Effect of salt stress on antioxidant enzymes and lipid peroxidation in leaves in two contrasting corn, ‘Lluteño’ and ‘Jubilee’

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Salinity is an important problem to crop production; affecting photosynthesis process which favors the production of reactive oxygen species. Plants have generated adaptation strategies to prevent oxidative damage caused by salinity. In this study we evaluated the effect of salinity on lipid peroxidation and antioxidant enzymes in leaves of corn (Zea mays L.) plants ‘Lluteño’ (adapted to salinity) and ‘Jubilee’ (improved variety). ‘Lluteño’ is the only one corn capable to prosper in the Valley of Lluta under saline conditions (EC = 9.1 dS m⁻¹) and the most widely cultivated crop in terms of area in the desert of northern Chile. Plants of 21 d old were subjected for 15 d to two saline treatments: 50 and 100 mM NaCl. Salinity caused a significant reduction in plant biomass, ca. 65% in ‘Jubilee’ and 20% in ‘Lluteño’ (P ≤ 0.05). The biomass reduction and oxidative damage to cell membranes caused by the generation of peroxides was significantly less in ‘Lluteño’. The activity of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) in ‘Lluteño’ was significantly greater than in ‘Jubilee’ in all treatments, while glutathione reductase (GR) had greater activity in ‘Jubilee’. However, most enzymes studied were adversely affected by salinity compared to the control. These results indicate that tolerance to salinity exhibited by ‘Lluteño’ should in part due to the high activity that exhibit antioxidant enzymes compared to ‘Jubilee’.

Key words: Antioxidant enzymes, lipid peroxidation, salt stress, Zea mays.

INTRODUCTION

Salinity causes a decrease in the growth of cultivated plants, since salts affect a number of physiological processes such as photosynthesis, stomatal conductance, osmotic adjustment, ion absorption, protein and nucleic acid synthesis, enzymatic activity, and hormone balance (Hernandez et al., 2000). It also affects transport of ions and water, which promotes ionic toxicity and nutritional imbalance (Munns and Tester, 2008).

The effects of salinity also have an oxidative component derived from the limitation of the photosynthetic rate which occurs in conditions of drought and salinity (Lawlor, 1995; Filella et al., 1995; Munns, 2002). If under saline stress plants are not capable of photosynthetic transformation of all the solar energy they receive, the energy excess may produce an increase in singlet and triplet forms of chlorophyll and singlet oxygen. The decrease of NADP⁺ pool with excess excitation energy causes an increase in the flow of electrons from the donor part of photosystem I (PSI) to oxygen, generating reactive oxygen species (ROS) (Johnson et al., 2003), produced principally in chloroplasts, which provoke metabolic disorders such as oxidation of membrane lipids, proteins and nucleic acids (Imlay, 2003).

Plants have enzymatic and non-enzymatic anti-oxidation mechanisms which may counteract adverse effects of salinity. The former include enzymes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR); while the latter include compounds such as ascorbate, glutathione, flavonoids, and vitamins C and E (Noctor and Foyer, 1998). Therefore, an imbalance between production of free radical species and systems of cellular antioxidant defense would produce the appearance of oxidative stress. The cell damage which results from an excess of free oxygen radicals has been explained as a consequence of an alteration of cell membrane produced by oxidation of acids of the lipid bilayer, a process known as lipid peroxidation. This generates changes in chemical composition and deterioration in ultrastructure of cell membranes, decreasing their fluidity, altering their permeability, and inactivating enzymes and membrane-linked receptors (Mansour and Salama, 2004).

Since salinity is an important cause of oxidative stress, in this study we evaluated the activity of antioxidant enzymes expressed in two corn (Zea mays L.) cultivars: ‘Lluteño’ (the only one corn capable to prosper in the
valley of Lluta under saline conditions -Ec = 9.1 dS m⁻¹- and the most widely cultivated crop in terms of area in the desert of northern Chile and ‘Jubilee’ (improved variety, normally cultivated in the North of Chile in non-saline soils), which have different levels of tolerance to salt stress (Carrasco-Ríos et al., 2013). The specific objectives of the study were: i) to evaluate the oxidative damaged caused by salinity, and ii) evaluate the relative importance of the enzymatic antioxidant mechanisms in salt tolerance.

**MATERIAL AND METHODS**

**Plant material and experimental conditions**
The study was performed at the Experimental Station of the Tarapacá University (18°31’2” S, 70°10’34” W) using young plants of ‘Lluteño’ and ‘Jubilee’ corn, this last is a corn variety bred (Syngenta, Wilmington, Delaware, USA) commercialized in Chile by Bioamerica S.A. Seeds were sown in plastic pots containing perlite-vermiculite 1:1 (v/v) as substrate. To keep humidity, pots were covered with a plastic wrap until epicotyls appeared 4 to 5 d after sowing. Then plants were cultivated for 2 wk under greenhouse conditions where mean maximum temperature was 35 °C and mean relative humidity was 34%. Along this time, plants were cultivated in hydroponic conditions using a non-saline Hoagland solution (Arnon and Hoagland, 1940). Twenty one days after sowing, plants were randomly placed outdoors and subjected to the treatments for 15 d (Table 1). During the experiment, midday photosynthetically active radiation (PAR) intensity was 2200 µmol m⁻² s⁻¹.

**Dry matter accumulation and Na content measurements**
After 15 d of saline treatments, five plants per treatment were harvested and divided into leaves, stems, and roots, for their respective fresh (FW) and dry weight (DW) determination. Dry weight was obtained after drying each part of the plant in an oven at 80 °C for 48 h. Accumulation of DM was expressed as the plant weight at the end of treatments minus the weight of plants recorded at the beginning of treatments. For Na⁺ analysis, samples of dried leaves and roots were ashed in a furnace at 500 °C for 6 h. Contents of Na⁺ were determined by flame emission photometry (PFP7, Jenway, Stone, Staffordshire, UK).

**Table 1. Description of treatments applied during 15 d.**

| Treatments         | NaCl          | EC            |
|--------------------|---------------|---------------|
| T1 (low salinity)  | Hoagland’s solution, 0 mM NaCl | 2.99 |
| T2 (intermediate)  | Hoagland’s solution, 50 mM NaCl | 8.75 |
| T3 (high salinity) | Hoagland’s solution, 100 mM NaCl | 13.01 |

EC: electrical conductivity.

**Determination of relative membrane permeability, hydrogen peroxide, malondialdehyde, and ascorbic acid**
Relative membrane permeability (EC %) was calculated from measurements of electrolytic conductivity (Yang et al., 1996). The hydrogen peroxide (H₂O₂) content was colorimetrically measured as described by Patterson et al. (1984). The content of H₂O₂ was calculated by comparison with a standard calibration curve, previously plotted by using different concentrations of H₂O₂. Oxidative damage to leaf lipids resulting from salt stress was estimated by the total content of 2-thiobarbituric acid reactive substances expressed as equivalents of malondialdehyde (MDA). The content of MDA was calculated using its absorption coefficient (ε) and expressed as nmol MDA g⁻¹ fresh mass (Cakmak and Horst, 1991). To extract and quantify the total ascorbic acid content (AsA and its oxidized form dihydroascorbic acid DHA) we used the method of Gossett et al. (1994), based on the reduction of Fe³⁺ to Fe²⁺ by AsA in an acid solution; absorption was measured at 525 nm and compared with a calibration curve previously prepared with AsA using the same procedure. Reduced AsA was quantified using the above procedure but using 0.1 mL distilled H₂O instead of dithiothreitol (DTT). Concentration of DHA was calculated as the difference between total AsA and reduced AsA. Results were expressed in nmol g⁻¹ FW.

**Enzyme determinations**
Proteins were extracted from approximately 0.25 g fresh material and the slurry frozen at -80 °C based on the method of Ferrario-Mery et al. (2002). The slurry was desalted with molecular exclusion columns packed with Sephadex G-25, diluting the sample with 100 mM Tris-HCl buffer (pH 8.0) and quantified in a molecular absorption spectrophotometer using the bicinchoninic acid method (Brown et al., 1989). Superoxide dismutase (SOD) activity was determined by measuring its capacity to inhibit the photochemical reduction of the chloride of nitroblue tetrazolium (NBT) (Giannopolitis and Ries, 1977). Absorbance was measured at 560 nm; one unit of SOD activity (U) was defined as the quantity required to inhibit 50% of photoreduction rate of NBT. Results were expressed as U mg⁻¹ protein. Catalase (CAT) activity was determined by the method of Beers and Sizer (1952). We monitored decrease of H₂O₂ by the absorbance at 240 nm for 1 min from the moment H₂O₂ was added. The difference in absorbance (AA240) was divided by the coefficient of molecular extinction of H₂O₂ (36 mM⁻¹ cm⁻¹); CAT activity was expressed as μmol H₂O₂ min⁻¹ mg⁻¹. Guaiacol peroxidase (GPX) activity was determined by the method of Urbanek et al. (1996). The oxidation of guaiacol (formation of tetraguaiacol) was monitored by the absorbance at 470 nm for 1 min from the moment H₂O₂ was added. The difference in absorbance (ΔA470) was divided by the coefficient of molecular extinction
of tetraguaiacol (26.6 mM⁻¹ cm⁻¹). The determination of ascorbate peroxidase (APX) activity was expressed as µmol H₂O₂ min⁻¹ mg⁻¹ protein, taking into account that 1 mol ascorbate is needed to reduce 1 mole H₂O₂ (McKersie and Leshem, 1994). The glutathione reductase (GR) activity was done using the method of Foyer and Halliwell (1976). The oxidation rate of NADPH was monitored by the absorbance at 340 nm for 1 min from the moment NADPH was added. The difference in absorbance (ΔA340) was divided by the coefficient of molecular extinction of NADPH (6.22 mM⁻¹ cm⁻¹); GR activity was expressed as µmol of NADPH min⁻¹ mg⁻¹ protein.

Experimental design and statistical analysis
A factorial design in randomized complete block was used, with two factors: cultivars (‘Lluteño’ and ‘Jubilee’) and salinity (0, 50, and 100 mM NaCl) and five replicates for the measurements. All data were analyzed statistically by ANOVA using the Statgraphics Plus program 5.1 version (StatPoint Technologies, 2001). The Tukey’s test was used for multiple comparisons with a 95% level of significance.

RESULTS AND DISCUSSION

Effect of salinity on dry matter accumulation and Na⁺ content
The increase of salts in the irrigation solution caused a significant decrease in growth of both corn cultivars. This effect was more pronounced in ‘Jubilee’, whose growth was reduced by 52.3% with 50 mM NaCl and 63.3% with 100 mM NaCl compared to the control, while the reduction in growth of ‘Lluteño’ was only 20.3% and 25.4% with 50 and 100 mM NaCl, respectively (Figure 1A).

We observed wilting and leaf rolling only in ‘Jubilee’ with high salinity, but without necrosis of the leaf lamina. It has been reported that when cultivated at a concentration of soil salts of 40 mM, which generates an osmotic pressure of approximately 0.2 MPa, the majority of plant species reduce their production by 50% (Munns and Tester, 2008). Leaves and stems are the plant organs most sensitive to this osmotic effect, which has been observed in corn even in the absence of nutritional deficiencies and ionic toxicity (Hu et al., 2007). Root growth in ‘Lluteño’ appeared to be less affected by salinity than the aerial tissues (Carrasco-Ríos et al., 2013). In corn, for example, root cells are capable of recovering their turgor after an osmotic shock at a concentration of 150 mM NaCl, while leaves do not recover completely (Frensh and Hsiao, 1994). However, mechanisms involved in this response are still unknown (Munns and Tester, 2008).

The growth inhibition produced by NaCl affect the entire plant in ‘Jubilee’, while it only affected leaf growth in ‘Lluteño’; DM accumulation in stems and roots did not vary significantly.

One of the criteria which indicate tolerance to salinity is the maintenance of the development of the root system. As the principal organ of absorption of water and ions, roots are very important in short and long term responses to salt stress; both anatomical and morphological characteristics of roots may affect capacity of adaptation of plants to salinity (Reinhardt and Rost, 1995; Maggio et al., 2001).

NaCl in the irrigation solution induced an increase in the concentration of Na⁺ in leaves of both cultivars (Figure 1B) and roots (data not shown), especially in roots. It is known that Na concentration around the roots produces an increase in the electrochemical potential for Na, which favors its passive transport from the exterior to the cytosol (Blumwald, 2000; Apse and Blumwald, 2002). ‘Lluteño’ accumulated less Na⁺ in leaves than ‘Jubilee’. Only 12.7% and 37.1% of total Na that ‘Lluteño’ plants contained at intermediate and high salinity was transported to leaves, while in ‘Jubilee’ these percentages were significantly greater, 44.6% and 62.1%, respectively.
Effect of salinity on peroxide content
Hydrogen peroxide is one of the most abundant and most stable ROS; it regulates several metabolic pathways acting as a molecular signal in processes of defense and development (Neill et al., 2002; Slesak et al., 2007). However, an increase in the H$_2$O$_2$ concentration in plant tissues may prejudice their growth due to the damage it produces in macromolecules, especially membrane lipids (Johnson et al., 2003). Figure 2A shows that salinity induced H$_2$O$_2$ production, which was double that control at intermediate salinity in both cultivars. However, at high salinity the peroxide content of ‘Jubilee’ was 8 times greater than control, while ‘Lluteño’ was only 3.6 times greater. These results demonstrate that salinity conditions employed in this study induced oxidative damage in the studied cultivars, and that ‘Jubilee’ was more affected by high salinity.

Effect of salinity on membrane permeability
The leak of solutes was evaluated to determine damage to the cell membrane provoked by NaCl. Figure 2B shows that the loss of solutes increased with increasing salinity; electrolyte loss was greater in ‘Jubilee’. Several studies have demonstrated that plasma membrane is the part of the cell that first confronts salt, and that it must be less susceptible and maintain its integrity in conditions of high salt to fulfill its selective function, regulation of the passage of water, ions, metabolites and electrochemical potential (Spector and Yorek, 1985). For this reason the stability of plasma membrane has been widely used to differentiate cultivars which are tolerant or sensitive to salinity. In many cases high membrane stability is directly correlated with tolerance to abiotic stress (Mansour, 1997; Masood et al., 2006).

Effect of salinity on MDA content
Malondialdehyde is produced when the polyunsaturated fatty acids of the plasma membrane are peroxidized, and represents the expression of the oxidative damage. The increase in salinity induced an increase in MDA content in both corn cultivars (Figure 2C), especially at high salinity; ‘Jubilee’ had a greater degree of lipid peroxidation in the cell membrane than ‘Lluteño’ at high salinity. A number of studies have shown that salt stress produces alterations in structure and composition of plasma membrane lipids, such as the increase in degree of saturation of free fatty acids and increase in free sterols, which leads to a decrease in the fluidity of cell membrane (Mansour et al., 2005). However, it is not clear whether the saturation of membrane lipids has an adaptive value, if there is a correlation between a specific class of lipids and salt tolerance, or if it is just a negative effect of salinity (Mansour and Salama, 2004).

Salinity appears to have produced lower levels of lipid peroxidation and less loss of electrolytes in ‘Lluteño’ than in ‘Jubilee’, which is related to the greater salt tolerance of ‘Lluteño’. Similar results were reported by Azevedo Neto et al. (2006), who found that the peroxidation of lipids was increased by salinity in sensitive corn genotypes.

Effect of salinity on the activity of SOD, CAT, and GPX
Superoxide dismutase activity decreased with increasing salinity in both corn cultivars; however, ‘Lluteño’ had a specific activity 1.5 to 1.7 times greater than ‘Jubilee’ in all treatments (Figure 3A). Superoxide dismutase is the main enzyme which traps ROS, and is found in almost all cell compartments. High SOD levels have been found in salt-resistant plants (Gosset et al., 1994; Hernandez et al., 1995a; 1995b), and constitutive high levels of this enzyme...
are correlated with salt tolerance (Acar et al., 2001). It is well known that SOD converts superoxide (O$_2^-$) radical to H$_2$O$_2$; thus an increase in H$_2$O$_2$ production will require an increase in the capacity of rapid removal of the H$_2$O$_2$ generated by SOD. Catalase participates in this action, its activity increased in 'Lluteño' in saline treatments and was 50% greater than the activity in 'Jubilee' in all treatments (Figure 3B). By contrast, in 'Jubilee' there was no significant variation in CAT activity under saline conditions.

Corn plants have three known isoforms of catalase: cat-1, cat-2 (peroxisome and cytosol), and cat-3 (mitochondria) located on different chromosomes and regulated independently (Polidoros and Scandalios, 1999). Catalase is indispensable in detoxification when ROS levels increase due to stress (Foyer et al., 1994; Willekens et al., 1997).

The increase of CAT in ‘Lluteño’ agrees with the results of Lopez-Huertas et al. (2000), who showed that H$_2$O$_2$ induces peroxisome gene biogenesis in plant and animal cells. These authors suggested that a number of stress situations which cause the production of H$_2$O$_2$ can be mitigated by proliferation of peroxisomes generated to restore the cell redox equilibrium.

The guaiacol peroxidases (GPX), which are also involved in the catabolism of H$_2$O$_2$ in the cytosol, vacuole and cell wall (Asada, 1992), decreased their activity under saline conditions in ‘Lluteño’. By contrast, GPX levels increased significantly with saline conditions in ‘Jubilee’; however, the specific activity of these enzymes was greater in ‘Lluteño’ in all treatments.

Effect of salinity on the activity of APX and GR

Ascorbate peroxidase (APX) is involved in the ascorbate-glutathione cycle, which has an important role in the protection of plants from the H$_2$O$_2$ that results from normal photosynthetic activity and in situations of environmental stress (Noctor and Foyer, 1998). Activity of APX did not vary significantly in ‘Jubilee’ due to salinity, while the activity decreased by about 35% in ‘Lluteño’ with high salinity (Figure 4). Ascorbate peroxidase uses ascorbate (AsA) as an electron donor; AsA content was affected by high salinity conditions in ‘Lluteño’, however, there was an increase in this compound in ‘Jubilee’ (Table 2). Since AsA may eliminate O$_2^-$, OH$^-$ and oxygen singlets (O$^3$) directly as well as reducing H$_2$O$_2$ to H$_2$O via the ascorbate-glutathione cycle (Noctor and Foyer, 1998). Ascorbate may also remove oxygen free radicals directly with or without enzymatic catalysis, recycled to its reduced form tocopherol (Zhang, 2013). Furthermore, chloroplast AsA acts as a cofactor for violaxanthin deepoxidase, an enzyme involved in xanthophyll cycle-mediated photoprotection (Smirnoff and Wheeler, 2000). While the increase of AsA in plants of ‘Jubilee’ could suggest an important role of this antioxidant in salt stress, you may also be an indicator of photoinhibition (Smirnoff, 2011).

The content of DHA, a compound which recycles AsA via dehydroascorbate reductase (DHAR), decreased significantly in ‘Lluteño’ with 100 mM NaCl, while in ‘Jubilee’ DHA decreased by 2.8 and 4.6 times the control value under saline conditions (Table 2).

The regeneration of AsA from DHA is a process of considerable importance in the anti-oxidation response.
Dehydroascorbate reductase is involved in this process, using the reducing power of reduced glutathione; the resulting oxidized glutathione is recycled by the action of glutathione reductase (GR). Therefore the increase in GR activity observed in both corn cultivars due to salinity is directly correlated with the lower DHA content shown by both varieties, although the activity of this enzyme was significantly greater in ‘Jubilee’. Gosset et al. (1994; 1996) showed that salt-tolerant cotton varieties had a high GR activity, favoring a greater AsA/DHA ratio as we found in ‘Jubilee’ (Table 2). Thus ‘Jubilee’ appears to use principally the ascorbate-glutathione cycle to deactivate the ROS generated by energy-producing pathways under conditions of saline stress. This alternative mechanism for removing H$_2$O$_2$ could compensate for the low CAT activity that presents the ‘Jubilee’ (Figure 3).

A number of studies have indicated that salt-tolerant plants can induce activity of antioxidant enzymes (Masood et al., 2006; Ashraf and Ali, 2008), and there are also several reports of wild crops which have prospered in conditions of natural stress which have enzymatic antioxidant mechanisms which are constitutively superior than cultivated varieties of these crops (Shalata and Tal, 1998). The characteristics we report for ‘Lluteño’, including enzymatic anti-oxidant mechanisms, the ability to restrict the passage of salts to the aerial tissues and/or utilize them for osmotic adjustment, maintain a greater concentration of K$^+$ and Ca$^{2+}$ in leaves in relation to the increase of Na$^+$ may all contribute to salt tolerance that this corn variety possesses (Carrasco-Ríos et al., 2013).

**CONCLUSION**

The saline conditions applied in this study induced the formation of reactive oxygen species (ROS) and lipid peroxidation in both corn cultivars. However, ‘Lluteño’ has an enzymatic anti-oxidation system which is constitutively more active than that of ‘Jubilee’. Consequently this characteristic may be considered to be a defense mechanism which contributes to the tolerance of the oxidative effect of salinity, and thus the oxidative damage in ‘Lluteño’ was less than in ‘Jubilee’.

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