Reprinted from the Soil Science Society of America Journal
Volume 43, no. 3, May-June 1979
677 South Segoe Rd., Madison, WI 53711 USA

Effects of Calcium, Manganese, and Aluminum on Growth of Rhizobia in Acid Media[1]

H. H. KEYSER AND D. N. MUNNS[2]

ABSTRACT

Growth studies were done in defined liquid media to assess 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 4.5 and 10 strains of *Rhizobium 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 three cowpea strains at pH 4.6, *low P (10 μM)* limited maximum population density in all three strains. Low Ca limited it in one strain.

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 4.8. High Mn and low Ca slowed growth of some strains, but Mn stopped growth of none and low Ca stopped growth of only three strains. Neither was as severe a stress as 25-50 *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 three cowpea strains, by a factorial experiment with three Ca levels (50-1,000 *μM)* and four Al levels (0-100 *μM),* at pH 4.5 in liquid media. Calcium had a statistically significant protective effect against AI in two strains, but the effects were small and probably of no biological or practical significance.

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

Additional Index Words: acidity, calcium, manganese, aluminum, rhizobia, cowpea miscellany, *Rhizobium japonicum*

. Munns, D. N., and H. H. Keyser. 1979. Effects of calcium, manganese and aluminum on growth of rhizobia in acid media. Soil Sci. Soc. Am. J. 43:500-503.

T HE REQUIREMENT for Ca as an essential nutrient for rhizobia is quite small, as determined in liquid media (Vincent 1962). Vincent (1962) showed the Ca requirement to be about 25 $_{\mu}$M (micromoles/liter) for normal growth, and found no effect of pH down to 5.5 on the response to Ca from 0.1 to 10 mM (millimoles/liter) for *Rhizobium trifolii* at various pH down to 4.5 which stopped growth. However, Rerkasem (1977)[3] reported 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 nonsymbiotic legumes (Munns, 1965), there is little information of such an interaction on rhizobia. The one relevant study is that of Rerkasem (1977)[3] where 1 m*M* 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, 1966). Rhizobia can tolerate very high levels of Mn in artificial media (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 on rhizobia in acid media of low Ca and high Mn alone and in combination with high Al, (ii) to compare these effects with those of low P and low P + high Al from Keyser and Munns (1979), and (iii) to determine any effects of increasing Ca levels on the response to Al among rhizobia.

MATERIALS AND METHODS

Rhizobia and Culture Media-Our previous paper (Keyser and Munns, 1979) lists sources of rhizobia, and particulars of media preparation, adjustment of Al and pH, and counting of viable cells. The basal solution in all treatments is as follows: Mannitol 10g/liter, Na-glutamate l.lg/liter; salts (μM)· $MgSO_4$ 300, Ferric EDTA 100, KCl 10, $MnC1_2$ 1, $ZnSO_4$ 0.4, $CuCl_2$ 0.1, Na_2Mo0_4 0.02, $Co(NO_3)_2$ 0.002, distilled water. Also, for strains which demonstrated a response to growth factors, 1 ppm thiamine and 0.1 ppm biotin were added. Specific additions to the basal solution for the different treatments are listed 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 290-Erlenmeyer flasks, plugged with cotton, covered with a small beaker, and autoclaved for 20 min. Bacteria from agar slopes of similar age were suspended and serially diluted so that delivering 1 ml to treatments gave an initial density of about 10^3 cells/ml. The diluent was basal solution adjusted to pH 4.6. Population density was determined as total viable cells. Population density at time zero was determined directly from the inocula. Inoculated cultures were incubated at 25°C on a slowly reciprocating shaker. In sampling for population density, 1 ml of media was aseptically removed.

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 1. The treatments were adjusted to pH 4.5 for cowpea miscellany and 4.8 for *R. japonicum* strains (pH 4.5 was found to be too stressful for many of the *R. japonicuin* strains) (Keyser and Munns, 1979). 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 cultures tubes. The inocula diluent was basal solution adjusted to the same pH as that of the given medium. The incubation conditions were the same as in Experiment A, and 0.1-ml of media was removed at each sampling for population density.

[1]Contribution from Department of Land, Air and Water Resources, University of California, Davis CA 95616. Supported by grants from U.S. Agency for International Development (NifTAL Project) and NSF/RANN. Received 21 July 1978. Approved 14 Feb. 1979.

[2]Postgraduate Research Scientist and Professor of soil science, respectively. Senior author is now with U.S. Department of Agriculture, SEA, AR, Cell Culture :, Nitrogen Fixation Lab., BARC-West, Beltscille, Md 20705.

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

Table 1—Media used for different experimental treatments.

Experimental treatment	Additions to basal† solution (*μM*)							
	$CaCl_2$	$MnCl_2$	$Alk(SO_4)_2$	KH_2PO_4	K_2HPO_4	K_2SO_4	KCl	pH‡
			Experiment A					
Full nutrients	300	0	0	500	500	0	0	4.6
Low P	300	0	0	10	0	750	0	4.6
High Mn	300	200	0	500	500	0	0	4.6
Low Ca	50	0	0	500	500	0	0	4.6
			Experiment B					
High Mn	300	200	0	500	500	0	0	4.5/4.8§
Low Ca	50	0	0	500	500	0	0	4.5/4.8
Combined factors	50	200	25/50¶	5	0	0	1,500	4.5/4.8
			Experiment C					
Ca × Al factorial	50, 250 or 1,000	0	0, 25, 50 or 100	5	0	0	1,500	4.5

† See Materials and Methods section.
‡ pH adjusted with HCl.
§ pH 4.5 for test with cowpea miscellany and 4.8 with *R. japonicum*.
¶ 25 *μM* Al for test with *R. japonicum* and 50 *μM* with cowpea miscellany.

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. The incubation conditions were the same as in Experiment A, and 0.1-ml of media was removed at each sampling for population density.

RESULTS AND DISCUSSION

At low pH, 50 *μM* Ca and 200 p.M Mn imposed little, if any, stress to the majority of cowpea miscellany and R. *japonicum* strains (Fig.l and 2, Tables 2 and 3). Results from Experiment A (Fig. 1) show that while 10 ,*μM* P limited population density in all three strains, 200 *μM* Mn did not, and 50 μM Ca did so only for strain TAL 11. The data suggest high Mn may have slowed early growth rate for TAL 169N and TAL 11 (Fig. lb and lc). The turbidity tests of Experiment B (Table 2) also demonstrate the fairly uniform tolerance of low Ca and high Mn. Of the strains previously determined as acid tolerant but Al sensitive (Keyser and Munns, 1979), three were sensitive to low Ca, whereas none of the Al-tolerant strains were sensitive to the Mn or Ca.

Table 3 shows that while Al is the most severe single stress to the rhizobia, an additive negative effect is found for a few stains (172, M3, 61A101, and 61A112) when low Ca and high Mn are also present with the Al. This may be of significance since all these factors could occur together in acid soils (Munns, 1977, a&b).

Compared with soil solution analyses from a wide spectrum of soils, 50 *μM* Ca is realistically low (Reisenauer, 1966; Gilman and Bell, 1978). Vincent (1962) reported that Ca deficiency for several strains did not occur above a level of 25 *μM* at pH 5.5, however we found three strains which did not make turbid growth at pH 4.5 with 50 *μM* Ca. These same strains were able to make turbid growth with 300μ*M* Ca (in the high Mn treatment; also Keyser and Munns, 1979.) A similar response has been found by Rerkasem (1977)[3] for some fast growing rhizobia. While it is difficult to find data on soil solution Mn analyses, the 200-*μM* level tested here has been shown to be inhibitory to several legumes grown nonsymbiotically in solution culture (Morris and Pierre, 1949; Andrew and Hegarty, 1969). Rhizobia have been shown to tolerate levels of Mn up to 16 mM in media, but not in media as acid as reported here (Masterson, 1968; Holding and Lowe, 1971). Further, comparable levels of both these acidity factors (Ca and Mn) are known to adversely affect either the nodulation, nodule function or growth of symbiotic and nonsymbiotic legumes that are hosts for these strains (Andrew and Hegarty, 1969; Lowther and Loneragan, 1970; Munns, 1977a & b; Andrew, 1978). Therefore, under acid conditions the tolerance to low Ca or high Mn among most slow

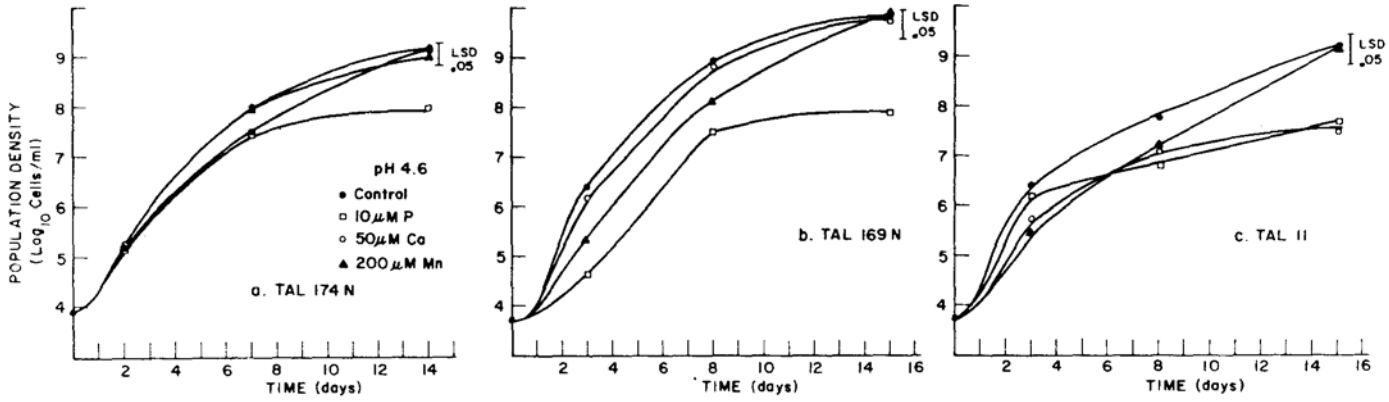

Fig. 1—Response of three cowpea rhizobia to P. Ca and Mn (Exp. A).

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*			
				50 μM Ca		200 μM Mn	
	Tested	Sensitive to Ca	Sensitive to Mn	x§	r¶	x	r
		Cowpea miscellany					
Sensitive to pH 4.5- μM P‡	9	--	--	--	--	--	--
Tolerant of pH 4.5, sensitive to 50 μM Al	10	3	0	8.4	6–12	11.4	7–24
Tolerant of 50 μM Al	13	0	0	8.1	6–15	9.2	7–15
		R. japonicum					
Tolerant of pH 4.8, sensitive to 25 μM Al	3	0	0	10.3	10–11	10.0	10
Tolerant of 25 μM Al	7	0	0	8.6	5–10	8.6	5–10

† Mean initial densities; cowpea miscellany $10^{3.3\pm0.8}$ cells/ml: *R. japonicum* $10^{3.3\pm0.3}$ 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).
§ Mean.
¶ Range.

Table 3—Response to individual and combined acidity factors among rhizobia (Exp. B).

	Time to turbidity, in days				
Strains Strains	5 μM P†	50 μM Ca	200 μM Mn	50 μM Al†	Combined factors
		Cowpea miscellany (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).

growing rhizobia strains appears at least equal or superior to that of the host plant.

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

The statistical analysis for TAL 11 shows no main or interaction effects of Ca. All levels of added Al caused a significant early reduction in population density, with the 25 μM Al treatment thereafter showing a faster growth rate than the two higher levels (Fig. 3a). The low P level in this trial limits total cell number and therefore prevents the response to Ca that TAL 11 showed in Experiment A.

For TAL 189 (Fig. 3b), the initial large decrease in viability occurred only at the two highest Al levels; however, this strain was able to recover rather well,

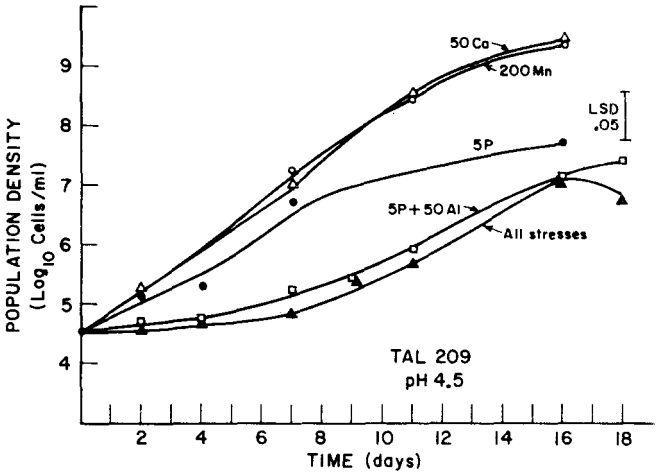

Fig. 2—Response of strain TAL209 to individual and combined acidity factors (Exp. B).

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 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 two higher Ca levels at the same Al levels. However, the 50μM Al level at the lowest Ca addition grew slightly faster than at the two higher Ca levels, so that a meaningful trend is not apparent.

For TAL 425, 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 (Fig. 3c).

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 TAL 189 and TAL I 1 were probably due to death of cells, not to clumping. Aluminum-induced clumping, observed by

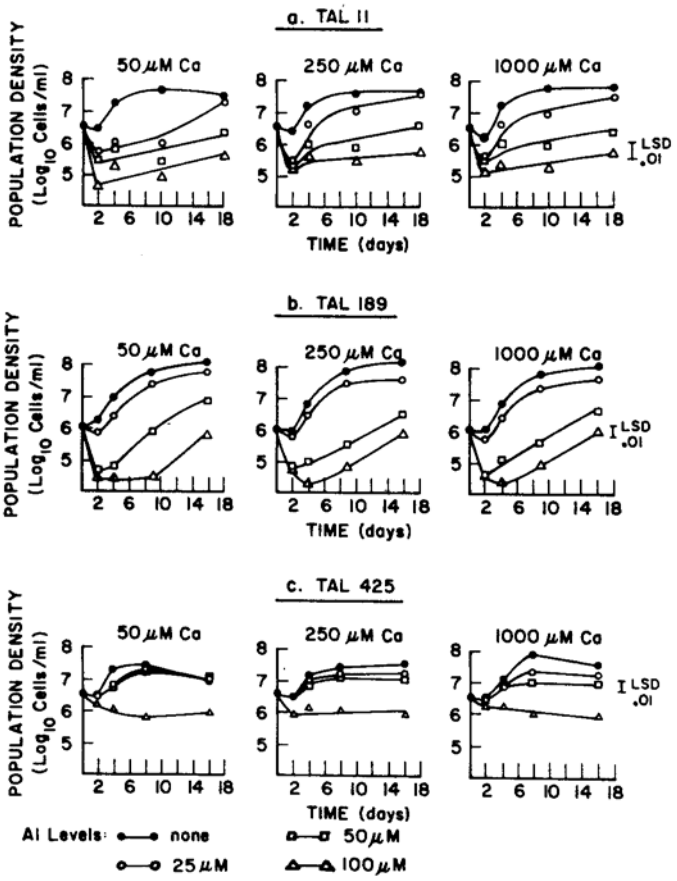

Fig. 3—Response of three cowpea rhizobia to factorial Ca and Al levels (Exp. C).

Rerkasem (1977) at very high Al concentration (1 mM), was restricted to fast-growing strains, 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.

For growth of legumes in nutrient solution, Ca levels from 1 to 5 mM can ameliorate the effects of Al (Munns, 1965), but for the three 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).[3] 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, 1954). 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.

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.

ACKNOWLEDGMENTS

The authors wish to thank Drs. Deane Weber, Joe Burton, Victor Reyes, Tow Wacek, and Dick Date for supplying strains of rhizobia, and Julia Hoheuberg, David Lauter, and Peter Vonich for their valuable technical assistance.

Table 4—Analysis of variance for Ca × Al trial (Exp. C).

Source	df	Strains		
		TAL 11	TAL 189	TAL 425
		F ratio†		
Ca	2	2.36	0.17	4.16*
Al	3	332.6 ***	2,735.00***	481.00***
Time	3	124.6 ***	1,381.00***	136.00***
Ca × Al	6	1.09	2.98*	2.81*
Ca × Time	6	0.55	2.10	2.87*
Al × Time	9	11.21***	67.2 ***	33.22***
Ca × Al × Time	18	0.97	3.29***	4.39***
Error	96			

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

LITERATURE CITED

1. Adams, F. 1974. Soil solution. p. 441-481. *In* E. W. Carson (ed.) The plant root and its environment. Univ. Press of Virginia, Charlotteville.
2. Andrew, C. S., and M. P. Hegarty. 1969. Comparative responses to Mn excess of 8 tropical and 4 temperate pasture legume species. Aust. J. Agric. Res. 20:687-96.
3. Andrew, C. S. 1978. Mineral characterization of tropical forage legumes. p. 93-112. *In* C. S. Andrew and E. J. Kamprath (eds.) Mineral nutrition of legumes in tropical and subtropical soils. CSIRO, Melbourne, Australia.
4. Dobereiner, J. 1966. Manganese toxicity effects on nodulation and nitrogen fixation of beans in acid soils. Plant Soil 24:153-166.
5. Gillman, G. P., and L. C. Bell. 1978. Soil solution studies on weathered soils from tropical north Queensland. Aust. J. Soil Res. 16:67-77.
6. Holding, A. J., and J. F. Lowe. 1971. Some effects of acidity and heavy metals on the Rhizobium-leguminous plant association. p. 153-166. *In* T. A. Lie and E. G. Mulder (eds.) Biological nitrogen fixation in natural and agricultural habitats. Plant and Soil Spec. Vol.
7. Keyser, H. H., and D. N. Munns. 1979. Tolerance of rhizobia to acidity, aluminum, and phosphate. Soil Sci. Soc. Am. J. 43:519-523 (this issue).
8. Loneragan, J. F., and E. J. Dowling. 1958. The interation of Ca and H ions in the nodulation of subterranean clover. Aust. J. Agric. Res. 9:464-472.
9. Lowther, W. L., and J. F. Loneragan. 1970. Calcium in the nodulation of legumes. Proc. 11th Inter. Grasslands Congr. p. 446-450.
10. Masterson, C. L. 1968. Effects of some soil factors on R. trifolii. Int. Congr. Soil Sci., Trans. 9th (Adelaide) 11:95-102. 11. Morris, H. D., and W. H. Pierre. 1949. Minimum concentrations of manganese necessary for injury to various legumes in culture solutions. Agron. J. 41:107-112.
12. Munns, D.. N. 1965. Soil acidity and growth of a legume. II. Aust. J. Agr. Res. 16:743-755.
13. Munns, D. N. 1977a. Mineral nutrition and the legume symbiosis. p. 353-392. *In* R. W. F. Hardy and A. H. Gibson (eds.) Treatise on dinitrogen fixation. Section IV, Wiley & Sons, New York.
14. Munns, D. N. 1977b. Soil acidity and related factors. p. 211-236. *In* J. M. Vincent, A. S. Whitney and J. Bose (eds.) Exploiting the legume-Rhizobium symbiosis in tropical agriculture. Univ. Hawaii Coll. Trop. Agr. Misc. Publ. 145.
15. Reisenauer, H. M. 1966. Mineral nutrients in soil solution. p. 507-508. *In* P. L. Altman and D. S. Dittman (eds.) Environmental Biology. Fed. Am. Soc. for Exp. Biol. Bethesda, MD.
16. Vincent, J. M., and L. W. Waters. 1954. The root nodule bacteria as factors in clover establishment in the red basaltic soils of the Lismore District, New South Wales. It. Survival and success of inocula in laboratory trials. Aust. J. Agric. Res. 5:61-76.
17. Vincent, J. M. 1962. Influence of Ca and Mg on growth of Rhizobium. J. Gen. iMicrobiol. 28:653-663.