HRI's Mission:

To direct, fund, promote and communicate horticultural research, which increases the quality and value of ornamental plants, improves the productivity and profitability of the nursery and landscape industry, and protects and enhances the environment.

The use of any trade name in this article does not imply an endorsement of the equipment, product or process named, nor any criticism of any similar products that are not mentioned.

Nodulation and Growth of *Alnus nitida* and *Alnus maritima* Inoculated with Species-specific and Nonspecific *Frankia*[1]

James A. Schrader and William R. Graves[2]
Department of Horticulture, Iowa State University, Ames, IA 50011-1100

Abstract

Actinorhizal plants form N_2-fixing symbioses with soil-borne bacteria of the genus *Frankia*. Potential exists for development of sustainable, actinorhizal nursery crops that obtain most of their required N through N_2 fixation, but information on host-symbiont specificity, presence of compatible *Frankia* in soils, and techniques to inoculate during plant production is lacking. Our objectives were to determine the effect of inoculum type and source and the effect of supplemental N on nodulation, growth, and N content of two actinorhizal species, *Alnus nitida* (Spach) Endl. and *Alnus maritima* (Marsh.) Muhl. ex Nutt. Plants of both species were subjected to one of four inoculum treatments (two crushed-nodule inocula: species-specific and cross inoculation, and two soil inocula: soil collected beneath native *Alnus rubra* Bong. in Washington state and native prairie soil from Iowa), were supplied fertilizer with or without N, and were grown in a greenhouse for 22 weeks. Inoculated plants nodulated, grew larger and faster, and accrued greater N content than uninoculated controls in both fertilizer treatments. Plants that received species-specific inoculum grew larger, acquired more dry weight from symbioses, and accumulated higher N content than cross-inoculated plants. Plants of *A. nitida* inoculated with soil from Washington state grew larger and accumulated more dry weight from symbioses than those inoculated with prairie soil, but *A. maritima* grew similarly with soil inoculum from both sources. Our results demonstrate that *A. nitida* and *A. maritima* can benefit from N_2-fixing symbiosis during production and that potential exists for development of superior inocula and inoculation techniques.

Index words: symbiotic nitrogen fixation, actinorhizal plants, sustainable nursery production, plant-microbe interaction, host-symbiont compatibility and efficiency.

Species used in this study: *Alnus nitida* (Spach) Endl. (Himalayan alder); *Alnus maritima* (Marsh.) Muhl. ex Nutt. subsp. *oklahomensis* J.A.Schrad. & W.R.Graves (seaside alder, Oklahoma alder); *Frankia* Brunchorst spp.

Significance to the Nursery Industry

The need to develop sustainable nursery and landscape practices is of utmost importance due to unpredictable and rising resource costs and the need to enhance ecosystem services [the benefits humans obtain from ecosystems (10)]. Consumers continue to demand affordable, vigorous plants from the nursery industry, but environmental concerns have caused both producers and consumers to prefer cultural practices that minimize environmental impacts. While consistent production of nursery crops has traditionally depended on high input of N fertilizer, which is costly both economically and environmentally, lower-input production methods must be developed for the industry to progress toward sustainability. N_2-fixing species have been utilized as sustainable agronomic and vegetable crops for generations, but their potential as eco-friendly alternatives to high-input nursery crops is just now being realized. The goals of our research were to evaluate the capacity of actinorhizal species to provide for their N requirements through symbiotic N_2 fixation and to determine the importance of inocula type, species specificity, and supplemental N for development of effective N_2-fixing symbioses in seedlings of *A. nitida* and *A. maritima*. Our results demonstrated that proper inoculation of actinorhizal species can reduce their need for N input during production. Our findings also indicated that *Frankia* strains compatible with *A. nitida* and *A. maritima* are common in soils, but host specificity affects symbiotic efficiency. Superior symbioses can be formed by inoculation with soils housing highly effective *Frankia* or with species-specific inoculum prepared from nodules of plants growing in native soils. These results provide important information for the development of low-input production protocols for actinorhizal nursery crops and provide basic knowledge concerning host-symbiont specificity and efficiency in two promising actinorhizal taxa.

Introduction

Actinorhizal species are trees and shrubs that function as pioneer plants on N-poor soils. Their potential to provide much of their N requirements through N_2-fixing symbioses with soil-borne *Frankia* bacteria makes them logical candidates for development as sustainable crops for landscaping. If attractive actinorhizal species can be selected and cultural methods developed to maximize the benefit of their N_2-fixing symbioses, growers should be able to reduce N input during production. Of the more than 200 taxa known to form actinorhizal symbioses, species in the genus *Alnus* demonstrate the highest rates of N_2 fixation and are worthy of increased ornamental use (5, 6, 15, 33). Two autumn-blooming members of the genus, *Alnus maritima* (seaside alder) and *Alnus nitida* (Himalayan alder), are under horticultural evaluation and are gaining in popularity. *Alnus maritima*, native to Oklahoma, Georgia, Maryland, and Delaware, is a stress-resistant, large shrub especially adapted to full sun and wet soils (9, 29). *Alnus nitida*, native to India and Nepal, is the largest of the alders, exceeding 30 m (98 ft) in height. With slightly bluish leaves accented by red stems and twigs, its potential as a fast-growing landscape species is gaining recognition in Australia (14). The pendulous catkins of both species are a unique and striking feature in autumn, and their persistent,

[1]Received for publication July 2, 2007; in revised form September 10, 2007. We gratefully acknowledge the assistance and technical support of M.S. Helgeson, R. Hummel, S.P. Kulshrestha, H.B. Naithani, A. Nakra, S.S. Samant, J. Sharma, Y.P. Singh, and the staff and students of B. Pant Institute of Himalayan Environment and Development, Himachal Unit, Mohal-Kullu, Himachal Pradesh, India.

[2]Assistant Scientist and Professor, respectively.

cone-like strobili provide ornamental appeal during winter. Ample genetic diversity exists within both species to facilitate plant breeding and cultivar selection (12, 27, 28).

Our objectives were to evaluate the potential for *A. nitida* and *A. maritima* to provide for their N requirements through actinorhizal symbioses; to determine the effects of inocula type and source on nodulation, growth, and N content of plants; and to determine if supplemental N is essential for proper growth and nodulation of young seedlings. While symbionts of many N_2-fixing taxa are limited in geographic distribution, *Frankia* compatible with *Alnus* spp. are believed to be both cosmopolitan and ubiquitous in soils, present even in areas with no native *Alnus* spp. (2, 18, 20). Nonetheless, *Frankia* are normally more abundant in soils where host plants are indigenous (3, 34), and *Frankia* strains capable of nodulating *Alnus* spp. vary in symbiotic efficiency (11, 21, 36). To evaluate the importance of these issues in the symbioses of *A. nitida* and *A. maritima*, we tested the nodulation capacity and symbiotic efficiency of *Frankia* from crushed nodules of both species (species-specific and cross inoculation) and from two soils, one collected beneath indigenous *A. rubra* in Washington state and one collected from an Iowa prairie devoid of actinorhizal species.

Another factor that influences the establishment and efficiency of N_2-fixing symbioses is N content in the root zones of the host plants. High concentrations of N restrict nodule formation and activity in both legumes and actinorhizal species (8, 31). Research examining the effect of N concentration on nodulation and N_2-fixation of *A. maritima* shows that daily fertigation with ammonium-nitrate concentrations as low as 0.25 mM (≈ 7 ppm N) reduces nodulation, and daily ammonium-nitrate applications ≥ 8 mM (≥ 224 ppm N) inhibit nodulation (17). While established seedlings require N concentrations to be relatively low to form optimal N_2-fixing symbioses, other research with *A. maritima* indicates that young seedlings require greater N concentrations between the time of germination and formation of their first true leaves. Schrader (24) reported that survival at 60 days after germination declined from 87 to 72% for *A. maritima* seedlings growing in medium with initial N concentrations of 48.6 g/kg (4.9%) and 6.3 g/kg (0.63%), respectively. Therefore, we included two fertilizer treatments (one with and one without N) in factorial combination with species and inoculation treatments to help determine effects of supplemental N on nodulation and early growth of *A. maritima* and *A. nitida* seedlings.

Materials and Methods

Strobili of *A. nitida* were collected from one plant growing along the Beas River in Himachal Pradesh, India (32°06.138' N, 77°08.404' E) in November 2005, and strobili of *A. maritima* subsp. *oklahomensis* were collected from one tree growing along the Blue River in Johnston County, OK (34°20.045' N, 96°35.681' W) in November 2002. Seeds of both species were extracted by gently crushing the strobili and sorting the seeds from the bracts. On January 4, 2006, seeds were surface sterilized with 10% chlorine bleach solution and placed in moist stratification at 4C (39F) for 3 weeks, then held for 12 days in germination conditions [24C (75F) in the dark] as described by Schrader and Graves (26).

Two types of *Frankia* inoculum (crushed-nodule and soil) were prepared from samples collected from four sources. In November 2005, nodules for crushed-nodule inoculum were collected from the roots of indigenous *A. nitida* growing west of the Beas River in Himachal Pradesh, India (31°53.048' N, 77°05.204' E). In December 2005 nodules were collected from the roots of indigenous *A. maritima* growing along the Blue River in Johnston County, OK (34°19.589' N, 96°35.556' W). Nodules were transported on ice to our research facility in Ames, IA, and crushed-nodule inoculum was produced for each species by preparing a buffered slurry [phosphate buffer, pH 7.3 (19)] that was mixed with fine vermiculite to form a saturated inoculation medium. Control medium for this inoculum type was vermiculite saturated with distilled, de-ionized water (dd H_2O). Soil for the two treatments of soil inocula was collected from the upper 20 cm (7.9 in) of the soil profile from within the dripline of native *A. rubra* in Pierce County, WA (47°10.634' N, 122°19.150' W) and from an undisturbed prairie site in Boone County, IA (Boone Prairie, 42°03.182' N, 93°49.358' W), devoid of actinorhizal species. Soil from the Washington site was used to help determine if compatible *Frankia* are present in soils inhabited by *Alnus* spp. other than *A. nitida* and *A. maritima*. Prairie soil was used to help determine if compatible *Frankia* are present in areas with no host plants. Control medium for the soil inocula treatments was agricultural soil collected near Ames, IA, and sterilized by autoclaving for 3 hours. Initial N, P, and K contents of the three soils were analyzed at the Soil and Plant Analysis Laboratory, Iowa State University, Ames, IA (Table 1).

The experiment was conducted in a glass-glazed greenhouse with 384 experimental units arranged in a completely randomized design. An experimental unit consisted of one seedling growing in a 15-cm polypropylene pot [top diameter 15 cm (5.9 in), bottom diameter 10.5 cm (4.1 in), height 14.5 cm (5.7 in)] containing 1100 cm^3 (67.1 in^3) of sterile coarse perlite, with 30 cm^3 (1.8 in^3) of inoculum positioned in the top-center of the perlite. On February 6, 2006 (10 days after most seeds had germinated), seedlings were transplanted from germination plates (Petri plates containing moist filter paper) into the inoculation medium of experimental units by using sterilized tweezers. The factorial treatment design consisted of two species (*A. maritima* and *A. nitida*), two types of inocula (soil or crushed nodule), three source treatments for each inoculum type (species-specific, cross inoculation, or sterilized control for crushed nodules; Washington soil, prairie soil, or sterilized soil control), and two types of fertilizer [25% Hoagland solution with or without N (13)] for a total of 24 treatment combinations (16 replicates per treatment combination). Fertilizer with N provided 52.6 mg/liter (ppm) N in the form of NO_3. Plants were fertigated with 200 cm^3 (12.2 in^3) of their assigned Hoagland solution on the first day of the experiment and every 2 weeks thereafter.

Table 1. Initial nitrogen, phosphorus, and potassium contents of three soils used to inoculate *Alnus maritima* and *Alnus nitida*.

Soil	Nitrogen (%)	Phosphorus (ppm)[z]	Potassium (ppm)
Control[y]	0.17	28	76
Washington state	1.82	7	114
Prairie in Iowa	0.15	< 1	80

[z]ppm = parts per million or mg/liter.

[y]Control inoculum was agricultural soil collected near Ames, IA, and sterilized by autoclaving for 3 hours.

To reduce the risk of cross contamination during irrigation, each experimental unit was placed in an individual irrigation tray [diameter = 15 cm (5.9 in), depth = 3.5 cm (1.4 in)] and was irrigated every other day between fertilizer treatments by filling the tray with distilled water. Inoculated seedlings were grown under a 16-hour photoperiod during which solar radiation was supplemented with six 400-W high-pressure sodium lamps. Air temperature was maintained at 26 ± 5C (79 ± 9F), and relative humidity ranged from 40 to 86% during treatments.

After 7 weeks, half of the plants (eight per factorial treatment combination) were harvested to obtain preliminary size measurements and to provide initial data for calculating growth rates. The remaining plants were harvested 15 weeks later (total experimental duration = 22 weeks). At both harvests, plants were measured for number of nodule units [nodule tissue equal to a 3.2 mm (1/8 in) diameter sphere] per plant, shoot height, plant weight after drying tissue for 3 days at 67 C (153 F), and total N concentration of leaves. These measurements were used to calculate plant weight resulting from symbiosis (dry weight of nodulated plant minus mean dry weight of control plants), percentage of plant weight from symbiosis (dry weight from symbiosis ÷ total plant dry weight × 100), and relative growth rate (*ln* of final dry weight, minus *ln* of initial dry weight, over the 15 weeks between harvests). Total leaf N percentages were the mean of two samples taken randomly from ground and homogenized leaf tissue from all experimental units within each factorial treatment. Leaf N analyses were conducted at the Soil and Plant Analysis Laboratory, Iowa State University.

Data analysis. Data were analyzed for main effects, interactions, and mean-separation statistics by using the general linear models (GLM) procedure and the least significant difference (LSD) option of SAS/STAT®, Version 6.12 (23). Data sets were tested for homogeneity of variance by using Levene's test (22), and non-homogeneous data were transformed by a log or square-root function. Means were calculated from raw data, and the mean-separation statistics were calculated from raw or transformed data as necessary.

Results and Discussion

Inoculation with *Frankia* increased growth and N content of *A. maritima* and *A. nitida* seedlings. Across species and fertilizer treatments, inoculated plants accumulated nearly three times the dry weight, grew at nearly twice the relative rate, and accrued greater leaf N content than that of uninoculated controls (Table 2, Main effects of inoculation). Control plants formed no nodules, verifying the effectiveness of the experimental methods and ensuring that nodules in other treatments were the result of inoculation rather than contamination. Across inoculation and fertilizer treatments, *A. nitida* averaged more nodules per plant and grew larger than *A. maritima*, but relative growth rate, percentage of plant weight attributed to symbiosis, and leaf N content were similar between the two species (Table 2, Main effects of species). Previous research has demonstrated that *A. nitida* is a faster-growing species than *A. maritima* under most conditions (25). Therefore, the difference in nodule number between the two species across inoculation and fertilizer treatments might be explained by plant size and absolute growth rate, rather than differences in host-symbiont compatibility or symbiotic efficiency. Across species and inoculation treatments, plants that received N fertilizer grew faster and larger than those that received N-free fertilizer, but treatment with N fertilizer had no effect on the percentage of plant weight attributed to symbiosis or leaf N content (Table 2, Main effects of fertilizer type).

Along with the main effects of species, inoculation, and supplemental N, analysis of variance (ANOVA, $P < 0.0001$) revealed important interactions among factorial treatments. Species-specific inoculum and Washington-soil inoculum evoked the most nodules and stimulated the greatest increases in plant height and weight, weight and percentage of weight from symbiosis, and relative growth rate (Table 2, Effects of inoculation type). Cross-inoculated plants (crushed-nodule inoculum from other *Alnus* sp.) were smaller, but had similar nodule counts and similar relative growth rates compared to plants that received species-specific inoculum and soil inoculum from Washington. Plants inoculated with prairie soil were small but had similar relative growth rates and similar N content compared to plants that received species-specific inoculum and soil inoculum from Washington. Results for inoculum-by-fertilizer factorial treatments revealed that species-specific inoculation was more effective than cross inoculation within each fertilizer treatment (Table 2, Factorial with fertilizer type). While cross inoculation and species-specific inoculation evoked similar numbers of nodules, plants treated with species-specific inoculum grew to nearly twice the height and weight and accrued greater N content in their leaves than cross-inoculated plants. Plants treated with species-specific inoculum also weighed more and had a higher percentage of weight from symbiosis than cross-inoculated plants within each fertilizer treatment. Results were similar when analyzed in full factorial and assessed separately for each species (Table 3). We conclude that, although nodulation is easily achieved by cross inoculation between *A. maritima* and *A. nitida*, and the symbionts are compatible, the resulting symbiosis is not highly effective in providing the N requirements of the plant. Nonetheless, symbiosis from cross inoculation increased growth and N content of plants of both species compared to that of uninoculated controls (Tables 2 and 3). These results are consistent with prevailing views regarding host specificity. *Frankia* capable of forming N_2-fixing symbioses with an *Alnus* sp. have been classified as members of the *Alnus-Myrica-Comptonia* host-specificity group (HSG 1) and are believed capable of nodulating species of these three genera (3, 32). While cross inoculation with *Frankia* strains of HSG 1 commonly induces nodulation of *Alnus* spp., symbiosis efficiency varies extensively (4), an insight that helps explain the high level of nodulation but low symbiotic efficiency shown by cross inoculated *A. maritima* and *A. nitida* (Tables 2 and 3).

Our results demonstrate that highly effective N_2-fixing symbioses can be established with *A. maritima* and *A. nitida* by inoculation with non-native soils. While a species-by-treatment interaction resulted in more growth for *A. maritima* inoculated with species-specific inoculum compared to plants inoculated with soil from Washington (Table 3), Washington-soil inoculum induced more growth than species-specific inoculum for *A. nitida* (Table 3) and across species under the N-free fertilizer treatment (Table 2). Growth and N content of plants treated with Washington soil and species-specific inoculum were similar when results were evaluated across species and fertilizer treatments (Table 2). Plants inoculated with Washington soil most likely received a slight nutrient

Table 2. Main effects and factorial treatment effects of inoculation and fertilizer type on nodulation, growth, and leaf N concentration of *Alnus nitida* and *Alnus maritima*. Plants were treated with four types of inoculum and two types of fertilizer and grown in a greenhouse for 22 weeks.

	Nodule count	Shoot height (cm)	Plant dry weight (g)	Plant dry wt. from symbiosis (g)	Percentage of plant dry wt. from symbiosis (%)	Relative growth rate[z] (g/g)	Total leaf N (%)
Main effects of inoculation							
Control	0b[y]	12.5b	1.4b	0.0b	0b	1.9b	1.1b
Inoculated	23.5a	21.2a	4.1a	2.7a	56a	3.6a	1.9a
Main effects of species							
Alnus nitida	21a	21.6a	4.6a	2.9a	40a	3.4a	1.4a
Alnus maritima	12b	16.7b	2.2b	0.9b	31a	2.7a	1.4a
Main effects of fertilizer type							
N-free	6.2b	7.3b	0.3b	0.3b	36a	1.7b	1.4a
With N	26.2a	30.3a	6.1a	3.4a	35a	4.4a	1.7a
Effects of inoculation type							
Control inoculum[x]	0c	13.2bc	1.8bc	0.0d	0c	2.2b	0.9c
Control soil	0c	11.7c	1.1c	0.0d	0c	1.7b	1.2b
Cross inoculation	22ab	16.2bc	2.7b	1.4b	48b	3.1ab	1.6b
Species-specific inoculation	26a	25.5a	5.2a	3.3a	69a	3.5a	2.0a
Washington soil	28a	25.0a	5.4a	4.3a	74a	4.7a	2.0a
Prairie soil	18b	18.2b	3.1b	2.0b	34b	3.0ab	2.0a
Factorial with fertilizer type							
Control inoculum, N-free	0d	4.7e	0.04f	0.0e	0e	0.4e	[w]
Cross inoculation, N-free	7c	5.9e	0.1e	0.1e	53bc	2.1c	1.6b
Species-specific inoculation, N-free	14c	10.2d	0.4e	0.3d	82a	2.0cd	2.0a
Control inoculum, with N	0d	22.4c	3.6c	0.0e	0e	4.0b	0.9c
Cross inoculation, with N	38ab	27.5b	5.3b	2.8bc	33cd	4.2ab	1.5b
Species-specific inoculation, with N	39ab	40.8a	10.0a	6.3a	55b	5.1a	2.1a
Control soil, N-free	0d	5.3e	0.1e	0.0e	0e	0.3e	1.0c
Washington soil, N-free	14c	12.9d	1.3d	1.5c	59b	4.3ab	1.9a
Prairie soil, N-free	2d	5.0e	0.1e	0.03d	21d	1.3cd	1.7b
Control soil, with N	0d	20.0c	2.1cd	0.0e	0e	3.1bc	1.3bc
Washington soil, with N	44a	38.7a	9.4a	7.1a	65ab	5.2a	2.0a
Prairie soil, with N	36b	32.2b	6.2b	3.9b	55b	4.6a	2.2a

[z]Relative growth rate = increase in dry weight (grams) per gram of plant dry weight over a 15-week period.

[y]Mean separation within each column and treatment heading by Fisher's least significant difference, $P \leq 0.05$. Mean-separation statistics were assessed separately for each category. n = 128 for inoculated and n = 64 for controls under the category main effects of inoculation. n = 96 for main effects of species and main effects of fertilizer type categories. n = 32 for effects of inoculation type and n = 16 for inoculation type in factorial with fertilizer type.

[x]Species-specific inoculation, and cross inoculation were crushed-nodule inoculation treatments in which nodule contents were mixed with a buffer and sterilized vermiculite to form a saturated medium. Species-specific inoculations were treatments given to the same species from which nodules were collected. Cross inoculations were given to a different species from which nodules were collected (i.e. *A. maritima* nodules to inoculate *A. nitida* seedlings and *A. nitida* nodules to inoculate *A. maritima* seedlings). Control inoculum was sterilized vermiculite saturated with dd H_2O. Washington soil inoculum was soil collected from the root zone of *A. rubra* in Pierce County, WA; prairie soil inoculum was soil from a prairie in Iowa that was devoid of actinorhizal species; and control soil was sterilized agricultural soil from Iowa.

[w]Quantity of tissue inadequate for analysis.

advantage over those given other treatments due to the greater N and K contents of the Washington soil (Table 1). Nonetheless, the beneficial effects of the symbiosis derived from this inoculum were evident in the high nodule count, plant weight from symbiosis, and percentage of plant weight from symbiosis, which were similar to those of plants inoculated with species-specific inoculum (Tables 2 and 3). Any benefit from the higher nutrient content of Washington soil probably was small and early in the experiment for plants that received N fertilizer because the 25% Hoagland solution provided 52.6 mg/liter (ppm) N, 7.8 mg/liter (ppm) P, and 58.7 mg/liter (ppm) K every 2 weeks. The nutrient advantage from Washington soil was likely greater for plants receiving N-free fertilizer. This may help explain why plants of *A. nitida* in the Washington-soil treatment grew larger than those in the species-specific inoculation treatment when plants received fertilizer without N (Table 3), a trend also apparent when results were analyzed across species (Table 2).

Our results indicate that *Frankia* compatible with *A. maritima* and *A. nitida* are common in soils, findings consistent with research evaluating the distribution of other actinorhizal symbionts (2, 18, 20). We included soil from an Iowa prairie as an inoculum treatment to evaluate whether compatible *Frankia* are present in soils devoid of actinorhizal species. Records show that the prairie site we sampled was never opened to modern cultivation (7) and therefore has likely been continuous prairie lacking in actinorhizal species for approximately 8000 years (16, 35). Inoculation with prairie soil caused nodules to form on both species and increased growth or relative growth and N content of plants over that

Table 3. Effects of 24 factorial treatments of species, inoculation type, and fertilizer type on nodulation, growth, and leaf N concentration of *Alnus nitida* and *Alnus maritima*. Plants were treated with four types of inoculum and two types of fertilizer and grown in a greenhouse for 22 weeks.

Treatment	Nodule count	Shoot height (cm)	Plant dry weight (g)	Plant dry wt. from symbiosis (g)	Percentage of plant dry wt. from symbiosis (%)	Relative growth rate[z] (g/g)	Total leaf N (%)
Alnus nitida							
Control inoculum, N-free[y]	0g[x]	3.8g	0.04e	0.0	0	0.5	[w]
Cross inoculation, N-free	7f	5.2g	0.1e	0.08g	64b	2.8	1.5
Species-specific inoculation, N-free	19e	10.3e	0.5e	0.4ef	86a	1.4	2.2
Control inoculum, with N	0g	18.2de	3.9c	0.0	0	4.3	0.9
Cross inoculation, with N	43bc	37.8b	9.2b	5.4cd	44c	4.9	2.1
Species-specific inoculation, with N	46b	47.8a	12.3a	8.5a	65ab	5.1	2.4
Control soil, N-free	0g	4.5g	0.04e	0.0	0	0.01	[w]
Washington soil, N-free	25e	19.0de	2.9cd	2.8d	66ab	5.9	2.1
Prairie soil, N-free	2fg	4.1g	0.1e	0.01g	17e	1.2	[w]
Control soil, with N	0g	22.5d	2.8cd	0.0	0	3.8	1.5
Washington soil, with N	65a	48.2a	14.4a	11.6a	78a	6.1	2.1
Prairie soil, with N	49b	37.5b	8.5b	5.7c	62b	4.8	2.3
Alnus maritima							
Control inoculum, N-free	0g	5.2g	0.04e	0.0	0	0.4	[w]
Cross inoculation, N-free	7f	6.4f	0.1e	0.04g	45c	1.3	1.7
Species-specific inoculation, N-free	9f	10.2e	0.24e	0.2f	78a	2.6	1.7
Control inoculum, with N	0g	23.7d	3.6c	0.0	0	3.7	0.9
Cross inoculation, with N	35cd	21.6d	2.3d	0.5g	22d	3.5	1.0
Species-specific inoculation, with N	34d	36.0b	8.4b	4.8cd	49bc	5.0	1.8
Control soil, N-free	0g	5.6g	0.08f	0.0	0	0.5	1.0
Washington soil, N-free	6f	8.6ef	0.2e	0.1g	53b	2.6	1.7
Prairie soil, N-free	2fg	5.7fg	0.2e	0.04g	24e	1.4	1.7
Control soil, with N	0g	19.4de	1.9de	0.0	0	2.5	1.1
Washington soil, with N	26e	30.4c	4.9c	3.2d	54b	4.3	2.0
Prairie soil, with N	25e	28.0c	4.3c	2.5de	49bc	4.5	2.0

[z]Relative growth rate = increase in dry weight (grams) per gram of plant dry weight over a 15-week period.

[y]Species-specific inoculation, and cross inoculation were crushed-nodule inoculation treatments in which nodule contents were mixed with a buffer and sterilized vermiculite to form a saturated medium. Species-specific inoculations were treatments given to the same species from which nodules were collected. Cross inoculations were given to a different species from which nodules were collected (i.e. *A. maritima* nodules to inoculate *A. nitida* seedlings and *A. nitida* nodules to inoculate *A. maritima* seedlings). Control inoculum was sterilized vermiculite saturated with dd H_2O. Washington-soil inoculum was soil collected from the root zone of *A. rubra* in Pierce County, WA; prairie-soil inoculum was soil from a prairie in Iowa devoid of actinorhizal species; and control soil was sterilized agricultural soil from Iowa.

[x]Mean separation within each column by Fisher's least significant difference test, $P \leq 0.05$. Mean-separation statistics were assessed across species (n = 8).

[w]Quantity of tissue inadequate for analysis.

of controls in both fertilizer treatments (Tables 2 and 3). The omnipresence of viable actinorhizal symbionts in soils (2, 18, 20) contrasts the ecology of some N_2-fixing legumes, such as *Maackia amurensis* Rupr. & Maxim (Amur maackia), in which compatible symbionts appear more isolated to soils where the legume is native or in the root zone of planted or established N_2-fixing plants of their genus or rhizobial compatibility group (1, 30). Although *Frankia* compatible with *A. maritima* and *A. nitida* appear broadly distributed, our results also indicated that symbiotic efficiency varies among *Frankia* strains inhabiting soils from different geographical regions. Symbioses initiated by inoculation with soil from Washington were superior to those of prairie soil for nearly all parameters of nodulation and growth of *A. nitida* (Table 3). This trend was also apparent for *A. maritima*, although means were not separate at $P \leq 0.05$.

The increase in growth and N content of inoculated plants of *A. maritima* and *A. nitida* in both fertilizer treatments illustrates the potential for low-input production of actinorhizal species for managed landscapes. The best overall growth and N content for both species occurred when plants were inoculated and provided with a low level of N fertilizer (25% Hoagland, every 2 weeks). Although high levels of N fertilizer can inhibit nodulation and N_2 fixation (8, 17), at least some additional N will be required for optimum nodulation and growth of *A. maritima* and *A. nitida*. Our results were consistent with those of Schrader (24) that showed poor growth and development of *A. maritima* during the first 60 days after seed germination when the root medium was very low in N [≤ 6.3 g/kg (≤ 0.63%)]. In the present study, the N concentration of the root medium in the N-free fertilizer treatment was extremely low [≤ 1.7 g/kg (≤ 0.17%) for all inoculation treatments except Washington soil]. The increase in growth and N content of inoculated plants in the N-free treatment indicates that N_2-fixing symbiosis may help plants survive and grow under severe N stress, but the N concentration of the root medium must be greater during early growth to optimize nodulation and maximize the benefits of N_2-fixing symbiosis for nursery production (Table 2). Starting inoculated plants in a medium containing supplemental macro- and microelements should supply all the nutrients needed for establishment; thereafter, nodulated plants can

grow to marketable size and beyond without addition of fertilizer, a scenario that we have observed in preliminary greenhouse trials.

Finding sources of inoculum that can initiate symbioses beneficial to *A. maritima* and *A. nitida* should not be difficult because compatible *Frankia* strains were present in both soils we used, neither of which was from a site where *A. nitida* or *A. maritima* are native or have been planted. Differences in the effectiveness of our inoculation treatments indicate that superior strains of *Frankia* and methods of inoculation may be discovered and developed. The marginally better nodulation and growth of *A. nitida* inoculated with soil from Washington compared to *A. nitida* treated with species-specific, crushed-nodule inoculum suggest that *Frankia* strains superior to those in native Asian soils might be present in North America. The ease of preparation and inoculation with soil (refrigerate inoculum until use, add water to saturation, sow seeds or plant seedlings in inoculum sufficient to surround roots) and the high level of effectiveness that can be achieved (comparable or better than species-specific, crushed nodule inoculum) may make soil application the preferred method for inoculations in nurseries. Future research for optimizing sustainable production of these species should examine the effects of root medium type, supplement type, and form of N. Alternative soil sources should be screened for potentially superior *Frankia* strains, and long-term studies should be designed to quantify the benefits of symbiotic N_2 fixation in actinorhizal species during nursery production, distribution, landscape establishment, and sustainable use in managed landscapes.

Literature Cited

1. Batzli, J.M., W.R. Graves, and P. van Berkum. 1992. Isolation and characterization of rhizobia effective with *Maackia amurnesis*. J. Amer. Soc. Hort. Sci. 117:612–616.

2. Benecke, U. 1969. Symbionts of alder nodules in New Zealand. Plant and Soil 30:145–149.

3. Benson, D.R. and W.B. Silvester. 1993. Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol. Rev. 57:293–319.

4. Bousquet, J. and M. Lalonde. 1990. The genetics of actinorhizal Betulaceae. *In*: C.R. Schwintzer and J.D. Tjepkema (eds.). The Biology of *Frankia* and Actinorhizal Plants. Academic Press, San Diego.

5. Busse, M.D. 2000. Ecological significance of nitrogen fixation by actinorhizal shrubs in the interior forests of California and Oregon. U.S. Dept. Agric. Forest Serv. Gen. Tech. Rpt. PSW-GTR-178.

6. Del Tredici, P. 1995. A nitrogen fixation: The story of the *Frankia* symbiosis. Arnoldia 55(4):26–31.

7. Freckmann, R.W. 1966. The prairie remnants of the Ames area. Proc. Iowa Acad. Sci. 73:126–136.

8. Gentili, F. and K. Huss-Danell. 2003. Local and systemic effects of phosphorus and nitrogen on nodulation and nodule function in *Alnus incana*. J. Expt. Bot. 54:2757–2767.

9. Graves, W.R. and J.A. Schrader. 2004. 'September Sun' seaside alder, an autumn-blooming shrub native to North America. HortScience 39:438–439.

10. GreenFacts asbl/vzw. 2001–2007. GreenFacts Glossary. Accessed September 4, 2007. http://www.greenfacts.org/glossary/def/ecosystem-services.htm

11. Hall, R.B., H.S. McNabb, Jr., C.A. Maynard, and T.L. Green. 1979. Toward development of optimal *Alnus glutinosa* symbioses. Bot. Gaz. 140:S120–S126.

12. Handa, A.K., S.K. Chauhan, and P.L.Gautam. 1997. Variation studies in natural stands of *Alnus nitida* in Kullu Valley. Crop Improv. 24:35–38.

13. Hoagland, D.R. and D.I. Arnon. 1950. The water-culture method for growing plants without soil. Calif. Agric. Expt. Sta. Circ. 347:1–32.

14. Hossain, T., P. Abell, N. Hulse, K. Prakash, H. Costa, D. Waterhouse, K. Dawes-Read, and B. Blazey (eds.). 2002. Plant Varieties J. 15 (4):95.

15. Huss-Danell, K. 1997. Actinorhizal symbioses and their N_2 fixation. New Phytol. 136:375–405.

16. King, J.E. 1981. Late Quaternary vegetational history of Illinois. Ecol. Monogr. 51:43–62.

17. Laws, M.T. and W.R. Graves. 2005. Nitrogen inhibits nodulation and reversibly suppresses nitrogen fixation in nodules of *Alnus maritima*. J. Amer. Soc. Hort. Sci. 130:496–499.

18. Maunuksela, L., K. Zepp, T. Koivula, J. Zeyer, K. Haahtela, and D. Hahn. 1999. Analysis of *Frankia* populations in three soils devoid of actinorhizal plants. FEMS Microbiol. Ecol. 28:11–21.

19. Maynard, C.A. 1980. Host-symbiont interactions among *Frankia* strains and *Alnus* open-pollinated families. PhD Dissertation. Iowa State University, Ames, IA.

20. Paschke, M.W. and J.O. Dawson. 1992. The occurrence of *Frankia* in tropical forest soils of Costa Rica. Plant and Soil 142:63–67.

21. Prat, D. 1989. Effects of some pure and mixed *Frankia* strains on seedling growth in different *Alnus* species. Plant and Soil 113:31–38.

22. SAS Institute Inc. 1989. SAS/STAT® user's guide. Version 6. 4th ed. SAS Inst. Inc., Cary, NC.

23. SAS/STAT® Software, Version 6.12 of the SAS System for Power Macintosh. Copyright© 1989–96. SAS Inst. Inc., Cary, NC.

24. Schrader, J.A. 1999. Propagation of *Alnus maritima* from its three disjunct populations. MS Thesis. Iowa State University, Ames, IA.

25. Schrader, J.A., S.J. Gardner, and W.R. Graves. 2005. Resistance to water stress of *Alnus maritima*: Intraspecific variation and comparisons to other alders. Environ. Expt. Bot. 53:281–298.

26. Schrader, J.A. and W.R. Graves. 2000. Seed germination and seedling growth of *Alnus maritima* from its three disjunct populations. J. Amer. Soc. Hort. Sci. 125:128–134.

27. Schrader, J.A. and W.R. Graves. 2002. Infraspecific systematics of *Alnus maritima* (Betulaceae) from three widely disjunct provenances. Castanea 67:380–401.

28. Schrader, J.A. and W.R. Graves. 2004. Systematics of *Alnus maritima* (seaside alder) resolved by ISSR polymorphisms and morphological characters. J. Amer. Soc. Hort. Sci. 129:231–236.

29. Schrader, J.A., W. R. Graves, S. Rice, and P. Gibson. 2006. Differences in shade tolerance help explain varying success of two sympatric *Alnus* species. Intern. J. Plant Sci. 167:979–989.

30. Schrader, J.A., M. Kroggel, and W.R. Graves. 2007. Nodulation and nitrogen-fixing capacity of rhizobial isolates from China in symbiosis with *Maackia amurensis*. J. Environ. Hort. 25:47–50.

31. Streeter, J. 1988. Inhibition of legume nodule formation and N_2 fixation by nitrate. Crit. Rev. Plant Sci. 7:1–23.

32. Tjepkema, J.D., C.R. Schwintzer, and D.R. Benson. 1986. Physiology of actinorhizal nodules. Annu. Rev. Plant Physiol. 37:209–232.

33. Tripp, K.E. 1995. Hardy Asian alders. Arnoldia 55(4):17–25.

34. Van Dijk, C. 1979. Endophyte distribution in the soil, pp. 84-94. *In*: J.C. Gordon, C.T. Wheeler, and D.A. Perry (eds.). Symbiotic Nitrogen Fixation in the Management of Temperate Forests. Oregon State University Press, Corvallis, OR.

35. Van Zant, K. 1979. Late-glacial and postglacial pollen and plant macrofossils from Lake West Okoboji, northwestern Iowa. Quaternary Res. 12:358–380.

36. Verghese, S. and A.K. Misra. 2002. *Frankia*-actinorhizal symbiosis with special reference to host-mycrosymbiont relationship. Current Sci. 83:404–408.