		

[a] Expected values when interaction is not present.

[b] σ_s^2: split–plot error; φ_S: slope effect; σ_w^2: whole plot error; φ_T: treatment effect.

Because we measured only one slope, there is no way to estimate variation between slopes; thus we cannot estimate the whole plot error term to test the

Table 2
Treatment comparisons for red alder[a]

Treatment comparisons	Plant biomass (*P*-value)	Acetylene reduction (*P*-value)
Treatment 2 vs. Treatment 3	0.0414[a]	0.0038[a]
Treatment 3 vs. Treatment 4	0.4717	0.0016[a]
Treatment 3 vs Treatment 5	0 3976	0.0178[a]
Treatment 5 vs. Treatment 6	0.8086	0.0110[a]
Treatment 6 vs. Treatment 7	0.6309	0.4703
Treatment 7 vs. Treatment 8	0.9202	0.5151

[a] Significant differences.

whole plot effect, or the whole plot by split–plot interaction.

Where ANOVA showed significant treatment effects (alder only), Fisher's protected LSD was used to make treatment comparisons (Table 2). Each comparison tests the effect of adding a single factor (or in one case two factors) to a preexisting set of factors (Table 2).

3. Results

At harvest, a striking location effect was easily seen. Whereas alder seedlings were larger when grown in upper slope soil, snowbrush seedlings were larger on soil from the bottom slope. Where there were significant treatment effects for alder, the one-way ANOVA analysis was performed first by treatment and then by location. In the first analysis, each treatment was analyzed separately with regard to seedling response to location, with trees used as replicates; in the second analysis, all trees were pooled by location, regardless of treatment. We were able to pool trees because the pattern of significant location effects was the same for all treatments. There was no significant treatment effect for snowbrush; we therefore tested location effects by treating treatments within a given location as replicates and compared locations using a one-way ANOVA. Because slope positions were not replicated, these ANOVAs do not permit inferences about slope position in general. Rather, inferences are restricted to how seedlings or groups of seedlings (the replicates) differ in growth among the four specific locations within the clearcut.

Seedlings grown in pasteurized soils (Treatment 1, Table 1) did not survive and were not included in the analyses.

3.1. Red alder

3.1.1. Treatment effects

Table 1 shows in detail the eight treatments used in the alder and snowbrush experiments. Table 2 shows *P*-values for the six treatment comparisons. Treatments significantly influenced both seedling biomass ($P = 0.0202$) and acetylene reduction ($P = 0.0001$). According to the Characteristic Root Test, the location at which soils were collected within the clearcut did not influence seedling response to treatments.

Seedlings given *Frankia* and macronutrients (Treatment 3) had significantly greater biomass ($P = 0.0414$) and reduced more acetylene ($P = 0.0038$) than seedlings grown in unsterile soil without additions (Treatment 2) (Table 2; Fig. 1). No treatments significantly increased biomass beyond that attained by adding *Frankia* and macronutrients; however, acetylene reduction was significantly influenced by the *Alpova* mycorrhizae (Treatment 5), micronutrients (Treatment 4), and interactions between these two factors (Table 2; Fig. 1B). Adding *Alpova* (Treatment 5) increased acetylene reduction by 33% over that attained with *Frankia* and macronutrients alone (Treatment 3, $P = 0.0178$; Fig. 1B); when combined, *Frankia*, macronutrients, and the mycorrhizal fungus (Treatment 5) increased acetylene reduction by 136% over the controls (Fig. 1B).

In contrast, adding micronutrients to *Frankia* and macronutrients (Treatment 4) completely negated the positive effect of the *Frankia* and macronutrients (Treatment 3, $P = 0.0016$), reducing acetylene reduction by nearly one-half. The presence of *Alpova* appeared to buffer the negative effects of micronutrients at least somewhat (Treatment 6); although acetylene reduction was reduced when both micronutrients and *Alpova* were added (Treatment 6 versus

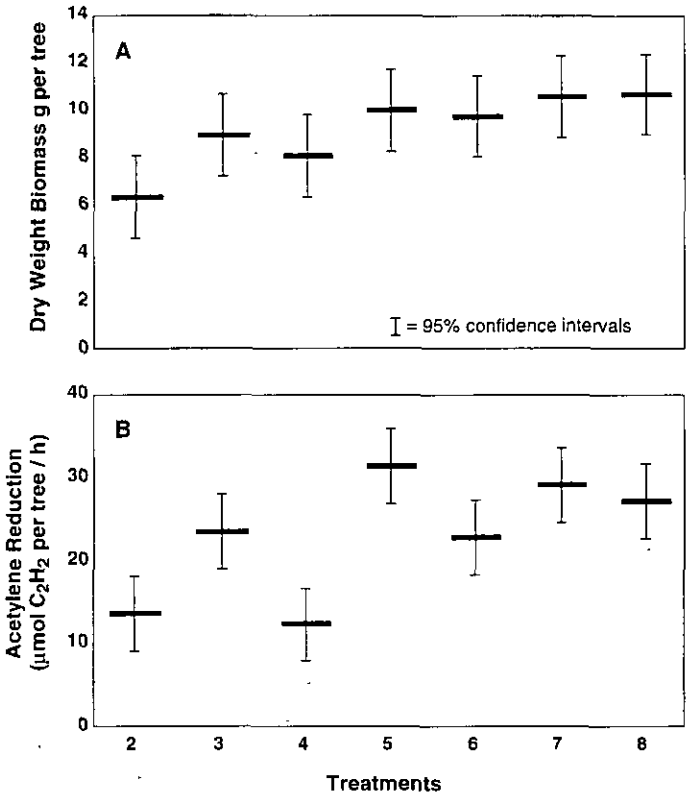

Fig. 1. Means and 95% confidence limits for (A) dry weight biomass (g per tree) and (B) acetylene reduction (μmol C_2H_2 per tree/h) of red alder grown on soils with different treatments

Treatment 5, $P = 0.011$), the level remained well above that attained when micronutrients were added without *Alpova* (Treatment 4). *Pseudomonas* inoculation (Treatment 7) did not promote the effects of macronutrients and mycorrhizae in either plant biomass production or acetylene reduction (Table 2). Seedling biomass was positively correlated with acetylene reduction ($r = 0.59$, $P = 0.0001$).

3.1.2. Slope location effects

When the one-way ANOVA was performed for slope location effect (all trees from one location pooled together, regardless of treatments), both seedling dry weight and acetylene reduction showed a significant location effect (both $P = 0.0001$). Seedling responses for both variables were higher in soil from the two upslope positions than in soil from lower slopes (Fig. 2)

3.2. Snowbrush

3.2.1. Treatment effects

None of the four variables studied for snowbrush showed a significant treatment effect (Fig. 3). There was no significant interaction between treatment and location.

Correlations for the three snowbrush response variables were each statistically significant. Nodule weight correlated positively with plant biomass ($r = 0.87$, $P = 0.0001$). Plant acetylene reduction correlated positively with plant biomass ($r = 0.75$, $P = 0.0001$) and with nodule weight ($r = 0.45$, $P = 0.0001$).

3.2.2. Slope location effects

Slope location strongly affected all four variables: plant biomass ($P = 0.0001$), nodule weight ($P = 0.0001$), acetylene reduction/g of nodule

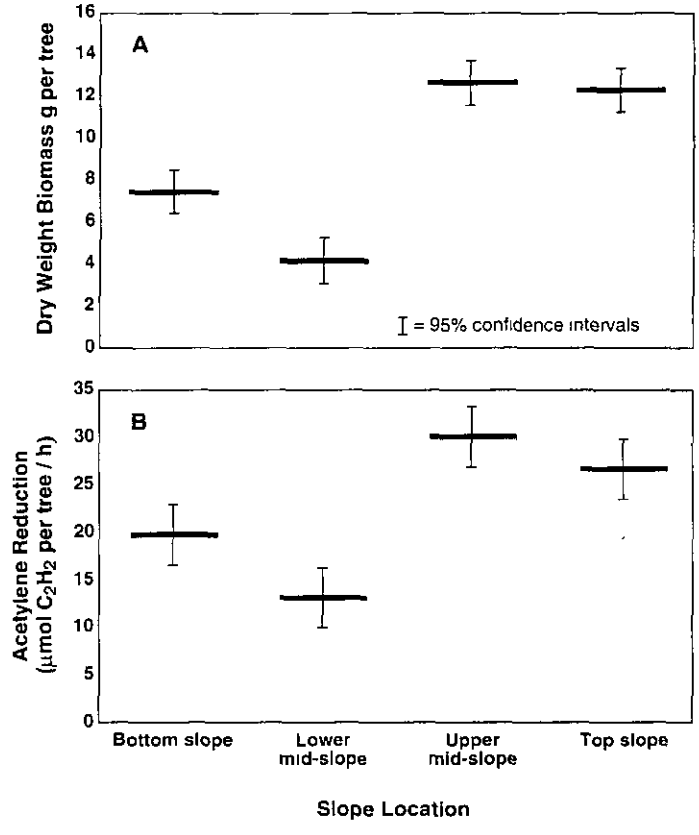

Fig. 2. Means and 95% confidence limits for (A) dry weight biomass (g per tree) and (B) acetylene reduction (μmol C_2H_2 per tree/h) of red alder grown on soils from different slope locations

($P = 0.0001$), and acetylene reduction ($P = 0.0006$). Whereas alder seedling responses were higher on upper slope soils, snowbrush biomass, acetylene reduction, and nodule weight were highest in soils from lower slopes (Fig. 4A–C). Acetylene reduction/g nodule, on the other hand, was highest on upper slope soils (Fig. 4D).

4. Discussion

4.1. Treatment effects

Our hypothesis that mycorrhizal fungi and fluorescent *Pseudomonas* would increase nodulation and N fixation was partially supported for red alder, but not for snowbrush. Alder nitrogenase activity, as measured by acetylene reduction, was significantly influenced by *Frankia* and macronutrients, micronutrients, the EM fungus *Alpova*, and by interaction between micronutrients and *Alpova*. *Pseudomonas* inoculation, on the other hand, did not affect any of the measured variables. There were no significant treatment effects for snowbrush.

Red alder seedlings receiving *Frankia* and macronutrients produced more biomass and reduced more acetylene than the seedlings grown without additions. None of the other treatments further increased biomass. The small size of the Leach tubes may have prevented biomass from responding to the further treatments. Rojas et al. (2001) reported that greenhouse grown snowbrush plants (1-year-old) produced more biomass and nodules and fixed more nitrogen when grown in 590 ml D-cell tubes than in 150 ml

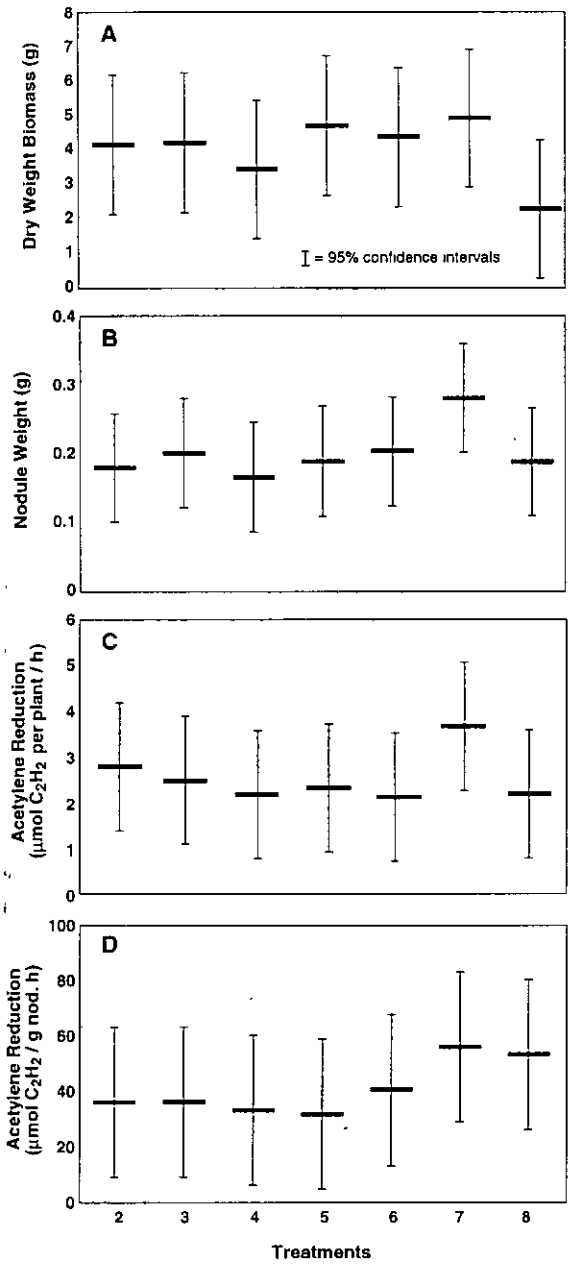

Fig. 3. Means and 95% confidence limits for (A) dry weight biomass (g per plant), (B) nodule weight (g per plant); (C) acetylene reduction (μmol C_2H_2 per plant/h), and (D) acetylene reduction/g nodule (μmol C_2H_2/g nodule h) of snowbrush grown on soils with different treatments.

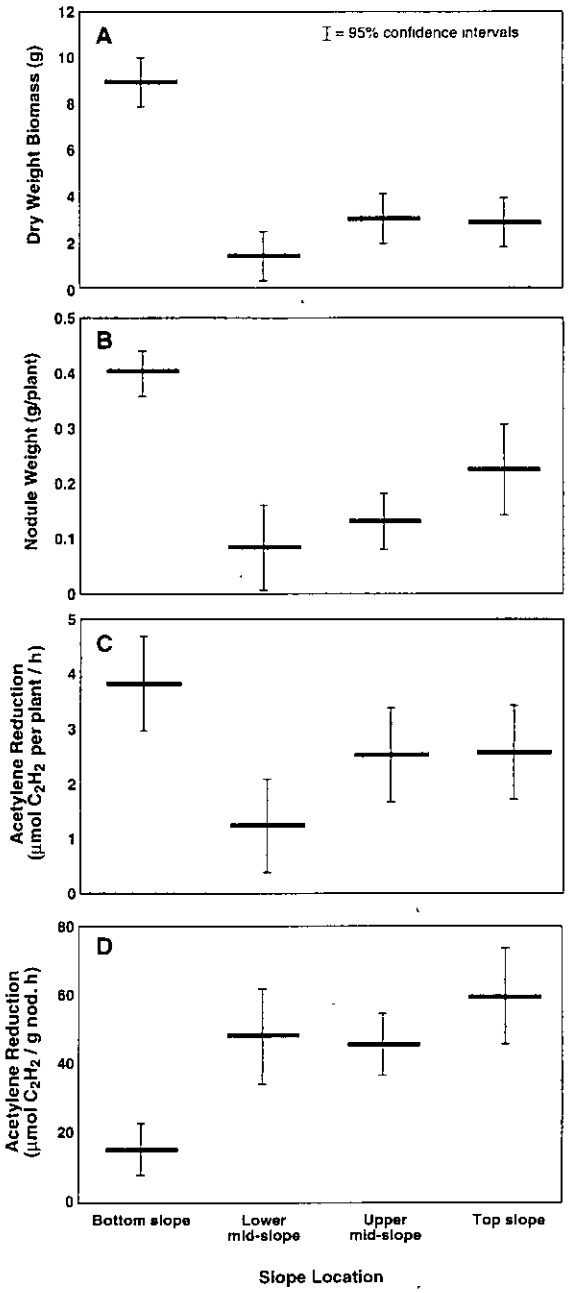

Fig. 4. Means and 95% confidence limits for (A) dry weight biomass (g per plant); (B) nodule weight (g per plant); (C) acetylene reduction (μmol C_2H_2 per plant/h), and (D) acetylene reduction/g nodule (μmol C_2H_2/g nodule h) of snowbrush grown on soils from different slope locations.

Ray Leach tubes, and work by Koo (1989) has shown that red alder plants develop water stress when grown in small containers.

Nitrogen fixation measured by acetylene reduction in red alder correlated positively with seedling biomass. A positive relation between biomass and N fixation has been seen in many studies and is usually explained by two things: seedlings are N limited, and bigger seedlings have more energy to devote to their diazotrophs (Arnone and Gordon, 1990). The link between *Frankia* and seedling biomass is obviously through the N supplied by the *Frankia* (unless the diazotroph has some other effect that we do not know about). Macronutrients could influence either N fixation (and through that, seedling biomass) or seedling biomass (and through that, N fixation).

Red alder seedlings grown in pasteurized soils did not survive and therefore did not fix nitrogen. On the other hand, seedlings grown in non-pasteurized soils with no additions survived, produced nodules, and fixed nitrogen, indicating that *Frankia* was still present in the clearcut at the time we collected our soil samples, even though actinorhizal plants had probably been absent for hundreds of years. The occurrence of *Frankia* in soils without hosts is probably due to the ability of this microorganism to survive either saprophytically (Smolander and Sundman, 1987; Li et al., 1997), or through extended dormancy (Molina et al., 1994). Dispersal by terrestrial vertebrates and/or invertebrates is also possible (Li et al., 1997). Previous work using soils from our site and a nearby Douglas-fir plantation and old growth forest showed that nodulation of both red alder and snowbrush varied widely among sample points within each area (Rojas et al., 2001).

Like seedling biomass, nitrogen fixation rates (acetylene-reducing rates) increased significantly when *Frankia* and macronutrients were added to unpasteurized soil. Because *Frankia* and macronutrients were always added together, we cannot separate their effects. However, it is reasonable to speculate that the two acted synergistically to increase nitrogenase activity, *Frankia* through increased nodulation and macronutrients through effects on plant vigor (which enhances rates of N fixation) (Huss-Danell, 1986; Sharma, 1988; Jha et al., 1993) and perhaps also through effects on nodulation and nitrogenase activity per unit nodule weight (Righetti et al., 1986; Lynd and Ansman, 1989; Crannell et al., 1994; Sprent, 1995).

Originally, we had expected mycorrhizae to benefit VAM snowbrush more than EM alder, but in fact it was only alder's nitrogenase activity that was influenced by a mycorrhizal fungus. Work elsewhere has shown that mycorrhizae increase rates of nitrogen fixation both in legumes and actinorhizal associations (Rose and Youngberg, 1981; Gardner et al., 1984; Hayman, 1987). In our study, when *Alpova* was added with *Frankia* and macronutrients, red alder seedlings reduced 33% more acetylene than the seedlings grown with *Frankia* and macronutrients alone, and 136% more acetylene than seedlings grown in non-pasteurized soils. But giving VAM to snowbrush had no effect, even though VAM inocula were low at the study site. A random sample obtained from the site in 1990 showed that colonization of snowbrush seedling root systems by mycorrhizal fungi averaged 6–26% (Cazares, personal communication). This range corresponds to class 2 of the classification method used by the USDA Institute for Mycorrhizal Research and Development in Athens, Georgia (Kormanik and McGraw, 1982), which is generally considered low for a VAM plant (Cazares, personal communication). In contrast, Rose and Youngberg (1981) found that 1-year-old nodulated snowbrush plants infected with both *Frankia* and the VAM *Glomus gerdemannii* had, on average, 80% of their roots colonized by *Glomus*, whereas plants inoculated with *Glomus* alone had only 45% of roots colonized; they reported increases in shoot, root, and nodule biomass, along with increased nitrogenase activity, on dually infected plants.

Clearly, actinorhizals as well as legumes benefit from the tripartite association (plant, diazotroph, mycorrhizal fungus). The plant provides reduced forms of carbon and a shelter in the form of a root nodule for the diazotroph, *Frankia* fixes N, and the mycorrhizal fungus gathers soil minerals such as P (Abuzinadah and Read, 1986a,b; Janos, 1987; Allen, 1991). Consequently, N and P, the two most important macronutrients for plant growth, are provided to the host plant by these two symbionts (Rose and Youngberg, 1981). Because of its extensive hyphal networks and hyphal surface area, the fungus also helps the plant absorb water (Molina et al., 1994; Perry, 1994).

Nitrogenase activity of red alder seedlings was also influenced by interactions between micronutrients and the mycorrhizal fungus (*Alpova*). Contrary to our expectations, adding micronutrients to the combination

of *Frankia* and macronutrients essentially eliminated the beneficial effect of these additions. With *Alpova* in the system, micronutrients still lowered acetylene reduction, but not to the degree that it was lowered without *Alpova*. Apparently the mycorrhizal fungus was able to buffer the deleterious effect of micronutrients to some degree. The negative effect of micronutrients on nitrogenase activity was unexpected; Co, Cu, Fe, B, Mo, and Ni are known to be important in nodule formation and nitrogenase activity of legumes (O'Hara et al., 1988a,b), although we are not aware of similar work in actinorhizal plants.

Accordingly, we hypothesized that micronutrient fertilization would substitute at least in part for mycorrhizal fungi and *Pseudomonas*. It is not clear why the opposite occurred. Micronutrients were added at rates recommended for *Alnus* species, but recommended rates of fertilization are often considerably higher than those occurring naturally and can result in unnatural plant responses (Ingestad, 1982). It is possible that the relatively large pulse added as fertilizer induced a chemical or biological imbalance in seedling rhizospheres, perhaps favoring the growth of competitive or pathogenic microorganisms. The apparent buffering effect of *Alpova* could be due to the ability of the fungus to protect the plant by increasing its resistance to toxins and soil pathogens (Marx, 1972; Marx and Krupa, 1978).

Contrary to our expectations, *Pseudomonas* did not affect acetylene reduction or plant biomass. We expected *Pseudomonas* to increase rates of N fixation by producing siderophores, which are involved in plant iron nutrition (Torres et al., 1986), and Fe is an essential component of the nitrogenase enzyme (Atlas and Bartha, 1987; Sprent, 1987; Sprent and Sprent, 1990). Soils of the study site may already have abundant *Pseudomonas*. Also, mycorrhizal fungi produce siderophores (Szaniszlo et al., 1981; Powell et al., 1982; Perry et al., 1984; Watteau and Berthelin, 1990) and may have provided seedlings with sufficient Fe.

4.2. Slope location effects

Snowbrush responses in the greenhouse were very similar to those in the field. Although snowbrush normally regenerates abundantly following clearcutting and slash burning (Swanson et al., personal communication), cover on the study site remained low. At the time of soil collection, snowbrush was unevenly distributed, with a few large plants growing on flats at slope bottoms and scattered small plants elsewhere. Spot surveys 8 years after broadcast burning showed that the larger plants at slope bottoms were nodulated, but the small plants growing on mid- and upper slopes were not.

We cannot definitely determine what is causing snowbrush to nodulate and grow better at slope bottoms than on upper slopes, but we do know, since the same pattern occurred in the greenhouse, that the cause must be related to soil. The poor performance of snowbrush on upper slopes may be related to effects of clearcutting and slash burning; the site was subjected to an intense broadcast burn immediately after the end of logging in 1981 (Franklin et al., personal communication).

Rojas et al. (2001) did a soil bioassay of an age sequence of stands using red alder and snowbrush at the H.J. Andrews Experimental Forest and found that for red alder, plant biomass and nodule weight were highest in clearcut soils, but for snowbrush, they were highest in soils from the 20-year-old Douglas-fir stand, where thick snowbrush cover apparently served as a good source of *Frankia* inoculum. Red alder and snowbrush responded differently to different conditions; factors that produced the best growth and nodulation in one species did not produce the best results in the other. This was true both within a site and between sites.

Early successional nitrogen-fixing plants such as red alder and snowbrush play an important ecological role in the long term productivity of a site. These early successional plants are known to possess some "biological imprints" (microflora shared between tree species) (Perry et al., 1989) that will be shared with their associated conifer seedlings for their mutual benefit. At our site, the benefit of the nitrogen-fixing association was probably first reestablished at the bottom of the slope, where growing conditions for plants were more favorable; moisture was less limiting there than on mid- and upper slopes. Because of the ecological importance of snowbrush on such sites, delays in its establishment at mid- and upper slopes may have profound effects on the long term N and C budgets of these ecosystems.

Even though red alder was not physically present at the site, a strain of *Frankia* that preferentially

nodulates red alder over snowbrush apparently was, especially on mid- and upper slopes, where the strain that nodulates snowbrush was less abundant. This suggests that different *Frankia* strains may have different adaptations, which in turn could reflect a survival mechanism of the nitrogen-fixing endophyte (Molina et al., 1994).

We observed poor correlation between red alder and snowbrush in nodulation—that is, sometimes alder nodulated relatively well and snowbrush did not, or vice versa. One possibility is that there are different *Frankia* strains with different plant preferences distributed heterogeneously across the landscape, perhaps because of differing adaptations but possibly due to purely random factors. For whatever reason, the heterogeneous distribution of endophytes could produce a corresponding heterogeneous distribution of host plants (although no alder occurred in the clearcut). Another possibility is that the endophytes are the same, but the plant species differ in some triggering factor necessary for nodulation, and the triggering factor (if it exists) was distributed heterogeneously.

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