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# Nodulation and Nitrogen Fixation Efficacy of *Rhizobium fredii* with *Phaseolus vulgaris* Genotypes

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***Phaseolus* plant introduction (PI) genotypes (consisting of 684 *P. vulgaris*, 26 *P. acutifolius*, 39 *P. lunatus*, and 5 *P. coccineus* accessions) were evaluated for their ability to form effective symbioses with strains of six slow-growing (*Bradyrhizobium*) and four fast-growing (*Rhizobium fredii*) soybean rhizobia. Of the 684 *P. vulgaris* genotypes examined, three PIs were found to form effective nitrogen-fixing symbioses with the *R. fredii* strains. While none of the *Bradyrhizobium* strains nodulated any of the genotypes tested, some produced large numbers of undifferentiated root proliferations (hypertrophies). A symbiotic plasmid-cured *R. fredii* strain failed to nodulate the *P. vulgaris* PIs and cultivars, suggesting that *P. vulgaris* host range genes are Sym plasmid borne in the fast-growing soybean rhizobia.**

Members of the genera *Rhizobium* and *Bradyrhizobium* are nitrogen-fixing soil bacteria which have the ability to form root nodule symbioses with leguminous plants. The genus *Rhizobium* contains the four fast-growing species *R. fredii*, *R. leguminosarum* (consisting of three biovars: *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *phaseoli*, and *R. leguminosarum* bv. *viceae*), *R. loti*, and *R. meliloti*, while the genus *Bradyrhizobium* contains the single slow-growing species *B. japonicum* and other slow-growing root nodule bacteria yet to be assigned species status (11). The current classification scheme bases species designation mostly on the ability of a specific bacterium to nodulate a given legume host and divides the rhizobia into several plant infection or cross-inoculation groups (11, 21). Although this scheme does have certain practical applications, there are some major problems associated with these divisions (21). While some rhizobial strains can be defined by their ability to nodulate a relatively small number of host plants, others can promiscuously nodulate many different legumes.

The rhizobia that nodulate *Phaseolus* spp. have been divided into two distinct groups (1, 2, 6). *Phaseolus vulgaris* (common bean) and *P. coccineus* (scarlet runner bean) are nodulated by fast-growing *R. leguminosarum* bv. *phaseoli* isolates, while *P. lunatus* and *P. acutifolius* (tepary bean) are nodulated by slow-growing *Bradyrhizobium* spp. isolates. Although *P. coccineus* is relatively specific in its rhizobial requirements, *P. vulgaris* has been reported to be rather promiscuous (5–7). Reports that *B. japonicum* strains can form symbioses with *P. vulgaris* have been contradictory. While results of studies by Ishizawa (10) and Graham and Parker (7) have indicated that several *B. japonicum* isolates nodulate *P. vulgaris*, Taha et al. (19) and Barua and Bhaduri (2) have reported that the *B. japonicum* isolates they examined failed to nodulate the tested *P. vulgaris* genotypes. In addition, it has been reported (2, 10, 12, 13) that *B. japonicum* isolates are also capable of nodulating several other *Phaseolus* spp. (*P. aureus*, *P. mungo*, *P. calcaratus*, *P. sublobatus*, *P. trilobus*, *P. aconitifolius*, *P. atropurpureus*, and *P. lathyroides*). These host plants, however, have since been reclassified as belonging to the genera *Vigna* and *Macroptilium* (1).

Since many *Phaseolus* species have been transferred into other host genera and since there are contradictory reports as to the rhizobial requirements of the currently classified *P. vulgaris* genotypes, it was of interest to determine whether *B. japonicum* and *R. fredii* (the fast-growing soybean-nodulating bacteria [17]) are capable of forming effective nitrogen-fixing symbioses with a variety of genetically and geographically distinct *Phaseolus* genotypes.

## MATERIALS AND METHODS

**Primary screening experiments.** Initial experiments were designed to determine whether the fast- and slow-growing soybean rhizobia could effectively nodulate a large number of geographically (and presumably genetically) diverse *P. vulgaris*, *P. lunatus*, *P. coccineus*, and *P. acutifolius* genotypes. Inoculum for these studies consisted of an equal (vol/vol) mixture of the *B. japonicum* strains USDA 31, USDA 76, USDA 110, USDA 122, USDA 123, and USDA 138 and the *R. fredii* strains USDA 192, USDA 201, USDA 205, and HH103. Cultures were grown individually in yeast extract-mannitol medium (20) to the early stationary phase and combined to form a mixed inoculum consisting of approximately $10^8$ cells of each strain per ml. *R. leguminosarum* bv. *phaseoli* USDA 2667 was used as a positive control for the *Phaseolus* genotypes. Surface-sterilized seeds of 754 *Phaseolus* plant introductions (PIs), consisting of 684 *P. vulgaris*, 26 *P. acutifolius*, 39 *P. lunatus*, and 5 *P. coccineus* genotypes (obtained from Richard Hannan, Regional Plant Introduction Station, Agricultural Research Service, U.S. Department of Agriculture, Pullman, Wash.), were planted in 4.8-liter plastic pots containing sterilized vermiculite, as described previously (3). Ten different PIs were planted in each pot in separate "hills" (3) by using six seeds of each PI per hill and were inoculated with 1.0 ml of the mixed soybean rhizobia inoculum. Pots containing *Glycine max* cv. Essex, Corsoy, Peking, Jupiter, and Williams served as positive inoculation controls. Plants were watered with nitrogen-free plant nutrient solution (4), thinned to two plants per hill 10 days after planting, and grown in a greenhouse with supplemental lighting to extend the photoperiod to 18 h (3). Day and night temperatures were maintained at 25 ± 5°C. Plants were harvested 5 weeks after inoculation. The root systems were examined for the pres-

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ence of nodules, and plants were scored for visual symptoms of nitrogen deficiency (top color). Nodule occupants were determined by using strain-specific fluorescent antibodies that were produced as described by Schmidt et al. (16).

**Secondary screening.** Five of the *P. vulgaris* PI genotypes (PIs 181786, 209483, 209484, 209491, and 219701) identified in the initial screening experiment were reexamined for their nodulation and nitrogen fixation responses when inoculated separately with the individual fast- and slow-growing soybean rhizobia. A randomized complete block experimental design was used that consisted of a split plot arrangement of treatments with three replications. Whole plots (pots) were inoculation treatments and subplots were genotypes. Pots (4.8 liters) containing sterilized vermiculite were planted with one hill each of the five *P. vulgaris* PI genotypes; *P. vulgaris* cv. Kentucky Wonder, Great Northern, and C-20; and *G. max* cv. Peking. Three seeds per hill were planted, and hills were individually inoculated with about $10^9$ cells of the *R. fredii* or *B. japonicum* strains that were used in the primary screening. Uninoculated pots served as negative controls. A *R. leguminosarum* bv. *phaseoli* strain mixture (consisting of an equal mixture of the effective strains USDA 2667, USDA 2674, and USDA 2676) served as the positive control for the *P. vulgaris* and *P. coccineus* PIs. Growth conditions were as described above, and plants were harvested 30 days after inoculation. Nodules were counted and weighed, and nitrogen fixation effectiveness was determined by using the acetylene reduction assay of Hardy et al. (8).

**Nitrogen fixation effectiveness of *R. fredii* isolates with *P. vulgaris* PIs.** To determine the nitrogen fixation efficacy of the *R. fredii-P. vulgaris* symbiosis, *R. fredii* strains were inoculated onto three *P. vulgaris* PIs (209483, 209484, and 209491), and total shoot nitrogen (N accumulation) was measured at the time of harvest. The randomized complete block experimental design described above was used. Surface-sterilized seeds of the three *P. vulgaris* PI genotypes, *P. vulgaris* cv. Kentucky Wonder and C-20, and *G. max* cv. Peking and Williams were planted (three seeds of each genotype per hill) in sterilized vermiculite. Hills were inoculated with about $10^9$ cells of *R. fredii* USDA 192, USDA 201, USDA 205, or HH103 or the *R. leguminosarum* bv. *phaseoli* strain mixture described above. At 10 days after planting, plants were thinned to one per hill and grown as described above. Thirty days after inoculation nodules from each plant were removed, dried, and weighed. The plant tops above the cotyledonary node were excised and dried for subsequent total N concentration analysis of duplicate samples by using a nitrogen analyzer (Erba) (3).

**Involvement of the *R. fredii* Sym plasmid in *P. vulgaris* nodulation.** Since the *R. fredii* strains effectively nodulated some of the *P. vulgaris* genotypes in vermiculite, it was of interest to determine whether these rhizobia were also capable of nodulating *P. vulgaris* in soil. We also investigated the possibility that the genetic determinants for *Phaseolus* nodulation were located on the large symbiotic (Sym) plasmid in the *R. fredii* strains. Surface-sterilized seeds of *P. vulgaris* PI 209483, *P. vulgaris* cv. Kentucky Wonder, and *G. max* cv. Peking were planted in 2.2-liter plastic pots containing Monmouth fine sandy loam soil (Alfic Normudult). This soil is essentially free of soybean-nodulating rhizobia (3). Three seeds of each genotype were planted per hill and inoculated with about $10^9$ cells of *R. fredii* USDA 201, USDA 205, or USDA 2051AO3 (a Sym plasmid-cured derivative of USDA 205 [14]) or the *R. leguminosarum* bv. *phaseoli* strain mixture used as described above. Uninoculated pots served as negative controls. Pots were inoculated in triplicate. Plants were grown as described above and harvested 30 days after inoculation. The occupants of 25 nodules from each plant genotype-inoculation treatment were determined by using strain-specific fluorescent antibodies (16).

TABLE 1. Nodulation of *P. vulgaris* genotypes by strains of *R. fredii*

| Genotype | Nodule dry wt produced by strains[a]: | | | |
|---|---|---|---|---|
| | USDA 192 | USDA 201 | USDA 205 | HH103 |
| PI 209483 | 25 a | 35 ab | 16 ab | 26 b |
| PI 209491 | 18 ab | 35 ab | 13 bc | 17 bc |
| PI 209484 | 18 ab | 44 a | 23 ab | 12 bc |
| PI 219701 | 7 bc | 8 c | 6 c | 7 c |
| PI 181786 | 2 c | 8 c | 0 c | 0 c |
| Great Northern | 6 bc | 9 c | 3 c | 0 c |
| Kentucky Wonder | 2 c | 1 c | 3 c | 0 c |
| C-20 | 2 c | 4 c | 0 c | 0 c |
| *G. max* cv. Peking | 21 a | 29 ab | 32 a | 45 a |

[a] Values are milligrams per plant and are the means of three replicates. *R. leguminosarum* bv. *phaseoli* USDA 2667 produced an average of 40 mg (dry weight) of nodules per plant on the *P. vulgaris* PIs and 38 mg per plant on the commercial genotypes. Numbers within a single column that are not followed by the same letter differ significantly at the 0.05 probability level, as determined by Duncan's New Multiple Range Test.

## RESULTS AND DISCUSSION

Of the 754 *Phaseolus* genotypes examined, only the *P. vulgaris* PIs formed nodules with the mixed soybean rhizobia inoculum. Nine of the *P. vulgaris* genotypes (PIs 165038, 181785, 181786, 209259, 209483, 209484, 209491, 209803, and 219701) had 5 to 10 nodules per plant and formed effective nitrogen-fixing symbioses, as indicated by plant top and nodule color. The nodule occupancy of six of the nine effective *P. vulgaris* PIs was determined, and nodules were found to be occupied only by the *R. fredii* isolates. Over 78% of the nodules examined were formed by *R. fredii* USDA 201; and the remainder were formed by strains USDA 192, USDA 205, and HH103 (data not shown). While an additional 78 of the PIs formed one to four small nodules with the soybean rhizobia inoculum, the symbioses were judged to be ineffective (nodules were small and white, and the plants were chlorotic). These genotypes were not examined further. Most of the *P. vulgaris* PIs (693) failed to nodulate when they were inoculated with the fast- and slow-growing soybean rhizobia. Interestingly, 516 of these PIs had large numbers of undifferentiated proliferations (hypertrophies) on their primary and secondary roots. These hypertrophies were white and visually appeared to lack the organizational structure characteristic of nodules. None of the 26 *P. acutifolius*, 39 *P. lunatus*, or 5 *P. coccineus* genotypes examined formed nodules with the mixed soybean rhizobia inoculum.

Results of the secondary screening (Table 1) indicated that of the five PIs that were identified in the initial experiment, three were abundantly nodulated by the *R. fredii* strains. When inoculated with the *R. fredii* strains, the nodule dry weights of the three PIs (209483, 209484, and 209491) were significantly greater than those produced on the three commercial *P. vulgaris* cultivars (Table 1). In the secondary screening, two of the PIs (219701 and 181786) had nodule masses that were not significantly different from those of the commercial *P. vulgaris* genotypes, and consequently, they were not used in subsequent studies. Generally, the *R. fredii* isolates (with the exception of strain HH103) produced about

TABLE 2. Nodulation and nitrogen accumulation of *P. vulgaris* genotypes inoculated with strains of *R. fredii*

| Genotype | Nodulation and N accumulation of strains[a]: | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | USDA 192 | | | USDA 201 | | | USDA 205 | | | HH103 | | | *R. leguminosarum*[b] | | |
| | Nodule | | Plant N | Nodule | | Plant N | Nodule | | Plant N | Nodule | | Plant N | Nodule | | Plant N |
| | No. | Wt | | No. | Wt | | No. | Wt | | No. | Wt | | No. | Wt | |
| PI 209483 | 47 a | 63 a | 18 a | 25 ab | 58 a | 24 a | 54 a | 125 a | 39 a | 23 ab | 62 ab | 17 a | 76 b | 147 bc | 44 b |
| PI 209484 | 8 b | 13 b | 7 bc | 41 a | 61 a | 13 ab | 25 bc | 22 b | 6 c | 29 ab | 35 abc | 6 c | 124 a | 200 ab | 42 b |
| PI 209491 | 10 b | 14 b | 6 bc | 27 ab | 70 a | 23 a | 37 ab | 37 ab | 5 c | 47 a | 44 ab | 6 c | 68 b | 164 b | 35 b |
| C-20 | 5 b | 7 b | 5 c | 15 b | 29 ab | 5 b | 24 bcd | 20 b | 5 c | 16 ab | 26 bc | 5 c | 92 ab | 106 c | 31 b |
| Kentucky Wonder | 5 b | 7 b | 6 bc | 5 b | 4 b | 6 b | 5 de | 9 b | 8 c | 0 b | 0 c | 7 bc | 103 ab | 229 a | 59 a |

[a] Nodule No., number of nodules per plant; Nodule Wt, dry weight of nodules, in milligrams per plant; and Plant N, total nitrogen accumulated, in milligrams per plant. Uninoculated controls accumulated an average of 4.6 mg per plant (with a range of 4 to 5 mg per plant) of total nitrogen across all genotypes. Numbers within a single column that are not followed by the same letter differ significantly at the 0.05 probability level, as determined by Duncan's New Multiple Range Test.

[b] Consisting of an equal (vol/vol) mixture of *R. leguminosarum* bv. *phaseoli* USDA 2667, USDA 2674, and USDA 2676.

as much nodule mass on the three PIs as they did on their preferred host, *G. max* cv. Peking. All of the *R. fredii* strains produced effective symbioses with the *P. vulgaris* PIs, as determined by using the acetylene reduction assay and by plant top color (data not shown). The commercial *P. vulgaris* cv. Great Northern, Kentucky Wonder, and C-20 nodulated poorly or not at all when inoculated with the *R. fredii* strains. In addition, none of the plants formed nodules when inoculated with the *Bradyrhizobium* strains. These results suggest that the *R. fredii* strains did not merely outcompete the slow-growers for nodulation in the initial screening experiment. The individual inoculations also indicated that the root hypertrophies seen in the primary screening were produced by the *B. japonicum* strains (data not shown).

There were significant genotype × strain interactions between the *R. fredii* strains and the PIs for nodule number, nodule weight, and aboveground nitrogen accumulation (Table 2). In all of the *R. fredii* treatments, with the exception of strain USDA 201, PI 209483 accumulated significantly more N than any of the other genotypes tested did. Our results also suggest that the superior nitrogen accumulation of PI 209483 with the *R. fredii* strains must be specific, since *P. vulgaris* cv. Kentucky Wonder accumulated more N with the *R. leguminosarum* bv. *phaseoli* strains than with the other PIs tested. In addition to the superior $N_2$-fixing capacity of PI 209483, the *R. fredii* strains generally produced more nodule mass on this genotype than on the other *P. vulgaris* hosts. Although the tested PIs nodulated quite well with all of the *R. fredii* strains, there was considerable variation with respect to nodule number. While *R. fredii* USDA 192 and USDA 205 produced significantly more nodules on PI 209483 than the other genotypes did, strains USDA 201 and HH103 produced more nodules with *P. vulgaris* PIs 209484 and 209491, respectively. All of the *P. vulgaris* genotypes accumulated significant amounts of nitrogen and were abundantly nodulated when inoculated with the *R. leguminosarum* bv. *phaseoli* strain mixture.

Both USDA 201 and USDA 205 were capable of effectively nodulating PI 209483 in soil. On PI 209483, strains USDA 205 and USDA 201 occupied approximately 68 and 88% of the nodules, respectively. The remainder of the nodules were apparently produced by the low-level, indigenous *R. leguminosarum* bv. *phaseoli* population which was present in this soil. In addition, while strain USDA 201 nodulated *P. vulgaris* cv. Kentucky Wonder in Monmouth soil, the symbiosis produced was generally ineffective. This is essentially the same result that was obtained with vermiculite-grown plants. The Sym plasmid-cured strain 2051AO3 failed to nodulate either of the *P. vulgaris* genotypes or *G. max* cv. Peking in soil, suggesting that genes controlling the nodulation of both hosts are located on the large symbiotic plasmid in *R. fredii*.

In summary, our results suggest that the host genotype plays a major role in determining the outcome of the *P. vulgaris-R. fredii* symbiosis. When inoculated with the *R. fredii* strains, there was considerable *Rhizobium* strain by host genotype interaction for nodulation and nitrogen fixation; however, one of the *P. vulgaris* PIs, 209483, generally accumulated more nitrogen than the other genotypes did. The *P. vulgaris* genotypes (PIs 209483, 209484, and 209491) which had the greatest nodule number and weight and accumulated the most nitrogen were all collected from Costa Rica, indicating, perhaps, that there is a relationship between the geographic origin of the selected PIs and nitrogen fixation effectiveness. Interestingly, most of the *R. fredii* strains produced about as much nodule mass on PI 209483 as they did on their preferred host, *G. max* cv. Peking, indicating that *R. fredii* nodulation genes can function efficiently with genetically dissimilar host plants. Thus, *R. fredii* appears to represent a unique group of microsymbionts in that they have the serological (15) and symbiotic characteristics of two widely divergent genera of root nodule bacteria.

While some investigators have reported (7, 10) that strains of *B. japonicum* and *Bradyrhizobium* spp. (18) are capable of nodulating *P. vulgaris*, results of our studies, which were done with a wide variety of geographically and genetically diverse *Phaseolus* germplasm and serologically and genetically distinct bradyrhizobia (representing the members of six serogroups [15] and two DNA homology groups [9]), failed to show such a symbiotic relationship. One possible explanation for these contradictory results is that many of the genotypes used in previous studies have since been reclassified as belonging to the plant genera *Vigna* and *Macroptilium* (1), and both of these hosts have been shown to form symbioses with a large number of rhizobial species and strains (7, 20).

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