Concomitant accumulations of ions, osmoprotectants and antioxidant system-related substances provide salt tolerance capability to succulent extreme-halophyte Scorzonera hieraciifolia

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Abstract

Halophytes adapting to live in salinized areas can activate some tolerance mechanism through signal compounds to cope with salinity. However, the role of co-activity of signal compounds in salt tolerance of halophytes is not yet fully understood. We have firstly detected that *Scorzonera hieraciifolia* with fleshy shoots is a succulent extreme-halophyte and researched the changes in signal compounds involved in the salt tolerance mechanism, including inorganic ions, osmoprotectants and substances related to antioxidant system. The levels of signal compounds such as calcium, magnesium, proline, soluble sugar, hydrogen peroxide, superoxide, ascorbate and glutathione concomitantly increased when thickness of shoot tissues enhanced under excess salinity. There were 3.3-fold, 5-fold, 8-fold and 10-fold enhancements in the levels of inorganic ions (Ca^{2+} and Mg^{2+}), hydrogen peroxide, ascorbate and glutathione in the shoots treated with excess salinity, respectively. Contents of sodium, potassium and chlorine, and antioxidant enzyme activities, superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, catalase and glutathione reductase, also increased in the salinized shoots. Western blot analysis showed that the increases in antioxidant enzyme activities were consistent with increases in their protein contents.

The results suggest that extraordinary salt tolerance capacity in *Scorzonera hieraciifolia*, a succulent extreme-halophyte can be improved by modulated accumulations of signal compounds, especially calcium, magnesium, osmoprotectants, reactive oxygen species and antioxidant substances. Moreover, massive induction of antioxidant enzymes can make strong contributions to salt stress tolerance of *S. hieraciifolia*.

Keywords Extreme-halophyte [?] signal compound [?] salt tolerance [?] succulent [?] antioxidant system [?] osmoprotectant

Introduction

Salt stress, one of the abiotic stresses has become a common problem for crops all over the world, especially in recent years. Salinity can cause osmotic and ionic imbalance, functional and structural protein damage and membrane injury in plants. However, plants can promote some tolerance mechanism linked with signal transduction, reactive oxygen species (ROS) production, induction of antioxidants, synthesizing of osmoprotectants, controlling ion absorption, and expression of salt responsive genes and transcription factor to cope with salinity (Gupta and Huang 2014; Mishra and Tanna 2017). In addition, plants adapted to live in salinized areas may exhibit physiological and morphological changes such as leaf shedding and succulent structures (Mishra and Tanna 2017).

Signal compounds, a member of signaling networks, participate in the growth and developmental responses of plants to environmental factors. One of them, ascorbic acid (ASC) reported in recent years, may trigger cytosolic calcium elevation, contribute to the propagation of ROS/Ca^{2+} signals in plant tissue and thus

ascorbate may be linked to calcium signaling. Ascorbate, a major antioxidant in plants, hypothetically acts as an extracellular signaling and regulatory molecule having high importance for a number of physiological functions (Makavitskaya et al. 2018). The role of glutathione (GSH) as a key-redox signaling component was also established in activation of various defense mechanisms against unfavorable stress factors. In this signaling pathway, GSH can interact with other established signaling molecules including ROS (Ghanta and Chattopadhyay 2011). As the same way, some osmoprotectants such as proline and soluble sugar play signal role in abiotic stress tolerance of maize seedlings (Hayat et al. 2012; Altuntaş et al. 2019). On the other hand, osmoprotectants accumulated in plants during water deficiency and salinity help plants to avoid ion toxicity and maintain water uptake under the stress conditions without preventing the normal metabolic processes. Like organic solutes (soluble sugars, proline, betaine, glycerol, and other low molecular weight metabolites), inorganic ions (Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^-) also have a osmoprotectant property and play a key role in osmotic adjustment to maintain water uptake (Zhou and Yu 2009; Chen and Jiang 2010). As seen, signaling roles of some organic osmoprotectants and calcium, an inorganic osmoprotectant were well known in plants. Although, putative second messenger function of magnesium has been currently reported in an animal cell (Stangherlin and O'Neill 2018), no the signaling roles of magnesium and other inorganic ions in responses of plants to abiotic stresses have been clarified yet.

The osmotic regulation by accumulation of inorganic anions and cations in the vacuole is the most important feature of succulent halophytes (Shabala and Mackay 2011). It was reported that detrimental effects of increased Na^+ and Cl^- contents were prevented by enhanced uptake of ions such as Ca^{2+}, Mg^{2+} and K^+ in extreme halophyte two grasses (Mangalassery et al. 2017). Conversely, Ahmad et al. (2013) noted that Ca^{2+}, Mg^{2+} and K^+ contents declined while Na^+ concentration was increasing in shoot of *Salicornia persica*. They suggested that succulent halophytic *Salicornia persica* may use sodium ion for acceleration of water uptake under water shortage, to regulate sodium concentration in cellular spaces. On the other hand, in plant cells, ensuring potassium hemostasis in a salinity environment is a key factor in detecting capability of salt tolerance. Moreover, the plants maintaining cytosolic K^+/Na^+ ratio can have salt tolerance capacity (Liang et al. 2018). In the light of these records, the contribution of variations in the mineral ion levels to the salinity tolerance of halophytes exposed to high salt concentrations is not well understood.

High salt concentrations in the living environment can cause hyperosmotic stress as well as ion imbalance in plants. A result of these primary effects, oxidative stress can occur as a secondary effect (Gupta and Huang 2014). However, the induction of antioxidant defense system consisting of enzymatic and non-enzymatic components maintains balance of reactive oxygen species, which include hydrogen peroxide (H_2O_2), superoxide radical ($O_2[?]^-$), hydroxyl free radicals (*OH) and singlet oxygen ($^1O_2$) within the cell. Enzymatic antioxidants such as superoxide dismutase (SOD) (EC 1.15.1.1), catalase (CAT) (EC 1.11.1.6), guaiacol peroxidase (GPOD) (EC. 1.11.1.7), ascorbate peroxidase (APX) (EC 1.11.1.11), glutathione reductase (GR) (EC 1.6.4.2) and non-enzymatic antioxidants such as ascorbate, glutathione, carotenoids and polyphenols scavenge different types of ROS (Gupta and Huang 2014). Ascorbate is considered as the most popular and powerful ROS detoxifying compound because of its electron donor ability in a number of enzymatic and non-enzymatic reactions (Gupta and Huang 2014). Well managed oxidative stress significantly contributed to the tolerance capacity of salinized plants by keeping cellular ROS levels in balance (Gupta and Huang 2014). In this context, salt tolerance capacity of plant species is closely related to maintaining the effectiveness of the antioxidant system.

Some studies have been conducted on the salinity tolerance mechanism of non-succulent or succulent halophytes. For instance, Shabala and Mackay (2011) recorded that succulent structures were associated with an increase in mesophyll cell size and the relative size of their vacuoles, a decrease in surface area per tissue volume, and high water content per unit surface area. Zeng et al. (2018) investigated some antioxidant enzyme activities in succulent halophyte *Carpobrotus rosii* suggested that salt stress resulted in significant increases in activities of major antioxidant enzymes, such as APX, CAT and GR and decrease in SOD activity in the mesophyll tissue. Likewise, non-succulent extreme-halophyte *Thellungiella parvula*, a halophytic relative of Arabidopsis (*Arabidopsis thaliana*), could regulate ion hemostasis by providing osmotic adjustment and alleviate hazardous effects of excess salinity by inducing antioxidant system (Uzilday et al. 2015).

However, more research should be performed on signal compounds to explain the salt tolerance mechanism of succulent-extreme halophytes.

Scorzonera hieraciifolia is an endemic halophyte species that is mainly distributed in salt habitats in Irano-Turanian regions. It has been currently reported that *S. hieraciifolia* is a medicinal plant having in vitro antioxidant and anti-inflammatory activity (Sarı et al. 2019). We have observed fleshy shoots in *S. hieraciifolia* in the habitat. Understanding of the salt tolerance mechanism of *S. hieraciifolia*, a medicinal succulent extreme-halophyte, may provide a contribution to cultivation of medicinal plants in salinized areas.

The aim of the present study is to elucidate the signaling compounds involved in responses of the succulent extreme-halophytes to salt stress, including osmotic regulation and induction of antioxidant system. Firstly, extreme halophyte, *Thellungiella parvula* (syn. *Eutrema parvulum*), closely related to Arabidopsis (Uzilday et al. 2015), is used in the present research to determine whether *S. hieraciifolia* is an extreme-halophyte. Based on the measurement of some stress parameters indicating stress damage such as relative water content, lipid peroxidation and H_2O_2 content by comparing to *Thellungiella parvula* under different salinity conditions (0, 150, 300, 450 and 600 mM), it was determined that *S. hieraciifolia* was an extreme-halophyte. We hypothesized that extraordinary salt tolerance of succulent extreme-halophytes can be provided by combined induction of multiple signal compounds especially calcium, magnesium, osmoprotectants, reactive oxygen species and antioxidant substances. We also tested the hypothesis that induction of antioxidant system can make strong contributions to salt stress tolerance of S. *hieraciifolia*.

Materials and methods

Plant materials and sterilization

Scorzonera hieraciifolia L. achenes were collected from Sivas Tödürge Lake (İran-Turan Region, Turkey) and brought to the laboratory. *Thellungiella parvula* (synonym, *Eutrema parvulum*) seeds gently requested and acquired from Prof. Dr. İsmail TURKAN, Ege University, İzmir, TURKEY. The mature achenes of *S. hieraciifolia* were carefully separated from their pappus bristles. The achenes were stratified for 2 d at 4°C in petri dishes containing double-layer wet filter paper. After that, the achenes were treated with 10% (v/v) sodium hypochlorite (NaOCl) solution for sterilization, followed by three rinsing stages with sterile distilled water.

To determine whether *S. hieraciifolia* is an extreme halophyte plant, we compared *S. hieraciifolia* with the model plant for extreme halophytes, *T. parvula*, closely related to *Arabidopsis thaliana*. Similarly, *T. parvula* seeds were treated with 10% (v/v) NaOCl solution for sterilization followed by three rinsing stages with sterile distilled water.

Media, culture conditions and stress treatments

The sterilized *S. hieraciifolia* achenes and *T. parvula* seeds aseptically germinated at MS medium (pH 5.8) (Murashige and Skoog 1962) which is sterilized by autoclaving for 15 min at 121°C and 1 atm pressure. The nutrient basal medium is consisted of MS including vitamins (4.95 g L^{-1}), 1% sugar (w/v) and 0.8% agar (w/v). This basal medium was supplemented with growth regulators including naphthalene acetic acid (NAA, 0.1 mg L^{-1}), kinetin (1 mg L^{-1}), isopentenyl adenine (2IP, 2 mg L^{-1}) at different developmental stage of the plants. *S. hieraciifolia* and *T. parvula* plants were grown until their shoots develop (for 45 days and 35 days, respectively), at 24±2 °C, on light intensity 400-430 µmol photons $m^{-2}s^{-1}$ and 16h light/8 h dark photoperiod. Subsequently, *S. hieraciifolia* and *T. parvula* plants were exposed to salt stress through 0, 150, 300, 450 and 600 mM NaCl supplements to MS media for 7 d. Salt concentrations were gradually enhanced by 150 mM NaCl supplements with 2 d intervals until reaching the last salinity levels (150, 300, 450 or 600 mM). Thus, gradually increasing salt concentrations were applied to the plants with transferring of the plants from previous salt concentration to the next concentration. Five different NaCl treatment groups (0, 150, 300, 450 and 600 mM NaCl) were constituted for *S. hieraciifolia* and *T. parvula*. Morphological observations and, following measurements and analysis were conducted.

Stress determination parameters

To determine whether *S. hieraciifolia* is an extreme halophyte, we comparatively observed morphological variations and determined changes in some stress parameters such as relative water content (RWC), lipid peroxidation and H_2O_2 content in *S. hieraciifolia* and *T. parvula*.

Relative water content

The relative water content of *S. hieraciifolia* and *T. parvula* shoots were measured according to Castillo (1996). After shoot fresh weight (FW) was immediately noted, the samples were incubated for at 4°C 16 h into distilled water. Shoots were then blotted and shoot turgid weight (TW) was measured. To measure the shoot dry weight (DW), samples were continuously incubated in an oven at 80 °C until achieving a constant dry weight. The dry weights were recorded. The shoot RWC was calculated using following formula: RWC (%) = (FW- DW) / (TW-DW) x 100

Lipid peroxidation

The levels of lipid peroxidation in *S. hieraciifolia* and *T. parvula* shoots were determined to assess the membrane damage in terms of thiobarbituric acid reactive substances (TBARS) according to the method by Heath and Packer (1968). The shoot samples (0.1 g) were homogenized 0.1% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 15,000 g for 5 min. The supernatant (1 ml) was mixed with 4 ml 0.5% (w/v) thiobarbituric acid in 20 % TCA. The mixture was heated at 95 °C for 30 min and then cooled on ice bath. The absorbance of the supernatant 532 and 600 nm was read and the value of 600 nm was subtracted from 532 nm value. The amount of MDA-TBA complex was calculated from the extinction of 155 $mM^{-1}cm^{-1}$.

H_2O_2 content

Hydrogen peroxide (H_2O_2) content was determined using a modified method of Velikova et al. (2000). *S. hieraciifolia* and *T. parvula* samples were extracted with 5 % cold TCA (w/v) with activated charcoal. After the extract was centrifuged, 10 mM potassium phosphate (K_2HPO_4) buffer (pH 7.0) and 1 M potassium iodide (KI) were added to 1 ml supernatant. The absorbance was then read at 390 nm.

Succulence measurements

We measured succulence degree, shoot thickness and plant area to determine the shoot succulence in extreme-halophyte *S. hieraciifolia*. The shoot succulence degree (SSD) was performed according to Qi et al. (2008). After measuring fresh weights and dry weights of *S. hieraciifolia*, the shoot succulence degree was expressed as the ratio of these two values to each other.

To determine shoot thickness (ST), all shoots of each treatment were transversally cut with the help of a razor blade in *S. hieraciifolia*. Subsequently, widths of the shoots were measured at several points under light microscope (Olympus, CX21) with ocular meter and it was calculated by averaging these values.

The images of each plant area (PA) were determined by "ImageJ" Software program. In this program, the images had to be in a certain format (8-bit grayscale, 16-bit gray scale, etc.). Image-Type 8-bit option which is appropriate to *S. hieraciifolia* was selected and the scale was adjusted by entering the reference range 1 cm from the Analyze-Set Scale option. The area calculation for each shoot was performed from the Analyze-Tools-ROI Manager step.

Determination of osmoprotectants

Changes in total soluble sugar and proline contents in *S. hieraciifolia* exposed to enhanced salinity were determined.

Soluble sugar

To detect soluble sugar content, dry samples of *S. hieraciifolia* (0.1 g) were homogenized with 5 ml 70% ethanol, the homogenate was boiled at 80 °C for 3 min and centrifuged at 10,000g for 5 min. For spectrophotometric measurement, 900 µl of distilled water was added to 100 µl of the supernatant and diluted. 1 ml of 5% phenol was added to this mixture and 5 ml of 96% sulfuric acid was added and then the mixture was homogenized by vortex. The mixture was cooled to room temperature and the absorbance of the mixture was recorded at 490 nm (Dubois 1956).

Proline

Proline content was measured according to Bates et al. (1973). Oven dried shoots (0.1 g) were homogenized with 3% sulfosalicylic acid (5ml) and filtrated. One ml of filtrate, equal one ml of glacial acetic acid and ninhydrin reagent were mixed and subsequently incubated for 1 h at 100°C. After that, the test tubes were placed in an ice bath in order to stop reaction. Toluene were added to the samples and shaken by a vortex. The UV-VIS spectrophotometer (Nicolet Evolution 100, Thermo) recorded absorbance of the toluene phase at 520 nm. A standard curve was used to determine the proline concentration.

Inorganic ions

Using dry extracts of the shoots, anion-cation analyzes were performed with the Dionex ICS-5000 system (Haddad 1997). Analysis of chloride ion content was performed with Dionex Ion Pac AS 9 HC (4x250 mm) separation column and 10 mM sodium carbonate fluid phase (flow rate: 1 ml/min). Analysis of the cation ion contents (Na^+, K^+, Mg^{2+}, Ca^{2+}) was carried out with Dionex Ion Pac CS 12 A (3x150 mm) column and 20 mM meta sulfonic acid conductor phase (flow rate: 0.5 ml/min). Dionex CERS 500 (2 mm) was used as cation suppressor column and Dionex AERS 500 (4 mm) was used as anion suppressor column. Samples were given to the column in 20 µl volumes by auto-sampler. Thermo Scientific conductivity detector was used in the system and calculations were made with Chromeleon Software System.

Antioxidant system-related substances

ROS levels

Superoxide release to the medium was determined spectrophotometrically, using the tetrazolium salt XTT {(2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} (Schopfer et al. 2001). The shoots of *S. hieraciifolia* (0.5 g) were cut into small pieces and were vacuum-infiltrated for 20 min with 5 ml of 10 mM Na-citrate buffer (pH 7.0) containing 500 µM XTT, with/without 3.5 U ml^{-1} superoxide dismutase in dark. The samples were kept in this buffer for 2 hours. The increase in XTT reduction in the shoots was recorded at 470 nm in a spectrophotometer.

Histochemical detection of H_2O_2

As mentioned above the production of H_2O_2 was determined both spectrophotometrically and it was visualized in vivo by 3,3'-diaminobenzidine (DAB) staining methods (Daudi et al. 2012). *S. hieraciifolia* shoots were exposed to DAB prepared in 0.05% Tween 20 (v/v) and 10 mM sodium phosphate buffer (pH 7.0). The shoots were then placed in test tubes and incubated at 80-100 rpm in a shaker. After incubation, the shoots were boiled in a water bath at 90-95 °C for 15±5 min in bleach solution; ethanol: acetic acid: glycerol (3: 1: 1). Color changes were observed in the shoots.

Ascorbate and glutathione contents

Ascorbate concentration was determined according to Liso et al. (1984). *S. hieraciifolia* shoots (0.25 gr) were homogenized with 5 ml, 5 % (w/v) m-phosphoric acid and the extract was centrifuged at 10,000g for

4 min. Sample (70 µl) was added to 3 ml of reaction medium containing 0.1 M citrate-0.2 M phosphate buffer (pH 6.2). The initial absorbance was recorded at 265 nm and then the ascorbate concentration was determined by reading the reduction of 5 min after the addition of two units of ascorbate oxidase to the reaction medium. After ascorbate oxidation was completed, ascorbate oxidase was inhibited with 10 mM sodium azide. Dithiothreitol (DTT) (2.5 mM) was added to the medium and following reduction (3 min) with DTT, the absorbance was recorded again at 265 nm.

Glutathione content was determined according to the total glutathione assay kit (Northwest Life Science Specialties, LLC.) according to the instructions of the producer firm. Glutathione was measured using a reaction mixture containing 250 mM K_2HPO_4/KH_2PO_4 (pH 7.5), 200 µM NADPH, 600 µM DTNB, 25 µl extract and 0.3 U GR. The change in absorbance was observed at 412 nm for 3 min. GSH concentration was calculated on the standard graph obtained by using GSH at 0-5 µM concentrations.

Antioxidant enzyme assays and protein determination

For superoxide dismutase, catalase and guaiacol peroxidase extractions, the shoot tissues (0.1 g) were homogenized with a 50 mM sodium phosphate buffer (pH 7.8) with 1 mM ethylenediaminetetraacetic acid (EDTA) and polyvinylpolypyrrolidone (1%). For determination of APX activity, 2 mM of ascorbate was added into the sodium phosphate buffer. The samples were centrifuged at 15,000g for 15 min.

Superoxide dismutase activity was determined according to the method of Beauchamp and Fridovich (1971). Reaction was initiated by the addition of 2 µM riboflavin to 1 ml reaction medium containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 75 µM nitro blue tetrazolium (NBT), and 50 µl extract. The absorbance values at 560 nm were determined after the mixture was exposed to white light at 375 µmol m^{-2} s^{-1} for 10 min. One unit (U) of SOD activity was defined as the amount of enzyme needed to bring about 50% inhibition of the NBT photoreduction rate.

Catalase activity was determined by measuring the decrease in reaction time of 1 ml at 240 nm for 5 min, containing 50 mM potassium phosphate buffer (pH 7.0), 30 mM H_2O_2 and 20 µl enzyme extract. Catalase activity was calculated using the 39.4 $mM^{-1}cm^{-1}$ epsilon coefficient for H_2O_2 (Bergmeyer and Graßl 1983).

Ascorbate peroxidase activity was determined with decrease at 290 nm (Nakano and Asada 1987). APX activity was determined by measuring a 1 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 250 µM ascorbate, 5 mM H_2O_2 and 20 µl enzyme extract. APX activity was calculated using the 2.8 $mM^{-1}cm^{-1}$ epsilon coefficient for ASC.

Guaiacol peroxidase activity was measured by increase in absorbance at 470 nm (25°C, e = 26.6 $mM^{-1}cm^{-1}$) in a 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 5 mM guaiacol, 15 mM H_2O_2 and 50 µl of enzyme extract (Urbanek et al. 1991).

Glutathione reductase activity was determined spectrophotometrically according to Foyer and Halliwell (1976). GR was assayed by the fall in absorbance at 340 nm as NADPH was oxidized. The assay contained 50 mM Tris-HCl (pH 7.8), 150 µM NADPH, 500 µM oxidized glutathione (GSSG) and 50 µl extract. The activity of GR was calculated using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ for NADPH at 340 nm.

Antioxidant enzyme activities were presented on a protein basis. Protein content was determined according to Bradford (1976), using BSA as a standard.

Western blot analysis of antioxidant enzymes

Protein extractions of SOD, CAT, glutathione peroxidase (GPX; E.C. 1.11.1.9) and GR were performed using 4X PEB (protein extraction buffer, AS08 300 Agrisera Inc.). Samples were dissolved in an equal volume of sample buffer (2X Laemmli sample buffer 1610737 Bio-Rad) and heated at 95 °C for 5 min.

Separation of total proteins (30 µg) by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, (TGX Stain-Free Precast Protein Gels, 4568095 Bio-Rad) was performed by electrophoresis (Mini-PROTEAN Tetra Cell system, 165800 Bio- Rad) at room temperature. The marker (Precision Plus Protein Western C Blotting Standards, 1610376 Bio-Rad) was used to determine the molecular weight of the proteins. Proteins separated in gel electrophoresis were transferred to the PVDF membrane (Trans-Blot Turbo Mini PVDF Transfer Packs, 1704156 Bio-Rad) using the Trans-Blot Turbo Transfer System (1704155 Bio-Rad). After transfer, the membrane was blocked at 4°Cwith 2.5% milk powder in TBS. Fe-SOD Chloroplastic Fe-Dependent Superoxide Dismutase (A S06 125) for SOD, Glutathione Peroxidase Chloroplastic (A S04 055) for GPX, and, Catalase (A S09 501) for CAT were primary used antibodies and they incubated overnight at 4 °C. After incubation, the membranes were incubated with goat anti-rabbit IgG-HRP secondary antibodies (AS09 602 Agrisera) for 4 h at room temperature. The density of the scanned protein bands was calculated with Image Lab Software (1709690, Bio-Rad).

Statistical analysis

All experiments were carried out five times with five biological replicates. All results were presented as means ± standard deviation. All physiological data were processed with one-way analysis of variance ($\alpha = 0.05$) using the SPSS Ver. 15.0 software for Microsoft Windows (SPSS Inc., Chicago, USA). Mean differences were determined with the Duncan multiple comparison test at $\alpha = 0.05$.**Results**

Determination of extreme halophyte characteristic of *S. hieraciifolia*

Morphological observations

It was observed that *S. hieraciifolia* grown on the MS media could survive under the excess salt stress condition (600 mM NaCl) though chlorosis. Conversely, *T. parvula* could withstand the 450 mM NaCl treatment but not the 600 mM NaCl treatment. The 300 mM NaCl caused shoot succulence and the shoot succulence increased in accordance with the enhanced NaCl concentration in *S. hieraciifolia*. Additionally, the shoot length was down while shoot number increased in the 300 mM NaCl treatment in *S. hieraciifolia* in comparison with the 0 mM NaCl treatment. *S. hieraciifolia* and *T. parvula* displayed optimal growth at 300 mM NaCl (Fig. 1 A-D).

Effects of salinity on relative water content

The relative water content (%) of *S. hieraciifolia* shoots significantly enhanced at all levels of salinity in comparison with the 0 mM NaCl treatment. High increases in RWC were determined in the 450 and 600 mM NaCl treatments. Conversely, in *T. parvula*, the RWC value decreased while salt concentration was increasing. The highest decrease was determined the shoots treated with 450 mM NaCl in *T. parvula* (Table 1).

Effects of salinity on lipid peroxidation

Lipid peroxidation enhanced in the shoots treated with 300, 450 and 600 mM NaCl, while 150 mM NaCl treatment had no significantly effect in comparison with 0 mM NaCl in *S. hieraciifolia*. However, in *T. parvula*, TBARS content was increased in all salt treatments compared to the 0 mM NaCl treatment and the highest TBARS content was observed in the 450 mM salt treatment. As mentioned above, the shoots of *T. parvula* lost their vitality in the 600 mM NaCl treatment. The rates of increase of TBARS content in both plants were same (1.1-fold) in the 300 mM NaCl treatments where observed the optimum growth. However, there were 2.9-fold and 3.8-fold increases in *S. hieraciifolia* and *T. parvula* in the 450 mM NaCl treatments, respectively (Table 1).

Effects of salinity on H_2O_2 content

H_2O_2 contents gradually increased in *S. hieraciifolia* and *T. parvula* while salt concentration was increasing. H_2O_2 contents and the rates of the increases observed in *T. parvula* were higher than those of *S. hieraciifolia*. For example, there were 2.4-fold increase in *T. parvula* and 1.8-fold in *S. hieraciifolia* in the 300 mM NaCl treated shoots as compared with non-saline conditions. Likewise, there were 8.3-fold and 4.4-fold enhancements in H_2O_2 contents of the shoots treated with 450 mM NaCl in *T. parvula* and *S. hieraciifolia*, respectively (Table 1).

Determination of shoot succulence in *S. hieraciifolia*

Fleshy shoots were observed at 300 mM and higher salt concentrations in *Scorzonera hieraciifolia*. Accordingly, the shoot succulence degree increased in the 300 mM NaCl treatment in comparison with the non-stressed shoots. The succulence degrees were high in the 450 and 600 mM NaCl treatments (Table 2). Likewise, the shoot thickness increased depending on salt concentration. The highest increase in the shoot thickness was observed in the 600 mM NaCl treated shoots. As compared with the 0 mM NaCl treatment, there were 2.4-fold and 9-fold enhancements in the thicknesses of shoots treated with 300 mM and 600 mM NaCl, respectively (Table 2).

The salt treatments also increased the plant area. The highest plant area was detected in plants treated with 300 mM NaCl (Table 2). There was 5.4-fold and 2.7-fold enhancements in the plant areas treated with 300 mM and 600 mM NaCl, respectively (Table 2).

Effects of salinity on osmoprotectants

Soluble sugar and proline contents

Both total soluble sugar and proline contents increased by the all salt treatments compared to the non-stressed shoots in *Scorzonera hieraciifolia*. Moreover, the highest increases in the contents of proline (2.6-fold) and total soluble sugar (1.4-fold) were observed in the 600 mM salt treated seedlings in comparison with the 0 mM NaCl treatment. There were 1.2-fold and 1.3-fold increases in proline and total soluble sugar contents of the shoots treated with 300 mM NaCl, respectively (Fig. 2A, B).

Ion contents

K^+ ion concentration increased at all NaCl concentrations in comparison with the non-stressed shoots. K^+ content was high in the 150 and 300 mM NaCl treated shoots. Na^+ and Cl^- contents increased gradually with the enhanced NaCl concentrations as compared to 0 mM NaCl treatment. The highest Na^+ and Cl^- contents were detected in the shoots exposed to 600 mM NaCl. Ca^{2+} and Mg^{2+} ion contents significantly increased in all salt treatments as compared with non-saline condition. The highest increases in Ca^{2+} and Mg^{2+} contents were determined in the 600 mM NaCl treated shoots and the rate of increase of both Ca^{2+} and Mg^{2+} was recorded by 3.3-fold (Table 3).

Effects of salinity on antioxidant system-related signal compounds

ROS levels

Superoxide ($O_2[?]^-$) level in the shoot of *S. hieraciifolia* increased in parallel with the increasing salt level. The highest superoxide level was detected in the shoots treated with 600 mM NaCl (Fig. 3).

The changes in H_2O_2 level in the shoot tissues of *S. hieraciifolia* was also determined with the observation of light brown by DAB staining. We observed that all salt treatments increased H_2O_2 level in comparison with the non-stressed shoots (Fig. 4).

Ascorbate and glutathione contents

The total ascorbate concentration no significantly changed in the shoots treated with 150 mM NaCl. However, the 300 mM NaCl and further salt concentrations (450 and 600 mM) significantly increased the total ascorbate content in comparison with 0 mM NaCl treatment. As compared to the 0 mM NaCl treatment, there were 2.2-

fold and 8-fold increases in ASC content of the shoots treated with 300 mM and 600 mM NaCl, respectively (Fig. 5A).

Total glutathione content gradually enhanced while salt concentration was increasing in *S. hieraciifolia*. There were 3.4-fold and 10-fold increases in GSH content of the shoots treated with 300 mM and 600 mM NaCl in comparison with the 0 mM NaCl treatment respectively (Fig. 5B).

Effects of salinity on antioxidant enzyme activities

The activities SOD and APX significantly enhanced while NaCl concentrations were increasing. The highest increases in the activities of SOD and APX were recorded in the 600 mM NaCl treatment (Fig. 6 A). The catalase activity increased in the 150 and 300 mM NaCl treatments but it decreased in the 450 and 600 mM NaCl treatments (Figure 6 B). There was no significant change in GPOD activity in the shoots treated with 150 mM NaCl, while the activity increased in 300 mM, (1.7-fold), 450 mM (3.4-fold) and 600 mM (4-fold) NaCl treatments as compared to the 0 mM NaCl treatment (Figure 6 B). As for glutathione reductase, the activity increased under the all salt stress conditions. The rate of the increase was high (2.5-fold) in the high (600 mM) salt treatment (Fig. 6 B).

Effect of salinity on antioxidant enzyme contents

The increases in the SOD activity were consistent with increases in Fe-SOD contents in *S. hieraciifolia*. Fe-SOD content increased in the all salt treatments compared to 0 mM NaCl treatment and, Fe-SOD content was high in the shoots treated with 450 and 600 mM NaCl (Figure 7). The protein content of catalase distinctively enhanced in the shoots treated with 300 mM NaCl. Similar to the CAT activity, there was a decrease trend in the shoots exposed to 450 mM NaCl. Also, CAT content no significantly changed in the 150 and 600 mM salt treatments (Figure 6). Glutathione peroxidase content increased in all salt treatments in comparison with the non-stressed shoots. GPX content was especially high in the 300 mM NaCl and upper salt treatments (Fig. 7). GR content increased in the shoots treated with 150, 300 and 450 mM NaCl compared to the non-stressed shoots. There was a significant increase in the GR protein content in the 300 mM treatment in *S. hieraciifolia* (Fig. 7).

Discussion

We detected that *S. hieraciifolia* displayed optimal growth at the 300 mM NaCl concentration, as similar to *Salvadora persica*, stem-succulent extreme-halophyte, reported by Aghaleh et al. (2009). *S. hieraciifolia* was able to survive, even at the 600 mM NaCl concentration in spite of chlorosis. Likewise, Aghaleh et al. (2009) recorded that succulent halophyte, *Salvadora persica* was able to survive at 600 mM salt, which is a dose higher than the concentration of salt in seawater. *T. parvula* also displayed optimal growth at the 300 mM NaCl but it was unable to tolerate the 600 mM NaCl concentration in MS media. Accordingly, Uzilday et al. (2015) showed that *T. parvula* grown in a media including peat moss, vermiculite and soil mix could withstand 300 mM NaCl treatment. These results indicated that the maximum salt concentration that could be tolerated by halophytes may depend on the plant species, the presence of succulent structures, severity of the stress treatments and media exposed to the stress.

The relative water content increased in accordance with increased salt concentrations in *S. hieraciifolia* with fleshy shoots. Conversely, RWC value decreased with increasing salt concentration in *T. parvula*. Our data in *T. parvula* is consistent with previous reports that RWC and osmotic potential decreased in extreme halophyte, *Salsola crassa* (Yildiztugay et al. 2014). Increase in RWC indicated that succulent extreme-halophytes could dilute excess salt in their succulent structures and they could promote a mechanism of salt tolerance.

Amount of thiobarbituric acid reactive substances that is the product of lipid peroxidation, and ROS levels in plant tissues are important parameters indicating the stress damage of plants (Gupta and Huang 2014). Uzilday et al. (2015) reported no significant change in membrane damage in shoots of *T. parvula* grown

in saline soil (300 mM NaCl). However, our findings obtained in MS media showed that TBARS content and H_2O_2 level increased by enhanced salt concentrations in *T. parvula*, and the rates of the increases in *T. parvula* were higher than those of *S. hieraciifolia*. Likewise, Uzilday et al. (2015) showed that H_2O_2 content significantly increased by the 300 mM NaCl treatment but no changed by low salt (50 and 200 mM NaCl) treatments in T. *parvula*. In the light of all this information on morphological observations, RWC, TBARS and H_2O_2 content we can say that *S. hieraciifolia* can be capable of tolerating high salt conditions and thus it can have characteristic properties of extreme-halophytes like *T. parvula*.

We also determined shoot succulence in *S. hieraciifolia* by measuring changes in the shoot succulence degree, shoot thickness and plant area under increased salinity conditions. We observed the increases in these parameters. Likewise, Parida et al. (2016) reported that succulence degree increased when salinity was increasing in extreme-halophyte, *Salvadora persica*. Increased shoot thickness and plant area due to increased salt concentration supported the idea that *S. hieraciifolia* displayed a succulent shoot structure under salt stress.

Plants supply osmotic adjustment via two processes under high saline environment: ion accumulation in the vacuole and synthesis of compatible solutes in cytosol (Zhou and Yu 2009; Chen and Jiang 2010; Shabala and Mackay 2011). Our results showed that proline content and total soluble sugars significantly increased in *S. hieraciifolia* shoots at all levels of salinity. Increased level of organic osmolytes in response to salt stress has been reported in many succulent halophytes. For instance, proline content increased in succulent extreme-halophyte, *Salvadora persica* under NaCl stress conditions (Parida et al. 2016). Likewise, Liang et al. (2018) suggested that the soluble sugar content of Arabidopsis overexpressing salt-related wheat *TaSST* gene was significantly higher than that of wild-type and the transgenic plants resisted external salt stress by accumulating soluble sugars. The regulated accumulation of total soluble sugar and proline with signaling functions may contribute to the osmotic adjustment to continue water uptake in *S. hieraciifolia* under excess salinity.

Our findings showed that K^+ content increased in all salt treatments and the increase was high in the 150 and 300 mM NaCl treated shoots. Levels of Na^+ and Cl^- ions also increased parallel with the increase in NaCl concentrations in the shoots. Moreover, we observed 3.3-fold increases in both Ca^{2+} and Mg^{2+} contents of the 600 mM NaCl treated shoots (Table 3). The same increase rate pointed out that Mg^{2+} may have a secondary messenger function similar to Ca^{2+} and affect uptake of other inorganic ions under salt stress in plants. Magnesium also led to the increase in the osmotic potential in plant tissues to maintain water uptake from medium to the tissues (Ahmad et al. 2013). Therefore, in the present study, the possible signaling role of Mg^{2+} reported earlier in animals has been expressed for the first time in plants. Additionally, based on the increases in Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^- contents, we can say that *S. hieraciifolia* can provide osmotic adjustment by using inorganic ions and induce water uptake under excess salinity.

Our results showed that increased salinity enhanced the amounts of superoxide and the H_2O_2 as a sign that the shoots were subjected to oxidative stress. The highest superoxide and H_2O_2 levels were detected in the 600 mM NaCl treated shoots. Uzilday et al. (2015) showed that H_2O_2 concentration of *T. parvula* treated with 300 mM NaCl was significantly promoted. It was also reported that ROS level increased in extreme halophyte *Salsola crassa* by Yildiztugay et al. (2014). However, moderated ROS levels in the plant tissues can act signaling role for the induction of antioxidant system under excess salinity.

Cellular antioxidant system through the activation of non-enzymatic or enzymatic antioxidants is crucial for the maintenance of cellular redox homeostasis required for salt adaptation or tolerance response in different plants (Gupta and Huang 2014). There were 2.2-fold and 8-fold increases in ASC content of the shoots treated with 300 mM and 600 mM NaCl, respectively. ASC contributes to ROS-scavenging and salt tolerance (Gupta and Huang 2014). We observed that like ASC, GSH content increased by salt stress and there were 3.4-fold and 10-fold increases in GSH content in the 300 mM and 600 mM NaCl treatments, respectively. GSH has been reported to play a protective role in salt stress tolerance by maintaining redox status and plants adapted to stress conditions had higher GSH levels (Gupta and Huang 2014). Coherently with our results, it was reported that total ASC and total GSH contents significantly increased in extreme

halophyte, *Salsola crassa*, treated with 1000-1500 mM NaCl (Yildiztugay et al. 2014). Therefore, we can say that the antioxidant signal compounds, ASC and GSH could activate the signaling network to stimulate the antioxidant system responses to provide salt tolerance to *S. hieraciifolia*.

Antioxidant enzymes such as SOD activity was increased under salt stress conditions in *S. hieraciifolia*. There were 1.6-fold and 2.4-fold enhancements in SOD activity of the shoots treated with 300 mM and 600 mM NaCl, respectively. Increase in SOD activity was recorded in *T. parvula* during salt stress (Uzilday et al. 2015). CAT activity enhanced in the shoots treated with 150 mM and 300 mM NaCl although the activity declined under high salt stress conditions. Similar to our results, Parida and Jha (2010) reported that CAT activity decreased under high salinity (400 mM and 600 mM NaCl) in succulent extreme-halophyte *Salicornia brachiate*. Increased CAT activity under salt stress indicated that CAT can be effective in scavenging of H_2O_2 in *S. hieraciifolia*. Moreover, GPOD activity increased by 1.7-fold at 300 mM NaCl and 4-fold at 600 mM NaCl in *Scorzonera hieraciifolia*. The activity of GR playing an important role in controlling endogenous H_2O_2 content through a redox cycle containing glutathione and ascorbate (Gupta and Huang 2014), increased under salt stress in *S. hieraciifolia*. There were 1.3-fold and 2.5-fold enhancements in the GR activities in the 300 mM and 600 mM NaCl treatments, respectively. Also, APX activity showed significant stimulation in all treatments of salinity and, the activity increased by 1.3-fold and 1.5-fold at 300 mM and 600 mM NaCl, respectively. In the light of these findings, we can say that succulent extreme-halophytes can improve their salt tolerance capacity through the powerful antioxidant enzyme system.

We also detected here the changes in the contents of some antioxidant enzymes. As known, in higher plants, SOD is divided into three groups: manganese SOD (Mn-SOD), iron SOD (Fe-SOD), and copper/zinc SOD (Cu/Zn-SOD), according to the different metal ions, bound by its auxiliary sites (Wang et al. 2016). Fe-SOD and GPX protein contents increased in the 300 mM NaCl treatments in comparison with the unstressed treatment and the highest increases were recorded in the 600 mM NaCl treatment. CAT content was also high in the 300 mM NaCl treatment. The results point out the fact that the antioxidant enzyme system act as a defense arsenal for building an adaptive mechanism under high salinity in succulent extreme-halophytes. Also, GR protein content enhanced in all salt treatments except 600 mM NaCl treatment. Previous studies have reported that GR has a protective role against salinity-induced oxidative damage in different plant species (Zeng et al. 2018; Parida and Jha 2010). High antioxidant enzyme activities and the protein contents in *S. hieraciifolia* exposed to excess salinity showed that the plant can effectively induce the antioxidant system to survive excess salt. Moreover, there could be essential protective roles of antioxidant enzyme activities in conjunction with their content in the scavenging processes in *S. hieraciifolia*.

Conclusion

Our results showed that succulent *Scorzonera hieraciifolia* with antioxidant and anti-inflammatory activity may be extremely salt tolerant that could survive at excess salinity (600 mM NaCl) conditions. *S. hieraciifolia* displays optimal growth at 300 mM NaCl in the MS media. *S. hieraciifolia*, can display shoot succulence by up-taking water effectively. The combined accumulations of signal compounds involved in osmoregulation such as proline, total soluble sugar, calcium and magnesium can provide the exceptional salt tolerance to *S. hieraciifolia*. Also, ASC can contribute activation of ROS and Ca^{2+} signaling under salt stress in succulent extreme-halophyte *Scorzonera hieraciifolia*. The modified accumulation of ASC, GSH and other signaling compounds may trigger the propagation of salt tolerance mechanism. Therefore, the exceptional salt tolerance of succulent extreme-halophyte *S. hieraciifolia* is achieved by regulated accumulation of combined signal compounds, involved in osmoregulation and induction of antioxidant system. Concomitant increases in antioxidant enzyme activities, antioxidant enzyme contents and the shoot succulence in the salinized plants showed that *S. hieraciifolia* may use the comprehensive strategy when coping with salinity.

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Author contributions

CA performed the all experiments and wrote the manuscript; RT analyzed all data, read and edited the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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TABLES

Table 1 Alterations in some stress-determining parameters in the shoots of *Scorzonera hieraciifolia* and *Thellungiella parvula* treated with 0, 150, 300, 450 and 600 mM NaCl. Means ± SD. Means followed by different letters are significantly different according to Duncan's test at P £ 0.05.

NaCl	***Scorzonera hieraciifolia***		***Thellungiella parvula***
(mM)	**RWC** (%)	**TBARS** (μmolg^{-1}DW)	**H_2O_2** (μmolg^{-1}DW)
0	73.51±0.8 d	2.8±0.09 d	6.3±0.01 e
150	76.44±0.8 c	2.7±0.08 d	8.7±0.01 d
300	82.84±0.8 b	3.2±0.08 c	11.5±0.05 c
450	92.07±0.6 a	8.3±0.25 b	28.0±0.05 b
600	94.1±1.0 a	9.1±0.12 a	32.2±0.07 a

NA: non-alive

Table 2 Changes in parameters related to shoot succulence in *S. hieraciifolia*. Means ± SD. Means followed by different letters are significantly different according to Duncan's test at P £ 0.05. SSD: shoot succulence degree, ST: shoot thickness, PA: plant area, DW: dry weight.

NaCl (mM)	SSD	ST (μm)	PA (cm^2 plant^{-1})
0	46±2.5 c	39.2±0.3 e	2.31±0.6 d
150	47±1.1 c	52.8±0.5 d	3.81±0.4 c
300	69±1.4 b	92.5±4.1 c	12.5±2.1 a

450	86±2.5 a	311±4.8 b	8.08±2.1 b
600	88±2.7 a	351±7.3 a	6.28±0.8 b

Table 3 Alterations in ion contents in the shoots of *S. hieraciifolia*. Means ± SD. Means followed by different letters are significantly different according to Duncan's test at P £ 0.05.

NaCl (mM)	$\mathbf{K^+}$	$\mathbf{Na^+}$	$\mathbf{Ca^{++}}$	$\mathbf{Mg^{++}}$	$\mathbf{Cl^-}$
0	198.6±0.7 d	5.01±0.01 e	8.72±0.4 e	0.37±0.03 d	1.76±0.2 e
150	373.1±10 a	46.7±0.06 d	23.6±0.9 b	1.18±0.06 a	18.9±2.2 d
300	364.1±0.5 a	76.9±0.1 c	22.5±0.3 c	0.94±0.01 b	24.2±1.2 c
450	269.9±0.9 c	103.6±0.9 b	20.6±0.4 d	0.82±0.03 c	26.3±0.1 b
600	288.9±3.5 b	144.5±1.8	28.7±0.5 a	1.22±0.04 a	28.6±1.5 a

Fig. 1 Morphological alterations in the shoots of *Scorzonera hieraciifolia* and *Thellungiella parvula* treated with 0, 150, 300, 450 and 600 mM NaCl (A, B). *S. hieraciifolia* grown at 300 mM NaCl (C). *S. hieraciifolia* grown at 600 mM NaCl (D).

Fig. 2 Alterations in osmoregulation related signal compounds, total soluble sugar (A) and proline (B), in the shoots of *S. hieraciifolia*. Vertical bars represent standard deviations. Different letters denote significant differences among all treatments at P<0.05.

Fig. 3 Alterations in superoxide level in the shoots of *S. hieraciifolia*. Vertical bars represent standard deviations. Different letters denote significant differences among all treatments at P<0.05.

Fig. 4 Alterations in H_2O_2 content detected by DAB staining in the shoots of *S. hieraciifolia*. Vertical bars represent standard deviations. Different letters denote significant differences among all treatments at P<0.05.

Fig. 5 Alterations in non-enzymatic antioxidant signal compounds, ascorbate (A) and glutathione (B), in the shoots of *S. hieraciifolia*. Vertical bars represent standard deviations. Different letters denote significant differences among all treatments at P<0.05.

Fig. 6 Alterations in antioxidant enzyme activities, superoxide dismutase, ascorbate peroxidase (A), glutathione reductase, catalase and guaiacol peroxidase (B), in the shoots of *S. hieraciifolia*. Vertical bars represent standard deviations. Different letters denote significant differences among all treatments at P<0.05.

Fig. 7 Alterations in antioxidant enzyme contents, Fe-superoxide dismutase, catalase glutathione peroxidase and glutathione reductase, in the shoots of *S. hieraciifolia*. Vertical bars represent standard deviations. Different letters denote significant differences among all treatments at P<0.05.