PN-AAP-192
ISN: 33194

Reprinted from the *Soil Science Society of America Journal*
Volume 47, no. 5, September-October 1983
677 South Segoe Rd., Madison, WI 53711 USA

Selecting *Rhizobium meliloti* for Inoculation of Alfalfa Planted in Acid Soils[1]

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ABSTRACT

A study was conducted to obtain *Rhizobium meliloti* strains suitable for use with alfalfa grown in acid soils. Thirteen strains of *R. meliloti* were examined for their ability to grow in acidified culture medium, and seven of these were characterized for the ability to survive in acid and limed nonsterile soils or grow in the presence of the host legume, *Medicago sativa* L. The pH values of the most acid, defined medium that permitted growth of the bacteria from a small inoculum ranged from pH 5.3 to 6.0. For *R. meliloti* 411SE1 and GH1-1SE1, the minimum pH that allowed for growth, the critical pH, was not a dependable indicator of survival in a more acid medium. Strains of *R. meliloti* with relatively low critical pH values survived better in a limed soil but not in acid soils than strains with higher critical pH values. Three strains of *R. meliloti* previously identified as good inoculants for alfalfa in acid soils did not consistently survive better than other strains in a planted or unplanted acid soil of pH 5.3. However, the plants increased the population densities of these three strains more than other strains. These results suggest that *R. meliloti* strains suitable for inoculation of alfalfa in acid soils may be selected not by simple saprophytic properties but by their stimulation by the host legume in acid soils.

Additional Index Words: commensalism, survival, microbial ecology.

Lowendorf, H.S., and M. Alexander. 1983. Selecting *Rhizobium meliloti* for inoculation of alfalfa planted in acid soils. Soil Sci. Soc. Am. J. 47:935–938.

IN ACID SOILS, alfalfa (*Medicago sativa* L.) grows poorly, *Rhizobium meliloti* is found in low numbers or not at all, and nodulation and N fixation by the symbiosis are relatively poor (4, 9, 12, 13). Yet, because of the usually high yield and N fixation by alfalfa, many attempts are being made to find acid-tolerant alfalfa varieties and *R. meliloti* strains (2, 3, 7, 11, 14).

In alfalfa fields, the numbers of *R. meliloti* decline substantially in soils below pH 6.0 (6, 16). Attempts to find acid-tolerant strains of this species often involve seeking isolates that grow well in acid culture media. Fred and Davenport (4) and Graham and Parker (5) found that *R. meliloti* proliferated in culture with pH values as low as 4.9 and 4.5, respectively. More recently, Barber (1) and Rakotoarisoa et al. (14) reported that they obtained mutants of *R. meliloti* that could grow better in culture than the parent strains at a pH as low as 5.5 and 5.2, respectively. Yet, the relationships between growth in pure culture at low pH and survival, growth, nodulation of alfalfa, and N fixation in acid soils are at best obscure. It has been reported that two strains of *R. meliloti* survived well and even reproduced in sterile, acid soils well below the pH values, 5.9 and 5.3, at which the strains could grow in culture (11). In nonsterile soils, however, neither strain survived well.

The present study was conducted to determine the relationship between sensitivity of *R. meliloti* to acidity in culture medium and its survival in unplanted and planted acid soils and to explore ways to select strains of *R. meliloti* that form an effective symbiosis with alfalfa in acid soils.

[1] Contribution from the Dep. of Agronomy, Cornell Univ., Ithaca, NY 14853. Supported by U.S. Agency for International Development Grant no. AID/DSAN-G-0090. The conclusions in this publication do not necessarily represent those of the granting agency. Received 6 Jan. 1983. Approved 12 Apr. 1983.

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MATERIALS AND METHODS

Rhizobium meliloti strains 411SE1 and GH1-1SE1, both resistant to 1.0 mg of streptomycin and 50 μg of erythromycin/mL, were obtained as described earlier (11). *Rhizobium meliloti* Aur, CF-1, and GH2-6 were isolated from Lima silt loam (fine-loamy, mixed, mesic Glossoboric Hapludalf, pH 7.6), Williamson very fine sandy loam (coarse-silty, mixed, mesic Typic Fragiochrept, pH 6.1), and Mardin channery silt loam (fine-loamy, mixed, mesic Typic Fragiochrept, pH 4.7), respectively, from New York. *Rhizobium meliloti* 102F66, 104A13, 102F34, 102F51, and 102F77 were obtained from J. C. Burton, and *Rhizobium meliloti* NRG-43, NRG-185, and BALSAC were provided by R. S. Smith, both of Nitragin Co., Milwaukee, Wis. Antibiotic-resistant isolates were derived from the strains as described previously (10). These strains grew on an agar medium containing 1.0 mg of streptomycin and 50 μg of erythromycin/mL and are designated by adding the suffix "SE" with a numeral. All mutants retained the infective and N-fixing capacities of the parent cultures as determined by inoculation onto *M. sativa* 'Iroquois' growing in disposable plastic pouches (American Scientific Products, Rochester, N.Y.). The parent cultures were maintained on yeast extract-mannitol (YEM) agar slants (17), and the mutant cultures were maintained on YEM agar supplemented with streptomycin and erythromycin.

Tests for acid sensitivity in culture were carried out by inoculating *Rhizobium meliloti* into a defined medium (11), which was adjusted to various pH values with 1.0*M* HCl, in duplicate culture tubes. The inoculum, which provided an initial density of 10^3 to 10^4 cells/mL, originated from a culture growing in the defined medium. The tubes were incubated at 29° C on a reciprocal shaker operating at 120 cycles/min, and the presence or absence of growth was determined by visual assessment of turbidity. The lowest pH at which growth was evident within 4 weeks was designated the critical pH (4). For assessment of survival of *Rhizobium meliloti* in culture at pH values below the critical pH, duplicate 125-mL Erlenmeyer flasks containing 50 mL of defined medium were inoculated as above. Rhizobial numbers were determined from 1.0-mL samples followed by serial dilution in a sterile saline (11) and spreading on plates of YEM agar.

The soils used were Mardin channery silt loam and Langford channery silt loam (coarse-loamy, mixed, mesic Aquept ic Fragiudalf) from New York and Durham sandy loam (fine-loamy, siliceous, thermic Typic Hapludult) from North Carolina. Soils were chosen to have pH values in a range that allowed for good to poor survival of *Rhizobium meliloti* (11). Samples of soils were air dried, passed through a 2-mm sieve, and stored at room temperature in double plastic bags. The pH values were determined by electrode in 1:1 soil-to-water suspensions. The Mardin soil was amended with 5.0 g of $Ca(OH)_2$/kg of air-dried soil.

For studies of bacterial survival, portions of air-dried, nonsterile soil equivalent to 10 g of oven-dried soil were placed in sterile 160-mL dilution bottles, and before inoculation, the initial water content was adjusted with sterile, distilled water so that during incubation the water content was 25% (wt/wt), except as otherwise indicated. The inoculation, incubation, sampling, and counting procedures were as described previously (11). Except as indicated, triplicate samples of soils were used. Some of the data were subjected to a logarithmic transformation for statistical analysis; for this purpose, the ratios of the final numbers to the initial numbers of the acid-tolerant and the acid-sensitive species were compared. The data were treated similarly for the ratios of the counts in the presence and absence of plants. A *t*-test was used with a pooled variance.

To determine rhizobial numbers in the presence of host plants, 20 seeds of *M. sativa* 'Iroquois' were added to the soil immediately after inoculation. The bottles were gently tapped to cover the seeds with soil, capped with Parafilm, and incubated under Gro-lux, wide spectrum fluorescent lamps at 24°C and 63 to 81 μmol $m^{-2}s^{-1}$ (photon flux at 400 to 700 nm) as measured by a LI-COR meter with a LI-188B sensor (LI-COR, Inc., Lincoln, Nebr.). Control bottles without seeds received the same treatment. The bottles were sampled 10 d after inoculation, a period of time sufficient to allow the rhizobial populations to reach their maximum size (11). No significant difference was found between the dry weights of alfalfa seedlings grown in the acid and limed soils.

Table 1—Critical pH of *R. meliloti* strains.

Strain	Critical pH
102F66	6.0
NRG-43	6.0
411SE1	5.9
Aur	5.9
GH2-6	5.7
CF-1	5.6
104A13	5.3-5.6†
102F34	5.3-5.6†
102F51	5.3-5.6†
102F77	5.3-5.6†
NRG185	5.6
BALSAC	5.4
GH1-1SE1	5.3

† Range given because of imprecision of particular test.

RESULTS AND DISCUSSION

The critical pH values for the *Rhizobium meliloti* strains used in these experiments are given in Table 1. The lowest critical pH was 5.3, the highest 6.0. The behavior of two of these strains, 411SE1 and GH1-1SE1, in acid and limed soils has been described previously (11).

To compare the fates of these two strains at pH values above and below the critical pH, they were inoculated into the defined medium adjusted to various pH values. In the solutions initially at pH 6.3, both strains grew (Fig. 1). The doubling time for *Rhizobium meliloti* 411SE1 was 4.1 h, and the final pH was 6.4. The doubling time for *Rhizobium meliloti* GH1-1SE1 was 3.2 h, and the final pH, 8.3. In media with initial pH values of 5.2, 5.0, and 4.8, *Rhizobium meliloti* 411SE1 did not increase in numbers, and its population density was relatively constant for the 14-d period. The final pH of the medium in each instance rose 0.2 units. *Rhizobium meliloti* GH1-1SE1 grew slowly at pH 5.2, and its doubling time was 27 h. At pH 5.0 and 4.8, the numbers of the latter strain declined slowly for 3 d, and then the average counts increased slightly. However, in only one-half of the flasks at these two pH values did the numbers increase. In the remaining flasks, the numbers continued to decline. For media with initial pH values of 5.2, 5.0, and 4.8 that were inoculated with strain GH1-SE1, the final pH values were 8.4, 5.0, and 4.9, respectively. Because the critical pH for *Rhizobium meliloti* GH1-1SE1 was earlier found to be 5.3, its growth at pH 5.2 and, in some flasks, at pH 5.0 and 4.8, probably reflects the fact that the inoculum density in the present experiment (10^5/mL) was about 10-fold greater than used in establishing the critical pH. At high cell densities, the bacteria may modify the medium sufficiently or become adapted to allow growth.

In separate experiments, the numbers of *Rhizobium*

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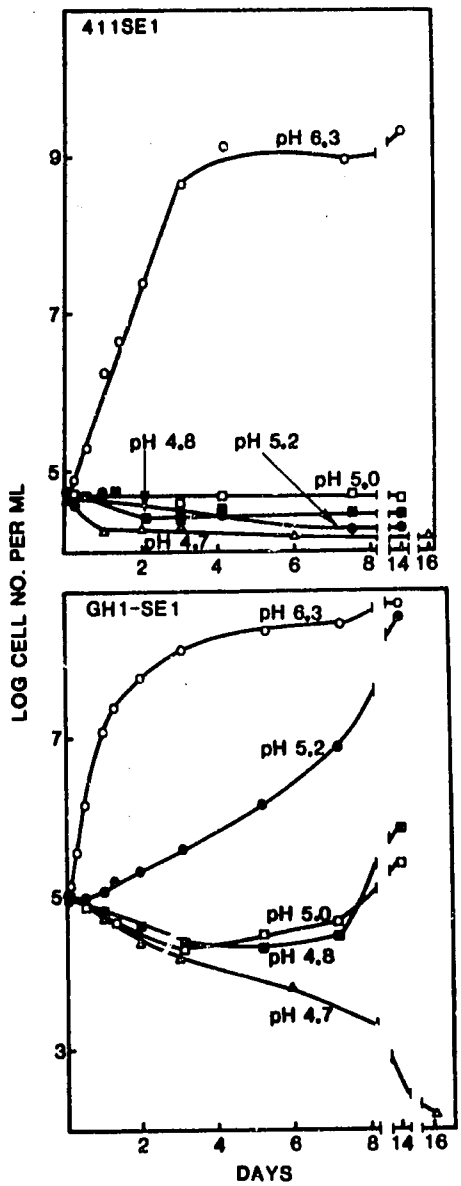

Fig. 1—Changes in population density of two *Rhizobium meliloti* strains in a defined medium at various pH values.

meliloti GH1-SE1 declined slowly but consistently at pH 4.7, whereas *Rhizobium meliloti* 411SE1 survived well. Because *Rhizobium meliloti* 411SE1, despite its higher critical pH, survived as well as or better than *Rhizobium meliloti* GH1-1SE1 at pH values below 5.0, it may be inappropriate to predict survival of *Rhizobium meliloti* in media at low pH values on the basis of a comparison of critical pH values. These results are consistent with earlier findings that the two strains in question survived in sterile soils at pH values considerably below their critical pH (11).

A comparison was made of the survival in an acid and a limed soil of antibiotic-resistant isolates of three strains with different critical pH values. Counts were performed on duplicate samples of soil. In the pH 6.4 soil, numbers of the three strains fell slowly in a 21-d period by 4- to 25-fold from the initial density (Fig. 2). In the pH 5.0 soil, in contrast, the cell densities of the strains fell from three to greater than four orders of magnitude. No major difference in survival in the

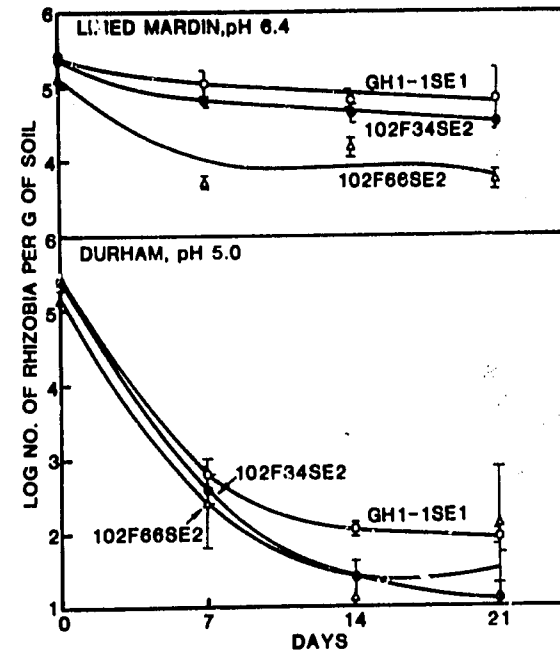

Fig. 2—Survival of three *Rhizobium meliloti* strains in an acid and a limed soil at 30% (wt/wt) moisture. Each point represents the average of duplicate samples. Error bars represent 1 SD.

nonsterile acid soil was observed among the three strains. These results confirm the view that selection of *Rhizobium meliloti* strains for survival in acid soils based on the critical pH is unwarranted (11, 14). Because it has been shown that *Rhizobium phaseoli* strains can be selected for survival in acid soils based upon the criterion of critical pH (10), the present results point to a physiological and ecological distinction between *Rhizobium phaseoli* and *Rhizobium meliloti*, a distinction that can be added to the symbiotic, genetic, and biochemical differences already reported (8, 18).

Yields of alfalfa grown in soils of pH values between 5.0 and 6.0 were increased more by *Rhizobium meliloti* NRG-43, BALSAC and NRG-185 than by acid-sensitive strains, but the yields were the same at pH 6.7 (15) (W. A. Rice, personal communication). To determine the behavior of the test strains in acid soils, antibiotic-resistant isolates of these strains were obtained. These isolates and two other strains, which were used for comparative purposes, were inoculated into soils of pH 6.4 and 5.3. Counts made on day 28 showed that cell numbers of *Rhizobium meliloti* 102F66SE2 and NRG-43SE1, strains with relatively

Table 3—Numbers of *R. meliloti* in soils in the presence or absence of *M. sativa*.

		No. of cells/g of dry soil at day 10			
	Initial	Mardin, pH 6.4		Langford, pH 5.3	
Strain	count (per g of soil)	No plant	With plant	No plant	With plant
102F66SE2	90 700	ND†	ND	6 980	55 900
NRG-43SE1	377 000	39 600	732 000	4 150	126 000
104A13SE2	399 000	ND	ND	31 800	41 700
102F34SE2	63 200	ND	ND	23 000	77 300
NRG-185SE2	132 000	ND	ND	4 480	362 000
BALSAC-SE1	437 000	154 000	362 000	6 280	226 000
GH1-1SE1	449 000	613 000	1 690 000	38 000	267 000

† Not determined.

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Table 2—Number of *R. meliloti* surviving for 28 d in two soils.

Strain	No. of cells/g of dry soil		
	Initial count	Mardin (pH 6.4) at day 28	Langford (pH 5.3) at day 28
102F66SE2	303 000	567	83
NRG-43SE1	287 000	33	167
NRG-185SE2	196 000	39 900	667
BALSAC-SE1	179 000	19 800	600
GH1-1SE1	443 000	57 500	83

high critical pH values, dropped by nearly three to four orders of magnitude in both acid and limed soils (Table 2). On the other hand, the numbers of *Rhizobium meliloti* GH1-1SE1, BALSAC-SE1, and NRG-185SE2 fell about one order of magnitude in the limed soil and up to four orders of magnitude in the acid soil. The latter three strains have lower critical pH values and also survived better in limed soil than the other two strains, results consistent with those reported previously (11). The three "acid-tolerant" strains, *Rhizobium meliloti* NRG-43SE1, NRG-185SE2, and BALSAC-SE1, survived slightly better than the remaining strains in the pH 5.3 soil (α, 0.05); such results were not consistently obtained as shown in the following experiment, and the final population densities were still significantly lower than those in the pH 6.4 soil.

If good survival in unplanted acid soil is not a trait of the "acid-tolerant" *Rhizobium meliloti* strains and does not substantially differentiate them from strains not chosen for acid tolerance, then perhaps the former strains respond more to exudates in the alfalfa rhizosphere. To test this possibility, the three "acid-tolerant" strains and four strains not considered acid tolerant were inoculated into an acid soil in the presence or absence of germinating alfalfa seeds. The changes in population density of three of the strains were also followed in limed Mardin soil. In the pH 6.4 soil in the absence of plants, the two strains with low critical pH, *Rhizobium meliloti* NRG-185SE2 and GH1-1SE1, survived better than the one with higher critical pH, *Rhizobium meliloti* NRG-43SE1 (Table 3). These results are similar to those found previously. The presence of alfalfa increased the numbers of *Rhizobium meliloti* NRG-43SE1 and GH1-1SE1 and reduced the extent of decline of *Rhizobium meliloti* BALSAC-SE1.

Although comparisons among strains were difficult to make because the initial numbers varied considerably, the percent of the inoculated cells surviving in the pH 5.3 soil in the absence of plants was remarkably consistent at 1.1 to 8.5%, except for *Rhizobium meliloti* 102F34SE2. Thus, poor survival in unplanted acid soil is found to be general among the *Rhizobium meliloti* strains tested. In fact, among all the strains tested, the final cell densities were lowest for the "acid-tolerant" strains.

As in the limed Mardin soil, the presence of growing plants improved survival and, for *Rhizobium meliloti* NRG-185SE1 and 102F34SE2, actually led to a net increase in numbers of viable bacteria in Langford soil, pH 5.3. However, in an earlier study, survival was not improved in the rhizosphere of alfalfa in a soil of pH 4.6 (11). Thus, a stimulation is not observed if the soil acidity is too great. The relative stimulation of rhizobial numbers by host plants in the Langford soil was greatest for the strains that had been selected for "acid tolerance" (i.e., *Rhizobium meliloti* NRG-43, BALSAC, and NRG-185—ranging from 30- to 80-fold compared to 1.3- to 8-fold for the remaining strains. The difference between the acid-tolerant and nontolerant groups in response to the presence of alfalfa was statistically significant (α, 0.01). The magnitude of stimulation was independent of the critical pH of the strain.

It is concluded that strains of *Rhizobium meliloti* that had previously been chosen for use as inoculants because of their ability to increase alfalfa yields in acid soils (R. S. Smith, personal communication) are not exceptional in critical pH or their ability to survive in planted or unplanted acid soil. However, these strains responded more than other strains to the presence of growing alfalfa in acid soil. If the cause of this response can be established and the process reproduced in culture, then a simple procedure may be developed to select acid-tolerant *Rhizobium meliloti* strains.

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Grant/Contract Number: AID/DSAN-G-0090 (211-(d) grant

Authors: Lowendorf, Henry S. and Martin Alexander. 1983

Title: Selecting Rhizobium meliloti for Inoculation of Alfalfa Planted in Acid Soils

Publisher ~~Ed.~~: Soil Science Soc. Amer. Journ 47: 935-938.

Estimated Publication Date:

Estimated Demand for number of copies requested thru S&T/DIU

Type of Audience

Comments:

6