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Comparative study of nitrogen fixation and carbon metabolism in two chick-pea (*Cicer arietinum* L.) cultivars under salt stress

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Abstract

Two cultivars of *Cicer arietinum* with differential tolerance to salinity have been compared by analysing growth, photosynthesis, nodulation, nitrogenase activity, and carbon metabolism in the nodule cytosol. The aim was to help elucidate the relationships between, on the one hand, sucrose and malate metabolism in nodules and, on the other, the inhibition of nitrogen fixation under salt stress. Chick-pea cultivars Pedrosillano (sensitive) and ILC1919 (tolerant) inoculated with *Mesorhizobium ciceri* strain Ch-191 were grown in a controlled environmental chamber and were treated with salt (0, 50, 75, and 100 mM NaCl) from sowing to harvest time (28 d). Plant growth and photosynthesis were more affected by salt in Pedrosillano than in ILC1919. Also the effect of salt on nodulation and nitrogen fixation was much more pronounced in Pedrosillano. The increase in nodular mass in ILC1919 can partially counteract the inhibition of nitrogenase activity. The enzymes of sucrose breakdown were inhibited by NaCl, but in ILC1919 a rise in alkaline invertase was observed with salinity, which could compensate for the lack of the sucrose synthase hydrolytic activity. The activity of PEPC was stimulated by salt in ILC1919. Also, this cultivar showed higher malate concentrations in root nodules.

Key words: Chick-pea, salinity, nitrogen fixation, carbon metabolism, malate.

Introduction

Salt stress is known to depress greatly the growth and symbiotic performance of nodulated legumes, and soil salinity is one of the major environmental constraints on agriculture in many regions of the world (Boyer, 1982; Serrano and Gaxiola, 1994). Chick-pea (*Cicer arietinum* L.) nodules are indeterminate (Hirsch, 1992) and export fixed N as amides and ureides (Schubert, 1986). This legume is considered to be sensitive to salt stress, which affects its growth (Dua, 1992; Rao and Sharma, 1995), nodulation and nitrogenase activity (Elsheikh and Wood, 1990; Sheokand *et al.*, 1995), and photosynthesis and carbon metabolism in root nodules (Soussi *et al.*, 1998). Nevertheless, some authors have reported that nitrogen fixation in indeterminate nodules is more tolerant of salt stress that in determinate ones (Bordeleau and Prevost, 1994; Sinclair and Serraj, 1995).

The microsymbiont, i.e. the bacteroid, depends upon oxygen and photoassimilates supplied by the host plant. The photosynthate translocated to the root nodules, mainly sucrose (Gordon *et al.*, 1992), is metabolized to produce dicarboxylic acid, mainly malate, which is the preferred respiratory substrate for bacteroids (Kim and Copeland, 1996). Although photosynthesis is inhibited by salt, limitation in the photosynthate supply to the nodules does not appear to inhibit nitrogenase, given that stress promotes an accumulation of soluble sugar in nodules (Fougère *et al.*, 1991; James *et al.*, 1993). In addition, it has been observed that the reduction of nitrogenase activity under salt stress was associated with the decrease in sucrose synthase (SS) expression (Gordon *et al.*, 1997).

Another possible explanation for the inhibition of nitrogenase activity is that the supply of malate to bacteroids is limited under saline conditions (Delgado *et al.*, 1993). The production of malate in legume nodules

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Abbreviations: ADH, alcohol dehydrogenase; AI, alkaline invertase; ARA, acetylene reduction activity; ARAP, total ARA per plant; LSD, least significant difference; MDH, malate dehydrogenase; NDW, dry weight of nodules; NN, nodule number; PDW, plant dry weight; PEPC, phosphoenolpyruvate carboxylase; SS, sucrose synthase; TSS, total soluble sugars.

is mediated by the phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) activities. Malate concentration reportedly diminishes with salt stress, but under these conditions PEPC activity increased in nodules of pea (Delgado *et al.*, 1993) and of alfalfa under drought stress (Irigoyen *et al.*, 1992*a*).

Various researchers have found that salt stress reduced the oxygen permeability in soybean nodules (Serraj *et al.*, 1994; Fernandez-Pascual *et al.*, 1996). Reduced oxygen diffusion through the nodule cortex under drought-stress has also been reported (Weisz and Sinclair, 1987; Guérin *et al.*, 1990), a condition that depresses nodule respiration due to enforced O_2 limitation (Walsh, 1995). On the other hand, a stimulation of the fermentative metabolism in nodules, probably due to the lower oxygen concentration, has been observed (Irigoyen *et al.*, 1992*a*). It has also been suggested that malate derived from oxaloacetate synthesized via PEPC may be a factor in the regulation of the variable O_2 diffusion barrier (Vance, 1998).

The accumulation of some organic solutes under saline conditions has been considered an adaptation of plants against osmotic stress (Fougère *et al.*, 1991; Delauney and Verma, 1993), but other authors (Waisel, 1989; Petrusa and Winicov, 1997) have reported a negative relationship between salt tolerance and proline accumulation.

In a previous work (Soussi *et al.*, 1998) the response of *Cicer arietinum* cv. ILC1919 to salt stress applied at the vegetative growth period was studied. In the present work, the salt was supplied at the beginning of cultivation, and the ILC1919 cultivar was compared with cv. Pedrosillano, which presents a different degree of sensibility to salinity. Growth, photosynthesis, nodulation, nitrogenase activity, and carbon metabolism in the nodule cytosol have been analysed. In addition, the activity of SS, alkaline invertase (AI), PEPC, MDH, and alcohol dehydrogenase (ADH) have been assessed to help elucidate the relationships between, on the one hand, sucrose and malate metabolism in nodules and, on the other, the inhibition of N_2 fixation under this type of stress.

Materials and methods

Plants, bacteria and growth conditions

Chick-pea (*Cicer arietinum* L.) cv. ILC1919 was obtained from ICARDA and Pedrosillano from Sierra Nevada (Spain). In previous experiments (data not shown) Pedrosillano was more sensitive to salinity than ILC1919 in growth and nitrogen fixation. These cultivars also differed in the seed dry weight, ILC1919 weighing 0.203 ± 0.015 g, and Pedrosillano 0.261 ± 0.011 g ($n = 15$). Seeds were surface-sterilized in 30% (w/v) H_2O_2 for 2 min, rinsed with sterile water and germinated in moist autoclaved vermiculite at 26 °C for 48 h. Germinated seeds were then transplanted to modified Leonard jars (Caba et al., 1990), and each seed was inoculated with 1 ml of a suspension (*c.* 10^9 cell ml^{-1}) of *Mesorhizobium ciceri* strain Ch-191 (procured from ICARDA), which, according to previous experiments, forms effective symbioses with both Pedrosillano and ICL1919 cultivars. Cultivation and growth conditions were as described previously (Soussi et al., 1998).

Salt stress was induced at transplanting by adding three treatments of NaCl (50, 75 and 100 mM) to the growth medium, while control plants were grown in a NaCl-free solution. Plants were harvested 4 weeks after transplanting and six plants per treatments were used to record shoot, root and nodule dry weights (after drying for 24 h at 70 °C) and to assay nitrogen fixation.

Nitrogen fixation assays

Nitrogenase (EC 1.7.99.2) activity was determined by acetylene reduction on nodulated root portion of the plants as previously described (Herdina and Silsbury, 1990). The aliquots were analysed for ethylene in a Perkin Elmer 8600 gas chromatograph equipped with a Poropak R Column (Ligero *et al.*, 1986).

Preparation of cell-free extracts

Nodule extracts (0.5–1 g) for determining PEPC, MDH and ADH were prepared with 100 mM maleic acid–KOH pH 6.8 (Caba *et al.*, 1990). The homogenate was centrifuged at 30 000 *g* at 2 °C for 30 min, and the supernatant was used for the enzyme assay. For the extraction of SS, 50 mM K-phosphate buffer (pH 8.0) containing 1 mM EDTA and 20% ethylene glycol (Morell and Copeland, 1985) was used. For AI the extraction medium consisted of 50 mM MOPS, 10 mM DTT and 4 mM $MgCl_2$.

Enzyme assays

The activity of PEPC (EC 4.1.1.31) and MDH (EC 1.1.1.37) were assayed spectrophotometrically as described earlier (Soussi *et al.*, 1998). For the assay of ADH (EC 1.1.1.1) the reaction medium was as determined by the method of Thynn and Werner (Thynn and Werner, 1997). The SS activity (EC 2.4.1.13) was assayed by monitoring the reduction of NAD^+ (Morell and Copeland, 1985). The reaction mixture included 2 mM UDP, 1.5 mM NAD^+ and 5 U of UDPG dehydrogenase (SIGMA) in 100 mM bicine-KOH (pH 8.5).

For the assay of AI (EC 3.2.1.26) (modified from Gonzalez *et al.*, 1995), a medium of 50 mM imidazole pH 8.5, 5 mM $MgCl_2$, 1 mM NAD, 1 mM ATP, and 2 units per ml hexokinase, phosphoglucose isomerase and glucose 6 phosphate dehydrogenase was used.

Chlorophyll and Rubisco

After extraction with 80% acetone, the chlorophyll content was analysed (Arnon, 1949). For ribulose 1,5 biphosphate carboxylase (Rubisco; EC 4.1.1.39) the extraction procedure of Keys and Parry was followed (Keys and Parry, 1990). Rubisco activity was immediately measured in glass scintillation vials by adding a 25 µl aliquot of the supernatant to a reaction solution containing 100 mM bicine (pH 8.2), 20 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 20 mM $NaH^{14}CO_3$ (0.5 µCi μmol^{-1}), and 0.5 mM RuBP. This activity was assayed by determining the incorporation of $^{14}CO_2$ into the acid-stable product by liquid-scintillation counting.

Proline, sugar and malate determination

The proline content in nodule and leaf extracts was determined following the procedure of Irigoyen *et al.* using ninhydrin reagent (Irigoyen *et al.*, 1992*b*). Assay of soluble sugar followed a colorimetric method (Irigoyen *et al.*, 1992*b*). For the

calculation of the proline and sugar concentrations a standard curve was prepared with L-proline (SIGMA) and glucose, respectively. Malate was determined enzymatically according to the procedure of Bergmeyer and Bernt (Bergmeyer and Bernt, 1974).

Statistical design and analysis

The experimental layout was a randomized block design. All values are means of eight replicates per treatment, and the results were subjected to a two-way analysis of variance with the least significant (LSD) test between means.

Results

The adverse effect of salinity on plant growth was significant in both Pedrosillano and ILC1919 cultivars (Table 1). Growth inhibition proved greater in Pedrosillano, the lowest salt concentration causing a 22% decrease while in ILC1919 the effect was negligible. With 100 mM NaCl, growth declined 41% in the former cultivar but only 20% in the latter.

The nodule number (NN) was similar in control plants of both cultivars (Table 1). The variations of this parameter with salt were significant only at 100 mM in Pedrosillano, while in ILC1919 they were significant at 75 and 100 mM, the NN values being lower in ILC1919 than in Pedrosillano. Also, the NN was lower in ILC1919, except for 100 mM when it was lower in Pedrosillano, but the decreases caused by 75 and 100 mM were significant only in the latter cultivar. The mean nodule dry weight (Table 1) was reduced by 100 mM NaCl, and did not vary significantly with lower salt concentrations in Pedrosillano, while in ILC1919 this parameter rose with respect to control with all salt concentrations.

The effect of salt on total nitrogenase activity per plant (ARAP) differed for each cultivar. At 50 mM the decrease of ARAP in Pedrosillano plants was not significant (8% with respect to control), while ILC1919 registered an apparent increase of 31% (Table 1). Nevertheless, the negative effects of higher salt dosages were more pronounced in Pedrosillano than in ILC1919, showing the latter cultivar to be more salt tolerant than the former in growth as well as in nitrogen fixation. In the analysis of variance, the data for growth and nitrogen fixation confirmed a significant effect of the variety–salinity interaction.

The activity of Rubisco was similar in the control plants of both cultivars (Fig. 1). With 50 mM NaCl, a considerable activation of Rubisco was detected in Pedrosillano, while in ILC1919 the effect of this concen-

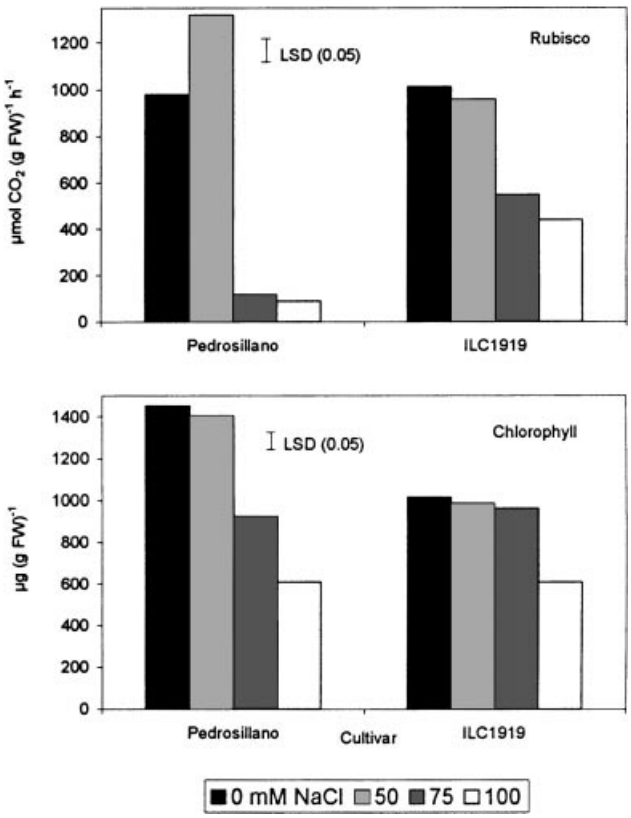

Fig. 1. Rubisco activity and chlorophyll content in leaves of two *Cicer arietinum* L. cultivars inoculated with strain Ch-191 of *Mesorhizobium ciceri*. A 28 d period of salt stress (by application of 50, 75 and 100 mM NaCl) was induced at sowing.

Table 1. *Effect of salt stress on growth and nitrogen fixation parameters in plants of two cultivars of* Cicer arietinum

PDW, plant dry weight (g plant^{-1}), NN, nodule number, NDW, nodule dry weight (g plant^{-1}), Mean NDW, mean nodule dry weight (mg), ARAP, nitrogenase activity per plant (µmol C_2H_4 (plant)$^{-1}$h^{-1}) in plants of two *Cicer arietinum* cultivars at the onset of flowering, with different dosages of NaCl from the beginning of cultivation.

Cultivar	NaCl (mM)	PDW	NN	NDW	Mean NDW	ARAP
Pedrosillano	0	1.11 e[a]	39 cd	0.039 e	1.00 b	1.27 c
	50	0.86 bc	42 d	0.049 f	1.17 b	1.16 c
	75	0.81 b	33 bc	0.036 de	1.09 b	0.49 b
	100	0.65 a	29 b	0.014 a	0.48 a	0.06 a
ILC1919	0	0.98 d	38 cd	0.022 b	0.58 a	1.15 c
	50	0.93 cd	33 bc	0.033 cd	1.00 b	1.52 d
	75	0.94 cd	20 a	0.031 cd	1.55 c	0.59 b
	100	0.78 b	19 a	0.028 c	1.47 c	0.43 b
LSD (0.05)		0.09	6	0.005	0.24	0.20

[a]Means in a column followed by the same letter are not significantly different as determined by an LSD ($P \leq 0.05$).

tration was not significant. With 75 and 100 mM the inhibition of this activity in Pedrosillano was about 80%. In ILC1919, the fall in Rubisco levels with these concentrations was 47% and 57%, respectively. The chlorophyll content (Fig. 1) remained higher in the leaves of Pedrosillano control plants, and the effect of 50 mM was not significant in either cultivars. In Pedrosillano, 75 and 100 mM resulted in a reduction of 36% and 58%, while in ILC1919 75 mM caused no effect, and 100 mM decreased this parameter by 35%.

The enzyme activities of sucrose breakdown (Fig. 2) in nodules of ILC1919, in general, showed higher values than in Pedrosillano. Salt only slightly stimulated SS activity at 50 mM, and at 75 and 100 mM, and inhibition was more pronounced in Pedrosillano than in ILC1919. The response of AI activity to salt treatment (Fig. 2) differed for that of SS; in Pedrosillano no significant effects were noted, while in ILC1919 salt stimulated this activity at all levels.

The activity of PEPC (Fig. 3) in the nodule cytosol of Pedrosillano was stimulated by less than 10% with 50 and 75 mM NaCl, and inhibited by more than 20% with 100 mM. However, in ILC1919 this activity rose about 37% with respect to controls with all salt concentrations.

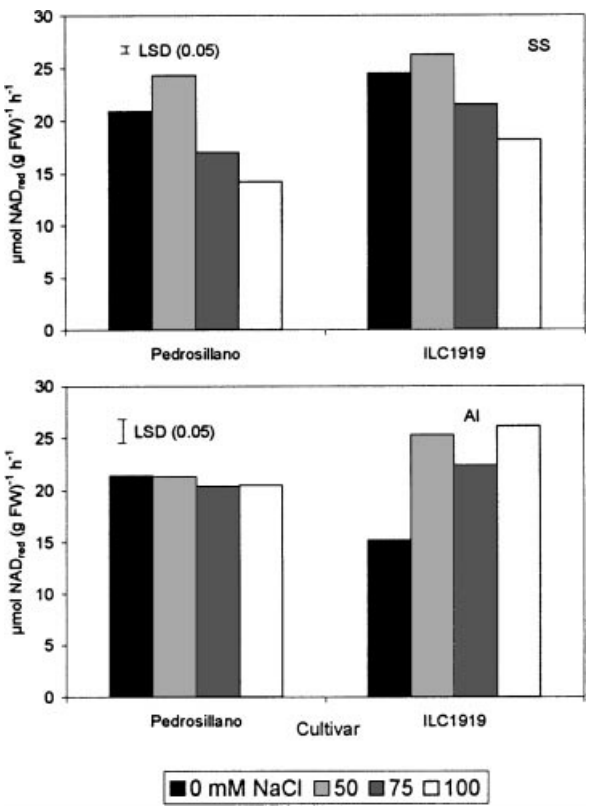

Fig. 2. Activities of SS and AI in root nodules of two *Cicer arietinum* L. cultivars inoculated with strain Ch-191 of *Mesorhizobium ciceri*. A 28 d period of salt stress (by application of 50, 75 and 100 mM NaCl) was induced at sowing.

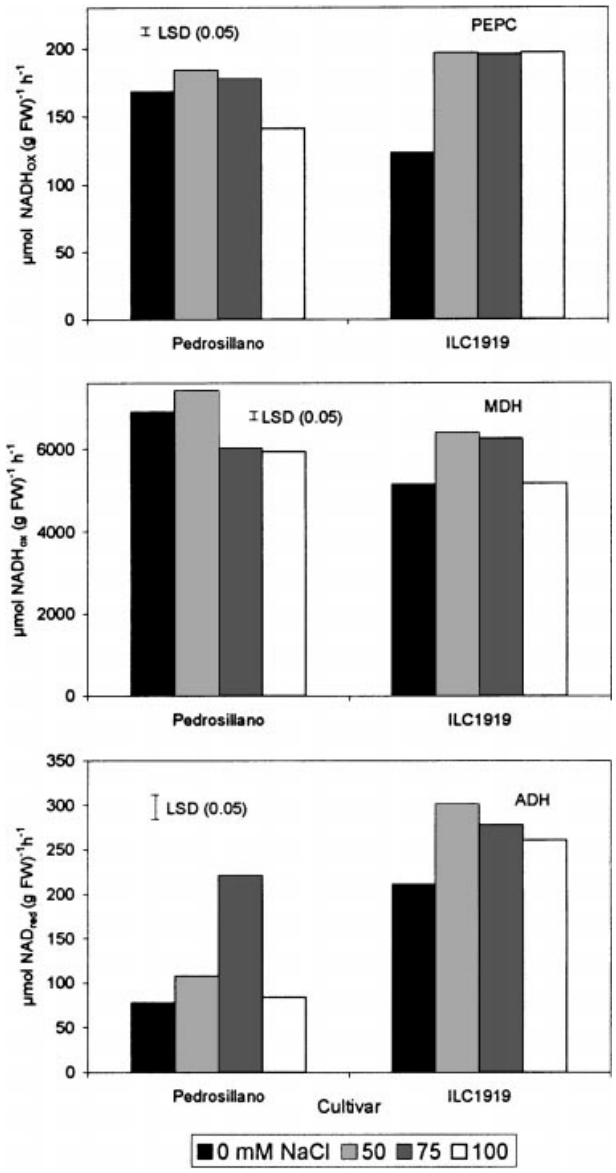

Fig. 3. Activities of PEPC, MDH and ADH in root nodules of two *Cicer arietinum* L. cultivars inoculated with strain Ch-191 of *Mesorhizobium ciceri*. A 28 d period of salt stress (by application of 50, 75 and 100 mM NaCl) was induced at sowing.

The MDH values in Pedrosillano fell by 13% with 75 and 100 mM (Fig. 3). In ILC1919, this activity intensified with 50 and 75 mM but did not vary with 100 mM.

A high value of constitutive ADH activity was found in this species (Fig. 3). The value under control conditions was about three times higher in ILC1919 than in Pedrosillano. Salt stress boosted this activity, the increase being higher in Pedrosillano; with 75 mM the increase reached almost three times the control value, while in ILC1919 the highest increase was less than 40% with 50 mM, and lower with 75 and 100 mM.

The two cultivars differed remarkably in proline accumulation in leaves under saline conditions (Fig. 4). While

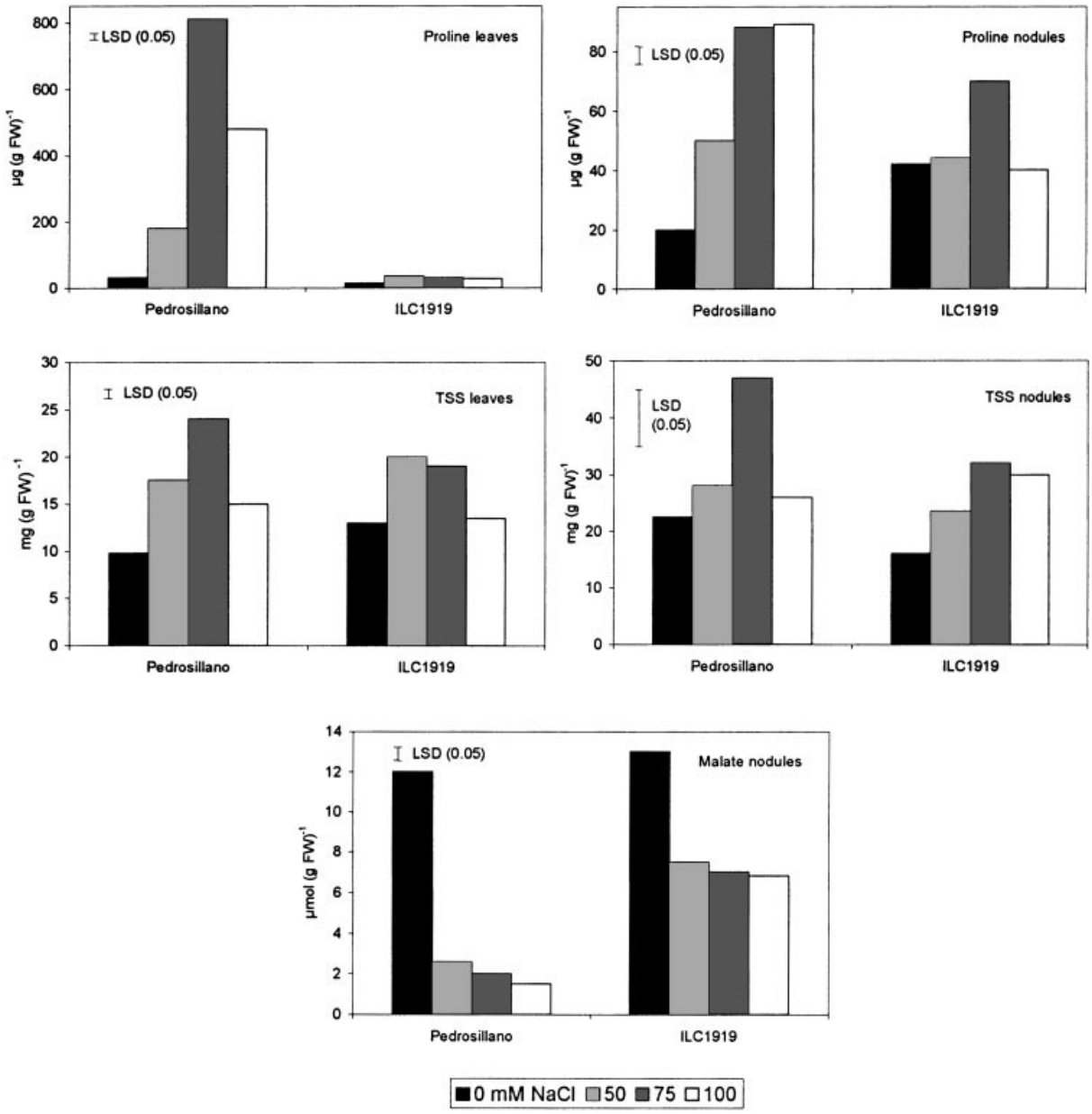

Fig. 4. Proline and TSS content in nodules and leaves, and malate concentration in root nodules of two *Cicer arietinum* L. cultivars inoculated with strain Ch-191 of *Mesorhizobium ciceri*. A 28 d period of salt stress (by application of 50, 75 and 100 mM NaCl) was induced at sowing.

Pedrosillano showed a rise in proline concentration of more than 20 times with 75 mM NaCl, in ILC1919 this parameter did not significantly vary ($P \leq 0.05$), maintaining levels similar to those of Pedrosillano control plants. Also, the proline content in Pedrosillano nodules (Fig. 4) increased sharply with all NaCl dosages, while in ILC1919 the content rose significantly only at 75 mM.

Both cultivars showed the same range of total soluble sugars in leaves (Fig. 4). The concentrations of these solutes increased with salt, the values being higher in the leaves of Pedrosillano with 75 mM. Also, the accumulation of these compounds in nodules registered similar levels in both cultivars, with a greater increase in Pedrosillano with 75 mM. Malate content in nodules (Fig. 4) declined with salinity, but the concentrations were higher in ILC1919 with all treatments.

Discussion

The results presented here confirmed that cv. ILC1919 is more tolerant, and cv. Pedrosillano more sensitive to salt (Table 1). In ILC1919 the decrease in growth proved significant only at the highest salt dosage, in agreement with previous results for short-term stress (Soussi *et al.*, 1998).

It is well known that rhizobia can survive under much higher salt concentrations than its specific host legume

(Nair *et al.*, 1993; Cordovilla *et al.*, 1996). It has been recorded that nodulation was inhibited by salt in soybean plants inoculated with two strains of *Bradyrhizobium* with different degrees of salt tolerance, producing no significant differences in nodulation (Elsheikh and Wood, 1995).

In the present work, a clear difference was observed in nodulation between the sensitive and the tolerant cultivar (Table 1). Data for the tolerant cultivar reflected an increase in total nodule dry weight under salt stress, which could partly offset the inhibition of nitrogenase activity (Yousef and Sprent, 1983), while the nodule number decreased with salinity (Table 1). Under saline conditions, which are unfavourable for nodule initiation (Singleton *et al.*, 1982; Zahran and Sprent, 1986), the cultivar ILC1919 showed a surge in nodule growth, and, subsequently, in total nitrogenase activity due to an increase in mean nodule dry weight. On the other hand, the sensitive cv. Pedrosillano showed lower ARAP values than ILC1919 with salt treatments. Thus the greater performance of symbiosis under saline conditions seems to be determined mainly by the tolerance of the legume host-plant (Velagaleti and Marsh, 1989).

The photosynthetic parameters also proved more sensitive in Pedrosillano (Fig. 1). The effect of salt on these parameters resembled that on nitrogenase activity, in contrast to when the salt was applied to chick-pea plants during the vegetative growth period 17–31 d after sowing (Soussi *et al.*, 1998). Therefore, the effect of salinity on these parameters depends on the timing of salt application.

The enzyme activities of sucrose breakdown reflected a noteworthy difference between the two cultivars (Fig. 2). In nodules of both cultivars SS activity was inhibited by salt, but to a greater extent in the sensitive cv. Pedrosillano. A similar finding has been reported in nodules of soybean, pea and common bean under water stress (Gordon *et al.*, 1997; Gonzalez *et al.*, 1998; Ramos *et al.*, 1999). Also, these authors reported that AI was not significantly inhibited by water stress, a result consistent with the data of the present work for AI in nodules of Pedrosillano. However, in ILC1919 nodules, AI was stimulated by salt stress (Fig. 2). On the other hand, in nodules of pea and common bean the values of AI reported are less than half those of SS (Gonzalez *et al.*, 1998; Ramos *et al.*, 1999), while in these chick-pea nodules the AI values were similar in magnitude to those of SS activity. Therefore, in the chick-pea, AI is more important to sucrose metabolism than in other legumes. In addition, the increase recorded in the more tolerant cultivar subjected to salinity, which could compensate for the depressed hydrolytic SS activity, implies a response characteristic in this species, which deserves a more thorough study.

These results support only in part the hypothesis of Gonzalez *et al.* which suggests that carbohydrate accumulation under drought stress is due to the inhibition of the enzymes involved in sucrose breakdown (Gonzalez *et al.*, 1995). Indeed, the sensitive cultivar, which showed the lower SS and AI activities, accumulated more sugars with 75 mM NaCl (Fig. 4), while the more tolerant one exhibited a lower concentration of these compounds under the same treatment. However, the more tolerant cultivar registered values similar to those of the sensitive cultivar in the other treatments. In addition, this result contradicts the attribution of the osmoregulatory function to the accumulation of total soluble sugars in chick-pea under salt stress (Sheokand *et al.*, 1995) or, at least, this function does not appear to be a determining factor of salt tolerance.

The values of PEPC activity in the nodule cytosol increased with salt in ILC1919 (Fig. 3), as previously reported (Irigoyen *et al.*, 1992*a*; Delgado *et al.*, 1993) and were higher in the tolerant cultivar under saline conditions, and this could explain the higher concentrations of malate detected in root nodules of ILC1919. The MDH activity showed values some 40 times higher than those of PEPC and therefore does not constitute a limiting factor for malate production. The salinity did not significantly alter MDH activity, a finding consistent with the results of Gonzalez *et al.* (Gonzalez *et al.*, 1998) for nodules of pea under drought stress.

In the present work, *Cicer arietinum* nodules showed a high constitutive ADH activity (Fig. 3), as reported by Thynn and Werner, who suggest that the activity of ADH, a key enzyme of anaerobic metabolism in prokaryotes and eukaryotes, can be used as a bioindicator for low oxygen concentrations (Thynn and Werner, 1997). Salinity promoted this activity in both cultivars, indicating lower oxygen permeability, and suggesting an increase in the O_2-diffusion barrier (Serraj *et al.*, 1995; Fernandez-Pascual *et al.*, 1996). It has been suggested that PEPC may be involved in the active regulation of the turgescence/osmocontraction of cells of the inner cortex, which is one of the proposed mechanism of the oxygen diffusion barrier (Drevon *et al.*, 1998). In this light, the boosted PEPC activity in the tolerant cultivar could improve regulation of oxygen diffusion.

The proline content was stimulated by salt in leaves and in the nodule cytosol (Fig. 4), more in the sensitive cultivar than in the tolerant one, and this difference was especially significant in leaves. These results confirm the negative relationship between salt tolerance and proline accumulation (Petrusa and Winicov, 1997), suggesting that this parameter could be a good index of sensitivity to saline stress in this species.

In summary, it is concluded that the tolerance to salinity in chick-pea is related to a higher level of Rubisco, and higher values of enzyme activities of sucrose breakdown in nodule cytosol. Salinity increases the oxygen-diffusion barrier, which enhances the enzyme activities of

anaerobic metabolism. The inhibition of nitrogenase by salt stress may be a consequence of the decrease in malate content in nodules, and it could be offset in the tolerant cultivar by an increase in mean nodule weight.

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References

Arnon DI. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* **24,** 1–15.

Bergmeyer HU, Bernt E. 1974. L-glutamate UV-assay with glutamate dehydrogenase and NAD. In: Bergmeyer HU, ed. *Methods of enzymatic analysis*, Vol. III. New York: Academic Press, 1704–1708.

Bordeleau LM, Prévost D. 1994. Nodulation and nitrogen fixation in extreme environments. *Plant and Soil* **161,** 115–125.

Boyer JS. 1982. Plant productivity and environment. *Science* **218,** 443–448.

Caba JM, Lluch C, Hervás A, Ligero F. 1990. Nitrate metabolism in roots and nodules of *Vicia faba* in response to exogenous nitrate. *Physiologia Plantarum* **79,** 531–539.

Cordovilla MP, Ligero F, Lluch C. 1996. Growth and nitrogen assimilation in nodules in response to nitrate levels in *Vicia faba* under salt stress. *Journal of Experimental Botany* **47,** 203–210.

Delauney AJ, Verma DPS. 1993. Proline biosynthesis and osmoregulation in plants. *The Plant Journal* **4,** 215–223.

Delgado MJ, Garrido JM, Ligero F, Lluch C. 1993. Nitrogen fixation and carbon metabolism by nodules and bacteroids of pea plants under sodium chloride stress. *Physiologia Plantarum* **89,** 824–829.

Drevon JJ, Frangne N, Fleurat-Lessard, Payre H, Ribet J, Vadez V, Serraj R. 1998. Is nitrogenase-linked respiration regulated by osmocontractile cells in legume nodules? In: Elmerich C, Kondorosi A, Newton WE, eds. *Biological nitrogen fixation for the 21st century*. Dordrecht: Kluwer Academic Publishers, 465–466.

Dua RP. 1992. Differential response of chickpea (*Cicer arietinum*) genotypes to salinity. *Journal of Agricultural Science* **119,** 367–371.

Elsheikh EAE, Wood M. 1990. Effect of salinity on growth, nodulation and nitrogen yield of chickpea (*Cicer arietinum* L.). *Journal of Experimental Botany* **41,** 1263–1269.

Elsheihk EAE, Wood M. 1995. Nodulation and N_2 fixation by soybean inoculated with salt-tolerant rhizobia or salt-sensitive bradyrhizobia in saline soil. *Soil Biology and Biochemistry* **27,** 657–661.

Fernández-Pascual M, De Lorenzo C, De Felipe MR, Rajalakshmi S, Gordon AJ, Thomas BJ, Minchin FR. 1996. Possible reasons for relative salt stress tolerance in nodules of white lupin cv. Multolopa. *Journal of Experimental Botany* **47,** 1709–1716.

Fougère F, Le Rudulier D, Streeter JG. 1991. Effects of salt stress on amino acid, organic acid, and carbohydrate composition of roots bacteroids and cytosol of alfalfa (*Medicago sativa* L.). *Plant Physiology* **96,** 1228–1236.

Gonzalez A, Gordon AJ, James CL, Arrese-Igor C. 1995. The role of sucrose synthase in the response of soybean nodules to drought. *Journal of Experimental Botany* **46,** 1515–1523.

Gonzalez EM, Aparicio-Tejo PM, Gordon AJ, Minchin FR, Royuela M, Arrese-Igor C. 1998. Water-deficit effects on carbon and nitrogen metabolism of pea nodules. *Journal of Experimental Botany* **49,** 1705–1714.

Gordon AJ, Minchin FR, Skot L, James CL. 1997. Stress-induced declines in soybean N_2 fixation are related to nodule sucrose synthase activity. *Plant Physiology* **114,** 937–946.

Gordon AJ, Thomas BJ, Reynolds PHS. 1992. Localisation of sucrose synthase in soybean root nodules. *New Phytologist* **122,** 35–44.

Guérin V, Trinchant JC, Rigaud J. 1990. Nitrogen fixation (C_2H_2 reduction) by broad bean (*Vicia faba* L.) nodules and bacteroids under water-restricted conditions. *Plant Physiology* **92,** 595–601.

Herdina, Silsbury JH. 1990. Estimating nitrogenase activity of Faba bean (*Vicia faba* L.) by acetylene reduction (AR) assay. *Australian Journal of Plant Physiology* **17,** 489–502.

Hirsch AM. 1992. Developmental biology of legume nodulation. *New Phytologist* **122,** 211–237.

Irigoyen JJ, Emerich DW, Sánchez-Díaz M. 1992*a*. phosphoenolpyruvate carboxylase, malate and alcohol dehydrogenase activities in alfalfa (*Medicago sativa*) nodules under water stress. *Physiologia Plantarum* **84,** 61–66.

Irigoyen JJ, Emerich DW, Sánchez-Díaz M. 1992*b*. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiologia Plantarum* **84,** 55–60.

James EK, Sprent JI, Hay GT, Minchin FR. 1993. The effect of irradiance on the recovery of soybean nodules from sodium chloride induced senescence. *Journal of Experimental Botany* **43,** 777–788.

Keys JA, Parry MAJ. 1990. Ribulose biphosphate carboxylase/oxygenase and carbonic anhydrase. In: *Methods in plant bochemistry*, Vol. 3. London: Academic Press, 1–14.

Kim SA, Copeland L. 1996. Enzymes of poly-β-hydroxybutyrate metabolism in soybean and chickpea bacteroids. *Applied and Environmental Microbiology* **62,** 4186–4190.

Ligero F, Lluch C, Olivares J. 1986. Evolution of ethylene from root of *Medicago sativa* plants inoculated with *Rhizobium meliloti*. *Journal of Plant Physiology* **125,** 361–365.

Morell M, Copeland L. 1985. Sucrose synthase of soybean nodules. *Plant Physiology* **78,** 149–154.

Nair S, Jha PK, Babu CR. 1993. Induced salt-tolerant rhizobia, from extremely salt-tolerant *Rhizobium* gene pools, form reduced but effective symbiosis under non-saline growth conditions of legume host. *Microbios* **74,** 39–51.

Petrusa LM, Winicov L. 1997. Proline status in salt-tolerant and salt-sensitive alfalfa cell lines and plants in response to NaCl. *Plant Physiology and Biochemistry* **35,** 303–310.

Ramos MLG, Gordon AJ, Minchin FR, Sprent JI, Parsons R. 1999. Effect of water stress on nodule physiology and biochemistry of a drought-tolerant cultivar of common bean (*Phaseolus vulgaris* L.). *Annals of Botany* **83,** 57–63.

Rao DLN, Sharma PC. 1995. Alleviation of salinity stress in chickpea by *Rhizobium* inoculation or nitrate supply. *Biologia Plantarum* **37,** 405–410.

Schubert KR. 1986. Products of biological nitrogen fixation in higher plants: synthesis, transport and metabolism. *Annual Review of Plant Physiology* **37,** 539–574.

Serraj R, Fleurat-Lessard P, Jaillard B, Drevon JJ. 1995. Structural changes in the inner-cortex cells of soybean root-nodules are induced by short-term exposure to high salt or oxygen concentrations. *Plant, Cell and Environment* **18,** 455–462.

Serraj R, Roy G, Drevon JJ. 1994. Salt stress induces a decrease in the oxygen uptake of soybean nodules and in their permeability to oxygen diffusion. *Physiologia Plantarum* **91,** 161–168.

Serrano R, Gaxiola R. 1994. Microbial models and salt stress tolerance in plant. *Critical Reviews in Plant Science* **13,** 121–138.

Sheokand S, Dhandi S, Swaraj K. 1995. Studies on nodule functioning and hydrogen peroxide scavenging enzymes under salt stress in chickpea nodules. *Plant Physiology* **33,** 561–566.

Sinclair TR, Serraj R. 1995. Dinitrogen fixation sensitivity to drought among grain legume species. *Nature* **378,** 344.

Singleton PW, Swaify AE, Bohlool BB. 1982. Effect of salinity on *Rhizobium* growth and survival. *Applied and Environmental Microbiology* **44,** 884–890.

Soussi M, Ocaña A, Lluch C. 1998. Effect of salt stress on growth, photosynthesis and nitrogen fixation in chick-pea (*Cicer arietinum* L.). *Journal of Experimental Botany* **49,** 1329–1337.

Thynn M, Werner D. 1997. Chickpea root and nodule alcohol dehydrogenase activities as very reactive systems monitoring oxygen concentrations. *Journal of Applied Botany-Angewandte Botanik* **71,** 68–69.

Vance C. 1998. Legume symbiotic nitrogen fixation: agronomic aspects. In: Spaink HP, Kondorosi A, Hooykaas PJJ, eds. *The Rhizobiaceae*. Dordrecht: Kluwer Academic Publishers, 482–525.

Velagaleti RR, Marsh S. 1989. Influence of host cultivars and *Bradyrhizobium* strains on the growth and symbiotic performance of soybean under salt stress. *Plant and Soil* **119,** 133–138.

Waisel Y. 1989. Screening for salt resistance. In: Laudelout H, ed. *Proceedings of the 21st Colloque International*. Bern: Potash Institute, 143–155.

Walsh KB. 1995. Physiology of the legume nodule and its response to stress. *Soil Biology and Biochemistry* **27,** 637–655.

Weisz PR, Sinclair TR. 1987. Regulation of soybean nitrogen fixation in response to rhizosphere oxygen. I. Role of nodule respiration. *Plant Physiology* **84,** 900–905.

Yousef AN, Sprent JI. 1983. Effects of NaCl on growth, nitrogen incorporation and chemical composition of inoculated and NH_4NO_3 fertilized *Vicia faba* L. plants. *Journal of Experimental Botany* **34,** 841–950.

Zahran HH, Sprent JI. 1986. Effects of sodium chloride and polyethylene glycol on root-hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*. *Planta* **167,** 303–309.