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Different responses of the halophyte *Carex pumila* to salt stress

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Abstract

Salt stress is one of the most important stresses that affect coastal vegetation. A halophyte *Carex pumila* plays a crucial role in the maintenance of fragile ecosystems in coastal areas. Thus, understanding the mechanism of *C. pumila* responses to salt stress is a prerequisite for the conservation and utilization of this species. After treatment with 200, 300, 400, 500 mM NaCl for 60 h, *C. pumila* leaves displayed a decline in the relative water content and an increase in salt injury index. Osmolyte accumulation, as a function of osmotic adjustment, and antioxidant enzyme activities were maintained under salinity, even at a high NaCl concentration. High NaCl concentrations severely affected the photosystem II, the JIP-test indicated a significant decrease in performance indexes and quantum efficiencies and an increase in phenomenological fluxes. Metabolic analyses showed the changes of 39 metabolites, including 16 kinds of organic acids, 9 kinds of amino acids, 9 kinds of sugars, 3 kinds of sugar alcohols, and 2 amines. The identified metabolites were mainly involved in the glycolysis, pentose phosphate pathway, and tricarboxylic acids cycle.

Additional key words: antioxidant enzymes, chlorophyll *a* fluorescence, metabolites, NaCl, osmotic adjustment.

Introduction

The coastal zone has its special vegetation ecosystem that plays an important role in coastal soil and water conservation and most beach plants act as “bioshields” for beach protection, inhibition of coastal erosion, and sandstorms reduction (Dahdouh-Guebas and Koedam 2006). However, coastal beaches are narrow and isolated fragmented habitats that limit the size of plant populations and allow plant populations to be naturally constrained in gene exchange and survival (Hämmerli and Reusch 2003, Feagin *et al.* 2010). Coastal habitats are fragile, and, currently, the coastal beach plants are increasingly suffering from serious human encroachment (Rodgers *et al.* 2009, Iwasato and Nagamatsu 2018) and affected by various abiotic stresses including salt stress.

Many coastal halophytes have formed different salt tolerance mechanisms during long-term adaptive evolution. The effects of salinity on plants include reduced growth, ion toxicity, osmotic stress, mineral deficiencies,

nutritional disorder, photosynthetic imbalance, and combinations of these effects (Evelin *et al.* 2009, Jenkins *et al.* 2010, D’Amelia *et al.* 2018, Morton *et al.* 2019). Salt stress causes oxidative damage by excessive production of reactive oxygen species (Zheng *et al.* 2009, Zhu *et al.* 2019), and superoxide dismutase (SOD) is a major scavenger of O²⁻, as it catalyzes the dismutation of superoxide radical anions into O₂ and H₂O₂ (Scandalios 1993). The produced H₂O₂ is then dismutated into H₂O and O₂ by CAT and POD (Parida *et al.* 2004, Garg and Manchanda 2009). Salt stress also affects plant photosynthesis, including the function of chloroplasts (Allakhverdiev *et al.* 2000, Zhang *et al.* 2018). Metabolomics, as the end products of cellular regulatory processes, are crucial functional genomics tools for understanding plant response to salt stress (Lu *et al.* 2002, D’Amelia *et al.* 2018). Plants regulate the content of multiple metabolites to protect them from the detrimental effects of salinity (Kumari *et al.* 2015). Many studies have revealed that small soluble metabolites, including amino acids, such as proline, pipercolic acid, sugars,

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Abbreviations: CAT - catalase; DI - injury index; EL - electrolyte leakage; GC-MS - gas chromatography - mass spectrometry; MDA - malondialdehyde; OJIP - chlorophyll *a* fluorescence induction transient; POD - peroxidase; PS II - photosystem II; RWC - relative water content; SOD - superoxide dismutase; TCA - tricarboxylic acid; CK - control treatment.

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such as glucose, fructose, sucrose, trehalose, raffinose, and fructans, sugar alcohols, such as sorbitol, mannitol, glycerol, inositol, and methylated inositols, polyamines, such as putrescine, spermidine, and spermine participate in plants response to abiotic stresses (Mansour 2000, Stitt and Hurry 2002, Chen and Murata 2008, Ferchichi *et al.* 2018).

Carex pumila, (commonly known as draft sedge) is a widely distributed herb that is adapted to the coastal beach habitats (Burgess 1984). *C. pumila* is widely distributed for example in New Zealand, Australia, Chile, China, Japan, and Korea. As a clonal plant, *C. pumila* has large underground rhizome system, which plays an important role in the protection of coastal sands and soils. Unfortunately, because of the special nature of coastal habitats as well as other abiotic stress effects, the *C. pumila* population has gradually declined in China's coastal regions and has even disappeared in some regions. Sand dune vegetation has often been considered to be non-halophytic (Rozema *et al.* 1983), this is because sand salinity of the coastal zone is normally around 1 % (Hope-Simpson 1955) and sand is a highly porous medium which allows rapid salt leaching by rainwater. However, rain leaching is less effective in dry summer weather and in arid regions. Salinity may also be high in dune hollows, of up to 3 - 5 % NaCl (Sykes and Wilson 1989). As a halophyte, *C. pumila* has been previously reported by Sykes and Wilson (1989) to survive up to 2 % salt for a prolonged period. However, the physiological and metabolic mechanisms of salt tolerance in *C. pumila* have not been reported. Thus, understanding the mechanism of *C. pumila* response to salt stress is important for the conservation and utilization of *C. pumila* and for breeding of salt-tolerant crops.

This study was designed to investigate the mechanism of *C. pumila* response to salt stress. We particularly focused on the modulations of antioxidant enzymes activities, photosynthesis, and metabolisms. This study provides some insights into the possible mechanisms of salt tolerance ability in *C. pumila*.

Materials and methods

Plants and growth conditions: The *C. pumila* used in this study was collected from wild fields of Taohua Island, Zhejiang Province, China (latitude 29°48'59"N and longitude 122°17'43"E). The average salinity of habitat is about 1.231 %, and the salt content of seawater is about 2.4 %. About 15 cm rhizome were reserved and planted in triangular bottle (150 cm³ and wrapped in foil) with Hoagland's nutrient (Hoagland and Arnon 1950, Shahverdi *et al.* 2019) in incubator (CIMO-GZX-250BSH-III, Shanghai, China), which was set at a temperature of 26 °C, an irradiance of ~250 μmol(photons) m⁻² s⁻¹, a 16-h photoperiod, and a ~60 % relative humidity. The nutrient solution was replaced every 5 d and aerated 3 times per day for 30 min. One-month-old *C. pumila* healthy plants of a similar size were used for the next salt treatments.

Salt treatments and experimental design: The plants were subjected to five salinity levels: 0 (control), 200, 300, 400, and 500 mM NaCl. Salinity was increased every 8 h by increments of 100 mM NaCl until final salinity was achieved. After that, plants were exposed to the final NaCl concentrations for 60 h. During the period of salt exposure, the plants were maintained in the incubator under the same environmental conditions as mentioned above. At the end of the experiment, all leaves were sampled from the plants and then immediately frozen in liquid nitrogen and stored at -80 °C for further analyses. The salt treatments were arranged in a randomized complete block design with three replicates.

Salt injury (DI) index was scored for visible symptoms on a 1 - 4 scale as follows: 1 - no injury; 2 - browning on shoot-tips and leaf edges or slightly curling or drooping; 3 - serious leaves curling or drooping or/and wilting; 4 - dead. Following this scale, salt injury index (DI) was calculated according to the following formula: $DI = \sum(n_i \times i)/N$, where n_i is the number of explants receiving the mark "i" (from 1 to 4) and N is the total number of explants in each NaCl concentration (Sivritepe *et al.* 2008).

Quantification of electrolyte leakage: The relative electrolyte leakage (EL) was expressed as the ratio of initial conductivity to the conductivity after boiling and measured by a conductivity meter (Mettler Toledo-FE30-Plus, Shanghai, China). About 0.1 g of leaves was washed three times with deionized water and cut to about 0.5 cm long fragments. The fragments were transferred into a centrifuge tube (50 cm³) which was filled with 15 cm³ of deionized water, and then the initial conductivity (CA) was measured after being shaken for 24 h at room temperature. The samples were then heated to 95 °C for 30 min, and total conductivity (CB) of the bathing solution was then measured after cooling. Electrolyte leakage was calculated from the following equation: $EL [\%] = (CA/CB) \times 100$.

Relative water content: The surface of the leaves was quickly washed with deionized water, the fresh mass (FM) was measured immediately and the dry mass (DM) was weighed after drying at 80 °C for 15 h. Relative water content (RWC) was calculated from an equation: $RWC [\%] = (FW-DW)/FM \times 100$.

Malondialdehyde content was determined by the thiobarbituric acid (TBA) method according to Liu *et al.* (2016). About 1 cm³ of crude enzyme solution was added into 2 cm³ of malondialdehyde (MDA) reaction buffer, which included 0.5 % (v/v) TBA and 20 % (v/v) trichloroacetic acid. The mixture was heated at 95 °C for 30 min and then cooled to room temperature before being centrifuged at 12 000 g and 20 °C for 10 min. The absorbance (A) of the supernatant at 532 and 600 nm was measured using a spectrophotometer (TU-1810, Beijing, China). The MDA content was calculated as $[(A_{532} - A_{600}) \times L] / 1 \times \epsilon \times FM$, where L was the volume of the extract solution; l indicates the thickness of the cuvettes; ϵ was the coefficient of absorbance of 155 mM⁻¹ cm⁻¹.

Enzyme extraction and activities: To extract crude enzymes, about 0.3 g of leaves were ground to a fine powder in liquid nitrogen and then mixed with 4 cm³ of pre-cooled 150 mM sodium phosphate buffer (pH 7.0). The homogenate was transferred to 10-cm³ centrifuge tube for centrifugation (12 000 g, 4 °C, 20 min). The supernatant was collected for subsequent assays.

The superoxide dismutase (SOD; EC 1.15.1.1) activity was measured according to the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) (Fan *et al.* 2014). The reaction mixture (3 cm³) contained 2.2 cm³ of phosphate buffer (50 mM, pH 7.8), 0.1 cm³ of 3 μM EDTA-Na₂, 0.2 cm³ of 195 mM methionine (MET), 0.2 cm³ of 1.125 mM NBT, 0.2 cm³ of 0.06 mM riboflavin, and 0.1 cm³ of enzyme extract. The reaction mixture was kept 30 cm below the light source for 60 min. All reactants without NBT which served as light blank (CK0) were kept along with samples. The reaction mixture without enzyme extract incubated in the dark served as a dark blank (CKmax). The NBT reduction was estimated by monitoring the change in absorbance at 560 nm. The amount of enzyme extract that produced 50 % inhibition of NBT reduction was considered as 1 unit (U) of SOD activity.

The catalase (CAT; EC 1.11.1.6) activity was estimated by Fan *et al.* (2014) method with some modifications. Activity was measured in terms of the degradation of hydrogen peroxide. The reaction mixture contained 1.9 cm³ of phosphate buffer (50 mM, pH 7.4), 1 cm³ of 45 mM H₂O₂, and 0.1 cm³ of enzyme extract. Activity was measured by the decrease in absorbance at 240 nm at 1-min intervals for 3 min. A unit of CAT activity was defined as the absorbance change of 1 unit per minute.

The peroxidase (POD; EC 1.11.1.7) activity was measured by the method described by Fan *et al.* (2014). The reaction mixture contained 0.05 cm³ of crude enzyme solution, 1 cm³ of guaiacol (0.25 %, dissolved in 50 % ethanol), 1.85 cm³ of acetic acid buffer (pH 5.0), and 0.1 cm³ of 0.75 % (v/v) H₂O₂. Absorbance at 460 nm at 1-min intervals was measured for 3 min. A unit of POD activity was defined as the absorbance change of 1 unit per minute.

Chlorophyll *a* fluorescence transient (OJIP) curves:

The shape of the OJIP curve is very sensitive to environmental stresses. The OJIP transients of cells after salt stress treatments were measured using pulse-amplitude modulation (PAM) fluorimeter (*PAM 2500*, Heinz Walz, Effeltrich, Germany) with a high time resolution (10 μs). In this study, the leaves were kept in dark for 20 min before the measurements were started. The OJIP transients were triggered by a red radiation of 3000 μmol(photons) m⁻² s⁻¹ by the *PAM 2500* through an array of light-emitting diodes. The fluorescence was subsequently measured and digitized between 10 μs and 300 ms. The OJIP transients were analyzed by the JIP-test method described by Liu *et al.* (Liu *et al.* 2016).

Metabolite extraction and derivatization: Extraction and derivatization of metabolites from samples for gas

chromatography - mass spectrometry (GC-MS) analysis were performed by using a modified method that was previously outlined by Hu *et al.* (Hu *et al.* 2016). For each sample, about 0.3 g of fresh leaves were ground to a fine powder in liquid nitrogen, transferred into a centrifuge tube (15 cm³), and then extracted in 4.2 cm³ of 80 % (v/v) aqueous methanol under intensive oscillation (200 g) at 25 °C for 2 h. After extraction, 60 mm³ of ribitol solution (2 mg cm⁻³) as an internal standard was added and incubated in a metal bath at 70 °C for 15 min. The tube was centrifuged at 12 000 g and 20 °C for 5 min, the supernatant was decanted to new 10-cm³ tubes, and 4.5 cm³ of water and 2.25 cm³ of chloroform were added. The mixture was vortexed and centrifuged at 8 000 g for 15 min. The supernatant was the polar phase, which was collected for derivatization.

The polar phase supernatant (400 mm³) was transferred into 1.5 cm³ high-performance liquid chromatography (HPLC) vials and dried in a vacuum concentrator (*Labconco Corporation*, Kansas City, KS, USA) overnight. The dried residue was redissolved and derivatized under intensive oscillation (200 g) at 37 °C (in 110 mm³ of 20 mg cm⁻³ methoxyamine hydrochloride in pyridine) for 2 h followed by a 2 h treatment at 37 °C with 60 mm³ of N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA).

Metabolite data processing and analysis: The metabolites were measured by using GC-MS (*Agilent 7890A/5975C*, *Agilent Technologies*, Palo Alto, CA, USA) system based on the method of Xie *et al.* (2014). The 1 mm³ of the derivatized extract was injected into a *DB-5MS* capillary (30 m × 0.25 mm × 0.25 μm, *Agilent J&W GC* column) (Xie *et al.* 2014). The GC temperature was programmed as follows: 70 °C for 5 min and then an increase by a 5 °C per min rate to 300 °C and hold for 10 min. Subsequently, after 1 min of injection, the electron current was set at 70 eV, the injection temperature was adjusted to 280 °C, and the oven temperature was ramped up to 290 °C. The ion source temperature was 230 °C. After the solvent delay time of 5 min, the scanning speed was conducted at 0.6 scans s⁻¹ with a full scan mode (m/z 30/650).

The metabolites were identified by the application of the *Agilent MSD Productivity Chemstation* software and coupled with the commercially available compound libraries (*NIST 11*) (Gaithersburg, MD, USA). Relative quantification of the metabolites was determined according to the value of internal standard ribitol. The hierarchical clustering analysis (HCA) was done by using the *MetaboAnalyst* webpage (<http://www.metaboanalyst.ca/MetaboAnalyst/>) (Xia *et al.* 2009). Finally, we calculated the log-transformed response ratio of each identified substance before statistical estimation.

Statistical analysis: The experiments in this study were repeated three times and the data shown are the means ± SDs. The data were analyzed by one-way analysis of variance (*ANOVA*) followed by the least significant difference test at $\alpha = 0.05$ level using the statistical software *SPSS 20.0*.

Results

Compared with the control, there was no obvious morphological change at 200 mM NaCl, a few leaves dropped under 300 mM NaCl. However, the leaves turned yellow and curly after treatment with 400 and 500 mM NaCl (Fig. 1 Suppl.). Salt injury index showed no change at 200 mM NaCl and a considerable increase from 300 to 500 mM NaCl as compared to the control plants. (Fig. 1A). Moreover, leaf RWC decreased with an increase in the salinity which was especially significant at 300 to 500 mM NaCl (Fig. 1B).

The content of MDA, a product of lipid peroxidation, and EL reflect the extent of damage in the cell membranes. In this study, MDA and EL showed no change in 200 mM NaCl compared to the control, but gradually increased with increasing salt concentration. The highest MDA and EL values were reached at 500 mM NaCl but there was no significant difference between 400 and 500 mM NaCl (Fig. 1 C,D).

As compared to the control plants, antioxidative activities showed no remarkable changes at 200 mM NaCl (Fig. 2). The SOD activity also showed no change at 300 mM NaCl but significantly increased at 400 and especially at 500 mM NaCl (Fig. 2A). The POD activities similarly increased at 300 and 400 mM NaCl and further increased at 500 mM NaCl (Fig. 2B). The leaf CAT activities were significantly higher at 300 to 500 mM NaCl compared with the control, but the changes were not

significant among 300, 400, and 500 mM NaCl treatments (Fig. 2C).

There were no significant changes in the original fluorescence with increasing salinity, but the fluorescence yield at the J, I, and P phases (J-P curve) clearly changed. The J-P curve was higher in the 200 and 300 mM NaCl than in the control and the curve at 200 mM NaCl was higher than at 300 mM NaCl. At 400 and especially 500 mM NaCl, J-P curves were below the control (Fig. 3).

For the effects of salinity on electron transport at the acceptor side of photosystem (PS) II, ϕP_o , Ψ_o , and ϕE_o were compared under different NaCl concentrations. We observed that ϕP_o , Ψ_o , ϕE_o were unaffected up to 400 mM NaCl, but were significantly decreased at 500 mM NaCl. For the effect of salinity stress on the energy flux (per reaction center, RC), for electron transport (ET/RC), and dissipation (DlO/RC), there were no significant differences up to 400 mM NaCl, but increased DlO/RC and decreased ETo/RC were observed at 500 mM NaCl (Table 1 Suppl.).

The effects of salinity stress on the number of phenomenological fluxes for trapping per cross-section (TR_o/CS_o) and dissipation per cross-section (DI_o/CS_o), were not significantly different up to 400 mM NaCl, but increased the DI_o/CS_o and decreased TR_o/CS_o in 500 mM NaCl concentration was observed. High salinity stress (500 mM NaCl) significantly affected the PS II behavior, as quantified by the performance index; PI_{ABS} and PI_{CS} were decreased by 70 and 67 %, respectively, in 500 mM NaCl compared with the control (Table 1 Suppl.).

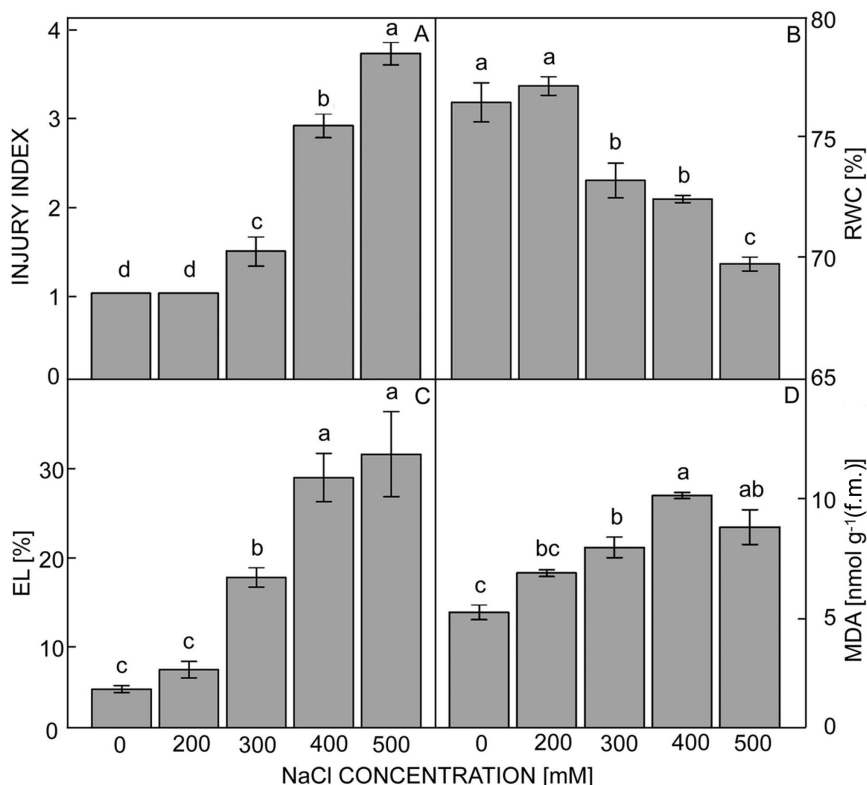


Fig. 1. The effects of salt stress on salt injury index (A), relative water content (RWC, B), electrolyte leakage (EL, C), and malondialdehyde (MDA) content (D) in *Carex pumila* leaves under different NaCl concentration (0, 200, 300, 400, and 500 mM) for 60 h. Means \pm SDs, $n = 3$, different letters indicate significant differences between treatments ($P < 0.05$) based on the least significant difference test.

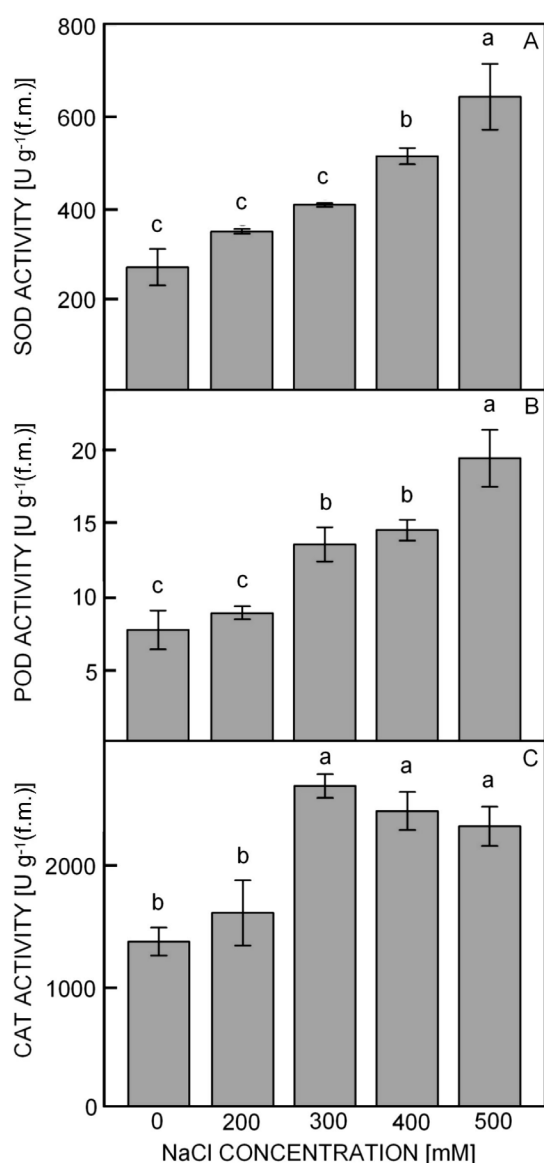


Fig. 2. Changes in superoxide dismutase (SOD, A), peroxidase (POD, B), and catalase (CAT, C) activities in the leaves of *Carex pumila* induced by different NaCl concentrations for 60 h. Means \pm SDs, $n = 3$, different letters indicate significant differences between treatments ($P < 0.05$) based on the least significant difference test.

Based on the analysis by GC-MS, a total of 39 kinds of differentially accumulated metabolites were identified and quantified, which included 9 amino acids, 16 organic acids, 9 sugars, 3 sugar alcohols, and 2 amines (Fig. 4).

The amount of most amino acids and their derivatives were higher in NaCl-treated plants than in the control group, and these were important products of amino acid metabolism and synthesis. The content of organic acids could be divided into two categories. The first category was increased under salt stress and these products were mostly produced by the TCA cycle. The second group was decreased under salt stress and these were products of fatty acid metabolism. The content of majority sugars and sugar alcohols was higher in salinity-treated plants and these

products are mainly involved in galactose metabolism. The content of amines was decreased by salt stress and mainly involved in nitrogen metabolism (Table 2 Suppl.).

Additionally, 17 metabolites comprising eight amino acids, five sugars, two sugar alcohols, and two organic acids assigned to glycolysis, tricarboxylic acid (TCA) cycle, and oxidative pentose phosphate pathway were identified (Fig. 2 Suppl., Table 3 Suppl.).

Discussion

Halophytes adapt to the salt environment through regulation of physiology and metabolism (Flowers and Colmer 2015). In our study, we found that *C. pumila* can survive stably in the coastal sandy land, and revealed the possible salt tolerance mechanism of halophytes based on its physiological, photosynthetic, and metabolite responses under salt stress.

Halophytes can tolerate and complete their life cycle at salt concentrations of 200 mM NaCl (Flowers and Colmer 2015). In the present study, *C. pumila* was defined as an obligate halophyte which can survive in 2% (about 350 mM NaCl) salinity, despite a decline in plant growth (Sykes and Wilson 1989). In this experiment, we observed that 300 mM NaCl induced no obvious morphological change, salt injury index gradually increased with an increase in salinity after 300 mM treatment. Plant water status is highly sensitive to salinity and, therefore, a dominant factor in determining the plant responses to stress (Meng *et al.* 2017). Regulation of water balance measured by leaf RWC is considered to be one of the most important adaptation measures to salt stress (Noreen *et al.* 2010, Ahmad *et al.* 2012, Franzini *et al.* 2019). In this work, significant declines in RWC were observed at 400 and 500 mM NaCl which was consistent with previous reports (Hu *et al.* 2012, Liu *et al.* 2016, Bela *et al.* 2018). This result corresponded with the ability of *C. pumila* to survive at the measured NaCl concentration in the field investigation. The stress time of this treatment was only 60 h, because we considered that *C. pumila* was mainly affected by the regular stress from seawater tides. In our continuous salt stress experiment, it was found that *C. pumila* showed obvious morphological (severe water loss in the leaves) changes under 300 mM salt stress at about 100 h. However, we still think that *C. pumila* could be a very good halophyte for the restoration of the coastal zone.

Membrane lipid peroxidation characterized by MDA content and EL has been often used as salt stress biomarkers (Ahmad *et al.* 2012, Hossain *et al.* 2019). In our study, both markers significantly increased under 300 to 500 mM NaCl, but showed no significant change at 200 mM NaCl. This result implied that under higher salinity (300 - 500 mM NaCl), *C. pumila* exhibited enhanced lipid peroxidation and oxidative damage. Such a pattern of MDA accumulation has already been observed in other halophytes such as *Salicornia persica* and *S. europaea* (Aghaleh *et al.* 2009).

It is well known that salinity increased accumulation

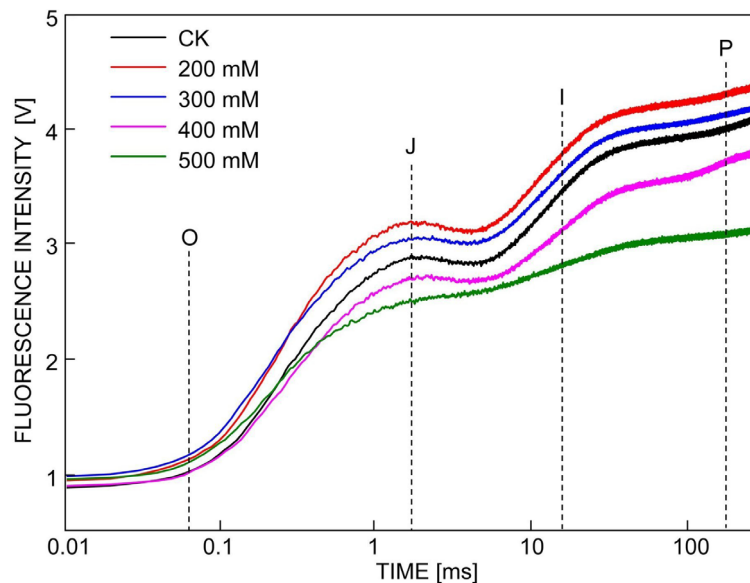


Fig. 3. Chlorophyll *a* fluorescence induction transients (OJIP) in *Carex pumila* leaves after different salt treatments (0, 200, 300, 400, and 500 mM NaCl) for 60 h. The leaves were vacuum-infiltrated for 20 min in the dark. O - minimal reliable recorded fluorescence at 20 μ s, J - fluorescence intensity at 2 ms, I - fluorescence intensity at 30 ms, P - maximal recorded fluorescence intensity.

of reactive oxygen species (ROS; Hernández *et al.* 2002, Hasanuzzaman *et al.* 2018), which can damage proteins, lipids, and nucleic acids (Foyer and Noctor 2005). The role of antioxidant enzymes (such as SOD, CAT, and POD) in salt-induced ROS scavenging has been demonstrated also in halophytes (Shabala 2013, 2016, Muchate *et al.* 2016, Rangani *et al.*). Among all the antioxidative enzymes, SOD is in the first line of defense converting superoxide to the less toxic H_2O_2 (Alscher *et al.* 2002, Bose *et al.* 2014). Enhanced H_2O_2 content is dismutated to H_2O and O_2 with the help of POD and CAT (Gao *et al.* 2020). An increase in the CAT activity has been shown as a measure of antioxidant defense in halophytes (Sekmen *et al.* 2012, Hossain *et al.* 2019). In our experiment, the SOD and POD activities gradually increased under 300 to 500 mM NaCl concentrations. It has been reported that exposure to salt stress in many plant species causes elevated activities of antioxidant enzymes, *e.g.* in *Vigna radiata* (Nazar *et al.* 2011), *Chenopodium quinoa* (Amjad *et al.* 2015), *Oryza sativa* (Lee *et al.* 2001), *Triticum aestivum* (Amjad *et al.* 2015), and *Sesuvium portulacastrum* (Muchate *et al.* 2016). However, in *C. pumila*, the CAT activity was enhanced significantly at 300 mM NaCl and the activity remained constant until 500 mM NaCl, as observed in quinoa (Amjad *et al.* 2015), indicating that CAT had a high capacity for the decomposition of H_2O_2 generated by SOD. The tolerance to high salinity in *C. pumila* derives largely from the constitutively high antioxidative enzymatic activities and CAT may be of major significance in the salt adaptive mechanism.

Salinity affects plant growth as a result of alterations in many physiological processes including photosynthesis (Xia *et al.* 2009, Kalaji *et al.* 2011, Zhang *et al.* 2018). Chlorophyll *a* fluorescence was regarded as a crucial tool for investigation of the photosystem II under abiotic stress conditions (Chen *et al.* 2013). In this study, the OJIP

transient curve declined under high NaCl concentrations (400 and 500 mM). The PI_{ABS} and PI_{CS} as a sensitive indicator of photosynthetic performance based on light absorption (Bi *et al.* 2016, Hu *et al.* 2016), significantly decreased under high salinity, which suggested that salinity stress may inhibit electron transport at the donor side of PS II. Hagemyer (1999) and Perales-Vela *et al.* (2007) reported that to protect the plant growth from the extremely adverse environmental condition, excess excitation energy was transformed into thermal dissipation so as to maintain the energy balance between absorption and utilization. Thus the PI_{ABS} and PI_{CS} value also confirmed this finding. This result is consistent with the observation that PS II was highly susceptible to salinity stress (Corney *et al.* 2003, Xia *et al.* 2009). Simultaneously, the quantum yields of primary photochemistry (ϕPo), the quantum yield for electron transport (ϕEo), and the efficiency per trapped excitation (Ψo) were also decreased by high salinity. These parameter can avail reliable information about electron transport activity at the acceptor side of PS II (Wang *et al.* 2011, Zhang *et al.* 2018). Furthermore, the specific energy fluxes (DI_o/RC and ET_o/RC) and phenomenological fluxes (TR_o/CS_o and DI_o/CS_o) of *C. pumila* leaves were markedly changed under high salinity (400 - 500 mM NaCl), and those fluxes had a vital effect on the photosynthetic activity and also revealing that salt stress inhibited the energy absorption and dissipation in *C. pumila* to reduce the excessive energy production. However, in this study, the OJIP transient curve were higher under low NaCl concentration (200 - 300 mM) than in the control group, but all the JIP-tests had no significant changes. This result indicated that low salinity may have little effect on *C. pumila* in quantum efficiencies and the activity of PS II reaction center. Therefore, this may also be the result of continuous adaptation and evolution of *C. pumila* in the long-term salt environment.

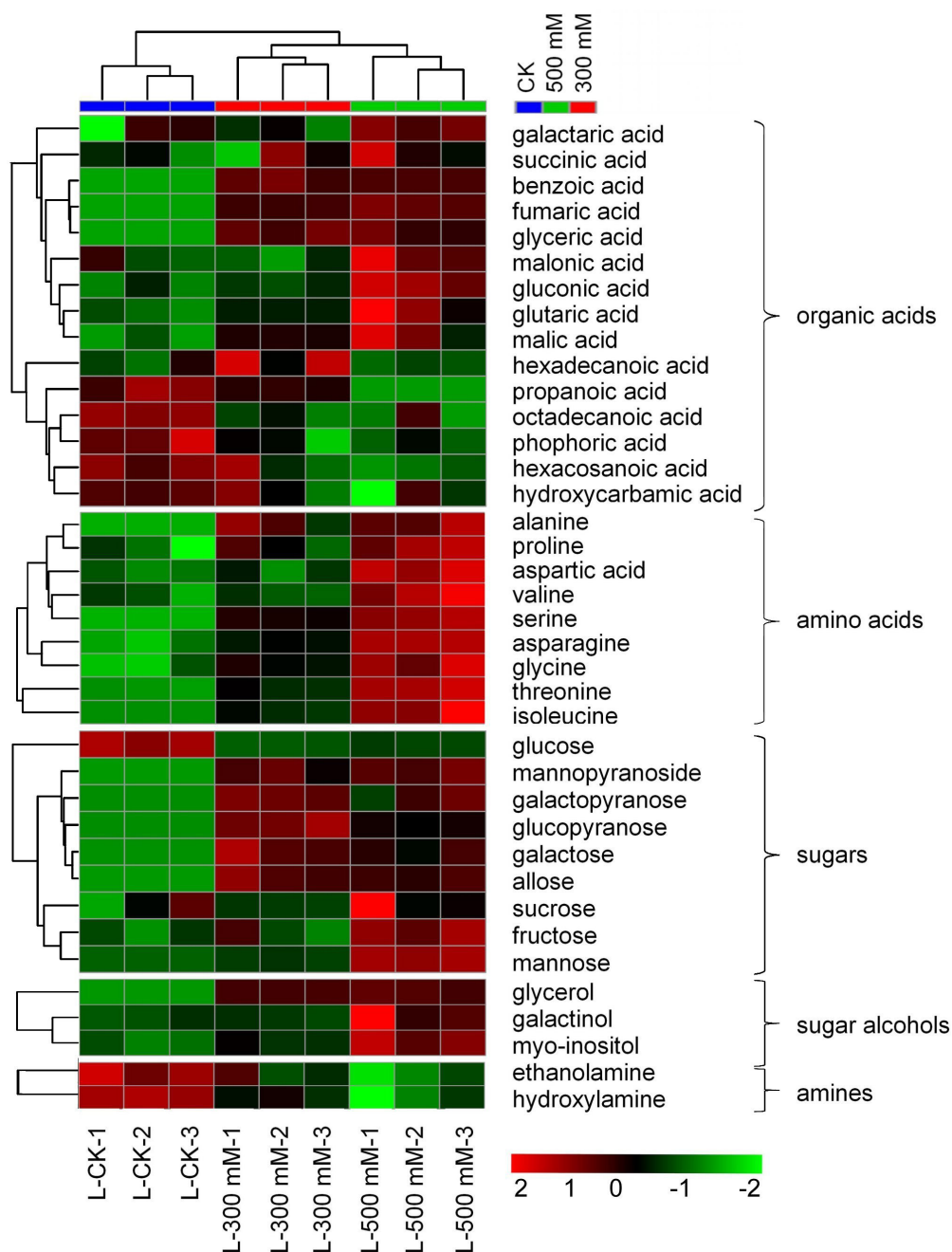


Fig. 4. The heat map of hierarchical cluster analysis of 39 metabolites in the leaves of *Carex pumila* in response to salt stress. The plants were treated with different NaCl concentrations (0, 300, and 500 mM) for 60 h, CK - control. Mean values were calculated from three independent experiments.

Metabolomics can perform a comprehensive analysis of a large number of metabolites, and is one of the fastest analytical methods for studying species under stress to determine products involved in metabolic responses (D'Amelia *et al.* 2018, Zhang *et al.* 2019). Plants respond to up and down regulation of multiple metabolites under salt stress, thereby preventing salt damage (Kumari *et al.* 2015, Ghatak *et al.* 2018). In this study, we found out that 39 metabolites were differentially expressed compared with control plants. Among these metabolites, there are amino acids, such as proline, alanine, arginine, serine,

threonine, which play major roles in osmoregulation during salt stress (Slama *et al.* 2015). Sugars and sugar alcohols constantly accumulate under salt stress and our experiment suggested that sugars frequently accumulated in plants under salinity. Previous studies have reported that sugars played major roles as osmoprotectant, carbon storage, and scavenger of ROS accumulation (Kumari *et al.* 2015, Zhang *et al.* 2019, Li *et al.* 2020). Sugar alcohols interact with proteins, enzymes, and membranes for protection of cellular structures and in signaling (Valluru and Van den Ende 2011). Organic acids are often considered to

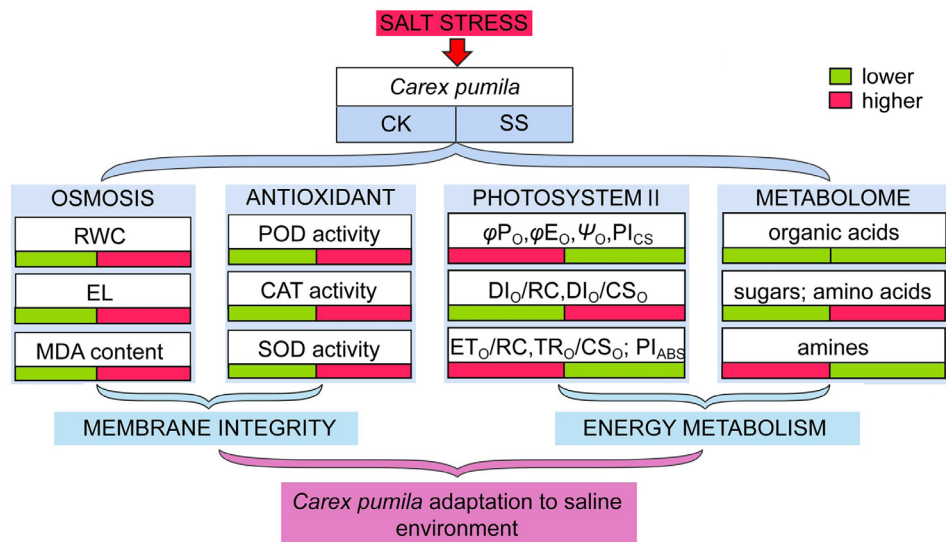


Fig. 5. The proposed model of *Carex pumila* physiological, photosynthetic, and metabolic responses under salt stress. The left boxes represent the control group (CK) and the right boxes represent the salt stress (SS). Different alterations are marked with different colours; green represents a decrease and red an increase in given characteristics. For the abbreviations - see the text.

maintain the osmotic potential (Rybka and Nita 2015, Chen *et al.* 2019, Wang *et al.* 2019). Among them, fatty acids are important phase solvent, especially palmitic acid and hexacosanoic acid, participating in membrane lipid bilayer flow and signal transduction (Guo *et al.* 2017). The metabolites exhibited significantly higher content under salt stress conditions. These results indicated that the widespread effects of salt treatment on carbon metabolism, amino acid metabolism, nitrogen metabolism, and fatty acid metabolism might play some role in salt stress resistance in *C. pumila* (Table 3 Suppl.). In our study, it can be found that under salt stress, many metabolites involved in a variety of physiological metabolic pathways respond to the salt environment. Our research only provides an analysis from the metabolic pathway, and, in the future, it should be combined with the molecular research to jointly reveal its salt tolerance mechanism.

Conclusions

This study revealed adaptation mechanisms of the salt-tolerant halophyte *C. pumila*. It might be attributed to the different responses of *C. pumila* to salt stress, including alterations of osmotic adjustment, antioxidant system activity, PS II performance, and metabolic homeostasis (Fig. 5). This result provides a theoretical basis for the selection of plant material for breeding of salt-tolerant plants and for ecological restoration in coastal areas.

References

Aghaleh, M., Niknam, V., Ebrahimzadeh, H., Razavi, K.: Salt stress effects on growth, pigments, proteins and lipid peroxidation in *Salicornia persica* and *S. europaea*. - Biol.

Plant. **53**: 243-248, 2009.

- Ahmad, P., Kumar, A., Ashraf, M., Akram, N.A.: Salt-induced changes in photosynthetic activity and oxidative defense system of three cultivars of mustard (*Brassica juncea* L.). - Afr. J. Biotechnol. **11**: 2694-2703, 2012.
- Allakhverdiev, S.I., Sakamoto, A., Nishiyama, Y., Inaba, M., Murata, N.: Ionic and osmotic effects of NaCl-induced inactivation of photosystems I and II in *Synechococcus* sp. - Plant Physiol. **123**: 1047-1056, 2000.
- Alscher, R.G., Erturk, N., Heath, L.S.: Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. - J. exp. Bot. **53**: 1331-1341, 2002.
- Amjad, M., Akhtar, S.S., Yang, A., Akhtar, J., Jacobsen, S.E.: Antioxidative response of quinoa exposed to iso-osmotic, ionic and non-ionic salt stress. - J. Agron. Crop Sci. **201**: 452-460, 2015.
- Bela, K., Riyazuddin, R., Horváth, E., Hurton, Á., Gallé, Á., Takács, Z., Zsigmond, L., Szabados, L., Tari, I., Csiszár, J.: Comprehensive analysis of antioxidant mechanisms in *Arabidopsis* glutathione peroxidase-like mutants under salt- and osmotic stress reveals organ-specific significance of the AtGPXL's activities. - Environ. exp. Bot. **150**: 127-140, 2018.
- Bi, A., Fan, J., Hu, Z., Wang, G., Amombo, E., Fu, J., Hu, T.: Differential acclimation of enzymatic antioxidant metabolism and photosystem II photochemistry in tall fescue under drought and heat and the combined stresses. - Front. Plant Sci. **7**: 453-453, 2016.
- Bose, J., Rodrigo-Moreno, A., Shabala, S.: ROS homeostasis in halophytes in the context of salinity stress tolerance. - J. exp. Bot. **65**: 1241-1257, 2014.
- Burgess, R.E.: The life history strategy of *Carex pumila* Thunb. (*Cyperaceae*), a rhizomatous perennial pioneer species on the sand plains of the dune system of coastal Manawatu. - Dissertations & Theses, Massey University, Palmerston North 1984.
- Chen, C., Liu, H., Wang, C., Liu, Z., Liu, X., Zou, L., Zhao, H., Yan, Y., Shi, J., Chen, S.: Metabolomics characterizes metabolic changes of *Apocyni Veneti* folium in response to salt stress. - Plant Physiol. Biochem. **144**: 187-196, 2019.
- Chen, K., Chen, L., Fan, J., Fu, J.: Alleviation of heat damage

- to photosystem II by nitric oxide in tall fescue. - *Photosynth. Res.* **1**: 21-31, 2013.
- Chen, T.H., Murata, N.: Glycinebetaine: an effective protectant against abiotic stress in plants. - *Trends Plant Sci.* **13**: 499-505, 2008.
- Corney, H.J., Sasse, J.M., Ades, P.K.: Assessment of salt tolerance in eucalypts using chlorophyll fluorescence attributes. - *New Forest.* **26**: 233-246, 2003.
- D'Amelia, L., Dell'Aversana, E., Woodrow, P., Ciarmiello, L. F., Carillo, P.: *Metabolomics for Crop Improvement against Salinity Stress. (Salinity Responses and Tolerance in Plants. Vol. 2.)* - Springer-Verlag, Cham - Berlin - Heidelberg 2018.
- Dahdouh-Guebas, F., Koedam, N.: Coastal vegetation and the Asian tsunami. - *Science* **311**: 37-38, 2006.
- Evelin, H., Kapoor, R., Giri, B.: *Arbuscular mycorrhizal fungi in alleviation of salt stress: a review.* - *Ann. Bot.* **104**: 1263-1280, 2009.
- Fan, J., Ren, J., Zhu, W., Amombo, E., Fu, J., Chen, L.: Antioxidant responses and gene expression in bermudagrass under cold stress. - *J. amer. Soc. hort. Sci.* **139**: 699-705, 2014.
- Feagin, R.A., Mukherjee, N., Shanker, K., Baird, A.H., Cinner, J., Kerr, A.M., Koedam, N., Sridhar, A., Arthur, R., Jayatissa, L.P.: Shelter from the storm? Use and misuse of coastal vegetation bioshields for managing natural disasters. - *Conserv. Lett.* **3**: 1-11, 2010.
- Ferchichi, S., Hessini, K., Dell'Aversana, E., D'Amelia, L., Woodrow, P., Ciarmiello, L.F., Fuggi, A., Carillo, P.: *Hordeum vulgare* and *Hordeum maritimum* respond to extended salinity stress displaying different temporal accumulation pattern of metabolites. - *Funct. Plant Biol.* **45**: 1096-1109, 2018.
- Flowers, T.J., Colmer, T.D.: Plant salt tolerance: adaptations in halophytes. - *Ann. Bot.* **115**: 327-331, 2015.
- Foyer, C.H., Noctor, G.: Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. - *Plant Cell Environ.* **28**: 1056-1071, 2005.
- Franzini, V.I., Azcón, R., Ruiz-Lozano, J.M., Aroca, R.: Rhizobial symbiosis modifies root hydraulic properties in bean plants under non-stressed and salinity-stressed conditions. - *Planta* **249**: 1207-1215, 2019.
- Gao, Y.F., Liu, J.K., Yang, F.M., Zhang, G.Y., Wang, D., Zhang, L., Ou, Y.B., Yao, Y.A.: The WRKY transcription factor WRKY8 promotes resistance to pathogen infection and mediates drought and salt stress tolerance in *Solanum lycopersicum*. - *Physiol. Plant.* **168**: 98-117, 2020.
- Garg, N., Manchanda, G.: ROS generation in plants: boon or bane? - *Plant Biosystem.* **143**: 81-96, 2009.
- Ghatak, A., Chaturvedi, P., Weckwerth, W.: *Metabolomics in plant stress physiology.* - *Adv. Biochem. Eng. Biotechnol.* **164**: 187-236, 2018.
- Guo, R., Shi, L., Yan, C., Zhong, X., Gu, F., Liu, Q., Xia, X., Li, H.: Ionic and metabolic responses to neutral salt or alkaline salt stresses in maize (*Zea mays* L.) seedlings. - *BMC Plant Biol.* **1**: 1-13, 2017.
- Hagemeyer, J. *Ecophysiology of plant growth under heavy metal stress.* - In: Hagemeyer, J.: *Heavy Metal Stress in Plants.* Pp. 201-222. Springer-Verlag, Berlin - Heidelberg 1999.
- Hämmerli, A., Reusch, T.: Inbreeding depression influences genet size distribution in a marine angiosperm. - *Mol. Ecol.* **12**: 619-629, 2003.
- Hasanuzzaman, M., Oku, H., Nahar, K., Bhuyan, M.B., Al Mahmud, J., Baluska, F., Fujita, M.: Nitric oxide-induced salt stress tolerance in plants: ROS metabolism, signaling, and molecular interactions. - *Plant Biotechnol. Rep.* **12**: 77-92, 2018.
- Hernández, J.A., Almansa, M.S.: Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves. - *Physiol. Plant.* **115**: 251-257, 2002.
- Hoagland, D.R., Arnon, D.I.: The water-culture method for growing plants without soil. - *Circular Calif. Agr. Exp. Sta.* **347**: 1-39, 1950.
- Hope-Simpson, R.B.J.F.: Downs and dunes: their plant life and its environment. by Edward Salisbury. - *J. Ecol.* **43**: 311-315, 1955.
- Hossain, M.S., Hasanuzzaman, M., Sohag, M.M.H., Bhuyan, M.B., Fujita, M.: Acetate-induced modulation of ascorbate: glutathione cycle and restriction of sodium accumulation in shoot confer salt tolerance in *Lens culinaris* Medik. - *Physiol. mol. Biol. Plants* **25**: 443-455, 2019.
- Hu, L., Hu, T., Zhang, X., Pang, H., Fu, J.: Exogenous glycine betaine ameliorates the adverse effect of salt stress on perennial ryegrass. - *J. amer. Soc. hort. Sci.* **137**: 38-46, 2012.
- Hu, Z., Fan, J., Xie, Y., Amombo, E., Liu, A., Gitau, M.M., Khaldun, A., Chen, L., Fu, J.: Comparative photosynthetic and metabolic analyses reveal mechanism of improved cold stress tolerance in bermudagrass by exogenous melatonin. - *Plant Physiol. Biochem.* **100**: 94-104, 2016.
- Iwasato, M., Nagamatsu, D.: Plant species diversity and habitat conditions in a protected large coastal dune area of western Japan. - *Lands Ecol. Eng.* **14**: 99-113, 2018.
- Jenkins, S., Barrett-Lennard, E.G., Rengel, Z.: Impacts of waterlogging and salinity on puccinellia (*Puccinellia ciliata*) and tall wheatgrass (*Thinopyrum ponticum*): zonation on salt land with a shallow water-table, plant growth, and Na⁺ and K⁺ concentrations in the leaves. - *Plant Soil* **329**: 91-104, 2010.
- Kalaji, H.M., Bosa, K., Kościelniak, J., Żuk-Golaszewska, K.: Effects of salt stress on photosystem II efficiency and CO₂ assimilation of two Syrian barley landraces. - *Environ. exp. Bot.* **73**: 64-72, 2011.
- Kumari, A., Das, P., Parida, A.K., Agarwal, P.K.: Proteomics, metabolomics, and ionomics perspectives of salinity tolerance in halophytes. - *Front. Plant Sci.* **6**: 537-537, 2015.
- Lee, D.H., Kim, Y.S., Lee, C.B.: The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza sativa* L.). - *J. Plant Physiol.* **158**: 737-745, 2001.
- Li, Z., Cheng, B., Zeng, W., Zhang, X., Peng, Y.: Proteomic and metabolomic profilings reveal crucial function of γ -aminobutyric acid (GABA) on regulating ionic, water, and metabolic homeostasis in creeping bentgrass under salt stress. - *J. Proteome Res.* **19**: 769-780, 2020.
- Liu, A., Hu, Z., Bi, A., Fan, J., Gitau, M.M., Amombo, E., Chen, L., Fu, J.: Photosynthesis, antioxidant system and gene expression of bermudagrass in response to low temperature and salt stress. - *Ecotoxicology* **25**: 1445-1457, 2016.
- Lu, C., Vonshak, A.: Effects of salinity stress on photosystem II function in cyanobacterial *Spirulina platensis* cells. - *Physiol. Plant.* **114**: 405-413, 2002.
- Mansour, M.: Nitrogen containing compounds and adaptation of plants to salinity stress. - *Biol. Plant.* **43**: 491-500, 2000.
- Meng, R., Saade, S., Kurtek, S., Berger, B., Brien, C., Pillen, K., Tester, M., Sun, Y.: Growth curve registration for evaluating salinity tolerance in barley. - *Plant Methods* **13**: 18-18, 2017.
- Morton, M.J., Awlia, M., Al-Tamimi, N., Saade, S., Pailles, Y., Negrão, S., Tester, M. Salt stress under the scalpel-dissecting the genetics of salt tolerance. - *Plant J.* **97**: 148-163, 2019.
- Muchate, N.S., Nikalje, G.C., Rajurkar, N.S., Suprasanna, P., Nikam, T.D.: Physiological responses of the halophyte *Sesuvium portulacastrum* to salt stress and their relevance for saline soil bio-reclamation. - *Flora* **224**: 96-105, 2016.
- Nazar, R., Iqbal, N., Syeed, S., Khan, N.A.: Salicylic acid alleviates decreases in photosynthesis under salt stress by enhancing nitrogen and sulfur assimilation and antioxidant

- metabolism differentially in two mungbean cultivars. - *J. Plant Physiol.* **168**: 807-815, 2011.
- Noreen, Z., Ashraf, M., Akram, N. Salt-induced regulation of some key antioxidant enzymes and physio-biochemical phenomena in five diverse cultivars of turnip (*Brassica rapa* L.). - *J. Agron. Crop Sci.* **196**: 273-285, 2010.
- Parida, A.K., Das, A.B., Mohanty, P.: Defense potentials to NaCl in a mangrove, *Bruguiera parviflora*: differential changes of isoforms of some antioxidative enzymes. - *J. Plant Physiol.* **161**: 531-542, 2004.
- Perales-Vela, H.V., González-Moreno, S., Montes-Horcasitas, C., Cañizares-Villanueva, R.O.: Growth, photosynthetic and respiratory responses to sub-lethal copper concentrations in *Scenedesmus incrassatulus* (Chlorophyceae). - *Chemosphere* **67**: 2274-2281, 2007.
- Rangani, J., Parida, A.K., Panda, A., Kumari, A.: Coordinated changes in antioxidative enzymes protect the photosynthetic machinery from salinity induced oxidative damage and confer salt tolerance in an extreme halophyte *Salvadora persica* L. - *Front. Plant Sci.* **7**: 50-50, 2016.
- Rodgers III, J.C., Murrah, A.W., Cooke, W.H.: The Impact of hurricane Katrina on the coastal vegetation of the Weeks Bay Reserve, Alabama from NDVI data. - *Estuaries Coasts* **32**: 496-507, 2009.
- Rozema, J., Van Manen, Y., Vugts, H., Leusink, A.: Airborne and soilborne salinity and the distribution of coastal and inland species of the genus *Elytrigia*. - *Acta bot. neerl.* **32**: 447-456, 1983.
- Rybka, K., Nita, Z.: Physiological requirements for wheat ideotypes in response to drought threat. - *Acta Physiol. Plant.* **37**: 97-97, 2015.
- Scandalios, J.G.: Oxygen stress and superoxide dismutases. - *Plant Physiol.* **101**: 7-12, 1993.
- Sekmen, A.H., Turkan, I., Tanyolac, Z.O., Ozfidan, C., Dinc, A.: Different antioxidant defense responses to salt stress during germination and vegetative stages of endemic halophyte *Gypsophila oblaunceolata* BARK. - *Environ. exp. Bot.* **77**: 63-76, 2012.
- Shabala, S.: Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. - *Ann. Bot.* **112**: 1209-1221, 2013.
- Shahverdi M. A., Omid H, Tabatabaei S. J.: *Stevia (Stevia rebaudiana* Bertoni) responses to NaCl stress: growth, photosynthetic pigments, diterpene glycosides and ion content in root and shoot. - *J. Saudi Soc. agr. Sci.* **18**: 355-360, 2019.
- Sivritepe, N., Erturk, U., Yerlikaya, C., Turkan, I., Bor, M., Ozdemir, F.: Response of the cherry rootstock to water stress induced *in vitro*. - *Biol. Plant.* **52**: 573-576, 2008.
- Slama, I., Abdelly, C., Bouchereau, A., Flowers, T., Savoure, A.: Diversity, distribution and roles of osmoprotective compounds accumulated in halophytes under abiotic stress. - *Ann. Bot.* **115**: 433-447, 2015.
- Stitt, M., Hurry, V.: A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in Arabidopsis. - *Curr. Opin. Plant Biol.* **5**: 199-206, 2002.
- Sykes, M., Wilson, J.: The effect of salinity on the growth of some New Zealand sand dune species. - *Acta Bot. Neerlandica* **38**: 173-182, 1989.
- Valluru, R., Van den Ende, W.: Myo-inositol and beyond-emerging networks under stress. - *Plant Sci.* **181**: 387-400, 2011.
- Wang, G., Chen, L., Hao, Z., Li, X., Liu, Y.: Effects of salinity stress on the photosynthesis of *Wolffia arrhiza* as probed by the OJIP test. - *Fresenius environ. Bull.* **20**: 432-438, 2011.
- Wang, Y., Zeng, X., Xu, Q., Mei, X., Yuan, H., Jiabu, D., Sang, Z., Nyima, T.: Metabolite profiling in two contrasting Tibetan hullless barley cultivars revealed the core salt-responsive metabolome and key salt-tolerance biomarkers. - *AoB Plants* **11**: plz021, 2019.
- Xia, J., Psychogios, N., Young, N., Wishart, D.S.: MetaboAnalyst: a web server for metabolomic data analysis and interpretation. - *Nucl. Acids Res.* **37**: 652-660, 2009.
- Xie, Y., Hu, L., Du, Z., Sun, X., Amombo, E., Fan, J., Fu, J.: Effects of cadmium exposure on growth and metabolic profile of bermudagrass [*Cynodon dactylon* (L.) Pers.]. - *PLoS ONE* **9**: e115279, 2014.
- Zhang, H., Li, X., Zhang, S., Yin, Z., Zhu, W., Li, J., Meng, L., Zhong, H., Xu, N., Wu, Y.: Rootstock alleviates salt stress in grafted mulberry seedlings: physiological and PS II function responses. - *Front. Plant Sci.* **9**: 1806, 2018.
- Zhang, Y., Li, D., Zhou, R., Wang, X., Dossa, K., Wang, L., Zhang, Y., Yu, J., Gong, H., Zhang, X.: Transcriptome and metabolome analyses of two contrasting sesame genotypes reveal the crucial biological pathways involved in rapid adaptive response to salt stress. - *BMC Plant Biol.* **19**: 66-66, 2019.
- Zheng, C., Jiang, D., Liu, F., Dai, T., Jing, Q., Cao, W.: Effects of salt and waterlogging stresses and their combination on leaf photosynthesis, chloroplast ATP synthesis, and antioxidant capacity in wheat. - *Plant Sci.* **176**: 575-582, 2009.
- Zhu, D., Hou, L., Xiao, P., Guo, Y., Deyholos, M.K., Liu, X.: VvWRKY30, a grape WRKY transcription factor, plays a positive regulatory role under salinity stress. - *Plant Sci.* **280**: 132-142, 2019.