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BRIEF COMMUNICATION

Role of nitric oxide under saline stress: implications on proline metabolism

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Abstract

The present work is focused on the possible relationship between nitric oxide and the induction of proline in response to salt stress. The plants were subjected to 100 mM NaCl and sodium nitroprusside (SNP; the donor of NO) at different concentrations. The plants showed lower NaCl-induced oxidative stress and proline accumulation after application of low concentrations of SNP together with the NaCl treatment. The reduction in the proline content was related to increased activity of proline dehydrogenase. These results suggest that the NO could be capable of mitigating damage associated with salt stress.

Additional key words: ornithine-δ-aminotransferase, proline dehydrogenase, Δ^1-pyrroline-5-carboxylate synthetase, sodium nitroprusside.

Salinity is one of the main environmental factors that severely limit crop productivity by depressing plant growth and interfering with photosynthesis, protein synthesis, and energy metabolism (Parida and Das 2005). To counter the effects of osmotic stress caused by salinity, plants have developed a number of mechanisms, such as the accumulation of compatible solutes and proline being perhaps the most widely distributed amino acid. In addition to being an osmotically active compound, it acts as a N storage, protects macromolecules against denaturation, reduces stress-induced acidification, serves as a substrate for respiration, contributes to membrane stability, and protects plants against damage induced by free-radicals (Delauney and Verma 1993, Hare and Cress 1997, Matysik *et al.* 2002, Ashraf and Harris 2004).

Some authors have demonstrated that this solute accumulates readily under stress conditions and that its accumulation is related to salinity-stress tolerance. Moreover, different studies with transgenic plants show that an increase in the proline content due to the overexpression of enzymes in charge of its synthesis improve salinity tolerance (Hmida-Sayari *et al.* 2005). However, other authors hold that proline is simply an indicator of salt stress, given that considerable increases have not been found to mediate in osmotic adjustment (Hare and Cress 1997) and this compound has been found even to increase in salt-sensitive varieties (Lutts *et al.* 1999). On the other hand, Hare and Cress (1997) propose that the proline synthesis can be more important than the accumulation itself, and therefore attention merely on the accumulation of the compound might be erroneous.

Another compound that has recently been proposed in the mediation of the response to salinity is NO. It is strongly reactive and can be toxic, however, it can act as a signal molecule in plants (Beligni and Lamattina 2001). Recently, numerous functions of NO have been discovered. It promotes germination, leaf extension and root growth, it delays leaf senescence and fruit maturation and acts as a signal in resistance to biotic stress (Delledonne *et al.* 1998).

The influence of this gas has been also demonstrated under abiotic stress. In this sense, it has been related to the response against drought stress by inducing stomatal closure as well as to salinity stress by inducing expression and activity of plasma membrane H^+-ATPase

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Abbreviations: BSA - bovine serum albumin; DM - dry matter; FM - fresh matter; GSA - glutamic-γ-semialdehyde; H_2O_2 - hydrogen peroxide; LOX - lipoxygenase; MDA - malondialdehyde; NO - nitric oxide; δ-OAT - ornithine-δ-aminotransferase; P5C - Δ^1-pyrroline 5-carboxylate; P5CDH - pyrroline-5-carboxylate dehydrogenase; P5CR - Δ^1-pyrroline-5-carboxylate reductase; P5CS - Δ^1- pyrroline-5-carboxilate synthetase; PDH - proline dehydrogenase; PMSF - phenylmethylsulfonyl fluoride; PPFD - photosynthetic photon flux density; RGR_L - leaf relative growth rate; ROS - reactive oxygen species; SNP - sodium nitroprusside.

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(Zhao *et al.* 2004) and increasing the activities of H^+-ATPase and H^+-PPase in the tonoplast (Zhang *et al.* 2006). Many studies have proposed that NO is an antioxidant, blocking chain reactions triggered by reactive oxygen species (ROS) under oxidative-stress conditions, or directly detoxifying free radicals (Beligni and Lamattina 2002, Tian and Lei 2006).

Therefore, the aim of the present work is to determine the capacity of NO to induce adaptive responses against salt stress and elucidate the possible involvement of NO in the proline metabolism.

Seeds of the Chinese cabbage (*Brassica rapa* L. cv. Onekilo) were germinated and seedlings were grown for 30 d in the experimental greenhouse in southern Spain (*Saliplant S.L.*, Granada). The 30-d-old seedling were transferred to a cultivation chamber under controlled environmental conditions under relative humidity of 60 - 80 %, day/night temperature 25/15 ºC, and 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 350 µmol m^{-2} s^{-1} (measured at the top of the plants with a *190 SB* quantum sensor, *LI-COR*, Lincoln, NE, USA). The plants were grown in *Vermiculite*-filled individual pots, each containing one plant. Over the entire experiment, all the plants received a nutrient solution (pH 5.0 - 5.5) consisting of: 6 mM KNO_3, 5 mM $CaCl_2$, 1 mM NaH_2PO_4, 2 mM $MgSO_4$, 0.025 mM H_3BO_3, 2 µM $MnCl_2$, 1 µM $ZnSO_4$, 0.1 µM $(NH_4)_6Na_7MoO_{24}$ and 0.25 µM $CuSO_4$, 10 µM Fe-EDDHA. The nutrient solution was renewed every 3 d and the *Vermiculite* partly rinsed with Millipore-filtered water in order to avoid nutrient accumulation.

At day 45 after sowing, the different treatments were applied together with the nutrient solution described above. NaCl was applied at a concentration of 100 mM and SNP, the NO-donor (Ignarro *et al.* 1980), in concentrations 0.25, 0.5, and 1 mM. The control treatment consisted of applying the complete nutrient solution without NaCl or SNP. The different treatments were maintained for 14 d, when most of the plants showed clear symptoms of NaCl toxicity. The six plants were used per treatment and the experiment was repeated three times under the same conditions.

Half of the plants were sampled on day 45 after sowing, immediately before the start of the salinity and SNP treatments (T_i) and the remaining plants were sampling on day 59 after sowing (day 14 of treatments, T_f). Fully expanded leaves from the middle parts of each plant were rinsed three times in distilled water after disinfection with non-ionic 1 % detergent. Fresh samples were used for analysis of lipoxygenase activity (LOX, EC 1.13.11.12), malondialdehyde (MDA), hydrogen peroxide and proline contents and activities of Δ^1-pyrroline-5-carboxylate synthetase (P5CS, E.C. 2.7.2.11/1.2.1.41), ornithine-δ-aminotransferase (δ-OAT, E.C 2.6.1.13) and proline dehydrogenase (PDH, E.C. 1.5.99.8). Another subsamples of leaves was dried in a force-air oven at 70 ºC for 24 h, and dry mass was recorded. The material was ground in a Wiley mill and then used for the analysis of nutrient concentration (Na^+, K^+ and Cl^-). Leaf relative growth rate (RGR_L) was calculated from the increase in dry matter (DM) of leaves between the beginning and at the end of treatments (Gutschick and Kay 1995).

For the MDA assay, leaves were homogenized with 5 cm^3 of 50 mM buffer solution containing 0.07 % of NaH_2PO_4 ·2 H_2O and 1.6 % Na_2HPO_4· 12 H_2O, ground with a mortar and pestle, and centrifuged at 20 000 *g* for 25 min (4 ºC). For measurement of MDA concentration, 4 cm^3 of 20 % trichloroacetic acid containing 0.5 % thiobarbituric acid was added to a 1 cm^3 of aliquot of the supernatant. The mixture was heated at 95 ºC for 30 min and then quickly cooled in an ice bath. After the tube was centrifuged at 10 000 *g* for 10 min, the absorbance of the supernatant was read at 532 nm *Helios* UV/visible spectrophotometer (thermo Electron Corporation, Cambridge, UK). The value for the nonspecific absorption at 600 nm was subtracted. The concentration of MDA was calculated using coefficient of absorbance of 155 mM^{-1} cm^{-1} (Fu and Huang 2001).

LOX activity was measured according to Minguez-Mosquera *et al.* (1993), using 50 mM K-phosphate buffer (pH 6.0) for extraction. The reaction mixture consisted of 0.2 cm^3 crude extract and 0.5 mM linoleic acid in 50 mM K-phosphate buffer (pH 6.0). The LOX activity was calculated following the rise in the extinction at 234 nm using a coefficient of 25000 M^{-1} cm^{-1}. The protein content of the extract was measured according to Bradford (1976) with bovine serum albumin as the standard.

The H_2O_2 content in leaf samples was measured as described by Mukherjee and Choudhuri (1983). Leaf samples were extracted with cold acetone to determine the H_2O_2 levels. An aliquot (1 cm^3) of the extracted solution was mixed with 0.2 cm^3 of 0.1% titanium dioxide in 20 % (v:v) H_2SO_4 and the mixture was then centrifuged at 6 000 *g* for 15 min. The intensity of yellow colour of the supernatant was measured at 415 nm and the H_2O_2 content was calculated from a standard curve.

Free proline was determined in 95 % ethanol extracts from leaves. After centrifugation at 3 500 *g* for 10 min the supernatant was stored at 4 ºC and proline content determined according to the method described by Paquin and Lechasseur (1979) and Irigoyen *et al.* (1992).

All operations during extraction of enzymes of proline metabolism were performed at 4 ºC. P5CS extraction was carried out according to Sumithra *et al.* (2006). Leaves were homogenized with extraction buffer containing 100 mM Tris-HCl (pH 7.5), 100 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF) and then centrifugated at 10 000 *g* for 15 min. The supernatant was used for enzyme assays. For δ-OAT and PDH extraction, leaves were homogenized in 100 mM K-phosphate buffer (pH 7.8). The homogenate was filtered and centrifuged at 12 000 *g* for 20 min (4 ºC) (Charest and Phan 1990). P5CS activity was measured as describe by Charest and Phan (1990). The reaction mixture (3 cm^3) contained: 100 mM Tris-HCl (pH 7.2), 25 mM $MgCl_2$, 75 mM sodium glutamate, 5 mM ATP and the enzyme extract. The reaction was initiated by the addition of 0.4 mM NADPH. The activity

Table 1. Leaf biomass [g(DM) leaf^{-1}] and RGR_L [g g^{-1}(DM) d^{-1}], leaf concentration of Na^+, Cl^-, K^+ [mg g^{-1}(DM)] and K^+/Na^+ ratio, MDA content [nmol g^{-1} (FM)], LOX activity [μmol(oxidised linoleic acid) mg^{-1}(protein) min^{-1}], H_2O_2 content [μmol g^{-1}(FM)], proline content [mg g^{-1} (FM), P5CS activity [nmol(NAPH) mg^{-1}(protein) min^{-1}], δ-OAT activity [μmol(NAD^+) mg^{-1}(protein) min^{-1}], and PDH activity [nmol(NADH) mg^{-1}(protein) min^{-1}] in plants subjected to salinity stress (100 mM NaCl) and different concentrations of SNP applied along with 100 mM NaCl. Means ± SE, n = 9. Values followed by the same letters within each row are not different using the Fisher's least-significant differences at 95 %. Levels of significance are represented by * - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$ and NS - not significant.

Parameters	Control	100 mM NaCl	NaCl + 0.25 mM SNP	NaCl + 0.50 mM SNP	NaCl + 1 mM SNP	$LSD_{0.05}$	
Leaf biomass	0.71 ± 0.10 a	0.52 ± 0.07 d	0.66 ± 0.08 b	0.67 ± 0.08 b	0.62 ± 0.07 c	0.02	***
RGR_L	0.09 ± 0.02 a	0.07 ± 0.01 d	0.08 ± 0.03 b	0.09 ± 0.03 b	0.08 ± 0.02 c	0.002	***
Na^+	4.44 ± 0.92 c	23.56 ± 1.92 a	19.68 ± 1.75 b	22.64 ± 2.01 a	24.09 ± 1.86 a	1.63	***
Cl^-	6.83 ± 0.95 b	25.37 ± 2.12 a	24.02 ± 2.05 a	25.46 ± 1.92 a	24.84 ± 2.01 a	1.54	***
K^+	54.07 ± 2.86 a	30.13 ± 2.21 c	34.87 ± 2.38 b	30.74 ± 1.86 c	29.99 ± 2.12 c	2.06	***
K^+/Na^+ ratio	12.19 ± 1.07 a	1.28 ± 0.18 c	1.78 ± 0.21 b	1.36 ± 0.15 c	1.25 ± 0.16 c	0.36	***
MDA	4.50 ± 0.37 d	8.22 ± 0.52 a	6.33 ± 0.49 c	6.18 ± 0.51c	7.48 ± 0.55 b	0.41	***
LOX activity	3.78 ± 0.21 bc	4.62 ± 0.19 a	3.65 ± 0.20 c	3.92 ± 0.17 b	4.75 ± 0.23 a	0.15	***
H_2O_2	13.37 ± 1.24 c	35.08 ± 2.08 a	29.28 ± 1.78 b	30.10 ± 2.04 b	30.33 ± 1.94 b	1.12	***
Proline	0.16 ± 0.05 c	0.98 ± 0.15 a	0.33 ± 0.11 bc	0.54 ± 0.14 b	0.43 ± 0.12 b	0.21	***
P5CS activity	20.58 ± 1.56 a	20.01 ± 1.62 a	22.67 ± 1.49 a	22.16 ± 1.43 a	21.10 ± 1.51 a	2.59	NS
δ-OAT activity	25.19 ± 1.87 a	13.50 ± 1.32 c	18.22 ± 1.47 b	18.26 ± 1.52 b	17.13 ± 1.50 b	2.92	***
PDH activity	5.95 ± 0.96 a	3.01 ± 0.77 c	3.99 ± 0.65 b	4.72 ± 0.82 b	2.13 ± 0.50 b	0.84	***

was measured as the rate of consumption of NADPH monitored by decreased in absorbance at 340 nm. δ-OAT was assayed following Mazelis and Fowden (1976). The reaction mixture contained 0.2 M Tris-HCl buffer (pH 7.8) containing 46.8 mM ornithine, 12.5 mM α-keto-glutarate and 0.125 mM NADH. The decrease in absorbance was recorded at 340 nm after starting the reaction by the addition of the enzyme extract. PDH activity was assayed by the reduction of NAD^+ at 340 nm (Charest and Phan 1990). The reaction mixture contained 0.15 M Na_2CO_3-HCl buffer (pH 10.3) containing 2.67 mM L-proline and 10 mM NAD^+.

For the determination of the total amounts of Na^+ and K^+, dry leaf (0.15 g) was subjected to sulfuric acid digestion in presence of H_2O_2, and diluted with distilled water. Total Na^+ and K^+ foliar contents were directly measured by flame spectrophotometry (Wolf 1982). Cl^- was analyzed after aqueous extraction of 0.15 g of dried and ground leaf material in 10 cm^3 of distilled water. Cl^- content was measured according Diatloff and Rengel (2001).

Analysis of variance (*ANOVA*) was used to assess the significance of treatment means. Differences between treatments were compared using the Fisher's least-significant difference test (LSD) at the 0.05 probability level.

In plants treated with 100 mM NaCl, both leaf biomass production as well as the RGR_L significantly declined (26 and 21 %, respectively, compared to control plants; Table 1). However, the 0.25 and 0.5 mM SNP application together with the 100 mM NaCl increased both parameters with respect to the application of 100 mM NaCl alone (Table 1). On the other hand, the 1 mM dosage of SNP applied together with 100 mM NaCl further reduced both parameters (Table 1). These results support the literature, in which SNP applied at high concentrations proved to be toxic (Beligni and Lamattina 2002, Tian and Lei 2006, Zhang *et al*. 2006, Víteček *et al*. 2007), implying the duality of NO effects, since at low concentrations it can be beneficial but at high concen-trations it can be toxic to plants.

One of the main harmful effects of salinity on plant growth is the excessive accumulation of Na^+ and Cl^- ions in leaves, which upsets nutritional balances due to decreased uptake of essential nutrients such as K^+ (Ashraf and Harris 2004, Juan *et al.* 2005). In agreement with above mentioned data, 100 mM NaCl significantly increased leaf concentrations of Na^+ and Cl^-, and decreased K^+ concentration with respect to control (Table 1). 0.25 mM SNP lowered the Na^+ concentration compared with 100 mM NaCl (Table 1), while at 0.5 mM SNP + 100 mM NaCl and 1 mM SNP + 100 mM NaCl Na^+ concentrations were similar to those at of 100 mM NaCl alone (Table 1). None of the SNP treatments combined with 100 mM NaCl significantly affected leaf Cl^- content (Table 1) and leaf K^+ content was affected only at 0.25 mM SNP (Table 1).

The ratio K^+:Na^+ has been proposed as an indicator of salinity tolerance (Juan *et al.* 2005). This index decreased considerably in the 100 mM NaCl treatment with respect to control (Table 1). However, the dosage of 0.25 mM of SNP applied together with 100 mM NaCl slightly raised this ratio above the value obtained in the plants treated exclusively with 100 mM NaCl (Table 1), while higher SNP dosages did not significantly affect this parameter with respect to 100 mM NaCl (Table 1).

Many studies show that salinity stress provokes lipid peroxidation due to an increased generation of ROS (Demiral and Türkan 2005). This peroxidation can be

quantified in terms of LOX activity and MDA content, as both of these parameters can be used as indicators of oxidative damage (Demiral and Türkan 2005). The 100 mM NaCl increased MDA concentration as well as LOX activity with respect to control (Table 1). All the SNP dosages applied jointly with 100 mM NaCl significantly reduced the MDA content (Table 1), while only 0.25 and 0.5 mM SNP lowered LOX activity (Table 1) while at the treatment 1 mM SNP + 100 mM NaCl the highest LOX activity was registered (Table 1). The 100 mM NaCl treatment significantly raised the leaf H_2O_2 concentration with respect to control (Table 1), and the application of SNP reduced the H_2O_2 content with respect to the plants treated only with NaCl (Table 1).

Proline is a compatible solute that accumulates in great quantities under osmotic stress and participates in osmoregulation and osmoprotection. However, its role in salinity-stress tolerance is not yet clear. Some authors hold that the increase in the proline content under salinity stress is related to stress tolerance (Tripathi *et al*. 2007) while others contend that proline accumulation is more an indicator of stress and that it can therefore not be used as a reliable criterion of salinity tolerance (Lutts *et al.* 1999). The 100 mM NaCl treatment significantly augmented the concentration of proline with respect to control (Table 1). However, all the SNP dosages combined with 100 mM NaCl significantly lower the proline content in comparison with the NaCl treatment alone (Table 1) and the 0.25 mM dosage of SNP was the most effective (Table 1).

In higher plants, proline can be synthesized from glutamate. The first reaction is catalysed by P5CS, which catalyses the transformation of glutamate to glutamic-γ-semialdehyde (GSA), which undergoes spontaneous cycling to Δ^1-pyrroline 5-carboxylate (P5C). The next step involves the enzyme Δ^1-pyrroline-5-carboxylate reductase (P5CR), which reduces P5C to proline. The synthesis pathway from ornithine requires δ-OAT, which catalyses the conversion of ornithine to GSA (Delauney *et al*. 1993). This compound is converted spontaneously into P5C, which follows the glutamate pathway. It has been demonstrated that proline synthesis depends on the availability of its precursor, P5C, given that transgenic tobacco plants with P5CR overexpressed gene did not register any increase in proline content despite undergoing a considerable increase in enzymatic activity (LaRosa *et al*. 1991). Therefore, it has been suggested that the limiting enzymes in the synthesis pathways from glutamate and ornithine are P5CS and δ-OAT, respectively (Kavi Kishor *et al.* 2005). For this reason, we studied the activity of both enzymes.

The P5CS activity showed no significant variations in the 100 mM NaCl treatment with respect to control (Table 1). With regard to the joint treatments of NaCl and SNP, a slight increase in P5CS activity was noted compared with the 100 mM NaCl alone, but this was not significant in any of the SNP dosages applied (Table 1), so that the different SNP treatments did not affect this biosynthetic pathway under salinity stress.

The synthesis pathway for ornithine, represented by the enzymatic activity of δ-OAT, was not visibly stimulated, either, in the 100 mM NaCl treatment, being significantly reduced with respect to the control treatment (Table 1). On the contrary, the activity of this enzyme increased with respect to the NaCl treatment at all the SNP dosages applied (Table 1). These results show that the application of SNP to the 100 mM NaCl treatment could activate the ornithine-synthesis pathway, although the SNP dosage does not appear to affect the activity of this enzyme, as no significant differences were found between the different SNP treatments.

Generally, it is assumed that proline accumulation is the result of increased synthesis together with a reduction in the degradation (Delauney and Verma 1993). For a better understanding of the response of proline metabolism to salinity stress, it was necessary to study the degradation pathway. Proline is oxidized to glutamate in mitochondria through the sequential action of two enzymes, PDH and pyrroline-5-carboxylate dehydrogenase (P5CDH). PDH catalyses the conversion of proline to P5C, and P5CDH the conversion of GSA to glutamate (Kavi Kishor *et al.* 2005).

It is believed that PDH catalyses the limiting step in proline degradation (Hare and Cress 1997). The results showed a significant reduction of PDH activity in the treatment 100 mM NaCl with respect to control (Table 1). On the contrary, the application of 0.25 and 0.5 mM of SNP together with 100 mM NaCl showed a significant rise in the activity of this enzyme compared to the treatment of 100 mM NaCl (Table 1). However, the highest SNP dosage used in our study (1 mM SNP) decreased PDH activity more than the NaCl treatment alone (Table 1).

These results suggest that the increase in proline content noted under 100 mM NaCl was due fundamentally to a depression of PDH activity (Table 1), and not to an increase in the activities of the enzymes responsible for the proline synthesis P5CS and OAT (Table 1). In plants treated with 100 mM NaCl and 0.25 and 0.5 mM SNP lower proline content was due to the stronger PDH activity (Table 1). The proline-degradation seems to be beneficial in the response to stress, given that the degradation of proline to glutamate generates reducing equivalents that support mitochondrial oxidative phosphorylation (Hare and Cress 1997). Furthermore, the PDH activity is capable of consuming O_2 (Hare and Cress 1997), and perhaps could reduce the oxidizing power of the cell and in turn possibly generate ROS (Rosales *et al.* 2007).

Our results indicate that proline accumulation might not be essential in salinity-stress tolerance, given that the treatments with greater proline accumulations did not present better tolerance. On the other hand, NO appears to be capable of mitigating damage associated with salinity stress by reducing oxidative stress and inducing proline degradation, mechanisms that permit the plant to adapt with greater facility under these conditions. In this sense, our data show that the dosage of 0.25 and 0.5 mM

SNP would be the most suitable for counteracting the damage associated with salinity, whereas higher dosages (1 mM SNP) proved less beneficial and even toxic.

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