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## Growth of *Rhizobium* in Soil Amended with Organic Matter<sup>1</sup>

J. J. PENA-CABRIALES AND MARTIN ALEXANDER<sup>2</sup>

### ABSTRACT

Small populations of *Rhizobium meliloti*, *R. phaseoli*, *R. japonicum*, and a strain of *Rhizobium* nodulating cowpeas failed to grow in moist unamended Lima silt loam at 29°C. The numbers of the four rhizobia increased if 0.5 or 1.0 but not 0.01 or 0.1% mannitol was added. The population of *R. phaseoli* and *R. meliloti* but not the other two bacteria then fell markedly after 2 d. A second addition of mannitol on day 4 did not result in a rise in the *R. meliloti* population. The addition of 1% mannitol also resulted in an increase in the total number of bacteria and protozoa. Addition of corn leaf residues to the soil did not stimulate *R. japonicum* or *R. lupini* but caused a fall in the abundance of *R. meliloti* and *R. trifolii*. Additions of alfalfa residues led to a reduction in the numbers of *R. meliloti*, *R. phaseoli*, *R. japonicum*, and the cowpea *Rhizobium* and an increase in the abundance of protozoa, but bacteriobionts, bacteriophages, and myxobacteria either could not be detected or did not respond. If the soil was supplemented with streptomycin, erythromycin, actidione, and nystatin, to which these rhizobia were resistant, the four strains proliferated, but growth in such treated soils was much greater if 0.1% mannitol was also added. Stimulation of the four rhizobia was greater in soils treated with the two antibacterial compounds than in Lima silt loam amended with the two compounds effective against eukaryotes. It is suggested that competition with bacteria and possibly grazing by protozoa limit the growth of small populations of *Rhizobium* in soil.

**Additional Index Words:** bacterial growth, competition, predation, root-nodule bacteria.

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THE ABILITY of *Rhizobium* to proliferate and persist in soil in the absence of the host plant is necessary because, if deliberate inoculation is not practiced, the bacterium must be present in numbers sufficiently large to nodulate the legume crop. *Rhizobium* grows in soil in the immediate vicinity of plant roots, and it is often considered to be stimulated more by legumes than by other plants (9, 20). However, controversy exists as to whether stimulation by individual species of legumes is specific for the infective rhizobia for that plant species (9, 10, 13).

*Rhizobium* is also able to grow in sterile peat or mixtures of soil and peat, which are used as carriers for legume inoculants. Van Schreven (17) showed that the proliferation of rhizobia in these materials depended on the type of peat used. The growth of *Rhizobium* in non-

sterile soil has received little attention. Chatel and Parker (2) noted that species and strains of *Rhizobium* differ in their capacity to colonize soil during the growing season. They observed that *Rhizobium lupini* attained larger numbers and proliferated more readily than did *Rhizobium trifolii*.

The present study was designed to assess the ability of *Rhizobium* to grow in amended soil and to establish factors that limit the proliferation of these bacteria in soil.

### MATERIALS AND METHODS

Samples of Lima silt loam (fine loamy, mixed, mesic Glosoboric Hapludalf, pH 7.2, 4.2% organic matter) from Aurora, N.Y., were collected from the surface 15 cm and passed through a 2-mm screen. The soil had water contents of 25.5, 21.2, and 18.3% at 0.33, 1.0, and 10 bars of tension, respectively. When sterile soil was used, the soil at 23% (wt/wt) moisture was autoclaved for 1 h on 3 consecutive days. Portions (10 g) of soil contained in 125-mL milk dilution bottles were adjusted to 23% (wt/wt) moisture with sterile distilled water and inoculated with 1.0 mL of the rhizobial suspension and treated with various amendments. The bottles were shaken by hand and then incubated vertically at 29°C. Triplicate bottles were sacrificed periodically for microbial counts. The soil or cell suspensions and dilutions were made in a sterile aqueous solution of 0.02% KH<sub>2</sub>PO<sub>4</sub> and 0.08% K<sub>2</sub>HPO<sub>4</sub>. Counts were performed with triplicate plates at each dilution, with 1.0 mL of dilutions of the soil suspension mixed into the agar.

Spontaneous mutants of *Rhizobium japonicum* ATCC 10324, *R. meliloti* 3DOa20, *R. trifolii* TK, *R. lupini* 3C2bl, and cowpea *Rhizobium* S57 that were resistant to 1.0 mg of streptomycin and 50 µg of erythromycin/mL were obtained by the method of Danso et al. (4). *Rhizobium phaseoli* 127K17, which was already resistant to these antibiotics at the same concentrations, was obtained from Dr. Carlos Ramirez-Martinez. The cowpea *Rhizobium* was originally isolated from Trinidad. The bacteria were grown in 200 mL of yeast extract-mannitol (YEM) broth (18) contained in 500-mL Erlenmeyer flasks and incubated at 30°C on a rotary shaker (120 rpm) either for 3

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<sup>2</sup> Graduate Student and Professor of Soil Science, respectively. The Senior Author is currently at Centro de Investigacion y de Estudios Avanzados del Instituto Politecnico Nacional—Unidad Irapuato, Apdo. Postal 629, Irapuato, Gto. 36500, Mexico.

d for *R. phaseoli*, *R. leguminosarum*, and *R. trifolii* or for 10 d for *R. japonicum*, *R. lupini*, and *Rhizobium* S57. The cells were collected by centrifugation and washed aseptically three times with sterile buffer solution. The number of rhizobia in soil was determined by plating serial dilutions of the soil on YEM agar containing 1.0 mg of streptomycin sulfate, 50 µg of erythromycin, 300 µg of cycloheximide, and 100 µg of nystatin/mL.

Fungi were enumerated on Martin's medium (11), and counts of total bacteria were made on nutrient agar. The method of Singh (15) was used to count protozoa. The petri dishes contained 15 mL of buffered saline agar (BSA), which was composed of the inorganic salts of YEM and 2% agar, and each glass ring inserted into the agar received 0.1 mL of a suspension containing approximately  $1 \times 10^8$  rhizobia and 0.5 mL of a soil dilution. After 5 d at 30°C, the 5 rings at each dilution were examined under low-power magnification for the presence of protozoa, and the final numbers were estimated from a most-probable-number table.

The double-layer technique of Stolp and Starr (16) was used for counting *Bdellovibrio*. The bottom layer consisted of BSA with 1.9% agar. The upper layer was yeast extract-peptone agar (0.3% yeast extract, 1.0% peptone, and 0.6% agar) that had been inoculated with approximately  $1 \times 10^9$  cells of the strain of *Rhizobium* that had been added to the soil. Examinations for characteristic *Bdellovibrio* plaques were made daily for 10 d.

The presence of bacteriophages was assayed by a double-layer technique (8). A 1:10 dilution of soil was centrifuged at  $3,000 \times g$  for 20 min to remove large particles, and 10-fold dilutions of the supernatant fluid were made in trypticase soy broth (Difco). The bottom layer of the plate was 20 mL of trypticase soy agar supplemented with 1.0 mg of streptomycin sulfate/mL. A drop of the *Rhizobium* culture and 0.5 mL of the soil dilution were added to tubes with 6.0 mL of molten BSA (containing 0.7% agar), the tubes were immediately mixed,

and their contents were poured onto the bottom layer. The plates were incubated for 5 to 10 d.

The attempts to enumerate myxobacteria were made by the method of Singh (14). A suspension (0.2 mL) containing approximately  $10^9$  cells of *Rhizobium* was spread on plates containing 0.5% NaCl in 1.5% water agar to make circles of 2.5-cm diameter, each of which was inoculated with a drop of a dilution or a crumb of soil. The plates were incubated for up to 3 weeks and examined for fruiting bodies.

In investigations of the effect of mannitol, the soil received 0.5 mL of a mannitol solution. When a second mannitol addition was made, 0.5 mL of a 20% mannitol solution (wt/vol) was added on day 4, the bottles were placed horizontally for 3 h to reduce the water content, and then they were again incubated vertically.

In tests of the influence of crop residues, leaves and stems of 1-year-old alfalfa or leaves of 2-month-old corn were dried at 70°C and ground to pass through a 2-mm sieve. The soils were thoroughly mixed with the plant residues (added to 1% by weight of soil) and then inoculated with 1.0 mL of a rhizobium suspension.

In studies in which antibiotics were added to soil, the test compounds were added to give 1.0 mg of streptomycin, 50 µg of erythromycin, 300 µg of actidione, and 100 µg of nystatin/

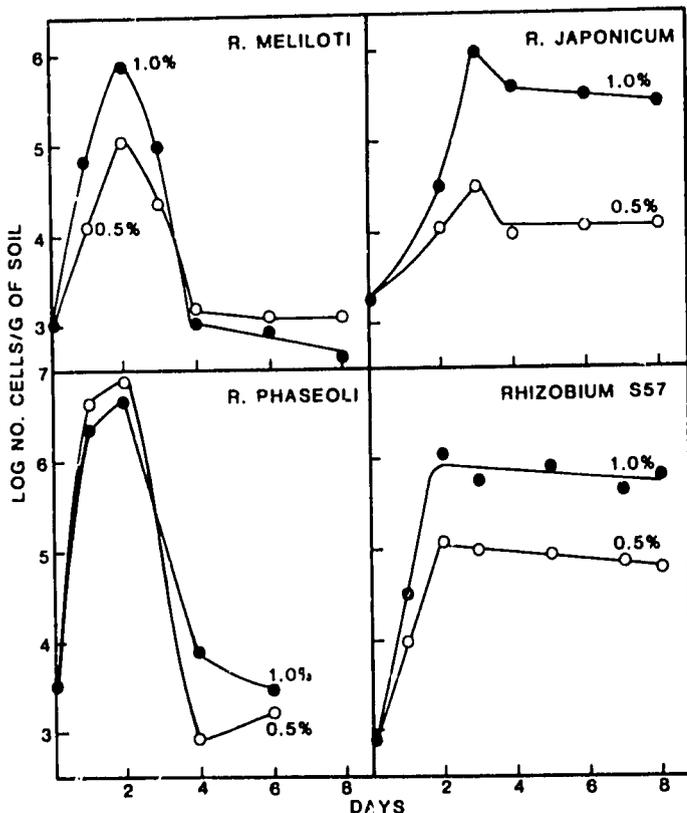


Fig. 1—Growth of four rhizobia in soil receiving 0.50 and 1.0% mannitol.

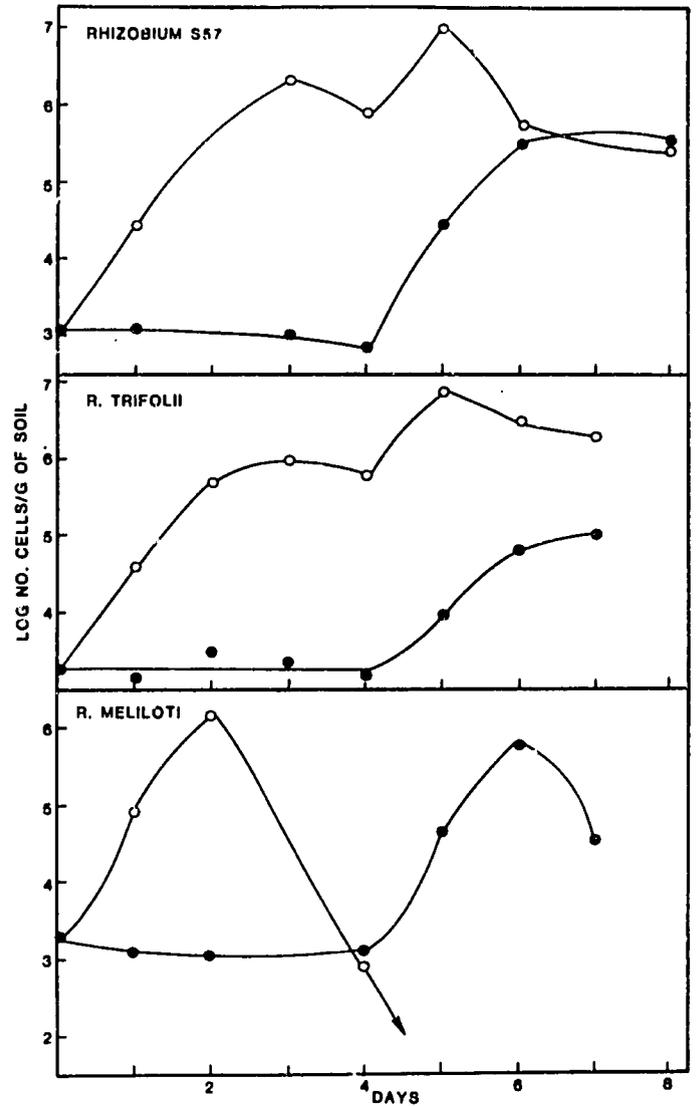


Fig. 2—Effect of the addition of mannitol on days 0 and 4 or day 4 only on the growth of *R. meliloti*, *R. trifolii*, and *Rhizobium* S57 in soil.

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g of soil. Then, 0.5 mL of a rhizobial suspension was added, and the bottles containing the soil were mixed by hand. The moisture content was adjusted with sterile water to 23% (wt/wt). The bottles were incubated horizontally at 29°C, and samples were taken regularly for rhizobial counts.

Soils and plates for counting were incubated at 29°C.

RESULTS

*Rhizobium phaseoli* and *R. japonicum* were added to separate samples of non-sterile soil at moisture levels of 15 and 25% (wt/wt) and at initial densities of 25,000 to 37,000/g. Daily counts of *R. phaseoli* for 6 d and *R. japonicum* for 5 d showed no statistically significant increase in numbers. In contrast, growth occurred when the bacteria were added to sterile soil at 25% moisture.

The effect of mannitol on growth in nonsterile soil was studied with inocula of 1,000 *R. meliloti*, 3,700 *R. phaseoli*, 1,800 *R. japonicum*, and 760 cowpea *Rhizobium* cells/g. Significant increases in cell numbers were not evident in 8 d (6 d for *R. phaseoli*) in soil receiving 0.01 or 0.10% mannitol. However, appreciable growth of all four rhizobia was evident if the soil received 0.50 or 1.0% mannitol (Fig. 1). After 2 or 3 d at the higher two sugar levels, the population size diminished, and this decline was especially marked for *R. meliloti* and *R. phaseoli*. The population sizes of the two slow-growing rhizobia did not show as marked a fall.

The effect of two additions of 1% mannitol on the growth of *Rhizobium* is shown in Fig. 2. The carbohydrate was added at day 4 in one set of soils (closed circles) and at days 0 and 4 in the second set (open circles). The population of *R. meliloti* rose following the first but not the second addition; on days 5, 6, and 7, the population in the soil receiving mannitol on days 0 and 4 was at levels below the sensitivity limits (100 cells/g) of the technique used. In contrast, the populations of *R. trifolii* and the cowpea *Rhizobium* remained high after both the first and second addition of sugar. Similarly, additions on only day 4 stimulated all three bacteria; however, the density of *R. meliloti* but not the other rhizobia fell during the test period.

The responses of two species of *Rhizobium*, total bacteria, and protozoa to the addition of 1.0% mannitol to

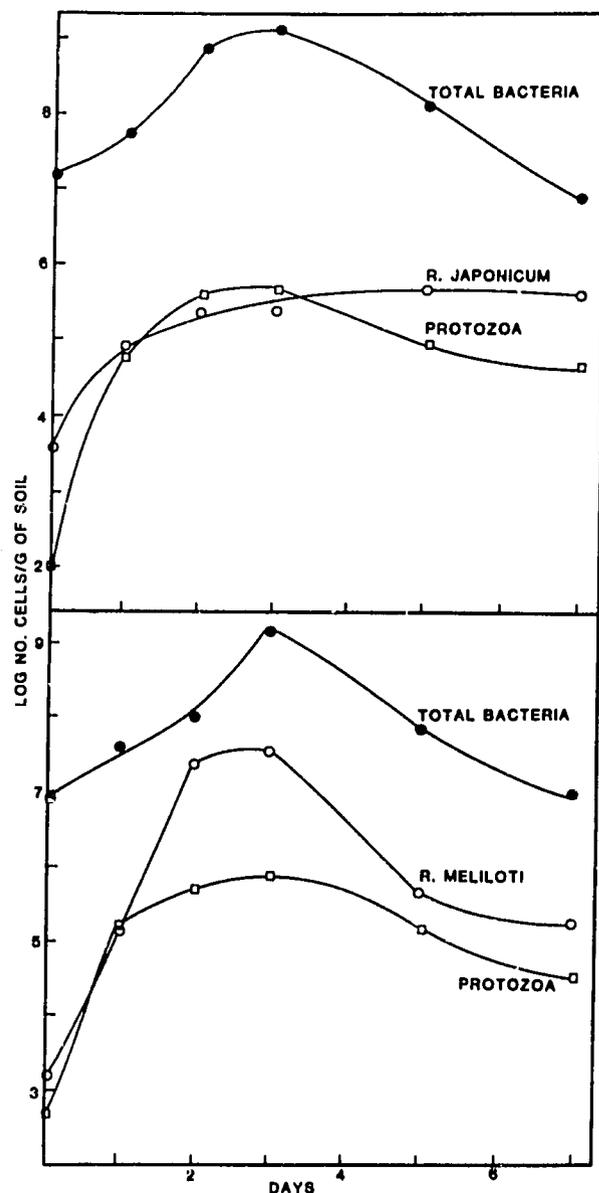


Fig. 3—Response of *R. japonicum* and *R. meliloti* to the addition of 1.0% mannitol to Lima silt loam.

Table 1—Effect of corn residues on populations of *Rhizobium* in soil.

Days	Residues added	No. of cells × 10 <sup>4</sup> per gram of soil							
		<i>R. japonicum</i>		<i>R. lupini</i>		<i>R. trifolii</i>		<i>R. meliloti</i>	
		Sterile soil	Nonsterile soil	Sterile soil	Nonsterile soil	Sterile soil	Nonsterile soil	Sterile soil	Nonsterile soil
0	-	12	10	9.7	10	29	32	26	26
	+	13	10	10	10	27	36	29	28
5	-	35	16	10	13	32	48	28	31
	+	17	20	12	14	34	25	34	51
10	-	18	24	12	16	47	26	32	38
	+	32	31	13	15	46	18	36	27
15	-	37	50	39	43	100	13	97	100
	+	41	55	45	59	130	5	120	<1
20	-	420	39	87	15	2,100	61	830	91
	+	380	29	100	17	1,900	<1	1,000	<1
25	-	790	38	600	97	9,700	57	7,200	74
	+	4,700	13	620	10	8,700	<1	8,300	<1
30	-	5,200	31	5,100	35	9,500	93	8,100	89
	+	7,000	30	3,900	46	10,000	<1	9,500	<1
35	-	6,700	21	4,800	95	ND†	ND	ND	ND
	+	12,000	39	10,000	83	ND	ND	ND	ND

† Not determined.

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the soil are shown in Fig. 3. The populations of *R. meliloti*, total bacteria, and protozoa increased during the first 3 d and then declined. Bdellovibrios, bacteriophages, and myxobacteria could not be detected, except for the finding of 16 and 20 bdellovibrios/g on day 5 in soil inoculated with *R. meliloti* and *R. japonicum*, respectively. On the other hand, the population of *R. japonicum* rose slowly with time and did not show an appreciable decline. However, the density of *R. meliloti* rose to well above  $10^7$ /g before declining to somewhat fewer than  $10^6$ /g, whereas the numbers of *R. japonicum*, possibly because of its slow growth, never reached  $10^6$ /g of soil.

The counts of *R. japonicum* and *R. lupini* in nonsterile soil amended with 1% corn leaf residues were the same as in unamended soil for the 35-d test period (Table 1). With *R. meliloti* and *R. trifolii*, on the other hand, the counts were the same in amended and unamended soil for the first 10 d; however, whereas the counts stayed at about that level in unamended soil, the numbers declined to  $< 1,000$ /g of soil, which was the sensitivity limit for counting, after about 2 weeks in the amended soil. The numbers remained at such low levels until the end of the 30-d test period. Thus, the faster-growing *R. trifolii* and *R. meliloti* were more susceptible than the slower-growing *R. japonicum* and *R. lupini* to the apparent killing agent arising from additions of the plant residues. Microorganisms are responsible for the decline because such a reduction in population was not observed in sterile soil amended with corn residues. In sterile soil, all four species grew and ultimately reached values of about  $10^7$ /g, but the counts for each species in amended soil were similar to those in soil not receiving the corn residues; hence, corn tissue is not a substrate for the rhizobia.

When ground alfalfa residues (1%) were used, a decline occurred in the population of all rhizobia (Table 2). Except for *R. phaseoli*, no such marked decline was evident in unamended soil. The number of *R. japonicum*, which was not suppressed in the soil receiving corn residues, also fell. The numbers of protozoa increased markedly following the addition. *Bdellovibrio*, myxobacteria, and bacteriophages were not found in the 1:100 dilution of soil, except for a count of 290 bdellovibrios/g in the amended soil at day 5.

A study was conducted of the effect on *Rhizobium* of suppressing components of the microbial community by additions to soil of a mixture of streptomycin, erythromycin, actidione, and nystatin. The addition of these antibiotics to the soil allowed the rhizobia to grow and increase in abundance (Table 3). The response to the

Table 3—Effect of adding antibiotics and mannitol to soil on growth of rhizobia.

Organism	Days	No. of cells $\times 10^3$ per gram of soil			
		No addition	Antibiotics	Antibiotics + mannitol	Mannitol
<i>R. phaseoli</i>	0	25	23	23	23
	1	28	71	310	34
	2	26	160	5,800	29
	4	17	180	6,200	27
<i>R. meliloti</i>	0	15	15	15	15
	1	10	45	180	12
	2	14	180	2,100	18
	4	10	160	1,900	20
<i>R. japonicum</i>	0	19	19	19	19
	1	15	160	210	14
	2	21	380	1,400	26
	4	18	500	15,000	24
<i>Rhizobium</i> S57	0	27	27	270	27
	1	19	180	320	20
	2	23	410	4,400	23
	4	29	600	8,500	21

inhibitors was enhanced by amending the soil with 0.1% (wt/wt) mannitol. As previously shown, this concentration of the carbohydrate was not sufficient by itself to permit an increase in the population size of *Rhizobium*.

To determine if this promotion of *Rhizobium* growth resulted from a suppression of bacteria or eukaryotic organisms, antibiotics acting on prokaryotic microorganisms (streptomycin and erythromycin) and on eukaryotic organisms (actidione and nystatin) were added separately to soil inoculated with a strain of *Rhizobium*. The antibiotics and mannitol were at the same concentrations as previously used. Treatment of soil with antibiotics acting on eukaryotic organisms allowed rhizobium to grow, and counts as high as  $1.5 \times 10^5$  to  $1.7 \times 10^6$  were attained (Table 4). The increase in population size because of addition of antibiotics acting on eukaryotic organisms was not as large as when antibacterial chemicals were added, the addition of which sometimes allowed the *Rhizobium* cell densities to exceed  $10^7$ /g.

## DISCUSSION

Rhizobia did not increase in abundance in unamended moist soil; however, growth occurred in autoclaved or mannitol-amended soil, suggesting that nutrient limitation, interactions with other organisms, or both were involved in holding rhizobial numbers in check. The failure of the rhizobial population to increase in soil amended with 0.01 or 0.1% mannitol suggests that the rhizobia are

Table 2—Effect of alfalfa residues on populations of *Rhizobium* and other microbial groups in soil.

Day	Residues added	No. of cells per gram of soil						
		<i>R. meliloti</i>	<i>R. phaseoli</i>	<i>Rhizobium</i> S57	<i>R. japonicum</i>	Total bacteria $\times 10^6$	Fungi $\times 10^4$	Protozoa
0	—	460	830	1,400	770	12	80	240
	+	460	830	1,500	770	14	230	300
1	—	760	660	3,900	460	ND†	ND	ND
	+	450	510	100	430	ND	ND	ND
3	—	560	400	2,300	350	19	120	1,000
	+	100	100	100	100	260	640	43,000
5	—	ND	390	1,500	510	26	190	ND
	+	100	100	100	100	43	270	83,000
7	—	ND	220	ND	ND	21	160	820
	+	100	100	100	100	89	180	7,300

† Not determined.

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**Table 4**—Populations of *Rhizobium* in soil receiving mannitol and antibiotics with mannitol.

Day	Chemical added	No. of cells $\times 10^4$ per gram of soil			
		<i>R. phaseoli</i>	<i>R. meliloti</i>	<i>R. japonicum</i>	<i>Rhizobium</i> S57
0	None	10	34	43	17
4	None	9.6	41	32	28
4	Mannitol	8.4	32	10	12
4	Mannitol, actidione, nystatin	150	190	1,700	1,000
4	Mannitol, streptomycin, erythromycin	7,700	13,000	25,000	44,000

poor competitors in soil. The large amounts of exogenous carbon as mannitol that had to be added to induce a response are in agreement with the data of Chowdhury (3), who observed increases in numbers of *R. trifolii* and *R. lupini* in non-sterile soil only if it was amended with carbohydrates at rates similar to those reported herein.

The growth rates in mannitol-amended soil were not greatly different among the strains of *Rhizobium* tested. The mean generation times observed were 4.9 h for *R. meliloti*, 4.6 h for *R. phaseoli*, 4.8 h for the cowpea *Rhizobium*, and 7.8 h for *R. japonicum*. These findings are in contrast with the common observation of appreciable differences in the growth rates of the so-called fast- and slow-growing strains of *Rhizobium* in culture media, for which mean values of 2 to 4 h for fast growers and 6 to 8 h for slow growers are commonly reported (19). Nevertheless, differences in final population sizes were evident between fast- and slow-growing strains of *Rhizobium* after growth in mannitol-amended soil. A population density of about  $10^6$  cells per gram of soil, below which predation may be of diminished importance (6, 7), was found for the slow-growing rhizobia after 7 d of incubation in mannitol-amended soil, but *R. meliloti* and *R. phaseoli* declined markedly after growing readily for 48 h in sugar-amended soil. The decline in numbers of total bacteria and the inability of the rhizobia to reach populations much in excess of about  $10^6$ /g may be a result of predation by protozoa. It is not clear why the population of *R. meliloti* and *R. phaseoli* in mannitol-amended soil fell below the presumed threshold level for protozoan predation (1). The data of Chowdhury (3) also show such an effect; i.e., the population of *R. lupini* was about  $10^6$  and that of *R. trifolii* was about  $10^4$  cells/g of soil 70 d after addition of a carbohydrate to soil receiving the same size inoculum of the two bacteria.

Although the increase in *Rhizobium* population depended on the availability of simple carbon compounds, rhizobia grew in nonsterile soil amended with low concentrations of mannitol provided that the microbiota was suppressed by antibiotics. The beneficial effect of antibacterial agents suggests that competition with bacteria for available carbon sources limits the growth of *Rhizobium*. Furthermore, the enhanced *Rhizobium* proliferation associated with suppressing eukaryotes indicates that either competition occurs with eukaryotes or pro-

tozoa are preying on the rhizobia. It has previously been reported that the inhibition of protozoa by actidione enhances rhizobial survival in soil (12) and that protozoa appear to be responsible for reducing rhizobial populations (5). Such a view, however, implies that rhizobia even at low densities are subject to predation, which is not consistent with most available data (1). It is also possible that compounds released from organisms killed by the chemicals toxic to eukaryotes increased the supply of nutrients for bacteria, thus permitting *Rhizobium* to grow.

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