

## Influence of *Bradyrhizobium japonicum* Location and Movement on Nodulation and Nitrogen Fixation in Soybeans

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Received 3 February 1989/Accepted 13 April 1989

The influence of seed and soil inoculation on bradyrhizobial migration, nodulation, and N<sub>2</sub> fixation was examined by using two *Bradyrhizobium japonicum* strains of contrasting effectiveness in N<sub>2</sub> fixation. Seed-inoculated strains formed fewer nodules on soybeans (mostly restricted to the tap and crown roots within 0 to 5 cm from the stem base) than did bradyrhizobia distributed throughout the soil or inoculated at specific depths. Nodulation was greater below the depths at which bradyrhizobial cells were located rather than above, even though watering was done from below to minimize passive bradyrhizobial migration with percolating water. The most profuse nodulation occurred within approximately 5 cm below the point of placement and was generally negligible below 10 cm. These and other results suggest that bradyrhizobial migration from the initial point of placement was very limited. Nevertheless, the more competitive strain, effective strain THA 7, migrated into soil to a greater extent than the ineffective strain THA 1 did. Nitrogen fixation resulting from the dual-strain inoculations differed depending on the method of inoculation. For example, the amount of N<sub>2</sub> fixed when both strains were slurried together onto the seed was about half that obtained from mixing the effective strain into the soil with the ineffective strain on the seed. The results indicate the importance of rhizobial distribution or movement into soil for nodulation, nodule distribution, strain competitiveness, and N<sub>2</sub> fixation in soil-grown legumes.

Although there have been many studies on the rhizosphere ecology of rhizobia in soil (4, 12, 16), only a limited number have examined the migration of rhizobia in the rhizosphere of legumes. Rhizobia move over only short distances and are largely aided by water flow through the soil (5, 10). Seed inoculation therefore results largely in crown nodulation, while native soil rhizobia (because of their relatively greater distribution in soil) form nodules distributed over the entire root system (5, 20). However, the interpretation that limited nodule distribution from seed inoculation is due to limited migration is questionable, because seed inoculation may also trigger nodulation-regulatory (suppression) processes that limit nodulation to the crown, even when rhizobia capable of nodulating other infectible sites are available (1, 11, 13, 15).

Many soils contain rhizobial strains that differ greatly in N<sub>2</sub>-fixing effectiveness (18), and early infection by ineffective strains may result in low N<sub>2</sub> fixation and poor yields (2, 18). Since the rhizosphere behavior of rhizobia may determine the nodulation capacity and thus the effectiveness of N<sub>2</sub> fixation in legumes, this preliminary study examined the approximate extent of the vertical migration of bradyrhizobial cells in the rhizosphere of soybeans and how this influences competition between different strains, nodulation, and N<sub>2</sub> fixation.

### MATERIALS AND METHODS

**Bradyrhizobial cultures and inoculant preparation.** *Bradyrhizobium japonicum* THA 7, effective (E) in N<sub>2</sub> fixation, and THA 1 and TAL 944, both ineffective (I) in N<sub>2</sub> fixation, were obtained from the Biological Nitrogen Fixation Resource Center for South and Southeast Asia, Bangkok, and were cultured individually in yeast extract-

mannitol broth (6) for 5 to 7 days to a cell density of about 10<sup>9</sup>/ml. For inoculating seeds, a peat-based inoculum of each strain was prepared by mixing 2 ml of the broth culture with 3 g of a gamma-irradiated peat. The suspension for inoculation of soil in each pot was prepared by diluting 2 ml of each broth culture with 200 ml of sterile distilled water.

**Plant culture and experimental treatments.** All three experiments were conducted in a glasshouse at the International Atomic Energy Agency Laboratory in Seibersdorf, Austria, from March to July 1987 by using a Typic Eutrocrepts soil which contained no indigenous *B. japonicum* (21). Nodulating soybean variety 129 and its non-nodulating isolate (as reference crop), both obtained from the University of Minnesota, were grown separately in plastic pots (16.6 cm in diameter and 17.5 cm high, with three holes at the bottom) containing 4 kg of soil-plus-sand mixture (2.5 kg of soil plus 1.5 kg of washed sand). To minimize the passive downward washing of bradyrhizobial cells associated with watering from above, watering was by capillary rise from a 2-cm-deep water reservoir contained in plastic saucers under each pot.

Soybean seeds of cultivar 129 were surface sterilized as described by Vincent (19) before inoculating or sowing. Four seeds were sown per pot, and seedlings were thinned to two 7 days after planting (DAP), at which time 25 ml of solution containing 10 atoms percent of <sup>15</sup>N excess (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (to give the equivalent of 10 ppm [10 mg/kg of soil] of N for the nodulating isolate and 30 ppm of N for the non-nodulating isolate) was poured on the surface of the soil. Pots in each experiment were arranged in a completely randomized design. The quantity of nitrogen fixed was estimated by using the A-value approach (8).

**Experiment 1.** The first study simulated a field situation in which either *B. japonicum* THA 7 (E) and THA 1 (I) were both inoculated onto the seed (S) or one was seed inoculated and the other was mixed throughout the soil (P). Peat-based inoculum was applied as a water slurry on surface sterilized

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seeds (approximately  $10^5$  cells per seed) at planting on 6 April 1987. For soil inoculation (representing indigenous rhizobia), 200 ml of bradyrhizobial suspension was added to 4 kg of soil-plus-sand mixture in three stages, as follows: (i) 60 ml of the inoculum was poured over 1.3 kg of soil in the pot; (ii) an additional 1.3 kg of soil was added into the pot, and 70 ml of the inoculum was poured over it; and (iii) the remaining 1.4 kg of soil was added as a top layer, and 70 ml of the inoculum was poured over this layer. The experiment was replicated three times, and plants were harvested sequentially at 45, 55, and 75 DAP.

**Experiment 2.** The next study examined how the location of bradyrhizobial strains influences nodulation along the root of soybean cultivar 129. Peat-based inoculum (5 g) of THA 7 containing approximately  $10^9$  cells per g of dry peat was spread superficially at planting (27 March 1987) in a thin layer across the pot, either at seed level, at 5 cm below the seed, or at 10 cm below the seed. For the 5- and 10-cm depth placements, soil to the desired depth was removed and then replaced after inoculation. Each treatment was replicated three times, and plants were harvested at 45, 55, and 75 DAP.

**Experiment 3.** The third study simulated an early contact of roots with an ineffective strain and examined how this affects subsequent nodulation and  $N_2$  fixation by an effective bradyrhizobial strain inoculated at different soil depths. *Bradyrhizobium* strain TAL 944 (I) was inoculated as a peat-based slurry onto soybean seeds (designated SI) and grown in soil into which THA 7 (E) had been spread (designated PE) at three layers as in experiment 2 or incorporated as a liquid suspension into the soil as in experiment 1. Treatments were replicated four times, and plants were harvested at 55 and 75 DAP.

**Harvests.** Plants were harvested by digging up roots carefully, washing them, and cutting them into four segments (0 to 5, 5 to 10, 10 to 15, and >15 cm from the crown of the root). Nodules from each section were counted and weighed while fresh, and where two strains were inoculated, the resistance of THA 7 to streptomycin was used to identify nodule-forming strains by growth of nodule extracts on yeast extract-mannitol agar containing 400  $\mu$ g of streptomycin per ml.

Plant tops were oven dried at 70°C for 24 h and milled to a <0.2-mm particle size in a Wiley mill. Both percent N and atoms percent  $^{15}N$  excess in plants were determined by the Dumas combustion method (7) and a VG-SIRA mass spectrometer coupled to an NA-1500 automatic N analyzer (Carlo-Erba, Middlewich, Cheshire, England).

## RESULTS

**Experiment 1: inoculation with *B. japonicum* THA 1, THA 7, or combinations of THA 1 and THA 7. (i) Nodule numbers, weight, distribution, and strain competition.** Total nodule numbers increased after the first and second harvests and, on the average, more than doubled between 45 and 75 DAP (Fig. 1). Significant treatment differences in nodule numbers occurred at each harvest. The SI plus PE (SI + PE) treatment usually nodulated best and at 75 DAP was approximately double that of the reverse treatment (SE + PI). The SE plus PE (SE + PE) treatment caused the formation of significantly more nodules (33 to 80%) ( $P < 0.05$ ) than the corresponding double inoculation with only the ineffective strain (SI + PI) at all harvests. The combined seed inoculation with both strains (SE + SI) gave the poorest nodulation.

Nodule weight for the SI + PI inoculation was the lowest ( $P < 0.05$ ) at the first harvest. However, despite the fact that

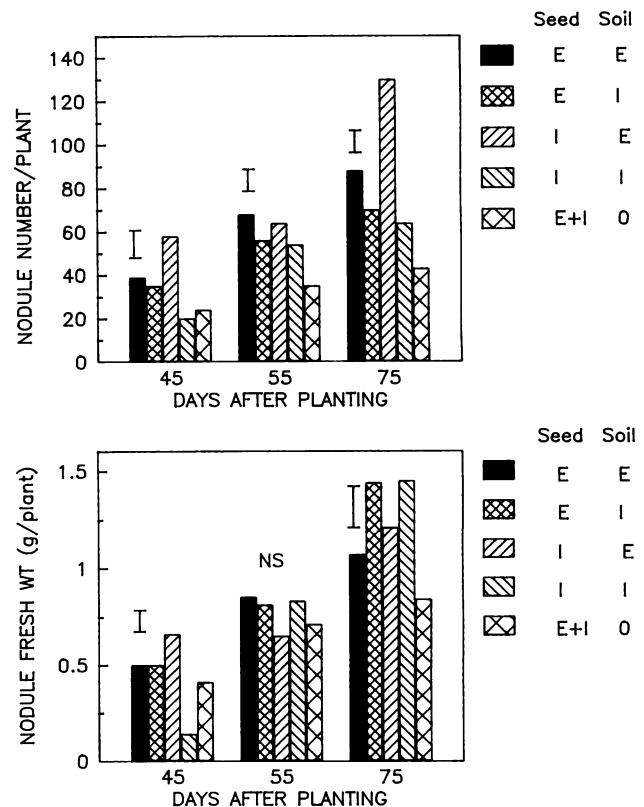


FIG. 1. Nodule numbers and weight from soybeans inoculated with different combinations of *B. japonicum* THA 7 (E) and THA 1 (I) on seed and into soil. Single lines represent the least significant difference at  $P < 0.05$ .

this treatment resulted in fewer nodules than most other treatments at 75 DAP, the nodules had the highest weight, being slightly higher than the weight of nodules formed after the SE + PI treatment (Fig. 1). Also, at the final harvest, apart from the SE + SI inoculation (which gave the lowest nodule weight), all treatments containing the ineffective strain gave higher nodule weights than the SE + PE inoculation.

Except for the double inoculation of the ineffective strain on seed and into soil (SI + PI), the 0- to 5-cm root segment bore the greatest number of nodules compared with other segments (Fig. 2). This was most pronounced for instances in which both strains were inoculated on the seed. Indeed, in treatments SE + PE, SI + PE, and SI + PI at 75 DAP, 65 to 67% of nodules occurred on the entire root, excluding the 0- to 5-cm zone, compared with the corresponding value of 34% for the seed-only inoculation treatment. Only a few nodules developed on the roots further than 15 cm from the stem base (Fig. 2).

With SE + SI and SI + PE inoculations, THA 7 (E) completely dominated nodulation, with THA 1 being almost completely excluded (Fig. 2). However, except for the 0- to 5-cm segment, the situation was reversed with the SE + PI combination; at 75 DAP, nodule occupancy by THA 7 below this zone was only 17%.

**(ii) Nitrogen fixation.** Negligible  $N_2$  was fixed by the dual inoculation of the ineffective strain on seed plus into soil (SI + PI) at each harvest (<10%) (Fig. 3), compared with high  $N_2$  fixation by the other treatments from the first harvest (mean, 61%) to the final harvest (mean, 77%). There were

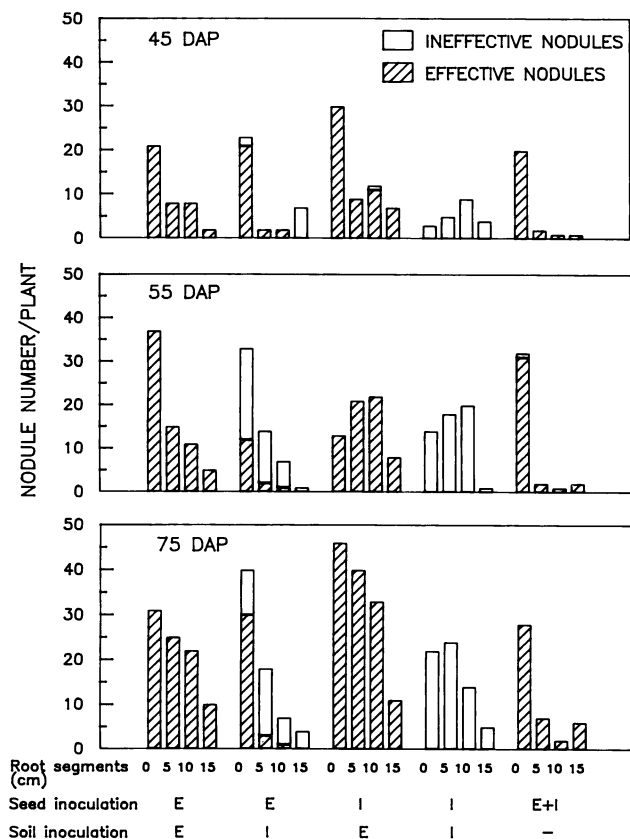


FIG. 2. Distribution of nodules on root segments when different combinations of *B. japonicum* THA 7 (E) and THA 1 (I) were inoculated on soybean seeds and into soil.

significant effects ( $P < 0.05$ ) of type of bradyrhizobial strain(s) and site of inoculation on total N<sub>2</sub> fixed. Although nodulation by the mixed strains inoculated on only the seed (SE + SI) was completely dominated by the effective strain (Fig. 2), the N<sub>2</sub> fixed by this treatment was approximately half that of the treatment with only the effective strain (SE + PE).

(iii) **Total N and dry matter yield.** Nitrogen accretion was consistently lowest with inoculation of only the ineffective strain both into soil and on the seed (SI + PI) (Table 1), and at the first and final harvests, the combined inoculation of the ineffective strain on seed and the effective one in soil (SI + PE) resulted in the highest levels of N. Indeed, treatments in which the E strain was applied to soil yielded consistently higher N than treatments in which the E strain was inoculated on the seed, although this was not statistically significant by 75 DAP. As with N yields, the SI + PE inoculation generally produced the highest dry matter yield, while the SI + PI inoculation gave yields averaging 53% of the average yield of the other treatments (Table 1). The SI + PE inoculation resulted in plants weighing approximately 25% more than those receiving the SE + PI treatment (i.e., by only reversing the site where each strain was inoculated).

**Experiment 2: inoculation with *B. japonicum* THA 7 at different soil depths.** (i) **Nodule numbers, weight, and distribution.** The depth of inoculation did not significantly influence nodule abundance and nodule weight at any harvest (data not presented). In general, there was a marked domination of nodulation by the 5-cm zone immediately below the

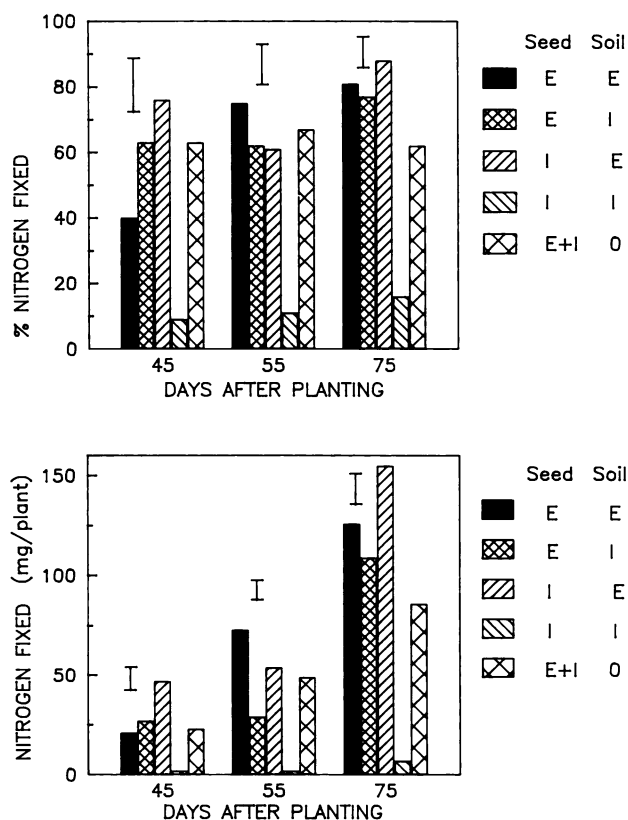


FIG. 3. Nitrogen fixation in soybeans after different combinations of seed and soil inoculations with *B. japonicum* THA 7 (E) and THA 1 (I). Single lines represent the least significant difference at  $P < 0.05$ .

level at which the inoculum was placed (Fig. 4). For the 5- and 10-cm placements, this layer accounted for 56 and 53%, respectively, of the nodules at 75 DAP. The corresponding values for the 5-cm zone above the point of inoculation were 16 and 6%, respectively.

(ii) **Nitrogen fixation.** Although the 5-cm-depth placement of inoculum resulted in greater N<sub>2</sub> fixation than the other treatments, the differences were only slight and never significant (data not presented).

(iii) **Total N and plant yield.** Nitrogen accumulation by the inoculated plants increased substantially after the 45- and

TABLE 1. Nitrogen and shoot yields of non-nodulating and nodulating soybeans after various inoculation treatments with *B. japonicum* THA 7 (E) and THA 1 (I)

Strain type inoculated into:		Total N (mg/pl) <sup>a</sup>			Shoot dry wt (g/pl) <sup>a</sup>		
Seed	Soil	45 <sup>b</sup>	55	75	45	55	75
E	E	48.7 ab	97.7 a	155.4 a	2.2 ab	4.2 a	6.4 a
E	I	44.2 b	45.0 c	142.5 a	1.8 abc	2.4 c	5.8 ab
I	E	62.5 a	89.1 ab	177.3 a	2.4 a	3.7 ab	7.2 a
I	I	22.7 c	29.9 c	49.9 b	1.5 c	2.3 c	3.3 c
E + I	— <sup>c</sup>	36.9 bc	75.6 b	136.1 a	1.6 bc	3.6 ab	5.5 ab
		48.0 ab	32.3 c	90.9 b	2.0 abc	2.6 bc	4.3 bc

<sup>a</sup> Values within a column followed by the same letter are not statistically different at the 0.05 level.

<sup>b</sup> DAP.

<sup>c</sup> —, Non-nodulating soybean isolate.

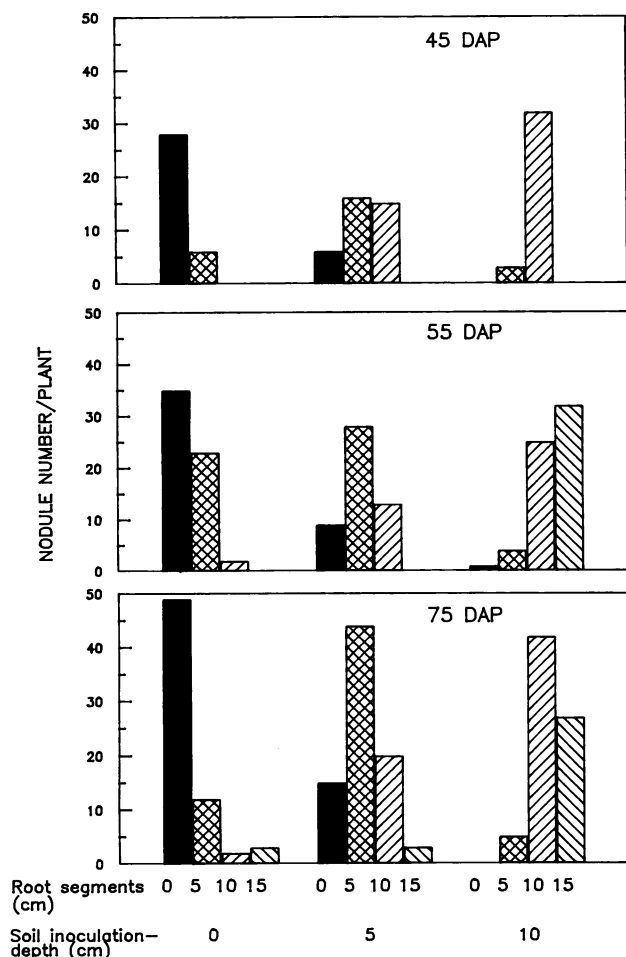


FIG. 4. Nodule distribution along segments of roots of soybeans grown in soil in which *B. japonicum* THA 7 (E) was located at a 0-, 5-, or 10-cm depth. (Missing bars indicate that no nodules were found on those segments of the root).

55-DAP harvests (Table 2) and was higher than that of the non-nodulating soybean isolate, which received three times more  $^{15}\text{N}$ -labeled fertilizer than the inoculated treatments. At 75 DAP, the 5-cm inoculation resulted in significantly higher ( $P < 0.05$ ) total N in plants than the 10-cm inoculation did. There were no significant differences in plant dry weight between treatments at any harvest (data not presented).

**Experiment 3: seed inoculation with TAL 944 and THA 7 inoculated into soil. (i) Nodulation.** Nodulation was domi-

TABLE 2. Nitrogen yield of non-nodulating and nodulating soybeans grown in soil in which *B. japonicum* THA 7 (E) was localized at various depths

Depth of inoculation (cm)	Total N (mg/pl) <sup>a</sup>		
	45 <sup>b</sup>	55	75
0	52.6 ab	95.2 a	155.1 ab
5	50.1 a	89.1 a	184.5 a
10	40.3 ab	86.1 a	136.4 b
— <sup>c</sup>	26.0 b	46.2 b	90.5 c

<sup>a</sup> Values within a column followed by the same letter are not statistically different at the 0.05 level.

<sup>b</sup> DAP.

<sup>c</sup> —, Non-nodulating soybean isolate.

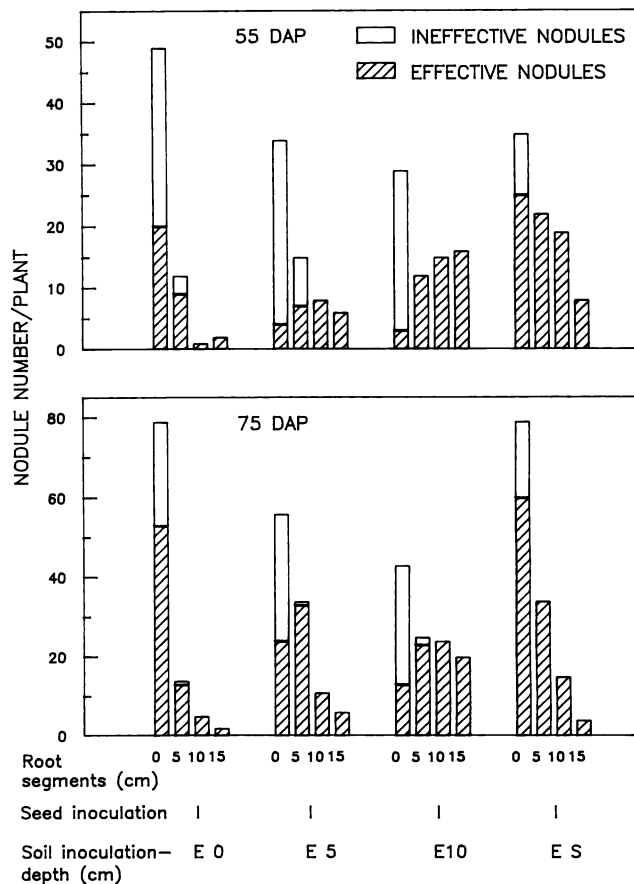


FIG. 5. Nodulation by *B. japonicum* THA 7 (E) or TAL 944 (I), with TAL 944 inoculated on the seed and THA 7 located at a 0-, 5-, or 10-cm depth or mixed into soil.

nated by the soil-inoculated THA 7, which formed 70 to 85% of all nodules (Fig. 5). Similar to the results obtained in experiment 2, nodulation was greatest within 5 cm from the point of inoculation. Nevertheless, the dual-strain inoculation resulted in a greater nodulation above the point of inoculation than when only THA 7 was similarly inoculated in experiment 2, suggesting a greater migration of THA 7 in the upward direction when the ineffective *Bradyrhizobium* strain was inoculated on the seed than when it was absent. Compared with nodulation by THA 7, nodulation by TAL 944 was virtually restricted to the topmost 5-cm root zone, even when THA 7 was placed at a 10-cm depth, and this was most pronounced when THA 7 was mixed into the soil.

**(ii) Nitrogen fixation.** The average levels of  $\text{N}_2$  fixed were 63% (50 mg per plant) at 55 DAP and 72% (99 mg per plant) at 75 DAP (data not presented). However, although mixing the inoculum with soil resulted in the greatest  $\text{N}_2$  fixation, both percent and total  $\text{N}_2$  fixed at each harvest were not significant at the 0.05 level.

**(iii) Total N and dry matter yield.** The mean N content almost doubled within the 20-day interval between harvests (Table 3). There were significant differences ( $P < 0.05$ ) in N yield between the non-nodulating isolate and the symbiotic plants at 55 DAP, and at 75 DAP, the non-nodulating soybean isolate contained only about half of the mean N in the symbiotic plants. The nodulated plants inoculated with supplemental THA 7 suspension or inoculated at the 5- or 10-cm depth derived similar amounts of N and some 19 to

TABLE 3. Nitrogen and shoot yield of non-nodulating and nodulating soybeans seed inoculated with *B. japonicum* TAL 944 (I) and grown in soil into which THA 7 (E) was mixed or localized at various depths

Depth (cm)	Total N (mg/pl) <sup>a</sup>		Shoot dry wt (g/pl) <sup>a</sup>	
	55 <sup>b</sup>	75	55	75
0	79.6 ab	111.4 b	4.9 a	6.5 b
5	72.6 b	151.9 a	4.1 a	7.5 a
10	76.9 b	152.8 a	4.5 a	8.3 a
Mixed	83.7 a	157.0 a	4.5 a	8.8 a
— <sup>c</sup>	55.1 c	73.3 c	4.3 a	6.7 b

<sup>a</sup> Values within a column followed by the same letter are not statistically different at the 0.05 level.

<sup>b</sup> DAP.

<sup>c</sup> —, Non-nodulating soybean isolate.

31% more N than when the supplemental THA 7 suspension was inoculated on the soil surface.

Dry matter yields did not differ significantly at the 0.05 level at the 55-DAP harvest (Table 3). At 75 DAP, the highest yield occurred where the bradyrhizobia had been inoculated directly into soil, and this was significantly greater ( $P < 0.05$ ) (about 35%) than with the THA 7 inoculation at the seed level.

## DISCUSSION

It is generally considered that early-formed nodules seriously suppress further nodulation, thus leading to restriction of nodules largely to the crown. In experiment 1, strain THA 7 almost completely excluded strain THA 1, and therefore nodulation from the seed treatment with both strains served as a control for the treatments with seed inoculation plus supplemental soil inoculation. This treatment gave up to twice as many nodules as the seed treatment alone, and these nodules were distributed over much larger parts of the root system (experiment 1). Although undoubtedly some feedback control of nodulation occurs, our data suggest that this was not great in soil, and we conclude from this that classical crown nodulation patterns are largely due to limited migration of rhizobia to other potential nodulation sites along the root. This is supported by the observation (experiment 1) that when THA 1 was completely mixed with the soil, it formed nodules on all portions of the root, compared with the virtual absence of THA 1 nodules when it was inoculated on the seed. This would account for the restriction of ineffective TAL 944 nodules to the topmost 5 cm of root when it was seed inoculated and when THA 7 was mixed into soil or placed at various locations in experiment 3. The dual-strain seed inoculation data (experiment 1) further suggest that THA 7 migrated into soil to a greater extent than THA 1.

The dominance of nodulation in the root zone immediately below the various inoculum placements in experiment 2 also indicates very limited migration of the bradyrhizobia in unsterilized soil (something on the order of 5 to 10 cm) and indeed suggests greater migration in the direction toward the root tip. In general, this might be expected, but caution must be exercised in interpreting nonoccurrence or the more meager occurrence of nodules above the point of inoculation as a result of lack of migration. Nodulation results from both the occurrence of rhizobia and the receptivity of potential infection sites, and it is possible that the receptivity may have been lessened to some extent in the older portion of the root.

Whether N<sub>2</sub> fixation is greater with nodule formation from seed inoculation or with that from soil inoculation is very much a matter of the soil populations necessary to give ready infection of the root. It is likely that bacteria such as *Rhizobium* spp. do not move through the soil to the root over large distances, i.e., the root must encounter the rhizobia (3), and relatively large populations may be needed to saturate available infectible sites over the root. Three general strategies are suggested: (i) developing rotation systems in which *Rhizobium* spp. would proliferate as rhizosphere microorganisms, (ii) selecting strains not only for efficiency of N<sub>2</sub> fixation but also for differences in ability to migrate in the rhizosphere (the data in experiments 1 and 3, for example, suggest that THA 7, in addition to being more effective, moved deeper into soil than did THA 1 or TAL 944), and (iii) increasing the migration of rhizobia in the rhizosphere, e.g., by inoculating as a water suspension (17) or applying heavy irrigation immediately after inoculation. This is supported by the data of Griffin and Quail (9), which showed that *Pseudomonas aeruginosa* did not migrate in a light sandy soil when water content was 28%. It moved little at 30 and 33% water contents but exhibited increased migration at 39% water content.

Again, although THA 7 was obviously capable of completely displacing THA 1 (experiment 1) when soil was perfused with THA 1 and seed was inoculated with THA 7, THA 1 dominated nodulation on the lower roots. This was no doubt due to the limited migration of seed-inoculated THA 7. Viewed in the context of perennial self-seeding legumes, this probably leads to vastly increased populations of naturally occurring poorly effective rhizobia throughout soil and very localized populations of the seed-inoculated effective strains (4). Coupled with the anticipated poor movement of *Rhizobium* spp. through soil, logistically the poorly effective local strains would be expected to dominate nodulation in subsequent seasons (4). Migration of bacteria in the rhizosphere appears to have been little studied. It could be due to active movement (3), passive movement with flowing water (14), or transportation by the elongating root (3). In the present study, passive movement with water was virtually eliminated. Two promising areas of research being studied in our laboratory at present are the possible selection of *Rhizobium* strains for high migration and the judicious use of irrigation to increase passive movement along the root.

## ACKNOWLEDGMENTS

We are grateful to H. Axmann and the analytical group of the FAO/IAEA Agricultural Biotechnology Laboratory in Seibersdorf for analytical and technical support, to Ruth C. Rossi for typing the manuscript, and to Maria Heiling for the graphics.

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