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Salt tolerance of nitrogen fixation in *Medicago ciliaris* is related to nodule sucrose metabolism performance rather than antioxidant system

Imène Ben Salah · Tarek Slatni · Alfonso Albacete · Mhemmed Gandour · Cristina Martínez Andújar · Hayet Houmani · Karim Ben Hamed · Vicente Martinez · Francisco Pérez-Alfocea · Chedly Abdelly

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Abstract In this study, the effect of 100 mM NaCl on physiological and biochemical responses were investigated in nodules of two *Medicago ciliaris* lines differing in salt tolerance (TNC 1.8 and TNC 11.9). Results showed that, on the basis of growth and nitrogen fixation, the line TNC 1.8 proved more salt tolerant than TNC 11.9. The salt-induced oxidative stress (membrane lipid peroxidation, leghemoglobin degradation, antioxidant activities reduction) occurred similarly in nodules of both lines. The tolerant line TNC 1.8 showed a better capacity to preserve higher sucrolytic activities and maintained higher nodule malate concentration, although total organic acids decreased in both lines. The higher amount of organic acids in the tolerant line seems to be related to its capacity to maintain higher NH_4 nodule concentration in comparison with the sensitive line. Although salt stress reduced concentrations of the majority of amino acid in both lines, the decrease of the most preponderant amino acids glycine, valine, aspartate and glutamate was more accentuated in the sensitive line TNC 11.9. However, alanine concentration increased in the nodules of this sensitive line, suggesting a higher incidence of stress-induced hypoxia. The present study provides further evidence that salt tolerance of nitrogen fixation in the tolerant line is linked to a more effective supply of malate to bacteroids which allows the synthesis of amino acids required to maintain both plant and nodule growth.

Keywords Amino acids · Nitrogen fixation · Organic acids · Oxidative stress · Salt stress · Sucrolytic activities

I. Ben Salah (✉) · T. Slatni · M. Gandour · H. Houmani · K. Ben Hamed · C. Abdelly
Laboratoire des Plantes Extrêmophiles, CBBC, BP 901, 2050 Hammam-Lif, Tunisia
e-mail: imen_ben_salah@yahoo.fr

A. Albacete · C. Martínez Andújar · V. Martinez · F. Pérez-Alfocea
Department of Plant Nutrition, CEBAS-CSIC, Campus de Espinardo, 30100 Murcia, Spain

1 Introduction

Salinization is expected to have overwhelming global effects, resulting in a 30% land loss within the next 25 years and up to 50% by the year 2050 (Wang et al. 2003). This constraint is considered one of the most threatening factors to the natural environment and to limiting agriculture crop production (Chinnusamy et al. 2005; Zadeh and Naeini 2007). Leguminous plants play a critical role in natural ecosystems and agriculture owing to their capacity to fix atmospheric nitrogen.

Symbiotic nitrogen fixation (SNF) process involves a mutualism between legume plants and soil bacteria 'rhizobia'. This interaction results in the formation of root-nodules, in which the bacteria reduce atmospheric nitrogen to ammonia which is used by the plant for the synthesis of a variety of amino acids, nucleic acids and other nitrogenous compounds (Appels and Haaker 1991). At the same time the bacteria are completely dependent on the host plant for carbon compounds, namely sucrose, and all other nutrients needed for energy production and carbon skeleton for SNF, ammonia assimilation, export of nitrogenous compounds and the building and the maintaining of the machinery of nitrogen fixation which constitutes the nodule (Arrese-Igor et al. 1999; Minchin and Witty 2005). The sucrose supplied to

nodules is cleaved by sucrose synthase and alkaline invertase into hexoses which are metabolized through the glycolytic pathway yielding C4-dicarboxylates. Among these C4-diarboxylates, malate has been suggested to be the preferred substrate for bacteroid respiration and to be involved in the regulation of oxygen diffusion (Gálvez et al. 2000).

In addition to having physiological effects, salt stress can induce senescence process in different plant tissues by enhancing production of toxic reactive oxygen species (ROS). NaCl-induced senescence of nodules and decrease in SNF is associated with simultaneous decrease in the levels of antioxidants and the autooxidation of oxygenated leghemoglobin to ferric leghemoglobin yielding O_2^{-}. This radical can dismutate to H_2O_2, which in turn may attack leghemoglobin, releasing catalytic Fe and producing the highly toxic HO• radical (Puppo and Halliwell 1988). Legume nodules possess a number of enzymatic mechanisms to prevent or limit the toxicity of ROS including superoxide dismutase, catalyse and peroxidase reducing the inherent danger of oxidative damage in the nodules (Jamet et al. 2003).

SNF has been described as being sensitive to salt stress (Jebara et al. 2005; López et al. 2008; Ben Salah et al. 2009). It has been suggested that the salt induced reduction of SNF was concomitant to a decrease in nodule sucrolytic activities, namely that of sucrose synthase (López et al. 2008; Ben Salah et al. 2009) causing a shortage of substrates for bacteroids respiration (Arrese-Igor et al. 1999). Other studies have demonstrated that the salt induced decrease in sucrose synthase activity was associated with an increase in alkaline invertase activity that, however, did not restore SNF (Soussi et al. 1999); thus, the role of sucrose synthase in the response to abiotic stresses was demonstrated (Arrese-Igor et al. 1999). Another major constraint for SNF is the enhanced ROS generation induced by salt stress and the impairment of antioxidant enzyme activities which leads to enhanced lipid peroxidation, protein degradation (namely leghemoglobin) and premature nodule senescence (Bianco and Defez 2009).

As a part of comparative study on the effects of salt stress on growth and nitrogen fixation in wild fodder legume *M. ciliaris*, we found that nitrogen fixing root-nodules were poorly supplied with sucrose and that the superiority of the tolerant line (TNC 1.8) was associated with its better ability to maintain higher nodule sucrolytic activities namely that of sucrose synthase and alkaline invertase (Ben Salah et al. 2009). We hypothesized therefore that the tolerant line TNC 1.8 would ensure a better supply of bacteroids in organic acids; this would allow an effective supply in nitrogen and thus the maintenance of growth under stress conditions. The objective of the present study was to address the effect of salt stress on antioxidant and sucrolytic activities and the repercussion on the concentration of tricarboxylic acid cycle acids and on amino acids synthesis and their relevance in the tolerance of SNF to salt stress.

2 Materials and methods

2.1 Biological materials and growth conditions

The two lines of *M. ciliaris* used in this study are originated from local populations from the edge of saline depression of Enfidha (TNC1.8) and non-saline habitat in Mateur (TNC11.9). Since the hard seed coat is impervious to water, seeds were scarified with concentrated H_2SO_4 for 40 min and were then washed 10 times with sterile distilled water and placed on sterile agar medium at 25°C in the dark. Three-day-seedlings were transferred sterile perlite and were inoculated with *Sinorhizobium medicae* CI 1.12/E22 strain suspension (about $10^8 mL^{-1}$) (Zribi et al. 2007). After 2 weeks plants were transferred in 250 mL glass bottle wrapped with aluminium foil to maintain darkness in the rooting environment containing Hewitt nutrient solution: KH_2PO_4 (1.60 mM), $MgSO_4$ (1.50 mM), K_2SO_4 (1.50 mM), $CaSO_4$ (3.50 mM), H_3BO_3 (4 μM), $MnSO_4$ (4 μM), $ZnSO_4$ (1 μM), $CuSO_4$ (1 μM), $CoCl_2$ (0.12 μM), $(Na)_6(Mo)_7O_{24}$ (0.12 μM). Urea (2 mM N) was added to the nutrient solution only during the first week of irrigation. A month later (after the appearance of functional nodules), plants were divided into 2 lots irrigated with nutrient solution supplemented with 0 mM NaCl (control plants) or 100 mM NaCl (salt-treated plants). The continuously aerated culture medium was renewed weekly. Plants were grown in a greenhouse at 28±5°C / 16±5°C (day/night) temperature, 40±5% / 80±5% (day/night) relative humidity, and 14 h light / 10 h dark regime. The plants were harvested 3 weeks after the start of salt treatment (at late vegetative stage). Plants were uprooted carefully and washed with distilled water.

2.2 Nitrogen-fixation assay and nitrogen determination

Nitrogen fixation was estimated by measuring "*in situ*" the acetylene reduction activity (ARA) at the end of the vegetative stage. To avoid nodule disturbance, the level of the N-free solution was lowered to 40% of the glass bottle volume one day before the assay. 10% of acetylene was added to the nodulated roots enclosed in the glass bottle. After 20 min of incubation, samples of 1 mL were withdrawn from the flask and ethylene was measured using a gas chromatograph (Cromatix KNK-2000) (Hardy et al. 1968). N was determined as described by Kjeldahl (1983). Nitrogen fixation was estimated as the difference between total N quantities (mg $plant^{-1}$) before and after salt treatment.

2.3 Ammonium determination

Free ammonium was extracted from frozen material, stored in liquid nitrogen, at 4°C with 0.3 mM H_2SO_4 and 0.5% (w/v) polyclar AT. The homogenate was centrifuged at 15 000 g for 15 min at 4°C. 100 µL of the supernatant was incubated in presence of 900 µL of phenol-hypochlorite reagent for 30 min at 37°C. Ammonium concentration was estimated measuring the absorbance at 620 nm and calculated from the calibration curve using ammonium sulphate (Weatherburn 1979).

2.4 Nutrients determination

The dried and ground samples (100 mg) were digested in microwave with HNO_3: H_2O_2 (5:3) according to the method of Oliva et al. (2003) and mineral concentration was determined by inductively coupled plasma (ICP) spectrometry.

2.5 Organic and amino acids determination

Organic acids were analysed according to Balibrea et al. (1997). The ethanol extracts (1:3, W:V) were directly filtered through 13 mm diameter and 0.45 mm pore size Millex filters (Millipore Co., Milford, MA, USA) and analyzed by HPLC (Shimadzu Co. Ltd., Kyoto, Japan). Organic acids were determined by using an ion exchange column Interaction ORH-801 (Interaction Chromatography Inc., San Jose´, CA, USA), an UV detector at 210 nm, a mobile phase with H_2SO_4 0.01 N at a flow rate of 0.6 mL min^{-1} and at 45.1°C. Quantification was performed by the external standard method by using a data analysis Chrompass program (Jasco Co. Ltd., Tokyo, Japan). Amino acids were analyzed in purified nodule extracts by method described by Godel et al. (1987) using combined orthophthaldehyde (OPA) and fluoreylmthyloxycarbonylchloride (FMOC-CI) precolumn derivatization with OPA in a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan). The injection volume was 10 µL and the samples were eluted by binary gradient during 40 minutes: mobile phase A (sodium acetate 3- hydrate 100 mM, tetrahydrofuran (THF) 0.3% and triethylamine (TEA) 0.018%) and mobile phase B (sodium acetate 3-hidrate 100 mM: acetonitrile: methanol 20:40:40), with a flow 1.2 mL min^{-1} at 30°C of temperature, and UV detection at 338 nm.

2.6 Sucrolytic and antioxidant enzymes extraction and assay

After harvest, nodules were immediately frozen in liquid nitrogen and stored at−80°C until analysis. For sucrose synthase and alkaline invertase, samples containing polyvinylpirrolydone (PVP) and Fontainebleau sand were homogenized in 1 mL of extraction buffer containing 50 mM HEPES (pH 7), 10 mM $MgCl_2$, 1 mM EDTA ($2H_2O$), 2.6 mM dithiothreitol (DTT), 10% ethylene glycol and 0.02% Triton X-100 (Pelleschi et al. 1997). After centrifugation at 20,000 g, the supernatant was desalted on G25-Sephadex column pre-equilibrated with 4 mL of the reaction buffer containing 50 mM HEPES pH 7, 2 mM $MgCl_2$, 1 mM Na_2EDTA, 2.6 mM dithiothreitol (DTT) and 0.1% bovine serum albumin (BSA). Sucrose synthase and alkaline invertase were assayed by an enzyme-linked assay monitoring NADH formation at 340 nm (Balibrea et al. 2003). Superoxide dismutase (EC 1.15.1.1) and peroxidase (EC 1.11.1.7) were extracted with 10% (w/w) polyvinylpyrrolidone in 50 mM K-phosphate buffer (pH 8) containing 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM phenyl-methyl-sulfonyl-fluoride (Scebba et al. 1999). The homogenate was centrifuged at 12 000 g for 30 min. The supernatant protein concentration was determined according to Bradford (1976). Spectrophotometric determination of superoxide dismutase activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Scebba et al. 1999). Peroxidase activity was assayed by monitoring the formation of tetragaiacol from guaiacol at 470 nm according to Srinivas et al. (1999).

2.7 Oxidative damage

Oxidative damage (estimated by membrane lipid peroxidation) was assessed by measuring the amount of malondialdehyde (MDA) in tissue. Fresh samples were homogenized in 0.1% (w/v) trichloroacetic acid solution. The homogenate was centrifuged at 15,000 g for 15 min. An aliquot of the supernatant was added to 0.5% thiobarbituric acid in 20% trichloroacetic acid. The mixture was incubated at 90°C for 30 min; the reaction was stopped by placing the reaction tubes in an ice water bath. Samples were centrifuged at 10,000 g for 5 min, and the absorbance of the supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated from the extinction coefficient 155 $mM^{-1} cm^{-1}$.

2.8 Leghemoglobin determination

Nodules were homogenised in Drabkin's reagent and leghemoglobin was quantified spectrophotometrically at A_{540} as described by Wilson and Reisenauer (1963). Bovine haemoglobin was used as a standard. Drabkin's solution was obtained by dissolving 52 mg of potassium cyanide, 198 mg of potassium ferricyanide and 1 mg of sodium bicarbonate in 1,000 ml of distilled water.

2.9 Statistical analysis

Analysis of variance (ANOVA) was used for the statistical analysis of data. Mean separation procedures were carried out using the multiple range tests with Fisher's least significant difference (LSD) procedure ($P<0.05$).

3 Results

3.1 Growth and symbiotic nitrogen fixation

The line from the salty biotope, TNC 1.8, showed the higher production of biomass when compared with the second line TNC 11.9 even in presence of 100 mM NaCl (Fig. 1A). The inhibitory effect of salt treatment on total biomass was detected in both lines but this effect was less pronounced in TNC 1.8. Salinity caused also a decrease in nodule dry matter in both lines; the depressive effect of salt was always more accentuated in TNC 11.9 (Fig. 1B). Specific nitrogen fixation, estimated by acetylene reduction assay was repressed in both lines; TNC 11.9 was more affected by salt than TNC 1.8 (Fig. 1C). This parameter seemed to be more affected by salt than nodule growth (reduction 2.4–1.9 times greater) (Fig. 1C). The amount of fixed nitrogen decreased in both lines (Fig. 1D) and TNC 1.8 showed higher values of N-fixed compared to the other line. Under salt stress, nodule NH_4 concentration was significantly more decreased in the sensitive line TNC 11.9 (Fig. 1E).

3.2 Nodule nutrients concentration

Under control and stressed conditions, nodules of both lines accumulated higher amounts of iron by comparison with the other micronutrients manganese, zinc and copper. Salt treatment decreased to the same extent both lines nodule concentration of potassium. Nodule micronutrient concentrations were unaffected by salt in both lines, except that of manganese which decreased in the tolerant line TNC 1.8, and zinc in the sensitive line TNC 11.9. No differences were observed for sodium accumulation in nodules of either line (Table 1).

3.3 Sucrolytic and antioxidant activities, nodule MDA and leghemoglobin concentration

Salt treatment led to a significant inhibitory effect on sucrose synthase activity in both lines particularly in the sensitive line TNC 11.9 (reduction of 81% against 43% in the tolerant line TNC 1.8). However, alkaline invertase activity was not altered by salt in the tolerant line TNC 1.8 whereas it was repressed by 77% in the sensitive line TNC 11.9. Salt treatment led to a significant inhibitory effect on both antioxidant enzymes

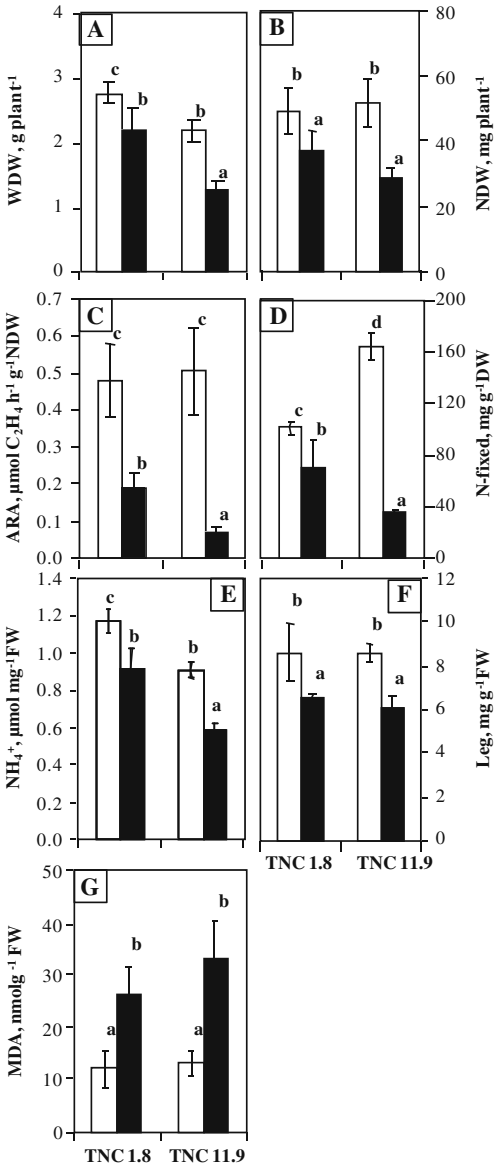

Fig. 1 Changes in whole plant dry weight (**A**), nodule dry weight (**B**), specific acetylene reduction activity (**C**), nitrogen fixation (**D**), NH_4^+ (**E**), leghemoglobin (**F**) and MDA concentration (**G**) in control (□) and salt-stressed (■) *Medicago ciliaris* lines. Mean values followed by the same letter are not significantly different at $P<0.05$ ($n=3$–7)

activities, superoxide dismutase and peroxydase, at the same extent (Table 2). To estimate Na toxicity-induced oxidative damage, the MDA formation was measured in *M. ciliaris* nodules (Fig. 1G). Under salt stress, MDA levels increased similarly in both lines. This oxidative damage was associated with decline in nodules leghemoglobin concentration in both lines; this reduction was comparable in both lines (Fig. 1F).

3.4 Nodule metabolite concentration

In absence of 100 mM NaCl and in both lines, succinate and malate were the most abundant organic acids (Table 3)

Table 1 Effect of salinity on K, Na, Fe, Mn, Cu and Zn concentration in nodules of *Medicago ciliaris* lines subjected to 21 days of treatment with or without 100 mM NaCl added to the nutrient solution

Mean values followed by the same letter are not significantly different at $P<0.05$.

		K ($mg g^{-1}DW$)	Na	Fe ($\mu g g^{-1}DW$)	Mn	Cu	Zn
Lines	NaCl (mM)						
TNC 1.8	0	28.2b	1.4a	631.5a	60.0b	36.9a	83.3b
	100	18.9a	13.6b	669.5a	36.7a	35.7a	113.4bc
TNC 11.9	0	36.1c	1.1a	634.1a	65.7b	35.4a	72.4b
	100	26.0b	13.8b	719.0a	59.0b	37.3a	56.4ab

and amounted to 90 and 84% of the total organic acid fraction, whereas oxalate and fumarate were less abundant and represented 3 and 2% of the total organic acid fraction in TNC 1.8 and TNC 11.9 nodules, respectively. Under stress conditions, citrate became the second abundant organic acid following succinate in both lines. Salt stress decreased the total organic acid concentration in both lines (by 40 and 48% in TNC 1.8 and TNC 11.9, respectively). Oxalate was not changed by salt stress in both lines. The concentration of all other organic acids was affected to the same extent in both lines except for the concentration of malate which decreased much higher in the sensitive line TNC 11.9 (reduction of 66 against 30% in the tolerant line TNC 1.8).

Amino acids which were determined in *M. ciliaris* nodules are listed in Table 3. Under control conditions and in both lines, the most abundant amino acids were alanine, glycine, asparagine, valine, aspartate, glutamate and glutamine. Salt stress induced a decrease in total amino acid concentration in the nodules of both lines to the same extent. This constraint did not affect the pattern of accumulation of amino acid concentration of both lines but it induced a decrease in the concentration of glycine, valine, aspartate, glutamate and other minor amino acids such as arginine, threonine, phenylalanine and methionine. This reduction was more pronounced in the sensitive line TNC 11.9. Asparagine was unmodified by salt stress in both lines; glutamine also remained not modified but this only in the tolerant line TNC 1.8. Alanine concentration increased under salt stress in the sensitive line TNC 11.9; whereas, it remained unchanged in the tolerant line.

4 Discussion

In this study, nodules of *M. ciliaris* lines subjected to salt stress showed a remarkable inhibition in their nitrogen fixation capacity (measured as the capacity to reduce C_2H_2). This depressive effect of salt was less pronounced in the line TNC 1.8 originating from the salty biotope. As reported previously (Ashraf and Bashir 2003; Jebara et al. 2005), nodule growth and functioning were more sensitive to salinity than were vegetative organs (Fig. 1A).

As Na negatively affects the acquisition and homeostasis of essential nutrients, salt stress could reduce nodule growth and functioning through its nutritional effect. A previous study showed that in both lines, 100 mM NaCl decreased nodule nutrients concentration particularly that of K, Ca and P (Ben Salah et al. 2009). O'Hara (2001) reported that the symbiotic legume-rhizobium association requires nutrients to a greater extent than both partners individually. The decrease in K and Ca concentration in both lines would lead (i) to a disturbance in the operation of the cortical diffusion barrier and in the control of the ion channel that facilitates voltage dependent transport of monovalent cations including NH_4 through the symbiosome membrane, and (ii) to nodules structure alteration, and would then be responsible for the reduction of nitrogenase activity (Tyerman et al. 1995; El-Hamdaoui et al. 2003). The reduction in Mn and Zn concentrations (in the tolerant and sensitive line, respectively) would have some repercussions on the antioxidant response of plants subjected to salt. In fact, these nutrients have been reported to have specific reactions in which the ion brings about optimum conformation of enzyme protein, such superoxide dismutase, and thus its activation and

Table 2 Effect of salinity on sucrolytic and antioxidant enzymes activities in nodules of *Medicago ciliaris* lines subjected to 21 days of treatment with or without 100 mM NaCl added to the nutrient solution

Lines	TNC 1.8		TNC 11.9	
NaCl (mM)	0	100	0	100
Sucrose synthase (nmol NADH $min^{-1}mg^{-1}$ protein)	38.7c	22.2b	50.7c	9.4a
Alkaline invertase (nmol NADH $min^{-1}mg^{-1}$ protein)	22.8a	20.3a	52.5b	12.3a
Superoxyde dismutase (U mg^{-1}protein)	9.2b	7.3a	10.6b	7.7a
Peroxydase (U mg^{-1}protein)	0.3b	0.2a	0.4b	0.3a

Mean values followed by the same letter are not significantly different at $P<0.05$.

Table 3 Effect of salinity on organic and amino acid concentration in nodules of *Medicago ciliaris* lines subjected to 21 days of treatment with or without 100 mM NaCl added to the nutrient solution

Lines	TNC 1.8		TNC 11.9	
NaCl (mM)	0	100	0	100
Organic acids (nmol g^{-1}FW)				
Oxalate	0.12 a	0.12 a	0.08 a	0.07 a
Citrate	0.44 a	0.81 ab	1.03 b	0.57 ab
Malate	0.84 bc	0.58 ab	1.14 c	0.38 a
Succinate	5.01 bc	2.33 a	5.79 c	3.20 ab
Fumarate	0.07 b	0.01 a	0.10 a	0.01 a
Total	6.47 bc	3.85 a	8.22 c	4.24 ab
Amino acids (nmol g^{-1}FW)				
Alanine	1.05 a	1.07 a	1.15 a	1.81 b
Glycine	0.87 c	0.53 b	0.79 c	0.18 a
Asparagine	0.44 a	0.58 a	0.45 a	0.41 a
Valine	0.40 bc	0.28 ab	0.49 c	0.17 a
Aspartate	0.27 b	0.13 a	0.23 b	0.15 a
Glutamate	0.15 c	0.12 b	0.13 cb	0.07 a
Glutamine	0.14 b	0.16 b	0.17 b	0.07 a
Serine	0.11 a	0.12 a	0.12 a	0.07 a
Arginine	0.12 c	0.07 b	0.07 b	0.04 a
Leucine	0.10 b	0.08 b	0.09 b	0.05 a
Threonine	0.11 b	0.06 a	0.14 c	0.06 a
Phenylalanine	0.10 b	0.07 a	0.14 c	0.08 ab
Cysteine	0.03 a	0.06 b	0.02 a	0.08 c
Proline	0.02 a	0.02 a	0.04 b	0.03 ab
Tyrosine	0.04 b	0.03 ab	0.03 a	0.03 ab
Lysine	0.03 ab	0.05 c	0.03 bc	0.02 a
Histidine	0.03 b	0.01 a	0.03 b	0.02 b
Methionine	0.02 d	0.01 b	0.02 c	0.01 a
Total	4.02 b	3.46 a	4.14 b	3.35 a

Mean values followed by the same letter are not significantly different at $P<0.05$.

enables electrons transport by valency change (Mengel and Kirkby, 2001). A differential distribution of the toxic ion Na across cell layers (uninfected and infected cells) between the two lines can not be ruled out, as reported by Abd-Alla et al. (2001). Unless Na is sequestrated in vacuoles, this ion would partially induce damage of cellular components, disturbance of enzymatic activities and overproduction of harmful reactive oxygen species (Munns and Tester 2008; Garg and Manchanda 2008).

In nodules of both lines, the decline in leghemoglobin concentration (Fig. 1F), but not in total soluble protein (data not shown) would be indicative of sensitivity of leghemoglobin to degradation compared with other cytosolic proteins. Leghemoglobin degradation could be due to the activation or de-localization of proteases located in the infected cells that display a high affinity for leghemoglobin, especially at the acidic intracellular pH of senescing nodules (Pladys et al. 1991) and would therefore provide catalytic Fe which reacts with H_2O_2 to produce the toxic HO• radical (Puppo and Halliwell 1988) and participate directly or indirectly (bound to phytoferritin) in lipid peroxidation as occurred in both lines (Fig. 1G). Therefore, it can be suggested that peribacteroid membrane is target of degradative reactions induced by salt stress in the nodules of both lines. The observed oxidative damage have been earlier reported in nodules of soybean, pigeonpea and pea subjected to salt stress (Borucki and Sujkowska 2008; Zilli et al. 2008; Garg and Manchanda 2008). This salt induced oxidative damage could be due to disturbance in the antioxidant defence mechanism namely in the antioxidant enzymes such some superoxide dismutase and peroxidase (Table 2). In both lines, the activity of these enzymes was decreased in presence of salt. This reduction could explain to some extent the depressive effect on the membranes structural integrity and on leghemoglobin concentration. However, the similar response of antioxidant enzymes in both lines suggests that the relative tolerance of TNC 1.8 is not linked to the performance of antioxidant system.

Sucrose synthase represents the key enzyme required for the maintenance of nitrogenase activity (Gordon et al. 1999) and it has been proven to be sensitive to a wide range of stresses such drought, nitrate, salt and defoliation (Ramos et al. 1999; López et al. 2008). In *M. ciliaris* lines, the salt constraint triggered a drastic inhibition of sucrolytic activities particularly in the sensitive line TNC 11.9 (Table 2). However, although the tolerant line TNC 1.8 maintained constant activity of alkaline invertase, nitrogenase activity was decreased. This implies that the lack of sucrose synthase activity could not be compensated for by alkaline/neutral invertase (Gordon et al. 1999). Because of the decreased sucrose synthase activity, the effectiveness of the nitrogen-fixing capacity of the rhizobial symbiont was decreased and thus altered plant development (Baier et al. 2007).

C4-dicarboxylates are the main products of sucrose breakdown supplied to bacteroids to fuel nitrogen fixation (Lodwig and Poole 2003). Malate and succinate are the preferred substrates to support SNF and are well transported across the peribacteroid membrane *via* dicarboxylate carrier with high affinity for these two organic acids (Arrese-Igor et al. 1999). Consistent with earlier report (Fougère et al. 1991), salt stress severely decreased the total organic acid concentrations in both lines. The concentration of malate was more reduced in the sensitive line TNC 11.9 whereas changes in concentration of other organic acids was similar in both lines which would highlight the determining involvement of this substrate to fuel SNF. Thus, it can be suggested that the relative tolerance of SNF in TNC 1.8 could be related to its capacity to supply bacteroids

effectively in malate. The maintenance of oxalate concentration in both lines (Table 3) could contribute in protecting nodules against the probable salt induced oxidative damage. Indeed, it has been suggested that the complete oxidation of oxalate was of interest in scavenging oxygen and limiting the generation of reactive oxygen species in the vicinity of bacteroids (Trinchant and Rigaud 1996).

The synthesis of organic acids is regulated in response to NH_4 (Vance and Gantt 1992). In fact, in non-effective nodules, NH_4 is involved in the expression of genes of nodule carbon metabolism enzymes (e. g. sucrose synthase, phosphoenolpyruvate carboxylase (PEPC) and PEPC-Kinase). The higher activities of sucrose synthase and alkaline invertase in *M. ciliaris* nodules of the tolerant line TNC 1.8 in response to salt stress (Table 2) would explain its better capacity to preserve nitrogen fixation and then to maintain an effective concentration of NH_4 (Fig. 1E) and organic acid (Table 3). Since primary assimilation of nitrogen is inseparably linked to carbon metabolism, the differential depressive effect of salt on bacteroid supply in organic acids would lead to a differential amino acid biosynthesis activity in root nodules of both lines.

Salt stress affected nodule concentration of some amino acids to a greater extent in the sensitive line TNC 11.9 than the tolerant line TNC 1.8 (Table 3). The pronounced increase in alanine observed in the sensitive line TNC 11.9, but not in the tolerant one, is a characteristic response to O_2 deficiency reported in many plant tissues (Streeter and Thompson 1972; Reggiani et al. 1988; Fan et al. 1997; Reggiani et al. 2000). Then, it could be suggested that in the sensitive line TNC 11.9 there was an establishment of an anaerobic metabolism in nodules likely following the salt induced decrease in the O_2 diffusion to the infected zone (Serraj et al. 1995). In contrast to ethanol, which diffuses out into the media, most of the alanine produced under anaerobic conditions is retained in tissues (Hoffman et al. 1986) and contribute to cytoplasmic homeostasis (Sakano and Tazawa 1984, 1985). The marked depressive effect of salt on glutamate synthesis in the sensitive line TNC 11.9 (probably because of the inhibition of enzymes involved in the glutamine synthetase/glutamate synthase pathway) could explain the higher reduction in the majority of amino acids in this line by comparison with the tolerant line TNC 1.8; glutamate which is assimilated is used by various transaminases to generate all of the other amino acids. Apart from being a central nitrogen metabolite in nodule cells, glutamate, together with glycine, is thought to be involved in the regulation of cytosolic concentration of Ca (Dennison and Spalding 2000; Dubos et al. 2003). Glycine might also function as signalling molecule and favour the growth of nodules (White 1939; Fries 1953; Skinner and Street 1953). This amino acid is also essential for the terminal step of glutathione synthesis (Noctor et al. 1997) and it represents the major component of glycine-rich proteins (it represents up to 60%) found in the cell walls of many higher plants (Ringli et al. 2001). Since, *M. ciliaris* nodules have indeterminate growth, the maintenance of a relatively higher glycine and glutamate concentration in the tolerant line TNC 1.8 could account for a better growth of nodules and then to better fix nitrogen.

The present data suggest that the tolerance of nitrogen fixation in TNC 1.8 is related to its capacity to maintain sucrolytic activities; this would allow the supply of bacteroids in organic acids, namely malate, and thus a better synthesis of amino acids required for the maintenance of nodule and plant growth. The knowledge of the regulatory mechanisms that simultaneously allow the maintenance of SNF activity and plant growth will help to improve the tolerance of these legumes to saline soils.

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