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RESEARCH ARTICLE

ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANT ACTIVITIES IN MAIZE (*ZEA MAYS* L.) UNDER SALT STRESS

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Abstract

The present study was carried out to determine the enzymatic antioxidants like superoxide dismutase (SOD EC, 1.15.1.1), catalase (CAT EC, 1.11.1.6), peroxidase (POD EC, 1.11.1.7) and non-enzymatic antioxidants such as ascorbic acid and □-tocopherol were analyzed in *Zea mays* L. in order to explore the plant's protective mechanism against reactive oxygen species under salt stress. These enzymes were examined and analyzed in 45 days old plants exposed to various concentrations of 25mM-150mM NaCl. The NaCl stress enhanced all the enzymatic and non-enzymatic antioxidant activities were augmented with increasing salinity compared with respective control. The results suggest that maize plants may increase the activity of antioxidant enzymes to have superior protection system against oxidative damage under NaCl stress.

Key words: Antioxidant enzyme, NaCl, Stress, Oxidative damage, Maize

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Introduction

Salinity is one of the most harmful factors responsible for decline in plant growth and yield (Shahbaz *et al.* 2012) by disconcerting numerous physiological and biochemical processes like photosynthesis, ion homeostasis and enzyme activities (Hasegawa 2013). It also causes the production of oxidative stress in plants by generation of reactive oxygen species (ROS) such as superoxide radicals (O_2^-), singlet oxygen, ($^1O_2$) hydrogen peroxides (H_2O_2), and hydroxyl radicals ($.OH^-$) (Sharma *et al.* 2012). These ROS can damage nucleic acids of cells as well as protein degradation and essential membrane lipids (Ozgur *et al.* 2013).

To repair the restrictive effects of reactive oxygen species, plants activate the antioxidant system that is involved in the detoxification of ROS. This system comprises of enzymatic and non-

enzymatic antioxidants. Of various enzymatic antioxidants, superoxide dismutase (SOD) converts superoxide to H_2O_2 in different sub-cellular compartments. H_2O_2 is further scavenged by catalase (CAT), ascorbate peroxidase (APX) and other non-enzymatic antioxidants including tocopherols, Carotenoids and ascorbate (Weisany *et al.* 2012) and they can scavenge ROS produced in plants under salinity (Batool *et al.* 2012).

Maize (*Zea mays* L.) is a genus of the family Graminae (Poaceae), commonly known as the grass family, and is considered as one of the most important cereal crops used for not only human consumption and animal feeding but also starch industry and oil production (Amin *et al.* 2007). Maize is C_4 plant and classified as moderately sensitive to salinity. There is significant reduction in the growth and yield for maize when grown under salinity condition (Ouda *et al.* 2008). It is the third most important cereal after wheat and rice in many parts of the globe, it is pompously called as " Queen of Cereals and "king of Fodder" and miracle crop (Rajurkar *et al.* 2011). The purpose of the present investigation was to evaluate anti-oxidative defense system stimulation against reactive oxygen species under salt stress.

Materials and Methods

The seeds of *Zea mays* were obtained from Syngenta Ltd. The seeds were shown in plastic pots dimensions of 22cm height and 26cm wide and filled with equal amount (1:1:1) of soil, sand and farm yard manure was mixed and mixture was used to fill these pots. The experiment was placed in a Completely Randomized Block Design with three replicates and each replicates consisted of seven pots. Five seeds for each pot were utilized and inundated with faucet water for 20days. After germination the seedlings were diminished to one plant for each pot. The salt treatments were started 20days after planting and it consists of 25mM, 50mM, 75mM, 100mM, 125mM and 150mM NaCl and 0mM served as control and imposed on plant to 10 days, on fifteenth day after salt treated examples were gathered for additional investigation.

Extraction of enzymes and assays

Enzymatic antioxidants

Two grams of young leaves were macerated to powder with liquid nitrogen with a mortar-pestle; then 0.1 g PVP and 5 ml of extraction buffer (consisting of 1 M Sucrose, 0.2 M Tris-HCl and 0.056 M β-Marcaptoethanol; pH changed at 8.5) was added and homogenized. The extracts were centrifuged at 10,000 rpm for 20 min at 48°C; supernatants were used as samples for enzyme assays.

Estimation of superoxide dismutase: (SOD: E. C. 1. 15.1.1)

Superoxide dismutase was assayed as described by Beauchamp & Fridovich (1971). The reaction mixture contained 1.17 µM × 10^{-6}M riboflavin, 0.1 M methionine, 2µM × 10^{-5}M potassium cyanide and 5.6 µM × 10^{-6}M Nitroblue tetra-zolium salt (NBT) dissolved in 3ml of 0.05 M sodium phosphate buffer (pH 7.8). Three ml of the reaction medium was added to 1 ml of enzyme extract. The combinations were enlightened in glass test containers of chose uniform thickness. The light was performed by two arrangements of Philips 40W fluorescent cylinders. The test tubes were arranged in a single row, with a set of tube lights fixed on either side. Illumination was started to initiate the reaction at 30˚C for an hour. Indistinguishable arrangements were held under dull filled in as spaces. The absorbance was read at 560nm in the spectrophotometer against the blank. Superoxide dismutase activity is expressed in units. One unit is defined as the amount of change in the absorbance by 0.1 per hour per mg protein under assay condition.

Estimation of catalase: (CAT: E.C.1.11. 1.6)

Catalase was measured according to Chandlee & Scandalios (1984) by change in absorbance at 240nm. An assay mixture contained 2.6ml of 50mM potassium phosphate buffer (pH7.0), 0.4ml of 15mM H_2O_2 and 0.04ml of enzyme extract. The decomposition of H_2O_2 was

followed by the decline absorbance at 240nm. The enzyme activity is expressed in units per min per mg protein.

Estimation of peroxidase: (POD: E.C.1.11.1.7)

Peroxidase activity was measured by Kumar & Khan (1982) following the change in absorbance at 470nm due to 2ml of 0.1 M phosphate buffer (pH 6.8), 1ml of 0.001 M pyrogallol, and 1ml of 0.0054 M hydrogen peroxide and 0.5ml of enzyme extract. The reaction mixture was incubated for 5 minutes at 25˚C, after which the reaction was terminated by adding 1ml of 2.5N sulphuric acid. The activity is expressed in unit per minute per mg protein.

Determination of non-enzymatic antioxidant Ascorbic acid (A_SA)

Ascorbic acid was analyzed by Omaye *et al.* (1979). Ascorbate was converted into dehydroascorbate on treatment with activated charcoal, which in turn reacted with 2,4-dinitrophenylhydrazine to form osazones. These osazones produce an orange coloured solution when dissolved in H_2SO_4, whose absorbance can be measured spectrophotometrically at 540nm.

Ascorbate was extracted from 1g of the plant sample using 4 per cent TCA and the volume was made up to 10 mL with the same. The supernatant got after centrifugation at 2000 rpm for 10 minutes was treated with a spot of initiated charcoal shaken overwhelmingly utilizing a cyclomixer and saved for 5 minutes. The charcoal particles were removed by centrifugation and aliquots were used for the estimation. Standard ascorbate ranging between 0.2 and 1.0 mL of the supernatant were taken. The volume was made up to 2.0 mL with 4 per cent TCA. DNPH reagent (0.5 mL) was added to all the cylinders, trailed by 2 drops of 10% thiourea arrangement. The substance were blended and hatched at 37°C for 3 hours bringing about the development of osazone gems. The crystals were dissolved in 2.5 mL of 85% H_2SO_4 in cold. To the blank alone, DNPH reagent and thiourea were added after cooled in ice and the absorbance was read at 540nm in a spectrophotometer. The concentrations of ascorbate in the samples were calculated using a standard curve and expressed in terms of mg/g FW of sample.

α-tocopherol

α-tocopherol was measured by Backer *et al.* (1980). Five hundred milligrams of fresh tissue was homogenized with 10 mL of a mixture of petroleum ether and ethanol (2:1. 6 v/v) and the concentrate was centrifuged at 10,000 rpm for 20 minutes and the supernatant was utilized for the assessment of □-tocopherol. To one mL of concentrate, 0.2 mL of 2% 2,2-dipyridyl in ethanol was added and blended altogether and kept in dull for 5 minutes. The subsequent red shading was weakened with 4 mL of refined water and blended well. The subsequent shading in the fluid layer was estimated at 520nm. The α-tocopherol content was calculated using a standard graph made with known amount of α-tocopherol.

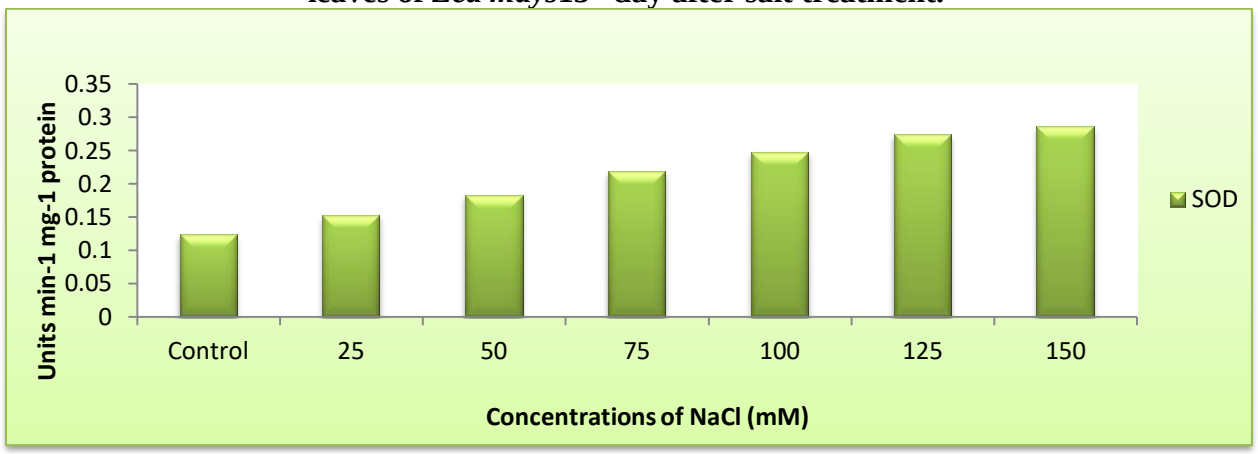
Statistical analysis

The results were analyzed by one-way ANOVA with the help of SPSS 16.0 software package. Means and standard deviation were calculated from three replications.

Results

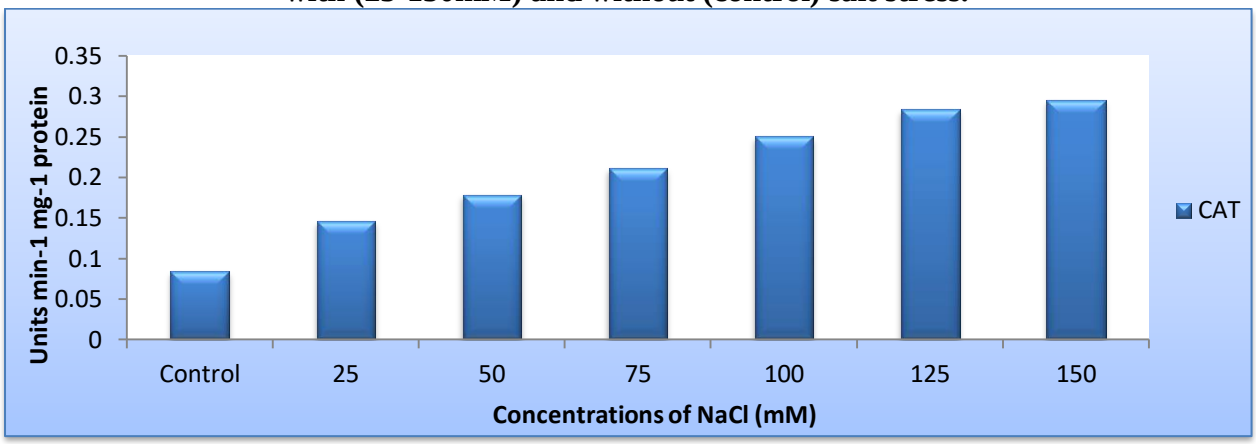
The enzymatic antioxidant activity like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) and non-enzymatic antioxidant activity such as A_SA and □-tocopherol showed fully different response under saline condition. According to our data revealed that these activities exhibited in response to the different concentrations of NaCl in the soil. The highest level of NaCl possessed maximum amount for both antioxidant activity increased in maize leaves compared with respective control plants. The highest values are recorded at 150mM of NaCl (Figure 1-5)

Fig: 1. Effect of different concentrations of NaCl treatment on SOD activity (units $min^{-1}mg^{-1}$ protein) in leaves of *Zea mays*15th day after salt treatment.



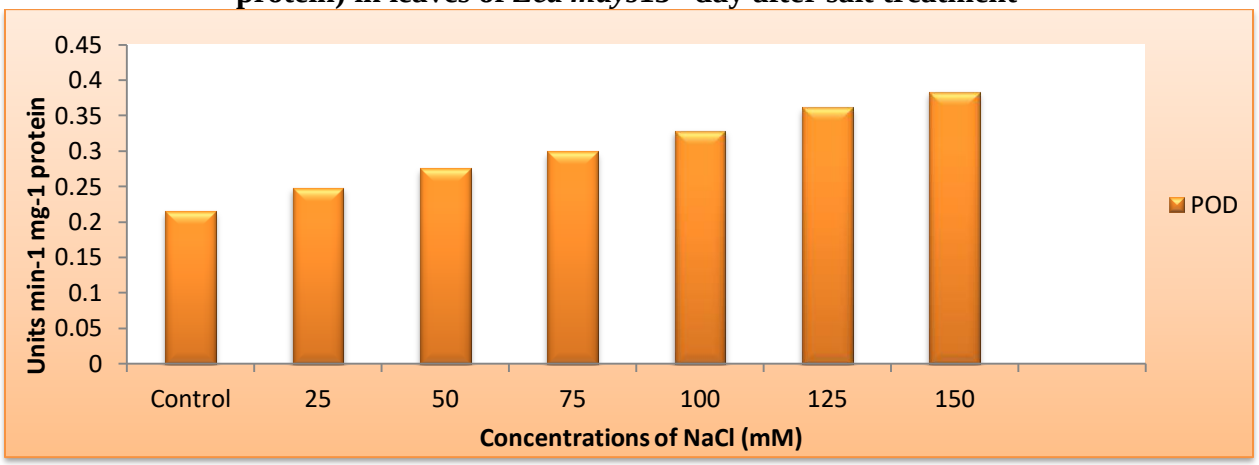
(Values are mean ± S.D. of 3 samples, n-3 and expressed in units min^{-1} mg^{-1} protein)

Fig: 2. Effects of NaCl on catalase activity (units min^{-1} mg^{-1} protein) of 15th day old maize plants grown with (25-150mM) and without (control) salt stress.



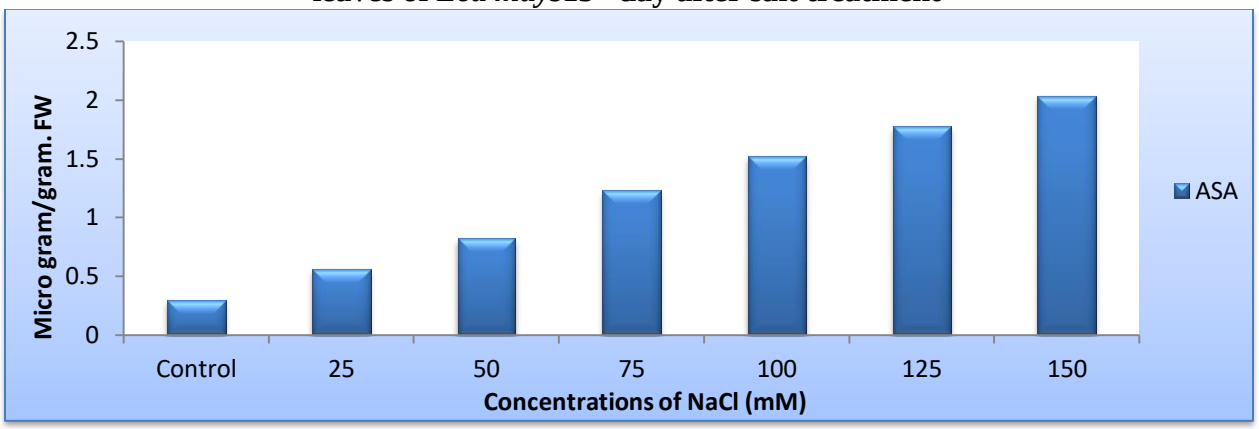
(Values are mean ± S.D. of 3 samples, n-3 and expressed in units min^{-1} mg^{-1} protein)

Fig: 3. Effect of different concentrations of NaCl treatment on Peroxidase activity (units $min^{-1}mg^{-1}$ protein) in leaves of *Zea mays*15th day after salt treatment



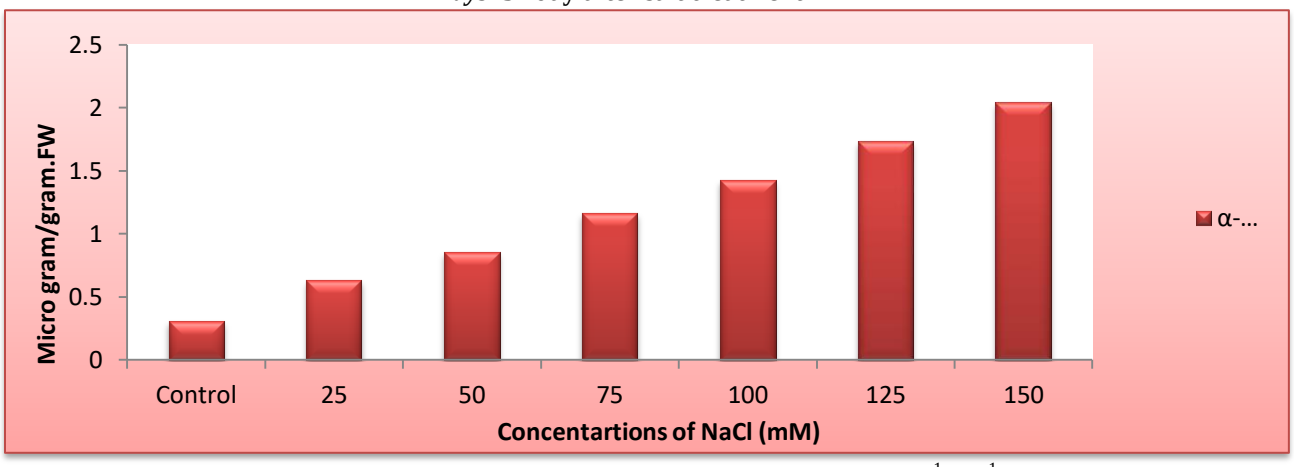
(Values are mean ± S.D. of 3 samples, n-3 and expressed in units min^{-1} mg^{-1} protein)

Fig: 4. Effect of different concentrations of NaCl treatment on A_SA content (Microgram/gram. FW) in leaves of *Zea mays*15th day after salt treatment



(Values are mean ± S.D. of 3 samples, n-3 and expressed in units min^{-1} mg^{-1} protein)

Fig: 5. Effect of different concentrations of NaCl treatment on α Tocopherol content (Microgram/gram. FW) in leaves of *Zea mays*15th day after salt treatment



(Values are mean ± S.D. of 3 samples, n-3 and expressed in units min^{-1} mg^{-1} protein)

Discussion

Plants have particular mechanisms to detoxify reactive oxygen species which include activation of antioxidant enzymes such as superoxide dismutase, catalase, peroxidase and those of the ascorbate–glutathione cycle (Smirnoff 2005) as well as non-enzymatic antioxidants such as flavones, tocopherols, anthocyanins, carotenoids and ascorbic acid (Mittler 2002). These antioxidant enzymes are known to significantly diminish the levels of O_2 and H_2O_2 in plants (Ali & Alqurainy 2006).

Sajjad & Pakniyat (2012) reported that SOD is one of the major anti-oxidative enzymes present in all aerobic organisms and largely in sub cellular elements that generate activated oxygen, furthermore this enzyme is concerned in dismutation of superoxide radicals to hydrogen peroxide and oxygen. The increase in hydrogen peroxide is extremely detrimental for the cells; therefore it gets dismutated to water and oxygen (Ashraf 2009). Lee *et al.* 2001 observed that salt stress has increased the activities of leaf mitochondrial Mn-SOD and chloroplastic Cu/Zn

SOD, which are considered the most important scavenger in the detoxification of active oxygen species in plants and offers protecting cells against super oxide-induced oxidative stress.

Catalase is usually located in a cellular organelle called the peroxisome (Alberts *et al.* 2002). Noreen *et al.* (2010) estimated that under saline stress enhance in catalase activity which reduces the toxic levels of H_2O_2 and protects the cell from oxidative damage and ROS detoxification, where it removes H_2O_2 fashioned throughout photorespiration and helping to overcome the destruction to tissue metabolism by reducing venomous levels of H_2O_2 (Sekmen *et al.* 2007). Our data showed that catalase activity in leaves of maize increased at 150mM NaCl. Similar results are also observed in sugar beet (Bor *et al.* 2003) and rice (Vaidyanathan *et al.* 2003).

POD is among the enzymes that scavenge H_2O_2 in chloroplasts, which is produced through dismutation of O_2^- catalyzed by SOD. In tolerant plant species, POD activity was found to be higher, providing a greater protection against the oxidative stress caused by salt stress (Asada & Takahashi 1987) and involved in stopping growth through the activation of tissue lignifications (Cavalcanti *et al.* 2004). Peroxidase activity increased with elevated exposure to salt indicating that the H_2O_2 produced during salt stress could be effectively removed by it. The increased total peroxidase activities in response to salinity have also been reported in rice (Lee *et al.* 2001) and wheat (Sairam *et al.* 2005). It is a well documented fact that in plants there exist a relationship between total peroxidase activities, changes in cell wall and oxidative stress under salt stress (Chen *et al.* 1993). This high activity of peroxidase may, therefore, be correlated with the capability of the cells to quash oxygen-free radicals, which can damage the cell compartment.

Ascorbate (AsA) is the most abundant, low molecular mass antioxidant that has a key role in defense against ROS caused by elevated level of NaCl and vast physiological processes in plants, including growth, differentiation, and metabolism (Wheeler *et al.* 1998). Ascorbic acid reacts with a range of ROS such as $^1O_2$, O_2^-, HO• and H_2O_2, which is the basis of its antioxidant action (Foyer 2004) and detoxify free radicals as well as oxidants (Li *et al.* 2012). Different studies showed that AsA content in leaves of stressed plants tends to increase with increasing levels of salt stress (Mohamed *et al.* 2010). Similar results are also observed in *Momordica charantia* (Agarwal & Shaheen 2007) and *Solanum melongena* (Shaheen *et al.* 2013). Tocopherols are non-enzymatic lipophilic antioxidants; they can scavenge not only a variety of reactive oxygen species but also free radicals particularly under stress conditions (Hollander-Czytko *et al.* 2005). It has been important role to protect lipids and other membrane components by physically quenching and chemically reacting with O_2 in chloroplasts, thus protecting the structure and function of PSII (Ivanov & Khorobrykh 2003). Generally, salt stress is known to enhance tocopherols in plants (Abbasi *et al.* 2007; Sorkheh *et al.* 2012).

Of the enzymatic antioxidants superoxide dismutase, catalase and peroxidase are considered to be the key enzymes, which play an effective role in plants' oxidative defense mechanism (Mittler 2002; Akram *et al.* 2012). In the present investigation, the activity of leaf SOD, CAT and POD, enzymes increased markedly in maize under saline conditions. Analogous to our results, salt stress-induced enhanced activities of these enzymes were observed in sunflower (Akram *et al.* 2012), pistachio plants (Abbaspour 2012) and egg plants (Shaheen *et al.* 2013). However, the reverse has been observed in other plant species with respect to these enzymes. For instance, the activities of antioxidant enzymes decreased in *Cynodon dactylon* (Hu *et al.* 2012).

Increase in ROS has been described in numerous species to be not only due to salt stress, but also as a response to other environmental stresses (Azevedo Neto *et al.* 2005; Koca *et al.* 2007; Willadino *et al.* 2011; Benzarti *et al.* 2012; Medeiros *et al.* 2012). Under such conditions, an increase in antioxidant enzyme systems thus reducing the contents of reactive oxygen species and decreasing damage to membranes in grasses (Azevedo Neto *et al.* 2005). The maize leaves is the most promising at controlling the ROS content due

to high antioxidant defense system when compared with non treated leaves, and a minimization of damage to the membrane is evidenced by lower MDA content. Lower MDA content than in the control, corresponding to an activation of enzymatic antioxidant system, was also observed by Sergio *et al.* (2012) in leaves of salt-stressed *Cichorium intybus* plants. The importance of the balance between ROS production and ROS scavenging in plant responses to abiotic stress is well established (Gill & Tuteja 2010; Miller *et al.* 2010). Overall, such mechanisms are intimately related to the increased expression levels of antioxidant enzymes involved in the cellular homeostasis counteracting the stress (Gill & Tuteja 2010). In summary, the result of this study indicates that increased activity of antioxidant defense system may be having a better protection against reactive oxygen species under salinity stress.

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