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OXIDANT AND ANTIOXIDANT ENZYME RESPONSE OF REDBINE SWEET SORGHUM UNDER NaCl SALINITY STRESS

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ABSTRACT

Sorghum bicolor is an indispensable source of bioactive compounds with benefits to human health. In present study, salt tolerance of Redbine variety of sweet sorghum was investigated via enzymatic and non-enzymatic antioxidants. Plants were supplied with Hoagland solution containing 0-200 mM salt concentrations for 10 days. Enzyme activities, chlorophyll and carotene contents were spectrophotometrically assessed. Root catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and proline amounts indicated increase in 50-150 mM doses revealing the protective response of the plant. As distinct from other parameters, superoxide dismutase (SOD) activity and the amount of membrane lipid peroxidation (malondialdehyde, MDA) revealed increase in all concentrations both in roots and leaves. APX, GR, glutathione S-transferase (GST), MDA, and Proline in leaves and roots were similar with increasing trend. However, SOD activity was the highest at 200 mM salt concentration. Redbine sweet sorghum reacted by increasing the synthesis of all chlorophylls and carotene against all concentrations. The plant indicated an effective response against salt stress. Increasing salt concentrations caused increase in chlorophyll and carotene contents indicating the effective role of carotenes in plant defense against salt stress. Although the antioxidant enzyme activities of Redbine sweet sorghum contribute to its response to salt stress, the situation seems to be not adequate at higher concentrations.
Additional keywords: Malondialdehyde, proline, salt stress

RESUMEN

Respuesta de enzimas oxidantes y antioxidantes del sorgo dulce var. Redbine bajo estrés salino de NaCl

El sorgo posee compuestos bioactivos benéficos para la salud humana. Se investigó la tolerancia al NaCl de la variedad Redbine a través de antioxidantes enzimáticos y no enzimáticos. Las plantas recibieron solución de Hoagland con 0-200 mM de la sal por 10 días y se evaluó la actividad enzimática y contenido de clorofila y carotenos. La catalasa (CAT), ascorbato peroxidasa (APX), glutatión reductasa (GR) y la prolina indicaron un aumento en dosis de 50-150 mM que indicó la respuesta protectora de la planta. La actividad de la superóxido dismutasa (SOD) y la cantidad de peroxidación lipídica de membrana (malondialdehído, MDA) revelaron un aumento en todas las concentraciones tanto en las raíces como en las hojas. APX, GR, glutatión S-transferasa (GST), MDA y prolina en hojas y raíces también mostraron tendencia creciente. La mayor actividad de SOD ocurrió a 200 mM de la sal y la síntesis de clorofilas y caroteno se produjo en todas las concentraciones, indicando que la planta mostró respuesta efectiva contra el estrés salino. Aunque las actividades enzimáticas antioxidantes del sorgo contribuyen a su respuesta al estrés salino, esto no parece ser adecuada a concentraciones más altas.
Palabras clave adicionales: Malondialdehído, proline, salt stress

INTRODUCTION

Sorghum species are among the most cultivated fifth cereals for obtaining human and animal nutrition, biofuels and also alcoholic beverages (Mutegi et al., 2010). In many parts of the world it is an indispensable origin of food and bioactive compound with benefits for health (De Morais et al., 2017). Sorghum is known to resist both biotic and abiotic stress factors. About 400 million hectares (25%) of the total area in the world is under salt threat. In this sense This species have received great interest due to their durability under stress situations. The effects of salt stress on plants varies according to their varieties, type of stress factors and exposure periods. Reactive oxygen species (ROS), generated by stress factors interrupts biochemical and physiological processes in organisms such as inhibition of transport and growth, alterations in

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chlorophylls and chloroplast pigments and accordingly in photosynthesis, lipid peroxidation, destabilization in nucleic acids, and enzyme inactivation (Karuppanapandian et al., 2011). Under stress conditions, the concentration of free radicals may increase and activate antioxidants such as CAT, SOD and peroxidases to remove ROS (Zhang et al., 2007). Plants have various antioxidant systems as enzymatic and non-enzymatic defense mechanisms to avoid destructive activities of ROS and other stress products. Among the enzymatic scavenging mechanisms APX (especially in chloroplast), GR (especially in mitochondria), CAT for effectively removal of H_2O_2, SOD for clearing the superoxide anions (OH^-, O^{2-}), and GST are available (Karuppanapandian et al., 2011). Due to scavenging characteristics of these enzymes, accumulation of oxygen radicals remains low under normal cellular conditions (Asada, 1984). Proline, commonly found in higher plants, under salt stress accumulates more than other amino acids. The resistance of plants on adverse conditions for detoxification varies from species to species depending on the production capacity of antioxidant enzymes. Little information is present about biochemical and physiological machinery of antioxidant enzymes under salt (NaCI) stress in sweet sorghum. Therefore, to contribute the sorghum ecosystem, we focused on studying the effect of salt stress on enzymatic and non-enzymatic antioxidative defense systems. The objective of this study was to investigate the scavenging activity of CAT, APX, GR, GST, SOD enzymes as well as the amount of proline, MDA as thiobarbituric acid reactive substances (TBARS) in root and leaf tissues and, Chlorophyll a, Chl b, total Chl and carotenes in leaf tissues of Redbine sweet sorghum under different NaCl treatments (0 -200 mM) and laboratory conditions.

MATERIALS AND METHODS

Experimental design. Trials were performed through cultivating 10 seeds in three plastic cultivation containers each (15 cm width, 5 cm depth) with hydroponic Hoagland solution in climate cabinet under controlled (25±1 °C, 70±5 % RH and 16/8 L/D) conditions to investigate the impact of NaCI (0, 50, 100, 150, 200 mM) on some physiological and biochemical processes. Treatments were repeated three times independently. Initially seedlings were allowed to grow for 10 days up to 10-15 cm. Then, they were exposed to NaCI concentrations at following 15 days. Hoagland solution without salt concentrations was used as control for 25 days. The plants were prevented from dehydration by daily replacement of Hoagland solution. Following the 15 days of salt stress, the root and leaf segments of 90 seedlings were separated into small pieces. 0.25-0.5 g of each tissue were wrapped in aluminum foil and socked with liquid nitrogen before storing at -20 °C.

Enzyme activity. Crude proteins were purified by the method of Misra and Gupta (2006) using the buffer containing PVP (1%), EDTA (0.1%), and potassium phosphate (0.1 M with pH 7.4). The amount of proteins (mg/ml) in samples were determined spectrophotometrically with two replications at 595 nm by the Bradford method (Bradford, 1976) using the BSA as the standard.

CAT. Sodium hydrogen phosphate ($NaHPO_4$, 20 mM with pH 7.5), H_2O_2 (20 mM), and 50 µl enzyme solution were used. The reaction was started with H_2O_2 (20 mM) through transferring 50 µl of extract into buffer (20 mM, pH 7.5), and absorbance was recorded at 240 nm for 3 minutes (Misra and Gupta, 2006). The molar absorption coefficient (ε) of H_2O_2 for catalase is 40 $mM^{-1}cm^{-1}$. The specific activity (unit/mg) in samples was estimated using the formula;

SA=[[(ΔAbs/min)/40]*(reaction volume (2 ml)/volume of crude enzyme)*(1/protein conc.)]*1000.

APX. The reaction was started by transferring 50 µl of enzyme extract into the solution containing $KHPO_4$ (50 mM, pH 7.0), ascorbic acid (0.15 mM), and H_2O_2 (20 mM) (Akbulut and Çakır, 2010). The absorbance at 290 nm in 2 mL cuvettes was observed and recorded for three minutes. The molar absorption coefficient (ε) of H_2O_2 at 290 nm is 2.8 $mM^{-1}cm^{-1}$. The specific activity (unit/mg) of the samples were estimated using the formula;

SA=[[(ΔAbs/min)/2.8]*(reaction volume (2 mL)/volume of crude enzyme)*(1/protein conc.)]*1000.

GR. The activity was determined using the method of Misra and Gupta (2006). The reaction was started by adding 50 µl crude extract into $KHPO_4$ (100 mM, pH 7.5) buffer with Na_2EDTA (0,1 mM), oxidized glutathione (1mM), and NADPH (0.1 mM) and absorbance was observed

for 5 min at 340 nm. The molar absorption coefficient (ε) of NADPH at 340 nm is 6.2 $mM^{-1}cm^{-1}$. The specific activity (unit/mg) of the samples were estimated using the formula;

SA= [[(ΔAbs/min)/6.2]*(reaction volume (3 ml)/volume of crude enzyme)*(1/protein conc.)]*1000

GST. The reaction was started by adding 50 µl of enzyme extract into $KHPO_4$ (100 mM, pH 7.5) with EDTA (0.1 mM), GSH (1 mM), CDNB (1 mM), and 0.1 mM NADPH. Changes in absorbance was recorded during 5 min at 25 °C at 340 nm. The molar absorption coefficient (ε) of NADPH at 340 nm is 6.2 $mM^{-1}cm^{-1}$ (Yilmaz et al., 2017). The specific activity (unit/mg) of the samples were estimated using the formula:

SA=[[(ΔAbs/min)/6.2]*(reaction volume (3 mL)/ volume of crude enzyme)*(1/protein conc.)]*1000

SOD. The activity was measured using the method of Giannopolitis and Ries (1977) in sodium phosphate buffer (20 mM, pH 7.5) with NBT (0.1 mM), riboflavin (0.005 mM), EDTA (0.1 mM), and methionine (10 mM). Absorbance at 560 nm was measured immediately after 15 min exposure of the solution containing 50 µl of enzyme extract to light source (500 lumen) with 20 cm distance. SOD standard (10-500 ng) was used for comparing the percent inhibition in samples.

Proline. Absorbance for proline in samples were measured spectrophotometrically at 520 nm against toluene using acid ninhydrin method (Tewari et al., 2002). Graph obtained using proline standard was used for estimating the amounts (nmol/g) in samples.

MDA. Lipid peroxidation in root and leaf tissues was measured through determining the MDA content spectrophotometrically at 532 nm in samples using the method of Madhava and Sresty (2000). Concentrations was calculated with extinction coefficient of 155 $mM^{-1}\cdot cm^{-1}$.

Determination of Chl a, b, total Chl and carotene. Chl and carotene contents in leaf tissues were searched using the method of Hiscox and Israelstam (1979). Fresh leaf tissue (100 mg) was transferred into a test tube (50 ml) and DMSO (7 mL) was added and incubated at 65 °C till the color was removed. Afterwards, the liquid part is transferred into a clean tube and completed to 10 mL with DMSO. Spectrophotometric measurements in samples were performed against DMSO at 647, 663 and 470 nm. The Chl (Chl a and Chl b) and carotene (Car) contents were determined as mg/g fresh weight using the following formulas;

Chl a=(12.25*A663)-(2.79*A647)

Chl b=(21.5*A647)-(5.1*xA663)

Total Chl=(7.15*A663)+(18.71*A647)

Car=[(1000*A470)-(1.82*Chl a)-(85.02*Chl b)]/198

Statistical analysis. Experimental data were subjected to analysis of variance (Anova) and Duncan's multiple range test using the SAS software v. 8.0 (Cary, NC, USA).

RESULTS

CAT. The activity both in root and leaf tissues indicated an increase up to a certain concentration of salt (150 mM in root, 100 mM in leaf) and then decreased depending on increased stress. The highest CAT activity was achieved at 150 mM concentration in root. An important point that stands out is that the CAT activity in roots were twice as much as the activity in leaves (Figure 1a).

APX. The activity in root and leaf tissues increased up to 150 mM salt concentration. However, an important difference between these tissues is that at 150 mM salt concentration nearly three-fold higher APX activity was observed in roots compared to that of leaves. At higher concentration (200 mM) the amount indicated a significant decrease (Figure 1b).

GR. Depending on increasing salt concentration, GR activities in root and leaf tissues rose continuously till 150 mM. On the other hand, the activity at 200 mM in both tissues indicated a significant decrease. GR activity in roots were 1.5-fold higher compared to that of leaves (Figure 1c).

GST. The activity in root and leaf tissues rose up to 100 mM concentration, and then especially in root, a considerable decrease was observed. However in leaf tissues such kind of decrease was not evident at 100, 150 and 200 mM concentrations, but rather a steady state situation was seen. While the activity of GST was higher in root at low concentrations (till 100 mM), at higher concentrations (150 and 200 mM) the activity was higher in leaf tissues (Figure 1d).

SOD. Different from other antioxidant enzymes, SOD activity indicated a continuous increase in leaf tissues. Specifically, after 150 mM, an excessive 8-fold increase was seen in leaves

compared to control group. However, although the SOD activity increased till 150 mM in root, this was lower than that of leaves. A decrease was seen at SOD activity after 150 mM salt concentration in root tissues (Figure 1e).

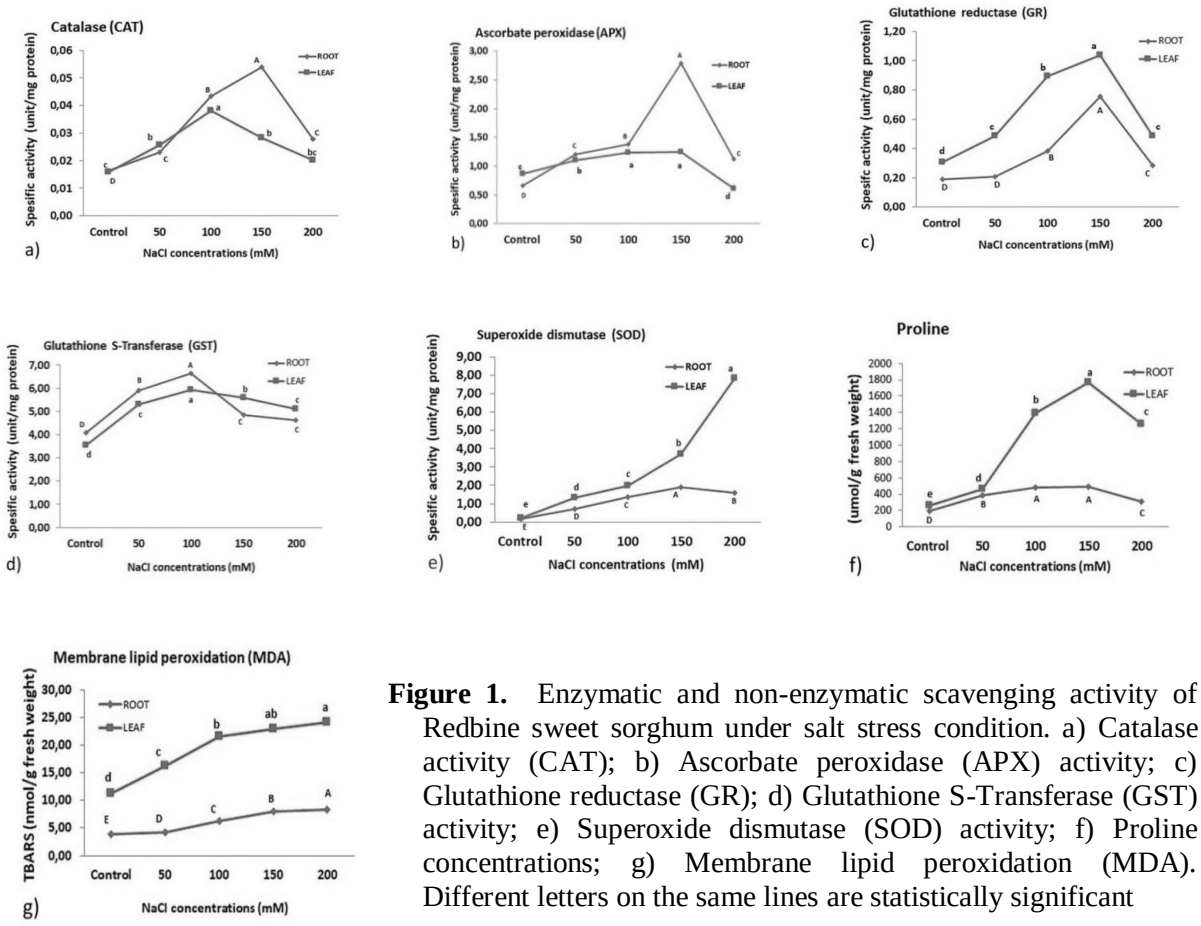

Figure 1. Enzymatic and non-enzymatic scavenging activity of Redbine sweet sorghum under salt stress condition. a) Catalase activity (CAT); b) Ascorbate peroxidase (APX) activity; c) Glutathione reductase (GR); d) Glutathione S-Transferase (GST) activity; e) Superoxide dismutase (SOD) activity; f) Proline concentrations; g) Membrane lipid peroxidation (MDA). Different letters on the same lines are statistically significant

Table 1. Chl a, Chl b, total Chl and carotene content in leaf tissues

NaCl Concentration	Chl a	Chl b	Total Chl	Carotene
0 mM	95.394 d	72.432 e	167.826 e	15.555 e
50 mM	99.104 d	84.906 d	184.010 d	19.858 d
100 mM	208.899 c	142.251 c	351.151 c	40.823 c
150 mM	309.846 b	197.427 b	507.274 b	81.000 b
200 mM	345.317 a	234.397 a	579.714 a	125.220 a

Values with different letters in the same column are statistically different according to Duncan's test ($P \leq 0.05$)

Proline. Depending on increasing salt concentrations, proline amount indicated an increase till 150 mM both in leaf and root tissues. The amount in leaves were nearly 5-fold higher compared to root proline levels. At the same time decrease was observed after 150 mM salt both in leaf and root tissues (Figure 1f).

MDA. MDA accumulation in root and leaf tissues indicated a continuous increase depending on increasing salt concentration. However, the amount of MDA in leaves were 6-fold higher than that of root tissues (Figure 1g).

Chlorophyll and carotene contents. Chl a, Chl b, total Chl and carotene contents increased with increasing salt concentrations. When compared with control, while the increase in Chl a, Chl b, and total Chl was nearly 3-fold high, carotene content was 8-fold high (Table 1).

DISCUSSION

In Redbine sweet sorghum the highest CAT activity was seen at 100 mM in leaf tissues and 150 mM in root tissues (Figure 1a). This was supported by the finding that CAT is one of the most efficient antioxidant enzymes, which prevents the cellular damage (Scandalios, 1993). Although there is not much work about enzyme activity in sweet sorghum, similar results were reported in studies with other plants as *Solanum* spp. (Abdel and Chaoxing, 2011), *Brassica juncea* (Mittal et al., 2012), *Oryza* seedlings (Nounjan et al., 2012) and *Glycine* (Arshi et al., 2012). In our previous study, the highest CAT activity in grain sorghum under salt stress was obtained at 100 mM NaCI concentration (Temizgul et al., 2016).

Plants respond to environmental stress via increasing the activity of APX as well as other scavenging enzymes (Shigeoka et al., 2002). In accordance with this, Menezes et al. (2004) reported that expression of APX under salt stressed conditions was up-regulated in early stages of rice seedlings (García et al., 2019). Similarly, in the present study considerable increase was observed with the highest level at 150 mM salt concentration both in leaf and root sections of Redbine sweet sorghum (Figure 1b). The plant responded as strong as possible till 150 mM as was the case for the study carried out by Yamane et al. (2010), but at 200 mM APX activity exhibited a sharp decrease and subsequent growth reduction indicated that the limiting value for tolerance has been exceeded.

GR activity in different species of plants revealed an increase under various abiotic stress conditions (Yousuf et al., 2012). Eyidogan and Öz (2007) reported an increased response of GR activity in leaf tissues of *Cicer arientinum* under salt stress. Also, in root tissues of the same plant GR activity was reported to be high under salt stress (Yousuf et al., 2012). AbdElgawad et al., (2016) reported that in maize plant, SOD, APX and GR activity specifically increased in root tissues under salt stress. In the present study, although the plant is different, the activity of GR was found to be significantly high in 150 mM salt concentration both in roots and leaves (Figure 1c). However in our previous study the highest GR activity in grain sorghum under salt stress was obtained at 100 mM NaCI concentration (Temizgul et al., 2016) indicating the higher tolerance of sweet sorghum against salt stress.

GSTs are well known enzymes for detoxification of potentially harmful xenobiotics into glutathione, thereby they can easily be removed from the cell (Yousuf et al., 2012). They may be important for acclimatization period of plants to unfavorable situations, yet Csiszár et al., (2014) indicated that it was not valid all the time for its various isoenzymes in terms of expression levels. In accordance with this, Sun et al., (2010) indicated that while some members of the GST gene family were down regulated under salt stress, others indicated no change. For example, salt-tolerant wild tomato and a salt-sensitive domesticated cultivar differed in their GST expression profiles (Sun et al., 2010). In our previous study, the highest root and leaf GST level was observed as 5.5 $u \cdot mg^{-1}$ in grain sorghum under salt stress with 150 mM. On the other hand, the similar level was observed at 100 mM for leaf tissues in Redbine sweet sorghum, but in root tissues it was higher (6.5 $u \cdot mg^{-1}$) (Figure 1d).

As the main detoxifying enzyme SOD converts O^{2-} radicals to H_2O_2, root SOD activity in Redbine sweet sorghum revealed remarkable increase at all concentrations indicating that excessive ROS accumulation was prevented and accordingly the cells were protected from oxidative damage. But, in leaf tissues a decrease was observed at 200 mM indicating that the maximal tolerance capacity was up to 150 mM concentration (Figure 1e). In a similar way Huang (2018) searched the enzyme activity in seedlings of sorghum cultivar and observed an increasing trend with increasing NaCl concentration, especially in salt-tolerant cultivar. They also reported that the salt-tolerant cultivar ensured considerably high antioxidant capacity and rapidly get rid of ROS. Thus, the ROS accumulation and consequently lipid peroxidation in cell membranes were prevented (Huang, 2018).

Proline is one of the common osmolytes maintaining fluid balance in plants and under

stress situations it is up-regulated and provides protection against damage (Hare and Cress 1997). Salt stress result in ion toxicity and osmotic stress through impairing the composition of cellular ions (Nounjan et al., 2012). To cope with the osmotic stress and resultant damage under salt stress, plants must try to produce and accumulate non-enzymatic antioxidant solutes as proline and glycine betaine, and ascorbate as well as other enzymatic antioxidants (Nounjan et al., 2012; Yildız et al., 2013; Gharsallah et al., 2016). Increase in endogenous level of proline helps plants in a positive way for the removal of ROS (Huang, 2018). In compliance with these results, in the present study Redbine sweet sorghum increased the amount of proline till 150 mM and then a significant decrease was observed (Figure 1f). The increased production of proline especially in leaf tissues till 150 mM concentration in Redbine sweet sorghum together with other antioxidants prevents severe losses in growth and yield and is a reliable indication of resistance to NaCI stress. Similarly, Yan et al. (2015) reported that proline content and relative water content in leaf tissues increase under salt stress.

Peroxidation of various molecules through oxidative damage in plant membranes is a major cause of salt stress (Hernandez and Almansa, 2002). Among them lipid peroxidation directly causes membrane disruption and is a reliable indication of the extend of oxidative damage (Demiral and Türkan 2005; Yadav, 2010). In present study the extend of lipid peroxidation was measured in roots and leaves of Redbine sweet sorghum in terms of MDA content and observed that it increased depending on the increasing salt concentrations both in root and leaf tissues (Figure 1g). In the same way, Balaji et al. (2013) reported a continuous increase of MDA content at 100 and 200 mM NaCI concentrations. In another study Huang (2018) reported that increasing treatment of NaCI concentrations resulted in raise in O^{2-} and MDA contents both in tolerant and sensitive cultivars. A remarkable point is that SOD and MDA continued to increase while the other antioxidants decreased after 150 mM. It is an indication that the damage to the cells at 200 mM concentration has reached very serious levels and the repair mechanisms are not enough to cope with the damage of salt stress in Redbine sweet sorghum.

Plants normally produce ROS through cellular metabolism, however, when they are subjected to stress the balance between their production and destruction deteriorates (Karuppanapandian et al., 2011). In such a case ROS should be scavenged through one or both of the enzymatic and non-enzymatic antioxidative systems. In accordance with this knowledge, the 3-fold increase in Chl a, b, and total Chl's and 8-fold increase in the amount of carotene together with increase in ROS scavenging enzyme activities in Redbine sweet sorghum indicates that this species efficiently uses both enzymatic and non-enzymatic defense system to overcome salt stress (Figure 1a-g, Table 1). Among these parameters especially the carotenes take part a role in metabolism for developing tolerance to oxidative stress (Karuppanapandian et al., 2011). Such a result indicates that the salt tolerance of Redbine sweet sorghum is quite high. In compliance with our results, researchers reported that tolerance of varieties to stress conditions are related with high amount of Chl and carotenes (Ramanjulu and Bartels 2002; Munné and Alegre 2004).

CONCLUSIONS

Increasing NaCI concentrations resulted in considerable increase in antioxidant enzyme activities, and also high level of proline and carotene levels indicated their effective role in defense against ROS under salt stress in Redbine sweet sorghum. However, although the antioxidant enzyme activities of Redbine sweet sorghum contribute to its response to salt stress, the situation seems to be not adequate at concentrations higher than 150 mM. Another remarkable point is that SOD (in leaf) and MDA continued to increase while the other antioxidants decreased after 150 mM concentration. It is an indication that the damage to the cells at 200 mM concentration has reached very serious levels and the repair mechanisms may not be not enough to overcome the damage of salt stress in Redbine sweet sorghum.

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