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Oxidative damage and antioxidant defenses as potential indicators of salt-tolerant *Cenchrus ciliaris* L. genotypes

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ABSTRACT

Oxidative stress was used as a tool for a differential characterization of salt-tolerant *Cenchrus ciliaris* L. genotypes, as part of a genetic improvement program. Four genotypes of *Cenchrus ciliaris* L. were subjected to gradual salinity stress. After 17 days of 300 mM NaCl treatment, the level of damage in morphological traits was lower in two genotypes, Americana and Biloela (named the salt-tolerant genotypes), than in Texas and Sexual (named the less salt-tolerant genotypes). Oxidative stress characters were evaluated at early time points of salt treatment. Thus, at 48 h, salt tolerance in Americana was correlated with a lower increase in total superoxide dismutase (SOD) activity but a higher increase in total catalase (CAT) activity than in the less tolerant Texas. Salt tolerance was accompanied by a decrease in oxidative damage, evaluated as foliar malondialdehyde (MDA) and $^{\bullet}O_2^-$ content in roots, in salt-tolerant Americana, as compared with the less salt tolerant, Texas. Moreover, in the more salt-tolerant Americana, the decrease in $^{\bullet}O_2^-$ in roots was associated with an enhanced total SOD activity. To validate oxidative damage characters they were measured in Biloela and Sexual, the other two genotypes with contrasting salt tolerance. We propose oxidative stress characters, particularly foliar MDA and root $^{\bullet}O_2^-$ content, as potential indicators of salt tolerance, since they allow a simple, rapid, and cost-effective identification of salt-tolerant *Cenchrus ciliaris* L. genotypes.

Introduction

Salinity is one of the most severe problems in agricultural production (Ashraf, 2009; Yamaguchi and Blumwald, 2005). Salt stress affects all the major processes in plants, such as growth, photosynthesis, protein synthesis, energy, and lipid metabolism (Parida and Das, 2005). Developing crops that can tolerate high levels of soil salinity has been mentioned as a practical contribution to the problem (Ashraf, 2009; Yamaguchi and Blumwald, 2005). To identify salt-tolerant genotypes, genetic improvement programs require the use of sound selection criteria (Ashraf, 2004). Since complex characters, such as yield, have a multigenic inheritance and environmental influence, the combined use of biochemical and physiological characters as tolerance indicators has been proposed as a reliable approach (Ashraf, 2004, 2009; El-Hendawy et al., 2007; Juan et al., 2005; Sairam et al., 2002). However, it has been mentioned that a successful selection of salt-tolerant genotypes depends on a consistent relationship between agronomical, physiological or biochemical markers and plant response to salinity (Ashraf, 2004; Juan et al., 2005).

Tolerance to salinity stress has been often associated with oxidative stress, since one of the consequences of exposure to salinity is the production of reactive oxygen species (ROS), such as superoxide radicals ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) (Ashraf and Foolad, 2007; Ashraf, 2009). ROS can damage almost every macromolecule (Apel and Hirt, 2004). It has been frequently reported that Cl^- toxicity during salinity stress somehow disrupts normal electron flow around photosystem II. Such a disruption would result in excess electron leakage, which in turn could increase the generation of ROS (Ashraf, 2009; Gossett et al., 1994). Superoxide dismutase (SOD) is the primary antioxidant enzyme. SOD converts $^{\bullet}O_2^-$ into H_2O_2, which is eliminated by catalase (CAT) and ascorbate peroxidase (APX), with the contribution of glutathione reductase (GR) (Asada, 2006; Niyogi, 1999; Noctor and Foyer, 1998). Increases in the activity of the enzymes SOD, APX, CAT, and GR under salt stress conditions have been reported for leaves of tolerant genotypes of *Triticum aestivum*, *Chloris gayana*, *Oryza sativa*, and *Setaria viridis* (Kim et al., 2004; Luna et al., 2002; Sairam et al., 2002, 2005; Vaidyanathan et al., 2003). Most of these studies suggest a correlation between tolerance to salinity stress and the presence of an efficient antioxidant system (Ashraf, 2009; Gossett

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et al., 1994; Hernandez et al., 1995; Luna et al., 2002; Mittova et al., 2003).

The present study was conducted as part of a genetic improvement program of *Cenchrus ciliaris* L., an important pasture grass used for cattle and sheep production in arid and semiarid regions worldwide (Griffa et al., 2006; Saini et al., 2007). While there is some evidence about tolerance of *Cenchrus ciliaris* L. to some abiotic stress factors (Ayerza, 1981; De Leon, 2004), a comprehensive study about salt tolerance as well as information about the relationship between salt tolerance and oxidative stress in this species is still lacking. In this work, genotypes of *Cenchrus ciliaris* L. subjected to a long-term salt treatment were evaluated for salt tolerance in terms of morphological traits. At early time points, their response to oxidative damage was measured as total SOD and CAT activities, two key antioxidant enzymes involved in salinity stress (Ashraf, 2009). Malondialdehyde (MDA), a product of membrane lipids peroxidation, was also selected on the basis of previous works (Luna et al., 2000, 2002) suggesting that foliar MDA content would be a good salinity tolerance marker in *Chloris gayana*. Nitrobluetetrazolium (NBT) reduction was used to evaluate $^{\bullet}O_2^-$ content in roots under salinity stress because it has been documented as a tool to identify individuals tolerant of aluminum stress (Maltais and Houde, 2002). The use of foliar MDA and the $^{\bullet}O_2^-$ content in roots as potential indicators of salinity tolerance in *Cenchrus ciliaris* L. genotypes is proposed.

Materials and methods

Plant material

In this study four genotypes of *Cenchrus ciliaris* L. – Americana (Am), Biloela (Bl), Texas 4464 (Tx), and Sexual line (Sx) – were grown in pots (30 cm in diameter), in greenhouse under natural light and day/night temperature of 30/15 °C and 65–75% relative humidity. Twenty days after sowing, seedlings were placed individually in holes of a Styrofoam board (20 plants per board); the boards were set on rectangular plastic trays (30 cm × 20 cm × 60 cm) filled with aerated Hoagland nutrient solution (Hoagland and Arnon, 1950). The plants were maintained under these conditions during 10 days. Salinization was accomplished by gradually adding 50 ml of 1 M NaCl per L of nutrient solution (100 mM every 48 h). Nutrient solution without NaCl was used as control. Evaporative conditions of nutrient solution were controlled regularly and the nutrient solution was renewed every 5 days. When the treatment reached 300 mM NaCl, samples (six plants per treatment) were collected at different times, frozen in liquid nitrogen and immediately used for biochemical determinations.

Analysis of oxidative damage and antioxidant defenses

Ion superoxide content was evaluated in root apexes at 24 h after 300 mM NaCl was reached. The excised root apexes (0.5–0.8 cm long) were incubated for 2 min in a 0.25% NBT solution, as described by Bielski et al. (1980) and Maltais and Houde (2002). In the presence of $^{\bullet}O_2^-$, NBT is reduced to blue formazan, a bluish purple compound. The reaction was stopped by submerging the roots in 37% formaldehyde; roots were placed on an agar base and photographed with a Panasonic camera (WV CD132LE) through the lens of a stereoscopic magnifying glass (Nikon SMZ-10; magnification 13×). Ion superoxide content in roots was evaluated as NBT reduction by visual score. To verify that the presence of blue formazan was due to $^{\bullet}O_2^-$, roots were incubated in 0.25% NBT and 0.25% $MnCl_2$, a competitive inhibitor of $^{\bullet}O_2^-$ (Schraudner et al., 1998); in this case, NBT reduction was not evident (data not shown), which supported the idea that in the salt responses $^{\bullet}O_2^-$ was involved. Also, $^{\bullet}O_2^-$ content was measured in leaves; however, differences in NBT reduction to blue formazan between salt and control treatments were not as evident as in roots (data not shown).

Lipid peroxidation and antioxidant enzyme activities were evaluated in another group of plants at 48 h after 300 mM NaCl was reached. Lipid peroxidation was determined as MDA content by the thiobarbituric acid method (Heath and Packer, 1968). For antioxidant enzyme activities, 100 mg of frozen leaf samples were ground to a fine powder in liquid nitrogen and homogenized in 50 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 1% PVPP (polyvinylpolypyrrolidone). Homogenates were centrifuged at 16 000 *g* for 25 min at 4 °C and the supernatant was used to determine protein concentration (Bradford, 1976) and antioxidant enzyme activity. Superoxide dismutase (SOD) activity was estimated according to the method described by Beauchamp and Fridovich (1973), which is based on the ability of this enzyme to inhibit NBT reduction. The reaction mixture (1 ml) consisted of 5 μl of enzymatic extract and a phosphate buffer solution (0.05 M, pH 7.8) that contained 13 mM methionine, 75 μM NBT, and 1 μM EDTA. The reaction was started by adding 4 μM riboflavin to the mixture and placing it under fluorescent lamps (360 nm) for 6 min. A complete reaction mixture without enzymatic extract, which gave the maximal color, served as the control of the reaction. The reaction was stopped by placing the tubes in the dark. Absorbance of the reaction mixture was read at 560 nm, and one unit of enzyme activity was defined as the amount of enzyme that reduced the optical density to 50% of the control (reaction mixture lacking enzyme). Specific enzymatic activity was expressed as SOD units per mg of protein. Catalase (CAT) activity was estimated as described by Chance and Maehly (1955). The reaction mixture (1 ml) consisted of 80 μl of enzymatic extract, phosphate buffer (0.05 M, pH 7.4) and 5 mM H_2O_2. The decrease in absorbance was due to the reduction of H_2O_2, and was detected spectrophotometrically every second at 240 nm during 30 s. CAT activity was calculated using the extinction coefficient of H_2O_2 (43.6 mM^{-1} cm^{-1}), and one unit of CAT activity was defined as the amount of enzyme required to reduce 1 nmol of H_2O_2 per minute. Specific enzymatic activity was expressed as CAT units per mg of protein.

Morphological traits

To determine salt-stress induced damage, the characters fresh weight of aerial part (FW) and height (H) were measured at the end of the experiment, 17 days after 300 mM NaCl was reached, in control and salt-stressed plants. These morphological traits were chosen following Griffa (2002), who found that FW and H showed significant differences between *Cenchrus ciliaris* L. genotypes and that these morphological traits were more reliable than others, like fresh weight of root, seedling height, root length, number of tillers and of leaves. Results were expressed as percentage of damage suffered by salt-treated plants compared with control plants, and were calculated as follows:

$$\text{percentage of damage} = \left[\frac{X_c - X_i}{X_c}\right] 100,$$

where X_c is the mean value of control plants and X_i is the value of each treated plant.

Statistical analyses

Data of morphological traits, expressed as percentage of damage, and data of biochemical parameters (MDA, SOD, and CAT activities), expressed as percentage of control (100%), were submitted to an analysis of variance (ANOVA) and the means were compared by DGC test (Di Rienzo et al., 2001) ($p < 0.05$), using InfoStat statistics software (InfoStat, 2007). No data transformation was

required because percentage of damage in morphological traits and data of biochemical parameters expressed as percentage of control were normally distributed. Standard error of mean was also calculated and is shown in figures.

Results and discussion

The use of physiological and biochemical criteria has been recommended to achieve a rapid and simple screening of highly salt-tolerant individuals (Ashraf, 2004; El-Hendawy et al., 2007; Juan et al., 2005; Sairam et al., 2002). Accordingly, the role of some enzymatic and non-enzymatic antioxidants as potential selection criteria for improving plant salt tolerance has been recently discussed (Ashraf, 2009). In the present work, we investigated differences in oxidative stress among *Cenchrus ciliaris* L. genotypes and related such differences to salt tolerance. Consequently, some oxidative damage characters, such as MDA and $^{\bullet}O_2^-$, are proposed as a tool to identify *Cenchrus ciliaris* L. genotypes of greater salt tolerance.

Long-term growth responses of *Cenchrus ciliaris* L. genotypes to salinity stress (300 mM NaCl for 17 days) were analyzed. The morphological traits FW and H exhibited a lower damage percentage in the genotypes Am and Bl than in Tx and Sx line (Fig. 1). This behavior was consistent with our previous results on survival of *Cenchrus ciliaris* L. genotypes to salinity stress (Griffa et al., unpublished). In that experiment the genotypes Am and Bl did not show signs (in terms of morphological traits) of salt stress at up to 600 mM NaCl; Tx and Sx line, however, showed a decrease in FW and H at 300 mM NaCl. Considering the previous and the present results, Am and Bl are considered more tolerant, whereas Tx and Sx line are regarded as genotypes less tolerant to salinity stress.

To analyze the behaviour of antioxidant defenses, we selected some key antioxidant enzymes, such as total SOD and CAT activity. They were evaluated at 48 h of 300 mM NaCl treatment in two genotypes with differential tolerance, Am (more tolerant) and Tx (less tolerant). SOD activity of salt-tolerant cultivars is generally expected to be substantially higher than that of sensitive ones. Increases in total SOD activity were recorded in the most salt-tolerant genotypes after a long salt treatment (Ashraf, 2009; Gossett et al., 1994; Kim et al., 2004; Sairam et al., 2002, 2005). In our study, however, already very early after the salinity stress was imposed, total SOD activity increased in both genotypes, the increase being significantly greater in the less salt-tolerant Tx than in Am (Fig. 2).

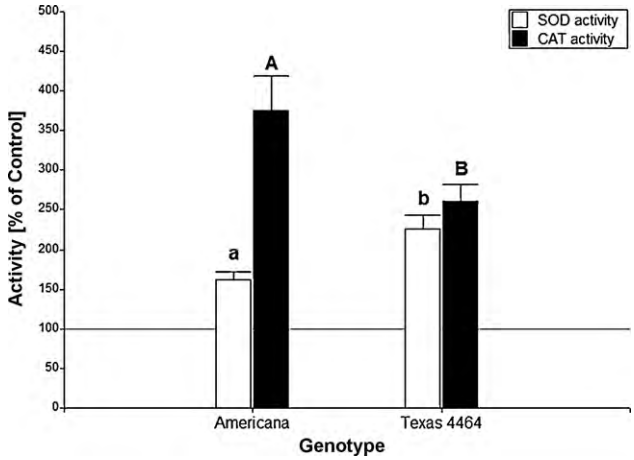

Fig. 2. Effect of NaCl on the activity of antioxidant enzymes, SOD and CAT, in leaves of the more tolerant genotype Americana and less tolerant genotype Texas 4464. Results are expressed as percentage of the control. Different letters indicate significant differences ($p < 0.05$).

Our results agree with Vaidyanathan et al. (2003), who found that total SOD activity was lower in the salt-tolerant *Oryza sativa* cultivar than in salt sensitive ones at early time points of salt treatment. Alves da Costa et al. (2005) observed a similar behaviour after a long salt treatment in sorghum genotypes differing in salt tolerance. On the other hand, total CAT activity also increased in both genotypes with respect to control, but the increment was significantly greater in the more salt-tolerant Am (Fig. 2). These data are consistent with other reports indicating that tolerance to salt stress is associated with an increase in CAT activity (Ashraf, 2009; Kim et al., 2004; Sairam et al., 2002; Vaidyanathan et al., 2003). However, even though total SOD and CAT activities increased in Tx, a higher level of oxidative damage than in Am genotype was observed. Thus, foliar MDA content was significantly high in the less tolerant genotype Tx (113% MDA increase) whereas Am showed only 27% of MDA increase with respect to control (Fig. 3). Since increased SOD activity implies the generation of H_2O_2, the increase in CAT activity in Tx genotype was possibly not sufficient to reduce damage under salinity stress. By contrast, a lower increase in foliar SOD activity in Am than in Tx might indicate a lower accumulation

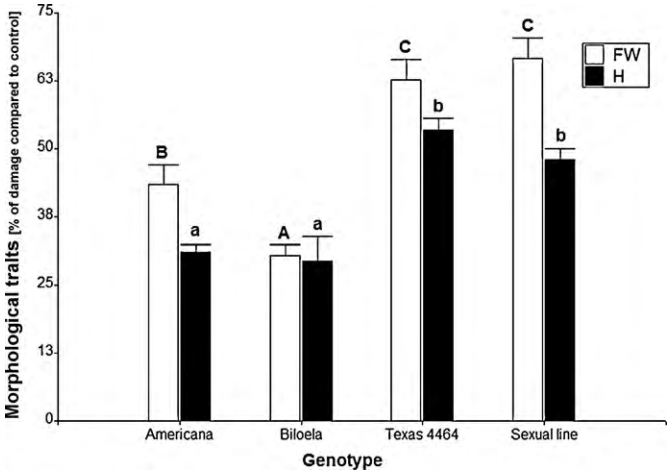

Fig. 1. Effect of NaCl on fresh weight (FW) and height (H) of the more tolerant genotypes Americana and Biloela and the less tolerant genotypes Texas 4464 and Sexual line. Values represent the percentage of damage with respect to the control. Different letters indicate significant differences ($p < 0.05$).

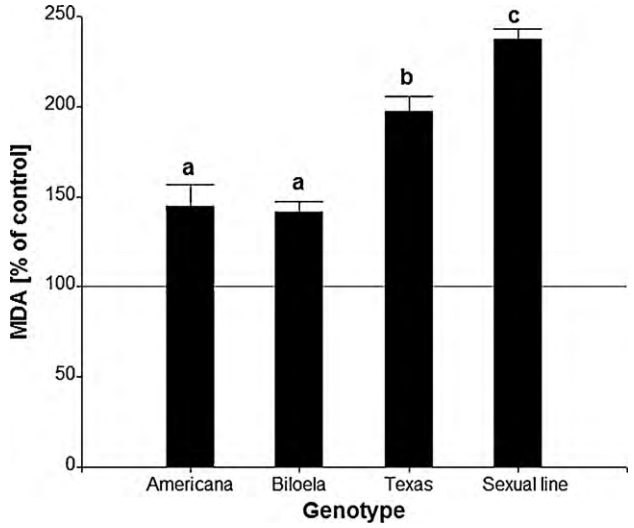

Fig. 3. Effect of NaCl on MDA content in leaves of the more tolerant genotypes Americana and Biloela and the less tolerant genotypes Texas 4464 and Sexual line. Results are expressed as percentage of the control. Different letters indicate significant differences ($p < 0.05$).

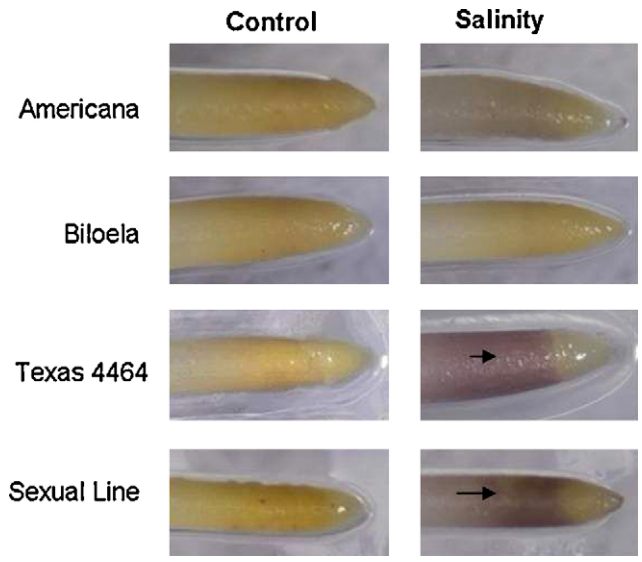

Fig. 4. Superoxide anion content in root apexes of the more tolerant genotypes Americana and Biloela and the less tolerant genotypes Texas 4464 and Sexual line under saline conditions. The bluish purple stain (→), Blue formazan, evidences ROS accumulation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

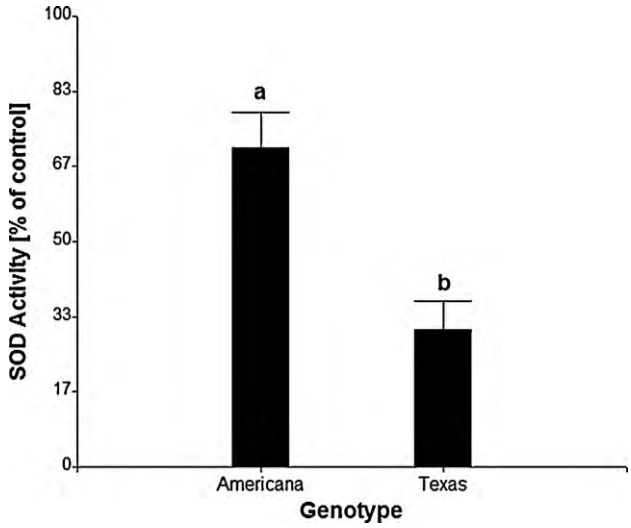

Fig. 5. Effect of NaCl on the activity of the antioxidant enzyme SOD in root apexes of the more tolerant genotype Americana and the less tolerant genotype Texas 4464. Results are expressed as percentage of the control. Different letters indicate significant differences ($p < 0.05$).

of H_2O_2, which in turn can be reduced by the higher increase in foliar CAT activity observed. Thus, a lower total SOD activity and a higher total CAT activity in Am than in Tx could be involved in highly efficient enzymatic detoxification of H_2O_2. Our data suggest that SOD/CAT relationship could be more important than the activities of a single enzyme in maintaining oxidative stress under control during salinity stress. Accordingly, a similar SOD/CAT relationship was observed in salt-tolerant and sensitive varieties of rice by Vaidyanathan et al. (2003) and in sorghum by Alves da Costa et al. (2005).

Some investigations have demonstrated that apexes of roots are very sensitive to oxidative damage during salinity stress. Increased ROS formation has been observed after exposure of *Arabidopsis* roots to high salt stress (Demidchik et al., 2003). Moreover, Mittova et al. (2003) and Khan et al. (2002) detected increased levels of H_2O_2 and lipid peroxidation in the roots of tomato and rice under salt stress. Interestingly, Mittova et al. (2004) reported increases in MDA and H_2O_2 contents and changes in the activity of antioxidant enzymes in the roots of a susceptible tomato genotype under salt stress conditions. Consequently, the behaviour of Am and Tx genotypes during salinity stress was compared by $^{\bullet}O_2^-$ accumulation in roots. Although $^{\bullet}O_2^-$ is not able to react directly with proteins or lipids, its protonated form (prehydroxyl radical) can bring about oxidative damage by increasing lipid peroxidation, hydrogen peroxide and hydroxyl radicals (Apel and Hirt, 2004; Asada, 2006; Hideg, 1997). Superoxide ion accumulation could be promoted by the disruption of root mitochondrial electron transport under salt stress conditions. This reactive anion is rapidly converted to H_2O_2 by spontaneous dismutation or by mitochondrial SOD activity. In our work $^{\bullet}O_2^-$ accumulation was evaluated as strong NBT reduction to blue formazan in roots under salinity stress, and NBT reduction seems to be associated to tolerance to salinity stress. Thus, at an early stage of salt treatment (24 h), in the root apex of the less tolerant genotype Tx a bluish purple compound was observed as a consequence of NBT reduction to blue formazan, suggesting $^{\bullet}O_2^-$ accumulation. By contrast, the root apex of the more tolerant genotype Am showed a very slight bluish purple compound (Fig. 4). It has been reported that root tissues are protected against NaCl stress by enhanced total SOD activity (Bandeolu et al., 2004; Demiral and Türkan, 2005; Mittova et al., 2004). To investigate if changes in $^{\bullet}O_2^-$ content in roots were regulated by total SOD activity, this enzyme was estimated in the roots of the genotypes Am and Tx. When exposed to saline stress, Am exhibited a non-significant decrease in SOD activity (Fig. 5), which could explain the slight build-up of $^{\bullet}O_2^-$ in the root apexes (Fig. 4). By contrast, SOD activity decreased markedly in the roots of Tx genotype under salinity conditions (Fig. 5), which is possibly related to the higher accumulation of this ROS than in Am (Fig. 4).

Efforts for genetic improvement in salinity tolerance require an efficient screening technique (Ashraf, 2004; Hameed and Ashraf, 2008). In the present work oxidative stress characters were evaluated as a tool to select more tolerant *Cenchrus ciliaris* L. genotypes. Both key antioxidant enzymes, total SOD and CAT activities, increased in Am and Tx genotypes (which have contrasting salt tolerance); however, while CAT activity increment was correlated with salt tolerance of both genotypes, total SOD activity was lower in the more tolerant Am than in the less tolerant Tx. These results show the variations of total antioxidant enzymes in relation to salt stress tolerance, as indicated in Ashraf (2009). Consequently, in agreement with some authors (Alves da Costa et al., 2005; Vaidyanathan et al., 2003) we suggest that SOD/CAT ratio could be a more useful salt tolerance biochemical marker than the activities of a single enzyme. However it should be noted that evaluating total SOD and CAT activities is time-consuming and costly; therefore the number of samples that can be processed is limited. By contrast, oxidative damage measured as early changes in foliar MDA content and $^{\bullet}O_2^-$ content in roots were correlated with salinity stress tolerance. Moreover, oxidative damage characters were measured in other genotypes with contrasting salt tolerance to validate their efficiency as salt tolerance indicators. Thus, the more salt-tolerant Bl showed a lower oxidative damage, evaluated as a reduction in foliar MDA and $^{\bullet}O_2^-$ accumulation in roots. By contrast, the less salt-tolerant Sx line evidenced higher oxidative damage, measured as increases in foliar MDA and $^{\bullet}O_2^-$ content in roots (Fig. 3 and 4). Foliar MDA content was consistent with salt tolerance in *Oryza sativa*, *Setaria viridis* and tomato cultivars (Juan et al., 2005; Kim et al., 2004; Vaidyanathan et al., 2003) and with findings from our previous work on salt tolerance in *Chloris gayana* diploids (Luna et al., 2000, 2002) and in *Chloris gayana* tetraploids (unpublished data). It should be noted that in the latter, foliar MDA showed that it can be a heritable character. All these evidences suggest that foliar MDA content level might be considered a suitable salinity toler-

ance indicator in *Cenchrus ciliaris* L. genotypes, as Luna et al. (2000) proposed for *Chloris gayana*. The other possible indicator of salt tolerance could be $^{\bullet}O_2^-$ content in roots. Evaluating $^{\bullet}O_2^-$ in roots was easier than in leaves and provided more reliable results, offering the possibility to screen numerous *Cenchrus ciliaris* L. salt-tolerant genotypes. To our knowledge, $^{\bullet}O_2^-$ in salt-treated roots till now has not been considered a salinity tolerance indicator, although NBT reduction in roots has been reported as an aluminum tolerance marker (Maltais and Houde, 2002). Interestingly, our results show that changes in $^{\bullet}O_2^-$ content in roots were associated with changes in total activity of SOD, the antioxidant enzyme that regulates $^{\bullet}O_2^-$ concentration. Furthermore, SOD activity in roots was correlated with salt tolerance, since the more salt-tolerant genotype Am exhibited the lowest accumulation of $^{\bullet}O_2^-$ in roots, as compared to the less salt-tolerant Tx. Overall, in this work we propose oxidative damage characters, particularly foliar MDA and root $^{\bullet}O_2^-$ content, as potential indicators of salinity tolerance because they allow a simple, rapid and cost-effective identification of salt-tolerant *Cenchrus ciliaris* L. genotypes.

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