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Calcium improves the leaf physiology of salt treated *Limonium stocksii*: A floriculture crop

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ABSTRACT

Calcium acts as a signaling molecule in many plants to improve resistance during unfavorable environments. *Limonium stocksii* (Boiss.) Kuntze seedlings were grown in 0 and 600 mmol L^{-1} NaCl (with and without additional 15 mmol L^{-1} Ca^{2+}) for 15 d. The effects of these treatments were studied on plant growth, leaf water relations, malondialdehyde (MDA) content, ion-flux, Na^+ secretion rate and photosynthesis. Plant biomass declined by 50% and MDA content increased by 50% in plants treated with 600 mmol L^{-1} NaCl for 15 d. Leaf water content (WC_{FM}) and relative water content declined and sap osmolality increased after 3 d of 600 mmol L^{-1} NaCl treatment. Leaf Na^+, Na^+/K^+ ratios and Na^+ secretion rate were increased in salinity treatment. Decrease in photosynthesis was coupled with lower stomatal conductance and intercellular CO_2, however, instantaneous water use efficiency was improved under salinity treatment. Efficiency of PSII, relative electron transport rate, photochemical quenching and non-photochemical quenching were reduced by salinity treatment compared to non-saline control. The Ca^{2+} application yielded 25% higher fresh mass at enhanced water contents while lowers tissue osmotic potential and membrane damage in plants exposed to high salinity. The Na^+ accumulation in the leaf was reduced, while the secretion was increased in the presence of additional Ca^{2+}. However, Ca^{2+} application did not improve either the photosynthetic gas exchange or light reactions of photosynthesis under saline conditions. Our results indicated that *L. stocksii* decreases its growth, water content, photosynthesis and dark respiration under increased leaf Na^+ concentrations. Whereas, application of Ca^{2+} enhanced plant salinity resistance by improving water balance, Na^+-secretion and membrane integrity.

1. Introduction

Success in the floriculture industry is largely based on the availability of new and attractive ornamental plant species (Morgan and Funnell, 2018). Due to the primary importance of their external appearance, ornamentals are traditionally irrigated with the highest quality water, as salinity usually affects their yield and quality (Niuet al., 2019). Soil salinization is one of the major environmental problems affecting plant productivity all over the world. About 10% of the total arable land was affected by salinity and sodicity and causing a loss of 1.5 million hectares per year, resulting in economic penalties worth billions of dollars (Shahid et al., 2018). Niu et al. (2019) noted that salinity could cause adverse effects on ornamental plants. A number of floricultural glycophytes were tested for their salt tolerance and the possibility of irrigating them with low quality brackish water (García-Caparrós et al., 2016).

The application of halophytic plants for landscaping and ornamental purposes seems to be a very attractive proposition for the economic utilization of saline soil (Ventura et al., 2015). Halophytes have various adaptive strategies when exposed to salinity such as the ability i) to relocate more energy towards osmotic adjustment, membrane transport and cellular defense mechanisms rather than growth, ii) for maintaining nutrient balance, iii) to minimize sodium toxicity in cytoplasm by exclusion from root, by vacuolar sequestration, and/or secretion with the help of salt glands and iv) to regulate CO_2 assimilation rate by stomatal closure and down regulating light harvesting mechanisms (Munns et al., 2020; Van Zelm, 2020).

Limonium species are known for high salt resistance and attractive flowers (Morgan and Funnell, 2018). *Limonium stocksii* (Boiss.) Kuntze (Plumbaginaceae), a salt secreting C3 perennial, is a good ornamental candidate for dry saline areas (Hameed et al., 2015). The evergreen

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L. stocksii is distributed along the coastal areas of Pakistan and India and produces beautiful flowers twice a year (June and November) despite lack of rainfall over consecutive years (*personal observation*). Profit margins could be substantially enhanced if *L. stocksii* could be grown using seawater and/or on saline land. Previously, reported that *L. stocksii* could survive salinity up to seawater concentrations by altering growth and ion transport (Zia et al., 2008; Hameed et al., 2015). However, the effect of salinity on photosynthesis, water relation and ion secretion mechanisms of plants of this species are still not well known.

Application of Calcium (Ca^{2+}) may ameliorate plant growth under salinity by acting as a signaling molecule, by participating in various developmental processes, and in photosynthesis, mineral nutrition and water transport (Pathak et al., 2020). Ca^{2+} is reported to counter the osmotic imbalance by the regulation of aquaporin function, stomatal conductance and, thus, transpiration (Gilliham et al., 2011; Guo et al., 2018). Cytoplasmic Ca^{2+} may control ion transport during salinity exposure and alleviates Na^+ toxicity in a variety of plant species (Pathak et al., 2020). It has also been suggested to restrict the entry of Na^+ through non-selective cation channels and inhibit K^+ loss from the cell by regulating K^+ efflux channels (Shabala et al., 2016). Consequently, the involvement of Ca^{2+} in the development of salt glands and ions secretion was previously reported (Ding et al., 2010).

Salt tolerance of *L. stocksii* was reported by Hameed et al. (2015), however, no information is available on the role of Ca^{2+} application in enhancing its salt tolerance. The specific focus of the present study was to evaluate the role of Ca^{2+} application on growth, water relations, ion homeostasis and photosynthesis of *L. stocksii* under saline conditions. Therefore, experiments were designed to test the following hypotheses: Ca^{2+} application will alleviate deleterious effects of salinity on growth of *L. stocksii* by reducing the osmotic stress and ionic toxicity and by improving photosynthesis under salinity.

2. Material and methods

2.1. Growth conditions

Seeds of *L. stocksii* were collected from saline areas spread between Manora Creek and Hawks Bay, Karachi, Pakistan (24° 52–647′N and 66° 53–321′E). Seeds were separated from the inflorescence, surface sterilized using Clorox (0.85% sodium hypochlorite) and stored dry at 4 °C. Growth experiments were performed in a greenhouse under ambient atmospheric conditions [temperature: 30 ± 2 °C; relative humidity: 55–65%; photoperiod 12 h day-night at 500–550 μmol m^{-2} s^{-1} photosynthetic photon flux rate (PPFR)]. Seeds were germinated in plastic trays filled with sandy clay soil. Healthy seedlings were transplanted in plastic pots (35 cm length, 11 cm diameter; three plants per pot) and irrigated with nutrient solution (half strength Hoagland solution; Epstein, 1972).

Experiment was started after 15 d of seedling acclimatization. It was based on three factors, 1) salinity (0 and 600 mmol L^{-1} NaCl), 2) calcium treatments (without and with additional 15 mmol L^{-1} $CaCl_2$), and 3) time intervals (0, 7 and 15 d). Therefore, seedlings were divided into four groups (0, 600 mmol L^{-1} NaCl -without additional Ca^{2+}; 0, 600 mmol L^{-1} NaCl -with additional 15 mmol L^{-1} Ca^{2+}; 75 seedlings per group), which were further distributed into three sub-groups (0, 7 and 15 days; 25 seedlings per sub-group). Seedlings of the non-saline groups (0 mmol L^{-1} NaCl) were irrigated only with nutrient solution, whereas, for salinity treated plants (600 mmol L^{-1} NaCl), salt concentration was stepwise increased by adding 50 mmol L^{-1} NaCl per day until the final concentration were reached.

2.2. Plant harvest, growth parameters and water relations

Leaf fresh mass (FM) and dry mass (DM) was recorded while fresh mass based leaf water content (WC_{FM}) was calculated using the equation: WC_{FM} = (FM - DM) / FM × 100. Leaf turgid mass (TM) was recorded after rehydration of fresh leaves in distilled water for 24 h at room temperature (~25 °C). Relative water content (RWC) of the leaves was calculated using the equation: RWC = (FM - DM) / (TM - DM) × 100

Pressed sap of fresh leaf material was obtained using MARKHART leaf press (LP 27; Wescor Inc., Logan, UT, USA), and used to determine osmolality with a vapor pressure osmometer (VAPRO-5520; Wescor Inc., Logan, UT, USA) (Gucci et al., 1991).

2.3. Lipid peroxidation

The oxidative degradation of lipids in leaf samples was estimated in terms of malondialdehyde (MDA) content (Heath and Packer 1968). Fresh leaf material (0.1 g) was homogenized with 1 mL trichloro-acetic acid (1% TCA) and centrifuged at 10,000 x *g* at 4 °C for 5 min (Sorval Evolution RC, Kendro, Newtown, CT, USA). The supernatant (1 mL) was mixed with 4 mL of 20% trichloro-acetic acid containing 0.5% 2-thiobarbituric acid and heated in a boiling water bath for 30 min. The mixture was cooled quickly in an ice bath and centrifuged at 10,000 x *g* for 10 min at 4 °C. The absorbance of the supernatant was read at 532 and 600 nm. The concentration of MDA was calculated using the extinction coefficient of 1.56 L mol^{-1} cm^{-1} and data expressed as μmol g^{-1}.

2.4. Cation content (Na^+ and K^+)

Cations (Na^+ and K^+) were measured in root and leaf tissues by using hot water extract method (Shoukat et al., 2020). The samples were oven-dried at 60 °C for 48 h then ground by ball milling, homogenized and hot-water extracts prepared with distilled water at 80 °C for 4 h. The extract was filtered and diluted with distilled water for measuring cations by atomic absorption spectrometry (AA-700; Perkin Elmer, Santa Clara, CA, USA).

2.5. Rate of Na^+ secretion

To determine the rate of Na^+ secretion, leaves were rinsed with 2 mL de-ionized water in Eppendorf tubes (Eppendorf, San Diego, CA, USA). Fully expanded young leaves of three plantlets were tagged from each treatment. Tagged leaves of each treatment were prewashed three days before the end of both experiments. The amount of secreted Na^+ was determined by atomic absorption spectrometer (AA-700; Perkin Elmer) and the area of rinsed leaves was determined by ImageJ software ver. 1.45 (http://rsb.info.nih.gov/ij/, accessed 10 September 2010). The rate of Na^+ secretion was expressed in mmol m^{-2} d^{-1}.

2.6. Leaf gas exchange, chlorophyll fluorescence and chlorophyll content

Leaf gas exchange was measured by portable infrared CO_2/H_2O gas exchange cuvette system LI-COR 6400XT (LI-COR, Lincoln, NE, USA) with CO_2 concentrations, flow rate and PPFR set to 400 ppm, 300 mL min^{-1} and 600 μmol m^{-2} s^{-1}, respectively. Net photosynthetic rate (P_N), dark respiration (Rd), transpiration (E), stomatal conductance (Gs) and intercellular carbon dioxide concentration (Ci) were estimated based on the measured CO_2 and water vapor exchange on the youngest fully emerged leaves. Intrinsic water use efficiency was calculated as $_{int}$WUE = PN/Gs. Relative chlorophyll content was analyzed by SPAD 502 (Konica Minolta, Inc., Tokyo, Japan).

Rapid light curves were measured with a pulse amplitude modulated chlorophyll fluorometer (PAM 2500, Heinz Walz GmbH, Effeltrich, Germany). Each rapid light curve exposed the leaf to seven incremental steps of irradiance ranging from 0 to 1330 μmol m^2 s^{-1} PPFR. Leaf temperature and PPFR was automatically measured by the calibrated PAM. The potential maximum photochemical quantum yield of PSII [Fv/Fm = (Fm-Fo)/Fm] was measured on dark adapted (approx. 25 min) leaves according to Kitajima and Butler (1975), where Fo denotes the minimum fluorescence at low modulated light (< 0.1 μmol m^{-2} s^{-1}) and Fm the maximal fluorescence signal at saturating light (10,000 μmol

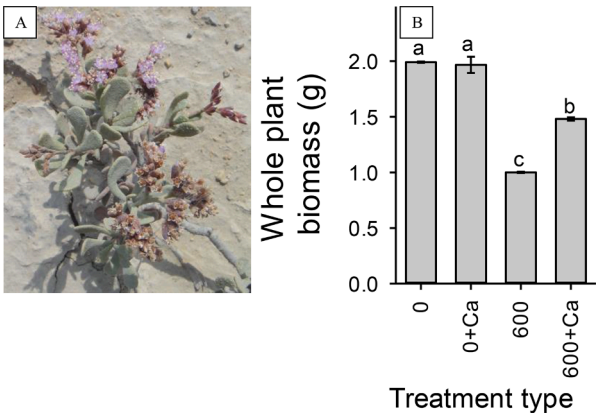

Fig. 1. *Limonium stocksii* (A) plant in natural field condition, (B) effect of salinity (0 and 600 mmol L^{-1} NaCl) and Ca^{2+}treatments (0 and 15 mmol L^{-1}) on means (± SE; $n = 4$) of whole plant fresh biomass after 15 d of experiment. Values with similar Bonferroni letters were not significantly different at $P < 0.05$ (left panel).

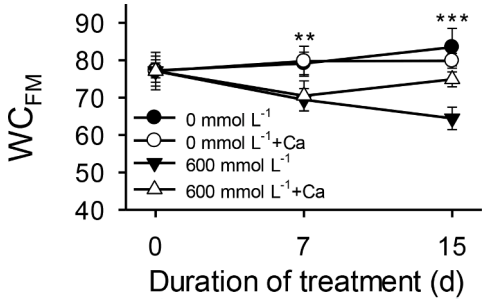

Fig. 2. Effect of salinity (0 and 600 mmol L^{-1} NaCl), Ca^{2+} treatments (0 and 15 mmol L^{-1}) and time interval (0, 7 and 15 d) on means (± SE; $n = 4$) of fresh mass based leaf water content (WC_{FM}) of *Limonium stocksii*. The asterisks indicate the significance of difference between means at the respective measurement days. (** = $P < 0.01$ and *** = $P < 0.001$).

m^{-2} s^{-1}, for 0.6 s). The effective photochemical quantum yield of PSII [Y (II)= F_m'-F_s/F_m'] and non-photochemical quenching (NPQ = F_m/F_m'−1) were calculated according to Genty et al. (1989) and Bilger and Björkman (1990), respectively, where F_s denotes the instantaneous

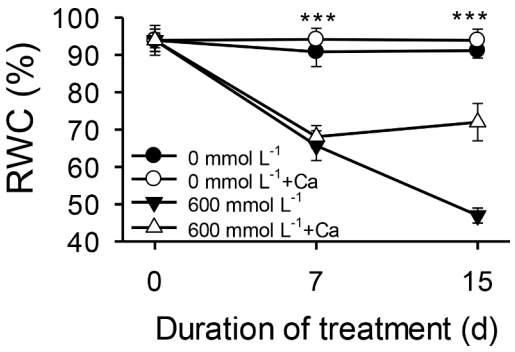

Fig. 3. Effect of salinity (0 and 600 mmol L^{-1} NaCl), Ca^{2+} treatments (0 and 15 mmol L^{-1}) and time interval (0, 7 and 15 d) on means (± SE; $n = 4$) of leaf relative water content (RWC) of *Limonium stocksii*. The asterisks indicate the significance of difference between means at the respective measurement. (*** = $P < 0.001$).

fluorescence signal just before a saturating pulse and F_m' is the respective maximum fluorescence signal of the illuminated leaf induced by a saturating light pulse. The relative fluorescence yield parameters [Y(II)], quantum yield of regulated non-photochemical energy loss in PSII [Y (NPQ) = (Fs/Fm') - (Fs/Fm)] and quantum yield of non-regulated non-photochemical energy loss in PSII [Y(NO)] = Fs/Fm] were calculated at PPFR 600 μmol m^{-2} s^{-1} according to Kramer et al. (2004). Photochemical quenching (qP) was calculated as (F_m'-F_s) / (F_m'-F_o') (Bilger et al., 1995). Relative electron transport rate (rETR; Krall and Edwards, 1992) was estimated as rETR = PPFR * YII * 0.5 * 0.84, where PPFR is the photosynthetic photon flux rate; 0.5 reflects the energy distribution between PSI and PSII assuming to be equal while 0.84 estimates a mean absorption coefficient.

2.7. Statistical analysis

Three-way analysis of variance (ANOVA) was used to test for significant effects of salinity, calcium, days of salinity treatment and their interactions on leaf water relations, ion content and photosynthetic parameters of *Limonium stocksii*. Statistical analysis was carried out by SPSS Statistics for Windows, version 16. 0 (SPSS Inc., Chicago, Ill., USA) for all parameters in this study ($P < 0.05$; $n = 4$; experimental design = CRD type). The Post-hoc Bonferroni's test was also used to evaluate significant differences between individual treatment means.

Table 1
Three-way ANOVA for parameters related to water relations, membrane damage, ion content, Na^+ secretion rate and leaf gas exchange in *Limonium stocksii* due to salinity (S), calcium (Ca), time (T) and their interactions.

ANOVA	Salinity (S)	Calcium (Ca)	Time (T)	S*Ca	S*T	Ca*T	S*Ca*T
Water relations and membrane damage							
WC_{FM}	56.02***	5.47*	6.46*	12.70**	16.08***	2.91	9.00**
RWC	296.45***	136.32***	156.50***	171.00**	191.27***	76.15***	185.00**
OP	29,735.37***	330.76***	89.76***	194.68***	63.84***	85.54***	92.76***
MDA	225.21***	32.22***	71.96***	14.33**	85.20***	19.27***	10.32**
Ion content and Na^+ secretion rate							
Na^+	1427.45***	13.89**	382.28***	28.65***	359.72***	7.33**	12.06***
K^+	7.60*	0.79	2.37	0.07	2.03	0.40	0.06
Na^+/K^+	406.99***	11.43**	96.82***	12.08**	101.88***	2.96	4.35*
Na^+ secretion	99.17***	199.95***	–	–	–	–	–
Leaf gas exchange							
P_N	56.53***	0.23	0.31	1.96	0.62	1.17	2.99
G_S	38.37***	0.02	3.24	0.35	3.72	2.38	2.56
Ci	1.24	0.05	0.06	0.13	1.77	2.51	0.56
E	84.31***	0.46	0.05	3.14	0.36	4.46	6.26
Ci/Ca	3.19	0.06	0.15	0.06	2.21	2.90	0.38
Rd	93.97***	1.71	6.61*	13.94**	9.89*	8.49*	11.31**
$_{int}$WUE	3.47	0.09	0.01	0.03	2.20	2.88	0.42
SPAD	2.18	2.02	0.18	2.02	0.18	4.10	1.39

(* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

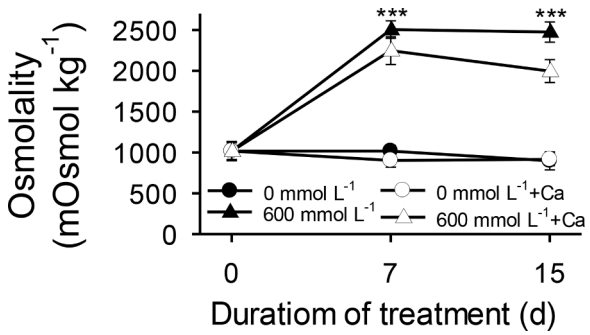

Fig. 4. Effect of salinity (0 and 600 mmol L^{-1} NaCl), Ca^{2+} treatments (0 and 15 mmol L^{-1}) and time interval (0, 7 and 15 d) on means (± SE; $n = 4$) of leaf osmolality of *Limonium stocksii*. The asterisks indicate the significance of difference between means at the respective measurement. (*** = $P < 0.001$).

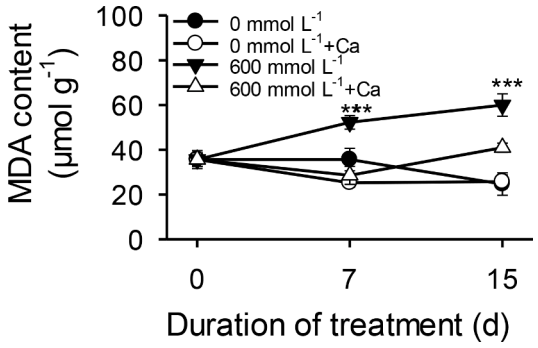

Fig. 5. Effect of salinity (0 and 600 mmol L^{-1} NaCl), Ca^{2+}treatments (0 and 15 mmol L^{-1}) and time interval (0, 7 and 15 d) on means (± SE; $n = 4$) of malondialdehyde content of *Limonium stocksii*. The asterisks indicate the significance of difference between means at the respective measurement. (*** = $P < 0.001$).

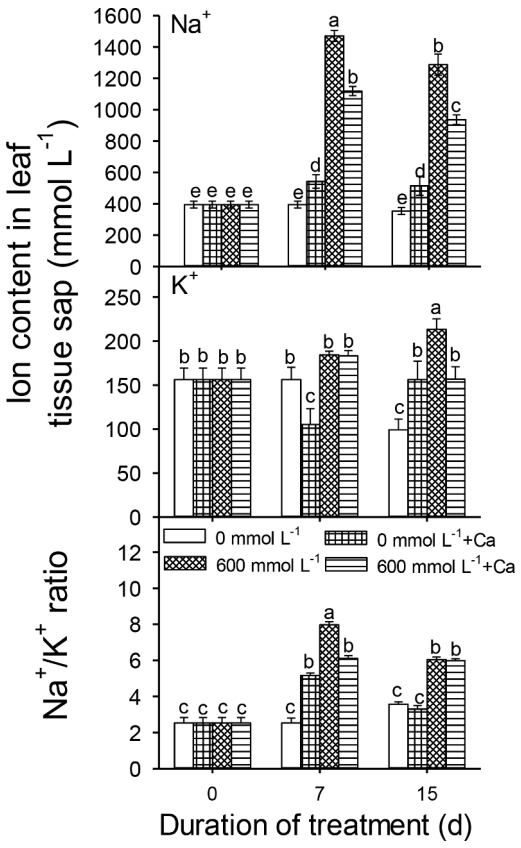

Fig. 6. Effect of salinity (0 and 600 mmol L^{-1} NaCl), Ca^{2+} treatments (0 and 15 mmol L^{-1}) and time interval (0, 7 and 15 d) on means (± SE; $n = 4$) of leaf ion (Na^+ and K^+) content and Na^+/K^+ ratio of *Limonium stocksii*. Values with similar Bonferroni letters at each salinity concentration were not significantly different at $P < 0.05$.

3. Results

3.1. Plant growth, leaf water relations and membrane damage

Salinity treatment significantly decreased plant biomass and growth was approx. 50% lower in plants cultivated at 600 mmol L^{-1} NaCl than in controls (Fig. 1). Whereas, the application of Ca^{2+} improved plant fresh mass by approx. 50% (Fig. 1) compared to plants grown without Ca^{2+} at the same salinity (600 mmol L^{-1}).

Leaf WC_{FM} of salinity grown plants declined after 7 d of NaCl treatment and was 15% lower than that of non-saline grown controls after 15 d (Fig. 2; Table 1). Leaf RWC decreased after 3 d of the NaCl treatment, and was 40% lower than that of controls at final harvest (Fig. 3; Table 1). Application of Ca^{2+} considerably enhanced WC_{FM} and RWC of leaf tissue in salinity when compared to the salt treatments without Ca^{2+} application (Figs. 2 and 3; Table 1).

Salinity treatment resulted in increased leaf sap osmolality, which was highest at the end of experiment (Fig. 4). However, Ca^{2+} application alleviated the effects of salinity on leaf osmolality (Fig. 4).

Salinity treatment leads to increase in leaf MDA content (Fig. 5; Table 1). However, Ca^{2+} application alleviated the effects of salinity on MDA content.

3.2. Leaf ion content and Na^+ secretion rate

Salinity treatment leads to an increase in leaf Na^+, K^+ content and Na^+/K^+ ratios (Fig. 6; Table 1). However, Ca^{2+} application alleviated leaf Na^+ and K^+ in salinity treated plants, thereby maintaining Na^+/K^+ ratio at final harvest.

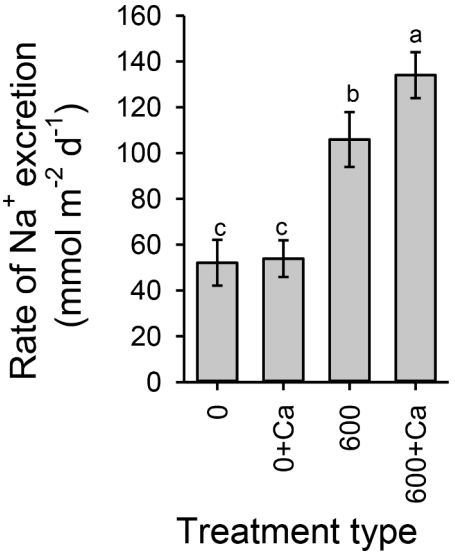

Fig. 7. Effect of salinity (0 and 600 mmol L^{-1} NaCl) and Ca^{2+} treatments (0 and 15 mmol L^{-1}) on means (± SE; $n = 4$) of sodium secretion rate of *Limonium stocksii* after 15 d of experiment. Values with similar Bonferroni letters were not significantly different at $P < 0.05$.

Table 2
Effect of salinity and Ca^{2+} application on net-photosynthesis (P_N), dark respiration (Rd), stomatal conductance (G_S), inter-cellular CO_2 (Ci), transpiration (E), inter-cellular / ambient CO_2 (Ci/Ca), intrinsic water use efficiency ($_{int}$WUE) and relative chlorophyll content (SPAD) of *Limonium stocksii* plants at different duration (7 and 15 d) of experiment. Values (means ± SE; $n = 4$) with similar Bonferroni letters were not significantly different at $P < 0.05$.

Days	NaCl (mmol L^{-1})	P_N (µmol CO_2 m^{-2} s^{-1})	Rd (µmol m^{-2} s^{-1})	G_S (mol H_2O m^{-2} s^{-1})	Ci (µmol CO_2 mol^{-1} air)	E (mmol H_2O m^{-2} s^{-1})	Ci/Ca	$_{int}$WUE (µmol CO_2 mol^{-1} $_{H2O}$)	SPAD
With calcium									
7d	0	12.3 ± 1.2a	−7.3 ± 0.9a	0.17±0.02a	229±05a	5.6 ± 0.7a	0.6 ± 0.0a	73±01b	59.2a
	0Ca	6.7 ± 1.3b	−3.1 ± 0.6b	0.07±0.01b	210±22a	2.8 ± 0.4b	0.6 ± 0.1a	93±12a	60.4a
	600	0.6 ± 0.2b	−2.2 ± 0.2b	0.01±0.00b	265±24a	0.4 ± 0.0b	0.7 ± 0.1a	65±15b	69.6a
	600Ca	2.4 ± 0.2a	−3.3 ± 0.4a	0.02±0.00a	186±04b	1.0 ± 0.1a	0.5 ± 0.0b	114±03a	59.2a
15d	0	10.6 ± 3.2a	−7.1 ± 0.6a	0.18±0.07a	249±01b	3.9 ± 1.0a	0.7 ± 0.0b	64±06a	59.2a
	0Ca	11.7 ± 1.3a	−7.5 ± 0.1a	0.24±0.03a	276±02a	4.8 ± 0.4a	0.8 ± 0.0a	48±01a	63.4a
	600	1.1 ± 0.3a	−2.2 ± 0.0a	0.01±0.00a	172±68a	0.3 ± 0.0b	0.4 ± 0.2a	130±43a	59.7a
	600Ca	1.4 ± 0.1a	−2.9 ± 0.3a	0.01±0.00a	220±10a	0.6 ± 0.1a	0.6 ± 0.0a	98±07a	65.5a

Salinity treatment resulted in high Na^+ secretion rate which was further increased by Ca^{2+} treatment (Fig. 7; Table 1).

3.3. Leaf gas exchange and chlorophyll fluorescence

Salinity treatment resulted in early decrease of gas exchange parameters (P_N, Rd, Gs and E) whereas, Ci decreased and $_{Int}$WUE increased at the end of the experiment (Table 2). However, Ca^{2+} treatment ameliorated salinity effects on gas exchange parameters only at 7 d of experiment. There was no change in leaf SPAD values throughout the experiment (Table 2).

The maximum photochemical efficiency in dark adapted condition (Fv/Fm) remained unaffected during the experiment (data not shown). Under saturating light, photochemical quenching (qP), which indicates the proportion of electrons going towards photochemistry, decreased under salinity (Fig. 8), whereas rETR was about two fold lower in salinity-treated plants than in controls (Fig. 8). NPQ as well as the corresponding leaf temperatures declined with the increase in salinity (Fig. 8). The relative yield parameters of Y(II) and Y(NPQ) decreased while Y(NO) increased with increase in salinity from 0 to 600 mmol L^{-1} NaCl (Table 3). Application of Ca^{2+} had similar effects under non-saline and saline treatments. In general, application of Ca^{2+} did not improve chlorophyll fluorescence parameters (Fig. 8; Table 3).

4. Discussion

Salinity is known to decrease the availability, influx, and transport of Ca^{2+} in the plant, while supplemental Ca^{2+} may help in alleviating salinity induced damages (Pathak et al., 2020; Ding et al., 2010). This study was performed to understand the role of additional Ca^{2+} in alleviating the inhibitory effect of salinity on growth and leaf physiology of *Limonium stocksii* - a potential horticultural plant.

4.1. Effects of Ca^{2+} on plant growth and leaf water relations of L. stocksii under saline conditions

Plant biomass is one of the most direct indicators of salt tolerance. In this study, *Limonium stocksii* appeared to be highly salt tolerant (up to 600 mmol L^{-1} NaCl) but with reduced growth. A 50% decrease in shoot length, but not in root length was reported for *L. stocksii* plants grown at 60 dS m^{-1} of either NaCl or sea-salt treatments (Zia et al., 2008; Hameed et al., 2015). In the present study, substantial growth reduction for survival at 600 mmol L^{-1} NaCl treatment could indicate higher energy allocation to various salinity resistance mechanisms (Munns et al., 2020). Adjusting a high root/shoot ratio could be an effective strategy of plant growing under harsh salinity conditions to maintain water and nutrient uptake and most of all, decrease transpirational water losses (Nackley and Kim, 2015). Decrease in leaf WC_{FM} and RWC of *L. stocksii*, from early phase (3 d) at 600 mmol L^{-1} NaCl treatment, indicated osmotic stress. In addition, high water saturation deficit (inverse of RWC), indicated turgor loss which reduced stomatal conductance, thereby restricting water vapor release and CO_2 uptake. Reduced growth of *L. stocksii* due to decreased CO_2 uptake could help to maintain plant water balance for long term survival under unfavorable conditions. Among different markers of osmotic stress, an increase in leaf osmolality indicates osmotic adjustment under saline conditions (Munns et al., 2020). In our study, leaf osmolality of 600 mmol L^{-1} NaCl treated plants was 5-folds higher than control, apparently by about 50% osmotic contribution of Na^+ (calculated on dry weight basis), although this calculation was not corrected for symplastic water volume. The major osmotic contribution of inorganic solutes (Na^+ and K^+ was approx. 55%) in case of *L. stocksii*, is a common characteristic of euhalophytes, which may account for lower metabolic cost towards osmolyte production (Munns et al., 2020). The contribution of cat- and anions must be approximately proportional for charge compensation (Munns and Tester, 2008). The major contribution of inorganic solutes such as Na^+ and Cl^- may also simply result form an incomplete ability of many halophytes to exclude these ions from shoots. The accumulation of compatible solutes (amino acids, tertiary sulphonium compounds, quaternary ammonium compounds, sugars and polyhydric alcohols) certainly does not contribute much to the overall osmotic adjustment (Al Hassan et al., 2017). The decline in RWC appears to reflect a reduced ability of *L. stocksii* to take up water from the soil due to its largely decreased water potential as a result of salinity. This may have directly, or indirectly, reduced stomatal conductance (low Gs) and hence CO_2 availability. Increased MDA in *L. stocksii* under high salinity treatment, could be associated with an insufficient antioxidant defense system (Hameed et al., 2015), which could in turn reduce bio-membrane stability.

Ca^{2+} application appears to stabilize membranes and proteins (reduced membrane damage) in *L. stocksii*, thus preventing a reduction in tissue water (as decline in water saturation deficit at increase WC_{FM}

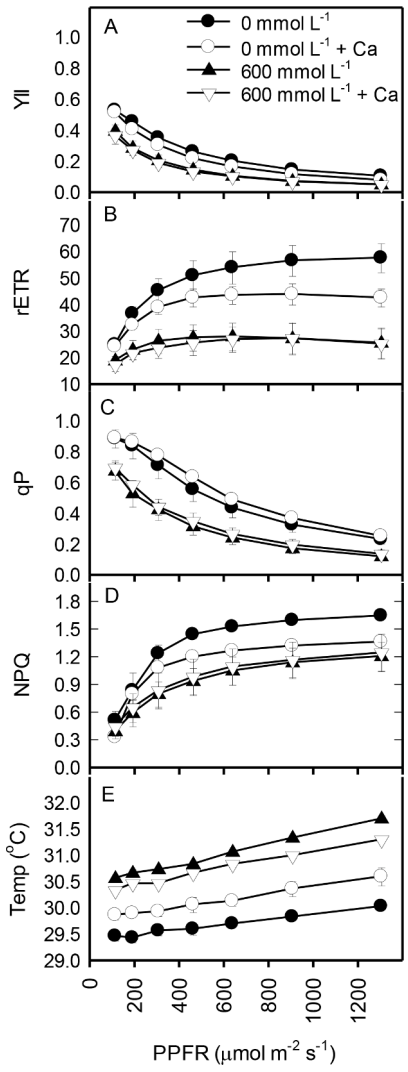

Fig. 8. Rapid light curves of (A) effective photochemical quantum yield of photosystem II (YII), (B) relative electron transport (rETR), (C) photochemical quenching (qP), (D) non-photochemical quenching (NPQ), and (E) leaf temperature of *Limonium stocksii* plants treated with 0 (circles) and 600 NaCl (triangles) after 15 d treatment ($n = 4$) without (black colored) and with addition of Ca^{2+} (white colored).

Table 3
Effect of salinity and Ca^{2+} application on actual quantum yields of PSII: Y(II); regulated Y(NPQ) and non-regulated Y(NO) non-photochemical energy dissipation (calculated at PPFR 600 µmol m^{-2} s^{-1}) of *Limonium stocksii* plants after 15 d of experiment. Values (means ± SE; $n = 4$) with similar Bonferroni letters were not significantly different at $P < 0.05$.

NaCl (mmol L^{-1})	Y(II)	Y(NPQ)	Y(NO)
0	0.26±0.03a	0.44±0.02a	0.30±0.01a
0Ca	0.22±0.02a	0.43±0.01a	0.36±0.01a
600	0.15±0.03a	0.41±0.04a	0.45±0.03a
600Ca	0.13±0.02a	0.42±0.04a	0.45±0.06a

and RWC) and enhance growth under salinity. Improved growth and water relations in Ca^{2+}-treated plant at 600 mmol L^{-1} NaCl could probably be due to an increase in the membrane water permeability and membrane integrity (Nedjimi and Daoud, 2009). Additional calcium prevented further increase in the MDA content of *L. stocksii* under salinity, which indicates an improvement in the antioxidant defense system as reported for other halophytes (Amor et al., 2010; Xu et al., 2017).

4.2. *Effects of Ca^{2+} on Na^+-flux and K^+-homeostasis in leaf tissue of L. stocksii under salinity*

Limonium stocksii maintained leaf K^+ concentration under long term (15 d) salinity exposure. Whereas, a substantial increase in leaf Na^+ indicates that the plants cannot effectively exclude Na^+ at root tissue, which is in agreement with the previously reported information about salt secreting dicotyledonous halophytes (Al Hassan et al., 2017). In addition, increase in Na^+/K^+ ratio exclusively was due to changes in Na^+ concentrations. Application of Ca^{2+} increased Na^+ secretion rate (around 15%), which could be one reasons to reduce leaf Na^+ and salt-stress damages in *L. stocksii.*

4.3. *Effects of Ca^{2+} on leaf gas exchange and photochemistry of L. stocksii under salinity*

Salinity affects photosynthesis through stomatal and non-stomatal limitation (Hasanuzzaman et al., 2018), most sub-tropical halophytes appear to display stomatal limitations under saline conditions (Moinuddin et al., 2017). Similarly, in this study increasing salinity lowered photosynthetic rates due to stomatal rather than biochemical limitation of photosynthesis (Ahmed et al., 2013; Moinuddin et al., 2017).

Various cellular components are sources of reactive oxygen species in plants under stress but chloroplasts are the most important of these sources (García-Caparrós et al., 2019). Halophytes such as the single-cell C_4 plant *Bienertia sinuspersici* reduce photosynthetic CO_2 fixation and leaf chlorophyll content but not growth under saline conditions to avoid light energy in excess of that required for photochemistry (Leisner et al., 2010). Carbon fixation reactions of photosynthesis are more sensitive to salinity than the light reactions resulting in ROS generation and therefore the need for alternative electron sinks for dissipating excess photon energy (Maricle et al., 2007; Moinuddin et al., 2017; Rasool et al., 2019). *Limonium stocksii*, a C_3 halophyte appeared to down regulate its photochemistry to avoid oxidative burst, although SPAD values showed little change in chlorophyll content, indicating the possibility of cytosolic processes including photorespiration as a source of reactive oxygen species.

External application of Ca^{2+} partially eased the gas exchange parameters of *L. stocksii*, only in the early phase of salt treatment possibly due to high $_{int}$WUE (Smith and Shortle, 2013). However, Ca^{2+} could not improve the effective photochemical efficiency under saline condition as reported in literature (Murillo-Amador et al., 2006).

5. Conclusions

Limonium stocksii appears to survive in high salinity (600 mmol L^{-1} NaCl; equal to sea water salinity) at the cost of plant growth due to decrease in WC_{FM}, photosynthesis and dark respiration along with increase in leaf Na^+ and energy demand. Whereas, application of Ca^{2+} enhanced salinity resistance and plant biomass by improving water balance, Na^+-secretion and membrane integrity. On the basis of our findings, we can suggest that *L. stocksii* can be cultivated in saline habitats.

CRediT authorship contribution statement

Muhammad Zaheer Ahmed: Conceptualization, Data curtion, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing. **Tabassum Hussain:** Data curtion, Methodology, Writing - original draft. **Salman Gulzar:** Data curtion, Methodology, Investigation, Writing - review & editing. **Muhammad Yousuf Adnan:** Formal analysis, Methodology, Writing - original draft. **Muhammad Ajmal Khan:**

Conceptualization, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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