Salt tolerance of *Centarea 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 *Centarea 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 isosmotic concentrations of NaCl (150 mM) and mannitol were compared and discussed in order to discriminate possible differences in *C. ragusina* response to toxic 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* 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.

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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 reox 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

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**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 to 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 rugosa 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 cuticule 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. rugosa 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. rugosa, 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. rugosa 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, LHCl, LHII and FNR), and certain detoxifying enzymes and antioxid. In our previous investigation, a great accumulation of H2O2 in mannitol-treated C. rugosa 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 lipoxigenase, 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. rugosa seeds were collected from their natural habitat near Dubrovnik (rock formation Konavoske stijene – locality Pašača). 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 (GAs), 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 mM−1), 300 (30.44 mM−1), 450 (44.15 mM−1) and 600 mM (55.08 mM−1) NaCl or 300 mM mannitol (2.38 mM−1), corresponding to osmotic potentials −0.85, −1.5, −2.44, −3.0 or −0.84 MPa, respectively. Control (2.52 mM−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. rugosa 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 HNO3 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 Axiosvert 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 (F0) 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 Fm). The radiation was maintained until both F and Fm were stable. Calculations of fluorescence parameters – maximum quantum yield of the PS II (Fi/Fm), the effective quantum yield of the PS II (ΔF/ΔFm), nonphotochemical quenching (NPQ) and relative electron transport rate (reETR) – 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 polyvinyl polypyrrolidone (PVPP, Sigma–Aldrich) and then centrifuged at 25,000 g for 30 min. Total protein concentration was determined using bovine albumin serum as standard. Aliquots of each homogenate were mixed with corresponding volumes of denaturing 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 bromophenol 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, electrophoresed 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 Na2HPO4, 17 mM NaH2PO4, 68 mM NaCl) pH 7.4 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 (LHCCI) (dilution 1:500) and mouse anti-light–harvesting complex I of PSII (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-dinitrophenyhydrazine (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 hydrazine.

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 Beaufhemp 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 H2O2 and was measured following the method of Aebi (1984). Lipoygenase 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 Cragusina 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 Cragusina 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 relative 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%
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. rugusina* 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. rugusina* 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 *Cragusina* 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 plastoglobuli (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 ΔF/ΔFm (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 *Cragusina* plants (Fig. 3a). When grown at 150 mM NaCl, a 35% and 30% decline of chlorophylls and carotenoids, respectively, in *Cragusina* plants was observed (Fig. 3a). At higher salinities, the reduction of Chl b and

![Fig. 1.](image)

**Fig. 1.** (a) RWC (%), (b) RGR (g), and (c) proline content of *C. rugusina* 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 period. Values are mean ± SE based on six replicates. Bars with different letters are significantly different at *p* < 0.05.

**Table 1**

Contents of Na, Cl, K, Ca and Mg (mg g⁻¹ DW) of *C. rugusina* plants after 15-day period growth.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Na</th>
<th>Cl</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.5 (0.1)c</td>
<td>18.8 (1.7)d</td>
<td>26.6 (2.8)a</td>
<td>4.0 (0.2)a</td>
<td>1.2 (0.2)a</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>70.1 (8.6)b</td>
<td>118.9 (6.0)c</td>
<td>19.1 (3.5)b</td>
<td>2.6 (0.2)c</td>
<td>0.8 (0.09)b</td>
</tr>
<tr>
<td>300 mM NaCl</td>
<td>138.0 (17.3)a</td>
<td>168.5 (5.9)b</td>
<td>16.3 (2.9)b</td>
<td>2.1 (0.1)bc</td>
<td>0.7 (0.04)b</td>
</tr>
<tr>
<td>450 mM NaCl</td>
<td>132.9 (11.5)a</td>
<td>192.3 (5.9)c</td>
<td>13.7 (0.8)b</td>
<td>1.8 (0.2bc)</td>
<td>0.6 (0.05)b</td>
</tr>
<tr>
<td>600 mM NaCl</td>
<td>120.4 (11.2)a</td>
<td>198.0 (20.6)a</td>
<td>12.3 (0.9)b</td>
<td>1.4 (0.1)c</td>
<td>0.6 (0.06)b</td>
</tr>
<tr>
<td>300 mM mannitol</td>
<td>1.2 (0.1)c</td>
<td>28.3 (5.0)d</td>
<td>14.3 (0.5)b</td>
<td>1.4 (0.2)c</td>
<td>0.6 (0.06)b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Root</th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0 (0.2)e</td>
<td>17.5 (2.6)d</td>
<td>30.3 (1.1)a</td>
<td>1.6 (0.03)a</td>
<td>1.2 (0.08)a</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>22.7 (0)de</td>
<td>55.2 (3.2)c</td>
<td>17.9 (1.2)c</td>
<td>1.0 (0.03)c</td>
<td>0.7 (0.02)c</td>
</tr>
<tr>
<td>300 mM NaCl</td>
<td>31.6 (2.1)c</td>
<td>86.5 (6.9)b</td>
<td>11.9 (0.9)d</td>
<td>1.0 (0.03)d</td>
<td>0.5 (0.03)e</td>
</tr>
<tr>
<td>450 mM NaCl</td>
<td>50.1 (3.1)bc</td>
<td>102.9 (11.8)b</td>
<td>9.4 (0.4)d</td>
<td>0.8 (0.05)d</td>
<td>0.4 (0.04)d</td>
</tr>
<tr>
<td>600 mM NaCl</td>
<td>82.2 (8.6)a</td>
<td>132.4 (8.6)a</td>
<td>6.8 (0.4)e</td>
<td>0.8 (0.04)d</td>
<td>0.4 (0.03)d</td>
</tr>
<tr>
<td>300 mM mannitol</td>
<td>2.2 (0.1)e</td>
<td>23.5 (3.4)d</td>
<td>22.5 (0.7)b</td>
<td>1.5 (0.08)ab</td>
<td>0.8 (0.05)b</td>
</tr>
</tbody>
</table>

Values represent mean ± S.D. (parenthesis) of 3 replicates. Different letters indicate significant difference at *p* < 0.01.
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, LHCII, LHII and FNR proteins

Large subunit of Rubisco accumulated less with increasing NaCl concentrations (Fig. 4). The intensity of other photosynthetic
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 LHCl protein was not detected under the treatment.

3.6. Carboxyl groups contents and LOX activity

The level of oxidatively damaged proteins, expressed as carboxyl groups content, in C. rugusina 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. rugusina 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).

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. rugusina 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
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 inductor (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 *Cragusina* 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 (Cavaleri and Huang, 1979; Dellauney 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).

Fig. 5. (a) carbonyl groups content (C), (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 period. Values are mean ± SE based on six replicates. Bars with different letters are significantly different at p < 0.05.
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. rugusina 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 water uptake, 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. rugusina 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 PSI 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 centralasisatica, Sorgum bicolor (Qiu et al., 2003; Netondo et al., 2004; Megdiche et al., 2008; Deg'IInnocenti et al., 2009). However, the unimpairred Fv/Fm values in salt-treated C. rugusina were accompanied by a significant decrease in effective quantum yield (ΔF/ΔFm) and reduced electron transport activity. The reductions in ΔF/ΔFm and reETR were correlated with an increase in NPQ which may indicate that reduced CO2 assimilation decreases demand for products of electron transport, and thus increases thermal dissipation of light energy at PSII 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 (Masojdeek 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 burst of oxidative stress. 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 PSI/PSII 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 LHCl, 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 (Alakherdiev 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 plastoglobulins 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, ΔF/ΔFm and reETR. The downregulation of PSII was paralleled with partial or total inhibition of LHCs and D1 proteins synthesis though the FNR and Rubisco protein contents were reduced less as well (Fig. 4). In the study of Lu and Zhang (1999) the loss of PSI 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 CO2 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 plastoglobulins observed under higher salt and mannitol treatments might be indication of stored lipid breakdown products (Paramonova et al., 2004; Bréhelin et al., 2007). The formation of plastoglobuli is thought to be linked to the breakdown of thylakoids that accompanies senescence (del Rio et al., 1998). The accumulation of microbodies such as glyoxysomes, observed under higher saline treatments is also indicative of senescence processes (Koyro, 1985).

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 Rio 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 (Kanoisky 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 malondialdehyde (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. rugusina 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 (Radic 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 H2O2, was recorded in both plant organs under all treatments except in C. rugosa leaves exposed to higher salt concentrations. The result implies that both salt or mannitol increase the formation of superoxides in C. rugosa leaves and roots probably as a consequence of the inhibition of Calvin cycle and increased respiration in root mitochondria, respectively (Moller, 2001; Mithoova et al., 2004). In plants, CAT, APX and GPX are considered the most important in degradation of H2O2 (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; Mithoova et al., 2003; Turkan et al., 2005; Ben Hamed et al., 2007).

The observed low levels of H2O2 in salt-treated C. rugosa leaves (Radic 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 H2O2 detoxification i.e. ascorbate consumption as an electron donor for APX. In response to higher saline conditions, APX and GPX (Radic et al., 2006) constituted the system responsible for the elimination of H2O2, 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. rugosa leaves under 450 and 600 mM NaCl. In roots, H2O2 was eliminated by CAT and GPX under lower and by APX under higher saline conditions, respectively. On the other hand, the significant increase in H2O2 (Radic et al., 2006) and carboxyl groups contents (Fig. 5) was recorded after 15-day period in response to mannitol despite increased GPX (Radic et al., 2006) and APX activities as well as high ascorbate content. Those results suggest that C. rugosa is equipped with balanced and responsive antioxidant system which proved to be highly inducible even under hypersalinity conditions induced by mannitol or hypoxic conditions reached at higher salinities.

In conclusion, that 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. rugosa 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. rugosa 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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