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Nodulation and Nitrogen-fixing Capacity of Rhizobial Isolates from China in Symbiosis with *Maackia amurensis*[1]

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Abstract

Maackia amurensis Rupr. & Maxim (Amur maackia) is a leguminous Asian tree capable of forming N_2-fixing symbioses with soil-borne *Bradyrhizobium* spp. This trait sets Amur maackia apart from many legumes now produced in North American nurseries. Two determinants of N_2-fixing capacity in legumes are the compatibility of the host plant and its bacterial microsymbiont and the metabolic efficiency of compatible bacteria. Our objectives were to isolate numerous rhizobia from the root zones of indigenous Amur maackia in China and to select isolates that form superior N_2-fixing relationships with inoculated seedlings. Soil samples collected in the Heilongjiang Province of China were used as inocula to establish nodules on seedlings. Putative rhizobia were isolated from these nodules and cultured. Inoculation of additional seedlings with 170 of these isolates evoked nodulation, confirming their identity as rhizobia. Isolates that induced the most nodules were evaluated further. All selected isolates increased growth and total N content of Amur maackia compared to uninoculated controls. Three of the isolates induced more root nodules, and four evoked a higher total N content in plants than did isolate USDA 4349, a previously characterized strain of *Bradyrhizobium* selected for Amur maackia. Our results demonstrate marked variation among rhizobia compatible with Amur maackia and illustrate the potential to inoculate plants in nurseries and landscapes with superior bacteria, a practice that could reduce fertilizer use and improve performance of trees in N-deficient soils.

Index words: *Bradyrhizobium*, nitrogen fixation, Amur maackia, Fabaceae, sustainable production, plant-microbe interaction.

Species used in this study: *Maackia amurensis* Rupr. & Maxim. (Amur maackia), *Bradyrhizobium* spp.

Significance to the Nursery Industry

Although several leguminous species, some of which benefit from N_2-fixing symbioses with rhizobia, have become important nursery crops, little information exists about how to optimize the legume-rhizobia relationship to benefit the nursery and landscape industry. If superior, compatible strains of rhizobia can be selected and protocols developed to maximize N_2-fixing capacity, growers may be able to reduce or eliminate N input during production, and the symbiotic plant should perform exceptionally well in landscapes with N-deficient soils. The goal of our research was to select superior strains of rhizobia for use in the production of Amur maackia, an Asian tree species with strong landscape potential. We isolated over 170 compatible rhizobia from the soils beneath indigenous Amur maackia in China, chose eight isolates with high nodulation capacity for further testing, and selected four of those isolates for their superior N_2-fixing symbioses with seedlings of Amur maackia. Our findings demonstrate that further research and development could lead to the marketing of inocula for use either during production of Amur maackia or after trees are established in landscapes. Inclusion of superior isolates of *Bradyrhizobium* in commercial inoculants will ensure maximal benefits of the resulting N_2-fixing symbioses.

Introduction

As the call for sustainable horticultural practices intensifies, there is a growing need for the development of nursery and landscape methods that will efficiently manage inputs of N fertilizer and minimize run-off and leaching into the environment. One approach that has made a significant impact within agronomic cropping systems is the utilization of legume species such as alfalfa (*Medicago sativa* L.) and soybean (*Glycine max* [L.] Merr.), which gain N through a symbiotic relationship with N_2-fixing bacteria (rhizobia) that function within nodules on their roots. The N applied in fertilizers is used inefficiently by plants, with less than 50% of N from fertilizers typically being used by agronomic crops to which it was applied (5). In contrast, all N produced during symbiotic N_2 fixation by legumes is assimilated by the plant (5). Legume-rhizobial symbioses vary widely across species in both compatibility of the plant host and its bacterial symbiont and in symbiotic efficiency (22), but superior rhizobia have been selected for many leguminous food crops (1, 2, 10, 13, 15). Although much less attention has been given to the use of N_2-fixing species by the nursery industry, the potential exists to develop nursery crops and protocols that benefit from the process of symbiotic N_2 fixation (5, 8). If plant and bacterial selection can be coordinated, and protocols developed to maximize N_2-fixing capacity in woody legumes capable of N_2 fixation, the resulting nursery crops could be marketed as sustainable and economical alternatives to higher-input components of managed landscapes.

One N_2-fixing legume gaining in popularity in the nursery industry is *Maackia amurensis* Rupr. & Maxim. (Amur maackia), a moderately sized tree with attractive bark, silvery foliage at bud break, and appealing late-season flowers borne on stiff, upright racemes (16). Early research suggests that rhizobia compatible with Amur maackia are not ubiquitous in soils but can be isolated from root zones of at least some established trees (4, 6). The variable symbiotic efficiency shown among the small number of isolates studied to date indicates the potential to select rhizobia with superior N_2-fixing capacity. Because Amur maackia is native to China and presumably co-evolved with the soil microorganisms there, we hypothesized that rhizobia from areas of China where Amur maackia is indigenous should form particularly

[1]Received for publication September 26, 2006; in revised form December 1, 2006. We gratefully acknowledge the assistance and technical support of Wang Qingcheng, Carol M. Foster, Edie Emmenegger, and Randy Mayer.

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effective symbioses. Therefore, our objectives were to: (1) establish a large collection of rhizobia from the root zones of Amur maackia in native stands in China; (2) assign those isolates to groups based on similarities in their traits; and, (3) use highly compatible representatives of the similarity groups to screen and select for rhizobial isolates that evoke the greatest number of nodules, greatest plant growth, and the highest N content in inoculated seedlings of Amur maackia.

Materials and Methods

Isolation and selection of compatible rhizobia. Soil samples were collected from Liangshui Research Forest (47°10' N, 128°53' E; mean annual precipitation = 724 mm [28.5 in], mean annual temperature = 2.8C [37F]) and the Maoershan Research Forest (45°30' N, 127°32' E; mean annual precipitation = 676 mm [26.6 in], mean annual temperature = –0.3C [31F]) in the Heilongjiang Province of China. Samples were taken from the upper 15 cm (5.9 in) of the soil profile, within the dripline of 14 trees of *M. amurensis* (seven indigenous to each forest) and transported on ice to our research facility in Ames, IA. Rhizobia were isolated according to the methods of Somasegaran and Hoben (22) and Vincent (25) by growing seedlings of Amur maackia in sterilized, perlite-filled 10-cm (4-in) standard clay pots inoculated with 64 cm^3 (3.9 in^3) of soil from China (three replicate pots for each of 14 samples). Non-inoculated controls were maintained and randomly arranged among other pots to verify proper protocol. After 10 weeks we harvested and surface sterilized up to five nodules from each plant, crushing each nodule and streaking the contents on a Petri plate (one nodule per plate) containing arabinose-gluconate (AG) medium (12). Cultures were incubated at 28 ± 1C (82.4 ± 1.8F) for 7 days, and over 200 individual colonies were isolated by streaking on a second Petri plate containing AG medium. Inocula from these single-colony isolates were used to establish cultures in 3 mL (0.18 in^3) of liquid AG medium held in 20 × 150 mm (0.79 × 5.9 in) culture tubes. The liquid cultures were incubated at 28 ± 1C (82.4 ± 1.8F) on a gyratory shaker platform (Lab-Line Instruments, Melrose Park, IL) at 1.7 oscillations/s for 7 days.

A subset of the >200 initial isolates was verified as rhizobial by using presumptive tests and aseptic inoculation procedures (22) to test for nodulation on half-sib seedlings of Amur maackia. To minimize the chance of contamination, inoculated seedlings were held in sterilized, #SP2 clay rose pots filled with coarse perlite and positioned in the opening of a pint (475 cm^3) wide-mouth Mason jar. The bottom of each jar contained 130 cm^3 (7.9 in^3) of sterilized, fine silica sand that was saturated with 250 cm^3 (15.3 in^3) of sterile, N-free 25% Hoagland solution (9), to which was added 1.25 mL/liter (1250 ppm) MES-Hepes and 0.25 mL/liter (250 ppm) 1-N NaOH to maintain a pH of 6.8. This growth system emulated the inoculation system of Leonard (14), but was comparatively economical and easy to construct. To reduce evaporation and to help prevent contamination by undesired bacteria, we placed a polyethylene sleeve [length: 40 cm (15.7 in), diameter: 10 cm (3.9 in)] over the inoculation jar and held the top of the sleeve partially closed with a staple. Inoculated seedlings (two growth units per isolate, with one seedling per unit) were grown in a glasshouse under a 16-hour photoperiod during which solar radiation was supplemented with two 400-W high-pressure sodium lamps arranged 1 m (3.3 ft) above the canopy. Air temperature was maintained at 25 ± 5C (77 ± 9F), and seedlings were provided with 100 mL (6.1 in^3) of N-free, 25% Hoagland solution every 10 days. Plants were harvested to assess nodulation after 48 days. The 50 isolates that evoked the greatest number of nodules per seedling were selected for further characterization to group them according to physiological traits and to determine generic classification.

Characterization and selection of rhizobia. The 50 isolates selected from the initial screening were characterized along with a previously characterized isolate of *Bradyrhizobium* (USDA 4349), which was used as a control and to assist in classification. USDA 4349 was isolated from the root zone of a cultivated Amur maackia planted outside its native range in China (4). Isolates were assessed for growth rate, effect on pH of the medium, and tolerance to NaCl according to the methods of Batzli et al. (4) and Norris (18). All tests were conducted in liquid medium with cultures adjusted to pH 6.8, agitated on a gyratory platform shaker at 1.7 oscillations/s, and incubated at 28 ± 1C (82.4 ± 1.8F). Isolates were grouped by cluster analysis of these characteristics, and one representative isolate was chosen from each group (four from each of the two forests) to undergo further testing for symbiotic efficiency with Amur maackia.

Quantification of symbiotic efficiency. The eight rhizobial isolates from China were evaluated for nodulation capacity and symbiotic efficiency along with USDA 4349 and MF-J, which was isolated from nodules on roots of *Maackia floribunda* (Miq.) Takeda in Japan and was shown to induce nodules on Amur maackia (7). Seedlings were inoculated as during earlier trials by using the inoculation-jar system. Rhizobia were cultured in liquid AG medium as during earlier trials, and serial dilutions were plated on AG with agar to determine cell density. Each experimental unit was inoculated with liquid cell culture diluted to provide 10^7 cells. Units were arranged on a glasshouse bench in a completely randomized design with 12 replicates for each of the 10 isolate treatments and an uninoculated control treatment. Plants were grown under a 16-hour photoperiod during which solar radiation was supplemented with four 400-W high-pressure sodium lamps arranged 1 m (3.3 ft) above the canopy. Experimental units were provided with 100 mL (6.1 in^3) of sterile, N-free 25% Hoagland solution every 10 days and, between those applications, with sterile, deionized water when the liquid in most jars was reduced to the level of the sand. After 14 weeks, plants were harvested and measured for number of nodules per plant, plant weight after drying tissue for 3 days at 67C (153F), and total N concentration of tissues. Total N was determined by using a modified micro-Kjeldahl digestion procedure (11, 17) in conjunction with a nitroprusside-salicylate assay (26) by using flow-injection analysis (23). Total N per plant was calculated by multiplying the N concentration of each plant by the plant dry weight, and N per nodule was calculated by subtracting the mean N per plant of the uninoculated controls from the N per plant of each inoculated seedling, then dividing that quantity by the number of nodules.

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 (21). Data sets were tested for homogeneity of variance by using Levene's test (20), and non-homogeneous data were transformed by a log or square-root function. Means were calculated from raw data, and mean-separation statistics were calculated from raw or transformed data as necessary. Ward's cluster analysis was performed by using JMP® software, Version 3.2.6 (1989–1999).

Results and Discussion

Isolation and selection of compatible rhizobia. The 'crushed-nodule' isolation technique of Vincent (25) was successful for isolating rhizobia from soil samples collected beneath indigenous Amur maackia in China. Isolation attempts with up to five nodules from each soil-inoculated seedling yielded >200 putative rhizobial isolates, 170 of which were authenticated as pure cultures of rhizobia. Of the 340 seedlings of Amur maackia that were aseptically inoculated with these cultures, only three seedlings, each from different isolate treatments, failed to nodulate. Absence of nodules on uninoculated controls confirmed the validity of the test by providing assurance that nodules resulted from inocula rather than from contamination. Fifty of the isolates that evoked the highest number of nodules on seedlings were selected for further characterization. Of these 50, 79% were from Liangshui Forest, suggesting that soils from this more northerly ecosystem may contain a higher percentage of highly compatible rhizobia than the soils of Maoershan Forest.

Characterization and selection of rhizobia. The 50 rhizobial isolates from China showed diversity in physiological characteristics, suggesting the presence of different strains and possibly different rhizobial species, but general similarities between the isolates and the previously selected *Bradyrhizobium*, USDA 4349, led us to conclude that all of the isolated strains are *Bradyrhizobium* spp. rather than members of another rhizobial genus such as *Rhizobium*. These results confirm the findings of Foster et al. (6) that showed nodulation of Amur maackia with several isolates of rhizobia and indicated that Amur maackia has a wide compatibility with diverse *Bradyrhizobium*. Our results also add to the number of reports indicating promiscuous nodulation of woody legumes by rhizobia within a genus, an observation first reported by Turk and Keyser (24). Cluster analysis based on standardized results of the three physiological parameters placed the 50 isolates into eight well-defined clusters. We selected one representative strain with high nodulation capacity from each of the clusters (four each from the two forests) to undergo testing for symbiotic efficiency.

Quantification of symbiotic efficiency. There were no differences in nodulation capacity among the eight representative isolates from Chinese soils (Table 1), but two of the new isolates (Maoershan-D and Maoershan-G2) and previously selected MF-J evoked more nodules on Amur maackia than did USDA 4349, a *Bradyrhizobium* previously selected for superior nodulation capacity and symbiotic efficiency (4). Nodules were absent from all uninoculated control plants. All of the rhizobial isolates led to higher plant dry weight than that of controls (Table 1), and plants inoculated with isolate MF-J weighed more than plants inoculated with Maoershan-B, Maoershan-G1, and USDA 4349. All of the rhizobia formed effective N_2-fixing symbioses with Amur maackia, evoking higher N per plant than that of uninoculated controls, and three of the isolates from the Liangshui forest, along with MF-J, showed superior N_2-fixing capacity (higher N per plant) than USDA 4349 and two of the strains from the Maoershan forest (Table 1). Three of the isolates from the Liangshui forest evoked higher N fixed per nodule than most of the isolates from the Maoershan forest. Analysis of provenance effects among the new rhizobial isolates from China showed no difference in nodulation capacity, but isolates from the Liangshui provenance evoked higher plant dry weight,

Table 1. Mean number of nodules, plant dry weight, and N content of *Maackia amurensis* (Amur maackia) inoculated with rhizobia isolated from soils of two Chinese forests (Liangshui and Maoershan) where Amur maackia is native. Isolate MF-J was previously isolated from nodules of *Maackia floribunda* from Japan (7), and isolate USDA 4349 is a previous selection from the root zone of a cultivated Amur maackia, planted outside its native range in China (4). Plants were grown in a glasshouse for 14 weeks and irrigated with N-free, 25% Hoagland solution.

Isolate	Number of nodules per plant	Plant dry weight (mg)	N per plant (µg)	N per nodule (µg)
Liangshui-A[z]	7.8ab[y]	349ab	596a	58a
Liangshui-C	7.8ab	312ab	576a	64a
Liangshui-E	8.7ab	313ab	559ab	51ab
Liangshui-F	7.8ab	346ab	624a	63a
Maoershan-B	7.1ab	262b	440b	38bc
Maoershan-D	9.4a	269ab	505ab	39bc
Maoershan-G1	8.4ab	245b	421b	32c
Maoershan-G2	9.8a	269ab	486ab	42abc
MF-J	10.5a	368a	650a	54ab
USDA 4349	5.9b	260b	414b	45abc
Control[x]	0.0c	130c	140c	
Provenance				
Liangshui	8.0a	330a	589a	59a
Maoershan	8.7a	261b	463b	38b

[z]Soil samples were collected in the Heilongjiang Province of China, from the Liangshui Research Forest (47°10' N, 128°53' E), and Maoershan Research Forest (45°30' N, 127°32' E). Letters (A–G) indicate isolates from different soil samples.

[y]Means within each column followed by the same letter are not different at $P \leq 0.05$ according to Fisher's least significant difference test. Mean-separation statistics were assessed separately for Isolate (N = 12) and Provenance (N = 48) categories.

[x]Control plants were uninoculated.

higher N per plant, and higher N per nodule than did isolates from the Maoershan forest, again suggesting that rhizobia from the Liangshui forest are better adapted for N_2-fixing symbioses with Amur maackia than are those from the Maoershan forest.

Although cross-inoculation success has been reported with a few other woody legumes (3), it is noteworthy that a rhizobial strain isolated from the nodules of a different species (MF-J from *M. floribunda*) formed a symbiosis with Amur maackia that was superior to symbioses involving some of our isolates from root zones of indigenous Chinese Amur maackia. This suggests that, although Amur maackia shows high general specificity and only forms effective symbioses with members of one genus of rhizobia (*Bradyrhizobium*) from soils of established trees (4), Amur maackia also is compatible with isolates adapted for symbioses with other *Maackia* spp. To discover optimum symbiotic partnerships, the specificity and effectiveness of rhizobial strains isolated from diverse soils and closely related host species should be examined.

Horticultural implications. In earlier research, Pai and Graves (19) found no net effect of nodulation on the growth of Amur maackia seedlings inoculated with rhizobial selection USDA 4349 and provided with low levels of supplemental N. Our results add to those earlier findings and reveal that, although USDA 4349 was the most effective isolate known at the time, this isolate is not optimal for horticultural use with Amur maackia. We also demonstrated that, in the absence of supplemental N, all seedlings infected and nodulated with selected rhizobial strains (including USDA 4349) grew larger and had higher N per plant than did non-nodulated seedlings. While it is likely that the growth potential of Amur maackia achieved by using generous but potentially wasteful amounts of N fertilizer cannot be achieved with production methods that rely solely on N_2 fixation (19), potential exists for the production of healthy, N_2-fixing nursery crops with little or no N input, and therefore, much lower economical and environmental costs. The ease with which effective rhizobia were isolated from soils under native plants and established in pure culture is a reassuring indication of the feasibility of developing commercial inocula for the production of Amur maackia that gain much or all of their N through N_2 fixation.

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