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Differential responses of components of the antioxidant defense system to high salinity stress in the lesser duckweed (*Lemna minor* L.)

Laszlo Fodorpataki[1,✉], Szabolcs Barna[1] and Botond Holinka[1]

SUMMARY. Salt stress causes oxidative damage in plants, and it induces protective mechanisms through enzymatic and non-enzymatic components of the antioxidant system. Different components of this system exhibit specific degrees of tolerance toward certain salt concentrations. Their differential responses may contribute not only to a better understanding of the functional interconnections in the antioxidant defense system, but also to a more efficient selection of physiological and biochemical markers of stress reactions of plants, in the effort for an early and precise bioindication of oxidative damage caused by high salinity of the environment. In this context, the molar ratio between the reduced and the oxidized form of ascorbic acid is a more sensitive marker of oxidative stress than the total amount of this vitamin in the biomass of lesser duckweed. Glutathione content exhibits a more moderate variation with increasing salt stress than the concentration of carotenoid pigments in the fronds exposed to constant photon flux density. From among the antioxidant enzymes, ascorbate peroxidase was found to be the most sensitive, and superoxide dismutase was the most resistant to oxidative stress caused by increasing salinity. Catalase and glutathione reductase activities decreased under severe salt stress. Efficiency of the antioxidant system can be monitored by membrane damage through lipid peroxidation. Antioxidants of duckweed are useful tools for indication of increasing salinity of aquatic environments.

Keywords: ascorbate, carotenoids, protective enzymes, salt stress.

Introduction

A large variety of environmental stress factors exerts convergent changes in plant metabolism by inducing oxidative damage, this is why antioxidant defense is a basic manifestation of cross-tolerance for different abiotic and biotic stresses.

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Oxidative stress is caused by over accumulation of harmful reactive oxygen species, generated by disturbances in basic physiological processes, such as the light reactions of photosynthesis, photorespiration, aerobic respiration, and other oxidative processes (Apel and Hirt, 2004; Pogany *et al.*, 2006; Smirnoff, 2005). High amounts of singlet oxygen, superoxide radical, hydrogen peroxide, alkyl-peroxides and hydroxyl radical are produced in plants exposed to drought, heavy metals, air pollutants, certain pesticides, excessive photon flux density and UV-B radiation, extreme temperatures, as well as by increased salinity of terrestrial and aquatic habitats (Fodorpataki *et al.*, 2014; Khanna-Chopra and Selote, 2007; Mittler, 2002; Wang *et al.*, 2009). While low concentrations of reactive oxygen species have a useful role in signaling of developmental and environmental changes, their high amounts are chemically harmful to vital biomolecules, such as unsaturated fatty acids in membrane lipids, chlorophylls, nucleic acids and proteins. As a physiological response to the oxidative damage, concerted changes occur in plant metabolism, in order to develop stress tolerance. The network of antioxidative protection processes, which relies on the pronounced metabolic plasticity of plants during hardening, represents a key mechanism of cross-tolerance, which enables plants to defend themselves against various environmental stress factors (Chattopadhyay, 2014; Laloi *et al.*, 2004; Shah *et al.*, 2001). The knowledge of how oxidative stress modulates plant metabolic processes during the physiological acclimation to adverse growth conditions, enables us to monitor the early effects of environmental changes, and to influence plant production in the direction of inducing accumulation of protective metabolites which not only confer a better survival of plants and a sustained primary production in terrestrial and aquatic ecosystems, but also possess health-promoting qualities for consumers, including humans (Mahmoudi *et al.*, 2010; Oh *et al.*, 2009; Pallag *et al.*, 2009; Rios *et al.*, 2008).

Salt stress is one of the most frequent environmental impacts that impair plant development in both aquatic and terrestrial habitats, in connection with global climate warming that enhances evaporation of water (Djanaguiraman and Prasad, 2013). Beside osmotic dehydration and chemical toxicity of sodium ions, salt stress increases the amount of reactive oxygen species in plants. This is why salinity tolerance is related to an increased amount and activity of antioxidants, while salt sensitivity is associated with down-regulation of protective enzymes involved in detoxification of oxygen radicals and peroxides (Bartha *et al.*, 2011; Bordi, 2010; Zushi *et al.*, 2009). An early detection of changes in the quality of aquatic environments, caused by increased salinity and by different agents of water pollution, is possible by using biochemical markers related to oxidative stress in test organisms such as various algae and duckweed (Fodorpataki and Bartha 2008; Karatas *et al.*, 2009; Radic *et al.*, 2011; Tkalec *et al.*, 2007; Zhang *et al.*, 2011).

The aim of the present work is to reveal differences in the activity of antioxidant enzymes and in the dynamics of protective biomolecules in the lesser duckweed exposed to different degrees of salt stress, in order to identify those biochemical markers that are most suitable for an efficient bioindication of oxidative stress induced by increased salinity of the aquatic environment.

Materials and methods

Plant material and growth conditions. Lesser duckweed (*Lemna minor* L.) individuals were collected from a small lake in Ernei (Mureş county, Romania), rinsed with 10 mM NaOCl for 30 s, and introduced in axenic cultures grown in Steinberg's inorganic nutrient medium (Fodorpataki *et al*., 2014) in an environmental test chamber (MLR-351H, Sanyo), at 22 ºC and a constant illumination with a photon flux density of 330 μM m^{-2} s^{-1} provided by white fluorescent lamps. Experimental variants were set up under the same conditions in Petri dishes, in 5 replicas, the starting cultures containing 500 individuals with one fully developed frond. Duckweed cultures were treated for 7 days with 40 mM, 80 mM, 120 mM or 160 mM of sodium chloride (p.a.), the control being grown in pure Steinberg solution.

Ascorbic acid content and reduced ascorbate to oxidized dehydroascorbate ratio. Ascorbic acid (vitamin C) content was determined according to Kampfenkel *et al.* (1995). 0.5 g of duckweed (fresh weight) was homogenized in a prechilled mortar with 4 mL of 6% trichloroacetic acic (TCA), than centrifuged for 15 min at 4 ºC with 15600 g. 200 μL of supernatant was introduced in sodium phosphate buffer (pH 7.4) containing TCA, dithiothreitol, ethanolic solution of 2,2'-dipyridyl, ortophosphoric acid, and completed with N-ethylmaleimide and iron(III) chloride. After 1 h incubation at 42 ºC with continuous mixing, absorbance of the mixture was measured at 525 nm. Standard curve was obtained with 25-100 nM ascorbic acid dissolved in 6% TCA. The assay is based on reduction of ferric ions to Fe(II) by reduced ascorbate, then Fe(II) forms a coloured complex with 2,2'-dipyridyl. Dehydroascorbate is reduced to ascorbate by dithiothreitol, the excess of the latter isremoved with N-ethylmaleimide, and total ascorbic acid is determined spectrophotometrically by the 2,2'-dipyridyl method. Concentration of dehydroascorbate is calculated from the difference of total ascorbic acid and reduced ascorbate (without pretreatment with dithiothreitol).

Glutathione content. Glutathione concentration in duckweed extract was determined according to Razinger *et al.* (2008). 0.5 g fresh plant material was homogenized in 3 ml of 5% sulfosalicylic acid and centrifuged for 10 min at 4 ºC with 14000 g. 0.1 mL of supernatant was supplemented with 2 μL 3-ethanolamine and 2 μL 2-vinylpyridine and incubated 1 h at room temperature for determining the oxidized glutathione. Glutathione concentration was measured as increase of absorbance at 412 nm as a result of reduction of 5,5-dithio-bis(2-nitrobenzoic acid), in a reaction mixture that also contained potassium phosphate buffer (pH 7.5), EDTA and NADPH. After incubation of the reaction mixture for 10 min at 30 ºC, 10 μL of glutathione reductase (50 units mL^{-1}) were added to 50 μL sample.

Carotenoid pigment determination. 0.25 g fresh weight of duckweed fronds were immersed in 5 ml dimethylformamide and kept for 48 h in darkness until complete extraction. The extract was centrifuged for 10 min at 4000 g, and the carotenoid content of the supernatant was determined spectrophotometrically (with a V-530 UV-Vis Spectrophotometer, Jasco), based on its absorbance at 480 nm (Zhang *et al*., 2013).

Ascorbate peroxidase (APX) activity. Determination of APX activity was performed through the oxidation of ascorbic acid initiated by addition of hydrogen peroxide, and measured by decrease in the absorbance of the reaction mixture at 290 nm. 0.5 g fresh duckweed was ground in a prechilled mortar with 5 mL extraction solution containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM Na_2-EDTA, 1 mM ascorbate and 2% water-soluble polyvinyl-pyrrolidone. The homogenate was centrifuged at 15000 g for 20 min, then 50 µL of supernatant was resuspended in a mixture of 1.75 mL phosphate buffer (pH 7.8) containing 1 mM Na_2-EDTA and 0.1 mL of 10 mM ascorbic acid. The reaction was initiated by the addition of 0.1 mL of 20 mM hydrogen peroxide, and after a period of 40 s the decrease of absorbance at 290 nm was measured for 3 min. Reference mixture contained distilled water instead of hydrogen peroxide, an extinction coefficient of 2.8 mM^{-1} cm^{-1} was used for ascorbic acid, and the APX activity was expressed as scavenged hydrogen peroxide in unit of time per unit of protein quantity.

Protein content of duckweed was determined with Bradford's method, using bovine serum albumine as standard (Bartha *et al.*, 2010).

Superoxide dismutase (SOD) assay. 0.5 g of fresh duckweed was ground in a prechilled mortar with 2.5 mL extraction solution consisting of 50 mM potassium phosphate buffer (pH 7), 1 mM Na_2-EDTA, 1 mM ascorbic acid and 2% water-soluble polyvinyl-pyrrolidone. The homogenate was centrifuged for 20 min at 15000 g and the obtained supernatant was used as the source of enzyme. Determination of SOD activity was based on the fact that in the presence of riboflavine and light, SOD inhibits the formation of formazane from nitro blue tetrazolium (NBT). 0.1 mL of enzyme extract from duckweed was introduced in 3 mL of reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 15 mM methionine, 0.1 mM Na_2-EDTA, then 0.1 mL of 5 mM NBT and 0.1 mL of 0.2 mM riboflavine were added. The mixture was intensely illuminated for 15 min, then absorbance of the generated formazane was measured at 560 nm. References were the mixtures kept in darkness, and blank samples contained no enzyme extract. One enzyme unit is the amount that inhibits by 50% the reduction of NBT to formazane in the presence of light. The specific SOD activity was expressed as enzyme units in 1 mg protein content of the plant extract, and protein content was determined as mentioned above for ascorbate peroxidase (Eraslan *et al*., 2007).

Catalase (CAT) activity. Enzymatic activity of catalase was determined spectrophotometrically, by measuring change of absorbance at 240 nm due to consumption of hydrogen peroxide during 1 min at 22 ºC, in a mixture containing plant extract corresponding to 10 µg protein in 1 mL of 50 mM potassium phosphate buffer (pH 7.5), and 0.1 mL of 200 mM hydrogen peroxide to start the reaction (Sairam *et al*., 2005). Protein content was determined as for ascorbate peoxidase.

Glutathione reductase (GR) assay. Glutathione reductase activity was determined by the increase in absorbance at 412 nm due to formation of 2-nitro-5-thiobenzoic acid by reaction of reduced glutathione with 5,5-dithio-bis(2-nitro-benzoic acid). The

reaction mixture contained homogenized plant extract corresponding to 10 μg protein, 1 mM EDTA, 2 mM NADPH and 15 mM 5,5-dithio-bis(2-nitrobenzoic acid) in 0.7 mL of 0.2 M potassium phosphate buffer (pH 7.5), and was incubated at 22 ºC for 40 min, than the reaction was started by addition of 50 μL of 20 mM oxidized glutathione (GSSG) and change of absorbance was monitored for 15 min (Panda *et al*., 2003).

Lipid peroxidation assay. Lipid peroxidation was measured as the amount of malondialdehyde determined by the reaction with thiobarbituric acid (TBA). 0.5 g of duckweed (fresh weight) was homogenized in 10 mL of 0.1% TCA and centrifuged at 15000 g for 15 min. 1 mL of supernatant was mixed with 4mL of 0.5% TBA dissolved in 20% TCA, the mixture was incubated at 95 ºC for 30 min and cooled instantly in ice bath. After centrifugation at 10000 g for 10 min, the absorbance of the supernatant was measured at 532 nm and corrected for 600 nm. Malondialdehyde content was calculated according to its absorbance coefficient of 155 mM^{-1} cm^{-1} (Panda *et al.*, 2003).

Statistical analysis. Experimental data were statistically analyzed in R environment (version 2.14.1), using one-way ANOVA and the post-hoc Tukey HSD test for the significance of differences between treatments. The results were expressed as the mean ± standard error, and a value of $P < 0.05$ was considered to be statistically significant.

Results and discussion

Oxidative stress is a common side effect of high salinity, along with osmotic stress leading to imbalanced water status and with chemical toxicity of excess sodium ions that accumulate over time in plant cell. Increased generation of reactive oxygen species is also associated with various other environmental stress factors, being a main feature responsible for development of cross tolerance towards different external constraints. Plants defend themselves against oxidative damage with the concerted action of interconnected enzymatic and non-enzymatic antioxidants, induced specifically by overproduction of certain reactive oxygen species (Gill and Tuteja, 2010). Carotenoid pigments (especially some xanthophylls) prevent overproduction of singlet oxygen. Ascorbate, glutathione, ascorbate peroxidase and glutathione reductase interact in scavenging excessive amounts of hydrogen peroxide, superoxide dismutase detoxifies superoxide radicals, while tocopherol deactivates hydroxyl and alkyl-peroxyl radicals. Catalase, peroxidases and peroxiredoxins also contribute to protection against inorganic and organic peroxides. The various components of the antioxidative defense system of plants are induced by different stress signals and exhibit different levels of sensitivity to various concentrations of reactive oxygen species related to the degree of stress. This is why under a given stress condition the amount or the activity of different antioxidants vary according to various patterns, indicating different levels of sensitivity, tolerance or resistance. As a consequence, various antioxidants have different indicative values as

functional markers of stress status, and knowledge of their differential behaviour under a range of stress conditions enables a better understanding of the mechanism of acclimation processes, a more sensitive evaluation of the quality of environment for given organisms, as well as more efficient procedures of phytoremediation using these organisms. In this context, the ubiquitous duckweed is a well-suited indicator of water pollution for freshwater ecosystems (Parra *et al.*, 2012).

Ascorbic acid (vitamin C) is the most abundant antioxidant in the water-soluble phase of different cell compartments, its highest amounts being found in chloroplasts. It participates in the Halliwell-Asada-Foyer redox chain that scavenges hydrogen peroxide. When duckweed plants were exposed for several days to different degrees of salt stress, their total ascorbic acid content registered a moderate, but statistically significant increase in the presence of 80 mM and 120 mM NaCl, with a further increment at 160 mM (Fig. 1).

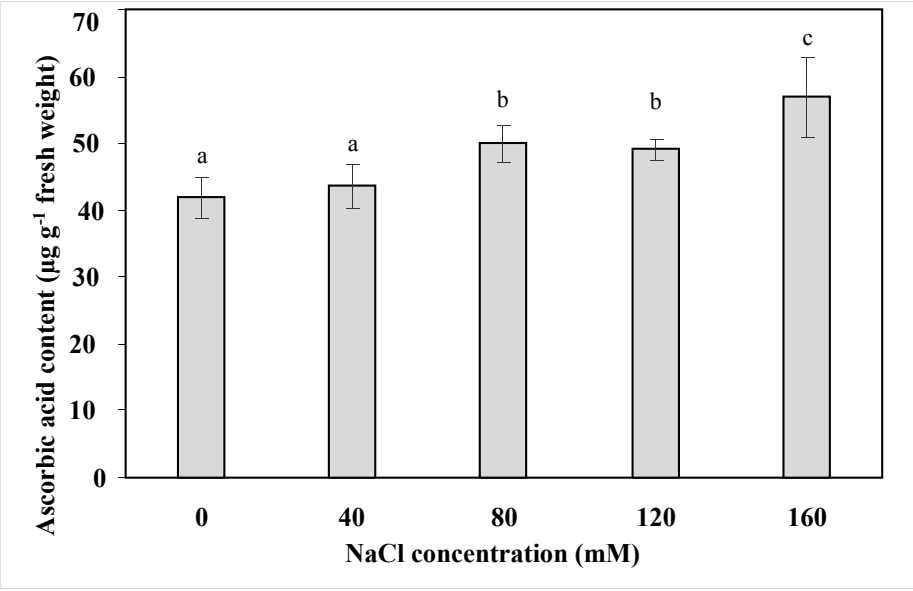

Figure 1. Ascorbic acid (vitamin C) content of duckweed (*Lemna minor* L.) fronds exposed for one week to different degrees of salinity stress. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

This result reflects that duckweed plants enhance the biosynthesis of vitamin C (from mannose and galactose) to cope with the increasing amount of hydrogen peroxide that has to be reduced to water. Increased ascorbic acid content was also reported in plants exposed to other stress conditions, such as high light intensity, UV irradiation, air pollution with sulfur dioxide, low temperature and drought stress (Kampfenkel *et al.*, 1995; Khanna-Chopra and Selote, 2007).

Because under several moderate stress conditions the total amount of ascorbic acid changes in a hardly detectable degree, the molar ratio between the reduced and the oxidized form of vitamin C might be a more sensitive marker of impaired metabolic homeostasis. In the case of duckweeds grown for one week in an aquatic environment containing increased amounts of sodium chloride, the reduced ascorbate to oxidized dehydroascorbate decreased in a higher extent than the variation of total ascorbic acid concentration, as salt stress became more intense (Fig. 2). While in control plants around 90% of vitamin C was in the reduced state, this percentage became reduced to 70-80% in the presence of 80 mM NaCl (moderate salt stress) and to approximately 50% at 160 mM NaCl (severe salt stress). For wheat it was demonstrated that in salinity tolerant genotypes this ratio exhibited a much moderate decrement than in susceptible ones (Sairam *et al*., 2005), while in lettuce the improved antioxidant capacity was correlated with maintainance of a higher ratio between the reduced and the oxidized form of ascorbic acid (Rios *et al*., 2008).

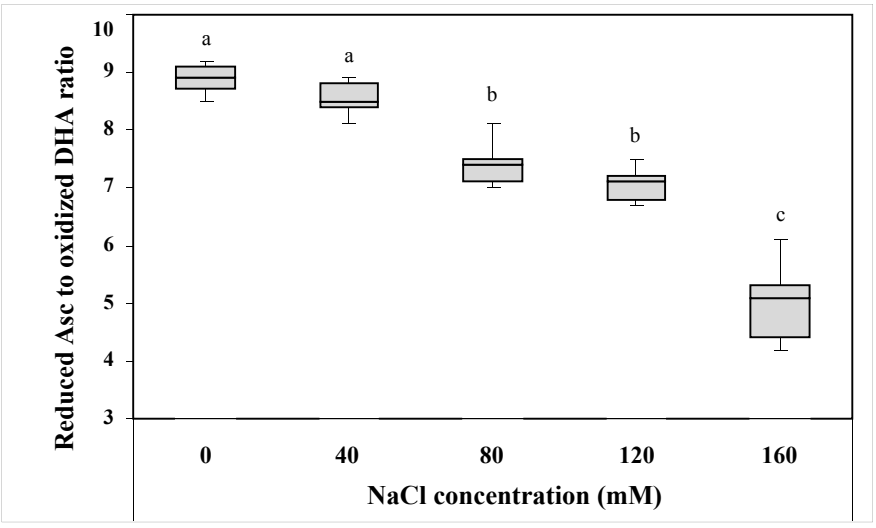

Figure 2. Molar ratio between the reduced ascorbate (Asc) and the oxidized dehydroascorbate (DHA) in fronds of duckweed exposed for one week to different concentrations of sodium chloride. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Along with ascorbic acid, glutathione (a tripeptide consisting of glutamic acid, cysteine and glycine) is another important non-enzymatic component of the antioxidant defense system, and plays a crucial role in the redox homeostasis of plant cells. By ensuring regeneration of the reduced vitamin C during the scavenging of hydrogen

peroxide molecules, glutathione ensures the continuous functioning of the Halliwell-Asada-Foyer redox chain, while it also contributes to protection of several functional proteins against oxidative damage (Chattopadhyay, 2014; Rouhier *et al*., 2008). Our experiments showed that the free glutathione level is kept at a constant level in cells of duckweed exposed to salinity, significantly higher glutathione content being registered only at salt concentrations that reached or exceeded 120 mM (Fig. 3). As a consequence, under the experimental conditions described above, glutathione level in duckweed is a less sensitive biochemical indicator of salt stress as compared to ascorbic acid. More intense changes in glutathione content were reported in lettuce leaves exposed to lower levels of salinity (Mahmoudi *et al*., 2010), and even for duckweed exposed for short time to cadmium toxicity (Razinger *et al*., 2008).

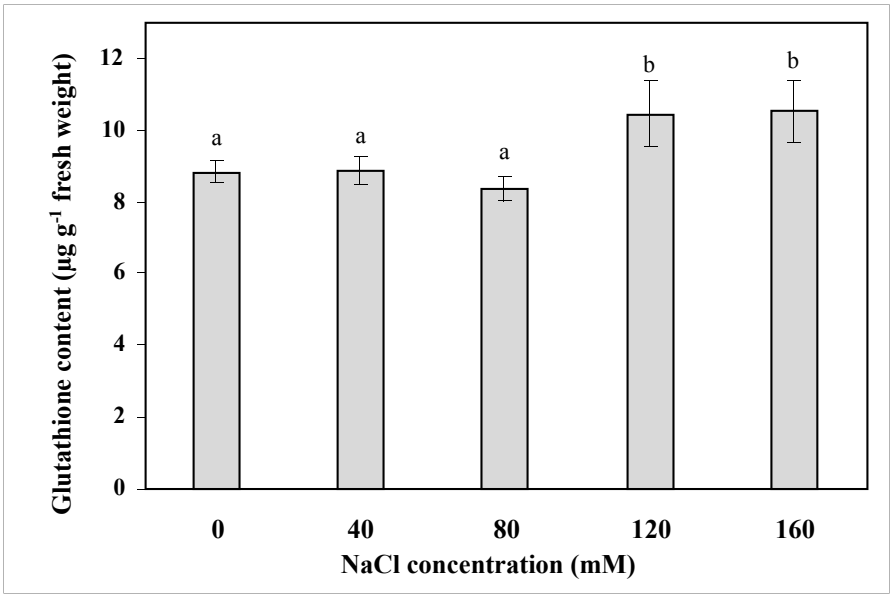

Figure 3. Glutathione content of duckweed (*Lemna minor* L.) fronds exposed for one week to different degrees of salinity stress. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Carotenoid pigments, synthesized by plants as accessory photosynthetic pigments of the thylakoid membranes of chloroplasts, also have an important antioxidative function, especially under excessive photon flux densities, when limitation of energy use in carbon assimilation leads to photooxidative damage. Increasing amounts of certain carotenoids (especially the zeaxanthin and antheraxantin components of the xanthophyll cycle) prevent formation of singlet oxygen by dissipating the excess light energy

absorbed by chlorophylls, and inactivate the newly generated singlet oxygen in a reaction followed by heat dissipation from the excited carotenoid molecule. As hydrophobic, membrane-integrated molecules, carotenoids play a determining role, together with vitamin E, in preventing oxidative damages of membrane lipids, thus in maintaining integrity, selective permeability and normal functions of biomembranes (Mittler, 2002; Zushi *et al*., 2009). Our results show that total carotenoid pigment content of duckweed plants is maintained at a relatively constant level until salt stress becomes severe (120-160 mM NaCl for 7 days), when carotenoid level significantly increases as part of the antioxidant defense mechanisms that tends to protect thylakoid membranes against photooxidative damage. The higher carotenoid content makes salt-stressed duck-weed a more valuable food source for its consumers, because carotenoids are general health-promoting metabolites for all living organisms (Smirnoff, 2005).

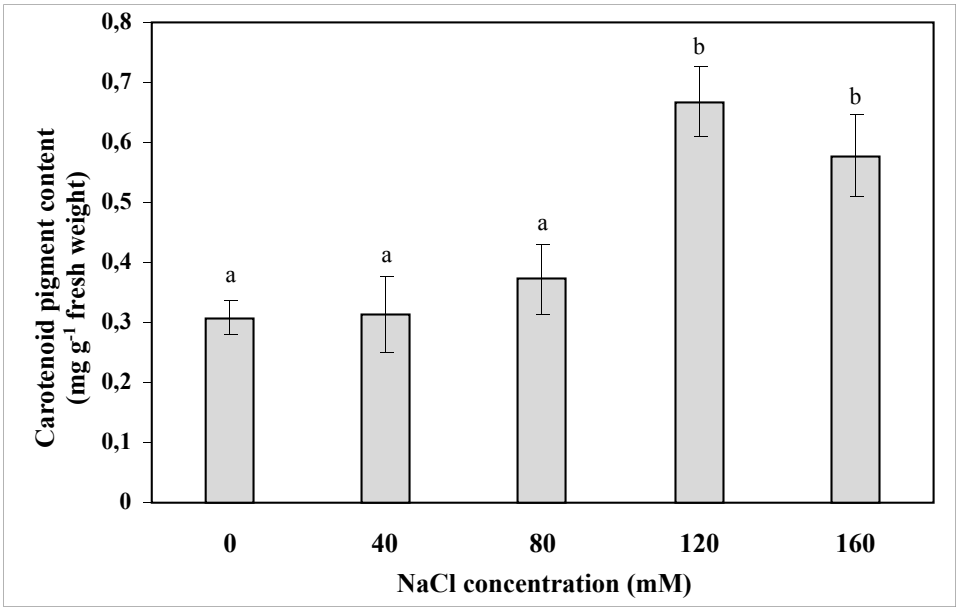

Figure 4. Carotenoid pigment content of duckweed (*Lemna minor* L.) fronds exposed for one week to different concentrations of sodium chloride. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Several non-enzymatic antioxidants act together with enzymes that catalyze the transformation of reactive oxygen species into nontoxic products. In many cases these enzymes are associated in functional complexes (metabolomes) to increase the efficiency of antioxidant protection. E. g. the hydrogen peroxide generated by superoxide dismutase is scavenged by ascorbate peroxidase, while dehydroascorbate reductase and glutathione

reductase regenerate the reduced form of ascorbate and glutathione needed for continuous protection against newly generated peroxide molecules. These enzymes have different levels of sensitivity towards oxidative stress and their catalytic activity is down- or up-regulated according to the redox state of the cell compartments in which their function is induced by several environmental constraints (Pogany *et al*., 2006).

Ascorbate peroxidase (APX) has different isozymes in chloroplasts, mitochondria, peroxisomes, cytosol and cell wall, the cytosolic one being mostly sensitive to oxidative stress. Our experiments revealed that APX activity is very sensitive to different degrees of salt stress, and therefore is a very suitable marker of stress tolerance when duckweeds are exposed for 7 days to various salinity levels. APX activity increased progressively with salinity up to 120 mM NaCl, registering significant differences among the plants exposed to different salt concentrations. Under conditions of severe salt stress caused by 160 mM NaCl, APX activity decreases below the values of control plants, reflecting its pronounced sensitivity to high salt stress (Fig. 5). This down regulation is most probably related to the lowered amount of reduced ascorbate under high salinity conditions, considering that APX activity is dependent on the concentration of reduced vitamin C. Its inhibited activity at 160 mM NaCl may be also related to overaccumulation of hydrogen peroxide, taking into account that APX scavenges hydrogen peroxide in its micromolar concentration range, being inactivated by higher amounts of this reactive oxygen derivative (Shigeoka *et al*., 2012).

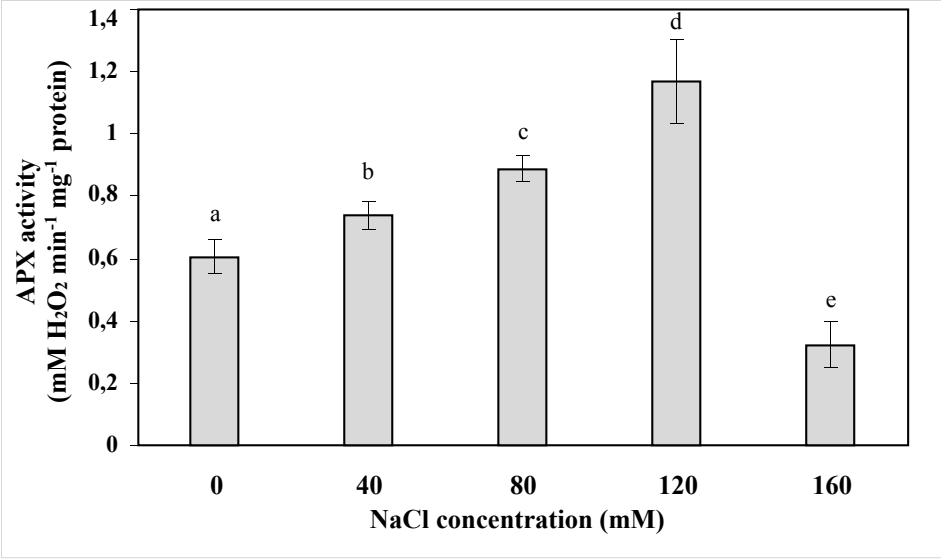

Figure 5. Hydrogen peroxide-scavenging activity of ascorbate peroxidase (APX) in fronds of duckweed exposed for one week to different degrees of salinity stress. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Superoxide dismutase (SOD) also has several isozymes in different plant cell compartments, and in contrast with ascorbate peroxidase, it is a very stable enzyme (because of very few α helices and several β sheets in its molecular structure) and exhibits a very high catalytic rate, being inactivated only by millimolar amounts of hydrogen peroxide that forms during conversion of superoxide radicals (Alscher *et al*., 2012). In this context, it is explainable that under our experimental conditions the SOD activity did not exhibit significant modification upon mild stress exerted by 40 mM NaCl, it increased moderately under exposure of duckweed to 80 mM and 120 mM NaCl, and at the very high salinity level of 160 mM, instead of inhibition (as in case of APX) it registered a significant increase in the catalytic activity (Fig. 6). Under conditions of more moderate salt stress, several authors have found in different terrestrial plants that SOD level does not change significantly (Bartha *et al*., 2011; Mahmoudi *et al*., 2010; Wang *et al*., 2009).

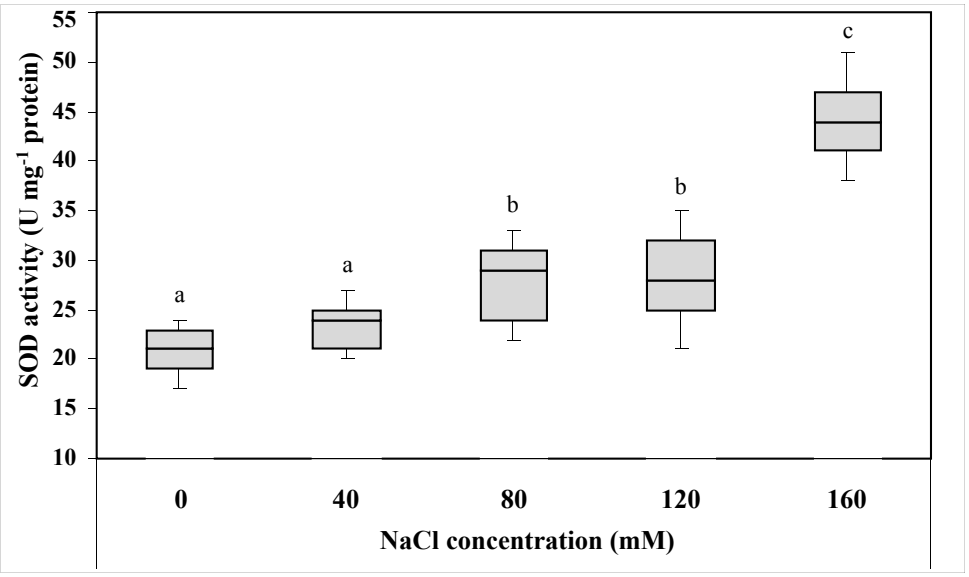

Figure 6. Superoxide dismutase (SOD) activity in duckweed (*Lemna minor* L.) fronds exposed for one week to different concentrations of sodium chloride. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

While ascorbate peroxidase regulates hydrogen peroxide level in the micromolar range, catalase (CAT) has a low affinity to hydrogen peroxide, so it scavenges it only when it accumulates in micromolar concentrations in micro-bodies of plant cells (peroxisomes, glyoxysomes, uricosomes). It is easily photo-inactivated, and as a compensation for this, it has a high turnover rate (Mittler, 2002). Because higher amounts

of hydrogen peroxide accumulate under more severe salt stress, in duckweed exposed for a period as long as 7 days to different salinity levels, one can notice that 40 mM and 80 mM NaCl do not induce significant changes in catalase activity as compared to control conditions, 120 mM NaCl induces an obvious increase of this enzyme's catalytic activity, while severe salt stress caused by exposure to 160 mM results in drastical inhibition of hydrogen peroxide-scavenging activity of CAT (Fig. 7). These results are in agreement with the sensitivity of this enzyme only to higher concentrations of hydrogen peroxide (generated by more severe salt stress), and they also suggest that catalase activity is not a suitable marker for distinction between different levels of moderate salt stress in duckweed, but it can indicate distinct levels of severe salt stress.

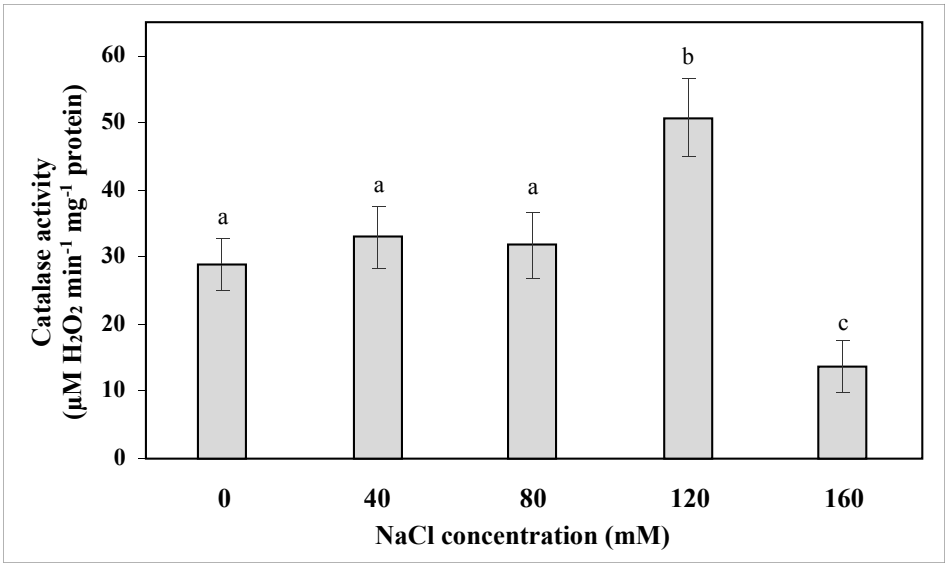

Figure 7. Enzymatic activity of catalase in fronds of duckweed exposed for one week to different degrees of salinity stress. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Glutathione reductase (GR) has an indirect role in the antioxidant defense: it does not anihilate any of the reactive oxygen species, but it regenerates the reduced form of glutathione, which is required for sustained scavenging of excess amounts of hydrogen peroxide in the Halliwell-Asada-Foyer redox chain, along with ascorbate (Chattopadhyay, 2014). In accordance with the results concerning variations of glutathione content of duckweed exposed to different degrees of salt stress, GS level exhibits no significant variation at lower salt concentrations (40 mM and 80 mM), and

it increases at 120 mM NaCl to ensure an efficient regeneration of the elevated amount of glutathione in the effort of plants to cope with oxidative stress associated with higher salinity. At even higher salt con-centration (160 mM), even though glutathione content continues to increase, GR activity declines significantly, probably because of enzyme damage caused by oxidative stress. Like catalase, this enzyme is not suitable for the early detection of milder salt stress, but its activity varies in a wide range when salinity reaches different higher values (Fig. 8). In another set of experiments, conducted also with duckwed, Zhang *et al*. (2013) have found that GR activity increases progressively with intensification of oxidative stress induced by water pollution with an organic xenobiotic substance, over a wide range of its concentrations. This substance probably caused a lower oxidative stress than the salt concentrations used in the present experiments.

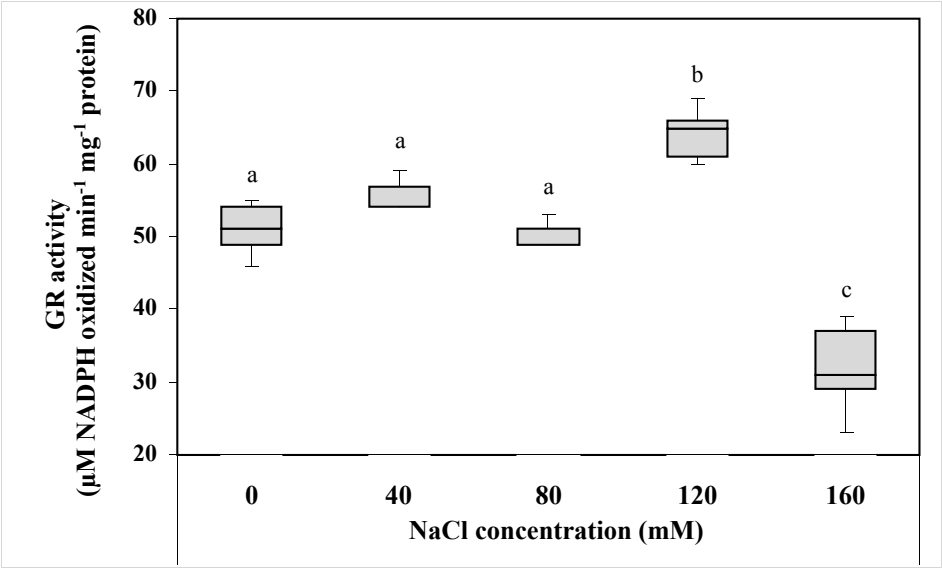

Figure 8. Glutathione reductase (GR) activity in duckweed (*Lemna minor* L.) exposed for one week to different concentration of sodium chloride. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Efficiency of antioxidant defense may be evaluated by the degree of membrane damage due to peroxidation of unsaturated fatty acids in the lipids structure of membranes. When environmental stress factors induce enhanced generation of hydrogen peroxide and alkyl peroxides, the excess amounts of these reactive oxygen species, if they are not quickly detoxified by specific enzymatic and non-enzymatic components, oxidative membrane damage results in formation of lipid peroxide derivatives, the most frequent and most toxic of these being the malondialdehyde (MDA). Our results

reflect that mild and moderate salt stress exerted for one week on duckweed plants does not significantly increase membrane damage by lipid peroxidation, because the concerted action of different antioxidants anihilates deleterious increment of peroxide concentrations. Enhanced membrane lipid peroxidation, manifested in increased generation of malondialdehyde and other related, thiobarbituric acid-reactive substances, occurs only upon exposure of duckweed to heavy stress condition induced by 160 mM sodium chloride (Fig. 9). This is in agreement with the results that indicated that ascorbate peroxidase, catalase and glutathione reductase activities markedly decrease at this salinity level, while superoxide dismutase activity, which generates hydrogen peroxide, increases. Even though ascorbic acid, glutathione and carotenoid pigment concentrations become higher, in lack of a proper antioxidant enzyme activity these protective molecules cannot prevent oxidative membrane damage if salt stress is pronounced. Similar results, when the antioxidant defense system could not prevent peroxidation of membrane lipids and oxidative stress became irreversibe, were reported especially for heavy metal toxicity in crop plants such as wheat, rice and lettuce (Eraslan *et al*., 2007; Panda *et al*., 2003; Shah *et al*., 2001).

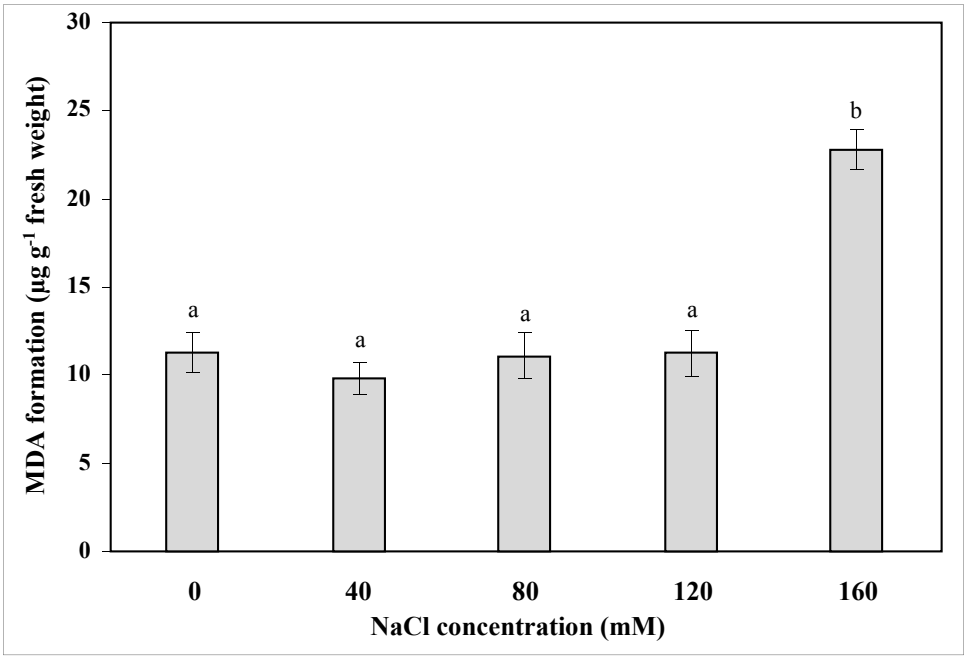

Figure 9. Degree of oxidative membrane damage by lipid peroxidation, revealed by accumulation of the toxic breakdown product malondialdehyde (MDA) in fronds of duckweed (*Lemna minor* L.) exposed for one week to different degrees of salinity stress. Bars represent ±SD from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Conclusions

Selected antioxidants of duckweed may be useful biochemical markers of oxidative stress induced by increased salinity of the aquatic environment. While the molar ratio between the reduced and the oxidized form of ascorbic acid (vitamin C) progressively decreases with enhanced salinity, the concentration of carotenoid pigments is increased only by pronounced salt stress caused by 120-160 mM sodium chloride in the nutrient solution. Enzymatic activity of ascorbate peroxidases is stimulated by milder salt stress, but it is decreased by salinity as high as 160 mM. In contrast, superoxide dismutase activity is significantly increased only by higher salt concentrations. Glutathione reductase and catalase activities are inhibited by salt stress induced with 160 mM sodium chloride, but are enhanced by moderately high salinity (120 mM). Oxidative membrane damaged, evaluated by the degree of peroxidation of unsaturated fatty acids, intensifies only at high salt concentrations, due to the effective protection ensured by different antioxidants at lower levels of salt stress. The ratio between reduced and oxidized form of ascorbic acid is a more sensitive stress marker than variation of the total amount of ascorbate. Moderate salt stress is indicated properly by increased ascorbate peroxidase activity, while pronounced salt stress may be monitored by high superoxide dismutase activity, by enhanced membrane lipid peroxidation and by increased carotenoid content of the duckweed biomass. The results demonstrate that the components of the antioxidative defense system behave differently under various degrees of oxidative damage caused by increased salinity, some of them being resistant to mild stress and tolerant to heavy stress, while others are tolerant to mild stress and sensitive to more severe stress conditions. In conclusion, measurement of changes in the dynamics of some selected antioxidants gives only a partial insight in the whole defense system against various degrees and forms of oxidative stress, and integrated stress tolerance of plants may be better evaluated if more enzymatic and non-enzymatic components of the antioxidant system are taken into account.

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