

Restriction of Nodulation by *Bradyrhizobium japonicum* Is Mediated by Factors Present in the Roots of *Glycine max*†

MICHAEL J. SADOWSKY,^{1*} RENEE M. KOSSLAK,² CEZARY J. MAĐRZAK,^{1,3}
BARBARA GOLIŃSKA,^{1,3} AND PERRY B. CREGAN⁴

Departments of Soil Science and Microbiology, University of Minnesota, St. Paul, Minnesota 55108¹; Department of Zoology and Genetics, Iowa State University, Ames, Iowa 50011²; Institute of Biochemistry and Biotechnology, University of Agriculture, Poznan, Poland³; and Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705⁴

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Reciprocal grafting experiments done using soybean plant introduction genotypes indicated that restriction of nodulation by *Bradyrhizobium japonicum* is determined by the genotype of the root and is dependent on plant growth temperature. Microscopic analyses indicated that the soybean plant introduction genotypes restrict nodulation of *B. japonicum* at symbiotic stages which occur both before and after the formation of nodule primordia.

Bradyrhizobium japonicum forms symbiotic nitrogen-fixing root nodules on soybean (*Glycine max* (L.) Merr.) plants. Results from many studies have indicated that soybean genotypes can differentially restrict nodulation by specific strains or serogroups of *Bradyrhizobium japonicum* (4, 5, 11, 21, 23). Several soybean genotypes (including plant introductions [PI] and cultivars) which restricted nodulation by *B. japonicum* USDA 123 have been identified (4). Soybean genotypes PI 377578, PI 371607, and PI 417566 were shown to restrict nodulation by different *B. japonicum* serocluster 123 isolates (5, 6, 11, 16, 18). PI 417566 also restricts nodulation by strain USDA 110 (13). We have reported that *B. japonicum* serogroup 123 strains containing the genotype-specific nodulation gene, *nolA*, have the ability to overcome nodulation restriction conditioned by PI 377578 (17) and that a transposon Tn5 mutant of strain USDA 110, D4.2-5, has gained the ability to nodulate PI 417566 (13).

While there have been several reports of genotype- and cultivar-specific interactions between leguminous plants and their respective symbionts (see reference 19 for a review), little information is available concerning factors affecting incompatible interactions between bradyrhizobia and soybean plants and the plant's response to infection by nodulation-restricted *B. japonicum* strains. Some strains, however, have been reported to produce infection threads and initiate the production of nodule primordia on the incompatible soybean hosts, *G. max* cultivars Hardee and Clark (*rj₁rj₁*) (14, 15). In addition, Delves et al. (7) reported that supernodulation and nonnodulation are shoot and root controlled, respectively, in *G. max* cv. Bragg.

In this study, we determined whether the genotype of the root or shoot of soybean PI 377578 and PI 417566 controls nodulation restriction, and we examined the influence of temperature on host-controlled nodulation restriction in the soybean-*B. japonicum* symbiosis.

Effect of temperature on host-controlled nodulation restriction. The influence of temperature on host-controlled nodulation restriction on soybeans was investigated in modified Leo-

nard jar assemblies (22) containing vermiculite and perlite (3:1). Leonard jars were planted with three surface-sterilized seeds each of *G. max* PI 377578 (4), *G. max* PI 417566 (5), *G. max* cv. Williams, or *G. max* cv. Hill (which contains the *Rj₄* allele) and thinned to two seedlings of each genotype 3 days after emergence. The plants were inoculated, in triplicate, with 1.0 ml (about 10⁷ cells) of AG-grown (18), stationary-phase *B. japonicum* cultures. Uninoculated plants served as negative controls, and the *B. japonicum* strains inoculated onto cv. Williams were the positive nodulation controls. After inoculation, the seeds were covered with vermiculite and a 1-cm layer of sterilized paraffin-coated sand. The plants were grown in growth chambers at 20, 25, or 30°C with an 18-h photoperiod and watered, alternately, with nitrogen-free nutrient solution (11) or tap water as needed. The plants were harvested 35 days after inoculation. The number of nodules produced and nodule dry mass were determined, and statistical significance was determined by analysis of variance and Duncan's new multiple range procedures of SAS (SAS Institute, Inc., Cary, N.C.).

The results shown in Table 1 indicate that the incubation temperature of the symbiotic system affects host-controlled nodulation restriction. On PI 377578, *B. japonicum* USDA 438 and USDA 61 produced significantly fewer nodules at 20°C than at 30°C. Strain USDA 123, however, produced significantly fewer nodules on PI 377578 at 30°C than at 20°C. Changes in incubation temperature did not significantly affect the number of nodules produced by strain USDA 438 on soybean cultivars Hill and Williams or that produced by strain USDA 123 on cv. Williams. Thus, our data indicate that the temperature effect on nodulation is not related to the infecting strain. Moreover, the numbers of nodules produced by *B. japonicum* USDA 61 and USDA 438 on cv. Hill, which contains the *Rj₄* allele, were not significantly affected by temperature, and both strains were restricted for nodulation at all of the tested temperatures. Strain USDA 123, however, produced significantly more nodules on cv. Hill at 25°C than at the other tested temperatures. Generally speaking, all three strains produced more nodules at 25°C, on the nonrestrictive host cv. Williams, although the numbers of nodules produced were not significantly different from those produced at the other tested temperatures. There was no correlation between the mass and number of nodules produced by the *B. japonicum* strains on

* Corresponding author.

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TABLE 1. Nodulation of soybean genotypes by *B. japonicum* USDA 123, USDA 438, and USDA 61 at three temperatures

Strain	Nodulation response ^a on <i>G. max</i> genotype:					
	PI 377578		cv. Hill		cv. Williams	
	No.	Mass	No.	Mass	No.	Mass
USDA 123						
20°C	24.3 A	70.0 A	19.7 B	58.3 B	25.0 A	56.3 B
25°C	21.5 A	53.0 AB	37.7 A	122.0 A	38.0 A	104.7 A
30°C	8.3 B	23.0 B	18.0 B	34.3 B	27.7 A	82.3 AB
USDA 438						
20°C	0.0 A	0.0 A	0.7 A	3.0 A	24.3 A	69.7 A
25°C	0.0 A	0.0 A	0.3 A	1.0 A	27.3 A	93.0 A
30°C	9.0 B	24.3 A	2.7 A	5.3 A	27.7 A	79.3 A
USDA 61						
20°C	1.0 B	9.3 B	4.7 A	17.7 A	22.7 A	80.3 A
25°C	0.7 B	30.0 AB	6.0 A	25.7 A	27.3 A	108.7 A
30°C	17.7 A	66.0 A	10.3 A	54.3 A	18.7 A	69.3 A

^a Values are means of three replicates. Nodule number and mass (in milligrams) values are per plant. For each strain, values within a column not followed by the same letter differ significantly ($P = 0.05$) as tested by Duncan's new multiple range test.

the hosts at any of the tested temperatures, although *B. japonicum* USDA 123 and USDA 61 produced significantly less nodule mass on PI 377578 at 30 and 20°C, respectively. Similarly, *B. japonicum* USDA 430 (formerly strain MN1-1c [11]) was significantly restricted for nodulation at 20 and 25°C (producing 7.7 and 12.3 nodules per plant, respectively) on PI 417566 but produced normal nodulation relative to that found on cv. Williams (27.3 nodules per plant) at 30°C. On the other hand, nodulation of cv. Williams by strain USDA 430 was not significantly different at any of the tested temperatures (mean, 23.6 nodules per plant), indicating that the temperature effect was specific for the PI 417566-USDA 430 symbiotic system. Moreover, a similar result was also found when strain USDA 110 was inoculated onto PI 417566 (data not shown). Nodule mass on PI 417566 was inversely related to temperature; significantly greater nodule mass was produced at the lower temperatures. The mass per nodule was 10 times greater at the nodulation-restrictive temperature of 20°C than at 30°C. This result is consistent with our previous observations that the few nodules produced on the nodulation-restricting soybean genotypes (at restrictive temperatures) are relatively large. Our results are in general agreement with those of Lie (12), who reported that temperature differentially affects the interaction of specific *Rhizobium leguminosarum* bv. *viciae* strains on peas and that a single dominant gene (*Sym 1*) conditions temperature-sensitive nodulation restriction.

Grafting experiments. To determine if the roots or shoots of *G. max* PI 377578 and PI 417566 control restriction of nodulation by *B. japonicum* strains, we performed reciprocal grafting experiments with the PI genotypes and cv. Kasota. For these analyses, seeds were surface sterilized in an atmosphere of Cl₂ gas for 15 h (9). The seeds were planted in sterile potting medium consisting of a 3:1 mixture of vermiculite and perlite and incubated in a plant growth chamber at 25°C (23°C at night), with full light intensity and an 18-h photoperiod. The seedlings were watered with sterile tap water as needed. Reciprocal plant grafts between 5-day-old seedlings of *G. max* cv. Kasota and 7-day-old seedlings of PI 417566 or PI 377578 were made as described (1). The stems (scions) of cv. Kasota seedlings were grafted onto the roots of either PI 377578 or PI

TABLE 2. Root-determined nodulation restriction of *B. japonicum* USDA 438 by *G. max* PI 377578

Plant genotype ^a	Mean no. of nodules ^b
Kasota (ungrafted)	48.2 A
PI 377578 (ungrafted)	0.0 B
Kasota/Kasota	44.7 A
PI 377578/PI 377578.....	0.5 ^c B
PI 377578/Kasota.....	38.2 A
Kasota/PI 377578.....	0.0 B

^a Plant genotypes separated by a slash represent grafted plants, in which the first genotype was used as the source of the plant stem (scion) and the second genotype was used as the source of the root system.

^b Values are means of at least three replicates (range, 3 to 6). Nodule number values are per plant. Values not followed by the same letter differ significantly ($P = 0.05$) as tested by Duncan's new multiple range test.

^c Mean of two replicates; one plant had one nodule.

417566, and the stems (scions) of PI 377578 or PI 417566 were grafted onto the roots of cv. Kasota seedlings. Intra-genotype grafts, plants that were not grafted, and uninoculated plants were included as controls. The grafted plants and controls were transferred to modified Leonard jar assemblies containing a 3:1 mixture of vermiculite and perlite, incubated at 20°C (18°C at night) with full light intensity and an 18-h photoperiod, and watered with nitrogen-free, plant nutrient solution (10) as needed. Seven days later, the replicate grafted plants (3 to 12 replicates) and nongrafted controls were inoculated with about 10⁸ cells per seedling of 4-day-old AG-grown *B. japonicum* USDA 110 or USDA 438. The plants were harvested 4 weeks after inoculation, and the number of nodules produced was determined. The grafting experiments were repeated two times. Statistical significance was determined by analysis of variance and Duncan's new multiple range procedures of SAS.

The results of these studies (Table 2) indicated that restriction of nodulation by *B. japonicum* USDA 438, which is conditioned by PI 377578, is controlled by the genotype of the root. Virtually no nodules (range, 0.0 to 0.5 nodules per plant) were produced on the hybrid plants containing the PI 377578 root system and either PI 377578 or cv. Kasota shoots or on the ungrafted PI 377578 control plants. On the other hand, the plants constructed by grafting cv. Kasota roots onto cv. Kasota or PI 377578 shoots exhibited nodulation which was not statistically different from that found with the ungrafted cv. Kasota control plants (38.2 to 48.2 nodules per plant). The results in Table 2 also show that the grafting of cv. Kasota shoots onto cv. Kasota roots had little effect on nodulation, indicating that

TABLE 3. Root-determined nodulation restriction of *B. japonicum* USDA 110 by *G. max* PI 417566

Plant genotype ^a	Mean no. of nodules ^b
Kasota (ungrafted)	49.3 A
PI 417566 (ungrafted).....	4.3 B
Kasota/Kasota	56.5 ^c A
PI 417566/PI 417566.....	7.3 B
PI 417566/Kasota.....	51.6 A
Kasota/PI 417566.....	2.2 B

^a Plant genotypes separated by a slash represent grafted plants, in which the first genotype was used as the source of the plant stem (scion) and the second genotype was used as the source of the root system.

^b Values are means of at least three replicates (range, 3 to 12). Nodule number values are per plant. Values not followed by the same letter differ significantly ($P = 0.05$) as tested by Duncan's new multiple range test.

^c Mean of two replicates.

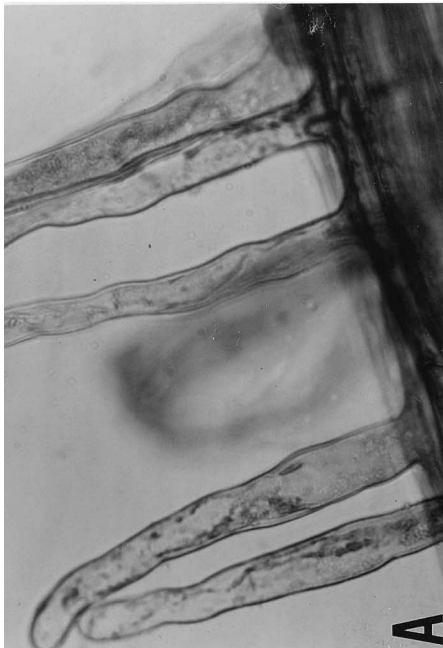
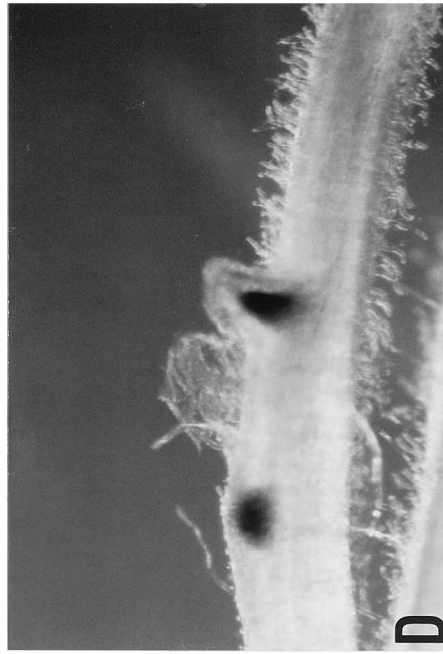
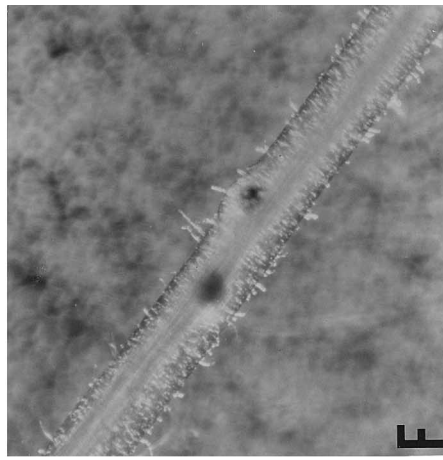


FIG. 1. Response of *G. max* PI 377578 to inoculation with compatible (strain 671-21) and incompatible (strain USDA 438) *B. japonicum* strains. (A) Root hairs of *G. max* PI 377578 from uninoculated control plants. (B) Root segment of uninoculated control plant stained with hematoxylin. (C) Root hair curling response observed when *G. max* PI 377578 was inoculated with nodulation-incompatible *B. japonicum* USDA 438. Inset shows infection thread. (D) Nodule primordium induced by *B. japonicum* USDA 438 on *G. max* PI 377578. The nodule primordium (dome-shaped meristematic zone) is easily distinguishable from the emerging lateral root (triangular apical meristem). (E) Root hair curling response observed when *G. max* PI 377578 was inoculated with nodulation-compatible *B. japonicum* 671-21. (F) Nodule primordia on *G. max* PI 377578 roots inoculated with *B. japonicum* 671-21.

grafting, per se, was not influencing nodulation ability. A similar result was found when grafting was done between PI 417566 and cv. Kasota and the plants were inoculated with *B. japonicum* USDA 110 (Table 3). The hybrid plants constructed by grafting cv. Kasota shoots onto PI 417566 roots restricted nodulation by *B. japonicum* USDA 110. These grafted plants had mean nodule numbers (2.2 nodules per plant) that were not significantly different from those of the grafted control plants (7.3 nodules per plant), which consisted of PI 417566 shoots grafted onto PI 417566 roots or ungrafted PI 417566 control plants (mean, 4.3 nodules per plant). The hybrid plants having cv. Kasota as the source of the root genotype produced a large number of nodules (mean, 54.0 nodules per plant), and the number of nodules produced was not significantly different from that produced on ungrafted cv. Kasota plants (mean, 49.3 nodules per plant). Taken together, the results of the grafting studies presented in Tables 2 and 3 indicate that host-controlled nodulation restriction conditioned by the PI genotypes is autonomous for the root and is independent of any putative factors expressed in stems (systemic factors). Similar results of root-controlled restriction of nodulation in soybeans have been reported by Balatti and Pueppke (2) for the interaction of *Rhizobium fredii* USDA 257 and the soybean cultivar McCall and by Caldwell and coworkers (3) for *B. japonicum* strains restricted for nodulation by the soybean Rj₂ allele. Delves et al. (7) reported that while nonnodulation in soybeans is controlled by the genotype of the root, the shoot governs the supernodulation phenotype. These results indicate that both root and shoot factors can influence the interaction of soybean genotypes and specific *B. japonicum* strains; however, it is the root which plays a major role in controlling nodulation restriction in the soybean PI genotypes which we examined.

Microscopic analysis of early stages of nodule formation.

While there have been several reports of soybean host-controlled restriction of nodulation by specific *B. japonicum* strains, there is little information available concerning the plant's response to inoculation with incompatible bradyrhizobia. To determine the symbiotic stage at which host-controlled restriction of nodulation begins, we inoculated PI 377578 and PI 417566 with incompatible or compatible *B. japonicum* strains and analyzed the plant's response to infection. Root hair curling responses and infection thread formation were analyzed as described (14). *G. max* cv. Williams, PI 377578, and PI 417566 seeds were surface sterilized (22) and pregerminated in sterile vermiculite. Two seedlings of each genotype were aseptically transferred to a sterile plastic growth pouch (Mega International, Minneapolis, Minn.) and inoculated with about 10⁸ cells of 4-day-old, AG-grown, washed, *B. japonicum* USDA 110, USDA 438, 671-21, or D4.2-5. *G. max* cv. Williams (or cv. Kasota) seedlings were inoculated with strain USDA 110, USDA 438, 671-21, or D4.2-5; seedlings of PI 377578 were inoculated with strain USDA 438 or 671-21; and PI 417566 seedlings were inoculated with strain USDA 110 or D4.2-5. Plants used for root hair analyses were harvested 4 days after inoculation, and plants used to determine the production of nodule primordia by PI 377578 and PI 417566, which were inoculated with compatible and nodulation-restricted strains, were prepared as described above. The plants were harvested

7 days after inoculation and processed as previously described (8). Uninoculated plants of each genotype served as negative controls.

The results for PI 377578 inoculated with *B. japonicum* USDA 438 and 671-21 are shown in Fig. 1. Although the PI genotype restricted nodulation by *B. japonicum* USDA 438, the nodulation process was not aborted until sometime after the formation of nodule primordia. While strain USDA 438 induced a root hair curling response (Fig. 1C), the formation of infection threads (Fig. 1C, inset), and the development of nodule primordia (Fig. 1D), the frequency at which these plant responses occurred on the PI genotype was reduced to about 10 to 30% of that seen on the nodulation-permissive hosts (cv. Williams or cv. Kasota) or on the PI genotype inoculated with the nodulation-competent, near-isogenic strain, 671-21 (Fig. 1E and F). No such plant responses were seen on the uninoculated control plants (Fig. 1A and B). The incompatible host-strain combinations also had many distorted root hairs. In addition, the incompatible host-strain combination had nodule primordia that were smaller, less developed, and located farther down the root system (below the root tip mark), indicating that although the infection process proceeds, it is slower or delayed relative to the infection process in nodulation-compatible combinations. A similar result was seen when PI 417566 was inoculated with the nodulation-restricted strain USDA 110 and the infection process was compared with that seen with strain D4.2-5 (data not presented). The overall results of these studies indicated that while the PI genotypes have the capacity to restrict nodulation by specific *B. japonicum* strains, the nodulation process is not totally aborted until sometime after the formation of nodule primordia and that the incompatible host-strain combinations are unable to continue a sustained program of infection. The frequency at which these plant responses occurred, however, was significantly reduced relative to that seen when the PI genotypes were inoculated with the nodulation-competent, near-isogenic strain, 671-21 or D4.2-5. The same general phenomenon has also been reported for the interaction of *B. japonicum* and *R. fredii* strains on other nodulation-restricting soybean genotypes (2, 14, 15). Our findings that few root hairs are curled and that infection threads and nodule primordia are infrequently produced with the incompatible soybean-*Bradyrhizobium* combinations suggest that host-strain incompatibility for nodulation is most likely governed by an autoregulatory feedback mechanism which has been proposed to operate after the initiation of the infection process (20).

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REFERENCES

- Albertsen, M. C., T. M. Curry, R. G. Palmer, and C. E. Lamotte. 1983. Genetics and comparative growth morphology of fasciation in soybeans [*Glycine max* (L.) Merr.]. Bot. Gaz. 144:263-275.

2. **Balatti, P. A., and S. G. Pueppke.** 1992. Differential sensitivity of *Rhizobium fredii* strains to nodulation blocking on McCall soybean: nodulation rates and efficiency. *Plant Physiol. Biochem.* **30**:193–199.
3. **Caldwell, B. E., K. Hinson, and H. W. Johnson.** 1966. A strain-specific ineffective nodulation reaction in the soybean *Glycine max* L. Merrill. *Crop Sci.* **6**:495–496.
4. **Cregan, P. B., and H. H. Keyser.** 1986. Host restriction of nodulation by *Bradyrhizobium japonicum* strain USDA 123. *Crop Sci.* **26**:911–916.
5. **Cregan, P. B., H. H. Keyser, and M. J. Sadowsky.** 1989. A soybean genotype that restricts nodulation of a previously unrestricted isolate of *Bradyrhizobium japonicum* serocluster 123. *Crop Sci.* **29**:307–312.
6. **Cregan, P. B., H. H. Keyser, and M. J. Sadowsky.** 1989. Host plant effects on nodulation and competitiveness of the *Bradyrhizobium japonicum* serotype strains constituting serocluster 123. *Appl. Environ. Microbiol.* **55**:2532–2536.
7. **Delves, A. C., A. Matthews, D. A. Day, A. S. Carter, B. J. Carroll, and P. M. Greshoff.** 1986. Regulation of the soybean-*Rhizobium* nodule symbiosis by shoot and root factors. *Plant Physiol.* **82**:588–590.
8. **Dudley, M. E., T. W. Jacobs, and S. R. Long.** 1987. Microscopic studies of cell division induced in alfalfa roots by *Rhizobium meliloti*. *Planta* **171**:289–301.
9. **Gamborg, O. L., B. P. Davis, and R. W. Stahlhut.** 1983. Somatic embryogenesis in cell cultures of *Glycine* species. *Plant Cell Rep.* **2**:209–212.
10. **Imbande, J.** 1986. Nitrate-ammonium ratio required for pH homeostasis in hydroponically grown soybean. *J. Exp. Bot.* **37**:341–347.
11. **Keyser, H. H., and P. B. Cregan.** 1987. Nodulation and competition for nodulation of selected soybean genotypes among *Bradyrhizobium japonicum* serogroup 123 isolates. *Appl. Environ. Microbiol.* **53**:2631–2635.
12. **Lie, T. A.** 1984. Host genes in *Pisum sativum* L. conferring resistance to European *Rhizobium leguminosarum* strains. *Plant Soil* **82**:415–425.
13. **Lohrke, S. M., and M. J. Sadowsky.** 1994. Isolation and characterization of genotype-specific nodulation gene from *Bradyrhizobium japonicum* strain USDA 110, p. 335. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
14. **Pueppke, S. G.** 1983. *Rhizobium* infection threads in root hairs of *Glycine max* (L.) Merr., *Glycine soja* Sieb & Zucc., and *Vigna unguiculata* (L.) Walp. *Can. J. Microbiol.* **29**:69–76.
15. **Pueppke, S. G., and J. H. Payne.** 1987. Responses of R_{j1} and r_{j1} soybean isolines to inoculation with *Bradyrhizobium japonicum*. *Plant Physiol.* **84**:1291–1295.
16. **Sadowsky, M. J., and P. B. Cregan.** 1992. The soybean *Rj4* allele restricts nodulation by *Bradyrhizobium japonicum* serogroup 123 strains. *Appl. Environ. Microbiol.* **58**:720–723.
17. **Sadowsky, M. J., P. B. Cregan, M. Gottfert, A. Sharma, D. Gerhold, F. Rodriguez-Quinones, H. H. Keyser, H. Henneke, and G. Stacey.** 1991. The *Bradyrhizobium japonicum nolA* gene and its involvement in the genotype-specific nodulation of soybeans. *Proc. Natl. Acad. Sci. USA* **88**:637–641.
18. **Sadowsky, M. J., R. E. Tully, P. B. Cregan, and H. H. Keyser.** 1987. Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybeans. *Appl. Environ. Microbiol.* **53**:2624–2630.
19. **Triplet, E. W., and M. J. Sadowsky.** 1992. Genetics of competition for nodulation. *Annu. Rev. Microbiol.* **46**:399–428.
20. **Vasse, J., F. de Billy, and G. Truchet.** 1993. Abortion of infection during the *Rhizobium meliloti*-alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. *Plant J.* **4**:555–566.
21. **Vest, G., D. F. Weber, and C. Sloger.** 1973. Nodulation and nitrogen fixation. *Agronomy* **16**:353–390.
22. **Vincent, J. M.** 1970. A manual for the practical study of root nodule bacteria. International Biological Programme Handbook 15. Blackwell Scientific Publications, Oxford.
23. **Vincent, J. M.** 1980. Factors controlling the legume-rhizobium symbiosis, p. 103–129. *In* W. E. Newton and W. H. Orme-Johnson (ed.), Nitrogen fixation, vol. II. University Park Press, Baltimore.