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Salt-Induced Changes in Antioxidative Enzyme Activities in Shoot Tissues of Two Atriplex Varieties

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Abstract

This study examined the influence of salt levels on antioxidant activity and content of carotenoids and anthocyanins of the *A. hortensis* leaves using two varieties: green orach (var. *purpurea*) and red orach (var. *rubra*). Seeds of *Atriplex* were exposed to 0, 90, 180 and 260 mM NaCl for 3 months and seeds were sown in an earthen pot. Overall levels of ascorbate peroxidase (APX) and glutathione reductase (GR) activity were significantly elevated. Salt stress caused a significant decline in tissue concentrations of catalase (CAT) and superoxide dismutase (SOD). However, 90 mM NaCl did not modify these parameters, which remains similar to control values. Activities of APX and CAT were increase whether the shoots of *A. hortensis* var. *purpurea* were grown in the presence of 180 mM NaCl. Thus although some indications of oxidative stress accompany exposure of this salt-tolerant *Atriplex* varieties to salinity, mechanisms appear to exist within its shoot tissue to permit the tolerance of such oxidative stress. High salt concentration in the culture medium provokes oxidative damage in *Atriplex* leaves and induces a general increase in antioxidant enzyme activity. In particular, NaCl toxicity decreased content of carotenoids. It also decreased the concentration of anthocyanin pigments in leaves of *Atriplex*. This work therefore provides a starting point towards a better understanding of the role of antioxidant enzyme in the plant response against salt stress.

Keywords: anthocyanins, antioxidant enzymes, *Atriplex hortensis*, carotenoids, Salinity stress

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; SOD, GR, glutathione reductase; superoxide dismutase

Introduction

Salt stress has become one of the most damaging environmental hazards to crop productivity all over the world (Ashraf and Ali, 2008). This adverse environmental condition impairs plant growth by both water deficit and ionic toxicity (Munns and Tester, 2008). Most plants can adapt to low or moderate salinities, but their growth is severely limited above 200 mM NaCl (Hasegawa *et al.,* 2000). Salinity stress leads to a series of changes in basic biosynthetic functions, including photosynthesis and photorespiration, and amino acid and carbohydrate synthesis (Seki *et al.,* 2002). To avoid the cellular damage due to reactive oxygen species (ROS) generation, plants produce a number of antioxidant enzymes that are induced and provide secondary protection against oxidative stress (Mittova *et al.,* 2003). It is generally accepted that stress-induced deregulation of plant metabolism leads to the enhanced production of ROS, the cellular titer of which is policed by the antioxidant system (Beak and Skinner, 2003). Both ROS and soluble antioxidants are involved in signaling processes in plants: the picture that is emerging suggests that relatively stable oxidants (H_2O_2) and antioxidants (ascorbate, glutathione) act as sensors of the 'oxidative load' on the cell (Noctor *et al.,* 2002). Salinity may lead to the production of ROS and tissue damage (Adams *et al.,* 2004). Antioxidant metabolism plays an important role in protecting plants from a wide variety of environmental stresses, such as drought, extreme temperatures, pollutants, ultraviolet radiation and high levels of light (Amirjani, 2010; Siringam *et al.,* 2011). The antioxidant systems, including antioxidants and antioxidant enzymes, can ameliorate the deleterious effects of ROS in vivo and in vitro. Antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) function, by catalyzing the decomposition of oxidants and free radicals. The ROS concentration in the tissues directly exposed to salt are strongly influenced by the coordinated action of different antioxidative enzymes (Munns and Tester, 2008). Recently, it has been reported that salinity provoked in plant leaves an imbalance between ROS production and antioxidant defenses, with the induction of oxidative stress (Valderrama *et al.,* 2006). To counteract the toxicity of ROS, defense systems that scavenge cellular ROS have been developed in plants to cope with oxidative stress via the nonenzymatic and enzymatic systems (Noctor and Foyer, 1998). Enhancement of antioxidant defense in plants can thus increase tolerance to different stress fac-

tors. Antioxidants (ROS scavengers) include enzymes such as CAT and SOD, as well as enzymes such as APX, GR, carotenoids, and anthocyanins. Photosynthesis provides a strong reducing power and a high risk for generation of ROS particularly under environmental constrains (Foyer *et al.,* 1994). Under unfavorable conditions, the biosynthesis and the activity of these antioxidants increase (Horling *et al.,* 2003; Mittler *et al.,* 2004; Rossel *et al.,* 2002) and stabilize the chloroplast redox poise (Asada, 2000; Foyer *et al.,* 1994).

The question of a functional significance of anthocyanin pigments in leaves has received substantial attention in the recent literature (Archetti *et al.,* 2009; Chalker-Scott, 1999; Manetas, 2006). Comparatively little attention has been given to the question of why only certain species change leaf colour from green to red during certain ontogenetic stages or seasons while others do not. Most research seeking to determine a functional role of anthocyanins in evergreen leaves has focused on their putative roles in photoprotection (Hughes *et al.,* 2005; Hughes and Smith, 2007; Kytridis *et al.,* 2008).

Anthocyanins are thought to minimize oxidative damage (Hughes *et al.,* 2005), and/or through neutralizing ROS directly as antioxidants (Kytridis and Manetas, 2006). Specifically, anthocyanin synthesis is known to be inducible under high salinity (Eryilmaz, 2006) and drought (Yang *et al.,* 2000). Furthermore, species with high levels of foliar anthocyanin seem to be common in environments characterized by low soil moisture (Spyropoulos and Mavormmatis, 1978), and are more tolerant of drought conditions (Paine *et al*., 1992). Carotenoids are responsible for quenching of singlet oxygen (Knox and Dodge, 1985) hence their comparative levels in a variety may determine its relative tolerance. Salt stress is a complex phenomenon that involves morphological and developmental changes. With the aim of obtaining a better comprehension of the effect of salinity on oxidative stress defense system, we have analyzed oxidative stress parameters, the behavior of the well-known antioxidant enzymes CAT, GR, APX and SOD, as well as the response of carotenoids and anthocyanins to different NaCl concentrations. In view of the above, the present study was designed to investigate the oxidative damage and antioxidant system of two *Atriplex* (*Atriplex hortensis*) varieties subjected to high salt levels in the medium. The results show that NaCl toxicity causes oxidative damage in *Atriplex* leaves and that antioxidant defences, particularly related to APX and GR, are increased.

Materials and methods

Plants and Growth Conditions

Atriplex hortensis (L.), a C_3 xero-halophyte of the family Chenopodiaceae, is an annual species that is well adapted to saline and drought conditions. Seeds of two varieties of *A. hortensis* were obtained from CN Seeds Ltd. (Ely, UK). The varieties used were *A. hortensis* var. *purpurea* (green) and *A. hortensis* var. *rubra* (red). The plants were cultivated in an experimental station and tested under greenhouse conditions between March and August in Tunis (10°14' E, 36°50' N). The plants were grown in randomised rows under mono-specific conditions (natural light, an average temperature of 28/20°C day/night, mean annual rainfall of 525 mm and a relative humidity of 65/90%).

Atriplex seeds were planted in pots 14 cm in diameter and 25 cm in depth; each pot containing 3.5 kg of soil. The soil was characterized as: sandy loam in texture with 52.3%, 10.5% and 12% of sand, silt, and clay, respectively; pH 8.75 and organic matter 1.5%. The field capacity (FC) of soil, measured according to the technique reported by Bouyoucos (1983), was 12%.

The salt concentration in the water solution was increased up to 260 mM NaCl following this scheme, to give salinity treatments of 0, 90, 180 and 260 mM NaCl. Salt stress was initiated 21 days after seed germination. Pots were arranged in a completely randomized design within treatment trays and the position of the trays was changed weekly to avoid a position effect in the greenhouse. Pots were placed in trays with standing salt stress. The same nutrients amount was used at any salinity level. During this period, the plants were watered with Hoagland nutrient solution once a week.

Analyses

For enzyme extracts and assays, leaves were sampled every 6 d. 0.2 g of leaves were frozen in liquid nitrogen and then ground in 4 mL solution containing 50 mM phosphate buffer (pH 7.0), 1% (w/v) polyvinylpolypyrrolidone, and 0.2 mM ascorbic acid (ASA). The homogenate was centrifuged at 15,000 *g* for 30 min, and supernatant was collected and used for enzyme assays.

SOD was assayed by the nitroblue tetrazolium (NBT) method as described by Gong *et al*. (2005). The reaction mixture (3 mL) contained 50 mM K-phosphate buffer, pH 7.3, 13 mM methionine, 75 mM NBT, 0.1 mM EDTA, 4 mM riboflavin and enzyme extract (0.2 mL). Riboflavin was added last, and the glass test tubes were shaken and placed under fluorescent lambs (60 mmol m^{-2} s^{-1}). The reaction occurred for 5 min and was then stopped by switching off the light. The absorbance was measured at 560 nm. Blanks and controls were run in the same manner, but without illumination and enzyme extractions, respectively. One unit of SOD was defined as the amount of enzyme that produced 50% inhibition of NBT reduction under the assay conditions.

APX activity was determined by following the decrease of ascorbate and measuring the change in absorbance at 290 nm for 1 min in 2 mL of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1 mMEDTA-Na_2, 0.5 mM ascorbic acid, 0.1 mMH_2O_2 and 50 mL of crude enzyme extract at 25°C. APX was determined according to Nakano and Asada (1981). The decrease in

ascorbate concentration was followed as a decline in the optical density at 290 nm, and activity was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1} at 290 nm) for ascorbate.

CAT activity was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 (Cakmak *et al*., 1993). The reaction mixture (3 mL) contained 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50 mL of crude enzyme extract at 25°C. The activity was calculated using the extinction coefficient (40 mM^{-1} cm^{-1}) for H_2O_2 (Kato and Shimizu, 1987).

Reduced glutathione (GSH) was assayed by the enzymatic recycling procedure in which it is sequentially oxidized by 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and reduced by nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of glutathione reductase according to Griffith (1980). The ground tissue (approximately 1 g fresh wt.) was, homogenized in 4 ml 5% sulfosalicyclic acid and centrifuged at 10,000 × *g* for 10 min. A 330 µl aliquot was removed and neutralized by addition of 18 µl 7.5 M triethanolamine. One 150 µl sample was then used to determine concentrations of sure GSH plus (GSSG). Another was pretreated with 3 µl 2-vinylpyridine for 60 min at 20°C to mask the GSH by derivatization and to allow the subsequent determination of GSSG alone.

Extraction and quantification of anthocyanins

For measurements of anthocyanin content, leaf discs were powdered in liquid nitrogen and extracted with methanol containing 1% HCl. Anthocyanins were extracted as previously described (Close *et al.,* 2000) before absorbance measurements at 530 and 657 nm. Quantitation of anthocyanins was performed using the following equation: Q (anthocyanins) = (A530 - 0.25 A657) × M^{-1}, where Q (anthocyanins) is the concentration of anthocyanins, A530 and A657 are the absorptions at the wavelengths indicated, and M is the fresh weight (in grams) of the plant tissue used for extraction. The numbers of samples used for the measurements are indicated in each figure. Error bars indicate the SD of the average anthocyanin contents.

Carotenoids pigments

At the end of the experiment period, carotenoids pigments in fully expanded leaves (a randomly selected mixture of old and young leaves) from each treatment were extracted using 0.05 g of fresh material in 10 mL of 80 % aqueous acetone. After filtering, 1 mL of the suspension was diluted with a further 2 mL of 80 % aqueous acetone, and carotenoids (C*x* + c) contents were determined with a spectrophotometer, using wavelength (470.0 nm). Concentrations of pigments [µg g fresh weight (f. wt)$^{-1}$] were obtained by calculation, using the method of Lichtenthaler (1987).

C_{x+c} = 1000 A470 - 2.270 C_a - 81.4 C_b/227

C_a = Chlorophyll a, C_b = Chlorophyll b, C_{x+c} = Carotenoids.

Statistical analysis

The experiment consisted of pots in a randomized complete block design with six replications (pots). Data were the means of five plants (± SE). Differences were analysed using one-way ANOVA, followed by *post-hoc* comparisons using Statistica Software (2007). Differences were considered significant for $p < 0.05$. All calculations were made using Statistica V6.1 software (StatSoft).

Results and discussion

Activities of antioxidant enzymes

In order to get further insight into the effect all salt-stress on oxidative stress parameters, CAT, APX, GR and SOD activities were determined. As shown in Fig. 1, a decrease of CAT activity occurred in plants treated with 90 and 260 mM NaCl, respectively, whereas no difference respect to controls for a var. *purpurea* were observed in 180 NaCl-treated plants. As shown in 260 mM NaCl caused a 43% and 33% enhancement of SOD activity of two varieties respect to controls. On the other hand, under 90, 180 and 260 mM NaCl, there was no significant difference in SOD activity, but a significant inhibition was observed respect to controls (Fig. 3). Analysis revealed that these activities are correlated with increase APX and GR activities, were increased under 90 mM NaCl, and no differences were observed respect to controls under 260 mM salt (Fig. 2 and 4). Treatment with 180 mM NaCl caused an increase in APX and GR activities of *A. hortensis* var. *purpurea* (green).

Activity of GR increased steadily with NaCl concentration and it nearly doubled in response to 90 mM NaCl for red variety. In contrast, there was not much increase in

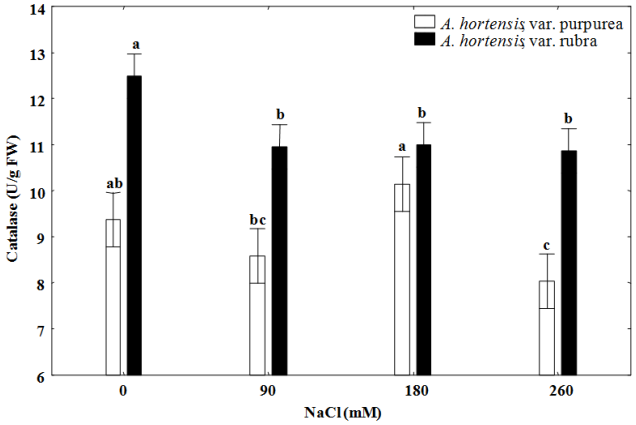

Fig. 1. Effect of different salt concentrations on catalase activity. Enzyme activity was measured as described in Experimental section. Columns represent means (n = 5) and error bars represent ±SE of the means. Columns for each variety with a different lower-case letter were significantly different at $p < 0.05$ compared to control

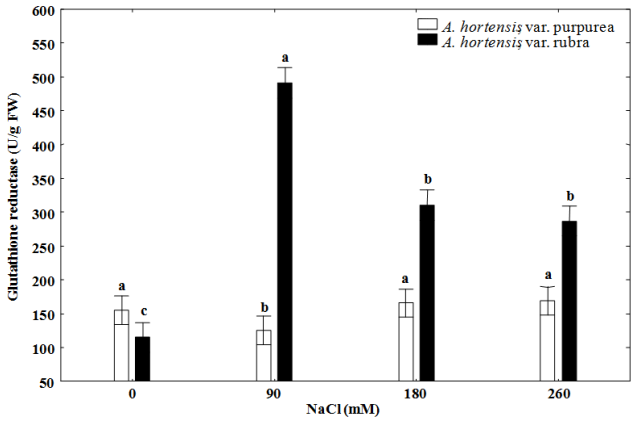

Fig. 2. Effect of different salt concentrations on glutathione activity. Enzyme activity was measured as described in Experimental section. Columns represent means (n = 5) and error bars represent ±SE of the means. Columns for each variety with a different lower-case letter were significantly different at $p < 0.05$ compared to control

the activity of GR for green variety at the concentrations (180 and 260 mM) compared with the control. However, at higher concentrations GR activity of var. *rubra* showed a significant ($p < 0.05$) decrease compared with the control. In this current work *Atriplex* varieties showed different responses to salt stress in terms of growth and antioxidative enzyme activity. Oxidative stress, which frequently accompanies high temperature, salinity, or drought stress, may cause denaturing of functional and structural proteins (Mandhania *et al.,* 2006; Shanker *et al.,* 2004). As a consequence, these diverse environmental stresses often activate similar cell signaling pathways (Foyer *et al.,* 1997; Zhu, 2001) and cellular responses, such as the production of stress proteins and up regulation of antioxidants (Vierling and Kimpel, 1992; Zhu *et al.,* 1997). A number of reports indicate that oxidative stress induces an increase in the responses of enzymatic systems linked to ROS-scavenging process (Jones and Smirnoff, 2005). In fact, GR is a key enzyme in providing protection against a variety of environmental and abiotic stresses (Dalton, 1995; Romero-Puertas *et al.,* 2006). Activity of GR and hence GSH production is generally elevated in plants upon exposure to xenobiotics and various environmental stresses (Foyer *et al.,* 1991). APX and GR which are, respectively, are responsible for H_2O_2 detoxification in green leaves (Foyer and Harbinson, 1994). APX is considered to be a key antioxidant enzyme in plants (Orvar and Ellis, 1997) and GR has a central role in maintaining the reduced glutathione (GSH) pool during stress (Pastori *et al.,* 2000). SOD is the major scavenger of superoxide ($O_2^{\cdot -}$) to form H_2O_2 and O_2, and plays an important role in defense activity against the cellular damage caused by environmental stress (Meloni *et al.,* 2003). In rice leaf, the salt, preferentially enhances the activities of SOD, APX and GPX, decreases the CAT one and has little effect on the activity of GR (Lee *et al.,* 2001). Gossett *et al.* (1994) reported that in cotton, NaCl increases the SOD, GPX and GR activities and decreases that of CAT and APX.

Anthocyanins and carotenoids content

In this study, the response of these two varieties to salinity was evaluated. Given that they have different color, we expected that there would be distinct differences in their metabolic responses to salinity. Anthocyanins are a functionally diverse group of secondary products with roles in pigmentation, plant-microbe interaction, and reproduction. In our experiment, the Anthocyanins pathway was induced during salinity stress in *A. hortensis* var. *rubra*, but not in *A. hortensis* var. *purpurea*. This study was undertaken to evaluate in *Atriplex* leaves, the response of

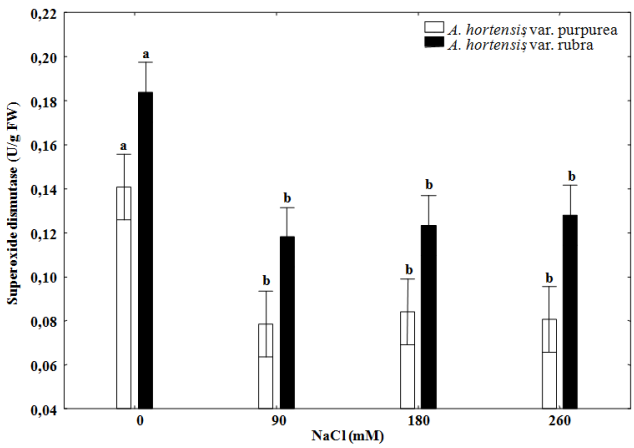

Fig. 3. Effect of different salt concentrations on superoxide dismutase activity. Enzyme activity was measured as described in Experimental section. Columns represent means (n = 5) and error bars represent ±SE of the means. Columns for each variety with a different lower-case letter were significantly different at $p < 0.05$ compared to control

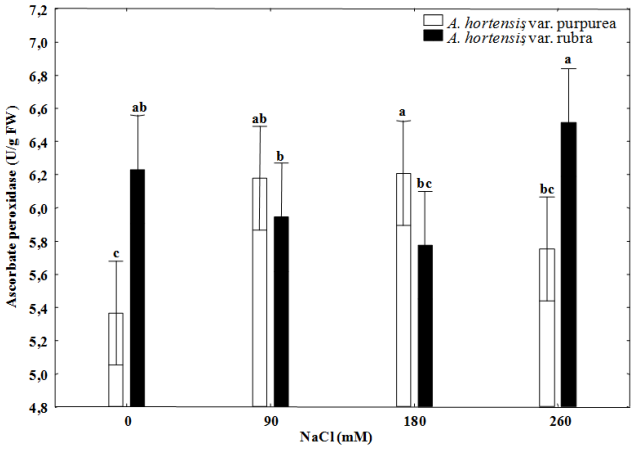

Fig. 4. Effect of different salt concentrations on ascorbate peroxidase activity. Enzyme activity was measured as described in Experimental section. Columns represent means (n = 5) and error bars represent ± SE of the means. Columns for each variety with a different lower-case letter were significantly different at $p < 0.05$ compared to control

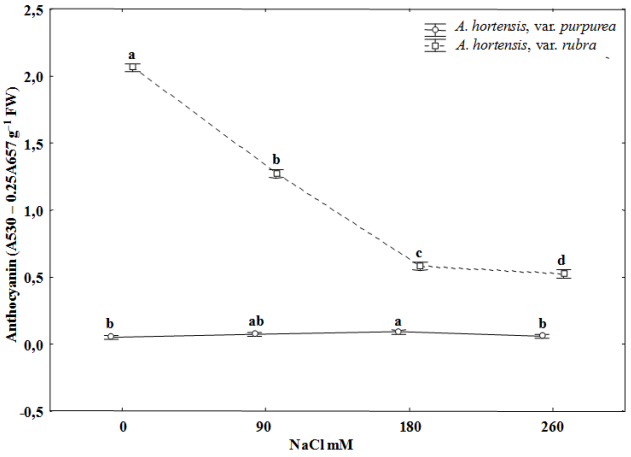

Fig. 5. Anthocyanins content in the leaves of *Atriplex hortensis* subjected to increasing salinity. Anthocyanin quantification was performed by a spectrophotometric method. Error bars represent standard deviation (n = 5). The experiments were repeated three times with similar results

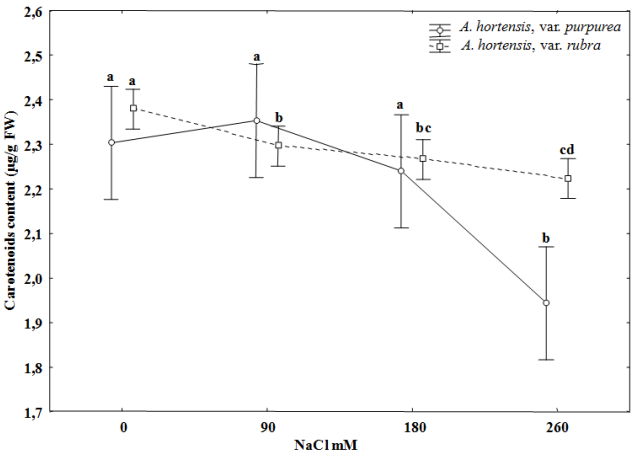

Fig. 6. Carotenoids content in the leaves of *Atriplex hortensis* subjected to increasing salinity. Data are mean ± SD (n = 5). Different letters indicate that the mean value is significantly different ($p < 0.05$) compared to control

carotenoid pigments to salinity. The results presented in this study showed that carotenoid pigments of var. *purpurea* leaves was induced by 260 mM NaCl, no induction was observed under 180 mM, and a decrease occurred in plants of var. *rubra* treated under salinity stress. According to our results, it can be suggested that under 90 mM NaCl the defense system of *A. hortensis* var. *rubra* can cope with carotenoid damage, under salt stress carotenoid pigments are induced in an attempt to protect the cell against this insult. Taken together, the present results allow us to conclude that under salinity anthocyanins amount and carotenoids content are enhanced in an attempt to protect leaves tissues against this insult. Inhibition of anthocyanins content demonstrated that this flavonoid plays a leading role in the defense mechanism against salinity, and it could be considered as essential component of defense system in plant tissues (Fig. 5). In addition, carotenoids concentrations were decreased in var. *rubra*, but not in var. *purpurea*. Content of anthocyanins decreased steadily upon exposure to salt stress and this decrease was statistically significant at $p < 0.05$ for the both varieties (Fig. 6). Anthocyanin pigments play many important eco-physiological roles in plants. Anthocyanins are a diverse group of secondary metabolites with a wide array of biological functions, including roles in stress protection (Winkel-Shirley, 2002). Carotenoids, being antioxidants, have the potential to detoxify the plants from the effects of ROS (Verma and Mishra, 2005). Salinity induced decline in carotenoid content has been reported in maize genotypes (Singh *et al.,* 2008) and wheat genotypes (Sairam *et al.,* 2002). Hernandez *et al.* (1995) have also suggested that carotenoids may be one of the required factors for salt tolerance in crop plants and therefore, carotenoid content may be helpful to differentiate between salt sensitive and tolerant cultivars. However, it remains to be elucidated the mechanisms underlying the coordinated regulation of the antioxidative enzyme activities under salt stress.

Conclusions

For successful scavenging of ROS by a scavenging system, some antioxidant enzymes must cooperate with each other. Exposure to salinity induced oxidative stress through the enhanced generation of ROS, which was accompanied by membrane damage, enhanced carotenoids levels, anthocyanins accumulation and by activation of antioxidant enzyme systems. Increased levels of scavenging enzymes indicate their induction as a secondary defense mechanism in response to salt stress.

However, more research about the reaction of Atriplex plant to other factors accompanying climate change (such as elevated temperature) and their interaction with salinity tolerance would be desirable.

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