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Growth of *Rhizobium* in Unamended Soil

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ABSTRACT

A study was conducted to determine the conditions in unamended soil that favored growth of *Rhizobium*. Moistening a dry soil resulted in growth of *R. japonicum* and a strain of *Rhizobium* nodulating cowpeas but not of *R. meliloti*. The extent of decline resulting from desiccation was usually greater than the increase following wetting. The extent of growth varied among soils. A freezing-thawing cycle did not promote growth of *R. phaseoli* and the cowpea *Rhizobium*. The latter bacterium was more tolerant to a freezing-thawing cycle than the former. *Rhizobium japonicum* and *R. phaseoli* grew readily in the presence of germinating seeds and developing root systems of soybeans, kidney beans, red clover, cowpeas, oats, wheat, and corn, the population size varied among the plant species, and legumes were no more stimulatory than nonlegumes. The numbers of *R. phaseoli* in the rhizosphere of several plants declined in 1 week, the abundance of *R. japonicum* fell after 6 weeks in the soybean rhizosphere, but the population size of the cowpea *Rhizobium* was high for at least 20 weeks. Counts of *R. japonicum* in soil increased after the soil was amended with soybean nodules. It is suggested that appreciable rhizobium growth in unamended soil occurs only in the presence of germinating seeds, growing roots, and decomposing nodules.

Additional Index Words: ecology, rhizosphere, seed stimulation, drying.

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ALTHOUGH rhizobia are common inhabitants of soil, evidence that they grow in this environment is scanty. This is surprising because of the importance of these bacteria to the growth of legumes and the nitrogen economy of many soils. However, Chowdhury (4) demonstrated that two species of *Rhizobium* responded to carbon and nitrogen additions to soil, but no growth was evident in unamended, nonsterile soil. Chatel et al. (2, 3) showed that *R. lupini* persisted longer and was a better colonizer of sandy soils than *R. trifolii*, and they found considerable differences in the ability of strains of *R. trifolii* to colonize soils. Growth rates in soil were determined by Bohlool and Schmidt (1) and Schmidt (16), who estimated that the doubling times of a strain of *R. japonicum* in two soils were 240 and 365 h. Growth of *Rhizobium* also occurs in the rhizosphere of legumes as well as nonleguminous plants (15), and Tuzimura and Watanabe (20) showed that rhizobia of different cross-inoculation groups were present in the rhizosphere of plants the bacteria could not infect. On the other hand, *R. meliloti* has been reported to be unable to colonize the clover rhizosphere (14).

The present study was designed to establish factors governing the growth of *Rhizobium* in soil. Consideration was given to circumstances possibly leading to increases in the availability of nutrients for the growth of rhizobia, specifically drying and wetting, freezing and thawing, and the presence of roots.

MATERIALS AND METHODS

The soils used were Lima silt loam (fine loamy, mixed, mesic Glossoboric Hapludalf, pH 7.2, 4.2% organic matter) from Aurora, N.Y., Williamson silt loam (coarse silty, mixed, mesic Typic Fragiochrept, pH 6.3, 2.9% organic matter) from Ithaca, N.Y., an unclassified histosol (pH 6.2, 31% organic matter) from Seneca Falls, N.Y., Holopaw sand no. 1 (loam, siliceous, hyperthermic Grossarenic Ochraqualf, pH 6.8, 0.62% organic matter) from a pasture in Citra, Fla., and Holopaw sand no. 2 (pH 6.4, 6.4% organic matter) from a hardwood forest in Citra, Fla. The soils were collected from the surface 15 cm and passed through a 2-mm sieve.

Spontaneous mutants of *R. japonicum* ATCC 10324, *R. meliloti* 3DOa20, and *Rhizobium* S57, the last being an isolate from Trinidad that nodulates cowpeas, resistant to 1.0 mg of streptomycin and 50 μg of erythromycin per mL were obtained by the method of Danso et al. (5). *Rhizobium phaseoli* 127K17, which was obtained from C. Ramirez Martinez, was already resistant to these antibiotics. The bacteria were grown in yeast extract mannitol (YEM) broth or agar (21). To count *Rhizobium*, YEM agar was amended with 5 μL of 95% ethanol containing 0.5% bromthymol blue, 1.0 mg of streptomycin sulfate, 50 μg of erythromycin, 300 μg of cycloheximide, and 100 μg of nystatin per mL.

To study the effect of drying and subsequent wetting, the bacteria were added to 10-g portions (dry weight) of Lima silt loam contained in 120-mL milk dilution bottles. The soil was allowed to dry at 29°C with the bottles in a horizontal position. After 21 d, the soil was moistened with 2.0 mL of sterile water, and the bottles were closed with a rubber stopper and incubated vertically for 3 to 4 d. Counts of rhizobia were performed on triplicate soil samples. A similar procedure was used for other soil types, but the length of the dry period was 10 d and 9.0 mL of water was added to the histosol.

Corn (*Zea mays* L.), oats (*Avena sativa* L.), wheat (*Triticum aestivum*), cowpeas (*Vigna unguiculata* [L.] Walp.) (var. Whippoorwill), kidney beans (*Phaseolus vulgaris* L.) (var. Red Kloud), red clover (*Trifolium pratense* L.) (var. Arlington), and soybeans (*Glycine max* [L.] Merr.) (var. Corsoy) were used to study the response of *R. phaseoli* and *R. japonicum* to germinating seeds and growing roots. Lima silt loam (30 g) contained in a plastic container (6 containers/plant species), 20 cm top diameter and 20 cm in height, was thoroughly mixed with a cell suspension of *Rhizobium*, and the moisture content was adjusted to 23% (wt/wt) with sterile water. The surfaces of the seeds were sterilized by the method of Vincent (21), and 10 seeds were planted in each container at a depth of 2 cm. The soils were incubated at 24 to 26°C, and counts were made of three seedlings at regular intervals. Before making the dilutions, the plant material was shaken for 1 min in a solution containing 0.08% K_2HPO_4 and 0.02% KH_2PO_4. The root system and adhering soil were dried at 105°C for 24 h, and counts are expressed per unit weight of dry material. Counts of bacteria in soil are also expressed on the basis of dry weight.

To study the duration of the rhizosphere effect, cowpeas and soybeans were grown in the summer in the greenhouse under natural light. Lima silt loam mixed with vermiculite (9:1, v/v) was added to plastic pots (23 cm top diameter, 21 cm in height), 2.0 kg/pot, and inoculated with a cell suspension of the ho-

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mologous *Rhizobium*. The inoculum was thoroughly mixed in the soil using a dry blender (Patterson Kelly Co., East Stroudsburg, Pa.), and two surface-sterilized seeds were planted in each pot. The temperatures were 27°C during the day and 21°C at night. At regular intervals, the root systems in three pots selected at random were removed and gently shaken for 1 min in a solution containing 0.08% K_2HPO_4 and 0.02% KH_2PO_4, and then counts were made.

To assess the effect of defoliation, cowpeas were grown for 4 weeks in Lima silt loam that had been inoculated as described above. The aerial portions of some of the plants were then cut off. At regular intervals thereafter, counts were made of the antibiotic-resistant rhizobia on the root systems in three pots of soil using both defoliated and nondefoliated plants.

In studies of the survival of rhizobia derived from nodules, approximately 5 g of fresh nodule tissue from 4-week-old soybean plants was crushed with a mortar and pestle in 50 mL of a sterile solution containing 0.08% K_2HPO_4 and 0.02% KH_2PO_4. This homogenate (1.0 mL) containing the antibiotic-resistant *R. japonicum* was added to bottles containing moist Lima soil or sterile sand equivalent to 10 g of dry material. The moisture content of the soil and sand after inoculation was 23 and 12% (wt/wt), respectively. The bottles were incubated at 29°C, and counts were made on triplicate bottles at different times. A similar procedure was used in tests with uncrushed soybean nodules, in which case nodule tissue or approximately three nodules (containing about 0.29 g of tissue) were added per bottle.

Table 1.—Effect of drying and wetting of Lima silt loam on *Rhizobium*.

No. of cells × 10^3/g soil

Day	*R. japonicum*	Cowpea *Rhizobium*	*R. meliloti*	*R. phaseoli*
0	830	480	1,100	300
21	2.7	36	1.5	<0.1
22	14	570	0.93	<0.1
23	12	360	0.67	<0.1
24	9.3	310	0.50	<0.1

Table 2.—Growth of cowpea *Rhizobium* in soils exposed to a drying and wetting cycle.

No. of cells × 10^3/g soil

Day	Williamson silt loam	Histosol	Holopaw sand No. 1	Holopaw sand No. 2
10	1.0	7.5	0.70	2.3
11	3.5	9.5	1.5	18
12	4.7	7.9	1.6	12

RESULTS

Drying and subsequent rewetting of soil had different effects on the four rhizobia. The dry soil was remoistened on day 21, at which time *R. japonicum* and the cowpea *Rhizobium* grew, the former increasing in number about fivefold and the latter about 15-fold in the 1st day after the dry soil was moistened (Table 1). This higher count was then maintained in the moist soil. In contrast, no growth of *R. meliloti* was evident when the soil was remoistened. *Rhizobium phaseoli* was particularly sensitive to desiccation, and the number of cells surviving the drying period was below the sensitivity limits of the counting procedure.

Soils varying in organic matter content were inoculated with the cowpea *Rhizobium* (10^6 cells per g) and allowed to dry. On day 10, water was added to bring the soil to field capacity. An appreciable increase in cell number was not detected in the histosol or in the sample of Holopaw sand (no. 1) with the low organic matter content (Table 2). However, the numbers rose by more than three- and sevenfold in Williamson silt loam and Holopaw sand no. 2, respectively (significant at $\alpha = 0.05$), these soils having organic matter contents of 6.3 and 6.4%, respectively.

A study was conducted of the effect of freezing and thawing because freezing-thawing cycles increase the availability of organic nutrients in soil (18). A sample of Lima silt loam was mixed with the inoculum, stored at −29°C for 14 d, and then incubated at 20°C. Under these circumstances, no increase in cell number was detected with the two test rhizobia (Table 3). It is evident, however, that the cowpea *Rhizobium* was more resistant to the freezing-thawing stress than *R. phaseoli*.

In tests of the influence of germinating seeds and emerging roots on *Rhizobium*, the initial counts were 2.5

Table 3.—Effect of freezing and thawing Lima silt loam on *Rhizobium*.

No. of cells × 10^3/g soil

Day	Cowpea *Rhizobium*	*R. phaseoli*
0†	480	310
14	290	0.60
15	77	<0.1
16	32	<0.1

† Before freezing. Subsequent counts were after soil had thawed.

Table 4.—Growth of *Rhizobium* in the presence of germinating seeds and emerging roots of different plants.†

Plant	No. of cells of *R. phaseoli* × 10^3/g of root and adhering soil				No. of cells of *R. japonicum* × 10^3/g of root and adhering soil			
	Day 2	Day 3	Day 5	Day 7	Day 2	Day 3	Day 6	Day 7
Red clover	26	79	22	0.47	35	59	66	330
Oats var. Garry	3.5	2.1	0.81	0.27	5.5	5.1	8.7	41
Oats var. Astro	1.3	2.0	0.33	8.3	240	13	22	79
Cowpea	3.1	2.0	17	18	2.2	2.2	6.8	20
Wheat var. Ticonderoga	4.3	4.0	0.23	0.30	4.0	3.5	7.6	18
Wheat var. Houser	1.4	1.3	3.2	1.8	1,400	1,500	150	63
Kidney beans	26	1.5	0.23	0.33	310	320	380	3.7
Soybeans	1.6	1.6	4.6	5.0	1.4	15	23	160
Corn var. Dominican Republic	21	17	8.5	4.0	10	11	12	2.1
Corn, line cross A632X634	1.5	2.7	24	5.6	420	400	410	130
Corn var. Cornell 281	20	17	26	3.0	740	620	630	460
None (soil only)	0.43	0.30	0.55	0.42	3.1	2.8	5.0	5.8

† Two means are significantly different ($\alpha = 0.05$) if one is 13 times larger than the other for *R. phaseoli* and 17 times for *R. japonicum*.

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$\times 10^3$ cells of *R. phaseoli* and 4.1 $\times 10^4$ cells of *R. japonicum* per g of soil. Seed germination of all plants was evident 3 d after planting. The germinating seeds and developing roots brought about considerable rhizobial growth (Table 4). Most of the increase in population size took place in the first 2 d. In many instances and especially with *R. phaseoli*, a decline in numbers in the rhizosphere was apparent after several days. This decline was sometimes quite large. The legumes as a group and even the homologous host legumes were not more stimulatory than the nonlegumes.

The results of a study of the duration of the root stimulation by soybeans and cowpeas are presented in Table 5. Cell densities of about 10^5 cells per g of dry soil plus roots were reached in the 1st week for both rhizobia. Thereafter, the populations of both species increased slowly with time. *Rhizobium japonicum* declined in abundance after about week 6, and this decline continued until a small number of viable cells remained by week 15. The decrease in rhizobial cells in the soybean rhizosphere preceded the decline in number of nodules. By week 13, senesced nodules represented about 75% of the total nodules, but young, small, pink nodules were also present throughout the root system; at this time, all the leaves were chlorotic, and the pods had become brown and dry. The cowpea *Rhizobium*, on the other hand, did not decline appreciably in abundance, and its population remained relatively constant from week 5 to 15; at week 20, the pods and 60% of the leaves were dry and brown, and the total number of nodules had declined even though the rhizobia were still abundant. However, the insides of most nodules were green, and only a few were still pink at week 20.

Table 5—Duration of the rhizosphere effect of soybeans and cowpeas.

	No. of cells × 10^6/g of roots and adhering soil		No. of nodules per plant	
Week	*R. japonicum*	Cowpea *Rhizobium*	Soybeans	Cowpeas
0	2.7	1.2	ND†	ND
1	100	89	ND	ND
2	130	16	ND	ND
3	240	24	ND	ND
4	520	280	62 ± 11‡	ND
5	640	1,400	96 ± 21	ND
6	580	1,800	87 ± 7	180 ± 13
10	7.9	ND	98 ± 20	ND
13	4.4	1,200	25 ± 12	165 ± 12
16	0.76	2,900	11 ± 5	250 ± 47
20	ND	630	ND	75 ± 15

† Not determined
‡ Mean ± SE

Table 6—Effect of cutting the aerial part of cowpeas on the population of cowpea *Rhizobium* in the rhizosphere.

	Aerial part removed		Aerial part not removed	
Days after cutting	No. of cells × 10^6/g of roots and adhering soil	Nodules per plant†	No. of cells × 10^6/g of roots and adhering soil	Nodules per plant†
0	290	180 ± 10	320	191 ± 25
4	740	157 ± 12	1,700	168 ± 16
7	1,500	60 ± 14	2,500	173 ± 19
12	2,400	2 ± 1	1,300	180 ± 13
18	1,700	0.5 ± 0.7	2,600	165 ± 12

† Mean number per plant ± SE

After the above-ground part of 4-week-old cowpeas were removed, the rhizobial population in the rhizosphere increased (Table 6). The population was maintained at a high level for a period of at least 18 d after removal of the aerial parts. The number of nodules, on the other hand, was appreciably reduced by removing the foliage. This decline in number of nodules was pronounced after the 4th day following cutting.

After adding soybean nodules to soil or sterile sand, *R. japonicum* proliferated and reached large numbers (Table 7). This population was still large at day 30. Following the addition of a nodule homogenate to nonsterile soil, however, the population declined until only 1% of the initial number remained at day 30. In contrast, much better survival was observed when the homogenate was added to sterile sand.

DISCUSSION

Because organic carbon in a form readily available to bacteria presumably is provided in soil by drying and subsequent wetting, freezing and thawing, and root excretions, experiments were conducted to determine whether the nutrients thus made available could be used to support the proliferation of *Rhizobium*. Growth of certain rhizobia occurred when the soil was moistened after a period of desiccation. It has been reported that the amount of nutrients made available during cycles of drying and wetting depends on the organic matter content of the soil and on the length of the drying period (17, 18). However, following drying and rewetting, *Rhizobium* was not found to grow significantly in the soil with the highest organic matter content. As a result of drying, moreover, not only is there presumably a greater availability of nutrients resulting from changes in the organic fraction of the soil, but the surviving rhizobia face a microflora somewhat smaller in numbers (19). Nevertheless, the potential for growth of *Rhizobium* in remoistened soils, even with the greater availability of substrates and reduced number of competitors, should be interpreted with some caution because the magnitude of the decline in the rhizobial populations during desiccation may be greater than the increase in numbers after the soil is moistened. The marked decline in rhizobia as soils dry is well established (9, 11).

Freezing and subsequent thawing, which also increase the amount of readily available carbon in soils (18), were not associated with increases in *Rhizobium* numbers. However, the declines occurring as a result of this stress were different for the two rhizobia tested.

Growth in the presence of germinating seeds or root systems of even nonleguminous plants was appreciable. Large numbers of rhizobia have previously been noted

Table 7—Numbers of *Rhizobium japonicum* in Lima silt loam or sterile sand amended with intact soybean nodules or nodule homogenates.

	No. cells × 10^6/g of soil or sand			
	From whole nodules		From nodule homogenates	
Day	Soil	Sterile sand	Soil	Sterile sand
0	0.010	0.016	100	100
3	0.12	0.17	140	120
10	0.61	1.0	18	180
20	0.43	5.7	8.9	170
30	0.48	7.9	1.0	150

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in the rhizosphere (15, 20). In fact, it has been proposed to use the rhizosphere effect of nonleguminous crops to enhance the establishment of a given strain of *Rhizobium* in soils not favorable to the colonization of *Rhizobium* (6). The two species of *Rhizobium* tested reached similar population levels, but the population of *R. phaseoli* often declined about the 3rd or 4th day after planting. This decline has been attributed to predation by protozoa in the rhizosphere of the host legume (8, 12). The data herein show that the duration of the stimulation by roots differs with different plant-rhizobia combinations. Thus, the rhizobial density in association with cowpea roots was maintained at a high level for a long time, whereas *R. japonicum* declined markedly as the plant aged. Reyes and Schmidt (13) also noted significant declines in the *R. japonicum* populations in the soybean rhizosphere as harvest time approached.

Nodules represent a potential source of rhizobia in soil. However, the time and pattern of nodule growth, decay, and shedding are not the same for all legumes. For alfalfa, the nodules remain attached to the root system, and their growth occurs after each clipping of the crop. In clover, in contrast, the nodules disappear after clipping, and new ones appear further down the root system. In soybeans, the nodules usually decay as the plants senesce (22). The loss of nodules may result in increases in the rhizobial populations in soils, as shown here. Wilson (23) also considered the nodule as a significant source of rhizobia in soil, and Paau et al. (10) proposed that persistent infection threads of senescing nodules may provide rhizobia to allow for perpetuation of the species. In the present study, it was observed that additions to soil of *Rhizobium* with intact nodules led to increases in the rhizobial population, whereas additions of a nodule homogenate resulted in a significant decline of the population. This decline probably occurred because protozoa reduced the large numbers in the homogenate and had no effect on the small numbers derived from nodules. However, the sizes of the final populations in soil were not appreciably different, whether they were derived from the homogenate or intact nodules.

Defoliation appears to be a significant factor in nodule longevity of many legumes (7). However, when the foliage of nodulated cowpeas was removed, the rhizobial population in the rhizosphere did not fluctuate appreciably, despite the marked reduction in nodule number. It is possible that the addition to soil of the rhizobia from nodules served to balance the number of bacteria in the rhizosphere that died because of the lack of nutrients that would have been provided by an actively photosynthesizing plant.

Based on the information in this study, it appears that *Rhizobium* is usually not capable of growing in nonrhizosphere soil, where a major stress is the lack of available nutrients. Drying followed by wetting may allow for proliferation in some soils, however. Growth is apparently chiefly associated with root excretions in the soils studied, although for brief periods, replication may be linked with organic nutrients derived from germinating seeds and decaying nodules.

Few strains of *Rhizobium* and few soils were investigated. Hence, additional strains and soils should be studied to confirm whether the conditions for growth postulated here are applicable under a wide range of natural conditions.

REFERENCES

1. Bohlool, B.B., and E.L. Schmidt. 1973. A fluorescent antibody technique for determination of growth rates of bacteria in soil. Bull. Ecol. Res. Comm. (Stockholm) 17:336–338.
2. Chatel, D.L., and R.M. Greenwood 1973. The colonization of host-root and soil by rhizobia. II. Strain differences in the species *Rhizobium trifolii*. Soil Biol. Biochem. 5:433–440.
3. Chatel, D.L., and C.A. Parker. 1973. The colonization of host-root and soil by rhizobia. I. Species and strain differences in the field. Soil Biol. Biochem. 5:425–432.
4. Chowdhury, M.S. 1977. Effect of soil antagonists on symbiosis. p. 385–411. *In* J.M. Vincent et al. (ed.) Exploiting the legume-*Rhizobium* symbiosis in tropical agriculture. Misc. publ. no. 145, College of Agric., University of Hawaii, Honolulu.
5. 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.
6. Diatloff, A. 1969. The introduction of *Rhizobium japonicum* to soil by seed inoculation of non-host legumes and cereals. Aust. J. Exp. Agric. Anim. Husb. 9:357–360.
7. Gibson, A.H. 1977. The influence of the environment and managerial practices on the legume-*Rhizobium* symbiosis. p. 393–450. *In* R.W.F. Hardy and A.H. Gibson (ed.) A treatise on dinitrogen fixation. Section IV: agronomy and ecology. Wiley-Interscience, New York.
8. Lennox, L.B., and M. Alexander. 1981. Fungicide enhancement of nitrogen fixation and colonization of *Phaseolus vulgaris* by *Rhizobium phaseoli*. Appl. Environ. Microbiol 41:404–411.
9. Lowendorf, H.S. 1980. Factors affecting survival of *Rhizobium* in soil. Adv. Microb. Ecol. 4 87–124.
10. Paau, A.S., C.B. Bloch, and W.J. Brill. 1980. Developmental fate of *Rhizobium meliloti* bacteroids in alfalfa nodules. J. Bacteriol. 143:1480–1490.
11. Pena-Cabriales, J.J., and M. Alexander. 1979. Survival of *Rhizobium* in soils undergoing drying. Soil Sci. Soc. Am. J. 43:962–966.
12. Ramirez, C., and M. Alexander. 1980. Evidence suggesting protozoan predation on *Rhizobium* associated with germinating seeds and in the rhizosphere of beans (*Phaseolus vulgaris* L.). Appl. Environ. Microbiol. 40:492–499.
13. Reyes, V.G., and E.L. Schmidt. 1979. Population densities of *Rhizobium japonicum* strain 123 estimated directly in soil and rhizospheres. Appl. Environ. Microbiol. 37:854–858.
14. Robinson, A.C. 1967. The influence of host on soil and rhizosphere populations of clover and lucerne root nodule bacteria in the field. J. Aust. Inst. Agric. Sci. 33:207–209.
15. Rovira, A.D. 1961. *Rhizobium* numbers in the rhizosphere of red clover and paspalum in relation to soil treatment and the numbers of bacteria and fungi. Aust. J. Agric. Res. 12:77–83.
16. Schmidt, E.L. 1974. Quantitative autecological study of microorganisms in soil by immunofluorescense. Soil Sci. 118:141–149.
17. Skyring, G.W., and J.P. Thompson. 1966. The availability of organic matter in dried and undried soil, estimated by an anaerobic respiration technique. Plant Soil 24:289–298.
18. Soulides, D.A., and F.E. Allison. 1961. Effect of drying and freezing soils on carbon dioxide production, available mineral nutrients, aggregation, and bacterial population. Soil Sci. 91:291–298.
19. Sparling, G.P., and M.V. Cheshire. 1979. Effects of soil drying and storage on subsequent microbial growth. Soil Biol. Biochem. 11:317–319.
20. Tuzimura, K., and I. Watanabe. 1962. The effect of rhizosphere of various plants on the growth of *Rhizobium*. Ecological studies of root nodule bacteria (Part 3). Soil Sci. Plant Nutr. (Tokyo) 8:153–157.
21. Vincent, J.M. 1970. A manual for the practical study of root-nodule bacteria. Blackwell Scientific Publications, Oxford.
22. Wilson, J.K. 1942. The loss of nodules from legume roots and its significance. J. Am. Soc. Agron. 34:460–471.
23. Wilson, J.K. 1934. Longevity of *Rhizobium japonicum* in relation to its symbiont on the soil. Cornell Univ. Agric. Exp. Sta. Memoir 162, Ithaca, N.Y.

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