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RESPONSE OF CHICKPEA AND SOYBEAN RHIZOBIA TO SALT: OSMOTIC AND SPECIFIC ION EFFECTS OF SALTS

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Summary—Chickpea *Rhizobium* strain 2-ICAR-SYR-Ch-184 showed different responses (in terms of growth and survival) to a range of different salts. The chloride ions of Na, K and Mg were found to be more toxic than the corresponding sulphate ions. The osmotic effect was studied using polyethylene glycol (PEG), glycerol and sorbitol to raise the osmotic pressure to −1.0 and −2.0 MPa. PEG was toxic even at low concentrations. However, there was good growth at −1.0 and −2.0 MPa using glycerol and sorbitol. Although, the sensitivity of the strain to NaCl was increased at higher osmotic pressures, the harmful effect of salts on the growth of this strain could be attributed more to the specific ion effect rather than the osmotic effect. The influence of various organic osmotica on the growth of chickpea rhizobia and soybean rhizobia and bradyrhizobia was studied in salt-stressed media. Chickpea *Rhizobium* strain 2-ICAR-SYR-Ch-Ch184 showed an increase in final cell density when glutamate was added to 0.34 M NaCl. All other strains showed no improvement in growth with the addition of glutamate. Glycine betaine did not relieve the inhibition of growth of all strains except for a slight improvement in the growth of the salt-sensitive soybean *Bradyrhizobium japonicum* strain RCR 3407 at 0.08 M NaCl. However, all tested strains used glycine betaine as a sole carbon source regardless of the presence or absence of NaCl.

INTRODUCTION

Detrimental effects of salts on microorganisms may be due to the toxicity of specific ions, elevation of osmotic pressure or the increase in alkalinity which may restrict the availability of water or influence cellular physiology and metabolic pathways (Botsford, 1984; Prior *et al.*, 1987). There is evidence that certain ions, particularly HCO_3^-, are more toxic than others (Botsford, 1984). Although many biochemical functions require specific inorganic ions, increasing the concentrations of these ions above normal intracellular concentrations may lead to disruption of metabolic functions by reducing the activities of enzymes (Yancy *et al.*, 1982). The ionic and osmotic effects may differ, and cannot easily be distinguished under saline conditions (Imhoff, 1986).

The presence of polyols such as glycerol and sorbitol in the growth media allow certain bacteria to grow and respire at higher osmotic pressures (lower osmotic potentials) than sucrose and NaCl (Prior *et al.*, 1987). The enhancement of growth of *Rhizobium* strains from different species, resulting from added glycine betaine and other betaines under saline conditions, has been reported (Bernard *et al.*, 1986; Sauvage *et al.*, 1983). However, Botsford (1984) reported that glycine betaine did not relieve the inhibition of growth of *Rhizobium meliloti* in NaCl. Betaine, in relatively low concentrations, relieved osmotic stress in different rhizobia in a minimal growth medium (Bernard *et al.*, 1986).

Our purpose was to (i) examine the effect of a range of salts on growth and survival of a strain of chickpea *Rhizobium*, (ii) to investigate whether the harmful effect of salts is due to a specific ion effect or an osmotic effect, and (iii) to examine the effect of externally added osmotica on salt tolerance.

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MATERIALS AND METHODS

General

Chickpea *Rhizobium* strains 2-ICAR-SYR-Ch-184 (Ch184) and 2-ICAR-MOR-Ch-192 (Ch192) were obtained from the International Centre for Agricultural Research in the Dry Areas (ICARDA). Slow-growing soybean *B. japonicum* strain RCR 3407 was obtained from Rothamsted Experimental Station, Harpenden, England. Fast-growing soybean *Rhizobium fredii* strains USDA 201 and USDA 208 were obtained from Dr H. Keyser, USDA. In this study, salt-tolerant (*Rhizobium*) strains are defined as those able to multiply in 0.34 M NaCl.

The strain preservation, the defined arabinose-galactose-glutamate medium and the measurement of viable cells were described by Elsheikh and Wood (1989). Maximum doubling times (h) were obtained from the exponential phases of the growth curves.

Specific ion effects

Seven different salts were used to determine their different specific ion effects on the growth and survival of strain Ch184. These salts were Na_2SO_4, NaCl, $MgSO_4$, $MgCl_2$, K_2SO_4, KCl and $CaCl_2$. Calcium sulphate was not used because of its low solubility. 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) (different salts have different molarities, for the toxic concentrations see Table 2). The autoclaved media were dispensed as 5 ml 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 cfu 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. The osmotic pressure of the toxic level (the lowest salt concen-

Table 1. The effect of different concentrations of salts on the growth of chickpea *Rhizobium* strain Ch184 (time in days for the strain to show turbidity)

Salt	Concentration (%)						
	0	1	1.5	2	2.5	3	3.5
Na_2SO_4	3	3	3	6	6	13	NG
NaCl	3	3	3	6	NG	NG	NG
$MgSO_4$	3	3	3	3	3	8	12
$MgCl_2$	3	3	3	6	NG	NG	NG
K_2SO_4	3	3	3	3	4	6	6
KCl	3	3	3	5	5	NG	NG
$CaCl_2$	3	3	3	3.5	NG	NG	NG

NG = no turbidity within 14 days.

tration in which no turbidity appeared) of these salts was calculated from the following equation (United States Salinity Laboratory Staff, 1954):

$$OP = 12.06\Delta T - 0.021(\Delta T)^2$$

where OP is the osmotic pressure and ΔT is the freezing point depression.

Osmotic effects

Sodium chloride was added before autoclaving to the defined medium to give final concentrations of 0.10 or 0.15 M (equivalent to 0.60 and 0.88% respectively) in addition to an unamended control. The osmotic pressure of the medium at each concentration of NaCl was either unadjusted or raised to −1.0 or −2.0 MPa using polyethylene glycol 200 (PEG), glycerol or sorbitol. The initial osmotic pressures of the unadjusted controls were −0.12, −0.50 or −0.68 MPa for unamended, 0.1 M and 0.15 M NaCl respectively (1.0 MPa = 10 bar). The osmotic pressures of the media were adjusted and checked psychrometrically (Prior *et al.*, 1987). Twenty five ml aliquots of the media were dispensed, in 100 ml conical flasks. The media were inoculated with a freshly prepared culture of strain Ch184 in defined medium to give 10^2–10^3 cfu ml^{-1}. The flasks were shaken (90 rev/min) at 25°C. Growth was measured by viable counts every 2 days for 14 days. Two replicates were included per treatment.

Effect of externally-added glutamate

Sodium glutamate was added as a filter-sterilized solution to defined medium containing 0.34, 0.51 or 0.60 M in addition to unamended control, to give final concentrations of 0.45, 1.8 or 3.6 g l^{-1} (equivalent to 2.6, 10.6 and 21.3 mM respectively). Twenty five ml aliquots were dispensed into 100 ml conical flasks and inoculated with freshly prepared cultures of the *Rhizobium* strains USDA 201, USDA 208, Ch184 or Ch192 in defined medium to give an initial count of 10^2–10^3 cfu ml^{-1}. They were incubated on a shaker (90 rev/min) at 25°C. Viable counts of duplicate cultures were made every 2 days for 14 days.

Effect of externally-added betaine

Glycine betaine was added to the defined medium containing 0, 0.34 or 0.51 M NaCl to give final concentrations of 0, 1 or 10 mM glycine betaine for strains Ch184, Ch192, USDA 201 and USDA 208. In addition, the salt-sensitive soybean *B. japonicum* strain RCR 3407 (Elsheikh and Wood, 1989) was used with NaCl concentrations of 0, 0.08 or 0.17 M. Details were otherwise as described for the previous experiment.

The ability of the four salt-tolerant strains of rhizobia to utilize glycine betaine as a carbon and energy source was investigated in the defined medium containing 0, 0.25 or 0.5 M NaCl with 0 or 50 mM glycine betaine. Glutamate, arabinose, and galactose were omitted from the defined medium. Details were as described for the previous experiments except that only initial (directly after inoculation) and final (after 14 days) viable counts were made.

RESULTS AND DISCUSSION

Specific ion effects

The chickpea *Rhizobium* strain Ch184 showed different responses to the different salts. The growth of the strain was unaffected at 1 and 1.5% concentrations of all salts (Table 1). Turbidity appeared at 2% of all salts, but was delayed at this concentration by all salts except $MgSO_4$, K_2SO_4 and to some extent $CaCl_2$. Growth was inhibited (as shown by the absence of turbidity) by all salts at different concentrations in the range 2.5–3.5%, except for $MgSO_4$ and K_2SO_4 which allowed turbidity to appear at 3.5%.

The critical concentrations for the cell growth in the different ions are shown in Table 2. As percent the anion had a greater effect on growth than the cation, and the chloride ion was more toxic than the sulphate ion. The osmotic pressures of the inhibitory concentrations of the different salts were also calculated (Table 2). This showed that the sulphates were toxic at much lower osmotic pressures than the chlorides, indicating that the osmotic effect was not the major factor determining toxicity. Yadav and Vyas (1973) reported that the salt effect on rhizobia appeared to be ion specific and not purely osmotic. Botsford (1984) reported that the inhibition of growth rate of the cells of *R. meliloti* was not influenced simply by increased osmotic pressure but by the particular ion present. The inhibition of growth of *R. meliloti* by various salts was found to vary depending on the salt (Botsford, 1984). The tolerance to NaCl of *Rhizobium* strains from different species was found to vary widely from 0.6 to 3.5% (equivalent to 0.102 and 0.599 M respectively); however, growth was completely inhibited by NaCl and KCl at −2.6 MPa (3.5% NaCl and 4.5% KCl) (Mary *et al.*, 1986).

Table 2. The inhibitory concentrations of different chloride and sulphate salts [in % and (M) molarity] on the growth of chickpea *Rhizobium* strain Ch184 and their corresponding osmotic pressures

		Na^+	K^+	Mg^{2+}	Ca^{2+}
(i)	Cl^-				
	(%)	2.5	3.0	2.5	2.5
	(M)	0.428	0.402	0.123	0.114
	(MPa)	−1.79	−1.66	−1.63	−1.33
(ii)	SO_4^{2-}				
	(%)	3.5	>3.5	>3.5	ND
	(M)	0.246	>0.200	>0.142	ND
	(MPa)	−1.21	<−1.01	<−0.73	ND

ND = not determined.

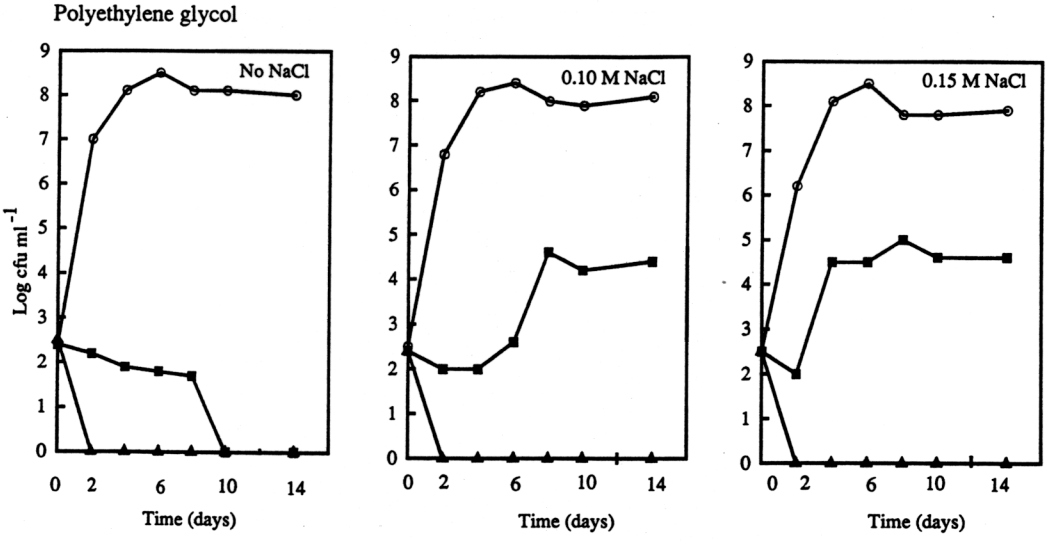

Fig. 1. The effect of osmotic pressure, adjusted using polyethylene glycol, on the growth of chickpea *Rhizobium* strain Ch184 under different concentrations of NaCl. (○) Unadjusted, (■) −1.0 MPa, (▲) −2.0 MPa. Two observations per mean.

Osmotic effects

Increasing the concentration of salt from 0.1 to 0.15 M in the absence of PEG caused a slight reduction in growth rate (Fig. 1). The added PEG decreased the growth at −1.0 MPa and inhibited the growth completely at −2.0 MPa regardless of the level of salt. The inhibition of growth by PEG at −1.0 MPa was greater with less salt, that is, where more PEG was added to obtain the required osmotic pressure. Strain Ch184 failed to survive for more than 2 days at −2.0 MPa at all concentrations of NaCl.

These results indicate that PEG is toxic in itself to this strain, because the strain multiplied well at higher osmotic pressures under different salts (Tables 1, 2 and Fig. 1). PEG is not suitable even for short-term studies on plants because of its immediate toxic effects (Termaat and Munns, 1986). Polyethylene glycol was found to interfere with ion transport (Yeo and Flowers, 1984), and to limit oxygen diffusion rates (Mexal *et al.*, 1975). The growth rate of *Escherichia coli* was found to be highly sensitive to PEG with growth inhibition occurring at −0.8 MPa compared with −4.0 MPa when using inorganic salts (McAneney *et al.*, 1982). The increase in cell death caused by addition of PEG may be attributed to (i) changes in the environmental matric potential causing changes in the cell wall permeability (McAneney *et al.*, 1982), (ii) a greater maintenance energy requirement by the organism under PEG stress (McAneney *et al.*, 1982), (iii) the accumulation of PEG in the space between the cell wall and cytoplasmic membrane affecting the periplasmic enzymes (Costerton, 1977), and (iv) PEG may cause ribosome precipitation for some organisms even at very low concentrations (Levenson *et al.*, 1984).

The effects of the three concentrations of NaCl, without any added glycerol (Fig. 2), were more or less similar to those in the PEG experiment (Fig. 1). The amount of glycerol added to the defined medium was greater when there was no salt added to the defined medium than with 0.10 M NaCl and the least amount was added with 0.15 M to adjust the osmotic level to −1.0 or −2.0 MPa. Although the strain was able to grow and multiply in all media even in those with high osmotic pressures (Fig. 2), there was an increase in lag time with increasing osmotic pressure at all salt concentrations. 0.15 M NaCl caused a reduction in growth rate at −2.0 MPa but not at −1.0 MPa (Fig. 2). This additive effect of salt and osmotic pressure was not observed at lower salt concentrations. Overall, these results show that this strain could tolerate osmotic stress of −2.0 MPa or more using glycerol to adjust the osmotic pressure. Also, the strong interaction between salt and osmotic pressure may indicate harmful effects of salts (specific ions) at higher osmotic pressures.

Mary *et al.* (1986) found that at similar osmotic pressures glycerol had the least inhibitory effect, while the inhibitory effect of NaCl and KCl were about equal. Similar results were reported for *R. meliloti* (Botsford, 1984), in which the osmotic pressure of the growth medium was increased 6-fold by the addition of glycerol without affecting the growth rate of the strain. To maintain turgor pressure in a saline environment considerable concentrations of salts have to be accumulated in cells (Imhoff, 1986). However, the organism must be able to control its volume or its internal turgor pressure (Brown *et al.*, 1986). Glycerol and other polyols were found to accumulate in salt-stressed microorganisms and plants. They assist in cell water retention while remaining compatible with macromolecular function (Brown *et al.*, 1986; Kenyon *et al.*, 1986). Glycerol was found to be produced by yeast as an intracellular, osmotically-active solute that also enables enzymes to function within the cell under conditions of high osmotic stress (Levenson *et al.*, 1984). The enzyme glucose-6-phosphate dehydrogenase, in algae, was

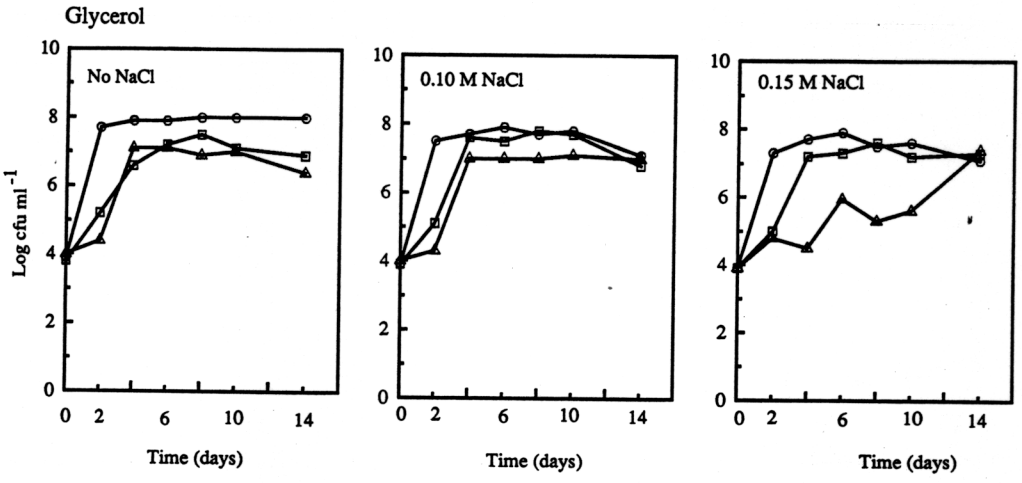

Fig. 2. The effect of osmotic pressure, adjusted using glycerol, on the growth of chickpea *Rhizobium* strain Ch184 under different concentrations of NaCl. (○) Unadjusted, (■) −1.0 MPa, (▲) −2.0 MPa. Two observations per mean.

not affected by high glycerol concentrations, in contrast to the deleterious effects of NaCl and KCl (Yancy *et al.*, 1982).

The effects of the three concentrations of NaCl, without any added sorbitol (Fig. 3), were similar to the PEG and glycerol experiments (Figs 1 and 2). The response to salt and osmotic pressure, adjusted using sorbitol, was similar to that observed with glycerol. The effect of 0.15 M NaCl was more severe at −2.0 MPa than at −1.0 MPa but osmotic pressure had less effect at lower salt concentrations.

Sorbitol was found to allow certain bacteria to grow and respire at higher osmotic pressures than sucrose or NaCl (Prior *et al.*, 1987). At similar osmotic pressures (−4.0 MPa), the growth of *Saccharomyces cerevisiae* was inhibited more by NaCl than sorbitol (Kenyon *et al.*, 1986). Sorbitol, like other polyols, was found to assist in cell water retention while remaining compatible with macromolecular function (Yancy *et al.*, 1982).

Effect of glutamate

Na-glutamate had no effect on enhancing the growth rate of strains Ch184, Ch192, USDA 201 and USDA 208 under salt stress (Table 3). The chickpea *Rhizobium* strain Ch184 failed to grow beyond 10^7 cfu ml^{-1} in the lowest concentration of Na-glutamate in 0.34 M NaCl (Fig. 4). However, when the concentration of Na-glutamate was increased to 10.6 and 21.3 mM the strain showed better growth than with 2.6 mM glutamate, and reached the maximum growth after 12 days from inoculation. It seems that this strain is glutamate dependent when it is subjected to concentrations of salts of about 0.34 M NaCl. There were no significant differences in growth of

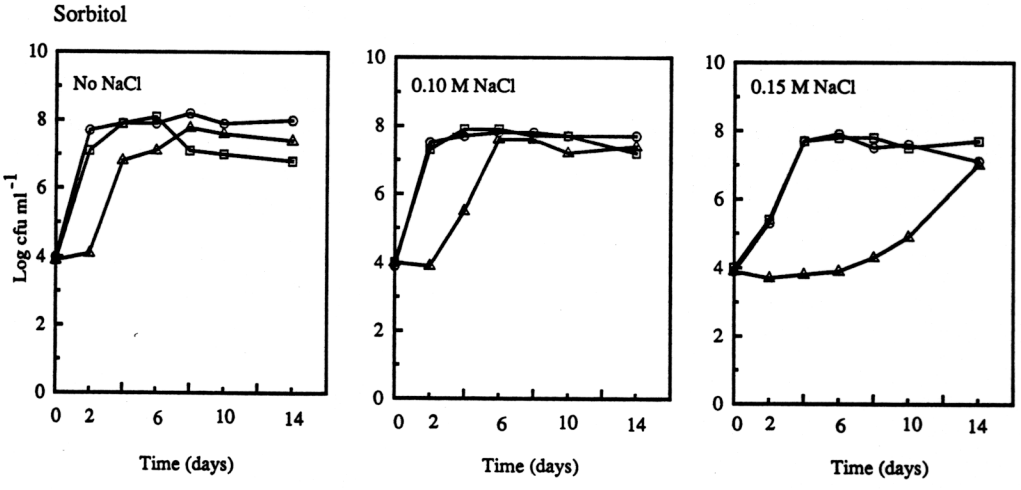

Fig. 3. The effect of osmotic pressure, adjusted using sorbitol, on the growth of chickpea *Rhizobium* strain Ch184 under different concentrations of NaCl. (○) Unadjusted, (■) −1.0 MPa, (▲) −2.0 MPa. Two observations per mean.

Table 3. The maximum doubling time (h) for chickpea rhizobia and soybean rhizobia and bradyrhizobia in different concentrations of glycine betaine and glutamate under NaCl stress

Strain	Salt (M)	Betaine (mM)			Glutamate (mM)		
		0	1	10	2.6	10.6	21.3
Chickpea							
Ch184	0.00	3.8	3.8	3.8	3.9	3.9	3.9
Ch184	0.34	9.6	9.6	9.6	9.6	9.6	9.6
Ch192	0.00	12.0	12.0	12.0	12.0	12.0	12.0
Ch192	0.34	16.3	16.3	16.3	16.5	16.5	16.5
Soybean							
USDA 201	0.00	4.8	4.8	4.8	4.8	4.8	4.8
USDA 201	0.34	13.0	13.0	13.0	13.0	13.0	13.0
USDA 208	0.00	4.5	4.5	4.5	4.5	4.5	4.5
USDA 208	0.34	9.6	9.6	9.6	9.6	9.6	9.6
RCR 3407	0.00	6.2	6.2	6.2	ND	ND	ND
RCR 3407	0.08	13	9.1	9.1	ND	ND	ND

ND = not determined.

the strain in 0.51 M NaCl (Fig. 4) and 0.60 M NaCl (data not shown) regardless of the concentration of Na-glutamate.

The lack of response or small response to glutamate in these strains was in agreement with other reports for non-halophilic bacteria under salt-stress (Measures, 1975; Strom *et al.*, 1986). Botsford (1984) reported that glutamate had no effect with NaCl, however, the addition of glutamate appeared to have a slightly stimulatory effect on the growth rate of *R. meliloti* in the presence of $MgCl_2$ and Na-acetate. The data presented here indicate that glutamate did not act as an osmoregulator for the tested strains with the exception of strain Ch184, in which the accumulation of glutamate (data not shown) may protect the cells from the effect of sodium.

Effect of betaine

No differences in growth of the salt-tolerant *Rhizobium* strains Ch184, Ch192, USDA 201 and USDA 208 were observed in the presence of the three betaine concentrations regardless of the presence or absence of NaCl (Table 3). Betaine did not improve growth of all salt-tolerant strains at 0.51 M NaCl (data not shown). The salt-sensitive soybean *Bradyrhizobium* strain RCR 3407 responded to the added glycine betaine (Table 3, Fig. 5). This was due to the faster growth at 0.08 M NaCl with 1.0 and 10.0 mM betaine compared to the control with no added betaine.

All salt-tolerant strains were able to grow in medium containing glycine betaine and lacking all other carbon sources, and turbidity (log cfu $ml^{-1} > 7.0$) was shown by the strains even when they were grown in 0.25 M NaCl with 50 mM glycine betaine (Table 4).

The slight improvement in the overall growth caused by glycine betaine addition observed here, in stressed medium with glutamate, galactose and arabinose present, is in agreement with reports for other bacteria such as *E. coli* (Larsen *et al.*, 1987) and different *Rhizobium* strains (LeRudulier and Bernard, 1986). Bernard *et al.* (1986) reported that only 4 out of 15 *Rhizobium* strains, including *R. japonicum* (now *B. japonicum*) and *Rhizobium leguminosarum*, showed a positive response to glycine betaine in salt-stressed media. The effect of glycine betaine on salt-tolerant strains is minimized or completely inhibited in the presence of other carbon sources.

Overall our results show that PEG is toxic for the chickpea *Rhizobium* strain Ch184, while glycerol and sorbitol seem to act as compatible solutes and allow cell function and metabolism to continue at higher osmotic pressures. The harmful effect of salts on the growth of this strain seems to be ion specific rather than purely osmotic, with the chloride ions more toxic than the sulphate ions of Na, K and Mg. However, the sensitivity of this strain to NaCl was increased at higher osmotic pressures. Externally added Na-glutamate or glycine betaine had no major effects on the response of these strains of rhizobia to salinity in the presence of other carbon sources in the media. The results show that a strain which is tolerant of NaCl in a glutamate-based defined medium should be tolerant of other salts. However, Elsheikh and Wood (1989) demonstrated that pH, temperature and carbon source may influence the response of chickpea rhizobia to salinity and should therefore, be considered when selecting strains for use in saline soils.

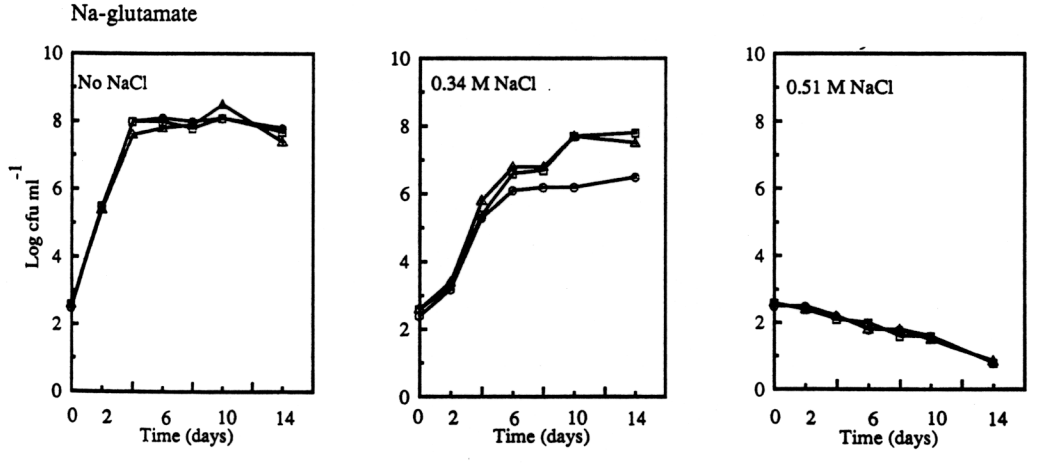

Fig. 4. The effect of Na-glutamate on the growth of chickpea *Rhizobium* strain Ch184 under different concentrations of NaCl. (○) 2.6 mM Galutamate, (□) 10.6 mM glutamate, (△) 21.3 mM glutamate. Two observations per mean.

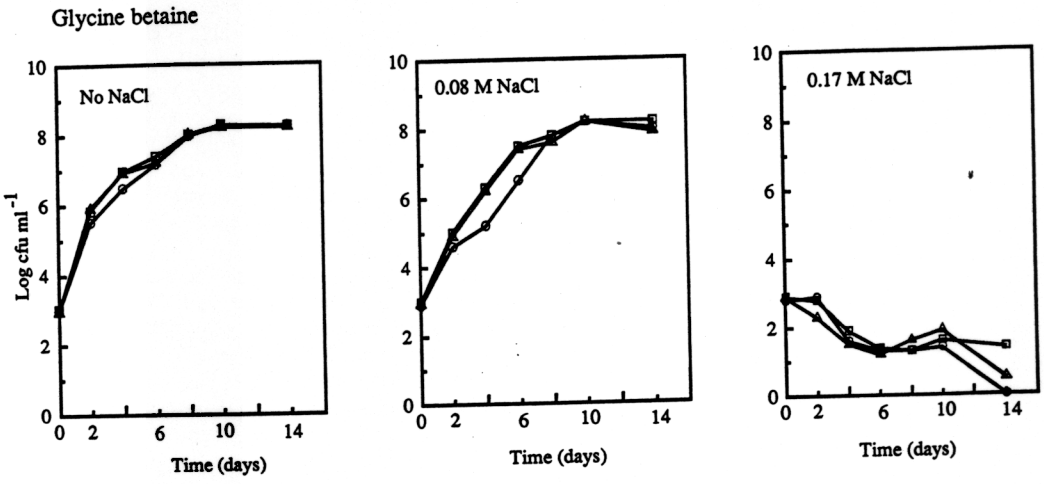

Fig. 5. The effect of glycine betaine on the growth of soybean *Bradyrhizobium* strain RCR 3407 under different concentrations of NaCl. (○) No glycine betaine, (□) 1 mM glycine betaine, (△) 10 mM glycine betaine. Two observations per mean.

Table 4. Growth of salt-stressed chickpea and soybean rhizobia on glycine betaine as a carbon source. Values are for log cfu ml^{-1} at the initial (I) and final (F) counts (two replications per mean)

Salt (M)	Betaine (mM)	Strain Ch184 I	Ch184 F	Ch192 I	Ch192 F	USDA 201 I	USDA 201 F	USDA 208 I	USDA 208 F
0.0	0	3.00	4.94	3.43	4.18	2.89	4.97	3.18	4.43
0.0	50	2.99	7.71	3.36	7.93	2.76	7.15	3.76	7.59
0.25	0	3.04	4.76	3.34	4.20	3.00	2.00	3.18	4.99
0.25	50	2.96	7.83	3.36	8.98	2.88	7.60	3.23	7.49
0.50	0	2.89	2.26	3.36	2.76	2.76	2.00	3.18	1.65
0.50	50	2.92	2.49	3.36	2.36	2.91	1.95	3.20	1.60

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