

Effects of salt stress on growth, photosynthesis and nitrogen fixation in chick-pea (*Cicer arietinum* L.)

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Abstract

Plants of chick-pea (*Cicer arietinum* L. cv. ILC1919) inoculated with *Mesorhizobium ciceri* strain ch-191 were grown in a controlled environmental chamber, and were administered salt (0, 50, 75, and 100 mM NaCl) during the vegetative period. Four harvests (4, 7, 11, and 14 d after treatment) were analysed. The aim was to ascertain whether the negative effect of saline stress on nitrogen fixation is due to a limitation on the photosynthate supply to the nodule or a limitation genase activity.

Plant growth was affected only by the highest NaCl concentration, whereas nitrogenase activity was affected from 50 mM. At the first harvest, Rubisco, PEPC and MDH activities in leaves rose with salt, but fell during the following harvests. The increase of PEPC and MDH in nodules at the two first samplings was clearly related to salt concentration. While 50 mM NaCl increased GS and GOGAT in nodules at some harvests, 100 mM strongly inhibited these activities at all the harvests. The accumulation of proline, amino acids and carbohydrates was clearly related to salt especially in the leaves, whereas in the nodules the protein content was boosted by salt. Although photosynthesis declined with NaCl, the response of nitrogen fixation to salt was more pronounced. This situation, together with carbohydrate accumulation, suggests that the lack of photosynthate does not cause the inhibition of nitrogenase activity under this type of stress. The similar trend observed for the PEPC-MDH pathway and the ARA support the hypothesis concerning the limitation in the supply of energy substrate, mainly malate, to the bacteroids. The accumulation of compatible sol-

utes is more a consequence of damage produced by salt stress than of a protective strategy.

Key words: Chick-pea, salt stress, nitrogen fixation, photosynthesis, ammonium assimilation.

Introduction

Chick-pea (*Cicer arietinum* L.) is an important crop in the Mediterranean area, offering high-quality protein and increasing the imput of combined N_2 into the soil. In this region, the chick-pea has been traditionally used in rotation with cereal crops, and the benefits of these practices are well known (Herridge *et al.*, 1995). However, salt sensitivity can adversely affect yield in this crop. Although the effect of salinity on growth, nodulation and N_2 fixation of this species has been studied (Lauter and Munns, 1986; Elsheikh and Wood, 1990), little is known of the physiological and biochemical responses of chickpea to salt stress concerning such aspects as photosynthesis, ammonium assimilation or compatible solute accumulation.

Several hypotheses have been advanced to explain the negative effect of salt on nitrogen fixation in plant legumes, diminished photosynthate supply to the nodule (Bekki *et al.*, 1987; Georgiev and Atkins, 1993), reduced supply of respiratory substrates to the bacteroids (Delgado *et al.*, 1993, 1994) and alterations in the oxygendiffusion barrier (Serraj *et al.*, 1994). The provision of substrates by the legume host in order to support N_2 fixation in the nodules, is an important facet of effective symbiosis, which has been studied more extensively in ureide-exporting nodules (Day and Copeland, 1991; Gordon and James, 1997). Salinity can seriously change

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Abbreviations: AA, amino acids; *ARA*, acetylene reduction activity; *ARAP*, total *ARA* per plant; GS, glutamine synthetase; LSD, least significant difference; MDH, malate dehydrogenase; NADH-GOGAT, NADH-dependent glutamate synthase; *NDW*, dry weight of nodules; *PDW*, plant dry weight; PEPC, phosphoenolpyruvate carboxylase; *RSR*, root to shoot ratio; Rubisco, ribulose 1,5 biphosphate carboxylase; *TSS*, total soluble sugar.

the photosynthetic carbon metabolism, leaf-chlorophyll content, as well as photosynthetic efficiency (Seeman and Critchley, 1985; Sharkey et al., 1985). However, salinity is known to boost the nodular carbohydrate content, and sucrose is the predominant carbohydrate in legume root nodules (Fougère et al., 1991; Gordon et al., 1997). In addition, phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) activities in root nodules produce malate, which is the preferred substrate for supporting nitrogenase activity (Delgado et al., 1993; Kim and Copeland, 1996). However, the effect of salt stress on both PEPC and MDH activities remains the subject of controversy (Bourgeais-Chaillou et al., 1992; Delgado et al., 1993). The stimulation of these activities under saline conditions may be a consequence of the increase in the oxygen-diffusion barrier (Irigoyen et al., 1992a; Delgado et al., 1993).

Salt reportedly promotes the accumulation of ammonium, nitrate and free amino acids in plants (Hatata, 1982; Pessarakli et al., 1989), and tends to depress the activity of the enzymes involved in ammonium assimilation. In Vicia faba, glutamine synthetase (GS) activity proved to be more inhibited by salinity than was NADH-glutamate synthase (NADH-GOGAT) activity (Cordovilla et al., 1994). Proline and carbohydrates are accumulated in plant tissues under saline stress, and these substances are suspected of contributing to osmotic adjustment (Fougère et al., 1991; Irigoyen et al., 1992b; Delauney and Verma, 1993). Recently, it has been suggested that proline, which is accumulated in nodules subjected to osmotic stress, could be utilized as an energy source for nitrogen fixation (Kohl et al., 1994; Pedersen et al., 1996).

However, the correlation between salt concentration and the concentration of these compounds is generally poor (Weimberg and Shanon, 1988; Cordovilla *et al.*, 1996).

In the present work, the response of *Cicer arietinum– Mesorhizobium ciceri* symbiosis to salt stress during the vegetative period when symbiosis is well established, has been studied. The aim is to ascertain whether the negative effect of salt stress on nitrogen fixation is due to a limitation on the photosynthate supply to the nodule or a limitation on the nodular metabolism which sustains nitrogenase activity. In this sense, the interaction between N fixation, photosynthesis, nitrogen and carbon metabolism has been investigated, and the relationship of these activities with changes in the levels both of sugars and of the proline contents in nodules and leaves of chick-pea plants subjected to salt stress has been examined.

Materials and methods

Plants, bacteria and growth conditions

Chick-pea (*Cicer arietinum* L. cv. ILC1919) was obtained from ICARDA. The plants were inoculated with *Mesorhizobium*

ciceri strain ch-191 procured from ICARDA. In previous experiments, the chick-pea variety and *Mesorhizobium* strain were selected as being salt tolerant (data not shown). In order to study the physiological response of the chick-pea to the salt concentrations habitually used in these types of studies (Fougère *et al.*, 1991; Cordovilla *et al.*, 1994; Fernandez-Pascual *et al.*, 1996), cv. ILC1919 has been chosen for its salt tolerance in terms of growth as well as nitrogen fixation.

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. The young seedlings were sown in a modified Leonard jars (Caba *et al.*, 1990) with nutrient solution (Rigaud and Puppo, 1975) containing 2 mM KNO₃, and fresh nutrient solution replaced the old every week. This N concentration stimulated plant growth, but did not inhibit nodule growth or activity (Caba *et al.*, 1990). Each seedling was inoculated with a *Rhizobium* suspension (*c*. 10⁹ cell ml⁻¹), and grown in a growth chamber under a 16/8 h light/dark cycle, 25/17 °C day/night temperature and at a relative humidity of 55–75%.

Once the symbiosis was well established (17 d after planting), the plants were subjected to salt stress by adding NaCl to the growth medium (50, 75 and 100 mM). Control plants were maintained in a NaCl-free solution.

Plants were harvested at 4, 7, 11, and 14 d after adding salt. Six plants were used per treatment and harvest. A sample of root with nodules of each plant was used for the nitrogenase assay and afterwards the nodules were detached, weighed, and dried at 70 °C for 24 h to calculate dry weight. Other samples of nodules from each plant were pooled and stored at -80 °C for the enzyme assays and analytical determinations. For these determinations in leaves, samples from each plant were pooled and frozen in liquid nitrogen until used. The fresh weight of roots, including the portions used for the nitrogenase assay, stems and leaves were recorded and then all the organs were dried at 70 °C for 24 h, and dry weight calculated.

Nitrogen fixation assays

Nitrogenase (EC 1.7.99.2) activity was determined by acetylene reduction on nodulated root portions of six plants, following the method of Herdina and Silsbury (1990). The nodulated root sample (1 g root plus nodules) of each plant was incubated at room temperature in vials containing C_2H_2 (10%, v/v) in air and sealed with serum caps. Aliquots of 0.2 ml were taken after 5 and 10 min incubation and analysed for ethylene in a Perkin Elmer 8600 gas chromatograph equipped with a Porapak R column (Ligero *et al.*, 1986).

Enzyme assays

Crude extracts were prepared by homogenizing 0.5-1 g fresh matter (nodules and leaves) with 100 mM maleic acid-KOH pH 6.8, (Caba *et al.*, 1990). The homogenate was centrifuged at 30 000 g for 30 min, and the supernatant assayed for enzyme activities of PEPC, MDH, GS, NADH-GOGAT, and protein content. All steps were carried out at 4 °C. Four replicates were performed for each enzyme-activity assay.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) and malate dehydrogenase (EC 1.1.1.37) were assayed spectrophotometrically, at 30 °C and 340 nm for 10 min in the assay media described below, in a final volume of 1 ml. For the PEPC, the assay medium consisted of 10 mM NaHCO₃, 10 mM MgCl₂, 0.2 mM NADH, and 4 mM PEP in 100 mM Bicine–KOH buffer (pH 8.5) optimized from Vance and Stade (1984); for MDH, the assay medium consisted of 5 mM MgCl₂, 0.2 mM NADH, and 1 mM oxalacetate in 100 mM Bicine–KOH

(pH 8.5). The reaction was started by adding the appropriate amount of extract.

The NADH-glutamate synthase (EC 1.4.1.14) activity was assayed by monitoring the oxidation of NADH (Groat and Vance, 1981). The decrease in absorbance was recorded for 10 min.

Glutamine synthetase (EC 6.3.1.2) was determined by the hydroxamate synthetase assay, adapted from Kaiser and Lewis (1984). Assays were optimized for the amount of enzyme to give a linear reaction within at least 30 min. Two blanks without enzyme and without L-glutamate were also analysed (Cordovilla *et al.*, 1994).

For Rubisco (EC 4.1.1.39) extraction, leaf tissue (0.3 g) was homogenized in 3 ml of 100 mM Bicine, pH 7.8, containing 10 mM MgCl₂, 1 mM EDTA, 5 mM DTT, and 2% (w/v) PVP (Keys and Parry, 1990). The homogenate was centrifuged at 35 000 g for 10 min. Rubisco activity was immediately measured in glass scintillation vials by the addition of a 25 μ l aliquot of the supernatant to a reaction buffer (1 ml) containing 100 mM Bicine (pH 8.2), 20 mM MgCl₂, 0.1 mM EDTA, 5 mM DTT, 20 mM NaH¹⁴CO₃ (0.5 μ Ci μ mol⁻¹), and 0.5 mM RuBP. After 30 s, the reaction was stopped by the addition of 0.2 ml 6 N HCl. Rubisco activity was assayed at 25 °C by determining the incorporation of ¹⁴CO₂ into acid-stable product by liquidscintillation counting.

Chlorophyll and protein determinations

After extraction with 80% acetone, the chlorophyll content was analysed according to Arnon (1979). Protein concentration in the different extracts was quantified by the dye-binding assay of Bradford (1976) using bovine serum albumin as a standard (Merck, fraction V).

Organic solute analysis

Proline (Irigoyen *et al.*, 1992*b*) and free amino acids (Yemm and Cocking, 1955) were measured in nodules and leaf extracts using ninhydrin reagent. For the calculation of the proline and amino-acid concentrations, a standard curve was prepared with L-proline and L-glutamine, respectively. Assay for soluble sugars followed the colorimetric methods of Irigoyen *et al.* (1992*b*).

Statistical design and analysis

The experimental design was a randomized complete block. The growth values and the parameters related to nitrogen fixation were means of six replicates per treatment. Four replicates were performed for the enzyme activity assays and protein content, and three replicates for sugar, amino acid and proline content. All results were subjected to a two-way analysis of variance with a least significant difference (LSD) test between means.

Results

The growth response to salt stress of chick-pea plants is represented in Table 1. These data clearly indicate that the dry weight of plant was not affected by 50 and 75 mM NaCl. With 100 mM, dry-mass accumulation decreased roughly 10% in all harvests except in the second, which decreased by 36%. With salinity, the root-to-shoot ratio increased at the first harvest, but decreased at the second, and remained unchanged at the third and fourth.

Salt treatment significantly (P < 0.05) reduced nodule

dry weight (Table 1) except at 7 d after treatment, when 50 and 75 mM NaCl boosted nodulation. Nitrogenase activity (Table 1) was inhibited by salt from the first harvest in plants given 75 and 100 mM, although the difference was statistically significant only from the second harvest on. With 50 mM, this effect began at the third harvest. Total ARA per plant showed a similar trend.

In control plants, GS and NADH-GOGAT activities in root nodules (Fig. 1) increased slightly with development, GOGAT registering a sharper increase at the fourth harvest. The activity of these enzymes involved in ammonium assimilation in nodules was affected by salt, 100 mM NaCl decreasing GS and NADH-GOGAT activities at all harvests. On the other hand, 50 mM stimulated these activities, registering a significant increase at the third harvest, but a decrease at the fourth. With 75 mM, there was little difference compared to control, except for GOGAT in the last harvest, which significantly disminished.

The GS activity in leaves (Fig. 1) was about 5-fold higher than in nodules at the first harvest, but contrary to the trend in the nodules, decreased with time. With 75 and 100 mM NaCl the activity declined. With 50 mM NaCl there was a notable rise at the second harvest, but not subsequently. NADH-GOGAT registered about half the activity detected in nodules and, like GS, diminished with time; 50 mM NaCl increased this activity at the first and second harvest, while 75 and 100 mM caused a decrease which proved especially significant in the first harvest.

Activity of ribulose 1,5 biphosphate carboxylase (Fig. 2), declined with development. The enzyme activity was stimulated by 50 and 75 mM only 4 d after salt treatment, whereas salt addition drastically inhibited the activity in the later harvests. The inhibition was stronger in plants given 100 mM NaCl, especially in the last three harvests. As with Rubisco, the content of total chlorophyll (Fig. 2) rose with salt in the first harvest, followed by a fall, but the differences were significant only at the first harvest, with 75 and 100 mM NaCl.

The specific activity of PEPC and MDH in nodules of control plants increased with time (Fig. 3). At 4 d after the addition NaCl, there was a pronounced rice in these activities, which intensified as the concentration of salt increased. With 100 mM, this stimulation was 87% for PEPC and 30% for MDH. However, in the following harvests these activities diminished, and in the last harvest this high salinity level caused a reduction of approximately 70% in PEPC and 27% in MDH. In the nodules, the values for MDH activities were about 50-fold higher than for PEPC, although the variations produced by salt were proportionally higher for PEPC activity.

The values of PEPC activity detected in the leaves of control plants (Fig. 3), though similar in range to those of the nodules, showed an opposite trend. As in the

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Table 1. Effect of four levels of sodium chloride on growth and nitrogen fixation parameter: PDW, plant dry weight (g plant⁻¹); RSR, root-to-shoot ratio; NDW, nodule dry weight (mg plant⁻¹); ARA, acetylene reduction activity (μ mol C₂H₄ [g NDW]⁻¹ h⁻¹); ARAP, ARA per plant (μ mol C₂H₄ plant⁻¹ h⁻¹)

Days after treatment	NaCl (mM)	PDW	RSR	NDW	ARA	ARAP
4	0	0.437	0.64	80	9.4	0.076
	50	0.519	1.19	77	9.9	0.078
	75	0.487	1.27	43	6.9	0.030
	100	0.378	0.87	24	4.5	0.011
7	0	0.768	1.08	101	39.7	0.408
	50	0.757	1.15	138	42.5	0.578
	75	0.680	0.89	188	25.1	0.463
	100	0.487	0.88	74	6.2	0.043
11	0	0.942	0.89	293	38.9	1.138
	50	0.991	0.84	231	18.3	0.417
	75	1.019	0.75	169	21.3	0.360
	100	0.839	0.94	147	6.2	0.087
14	0	1.077	1.11	290	49.7	1.447
	50	1.148	1.09	220	17.7	0.204
	75	1.051	1.09	177	9.3	0.333
	100	0.976	0.87	105	4.4	0.048
	LSD (0.05)	0.053	0.16	29	6.9	0.15



Fig. 1. Glutamine synthetase and NADH-glutamate synthase activities in root nodules and leaves of *Cicer arietinum* L. cv. ILC1919 inoculated with strain ch-191 of *Mesorhizobium ciceri*. 0 (\bullet), 50 (\diamond), 75 (\blacktriangle), and 100 (\Box) mM NaCl, were applied at the vegetative growth stage (17 d after planting).

nodules, salinity stimulated PEPC activity at the first harvest, but to a less pronounced degree. The MDH activity (Fig. 3) was about 3-fold higher in leaves than in nodules. This activity increased with 50 mM NaCl, while 75 mM caused no differences, and 100 mM significantly inhibited the activity only at the second harvest.

In the control plants, the proline content both in nodules and in leaves (Fig. 4), decreased steadily until day 11 after treatment. There was a strong accumulation of nodular proline with increasing salt levels, but not directly in proportion with salt concentration; in fact, in nodules the highest salinity levels caused the least increase,



Fig. 2. Rubisco activity and chlorophyll content of *Cicer arietinum* L. cv. ILC1919 inoculated with strain ch-191 of *Mesorhizobium ciceri*. 0 (\bullet), 50 (\diamond), 75 (\blacktriangle), and 100 (\Box) mM NaCl, were applied at the vegetative growth stage (17 d after planting).

except at the fourth harvest. In leaves, however, the content of this amino acid was directly related to salinity. In leaves, the accumulation of proline with salt reached far higher values than in root nodules.

The total amino-acid content in nodules as well as in leaves (Fig. 4), declined over time in control plants. The salt drastically reduced the free-amino-acid concentration in nodules at 7 d after treatment; this reduction was similar with 75 and 100 mM at 11 d after salt addition, whereas 50 mM NaCl resulted in a heavy accumulation of soluble amino acids at the third harvest. In leaves, the amino acid level was higher than in root nodules and accumulation with salt was clear at third and fourth harvest, but not at the first or second.

The total soluble sugar content in nodules (Fig. 4), increased in control plants from days 4 to 11, but then stabilized until day 14 after treatment. These solutes accumulated with salt addition. With 100 mM NaCl, the highest values were reached at day 14 after salt addition, while with 50 and 75 mM the sugar concentration was similar to the control at this time in nodules. The concentration of soluble sugars in leaves was 4- to 5-fold higher than in nodules. Salt increased this concentration, especially at 11 and 14 d after treatment.

At 4 and 7 d after NaCl addition, soluble protein in

nodules increased proportionally to NaCl levels (Fig. 4). Because of this increase, if the activity of certain enzymes (PEPC, MDH) are expressed on the basis of protein (data not shown), the increases found in these activities are much lower or even absent, suggesting that these increases are due to induction of these enzymes more than to activation. The protein content fell in the last harvest, more sharply as saline levels rose. The protein concentration in leaves was greater than in nodules (Fig. 4). In control leaves, this content declined with development. Salt caused an accumulation which was especially significant at 11 d after the addition of 50 mM NaCl, but there was not a clear relationship between salinity and protein content.

Discussion

In the present work, the effect of NaCl on chick-pea plants inoculated with a specific Mesorhizobium ciceri applied at the vegetative growth stage, has been investigated. The decrease in growth proved significant only with the highest dosage of salt (Table 1). Although the RSR increased with the salt treatments at the first sampling, a trend which can be considered a short-term response to salinity, increases in the following samplings were hardly significant. Depressive effects of NaCl on growth have been reported in legumes, as well as the chick-pea (Dua, 1992), soybean (Singleton and Bohlool, 1984) and faba-bean (Zahran and Sprent, 1986; Cordovilla et al., 1996). Salinity significantly reduced the nodule dry weight per plant; however, in the second harvest an increase was registered in nodule dry weight, which resulted in an ARA per plant equal to or higher than control. These results differ from those reported by Elsheikh and Wood (1990) and Sheokand et al. (1995) for other cultivar, indicating that cv. ILC1919 was more tolerant to salinity.

Considerable inhibition of nodulation and N₂-fixation under saline conditions has been reported by other workers (Bekki et al., 1987). According to Elsheikh and Wood (1990), nodulation and nitrogen fixation are apparently more salt sensitive than is growth, given that the values for the former two parameters were reduced even by the lowest NaCl concentration, while plant dry weight was affected only by 100 mM. The clear accumulation of carbohydrates in nodules under salinity (Fig. 4) suggests that the decline in nitrogenase activity (Table 1) was not caused directly by a lack of photosynthates (Seeman and Critchley, 1985; Hunt and Layzell, 1993). As in the case of drought stress, this accumulation may be due to the fact that sucrose content increased because the enzyme activities involved in sucrose breakdown were diminished (Gonzalez et al., 1995).

According to Cordovilla *et al.* (1994), salt stress in *Vicia faba* inhibited GS and NADH-GOGAT activities



Fig. 3. Phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) activities in root nodules and leaves of *Cicer arietinum* L. cv. ILC1919 inoculated with strain ch-191 of *Mesorhizobium ciceri*. 0 (\bullet), 50 (\diamond), 75 (\blacktriangle), and 100 (\Box) mM NaCl, were applied at the vegetative growth stage (17 d after planting).

in nodules at high NaCl concentrations, but in this work these activities were stimulated at 50 mM NaCl earlier in leaves than in nodules (Fig. 1). Stewart and Rhodes (1978) reported that an increase in external salinity stimulates the levels of glutamine synthetase of shoot tissue. These changes may reflect a tendency for the shoot to play a greater role in nitrogen assimilation under saline conditions. In these results, this increase occurs at the first two harvests, perhaps due to the ammonium and amide accumulation induced by stress (Hatata, 1982). On the other hand, neither GS not GOGAT correlated with *ARA*, a result consistent with a report by Gordon *et al.* (1997) working under drought conditions.

The photosynthetic carbon assimilation process in chick-pea cultivar ILC1919 was found to be greatly depressed by high levels of NaCl. However, stimulation of Rubisco by salt (Fig. 2) was noted 4 d after adding NaCl. Data show that photosynthesis was less sensitive than was nitrogen fixation; in fact, while the inhibition of Rubisco activity 4 d after adding 100 mM NaCl was 18%, the *ARA* was depressed by more than 50% (Table 1). In the last sampling, salt inhibited the fixation more than 90%, while Rubisco activity was reduced in 20% with respect to control. A similar finding was reported by Djekoun and Planchon (1991) in soybean under drought stress. Inhibition of Rubisco activity by salt may be due

to the sensitivity of this enzyme to chloride ions (Seeman and Critchley, 1985). The negative effect of salt on chlorophyll content has previously been described (Mostan *et al.*, 1988). In the present study, chlorophyll synthesis decreased and the proline content increased. Le Dily *et al.* (1993) suggested that these two compounds are formed from a common precursor, glutamate, a possibility that agrees with the negative correlation found in the present work between proline and chlorophyll content.

Phosphoenolpyruvate carboxylase and malate dehydrogenase activities in leaves were stimulated by salt at the first harvest (Fig. 3). The induction of PEPC under saline treatment was reported in leaves of CAM plants (Yen et al., 1995). Guerrier (1988) also proposed that the levels of PEPC activity could be used as biochemical indicator of salt tolerance. In legumes, stimulation of PEPC-MDH patways was also reported in root nodules of alfalfa under drought stress, perhaps caused by decreased O_2 avaibility (Irigoyen et al., 1992a). In this study, cytosolic PEPC in nodules increased at 4 and 7 d after adding salt and declined at the two last harvests. The positive and significant correlation between PEPC and MDH in nodules (r=0.73, P<0.01) indicated that these enzymes behaved similarly under salt stress. The trend of specific ARA was also similar to that of PEPC indicated by the



Fig. 4. Proline, soluble amino acid (AA), total soluble sugars (TSS) and protein content in nodules and leaves of *Cicer arietinum* L. cv. ILC1919 inoculated with strain ch-191 of *Mesorhizobium ciceri*. $0 (\bullet)$, $50 (\diamond)$, $75 (\blacktriangle)$, and $100 (\Box)$ mM NaCl, were applied at the vegetative growth stage (17 d after planting).

positive correlation found between these two parameters (r=0.57, P<0.05), contrary to earlier observations by Delgado *et al.* (1993) in *Pisum sativum*. Also, these authors reported a relationship between salinity and

PEPC activation, while the results of this study support this relationship only in part: the enzyme activity increased with salinity at the first harvest, but decreased at the last two. Therefore, the response of this activity to

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salt could depend on the timing of the salt application and the duration of exposure.

The accumulation of organic solutes under saline conditions have been well documented. It has been assumed that the accumulation of organic solutes in response to salt stress was involved in protection mechanisms such as the restoration of cell volume and turgor, the reduction of the cell damage induced by free radicals, the protection and stabilization of enzymes and membrane structures (Timasheff and Arakawa, 1989). The content of compatible solutes increased in the last samplings, whereas nodulation, nitrogen fixation, photosynthesis, PEPC, and MDH diminished. This supports the idea that the accumulation of compatible solutes is more a consequence of damage produced by salt stress than of a protective strategy. Similar results were reported by Pérez-Alfocea et al. (1994) in Lycopersicum esculentum and Lutts et al. (1996) in Oryza sativa L. In the present work, a negative correlation was found between ARA, PEPC, MDH, and the proline content in leaves. A negative relationship between salt tolerance and proline accumulation was also reported (Waisel, 1989; Petrusa and Winicov, 1997).

The response of *Cicer arietinum* cv. ILC1919 to salt applied at the vegetative growth stage includes the inhibition of nodulation and nitrogen fixation even at the lowest NaCl concentration, whereas growth was inhibited only by the highest. The response of nitrogen fixation to salt was more pronounced than the response of photosynthesis, which, together with the carbohydrate accumulation found in the nodule, suggests that the lack of photosynthate did not cause the inhibition in the nitrogenase activity under this type of stress. The similar tendency observed in the PEPC-MDH pathway and the ARA support the hypothesis of Delgado et al. (1993) concerning the limitation in the supply of energy substrate, mainly malate, to the bacteroids. On the other hand, the accumulation of organic solutes, in this case, reflects the damage caused by NaCl.

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