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Survival of *Rhizobium* in Soils Undergoing Drying

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Survival of *Rhizobium* in Soils Undergoing Drying[1]

J. J. PENA-CABRIALES AND M. ALEXANDER[2]

ABSTRACT

Streptomycin-resistant mutants of *Rhizobium* were used to study *Rhizobium* survival in soils undergoing drying. The decline in numbers of *R. japonicum* in soil was biphasic. The initial, rapid phase coincided with the time of major water loss, and the subsequent slow rate of decline of viability occurred as the soil lost little water. Similar population charges were found with other species of *Rhizobium*. The rate of dying of *R. japonicum* and *R. leguminosarum* during the second phase in soil at relatively low moisture levels was governed by the water content of the soil. In 10 days of drying, the cell numbers of the six *Rhizobium* species and a cowpea *Rhizobium* were reduced by two logarithmic orders of magnitude, and no differences in susceptibility were noted between the fast- and slow-growing rhizobia. Exposure of *R. japonicum* to several cycles of wetting and drying resulted in a further reduction in population size with each cycle. The percentage survivors of *R. japonicum* was not greatly affected by the time required for the drying but was different in different soils. Rhizobia that proliferated in soil showed somewhat better survival than those grown in culture and added to soil. Decayed organic residues afforded little protection against desiccation, but placing the root-nodule bacteria in a suspension of the polysaccharide they elaborate allowed for somewhat better survival.

Additional Index Words: desiccation, moisture effects, root-nodule bacteria, water loss.

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BOTH BIOLOGICAL and abiotic factors may govern the persistence or establishment of populations of *Rhizobium* in soil. Among the biological factors cited to account for the decline of rhizobia are predation by protozoa (4, 9), attack by *Bdellovibrio* (8), and parasitism by bacteriophages. The role of these agents under natural conditions still is not certain. However, protozoa feed avidly on rhizobia, but only when the prey population is higher than 10^6 cells/g of soil, whereas at lower rhizobial populations, the bacteria may reproduce at a rate sufficient to replace the cells that are destroyed by the predators (5, 6).

Drying is considered among the abiotic factors affecting *Rhizobium* survival in soil, and published data suggest a difference exists among species of *Rhizobium* in regard to their drought susceptibility. For example, Bushby and Marshall (1) observed that fast-growing strains of *Rhizobium* declined four orders of magnitude during the drying of a sandy soil, but the slow-growing strains declined by only two orders of magnitude. The present work was designed to establish quantitatively the effect on *Rhizobium* of the slow drying characteristic of soils in the field. Use was made of antibiotic-resistant strains whose populations in nonsterile soil could be estimated accurately even following marked declines in rhizobial populations. Emphasis was placed on the effect of constant P_w values of the soil. P_w is the grams of water in 100 g of oven-dry soil, expressed as a percent.

MATERIALS AND METHODS

Strains of *Rhizobium leguminosarum* 178, *R. trifolii* 412, *R. phaseoli* 391, *R. phaseoli* K17, *R. meliloti* 92, *R. japonicum* 382, *R. lupini* 306, *R. lupini* 386, and *Rhizobium* 393 of the cowpea group resistant to streptomycin were obtained by the method of Danso et al. (3). The nodulating capacity of the streptomycin-resistant strains was verified by testing them on their specific host plants. The bacteria were grown in yeast extract-mannitol (YEM) broth at 30°C on a rotary shaker (150 rpm) either for 4 days for *R. leguminosarum*, *R. phaseoli*, *R. trifolii*, and *R. meliloti*, or for 7 days for *R. japonicum*, *R. lupini*, and *Rhizobium* 393. The cells were collected by centrifugation at 4°C and washed 3 times in phosphate buffer (0.08% K_2HPO_4 and 0.02% KH_2PO_4).

The soil used, Lima silt loam, had a pH of 7.2, contained 4.2% organic matter, and had water contents of 25.5%, 21.2%, and 18.3% at 1/3, 1.0, and 10 bars of tension, respectively. The soil was collected from the top 15-cm, and it was passed through a 2-mm sieve before use. For the studies of survival, 1.0 ml of a washed cell suspension was added to 10 g of air dried soil contained in 125-ml bottles. The bottles were incubated horizontally at 30°C, and triplicate bottles were sampled at intervals. The number of surviving rhizobia was determined by plating serial dilutions of the soil on YEM agar containing 1.0 mg of streptomycin sulfate (Calbiochem, La Jolla, Calif.) and 300 μg of actidione/ml (ICN, Pharmaceutical, Inc., Cleveland, Ohio). The antibiotics were sterilized by filtration.

To determine whether allowing the rhizobia to grow in soil prior to desiccation affected their resistance, soil that had been autoclaved on 3 consecutive days was inoculated with 1.0 ml of a washed cell suspension. The soil was maintained at field capacity (P_w 25.5%) for 3 weeks to allow for growth of the rhizobia, and then, as the soil was allowed to dry out, plate counts were made.

An experiment was conducted to determine if the addition of the polysaccharide from a slimy strain of *R. trifolii* would change the behavior of *Rhizobium* during desiccation. The polysaccharide was isolated from the supernatant fluid after centrifugation of cells by the method of Humphrey and Vincent (7). Washed cells suspended in buffer or in the polysaccharide solution were inoculated into Lima silt loam, and the survival during desiccation was then evaluated.

For studies involving rapid drying, air was first passed over silica gel to absorb moisture and then through cotton, and finally this air was directed through a pasteur pipette to the bottom of inoculated soil to promote rapid drying. Plate counts were made at regular intervals.

To investigate the effect of constant P_w on survival, 90 bottles of Lima silt loam were inoculated with *R. leguminosarum* 178 and *R. japonicum* 382. Eighteen of the bottles were transferred immediately to a humidifier so that the initial P_w was retained. The soil samples in the remaining bottles were allowed to begin to dry. After 1 day and additional periods as appropriate, 18 of the bottles were transferred to another humidifier. Plate counts as well as moisture determinations of the soil were made of the samples being incubated in the humidifiers, which were glass desiccators containing water at the bottom.

RESULTS

All isolates of *Rhizobium* resistant to high concentrations of streptomycin were tested for their capacity to nodulate the host plant of the parent culture. Only one isolate of *R. phaseoli* failed to induce nodulation, and it was discarded. The rest of the strains retained

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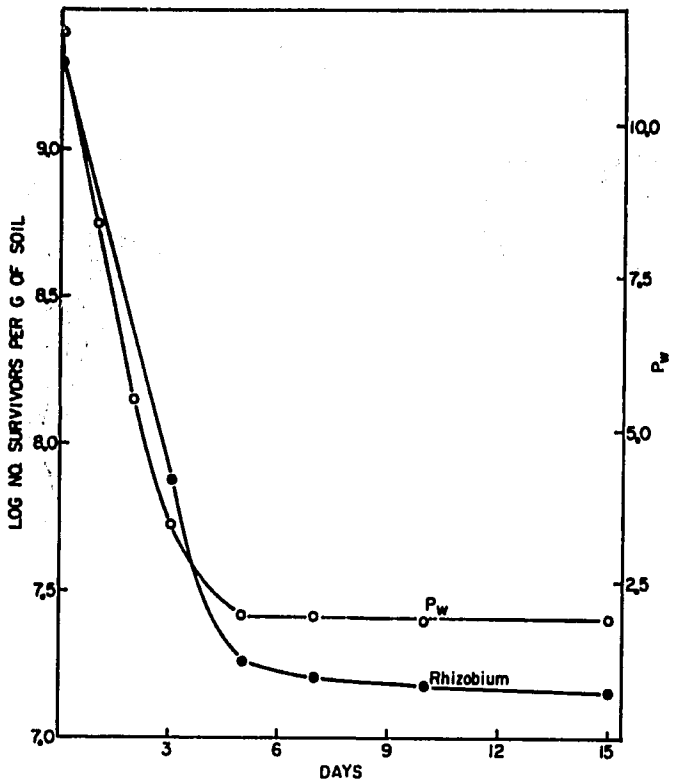

Fig. 1—Survival of *Rhizobium leguminosarum* 178 and moisture content of Lima soil undergoing drying.

their infectiveness. Sometimes, other soil microorganisms grew in the medium used for counting the rhizobia surviving in soil, but the capacity to nodulate was then used to confirm that a given survivor was indeed *Rhizobium*.

The root-nodule bacteria exhibited a consistent pattern of behavior as soil was desiccated (Fig. 1). The data here presented for *R. leguminosarum* are typical of the rhizobia. Two distinct phases were evident. In the first phase, the number of cells fell rapidly and extensively, and the rate was essentially exponential. In the second phase, the rate of bacterial decline was much slower. The first phase coincided with the loss of water from the soil, but the small decline in the rhizobial population in the second phase, which is evident in a linear plot but is made somewhat obscure in a logarithmic plot of the data, coincided with the time that the soil had reached a dry stage. The decline in numbers is not a result of the antibiotics in the agar killing drought-stressed survivors; thus, in studies in which rhizobia were dried in sterile soil, the numbers of survivors were not significantly different when counted in media with or without antibiotics.

Members of each species of *Rhizobium* were added to Lima silt loam, which was then allowed to dry in air. Under these circumstances, the patterns of population decline were the same as for *R. leguminosarum*, but the percentages of the original population that survived were somewhat different among the bacteria tested (Table 1). The values given, which are the means of triplicate determinations, are for incubations for 10 days at 29°C, but the same relative degrees of resistance were noted after 21 days. These data show that the population of *Rhizobium* was reduced at least

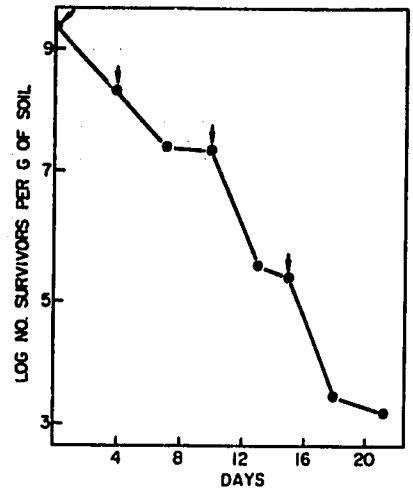

Fig. 2—Effect of several cycles of wetting and subsequent drying on the survival of *R. japonicum* in Lima silt loam. The arrows indicate the time when the soil was moistened and then a drying cycle initiated. Means are significantly different (95% level) if they differ by a factor of 1.16.

two orders of magnitude by a simple drying cycle. Because only a single strain of each species was used, it is not clear whether the differences in resistance are characteristic of individual strains or species. Nevertheless, it is evident that no clear difference in susceptibility to desiccation was found between strains of *Rhizobium* that belong to the fast- or the slow-growing group, in contrast with the findings of Bushby and Marshall (1, 2), who reported that the former were more susceptible.

To test the effects of additional wetting-drying cycles, Lima silt loam was inoculated with *R. japonicum* and allowed to dry. At three time intervals thereafter, water was added to bring the soil to a P_w level of 12%, and then the soil was allowed to dry out again. It is evident from Fig. 2 that the rhizobial population underwent a further decline when the soil was rewetted and then allowed to dry again. The moisture added was not sufficient to allow the cells to reproduce to a detectable extent, but its addition did allow for the subsequent death when the soil dried out. It is also evident that the pattern of survival on additional wetting/drying cycles was like the first, the population declining rapidly when the soil was losing water but remaining at a more constant value as the soil reached dryness.

To determine whether growth of the bacteria in soil affected their resistance to desiccation, two strains of *Rhizobium* were inoculated into sterile, autoclaved Lima silt loam. The bacteria were allowed to grow,

Table 1—Survival of rhizobia in Lima loam undergoing drying.

Species	Initial no. of cells ($\times 10^6$/g of soil)	% survivors after 10 days†
R. trifolii 412	800	0.92 a
R. meliloti 92	1,620	0.90 a
R. leguminosarum 178	1,820	0.84 a
R. japonicum 382	1,520	0.50 b
Rhizobium 393 (cowpea group)	1,980	0.47 bc
R. lupini 306	1,590	0.41 c
R. phaseoli 391	1,790	0.39 c

† Values followed by the same letter are not significantly different at the 95% level.

Table 2—Resistance to desiccation of *R. meliloti* and *R. lupini* after growing in soil.

Days after inoculation	No. × 10⁶/g of soil	
	R. meliloti 92	*R. lupini* 306
0	8.3	5.9
7	236	106
14	1,140	560
21†	1,720	660
24	283(16.5)‡	155(23.5)
26	160(9.3)	124(18.8)
31	36(2.1)	19.6(3.0)

† Beginning on day 21, the soil was allowed to dry.
‡ The numbers in parentheses indicate survivors as a percent of the counts at day 21.

and the soil was incubated for 3 weeks at 29°C. The moisture was maintained at a P_w value of 25.5%. The soil was then allowed to dry in air. The data in Table 2 show that allowing the organisms to grow in the soil rather than in solution appeared to increase their capacity to survive during the drying period Under these conditions, 2.1 and 3.0% of the cells of *R. meliloti* and *R. lupini*, respectively, survived, whereas in earlier experiments in which the bacteria did not grow in soil, only 0.90 and 0.51% of the cells of these two species survived.

In the investigations of Bushby and Marshall (1), the soil was dried rapidly by placing samples in a forced-draft oven, a treatment that might not mimic the rate of natural water loss. A study was thus conducted to establish the effect of rate of drying on the survival of *R. japonicum*. Rapid drying was achieved by passing dry air through the soil, and slow drying was the same procedure used before. It is evident from the results in Table 3 that the rapid drying did cause an appreciable change in the rate of decline of the rhizobial population. In the soil that lost water rapidly, the same two phases of the typical survival curve were observed, and the final population size in this soil sample was not appreciably different from that in the soil in which drying proceeded slowly. Such findings suggest that it is the drying process itself and not its length that causes the reduction in numbers.

A study was conducted to assess the effect of plant residues on the decline during the period of water loss. In this instance, fine quartz sand (not soil) with or without finely ground alfalfa residues (3% by weight) was used, and the initial counts were 1.12 to 1.32 × 10^9/g. After 10 days the percentage survivors was 0.77 and 1.98% for *R. leguminosarum* and 0.74 and 0.75% for *R. japonicum* in the absence and presence of the residues, respectively. Each value represents the mean of triplicate determinations, and values different by more than a factor of 1.23 are significantly different

Table 4—Survival in Lima silt loam of *Rhizobium trifolii* 412 added in suspensions of the bacterial polysaccharide.

Days	No. of cells × 10⁶/g of soil†	
	No polysaccharide	With polysaccharide
0	7.70	3.60
3	0.101(1.3)‡	0.123(3.4)
6	0.072(0.94)	0.091(2.5)
10	0.037(0.48)	0.074(2.1)

† Each value represents the mean of 3 replicates. Means are significantly different (95% level) if they differ by a factor of 1.18.
‡ The numbers in parentheses refer to percent of survivors.

Table 3—Effect of rate of drying on the survival of *R. japonicum* 382 in soil.†

Slow drying			Rapid drying		
Days	No. × 10⁶/g of soil	% survivors	Hours	No. × 10⁶/g of soil	% survivors
0	750	–	0	750	–
1	268	35.7	2	208	27.7
2	150	20.0	4	75	10.0
3	17.0	2.3	8	43	5.7
5	16.0	2.1	24	12	1.6
10	10.8	1.4	48	7.0	0.93

† Each value represents the mean of 3 replicates. Two means are significantly different (95% level) if they differ by a factor of 1.17.

at the 95% level. These data thus demonstrate that fresh plant remains have little or no protective effect.

The effect of decaying organic residues on the survival of *R. leguminosarum* was investigated. In this instance, fine quartz sand containing finely ground alfalfa residues (3% by weight) was inoculated with 0.01 g of soil. The mixture was moistened, incubated for 2 months at 29°C to permit decomposition, and then dried and inoculated with *R leguminosarum*. The initial counts were 1.84 × 10^9 and 2.12 × 10^9/g of sand and sand plus decayed residues, and 0.40 and 0.66% survivors, respectively, were found after 3 days. Hence, no appreciable protection was afforded by the decayed residues.

Much of the polysaccharide produced by *Rhizobium* during its growth in culture medium is removed during harvesting and washing of the cells, and hence the cells added to soil may not have the quantity of polysaccharide characteristic of the organism. When cells were suspended in their own polysaccharide and then inoculated into soil, their survival was improved to some extent (Table 4).

A comparison was made of the survival of *R. japonicum* added to four soils. The percentage of cells surviving the drying, whether the initial number of bacteria added was large or small, was similar in each soil (Table 5). However, a higher percentage of the inoculum remained viable in the Arkport and Lima soils, the pH values of which were higher and the organic matter levels of which were lower than the two soils in which fewer rhizobia survived.

The effect of a constant, relatively low P_w of soil on the survival of *R. leguminosarum* and *R. japonicum* is shown in Fig. 3. It is evident that survival at P_w

Table 5—Survival of *R. japonicum* 382 introduced into four soils at two cell densities (incubation for 10 days).†

Soil	Soil pH	Organic matter content,%	High initial density		Low initial density	
			No. × 10⁶/g of soil‡	% survivors	No. × 10⁶/g of soil‡	% survivors
Arkport fine sandy loam	6.2	1.5	1,130	1.7	71	2.28
Dalton silt loam	5.8	6.5	1,210	0.62	87	0.65
Hudson silty clay loam	5.9	5.3	1,120	0.92	85	0.63
Lima silt loam	7.2	4.2	1,290	2.6	100	2.43

† Each value represents the mean of triplicate determinations. Means are significantly different (95% level) if they differ by a factor of 1.29.
‡ Initial count.

of 11.3% is quite good, since about 30% of the initial population was still viable after 12 days. In contrast, only 0.4% were viable after 7 days in air-dried soil (P_w of 2.0%). When the P_w of the soil was reduced from its initial to lower values, the cells died at different rate constants, even though the soil was not air dry and its water content was kept constant.

Linear regressions of the logarithm of the number of survivors as a function of time were calculated for each survival curve at the different P_w values studied. The values for all counts that were made after the soil was placed at constant P_w were included in the regressions. The slopes of such curves were interpreted as the rate constants of dying. The rate constants were derived from the equation

$$N = N_o e^{-k'\Delta t}$$

where N is the number of survivors after a given time, N_o is the initial number of cells, k' is the rate of decline in percentage survivors per day, and t is time in days. Therefore, $\ln N = \ln N_o - k't$. These slopes are presented in Table 6. It can be seen that the slopes for the death rate constant increase as P_w increases to a maximum and then decrease again such that the slope at P_w of 11.3 and at P_w of 2.0 are similar. In general, the rate constants for death of *R. japonicum* were less than those obtained for *R. leguminosarum*. Because growth of the rhizobia may have occurred in some instances, the decline rates associated with drying may be even greater than those reported.

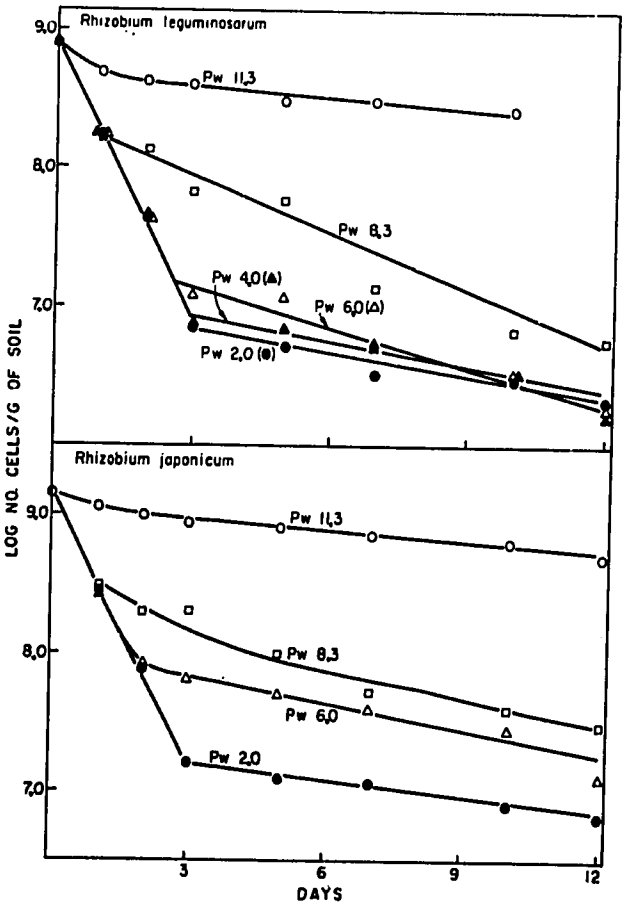

Fig. 5—Survival of *Rhizobium leguminosarum* (top) and *R. japonicum* (bottom) in Lima silt loam at different P_w values.

DISCUSSION

The data demonstrate that populations of *Rhizobium* initially fall markedly as soils undergo drying, but the rapid decline is followed by a period when a far smaller percentage of cells lose viability. A similar pattern of survival has been reported for *Rhizobium* applied to glass beads or seeds undergoing drying (10). Although it might seem that the slower rate of decline results from the existence of cells more resistant to desiccation than those that initially died, the results reported here indicate that this was not the case; the progeny of cells that had survived for 3 months in desiccated soil were not appreciably more resistant to drying in soil. Thus, intrinsic resistance to desiccation is not involved in the persistence of part of the population.

No significant difference in susceptibility to desiccation was found between the fast- and slow-growing rhizobia. These findings are in contrast with the report of a greater susceptibility to desiccation of the fast-growing group of rhizobia compared with the slow-growing rhizobia (1). The slow drying used in the present study, however, would appear to mimic more realistically the conditions in nature than the fast-drying technique used in those studies, but the reasons for the differences in the two studies are still obscure. It has been suggested that a low water content at low relative vapor pressure values probably enhances the survival of bacteria by reducing the activity of those enzymes capable of functioning at relatively low water activities (2).

The somewhat greater resistance of root nodule bacteria previously grown in sterile soil as compared to the same strain grown in culture medium and then inoculated into soil may be related to a protection of cells in microcolonies in the soil. The cells of the inner part of a microcolony are possibly better protected against desiccation than cells at the outside of a microcolony or those existing individually. In cells inoculated into soil and afterwards exposed to desiccation, such a mechanism of protection would not be operative.

The rate constants of dying were found to depend on the water content of the soil. At a P_w of 11.3%, the rhizobia were not significantly affected either by the low moisture level or by predators and parasites. Although protozoa destroy rhizobia in soil (5, 6), their predatory activity may have been restricted by the little available water or the reproduction of the rhizobia may have kept pace with feeding of the protozoa. On

Table 6—Effect of stable P_w on the rate constant of death in Lima loam.

P_w	Rate constant†	
	R. leguminosarum	*R. japonicum*
2.0	12.0	9.0
4.0	23.9	-‡
6.0	25.4	16.0
8.3	31.3	20.0
11.3	10.1	7.0

† Expressed as percent.
‡ Not determined.

the other hand, as the moisture level in the soils fell, the population of the root-nodule bacteria dropped abruptly. Yet, once the moisture level was maintained at a reasonably constant value, the period of precipitous decline ended and was replaced by a slow rate of dying, the rate of which was a function of P_w. It is possible that the rapid followed by a slow decline reflects the existence of two mechanisms for killing, but it is presently not clear what these mechanisms might be.

LITERATURE CITED

1. Bushby, H. V. A., and K. C. Marshall. 1977. Some factors affecting the survival of root-nodule bacteria on desiccation. Soil Biol. Biochem. 9:143–147.
2. Bushby, H. V. A., and K. C. Marshall. 1977. Water status of rhizobia in relation to their susceptibility to desiccation and to their protection by montmorillonite. J. Gen. Microbiol. 99:19–27.
3. Danso, S. K. A., M. Habte, and M. Alexander. 1973. Estimating the density of individual bacterial populations introduced into natural ecosystems. Can. J. Microbiol. 19: 1450–1451.
4. Danso, S. K. A., S. O. Keya, and M. Alexander. 1975. Protozoa and the decline of *Rhizobium* populations added to soil. Can. J. Microbiol. 21:884–895.
5. Habte, M., and M. Alexander. 1978. Mechanisms of persistence of low numbers of bacteria preyed upon by protozoa. Soil Biol. Biochem. 10:1–6.
6. Habte, M., and M. Alexander. 1978. Protozoan density and the coexistence of protozoan predators and bacterial prey. Ecology 59:140–146.
7. Humphrey, B. A., and J. M. Vincent. 1959. Extracellular polysaccharides of *Rhizobium*. J. Gen. Microbiol. 21:477–484.
8. Parker, C. A., and P. L. Grove. 1970. *Bdellovibrio bacteriovorus* parasitizing *Rhizobium* in Western Australia. J. Appl. Bacteriol. 33:253–255.
9. Sardeshpande, J. S., R. H. Balasubramanya, J. H. Kulkarni, and D. J. Bagyaraj. 1977. Protozoa in relation to *Rhizobium* S-12 and *Azotobacter chroococcum* in soil. Plant Soil 47:75–80.
10. Vincent, J. M., J. A. Thompson, and K. O. Donovan. 1962. Death of root-nodule bacteria on drying. Aust. J. Agric. Res. 13:258–270.