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Genotypic Divergence, Photosynthetic Efficiency, Sodium Extrusion, and Osmoprotectant Regulation Conferred Salt Tolerance in Sorghum

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ABSTRACT

Salt stress is one of the major limitations to modern agriculture that negatively influences plant growth and productivity. Salt tolerant cultivar can provide excellent solution to enhance stress tolerance with plant fitness to unfavorable environments. Therefore, this study was aimed to screen salt tolerant sorghum genotypes through evaluating of different morphological, biochemical, and physiological attributes in response to salinity stress. In this study, we have been evaluated total six sorghum genotypes including Hybrid sorgo, Debgiri, BD-703, BD-706, BD-707, and BD-725 under salt stress (12 dS m⁻¹ NaCl). The response variables included length and weight of root and shoot, root: shoot ratio (RSR), photosynthesis (*A*), transpiration rate (*E*), elemental concentrations (K⁺, Na⁺ and K⁺/Na⁺), photochemical efficiency of photosystem II (*F_v*/*F_m*), water use efficiency (WUE) and pigment content (chlorophyll a, and b). The results revealed that saline environment significantly reduced all response variables under study of sorghum genotypes, however, Hybrid sorgo remained unmatched by recording the maximum root and shoot traits. The same genotype recorded higher photosynthetic efficiency which was attributed to Na⁺ extrusion, K⁺ uptake and higher K⁺/Na⁺ ratio (1.8 at stress), while these mechanisms were not fully active in rest of genotypes. Moreover, this study also implies the involvement of proline in imparting tolerance against saline environment in Hybrid sorgo genotype. Overall, BD-703 remained the most salt sensitive genotype as evident from the minimum morphological growth traits and the least biosynthesis of



osmoprotectants. These findings open new research avenues for salt stress alleviation by identifying elite salt-tolerant genotypes of sorghum for breeding programs.

KEYWORDS

Salinity tolerance; sorghum; photosynthesis; proline; ion accumulation; genotypic variance

1 Introduction

The addition of excess sodium salts (e.g., NaCl, NaSO₄, NaHCO₃, etc.) to agricultural soils is a major threat to agricultural production that inhibits plant growth and declines yield [1]. About 6% of the world's soil and 20% of irrigated soil are salt-affected, which is expected to double by 2050 [2]. The agricultural soil is becoming more saline due to the addition of a disproportionate amount of several water-soluble salts. Salts of different compounds such as sodium, potassium, magnesium, calcium, sulfates, carbohydrates, bicarbonates, and chlorides are the primary cause of soil salinization [3–5]. Among them, the critical factor is several forms of sodium salts (NaCl, NaSO₄, Na₂CO₃, and NaHCO₃) primarily deposited to the soil by sodium-containing fertilizers and irrigation water [3–5]. The SS impairs a series of physiological processes and induces numerous molecular alterations in crop plants [6,7]. A few of the most pronounced salinity-induced alterations include inhibition of stomatal regulation, malfunctioning of photosynthetic machinery, and electron transport chain, which reduce the photosynthesis process and hamper plant growth [8,9]. More importantly, a higher accumulation of Na⁺ in the cytosol is responsible for creating toxicity in cells [3]. Additionally, the excessive accumulation of Na⁺ leads to disruption of metabolic processes such as osmotic imbalances and synthesis of reactive oxygen species (ROS) in crop plants. These metabolic disruptions lead to cellular injury, lipid peroxidation, oxidative stress, and even plant death [7,10].

To cope with SS induced disruptions, plants accumulate several osmoprotectants (proline, soluble sugar, and glycine betaine, etc.) [11–14], which act as defense molecules for enhancing plants' acclimation through osmotic homeostasis. Moreover, plants have evolved antioxidant defense systems that protect from cellular injury and lipid peroxidation under saline conditions [10]. Furthermore, plants tend to throw out sodium ions (Na⁺) from cells to escape from salt toxicity which is known as the ions extrusion mechanism [7]. Plants which exhibit these potential physiological adaptations during salt stress are salt resistant, so it is necessary to consider these physiological adaptations during the screening of crop genotypes for getting higher salt resistance. Thus, to ensure food security for the ever-increasing population, it is necessary to select crop genotypes based on their adaptability potential for countering the deleterious effects of soil salinity.

Sorghum [*Sorghum bicolor* (L.) Moench] is a coarse grain crop of the Poaceae family that is being cultivated widely in warm, arid regions under both irrigated and rainfed conditions [15–22]. Although it can tolerate soil salinity up to 6.8 dSm⁻¹ [23], higher salinity levels reduce its yield by 16%–63% [24]. Previously, it has been suggested to explore the genotypic variations in sorghum for screening out tolerant genotypes [25–28]. The research gaps exist pertaining to sorghum genotypes' response in terms of morphological, physiological, and biochemical alterations under saline conditions. Thus, it was hypothesized that sorghum genotypes might respond differently to induced SS, and different morphological and physiological traits might assist in screening out the salt-tolerant genotypes. Moreover, it is expected that salt-tolerant sorghum genotypes may be grown in bare lands, which are fallow due to high soil salinity and may contribute to the global food security. Sorghum is the 5th most cultivated grain crop in the world. Drought tolerance efficiency is highly recognized compared to salt. However, sorghum

cultivation is effective in saline-affected areas, though it has not yet been well established. Therefore, screening of salt-tolerant efficient genotypes will encourage us to enhance salt tolerance in sorghum through an appropriate breeding program. Therefore, this study was designed to screen out salt-tolerant sorghum genotypes from the pool of promising high-yielding cultivars based on different morphological, biochemical, and physiological attributes in response to salinity stress. We found that the Hybrid sorghum genotype showed better salt adaptation against salt stress. This study would be helpful to the sorghum breeders and farmers for improving agricultural traits and enhancing salt stress tolerance in sorghum.

2 Materials and Methods

2.1 Plant Materials and Growth Conditions

The trial was conducted at the Department of Crop Botany, Bangladesh Agricultural University, Bangladesh. The planting material included viable seeds of sorghum genotypes (Hybrid sorgho, Debgiri, BD 703, BD 706, BD 707, and BD 725). Among the six genotypes, Debgiri and Hybrid sorgho were collected from India and Japan, respectively, while the rest were collected from the seed bank of Bangladesh Agricultural Research Institute (BARI). The experiment was conducted in a completely randomized design (CRD) with three replications. Sorghum seeds were surface-sterilized with a 5% NaOCl (sodium hypochlorite) solution and then rinsed thrice with distilled water. The seeds were placed into a net for germination for four days. Thereafter, sorghum seedlings were transferred to the plastic box (area $70\text{ cm}^3 \times 24\text{ cm}^3 \times 23\text{ cm}^3$ having the size of 32 L) containing a hydroponic system containing a perforated styrofoam tray ($9 \times 3 = 27$ holes) (Fig. 1). Pre-germinated 5-day-old seedlings were planted into the holes of the tray and supplied with the half-strength Hoagland's nutrient solutions [29] containing 1 mM K_2SO_4 , 0.2 mM KH_2PO_4 , 2 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 μM H_3BO_3 , 200 μM Fe-EDTA, 2 μM $\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$, 0.3 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.5 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.01 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$. The pH of the hydroponic solution was maintained at 6.0. The sorghum seedlings were cultivated in a control growth chamber having a constant temperature of 25°C , white fluorescent light ($300\text{--}350\ \mu\text{mol m}^{-2}\text{ s}^{-1}$) for 16 and 8-h dark cycles, and 60%–65% relative humidity. The 4-week-old seedlings were exposed to NaCl stress (Electrical Conductivity value of $12\ \text{dS m}^{-1}$), while the control treatment was also maintained in a standard nutrient solution (control). The salt treatment was terminated after a week. In order to remove salt ions from the root surface, the plant roots were washed properly with distilled water. The samples of control and salt-treated sorghum plants were harvested separately and kept at -80°C until further analyses.

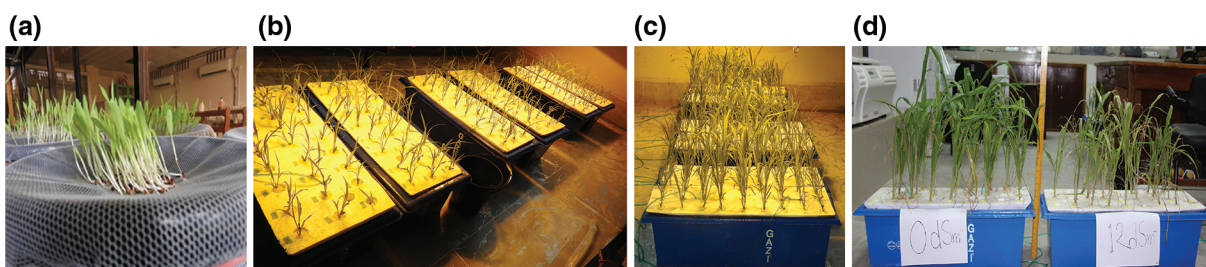


Figure 1: Laboratory arrangements of (a) sorghum seedlings, (b) set up of the hydroponic system, (c) growth condition of the sorghum seedlings inside the growth chamber, and (d) comparison of plants growing in controlled and stressed conditions

2.2 Morphological Parameters

The length (mm) of roots (RL) and shoots (SL) were recorded using a ruler scale, while fresh weights (g) of roots (RFW) and shoots (SFW) were taken immediately using a precision digital balance. The plant samples were dried in electronic oven at 70°C for 72 h, and the root dry weight (RDW) and shoot dry

weight (SDW) was also recorded. The total fresh weights (TFW) were obtained by aggregating RFW and SFW. The root-shoot dry weight ratio (RSR) was calculated using the following formula:

$$RSR = \frac{RDW}{SDW} \quad (1)$$

2.3 Photosynthesis Indices and Water Use Efficiency

The photosynthesis indices were recorded using a photosynthetic machine (LCi-SD photosynthetic system, ADC BioScientific, Ltd., Hertfordshire, UK). The gaseous exchange traits, including A (rate of photosynthesis), g_s (stomatal conductance), and E (rate of transpiration) were measured at $350 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD in ambient temperature. For A determination, the leaves' internal CO_2 level was maintained at 400 ppm. The topmost leaves were chosen for the assessment in all growth conditions. The instantaneous water use efficiency (WUE_{ins}) was obtained by following the protocol of Huang et al. [30]. The maximal photochemical efficiency of photosystem II (F_v/F_m) was recorded in every genotype. The F_v/F_m was recorded in sorghum leaves using a fluorometer (Pocket PEA, Hansatech, Norfolk, UK), and the data were recorded from control and salt-treated leaves. The plants were adapted at dark for 30 min prior to data recording. The Pocket PEA was used to quantify the F_v/F_m at saturated $3500 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD [31].

The measurement of leaf chlorophyll content (Chl a, b) from control and salt-treated plants was performed. Fifty milligrams of fresh leaves tissue were homogenized with 10 mL of 80% acetone assay [32], which was incubated at 65°C for 30 min in the dark. The absorbance of the solution was measured using UV-vis spectrophotometry (DR6000, Hach, Germany). The readings of the solution were taken at 647 and 663 nm, respectively. Total Chl (a+b) was calculated as the sum of Chl a and b, while the ratio of chlorophyll a and b (Chl a/b) was also estimated.

2.4 Ion Concentration

The ions concentration was determined according to standard protocols [33]. Sorghum seedlings' roots and shoots were separated and washed thrice with Milli-Q water to remove salt from the surface. Plant samples were dried in an oven for 72 h at 70°C and subjected to digestion using a solution ($\text{HClO}_4/\text{HNO}_3$, 1:3 v/v) [34]. The elemental concentrations (K^+ , Na^+) were measured by atomic absorption spectrophotometer (Perkin-Elmer 2380, Perkin Elmer, Rutherfordton, USA).

2.5 Proline Accumulation

The proline accumulation was estimated according to the standard methods [3,35] by taking 50 mg plant tissues which were homogenized using 10 mL of 3% sulfosalicylic acid and centrifuged at 10,000 rpm for 10 min. The supernatant, acid ninhydrin, and glacial acid were mixed 1:1:1 in a new tube and incubated at 95°C water bath for an hour, and subsequently transferred in the tubes in an ice bath for 5 min. Toluene was supplemented to the mixture and shaken well. Two phases (aqueous and oil) were developed in the mixture, and the solution from the upper phase was measured using UV-vis spectrophotometric (DR6000, Hach, Germany); absorbance was taken at 520 nm. The proline content was calculated using L-proline by following the standard method.

2.6 Screening of Salt-Tolerant Genotypes

The salt-tolerant genotype was screened using the STIs of each trait were calculated according to the method [36] using the following formula:

$$STI = \frac{Dc \times Ds}{(Xc)^2} \quad (2)$$

where, D_c = data of a genotype belonging to the control environment; D_s = data of the same genotype belonging to the stress environment, and X_c = average data of genotypes belonging to the control environment.

2.7 Heatmap and Principal Component Analysis (PCA)

To determine the response patterns of different physiological traits of six sorghum genotypes, the standardized STI values were used to construct a two-way hierarchical clustering heat map using the open-source statistical package *Complex Heatmap* in the R program (R version 4.1.3). The principal component analysis (PCA), biplot, and correlation–matrix scatter plot were generated using the R program.

2.8 Statistical Analysis

The MINITAB software (Minitab version 20) was used to compare the treatment means, while data were subjected to two–way (genotype and salt stress) and one–way (genotype with STI values) analysis of variance (ANOVA) with a significant level of $p < 0.05$. All the results were presented as mean value \pm standard error of three replications.

3 Results

3.1 Morpho-Physiological Traits

Salt stress-induced phenotypic variations in the root (RL) and shoot (SL) length, and their fresh (RFW and SFW) and dry (RDW and SDW) weights among the six sorghum genotypes have been demonstrated in Fig. 2. All genotypes regulated the morpho-physiological traits in response to salinity compared to control plants (Table 1). The genotype (G) and stress (S) interactions were highly significant for most of the morpho-physiological traits except the F_v/F_m and stomatal conductance (Table 1). However, the Hybrid sorgo genotype remained unmatched in mitigating salt stress compared to other sorghum genotypes. After salt treatment, RL and SL showed significant variation between the control (414.1 and 602.0 mm, respectively) and salt-stressed (346.3 and 558.3 mm, respectively) genotypes (Fig. 2). The RL of 337.1 and 455.7 mm for BD-703 and Debgiri, respectively, under control conditions, while the corresponding figures for BD-703 and Hybrid sorgo were 271 and 411 mm, respectively (Table 2). Similarly, SL ranged from 559.9–658 mm under the control condition and 522.2–610 mm under the stress condition (Table 2). Moreover, the lowest reduction of RL and SL due to salt stress was found in Hybrid Sorgo, and they are 9.8% and 2.94%, respectively.

The salt stress negatively affected SFW and RFW (Table 1) as salt-stressed plants produced a significantly lower SFW (4.95 g) and RFW (2.8 g) compared to control plants (10.2 and 4.03 g, respectively) (Fig. 2). The maximum SFW was observed in Hybrid sorgo (6.27 g) and minimum in BD-706 (3.39 g), while RFW was maximum in Hybrid sorgo (3.84 g) and minimum in BD-707 (1.86 g) under SS condition (Table 2). Again, the mean values of SDW and RDW under stress conditions (0.74 and 0.17 g, respectively) were significantly lower than the control plants (1.25 and 0.31 g, respectively) (Fig. 2). The SDW and RDW ranged from 0.55–0.95 g and 0.09–0.28 g under salt-stressed conditions (Table 2). The TFW, TDW, and RSR were significantly affected by salinity compared to control plants (Table 1). The average TFW, TDW, and RSR of each genotype were substantially higher in control plants (14.2, 1.57, and 0.25 g, respectively) in comparison to the stressed plants (7.75, 0.91, and 0.22 g, respectively) (Fig. 2). The TFW, TDW, and RSR showed significant variations, while the TFW and TDW were maximum in Hybrid sorgo (10.1 and 0.28 g, respectively) and minimum in BD-707 (5.54 and 0.09 g, respectively) under SS (Table 2). The RSR showed a similar fashion as RSR ranged from 0.19–0.34 under the control condition, while their values were 0.17–0.30 under the SS condition (Table 2).

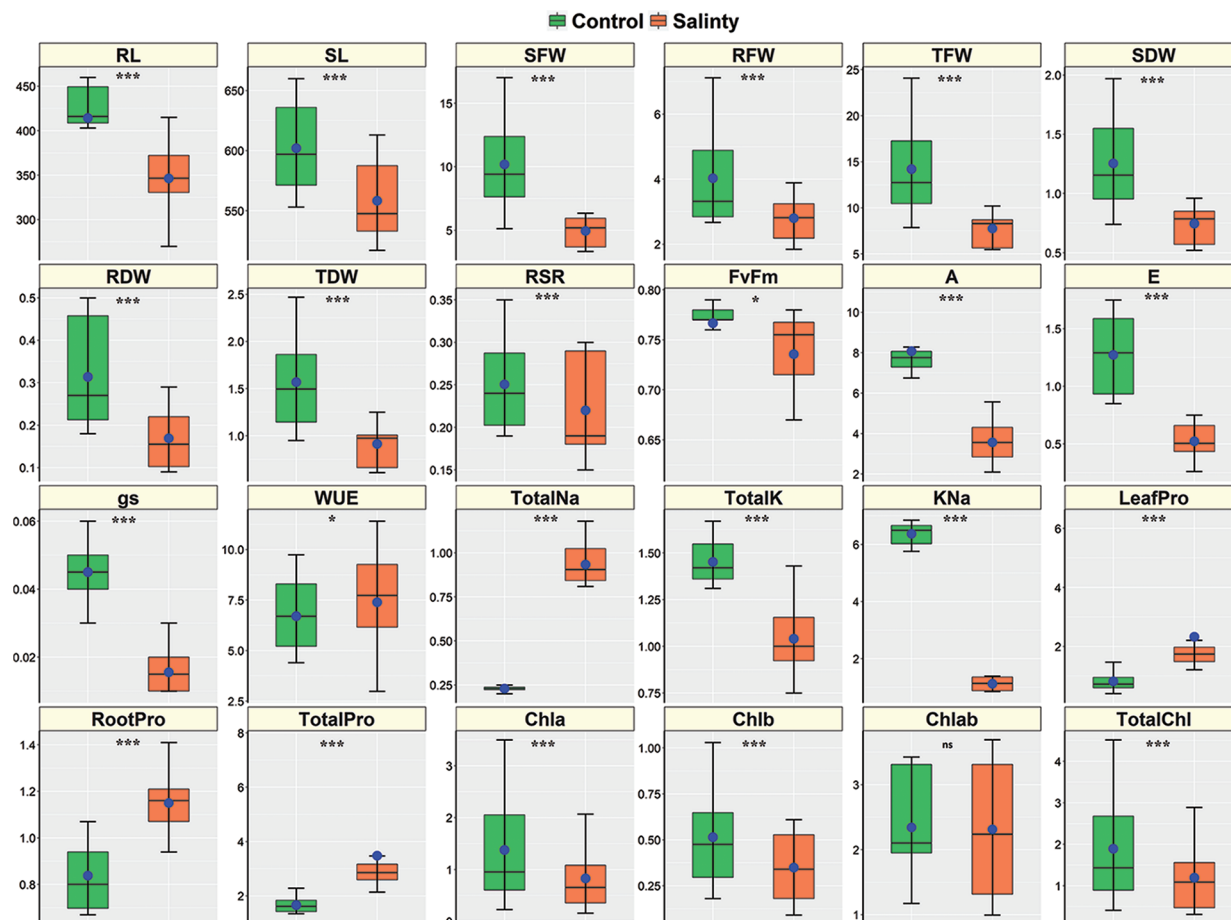


Figure 2: Boxplots demonstrating the descriptive statistics of morpho-physiological and biochemical traits of 6 sorghum genotypes grown in control and salt conditions under a hydroponic system. The treatment means are denoted with blue dots, while the medians are characterized by the white and thick horizontal box dividing lines. The higher and lower whiskers and the box boundaries of ups and down denote the Q3 (75th percentile), Q1 (25th percentile), maximum (Q1+1.5 IQR), and minimum (Q1-1.5 IQR) values, respectively. IQR denotes Interquartile Range. *, *** and ns denote significant variation between treatments at 5%, 0.1% levels of probability, and non-significant, respectively

Table 1: Analysis of Variance (ANOVA) for various studied traits in six sorghum genotypes growing in control and saline environments

Parameters	Abbreviations	Sources of variation			STI
		Genotype (G)	Stress (S)	G × S	
Root length	RL	***	***	***	***
Shoot length	SL	***	***	***	***
Shoot fresh weight	SFW	***	***	***	***
Root fresh weight	RFW	***	***	***	***
Total fresh weight	TFW	***	***	***	***

(Continued)

Table 1 (continued)					
Parameters	Abbreviations	Sources of variation			STI
		Genotype (G)	Stress (S)	G × S	
Shoot dry weight	SDW	***	***	***	***
Root dry weight	RDW	***	***	***	***
Total dry weight	TDW	***	***	***	***
Root Shoot Ratio	RSR	***	***	***	***
F_v/F_m	F_v/F_m	ns	*	ns	ns
Photosynthesis rate	<i>A</i>	***	***	***	***
Transpiration rate	<i>E</i>	***	***	***	***
Stomatal conductance	g_s	***	***	ns	ns
Instantaneous WUE	WUE_{ins}	***	*	***	***
Chlorophyll a	Chl a	***	***	***	***
Chlorophyll b	Chl b	***	***	***	***
Total chlorophyll	TotalChl	***	***	***	***
Chlorophyll a/b	Chl ab	***	ns	***	***
Na ⁺ content	Na ⁺	***	***	***	*
K ⁺ content	K ⁺	***	***	***	***
K ⁺ /Na ⁺	K ⁺ /Na ⁺	***	***	***	***
Leaf proline	LeafPro	***	***	***	***
Root proline	RootPro	***	***	***	ns
Total proline	TotalPro	***	***	***	***

Note: *, **, and *** denote 5%, 1%, and 0.1% levels of probability. And nsdenotes non-significant.

Table 2: Effects of salinity on growth attributes of six sorghum genotypes exposed to control and salt stress

Genotypes	Salinity (dS m ⁻¹)	RL (mm)	SL (mm)	SFW (g)	RFW (g)	SDW (g)	RDW (g)	TFW (g)	TDW (g)	RSR (g)
H. Sorgo	0	455.7 a	607.9 c	11.24 c	3.72 d	1.34 c	0.46 b	14.96 c	1.80 b	0.34 a
	12	411.0 b	590.0 d	6.27 e	3.84 c	0.95 d	0.28 d	10.11 e	1.23 c	0.30 b
Debgiri	0	452.3 a	658.0 a	16.94 a	7.09 a	1.95 a	0.49 a	24.03 a	2.44 a	0.25 c
	12	373.8 d	610.0 c	5.18 g	3.25 e	0.76 f	0.22 e	8.43 g	0.98 e	0.30 b
BD 703	0	337.1 de	568.3 ef	7.67 d	2.93 f	0.96 d	0.22 e	10.60 d	1.18 cd	0.23 d
	12	271.0 f	522.2 g	5.20 g	2.90 f	0.82 e	0.16 g	9.10 h	0.98 e	0.19 ef
BD 706	0	409.0 c	559.9 f	7.62 d	2.83 fg	0.97 d	0.18 f	10.45 d	1.15 d	0.19 ef
	12	330.0 e	534.9 g	3.39 i	2.18 i	0.55 g	0.10 h	5.57 j	0.65 f	0.19 ef
BD 707	0	417.2 b	581.3 de	5.18 g	2.70 h	0.75 f	0.22 e	7.88 i	0.97 e	0.29 b
	12	343.7 de	536.2 g	3.68 h	1.86 j	0.55 g	0.09 h	5.54 j	0.64 f	0.18 f
BD 725	0	413.4 b	636.7 b	12.38 b	4.90 b	1.55 b	0.31 c	17.28 b	1.86 b	0.20 e
	12	348.2 d	556.3 f	5.95 f	2.76 gh	0.85 e	0.15 g	8.71 f	1.00 e	0.18 f

Note: H. Sorgo denotes Hybrid Sorgo. Different letters on treatment means represent the significant difference at 5% levels of significance.

3.2 Photosynthesis, Stomatal Conductance, and Transpiration

Salt stress considerably declined the rate of photosynthesis (A) and rate of transpiration (E) in all sorghum genotypes (Fig. 3). However, the decrement of stomatal conductance (g_s) was not statistically significant in all the genotypes. The genotype Hybrid sorgho remained superior in terms of photosynthesis parameters under study. The reduction rate of photosynthesis due to salt stress was minimum in Hybrid sorgho, which is 39.8%. The performance of g_s in Hybrid sorgho was also better compared to the rest of the genotypes (Fig. 3b). However, the E rate was significantly higher in Debgiri under salt stress, while BD-703 showed the lowest E rate under SS (Fig. 3b).

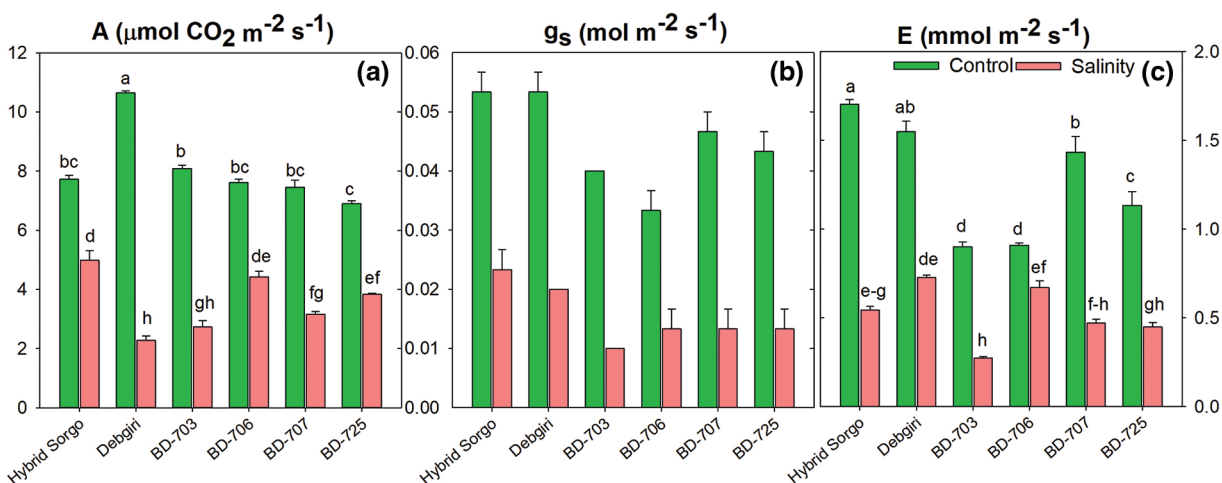


Figure 3: The (a) rate of photosynthesis (A ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), (b) stomatal conductance (g_s ; $\text{mol m}^{-2} \text{ s}^{-1}$), and (c) rate of transpiration (E ; $\text{mmol m}^{-2} \text{ s}^{-1}$) of six sorghum genotypes growing in control and salt-stressed conditions. Vertical bars on top of each bar are SEM ($n = 3$). Different letters on treatment means represent the significant difference at 5% levels of significance. The parameters which lack letters represent non-significant

3.3 Quantum Yield of PSII (F_v/F_m) and Water Use Efficiency (WUE)

The rate of F_v/F_m and WUE were differentially regulated under salt stress in sorghum genotypes. The F_v/F_m level was significantly regulated in Debgiri, BD-703, and BD-725, while no considerable variation was observed in the rest of the genotypes (Fig. 4a). The WUE was higher in Hybrid sorgho and BD-703 than in the rest of the sorghum genotypes (Fig. 4b). Under salt stress, the WUE was increased in Hybrid sorgho than in control, and Hybrid sorgho showed 44.4% higher WUE than the control condition.

3.4 Alteration of Leaf Pigments During Salt Stress

The leaf pigments content was significantly reduced in all genotypes due to salinity as leaf Chl a, Chl b, Total Chl as well as Chl a/b ratio were significantly higher in non-stressed plants compared to salt-stressed plants (Fig. 5). Significant genotypic variations were observed in Chl a, Chl b, total Chl, and Chl a/b among the genotypes because Chl a level severely declined in BD-703, while Hybrid sorgho exhibited non-significant variation of Chl a level in the saline environment (Fig. 5a). The BD-706 genotype showed a significant reduction of Chl b than the other genotypes, while Hybrid sorgho showed no significant variation under control and SS conditions (Fig. 5b). The same pattern was observed for total Chl, which was the sum of Chl a and Chl b (Fig. 5c). The Chl a and Chl b ratios were differently regulated in sorghum genotypes. The lowest Chl ratio was found in BD-703 under SS, and it was oppositely altered between BD-706 and BD-725 (Fig. 5d). However, no significant difference was observed in Hybrid sorgho under SS and normal conditions.

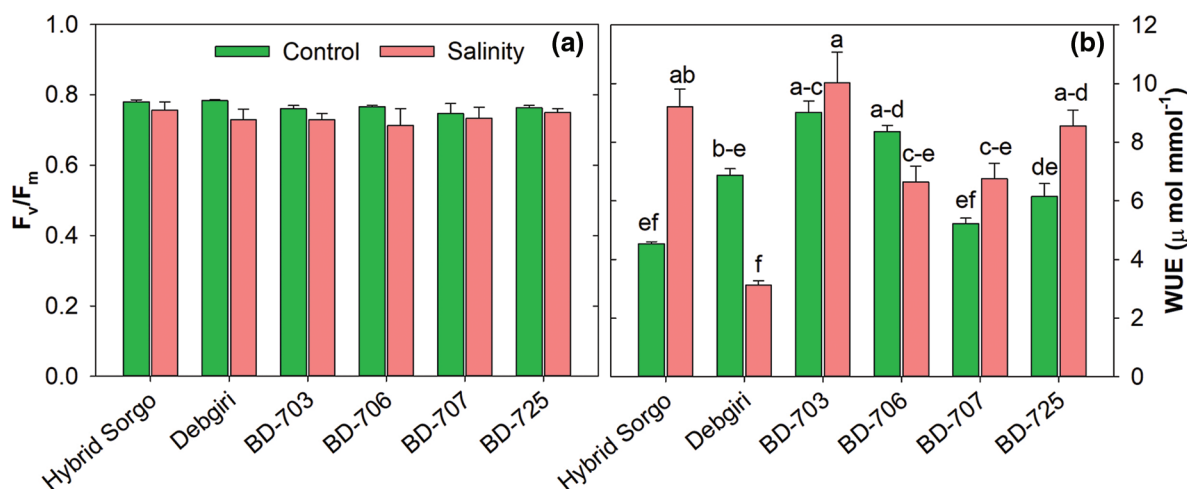


Figure 4: (a) Maximum photochemical efficiency of PS II (F_v/F_m) and (b) Instantaneous Water use efficiency (WUE_{ins} ; $\mu\text{mol mmol}^{-1}$) of six sorghum genotypes exposed to control and salt stress conditions. Vertical bars on top of each bar are SEM (n = 3). Different letters on treatment means represent the significant difference at 5% levels of significance. The parameters which lack letters represent non-significant

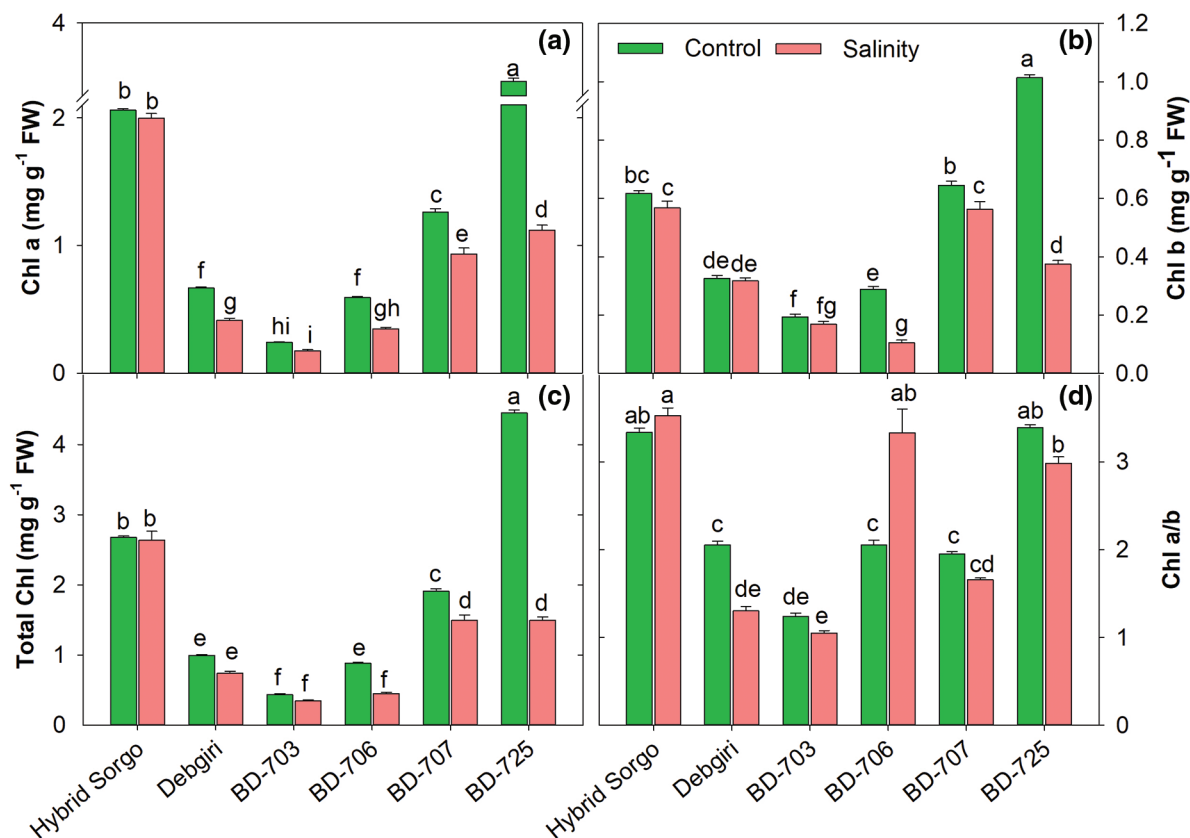


Figure 5: (a) The chlorophyll a (Chl a; mg g^{-1} FW), (b) chlorophyll b (Chl b; mg g^{-1} FW), (c) total chlorophyll (TotalChl; mg g^{-1} FW), and (d) ratio of Chl a over Chl b (Chl a/b) of six sorghum genotypes grown in control and salt stress conditions. Vertical bars on top of each bar are SEM (n = 3). Different letters on treatment means represent the significant difference at 5% levels of significance

3.5 Regulation of Ion Concentrations

The sodium (Na^+) and potassium (K^+) accumulations were differently altered during salt stress in sorghum genotypes (Fig. 3). The highest Na^+ concentration was found in BD-706 among the six sorghum cultivars (Fig. 3a). However, the Na^+ level was significantly lower in Hybrid sorgho compared to BD-706. The K^+ accumulation significantly increased in Hybrid sorgho under salt stress (Fig. 6b). The K^+/Na^+ ratio was higher in control plants than salt-stress plants (Fig. 6c). The K^+/Na^+ was highest, and almost similar accumulation patterns were observed in Hybrid sorgho and BD-703 under salt stress.

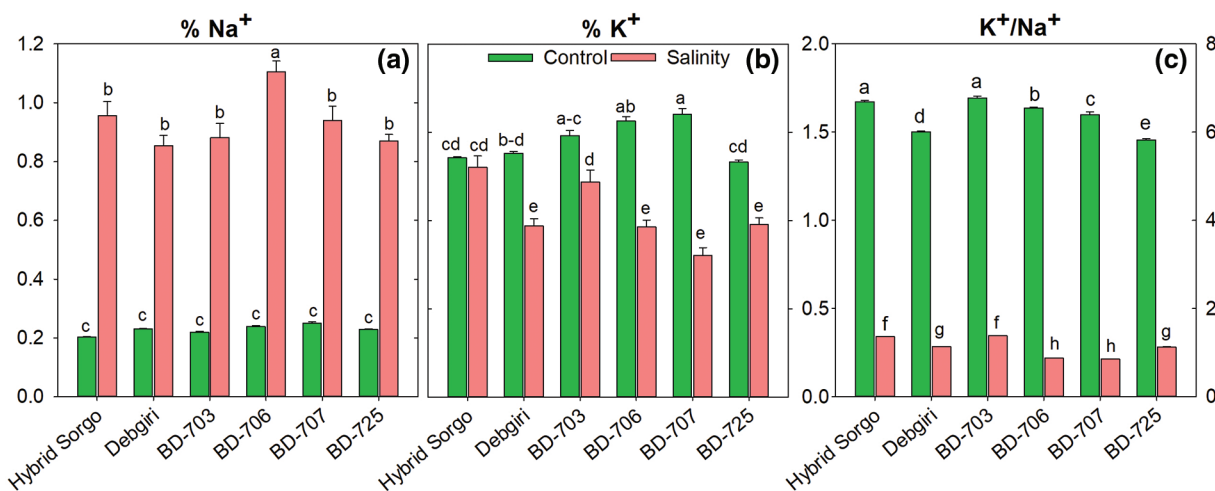


Figure 6: (a) The Na^+ concentrations (%), (b) K^+ concentrations (%), and (c) ratio of K^+ over Na^+ (K^+/Na^+) of six sorghum genotypes grown in two stress conditions (control and salt). Vertical bars on top of each bar are SEM ($n = 3$). Different letters on treatment means represent the significant difference at 5% levels of significance

3.6 Accumulation of Proline under Salt Stress

The proline concentration was significantly enhanced under salt stress compared to normal conditions (Fig. 7). The leaf proline accumulation was significantly higher in Hybrid Sorgho under SS (Fig. 7a). The root proline was considerably accumulated in Hybrid Sorgho, while the genotypes Debgiri, BD-707, and BD-725 showed a similar accumulation pattern in roots under SS (Fig. 7b). The highest total proline accumulation was found in the Hybrid Sorgho genotype (Fig. 7c).

3.7 Variations in Stress Tolerance Indices (STI)

The analyses of the hierarchical clustering heatmap of genotypes and several physiological traits were presented in Fig. 8. A total of 24 different traits were presented in the heatmap, in which the highest number of traits (total 15) showed up-regulation patterns in Hybrid Sorgho (Fig. 8a). The genotypes and observed parameters were clustered using STI, and the number of clusters was fixed by the gap statistic method before clustering. The hierarchical clustering heatmap of genotypes and traits, along with the dendrogram, is depicted in Fig. 8a. Six (6) sorghum genotypes were clustered into three row-clusters according to the existing variations in the different traits (Hybrid Sorgho in Cluster-2, BD-703 and BD-707 in Cluster-3, and the other three were in Cluster-1). Similarly, the parameters were clustered into three column-clusters where Cluster-1, Cluster-2, and Cluster-3 comprised 12, 4, and 8 traits, respectively (Fig. 8a). The highly linked parameters, for instance, Chl b, Chl a, TotalChl, RDW, RFW, SFW, SDW, RSR, TFW, F_v/F_m , A , and WUE_{ins} were assembled in Cluster-1; K^+ (TotalK), K^+/Na^+ (KNa), TDW and RootPro under Cluster-

2 and E, Chl a/b (Chlab), RL, LeafPro, TotalPro, g_s, SL and Na⁺ (TotalNa) were compiled in Cluster-3 (Fig. 8a).

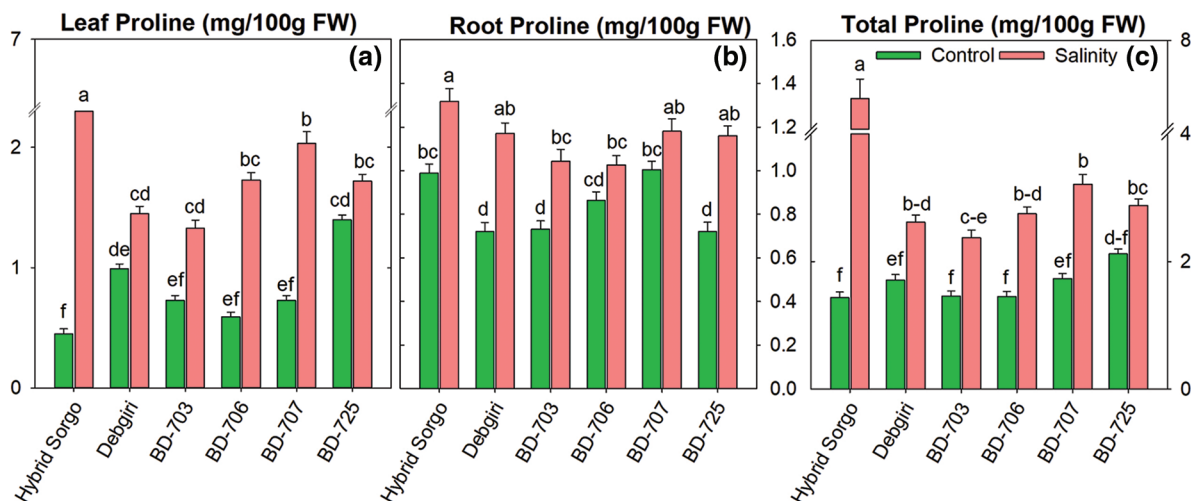


Figure 7: The (a) leaf proline (LeafPro; mg/100g FW), (b) root proline (RootPro; mg/100g FW), and (c) total proline (TotalPro; mg/100g FW) concentrations in sorghum genotypes grown in control and stress environments. Vertical bars on top of each bar are SEM (n = 3). Different letters on treatment means represent the significant difference at 5% levels of significance

According to the STI values, the genotype Hybrid sorgho in Cluster-2 showed a greater extent of salt tolerance, followed by Cluster-3 and Cluster-1 (Fig. 8b). Out of 24 traits, the lowest STI values for Na⁺ were considered as salt-tolerant, while the greater STI values in the other 24 parameters, including proline, were depicted as tolerance attributes. The STI values of TDW and root-Pro were higher in Cluster-1 and subsequently in Cluster-2 and Cluster-3 (Fig. 8b). Additionally, the maximum STI of K⁺ and K⁺/Na⁺ were observed in Cluster-2, followed by Cluster-1 and Cluster-3. The STI values of the leaf and total proline in the clusters were ranked as Cluster-2 > Cluster-3 > Cluster-1 (Fig. 8b). However, the heat map illustrated that the genotype under Cluster-2, such as Hybrid sorgho, exhibited higher salt tolerance compared to the genotypes belonging to Cluster-1 and Cluster-3 (Fig. 8b).

3.8 Principal Component Analysis

The PCA results exhibited that the leading two principal components (PCs) are responsible for 68.9% of the overall dissimilarity (Fig. 9). The PC1 and PC2 were considered to construct PCA-biplot. In PCA-biplots, PC1 exhibited 51.7% of the total variation, while PC2 exhibited 17.2% variability among the traits. The first biplot showed the variations and correlation between the treatments. Traits include Total Na⁺, root Pro, leaf Pro, and total Pro were nearly located in the same, which indicated a strong positive correlation and contribution to SS (Fig. 9a). The genotypic variance and/or correlations with the total 24 traits were also clarified. The genotypes Hybrid sorgho and BD-725 clustered in the same plot, while Debgiri, BD-725, and BD-707 were clustered in another plot (Fig. 9b). These results indicate a strong positive correlation between the Hybrid sorgho and BD-725 in terms of salt tolerance.

3.9 Correlation among the Traits

Most of the morpho-physiological and biochemical traits were correlated with each other (Fig. 10). However, the correlation levels were different among the traits. According to the results of Pearson's

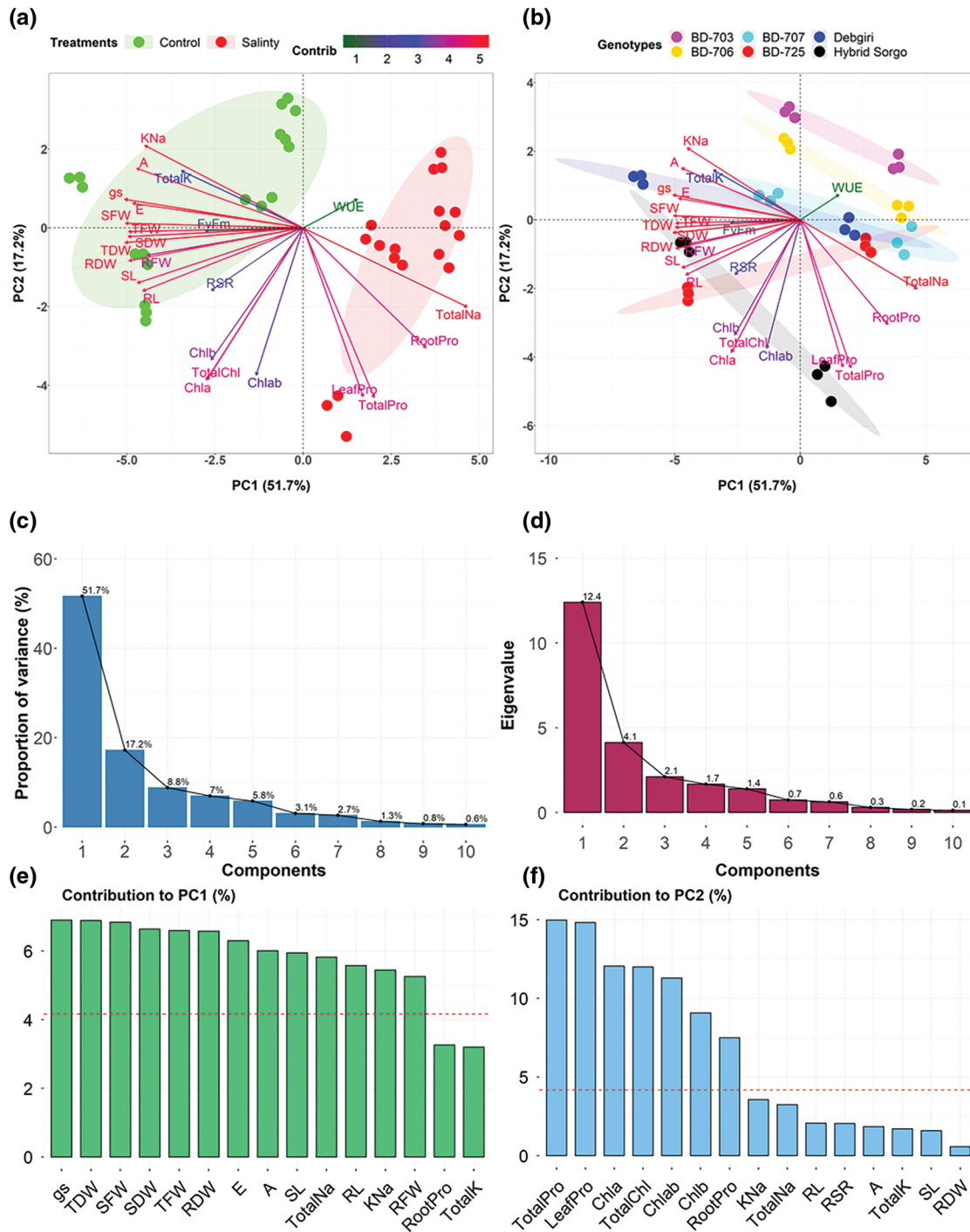


Figure 9: (a) Principal component analysis (PCA)-biplot of 6 sorghum genotypes based on the variance in 24 traits grown under stressed and non-stressed conditions. (b) Distribution of genotypic variance in different ordinates according to PC1 and PC2. (c) Proportion of variance (%) and (d) Eigenvalues of first 10 components derived from PCA. Contribution (%) of the top 15 observed traits to (e) PC1 and (f) PC2. In PCA, PC1, as well as PC2 described 51.7% and 17.2% of the total dissimilarity, respectively. Arrows indicate the strength of the trait influence on the first two PCs. The contribution of the traits to the first two components in PCA was denoted by the different color intensities and lengths of the arrows. The darker red and long arrows indicate a higher contribution, while the darker green and shorter arrows indicate a lower contribution of the variables

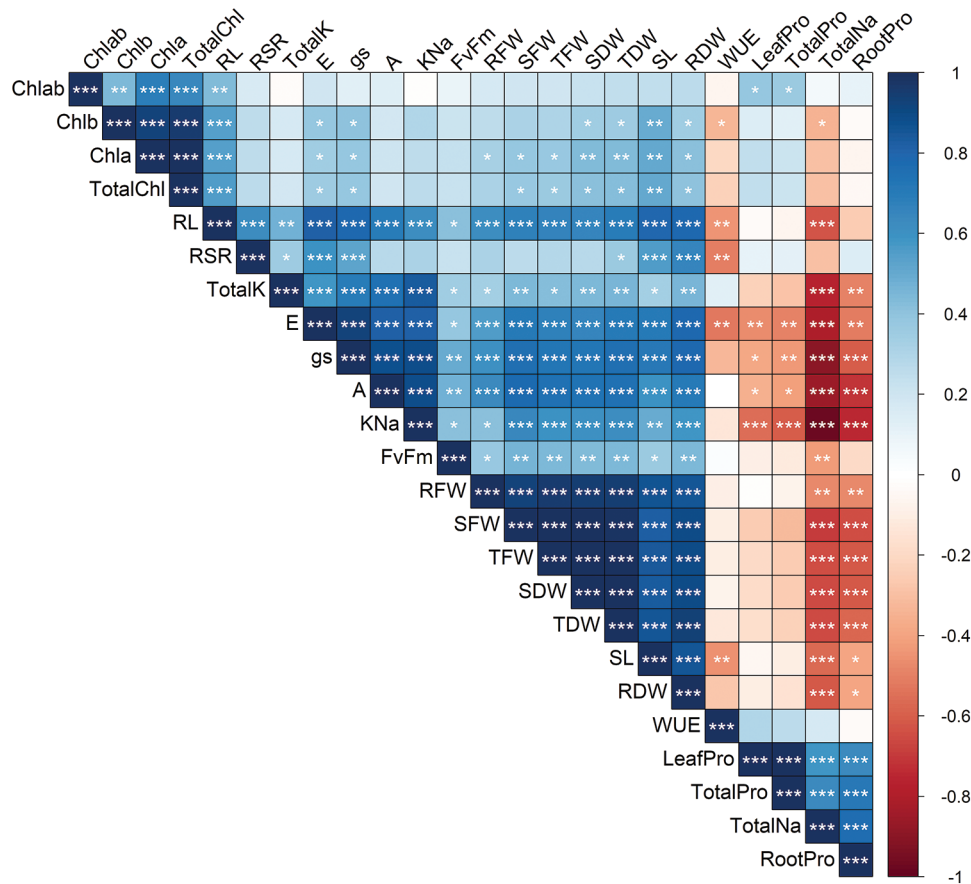


Figure 10: Correlation matrix of the measured 24 traits in six sorghum genotypes grown in control and salt stress conditions based on Pearson’s correlation analysis. Red and Blue shades specify negative and positive correlations, respectively. The higher the color intensity, the higher the correlation coefficient. Asterisks denote a significant correlation between traits, and *, **, and *** specify 5%, 1%, and 0.1% levels of significance, respectively. Insignificant ($p \geq 0.05$) correlations are specified by blank blocks

4 Discussion

The study was conducted to screen out the salt-tolerant genotypes of sorghum based on morphological, physiological, and biochemical traits, while the results revealed a combination of different mechanisms associated with salt-stress tolerance in sorghum. These research findings highlighted several morphological, physiological, and biochemical traits which were linked to salt tolerance.

4.1 Genotypic Variations, Robust Growth, and Photosynthetic Efficiency Confer Salt Tolerance

Our study exhibited Hybrid sorgho as the superior genotype that potentially maintained growth-related traits in a saline environment. This might be ascribed to the regulation of Chl in salt-stressed plants. A noticeable reduction of plant biomass was observed for the rest of the sorghum genotypes, confirming morphological inhibition as one of the adverse effects of SS. These findings are in concurrence with previously reported results where inhibition of growth attributes was closely linked to the sensitivity of crop plants to salt toxicity [3]. These findings also state that the salinity of NaCl salt gives rise to shortened growth attributes in stress conditions than control. These salinity-induced shortened growths might have happened to the declined cell elongation rate and a smaller number of elongated cells

[37–39]. Cell elongation in salinity might be hampered due to a decline in the transportation of crucial ions like NO_3^- as well as a higher rate of absorption of Na^+ ions in plants under elevated salt stress [29]. Furthermore, declined turgor pressure, in addition to the restricted availability of water to the cell, might be another reason for the declined fresh weight of the plant [40–42]. However, growth reduction was also reported to be the direct result of physiological damage under salt toxicity [43]. Likewise, our results regarding better morphological growth traits of salt-tolerant genotype (Hybrid sorgo) could also be explained by the regulation of the photosynthesis process under SS [7]. High photosynthesis and transpiration maintaining the ability of plants under SS has already been recognized as one of the most pronounced strategies of crop plants in order to offset the deleterious effects of the saline environment [44].

4.2 Sodium Extrusion

Although optimum accumulation of Na^+ results in the balancing of osmoregulation, however over-accumulation in the cytosol can be toxic to plants by inhibiting K^+ transport and disturbing K^+ in cells [45]. Salt-tolerant plants inhibit Na^+ or push it out from cells to mitigate salt toxicity [3]. Our study indicated that Hybrid sorgo seedlings accumulated low Na^+ and high K^+ , suggesting it is one of the key mechanisms of Hybrid sorgo for maintaining lower Na^+ that led to better adaptation under SS. These results are supported by previously reported conclusions whereby the extrusion of salt ions was declared responsible for imparting salinity tolerance in crop plants [46]. In addition, salt tolerance often depends on the ability to maintain K^+/Na^+ ratio through declining Na^+ and enhancing K^+ and in halophytes [47]. In this study, the ability of Na^+ and K^+ homeostasis in Hybrid sorgo enhanced adaptation to SS, while the rest of the sorghum genotypes showed severe morphological and photosynthetic disruptions. A recent study suggested that high Na^+ accumulation led to disruption of metabolic processes and reduced plant growth and productivity [3]. However, our findings have explicitly indicated that the mechanism of Na^+ ion exclusion was fully active in Hybrid sorgo, while the K^+ depletion (losses of K^+ from the tissue due to a high level of Na^+) hypothesis was applicable for the rest of the five sorghum genotypes.

4.3 Regulation of Osmoprotectants

In the saline environment, plants tend to accumulate several compatible solutes (proline, soluble sugar, glycine betaine, etc.), which protect cells from the adverse effects of osmotic stress [3,48]. Proline (Pro) serves as a beneficial safeguard for plants exposed to salinity stress by preventing cellular damages [49]. Besides contributing as an excellent osmolyte, Pro acts as an antioxidative defense molecule, metal chelator, and signaling molecule in salt-affected plants [6,50]. In this study, Pro accumulation was considerably higher in Hybrid sorgo than in the rest of the genotypes under SS. However, this observation suggests that a high concentration of Pro provided more support to Hybrid sorgo for coping with salt-induced osmotic stress, while the rest of sorghum genotypes were severely affected by salt-induced toxicity. These results indicate that 12 dS m^{-1} was above the salinity tolerance potential of the remaining five genotypes of sorghum which must have suffered oxidative damages.

Principal component analysis (PCA)-biplot is a multivariate analysis that facilitates the main characters for the selection of salt-tolerant genotypes by combining two dimensions (traits and objects) and minimizing variations [51]. The PCA revealed that the traits include TDW, g_s , SFW, SDW, TFW, RDW, E , A , SL, total Na, RL, KNa, RFW, TotalPro, LeafPro, Chl a, total Chl, and root Pro were correlated differentially among the genotypes. In this study, the correlation pattern disclosed a considerable and strong positive relationship between K^+/Na^+ and almost all of the traits except for Na^+ and proline concentrations (Fig. 10). Similarly, A , E , and g_s maintained a negative correlation with total Na^+ . Interestingly, similar trends were recorded for the total proline concentrations. Therefore, considering PCA and correlation analyses, the proline accumulation, ion concentration, and photosynthetic regulation-related traits might be effective as evidence for screening salt tolerant genotypes as suggested by a number of previous researches

[4,13,52,53]. Moreover, underlying mechanisms that enable crop plants to cope with the adverse effects of salinity by maintaining photosynthetic efficiency, salt extrusion, and biosynthesis of osmoprotectants need further research [26,54–57].

5 Conclusions

This work unveils the mechanistic basis of salt tolerance in sorghum. Differential regulations of morphological and physiological traits among the six sorghum genotypes indicated their sensitivity in response to salt stress. It was inferred that the Hybrid sorgho genotype surpassed the rest of the genotypes in terms of salt tolerance by maintaining morphological growth, physiological functioning, and photosynthetic activity in a saline environment. Additionally, significantly higher salt extrusion and K^+ uptake by Hybrid sorgho were attributed to higher salt tolerance. This study also implies the involvement of osmoprotectants (e.g., proline) in imparting tolerance against excessive salts. The traits associated with genotypic variation in salt stress tolerance provide meaningful information to breeders and farmers to improve sorghum production on saline soils. These findings open up new avenues for research into salt stress alleviation and help to screen elite genotypes for sorghum breeding programs. However, these combined insights may encourage us to set more suitable approaches for alleviating salt stress in sorghum and other plant species.

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