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Physiological and antioxidative responses of a halophytic grass *Leptochloa fusca* L. kunth (*Kallar grass*) to salinity

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Abstract

In this study, the effects of salt stress on growth and some physiological parameters of halophytic plant, *Leptochloa fusca* L. Kunth, were investigated. The seedlings were irrigated with half-strength Hoagland solution and then treated with different concentrations of NaCl (0, 100, 300, 500 and 700 mM) for 15 days. The fresh and dry weights of both roots and shoots were unchanged at 100 mM NaCl and decreased at higher concentrations. Relative water content significantly decreased at high NaCl treatments. The significant increase in the contents of chlorophylls and carotenoids due to NaCl stress was observed in all salinity treatment except for100 mM NaCl. Leaf Na^+/ K^+ ratio increased with increase in medium salinity. Moderate (300 mM) and high (700 mM) salinities dramatically increased proline content as compared to that of the control group. With a progressive increase in salinity, catalase, ascorbat peroxidase and guaiacol peroxidase activities also increased gradually in this species. It seems that antioxidant enzyme activity for scavenging reactive oxygen species and proline accumulation for osmotic adjustment play an essential protective role in *L. fusca* under salinity stress. In summary, these data indicate that salinity tolerance is well programmed in *L. fusca* allowing adaptation to harsh environmental conditions in the natural habitat.

Key words: Antioxidant enzyme, Proline, Salinity, Growth, Na/K ratio

Introduction

One of the most important environmental problems in plant growth process is salinity. In the arid and semi-arid regions, planting salt-tolerant species is a favorite way to restore salt-affected degraded lands (Ladeiro, 2012).

Leptochloa fusca L. Kunth, commonly known as kallar grass is a high salt tolerant plant, which grows in saline soils. This grass has C_4 photosynthetic pathway and has reported to harbor N_2^- fixing bacteria in or around their roots (Reinhold, 1987). Its habitat includes Pakistan, India, Asia, Africa and Australia (Bors, 1982). The grass is currently of great interest because of high salinity tolerance and potential as a fodder crop. These specifications make Kallar grass a suitable species for the economic utilization of salt-affected soils (Sandhu *et al*., 1981) and plant establishment in the saline-sodic soils. The existence of salt gland and selective secretion of Na^+ and Cl^- by *L. fusca* as a powerful strategy to cope with salt stress has been reported (Al Hassan, 2017).

Plants respond to salinity by different mechanisms. The most important strategies are the synthesis of a compatible solute such as proline, compartmentation of ions and production of antioxidants. In the other words, salt-tolerant plants must be able to regulate the ion and water movements and scavenge reactive oxygen species (Slama *et al*., 2015). The accumulation of Na^+ and/or Cl^- causes ROS production especially in photosynthetical and respiratory electron transport chain and agitates the normal status of the plant cells plasma membrane. The reactive oxygen species such as superoxide anion and H_2O_2 damages lipids, proteins, DNA and RNA in the cells (Turkan and Demiral, 2009). In stress condition, plants increase the non-enzymatic antioxidants and activity of antioxidant enzymes such as peroxidase (POX), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). SOD converts superoxide to H_2O_2 which is further scavenged by other enzymes such as POX and CAT (Caverzan *et al*., 2016). One of the Asada- Halliwell cycle enzymes is APX, which decomposes H_2O_2 in different cellular compartments. Removing ROS is a basic way to protect the integrity of cell structures and keep the action of various metabolic pathways (Jaleel *et al*., 2008). Hence, salt tolerant plants survive in salt stress conditions via different mechanisms such as ion exclusion, osmotic regulation, and antioxidant resistance.

Halophytes have physiological and biochemical mechanisms which enable them to survive in high saline conditions. In this research, *L. fusca* responses to salinity stress were investigated at physiological, biochemical, and morphological levels to identify

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alternations caused by salinity and adaptive mechanisms in this species. The studied plant is a unique member of poaceae family, which can establish in saline lands as an appropriate rangeland plant. It is important to understand our natural world and use some of halophyte plants as forages and possibility to utilize this plant in saline rangelands to remediate salinity.

Materials and methods

Plant material and culture condition: Kallar grass (*L. fusca*) seeds were soaked in water for 2 hrs. and then about 15 seeds were germinated in pots containing perlite. The seedlings were subjected to 16 hrs. photoperiod, 25/16°C day/night temperature. The pots were irrigated daily with 75 ml half-strength Hoagland solution (pH 5.5) for 15 days. After 15 days, seedlings were treated with Hoagland nutrient solution containing 0, 100, 300, 500, and 700 mM NaCl (pH 6.0). Seedlings were harvested 15 days after NaCl treatment and were stored at -20°C until further analysis.

Growth and relative water content assay: Shoot and root dry weight (DW) was evaluated after the plant samples were dried at 70°C for 24 hrs. Leaf relative water content (RWC) was estimated according to the method of Barrs and Weatherly (1973). It was calculated by following formula:

RWC = [(fresh mass - dry mass) / saturated mass-dry mass] ×100

Photosynthetic pigments measurement: Chlorophylls (Chl) and carotenoids (Car) contents were extracted using 80% acetone. The supernatant absorbance was measured at appropriate wavelengths (665, 645 and 470 nm). The chlorophylls and carotenoids concentrations were calculated according to the method of Lichtenthaler (1983).

Na^+ and K^+ content: The total amounts of K^+ and Na^+ were determined by flame photometry according to Chapman and Pratt (1961).

Proline content assay: Determination of free proline content was performed according to Bates *et al*. (1973). L-proline (concentrations of 0, 2, 4, 8, 16, 32 mM) used as a standard. Proline concentration was determined using calibration curve ($Y = 0.0093\ X + 0.0022$, $R^2 = 0.983$).

Lipid Peroxidation assay: Malondialdehyde (MDA) was used as an indicator of membrane lipid peroxidation (Heath and Packer, 1968). For the calculation of MDA, 1 ml of leaf extract, was mixed with 4 ml 20% (w/v) trichloroacetic acid containing 5% (w/v) thiobarbituric acid (TBA) and then centrifuged. The absorbance of supernatant was read at 532 nm and the MDA concentration was estimated by using an extinction coefficient (ε) of $1.55 \times 10^5\ M^{-1}\ cm^{-1}$.

Protein content and antioxidative enzymes activity assay: Leaves (500 mg) were homogenized in 50 mM potassium phosphate buffer (pH=7) containing 1% soluble polyvinylpyrrolidone (PVP) and 1 mM ethylenediamine tetra acetic acid (EDTA). The homogenate was centrifuged at 20,000 g (20 min). All operations were performed at 4°C. The supernatant was used for enzyme activity assay.

Total protein content of the extracts was determined according to the spectrophotometric method of Bradford (1976), using bovine serum albumin (BSA) as the standard.

Ascorbate peroxidase (APX) was determined spectrophotometrically from the decrease in absorbance at 290 nm (extinction coefficient of 2.8 $mM^{-1}\ cm^{-1}$ was used for ASA). The reaction solution contained 50 mM potassium phosphate buffer (pH=7), 0.5 mM ascorbate, 0.1 mM H_2O_2 and 150 µl enzyme extract. An enzyme activity unit is an enzyme that oxidizes 1 µM of ascorbic acid in 1 minute (Nakano and Asada, 1981).

The GPX activity was determined by using the method of Plewa *et al*. (1999) following the formation of tetraguaiacol by monitoring the increase in absorbance at 470 nm (extinction coefficient of 25.5 $mM^{-1}\ cm^{-1}$ for tetraguaiacol). One unit of enzyme activity is an enzyme that oxidizes 1 µM of guaiacol in 1 minute.

Catalase activity was assayed by decrease absorption of H_2O_2 at 240 nm using the extinction coefficient of 40 $mM^{-1}\ cm^{-1}$ for H_2O_2 (Dhindsa *et al*., 1981). One enzyme activity unit is an enzyme that breaks up 1 ml of H_2O_2 within a minute.

Statistical analysis: The experiments were performed in a completely randomized design. All treatment were carried out in three biological replicates. Analysis of variance was performed using the ANOVA procedure. Statistical analyses were performed via the SPSS software. Duncan's multiple Range Tests showed significant differences between means. P values less than 0.05 were considered statistically significant.

Results

Growth and relative water content: As shown in Fig. 1 fresh and dry weight of shoots, fresh and dry weight of roots and shoot length significantly decreased in all NaCl treatments except 100 mM NaCl. RWC decreased only in high concentration of NaCl (500 and 700 mM) compared to the control group. The plants were treated with high salinity concentrations (500 and 700 mM NaCl) and some salt precipitations were observed over the leaf and shoot. There was no sign of chlorosis in these treatments.

Photosynthetic pigments: Chlorophyll a, b, total chlorophylls and carotenoids contents increased significantly in plants after treatment with 300, 500, and 700 mM NaCl (Fig. 2).

Na^+ and K^+ content: The shoot K^+ content was slightly changed in all salinity treatments, but there was a significant decrease in 300 mM NaCl (Fig. 3 a). Shoot Na^+ content was significantly increased with increasing NaCl concentration in the medium (Fig. 3 b). The root K^+ content significantly decreased in 500 mM NaCl (Fig. 3 c). As it is shown in Fig. 3 d, the root Na^+ content increased in NaCl treatment especially in 700 mM NaCl. The Na^+/K^+ ratio increased significantly in

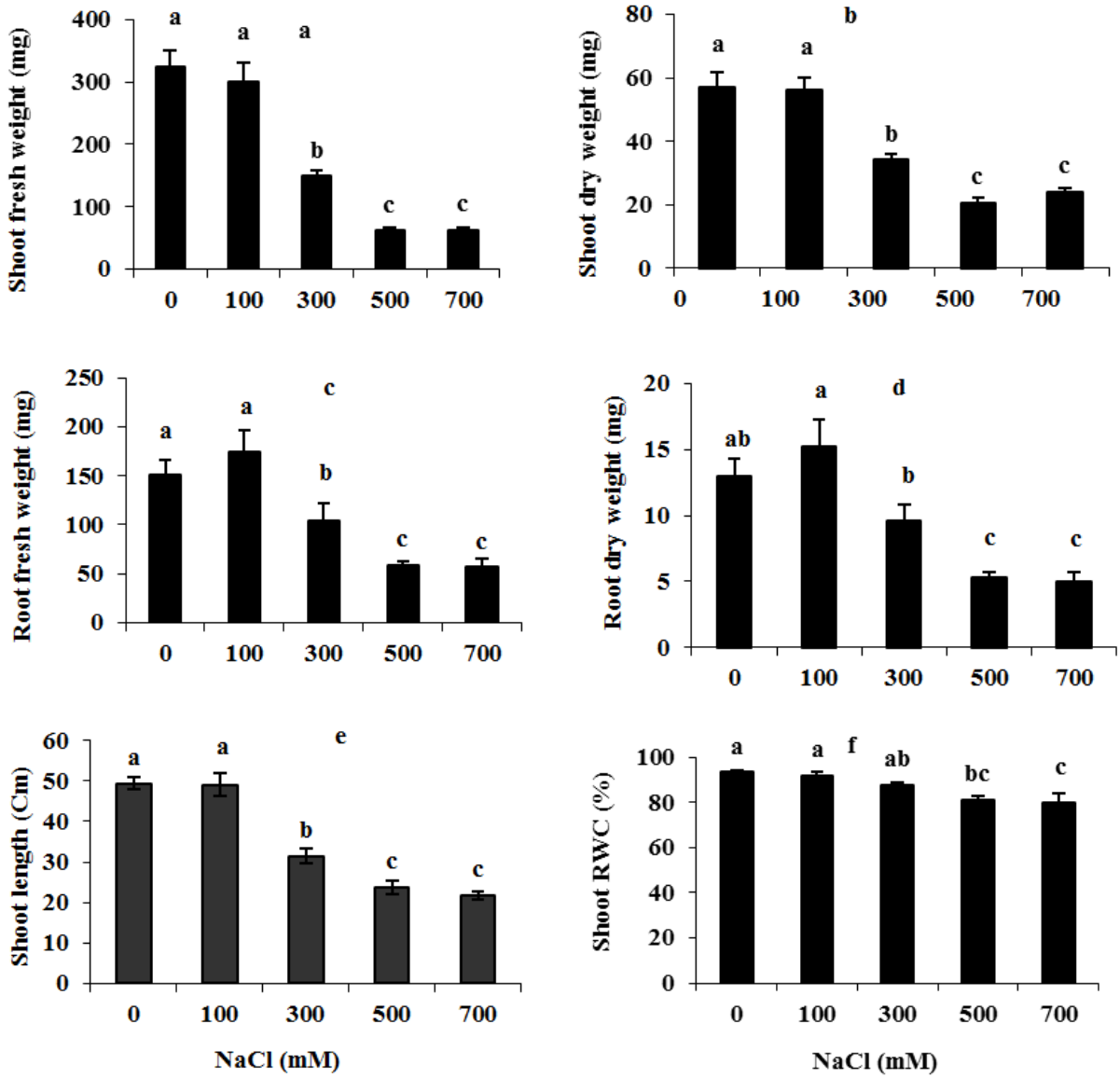

Figure 1- shoot fresh and dry weight (a, b), root fresh and dry weight (c, d), shoot length (e) and shoot relative water content in seedlings (30 days) of *L. fusca* after 15 days of NaCl stress. Each value represents the mean of three replicates. *Bars* indicate SE. Different letters over the bars indicate significant differences (P<0.05).

shoots and roots of plants in all treatments except for 100 Mm NaCl (Fig. 3 e and f). Generally, as shown in Fig. 3, the root Na^+ and K^+ content was higher than the shoot.

Proline content: Proline content increased in shoot of plants, which were treated with NaCl. However, 100 mM NaCl had no significant effects on the proline content. A clear increase in proline concentration was detected in shoots of plants which were treated with700 mM NaCl (9.74 mM/g FW in 700 mM NaCl compared to 0.11 mM/g FW in control) (fig. 4).

Lipid peroxidation: Lipid peroxidation in leaves of *L. fusca* was measured as MDA content shown in Fig. 5. The level of lipid peroxidation increased with increase in NaCl concentration except for 100 mM NaCl.

Protein content and antioxidative enzymes activity: As observed in Fig. 6 a, salinity treatment had no significant effects on total protein content in 100, 500 and 700 mM NaCl. However, the total content of soluble protein significantly increased only in 300 mM NaCl treatment compared to the control.

The effect of different salinity treatment on the activities of CAT, APX, and GPX in *L. Fusca* are shown in Figure 6. The activity of CAT increased in all salinity treatments, but this increase was not significant in 100 mM NaCl treated plants (Fig. 6 b). The maximum activity of this enzyme was observed at 500 mM NaCl (about 8 fold compared to the control plants). APX activity significantly increased in all NaCl treated plants except for 100 mM NaCl treatment. In 100 mM NaCl trteatment, the activity of this enzyme decreased compared with the control plants (Fig. 6 c).

GPX activity significantly increased in 500 and 700 mM NaCl treatments but significantly decreased in 300

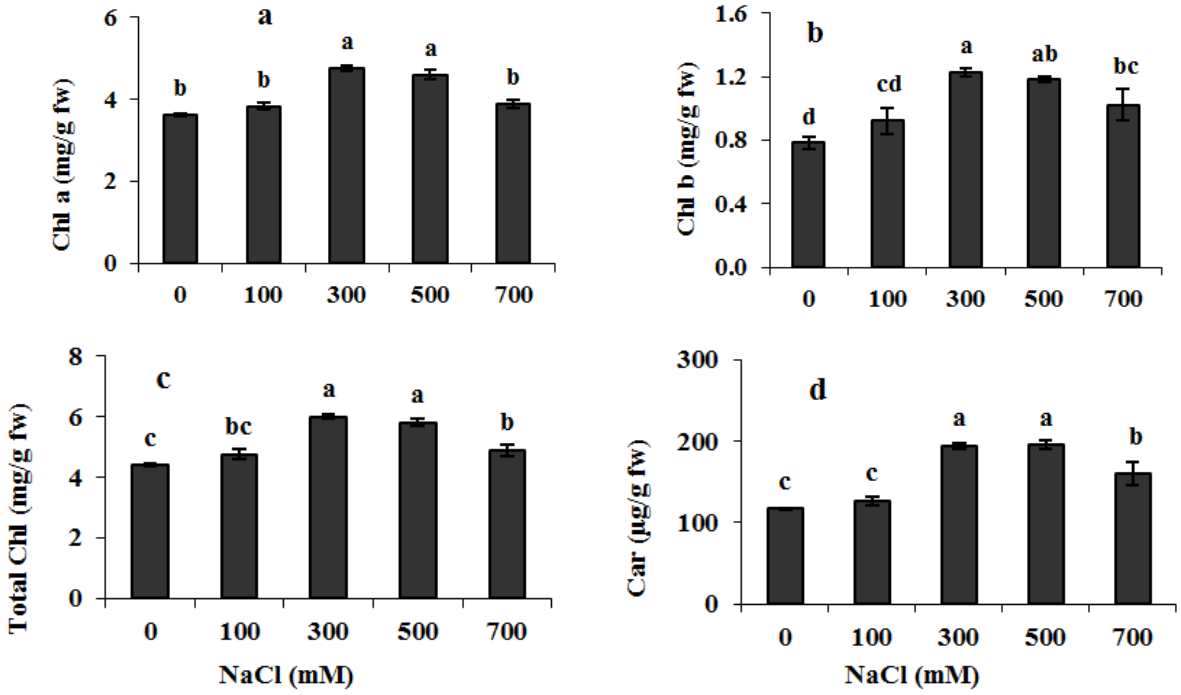

Figure 2- Chl *a* (a), Chl *b* (b), total chlorophyll (c) and Carotenoids (d) content in seedlings (30 days) of *L. fusca* after 15 days of NaCl stress. Each value represents the mean of three replicates. *Bars* indicate SE. Different letters over the bars indicate significant differences (P<0.05).

mM NaCl (Fig. 6 d). The maximum activity of all antioxidant enzyme obtained in 500 mM NaCl treatment.

Discuttion

In the present study, the effects of salinity stress on growth and physiological responses of halophytic grass *L. Fusca* were investigated. Some of the monocotyledonous (Poaceae) species can tolerate a large range of salt tolerance (Rozema and Schatb, 2013). The growth of *L. fusca* at 100 mM NaCl was not significantly changed and it seemed that *L. fusca* tolerates this condition, while moderate salinity treatment (300 mM NaCl) significantly decreased plant growth. The decrease in plant growth in this halophytic plant at high salinity is a common event and reported for dicotyledonous halophytes such as *Salicornia* species (Aghaleh *et al*., 2011) and monocotyledonous halophytes such as *Aeluropus lagopoides* (Sobhanian *et al*., 2010). In high salinity treatments (500 and 700 mM), RWC of *L. fusca* decreased. In this state, RWC reduced to below 13 and 16% of the control condition. It seems that high salinity increases Na^+ and Cl^+ ion in soil and decreases Ψw and water uptake. This is a common response to salinity similar to those reported for other species (Suarez and Medina, 2008). In salinity conditions, water content of the plant, water uptake and plant growth decreases. In addition, the excessive amounts of salt can disturb the cells respiration and decrease the uptake of essential nutrients, which reduced the growth of plants (Parihar *et al*., 2015).

The degradation and decreases of chlorophylls is one of the visible symptoms of salt stress (Parida *et al*., 2002; Mittal, 2012). However, in *L. fusca*, 300 mM NaCl and higher salinity treatments cause a remarkable increase in chlorophylls and carotenoid contents when compared to the control plants. Also, total Chl content incensement under salt stress was observed in *Chenopodium album* (Yao, 2010) and *Phaseolus vulgaris* (Taibi *et al*., 2016). In this experiment, carotenoid content increased at moderate and high salinity treated plants. In plant cells, carotenoids are protectant agents, which preserve chloroplast against photo-oxidative stress (Ashraf, 2009). Therefore, it seems that the increase of carotenoids in this plant under salt stress condition probably is the protective mechanism.

Na^+ content in all NaCl treated plant increased in roots and shoots when compared to the control group. The current results showed that there was no any significant changes in Na^+ concentration between 100, 300 and 500 mM NaCl treatment. This observation confirms the remove of extra Na^+ through white bladder hair that we observed on the top of leaves which it seems is the effective strategy in *L. fusca* restriction.

In *L. fusca*, Na^+/K^+ ratios generally increased as salinity increased. It ranged from 0.33 to 0.93 in roots and from 0.29 to 1.35 in shoots, which are similar to those reported in other members of the Poaceae such as *Odyssea paucinervis* (Naidoo *et al*., 2008). Halophytic monocotyledons (Poaceae) have a much higher K^+ over Na^+ selectivity in comparison with dicotyledonous halophytes. They may rely on maintenance K^+ homeostasis and/or Na^+ exclusion under salt stress.

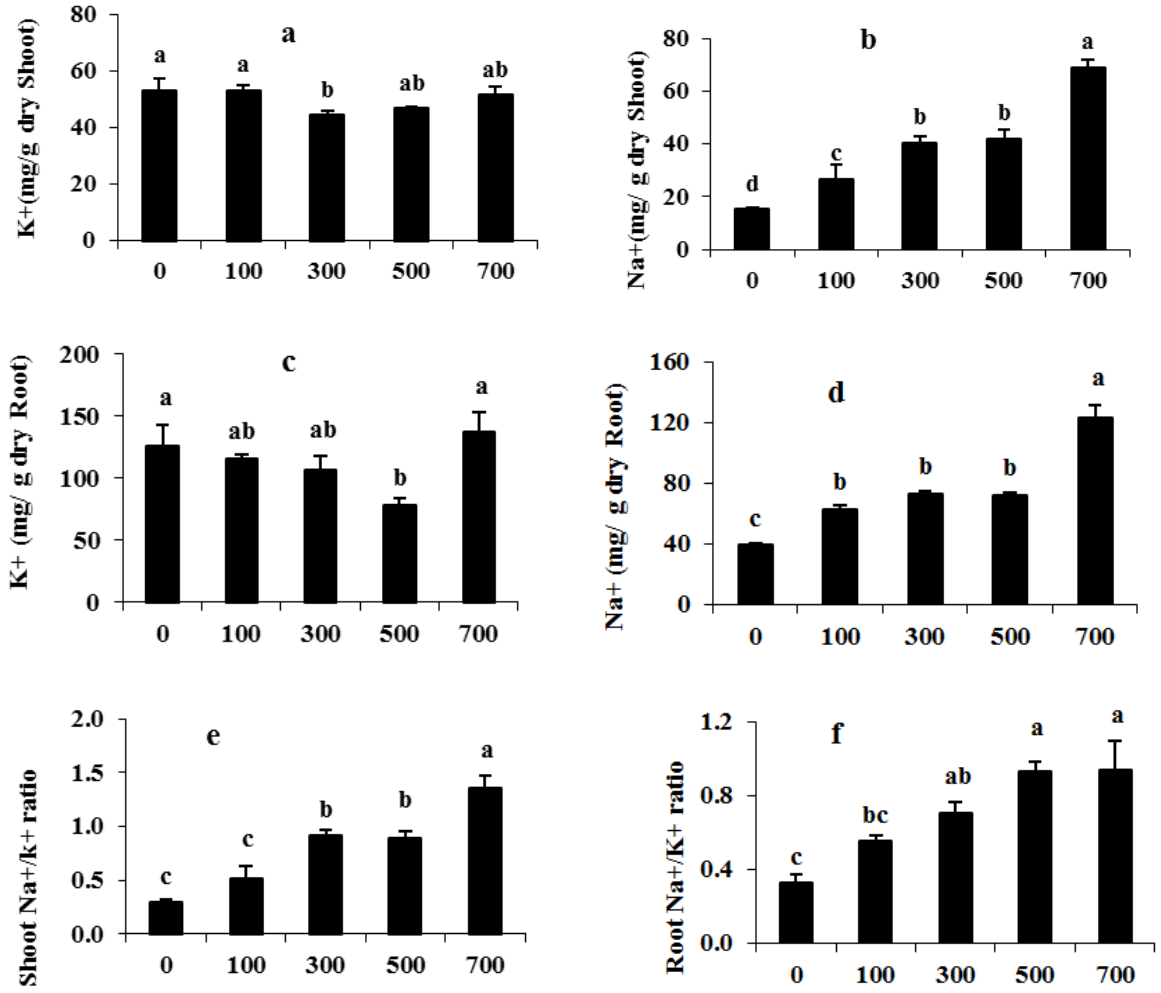

Figure 3- Change in ion concentrations of Na^+ and K^+ in shoots and roots of *L. fusca* seedlings (15 days) after 15 days of NaCl stress. Each value was the means ± SE of three replicates. Different letters indicate significant differences (P<0.05).

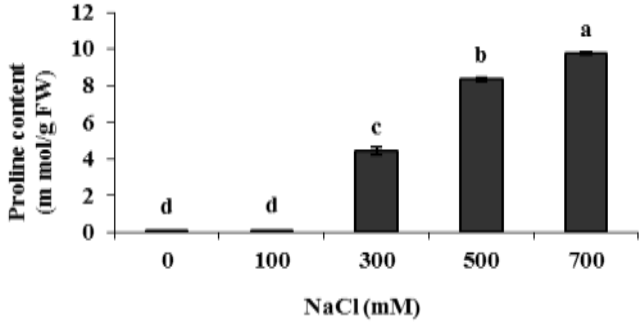

Figure 4- Proline content in seedlings of *L. fusca* seedlings (15 days) after 15 days of NaCl stress. Each value was the means ± SE of three replicates. Different letters indicate significant differences (P<0.05).

However, Na^+ excretion has been reported for many Poaceae in salt lands. Na^+ exclusion in halophytes shows that production of osmotic solutes supports a balance of internal and external water potentials in a saline habitat (Lugan *et al*., 2010).

Proline content dramatically increased in 300, 500, and 700 mM salinity treatments. Proline accumulation has been mostly reported as a resistant mechanism in response to hyperosmotic conditions for example in *Atriplex nummularia* (Hussin *et al*., 2013), *Spartina alterniflora* (Li *et al*., 2010) and *Odyssea paucinervis* (Naidoo *et al*., 2008). In *L. fusca* high concentration of proline accumulated in the high salinity level (700 mM) and its concentration was sufficient to account for contribution to the osmotic adjustment. The increase of proline accumulation can be attributed to the up-regulation of biosynthetic gene expression and a decrease in proline consumption as well as down-

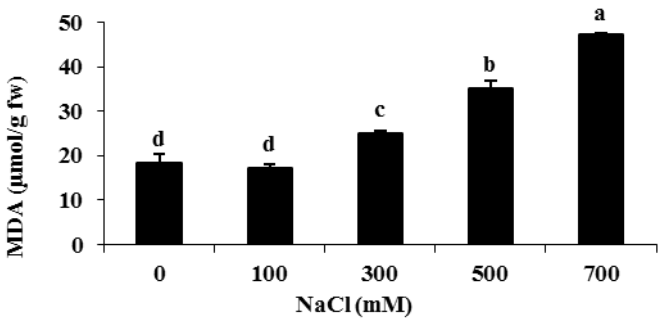

Figure 5- Malondealdehyde content of *L. fusca* seedlings (15 days) after 15 days of NaCl stress. Each value was the means ± SE of three replicates. Different letters indicate significant differences (P<0.05).

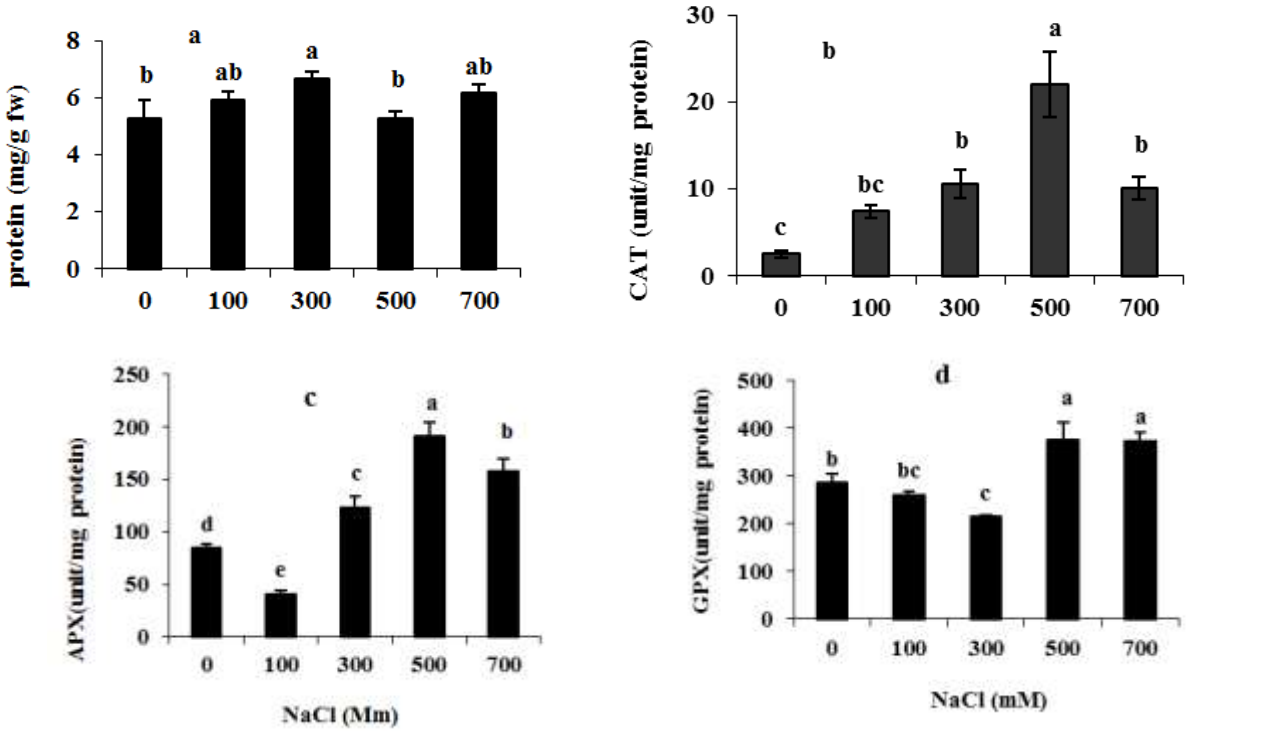

Figure 6- Changes in the protein (a) and activity of CAT (b), APX (c) and GPX (d) enzymes of *L. fusca* seedlings (15 days) after 15 days of NaCl stress. Each value was the means ± SE of five replicates. Different letters indicate significant differences (P<0.05).

regulation of catabolism of this amino acid (Czarnocka and Karpinski, 2018). Proline is known as a compatible osmolyte. It removes free radicals, protects enzymes and stabilizes cell redox in stress conditions (Verbruggen and Hermans, 2008). Hasanuzzaman *et al.* (2014) have shown that proline increases rice seedlings' tolerance to salt-induced oxidative damage by up-regulating their antioxidant defense system.

Total protein content in all NaCl treatments slightly increased. Increase in total soluble protein content under salinity stress was also reported for another halophyte grass such as *Aeluropus Lagopoides* (Sobhanian *et al.*, 2010) and *Brassica juncea* (Mittal *et al.*, 2012). The increase in protein content could be attributed to the increase in protein biosynthesis for acclimation to new conditions, sustain photosynthesis under salt stress, biosynthesis of anti-oxidative enzymes and other stress-induced proteins upon stress exposure.

Peroxidation of lipid membranes causes damage in many cellular compartments under salt stress conditions (Taibi *et al.*, 2016). The results of our experiment showed that with increase in NaCl concentrations, MDA content increased. In stress conditions, MDA plays an important role in disturbing membrane integrity and in controlling programmed cell death in plants. Therefore, maintaining the minimum amounts of this molecule under salinity stress is a key factor in keeping plants healthy and in their subsequent acquisition of tolerance to salinity.

In the salt stress conditions, formation of ROS such as hydrogen peroxide, superoxide, and hydroxyl radicals are induced. The SOD converts superoxide into H_2O_2, which is detoxified by CAT and peroxidases. Glutathione reductase (GR) and APX reduce H_2O_2 to

water through the Asada-Halliwell pathway (Kanwar *et al*., 2015). It has been shown that H_2O_2 accumulation in the leaves of *Oryza sativa* and *Vigna catjang* under salinity stress is due to the decrease in CAT activity (Singha and Choudhuri, 1990). In ths research, CAT activities decreased significantly in the the plants which were subjected to the 700 mM NaCl (compare to 500 mM) suggesting it may promote H_2O_2 accumulation (Fig. 6 b), and hydroxyl radicals ($OH^{\bullet}$) formation. Since $OH^{\bullet}$ radicals react with most compounds present in biological systems and damage biological membranes, they might precipitate lipid peroxidation and membrane damage in the high salinity.

GPX activity significantly decreased by 300 mM NaCl treatment, but at high salinity treatments increased markedly. It can be concluded that GPX had an important role in salt tolerance only at the high level of salt stress such as 500 and 700 mM in the present study. APX activity significantly increased during NaCl treatment except at 100 mM NaCl. Yao *et al*. (2010) reported higher activity of GPX, APX and CAT in *Chenopodium album* under salinity stress conditions.

In addition, it has been reported that ROS scavengers are linked to antioxidative enzymes activities and existence of osmo-protectant compounds solutions such as proline (Choudhury *et al*., 2017). The present findings showed that enzymes involved in ROS-scavenging could be considered a portion of the general adaptive mechanism of *L. fusca* against salinity in this species.

Conclusion

This study helps us to understand more about biochemistry and physiology of the *L. fusca* tolerance to salt stress. The current results demonstrated that *L. fusca* could tolerate high salinity because of the increase in chlorophyll and carotenoid, maintenance of constant tissue K^+ level, proline accumulation and an increase in antioxidant activities. The results, also, showed that low level of salinity (100 mM) had no significant effects on growth of *L. fusca* and furthermore, this halophyte grass can grow at moderate (300 mM) and can survive at extreme salinities (500 and 700 mM). The analysis of the data from this research support the idea that this halophytic grass is a valuable plant model for understanding plant strategies under salt stress and can help to improve salt tolerance of crops in future.

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