Changes in tomato leaves induced by NaCl stress: leaf organization and cell ultrastructure

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Abstract

The alterations of organization of leaf tissues and cell ultrastructure as a consequence of salt stress (75 and 150 mM NaCl) were studied in two tomato (*Lycopersicum esculentum* Mill.) cultivars showing different salinity tolerance. The salinity brought changes in cell shape, volume of intercellular spaces and chloroplast number, shape and size. These characteristics were specific in each cultivar. The ultrastructural changes were also different in the two tomato cultivars studied and the most important ones were in the number and size of starch granules in chloroplasts, the number of electron-dense corpuscules in the cytoplasm, the structure of mitochondria, and number of plastoglobuli.

Additional key words: chloroplasts, *Lycopersicum esculentum*, mitochondria, plastoglobuli.

Introduction

The plants as all living organisms, possess an adaptation capacity, according to the environment, in which they are developed. This capacity is variable and is not showed until the plant is submitted to a determined stress, *e.g.* saline stress. Salt stress involves both osmotic stress and ionic toxicity. Salt stress causes alterations in plant cell structure and function (McKersie and Leshem 1994, Rusch *et al.* 1996, Mansour *et al.* 2000, Hernández *et al.* 2001, 2002). It has long been recognised that salt tolerance is a multigenic trait. It requires at least capacity for osmotic adjustment by synthesis of compatible solutes (osmoprotectants) to retain the water potential of the cytosol, and for effective exclusion of salt or for its intracellular compartmentation.

The saline excess in the soil inhibits crop growth and yield due to different reasons and is shown in many aspects of the plants. Many salt-tolerant plants exhibit morphological adaptations; for example, specialised bladder cells for accumulation of salt or glands for salt excretion and so on.

In spite that tomato is a crop largely distributed in many world regions, the anatomical and ultrastructural studies on .tomato related to abiotic stresses are limited (Guerrier *et al.* 1998, Mäkelä *et al.* 2000, Sam *et al.* 2000, 2001). The aim of this study was to analyse the alterations in the tissue organization and cell ultrastructure in young leaves of two tomato cultivars as a consequence of NaCl stress.

Materials and methods

Plant material and treatments: Tomato (*Lycopersicon esculentum* Mill.) cv. Cambell-28 (C-28) and cv. Mariela, seeds were sown in pots containing turf/perlite, (3:1 v/v) in greenhouse (day/night temperature 24/18 °C, relative humidity 80 - 85 %, maximum photosynthetically active radiation, PAR, 1600 μmol m^{-2} s^{-1}, 12-h photoperiod).

Twenty days after germination the treatments were given to plants in both cultivars for 10 d. Ten pots,

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each with a plant of each cultivar were arranged in a plastic tray with a level of water to cover the roots only, which was the control treatment; the other two treatments were equally imposed, but with 75 or 150 mM NaCl solutions in trays.

Selection of samples and processing for light and electron microscopy: Young leaves were excised from the control and treated plants, small portions without the central vascular tissues were cut and processed for electron microscopy. Samples were taken from 3 control plants and 3 plants of each treatment (75 and 150 mM NaCl), and 3 different leaves were excised from each plant. Leaf samples were fixed in glutaraldehyde/ osmium, dehydrated and embedded in Spurr resin for transmission electron microscopy, according to Sam *et al.* (2001).

Semithin sections of 2 μm were cut with a glass knife in a *LKB Nova* (Uppsala, Sweden) ultramicrotome, mounted on glass slides and stained with toluidine blue. The sections were photographed in a *Zeiss* (Jena, Germany) photo-microscope.

Protein electrophoresis: Electrophoresis was realized as had previously been described (Testillano et al. 1993). The tissue homogenisation was performed in a cracking buffer (20 mM Tris, pH 8.0, 2% sodium dodecyl sulphate (SDS), 1 % β-mercaptoethanol, 1 mM phenylmethyl-sulphonylfluoride (PMSF)). The homogenate was heated at 100 °C and, after cooling to room temperature, centrifuged for 15 min at 8 160 *g*. The supernatants were collected and stored at -20 °C. Protein content was measured by the dye-binding method of Bradford (1976). The samples and standards were separated by electrophoresis on 12 % SDS-PAGE gels in a *BIO-RAD* (Hercules, CA, USA) system. The resultant gels were stained with Coomassie blue. Images from gels were captured using a *Hewlett-Packard ScanJet 6200C* flat-bed scanner.

Results

In dorsiventral tomato leaf of the control plants a similar organization was observed in both cultivars, C-28 and Mariela (Fig. 1*a-d*). There were 1 to 2 palisade and 3 to 4 spongy parenchyma layers with large intercellular spaces; in the boundary zone of these parenchyma, dispersed silex granules were observed (Fig. 1*c*). In both cultivars, cells of the mesophyl parenchyma (Fig. 1*b,d*) exhibited numerous chloroplasts containing abundant starch granules.

The structure of the leaves changed after treatment with NaCl in both cultivars studied. The changes observed in the mesophyl were similar after the treatment with 75 mM (Fig. 1*e-h*) and 150 mM NaCl (data not shown). The leaf sections of plants treated with 75 mM NaCl of C-28 (Fig. 1*e,f*) and Mariela (Fig. 1*g,h*) showed larger intercellular spaces in the palisade parenchyma, specially in the case of Mariela leaves (Fig. 1*g*). Palisade cells were thicker and shorter than those in control plants; however, in the spongy parenchyma, the differences were not evident.

In control plant leaves of C-28 (Fig. 2*a*) and Mariela (Fig. 2*b*), the ultrastructure of parenchymatic cells showed chloroplasts with large starch granules, some plastoglobules and well developed intergranal thylakoids and grana; the nucleus (N) was elongated and exhibited a conspicuous nucleolus, and some dense peripheral chromatin masses (Fig. 2*a*).

The shape and size of chloroplasts also changed after the NaCl treatments, the modifications being different in the two cultivars. In parenchymatic cells of C-28 treated plants, chloroplasts were bigger and almost spherical, in some cases, with large quantity of starch granules and plastoglobules; these cells also displayed cytoplasmic regions as peripheral thin layers without chloroplasts (Figs. 1*f*, 2*c*). The chloroplasts of Mariela plants treated with 75 mM NaCl (Fig. 1*h*) showed great differences from the control plants, they were smaller with a flat shape with evident plastoglobules and contained less starch granules (Fig. 1*h*). The cells also exhibited large vacuoles and electron-dense corpuscles in the cytoplasm of parenchyma cells (Fig. 2*d*). The electron dense corpuscles had a homogeneous density and lacked membranes, being their size between 200 and 600 nm; they were not associated to chloroplasts and appeared free in cytoplasmic regions where these organelles were not observed (Fig. 2*f*).

Some changes were specifically observed in the cells of Mariela but not in C-28. Parenchymatic cells from plants treated with 75 mM and 150 mM NaCl contained rounded and elongated mitochondria of irregular shape with membrane invaginations and cytoplasmic digitations (Fig. 2*e*) which had not been observed in cells of control plants.

The polyacrylamide electrophoresis provided the general profile of total proteins in leaves of each tomato cultivar, being very similar between both cultivars and showing the most abundant bands below 60 kDa in both cultivars studied (Fig. 3). After the two NaCl treatments, a general increase in content of proteins of different molecular masses was observed (Fig. 3).

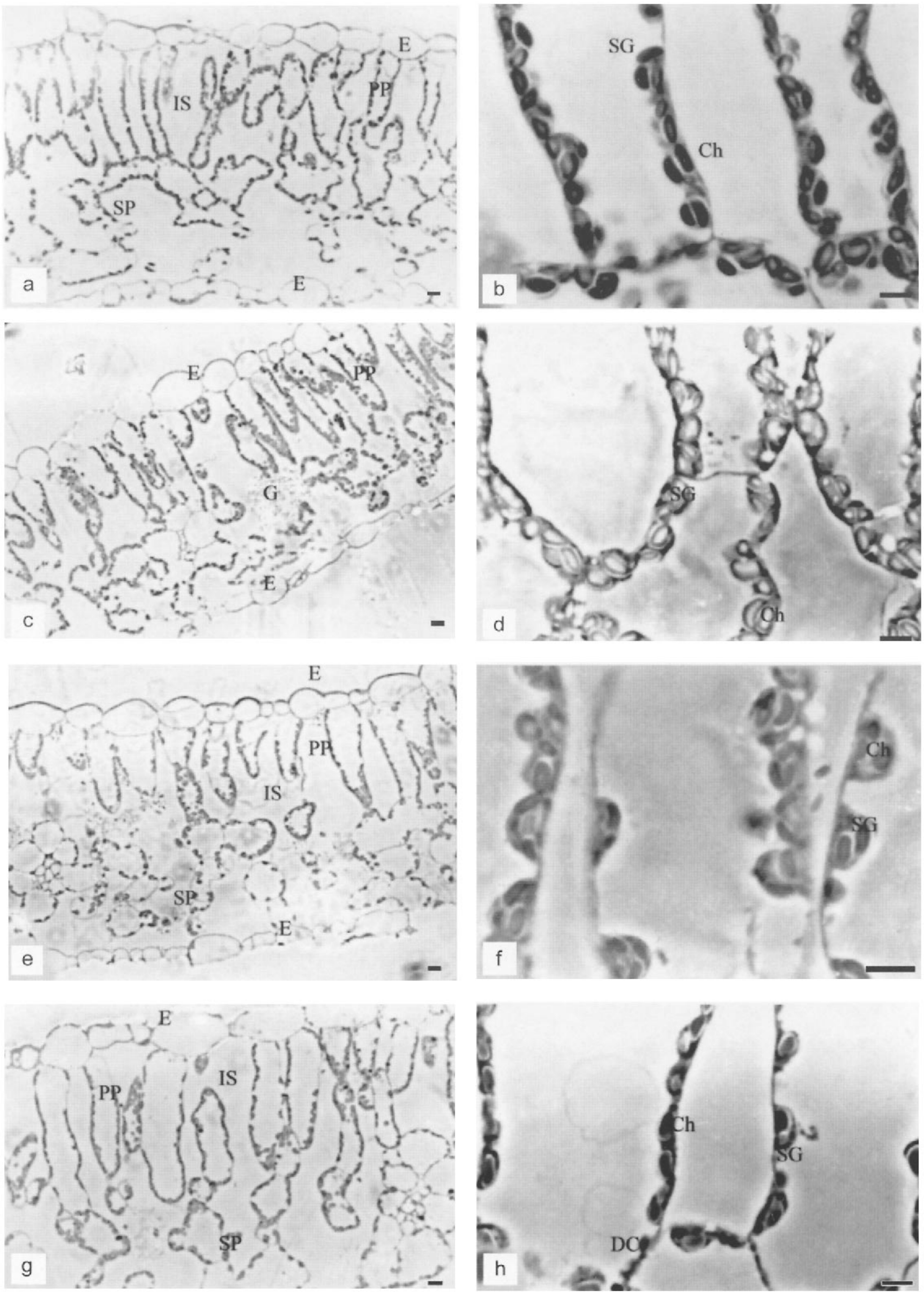

Fig. 1. Leaf cellular organization in transversal sections of control and salt-treated plants of tomato cvs. C-28 and Mariela. Control leaves of C-28 (*a*, *b*) and Mariela (*c*, *d*). Typical mesophyll cells with a palisade parenchyma (PP; 1 - 2 layers) and a spongy parenchyma (SP; 3 - 4 layers), with intercellular spaces (IS) among them, and sylex granules (G). Leaves from 75 mM NaCl treated plants of C-28 (*e*, *f*) and Mariela (*g*, *h*). After the treatment, intercellular spaces (IS) are larger and more numerous. In C-28, chloroplasts are almost spherical and decrease in number (*f*), whereas in Mariela they are flat (*h*). E - epidermis, Ch - chloroplasts, SG - starch granules. *Bars* represent 10 μm.

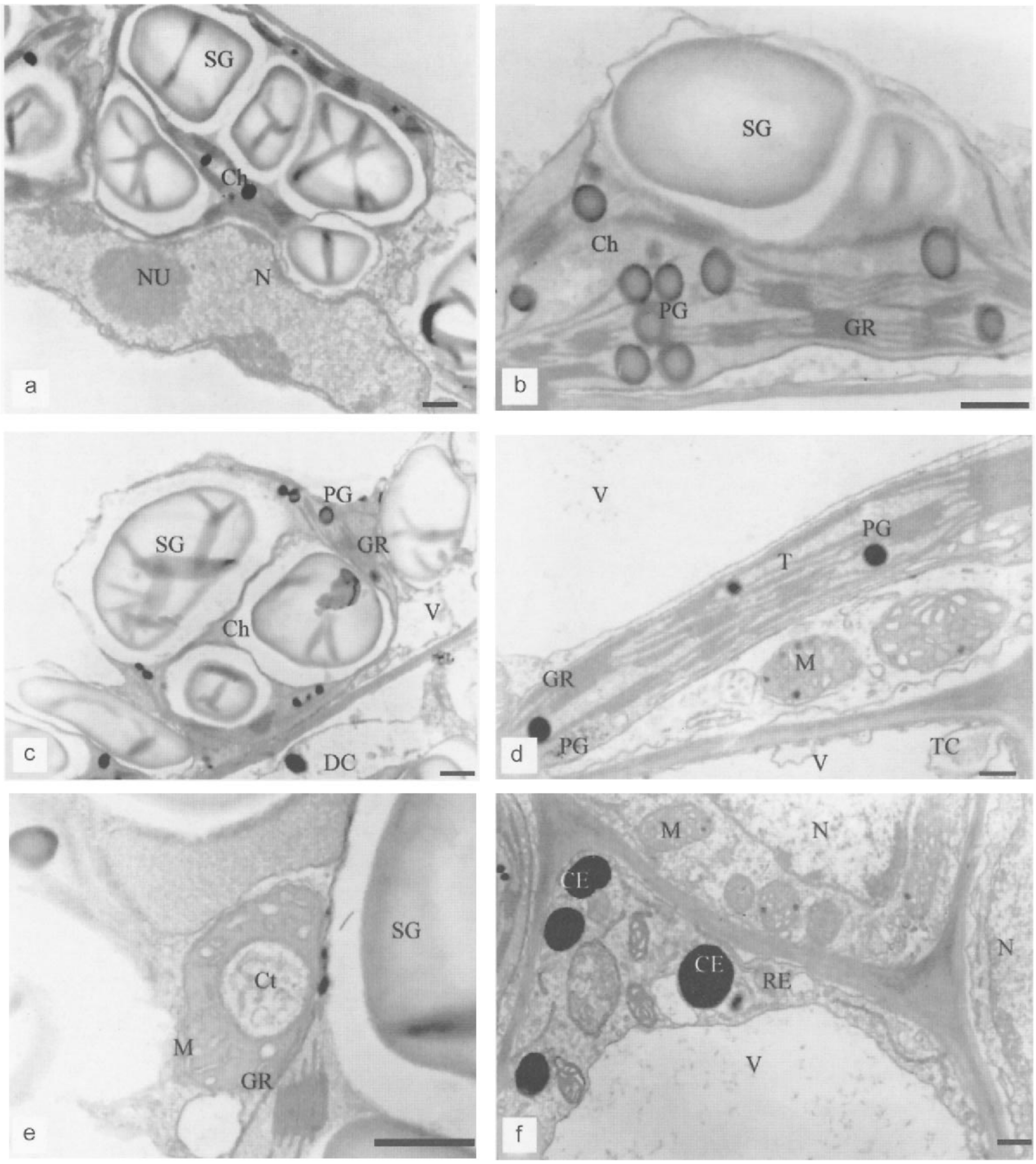

Fig. 2. Ultrastructure of leaf parenchymatic cells of control and salt-treated plants of tomato cvs. C-28 and Mariela. Leaf cell of control plant of C-28 (*a*) and Mariela (*b*) contain large chloroplasts with many starch granules (SG) and well developed intergranal thylakoids and grana (GR). The nucleus (N) is elongated, with a dense nucleolus (NU). In Mariela many plastoglobules (PG) are observed. Leaf cells of plants treated with 75 mM NaCl of C-28 (*c*) show bulky chloroplasts with large starch granules and some electron-dense corpuscles (DC) in the cytoplasm. In NaCl-treated Mariela plants chloroplasts appear flat with several plastoglobules (PG) and without starch granules; cells also show several mitochondria (M) with electron dense corpuscules at their interior, and large vacuoles (V) (*d*, *e*). Some mitochondria (M) show cytoplasmic digitations (Ct) and various electron dense corpuscles (DC) are observed in the cytoplasm (*f*). ER - endoplasmic reticulum, TC - transfer cell. *Bars*: *a*, *c*: 1 μm; *b*, *d*, *e*, *f*: 0.5 μm.

Discussion

In this work, changes in the structural organization of young leaves, induced by NaCl treatments are reported in two tomato cultivars in comparison with non-treated plants. Different effects of salinity in the structure of leaf cells and other plant tissues have been reported in several plant species (Babber *et al.* 2000, Dekov *et al.* 2000, Mäkelä *et al.* 2000, Muthukumurasamy *et al.* 2000).

Various modifications in the shape and size of mesophyll cells have been reported after salt stress. Voronin *et al.* (1995) reported that in some C_4 plant species, an increase in the volume of parenchymatic cells due to salinity was observed. In plants of *Arabidopsis thaliana,* Burssens *et al.* (2000) saw in cotyledons stressed with 1 % NaCl, a swelling of the palisade and spongy parenchyma cells, and in the leaves formed during the stress, the epidermal, spongy and palisade cells were radially swollen. Allakhverdiev *et al.* (2000) said that salt stress also decreased cytoplasmic volume in *Synechococcus* sp., and suggested that NaCl had both, osmotic and ionic effects, which decreased the amount of water in the cytosol, and rapidly increased the concentration of salts in cytosol. In spinach leaves exposed during 20 d to salt stress the anatomical changes were the decrease in palisade and spongy parenchyma cells and total mesophyll thickness and intercellular space (Delfine *et al.* 1998); however, in cucumber plants the salt stressed leaves had shorter palisade cells, but the spongy mesophyll was thicker, and had less air spaces than in the control plants (Lechno *et al.* 1997).

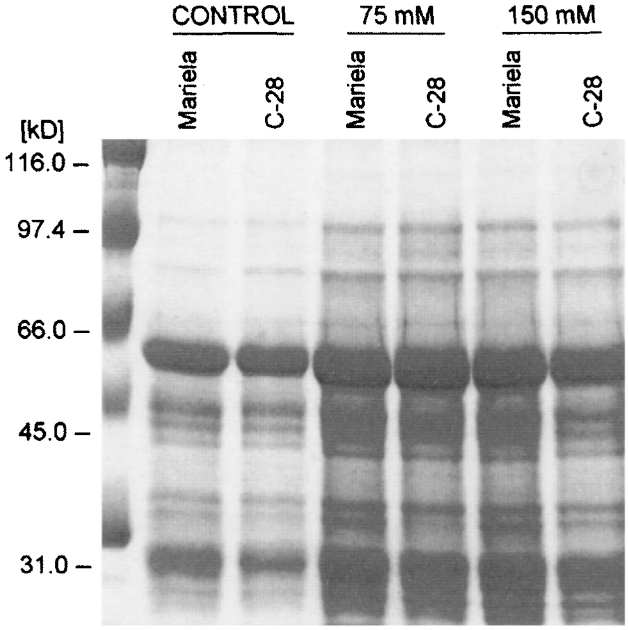

Fig. 3. SDS-PAGE gels stained with Coomasie blue for total proteins visualisation. First lane corresponds to molecular mass standards representing from top to bottom: 116, 97.4, 66, 45, and 31 kDa. The intensity of the different bands in the samples of both cultivars after NaCl treatment was higher than in the control samples.

In the results shown here, in tomato, the changes observed in the leaf tissues after the application of NaCl treatments also affected the organization of the palisade and spongy mesophyll, increasing the intercellular spaces and changing the cell shape, specially in Mariela plants. These results support the effect of NaCl treatments on the structural organization of leaves and illustrate the specific changes produced in two tomato cultivars, C-28 and Mariela, for the first time.

Our results in tomato reveal changes in the presence of plastoglules and electron-dense corpuscles, as well as invaginations of the mitochondria membranes, associated with salt stress. Concerning the plastoglobules, Pääkkönen *et al.* (1998) have said that drought increased plastoglobule size and reduced the proportion of thylakoids with respect to control treatment. On the other hand, due to salinity, electron-dense corpuscles could be also seen in the mitochondria, where swollen crests were observed. The plasmalemma and other cellular membranes are reported to be affected by salt and drought stress in various plant species (Dekov *et al.* 2000). These stresses produced in some cases plasmatic membrane invaginations (Serraj *et al.* 1995, Niu *et al.* 1996). Our results indicated modifications in the shape of mitochondria, some of which presented membrane invaginations.

The cellular responses to salt stress are very complex and include the induced expression of new genes and proteins (Gaxiola *et al.* 1992, El-Aref and Hamada 1998, Gil-Mascarell *et al.* 1999, Hassanein 1999). Concerning the protein content, our results indicated some changes in the protein content of leaves of salt-treated plants in comparison with control plants (an increase in the intensity of numerous bands). This fact suggested that new protein synthesis could occur as a response to the salt stress. Further studies will be needed to identify the putative new salt-induced proteins.

The growing of different plant species under salinity is genetically controlled (Gong *et al.* 2001), and salt tolerance relationship between genotypes may change with time (Cramer *et al.* 1994). The two cultivars analyzed in this work displayed some differences in the structural changes accompanying salt-treatment, and possibly this could be related to salt tolerance. Our results indicate that NaCl stress provoked changes in tissues and cells, and these changes were not equal in both cultivars, until the NaCl concentration used in this study. Some of them, like the increase in cytoplasmic electron-dense corpuscles and changes of chloroplast shape, were more intense in Mariela plants. These changes could be related to cultivar tolerance since Mariela grew better under stressed conditions such as high temperatures and saline soil (personal comunication of Mariela author). Further studies on the structural changes induced by salt stress in other tomato cultivars will shed ligth to the question whether some of them would be taken as tolerance salinity indicators.

References

Allakhverdiev, S.I., Sakamoto, A., Nishiyama, Y., Inaba, M., Murata, N.: Ionic and osmotic effects of NaCl-induced inactivation of photosystems I and II in *Synechococcus* sp. - Plant Physiol. **123**: 1047-1056, 2000.

Babber, S., Sheokand, S., Malik, S.: Nodule structure and functioning in chickpea *(Cicer arietinum)* as affected by salt stress. - Biol. Plant. **43**: 269-273, 2000.

Bradford, M.M.: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem **72**: 248-254, 1976.

Burssens, S., Himanen, K., Van de Cotte, B., Beeckman, T., Van Montagu, M., Inze, D., Verbruggen, N.: Expression of cell cycle regulatory genes and morphological alterations in response to salt stress in *Arabidopsis thaliana*. - Planta **211**: 632-640, 2000.

Cramer, G.R., Alberico, G.J., Schmidt, C.: Leaf expression limits dry matter accumulation of salt-stressed maize. - Amer. J. Bot. **21**: 663-674, 1994.

Dekov, I., Tsonev, T., Yordanov, I.: Effects of water stress and high-temperature stress on the structure and activity of photosynthetic apparatus of *Zea mays* and *Helianthus annuus*. - Photosynthetica **38**: 361-366, 2000.

Delfine, S., Alvino, A., Zacchini, M., Loreto, F.: Consequence of salt stress on conductance to CO_2 diffusion, Rubisco characteristics and anatomy of spinach leaves. - Aust. J. Plant Physiol. **25**: 395-402, 1998.

El-Aref, H.M., Hamada, A.M.: Genotypic differences and alterations of protein patterns of tomato explants under copper stress. - Biol. Plant. **44**: 555-564, 1998.

Gaxiola, R., De Larrinoa, I.F., Villalba, J.M., Serrano, R.: A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. - EMBO J. **11**: 3157-3164, 1992.

Gil-Mascarell, R., López-Coronado, J.M., Bellés, J.M., Serrano, R., Rodríguez, P.L.: The *Arabidopsis HAL2*-like gene family includes a novel sodium-sensitive phosphatase. - Plant J. **17**: 373-383, 1999.

Gong, Z., Koiwa, H., Cushman, M.A., Ray, A., Bufford, D., Korzeda, S., Matsumoto, T.K., Zhu, J., Cushman, J.C., Bressan, R.A., Hasegawa, P.M.: Genes that are uniquely stress regulated in salt overly sensitive (SOS) mutants. - Plant Physiol. **126**: 363-375, 2001.

Guerrier, G.: Proline accumulation in leaves of NaCl-sensitive and NaCl-tolerant tomatoes. - Biol. Plant. **40**: 623-628, 1998.

Hassanein, A.M.: Alterations in protein and esterase patterns of peanut in response to salinity stress. - Biol. Plant. **42**: 241-248, 1999.

Hernández, J.A., Almanza, M.S.: Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves. - Physiol. Plant. **115**: 251-257, 2002.

Hernández, J.A., Ferrer, M.A., Jimenez, A. Ros-Barceló, A., Sevilla, F.: Antioxidant systems and O_2^{-}/H_2O_2 production in the apoplast of *Pisum sativum* L. leaves: its relation with NaCl-induced necrotic lesions in minor veins. - Plant Physiol. **127**: 817-831, 2001.

Lechno, S., Zamski, E., Tell-Or, E.: Salt stress-induced responses in cucumber plants. - J. Plant Physiol. **150**: 206-211, 1997.

Mäkelä, P., Kärkkäinen, J., Somersalo, S.: Effect of glycinebetaine on chloroplast ultrastructure, chlorophyll and protein content, and RuBPCO activities in tomato grown under drought or salinity. - Biol. Plant. **43**: 471-475, 2000.

Mansour, M.M.F., Van Hasselt, P.R., Kuiper, P.J.C.: NaCl effects on root plasma membrane ATPase of salt tolerant wheat. - Biol. Plant. **43**: 61-66, 2000.

McKersie, B.D., Leshem, Y.Y.: Stress and Stress Coping in Cultivated Plants. - Kluwer Academic Publishers, Dordrecht 1994.

Muthukumarasamy, M., Dutta Gupta, S., Panneerselvam, R.: Enhancement of peroxidase, polyphenol oxidase and superoxide dismutase activities by triadimefon in NaCl stressed *Raphanus sativus* L. - Biol. Plant. **43**: 317-320, 2000.

Niu, X., Damsz, B., Kononowicz, A.K., Bressan, R.A., Hasegawa, P.M.: NaCl-induced alterations in both cell structure and tissue-specific plasma membrane H^+-ATPase gene expression. - Plant Physiol. **111**: 679-686, 1996.

Pääkkönen, H., Vahala, J., Pohjolai, M., Holopainen, T., Kärenlampe, L.: Physiological, stomatal and ultrastructural ozone responses in birch (*Betula pendula* Roth.) are modified by water stress. - Plant Cell Environ. **21**:671-684, 1998.

Rusch, T., Kirsch, M., Löw, R., Lehr, A., Virreck, R., Zhigang, A.: Salt stress responses of higher plants: the role of proton pumps and Na^+/H^+ antiporters. - J. Plant Physiol. **148**: 425-433, 1996.

Sam, O., Jeréz, E., Dell'Amico, J., Ruiz-Sanchez, M.C.: Water stress induced changes in the anatomy of tomato leaf epidermis. - Biol. Plant. **43**: 275-277, 2000.

Sam, O., Núñez, M., Ruiz-Sánchez, M.C., Dell'Amico, J., Falcón, V., De La Rosa, M.C., Seoane, J.: Effect of a brassinosteroid analogue and high temperature stress on leaf ultrastructure of *Lycopersicon esculentum*. - Biol. Plant. **44**: 213-218, 2001.

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

Testillano, P.S., Sánchez-Pina, M.A., Olmedilla, A., Fuchs, J.P., Risueño, M.C.: Characterization of the interchromatin region as the nuclear domain containing snRNPs in plant cells. A cytochemical and immunoelectron microscopy study. - Eur. J. Cell Biol. **61**: 349-361, 1993.

Voronin, P.Y., Manzhulin, A.V., Myasoedov, N.A., Balnokin, Y.V., Terentieva, E.I.: Morphological types and photosynthesis of C_4 plants leaves under long-term soil salinity. - Russ. J. Plant Physiol. **42**: 310-320, 1995.