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INFLUENCE OF ACTINOMYCETES ON *FRANKIA* INFECTION, NITROGENASE ACTIVITY AND SEEDLING GROWTH OF RED ALDER

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Summary—The influence of actinomycetes isolated from different sources was examined in respect to acetylene reduction, nodule weight and total biomass of red alder (*Alnus rubra* Bong.) seedlings. Soil samples and nodulated roots of young red alder were collected from a 5 yr old clearcut on Marys Peak, Ore. Nitrogen-fixing *Frankia* was isolated from root nodules, and other actinomycetes from nodule surfaces, roots and soil. Ten morphologically-different actinomycetes from each of the three sources were selected for the experiment. Red alder seedlings were greenhouse-grown in a 1:1 soil mixture of peat moss and vermiculite. After 1 month, seedlings were either inoculated with *Frankia* and an actinomycete isolate from one of the 30 colonies or inoculated with *Frankia* only. A control group was not inoculated. Five months after inoculation, seedlings inoculated with isolates from nodule surfaces had slightly greater nodule weight than seedlings inoculated with isolates from roots, but the source of isolate did not appear to influence other measured variables. However, the influence of the different isolates on C_2H_2 reduction g nodule^{-1}, nodule weight seedling^{-1} and total seedling weight varied significantly. Acetylene reduction seedling^{-1} did not differ. Seedling capacity for N_2 fixation was not associated with reduced seedling growth. With 5 of the 30 isolates, seedlings had greater C_2H_2 reduction g nodule^{-1} than those inoculated only with *Frankia*, and with one isolate, they had lower nodule weight. By far the major influence of actinomycetes was reduced seedling weight. Of 30 *Frankia*-actinomycete mixtures, 13 were associated with seedlings that were significantly smaller than seedlings inoculated with *Frankia* alone.

INTRODUCTION

Actinorhizal red alder (*Alnus rubra* Bong.), an important hardwood species in the northwestern U.S.A., covers 13% of the coastal commercial forest land of Oregon and Washington (Resch, 1988). The capacity of red alder to assimilate atmospheric nitrogen and to improve soil fertility make it a valuable component of Pacific Northwest ecosystems, which are limited by N supply (Cole *et al.*, 1978; DeBell, 1979; Perry and Rose, 1983). Actinomycetes are a poorly-understood but potentially important components of forest-soil microflora. They are well known for the production of substances that inhibit growth of fungi, bacteria and yeasts (Alexander, 1977), and have been shown to have allelopathic effects not only on other microorganisms but on higher plants (Friedman *et al.*, 1989).

Streptomyces griseoloalbus isolated from surfaces of nodules on *Ceanothus velutinus* has been shown to inhibit 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 shrub *Coridothymus capitatus* is related to allelopathic effects of actinomycetes living in soils around the shrub. Recently, actinomycetes have been related to reforestation failures in southwestern Oregon, and it has been conjectured that when reforestation is delayed, phytotoxic actinomycetes interfere with the survival and growth of Douglas-fir seedlings (Friedman *et al.*, 1989).

We hypothesized that actinomycetes isolated from root surfaces, nodule surfaces and soil would differ in their effect on nodulation, total seedling biomass and N_2 fixation. To test this hypothesis, we conducted a greenhouse experiment in which red alder seedlings were inoculated with *Frankia* and other actinomycetes isolated from open soil and from nodule and root surfaces of red alder seedlings.

MATERIALS AND METHODS

Study area

Soil samples and nodulated roots of red alder seedlings were collected in June 1987 from a 5 yr old clearcut on Marys Peak, 32 km southwest of Corvallis, Ore. (44°30′N, 123°33′W). Summers in the area are dry and winters wet, with an average annual precipitation of 2032 mm. The deep and well-drained soils, classified in the Klickitat and Kilchis series (Knezevich, 1975), are formed from intrusive and basalt rocks. The study site, 1000 m above sea level, is in the western hemlock zone described by Franklin

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and Dyrness (1973). Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] and western hemlock [*Tsuga heterophylla* (Raf.) Sarg.] are dominant in undisturbed forests, but western redcedar (*Thuja plicata* Donn), red alder, and bigleaf maple (*Acer macrophyllum* Pursh) are also present. Salal (*Gaultheria shallon* Pursh), vine maple (*Acer circinatum* Pursh), salmonberry (*Rubus spectabilis* Pursh) and swordfern [*Polystichum munitum* (Kaulf.) Presl] are typical understory plants.

Samples were collected within an area of *ca* 0.1 ha that was relatively flat (< 10% slope). Logs had been piled on the area during clearcutting, and organic layers were largely lost as they became mixed with mineral soil in the process. Scattered red alder seedlings had colonized the site after it was clearcut.

Sample collection

Nodulated roots were dug from 15 red alder seedlings within the 0.1-ha area. Soil was collected at 10–20 cm depth from the root zone of each of the 15 seedlings and at the same depth at least 4 m from the nearest alder in what is hereafter referred to as "open soil". Samples were kept on ice and returned to the laboratory within 2 h of collection.

Laboratory procedure for Frankia

Within 24 h of collection, *Frankia* vesicle clusters were isolated from nodules by filtration (Benson, 1982). Nodules were rinsed with tap water; stirred 15 min for surface sterilization in a beaker containing 100 ml distilled water, 100 ml Sani-clor bleach (sodium hypochlorite 6%), and a drop of Tween 20 (polyoxyethylene sorbitan monolaurate); then 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 first through a 52 μm mesh screen, then a 20 μm mesh screen.

Frankia vesicles retained on the 20 μm mesh screen were transferred to test tubes containing N-free BAP medium for *Frankia* (Murry *et al.*, 1984). The test tubes were kept at 30°C for 4 weeks and each day shaken by hand. After 2 weeks, *Frankia* had formed a white precipitate on the bottom of the 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 test tubes containing the BAP medium with 5.0 mM of NH_4Cl. The test tubes were kept 6 weeks at 30°C and were again shaken daily.

Laboratory procedure for actinomycetes

Actinomycetes were isolated from root surfaces, nodule surfaces and open soil by the procedure of Friedman *et al.* (1989). Of the 229 pure-culture actinomycete isolates recovered, 131 were from root surfaces, 52 from soil and 46 from nodule surfaces. From these, 30 morphologically-different colonies were selected for the experiment, 10 from each source. Each colony was transferred to a 500-ml Erlenmeyer flask containing 250 ml of malt yeast medium without agar and grown at 25°C for 11 days. Each flask was well shaken by hand twice a day.

After 11 days, the liquid medium was decanted, and each of the 30 cultures was centrifuged (4340 × G) and washed with sterile, distilled water, after which the *Frankia* pellet obtained was ground in a sterile tissue grinder. 1 ml from the resulting solution was added to a sterile test tube.

The *Frankia* culture was washed three times with sterile, distilled water and ground in a tissue grinder. The resulting solution was added to 500 ml of sterile, distilled water. 19 ml of this solution was added to each of the 30 tubes containing the individual actinomycete suspensions. Four other tubes contained only *Frankia*.

Plant culture

Red alder seeds from the stock of the Forestry Sciences Laboratory in Corvallis, Ore. were surface sterilized in 30% H_2O_2 containing a drop of Tween 20 for 25 min. After sterilization, the seeds were placed on a moist filter paper in a Petri dish exposed to light at room temperature until they germinated.

Seedlings were greenhouse-grown in a pasteurized 1:1 mixture of peat moss and vermiculite. Five germinating seeds were planted in surface-sterilized Ray Leach tubes (150 ml capacity) and covered with a thin layer of sterilized silica. At 3 weeks, seedlings were thinned to one per tube, and at 1 month, all but controls were inoculated with 1 ml of well-shaken inoculum suspension, equivalent to 50 μl packed cell volume (3000 rev min^{-1}, 15 min) (Vogel and Dawson, 1985). Of 320 seedlings, 10 were not inoculated, 10 were inoculated with *Frankia* alone and 300 were inoculated with *Frankia* and one of the 30 actinomycete isolates in mixture (10 seedlings for each of the 30 isolates).

One week after inoculation and every week until harvest, each seedling was fertilized with 10 ml of N-free mineral solution (Prégent and Camiré, 1985).

Seedlings were grown until harvest at 6 months with a 24–18°C (day–night) regime, a 16-h photoperiod maintained by sodium-vapor lamps at 11,000 lx, and twice-a-day watering. Those 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 were systematically rotated to different bench positions once weekly to minimize differences due to greenhouse location.

Data collection and analyses

Three variables were measured: nitrogenase activity (acetylene reduction), oven-dry weight of nodules, and oven-dry total seedling biomass.

For measurement of nitrogenase activity, each seedling was placed in a 400-ml plastic tube in which the root system was sealed from the rest of the plant

by a rubber stopper and a caulking agent. Commercially purified C_2H_2 (40 ml) was injected into the tube through a plastic syringe. After 2 h, a 0.1-ml gaseous sample from each tube was removed and analyzed for C_2H_4 and C_2H_2 with a Hewlett-Packard 5830A g.c. fitted with a 2 m × 2.1 mm, 80–100 mesh, Porapack R column; oven temperature 70°C. Injection temperature and f.i.d. temperature were adjusted to 100°C. Flow rate of the N_2 carrier gas was adjusted to 40 ml min^{-1}.

For measurement of nodule weight and total seedling biomass, nodules were first excised from roots; then nodules, roots, and tops were dried in an oven at 70°C for 3 days. Finally, nodules, leaves, stems and roots of each seedling were separately weighed.

Statistical analyses were made with Statgraphics (version 2.6). Analysis of variance of the 30 actinomycete treatments was made with a design in which isolates were nested within isolate source (nodule surface, root surface, open soil). A simple one-way analysis of variance was performed on the 30 actinomycete and the *Frankia*-only control treatments. Seedlings not inoculated with *Frankia* survived and grew 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 seedlings receiving the various actinomycete treatments with those inoculated with *Frankia* only. In order to summarize the overall effect of actinomycete isolates, contrast analysis (Petersen, 1985) was used to compare all plants inoculated with actinomycetes to plants inoculated with *Frankia* only. Three correlations were also performed: seedling biomass on C_2H_2 reduction, C_2H_2 reduction on nodule weight, and nodule weight on seedling biomass.

RESULTS

The source of isolate did not influence seedling weight or C_2H_2 reduction g $nodule^{-1}$ or $plant^{-1}$, but seedlings inoculated with isolates from nodule surfaces produced the greatest mean nodule weight, 90.5 mg, as opposed to 84.3 mg with soil ($P = 0.056$) and 80.3 mg with root-surface actinomycetes ($P = 0.003$). Nodule weight, seedling biomass and rate of C_2H_2 reduction expressed in C_2H_2 μmol g $nodule^{-1}$ h^{-1} varied significantly among actinomycete isolates within a source, while C_2H_2 reduction expressed in μmol C_2H_2 $plant^{-1}$ h^{-1} did not (Table 1).

With 15 of the 30 actinomycete isolates, one or more responses of seedlings differed significantly ($P < 0.05$) from those of seedlings inoculated with *Frankia* only (Figs 1–3); contrast analyses showed that actinomycetes as a group had a significant influence on plant biomass ($P = 0.009$), but not on any other variable. However, there was considerable variation among isolates in their effect on plants. No isolate influenced C_2H_2 reduction $plant^{-1}$ (Fig. 1). Five isolates were associated with significantly greater C_2H_2 reduction g $nodule^{-1}$; none with a decrease (Fig. 1). With one isolate, nodule weight decreased (Fig. 2).

With each of 13 *Frankia*-actinomycete mixtures, total biomass of seedlings was significantly lower than biomass of seedlings inoculated with *Frankia* only, and biomass was higher with none of the mixtures (Fig. 3). Of the 13 isolates associated with the lowest biomasses, five were from open soil, six from root surfaces, and two from nodule surfaces. A chi-square goodness-of-fit test of the proportion of inhibitory isolates from nodule surfaces and the proportion from root surfaces and soil indicated a significant difference ($P = 0.05$) between the two groups. Three of the five isolates associated with greater C_2H_2 reduction g $nodule^{-1}$ were also associated with lower seedling biomass.

The measured variables showed only two statistically significant correlations: nodule weight correlated positively with seedling biomass ($r = 0.52$, $P < 0.0001$), and seedling biomass correlated positively with C_2H_2 reduction $plant^{-1}$ ($r = 0.30$, $P < 0.0001$).

Seedlings not inoculated with *Frankia* were weak and yellowish and averaged only 12 mg oven-dry biomass. Although none of these seedlings were nodulated, they nevertheless reduced small amounts of C_2H_2 (0.3 vs 3.4 μmol h^{-1} for inoculated plants), probably because of free-living or associative N_2 fixing bacteria. Contamination due to watering in the greenhouse is common.

DISCUSSION

Our hypothesis that actinomycete isolates from different sources would differ in their effects on alder

Table 1. Analysis of variance of measured variables for red alder seedlings inoculated with actinomycete colonies isolated from nodule surfaces, root surfaces or open soil

								C_2H_2 reduction					
		Nodule weight			Total plant weight			μmol C_2H_2 g $nodule^{-1}$ h^{-1}			μmol C_2H_2 $plant^{-1}$ h^{-1}		
Source	d.f.	MS	F	P-value	MS	F	P-value	MS	F	P-value	MS	F	P-value
Isolation source*	2	2.7×10^{-3}	2.77	0.08	1.29	1.47	0.248	292.6	0.45	0.642	3.30	1.32	0.284
Isolates w/i source*	27	9.7×10^{-4}	1.97	0.004	0.87	2.48	0.0001	652.6	2.78	0.00001	2.51	1.02	0.441
Error	270	4.9×10^{-4}			0.35			235.0			2.47		

*Source = nodule surface, root surface or open soil.

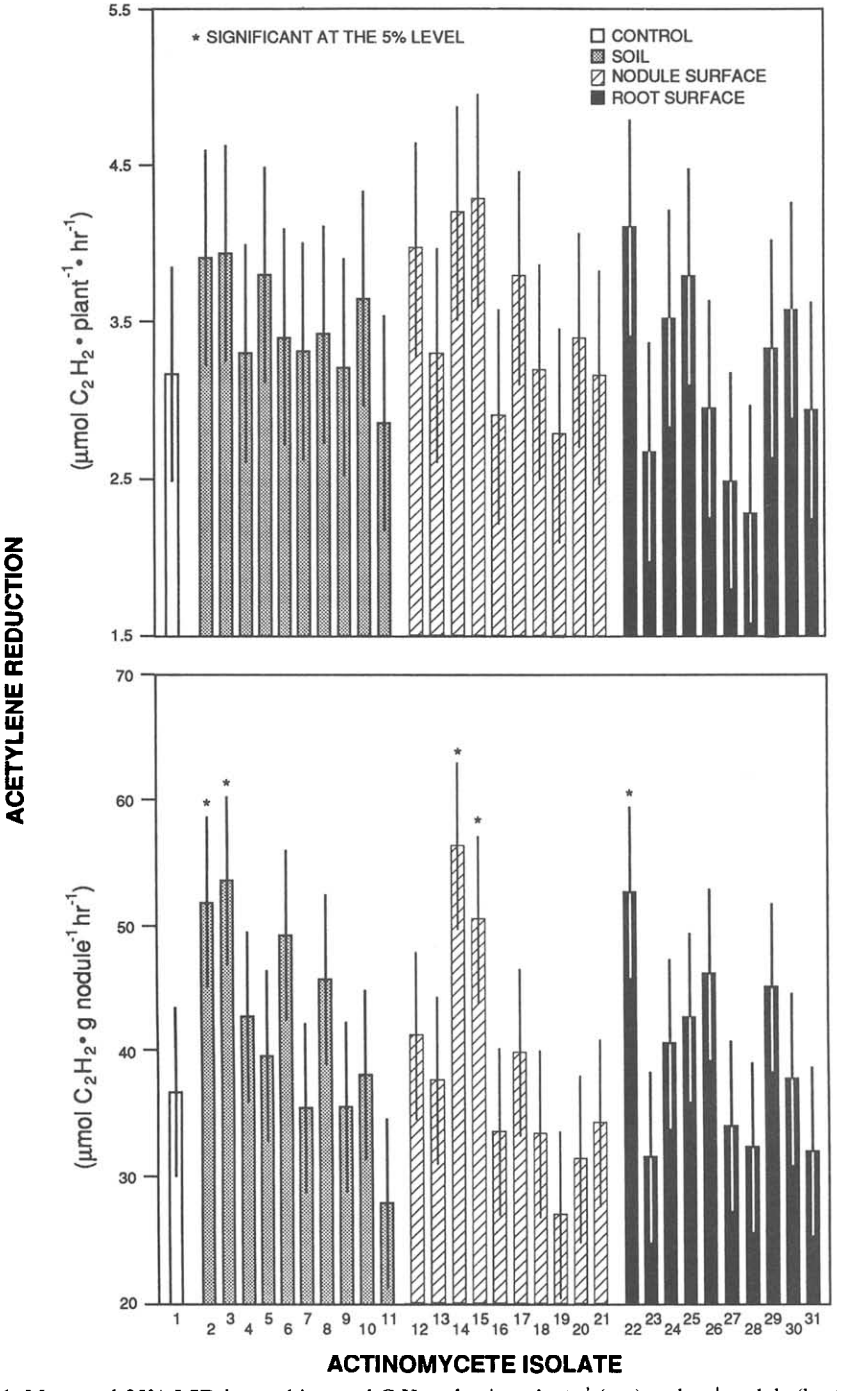

Fig. 1. Mean and 95% LSD interval in μmol C_2H_2 reduction plant^{-1} (top) and g^{-1} nodule (bottom) for red alder seedlings inoculated with individual actinomycete colonies (total = 30) isolated from three sources.

and the alder-*Frankia* mutualism was supported only in the case of nodule weight. Other investigators have found that actinomycete isolates that express allelopathy are in higher proportion in soils that are not occupied by perennial plants than in soils that are. Perry and Rose (1983), in a southern Oregon study, found that the percentage of *Streptomyces* spp antagonistic to the fungal pathogen *Phellinus weirii* and to the mycorrhizal fungus *Laccaria laccata* increased in soils that had been either logged or logged and burned. Friedman *et al.* (1989) found that 22.8% of the actinomycetes isolated from an unreforested clearcut inhibited seed germination of the test plants *Anastatica hierochuntica* L. or *Lactuca sativa* L.; whereas only 9.2% of isolates from an adjacent forested area did. In that study, 4% of

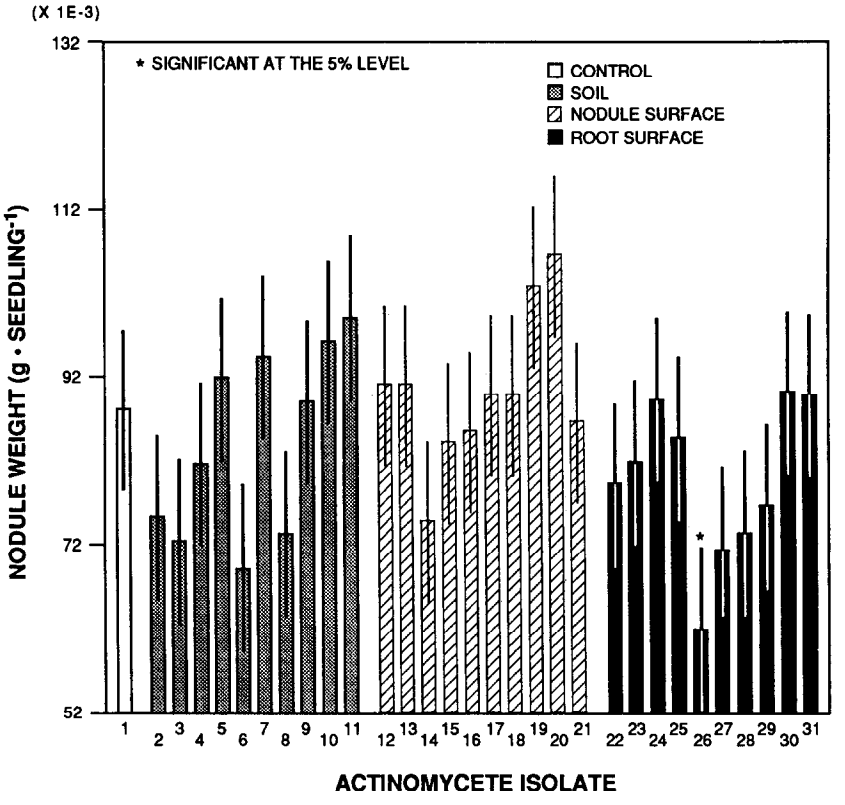

Fig. 2. Mean and 95% LSD interval in oven-dry nodule weight for red alder seedlings inoculated with individual actinomycete colonies (total = 30) isolated from three sources.

isolates from the clearcut and 2.6% from the forest reduced *in vitro* growth of the ectomycorrhizal fungi *Laccaria laccata* and *Hebeloma crustuliniforme*. Our ability to detect significant differences between isolate sources with regard to their effect on plant growth and C_2H_2 reduction was greatly reduced by the large variability among isolates within a source.

Even with no difference in the proportion of isolates expressing allelopathy, high numbers of actinomycetes may create a greater total inhibitory effect in soils not influenced by perennial plants than in soils beneath perennials. Friedman *et al.* (1989) isolated more than twice as many actinomycete colonies from an unreforested clearcut than from an adjacent forest. In the southwest Oregon clearcut studied by Borchers and Perry (1990), 10 times more actinomycetes were isolated from soils beneath annual grasses than from beneath sprouting hardwood trees. The greater abundance of actinomycetes in areas with little plant cover may be due to their ability to tolerate environmental extremes and to degrade complex substrates such as lignin (Crawford and Crawford, 1980; Friedman *et al.*, 1989).

Nearly half of the isolates we investigated reduced seedling growth. Further work will be needed to elucidate the mechanism of inhibition; however, it was not related to seedling N_2 fixation, which was unaffected by actinomycetes. Previous investigators have reported that actinomycetes and other unidentified soil microorganisms inhibit seed germination and growth of different plants. DeFrank and Putman (1985), isolating actinomycetes from nine different soils, found that 8 isolates of a total of 120 inhibited germination and growth of cucumber and barnyard grass. Katz *et al.* (1987) reported that some soil-borne actinomycetes isolated from samples around the shrub *Coridothymus capitatus* inhibited seed germination and seedling growth of *Lactuca sativa* and *Anastatica hierochuntica*. Similarly, unidentified soil microorganisms inhibited seed germination and growth of herbaceous vegetation under the canopy of mature shrubs of *Adenostoma fasciculatum* in California (Kaminsky, 1981). Gray (1955) reported that 20 μg streptomycin sulfate ml^{-1} caused as much as 50% inhibition of root growth of germinating tomato plants, and 25 μg streptomycin ml^{-1} inhibited root and stem growth of larger plants. Interestingly, both Gray (1955) and Brian (1956) found that inhibition of plant growth by streptomycin was reversed by calcium and manganese, especially the latter. Accumulation of those nutrients in root zones may protect plants from allelochemicals produced by actinomycetes, and may explain, in part at least, the relatively low abundance of actinomycetes in soils beneath perennial plants (Borchers and Perry, 1990).

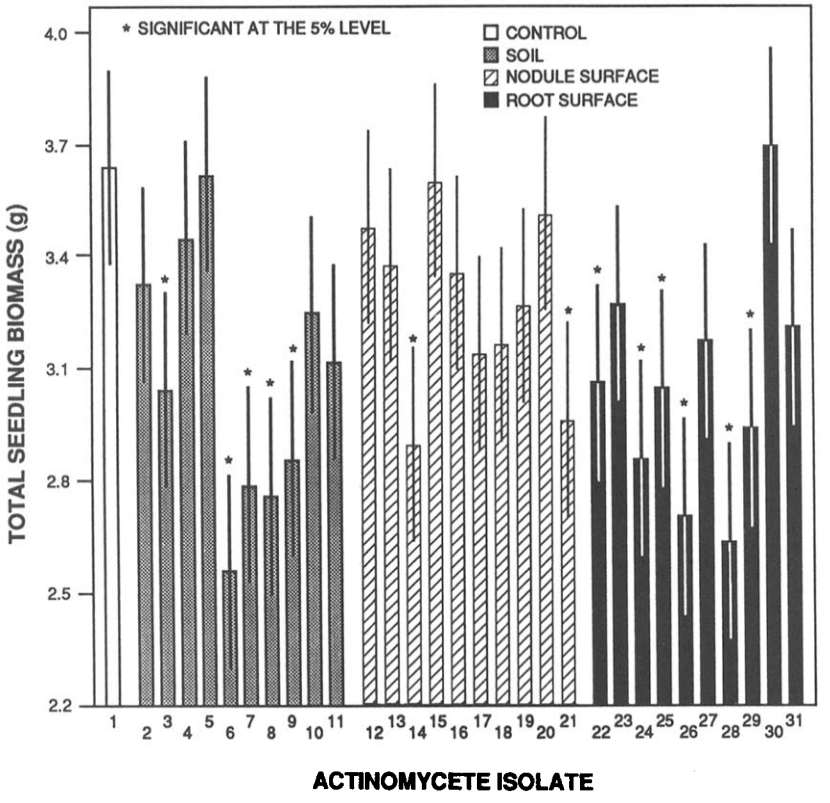

Fig. 3. Mean and 95% LSD interval in total oven-dry biomass of red alder seedlings inoculated with individual actinomycete colonies (total = 30) isolated from three sources.

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REFERENCES

Alexander M. (1977) *Introduction to Soil Microbiology*. Wiley, New York.

Benson D. R. (1982) Isolation of *Frankia* strains from alder actinorhizal root nodules. *Applied and Environmental Microbiology* **44,** 461–465.

Borchers S. L. and Perry D. A. (1990) Growth and ectomycorrhiza formation of Douglas-fir seedlings grown in soils collected at different distances from pioneering hardwoods in southwest Oregon clear-cuts. *Canadian Journal of Forest Research* **20,** 712–721.

Brian P. W. (1956) Effects of antibiotics on plants. *Annual Review of Plant Physiology* **8,** 413–426.

Cole D. W., Gessel S. P. and Turner J. (1978) Comparative mineral cycling in red alder and Douglas-fir. In *Utilization and Management of Alder* (D. G. Briggs, D. S. DeBell and W. A. Atkinson, Eds), pp. 327–336. Pacific Northwest Forest and Range Experiment Station, Portland, Ore. General Technical Report PNW-70.

Crawford D. L. and Crawford R. L. (1980) Microbial degradation of lignin. *Enzyme and Microbial Technology* **2,** 11–22.

DeBell D. S. (1979) Future potential for use of symbiotic nitrogen fixation in forest management. In *Symbiotic Nitrogen Fixation in the Management of Temperate Forests* (J. C. Gordon, C. T. Wheeler and D. A. Perry, Eds), pp. 451–466. Forest Research Laboratory, Oregon State University, Corvallis, Ore.

DeFrank J. and Putnam A. R. (1985) Screening procedures to identify soil-borne actinomycetes that can produce herbicidal compounds. *Weed Science* **33,** 271–274.

Franklin J. F. and Dyrness C. T. (1973) *Natural Vegetation of Oregon and Washington*. Oregon State University Press, Corvallis, Ore.

Friedman J., Hutchins A., Li C. Y. and Perry D. A. (1989) Actinomycetes inducing phytotoxic or fungistatic activity in a Douglas-fir forest and in an adjacent area of repeated regeneration failure in southwestern Oregon. *Biologia Plantarum* **31,** 487–495.

Gray R. A. (1955) Inhibition of root growth by streptomycin and reversal of the inhibition by manganese. *American Journal of Botany* **42,** 327–331.

Kaminsky R. (1981) The microbial origin of the allelopathic potential of *Adenostoma fasciculatum* H & A. *Ecological Monographs* **51,** 365–382.

Katz D. A., Sneh B. and Friedman J (1987) The allelopathic potential of *Coridothymus capitatus* L. (Labiatae). Preliminary studies on the roles of the shrub in the inhibition of annuals germination and/or to promote allelopathically active actinomycetes. *Plant and Soil* **98,** 53–66.

Knezevich C. A. (1975) Soil Survey of Benton County Area, Ore. USDA Soil Conservation Service, Washington, D.C.

Murry M. A., Fontaine M. S. and Torrey J. G. (1984) Growth kinetics and nitrogenase induction in *Frankia* sp. HFPArl 3 grown in batch culture. *Plant and Soil* **78,** 61–78.

Perry D. A. and Rose S. L. (1983) Soil biology and forest productivity: opportunities and constraints. In

I.U.F.R.O. Symposium on Forest Site and Continuous Productivity (R. Ballard and S. P. Gessel, Eds), pp. 229–238. Pacific Northwest Forest and Range Experiment Station, Portland, Ore.: General Technical Report PNW-163.

Petersen R. G. (1985) *Design and Analysis of Experiments*. Dekker, New York.

Prégent G. and Camiré C. (1985) Mineral nutrition, dinitrogen fixation, and growth of *Alnus crispa* and *Alnus glutinosa*. *Canadian Journal of Forest Research* **15**, 855–861.

Resch H. (1988) *Red Alder: Opportunities for Better Utilization of a Resource*. Special Publication 16. Forest Research Laboratory, Oregon State University, Corvallis. Ore.

Rose S. L., Li C. Y. and Hutchins A. S. (1980) A streptomycete antagonist to *Phellinus weirii*, *Fomes annosus*, and *Phytophthora cinnamomi*. *Canadian Journal of Microbiology* **26**, 583–587.

Vogel C. S. and Dawson J. O. (1985) Effect of juglone on growth *in vitro* of *Frankia* isolates and nodulation of *Alnus glutinosa* in soil. *Plant and Soil* **87**, 79–89.