

AN ABSTRACT OF THE THESIS OF

Nestor S. Rojas-Melo for the degree of Master of Science
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Title: Influence of Soil and Rhizosphere Actinomycetes
on Frankia Infection and Nitrogenase Activity
in Alnus Rubra Bong

Signature redacted for privacy.

Abstract approved:

David A. Perry

Nodulated roots of young red alder trees and soil samples were collected from a 5 year old clearcut on Mary's Peak, Oregon. Frankia was isolated from root nodules, and actinomycetes from nodules, roots, and soil. A total of 229 pure culture actinomycete isolates were recovered: 131 colonies from root surfaces, 52 colonies from soil, and 46 colonies from nodule surfaces. Thirty morphologically different colonies were selected for the experiment, 10 from each source. Red alder seedlings were grown in the greenhouse for 6 months. They were placed in Ray Leach tubes containing a 1:1 soil mixture of peat moss and vermiculite. Seedlings were inoculated with a Frankia-actinomycete combination when they were 1

month old. N-free mineral solution was applied weekly. The study consisted of thirty-two treatments, thirty in which seedlings were inoculated with Frankia plus one of thirty actinomycete isolates (10 each from nodule surfaces, root surfaces, and open soil), one inoculated with Frankia only, and one in which seedlings were inoculated with neither Frankia nor an actinomycete. Each inoculation treatment was replicated on ten seedlings. Three variables were measured: acetylene reduction, oven-dry nodule weight, and total dry biomass of seedlings. Actinomycete colonies varied significantly in their influence on nodule weight per seedling, acetylene reduced per gram nodule, and total seedling weight, but not in their effects on acetylene reduced per seedling. Colonies isolated from nodule surfaces produced slightly greater nodule weights on seedlings than colonies isolated from roots, but colonies from the three sources (nodules, roots, and open soil) did not differ in their effects on the other measured variables. Compared to seedlings inoculated only with Frankia, five of the thirty actinomycete colonies that were tested enhanced acetylene reduction per gram nodule; one colony reduced nodule weight, and none altered the amount of acetylene reduced per seedling. By far the major

influence of actinomycetes was to reduce seedling weight,
13 of 30 colonies producing seedlings that were smaller
than those inoculated only with Frankia.

Influence of Soil and Rhizosphere Actinomycetes
on Frankia Infection and Nitrogenase Activity
in Alnus rubra Bong

by

Nestor S. Rojas-Melo

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I dedicate this thesis to my wife Rosa and to my parents for their constant support.

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INFLUENCE OF SOIL AND RHIZOSPHERE ACTINOMYCETES
ON FRANKIA INFECTION AND NITROGENASE ACTIVITY
IN ALNUS RUBRA BONG

INTRODUCTION

Understanding microbial interactions in soil, with special reference to the response of trees, may yield insights into how to better manage forests on a sustainable basis.

Actinorhizal red alder (Alnus rubra Bong.) is an important hardwood species in the northwestern United States; it covers 13% of the coastal commercial forest land of Oregon and Washington (Resch, 1988). Red alder's ability to assimilate nitrogen and its potential to improve soil fertility make this tree a valuable component of Pacific Northwest ecosystems, which are limited by nitrogen (Cole et al., 1977; DeBell, 1979; Perry and Rose, 1983). Actinomycetes are a poorly understood but potentially important component of forest soil microflora. They are well known for the production of substances which inhibit growth of fungi, bacteria and yeasts (Alexander, 1977). Recently, actinomycetes have been related to reforestation failures in Southwestern Oregon; it has been conjectured that after a delay in reforestation, phytotoxic actinomycetes interfere with the reforestation of Douglas-fir seedlings (Friedman et al., 1989).

We hypothesized that actinomycetes isolated from root surfaces, nodule surfaces, and soil differ in their effect on nodulation, total seedling biomass, and nitrogen fixation. To test this hypothesis, a greenhouse experiment was conducted, in which red alder seedlings were inoculated with Frankia and actinomycetes isolated from nodule and root surfaces of red alder seedlings, and from soil.

MATERIALS AND METHODS

Study Area

Nodulated roots of red alder seedlings and soil samples were collected in June 1987 from a 5 year old (approximate) clearcut on Mary's Peak, Oregon. The area is located 32 km southwest of Corvallis (44° 30'N, 123° 33'W). Summers are dry and winters wet, with an average annual precipitation of 2,032 mm. Soils in the area have been classified in the Klickitat and Kilchis series (Knezevich, 1975). Formed from intrusive and basalt rocks, these soils are deep and well drained. The study site, at an elevation of 1,000 meters above sea level, is in the Western Hemlock zone described by Franklin and Dyrness (1973). Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) and Western hemlock (Tsuga heterophylla (Raf.) Sarg.) are the dominant trees in undisturbed forests, but western redcedar (Thuja plicata Donn.), red alder and bigleaf maple (Acer macrophyllum Pursch.) are also present. Salal (Gaultheria shallon Pursch.), vine maple (Acer circinatum Pursch.), salmonberry (Rubus spectabilis Pursch.), and swordfern (Polystichum munitum (Faulf.) Presl) are typical understory plants.

Samples were collected within an approximately 0.1 ha area, which was relatively flat (< 10 % slope). The area had been used to pile logs during clearcutting, and organic layers were largely lost as mixed with

mineral soil during this process. Scattered red alder seedlings had colonized the site following the clearcut.

Sample Collection

Nodulated roots were dug from 15 red alder seedlings within a 0.1 ha area. Soil was collected at 10 to 20 cm depth from the root zone of each of the 15 seedlings and also from areas at least 4 m from the nearest alder (hereafter referred to as "open soil"). Samples were kept on ice and returned to the laboratory within 2 hours of collection.

Laboratory Procedure for Frankia

Within 24 hrs. of collection, Frankia vesicle clusters were isolated from nodules using the filtration procedure described by Benson (1982). Following an initial rinse with tap water, nodules were surface sterilized by stirring for 15 minutes in a beaker containing 100 ml of distilled water, 100 ml of Sani-clor bleach (sodium hypochlorite 6%), and a drop of tween 20 (polyoxyethylene sorbitan monolaurate). Following sterilization, nodules were rinsed with distilled water. Nodule lobes, which had been separated from their tips, were ground in a tissue grinder containing distilled water. The resulting solution was filtered using two plastic syringe barrels; the filtration was done first

through a 52-um mesh screen, then through a 20-um mesh screen. We sterilized both syringes for 3 minutes in a 1:1 solution of distilled water and Sani-clor bleach. Unless otherwise stated, all procedures were conducted under sterile conditions.

After filtration, Frankia vesicles were retained on the 20-um mesh screen as liquid suspension. This suspension was transferred to a dry Petri dish and inoculated into 10 test tubes containing an N-free, modified BAP medium (BAP) for Frankia (medium modified from Murry et al., 1984), with the following composition: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -- 0.2 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ -- 0.07 mM; FeNa EDTA -- 10 mg/l; micronutrients -- 1 ml of (g/l) H_3BO_3 : 2.86, $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$: 1.81, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$: 0.22, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$: 0.08, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$: 0.025, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$: 0.001; and vitamins -- 1 ml of (mg/100 ml) Thiamin HCl : 10, Pyridoxine HCl : 50, Nicotinic acid : 50, Biotin : 22.5, Folic acid : 10, Ca pantothenate : 10, riboflavin : 10. Ten mM of phosphate buffer and 5.0 mM of organic acid (Na Propionate) adjusted to pH 6.7 were also added to the solution.

The 10 test tubes were left in a 30°C-incubator for 4 weeks, during which time tubes were shaken by hand daily. After 2 weeks, Frankia had formed a white precipitate on the bottom of each of the 10 test tubes.

After 4 weeks on N-free BAP, Frankia cultures were

washed with distilled water and ground in a tissue grinder. The resulting solution was added to an Erlenmeyer flask containing 100 ml of distilled water, and this mixture then divided into 60 test tubes containing the previously described BAP with 5.0 mM of NH_4Cl . The test tubes were left for 6 weeks in a 30°C-incubator. The tubes were shaken by hand daily.

Laboratory Procedure for Actinomycetes

Actinomycetes were isolated from root surfaces, nodule surfaces, and open soil, using the procedure described by Friedman et al. (1989). Three 125 ml Erlenmeyer flasks were magnetically stirred for 20 minutes, each of them containing 100 ml of sterile distilled water plus 1 g of nodules, 1 g of roots, or 0.1 g of open soil. Aliquots of 25 and 50 μl from the three Erlenmeyer flasks were spread on plates containing either tap water agar adjusted to pH 10.5 using 1N NaOH, or sodium albuminate agar adjusted to pH 6.8 using 1N HCl (Friedman et al., 1989). The components for the sodium albuminate agar were glucose -- 1 g; agar -- 15 g; egg albumin -- 0.25 g in 10 ml of 0.1N NaOH; K_2HPO_4 -- 0.618 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -- 0.4 g; $\text{Fe}_2(\text{SO}_4)_3$ -- 0.01 g; and H_2O -- 990 ml.

After incubating one week at 25°C, actinomycete colonies isolated from both media were transferred

individually to Petri dishes containing Malt Yeast agar (pH 7.0). The components for the Malt Yeast agar were malt extract -- 10 g; yeast extract -- 4 g; agar -- 15 g; CoCl_2 1% -- 1 ml; tap water -- 1 l. Plates showing contamination after 7 days were discarded, and the isolation procedure repeated.

A total of 229 pure culture actinomycete isolates were recovered: 131 colonies from root surfaces, 52 colonies from soil, and 46 colonies from nodule surfaces. Of this total, 30 morphologically different colonies were selected for the experiment, 10 from each source. These 30 colonies were transferred to separate 500 ml cotton-plugged Erlenmeyer flasks, each containing 250 ml of Malt Yeast medium without agar. The colonies were incubated at 25°C for 11 days. Each flask was well shaken by hand twice a day.

After 11 days, the liquid medium was decanted off, and colonies washed by centrifugation at 4340 x G in an automatic refrigerated centrifuge for two 30-minute periods, following which they were ground in a sterile tissue grinder. One ml from the resulting solution was added to a sterile test tube to which the previously isolated Frankia was subsequently added. This procedure was done for each of the 30 actinomycete isolates.

Before being added to the actinomycete isolates, Frankia was washed three times with sterile distilled

water, ground in a tissue grinder, and the resulting solution added to 500 ml of sterile distilled water. Nineteen ml of this solution were added to each of the 30 test tubes containing the actinomycete colonies. Four tubes contained only Frankia.

Plant Culture

Red alder seeds from the Forest Science Laboratory stock in Corvallis were surface sterilized in 30% hydrogen peroxide for 5 minutes. After sterilization, the seeds were placed in a Petri dish containing wet filter paper and left in a water bath at room temperature until germination. Sterile distilled water was added to keep the filter paper wet.

Seedlings were grown in a 1:1 autoclaved soil mixture of peat moss and vermiculite. Five germinating seeds were planted in surface sterilized Ray Leach tubes (150 cc capacity). A thin layer of sterilized silica was used to cover the seeds. At three weeks of age, seedlings were thinned to one per tube. Of the 320 seedlings used in this experiment, 10 were not inoculated, 10 were inoculated only with Frankia and the remaining 300 inoculated with mixtures of Frankia and actinomycetes (10 seedlings with each of the 30 actinomycete isolates). Seedlings were inoculated when 1 month old. Each test tube was well shaken by hand, and

1 cc of inoculum suspension equivalent to 0.05 ml of packed cell volume (3,000 rpm, 15 min) (Vogel and Dawson, 1985) was inoculated into each seedling in the greenhouse. One week after inoculation, each seedling was fertilized using a 10 cc mineral solution without nitrogen (Pregent and Camire, 1985). This procedure was repeated once per week until harvesting. The composition for the nutrient solution, in mmoles/l, was: $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ -- 1; K_2SO_4 -- 1.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ -- 4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -- 1; and, in umoles/l, NaFe EDTA -- 89.5; H_3BO_3 -- 46.2; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ -- 9.1; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ -- 0.5; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ -- 0.3; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ -- 0.8; ZnCl_2 -- 0.8.

Seedlings were grown in the greenhouse for 6 months. The temperature was kept at 24°C during the day and at 18°C during the night. A 16-hour per day photoperiod was maintained by using sodium-vapor lamps at 11,000 Lux. Seedlings were watered twice a day until harvest.

Seedlings receiving different inoculation treatments were separated by at least 20 cm on the greenhouse benches in order to reduce the possibility of cross contamination, and systematically rotated to different bench positions once weekly to minimize differences due to greenhouse location.

Data Collection and Analyses

Three variables were measured: Acetylene reduction,

oven-dry nodule weight, and total dry biomass of seedlings. In order to measure acetylene reduction, each seedling was placed in a 400 ml plastic tube, the root system was sealed from the rest of the seedling using a rubber stopper and a caulking agent, and 40 ml of prepurified C_2H_2 were injected into the tube through a plastic syringe. Seedlings were incubated for 2 hours, after which 2 ml of air was extracted into a vacutainer. To determine the total area of C_2H_4 formed, 0.1 ml of gaseous samples were injected into a model 5830 Hewlett-Packard gas chromatograph with an integrator. These results were then converted to umoles of C_2H_2 reduced per g oven-dry nodule per hour.

In order to measure oven-dry nodule weight and total oven-dry biomass, nodules were first excised from the roots; then nodules, roots, and tops were dried in an oven at $70^{\circ}C$ for 3 days. After this period, the nodules, leaves, stems, and roots of each seedling were weighed.

Statistical analyses were done using Statgraphics (version 2.6). Two types of ANOVA were performed. The thirty actinomycete treatments were analyzed using a nested design in which isolates were nested within the source of the isolate (nodule surface, root surface, or open soil). In addition, a simple one-way ANOVA was performed on the thirty actinomycete treatments plus the Frankia-only control (seedlings not inoculated with

Frankia grew and survived very poorly and were not included in the analyses). Fisher's protected LSD, calculated from the error variance of the one-way ANOVA, was used to compare the various actinomycete isolates to the treatment inoculated with Frankia only. Three correlations were also performed; seedling biomass on acetylene reduction, acetylene reduction on nodule weight and nodule weight on seedling biomass.

RESULTS

Rate of acetylene reduction (umoles C_2H_2 reduced per gram of nodule per hour), nodule weight, and seedling biomass, all varied significantly depending on actinomycete isolate, while acetylene reduction expressed as umoles C_2H_2 reduced per plant per hour did not (Table 1). Source of isolate (nodule surface, root surface, or soil) did not influence seedling weight, acetylene reduction per gram nodule weight or acetylene reduction per plant. But source had a significant effect ($P < 0.10$) on nodule weight; seedlings inoculated with isolates from nodule surfaces produced the greatest nodule weights (Table 2). Of the thirty actinomycete isolates, fifteen produced one or more seedling responses that differed ($P < 0.05$) from those of seedlings inoculated with Frankia only (Figures 1, 2, 3, 4). However, the effect of actinomycetes was quite different for the different seedling variables. Five isolates produced a significant increase in C_2H_2 reduction per gram nodule weight, while none produced a decrease (Figure 1). One isolate decreased total nodule weight (Figure 2), and none influenced C_2H_2 reduced per plant (Figure 3). Actinomycetes had a strong negative effect on total seedling biomass, thirteen of the isolates reducing it while none enhanced it (Figure 4). Of the five isolates that increased C_2H_2 reduced per gram

nodule, three also reduced total seedling biomass. None of the five influenced nodule biomass. Of the 13 isolates that reduced seedling biomass, 5 were from open soil, 6 from root surfaces, and 2 from nodule surfaces. We used the χ^2 goodness of fit statistic to test whether the proportion of inhibitory isolates differed between those isolated from nodule surfaces as compared from soil and root surfaces. A χ^2 value of 6.24 was calculated, which indicates that the number of inhibitory isolates differed between the two groups at the $p = 0.05$ level of significance.

In general, the measured variables did not correlate with one another. However, two correlations were statistically significant but with low explanatory power. Nodule weight correlated positively to seedling biomass ($r^2 = 0.27$, $P < 0.0001$), and seedling biomass correlated positively to acetylene reduction per seedling ($r^2 = 0.09$, $P < 0.0001$).

Seedlings not inoculated with Frankia did not grow. These seedlings were weak and yellowish in color averaging only 0.012 g oven-dry biomass per seedling. Although none of the uninoculated seedlings were nodulated, they did reduce very small amounts of C_2H_2 (0.3 umoles C_2H_2 reduced per plant per hour versus 3.4 umoles C_2H_2 reduced per inoculated plant per hour). This was probably due to free-living or associative

nitrogen fixing bacteria. Contamination in the greenhouse is common, especially due to watering.

DISCUSSION

Actinomycetes have been shown to have allelopathic effects on other microorganisms and on higher plants. Streptomyces griseolobus isolated from surfaces of Ceanothus velutinus nodules inhibited growth of the root rot fungi Phellinus weirii, Fomes annosus, and Phytophthora cinnamomi (Rose et al., 1980). Work by Katz et al. (1987) suggests that suppression of annuals around the desert shrubs Coridothymus capitatus is related to allelopathic effects of actinomycetes living in soils around the shrub.

Vegetation type can have a strong influence on both, the population density of soil actinomycetes and the degree of allelopathy that they express. Hutchins and Li (1981), in a study using soils from under conifer and alder stands, found that conifer soil contained higher numbers of Streptomyces spp. than alder soil, and a greater percentage of Streptomyces isolated from conifer soil were antagonistic to Phellinus weirii. They attributed the difference to soil pH, being 5.3 in conifer soil and 4.6 in alder soil.

Perry and Rose (1983) in a study done in southern Oregon found an increase in the percentage of Streptomyces spp. antagonistic to the pathogen Phellinus weirii and to the mycorrhizal fungus Laccaria laccata in soils that had been logged or logged and burned.

Recently, soil-borne actinomycetes have been related to reforestation failures in southwestern Oregon; there it has been conjectured that after a delay in reforestation, phytotoxic actinomycetes interfere with the regeneration of Douglas-fir (Friedman et al., 1989).

In our study, a high proportion--nearly fifty percent of the isolates used--reduced seedling growth. Further work will be needed to elucidate the mechanism of inhibition, however it was not related to seedling nitrogen fixation, which was unaffected by actinomycetes. Although ANOVA did not show the source of actinomycete isolate to have a statistically significant effect on seedling biomass, the χ^2 test indicates that a disproportionate number of the inhibitory isolates were from either open soil or root surfaces rather than from nodule surfaces. Apparently, there is some discrimination against inhibitory isolates on nodule surfaces.

Table 1. Summary of results from ANOVA for measured variables

Source	d.f.	Nodule weight		Total plant weight		C ₂ H ₂ reduction per gram nodule per hour		C ₂ H ₂ reduction per plant per hour	
		MS	F	MS	F	MS	F	MS	F
Isolation source	2	2.7x10 ⁻³	2.77*	1.2870	1.47	292.6394	0.45	3.3049	1.32
Isolates w/i source	27	9.7x10 ⁻⁴	1.97***	0.8748	2.48***	652.5931	2.78***	2.5071	1.02
Error	270	4.9x10 ⁻⁴		0.3528		234.9531		2.4676	

***Significant at the 1% level

*Significant at the 10% level

Table 2. Oven-dry weight (g) of nodules of red alder seedlings inoculated with *Frankia* and with actinomycetes isolated from either nodule surfaces, root surfaces, or soil. (Means followed by same letter do not differ at $P=0.05$).

Source of isolation	Actinomycetes from nodule surfaces	Actinomycetes from soil	Actinomycetes from root surfaces	Pooled standard error
Mean dry weight (g)	0.0905 a	0.0843 a,b	0.0803 b	0.0031

FIGURE 1. MEANS AND NINETY FIVE PERCENT LSD INTERVALS FOR ACETYLENE REDUCTION PER GRAM NODULE

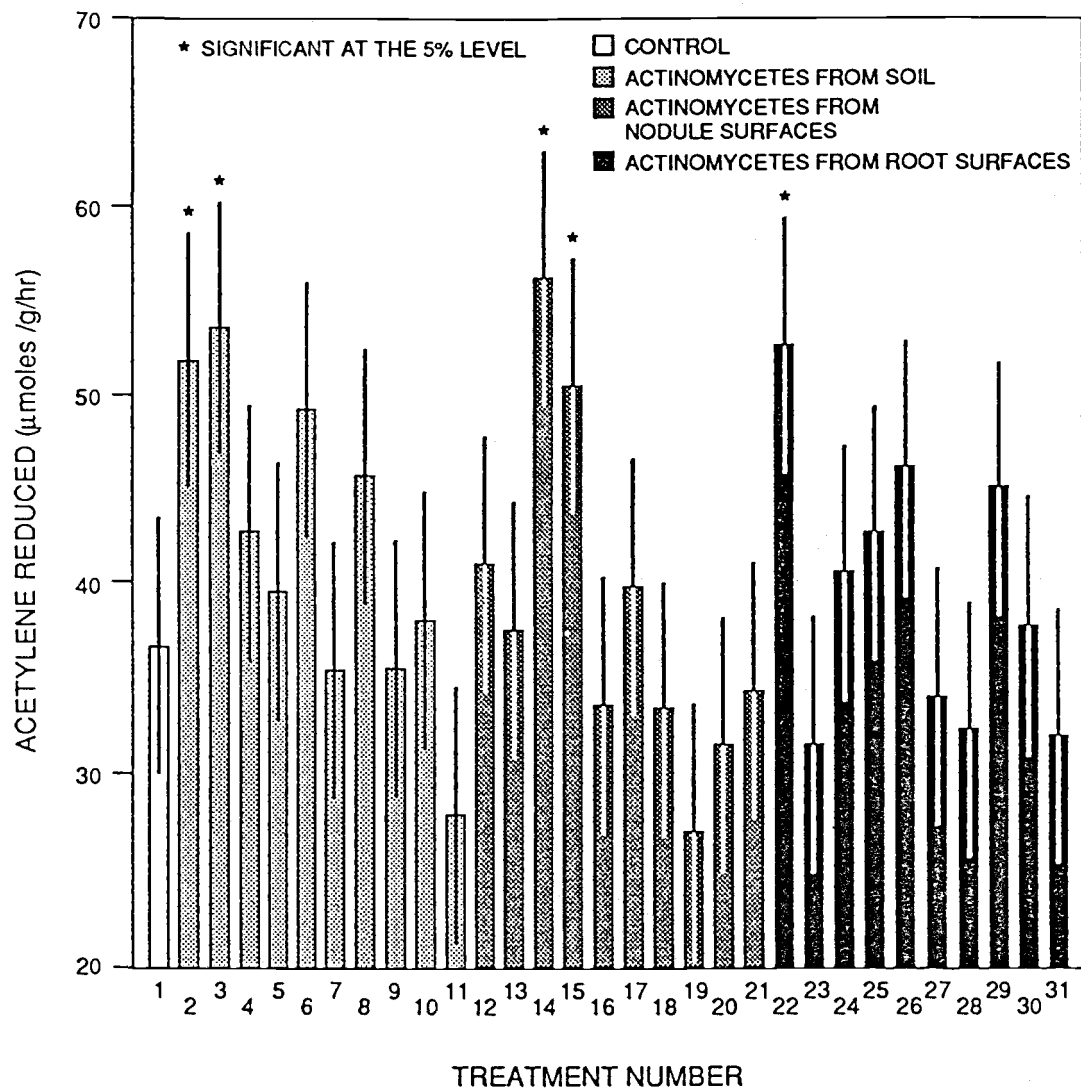


FIGURE 2. MEANS AND NINETY FIVE PERCENT LSD INTERVALS FOR OVEN-DRY NODULE WEIGHT

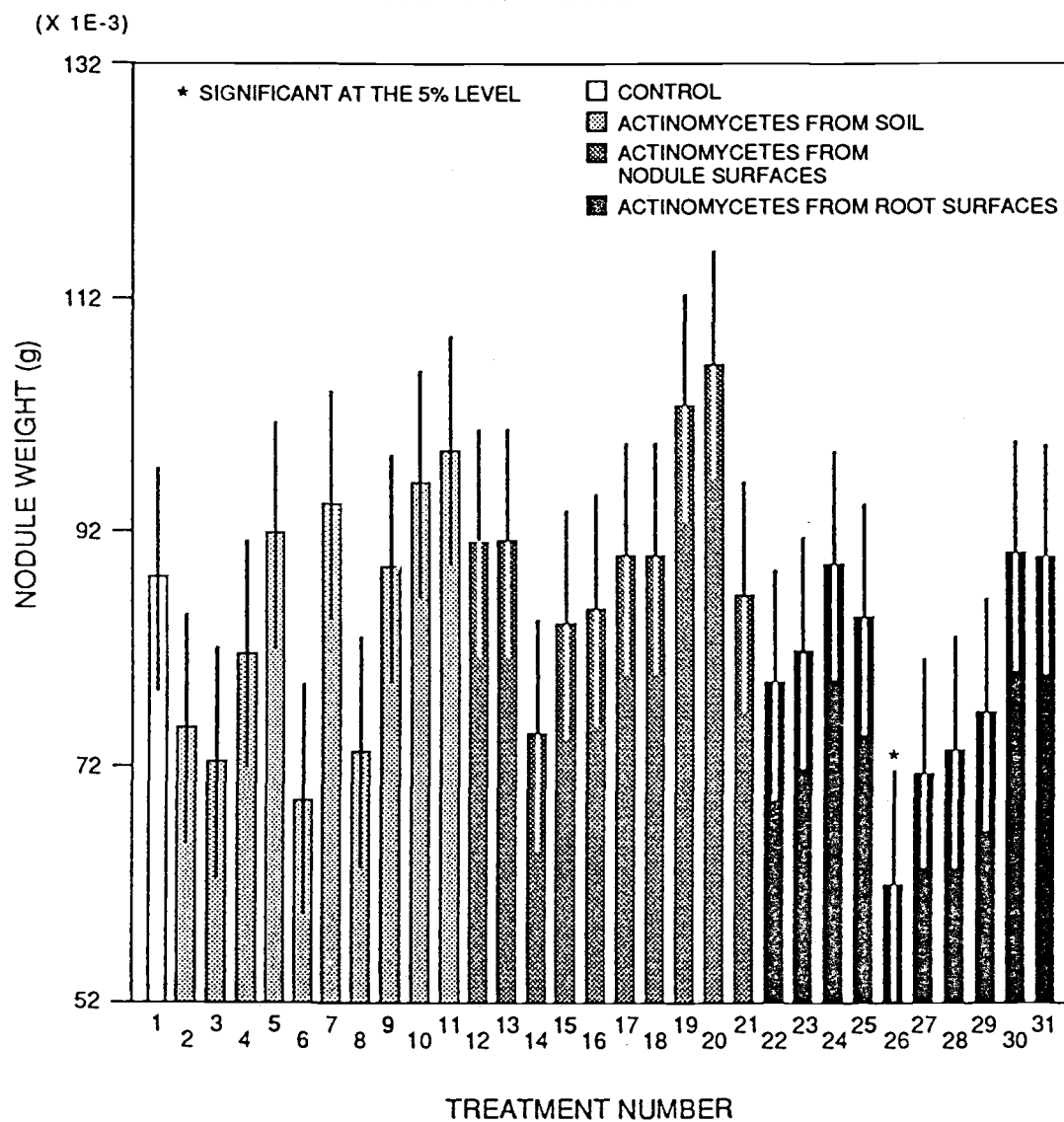


FIGURE 3. MEANS AND NINETY FIVE PERCENT LSD INTERVALS FOR ACETYLENE REDUCTION PER PLANT

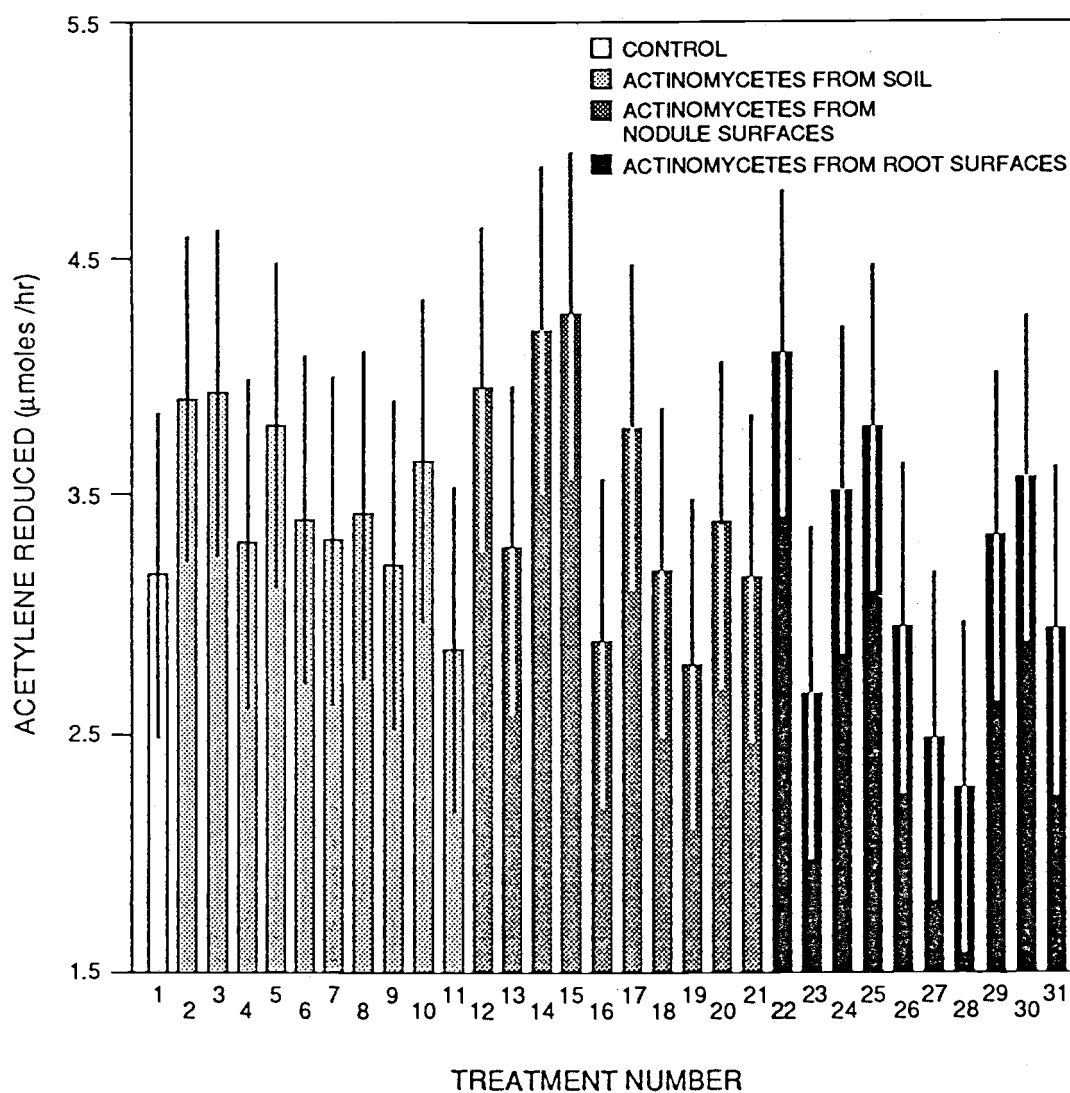
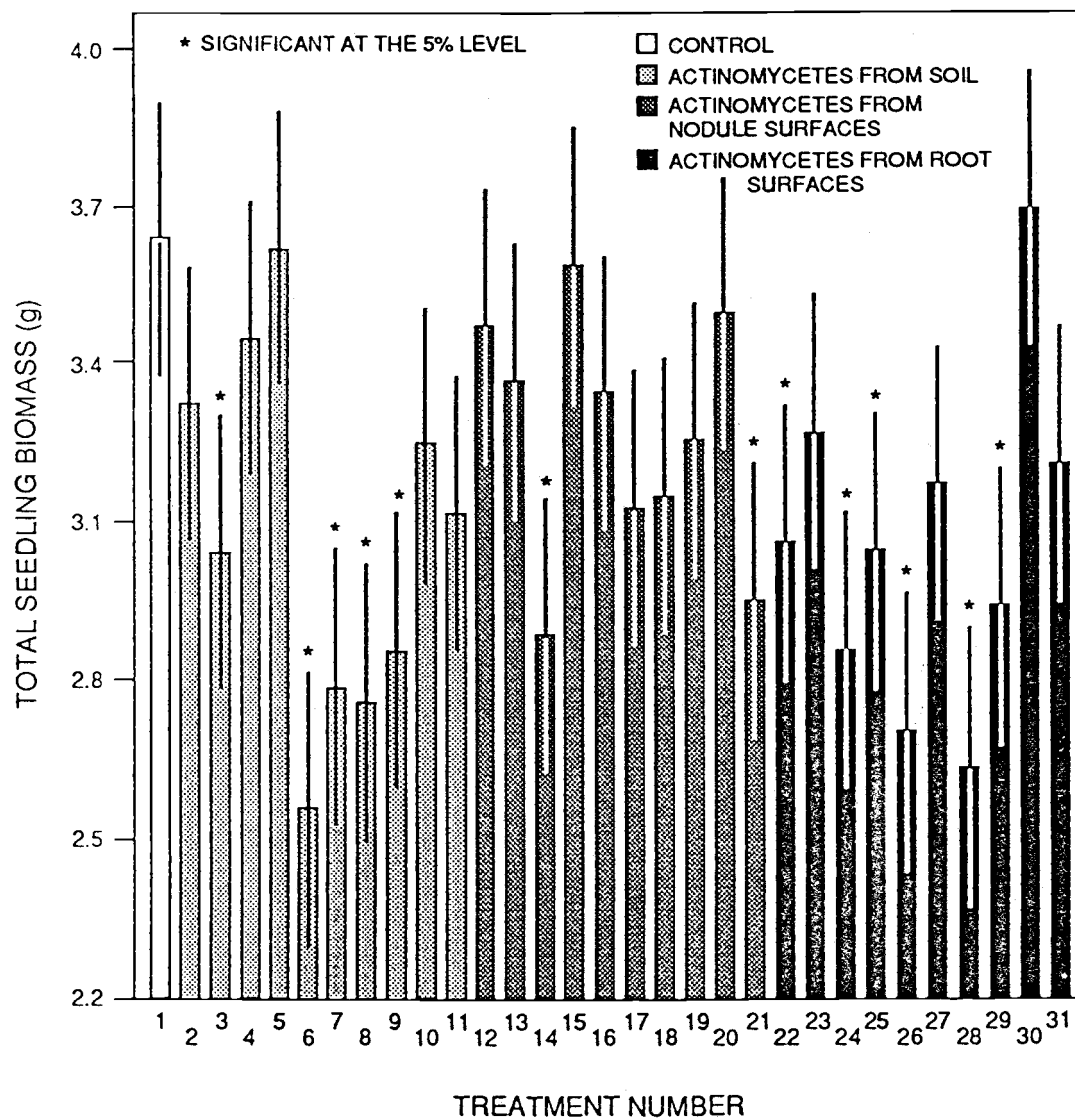


FIGURE 4. MEANS AND NINETY FIVE PERCENT LSD INTERVALS FOR TOTAL OVEN-DRY BIOMASS



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