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Differences in photosynthetic syndromes of four halophytic marsh grasses in Pakistan

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Abstract Salt-tolerant grasses of warm sub-tropical ecosystems differ in their distribution patterns with respect to salinity and moisture regimes. Experiments were conducted on CO_2 fixation and light harvesting processes of four halophytic C_4 grasses grown under different levels of salinity (0, 200 and 400 mM NaCl) under ambient environmental conditions. Two species were from a high saline coastal marsh (*Aeluropus lagopoides* and *Sporobolus tremulus*) and two were from a moderate saline sub-coastal draw-down tidal marsh (*Paspalum paspalodes* and *Paspalidium geminatum*). Analyses of the carbon isotope ratios of leaf biomass in plants indicated that carbon assimilation was occurring by C_4 photosynthesis in all species during growth under varying levels of salinity. In the coastal species, with increasing salinity, there was a parallel decrease in rates of CO_2 fixation (A), transpiration (E) and stomatal conductance (g_s), with no effect on water use efficiency (*WUE*). These species were adapted for photoprotection by an increase in the Mehler reaction with an increase in activity of $PSII/CO_2$ fixed accompanied by high levels of antioxidant enzymes, superoxide dismutase and ascorbate peroxidase. The sub-coastal species *P. paspalodes* and *P. geminatum* had high levels of carotenoid pigments and non-photochemical quenching by the xanthophyll cycle.

Introduction

Coastal and near coastal sub-tropical ecosystems are characterized by high soil salinity ranging from $\sim$50 to 200 dS m^{-1} (ca. 516–2064 mM NaCl) (Gul and Khan 1994; Gulzar and Khan 1994). Most species occurring in theses saline habitats are C_4 plants, which are considered better competitors in these harsh ecosystems (Khan and Gul 2002; Akhani 2006; Khan and Qaiser 2006; Grigore et al. 2014).

Salinity can inhibit photosynthesis by stomatal or non-stomatal limitations within photosynthetic cells (Lovelock and Ball 2002). This may occur by limiting the uptake of water and causing water stress, by interference with the uptake of essential elements and ion homeostasis, and by accumulation of salt in the cytoplasm of photosynthetic cells (functions of plant proteins are not compatible with high levels of NaCl). In photosynthetic tissue, high levels of salt can affect the levels of photosynthetic enzymes (Leisner et al. 2010, Sobhanian et al. 2010; Koyro et al. 2013) and cause dissociation and inactivation of the O_2 evolving complex of PSII (Murata et al. 1992), while there is generally less effect on the light harvesting complexes (Osmond 1994).

There are innate differences in the mechanism of photosynthesis between C_3 and C_4 species which result in differences in the capacity for photosynthesis (e.g., on a

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soluble protein, chlorophyll or leaf area basis) (Ehleringer and Pearcy 1983; Henderson et al. 1992; Nippert et al. 2007). C_4 species are adapted to dominate and carry out photosynthesis under conditions where carbon dioxide (CO_2) is limiting, primarily in warm, dry and often salinized places as they have a biochemical CO_2 pump (Nayyar and Gupta 2006). High temperature causes a dramatic increase in the level of photorespiration in C_3 plants (by decreasing CO_2 fixation and increasing RuBP oxygenase activity) (Sage 2002; Leegood and Edwards 1996). Limited availability of CO_2 can occur where the soil water potential decreases and lowers stomatal conductance (g_s) to CO_2 (e.g., limited rainfall or high salinity which decreases soil water potential) (Edwards and Walker 1983; Sage and Stata 2015).

In C_4 plants, there is cooperative function of the C_4 cycle with the C_3 cycle (Edwards et al. 2001; Voznesenskaya et al. 2002). Leisner et al. (2010) reported that greater sensitivity of the C_4 cycle enzymes than those of the C_3 cycle is responsible for low photosynthetic rates under salt stress. The efficiency and rate of photosynthesis are decreased in the presence of excess light (Erickson et al. 2015) and, more so under saline conditions which result in limited CO_2 fixation in comparison with the light harvesting ability (Maricle et al. 2007; Munns and Tester 2008). Protection of the photosynthetic apparatus may occur in many ways, e.g., by heat dissipation through the xanthophyll cycle, which is considered the first line of defense against damage by excessive excitation energy (Horton et al. 1996; Owens 1996; Müller et al. 2001; Terashima et al. 2009; Erickson et al. 2015). The water–water cycle is the second line of defense to consume excess electrons at PSI particularly in halophytes (Mehler 1957; Lovelock and Ball 2002; Cruz et al. 2005; Jithesh et al. 2006; Maricle et al. 2007; Seckin et al. 2010; Erickson et al. 2015). Another means of preventing over-reduction of the electron transport chain and protecting other components of photochemistry is inactivation of PSII (Jaleel et al. 2009; Taiz and Zeiger 2010).

Previous studies on salt tolerance of cordgrasses indicated the utilization of alternate electron sinks for dissipation of excess light energy, which varied between species (Maricle et al. 2007), as well as among different accessions (Mateos-Naranjo and Redondo-Gómez 2016). Few reports are available on the responses of photosynthesis and mechanisms of protection against stress in salt-tolerant grasses from warm sub-tropical saline ecosystems. Moinuddin et al. (2014) reported on differences between two coastal (*Aeluropus lagopoides* and *Sporobolus tremulus*) and two sub-coastal C_4 grasses (*Paspalum paspalodes* and *Paspalidium geminatum*) in Pakistan in their response to saline conditions, including leaf succulence, osmotic potential, and growth. Here we study the effects of salinity

on parameters associated with photosynthesis and transpiration in these C_4 grasses, *A. lagopoides* (NAD-ME; δ^{13}C: -13.3 ‰; Pyankov et al. 2010), *S. tremulus* (species in genus having C_4 anatomy; Baaijens and Veldkamp 1991 and NAD-ME/PEP carboxykinase; Sage et al. 1999), *P. paspalodes* (NADP-ME; δ^{13}C: -10.3 ‰; Pyankov et al. 2010) and *P. geminatum* (genus NADP-ME; Sage et al. 1999; C_4 anatomy; Rondeau et al. 2005). The coastal species grow in higher salinity (EC: 12–54 dS m^{-1}) than the sub-coastal species (EC: 7–10 dS m^{-1}); for habitats see http://www.tropicos.org/Project/Pakistan. Coastal species are salt excreting, sub-coastal not salt excreting (Moinuddin et al. 2014 personal observations). We hypothesize that these halophytes will have tolerance to saline conditions by engaging various photoprotective mechanisms, that the photosynthetic apparatus will be altered with decreases in carbon assimilation and transpiration affecting water use efficiency, and that differences will be observed between individuals and between species which grow in coastal versus sub-coastal habitats.

Materials and methods

Ramets of the sub-coastal species *P. paspalodes* (Michx.) Scribner and *P. geminatum* (Forssk.) Stapf. were collected from Korangi Industrial area, Karachi (24°51′03.2N; 67°05′60.4E). These inland water channels were also periodically flooded due to tidal sea water intrusions. The coastal species *A. lagopoides* (Linn.) Trin. ex Thw (low marsh grass) and *S. tremulus* (Willd.) Kunth (high marsh grass) were collected from Manora Creek near Sandspit, Karachi (24°49′06.70″N; 66°56′06.80″E). Ramets with about 8–10 tillers each were planted in plastic pots (26 cm high ×20 cm dia.) during the first week of February 2009 in sand culture and these pots were sub-irrigated by placing in plastic trays containing two liters of half strength Hoagland solution (Epstein 1972) and allowed to establish for 1 month. Plants were grown under saline conditions for 45 days at maximum light intensity of ∼2000 μmol m^{-2} s^{-1} (see Suppl. Fig. 1 for details of precipitation and temperature ranges during the study year). Then, salt treatments (0, 200 and 400 mM NaCl) were imposed and, for the two NaCl treatments, to avoid osmotic shock, 100 mM NaCl was added per day until final concentrations were achieved (see Moinuddin et al. 2014). Final concentrations in trays used for sub-irrigation were maintained daily by adding distilled water to compensate for evaporation. In plastic pots this was achieved by flushing soil from above with the respective irrigation solution at 3–4-day intervals allowing them to drip from below. Fresh treatment solutions were added every fifth day. Photosynthesis parameters were measured after ∼40 day of growth after final salinity concentrations were achieved.

The Li-Cor 6400XT gas exchange system (LI-COR Biosciences Inc. 2008) was used to measure rates of photosynthesis on fully expended leaves by providing 370 µmol mol^{-1} CO_2, with a flow rate of 500 µmol s^{-1}. All readings were taken between 11:00 am and 02:00 pm at ~ 37 °C, at 50–60 % relative humidity and light intensities of ~ 1200 µmol m^{-2} s^{-1} were maintained inside the leaf chamber by tilting it laterally against the sunlight. Two or more leaves were used to completely cover the 2×3 cm Li-Cor sample chamber. In some cases, digital images of leaves placed in the sample chamber were taken and the projected leaf area calculated with the help of ImageJ 1.43u software (NIH, USA).

Chlorophyll fluorescence parameters were measured on mature fully expanded leaves of plants grown under ambient conditions after 40 days of salt treatment with a PAM-2500 fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Stern–Volmer type non-photochemical fluorescence quenching (NPQ) was estimated according to Bilger and Björkman (1990), maximum intrinsic quantum yield of PS II (F_v/F_m) by Kitijama and Butler (1975), effective quantum yield of PS II (ΦII) by Genty et al. (1989), coefficient of photochemical fluorescence quenching (qP) as formulated by van Kooten and Snel (1990), the ratio of photochemistry per CO_2 fixed (ΦII/ΦCO$_2$*) and the relative yields of photochemistry (φII), non-photochemical quenching (φNPQ), and non-regulated quenching (φNO) by Laisk et al. (1997) as formulated by Genty et al. (1996). Light energy absorbed by PSII is dissipated by three general processes which add up to unity (Kramer et al. 2004), and can be expressed as:

$$\phi II + \phi NPQ + \phi NO = 1$$

φII is the yield of photochemistry, φNPQ is the yield for dissipation by down regulation of PSII and φNO is the yield of other non-photochemical losses. Light intensity was set at 1200 µmol m^{-2} s^{-1} PPFD (this level was saturating for non-saline controls of the four test species based on photosynthetic light response curves by LiCOR-6400XT). All PPFD measurements were taken with the help of the fiber optics light source of PAM-2500 on leaves placed in the 2010-A distance clip at 60° angle to the fluorescence probe.

The photosynthetic pigments (chlorophyll a and b, and carotenoids) were extracted from 100 mg of fresh leaf material in 7 ml of 80 % (v/v) ice-cold acetone and levels analyzed spectrophotometrically using the equations of Lichtenthaler (1987). Leaf antioxidant enzymes were extracted according to Polle et al. (1994) after 40 days under saline conditions. Activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed by Beauchamp and Fridovich (1971), catalase (CAT, EC 1.11.1.6) activity according to Abey (1984) and ascorbate peroxidase (APX,

EC 1.11.1.11) activity was measured by the procedure of Nakano and Asada (1981). Total soluble proteins in the extracts were determined by Bradford (1976) method, and enzymes activities were expressed per mg protein.

To determine the leaf carbon isotope composition as a measure of the form of photosynthesis during plant growth, fully expanded mature leaves (2–3) were taken from plants after 45 days of growth under salinity treatments. Leaves were oven dried at 70 °C for 24 h. Samples were then finely ground using Mixture Mill (Model MM-400, Retsch, Germany). Carbon isotope discrimination values (δ^{13}C measured in parts per thousand) were determined on dried leaves using a standard procedure relative to Pee Dee Belemnite (PDB) limestone as the carbon isotope standard (Bender 1971; Bender et al. 1973). Two-way ANOVA's were used to compare the effects of species, salinities and their interactions on various leaf-level physiological parameters. A post hoc Bonferoni test was used to test for significant ($P < 0.05$) differences across salinity treatments within each species. Correlations between parameters were calculated using Pearson's correlation method.

Results

### Effects of salinity on photosynthetic pigments

In the coastal species *A. lagopoides*, with increasing salinity, there was a significant decrease in chlorophyll a, chlorophyll b, and chlorophyll (a + b), and under high salinity in the chlorophyll a/b ratio, while the carotenoid content was very low and not affected by salinity (Fig. 1). In the coastal species *S. tremulus*, there were no significant effects of salinity on these pigments, and, as in *A. lagopoides*, the carotenoid content was low.

In the sub-coastal species *P. paspalodes*, the mean values for chlorophyll content was lower at 400 mM NaCl; however, not significant at $P < 0.05$; the carotenoid content was very high compared to the coastal species and not affected by salinity (Fig. 1). In the sub-coastal species *P. geminatum*, there was a strong reduction in the chlorophyll content at both 200 and 400 mM NaCl, a reduction in the chlorophyll a/b ratio, while the carotenoid content was high and unaffected by salinity.

Effects of salinity on gas exchange

Leaf gas exchange parameters of the four species were measured on a leaf area basis to determine capacity for CO_2 assimilation and *WUE* of these species under increasing salinities (Fig. 2).
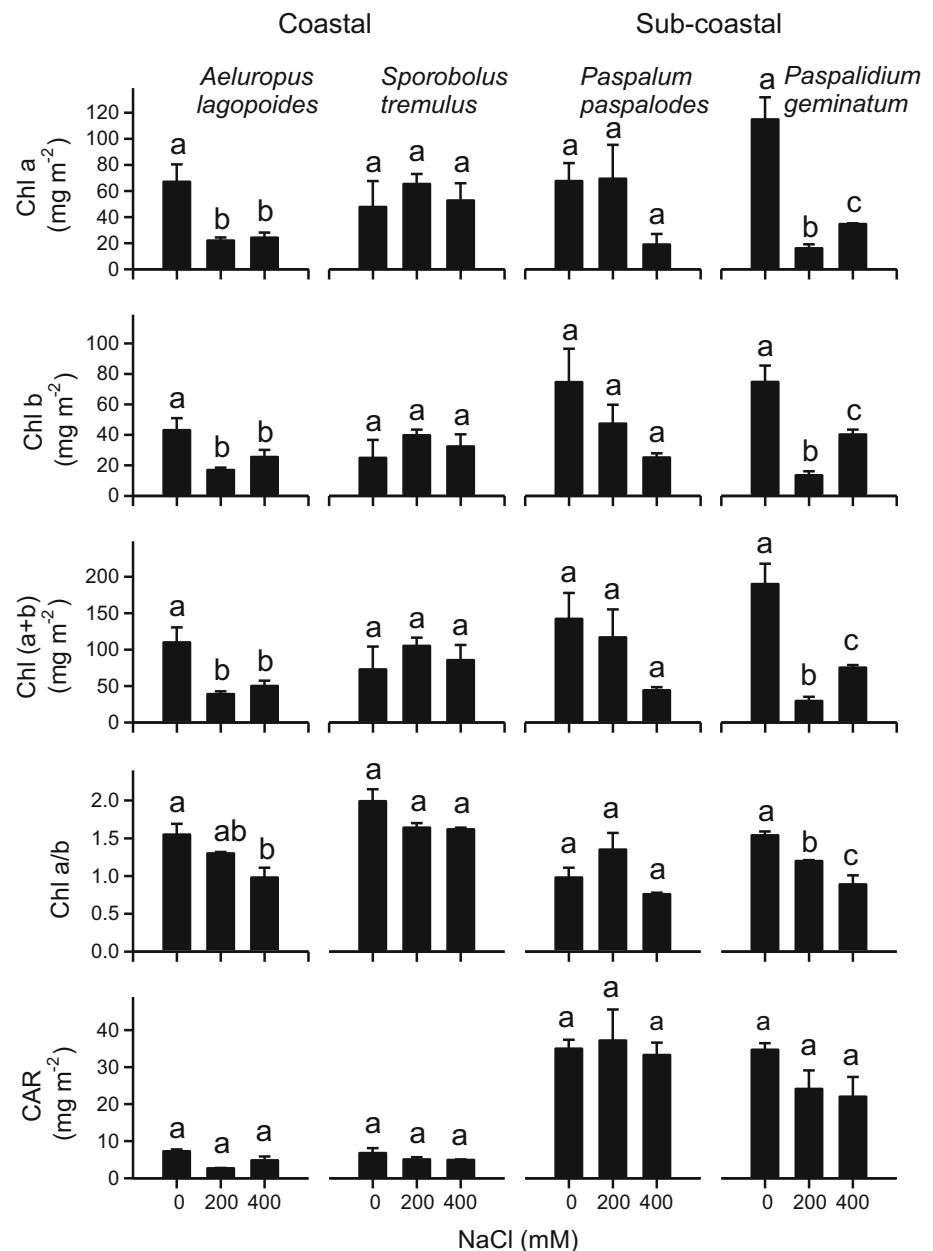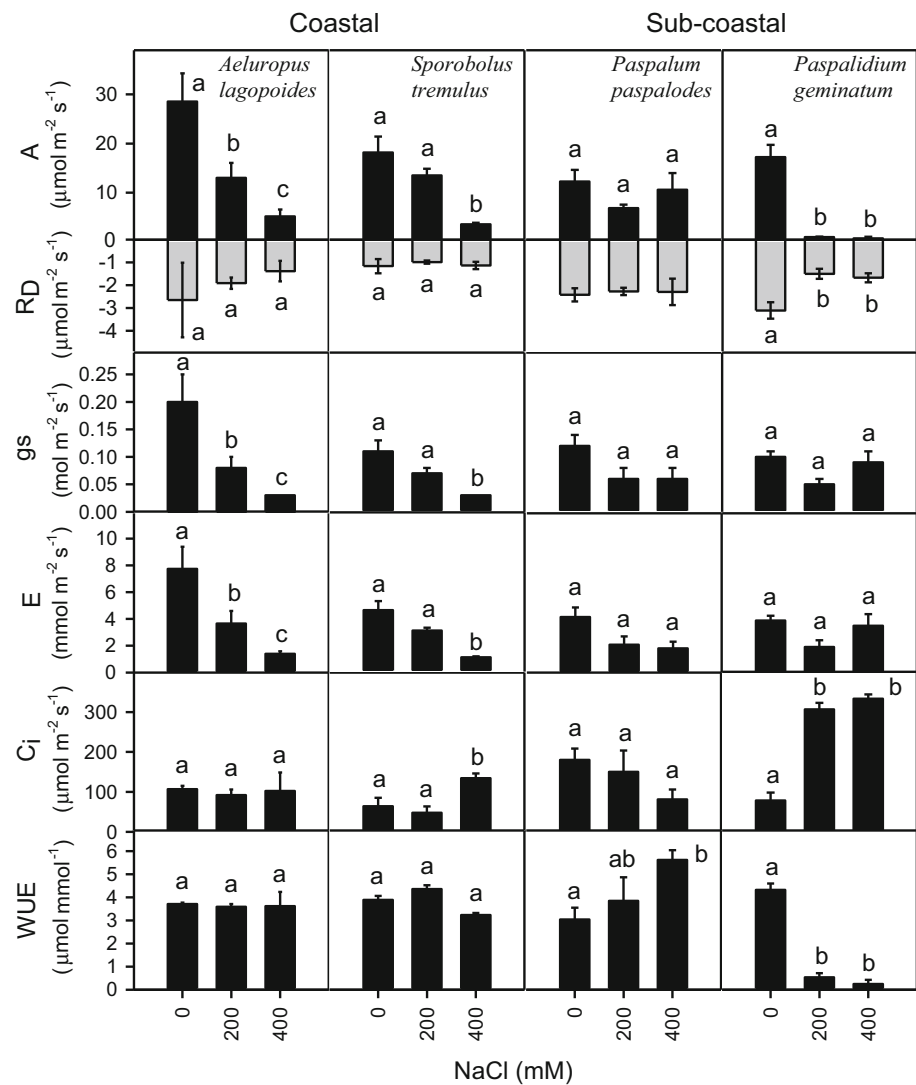
Effect of salinity on A and R_D

The two coastal species, *A. lagopoides* and *S. tremulus*, had a similar decrease in the rate of photosynthesis (A) with increasing levels of NaCl (P < 0.05). *A. lagopoides* had the highest rate of photosynthesis without NaCl. In the sub-coastal species *P. paspalodes*, there was no significant effect of salinity on A, while *P. geminatum* had the strongest decrease in A among the species under both moderate and high NaCl treatment. Results of a two-way ANOVA did not shown any difference in rates of dark respiration (R_D) due to increasing salinity, either between species or their interaction at P < 0.05, except in *P. geminatum* where

there was a significant decrease in R_D under saline conditions.

Effect of salinity on stomatal conductance (g_s), transpiration (E) and intercellular CO_2 concentration (C_i)

There was a significant decline in g_s and E (P < 0.05) in the coastal species with increasing salinity; while there were no significant differences in the sub-coastal species. Mean values of intercellular CO_2 concentration (C_i) did not vary significantly (P < 0.05) with increase in NaCl treatments in *A. lagopoides*; but, C_i increased in *S. tremulus* (P < 0.05) at 400 mM NaCl (Fig. 2). In *P. paspalodes*, C_i

Fig. 2 Effect of salinity (0, 200 and 400 mM NaCl) on the rate of net photosynthesis (A; μmol CO_2 m^{-2} s^{-1}), respiration (R_D; μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}), transpiration (E; mmol H_2O m^{-2} s^{-1}), intercellular CO_2 concentration (C_i; μmol m^{-2} s^{-1}) and water use efficiency (WUE; μmol CO_2 fixed mmol^{-1} H_2O transpired) of *A. lagopoides*, *S. tremulus*, *P. paspalodes* and *P. geminatum*. Bars represent mean ± standard error (n = 3). Different letters over bars indicate significant differences by a post hoc bonferroni test at $P < 0.05$

was unaffected with salinity; but, in *P. geminatum* C_i increased significantly ($P < 0.001$) at 200 and 400 mM NaCl in comparison to the non-saline treatment.

Effect of salinity on water use efficiency (WUE)

In the two coastal species, WUE remained unaffected with increasing levels of NaCl (mean value of ~4 μmol CO_2 fixed mmol^{-1} H_2O transpired). In the sub-coastal species *P. paspalodes*, WUE increased progressively with increase in NaCl from ~4 μmol CO_2 mmol^{-1} H_2O to a maximum of ~6 μmol CO_2 mmol^{-1} H_2O. In *P. geminatum* there was a dramatic drop in WUE ($P < 0.001$) in the 200 and 400 mM NaCl treatments (~0.5 μmol CO_2 mmol^{-1} H_2O, Fig. 2).

In general, the leaf gas exchange parameters of the four grasses, i.e., R_D, g_s, E and WUE were positively correlated with A, while C_i was negatively correlated with A and WUE (Suppl. Table 1).

Effects of salinity on chlorophyll fluorescence

In the non-saline treatments, F_v/F_m, which measures the maximum quantum yield of photochemistry, was 0.62 in the coastal species and 0.70 in their sub-coastal counterparts (Fig. 3). In the coastal grasses F_v/F_m increased slightly in *S. tremulus* at 200 mM NaCl, while there was a progressive decrease in F_v/F_m in *A. lagopoides* ($P < 0.05$) to ~0.45 at 400 mM NaCl. There was no effect on F_v/F_m with 200 and 400 mM NaCl treatments in the sub-coastal grasses.

In the coastal species, φII increased from treatment with no salt to 200 mM NaCl, with a substantial drop in values at 400 mM NaCl, while there was no significant effect of salinity on φII in the sub-coastal species. A similar trend was observed in the qP values in all species and across all salinities tested (Fig. 3). In the coastal species *A. lagopoides* and *S. tremulus*, ΦPSII/ΦCO$_2$* ratios progressively

Fig. 3 Effect of salinity (0, 200 and 400 mM NaCl) on maximum intrinsic quantum yield of photochemistry (F_v/F_m), effective quantum yield of PSII (ϕII), quantum efficiency of photochemistry compared to that of gross rate (net + dark respiration) of carbon fixation ($\phi II/\phi CO_2*$), coefficient of photochemical fluorescence quenching (qP), non-photochemical quenching (NPQ) and leaf temperature (T) of A. lagopoides, S. tremulus, P. paspalodes and P. geminatum. Bars represent mean ± standard error (n = 3). Different letters over bars indicate significant differences by a post hoc Bonferroni test at P < 0.05
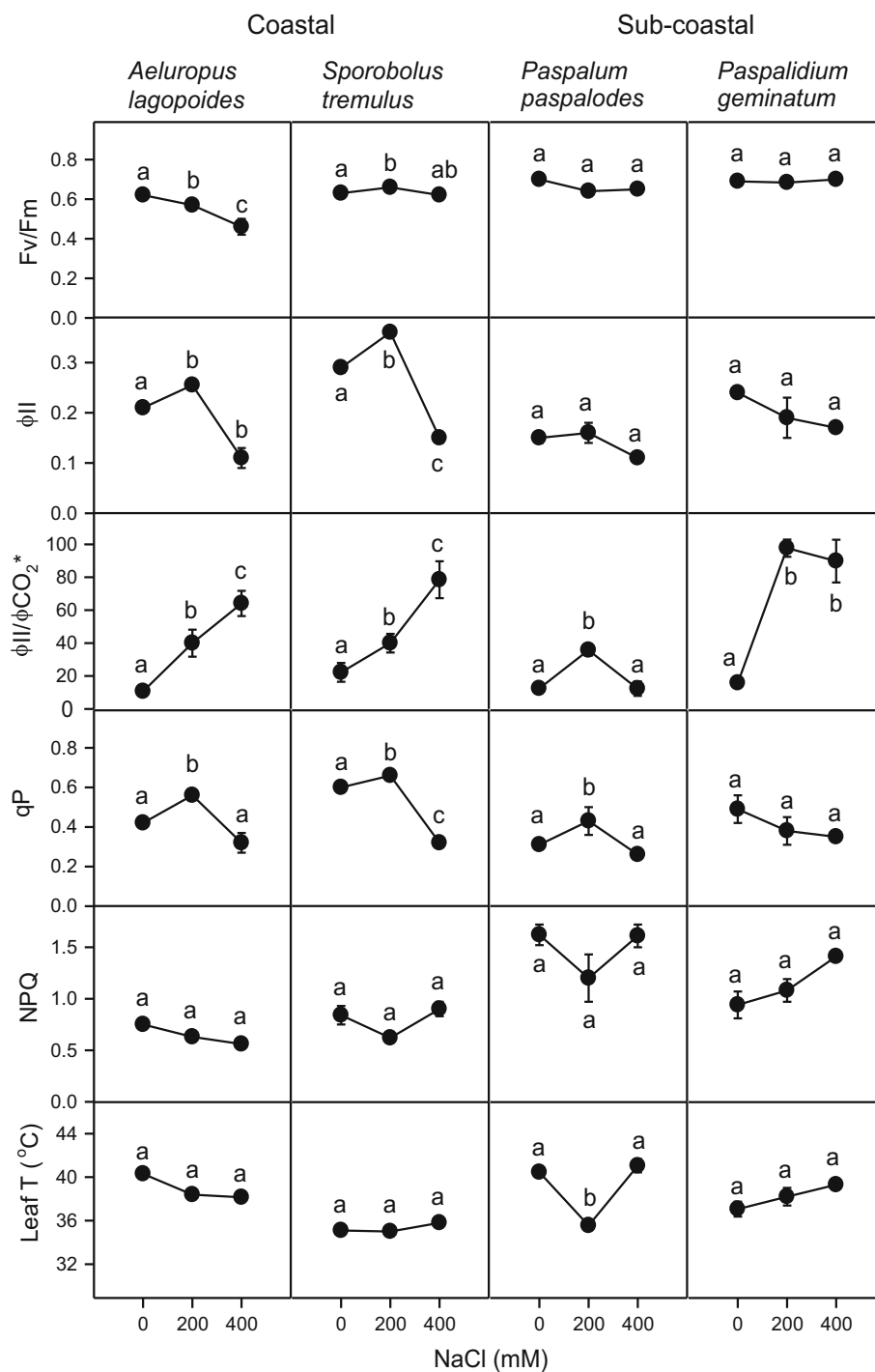
increased with increasing salinity. In the sub-coastal species, ΦPSII/ΦCO₂* increased only transiently in P. paspalodes while there was a large increase in ΦPSII/ΦCO₂* in P. geminatum at 200 and 400 mM NaCl (Fig. 3). There was no significant change in NPQ values with increasing NaCl treatments or between the test species; but NPQ values were higher in the sub-coastal than in the coastal species.

Changes in relative fluorescence yields of photochemistry (ϕII), non-photochemical quenching (ϕNPQ), and non-regulated quenching (ϕNO) were also measured for plants of the four species in the salinity trials (Table 1). Results of a two-way ANOVA of ϕNPQ showed significant effects of species (P < 0.001), salinity (P < 0.001) and their interactions (P < 0.001). ϕNPQ values were higher in the sub-coastal species than in the coastal species,

Table 1 Effect of salinity (0, 200 and 400 mM NaCl) on effective photochemical quantum yield of PS II (φII), quantum yield of non-regulated heat dissipation fluorescence emission (φNO) and quantum yield of light-induced (ΔpH- and zeaxanthin-dependent) non-photochemical fluorescence quenching (φNPQ) of *A. lagopoides*, *S. tremulus*, *P. paspalodes* and *P. geminatum*

Species	NaCl	φII	φNPQ	φNO
Coastal				
Aeluropus lagopoides	0	0.21 ± 0.01a	0.34 ± 0.00a	0.46 ± 0.00a
	200	0.26 ± 0.01b	0.29 ± 0.04a	0.46 ± 0.03a
	400	0.11 ± 0.03c	0.33 ± 0.03a	0.57 ± 0.01b
Sporobolus tremulus	0	0.29 ± 0.01a	0.33 ± 0.02a	0.40 ± 0.01a
	200	0.36 ± 0.00b	0.24 ± 0.00b	0.40 ± 0.00a
	400	0.15 ± 0.01c	0.40 ± 0.01c	0.45 ± 0.02b
Sub-coastal				
Paspalum paspalodes	0	0.15 ± 0.01a	0.54 ± 0.00a	0.33 ± 0.01a
	200	0.27 ± 0.11b	0.50 ± 0.04a	0.43 ± 0.05b
	400	0.11 ± 0.00c	0.55 ± 0.01a	0.35 ± 0.01a
Paspalidium geminatum	0	0.26 ± 0.04a	0.37 ± 0.04a	0.37 ± 0.00a
	200	0.19 ± 0.04ab	0.43 ± 0.04ab	0.38 ± 0.00a
	400	0.17 ± 0.00b	0.48 ± 0.00b	0.34 ± 0.00b

Bars represent mean ± standard error ($n = 3$). Different letters over bars indicate significant differences by a post hoc Bonferroni test at $P < 0.05$

particularly under saline treatments; while, the values of φNO were higher in the coastal than in the sub-coastal species with little variation in values with increasing salinity (Table 1). In this study, φPSII was highly correlated with qP while both of these parameters were negatively correlated with leaf temperature (Suppl. Table 1).

Effects of salinity on δ13C values

There were significant differences in $\delta^{13}C$ values ($P < 0.001$) among species, and with the species x salinity interactions ($P < 0.001$); but, not with changes in salinity (Fig. 4; Table 2). With growth under increasing levels of salinity, $\delta^{13}C$ values in the coastal plants, increased in *A. lagopoides* and remained unchanged in *S. tremulus*, while $\delta^{13}C$ values decreased in the sub-coastal species at 400 mM NaCl (Fig. 4).

Effects of salinity on antioxidant enzyme activity

In the coastal species, SOD activity (per mg soluble protein), increased ($P < 0.05$) progressively with increasing

salinities (0 to 400 mM NaCl) resulting in high activities (Fig. 5). There was a transient increase in the sub-coastal species at 200 mM NaCl, and *P. geminatum* had the lowest SOD activities across all salinity treatments. APX activities were much higher ($P < 0.001$) in the coastal species than in the sub-coastal species; the activities decreased in all species with increasing salinity treatments (Fig. 5). Compared to coastal grasses, CAT activity was low in the sub-coastal grasses ($P < 0.01$) and the activity decreased to a low level with increasing salinity (Fig. 5).

Discussion

The effects of salinity on function of photosynthesis and water use efficiency was studied in leaves of coastal and sub-coastal marsh grasses in Pakistan by growing plants in containers outdoors under varying levels of salinity. Analyses were made on leaf carbon isotope composition, leaf gas exchange, and partitioning of absorbed energy between photochemical and nonphotochemical processes.

Fig. 4 Effect of salinity (0, 200 and 400 mM NaCl) on leaf $\delta^{13}C$ values of *A. lagopoides*, *S. tremulus*, *P. paspalodes* and *P. geminatum*. *Bars* represent mean ± standard error ($n = 3$). *Different letters* under *bars* indicate significant differences by a post hoc Bonferroni test at $P < 0.05$
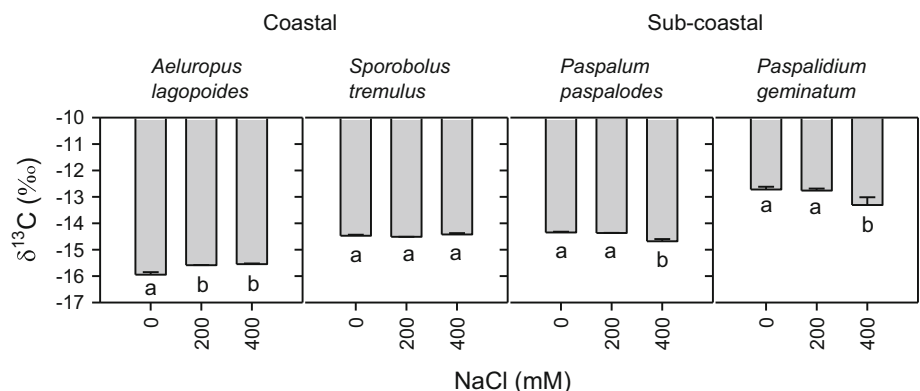
Table 2 Results of two-way analysis of variance indicating the effects of plant species (Sp), salt treatment (NaCl) and their interaction (Sp × NaCl) on various leaf parameters at $P < 0.05$

Parameter	Sp	NaCl	Sp × NaCl
^{13}C	32.60***	4.62*	10.31***
Osmol	11.63***	69.50***	2.98*
Chl a	0.79 ns	5.36*	2.15*
Chl b	1.94 ns	4.50*	1.91 ns
Tot Chl	0.97 ns	5.14*	0.99 ns
CAR	51.25***	1.12 ns	0.42 ns
Tot Chl/CAR	8.86***	1.12 ns	3.43*
Chl a/b	18.59***	10.99***	2.18*
A	6.79**	31.90***	4.05**
R_D	2.82 ns	2.08 ns	0.60 ns
g_s	1.55 ns	15.16***	2.65*
E	2.81*	20.27***	3.30*
C_i	21.33***	4.79*	10.11***
WUE	21.10***	3.07 ns	13.29***
qP	7.40**	20.23***	2.24 ns
NPQ	3.86*	0.32 ns	0.56 ns
Temp	44.23***	22.40***	13.25***
F_v/F_m	0.72 ns	0.90 ns	0.72 ns
$\phi II/\phi CO_2$*	1.13 ns	6.93**	6.52***
ϕII	2.05 ns	10.96***	1.33 ns
ϕNO	19.25***	3.46*	3.88*
ϕNPQ	30.12***	4.88*	1.69 ns
SOD	1.72 ns	1.30 ns	2.29 ns
APX	53.30***	11.10***	2.82*
CAT	123.12***	1.78 ns	5.81**

Numbers represent F values; *Osmol* (osmolality) data in Suppl. Fig. 2

ns non-significant

* Significant at $P < 0.05$

** Significant at $P < 0.01$

*** Significant at $P < 0.001$

Effect of salinity on carbon isotope composition

Carbon isotope discrimination ($\delta^{13}C$) of leaf dry biomass has been extensively used to differentiate between C_3 and C_4 plants (Farquhar 1983; Farquhar et al. 1989; Taiz and Zeiger 2010). With respect to C_4 function, increased leakiness of CO_2 from bundle sheath cells will cause more discrimination against $^{13}CO_2$ and more negative $\delta^{13}C$ values. Increased leakiness can occur by lower resistance to leakage of CO_2 from bundle sheath cells due to structural changes, and by changes in biochemistry with increased ratio of C_4 cycle turnover to CO_2 fixation by Rubisco. Thus, in studies with C_4 species, $\delta^{13}C$ values can provide some insight into C_4 function under different environmental conditions (Henderson et al. 1992; Kubasek et al. 2007).

In the current study, all four halophytic species had C_4 type $\delta^{13}C$ values which indicates C_4 photosynthesis is functioning under saline conditions in different biochemical subtypes; coastal species *A. lagopoides* (NAD-ME); *S. tremulus* (NAD-ME/PEPCK); and sub-coastal species *P. paspalodes* and *P. geminatum* (both NADP-ME). While C_4 type $\delta^{13}C$ values were observed, there were some differences between species. The most negative value was in the coastal species *A. lagopoides* without salt where there was a shift to more positive C isotope values in plants grown under saline conditions which suggests increased effectiveness of Rubisco in capturing CO_2 delivered by the C_4 cycle. A similar shift to more positive values under saline conditions was also observed in a study with the chenopod halophyte C_4 *Bienertia sinuspersici* (NAD-ME type) with increasing salinity from 0 to 400 mM (Leisner et al. 2010). In *A. lagopoides* (Sobhanian et al. 2010) and *B. sinuspersici* (Leisner et al. 2010) there is an up-regulation of enzymes in the C_4 cycle which in part may be to compensate for reduced stomatal conductance and availability of CO_2 and susceptibility of enzymes of the carboxylation phase of the C_4 cycle in the mesophyll cells to inactivation by increasing levels of NaCl. In the coastal species *S. tremulus* there was no effect of varying salinity on $\delta^{13}C$. In the two sub-coastal species, there was no difference between treatments without salt versus 200 mM salt, while $\delta^{13}C$ values were more negative under 400 mM NaCl suggesting some decrease in efficiency of function of C_4. The effect of salinity on $\delta^{13}C$ values may in part be related to the water status of leaves. With increasing salinity, the carbon isotope values (Fig. 4) and the leaf succulence-RWC (Suppl. Fig. 3) increased in *A. lagopoides,* decreased in *P. germinatum*; while, with increasing salinity there was little or no effect on $\delta^{13}C$ values or succulence-RWC in *S. tremulus* and *P. paspalodes.* Thus, more positive $\delta^{13}C$ values, may be suggestive of decreased leakiness of CO_2 being correlated with change in succulence, and vice versa.

In studies by Kubasek et al. (2007), similar estimates of leakiness from bundle sheath cells of C_4 plants grown under different conditions were obtained from analysis of carbon isotope discrimination in leaf biomass with that in short-term measurements from gas exchange. However, since non-photosynthetic processes can also contribute to more negative carbon isotope values in biomass (e.g., 1–2 ‰) due to fractionation occurring during respiration and biosynthesis of lignin and lipids (Henderson et al. 1992; Kubasek et al. 2007), online short-term measurements can provide additional information (Cousins et al. 2008). Further insight into the function of C_4 under different levels of salinity in the coastal and sub-coastal species may be obtained by online measurements of carbon isotope discrimination along with analyses of levels of photosynthetic enzymes and leaf anatomy.

Fig. 5 Effect of salinity (0, 200 and 400 mM NaCl) on the in vitro activity (U mg^{-1} protein) of catalase (CAT), ascorbate peroxidase (APX) and super-oxide dismutase (SOD) in leaf extracts of *A. lagopoides*, *S. tremulus*, *P. paspalodes* and *P. geminatum*. Bars represent mean ± standard error (*n* = 3). *Different letters* over *bars* indicate significant differences between salinity treatments by a post hoc bonferroni test at $P < 0.05$
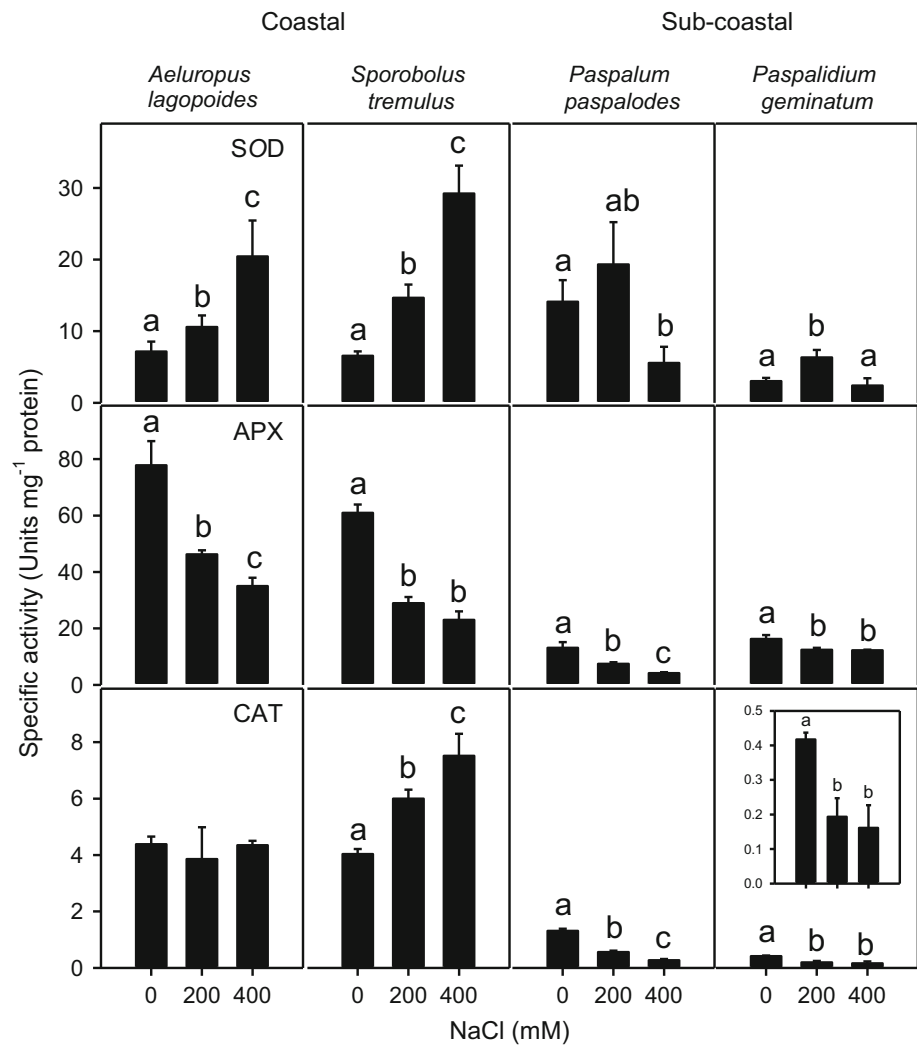
Effect of salinity on gas exchange

Our results indicate that the coastal species *S. tremulus* and *A. lagopoides* respond in a very similar way in gas exchange, with photosynthesis, stomatal conductance and transpiration decreasing with increasing salinity treatments; a similar trend was observed in *A. lagopoides* grown under saline conditions for 15 days (Ahmed et al. 2013). With increasing salinity there was no effect on *WUE* and little, to no, effect on intercellular CO_2 concentrations (C_i); the results indicate salinity causes a parallel decrease in loss of photosynthesis and transpiration which suggest both stomatal and biochemical limitations on carbon assimilation. There is no evidence of dehydration in these species as salinity causes an increase in leaf osmolality and RWC and succulence in *A. lagopoides*, while having no effect on leaf succulence and RWC in *S. tremulus* (Moniuddin et al. 2014; Suppl. Figs. 2, 3). The strong drop in stomatal conductance in the coastal species with increasing salinity

may help maintain succulence and RWC. Succulence allows plants to withstand large variations in soil salinity and moisture (Gulzar and Khan 2006; Edwards and Ogburn 2012) and, in halophytes, greater accumulation of Na$^+$ (salt includers; Hussin et al. 2013) accompanies higher leaf succulence under saline conditions (Jennings 1968). Somewhat similar responses were also reported for the halophytic shrubs *Sarcocornia fruticosa* and *Halimione portulacoides* where reduction in photosynthetic efficiency appeared to result from PSII damage rather than from effects on stomata or compromised water status (Duarte et al. 2013).

The gas exchange responses of the two sub-coastal species were very different. In *P. paspalodes*, there was no significant effect on *A* under saline conditions. Unlike the other species, there was a significant increase in WUE with increasing salinity, which coincided with apparent decreases in stomatal conductance (g_s), transpiration rate (*E*), and intercellular CO_2 concentration (C_i). A decrease in

g_s can effect E (a biophysical process) more than A (biophysical/biochemical process) which results in an increase in WUE. With increasing salinity leaf osmolality increases (Suppl. Fig. 2), and there is no effect on leaf RWC (Suppl. Fig. 3; Moinuddin et al. 2014). The favorable gas exchange features of *P. paspalodes*, may account for it having the highest growth among these four species under high salinity (Moinuddin et al. 2014). In the sub-coastal species *P. geminatum*, there was an opposite effect as increasing salinity caused a severe loss in photosynthetic rate, with no significant effect on stomatal conductance and transpiration rate; this resulted in a dramatic drop in WUE. The large rise in C_i suggests a large effect of salinity on the process of carbon assimilation. Saline conditions cause a decrease in RWC and leaf succulence with increasing salinity in *P. geminatum* (Suppl. Fig. 3; Moinuddin et al. 2014) which suggests inhibition of photosynthesis and growth by dehydration. The differences in response of these four species to salinity cannot be accounted for by information on effects of salinity on levels of Na^+, compatible solutes, and some essential nutrients (Moinuddin et al. 2014). With increasing salinity they found a similar increase in Na^+ and the compatible solute glycine betaine in the shoots, with maintenance of homeostasis of the essential nutrients K^+, Ca^{2+} and Mg^{2+}.

Other studies have shown differences in the response of WUE in halophytes to saline conditions depending on how salinity affects photosynthesis and stomatal conductance (Lovelock and Ball 2002). Salinity caused a concomitant decrease in the rate of photosynthesis and stomatal conductance in some halophytic grasses with little effect on WUE (e.g., *Pennisetum clandestinum, Odyssea paucinervis, Spartina alterniflora, Spartina patens;* Naidoo and Mundree 1993; Radhakrishnan et al. 2006; Maricle et al. 2007); whereas, in *Spartina densiflora* and *Distichlis spicata* (Maricle et al. 2007) there is inhibition of photosynthesis without a decrease in stomatal conductance which resulted in a decrease in WUE.

Effect of salinity on chlorophyll content and partitioning of absorbed energy on photochemical and nonphotochemical processes

There were differences among the species in the effect of growth under saline conditions on chlorophyll content. With increasing salinity, there was no loss in chlorophyll per unit leaf area in the coastal species *S. tremulus* while the other species had substantial loss of chlorophyll. In coastal species *A. lagopoides* which has substantial tolerance to salt, the decrease in chlorophyll content may be related to acclimation to reduce light absorption and provide photoprotection (Wang et al. 2003). The sub-coastal

species *P. geminatum* had a major loss of both chlorophyll and capacity for photosynthesis at 200 and 400 mM NaCl. Pigment degradation in *P. geminatum* could be related to water deficit as indicated by the very low RWC (Suppl. Fig. 3) or direct effects of salinity on the chloroplast (e.g., salt inactivation of the O_2 evolving complex of PSII; Murata et al. 1992). Loss of chlorophyll has often been associated with salinity stress (Lee et al. 2004; Naz et al. 2010; Ferrante et al. 2011). This decrease has been attributed to direct salt effects on chlorophyll degradation due to weakening bonds of the pigment–protein–lipid complex (Levitt 1980), to an increase in chlorophyllase activity (Iyengar and Reddy 1996), or inhibition of *de novo* synthesis of proteins associated with chlorophyll molecules (Jaleel et al. 2007). Loss of chlorophyll could be limiting for photosynthesis if salinity does not cause other damage to the photosynthetic apparatus. However, if high salinity limits photosynthesis by affecting enzymes of carbon assimilation, photophosphorylation, or the electron transport chain (e.g., the O_2 evolving complex), then loss of light harvesting chlorophyll may contribute to photoprotection from excess light. To protect the photosynthetic tissue against high salinity, buffering is needed, e.g., by dilution through higher water content (succulence), storage of salt in vacuoles, synthesis of compatible solutes, salt secretion, or by maintaining high Mg^{++} for maintaining chlorophyll integrity (Salama et al. 1994; Munns and Tester 2008; Hameed and Khan 2011).

The maximum quantum yield of photosystem II (F_v/F_m) provides a measure of photoinhibition (Maxwell and Johnson 2000). The decrease in F_v/F_m ratio in *A. lagopoides* with increasing salinity suggests that there was some photoinhibition, while no decrease was seen in the other three species. Although most salt-tolerant species tend to maintain high F_v/F_m ratios under stressful conditions (Wei et al. 2006; Maricle et al. 2007; Naidoo et al. 2008; Stepien and Johnson 2009), lower F_v/F_m could also be a means of down-regulating light harvesting reactions and thus minimizing ROS production.

The proportion of open PSII reaction centers (qP, photochemical quenching) and yield of PSII (ϕII), increased significantly in the coastal species with moderate salinity before declining at the highest salinity treatment. In the sub-coastal species *P. geminatum* and *P. paspalodes*, there was less effect. In C_4 plants there is generally a tight correlation between flux of electrons through linear electron flow and CO_2 fixation, which is reflected in the linear relationship between the quantum yield of PSII (ϕ_{II}) with the carboxylation efficiency (ϕ_{CO_2*}) (Oberhuber et al. 1993). In the present study, with increasing salinity there is a decrease in A (resulting in a decrease in ϕ_{CO_2*}) while there is not a parallel reduction in ϕII. Rather there is a
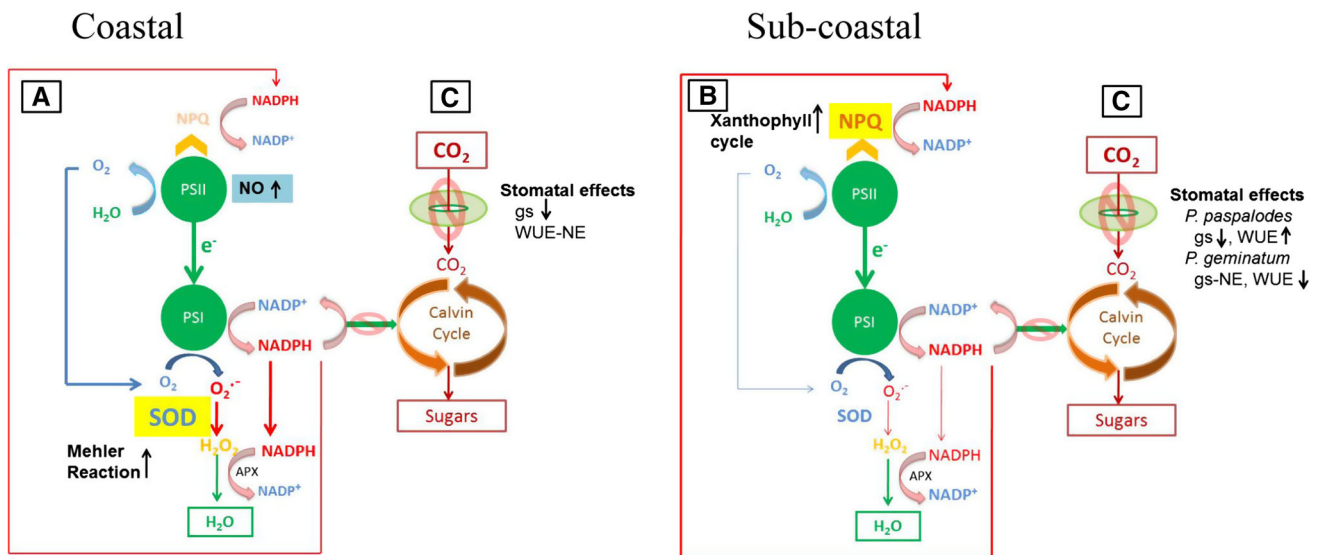
Coastal

Sub-coastal

Fig. 6 Graphical summary of key findings showing: (*A*) higher use of the Mehler reaction, SOD and APX and NO dissipation of energy, in coastal grasses, (*B*) enhanced NPQ and xanthophyll cycle in sub-coastal grasses. (*C*) Change in stomatal conductance, CO_2 assimilation and water use efficiency. *NE* no effect

strong increase in the $\phi_{II}/\phi_{CO_{2*}}$ ratio in the coastal grasses with increasing salinity which suggests they employ the Mehler reaction. Induction of alternative electron flow under saline conditions was also found in some halophytic grasses (*Spartina* spp. and *D. spicata*) by Maricle et al. (2007) and some Amaranths (*S. fruticosa* and *H. portulacoides*) by Duarte et al. (2013). In the sub-coastal grass *P. paspalodes* the $\phi_{II}/\phi_{CO_{2*}}$ ratio was relatively low indicating limited alternative electron flow, while in *P. geminatum* with increasing salinity there was severe inhibition of photosynthesis resulting in a very low $\phi_{CO_{2*}}$ resulting in an increase in the $\phi_{II}/\phi_{CO_{2*}}$ ratio. Enzymes which eliminate ROS which are produced under stress are located in various subcellular compartments (Mittler 2002). The induction of the Mehler reaction under stress could help dissipate excess solar energy; but protection against damage requires removing reactive oxygen species; i.e., superoxide via SOD, hydrogen peroxide via ascorbate peroxidase in the chloroplast, and hydrogen peroxide via catalase outside the chloroplast. Compared to the sub-coastal species, the coastal species *A. lagopoides* and *S. tremulus* have high levels of these enzymes, which may protect against damage via the Mehler reaction and support this alternative electron flow. The sub-coastal species *P. geminatum* had an increase in $\phi_{II}/\phi_{CO_{2*}}$ suggestive of alternative electron flow where CO_2 assimilation was restricted; it has low levels of these three antioxidant enzymes under saline conditions which may result in damage by ROS and account for the severe loss in capacity for photosynthesis. In this study the results indicate ϕ_{II} is largely accounted for by PSII activity supporting C_4 photosynthesis and the Mehler reaction.

Under conditions where plants experience excess light, energy dependent NPQ is considered a major line of defense against photodamage. In measurement of photosynthesis at 1200 PPFD there was substantial dissipation of energy by NPQ (from 24 up to 60 % across salinity treatments, Table 1). However, the mean % dissipation via NPQ across treatments (0–400 mM NaCl) was lower in the coastal (32 %) than in the sub-coastal species (48 %); and, the carotenoids which can provide photoprotection (e.g., β-carotene, xanthophyll pigments) are much lower in the coastal species. The xanthophyll pigments violaxanthin and zeaxanthin which mediate thylakoid acidification and NPQ (Bilger and Bjorkman 1994) and the presence of PsbS, a specialized pigment binding protein (Li et al. 2000) are involved in thermal dissipation through non-photochemical quenching in higher plants. There was little to no effect of increasing salinity on the level of NPQ. Sub-coastal grasses showed greater reliance on NPQ than on NO in comparison to the coastal grasses. In another study on halophytic grasses, Maricle et al. (2007) found with increase in salinity from 0 to 30 % there was no effect on NPQ in *S. alterniflora* and *S. densiflora*, while NPQ increased in *S. patens* and *D. spicata*. Differences in the use of NPQ among two co-occurring dicotyledonous salt marsh species concomitant with overall reduced levels of photosynthetic pigments were also reported by Duarte et al. (2013). In the current study, while ϕNPQ was higher in the sub-coastal grasses, the mean fraction of energy dissipated by ϕNO across NaCl treatments was higher in coastal grasses (46 %) than sub-coastal grasses (37 %) (Table 1). The results indicate ϕNO is also a significant contributor to

nonphotochemical dissipation of energy. One potential contributor to φNO is dissipation of energy from singlet O_2 by ß-carotene (which can be generated from triplet state chlorophyll formed by over-reduction on the acceptor side of PSII and charge recombination in the reaction center, Noguchi 2002; Krieger-Liszkay 2004). Otherwise, singlet O_2 can damage the D1 protein of the reaction center and cause photoinhibition (e.g., possibly accounting for the decrease in F_v/F_m with increasing salinity in *A. lagopoides*).

These results show there are major differences in absorption and use of solar energy in photosynthesis between these important coastal and sub-coastal C_4 grasses which grow in salt marshes in Pakistan (Fig. 6). This includes differences in WUE, levels of photosynthetic pigments and in mechanisms for dissipating excess light energy under increasing salinities. The coastal species which grow in more stressful conditions, with fluctuations in salinity and moisture, are adapted to dissipate excess energy via alternative electron flow in the Mehler reaction, along with dissipating some energy by NPQ. Their leaves are also salt-excreting which may increase their tolerance to high salinity. The sub-coastal species have lower alternative flow, with a higher level of carotenoids and energy dissipation by NPQ. Studies on additional species are needed to determine if differences in adaptations between species are related to habitat, or evolution of different means of tolerating salinity. In family Poaceae, halophytes have repeatedly evolved in C_4 lineages more frequently than in C_3 groups (Bromham and Bennett 2014).

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