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Identification of *Rhizobium phaseoli* Strains That Are Tolerant or Sensitive to Soil Acidity

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A study was conducted to determine whether the survival of *Rhizobium phaseoli* in acid soils could be predicted on the basis of the tolerance of the organism to acidity in culture. Of 16 strains tested, all grew in culture at pH 4.6, but only those that grew at pH 3.8 survived in soils having pH values of 4.1 to 4.6. Strains that tolerated the lowest pH values in culture were tolerant of the highest aluminum concentrations. In one acid soil, an acid-tolerant strain was unable to survive in numbers greater than 100/g, but the poor survival was not related to the level of extractable aluminum or manganese in the soil. Reproduction of an acid-tolerant strain of *R. phaseoli* was enhanced in the rhizosphere of *Phaseolus vulgaris* in both acid and limed soils, but stimulation of an acid-sensitive strain by the plant occurred only in the limed soil. These results indicate that cultural tests can be used to predict the ability of *R. phaseoli* to survive in acid soil.

The common bean, *Phaseolus vulgaris*, is an important food in tropical regions of North and South America, Asia, and Africa. Per capita consumption of beans is estimated to average between 5 and 25 kg/year in tropical North and South America, for example, and this legume provides 20% or more of the total protein intake per person (17). Beans in Latin America are typically grown on infertile acid soils, of which over 10^9 ha (10^{13} m^2) have been identified (8, 19). Worldwide, nearly 1.6×10^9 ha (1.6×10^{13} m^2) of tropical soils are classed as highly infertile, acid Oxisols or Ultisols (19).

To obtain satisfactory yields of *P. vulgaris* on such acid soils, varieties are desired that are tolerant to the stresses associated with these soils. These stresses include low pH and high levels of aluminum and manganese. Some bean cultivars produce far higher yields than others in aluminum-rich, acid Oxisols with lower lime inputs (20), which indicates that breeding beans for cultivation in these soils can provide better adapted varieties. In addition, to obtain maximum yields and optimum rates of biological nitrogen fixation from the symbiosis between *P. vulgaris* and *Rhizobium phaseoli*, strains of the root nodule bacteria must be found that not only are active in nitrogen fixation but also survive well in the acid soils, multiply in the rhizosphere of the growing plant, and infect its roots when competing with indigenous microorganisms.

Little is known about the effect of soil acidity on this economically important *Rhizobium* species or about the ranges of soil acidity tolerance that exists among strains. It has been noted, however, than an *R. phaseoli* strain that grows in culture medium at pH 4.4 survives poorly in acid soils, although a strain that nodulates cowpeas and grows in culture at pH 4.2 survives well (14). This paper is concerned with the sensitivity of *R. phaseoli* to soil acidity. The results indicate that simple cultural methods can be used to distinguish strains tolerant of soil acidity from strains that are sensitive.

MATERIALS AND METHODS

R. phaseoli 523 was originally obtained from J. C. Burton as strain 127K17. *R. phaseoli* 6-3 was isolated from a New York soil (pH 7.6), and *R. phaseoli* strains C12, C09, CAR36, 442, and CIAT 161 were provided by R. H. Miller. Antibiotic-resistant isolates derived from these strains were obtained by transferring portions of dense cultures growing actively in yeast extract-mannitol (YEM) broth (21) to plates of YEM agar containing 1.0 mg of streptomycin sulfate per ml. Colonies isolated from these plates were inoculated, either directly or after growth in YEM broth, onto YEM agar containing 1.0 mg of streptomycin and 50 μg of erythromycin per ml. Mutants resistant to both antibiotics (named by adding SE and a number after the parent strain designation) were inoculated onto *P. vulgaris* var. Red Kloud growing in disposable plastic pouches (American Scientific Products, Rochester, N.Y.) to determine whether the infectivity and effectiveness of the parent culture were maintained. The parent cultures were maintained on YEM agar, and the mutants were maintained on YEM agar supplemented with the appropriate antibiotics. In contrast to the results of Date and Halliday (4), who found that some strains nodulating *Stylosanthes* spp. grow only in an acid medium, all strains used in this study grew well in YEM at neutral pH, although some grew better at pH

TABLE 1. Chemical and physical properties of soils used

Soil	pH	Organic matter (%)	Exchangeable cations (meq/100 g)				KCl-extractable ions (μg/g)		Composition (%)		
			Mg	Ca	K	H	Al	Mn	Sand	Silt	Clay
Mardin	4.3	4.0	0.25	2.38	0.18	18	170	60	26.7	53.4	19.9
Piarco	4.1	1.8	0.25	0.76	0.08	6	200	1	29.1	49.9	21.0
Windsor	4.6	2.6	0.02	0.10	0.02	13	83	1	91.9	3.4	4.7

5.0 (e.g., *R. phaseoli* C12). However, like the strains nodulating *Stylosanthes* spp., those nodulating *P. vulgaris* increased the pH of the defined medium from 4.7 to between 5.1 and 8.1, depending on the strain.

Soil samples were air dried, passed through a 2-mm sieve, and stored at room temperature in double plastic bags. The soils used were Mardin channery silt loam (fine-loamy, mixed, mesic, Typic Fragiochrept) from New York, Windsor loamy fine sand (mixed, mesic, T ic Udipsamments) from New York, and Piarco fine sand (fine, sandy, clayey, kaolinitic, isohyperthermic, Aquoxic Tropudult) from Trinidad. Some of the properties of these soils are shown in Table 1. The pH values were determined with electrodes in 1:1 soil-in-water suspensions. The other analyses were performed as described by Greweling and Peech (10) and Dower and Olson (6). When soils were to be sterilized, they were subjected to 10 Mrad of gamma irradiation from a ^{60}Co source (14). For some experiments, samples of the Mardin or Piarco soil were amended with 5.0 or 0.79 g, respectively, of $Ca(OH)_2$ per kg of air-dried soil. The soils were autoclaved before they were discarded.

Tests of acid sensitivity in culture were carried out by inoculating *R. phaseoli* into a defined medium (14). The initial count was 10^3 to 10^4/ml. Growth was determined by visual assessment of turbidity. The lowest pH at which growth was evident within 4 weeks was designated the critical pH (7). Aluminum sensitivity was determined by adding various amounts of $AlCl_3$ to the defined medium, which contained 10 μM phosphate and had been adjusted to pH 4.7 (11, 16). The test bacteria were inoculated into duplicate culture tubes containing the medium, the tubes were incubated at 29°C on a reciprocal shaker operating at 120 cycles per min, and the presence or absence of growth was observed.

TABLE 2. *R. phaseoli* critical pH and tolerance of aluminum in a defined medium (pH 4.7)

Strain	Critical pH	$AlCl_3$ (μM)[a]
6-3	4.6	5
CIAT 161	4.6	10
442	4.4	0
523	4.4	10
C09	3.8[b]	50
C12	3.8[b]	50
CAR36	3.8[b]	50

[a] Highest concentrations at which growth occurred.
[b] Lowest pH tested.

For survival studies, portions of air-dried soil equivalent to 10 g of oven-dried soil were placed in sterile 160-ml dilution bottles, the initial water content was brought to 10 or 20% (wt/wt) with sterile distilled water, and the soil was incubated for 3 to 5 days before inoculation. The inoculum added to the soil was grown, and the bottles were sampled in duplicate by the procedures of Lowendorf et al. (14). Rhizobia added to the sterile soil were counted on duplicate plates of YEM agar incubated at 29°C. In experiments with nonsterile soils, rhizobia were counted on YEM agar supplemented with (per milliliter) 1.0 mg of streptomycin, 50 μg of erythromycin, 250 μg of cycloheximide, and 50 μg of nystatin. In these experiments, as few as 13 rhizobia per g of soil could be detected in this medium.

To determine rhizobial population densities in the presence of a growing plant, 20 g of dry, nonsterile soil contained in a dilution bottle was brought to 20% moisture (wt/wt). After 5 days, 2.0 ml of a rhizobial suspension was mixed with the soil, and a single seed of *P. vulgaris* was placed on the soil surface. The seed was covered with soil by gently tapping the bottle. The bottles were covered with Parafilm and incubated at 24°C and a light intensity of 165 to 240 μmol m^{-2} s^{-1} (photon flux at 400 to 700 nm) under Gro-lux wide-spectrum fluorescent lamps. Control bottles received the same treatment without the seeds. A sample consisted of all soil in a single bottle, and the first dilution was made by adding the dilution liquid (14) to the bottle containing plant and soil. After this suspension was shaken, the plant was removed, washed free of soil, and dried at 110°C for 15 to 18 h. Root and shoot weights were then determined. All treatments were conducted in duplicate.

RESULTS

All 16 strains tested in the defined medium grew at pH 4.6 or above. Representative data showed that some grew at pH 3.8, which was the lowest pH tested (Table 2). Because the minimum pH value for growth of the most acid-tolerant strains was not determined, the value of pH 3.8 was taken as the critical value for these strains to estimate an average critical pH; in this way, the average critical pH for the 16 strains of *R. phaseoli* was determined to be 4.3 (±0.3 [standard deviation]). This value is close to pH 4.2, the value given by Fred and Davenport (7).

We further studied the ability of some strains to grow in the presence of aluminum, which is toxic to beans and most crop plants and is

2

TABLE 3. Survival of *R. phaseoli* in two sterile soils

Strain	Limed Mardin soil, pH 6.3		Windsor soil, pH 4.6	
	Initial no. ($\times 10^6$/g)	% Surviving at 20 days	Initial no. ($\times 10^6$/g)	% Surviving at 8 days
6-3	35	290	39	1.2
CIAT 161	17	470	76	5.3
442	110	79	69	0.78
523	200	30	50	0.82
C09	310	46	120	22
CAR36	350	30	130	30

especially important in acid soils. The defined medium was adjusted with 1 N HCl to pH 4.7, a value at which all strains grew in the absence of aluminum, and the phosphate concentration was reduced to 10 μM to avoid precipitating the aluminum. $AlCl_3$ was added to achieve a final concentration of 0, 5, 10, 25, 50, or 100 μM. Some strains did not grow at 5 μM $AlCl_3$, some proliferated at 10 but not at 25 μM $AlCl_3$, and some grew at 50 but not at 100 μM $AlCl_3$ (Table 2). Strains that grew at the most acid pH values also grew in the presence of the higher concentrations of aluminum. In addition, aluminum did not substantially increase the lag time or the doubling time of the aluminum-tolerant strains: the appearance of turbidity, which was evident at 3 days in the absence of aluminum, was either not delayed at all or delayed at most until 4 days after inoculation in the presence of 50 μM $AlCl_3$.

To determine how physical and chemical characteristics of the soils affected rhizobial survival, six of the strains were inoculated into sterilized soils at a final moisture content of 30% (wt/wt). In a soil adjusted to pH 6.3, all strains survived well after 20 days of incubation at 29°C (Table 3). No population declined below 30% of the inoculated number, and *R. phaseoli* CIAT 161 and 6-3 increased in abundance. When these six strains were inoculated into a highly acid soil, however, survival of some was greatly reduced. In only 8 days, populations of the four strains more sensitive to acidity in the defined medium (6-3, CIAT 161, 442, and 523) were reduced to 0.78 to 5.3% of the initial count. On the other hand, numbers of the two strains more tolerant of acidity and aluminum in culture medium were only reduced to 22 or 30% of the numbers inoculated. Thus, it appears that the strains more tolerant of acidity and aluminum in culture are also better able to survive in sterile acid soil.

To study the survival of *R. phaseoli* in nonsterile soils, two antibiotic-resistant isolates were used. These bacteria had the same acid tolerance in medium as did the parent cultures. The organisms were grown in YEM broth, and the cells were inoculated into one limed and three highly acid soils. The final soil moisture content was 20% (wt/wt). *R. phaseoli* 523SE5 was acid sensitive and C12SE1 was acid tolerant by all tests described above. In the soil of high pH, the numbers of *R. phaseoli* 523SE5 increased about 10-fold before reaching a reasonably constant density (Fig. 1). In the three acid soils, the population of this strain decreased rapidly by 3 or more orders of magnitude in 2 to 4 weeks. Thus, in nonsterile soils, as well as in

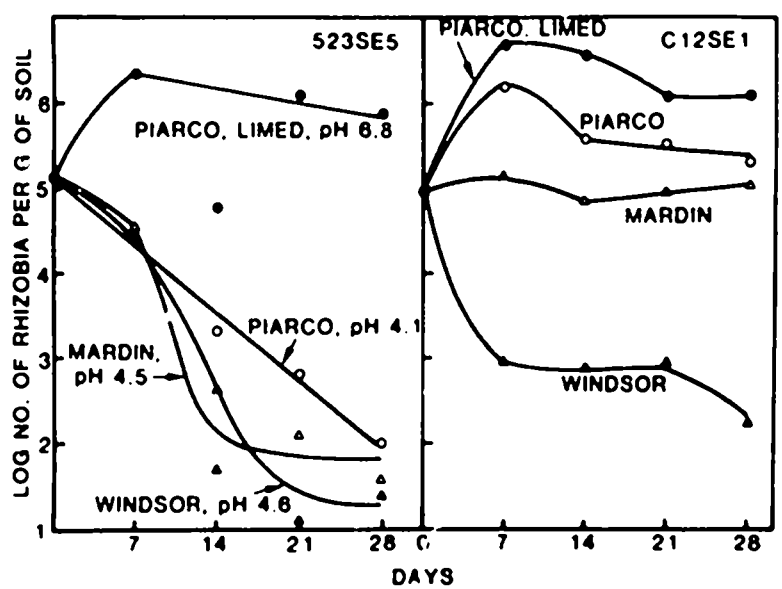

FIG. 1. Changes in populations of two *R. phaseoli* strains inoculated to four nonsterile soils.

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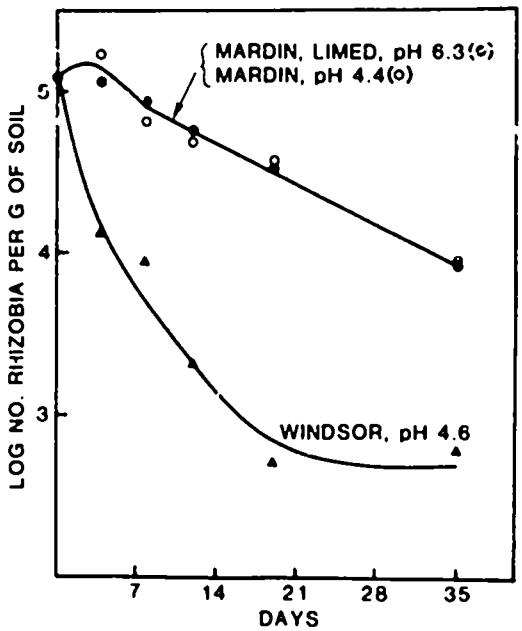

FIG. 2. Changes in populations of acid-tolerant *R. phaseoli* C09SE1 in nonsterile acid and limed soils.

sterile soil and culture medium. *R. phaseoli* 523 and the antibiotic-resistant isolate derived from it did not tolerate high acidity. In contrast, the abundance of *R. phaseoli* C12SE1 remained essentially constant for 4 weeks in the limed soil or the soils at pH values of 4.1 and 4.5. However, in acid Windsor soil, strain C12SE1 declined rapidly by 2 orders of magnitude. Thus, this strain survived well in most but not all of the acid soils tested.

Two of the other acid-tolerant isolates of *R. phaseoli*, strains CAR36SE1 and C09SE1, behaved similarly to strain C12SE1: they survived well in limed or unlimed Mardin soil and poorly in Windsor soil (all at 30% moisture). The population changes of one of the strains are shown in Fig. 2. Hence, a stress in addition to acidity is present in Windsor soil but not in the other acid soils examined.

It was shown above that small numbers of the acid-sensitive *R. phaseoli* 523SE5 survived in acid soils for at least 28 days. The bacteria may have survived in soil microsites at pH values higher than the pH of the bulk soil (14). However, this persistence is not prolonged. Bacterial numbers fell continuously in acid Piarco soil at 20% (wt/wt) moisture from an initial density of 10^7/g to undetectable levels at 9 weeks (Fig. 3). The sensitivity limit for counting was 13 cells per g of soil. In contrast, *R. phaseoli* 523SE5 in limed Piarco soil and strain C12SE1 in acid and limed Piarco soil maintained relatively high population densities for the same period of time.

A study was performed to determine whether roots of a growing bean plant could protect the acid-sensitive strain of *R. phaseoli* from elimination in an acid soil. The plant yields were not significantly different in acid and limed soils, and the mean weights were 317 and 305 mg, respectively. In the limed soil in the absence of a plant, acid-sensitive strain 523SE5 numbers remained essentially constant for the 12 days of the experiment (Fig. 4). In the presence of a plant, the abundance of this strain increased about 10-fold. In the acid soil, however, the density of this strain declined steadily in the absence or presence of its host plant. Therefore, the host plant did not protect this strain in the acid soil. In contrast, the population of acid-tolerant *R. phaseoli* C12SE1 remained large in the absence of plants in both acid and limed soils, and growth of the bacteria was stimulated by the plant in both soils.

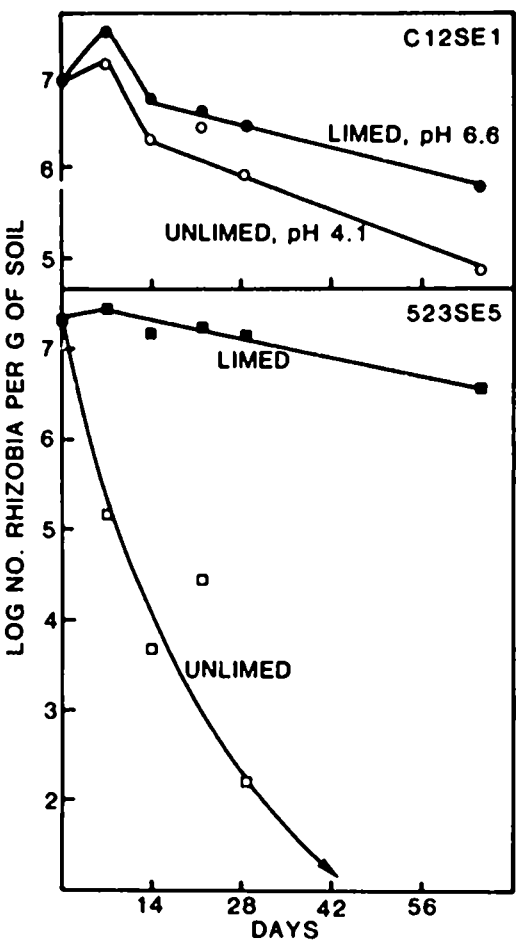

FIG. 3. Changes in populations of acid-tolerant and -sensitive strains of *R. phaseoli* inoculated at high densities into acid and limed Piarco soils.

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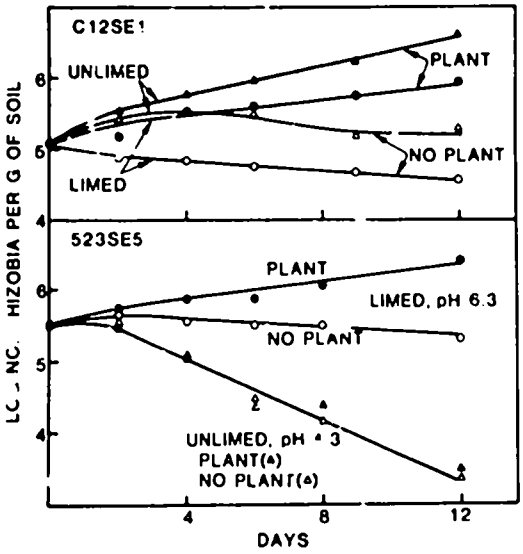

FIG. 4. Effects of *P. vulgaris* growth on populations of acid-sensitive and -tolerant strains of *R. phaseoli* in acid and limed Mardin soils.

DISCUSSION

The development of procedures for testing the behavior of *Rhizobium* strains in soil is considered to be important (2, 9, 12, 13), but attempts to screen fast-growing rhizobia for use on crops grown in acid soils have not been successful (1, 3, 14, 18). The results presented here indicate that survival of *R. phaseoli* strains in at least some acid soils can be predicted from the ability to grow in defined, acidified medium. Thus, the only strains that survived well in a sterilized soil of pH 4.6 were those that had grown at pH 3.8 in culture, although all strains grew at pH 4.6. In contrast, two strains of *R. meliloti* survived and multiplied in sterilized soils at pH values below the critical value for these strains, possibly because of microsites of higher pH in which the bacteria proliferated (14). Although microsites of higher pH probably exist in the soils used in this study, the strains of *R. phaseoli* with a critical pH of ≥4.4 were unable to grow in them. Alternatively, some factor other than the hydrogen ion concentration governs the survival of *R. phaseoli* in highly acid soils.

The only strains that survived in nonsterile acid soils were those that grew at pH 3.8 in culture and survived well in sterile acid soil. The less acid-tolerant strain, 523SE5, did not survive in any of the acid soils and, even at high inoculum levels, disappeared from the acid soil in 9 weeks. As a consequence of these findings, a procedure for screening strains of *R. phaseoli* for survival in acid soils can be based on ability to grow in culture at pH 3.8.

Strains that survived in other acid soils failed to survive in the Windsor soil. Hence, this soil has an additional stress, the identity of which is unknown. The concentrations of KCl-extractable aluminum and manganese did not seem to be responsible for the inhibition because the levels of these cations were greater in the two other acid soils, both of which permitted survival of the acid-tolerant *R. phaseoli*. The concentrations of the extractable aluminum and manganese in the limed soils were less than 1.0 μg/g (data not shown). Nevertheless, the extractable aluminum and manganese may not accurately reflect concentrations affecting the bacteria. The Windsor soil is also notable because it contains the highest proportion of sand of the soils studied. Rhizobial survival is reported to be poor in sandy soils subjected to drying or high temperatures (13), and perhaps soil texture also affects the tolerance of the root nodule bacteria to soil acidity.

It may be possible to use differences in survival resulting from liming to determine the factors responsible for the decline of the sensitive rhizobia. *R. phaseoli* C12SE1, which was generally tolerant of acid soils, survived better in limed than unlimed Piarco soil but more poorly in limed than unlimed Mardin soil. However, the cause of such differences in survival resulting from lime-induced changes in soil properties is still unknown.

The behavior of the more acid-sensitive *R. phaseoli* strain was not tested in soils of pH values between 4.6 and 6.3. Therefore, we do not know the soil pH below which survival of the acid-sensitive *R. phaseoli* is likely to be poor. However, the survival of an acid-sensitive strain of *R. phaseoli* has been found to improve as the pH of a sterile acid soil is raised with incremental additions of lime (14).

The poor survival of the acid-sensitive *R. phaseoli* in acid soils in the presence of growing *P. vulgaris* contrasts with the results of Mulder et al. (15), who found that the numbers of rhizobia in acid soils are higher in the presence of legume roots than in their absence. In the presence of *P. vulgaris* roots, *R. phaseoli* 523SE5 was still subject to the stress of the acid soil, although strain C12SE1 was able to multiply under the same conditions. Similarly, strains of *R. meliloti* that do not survive in acid soils do not persist in the rhizosphere of *Medicago sativa* (14). Thus, there is presently no way to predict the strains of *Rhizobium* for which the stresses in acid soil are alleviated by roots.

Nodulation of and nitrogen fixation by bean plants were not tested in this study except to confirm the effectiveness of the bacteria. However, bean plants are nodulated abundantly in soil at pH values as low as 4.2 (5), and Spain et

5

al. (20) reported high yields of black bean in soils at pH values of 4.3 and 4.7 and exchangeable aluminum levels of 3 and 2 meq/100 g, respectively. Because the plants tolerate such pH and aluminum stresses, it is of practical importance to identify strains of *R. phaseoli* that are also tolerant.

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6