Influence of vegetation type and madrone soil inoculum on associative nitrogen fixation in Douglas-fir rhizospheres[1]

M. P. AMARANTHUS[2]

Department of Forest Science, College of Forestry, Oregon State University, Corvallis, OR 97331, U.S.A.

C. Y. LI

Forestry Sciences Laboratory, Pacific Northwest Research Station, USDA Forest Service, Corvallis, OR 97331, U.S.A.

AND

D. A. PERRY

Department of Forest Science, College of Forestry, Oregon State University, Corvallis, OR 97331, U.S.A.

Received February 3, 1988

Accepted October 18, 1989

AMARANTHUS, M. P., LI, C. Y., and PERRY, D. A. 1990. Influence of vegetation type and madrone soil inoculum on associative nitrogen fixation in Douglas-fir rhizospheres. Can. J. For. Res. **20**: 368–371.

Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings grown on a site cleared of whiteleaf manzanita (*Arctostaphylos viscida* Parry) and an adjacent, cleared, annual grass meadow were either inoculated with 100–120 mL per seedling of pasteurized or unpasteurized soil from a nearby Pacific madrone (*Arbutus menziesii* Pursh) stand or left uninoculated. After one growing season, Douglas-fir seedling whole-plant soil systems were assayed for nitrogenase activity by the acetylene reduction method. The rate of acetylene reduction in rhizospheres of uninoculated seedlings from the manzanita site (1.40 ± 0.44 nmol·h^{-1}) was significantly higher than that of uninoculated seedlings from the meadow site (0.67 ± 0.15 nmol·h^{-1}). Unpasteurized madrone soil increased the rate of acetylene reduction over 500% for inoculated seedlings grown on the manzanita site, but decreased it by 80% for those grown on the meadow site. The madrone soil influence was apparently biotic: pasteurized, madrone soil did not have a significant effect. No acetylene was reduced in soil without seedlings. *Azospirillum* sp., a microaerophilic nitrogen (N_2) fixing bacterium, was isolated from within the mycorrhizae of inoculated seedlings harvested from the manzanita site. These results suggest that early successional ectomycorrhizal shrubs and hardwood trees may be important in maintaining mycorrhizal fungi and associated N_2 fixers after severe disturbance.

AMARANTHUS, M. P., LI, C. Y., et PERRY, D. A. 1990. Influence of vegetation type and madrone soil inoculum on associative nitrogen fixation in Douglas-fir rhizospheres. Can. J. For. Res. **20** : 368–371.

Des semis de Sapin Douglas (*Pseudotsuga menziesii* (Mirb.) Franco) cultivés sur une station éradiquée d'*Arctostaphylos viscida* Parry, et sur une station adjacente d'une prairie annuelle, ont été inoculés avec chacun 100–120 mL par semis d'un sol pasteurisé ou non pasteurisé d'un peuplement d'*Arbutus menziesii* (Pursh) ou laissé non inoculé. Après une saison de croissance, les systèmes sol des plants entiers de Sapin de Douglas ont été évalués pour l'activité nitrogénase par la méthode de la réduction de l'acétylène. Le taux de réduction d'acétylène dans les rhizosphères de semis non

[1]Paper No. 2335 of the Forest Research Laboratory, Oregon State University, Corvallis.

[2]Author to whom all correspondence should be addressed.

Printed in Canada / Imprimé au Canada

inoculés de la station d'*Arctostaphylos* (1,40 ± 0,44 nmol·h^{-1}) était significativement plus élevé que les semis non inoculés de la station de prairie (0,67 ± 0,15 nmol·h^{-1}). Le sol non pasteurisé d'*Arbutus* a augmenté le taux de réduction d'acétylène de plus de 500% pour les semis inoculés cultivés sur la station d'*Arctostaphylos*, mais a diminué celui-ci de 80% pour ceux cultivés sur la station en prairie. L'influence du sol à *Arbutus* était apparemment biotique : pasteurisé, le sol à *Arbutus* n'a pas présenté d'effet significatif. L'acétylène n'était pas réduit dans le sol sans semis, *Azospirillum* sp., une bactérie microacrophilique fixatrice d'azote (N_2), a été isolée des systèmes mycorhiziens des semis inoculés récoltés de la station d'*Arctostaphylos*. Ces résultats suggèrent que les arbustes ectomycorhizés des stades premiers de succession et les arbres feuillus peuvent être importants dans le maintien des champignons mycorhizateurs et les fixateurs de N_2 associés après une perturbation sévère.

[Traduit par la revue]

Introduction

Nitrogen is generally believed to be the most limiting nutrient in Pacific Northwest forests (Johnson *et al.* 1982). In the absence of symbiotic N_2-fixing plants, known natural inputs are far below those required for good tree growth, and productivity is believed to be maintained by efficient cycling. Associative N_2 fixation (N_2 fixation in association with roots and mycorrhizae) was first suggested by Richards and Voigt (1964), and the phenomenon seems well established (Chatarpaul and Carlisle 1983; Dawson 1983; Florence and Cook 1984; Cracknell and Lousier 1986; Li and Hung 1987). However, its ecological significance is largely unknown. Among the important research questions are how rates of associative fixation vary with habitat and among different tree species, and what role associative fixation plays in restoring site N after severe disturbance. Intense burns often result in large losses of N, and the mechanisms by which this lost site N is restored are significant for maintaining long-term forest productivity.

In this study, we compare nitrogenase activity in the rhizospheres of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings grown on two adjacent, cleared sites previously supporting very different vegetation types in southwest Oregon: a whiteleaf manzanita (*Arctostaphylos viscida* Parry) stand and a meadow of mixed annual grasses. Previous work had shown that seedlings planted on the cleared manzanita site had significantly greater growth and survival, and formed different mycorrhiza types, than seedlings on the meadow site (Amaranthus and Perry 1989). Addition of small amounts of unpasteurized soil from a nearby Pacific madrone (*Arbutus menziesii* Pursh) stand further enhanced differences. Others have found that associative N_2 fixation is stimulated by certain types of mycorrhizae (Bevege *et al.* 1978; Li and Hung 1987). In the present study we test the hypothesis that seedlings planted on the cleared manzanita site have significantly greater rhizosphere N_2 fixation than seedlings on the meadow site and that small amounts of unpasteurized soil from a nearby Pacific madrone stand accentuate these differences.

Methods

Site descriptions

The study was conducted in a small valley in the Siskiyou Mountains of southwest Oregon. Annual precipitation averages 65 cm, <10% falling from mid-May through mid-September. Plots were installed on a toe slope (southwesterly aspect, <10% slope) just above the valley bottom at about 385 m elevation within a dense stand of whiteleaf manzanita established by a fire that burned the valley in 1938, and in a meadow that was part of an old homestead dating from 1923. The manzanita stand contained virtually no other plant species. The meadow was stocked with various annual grasses and herbs, primarily hedgehog dogtail (*Cynosurus echinatus* L.), catchweed bedstraw (*Galium aparine* L.), roughstalk bluegrass (*Poa trivialis* L.), and *Anthriscus* spp. Soils in both vegetation types are fine loamy, mixed mesic Ultic Haploxeralfs, formed in granitic colluvium, and underlain by weathered granitic bedrock at 60–100 cm depth. Surface layers (to 18 cm) are dark greyish brown to brown sandy loams with fewer than 10% rock fragments. Sand, silt, and clay constitute, respectively, 52, 24, and 24% on the manzanita site and 54, 24, and 22% on the meadow site.

Field procedure

Seedlings used in the present study were part of a larger study described in Amaranthus and Perry (1989). In spring 1985, the manzanita site was cleared with chainsaws, and both the manzanita and meadow sites were scalped with hoes. Five replicate blocks were installed per vegetation type, each block receiving three soil treatments: (*i*) unpasteurized soil transferred from a nearby Pacific madrone stand; (*ii*) pasteurized soil (steam heated at 70°C for 3 h) transferred from the same location; and (*iii*) no transfer of soil (control). For the treatments receiving transfer soils, 100–120 mL of soil was added to the planting hole of each seedling at the time of planting. Transfer soils were collected from the feeder root zone of 12 madrones selected randomly, except that trees with nearby conifers were excluded. Soil from all madrones was combined and half was pasteurized. Nonmycorrhizal 1-0 Douglas-fir seedlings (nine per treatment per block) grown in Ray Leach fir cells were planted in a 3 × 3 array, with 40 cm between seedlings within a given treatment and 1 m between treatments.

Soil macronutrients and pH, determined from 12 samples collected randomly at the 15 cm depth from both sites (plus the madrone stand from which transfer soils were collected), did not differ significantly (Amaranthus and Perry 1989). Soil moisture content, measured gravimetrically on samples collected to the 35 cm depth at 2–3 week intervals between April 1 and October 1, 1985, was virtually identical on the manzanita and meadow sites throughout the 1985 growing season (Amaranthus and Perry 1989).

N_2-fixation analysis

One year following outplanting (April 1986), 42 seedlings from the manzanita and meadow sites (7 seedlings per treatment per site) were carefully excavated with their root systems and the surrounding soil intact. Samples of soil without roots, excavated at least 0.5 m from the nearest planted seedling in treatment plots, were collected and combined to serve as a control. Seedlings and soil were placed in 1150-mL pyrex tubes. Rubber stoppers were split to allow insertion of stems, then sealed with artist's clay and paraffin wax.

The whole-plant soil system was assayed for N_2 fixation by the acetylene reduction method (Döbereiner 1980; Hable 1983). A 7-mm gas port plugged with a serum stopper was located on the top of the rubber stopper. Ten percent of the gas in the tube was replaced with acetylene. Gas samples were withdrawn after 48 h and analyzed on a Hewlett-Packard 3830 gas chromatograph[3] equipped with a flame ionization detector and a 2 m × 2.1 mm column packed with 80- to 100-mesh Porapak R. Three seedlings per treatment per site in soil without acetylene injection, and seven

[3]The mention of trade names or commercial products does not constitute endorsement or recommendation for use by the authors or their institutions.

TABLE 1. Mean acetylene reduction rates (nmol/(h·seedling)) in Douglas-fir seedling rhizospheres, by vegetation type and soil treatment, in southwest Oregon

Soil treatment	Vegetation type		
	Manzanita	Meadow	p*
No soil transfer	1.40 (0.44)*a*	0.67 (0.15)*a*	<0.05
Unpasteurized madrone soil	7.55 (2.96)*b*	0.13 (0.13)*b*	<0.01
Pasteurized madrone soil	2.41 (0.48)*a*	0.85 (0.48)*ab*	<0.05

NOTE: Within each vegetation type (column), values followed by different lowercase letters are significantly different (Tukey test, $p < 0.05$). SE is given in parentheses.
*Levels at which the vegetation types (values within a row) are significantly different.

combined samples per site of soil without roots with and without acetylene injection, served as controls.

Mycorrhizae of the three seedlings with highest total nitrogenase activity were investigated for the presence of associative N_2-fixing bacteria. All three seedlings were from the manzanita site, two from the unpasteurized madrone soil transfer treatment and one from the control. Mycorrhizae, detached from roots and thoroughly washed under tap water to remove soil particles, were identified as *Rhizopogon vinicolor* based upon color and morphology. They were then immersed in 1% chloramine-T for 1 h, shaken frequently, and washed in five changes of sterile distilled water (Li and Hung 1987). The mycorrhizae were aseptically cut into 0.2- to 0.5-cm segments, which were placed onto a N-free semisolid agar medium with malate as a carbon source in a test tube and incubated at 30°C (Döbereiner 1980). This method was adopted to isolate N_2-fixing *Azospirilla* from the thoroughly surface sterilized mycorrhizae. Although a few other bacteria may have survived the surface sterilization, they likely had little ecological significance.

Four pure cultures of *Azospirillum* sp., the only free-living N_2 fixer isolated from the Douglas-fir mycorrhizae, were tested for acetylene reduction (Li and Hung 1987). An aqueous bacterial suspension (0.01 mL), prepared by suspending the isolate in sterile distilled water, was inoculated into serum bottles that contained 20 mL Döbereiner's N-free liquid medium supplemented with yeast extract and vitamins (Barber and Evans 1975). The bottles were capped with sterile serum stoppers and incubated under microaerophilic conditions (99% N_2 + 1% O_2) at 30°C. After 3–4 days, acetylene reduction, expressed per unit of bacterial protein, was determined by the aforementioned procedures. Bacterial protein was determined by harvesting cells within serum bottles and washing them with cold 5% trichloroacetic acid. Cell protein was solubilized in 0.5 M NaOH in a boiling water bath for 10 min (Agarwal and Keister 1983) and measured by the modified Lowry method (Markwell *et al.* 1978). This experiment was replicated 3 times. The Döbereiner's N-free medium with and without acetylene and the same medium with *Azospirillum* but without acetylene served as controls.

Data analysis

Data were subjected to an analysis of variance. To compensate for the lognormal distribution, we used log-transformed values for acetylene reduction (Steel and Torrie 1980). Tukey's multiple range test was used to examine differences among means.

Results and discussion

The acetylene reduction rate was significantly greater in the rhizospheres of uninoculated seedlings from the manzanita site than from the meadow (1.40 vs. 0.67 nmol·h^{-1}; Table 1). Inoculation with unpasteurized madrone soil had a drastically different effect on seedlings from the two sites: added to manzanita soil, unpasteurized madrone soil increased the acetylene reduction rate more than 500%, compared with that of uninoculated seedlings; added to meadow soil, it reduced the acetylene reduction rate by 80%. Pasteurized madrone soil did not significantly influence acetylene reduction in either vegetation type, and no acetylene was reduced in controls with soil without roots.

Differences in acetylene reduction rates correlated well only with numbers of tips colonized by mycorrhiza type A, which we identified as *Rhizopogon vinicolor* based on color and morphology. *Rhizopogon vinicolor* mycorrhizae were much more numerous on the manzanita than on the meadow site. After 3 days, incubation of mycorrhizae formed by *R. vinicolor* produced bacterial colonies with a distinctive white pellicle and other characteristics typical of the genus *Azospirillum* (Krieg and Döbereiner 1984; Bashan and Levanony 1985). The four isolates tested for nitrogenase activity, all from mycorrhizae identified as *R. vinicolor* from the manzanita site, formed an average of 60 nmol (SE = 4 nmol) of acetylene per hour per milligram of bacterial protein. *Azospirillum* species have previously been isolated from conifer rhizospheres (Florence and Cook 1984; Li and Hung 1987). This genus also occurs in association with grasses and cereal grains (e.g., Von Bulow and Döbereiner 1975; Barber *et al.* 1976; Nayak and Rao 1977; Tjepkema and van Berkum 1977).

The striking, opposite effect of madrone soil on associative N_2 fixation on the two sites was parelleled by differential effects on mycorrhiza formation. Unpasteurized madrone soil enhanced formation by *R. vinicolor* on seedlings on the manzanita site and also induced a new, unidentified mycorrhiza type (Amaranthus and Perry 1989). In contrast, unpasteurized madrone soil did not affect formation of mycorrhizae by *R. vinicolor* in the meadow, and formation by the new, unidentified type was much lower than on the manzanita site. There appears to be a link between differences in mycorrhiza types and associative N_2 fixation for the two sites.

The madrone soil influence was apparently biotic: pasteurized, madrone soil did not affect associative N_2 fixation on either site. The benefit of madrone soils on the manzanita site could reflect high levels of *Azospirillum* or other N_2-fixing bacteria beneath madrone; however, that madrone soil lowered N_2 fixation when added to the meadow site is not so readily explained. Both madrone and manzanita are ericaceous and ectomycorrhizal and may support similar, relatively compatible, soil microbial communities, whereas annual grasses might support different communities. Possibly, organisms in the madrone soil suppress indigenous N_2 fixers in meadow soils. Fallik *et al.* (1988) showed that high concentrations of fluorescent *Pseudomonas* (not compared in this study) decreased the effectiveness of *Azospirillum brasilensis* in maize rhizospheres.

Others have suggested a relationship between ectomycorrhizae and associative N_2 fixation. *Rhizopogon roseolus* was associated with increased N content of Monterey pine (*Pinus radiata* D. Don) seedlings (Richards and Voigt 1964), although this may have been due to enhanced uptake of soil N rather than associative fixation (Dawson 1983). Bevege *et al.* (1978) reported that ectomycorrhizae stimulated associative N_2 fixation by Caribbean pine (*Pinus caribaea* Morelet) and slash pine (*Pinus elliottii* Engelm.). Evidence also suggests that there is some degree of preferential association between N_2-fixing bacteria species and mycorrhizal fungus species. Li and Hung (1987) isolated *Azospirillum* spp. from mycorrhizae formed by *R. vinicolor* and *Thelephora* spp., and *Clostridium* spp. from mycorrhizae formed by *Laccaria laccata* and *Hebeloma crustuliniforme*. Nitrogen-fixing bacteria have also been isolated from sporocarps of various mycorrhizal fungi (Li and Castellano 1987) and from feces of mammals that feed on sporocarps (Li *et al.* 1986).

On an area basis, amounts of N added to ecosystems through associative fixation are probably much less than those added by the symbiotic N_2 fixers *Rhizobium* spp. and *Frankia* spp. However, because associative fixation is "targeted" within the rhizosphere, its significance to trees may be greater than indicated by simply calculating inputs on a kilogram per hectare basis. On N-limited sites, even small amounts of N added by bacteria living in association with mycorrhizae could enhance the performance of individual plants. Seedling growth and survival were, in fact, much better on the manzanita site than in the meadow. By the end of the second growing season, survival averaged 92% in the former compared with 42% in the latter (Amaranthus and Perry 1989). Seedling growth paralleled associative N_2 fixation. Average basal-area growth was 2 times greater on the manzanita site than the meadow site for uninoculated seedlings and 4 times greater for seedlings inoculated with unpasteurized madrone soil.

Molina and Trappe (1982) suggest that manzanita roots provide a reservoir of ectomycorrhizal fungi for conifer seedlings establishing in clear-cuts. Our work indicates that both manzanita and madrone provide not only mycorrhizae, but also associative N_2 fixers as well. Because heavy N losses accompany intense burns, mechanisms by which site N_2 is restored following natural fire or broadcast burning are significant for long-term forest productivity. Early successional ectomycorrhizal shrubs and hardwood trees may therefore be important in maintaining mycorrhizal fungi and associated N_2 fixers and may contribute to replenishing soil N following disturbance.

AGARWAL, A.K., and KEISTER, D.L. 1983. Physiology of ex planta nitrogenase activity in *Rhizobium japonicum*. Appl. Environ. Microbiol. **45**: 1592–1601.

AMARANTHUS, M.P., and PERRY, D.A. 1989. Interaction effects of vegetation type and Pacific madrone soil inocula on survival, growth, and mycorrhiza formation of Douglas-fir. Can. J. For. Res. **19**: 550–556.

BARBER, L.E., and EVANS, H.J. 1975. Characterization of a nitrogen-fixing bacterial strain from the roots *Digitaria sanguinalis*. Can. J. Microbiol. **22**: 254–260.

BARBER, L.E., TJEPKEMA, J.D., RUSSELL, S.A., and EVANS, H.J. 1976. Acetylene reduction (nitrogen fixation) associated with corn inoculated with *Spirillum*. Appl. Environ. Microbiol. **32**: 108–113.

BASHAN, Y., and LEVANONY, H. 1985. An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*. Can. J. Microbiol. **31**: 947–952.

BEVEGE, D.L., RICHARDS, D.N., and MOORE, A.W. 1978. Nitrogen fixation associated with conifers. C.S.I.R.O. Div. Soils. Div. Rep. No. 26.

CHATARPAUL, L., and CARLISLE, A. 1983. Nitrogen fixation: a biotechnological opportunity for Canadian forestry. For. Chron. **59**: 249–250.

CRACKNELL, P.C., and LOUSIER, J.D. 1986. Possible contribution of N_2-fixing *Azospirillum* spp. to conifer seedling vigor. *In* Conference on Roots in Forest Soils: Biology and Symbioses (Abstracts), 4–8 Aug. 1986, University of Victoria, Victoria.

DAWSON, J.O. 1983. Dinitrogen fixation in forest ecosystems. Can. J. Microbiol. **29**: 979–992.

DÖBEREINER, J. 1980. Forage grasses and grain crops. *In* Methods for evaluating biological nitrogen fixation. *Edited by* F.J. Bergersen. John Wiley & Sons, New York. pp. 535–555.

FALLIK, E., OKON, Y., and FISCHER, M. 1988. Growth response of maize roots to *Azospirillum* inoculation: effect of soil organic matter content, number of rhizosphere bacteria and timing of inoculation. Soil Biol. Biochem. **20**: 45–49.

FLORENCE, L.Z., and COOK, F.D. 1984. Asymbiotic N-fixing bacteria associated with three boreal conifers. Can. J. For. Res. **14**: 595–597.

HABLE, M. 1983. Apparatus for the nitrogenal (C_2H_2–C_2H_4) assay of intact whole-plant soil systems. Soil Biol. Biochem. **15**: 719–720.

JOHNSON, D.W., COLE, D.W., BLEDSOE, C.S., CROMACK, K., JR., EDMONDS, R.L., GESSEL, S.P., GRIER, C.C., RICHARDS, B.N., and VOGT, K.A. 1982. Nutrient cycling in forests of the Pacific Northwest. *In* Analysis of coniferous forest ecosystems in the western United States. *Edited by* R.L. Edmonds. Hutchinson Ross, Stroudsberg, PA. pp. 186–232.

KRIEG, N.R., and DÖBEREINER, J. 1984. Genus *Azospirillum*. *In* Bergey's manual of systematic bacteriology. Vol. 1. *Edited by* N.R. Krieg. Williams and Wilkins, Baltimore. pp. 94–104.

LI, C.Y., and CASTELLANO, M.A. 1987. *Azospirillum* isolated from within sporocarps of the mycorrhizal fungi *Hebeloma crustuliniforme*, *Laccaria laccata*, and *Rhizopogon vinicolor*. Trans. Br. Mycol. Soc. **88**: 563–565.

LI, C.Y., and HUNG, L.L. 1987. Nitrogen-fixing (acetylene-reducing) bacteria associated with ectomycorrhizae of Douglas-fir. Plant Soil, **98**: 425–428.

LI, C.Y., MASER, C., and FAY, H. 1986. Initial survey of acetylene reduction and selected microorganisms in the feces of 19 species of mammals. Great Basin Nat. **46**: 646–650.

MARKWELL, M.A.K., HAAS, S.M., BIEBER, L.L., and TOLBERT, N.E. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**: 206–210.

MOLINA, R., and TRAPPE, J.M. 1982. Lack of mycorrhizal specificity by the ericaceous hosts *Arbutus menziesii* and *Arctostaphylos uva-ursi*. New Phytol. **90**: 495–509.

NAYAK, D.N., and RAO, V.R. 1977. Nitrogen fixation by *Spirillum* sp. from rice roots. Arch. Microbiol. **115**: 359–360.

RICHARDS, B.N., and VOIGT, G.N. 1964. Role of mycorrhizae in nitrogen fixation. Nature (London), **201**: 310–311.

STEEL, R.G.D., and TORRIE, J.H. 1980. Principles and procedures of statistics—a biometrical approach. 2nd ed. McGraw-Hill, New York.

TJEPKEMA, J., and VAN BERKUM, P. 1977. Acetylene reduction by soil cores of maize and sorghum in Brazil. Appl. Environ. Microbiol. **33**: 626–629.

VON BULOW, J.F.W., and DOBEREINER, J. 1975. Potential for nitrogen fixation in maize genotypes in Brazil. Proc. Natl. Acad. Sci. U.S.A. **72**: 2389–2393.