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Effects of osmotic stress on antioxidative system of duckweed (*Lemna minor* L)

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Abbreviations:
APOX – ascorbate peroxidase;
CAT – catalase;
POX – pyrogallol peroxidase;
MDA – malondialdehyde;
PEG – polyethylene glycol

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Abstract

Background and Purpose: *It is known that osmotic stress may cause damage to cells by inducing active oxygen species production or by disrupting detoxification mechanisms. We hypothesize that increased activity of antioxidant enzymes in duckweed (*Lemna minor *L.) provides a mechanism of tolerance to osmotic stress.*

Material and Methods: *Plants were subjected to NaCl- (50 mM), mannitol- (50 and 100 mM) and polyethylene glycol-mediated osmotic stress (PEG, 50 mM) for a period of 15 days. The responses of antioxidative system including also changes in growth, proline content and the extent of oxidative damage in terms of malondialdehyde, H_2O_2 and chlorophyll a to chlorophyll* b *(chl* a/b*) and chlorophyll* a+b *to carotenoids (chl* a+b/*car) ratios were studied.*

Results and Conclusion: *Iso-osmolar concentrations of salt and mannitol significantly reduced relative growth rate compared to control plants while osmotic shock mediated by PEG caused complete cessation of growth. Proline content increased with the severity of osmotic stress showing the highest values in PEG-treated plants. The proline accumulation upon PEG stress was paralleled by equal increase of ascorbic acid pool. Catalase, ascorbate peroxidase and non-specific peroxidase activities showed considerable increase under all osmotic agents, especially PEG. The increased enzyme activities coincided with unchanged H_2O_2 levels, chl a/b and chl a+b/car under mannitol and salt stress. Despite the highest induction of antioxidative defense, a marked increase in lipid peroxidation and H_2O_2 level as well as decrease of chl* a/b *and chl* a+b/*car accompanied PEG treatment. These results suggested that induction of antioxidant defences is at least one component of the tolerance mechanism of plants to long-term osmotic stress.*

INTRODUCTION

Salinity and drought represent serious problems worldwide negatively influencing plant growth in agricultural and natural ecosystems. According to Munns *(1)* growth is limited predominantly by osmotic effect, common both to salinity and drought, while salt-specific effects develop with time. Depending on the species, plants deal with such effects in very versatile manners including intracellular compartmentation of salt and osmotic adjustment achieved by accumulation of inorganic ions or organic solutes such as proline *(2)*. However, osmotic stress might also cause stomatal closure and reduce CO_2 fixation lead-

ing to a shortage of $NADP^+$ which serves as an electron acceptor in photosynthesis. Consequent leaking of electrons from electron transport chains to molecular oxygen enables increased production of reactive oxygen species (ROS) such as singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals which can be also formed in mitochondria, peroxisomes and plasma membranes *(3, 4)*. These cytotoxic ROS are highly reactive and can disturb normal metabolic processes through oxidative damage of lipids, proteins and nucleic acids. As a counter-act, plants have evolved efficient defense mechanisms consisting of low molecular mass antioxidants – ascorbic acid, carotenoids, proline, tocopherols and ROS-interacting enzymes – superoxide dismutase (EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and a variety of peroxidases (ascorbate peroxidase – APOX, EC 1.11.1.11; non-specific peroxidase – POX, EC 1.11.1.7). Aside from acting as an osmolyte and an antioxidant, many other functions have been assigned to proline such as stabilization of proteins and regulation of the cytosolic pH value *(5)*. Many studies show positive correlation between enhanced antioxidative system and increased resistance to osmotic stress and many other environmental stresses *(6, 7, 8)*.

Duckweed (*Lemna minor* L.) is a small floating macrophyte common to a wide range of rivers and lakes all over the world *(9)*. Many advantages such as rapid growth, genetically homogenous populations, easiness of culture and sensitivity to many toxic substances make duckweed a suitable plant model. The relative degree of *L. minor* tolerance to osmotic stress was assessed by studying the changes in antioxidant system and chl *a/b* and chl *a+b*/car ratios *(10)*. Several osmotic agents differing by their physico-chemical properties have been used to simulate effects of osmotic stress in vitro – PEG (non-ionic-impermeable), mannitol (non-ionic-permeable) and NaCl (ionic-permeable). As PEG simulates water deficit conditions in cultured cells in a similar manner to that observed in the cells of intact plants subjected to true drought conditions, the use of PEG of high molecular weight (6000 or 8000) is recommended *(11)*. A few studies carried out on *Lemna* plants correlated activation of antioxidant system to oxidative injury caused by short-termed NaCl stress *(12)*, as well as reduction of growth to proline accumulation in response to different osmotic agents *(13)*. However, involvement of antioxidative defense system in mechanisms of oxidative damage by PEG- and mannitol-induced osmotic stress has not yet been studied in *L. minor* plants. We hypothesized that duckweed tolerance to osmotic stress could be associated with the maintenance of or induction of antioxidative system. Hence, this study reports 1) a comparative analysis of the changes in the non-enzymatic (ascorbic acid and carotenoids) and enzymatic (activities of CAT, APOX and POX and their electrophoretic patterns) antioxidative components in test plants upon PEG and iso-osmolar concentrations of mannitol and NaCl, and 2) a possible induction of oxidative stress upon PEG and mannitol.

MATERIALS AND METHODS

Plant material and culture

Lemna minor L. was originally collected from Botanical Garden, Faculty of Science, University of Zagreb. Plants were sterilized according to Krajnčič and Devidé *(14)* and maintained as stock cultures on Pirson-Seidel nutrient medium *(15)*. The pH value of nutrient media was adjusted to 4.55 with 0.1 M KOH and media autoclaved at 118 kPa and 120 °C for 20 mins. The cultures were grown under a 16 hour photoperiod of fluorescent light (80 $\mu Em^{-2}s^{-1}$) at 24±2 °C.

Growth parameters

Individual healthy colonies with 2-3 fronds (from stock cultures) were transferred to 100 cm^3 Erlenmayer flasks containing 60 cm^3 Pirson-Seidel (PS) nutrient medium each supplemented with 50 mM NaCl, 50 mM mannitol (M_{50}), 100 mM mannitol (M_{100}) or 50 mM PEG (MW 6000) except in the case of control. The culture flasks were manually shaken once a day. Frond numbers were monitored during a 15-day period, on days 0, 3, 5, 8, 10,12 and 15 (n). Relative frond number (RFN) was calculated using the equation *(16)*: RFN = (frond No. at day n – frond No. at day 0)/ frond No. at day 0. Relative growth rate (RGR) was based on fresh mass (f.m.) determined after 15 days of experiment and calculated using the following formula: RGR = [ln (final weight) – ln (initial weight)]/ weeks.

Chlorophyll and carotenoid content

Photosynthetic pigments were measured according to the method described by Arnon *(17)*. In short, 30 mg fresh samples were homogenized with 80% (w/v) cold acetone, centrifuged at 5000 × *g* for 10 min and absorbances of the supernatant at 663, 646 and 470 nm were read. Chlorophyll *a*, *b* and total carotenoid content was determined according to Lichtenthaler *(18)*.

MDA and H_2O_2 content

Lipid peroxidation was determined by estimating the amount of malondialdehyde (MDA) content using the thiobarbituric acid method described by Heath and Packer *(19)*. The crude extracts were mixed with 0.25% (w/v) thiobarbituric acid solution containing 10% (w/v) trichloroacetic acid, heated at 95 °C for 30 min and the reaction was stopped in an ice bath. The cooled mixtures were centrifuged at 10000 × *g* for 10 min and the MDA content calculated from the absorbance at 532 nm (correction was done by substracting the absorbance at 600 nm for non-specific turbidity) by using extinction coefficient of 155 $mM^{-1}cm^{-1}$. H_2O_2 was extracted from 100 mg fresh tissue in ice-cold acetone and estimated according to the method of Mukherjee and Choudhuri *(20)*. After addition of titanyl-sulphate and conc. NH_4OH solution, the formed peroxide-titanium precipitate was dissolved in 2 M H_2SO_4 and absorbance of the mixtures read at 415 nm. The H_2O_2 content was calculated from a standard curve and expressed as $\mu mol\ (H_2O_2)\ g^{-1}$ (f.m.).

Ascorbate content

scorbate was isolated by extraction with 6% trichloroacetic acid from 100 mg plant tissue, following the method of Mukherjee and Choudhuri *(20)*. Extracts were mixed with 2% dinitrophenyl hydrazyne followed by the addition of 1 drop of 10% thiourea solution and boiled for 15 min in a water bath. After adding 80% (w/v) H_2SO_4 (in an ice-bath), the absorbance of the mixtures containing hydrazone complex was read at 530 nm. Ascorbate concentration was determined using calibration curve and expressed as μmol (ascorbate) g^{-1} (f.m.).

Proline content

Free proline content was measured by the method of Bates *et al. (21)*. Plant tissue (100 mg) was homogenized in 3% (w/v) sulphosalycylic acid and centrifuged at 700 × *g* for 3 min. After addition of ninhydrin reagent, mixtures were heated at 100 °C for 1 h and cooled in an ice-bath. The chromophore obtained was extracted from liquid phase with toluene and the absorbance of organic layer was read at 520 nm. Proline concentration was determined from calibration curve using L-Proline as standard and expressed as nmol (proline) g^{-1} (f.m.).

Enzyme determinations

For enzyme analysis, cultures were started by transferring 5–10 healthy colonies with 2–3 fronds from stock cultures in 250 cm^3 Erlenmayer flasks containing 100 cm^3 PS medium supplemented with NaCl, mannitol and PEG-6000 in the same concentrations as in growth experiments. Plant tissue (100 mg) was homogenized in 1 cm^3 100 mM potassium phosphate (KPO_4) buffer (pH 7) including 1 mM EDTA and polyvinylpolypyrrolidone (PVPP), using pre-chilled mortars and pestles. The homogenates were centrifuged at 22000 × *g* for 20 min at 4 °C. The supernatant was used for enzyme activity and protein content assays. Total soluble protein contents of enzyme extracts were estimated according to Bradford *(22)* using bovine albumine serum (BSA, Sigma) as a standard.

Ascorbate peroxidase activity was determined according to Nakano and Asada *(23)*. The ascorbate oxidation was followed at 290 nm and its concentration calculated using the molar extinction coefficient (ε = 2.8 $mM^{-1}cm^{-1}$). Corrections were done for low, non-enzymatic oxidation of ascorbate by H_2O_2. One enzyme unit was defined as μmol (oxidized ascorbate) g^{-1} (f.m.) per min.

Catalase (CAT) activity was determined by the decomposition of H_2O_2 and was measured spectrophotometrically by following the decrease in absorbance at 240 nm *(24)*. Activity was calculated using the extinction coefficient (ε = 0.04 $mM^{-1}cm^{-1}$) and μmol (H_2O_2 decomposed) g^{-1} (f.m.) min^{-1} was defined as unit of CAT.

The activity of non-specific peroxidase was measured by monitoring the formation of purpurogallin at 430 nm (ε = 2.47 $mM^{-1}cm^{-1}$) according to Chance and Maehly *(25)*. The reaction mixture contained 50 mM KPO_4 buffer (pH 7), 1mM H_2O_2, 20 mM pyrogallol and enzyme extract.

Specific enzyme activity for all enzymes was expressed as units mg^{-1} protein.

Isoenzyme profile of APOX, POX and CAT

Samples of L. *minor* extracts were separated by non-denaturating gel electrophoresis in 10% (APOX) or 7% (CAT) polyacrylamide slab gels at pH 8.3 and 4 °C according to Laemlli *(26)*. Equal amounts of protein (50 μg for APOX, 40 μg for CAT, 30 μg for POX) were loaded onto each lane. For detection of APOX isoforms, the gels were first soaked in 50 mM KPO_4 buffer (pH 7) containing 2 mM ascorbate for 30 min (3 x 20 min). The gels were then incubated in the same buffer including 4 mM ascorbate and 2 mM H_2O_2 for 20 min, briefly washed and the achromatic bands visualized in 50 mM KPO_4 buffer (pH 7.8) containing 2.45 mM NBT and 28 mM TEMED. Following 30 min incubation in 50 mM KPO_4 buffer (pH 7.0), visualization of POX activity was performed in the same buffer containing 20 mM pyrogallol and 4 mM H_2O_2 *(27)*.

Staining for CAT was carried out as described by Woodbury *et al. (28)*. After 3 washes for 15 min in distilled water, the gels were soaked in 5 mM H_2O_2 for 10 min. After a brief rinse, the gels were incubated in 1% (w/v) ferric chloride / 1% potassium ferricyanide solution and CAT isozymes appeared as colorless bands on a dark green field.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Statistica 7.1. (StatSoft, Inc.) software package, and differences between corresponding controls and exposure treatment were considered as statistically significant at $P<0.05$. Each data point is the average of nine replicates ($n=9$).

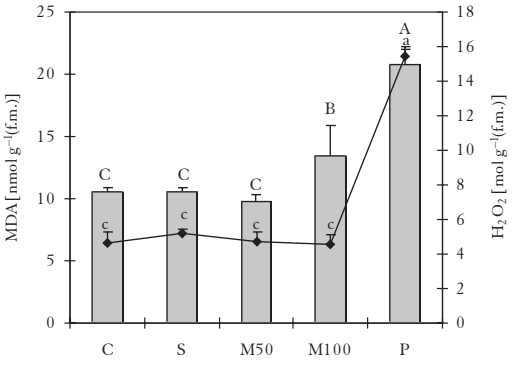

Figure 1. *H_2O_2 (line) and MDA (column) contents in* L. minor *plants under control (C) and stress – 50mM NaCl (S), 50 mM (M_{50}) or 100 mM mannitol (M_{100}) and 50mM PEG – conditions after 15-day growth period. Values are mean ± S.E. based on nine replicates. Bars with different letters are significantly different at* $p < 0.05$.

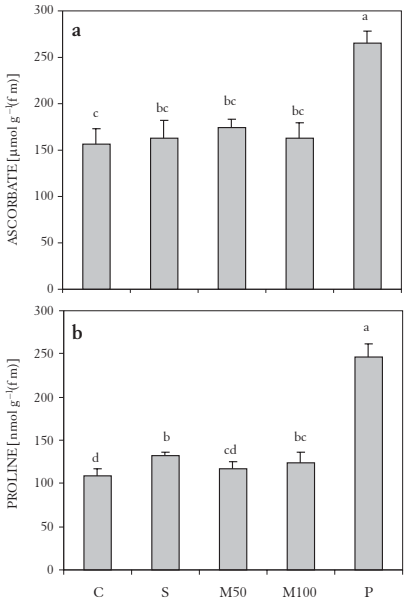

Figure 2. *Ascorbic acid (a) and free proline (b) contents under control (C) and stress – 50 mM NaCl (S), 50 mM (M_{50}) or 100 mM mannitol (M_{100}) and 50mM PEG – conditions after 15-day growth period. Values are mean ± S.E. based on nine replicates. Bars with different letters are significantly different at* $p < 0.05$.

RESULTS

Effects of NaCl, mannitol and PEG on *L. minor* growth, in terms of relative frond number and relative growth rate are shown in Table 1. Application of 50 mM mannitol caused relative frond number retardation from day 12, but had no effect on relative growth rate. Iso--osmolar concentrations of NaCl, and mannitol significantly reduced relative growth rates and relative frond numbers (from day 8), both to almost the same extent. PEG caused total growth inhibition.

Chlorophylls and carotenoid contents displayed the highest values in response to mannitol, no significant change upon NaCl and marked decrease upon PEG treatments (Table 2). Ch *a*/*b* and chl *a*+*b*/car ratios decreased for 18% and 8%, respectively, in *L. minor* plants exposed to PEG.

The changes of MDA and H_2O_2 contents under tested osmotic agents are given in Figure 1. The plants treated with 50 mM NaCl and mannitol exhibited no increase in H_2O_2 and MDA levels, while 100 mM mannitol treatment increased MDA formation by nearly 27% compared to control, though it did not change H_2O_2 content. Maximum MDA (105%) and H_2O_2 (233%) values, as compared to control, were observed under PEG stress, indicating high extent of lipid peroxidation.

Ascorbic acid and proline contents are shown in Figure 2. Ascorbic acid pool markedly (71% compared to control) increased under PEG treatment while other osmotic agents exhibited values similar to control (Figure 2A). Iso-osmolar NaCl and mannitol treatments significantly increased proline content in *L. minor* plants, but not to such a high level as PEG (126% when compared to control) (Figure 2B).

Figures 3A-C describe the effect of osmotic stress on APOX, POX and CAT activities in *L. minor* plants after 15 days of exposure to tested osmotic agents. POX activity of plants exposed to NaCl was similar to control but increased by 31% and 70% under 50 and 100 mM mannitol, respectively, and 87% under PEG treatment (Figure 3A). All osmotic agents significantly increased APOX activity in tested plants – NaCl and higher concentration of mannitol by 45%, 50 mM mannitol by 32%, and PEG by 106% (Figure 3B). The induction of CAT was most conspicuous under PEG and 50 mM mannitol treatments – approximately 40% increase compared to control (Figure 3C). Higher concentration of mannitol and NaCl increased the activity of the enzyme by approximately 30% and 20%, respectively. The POX, APOX and CAT isoenyzme patterns of *L. minor* plants subjected to different osmotic treatments are presented in Figure 4. In total, four POX isoenzymes could be discerned on native gels (Figure 4A). Isoenzyme POX4 was not present in control but appeared upon stress imposition. The intensity of isoenzyme POX4 increased in the order NaCl < M_{50} < M_{100} < PEG.

TABLE 1.

Growth of L. minor plants grown in PS medium without (control) or with 50 mM NaCl, 50 mM mannitol (M50), 100 mM mannitol (M100) and 270 gl^{-1} PEG for a period of 15 days.

	Relative growth rate (after 15 days)	Relative frond number					
		3rd day	5th day	8th day	10th day	12th day	15th day
Control	0.189 ± 0.014^{a}	0.22 ± 0.25^{ab}	1.00 ± 0^{a}	3.19 ± 0.36^{a}	5.91 ± 0.75^{a}	10.45 ± 1.25^{a}	19.90 ± 1.88^{a}
NaCl	0.162 ± 0.011^{b}	0.17 ± 0.21^{ab}	0.86 ± 0.31^{a}	2.38 ± 0.67^{b}	4.75 ± 0.34^{b}	7.78 ± 0.79^{b}	14.96 ± 2.04^{b}
M50	0.180 ± 0.008^{a}	0.42 ± 0.47^{a}	1.00 ± 0.15^{a}	2.88 ± 0.19^{a}	5.32 ± 0.94^{ab}	8.49 ± 0.90^{b}	16.26 ± 2.11^{b}
M100	0.160 ± 0.009^{b}	0.25 ± 0.42^{ab}	0.94 ± 0^{a}	2.75 ± 0.27^{b}	5.02 ± 0.68^{b}	7.90 ± 0.42^{b}	15.17 ± 1.33^{b}
PEG	0^{c}	0^{b}	0^{b}	0^{c}	0^{c}	0^{c}	0^{c}

Values are means of six replicates ± S.D. per treatment. Means in each columns followed by different letters are significantly different ($p<0.05$).

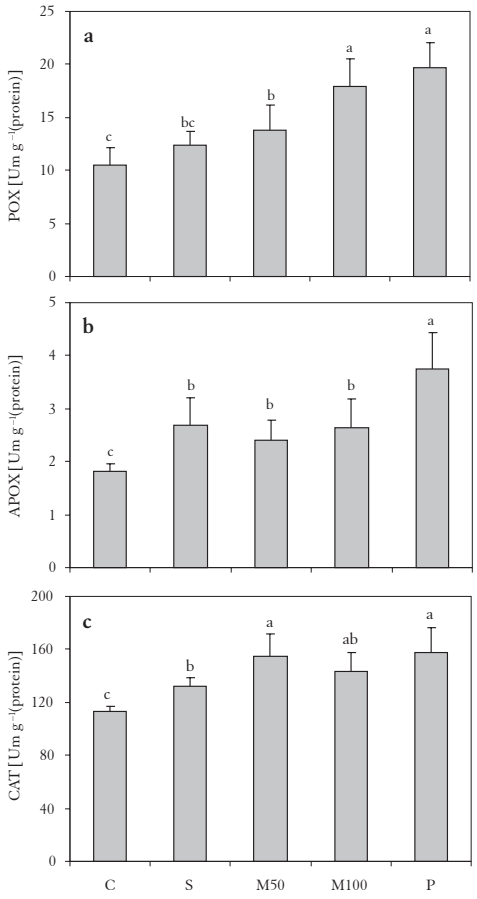

Figure 3. *Non-specific peroxidase (a), ascorbate peroxidase (b) and catalase (c) activities under control and stress conditions after 15-day growth period. Values are mean ± S.E. based on nine replicates. Bars with different letters are significantly different at* $p < 0.05$. *For abbreviations see Figure 2.*

The intensity of two observed APOX isozymes increased according to the change in enzyme activity, being the most intense upon PEG-induced stress (Figure 4B). Out of three observed catalase isozymic bands, CAT2 was missing in control and CAT3 accumulation increased under treatments (Figure 4 C).

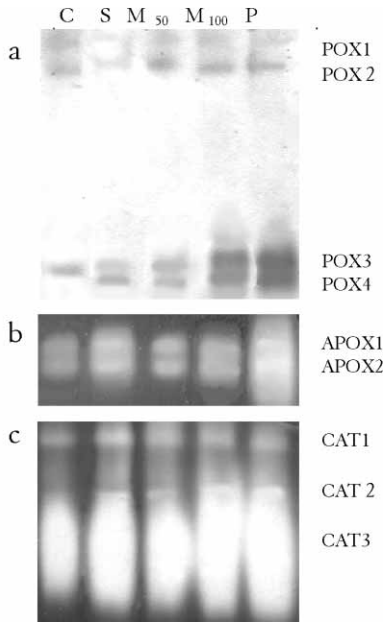

Figure 4. *Electrophoretic patterns of non-specific peroxidase (a), ascorbate peroxidase (b) and catalase (c) isoenzymes under control and stress conditions during 15-day growth period. For abbreviations see Figure 2.*

DISCUSSION

In the present study, osmotic agents PEG, NaCl and mannitol induced differential antioxidant and growth responses in model plant system *L. minor*. Long-term exposure (15 days) to mild osmotic treatment caused inhibition of clonal reproduction (presented as relative frond number) by nearly 20%, but had no effect on relative growth rate (based on fresh mass). The application of iso-osmolar NaCl and mannitol concentrations revealed no significant difference between the effects of these two osmotic agents on growth – in both cases relative frond numbers and relative growth rates were affected by approximately 25% and 15%, respectively (Table 1). In addition, the most negative impact on *L. minor* growth induced by non-ionic-impermeable PEG suggests it is the osmotic and not ionic component of salinity that is responsible for considering duckweed a salt-sensitive plant

TABLE 2.

Content of chlorophyll a, b and carotenoids [mg/g (f.m.)], chlorophyll *a*+*b* ratio and chlorophyll *a*+*b*/carotenoids ratio in L. minor plants subjected to different osmotica for 15-day period. For abbreviations see Table 1.

Tested media	chl *a*	chl *b*	carotenoids	chl *a*/*b*	chl *a*+*b*/carotenoids
Control	0.58±0.02^{b}	0.20±0.01^{b}	0.27±0.01^{b}	2.91±0.15^{a}	2.88±0.16^{a}
NaCl	0.56±0.07^{b}	0.18±0.02^{b}	0.27±0.01^{b}	2.82±0.08^{a}	3.00±0.21^{a}
M50	0.64±0.03^{a}	0.22±0.02^{a}	0.30±0.01^{a}	2.96±0.32^{a}	2.91±0.16^{a}
M100	0.65±0.02^{a}	0.23±0.01^{a}	0.31±0.02^{a}	2.88±0.16^{a}	2.89±0.07^{a}
PEG	0.47±0.05^{c}	0.19±0.02^{b}	0.24±0.03^{c}	2.40±0.10^{b}	2.66±0.08^{b}

Values are means of six replicates ± S.D. per treatment. Means in each column followed by different letters are significantly different ($p<0.05$).

species *(29)*. Similar NaCl/mannitol and PEG effect on *L. minor* growth were obtained by Frick and Golt *(13)* who also noticed correlation between decreased growth rate and increased proline accumulation. Our data confirmed these results since the highest proline content with maximum growth inhibition was observed under PEG and the lowest with a minimal effect on growth under 50 mM mannitol (Figure 2B). It has already 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 and thus tend to lead to growth inhibition *(30, 31)*.

Besides their primary effects on growth and photosynthesis, the negative effects of salt and water stresses may be in part a consequence of oxidative damage to important molecules, resulting from the imbalance between ROS formation and antioxidant defenses. In this study, all osmotic agents increased APOX, CAT and POX activities in *L. minor* plants, but the induction of studied enzymes was most conspicuous under PEG-induced stress (Figure 3A-C). As a confirmation, a new CAT isoform and increased accumulation of APOX and CAT isoforms were revealed by native PAGE under stress conditions (Figure 4). Increase of APOX, CAT and POX, as major H_2O_2-detoxifying enzymes, has been related to better salt- and water deficit-resistance in many plant species *(8, 32)*. Comparing activities of studied enzymes in osmotically stressed *L. minor* plants, POX and especially APOX activities were greater than CAT activity. This may be so because 1) peroxidases have much higher affinity for H_2O_2 than catalase, and 2) ascorbate-peroxidase is regarded as one of the most widely distributed antioxidant enzymes in plant cells, being present in the cytosol, chloroplasts, microbodies, mitochondria, as well as in the cell wall *(33)*.

MDA, a secondary end product of free radical induced lipid peroxidation and H_2O_2 contents are taken as measures of the stress-induced damage at cellular level *(34)*. H_2O_2 is a natural plant metabolite that exerts its detrimental role predominantly by generation of highly reactive hydroxyl radicals which then initiate lipid peroxidation. On the other hand, H_2O_2 acts as a second messenger to regulate the gene expression of some antioxidative enzymes in plant cells, and a transient increase of H_2O_2 is observed during the early stages of oxidative stress *(35)*. Panda and Upadhyay *(12)* reported considerable H_2O_2 accumulation in duckweeds exposed to short--term salinity, which was accompanied by a decrease in CAT activity. Thus, in the present study, H_2O_2 might have up-regulated antioxidative system of *L. minor* plants under NaCl- and mannitol- induced stresses since after a 2-week-period CAT, APOX and POX activities were enhanced to such an extent that they in turn ensured unchanged H_2O_2 levels (Figure 1). It has also been shown that ascorbate and carotenoids are able to interact directly with ROS (superoxide, hydroxyl radicals and singlet oxygen), preventing them from initiating lipid peroxidation *(36)*. Thus, together with the above mentioned results, elevated and/or unaffected carotenoid and ascorbate contents as well as unchanged ratios of chl *a/b* and chl *a+b*/car speak against oxidative damage under moderate osmotic stress, even though the MDA content increased by 27% in response to 100 mM mannitol. However, it is evident that in spite of remarkably high ascorbate and antioxidant activity, PEG treatment equally increased MDA and H_2O_2 levels and decreased chl *a* and carotenoid contents, suggesting that the extent of oxidative injury is much higher than the duckweed defense mechanisms. Also, the ratios of chl *a/b* and chl *a+b*/car decreased under PEG-mediated stress, which implies slower degradation of chl *b*. Chl *b* plays an important role in stabilizing apoproteins and the assembly of light harvesting complex *(37)*. Several studies have shown that PEG promotes plant senescence *(38, 39)*. Generally, chl breakdown is a common event during senescence and it is assumed that chl loss is linked to protein degradation *(40)*. An increased rate of senescence and activity of different proteolytic enzymes was noticed in wheat and rice exposed to osmotic stress *(41)*. In our study, PEG may have promoted premature senescence also because that osmotic agent caused not only the degradation of chl but also of protein content in duckweed plants (data not shown).

It is noteworthy that massive proline accumulation observed under PEG-induced stress could not ameliorate consequent oxidative damage and inhibitory effects on *L. minor* growth, though it might have contributed to more efficient antioxidant enzyme activity *(42)*. Sharma and Dubey *(33)* found that addition of external proline prevents APOX inactivation under PEG-induced water deficit and suggested the protective role of proline for enzyme activity under osmotic stress conditions. Effects of PEG as an osmotic agent were far more severe than those of mannitol during 15 days of stress imposition, probably because of slight absorption of mannitol. While PEG cannot enter the pores of plant cells, mannitol has been shown to be taken up by plant cells where it can serve as a compatible solute *(43, 44)*. This could be the reason for the absence of, or a low extent of, lipid peroxidation under mannitol-induced osmotic stress.

In conclusion, our hypothesis was confirmed by the results of this study which showed that tolerance of *L. minor* to long-term moderate osmotic stress was closely related to stimulation of antioxidant defense mechanisms. The results also show duckweed capacity to cope with severe osmotic stress though at the expense of complete growth cessation.

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