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Osmotic versus toxic effects of NaCl on pepper plants

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Abstract

Water relations, mineral composition, growth and root morphology were studied in pepper plants (*Capsicum annuum* L. cv California Wonder). Two NaCl concentrations (30 and 60 mM) and two nutrient solutions in which the concentrations of macronutrients were increased were used to assess the ionic and osmotic effects of NaCl in these plants. The hydraulic conductivity (L_o), stomatal conductance (g_s), percentage of open stomata and pressure potential (Ψ_p) decreased with all treatments, in a similar way for 30 mM NaCl and for its iso-osmotic solution of macronutrients, however, the decrease was higher for 60 mM NaCl than for its iso-osmotic solution. Ion analyses also revealed that nutrient concentrations were altered greatly at 60 mM NaCl. Also, changes in morphology, such as increases in cortex cell size and in intercellular spaces, were detected. Therefore, at low salinity, the effect of NaCl was mainly osmotic, however, under higher salinity also the toxicity of Na^+ and Cl^- participate.

Additional key words: anions, *Capsicum annuum*, cations, hydraulic conductivity, morphology, osmotic potential, pressure potential, stomatal conductance, water potential, water uptake.

Introduction

Global scarcity of water resources and the increased salinization of soil and water are having a high impact on agricultural productivity. Crop performance may be adversely affected by salinity as a result of nutritional disorders. These disorders may derive from the effect on nutrient availability, competitive uptake, transport or partitioning within the plant. Although plants in nature have evolved several adaptive mechanisms to cope with the presence of salts in their environment, the understanding of these mechanisms still remains incomplete. The 'osmotic effect' involves limited water absorption due to salinity in the rhizosphere and the 'ionic effect' consists of intracellular toxicity or imbalance due to excess ions (Greenway and Munns 1980, Zhu 2001). Under salt stress, cells must develop a sufficiently low osmotic potential to absorb water, either through the uptake of ions from the medium or by synthesis or transport of organic compounds (Zimmerman 1978, Carvajal *et al.* 1999). Osmotic adjustment is defined as a decrease in the osmotic potential of cell sap in order to avoid the loss of water (Blum *et al*. 1996) and maintain pressure potential (Morgan 1984, Karimi *et al*. 2005). In this process, the antioxidant activities of some osmolytes are also important (Mandhania *et al*. 2006). It has been suggested that the effects of salinity on water uptake and transport are due to the both osmotic stress and ionic imbalance caused by the high apoplastic concentrations of Na^+ and Cl^- (Navarro *et al*. 2000, Cabañero *et al.* 2003, Martínez-Ballesta *et al*. 2000, 2004). The large reductions observed in root hydraulic conductance under salinity could be closely related to the decrease in the amount or activity of aquaporins in the root plasma membrane (Carvajal *et al.* 1999, Martínez-Ballesta *et al*. 2000). It has been proposed that growth suppression may be a non-specific effect of salts, depending more on the total concentration of soluble salts than on specific ions (Maas and Niemann 1978).

Roots take up water and necessary solutes from the soil, and simultaneously avoid the influx of unnecessary solutes into the stele. Under salinity stress, the only organ that is directly exposed to salt is the root. Thus, it is important to understand the developmental and anatomical changes in the barriers that protect the roots against an influx of salt (Cachorro *et al.* 1995, Karahara 2004).

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Abbreviations: g_s - stomatal conductance; L_0 - root hydraulic conductance; Ψ_π - leaf osmotic potential; Ψ_p - pressure potential; Ψ_w - leaf water potential.

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The aim of this work was to study the effect of NaCl at two concentrations and two iso-osmotic nutrient solutions, in which the concentrations of macronutrients had been increased, on pepper (*Capsicum annuum* L.) plants. This arrangement could help to discriminate between the osmotic and specific ionic effects. For this, water relations, osmotic adjustment, root anatomical changes and plant nutrient composition were measured after seven days of treatment.

Materials and methods

Plant culture: The experiments were carried out using 21-d-old pepper seedlings (*Capsicum annuum* L. cv. California). Pepper seeds were imbibed in deionised water for 24 h. After this, seeds were germinated in trays containing *Vermiculite*, in darkness at 28 ºC, inside a germination chamber, for 4 or 5 d. Plants were then placed in 15-dm^3 containers (about 40 plants per container), with a modified Hoagland nutrient solution having macronutrients at half the normal concentration (0.5 H, Table 1), with continued aeration. They were transferred to the environmental chamber, with a 16-h photoperiod and day/night air temperature of 25/20 ºC, and relative humidity 60/80 %. The photosynthetically active radiation (PAR) was 400 μmol m^{-2} s^{-1}, provided by a combination of fluorescent tubes (*Philips TLD 36/83* and *Sylvania F36 W/GRO*) and metal halide lamps (*Osram HQ1*, 400 W). Nutrient solution was changed every 4 d and the pH was checked and adjusted daily. After 21 d, the plants were separated in groups of 10 in containers and subjected to the following treatments (Table 1) for 7 d: control: 0.5 Hoagland nutrient solution (H) (Ψ_π= -0.029 MPa), addition of 30 mM NaCl to 0.5 H (Ψ_π= -0.017 MPa), addition of 60 mM NaCl to 0.5 H (Ψ_π= -0.290 MPa), 3.0 H (Ψ_π= -0.170 MPa), and 5.5 H (Ψ_π= -0.290 MPa).

Table 1. Macronutrient composition of the solutions used in the treatments.

[mM]	0.5 H	3.0 H	5.5 H
KNO_3	7.0	42	77.0
$Ca(NO_3)_2$	3.5	21	38.5
KH_2PO_4	2.0	12	22.0
$MgSO_4$	0.5	3	5.5

Root hydraulic conductance: The hydraulic conductance of pepper roots was measured by natural exudation. The aerial part of the plant was removed, leaving a little part of the stem immediately below the leaves, which was sealed with silicone grease into plastic tubes. After 3 h (depending on whether the treatment was saline or not), the exudate was collected using a Pasteur pipette and transferred to an Eppendorf tube. The sap was weighed and the roots were removed and weighed. Xylem sap (Jv) was expressed as [mg g^{-1}(root f.m.) min^{-1}]. Samples of sap (0.1 cm^3) were measured using an osmometer (*Digital Osmometer, Röebling*, Berlin, Germany). The osmotic potential difference between the xylem sap and the external solution, $\Delta\Psi_\pi$, was calculated. The hydraulic conductance,
L_0 [mg g^{-1}(root f.m.) min^{-1} MPa^{-1}] = $J_v/\Delta\Psi_\pi$.

Stomatal conductance: g_s [mmol m^{-2} s^{-1}] was measured daily on attached two fully-expanded leaves of each plant with a porometer (*AP4*, *Delta-T Devices*, Cambridge, UK), in the middle of the light period.

Leaf water and osmotic potentials: The leaf water potential (Ψ_w) of the most recent fully-expanded leaves was measured using a pressure chamber technique (Turner 1988). The same leaves were then put in Eppendorf tubes with holes at the bottom and rapidly frozen. These tubes were then centrifuged twice, at 4 000 *g* for 4 min (4 ºC), using a *Hettich-Universal 32R* centrifuge, in such a way that all sap was extracted from samples. The osmotic potential (Ψ_π) of the leaf sap was measured with an automatic freezing-point depression osmometer (*Digital Osmometer*). Pressure potential (Ψ_p) was calculated as the difference between leaf water potential and osmotic potential.

Water uptake and plant growth: The water uptake [cm^3 plant^{-1} d^{-1}] was measured gravimetrically during the 7th day after the treatments application, at the middle of the light period. The plant growth was determined by measuring their fresh mass daily.

Ion analysis in leaf and root dry matter: Anions were extracted from 0.05 g of ground material with 10 cm^3 of deionised water. This extract was diluted and injected into a *Dionex-D-100* (Sunnyvale, CA, USA) ion chromatograph, using an *Ionpac AS12A* (Sunnyvale, CA, USA) 4 mm (10-32) column and guard column. The anions were detected with a conductivity detector and quantified by comparing peak areas with those of known standards. For cation analysis, plant material, separated into shoots and roots, was lyophilised for 3 d. Chemical analyses were carried out after digestion with HNO_3-$HClO_4$ (2:1). Sodium, K, Ca and Mg concentrations were determined by atomic-absorption spectrometry (*Perkin-Elmer 5500*, Norwalk, CT, USA).

Anatomical studies: For the observation of cellular size and shape and the area of the intercellular spaces of young roots, thin sections were analysed. Root sections, 0.3 cm long and cut 0.2 mm from the tips, were fixed and imbedded in 5 % glutaraldehyde plus 7 % *p*-form-aldehyde in 0.2 M phosphate buffer, pH 7.2 (Karnowsky

1965). Root pieces were submerged in the fixed solution. After being placed under vacuum for a few minutes, they were maintained at 4 ºC for 10 h. Then, the sections were washed in 0.1 M cacodilate buffer + 8 % sucrose, at 4 ºC for 8 h. After this, the samples were submerged in 1 % osmium tetroxide, at 4 ºC, for 2 - 3 h. Then, they were washed in cacodilate buffer + 8 % sucrose. The fixed samples were dehydrated in a series of ethanol solutions (from 30 to 100 %), being immersed in all of these for 50 min. They were washed two times (30 min), then propylene oxide and Spurr resin, 1:1 and 1:2 (120 min each time), was added, before imbedding in Spurr resin at 100 %. The sections, cut in pieces of 1 μm thickness and were analysed under a *Leica D6330* microscope (Wetzlar, Germany) connected to a computer (*Leica Q-500* computer programme) *via* a video-camera to obtain images.

Data analysis: All data were subjected to analysis of variance and the Tukey test for mean significance, using the *SPSS* software package (*version 12*).

Results

The fresh mass of pepper plants, measured daily, increased at a lower rate at all treatments (Fig. 1) than in control. This decrease was in order 60 mM NaCl > 5.5 H > 30 mM NaCl > 3.0 H. The significant differences started to appear 7 d after treatments commenced. From 6 d the mass of the plants treated with 60 mM NaCl remained constant.

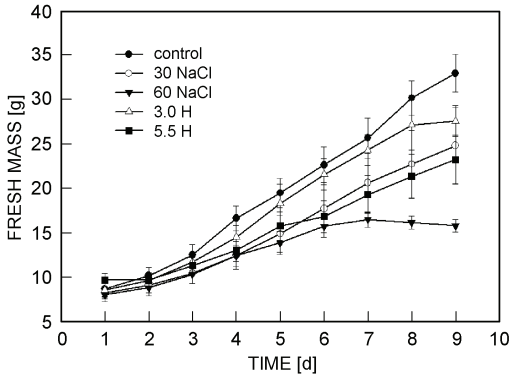

Fig. 1. Fresh mass of pepper plants growing under five treatments, consisting of the control (0.5 H), two salinity levels (30 and 60 mM NaCl) and two corresponding iso-osmotic nutrient solutions, 3.0 H and 5.5 H (n = 5, means ± SE).

The water uptake of pepper plants decreased progressively when salinity was increased (Fig. 2*A*). However, there were no significant differences between the iso-osmotic solutions and 30 mM NaCl. The hydraulic conductance of roots, L_0, (Fig. 2*B*) showed a great reduction in all treated pepper plants. However, the decrease was greatest in those plants treated with 60 mM NaCl, being similar for the rest of the treatments, with no significant differences among them. The stomatal conductance (Fig. 2*C*) was reduced as salinity was increased, especially with the 60 mM NaCl treatment. For the iso-osmotic treatments, plants treated with 3.0 H and 5.5 H showed similar values to 30 mM NaCl.

The leaf water potential (Fig. 3*A*) significantly decreased with 60 mM NaCl. The rest of the treatments did not produce significant differences from the control, although the 30 mM NaCl and 3.0 H treatments showed values similar to 60 mM NaCl. The osmotic potential (Fig. 3*B*) had a significant increase with both iso-osmotic solutions. The two salinity treatments did not show significant differences from the control or from each other. Leaf pressure potential (Fig. 3*C*) was not significantly altered in plants grown under salinity or treated with iso-osmotic solutions, although the increase was lower with salinity treatments.

The cation concentrations in the shoot dry matter were analysed (Fig. 4). As expected, the sodium concentration in the shoot significantly increased with the

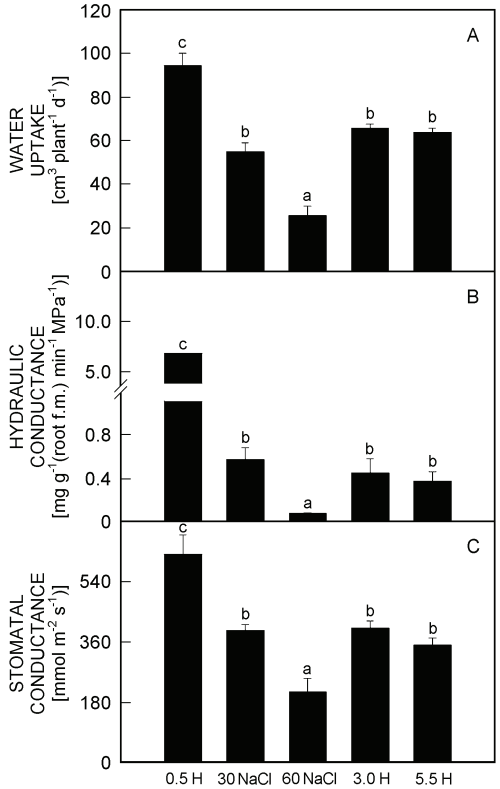

Fig. 2. Water uptake (*A*), root hydraulic conductance (*B*), and stomatal conductance (*C*) of pepper plants under five treatments, consisting of the control (0.5 H), 30 and 60 mM NaCl and corresponding iso-osmotic nutrient solutions, 3.0 H and 5.5 H, measured at the 7th day of the treatments (n = 5, means ± SE).

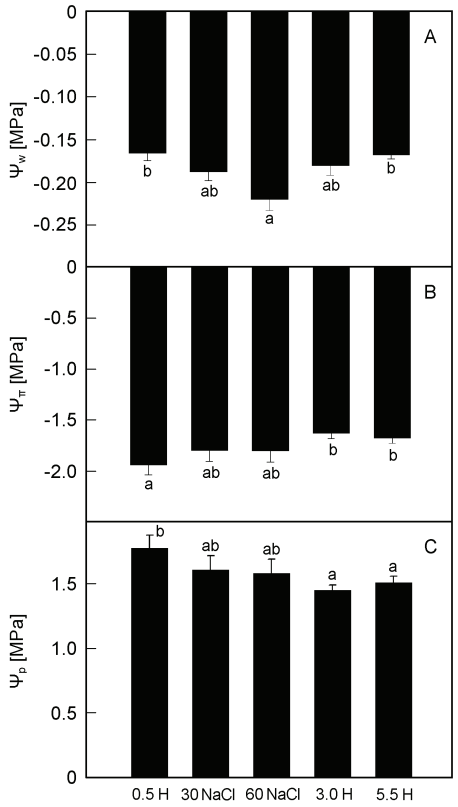

Fig. 3. Water potential, Ψ_w (*A*), osmotic potential, Ψ_π (*B*) and pressure potential, Ψ_p (*C*) of leaves from pepper plants grown under five treatments, consisting of the control (0.5 H), two salinity levels (30 and 60 mM NaCl) and two corresponding iso-osmotic nutrient solutions, 3.0 H and 5.5 H (n = 5, means ± SE).

salinity treatments, but it was unchanged at 3.0 H and 5.5 H. The K^+, Ca^{2+} and Mg^{2+} concentrations decreased with the NaCl treatments, while there were no significant differences for the iso-osmotic solutions with regard to the control. The root (Fig. 4) showed a significant and progressive increase of Na^+ as the salinity level was increased. The root concentrations of K^+, Ca^{2+} and Mg^{2+} were increased for the treatments in which the concentrations of macronutrients were increased. However, while for the salinity treatment the concentrations of K^+ and Ca^{2+} were similar to the control, the Mg^{2+} concentration increased with respect to the control.

Considering the anions (Fig. 5), there was a significant increase in shoot and root Cl^- as salinity increased in the nutrient solution. However, there were no significant differences between the treatments in which the macronutrient concentrations were increased and the control. Nitrate was increased significantly in treatment 5.5 H for shoots and in 5.5 H and 3.0 H for roots. There were no changes for the salinity treatments except a decrease at 60 mM NaCl. The phosphate and SO_4^{2-} concentrations in shoots and SO_4^{2-} in roots did not change with respect to the control. However, root PO_4^{3-} showed a significant decrease with 60 mM NaCl and an increase with 5.5 H.

The intercellular spaces (Fig. 6*A*) increased significantly at all treatments, the values obtained for 5.5 H being double those of the control. Also, the area of cortical cells (Fig. 6*B*), increased at all the treatments, being similar for 30 mM NaCl and 3.0 H, higher for 60 mM NaCl and, again, doubled the control values for 5.5 H.

Discussion

Increased osmolarity of the nutrient solutions resulted in a slower plant growth and in ionic composition. Taking into account that pepper is considered as moderately sensitive to salt stress (Rhoades *et al.* 1992), the growth was highly delayed with 60 mM NaCl (Fig. 1), but its iso-osmotic macronutrient solution, 5.5 H, reduced growth to a lesser extent. This suggests that the reduced growth observed with 60 mM NaCl was influenced by ion toxicity in addition to an osmotic effect. However, at low salinity, the effects can be attributed only to an osmotic effect since the growth was similar after the 8th day for 30 mM NaCl and 3.0 H. This toxic effect of NaCl has been reported previously to be due to an accumulation of Na^+ and Cl^- ions (Munns *et al.* 2002, 2005). This effect, noticeable after 8 d of treatment, also points out the time-effect of these treatments.

A great and significant reduction of L_0 was observed with all treatments (Fig. 2*B*). In a previous report (Martínez-Ballesta *et al.* 2000), it has been suggested that negative effects of NaCl on L_0 are due to both osmotic stress and an ionic imbalance caused by the high apoplastic concentrations of Na^+ and Cl^-. These effects could have caused the decrease of L_0 in our salt-treated plants, and decreases in L_0 with the iso-osmotic (high-osmolarity) macronutrient solutions might have been due to osmotic effects. Martínez-Ballesta *et al*. (2004) pointed out that NaCl could influence negatively the function of aquaporins and so reduce L_0 values, since these water channels could be strongly reduced in number or, if present, could be non-functional in salt-stressed pepper plants. Aquaporins mediate the mass flow of water within the plant and the abundance of the proteins is a critical parameter with respect to understanding their function at the tissue, cell or subcellular level. Reduced water uptake under salinity may prevent a mass flow of salt towards the shoots (Luu and Maurel 2005). This might be a mechanism useful in the protection of pepper shoots from salt injury. However, the fact that the treatments in which the concentrations of macronutrients were increased also reduced L_0 has to be addressed, since in a previous report no effect was observed after a shorter time (Carvajal *et al*. 1999).

In this work, g_s decreased at the 7th day with all treatments, but the lowest value was reported with

60 mM NaCl. Partial stomatal closure (by 50 %, Fig. 4) occurred in pepper plants with high salinity, as reported previously (Bethke and Drew 1992, Cabañero *et al.* 2003). This response could have been due to a direct inhibition of stomatal opening by an elevated apoplastic Na^+ concentration (Parera *et al*. 1994). Other authors have related reductions in photosynthetic capacity and stomatal conductance with high concentrations of Cl^- (Bañuls *et al*. 1997, García-Sánchez *et al.* 2002). It has been reported that salinity and osmotic stress produced a rapid plant response, consisting of stomatal closure to reduce water loss from plants (Luan 2002). However,

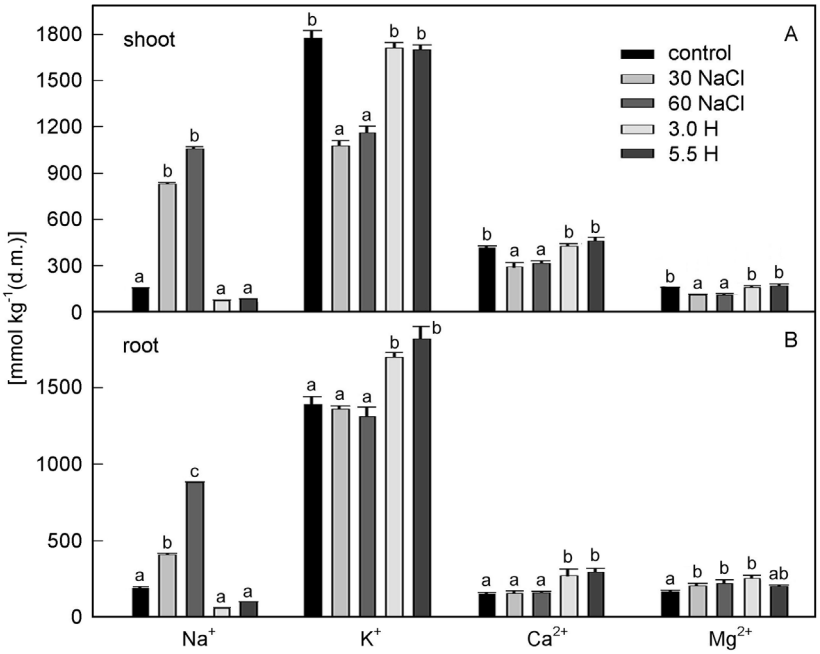

Fig. 4. Concentrations of cations in the shoot (*A*) and roots (*B*) of pepper plants growing under five treatments, consisting of the control (0.5 H), two salinity levels (30 and 60 mM NaCl) and two corresponding iso-osmotic nutrient solutions, 3.0 H and 5.5 H, at the seventh day under the treatments (n = 5, means ± SE).

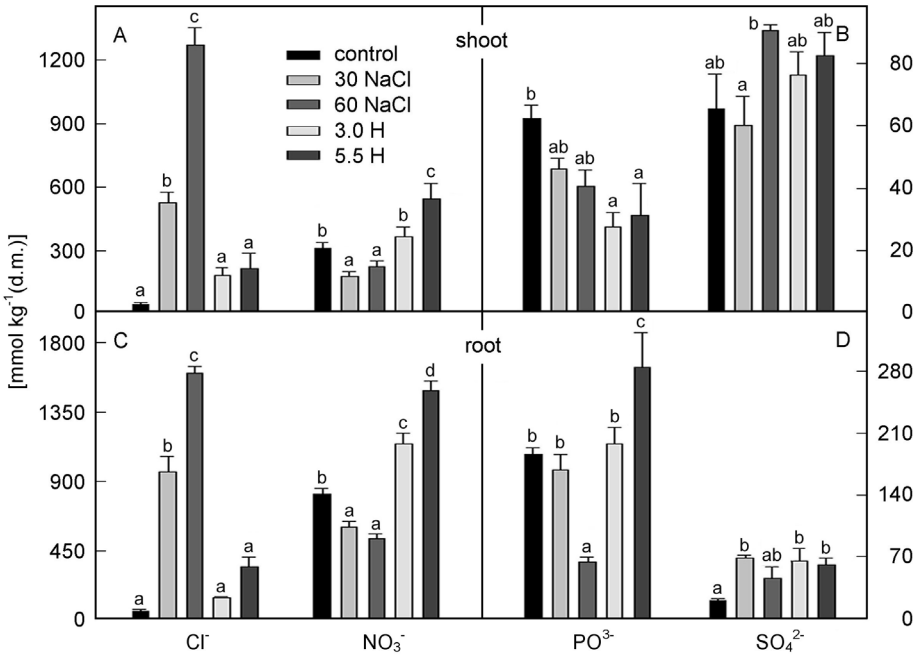

Fig. 5. Concentrations of anions in the shoot (*A*,*B*) and roots (*C*,*D*) of pepper plants growing under five treatments, consisting of the control (0.5 H), two salinity levels (30 and 60 mM NaCl) and two corresponding iso-osmotic nutrient solutions, 3.0 H and 5.5 H, at the seventh day under the treatments (n = 5, means ± SE).

g_s could be decreased as a result of changes in L_0 (Carvajal *et al.* 1996, Navarro *et al*. 2003). In the same way, it has been hypothesised that lowered water potentials in roots could trigger a signal from root to shoot to reduce g_s (Zhang and Davies 1991).

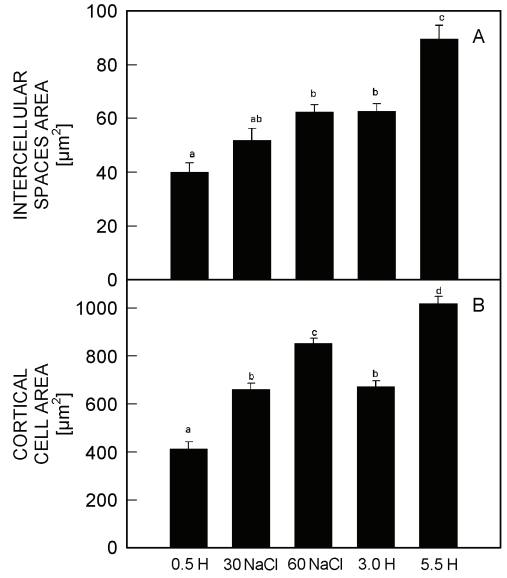

Fig. 6. Intercellular spaces between root cortical cells (*A*) and area of root cortical cells (*B*) of pepper plants growing under five treatments, consisting of the control (0.5 H), two salinity levels (30 and 60 mM NaCl) and two corresponding iso-osmotic nutrient solutions, 3.0 H and 5.5 H, at the seventh day of the treatments (n = 5, means ± SE).

Osmotic adjustment involves the net accumulation of solutes in cells in response to a fall in the water potential of their environment. As a consequence of this net accumulation, the cell osmotic potential is lowered, and pressure potential tends to be maintained (Blum *et al*. 1996). In our experiment, the NaCl and high concentration of macronutrients decreased markedly the leaf water potential and this change was not compensated by a reduction in leaf osmotic potential (Fig. 3*A*), the values were slightly higher compared to control plants. Thus, Ψ_p was not maintained and osmotic adjustment was not sufficient to offset the reduction in leaf water potential in stressed plants. The changes observed in all the water relations in the plants have been seen previously in pepper plants (Navarro *et al*. 2003, Martínez-Ballesta *et al*. 2004) and concluding that these plants are unable to adjust osmotically. In any case, the rate of acclimation to salinity and the duration of the salt treatment are clearly key factors influencing the plant response, but plant age and size are also likely to be involved (Franco *et al*. 1993, De Pascale *et al*. 2003).

Under the NaCl treatments, an increase in the Na^+ and Cl^- contents in the roots and shoot of salinized plants was found (Fig. 4). Cl^- increased significantly in tissues with NaCl treatment, but the concentration of Na^+ was lower than that of Cl^-. These results are consistent with Navarro *et al.* (2003) and Blom-Zandstra *et al.* (1998). The later, described the presence of Na^+ re-circulation system in pepper, which protects the photosynthetic tissue from the toxic effects of Na^+. Salinity had also a significant effect on the shoot concentrations of K^+, Ca^{2+} and Mg^{2+}, which decreased as the concentration of Na^+ increased. This has been reported widely and described as a mechanism of competition between cations (Bayuelo-Jiménez *et al.* 2003, Kaya *et al.* 2003b, Navarro *et al.* 2000). In addition, although it has been reported that a luxury consumption of macronutrients and its antagonist effect should be taken into account when concentrated nutrient solution are used (Morard *et al*. 2004), in our experiments, when 3 H and 5.5 H were applied, no alteration of the cation concentrations was observed, supporting the hypothesis that when the ratio of the different cations in the nutrient solution is maintained, no toxic effect or imbalance is observed (Carvajal *et al*. 2000).

A significant decrease in the NO_3^- content (Fig. 5*A*, 5*C*) in the shoot and root of salt-stressed plants was observed. Botella *et al*. (1994) described that NO_3^- uptake is reduced by salinity and Kaya *et al.* (2003a) found lower leaf nitrogen concentrations with NaCl treatments. The fact that the concentration in roots was higher than in leaves could be due to the assimilation processes (Flores *et al*. 2004). The increased contents of NO_3^- with the 5.5 H treatment could be due to the high content of NO_3^- in that nutrient solution. The exact mechanism by which NaCl influences P uptake is unknown, but Navarro *et al*. (2001) hypothesised that high concentrations of NaCl decrease the mobility of P stored in the vacuole and inhibit export from this storage compartment to other parts of the plant, which is in agreement with our results in roots, since the lowest concentration was found at 60 mM NaCl.

Direct exposure of the root cortex to NaCl led to several structural changes in cells (Koyro 1997). In pepper, significant increases in cortical cell size and in intercellular spaces, as a consequence of the salt stress, have been found. This has been described as a typical response to salinity (Molassiotis *et al.* 2006). Also, the stressed plants showed irregular cell shapes, which may be related to the decrease in pressure potential. However, decrease in pressure potential also occurred in the plants treated with high concentrations of macronutrients, but these cortical cells were round in shape. Smaller intercellular spaces have been related to NaCl-resistance in plants, to avoid toxic ions passing through the cortex to the stele (Cachorro *et al.* 1995). Therefore, the fact that the intercellular spaces in our plants were bigger could be related to the sensitivity to salinity stress (García-Sanchez *et al*. 2000). Also, the fact that the intercellular spaces were increased significantly in treatments 3.0 H and 5.5 H could indicate the need of the plants to transport such high amounts of nutrients directly to the xylem. Bahaji *et al.* (2002) described, for rice, that the epidermis as well as the two exodermal layers were affected by both saline and osmotic stress; these three cell layers appeared disorganised and with bigger cells.

In conclusion, pepper plants, moderately sensitive to salt stress, showed a different response depending on the salinity level. At low salinity, the effect can be compared to that observed with the iso-osmotic solution, indicating that mainly an osmotic effect occurs. However, the fact that the alteration in all parameters was higher for 60 mM NaCl than for its iso-osmotic solution leads us to affirm that, at high salinity levels, the effect is due to the toxicity of Na^+ and Cl^-. This fact complicates the picture concerning the regulation of the different processes during the response of plants to stresses.

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Advances in technology have brought about dramatic advances in DNA sequencing. After large-scale sequencing of first model plant *Arabidopsis thaliana,* the second generation models such as rice, *Medicago*, *Lotus* and poplar have been promoted. The need for improvement in all crops is really desired and the huge volume of information flowing from the model sequencing projects should be closer associated with crop biology and plant breeding. This book documents achievements, pitfalls and prospects of model plant research in the past to decades.

The editors and authors are authorities in particular fields included in this book. The book is divided into 11 chapters. The editors summarize progress in plant genomics during last decades in first chapter. Five chapters give comprehensive overview of current knowledge status for model plant genomes. Rice, *Brassica* and *Arabidopsis*, *Medicago trunculata* and *Brachypodium distachyon* are described from its origin, through breeding, genetic and physical mapping to utilization for crop improvement. Dormancy and germination of plants are discussed in Chapter 2. Model of green alga *Chlamydomonas* serve as a tool for nitrate assimilation pathway studies in Chapter 7. Plant defense responses and defence signalling are theme of Chapter 8 and Chapter 9. The Chapter 10 deals with identification of heat-shock factor regulated genes. Last chapter is aimed at low temperature tolerance in plants.

Text is supplemented by tables and figures. Each chapter is concluded with a comprehensive list of references. Carefully done subject index saves readers time. Those features, together with newest information presented on the subject of each chapter, will make this book useful reading material for advanced students as well as researchers who have a keen interest in the recent progress in plant genomics.

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