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ORIGINAL ARTICLE

Physiological and biochemical responses of two maize cultivars (Corralejo and Tlaltizapon) under salt stress

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The aim of the present work was to study the effect of different concentrations of NaCl (0, 50 and 100mM) in two cultivars of maize (Corralejo and Tlaltizapan), on their nutritional and photosynthetic comportment. The measures focused on the physiological parameters (growth weight, hydration and nutritional status of plants) and biochemical (chlorophyll, PEPC activity, activity of some anti-oxidant enzymes and lipid peroxidation). Analysis of morphological parameters showed a yellowing of the extremity of leaves, at 100 mM of NaCl. These visual symptoms are associated with a decrease of chlorophyll. A decrease in potential growth was found in two cultivars, but less significant in Corralejo. The best salt tolerance of the latter was due to a better hydration of the leaves, to a lesser accumulation of Na^+ and Cl^- in its leaves and a better selectivity K/Na. To identify the biochemical characteristics associated with the physiological behavior, we conducted measures activity of PEPC, the protein, catalase and peroxidase on the fourth leaf from the bottom. A negative correlation between the activity of PEPC and Na+ amount was found at 50 mM in the sensitive cultivar and at 100 mM of NaCl in tolerant cultivar Corralejo. Furthermore, the antioxidant response was marked by a greater accumulation of malondialdehyde, in Tlaltizapan at 100 mM of NaCl. At the same concentration, catalase peroxidase and SOD activities weren't decreased in this cultivar. This suggests that salt has created a stress oxidative state only in Tlaltizapan leaves. These results showed a better performance of Corralejo cultivar compared to Tlaltizapan cultivar, at 50 and 100 mM of NaCl.

Key words: maize, growth weight, hydration, lipid peroxidation, proline, chlorophyll, salt stress.

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Salt stress in soil is one of the major stresses especially in arid and semi-arid regions and affects plant production in many parts of the world, particularly on irrigated land (Koca *et al.,* 2007). It is estimated that 20% of the irrigated land in the world is presently affected by salinity (Yeo, 1999). This reduction in plant growth in saline environments could be due to either adverse water relations or the toxic effects of Na^+ and Cl^- ions accumulation on metabolism (Yeo and Flowers, 1983).

Many crop species are sensitive to high concentrations of salt with negative impacts on agricultural production. Maize (*Zea mays L.*) is considered a moderately salt-sensitive plant, (Mass and Hoffman, 1977). Salt resistance of plants is a complex phenomenon that involves biochemical and physiological processes as well as morphological and developmental changes (Munns, 2002; Pitann *et al.,* 2009). Plant salt tolerance has generally been studied in relation to regulatory mechanisms of ionic and osmotic homeostasis (Ashraf and Harris, 2004). The decrease in uptake of K^+, Mg^{2+}, Ca^{2+} and thereby as well as in growth at higher sodium concentration has also been reported (Koca *et al.,* 2007). In addition, salt stress, like other abiotic stresses, also leads to oxidative stress through an increase in reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) (Neill *et al.,* 2002).

These oxygen species are highly cytotoxic and can seriously react with vital biomolecules such as lipids and proteins (Imlay, 2003) causing lipid peroxidation, protein denaturing and DNA mutation (Breusegem *et al.,* 2001; Quiles and Lopez. 2004). Evidence suggests that membranes are the primary sites of salinity injury to cells and organelles (Candan and Tarhan, 2003) because ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasmalemma or intracellular organelles (Karabal *et al.,* 2003). Fortunately, plants have developed various protective mechanisms to eliminate or reduce ROS, which are effective at different levels of stress-induced deterioration (Beak and Skinner, 2003). The enzymatic antioxidant system is one of the protective mechanisms including CAT (Kono and Fridovich, 1983) and POX (Gara *et al.,* 2003).

Despite the importance of this cereal in Tunisia, until now, we do not have a veritable maize culture. This leads us to think of integrating foreign varieties that could ameliorate productivity and adapted to the conditions of drought and salinity. Therefore, the aim of this study was to evaluate the effects of salt stress on growth and on anti-oxidative enzymes activity in leaves of tow maize cultivars, in order to better understand the physiological and biochemical mechanisms of salt tolerance.

MATERIALS AND METHODS

Plant growth

Seeds of maize (cv. Corralejo and Tlaltizapon*)* were imbibed for one hour in deionized water at 4°C, and then sown in Petri dishes with wet filter paper for germination in the dark at 25°C. Five days after germination; seedlings were transferred into pots containing nutrient solution of Hoagland and Arnon (1940) in a growth room. Photoperiod was 16 h with

150 µmol m^{-2} s^{-1} PAR at the plant level. Day and night temperature and relative humidity regimes were 22/18°C and 60/80%, respectively. Seventeen days after germination, an initial harvest was performed. Then, three treatments were started. In the first, seedlings were cultivated in the same nutrient solution and considered as control. In the other treatment, 50 and 100 mM NaCl was added to the medium. The final harvest was performed after 15 days of treatment. For each treatment, 8 plants were taken and the fresh weights of leaves, leave order 4, stems and roots were weighed. The samples were then oven-dried at 70°C for 72 h for the determination of DW. Besides, fresh samples from each plant were immediately frozen in liquid nitrogen and stored at -80°C until performing biochemical analysis.

Ionic status

Aliquots (0.2 g) of oven-dried ground material of all organs were digested with 25 ml nitric acid (HNO_3) 0.5%. Sodium (Na^+) and potassium (K^+) content were measured in the digests with a flame emission photometer (Jenway PFP7). Chloride (Cl^-) content of the digested extracts was determined using a chloride analyser.

Lipid peroxidation

The level of lipid peroxidation was determined by a procedure based on the method of Heath and Packer (1968). 0.5 g fresh samples were ground in 5 ml of ice-cold phosphate buffer solution (0.05 mM, pH 7.8) containing 1% polyvinylpyrrolidone (w/v) (PVP). The homogenate was centrifuged at 10,000 g for 30 min. 2 ml of supernatant was mixed with 2 ml of thiobarbituric acid (TBA) (0.5% TBA, 20% trichloroacetate TCA). The mixture was heated at 100°C for 30 min, chilled on ice, and then centrifuged at 1000 g for 10 min. Absorbance of the supernatant was measured at 532 nm and adjusted for non-specific absorbance at 510 nm and 560 nm.

Electrolyte leakage

The electrolyte leakage (EL) was determined as described by Dionisio-Sese and Tobita (1998). Leaves 4 discs of fresh seedlings were cut into 2–3mm pieces and placed in test tubes containing 10 ml distilled water. The tubes were incubated in a water bath at 32°C for 2 hours and the initial electrical conductivity of the medium (EC1) was measured. The samples were autoclaved at 121°C for 20 min to release all electrolytes; cooled to 25°C and the final electrical conductivity (EC2) was measured. The EL was calculated from EL = (EC1/ EC2)100.

Protein determination and enzyme assays

Fresh material of the leaves 4 obtained at 15 days after salt treatment were excised and immediately placed in liquid nitrogen. They were later ground in a 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 5% glycerol and 5% polyvinylpolypyrrolidone, and centrifuged for 20 min at 15000×g. The supernatant was used for determination of soluble proteins and enzymatic activities. The concentration of proteins was determined according to the method of Bradford (1976).

CAT activity was measured according to the modified method of Aebi (1984). The reaction mixture consisted of 25 mM potassium phosphate buffer (pH 7.0), 30 mM H_2O_2 and enzyme extract. The

decomposition of H_2O_2 was followed by measuring the decrease in absorbance at 290 nm. Total peroxidase activity was assayed using guaiacol as an electron donor, with a reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 5 mM H_2O_2 and 10mM guaiacol, a method derived from Fielding and Hall (1978). The increase of absorbance, due to tetraguaiacol formation, was recorded at 470 nm through 3 min. All enzyme activities were expressed per mg of total proteins.

Enzymatic activity determination on frost of polyacrylamide

In our experiences, the native frosts used, don't contain any denaturing agents and permit to make migrate the native proteins. It will allow surveying the raw state activity and the total molecular weight of the total protein. The isoforms of superoxide dismutase (SOD) were separated by gel electrophoresis of the supernatant (stacking gel: 5% acrylamide, pH 6.8, 0.5M Tris-HCl buffer; resolving gel: 12%, pH 8.8, 1.5M Tris-HCl buffer).

After the migration, the native gels 12% is incubated in the dark for 20 min in 2.45mM Nitro Blue Tetrazolium pH 7.8, 36mM phosphate buffer containing 28mM TEMED and 28 mM riboflavin. Thereafter, the gels were exposed to intense white light for 20 min in Phosphate of potassium (36 Mm, pH 7.8), TEMED (28 Mm) and Riboflavine (28 µM). SOD activity appears as bleached bands on the gels. Cu/Zn-SOD are inhibited by KCN and H_2O_2 and Fe-SOD are inactivated by H_2O_2, whereas Mn-SOD are resistant to both inhibitors (Fridovich, 1989).

Statistical analysis

Statistical analysis performed with Statistica™ software, using two-way ANOVA and Newman-Keuls test for post-hoc mean comparison. The ANOVA was performed over the whole set of data.

RESULTS

Growth and relative water contents

The growth responses to salt stress of two cultivars of maize were represented in Table I. At 0 mM NaCl, all cultivars produced the same total dry weight (1.9 g and 1.7 g respectively for Corralejos and Tlaltizapan). At 100 mM, the whole plant biomass decreased by 31% and 45%, respectively, in Corralejo and Tlaltizapon in comparison to the control. Considering plant organs, Table I shows that leave growth is the most affected by salinity followed by roots and stems. In addition, the applied NaCl inhibit the growth of maize plant and caused the decrease in the length of the aerial part with leaf yellowing especially in Tlaltizapon.

Particular interest is granted to the leaf of the order 4, this is the first leaf which appears just after salt treatment. Dry weight of leaves 4 was drastically reduced by 54% and 30% under 100 mM NaCl, respectively in Tlaltizapon and Corralejo. The 50 mM NaCl had non-significant effects on dry weight of these leaves.

Water content of three organs of Corralejo and Tlaltizapon (Table I) depended poorly on the treatments. At 100 mM, salt treatment induced no significant effect on leaf tissue hydration in Corralejo, but affected it (-57 %) in Tlaltizapon. In Corralejo as well as in Tlaltizapon, the hydration of the roots seems

insensible to salt stress. On the other hand, the water content in the leaves order 4 decreased only in Tlaltizapan (-20%).

Ion accumulation

In this study, Na^+ accumulation in Corralejo as well as Tlaltizapon showed a positive relationship with NaCl concentrations added in the culture medium. Under NaCl treatment, a significant variation in the areal part (leaves, leave 4 and stem) and roots Na^+ content was observed (Table II). In the two cultivars, the roots Na^+ content was greater than that of the leaves. Moreover, at 100 mM, the Na^+ content was more important in Corralejo roots than in Tlaltizapon. In leaves, salt treatment increased Na^+ content, especially in Tlaltizapon.

Chloride uptake showed important variations according to the NaCl concentration. Cl^- is accumulated in the same way as Na^+ in the different organs of maize plants, according to a decreasing pressure gradient of the roots toward the leaves. At 100 mM of NaCl, Corralejo plants accumulate less Cl^- in their leaves and more in their roots. In addition, both cultivars accumulated more Na^+ and Cl^- in their leaves of order 4.

Salt treatment significantly reduced K^+ content in all organs, but differently according to the varieties: the main reduction concerned leaves and stems in Tlaltizapon.

Lipid peroxidation

Membrane lipid peroxidation in the leaves 4, first leaf appear just after salt treatment, of two maize cultivars was assessed by the content of MDA and the EL (Figure 1). Under non-saline conditions, MDA concentration was similar in leaves of both cultivars. Salt treatment increased MDA concentration in Tlaltizapon, reaching 4,80 $\mu mol.g^{-1}$ FW at 100 mM NaCl. However no significant effect was observed in leaves of Corralejo. These results are confirmed by electrolyte leakage. Results showed that salt stress impaired membrane permeability by increasing ion leakage in the leaves of both cultivars, but highly in Tlaltizapon.

Protein content and antioxidant Enzyme Activity

Protein contents in the leaves 4 of two cultivars after 12 day of NaCl treatment are given in Figure 2. The results show that protein content was higher in the leaves of Corralejo, under control as well as salt conditions. NaCl treatment did not significantly affect protein content in both cultivars.

Activities of CAT and POX, (Figure 2) showed that, CAT activity in Corralejo leaves was significantly enhanced by 100 mM NaCl, however in seems it remain unchanged at 50 mM. in Tlaltizapon, CAT activity was not affected by salt treatment. As for CAT activity, only 100 mM NaCl significantly increased the activity of POX in leaves of Corralejo cultivar, however it was not significantly affected in Tlaltizapon.

The phosphoénolpyruvate carboxylase or PEPC

PEPC activity was also measured in leaves 4 extracts from the two cultivars (Figure 3). In control medium, the two cultivars of plants present an equivalent PEPC activity. The presence of salt in the nutrition solution reduced activity of this enzyme, in the both maize cultivar. This reduction increased with NaCl concentration and reached 71% and 79% at 100 mM NaCl, respectively in Corralejo and Tlaltizapan.

Enzymatic activity in polyacrylamide gel

There were striking differences in antioxidant enzyme activities between the two cultivars with increasing NaCl concentration (Figure 3). In Corralejo, we showed any remarkable change of SOD activity, whereas it decreases strongly to 50 mM and 100 mM of NaCl in Tlaltizapan. The tests of inhibition, by cyanide and H_2O_2 show that the band of the SOD detected is a Cu/Zn-SOD. The analysis of the Electrophoregrams to Basic guaiacol peroxidase was represented as two bands, in the both of cultivars. The activity of the isoforme 1 rest insensible to salt in Corralejo, it increased in this cultivar but decreased with increasing of NaCl concentration in Tlaltizapan. As for the isoforme 2, salt stress affects the activity of this enzyme only at Tlaltizapan.

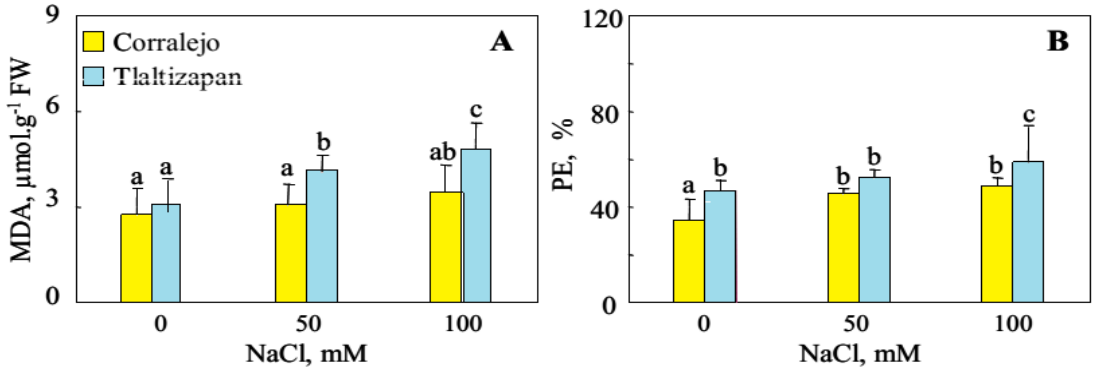

Figure 1. Concentration of malondialdehyde (MDA) (A) and electrolyte leakage levels (EL) (B) in leaves, stems and roots of tow cultivar of maize plants, after 15 days of growth under NaCl (0, 50mM and 100mM NaCl). Data are means of 6 replicates. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$.

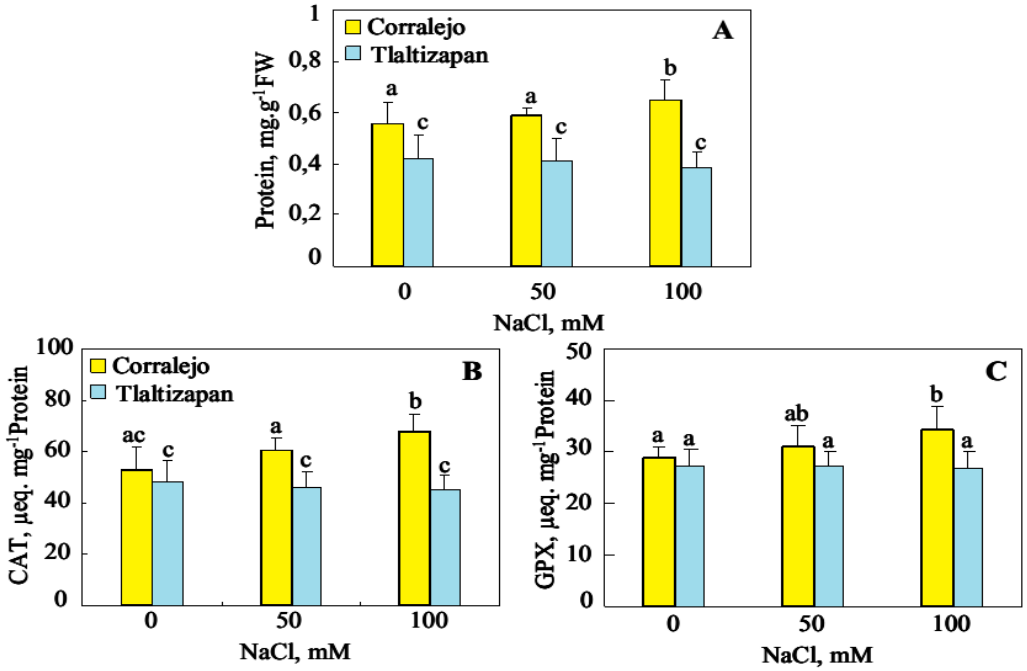

Figure 2. Total protein contents(A), catalase (B) and Guaiacol peroxidase (C) activities in leave in leave, stem and roots of tow cultivar of maize plants grown for 12 days in presence of different NaCl concentrations (0, 50mM and 100 mM NaCl). Data are means of 4 replicates. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$.

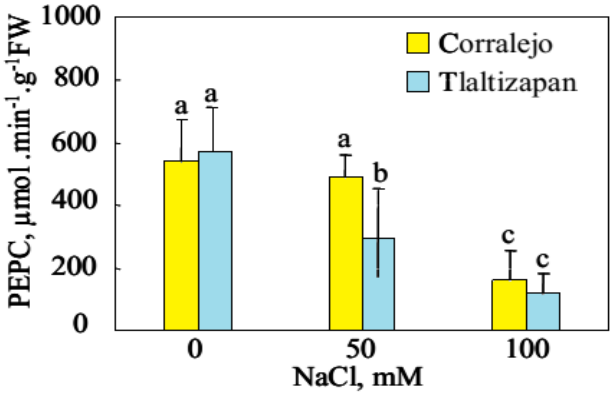

Figure 3. Effect of different levels of salinity (0, 50 and 100 mM NaCl) on the rate of phosphoenolpyruvate carboxylase kinase activity in leaves 4 of tow cultivar of maize (Corralejo and Tlaltizapon), after 15 days of growth under NaCl (0, 50mM and 100mM NaCl)). Data are means of 4 replicates. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$

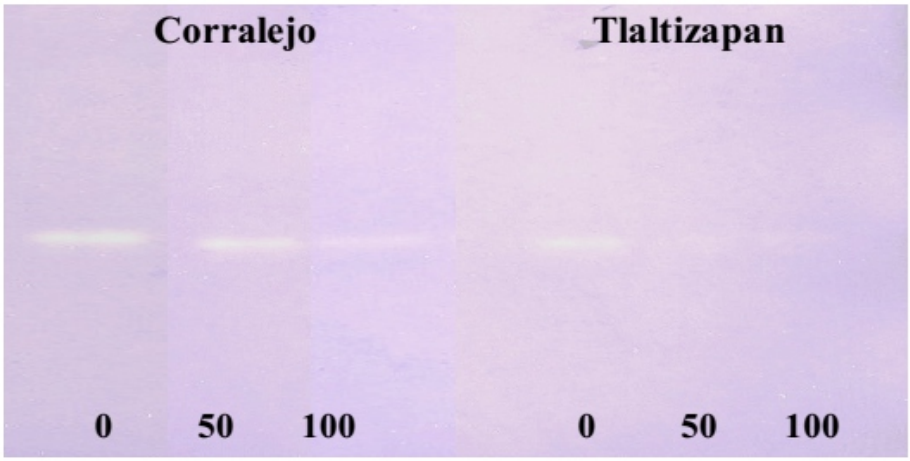

Figure 4. Superoxide dismutase (SOD) native polyacrylamide gel in tow cultivar of maize (Corralejo and Tlaltizapon) plants grown for 12 days under different NaCl concentrations (0, 50mM and 100 mM NaCl).

Table 1. Dry weights (DW) and relative water contents (RWC) in leave, leave4, stem, and roots maize plants (Corralejo and Tlaltizapon) after 15 days of growth under NaCl (0, 50 and 100 mM NaCl) conditions.

NaCl, mM		0 mM NaCl		50 mM NaCl		100 mM NaCl	
		Corralejo	Tlaltizapon	Corralejo	Tlaltizapon	Corralejo	Tlaltizapon
Dw, g	Leaves	0.857±0.12a	0.765±0.08ab	0.735±0.11ab	0.658±0.11ab	0.591±0.17bc	0.423±0.08c
	Leaves 4	0.254±0.06ab	0.287±0.05a	0.290±0.08a	0.207±0.04ab	0.175±0.05bc	0.133±0.02c
	Stems	0.508±0.09ab	0.538±0.15ab	0.688±0.17a	0.452±0.09b	0.452±0.05b	0.237±0.06c
	Roots	0.493±0.10a	0.383±0.03abc	0.428±0.08ac	0.367±0.06c	0.275±0.05bcd	0.248±0.05d
RWC ml.g^{-1} DW	Leaves	6.01±0.43a	7.80±1.00b	6.0±0.11a	6.301±1.12a	5.8±2.70a	3.41±0.65c
	Leaves 4	7.94±0.87abc	8.66±0.77b	6.99±0.72ac	8.28±0.44ab	7.33±0.96ac	6.88±0.84c
	Stems	12.71±2.73ab	16.02±1.52c	11.43±1.17ab	14.02±3.02d	8.45±0.79e	11.63±2.41ab
	Roots	14.33±1.98a	13.41±1.44b	13.11±1.42b	13.30±1.71b	12.60±2.07bc	12.02±1.72c

Table 2. Na^+, K^+ and Cl^- content in leave, leave4, stem, and roots maize plants (Corralejo and Tlaltizapon) after 15 days of growth under NaCl (0, 50 and 100 mM NaCl) conditions.

NaCl, mM		0 mM NaCl		50 mM NaCl		100 mM NaCl	
		Corralejo	Tlaltizapon	Corralejo	Tlaltizapon	Corralejo	Tlaltizapon
Na^+, meq.g^{-1} MS	Leaves	0.02±0.00a	0.003±0.00a	0.807±0.13b	0.982±0.23c	1.474±0.15d	2.010±0.23e
	Leaves 4	0.035±0.06a	0.01±0.007a	0.986±0.06b	1.324±0.04c	1.384±0.06c	1.910±0.11d
	Stems	0.017±0.00a	0.02±0.005a	1.046±0.09b	1.938±0.15c	1.747±0.25c	2.259±0.16d
	Roots	0.022±0.00a	0.01±0.005a	2.007±0.024b	2.009±0.22b	3. 005±0.09c	2.342±0.12d
K^+, meq.g^{-1} MS	Leaves	1.115±0.06a	1.305±0.13a	0.853±0.073b	0.748±0.18b	0.209±0.03c	0.243±0.07c
	Leaves 4	1.056±0.15a	1.301±0.18d	0.596±0.03b	0.655±0.02b	0.376±0.04c	0.397±0.07c
	Stems	1.076±0.22ac	1.240±0.18a	0.780±0.07b	0.924±0.09c	0.555±0.03d	0.543±0.06d
	Roots	0.722±0.04ab	0.747±0.08b	0.501±0.04c	0.416±0.08c	0.234±0.02d	0.198±0.04d
Cl^-, meq.g^{-1} MS	Leaves	0.051±0.01a	0.082±0.01a	0.251±0.01b	0.388±0.01c	0.629±0.04d	1.138±0.01e
	Leaves 4	0.015±0.04a	0.025±0.01a	0.514±0.02b	0.641±0.02c	1.114±0.02d	1.010±0.03d
	Stems	0.043±0.01a	0.049±0.00a	0.456±0.01b	0.815±0.04c	0.748±0.01d	0.931±0.03e
	Roots	0.034±0.01a	0.022±0.01a	0.594±0.02b	0.501±0.03b	1.623±0.09c	1.113±0.06d

Data are means of 8 replicates ± standard error. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$.

DISCUSSION

Soil salinity is a prevalent abiotic stress for plants that generally leads to growth arrest and even plant death (Munns and Tester, 2008). Growth inhibition is a common response to salinity; plant growth is one of the most important agricultural indices of salt stress tolerance as indicated by different studies (Parida and Das, 2005). Our results showed that the NaCl salinity reduced growth of the studied species, and the extent of reduction was difference among the cultivars. Tlaltizapan showed a higher growth reduction under salt conditions as compared to Corralejo.

Indeed, leaves growth of Tlaltizapan was more affected than that of Corralejo. Beatriz *et al.* (2001) showed that inhibition of leaves biomass is one of the primary effects of salt stress and is probably due to inhibition of leaves elongation induced by salt stress in maize (*Zea mays*). Variance analysis showed that relative growth rate values of cultivars were affected significantly by salt treatment. From our results, it may be expressed that Corralejo is more tolerant than Tlaltizapan. Similar results obtained from researches on maize were reported by other researchers (Ashrafuzzaman *et al.,* 2003; Neto *et al.,* 2004).

Increasing levels of NaCl induced a progressive absorption of Na and Cl in plant, agreeing with Turan *et al.* (2007a, b). Excessive Na concentration in the plant tissue hinders nutrient balance, and causes toxicity (Bernstein, 1963). Accumulation of Cl in the root tissue is disruptive of the membrane uptake mechanisms, and increased translocation of Cl to the shoots (Yousif *et al.,* 1972). When NaCl was applied to the soil, the levels of K in plant were reduced in accordance with the antagonism between Na and K (Azevedo and Tabosa, 2000). Cramer *et al.* (1985) showed that excess NaCl leads to the loss of potassium due to membrane depolarization by sodium. In Tlaltizapan plants, leaves are more vulnerable than roots to Na^+, simply because Na^+ and Cl^- accumulate to higher levels in leaves than in roots.

Contrary, Corralejo developed a physiological strategy of tolerance limiting the accumulation of Na and Cl in leaves. These results are consistent with that of Tester and Davenport (2003).

To date, very little is known about the toxic effects of Cl^- ions in the plant cell (Teakle and Tyerman, 2010). It is also reported that sensitivity of some crops to salinity is due to the inability to keep Na^+ and Cl^- out of transpiration streams (Gorham *et al.,* 1990). Starting from the physiological part, we can say that leaves 4 enables us to compare the two cultivars. That is why the biochemical part focuses on this leaf unjustly especially as it is the first leaf appear just after salt treatment.

Effect of salt stress on the plant tissues were determined by measuring of MDA content and the solute leakage from the cells. Our results showed that electrolyte leakage and lipid peroxidation increased as the stress level rose up, only in Tlaltizapan plants. The increase in MDA content was a clear indication for oxidative stress in these plants, which thus seemed to suffer from a more acute saline aggression than Corralejo cultivar. It has been reported that MDA content was correlated with leaf H_2O_2 accumulation in two corn varieties (Hajlaoui *et al.,* 2009).

Excess of ROS causes phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation (Pitzschke and Hirt, 2006). To protect themselves against these toxic oxygen intermediates, a simultaneous increase antioxidant system such as catalase and peroxidases in salt treated plants. Our results show that salt treatment significantly increased CAT and GPX activities in Corralejo in the presence of 100 mM NaCl. However, this response was not observed in Tlaltizapon. A decline in CAT and GPX activities has been described in basilica plants cultivated at 50 mM NaCl levels (Attia *et al.,* 2009). This can be attributed either to inhibition of catalase biosynthesis or to inactivation of this enzyme (Feierabend and Dehne, 1996). The decrease of catalase and other peroxidase activity, as observed in our work, is expected to lead to over accumulation of H_2O_2 and damaging membrane permeability, as described previously. Such a situation has been described by several authors (Gupta and Datta, 2003), and discussed in relation to the signaling role of H_2O_2 for morphogenesis. As a result, SOD (superoxide dismutase) increased in Corralejo with the increase of salt stress, Esfandiari *et al.* (2007) show that SOD (superoxide dismutase) increased in Sardari with the increase of salt stress, while in the case of Alvand, SOD showed constant activity at all salt stress levels. The superoxide dismutase (SOD) can be found in various cell compartments and it catalyses the disproportion of two $O_2^{\cdot-}$ radicals to H_2O_2 and O_2 (Scandalios, 1993).

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