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RESPONSE OF CHICKPEA AND SOYBEAN RHIZOBIA TO SALT: INFLUENCE OF CARBON SOURCE, TEMPERATURE AND pH

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

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

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Summary—Chickpea and soybean rhizobia showed patterns of utilization of carbon sources characteristic of fast-growers in the absence of NaCl. *Bradyrhizobium japonicum* strain RCR3407 shared some characteristics of both fast- and slow-growers. Out of four chickpea rhizobia and four soybean rhizobia and bradyrhizobia only chickpea *Rhizobium* strain 2-ICAR-MOR-Ch-192, soybean *Rhizobium* strains USDA 191 and USDA 201 were able to utilize some carbon sources in the presence of 0.25 M NaCl. All chickpea and soybean rhizobia were able to grow at 25 and 37°C in YEMA (yeast extract mannitol agar), but not at 45°C. The effect of four temperatures, three pH values and three NaCl concentrations on growth and multiplication of chickpea *Rhizobium* strain 2-ICAR-SYR-Ch-184 showed that salt-stress was more severe at alkaline pH and lower temperatures. The results indicate that tolerance to salt by rhizobia is dependent upon pH, temperature and carbon source.

INTRODUCTION

Members of the genera *Rhizobium* and *Bradyrhizobium* are symbiotic nitrogen–fixing bacteria which are able to invade and form nodules on the roots of leguminous plants. Fast-growing rhizobia are nutritionally diverse in their utilization of carbon sources, whereas slow-growing bradyrhizobia appear to be nutritionally fastidious (Stowers, 1985). Detrimental effects of salt on the survival and growth of *Rhizobium* have been reported (Yap and Lim, 1983). High concentrations of NaCl reduce the number of rhizobia in legume inoculants (Steinborn and Roughley, 1975). However, it is not known how salinity affects utilization of carbon sources or how different carbon sources affect the response to salt by rhizobia.

In saline soils the pH is usually <8.5, and pH may influence the response of rhizobia to salinity. Temperature is another major environmental factor controlling the survival and establishment of rhizobia in culture and in soil. High temperature may be associated with saline soils. With a few exceptions (LaFavre and Eaglesham, 1986), a positive correlation has been found between the ability of strains to nodulate at high temperatures and their ability to grow at high temperatures (Munevar and Wollum, 1981a,b; Eaglesham and Ayanaba, 1984). A better understanding of the factors affecting the response of rhizobia to salinity may assist in the selection of inoculants for use with legumes in saline soils.

Our aim was to examine the patterns of carbon utilization by strains of rhizobia and bradyrhizobia in saline and non-saline conditions, and to investigate the interactions between temperature, pH and salt concentrations on the growth and multiplication of one of these strains.

*To whom all correspondence should be addressed.

MATERIALS AND METHODS

General

Six strains of chickpea rhizobia were obtained from the International Centre for Agricultural Research in the Dry Areas (ICARDA). These strains were 2-ICAR-SYR-Ch-178 (Ch178), 2-ICAR-SYR-Ch-179 (Ch179), 2-ICAR-SYR-Ch-184 (Ch184), 2-ICAR-SYR-CH-185 (Ch185), 2-ICAR-UNK-Ch-191 (Ch191) and 2-ICAR-MOR-Ch-192 (Ch192). All these are fast-growers (*Rhizobium* spp). Slow-growing soybean *Bradyrhizobium japonicum* strain RCR3407 was obtained from Rothamsted Experimental Station, Harpenden, England; fast-growing soybean *Rhizobium fredii* strains USDA 191, 201, 205 and 208 were obtained from Dr H. Keyser, USDA. Strains were maintained at 4°C on yeast extract mannitol (YEM) agar slopes incorporating 3 g $CaCO_3\,l^{-1}$ (Vincent, 1970).

A defined growth medium was used 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; $CoCl_2 \cdot 6H_2O$, 0.005. After autoclaving, Na-glutamate (1.8 g l^{-1}) (which acts as a pH buffer and N source), arabinose (0.3 g l^{-1}) and galactose (0.3 g l^{-1}) were added as 0.2 μm filter-sterilized solutions. Viable counts were performed using standard serial dilutions and the drop count method (Hobben and Somasegaran, 1982). In all experiments strains were grown to turbidity in defined medium on a rotary shaker at 90 rev/min at 25°C for 3–5 days.

Carbon utilization

The nutritional characteristics of chickpea rhizobia strains Ch184, Ch185, Ch191 and Ch192 and soybean *B. japonicum* strain RCR 3407, and soybean rhizobia strains USDA 191, USDA 201 and USDA 208

Table 1. Utilization of carbon sources by strains of chickpea rhizobia and soybean rhizobia and bradyrhizobia in the absence of additional salt (C) or with 0.25 M NaCl (S) in modified YEMA at 25°C. Six replicates per treatment

	Chickpea rhizobia								Soybean rhizobia							
	Ch 184		Ch 185		Ch 191		Ch 192		RCR 3407		USDA 191		USDA 201		USDA 208	
	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S
No carbon source	1+	–	1+	–	1+	–	1+	1+	1+	–	1+	1+	1+	–	1+	–
Arabinose	2+	–	2+	–	2+	–	2+	1+	2+	–	1+	1+	1+	–	2+	–
Cellobiose	2+	–	2+	–	2+	–	2+	2+	2+	–	2+	2+	2+	1+	2+	–
Dulcitol	1+	–	2+	–	2+	–	1+	–	1+	–	1+	1+	1+	–	1+	–
Fructose	2+	–	2+	–	2+	–	2+	2+	2+	–	2+	1+	2+	–	2+	–
Fumarate	1+	–	–	–	1+	–	1+	–	–	–	2+	1+	2+	–	1+	–
Galactose	2+	–	2+	–	2+	–	2+	2+	2+	–	2+	2+	2+	1+	2+	–
Glucose	2+	–	2+	–	2+	–	2+	1+	2+	–	2+	1+	2+	1+	2+	–
Glutamate	2+	–	1+	–	2+	–	1+	1+	1+	–	2+	–	2+	1+	2+	–
Glycerol	2+	–	1+	–	1+	–	1+	1+	2+	–	2+	1+	2+	–	2+	–
Lactose	2+	–	2+	–	1+	–	2+	1+	2+	–	2+	1+	2+	1+	2+	–
Malic acid	1+	–	–	–	1+	–	1+	–	–	–	1+	–	1+	–	1+	–
Maltose	2+	–	2+	–	2+	–	2+	2+	2+	–	2+	2+	2+	2+	2+	–
Mannitol	2+	–	2+	–	2+	–	2+	2+	2+	–	2+	2+	2+	1+	2+	–
Mannose	2+	–	2+	–	2+	–	2+	1+	2+	–	2+	1+	2+	–	2+	–
Rhamnose	2+	–	2+	–	2+	–	2+	2+	2+	–	2+	2+	2+	2+	2+	–
Sorbitol	2+	–	2+	–	2+	–	2+	2+	1+	–	2+	2+	2+	1+	2+	–
Succinic acid	1+	–	–	–	1+	–	1+	–	–	–	2+	–	2+	–	1+	–
Sucrose	2+	–	2+	–	2+	–	2+	2+	2+	–	2+	1+	2+	1+	2+	–
Trehalose	2+	–	2+	–	2+	–	2+	1+	2+	–	2+	–	2+	–	2+	–
Trisodium citrate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Xylose	2+	–	2+	–	2+	–	2+	–	2+	–	2+	–	2+	–	2+	–

–, 1+, 2+ indicate colonies of 0, 0.5–2, >2 mm dia respectively within 14 days.

were determined on an agar medium supplemented with various carbon sources. A modified YEMA (MYEA) (Stowers and Eaglesham, 1984) was used with the following composition ($g l^{-1}$): 0.5 K_2SO_4; 0.8 $MgSO_4 \cdot 7H_2O$; 0.2 NaCl; 0.1 $FeCl_3 \cdot 6H_2O$; 1.0 yeast extract (YE). The pH was adjusted to 6.8. Sodium chloride was added before autoclaving to give a final concentration of 0.25 M in addition to the control in which no NaCl was added. Carbon sources (Table 1) were prepared as 10% (w/v) solutions sterilized by 0.2 μm filtration or by autoclaving (glycerol), and each one was added to sterile MYEA to give a final concentration of 1% (w/v). Carbon sources were omitted in controls. Strains were transferred from defined medium to MYEA plates using a multi-point inoculator. Six replicates were included per treatment. The plates were held at 25°C and checked daily for growth for 14 days. Any growth on the control plates was attributed to utilization of yeast extract as a sole carbon source. Colonies of 0, 0.5–2, >2 mm dia were designated—, 1+ and 2+ respectively.

An experiment was carried out in the defined medium lacking the three carbon sources, to compare growth of strains in liquid and solid media. Sodium chloride was added before autoclaving as for the previous experiment. Carbon sources (Table 2) were added as filter-sterilized solutions to the medium after autoclaving. The media were inoculated with either strain Ch192 or USDA 201 to give an initial count of 10^2–10^3 cfu ml^{-1}. Two replicates were included per treatment. The liquid media were checked daily for the appearance of visible turbidity for 14 days at 25°C. Final viable counts were determined on YEMA from the media in which no turbidity was observed after 14 days.

Effect of temperature, pH and salt

All strains were screened for their temperature tolerance. The strains were streaked on YEMA plates and held at 25, 37 or 45°C. The plates were checked twice a day for 14 days. Growth was scored visually for any colony ⩾2 mm dia. Three replicates were included per treatment.

A factorial experiment was carried out to determine the effect of four temperatures, three pH values and three concentrations of NaCl on the growth and multiplication of chickpea *Rhizobium* strain Ch184. Two replicates were included per treatment. Sodium chloride was added to the basal medium before autoclaving to give final concentrations of 0.34 or 0.51 M in addition to a control with no added NaCl. The pH was adjusted (using HCl or NaOH) to 6.3, 7.3 or 8.3. Fifteen ml of each sterilized medium was dispensed into sterile Universal bottles and inoculated with a diluted culture of a strain to give 10^2–10^3 cfu ml^{-1}. They were held at 19, 26, 33 or 40°C. Viable counts were made daily for 12 days.

Table 2. Utilization of carbon sources by a strain of chickpea rhizobia (Ch192) and a strain of soybean rhizobia (USDA 208) in the absence of salt (C) or with 0.25 M NaCl (S) in defined medium at 25°C. Figures are time (days) taken to show visible turbidity or the final viable counts (log cfu ml^{-1} in parentheses). Two replicates per treatment

	Ch192		USDA 201	
Carbon source	C	S	C	S
Fructose	5.0	10	4.0	(<1)
Galactose	6.0	ND	4.0	ND
Glucose	5.0	(<1)	4.0	(<1)
Lactose	7.0	(4.30)	4.0	9.0
Maltose	10.0	10.0	4.0	5.0
Rhamnose	8.0	12.0	4.0	4.0
Sorbitol	5.0	12.0	4.0	4.0
Sucrose	5.0	12.0	4.0	4.0
Trisodium citrate	ND	(2.62)	(2.12)	(2.12)
Xylose	6.0	(4.40)	4.0	(1.30)

ND = Not determined.

Table 3. The effect of temperature on growth of chickpea rhizobia and soybean rhizobia and bradyrhizobia measured as time (days) taken to form colonies >2 mm diameter on YEMA. Three replicates per treatment

Strain	Temperature		
	25°C	37°C	45°C
Chickpea rhizobia			
Ch178	2.5	4.0	NG
Ch179	2.5	5.0	NG
Ch184	2.0	2.0	NG
Ch185	3.0	5.0	NG
Ch191	3.0	5.0	NG
Ch192	3.0	5.0	NG
Soybean rhizobia			
USDA 191	2.0	2.0	NG
USDA 201	2.0	2.5	NG
USDA 205	2.0	2.5	NG
USDA 208	2.0	2.5	NG
Soybean bradyrhizobia			
RCR 3407	4.0	5.0	NG

NG = No growth.

RESULTS

Carbon utilization

Strains of chickpea and soybean rhizobia and bradyrhizobia showed different responses to different carbon sources. All strains showed limited growth (1+) on control MYEA from which carbon sources were omitted (Table 1). Utilization of carbon sources therefore resulted in greater growth (2+) than the control. However, all strains were inhibited by trisodium citrate in the presence or absence of NaCl.

Rhizobium fredii strains USDA 191, USDA 201 and USDA 208 showed consistent carbon utilization patterns (Table 1). These strains utilized cellobiose, fructose, galactose, glucose, glutamate, glycerol, lactose, maltose, mannitol, mannose, rhamnose, sorbitol, sucrose, trehalose and xylose. However, these strains were unable to utilize dulcitol and malic acid and they varied in their utilization of fumarate and succinic acid. With the exception of *R. fredii* strain USDA 201, these strains were unable to utilize arabinose.

The slow-growing *B. japonicum* strain RCR3407 showed intermediate characteristics in its utilization of carbon sources (Table 1). It was able to utilize glycerol, lactose, maltose, sucrose, trehalose and cellobiose which are usually associated with fast-growers (Sadowsky *et al.*, 1983; Stowers and Eaglesham, 1984). This strain failed to utilize trisodium citrate, dulcitol or fumarate which are normally utilized by slow-growers.

The fast-growing chickpea rhizobia were all similar in their carbon utilization. However, the intensities of utilization varied from one carbon source to another with the strain tested. These strains showed a pattern (with different intensity) of utilization of carbon sources similar to that of the *R. fredii* strains and typical of fast-growers (Stowers, 1985; Stowers and Eaglesham, 1984). The strains did not respond or were inhibited in the presence of fumarate, succinic acid or trisodium citrate. These strains also varied in their utilization of dulcitol, glutamate and glycerol (Table 1).

In the case of the salt-amended media, all strains except Ch192 and USDA 191 showed no growth on control MYEA (Table 1). Any growth of these strains (1+ or 2+) in the presence of salt, therefore indicates utilization of the carbon source present. *Rhizobium fredii* strain USDA 208 and *B. japonicum* strain RCR3407 were inhibited by salt irrespective of carbon source. Strain USDA 191, which utilized yeast extract in the presence of salt, utilized only cellobiose, galactose, maltose, mannitol, rhamnose and sorbitol in salt-treated MYEA, while strain USDA 201 utilized glucose, glutamate, lactose and sucrose in addition to these. With the exception of Ch192 all chickpea rhizobia were completely inhibited by 0.25 M NaCl regardless of carbon source. Chickpea *Rhizobium* strain Ch192, which also utilized yeast extract in salt-treated MYEA, utilized cellobiose, fructose, galactose, maltose, mannitol, rhamnose, sorbitol or sucrose in the presence of NaCl. Some carbon sources, e.g. malate and succinate inhibited utilization of yeast extract when salt was present.

Table 2 shows the responses of *R. fredii* strain USDA 201 and chickpea *Rhizobium* strain Ch192 to 10 carbon sources in liquid basal medium in the presence and absence of NaCl. The results agree with those in Table 1 and, hence, confirm the findings in the solid medium. However, a minor difference was observed in the response of strain USDA 201 to lactose in 0.25 M NaCl. Differences between liquid and solid media were observed by Chakrabarti *et al.* (1981).

Effect of temperature, pH and salt

None of the strains grew at 45°C. All strains grew at 25°C, and *B. japonicum* strain RCR 3407 grew slower than strains of *Rhizobium*. All strains grew at 37°C, but growth of all strains except Ch184 and USDA 191 was reduced compared to that at 25°C (Table 3).

In the absence of salt, Ch184, a strain of fast-growing chickpea *Rhizobium*, multiplied in defined medium (Fig. 1). Multiplication rate was reduced at 40°C and there was a prolonged lag at 19°C at pH 7.3 and 8.3. At 0.51 M NaCl multiplication was inhibited at all temperatures and all pH values. At 0.34 M NaCl the multiplication rate was reduced compared to the control with no added salt. At all temperatures except 19°C, the effects of 0.34 M NaCl were similar at pH 6.3 and 7.3, and were more severe at pH 8.3. Furthermore, the inhibitory effect of 0.34 M NaCl was less at 40°C than at 19 or 26°C and further reduced at 33°C to allow a cell density after 12 days equal to that of the control treatment without added salt.

DISCUSSION

All fast-growing chickpea and soybean rhizobia utilized cellobiose, fructose, galactose, glucose, maltose, mannitol, mannose, rhamnose, sorbitol, sucrose, trehalose and xylose. This is a characteristic of fast-growing strains (Stowers, 1985; Stowers and Elkan, 1984). Chakrabarti *et al.* (1981) showed a wide variation in the response of 85 strains of various *Rhizobium* species, to carbon sources. *Rhizobium fredii* strains respond differently to different carbon sources in the absence of salt. *Rhizobium fredii* strain USDA 191 has a doubling time of 4.0, 4.0 and 6.9 h in minimal media containing glutamate–mannitol,

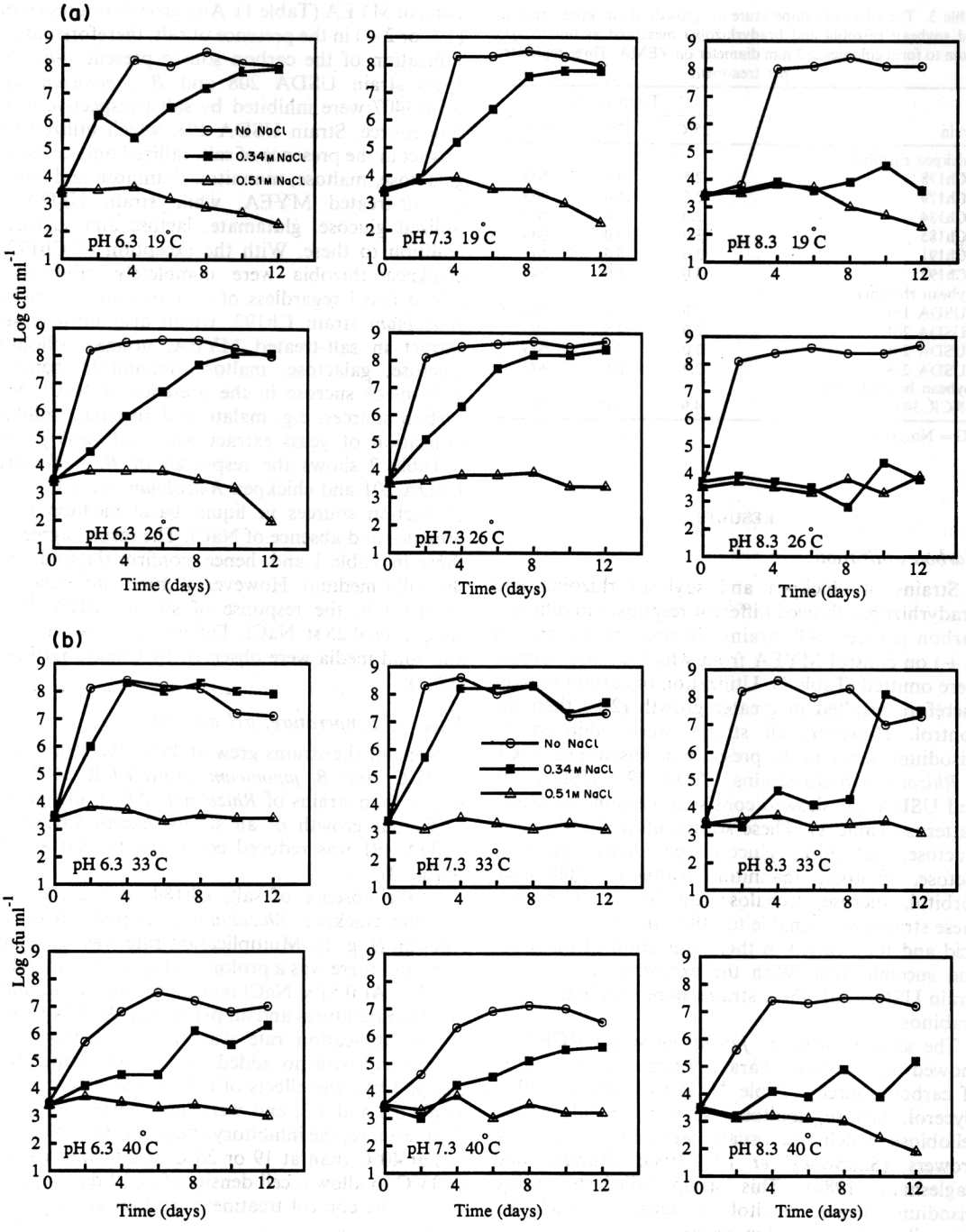

Fig. 1. The effect of temperature, pH and salt on the growth and multiplication of chickpea *Rhizobium* strain Ch184. Two observations per mean.

glutamate–succinate or aspartate–lactate, respectively; *B. japonicum* strain USDA 110 gave doubling times of 15.2, 16.0 and 12.4 h respectively in the same media (Yelton *et al.*, 1983).

Bradyrhizobium japonicum strain RCR 3407 showed mixed characteristics of both fast- and slow-growing rhizobia. Stowers and Elkan (1983) reported that cowpea *Rhizobium* strain BATi 1 has mean generation times of 3.2 and 4.0 h with glucose and mannitol respectively. This strain had the ability to utilize trehalose, citrate, malate, fumarate or dulcitol which are characteristics of slow growers and they concluded that this strain was an intermediate type sharing characteristics of fast- and slow-growers. Stowers (1985) reported similar results for different strains.

Data from our experiments indicate that carbohydrate utilization in *Rhizobium* is affected by the salinity of the growth medium. The data obtained using MYEA were confirmed by the results obtained

using broth cultures (Table 2). The inhibition of the growth and multiplication by salts may be due to the inhibition of activity of specific enzymes by certain mineral ions (Greenway and Munns, 1980). Rhizobia under salt-stress accumulate products such as glutamate (Hua *et al.*, 1982). This accumulation may protect cells from the harmful effects of salts, but may also inhibit enzyme activity at high concentrations (Hua *et al.*, 1986). The different abilities of rhizobia to accumulate metabolites under salt-stress are reflected in their utilization of different carbon sources under salt-stress.

Different responses to the same concentration of salt can occur in media of different composition. Sadowsky *et al.* (1983) reported the tolerance of *R. fredii* strains to 2% (0.34 M) NaCl in YEMA while the same strains failed to grow in 2% NaCl when Stowers and Eaglesham (1984) used modified YEMA in which yeast extract was increased to 1.0 g l^{-1}. Our results indicate that the carbon source can also affect the tolerance of *Rhizobium* to salt. Furthermore, strain Ch184 which was salt-sensitive in MYEA, had been identified as salt-tolerant (data not presented) as confirmed in Fig. 1. Overall, the most salt-tolerant strain of chickpea rhizobia was Ch192 and the most tolerant strain of soybean rhizobia was USDA 191.

The strains of rhizobia and bradyrhizobia used here could only tolerate 37, not 45°C, and strains differed in their response to 37°C. La Favre and Eaglesham (1986) reported strains of cowpea rhizobia which tolerate up to 43°C, and Karanja and Wood (1988) found strains of *R. leguminosarium* biovar *phaseoli* which could multiply at 47°C. The optimum temperature for 42 *B. japonicum* strains was found to vary between 27.7 and 35.2°C (Munevar and Wollum, 1981b).

Factorial experiments using multiplication of *Rhizobium* as the criterion for tolerance of a particular stress are likely to indicate the hidden harmful effects of such a stress. Results here show an interaction between pH, temperature and salinity which might explain the poor *Rhizobium* populations often found in saline soils. The strain of chickpea *Rhizobium* used here was more sensitive to salt-stress at alkaline pH values at temperatures higher or lower than the optimum (33°C). The results should assist with development of criteria for the selection of rhizobia for use with legumes in salt-affected soils.

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