Tolerances to Soil Acidity Factors Among Rhizobia

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ABSTRACT

Tolerance of low pH (4.5), low phosphate (5-10 µM), and high Al (50 µM) was assessed in 10 strains of cowpea rhizobia by detailed growth studies based on viable counts in defined liquid glutamate/mannitol media, and in 65 strains of cowpea rhizobia and R. japonicum by a rapid method based on attainment of turbidity from a small inoculum. Strains varied in response. Aluminum was the most severe stress.

Low P (as compared with 1000 µM) limited total attainable population density to 5 x 10^7 cells/ml, and slowed the growth of some strains. Acidity increased lag time or slowed growth of most strains, and stopped growth of about 30% of them. Tolerance of acidity did not necessarily entail tolerance of the Al that would normally be associated with soil pH 4.5. Aluminum (50 µM) increased lag or slowed growth of almost all the strains tolerant of low pH, and virtually stopped growth of half of them.

Despite weak buffering, pH was adequately controlled by working at low population density, beginning with inoculation at 10^3 cells/ml, because the pH did not rise by 0.1 unit until cultures had become almost visibly turbid at 10^7 cells/ml. Thus the rhizobia had to make 1000-fold growth under the stress before they could significantly raise pH and precipitate Al. Tolerant strains therefore had a real tolerance that might be expressed in buffered soil environments. A valid rapid screening can be based on ability to attain visible

turbidity in culture under acid or Al-stress, so long as inoculum level is small (<< 10^5 cells/ml).

Growth studios were done in defined liquid media to assess likely effects of Mn toxicity and Ca deficiency associated with soil acidity. The study included 23 strains of cowpea rhizobia previously found capable of growth at pH 4.5 and 10 strains of R. japonicum tolerant of pH 4.8. The low level of Ca (50 µM) represented the extreme low range in soil solutions, and the high level of Mn (200 µM) has been found toxic to legume hosts of the strains tested.

In a detailed growth study of 3 cowpea strains at pH 4.6, low P (10 µM) limited maximum viable cell density in all 3 strains. Low Ca limited it in 1 strain. High Mn reduced growth rates of 2 strains.

A rapid screening method based on attainment of turbidity from a small inoculum was applied to the cowpea rhizobia at pH 4.5 and soybean rhizobia at pH 4.8. High Mn and low Ca slowed growth of only 3 strains. Neither was as severe a stress as 50 µM Al, simultaneously observed and previously reported. All strains tolerant of Al were tolerant of Mn and low Ca.

Possible amelioration of Al toxicity by Ca was tested in 3 cowpea strains, by a factorial experiment with 3 Ca levels (50-1000 µM) and 4 Al levels (0-100 µM), at pH 4.5 in liquid media. Calcium had statistically significant pretective effect against Al in two strains, but the effects were small and probably of no biological or practical significance.

In acid soils, Al toxicity and acidity itself are probably more important limiters of rhizobal growth than Mn toxicity and Ca deficiency.

As a preliminary investigation into rhizobial adaptation to acid-Al stress, ten strains were introduced, each at widely varying inoculum levels, into pH 4.5-5 µM P - 50 µM Al broth medium. Five strains attained relatively high population levels in 17 days from very few initial cells, three strains needed higher initial numbers to make net growth, and then had slower growth rates than the first group, and one strain showed death of cells at all inoculum levels. It was concluded that the mechanism of Al-adaptation was not spontaneous mutation, being due either to the existence of genetic variants within a population or the phenotypic adaptation of similar cells.

In greenhouse pot trials, 25 strains of rhizobia were tested on 3 host varieties of Vigna unguiculata (cowpea) on 2 acid soils. The symbiotic effectiveness of strains at pH 4.6 and 6.0-6.2 was compared with their known tolerances to acidity and Al in defined laboratory media. One soil was naturally acid at pH 4.6, and the other was acidified to this pH with $Al_2(SO_4)_3$. Large differences were found between strains in ability to produce good plant growth at low pH. The general effects of acidity on both soils was reduced plant growth associated with decreased nodule abundance and mass, as compared with that at pH 6.0-6.2. Symbiotic growth at low Ph in the soil which received $Al_2(SO_4)_3$ was more inhibited than in the soil with the same pH but lower available Al. Most importantly, the strains which gave the

best yields in both soils at pH 4.6 were all tolerant of acid and Al in laboratory media. The prescreening of strains in various acid broth media appears to be a promising, worthwhile procedure in selecting tolerant candidate strains for use in acid soils.

INTRODUCTION AND LITERATURE REVIEW

This thesis reports on the effects of certain soil acidity factors upon the growth and symbiotic performance of slow growing strains of the symbiotic, nitrogen-fixing bacteria Rhizobium.

The natural, nonsymbiotic habitat of rhizobia is the soil, and the immediate environment is that of the soil solution. In an acid soil, besides the high hydrogen ion activity in solution, there are several mineral elements whose pH-dependent solubility and reaction with the soil matrix can produce relative toxic or deficient growth effects on higher plants (Pearson and Adams 1967; Pearson 1975; Kamprath 1972). Presumably these same factors could be stressful in rhizobia also, though much less information is available on this (Munns 1977b; 1978; Rerkasem 1977; Carvalho 1978).

The most commonly reported mineral elements producing these effects in acid soils are calcium, magnesium, phosphorus, and molybdenum in the deficiency category, and aluminum, manganese, and hydrogen-ion in the toxicity category (Pearson and Adams 1967; Pearson 1975; Munns 1978). Though any one of these soil acidity factors alone can produce a biological stress, in many acid soils several of them occur together, and would then interact in their effects on the plant or microbe (Munns 1977b; Pearson 1975).

The factors chosen for study of their effects on rhizobia reported in this thesis are calcium, phosphorus, manganese, and aluminum, at low pH in pure culture studies, as well as the effects of two acid-infertile soils.

The Bacterium

The Eubacteriales genus Rhizobium is characterized as gram-negative, nonspore-forming, short rods, about 0.5 to 0.9 by 1.2 to 3.0 µM, though it is commonly pleomorphic under adverse stress condtions (Jordon and Allen 1975; Vincent 1962). Members of the genus characteristically invade roots of legumes and induce root nodules to form, and the different strains exhibit host range affinities. The nodule bacteroids are involved in fixing molecular nitrogen into combined forms utilizable by the host plant (Vincent 1970).

The bacteria are chemoorganotrophs with a respiratory metabolism. They are aerobic, and often able to produce excellent growth under 0_2 tensions less than 0.01 atmosphere. The optima for temperature and pH is 25-25°C and 5.0-8.5 respectively. Most satisfactory growth is provided by media containing yeast or other plant extracts, and yeast extract-mineral salts media containing mannitol or glucose are among the most conventional (Jordan and Allen 1975).

The taxonomic division of Rhizobium (Jordon and Allen 1975) is based upon host affinity for nodulation, and two major groups are recognized:

Group l - 2 to 6 peritrichous flagella, rapid growth on yeast extract media.

R. leguminosarum	R. trifolii
R. phaseoli	R. meliloti

Group 2 - polar or subpolar flagellum, slow growth on yeast extract media.

R. japonicum R. lupins

Another large group of mostly slow growing strains is recognized by many, referred to as the "cowpea rhizobia" or the "cowpea miscellany", as well as a smaller, odd group termed the lotus rhizobia (Vincent 1970, 1974).

The representative preferred hosts for all of the species are compiled elsewhere (Jordon and Allen 1975; Vincent 1970, 1974).

As Vincent (1974) points out, the distinction between the fast and slow growing rhizobia is well established on the basis of growth characteristics, acid production in synthetic media, utilizable carbon source, complement of glycolytic enzymes and internal antigens, as well as evidence from numerical taxonomy.

A Review of the Pertinent Literature

pH

Of all the soil acidity factors, the effects of pH per se on rhizobia has been the most thoroughly researched. Early work on the response of several strains to low pH liquid media revealed that the different species of rhizobia had different critical pH for growth, R. meliloti being the most acid sensitive with a critical pH of 4.9, and the slow growing R. japonicum and R. lupini were the most acid

tolerant, having critical pH of 3.3 and 3.15 respectively (Fred and Davenport 1918).

From a study of 8 strains of R. japonicum, Wright (1925) found the critical pH range in liquid media to be 4.1 to 4.5, though 5 of the 8 strains showed net viability decrease at pH less than 4.4 within 2 weeks.

Fairly good agreement with these values was found by Bryan (19.23) who introduced strains of different species into acid soils and found the critical pH for their recovery to be 5.0 for R. meliloti, 4.5-4.7 for R. trifolii, and 3.5-3.9 for R. japonicum. Comparing 2 fast growing species for acid tolerance in liquid media, Jensen (1942) confirmed that R. trifolii is more tolerant than R. meliloti, but both were inhibited below pH 5.

The most extensive investigation of the sensitivity to pH among rhizobia was conducted by Graham and Parker (1964) who tested 79 strains in liquid medium and found the general order of tolerance to low pH to be R. meliloti other fast growers slow growers. Of great significance was the demonstration of differential strain tolerances within each species. Their findings, *as* well as more recent reports (Graham and Hubbell 1975; Broughton et al. 1975) puts the critical pH range for the more tolerant slow growers at 4.3-4.9, a range more conservative than the earlier studies.

The results from several studies on the effects of acidity as tested in soil give good agreement with artificial media tests concerning the more important observations; the critical pH ranges, the relative tolerances between rhizobium species, and within species

the existence of strain to strain differences. Studies on isolates of the slow grower R. japonicum from Iowa soils show that some strains (sero-groups) are indeed acid tolerant with a lower pH limit for growth of 4.0, while there exist large sero-group differences in adaptation to soils in the 7.5-8.0 range (Damirgi et al. 1967; Ham et al. 1971). Among the fast growers, R. trifolii is found to persist better in soils at a lower pH than R. meliloti, though they both exhibit increased population levels in more neutral soils (Jones 1966; Jensen 1969; Munns 1965a; Nutman and Ross 1969; Peterson and Gooding 1941). Even the acid sensitive R. meliloti is found to have significant strain differences to survival in acid soil (Munns 1965a; Robson and Loneragan 1970a) so that these authors noted the possible benefits accruing from selection of R. meliloti strains for their performance in acid soils. Also, Lie (1971) found the same strain differentiations occurring at low pH among R. leguminsoraum, and these differences were not predictable from the strain's symbiotic performance at a neutral pH. Related studies of the cowpea type rhizobia show that in this acid tolerant group there also exists strain variation (Norris 1973; Rerkasem 1977).

The general association of the most acid tolerant rhizobia with the slow growth habit was a fundamental tenet in Norris' hypothesis (Norris 1965) that the prototype Rhizobium was a cowpea type organism that evolved in the wet, acid soils of the tropics, along with the tropical legumes, this group also supposedly being the more primitive line of the host plant family. Norris associated a broad host-range compatibility with this primitive condition of the symbiosis. From

tests on over 700 strains, he emphasized that the slow growing strains produced a net alkaline reaction on agar medium and that they were associated with, or isolated from, the most acid tolerant hosts, as well as themselves being the most acid tolerant group of rhizobia, and that there would be an ecological advantage for an alkaline producing strain over an acid producing strain (the fast grower) in an acid soil. Though there are reports giving support for some aspects of his hypothesis (Norris 1973; Brockwell et al. 1966; Rerkasem 1977), Parker (1968) has challenged many theoretical aspects of the hypothesis, and specifically Parker (1971) was able to demonstrate that the relative pH alteration of media is very dependent on its composition and that it would be unreliable to assume that the rhizobia produce similar alterations of pH in the buffered soil itself. Neither slow nor fast growing rhizobia were able to alter the pH of an unmodified soil extract solution, despite good growth, thereby showing no relationship with their respective abilities to change the pH in conventional media. Also, Munns et al. (1978) in a screening of 40 strains of the cowpea miscellany found only a weak association between alkali production in media and symbiotic acid tolerance, alkali production being a very imprecise indicator of acid tolerance. There appears to be little disagreement over the relative acid tolerances of the species of Rhizobium, but there is not enough information available concerning the evolution of the symbiosis or the bacterial mechanisms of acid tolerance to put these together into a unifying concept that relates them all.

Several soil studies have shown that applied rhizobia fail to persist or colonize in acid soils (Vincent and Waters 1954a & b; Vincent 1958; Robson and Loneragan 1970a; Mulder et al. 1966). Related research has shown that large innoculum levels can somewhat replace the beneficial effect of timing (Spencer 1950; Vincent and Waters 1954b; Mulder and Van Veen 1960; Robson and Loneragan 1970a; Rerkasem 1977), this being demonstrated for R. trifolii, R. meliloti, and the cowpea miscellany.

There are reports in the literature that poorly effective, indigenous, strains of rhizobia are more common in acid soils, with the effectiveness increasing in neutral soils (Holding and King 1963; Jones 1966; Jones and Burrows 1969). Also, Van Schreven (1972) demonstrated differences in strain response to the effect of subculturing at low pH on agar media for 300 days on this subsequent effectiveness. Holding and King (1963) suggested that the more ineffective strains found at low pH may be more tolerant of elements such as Mn and Al as compared with more effective strains. While all the above work on the relationship between ineffectiveness and soil acidity has been done with R. trifolii, Munns et al. (1978) working with the cowpea miscellany group found no trend for the symbiotic acid tolerant strains to be reduced in comparative effectiveness.

Abundant literature exists on the effects of acidity on different aspects of the symbiosis as separate from effects on the Rhizobium per se (Vincent 1965; Andrew 1976a; Jensen 1943, 1947; Lowther and Longeragan 1970; Munns 1968), and the reader is directed to excellent,

current review on this subject (Andrew 1978; Munns 1977a & b, 1978; Lie 1971, 1974; Rerkasem 1977; Carvalho 1978).

Calcium

The requirement for Ca as an essential nutrient for rhizobia is quite small, as determined in liquid media (Loneragan and Dowling 1958; Norris 1959; Bergersen 1961; Vincent 1962). Vincent (1962) shows that the Ca requirement is about 25 μM for normal growth in liquid culture. In that study he also found no effect of pH down to 5.5 on the response to Ca levels. Humphrey and Vincent (1962) have shown that the Ca is an essential component of the cell wall fraction of the Rhizobium.

While Bergersen (1961) did find differences between strains in growth characteristics in low Ca broth, Norris (1959) reported differences in Ca requirement between fast and slow growing rhizobia when studied in electrodialyzed clay systems. Rerkasem (1977) points out that the differential Ca requirements noted by Norris may only reflect a difference in ability to obtain or retain calcium in those conditions. Nonetheless, she reports that many fast growers responded to increased Ca at pH 3.9, while many of the slow growers studied showed no such response above pH 2.8. Also, Lowther and Longeragan (1970) obtained a positive response to Ca for the fast growing R. trifolii in the rhizosphere of subclover in solution culture at pH 5.0.

Reports are scant on the effects of neutral calcium salts on rhizobia in soil. Albrecht and Davis (1929) reported better survival of rhizobia in soil, as assessed by nodulation on a second crop of

soybeans, when $CaCl_2$ was supplied. Anderson and Maye (1952) suggested that the gradual improvement of modulation on an acid soil might be attributable to the Ca or P applied in the basal superphosphate fertilizer used. However, recent work of Rerkasem (1977) showed no effect of $CaS0_4$ addition on growth or survival of either a fast or slow growing cowpea rhizobium in soil at pH 4.5.

The low Ca requirement for rhizobia appears below that needed for adequate nodule initiation and function, and general symbiotic growth, those aspects of the slymbiosis as affected by Ca being reviewed elsewhere (Munns 1977a & b, 1978; Andrew 1978; Robson 1978). It appears then that a deficiency of Ca in acid soil would probably have deleterious effects on modulation and legume growth before it would limit rhizobial growth and survival, but there is insufficient data on important rhizosphere effects to make this a definite conclusion (Munns 1977b).

Phosphorus

Phosphorus as a soil acidity factor is important because it is often limiting to plat growth, being available at low levels in acid soils (Hsu 1965; Fox 1978; Kamprath 1973; Sanchez 1976). Though it is accepted as an essential nutrient for rhizobia, the quantitative requirement for P in the free living state has not been reported. Luria (1960) reports the range of dry weight P content in bacteria to be 2 to 6 percent.

Truesdell (1917) added 3 levels of phosphate, as the Na, K or Ca salt to sterilized soil, and between the 2 highest levels obtained large growth responses with introduced R. meliloti. Kamata (1962)

reported that some strains of R. japonicum did not nodulate P-deficient soybean roots, and this was correlated to the relative P response of the two strains in culture media. Clearly the quantitative response of rhizobia to P in synthetic media and soil needs further elucidation.

In the legume-Rhizobium symbiosis, phosphorus is required in the ATP functioning in the nitrogen fixation process (Bergersen 1971). There are reports of added P improving nodulation, but no clear indication that these are not simply due to improved host growth (Munns 1977b; Carvalho 1978; Fox 1978; Andrew and Jones 1978).

Manganese

In acid soils, manganese toxicity can be a stress factor to plant growth (Pearson 1975; Pearson and Adams 1967; Sanchez 1976). While it is difficult to find data on soil solution concentrations associated with manganese toxicity, Asher and Edwards (1978) say that a value of about 100 μM is sufficient to cause toxicity to leguminous plants, and this is in fair agreement with solution levels found in acid, Mn-toxic soils (Morris 1948). Studies on the effects of Mn on nonsymbiotic legumes in solution culture show that most species are reduced in growth at manganese concentrations between 100 and 200 μM (Morris and Pierre 1949; Andrew and Regarty 1969).

Wilson and Reisenauer (1970) demonstrated a lower optimum growth range for Mn by rhizobia to be between 0.1 and 1 μM, and found no depressive effect of the highest level, 10 μM.

Two strains of R. phaseoli have been reported to differ in tolerance to Mn at very high levels, up to 400 ppm in culture media

(Dobereiner 1966). However, when these same strains were used as inocula in acid soil with manganese added to inhibitory level, they displayed different effects on reduction in nodule numbers or in nitrogen fixation per unit nodule weight, indicating Mn toxicity effects are different for bacterial growth than for the symbiotic relationships. The author also stressed the importance of selecting rhizobia for their tolerance to soil acidity when selecting inocula for such problem soils.

Masterson (1968) found that R. trifolii isolates from mineral acid soils were less effective on their host than isolates from acid peat soils, implying that the more tolerant strains were less effective. He suggested the peat may provide some protection from high levels of Mn, Al or Fe found at low pH. He also reported data of Sherwood where culturing strains of R. trifolii on media with 800 to 1500 ppm Mn showed little effect, and in some cases raised the effectiveness. Related to these observations are those of Holding and Lower (1971) showing that high levels of Mn (16 mm) in continuous culture can reduce the symbiotic effectiveness of R. trifolii. The effect was transient though, in that good effectiveness was recoverable upon transfer to media low in Mn.

In studying the effects of Mn on nitrogen fixation in Beijerinckia and Azotobacter, Becking (1961) found differential strain tolerance to 20 ppm Mn for Azotobacter, and the Beijerinckia strain was little affected by 40 ppm, except at a low pH of 3.0-3.2.

The effects of Mn on the symbiotic and nonsymbiotic legume are reported elsewhere (Andrew and Regarty 1969; Robson and Loneragan

1970b; Vose and Jones 1963; Morris and Pierre 1949; Souto and Dobereiner 1969; Dobereiner and Aronovich 1965; Dobereiner et al. 1965), as well as in recent reviews (Andrew 1978; 1976; Munns 1978). A survey of this literature reveals that Mn as a soil acidity factor can be quite limiting to symbiotic legumes, and that the effects could be more clearly understood with more knowledge of the effects of Mn at low pH on the rhizobia.

Aluminum

The high levels of aluminum that occur in soil solutions at low pH can be quite toxic to plant growth, and its abundance on the exchange sites of acid mineral soils make it the most important soil acidity factor (Kamprath 1972; Foy 1976; Pearson 1975; Andrew 1978). The few reports dealing with the effects of Al on rhizobia and other, microorganisms are reviewed here.

The important effects of Al on the functioning of the symbiotic and nonsymbiotic legume are detailed elsewhere (Munns 1965b; Carvalho 1978; Andrew et al. 1973; Foy and Brown 1964; Foy et al. 1969), and discussed in recent reviews (Asher and Edwards 1978; Andrew 1978). The work of Carvalho (1978) alone shows clearly that Al is more detrimental to symbiotic versus nonsymbiotic growth for Stylosanthes, though there was a range of tolerance among the 6 species tested. And since only 1 rhizobial strain was used, it merits further research to see if strains with superior Al-tolerance could be identified and compared with less tolerant strains in their symbiotic performance with a tolerant host.

Much of the work on Al suffers from lack of control of Al concentration and pH (Munns 1965b; Andrew 1978). In studies concerning the levels of P and Al available in nutrient solutions at low pH, Munns (1965b) reported that if P was kept below 50 µM, (1.5 ppm) at pH 4, or below 10 PM at pH 4.5, then Al concentrations on the order of 100 µM (2.7 ppm) could be maintained without reaction with phosphate. Andrew (1978) pointed out that many reports on the effects of Al on plants have utilized treatments that greatly exceeded the solubility limitations in the pH-P-Al system, and notes that even at low pH, Al concentrations in soil solutions rarely exceed 4 ppm.

Early research by Whiting (1923) demonstrated that rhizobia were not inhibited in their growth by addition of insoluble Al salts, but were inhibited by Al salts which lowered the pH of the media, though no data was given.

The existence of differential species tolerance to Al among Azotobacter has been shown by Katznelson (1940). In liquid media, with a pH range from 4 to 7, and with Al up to 100 ppm, A. indicum was much more tolerant than A. chroccoccum, the former not being seriously inhibited except at pH 4.0 with 100 ppm Al. The effects of Al at low pH were clear, if not the actual aluminum levels; many of the treatments probably exceeded the solubility of gibbsite.

In a similar study, Becking (1961) investigated effects of Al on the nitrogen fixation (growth) of 2 strains of Beijerinckia and one of Azotobacter in liquid media. The results showed the superior tolerance of Beijerinckia over Azotobacter, although the Al standard

solution was neutralized before addition, and the organisms lowered the pH of the medium.

Chlorella pyrenoidosa, a green alga, has been reported by Foy and Gerloss (1972) to be quite tolerant of aluminum, and a strain having yet greater tolerance was obtained by adaptation to increasing Al stress. Again, while the relative effects of A1 were clear, the quantitative levels of Al reported are open to question because some of the treatments would have exceeded the solubility of variscite.

The only direct studies of effectively controlled Al level on rhizobia relate to survival, not growth rate. Rerkasem (1977) has shown that a slow growing strain of cowpea rhizobia that was little affected by introduction to soil with pH 4.5 displayed a decrease in viability when in the same soil acidified to pH 3.9-4.1 with aluminum sulphate, while a fast cowpea strain was inhibited by pH 4.5 alone. The presence of the rhizosphere environment of the host root enabled both strains to better survive in acid soil. Also, in short term tests on retention of viability (20 minutes) over a wide pH range, she also found that many slow growers were unaffected by low pH or high Al, while the fast growers were inhibited by the acidity itself, with no extra detrimental effect of the Al. The addition of 1 MM $Al_2(SO_4)_3$ caused all 12 strains of the fast growers to flocculate, whereas none of the 12 slow growers were flocculated, the difference between the two groups probably reflecting some basic characteristic as cell surface properties.

A recent study by Carvalho (1978) investigated the effects of Al on the symbioses of 6 Stylosanthes species with the slow growing,

cowpea strain C8756. In looking at the effect of Al on the microbial component of the system, she compared the viability of the strain in nutrient solutions at pH 4.5 and with none, 25 or 100 μM Al and found no differences, though all treatments displayed a slow decrease in population density through 8 days.

While the studies of Rerkasem and Carvalho show that some rhizobia can survive at high levels of Al, there is insufficient evidence to establish Al-tolerance in rhizobia as distinct from acid-tolerance, and no data concerning effects on their growth rate.

SECTION 1

EFFECTS OF ACIDITY, ALUMINUM AND PHOSPHATE ON GROWTH OF RHIZOBIA

INTRODUCTION

Effects of acidity on species of *Rhizobium* are well documented. Pure culture studies (Fred and Davenport 1918; Wright 1925; Jensen 1942; 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 (Bryan 1923; Damirgi et al. 1967; Jensen 1969; Nutman and Ross 1969; Norris 1973; Rerkasem 1977). Also within each species, important strain to strain variation has been demonstrated (Graham and Parker 1964; Munns 1965a; Damirgi *et al*. 1967; Ham *et al*. 7.971; Lie 1971; Norris 1973; Rerkasem 1977).

Besides low pH per se, acid mineral soils have low levels of phosphorus and high levels of aluminum (Hsu 1965; Kamprath 1973; Pearson 1975; Probert 1978). There has been little research on the effects of these two soil factors on rhizobia. The reports on effects of P on rhizobia are limited to an early study showing positive growth response to P additions in soil (Truesdell 1917), a report relating inability to *R. japonicum* strains to nodulate P-deficient soybeans

with their relative response to P in culture media (Kamata 1962), and a report demonstrating that three strains of rhizobia were better than two other common bacteria at taking up P from very low concentrations in solution (Werner and Berghauser 1976). Concerning Al, Whiting (1923) stated that Al salts inhibited rhizobial growth only when they lowered the pH of the culture media. Recent studies (Rerkasem 1977; Carvalho 1978) 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 (acid, P and Al) according to their inhibitory effects on rhizobial growth.

MATERIAL AND METHODS

Rhizobia

The 65 strains of rhizobia were obtained from three sources: University of Hawaii NifTAL Project, Paia, Hawaii; CSIRO Division of Tropical Crops and Pastures, Brisbane, Australia; 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, and those with a prefix CB are from

CSIRO. Most of the strains have been shown to be effective on at least one of the following legume hosts; Vigna unguiculata, V. radiata, Arachis hypogea, Stylosanthes guyanensis. Cultures were maintained on yeast mannitol agar slopes, refrigerated, with 3-monthly renewal and purity checks (Vincent 1970).

Culture Media

The media, all liquid, are described in Table 1. Acid media were acidified with RU before autoclaving. Aluminum was added after autoclaving as a 5 mM $AlK(S0_4)_2$ or $AlCl_3$ solution sterilized by passage through a membrane filter with 0.20 uM openings. Measurements of pH on additional samples just prior to inoculation showed that neither autoclaving nor addition of Al changed the pH of the acid solutions. Aluminum and phosphate levels were designed to avoid precipitation of aluminum phosphate, according to published solubility criteria and experimental work in similar solutions (Munns 1965a). Samples of uninoculated medium (c) in experiment A 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 analysis indicated 95% of the added P and all of 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 minutes. Bacteria from agar

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, $Ca(NO_3)_2$ 0.002, distilled water.

Experimental Treatment	Additions to Basal Solution	pH
Exp. 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 glutamate (1.1 g/liter)	4.6
(c) high Al	(b) above + 50 μM $AlK(SO_4)_2$	4.6
Exp. 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 + 1500 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.

slopes of similar age were suspended and serially diluted so that introducing l ml into flasks would give an initial density of 10^3 to 10^4 cells per ml. The diluent contained equivalent concentrations of $MgS0_4$ and $CaCl_2$ at an ionic strength similar to that of the media. One ml of the dilution was used to inoculate each replicate treatment. The inoculated cultures were then incubated on a slowly reciprocating shaker in a constant temperature room at 25°C.

Rhizobial population density was determined by the agar plate method (Vincent 1970) for total viable cells, after serial dilution in routine yeast mannitol medium. Counts were made on duplicate plates of 30 to 300 colonies per plate after 6 to 10 days' incubation. Colonies were counted under a low-power dissection microscope. Where the highest dilution exceeded 300 colonies per plate, a transparent square-centimeter grid was placed under the plate and individual squares totalling 10% of the plate area were counted. 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 rhizobial density and pH. In-sampling for cell density, either 1 ml or 0.1 ml was taken using a sterile serological pipette. To saturate the inner wall of the pipette with bacteria, the sample was drawl up and released eight times before transferring the final sample to diluents or petri plate for counting. In sampling for pH, a 3 ml volume was removed aseptically and tested with a combination glass electrode.

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), adjusted to pH 4.8, and a medium of the same composition with 25 µM Al at pH 4.8. All strains were examined twice daily for detectable 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.

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 10x135 mm screw-cap culture tubes. These tubes were incubated in slanted position atop 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 supposed, however, that if a culture inoculated at only 10^3 or 10^4 cells/ml attained visually detectable turbidity, about 10^7 cells/ml, this would indicate considerable growth, perhaps with no significant shift from nominal pH and therefore no precipitation of Al, at least during the earlier stages. It was necessary to establish two relationships: (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 growth occurred prior to any change in pH by alkali-producing strains, or only after it. Therefore paired counts and pH, and counts and turbidity observations, were taken n 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. 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 became 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) 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 Al as either $AlCl_3$ or $AlK(SO_4)_2$ (fig. 4). Treatments were in triplicate. Culture volume was 5 ml.

RESULTS

Relationships off Turbidity and pH-Change with Growth

TABLE 2. RELATIONSHIPS BETWEEN TURBIDITY, pH-CHANGE, AND POPULATION DENSITY.

	no. of tests	$\log_{10}$ cells/ml		
		mean	std. dev'n	range
(a) population density required for detectable turbidity	19	6.8	0.39	6.0-7.6
(b) population density required to raise pH by 0.1 unit	13	6.7	0.37	6.1-7.2

Table 2a shows cell density associated with detectable turbidity in acid media was consistently ~10^7 cells/ml. All cultures in this investigation were inoculated at levels between 10^3 and 10^5 cells/ml, most below 10^4. Therefore, from 2 to 4 orders of magnitude of growth occurred before the culture became turbid. With the restriction that initial inoculum level has to be < 10^5 cells/ml, as determined by viable count, attainment of visually detectable turbidity is a reliable criterion of significant growth.

Table 2b shows that the population required to significantly raise the pH of the medium was also about 10^7 cells/ml. 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. Evidently, strains which made good growth in acid or acid-Al media could increase in number by up to 4 orders of magnitude before appreciably changing the pH and precipitating Al. This reflects real tolerance to the stress factors. The 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 figs. 2 and 3. All the strains were in the cowpea miscellany. Marked variation between 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.

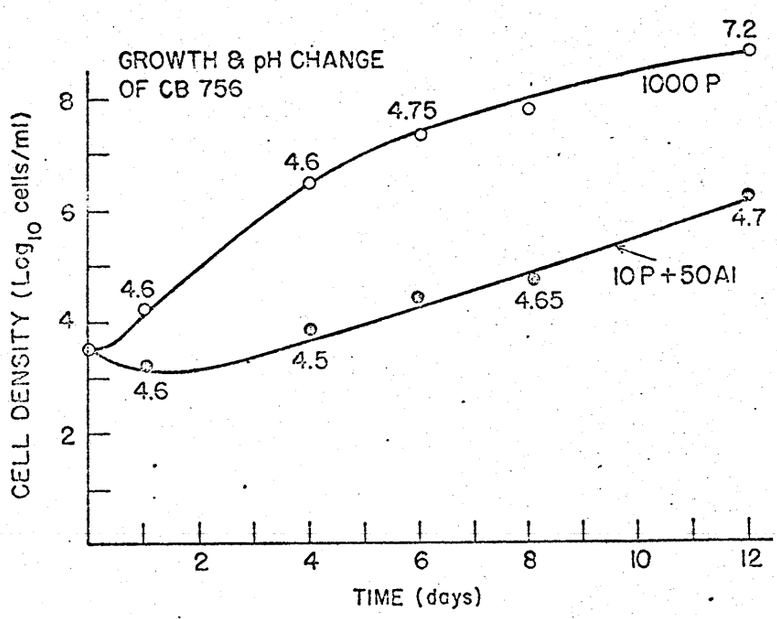


Figure 1 - Growth and pH change in liquid media for strain CB756. P and Al levels given as μM.

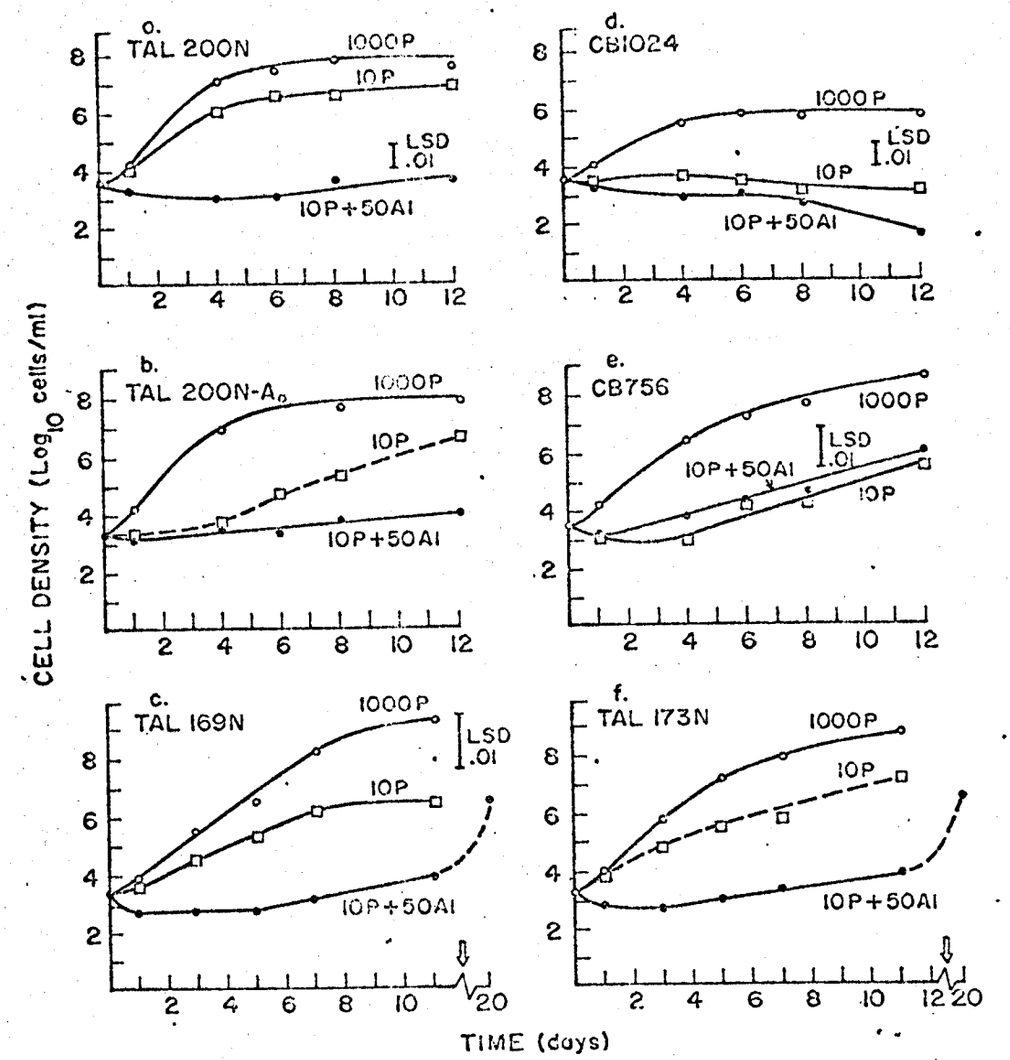


Figure 2 - Response of 6 cowpea miscellany strains to P and Al, given in μM. Initial pH 4.6 (Experiment A).

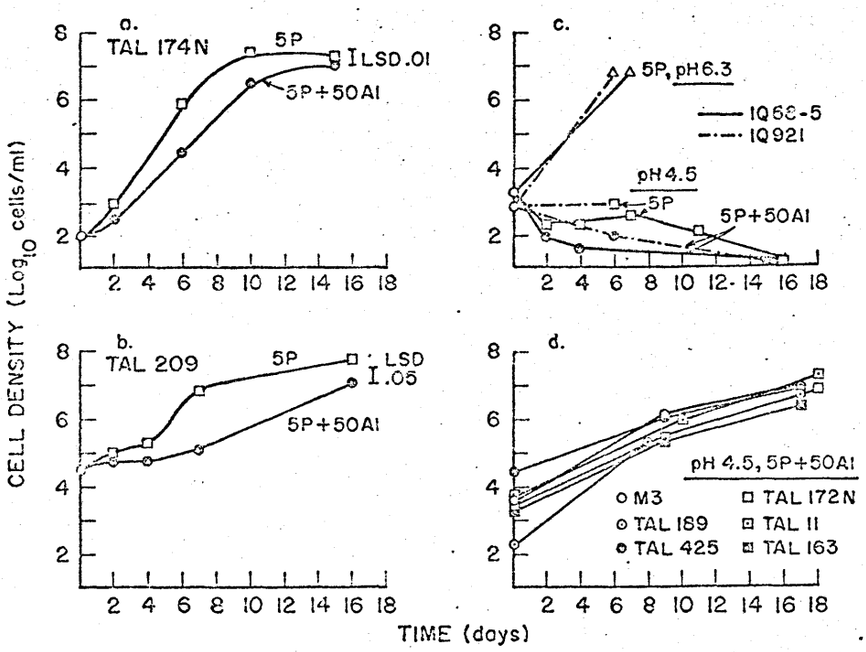


Figure 3 - Response of several cowpea miscellany strains to pH, P and Al. P and Al levels given in μM. Initial pH 4.5, except as noted (Experiment B).

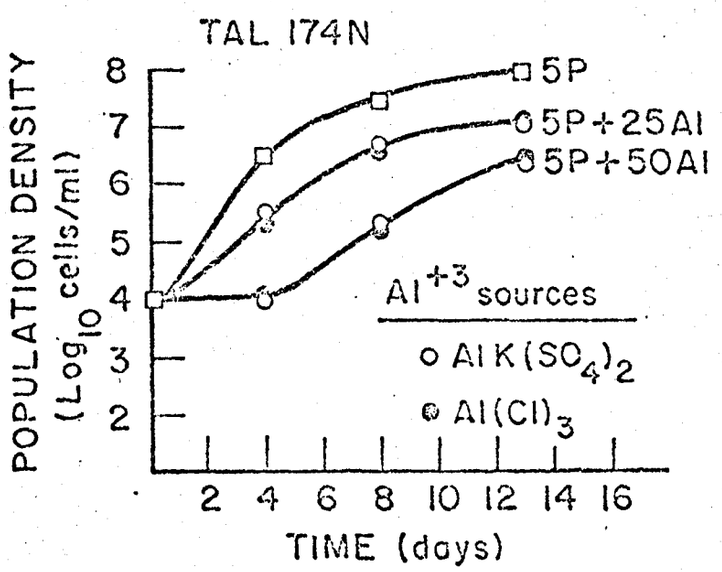


Figure 4 - Response of TAL174N to different Al sources. P and Al given in µM. Initial pH 4.5, with Fe as 5 µM $FeCl_3$.

Two straits, IQ68-5 and IQ921, were acid sensitive, unable to grow at pH 4.5 (fig. 3c). Strain CB1024 was semi-sensitive to acid, unable to make vigorous growth at pH 4.6 even with 1 mM P (fig. ld). This strain responded slightly to inclusion of vitamins in non-stress medium, but not at low pH. In the remaining, acid-tolerant strains, the presence of 50 µM Al was clearly the most severe stress.

Lag periods in low-P media were usually less that 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 at 10 µM p, so that the dotted line indicating that growth curve is approximate. In general, replicates varied in growth 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 $\pm$ 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 13.4 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^{u89} cells/ml, P apparently becoming exhausted at this level.

TABLE 3. TOLERANCE TO pH, P, AND Al STRESS AMONG 52 RHIZOBIA OF THE COWPEA MISCELLANY†.

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

† Based on attainment of turbidity within 25 days from mean initial density of $10^{3.14 \pm 0.67}$ cells/ml (Exp. B).

TABLE 4. TOLERANCE TO pH, P, AND Al STRESS AMONG 13 STRAINS OF *R. 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
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 $10^{3.14 \pm 0.42}$ cells/ml (Exp. B).

Figure 4 shows the results of adding Al as two different 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 3 lists tolerances to the various factor combinations among strains of the cowpea miscellany. The data were combined from detailed sampling trials and the screening trials with growth assessed by visual turbidity. The data are based on 25 days' growth from an average inoculum level of $10^{3.14 \pm 0.67}$ cells/ml. With 5 µM P, the average time to reach turbidity was 8.3 days at pH 6.3, 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.

Table 4 shows a similar summary for R. japonicum. The time period was again 25 days, and the inoculum level was $10^{3.14 \pm 0.42}$ cells/ml. 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 tunes were respectively 5.2 and 7.3 days.

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 do verify recent evidence that some strains of slow-growing rhizobia can survive high concentrations of Al (Rerkasem 1977; Carvalho 1978); but the large reduction in growth rate shown here could be critical for colonization

of soil and rhizosphere, and for induction of modulation (Munns 1968a; Vincent 1974; Dart 1976). Aluminum has been clearly shown to inhibit modulation of Stylosanthes spp. (Carvalho 1978).

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 solutions (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 half 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 (1000 µM) P concentration precludes the existence of toxic Al concentration and in any case is only remotely likely to exist in soil.

The low P concentration itself inhibited growth of some strains, but with less severity than acid or Al. According to data from soil studies (Fox et al. 1978), the 5 µM P concentration would not be deficient for plant growth. In fact, most soil solutions contain P at concentrations <1 µM (Reisenauer 1966; Gilman and Bell 1978). Soils, unlike the test media, are buffered with respect to phosphate. Only in laboratory media would rhizobia normally encounter the extremely

high concentration of 1000 µM. As Parker et al. (1977) pointed out, conventional bacteriological media, which are absurdly rich, may encourage rhizobial dependence on luxury levels of nutrition. The data here suggest that the large majority of rhizobia could grow adequately at P levels 100 to 1000-fold more dilute than in common media (Vincent 1970; Bergersen 1961). Routine use of media at more dilute nutrient levels might keep in check any tendency of the bacteria to develop luxury dependence.

Comparison of tolerance categories for the two groups of rhizobia suggest that the cowpea rhizobia have more tolerance to Al. 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 (Munns 1965b; Andrew et al. 1973; Carvalho 1978); and third, Al at realistic concentrations appeared to be more commonly a severe stress than low pH or low P at the levels tested here.

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; Jones 1966; 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 expedient 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 figs 4 and 2a). This may be an effect of EDTA. Ferric EDTA was used because if 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) and Parker et al. (1977). Introduced bacteria often are unable to tolerate biotic or abiotic stresses in a new environment (e.g., 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.

SECTION II

EFFECTS OF CALCIUM AND MANGANESE IN COMBINATION WITH OTHER SOIL ACIDITY FACTORS ON GROWTH OF RHIZOBIA

INTRODUCTION

The requirement for Ca as an essential nutrient for rhizobia is quite small, as determined in liquid media (Loneragan and bowling 1958; Norris 1959; Bergersen 1961; Vincent 1962). Vincent (1962) showed the Ca requirement to be about 25 µM for normal growth, and found no effect of pH down to 5.5 on the response to Ca. Loneragan and Dowling (1958) found no response to Ca from 0.1 to 10 mM for R. trifolii at various pH down to 4.5 which stopped growth. However, Rerkasem (1977) reports that Ca prevented the effects of moderate acidity for fast growing rhizobial strains, while cowpea miscellany strains were more tolerant of acidity and displayed no response to Ca at low pH. Further, in soil at pH 4.5, addition of a neutral Ca salt did not affect growth or survival of a fast or a slow grower, but did improve the growth of the fast grower in the rhizosphere.

While Ca can partially ameliorate the inhibitory effects of Al on non-symbiotic legumes (Munns 1965b; Foy et al. 1969; Lund 1970), there is little information of such an interaction on rhizobia. The one relevant study is that of Rerkasem (1977) where 1 mM Ca prevented the decline in viability of a fast grower in solution at pH 4.3, but did

not overcome the negative effects of Al addition. A slow grower that was not affected by the acidity or Al did not respond to Ca either.

Rhizobium strains differ in their tolerance to acid soils with Mn toxicity (Dobereiner et al. 1965; Dobereiner 1966). Rhizobia can tolerate very high levels of Mn in artificial media (Dobereiner 1966; Ma Masterson 1968; Holding and Lowe 1971) but there appears to be no information from actual growth studies concerning effects of high Mn at low pH.

The objectives of this research were (i) to examine the effects of low Ca on rhizobia at low pH, and at low pH in combination with high Mn and Al and low P, (ii) to examine the effects of high Mn at low pH, and at low pH with high Al and low Ca and P, (iii) to compare these effects with those of low P and low P + high Al from Section I and (iv) to determine any effects of increasing Ca levels on the response to Al among rhizobia.

MATERIALS AND METHODS

Rhizobia and Culture Media

Section I and the Appendix lists sources of rhizobia, and particulars of media preparation, adjustment of Al and pH, and counting of viable cells. The media, all liquid, are described in Table 1.

Experiment A

Three strains from the cowpea miscellany were selected for growth studies in defined media at pH 4.6. Four treatments were imposed (Table 1). Media were dispensed in triplicate 50 ml volumes in 250

TABLE 1. MEDIA

Basal solution: mannitol 10 g/L, Na-glutamate 1.1 g/L; salts (μM); $MgSO_4$ 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. Also, for rhizobial strains which demonstrated a response to growth factors in defined medium, 1 ppm thiamine and 0.1 ppm biotin were added.

Experimental Treatment	Additions to Basal Solution	pH*
Exp. A.		
(a) full nutrients	500 M KH_2PO_4 + 500 M K_2HPO_4 + 300 M $CaCl_2$	4.6
(b) low P	10 M KH_2PO_4 + 300 μM $CaCl_2$ + 0.75 mM K_2SO_4	4.6
(c) high Mn	as (a) above + 200 μM $MnCl_2$	4.6
(d) low Ca	as (a) above except with only 50 μM $CaCl_2$	4.6
Exp. B.		
(a) high Mn	as (c), Exp. A, above	4.5 or 4.8†
(b) low Ca	as (d), Exp. A, above	4.5 or 4.8
(c) combined factors	1.5 mM KCl + 5 μM KH_2PO_4 + 50 μM $CaCl_2$ + 200 μM $MnCl_2$ + 25 or 50 μM $AlK(SO_4)_2$§	4.5 or 4.8
Exp. C.		
Ca x Al	$CaCl_2$: + 50, 250 or 1000 μM $AlK(SO_4)_2$: + 0, 50 Or 100 M	4.5

* pH adjusted with HCl.

† pH 4.5 for tests with cowpea miscellany and 4.8 with R. japonicum.

§ 25 M Al for tests with R. japonicum and 50 M with cowpea miscellany.

Erlenmeyer flasks, plugged with cotton, covered with a small beaker, and autoclaved for 20 minutes. Bacteria from agar slopes of similar age were suspended and serially diluted so that delivering 1 ml to treatments gave an initial density of about 10^3 cells/ml. The diluent was basal solution adjust to pH 4.6. Population density was determined as viable cells.

Experiment B

Forty-two strains of rhizobia, 32 from the cowpea miscellany and 10 from R. japonicum were tested for tolerance to high Mn (200 µM) and low Ca (50 µM) (Table 1). Five of the Al-tolerant cowpea miscellany strains and all 10 of R. japonicum were further tested in a combination medium having the low Ca and high Mn along with low P (5 µM) and high Al (25 or 50 µM) (Table l). The treatments were adjusted to pH 4.5 for cowpea miscellany and 4.8 for R. japonicum strains. In the combination treatment the Al levels were 50 µM for the cowpea group and 25 µM for R. japonicum. All strains were examined twice daily for detectable turbidity over a 25 day period. One strain was sampled for detailed study over an 18 day period. Duplicate 5 ml volumes were dispensed in screw cap culture tubes, and the diluent was basal solution adjusted to the same pH as that of the given medium.

Experiment C

Three strains from the cowpea miscellany were tested in a factorial combination of 3 Ca and 4 Al levels at pH 4.5 (Table 1). Samples were taken over the 2 1/2 week growth period for viable counts. Triplicate 5 ml volumes were dispensed in screw cap culture tubes, and the diluent was basal solution adjusted to pH 4.5.

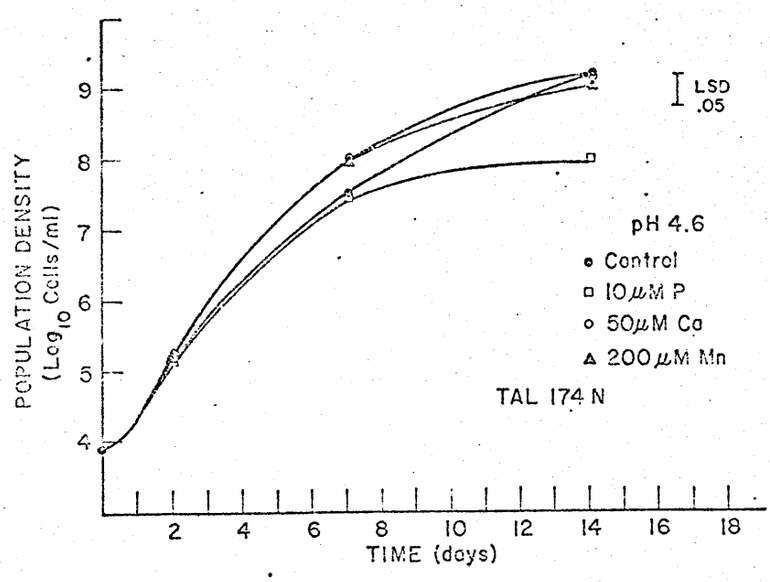


Figure 1 - Response of strain TAL174N to P, Ca and Mn (Experiment A).

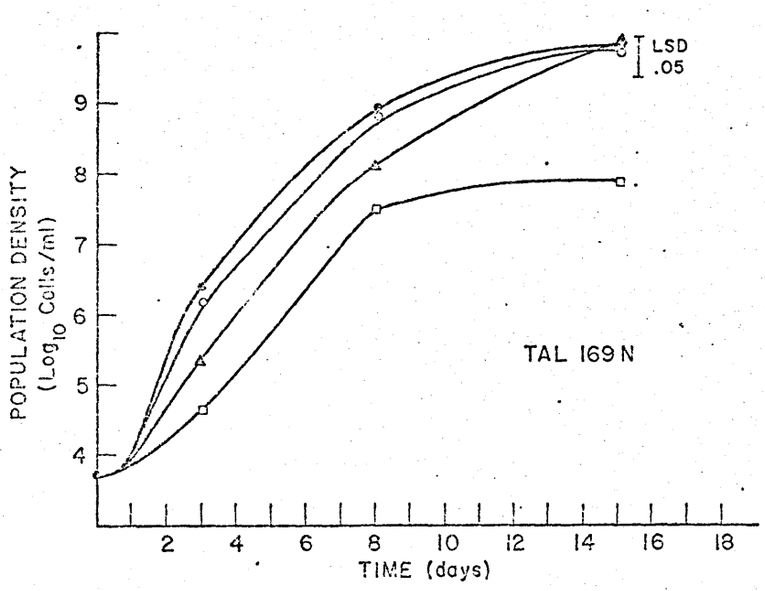


Figure 2 - Response of strain TAL169N to P, Ca and Mn (Experiment A).

(For meaning of symbols see Figure 1.)

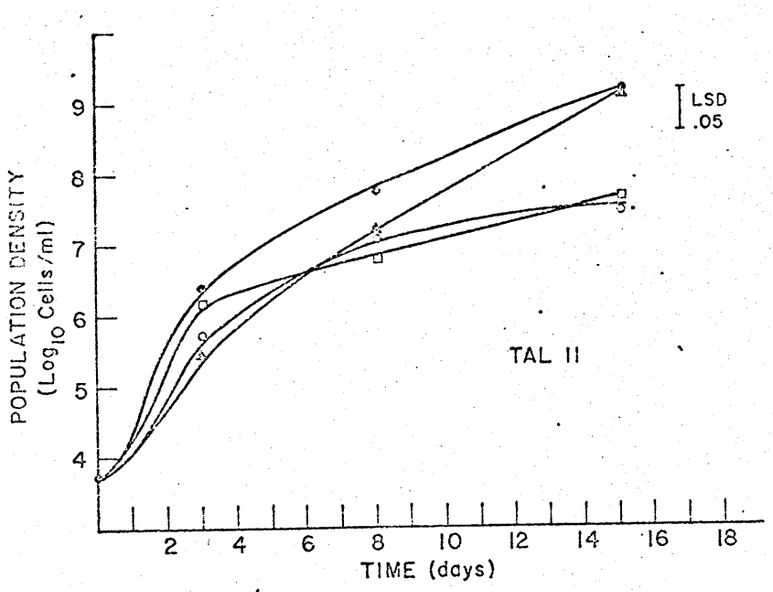


Figure 3 - Response of strain TAL11 to P, Ca and Mn (Experiment A).

(For meaning of symbols see Figure 1.)

RESULTS

Results of the growth studies in Experiment A are shown in figures 1 to 3. The 10 µM P medium limited final cell density in all 3 strains, as did 50 µM Ca for TAL11 (fig. 3). High Mn (200 µM) did not limit final density in any strain, but did reduce growth rate of TAL11 and TAL169. Sampling dates were not frequent enough for calculation of growth rates, but relative differences in rates are apparent in the growth curves.

Tables 2 and 3, and fig. 4 show results of Experiment B. In general, 50 µM Ca or 200 µM Mn had much less effect than 50 µM Al (Section I), though for some strains their combination in the presence of Al had effects beyond that of the Al alone.

Table 2 lists tolerances to low Ca and high Mn among the 2 groups of rhizobia. For comparative purposes, tolerance was judged as in Section I, i.e., by the strain's ability to achieve turbidity in 25 days from an initial low level inoculum. For the cowpea miscellany 2 strains previously shown to be acid-sensitive at 5 µM P were able to make turbid growth at pH 4.5 and 200 µM Mn, albeit only after 23 days. Since this high Mn medium contained 1 mM P, these 2 strains appear to be more tolerant of acidity with very high P. The remaining strains assessed as acid-sensitive with 5 µM P were also sensitive here with high Mn. Of the strains previously recorded as acid tolerant but Al-sensitive, none were sensitive to high Mn, while 3 were sensitive to the low Ca. Of the Al-tolerant strains, none were sensitive to either the Mn or Ca. Overall, Ca and Mn had little effect on growth rate in the R. japonicum strains tested.

TABLE 2. RESPONSE TO LOW Ca AND HIGH Mn AMONG RHIZOBIA IN DIFFERENT TOLERANCE CATEGORIES (EXP. B).

	Number of strains			Time to turbidity, in days Based on 25 days growth*					
				50 µM Ca			200 µM Mn		
	tested	sensitive to Ca	sensitive to Mn	x	(s.d.)	r	x	(s.d.)	r
Cowpea misc.									
sensitive to pH 4.5-5 µM P†	9	-	-	-	-	-	-	-	-
tolerant of pH 4.5, sensitive to 50 µM Al	10	3	0	8.4	(2.1)	6-12	11.4	(3.9)	7-24
tolerant of 50 µM Al	13	0	0	8.1	(2.7)	6-15	9.2	(2.8)	7-15
R. japonicum									
tolerant of pH 4.8, sensitive to 25 µM Al	3	0	0	10.3	(0.6)	10-11	10	(0)	10
tolerant of 25 µM Al	7	0	0	8.6	(2.4)	5-10	8.6	(2.4)	5-10

* Mean initial densities: cowpea miscellany $10^{3.32 \pm 0.76}$ cells/ml; R. japonicum $10^{3.34 \pm 0.33}$ cells/ml.

† Two of these strains could grow slowly (23 days to turbidity) at pH 4.5 in the 200 µM Mn medium (containing 1 mM P).

Symbols: x is mean; s.d. is standard deviation, and r is range.

TABLE 3. RESPONSE TO INDIVIDUAL AND COMBINED ACIDITY FACTORS AMONG RH. .IA (EXP. 3).

Strains	Time to turbidity, in days. Based on 25 days' growth				
	5 µM P*	50 µM Ca	200 µM Mn	50 µM Al*	combined factors
Cowpea misc. (pH 4.5)					
163	7	7	7	17	17
425	7	7	7	14	14
209	8	7	7	14	15
172	7	7	7	14	25
M3	7	7	7	15	17
mean	7.2	7	7	14.8	17.6
R. japonicum (pH 4.8)				25 µM Al	
61A101	10	5	5	20	>25
61A112	10	10	10	20	>25
61A124	22	10	10	>25	>25
61A144	10	10	10	20	20
61A150	20	10	10	>25	>25
Allen 519	10	10	10	25	25
Allen 511	12	10	10	20	20
Allen 542	8	10	10	nd	nd
USDA 110	10	5	5	20	20
CB 1809	12	11	10	>25	>25
mean	12.4	9.0	8.8	>20	>20

* Values from simultaneous studies reported in Keyser and Munns (1979).

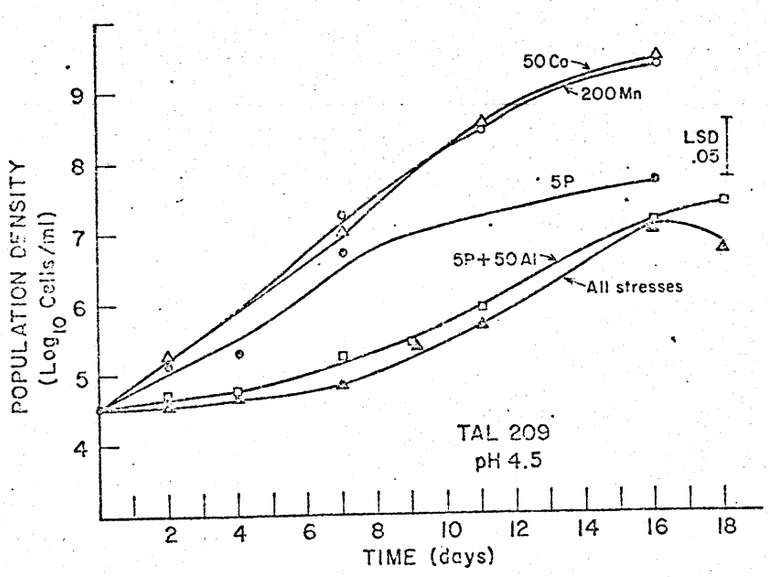


Figure 4 - Response of strain TAL209 to individual and combined acidity factors (Experiment B).

Table 3 lists the time taken to achieve turbid growth in the different stress media, mainly for comparison with the results of the combined factors treatment. For the 5 cowpea strains low Ca and high Mn provide little stress, but strains 172 and M3 displayed a significant inhibition beyond that of the Al alone when the low Ca and high Mn were also included. This was also noted for 2 R. japonicum strains.

The results from the Ca x Al trial are shown in figures 5 through 7, and a summarized analysis of variance is given in Table 4. Though statistical analysis indicates significant Ca interaction effects for 2 of the 3 strains, inspection of the growth curves suggests that Ca offers too little protection against Al to be biologically significant.

The statistical analysis for TAL11 shows no main or interaction effects of Ca. All levels of added Al caused a significant early reduction in viable density, with the 25 µM Al treatment thereafter showing a faster growth rate than the 2 higher levels. The low P level in this trial limits total cell number and therefore prevents the response to Ca that TAL11 showed in Experiment A.

For TAL189, the initial large decrease in viability occurred only at the 2 highest Al levels; however, this strain was able to recover rather well, though at lower growth rates. Statistically, the effects here are also largely due to Al levels and time, but there were smaller effects of Ca in first and second order interactions. The Al x Ca effect appears to be due to a slight progressive response to increased Ca levels only at the highest level of Al (100 µM), this

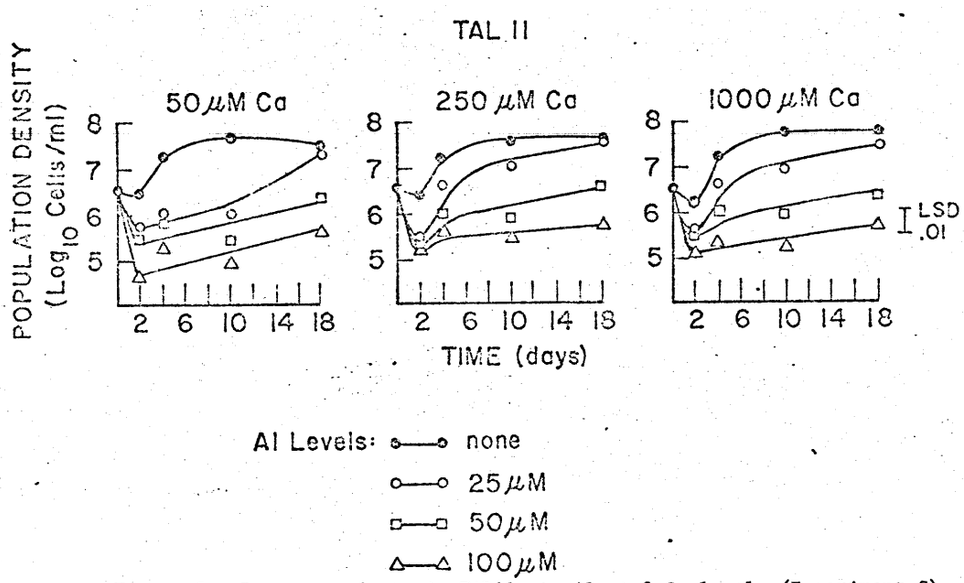


Figure 5 - Response of strain TAL11 to Al at 3 Ca levels (Experiment C).

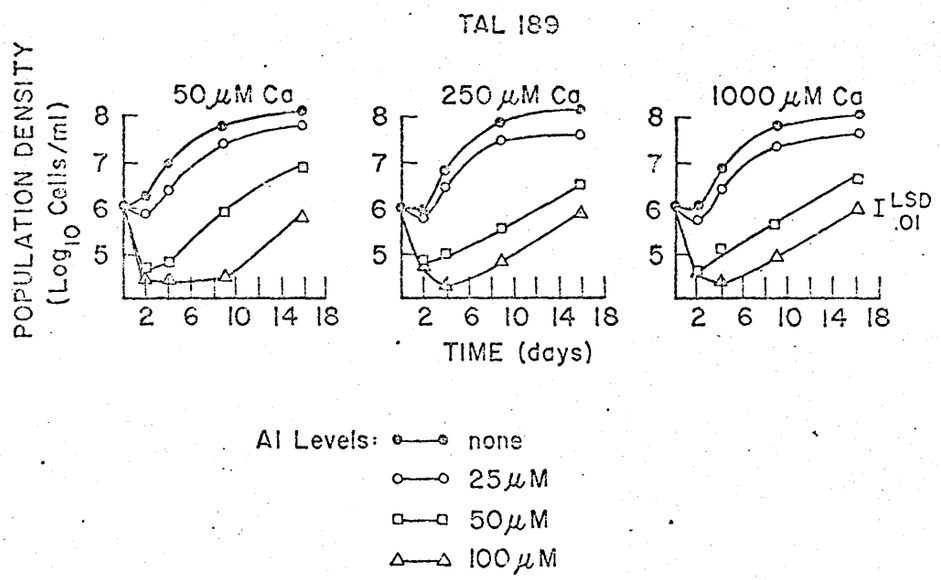


Figure 6 - Response of strain TAL189 to Al at 3 Ca levels (Experiment C).

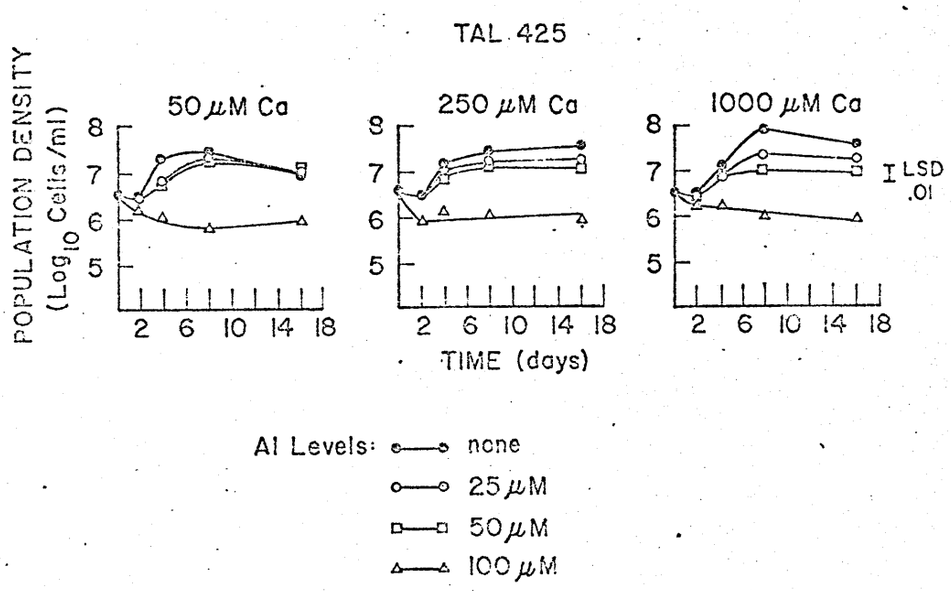


Figure 7 - Response of strain TAL425 to Al at 3 Ca levels (Experiment C).

TABLE 4. ANALYSIS OF VARIANCE FOR Ca x Al TRIAL (EXP. C)

		Strains		
Source	df	TAL11	TAL189	TAL524
			F ratio	
Ca	2	2.36	0.17	4.16
Al	3	332.6 ***	2,735. ***	418. ***
Time	3	124.6 ***	1,381. ***	136. ***
Ca x Al	6	1.09	2.98*	2.81*
Ca x Time	6	0.55	2.10	2.87*
Al x Time	9	11.21***	67.2 ***	33.22***
Ca x Al x Time	18	0.97	3.29***	4.39***
error	96			
$LSD_{.01}$ Ca x Al x Time in cells/ml		$10^{0.56}$	$10^{0.27}$	$10^{0.28}$

Significance at probability levels of 0.05, 0.01, and 0.001 are indicated by 1, 2, and 3 asterisks, respectively.

determined from comparing all Ca-Al means averaged over time. From inspection of all individual means, the significant second order interaction appears to be due to the longer lag period in the lowest Ca and highest Al level as compared to the 2 higher Ca levels at the same Al level. However, the 50 µM Al level at the lowest Ca addition grew slightly faster than at the 2 higher Ca levels, so that a meaningful trend is not apparent.

For TAL425, the results are more statistically complicated. The simple features are that even at 100 µM Al there was comparatively little initial decline in viability, there was good early growth with up to 50 µM Al, and the Al-free treatment displayed the greatest Ca response. From inspection of the appropriate means, the first and second order Ca interactions appear to be due to the combination of the increasing response to Ca for the 0 and 25 µM Al treatments, and the slightly contrasting behavior over the Ca range at 100 µM Al. While this strain displayed the greatest Ca effects on Al response, the dominating effects of Al level are still clear.

In the Ca x Al trial, Al activities were calculated using the first approximation of the Debye-Huckel equation (Adams 1974). Increasing Ca concentrations did not seriously lower Al activities through an effect of ionic strength. In the 25 µM Al treatment, the Al activity ranged from 11.4 µM at the lowest Ca level, to 10.5 at the highest Ca. The corresponding ranges of Al activity were 22.7 to 20.8 for the 50 µM Al media, and 45.0 to 41.4 for the 100 µM Al media.

The initial declines in counts for strains TAL189 and TAL11 were probably due to death of cells, not to clumping. Aluminum-induced

clumping, observed by Rerkasem (1977) at high Al concentration (1 mM), was restricted to fast growing strain, not slow growers. Further, phase-contrast microscopy of the suspensions in the Ca x Al trial indicated that almost all cells were isolated from each other.

DISCUSSION

At low pH, 50 µM Ca and 200 µM Mn imposed little if any stress to the majority of cowpea miscellany and R. japonicum strains. The data confirm earlier work of Vincent (1962) where Ca deficiency for several strains did not occur above a level of 25 µM at pH 5.5, though in the present study a few acid-tolerant strains were detected which did not make turbid growth with only 50 µM Ca. Since these same strains were able to make turbid growth at low pH with 300 µM Ca (in the high Mn treatment; also in Section I) it appears that some strains will respond to Ca beyond a level of 50 µM at pH 4.5. A similar response was found by Rerkasem (1977) for some fast growing rhizobia.

None of the acid-tolerant strains were limited in final densities by the high Ma level, though some did display early depressive effects on growth rates. Rhizobia have been shown to tolerate levels up to 16 mM in artificial media (Dobereiner 1966; Masterson 1968; Holding and Lowe 1971).

Comparison with results of Section I implies that Al is the most severe stress at the levels tested, and all strains tolerant of Al were also tolerant of low Ca and high Mn. An additive negative effect of these combined factors was found for a few of the Al-tolerant

strains. This may be of significance since all these factors could occur together in an acid soil (Munns 1977a and b).

For growth of legumes, Ca levels from 1 to 5 mM can ameliorate the effects of Al (Munns 1965b; Foy et a1. 1969; Lund 1970), but for the 3 rhizobia tested here any beneficial effects of increased Ca up to 1 MM were slight. This agrees with similar observations on other Al-tolerant strains (Rerkasem 1977). Also, as Helyar (1978) pointed out, some plant studies claiming protective effects of high Ca did not take into account large effects of Ca salts on the activity of Al. Mostly, the data here confirm that Al can be quite inhibitory to rhizobia, in some strains causing an initial decline in viability as well as an increased lag period and a reduced growth rate. An early decline in viability has also been demonstrated in acid soil (Vincent and Waters 1954a). Ability of strains to recover after a large initial decline in viability in the presence of Al may imply physiological adaptation or selection of genetically tolerant variants.

Compared with soil solution analyses from a wide spectrum of soils, 50 µM Ca is very low (Reisenauer 1966; Gilman and Bell 1978). It is difficult to find data on soil solution Mn values associated with Mn toxicity, but the 200 µM level tested here can be inhibitory to several legumes grown non-symbiotically in solution culture (Morris and Pierre 1949; Andrew and Hegarty 1969). Further, comparable levels of these acidity factors are known to adversely affect the nodulation, nodule function, or growth of legumes, including those that are hosts for the slow growers such as Glycine spp., Vigna unguiculata, Arachis

hypogea, and Stylosanthes spp. (Armiger et al. 1968; Andrew and Hegarty 1969; Lowther and Loneragan 1970; Andrew et al. 1972; Munns 1977, 1978; Andrew 1978; Carvalho 1978). Therefore, under acid conditions the tolerance to low Ca or high Mn among most slow growing rhizobial strains appears at least equal or be superior to that of the host plants, while tolerance to Al is less common.

Finally, if the more important tolerances among strains could be verified in the soil environment, then the ability to identify these tolerances for a given strain would be a valuable aid in interpreting effects of such soil acidity factors on the legume-Rhizobium symbiosis.

SECTION III

ADAPTATION TO ALUMINUM BY RHIZOBIA

INTRODUCTION

Adaptation among microorganisms to various stresses has been reviewed by Stanier (1953) and Dean and Hinshelwood (1953). From their discussions it seems justifiable to define microbial adaptation as a population's gain is ability to function under stress. Further, there appear to be two prominent types of adaptive mechanisms, though they may not be entirely mutually exclusive; (1) selective adaptation, being the selection under stress of genetic variants already existing in a population, or of new mutants, and (2) physiological adaptation, being the variable, phenotypic adaptation of individual cells of the same genotype.

Three important phenomena of acid-Al stress on rhizobia were observed in Sections I and II. First, some strains displayed early death of cells with no recovery. Second, there was most often a lengthened lag period, this varying between-strains; and in some cases within strains at small inocula levels. During the lag phase some strains which eventually recovered showed an early decline in cell numbers. Third, there was strain-dependent regrowth after the lag phase, quite often at reduced growth rates. All of these observations have been found with microbes under stress conditions in general (Jackson and Hinshelwood 1950; Baskets 1952; Doudoroff 1940). Specifically, Foy and Gerloff (1972) reported slow growth at high Al

levels for a tolerant Chlorella species. In a study of NaCl stress to rhizobia, Steinborn and Roughley (1975) attributed the longer time for strains to grow at high concentrations to the time necessary for adaptation. The number of cells of a R. meliloti strain able to grow on 3% NaCl agar was only one-tenth to one-twentieth of the population.

As a preliminary investigation into the nature of such adaptation an experiment was set up. Several strains of rhizobia were tested for growth from widely varying inoculum levels in Al-containing medium. This approach should demonstrate whether or not growth is due to the selective adaptation of new mutants.

MATERIALS AND METHODS

Ten straits of Rhizobium, 9 of the cowpea miscellany, and 1 R. japonicum were tested in pH 4.5-5.0 μM Al medium, the same composition as in treatment (d), experiment B of Section I. For each strain 4 levels of inoculum were introduced by 100-fold serial dilutions of a high density suspension. All strains were tested in duplicate at each inoculum level. Strains TAL174N, TAL98A, and TAL209 were tested in 20 ml volumes in 50 ml tubes. Viable counts were taken initially on all inocula and at day 17. The diluent composition was that of the medium less the Al. Details regarding medium preparation, growth conditions and counting are described in Experiment A of Section I.

RESULTS AND DISCUSSION

The results are shown in Table 1. Three types of intrastrain response emerge. Strains TAL174N, TAL209, M3, TAL169N and CB756 were

Table 1. Growth of 10 strains in A1 medium from different inoculum levels.

(log_{10} cell numbers, viable count)

	initial, delivered	at 17 days			initial, delivered	at 17 days	
		total	per ml*			total	per ml
TAL174	6.6	7.8	6.5	TAL98	4.8	7.6	6.8
	4.6	7.4	6.1		2.8	5.9	5.1
	2.9	7.9	6.6		0.8	3.0	2.2
	0.8	8.0	6.7		<0	---	<1
TAL209	6.5	8.4	7.1	TAL98A	5.7	6.8	5.5
	4.4	8.4	7.1		3.8	6.5	5.3
	2.4	8.4	7.1		1.6	---	<1
	0.4	8.2	6.9		<0	---	<1
M3	5.2	8.4	7.7	TAL421	6.1	7.5	6.7
	3.2	7.8	7.0		4.1	5.8	5.0
	1.2	8.1	7.3		2.1	4.2	3.4
	<0	---	<1		<0	---	<1
TAL169	6.1	8.5	7.8	3I6n12	5.9	8.5	7.7
	4.1	8.7	7.9		3.9	6.8	6.0
	2.0	8.6	7.8		1.3	---	<1
	0.15	7.5	6.7		<0	---	<1
CB756	5.7	8.8	8.0	USDA110	6.3	4.8	4.0
	3.7	8.9	8.1		4.2	3.3	2.5
	1.7	8.8	8.1		2.1	---	<1
	<0	---	<1		0.3	---	<1

* Maximum cell densities were limited by the low P level (5 μM).

able to attain high levels from only a few initial cells in the Al medium. Strains TAL98, TAL98A, TAL421 and n12 had a slower growth rate than the first group, and some did not grow from very low initial levels. R. japonicum US110 showed a reduction in cell numbers at all inoculum levels.

All strains in the first group were assessed as Al-tolerant in Section I, judged from turbid growth and viable counts from inocula at 10^3 cells/ml. A simple interpretation is that these strains contain a large population of cells with the ability to adapt rapidly in Al-stressed medium. Their response at very low inocula levels indicates little if any death of cells during an adaptive period, or alternatively, few cells genetically incapable of adapting.

Contrasting this is the behavior of R. japonicum US110, which was judged sensitive to pH 4.5 (Section I). It shows death of cells at all levels of addition, being unable to adapt to the acid-Al medium within 17 days.

Of the remaining strains, TAL421 and n12 were rated Al-tolerant (Section I). TAL421 has a very slow growth rate in Al for an adapted strain. Strain nl2 also has a slow growth rate, and no growth from the addition of only 20 cells ($10^{1.3}$). This may be due to death of cells during an adaptation period or alternatively, few cells genetically capable of adapting. TAL98A received separately from 98, subcultured from the same parental strain. While both show slow growth rates as compared to the most tolerant group, TAL98 made growth from fewer cells than 98A. This difference is probably of no significance. Even from the same dilution, Jackson and Hinshelwood

(1950) found variation in a strain of bacteria adapting to ammonium sulphate.

From the data here it seems clear that the adaptation to Al stress is not due to the occurrence of de novo mutants. Stanier et al. (1976) and Brock (1974) put the frequency of new mutants at 10^{-8} to 10^{-9}, and the probability of this accounting for the growth of even the less tolerant strains is very small indeed. The argument for selective adaptation of existing genetic variants is inconclusive from this data. The performance of the 5 most tolerant strains suggest they have a uniform population in respect to adaptation abilities. While some of the other strains did not make growth from very low levels, it can be argued that this was due to identical cells which did not survive an adaptation period (Jackson and Hinshelwood 1950), compatible with the mechanism of physiological adaptation.

This study concludes that the mechanism of Al-adaptation is not one of spontaneous mutation. It shows that large differences exist between strains in ability to adapt. Also, there appear to be degrees of adaptiveness, expressed in relative rates of growth from a given inoculum level, as well as ability to grow from very few initial cells. To elucidate the adaptive mechanism responsible for tolerance, further studies can now focus on discriminating between the existence of genetic variants already present or that of phenotypic adaptation of similar cells. This would require determining the stability of adapted cells, using such techniques as replica plating and ability of cells to be trained for adaptation and to retain the adaptation in the absence of the inducing stress.

SECTION IV

RELATIONSHIP BETWEEN RHIZOBIAL TOLERANCES IN PURE CULTURE AND SYMBIOTIC PERFORMANCE IN ACID SOIL

INTRODUCTION

Differential strain tolerance to soil acidity has been demonstrated for different rhizobia species (Munns 1965a; Lie 1971; Rerkasem 1977; Munns et al. 1978). Lie (1971) reported that a R. leguminosarum strain that gave the best symbiotic performance at pH 4.6 was a comparatively mediocre strain at neutral pH. This observation was confirmed by Munns et al. (1978) on several strains of the cowpea miscellany, where effect of acidity on a strain's performance bore no relationship to its effectiveness at higher pH.

The detrimental effects of Al on symbiotic legumes are attributed to reduced nodulation and plant growth (Sartain and Kamprath 1975; Carvalho 1978). For Stylosanthes species, Carvalho (1978) demonstrated that the nitrogen fixation activity of nodules formed in the absence of Al was not reduced upon exposure to levels up to 100 µM, but nodule formation was. Increased nodulation of soybeans was partly attributed to reduced Al saturation in soil from liming, while it was also highly correlated with root Ca level (Sartain and Kamprath 1975). Differential symbiotic performance of rhizobia under conditions of Al stress have not been demonstrated.

This section describes the symbiotic performance in soil of 25 strains of rhizobia on 3 varieties of Vigna unguiculata (cowpea).

Comparative effectiveness of a strain at low pH with its effectiveness at a more neutral pH (symbiotic acid tolerance) was then compared with the strains' unknown tolerances to acidity and Al in pure culture, as determined in Sections I and II.

MATERIALS AND METHODS

Two greenhouse pot trials were conducted to test the symbiotic acid tolerance of several rhizobial strains. Three cultivars of the acid tolerant legume V. unguiculata were grown on two soils; one variety on the first soil, and 2 other varieties on the second soil. The treatments for each soil were a low pH of 4.6 and a more neutral pH of 6.0-6.2. Previous experience with the two soils showed them to be very nitrogen deficient and naturally acid.

Seed of Vigna unguiculata cv. Blackeye 5 (BE5) was obtained from the California Crop Improvement Association, Agronomy Department, University of California, Davis. Seed of varieties TVu 1190 and TVu 4557 were supplied by the International Institute of Tropical Agriculture, Ibadan, Nigeria, by way of the University of Hawaii NifTAL Project, Paia, Maui. The day of planting, seeds were surface sterilized by submergence in 30% H_20_2 for 5 minutes, and thoroughly rinsed with distilled water.

Twenty-five strains of rhizobia were tested, and are listed in the Appendix. Thirteen strains were used as inocula for a trial with BE5, and 23 strains with the TVu varieties, with only 2 strains tested on BE5 not also tested on the other 2 varieties. All of the strains were tested for tolerance to acidity and Al in pure culture in

Sections I and II. For inoculation, cultures from agar slants were suspended in full nutrient broth (treatment b, Sec. B, Sec. I), diluent, and delivered to seed in pots immediately before it was covered with soil. Viable counts of inoculum showed that $10^{4.9 \pm 0.25}$ cells per seed was applied in the BE5 trial, except for strains TAL163 and TAL98 which, not by design, received $10^{6.4}$ and $10^{6.2}$ cells per seed, respectively. In the trial with the TVu varieties $10^{5.9 \pm 0.5}$ cells/seed was applied. In each trial, a given strain was applied at the level for all treatments with that strain.

The soil used in the trial with the BE5 variety was Goldridge loamy fine sand (Typical Hapludult, fine loamy, mixed, mesic), B-horizon material collected from Sebastopol, California. It had a saturation paste pH of 4.6, and levels of available cations as listed in Table 1. Mineralogical analysis of the clay fraction showed it contained predominately kaolinite. Two pH treatments were imposed; the unadjusted pH of 4.6 and a pH of 6.0 from the addition of $CaCO_3$ Limed treatments received 0.9 grams lime per kg soil. Basal nutrients applied to all treatments were 10 mMoles KH_2P0_4, 2 mMoles K_2SO_4, 10 mg Zn and 0.1 mg Mo per kg soil. Nutrients and lime were added to 1.9 kg soil in plastic pots, mixed, watered, and incubated for a week before planting. The final pH of each treatment was checked the day before planting. Combined nitrogen treatments received 12 mMoles NH_4N0_3 per pot, in split applications. A zero control, with no inoculum or nitrogen, was included, and all treatments were replicated. Six seeds were planted per pot, and thinned upon emergence to 3 uniform seedlings.

The soil used in the trial with the TVu varieties was Josephine silt loam (Typic Haploxerult, fine loamy, mixed, mesic), B-horizon material collected near Georgetown, California. Mineralogical analysis of the clay fraction showed it contained predominately kaolinite and oxides. The saturation paste pH of the soil was 5.4, and available cations are listed in Table 1. Two soil pH treatments were imposed; (i) pH 4.6 by addition of 23.1 me Al as $Al_2(SO_4)_3$ per kg soil, and (ii) pH 6.2, by addition of 10.6 me $C0_3$ as $CaCO_3$ per kg soil. Basal nutrients applied to all pots were the same as for the Goldridge soil, except that 12 mMoles KH_2PO_4 per kg soil was added, and each pot also received 1.9 kg soil. Nutrients and lime were mixed, watered and incubated for 10 days with a final pH check before planting. Combined nitrogen treatments received a total of 16 mMoles NH_4N0_3 in split applications. A zero control was also included. Nine strains, the +N and zero controls were tested in triplicate, and the remaining 14 strains in duplicate. Four seeds of each variety were planted in the same pot, and thinned upon emergence to two plants per variety.

Greenhouse soil temperatures varied between 29°C (day) and 21°C (night). Pots were watered with distilled water, and maintained between 75 to 100% of the determined field capacity for each soil. Observations were taken daily on plant color and relative size of foliage. At 45 days in the trial with BE5, and at 42 days in the trial with the TVu varieties, plants were washed out of the soil, bagged in plastic and refrigerated until examined for nodule number and nodule fresh weight. Shoots were separated and dried at 75°C for weighing.

Table 1. Soil Properties - Cation Analyses

	Exchangeable* me/kg					Al saturation†	Al in 0.01 M§ $CaCl_2$ extract
	Ca	Mg	Na	Al	Mn	%	µM
Goldridge soil							
pH 4.8, unadjusted	2.0	1.0	.35	.70	<.06	17.3	12.8
pH 6.0, limed	2.6	1.0	.38	.10	<.06	0.02	<1
Josephine soil							
pH 4.6, + $Al_2(SO_4)_3$	1.6	.39	.30	1.9	.12	45.1	58.7
pH 6.2, limed	2.3	.34	.33	.11	.07	0.03	<1

* Cations extracted in 1 M KCl with a 10:1 solution to soil ratio. All cations except Al determined by atomic absorption spectrometry. Al determined by the colorimetric method of Frink and Peech (1962).

† Exchangeable Al divided by the sume of exchangeable cations analyzed.

§ Extracted with a 10:1 solution to soil ratio.

Analysis of variance was performed on plant yield and nodule number, and for the TVu varieties also on nodule fresh weight. To equalize variances when necessary, the transformations $1+\log_{10}$ yield or $\log_{10}$ nodule number were made for analysis. Plant yield analyses were used to test associations between rhizobial tolerances in defined acid media with symbiotic acid tolerance, as defined in the next section.

RESULTS

BE5 variety-Goldridge soil

Plants became yellow, and with effective nodulation became green at the 2nd trifoliate leaf stage (about 2 weeks) in the limed treatments. The earliest greening of plants at low pH was 3 days behind that with lime, and some of the more sensitive strains were 2 weeks later.

Figure 1 summarizes the plant yield and nodule number data for the strain in both pH treatments. Figure 2 compares the strain dependent yield at pH 6.0 with that at 4.6.

Plant yield analyses from both treatments were compared using Duncan's new multiple range test (Steel and Torrie 1960). At pH 6.0, strains TAL11 and 207 did not yield significantly better than the uninoculated zero control, and were considered ineffective on this variety. Of the remaining strains, TAL189, 173, 174, 303, 163 and 172 were considered highly effective, not significantly (5% level) different from the combined N treatment. Strains CB1024, TAL98, 169, 170 and 171 were moderately effective at high pH, being significantly better than the zero control, but significantly poorer than the

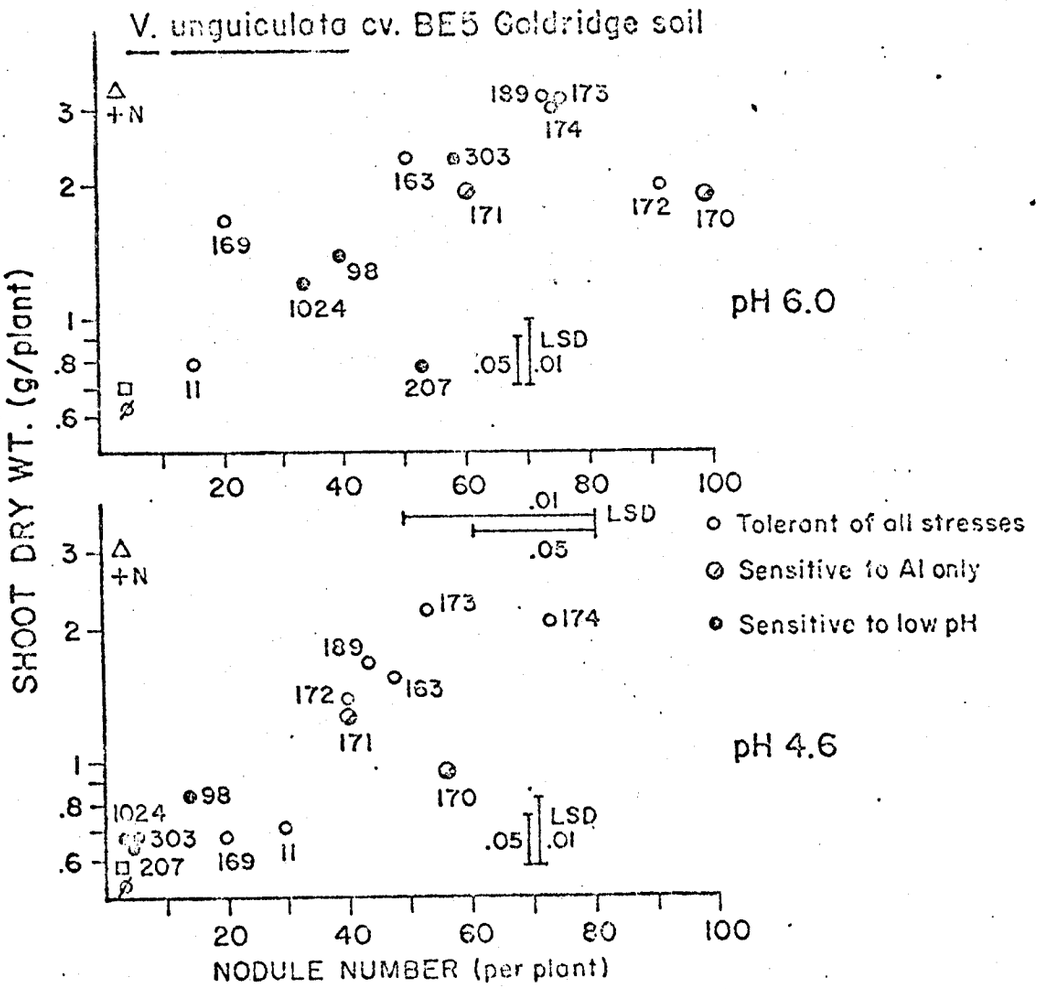


Figure 1 - Comparison of rhizobial strain performance on cv. BE5 in acid and limed Goldridge soil. Shading key refers to tolerances in defined media. Symbol Ø is the zero control, +N the combined nitrogen control. Strains are listed without prefixes (See Table 2).

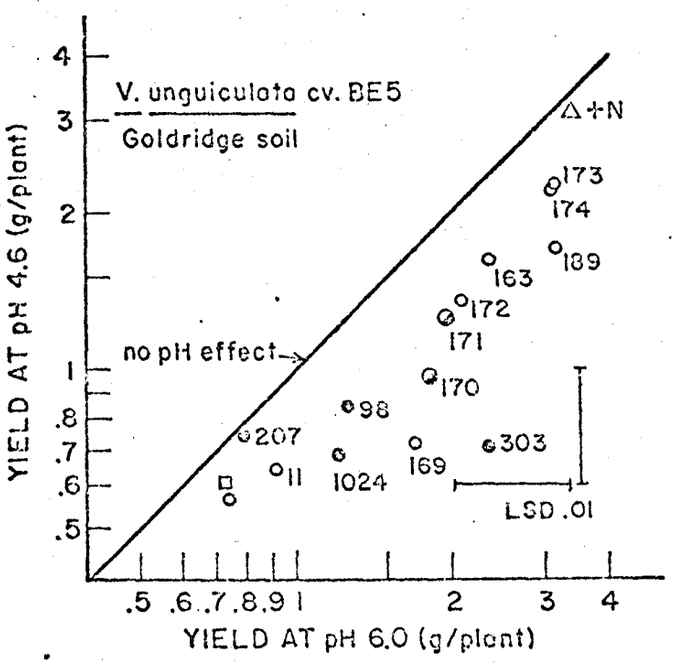


Figure 2 - Comparison of plant yield to pH 6.0 and 4.6 for different rhizobial strains on BE5. For meanings of symbols and shading see Figure 1.

combined N and the highly effective strains. For effective strains, there is a good relationship between nodule number and plant yield. The highly effective group contained one strain which was acid sensitive in media (TAL303), but obviously had no problem in being able to adequately survive a soil pH of 6.0. The moderately effective group contained strains in all 3 media tolerance categories.

At pH 4.6, most strains displayed reduced effectiveness and nodule number. Here, only strains TAL173 and 174 remained highly effective. Moderately effective at low pH were TAL189, 163, 171, and 172, while TAL169, 303, 98, 170, and CB1024 were affected to the extent of being completely ineffective.

The most notable decline in yield due to acidity is found with TAL303, which was highly effective at pH 6.0 and ineffective at 4.6. The large reduction in modulation with this strain suggests it survived poorly in the acid soil, which agrees with its tolerance rating in media. However, even the very tolerant TAL169 which had few nodules at pH 6.0 behaved similarly to TAL303 at low pH. More importantly, all the moderately and highly effective strains at low pH were acid tolerant in media, and all but one (TAL171) were tolerant of Al.

At low pH, the relationship between yield and nodule abundance appears to be a closer one than at high pH. This may be due to a strain's ability to multiply at low pH to sufficient levels for adequate modulation, though it might also reflect strain-dependent limitation in other steps involved in modulation. With the important

Table 2. Rhizobial association between tolerances in media and in so r the BE5 variety.*

A. Comparison of 2 populations for relative symbiotic acid tolerance.†

	acid tolerance quotient $\frac{(Yield-Yield\emptyset)\ 4.6}{(Yield-Yield\emptyset)\ 6.0}$§
1. Strains sensitive to Al in media	
CB1024 = 1024	0.141
TAL98 = 98	0.604
TAL303 = 303	0.131
TAL171 = 171	0.743
TAL170 = 170	0.327
mean	0.389
standard deviation	0.275
2. Strains tolerant to Al in media	
TAL169 = 169	0.199
TAL163 = 163	0.790
TAL172 = 172	0.742
TAL173 = 173	0.876
TAL174 = 174	0.875
TAL189 = 189	0.692
mean	0.696
standard deviation	0.254

Student's t test for mean difference of the 2 populations: t = 1.90, nonsignificant.

B. Comparison of 2 populations for association between tolerance in media and tolerance in soil.Λ

Fraction of strains tolerant in media also tolerant in soil: 5/6
Fraction of strains sensitive in media also sensitive in soil: 5/5

Chi-squared test of association using Yeats correction factor: $\chi^2 = 4.69\nabla$

* Only strains shown to be effective at high pH are tested here.

† As shown, the strains are grouped according to their tolerance in media. To better equalize frequencies of the classes, it was decided to combine the strains sensitive to Al but acid-tolerant with those that were acid-sensitive. This applies to both comparison A and B.

Footnotes to Table 2 continued.

§ The acid tolerance quotient for a strain is defined as the net growth (corrected for the growth of the zero control) at low pH divided by the net growth at high pH. The data are from the mean yield per plant on the transform.

Λ Tolerance in soil is defined as a yield not significantly different (5% level using Duncan's multiple range test) from the yield of the best strain at pH 4.6. Strains that gave significantly poorer yields were classed as sensitive in soil.

∇ Significant at the 5% level.

exception of TAL303, there was also a trend for the strains which performed best at high pH to do likewise at low pH.

The nodule fresh weight data related very closely with that of the nodule number data for all treatments. There was very little compensation in size for reduced abundance. Therefore, nodule weight data are not presented as they would add little information.

Table 2 shows the results of comparing 2 rhizobia populations, based on their tolerances in media, for their symbiotic acid tolerance expressed as an acid tolerance quotient, and for the association of tolerance in media and in soil based on counts of observed and expected frequencies. The t test for differences between the two populations for a mean acid tolerance quotient was not significant, while the X^2 test assuming non-association between tolerance in media with that in soil was significant at the 5% level, indicating the association of the 2 properties.

Figure 2 shows in a different way the extreme acid sensitivity of TAL303, indicated by its vertical displacement from the no pH-effect diagonal. For all strains it shows their yield performance at both pH 6.0 and 4.6. Plate 1 shows the marked N response of BE5 on this soil, and Plates 1 to 5 show the effects of acidity on top growth and modulation for strains TAL173N, TAL174N, TAL98 and TAL303.

TVu 1190 variety - Josephine soil

Effective modulation became apparent from the green color of previously yellow plants at the second trifoliate leaf stage in the lime treatment. As in the BE5 variety trial, there were varying delays among the strains in this color change at low pH.



Plate 1 - The response of top growth of BE5 to acidity and nitrogen in the Goldridge soil (collected in Sebastopol, California).





Plate 2 - The effect of soil acidity on top growth (top) and nodulation (bottom) on BE5 with strain TAL173N in Goldridge soil.



Plate 3 - The effect of soil acidity on top growth (top) and nodulation (bottom) on BE5 with strain TAL174N in Goldridge soil.



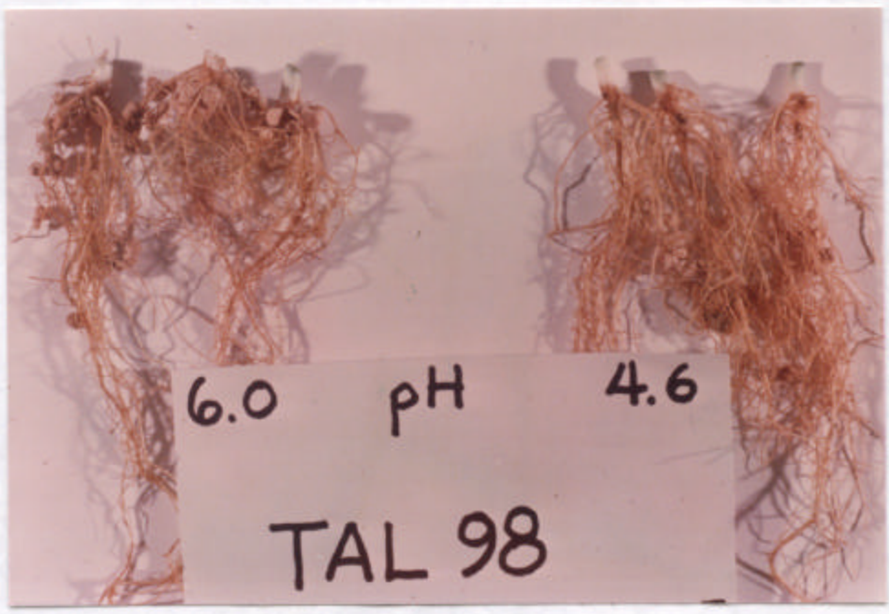


Plate 4 - The effect of soil acidity on top growth (top) and nodulation (bottom) on BE5 with strain TAL98 in Goldridge soil.





Plate 5 - The effect os soil acidity on top growth (top) and nodulation (bottom) on BE5 with strain TAL303 in Goldridge soil.

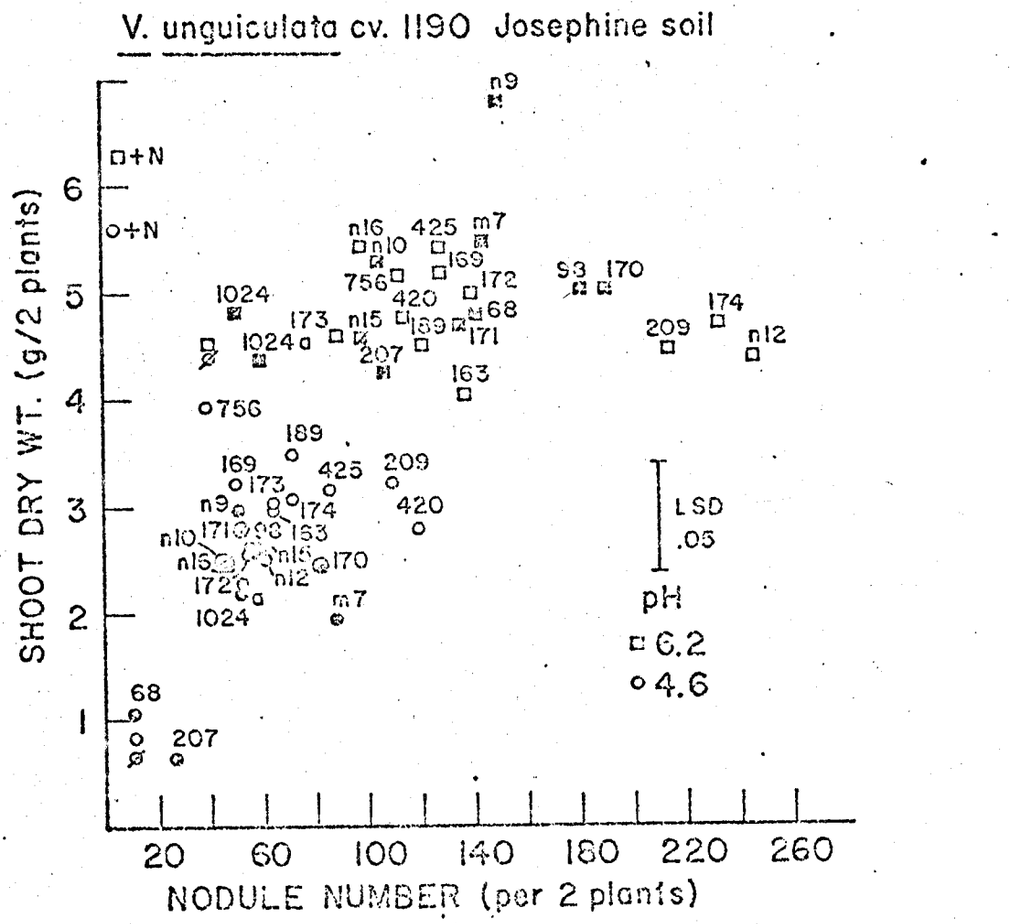


Figure 3 - Comparison of rhizobial strain performance on cv. TVu 1190 in acid and limed Josephine soil. Mean nodule numbers are significantly different from each other (5% level) if they differ by a factor of 3.0. Symbol ϕ is the zero control, +N the combined N control. Shading refers to strain tolerances in defined media; no shading (except for ϕ and +N) denotes tolerance of all stresses, half shading denotes sensitivity to Al only, and full shading denotes sensitivity to low pH.

Figure 3 shows the yield and nodule abundance due to each strain in both pH treatments. Three main features of this data are apparent. Firstly, there is the 2 pH groupings of the strains for both yield and nodule abundance, both being larger at pH 6.2. Secondly, the yield from a given strain at low pH had no useful relationship to its yield at high pH. Thirdly, at pH 4.6, the highest yielding (greater than 3 g/2 plants) strains were all tolerant of Al in media; CB756, TAL189, 209, 425, 169, 173 and 174. At this pH the soil has a high level of Al and these media-tolerant strains proved to be tolerant symbiotically. That this occurred over a fairly large nodule abundance range implies that high tolerance involves other factors besides just good survival or ineffectiveness as indicated by nodule number. Other Al-tolerant strains, such as n12 and TAL172 had a more reduced yield at a nodule abundance equal to that of CB756 and TAL169. Also, the acid-sensitive strains CB1024 and CB1024a yielded only 50% as much at pH 4.6 as at 6.2 while having the same nodule number. But as seen in fig. 4, they were much smaller nodules.

Figure 3 also shows that this soil contained indigenous rhizobia which were effective at pH 6.2. Analysis of yield data with use of Duncan's multiple range test showed that only one applied strain, n9, was significantly better than the zero control at high pH. However, at low pH, 20 of the 23 strains were significantly better, so that the indigenous rhizobia were sensitive to the soil acidity complex.

Figure 4 shows the relationship between yield and nodule mass. Similar trends are seen in the data as with nodule number; a general yield response with increasing nodule mass per plant, the 2 clusters

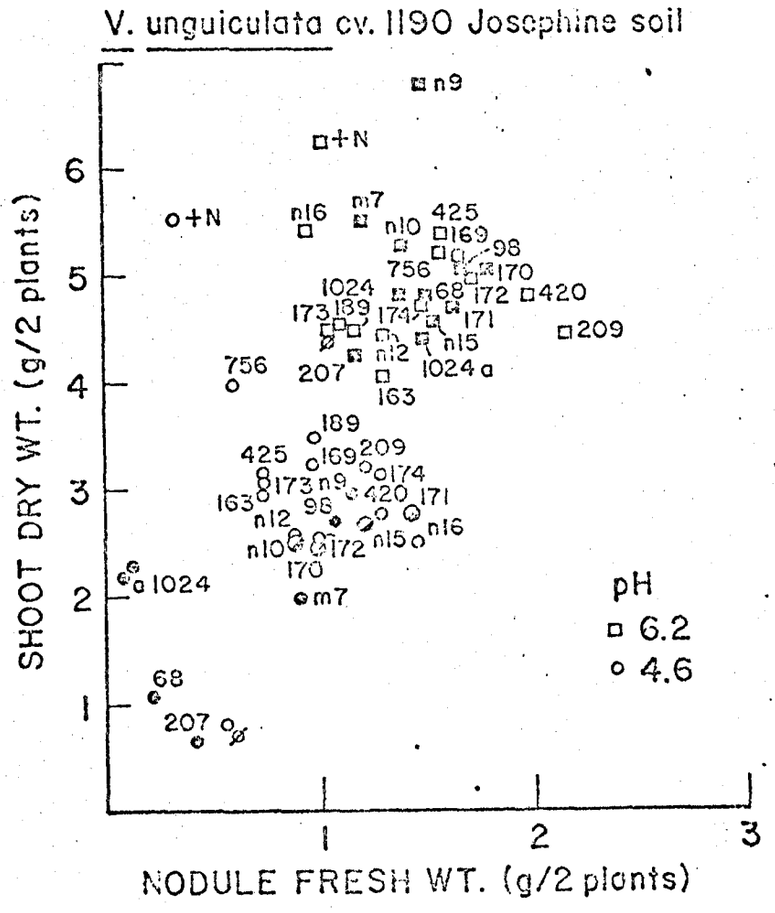


Figure 4 - Relationship of yield and nodule mass on TVu 1190. For meaning of symbols and shading see Figure 3. Mean nodule weights are significantly different from each other (5% level) if they differ by a factor of 1.4.

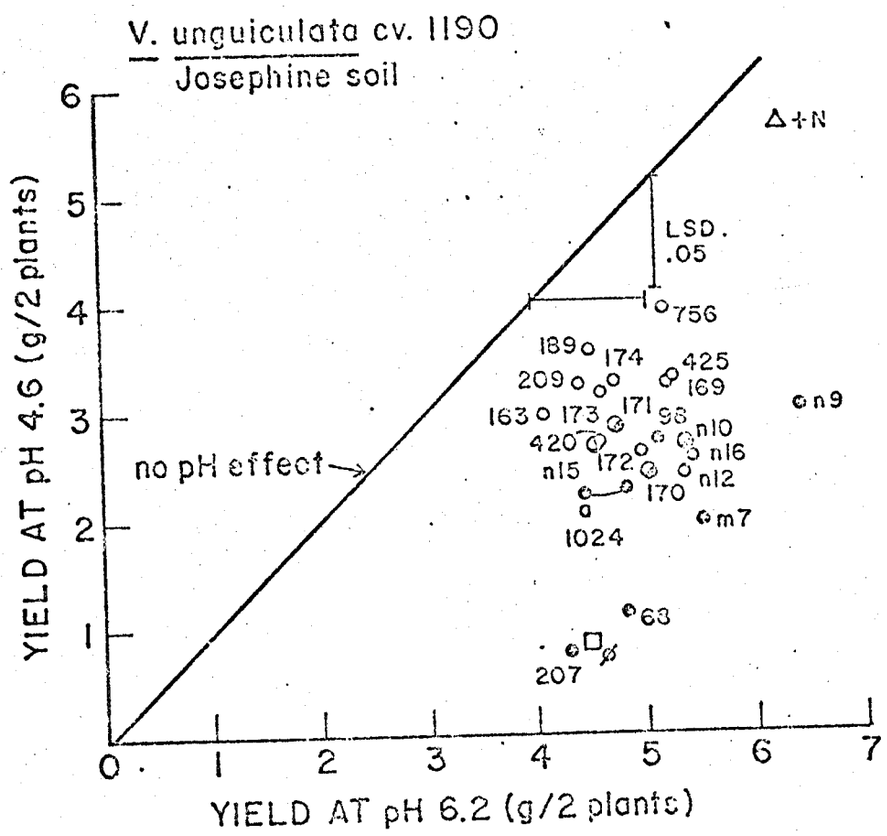


Figure 5 - Comparison of plant yield at pH 6.2 and 4.6 for different rhizobial strains on TVu 1190. For meaning of symbols and shading see Figure 3.

of treatment pH with a general reduction in mass at low pH, and the unrelatedness of nodule mass at high pH with that at low pH for a given strain. While nodule abundance and mass generally decreased together at low pH, the average nodule size tended to increase slightly for the majority of strains, but exceptions are seen as for the CB1024 strains. Also, the best yielding strain at low pH, CB756, had relatively low nodule numbers and mass indicating a very high specific N-fixation effectiveness for these conditions.

Figure 5 compares the yield of strains at both pH treatments. Distance to the right relates to effectiveness at high pH, while vertical distance from the diagonal indicates relative acid tolerance for a strain. It emphasizes again that the best yielding strains in the stress condition in soil are those which were identified as the most tolerant of stresses in laboratory media. Strain n9 was classed as acid-sensitive in media, and while it was better than all strains at pH 6.2, it showed greater than 50% yield reduction at 4.6. Still, it managed to do comparatively well at low pH, especially among the acid-sensitive strains.

Table 3 lists the results of the statistical tests of association as applied in the BE5 trial. The t test for mean difference of the 2 populations for net acid yield is highly significant. The chi-squared test of association of tolerance in media with that in soil was not significant, the value occurring at about the 6.5% probability level. It is obvious though that the majority of strains were associated for these tolerances.

TVu 4557 variety - Josephine soil

Table 3. Rhizobial association between tolerances in media and in soil with the TVu 1190 variety.*

A. Comparison of 2 populations for relative symbiotic acid tolerance.†

	net acid yield / (Yield-YieldØ) 4.6
1. Strains sensitive to Al in media	
TAL207 = 207	- 0.18
IQ68-5 = 68	0.23
CB1024 = 1024	1.47
CB1024a - 1024a	1.36
m7	1.12
n9	2.12
TAL98 = 98	1.84
TAL170 = 170	1.60
TAL171 = 171	1.96
n10	1.66
n15	1.82
mean	1.36
standard deviation	0.72
2. Strains tolerant of Al in media	
n12	1.69
n16	1.66
TAL172 = 172	1.71
TAL420 = 420	1.92
TAL163 = 163	2.10
TAL209 = 209	2.38
TAL189 = 189	2.65
TAL173 = 173	2.22
TAL174 = 174	2.28
TAL169 = 169	2.40
CB756 = 756	3.09
TAL425 = 425	2.34
mean	2.20
standard deviation	0.42

Students t test for mean difference of the 2 populations:
$t = 3.36\Lambda$

B. Comparison of 2 populations for association between tolerance in media and tolerance in soil.∇

Fraction of strains tolerant in media also tolerant in soil: 9/12
Fraction of strains sensitive in media also sensitive in soil: 8/11

Chi-squared test of association using Yeats correction factor:
$\chi^2 = 3.50$ nonsignificant (3.8 required at 5% level).

Footnotes for Table 3.

* See footnote 1, Table 2.

† See footnote 2, Table 2.

§ Net acid yield is defined as the net growth (corrected for the growth of the zero control) at pH 4.6. The effectiveness of the indigenous rhizobia (zero control) at high pH made the use of the acid tolerance quotient (Table 2) less desirable.

Λ Significant statistically at the 1% level.

∇ Significant statistically at the 1% level.

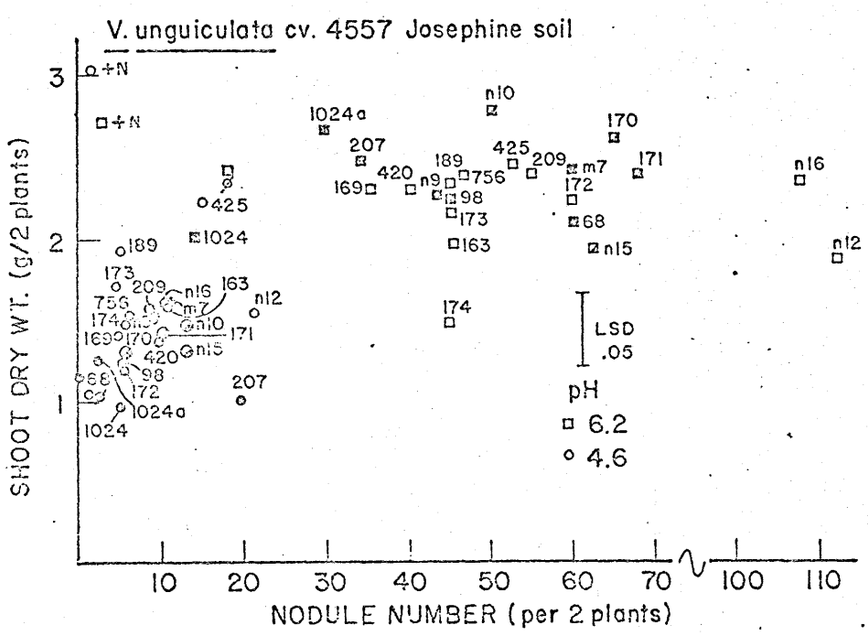


Figure 6 - Comparison of rhizobial strain performance on cv. 4457 in acid and limed Josephine soil. For meaning of shading and symbols see Figure 3. Mean nodule numbers are significantly different from each other if they differ by a factor of 3.0.

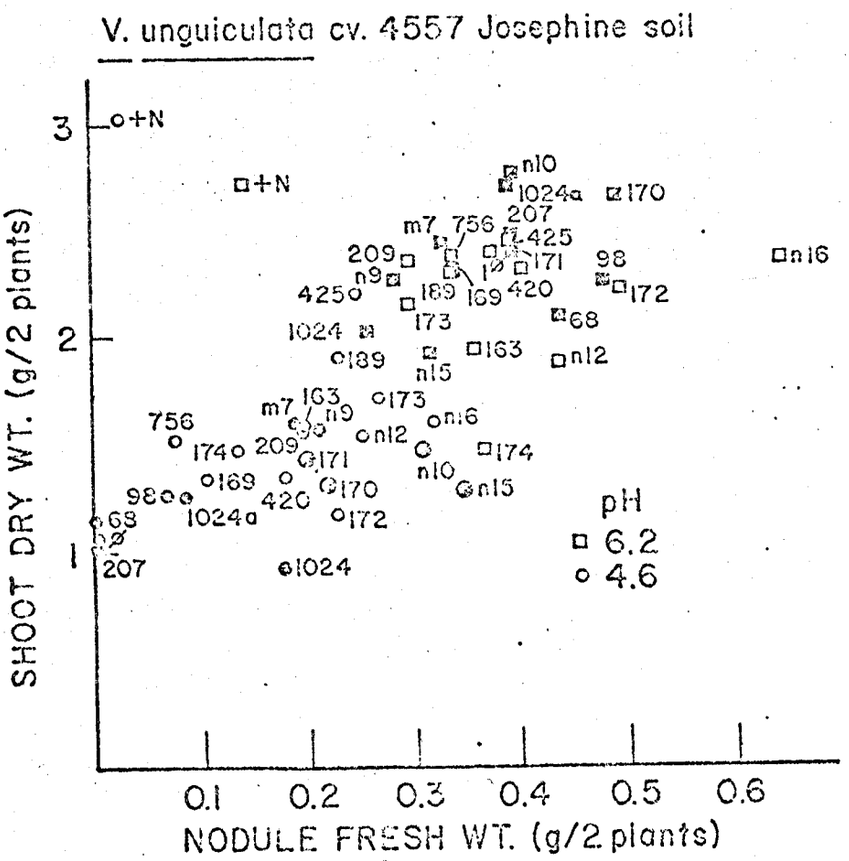


Figure 7 - Relationship of yield and nodule mass on TVu 4557 on Josephine soil. For meaning of symbols and shading see Figure 3. Mean nodule weights are significantly different from each other (5% level) if they differ by a factor of 3.2.

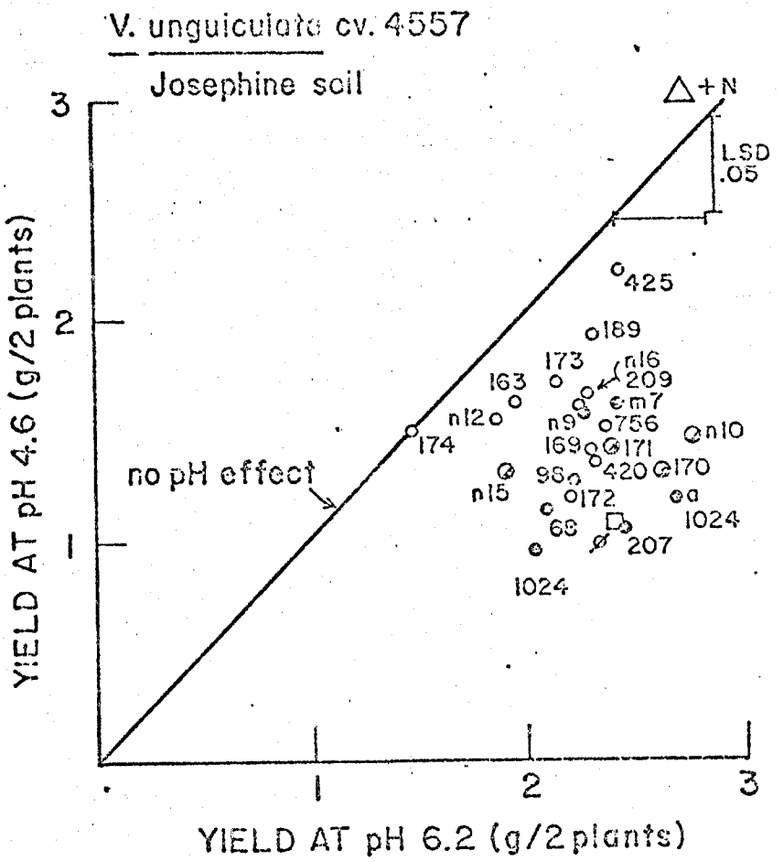


Figure 8 - Comparison of plant yield at pH 6.2 and 4.6 for different rhizobial strains on TVu 4557. For meaning of symbols and shading see Figure 3..

Table 4. Rhizobial association between tolerances in media and in soil with TVu 4557 variety.*

A. Comparison of 2 populations for relative symbiotic acid tolerance.†

	net acid yield § / (Yield-YieldØ) 4.6
1. Strains sensitive to Al in media	
TAL207 = 207	- 0.03
IQ68-5 = 68	0.09
CB1024 = 1024	- 0.11
CB1024a = 1024a	0.21
m7	0.55
n9	0.48
TAL98 = 98	0.20
TAL170 = 170	0.24
TAL171 = 171	0.37
n10	0.41
mean	0.25
standard deviation	0.19
2. Strains tolerant to Al in media	
n16	0.56
TAL172 = 172	0.12
TAL420 = 420	0.30
TAL163 = 163	0.54
TAL209 = 209	0.53
TAL189 = 189	0.85
TAL173 = 173	0.65
TAL169 = 169	0.32
CB756 = 756	0.46
TAL425 = 425	1.14
mean	0.55
standard deviation	0.29

Students t test for mean difference of the 2 populations:
$t = 2.70\Lambda$

B. Comparison of 2 populations for association between tolerance in media and tolerance in soil.∇

Fraction of strains tolerant in media also tolerant in soil: 7/10
Fraction of strains sensitive in media also sensitive in soil: 7/10

Chi-squared test of association using Yeats correction factor:
$\chi^2 = 1.80$, nonsignificant

Footnotes for Table 4.

* See footnote 1, Table 2.

† See footnote 2, Table 2.

§ See footnote 3, Table 3.

Λ Significant statistically at the 5% level.

∇ For this variety, tolerance in soil was defined as a yield not 1.5 times significantly different from the yield of the best strain at pH 4.6. The factor 1.5 was used since the yield of the best strain at 4.6 was much higher than for the bulk of the strains.

The time sequence of modulation in both treatments was much the same as for the previous 2 varieties.

Figure 6 summarizes the yield response from the strains as related to nodule number, and in Fig. 7 as related to nodule mass. The trends are much the same as for the 1190 variety; a general yield response with increasing nodule mass and number, these both being reduced by acidity; the greater sensitivity to acidity of the symbioses as compared with the nonsymbiotic condition (+N); the unrelatedness of yield at pH 6.2 to that at 4.6 for a given strain; and the majority of the best yielding strains at low pH were previously determined to be most tolerant of soil acidity factors.

The indigenous rhizobia in the Josephine soil were also effective on this variety at pH 6.2. Analysis of the yield data using Duncan's multiple range test showed that at high pH no strain was significantly better (5% level) than this zero control, and 3 strains were significantly poorer, being then considered ineffective on this variety -n15, n12, and TAL174. At low pH, 10 of the strains were significantly better than the zero control, though only 2 gave absolute lower yields than the zero control. The remaining strains were not significantly different from the control.

Figure 8 shows the comparative yields at pH 6.2 and 4.6 for each strain. Ignoring the ineffective strains (n12, n15, and TAL174), those with the least relative yield reduction due to effects of soil acidity are those determined as tolerant of acidity and aluminum. Again, the agreement between tolerances in media and soil is by no means complete; strains M7 and n9, which were acid sensitive in media,

yielded better at low pH than such acid-Al tolerant strains as CB756, TAL169, 172 and 420, while all had about the same effectiveness at pH 6.2.

Table 4 lists the results of association tests between tolerance in media and soil. As was found with variety 1190, the t test shows a significant difference between the 2 populations for net yield of plants at low pH, while the X^2 test of association of tolerances is not significant.

DISCUSSION

The data presented here show that there is large strain differences among rhizobia of the cowpea miscellany for symbiotic acid tolerance on different varieties of V. unguiculata. Soil analytical data indicate that the main stress in the acid Goldridge soil is that of pH per se, while in the acidified Josephine it is the high level of exchangeable and soluble Al. Nonetheless, strains which gave the best yield on varieties on either soil at low pH had the common property of being the most tolerant of acidity factors as tested in pure culture, especially to Al.

On studies of symbioses of V. radiata (mung bean) in the Goldridge soil, Munns et al. (1978) confirmed that it was not necessary to sacrifice effectiveness to get acid tolerance. This was also found in the present study on this same soil with the BE5 variety of cowpea.

On all 3 varieties, the effects of the soil acidity complex were to generally reduce yield by reducing nodule abundance and mass on the

N-deficient soils. The more severe effects of low pH on the symbiotic growth of the TVu varieties than with BE5 is attributed to the high Al level in the Josephine soil. No nitrogen analysis of plant material was done, as it was clear that the growth response of the initially yellow plants on these soils was due to the availability of N through rhizobial association. Also, Munns et al. (1978) have already shown that the yield of symbiotic V. radiata in the Goldridge soil correlated well with %N in the tops. Therefore, the inhibitory effects of soil acidity seen here are attributable directly to effects on the overall performance of the Rhizobium.

The drastic effects of pH and Al on nodulation seen here are in agreement with previous studies (Sartain and Kamprath 2975; Carvalho 1978; Munns et al. 1978). Also, from this present study there is good evidence that nodulation is reduced because growth of some strains of rhizobia is impeded by acidity and/or Al.

The best agreement between strain tolerance in media and in soil is seen with the highest and lowest yielding strains at low pH. Tolerance in media was for survival and growth alone. For symbiotic performance in a stressed soil, other events of modulation or nodule function may be limited by acidity or Al for a giver strain, a limitation not related to the ability to make growth in their presence. This may be why some effective (pH 6) and tolerant (in laboratory media) strains perform poorly in acid soil, despite adequate modulation. Also, some strains classed as acid and Al sensitive may simply survive better in stressed soil than pure culture

studies would indicate. Tests of strain survival alone in pH-Al stressed soil could clear up some of these points.

Spain (1975) has discussed the forage potential of acid, allic soils in South America. He notes that there are several tolerant legumes that have promise for good growth on such soils, provided they are effectively nodulated. If indigenous, effective rhizobia are not present in these soils, any inoculum for these plants would have to have the property of Al-tolerance for adequate saprophytic competence of the bacteria and subsequent host growth on such soils.

This is the first reported investigation of the relationship of rhizobial tolerances to acidity factors in media with that symbiotically in acid soil. Similar work is underway in Colombia (J. Halliday, personal communication) and it does not seem unlikely that with experience better relationships will be found. Even at this early stage of development of such screening of strains for defined tolerances, it appears to be a promising technique for use in selection of inocula for acid, unlimed soils. The value of screening strains for acidity related tolerances in defined laboratory media is seen in that if a decision was made to eliminate Al-sensitive strains for use in acid soil trials, then 60 to 70% of the poorest strains would have been retained for further testing in soil, none of the best yielding strains would have been eliminated, and this is the right kind of error to have for a pre-screening procedure. Obviously, the most critical test of the validity of such pre-screening would be its relation with strain performance in field trials.

APPENDIX

RHIZOBIAL STRAINS

Strain	Source*	Sections and experiments in which tested	Tolerance rating†
Cowpea miscellany			
h4=3I6h4	1	IB, IIB	iii
h7=3I6h7	1	IB	iii
n9=3I6n9	1	IB, IIB, IV	i
n10=3I6n10	1	IB, IIB, IV	ii
n12=3I6n12	1	IB, III, IV	iii
n15=3I6n15	1	IB, IIB, IV	ii
n16=3I6n16	1	IB, IV	iii
n22=3I6n22	1	IB	ii
m2=TAL437	2	IB, IIB	ii
m3=TAL438	2	IB, IIB, III	iii
m4=TAL439	2	IB	iii
m5=TAL440	2	IB, IIB	i
m6=TAL441	2	IB, IIB	i
m7=TAL442	2	IB, IV	i
m8=TAL443	2	IB, IIB	ii
11=TAL11	3	IB, IIA, IIB IIB, IV	iii
32H1	4	IB, IIB	iii
68=IQ68-5=TAL463	5	IB, IIB, IV	i
98=TAL98=CIAT239	6	IB, III, IV	i
98A=TAL98A=TAL98=CIAT239§	6	IB, III	i
108=IQ108	5	IB, IIB	i
163=TAL163	3	IB, IIB, IV	iii
169=TAL169=TAL169N=176A22	7	IA, IB, IIA, IIB, III, IV	iii
170=TAL170=176A23	7	IB, IIB, IV	ii
171=TAL171=176A27	7	IB, IIB, IV	iiV
172=TAL172=176A28	7	IB, IIB, IV	iii
173=TAL173=TAL173N=176A30	7	IA, IB, IIB, IV	iii
174=TAL174=TAL174N=176A32	7	IB, IIA, IIB, III, IV	iii
189=TAL189-176A31	7	IB, IIC, IV	iii
200N=TAL200N=150C6	7	IA, IB, IIB	ii
200NA=TAL200NA=150C6§	7	IA, IB, IIB	ii
207=TAL207	3	IB, IV	i
209=TAL209	3	IB, IIB, III, IV	iii
281=TAL281	3	IB	iii
295A=TAL295A	3	IB, IIB	ii
303=TAL303A	3	IB, IV	i

Strain	Source*	Sections and experiments in which tested	Tolerance rating†
305=TAL305	3	IB	ii
306=TAL306	3	IB	ii
420=TAL420=THA301	8	IB, IIB, IV	iii
421=TAL421=THA302	8	IB, III	iii
422=TAL422=THA303	8	IB, IIB	ii
423=TAL423=THA304	8	IB, IIB	iiV
425=TAL425=THA306	8	IB, IIB, IIC, IV	iii
756=CB756	9	IA, IB, III, IV	iii
1024=CB1024	9	IA, IB, IIB, IV	i
1024a=CB1024a=CB1024A	9	IB, IV	i
921=IQ921	5	IB, IIB	i
176A38	7	IB, IIB	ii
127D5	7	IB, IIB	iii
TAL175A=8A11	7	IB, IIB	iii
TAL176A=8A14	7	IB, IIB	iii
TAL236A	3	IB	iii
TAL307	3	IB, IIB	iii
R. japonicum			
61A101	4	IB, IIB	ii
61A112	4	IB, IIB	ii
61A124	4	IB, IIB	i
61A144	4	IB, IIB	ii
61A150	4	IB, IIB	i
Allen 511	10	IB, IIB	i
Allen 519	10	IB, IIB	ii
Allen 527	10	IB	ii
Allen 542	10	IB, IIB	iii
CB1809=TAL379	11	IB, IIB	i
CC709	12	IB	iii
TAL102=110=IITA 18	13	IB	iii
USDA110=110	14	IB, IIB, III	i

* Sources: 1. U.S. Department of Agriculture, Beltsville, Maryland. 2. Univ. of Philippines, Los Banos, via Univ. Hawaii NifTAL Project, Paia, Hawaii. 3. NifTAL isolates. 4. Nitragin Co. Inc., Milwaukee, WI. 5. Iraq Ministry of Agriculture (via NifTAL). 6. Centro Internacional de Agricultura Tropical, Cali, Colombia (via NifTAL). 7. Nitragin Co., Milwaukee, WI (via NifTAL). 8. Thai Min. of Agriculture, Bangkok (via NifTAL). 9. CSIRO Division of Tropical Crops and Pastures, Brisbane, Australia. 10. From the collection of O. N. Allen (via NifTAL). 11. CSIRO, Brisbane (via NifTAL). 12. CSIRO, Canberra, Australia from J. M. Vincent. 13. USDA, Beltsville (via NifTAL, via I.I.T.A., Ibadan, Nigeria). 14. USDA, Beltsville from R. Valentine, Univ. of California, Davis.

† Tolerance ratings: Based on test in Sections I and II, strains were assigned to the following categories; i=sensitive to pH 4.5, ii=tolerant of pH 4.5, sensitive to 50 µM Al, iii=tolerant of pH 4.5 and 50 µM Al.

§ TAL98A and TAL200NA were reselections at NifTAL of TAL98 and TAL200N respectively.

Λ CB1024a was a separate culture received subsequent to that of CB1024.

∇ TAL171 and TAL423 were tolerant of pH 4.5 with 1 mM P and 200 µM Mn (see Section II).

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