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Tolerance of Rhizobia to Acidity, Aluminum, and Phosphate[1]

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ABSTRACT

Low levels of phosphorus and high levels of aluminum are important soil acidity factors for the growth of higher plants; however, very little is known about their effects on the soil rhizobia. The present study was conducted to determine the relative effects of acidity, P, and Al on rhizobia. Tolerance of low pH (4.5), low P (5-10 μM), and high Al (50 μM) was assessed for 10 strains of cowpea rhizobia by detailed growth studies in defined liquid media. Tolerances to these factors were determined for 65 strains of cowpea rhizobia and *Rhizobium japonicum* by a rapid method based on attainment of turbidity from a small inoculum. Strains varied in response. Low P (as compared with 1,000 μM) limited total attainable population density to 5×10^7 cells/ml, and slowed the growth of some strains. Acidity generally increased lag time or slowed growth of most strains, and stopped growth of about 50% of them. Tolerance of acidity did not necessarily entail tolerance of Al. Aluminum (50 μM) increased the lag time or slowed growth of almost all strains tolerant of low pH. It virtually stopped growth of 40% of the strains.

With our system the rhizobia had to make 1,000-fold growth in the stress media before they could significantly raise pH and precipitate Al. A valid rapid screening can be based on ability to attain visible turbidity in culture under acid or Al-stress, so long as initial density is small ($<<10^6$ cells/ml). The cowpea rhizobia tended to have more tolerance to Al than *R. japonicum* and overall Al was a more severe stress than low pH or low P.

Additional Index Words: acidity, phosphorus, aluminum, rhizobia, cowpea miscellany, *Rhizobium japonicum*.

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EFFECTS OF ACIDITY on species of *Rhizobium* are well documented. Pure culture studies (Graham and Parker, 1964) have shown the critical low pH range for growth is from about 4.0 to 6.0, with the slower growing *R. japonicum*, *R. lupini*, and cowpea miscellany being in general more acid tolerant than the others, and *R. meliloti* being the most acid sensitive. These observations on relative tolerance of the species agree with studies in acid soils (Damirgi et al., 1967; Jensen 1969). Within each species, important strain-to-strain variation has been demonstrated (Graham and Parker, 1964; Munns, 1965a).

Besides low pH per se, acid mineral soils have low levels of phosphorus and high levels of aluminum (Kamprath, 1973; Pearson, 1975). There has been little research on the effects of these two soil factors on rhizobia. An early study of effects of P on rhizobia showed positive growth response to P additions in soil (Truesdell, 1917). Kamata (1962) related the ability to nodulate P-deficient soybeans with the rhizobial strains relative response to P in culture media. Werner and Berghauser (1976) demonstrated that three strains of *Rhizobium* were better than two other common bacteria at taking up P from very low concentrations in solution. Recent studies (Rerkasem, 1977; de Carvalho, 1978)[3,4] have shown that some rhizobia can indeed survive high Al concentrations at low pH in both solution media and soil. However, there is still insufficient evidence to establish Al-tolerance in rhizobia as distinct from tolerance of low pH. There are no data concerning effects of Al on rhizobial growth rate.

The objectives of this investigation were (i) to determine the effects of low P and high Al on the survival and growth rate of some rhizobia at low pH, (ii) to examine the relationship between acid-tolerance and Al-tolerance, and (iii) to rate the probable importance of the three stresses (acidity, P, and Al) according to their inhibitory effects on rhizobial growth.

MATERIALS AND METHODS

Rhizobia

The 65 strains of *Rhizobium* were obtained from three sources: University of Hawaii NifTAL Project, Paia, Hawaii; CSIRO Division of Tropical Crops and Pastures, Brisbane, Australia; and USDA Cell Culture and Nitrogen Fixation Laboratory, Beltsville, Maryland. Of the strains specifically identified in this report, those with a prefix TAL, IQ, or M are from NifTAL,

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[3] B. Rerkasem. 1977. Differential sensitivity to soil acidity of legume-*Rhizobium* symbioses. Ph.D. Thesis. Univ. of W. Australia, Nedlands.

[4] M. M. de Carvalho. 1978. A comparative study of the response of six *Stylosanthes* species to acid soil factors with particular reference to aluminum. Ph.D. Thesis. Univ. of Queensland, Australia.

Table 1—Media.

Basal solution: mannitol 10 g/liter; salts (μM) $MgSO_4$ 300, $CaCl_2$ 300, ferric EDTA 100, KCl 10, $MnCl_2$ 1, $ZnSO_4$ 0.4, $CuCl_2$ 0.1, Na_2MoO_4 0.02, $Co(NO_3)_2$ 0.002, distilled water.

Experimental treatment	Additions to basal solution	pH
	Experiment A	
(a) Full, defined, acid	500 μM KH_2PO_4 + 500 μM K_2HPO_4 + Na glutamate (1.1 g/liter)	4.6†
(b) Low P, acid	5 μM K_2HPO_4 + 5 μM KH_2PO_4 + 750 μM K_2SO_4 + Na gluatmate (1.1 g/liter)	4.6
(c) High Al	(b) above + 50 μM $AlK(SO_4)_2$	4.6
	Experiment B	
(a) Yeast	500 μM KH_2PO_4 + 500 μM K_2HPO_4 + yeast extract (1 g/liter)	6.3
(b) Full, defined‡	500 μM KH_2PO_4 + 500 μM K_2HPO_4 + Na glutamate (1.1 g/liter)	6.3
(c) Low P	5 μM KH_2PO_4 + 1,500 μM KCl + Na glutamate (1.1 g/liter)	6.3
(d) Low P, acid	as (c), Exp. B. above	4.5
(e) High Al	as (d), + 50 μM $AlK(SO_4)_2$	4.5

† Media acidified to pH 4.5 or 4.6 with HCl.
‡ Some strains required growth factors for good growth in defined media. When necessary, 1 ppm Thiamine, 1 ppm Ca-pantothenate, and 0.1 ppm biotin were included.

and those with a prefix CB are from CSIRO. Most of the strains were effective on at least one legume host.

Culture Media

The media, all liquid, are described in Table 1. Acid media were acidified with HCl before autoclaving. Aluminum was added after autoclaving as 5 mM $AlK(SO_4)_2$ or $AlCl_3$ solution sterilized by filtration. Measurements of pH just prior to inoculation showed that neither autoclaving nor addition of Al changed the pH. Aluminum and phosphate levels were designed to avoid precipitation of aluminum phosphate (Munns, 1965a). Samples of uninoculated medium (c) in experiment A (Table 1) were taken for analysis after centrifugation (Munns, 1965b) and analyzed for P by the colorimetric method of Watanabe and Olsen (1965) and Al by the 8-quinolinol method of Frink and Peech (1962). The analyses indicated 95% of the added P and all the added Al recoverable in solution in the supernatant.

Experiment A

Six strains from the cowpea miscellany were selected for growth studies in defined liquid media at pH 4.6. Three treatments were imposed (Table 1). Media were dispensed in duplicate 100-ml volumes into 250-ml Erlenmeyer flasks, plugged with cotton, covered with a small beaker, and autoclaved for 20 min. Bacteria from agar slopes of similar age were suspended and diluted so that 1 ml would provide an initial density of 10^3 to 10^4 cells per ml of media. The diluent contained equivalent concentrations of $MgSO_4$ and $CaCl_2$ at an ionic strength similar to that of the media. The cultures were incubated on a slowly reciprocating shaker in a constant temperature room at 25°C.

Rhizobial population density was determined by the agar pour plate method for total viable cells. Viable counting, though tedious, was necessitated by the low population densities required to maintain control of pH and Al (see below).

At intervals throughout the first 12 days of the trial, all treatments were sampled for measurement of rhizobial density and pH.

Experiment B

Sixty-five strains of rhizobia, 52 from the cowpea miscellany and 13 from *R. japonicum*, were tested for response to low pH, low P, and high Al in liquid media. Five treatments were applied (Table 1). In addition, the strains of *R. japonicum* were also screened in medium (d) (Table 1), adjusted to pH 4.8, and a medium of the same composition with 25 μM Al at pH

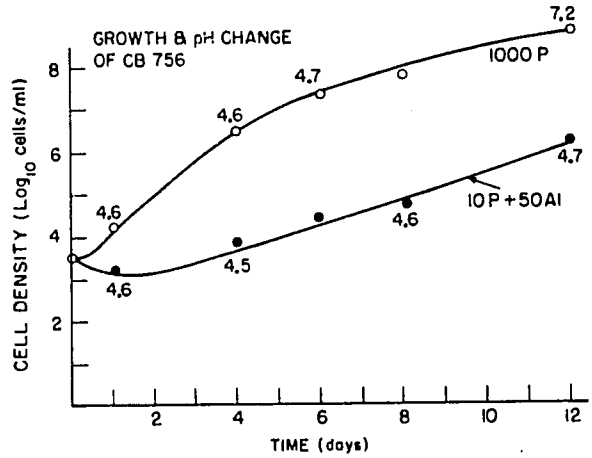

Fig. 1—Growth and pH change in liquid media for strain CB756. P and Al levels given in μM.

4.8. All strains were examined twice daily for detectable (by eye) turbidity, over a 25-day period. A few strains were selected for detailed sampling for viable cell count and pH change over an 18-day period. A complete listing of the strains is too lengthy to present here, but is available upon request from the senior author.

Media, inoculation, growth conditions, and counting were as in Experiment A, except that 50 ml volumes in 100-ml flasks were used for the cultures subject to detailed sampling, and the rest of the units were 5-ml volumes dispensed into 10 by 135-mm screw-cap culture tubes. These tubes were incubated in a slanted position on the shaker.

Visually Detectable Turbidity as a Measure of Growth

This screening trial required a reliable measure of growth less cumbersome and time-consuming than viable-counting. Nephelometry would be too insensitive, requiring densities of the order 10^7 per ml or higher (Vincent, 1970). It was assumed, however, that if a culture inoculated at only 10^3 or 10^4 cells/ml attained visually detectable turbidity, this would indicate considerable growth. A significant pH change would not be expected before considerable growth occurred. It was necessary to establish these assumptions: (i) the correlation of cell density with visual turbidity, and (ii) the dependence of pH change on the increase in cell density. The first relationship would yield an average cell density associated with attainment of turbidity. The second would indicate whether any change in pH occurred before attainment of turbidity. Therefore matched counts, pH, and turbidity observations, were taken in both experiments, A and B. In studying pH change with growth, 13 separate pairs of samples were taken from 6 different acid media for 7 strains. Attainment of visual turbidity was assessed by eye inspection against a diffuse-light background of inoculated units as compared to an uninoculated unit. For measuring cell density attained at turbidity, 19 separate samples were taken from 5 different acid media for 11 strains. The acid media here included a low-Ca medium (50 μM Ca) and a high-Mn medium (200 μM Mn), both at pH 4.5.

Not all 65 strains were screened at one time: a trial usually included 15 to 20 strains. To test consistency of treatment effects in time between trials, strains TAL174N and TAL209 were included in all treatments in each screening trial. They consistently become turbid at about the same growth time in a given treatment.

Sub-Experiment

To verify that the effect of $AlK(SO_4)_2$ was due to the Al moiety, its effect on TAL174N was compared with that of $AlCl_3$. Medium (d) (Table 1) of experiment B, modified by supplying Fe as $FeCl_3$ (5 μM) instead of Fe(III)EDTA, received no Al or additions of 25 and 50 μM as either $AlCl_3$ or $AlK(SO_4)_2$ (Fig. 4). Treatments were in triplicate. Culture volume was 5 ml.

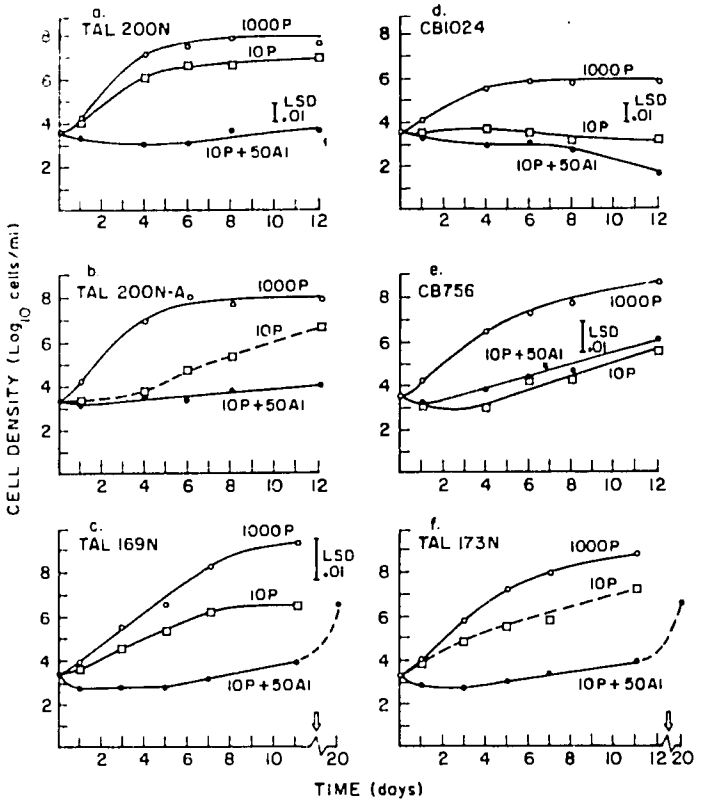

Fig. 2—Response of 6 cowpea miscellany strains to P and Al, given in μM.

RESULTS

Relationships of Turbidity and pH Change with Growth

The cell density associated with detectable turbidity in acid media was consistently $\sim 10^7$ ml (mean of $10^{6.8 \pm 0.4}$, with a range of $10^{6.0-7.6}$). All cultures in this investigation were inoculated at levels between 10^3 and 10^5 cells/ml, most below 10^4. Therefore, from 100- to 10,000-fold growth occurred before the culture became detectably turbid. The population required to significantly raise the pH of the medium was also about 10^7 cells/ml (mean of $10^{6.8 \pm 0.4}$, with a range of $10^{6.1-7.2}$). An example of pH change during growth in acid media is given in Fig. 1. Though almost all the strains eventually produced an alkaline reaction, measurements taken throughout the growth period showed that the rhizobia had to make several-fold growth before changing pH by 0.1 unit. The pH rose rapidly only when densities $\geq 10^7$/ml were reached. Thus, growth reflected real tolerance to the stress factors because rhizobia did not first raise the pH and then grow.

Detailed Growth Studies

Results of the detailed growth studies in experiments A and B are shown in Fig. 2 and 3. All the strains were in the cowpea group. Marked variation among strains in response to both P and Al is manifested in varying lag times, as well as reduced growth rates whether or not preceded by a lag.

Two strains, IQ68-5 and IQ921, were acid sensitive, unable to grow at pH 4.5 (Fig. 3c). Strain CB1024

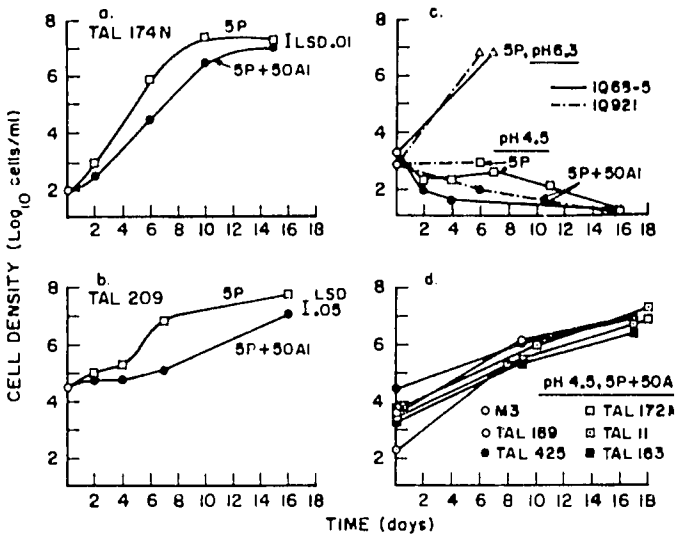

Fig. 3—Response of several cowpea miscellany strains to pH, P and Al. P and Al levels given to μM. Initial pH 4.5, except as noted (Exp. B).

was semi-sensitive to acid, unable to make vigorous growth at pH 4.6 even with 1 mM P (Fig. 2d). This strain responded slightly to inclusion of vitamins in nonstress medium, but not at low pH. Among acid-tolerant strains the presence of 50 μM Al was clearly the most severe stress.

Lag periods in low P media were usually less than in Al media, but large variation was evident. Strains TAL200N (Fig. 2b) and TAL173N (Fig. 2f) both displayed a 4-day difference in lag time between replicates of 10 μM P, so that the dotted line indicating their growth is approximate. In general, replicates varied much less in nonstressed treatments than in treatments that imposed P or Al stress.

Generation times were calculated over the period of exponential growth, usually from day 1 to day 4, following any lag period. For acid-tolerant strains, the mean generation time in 1 mM P was 8.5 ± 1.2 hours. In low P media (5 or 10 μM), generation times ranged from 10 hours for TAL174N (Fig. 3a) to 23 hours for CB756 (Fig. 2e). In 50 μM Al the generation times ranged from 13 hours for TAL174N (Fig. 3a) to over 99 hours for TAL200N (Fig. 2a).

Figure 3d shows the response to 50 μM Al by six strains that had similar high tolerance, comparable to strains TAL209 (Fig. 3b) and CB756 (Fig. 2e). In low-P media, with or without Al, the maximum cell density attainable usually was between 10^7 and 10^8 cells/ml: apparently P was exhausted.

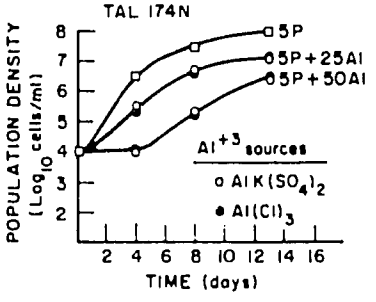

Fig. 4—Response of TAL174N to different Al sources. P and Al given in μM. Initial pH 4.5, with Fe as 5 μM $FeCl_3$.

Figure 4 shows the results of adding Al as two sources on growth of TAL174N. The equal effect of both sources verifies that the inhibitory effect of the Al compounds was due to Al.

Table 2 lists tolerances to the various factor combinations for all strains. The data were combined from experiments A and B. For the cowpea miscellany with 5 μM P, the average time to reach turbidity was 8.3 days at pH 6.5, 10.2 days at pH 4.5, and 13.4 days at pH 4.5 + 50 μM Al. These values are derived only from the strains that grew in the given medium within 25 days. For *R. japonicum* at 5 μM P, the average time to achieve turbidity was 7.0 days at pH 6.3, 11.3 days at pH 4.8, 17.3 days at pH 4.8 + 25 μM Al, and 15 days at pH 4.5 + 50 μM Al. In yeast and control defined media, the times were 5.2 and 7.3 days, respectively.

DISCUSSION

The data show that Al is a potent stress to the growth of free living rhizobia. Even for tolerant strains, Al reduced growth rate, and often lengthened lag phase. The data verify recent evidence that some strains of slow-growing rhizobia can survive high concentrations of Al (Rerkasem 1977; de Carvalho 1978);[3,4] but the large reduction in growth rate shown here could be critical for colonization of soil and rhizosphere, and for induction of nodulation (Munns, 1968; Vincent, 1974). Aluminum has been clearly shown to inhibit nodulation of *Stylosanthes* spp. (de Carvalho 1978).[4]

The Al concentrations imposed in these trials were chosen from data of displaced solutions from acid soils (Pearson, 1975; Pearson and Adams, 1967). Also, the 50-μM concentration corresponded to an Al activity of 21.8 μM calculated from the first-approximation Debye-Huckel equation (Adams, 1974), and this activity is well within the range found in acid soil solution (Pearson, 1975). Thus, the 50-μM Al level is a realistic one that rhizobia might encounter in acid soil.

Acidity itself was a severe stress, preventing growth of about one-third of the rhizobia. Tolerance of acidity did not necessarily confer tolerance of Al; about 40% of the strains tolerant of pH 4.5 could not tolerate the Al toxicity that would normally be associated with the acidity in soil.

Tolerance to both acidity and Al was rated at low (5-10 μM) P because the high (1,000 μM) P concentration precludes the existence of toxic Al concentration and is not likely to exist in soil.

The low P concentration itself inhibited growth of some strains, but with less severity than acid or Al. Most soil solutions contain P at concentration ≤ 1 μM (Reisenauer, 1966). Soils, unlike the test media, are buffered with respect to phosphate. Only in laboratory media would rhizobia normally encounter the extremely high concentration of 1,000 μM.

Comparison of Al tolerance among the two groups of rhizobia suggests that the cowpea rhizobia are more tolerant to Al than soybean rhizobia. However, there were perhaps too few strains on which to judge *R. japonicum*. Nonetheless, both groups show similar features. First, within each group there is strain-to-strain variation in tolerance; second, acid-tolerance and Al tolerance are separate, as they are for higher plants (Andrew et al., 1973); and third, Al at realistic concentrations appeared to be more commonly a severe stress than low pH or low P.

This study was limited to slow-growing rhizobia. Some fast-growing strains might also have useful acid and Al tolerance. Studies from soils acid enough to support moderate concentrations of soluble Al suggest that *R. trifolii* might contain such strains (Munns, 1965a; Jensen, 1969).

The screening procedure of experiment B may prove to be a useful and simple method for detecting tolerant strains. It obviates the need for pH control during growth by the simple precaution of using a small inoculum, so that population density remains too small to raise the pH and precipitate Al until detectable turbidity is approached. Since Parker (1971) has shown that pH change in media by rhizobia is a function of organic composition, an improvement in the screening procedure might be to alter the media to prevent a large pH change. A desirable modification also might be reduction of the EDTA concentration to 10 μM. The response of TAL174N to 50 μM Al was less in the presence of Fe(III) EDTA than in the presence of $FeCl_3$ (compare Fig. 4 and 2a). This may be an effect of EDTA. Ferric EDTA was used because it would not interfere with availability of P, whereas $FeCl_3$ could exceed the solubility of $Fe(OH)_2H_2PO_4$ (Norvell, 1972). The stability constant of $Fe^{III}EDTA$ so greatly exceeds that of AlEDTA that essentially no Al complex should form at pH 4.5 (Norvell, 1972). However, after making sufficient growth in the presence of Al, the rhizobia might separate and absorb enough Fe to allow EDTA to start complexing Al. If so, the use of EDTA would diminish the effects of Al.

The importance of saprophytic competence in rhizobia has been emphasized by Chatel et al. (1968). Introduced bacteria often are unable to tolerate biotic or abiotic stresses in a new environment (Alexander, 1971). Identification of strains of *Rhizobium* having superior tolerance to mineral stresses may be a step towards improving chances of selecting successful inoculants for acid infertile soils. It now needs to be shown if rhizobial tolerances to acidity factors in pure culture relate to their tolerance in soil.

Table 2—Tolerance of pH, P and Al stress among 65 strains of rhizobia.†

Cowpea miscellany, pH 4.5

Category	Number of strains	% of total
Sensitive to pH 4.5	14	27
Tolerant of pH 4.5, sensitive to 50 μM Al	13	25
Tolerant of 50 μM Al	25	48

Rhizobium japonicum

pH 4.8			pH 4.5		
Category	Number of strains	% of total	Category	Number of strains	% of total
Sensitive to pH 4.8	0	0	Sensitive to pH 4.5	5	38
Tolerant of pH 4.8, sensitive to 25 μM Al	5	38	Tolerant of pH 4.5, sensitive to 50 μM Al	5	38
Tolerant of 25 μM Al	8	62	Tolerant of 50 μM Al	3	23

† Based on attainment of turbidity within 25 days from mean initial density of 10^{5} cells ml (Exp B)

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