Growing interest in red alder (Alnus rubra Bong.) for land-reclamation and commercial use has led to investigations of nursery production methods. Pure culture Frankia inoculum has been used to increase red alder seedling nutrition, resulting in improved cost efficiency and production quality.

We investigated the effects of altered pH from applied calcium amendments on inoculum effectiveness at the Mima, WA nursery site. Calcium oxide was used to increase pH from 5.6 to 7, and CaCl$_2$ was used as a calcium control. Seedling numbers, height, root and shoot weights, and percentage of nodulated plants were determined at mid-season and at lifting. A companion study on the effects of pH adjustment with HCl was done at an Oregon nursery.

A significant ($P < 0.01$) interaction between inoculum rate and soil treatment on number of plants nodulated was found at the Mima site. The CaCl$_2$ soil amendment temporarily lowered pH to 4.5 when the plots were inoculated.
and seeded. This treatment had the greatest percentage of seedlings nodulated at mid-season and at lifting, indicating a possible interaction between calcium and low soil pH, within the pH range studied. To our knowledge, this is the first report of increased nodulation for red alder at pH 4.5, relative to that seen at 5.6 or 7.2. At lifting, the CaCl$_2$ treatment had approximately twice as many nodules per plant as the CaO or control treatments. The number of packable seedlings was greatest at the highest inoculum rate and the CaCl$_2$ treatment had on average 20 more packable seedlings per plot (P < 0.05) than control or CaO treatments.

Effects of amendments on Mima nursery soil and length of time inoculum remains effective at nodulation were examined in a subsequent greenhouse pot experiment. Soil CaCl$_2$ treatment effects obtained in the nursery production field trial could not be reproduced in the greenhouse because of poor aeration and drainage in these pots. Individual additions of HCl and CaO increased both the percentage of plants nodulated and root dry weights ($\alpha = 0.10$). The nodulation effectiveness of the applied Frankia inoculum decreased within 14 days and neither HCl nor CaO treatment could significantly prolong the nodulation capacity.

Our results from field and greenhouse studies indicated that the inhibitory effects of low soil pH on nodulation may be improved by addition of calcium amendments without increasing pH. Low soil pH has often been correlated to low base saturation and generally, lower calcium availability. Our results suggest that the effects of calcium amendments may be independent of pH. We propose that low soil pH in conjunction with moderate to high calcium at the time of nodule
initiation may provide good conditions for nodulation. We found that separate additions of both HCl and CaO increased nodulation in fertile nursery soils. This interpretation is consistent with the interaction found when CaCl$_2$ was applied in the nursery field trial.
Soil pH and Calcium Effects on Nodulation of Nursery

Grown Red Alder

by

Wanda K. Crannell

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SOIL pH AND CALCIUM EFFECTS ON NODULATION OF NURSERY GROWN RED ALDER

Chapter I

INTRODUCTION

Red alder (Alnus rubra Bong.), previously controlled as a weed species in the Pacific Northwest (PNW), is now managed for its ability to increase soil fertility, harvested for fiber and furniture, and planted for land-reclamation purposes and its relative resistance to the root rot fungus Phellinus weirii. With the increased demand for this fast growing tree species, many nurseries have begun large-scale production trials of red alder seedlings for use in commercial out-plantings. Current nursery practices often use Frankia, a N₂-fixing actinomycete, to enhance nodulation of nursery stock. There are 24 genera of plants, known as actinorhizal plants, which can be nodulated by Frankia strains. Early nodulation and nitrogen fixed in root nodules through the symbiotic relationship between Frankia and red alder increases seedling health, and decreases the time before seedlings are large enough for commercial use. Crushed nodule suspensions or pure cultures of Frankia applied with a peat carrier are used to inoculate nursery soil before seeding red alder. Both inoculum preparations are costly and time-consuming to produce. Endeavors which reduce the amount of inoculum required for reliable nodulation or enhance the inoculum’s
ability to nodulate seedlings have the potential to reduce costs associated with nursery production of red alder seedlings.

Many nodulating, actinorhizal plants increase base saturation of soil after colonization of low nutrient sites. In particular, exchangeable calcium concentrations tend to increase after establishment by Frankia-nodulated species (Scott, 1973; Sharma et al., 1985). The effect of increased bases in the soil surface layers on subsequent nodulation is often beneficial (Scott, 1973). Similar advantageous effects of calcium on nodule initiation by Rhizobium on legumes have long been known (Loneragan and Dowling, 1958; Lowther and Loneragan, 1968; Munns, 1970). Studies from the late 1950's demonstrate an interaction between acidity and calcium on nodulation and indicate that the calcium requirement for nodulated plants is greater than for the host or Rhizobium alone (Loneragan and Dowling, 1958). Later it was shown that at lower pH, a higher calcium concentration is needed for nodulation, and CaCl₂ or CaSO₄ are equally effective at increasing nodulation between pH 4.8 and 5.6 (Munns, 1970).

Nutrient requirements and pH optima for actinorhizal plants are not well established and much of the research is based on plants grown in solution culture where nutrients are easily acquired. Results obtained in solution cultures may not relate well to plants grown in soil. Solution-grown, non-nodulated Myrica gale L. plants supplied with adequate nitrate grow best at pH 3.3, whereas nodulated plants grow well at pH 5.4 (Bond, 1951). Ferguson and Bond (1953) found that nodulation of Alnus glutinosa is favorable over the pH range of 5.4 to 7.0, but
that non-nodulated plant growth is favored at lower pH (5.2-5.4). It is commonly accepted that actinorhizal host plant growth is more acid-tolerant than that of the nodulating endophyte (Akkermans and van Dijk, 1981). In general, nodulation of alder by Frankia in solution culture is believed to increase with increasing pH, and is best close to neutrality (e.g. Quispel, 1958).

Using greenhouse and growth chamber experiments, Scott (1973) observed that both CaCO$_3$ and CaSO$_4$ significantly increased both nodule numbers and nodule mass on Ceanothus velutinus (snowbrush) roots. The increase caused by CaSO$_4$ was greater than that from CaCO$_3$ but differences obtained could not be attributed to meeting a sulfur deficiency in that treatment. Scott’s results may have been caused by a direct calcium effect or a combined calcium and pH effect. Gypsum (CaSO$_4$) is used to supply calcium to soil and often temporarily, lowers soil pH, whereas CaCO$_3$ increases soil pH, and is much less soluble. The contrasting effect on soil pH and availability of many nutrients between CaCO$_3$ and CaSO$_4$ treatments may explain the observed increase in growth and nodulation. At pH 7, the availability of many micronutrients in soil is low. However, the calcium requirement for some nodulated actinorhizal plants may be high.

Good growth of nodulated Casuarina equisetifolia requires relatively high amounts of calcium (Yadav, 1983). Pot experiments of calcium-deficient, Frankia-inoculated, red alder show decreased foliar nitrogen and calcium in plant tissue (Hughes and Gessel, 1967). And, Scott (1973) found that an increase in
total nitrogen and calcium paralleled the increase in nodulation. Research regarding soil pH and calcium requirements for nodulation of actinorhizal species suggests that the interaction of calcium and pH may impact nodulation of some Frankia-nodulating plant species. This effect is well documented for Rhizobium (Loneragan and Dowling, 1958; Lowther and Loneragan, 1968; Munns, 1970).

The research presented in this thesis is intended to determine whether the addition of pH-altering calcium amendments to fumigated nursery soil can enhance the nodulation effectiveness of applied Frankia inoculum, using current nursery practices for the production of bare-root, red alder seedlings. Results from field production trials at two Weyerhaeuser nursery sites are presented. A subsequent greenhouse study using soil collected from one of the nursery sites was used to evaluate the length of time the inoculum remains effective at nodulating red alder and whether changes in soil pH or addition of calcium amendments can influence this parameter.
Chapter II

CALCIUM AND pH INTERACTION ON ROOT NODULATION OF NURSERY GROWN RED ALDER (Alnus rubra Bong.) SEEDLINGS BY FRANKIA

Summary

Growing interest in red alder (Alnus rubra Bong.) for land-reclamation and commercial use has led to investigations of nursery production methods. Pure culture Frankia inoculum has been used to enhance red alder seedling growth, resulting in better cost efficiency and production quality. The effects of altered pH from applied calcium amendments on inoculum effectiveness at a Washington state nursery site were examined. Calcium oxide was used to increase pH from 5.6 to 7, and CaCl₂ was used as a calcium control. A randomized complete block design was used in a factorial experiment, with nine treatments: control, CaCl₂, and CaO (equimolar calcium concentrations for calcium treatments); each soil treatment at 0, 20, and 200 µL packed cell volume m⁻³ Frankia inoculum rates. Soil samples were collected throughout the year for pH measurements. Seedling numbers, height, root and shoot weights, and percentage of nodulated plants were determined at mid-season and at lifting. A companion study on the effects of pH adjustment with HCl was done at an Oregon nursery. Greater seedling nodulation at mid-season paralleled increased height differences at lifting. A significant (P <
0.01) interaction between inoculum rate and soil treatment on the number of plants nodulated was found at the Washington site. The CaCl$_2$ soil amendment temporarily lowered pH to 4.5 when the plots were inoculated and seeded. This treatment also had the greatest percentage of seedlings nodulated at mid-season and at lifting, indicating a possible interaction between calcium and low soil pH, within the pH range studied. To our knowledge, this is the first report of increased nodulation for red alder at pH 4.5, relative to that seen at pH 5.6 or 7.2. We found that the numbers of packable seedlings were greatest at the highest inoculum rate and that the CaCl$_2$ treatment had on average 20 more packable seedlings per plot ($P < 0.05$) and nearly twice as many nodules per plant as control or CaO treatments.
Introduction

Increased demand for commercial sources of red alder has prompted extended nursery production. The actinomycete Frankia, a N$_2$-fixing symbiont of red alder (Alnus rubra Bong.), is always found in root nodules of natural stands of red alder (Bond, 1976). Nitrogen fixed through this symbiosis assists red alder growth and enhances soil fertility. Nursery production can be enhanced by use of Frankia to inoculate growing red alder (Hilger et al., 1991; Martin et al., 1991; Wheeler et al., 1991).

Nursery soils are routinely fumigated to control pathogens. Fumigation reduces indigenous microbial populations, and consequently, a source of Frankia inoculum is often required. Inoculum, applied to nursery beds either as a crushed nodule suspension or as a pure culture, has been used to increase red alder seedling packable numbers, size, and vigor (Hilger et al., 1991; Martin et al., 1991). Nodulated seedlings have been shown to better survive out-planting from nursery to the field than non-nodulated stock and this growth benefit can last for several years (Prat, 1992). Collecting and preparing nodules for crushed nodule suspensions is laborious and there is a possibility of introducing pathogens or inhibitory compounds. Pure culture Frankia production is tedious and time-consuming, however, and even intensive efforts result in low yields. Consequently, methods that increase inoculum efficiency in nodulating bare-root
red alder seedlings are important for reducing costs associated with inoculum preparation and use.

Conflicting reports exist for the pH and nutrient conditions required for optimum nodulation of alder by *Frankia*. Best growth of *Frankia* in pure culture occurs between pH 6 and 8 (Burggraaf and Shipton, 1982; Shipton and Burggraaf, 1983). Several studies report different pH ranges for optimum nodulation of alder under different growth conditions. In liquid culture between pH 3.5 to 6, there was no difference in the number of nodules formed; at pH greater than 6, more nodules were formed; and at pH above 7, nodules were seen earlier (Quispel, 1958). Seedlings grown in liquid culture and inoculated with *Frankia* increased in nodulation between pH 3.5 and 5.8, with the greatest number and fastest formation at 5.8 (Smolander et al., 1988). The greatest nodulation of seedlings grown in solution culture was at pH 7.0 with pure culture *Frankia* inoculum, however, nodulation increased when a "helper" bacteria (*Pseudomonas cepacia*) was added to the inoculum and the pH optimum shifted to between 5.5 and 6.5 (Knowlton and Dawson, 1983). Generally, in solution culture between pH 3.5 and 7, a positive correlation of pH with nodulation exists, except that when a "helper" bacteria is added, the optimum pH is reduced below 7. The lowered optimum pH in non-sterile condition may be more reflective of soil situations.

There was a positive correlation between soil pH (range of 3.4 to 4.4) and the number of nodulation units formed in alder grown in liquid culture and inoculated with soil dilutions (Smolander and Sundman, 1987). In a nutrient
application study with peat limed to pH 4.7 and 5.4, little pH-related difference was seen in nodulation, growth rate, or nitrogen content of plants with or without added nitrogen and with repeated nutrient doses (Granhall et al., 1983). However, with a single nutrient dose and no added nitrogen, the seedling growth rate was greater at pH 5.4 than 4.7. In a greenhouse experiment, Griffith and McCormick (1984) found that nodulation increased when low pH (3.6-4.7) mine soils were relimed to pH 7, five weeks after inoculation and just before seeding. When inoculated and seeded concurrently, greatest nodulation occurred between pH 5.5 and 7.2 for these soils. However, their study also showed that there was no difference in nodulation between pH 4.5 and 7.3 for a forest soil. Survival of Frankia, measured by nodulation capacity, was increased in fine sand, limed to pH 6.8 compared to 4.2 (Smolander et al., 1988). The relationship between nodulation capacity and soil pH between 4.9 and 7.2 was negative for black alder and was optimum at pH 4.9 (Zitzer and Dawson, 1992). However, Smolander (1990) showed a positive correlation between nodulation capacity and soil pH from 3.4-5.7. Liming of soils with initial pH below approximately 4.5 can increase nodulation, however, soils with initial pH values above 4.5 can give mixed results (Griffith and McCormick 1984; Smolander et al., 1988, Granhall et al., 1983). There is evidence to indicate that nodulation of alder may be increased by increasing pH with lime in some soils.

The lack of field trials and the differences observed for growth conditions and optimum nodulation pH make liming requirements and nutrient
recommendations difficult for optimizing nodulation in the field. The objective of this study was to examine nodulation of red alder seedlings after adjusting soil pH at nursery sites where red alder has previously been grown. Calcium oxide was used to increase soil pH and CaCl₂ was used as a calcium control at one site. Another site with an initial soil pH of 6.6, presumably near optimum for nodulation, was adjusted with HCl to examine the effects of increased soil acidity.
Materials and Methods

Site

Mima. We conducted most of this field experiment at the Weyerhaeuser Mima Nursery near Centralia, Washington (46° 52’ N, 123° 3’ W). The soil series is a Nisqually loamy sand, high in organic matter (8%) and is a sandy, mixed, mesic Pachic Xerumbrept (Thurston County Soil Survey, 1990). It was fumigated in the fall with 389 kg ha⁻¹ methyl-bromide/chloropicrin, injected at 150 to 200 mm, and retained for one week with a tarpaulin. Plots were laid out in a 120 cm-wide bed. Each plot was 60 cm long and was separated by a 15-cm buffer strip. The initial soil pH of this site was 5.6.

Aurora. Another study site was located in the Willamette Valley at the Weyerhaeuser Nursery near Aurora, Oregon (45° 15’ N, 122° 40’ W). The soil series is a Canderly (formerly Hillsboro) sandy loam, 4-6% organic matter, and is a coarse-loamy, mixed, mesic Ultic Haploxerol (Clackamas County Soil Survey, 1985). This site was fumigated and plots laid-out similarly to the Mima, WA site. The initial soil pH of this site was 6.6. During most years, red alder grown at the Aurora nursery site grow faster and have a longer growing season than seedlings grown at the Mima nursery. Because seedlings often become too tall to be efficiently handled at lifting, under-cutting of roots is a standard nursery practice at the Aurora site. The largest seedlings have the greatest amount of roots removed by under-cutting and must replace lost roots before above-ground
biomass can continue to increase. Before the mid-season sampling date, the seedling roots were under-cut at 4-5 cm below the soil surface. All nodulation data for the Aurora site are for nodulation of roots remaining after under-cutting.

Experimental design

**Mima.** A randomized complete block design in a 3 x 3 factorial experiment consisting of nine treatments was used: control, CaCl$_2$, or CaO; each at 0, 20, or 200 µL packed cell volume (pcv) m$^{-2}$ Frankia inoculum levels (Martin et al., 1991). Each plot consisted of one inoculum rate and one soil treatment. Treatments were distributed in four blocks for a total of 36 plots.

**Aurora.** A randomized complete block design in a 2 x 3 factorial experiment consisting of six treatments was used: control or HCl; each at 0, 20, or 200 µL pcv m$^{-2}$ Frankia inoculum levels. Each plot consisted of one inoculum rate and one soil treatment. Treatments were distributed in four blocks for a total of 24 plots.

Plot preparation

**Mima.** Soil amendments were sieved through a 2-mm sieve and broadcast in granular form at 250 g CaO per plot and 655.4 g CaCl$_2$·2H$_2$O per plot (equimolar calcium treatments) three weeks before inoculation. Plots were then raked to approximately 4 cm depth with a wide-tooth garden rake. Control plots were also raked at this time.
Prior to inoculation, six randomly selected plots were bioassayed for nodule-forming *Frankia* infective units (I U) in the top 8 cm of soil, using the MPN (most-probable-number) technique (Hilger et al., 1991). Fumigation decreased indigenous populations below the detection limit (0.16 I U g\(^{-1}\) soil) of the bioassay, thus subsequent nodulation was most likely due to applied inoculum.

**Aurora.** Six mL of 12 N HCl was added to 1 L of deionized water for a total of 72 mmol of HCl applied per treated plot. The HCl treated plots were sprinkled with this solution using a large watering can. The control plots received 1 L of deionized water applied in the same way.

_Inoculum preparation_

**Both sites.** *Frankia* cells (strain ArI5) were cultured in BAP medium (Murry et al., 1984), prepared and applied as reported in methods by Martin et al. (1991). The Mima plots were inoculated three weeks after soil amendments were applied, on April 30, 1991, with a pure culture strain of peat-stabilized *Frankia*. These plots were then raked to 4-cm depth immediately before 0.37 g of seeds per plot were sprinkled over the surface and plots were covered with Reemay (Ken-Bar Inc., 24 Gould St., Reading, MA 01867). The Aurora plots were inoculated and seeded on May 21, 1991, two weeks after soil treatments were applied, in the same way as the Mima plots. Plots were inoculated, seeded, and covered with Reemay. The Reemay was removed after seedlings were 7-8 cm tall.
Measured variables

**Mima.** Extractable soil calcium levels and cation exchange capacity (CEC) were measured as outlined by Horneck et. al. (1989) by Oregon State University Central Analytical Laboratory (Corvallis, OR) on combined surface soil from similar treatment plots.

Chloride analysis was performed on combined plot soil from similar treatments taken from beds at the inoculation and seeding date. Soil samples were extracted with deionized water (10 g soil to 100 mL water), mechanically shaken for 30 min, and filtered through a 2-μm filter disc. The filtered extract was analyzed by ion chromatography by Dionex 2000i (Dionex Corp. Sunnyvale, CA) fitted with an anion exchange column.

**Both sites.** Soil samples from the top 7-8 cm of each plot were collected periodically throughout the experiment. Soil solution pH values were determined by 30-min mechanical shaking of 2:1 (v/v) mixtures of deionized water:soil, which were allowed to settle for 30 min before measuring solution pH with an Orion Research pH Meter (model SA250) and combination electrode.

Percentage of nodulated plants, number of nodules per plant, shoot height, and root and shoot dry weights were measured at mid-season and at lifting. Separate counts were kept for nodules in the first 3.5 cm of root below the root collar and for nodules below that depth. A randomly-selected, 10% sample of seedlings was taken from each plot at mid-season. Mid-season sampling date was August 14, 1991 (100 days after seeding) at Mima and August 21, 1991 (90 days...
after seeding) at Aurora. A randomly-selected 10%, size-stratified, sample was taken from each plot at lifting. Harvested seedlings were separated into two categories: culls and packable. To meet the packable criteria, seedlings had to be both greater than 40 cm in height and greater than 4 mm in root collar diameter. The total number and packable number of seedlings per plot were determined at lifting. Lifting date for the Mima site was February 5, 1992 and for the Aurora site was January 20, 1992.

Statistical significance of the differences between the means was tested using analysis of variance and general linear model followed by Tukey's test ($\alpha = 0.05$) using SAS (SAS Institute, Cary, N.C.). Regression analysis was done with Quattro Pro (Borland International, Inc., Scotts Valley, CA.).
Results

*pH and calcium amendments*

Mima. The CaO treatment increased soil pH from 5.6 to between 6.8 and 7.4 at inoculation and seeding time and maintained a higher pH throughout the experiment (Fig. II. 1). In the CaCl₂ treatment, there was an initial, short-term drop from pH 5.6 to 4.5 at the time of inoculation and seeding. However, before the seedlings were more than 8 cm tall, the pH had reached that of control plots (Fig. II. 1). The initial short-term drop in pH for the CaCl₂ treatment is best explained by Ca²⁺ replacing H⁺ and aluminum hydroxides on the exchange sites and increasing soil solution acidity. When the plots were inoculated and seeded, the excess H⁺ had not completely leached through the soil profile, causing the temporary decrease in soil pH (Fig. II. 1).

Soil extractable calcium increased from 3.3 meq Ca 100 g⁻¹ soil (control) to 29.8 meq Ca 100 g⁻¹ soil in the CaO treatment at the time the beds were inoculated and seeded. Undissolved CaO pellets still remaining in the soil caused extractable soil calcium to be greater than the CEC of the soil (11.7 meq 100 g⁻¹ soil) over the course of the experiment (e.g., 15.9 meq Ca 100 g⁻¹ soil, at lifting). In the CaCl₂ treatment, extractable soil calcium increased to 8.6 meq Ca 100 g⁻¹ soil at the time these plots were inoculated and seeded but dropped to 4.7 meq Ca 100 g⁻¹ soil within 56 days after inoculation and seeding. At lifting the extractable calcium for this treatment was 4.9 meq Ca 100 g⁻¹ soil, still above that
of the control (3.2-3.7 meq Ca 100 g⁻¹ soil) but much lower than the CaO treatment, because CaCl₂ is much more soluble than CaO.

Chloride was on average 0.296 meq 100 g⁻¹ soil (109 ppm) for the CaCl₂ treatment when the plots were inoculated and seeded as compared with between 0.008 to 0.017 meq Cl⁻ 100 g⁻¹ soil (3-6 ppm) at the same time for control and CaO treatments. The chloride concentration in the CaCl₂ treatment probably returned to background level quickly, because Cl⁻ is very mobile in acidic, irrigated, well-drained soil such as this.

**Aurora.** The HCl-treated plots had a short-term drop in pH from 6.6 (control) to 5.9 at the time the plots were inoculated and seeded. Thirty-seven days later the pH was 6.4, similar to control plots.

*Growth and nodulation*

**Mima.** There were no significant block effects (P < 0.05). No significant (P < 0.05) inoculum rate X soil treatment interactions were found for: shoot height, number of nodules per plant, or root and shoot weights; therefore the effects of these two factors were evaluated independently (Table II. 1). However, a significant interaction (P < 0.01) was found on percentage of plants nodulated at mid-season and on percentage nodulation of the top portion of roots at lifting (Fig. II. 2. A, B, C). Inoculation rate had the most pronounced effect on growth and nodulation (Table II. 1). At mid-season, all measured variables except dry weights, which were less sensitive than wet weights (data not shown), were
significantly greater at each higher inoculation rate ($\alpha = 0.05$). At lifting, shoot height and shoot dry weight were significantly increased with each increase in inoculation rate (Table II. 1) and percentage nodulation was significantly greater for the 20 and 200 $\mu$L pcv m$^{-2}$ inoculation rates but not different from each other ($\alpha = 0.05$, Fig. II. 2. D). At lifting, the average number of nodules per plant was significantly greater at the highest inoculum level and showed no difference between 0 and 20 $\mu$L pcv m$^{-2}$ inoculum rates (Table II. 1). Differences at mid-season were more pronounced but trends were comparable to those at lifting (Table II. 1) and size and weight differences paralleled differences in percentage nodulation. At mid-season, percentage nodulation and number of nodules per plant were significantly ($\alpha = 0.05$) increased by soil CaCl$_2$ treatment (Table II. 1 and Fig. II. 2. A and C). Shoot height was greater than control for the two calcium treatments ($\alpha = 0.05$, Table II. 1). At lifting, percentage nodulation on surface roots was increased by soil treatment with CaCl$_2$ ($\alpha = 0.05$, Fig. II. 2. B).

Correlations of percentage of plants nodulated with shoot heights were greater when only the top portion of roots were examined at lifting ($R = 0.91$) compared to whole root analysis ($R = 0.75$). When whole roots were examined at lifting, little difference in percentage nodulation was observed (Fig. II. 2. D), even though shoot heights at the highest inoculum level were double those of the lower inoculum levels (Table II. 1). When only the top portion of roots (3.5 cm below the root collar) was examined at lifting, however, greater differences in
percentage nodulation were observed due to treatments (Fig. II. 2. B.) and this evaluation was more reflective of shoot heights measured at lifting.

The highest inoculation rate resulted in greater than 95% nodulation of seedlings at mid-season (Fig. II. 2. A and C), potentially masking differences from soil treatments. The CaCl$_2$ treatment at the intermediate inoculum rate of 20 $\mu$L pcv m$^{-2}$ had significantly higher percentage nodulation than control or CaO amended plots at mid-season and lifting ($\alpha = 0.05$, Fig. II. 2. A, B, C). This difference was not significant for the control or the 200 $\mu$L pcv m$^{-2}$ inoculum rates. The CaCl$_2$ treatment at lifting had on average 20 more packable seedlings per plot (approximately 10% greater) than the control or CaO treatments ($P < 0.05$).

At lifting, root-to-shoot ratio by dry weight was higher at the lowest (control) inoculum rate ($\alpha = 0.05$) but there was no significant effect from soil treatment (data not shown). There was no significant inoculum rate or soil treatment effect on root-to-shoot ratio at mid-season.

Aurora. No significant interactions or block effects were observed at this site ($P < 0.05$). At mid-season, percentage of plants nodulated, average number of nodules formed per plant, and shoot height were all significantly increased by the highest inoculum level but there was no difference between 0 and 20 $\mu$L pcv m$^{-2}$ inoculum rates for these same parameters ($\alpha = 0.05$, Table II. 2). Inoculum rate or soil treatment had no effect on root-to-shoot ratio at either sampling time ($\alpha = 0.05$, data not shown). The only effect of the HCl treatment was an increase in
both number of nodules per plant and shoot height at mid-season ($\alpha = 0.05$, Table II. 2). When only the top portion of roots was examined for percentage nodulation, differences due to inoculum rate were still observed at lifting even though shoot heights were similar (Table II. 2). When whole roots were examined at lifting, no difference in percentage nodulation was observed (Table II. 2).
Discussion

Site differences

Work at the Mima site by Martin et al. (1991) indicates that early nodulation is responsible for differences obtained in seedling sizes at lifting. Seeds germinating on the soil surface have roots that grow through the applied inoculum and thus become nodulated and begin N₂-fixation. Early nodulation was evaluated by examining roots for nodules just below the soil surface at mid-season. Our results confirm that early nodulation benefits are sustained at lifting for this site. Predictions about lifting-date seedling sizes may be made by analyzing seedling samples at mid-season. We found that approximately 85% nodulation at mid-season seemed necessary to produce packable (greater than 40 cm in height) seedlings by lifting. This is much higher than the 30% reported by Martin et al. (1991), where shorter seedlings (28.8 cm) were considered packable, and also may be because of differences in applied inoculum batch and yearly variations in growth conditions.

Results for the Aurora site indicate that early nodulation is not as important for producing large seedlings at lifting as for the Mima site. Even though there were differences in shoot height and percentage nodulation on whole roots at mid-season, there were no differences in either variable at lifting. When only the top part of the roots was examined at lifting some differences in percentage nodulation were still observed (Table II. 2). The under-cutting of roots
at this site probably decreased the size advantage of seedlings that were nodulated early, giving the uniform shoot heights at lifting shown in Table II. 2. The milder climate and the potentially higher indigenous populations, as indicated by mid-season data on whole roots showing 33 % nodulation at Aurora and only 8 % at Mima, a four-fold increase, at the 0 µL pcv m$^{-2}$ inoculum rate (Table II. 2 and Fig. II. 2. C), also may have contributed to differential site responses. Perhaps a much lower level of nodulation is required at mid-season to produce large, packable seedlings at lifting for the Aurora site. Treatment effects indicate that this level may be as low as 15 %. In spite of under-cutting at the Aurora site, shoot heights at the 0 µL pcv m$^{-2}$ inoculum rate were similar to those found at the Mima site at the 200 µL pcv m$^{-2}$ inoculum rate. Although site differences and management influences should not be over-looked, these findings suggest that conditions mimicking those at the Aurora site may lead to higher productivity.

_Treatment effects_

The effects of soil amendments (CaCl$_2$, CaO, and HCl) on nodulation may have been directly caused by changes in soil pH, Ca$^{2+}$, Cl$^-$, or some combination of these factors. Indirect effects of these amendments altering the availability of other nutrients cannot be excluded, however. The effect, if any, of the chloride is unknown and its potential effect on the increased nodulation observed in this study is uncertain. However, plants from the HCl treated plots at the Aurora site and CaCl$_2$ treated plots at Mima were not adversely affected in growth or
nodulation (Tables II. 1 and 2). Chloride concentrations at the Mima site were between 3 and 109 ppm which fall above requirement and well below toxicity levels (Tisdale et al., 1985). Therefore, a positive effect on growth or nodulation within this chloride range cannot be ruled out, although the duration of this effect would be short because chloride leaches rapidly.

In our study, independent treatments of both HCl and CaCl₂ caused temporary decreases in pH at the time the plots were inoculated and seeded. Without exception, there were more nodules per plant at mid-season and at lifting in these two treatments, although differences were not always significant (Tables II. 1 and 2). Cleland (1987) demonstrated the possibility that external acidity can help loosen cell walls by acid-induced cleavage of hemicellulose bonds. Nodule initiation and penetration by hyphae of the host cell wall could be benefited by decreased pH and subsequent cell wall loosening.

Several studies have shown a positive correlation between increasing pH and greater nodulation under a variety of growth conditions and with different inoculum preparations, in solution (Quispel, 1958; Knowlton and Dawson, 1983; Smolander et al., 1988) and in soil (Griffiths and McCormick, 1984; Smolander and Sundman, 1987; Smolander et al., 1988; Smolander 1990). Some studies, however, have not observed that nodulation capacity increases as soil pH increases between 3.5 and 7 (Granhall et al., 1983; Griffiths and McCormick, 1984; Zitzer and Dawson, 1992). It also is well established that infective Frankia units and nodulated alder roots can be found in acid soils with pH lower than 4.0.
(Smolander and Sundman, 1987; Paschke and Dawson, 1992; and Weber et. al, 1988). Thus, low pH by itself may not be the determining factor regulating alder nodulation. Lower soil pH is associated with lower base saturation, and hence low calcium availability. Thus, it is possible that other factors such as available calcium or base saturation are more important for alder nodulation.

Knowlton and Dawson (1983) investigated calcium effects on nodulation of seedlings grown in solution and found no effect of increased calcium at neutral pH, however, low pH and calcium interactions were not studied. Our results show that increased calcium at pH 4.5 at the time of inoculation and seeding (CaCl₂ treatment) increased nodulation relative to that seen at pH 5.6 (control) or 7.2 (CaO treatment). Within our study range, increased nodulation on red alder at pH 4.5, to our knowledge, has not been shown before. Smolander (1990) found that both the sum of available base cations and pH were significantly related to nodulation of alder. These were relatively fertile sites with either Betula pendula or Alnus incana stands. Birch sites had higher average soil pH than alder sites (4.5 vs. 3.9), had more exchangeable calcium, and greater nodulation capacity.

Various environmental factors including pH and nutrient availability may influence nodule initiation sites or trigger spore germination. Spores have been shown to be more effective at nodulation than hyphae (Burleigh and Torrey, 1990). Calcium, in conjunction with low soil pH, may facilitate the number of nodule initiation sites. Similar interactions have been shown in legume-Rhizobium symbioses (Loneragan and Dowling, 1958; Lowther and Loneragan, 1968; Munns,
1970). In our study there were approximately twice as many nodules per plant at lifting for the CaCl₂ treatment (Table II. 1). The results presented here indicate a pH-calcium relationship and that the effects of low pH on nodulation may be at least partially ameliorated by calcium addition without increasing pH.
Table II. 1. Growth and nodulation parameters for red alder seedlings grown at the Mima site.

<table>
<thead>
<tr>
<th>Main Effects</th>
<th>Mid-season*</th>
<th>Lifting*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nodulation</td>
<td>Dry Weight (g)</td>
</tr>
<tr>
<td></td>
<td>Shoot height (cm)</td>
<td>top</td>
</tr>
<tr>
<td>Inoculum level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µL m⁻²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10.2c</td>
<td>0.03c</td>
</tr>
<tr>
<td>20</td>
<td>12.4b</td>
<td>0.8 b</td>
</tr>
<tr>
<td>200</td>
<td>19.5a</td>
<td>3.7 a</td>
</tr>
<tr>
<td>Soil Treatment</td>
<td>Control</td>
<td>13.0b</td>
</tr>
<tr>
<td></td>
<td>CaO</td>
<td>14.5a</td>
</tr>
<tr>
<td></td>
<td>CaCl₂</td>
<td>14.5a</td>
</tr>
</tbody>
</table>

* Data with same letters grouped by main effects are not significantly different at p<0.05 by Tukey’s test.

** Nodulation on root portion 3.5 cm below root collar.

*** Nodulation on whole roots.
Table II. Growth and nodulation parameters for red alder seedlings grown at the Aurora site.

<table>
<thead>
<tr>
<th>Main Effects</th>
<th>Mid-season*</th>
<th>Lifting*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum Level (μL m⁻²)</td>
<td>Shoot height (cm)</td>
<td>Nodulation ( # / plant)</td>
</tr>
<tr>
<td>0</td>
<td>17.1b</td>
<td>0.4b 0.5b</td>
</tr>
<tr>
<td>20</td>
<td>18.8b</td>
<td>0.6b 0.8b</td>
</tr>
<tr>
<td>200</td>
<td>25.3a</td>
<td>2.7a 2.8a</td>
</tr>
<tr>
<td>Soil Treatment</td>
<td>Control</td>
<td>19.1b 1.1b 1.2b</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>21.7a 1.4a 1.5a</td>
</tr>
</tbody>
</table>

* Data with same letters grouped by main effects are not significantly different at p<0.05 by Tukey's test.

** Nodulation on roots 3.5 cm below root collar.

*** Nodulation on whole roots.
Fig. II. 1. Soil pH at the Mima Washington nursery site after calcium soil amendments were applied. The arrow indicates the date the plots were inoculated and seeds were applied. Bars indicate standard deviations.
Fig. II. 2. Nodulation as affected by inoculum level and calcium amendments, Mima site. Proportion of plants nodulated to 3.5 cm (A and B) and whole roots (C and D), at mid-season (A and C) and at harvest (B and D). The units for inoculum rate are: \( \mu L \) packed cell volume m\(^{-2} \) soil surface. The x-axis is nonlinear and the arrows indicate a significant treatment effect (Tukey’s \( \alpha = 0.05 \)). Significant inoculum rate x soil amendment interactions were found on data in A, B, and C (\( P < 0.01 \)).
References


Chapter III

PURE CULTURE, PEAT-STABILIZED, FRANKIA INOCULUM RAPIDLY DECREASE IN INFECTIVITY IN FUMIGATED NURSERY SOIL

Summary

Nursery soils are routinely fumigated to control pathogens. Fumigation reduces indigenous microbial populations, and consequently, a source of Frankia inoculum is often required to obtain good nodulation and growth of nursery grown red alder (Alnus rubra Bong.) seedlings. It is unknown how long applied Frankia inoculum remains effective in nodulating roots in fumigated nursery soil. Fumigated soil collected from the Mima, WA nursery site was used in a randomized, 4 x 5 factorial design in a greenhouse experiment. The four soil treatments were: control, HCl, CaO, and CaCl$_2$; each seeded with red alder in Frankia-inoculated pots on day 0 (day inoculum was applied), day 2, day 7, and day 14, as well as an uninoculated control (seeded on day 0). By observing root nodulation of seedlings from pots seeded at various times after inoculation, we hoped to determine the length of time that peat-stabilized Frankia inoculum remained highly effective at nodulation and effects on nodulation caused by soil treatment with calcium and pH-altering soil amendments. Seedlings were harvested 15 weeks after inoculation and were evaluated for: shoot height, percentage of plants nodulated, number of nodules formed per plant, and root and
shoot dry weights. All seedling roots were well below the depth of the applied inoculum indicating that the percentage of plants nodulated was unlikely to increase with more time. Seedlings did not survive in the CaCl₂-treated pots because of poor drainage and aeration. All measured variables were lowest in the uninoculated control. Seeding pots 14 days after inoculation decreased all measured variables ($\alpha = 0.10$) compared to pots seeded the day the inoculum was applied. Independent additions of HCl and CaO treatments increased both percentage of plants nodulated and root dry weights ($\alpha = 0.10$), suggesting these effects may be independent of pH in this soil.
Introduction

*Frankia* inoculum, applied to nursery beds either as crushed nodule suspension or as pure culture, has been used to increase red alder seedling packable numbers, size, and vigor (Hilger et al., 1991; Martin et al., 1991; Wheeler et al., 1991; Chapter II). Nodulated seedlings have been shown to better survive transplanting from nursery to the field than non-nodulated stock and the growth benefit can last for several years (Burgess et al., 1986; Prat, 1992). Peat carrier has been used to increase inoculation success with *Frankia* on red alder (*Alnus rubra* Bong.) in fumigated nursery beds (Martin et al., 1991).

Considerable research investigating methods to improve nodulation and plant health has been conducted. Much of this work concerns calcium and pH influences on nodulation. Our initial nursery field experiment (Chapter II) indicated that an initial decrease in pH, combined with the addition of calcium at the time of nodule initiation, may increase red alder seedling growth and nodulation using current nursery production practices. Similar results were reported for a greenhouse study with *Frankia*-inoculated snowbrush (Scott, 1973).

Hughes and Gessel (1967) demonstrated that under calcium-deficient conditions, inoculated red alder seedlings not only had lower foliar concentrations of calcium but were also lower in nitrogen. Scott (1973) found that the calcium levels in seedlings from both CaCO$_3$ and CaSO$_4$ treatments were nearly twice that of control seedlings. His work also showed that the N content of plants tended to
increase with calcium addition and this followed the trend in nodulation. The mechanism for calcium-induced increases in growth, nodulation, and N content of seedlings has not been defined. The greater number of nodules induced by calcium treatments led Scott (1973) to conclude that calcium initiated more nodule infection sites and thereby increased the amount of N available for plant growth.

A preliminary bioassay study that estimated *Frankia* numbers by the most-probable-number (MPN) method (Hilger et al., 1991) in the nursery field experiment (Appendix 1) suggested that CaCl₂ treated plots had more infective units than control or CaO treated plots (Pr > F 0.14). However, these differences were not significant at $\alpha = 0.05$, possibly because of poor detection limits and high variability. We found no trend between the number of infective units and time after inoculum application in soil samples collected 56 and 106 days after inoculation. The low number of infective units measured or the possibility that the applied *Frankia* decreased in infectivity before the first soil collection made it impossible to determine whether soil treatments influenced the length of time the inoculum remained effective at nodulation using the MPN bioassay technique.

These results led us to initiate a study that employed an alternative method to MPN analysis to determine the length of time the inoculum remained effective at nodulation. Soil collected from the Mima nursery site was used in a greenhouse experiment where seeds were applied to the soil surface at the time of inoculation (day 0), and 2, 7, and 14 days after inoculation (day 2, 7, and 14). HCl, CaO, and CaCl₂ amendment effects on nodulation were also investigated.
The effects of pH on seed germination of red alder in Perlite™ were examined in a subsequent investigation where HCl was used to adjust solution pH.
Materials and Methods

Experimental design

Six pots per treatment (120 total) were tested in a completely random, factorial design. Treatments consisted of 24 non-inoculated pots seeded at day 0, each with HCl, CaCl₂, CaO, or control (no amendment applied), and 96 inoculated pots with each of the applied soil amendments and control. The inoculated pots were seeded at day 0 (time inoculum was applied), day 2, day 7, or day 14. Pots were randomly distributed on one bench in the greenhouse.

Soil treatments and greenhouse operations

Fumigated soil collected from the Mima, WA nursery site (Chapter II) was distributed in 11.5 x 11.5 x 15 cm deep pots. An equal volume of soil was added to each pot, bringing the soil level to approximately 6 cm below the rim. Soil amendments were applied to the surface and then mixed in to about 4 cm soil depth. Equimolar amounts of calcium were applied for the calcium treatments (9.0 grams of CaCl₂·2H₂O and 3.4 grams of CaO), to approach similar surface area amounts as in the previous field experiment (Chapter II). HCl-treated pots received 1.4 mmol HCl (applied in 100 mL water) before each seeding day. Between 10 and 15 red alder seeds per pot were applied on March 24, 1993, day 0 (day of inoculation), 2, 7, or 14.
Pots were placed under a Reemay (Ken-Bar Inc., 24 Gould St., Reading, MA 01867) covering after they were seeded. The Reemay was removed once seedlings from the day 14 seeding were more than 4 cm in height, 7 weeks after inoculation. Pots were watered every 2-3 days. Greenhouse temperatures were between 11.1 and 27.8°C during the experiment.

Inoculum preparation

*Frankia* cells (strain Ar15) were cultured in BAP medium (Murry et al., 1984), prepared and applied as reported in methods by Martin et al. (1991). Peat-stabilized *Frankia* inoculum (20 g per pot of 70% moisture content peat) at 20 μl packed cell volume m⁻² (Martin et al., 1991) was spread on the surface of each pot immediately before seeds were applied to day 0 pots.

Thinning and harvest

When seedlings were well established, they were thinned to four plants per pot on June 1, 1993, 66 days after inoculation. Seedlings were harvested on July 8, 1993, 103 days after inoculation. Shoot height, number of nodules per plant, percentage nodulation, and root and shoot dry weights were measured.

Statistical significance of the differences between the means was tested using analysis of variance and general linear model followed by Tukey’s test (α = 0.05 and 0.10) using SAS (SAS Institute, Cary, N.C.).
Seed germination in Perlite™

Sorted red alder seeds containing no visible defects were selected for this seed germination study. We evaluated germination of three replicates each at five solution pH values. Equal volume amounts (approximately 500 mL) of Perlite™ obtained from the Portland Perlite Company, Portland, OR, were placed in 15 clear, 16 x 16 cm polystyrene containers with hinged lids. The containers were filled with approximately 2.5 cm of Perlite™. Three sets of solutions each containing: 1, 5, 25, 50, and 75 mLs of 0.1 N HCl each brought to 300 mL total volume were prepared. Each solution was poured over the Perlite™ in one container and allowed to equilibrate for 24 hr. Twenty seeds per container were placed on top of the Perlite™ and allowed to germinate on a laboratory bench. Six days after seeding, 150 mL of distilled water was added to each container. Percentage seed germination was recorded after 12 days. Solution pH was measured with an Orion Research pH Meter (model SA250) and combination electrode.
Results

*Seed germination in Perlite™*

The solution pH values in Perlite™ did not vary by more than 0.6 units within each treatment. More seedlings germinated at pH 6.8 than at any other pH tested (range: 2.8-7.3) (Fig. III. 1). The percentage of seeds germinating did not significantly decrease until solution pH was lower than 3.7 (Fig. III. 1).

*Greenhouse experiment*

Because no interactions were found between days seeded after inoculum application and soil treatment for those variables evaluated (shoot height, number of nodules per plant, percentage of plants nodulated, or root and shoot dry weights), the effects of these two variables were evaluated independently (Table III. 1). Pots receiving no inoculum had the lowest shoot height, number of nodules per plant, percentage of plants nodulated, and root and shoot dry weights ($\alpha = 0.10$, Table III. 1). All measured variables were lower when pots were seeded 14 days after the inoculum was applied as compared to pots seeded and inoculated the same day ($\alpha = 0.10$, Table III. 1). HCl and CaO treatments increased both the percentage of plants nodulated and root dry weights ($\alpha = 0.10$, Table III. 1).

All of the CaCl$_2$ treated pots were lost because the roots of newly germinating seedlings were unable to penetrate the soil. Soil in these pots became...
very dark and dense shortly after the CaCl₂ treatment and water were added. All pots showed some signs of poor drainage, but the CaCl₂ treatments were the most affected. We suspect that restricted aeration and drainage caused by the rapid solubility of the CaCl₂ treatment were the cause of the dense soil structure and the subsequent rotting of new roots on the soil surface for this treatment. The few plants which did survive to harvest in the CaCl₂ treatment exhibited severe Cl⁻ toxicity symptoms as described by Parker et al., 1986. There was no equilibration or potential leaching time allowed after the soil amendments were applied to pots, therefore the Cl⁻ concentration for this treatment (estimated above 3000 ppm) was well above most reported plant toxicity levels (Jackson, 1986).
Discussion

Nodulation of red alder by *Frankia* consists of many phases involving: seedling establishment, root-hair curling, and penetration of roots by hyphae leading to nodule formation. Because the seeds are applied to the soil surface after inoculation of *Frankia*, seed germination is the first necessary stage. We found that HCl-treated pots established seedlings at least 1 to 2 days earlier than control or CaO treated pots (personal observation). The temporary decrease in the pH of the CaCl₂ treatment from pH 5.6 to pH 4.5 observed in the field experiment (Chapter II) may have been beneficial for seed germination. Optimal pH for seed germination of alder may be low and may also be species-dependent. Schalin (1967) found that grey alder (*Alnus incana*) germinated best at pH 5 whereas black alder (*Alnus glutinosa*) germinated best at pH 4. Our study showed that percentage germination of red alder seeds sprouted in HCl amended Perlite™ was not reduced until pH was lower than 3.7 (Fig. III. 1), however, earlier germination at lower pH was not detected in the Perlite™ study. The earlier seedling establishment observed for HCl-treated pots in the greenhouse study may have been caused by differential soil structure in pots caused by soil treatments. Or, by other undetermined factors that may differ between soil and Perlite™.

Viability of applied *Frankia* inoculum could influence subsequent nodulation stages. Stowers and Smith (1985) found better growth of alder in containers inoculated at seeding date than seedlings inoculated 6 weeks later. Our
results show that seeding on inoculation date produced an 83 percent increase in the percentage of nodulated seedlings than those seeded 14 days after soil inoculation (Table III. 1). Because it appears that the applied inoculum decreases in infectivity rather quickly and that the best growth benefits occur from early nodulation, the closer the inoculum is applied to seed germination, the better the nodulation and growth that can be obtained.

For pots seeded 14 days after the inoculum was applied, the percentage of plants nodulated was unlikely to increase beyond harvest date because roots were well below the depth of the applied inoculum. Seedling sizes and age at harvest in this study were similar to those at mid-season in the field study (Chapter II). Previous work at the Mima site has shown that early nodulation benefits observed as early as 10 weeks were sustained to lifting (Chapter II; Hilger et al., 1991; Martin et al., 1991). It was shown that nodulation at mid-season could be used to reflect seedling sizes at harvest in this soil (Chapter II; Martin et al., 1991). Therefore, evaluation of nodulated plants after 13 to 15 weeks growth should be indicative of later seedling health.

Our results showed that both CaO and HCl treatments increased the percentage of plants nodulated (Table III. 1) in this relatively fertile nursery soil. Soils with low pH are often associated with low base saturation. However, the initial soil pH of this site was 5.6 and the base saturation was relatively high, almost 40%. The amount of HCl added in our study was based on a previous soil titration which reduced pH from 5.6 to 4.7 in the Mima soil. We believe that
within this pH range, nodulation by *Frankia* on red alder may be enhanced at low 
PpH provided that moderate to high levels of calcium are available. Even though 
equal surface area amounts of CaO were applied in the field experiment (Chapter 
II) and this greenhouse experiment, the later was not allowed to equilibrate before 
inoculation and probably had not reached pH 7 at the time of seeding. It is likely 
that the unequilibrated CaO treatment still had soil microsites at the control pH 
(5.6) as well as greater access to soluble calcium. In the nursery field trial 
(Chapter II), where the soil was allowed to equilibrate before inoculation, the effect 
of the added CaO and subsequent increase in pH above 7 could have decreased 
the uptake of many nutrients including: B, Co, Cu, Zn, Fe, and Mn. Because of 
excess free carbonates, phosphate may have been less available for this treatment. 
A reduction in availability of some nutrients may explain why the number of 
 nodules and percentage nodulation were not significantly increased for the CaO 
treatment in our previous field study (Chapter II). Vigorous seedling growth 
obtained by adequate access to essential soil nutrients is probably necessary for 
good nodulation. Calcium availability also appears to play a significant role in 
nodulation.

Calcium availability and soil pH changes could affect *Frankia*, the plant, 
or both. The role of pH or calcium on spore germination is unknown. If one or 
either of these factors can influence spore germination, this could increase 
nodulation because spores can be 1000 times more infective than hyphae 
(Burleigh and Torrey, 1990). However, the inoculum used in our experiments
were cultured in a relatively nutrient poor media which does not tend to produce cultures which contain many spores (personal observations). Therefore, this was probably not responsible for the increase in nodulation observed with the calcium or HCl amendments.

The calcium requirement for nodulated plants may be high and calcium may increase nodulation. Good growth of nodulated *Casuarina equisetifolia* requires relatively high amounts of calcium (Yadav, 1983). Hilger and Myrold (1992) found that *Frankia* infective units, as measured by MPN bioassays, increase by liming but that the number of genomic units, as measured by nested polymerase chain reaction (PCR) using 16S rRNA primer, are not changed. Genomic units represent total *Frankia* DNA in soil and are not necessarily related to the number of *Frankia* capable of forming nodules, where as MPN analysis uses nodulation to determine infective *Frankia* populations. Thus, the increase in infective units with lime measured by the MPN technique indicates that lime increases nodulation.

Our results suggest that calcium influences nodulation capacity. Smolander's (1990) study found a statistically significant effect on nodulation capacity with the sum of available base cations. These were relatively fertile sites and the average pH and extractable calcium were higher for the birch sites than for the alder sites. Soil collected under *Betula* stands has greater nodulation capacity than soil collected from under *Alnus* stands (pH range 3.3-5.7) (Smolander, 1990). The positive effect on nodulation observed under birch sites
may be caused by birch’s influence on available calcium. Survival of *Frankia*, measured by nodulation capacity, is greater in limed peat than in limed sand (Smolander et. al, 1988). However, before liming, the peat had greater than four times the extractable calcium than the sand and a lower initial pH (2.9 vs 4.2), therefore more lime was probably needed to increase soil pH above 6, in their study. Increased nodulation could have been caused by higher available calcium, if calcium has the ability to increase the number of infection sites as we suspect.

The frequency of nodulation on *Ceanothus velutinus* Dougl., from ten sites in the Pacific Northwest was correlated with soil base saturation and accounted for 78% of the variation found (Scott, 1973).

Nodule formation consists of many phases including: infection of root hairs, penetration of host cells, and host reactions leading to nodule formation. Influences from environmental factors on these stages are not well known. High concentration of calcium at the time of nodule initiation has been shown to increase the number of nodules formed by *Rhizobium* on subterranean clover (Lowther and Loneragan, 1968). Removal of plants from high calcium to lower calcium did not decrease the number of nodules formed, whereas plants grown in low calcium and transferred to high calcium did not increase the number of nodules formed. These investigators concluded that calcium increased initiation sites for nodulation. The positively charged calcium cation could act as a binder between negatively charged *Frankia* and plants roots. The influence of calcium in nodule initiation may have a greater impact on *Frankia* than on *Rhizobium*,
because *Frankia* do not produce a thick, polysaccharide layer on the outside of their cells, which may help *Rhizobium* to adhere to root surfaces.

Scott (1973) also concluded that the role of calcium in nodulation is to increase initiation sites. His greenhouse study with *Frankia*-inoculated snowbrush in soil with an initial pH of 6.1, where CaCO$_3$ or CaSO$_4$ amendments had been added, found that both calcium amendments tended to increase plant weights and nodule numbers and mass, but that the CaSO$_4$ treatment was consistently, significantly better. The addition of the CaSO$_4$ lowered soil pH from 6.1 to 5.6 and the CaCO$_3$ increased soil pH. In a subsequent experiment using H$_2$SO$_4$, Scott (1973) concluded that the increase in nodulation caused by CaSO$_4$ was not attributable to meeting a sulfur demand in that treatment. The positive effect on nodulation found with the combination of high calcium and lower pH is consistent with results presented here and in Chapter II.

The establishment of actinorhizal root nodules involves penetration of host cell walls and intracellular colonization by the nitrogen-fixing endosymbiont. Plant-microbe interactions leading to nodule formation have not been clearly defined. Changes in soil pH may provide an optimal environment for extracellular enzymes which may help *Frankia* to penetrate the root. Cleland (1987) demonstrated the possibility that external acidity can help loosen cell walls by acid-induced cleavage of hemicellulose bonds. Although differences were not always significant, HCl and CaCl$_2$ treatments which showed temporary decreases in pH at the time plots were inoculated and seeds were applied in our nursery
field trials (Chapter II) as well as HCl treatment in this study, had, without exception, more nodules per plant (Tables: (Chapter II. 1 and 2), and III. 1). Nodule initiation and penetration by hyphae of the host wall could be benefited by decreased pH and subsequent cell wall loosening.

It is interesting to note the solubilities of the calcium amendments applied in this study, the field study (Chapter II), and Scott’s (1973) greenhouse experiments:

\[ \text{CaCl}_2 \cdot 2\text{H}_2\text{O} \gg \text{CaSO}_4 \gg \text{CaO} \gg \text{CaCO}_3. \]

Both calcium chloride and calcium sulfate performed better at enhancing nodulation than the liming materials used in their respective studies. This would support the hypothesis that high concentrations of available calcium at the time of nodule initiation can increase the number of infection sites. In addition, both CaCl\textsubscript{2} and CaSO\textsubscript{4} tend to temporarily decrease soil pH whereas CaO and CaCO\textsubscript{3} are used to increase soil pH. Therefore, if the decrease in pH is not beneficial to nodulation, it certainly does not appear to be inhibitory, within the range studied. Our results suggest that within this pH range nodulation can be increased by calcium and this effect may be enhanced at lower pH. The effectiveness of Frankia inoculum applied to nursery soils may be increased by increasing calcium availability at the time of nodule initiation. Furthermore, seedling success may be improved by application of calcium to poor soils intended to be used for alder plantations.
Table III. 1. Growth and nodulation parameters for greenhouse grown red alder seedlings.

Data with same letters grouped by main effects are not significantly different at p<0.10 by Tukey’s test.

<table>
<thead>
<tr>
<th>Main Effects</th>
<th>Shoot height (cm)</th>
<th>Nodulation</th>
<th>Dry Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td># / plant</td>
<td>Root</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(%)</td>
<td>Shoot</td>
</tr>
<tr>
<td><strong>Days Seeded after Soil Inoculation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Inoculum</td>
<td>6.8c</td>
<td>0.1c</td>
<td>6.9c</td>
</tr>
<tr>
<td>0</td>
<td>11.1a</td>
<td>2.1a</td>
<td>87.5a</td>
</tr>
<tr>
<td>2</td>
<td>10.3ab</td>
<td>2.2a</td>
<td>75.0a</td>
</tr>
<tr>
<td>7</td>
<td>9.9ab</td>
<td>1.7ab</td>
<td>69.4ab</td>
</tr>
<tr>
<td>14</td>
<td>8.6bc</td>
<td>1.0b</td>
<td>47.7b</td>
</tr>
<tr>
<td><strong>Soil Treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.7a</td>
<td>1.6a</td>
<td>60.4b</td>
</tr>
<tr>
<td>HCl</td>
<td>9.8a</td>
<td>1.9a</td>
<td>77.4a</td>
</tr>
<tr>
<td>CaO</td>
<td>10.3a</td>
<td>1.7a</td>
<td>71.9a</td>
</tr>
</tbody>
</table>
Fig. III. 1. Relationship between percentage of red alder seedlings germinated in Perlite™ and pH. Bars indicate standard deviation.
References


Jackson T.L. (1986) In Chloride and Crop Production. Published by Potash and Phosphate Institute Atlanta, GA.


Chapter IV

SUMMARY AND CONCLUSIONS

Red alder (*Alnus rubra* Bong.) is currently being harvested, planted, and utilized for a variety of commercial purposes. Nurseries in the Pacific Northwest are now involved in intensive production of this species. Pure culture *Frankia* inoculum has been used to increase red alder seedling nodulation, resulting in improved cost efficiency and production quality. *Frankia* production for nursery inoculation is expensive and time-consuming. However, nodulated seedlings have much greater survival success once they are out-planted. Addition of soluble calcium amendments to fumigated nursery soil before nodule initiation has the potential to increase the applied *Frankia* inoculum efficiency and this effect may be enhanced at pH below 7. Consequently, the percentage of plants nodulated and the number of high-quality packable seedlings obtained from nursery production may be increased.

Generally, nursery soils in the Pacific Northwest have a high nutrient status and greater base saturation than soils to which red alder are out-planted. Good nodulation of nursery stock can be essential to the survival of newly planted seedlings. HCl addition to two relatively fertile nursery soils (base saturation: 40% and 48%) increased nodulation in soils with good calcium availability.
Calcium addition, in form of CaO in the greenhouse study and CaCl₂ in the field trial, to fumigated nursery soil from the Mima site also increased the percentage of plants nodulated. However, CaO addition in the field trial where soil was allowed to equilibrate to pH 7.2 before inoculation did not significantly increase nodulation. Whereas CaO addition in the greenhouse study, which was not allowed to equilibrate before inoculation, did significantly increase the number of plants nodulated. Furthermore, the addition of CaCl₂ in the field trial, which temporarily decreased soil pH at the time of inoculation and seeding and increased base saturation to 75%, significantly increased the percentage of plants nodulated. This treatment also produced more seedlings at the intermediate inoculum rate comparable in size to those obtained at an inoculum rate an order of magnitude greater. Our results demonstrate that in fumigated nursery soil between pH 4.5-7 nodulation of red alder may be increased by calcium and this effect may be enhanced at lower pH. Thus, the potential exists to increase seedling nodulation and success in low pH soils where aluminum and H⁺ toxicity are not limiting plant growth by increasing calcium availability without increasing pH.


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APPENDIX
APPENDIX

APPENDIX I

Bioassay

Soil samples collected from all treatments from two of the four blocks were bioassayed using the MPN method (Hilger et al., 1991): once after the Reemay was removed, 56 days after inoculation and seeding, and again at the mid-season sampling date, 106 days after inoculation.

MPN bioassay of soil collected after the Reemay was removed could only detect a significant difference at the 200 µl packed cell volume m⁻² inoculum rate (Pr > F 0.02). Infective units were increased due to the highest inoculum rate. The greatest number of infective units per gram of soil was seen with the CaCl₂ soil treatment, but the difference from the other two soil treatments was not highly significant (Pr > F 0.14). By mid-season, there were no significant differences detected by MPN analyses due to soil treatment or inoculum level at α = 0.05 level.
Table A. 1. Infective Frankia units per gram of soil collected from the Mima site, MPN method bioassay results.

<table>
<thead>
<tr>
<th>Inoculum Level (μL mL⁻¹)</th>
<th>Soil Treatment</th>
<th>After Remay Removed</th>
<th>Mid-season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Block A</td>
<td>Block B</td>
</tr>
<tr>
<td>0</td>
<td>Control</td>
<td>&lt;dL*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>CaO</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>CaCl</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>20</td>
<td>Control</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>CaO</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>CaCl</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>200</td>
<td>Control</td>
<td>3.00</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>CaO</td>
<td>0.30</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>CaCl</td>
<td>3.50</td>
<td>10.70</td>
</tr>
</tbody>
</table>

*dL = detection limits 0.16 IU per gram of soil.

After the reemay was removed, infective units were greatest at the highest inoculum rate (Pr>F 0.02).

The greatest number of infective units was seen with the CaCl₂ treatment, however not significant (Pr>F 0.14).