

Response of Soybean to Inoculation with Efficient and Inefficient *Bradyrhizobium japonicum* Variants

Rebecca A. Champion, James N. Mathis,* Daniel W. Israel, and Patrick G. Hunt

ABSTRACT

Soybean [*Glycine max* (L.) Merr.] plants are grown in soils containing large indigenous bradyrhizobial populations. Individual strains within these populations differ in symbiotic efficiency and competitiveness for nodule occupancy. The purpose of this study was to determine the effect of mixed inocula of an efficient and an inefficient variant of *Bradyrhizobium japonicum* on symbiotic performance in soybean. Lee soybean was initially inoculated with either an efficient or an inefficient USDA 110 colony morphology variant. The opposite variant was then applied after 0, 2, 4, 8, or 13 d. Delayed inoculation with the efficient variant following the inefficient variant resulted in progressively decreased symbiotic performance. In a subsequent competition experiment, soybean cultivars Lee and Ransom were inoculated with efficient and inefficient variants at ratios of 1:1, 1:10 and 10:1. More nodules were formed by the efficient variant than were expected by chance. Significantly reduced dry weight and whole plant N contents were noted when the inefficient variant was present in more than 50% of the nodules. Split-root experiments were conducted and either type of variant was capable of inhibiting the other after a 7-d delay in inoculation. In contrast, when both sides of the split-root were simultaneously inoculated, nodule numbers were similar; however, nodules formed by the efficient variant were larger. This increase in size indicated a preferential partitioning of photosynthate to the nodules formed by the efficient variant. These results together indicate that N₂ fixation is enhanced with increased nodule occupancy by superior variants due to more effective strain-cultivar interactions.

THE RHIZOSPHERE is an area where there is competition between strains of rhizobia for infectivity of the legume host. In previous studies the investigation of the competition phenomenon has involved the use of native strains, genetically diverse strains, or isogenic strains. Singleton and Stockinger (1983) inoculated soybean plants with different ratios of ineffective *B. japonicum* SM-5 and effective *B. japonicum* CC709 and observed that the average mass of effective nodules was 2.5 times the average mass of ineffective nodules. This observation is consistent with the inference that the plant may selectively channel photosynthate to effective nodules.

Kosslak and Bohlool (1985) compared the competition of *B. japonicum* USDA 123 and *B. japonicum* USDA 110 under various conditions: (i) soil type, (ii) vermiculite-amended soil, (iii) addition of antibiotic-producing actinomycetes, and (iv) N addition. They concluded that when the seeds were preexposed for 72 h to a specific bacterium the nodule occupancy of that bacterium was increased in all cases by 30 to 50%.

R.A. Champion, Dep. of Biology, Kennesaw State College, Marietta, GA 30061; J.N. Mathis, Dep. of Biology, West Georgia College, Carrollton, GA 30118; D.W. Israel, USDA-ARS and Dep. of Soil Science, North Carolina State Univ., Raleigh, NC 27695-7169; and P.G. Hunt, USDA-ARS, Coastal Plains Res. Ctr., Florence, SC 29502-3039. Contribution of the Georgia Inst. of Technology and the USDA-ARS. Received 29 Apr. 1991. *Corresponding author.

Published in *Crop Sci.* 32:457-463 (1992).

Pierce and Bauer (1983) observed that inoculation of soybean by an effective strain 15 h prior to a subsequent inoculation with the same strain resulted in a lack of nodulation by the second inoculum. This inhibition was not elicited by heterologous rhizobia (*Rhizobium leguminosarum* or *R. trifolii*) or by a UV-killed dose of the homologous strain of *B. japonicum*. They suggested that the initial inoculation elicited a rapid response in the plant that suppressed further nodulation.

Bhuvanewari et al. (1980) have suggested that nodulation inhibition may be a mechanism by which the host plant prevents excessive nodulation. Smith and Wollum (1989) in a study with six different *B. japonicum* strains observed that taproot nodules are formed first and an inverse relationship exists between the numbers of tap- and lateral-root nodules. They suggested that this inverse relationship may be a plant host mechanism to control the extent of nodulation. Sargent et al. (1987) investigated nodulation using Tn5-induced mutants of *R. trifolii* ANU843 impaired in their ability to nodulate subterranean clovers. In this near-isogenic system, it was found that 24 h after inoculation, bacteria with normal nodulation ability inhibited subsequent nodulation; however, exposure to bacteria that lacked normal nodulation ability did not inhibit subsequent nodulation.

The purpose of this study was to use naturally occurring, genetically related (Mathis et al., 1986a,b,c), easily differentiated, colony morphology variants of *B. japonicum* USDA 110 to (i) further characterize the effects that N₂ fixation ability has on competition for nodule occupancy and (ii) determine how timing of nodule formation and mixed populations of efficient and inefficient nodules influence N₂ fixation. This study, unlike previous studies, used closely related variants to systematically analyze the effects on N₂ fixation by various delays (0, 2, 4, 8 and 13 d) in inoculation with efficient or inefficient variants followed by the opposite type of variant.

MATERIALS AND METHODS

Soybean Germplasm, Bacterial Strains, and Growth Conditions

All experimental designs were randomized complete block; experiments were performed in a greenhouse during 1988. Temperatures were 21 to 36°C, and the relative humidity was 55%. To ensure flower repression, metal halide lights were used to extend day lengths to 18 h. These lights provided $\approx 75 \mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation. Plants were grown either: (i) hydroponically in 6-L pots filled with sterile horticultural perlite supplemented with 300 g of granulated oyster-shells (Israel et al., 1986) or (ii) in the Singleton split-root system (Singleton and Stockinger, 1983) as modified by Hunt et al. (1987). All pots and split-root fixtures were sterilized with a 1% dilu-

Abbreviations: CFU, colony-forming units; DAI, days after inoculation; DAP, days after planting; rpm, revolutions per minute; YEG, yeast-extract gluconate; YEM, yeast-extract mannitol.

tion (v/v) of commercial bleach (5.25% NaOCl, w/v) solution prior to planting.

Seeds of the soybean cultivars Lee, Ransom and Pickett were obtained from Dr. Joe Burton (USDA-ARS, and North Carolina State Univ.). In all experiments, seeds were pre-germinated for 72 h at 25°C in sterile coarse horticultural vermiculite (moistened with 0.5 mM calcium sulfate). In pot culture, the Lee and Ransom soybean seedlings initially received 250 mL of N-free nutrient solution two times a day for 5 d (Israel et al., 1986). Subsequently, the pots were flushed with water in the morning and afternoon. Nitrogen-free nutrient solution (500 mL) was applied after the afternoon flushing. Nonnodulating Lee soybean isolines were used as controls. These controls showed N levels equivalent to seed N, which indicated that all N accumulation in nodulated plants was from symbiotic N₂ fixation. In the split-root experiments, the seeds were germinated as described above, and the root tip was excised to promote root branching down each side of the split-root apparatus. The seedling was then planted in the split-root apparatus containing vermiculite which was soaked overnight in the N-free nutrients used above. Each plant was allowed to grow 7 d prior to inoculation. This growth period allowed the roots to grow into each side of the split-root system prior to inoculation. Uninoculated controls were also used, and no nodules developed on these controls. Plants were watered through straws every other day with bradyrhizobia-free deionized distilled water.

Bacterial cultures of *B. japonicum* USDA 110 derivatives I-110 and L2-110 were isolated by Kuykendall and Elkan (1976). Variant L2-110 lacks symbiotic nitrogen fixation but utilizes mannitol and produces large, slimy colonies on yeast-extract mannitol agar. Variant I-110 does not utilize mannitol, produces nonslimy colonies on YEM, and is efficient at symbiotic N₂ fixation (Mathis et al., 1986a,b,c). Both of these colony morphology variants have been shown to maintain their respective abilities to fix N₂ and utilize mannitol through repeated plant passage and culturing (J.N. Mathis, unpublished data, 1988). In all the experiments, plants were inoculated with bacterial cultures which had been incubated at 25 °C on a gyratory shaker (150 rpm) in yeast extract gluconate broth (Mathis et al., 1986b). Stationary-phase cultures (2 to 3 × 10⁹ CFU mL⁻¹) were used. Pot cultured plants were inoculated by dipping the roots for 2 to 3 s in the appropriate culture. Immediately following planting, there was an application of 2.5 mL of culture to the base of each seedling. The split root cultures were inoculated with 1.0 mL of the appropriate culture being applied to the proper side through plastic straws.

At the completion of each experiment, bacteria were purified from nodules of various strain-cultivar combinations (Mathis et al., 1986b). Bacteria were streaked on YEM plates supplemented with bromthymol blue. These plates were used to distinguish mannitol-utilizing and non-mannitol-utilizing colonies (Mathis et al., 1986a).

Dry Weight, Total Nitrogen, and Statistics

At the completion of each experiment, plant material was dried at 65°C for 48 h, weighed and ground. Nitrogen was determined by a semi-micro-Kjeldahl technique (Glowa, 1974; Nelson and Sommers, 1973). Since all plants were grown in a N-free culture system, accumulation of N represented biological N₂ fixation. All data were subjected to analysis of variance using the Statistical Analysis System (Goodnight, 1982). Programs for analysis of variance, linear regression, standard deviations and chi-square were used for various parameters.

Delayed Inoculation Experiment

The first experiment was performed from May to July 1988. 'Lee' soybean was used with a nonnodulating Lee

isoline (Lee non-nod) as a minus-N control. Each treatment consisted of eight replicates with two seedlings per pot. After 14 d, the seedlings were thinned to one per pot. Four replicates of each treatment were harvested after 28 d. The remainder were harvested 45 DAI.

On Day 0, the planting date, four different groups were planted: (i) control plants that were either left inoculated (Lee non-nod) or were inoculated with only one variant, I-110 or L2-110, (ii) plants inoculated simultaneously with I-110 and L2-110, (iii) plants inoculated only with I-110 that would later be cross inoculated with L2-110, and (iv) plants inoculated with only L2-110 that would later be cross inoculated with I-110. Eight pots each of uninoculated Lee non-nod seedlings, L2-110 inoculated seedlings and I-110 inoculated seedlings were used as controls. For the treatment plants, a replicate set of eight pots were inoculated by dipping the seedlings into a beaker that contained a 50:50 mixture of stationary phase cultures of L2-110 and I-110. Plate counts subsequently revealed the two stationary phase cultures contained 3 × 10⁹ CFU mL⁻¹. These were designated Day 0 plants, since they were simultaneously inoculated with I-110 and L2-110. Also on Day 0, the other treatment plants were inoculated by dipping half of the remaining seedlings into a stationary phase culture of L2-110 and the other half into a stationary phase culture of I-110. These pots were divided into four groups of replicate sets of eight. The plants were later cross-inoculated with the opposite type of variant on a delayed schedule of 2, 4, 8 or 13 d. For cross inoculation, 2.5 mL of the appropriate culture was applied to the base of each treatment plant.

After 28 d, one entire set of replicates was harvested. The nodule number, fresh nodule mass, dry root mass, and dry shoot mass was measured. To determine the occupant bacteria, 25 nodules from each plant were randomly selected, surface sterilized, crushed, and plated on YEM bromothymol blue-supplemented agar plates. Nitrogen content of the shoots and roots were determined as described above. After 45 d, the other set of replicates was harvested and the same dry weight and N determinations performed.

Competition Experiment

A competition experiment was designed, competing I-110 with L2-110, to further clarify the process of strain selection. The purpose of this experiment was to determine if nodulation by the more efficient strain was favored. The plants in this experiment were grown in minus-N pot culture in a greenhouse during June to July 1988. Each treatment consisted of eight replicates of cultivars Lee and Ransom. Cultures of L2-110 and I-110 were grown to stationary phase in YEG media. The cultures were diluted to 10⁶ and 10⁷ CFU mL⁻¹ in a basal medium of sterile salts (Mathis et al., 1986b). Each variant combination was competed at the following ratios: (i) 1:1, (ii) 1:10, and (iii) 10:1. Before being planted, the seedlings were dipped into a mixture of the proper dilution. After planting, 2.5 mL of additional inoculum was added to the base of each seedling.

The shoots and roots were harvested 34 DAP. They were then dried and weighed, and their N contents were determined. To screen for nodule occupancy, 25 nodules from each replicate were randomly selected and plated onto YEM plates containing bromthymol blue.

Split-Root Assay

Two split-root assays were also performed to determine the ability of the two types of *B. japonicum* USDA 110 variants to block nodulation by the opposite type of variant. In the first split-root experiment, there was a seven day delay between inoculations. On Day 0 (7 DAP), the first side of three replicates each of Lee, Ransom, and Pickett

cultivars was inoculated with 1.0 mL of I-110. In addition, three replicates of each cultivar were inoculated on the first side with 1.0 mL of L2-110. Seven days later (14 DAP) the second side of each replicate was inoculated with 1.0 mL of the opposite variant. For controls, a seedling of each cultivar was left uninoculated. These plants were grown in a greenhouse from October to November 1988. At 30 DAP, seedlings were harvested and the roots were washed with deionized water. Presence or absence of chlorosis was noted as an indication of biological N_2 fixation. The number of nodules and fresh mass were determined for each side. Three randomly selected nodules from each side were screened for occupancy by the appropriate variant. Plants were separated into roots (for each side) and shoots. Dry weights and N contents were determined as described above.

In the second split-root experiment, four seedlings of each cultivar (Lee, Ransom and Pickett) were simultaneously inoculated 7 DAP with I-110 on one side and L2-110 on the other side. A control seedling of each cultivar was left uninoculated. The plants were harvested 21 DAP, and the presence or absence of chlorosis noted. The number and fresh mass of the nodules from each side were determined. Plants were separated into roots (for each side) and shoots. Three randomly selected nodules from each side were screened for occupancy by the appropriate variant. Dry weights and N contents were determined as described above. These plants were grown in a greenhouse during November to December 1988.

RESULTS

Effect of Delayed Inoculation with Efficient or Inefficient *B. japonicum* USDA 110 Variants in Lee Soybean

When plants were inoculated with the efficient I-110 variant, nodulation by the inefficient L2-110 variant was completely inhibited after 8 d (Table 1). When plants were inoculated with the L2-110 variant, a 13-d delay was required for complete inhibition of nodulation by I-110 (Table 1). Plants simultaneously inoculated with equal numbers of I-110 and L2-110 cells were found to have 61% of the nodules formed by the N_2 -fixing I-110 variant. When comparing all treatments (28 and 45 DAI) where I-110 was the initial inoculum, there were no statistically significant differences in N content, N concentration, and plant dry weight. In contrast when L2-110 was the initial in-

oculum, statistically significant decreases in each of the symbiotic parameters were found. The effects on N concentration and content were linear with respect to decreased I-110 nodule occupancy as determined by regression analysis. Plants that were inoculated with strain I-110 13 d after inoculation with strain L2-110 had 0% nodule occupancy by strain I-110 at 28 DAI. These plants did not exhibit significantly greater whole-plant N content or dry weight than nonnodulated control plants at 28 DAI; however, whole-plant N content and dry weight had increased threefold and twofold, respectively, in these treatments at 45 DAI. These values were significantly greater than those of non-nodulated control plants (Table 1). One possible explanation for this result was that N accumulation increased due to increased nodule occupancy by strain I-110 over time. In order to test this hypothesis a second experiment was performed, in which nodule occupancy was determined at 26 and 45 DAI for plants initially inoculated by L2-110 and then by I-110 7 d later. Nodule occupancy by I-110 increased from 4 ± 1 to $14 \pm 2\%$ per plant between 26 and 45 DAI. The additional I-110 nodules found at 45 DAI were determined to be on the peripheral portion of the root system. Dry weight data demonstrated that these nodules had provided little nitrogen to their host plants (possibly due to their recent formation) since these plants only weighed 0.06 g more than nonnodulated control plants. As a separate control in this experiment, plants were nodulated by I-110 followed 7 d later by I-110. These plants were found to have 100% of their nodules formed by L2-110 at both 26 and 45 DAI. Dry weight data for these plants were similar to those found in Table 1 (data not shown).

Competition between I-110 and L2-110 on Soybean Cultivars Lee and Ransom

When variants I-110 and L2-110 were applied in approximately equal numbers on cultivars Lee and Ransom, I-110 occurred in 68 and 89% of the nodules, respectively (Table 2). When I-110 and L2-110 were competed at a 10:1 ratio favoring L2-110 on cultivars Lee and Ransom, the nodule occupancy by I-110 was 50 and 49%, respectively. These values

Table 1. Effect of delayed inoculation with efficient (I-110) and inefficient (L2-110) *Bradyrhizobium japonicum* USDA 110 variants on nodule occupancy and symbiotic effectiveness in Lee soybean. Plants were harvested at 28 and 45 days after inoculation (DAI).

Strain (1st/2nd side)	Inoculation delay	28 DAI				45 DAI		
		Occupancy of I	Dry weight	N concentration	N content	Dry weight	N concentration	N content
		d	%	g	g kg ⁻¹	mg	g	g kg ⁻¹
I/-	—	100	1.22	38.4	46.9	10.66	32.7	348.6
I/L2	0	61	1.02	37.2	38.0	7.65	33.4	255.6
I/L2	2	86	1.19	38.5	45.8	9.51	33.6	319.4
I/L2	4	83	1.09	39.8	43.4	8.63	33.2	287.0
I/L2	8	100	1.04	39.7	41.3	9.30	31.5	292.6
I/L2	13	100	0.92	37.4	34.4	8.39	33.1	277.5
L2/-	—	0	0.67	12.1	8.1	1.16	13.3	15.4
L2/I	0	61	1.02	37.2	38.0	7.65	33.4	255.6
L2/I	2	67	0.68	32.6	22.2	4.51	30.1	135.7
L2/I	4	22	0.65	18.5	12.0	1.95	27.1	52.8
L2/I	8	6	0.62	11.9	7.4	2.11	28.7	60.6
L2/I	13	0	0.67	11.6	7.8	1.25	18.6	23.2
Control†	—	—	0.60	10.8	6.5	0.76	10.8	8.2
LSD 0.05	—	12	—	4.3	11.7	1.79	21.8	69.0

† Control plants were a cultivar Lee isolate that is not nodulated by *B. japonicum* (Lee non-nod).

Table 2. Competition between *Bradyrhizobium japonicum* USDA 110 variants I-110 (efficient) and L2-110 (inefficient) on soybean cultivars Lee and Ransom. Plants were harvested 34 days after planting (DAP).

Cultivar	Competition	Competition ratio	Occupancy of I†	Whole plant dry wt.	Whole-plant N content
		CFU‡ mL ⁻¹	%	g	mg
Lee	I/L2	10 ⁷ /10 ⁷	68	2.4 ± 0.4	90.3 ± 19.6
Lee	I/L2	10 ⁶ /10 ⁷	50**	1.4 ± 0.1	44.2 ± 10.0
Lee	I/L2	10 ⁷ /10 ⁶	100	2.7 ± 0.3	100.1 ± 9.7
Ransom	I/L2	10 ⁷ /10 ⁷	89**	1.7 ± 0.3	61.8 ± 16.1
Ransom	I/L2	10 ⁶ /10 ⁷	49**	1.2 ± 0.2	32.9 ± 7.0
Ransom	I/L2	10 ⁷ /10 ⁶	96	1.8 ± 0.4	65.8 ± 17.6

** Statistically significant at the 0.01 level of confidence.

† Percent nodule occupancy was analyzed with a chi-square test using the ratio of each inoculum strain to derive the expected values.

‡ Colony-forming units.

were statistically different from expected values as determined by chi-square analysis (at the 0.01 confidence level). The drop in nodule occupancy by I-110 resulted in significant decreases in plant N and dry weight (Table 2). When the variants were applied to cultivars Lee and Ransom in a 10:1 ratio favoring I-110, respectively 100 and 96% of the nodules were occupied by I-110. This pattern corresponded to a significant increase in dry weight and plant N when compared with plants that contained ≈50% I-110 nodules. Collectively, these data indicate I-110 to be more competitive for nodule occupancy than L2-110.

Split-Root Experiments

Split-root experiments were performed to determine what influence the host plant has on which variant competes most effectively for nodule occupancy (Table 3 and 4). In these experiments, when I-110 was the initial inoculum, and L2-110 inoculation followed 7 d later, no chlorosis was noted in cultivars Lee, Ransom, and Pickett (Table 3). Chlorosis was noted in these cultivars when L2-110 was the initial inoculum and I-110 followed 7 d later. As expected, each of the uninoculated control plants was chlorotic. In comparing the nodule numbers on each side of the split-root system, it was noted that for all cultivars the number of nodules was very low on the side inoculated later (Table 3). In all trials, plants inoculated first with I-110 had no nodules on the roots receiving the secondary inoculation. All the cultivars initially inoculated with L2-110 and then inoculated with I-110 7 d later had I-110 nodules on the roots receiving the secondary inoculation, but in greatly reduced numbers (Table 3). In all treatments, total dry weights were not significantly different from one another, regardless of whether chlorosis was noted (Table 3). This result was probably due to the restricted plant growth that occurs in the split-root apparatus. However, all three cultivars initially inoculated on one side with I-110, followed 7 d later by inoculation with L2-110 on the second side, had considerably higher amounts of total N than plants inoculated with the reverse sequence (Table 3). Since the roots had limited growth in the split-root apparatus, the roots had similar amounts of total N in both treatments. These levels of N were similar to those found in the roots of control plants. Therefore, the shoots of plants inoculated initially with I-110 contained significantly more N than either control plants or plants inoculated

initially with L2-110. Plants initially inoculated on one side with L2-110 had similar amounts of total and shoot N when compared with uninoculated control plants.

In a second split-root experiment, plants were simultaneously inoculated by the two variants, L2-110 and I-110, on opposite sides of the split-root apparatus (Table 4). In these experiments, no apparent differences in nodule numbers on each side of the split-root were noted (Table 4). This indicated that N₂ fixation ability by the different variants had no effect on nodule formation. However, for each cultivar, the fresh nodule mass was at least threefold higher on the I-110 side of the system than on the L2-110 side. For each of the cultivars, N contents were similar for roots on both sides of the split-root, whether I-110 or L2-110 was placed on that side. Dry weight data for each cultivar were similar to those found in Table 3 and were similar to those of uninoculated controls (data not shown). In contrast, whole-plant N accumulation was significantly higher for inoculated plants than for uninoculated controls. The majority of this additional N accumulated in the shoot.

DISCUSSION

Other investigators have previously demonstrated that initial inoculation by one strain of *Bradyrhizobium* inhibits nodulation by a subsequent strain (Kosslak and Bohlool, 1984). In our experiment using efficient and inefficient *B. japonicum* USDA 110 colony morphology variants, a similar nodulation blockage was observed for pot-cultured soybean plants (Table 1). Furthermore, the delayed inoculation experiment showed that efficient variants were capable of blocking nodulation in a shorter time frame than inefficient variants. An 8-d delay in inoculation was required for complete blockage of L2-110 nodule occupancy by I-110, in contrast to the 13-d delay required for complete blockage of I-110 nodule occupancy by L2-110 (Table 1). Similar results were obtained when soybeans were cultured in the split-root apparatus that prevented interaction between the two strains. With this system, nodulation by the inefficient variant was completely blocked when the efficient variant was the initial inoculum. The inefficient variant, however, was unable to completely block nodulation by the efficient variant following a 7-d delay (Table 3). Nodulation blockage appears to be a host-plant response, which is more pronounced when the efficient rather than in-

Table 3. Nodulation and plant growth responses to inoculation with efficient (I-110) and inefficient (L2-110) *Bradyrhizobium japonicum* USDA 110 variants following a 7-day delay in inoculation with the opposite type of strain in a split-root apparatus. Plants were harvested 30 days after planting (DAP).

Strain (1st/2nd side)	Cultivar	Nodule number		Total dry wt. g	N content			Total N
		1st side	2nd side		Shoot	Root		
						Side 1	Side 2	
I/L2	Lee	49 ± 20	0 ± 0	0.9 ± 0.3	22.8 ± 8.6	1.9 ± 1.0	1.3 ± 0.6	26 ± 10
L2/I	Lee	48 ± 5	5 ± 1	0.8 ± 0.1	5.6 ± 1.2	1.1 ± 0.1	1.3 ± 0.1	8 ± 1
Control†	Lee	0	0	0.5	4.3	2.3‡		7
I/L2	Ransom	18 ± 3	0 ± 0	0.4 ± 0.1	23.0 ± 12.0	0.7 ± 0.2	0.7 ± 0.1	24 ± 12
L2/I	Ransom	38 ± 21	3 ± 2	0.4 ± 0.2	1.9 ± 1.7	1.3 ± 0.9	0.6 ± 0.3	4 ± 2
Control†	Ransom	0	0	0.5	3.4	2.5‡		6
I/L2	Pickett	84 ± 21	0 ± 0	1.0 ± 0.2	28.2 ± 4.2	2.0 ± 0.5	1.8 ± 0.6	32 ± 6
L2/I	Pickett	45 ± 16	3 ± 3	0.6 ± 0.3	5.3 ± 1.9	1.2 ± 0.6	2.2 ± 0.8	9 ± 2
Control†	Pickett	0	0	0.8	6.0	3.5‡		10

† One uninoculated control plant was used for each cultivar.

‡ For control plants in this experiment roots were pooled into one sample; several previous split-root experiments indicate that root masses were approximately equal (± 0.1 g dry wt.).

efficient variant is initially associated with the plant host.

In a similar manner, the more efficient variant was more competitive for nodule occupancy when entire root systems were inoculated with a mixture of efficient and inefficient variants (Table 2). Such results may suggest that either the host plant selects the most efficient strain from a population composed of strains with differences in efficiency (Robinson, 1969) or that this selection depends on both the competitiveness of the bacterial strains and the host-strain interaction (Ames-Gottfred and Christie, 1989). In both delayed inoculation experiments (Table 1) and delayed split-root inoculation experiments (Table 3), either the efficient or inefficient variant could elicit a plant response that inhibits nodulation by the other type of variant. In simultaneous split-root experiments, it was noted that equal numbers of efficient and inefficient nodules were formed on each side of the split-root (Table 4). Since inefficient variants elicit similar nodulation inhibition responses and are equally capable of nodule formation, we interpret our results to be more consistent with the latter view that selection is a host-strain interaction (Ames-Gottfred and Christie, 1989). The fact that host-strain interactions exert an influence on nodule occupancy has also been demonstrated by the observation that certain soybean cultivars suppress nodulation by specific *B. japonicum* strains (Caldwell, 1966; Caldwell and Vest, 1968; Vest and Caldwell, 1972; Cregan et al., 1989). These data together emphasize the importance of host-strain interactions in the infection process.

In the simultaneously inoculated split-root experiments, similar nodule numbers were found on each side (Table 4). However, the nodules on the efficient side were considerably larger than the nodules on the inefficient strain side. These data may indicate that more photosynthate was partitioned to the nodules containing efficient variants (Table 4). These data are similar to those of Singleton and Stockinger (1983), who also found that superior N₂-fixing strains preferentially receive carbohydrate from their host plant.

A reduction in N₂ fixation was clearly noted in plants that had higher percentages of nodules occupied by inefficient variants (Table 1 and 2). This results was found both in plants initially inoculated with L2-110 in pot culture (Table 1) and in plants with higher percentages of L2-110 as a result of a 10:1 ratio favoring L2-110 in competition studies (Table 2). As the percentage of nodules inhabited by inefficient variants increased, a linear decrease in N concentration and content was noted for the *B. japonicum*-soybean symbioses (Table 1 and 2) (dry weight also declined non-linearly). In other work, Cregan et al. (1989) have demonstrated that complex relationships exist between competitiveness and effectiveness. They noted that some relatively less efficient strains are found to be extremely competitive. Since soybean in the USA is grown in soils that contain large indigenous populations of strains, less efficient than the best strains, our data along with the observations of Cregan et al. (1989) clearly support the importance of early nodulation by strains which are both symbiotically efficient and able to effectively compete for nodule occupancy.

Table 4. Nodule occupancy and nitrogen content following simultaneous inoculation with efficient (I-110) and inefficient (L2-110) *Bradyrhizobium japonicum* USDA 110 variants in a split-root apparatus. Plants were harvested 21 days after planting (DAP).

Cultivar†	Nodules		Nodule fresh wt.		N content			Whole plant
	L2 side	I side	L2 side	I side	Shoot	Root		
						L2 side	I side	
	no.				mg			
Lee	10 ± 7	13 ± 10	32 ± 30	118 ± 69	13.2 ± 5.4	1.0 ± 0.3	1.1 ± 0.3	15.2 ± 5.8
Ransom	20 ± 12	12 ± 8	40 ± 20	120 ± 70	12.8 ± 5.9	1.3 ± 0.1	1.1 ± 0.4	15.2 ± 6.1
Pickett	19 ± 13	26 ± 19	20 ± 9	160 ± 29	16.1 ± 2.8	1.2 ± 0.2	1.5 ± 0.4	18.8 ± 3.3

† One uninoculated control plant was used for each cultivar. These controls were not nodulated, appeared chlorotic, and had N content values equivalent to that found in the seeds of each cultivar indicating a lack of biological N₂-fixation.

Another conclusion from our work is that nodulation is a continuous process. A similar finding has recently been reported by Wadisirisuk et al. (1989) and Smith and Wollum (1989). These investigators correlated nodulation with root growth. Our data indicate that nodules may be continuously formed during growth and development. During later stages of growth, nodules were formed on the periphery of lateral roots in plants initially inoculated with L2-110 followed 7 d later by I-110. The low levels of biological N₂ fixation, however, indicate that these nodules were not important to the overall N status of these plants at the time of harvest (45 DAI). Again these observations demonstrate the importance of having efficient, competitive strains within the rhizosphere that can form nodules early in plant growth and development.

The major implication that can be drawn from our work is that enhanced N₂ fixation should result if an efficient strain is established early. Recent field experiments by Paau (1989) suggest that improved rhizobial inoculants can have a positive effect on crop yield. This demonstrates a clear need to develop symbiotically superior strains which are more competitive for nodule occupancy under field conditions. In addition to symbiotic competence and competitiveness, the ability of these strains to survive and proliferate in the field should also be considered. Several recent studies have indicated that bacterial location in the rhizoplane is essential for bacterial-root contact and successful nodulation (Liu et al., 1989; McDermott and Graham, 1989; Wadisirisuk et al., 1989). To increase the number of efficient rhizobia in the plant rhizosphere, one approach may be to use a water-suspension inoculum (Rogers et al., 1982) or fluid drilling using a gel as a bacterial carrier (Jawson et al., 1989). A combination of several approaches may be beneficial. Our results and data from other investigators (Kvien et al., 1981; Singleton and Stockinger, 1983) indicate that the optimal number of efficient vs. inefficient nodules is >50% (Table 2). Determining mechanisms responsible for preferential nodulation of plants by more efficient strains in mixed populations will be a key to the improvement of soybean-*B. japonicum* symbioses under agricultural conditions.

ACKNOWLEDGMENTS

The authors would like to express their thanks to Dr. Tommy Carter (USDA-ARS and Department of Crop Science, North Carolina State University) for advice and assistance with statistical analysis and Terry Matheny (USDA-ARS, Coastal Plains Research Center, Florence, SC) for assistance with the modified split-root procedure. The authors would also like to acknowledge technical assistance by Joy Smith, Julie Danielly, Ruth Lamprey, Elizabeth Walton, Patricia Cook, and Karen O'Kelley. The work of Alfreda Reynolds in the final steps of manuscript preparation is also gratefully acknowledged. Partial support for this work came from DHHS/PHS/NIH grant 2S07RR07024-23 awarded to J.N. Mathis while at the Georgia Institute of Technology.

REFERENCES

Ames-Gottfred, N.P., and B.R. Christie. 1989. Competition among strains of *Rhizobium leguminosarum* biovar *trifolii* and use of

- a diallel analysis in assessing competition. *Appl. Environ. Microbiol.* 55:1599-1604.
- Bhuvanewari, T.V., B.G. Turgeon, and W.D. Bauer. 1980. Early events in the infection of soybean [*Glycine max* (L.) Merr.] by *Rhizobium japonicum*: I. Localization of infectible root cells. *Plant Physiol.* 66:1027-1031.
- Caldwell, B.E. 1966. Inheritance of a strain-specific ineffective nodulation in soybean. *Crop Sci.* 6:427-428.
- Caldwell, B.E., and G. Vest. 1968. Nodulation interactions between soybean genotypes and serogroups of *Rhizobium japonicum*. *Crop Sci.* 8:680-682.
- 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.
- Glowa, W. 1974. Zirconium dioxide, a new catalyst in the Kjeldahl method for total nitrogen determination. *J. Assoc. Off. Agric. Chem.* 57:1228-1230.
- Goodnight, J.A. 1982. SAS users guide: Statistics. SAS Inst., Cary, NC.
- Hunt, P.G., M.J. Kasperbauer, and T.A. Matheny. 1987. Nodule development in a split-root system in response to red and far-red light treatment of soybean shoots. *Crop Sci.* 27:973-976.
- Israel, D.W., J.N. Mathis, W.M. Barbour, and G.H. Elkan. 1986. Symbiotic effectiveness and host-strain interactions of *Rhizobium fredii* USDA 191 on different soybean cultivars. *Appl. Environ. Microbiol.* 51:898-903.
- Jawson, M.D., A.J. Franzluebbers, and R.K. Berg. 1989. *Bradyrhizobium japonicum* survival in and soybean inoculation with fluid gels. *Appl. Environ. Microbiol.* 55:617-622.
- Kosslak, R.M., and B.B. Bohlool. 1984. Suppression of nodule development on one side of a split-root system of soybean caused by prior inoculation of the other side. *Plant Physiol.* 75:125-130.
- Kosslak, R.M., and B.B. Bohlool. 1985. Influence of environmental factors on interstrain competition in *Rhizobium japonicum*. *Appl. Environ. Microbiol.* 49:1128-1133.
- Kuykendall, L.D., and G.H. Elkan. 1976. *Rhizobium japonicum* derivatives differing in nitrogen-fixing efficiency and carbohydrate utilization. *Appl. Environ. Microbiol.* 32:511-519.
- Kvien, C.S., G.E. Ham, and J.W. Lambert. 1981. Recovery of introduced *Rhizobium japonicum* strains by soybean genotypes. *Agron. J.* 73:900-905.
- Liu, R., V.M. Tran, and E.L. Schmidt. 1989. Nodulating competitiveness of a nonmotile Tn7 mutant of *Bradyrhizobium japonicum* in nonsterile soil. *Appl. Environ. Microbiol.* 55:1895-1900.
- Mathis, J.N., W.M. Barbour, T.B. Miller, D.W. Israel, and G.H. Elkan. 1986a. Characterization of a mannitol-utilizing, nitrogen-fixing *Bradyrhizobium japonicum* USDA 110 derivative. *Appl. Environ. Microbiol.* 52:81-85.
- Mathis, J.N., D.W. Israel, W.M. Barbour, B.D.W. Jarvis, and G.H. Elkan. 1986b. Analysis of the symbiotic performance on *Bradyrhizobium japonicum* USDA 110 and derivative I-110, and discovery of a new mannitol utilizing nitrogen fixing USDA 110 derivative. *Appl. Environ. Microbiol.* 52:75-80.
- Mathis, J.N., L.D. Kuykendall, and G.H. Elkan. 1986c. Restriction endonuclease and *nif* homology patterns of *Bradyrhizobium japonicum* USDA 110 derivatives with and without nitrogen fixation competence. *Appl. Environ. Microbiol.* 51:477-480.
- McDermott, T.R., and P.H. Graham. 1989. *Bradyrhizobium japonicum* inoculant mobility, nodule occupancy, and acetylene reduction in the soybean root system. *Appl. Environ. Microbiol.* 55:2493-2498.
- Nelson, D.W., and L.E. Sommers. 1973. Determination of total nitrogen in plant material. *Agron. J.* 65:109-112.
- Paau, A.S. 1989. Improvement of *Rhizobium* inoculants. *Appl. Environ. Microbiol.* 55:862-865.
- Pierce, M., and W.D. Bauer. 1983. A rapid regulatory response governing nodulation in soybean. *Plant Physiol.* 73:286-290.
- Robinson, A.C. 1969. Competition between effective and ineffective strains of *Rhizobium trifolii* in the nodulation of *Trifolium subterraneum*. *Aust. J. Agric. Res.* 20:827-841.
- Rogers, D.D., R.D. Warren, and D.S. Chamblee. 1982. Remedial post-emergence legume inoculation with *Rhizobium*. *Agron. J.* 74:613-619.
- Sargent, L., S.Z. Huang, B.G. Rolfe, and M.A. Djordjevic. 1987. Split-root assays using *Trifolium subterraneum* show that *Rhizobium* infection induces a systemic response that can inhibit nodulation of another invasive *Rhizobium* strain. *Appl. Environ. Microbiol.* 53:1611-1619.
- Singleton, P.W., and K.R. Stockinger. 1983. Compensation against ineffective nodulation in soybean. *Crop Sci.* 23:69-72.
- Smith, G.B., and A.G. Wollum. 1989. Nodulation of *Glycine*

max by six *Bradyrhizobium japonicum* strains with different competitive abilities. *Appl. Environ. Microbiol.* 55:1957-1962.

Vest, G., and B.E. Caldwell. 1972. *Rj4-a* gene conditioning ineffective nodulation in soybean. *Crop Sci.* 12:692-693.

Wadisirisuk, P., S.K.A. Danso, G. Hardarson, and G.D. Bowen. 1989. Influence of *Bradyrhizobium japonicum* location and movement on nodulation and nitrogen fixation in soybeans. *Appl. Environ. Microbiol.* 55:1711-1716.