DOI: 10.1007/s11099-016-0234-6 PHOTOSYNTHETICA 55 (1): 96-106, 2017

Effect of NaCl-induced changes in growth, photosynthetic characteristics, water status and enzymatic antioxidant system of *Calligonum caput-medusae* seedlings

Y. LU[*,**,#], J.-Q. LEI[*,**,#], F.-J. ZENG[*,**,#,+], B. ZHANG[*,**,#], G.-J. LIU[*,**,#], B. LIU[*,**,#], X.-Y. LI[*,**,#]

State Key Laboratory of Desert and Oasis Ecology, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, 830011 Urumqi, China[*]
Cele National Station of Observation and Research for Desert-Grassland Ecosystems, 848300 Cele, China[**]
Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, 830011 Urumqi, China[#]

Abstract

Calligonum caput-medusae is known to grow well when irrigated with water containing NaCl. The aim of this study was to investigate ecophysiological responses of *C. caput-medusae* to different NaCl concentrations. In our study, we examined the effect of 0, 50, 100, 200, and 400 mM NaCl. Our results demonstrated that maximum seedling growth occurred at 50 mM NaCl. Photosynthetic parameters, such as the photosynthetic pigment content and gas exchange parameters, correlated with growth response. High salinity (≥100 mM NaCl) resulted in a significant reduction of the plant growth. Similarly, marked declines in the pigment content, maximal efficiency of PSII photochemistry, net photosynthetic rate, transpiration rate, and stomatal conductance were also detected. However, intercellular CO_2 concentration showed a biphasic response, decreasing with water containing less than 200 mM NaCl and increasing with NaCl concentration up to 400 mM. Water-use efficiency and intrinsic water-use efficiency exhibited the opposite response. The reduction of photosynthesis at the high NaCl concentration could be caused by nonstomatal factors. High salinity led also to a decrease in the relative water content and water potential. Correspondingly, an accumulation of soluble sugars and proline was also observed. Na^+ and Cl^- concentrations increased in all tissues and K^+ concentrations were maintained high during exposure to NaCl compared with the control. High salinity caused oxidative stress, which was evidenced by high malondialdehyde and hydrogen peroxide contents. In order to cope with oxidative stress, the activity of antioxidative enzymes increased to maximum after 50 mM NaCl treatment. The data reported in this study indicate that *C. caput-medusae* can be utilized in mild salinity-prone environments.

Additional keywords: adaptation; chlorophyll content; lipid peroxidation; reactive oxygen species; salinity stress, gas exchange, chlorophyll fluorescence.

Introduction

Salinity is considered the primary environmental stress that negatively impacts plant growth and metabolism, particularly in the arid and semiarid areas of the world (Munns and Tester 2008). In China, 36 million hectares has is already saline (Yang 2008). The semiarid region of China is one of the regions most seriously affected by soil salinization (Wang and Jia 2012). However, before saline soils can be developed and utilized, methods, which plants employ to respond and adapt to saline stress, must be elucidated (Yıldıztugay *et al.* 2011).

High salinity can be regarded as a very serious environmental stress that imposes both osmotic stress and ionic toxicity, resulting in the decrease of plant growth, reduction in total chlorophyll content, and inhibition of

Received 14 June 2015, *accepted* 6 April 2016, *published as online-first* 26 April 2016.
[+]Corresponding author; phone: +86-991-7885442; e-mail: zengfj@ms.xjb.ac.cn
Abbreviations: APX – ascorbate peroxidase; CAT – catalase; Car – carotenoids; Chl – chlorophyll; C_i – intercellular CO_2 concentration; DM – dry mass; E – transpiration rate; FM – fresh mass; F_v/F_m – maximal quantum yield of PSII photochemistry; g_s – stomatal conductance; MDA – malondialdehyde; P_N – net photosynthetic rate; POD – guaiacol peroxidase; ROS – reactive oxygen species; RWC – relative water content; SOD – superoxide dismutase; WUE – water-use efficiency; WUE_i – intrinsic water-use efficiency; ψ_w – water potential.
Acknowledgments: This work was sponsored by the Chinese National Natural Scientific Foundation (41301103) and Science and Technology Service Network Initiative (KFJ-SW-STS-176).

photosynthesis (Feng *et al.* 2014). Salt-tolerant plants have developed a series of physiological responses that enable them to tolerate salinity stress (Munns and Tester 2008). One of these responses, osmotic regulation, which includes osmoprotectants, such as soluble sugars and proline, is an important physiological mechanism for salt adaptation in many plants (Mišić *et al.* 2012, Parida and Jha 2013). Moreover, salinity can cause oxidative stress through excessive production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2) (Koyro *et al.* 2013, Yıldıztugay *et al.* 2013). The accelerated accumulation of ROS is able to induce oxidative damage to many cellular constituents, such as proteins, membrane lipids, and nucleic acids (Mittler 2002). One of the most destructive impacts of oxidative damage is the peroxidation of membrane lipids, which leads to the accompanying generation of malondialdehyde (MDA) (Sekmen *et al.* 2012, Koyro *et al.* 2013). Consequently, a high MDA content is a helpful biomarker of lipid peroxidation and is therefore often used to detect oxidative stress induced by salinity (Hernández and Almansa 2002). To control the concentration of ROS, plants possess a well-developed and intricate antioxidant defense system involving enzymatic and nonenzymatic antioxidative processes (Blokhina *et al.* 2003). The antioxidative enzymes include superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (POD; EC 1.11.1.7), and ascorbate peroxidase (APX; EC 1.11.1.11). These enzymes can remove, neutralize, and/or eliminate oxidative species (Mittler 2002, Blokhina *et al.* 2003).

Calligonum caput-medusae is a perennial desert shrub belonging to the family Polygonaceae. The restoration project used it as a pioneer framework for sand-fixation species because of its high tolerance to drought, saline-alkaline soils, and sand burial (Zhu *et al.* 2014). *C. caput-medusae* was planted in the Taklimakan Desert Highway shelter belt (TDHS) to prevent sand drift onto the highway and was irrigated with high saline groundwater [2.8–29.7 g(NaCl) l^{-1}] (Fig. 1S*A*, *supplement available online*) (Han *et al.* 2010, Li *et al.* 2015). Within the shelter belt planted with *C. caput-medusae*, *Haloxylon* and *Tamarix* genera contribute nutrients to the soil, which are significantly accumulated at the soil surface (crust and 0–10 cm soil layers). Although irrigated with saline water, the soil salinization does not increase within the 40–60 cm of soil depth, where abundant lateral roots germinate and extend horizontally. After seven years, a planted structured soil under the shelter belt has progressively formed, as indicated by increased soil aggregate size and stability (Li *et al.* 2015). The natural vegetation around the Cele oases southwest of Taklimakan is dominated by perennial species, including *C. caput-medusae* (Fig. 1S*B*). The most likely water source for these oases is groundwater characterized by high pH and elevated sodium chloride concentrations (Arndt *et al.* 2004). However, little information has been reported on the biophysiological mechanisms *C. caput-medusae* uses in its adaptation to a salt environment. Hence, it is necessary to examine the relationship between *C. caput-medusae* and salinity stress in order to conserve arid and semiarid lands. In the current study, we were interested in: (*1*) evaluating salinity stress on the growth of *C. caput-medusae*; (*2*) determining the level of antioxidative enzyme activities (*i.e.*, SOD, CAT, POD, and APX), and the soluble sugar and proline content in an assimilating branch of *C. caput-medusae*. We also evaluated relative water content (RWC), water potential (ψ_w), H_2O_2 content, lipid peroxidation, photosynthetic pigment content, and gas-exchange characteristics of *C. caput-medusae*. The aim of this study was to examine in detail the stress caused by salinity and the level of tolerance and detoxification strategy employed by *C. caput-medusae*.

Materials and methods

Plant material and experimental treatments: The seeds of *C. caput-medusae* were collected in November 2012 from a desert-oasis transitional zone near the Cele Research Station of Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, which is located in the Cele oasis (80°03′24″–82°10′34″E; 35°17′55″–39°30′00″N; 1,340–1,380 m a.s.l.). Considerable quantities of seeds were collected from more than 30 randomly selected plants. After collection, the seeds were pooled. A pot experiment was conducted in a field of the Cele Research Station from 5 May to 5 August 2013. During the experimental period, temperature ranged from 15 to 40°C during day and from 10 to 25°C during night. Relative humidity ranged from 20 to 60%, and the precipitation was approximately 30.1 mm. The seeds were evenly planted on 5 May into plastic pots (diameter of 40 cm, height of 32 cm) filled with 20 kg of soil, which was collected from a desert-oasis transitional zone (sampling depth of 0–30 cm) and previously passed through a sieve of 2 mm. Soils were aeolian loose sediments, and soil texture was highly homogeneous. Based on a particle size, the main fraction was silt (> 88%), with sand (particle size 63–2,000 μm) and clay (particle size < 2 μm) generally accounting for less than 5% of soil texture (Thomas *et al.* 2006). Further analysis of the soil revealed the following: pH 8.52 (soil:water ratio of 1:5), electrical conductivity of 0.54 mS cm^{-1}, bulk density of 1.494 g cm^{-3}, total salinity of 1.749 g kg^{-1}, 1.462 g(organic matter) kg^{-1}, 0.848 g(organic carbon) kg^{-1}, 0.089 g(total nitrogen) kg^{-1}, 0.571 g(total phosphorus) kg^{-1}, 15.766 g(total potassium) kg^{-1}, 12.992 mg(available nitrogen) kg^{-1}, 2.362 mg(available phosphorus) kg^{-1}, and 224.751 mg(available potassium) kg^{-1}. In order to protect the plants from being burned by high temperature, the pots were

embedded in the soil. When seedlings grew for 30 d, they were thinned to retain one uniform plant per pot.

Irrigation with saline water began on 5 July. Saline growing conditions were simulated by applying NaCl to deionized fresh water at five concentrations (0, 50, 100, 200, and 400 mM). In order to avoid salinity shock to the plants, the NaCl concentration was increased stepwise in aliquots of 50 mM every day until the appropriate concentration was attained. Each treatment included 12 pots. After 30 d, the plants were harvested for analysis.

Measurements of growth: The number of first branches was recorded and shoot height, east-west canopy diameter (D_{EW}), north-south canopy diameter (D_{NS}), and assimilating branch length were measured with a tapeline. Basal diameter and assimilating branch diameter were measured with a vernier caliper. Canopy area was calculated from the equation: canopy area = $[(1/4)\pi \times D_{EW} \times D_{NS}]$ (Jia *et al.* 2009). After a quick wash with deionized fresh water, seedlings were gently dried on blotting paper and the assimilating branches, stems, and roots were separated immediately and individually weighed for fresh mass (FM). Plants were dried in an oven at 70°C until constant dry mass (DM) was obtained. Each treatment was replicated with six seedlings. Dry samples were used to determine ion content on a DM basis.

Ion concentration analysis: Finely ground, oven-dried tissue (0.1 g) was digested overnight with 25 ml of 0.1 M HNO_3 at room temperature (Sibole *et al*. 2003). Contents of Na^+ and K^+ in the acid extract were determined using an inductively coupled plasma-optical emission spectrometer (*ICP-OES, Agilent 735*, Santa Clara, CA, USA), while Cl^- concentration was determined in the same extract with a chloride meter (*Jenway PC LM3*, London, UK).

Photosynthetic pigment content and maximal quantum yield of PSII photochemistry (F_v/F_m): Photosynthetic pigment content was extracted with 80% acetone. The clear supernatant fraction gained after centrifugation at 480 × g for 3 min was used for spectrophotometrical (*UV752 N*, *Shanghai Precision & Scientific Instrument Co., Ltd.*, China) estimation of chlorophyll (Chl), using the extinction coefficients and equations determined by Lichtenthaler (1987).

F_v/F_m was measured using a portable fluoremeter (*OS-30, Opti-sciences*, USA) at 11:00 a.m. local time on a clear day. Chl fluorescence was measured in assimilating branches of the same plant and six plants were chosen for each treatment. Assimilating branches were dark-adapted for 15 min using clips in a dark room. Branches were exposed to saturating light at an intensity of 3,000 μmol(photon) m^{-2} s^{-1} for 2 s. After the dark adaptation, the parameters F_v and F_m were measured, and the F_v/F_m ratio was calculated.

Gas exchange: Net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured using a portable photosynthesis system (*LI-6400, LI-COR*, Lincoln, NE, USA) between 11:00 and 11:30 h. Six plants were selected for each treatment under the following atmospheric environments: PAR of 1,353 ± 66 μmol(photon) m^{-2} s^{-1}, CO_2 concentration of 386. 5 ± 3.3 μmol mol^{-1}, temperature of 31.1 ± 1.1°C, relative humidity (RH) of 30.1 ± 0.8%. Water-use efficiency (WUE) was calculated as the ratio of P_N/E, and intrinsic water-use efficiency (WUE_i) was calculated as the ratio of P_N/g_s.

RWC and ψ_w of assimilating branches: RWC was measured according to Smart and Bingham (1974). RWC was calculated using the following formula: RWC = (FM – DM)/(TM – DM) × 100%. Turgid mass (TM) was obtained after floating assimilating branches on distilled water for 4 h at 20°C in darkness. Assimilating branch ψ_w was measured on a freshly cut assimilating branch by a *WP4* dewpoint water potential meter (*Decagon Devices, Inc*., Pullman, WA, USA).

Total soluble sugars and proline content determination: The concentration of soluble sugars was measured by the anthrone method (Palma *et al.* 2009). Approximately 0.15 g of fresh assimilating branches was sheared into fine pieces and incubated in 15 ml of distilled water in a water bath at 100°C for 1 h. This extraction solution (0.05 ml) was added to 0.15 ml of distilled water and reacted with 0.05 ml of anthrone ethyl acetate. To develop colour, 0.5 ml of concentrated sulphuric acid was added to the mixture, which was then immediately boiled for 1 min. The absorbance of the solution was measured at 630 nm (*UV752 N*, *Shanghai Precision & Scientific Instrument Co., Ltd.*, China) and the concentration of soluble sugars determined using a standard curve prepared from a sucrose standard (*Sangon*, Shanghai, China).

The proline concentration was determined by the method of Bates *et al.* (1973). Approximately 0.15 g of fresh assimilating branches was ground in a mortar with 3 ml of 120 mM sulfosalicylic acid and the homogenate was centrifuged at 10,000 × *g* for 15 min. Two milliliters of the supernatant fraction was added to 2 ml of glacial acetic acid and 2 ml of acid ninhydrin and then boiled at 100°C for 1 h. The reaction was stopped by cooling in an ice bath, and the sample was extracted with 4 ml of toluene. The absorbance of the organic phase was determined at 520 nm (*UV752 N*, *Shanghai Precision & Scientific Instrument Co., Ltd*, China) and the proline concentration determined using a standard curve prepared from an L-proline standard (*Sangon*, Shanghai, China).

MDA and H_2O_2 content: MDA is a product of lipid peroxidation through the thiobarbituric acid (TBA) reaction. Fresh assimilating branches (0.3 g) were homogenized with 5 ml solution containing 0.5% TBA and 20% trichloroacetic acid (TCA). The mixture was heated

at 95°C for 30 min, and then the reaction was stopped by quickly cooling it in an ice bath. The cooled mixture was centrifuged at 5,000 × *g* for 15 min. The absorbance of the supernatant fraction at 532 and 600 nm was determined (*UV752 N*, *Shanghai Precision & Scientific Instrument Co., Ltd.*, China), and MDA content was calculated by its molar extinction coefficient, 155 $mmol^{-1}$ cm^{-1} (Kosugi and Kikugawa 1985).

H_2O_2 concentration was measured by the method of Sergiev *et al.* (1997). Fresh assimilating branches (0.3 g) were ground in an ice bath mortar with 2 ml 0.1% (w/v) TCA, and the homogenate was centrifuged at 12,000 × *g* for 15 min. One milliliter supernatant fraction was mixed with 1 ml of 10 mM potassium phosphate buffer (pH 7.0) and 2 ml of 1 M KI. The absorbance of the solution was determined at 390 nm (*UV752 N*, *Shanghai Precision & Scientific Instrument Co., Ltd.*, China) and the H_2O_2 concentration determined using a standard curve prepared from an H_2O_2 standard (*Sangon*, Shanghai, China).

Enzyme extraction and assays: The supernatant fraction used for measuring soluble protein concentration and SOD, POD, CAT, and APX activities were obtained according to the method of Lu *et al*. (2010) using a spectrophotometer (*UV752 N*, *Shanghai Precision & Scientific Instrument Co., Ltd.*, China).

SOD (EC 1.15.1.1) activity was determined by the method of Beauchamp and Fridovich (1971) in terms of its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. The reaction mixture of 3 ml contained 50 mM potassium phosphate buffer (pH 7.8), 75 μM NBT, 13 mM L-methionine, 0.1 mM EDTA, 0.002 mM riboflavin, and 50 μl enzyme extract. Reactions were carried out at 25°C, under cool white fluorescent light for 10 min. One unit of SOD was defined as the enzyme amount causing 50% inhibition reduction of NBT and the enzyme activity was expressed in units per mg of protein per min.

CAT (EC 1.11.1.6) activity was determined by measuring the consumption of H_2O_2 according to the method of Aebi (1984). The decomposition of H_2O_2 was followed by absorbance decrease at 240 nm for 90 s and was calculated per 60 s. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and 50 μl enzyme extract. Addition of H_2O_2 started the reaction. CAT activity was calculated using the extinction coefficient (0.036 mM^{-1} cm^{-1}). One unit of CAT activity was defined as 1 μmol(H_2O_2 destroyed) per min and the enzyme activity was expressed as units per mg of protein.

POD (EC 1.11.1.7) activity was measured in accordance with the method of Chance and Maehly (1955). The reaction mixture consisted of 50 μl of 20 mM guaiacol, 2.8 ml of 10 mM potassium phosphate buffer (pH 7.0), and 50 μl of enzyme extract at 25°C. The reaction was initiated *via* the addition of 20 μl of 40 mM H_2O_2. Increases in absorbance at 470 nm were recorded. POD activity was calculated using an absorption coefficient (26.6 mM^{-1} cm^{-1}) for tetraguaiacol. One unit of POD activity was defined as 1 μmol(tetraguaiacol formed) per min and the enzyme activity was expressed as units per mg of protein.

APX (EC 1.11.1.11) activity was assayed according to the method of Nakano and Asada (1981). Following the decrease of ascorbate and measuring the change in absorbance at 290 nm for 1 min in 3 ml of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA-Na_2, 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 50 μl of crude enzyme extract at 25°C. APX activity was calculated from the extinction coefficient (2.8 mM^{-1} cm^{-1}) for the ascorbate. One unit of APX enzyme was defined as 1 μmol(ascorbate oxidized) per min and the enzyme activity of the extract was expressed as units per mg of protein.

Proteins were quantified by the method of Bradford (1976) using bovine serum albumin as the standard protein.

Statistical analysis: Six independent experiments were performed in order to test the effect of NaCl treatment on plant growth and selected physiological parameters. Data used here were means ± SD. Mean values were separated by *Fisher*'s LSD test at the 0.05 probability level. A linear regression analysis was adopted to evaluate the relationship between NaCl concentration and tested parameters at different salinities. Calculations were carried out with *SPSS Inc., version 13* (Chicago, USA).

Results

Growth parameters: Plant growth parameters reached their peak value at 50 mM NaCl, and then decreased progressively with increasing NaCl concentrations (≥100 mM) (Table 1). Peak values were significantly higher than those of the plants receiving no NaCl (control). The results showed that *C. caput-medusae* required 50 mM NaCl to express its maximal growth potential. The shoot:root ratio was markedly lower than that of the control under high salinity stress (≥100 mM NaCl).

Photosynthetic pigment content and F_v/F_m: Chl, carotenoid (Car) content, and F_v/F_m values were maximal in assimilating branches of *C. caput-medusae* treated with 50 mM NaCl and reduced gradually with elevated NaCl concentration (≥100 mM) (Table 2). The Car/Chl ratio increased with increasing salinity.

Gas exchange: The plants grown under 50 mM NaCl concentration showed peak values for P_N, E, and g_s

(Table 3). However, increased salinity stress (≥100 mM NaCl) led to progressive reduction in P_N, E, and g_s compared with the control. WUE and WUE_i rose up to their maximum values with 200 mM NaCl treatment and then started to decline as the NaCl concentration increased to 400 mM NaCl. C_i initially decreased and then increased, the maximum value was recorded at 50 mM NaCl treatment, and the minimum value was observed at 200 mM NaCl treatment.

Table 1. Effect of NaCl treatments on growth of *Calligonum caput-medusae*. Values indicate the changes in growth parameters after 30 d of NaCl treatments. Means ± SD, $n = 6$. Values in rows followed by different *lowercase letters* are significantly different at $p<0.05$ according to *Fisher*'s LSD test.

Parameter	NaCl concentration [mM]				
	0	50	100	200	400
Plant shoot height [cm]	63.8 ± 3.51^{d}	71.5 ± 2.65^{e}	54.8 ± 2.51^{c}	42.3 ± 3.30^{b}	35.5 ± 3.11^{a}
Crown area [cm^2 per plant]	962 ± 85.6^{c}	1522 ± 99.3^{d}	339 ± 12.2^{b}	151± 6.6^{a}	90 ± 4.5^{a}
Basal diameter [mm]	3.75 ± 0.279^{d}	5.13 ± 0.209^{e}	3.33 ± 0.133^{c}	2.63 ± 0.171^{b}	2.23 ± 0.199^{a}
First branch number per plant	17.5 ± 1.29^{d}	26.0 ± 1.83^{e}	11.5 ± 1.25^{c}	8.3 ± 0.96^{b}	6.0 ± 0.82^{a}
Assimilating branch length [cm]	30.5 ± 1.73^{d}	35.5 ± 2.08^{e}	20.3 ± 1.63^{c}	14.8± 1.26^{b}	9.4 ± 1.29^{a}
Assimilating branch diameter [mm]	1.42 ± 0.043^{d}	1.61 ± 0.053^{e}	1.26 ± 0.048^{c}	1.16 ± 0.056^{b}	0.91 ± 0.042^{a}
Assimilating branch fresh mass [g per plant]	6.02 ± 0.478^{d}	10.03 ± 0.784^{e}	4.95 ± 0.396^{c}	2.88 ± 0.174^{b}	1.80 ± 0.210^{a}
Stem fresh mass [g per plant]	4.36 ± 0.306^{c}	7.02 ± 0.507^{d}	2.11 ± 0.212^{b}	1.48 ± 0.167^{a}	1.14 ± 0.112^{a}
Root fresh mass [g per plant]	5.92 ± 0.246^{d}	10.32 ± 0.665^{e}	5.31 ± 0.317^{c}	3.51 ± 0.373^{b}	2.53 ± 0.201^{a}
Assimilating branch dry mass [g per plant]	1.42 ± 0.141^{c}	2.79 ± 0.264^{d}	1.13 ± 0.128^{b}	0.57 ± 0.042^{a}	0.37 ± 0.052^{a}
Stem dry mass [g per plant]	1.93 ± 0.179^{c}	3.97 ± 0.242^{d}	1.04 ± 0.105^{b}	0.80 ± 0.048^{a}	0.71 ± 0.041^{a}
Root dry mass [g per plant]	2.85 ± 0.225^{c}	5.48 ± 0.499^{d}	2.60 ± 0.218^{bc}	2.20 ± 0.139^{b}	1.32 ± 0.047^{a}
Fresh shoot/root ratio	1.75 ± 0.059^{a}	1.65 ± 0.036^{a}	1.33 ± 0.052^{b}	1.25 ± 0.046^{c}	1.16 ± 0.049^{d}
Dry shoot/root ratio	1.18 ± 0.048^{a}	1.23 ± 0.022^{a}	0.83 ± 0.023^{b}	0.63 ± 0.034^{c}	0.81 ± 0.039^{b}

Table 2. Effect of NaCl treatments on photosynthetic pigment content and F_v/F_m in assimilating branch of *Calligonum caput-medusae*. Values indicate the changes in Chl *a*, Chl *b*, Chl *a* + *b*, Car, Chl *a*/*b*, Car/Chl, and F_v/F_m after 30 d of NaCl treatments. Means ± SD, $n = 6$. Values in a column followed by different *lowercase letters* are significantly different at $p<0.05$ according to *Fisher*'s LSD test. Car – carotenoids; Chl – chlorophyll; FM – fresh mass; F_v/F_m – maximal quantum yield of PSII photochemistry.

NaCl [mM]	Chl *a* [mg g^{-1}(FM)]	Chl *b* [mg g^{-1}(FM)]	Chl (*a*+*b*) [mg g^{-1}(FM)]	Car [mg g^{-1}(FM)]	Car/Chl	F_v/F_m
0	0.429 ± 0.016^{c}	0.067 ± 0.004^{c}	0.501 ± 0.025^{c}	0.024 ± 0.001^{c}	0.049 ± 0.001^{a}	0.814 ± 0.013^{d}
50	0.446 ± 0.037^{c}	0.067 ± 0.006^{c}	0.506 ± 0.045^{c}	0.025 ± 0.001^{c}	0.049 ± 0.003^{a}	0.815 ± 0.012^{d}
100	0.253 ± 0.024^{b}	0.066 ± 0.008^{c}	0.330 ± 0.047^{b}	0.018 ± 0.001^{b}	0.055 ± 0.005^{b}	0.755 ± 0.013^{c}
200	0.217 ± 0.022^{ab}	0.051 ± 0.006^{b}	0.268 ± 0.027^{ab}	0.016 ± 0.001^{a}	0.060 ± 0.003^{b}	0.704 ± 0.010^{b}
400	0.187 ± 0.017^{a}	0.038 ± 0.004^{a}	0.213 ± 0.020^{a}	0.014 ± 0.001^{a}	0.067 ± 0.002^{c}	0.658 ± 0.011^{a}

Table 3. Effect of NaCl treatments on gas-exchange parameters in assimilating branch of *Calligonum caput-medusae*. Values indicate the changes in P_N, E, g_s, WUE, WUE_i, and C_i after 30 d of NaCl treatments. Means ± SD, $n = 6$. Values in a column followed by different *lowercase letters* are significantly different at $p<0.05$ according to *Fisher'*s LSD test. C_i – intercellular CO_2 concentration; E – transpiration rate; g_s – stomatal conductance; P_N – net photosynthetic rate; WUE – water-use efficiency; WUE_i – intrinsic water-use efficiency.

NaCl	P_N [μmol m^{-2} s^{-1}]	E [mmol(H_2O) m^{-2} s^{-1}]	g_s [mol(H_2O) m^{-2} s^{-1}]	WUE [μmol(CO_2) mmol(H_2O)$^{-1}$]	WUE_i [μmol(CO_2) mol(H_2O)$^{-1}$]	C_i [μmol(CO_2) mol^{-1}]
0	9.06 ± 0.606^{d}	5.67 ± 0.508^{d}	0.163 ± 0.007^{d}	1.602 ± 0.035^{a}	55.43 ± 2.508^{a}	231.4 ± 8.02^{cd}
50	11.33 ± 0.875^{e}	6.91 ± 0.359^{e}	0.181 ± 0.005^{e}	1.638 ± 0.042^{a}	62.59 ± 3.155^{b}	240.7 ± 7.09^{d}
100	7.72 ± 0.431^{c}	4.47 ± 0.287^{c}	0.118 ± 0.006^{c}	1.727 ± 0.037^{b}	65.35 ± 2.683^{b}	210.2 ± 9.61^{b}
200	3.85 ± 0.267^{b}	1.93 ± 0.148^{b}	0.054 ± 0.003^{b}	2.001 ± 0.036^{d}	71.68 ± 1.982^{bc}	194.5 ± 5.51^{a}
400	2.68 ± 0.168^{a}	1.24 ± 0.998^{a}	0.035 ± 0.003^{a}	1.913 ± 0.047^{c}	67.22 ± 3.84^{b}	229.6 ± 6.03^{c}

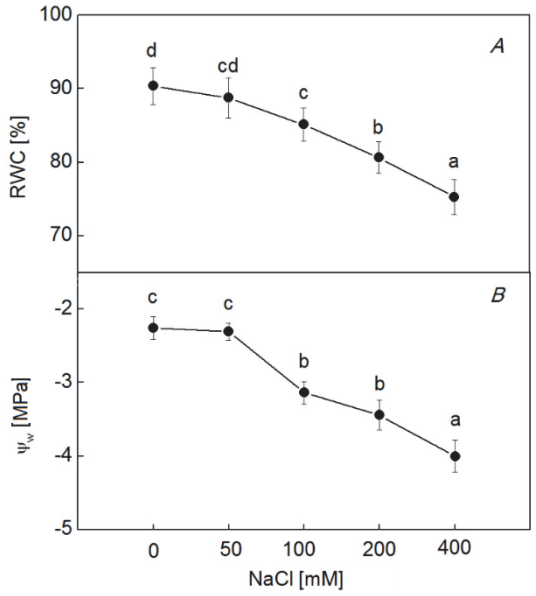

Fig. 1. Effect of NaCl treatment (0, 50, 100, 200, and 400 mM for 30 d) on *A*: relative water content (RWC) and *B*: water potential (ψ_w) in assimilating branches of *Calligonum caput-medusae*. Values are means ± SD (n = 6). Means followed by different *lowercase letters* are significantly different at p<0.05 according to *Fisher*'s LSD test.

RWC and ψ_w: RWC and ψ_w in assimilating branches of *C. caput-medusae* were significantly affected by salinity, both decreasing with increasing NaCl concentration (Fig. 1*A,B*). RWC and ψ_w were reduced by 1.8–16.7% and 2.1–77.1% with increased NaCl concentration, respectively, compared with the control.

Total soluble sugar and proline content: Total soluble sugar and proline content exhibited minor changes when the NaCl concentration was increased from 0 mM to 50 mM. However, both increased markedly with elevated NaCl concentration (≥100 mM), rising up to 18.9%–41.1% and 27.3%–85.2%, respectively, compared with the control (Fig. 2*A,B*).

Responses of ion concentration to NaCl treatment: Seedlings exposed to NaCl-containing solutions accumulated higher amounts of Na^+ in assimilating branches compared to that in stems and roots. The Na^+ concentration in assimilating branches varied from 4.19 to 10.24 mg g^{-1} at 50 and 400 mM NaCl, respectively, whereas that in the stems ranged from 3.21 to 8.83 mg g^{-1}, and that in the roots ranged from 2.16 to 4.71 mg g^{-1} (Fig. 3*A*). The low NaCl concentration (50 mM) had no significant effect on K^+ concentration in assimilating branches and stems, while higher K^+ concentrations were observed at higher NaCl concentrations compared with the controls. The highest contents were observed at 100 mM NaCl (Fig. 3*B*). Cl^- concentrations in seedlings displayed a similar pattern as that observed for Na^+ (Fig. 3*C*).

H_2O_2 and MDA contents: H_2O_2 and MDA content were not affected by a low NaCl concentration (50 mM), however, both significantly increased at elevated NaCl concentrations (≥100 mM) (Fig. 4*A,B*). With 400 mM NaCl treatment, the H_2O_2 and MDA concentrations were approximately 58.1 and 121.2% higher than that of the control, respectively.

Responses of antioxidative enzymes to NaCl treatment: In *C. caput-medusae* grown under increased NaCl concentrations, the activities of SOD, CAT, POD, and APX showed variable responses (Fig. 5). SOD and CAT activities reached their peak at 50 mM NaCl treatment, increasing by 11.2 and 34.5%, respectively, and then decreased gradually compared with the control (Fig. 5*A,B*). POD and APX activities reached their maximum value at 50 mM NaCl treatment and later decreased gradually up to 200 mM NaCl. With 100 or 200 mM NaCl, POD and APX activities were significantly higher than that of the control. With 400 mM NaCl, POD markedly decreased by 37.4% and APX declined slightly by 14.3% compared with the control (Fig. 5*C,D*).

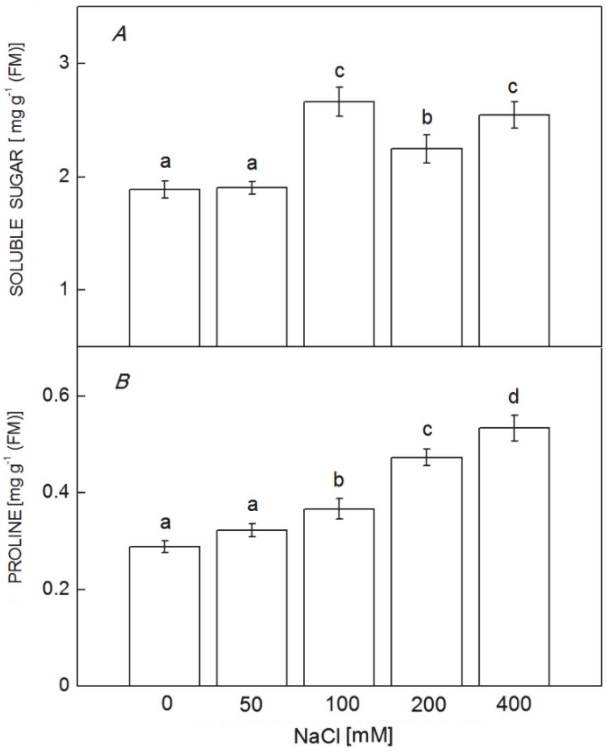

Fig. 2. Effect of NaCl treatment (0, 50, 100, 200, and 400 mM for 30 d) on *A*: soluble sugar and *B*: proline content in assimilating branches of *Calligonum caput-medusae*. Values are means ± SD (n = 6). Means followed by different *lowercase letters* are significantly different at p<0.05 according to *Fisher*'s LSD test.

Discussion

C. caput-medusae has been reported to grow well under saline-water irrigation (Li *et al.* 2015). In this study, the growth of *C. caput-medusae* seedlings was significantly accelerated at 50 mM NaCl. This finding is in agreement with previous reports regarding halophytic plants that achieve their optimal growth in the presence of salts (Hussin *et al.* 2013, Sharma and Ramawat 2014). These data indicate that *C. caput-medusae* possesses moderate salt tolerance and may be an effective means to improve saline soil in the southern edge of the Taklimakan Desert. In this study, ground water is the only reliable source of moisture and is moderately saline (Arndt *et al.* 2004). The substantial decline in seedling growth, demonstrated in this study with 100 mM NaCl, was also observed in *Periploca sepium* and *Cichorium intybus* (Sun *et al.* 2011, Sergio *et al.* 2012). Salinity could reduce the ability of plants to acquire water, which rapidly causes declines in their growth rate, as well as a suite of metabolic changes (Munns 2002). The shoot:root ratio has been reported to be a reliable indicator to assess salt or drought tolerance (Kramer 1983, Munns 2002). Our data showed that the shoot:root ratio of *C. caput-medusae* declined markedly under salinity stress, suggesting that root growth was less adversely influenced by NaCl than that of shoots. A decline in shoot:root ratio is regarded as an adaptive mechanism (Gorham *et al.* 1985, Munns 2002) as it leads both to increased surface area for water extraction by the roots and lowers water loss through reduced transpiration area.

Several studies have reported that plant growth relies on photosynthetic capacity. Therefore, abiotic stresses that influence growth should also affect photosynthesis (Dubey 1997). In our study, changes in growth paralleled salinity-induced changes in photosynthetic pigment contents (Chl *a*, *b* and Car). Photosynthetic pigments have been reported to play an important role in maintaining the photosynthetic

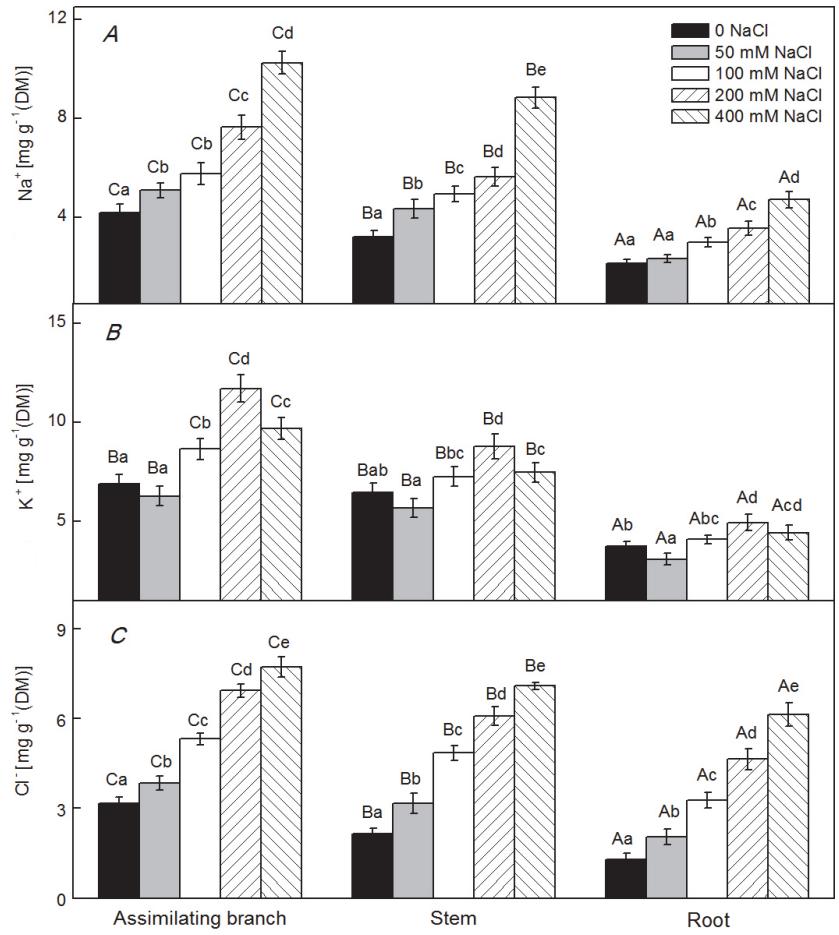

Fig. 3. Effect of NaCl treatment (0, 50, 100, 200, and 400 mM for 30 d) on *A*: Na^+ content, *B*: K^+ content, and *C*: Cl^- content in assimilating branches, stems, and roots of *Calligonum caput-medusae*. Values are means ± SD (n = 6). Different *lowercase letters* indicate significant differences of Na^+, K^+, and Cl^- concentrations in the same organ across the range of NaCl concentration, and different *uppercase letters* indicate significantly different Na^+, K^+, and Cl^- concentrations in different organ at a given NaCl concentration at $p<0.05$ according to *Fisher*'s LSD test.

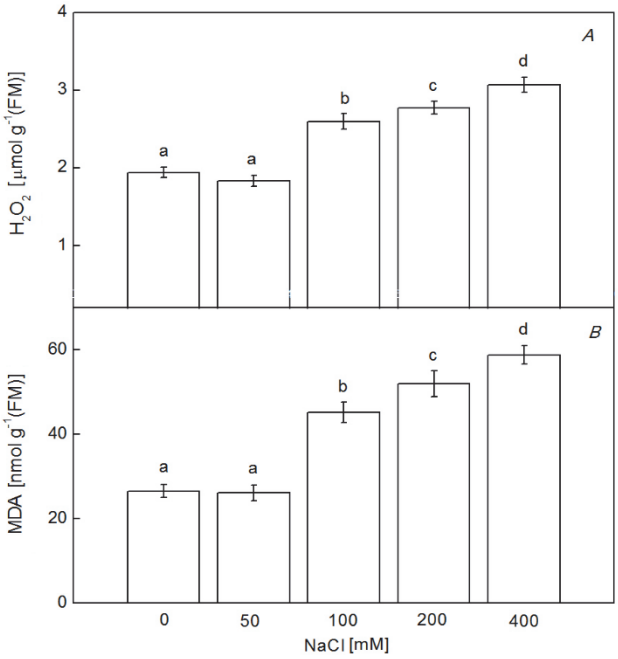

Fig. 4. Effect of NaCl treatment (0, 50, 100, 200, and 400 mM for 30 d) on *A*: hydrogen peroxide (H_2O_2) and *B*: malondialdehyde (MDA) content in assimilating branches of *Calligonum caput-medusae*. Values are means ± SD (n = 6). Means followed by different *lowercase letters* are significantly different at p<0.05 according to *Fisher's* LSD test.

capacity of plants (Dubey 2005). Chl and Car are the main photosynthetic pigments of higher plants. The concentrations of Chl and Car in *C. caput-medusae* slightly increased by lower salinity, while both of them were significantly reduced by increased salinity. PSII had been previously reported to play an important role in the mechanism of photosynthesis in plants subjected to abiotic stresses, including salinity stress (Baker 1991). The functionality of PSII can be evaluated by measuring different parameters of Chl fluorescence. The F_v/F_m is frequently used as an indicator of photoinhibition (Hichem *et al.* 2009), and the F_v/F_m ratio of 0.8 or above is a characteristic of a healthy plant (Dan *et al.* 2000). In our study, the F_v/F_m ratios in the control plants and those exposed to lower concentrations of NaCl (50 mM) were higher than 0.8, while the ratio was lesser than 0.8 with higher NaCl exposure (≥100 mM). This finding suggests that the high NaCl concentration can be considered a photosynthetic stressor in *C. caput-medusae*. The decline of the Chl content followed by suppression of photochemical reactions regulated by PSII suggests that inactivation in PSII could be caused by damage to the chloroplasts caused by thylakoid swelling, envelope decomposition, and instability of pigment-protein complexes (Prasad and Pardha Saradhi 2004). As shown in the current study, high NaCl concentrations (≥100 mM) significantly reduced photosynthesis. The decline in photosynthesis in *C. caput-medusae* after exposure to 100 or 200 mM NaCl might be attributable to stomatal factors, namely stomatal closure and reduced carbon absorption. Effects on photosynthesis after exposure to 400 mM NaCl could occur due to nonstomatal factors, such as a decline in photosynthetic pigments, reduction in RWC, and salinity-induced oxidative stress (Huang *et al.* 2014). Stomatal closure decreases loss of water by transpiration, leading to a decline in *E*. It has been reported that the reduction in g_s under mild stress may be a protective mechanism against stress by allowing the plant to conserve water, consequently improving plant WUE and WUE_i (Chaves *et al.* 2009).

Water distribution in plants is influenced by salinity (Gorai and Neffati 2011, Sai Kachout *et al.* 2011). In *C. caput-medusae*, leaf ψ_w reached a minimum value (below –4.01 MPa) at the highest salt concentration (400 mM NaCl). Similar responses have been found in other plants, such as *Reaumuria vermiculata* (Gorai and Neffati 2011) and *Atriplex halimus* (Hassine and Lutts 2010), where leaf ψ_w decreases with increasing salinity level. This reduction was shown to be due to decreases in RWC and leaf turgor potential (Gimeno *et al.* 2012). Given that plants accumulate nontoxic compatible solutes, which allow turgor and water content to be maintained (Hare *et al.* 1998), the accumulation of proline and sucrose may be an adaptive mechanism to prevent loss of water. It has been reported that proline helps plants maintain their cell turgor, protects membrane integrity, and prevents protein denaturation under various environmental stresses (Hong *et al.* 2000). Sucrose has often been proposed as one of the osmolytes that enables the maintaining of turgor and sufficient hydration during water loss (Sánchez *et al.* 2004). An increase in proline, sucrose, and soluble sugars has been widely reported under various stresses, including salinity stress (Gorai and Neffati 2011, Lokhande *et al.* 2013). In this study, significant accumulations of soluble sugars and proline in *C. caput-medusae* were observed under salinity stress compared with the control. This indicates that soluble sugars and proline might play an important role in osmotic adjustment following exposure to high salinity.

Na^+ and Cl^- are the main toxic ions in saline soil. High Na^+, often in conjunction with Cl^-, leads to specific ion toxicity and plant growth inhibition (Ashraf and Harris 2004). The influx of Na^+ competes with K^+, thus leading to a decrease in K^+ absorption during salt stress (Serrano and Rodriguez-Navarro 2001). However, the K^+ content in response to elevated NaCl concentration did not decrease compared with the control in our study, suggesting that K^+ uptake by *C. caput-medusae* did not compete with Na^+. This result is consistent with a study of *Caragana korshinskii* (Yan *et al.* 2012). The capacity to sustain a

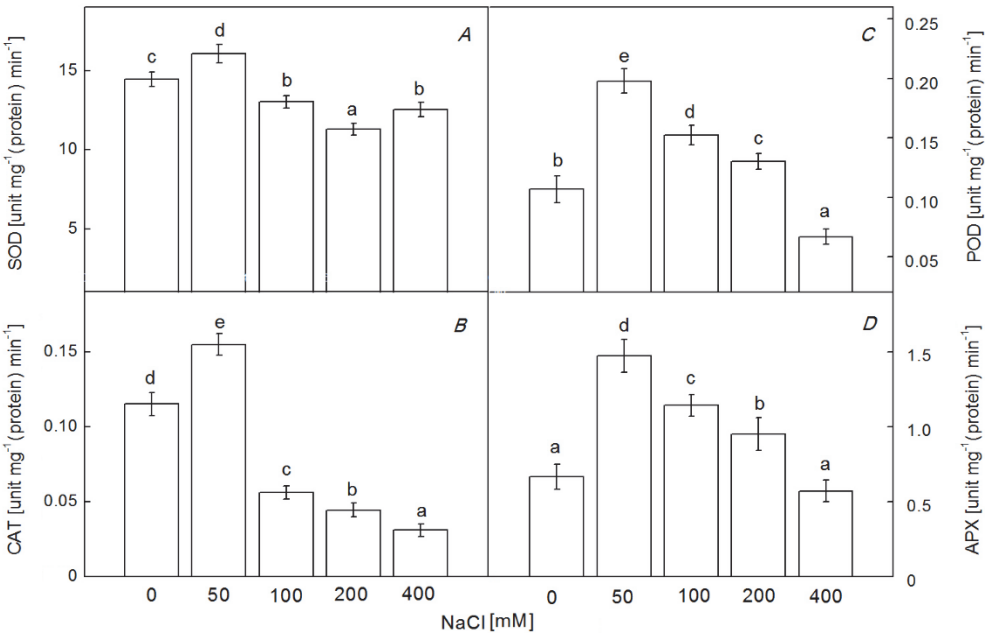

Fig. 5. Effect of NaCl treatment (0, 50, 100, 200, and 400 mM for 30 d) on *A*: superoxide dismutase (SOD), *B*: catalase (CAT), *C*: guaiacol peroxidase (POD), and *D*: ascorbate peroxidase (APX) in assimilating branches of *Calligonum caput-medusae*. Values are means ± SD (n = 6). Means followed by different *lowercase letters* are significantly different at p<0.05 according to *Fisher*'s LSD test.

high cytosolic K^+/Na^+ ratio is a key feature of plant salinity tolerance (Amor *et al.* 2005); thus, regulation of K^+ transport under salt stress is critical. In our study, the data showed that *C. caput-medusae* had a capacity to regulate K^+ transport under salt stress, despite increased Na^+ and Cl^- concentrations.

In higher plants, salinity induces oxidative stress by production of ROS (Sekmen *et al.* 2012, Guzmán-Murillo *et al.* 2013). Protonation of $O_2^{\cdot-}$ can generate the hydroperoxyl radical (H_2O_2 and $^{\cdot}OH$), which can transform fatty acids into harmful lipid peroxides, thus destroying biological membranes (Grant and Loake 2000). MDA production is a consequence of lipid peroxidation in plants during oxidative stress and is regarded as a biomarker of the degree of oxidative stress (Castelli *et al.* 2010). In *C. caput-medusae*, high concentrations of NaCl markedly elevated the production of H_2O_2 and, correspondingly, caused lipid peroxidation as revealed by an accumulation of MDA. This is in agreement with the results of salinity stress on other higher plants (Sekmen *et al.* 2012, Yıldıztugay *et al.* 2013).

To mitigate the damage caused by accumulated ROS under stressful environments, plants have evolved an efficient antioxidative enzyme system that includes SOD, POD, CAT, and APX (Mittler 2002, Blokhina *et al.* 2003). SOD is a crucial antioxidant enzyme that works as an $O_2^{\cdot-}$ scavenger in living organisms by rapidly transforming $O_2^{\cdot-}$ into H_2O_2 and O_2. SOD is considered a front-line sentinel against oxidative stress induced by ROS. The product of SOD activity is H_2O_2, which is itself toxic and must be eliminated by conversion to H_2O in subsequent reactions. In plants, CAT, POD, and APX play an important role in the detoxification of H_2O_2 (Mittler 2002, Blokhina *et al.* 2003). Several studies have shown that H_2O_2 can induce and stimulate production of ROS, leading to an enhancement in SOD, POD, CAT, and APX activities as a defense system (Sergio *et al.* 2012, Agrawal *et al.* 2013). Our results showed an increase in SOD activity under lower NaCl exposure (50 mM), which was accompanied by an increase in POD, CAT, and APX activities. Correspondingly, an increase in SOD, CAT, POD, and APX activities under lower NaCl concentration exposure ensured the effective scavenging of ROS, which was confirmed by a decreased generation of H_2O_2. The results of this study indicated that *C. caput-medusae* is equipped with a responsive and efficient antioxidative enzyme system, which proved to be moderately inducible under hyperosmotic and/or hyperionic situations triggered by NaCl. However, the significant accumulation of H_2O_2 detected under high NaCl exposure (≥100 mM) could occur due to a progressive decline in the activities of CAT, POD, and APX. In our study, a marked increase in CAT activity was observed only at 50 mM NaCl exposure compared with control, indicating that CAT activity participated in the H_2O_2-elimination process only under a low level of oxidative stress induced by salinity. Thus, in response to higher saline stress, CAT was inhibited, possibly as a result of ROS-triggered inhibition. On the contrary, notable increases in POD and APX activities were detected under exposure to high NaCl concentrations (100 or 200 mM) compared with control. These result suggest that in *C. caput-medusae*, H_2O_2 was scavenged by

CAT at lower and by POD and APX at higher saline stress.

In summary, *C. caput-medusae* possesses the capacity for moderate salt tolerance, indicating that it can adapt well to 50 mM NaCl. However, higher salt concentrations (≥100 mM NaCl) resulted in the accumulation of ROS, which led to high contents of MDA. Usually, MDA causes damage to lipids, particularly in the thylakoids, which has a negative influence on photosynthetic activity (Havaux and Niyogi 1999) and leads to a reduction in the F_v/F_m ratio and growth. However, *C. caput-medusae* is capable of specific adaptations to protect itself against saline stress. These adaptations include modulating the activities of antioxidative enzymes and the utilization of osmoprotectants. SOD, POD, CAT, and APX were effective in eliminating ROS after exposure to lower NaCl concentration (50 mM), which was shown by the maintenance of MDA and H_2O_2 contents. At higher NaCl concentrations (100 or 200 mM), the observed induction of POD and APX might be insufficient to scavenge the increased production of ROS as evidenced by the accumulation of MDA and H_2O_2. Moreover, in addition to the induced enzymatic antioxidant system (SOD, POD, CAT, and APX), the significant accumulation of soluble sugars and proline could play an important role in salt tolerance through osmotic adjustment.

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