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ANTIOXIDANT ACTIVITY AND POLYPHENOLIC CONTENT OF *PHRAGMITES KARKA* UNDER SALINE CONDITIONS

ZAINUL ABIDEEN[1], MUHAMMAD QASIM[1], AYSHA RASHEED[1], M. YOUSUF ADNAN[1], BILQUESS GUL[1*] AND M. AJMAL KHAN[2]

[1]*Institute of Sustainable Halophyte Utilization (ISHU), University of Karachi, Karachi-75270, Pakistan*
[2] *Qatar Shell Professorial Chair in Sustainable Development, Department of International Affairs, College of Arts and Sciences, Qatar University, Doha -2713, Qatar*
**Corresponding author, email: bilqueesgul@uok.edu.pk; phone: +9221-34820253; fax: +9221-34820258*

Abstract

Phragmites karka (Retz.) Trin. ex Steud. is a halophytic grass found in inland saline marshes potentially useful for biofuel industry. Growth, polyphenol content and antioxidant activity in different plant parts were investigated after 45 days NaCl (0,100, 200 and 400 mM) treatments. An increased in polyphenolic content (5.06 to 12.81 mg GAE g^{-1} DW) and antioxidant activity (0.64-3.21 IC_{50} mg ml^{-1} for DPPH and 9.09-17.91 mM Fe^{+2} g^{-1} DW for FRAP) was observed with the increasing salt concentrations among different plant parts. Increase in plant biomass, phenolic content and antioxidant activity with lower MDA at 100 mM NaCl indicates a strong protection against oxidative damage. Leaves exhibited highest polyphenol and antioxidant activity, followed by stem and root. Coefficient of regression shows the high predictability of antioxidant activity (0.705) and phenolic contents (0.763) with an increase in salinity. Our data indicates a link between production of polyphenolic antioxidants and salt stress in *P. karka* which indicates salinity as an effective tool to produce antioxidant rich biomass for industrial purposes.

Key words: Bioethanol, DPPH, FRAP, Halophyte, Medicinal plant, Salinity.

Introduction

Environmental stress (salinity, drought, high light intensity and nutrient deficiency etc.) could result in oxidative burst leading to the production of reactive oxygen species (ROS) which could bring about biochemical and physiological changes resulting in low biomass production in plants (Serrato *et al*., 2004; Borsani *et al*., 2005; Miao *et al*., 2006; Abbasi *et al*., 2007; Giraud *et al*., 2008). Synthesis of secondary metabolites such as phenolic compounds with free radical quenching activity serves as important tool in plant defense strategy (Rice-Evans *et al*., 1997; Meot-Duros & Magne, 2009). These compounds are known to induce higher antioxidant activity due to their redox properties along with metal chelation, hydrogen donation and singlet oxygen quenching (Rice-Evans *et al*., 1995). Beside antioxidant, they serve as antiviral, anticancer, antiallergic, anti-inflammatory and cardio-protective agents (Wong *et al*., 2006; Raghu *et al*., 2013). Recently, interest has been shifted towards application of phenolic compounds in food, pharmaceutical and cosmetic industry (Ksouri *et al*., 2009; Oueslati *et al*., 2012).

Halophytes (plants naturally adapted to saline conditions) are reported to be a potential source of food, edible oil, fodder, medicine and biofuel (Weber *et al*., 2007; Qasim *et al*., 2010; 2011; 2014; Abideen *et al*., 2012; Koyro *et al*., 2013). Cultivation of these plants on degraded saline lands with brackish water would spare arable land and fresh water for conventional agriculture (Khan *et al*., 2009; Gul *et al*., 2013). They are not only known to produce considerable biomass but are equipped with efficient antioxidant system to balance ROS production and quenching (Ksouri *et al*., 2008). Such phytochemicals (flavonoids, terpenoids, tannins, alkaloids etc) from halophytes are also reported to have various health benefits (Ksouri *et al*., 2012; Joseph *et al*., 2013).

Phragmites karka, a perennial halophytic grass, usually grow as pure population in flooded saline habitats (Zehra *et al*., 2012) could attain a height of about 5-7 meters with rapid growth rate (Abideen *et al*., 2011). Seeds of this perennial halophytic grass could germinate even in 500 mM NaCl indicating the possibility of establishment under highly saline conditions (Zehra & Khan, 2007). This plant species is traditionally used as a remedy for diabetes, and also known for its diuretic properties (Sharma & Pegu, 2011). *Phragmites karka* is capable of producing lignocellulosic biomass for bio-ethanol production (Abideen *et al*., 2012, 2014; Gul *et al*., 2013). The consideration that plants with high lignocellulosic content are promising sources of antioxidant compounds (Dominguez *et al*., 2001) makes *P. karka* an interesting candidate for research on this aspect. Further, it is also reported that production of secondary metabolites could be increased with increasing salt concentration of growth medium (Oueslati *et al*., 2012; Alhdad *et al*., 2013). Therefore, present study was designed to investigate the antioxidant capacity and polyphenolic content of *P. karka* under different salinity regimes. We hypothesized that the salt stress would increase polyphenol accumulation in different plant parts which could ultimately enhance their antioxidant activity.

Materials and Methods

Plant material and culture conditions: Seeds of *P. karka* were collected in February 2010 from University of Karachi campus, separated from inflorescence, cleaned and sown in quartz sand for germination. After 7 weeks, five equal sized plants pot^{-1} were selected and grown in a greenhouse under ambient conditions (with 1200-1600 µmol m^{-2} s^{-1} PAR) with quick check system (Koyro, 2003) using basic nutrient solution (Epstein, 1972). Additional iron was supplemented (as Fe_2SO_4) twice a week to avoid iron deficiency. After seedling establishment (5 weeks) plants were treated with 0, 100, 200 and 400 mM NaCl. A gradual increase of 50 mM NaCl day^{-1} was initiated to avoid salt shock. Plants were harvested 45 days after highest salinity was achieved.

Lipid peroxidation: Lipid peroxidation using malonyldialdehyde (MDA) content as a damage marker was determined in leaves by the method of Hernandez *et al*. (2001). Briefly, 2 mg of fresh samples were homogenized in 2 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 x g for 10 min at 4°C. A 0.5 ml aliquot of the supernatant was mixed with 1.5 ml of 0.5% thiobarbituric acid (TBA) prepared in TCA (20%) and incubated at 90°C for 20 min. After terminating the reaction in ice bath, samples were centrifuged at 10,000 x g for 5 min. The absorbance of supernatant was measured at 532 nm. After subtracting the non-specific absorbance at 600 nm, MDA concentration was determined using the extinction coefficient 155 mM^{-1} cm^{-1}.

Extraction of plant material for other biochemical tests: Finely ground plant material (0.5 g) was extracted with 10 mL of methanol (80%) at 40°C for 3 hours using shaking water bath (GFL-1092). The samples were then cooled and centrifuged at 4500 rpm for 15 min. The supernatant was recovered and used for further analysis.

Determination of total phenolic content: Total phenolic content (TPC) was estimated using the Folin-Ciocalteu colorimetric method described by Singleton & Rossi (1965). Briefly, the Folin-Ciocalteu (0.2 N) was added in each extract or standard. Saturated sodium carbonate (75 g L^{-1}) was used to neutralize the reaction and left for 90 min at room temperature. The absorbance was measured at 760 nm with a spectrophotometer (DU530 Beckman Coulter UV/Vis). Gallic acid was used to prepare standard curve and results were expressed as mg gallic acid equivalent (GAE) per gram of dry weight (mg GAE g^{-1} DW).

Free radical-scavenging ability by the use of a stable DPPH radical: Antioxidant activity using 1,1 - diphenyl-2-picrylhydrazyl (DPPH) radical was determined by the method of Brand-Williams *et al*. (1995). DPPH reagent (100 μM) was added in a same proportion with sample extract or standard. The reaction mixture was incubated in dark for 30 min and absorbance was measured at 517 nm. The anti-radical activity was expressed as IC_{50} mg ml^{-1} (IC_{50} is the concentration of extract required for 50% inhibition of DPPH radical) where lower value corresponds to a higher antioxidant activity (Patro *et al*., 2005).

Ferric reducing antioxidant power (FRAP) assay: The FRAP assay is based on the ability of plant extract to reduce Fe^{3+} to Fe^{2+} ions (Benzie & Strain, 1999). Briefly, 2 ml of FRAP reagent was added to the appropriate concentration of sample extract and incubated for 5 min at room temperature. After incubation the absorbance was measured at 593 nm and $FeSO_4$ was used as standard. The results were expressed as mMol Fe^{+2} g^{-1} DW.

Statistical analysis: Statistical analyses were performed using two-way ANOVA. Differences among means (± S.E) were compared using Bonferroni test ($p<0.05$). Coefficient of determination (R^2) was computed to determine the predictability of antioxidants to those of salinity concentrations. All statistical analyses were carried out by using SPSS (SPSS for windows ver. 2011).

Results

Effect of NaCl concentration on plant biomass: An optimum shoot fresh weight of *Phragmites karka* was recorded at 100 mM which was considerably reduced in 200 mM (28%) and 400 mM NaCl (69%). Root fresh weight significantly reduced only in 400 mM NaCl (Fig.1).

NaCl effect on MDA (lipid peroxidation) and polyphenol content: A significant variation in MDA content was found under saline treatments (Fig. 2). It was 32% lower in 100 mM however a progressive increase was observed with further increase in salinity and approaching 36% higher in 400 mM NaCl in comparison to non-saline control.

Polyphenol content (TPC) in all plant parts (leaf, stem and roots) significantly increased with increasing NaCl concentration with leaf representing highest values (Table 1). Polyphenol accumulation among different organs also varied (5.06-12.81 mg GAE g^{-1} DW) under all salinity treatments (Table 1).

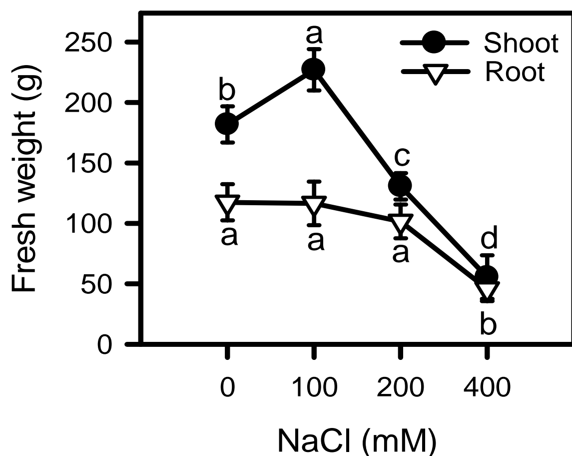

Fig 1. Shoot and Root biomass production of *Phragmites karka* irrigated for 45 days with a nutrient solution containing 0, 100, 200, and 400 mM NaCl. Means (n = 5) followed by at least one same letter are not significantly different at $p<0.05$.

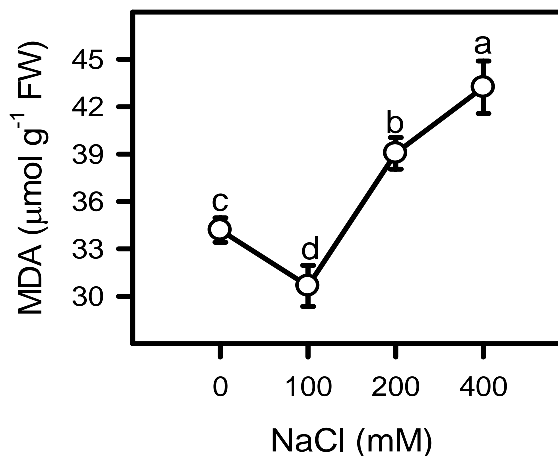

Fig. 2. Malonyldialdehyde (MDA) concentrations (μmol g-1 FW) of *Phragmites karka* leave in response to NaCl. Means (n = 5) followed by bars with different letters are significantly different ($p<0.05$).

Table 1. Total phenolic content (TPC), DPPH scavenging activity and Ferric reducing capacity (FRAP) in leaf, stem and root of *Phragmites karka* under various NaCl treatments.

	NaCl (mM)	Leaf	Stem	Root
TPC ($mg\ g^{-1}$ DW)	0	7.58 (0.41) ^{a}	6.16 (0.15) ^{c}	5.06 (0.16) ^{a}
	100	9.09 (0.32) ^{b}	7.54 (0.20) ^{c}	6.78 (0.34) ^{b}
	200	11.09 (0.30) ^{c}	9.53 (0.40) ^{b}	8.47 (0.48) ^{c}
	400	12.81 (0.03) ^{d}	11.80 (0.36) ^{a}	10.56 (0.28) ^{d}
DPPH (IC_{50} $mg\ ml^{-1}$)	0	1.94 (0.01) ^{d}	2.41 (0.02) ^{d}	3.21 (0.01) ^{d}
	100	1.56 (0.02) ^{c}	1.92 (0.01) ^{c}	2.28 (0.02) ^{c}
	200	1.15 (0.01) ^{b}	1.64 (0.01) ^{b}	1.90 (0.02) ^{b}
	400	0.64 (0.03) ^{a}	0.94 (0.03) ^{a}	1.20 (0.01) ^{a}
FRAP (mMol Fe^{+2} g^{-1} DW)	0	11.72 (0.26) ^{a}	9.09 (0.41) ^{a}	11.21 (0.35) ^{a}
	100	13.59 (0.29) ^{b}	12.11 (0.39) ^{b}	12.75 (0.25) ^{b}
	200	16.30 (0.49) ^{c}	12.76 (0.50) ^{b}	13.74 (0.33) ^{b}
	400	17.91 (0.19) ^{c}	15.90 (1.13) ^{c}	15.21 (0.14) ^{c}

All values are mean (± standard error) of three replicates where similar letter are not significantly different at p< .05.

Table 2. Results of two-way ANOVA of plant characteristics by salinity (S), Parts (P) and their interaction (S x P).

	TPC		DPPH		FRAP	
	SS	**F value**	**SS**	**F value**	**SS**	**F value**
S	150.80	172.29***	12.02	4.00***	154.11	79.35***
P	35.58	60.99***	4.08	2.04***	36.61	28.28***
S x P	0.41	0.23^{ns}	0.50	0.08***	10.31	2.65*

Key: *** (p<0.0001), ** (p<0.001), * (p<0.05), ns (non significant)

Table 3. Coefficient of determination (R^2) for total phenols (TPC) and antioxidant assays (DPPH and FRAP) against different organs of *Phragmites kaka.*

		DPPH	FRAP	TPC
Leaf	FRAP	0.95		
	TPC	0.95	0.9	
	Salinity	0.98	0.9	0.92
Stem	FRAP	0.83		
	TPC	0.98	0.83	
	Salinity	0.99	0.82	0.95
Root	FRAP	0.91		
	TPC	0.91	0.87	
	Salinity	0.94	0.9	0.93

Salinity and antioxidant capacity assay

Radical scavenging activity by using DPPH assay: The DPPH radical scavenging activity of *P. karka* gradually increased with increasing NaCl concentrations (p<0.05; Table, 1). Significant variations among antioxidant activity of different plant organs were found with leaf representing highest values (19-113%) followed by stem (20-90%) and root (29-105%; Table, 1).

Ferric reducing antioxidant power (FRAP) assay: Ferric reducing antioxidant power (FRAP) increased with salinity (100 to 400 mM NaCl). The highest promotion was found in leaf (13-34%) than both stem (5-42%) and root (12-26%) representing considerable variation among different organs (Table 1).

Statistical analysis

Analysis of Variance (ANOVA) among studied parameters: For phenolic content, two-way ANOVA showed a significant individual effect of both salinity (S) and plant parts (P) but their interactions (S × P) non-significant (Table 2). Similar results were observed for antioxidant capacity (DPPH and FRAP) showing a significant individual effect with salinity (S) and plant parts (P) however, in this case their interaction (S × P) was also significant (Table 2).

Relationship between antioxidant capacity and total phenolic content: Coefficient of regression (R^2) explains that variation in total leaf, stem and root phenolic content contributed to an increased antioxidant activity (both DPPH and FRAP) with average R^2 values of higher than 0.9 and regression values were also highly significant (Table 3).

Discussion

The effect of salinity on growth of *Phragmites karka* could be related to phenolic content as well as antioxidant activity. Some halophytes generally optimize their growth in moderately saline conditions and higher salinities cause growth reduction (Flowers & Colmer, 2008). *Phragmites karka* showed a typical halophytic response with optimum growth in 100 mM NaCl which decreased in higher salinities as observed in other species of the same genus (*P. australis*; Gorai *et al*., 2010). This increase in biomass at 100 mM could be attributed to better protection against oxidative damage as indicated by lower MDA and higher antioxidant (polyphenol) production as shown in *Cakile maritima* (Ksouri *et al*., 2007).

An increased synthesis of phenolic antioxidant is considered a general plant response to minimize oxidative damage under stressed environments (Agati & Tattini, 2010; Pollastri & Tattini, 2011). Structural configuration of phenolic compounds (aromatic ring(s) and free hydroxyl groups) provides ideal chemistry to detoxify ROS (Rice-Evans *et al*., 1996; Bors *et al*., 2002). Total phenolic contents in *P. karka* was comparable with several glycophytes (6-13 mg g^{-1}; Chu *et al*., 2002; Zhou & Yu, 2006) and even higher than some halophytes (3.77-10.12 mg g^{-1}; Benhammou *et al*., 2009). *Phragmites karka* gradually enhanced polyphenol content when exposed to saline conditions as reported for various other species (Oueslati *et al*., 2012; Alhadad *et al*., 2013; Pirie *et al*., 2013) which might be linked with up-regulation of phenylalanine ammonia lyase (PAL) activity (Faller & Fialho, 2010). Some glycophytic crops (peppers, sugar cane and strawberry) and halophytic plants (*Aegiceras corniculatum, Suaeda maritima* and *Carpobrotus rossii*) are also reported to increase phenolic content under saline conditions (Agastian, *et al*., 2000; Muthukumarasamy *et al*., 2000; Bano *et al*., 2003; Navarro *et al*., 2006; Wahid & Ghazanfer, 2006; Keutgen & Pawelzik, 2008; Parida & Jha, 2010; Pirie *et al*., 2013). The increased phenolic accumulation in above ground parts of *P. karka* (leaves and stem) could be attributed to protect photosynthetic machinery under high light intensity (1200-1600 µmol $m^{-2}s^{-1}$ PAR). Since light harvesting complex is a major site for ROS production in plants grown under salt stress conditions (Falleh *et al*., 2011). This organ dependent response helps plant to protect their leaves from photo-oxidation (Niknam & Ebrahimzadeh, 2002) as they are active source of primary metabolism.

Our results also indicate the potential contribution of antioxidant capacity towards salinity tolerance by regulating ROS levels within safe limits (Kang & Saltveit, 2002). The effective antioxidant system of *P. karka* may contribute to properly manage salt stress which ultimately results in better growth at 100 mM NaCl. However increased antioxidant activity with reduced plant growth in salinities higher than 100 mM NaCl could be related to an imbalance between ROS generation and detoxification which is well reflected in MDA content.

Significantly high values of coefficient of regression (R^2) among salinity, antioxidant capacity and polyphenols in *P. karka* indicate a strong relationship among each other (Katalinic *et al*., 2006; Wong *et al*., 2006).

In general higher polyphenol accumulation is related to higher antioxidant capacity in most of the halophytes (Ksouri *et al*., 2007, 2008; Alhdad *et al*., 2013). The antioxidant activity of *P. karka* is well comparable with other halophytes (*Cakile maritime)* however, its phenolic content (7.5-12.8 mg g^{-1}) was much lower than *Cakile maritime* (31-66 mg g^{-1}; Ksouri *et al*., 2007). That might indicate the presences of compounds with much greater antioxidant activity which would be an interesting hypothesis for future studies. Therefore, characterization and detailed screening of secondary compounds may lead to develop natural products of high economic and industrial value.

Conclusion

Current investigation indicates that halophyte like *P. karka* could serve as a source of natural antioxidants. When grown in 100 mM NaCl an effective antioxidant system with high polyphenol content contributed to increased plant biomass. Whereas, increased phenolic content at higher salinities was not sufficient to maintain a balance between ROS production and detoxification result in reduced plant biomass. Salt treatment in *P. karka* is linked with increased production of polyphenolic antioxidants which presents a potential to utilize saline resources by growing halophytes of high economic value.

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