INT. J. BIOL. BIOTECH., 16 (3): 631-639, 2019.

SALINITY RESISTANCE IS LINKED WITH ANTIOXIDANT ACTIVITY, PIGMENTATION PATTERN AND ANATOMICAL ADJUSTMENTS IN *PHRAGMITES KARKA* (Retz.) Trin. ex Steud.

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ABSTRACT

Halophytes can be good choice to exploit saline resources for economical purposes. *Phragmites karka* (Retz.) Trin. ex Steud., is an invasive perennial grass potentially useful for bioenergy and medicinal purposes. It is widely distributed from saline wetlands to dry habitats and resist NaCl at both seed germination and growth levels. Some eco-physiological mechanisms of *P. karka* are reported in literature, however, effect of salinity on its anatomical features, water relations, leaf pigments and activity of antioxidants are not well understood. Plants were grown in plastic pots containing sandy soil and sub-irrigated with Hoagland solution under 0, 100 and 300 mM NaCl for two months. Fresh and dry biomass, plant length, leaf and stomata number were decreased with increasing salinity. Midrib thickness improved at 100 mM NaCl, while area of aerenchyma and leaf osmotic potential were unchanged. Water and osmotic potentials were decreased with increasing NaCl, compared to control treatment. Total antioxidant capacity (TAC), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), anthocyanins, betacyanins and chlorophylls were increased in salinity treatments. These results indicate that survival of *P. karka* could be associated with enhanced antioxidant activity, pigmentation pattern and anatomical adjustment in saline condition.

Key words: Antioxidant, halophyte, leaf anatomy, pigments, salinity, water potential.

INTRODUCTION

Different abiotic stresses, like salinity and drought, directly affects plant growth and productivity. Soil salinity is increasing gradually and encroaching upon cultivated lands especially in arid and semi-arid regions of the world (Gupta and Huang, 2014). More than 800 million hectares of global lands are salt affected, which is considered as a major problem for plant cultivation (Munns and Tester, 2008). Around 1% of the total plants in the world are halophytes, which can complete their life cycle naturally in hyper saline conditions (Flowers and Colmer, 2008). Some of these plants have immense potential to grow in saline degraded/ marginal lands and produce biomass of economic value (Roy and Chakraborty, 2014).

Saline soil is characterized by high Na^+ and Cl^- concentration that can cause physiological water stress to growing plants. Glycophytes usually unable to absorb sufficient water from such soils, which create ionic imbalance and toxicity. However, salt resistant plants are well adapted to such conditions (Tester and Davenport, 2003). These plants regulate water relation parameters by accumulating organic and inorganic osmolytes, which are prerequisite to maintain optimal leaf turgidity and water potential. They also modify leaf anatomical features, such as number and size of stomata in order to control transpiration and avoid excessive ion influx to above ground tissues (Srinivas *et al*., 2018). Chlorophylls and other pigments are directly related to net photosynthetic rate and carboxylation efficiency. Nevertheless, these plants can efficiently manage oxidative stress by manipulating the status and activity of antioxidants by production of bioactive secondary metabolites, enzymes and substrates (Rangani *et al*., 2016).

Phragmites karka is a salt tolerant grass potentially useful for economic purposes, like other halophytes which can be used as food, fodder, edible oil, medicine, landscape development and bioenergy feedstock (Abideen *et al*., 2015; Soni and Dahiya, 2015). This grass has a wide distribution in different countries of Asia, Arabia, Africa and Europe. Its above ground biomass has suitable characteristics to be used as energy feedstock and for obtaining medicinal compounds (Soni and Dahiya, 2015; Zehra *et al*., 2013). Young leaves are traditionally used as a cattle fodder and long hollow stem is used in making musical instruments (Zehra *et al*., 2013). *Phragmites karka* is mostly distributed in coastal and inland marshy habitat and attain up to 8-10 m high (Zehra *et al*., 2013). It can tolerate 80% (400 mM NaCl) to 100% (500 mM NaCl) sea water salinity at growth and germination levels (Abideen *et al*., 2014; Zehra and Khan, 2007). *Phragmites karka* can survive in salinity for long durations by changes in bulk elastic modulus and photosynthetic rate that are vital for growth and biomass production in natural ecosystems

(Shoukat *et al*., 2019). *Phragmites karka* also increase polyphenol and antioxidant activities to protect plants from oxidative stress under saline conditions (Abideen *et al*., 2015). This study was conducted to investigate the unexplored anatomical features, pigments concentration and antioxidant responses of *P. karka*, under saline conditions.

MATERIALS AND METHODS

Cultural conditions

Fresh seeds of *Phragmites karka* (Retz.) Trin. ex Steud., were collected from populations located in marshy habitats of University of Karachi in December 2014. Seeds were sown in plastic trays filled with loamy soil containing farm manure and supplied with water for germination. After one month, healthy seedlings of similar size and vigor were transplanted into plastic pots (10 x 25 cm) containing sand loamy soil (3 individuals per pot) and sub-irrigated with Hoagland's nutrient solution. Plants were grown in ambient conditions (temperature: 37 ± 1 ^{o}C; relative humidity: 50 ± 5 %; light intensity: 1000-1200 µmol m^{-2} s^{-1}) in semi-controlled greenhouse. Plants were exposed to different saline treatments (0, 100 and 300 mM NaCl), after 30 days of seedling acclimatization. Plants were harvested after 2 months of salinity treatments.

Growth parameters

Plant fresh and dry weights, shoot and root lengths, number of tillers, green and yellow leaves were recorded. Freshly collected plant samples were cleaned and placed in oven at 60^{o}C until constant weight for dry weight determination. A portion of plant's fresh sample were immediately frozen in liquid nitrogen and stored at 0 ^{o}C for biochemical analysis. All experiments were performed on five biological replicates.

Stomatal distribution, aerenchyma area and midrib thickness

In daylight, nail paint was applied on the lower surface of fresh leaves to get the epidermal impression. After 10 min, leaves were peeled off and transparent nail paint was set on glass slides. Number of stomata under the light microscope were observed. Section cutting method was applied for the study of aerenchyma and midrib thickness. Fresh and healthy green leaves were cut into thin slices and kept in water. Slides were prepared to observe aerenchyma and midrib parameters under light microscope.

Water relations

Leaf water and osmotic potentials were determined according to (Gucci *et al*., 1991), using HR 33 T Dew point micro-voltmeter (Wescor Incorp. Logan, UTAH, USA). Leaf discs (5 mm diameter) were inserted into sample chamber (C-52) and allowed thermal equilibration. After equilibration water potential was measured in microvolts and finally converted into MPa using regression equation of standard curve plotted with different NaCl solutions. For osmotic potential, filter paper disks of the same size were immersed in leaf sap and inserted into C-52 sample chamber and osmotic potential was recorded in a similar way as mentioned for water potential.

Chlorophyll, carotenoids, anthocyanin, flavonol glycoside, β cyanin and lycopene

Chlorophylls and carotenoids were estimated by the method of Lichtenthaler (1987). Anthocyanin and flavonol glycoside (Ganjewala *et al*., 2008), betacyanins (Stintzing *et al*., 2005) and lycopene (Barros *et al*., 2007) were also estimated by using standard protocols.

Antioxidant activity

Antioxidant activity was determined in terms of radical scavenging activity using ABTS radical (Re *et al*., 1999). Total antioxidant capacity was also determined using ammonium molybedate method (Prieto *et al*., 1999).

Statistical analyses

The study was conducted on five biological replicates and data is presented as means ± standard error. SPSS (version 20) was used to check the significant differences among means at $P< 0.05$ by Bonferroni post-hoc test. All graphs were plotted using Sigmaplot (version 12.5).

RESULTS AND DISCUSSION

Effect of salinity on plant growth

Plants develop several physiological and anatomical adaptations for growth and survival under saline conditions (Blum, 2018). This study investigated whether the anatomical features, water relations and leaf pigments are related to the biomass production of a giant biofuel grass in response to salt stress. We found that, biomass of *P. karka* decreased with increasing NaCl (Abideen *et al*., 2014), which is not according to the previous reports showing growth stimulation at 100 mM NaCl (Abideen *et al*., 2014; Shoukat *et al*., 2019). Such a growth reduction in this study was probably related to the effect of high temperature and light intensity coupled with saline condition (Shoukat *et al*., 2019). Abideen *et al*. (2014) grew *P. karka* in a completely controlled greenhouse using a quick check irrigation system with periodic flooding (twice a day), under low light (200-250 µmol m^{-2} s^{-1}) and temperature (25 ºC). In contrast, this experiment was conducted in a semi-controlled netted greenhouse, where *P. karka* was grown in ambient environmental conditions with high light intensity (1200-1400 µmol m^{-2} s^{-1}) and temperature (42 ± 5 ^{o}C). These differences in cultural conditions would be the major possible factors that have been reflected in plant growth at moderate salinity. However, results of this study are in accordance with the growth responses of other halophytic grasses like *Sporobolus tremulus* (Moinuddin *et al*., 2014), *Andropogon greenwayi* (Hamilton *et al*., 2001), *Desmostachya bipinnata* (Asrar *et al*., 2017), *Panicum turgidum* (Al-Khateeb, 2006), *Pennisetum purpureum* (Wang *et al*., 2002), *Spartina pectinate* and *Panicum virgatum* (Kim *et al*., 2012).

Plant length and number of green leaves of *P. karka* were decreased with increasing NaCl concentrations (Fig. **2**). Leaf shrinkage, yellowing, shedding and death directly inhibit further cell division and elongation in many plants (Liu *et al*., 2013). For instance, number of leaf and foliage area of *Spergularia marina* (Curtis and Lauchli, 1987) and *Pennisetum purpureum* (Wang *et al*., 2002) reduced under salt stress. Reduction in leaf area avoids excess transpiration and water loss through aerial parts. Such a transpirational regulation helps to maintain concentrations of Na^+ and Cl^- under sub-toxic levels by sequestering these ions in root (Munns and Tester, 2008). In addition, *P. karka* also limit root development, especially at high salinity to reduce the overall load of toxic ions as reported earlier (Abideen *et al*., 2014). Other salt tolerant grasses like *Spartina anglica*, *Puccinellia maritima* and *Elymus pycnanthus* also reduced their upstream ion flux under high salinity (Bouma *et al*., 2001).

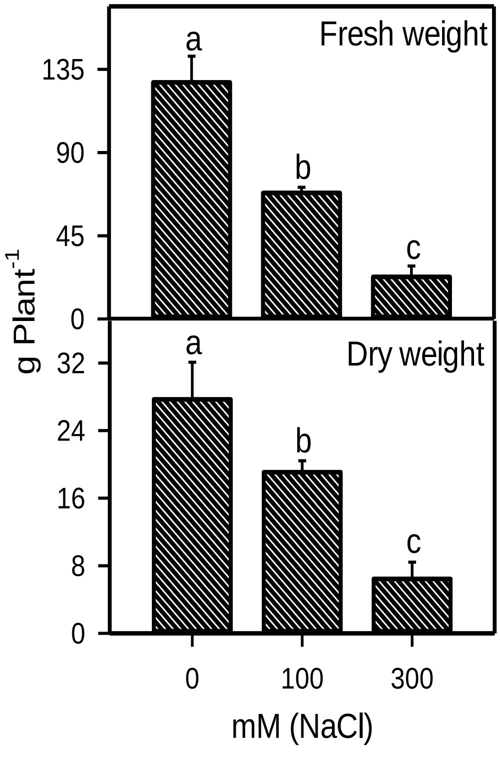

Fig. 1. Fresh and dry weights of shoot and root of *Phragmites karka* in response to 0, 100 and 300 mM NaCl treatments. Means of five replicates (n=5) with different letters are significantly different $P< 0.05$ (Bonferroni).

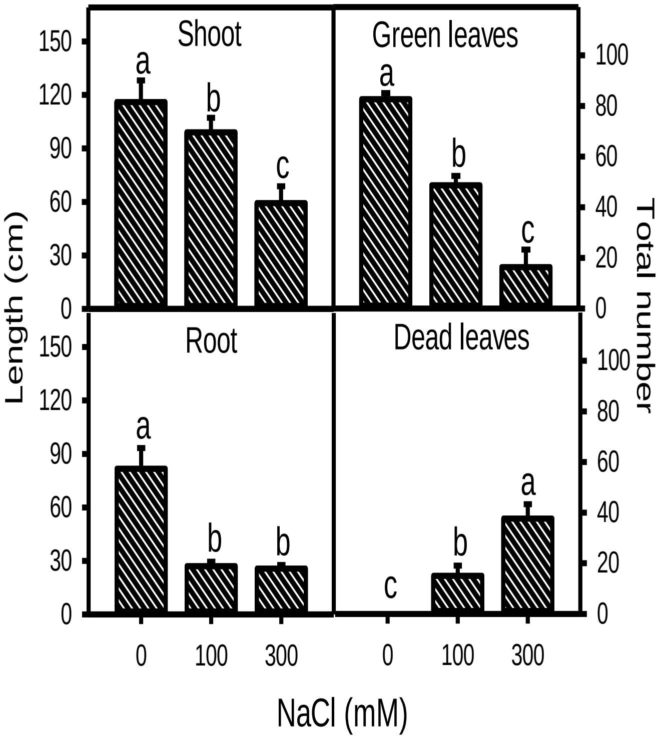

Fig. 2. Shoot and root lengths and number of green and dead leaves of *Phragmites karka* in response to 0, 100 and 300 mM NaCl treatments. Means of five replicates (n=5) with different letters are significantly different $P< 0.05$ (Bonferroni).

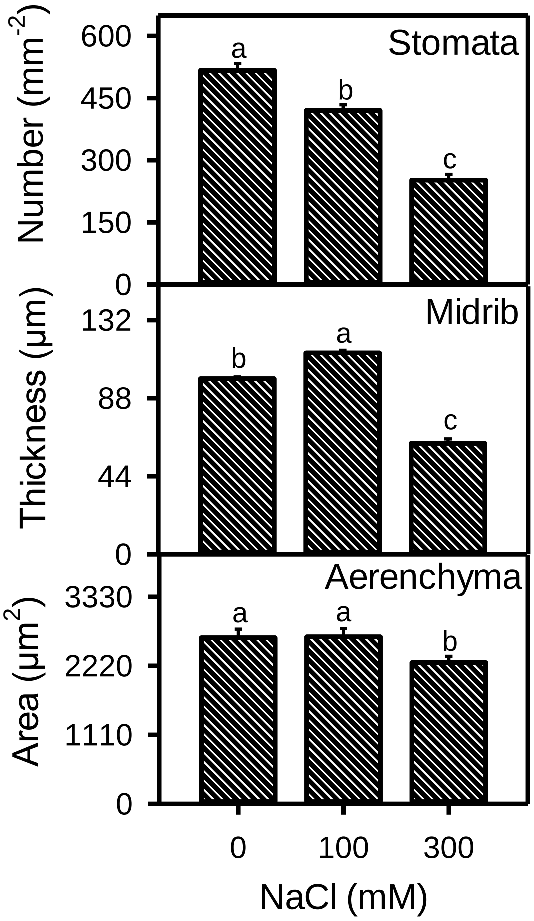

Fig. 3. Number of stomata, midrib thickness, and area of aerenchyma of *Phragmites karka* in response to 0, 100 and 300 mM NaCl treatments. Means of five replicates (n=5) with different letters are significantly different *P< 0.05* (Bonferroni).

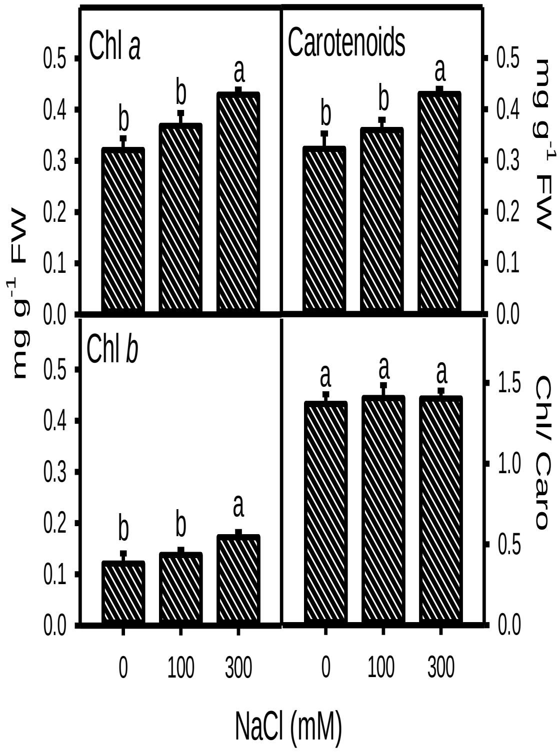

Fig. 4. Pigments (Chl *a*, Chl *b*, carotenoids and Chl/Car) of *Phragmites karka* in response to 0, 100 and 300 mM NaCl treatments. Means of five replicates (n=5) with different letters are significantly different *P< 0.05* (Bonferroni).

Effect of salinity on plant water relations

Leaf water and osmotic potentials of *P. karka* decreased linearly as the salinity of the medium increased (Table **1**) as reported earlier in salt resistant grasses like *Sporobolus virginicus* (Bell and O'Leary, 2003), *Odyssea paucinervis* (Naidoo *et al*., 2008), *Posidonia oceanica* and *Cymodocea nodosa* (Sandoval-Gil *et al*., 2012), *Panicum turgidum* (Koyro *et al*., 2013), *Aeluropus lagopoides*, *Sporobolus tremulus* and *Paspalidium geminatum* (Moinuddin *et al*., 2014). Maintenance of plant osmotic potential and turgor is important for plant to survive under saline habitats (Asrar *et al*., 2017). However, high salt concentration in soil makes it difficult for plant to absorb sufficient water, which leads to tissue dehydration and turgor loss (Abideen *et al*., 2014). Both these factors affect negatively on growth of *P. karka*, especially under high salinity (Munns and Tester, 2008). In addition, *P. karka* triggered leaf shedding to avoid excessive accumulation of toxin ions. Such a strategy is reported in many plants like *Aster tripolium* (Ueda *et al*., 2003), *Plantago coronopus* (Koyro, 2006), *Thellungiella halophila* (Ghars *et al*., 2008).

Effect of salinity on leaf anatomy

Plants acquire suitable changes in leaf anatomy to prevent water loss, when growing under saline and dry habitats. Halophytes are naturally adapted to modify stomatal morphology in order to conserve water and improve carboxylation efficiency (Akcin *et al*., 2017). *Phragmites karka* employed a similar strategy by gradually decreasing stomatal number with increasing salinity (Fig. **3**), as found in *Salicornia freitagii*, *Sporobolus ioclados* and *Imperata* *cylindrica* (Akcin *et al*., 2017; Cavusoglu *et al*., 2008; Polic *et al*., 2009). On the other hand, midrib thickness of *P. karka* leaf increased at 100 mM NaCl and followed a decline at 300 mM NaCl, as compare to control (Fig. **3**). Higher midrib thickness provide mechanical support as well as transportation of sugars, hormones and water in plants, which is associated with photosynthesis rate (Kehr and Buhtz, 2007). Decline midrib area was reported earlier in *Hordeum vulgare* and *Spergularia marina*, which is observed in *P. karka* under 300 mM NaCl (Akcin *et al*., 2015; Bijanzadeh and Kazemeini, 2014). *Phragmites karka* significantly reduced area of aerenchyma at high salinity to avoid excessive salt accumulation, as vacuole of aerenchyma provides site for toxic ions storage. In addition, small aerenchyma also helps in limiting water uptake from roots, as salt binds with water molecules and hamper water absorption (Akcin *et al*., 2015; Leite *et al*., 2017).

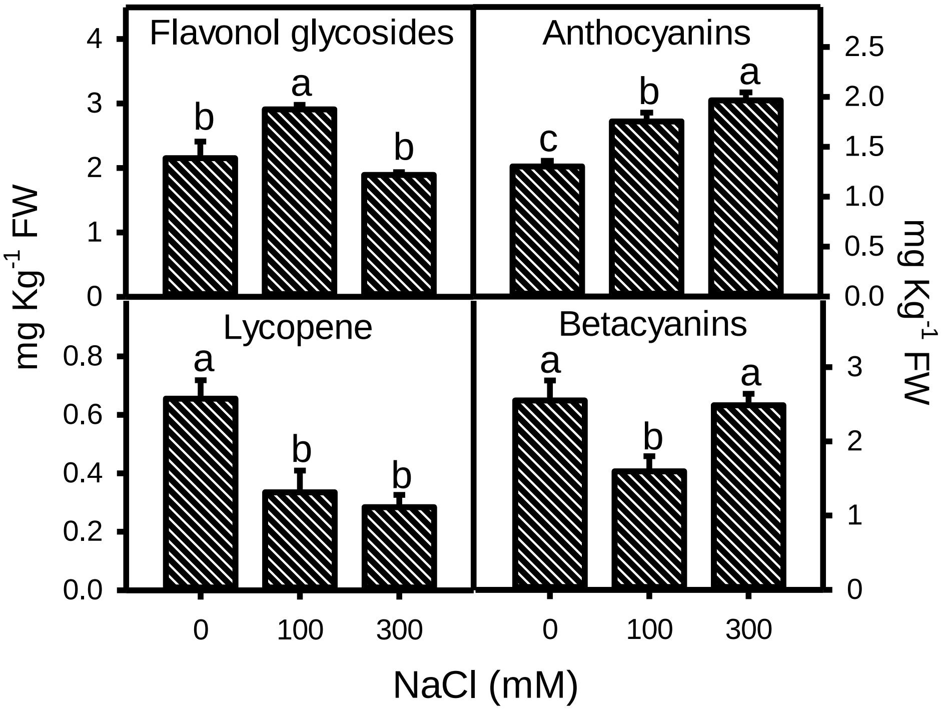

Fig. 5. Pigments (flavonol glycoside, lycopene, anthocyanin and β-cyanin) of *Phragmites karka* in response to 0, 100 and 300 mM NaCl treatments. Means of five replicates (n=5) with different letters are significantly different P< 0.05 (Bonferroni).

Table. 1. Diurnal water relation parameters of *Phragmites karka* leaves in response to 0, 100 and 300 mMNaCl treatments.

	Control		
mM NaCl	**0**	**100**	**300**
WP	-0.86 ± 0.04b	-1.1 ± 0.02a	-1.43 ± 0.03a
OP	-1.84 ± 0.02b	-1.94 ± 0.03b	-2.03 ± 0.02a
TP	0.97 ± 0.02a	0.84 ± 0.06b	0.59 ± 0.04c

WP = Water potential; OP= Osmotic potential; TP= Turgor potential. Means of five replicates (n=5) with different letters are significantly different $P< 0.05$ (Bonferroni).

Effect of salinity on leaf pigments

Leaf pigments of *P. karka* including chlorophyll *a*, *b*, carotenoids, and anthocyanin were increased at 300 mM NaCl, compared to control (Fig. **4** and **5**). Increased accumulation of chlorophyll might be linked with chloroplast development under saline condition as found in *Plantago coronopus* and *Paspalum vaginatum* (Pompeiano *et al*., 2014; Uddin *et al*., 2011). High levels of chlorophyll could be associated with increase mesophyll thickness and number of thylakoids, under high salinity (Pompeiano *et al*., 2016). Unchanged chlorophyll at 100 mM NaCl might be linked with increased light absorption to improve photosynthesis efficiency. On the other hand, increase in carotenoid content help plant to minimize the formation of singlet oxygen species (Asrar *et al*., 2017) and protect photosynthetic machinery under stress (Fazeli *et al*., 2006). Raised contents of chlorophylls and carotenoids were also found in *Zoysia japonica* (Pompeiano *et al*., 2014), *Paspalum scrobiculatum* (Shonubi and Okusanya, 2007) and *Paspalum vaginatum* (Pompeiano *et al.,* 2016) in response to salt stress.

Salt stress also increased accumulation of anthocyanins in *P. karka*, while betacyanins and flavonol glycosides were unaffected and lycopene decreased at high salinity. High levels of anthocyanins and betacyanins were found in *Nicotiana tabacum* (Naing *et al*., 2017) *Arabidopsis thaliana* and *Eremochloa ophiuroides* (Ahn *et al*., 2015; Kim *et al*., 2017; Oh *et al*., 2011) under stress. Anthocyanin have a protective role against high light and temperatures that provides protection to cellular membranes, organelles, and genetic material. Accumulation of anthocyanin helps in nitrogen absorption, which is linked with chlorophyll stability under stress (Ghassemi *et al*., 2019). Additionally, anthocyanin and betacyanins has strong antioxidant effects and their elevated levels under stress has been reported in many plants. Accumulation of these antioxidants help in plant stress tolerance by facilitating redox balance and proper cellular functioning. These compounds also participates in heat dissipation and provide protection to photosynthetic apparatus (Asrar *et al*., 2017).

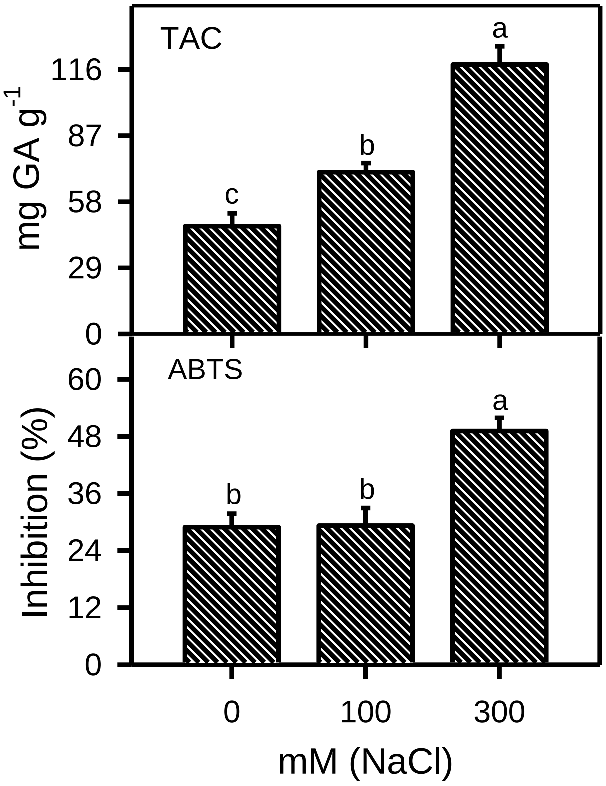

Fig. 6. Antioxidant enzymes (ABTS and TAC) of *Phragmites karka* in response to 0, 100 and 300 mM NaCl treatments. Means of five replicates (n=5) with different letters are significantly different *P< 0.05* (Bonferroni).

Effect of salinity on antioxidant activities

Antioxidant activity (ABTS and TAC) increased at high salinity compared to control (Fig. **6**). Increased antioxidant activity was reported in *Pluchea lanceolata, Ipomoea pes-caprae, Thespesia populneoides*, *Suaeda fruticosa, Salvadora persica* (Qasim *et al*., 2017), *Crithmum maritimum* (Meot-Duros *et al*., 2008) and *Cakile maritima* (Ksouri *et al*., 2007) under saline conditions. Accumulation of antioxidant compounds indicates a possible role of these metabolites against salt induced oxidative stress as reported by Abideen *et al*., (2015). Synthesis of antioxidants serves as important tool of plant defense under salt stress. Higher antioxidant production in salinity suggests that *P. karka* have an efficient antioxidant mechanism to scavenge ROS. Increased antioxidant activity of *P. karka* is linked with polyphenol accumulation, which is associated to protect photosynthetic machinery. Such a protection is become crucial when plants were growing under high light intensity, as in this experiment, since light harvesting complex is a major site for ROS production (Falleh *et al*., 2011).

CONCLUSIONS

Phragmites karka can resist salinity by adjusting anatomical features (such as reduction of stomatal number and area of aerenchyma), osmotic potential and pigmentation pattern (enhanced anthocyanin, betacyanin, carotenoids, chl *a* and *b*). These pigments and other bioactive compounds provide antioxidant defense to detoxify ROS and enables *P. karka* to tolerate salinity and produce biomass of economic importance.

ACKNOWLEDGMENT

This study was conducted under the Project # 20-4361/NRPU/R & D/HEC/14: **Assessing and improving bioethanol production potential of selected halophytes**, funded by Higher Education Commission (HEC) of Pakistan.

CONFLICT OF INTEREST

Authors have no conflict of interest to the manuscript.

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(*Accepted for publication May 2019*)