

An Abstract of the Thesis of

Nestor S. Rojas-Melo for the degree of Doctor of Philosophy in Forest Science
presented on July 29, 1997.

Title: Study of Some Biological and Nutritional Factors that Influence Nodulation
by *Frankia* on Red Alder (*Alnus rubra*) and Snowbrush (*Ceanothus velutinus*)
Within Three Age-Class Douglas-Fir Forests in H. J. Andrews Experimental
Forest, Oregon.

Abstract approved by: _____ Signature redacted for privacy.


David A. Perry

Bioassays using red alder and snowbrush plants grown in soils collected from a clearcut, a young Douglas-fir plantation, and an old-growth stand were conducted. Sites are located at the Andrews Experimental Forest, Oregon. In the first bioassays, more alder than snowbrush plants survived and nodulated. Of the plants that survived, more red alder plants nodulated when grown in clearcut soils than in other soils, and more snowbrush nodulated when grown in soils from the young stand. With the exception of acetylene reduction per plant, response variables differed among the three sites, however soil samples within sites were also a significant source of variation. Red alder biomass and nodule weight were highest when plants were grown in clearcut soils. Snowbrush biomass and nodule weight were highest when grown in soils from the young stand. The biomass of snowbrush plants grown in clearcut soils averaged higher in bottom slope soils than in soils from any other position.

Two additional bioassays using red alder and snowbrush plants consisted of adding sequentially to clearcut soils *Frankia* plus macronutrients, micronutrients, mycorrhizal fungi, and *Pseudomonas fluorescens*. There was no interaction between treatment and location for either species. There were no significant treatment effects for snowbrush, but there were significant treatment effects for red alder. Red alder seedlings given *Frankia* and macronutrients had greater biomass and reduced more acetylene than seedlings grown without additions. Adding *Alpova diplophloeus* increased acetylene reduction by 33% over that attained with *Frankia* and macronutrients alone. The combined effect of *Frankia*, macronutrients and the mycorrhizal fungus was to increase acetylene reduction by 136% over controls. Adding micronutrients to *Frankia* and macronutrients reduced acetylene reduction by nearly one-half, completely negating the positive effect of the *Frankia* and macronutrients. The presence of the mycorrhizal fungus appeared to buffer the negative effects of micronutrients. Red alder seedlings grown in upper slope soil had greater biomass and reduced more acetylene than seedlings grown in down slope soil. In contrast, snowbrush plants grown in bottom slope soil had greater biomass, nodule weight, and reduced more acetylene than seedlings grown in any of the other slope positions. Because slope positions were not replicated, any conclusions we draw apply only to the four locations we measured on the single slope.

**Study of Some Biological and Nutritional Factors That Influence Nodulation
by *Frankia* on Red Alder (*Alnus Rubra*) and Snowbrush (*Ceanothus
Velutinus*) Within Three Age-Class Douglas-Fir Forests in H. J. Andrews
Experimental Forest, Oregon.**

by

Nestor S. Rojas-Melo

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CONTRIBUTION OF AUTHORS

Dr. C. Y. Li was involved in the design of the experiments and data collection, provided technical assistance, and laboratory and greenhouse space.

Dr. Lisa Ganio assisted with data analysis and interpretation.

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Study of Some Biological and Nutritional Factors That Influence Nodulation by *Frankia* on Red Alder (*Alnus Rubra*) and Snowbrush (*Ceanothus Velutinus*) Within Three Age-Class Douglas-Fir Forests in H. J. Andrews Experimental Forest, Oregon.

Chapter 1. General Introduction

In the Pacific Northwest of North America, woody actinorhizal plants such as *Ceanothus sp.* and *Alnus sp.* are important contributors to the nitrogen inputs to forest ecosystems (Perry, 1994). *Ceanothus sp.* are commonly found on dry and middle elevation forest sites while *Alnus sp.* are found in coastal and mesic low and high-elevation forests (Conard *et al.*, 1985; Johnson, 1968; Harrington, 1994). Both, *Ceanothus sp.* and *Alnus sp.* are early colonizers after disturbances such as clearcuts and fires. In Oregon, we find eight species of *Ceanothus*, all colonizers in stressed sites (Rose and Youngberg, 1981). Snowbrush (*Ceanothus velutinus*) is especially important in forest ecosystems due to its abundant regeneration after clearcuttings. Of the *Alnus* species, red alder (*Alnus rubra*) has also an important ecological value in forestry. Red alder, which grows and dominates sites quickly, covers 13% of the coastal commercial forest land of Oregon and Washington (Resch, 1988).

Actinorhizals as well as legumes develop a tripartite association among the plant, the diazotroph and the mycorrhizal fungus. Snowbrush and red alder contribute to the nitrogen balance of forest ecosystems through a root symbiosis with an actinomycete like bacterium of the genus *Frankia*. As a result of this

symbiotic relationship, plants produce nodulated roots which allow them to fix atmospheric nitrogen (Paul and Clark, 1989). Due to their ability to assimilate nitrogen, actinorhizals can regenerate easily in nitrogen deficient soils. They can even improve the soil fertility. Therefore, their presence in forest lands, where nitrogen is often a limiting factor, is important (Perry, 1994). Besides *Frankia*, actinorhizal plants can also be colonized by ectomycorrhizal (EM) fungi, vesicular-arbuscular mycorrhizal (VAM) fungi, or both. Snowbrush is colonized by VAM fungi, while red alder can be colonized by either EM or VAM fungi (Rose, 1980). A mycorrhiza is a symbiotic association between a root and a fungus. Among the benefits obtained from this association are: Increased water and nutrient uptake and increased resistance to toxins and pathogens (Marx and Krupa, 1978; Harley and Smith, 1983; Nelsen, 1987). Mosse (1973) suggests that mycorrhizae aid in the uptake of P, S, and minor elements, while Barea and Azcon-Aguilar (1983) have suggested that vesicular-arbuscular mycorrhizas promote plant growth, nodulation and nitrogen fixation in both legumes and actinorhizal plants.

Pseudomonas sp., another common type of forest soils microorganism, have also been observed to promote nodulation in *Alnus rubra* (Knowlton and Dawson, 1983; Knowlton *et al.*, 1980). The increase in nodulation might be related to the *Pseudomonas* ability in producing siderophores, which are Fe⁺⁺⁺ chelators (Torres *et al.*, 1986). Perry *et al.* (1984), working with 10 different sites of undisturbed, logged and broadcast-burned, and logged-unburned areas in

Oregon, found that the greatest reduction in concentration of siderophores was produced when slash had been burned.

The nitrogenase enzyme, which catalyses the reduction of N_2 to NH_3 , consists of two proteins: the Fe-protein, and the Mo-Fe protein. The Fe protein contains atoms of Fe and S; while the Mo-Fe protein, contains atoms of Fe, S and Mo (Paul and Clark, 1989; Glass, 1989). The location of the reduction of N_2 on the enzyme is where Mo is found (Sprent, 1987). There are also other nutrients, besides Mo and Fe, considered important for the N_2 -fixation or the symbiont or the host plant: Co is considered as an essential micronutrient for the microorganisms involved in N_2 -fixation, and, in legumes, Co is important for nodule initiation (O'Hara *et al.*, 1988a,b). Also in legumes, Cu and Fe are involved in nodule development (O'Hara *et al.*, 1988). Ca and V are important too. V can replace Mo in the nitrogenase, but it is less effective (Alexander, 1977). In addition to micronutrients, soil temperature, soil moisture, calcium, phosphorus, magnesium and sulfur have been shown to influence nodulation and N-fixation in both *Alnus* and *Ceanothus* species (Wollum and Youngberg, 1969; McNabb and Cromack, 1983; Kummerow *et al.*, 1978; Sharma, 1988; Huss-Dannell, 1986; Jha *et al.*, 1993; Waring and Schlesinger, 1985; Perry, 1994).

Background (The "Snowbrush Study"). Supported by the Long-Term Ecological Research (LTER), a group of forest ecologists began the "Snowbrush study" in 1981 at the H. J. Andrews Experimental Forest in Blue

River, Oregon. Formerly occupied by an old-growth Douglas-fir, western hemlock, and western red cedar forest, the site was clearcut in 1981, and logging slash broadcast burned in 1982. The site was burned after the clearcut in order to stimulate germination of snowbrush seeds, which exist as a seedbank in the forest floor. Nine hundred Douglas-fir seedlings per ha were planted six months after slash burning.

Originally, the study design consisted of 4 replications of 4 treatments. The primary variable was *Ceanothus* density. Three levels were included: Zero, 750/ha, and 1500/ha. The fourth treatment was zero *Ceanothus*, but with annual N fertilization at rates equivalent to rates at which *Ceanothus* is expected to add N.

Ten years after the "Snowbrush study" began, most of the plants in the field were not nodulated. In past surveys, nodulated plants were found only on the bottom of the slope where snowbrush germinated right after the broadcast burning (P. Sollins, personal communication). Over the years, forest ecologists have speculated about the reasons for the lack of nodulation in the snowbrush plants, but no conclusive evidence has been yet demonstrated. Earlier studies have hypothesized that the poor performance of snowbrush at the site was primarily due to a heavy browsing by an increase population of elk at the Andrews Forests (Swanson *et al.*, 1987).

In the fall of 1990 we sampled from within three sites on the H. J. Andrews Experimental Forest (western Cascade Range, Oregon): (a) an 8-year-

old clearcut planted with Douglas-fir, also referred to as the "snowbrush" site; (b) an adjacent 20-year-old Douglas-fir plantation with an understory of snowbrush, and (c) an old-growth stand dominated by Douglas-fir. All sites were within 1 km of one another. The objective of this first study was: To determine colonization of *Frankia* on snowbrush and red alder grown in soils of three age-class Douglas-fir forests in H. J. Andrews Experimental Forest, Oregon.

In the fall of 1992, we sampled again but this time only from within the clearcut. The objective of the second study was: To determine whether the ability of snowbrush and red alder to nodulate and fix N in soils from an Oregon clearcut was influenced by various combinations of *Frankia* plus macronutrients, mycorrhizal fungi, *Pseudomonas fluorescens*, and micronutrients.

Four greenhouse bioassays were conducted: two with red alder and two with snowbrush.

Chapter 2

**Study of the Population Density of *Frankia* of Snowbrush
and Red Alder in Soils of Three Age-Class Douglas-Fir Forests
in H. J. Andrews Experimental Forest, Oregon:
Differences Among Slope-Related Patterns Within Age Classes.**

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ABSTRACT

Plant biomass, acetylene reduction per plant and per gram of nodule, and nodule weight were measured in greenhouse bioassays using red alder and snowbrush plants. Soils were collected from an 8-year-old clearcut planted with Douglas-fir, an adjacent old-growth forest, and an adjacent 20-year-old Douglas-fir plantation with an understory of snowbrush. All three sites are located at the H. J. Andrews Experimental Forest, Oregon. Ten soil samples were collected from within each of the three stands. Plants were grown in a mix of soil-vermiculite-perlite (2:1:1). Snowbrush plants were grown for a year while the alder plants were grown for six months. An N-free mineral solution (Pregent and Camire, 1985) was used to fertilize plants. More alder than snowbrush plants survived and nodulated. The alder experiment consisted of 360 seedlings of which 23 seedlings did not survive. Of the 299 survivors, 89% produced nodules. The snowbrush experiment consisted of 300 plants of which 63 plants did not survive and only 59 survivors (25%) produced nodules. Of the plants that survived, more red alder plants nodulated (100%) when grown in clearcut soils than in other soils, and more snowbrush nodulated (51%) when grown in soils from the adjacent 20-year-old Douglas-fir plantation. With the exception of acetylene reduction per plant, response variables differed among the three sites, however soil samples within sites were also a significant source of variation for all variables. Red alder biomass and nodule weight were highest when plants

were grown in clearcut soils. Snowbrush biomass and nodule weight were highest when grown in soils from the adjacent 20-year-old Douglas-fir plantation. The biomass of snowbrush plants grown in clearcut soils averaged higher in bottom slope soils than in soils from any other position within the clearcut. Correlations between plant biomass, acetylene reduction, and nodule weight were positive and statistically significant for both species.

INTRODUCTION

In the Pacific Northwest of North America, actinorrhizal plants such as *Ceanothus sp.* and *Alnus sp.* are the primary sources of nitrogen for forest ecosystems (Waring and Schlesinger, 1985; Perry, 1994). *Ceanothus sp.* are commonly found on relatively dry sites at midelevations, while *Alnus sp.* are found on more mesic sites across a range of elevations (Franklin and Dyrness, 1973; Conard *et al.*, 1985; Johnson, 1968; Harrington, 1994). Both *Ceanothus sp.* and *Alnus sp.* are early colonizers after disturbances such as clearcuts and fires. Of eight species of *Ceanothus* in Oregon, snowbrush (*Ceanothus velutinus*) is especially important in forest ecosystems due to its widespread occurrence and abundant regeneration after fire. Of the *Alnus* species, red alder (*Alnus rubra*) has an important ecological value in forestry as well, both because of its nitrogen-fixing capability and its commercial value. Red alder, which grows fast and dominates sites quickly, covers 13% of the coastal commercial forest land of Oregon and Washington (Resch, 1988).

Snowbrush and red alder contribute to the nitrogen balance of forest ecosystems through a root symbiosis with a nitrogen-fixing actinomycete in the genus *Frankia*. Four host specificity groups for *Frankia* have been recognized by Baker (1987). *Frankia* strains that nodulate *Alnus sp.* have been grouped with the strains that nodulates *Myrica sp.*. Baker (1987) was not able to group

Ceanothus sp. because isolation of *Frankia* from *Ceanothus* nodules has not been successful.

The presence of actinorhizal plants in Pacific Northwest forests is important because these forests are N limited (Perry, 1994). Annual nitrogen fixation rates for red alder have been estimated at about 100 kg/ha/year (Bormann and DeBell, 1981), while estimates for snowbrush average about 80 kg/ha/year (Cromack *et al.*, 1979).

Actinorhizal plants in the Pacific Northwest are pioneers occupying sites that have not supported actinorhizal plants for hundred of years, which raises the question of the degree to which *Frankia* survives in the absence of hosts. Past studies in the Pacific Northwest generally find abundant nodulation on alders but variable nodulation on snowbrush (Wollum *et al.*, 1968; Youngberg and Wollum, 1976; Youngberg *et al.*, 1979; Zavitkovski and Newton, 1967; Zavitkovski and Newton, 1968; and Binkley, 1981). Wollum *et al.* (1968) found that snowbrush nodulated better when growing on sites formerly occupied by mid-aged conifer stands than when growing on sites formerly occupied by old-growth, and hypothesized that *Frankia* declined over time in the absence of host plants.

Li *et al.* (1997) grew red alder seedlings in the greenhouse using a potting substrate containing coarse woody debris (CWD) collected from two sites located in the Coast Range of Oregon. One site was an old-growth mixed conifer stand with no actinorhizal plants, the other a clearcut with naturally

regenerated red alder. While alder seedlings grown on CWD from the clearcut grew better and fixed more nitrogen than seedlings grown on CWD from the old-growth, CWD from both induced root nodulation. Li *et al.* (1997) concluded *Frankia* lived saprophytically in CWD, and perhaps was dispersed by vertebrates and/or invertebrates. Smolander and Sundman (1987) grew alder seedlings in soils collected from under birch stands in Finland and recognized the presence of a *Frankia* strain that was unable to survive in the absence of its host and a *Frankia* strain that was able to survive saprophytically without its host.

The objective of this study was: To determine colonization of *Frankia* on snowbrush and red alder grown in soils of three age-class Douglas-fir forests in H. J. Andrews Experimental Forest, Oregon. We hypothesized that:

a) nodule biomass and acetylene reduction, for both snowbrush and red alder plants would be greatest in soils from an adjacent 20-year-old Douglas-fir plantation with an understory of snowbrush. We expected that the thick snowbrush cover present in the understory of the adjacent 20-year-old Douglas-fir plantation would serve as a good source of *Frankia* inoculum for snowbrush. We also expected that some of the *Frankia* strains nodulating snowbrush would also nodulate red alder.

b) nodule biomass and acetylene reduction for both snowbrush and red alder would be intermediate in soils from an 8-year-old clearcut planted with Douglas-fir. We expected that the presence of scattered

snowbrush plants in the clearcut would help maintain *Frankia* populations in the soil. It was also our expectation that *Frankia* spores would be transported into the clearcut by dispersing agents such as terrestrial vertebrates and/or invertebrates.

c) nodule biomass and acetylene reduction, for both snowbrush and red alder would be least in soils from an old-growth stand dominated by Douglas-fir up to several hundred years of age because actinorhizal plants have likely been absent from this site for hundreds of years.

Two greenhouse bioassays, one with red alder the other with snowbrush, were conducted to test these hypotheses.

MATERIALS AND METHODS

Site Description

We sampled from within three sites on the H. J. Andrews Experimental Forest (western Cascade Range, Oregon): (1) an 8-year-old clearcut planted with Douglas-fir; (2) an adjacent 20-year-old Douglas-fir plantation with an understory of snowbrush, and (3) an old-growth stand dominated by Douglas-fir. All sites were within 1 km of one another, in the western hemlock zone of Franklin and Dyrness (1973). The H. J. Andrews Experimental Forest is located 80 km east of Eugene, Oregon, on the Blue River Ranger District of the Willamette National Forest (lat. 44° 15' N., long. 122° 10' W.). Climate is mild, with dry summers which last for about 3 months and wet winters. Precipitation in this area averages 2400 mm a year, most falling in the winter and early spring as either rain or snow (Waring *et al.*, 1978). The mean annual temperature is 8.5 degrees C, with a July mean of 18°C and a January mean of 1° C. Shallow soils derived from ash flow, mudflow, and stream deposits are found at lower elevations, while deeper soils derived from volcanic ash and andesite lava flows are found at middle and higher elevations (Swanson and James, 1975). These soils are classified as Inceptisols, with some Alfisols and Spodosols also present (Brown and Parson, 1973).

The clearcut, also referred to as the "snowbrush" site, is at 890 m above sea level. The site is southeast facing, with an average 22 degree slope.

Formerly occupied by an old-growth Douglas-fir, western hemlock, and western red cedar forest, the site was clearcut in 1981, and logging slash broadcast burned in 1982. Nine hundred Douglas-fir seedlings per ha were planted six months after slash burning. Although normally regenerating abundantly following clearcutting and slash burning on sites such as this (Swanson *et al.*, 1987), snowbrush cover on the study site remained quite 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. Planted Douglas-fir has grown quite well on the site.

The adjacent 20-year-old Douglas-fir plantation with an understory of snowbrush, also referred to as the "young stand", is at 976 m above sea level, approximately 1000 m from the other two stands. The site is southwest facing, with an average 13 degree slope. There was abundant snowbrush cover in the understory of this site at the time of the sampling. The adjacent old-growth forest is at 1000 m above sea level and also southwest facing, with an average 5 degree slope. The old-growth stand, directly adjacent to the upper boundary of the clearcut, is dominated by Douglas-fir trees up to several hundred years of age; also present are mature trees of western hemlock and western red cedar. The understory consists of scattered shrubs composed mostly of vine maple, huckleberry, rhododendron, oxalis sp., pacific yew, and Oregon grape.

Soil Collection

Ten soil samples (replicates) were collected from two transects in each site in early November of 1990. In the clearcut, the first transect originated at a randomly chosen point on a flat at the base of a 22 degree slope and extending 60 m up-slope at right angles to the contour. The second transect was installed parallel to and 30 m distant from the first. Five sampling points were selected along each transect, one each at bottom slope (bs-1), lower mid-slope (ms-2), mid-slope (ms), upper mid-slope (ms-3), and the top of the slope (us-5). At each sampling point, soils were collected at four spots surrounding the point and pooled to make a single sample. Soil samples were taken at 90° intervals and at 1 m from the sample point center. This sampling scheme was chosen so that replicate samples reflected a range of slope positions within the clearcut.

The same procedure was used in the young stand and old-growth stand, except that the slopes in the young stand and old-growth stand were less steep than in the clearcut.

All soil samples were obtained from mineral soil to a 15 cm depth. Samples were labeled and kept inside of a cooler covered with ice and taken to the laboratory on the same day of sampling, where they were stored in a cold room at 4° C until further processed.

Plant Culture

Two greenhouse bioassays were conducted, one with red alder and the other with snowbrush. Red alder seeds were obtained from Brown Seed Company, 12101 N.E. 28th Street. Vancouver, WA 98668. Seed zone 042, elevation 2501-3000 feet, lot number B201-1987. Red alder seeds were surface sterilized with 30% hydrogen peroxide for 15 minutes and then rinsed with sterilized distilled water. Snowbrush seeds were collected from the "snowbrush" site and its vicinity at the H. J. Andrews Forest. Snowbrush seeds contained in a mesh tea infuser were immersed in boiling water, for five minutes, then soaked overnight in tap water. The following morning, seeds were surfaced sterilized with 30% hydrogen peroxide for 15 minutes 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 (4⁰ C) for three months for stratification.

Five germinating snowbrush seeds were planted in 150 ml Ray Leach tubes containing a mix of soil-vermiculite-perlite (2:1:1); five surface sterilized red alder seeds were also planted in Ray Leach tubes containing the same soil mix. Both were covered with sterilized silica. At four weeks, tubes were thinned to one plant each. The alder bioassay was initiated with 360 seedlings (12 for each of the 30 soil samples), and the snowbrush bioassay with 300 (10 for each soil sample).

The following conditions were maintained during the time the plants were growing in the greenhouse: room temperature was kept at 21°C during the day and at 16°C during the night and 11,000 lx sodium-vapor lamps kept a 14-hour photoperiod each day. If needed, plants were watered twice daily, otherwise watering was performed once per day only. To avoid cross contamination from splash during irrigation, different treatments were separated by at least 20 to 30 cm. In order to minimize location effect, plants were rotated to different bench locations once or twice a week.

An N-free mineral solution (Pregent and Camire, 1985) was used to fertilize plants. Beginning at six weeks after planting, 10 ml of a 1/4 dilution of full strength solution was used to fertilize each plant weekly until harvest.

Data Collection

Alder plants were harvested after 24 weeks and snowbrush plants after 48 weeks. Four variables were measured for each species: total plant biomass, acetylene reduction per plant, acetylene reduction per gram of nodule and nodule weight.

At harvest, nodules were picked from roots and transferred to test tubes. Roots and tops (leaves and stems) were separated and stored in individual paper bags. Nodules, roots, and tops were oven dried for three days at 80°C prior to weighing.

Prior to harvesting, we measured nitrogenase activity using the acetylene reduction technique as described by Koo (1989), and Rojas *et al.* (1992), in which whole root systems of living plants are assayed. Each red alder plant was placed inside of a 525-ml plastic tube (PVC) in such a way that the root systems were sealed from the plant tops using a rubber stopper perforated to let the plant stem go through. Snowbrush plants were also inserted into a PVC tube, but in this case whole plants (roots plus tops) were contained within the tube (because the small size of snowbrush plants allowed this). Each tube containing one whole snowbrush plant was sealed with a regular (non-perforated) rubber stopper. Sealed plastic tubes containing the root systems of a red alder plant or the roots and shoots of a snowbrush plant were injected with commercially purified acetylene to 10% of the total gas volume of the tube. After 2 hours 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 5830A gas chromatograph fitted with a 2.0 m x 2.1 mm, 80-100 mesh, Porapak R filled column, oven temperature at 70^o C. Injection temperature and flame-ionization detector temperature were each adjusted to 100^o C. Flow rate of the nitrogen carrier gas was adjusted to 40 ml per min. (Rojas *et al.*, 1992; Koo, 1989; Li and Castellano, 1987).

Data Analysis

Statistical analyses were performed using SAS (SAS Institute Inc. Cary, North Carolina) for windows, version 6.10. Data were analyzed using a model for a completely randomized design with subsampling (Petersen, 1985). In the model, red alder seedlings or snowbrush plants were nested within soil samples and soil samples were nested within stand locations (clearcut, old-growth, or young stand).

Because the design was unbalanced (unequal number of trees in different soil samples) the regular F statistic (Mean Square for Effect / Mean Square for Error) does not have an F-distribution. We used approximate F-tests as described by Steel and Torrie (1980) and Littell *et al.* (1991) to test for location effect and for significant sources of variation.

Logarithmic transformation (Log_{10}) was used for plant biomass, nodule weight, acetylene reduction per plant and per gram of nodule for snowbrush in order to satisfy the ANOVA assumptions of constant variance and normality. Transformation was not necessary for red alder.

Pearson correlations between plant biomass, acetylene reduction, and nodule weight were also calculated.

In order to determine if biomass and acetylene reduction of red alder and snowbrush plants differed among slope positions within sites, we used a one-way ANOVA to test for differences among positions. If the ANOVA indicated significant differences, we used the Bonferroni method as a mean

separation technique ($\alpha = 0.005$). Because soils for this experiment were collected from two parallel transects within each site, there were two replications of each slope position.

RESULTS

Differences in Survival and Nodulation Percentage

Red alder. Three hundred and thirty seven of the 360 red alder seedlings survived, 103 on clearcut soils (86%), 119 on old-growth soils (99%), and 115 on young stand soils (96%) (Table 2.1). All surviving seedlings in clearcut soils nodulated, while 77% of survivors nodulated in old-growth soils, and 90% of survivors nodulated in young stand soils.

Snowbrush. 93% of snowbrush plants survived on clearcut soils, 74% on old-growth soils, and 70% on young stand soils (Table 2.1). Nodulation was low in soils from all sites. Of the 237 plants included in this experiment, only 59 produced nodules, 21 (23%) in clearcut soils, 2 (3%) in old-growth soils, and 36 (51%) in young stand soils.

Differences Among Age Classes

Red alder. Acetylene reduction per plant did not differ significantly ($p=0.6549$) among the soils from the three locations (Figure 2.1), but nodule weight and acetylene reduction per gram of nodule did differ among locations at the $p=0.05$ level, and plant biomass differed at the $p=0.10$ level. Plant biomass and nodule weight were highest in clearcut soils (Figures 2.2 and 2.3), while acetylene reduction per g nodule was lowest in clearcut soils (Figure 2.4). Soil samples

Table 2.1. Percentage of snowbrush and red alder plants nodulating in soils of three age-class Douglas-fir forests.

Stand	Snowbrush		Red alder	
	Nodulating plants/ Total # of plants	Percentage of nodulating plants (%)	Nodulating plants/ Total # of plants	Percentage of nodulating plants (%)
Clearcut	21/93	23	103/103	100
Old-growth	2/74	3	92/119	77
Young stand	36/70	51	104/115	90
Total	59/237	25	299/337	89

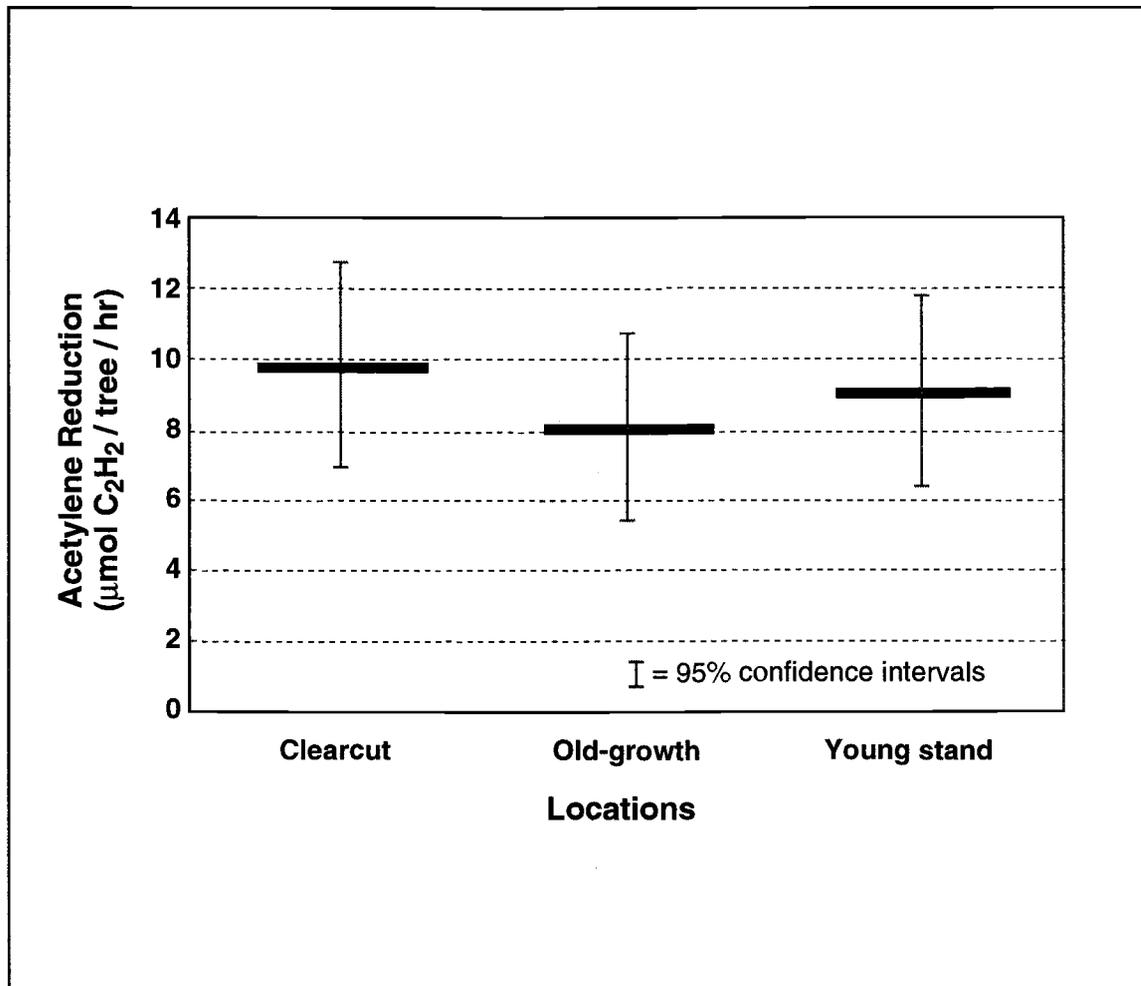


Figure 2.1. Means and 95% confidence limits for the acetylene reduction ($\mu\text{mol C}_2\text{H}_2/\text{tree/hr}$) of red alder seedlings grown on soils of the clearcut, old-growth, or young stand (using all plants).

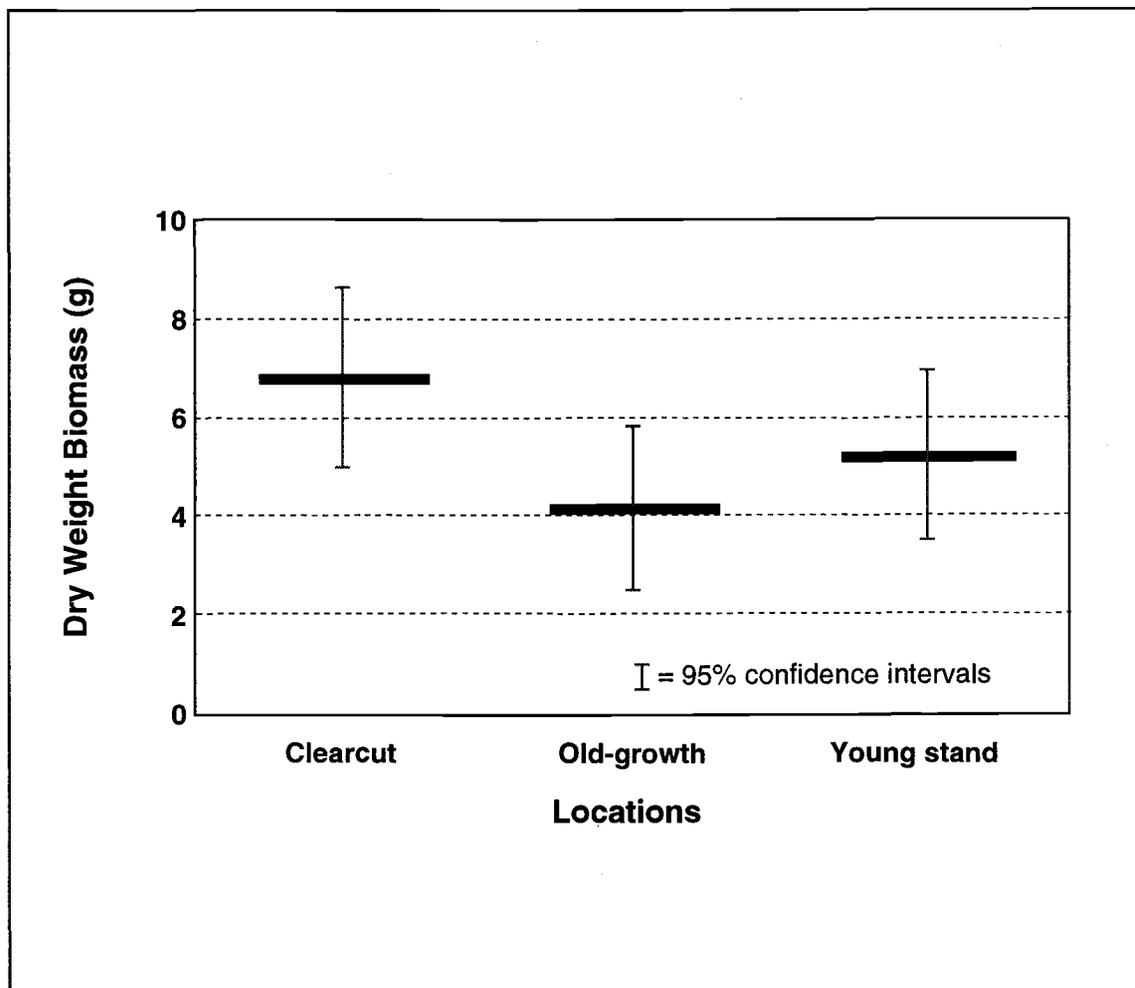


Figure 2.2. Means and 95% confidence limits for the biomass (g/tree) of red alder seedlings grown on soils of the clearcut, old-growth, or young stand.

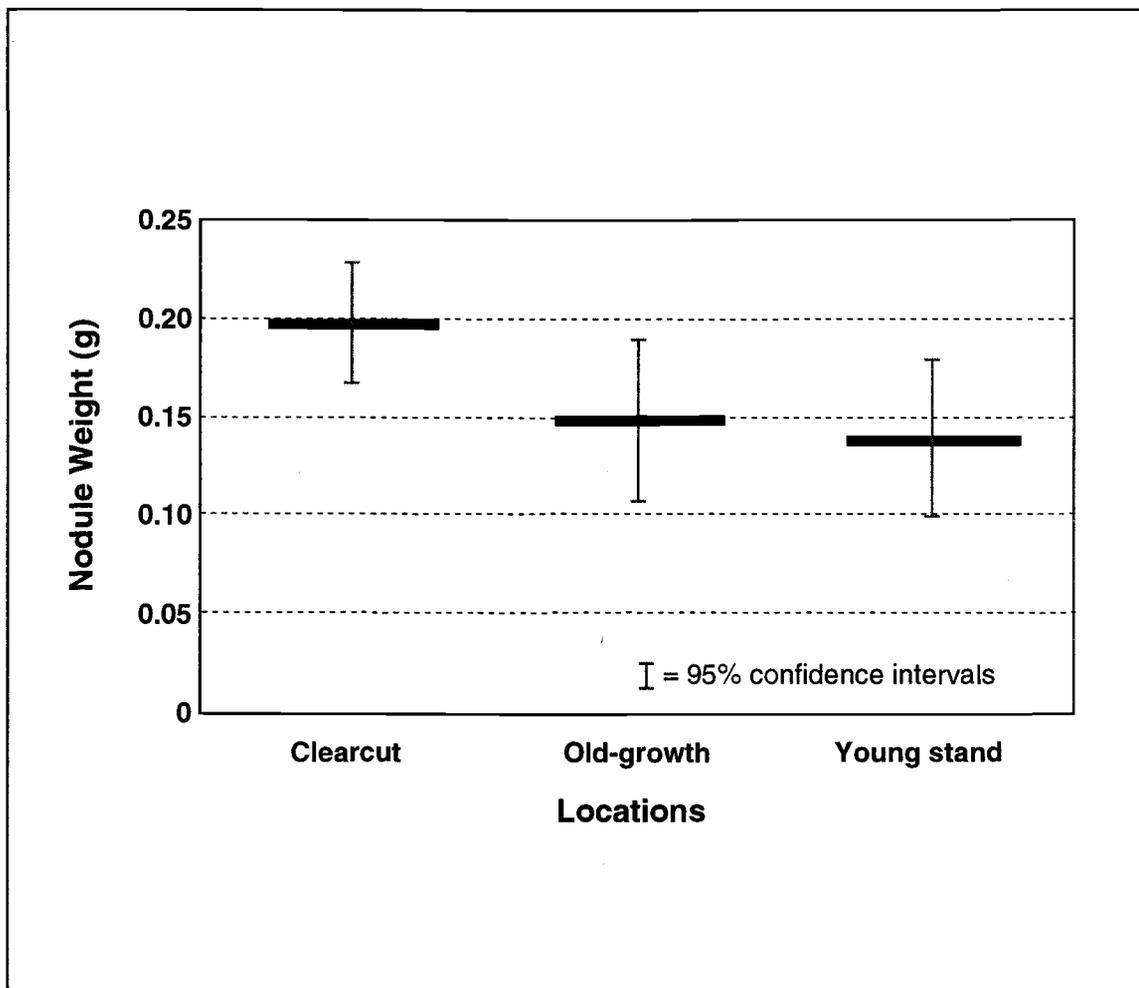


Figure 2.3. Means and 95% confidence limits for the nodule weight (g/tree) of red alder seedlings grown on soils of the clearcut, old-growth, or young stand (using all plants).

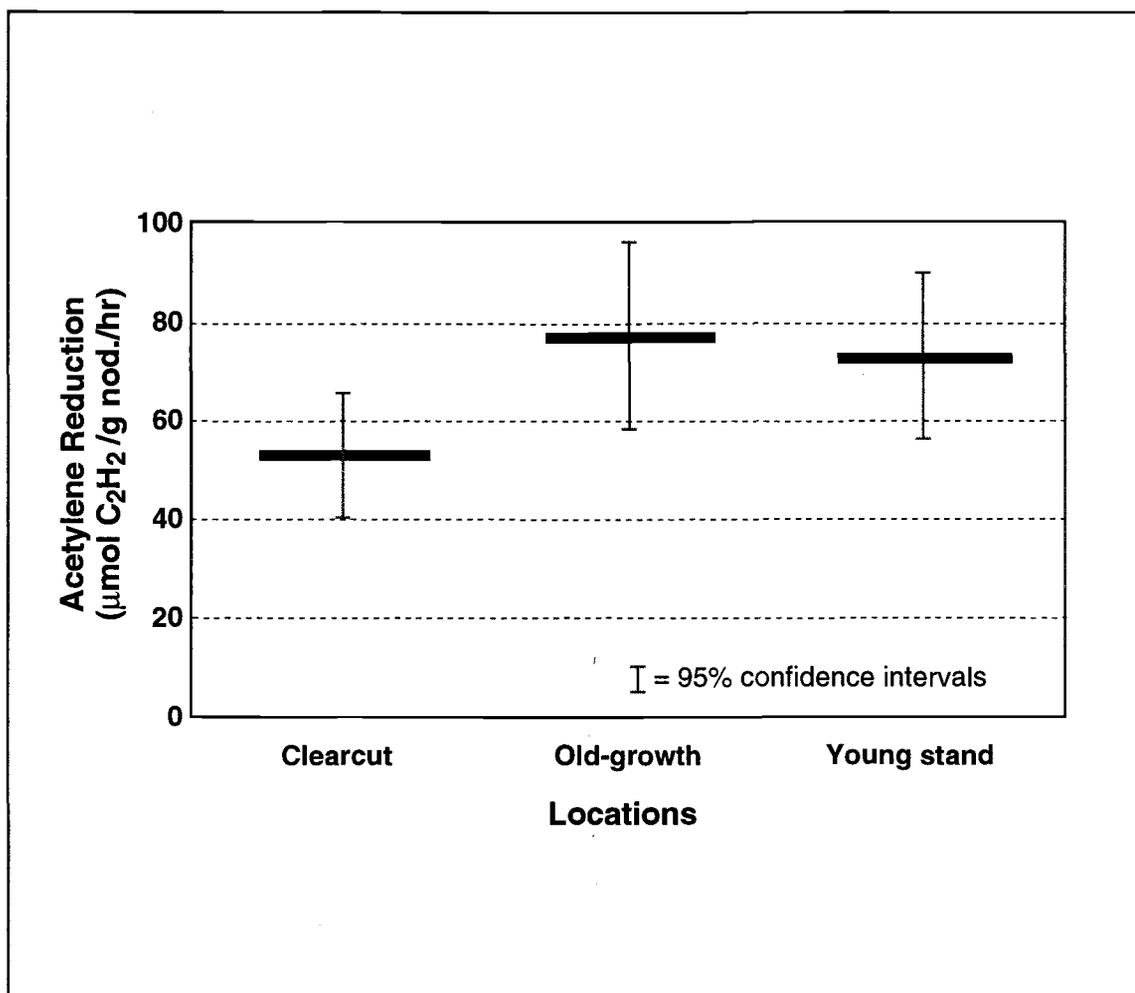


Figure 2.4. Means and 95% confidence limits for the acetylene reduction per g nodule ($\mu\text{mol C}_2\text{H}_2/\text{g nodule/hr}$) of red alder seedlings grown on soils of the clearcut, old-growth, or young stand.

within locations were a significant ($p < 0.0001$) source of variation for alder biomass, nodule weight, and acetylene reduction per plant and per gram of nodule (Tables 2.2, 2.3, 2.4 and 2.5).

All tested correlations among alder response variables were statistically significant (statistically non-zero) (Table 2.6). Nodule weight correlated positively with plant biomass ($r = 0.60$, $p = 0.0005$), while acetylene reduction per plant correlated positively with plant biomass ($r = 0.86$, $p = 0.0001$) and, more weakly, with nodule weight ($r = 0.41$, $p = 0.0279$).

Snowbrush. As with red alder, acetylene reduction per plant did not differ significantly among the soils from the three locations ($p = 0.8529$) (Figure 2.5), while plant biomass, nodule weight, and acetylene reduction per gram of nodule did ($p = 0.0379$, $p = 0.0226$, and $p = 0.0076$ respectively). The two plant species differed, however, in their response to soils from the different locations.

Whereas, alder biomass and nodule weight were highest in clearcut soils, for snowbrush these two variables were highest in young stand soils (Figures 2.6 and 2.7). Acetylene reduction per g nodule was highest in old-growth soils (Figure 2.8). As with red alder, soil samples within stands were a significant ($p < 0.0001$) source of variation for snowbrush biomass, nodule weight, and acetylene reduction per plant and per gram of nodule (Tables 2.7, 2.8, 2.9 and 2.10).

All tested correlations among snowbrush variables were statistically significant (statistically non-zero) (Table 2.11). Plant biomass correlated

Table 2.2. Analysis of variance for red alder biomass.

Source	d.f.	MS	E (MS)	F*	P-value
Total	331				
Locations	2	189.99	$\sigma^2 \text{ tree} + 10.774 \sigma^2 \text{ ss} + \varphi \text{st}$	2.53	0.0984
Soil samples w/i locations	27	76.91	$\sigma^2 \text{ tree} + 11.042 \sigma^2 \text{ ss}$	27.47	< 0.0001
Trees w/i soil samples w/i locations	302	2.80	$\sigma^2 \text{ tree}$		

φst : Effect due to locations.

$\sigma^2 \text{ tree}$: Variance component due to trees grown in the same soil.

$\sigma^2 \text{ ss}$: Variance component due to soil samples.

F*: Approximate F statistic based on E (MS).

Table 2.3. Analysis of variance for red alder nodule weight (using all plants).

Source	d.f.	MS	E (MS)	F*	P-value
Total	298				
Locations	2	0.085	$\sigma^2 \text{ tree} + 6.5671 \sigma^2 \text{ ss} + \phi \text{st}$	6.26	0.0045
Soil samples w/i locations	26	0.0179	$\sigma^2 \text{ tree} + 10.216 \sigma^2 \text{ ss}$	3.09	< 0.0001
Trees w/i soil samples w/i locations	270	0.0058	$\sigma^2 \text{ tree}$		

ϕst : Effect due to locations.

$\sigma^2 \text{ tree}$: Variance component due to trees grown in the same soil.

$\sigma^2 \text{ ss}$: Variance component due to soil samples.

F*: Approximate F statistic based on E (MS).

Table 2.4. Analysis of variance for red alder acetylene reduction (using all plants).

Source	d.f.	MS	E (MS)	F*	P-value
Total	336				
Locations	2	85.48	$\sigma^2 \text{ tree} + 11.032 \sigma^2 \text{ ss} + \phi \text{st}$	0.43	0.6549
Soil samples w/i locations	27	199.75	$\sigma^2 \text{ tree} + 11.216 \sigma^2 \text{ ss}$	15.67	< 0.0001
Trees w/i soil samples w/i locations	307	12.75	$\sigma^2 \text{ tree}$		

ϕst : Effect due to locations.

$\sigma^2 \text{ tree}$: Variance component due to trees grown in the same soil.

$\sigma^2 \text{ ss}$: Variance component due to soil samples.

F*: Approximate F statistic based on E (MS).

Table 2.5. Analysis of variance for red alder acetylene reduction per g nodule.

Source	d.f.	MS	E (MS)	F*	P-value
Total	298				
Locations	2	12644.91	$\sigma^2 \text{ tree} + 6.5671 \sigma^2 \text{ ss} + \phi \text{st}$	4.44	0.0191
Soil samples w/i locations	26	3787.93	$\sigma^2 \text{ tree} + 10.216 \sigma^2 \text{ ss}$	3.29	< 0.0001
Trees w/i soil samples w/i locations	270	1149.91	$\sigma^2 \text{ tree}$		

ϕst : Effect due to locations.

$\sigma^2 \text{ tree}$: Variance component due to trees grown in the same soil.

$\sigma^2 \text{ ss}$: Variance component due to soil samples.

F*: Approximate F statistic based on E (MS).

Table 2.6. Correlation coefficients for red alder response variables.

	Nodule Weight	Acetylene Reduction/plant
Plant Biomass	0.60 (p=0.0005)*	0.86 (p=0.0001)
Nodule Weight		0.41 (p=0.0279)

*p-value for testing H_0 : Correlation coefficient = 0

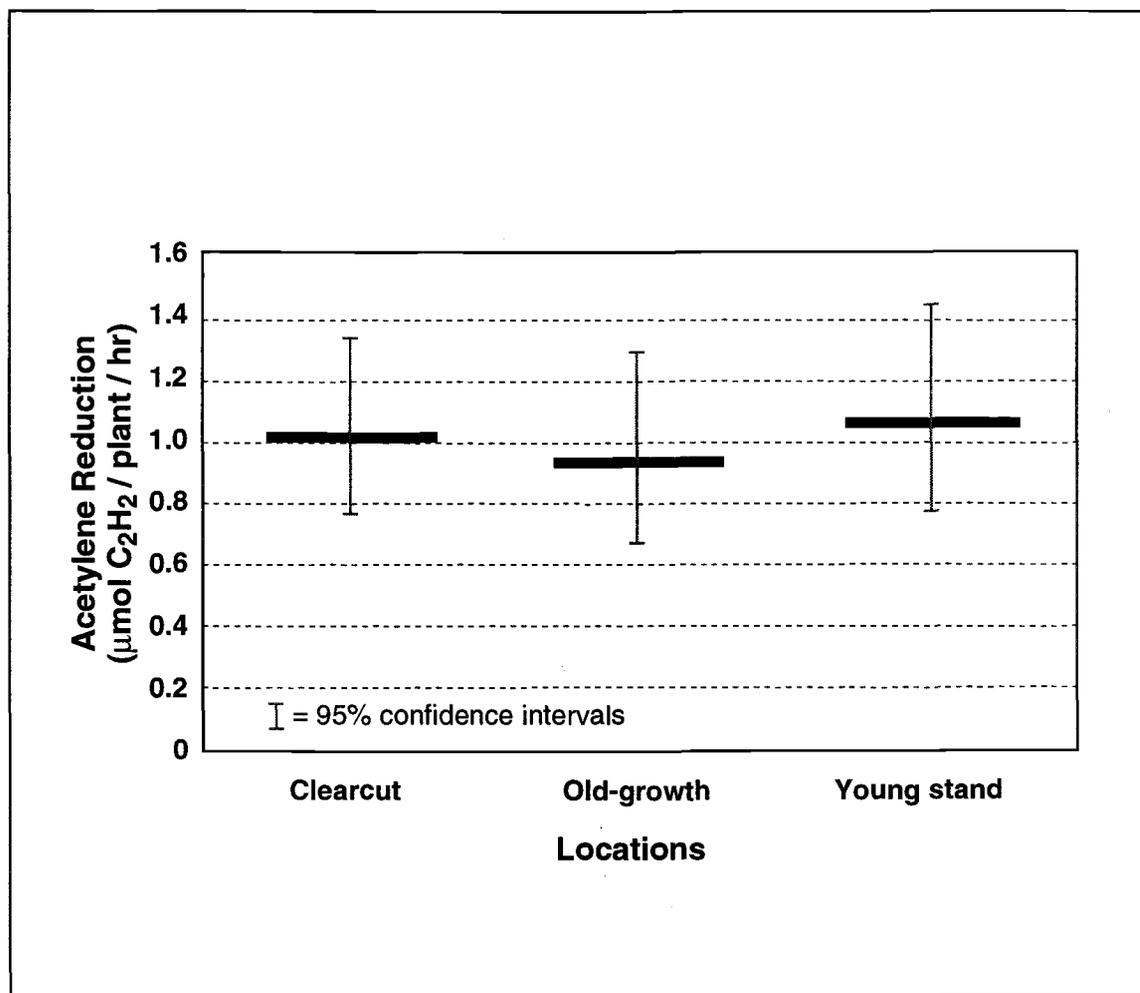


Figure 2.5. Means and 95% confidence limits for the acetylene reduction ($\mu\text{mol C}_2\text{H}_2 / \text{plant} / \text{hr}$) of snowbrush plants grown on soils of the clearcut, old-growth, or young stand (using all plants).

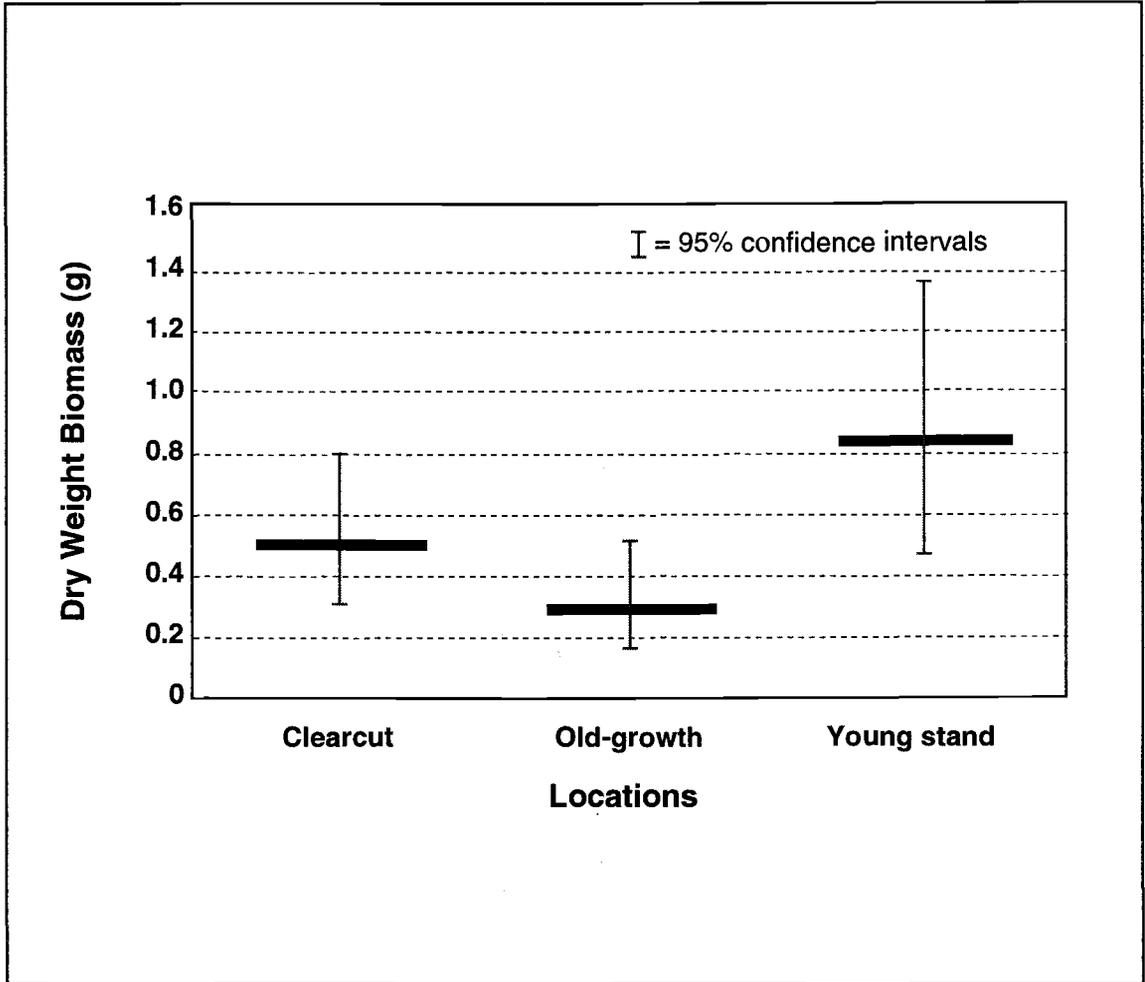


Figure 2.6. Means and 95% confidence limits for the biomass (g/plant) of snowbrush plants grown on soils of the clearcut, old-growth, or young stand.

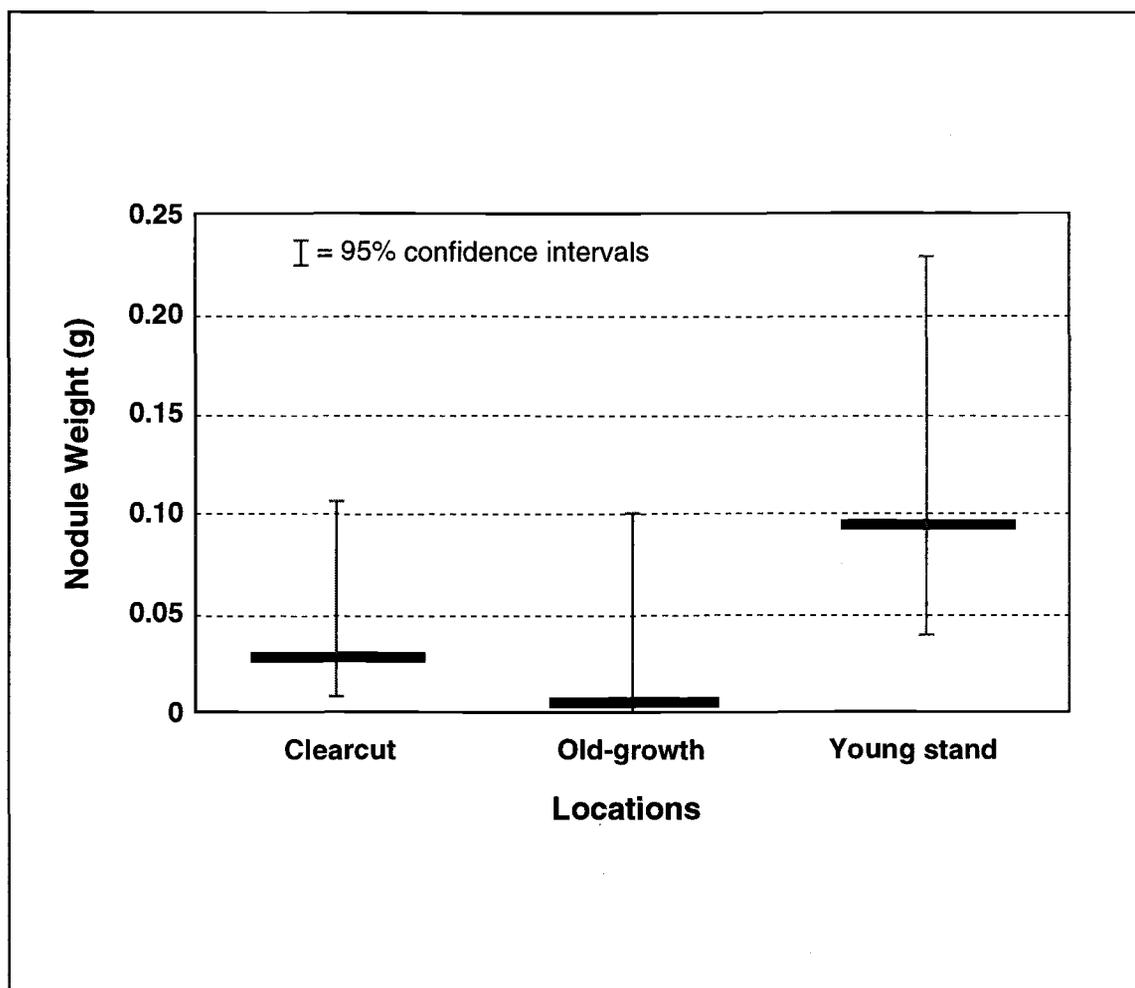


Figure 2.7. Means and 95% confidence limits for the nodule weight (g/plant) of snowbrush plants grown on soils of the clearcut, old-growth, or young stand (using all plants).

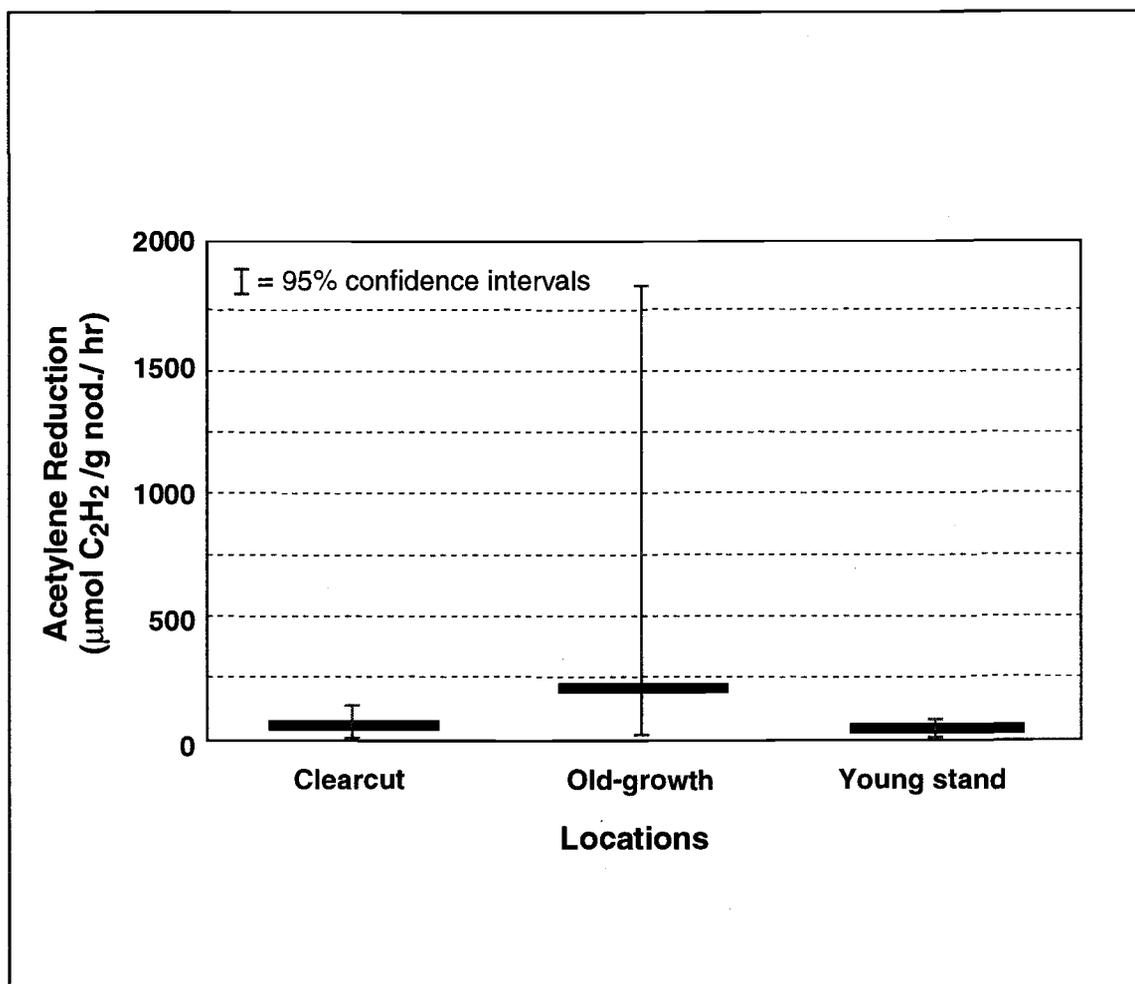


Figure 2.8. Means and 95% confidence limits for the acetylene reduction per g nodule ($\mu\text{mol C}_2\text{H}_2/\text{g nodule/hr}$) of snowbrush plants grown on soils of the clearcut, old-growth, or young stand.

Table 2.7. Analysis of variance for snowbrush $\text{Log}_{10}(\text{biomass})$.

Source	d.f.	MS	E (MS)	F*	P-value
Total	236				
Locations	2	3.019	$\sigma^2 \text{ plant} + 7.5408 \sigma^2 \text{ ss} + \phi \text{st}$	3.72	0.0379
Soil samples w/i locations	26	0.869	$\sigma^2 \text{ plant} + 8.1221 \sigma^2 \text{ ss}$	15.24	< 0.0001
Plants w/i soil samples w/i locations	208	0.057	$\sigma^2 \text{ plant}$		

ϕst : Effect due to locations.

$\sigma^2 \text{ plant}$: Variance component due to plants grown in the same soil.

$\sigma^2 \text{ ss}$: Variance component due to soil samples.

F*: Approximate F statistic based on E (MS).

Table 2.8. Analysis of variance for snowbrush $\text{Log}_{10}(\text{nodule weight})$ (using all plants).

Source	d.f.	MS	E (MS)	F*	P-value
Total	58				
Locations	2	2.77	$\sigma^2 \text{ plant} + 2.4782 \sigma^2 \text{ ss} + \phi \text{st}$	5.28	0.0226
Soil samples w/i locations	10	0.87	$\sigma^2 \text{ plant} + 4.5452 \sigma^2 \text{ ss}$	7.91	< 0.0001
Plants w/i soil samples w/i locations	46	0.11	$\sigma^2 \text{ plant}$		

ϕst : Effect due to locations.

$\sigma^2 \text{ plant}$: Variance component due to plants grown in the same soil.

$\sigma^2 \text{ ss}$: Variance component due to soil samples.

F*: Approximate F statistic based on E (MS).

Table 2.9. Analysis of variance for snowbrush Log_{10} (acetylene reduction)(using all plants).

Source	d.f.	MS	E (MS)	F*	P-value
Total	236				
Locations	2	0.045	$\sigma^2 \text{ plant} + 7.5408 \sigma^2 \text{ ss} + \phi \text{st}$	0.16	0.8529
Soil samples w/i locations	26	0.293	$\sigma^2 \text{ plant} + 8.1221 \sigma^2 \text{ ss}$	6.23	< 0.0001
Plants w/i soil samples w/i locations	208	0.047	$\sigma^2 \text{ plant}$		

ϕst : Effect due to locations.

$\sigma^2 \text{ plant}$: Variance component due to plants grown in the same soil.

$\sigma^2 \text{ ss}$: Variance component due to soil samples.

F*: Approximate F statistic based on E (MS).

Table 2.10. Analysis of variance for snowbrush Log_{10} (acetylene reduction per g nodule).

Source	d.f.	MS	E (MS)	F*	P-value
Total	58				
Locations	2	1.74	$\sigma^2 \text{ plant} + 2.4782 \sigma^2 \text{ ss} + \phi \text{st}$	7.27	0.0076
Soil samples w/i locations	10	0.3846	$\sigma^2 \text{ plant} + 4.5452 \sigma^2 \text{ ss}$	5.89	< 0.0001
Plants w/i soil samples w/i locations	46	0.0653	$\sigma^2 \text{ plant}$		

ϕst : Effect due to locations.

$\sigma^2 \text{ plant}$: Variance component due to plants grown in the same soil.

$\sigma^2 \text{ ss}$: Variance component due to soil samples.

F*: Approximate F statistic based on E (MS).

Table 2.11. Correlation coefficients for snowbrush response variables.

	Nodule Weight	Acetylene Reduction/plant
Plant Biomass	0.84 (p=0.0003)*	0.72 (p=0.0001)
Nodule Weight		0.73 (p=0.0044)

*p-value for testing H_0 : Correlation coefficient = 0

positively with nodule weight ($r=0.84$, $p=0.0003$) and with plant acetylene reduction ($r=0.72$, $p=0.0001$), while nodule weight correlated positively with plant acetylene reduction ($r=0.73$, $p=0.0044$).

Slope-Related Differences Within Age Classes

Red alder. When looking at the patterns of within stand variation (slope position) for red alder biomass, and whole plant acetylene reduction, we observed no difference between the means at five positions (bottom slope, lower mid-slope, mid-slope, upper mid-slope, and top of the slope) sampled along the two transects for any of the three stands studied.

Snowbrush. Snowbrush biomass and plant acetylene reduction in young stand and old-growth showed no significant differences among slope positions. However the one-way ANOVA for plant biomass in the clearcut indicated that some position means differed ($p=0.0049$) (Table 2.12 shows the p-values for the ten position comparisons). The biomass of snowbrush plants grown in clearcut soils averaged higher in bottom slope (bs-1) soils than in soils from any other position within the clearcut (Figure 2.9). Snowbrush plants grown in bottom slope soils produced more biomass than plants grown in soils from mid-slope (ms) ($p\text{-value} = 0.0018$), lower mid-slope (ms-2) ($p\text{-value} = 0.0044$), or upper mid-slope (ms-3) ($p\text{-value} = 0.0011$). Snowbrush plants grown in top slope soils (us-5) produced more biomass than plants grown in upper mid-slope soils (ms-3) (p -

Table 2.12. Position comparisons for the dry weight biomass of snowbrush plants grown in clearcut soils.

Position Comparisons			Plant Biomass (p-value)
Bottom slope (bs-1)	vs.	mid-slope (ms)	0.0018
Bottom slope (bs-1)	vs.	lower mid-slope (ms -2)	0.0044
Bottom slope (bs-1)	vs.	upper mid-slope (ms-3)	0.0011
Bottom slope (bs-1)	vs.	top of the slope (us-5)	0.0721
Mid-slope (ms)	vs.	lower mid-slope (ms-2)	0.3050
Mid-slope (ms)	vs.	upper mid-slope (ms-3)	0.5269
Mid-slope (ms)	vs.	top of the slope (us-5)	0.0129
Lower mid-slope (ms-2)	vs.	upper mid-slope (ms-3)	0.1281
Lower mid-slope (ms-2)	vs.	top of the slope (us-5)	0.0460
Upper mid-slope (ms-3)	vs.	top of the slope (us-5)	0.0066

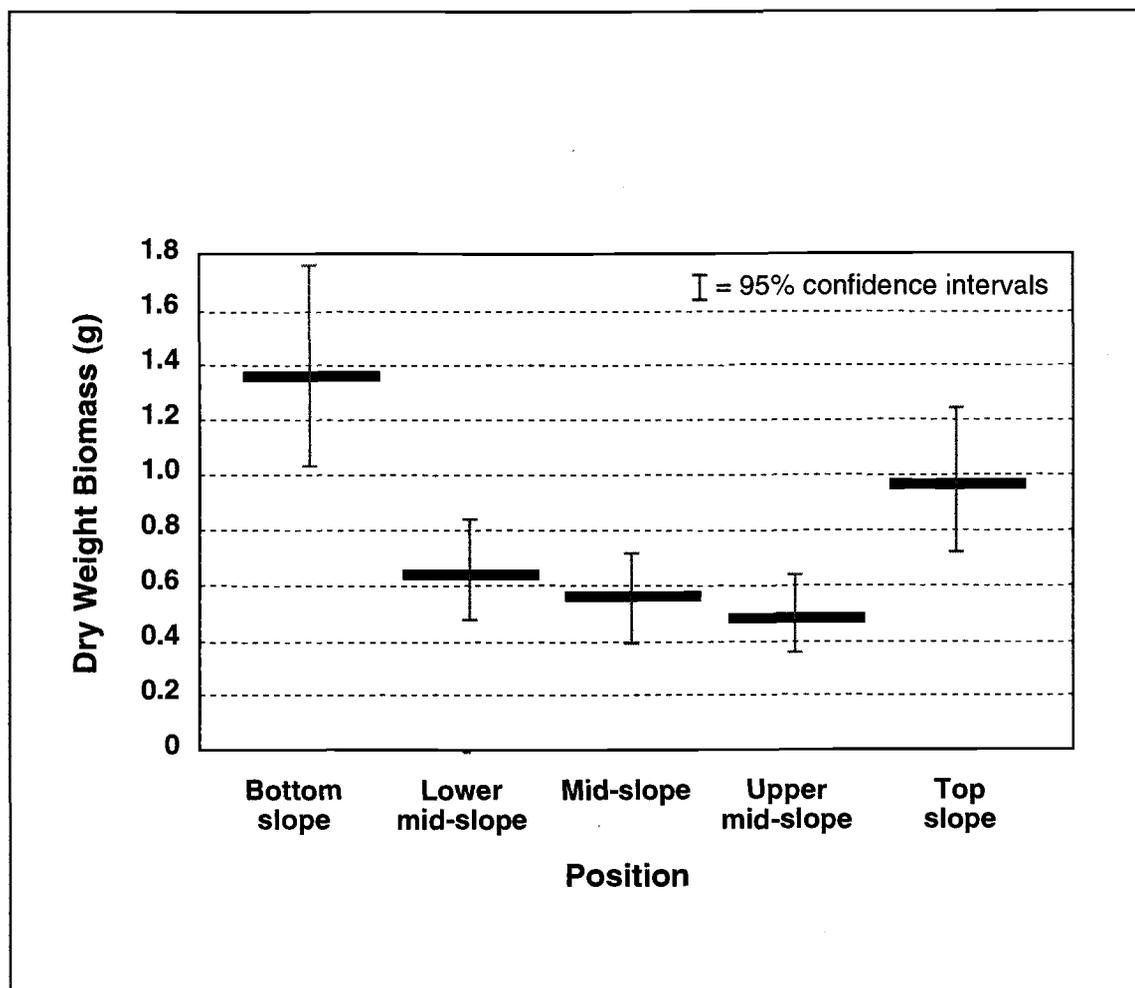


Figure 2.9. Means and 95% confidence limits for the dry weight biomass (g/plant) of snowbrush plants grown in clearcut soils by position.

value = 0.0066). Acetylene reduction in the clearcut showed no significant differences among positions.

DISCUSSION

When we account for differences in the number of plants nodulating, the sites and species are very different. Red alder plants nodulated well in soils from all three stands. The highest percentages of nodulating red alder seedlings were obtained in soils from the clearcut stand (100%) and in soils from the young stand (90%). Furthermore, soils from the old-growth stand also produced a relatively high red alder nodulation percentage (77%). Snowbrush plants, on the other hand, nodulated poorly in soils from all three stands. Fifty one percent of snowbrush plants formed nodules in the young stand soils, 23% in the clearcut soils, and only a 3% in the old- growth soils.

Our hypothesis that nodule biomass and acetylene reduction for both snowbrush and red alder would be greatest in soils from the adjacent 20-year-old Douglas-fir plantation with an understory of snowbrush, intermediate in soils from the "snowbrush" study site, and least in soils from the adjacent old-growth forest was partially supported in the case of snowbrush, but not in the case of red alder. Acetylene reduction per plant (nodulated and non-nodulated) did not vary significantly among the soils from the three sites, for either plant species, but plant biomass, nodule weight (using all plants), and acetylene reduction per gram of nodule did. For both species, nodule weight, acetylene reduction per

plant and per gram of nodule and plant biomass were highly variable within stands.

As expected, snowbrush plants produced more nodules and biomass when grown in young stand soils than when grown in soils from the other locations (nodulation calculated using all plants). At the time sampling was done, the adjacent 20-year-old Douglas-fir plantation had a healthy and thick snowbrush cover which probably served as a good source of *Frankia* inoculum. However, non-nodulated snowbrush plants may have had higher levels of rhizosphere nitrogen fixation (associative) on clearcut and old-growth soils than on young stand soils indicating snowbrush could compensate to some degree for reduced nodulation. Contrary to our expectations, red alder plants produced more nodules and biomass when grown in clearcut soils than when grown in soils of any other location (nodulation calculated using all plants). But red alder plants exhibited the lowest rate of acetylene reduction per g of nodule when grown in clearcut soils. This is most likely due to the well known inverse relation between nodule weight and nitrogenase activity per unit weight (Wheeler *et al.*, 1981; Sempavalan *et al.*, 1995).

In order for red alder or snowbrush plants to grow, nodulate, and fix atmospheric nitrogen on soils from the clearcut or the adjacent old-growth stand, soil *Frankia* populations must either have been able to survive without hosts and stayed infectious until actinorhizal plants established, or dispersed onto the sites from elsewhere. The occurrence of *Frankia* in soils without hosts is probably

due to the ability of this microorganism to survive saprophytically in the soil (Smolander and Sundman, 1987; Li *et al.*, 1997), or by becoming dormant (Molina *et al.*, 1994). It is also possible that *Frankia* spores were introduced into these stands by an unknown dispersal agent. Research conducted elsewhere has identified some vertebrates and invertebrates as being good dispersal agents of *Frankia* spores (Reddell and Spain, 1991; Paschke and Dawson, 1993; Burleigh and Dawson, 1995; Li *et al.*, 1997).

If nodulation potential for snowbrush in the old-growth stand formerly occupying the clearcut was similar to that in the adjacent old-growth (where only 3% of plants nodulated), then populations of snowbrush-nodulating *Frankia* have either increased since clearcutting, or the nodulation potential of existing populations has increased. The nodulation percentage differed among slope positions in the clearcut soils: of the 21 snowbrush plants (23%) that nodulated in the clearcut, 12 were from bottom slope, 3 were from lower mid-slope, 1 was from upper mid-slope, and 5 were from the top of the slope. This tells us that *Frankia* populations at the bottom of the slope had been rebuilt by the presence of healthy snowbrush plants, and that at mid- and upper slope some other factor is involved (e.g. dispersal).

Except for acetylene reduction per plant, which did not vary significantly between the three age-class Douglas-fir forests studied here (when calculated using all plants), the two actinorhizal plant species responded quite differently in our studies. Snowbrush plants did better when grown in young stand soils, while

red alder did better when grown in clearcut soils. As noted earlier, snowbrush was not growing well in the clearcut. Heavy browsing by a high population of elk is one possible explanation for this (Swanson *et al.*, 1987). However, the pattern of plant responses from our greenhouse experiments suggests poor growth of snowbrush was related to soil factors.

The strain(s) of *Frankia* that induces root nodulation on snowbrush and the strain(s) of *Frankia* that induces root nodulation on red alder probably differ from each other in several ways. These two (or more) *Frankia* strains may be to some extent plant specific, meaning they may not cross-inoculate their respective hosts; even though, we have good reasons to believe that some of the *Frankia* strains that nodulated snowbrush also nodulated red alder since red alder nodulated well in young stand soils (which supported a healthy stand of snowbrush but no alder). Nutritional demands of both the plants and *Frankia* could also be different, explaining perhaps their hosts differences regarding sites preferences, one doing better at the clearcut (alder), the other doing better at the young stand (snowbrush). Rojas *et al.* (1997, chapter 3 in this thesis) working with soils collected from four locations along a slope transect from the same clearcut also found strong differences in nodulation between snowbrush and red alder: Alder nodulating more readily at mid- and upper slope and snowbrush nodulating more readily at the bottom of the slope. Rojas *et al.*, (1997) speculate that the existence of strains with different adaptations may reflect a survival mechanism of the nitrogen-fixing endophyte *Frankia*.

One of the most interesting findings is that red alder and snowbrush are not responding to the different sites in the same way; the same seems to be true within the clearcut. The biomass of snowbrush plants grown in clearcut soils averaged highest in bottom slope soils reinforcing the pattern observed in the field and also what we had observed in the experiments reported in chapter 3. Better understanding the factors influencing nodulation and nitrogen-fixation of actinorhizal plants may yield important insights into the long-term carbon dynamics of these N-limited ecosystems.

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Chapter 3

Interactions Among Soil Biology, Nutrition, and Performance of Actinorhizal Plant Species in an Oregon Clearcut.

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ABSTRACT

Two greenhouse bioassays using red alder and snowbrush plants consisted of adding sequentially to soils *Frankia* plus macronutrients, micronutrients, mycorrhizal fungi, and *Pseudomonas fluorescens*. The influence of these treatment factors was examined with respect to acetylene reduction, nodule weight and total biomass. 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 transect on a slope gradient, one each at bottom slope, lower mid-slope, upper mid-slope, and the top of the slope. Nitrogen-fixing *Frankia* were isolated from root nodules of red alder collected near the study site. Fresh crushed nodules, from snowbrush growing on the study site, were used as *Frankia* inoculum for snowbrush. *Pseudomonas fluorescens* were isolated from nodule surfaces, rhizosphere soils, and root surfaces of snowbrush plants. Snowbrush and red alder plants were greenhouse-grown in a mix of soil-vermiculite-perlite (2:1:1) for 12 and 6 months respectively. At five weeks plants were inoculated with *Frankia* and a bacterial (*Pseudomonas*) suspension. Red alder plants were also inoculated with spores of *Alpova diplophloeus* and snowbrush with *Glomus intraradix*. There was no interaction between treatment and location for either species. There were no significant treatment effects for snowbrush, but there were significant treatment effects for red alder. Red alder seedlings given *Frankia*

and macronutrients had greater biomass and reduced more acetylene than seedlings grown without additions. Adding *Alpova diplophloeus* increased acetylene reduction by 33% over that attained with *Frankia* and macronutrients alone. The combined effect of *Frankia*, macronutrients and the mycorrhizal fungus was to increase acetylene reduction by 136% over controls. Adding micronutrients to *Frankia* and macronutrients reduced acetylene reduction by nearly one-half, completely negating the positive effect of the *Frankia* and macronutrients. The presence of the mycorrhizal fungus appeared to buffer the negative effects of micronutrients. *Pseudomonas* inoculation did not affect any of the measured variables. The two plant species differed in their response to soil from different slope positions. Red alder seedlings grown in upper slope soil had greater biomass and reduced more acetylene than seedlings grown in down slope soil. In contrast, snowbrush plants grown in bottom slope soil had greater biomass, nodule weight, and reduced more acetylene than seedlings grown in any of the other slope positions.

INTRODUCTION

Actinorhizal plants (i.e. nodulated by actinomycetes in the genus *Frankia*) are the primary sources of biologically-fixed nitrogen (N) in many temperate and boreal forest ecosystems and may be responsible for the majority of N inputs from all sources in northern forests with low N inputs in precipitation (e.g. the Pacific Northwest of North America) (Perry, 1994). Predominant actinorhizal plants in the Pacific Northwest include eight species in the genus *Ceanothus*, which are found in mesic to dry sites at mid-elevations (Rose and Youngberg, 1981; Conard *et al.*, 1985), and four *Alnus* species, which occur in relatively mesic sites across a range of elevations (Hibbs *et al.*, 1994). *Alnus rubra* (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), while *Ceanothus velutinus* (snowbrush) is common on disturbed sites throughout the central Cascades and eastward into the northern Rocky Mountains. As is commonly the case with actinorhizal plants, *Ceanothus* and *Alnus* species are aggressive pioneers following disturbance, a trait that has made them unpopular with many foresters.

Because N frequently limits net primary productivity in northern forests not impacted by pollution (Perry, 1994), understanding the environmental factors that influence the presence and N-fixing activity of actinorhizal plants is central to understanding long-term carbon budgets of these ecosystems. Soil

temperature, soil moisture, calcium, phosphorus, magnesium and sulfur have been shown to influence nodulation and N-fixation in both *Alnus* and *Ceanothus* species (Wollum and Youngberg, 1969; McNabb and Cromack, 1983; Kummerow *et al.*, 1978; Sharma, 1988; Huss-Danell, 1986; Jha *et al.*, 1993; Waring and Schlesinger, 1985). In addition to macronutrients, nodule development and nitrogen fixation in legumes requires the micronutrients Co, Cu, Fe, B, Mo, and Ni, and the first three have been found to 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 has been shown to limit asymbiotic N-fixation in the Pacific Northwest (Silvester, 1989).

Soil microbes may influence symbiotic N-fixation in various ways, most directly through the availability of the N-fixing diazotroph. There is an abundant literature on the occurrence of *Frankia* in soils with and without hosts. For example, research conducted by Smolander and Sundman (1987) in Finland showed that *Alnus* seedlings grown in soils collected from a birch stand devoid of actinorhizal plants formed more nodules than seedlings grown in soils collected from an *Alnus incana* stand, suggesting that *Frankia* lives saprophytically in soils devoid of actinorhizals.

Past studies in the Pacific Northwest generally find abundant nodulation on alders but variable nodulation on snowbrush when growing in

clearcuts (Wollum *et al.*, 1968; Youngberg and Wollum, 1976; Youngberg *et al.*, 1979; Zavitkovski and Newton, 1967; Zavitkovski and Newton, 1968; and Binkley, 1981). Wollum *et al.* (1968) found that snowbrush nodulated better when growing on sites formerly occupied by mid-aged conifer stands than when growing on sites formerly occupied by old-growth, and 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 using red alder and snowbrush, in which nodulation was highly variable within stands and varied between old-growth, a twenty-year-old conifer plantation intermixed with snowbrush, and a recent clearcut (the site of the study reported here) (Rojas *et al.*, 1997, chapter 2 in this thesis).

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* (Knowlton and Dawson, 1983; Knowlton *et al.*, 1980), a common soil microbe that produces the strong Fe⁺⁺⁺ 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 snowbrush and red alder to nodulate and fix N in soils from an Oregon clearcut was enhanced by amending soils with various combinations of *Frankia* plus macronutrients, mycorrhizal fungi, *Pseudomonas fluorescens*, and micronutrients. The research was stimulated by a widespread failure of snowbrush seedlings that germinated following clearcutting and burning to grow and survive. In addition, though, red alder occurs in moist microsites near the study site; it has not colonized the clearcut. Previous work using soils from this site found nodulation of both snowbrush and red alder to vary widely among sample points (Rojas *et al.*, 1997, chapter 2 in this thesis).

We speculated 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 *Pseudomonas fluorescens* to clearcut soils would increase nodulation and N-fixation of both plant species, the latter through its ability to produce HS. Work elsewhere has shown that both vesicular-arbuscular (VAM) and ectomycorrhizal fungi (EM) may be lost in erosion from disturbed sites (Valdes, 1986; Amaranthus and Trappe, 1993). Perry *et al.* (1984) found decreased concentrations of the Fe chelator, HS, in soils from 8 of 10 Oregon clearcuts, with largest decreases on sites that had been broadcast burned. We expected mycorrhizae to benefit VAM snowbrush more than EM alder for two reasons. First, the finding by Amaranthus and Trappe (1993) that more VAM than EM

inocula was lost in erosion from a disturbed site. Second, findings by Miller *et al.* (1992) suggest that alder seedlings would not benefit from inoculation with a mycorrhizal fungus as snowbrush would, since clearcut soils in their study seem to be well supplied with *Alpova* spores. Since mycorrhizal fungi function in nutrient gathering and *Pseudomonas* in solubilizing Fe, we expected the addition of micronutrients to substitute, in part at least, for these two groups of microorganisms.

MATERIALS AND METHODS

Study Area

Soils used for the greenhouse bioassays were collected in October of 1992 from the "snowbrush" site. We sampled from within a 10-year-old clearcut planted with Douglas-fir on the H. J. Andrews Experimental Forest. The H. J. Andrews Experimental Forest is located in the Cascade Mountains, 80 km east of Eugene, Oregon, on the Blue River Ranger District of the Willamette National Forest. Precipitation in this area averages 2400 mm a year, most falling in the winter and early spring as either rain or snow (Waring *et al.*, 1978). The mean annual temperature is 8.5 degrees C, with a July mean of 18^o C and a January mean of 1^o C. Shallow soils derived from ash flow, mudflow, and stream deposits are found at lower elevations, while 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, referred to as the "snowbrush" site, is 890 m above sea level, in the western hemlock zone of Franklin and Dyrness (1973). The site is southeast facing, with an average 22 degree slope. Formerly occupied by an old-growth Douglas-fir, western hemlock, and western red cedar forest, the site was clearcut in 1981, and logging slash broadcast burned in 1982. Nine

hundred Douglas-fir seedlings per ha were planted six 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 were nodulated, but the small plants growing on mid- and upper slopes were not. Planted Douglas-fir has successfully established on the site.

Soil Collection

Soils were collected along a transect originating at a randomly chosen point on a flat at the base of a 22 degree slope and extending 60 m up-slope at right angles to the contour. Four sampling points were selected along the transect, one each at bottom slope (bs-1), lower mid-slope (ms-2), upper mid-slope (ms-3), and the top of the slope (us-5). At each sampling point, soils were collected at four spots surrounding the point and pooled to make a single sample. Soil samples were taken at 90° intervals and at 1 m from the sample point center. This sampling scheme was chosen so that replicate 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 15 cm depth. Samples were labeled and transferred immediately to a cooler in which they were covered with ice, and taken to our laboratory at Oregon State University on the same day of sampling. In the laboratory, samples were stored in a cold room at 4° C until further processed.

Isolation and cultivation of *Frankia*

Frankia for use in inoculations of red alder were isolated from nodules of red alder collected near the study site. A modified Benson's (1982) filtration procedure, described by Rojas *et al.* (1992) and Molina *et al.* (1994), was used to isolate *Frankia* in the N-free defined liquid medium BAP (Murry *et al.*, 1984) at 30° C. *Frankia* was then transferred to BAP medium containing 5.0 mM of NH₄Cl and incubated for inoculum preparation. Isolation of *Frankia* from snowbrush nodules was not successful; therefore, we used fresh crushed nodules from snowbrush growing on the study site as *Frankia* inoculum for snowbrush.

Isolation of *Pseudomonas*

Three isolation sources (nodule surfaces, rhizosphere soils, and root surfaces of snowbrush plants) were chosen for the isolation of fluorescent *Pseudomonas* (*Pseudomonas fluorescens*) colonies. Several snowbrush plants were collected from the site with their root systems and nodules still attached. In order to obtain individual plants with their nodulated root systems as intact as possible, soil was carefully removed from around each plant. Entire plants along with soil adhering to roots were placed in plastic bags and brought back to the laboratory.

Fluorescent *Pseudomonas* were isolated following the procedure of Geels and Schippers (1983). Rhizosphere soils were obtained by carefully

shaking by hand the nodulated root systems of individual snowbrush plants and by removing (also by hand) attached soil particles when necessary.

Rhizosphere soils from all plants were collected in a sterile beaker, pooled to make a single sample and sieved through a 2 mm screen, and nodules and fine roots were carefully washed until free of attached soil particles. A prescription bottle with 20 grams sieved rhizosphere soils and 100 ml sterile distilled water was vigorously shaken for 15 minutes on a shaker. This procedure was repeated with prescription bottles containing 5 grams nodules or fine roots in 100 ml sterile distilled water. Serial dilutions, 1:500 for rhizosphere soils and 1:20 for nodules and fine roots, were made with sterile distilled water. Dilutions were plated out with the modified King's medium (Geels and Schippers, 1983) composed of: 20.0 g Proteose peptone #3; 15.0 g Bacto agar; 10.0 g Glycerol; 1.5 g K_2HPO_4 ; 1.5 g $MgSO_4 \cdot 7H_2O$; and 1000 ml distilled water with 100 ppm Cycloheximide; 50 ppm Ampicillin and 12.5 ppm Chloramphenicol. Fluorescent *Pseudomonas* was grown in the modified King's liquid medium for 3 days at 30° C. Colonies developed on the medium were examined under ultraviolet light at 366 nm. Colonies isolated from nodule surfaces fluoresced most intensely under ultraviolet light. The bacterial cells were harvested and washed three times with 0.01M phosphate buffer, pH 6.7.

Plant Culture

Two greenhouse bioassays were conducted, one with red alder and the other with snowbrush. Red alder seeds were obtained from Brown Seed Company, 12101 N.E. 28th Street. Vancouver, WA 98668. Seed zone 042, elevation 2501-3000 feet, lot number B201-1987. Red alder seeds were surface sterilized with 30% hydrogen peroxide for 15 minutes 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 five minutes, then soaked overnight in tap water. The following morning, seeds were surfaced sterilized with 30% hydrogen peroxide for 15 minutes 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 three months for stratification. Seeds began germinating after three months.

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

As mentioned earlier, two greenhouse bioassays were used in this study: One with snowbrush plants, the other with red alder plants. Snowbrush plants were grown for a year and alder plants for six months. The following conditions

were maintained during the time the plants were growing in the greenhouse: room temperature was kept at 21° C during the day and at 16° C during the night and 11,000 lx sodium-vapor lamps kept a 14-hour photoperiod each day. If needed, plants were watered twice daily; otherwise, watering was performed once per day only. To avoid cross contamination from splash during irrigation, different treatments were separated by at least 20 to 30 cm. In order to minimize location effect, plants were rotated to different bench locations once or twice a week.

Treatments.

The experiment consisted of adding to soils the following factors known to influence nodulation and nitrogen fixation: *Frankia*, macronutrients, micronutrients, mycorrhizal fungi, and *Pseudomonas fluorescens*. A complete factorial design was not possible because the size of the experiment would have been prohibitive, rather, we added treatment factors sequentially. All seedlings except controls received *Frankia* and macronutrients. Depending on treatment, seedlings additionally received mycorrhizal fungi, micronutrients, mycorrhizal fungi and micronutrients, mycorrhizal fungi and *Pseudomonas fluorescens* or mycorrhizal fungi plus micronutrients plus *Pseudomonas* (treatments are shown in Table 3.4). Ten seedlings per treatment were used in the alder experiment; while 12 seedlings per treatment were used in the snowbrush experiment.

Control soils were pasteurized in the laboratory over a 3-day period. Each day, the soil mixture was left in a steam bath for 1/2 hour after soils had reached a temperature range of 60° to 70° C. After cooling overnight, the procedure was repeated until three successive pasteurizations were complete.

At five weeks, all red alder seedlings except controls were inoculated with a 0.25 μ l packed *Frankia* cell per ml sterile distilled water and snowbrush seedlings (except controls) were inoculated with a crushed-nodule suspension. Seedlings receiving *Pseudomonas* were inoculated with 1 ml of bacterial suspension (1×10^6 CFU). Red alder seedlings receiving mycorrhizal fungi were inoculated with 1 ml of an inoculum suspension containing 290000 spores of the ectomycorrhizal fungus, *Alpova diplophloeus* which is host specific to alder (Molina, 1979; Molina, 1981; Molina *et al.*, 1994), and snowbrush plants receiving mycorrhizal fungi were inoculated with 1 gram soil containing the vesicular-arbuscular mycorrhizal fungus, *Glomus intraradix*. A total of 320 seedlings were used in the red alder experiment, and 384 seedlings in the snowbrush experiment.

Beginning at six 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: $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; K_2SO_4 ; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Micronutrients were added to the appropriate

treatments as: NaFe EDTA; H_3BO_3 ; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; and ZnCl_2 .

Data collection

Two variables were measured in the alder experiment: total plant biomass and acetylene reduction per plant. Four variables were measured in the snowbrush experiment: total plant biomass, acetylene reduction per plant, acetylene reduction per gram of nodule, and nodule weight.

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

Prior to harvesting, we measured nitrogenase activity in intact root systems (with nodules still attached), using the acetylene reduction technique as described by Koo (1989), and Rojas *et al.* (1992). Each red alder plant was placed inside of a 525-ml plastic tube (PVC) in such a way that the root systems were sealed from the plant tops using a rubber stopper perforated to let the plant stem go through. Sealed plastic tubes containing the root systems were injected, using a syringe, with prepurified acetylene to 10% of the total gas volume of the tube. After 2 hours 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 (G.C.) fitted with a 2.0 m x 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 per min. (Li and Castellano, 1987; Koo, 1989; Rojas *et al.*, 1992).

Since the morphology of snowbrush is different from that of red alder, a slightly different approach was used with that species. Each snowbrush plant, with soil adhering to roots and shoots attached, was inserted into a 900 ml Mason jar. Each jar containing one whole plant was sealed with a lid into which a serum stopper had been inserted. Forty ml of air was withdrawn from the jar using a syringe, and immediately after 40 ml of prepurified acetylene was injected. Following 2 hours incubation at room temperature, a 0.1 ml gas sample was withdrawn and analyzed for acetylene and ethylene in a G.C..

Data analysis

Statistical analyses were performed using SAS (SAS Institute Inc. Cary, North Carolina) for windows, version 6.10. The distribution of the residuals and the test for normality showed that transformations were not necessary for any of the variables.

Data were analyzed 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 cases such as ours, 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 is:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \lambda_{ij}\delta_j + \varepsilon_{ijk}$$

The following null (H_0) and alternative (H_A) hypotheses were tested by the characteristic root test; $H_0 : \lambda = 0$, and $H_A : \lambda \neq 0$. 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	df	$E(MS)^*$	$E(F)^*$
Total	27		
Slope position	3	$\sigma_s^2 + \sigma_w^2 + \varphi_s$	
Treatment	6	$\sigma_s^2 + \varphi_T$	$\frac{\sigma_s^2 + \varphi_T}{\sigma_s^2}$
Error	18	σ_s^2	

* Expected values when interaction is not present.

σ_s^2 = split-plot error

φ_s = slope effect

σ_w^2 = whole plot error

φ_T = treatment effect

Because we did not measure more than one slope in our study, there is no way to estimate variation between slopes. So we can not estimate the whole plot error term to test the whole plot effect, or the whole plot by split-plot interaction.

Seedlings grown in pasteurized soils (treatment 1, Table 3.4) did not survive and were not included in the analyses.

In the case of significant treatment effects in ANOVA (alder only), Fisher's protected LSD was used to make the treatment comparisons shown below. Each comparison tests the effect of adding a single factor (in one case two factors) to a preexisting set of factors. In the following, the added factor(s) in each comparison is (are) underlined.

No additions (Trt. 2) vs. Frankia plus macronutrients added (Trt. 3).

Frankia plus macronutrients added (Trt. 3) vs. *Frankia*, macronutrients, and micronutrients added (Trt. 4).

Frankia plus macronutrients added (Trt. 3) vs. *Frankia*, macronutrients, and mycorrhizae added (Trt. 5).

Frankia, macronutrients, and mycorrhizae added (Trt. 5) vs. *Frankia*, macronutrients, mycorrhizae and micronutrients added (Trt. 6).

Frankia, macronutrients, and mycorrhizae added (Trt. 5) vs. *Frankia*, macronutrients, mycorrhizae and Pseudomonas added (Trt. 7).

Frankia, macronutrients, mycorrhizae and *Pseudomonas* added (Trt. 7) vs. *Frankia*, macronutrients, mycorrhizae, *Pseudomonas* and micronutrients added (Trt. 8).

At harvest, a striking location effect was easily seen. In the case of snowbrush, seedlings appeared much larger when grown in soil from the bottom slope than when grown in soil from other locations. In the case of alder, seedlings grown in upper slope soil appeared larger. Because there was no significant treatment effect for snowbrush, we tested location effects by treating treatments within a given location as replicates and compared locations using a one-way ANOVA. In the case of alder, where there were significant treatment effects, the one-way ANOVA analysis was first performed by treatment and secondly by location. In the first analysis (by treatment), each treatment was analyzed separately with regard to seedling response to location using trees as replicates; while in the second analysis (by location), all trees were pooled together by location, regardless of their treatments (without separating trees by treatments). We were able to pool trees together regardless of their treatments because the pattern of significant location effects was the same for all treatments. Since slope positions were not replicated, these ANOVAs do not permit inferences about slope position in general. Rather, inferences are restricted to how seedlings or group of seedlings (the replicates) differ in growth among the four specific locations within the clearcut.

RESULTS

Red alder

Treatment effects: Treatments significantly influenced both acetylene reduction and seedling biomass ($p=0.0001$ and $p=0.0202$, respectively; Tables 3.1 and 3.2). According to the Characteristic Root Test, the location at which soils were collected within the clearcut did not influence seedling response to treatments. Table 3.3 shows the p -values for the six treatment comparisons (Table 3.4 shows in detail the eight treatments used in the alder and snowbrush experiments). 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). No other treatments increased biomass beyond that attained by adding *Frankia* and macronutrients (Figure 3.1), however, acetylene reduction was significantly influenced by *Alpova*, micronutrients, and interactions between these two factors. Adding *Alpova* increased acetylene reduction by 33% over that attained with *Frankia* and macronutrients alone (treatment 5 versus 3, $p=0.0178$; Table 3.3, Figure 3.2); the combined effect of *Frankia*, macronutrients and the mycorrhizal fungus was to increase acetylene reduction by 136% over controls. In contrast, adding micronutrients to *Frankia* and macronutrients reduced acetylene reduction by nearly one-half, completely negating the positive effect of the *Frankia* and macronutrients (treatments 4 versus 3, $p=0.0016$). The

Table 3.1. Analysis of variance for red alder acetylene reduction.

Source	d.f.	MS	F	P-value
Total	27			
Slope position	3	340.86		
Treatment	6	221.79	12.12	0.0001
Error	18	18.29		

Table 3.2. Analysis of variance for red alder biomass.

Source	d.f.	MS	F	P-value
Total	27			
Slope position	3	115.71		
Treatment	6	9.41	3.40	0.0202
Error	18	2.77		

Table 3.3. Treatment comparisons for red alder.

Treatment comparisons	Acetylene reduction (p-value)	Plant biomass (p-value)
No additions (Trt.2) vs <i>Frankia</i> + macronutrients added (Trt.3)	0.0038	0.0414
<i>Frankia</i> + macronutrients added (Trt.3) vs <i>Frankia</i> , macronutrients, and micronutrients added (Trt.4)	0.0016	0.4717
<i>Frankia</i> + macronutrients added (Trt.3) vs <i>Frankia</i> , macronutrients, and mycorrhizae added (Trt.5)	0.0178	0.3976
<i>Frankia</i> , macronutrients, and mycorrhizae added (Trt.5) vs <i>Frankia</i> , macronutrients, mycorrhizae, and micronutrients added (Trt 6)	0.0110	0.8086
<i>Frankia</i> , macronutrients, and mycorrhizae added (Trt.5) vs <i>Frankia</i> , macronutrients, mycorrhizae, and <i>Pseudomonas</i> added (Trt.7)	0.4703	0.6309
<i>Frankia</i> , macronutrients, mycorrhizae, and <i>Pseudomonas</i> added (Trt.7) vs <i>Frankia</i> , macronutrients, mycorrhizae, <i>Pseudomonas</i> , and micronutrients added (Trt.8)	0.5151	0.9202

Table 3.4. 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	+ <i>Frankia</i> + Macronutrients
4	+ <i>Frankia</i> + Macronutrients + Micronutrients
5	+ <i>Frankia</i> + Macronutrients + Mycorrhizae
6	+ <i>Frankia</i> + Macronutrients + Micronutrients + Mycorrhizae
7	+ <i>Frankia</i> + Macronutrients + Mycorrhizae + <i>Pseudomonas</i>
8	+ <i>Frankia</i> + Macronutrients + Mycorrhizae + <i>Pseudomonas</i> + Micronutrients

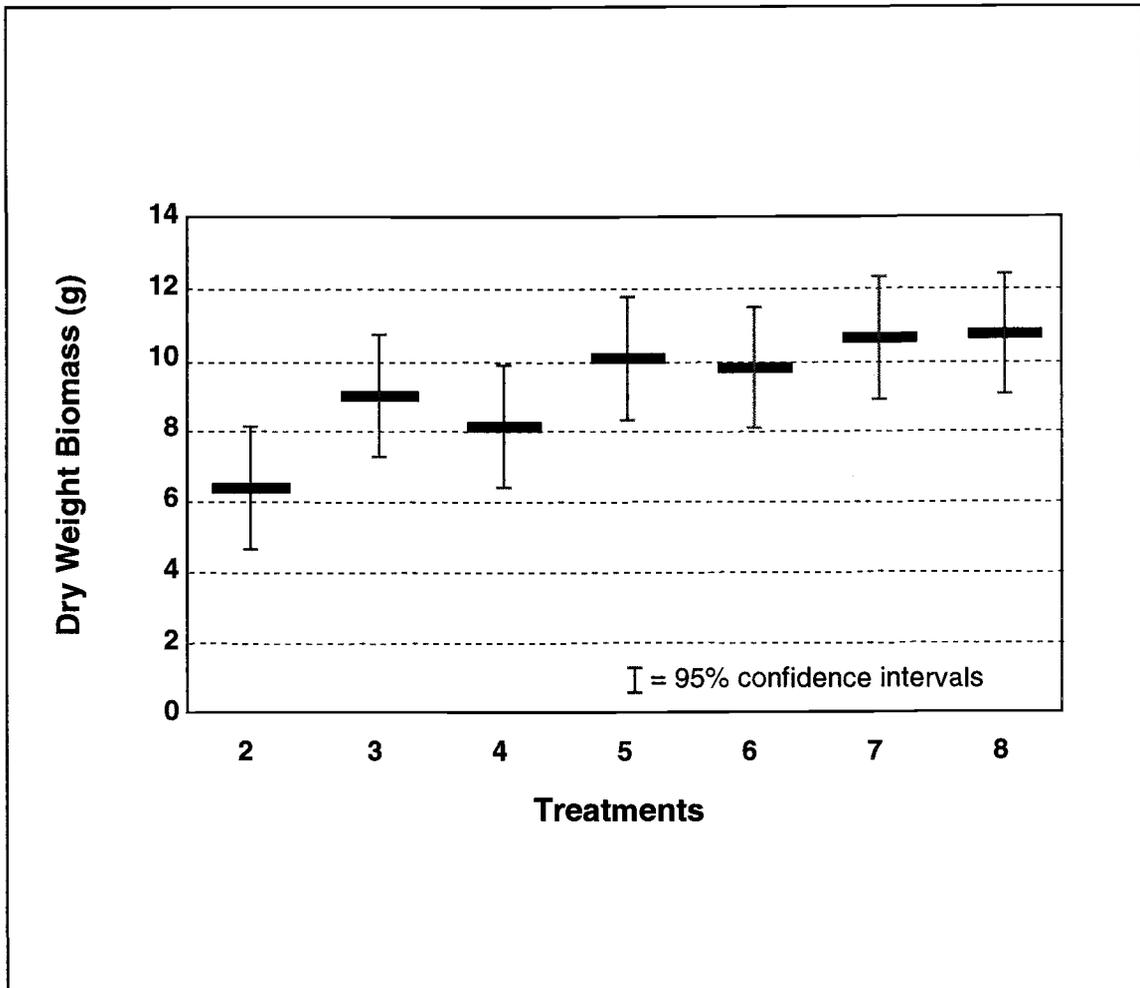


Figure 3.1. Means and 95% confidence limits for the dry weight biomass (g/tree) of red alder seedlings by treatment.

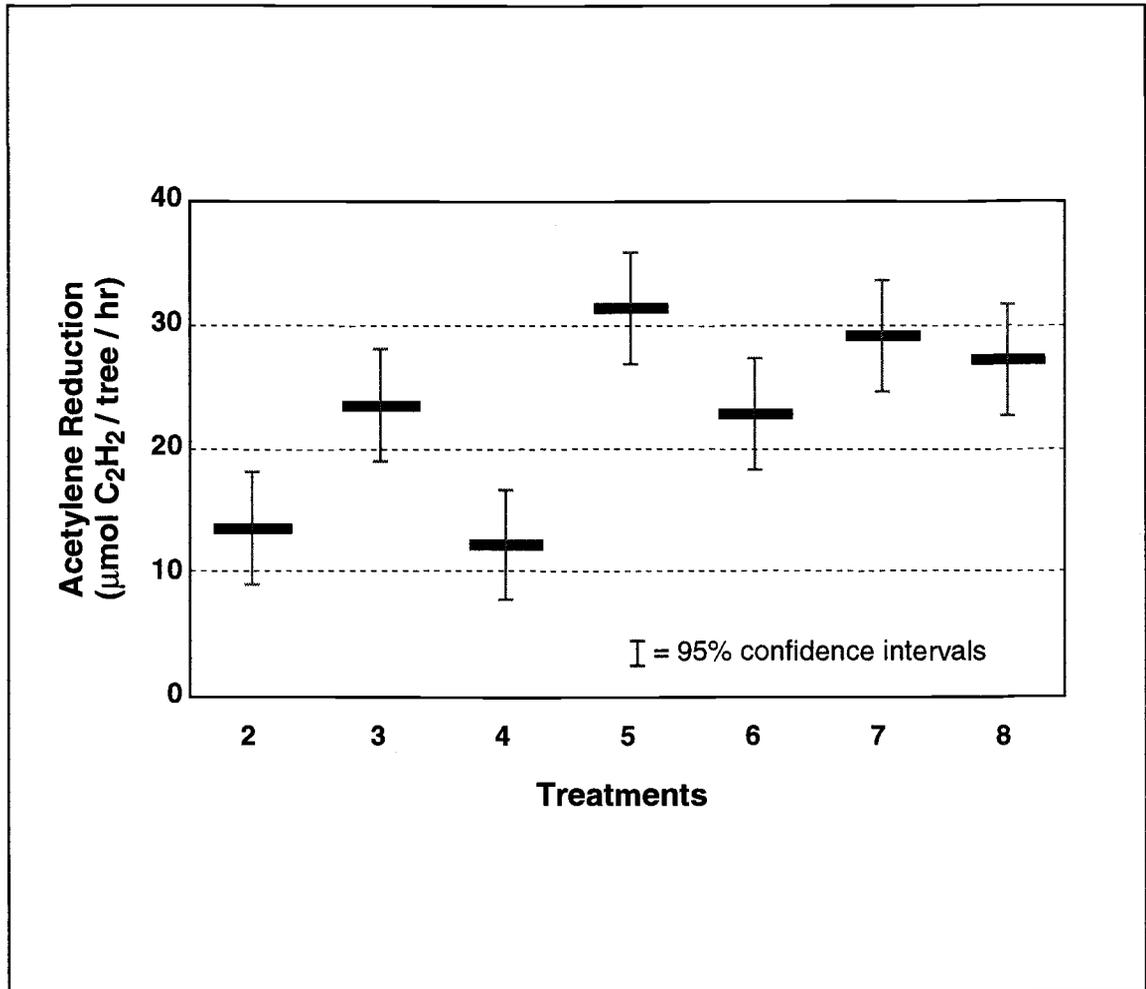


Figure 3.2. Means and 95% confidence limits for the acetylene reduction ($\mu\text{mol C}_2\text{H}_2/\text{tree/hr}$) of red alder seedlings by treatment.

presence of *Alpova* appeared to buffer the negative effects of micronutrients at least somewhat. Acetylene reduction was reduced by adding micronutrients to *Frankia*, macronutrients, and *Alpova* (treatments 6 versus 5, $p=0.011$); however, the level remained well above that attained when micronutrients were added without *Alpova*.

Acetylene reduction correlated positively with seedling biomass ($r=0.59$, $p=0.0001$).

Location effects: When the one-way ANOVA was performed for 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 $p=0.0001$) (Tables 3.5 and 3.6). Seedlings responses for both variables were higher in higher-mid (ms-3) and upper slope (us-5) soil than in lower-mid (ms-2) or bottom slope (bs-1) soil (Figures 3.3 and 3.4).

Table 3.5 . Analysis of variance for red alder biomass.

Source	d.f.	MS	F	P-value
Total	241			
Location	3	999.18	58.83	0.0001
Error	238	16.98		

Table 3.6. Analysis of variance for red alder acetylene reduction.

Source	d.f.	MS	F	P-value
Total	173			
Location	3	2422.89	22.39	0.0001
Error	170	108.23		

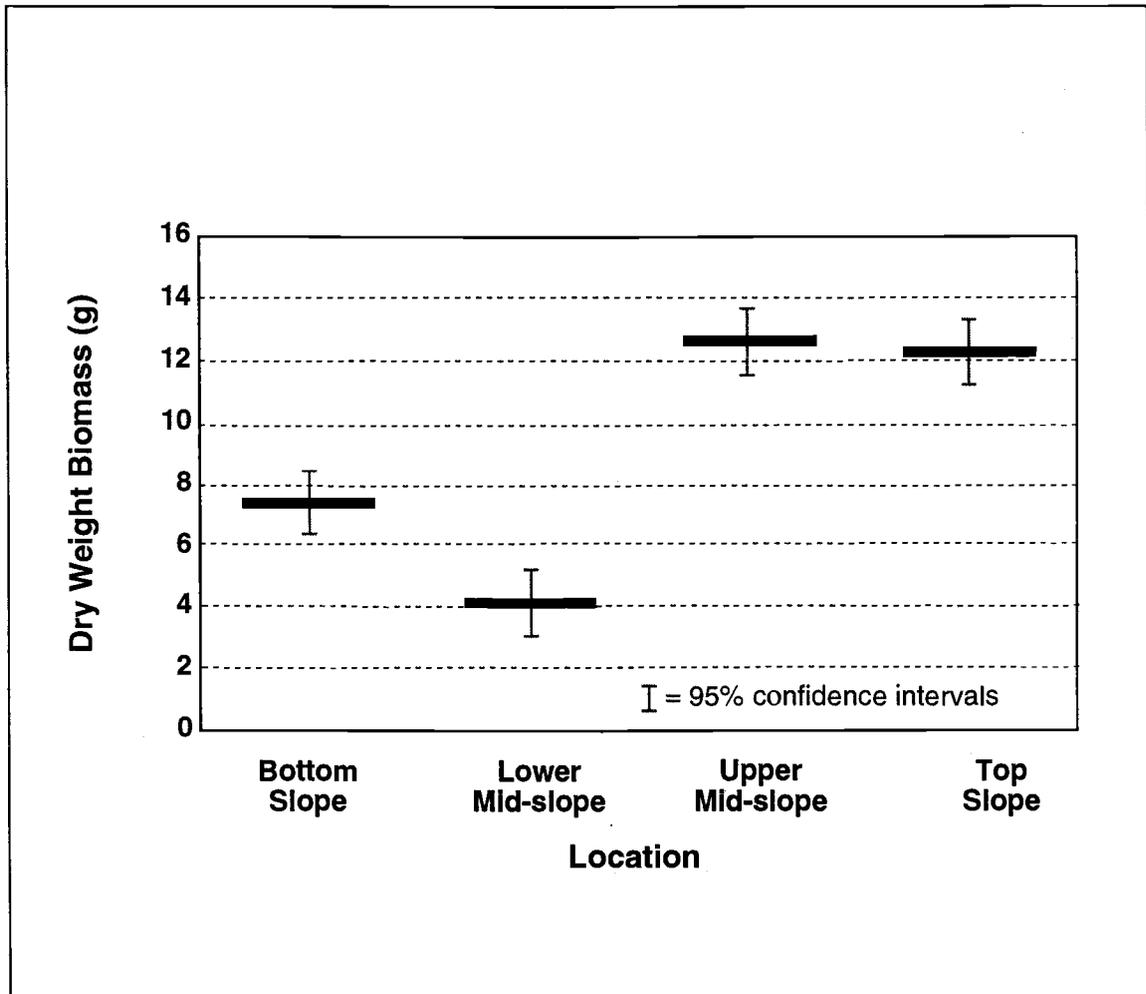


Figure 3.3. Means and 95% confidence limits for the dry weight biomass (g/tree) of red alder seedlings by location.

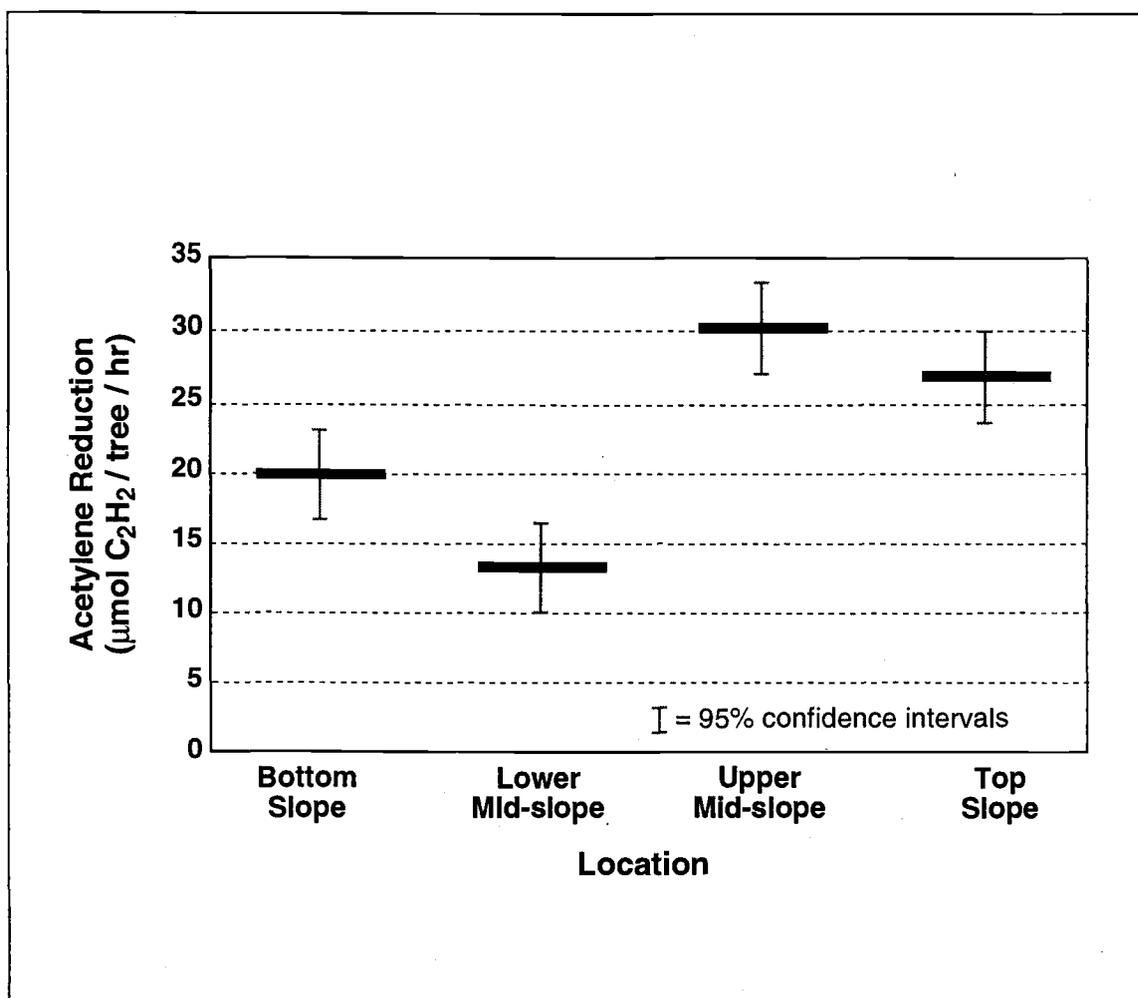


Figure 3.4. Means and 95% confidence limits for the acetylene reduction ($\mu\text{mol C}_2\text{H}_2/\text{tree/hr}$) of red alder seedlings by location.

Snowbrush

Treatment effects: None of the four variables studied for snowbrush showed a significant treatment effect (Tables 3.7, 3.8, 3.9 and 3.10, Figures 3.5, 3.6, 3.7 and 3.8). There was no significant interaction between treatment and location.

All three correlations of snowbrush response variables turned out to be 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 ($r=0.45$, $p=0.0001$).

Location effects: Location strongly affected all four variables: acetylene reduction ($p=0.0006$), plant biomass ($p=0.0001$), acetylene reduction per g of nodule ($p=0.0001$), and nodule weight ($p=0.0001$) (Tables 3.11, 3.12, 3.13 and 3.14). Whereas, in the alder experiment, seedling responses were higher in higher-mid (ms-3) and upper slope (us-5) soil, in the snowbrush experiment, plant biomass, acetylene reduction and nodule weight were highest in bottom slope (bs-1) soil (Figures 3.9, 3.10 and 3.11). Acetylene reduction per g nodule, though, showed an opposite trend being lowest in bottom slope (bs-1) soil (Figure 3.12).

Table 3.7. Analysis of variance for snowbrush biomass.

Source	d.f.	MS	F	P-value
Total	27			
Slope position	3	81.37		
Treatment	6	3.28	0.88	0.5288
Error	18	3.73		

Table 3.8. Analysis of variance for snowbrush nodule weight.

Source	d.f.	MS	F	P-value
Total	26			
Slope position	3	0.138		
Treatment	6	0.006	1.00	0.4552
Error	17	0.006		

Table 3.9. Analysis of variance for snowbrush acetylene reduction.

Source	d.f.	MS	F	P-value
Total	27			
Slope position	3	8.08		
Treatment	6	1.19	0.67	0.6778
Error	18	1.79		

Table 3.10. Analysis of variance for snowbrush acetylene reduction per g nodule.

Source	d.f.	MS	F	P-value
Total	27			
Slope position	3	1412.37		
Treatment	6	388.84	0.58	0.7417
Error	18	670.56		

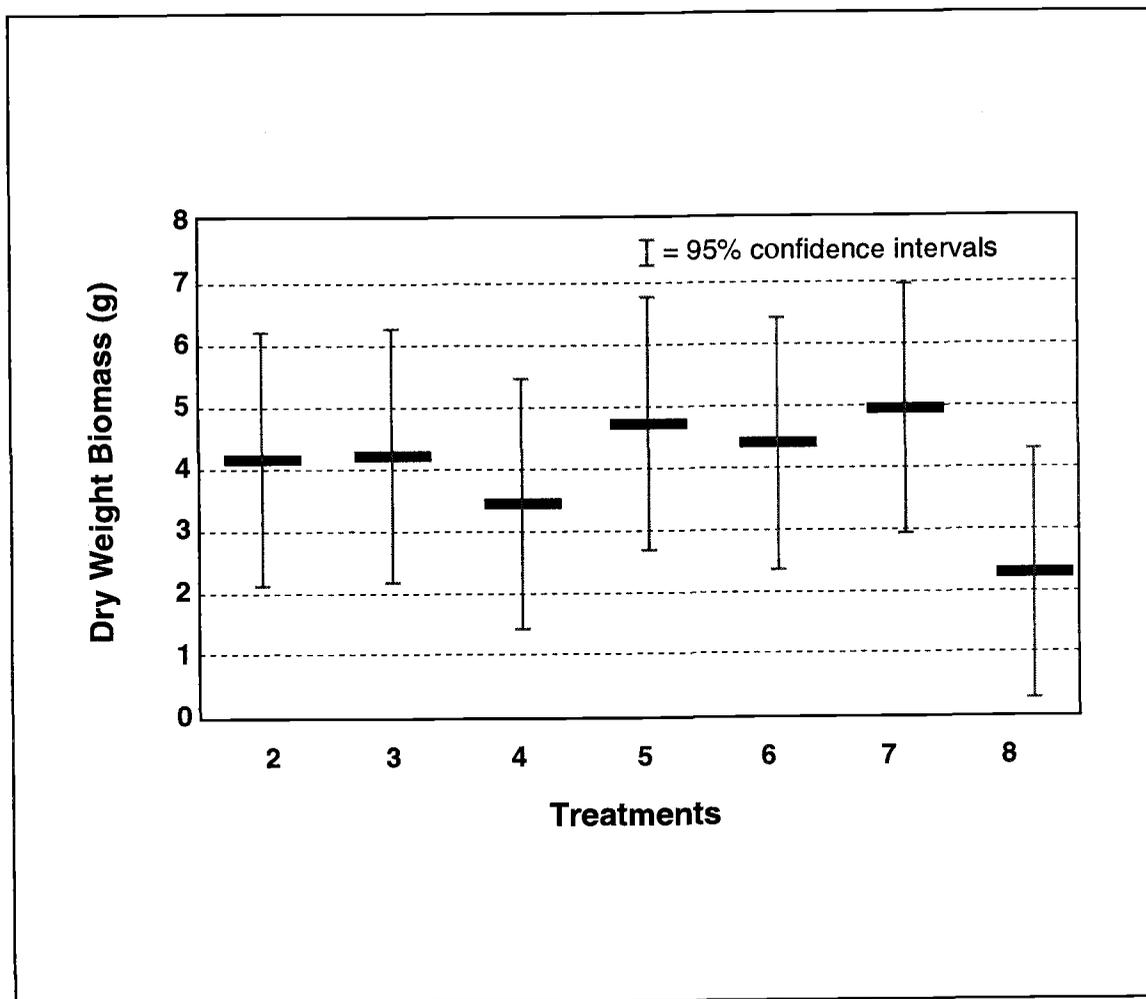


Figure 3.5. Means and 95% confidence limits for the dry weight biomass (g/plant) of snowbrush plants by treatment.

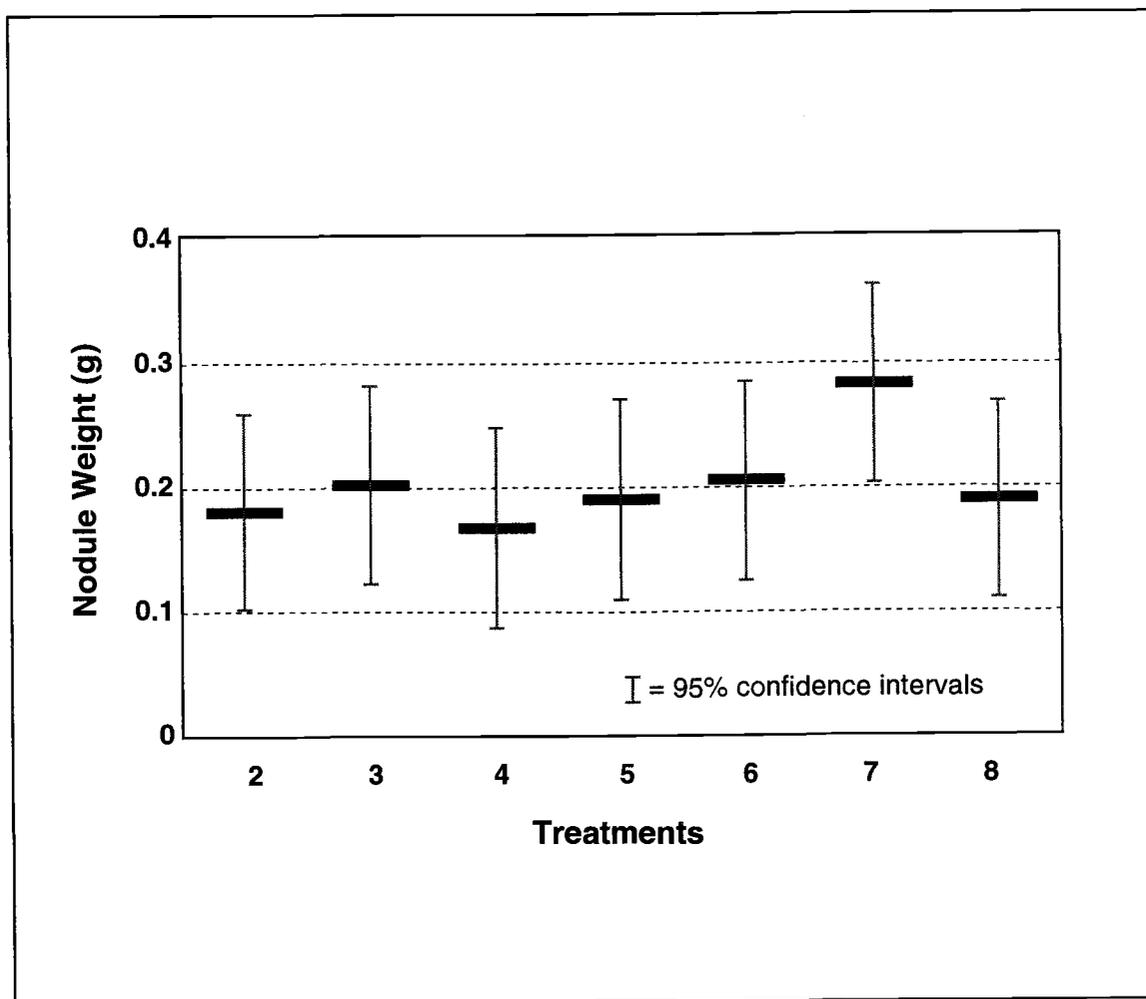


Figure 3.6. Means and 95% confidence limits for the nodule weight (g/plant) of snowbrush plants by treatment.

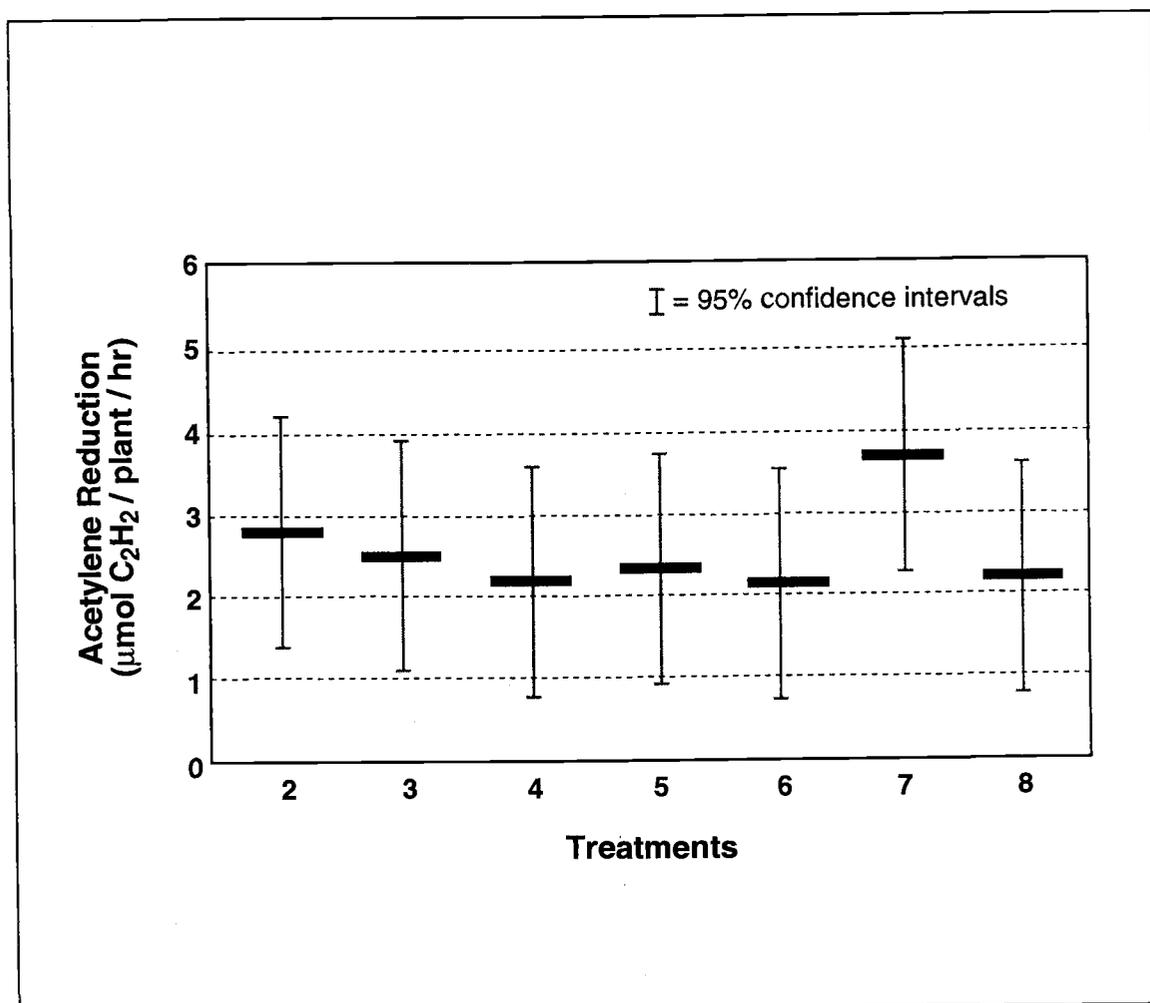


Figure 3.7. Means and 95% confidence limits for the acetylene reduction ($\mu\text{mol C}_2\text{H}_2 / \text{plant/hr}$) of snowbrush plants by treatment.

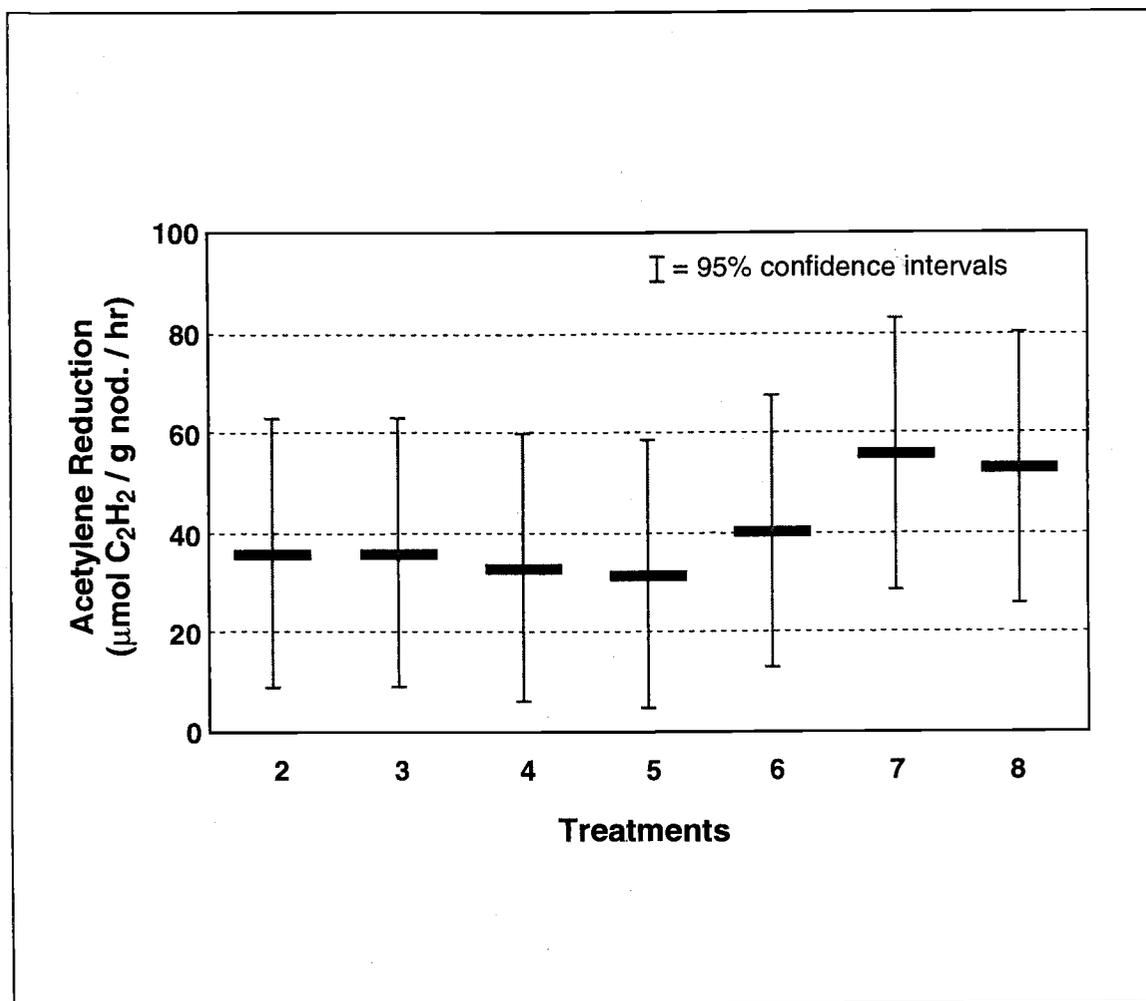


Figure 3.8. Means and 95% confidence limits for the acetylene reduction per g nodule ($\mu\text{mol C}_2\text{H}_2/\text{g nodule}/\text{hr}$) of snowbrush plants by treatment.

Table 3.11. Analysis of variance for snowbrush acetylene reduction.

Source	d.f.	MS	F	P-value
Total	317			
Location	3	88.98	5.94	0.0006
Error	314	14.99		

Table 3.12. Analysis of variance for snowbrush biomass.

Source	d.f.	MS	F	P-value
Total	317			
Location	3	898.75	38.28	0.0001
Error	314	23.48		

Table 3.13. Analysis of variance for snowbrush acetylene reduction per g nodule.

Source	d.f.	MS	F	P-value
Total	144			
Location	3	14515.43	15.86	0.0001
Error	141	915.23		

Table 3.14. Analysis of variance for snowbrush nodule weight.

Source	d.f.	MS	F	P-value
Total	143			
Location	3	0.88	30.59	0.0001
Error	140	0.03		

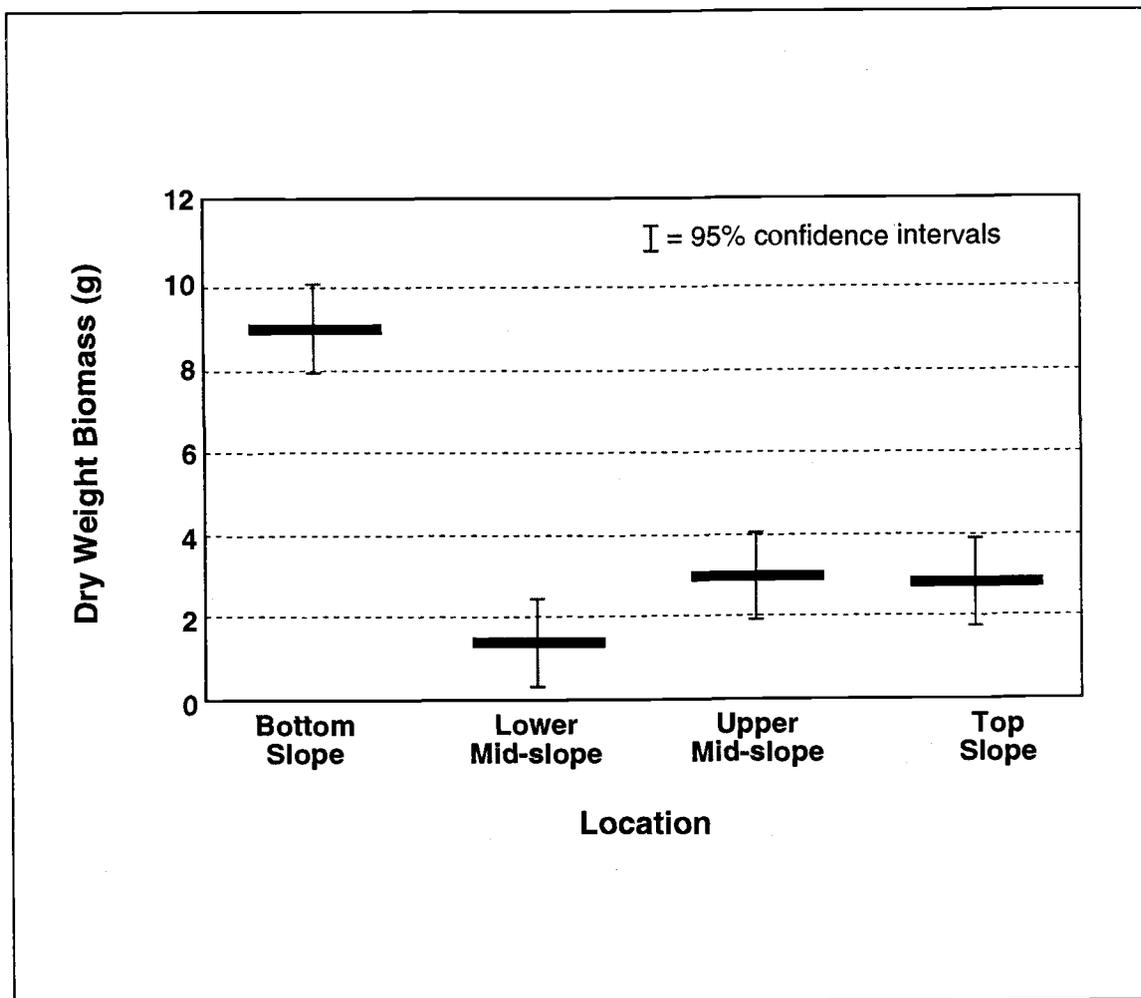


Figure 3.9. Means and 95% confidence limits for the dry weight biomass (g/plant) of snowbrush plants by location.

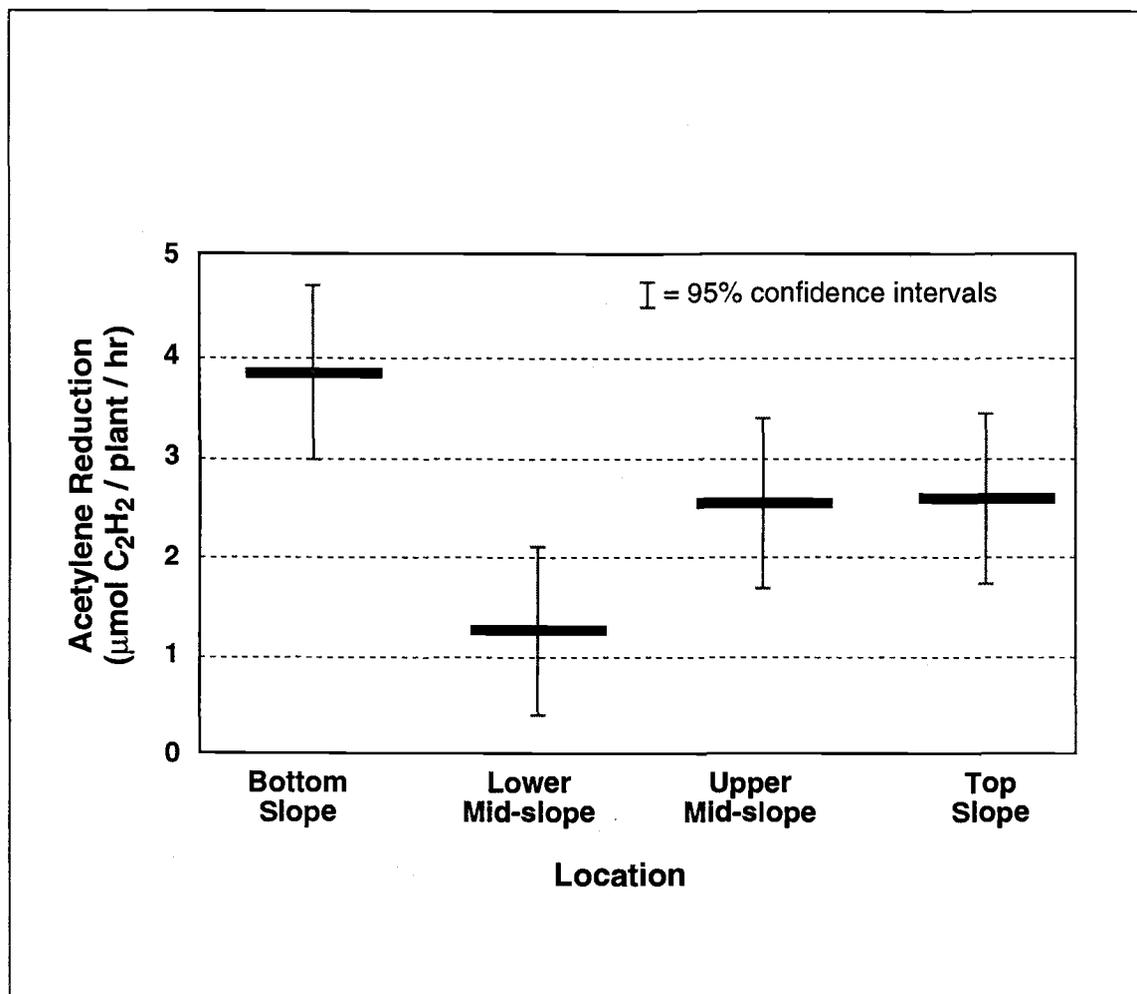


Figure 3.10. Means and 95% confidence limits for the acetylene reduction ($\mu\text{mol C}_2\text{H}_2/\text{plant/hr}$) of snowbrush plants by location.

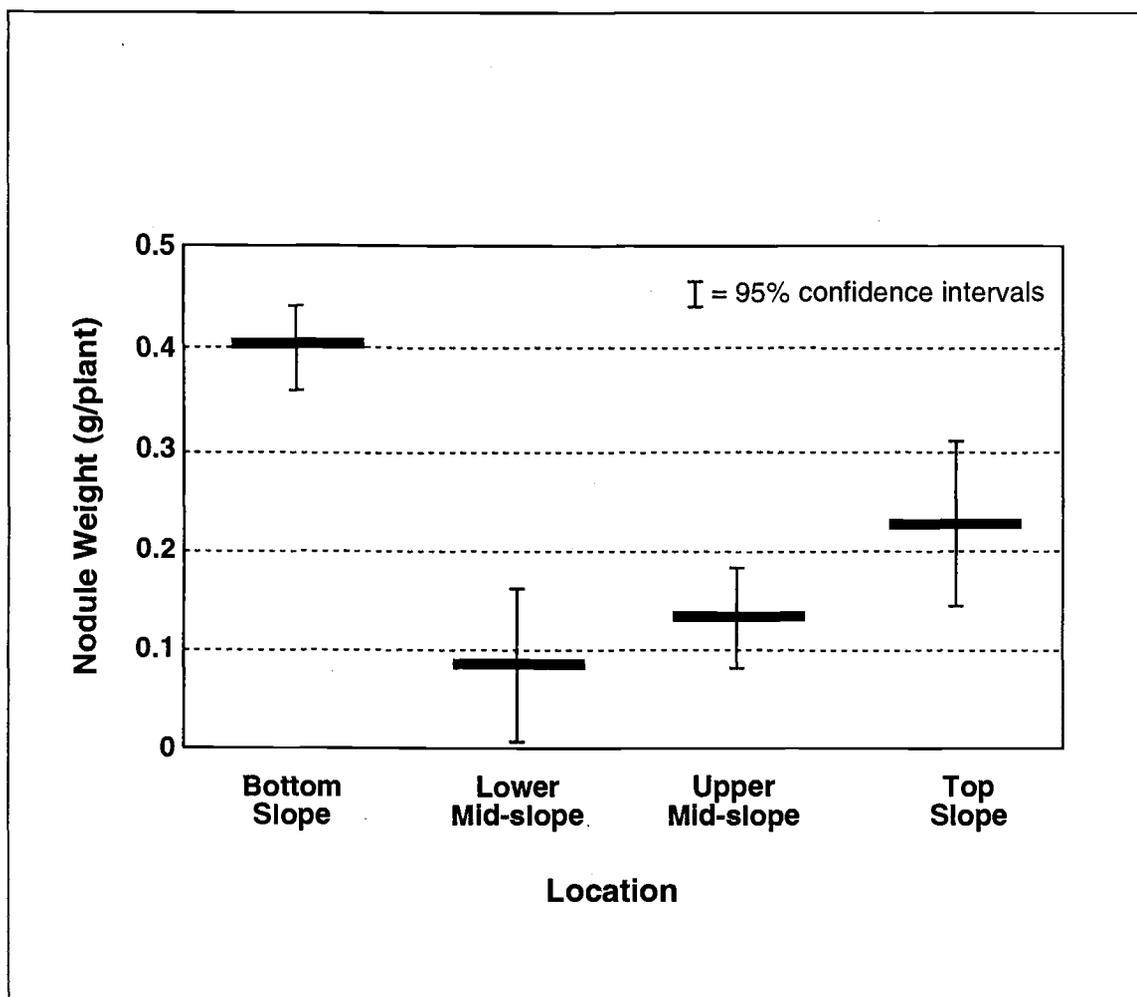


Figure 3.11. Means and 95% confidence limits for the nodule weight (g/plant) of snowbrush plants by location.

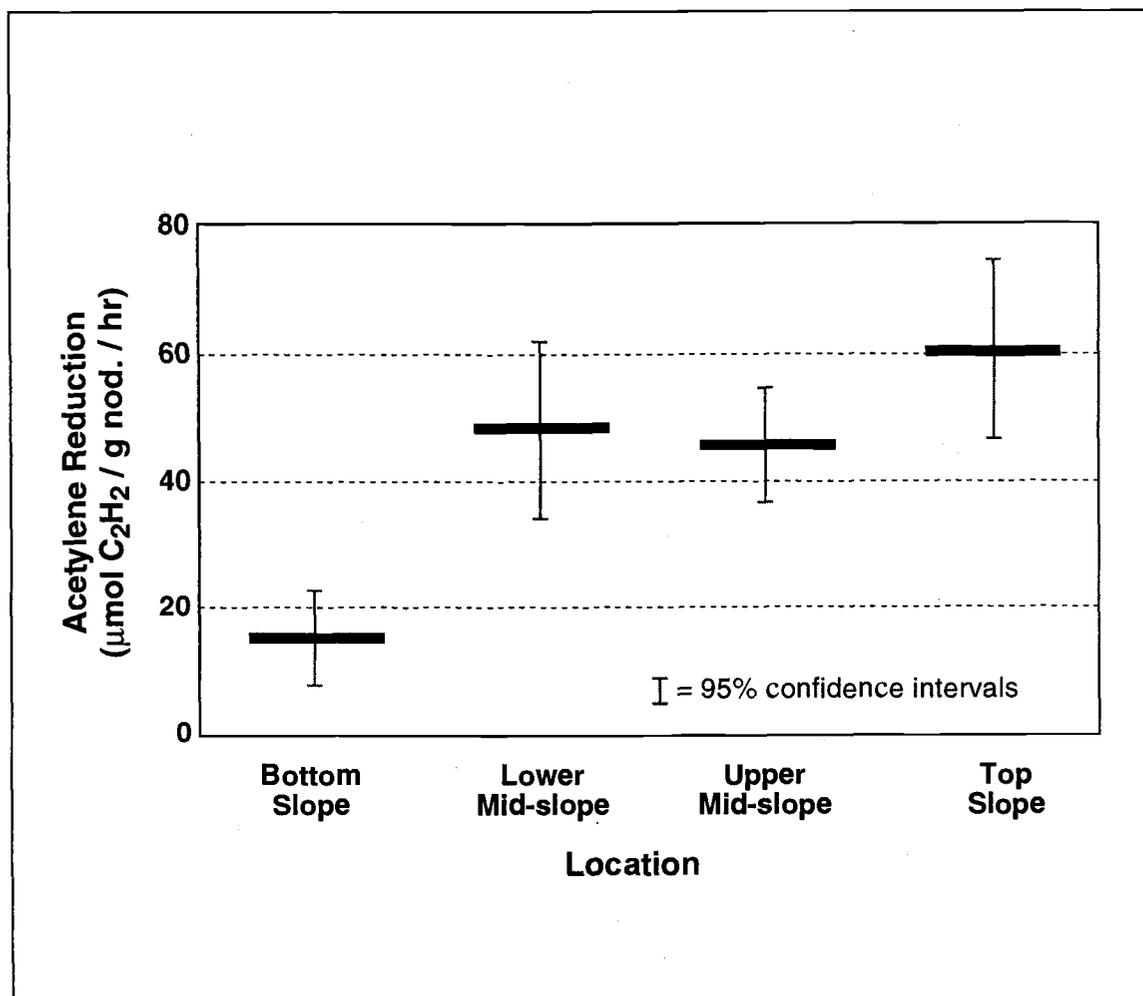


Figure 3.12. Means and 95% confidence limits for the acetylene reduction per g nodule ($\mu\text{mol C}_2\text{H}_2/\text{g nodule/hr}$) of snowbrush plants by location.

DISCUSSION

Treatment Effects

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

Red alder seedlings receiving *Frankia* and macronutrients produced more biomass and reduced more acetylene than the seedlings grown without additions. None of the treatments increased red alder biomass beyond that attained by adding *Frankia* and macronutrients alone. It is possible the small size of the Leach tubes prevented plant biomass from responding to treatments other than additions of *Frankia* and macronutrients. Rojas et al (1997, chapter 2 in this thesis) report that 1 year old greenhouse grown snowbrush plants produced more biomass and nodules, and fixed more nitrogen when grown in 590 ml D-cell tubes than when grown in 150 ml Ray Leach tubes, and work by

Koo (1989) has shown that red alder plants develop water stress when grown in small containers.

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 being N limited and bigger seedlings having more energy to devote to their diazotrophs (Arnone and Gordon, 1990). The link between *Frankia* and seedling biomass is obviously directly through N supplied by the former (unless the diazotroph has some other effect we don't know about), while 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 (treatment 1) did not survive, did not produce nodules and, therefore, did not fix nitrogen. On the other hand, seedlings grown in non-pasteurized soils with no additions (treatment 2) 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 from the site for hundreds of years. The occurrence of *Frankia* in soils without hosts is probably due to the ability of this microorganism to survive either by living 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, a nearby

old-growth forest and a nearby Douglas-fir plantation found nodulation of both snowbrush and red alder to vary widely among sample points within each area (Rojas *et al.*, 1997, chapter 2 in this thesis).

As with seedling biomass, adding *Frankia* and macronutrients to unpasteurized soil significantly increased rates of acetylene reduction. Because these two factors 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) (Wollum and Youngberg, 1969; McNabb and Cromack, 1983; Kummerow *et al.*, 1978; Sharma, 1988; Huss-Danell, 1986; Jha *et al.*, 1993; Waring and Schlesinger, 1985), and perhaps also through effects on nodulation and nitrogenase activity per unit nodule weight (Chatarpaul and Carlisle, 1983; El-Hassanin and Lynd, 1985; Righetti *et al.*, 1986; Lynd and Anzman, 1989; Crannell *et al.*, 1994; Sprent, 1995).

Originally, we had expected mycorrhizae to benefit VAM snowbrush more than EM alder, but our results do not support our expectations, since it was only alder's nitrogenase activity that was influenced by the addition of a mycorrhizal fungus. Work elsewhere has shown that mycorrhizae increase rates of nitrogen fixation both in legumes and actinorhizal associations (Hayman, 1987; Rose and Youngberg, 1981; Gardner *et al.*, 1984). In our study, when *Alpova* was added with *Frankia* and macronutrients (treatment 5), red alder

seedlings reduced 33% more acetylene than the seedlings grown with *Frankia* and macronutrients alone (treatment 3), and 136% more than seedlings grown in non-pasteurized soils (treatment 2). But, giving VAM to snowbrush had no effect; even though, VAM inocula was low at the study site. A random sample obtained from the site in 1990 showed that the average percentage of snowbrush seedling root systems colonized by mycorrhizal fungi was 6%-26%(E. Cazares, personal communication). A range of 6%-26% colonization 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 (E. Cazares, personal communication). In contrast, Rose and Youngberg (1981) studying the effect of dual infection (*Frankia* and VA *Glomus gerdemannii*) on snowbrush seedlings found that one year old nodulated plants had, on average, 80% of their roots colonized by *Glomus*, while plants inoculated with *Glomus* only had 45% of their roots colonized by the fungus. Rose and Youngberg (1981) reported increases of shoot, root, and nodule biomass and 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 phosphorus (P) (Allen, 1991; Janos, 1987; Abuzinadah and Read, 1986a, b). 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). In addition to gathering P, the fungus protects the plant from soil pathogens (Marx, 1972) and, because of its extensive hyphal networks and hyphal surface area, the fungus also helps the plant to gather water (Perry, 1994; Molina *et al.*, 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 the latter factors. With *Alpova* in the system, micronutrients also lowered acetylene reduction (treatment 6), but not to the degree that occurred 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 because 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), though we are not aware of similar work in actinorhizal plants. Accordingly, we hypothesized that micronutrient fertilization would substitute in part at least 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 occurs naturally, and can result in unnatural plant responses (Ingestad, 1982). It is possible 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, since by producing siderophores these soil microorganisms are involved in plant iron nutrition (Torres *et al.*, 1986), and Fe is an essential component of the nitrogenase enzyme (Sprent, 1987; Atlas and Bartha, 1987; Sprent and Sprent, 1990). Soils of the study site may have abundant *Pseudomonas*. Also, mycorrhizal fungi produce siderophores (Szaniszlo *et al.*, 1981; Powell *et al.*, 1982; Perry *et al.*, 1984; Reid *et al.*, 1984; Watteau and Berthelin, 1990), and may have provided seedlings with sufficient Fe.

Location Effects

Snowbrush plants reduced more acetylene, produced more nodules and produced more biomass when grown in bottom slope (bs-1) soil than when grown in soil from any other location; while plant biomass, acetylene reduction and nodule weight averaged lowest in lower mid-slope soil (location ms-2). The low rate of acetylene reduction per g nodule for snowbrush growing in bottom slope soil reflects the commonly observed inverse relation between nodule

weight and nitrogenase activity per unit weight (Wheeler *et al.*, 1981; Sempavalan *et al.*, 1995). Red alder, on the other hand, behaved quite differently. Red alder seedlings reduced more acetylene and produced more biomass when grown in upper mid-slope (ms-3), and top of slope (us-5) soil than when grown in bottom slope (bs-1), or lower mid-slope (ms-2) soil.

Snowbrush responses in the greenhouse were very similar to what we had observed in the field. Although normally regenerating abundantly following clearcutting and slash burning on sites such as this (Swanson *et al.*, 1987), snowbrush cover on the study site remained quite 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 conclude for sure what is causing snowbrush to nodulate and grow better at slope bottoms and do poorly at mid- and upper slopes, but we do know, since the same pattern occurred in the greenhouse, the cause must be a soil factor. It is possible that the lack of growth and nodulation of snowbrush at mid- and upper slopes is related to impacts on soils associated with the clearcutting and slash burning that took place at the "snowbrush" site. Earlier reports have indicated that the site was subjected to a very intense broadcast burning that took place immediately after the logging operation had concluded in 1981 (Franklin *et al.*, 1985).

Rojas *et al.* (1997, chapter 2 in this thesis) did a soil bioassay of an age sequence of stands using snowbrush and red alder. For the bioassay, soils were collected at the H. J. Andrews Experimental Forest, from a 20-year-old Douglas-fir plantation with an understory of snowbrush, an old-growth stand dominated by Douglas-fir, and from a recent clearcut planted with Douglas-fir (the site of the study reported here). Rojas *et al.* (1997) found that nodule weight and plant biomass for snowbrush 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 for snowbrush. In contrast, nodule weight and plant biomass for red alder were highest in clearcut soils (Rojas *et al.*, 1997). Snowbrush and red alder responded differently to different conditions; what produced best growth and nodulation in one species did not produce best growth and nodulation in the other. This was true both within a site and between sites.

Early successional nitrogen fixing plants, such as snowbrush and red alder, play an important ecological role in the long term productivity of a site. These early successional plants are known to possess some "biological imprints" (Perry *et al.*, 1989) that will be shared with their associated conifer seedlings for their mutual benefit. At the "snowbrush" site, the mutually reinforcing positive feedback was probably first reestablished at the bottom of the slope where growing conditions for plants were more favorable, at least moisture was less limiting there than at mid- and upper slopes, explaining why snowbrush did

better at slope bottoms. Because of the ecological importance of snowbrush on sites such as this, delays in its establishment at mid- and upper slopes may have profound impacts on the long-term nitrogen and carbon 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 is less abundant. This suggests different *Frankia* strains have different adaptations, which in turn could reflect a survival mechanism of the nitrogen-fixing endophyte (Molina *et al.*, 1994).

We observed (in both studies) poor correlation between red alder and snowbrush in nodulation, i.e. points in which alder nodulated relatively well snowbrush did not and vice versa. What are possible explanations?. One is the one that we already have given, there are different *Frankia* strains with different preferences for plant species. If true, the different strains are distributed heterogeneously across the landscape, perhaps because of differing adaptations, though it could also be due to purely random factors. For whatever reason, the heterogeneous distribution of endophytes could, in theory at least, produce a corresponding heterogeneous distribution of host plants (though no alder occurred in the clearcut). Another possible explanation is that the endophytes are the same, but the plant species differ in some triggering factor

necessary for nodulation, and that triggering factor (if it exists) was distributed heterogeneously.

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Chapter 4. General Conclusions

First Study (Chapter 2). The following conclusions were obtained from the first study:

(1) When we account for differences in the number of plants nodulating, the sites and species are very different: Red alder plants nodulated well in soils from all three stands. On the other hand, snowbrush plants nodulated poorly in soils from all three stands.

(2) The nodulation percentage for snowbrush differed among slope positions in the clearcut soils.

(3) Our hypothesis that nodule biomass and acetylene reduction for both snowbrush and red alder would be greatest in soils from the adjacent 20-year-old Douglas-fir plantation with an understory of snowbrush, intermediate in soils from the "snowbrush" study site, and least in soils from the adjacent old-growth forest was partially supported in the case of snowbrush, but not in the case of red alder.

(4) As expected, snowbrush plants produced more nodules and biomass when grown in young stand soils than when grown in soils from the other stands.

(5) Contrary to our expectations, red alder plants produced more nodules and biomass when grown in clearcut soils than when grown in soils of any other location.

(6) Except for acetylene reduction per plant, which did not vary significantly between the three age-class Douglas-fir forests studied here, the two actinorhizal plant species responded quite differently in our studies: Snowbrush plants did better when grown in young stand soils, while red alder did better when grown in clearcut soils.

(7) The pattern of plant responses from our greenhouse experiments suggests poor growth of snowbrush was related to soil factors.

(8) We have good reasons to believe that some of the *Frankia* strains that nodulated snowbrush also nodulated red alder since red alder nodulated well in young stand soils (which supported a healthy stand of snowbrush but no alder).

(9) Red alder and snowbrush are not responding to the different sites in the same way; the same seems to be true within the clearcut: The biomass of snowbrush plants grown in clearcut soils averaged highest in bottom slope soils reinforcing the pattern observed in the field and also what we had observed in the experiments reported in chapter 3.

Second Study (Chapter 3). The following conclusions were obtained from the second study:

A. Treatment Effects. (1) Our hypothesis that mycorrhizal fungi and *Pseudomonas fluorescens* would increase nodulation and N-fixation of both red alder and snowbrush plants was partially supported in the case of red alder, but not in the case of snowbrush.

(2) Alder nitrogenase activity, as measured by acetylene reduction, was significantly influenced by: *Frankia* and macronutrients, the EM fungus *Alpova*, micronutrients and by interaction between micronutrients and *Alpova*.

(3) Red alder seedlings given *Frankia* and macronutrients had greater biomass than seedlings grown in unsterile soil without additions.

(4) *Pseudomonas* inoculation, on the other hand, did not affect any of the measured variables.

(5) Acetylene reduction in red alder correlated positively with seedling biomass.

(6) 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 from the site for hundreds of years.

B. Location Effects. (1) Since slope positions were not replicated, ANOVAs do not permit inferences about slope position in general. Rather, inferences are restricted to how seedlings or group of seedlings differ in growth among the four specific locations within the single clearcut.

(2) Snowbrush plants reduced more acetylene, produced more nodules and produced more biomass when grown in the bottom slope soil than when grown in the soil from any other location.

(3) Red alder, on the other hand, behaved quite differently. Red alder seedlings reduced more acetylene and produced more biomass when grown in

the upper mid-slope and top of slope soil than when grown in the bottom slope, or lower mid-slope soil.

(4) Snowbrush responses in the greenhouse were very similar to what we had observed in the field.

(5) We cannot conclude for sure what is causing snowbrush to nodulate and grow better at the slope bottom and do poorly at mid- and upper slope, but we do know, since the same pattern occurred in the greenhouse, the cause must be a soil factor. It is possible that the lack of growth and nodulation of snowbrush at mid- and upper slopes is related to impacts on soils associated with the clearcutting and slash burning that took place at the "snowbrush" site.

(6) The presence of healthy snowbrush plants established at the bottom of the slope on the "snowbrush" site probably helped rebuild *Frankia* populations there.

(7) Snowbrush and red alder responded differently to different conditions; what produced best growth and nodulation in one species did not produce best growth and nodulation in the other. This was true both within a site and between sites.

(8) 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 is less abundant.

(9) We observed (in both studies) poor correlation between red alder and snowbrush in nodulation, i.e. points in which alder nodulated relatively well snowbrush did not and vice versa.

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