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Salt tolerance of *Centaurea ragusina* L. is associated with efficient osmotic adjustment and increased antioxidative capacity

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SUMMARY

The present study investigated the effects of salinity on the perennial species *Centaurea ragusina* L. interesting as a potential cash crop plant. Plants grown in culture conditions were subjected to increasing salt (0–600 mM NaCl) or mannitol (300 mM) treatments for two weeks. Effects of isoosmotic concentrations of NaCl (150 mM) and mannitol were compared and discussed in order to discriminate possible differences in *C. ragusina* response to ionic (NaCl) and osmotic (mannitol) components of salinity. *C. ragusina* plants used Na and to a lesser extent Cl ions as a primary osmotica though with higher salinity, proline accumulation increased as well. Concurrently, with increasing salinity significant reductions in plant K, Mg and Ca concentrations occurred. In addition, lower salt concentrations induced leaf succulence and increased leaf relative water content (RWC). A threshold salinity above which *C.ragusina* L. showed signs of damage and growth inhibition was reached at 300 mM. Activities of superoxide dismutase, catalase and ascorbate peroxidase in salinized plants seem to play an essential protective role in the scavenging processes. Regardless of the high induction of antioxidative system and massive proline accumulation, mannitol caused decrease of RWC and oxidative damage to proteins and lipids. Considering the abundance of some *photosynthetic proteins* (Rubisco, D1, LHCI, LHII and FNR) and PSII efficiency, it can be concluded that both salt and mannitol impaired photosynthesis in *C. ragusina* though salt to a much lesser extent. The results suggest that the major reason for the particular threshold of salinity tolerance in *C. ragusina* can be attributed to limited dilution capacity of succulent tissue. The tolerance strategies of *C. ragusina* to moderate salinity seem to include osmotic adjustment achieved through salt ions uptake as a dominant strategy but also highly inducible antioxidative defense.

1. Introduction

Salinity can inhibit plant growth due to various factors, including ion toxicity, changes in the water relations, impairment of mineral nutrition and inactivation of photosynthetic machinery. The extent to which each of these factors can affect growth depends on adaptations to both low water potentials and high sodium concentrations (Munns, 2002). Salt-induced osmotic and ionic stress disturb the cellular redox balance causing over reduction of photosynthetic electron transport chain and thus amplifying production of reactive oxygen species (ROS). Some of the excessive energy not utilized in photochemistry is quenched into chlorophyll fluorescence to minimize damage to photosystems, particularly in PSII and subsequent electron carriers (Krause and Weis, 1991).

The highly reactive ROS are cytotoxic when overly produced and can damage lipids, proteins, nucleic acids and photosynthetic components. Polyunsaturated fatty acids of plasma membrane are among the more susceptible biological molecules to oxidation. They can easily be oxidized into their corresponding hydroperoxides by ROS or enzymatically by lipoxygenase (LOX, EC 1.13.11.12). Detoxification of ROS in plants is controlled by enzymatic defense systems such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), a variety of peroxidases (ascorbate peroxidase – APX, EC 1.11.1.11, guaiacol peroxidase – GPX, EC 1.11.1.7) and non-enzymatic ones of which ascorbate is the most abundant (Parida and Das, 2005).

The ability of plants to survive and maintain their growth under saline conditions is known as salt tolerance. In summary, mechanisms of salt tolerance are of two main types: those minimizing the

Abbreviations: FNR, Ferredoxin (Fd): NADPH oxidoreductase; LHCI, light harvesting complex of photosystem I; LHCII, light harvesting complex of photosystem II; PSII, photosystem II; Rubisco, large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase.

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entry of salt into the plant (or at least their accumulation in photosynthetic tissues) and those minimizing the concentration of salt in the cytoplasm (Munns, 2002). This corresponds with two major adaptive strategies of plants to tolerate high environmental salinity: 1) stress avoidance, related to different physical, physiological and/or metabolic barriers with which the adverse effects of stress are ameliorated, and 2) stress tolerance, related to adaptive mechanisms which enable successful survival despite the influence of stress internally. Salt tolerant species are often able to accumulate high concentrations of salts in their tissues for osmotic adjustment through the compartmentalization of ions in vacuoles and the production of compatible solutes such as proline in the cytoplasm (Parida and Das, 2005). Proline may also protect enzymes (proteins) from oxidative damage under salinity or dehydration stress (Ghoulam et al., 2002). Earlier studies have suggested that tolerance of plants to salt stress is associated with the induction of antioxidant enzymes (Bor et al., 2003; Ben Amor et al., 2005).

Centaurea ragusina L. is a Croatian perennial plant which is, like some other species in Centaurea genus (Pieroni et al., 2002; Rusak et al., 2002; Arif et al., 2004; Naab et al., 2008) interesting as a potential cash crop plant due to a number of bioactive phytochemicals with potential medicinal or pharmaceutical applications (unpublished data). It thrives along the vertical limestone cliff-faces of its native habitat – coast of the Adriatic Sea and on some islands and is thus simultaneously affected by high light irradiance, drought and salt. Accordingly, it has been described as a halophyte with certain xeromorphic characteristics, such as very dense gray-white hair cover, thick cuticle and palisade parenchyma more developed than spongy parenchyma, which serves as water storing tissue (Bačić et al., 1997). However, the tolerance of that plant species to salinity has yet to be estimated.

The observation of salt induced succulence (Radić et al., 2006) implied that *C. ragusina* has no exclusion mechanism at root level to avoid excess accumulation of salt ions in leaves. Succulence is considered as an adaptation tolerance characteristic of halophytes in terms of conservation of internal water, efficient water storage and dilution of accumulated salts (Flowers et al., 1986; Wang et al., 2012).

Based on the previous results (Radić et al., 2006) and regarding the natural habitat of *C. ragusina*, we assumed its tolerance to salinity as well as to oxidative stress. In this study we aimed to clarify physiological strategies leading to the salinity tolerance of this species. Moreover, we discuss differences in *C. ragusina* adaptation mechanisms in response to salt or mannitol stress by comparing certain morpho-physiological and biochemical parameters – leaf anatomy, ion distribution, proline content, photosystem II efficiency, levels of some *photosynthetic proteins* (Rubisco LSU, D1, LHCI, LHII and FNR), and certain detoxifying enzymes and antioxidants. In our previous investigation, a great accumulation of H_2O_2 in mannitol-treated *C. ragusina* was noticed while salt did not affect the level of that oxygen species after 2-week period (Radić et al., 2006). Here, the activity of lipoxygenase, the hydroperoxide generating enzyme, and carbonyl groups content, an indicator of oxidative damage to proteins, were also investigated.

2. Material and methods

2.1. Plant material and culture conditions

C. ragusina seeds were collected from their natural habitat near Dubrovnik (rock formation Konavoske stijene – locality Pasjača). The sterilized seeds were germinated in containers filled with MS ½ medium containing 0.1 g L^{-1} myo-inositol, 0.1 mg L^{-1} thiamine × HCl, 0.5 mg L^{-1} pyridoxine × HCl, 0.5 mg L^{-1} nicotinic acid, 2.9 mM gibberellic acid (GA3), 0.5 mM 6-benzylaminopurine (BA), 30 g L^{-1} sucrose and 8 g L^{-1} agar (Murashige and Skoog, 1962). Four-week old plants were subcultured to liquid MS ½ medium and, following root initiation, were transferred to the same composition media supplemented with 150 (17.51 mS cm^{-1},), 300 (30.4 mS cm^{-1},), 450 (44.1 mS cm^{-1},) and 600 mM (55.0 mS cm^{-1},) NaCl or 300 mM mannitol (2.38 mS cm^{-1},) corresponding to osmotic potentials −0.85, −1.5, −2.44, −3.0 or −0.84 MPa, respectively. Control (2.52 mS cm^{-1}, −0.14 MPa) plants were kept in nutrient solution without salt or mannitol. All analyses were performed after 15 days of further growth. The plants were grown in a growth chamber at 24 ± 2 °C under a 16:8 h light:dark period of cool fluorescent light (90 μE $m^{-2}s^{-1}$).

2.2. Relative growth rate, relative water content and proline content

Relative growth rate (RGR) was expressed as a (DWt − DWo)/DWo where DWo is the dry weight just before salt treatment and DWt is the dry weight after 15 days of salt treatment. Dry weight (DW) was measured after oven-drying samples at 70 °C for 48 h. Relative water content (RWC) was calculated as: RWC (%) = (FW − DW)/FW × 100. Prior to determination of fresh weight, shoots and roots were washed with distilled water and dried with towels. Free proline content was measured by the method of Bates et al. (1973) using the ninhydrin reagent. Proline concentration was read at 520 nm and determined from calibration curve using L-Proline (Sigma–Aldrich) as standard and expressed as nmol proline/g fresh weight.

2.3. Ion analysis

The Na and K, Ca and Mg contents in the *C.ragusina* roots and shoots were determined by flame (PerkinElmer AA 600; Waltham, MA, USA) and graphite furnace atomic absorption spectrophotometer (PerkinElmer AA 300) respectively, after microwave wet digestion (Anton Paar Multiwave 3000, Graz, Austria, EU) of the dried and powdered material in 10 ml of supra-pure concentrated HNO_3 at 230 °C. Estimation was carried out in triplicate. Cl was determined by the application of the oxygen flask method followed by a mercurimetric titration with mercuric perchlorate and diphenilcarbazone as an indicator (Schöniger, 1955).

2.4. Light microscopy (LM) and transmission electron microscopy (TEM)

For ultrastructural analyses, small pieces of tissue were fixed for 30 min with 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 2 °C. Upon rinsing with the cacodylate buffer, the material was postfixed for 2 h with 1% (vol. ratio) osmium tetroxide in the same buffer at 2 °C. The material was dehydrated through an ethanol series and embedded in Spurr's resin. Semi-thin sections of fixed material were stained with 2% toluidine blue and examined using light microscope "Zeiss Axiovert 35". Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a FEI Morgagni 268D electron microscope operated at an accelerating voltage of 70 kV.

2.5. Measurements of chlorophyll fluorescence, chlorophyll and carotenoid content

In vivo chlorophyll *a* fluorescence was measured at room temperature with a portable fluorometer (PAM-2000, Walz, Germany) connected to a notebook computer with data acquisition software (DA-2000, Heinz, Walz). The plant material was dark-adapted for approximately 30 min before measurement. Estimation was carried out in triplicate. The minimal (F_o) and maximal fluorescence

levels were measured in dark-adapted leaves. The leaves were then continuously illuminated with white actinic light (photosynthetic photon flux density of 200 μmol m^{-2} s^{-1}) and the same parameters were measured (*F* and *F′m*). The radiation was maintained until both *F* and *F′m* were stable. Calculations of fluorescence parameters – maximum quantum yield of the PS II (*Fv*/*Fm*), the effective quantum yield of the PS II ($\Delta F/F'm$), nonphotochemical quenching (NPQ) and relative *electron transport rate* (rel*ETR*) – were made according to Maxwell and Johnson (2000).

Fresh material of adult leaves was extracted in 80% acetone, and contents of chlorophyll *a* (Chl a), chlorophyll *b* (Chl b), and carotenoids (Car) were calculated according to Lichtenthaler (1987).

2.6. Immunodetection of Rubisco, D1, LHCI, LHII and FNR

In order to determine the abundance of proteins involved in photosynthesis, leaf samples were homogenized in Tris–HCl extraction buffer pH 8 containing 17.1% (w/v) sucrose, 0.1% (w/v) ascorbic acid, 0.1% (w/v) cysteine-hydrochloride (Sigma–Aldrich) with addition of polyvinylpolypyrrolidone (PVPP, Sigma–Aldrich) and then centrifuged at 25,000 *g* for 30 min. Total protein concentration was determined using bovine albumine serum as standard. Aliquots of each homogenate were mixed with corresponding volumes of denaturating 0.065 M Tris–HCl buffer containing 6% (w/v) sodium dodecyl sulphate (SDS, Sigma–Aldrich), 6% (v/v) β-mercaptoethanol (Sigma–Aldrich), 30% (v/v) glycerol and 0.01% (w/v) of bromphenol blue, boiled for 5 min and loaded on the gel. The samples were separated by SDS-polyacrylamide gel electrophoresis in 12% (w/v) resolving gels and, subsequently, electroblotted onto the nitrocellulose membrane (0.45 μm, Bio-Rad). The membranes were blocked with 20.5% (w/v) non-fat powdered milk solution made in Phosphate buffered saline (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl) pH7.4 containing 1% (v/v) of Tween 20 (Sigma-Aldrich) and incubated overnight with the following antibodies raised against the pea proteins: rabbit anti-large subunit Rubisco (dilution 1:1000); rabbit anti-D1 protein (dilution 1:2500), rabbit anti-Ferredoxin (Fd): NADPH oxidoreductase (FNR) (dilution 1:1000), rabbit anti-light-harvesting complex II of PSII (LHCII) (dilution 1:500) and mouse anti-light-harvesting complex I of PSI (LHCI) (dilution 1:500). Detection of immunoreactive proteins was achieved by using alkaline phosphatase-linked secondary antibodies (dilution 1:30,000 anti-rabbit IgG from Sigma). The membranes were developed with BCIP/NBT (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma–Aldrich).

2.7. Carbonyl groups and ascorbate contents

The amount of protein oxidation was estimated by the reaction of carbonyl groups (C=O) with 2, 4-dinitrophenylhydrazine (Sigma–Aldrich), as described in Levine et al. (1990). Ascorbate was estimated according to the method of Mukherjee and Choudhouri (1983) using trichloroacetic acid and dinitrophenyl hydrazyne.

2.8. Analysis of SOD, APX, CAT and LOX activities

Shoot or root tissue was homogenized in 50 mM KPO4 buffer (pH 7) including 5 mM sodium ascorbate, 1 mM ethylene diamine tetraacetic acid (Sigma–Aldrich) and PVPP. The homogenates were centrifuged (Sigma 3K18 Centrifuge, Germany) at 25,000 *g* for 30 min at 4 °C and supernatants used for enzyme activity and protein content assays. Total soluble protein contents of the enzyme extracts were estimated according to Bradford (1976) using bovine albumine serum (Sigma–Aldrich) as standard. The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (Sigma–Aldrich) following the method of Beauchamp and Fridovich (1971). One unit of SOD was taken as the volume of the enzyme extract causing 50% inhibition of nitroblue tetrazolium reduction. Ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981). Catalase (CAT) activity was determined by the decomposition of H_2O_2 and was measured following the method of Aebi (1984). Lipoxygenase activity was determined using linolenic acid as substrate according to Axelrod et al. (1981).

2.9. Statistical analysis

For each analysis, data were compared by analysis of variance (ANOVA) with Duncan multiple Post Hoc test using the STATISTICA 10 (StatSoft, Inc., USA) software package; differences between the corresponding controls and exposure treatments were considered as statistically significant at $P < 0.05$. Each data point is the average of six replicates (n = 6), unless stated otherwise.

3. Results

3.1. Relative growth rate, relative water content and proline content

Mannitol and 150 mM NaCl resulted in opposite effects on the growth of *C.ragusina* plants (Fig. 1) – the first caused growth inhibition (60 and 20% decrease of shoot and root RGR, respectively, compared to control) and the latter caused growth stimulation (almost two-fold increase of shoot and root RGR compared to control). Plant growth was not affected by 300 mM NaCl while higher NaCl treatments, especially 600 mM, caused a significant decrease in the growth of *C.ragusina* plants (Fig. 1b). Less conspicuous effects of salt were observed on plant relative water content (Fig. 1a). Shoot RWC of plants exposed to 150 and 300 mM NaCl showed increase by 3 and 2%, respectively, while 600 mM NaCl decreased shoot RWC by 2% compared to control. A decrease of shoot RWC was highest following mannitol treatment (13% compared to control). Although root RWC was not affected by the lowest salt concentration, with the increase of salt concentration, it gradually declined (3–6% in comparison to control). Mannitol-treated plants also showed 6% lower RWC of roots. Regarding proline, the content of the amino acid was much higher in roots than in shoots of control plants. Salinity had a significant effect on proline content in roots and especially in shoots (Fig. 1c). Proline content substantially increased with an increase in salinity, the increase ranging from two-fold (at 150 mM) to 25-fold (at 600 mM) in shoots and from 50% (at 150 mM) to three-fold (at 450 mM) in roots. Mannitol caused 20- and three-fold increase in shoot and root proline content, respectively.

3.2. Contents of ions

The content of chloride in control plants was approximately ten times higher than that of sodium. Nevertheless, the contents of both ions, especially of Na, increased dramatically with the amount of salt added (Table 1). Leaf Na content reached maximum at 300 mM NaCl – it increased up to 92 times compared to that of the control while root Na increased significantly with an increase in salt concentration showing the highest value at 600 mM NaCl (41-fold increase compared to control). Content of Cl in salt-treated plants increased markedly with an increase in salt concentration; the leaf Cl was 6 to 10-fold higher and that of root 3 to 8-fold higher than in respective controls. The contents of K, Ca and Mg in salt-treated plants decreased according to increasing salt concentrations. The decline of those ions was sharper in roots than in shoots with the exception of Ca; with increased salinity K dropped for 30–50% in shoots and 40–78% in roots, Mg dropped for 30–50% in shoots and 40–67% in roots and Ca dropped for 35–65% in shoots and 25–56%

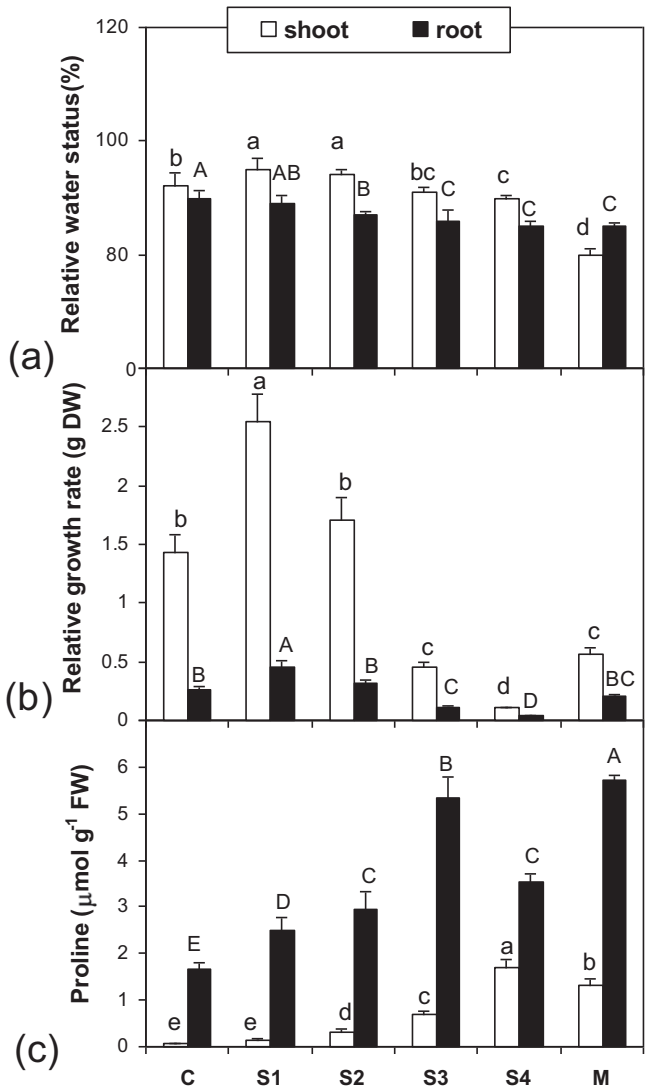

Fig. 1. (a) RWC (%), (b) RGR (g), and (c) proline content of *C. ragusina* shoots and roots under control (C) and stress – 150 mM NaCl (S1), 300 mM NaCl (S2), 450 mM NaCl (S3), 600 mM NaCl (S4), 300 mM mannitol (M) – conditions during the 15-day growth peroid. Values are mean ± SE based on six replicates. Bars with different letters are significantly different at $p < 0.05$.

in roots. No significant differences in the contents of Na and Cl were observed between control and mannitol-treated plants. The non-ionic osmoticum also caused a decrease of K, Ca and Mg which was more prominent in shoots.

3.3. Leaf morphology and anatomy

Exposure of *C. ragusina* to NaCl induced development of *leaf succulence* (Fig. 2). Palisade and spongy cells inflated under saline conditions, especially under 150 mM NaCl, (Fig. 2b) thus increasing the mesophyll thickness of *C. ragusina* leaves. The rise in leaf thickness was accompanied by a decline in intercellular spaces in mesophyll tissues (Fig. 2b). Contrary to salt, mannitol caused partial detachment of plasma membrane (Fig. 2c, i) and shrinkage of protoplasts indicating plasmolysis. The observation was supported by the results of RWC and wilting of *C.ragusina* leaves (Fig. 2). The chloroplasts of the control plants were mostly oval-shaped with a few starch grains (Fig. 2d) and their thylakoid membranes were well developed (Fig. 2d). Under saline conditions, in addition to normally developed chloroplasts, the vesiculated chloroplasts with a fewer number of starch grains but relatively still intact thylakoids were noticed (Fig. 2e). Such chloroplasts (Fig. 2e) were rarely observed at 150 mM NaCl while their number increased at 300 mM NaCl. Under higher salt treatments (450 and 600 mM NaCl), thylakoid membranes showed great dilations and undulated thylakoid areas developed (Fig. 2f). The proportion of glyoxysomes (Fig. 2g) increased with an increase in salt concentration. Also, salinity induced vesiculation in the mesophyll cells (Fig. 2h). Vesicles were mostly fused with plasma membrane (Fig. 2f, h). Mannitol-induced osmotic stress also increased the number of membrane vesicles as well as of plastoglobuls (Fig. 2i). However, the chloroplasts of mannitol-treated plants exhibited thylakoid membranes with visible grana and stroma thylakoids.

3.4. Efficiency of PSII and content of chlorophylls and carotenoids

The maximal efficiency of PSII (*Fv*/*Fm*) of salt-treated plants decreased (by 14% compared to control) only in response to the highest salt concentration, while mannitol suppressed *Fv*/*Fm* for 45% in comparison to control (Fig. 3c). Under saline conditions, the $\Delta F/F'm$ (Fig. 3c) as well as *ETR* (Fig. 3d) dropped by a similar amount with increasing salt concentration; the reduction of the parameters was in the order 25–35% under lower NaCl treatments, 60–70% under higher NaCl treatments and 80% under mannitol (Fig. 3c). Contrary to that, nonphotochemical quenching (NPQ) showed an increase in the range of 46–78% under saline treatments (Fig. 3c). There was no significant change in the NPQ of mannitol-stressed leaves (Fig. 3c).

Rising NaCl salinity also caused a continual decrease in chlorophyll a, chlorophyll b, and carotenoid content of *C.ragusina* plants (Fig. 3a). When grown at 150 mM NaCl, a 35% and 30% decline of chlorophylls and carotenoids, respectively, in *C.ragusina* plants was observed (Fig. 3a). At higher salinities, the reduction of Chl b and

Table 1
Contents of Na, Cl, K, Ca and Mg (mg g^{-1} DW) of C. *ragusina* plants after 15-day period growth.

	Treatments	Na	Cl	K	Ca	Mg
Shoot	Control	1.5 (0.1)c	18.8 (1.7)d	26.6 (2.8)a	4.0 (0.2)a	1.2 (0.2)a
	150 mM NaCl	70.1 (8.6)b	118.9 (6.0)c	19.1 (3.5)b	2.6 (0.2)b	0.8 (0.09)b
	300 mM NaCl	138.0 (17.3)a	168.5 (5.9)b	16.3 (2.9)b	2.1 (0.1)bc	0.7 (0.04)b
	450 mM NaCl	132.9 (11.5)a	192.3 (5.9)a	13.7 (0.8)b	1.8 (0.2bc)	0.6 (0.05)b
	600 mM NaCl	120.4 (11.2)a	198.0 (20.6)a	12.3 (0.9)b	1.4 (0.1)c	0.6 (0.06)b
	300 mM mannitol	1.2 (0.1)c	28.3 (5.0)d	14.3 (0.5)b	1.4 (0.2)c	0.6 (0.06)b
Root	Control	2.0 (0.2)e	17.5 (2.6)d	30.3 (1.1)a	1.6 (0.03)a	1.2 (0.08)a
	150 mM NaCl	22.7 (0.8)d	55.2 (3.2)c	17.9 (1.2)c	1.2 (0.03)c	0.7 (0.02)c
	300 mM NaCl	31.6 (2.1)c	86.5 (6.9)b	11.9 (0.9)d	1.0 (0.03)d	0.5 (0.03)e
	450 mM NaCl	50.1 (3.1)b	102.9 (13.8)b	9.4 (0.4)d	0.8 (0.05)d	0.4 (0.04)d
	600 mM NaCl	82.2 (8.6)a	132.4 (8.6)a	6.8 (0.4)e	0.8 (0.04)d	0.4 (0.03)d
	300 mM mannitol	2.2 (0.1)e	23.5 (3.4)d	22.5 (0.7)b	1.5 (0.08)ab	0.8 (0.05)b

Values represent mean ± S.D. (parenthesis) of 3 replicates. Different letters indicate significant difference at $p < 0.01$.

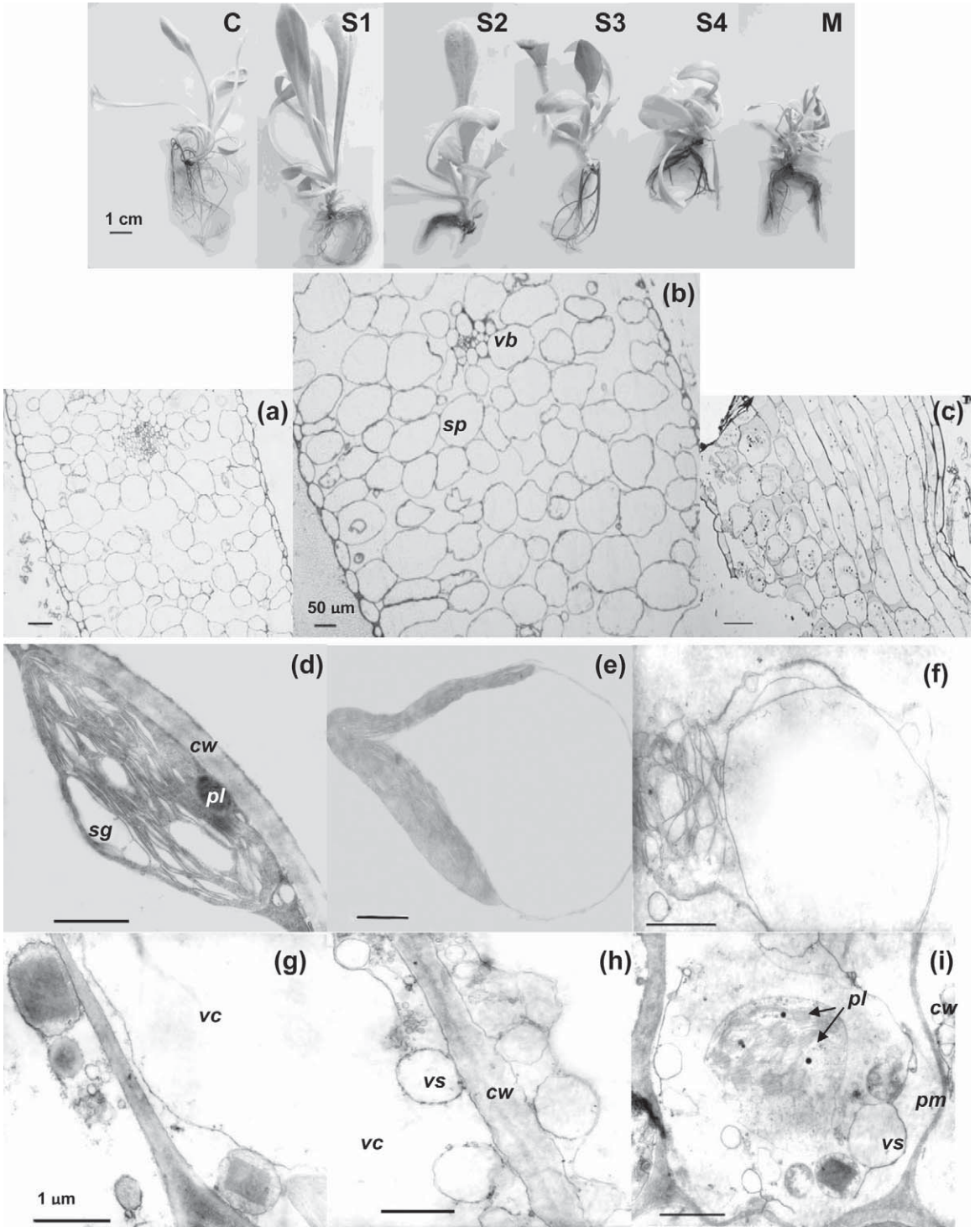

Fig. 2. Uppermost row – morphology of *C. ragusina* under control (C) and stress – 150 mM NaCl (S1), 300 mM NaCl (S2), 450 mM NaCl (S3), 600 mM NaCl (S4), 300 mM mannitol (M) – conditions during the 15-day growth period. LM photographs: cross-sections of leaves (a) control, (b) 150 mM NaCl – with strongly developed, inflated spongy parenchyma, (c) mannitol. TEM photographs: chloroplast of (d) control, (e) 300 mM NaCl-treated and (f) 450 mM NaCl-treated plants; (g) glyoxysomes in NaCl-treated cells, (h) vesiculation in NaCl-treated cells, and (i) mannitol-treated cell. sp, spongy parenchyma; vb, vascular bundle; vc, vacuole; cw, cell wall; pm, plasma membrane; vs, vesicle; sg, starch grain; pl, plastoglobul.

Car exceeded 50% in comparison to control. Under saline conditions, degradation of Chl b was faster (38% of the control under 600 mM NaCl) than that of Chl a and Car. Manitol decreased Chl a, Chl b and Car by 35, 12 and 22%, respectively, compared to control. Plants grown at higher salinities, showed an increase in Chl a/b ratio which was significant under 600 mM NaCl (Fig. 3b). Opposite to that, mannitol caused a 37% decrease of the ratio. The Chl a + b/Car ratio was not influenced either by NaCl or mannitol (Fig. 3b).

3.5. Rubisco, D1, LHCI, LHII and FNR proteins

Large subunit of Rubisco accumulated less with increasing NaCl concentrations (Fig. 4). The intensity of other photosynthetic

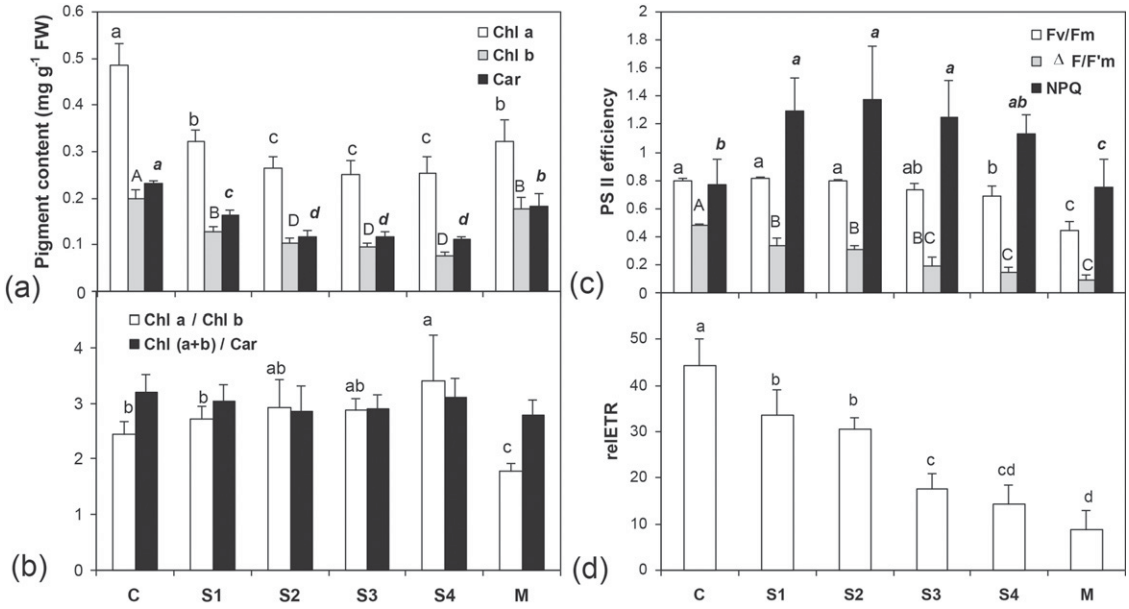

Fig. 3. (a) Chl a, Chl b and carotenoids content, (b) Chl a/b ratio and Chl a + b/Car ratio, (c) PS II efficiency – *Fv*/*Fm*, Δ*F*/*F*′*m* and NPQ (d) rel*ETR* of *C. ragusina* leaves under control (C) and stress – 150 mM NaCl (S1), 300 mM NaCl (S2), 450 mM NaCl (S3), 600 mM NaCl (S4), 300 mM mannitol (M) – conditions during the 15-day growth peroid. Values are mean ± SE based on six replicates. Bars with different letters are significantly different at $p < 0.05$.

protein bands started to decline from 300 mM NaCl with FNR showing the specific degrading pattern. Mannitol caused lesser accumulation of photosynthetic proteins compared to control while LHCI protein was not detected under the treatment.

3.6. Carbonyl groups contents and LOX activity

The level of oxidatively damaged proteins, expressed as carbonyl groups content, in *C.ragusina* shoots increased by 49 and 47% under 450 and 600 mM NaCl, respectively, compared to control (Fig. 5a). In roots, only the highest salt concentration caused protein damage (2-fold increase of C=O content in comparison to control). Mannitol-treated plants showed significant increase of C=O content in both shoots and roots (Fig. 5a).

The activity of LOX in *C. ragusina* shoots started to increase considerably from 450 mM NaCl and peaked at 600 mM NaCl while in roots only the highest salt concentration caused significant increase of the enzyme (Fig. 5b). Under mannitol treatment, the increase of LOX activity in both shoots and roots exceeded 80% in comparison to control (Fig. 5b).

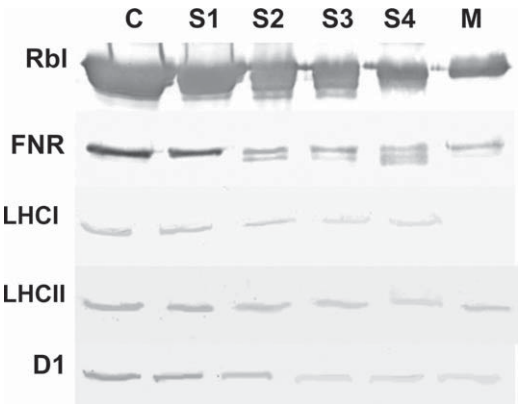

Fig. 4. Immunodetection of RbI, FNR, LHCI, LHCII and D1 proteins of *C. ragusina* leaves under control (C) and stress – 150 mM NaCl (S1), 300 mM NaCl (S2), 450 mM NaCl (S3), 600 mM NaCl (S4), 300 mM mannitol (M) – conditions during the 15-day growth peroid.

3.7. Antioxidative enzymes and ascorbate content

Activity of SOD in shoots was significantly increased in response to lower NaCl treatments (Fig. 5d). In roots, the activity of SOD increased with increasing salt concentrations reaching the maximum at 600 mM NaCl. The production of superoxide in mannitol-treated plants was approximately 2-fold higher than in control plants (Fig. 5d). Lower salt concentrations significantly increased the activity of CAT in both shoots and roots while higher ones did not affect the activity of the enzyme (Fig. 5e). Mannitol caused the induction of CAT in shoots while in roots the activity was similar to control (Fig. 5e). The activity of APX in shoots was significantly higher under 150, 300 and 450 mM NaCl than in control (Fig. 5f). In roots, higher salt concentrations induced APX activity but only 450 mM NaCl significantly. Mannitol increased APX activity in both shoots and roots by approximately 90% compared to control (Fig. 5f).

The content of ascorbate in shoots was significantly lower (by approximately 40%) under 150, 300 and 450 mM NaCl than in control (Fig. 5c). The content of ascorbate in roots was affected only by the highest NaCl concentration (27% lower value than the control). In shoots, mannitol increased ascorbate content by 43% while in roots the content of the antioxidant was lowered by 25% in comparison to control.

4. Discussion

Upon exposure to NaCl, *C. ragusina* accumulated salt ions (Table 1), increased leaf thickness and produced succulent shoots regardless of the salt concentration (Fig. 2). The increased thickness of succulent leaves can mainly be attributed to enlarged mesophyll cells, which have absorbed water along with salt ions and increased the size of their vacuoles (Munns, 2002). Raising the concentration of NaCl in hydroponic solutions resulted in greater leaf succulence and greater mesophyll thickness for many salt-tolerant plants but some non-halophytic species as well (Longstreth and

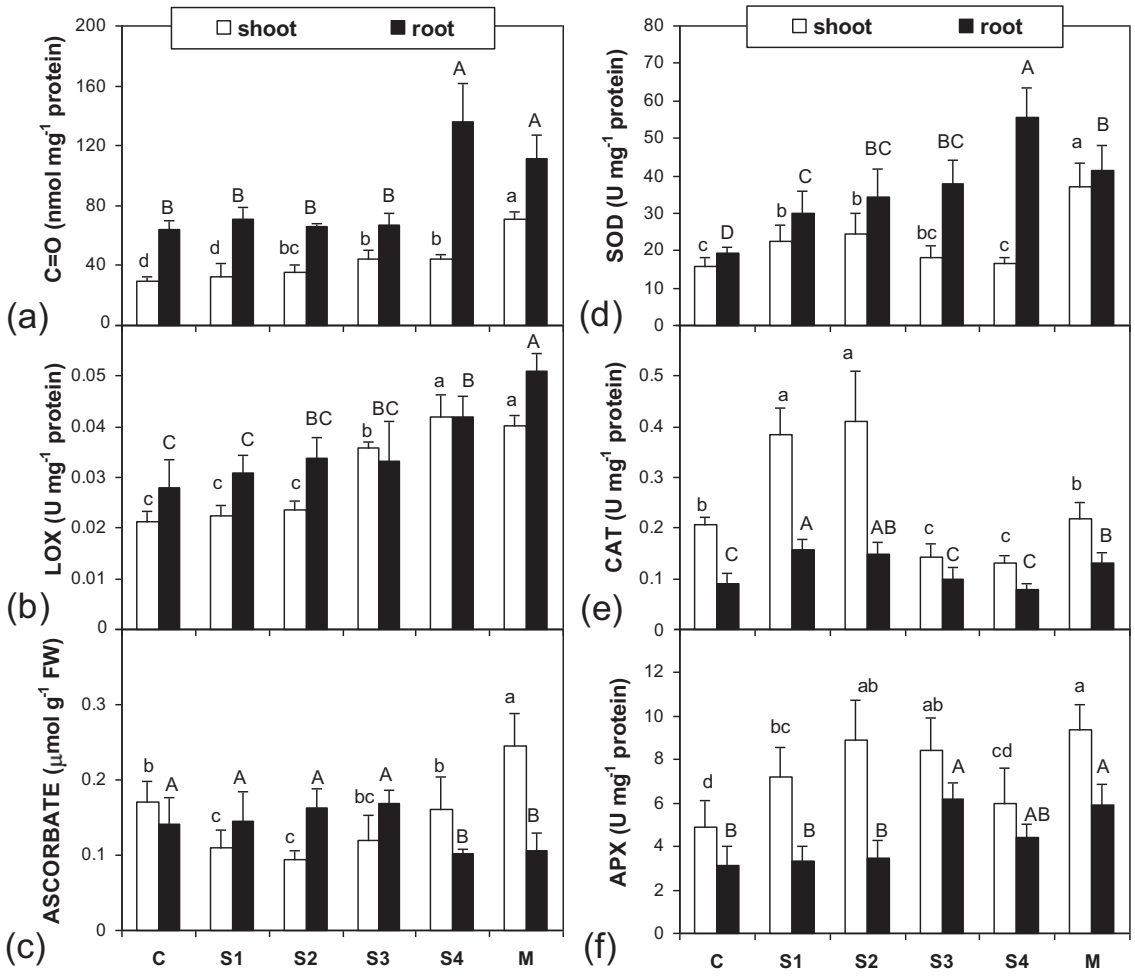

Fig. 5. (a) carbonyl groups content (C=O), (b) LOX activity, (c) ascorbate content, (d) SOD activity, (e) CAT activity, and (f) APX activity of *C. ragusina* shoots and roots under control (C) and stress – 150 mM NaCl (S1), 300 mM NaCl (S2), 450 mM NaCl (S3), 600 mM NaCl (S4), 300 mM mannitol (M) – conditions during the 15-day growth peroid. Values are mean ± SE based on six replicates. Bars with different letters are significantly different at $p < 0.05$.

Nobel, 1979; Maggio et al., 2000; Silveira et al., 2009). With respect to distribution of salt ions in different tissues, a prevalent accumulation of salt ions in leaves versus roots suggests *C. ragusina* to be an includer (Yeo, 1983). However, regarding the type of inorganic ions preferentially accumulated, *C. ragusina* employed Na as a primary osmolyte. Thus, in *C. ragusina* osmotic adjustment seems to be mainly achieved by sodium and, to a lesser extent, chloride which is consistent with previous studies on salt tolerant species (Wang et al., 1997; Khan et al., 2000; Ueda et al., 2003; Silveira et al., 2009). The accumulation of salt ions expanded leaf cells, positively affected plant growth and water content but only at 150 mM NaCl (Fig. 1). A threshold value for salt tolerance of the species was reached at 300 mM NaCl as above that concentration, inhibition of growth was observed. Similar growth pattern – growth stimulation under relatively low salinity and growth reduction under high salinity – was observed in many salt tolerant and halophytic species (Bajji et al., 1998; Short and Colmer, 1999; Koyro, 2006). These results infer that *C. ragusina* cells seem to have an efficient mechanism to adjust osmotically and that leaf turgor is not a limiting factor for growth at moderate salinity levels. The reason for growth restriction at higher salinities might lie either in limited dilution capacity of succulent tissue and consequent saturation of the solute uptake system or in excessive demand on the energy requirements of such systems (Munns, 2002). In addition, the concomitant increase of proline with increasing salinity might have contributed to negative growth trend. It has been shown that metabolic costs for osmotic adjustment achieved by accumulation of synthesized organic solutes such as proline are much higher than using NaCl for the same purpose (Munns, 2002). The massive proline accumulation might have contributed to mannitol-induced growth inhibition of *C. ragusina* as well. In contrast to 150 mM NaCl, iso-osmotic mannitol restricted growth to the same level as 450 mM NaCl and significantly decreased leaf water content as well as leaf thickness (Fig. 1). Thus, under osmotic stress caused by mannitol the increasing proline content did not result in an osmotic adjustment level preventing plant biomass reduction and water loss. Similar effects of water stress induced by either plant non-irrigation or by use of osmoticum such as mannitol were observed in other salt-tolerant species as well (Ueda et al., 2003; Slama et al., 2007b). Decrease of fresh weight and water content, as opposed to sharp increase in proline was also reported under mannitol-induced stress in halophyte *Sesuvium portulacastrum* (Slama et al., 2007a). Our results indicate that in *C.ragusina* osmotic adjustment through inorganic ion uptake is more efficient than adjustment through the production of proline. Several authors have noticed that significant proline accumulation generally occurs only after a threshold of drought or salt stress is exceeded (Cavalieri and Huang, 1979; Delauney and Verma, 1993; Hester et al., 2001). Therefore, in the case of *C. ragusina* proline seem to serve other roles, such as a radical scavenging, protection of cellular macromolecules, storage of nitrogen or maintenance of cellular pH (Verbruggen and Hermans, 2008).

Other factors, such as nutrient deficiencies, may also play an important role (Marschner, 1995) in growth retardation. Disturbance of nutrient balance is a usual consequence of either salinity or drought, irrespective of plant species salt tolerance (Wang et al., 1997; Zekri, 1995; Ghoulam et al., 2002; Slama et al., 2007a). Similar results were also obtained in our study as both salt and mannitol decreased K, Ca and Mg contents in *C.ragusina* plants. Drought and salinity are found to disturb the mineral-nutrient relations in plants through their effects on nutrient availability, transport, and partitioning in plants (Hu and Schmidhalter, 2005). In the present study, the effect of iso-osmotic NaCl and mannitol on K, Mg and Ca contents was quite different with respect to plant organ - the nutrients in shoots were more affected by mannitol and those in roots were more affected by salt. Moreover, the mannitol-induced imbalance of leaf nutrients was comparable to that caused by higher salt concentrations. Such effects of higher salt treatments and mannitol might be attributable to reduced nutrient uptake by the roots and transport from the roots to shoots, respectively, as a result of impaired active transport, reduced Ca content and consequent increased membrane permeability (Alam, 1999). In addition, loss of turgor and resultant dehydration could have contributed to mannitol-induced nutrient deficiency in *C. ragusina* leaves. Nevertheless, as both drought and salinity cause a similar effect on plant growth through a water deficit, K is equally important to maintain the turgor pressure of the plant under either stresses. Decrease of Mg uptake induced by salt and mannitol might have contributed to decreased chlorophyll content and thus impair photosynthetic machinery (Hu and Schmidhalter, 2005).

Several studies have suggested that PSII is highly resistant to salinity and drought tress (Lu and Zhang, 1998; Cornic and Fresneau, 2002; Debez et al., 2008). In the present study, with the exception of the highest salt concentration, saline conditions did not influence *Fv*/*Fm* ratios, measured after dark adaptation, indicating that salinity does not induce sustained photodamage (Fig. 3). Unchanged Fv/Fm values were also reported in *Cakile maritima*, *Hordeum maritimum*, *Atriplex centralasiatica*, *Sorgum bicolor* (Qiu et al., 2003; Netondo et al., 2004; Megdiche et al., 2008; Degl'Innocenti et al., 2009). However, the unimpaired *Fv*/*Fm* values in salt-treated *C. ragusina*, were accompanied by a significant decrease in effective quantum yield ($\Delta F/F'm$) and reduced electron transport activity. The reductions in $\Delta F/F'm$ and relETR were correlated with an increase in NPQ which may indicate that reduced CO_2 assimilation decreases demand for products of electron transport, and thus increases thermal dissipation of light energy at the antennae. Although, other mechanisms involved in energy dissipation related to a transmembrane proton gradient generated by ATPase activity and uncoupling of electron transport that may lead to oxidative stress, could be also involved (Maxwell and Johnson, 2000; Calatayud and Barreno, 2004). Structural changes of chloroplasts (Fig. 2) as well as decline of chlorophylls and carotenoids (Fig. 3) observed under saline conditions might have contributed to impairment of reaction centers of PSII either directly (Masojidek and Hall, 1992) or via an accelerated senescence (Kura-Hotta et al., 1987). The Chl a/b ratio and the frequency of vesiculated chloroplasts started to increase from 300 mM NaCl and above that concentration, salt caused damage to thylakoid membranes and decreased the grana stacks, which may finally cause the disturbance or inhibition of photochemical reactions. Moreover, these chloroplasts usually contained little or no starch, suggesting low photosynthetic activity (Barhoumi et al., 2007). An increase in the Chl a/b ratio under higher salinities might imply a shift in the PSII/PSI ratio (Varadi et al., 2003) or rather decrease in the LHC components as Chl b is mainly located in the complexes (Durnford et al., 2003). This view is in agreement with our results which showed lesser abundance of LHCI, LHC II and D1 proteins starting from 300 mM NaCl (Fig. 4). Salt stress was found to inhibit the repair of the photodamaged PSII through inhibition of the synthesis of proteins de novo and, in particular, the synthesis of the D1 protein (Allakhverdiev et al., 2002). Furthermore, decline of FNR and Rubisco enzymes noted from 300 to 600 mM NaCl indicate lesser NADPH utilization and photosynthetic carbon reduction which might led to reduced photosynthesis and growth (Woodrow and Berry, 1988). Contrary to higher salt concentrations, 150 mM NaCl did not affect the abundance of immunodetected proteins. Also, the occurrence of microbodies as well as of plastoglobuls was similar as in cells of control plants. Still, declined chlorophylls and carotenoids and the occurrence of modified chloroplasts might have been the reason for downregulation of PSII. Compared to salt, mannitol-induced osmotic shock caused much greater disturbance to PSII; NPQ of mannitol-treated leaves showed no apparent change compared to control, though the value of the parameter was rather high in comparison to greatly decreased values of *Fv*/*Fm*, $\Delta F/F'm$ and relETR. The downregulation of PSII was paralleled with partial or total inhibition of LHCs and D1 proteins synthesis though the FNR and Rubisco proteins accumulated less as well (Fig. 4). In the study of Lu and Zhang (1999) the loss of PSII chemistry under water stress has been associated with the loss or decline in D1 and D2 proteins of PSII. The drastic effects of mannitol on PSII and some photosynthetic proteins might be ascribed to decrease in RWC which has been known to induce stomatal closure (Cornic and Fresneau, 2002; Reddy et al., 2004). Consequent inhibition of CO_2 assimilation, coupled with the changes in photosystem activities and photosynthetic electron transport capacity, results in accelerated production of active oxygen via the chloroplast Mehler reaction (Asada, 1999).

Both salt and mannitol increased the amount of membrane vesicles (Fig. 2) which increase the membrane surface area and are often discussed in connection with processes such as transport, storage, and NaCl compartmentation (Koyro, 2002; Kurkova et al., 2002; Mitsuya et al., 2002). Higher salt concentrations and mannitol could affect thylakoid membranes by disrupting lipid bilayer or lipid–protein associations and thus impair electron transport activity (Reddy and Vora, 1986). Increased number of plastoglobuls observed under higher salt and mannitol treatments might be indication of stored lipid breakdown products (Paramonova et al., 2004; Bréhélin et al., 2007). The formation of plastoglobuli is thought to be linked to the breakdown of thylakoids that accompanies senescence (del Río et al., 1998).

The accumulation of microbodies such as glyoxysomes, observed under higher saline treatments is also indicative of senescent processes (Koyro, 1997).

Beside several senescence-promoting compounds such as ethylene and jasmonic acid, important factors in plant senescence are reactive oxygen species and LOX activity which, among other, has an important role in the breakdown of membrane lipids (del Río et al., 1998). Lipoxygenase catalyzes the hydroperoxidation of polyunsaturated fatty acids with oxygen to give hydroperoxide products which can undergo autocatalytic degradation, producing radicals and thus initiating the chain reaction of lipid peroxidation. In addition, LOX-mediated formation of singlet oxygen and superoxide has been shown (Kanofsky and Axelrod, 1986; Lynch and Thompson, 1984). Therefore, a reduced or unchanged LOX activity under stress conditions can be considered as beneficial for plants as LOX are oxidative enzymes. In a salt-tolerant tomato (Mittova et al., 2002) or drought-tolerant chives (Egert and Tevini, 2002) decreased or reduced LOX activity has been accompanied with unchanged malondyaldehide (indicator of lipid peroxidation) content and vice versa, increased LOX activity in drought-stressed olive (Sofo et al., 2004) was paralleled with increase of MDA content. Here, LOX activity (Fig. 5) of both shoots and roots was not affected by lower NaCl treatments while it increased in response to mannitol and higher salt concentrations. However, although a significant increase in the MDA content was observed in *C. ragusina* exposed

to either salt or mannitol following 10 days of stress, the level of lipid peroxidation in either case was unaffected after 15-day period (Radić et al., 2006).

Some evidence suggests that resistance to oxidative stress achieved through efficient antioxidant defense mechanisms, may, at least in part, be involved in salt stress tolerance (Meloni et al., 2003; Ben Hamed et al., 2007; Ben Amor et al., 2005). In this study, induction of SOD, an enzyme which eliminates superoxide and simultaneously produces H_2O_2, was recorded in both plant organs under all treatments except in *C. ragusina* leaves exposed to higher salt concentrations. The result implies that both salt or mannitol increase the formation of superoxides in *C. ragusina* leaves and roots probably as a consequence of the inhibition of Calvin cycle and increased respiration in root mitochondria, respectively (Møller, 2001; Mittova et al., 2004). In plants, CAT, APX and GPX are considered the most important in degradation of H_2O_2 (Parida and Das, 2005). Both salt and mannitol induced the activity of the enzymes, but to a different extent in plant organs. The enhancement in SOD, CAT and POX activities under salt and water stress was also reported in a number of halophytic and non-halophytic species (Broetto et al., 2002; Mittova et al., 2002; Meloni et al., 2003; Türkan et al., 2005; Ben Hamed et al., 2007).

The observed low levels of H_2O_2 in salt-treated *C. ragusina* leaves (Radić et al., 2006) were maintained by APX and CAT activities which were increased only up to 300 mM NaCl. The increased APX activity coincided with lowered ascorbate levels (Fig. 5) which indicate enzymatic H_2O_2 detoxification i.e. ascorbate consumption as an electron donor for APX. In response to higher saline conditions, APX and GPX (Radić et al., 2006) constituted the system responsible for the elimination of H_2O_2, while CAT activity was inhibited, possibly as a result of ROS-induced degradation. That hypothesis was corroborated with increased level of oxidatively damaged proteins (Fig. 5) recorded in *C. ragusina* leaves under 450 and 600 mM NaCl. In roots, H_2O_2 was eliminated by CAT and GPX under lower and by APX under higher saline conditions, respectively. On the other hand, the significant increase in H_2O_2 (Radić et al., 2006) and carbonyl groups contents (Fig. 5) was recorded after 15-day period in response to mannitol despite increased GPX (Radić et al., 2006) and APX activities as well as high ascorbate content. Those results suggest that *C.ragusina* is equipped with balanced and *responsive* antioxidant system which proved to be highly inducible even under hyperosmotic conditions induced by mannitol or hyperionic conditions reached at higher salinities.

In conclusion, the salt tolerance of this plant species can probably be attributed to its ability to: (1) develop *leaf succulence* thus maintaining convenient tissue water supply, (2) accumulate and compartmentalize salt ions into the vacuoles, (3) exhibit high antioxidant enzyme activities preventing the toxic buildup of ROS. Nevertheless, *C. ragusina* seems to be efficient at compartmentalizing and/or diluting salt ions only at saline levels not greater than external 300 mM NaCl. The particular threshold of *C. ragusina* salinity tolerance probably lies in limited dilution capacity of succulent tissue and consequent saturation of the solute uptake system thus causing both hyperosmotic and hyperionic stress, or in excessive demand on the energy requirements of such systems.

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References

Aebi, H., 1984. Catalase *in vitro*. Methods in Enzymology 105, 121–126.

Alam, S.M., 1999. Nutrient uptake by plants under stress conditions. In: Pessarakli, M. (Ed.), Handbook of Plant and Crop Stress. Marcel Dekker, New York, pp. 285–314.

Allakhverdiev, S.I., Nishiyama, Y., Miyairi, S., Yamamoto, H., Inagaki, N., Kanesaki, Y., Murata, N., 2002. Salt stress inhibits the repair of photodamaged photosystem II by suppressing the transcription and translation of psbA genes in Synechocystis. Plant Physiology 130, 1443–1453.

Arif, R., Küpeli, E., Ergun, F., 2004. The biological activity of *Centaurea* L. species. G.U. Journal of Science 17, 149–164.

Asada, K., 1999. The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annual Review of Plant Physiology and Plant Molecular Biology 50, 601–639.

Axelrod, B., Cheesebrough, T.M., Laakso, S., 1981. Lipoxygenase from soybeans. EC 1. 13. 11. 12 linoleate:oxygen oxidoreductase. Methods in Enzymology 71, 441–451.

Bačić, T., Vidović, D., Popović, Ž., 1997. Contribution to knowledge about the leaf anatomy of the Croatian endemic taxon *Centaurea ragusina* L. subsp. *ragusina*. Natura Croatica 6, 367–380.

Bajji, M., Kinet, J.M., Lutts, S., 1998. Salt stress effects on roots and leaves of *Atriplex halimus* L. and their corresponding callus cultures. Plant Science 137, 131–142.

Barhoumi, Z., Djebali, W., Chaïbi, W., Abdelly, C., Smaoui, A., 2007. Salt impact on photosynthesis and leaf ultrastructure of *Aeluropus littoralis*. Journal of Plant Research 120, 529–537.

Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water stress studies. Plant and Soil 39, 205–207.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assay and an assay applicable to PAGE. Analytical Biochemistry 44, 276–287.

Ben Amor, N., Ben Hamed, K., Debez, A., Grignon, C., Abdelly, C., 2005. Physiological and antioxidant responses of the perennial halophyte *Crithmum maritimum* to salinity. Plant Science 168, 889–899, http://www.sciencedirect.com/science/article/pii/S0168945204004741 - aff1.

Ben Hamed, K., Castagna, A., Salem, E., Ranieri, A., Abdelly, C., 2007. Sea fennel (*Crithmum maritimum* L.) under salinity conditions: a comparison of leaf and root antioxidant responses. Plant Growth Regulation 53, 185–194.

Bor, M., Özdemir, F., Türkan, I., 2003. The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. Plant Science 164, 77–84.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Bréhélin, C., Kessler, F., van Wijk, K.J., 2007. Plastoglobules: versatile lipoprotein particles in plastids. Trends in Plant Science 12, 260–266.

Broetto, F., Lüttge, U., Ratajczak, R., 2002. Influence of light intensity and salt-treatment on mode of photosynthesis and enzymes of the antioxidative response system of *Mesembryanthemum crystallinum*. Functional Plant Biology 29, 13–23.

Calatayud, A., Barreno, E., 2004. Response to ozone in two lettuce varieties on chlorophyll a fluorescence, photosynthetic pigments and lipid peroxidation. Plant Physiology and Biochemistry 42, 549–555.

Cavalieri, A.J., Huang, A.H.C., 1979. Evaluation of proline accumulation in the adaptation of diverse species of marsh halophytes to the saline environment. American Journal of Botany 66, 307–312.

Cornic, G., Fresneau, C., 2002. Photosynthetic carbon reduction and carbon oxidation cycles are the main electron sinks for photosystem II activity during a mild drought. Annals of Botany 89, 887–894.

Debez, A., Koyro, H.W., Grignon, C., Abdelly, C., Huczermeyer, B., 2008. Relationship between the photosynthetic activity and the performance of Cakile maritime after long-term salt treatment. Physiologia Plantarum 133, 373–385.

Degl'Innocenti, E., Hafsi, C., Guidi, L., Navari-Izzo, F., 2009. The effect of salinity on photosynthetic activity in potassium-deficient barley species. Journal of Plant Physiology 166, 1968–1981.

del Río, L.A., Pastori, G.M., Palma, J.M., Sandalio, L.M., Sevilla, F., Corpas, F.J., Jiménez, A., López-Huertas, E., Hernandéz, J.A., 1998. The activated oxygen role of peroxisomes in senescence. Plant Physiology 116, 1195–1200.

Delauney, A.J., Verma, D.P.S., 1993. Proline biosynthesis and osmoregulation in plants. Plant Journal 4, 215–223.

Durnford, D.G., Price, J.A., McKim, S.M., Sarchfield, M.L., 2003. Light-harvesting complex gene expression is controlled by both transcriptional and post-transcriptional mechanisms during photoacclimation in *Chlamydomonas reinhardtii*. Physiologia Plantarum 118, 193–205.

Egert, M., Tevini, M., 2002. Influence of drought on some physiological parameters symptomatic for oxidative stress in leaves of chives (*Allium schoenoprasum*). Environmental and Experimental Botany 48, 43–49.

Flowers, T.J., Hajibagheri, M.A., Clipson, N.J.W., 1986. Halophytes. The Quarterly Review of Biology 61, 313–337.

Ghoulam, C., Foursy, A., Fares, K., 2002. Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. Environmental and Experimental Botany 47, 39–50.

Hester, M.W., Mendelssohn, I.A., McKee, K.L., 2001. Species and population variation to salinity stress in *Panicum hemitomon*, *Spartina patens*, and *Spartina alterniflora*: morphological and physiological constraints. Environmental and Experimental Botany 46, 277–297.

Hu, Y., Schmidhalter, U., 2005. Drought and salinity: a comparison of their effects on mineral nutrition of plants. Journal of Plant Nutrition and Soil Science 168, 541–549.

Kanofsky, J.R., Axelrod, B., 1986. Singlet oxygen production by soybean lipoxygenase isozymes. Journal of Biological Chemistry 261, 1099–1104.
Khan, M.A., Ungar, I.A., Showalter, A.M., 2000. The effect of salinity on the growth, water status, and ion content of a leaf succulent perennial halophyte, *Suaeda fruticosa* (L.) Forssk. Journal of Arid Environment 45, 73–84.
Koyro, H.-W, 1997. Ultrastructural and physiological changes in root cells of Sorghum plants (*Sorghum bicolor* × *S. sudanensis* cv. Sweet Sioux) induced by NaCl. Journal of Experimental Botany 48, 693–706.
Koyro, H.-W., 2002. Ultrastructural effects of salinity in higher plants. In: Laüchli, A., Lüttge, U. (Eds.), Salinity: Environment—Plants—Molecules. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 139–157.
Koyro, H.-W., 2006. Effect of salinity on growth, photosynthesis, water relations and solute composition of the potential cash crop halophyte *Plantago coronopus* (L.). Environmental and Experimental Botany 56, 136–146.
Krause, G.H., Weis, E., 1991. Chlorophyll fluorescence and photosynthesis: the basics. Annual Review of Plant Physiology and Plant Molecular Biology 42, 313–349.
Kura-Hotta, M., Satoh, K., Katoh, S., 1987. Relationship between photosynthesis and chlorophyll content during leaf senescence of rice seedlings. Plant and Cell Physiology 28, 1321–1329.
Kurkova, E.B., Kalinkina, L.G., Baburina, O.K., Myasoedov, N.A., Naumova, T.G., 2002. Responses of *Seidlitzia rosmarinus* to salt stress. Biological Bulletin 29, 221–228.
Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods in Enzymology 186, 464–478.
Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods in Enzymology 148, 350–382.
Longstreth, D.V., Nobel, P.S., 1979. Salinity effects on leaf anatomy. Plant Physiology 63, 700–703.
Lu, C., Zhang, J., 1998. Thermostability of photosystem II is increased in salt-stressed sorghum. Australian Journal of Plant Physiology 25, 317–324.
Lu, C., Zhang, J., 1999. Effects of water stress on photosystem II photochemistry and its thermostability in wheat plants. Journal of Experimental Botany 50, 1199–1206.
Lynch, D.V., Thompson, J.E., 1984. Lipoxygenase-mediated production of superoxide anion in senescing plant tissue. FEBS Letters 173, 251–254.
Maggio, A., Reddy, M.P., Joly, R.J., 2000. Leaf gas exchange and solute accumulation in the halophyte *Salvadora persica* grown at moderate salinity. Environmental and Experimental Botany 44, 31–38.
Marschner, H., 1995. Mineral Nutrition of Higher Plants, second ed. Academic Press, London.
Masojidek, J., Hall, D.O., 1992. Salinity and drought stress are amplified by high irradiance in sorghum. Photosynthetica 27, 159–**1**71.
Maxwell, K., Johnson, G.N., 2000. Chlorophyll fluorescence—a practical guide. Journal of Experimental Botany 51, 659–668.
Megdiche, W., Hessini, K., Gharbi, F., Jaleel, C.A., Ksouri, R., Abdelly, C., 2008. Photosynthesis and photosystem 2 efficiency of two salt-adapted halophytic seashore *Cakile maritima* ecotypes. Photosynthetica 46, 410–419.
Meloni, D.A., Oliva, M.A., Martinez, C.A., Cambraia, J., 2003. Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environmental and Experimental Botany 49, 69–76.
Mitsuya, S., Yano, K., Kawasaki, M., Taniguchi, M., Miyake, H., 2002. Relationship between the distribution of Na and the damages caused by salinity in the leaves of rice seedlings grown under a saline condition. Plant Production Science 5, 269–274.
Mittova, V., Tal, M., Volokita, M., Guy, M., 2002. Salt stress induces up-regulation of an efficient chloroplast antioxidant system in the salt-tolerant wild tomato species *Lycopersicon pennellii* but not in the cultivated species. Physiologia Plantarum 115, 393–400.
Mittova, V., Guy, M., Tal, M., Volokita, M., 2004. Salinity upregulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. Journal of Experimental Botany 55, 1105–1113.
Møller, I.M., 2001. Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annual Review of Plant Physiology and Plant Molecular Biology 52, 561–591.
Mukherjee, S.P., Choudhouri, M.A., 1983. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. Physiologia Plantarum 58, 166–170.
Munns, R., 2002. Comparative physiology of salt and water stress. Plant, Cell & Environment 25, 239–250.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology 15, 473–497.
Naab, O.A., Tamame, M.A., Caccavari, M.A., 2008. Palynological and physicochemical characteristics of three unifloral honey types from central Argentina. Spanish Journal of Agricultural Research 6, 566–576.
Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant and Cell Physiology 22, 867–880.
Netondo, G.W., Onyango, J.C., Beck, E., 2004. Sorghum and salinity: II. Gas exchange and chlorophyll fluorescence of sorghum under salt stress. Crop Science 44, 806–811.
Paramonova, N.V., Shevyakova, N.I., Kuznetsov, V.V., 2004. Ultrastructure of chloroplasts and their storage inclusions in the primary leaves of *Mesembryanthemum crystallinum* affected by putrescine and NaCl. Russian Journal of Plant Physiology 51, 86–96.
Parida, A.K., Das, A.B., 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxicology and Environmental Safety 60, 324–349.
Pieroni, A., Janiak, V., Dürr, C.M., Lüdeke, S., Trachsel, E., Heinrich, M., 2002. *In vitro* antioxidant activity of non-cultivated vegetables of ethnic Albanians in southern Italy. Phytotherapy Research 16, 467–473.
Qiu, N., Lu, Q., Lu, C., 2003. Photosynthesis, photosystem II efficiency and the xanthophyll cycle in the salt-adapted halophyte *Atriplex centralasiatica*. New Phytologist 159, 479–486.
Radić, S., Radić-Stojković, M., Pevalek-Kozlina, B., 2006. Influence of NaCl and mannitol on peroxidase activity and lipid peroxidation in *Centaurea ragusina* L. roots and shoots. Journal of Plant Physiology 163, 1284–1292.
Reddy, M.P., Vora, A.B., 1986. Changes in pigment composition, Hill reaction activity and saccharides metabolism in Bajra (*Pennisetum typhoides* S & H) leaves under NaCl salinity. Photosynthetica 20, 50–55.
Reddy, A.R., Chaitanya, K.V., Vivekanandan, M., 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. Journal of Plant Physiology 161, 1189–1202.
Rusak, G., Robinson, N., Pepeljnjak, S., 2002. Antibacterial and antifungal activity of extracts and quercetagetin derivate isolated from *Centaurea rupestris* L. (Asteraceae). Acta Biologica Cracoviensia Series Botanica 44, 169–174.
Schöniger, W., 1955. Eine mikroanalytische Schnellbestimmung von Halogen in organischen Substanzen. Microchimica Acta 43, 123–129.
Short, D.C., Colmer, T.D., 1999. Salt tolerance in the halophyte *Halosarcia pergranulata* subsp. *pergranulata*. Annals of Botany 83, 207–213.
Silveira, J.A.G., Araújoa, S.A.M., Lima, J.P.M.S., Viégas, R.A., 2009. Roots and leaves display contrasting osmotic adjustment mechanisms in response to NaCl-salinity in *Atriplex nummularia*. Environmental and Experimental Botany 66, 1–8.
Slama, I., Ghnaya, T., Hessini, K., Messedi, D., Savoure, A., Abdelly, C., 2007a. Comparative study of the effects of mannitol and PEG osmotic stress on growth and solute accumulation in *Sesuvium portulacastrum*. Environmental and Experimental Botany 61, 10–17.
Slama, I., Ghnaya, T., Messedi, D., Hessini, K., Labidi, N., Savoure, A., Abdelly, C., 2007b. Effect of sodium chloride on the response of the halophyte species *Sesuvium portulacastrum* grown in mannitol-induced water stress. Journal of Plant Research 120, 291–299.
Sofo, A., Dichio, B., Xiloyannis, C., Masia, A., 2004. Lipoxygenase activity and proline accumulation in leaves and roots of olive trees in response to drought stress. Physiologia Plantarum 121, 58–65.
Türkan, I., Bor, M., Özdemir, F., Koca, H., 2005. Differential response of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. Plant Science 168, 223–231.
Ueda, A., Kanechi, M., Uno, Y., Inagaki, N., 2003. Photosynthetic limitations of a halophyte sea aster (*Aster tripolium* L.) under water stress and NaCl stress. Journal of Plant Research 116, 65–70.
Varadi, G., Polyanka, H., Darko, E., Lehoczki, E., 2003. Atrazine resistance entails a limited xanthophylls cycle activity, a lower PSII efficiency and altered pattern of excess excitation dissipation. Physiologia Plantarum 118, 47–56.
Verbruggen, N., Hermans, C., 2008. Proline accumulation in plants: a review. Amino Acids 35, 753–759.
Wang, L.-W., Showalter, A.M., Ungar, I.A., 1997. Effect of salinity on growth, ion content and cell wall chemistry in *Atriplex prostrata* Boucher. American Journal of Botany 84, 1247–1255.
Wang, D., Wang, H., Han, B., Wang, B., Guo, A., Zheng, D., Chongjing, L., Chang, L., Peng, M., Wang, X., 2012. Sodium instead of potassium and chloride is an important macronutrient to improve leaf succulence and shoot development for halophyte *Sesuvium portulacastrum*. Plant Physiology and Biochemistry 51, 53–62.
Woodrow, I.E., Berry, J.A., 1988. Enzymatic regulation of photosynthetic CO_2 fixation in C3 plants. Annual Review of Plant Physiology and Plant Molecular Biology 39, 533–594.
Zekri, M., 1995. PEG stress altered citrus root and leaf mineral concentrations. Journal of Plant Nutrition 18, 1087–1102.
Yeo, A.R., 1983. Salinity resistance: physiologies and prices. Physiologia Plantarum 25, 214–222.