Research Article

Ramazan Beyaz* and Hakan Kır

Physio-biochemical analyses in seedlings of sorghum-sudangrass hybrids that are grown under salt stress under in vitro conditions

In vitro koşullarda tuz stresi altında yetişen sorgum-sudangrass hibritlerin fidelerinde fizyo-biyokimyasal analizler

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Abstract

Objective: This study was conducted to analyze the physio-biochemical responses of two sorghum-sudangrass (*Sorghum bicolor* × *Sorghum Sudanese* Stapf.) hybrid ("Aneto" and "Sugar Graze") seedlings exposed to salt stress.
Materials and methods: Sorghum-sudangrass hybrid seeds sown in MS medium containing 50 and 100 mM NaCl. The activity of antioxidant enzymes (SOD, CAT, GR, APX), chlorophyll (a, b, and total), malondialdehyde (MDA), and proline levels measured in 14 days old seedlings.
Results: As a result of the study, the activity of antioxidant enzymes (CAT, SOD, APX, and GR), malondialdehyde (MDA), proline and chlorophyll contents of seedlings of cv. "Aneto" increased. On the other hand, SOD activity, proline, and chlorophyll content increased while CAT, APX, GR activity, and malondialdehyde (MDA) content decreased in seedlings of cv. "Sugar graze".
Conclusion: Overall, the results showed that the cv. "Aneto" was less affected by the adverse effects of salt stress than the cv. "Sugar graze". This study is essential for revealing biochemical responses of 14 days old Sorghum-Sudanese hybrid seedlings against salt stress. These study findings can use in breeding programs for sorghum plants.

Keywords: Salt (NaCl) stress; Antioxidant enzymes; Proline; Malondialdehyde (MDA); Chlorophyll.

***Corresponding author: Ramazan Beyaz,** Department of Soil Science and Plant Nutrition, Faculty of Agriculture, Kırşehir Ahi Evran University, Kırşehir, Turkey, e-mail: ramazanbeyaz@gmail.com. https://orcid.org/0000-0003-4588-579X
Hakan Kır: Department of Field Crops, Faculty of Agriculture, Kırşehir Ahi Evran University, Kırşehir, Turkey. https://orcid.org/0000-0002-3124-0491

Öz

Amaç: Bu çalışma, tuz stresine maruz burakılan iki sorgum-sudangrass (*Sorghum bicolor* × *Sorghum sudanense* Stapf.) hibrit ("Aneto" ve "Sugar Graze") fidelerinin fizyo-biyokimyasal tepkilerini analizi için yapılmıştır.
Gereç ve Yöntem: Sorgum-sudangrass hibrid tohumları içerisinde 50 ve 100 mM NaCl içeren MS ortamlarına ekilmiştir. 14 günlük fidelerde, antioksidant enzimlerin aktivitleri (SOD, CAT, GR, APX), klorofil (a, b ve toplam), malondialdehit (MDA) ve prolin seviyeleri ölçülmüştür.
Bulgular: Çalışma sonucunda, "Aneto" çeşidinin fidelerinde, antioksidant enzimlerin (CAT, SOD, APX ve GR) aktivitesi, malondialdehit (MDA), prolin ve klorofilin içerikleri artmıştır. Diğer taraftan, "Sugar graze" çeşidin fidelerinde, SOD aktivitesi, prolin ve klorofil içeriği artarken, CAT, APX, GR aktivitesi ve malondialdehit (MDA) içeriği azalmıştır.
Sonuç: Genel olarak, sonuçlar tuz stresinin olumsuz etkilerinden "Sugar graze" çeşidi ile kıyaslandığında "Aneto" çeşidinin daha az etkilendiği göstermiştir. Bu çalışma, 14 günlük Sorgum-Sudanotu melez fidelerin tuz stresine karşı biyokimyasal tepkilerini ortaya çıkarması bakımından önemlidir. Sorgum bitki için ıslah programlarında bu çalışma bulguları kulanılabilir.

Anahtar kelimeler: Tuz (NaCL) stresi; aktioksidan enzimler; prolin; malondialdehid (MDA); klorofil.

Introduction

Salinity is a major problem in agriculture and crop production [1]. It is known to limit plant performance through a decrease in plant growth and yield [2]. More 6% of the land in the entire world (more than 800 million ha of land) is affected by salt [3]. Wang et al. [4] and Jha et al. [5] highlighted emphasized the increasing ratios of salt-stressed areas and predicted an almost 30% loss in land based on salt stress in the next 25 years. Therefore, this environmental stress factor continuously increases its

impact. Although there are many ways to fight this stress factor, the farming salt-tolerant plants has been considered as the most economical and effective way to utilize saline-alkali land. Salinity stress influences a plant at its all stages of growth; however, for most plants, stages of seed germination and seedling growth are known to be more susceptible [6, 7]. Moreover, the stages of germination and early seedling are indications of plant growth reactions to salt stress [6]. Salt tolerance in plants is a complicated issue that is dependent on several associated factors regarding biochemical and physiological processes [8]. Therefore, revealing the physio-biochemical mechanism of salt tolerance is needed to improve the tolerance of plants to salt stress [9]. Salinity influences many aspects of plant physio-biochemical processes including antioxidant enzymes' activities, proline accumulation and photosynthesis. Many studies showed that these physio-biochemical processes are related to salt tolerance in many plants, and they affect the plant's acclimation in saline environments [6, 10, 11].

Sorghum is a C4 plant, known by its high photosynthetic capacity and high yield and its high tolerance of stressors (drought, radiation and solar heat), including salt [2, 12]. However, salt stress gravely influences the growth and development of sorghum, which is a significant plant grown in arid and semiarid regions [13]. Sorghum's tolerance to high-salinity conditions appears to differ based on the genotype [14]. Salt stress tolerance in types (grain sorghum, forage or sweet sorghum, sudangrass and sorghum-sudangrass hybrids) of sorghum plants has been investigated in previous studies by Chaugool et al. [14], de Lacerdaa et al. [15], Yan et al. [16] and Patane et al. [17]. However, there is limited information about the physio-biochemical responses of sorghum-sudangrass hybrids to salt stress. Therefore, here, the effects of salt (NaCl) stress on physio-biochemical variables were characterized on two sorghum-sudangrass hybrids' seedlings under in vitro conditions. The significance of this study was that it revealed the physio-biochemical responses of Sorghum-Sudangrass hybrids' seedlings in their early growth stage to salt stress.

Materials and methods

Plant materials

Seeds of two sorghum-sudangrass hybrids ("Aneto" and "Sugar graze") (*Sorghum bicolor* × *Sorghum sudanense* Stapf.) were utilized as plant material in this study. The seeds were obtained from the Field Crop Central Research Institute (Ankara, Turkey) and the Ulusoy Seed Company (Ankara, Turkey).

Seed surface sterilization, germination of seeds in in vitro conditions, in vitro application of salt stress

The sorghum (cv. "Aneto" and cv. "Sugar graze") seeds were kept in a 50% commercial bleach solution (ACE-Turkey, containing 5% sodium hypochlorite) for 20 min for surface sterilization and then rinsed thrice by sterile distilled water. The sterilized samples were planted into an MS (Murashige and Skoog) [18] basal medium containing 3% sucrose and 50 and 100 mM of sodium chloride (NaCl). It was solidified by 0.65% agar. All the cultures were kept under white fluorescent light (27 μmol m^{-2} s^{-1}) in a light-dark cycle of 16 h of light and 8 h of dark at 24 ± 1°C.

Biochemical analysis

Contents of chlorophyll and malondialdehyde (MDA) proline, and activities of antioxidative enzymes (CAT, SOD, GR and APX) were evaluated in the shoot tissues of 14-day-old sorghum seedlings (Figure 1).

Chlorophyll content

The protocol proposed by Curtis and Shetty [19] was used to determine the chlorophyll a, chlorophyll b and total chlorophyll compositions of the shoot of the plants on which different doses of NaCl were applied. By following the protocol, 50 mg of green material was added into 3 mL of methanol and stored at 23°C in darkness for 2 h to allow the chlorophyll in the green material to dissolve into the methanol. Then, the optic density (OD) of 1.5 mL of the liquid part (the chlorophyll-containing methanol) was determined at 650 and 665 nm using a spectrophotometer, and the chlorophyll a, chlorophyll b and total chlorophyll quantities were determined in units of "μg chlorophyll/g of fresh tissue".

Measurement proline and lipid peroxidation (MDA content)

Drawing on Lutts et al.'s [20] method, the malondialdehyde (MDA) contents were identifined. Five milliliter of trichloroacetic acid (TCA) (0.1%) was added onto 200 mg

Figure 1: The 14-day-old sorghum hybrids seedlings that exposed to salt (NaCl) stress (0, 50, and 100 mM) under *in vitro* condititons.

of green leaves as a specimen, and later, centrifuged at 12,500 rpm for around 20 min. Three milliliter of the supernatant was obtained from 5 mL of each extract. Three milliliter of 0.1% thiobarbituric acid mixed with 20% trichloroacetic acid (w/v) was complemented to the same amounts of all supernatants. The A-absorbance of the specimens was measured spectrophotometrically at 532 and 600 nm using a spectrophotometer.

Following the method described by Bates et al. [21], using 3% sulfosalicylic acid to break fresh plant specimens, a proline assay was performed. The ninhydrin reagent was put to the tubes that had the crushed specimens, and then, put in a water bath at the temperature of 100°C for almost an hour. Following a cool-down process, 4 mL of toluene was put into each specimen. The specimens were examined at 520 nm.

Antioxidant enzyme analyses

To find out the effects of the enzyme in plants under high-salinity conditions, almost 0.5 g of fresh shoot specimens in liquid nitrogen were powdered in porcelain mortars and homogenized with 8 mL of a 50-mM phosphate buffer solution, which had 0.1 mM of Na-EDTA (pH of 7.6). The homogenized specimens were centrifuged at 15,000 g for 15 min, and the precipitates that were formed were utilized in the enzyme analyses. The specimens were stored at +4°C up to the point the enzyme analyses were conducted. In enzyme measurements, the ultimate volume values were received by utilizing the buffer solution.

Superoxide dismutase (SOD) activity

Following the methods described by Çakmak and Marschner and Çakmak et al., we examined the superoxide dismutase (SOD) activity [22, 23] through the decrease of NBT (nitro blue tetrazolium chloride) by O_{2^-} in light. All mixtures were introduced to the reaction medium. 0.1 mM of Na-EDTA, including a 50 mM (pH: 7.6) phosphate (P) buffer. The enzyme extract (25 to 100 µL) before 0.5 mL of 50 mM Na_2CO_3 (pH of 10.2), 0.5 mL of 12 mM of L-methionine, 0.5 mL and 75 µM of p-nitro blue tetrazolium chloride (NBT) and 10 µm of riboflavin were all put into the medium. Hence, the ultimate volume of the medium reached 5 mL. The specimens were in light under light for around 15 min, and calculations were performed at the wavelength of 560 nm.

Ascorbate peroxidase (APX) activity

The ascorbate peroxidase (APX) activity was calculated [22, 23] by focusing on the oxidation of ascorbate at

290 nm (E = 2.8 mM cm^{-1}). The ultimate volume of the reaction medium was set at 1 mL by adding 0.1 mM of EDTA including a 50-mM phosphate buffer (pH of 7.6), 0.1 mL and 10 mM of EDTA including 12 mM of H_2O_2, 0.1 mL and 0.25 mM of L-ascorbic acid and enzyme extract within the medium. Afterwards, the ascorbate contents were was calculated spectrophotometrically at 290 nm.

Glutathione reductase (GR) activity

The glutathione reductase (GR) activity was calculated using the technique proposed by Çakmak and Marschner [22] and Çakmak et al. [23], and the oxidation process of NADPH at the wavelength of 340 nm (E = 6.2 mM cm^{-1}). The ultimate volume of the reaction medium was set at 1 mL by introducing 0.1 mM of EDTA that included a 50-mM phosphor buffer (pH of 7.6), 0.1 mL and 0.5 mM of oxidized glutathione (GSSG), 0.1 mL and 0.12 mM of NADPH and enzyme extract within the medium. The level of NADPH oxidation was calculated spectrophotometrically at the wavelength of 340 nm.

Catalase (CAT) activity

The catalase activity (CAT) was measured based on the degradation rate of H_2O_2 at 240 nm (E = 39.4 mM cm^{-1}) [22, 23]. In this enzyme analysis, the ultimate volume of the reaction medium was set at 1 mL through introducing 0.1 mM of EDTA which had a 50-mM phosphate buffer (pH of 7.6), 0.1 mL and 100 mM of H_2O_2 and enzyme extract within the reaction medium.

Statistical analysis

The experiment was conducted according to the "Completely Randomized Block Design" concept. One-way ANOVA (Analysis of Variance) was performed on each experiment, and the mean values were compared using Duncan's multi-range tests with the "SPSS for Windows" software.

Results

The responses of antioxidant enzymes (CAT, SOD, GR and APX) in the shoot tissues of 14-day-old seedlings of cv. "Sugar graze" are presented in Table 1. The activity of

Table 1: Physio-biochemical responses of 14-day-old seedlings of sorghum (cv. "Sugar graze") to salt (NaCl) stress.

NaCl concentration (mM)	Activity of antioxidant enzymes				Content of (µmol/g of fresh tissue)		Content of chlorophyll (µg/g fresh tissue)		
	CAT** (µmol min^{-1} mg^{-1} FW)	SOD* (U min^{-1} mg^{-1} FW)	APX** (µmol min^{-1} mg^{-1} FW)	GR** (µmol min^{-1} mg^{-1} FW)	MDA*	Proline**	a**	b**	Total**
0	155.35 ± 0.0^{a}	330.15 ± 2.5^{b}	50.89 ± 1.7^{a}	171.02 ± 1.2^{a}	13.08 ± 1.2^{a}	2.16 ± 0.4^{c}	224.99 ± 1.9^{c}	226.82 ± 2.4^{a}	327.94 ± 4.4^{c}
50	119.12 ± 1.8^{b}	154.41 ± 2.1^{c}	32.60 ± 2.1^{b}	157.68 ± 2.2^{c}	10.60 ± 1.0^{a,b}	9.03 ± 1.7^{b}	588.44 ± 2.9^{b}	160.02 ± 1.7^{c}	440.61 ± 3.6^{b}
100	58.20 ± 1.9^{c}	358.60 ± 6.7^{a}	38.89 ± 6.0^{b}	164.53 ± 4.5^{b}	9.91 ± 1.5^{b}	19.87 ± 4.1^{a}	699.23 ± 1.3^{a}	214.20 ± 1.5^{b}	546.72 ± 2.6^{a}

MDA, Malondialdehyde; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase. The values indicated in each column with a different letter were statistically significant at $^{**}p < 0.01$ and $^{*}p < 0.05$ levels. The values represent the mean ± SD calculated on the basis of results of three replicate experiments.

antioxidant enzymes was significantly ($p < 0.01$) affected by salt stress in cv. "Sugar graze" seedlings. Our results showed that CAT, APX and GR activities reduced by 2.67, 1.31 and 1.04-fold, respectively. Moreover, SOD activity increased by 1.08-fold in comparison to the control group with the highest degree (100 mM) of salt treatment (Table 1). The contents of proline and chlorophyll (chl. a, and total chl.) significantly ($p < 0.01$) increased by 9.16, 3.12, and 1.66-fold in the highest degree (100 mM) of salt stress treatment in comparison to the control group. Furthermore, compared the highest degree (100 mM) of salt treatment, MDA and chl. b contents were reduced by 1.31 and 1.05-fold, respectively.

The changing in antioxidant enzyme (CAT, SOD, GR and APX) activities of the shoot tissues of 14-day-old seedlings of cv. "Aneto" are shown in Table 2. The salt stress significantly ($p < 0.01$, and $p < 0.05$ for CAT) affected the antioxidant enzyme activities in the seedlings of cv. "Aneto". It was found that 1.31, 1.95, 1.96 and 1.04-fold increasing in CAT, SOD, APX and GR activities, respectively, in comparison to the control group with the highest degree (100 mM) of salt treatment (Table 2). In terms of MDA, proline and chlorophyll contents (chl. a, chl. b and total chl.), 1.30, 3.31, 1.96, 1.29 and 1.62-fold increases were, respectively, in comparison to the control group with the highest degree (100 mM) of salt treatment.

Table 2: Physio-biochemical responses of 14-day-old seedlings of sorghum (cv. "Aneto") to salt (NaCl) stress.

NaCl concentration (mM)	Activity of antioxidant enzymes				Content of (µmol/g of fresh tissue)		Content of chlorophyll (µg/g fresh tissue)		
	CAT* (µmol min^{-1} mg^{-1} FW)	SOD** (U min^{-1} mg^{-1} FW)	APX** (µmol min^{-1} mg^{-1} FW)	GR** (µmol min^{-1} mg^{-1} FW)	MDA*	Proline**	a**	b**	Total**
0	41.19 ± 2.0^{b}	151.36 ± 7.2^{b}	27.31 ± 6.7^{b}	170.48 ± 2.5^{b}	7.26 ± 2.2^{b}	1.71 ± 0.0^{b}	246.63 ± 4.8^{c}	108.08 ± 1.7^{b}	224.16 ± 0.7^{b}
50	54.28 ± 6.2^{a}	96.50 ± 4.0^{c}	52.22 ± 4.2^{a}	171.20 ± 0.8^{b}	17.17 ± 3.5^{a}	2.98 ± 0.1^{b}	303.00 ± 8.8^{b}	60.08 ± 4.5^{c}	145.49 ± 1.0^{c}
100	54.14 ± 1.6^{a}	295.61 ± 4.3^{a}	53.52 ± 0.9^{a}	177.68 ± 0.4^{a}	9.47 ± 2.6^{b}	5.67 ± 1.9^{a}	470.17 ± 11.7^{a}	140.67 ± 2.3^{a}	364.39 ± 5.1^{a}

MDA, Malondialdehyde; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase. The values indicated in each column with a different letter were statistically significant at $^{**}p < 0.01$ and $^{*}p < 0.05$ levels. The values represent the mean ± SD calculated on the basis of results of three replicate experiments.

Discussion

The influence of various contents of NaCl (50 and 100 mM) was investigated on physio-biochemical changes in two sorghum-sudangrass (*Sorghum bicolor* × *Sorghum sudanense* Stapf.) hybrids' ("Aneto" and "Sugar graze") seedlings under in vitro conditions. The findings of present study show that the physio-biochemical responses of these hybrids to salt concentrations were different.

Activity of antioxidant enzymes

Abiotic stresses (including salt stress) lead to harm in plants in part by stimulating excessive production of reactive oxygen species (ROS), which causes cellular damage and prevention of physiological functions in plants. Collection of ROS such as hydroxyl radicals ($HO^{\cdot}$) and singlet oxygen ($^{1}O2$) in the cell end up with oxidation of different biochemical substances: lipids, proteins, DNA and

RNA [24]. On the other hand, plants have developed antioxidative processes to survive ROS production and accumulation processes. One of such processes is enzymatic components including catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR).

When exposed to salt stress, the antioxidative enzyme system (CAT, SOD, APX, and GR) increased in comparison to the control group with the highest degree (100 mM) of salt treatment seemed to function better in cv. "Aneto" than in cv. "Sugarcane" (Tables 1 and 2). Meanwhile, the decreased activities of CAT, APX and salt-treated GR in cv. "Sugar graze" indicated that these enzymes might not have an indispensable part for scavenging superoxide radicals under salt stress. Similarly, decreases in the activity of some antioxidant enzymes such as SOD and CAT were shown by Reddy et al. [13] and Sundar et al. [25] in sorghum plants that were exposed to salt and water stress, respectively. Dugasa et al. [26] reported that SOD, APX and CAT activity increased in wheat that combined species of sorghum genotypes. Sen and Alikamanoglu [27] stated that functions of the antioxidant enzymes SOD, CAT and POD increased in salt stress conditions in all wheat varieties. Reddy et al. [13] noted that SOD, CAT and GR activity increased in transgenic sorghum under salt stress. Additionally, Elsawy et al. [28] observed that salt treatment causes elevated antioxidant enzyme (CAT, APX, sPOD, GR and SOD) activities in barley cultivars.

Mitigation of oxidative harm by scavenging ROS by utilizing antioxidant enzymes is a significant tactic of plants for raising their tolerance to stress [13]. Kholova et al. [29] reported that functions of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) rose depending on salt stress levels in maize genotypes. The results suggested that the CAT-SOD-APX-GR system protects cv. "Aneto" from oxidative damage under salinity stress conditions, while these protections systems (except, SOD) are invalid for cv. "Aneto". Elsawy et al. [28] observed that antioxidant activity in general was higher in tolerant cultivars than sensitive varieties in barley (*Hordeum vulgare* L.) under salt stress. Molazem and Bashirzadeh [30] stated that antioxidant enzyme activity such as SOD in salinity-resistant varieties of maize increased sharply. In this study, the results showed that antioxidant enzymes (CAT, SOD, APX and GR) seem to be more active in cv. "Aneto" than in cv. "Sugar graze" under salt stress. Therefore, it may be speculated that cv. "Aneto" might be a salt tolerant hybrid due to a strong antioxidant mechanism.

Contents of proline, MDA and chlorophyll

Among the osmoprotectants, proline (Pro) is one of the more efficient osmolytes used for improving salt tolerance in crops [31]. In this study, the results clearly showed that proline accumulation significantly ($p < 0.01$) increased in both hybrids' ("Aneto" and "Sugar graze") seedlings under salt stress. However, the accumulation of proline was greater in cv. "Sugar graze". Bavei et al. [32] reported that proline contents increase with the increasing salt stress in different sorghum varieties. Colmer et al. [33] stated that salt stress causes effects on the contents of proline in *Sorghum bicolor* (cv. "Hegari") seedlings. The findings that were reached here were also in parallel to those obtained by Luo et al. [34] and Wang et al. [35] who emphasized increased proline accumulation in maize that combined species of sorghum under salt stress. Proline also interacts with cellular macromolecules including substances like enzymes/proteins and stabilizes the structure and function of such substances under stressors' effects, including NaCl [13, 29, 36]. Therefore, it may be speculated that the high proline accumulation in the seedlings of cv. "Sugar graze" was due to low activity of antioxidant enzymes like CAT, APX and GR in salinity stress conditions. On the other hand, the low proline accumulation in the seedlings of cv. "Aneto" was due to high activity of all antioxidant enzymes.

The primary marker of oxidative stress in plants is MDA, which is a degradation product of polyunsaturated fatty acids of bio-membranes [37]. MDA is considered as a suitable marker for membrane lipid peroxidation. In this study, the results showed that the MDA contents increased in cv. "Aneto" while decreasing in cv. "Sugarcane" in comparison to the control group with the highest degree (100 mM) of salt stress (Tables 1 and 2). Du et al. [38] reported that proline contents had negative correlations with MDA contents under water deficits. Therefore, this may explain the decreasing MDA contents in the seedlings of cv. "Sugar graze" with high proline accumulation (Table 1). On the other hand, increasing the MDA contents in cv. "Aneto" seedlings might explain the insufficient degree of proline accumulation. Maswada et al. [39] stated that MDA contents increase in sorghum seedlings under salt stress (150 mM).

Photosynthesis is tightly associated with the yield of plants but prone to be influenced by salt stress. Decline of photosynthetic capacity prevalently occurred in plants under salt stress, but the underlying processes are complicated and not clear. In this study, interestingly, chlorophyll contents (except, chl b. in cv. "Sugar graze") increased in both hybrid leaves under salt stress (Tables 1 and 2).

Shah et al. [40] reported that salinity-induced increases in pigment contents were enhanced in wheat. On the other hand, Yan et al. [16] reported that during salt treatment with 50 and 150 mM NaCl, the photosynthetic rate (Pn) decreased in sorghum. However, several studies have shown that salinity stress decreased the concentrations of chlorophyll contents in maize that combined species of sorghum under salt stress [29, 30]. The reasons for increased chlorophyll content in seedling might be as a part of the adaptive mechanism of sorghum hybrids. In this study, the chlorophyll contents seemed to have a higher in cv. "Aneto" than in cv. "Sugar graze" under salt stress. Therefore, it might be speculated that cv. "Aneto" is a more tolerant hybrid against salt stress.

Conclusions

In this study, the two sorghum-sudangrass hybrids reacted differently to different concentrations of NaCl. According to physio-biochemical responses, cv. "Aneto" seemed to be more tolerant to oxidative damage provoked by salt stress than cv. "Sugar graze." Based on the results of this study, SOD, proline and chlorophyll contents seem to be reliable physio-biochemical parameters in determining tolerant or susceptible hybrids in sorghum plants under salt stress.

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