Soil Biol. Biochem. Vol. 22, No. 3, pp. 343–347, 1990
Printed in Great Britain. All rights reserved
0038-0717/90 $3.00 + 0.00

SALT EFFECTS ON SURVIVAL AND MULTIPLICATION OF CHICKPEA AND SOYBEAN RHIZOBIA

E. A. E. ELSHEIKH and M. WOOD*

Department of Soil Science, The University of Reading, London Road, Reading RG1 5AQ, England

(*Accepted* 30 *August* 1989)

Summary—Fourteen strains of chickpea rhizobia, eight strains of soybean rhizobia and eight strains of soybean bradyrhizobia were screened for their tolerance to different concentrations of NaCl. Assessment of visible turbidity after 14 days indicated 15 strains of both chickpea and soybean rhizobia tolerant of 0.34 M NaCl. Most fast-growers were more salt-tolerant than the slow-growers. Three salt-tolerant strains were further tested for their tolerance to different salts. $MgCl_2$ was the most inhibitory salt, and soybean rhizobia were slightly more tolerant than chickpea rhizobia.

Prior exposure to salt did not alter the subsequent response to the same or different salts and the same or higher concentrations. A 2% mixture of salts more representative of conditions in saline soils had no effect on survival of strains which initially multiplied in 2% NaCl but then died.

INTRODUCTION

Soils in many parts of the Sudan suffer from salinity (Mustafa, 1984). This includes area where chickpea (*Cicer arietinum*) and soybean (*Glycine max*) are being introduced. Saline conditions may affect the legume–*Rhizobium* symbiosis by reducing the survival of rhizobia, inhibiting the infection process, affecting nodule function or reducing plant growth (Singleton *et al.*, 1982).

The tolerance of different species of *Rhizobium* to sodium chloride varies from 0.1 to 0.65 M (Tu, 1981; Bernard *et al.*, 1986). Graham and Parker (1964) showed that fast-growing, acid-producing strains of *Rhizobium* were generally more salt-tolerant than the slow-growing, alkali-producing strains. Salts vary in the intensity of their effect on growth and survival of rhizobia, for example, sodium acetate is more harmful than KCl and NaCl (Botsford, 1984), and $CaCl_2$ is more harmful than NaCl (Steinborn and Roughley, 1975). Yadav and Vyas (1973) reported that the salt effect appeared to be ion specific, with chlorides being more toxic than sulphates of Na, K and Mg. Mg ions inhibit growth at much lower concentrations than Na or K ions (Botsford, 1984). The effect of salts on growth has been attributed to the inhibition of the activity of specific enzymes by specific mineral ions (Greenway and Munns, 1980).

The aims of these experiments were to investigate:

(1) the response of chickpea and soybean rhizobia and soybean bradyrhizobia to different salts;
(2) the salt tolerance of the rhizobia after initial exposure to a sub-lethal concentration of salts;
(3) the survival of salt-tolerant strains during prolonged exposure to a salt-stressed medium.

MATERIALS AND METHODS

Fourteen strains of chickpea rhizobia [*Rhizobium* sp. (*Cicer*) all are fast growers] were obtained from the International Centre for Agricultural Research in the Dry Areas (ICARDA) (Table 1). The origin of these strains together with eight slow-growing soybean strains (*Bradyrhizobium japonicum*) and eight fast-growing soybean strains (*Rhizobium fredii*) are shown in Table 1. All strains were maintained at 4°C on yeast extract mannitol (YEM) agar slopes incorporating 3 g l^{-1} $CaCO_3$ (Vincent, 1970).

The basal medium used was modified Munns and Keyser (1981) medium with the following composition (μM): $CaCl_2 \cdot 6H_2O$, 1000; $MgSO_4 \cdot 7H_2O$, 500; KCl, 50; FeEDTA, 25; KH_2PO_4, 10; H_3BO_3, 10; $MnSO_4 \cdot 4H_2O$, 1; $ZnSO_4 \cdot 7H_2O$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.025; $CoCl_2 . 6H_2O$, 0.005. After autoclaving, sodium glutamate (1.8 g l^{-1}), arabinose (0.3 g l^{-1}) and galactose (0.3 g l^{-1}) were added as filter-sterilized solutions. Viable counts were made using the standard serial dilution and the drop count method (Hoben and Somasegaran, 1982).

NaCl was added before autoclaving to the basal medium to give final molar concentrations of 0 (1.5), 0.03 (4.7), 0.07 (8.2), 0.10 (11.6), 0.14 (14.2), 0.17 (17.2), 0.21 (20.6), 0.27 (24.6), 0.34 (32.5), 0.43 (39.2) and 0.51 (50.7), equivalent to 0–3%. The figures in parentheses are the equivalent electrical conductivities (EC) in dS m^{-1} at 25°C. The electrical conductivity was measured by a water digital analyser (PT1-20). The medium was dispensed as 5 ml aliquots into test tubes (15 mm dia). The medium was inoculated with a dilution of a basal medium culture of *Rhizobium* to give an initial count of 10^2–10^3 c.f.u. ml^{-1}, kept at 25°C and checked daily for visible turbidity for 14 days. Two replicates were included per treatment.

Seven different salts were used to determine their different specific ion effects on the growth and survival of strains Ch192, USDA 201 and USDA 208. These salts were Na_2SO_4, NaCl, $MgSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$, K_2SO_4 and KCl. The salts were added to the defined medium before autoclaving to give the following concentrations: 0, 1, 1.5, 2, 2.5, 3 or 3.5% (w/v). The autoclaved media were dispensed as 5 ml

*To whom all correspondence should be addressed.

aliquots into sterilized test tubes and inoculated with a freshly prepared culture of the appropriate *Rhizobium* in defined medium to give 10^2–10^3 c.f.u. ml^{-1} in each test tube. The cultures were grown at 25°C on a rotary shaker for 14 days. They were checked daily for visible turbidity. Two replicates were included per treatment.

Strains Ch184, Ch192, USDA 201 and USDA 208 were subcultured in salt-stressed media to examine for intra-strain variation in the response to salinity. Rhizobia were added to the basal medium (10^2–10^3 c.f.u. ml^{-1}) with the following concentrations of salts: 0.25 and 0.5 M NaCl; 0.13 and 0.25 M Na_2SO_4; 0.13 and 0.25 M $MgCl \cdot 6H_2O$. The control medium received no additional salts. The experiment was carried out as previously, but for 26 days. When turbidity appeared [only in the media containing 0.25 M NaCl, 0.13 M Na_2SO_4, 0.13 M $MgCl_2 \cdot 6H_2O$, or 0.13 M $MgSO_4 \cdot 7H_2O$ (Table 3)] these cultures were used to inoculate another complete set of salt treatments. The initial inoculum density was again 10^2–10^3 c.f.u. ml^{-1} and growth conditions and measurements as for the first exposure to salt. Final counts (where no turbidity was observed) were made on YEM agar plates.

The four strains were further used to test their ability to survive in the presence of different salts for a period of 60 days. Basal medium containing 0.34 M NaCl, 0.1 M $CaCl_2 \cdot 6H_2O$, or a mixture of 0.1 M NaCl, 0.04 M Na_2SO_4, 0.003 M $MgCl_2 \cdot 6H_2O$ and 0.02 M $CaSO_4$ (13:7:1:4 by weight respectively) was used. Each medium contained 2% salt, in addition to the control. The medium was inoculated with the appropriate strain to give an initial count of 10^6–10^7 c.f.u. ml^{-1}. The cultures were grown at 90 rev min^{-1} at 25°C. Two replicates were included per treatment. Viable counts were made directly after inoculation, and 5, 29 and 60 days after inoculation.

RESULTS AND DISCUSSION

The screening procedure based upon the assessment of visible turbidity (Table 1) indicated that all strains were tolerant of 0.07 M NaCl and all were sensitive to 0.43 M NaCl. The absence of visible turbidity within 14 days does not necessarily indicate inhibition of multiplication, but does demonstrate a reduction in multiplication rate or an increase in lag phase. The pH value of the medium was 6.8 at the start of the experiment and remained constant for 14 days. All of the 14 chickpea rhizobia strains (all were fast-growers, rhizobia) were tolerant of 0.21 NaCl, and ten of the strains were tolerant of 0.34 M NaCl. The soybean strains showed considerable variation in their response to NaCl. Most of the fast-growers (rhizobia) were more salt-tolerant than the slow-growers (bradyrhizobia). Five out of the eight soybean fast-growers could tolerate 0.21 M NaCl and 0.34 M NaCl. The slow-growers were sensitive to NaCl concentrations >0.14 M. All strains of bradyrhizobia from Rothamsted were sensitive to concentrations >0.1 M NaCl.

Wide variations in tolerance can be expected among strains of the same inoculation group (Yadav and Vyas, 1971). Graham and Parker (1964) showed that NaCl concentrations as high as 0.34 M and as low as 0.03 M could be lethal to rhizobia. Other workers have reported that the tolerance limits of rhizobia vary from 0.09 to 0.34 M NaCl (Rai *et al.*, 1985; Douka *et al.*, 1984), although Bernard *et al.* (1986) found strains tolerant of up to 0.65 M NaCl. Despite the differences in the areas from which the fast-growing strains of both chickpea rhizobia and soybean rhizobia were isolated, they showed similar responses to the added salt. Singleton *et al.* (1982) reported similar observations for soybean rhizobia and bradyrhizobia.

Table 1 shows that there was a greater proportion of sensitive strains among the members of the genus *Bradyrhizobium* (slow-growers) than among the members of the genus *Rhizobium* (fast-growers). Similar results were reported by Yelton *et al.* (1983) and Bernard *et al.* (1986). This is possibly due to the ability of rhizobia to influence the osmotic potential (Chen and Alexander, 1973) or accumulate particular or specific solutes (Hua *et al.*, 1982) more rapidly than bradyrhizobia.

The effect of different concentrations of different salts on chickpea *Rhizobium* strain Ch192 and soybean *Rhizobium* strain USDA 201 (both are fast-growers), selected from the initial screening experiment as tolerant of NaCl (Table 1), is shown in Tables 2 and 3. When salt concentrations were expressed on a w/v basis, NaCl, KCl and $MgCl_2$ appeared to be the most toxic salts and the chloride ions of Na, K and Mg were more toxic than the corresponding sulphate ions (Table 2). However, when expressing in terms of molarity $MgCl_2$ appeared to be much more toxic than NaCl and KCl (Tables 2 and 3). The results also demonstrate the misleading nature of results expressed in percentage of salt rather than the use of moler concentration. Comparisons between different salts must take into account differences in molecular weight. *R. fredii* strain USDA 208 showed similar results to that of strain USDA 201 (data not shown). All strains responded similarly to NaCl. *R. fredii* USDA 201 was more tolerant of $MgCl_2$ than Ch192. *Rhizobium* sp (*Cicer*) strain Ch192 behaved in the same way as *Rhizobium* sp. (*Cicer*) Ch184 (Elsheikh and Wood, 1989) except that it was more tolerant of Na_2SO_4 than Ch184.

Previous exposure to salt did not alter the subsequent response to the same or different salts at the same or higher concentrations (Table 3). Strains of chickpea rhizobia were not sub-cultured from $MgCl_2$ but were sub-cultured from $MgSO_4$ which was less toxic. In cases where no turbidity was observed, the final cell density was similar to or less than the initial cell density (Table 3), indicating that visible turbidity is a reliable indicator of multiplication in these experiments.

Overall, the magnesium ion appears to be more toxic than the sodium ion, and the chloride ion more toxic than the sulphate ion. $MgCl_2$ was reported to be more inhibitory than other salts at the same concentrations (Botsford, 1984; Yadav and Vyas, 1971; Elsheikh and Wood, 1989), however, Amara and Miller (1986) found the Mg ion to be the least inhibitory to growth, but at lower concentrations. The effects of NaCl and Na_2SO_4 are generally reported to be similar, although NaCl sometimes appears to be more toxic (Botsford, 1984; Yadav and

Table 1. Source and tolerance of NaCl (+ for growth, − for no growth) for chickpea rhizobia [*Rhizobium* sp. (*Cicer*)], soybean rhizobia (*R. fredii*) and bradyrhizobia (*B. japonicum*) in defined liquid medium, based on the presence or absence of visible turbidity from initial inoculum density of 10^2–10^3 c.f.u. ml^{-1} within 14 days at 25°C (means of two replications)

		Tolerance of NaCl (M)				
Strain	Source	0.07	0.14	0.21	0.34	0.51
Rhizobium sp. (Cicer)						
CP30	Syria	+	+	+	+	−
CP31	Syria	+	+	+	+	−
CP36	Syria	+	+	+	+	−
CP39	Syria	+	+	+	+	−
2-ICAR-LEB-Ch-174 (Ch174)	Lebanon	+	+	+	+	−
2-ICAR-LEB-Ch-175 (Ch175)	Lebanon	+	+	+	−	−
2-ICAR-SYR-Ch-177 (Ch177)	Syria	+	+	+	−	−
2-ICAR-SYR-Ch-178 (Ch178)	Syria	+	+	+	−	−
2-ICAR-SYR-Ch-179 (Ch179)	Syria	+	+	+	+	−
2-ICAR-SYR-Ch-183 (Ch183)	India	+	+	+	−	−
2-ICAR-MOR-Ch-184 (Ch184)	Morocco	+	+	+	+	−
2-ICAR-SYR-Ch-185 (Ch185)	Syria	+	+	+	+	−
2-ICAR-SYR-Ch-192 (Ch192)	Morocco	+	+	+	+	−
2-ICAR-UNK-Ch-191 (Ch191)	unknown	+	+	+	+	−
Total number of tolerant strains		14	14	14	10	0
Total number of sensitive strains		0	0	0	4	14
R. fredii						
USDA 192	USDA	+	−	−	−	−
USDA 193	USDA	+	+	+	+	−
USDA 194	USDA	+	−	−	−	−
USDA 201	USDA	+	+	+	+	−
USDA 205	USDA	+	+	+	+	−
USDA 208	USDA	+	+	+	+	−
USDA 217	USDA	+	+	+	−	−
USDA 257	USDA	+	+	+	+	−
Total number of tolerant strains		8	6	6	5	0
Total number of sensitive strains		0	2	2	3	8
B. japonicum						
RCR 3407	Rothamsted[1]	+	+	−	−	−
RCR 3827	Rothamsted	+	−	−	−	−
RCR 3824	Rothamsted	+	−	−	−	−
3Ib/143	Rothamsted	+	−	−	−	−
USDA 5	USDA[2]	+	−	−	−	−
USDA 110	USDA	+	−	−	−	−
USDA 122	USDA	+	−	−	−	−
USDA 136	USDA	+	−	−	−	−
Total number of tolerant strains		8	1	0	0	0
Total number of sensitive strains		0	7	8	8	8

[1] Rothamsted Experimental Station, Harpenden, England.
[2] United States Department of Agriculture.

Vyas, 1971). Soluble salts can have a specific effect due to the particular ion they contain being harmful to the organism, and a general effect due to the increase in the osmotic potential in the medium (Brown, 1976). Previous results for *Rhizobium* sp (*Cicer*) strain Ch184 indicated that for these organisms the specific ion effect is more harmful than the osmotic effect (Elsheikh and Wood, 1989).

The time taken for an inoculum to multiply is dependent partly on the size of the inoculum and

Table 2. The effect of different concentrations of salts on the growth of chickpea *Rhizobium* sp. (*Cicer*) strain Ch192 and soybean *R. fredii* strain USDA 201 (+ for growth, − for no growth) in defined liquid medium, based on the presence or absence of visible turbidity from initial inoculum density of 10^2–10^3 c.f.u. ml^{-1} within 14 days at 25°C (mean values of three replicates)

	Concentration (%)							
Salt	0	1.0	1.5	2.0	2.5	3.0	3.5	M[1]
Ch192								
Na_2SO_4	+	+	+	+	+	+	+	>0.246
NaCl	+	+	+	+	−	−	−	0.428
$MgSO_4$	+	+	+	+	+	+	+	>0.142
$MgCl_2$	+	+	+	+	−	−	−	0.123
K_2SO_4	+	+	+	+	+	+	+	>0.200
KCl	+	+	+	+	+	−	−	0.402
USDA 201								
Na_2SO_4	+	+	+	+	+	+	+	>0.246
NaCl	+	+	+	+	−	−	−	0.428
$MgSO_4$	+	+	+	+	+	+	+	>0.142
$MgCl_2$	+	+	+	+	+	−	−	0.148
K_2SO_4	+	+	+	+	+	+	+	> 0.200
KCl	+	+	+	+	+	−	−	0.402

[1] The inhibitory concentration of different salts in molar concentration.

Table 3. Effect of salts on multiplication of *Rhizobium* sp. (*Cicer*) strain Ch192 and *R. fredii* strain USDA 201 following culture in medium containing no additional salt (control), 0.25 M NaCl, 0.13 M Na_2SO_4, 0.13 M $MgSO_4$ or 0.13 M $MgCl_2$. Values are for days taken for visible turbidity to appear from initial inoculum density of 10^2–10^3 c.f.u. ml^{-1}. Figures in parentheses are final cell densities where no turbidity was observed (mean values of two replicates)

Prior exposure	No salt	NaCl (M) 0.25	NaCl (M) 0.5	Na_2SO_4 (M) 0.13	Na_2SO_4 (M) 0.25	$MgCl_2$ (M) 0.13	$MgCl_2$ (M) 0.25
Ch192							
Control	5.0	7.0	(2100)	7.0	11.0	(40)	(<10)
NaCl	4.5	7.5	(65,000)	5.0	10.5	(440)	(<10)
Na_2SO_4	4.5	5.0	(35,000)	5.0	10.5	(ND)	(<10)
$MgSO_4$	4.5	7.5	(64,000)	5.0	10.5	(160)	(<10)
USDA 201							
Control	3	5.0	(100)	5.0	11.0	4.0	(<10)
NaCl	3	4.5	(150)	4.5	10.5	4.5	(<10)
Na_2SO_4	3	4.5	(300)	4.5	10.5	4.5	(<10)
$MgCl_2$	3	4.5	(100)	4.5	10.5	4.5	(<10)
$MgSO_4$	3	4.5	(120)	4.5	10.5	4.5	(<10)

ND, Not determined

partly on the time taken for the culture to adapt to the new environment (Steinborn and Roughly, 1975). In this case the inoculum was always the same size and no adaptation occurred. Mutation for tolerance to environmental stress may be selected by sub-culturing, or adaptation may occur during a long period in stressed medium. For example, a strain of *R. meliloti* grew in 0.6 M NaCl after adaptation for 26 days (Steinborn and Roughley, 1975). However, Steinborn and Roughley (1975) did not find any

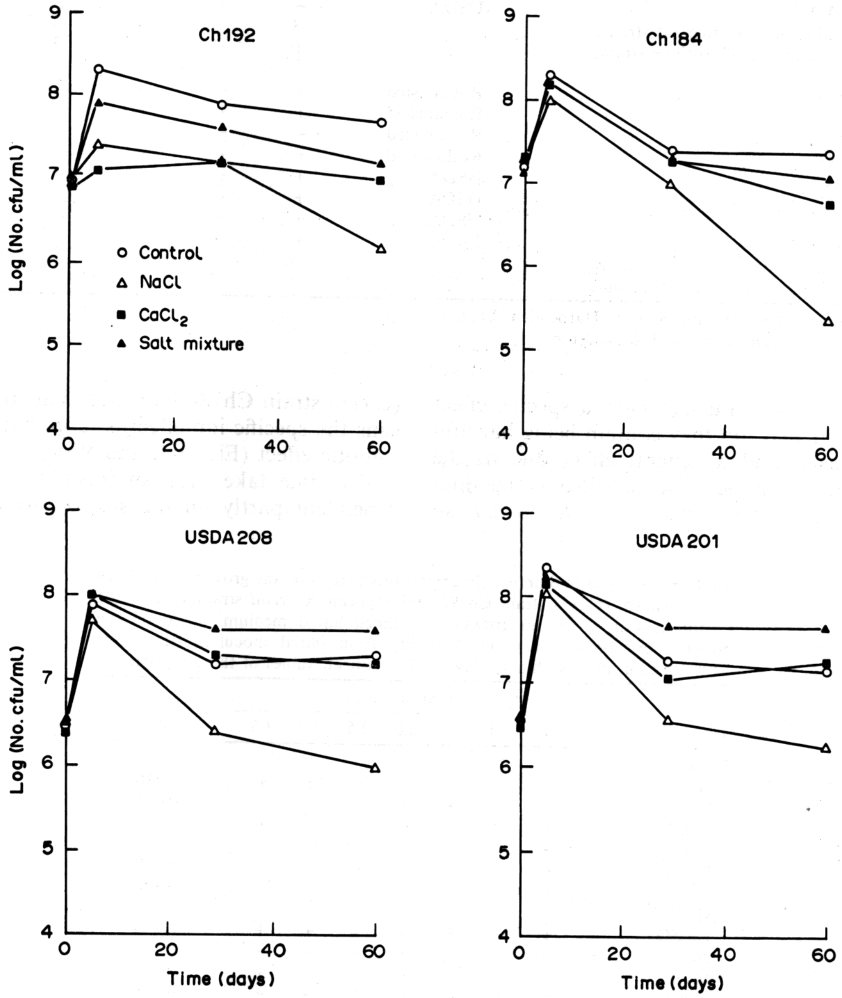

Fig. 1. Effect of different salts (2% final concentration) on survival of chickpea rhizobia strains Ch184 and Ch192 and soybean rhizobia strains USDA 201 and USDA 208 in defined liquid medium (mean values of two observations).

improvement in the growth of strains of *R. meliloti* when sub-cultured twice in salt-stressed medium. Tolerance to acid-aluminium stress is also a stable property within strains (Munns and Keyser, 1981; Wood and Cooper, 1985).

The 60-day survival study indicated that NaCl reduced the survival of all strains (Fig. 1). Although $CaCl_2$ was found to be more toxic than NaCl at the same percentage in broth and peat cultures (Steinborn and Roughley, 1975), $CaCl_2$ had no effect in this experiment. The mixture of salts imposed no additional stress above the control. This indicates that NaCl is more inhibitory than the other salts used in this experiment (in terms of percentage). The strains survived well in the mixture of salts compared to NaCl. The use of a mixture of salts (NaCl, $CaSO_4$, $MgCl_2$, Na_2SO_4) may provide a better indicator of the effect of salinity rather than the use of only one salt such as NaCl. However, strains which multiply and survive in NaCl are likely to tolerate the same percentage of any other single salt or combination of salts.

Acknowledgements—We thank the Sudanese Government for financial support. We are also grateful to Dr L. Materon (ICARDA) and Dr H. Keyser (USDA) for supplying cultures of chickpea rhizobia and soybean rhizobia respectively.

REFERENCES

Amara D. S. and Miller R. H. (1986) Effect of moisture and salt on selected *Rhizobium phaseoli* strains. *Mircen Journal* **2,** 373–382.

Bernard T., Pocard J., Perroud B. and LeRudulier P. (1986) Variation in the response of salt stressed *Rhizobium* strains to betaine. *Archives of Microbiology* **143,** 359–356.

Botsford J. L. (1984) Osmoregulation in *Rhizobium meliloti*: inhibition of growth by salts. *Archives of Microbiology* **137,** 124–127.

Brown A. D. (1976) Microbial water stress. *Bacteriological Reviews* **40,** 803–846.

Chen M. and Alexander M. (1973) Survival of soil bacteria during prolonged desiccation. *Soil Biology & Biochemistry* **5,** 213–221.

Douka C. E., Xenoulis A. C. and Paradellis T. (1984) Salinity tolerance of a *Rhizobium meliloti* strain isolated from salt affected soil. *Folia Microbiologica* **29,** 316–324.

Elsheikh E. A. E. and Wood M. (1989) Response of chickpea and soybean rhizobia to salt: osmotic and specific ion effects of salts. *Soil Biology & Biochemistry* **21,** 889–895.

Graham P. H. and Parker C. A. (1964) Diagnostic features in the characterization of root nodule bacteria of legumes. *Plant and Soil,* **20,** 383–396.

Greenway H. and Munns R. (1980) Mechanisms of salt tolerance in non-halophytes. *Annual Review of Plant Physiology* **31,** 149–190.

Hoben H. J. and Somasegaran P. (1982) Comparison of the pour, spread and drop plate methods for enumeration of *Rhizobium spp.* in inoculants made from pre-sterilized peat. *Applied and Environmental Microbiology* **44,** 1246–1247.

Hua S. S. T., Tsai V. Y., Lichens G. M. and Noma A. T. (1982) Accumulation of amino acids in *Rhizobium sp.* strain WR1001 in response to NaCl salinity. *Applied and Environmental Microbiology* **44,** 135–140.

Munns D. N. and Keyser H. H. (1981) Response of *Rhizobium* strains to acid and Al stress. *Soil Biology & Biochemistry* **13,** 115–118.

Mustafa M. A. (1984) Quality of bore hole waters and their effect on some characteristics of irrigated soils in Khartoum province *Sudan Agricultural Journal* **10,** 73–80.

Rai R., Nasar S. K. T., Singh S. J. and Prasad V. (1985) Interaction between Rhizobium strains and lentil (*Lens culinaris*) genotype under salt stress. *Journal of Agricultural Science Cambridge* **108,** 25–37.

Singleton P. W., Elswaify S. A. and Bohlool B. B. (1982) Effect of salinity on *Rhizobium* growth and survival. *Applied and Environmental Microbiology* **44,** 884–890.

Steinborn S. and Roughley R. J. (1975) Toxicity of sodium chloride ions to *Rhizobium spp.* in broth and peat cultures. *Journal of Applied Bacteriology* **39,** 133–138.

Tu. J. C. (1981) Effect of salinity on *Rhizobium*-root hair interaction, nodulation and growth of soybean. *Canadian Journal of Plant Science* **61,** 231–239.

Vincent J. M. (1970) *A Manual for the Practical Study of Root Nodule Bacteria.* Blackwell Scientific Publications, Oxford.

Wood M. and Cooper J. E. (1985) Screening clover and Lotus rhizobia for tolerance of acidity and aluminium. *Soil Biology & Biochemistry* **17,** 493–497.

Yadav N. K. and Vyas S. R. (1971) Response of root-nodule bacteria to saline, alkaline and acid conditions. *Indian Journal Agricultural Sciences* **41,** 875–881.

Yadav N. K. and Vyas S. R. (1973) Salts and pH tolerance of rhizobia. *Folia Microbiologica* **18,** 242–247.

Yelton M. M., Yang S. S., Edie S. A. and Lim S. T. (1983) Characterization of an effective salt tolerant fast growing strain of *Rhizobium japonicum. Journal of General Microbiology* **129,** 1537–1547.