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

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ABSTRACT

The effects of salt stress on growth and some physiological parameters were investigated in a holophytic plant, *Leptochloa fusca* L. Kunth. The seedlings were grown for 15 days in half-strength Hoagland solution and treated with different concentrations of NaCl (0, 100, 300, 500 and 700 mM) for 15 days. The fresh and dry weights of both root and shoot were unchanged at 100 mM NaCl and decreased at higher concentrations. Relative water content was decreased significantly at high NaCl treatments. A significant increase in the contents of chlorophylls and carotenoids due to NaCl stress was observed in all salinity treatment except for 100 mm NaCl. Leaf Na+/K+ ratio increased with increase in salinity in the medium. Moderate and high salinities dramatically increased proline content as compared with that of the control. With increased salinity, catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) activities also increased gradually in this species. It seems that antioxidant enzyme activity for scavenging reactive oxygen species and proline accumulation in 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, lipid peroxidation, proline, salinity tolerance.

Abbreviations: Car, Carotenoids; CAT, catalase; Chl, chlorophyll; DW, dry weight; FW, Fresh weight; H$_2$O$_2$, hydrogen peroxide; MDA, malondialdehyde; GPX, guaiacol peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; EDTA, ethylenediamine-N,N,N',N' tetraacetic acid; PVP, polyvinylpyrrolidone.

INTRODUCTION

Salinity is regarded as one of the most important environmental extremes that affect plant growth and metabolism adversely particularly in the arid and semi-arid regions in the world (Munns and Tester, 2008). In these regions, planting salt-tolerant species, particularly N$_2$-fixing species, is the most useful approach in rehabilitating salt-affected degraded lands (Oba et al., 2001).

*Leptochloa fusca* L. Kunth, commonly known as kallar grass is a highly salt tolerant plant which grows in saline sodic soil (EC22-40 ds/m). This grass has C$_4$ photosynthetic pathway (Zafa, 1984) and has been reported to harbor N$_2$-fixing bacteria in or around their roots (Bors, 1982). Its distribution is in Pakistan, India, Asia, Africa and Australia. The grass is currently of greatest interest because of high salinity tolerance and potential as a fodder crop. These characteristics make Kallar grass a possible candidate for the economic exploitation of waterlogged, salt-affected soils in tropical regions (Sandhu et al., 1981) and the establishment of a plant succession for the reclamation of saline-sodic soils (Sanhu and Malik, 1975). Existence of salt gland and selective secretion of Na$^+$ and Cl$^-$ by *L. fusca* as a powerful strategy to cope with salt stress were reported
(Wieneke et al., 1987). Adaptation to NaCl salinity is complex and comprises a wide range of morphological, physiological and biochemical mechanisms (Flowers and Colmer, 2008). Among these, selective accumulation or exclusion of ions, compartmentalization of ions at the cellular and whole-plant levels, synthesis of compatible solutes such as proline, and induction of antioxidant enzymes are crucial biochemical strategies (Torkan and Demiral, 2009).

Salt-tolerant plants, besides being able to regulate the ion and water movements, should also have a better antioxidant system for efficient removal of reactive oxygen species (ROS) which are formed by oxidative stress as a result of decreased stomatal conductivity under low water soil potential. Formation of ROS is also caused by ion toxicity due to the accumulation of Na+ and/or Cl− that disturbs redox status of the plasma membrane, and different compartments such as chloroplasts and mitochondria of the plant cells. Formation of ROS such as superoxide anion and H2O2 damage lipids, proteins, other vital molecules such as DNA and RNA in the cells (Torkan and Demiral, 2009). To survive under stress condition, plants possess non-enzymatic antioxidants such as ascorbic acid, α-tocopherol and reduced glutathione and enzymatic antioxidants such as SOD, POX, CAT, APX and GR. Among these, SOD decomposes O2− to H2O2 which is further scavenged by POX in extracellular space and cytosol; and mainly by CAT in peroxisomes. H2O2 is also decomposed by APX, one of the Asadae Halliwell cycle enzymes, in different cellular compartments. Hence, reacting directly or indirectly with ROS, enzymatic and non-enzymatic antioxidants contribute to maintaining the integrity of cell structures and the proper functions of various metabolic pathways (Jaleel et al., 2008). Thus, antioxidant resistance mechanisms, along with other strategies such as ion exclusion and osmotic adjustment may provide a strategy to enhance salt tolerance. To look into a special manner of salinity induced alternations and to elucidate adaptive mechanisms, we investigated the status of growth, free proline content, ion accumulation, and lipid peroxidation level and antioxidant enzymes activities in the leaves of L. fusca under salt stress. Using these species as a model, this can help to understand salt tolerance mechanisms and to identify salt-inducible genes in this halophyte plant belonging to Poaceae family which is an important family of crops, including wheat and rice, in the future.

**MATERIALS AND METHODS**

**Plant material and culture condition**

Kollar grass (L. Fusca) seeds were soaked in water for 2 h and then about 15 seeds were germinated in pots containing perlite and maintained under condition of 16 h 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, plants were treated with Hoagland nutrient solution (pH 6.0) containing 0, 100, 300, 500, and 700 mM NaCl. Plants were then harvested at 15th day after NaCl treatment and stored at -20°C until further analysis. Dry mass of leaves and roots was determined after drying the samples at 60°C for 48 h to constant mass.

**Dry weight and plant water relations**

For dry weight measurement, shoots of control and treated plants were oven dried at 60°C for 24 h. Leaf relative water content (RWC) was estimated according to Wheatherley (1973) and calculated as follows:

\[
\text{RWC} = \left[ \frac{\text{fresh mass} - \text{dry mass}}{\text{saturated mass} - \text{dry mass}} \right] \times 100
\]

Saturated mass of fresh seedlings was determined by keeping them in water for 24 h, followed by drying in a hot air oven (60°C for 48 h) until constant weight was achieved.

**Determination of pigments**

Chlorophylls (Chl a and Chl b) and carotenoids were extracted by pulverizing about 0.05 g of leaves in 80% acetone and quantified spectrophotometrically according to Lichtenthaler and Wellburn (1983).

**Determination of proline content**

Free proline content was determined according to Bates et al. (1973) using L-proline as a standard. A portion (500 mg) of liquid nitrogen powdered shoots was homogenized in 10 ml of 3% aqueous sulfosalicylic acid, and the homogenate was centrifuged at 2000 g for 5 min. The extract (2 ml) was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid and kept for 1 h at 100°C. Reaction was then stopped by using ice bath. The mixture was extracted with 4 ml of toluene and absorbance of the fraction with toluene aspirated from the liquid phase was read at 520 nm. Proline concentration was determined using a calibration curve.

**Lipid peroxidation**

Lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) following the method of Heath and Packer (1968). The seedlings (0.5 g) were homogenized in 5 cm³ of 0.1% (m/v) trichloro acetic acid (TCA). The homogenate was centrifuged at 10 000 g for 20
min. The supernatant (1 ml) was mixed with 4 ml of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA). The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000 g for 15 min, the absorbance of the supernatant was recorded at 532 and 600 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using the extinction coefficient of 155 mM⁻¹ cm⁻¹.

**Protein content and antioxidative enzymes activity**

For the determination of total protein content and enzyme activity, 1 g of fresh seedlings was homogenized in 3 ml of 25 mM potassium phosphate buffer (pH= 7) containing 3% poly vinyl poly pyrrolidone (PVP) and 0.06 g EDTA. The homogenate was centrifuged at 13000 g for 1 h and the supernatant was used for protein determination and enzyme assays. All the steps were carried out in ice bath. The total protein content of the extracts was determined according to the spectrophotometric method of Bradford (1976), using BSA as the standard.

APX (EC 1.11.1.11) activity was measured immediately in fresh extracts as described by Nakano and Asada (1981), using a reaction mixture (3 ml) containing 50 mM potassium phosphate buffer (pH=7.0), 0.1 mM H₂O₂, 0.5 mM ascorbate, 0.1 mM EDTA and 150 μl fresh extract. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (extinction coefficient of 2.8 mM⁻¹ cm⁻¹).

GPX (EC 1.11.1.7) activity was determined in the homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacyl (extinction coefficient of 26.6 mM⁻¹ cm⁻¹). Reaction mixture contained 20 μl of plant extract, 50 mM buffer K-phosphate pH= 7.0, 0.1 mM EDTA, 10 mM guayacol and 10 mM H₂O₂ (Plewa et al., 1999).

CAT (EC 1.11.1.6) activity was assayed by measuring the initial rate of H₂O₂ disappearance at 240 nm using the extinction coefficient of 40 mM⁻¹ cm⁻¹ for H₂O₂ (Velikova et al., 2000)

**Na+ and K+ content**

For the determination of the total amounts of K⁺ and Na⁺, dry shoot (0.25 g) was treated with nitric acid and diluted with distilled water. Total K⁺ and Na⁺ contents were directly measured by flame photometry.

**Statistical analysis**

All determinations were carried out in triplicate and data were subjected to analysis of variance. Analysis of variance was performed using the ANOVA procedure. Statistical analyses were performed according to the SPSS software. Significant differences between means were determined by Duncan’s multiple range tests. P values less than 0.05 were considered statistically significant.

**RESULTS**

**Growth and relative water content**

Plants were treated with 0, 100, 300, 500, 700 mM NaCl and harvested after 15 days. As shown in Figure 1, shoot length, the fresh and dry weight of shoot and root of plants decreased significantly in all NaCl treatment except 100 mM NaCl. However, in plants treated with 100 and 300 mM NaCl, morphological symptoms were not observed when compared with the control. While in plants treated with 500 and 700 mM NaCl leaf and shoot covered by the secreted material. There was no sign of chlorosis in these treatments.

The RWC decreased significantly only in 500 and 700 mM NaCl treated plants as compared with control plants (Figure 1F).

**Change in photosynthetic pigments**

Chlorophyll a, b, total chlorophyll and carotenoids increased significantly in plants treated with 300, 500 and 700 mM NaCl when compared with the control plants (Figure 2).

**Na+ and K+ content of Leaf and root**

Na⁺ content in leaves was significantly increased with increasing NaCl concentration. In root tissue, the amounts of Na⁺ also increased in NaCl treated plants when compared with the control plant (Figure 3b and d). NaCl treatment except at 300 mM concentration had no significant effects on the K⁺ content of the leaf. In plants treated with 300 mM NaCl, the amounts of K⁺ decreased significantly as compared with the control plants. In the root of the plants, the K⁺ content decreased only in 500 mM NaCl and in another treated plant NaCl had no significant effects on the K⁺ content of root. Leaves K⁺ content slightly changed in all salinity treatments except at 300 mM NaCl when compared with the control plants. There was a significant decrease in K⁺ content (Figure 3a).

The Na⁺/K⁺ ratio increased significantly in leaf and roots of plants in all NaCl treated plants except for 100 mM NaCl (Figure 3e and f). As shown in Figure 3, in all plants, the Na⁺ and K⁺ content of root tissue was more than the shoot tissue.
Proline content

Proline content increased in plants treated with NaCl salt. However, 100 mM NaCl had no significant effects on proline content. Proline content changed dramatically, as Figure 4 shows that the amount of free proline in the control plants was 0.11 (mM/g fw) and reached 9.74 (mM/g fw) in 700 mM NaCl. A clear increase in proline concentration was detected in shoots of plants treated with 300 mM NaCl, reaching a maximum value in 700 mM with 88-fold increase as compared with the control plants.

Lipid peroxidation

Lipid peroxidation in leaves of L. fusca measured as MDA content is given in Figure 2c. The level of lipid peroxidation increased with increase in concentration of NaCl in growth medium except for 100 mM NaCl.

Effect of NaCl on protein content and enzyme activities

As observed in Figure 5, salinity treatment had no significant effects on total protein content in 100, 500 and 700 mM concentrations. The total content of soluble protein was significantly increased only in 300 mM NaCl treatment when compared with the control. The activities of CAT, APX, GPX in L. fusca under the effect of different salinity treatment are shown in Figure 5.

The activity of antioxidant enzyme was measured in the
Figure 2: Carotenoid (a), total chlorophyll (b), Chl b (c) and Chl a (d) contents in seedlings of L. fusca under NaCl stress. Means ± SE of three replicates. Different letters indicate significant differences ($P < 0.05$).

control and salt stressed plants. The activity of CAT increased in all salinity treatments, but this increase was not significant in 100 mM NaCl treated plants. The maximum activity of this enzyme (with 8.5-fold as compared with the control plants) was observed in 500 mM NaCl.

APX activity increased significantly in all NaCl treated plants except for 100 mM NaCl treatment. In 100 mM NaCl, the activity of this enzyme decreased when compared with the control plants. GPX activity significantly increased in 500 and 700 mM NaCl but in 300 mM NaCl, the activity of this enzyme decreased significantly. Data in Figure 5 show that maximum activity of all antioxidant enzyme was in 500 mM NaCl treatment.

DISCUSSION

In the present study, the effects of salinity stress on growth and physiological responses of halophytic grass L. fusca were investigated. Levels of salt tolerance of the monocotyledonae (Poaceae) species span a large range of low, intermediate to high-salt tolerance, but do not include salt stimulated growth in non succulent halopytic poaceae (Rozema and Schat, 2013). Growth of L. fusca in 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. High salinity treatments significantly inhibited growth of the plant. Salt stress suppresses plant growth for two reasons: osmotic and ion excess effect (Munns and Genes, 2005). The decrease in plant growth for the halophytic plant in high salinity is a common event and reported for dicotyledinous halophytes such as Salicornia species (Aghaleh et al., 2011) and monocotyledonous halophytes such as Aeluropus lagopoides (Sobhianian et al., 2010). RWC of L. fusca decreased under high salinity treatments. In this state, RWC reduced to below 13 and 16% of the control ones. It seems that high salinity increase Na+ and Cl+ ion in solution, and decreasing Ψw and water uptake by plant become harder than normal condition, leading to a decrease in relative water content in L. fusca plants.

The breakdown of chlorophylls is one of the first visible symptoms and is closely linked to salt stress induction, (Khan, 2003) as a result of both decreased synthesis and increased degradation under salt stress (Parida et al., 2002; Mittal, 2012). However, in L. fusca, 300 mM NaCl and
Figure 3: Changes in ion concentrations in shoots and roots of L. fusca under NaCl stress. Means ± SE of three replicates. Different letters indicate significant differences (P < 0.05).

Figure 4: Proline in seedlings of L. fusca under NaCl stress. Means ± SE of three replicates. Different letters indicate significant differences (P < 0.05).
Figure 5: MDA in seedlings of *L. fusca* under NaCl stress. Means ±SE of three replicates. Different letters indicate significant differences (*P* < 0.05).

Figure 6: Changes in the protein (a) activities of CAT (b), APX (c) and GPX (d) enzymes in *L. fusca* subjected to various salt concentrations. Vertical bars indicate mean ± SE of three replicate.
higher salinity treatments cause a remarkable increase in chlorophylls and carotenoid content when compared with the control plants. Increased total Chl content under salt stress was also observed in *Chenopodium album* (Yao, 2010) and *Cucumis* sp. (Kusvuran, 2008). In *A. lagopoideis* under drought stress after 15 days, no significant reduction was observed (Mohsenzadeh et al., 2006). In this experiment, carotenoid content increased at moderate and high salinity treated plants. In plant cells, Car is a major chloroplastic antioxidant that protects the chloroplast from photo-oxidative stress (Ashraf, 2009). So it seems that the increase of Car in this plant under salt stress condition probably is the protective mechanism.

In this study, Na⁺ content in all NaCl treated plant increased in roots and shoot when compared with the control plants. Our result showed that there was no significant change in Na⁺ concentration between 100, 300 and 500 mM NaCl. This observation indicated that *L. fusca* salt tolerance to some extent was based on the restriction of Na⁺ uptake.

K⁺ concentration remains unchanged under most salinity treatments in both the roots and shoots. In *L. fusca*, Na⁺/K⁺ ratios generally increased with salinity increase and ranged from 0.33 to 0.93 in roots and 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 Poaceae, have a much higher K⁺ over Na⁺ selectivity than any dicotyledonous halophytes (Flowers and Colmer, 2008; Munns and Tester, 2008). This may suggest that Poaceae halophytes would rely on improving K⁺ homeostasis and/or Na⁺ exclusion under salt exposure, rather than enhanced capacity to sequester Na⁺ ions as in halophytic dicotyledonae. However, Na⁺ exclusion is surely the most important determinant of salt tolerance variation among Poaceae, there is strong evidence that “Na⁺ tissue tolerance” can play an important subsidiary role (Colmer et al., 2006). Na⁺ exclusion in salt tolerant Poaceae halophytes implies the production of organic osmotic solutes to maintain a balance of internal and external water potentials in a saline environment (Rozema and Schat, 2013).

Proline content dramatically increased in 300, 500 and 700 mM salinity treatment. Prolin accumulation is a common response to hyperosmotic salinity and considered to be involved in salt resistant mechanisms as reported for *Atriplex nummularia* (Hussin et al., 2012), *A. lagopoideis* (Sobhanian et al., 2010), *Spartina alterniflora* (Li et al., 2010), *O. paucinervis* (Naidoo et al., 2008), *Atriplex halimus* (Martínez et al., 2005). In *L. fusca*, high concentration of proline accumulated (85-fold) in the high salinity level and its concentration was sufficient to account for contribution to the osmotic adjustment. Increase in proline accumulation has been attributed to up regulation of biosynthetic gene expression (Kishor, 1995) and also decrease in proline consumption acid (Raymond and Smirnoff, 2002) as well as down regulation catabolism of the amino acid of (Borsan, 2005).

The individual role of proline under salinity stress in plants has been extensively studied (Kishor et al., 2005; Verbruggen and Hermans, 2008). It acts as a compatible osmolyte, free radical scavenger, enzyme protectant, cell redox balancer, cytosolic pH buffer and stabilizer for subcellular structures (Kishor et al., 2005; Verbruggen and Hermans, 2008) to bring about salinity tolerance. Moreover, it has been reported that Proline is involved in the synthesis of key proteins such as dehydrins that are necessary for stress responses (Khedr et al., 2003). Banu et al. (2009) have shown that proline provided a protection against NaCl-induced cell death by decreasing the level of reactive oxygen species (ROS) and lipid peroxidation as well as improvement of membrane integrity by increasing antioxidant gene. It showed that exogenous proline mitigated the adverse effects of salt stress by increasing antioxidant enzyme activities (Hoque et al., 2007). Total protein content in all salinity treatments slightly increased. Increase in total soluble protein content under salinity stress was reported for another halophyte grass *A. lagopoideis* (Sobhanian et al., 2010), *Brassica juncea* (Mittal et al., 2012) and *Hordeum vulgare* (Mehmet Ali et al., 2005). The increase in protein content could be attributed to the increase in protein biosynthesis for acclimation to new conditions and reprogramming, particularly to sustain photosynthesis under salt stress (Sobhanian et al., 2010) and increase protein synthesis of anti-oxidative enzymes and other stress-induced proteins upon stress activation (Ashraf and Harris, 2004). Peroxidation of lipid membranes of higher plants reflects free radical-induced oxidative damage at the cellular level under salt stress conditions (Halliwell, 1987; Demiral and Turkan, 2004). MDA is regarded as an indicator for evaluation of lipid peroxidation or damage to the plasma membrane and organelle membranes that increases with environmental stresses. The results of this experiment show that MDA accumulation is dose dependent and showed a progressive increase. Since salt stress can cause membrane damage and increase lipid peroxidation level, it stimulates the formation of ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals. Among ROS, superoxide is converted by the SOD enzyme into H₂O₂, which is further scavenged by CAT and different peroxidases. APOX and GR also play a key role by reducing H₂O₂ to water through the Halliwell–Asada pathway (Noctor and Foyer, 1998).

Singha and Choudhuri (1990) reported that H₂O₂ accumulation in the leaves of *Vigna* and *Oryza* seedlings under salinity stress was related to a decrease in CAT activity. Decreased CAT activities, in turn, might have promoted H₂O₂ accumulation in 700 mM NaCl when compared with 500 mM NaCl (Figure 6b), which could result in a Haber–Weiss reaction to form hydroxyl radicals (Bowler et al., 1992). Since OH• radicals are known to damage biological membranes and react with most compounds present in biological systems (Halliwell and
Gutteridge, 1989), they might have hastened lipid peroxidation and membrane damage in the high salinity.

GPX activity was significantly decreased by 300 mM NaCl treatment, but at high salinity, treatments increased significantly (Figure 5). Thus, it can be concluded that GPX had the 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 was significantly increased during NaCl treatment except in 100 mM NaCl. Increase in activity of CAT, APX and GPX in response to salinity stress in other halophyte species have also been reported (Yao et al., 2010; Aghaleh et al., 2011). Our findings imply that ROS-scapenging enzymes might be a part of the general adaptive strategy of plants exposed to salinity in this species.

It has been reported that, in some halophytes, scavenging of reactive oxygen species (ROS) is associated with both activity of the antioxidative enzymes and presence of osmoprotectant compounds such as proline (Xiong et al., 2002). Ion regulation and ability to maintain tissue K+ level over the wide range of salinity, as well as selective secretion of Na+ and Cl− (Wieneke et al., 1987) employed by L. fusca may contribute to high salinity tolerance strategies used by this species.

In summary, this study contributes to a better physiological and biochemical characterization of the L. fusca tolerance to salt stress. Our result demonstrated that L. fusca can tolerate high salinity because of the increase in chlorophyll and carotenoid, maintenance of constant tissue k+ level, proline accumulation and an increase in antioxidative activities. The result of the present study showed that low level of salinity had no significant effects on growth of L. fusca. However, this halophyte grass can grow at moderate and can survive at extreme salinities. The analysis support the idea that this grass is a valuable plant model for understanding plant strategies under salt stress and can help to improve salt tolerance of crops in the future.

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