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Population Densities of *Rhizobium japonicum* Strain 123 Estimated Directly in Soil and Rhizospheres†

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Rhizobium japonicum serotype 123 was enumerated in soil and rhizospheres by fluorescent antibody techniques. Counting efficiency was estimated to be about 30%. Indigenous populations of strain 123 ranged from a few hundred to a few thousand per gram of field soil before planting. Rhizosphere effects from field-grown soybean plants were modest, reaching a maximum of about 2×10^4 cells of strain 123 per g of inner rhizosphere soil in young (16-day-old) plants. Comparably slight rhizosphere stimulation was observed with field corn. High populations of strain 123 (2×10^6 to 3×10^6 cells per g) were found only in the disintegrating taproot rhizospheres of mature soybeans at harvest, and these populations declined rapidly after harvest. Pot experiments with the same soil provided data similar to those derived from the field experiments. Populations of strain 123 reached a maximum of about 10^5 cells per g of soybean rhizosphere soil, but most values were lower and were only slightly higher than values in wheat rhizosphere soil. Nitrogen treatments had little effect on strain 123 densities in legume and nonlegume rhizospheres or on the nodulation success of strain 123. No evidence was obtained for the widely accepted theory of specific stimulation, which has been proposed to account for the initiation of the *Rhizobium*-legume symbiosis.

Events that occur in the developing legume rhizosphere and in its adjacent soil are obviously critical to the establishment of the *Rhizobium*-legume symbiosis. The same factors which influence rhizobia in the soil are likely to be present and greatly intensified in the rhizosphere. Those additional factors that are brought into play in the rhizosphere by the legume root unquestionably exert effects that are central to an understanding of the ecology of the rhizobia and of the initiation of the symbiosis. The interactions of soil, plant, and rhizobia in the natural rhizosphere, however, have been virtually unstudied because of inadequate methodology (11).

The use of the plant-dilution assay (3) has provided evidence of marked increases of rhizobia in the rhizosphere of host legumes compared with nonrhizosphere soil (7, 8, 13). Such data have been interpreted as "specific stimulation" by a number of authors (2, 6, 16), i.e., as indicating that a host legume somehow selectively enhances the growth of its symbiont over other bacteria and other rhizobia. This selective effect is assumed to insure dominance of the appropriate *Rhizobium* at the nodulation site. A rhizosphere effect, however, merely indicates that the rhizobia, like many other soil bacteria, are able to profit from the more favorable conditions of the root zone. The nature of the rhizosphere response of a specific *Rhizobium* strain and its significance to the nodulation process are important unresolved problems.

Fluorescent antibody (immunofluorescence) techniques provide a new and unique capability to identify and enumerate specific bacteria directly in a natural environment. The method has been used for strain-specific detection and enumeration of rhizobia in soil (1, 9, 10, 12) but has not been applied to the rhizosphere. The objective of this investigation is to examine the dynamics of *Rhizobium japonicum* serotype 123 populations in the rhizosphere directly by means of immunofluorescence. *R. japonicum* strain 123 is commonly indigenous to soils of soybean fields in the north central region of the United States (4).

MATERIALS AND METHODS

R. japonicum strain 123 was grown and carried on yeast extract-mannitol medium (1). Fluorescent antibodies were prepared by the method of Schmidt et al. (12). The basic protocol for release of strain 123 from soil and its subsequent concentration on membrane filters followed the procedures for quantitative immunofluorescence outlined by Schmidt (10), as modi-

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fied slightly for the Waukegon soil. Modifications included various additions of a dispersing agent (Tween 80; Difco Laboratories) and an antifoam agent (AF72; General Electric Co.) during soil dispersion, as influencing recovery of *R. japonicum* 123 from sterilized soil. Recovery studies were carried out on partially moistened, 10-g samples of soil that had been distributed in test tubes, autoclaved for 20 min at 1 atmosphere, and inoculated with a log-phase culture of strain 123 at the rate of 10^4 to 10^5 cells per g. After overnight incubation, duplicate samples were extracted under varying test conditions, and fluorescent antibody counts of cells on the membranes (10) were compared with counts of the same suspensions plated on yeast extract-mannitol medium. Routine blending separations of strain 123 from nonsterile Waukegon soil were done with 6 drops of Tween 80 and 2 drops of antifoam agent AF72.

Routine fluorescent antibody counts were made on duplicate membrane filters after 10 ml of flocculated supernatant was passed through each filter. Staining was with fluorescent antibody diluted at least 1:4 in saline. Most counts were made on the basis of 25 fields per membrane, using a ×100 planapochromat oil immersion objective. At very low population densities a ×40 oil immersion planapochromat lens was used and 75 to 100 fields were counted per membrane to provide a roughly constant coefficient of variability (15). The immunofluorescence microscope was a Zeiss Universal equipped for epifluorescence with an FL500 reflector, a BG38 filter, and two fluorescein isothiocyanate filters as primary filters and a Zeiss 53 barrier filter.

Field experiments. Samples of Waukegan soil were taken before planting and during the early growing period of Chippewa soybeans at days 9, 16, and 30. The whole root system was dug carefully, and the soil loosened by gentle shaking was collected and designated the outer rhizosphere soil. Soil still adhering to the root and washed away by 20 min of shaking in a flask containing 3 to 5 g of 3-mm glass beads and enough deionized water to completely soak the roots was designated inner rhizosphere soil. Inner rhizosphere soil suspensions were subsampled before blending for dry weight determinations. There were eight replications. Immediately after harvest, with stubble still in place, the plant was pulled, and a soil sample was collected from the taproot site with a soil auger; this was designated the outer rhizosphere taproot sample. Outer rhizosphere lateral root samples were taken as composites of four auger samples obtained in a radius of 6 inches (ca. 15.2 cm) around a taproot site. These and the other field soil samples were replicated four times.

Pot experiments. Outer rhizosphere and inner rhizosphere soils of wheat and of nodulating and nonnodulating isolines of Chippewa soybeans grown in a greenhouse were compared at 7, 21, and 28 days after planting. Two seeds were planted uninoculated in large waxed-paper pots containing 1 kg of freshly collected Waukegan soil. Additions of inorganic and organic nitrogen, when made, were made as dibasic ammonium phosphate or soybean meal, both at 100 μg of N per g of soil. Soil moisture was maintained below field capacity. Pots were replicated four times. Loose soil not adhering to roots was combined for each pot as the outer rhizosphere sample. Root washings, as previously described, made up the inner rhizosphere sample.

RESULTS

Recovery of *R. japonicum* 123 from sterile Waukegon soil proved to be somewhat variable with respect to both fluorescent antibody and plate counts (Table 1). Data obtained in this experiment were comparable to those obtained in similar quantitative immunofluorescence experiments with *R. japonicum* strain 110 (10). Based on these results, the routine fluorescent antibody counting procedures used in this study were assumed to enumerate consistently at least 30% of the population of *R. japonicum* 123 in soil.

Numbers of strain 123 in the outer rhizosphere remained virtually unchanged during the early development of soybeans in the field (Table 2). The value for the inner rhizosphere, however, increased 5- to 15-fold over the outer rhizosphere

TABLE 1. *Recovery of R. japonicum 123 from sterile Waukegan soil as affected by treatment with Tween 80 surfactant and AF72 antifoam agent*

Treatment[a]		Counts (×10^4) per g of soil[b]		% Recovery[b]
Tween 80 (drops)	AF72 (drops)	Plate method	Filter method	
0	0	21.0	2.8	13.3
2	0	18.0	4.6	25.6
5	0	14.0	6.2	44.3
8	0	20.0	7.9	39.5
8	4	23.0	5.9	25.7
8	6	15.0	4.5	30.0
12	2	18.0	8.0	44.4
12	4	18.0	7.1	39.4

[a] There are 0.0214 and 0.0191 g per drop of Tween 80 and AF72, respectively.

[b] Average counts per gram of soil were 18.0 × 10^4 and 6.3 × 10^4 for the plate and filter methods, respectively. The average percent recovery was 35%. The averages do not include the untreated sample.

TABLE 2. *Populations of R. japonicum in field soil during early growth of soybeans*

Days after seeding	Counts (×10^3) per g of soil	
	Outer rhizosphere	Inner rhizosphere
9	3.9 (0.7)[a]	18.5 (8.4)
16	1.6 (0.4)	22.0 (7.3)
30[b]	1.4 (0.7)	1.4 (0.9)

[a] Values in parentheses are standard error.

[b] Counts were also made on days 0 and 30 in soil uninfluenced by any rhizosphere. Values were 2.7 × 10^3 and 0.4 × 10^3 cells per g of soil, respectively. Plants at 30 days had first and second trifoliate leaves fully developed.

figure during the initial growth of the plant before it declined to the same level as the outer rhizosphere value by day 30. The population of strain 123 in soil uninfluenced by the host roots ranged from a few hundred to a few thousand cells per gram and did not differ materially from the outer rhizosphere population of the young soybean plants.

The data in Table 3 are for the same plot at harvest and for plots treated with urea (100 kg/ha) before planting. These results primarily show the density of strain 123 in the soybean root zone at harvest and shortly thereafter. Plants were dry, and although root systems were still intact, the nodule remnants were dry, shriveled, and empty. Compared with the outer rhizosphere population of about 1,400 cells per g on day 30 (Table 2), the population of strain 123 increased by harvest time to 2×10^6 to 3×10^6 cells per g near the disintegrating taproot and to 8×10^4 to 30×10^4 cells per g in the vicinity of old lateral roots, probably due to the release of strain 123 from the senescent nodules. Populations were slightly lower in the nitrogen-fertilized plots. After 2 weeks the population of strain 123 in the lateral root zone was found to be essentially unchanged, whereas the taproot zone population had dropped sharply to about the same level as that of the lateral root zone. At 3 weeks after fall plowing, the population of strain 123 still remained relatively high at about 2×10^5 cells per g.

The effect of corn rhizospheres on the population of strain 123 as determined in a corn field adjacent to the soybean plot is shown in Table 4. Here the population in a fallow section of the field had stabilized to about 1.6×10^4 cells per g at harvest time. Outer rhizosphere soil influenced by corn roots had only slightly higher densities of strain 123. A similar slight rhizosphere effect for corn was seen in a nearby field where soil type was the same but the previous crop had been soybeans.

TABLE 3. *Population of native R. japonicum 123 in field soil in the vicinity of roots after soybean maturity*

Field condition and sampling period	Source of soil sample	Counts ($\times 10^4$) per g of soil
At harvest, plus nitrogen[a]	Taproots	179.4 (76.4)[b]
	Lateral roots	7.6 (3.4)
At harvest, minus nitrogen	Taproots	312.1 (57.2)
	Lateral roots	28.1 (16.2)
Two weeks after harvest	Taproots	41.3 (7.4)
	Lateral roots	29.1 (2.8)
After fall plowing[c]	Surface	24.0 (1.9)
	25-cm depth	21.2 (6.4)

[a] Urea (100 kg/ha) was applied before planting.
[b] Values in parentheses are standard errors.
[c] The sample was taken 3 weeks after harvest; plants were 120 days old at harvest.

TABLE 4. *Density of R. japonicum strain 123 at harvest time in field soil planted with corn*

Sample description	Sample location	Counts ($\times 10^4$) per g of soil
Fallow; adjacent to soybean plots	10-cm depth	1.6 (0.2)[a]
After harvest; adjacent to soybean plots; unplowed	Outer rhizosphere	2.4 (0.3)
After corn harvest; different location; plowed; previous crop, soybeans; same soil type	Outer rhizosphere[b]	1.1 (0.7)
	Surface	0.3 (0.1)

[a] Values in parentheses are standard deviations.
[b] Soil shaken loose from intact root system.

Additional studies in the greenhouse yielded data on the behavior of strain 123 in the rhizospheres of nodulating and non-nodulating soybean cultivars and wheat (Table 5). There was little difference between populations in the rhizospheres of nodulating and non-nodulating isolines of soybeans. The nodulating line value for 21 days was higher, perhaps due to broken nodules, as suggested by the large variability in replicate determinations. In any case, the population of strain 123 remained relatively low, and no dramatic increase was noticeable. Wheat roots also evidenced a rhizosphere effect with respect to strain 123. The effect of the outer rhizosphere was about the same as for the legume, whereas the response in the inner rhizosphere was only slightly less for the wheat than for soybeans.

Effects of organic and inorganic nitrogen additions on rhizosphere populations of strain 123 in pot experiments are shown in Table 6. The data indicate about a 10-fold increase in the outer rhizosphere compared with the baseline control (Table 5) and another 10-fold increase going from outer to inner rhizosphere. Such results are consistent with the outer field and greenhouse data and reflect no suppression of strain 123 by the nitrogen treatments. Moreover, there were no consistent differences with respect to plant.

Not only was strain 123 present in the rhizosphere of the nodulating soybeans at densities undiminished by nitrogen additions at the concentrations used in this experiment, but it was unaffected with respect to nodulating activity. The number of nodules per plant was about the same or higher for treated pots as compared with untreated pots (Table 7). All nodules sero-

TABLE 5. *Rhizosphere effect of wheat and nodulating and non-nodulating soybeans on R. japonicum 123 in Waukegon soil pot experiments*[a]

Days after seeding	Counts ($\times 10^3$) per g of soil					
	Outer rhizosphere			Inner rhizosphere		
	Wheat	Nodulating soybeans	Non-nodulating soybeans	Wheat	Nodulating soybeans	Non-nodulating soybeans
7	1.4 (0.8)[b]	2.8 (1.4)	2.7 (1.8)	7.8 (0.8)	12.4 (5.6)	9.0 (3.8)
21	5.4 (3.2)	4.5 (2.0)	2.4 (1.1)	10.6 (1.2)	138.0 (82.6)	23.3 (16.9)
28	1.3 (0.4)	3.8 (1.1)	3.2 (0.0)	14.8 (6.0)	64.0 (30.5)	54.3 (36.2)

[a] Control soil analyzed before and after the experiment had 300 counts per g of soil.
[b] Values in parentheses are standard errors.

TABLE 6. *Effect of soil treatment on rhizosphere population of R. japonicum 123 during early growth of soybeans and wheat in pot experiments using Waukegon soil*

Plant	Treatment[a]	Counts ($\times 10^3$) per g of soil					
		Outer rhizosphere			Inner rhizosphere		
		7 days	21 days	28 days	7 days	21 days	28 days
Soy, nodulating	NH_4^+	2.4 (1.1)[b]	2.9 (2.0)	3.8 (1.1)	63.5 (42.1)	79.3 (13.9)	22.0 (10.4)
	Soybean meal	3.9 (1.3)	6.2 (2.2)	7.5 (0.8)	26.9 (21.2)	21.0 (7.9)	43.8 (18.3)
Soy, non-nodulating	NH_4^+	1.7 (1.6)	6.0 (2.1)	4.6 (0.0)	24.1 (13.8)	13.0 (3.5)	92.3 (39.1)
	Soybean meal	2.5 (1.7)	3.2 (0.0)	1.0 (0.0)	13.8 (10.4)	11.3 (4.5)	43.0 (0.0)
Wheat	NH_4^+	2.6 (1.0)	4.6 (2.2)	3.1 (2.2)	27.3 (16.6)	14.3 (3.2)	10.8 (3.8)
	Soybean meal	1.3 (0.0)	5.7 (4.6)	2.2 (0.9)	22.8 (13.7)	15.0 (3.0)	10.7 (7.9)

[a] Dibasic ammonium phosphate or soybean meal at 100 μg of N per g of soil.
[b] Values in parentheses are standard errors.

typed in spot checks at each time period proved to be comprised of *R. japonicum* 123. Ammonia treatment before planting favored extensive root development, which may in turn have mobilized the bulk of the added nitrogen, both conditions leading to greater numbers of nodules. Apparently the mineralization rate of the nitrogen from soybean meal was so low as to have a negligible effect on nodulation.

TABLE 7. *Nodulation of soybeans as affected by nitrogen treatment*

Treatment	No. of nodules per plant at:		
	14 days	21 days	28 days
Control	18 (9)[a]	30 (11)	42 (19)
NH_4^+	11 (4)	48 (8)	109 (7)
Soybean meal	16 (5)	20 (5)	35 (11)

[a] Values in parentheses are standard errors.

DISCUSSION

This study constitutes the first direct examination of the population dynamics of a *Rhizobium* strain in developing rhizospheres under natural conditions. *R. japonicum* 123 is known to be well adapted to the soil investigated and to be so highly competitive as to form the majority of the nodules on soybeans grown in that soil (G. E. Ham, personal communication). The remarkable ability of this strain to compete for nodule sites is apparently not accomplished by massive increases in population in the developing host rhizosphere. Other direct evidence for this view is provided by the rare occurrence of strain 123 cells or microcolonies in microscopic scannings of the surface of stained and virtually undisturbed soybean roots (V. G. Reyes and E. L. Schmidt, manuscript in preparation).

Although stimulation of strain 123 was observed in the developing root system, the densities did not exceed a few hundred thousand cells per gram of the soil in contact with root surfaces. Such stimulation was unaffected by the plant (whether it was capable of nodulation or not) and was unaffected by nitrogen treatment. This suggests that strain 123 is responsive to general rhizosphere conditions and is competitive to some extent with other bacteria in the rhizosphere. Even at the maximal values encountered, however—perhaps 5×10^5 cells per g, assuming 30% counting efficiency—strain 123 must be numerically neglible in a total rhizosphere bacterial population that may be assumed to be at least 5×10^8 cells per g (5).

The preplanting density of serotype 123 in the soil studied ranged from a few hundred to a few thousand cells per gram at the beginning of the growing season. With the onset of plant root development, the population rose to about $2 \times$

10^4 cells per g of soil under the influence of plant roots (outer rhizosphere). Numbers increased dramatically only at plant maturity, when densities of 2×10^6 cells per g of soil were reached. This increase was apparently due to release of strain 123 from senescent nodules and was only temporary, declining by about 1 log within 3 weeks after soybean harvest.

The concept of specific stimulation, the strategy whereby the appropriate *Rhizobium* symbiont is selectively favored by its host root and responds with overwhelming growth in the host rhizosphere as a preliminary to successful nodulation, has been stated in considerable detail by Nutman (6). *R. japonicum* serotype 123 is successful as an indigenous soil organism and is highly successful in out-competing other soybean symbiont strains introduced as rhizobial inoculants (17); according to our data, however, strain 123 does not follow the scenario of specific stimulation. Rhizosphere stimulation of strain 123 was only slightly, if at all, greater for host than for nonhost plants, and the stimulation observed was not at all suggestive of a massive population expansion in the immediate vicinity of the roots. Experimental support for the specific stimulation hypothesis is meager, especially in terms of rhizobial response to rhizospheres under natural soil conditions. In several of the commonly cited studies involving natural soil, stimulation of rhizobia by nonlegume rhizospheres was in fact evident (7, 8, 14). Additional studies of strain 123 and other specific rhizobial strains that would provide equally good model systems for the study of prenodulation events in soil are obviously needed to resolve the inconsistencies and validate the projections found in the literature.

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