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NaCl-induced responses in giant duckweed (*Spirodela polyrhiza*)

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ABSTRACT

Salt accumulation leads to a decline in species richness in aquatic ecosystems. The effects of salt (NaCl) stress on plant growth and antioxidative responses in giant duckweed were investigated. NaCl significantly reduced photosynthetic pigment accumulation and inhibited plant growth. This inhibition in plant growth, may also provoke oxidative stress, enhancing malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents in plant tissues. The antioxidative enzymatic analysis showed a significant increase in superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD) activity, whereas glutathione reductase (GR) activities were marginally enhanced. POD is the most active enzyme in giant duckweed exposed to NaCl stress. These results suggest that an adapted reactive oxygen species (ROS) scavenging system could provide some protection against oxidative damage in giant duckweed plants under salt-stressed conditions.

Key words: antioxidative enzyme, peroxidase, salt stress, *Spirodela polyrhiza*,

INTRODUCTION

Plants respond and adapt to abiotic stresses by altering the biochemical and physiological pathways that reprogram the whole plant to attain a new metabolic and cellular homeostasis (Chaves et al. 2009). In crops, exposure to salt (NaCl) stress is a major factor in reducing yield worldwide (Yamaguchi and Blumwald 2005). Salt stress is known to cause morphological, biochemical, and physiological changes and often induces cellular damage that affects plant growth and development (Wang et al. 2003). Salt stress triggers the production of abscisic acid, a plant stress hormone that closes stomata when transported to guard cells and, in turn, results in reduced photosynthesis (Chaves et al. 2009). In addition to hormone production and photosynthetic inhibition, salt alters a broad range of metabolic processes, reduces enzyme activities and biochemical constituents, and causes the excessive generation of reactive oxygen species (ROS) in stressed plants (Allakhverdiev and Murata 2008). ROS production is a common feature of all aerobic organisms during normal growth, but environmental stresses, such as drought, salt, UV radiation, chilling, and heat shock, induce the overproduction of ROS, including hydrogen peroxide (H_2O_2), superoxide radical (O_2.-), and hydroxyl radical (·OH) (Mittler 2002). Oxidative damage of nucleic acids, proteins, and lipids through the alternation of cellular redox state or homeostasis is an important impact of ROS (Imlay 2003, Potters et al. 2010). Under environmental stress, the activity of antioxidative enzymes increases and is elevated even higher in stress-tolerant crops (Gueta-Dahan et al. 1997, Sairam et al. 1997, Sreenivasulu et al. 1999, El-Shabrawi et al. 2010). These enzymes play important roles in scavenging ROS through a series of biochemical reactions (Sairam and Tyagi 2004), including the dismutation of O_2.- to H_2O_2 by superoxide dismutase (SOD) and the detoxification of H_2O_2 by various enzymes, such as peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR; Sumithra et al. 2006). Under salt stress, antioxidative enzyme systems are similar among plants, and the defense mechanisms are shared by different plant species. Instead of antioxidative enzyme systems, nonenzymatic antioxidants such as proline, ascorbic acid, glutathione, and glycinebetaine also

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play a significant role against ROS (Mittler 2002, Ashraf and Harris 2004). However, responses to salt that reprogram the whole plant to attain a new metabolic and cellular homeostasis to cope with the upcoming stress are species-specific (Chaves et al. 2009).

Giant duckweed (*Spirodela polyrhiza*) is a free-floating freshwater macrophyte valuable in basic and environmental studies (Upadhyay and Panda 2010). The entire plant is dominant by a leaf-like structure called a "frond." Daughter fronds are produced asexually from reproductive pockets located approximately at the end of the mother frond. Although the mature frond of *S. polyrhiza* is small (4 to 10 mm long), it is the largest among the duckweed species (Oláh et al. 2008). Giant duckweed provides a high plant-protein food source for ducks and geese and may also be eaten by certain fishes. Giant duckweed, like other duckweeds, has evolved the ability to rapidly remove minerals necessary for its growth from the water on which it floats. This is a useful trait in bioremediation to reduce nutrients in sewage effluent (Hou et al. 2007). *Spirodela* has attracted recent attention because this rapidly growing macrophyte may provide the solution for absorbing atmospheric carbon dioxide, cycling ecosystem carbon, and producing biofuels (Michael et al. 2009). Giant duckweed was used as the test organism in this study because it is easy to culture and handle, has a rapid reproductive cycle and high growth rate in laboratory conditions, and is sensitive to various pollutants (Wang 1990, Oláh et al. 2008, Tkalec et al. 2008, Appenroth et al. 2010). Although the inhibitory effect of salinity on duckweed root growth has been studied in the past (Panda and Upadhyay 2003), specific knowledge about biochemical mechanisms of NaCl toxicity and salt-related responses in giant duckweed are largely unknown. Therefore, the aim of this study is to determine the effects of various growth and biochemical traits in axenic giant duckweed cultures under NaCl stress. The experimental results demonstrate the effect of NaCl on the growth, biochemical composition (photosynthetic pigments), ROS production, lipid peroxidation, and ROS scavenging of this plant. This study will enhance understanding of biochemical detoxification strategies that aquatic plant *S. polyrihza* adopts against stress induced by exposure to NaCl.

MATERIALS AND METHODS

Plant culture and treatments

Spirodela polyrhiza (L). Schleiden, clone DR (geographic isolate) plants were collected from a local stream and cultured under controlled conditions. They were sterilized with 1% NaOCl for 5 s and rinsed 5 times with sterilized distilled water. Collected plants were maintained as stock cultures in Schenk-Hildebrandt (SH) growth medium (Schenk and Hildebrandt 1972) and supplemented with 1% sucrose and 3 mM 2-(N-Morpholino)-ethanesulfonic acid (MES monohydrate); pH value of the growth medium was adjusted to 5.6 with 1N KOH. The medium was autoclaved at 118 kPa and 121 C for 20 min. The stock culture was kept in growth chambers with an 18 h light to 6 h dark cycle at 25 ± 1 C and irradiance of 50 mmol m^{-2} s^{-1}. Plants were subcultured at 2-week intervals.

Ten days (10 ± 2 d) before experiments, plants were further adapted to the modified SH growth medium (without 1% sucrose), called precultivation. Experimental cultures were started by inoculation of 45 healthy colonies with 3 fronds (growth experiments) or 60 plants of healthy colonies with 3 to 4 fronds (antioxidative analysis) from precultivation cultures. Plants were transferred to Erlenmeyer flasks (500 mL) containing SH medium at 25 ± 1 C in continuous cool white fluorescent light at 70 mmol m^{-2} s^{-1}. NaCl treatment was applied at concentrations 0, 100, 150, and 200 mM. After a 4 d exposure to NaCl, the fresh weight (FW), soluble protein, photosynthetic pigments, malondialdehyde (MDA), free proline, and H_2O_2 content of fronds were determined for growth experiments. Plant growth inhibition was estimated as the change in the fresh weight according to following formulas. Results were expressed as inhibition percentage.

Growth inhibition (%) = (1 - the fresh weight of NaCl-treated plants/the fresh weight of control plants) × 100.

To study time-dependent antioxidative enzyme induction, plants were exposed to 100 mM NaCl, and samples were collected at 0, 24, 48, 72, and 96 h after the onset of the exposure. NaCl-treated plant samples were collected for the determination of H_2O_2, $O_2^{\cdot-}$, and antioxidative enzymes. All experiments were repeated at least 3 times.

Determination of photosynthetic pigments

Giant duckweed fronds (0.1 g) were homogenized on ice with a Polytron homogenizer (Art-Miccra D-8) in 2 mL of cold 80% acetone. The homogenate was centrifuged at 10,000 g at 4 C for 5 min, and the absorbance of the supernatant was measured at wavelengths 470, 626, 645, 663, and 730 nm using a UV-VIS spectrophotometer (Unicam Helios b). Chlorophyll *a*, *b*, *a*/*b*, and carotenoid concentrations were calculated as described by Lichtenthaler (1987).

Determination of malondialdehyde and proline content

The level of lipid peroxidation was estimated indirectly *in vitro* as the formation of MDA content (the main aldehyde produced) that reacts with thiobarbituric acid reactive substances (TBARS), as described by Ortega-Villasante et al. (2005). Plant tissue samples (0.1 g) were homogenized on ice with a Polytron homogenizer (Art-Miccra D-8) in 2 mL of cold 80% (v:v) ethanol (Hodges et al. 1999), followed by centrifugation at 10,000 g at 4 C for 10 min. A 1 mL aliquot of plant extract was added to a test tube with 1 mL of 2-thiobarbiyuric acid (TBA) solution composed of 20% (w/v) trichloroacetic acid (TCA), 0.65% TBA and 0.01% butylated hydroxytoluene (BHT). Samples were then mixed vigorously, heated at 95 C in a water bath for 25 min, cooled, and centrifuged at 1630 g for 3 min. Absorbance of the supernatant was measured at 532 nm, and the value of nonspecific turbidity at 600 nm was subtracted. MDA equivalents were calculated using extinction coefficient of 155 $mM^{-1}cm^{-1}$ (Heath and Packer 1968) and expressed as nmol mg^{-1} protein.

Proline was determined according to Bates et al. (1973) with some modifications. Plant tissue samples (0.1 g) were homogenized (Polytron homogenizer, Art-Miccra D-8) with 2 mL of 3% (w:v) sulfosalicylic acid, followed by centrifugation

at 10,000 g at 4 C for 10 min. A 1 mL sample of plant extract was transferred to a test tube in which 1 mL of freshly prepared acid-ninhydrin solution (0.14 M ninhydrin in 6 mL of glacial acetic acid and 4 mL of 6 M phosphoric acid) and 1 mL of glacial acetic acid were added. Samples were then mixed and heated at 100 C in water bath for 1 h and cooled to room temperature. Samples were extracted with 4 mL toluene, and absorbance of toluene phase was read at 520 nm. Proline content was calculated using a standard curve prepared with known concentrations of proline.

Determination of hydrogen peroxide and superoxide radical

Hydrogen peroxide content was measured according to Velikova et al. (2000) and Sumithra et al. (2006). A 50 mg tissue sample was homogenized with a Polytron homogenizer (Art-Miccra D-8) in 0.1% TCA, and the homogenate was centrifuged at 10,000 g at 4 C for 15 min. The reaction mixture contained 0.5 mL of supernatant frond extract, 0.5 mL of potassium phosphate buffer (10 mM, pH 7.0) and 1 mL of KI (1 M). The reaction mix was allowed to stand in the dark for 1 h, and the absorbance was recorded at 390 nm. Hydrogen peroxide content was calculated using a standard curve prepared with known concentrations of H_2O_2.

The level of O_2.- was assayed spectrophotometrically by measuring the reduction of exogenously supplied nitroblue tetrazolium (NBT) according to Doke (1983). Five plants were immersed in 1.5 mL of mixture containing 0.01 M sodium phosphate buffer pH 7.8, 0.05% NBT and 10 mM NaN_3. After 60 min of incubation, 1.0 mL of the reaction solution was transferred into a test tube and heated at 85 C for 15 min. Then the solution was cooled and its absorbance at 580 nm was measured. NBT-reducing activity was expressed as the increase in A_{580} per hour per gram dry weight (DW).

Enzyme assay

Plant material (~0.2 g) was homogenized with a mortar and pestle under liquid nitrogen conditions in 0.8 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF), with the addition of ascorbic acid (ASC; 5 mM final concentration) for the APX assay. The homogenate was centrifuged at 15,000 g for 30 min at 4 C, and the supernatant was used for the enzyme assay. Protein content was quantified according to the method of Bradford (1976), using a standard curve generated with bovine serum albumin.

The APX (EC 1.11.1.11) activity was determined from the decrease in absorbance at 290 nm as described by Nakano and Asada (1981), with modifications. The reaction solution contained 50 mM potassium phosphate buffer (pH 7.0), 0.1m M EDTA, 0.5 mM ascorbic acid, and 0.1m M H_2O_2. The reaction was started by adding 50 to 100 mL of enzyme extract. The APX activity was calculated using the extinction coefficient 2.8 $mM^{-1}cm^{-1}$. One unit of APX activity was defined as the amount required to decompose 1 mmol of ascorbic acid oxidized per minute per milligram of protein. The CAT (EC 1.11.1.6) activity was determined by measuring the change of absorbance at 240 nm that accompanied the consumption of H_2O_2 (Beers and Sizer 1951). One mL of 10 mM buffered H_2O_2 (in potassium phosphate buffer; pH 7.0) was added to 1 mL of 50 mM potassium phosphate buffer (pH 7.0) and 0.1 mL of diluted sample. The decrease of absorbance at 240 nm (e = 40 $mM^{-1}cm^{-1}$) was measured. Total POD (EC 1.11.1.7) activity was determined as oxidation of guaiacol by H_2O_2 (Chance and Maehly 1955). The reaction mixture was 0.5 mL of 100 mM potassium phosphate buffer (pH 6.5), 0.1 mL of 10 mM buffered H_2O_2 (in potassium phosphate buffer; pH 6.5), 0.1 mL of 2.5% guaiacol, 0.25 mL of dH_2O and 10 to 50 mL of enzyme extract. The increase in absorbance at 470 nm was measured. One unit of enzyme activity was defined as the increase of 0.01 in absorbance per minute per gram fresh weight (Sreenivasulu et al. 1999). The GR (EC 1.6.4.2) activity was assayed as the increase of absorbance at 412 nm (e = 13.6 $mM^{-1}cm^{-1}$) resulting from the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to 2-nitro-5-thiobenzoic acid (TNB; Smith et al. 1988). The final reaction volume contained 1 mL 0.2 M potassium phosphate (pH 7.5) containing 1 mM EDTA, 0.5 mL 3 mM DTNB in 0.01 M phosphate buffer, 0.25 mL H_2O, 0.1 mL 2 mM NADPH, 0.05 mL plant crude extract, and 0.1 mL 20 mM oxidized glutathione (GSSG). The SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of NBT according to the method of Beauchamp and Fridovich (1971). The 3 mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 63 mM NBT, 0.1 mM EDTA, 2 mM riboflavin and 10 to 50 mL of enzyme extract. The reaction mixtures were illuminated at an intensity of 70 mmol $m^{-2}s^{-1}$ for 20 min. The absorbance of the supernatant at 560 nm was measured. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT. Activities of APX, CAT, and SOD were expressed in enzyme units per milligram of protein, and POD and GR were expressed in enzyme units per gram of fresh weight.

Gel electrophoresis

Plant extracts containing equal amounts of protein (20 mg/well) were subjected to nondenaturing, discontinuous mini-gel electrophoresis (Hoefer SE250) according to the method of Davis (1964). After electrophoresis, gels were rinsed and washed with phosphate buffer (pH 7.0). The CAT isozymes on nondenaturing gels were soaked in H_2O_2, rinsed with water, and stained in 1% potassium ferricyanide and 1% ferric chloride solution (Woodbury et al. 1971). To detect POD activity, gel was stained in 1 mM 3,3-diaminobenzidine (DAB) and 0.03% H_2O_2 (Adam et al. 1995). The SOD isozymes were separated and stained with NBT and riboflavin, as described by Beauchamp and Fridovich (1971). The gels were soaked in staining solution with inhibitors (2 mM KCN or 5 mM H_2O_2) prior to starting the reaction by irradiation with a fluorescent lamp to identify Cu/Zn-SOD, Fe-SOD and Mn-SOD on the gels.

Histochemical staining

Hydrogen peroxide can be histochemically localized *in vivo* by DAB staining (Thordal-Christensen et al. 1997). Plant

samples were washed with distilled water and then immersed in staining solution (0.1% [w/v] DAB, 10 mM MES, pH 6.5) for 8 h at 25 C in the light (Wohlgemuth et al. 2002). Stained samples were boiled twice in 95% ethanol for 10 min.

STATISTICS

Data presented here are the mean values ± standard error (SE) of at least 3 independent experiments. Statistical analysis was performed by computer software SPSS (ver.12: SPSS Inc., Chicago, IL, USA). All data were subjected to a one-way analysis of variance; mean differences were compared by the lowest standard deviations (LSD) test, and P-values <0.05 were considered significant. In all figures, the spread values are shown as error bars representing standard errors of the means.

RESULTS AND DISCUSSION

NaCl effects on plant growth and pigment contents

Growth of duckweed plants, as determined by an increase in fresh weight, was significantly reduced in a dose-dependent manner by NaCl treatments. The relative fresh weight of NaCl-treated plants was 24 to 37% below the control (Table 1). Under highest salt stress (200 mM), more growth retardation was observed in frond tissues. Fronds started to show signs of chlorosis as early as 2 d after exposure to NaCl treatments at 150 mM and 200 mM. Chlorosis was firstly observed on the edge of the mother fronds and, during further exposure, spread to their central parts. At the end of the experiment (day 4 after inoculation), both daughter and mother fronds (at 200 mM) were necrotic or completely yellow. During the experiment, the roots of these inoculated fronds (100 mM or above) became fragile. The fronds that developed during cultivation on NaCl-containing medium were green, fragmented, and smaller than those in the control colonies. Usually, these fragmented fronds contained short roots.

Reduction of photosynthetic pigment content is a physiological marker of abiotic stress in duckweed (Prasad et al. 2001, Hou et al. 2007, Tkalec et al. 2008, Appenroth et al. 2010). Giant duckweed fronds started to show signs of chlorosis after a 2 d exposure to NaCl treatments. Accordingly, chlorophyll *a* and *b* and carotenoid contents decreased significantly ($P < 0.05$) in the plants exposure to NaCl compared with the controls (Table 2). After treatments with 100, 150, and 200 mM NaCl for 4 d, the content of total chlorophyll decreased by about 16, 30, and 42%, respectively, and for carotenoids decreased about 9, 22, and 35%, respectively. This loss in pigment contents could be due to the damage of thylakoid membranes and direct oxidative breakdown of pigments and could induce the destruction of chloroplast or instability of pigment-protein complexes in photosynthetic reaction centers in giant duckweed. Similar results were reported by Rao and Rao (1981) in pigeon pea (*Cajanus indicus*) and gingelley (*Sesamum indicum*) under NaCl stress. Interestingly, chlorophyll *b* content is more sensitive to NaCl stress than either chlorophyll *a* content or carotenoids, as reported by Fodorpataki and Bartha (2008). Anthocyanin may play a role in protection against Cr (VI) stress in *S. polyrrhiza* (Oláh et al. 2008). Increased anthocyanin accumulation was not observed on the fronds of NaCl treated plants in our experiments (data not shown). These studies demonstrated that the contents of chlorophyll *a* and *b* and carotenoids decreased with the increased of NaCl concentration.

Effects of NaCl on levels of H_2O_2, malondialdehyde and proline

Levels of H_2O_2, MDA, and proline in fronds of *S. polyrhiza* were measured as an assay of NaCl-induced stress. Hydrogen peroxide content increased significantly in plants treated with NaCl when compared to the control plants (Table 3). The increase of H_2O_2 content (1.70 to 1.90folds) in treated plants was accompanied by elevated lipid peroxidation as evidenced by the change in MDA levels. MDA is the peroxidation product of polyunsaturated fatty acids of biomembranes (Imlay 2003, Södergren 2000). Under NaCl treatment, MDA content was altered during the experimental period in plants treated with salt (Table 3). Lipid peroxidation was higher at 200 mM NaCl treatment, while the levels of MDA in NaCl-treated plant tissues were statistically different at 100 and 200 mM when compared to the control plants.

Salt stress, which is frequently accompanied by osmotic stress and ion toxicity, may cause a loss of relative water content (RWC) in leaves and result in decreased cell turgor. Proline accumulation is a common characteristic in stressed monocot plants (Koskeroglu and Tuna 2010) and metal-stressed common duckweed (Panda and Upadhyay 2003, Radic´ et al. 2009) but does not seem to occur in giant duckweed under NaCl stress conditions. The experimental results indicated that proline was not significantly accumulated ($P < 0.05$) in NaCl-treated giant duckweed compared to control plants (Table 3). A similar result was also observed in barley

TABLE 1. GROWTH INHIBITION OF *SPIRODELA POLYRHIZA* CAUSED BY NACL.

Concentration	Growth inhibition (%)	Symptoms
NaCl (mM)		
0	0	Control plants
100	24.03 ± 6.68	Up to 15% of fronds chlorotic; necrosis occurs. Fronds size diminished.
150	28.31 ± 6.66	More than 30% of fronds chlorotic; necrosis were observed on the 2nd day of cultivation. Plants tended to be fragmented into single fronds.
200	36.97 ± 5.17	More than 60% of fronds chlorotic. Plants fragmented into single fronds. Fronds size diminished and severely injured.

Data shown are mean values ± SE of 3 independent experiments done in 3 replicates (n = 3) expressed as percent of control.

Table 2. Photosynthetic pigments in control and NaCl-treated *Spirodela polyrhiza* plants after a 4 d cultivation.

Treatment	Chlorophyll *a* (mg g^{-1}FW)	Chlorophyll *b* (mg g^{-1}FW)	Carotenoids (mg g^{-1}FW)
NaCl (mM)			
0	0.76 ± 0.02	0.27 ± 0.02	0.23 ± 0.02
100	0.65 ± 0.06*	0.21 ± 0.01*	0.21 ± 0.02
150	0.54 ± 0.05*	0.17 ± 0.03*	0.18 ± 0.02*
200	0.45 ± 0.05*	0.14 ± 0.02*	0.15 ± 0.02*

Data shown are mean values ± SE of 3 independent experiments done in 3 replicates (n = 3).
Values followed by an asterisk (*) in the same column are significantly different at $P < 0.05$.
FW = fresh weight.

Table 3. Effect of NaCl stress on physiological and biochemical parameters of plant tissues of *Spirodela polyrhiza*.

	H_2O_2 content (mmol g^{-1} FW)	MDA (nmol mg^{-1} Protein)	Proline (nmol g^{-1} FW)
Control	4.74 ± 0.38	0.35 ± 0.02	12.23 ± 1.30
NaCl (mM)			
100	8.04 ± 1.20	0.60 ± 0.01*	12.87 ± 1.44
150	9.03 ± 3.12*	0.54 ± 0.02*	12.74 ± 0.65
200	9.02 ± 1.07*	0.71 ± 0.04*	12.61 ± 0.36

Data shown are mean values ± SE of 3 independent experiments done in 3 replicates (n = 3).
Values followed by an asterisk (*) in the same column are significantly different at $P < 0.05$.
FW = fresh weight.

(Yamaya and Matsumoto 1989). Proline accumulation occurs in response to not only salinity stress but also to water deficit; therefore, the accumulation of proline is not a salt-tolerant specific event in stressed cells (Ashraf and Harris 2004). Other enzymatic complexes and nonenzymatic antioxidants might have been involved in osmotic adjustment through the accumulation of low mass molecular osmoprotectants under salt stress in giant duckweed and should be investigated in the future (Ashraf and Harris 2004).

The experimental results suggest that by the reduction of photosynthetic pigment contents and the increase in oxidative indicators such as MDA and H_2O_2 levels, the suppression of growth in NaCl-treated *Spirodela* plants could be, at least in part, attributed to the oxidative damage (Chaves et al. 2009).

Effects of NaCl on levels of ROS and antioxidant enzyme activities

Exposure of giant duckweed to 100 mM NaCl did cause about 24% inhibition in growth and 15% chlorosis to the fronds. To gain further insights on the plant response to NaCl, the ROS accumulation and the change of antioxidative enzyme activities in duckweed plants were measured. Under NaCl stress, an increase in superoxide content was evidenced within 24 h in plants as compared to control plants and reached its maximum at the 96 h time point. However, the superoxide levels were not statistically different at the 48 h and 72 h time points (Figure 1A). Hydrogen peroxide content increased significantly in plants treated with NaCl compared with control plants (Figure 1B), and levels of H_2O_2 in fronds of *S. polyrhiza* were detected histochemically (Figure 2). The increase of ROS such as H_2O_2 and O_2.- in NaCl-treated giant duckweed plants was accompanied by increasing antioxidative enzyme activities.

The activity of SOD increased significantly ($P < 0.05$; Figure 3A) in all plants treated with NaCl. In the course of the experiment, salt-induced O_2.- were converted by constitutive and induced levels of SOD in plant tissues. Proteome analysis of rice leaf sheathes under salt stress showed SOD was up-regulated (Abbasi and Komatsu 2004), but a chloroplast-specific form of SOD (Fe-SOD) was down-regulated in tobacco leaves (Razavizadeh et al. 2009). Superoxide dismutase converts O_2.- into H_2O_2, whereas CAT, GR, and peroxidase transforms H_2O_2 into H_2O and O_2 (Apel and Hirt 2004). An increase in the transformation capacity of O_2.- is accompa-

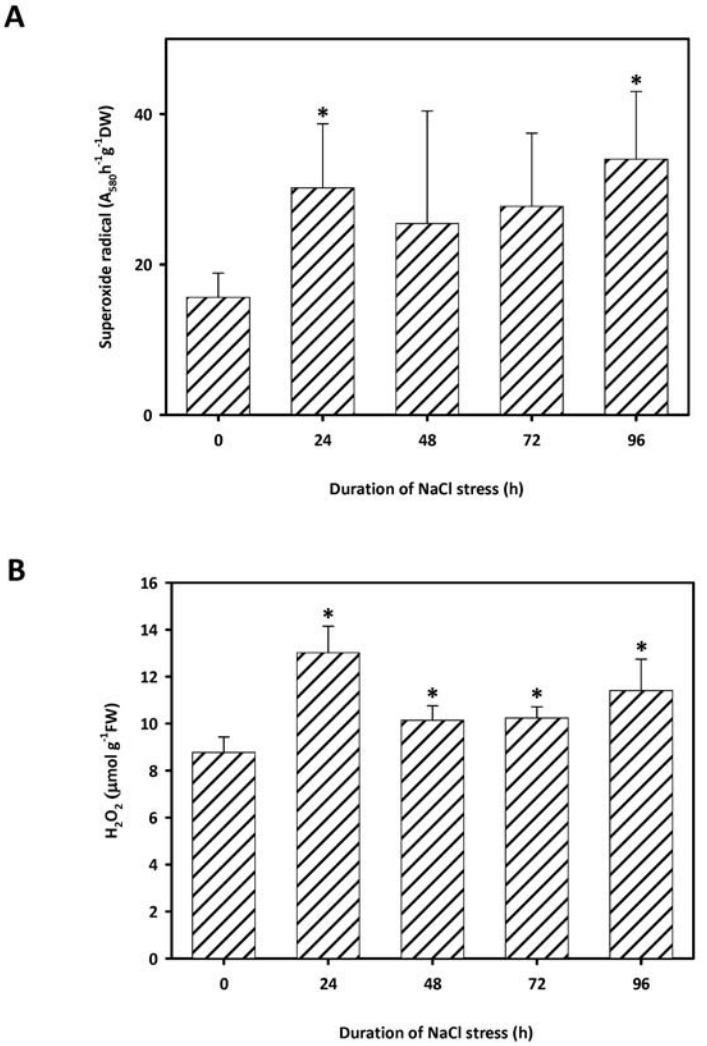

Figure 1. Effects of NaCl treatment on superoxide radical (A) and H_2O_2 (B) in *Spirodela polyrhiza*. Each value represents the mean ± SE (n = 3) obtained from 4 independent experiments. Columns marked by asterisks indicate a significant difference at $P < 0.05$.

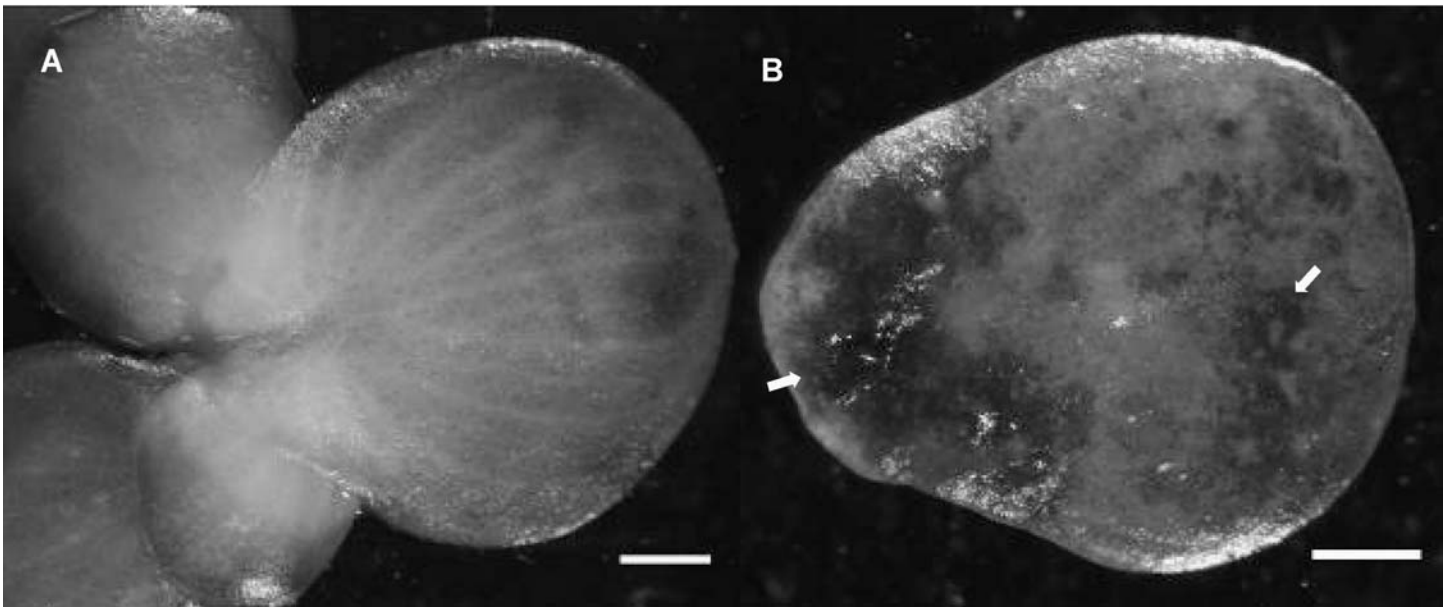

Figure 2. DAB-staining in fronds of *Spirodela polyrhiza* plants. Giant duckweed was cultured in modified SH solution for 2 weeks and transferred to the same solution (A) or the same solution containing 100 mM NaCl (B) for 4 d. Giant duckweed fronds were rinsed with distilled water and stained with 0.1% DAB (in 10 mM MES, pH 6.5) for 8 h. After treatment, fronds were transferred to 95% ethanol and boiled for 10 min and repeated 2 times. H_2O_2 was stained and localized in dark brown area and indicated by arrows. The scale bar represents 1 mm.

nied with an increase in the activity of H_2O_2-scavenging enzymes such as APX, CAT, GR, and POD in NaCl-treated giant duckweed plants. In our study, the CAT activity increased with salinity in plants treated with NaCl (Figure 3C). Salt was found to decrease the activities of CAT in root tissues of *Lemna minor* (Panda and Upadhyay 2003); in contrast, increased activity was determined in cadmium-treated plant extracts of common duckweed (Tkalec et al. 2008). Both SOD and CAT isozymes were analyzed, and their activities on native gels were detected in all treatments (Figure 3B and 3D).

Activity of APX increased significantly (except at the 24 h time point) in plants treated with salt when compared to the control plants (Figure 4A). Giant duckweed showed a 1.8-fold increase in APX activity at the 48 h time point compared to controls at 100 mM NaCl. Salt-induced changes in the activities of GR in duckweed plants (Figure 4B) increased significantly ($P < 0.05$) only in plants exposed to NaCl for 48 h compared with that in control plants. In *S. polyrhiza*, higher activities of CAT and APX with NaCl treatments indicates that these enzymes play crucial roles in scavenging excessive H_2O_2 in NaCl-treated giant duckweed plants. In plant cells, constitutive and induced APX uses ascorbic acid to reduce H_2O_2 to water (Noctor and Foyer 1998). Enhancement of APX activity is not only an important sign of salt tolerance in sesame plants (Koca et al. 2007) but also a possible sign of oxidative tolerance in cadmium-treated *Lemna* (Tkalec et al. 2008).

Total POD activity, which decomposes H_2O_2 produced by SOD, also increased significantly with respect to salt stress (Figure 5A). POD isozymes were detected using the nondenaturing PAGE in plant tissues of *S. polyrhiza* treated with NaCl during a 4 d period (Figure 5B). POD isozyme bands (I, II, and III) were enhanced, and the activity of isozyme VI was slightly detected in plants treated with NaCl, suggesting that isozyme VI genes are positively regulated under NaCl stress. These results indicate that the increase in the POD activity during a 4 d period was more pronounced in salt-stressed giant duckweed when compared to the other antioxidative enzymes, and was further corroborated by NaCl inducing a new isoperoxidase. Similar results in overexpression of POD isozymes under salt stress was reported in French beans (*Phaseolus vulgaris*), maize (*Zea mays*), and strawberry (*Fragaris* × *ananassa*; Nagesh Babu and Devaraj 2008, Tanou et al. 2009, Koskeroglu and Tuna 2010). The significance and function of the POD isozyme VI was not clear under salt stress in giant duckweed and should be investigated in the future.

In freshwater ecosystems, plants actively scavenged ROS during normal growth through low-molecular mass antioxidants (ascorbate, carotenoids, and glutathione) and ROS-scavenging enzymes (Scandalios 1993, Arora et al. 2002, Sairam and Tyagi 2004). However, when plants are subjected to environmental stresses, ROS production is high in plant tissues and overcomes the antioxidant system capacity; therefore, oxidative stress occurs and cytotoxic damage to proteins, DNA, and membranes may be observed (Arora et al. 2002, Sairam and Tyagi 2004). Studies have shown that salt-tolerant species increase their antioxidative enzyme activities and antioxidant contents in response to salt stress, whereas salt-sensitive species normally fail to do so (Koca et al. 2007, Yazici et al. 2007). Accumulation of NaCl in plants can modulate the activities of antioxidative enzymes, including SOD, CAT, and peroxidases (Panda and Upadhyay 2003, Sumithra et al. 2006, Tanou et al. 2009, Koskeroglu and Tuna 2010). Results presented here are similar to previous reports in which antioxidative enzymes, such as SOD, which converts

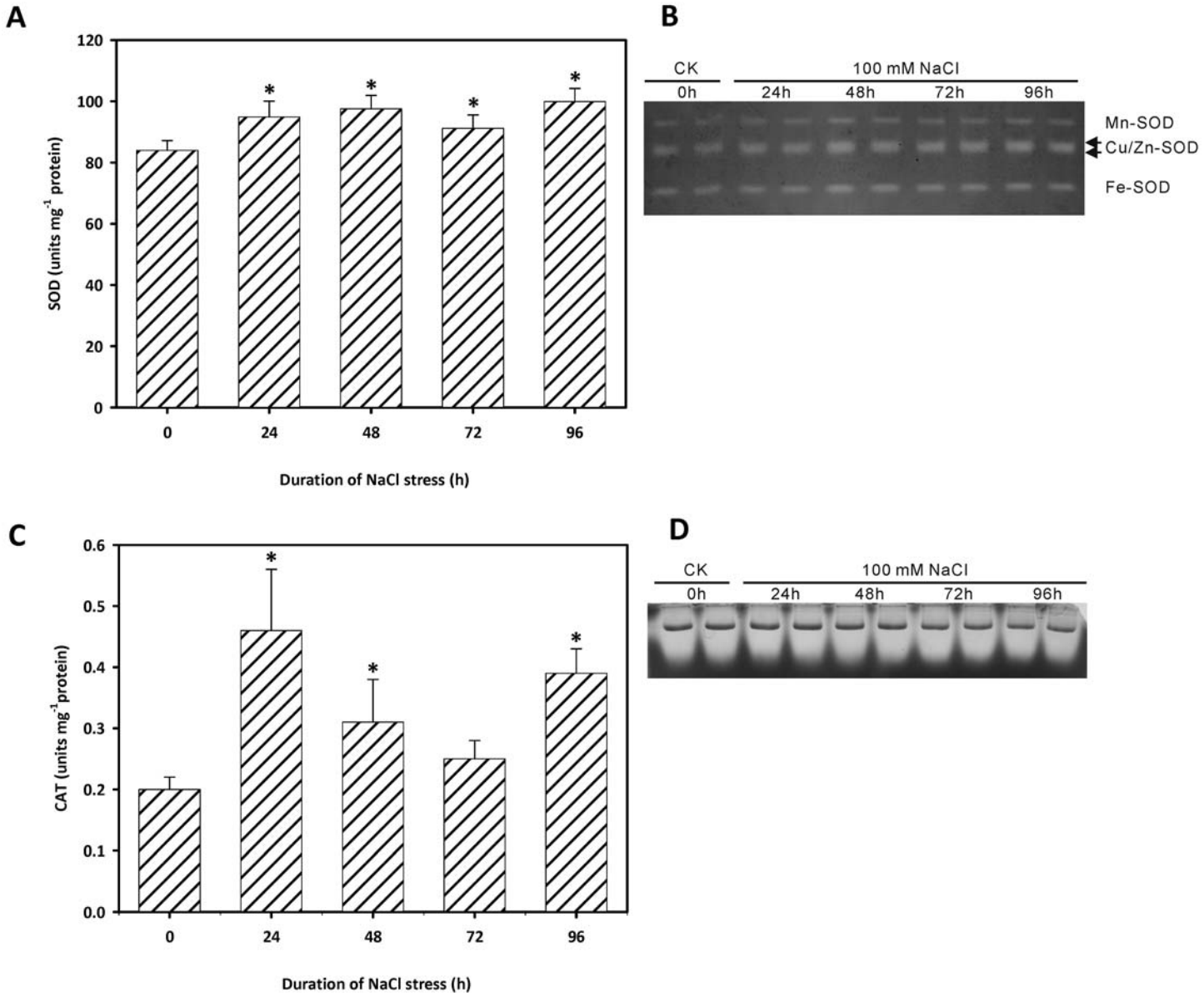

Figure 3. (A and B) Effects of NaCl treatment on superoxide dismutase (SOD); (C and D) catalase (CAT) activity in *Spirodela polyrhiza*. Enzyme activities were assayed and obtained from 4 independent experiments, and the means ± SE (n = 3) were reported. Columns marked by asterisks indicate a significant difference at $P < 0.05$. The activity of SOD (B) and CAT (D) on native gels was stained and visualized. SOD isoforms were identified according to their different sensitivities to KCN and H_2O_2 (see Materials and Methods). 20 mg of protein was loaded for each lane. CK: control plants; Mn-SOD: SOD ioszyme in mitochondria; Cu/Zn-SOD: SOD ioszyme in cytoplasm; Fe-SOD: SOD ioszyme in chloroplast.

superoxide radical to H_2O_2, were higher in samples of duckweed plants under NaCl stress conditions when compared to the controls. The enhanced activities of APX, CAT, and POD under salt stress and the change in isozyme patterns of POD indicated that an adaptive mechanism to reduce the ROS offers protection against oxidative damage in giant duckweed.

Salt stress is an important environmental factor that not only reduces plant growth and productivity (Boyer 1982) but also leads to a decline in species richness (Hart et al. 2003, Cao et al. 2007). Aquatic systems occupy the lowest areas in the landscape where salt can be accumulated (Hart et al. 2003, Brock et al. 2005). While salinization occurs from natural processes, the rate of salinization has increased significantly as a result of human activity, such as agricultural runoff, industrial discharge, and biological waste decomposition (Wang et al. 2008). Aquatic plants are different from terrestrial plants in salt-induced responses because salt stress overlaps with drought in terrestrial plants, but drought stress generally does not exist in the aquatic environment (Fodorpataki and Bartha 2008). Aquatic plants do not develop protective strategies against water deficit; therefore, freshwater macrophytes are more sensitive to increasing salinity, which may occur with intense evaporation of water from lakes or rivers, in relation with global warming, and to discharging saline water into wetlands (Wang et al. 2008). The timing of the pulse release and the growth stage of plants are impor-

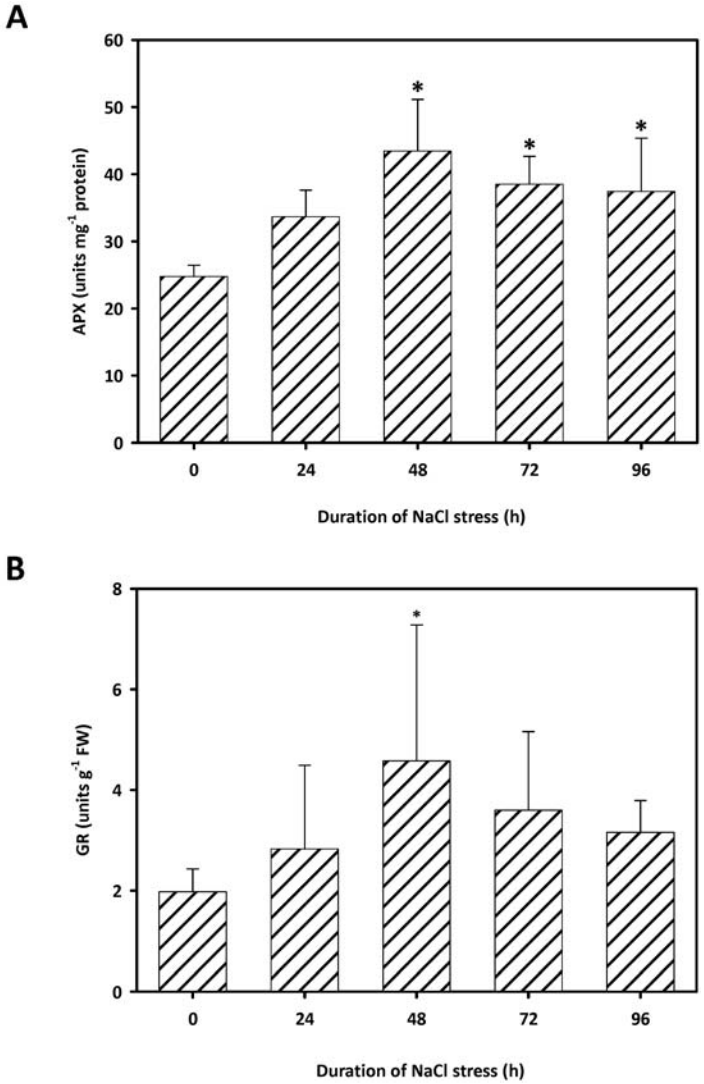

Figure 4. Effects of NaCl treatment on (A) ascorbate peroxidase (APX) and (B) glutathione reductase (GR) activity in *Spirodela polyrhiza*. Each value represents the mean ± SE (n = 3) obtained from four independent experiments. Columns marked by asterisks indicate a significant difference at $P < 0.05$.

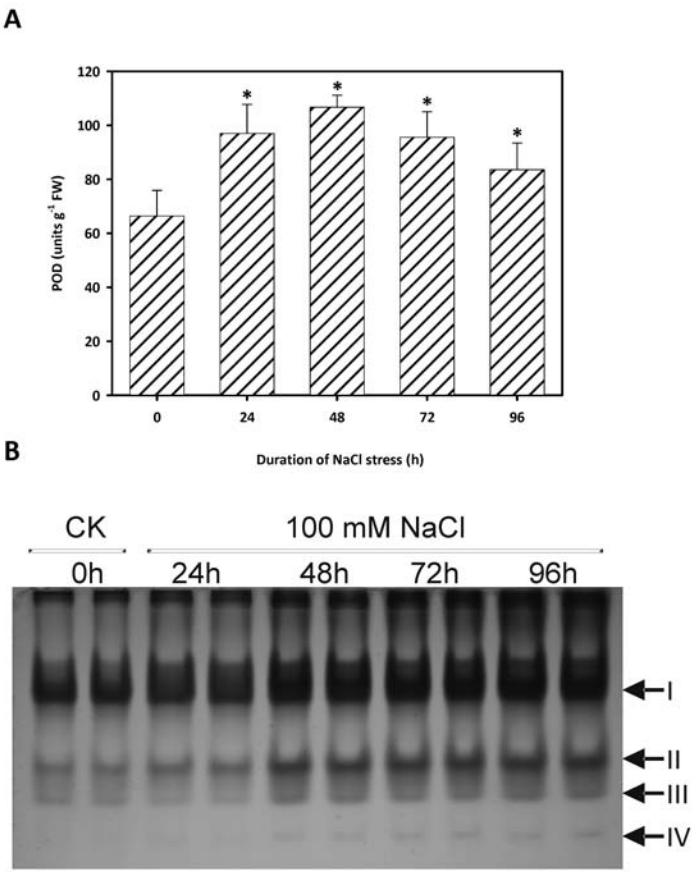

Figure 5. Effects of NaCl treatment on peroxidase (POD) activity in *Spirodela polyrhiza*. Guaiacol peroxidase (G-POD) activity (A) and the activity of POD (B) on native gel was stained and visualized (see Materials and Methods). The bands, which became enhanced in response to NaCl, are indicated by arrows. Each value represents the mean ± SE (n = 3) obtained from 4 independent experiments. Columns marked by asterisks indicate a significant difference at $P < 0.05$.

tant when saline water is discharged into wetlands (Warwick and Bailey 1998); therefore, studies on the responses of giant duckweed to increasing salinity are useful in developing water management programs to minimize salinity damage to sensitive species (Warwick and Bailey 1998, Goodman et al. 2010). A proper understanding of the biochemical response and adaptation mechanism of *S. polyrihza* under salt stress will help develop salt tolerant giant duckweed plants in the future.

In summary, growth inhibition in plant roots and shoots is a common response to soil salinity. In this study, giant duckweed, a fresh water plant lacking strategies against water stress, was analyzed for salt tolerance. The results showed that salt stress caused growth inhibition, reduced chlorophyll and carotenoid contents, and enhanced antioxidative enzyme (APX, CAT, SOD, and POD) activities in frond tissues (Figure 6). Although accumulation of proline within cytoplasm was not observed, the NaCl-induced increase in ROS content and MDA level indicates that oxidative damage is an important mechanism for salt toxicity in giant duckweed.

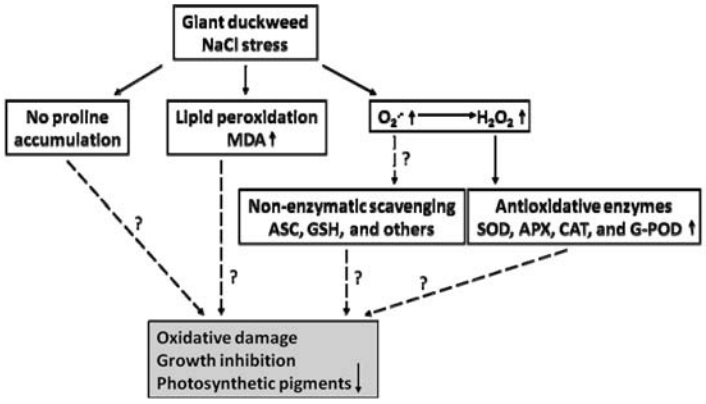

Figure 6. The summary of NaCl-induced stress response in *S. polyrhiza*. Solid line: data from this study; dot line: mechanism unknown. ASC: ascorbic acid; GSH: glutathione.

The increased activities of antioxidative enzyme such as peroxidase and a newly induced isoform of POD in NaCl-treated plants strongly suggest its crucial roles in *S. polyrhiza* defense against oxidative stress.

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