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Isolation and Characterization of Rhizobia Effective with *Maackia amurensis*

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Abstract. **Our objectives were to test whether *Maackia amurensis* Rupr. & Maxim. nodulates and fixes N and to characterize the N-fixing bacteria effective with this host. Soil samples were collected near diverse legume trees at arboreta and public gardens in the United States, Canada, and China. Seedlings of *M. amurensis* were grown for 6 weeks in a low-N, sterile medium and inoculated with soil samples. At harvest, nodules were found on the lateral and upper portions of root systems. Bacteria were isolated from nodules and subculture. Roots of seedlings inoculated with all 11 of these isolates nodulated and freed N, confirming that the isolates were rhizobial bacteria. Growth of isolates in axenic culture generally was poor when single sources of C were provided. Generation times of the isolates ranged from 6 to 10 hours, and all isolates raised the pH of culture media. Isolates were highly resistant to several antibiotics, showed no 6-phosphogluconate dehydrogenase (6PGD) or β– galactosidase activity, and were highly sensitive to NaCl. These results provide the first evidence that *M. amurensis* has the capacity to form N-fixing symbioses with rhizobial bacteria and indicate that the bacteria are *Bradyrhizobium* sp.**

Diversification of species is a primary goal of many municipal tree programs. The number of tree species considered capable of resisting environmental strains in cities is limited, and identification of additional stress-resistant genotypes is needed. Leguminous trees that fix N may be particularly useful in urban areas. The physical and chemical quality of soil at city planting sites often is poor (Craul, 1985), and insufficient N frequently limits the growth of landscape trees (Harris, 1992). Growth of N-fixing species may be less restricted by low soil N than the growth of other trees. These species also may accrue N in the soil that can benefit adjacent vegetation. In addition, inoculation of trees with rhizobia might permit a reduction in the quantity of N fertilizer used during nursery production, which could reduce costs and curtail the environmental damage caused by N leaching. Few of the legume tree species now in nursery production have been shown to fix N. All attempts to nodulate thornless honey locust *(Gleditsia triancanthos* L. var. *inermis* Wind.), redbud *(Cercis canadensis* L.), yellowwood *[Cladrastis kentukea* (Dum.-Cours.) Rudd], and Kentucky coffee tree *[Gymnocladus dioicus* (L.) K. Koch] have failed (Allen and Allen, 1981).

Maackia amurensis of the Papilionoideae subfamily of the Leguminosae is native to Korea and Manchuria. Named for Russian naturalist Richard Maack, *M. amurensis* resembles *Cladrastis* sp. and was previously named *C. amurensis* K. Koch. (Rehder, 1940). Trees have bronze, exfoliating bark and form creamy-white flowers in midsummer. They are not prone to insect or disease attacks and are cold hardy to U.S. Dept. of Agriculture (USDA) zone 4 (Dirr, 1990). Little is known about the capacity of *M. amurensis* to fix N. Lopatina (1931) found no nodules on roots of trees in the Soviet Union, and no nodulation was observed by Wilson (1945) after seedlings of *M. chinensis* Takeda. were inoculated with 106 diverse rhizobia. In 1970, however, Uemura reported nodules on *M. amurensis* var. *buergeri.* (Maxim.) C.K. Schneid in Japan through an unsubstantiated personal communication with Allen and Allen (1981). Because of this report, and the fact that modulation has been confirmed in 98% of the species in the Papilionoideae that have been observed (Allen and Allen, 1981), the first objective of this study was to test whether *M. amurensis* nodulates and fixes N in association with rhizobial bacteria.

The second objective of this project was to characterize the rhizobial bacteria that form symbioses with *M. amurensis.* Early investigators of N fixation regarded all rhizobia as a single species capable of modulating all legumes. During the last century, however, information on their host range and specificity has led to distinctions between these bacteria. Most recently, rhizobia have been placed in two genera, *Rhizobium* and *Bradyrhizobium,* based on their relative growth rates, physiology, and biochemical traits (Jordan, 1982). Characterization of the bacteria effective with *M. amurensis* was conducted to gather fundamental knowledge on the nature of these organisms and to provide practical information that will aid in their culture and use.

Materials and Methods

Isolation of rhizobia. Soil samples were shipped to us by cooperators at 15 arboreta and botanic gardens in the United States, Canada, and China (Table 1). Samples were taken at a depth of 4 to 6 cm adjacent to trunks of trees belonging to the

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Abbreviations: MAG, modified arabinose gluconate; NADP, nicotinamide adenine dimucleotide; PAR, photosythetically active radiation; YEM, yeast extract mannitol agar.

Table 1. Modulation and collection of *Maackia amurensis* isolates after inoculating seedlings with soil samples obtained from arboreta and botanic gardens.

Origin of soil sample	Nodulation[z]	Isolates recovered (no.)	USDA accession nos. assigned to isolates
Boston	+	0	
Clinton, Iowa	+	0	
Denver	−	0	
Ithaca, N.Y.	−	0	
Lexington, Ky.	+	0	
Lisle, Ill.	−	0	
Mentor, Ohio	−	0	
Miami	−	0	
Minneapolis	+	0	
San Francisco	−	0	
Tucson, Ariz.	+	0	
Washington, D.C.	+	4	4341, 4342, 4350, 4351
Wheaton, Md.	+	0	
Hamilton, Ont., Canada	−	0	
Nanjing, China	+	7	4343–4349

[z] + signifies nodulation; − signifies no nodulation.

Leguminosae. Only the samples from Washington, D.C., and Nanjing, China, were taken from the root zones of *M. amurensis.* Samples were held in the dark at 4C for up to 14 days until all were received.

Half-sib seeds of *M. amurensis* were scarified for 60 min in 18 M sulfuric acid and washed in sterile, deionized water. Seeds were placed on 8 g agar/liter in petri plates and germinated in the dark at 30C for 4 days. Two germinated seeds were sown aseptically in each of forty-eight 2.5-liter pots containing a sterile, soilless medium (Jiffymix, W.R. Grace, Cambridge, Mass.). Surface-sterilized, pregerminated seeds of *Sophora japonica* L. and *Robinia pseudoacacia* L. were sown in the same containers as part of separate experiments (Batzli, 1991). Three randomly chosen replicate containers were inoculated with 5 g of soil from each of the 15 samples. The inoculum was placed in the bottom of small furrows in which the seeds were sown. Three containers that were not inoculated served as controls. All containers were arranged randomly in a glasshouse under natural radiation in Sept. 1989. After 7 days, seedlings were thinned aseptically so that one of each species remained in each pot. Plants were irrigated with a N-free nutrient solution (Norris, 1964) when the surface of the medium appeared dry.

Plant root systems were separated from the medium after 6 weeks of growth and examined for modulation. Bacteria were isolated from nodules >3 mm in diameter (Vincent, 1970) and subculture on modified arabinose gluconate (MAG) agar plates (van Berkum, 1990). Cultures were incubated at 28C and stored on MAG agar slants at 4C.

To verify that the isolates were rhizobial, seedlings were inoculated in sterile growth pouches with paper towel matrices (Porter et al., 1966) that allowed root and nodule development to be monitored nondestructively. Seeds of *M. amurensis* were germinated aseptically and two were placed in each pouch and irrigated with the N-free nutrient solution. Axenic cultures of the 11 *M. amurensis* isolates were used to inoculate 10 ml of MAG broth that was placed on a shaker at 100 rpm for 5 days at 28C. Seedlings in pouches were inoculated by applying 1.5 ml of isolate culture at 10^8 cells/ml to the root zone. Three replicates of each isolate and three noninoculated controls were arranged randomly in a plant growth chamber at 30C with 16-h photoperiods at 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR) supplied by cool-white fluorescent lamps. The plants were grown for 6 weeks. Formation of root nodules was considered verification that the isolates were rhizobial. The isolates were deposited in the USDA National *Rhizobium* Collection, and they are cited herein according to their USDA accession numbers.

Characterization of rhizobia. Rhizobium leguminosarum biovar. *viceae* (USDA 2434) and *Bradyrhizobium japonicum* (USDA 97) obtained from the USDA National *Rhizobium* Collection were used as controls during characterization.

Colonies of *M. amurensis* and control isolates were examined for size, shape, color, and the production of exogenous polysaccharide gum after 5 days of growth on MAG agar. The gram stain reaction of each culture was observed after 5 days of growth in MAG broth medium at 28C (Vincent, 1970).

Intrinsic antibiotic resistance was determined on MAG agar plates, in duplicate, containing two concentrations (25 or 100 $mg \cdot liter^{-1}$) of rifampicin, streptomycin, spectinomycin, kanamycin, ampicillin, chloramphenicol, nalidixic acid, and tetracycline. All antibiotics were filter-sterilized (pore size 0.22 µm) and added to cooled, molten agar medium at ≈ 45C. Cultures were scored after 5 days of incubation at 28C: 0 = no growth, 1 = growth on 25 mg antibiotic/liter, and 2 = growth on 100 mg antibiotic/liter.

To determine the effect of the isolates on the pH of growth media, each isolate and the control strains were incubated in two replicate tubes containing bromthymol blue at 0.25 $mg \cdot liter^{-1}$ in yeast extract mannitol agar (YEM) (Vincent, 1970) for 7 days at 30C and scored visually based on color change of the media (Norris, 1965).

Tolerance to NaCl of each isolate and the control strains was determined by evaluating the development of distinct colonies after 7 days on MAG agar plates containing NaCl at 7, 8.5, 10, 12, or 15 $g \cdot liter^{-1}$. There were two replicate plates of each isolate at each concentration of NaCl.

Carbohydrate usage of the isolates was tested using MAG agar plates in which 1-(+)-arabinose, d-gluconate, and yeast extract were replaced with dl-malate, α– d(+)-glucose, sucrose, α– 1-rhamnose, d-fructose, d-gluconate (sodium salt), dextrose, d-(+)-mannose, myo-inositol, d-(–) ribose, maltose, α– lactose, d-mannitol, d-(+)-galactose, 1-(+)-arabinose, and yeast extract at 10 $g \cdot liter^{-1}$. The media were solidified with purified agarose at 8 $g \cdot liter^{-1}$. All carbohydrates were filter-sterilized (pore size 0.22 µm) before they were added to molten agar media at 45C. Complete MAG agar and MAG-purified agar with no C source were used as controls. Isolates were swabbed from MAG-agar slants and inoculated onto the surface of the carbohydrate plates. There were two replicate plates of each isolate for each carbohydrate treatment. Growth, defined by the formation of individual colonies, was observed after 7 days of incubation at 30C.

Activity of 6-phosphogluconate dehydrogenase (EC 1.1.1.44; 6PGD) was determined in cultures that had been grown for 3 days at 28C in yeast extract-glucose medium (Keele et al., 1969) using the cell-extraction method of Sadowsky et al. (1983). The activity of nicotinamide adenine dinucleotide phosphate (NADP)-linked 6PGD was measured through the reduction of NADP as described by Martinez-de Drets and Arias (1972). Activity of β– galactosidase (EC 3.2.1.23) was assessed by the formation of yellow pigment in cultures that had been grown in MAG broth for 5 days at 28C using the methods of Miller (1972).

Mean generation times were determined in cultures of *M. amurensis* isolates and control strains that had been grown in YEM broth for 5 days at 30C. Fractions of 0.2 ml of each culture at 10^8 cells/ml were used to inoculate 100 ml YEM broth in 300-ml Erlenmeyer flasks equipped with calorimetric tubes as side arms. There were two replicate flasks for each isolate and control strain culture. Cultures were grown on a shaker at 200 rpm at 30C. Absorbance was measured in Klett units twice per day for 7 days on a photoelectric calorimeter fitted with a green filter (Klett-Summerson, Model no. 800-3, New York). Mean generation times were calculated as described by Cooper (1971).

Host plant response. Leonard jars (Leonard, 1944) with volumes of 750 ml were filled with a mixture of 1 Beltsville sandy loam soil : 1 perlite (v/v). After the filled jars were autoclave, one aseptically germinated *M. amurensis* seedling was planted aseptically in each. Seedlings were inoculated with 1.5 ml (10^8 cells/ml) of bacterial broth as they were sown. There were five replicate seedlings treated with each of the 11 *M. amurensis* isolates and five noninoculated controls. Cultures had been grown for 5 days in MAG broth at 28C before use. Jars were arranged in a randomized complete-block design in a plant growth chamber at 30C with 16-h photoperiods of 450 µmol $m^{-2} \cdot s^{-1}$ PAR from cool-white fluorescent lamps.

Nitrogenase activity was estimated using the acetylene reduction assay (Hardy et al., 1968) as plants were harvested 60 days later. Root tissue was severed from the stem and washed gently to remove most of the growth medium. Root systems were placed singly in air-tight incubation vessels fitted with serum stoppers. Acetylene was added to each vessel to 10% by volume, and roots were incubated for 10 min. Reduction of acetylene to ethylene was determined on a Varian 3700 Gas Chromatograph (Varian, Walnut Creek, Calif.). Nodules were then separated from roots, and nodule and plant weights were determined after they were dried in a forced-air oven at 65C for 2 days. Nitrogen content of dried tissues from all plant parts was measured using a Carlo Erba Strumentazione Nitrogen Analyzer 1500 (Milan, Italy). Analysis of variance was conducted for dependent variables using the statistical analysis system (SAS, Cary, N.C.). Treatment means were separated using Fisher's least significant difference, and inoculated and noninoculated plants were compared using single-degree-of-freedom orthogonal contrasts.

Results

Isolation of rhizobia. Seedlings of *M. amurensis* grown in medium inoculated with soils from eight of the 15 locations formed nodules. Bacteria were isolated from nodules on plants grown in medium inoculated with soil from the U.S. National Arboretum, Washington, D.C., and Nanjing Botanic Garden (Table 1). Each isolate from China originated from a distinct nodule. However, each of two nodules on plants grown with soil from Washington, D. C., yielded bacterial cultures with two distinct colony morphologies. Therefore, although USDA 4341 and USDA 4342 originated from the same nodule and USDA 4350 and USDA 4351 originated from another, each was treated as a separate isolate. All 11 isolates were verified to be rhizobial after *M. amurensis* seedlings inoculated with them nodulated.

Characterization of rhizobia. The 11 isolates were gram-negative, short, rod-shaped bacteria. Most formed circular, raised, opaque to creamy-white, small colonies (0.5 to 1.5-mm diameter) with little gum. Colonies of one of the isolates, USDA 4349, were unusually large (1.5 to 2.5-mm diameter), circular, creamy-white, and produced gum. Mean generation times of the isolates ranged from 6 to 10 h, whereas the *Bradyrhizobium* and *Rhizobium* control had generation times of 14 and 4 h, respectively (Table 2).

All isolates had identical antibiotic resistance (Table 2). They were highly resistant to all antibiotics tested except kanamycin and nalidixic acid. This pattern was similar to the *Bradyrhizobium* control and in sharp contrast to the *Rhizobium* control (Table 2). Neither the isolates nor the *Bradyrhizobium* control grew on the lowest concentration of NaCl, 7 g·liter^{-1} (Table 2). On YEM agar slants containing 0.25 mg bromthymol blue/liter, *M. amurensis* isolates produced a deep-blue pigment, indicating that they raised the pH of the medium. Cultures of the *Bradyrhizobium* control had a similar effect, but the *Rhizobium* control lowered the pH of the medium (Table 2). Neither the *M. amurensis* isolates nor the *Bradyrhizobium* control showed 6PGD or β– galactosidase activities, but the *Rhizobium* control showed activity of both enzymes (Table 2).

Colony growth of all *M. amurensis* isolates was poor on all single-carbon sources compared to that on complete MAG agar. All isolates except USDA 4345, USDA 4348, and USDA 4349 grew only on MAG medium supplemented with yeast extract. USDA 4345, unlike the *Bradyrhizobium* control, grew on media with d-(+)-galactose, 1-(+)-arabinose, α– d-(+)-glucose, and yeast extract. USDA 4348 grew on media with d-(+)-galactose, α– d-(+)-glucose, and yeast extract. USDA 4349 grew on media with maltose, d-(+)-galactose, dextrose, α– d-(+)-glucose, and yeast extract. Unlike any *M. amurensis* isolate, the *Rhizobium* control grew on all C sources except dl-malate and d-gluconate.

Host plant response. All 11 *M. amurensis* isolates formed nodules on host seedlings. Mean acetylene reduction rates, nodule dry weight, and total N content of the inoculated and control plants varied significantly (Table 3). USDA 4342 was not sig-

Table 2. Biochemical and cultural characteristics of rhizobial control strains and isolates effective with *Maackia amurensis.*

	Strains/isolates		
Test	*M. amurensis* isolates	USDA 97[z]	USDA 2434[y]
pH reaction	Alkaline	Alkaline	Acid
6-PGD activity[x]	–	–	+
β-Galactosidase[x]	–	–	+
Generation time (h)	6–10	14	4
Intrinsic antibiotic resistance[w]			
Rifampicin	2	2	0
Streptomycin	2	2	1
Spectinomycin	2	2	1
Kanamycin	0	1	0
Ampicillin	2	2	0
Chloramphenicol	2	2	0
Nalidixic acid	1	2	0
Tetracycline	2	2	0
NaCl (%) tolerance[x]			
0.7	–	–	+
0.85	–	–	+
1.0	–	–	–
1.2	–	–	–
1.5	–	–	–

[z]Slow-growing *Bradyrhizobium japonicum*.
[y]Fast-growing *Rhizobium leguminosarum* biovar. *viceae*.
[x]+ = colony growth; – = no colony growth.
[w]0 = no growth at either level of antibiotic; 1 = growth on only 25 mg antibiotic/liter; 2 = growth on both 25 and 100 mg antibiotic/liter.

Table 3. Mean acetylene reduction rates, nodule dry weights, plant N content, and plant dry weight of *Maackia amurensis* seedlings inoculated with rhizobial isolates.

Isolate (USDA no.)	Acetylene reduction rate (μmol/plant per h)	Nodule dry wt (mg)	Plant N content (%)	Plant dry wt (mg)
4351	1.83 ab[z]	33.74 ab	3.20 bc	744
4350	2.56 ab	44.02 ab	3.48 ab	631
4349	3.14 a	43.40 ab	3.66 a	853
4348	2.25 ab	37.12 ab	3.43 abc	820
4357	2.05 ab	22.14 b	3.18 bc	530
4356	2.02 ab	46.86 a	3.10 c	491
4345	1.70 b	22.56 b	3.44 abc	680
4344	1.39 b	26.60 ab	3.38 abc	597
4343	1.75 b	38.26 ab	3.10 c	524
4342	1.34 bc	37.20 ab	2.47 d	564
4341	1.90 ab	35.28 ab	2.57 d	491
Control	0.0 c**	0.0 c**	2.36 d**	520

[z]Mean separation within columns by Fisher's LSD ($\alpha = 0.01$).
**Significant ($\alpha = 0.01$) orthogonal contrast analyses for inoculated vs. control plants.

nificantly different from the control seedlings for acetylene reduction rate and total plant N, and the N content of plants inoculated with USDA 4341 was similar to that of control plants (Table 3). Using single-degree-of-freedom contrast analyses, differences between the inoculated plants and the noninoculated controls were significant for acetylene reduction rate, nodule dry weight, and total N content. Mean plant dry weight was similar for inoculated and noninoculated plants.

Discussion

These results provide the first evidence that *M. amurensis* forms N-fixing symbioses with rhizobial bacteria. Its capacity to fix N represents an important attribute that may make *M. amurensis* a particularly important component of urban landscapes. The resistance of seedlings of *M. amurensis* to root-zone heat (Graves, 1992). and the ornamental traits, size, form, and resistance to pests and diseases of trees of this species (Dirr, 1990) suggest *M. amurensis* has potential for use along city streets and in other stressful environments. As a N-fixing species, trees of *M. amurensis* may be more capable than other species of sustaining growth when installed in the infertile soils common in urban areas (Craul, 1985). Further, production of this species in nurseries might require little or no input of N fertilizer. Because the commercial production and use of *M. amurensis* has been very limited, additional research is needed to document its performance in urban landscapes and to maximize the efficiency of N-fixing symbioses, both during production and after installation in the landscape.

Although inoculation with eight of the 15 soil samples caused modulation, isolates were recovered only from nodules on plants inoculated with samples collected beneath trees of *M. amurensis* in Washington, D.C., and Nanjing. Nodules from seedlings inoculated with these two soil samples were relatively large and had pink centers, indicating active leghemoglobin. Nodules from all the other sampling locations were smaller and had white centers, suggesting they were ineffective. Although a more thorough collection of *M. amurensis* rhizobia would be needed to determine the distribution of indigenous rhizobial populations in the soil, our results suggest bacteria that form effective symbioses with *M. amurensis* are not ubiquitous in soils and are found only in root zones of *M. amurensis* trees. The distribution of effective rhizobia in soils could have important consequences for horticulturists. If the bacteria are not present in soils in urban landscapes, the establishment of symbioses during nursery production might be critical to the performance of trees after installation.

Data on the characteristics of bacteria effective with *M. amurensis* are useful as indicators of isolate diversity and for classification of the organisms into the correct rhizobial genus. The 11 *M. amurensis* isolates had identical antibiotic resistance and all were very sensitive to NaCl (Table 2). These similarities suggest that all isolates might be a single strain. However, USDA 4349 had a unique colony morphology, and USDA 4345, USDA 4348, and USDA 4349 were capable of growth on media with different carbohydrate sources from the others. Whether any of the 11 isolates are the same strain remains in doubt, but our data provide two strong indications that all should be classified as *Bradyrhizobium* sp. First, the low tolerance to NaCl shown by the *M. amurensis* isolates is characteristic of members of this genus (Graham and Parker, 1964; Sadowsky et al., 1983). Second, the highly alkaline pH reaction of the isolates was similar to the *Bradyrhizobium* control and is consistent with Norris's (1965) hypothesis that nonacid-producing rhizobia grow slowly. A slow rate of growth is considered a trait of *Bradyrhizobium* (Jordan, 1982). Interestingly, *M. amurensis* isolates had generation times intermediate to the *Bradyrhizobium* and *Rhizobium* controls (Table 2), which are considered slow and fast growing, respectively. Such terms for describing growth rates, widely used to characterize rhizobia, appear to describe only a subset of these bacteria.

Although the growth rates of the *M. amurensi.s* isolates were intermediate to the fast- and slow-growing controls, other traits show similarity to slow-growing *Bradyrhizobium.* The *M. amurensis* isolates grew poorly on all the C sources except yeast extract. Studies conducted by Graham and Parker (1964) and Fred et al. (1932) showed that fast-growing strains in the genus *Rhizobium* are capable of growth on more carbohydrates than slow-growing rhizobia. Further, neither the *M. amurensis* isolates nor the slow-growing *Bradyrhizobium* control showed 6PGD or β– galactosidase activity. Consistent with these results, Sadowsky et al. (1983) and Martinez-de Drets and Arias (1972) found that only fast-growing rhizobia have 6PGD activity. Working with rhizobia effective with soybean *(Glycine max* L.), Sadowsky et al. (1983) found that fast-growing *Rhizobium* tend to have relatively high β– galactosidase activities, while activity is low in slow-growing *Bradyrhizobium.* Thus, the carbohydrate usage and enzyme activity of the *M. amurensis* isolates also indicate that they are *Bradyrhizobium* sp.

The host-plant response experiment confirmed that all 11 *M. amurensis* isolates nodulated seedlings. The mean acetylene reduction rates and total N of plants inoculated with the strains generally were higher than for the noninoculated controls (Table 3). However, acetylene reduction rates of roots inoculated with USDA 4342 and N content of plants inoculated with USDA 4341 and USDA 4342 were not significantly different from control seedlings. Additional research should explore this apparent variation between the efficiency of the isolates at fixing N with *M. amurensis* seedlings. Although inoculation with most isolates increased the N content of host seedlings, dry weights of inoculated plants were not significantly different from those of the noninoculated controls. At least two factors might have contributed to this finding. First, metabolic energy was required for the formation of nodules and the weight of these was not included in the plant dry weight data (Table 3). Second, we have observed that seedlings

of *M. amurensis* grow at a much slower rate than seedlings of other temperate leguminous trees. A longer growth period maybe needed in subsequent studies to demonstrate the effect of N fixation on dry-matter production in *M. amurensis.*

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