



# Salinity induced changes in light harvesting and carbon assimilating complexes of *Desmostachya bipinnata* (L.) Staph.



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## ABSTRACT

The present study was carried out to determine stomatal, photochemical and biochemical limitations of photosynthesis in *Desmostachya bipinnata* under saline conditions. We hypothesized that processes determining plant growth and photosynthetic rates will be differentially regulated under various salinity treatments. Plants were grown with 0 mM, 100 mM (moderate) and 400 mM (high) NaCl concentrations in a semi-hydroponic quick check system. Production of biomass was not affected by moderate salinity but a significant reduction was observed at high salinity. High salinity decreased stomatal conductance without affecting  $C_i$ . Dark respiration increased at moderate salinity compared to control and high salinity. Salinity treatments had no effect on the WUE. Photosynthetic efficiency was improved at moderate salinity compared to the control but was inhibited at high salinity. Net photosynthesis was similar to the control at moderate salinity but was inhibited with a further increase in salinity. Photosynthetic pigments, electron transport rate, and photochemical quenching were not affected at moderate salinity but declined significantly at high salinity. MDA content significantly increased at high salinity suggesting ROS accumulation. A linear decrease in  $V_{c,max}$ ,  $J_{max}$ , TPU and Rubisco content was observed with increasing salinity treatments. Relative expression of photosynthetic complex proteins was either enhanced (*D1*, *PetD*, *AtpA*) or unaffected (*PsbO*) with moderate salinity but decreased at high salinity with the exception of *AtpA*. Our data indicates that photosynthesis rates were maintained at moderate salinity. Damages to photochemical reactions and down-regulated expression of chloroplast proteins in combination with biochemical limitations, rather than stomatal limitations, restrained photosynthetic performance of *D. bipinnata* under high salinity.

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## 1. Introduction

Plants raised under saline conditions regulate their stomatal openings to minimize water loss, limit CO<sub>2</sub> uptake (i.e., stomatal limitation) and ultimately lower biomass accumulation (Kirschbaum, 2011; Chaves et al., 2009; Dodd, 2003). Reduced availability

of CO<sub>2</sub> alters the ratios of ATP/ADP and NADPH/NADP<sup>+</sup> (i.e., electron and proton acceptors), causing over-reduction of photosystems and producing reactive oxygen species (ROS) which damages photosynthetic machinery (Pinheiro and Chaves, 2011; Qiu et al., 2003). Therefore, limited water influx and ion toxicity under saline conditions reduces plant productivity (Munns and Tester, 2008).

**Abbreviations:** A, net photosynthesis; ATP, adenosine triphosphate; AtpA,  $\alpha$  subunit protein of ATP-synthase complex;  $C_i$ , intercellular CO<sub>2</sub>; Cyt b<sub>6</sub>f, cytochrome b<sub>6</sub>f complex; DTT, dithiothreitol; E, transpiration; ETR, electron transport rate;  $F_v/F_m$ , maximum photochemical quantum yield of PSII; HRP, Horseradish peroxidase;  $I_c$ , compensation irradiance;  $I_s$ , saturation irradiance;  $J_{max}$ , maximum rate of photosynthetic electron transport to regenerate RuBP;  $g_s$ , stomatal conductance; MDA, malondialdehyde; NADP<sup>+</sup>, nicotinamide adenine dinucleotide phosphate; NPQ, non-photochemical quenching; OEC, oxygen evolving complex; P<sub>i</sub>, inorganic phosphate; PEPC, phosphoenol pyruvate carboxylase; PSI, photosystem I; PSII, photosystem II; PetD, subunit D of photosynthetic electron transport protein (Cytb<sub>6</sub>f complex); PsbA, D1 protein of PSII reaction center complex; PsbO, PS II manganese-stabilizing protein of OEC; PAR, photosynthetically active radiation; qP, co-efficient of photochemical quenching; R<sub>d</sub>, dark respiration; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; ROS, reactive oxygen species; RuBP, ribulose-1,5-bisphosphate; SDS, sodium dodecyl sulfate; TBA, thiobarbituric acid; TBS, tris buffer saline; TCA, trichloroacetic acid; TPU, utilization of triose-phosphate to regenerate P<sub>i</sub>;  $V_{c,max}$ , the maximum rate of Rubisco carboxylase activity; WUE, water use efficiency (A/E); YII, effective quantum yield of PSII;  $\Phi_e$ , photosynthetic efficiency.

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The  $C_4$  mode of photosynthesis is a spatial and biochemical adaptation leading to  $CO_2$  accumulation with improved productivity and water use efficiency (Ashraf and Harris, 2013; Naidoo et al., 2012); therefore, ensuring survival of plants under harsh conditions such as salt stress (Sage et al., 2012). Plants with  $C_4$  photosynthesis tolerate soil salinity better than  $C_3$  and therefore are more likely to dominate saline habitats (Bromham and Bennett, 2014).

Photochemical and biochemical reactions in addition to stomatal limitations contribute significantly to photosynthetic performance of plants (das Neves et al., 2008). Damages in photochemical reactions might be associated with salt sensitivity of photosynthetic pigments and of chloroplast membrane protein complexes (Kosová et al., 2013). A decrease in chlorophyll concentration lowers consumption of photons for light driven electron transport in salt stressed plants (Megdiche et al., 2008) leading to photo-inhibition. Chlorophyll fluorescence and non-photochemical quenching (NPQ) are competitive processes aimed to quench light energy (Müller et al., 2001; Maxwell and Johnson, 2000). The efficiency of photosystem II (PSII) (Han et al., 2010), regulation of NPQ and fluorescence determines the levels of plant responses under saline conditions (Koyro et al., 2013; Yu et al., 2011; Genty et al., 1989).

A- $C_i$  curve measurements have been used to study biochemical reactions of photosynthesis (Flexas et al., 2004) which provides information about the maximum rate of Rubisco carboxylase activity ( $V_{c,max}$ ), rate of photosynthetic electron transport to regenerate RuBP ( $J_{max}$ ) and utilization of triose phosphates to regenerate  $P_i$  (TPU) (Von Caemmerer, 2000). An alteration in the efficiency of these processes due to toxic effects of salt impedes photosynthetic performance of plants (Zeng et al., 2010). Changes in structural composition of chloroplast proteins also affect the rate of photosynthesis under saline conditions (Yu et al., 2011; Sobhanian et al., 2010; Taylor et al., 2009). The major functional chloroplast protein complexes, i.e., PSI, PSII, ATP-synthase and  $Cytb_6/f$ , are involved in harvesting light energy (Dekker and Boekema, 2005). Several subunits that constitute these protein complexes such as *PsbO*, *D1* (*PsbA*), *PetD*, *AtpA* are reported to be altered under saline conditions along with Rubisco protein (Li et al., 2011; Pang et al., 2010; Sobhanian et al., 2010; Xu et al., 2010). Little information is available on linkages of photosynthesis to chloroplast proteins in salt tolerant monocots, therefore more data is needed.

Halophytes are distributed in saline environments and employ a series of inter-connected physiological, morphological and molecular responses for salt tolerance. *Desmostachya bipinnata* (L.) Stapf. (Poaceae) is a perennial  $C_4$  halophytic grass that is distributed in warm saline areas (Gulzar et al., 2007). It has a wide ecological distribution, ranging from Northern Africa, Sicily and Cyprus through the Middle East to Central Asia, Pakistan and India (Cope, 1982). This grass has been recognized as a candidate for fodder production (Khan et al., 2009), and is already being utilized in some areas for feeding animals (Galal and Shehata, 2013). Levels of salt tolerance and some physiological responses of *D. bipinnata* have been reported earlier (Adnan et al., 2016). The present study was aimed to determine relative contributions of stomatal, biochemical and photochemical factors in limiting photosynthesis and to evaluate and relate changes in the expression of chloroplast proteins with photosynthetic performance of *D. bipinnata* under saline conditions. Considering that halophytes show optimal growth with low to moderate concentrations of salt and inhibited growth with further increases in salt concentration (Flowers and Colmer, 2008; Glenn et al., 1999), we treated our test species with two different (moderate and high) NaCl concentrations.

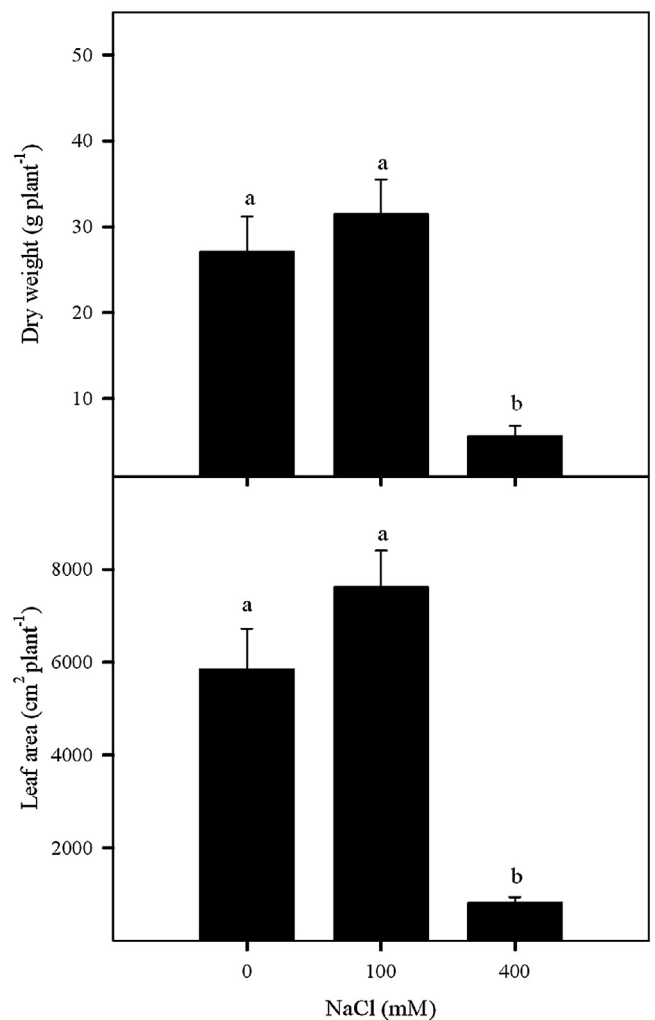
The following hypotheses were tested to address the objective of this study: (i) moderate salinity will promote growth of *D.*

*bipinnata*; (ii)  $CO_2$  uptake will be similar to non-saline control at moderate salinity; (iii) high salinity will decrease photosynthesis due to stomatal limitation; (iv) high salinity will reduce photochemical reactions; (v) moderate salinity will cause limitations to photosynthesis due to biochemical reactions and (vi) high salinity will reduce/alter the expression of chloroplast membrane protein complexes.

## 2. Materials and methods

### 2.1. Plant material and experimental conditions

Seeds of *D. bipinnata* were germinated in soil mixture (1:1 garden soil and manure) in trays placed in a growth chamber (30/20 °C and 16/8 h day/night). Three week old seedlings were grown in pots (6 × 10 cm) in a screen house under ambient conditions (temperature: 30 ± 2 °C, relative humidity: 40 ± 10%, PAR: 370 ± 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and watered with half strength of basic nutrient solution (Epstein, 1972). Similar sized seedlings (3–4 leaf stage) were transferred to pots containing Quartz sand (18 × 25 cm) in a semi-hydroponic Quick Check System (QCS, Koyro, 2006) under temperature: 37 ± 4 °C, relative humidity: 47 ± 12% and PAR:



**Fig. 1.** Dry weight and leaf area of *D. bipinnata* subjected to 0, 100 and 400 mM NaCl concentrations. Values represent the mean ± S.E. of three replicates (n=3). Different letters indicate significant difference due to salt treatments, according to Bonferroni's test ( $P < 0.05$ ).

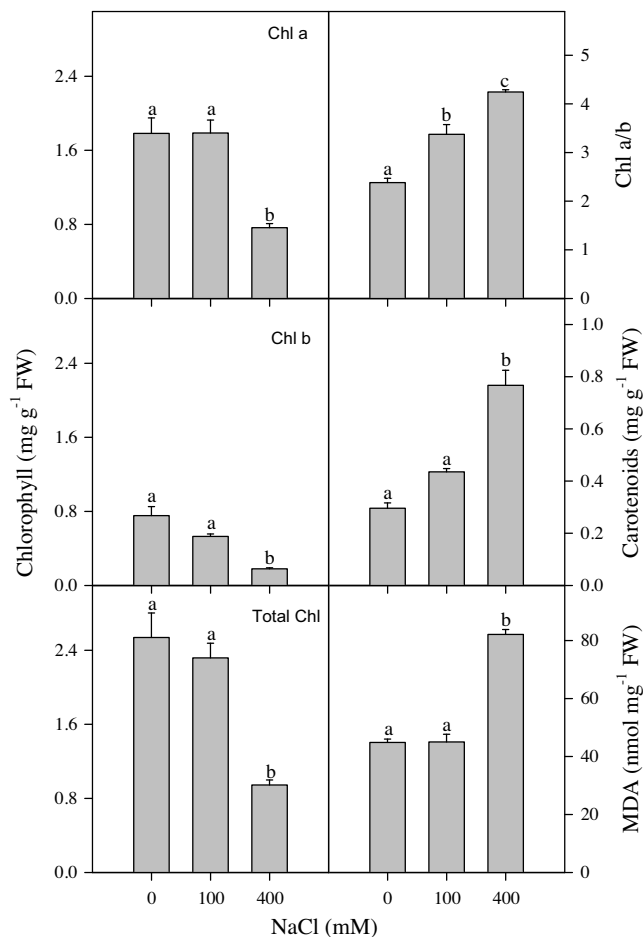
$1200 \pm 200 \mu\text{mol m}^{-2} \text{s}^{-1}$  after six weeks. Plants were irrigated for at least half an hour after every four hours and nutrient solution was allowed to drain from pots. After 2 weeks of acclimation, plants were treated with various NaCl (0, 100 and 400 mM) concentrations indicating control, moderate and high levels of salt, respectively. These treatments were selected based on results of preliminary experiments. The salinity of the nutrient solutions was increased gradually by adding 50 mM NaCl per day until the desired concentrations were attained. Solutions were changed every 2 weeks to maintain nutrient levels. The duration of NaCl treatments was 4 weeks.

## 2.2. Dry mass and leaf area measurements

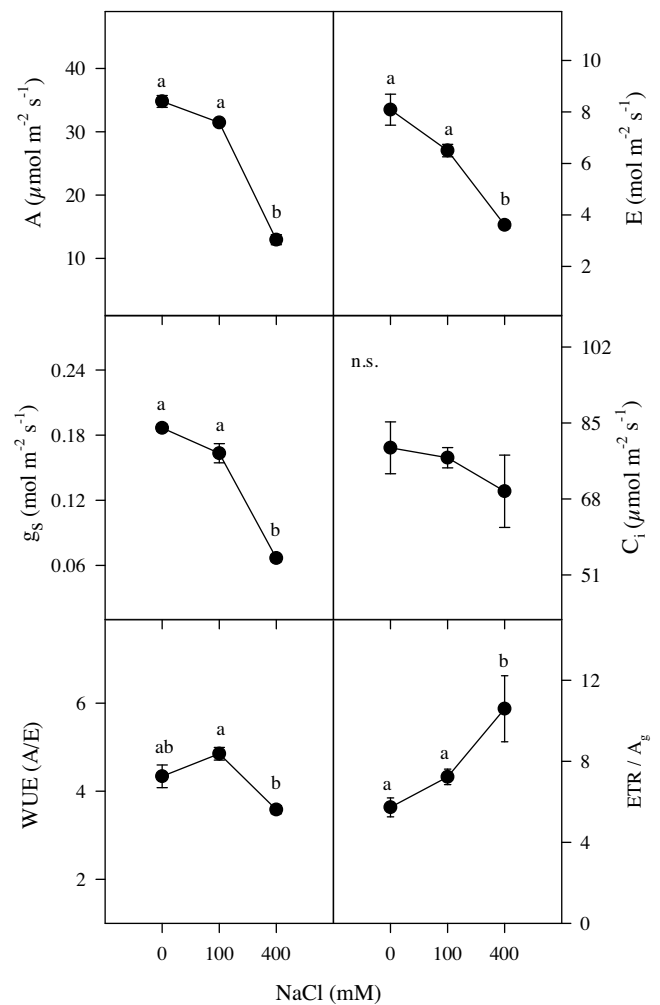
Three plants from each NaCl concentration were harvested at the end of the experiment. Weight was recorded after drying at  $60^\circ\text{C}$  in an oven (Thermo Scientific Heraeus, 6030, USA) until constant weight was obtained (about 48–72 h). Leaf area was calculated by using a portable leaf area meter (ADC Bio-Scientific Ltd. AM350, England).

## 2.3. Photosynthetic pigments

Fresh leaf tissue (100 mg) was homogenized with 80% acetone using a chilled mortar and pestle. After centrifugation (500g), the supernatant was collected and used for absorbance measurements



**Fig. 2.** Chlorophyll content (chl a, chl b and total chlorophyll), chlorophyll a/b ratio, total carotenoids and malondialdehyde (MDA) content of *D. bipinnata* at 0, 100 and 400 mM NaCl concentrations were determined. Values represent the mean  $\pm$  S.E. of three replicates ( $n=3$ ). Different letters indicate significant differences due to salt treatments, according to Bonferroni's test ( $P < 0.05$ ).



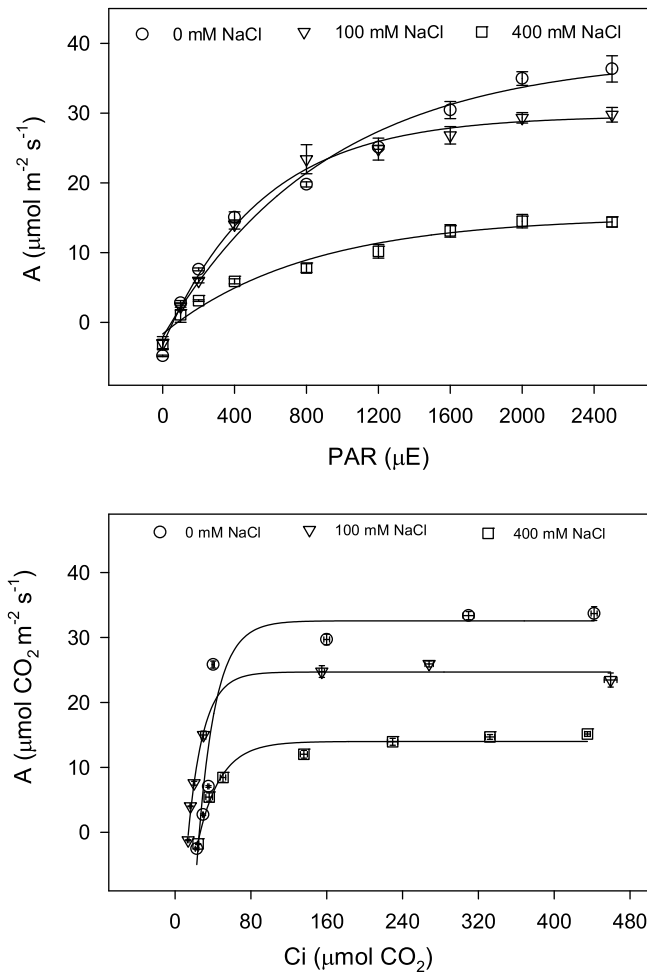
**Fig. 3.** Net photosynthesis (A), transpiration (E), stomatal conductance ( $g_s$ ), intercellular  $\text{CO}_2$  concentration ( $C_i$ ), water use efficiency (WUE) and  $\text{ETR}/A_{g_s}$  of *D. bipinnata* subjected to 0, 100 and 400 mM NaCl concentrations. Values represent the mean  $\pm$  S.E. of three replicates ( $n=3$ ). Different letters indicate significant differences due to salt treatments, according to Bonferroni's test ( $P < 0.05$ ). n.s. indicates no significant difference due to treatments.

at 663.2, 646.8 and 470 nm (Beckman-Coulter DU-730, UV-vis spectrophotometer). Three biological replicates for each treatment were used. Contents of chlorophyll a, b, total chlorophyll and total carotenoid were calculated with equations suggested by Lichtenthaler (1987).

## 2.4. Gas exchange and chlorophyll fluorescence measurements

Gas-exchange measurements were made using a portable photosynthetic system (LICOR-6400, Lincoln, NE, USA) on a fully expanded leaf at ambient  $\text{CO}_2$  partial pressure, humidity and temperature. Net photosynthesis (A), stomatal conductance ( $g_s$ ), intercellular  $\text{CO}_2$  ( $C_i$ ), transpiration (E) and water use efficiency (WUE) were recorded at saturation irradiance for each salinity treatment. Photosynthesis-irradiance response curves were plotted with PAR ( $0-2500 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ ). Dark respiration ( $R_d$ ), compensation irradiance ( $I_c$ ), saturation irradiance ( $I_s$ ) and photosynthetic efficiency ( $\Phi_c$ ) were estimated as described by Schulte et al. (2003). The electron transport rate to gross photosynthetic rate ratio ( $\text{ETR}/A_{g_s}$ ) was calculated according to Flexas et al. (1999).

The A- $C_i$  curve was constructed to determine the plant response to varying intercellular  $\text{CO}_2$  concentrations. Values for maximum



**Fig. 4.** Light response curve between net photosynthesis (A) and light intensities (PAR; 0–2500  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) and  $\text{CO}_2$  response curve between net photosynthesis (A) and variable intercellular  $\text{CO}_2$  concentrations on leaves of *D. bipinnata* under various NaCl concentrations (0, 100 and 400 mM). Values represent the mean  $\pm$  S.E. of three replicates ( $n=3$ ). Different letters indicate significant difference due to salt treatments, according to Bonferroni's test ( $P < 0.05$ ).

Rubisco carboxylase activity ( $V_{c,\text{max}}$ ), maximum rate of electron transport to regenerate RuBP ( $J_{\text{max}}$ ) and triose-phosphate utilization (TPU) were estimated according to Long and Bernacchi (2003).

Chlorophyll a fluorescence was measured using a pulse modulated chlorophyll fluorescence meter (2500 PAM, Walz, Germany). Before measurements, leaves were kept in the dark for 25 min. Minimal fluorescence ( $F_0$ ) with modulated light ( $< 0.1 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) and maximal fluorescence ( $F_m$ ) with saturating pulse ( $10,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 0.6 s) were determined to estimate maximum photochemical quantum yield of PSII ( $F_v/F_m = F_m - F_0/F_m$ ) according to Kitajima and Butler (1975). Later the leaves were exposed with actinic light ( $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) to determine steady-state ( $F_s$ ) and maximal fluorescence ( $F_m'$ ) in light-adapted leaves. Minimal fluorescence ( $F_0'$ ) was also calculated in light adapted leaves as described by Baker and Rosenqvist (2004). The effective photochemical quantum yield of PSII ( $Y_{II}$ ) and non-photochemical quenching (NPQ) were determined using these formulae, respectively:  $Y_{II} = F_m' - F_s/F_m'$  and  $NPQ = F_m/F_m' - 1$  (Genty et al., 1989). The coefficient of photochemical quenching ( $qP$ ) was calculated as  $(F_m' - F_s)/(F_m' - F_0')$  (Kooten and Snel, 1990; Schreiber et al., 1986). Electron transport rate (ETR; Krall and Edwards, 1992) was estimated as  $ETR = \text{PPFD} * Y_{II} * 0.5 * 0.84$ , where PPFD is the Photosynthetic Photon Flux Density incident on the leaf; 0.5:

factor applied assuming incident energy is equally divided between PSI and PSII and 0.84: indicates leaf absorbance used for green leaves. Temperature difference between air and leaf ( $\Delta T; ^\circ\text{C}$ ) was calculated as  $\Delta T = T_{\text{air}} - T_{\text{leaf}}$ .

## 2.5. Malonyldialdehyde (MDA) measurements

Fresh leaf (100 mg) was homogenized with 0.5% thiobarbituric acid (TBA) prepared in 20% trichloroacetic acid (TCA). The mixture was boiled at  $95^\circ\text{C}$  for 30 min and the reaction was terminated in an ice bath. After centrifugation (3000g), the supernatant was collected and absorbance at 532 and 600 nm was recorded (Beckman-Coulter DU-730, UV-vis spectrophotometer). MDA concentrations were determined after taking the difference of recorded absorbance using an extinction coefficient of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$  (Sairam and Sexena, 2000).

## 2.6. SDS-PAGE and Western blot analyses

Leaf samples (500 mg) were ground to fine powder with liquid nitrogen and solubilized in sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris-HCl (pH 6.8), 4% w/v SDS, 12% v/v glycerol, 50 mM dithiothreitol (DTT) and 0.01% bromophenol blue). Protein concentration was determined according to Bradford (1976), using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumen as a standard.

Proteins were separated by SDS-PAGE at 20 mA as described by Laemmli (1970). 5  $\mu\text{g}$  protein was loaded and separated at 20 mA per gel using a Mini-PROTEAN Tetra cell (BioRad, Hercules, CA, USA). After electrophoresis, gels were either stained with Coomassie Blue R-250 (for Rubisco (large subunit) and *PsbA*) or incubated in transfer buffer (48 mM Tris, 39 mM Glycine, 20% methanol, pH 9.2) for 30 min. After incubation, proteins were electro-blotted onto nitrocellulose membrane (Micron Separations, Westborough, MA) using a semi-dry trans-blotting system (Bio-Rad). Blots were then blocked with 3% skimmed milk in Tris buffered saline (TBS) (10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.5% Triton X-100) for 3 h at room temperature and probed with primary antibodies for individual chloroplast proteins (*PsbO*, *PetD* and *AtpA*) following the procedure described by Harlow and Lane (1988). Anti-Rabbit IgG coupled to horseradish peroxidase (HRP; Promega) was used as secondary antibody (dilution: 1:20,000). Positive signals were detected using SuperSignal<sup>®</sup> West Pico chemi-luminescent substrate (Thermo Scientific) according to manufacturer's instructions. Relative amounts of proteins were estimated after comparing intensities between treatments with CIS 1 D analysis software (Clinx, GenoSens Series, Gel documentation system). Intensity of protein from control treatment was assumed to present 100% protein.

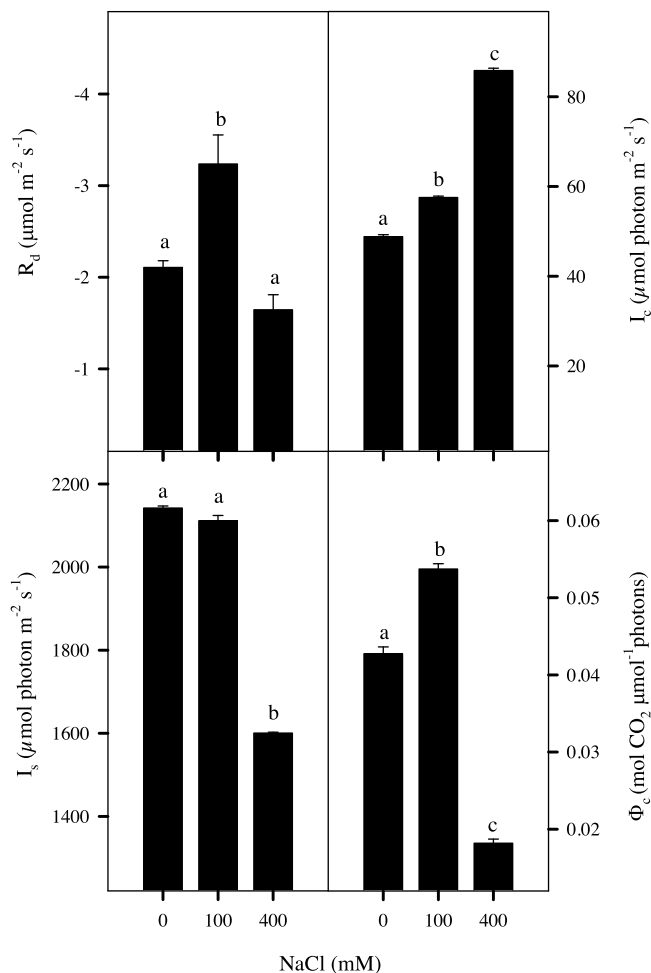
## 2.7. Statistical analyses

All values are presented as mean  $\pm$  standard error of mean (S.E.) of three replicates. Data were analyzed using statistical software, SPSS, version 11.0 (SPSS Inc., Chicago, USA). Analysis of variance (ANOVA) was used to test the significance of all parameters tested. Bonferroni test was carried out to determine if significant ( $p < 0.05$ ) differences existed among means.

## 3. Results

### 3.1. Growth parameters

Dry weight accumulation of *D. bipinnata* was significantly reduced (about 4.5 times) only at 400 mM NaCl in comparison to



**Fig. 5.** Dark respiration ( $R_d$ ), compensation irradiance ( $I_c$ ), saturation irradiance ( $I_s$ ) and photosynthetic efficiency ( $\Phi_c$ ) of *D. bipinnata* subjected to different NaCl concentrations. Values represent the mean  $\pm$  S.E. of three replicates ( $n=3$ ). Different letters indicate significant differences due to salt treatments, according to Bonferroni's test ( $P < 0.05$ ).

the control. Leaf area measurements showed a seven fold reduction at high salinity (Fig. 1).

### 3.2. Photosynthetic pigments, MDA, leaf gas exchange and chlorophyll fluorescence parameters

Moderate NaCl treatment (100 mM) did not have any significant effect on pigment concentration of *D. bipinnata* as compared to control. High NaCl treatment caused about 3 fold reduction in chlorophyll content and about 3 fold increase in carotenoids content in comparison to control plants. A progressive increase in the ratio of chlorophyll a to chlorophyll b was observed with increasing salinity. MDA content, indicating membrane lipid peroxidation, increased substantially (83%) under high salinity in comparison to the control (Fig. 2).

Exposure of *D. bipinnata* to 400 mM NaCl significantly decreased stomatal conductance ( $g_s$ ), net photosynthesis ( $A$ ) and transpiration ( $E$ ), while at moderate salinity none of these parameters were inhibited. Salinity treatments had no significant effect on water use efficiency ( $WUE$ ) and  $C_i$  values. The  $ETR/A_g$  ratio was increased by about 40% under high salinity in comparison to control (Fig. 3).

Analysis of light curves (Fig. 4) showed a salinity-induced shift towards higher compensation irradiance ( $I_c$ ). High salinity treatment increased  $I_c$  by almost 2 fold compared to non-saline

control plants. Irradiance where photosynthesis becomes saturated ( $I_s$ ) did not differ significantly between plants of control and moderate salinity treatments, but decreased significantly at high salinity. Rates of dark respiration ( $R_d$ ) were increased (54%) under moderate salinity, but the rates were similar in plants of control and high salinity treatments. Photosynthetic efficiency of plants was improved (26%) by 100 mM NaCl but it declined significantly (57%) by 400 mM NaCl (Fig. 5) as compared to control plants.

Changes in net photosynthesis as a function of increased  $\text{CO}_2$  concentration were analyzed to determine the contribution of biochemical limitations in rates of carbon assimilation under salinity treatment. Analyses of  $A-C_i$  curves revealed a progressive decrease in  $V_{c,max}$ ,  $J_{max}$  and  $TPU$  with an increase in salinity (Fig. 6).

Chlorophyll fluorescence parameters such as effective photochemical quantum yield of PSII ( $Y_{II}$ ), photochemical quenching ( $qP$ ), non-photochemical quenching ( $NPQ$ ), electron transport rate ( $ETR$ ) and temperature difference between leaf and air ( $\Delta T$ ) were not significantly different for non-saline or moderate salinity treated plants (Fig. 7). However, significant decreases in  $Y_{II}$  (42%),  $qP$  (28%) and  $ETR$  (42%) but increases in  $NPQ$  (36%) and  $\Delta T$  (60%) were recorded at 400 mM NaCl treatment in comparison to control plants. Salinity treatments had no significant effect on the  $F_v/F_m$  values.

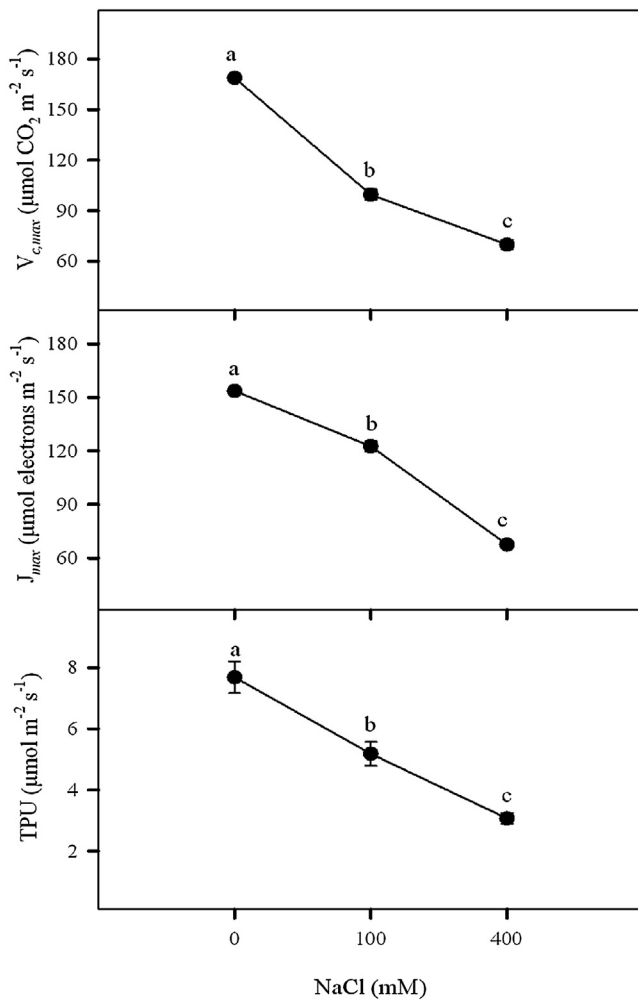
### 3.3. Analysis of chloroplast proteins

Chloroplast proteins, i.e., Rubisco, *PsbA*, *PsbO*, *PetD* and *AtpA* varied in their expression with the different salt treatments (Fig. 8). Rubisco protein showed progressive down-regulation with increasing NaCl concentration. Expression of *PetD* and *PsbA* proteins increased under moderate salinity, i.e., 138% and 103% of control but their levels were decreased with high salinity, i.e., 86% and 75% of the control, respectively. Relative abundance of *PsbO* protein declined (81% of that found in control) at high salinity but it remained unaffected at optimal salinity. Salinity treatments increased expression of extrinsic polypeptide *AtpA* by 137% and 138% at 100 and 400 mM NaCl treatments, respectively (Table 1).

## 4. Discussion

The present study was undertaken to document and compare the differential effect of various NaCl concentrations affecting photosynthesis of *D. bipinnata*, which ultimately influences growth and biomass production. Individuals of *D. bipinnata* in our experimental conditions show strong resistance to moderate salinity while plant growth substantially declined at high salinity. Salinity resistance in some halophytic grasses showed results similar to our data (Hussain et al., 2015; Naidoo et al., 2008), while a few other papers show growth stimulation at low salinity (Abideen et al., 2014; Bell and O'Leary, 2003). Growth stimulation under saline conditions is due to the level of salinity resistance of a given species, and is expected to show growth stimulation at some level of salinity. Therefore, it is very difficult to either confirm or reject our hypothesis "moderate salinity will promote growth of *D. bipinnata*".

Plants raised under moderate salinity did not show any effect on photosynthetic leaf area and chlorophyll content while  $chl a/chl b$  ratio and photosynthetic efficiency ( $\Phi_c$ ) were significantly increased. This increase in  $chl a/b$  ratio probably indicates changes in the size of antenna with respect to reaction centers (Ashraf and Harris, 2013). Slight effects of moderate salinity on the growth of halophytic grasses may be due to a predominant investment of energy on defense mechanisms rather than on biomass production under low stress (Hussain et al., 2015) or due to the management of water by reducing uptake which in turn reduces the assimilation of toxic ions (Naidoo et al., 2008). However, high salinity caused



**Fig. 6.** A-Ci curve was used to determine the following parameters; maximum rate of Rubisco carboxylase activity ( $V_{c,max}$ ), RuBP regeneration capacity mediated by the maximum rate of electron transport ( $J_{max}$ ) and  $P_i$  regeneration capacity mediated by utilization of triose phosphates (TPU) on leaves of *D. bipinnata* subjected to different NaCl concentrations (0, 100 and 400 mM). Values represent the mean  $\pm$  S.E. of three replicates ( $n=3$ ). Different letters indicate a significant difference due to salt treatments, according to Bonferroni's test ( $P < 0.05$ ).

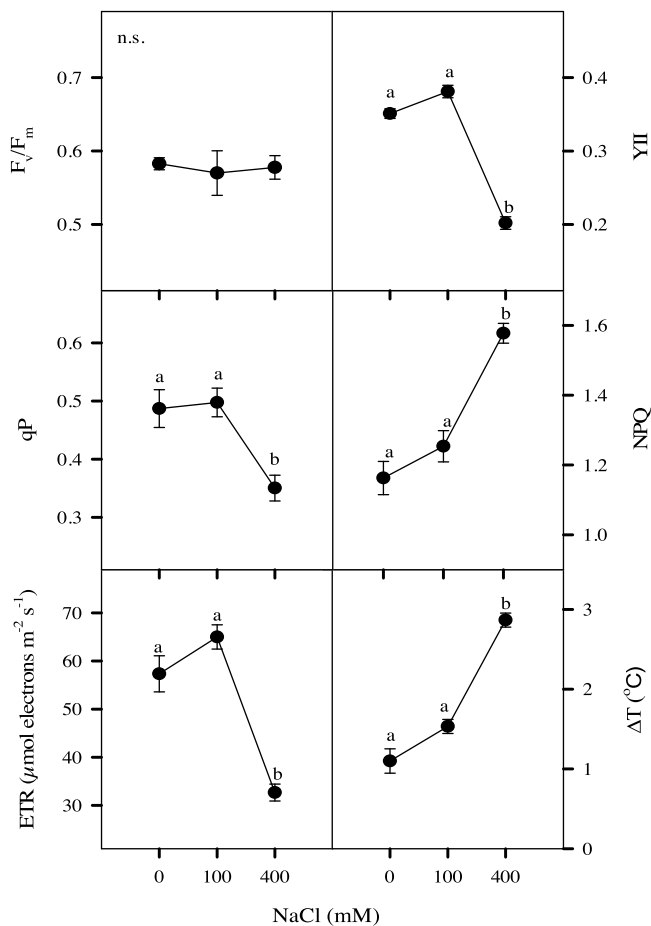
considerable reduction in leaf area, photosynthetic efficiency and chlorophyll content. Reduction in leaf area results in decreased transpiration to conserve water under high salinity but also reduces photosynthesis by limiting surface area for photosynthetic pigments (Ahmed et al., 2013; Koyro et al., 2013). Chlorophyll reduction caused either by reduced chlorophyll biosynthesis or degradation of existing chlorophyll (Ashraf and Harris, 2013) prompts structural changes in light harvesting complex, alters light fixation capacity and decreases photosynthetic efficiency (Duarte et al., 2013; Rabhi et al., 2012; Kocheva et al., 2004).

We noticed parallel decreases in stomatal conductance and transpiration but plant water use efficiency was maintained at high salinity. Stomatal closure to conserve water may result in low rates of photosynthesis. However, salinity treated plants of *D. bipinnata* showed turgor potential above the control levels (data not shown). Thus, reduced photosynthetic performance could not be attributed to disturbed water potential under high salinity. Instead, several studies exist that suggest diffusional limitations as a main cause of inhibited photosynthesis (Chaves et al., 2009; Pérez-López et al., 2012; Chen et al., 2015). An effective regulation of  $\text{CO}_2/\text{H}_2\text{O}$  gas

exchange is essential for survival of plants under stress conditions (Chaves et al., 2009). *WUE* of test species expressed an advantage for long-term survival under saline conditions especially at 100 mM NaCl as a high *WUE* is considered typical for  $C_4$  plants (Naidoo et al., 2012). *Desmostachya bipinnata* grown under high salinity maintained  $C_i$  but decreased the rate of photosynthesis. Our data is consistent with the paradigm that  $C_4$  plants do not suffer  $\text{CO}_2$  limitations due to efficient C uptake (Moinuddin et al., 2016; Koyro et al., 2013; Sage et al., 2012) and supports the hypothesis that “ $\text{CO}_2$  uptake will be similar to non-saline control at moderate salinity.” However, it disapproves our hypothesis that “high salinity will decrease photosynthesis due to stomatal limitation”. We suggest that the decline in net photosynthesis under salinity indicates non-stomatal (e.g., photochemical and biochemical) limitations of photosynthesis in our test species (Fig. 9).

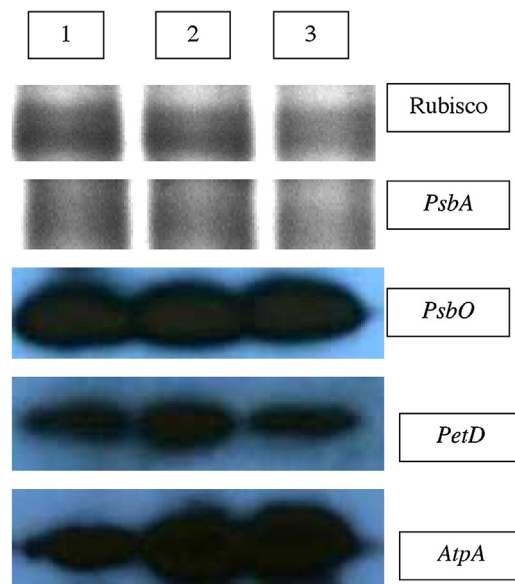
*Desmostachya bipinnata* showed a higher requirement for energy with increasing concentrations of NaCl in the media as indicated by an increase in compensation irradiance ( $I_c$ ). High salinity caused a reduction in photosynthetic machinery which can then be saturated by a small quantity of light (decreased level of  $I_s$ ); however, unutilized light by the photosystem may trigger photochemical damage (Athar and Ashraf, 2005). Our data is in agreement with several published reports (Eisa et al., 2012; Marín-Guirao et al., 2011; Shafer et al., 2011; Geissler et al., 2009). We observed an increase in non-photochemical quenching (*NPQ*),  $\Delta T$  and carotenoids content in 400 mM NaCl treated plants. Niyogi (1999) showed that carotenoid contributes in *NPQ* via the xanthophyll cycle. Increased levels of *NPQ* indicated the release of excessive light as heat to lessen the deleterious effects of salt on PSII (Duarte et al., 2013; Lambrev et al., 2012). It appears that this shift from light harnessing to light-protective mechanisms (e.g., reduction in chlorophyll while increases in carotenoids and *NPQ*) ensures survival of our test species under high salinity even with reduced carbon assimilation. However, continued exposure of the photosystems to excessive light ultimately compromised PSII performance as indicated by decreased *ETR* and *qP*. Our results are in agreement with some other studies (Pinheiro and Chaves, 2011; Lee et al., 2004). An increase in the *ETR/A\_g* ratio and restrictions in photochemical quenching during photosynthesis lead to release of excess electrons, which initiates ROS production in *D. bipinnata*. ROS induced lipid peroxidation (increase in *MDA* content) damages the integrity of plasma membranes and has other associated deleterious effects. Modifications in proteins of the PSII reaction center (e.g., *PsbA/D1* protein) and oxygen evolving complex (*OEC*) (e.g., *PsbO* protein) are known to enable PSII to cope with saline conditions (Pang et al., 2010; Askari et al., 2006). At moderate salinity, the level of *PsbO* remained unchanged and that of *D1* increased in *D. bipinnata*. The presence of these proteins prevented damage in PSII and hence increased photosynthetic efficiency. Reduced expression of *PsbO* and *D1* protein at high salinity reflects an alteration in the structure of *OEC* and also supports decreased *YII* and *qP*. Earlier studies indicated either an increase (Pang et al., 2010; Sengupta and Majumder, 2009) or unchanged expression of *PsbO* protein under salinity (Trotta et al., 2012). Findings of this study support our hypothesis that “high salinity will reduce photochemical reactions”.

Our results showed linear decreases in utilization of triose phosphate (TPU), maximum rate of Rubisco carboxylase activity ( $V_{c,max}$ ) and maximum rate of photosynthetic electron transport to regenerate Ribulose-1,5-bisphosphate ( $J_{max}$ ) with increasing salinity. Salinity in the medium reduces regeneration of  $P_i$  inhibiting synthesis of sucrose and starch as indicated by a reduced level of TPU (Long and Bernacchi, 2003). This reduction in starch production prevented growth stimulation at moderate salinity and caused growth inhibition at high salinity. We report here



**Fig. 7.** Effect of salinity on chlorophyll fluorescence was measured on leaves of *D. bipinnata*. Maximum quantum efficiency of PSII ( $F_v/F_m$ ), effective quantum yield of PSII ( $Y_{II}$ ), co-efficient of photochemical quenching ( $qp$ ) and non-photochemical quenching ( $NPQ$ ), electron transport rate ( $E_{TR}$ ) and temperature difference ( $\Delta T$ ) data representing the mean  $\pm$  S.E. of three replicates ( $n=3$ ). Different letters indicate significant difference due to salt treatments, according to Bonferroni's test ( $P < 0.05$ ). n.s. indicates no significant difference due to treatments.

salinity driven reduction in the amount of Rubisco (large subunit) in *D. bipinnata* that is considered as an important factor to influence its carboxylation capacity (Hu et al., 2013; Koyro et al., 2013). A decrease in  $V_{c,max}$  is also reported as a consequence of low  $\text{CO}_2$  (Long and Bernacchi, 2003). However, this is not the case for  $C_4$  plants as PEP-Carboxylase is functional to concentrate  $\text{CO}_2$  around Rubisco (Gowik and Westhoff, 2011). Khedr et al. (2011) demonstrated increased expression of PEP-carboxylase under saline conditions. Thus, unchanged levels of  $C_i$  in this study reinforce the need to highlight PEP-Carboxylase contribution in fixation of atmospheric  $\text{CO}_2$ . We observed a significant reduction in  $J_{max}$  at moderate salinity whereas the relative expression of  $cytb_6f$  complex (subunit IV: *PetD* protein) was increased. Proteins comprising cytochrome  $b_6f$  complex regulate proton gradient and electron flow between PSII and PSI (Choquet and Vallon, 2000) and alteration in abundance of these proteins under salt stress have been reported (Sobhanian et al., 2010; Xu et al., 2010). Our data indicates impaired electron transport of Calvin cycle at moderate salinity as  $E_{TR}$  affiliated with light reactions was found to be unchanged at moderate salinity. Moreover, PSII efficiency was not altered, which points towards a possibility that PSI instead of PSII is affected and therefore more research is needed to elucidate this mechanism. A decrease in  $J_{max}$  limited  $A$  as a consequence of



**Fig. 8.** Chloroplast proteins from leaves of *D. bipinnata* subjected to different NaCl concentrations. Rubisco (LSU) and *PsbA* are present on Coomassie blue-stained SDS-PAGE gels, while *PsbO*, *PetD* and *AtpA* are presented after immunoblotting with their specific antibodies. Lane 1–3 present 0, 100 and 400 mM NaCl, respectively.

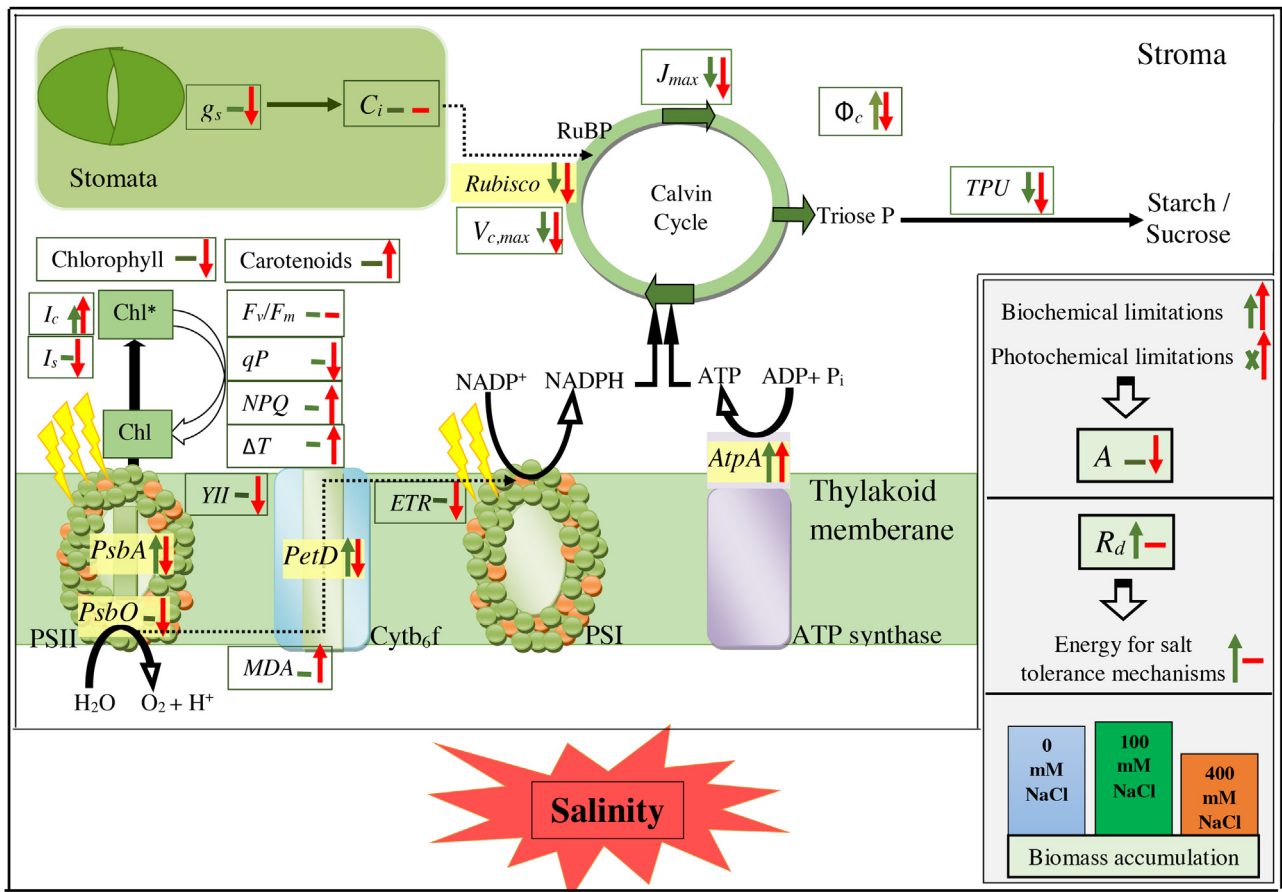
disturbed  $E_{TR}$  at high salinity.  $J_{max}$  reflects capacity to regenerate RuBP as indicated in other reports (Long and Bernacchi, 2003; Von Caemmerer, 2000). Disturbance in thylakoid reactions at high salinity was evident from hampered PSII activity and decreased expression of *PetD*. Any alteration in  $E_{TR}$  disturbs availability of electron acceptors (like  $\text{NADP}^+$ ) and utilization of ADP that ultimately limits RuBP regeneration (Lin et al., 2009). Therefore, based on the above assertions we can conclude that biochemical efficiency of photosynthetic apparatus under saline conditions was decreased due to co-limitation of  $V_{c,max}$ ,  $J_{max}$ , and  $TPU$ , verifying our hypothesis that “moderate salinity will cause limitations to photosynthesis due to biochemical reactions”.

*Desmostachya bipinnata* treated with moderate salinity showed a significant increase in the rate of dark respiration ( $R_d$ ), which may enhance consumption of carbohydrates for osmo-regulatory processes (Touchette, 2007). It is also proposed that increased  $R_d$  facilitates acquisition of more  $\text{CO}_2$  to attain a positive carbon balance (Hu et al., 2010). In addition, the regulation of the carbon fixation pathway ( $C_4$  metabolism) demands high respiratory rates to fulfill its high energy cost (Leegood, 2013; Sage, 2004). Levels of ATP synthase complex ( $\alpha$ -subunit: *AtpA* protein) were increased under saline conditions compared to control, however no difference was recorded between salinity treatments. This protein complex utilizes an electrochemical proton gradient from light dependent electron flow to synthesize ATP (Von Ballmoos et al., 2009). Increased accumulation of *AtpA* may enhance synthesis of ATP to meet increasing demands of energy for sustained salt

**Table 1**

Changes in the content of identified proteins in leaves of *D. bipinnata* under moderate and high salinity treatments following densitometric analysis. The values are given as % of control  $\pm$  S.E.

NaCl (mM)	Rubisco	<i>PsbA</i>	<i>PsbO</i>	<i>PetD</i>	<i>AtpA</i>
100	86 $\pm$ 5.4	103 $\pm$ 4.7	99.7 $\pm$ 2.7	138 $\pm$ 4.8	137 $\pm$ 7.3
400	74 $\pm$ 6.4	75 $\pm$ 3.7	81 $\pm$ 6.7	86 $\pm$ 5.0	138 $\pm$ 8.8



**Fig. 9.** Schematic presentation of salt induced alterations in photosynthetic performance of *D. bipinnata* subjected to moderate (100 mM) and high (400 mM) NaCl. Stomatal, biochemical and photochemical responses, and patterns of protein expression were represented by green and red colors (arrows/hyphens) for 100 mM and 400 mM NaCl, respectively. Responses determined in this study were presented in solid boxes. Analyzed proteins after integration to known locations were highlighted with yellow color. Arrows (up- or down- head) indicated increase or decrease in a response while hyphens indicated an unchanged response, in comparison to that of control plants. The length of the arrow increased with increasing response difference. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Abbreviations/symbols: stomatal conductance ( $g_s$ ), intercellular  $\text{CO}_2$  concentration ( $C_i$ ), dark respiration ( $R_d$ ), net photosynthesis ( $A$ ), fluorescence ( $F_v/F_m$ ), photochemical quenching ( $qP$ ), non-photochemical quenching ( $NPQ$ ), temperature difference between air and leaf ( $\Delta T$ ), compensation irradiance ( $I_c$ ), saturation irradiance ( $I_s$ ), effective quantum yield of PSII ( $Y_{II}$ ), electron transport rate ( $ETR$ ), malondialdehyde ( $MDA$ ), photosynthetic efficiency ( $\Phi_c$ ), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), maximum rate of Rubisco carboxylase activity ( $V_{c,max}$ ), ribulose-1, 5-bisphosphate (RuBP), inorganic phosphate ( $P_i$ ), maximum rate of photosynthetic electron transport to regenerate RuBP ( $J_{max}$ ), utilization of triose-phosphate to regenerate  $P_i$  ( $TPU$ ), adenosine triphosphate (ATP), adenosine diphosphate (ADP), nicotinamide adenine dinucleotide phosphate ( $\text{NADP}^+$ ), nicotinamide adenine dinucleotide phosphate-reduced ( $\text{NADPH}$ ), photosystem I (PSI), photosystem II (PSII), cytochrome  $b_6f$  complex ( $Cytb_6f$ ), D1 protein of PSII reaction center complex ( $PsbA$ ), manganese-stabilizing protein of oxygen evolving complex ( $PsbO$ ), subunit D of  $Cytb_6f$  complex ( $PetD$ ), and  $\alpha$  subunit protein of ATP-synthase complex ( $AtpA$ ).

resistance. Previous studies also documented a regulated expression of multiple subunits of this protein complex under salinity (Li et al., 2011; Yu et al., 2011). Differential expression of the investigated protein subunits i.e., *AtpA*, *D1*, *PsbO*, *PetD* and Rubisco LSU under salinity confirms our hypothesis that “high salinity will alter the expression of chloroplast membrane protein complexes”.

This study shows relative contribution of stomatal, photochemical and biochemical factors in limiting growth and photosynthetic performance of *D. bipinnata* under salinity. Fig. 9 compares salinity induced alterations in a schematic presentation. Results revealed that concentration of intercellular  $\text{CO}_2$  was unchanged under salinity and photosynthesis did not decline due to stomatal limitations. Plants treated with moderate salinity show growth and photosynthetic rates similar to controls by protecting photochemical reactions and thylakoid membrane protein complexes. An increase in respiratory rates exerts positive effects on plant growth

and metabolism by providing more energy. Photosynthetic efficiency of plants improves; however,  $A$  could not increase because of biochemical limitations. On the other hand, high salinity treatment significantly inhibits growth and photosynthetic performance of plants that seem to be associated with both photochemical and biochemical limitations. The reduced photosynthesis exposes plants to excessive irradiance and demands efficient photoprotective mechanisms. Increases in non-photochemical quenching and carotenoids content protect plants by dissipating excessive energy, but at the molecular level proteins comprising the photosynthetic apparatus are hampered. ROS are being produced damaging membrane integrity. In addition, respiratory rates are not sufficient to fulfill energetic demands and reduce the drastic effects of high concentrations of NaCl. These findings clearly indicate that *D. bipinnata* can be grown in areas of moderate salinity with optimal photosynthetic performance. We

realized that in addition to PSII dark reactions, PSI performance need to be investigated in order to gain new insights into the effects of salinity on photosynthesis.

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