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ORIGINAL ARTICLE

Effects of salt stress on physio-biochemical characters and gene expressions in halophyte grass *Leptochloa fusca* (L.) Kunth

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Abstract
Kallar grass (*Leptochloa fusca*) is a perennial C4 halophytic species with high salt tolerant. The present research was made to investigate the physio-biochemical characters and transcriptional changes of *L. fusca* under varying salinity levels (0–600 mM NaCl). The Na^+ level in shoots and roots increased significantly, whereas the K^+ content was maintained high in 300 mM NaCl and then declined with increasing salinity in both tissues. The content of proline in seedlings exposed to extreme salinity level was 15.5-fold higher than control. Photosynthetic pigments, total soluble proteins, PAL activity, and total phenolic compounds in salt-stressed plants increased gradually up to 450 mM and declined at 600 mM NaCl. High salt concentration led to oxidative stress that was manifested by increased MDA level. To tackle with oxidative damages, *L. fusca* enhanced the activity of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). Moreover, under NaCl stress, the mRNA levels of SOS1, PM H^+-ATPase, and NHX1 were up-regulated in both tissues, but higher in roots than in shoots. Our results demonstrate that *L. fusca* could use an osmotic adjustment, antioxidant defense system, and regulating the ion homeostasis as the most effective salt tolerance mechanisms for better plant growth under saline conditions.

Keywords Kallar grass · Halophyte · Salt stress · Physio-biochemical adaptations · Ion homeostasis

Abbreviations

MDA	Malondialdehyde
PM	Plasma membrane
FW	Fresh weight
TBA	Tribromoarsenazo
TCA	Trichloroacetic acid
PVP	Polyvinylpyrrolidone
BSA	Bovine serum albumin
NBT	Nitro blue tetrazolium
REST	Relative expression software tool

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Introduction

Soils salinity is certainly one of the most considerable environmental threats influencing millions of hectares of land worldwide and results in major decrements in area of arable land, crop yields and quality (Shabala 2013). The three damaging effects of salinity on plant growth have been connected to low external water potential (osmotic stress), severe toxicity of ions (salt stress) and nutrient deficiency, leading to a decrease in photosynthesis efficiency and other physiological disorders (Yang and Guo 2018). Moreover, salinity promotes overgeneration of reactive oxygen species (ROS) that induces oxidative damage in plant cells (Munns 2002). ROS are extremely reactive and their deleterious effects are because of their potential to give rise to peroxidation of lipid, protein denaturation, DNA damage, pigment breakdown, oxidation of carbohydrate, and enzymatic activity disturbance (Choudhury et al. 2017). According to the potential of plants to thrive on high salt medium, they are traditionally categorized as either glycophytes and halophytes (Shabala 2013). Halophytes that compose about 1% of the worldwide flora have developed different mechanisms that can withstand and reproduce in habitats with about 200 mM NaCl concentration or greater (Flowers and Colmer 2008). These salinity tolerance mechanisms consist of maintenance of intracellular ion homeostasis by membrane transporters (Apse and Blumwald 2007; Amtmann and Leigh 2010), regulation of osmolarity by synthesizing various osmoprotectants like polyols, soluble sugars, sugar alcohols, and proline (Hayat et al. 2012), induction of antioxidant enzymes, and antioxidant compounds (Ben Amor et al. 2006), induction of phytohormones and enzymes involved in their biosynthesis (Kaya et al. 2009) and regulating the expression of genes and transcription factors associated with plant salinity tolerance (Mishra and Tanna 2017).

Leptochloa fusca (L.) Kunth is a perennial C4 halophytic species from the Poaceae family and is widely spread in salt-affected regions of Pakistan and India. *L. fusca* is highly tolerant to high soil pH, salinity, and sodicity (Rauf et al. 2014). However, there are confined data on the molecular and physiological mechanisms of tolerance to salinity in *L. fusca*. To address the salt tolerance strategies in *L. fusca*, seedlings were grown under greenhouse conditions and the effects of varying salt treatments (0, 300, 450 and 600 mM NaCl) for 3 weeks on sodium and potassium contents, lipid peroxidation, photosynthetic pigments, accumulation of proline, status of enzymatic antioxidant, total phenolics and expression patterns of NHX1, SOS1 and plasma membrane (PM) H^+-ATPase genes were evaluated. Understanding the tolerance mechanisms of *L. fusca* to salinity will lead to efficient methods for breeding or genetically engineering of salinity tolerance in non-halophytic species.

Materials and methods

Growth conditions and treatments

Kallar grass seeds were kindly provided by the National Plant Gene Bank of Iran. The surface sterilized seeds were planted in 14-cm diameter plastic pots containing prewashed and nutrient-free sand. The seedlings were raised in a glasshouse at 25 °C and photoperiod of a 16 h and fed with ½ strength Hoagland solution (Hoagland and Arnon 1950) for 60 days before starting salt treatments. After this period, the seedlings were treated with nutrient solution containing varying NaCl levels (0–600 mM). To prevent osmotic shock, the treatments were gradually stepped up in increments of 75 mM day^{-1} until desired salinity levels of 300, 450, and 600 mM were obtained. For every treatment, five replicates were considered. Three weeks after reaching to final salt concentration, the shoots and roots were sampled, and maintained at − 80 °C in preparation for next analyses.

Determination of Na^+ and K^+ contents

The contents of Na^+ and K^+ were measured in the roots and shoots after digesting 0.1 g of the oven-dried tissues in 10 ml of 1 N HCl. The Na^+ and K^+ contents of the digested extracts were determined by a flame photometer (JENWAY, PFP-7, Staffordshire, UK).

Proline extraction and determination

The proline was extracted from fresh shoots (250 mg) in 10 mL 3% (w/v) sulphosalicylic acid and determined spectrophotometrically at A_{520} by the ninhydrin reagent and standard L-proline solutions (Bates et al. 1973).

Photosynthetic pigment determination

80% acetone extracts were prepared from the freshly collected shoot samples (0.25 g). The extract was then centrifuged at 3000 g, and the absorption of the extract was recorded at 646.8, 663.2 and 470 nm employing a UV/Vis spectrophotometer (Specord 200 Plus, Analytik Jena, Germany) against acetone as a blank (Lichtenthaler 1987).

Measurement of malondialdehyde (MDA) content

MDA content was measured by tribromo-arsenazo (TBA) colorimetry based on Sun et al. (2010). Fresh leaf (500 mg) was ground with the pre-chilled pestle in 10% trichloroacetic acid (TCA) and centrifuged for 15 min at 12,000 g. After that, 2 mL of extract was mixed with 2 mL of reaction solution comprising 10% TCA and 0.5% thiobarbituric acid (TBA). The obtained mixture was heated in boiled water for 30 min. After that the mixture immediately cooled, centrifuged, and the absorbance was checked at 450, 532, and 600 nm. The concentration of MDA was estimated as: 6.45 $(A_{532} - A_{600}) - 0.56A_{450}$.

Measurement of PAL enzyme activity

Extraction and measurement of PAL activity were done based on the Cahill and McComb (1992) method. Shoot samples (500 mg) were homogenized on ice with pre-chilled mortar using 4 ml of 0.1 M Tris–HCl buffer (pH 8.9) containing 10 mM 2-mercaptoethanol and 50 mg PVPP. The homogenates were centrifuged for 20 min at 13,000 g, at 4 °C, and the extract was exerted for assaying the PAL activity. The reaction solution including 500 µl of enzyme extract, 1000 µl of 80 mM borate buffer (pH 8.9), and 30 mM *L*-phenylalanine was incubated at 30 °C for 1 h. The reaction was ended by adding 1.5 ml of 2 M HCl and the amount of *trans*-cinnamic acid produced was determined at 290 nm employing a UV/Vis spectrophotometer (Specord 200 Plus, Analytik Jena, Germany).

Total phenolics assay

Total phenolic content was measured according to Slinkard and Singleton (1977) method using calibration curve of gallic acid. The extract (100 µL) was mixed with 4.5 ml of water and 1 mL of the Folin–Ciocalteu reagent in a 10 mL vial. After shaking 3 min, 300 µL of 2% Na_2CO_3 was added to the mixture and then incubated 2 h at 23 °C. The absorption was read at 760 nm employing a UV/Vis spectrophotometer (Specord 200 Plus, Analytik Jena, Germany) and the result was estimated as mg gallic acid/g of the FW.

Antioxidant enzyme assays

Shoot samples were homogenized with 2 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA-Na_2 and 0.5% PVPP under cold conditions. The mixture was centrifuged at 13000 g for 30 min at 4 °C and the extract was applied for measuring the activity of antioxidative enzymes. Total protein content determination was done based on Bradford assay (1976) with the use of BSA as the standard solution. The activity of APX (EC 1.11.1.1) was determined based on Nakano and Asada (1981) following the ascorbate oxidation by the decrement in absorption at 290 nm. The activity of CAT (EC 1.11.1.6) was analyzed by controlling the H_2O_2 absorbance reduction at 240 nm based on Beer and Sizer (1952) method. SOD (EC 1.15.1.1) activity was done spectrophotometrically based on its capability to inhibit the photochemical reduction of nitroblue tetrazolium (Dhindsa et al. 1981).

Statistical analysis

The data were analyzed with one-way ANOVA by the SAS 9.1, and the evaluation of mean values was done by Duncan's test at 1% significance level.

Quantitative real-time PCR analysis

The extracted RNAs were reverse transcribed to corresponding cDNAs with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) following the supplier's guidelines, later used as templates for qRT-PCR analysis. The qRT-PCR was done using SYBR Green PCR Master Mix on iQ5 real-time PCR detection system (BioRad, Hercules, CA, USA) based on the manufacturer's guideline. 18SrRNA was used as the housekeeping gene. The sequences of primers used in qRT-PCR are given in Table 1. The statistical analysis, ratios of expression, and confidence intervals for the results provided by relative expression software tool (REST) analysis. The qRT-PCR was done with a temperature profile of 94 °C for 3 min followed by 40 cycles at 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 45 s. After the ending of the program, analysis of melting curve was done to confirm the specificity of amplified products. Each qRT-PCR was done in triplicates.

Results and discussion

Effect of salt stress on Na^+ and K^+ accumulation

The amount of Na^+ and K^+ accumulated in shoot and root tissues, and the Na^+/K^+ ratio is shown in Table 2. In general, Na^+ accumulation exhibited a continuous increase in the shoot and root tissues. However, no statistically significant differences were detected between 450 and 600 mM NaCl in both tissues. The K^+ content increased substantially with increasing salinity in both tissues and reached the maximum value at 300 mM salt level and then diminished gradually in both tissues. The Na^+/K^+ ratio of *L. fusca* gradually increased in both tissues following treatment with NaCl. Nevertheless, in both tissues, there was no significant difference at low salt level (300 mM) compared to control. The continuous accumulation of Na^+ observed in *L. fusca* with increasing external NaCl concentration, is a hallmark of halophytes and indicates the

Table 1 Sequences of primers used for qRT-PCR analysis

Gene	Sequence (5´–3´)	Product size (bp)	Accession number
SOS1	F: CAAGGGTGACAGCCAGGA R: GAAGCCCCAAGAAGTGCC	154	KC525946
PM H^+-ATPase	F: TGCTACTGGTATTGTGCTTGG R: ATGCTCGCTGTCCCTGATAG	124	KM234610
NHX1	F: TTCTCACCGTGTTCTTCTGC R: GAGTGCATCCATACCGACATAG	187	JF933902
18SrRNA	F: ATGATAACTCGACGGATCGC R: CTTGGATGTGGTAGCCGTTT	169	KM036286

Table 2 Effect of different concentrations of NaCl on Na^+ and K^+ contents and Na^+/K^+ ratio of shoots and roots of *L. fusca* seedlings

NaCl (mM)	Shoots			Roots		
	Na^+	K^+	Na^+/K^+ ratio	Na^+	K^+	Na^+/K^+ ratio
0	1.6964c	2.4306b	0.6979c	1.7475c	1.925b	0.90779b
300	2.0164b	2.5535a	0.7896c	2.0565b	2.155a	0.9543b
450	2.30a	2.4136b	0.95293b	2.548a	1.815b	1.3994a
600	2.3695a	1.9289c	1.2284a	2.5505a	1.805b	1.42493a

Different letters represent statistically significant differences ($p < 0.01$), based on Duncan's test

effective sequestration of ions in the vacuoles to adapt to saline conditions (Munns 2002). There are numerous reports about the gradual increment of Na^+ concentration with increasing the salt level in halophytes (Theerawitaya et al. 2015; Yin et al. 2018). Under salinity, the halophytes strongly depend on the usage of inorganic ions (Na^+, K^+, and Cl^-), for maintaining the osmotic and turgor pressure of the shoots. The accumulation of inorganic ions as an osmolyte with low energy cost in the cytosol of halophytes lead to reduce their osmotic potential in response to rising salt and allow them to maintain adequate water intake and cell turgor required for continued growth and survival (Shabala 2013). The capability of plants to prevent the unfavorable effects of salinity strongly depends on their potassium nutrition and maintaining a high cytosolic K^+ concentration (Amtmann and Leigh 2010). Our results showed that in both tissues of *L. fusca*, the K^+ content in low NaCl concentration was increased compared to the control and then gradually diminished with increasing the Na^+ level in the growth environment. The decrement in K^+ content when seedlings were exposed to the external supply of Na^+ has been ascribed to the replacement of K^+ by Na^+. The leakage of potassium from the root can happen as a consequence of Ca^{2+} replacement by Na^+ (calcium maintains the integrity of the membrane and reduces the leakage of K^+ by protecting the membranes from detrimental effects of Na^+) (Tuna et al. 2007). Moreover, the uptake of Na^+ give rise to membrane depolarization and provokes K^+ loss through the activity of outward-rectifying K^+ channels (KORC) (Blumwald et al. 2000).

Effect of salt stress on photosynthetic pigments

The chlorophylls and carotenoids content significantly enhanced with increasing NaCl up to an optimal concentration of 450 mM and then dropped at high NaCl level (Table 3). In *L. fusca* chlorophylls and carotenoids underwent a gradual salt-dependent increase (+58% and +78% for total chlorophyll and +71% and +120% for carotenoids at 300 and 450 mM NaCl, respectively, Table 3). At higher salt level (600 mM) there was a reduction in the photosynthetic pigments. However, the concentration of the pigments were still 1.65- and twofold higher than the control for total chlorophyll and carotenoids, respectively. Chlorophylls are the main photosynthetic pigments, and the leaf chlorophyll content can be considered as a key biochemical marker of the photosynthetic capacity of the plant under various conditions (Ashraf and Harris 2013). It is known that in some halophytes, the chlorophylls are not affected by salinity or even stimulated in optimum salt concentrations whereas chlorophyll contents decrease in the majority of salt-sensitive species (Reginato et al. 2014). In the current research, *L. fusca* displayed a fine ability to tolerate raised salinity levels with increasing the chlorophyll concentration in comparison with control. Similar results were reported in *Eugenia myrtifolia* (Acosta-Motos et al. 2015) and *Aeluropus littoralis* (Talbi Zribi et al. 2017), etc. The chlorophyll a/b ratio was gradually increased by increasing the salinity. However, there were significant changes only at 450 and 600 mM salt levels. The rise of Chl a/b ratio following exposure to salinity which detected in our experiment is in accordance with the results of Rabhi et al. (2012) and indicates the rearrangement of the photosystem composition to prevent the of photoinhibition

Table 3 Effect of different concentrations of NaCl on leaf pigment contents in *L. fusca* seedlings

Pigment	0	300 mM NaCl	450 mM NaCl	600 mM NaCl
Chl *a* (mg g^{-1} FW)	1.5433d	2.46c	2.81a	2.7166b
Chl *b* (mg g^{-1} FW)	0.49d	0.7566b	0.82a	0.6533c
Chl *a* + Chl *b* (mg g^{-1} FW)	2.0333d	3.2166c	3.63a	3.37b
Chl *a*/Chl *b* ratio	3.1495c	3.2513bc	3.4268b	4.1582a
Car (mg g^{-1} FW)	0.3d	0.5133c	0.6633a	0.6266b
Car/Chl ratio	0.1475b	0.1595b	0.1818a	0.1839a

Different letters represent statistically significant differences ($p < 0.01$), based on Duncan's test risks (Bassi et al. 1997). Similar to chlorophylls, the carotenoid content of shoots increased by 1.7-, and 2.2- fold at 300 and 450 mM NaCl, respectively, compared to the control and then slightly declined at high NaCl level (600 mM) (Table 3). Carotenoids participate in quenching of singlet oxygen and scavenging of other ROS compounds that are produced during environmental stresses, including salinity (Bose et al. 2014). In agreement with our findings, numerous papers report the carotenoids accumulation in halophytes (Aghaleh et al. 2009; Rabhi et al. 2010). This may suggest the significant antioxidant role of carotenoids in detoxification of ROS in halophytes, including *L. fusca*. The carotenoid/chlorophyll ratio gradually increased and by 24% increase compared to the control, reached 0.184 at 600 mM NaCl. The elevated ratio of carotenoid/chlorophyll may constitute an approach to keep photosystems against photooxidation and emphasize that carotenoids in addition to their function as accessory pigments show antioxidant properties (Strzalka et al. 2003).

Effects of salt stress on lipid peroxidation

Our findings demonstrated that salinity had no significant effect on MDA content in *L. fusca* shoots up to 450 mM NaCl. Indeed, although there was a tendency of an increasing MDA with increasing salinity level, the effect was not statistically significant ($p < 0.01$). However, when the salt level was increased above 450 mM, the MDA content significantly enhanced and was approximately 31% higher than the control (Fig. 1a). MDA is a major cytotoxic product of lipid peroxidation and has frequently been applied as a biomarker to evaluate the sensitivity of plant to oxidative stress (Talbi Zribi et al. 2017). In *L. fusca*, the salinity concentration up to 450 mM did not significantly increase the MDA content of shoots, suggesting that the strategies that preserve the plant against salinity-induced oxidative damage are active in *L. fusca* shoots. However, high-level NaCl concentration significantly caused lipid peroxidation in *L. fusca*, as proposed by MDA accumulation, which is in agreement with former researches (Sekmen et al. 2012; Lu et al. 2017).

Effect of salt stress on proline content

Proline was progressively and significantly accumulated in *L. fusca* shoots with increasing concentration of NaCl. At 300, 450, and 600 mM NaCl, the proline concentration in shoots was 3.78-, 10.4- and 15.5-fold higher than control, respectively (Fig. 1b). Indeed, there was a linear association between proline accumulation and intensity of salinity in *L. fusca*. Under abiotic stress conditions, plants accumulate significant amounts of various kinds of osmoprotectants as a basic approach to avert the harmful effects of stress (Hayat et al. 2012). Proline, the most prevalent osmolyte accumulates in numerous plants along with increased salinity in the environment and could be assessed as a salinity tolerance index (Boscaiu et al. 2012). It has been cleared that proline assists plants to retain their cell turgor, prevent protein denaturation, and protect membrane integrity under different stresses (Hayat et al. 2012). The advantageous role of proline accumulation in tolerance to salinity has been proved in an

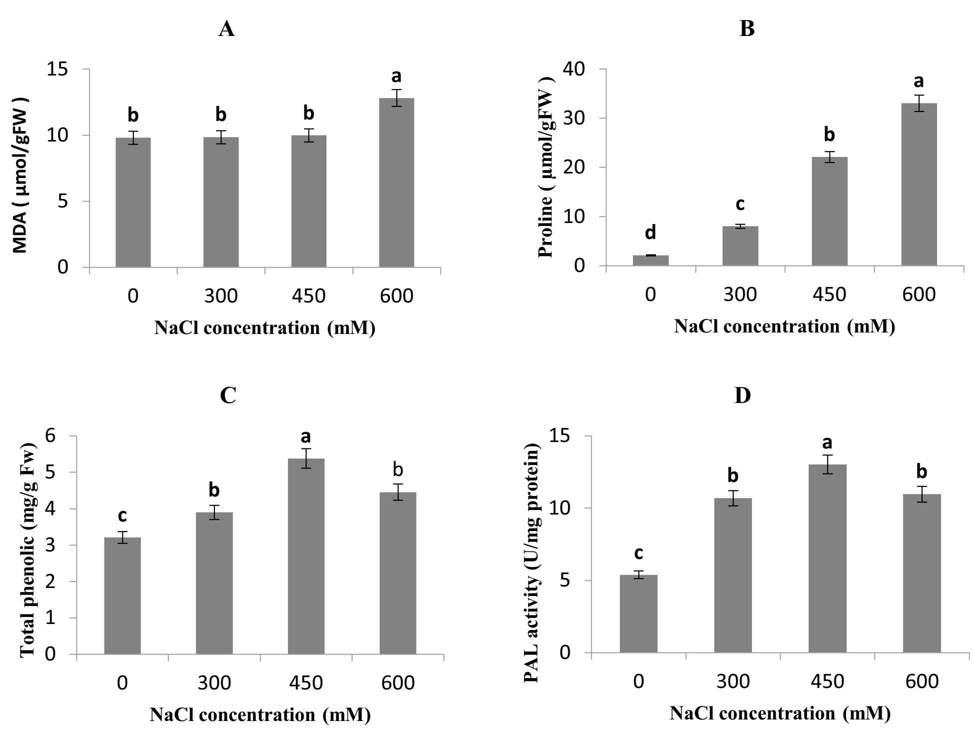

Fig. 1 Effect of different concentrations of NaCl on MDA content (**a**), proline content (**b**) total phenolic content (**c**), and activity of PAL (**d**) in the shoots of *L. fusca* seedlings. Values are mean ± SD ($n = 5$). Columns with same lowercase letter are not significantly different according to Duncan's test ($p < 0.01$)

extensive spectrum of halophytes (Theerawitaya et al. 2015; Lu et al. 2017). In our research, the considerable accumulation of proline in shoots of *L. fusca* was detected in response to salinity compared to the control. This suggests that following the exposure to high salinity, proline might play a remarkable role in the adjustment of osmotic pressure.

Effect of salt stress on total phenolic content and PAL activity

The total phenolic content and PAL activity increased with the increasing salt level, and the highest increment was seen in shoots exposed to 450 mM NaCl (1.67- and 2.42-fold higher than that of control, respectively). PAL enzyme activity and total phenolic of shoots significantly reduced at high salt level (600 mM). However, at this salt level, the activity of the enzyme and the content of phenolics were 103% and 38% higher than control, respectively (Fig. 1c, d). Phenolic compounds are well-known non-enzymatic chemical antioxidant playing a significant role in detoxifying free radicals induced under different constraints, including salinity (Reginato et al. 2014). PAL is the first enzyme and committed step in the phenylpropanoid metabolism, playing a substantial role in plants through adjusting the flow of carbon between the primary and secondary metabolism (Zhang and Liu 2015). Our findings are consistent with numerous reports which emphasize a positive association between increments in the PAL expression/activity and phenolics accumulation in developmental programming of plant and a variety of constraints, among them salinity (Boudet 2007; Falleh et al. 2012). This implies that enhanced PAL activity, which results in induction of the phenylpropanoid metabolism and subsequent increase of phenolics, represents a source of non-enzymatic antioxidants and protects *L. fusca* from salinity-induced oxidative damages.

Effect of salt stress on protein content and antioxidant enzyme activity

The total soluble protein content of shoots was gradually increased with the increased levels of salinity, peaked in 450 mM NaCl and after that reduced at 600 mM NaCl. However, no statistical difference was observed among 300, 450, and 600 mM NaCl Levels (Fig. 2a). The increment of protein content in salt-resistant species following exposure to salinity is not an unusual response (Yang et al. 2010; Ali et al. 2012). It can be inferred which an increment in the content of total soluble protein of *L. fusca* could be related to increases in protein biosynthesis for acclimation to new conditions and reprogramming, particularly to maintain the cellular integrity and photosynthesis under salinity (Sobhanian et al. 2010). Salinity caused a higher level of enzyme activity in shoots of *L. fusca* in comparison with controls. The activities of SOD and CAT were enhanced gradually by increasing salinity in growth medium and peaked at 450 mM NaCl (about 2.72- and 1.47-fold increase, respectively, compared to the control) and then declined at 600 mM NaCl, but still were higher than that of the control (Fig. 2b, c). The APX

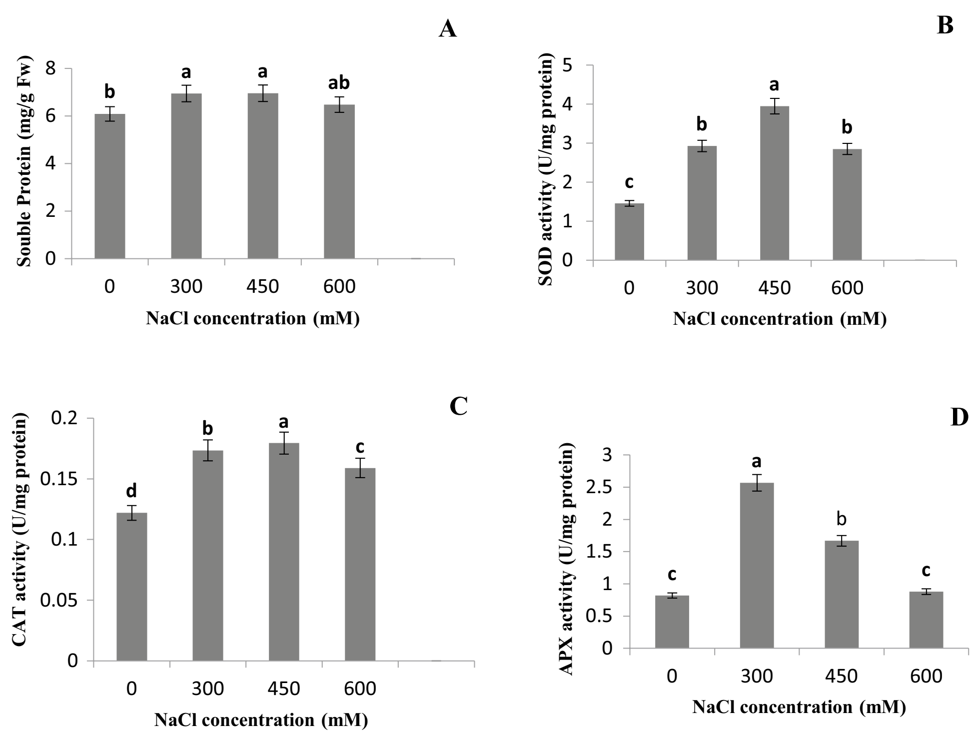

Fig. 2 Effect of different concentrations of NaCl on total soluble protein (**a**), SOD (**b**), CAT (**c**), and APX (**d**) in the shoots of *L. fusca* seedlings. Values are mean ± SD ($n = 5$). Columns with same lowercase letter are not significantly different according to Duncan's test ($p < 0.01$)

activity was significantly enhanced in seedlings exposed to NaCl and the activity of enzyme reached its maximum value at 300 mM NaCl (threefold higher than the control) and after that decreased gradually, and at 600 mM NaCl was equal to that of control (Fig. 2d). Salinity provokes the oxidative stress with the production of ROS in plant cells. To maintain the cellular components from damages induced by ROS accumulation, higher plants evolved a highly organized and effective enzymatic rapid response system which comprises SOD, CAT, and APX (Acosta-Motos et al. 2017). SOD is the most powerful antioxidant in the cell and considered as a first-line sentinel against oxidative damages caused by ROS compounds by rapidly transforming $O_2^{\cdot-}$ into H_2O_2 and O_2 (Miao and Clair 2009). Hydrogen peroxide (H_2O_2), which is generated by the activity of SOD, is still toxic and needs to be omitted in subsequent reactions by conversion to H_2O. In higher plants, some enzymes adjust intracellular levels of H_2O_2, but CAT and APX are the most prominent role in the degradation of H_2O_2. (Huang and Sikes 2014). Numerous researches have revealed a relation between the antioxidant capacity and salinity tolerance in many halophytes, including *Calligonum caput-medusae* (Lu et al. 2017), and *Bruguiera cylindrica* (Palliyath and Puthur 2018), etc. Our results generally demonstrated that salinity stress up to 450 mM led to SOD activity induction, which was affiliated with an increment in the activity of APX and CAT. An increment in the activity of SOD, CAT, and APX in low (300 mM) and moderate (450 mM) NaCl concentration suggested that *L. fusca* is equipped with an effective and responsive enzymatic ROS scavenging system which was confirmed by an unchanged level of MDA. However, a significant accumulation of MDA detected under high NaCl concentration (> 450 mM) in shoots of *L. fusca* could be related to a reduction in the activity of SOD, CAT, and APX enzymes. In our research, a notable increment in the activity of APX was noticed in low NaCl concentration (300 mM) compared to control, suggesting that APX activity probably involved in H_2O_2 scavenging only at low-level oxidative stress caused by salt stress. However, in response to higher salinity, ROS compounds trigger the inhibition of APX activity. In contrast, a considerable increase in CAT activity was detected under moderate NaCl concentration (450 mM) compared to the control. The results demonstrated that in *L. fusca*, H_2O_2 was detoxified by APX at lower and by CAT at higher NaCl stress.

Effect of salt stress on the expression pattern of sodium ion transporters

The results demonstrated that transcript levels of all studied genes were influenced by salinity in both tissues but much more increased in roots than in shoots. The expression levels of *Lf* SOS1 in 300 and 450 mM NaCl enhanced 1.35- and 2.28-fold in shoots and 1.5- and 2.5-fold in roots compared to the control, respectively (Fig. 3a). At high NaCl level, the expression of SOS1 in both tissues was equal to control. Under 300 and 450 mM of NaCl, the transcript levels of NHX1 in the shoots was up-regulated to about 1.5- and 2.2-fold higher than control and in the roots to about 2.2- and 6.7-fold higher than control, respectively. At 600 mM of NaCl, the mRNA levels of NHX1 in both tissues decreased, however, still were 1.5- and 1.22-fold higher than control in shoots and roots, respectively (Fig. 3b). In shoots tissue, the PM H^+-ATPase expression was increased by 1.15- and 1.4-fold at 300 and 450 mM NaCl, relative to control, respectively. In roots, tissue exposing to 300 and 450 mM NaCl enhanced the expression levels of H^+-ATPase by 1.6- and 1.9-fold higher than control, respectively. At higher concentrations, the mRNA levels of the PM H^+-ATPase were down-regulated in both tissues and remained statistically equal to that of control (Fig. 3c). During salt stress, halophytes utilize various mechanisms including controlled uptake of Na^+, Na^+ extrusion from the cell, and compartmentalization of Na^+ into the vacuole to cope with Na^+ toxic effects (Munns and Tester 2008). Under salinity, the Na^+ efflux from the cytosol and vacuolar Na^+ compartmentalization can be fulfilled with the Na^+/H^+ antiporter activity in the tonoplast (NHX1) and plasma membrane (SOS1). The driving force for Na^+ exclusion by the above-mentioned antiporters was created by the membrane H^+-ATPase and H^+-pyrophosphatase pumps (Shi et al. 2002; Almeida et al. 2017). The gene encoding SOS1 protein has been cloned from various species and, in a former study, we reported the partial cloning and gene expression analysis of the SOS1 gene from *L. fusca* during short-term salinity stress (Taherinia et al. 2015). In the current research, the higher transcript level of the SOS1 gene was detected in roots compared to shoots. The similar expression pattern in other halophytes such as *Puccinellia tenuiflora* (Guo et al. 2012) and *Aeluropus lagopoides* (Jannesar et al. 2014) was observed. The results indicated that Na^+ extruding from roots to soil or regulating long-distance transport of Na^+ from roots to shoots, and maintenance of low Na^+ level in the cytosol of the *L. fusca* shoots, especially in photosynthetic cells occurred through the activity of SOS1 (Shi et al. 2000; Apse and Blumwald 2007). The expression levels of PM H^+-ATPase were enhanced with salinity and the gene expression profile was similar to that of SOS1. Recently, we isolated and characterized the PM H^+-ATPase gene from *L. fusca* (data unpublished). The PM H^+-ATPase transcripts accumulation in roots and shoots of *L. fusca* following exposure to salinity indicates the necessity for this pump in these tissues during salinity adaptation. However, the high degree of salt-induced expression of the PM H^+-ATPase in roots compared to shoots could be related to the establishment and maintenance of the electrochemical gradient across the PM of the root cells to restrict the transport of harmful ions to

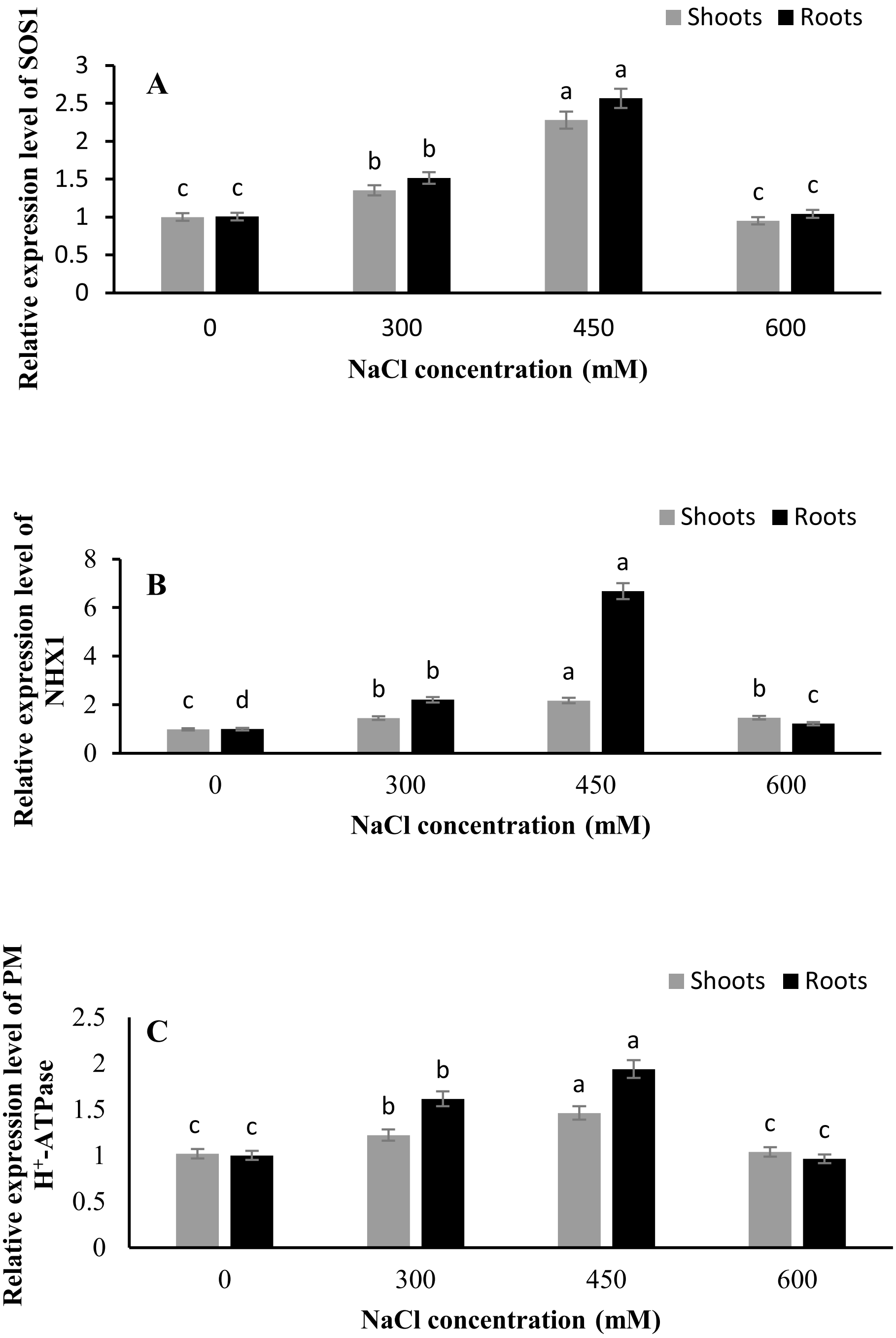

Fig. 3 Effect of different concentrations of NaCl on the relative mRNA level of SOS1 (**a**), NHX1 (**b**), and PM H^+-ATPase (**c**) in the shoots and roots of *L. fusca* seedlings. Data are mean ± standard error calculated from three independent biological replicates. Columns with different letters represent significant difference based on Duncan's test ($p < 0.01$)

the photosynthesizing tissues and adjust the ion homeostasis (Zhang et al. 1999). The NHX1 has been identified from different halophytes (Sanadhya et al. 2015). Rauf et al. (2014) cloned NHX1 gene from *L. fusca* and indicated that it could be used to enhance tolerance to salinity and drought in crops. In Arabidopsis *At*nhx1 mutant, the activity of vacuolar Na^+/H^+ antiporter substantially reduced, and the plant showed Na^+ sensitivity, which highlights the role of this antiporter under salinity (Apse et al. 2003). Our prior study indicated that the transcription of the vacuolar Na^+/H^+ antiporter in shoots of *L. fusca* was modified under short-term cadmium and salinity stresses (Adabnejad et al. 2015). In the current research, the mRNA level of NHX1 gene increased in both tissues but higher in roots than in shoots. The higher expression of *Lf*NHX1 in the roots resulted in slowing down the Na^+ translocation to shoots via sodium compartmentalization in the root vacuoles (Apse and Blumwald 2007). The coordinate inductions of SOS1, NHX1, and PM H^+-ATPase could support the idea that *L. fusca* has an organized mechanism that controls Na^+ influx and efflux and explains the ability of *L. fusca* to survive and maintain growth even under high salinity levels.

Conclusions

These results suggest that *L. fusca* tolerate salinity levels until 450 mM NaCl without showing significant physio-biochemical alterations and could accumulate large amounts of Na^+ in its shoots without damage. However, higher salt concentrations led to the accumulation of ROS, which was manifested by the high MDA accumulation. However, *L. fusca* can protect itself against salinity using numerous mechanisms. These adaptations consist of conservation of the photosynthetic pigments, capacity to enhance the activity of antioxidant enzymes and low levels of oxidative stress, sodium as well as proline accumulation for osmotic adjustment, increased PAL activity and phenolics against oxidative damage and coordinate inductions of SOS1, NHX1, and PM H^+-ATPase in both tissues as an efficient element to regulate the Na^+ accumulation in shoots.

Author contribution statement FM performed most of the experiments. HRK supervised the experimental design and wrote the manuscript. MM did some of the experimentation and provided reagents and materials. All authors reviewed and approved the final draft.

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