



Salicylic Acid Confers Salt Tolerance in Giant Juncao Through Modulation of Redox Homeostasis, Ionic Flux, and Bioactive Compounds: An Ionomics and Metabolomic Perspective of Induced Tolerance Responses

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Abstract

We investigated the stimulatory and/or inhibitory role of exogenous SA in alleviating the salt stress (250, 500 mM NaCl) in *Pennisetum giganteum* (Giant Juncao) through coordinated induction of redox homeostasis, ionic flux, and bioactive compounds. Salt stress radically impaired root and shoot (growth, fresh, and dry biomass as well as tolerance indices), leaf relative water content, and leaf chlorophyll *alb* ratio of Juncao due to higher Na⁺ and Cl⁻ accumulation followed by H₂O₂ generation, lipid peroxidation (MDA contents), and electrolyte leakage. However, the innate defense response of Juncao counteracted salt-induced damages by osmolytes accumulation combined with orchestrating antioxidants and ionic homeostasis mechanisms. Furthermore, the application of SA had an incremental impact on the development and productivity of high-salinity-exposed Juncao plants by increasing root length, plant biomass, tolerance indices, chlorophyll *alb* ratio, and protein contents. Furthermore, SA treatment considerably decreased Na⁺ and Cl⁻ toxicity by orchestrating antioxidant enzymes, ion transport, and secondary metabolism. Notably, the application of SA substantially mitigated the adverse effects of high salinity concentration (500 mM NaCl), owing to the simultaneous upregulation in enzymatic and non-enzymatic antioxidants, nutrient ion flux, alongside chlorogenic acid production. Thus, we concluded that SA enhanced the tolerance capability of Juncao plants in a NaCl concentration-dependent manner. The findings of this study will enable environmentalists and pharmacologists to gain dual farm-level benefits, including animal therapeutics and restoration of salinized soils for arable purposes.

Keywords Antioxidative defense systems · Bioactive compounds · Non-conventional crops · Saline agriculture · Salinity tolerance

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Introduction

Abiotic stressors are recurrently intensifying under the influence of geogenic (climate change, desertification, and seawater intrusion) as well as anthropogenic factors (rapid urbanization, industrialization, over-exploitation of agricultural inputs, and improper irrigation practices), posing huge threat to agricultural productivity, food security and socio-economic development worldwide (Chen et al. 2021; Jalil and Ansari 2019; Menhas et al. 2021). Among these stressors, salinity is unanimously accepted as one of the perilous environmental problems, negatively impacting plant's metabolic growth and development at both the physiological and biochemical levels and the capacity of the cropland, thus, limiting crop yields globally (Egamberdieva et al. 2019; Isayenkov and Maathuis 2019). Consequently, salinity is expected to result in over half of agricultural land loss by 2050 (Machado and Serralheiro 2017).

Furthermore, the catastrophic effects of salinity on plant developmental attributes are linked to hyperosmotic shock, hyperionic toxicity, and oxidative stress, eventually resulting in the cellular metabolic arrest and cell death (Abdelaal et al. 2020; Sharma et al. 2020). Meanwhile, higher salt concentration (in the growth media) compromises plant's coping mechanisms owing to a wide range of salinity-induced anomalies, including (a) oxidative damage to membranes and related cellular components via generation of reactive oxygen species (ROS) such as singlet oxygen ($^1\text{O}_2$), hydroxyl radical (OH), superoxide ($\text{O}_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) followed by lipid peroxidation, electrolyte leakage, protein denaturation, enzyme activity disruption, and DNA damage; (b) altered/unstable photosynthetic performance through stomatal or non-stomatal confines; (c) inhibition of photochemical routes by disturbing plant–water relationship and CO_2 assimilation, and (d) inadequate absorption and distribution of nutrients (particularly K^+ , Ca^{2+} , and Mg^{2+}), which regrettably leads to a substantial reduction in plant productivity (Ahanger et al. 2020; Kaya et al. 2020). Therefore, sustainable and integrative management practices are urgently needed to improve plant productivity and mitigate salinity-induced biotoxicity for addressing environmental sustainability concerns.

Nonetheless, state-of-the-art amelioratory technologies (such as microbial and molecular tools) for modulating plant growth and alleviating salinity-accrued harmful effects at the whole-plant level are not entirely practical and cost-effective (Shokat and Großkinsky 2019). Rationally, biostimulators, bioregulators, and/or phytohormones perform a crucial service in mediating plant cellular metabolism and conferring tolerance at the whole-plant

level under hostile conditions, ensuring self-modulation of growth (Nephali et al. 2020; Yu et al. 2020). Giant Juncao (*Pennisetum giganteum*; Family: Poaceae), a C4 perennial grass, is one of the major non-conventional crops in China and Africa, owing to its high ecological as well as agro-economic values, such as saline agriculture and simultaneous phytoremediation, bioenergy, biofertilizers, forage, mushroom cultivation, and production, among others (Hayat et al. 2020; Jia et al. 2020; Li et al. 2020). Likewise, Juncao has an intrinsic ability for yielding higher aboveground biomass, ROS detoxification, and halotolerance capability under extreme saline conditions, unlike low biomass halophytic and conventional glycophytic crops (Hayat et al. 2020). Although similar to other halophytic species of Poaceae (Adnan et al. 2021; Keshavarzi 2021), higher salinity levels (for longer-term in the growth media) have had inhibitory effects on the growth, development, and productivity of Juncao.

During the last couple of years, several biostimulants such as auxin (IAA) (Abdel Latef et al. 2021), abscisic acid (ABA) (Karimi et al. 2021), brassinosteroid (BR) (Alhaithloul and Soliman 2021; Rattan et al. 2020), cytokinins (CK) (Avalbaev et al. 2020), ethylene (Wang et al. 2020a), gibberellin (GA) (Wang et al. 2020b), melatonin (Kaya et al. 2020), methyl jasmonate (MeJA) (Alhaithloul and Soliman 2021), and salicylic acid (SA) (Hussain et al. 2021; Lamnai et al. 2021) ameliorated stress tolerance response, and/or adaptation mechanism as well as alleviated salinity-induced biotoxicity in diverse plant species. Salicylic acid (SA) is a phenolic-type ubiquitous phytohormone, an endogenous plant growth stimulator, and a multifaceted signaling molecule that performs paramount mediating roles in hormonal crosstalk, modulating a plethora of stress responses and adaptation mechanisms under environmental stressors, as well as reinforcing plant immunity via both local and systemic acquired resistance (SAR) against pathogenicity (Koo et al. 2020; Prakash et al. 2021).

The basic mechanisms underlying SA-induced regulatory/stimulatory effects on salinity-stressed plants entails: (1) enhancing growth rate, photosynthetic activity, and osmotic homeostasis maintenance by inducing osmolytes (proline and amines) accumulation, limiting K^+ leakage via a gated outwardly rectifying K (GORK) channel (Ghassemi-Golezani and Farhangi-Abriz 2018; Jayakanan et al. 2015); (2) intensification of enzymatic and non-enzymatic antioxidant activities followed by membrane protection (Esan et al. 2017; Ma et al. 2017; Sohag et al. 2020); (3) regulation of pernicious ion (Na^+ and Cl^-) toxicity combined with essential ions (K^+ , Ca^{2+} , and Mg^{2+}) uptake and their assimilation (Ghassemi-Golezani and Farhangi-Abriz 2018); (4) hormonal metabolism (Kudla et al. 2018; Mallahi et al. 2018); and (5) improved biosynthesis of various bioactive compounds/secondary

metabolites through PAL enzyme activity, e.g., polyphenols (Ali 2020; Koo et al. 2020). SA can be applied in different ways, such as seed soaking (priming), foliar (exogenous) application, or root-growing medium (Souri and Tohidloo 2019).

In particular, exogenous SA has been extensively found to enhance salinity tolerance in wide-ranging plant species, including barley (Pirasteh-Anosheh et al. 2021; Torun et al. 2020), basil (Kahveci et al. 2021), maize (El-Katony et al. 2019), mung bean (Hussain et al. 2021; Nawaz et al. 2021), mustard (Zaid et al. 2019), okra (Esan et al. 2017), pea (Ahmed et al. 2021), radish (Bukhat et al. 2020), rice (Jini and Joseph 2017), soybean (Farhangi-Abriz and Ghassemi-Golezani 2018), sunflower (Huang et al. 2021), tomato (El-Hady et al. 2021; Gharbi et al. 2018), and wheat (Ghafoor et al. 2020; Pirasteh-Anosheh et al. 2021). Additionally, Nadarajah et al. (2021) proposed that SA-induced tolerance to salinity is highly dependent on the severity and duration of stress, the developmental stage of plants at the time of SA application, the concentration of SA applied to the plant species, and the experimental conditions. Despite the aforementioned substantial studies on the ameliorative or stimulatory effects of SA on glycophytic and halophytic plants, the responses of non-conventional *quasi*-halophytic crops to foliar application of SA have not yet been reported under salinity stress. In the present manuscript, we hypothesized that an optimal concentration of SA could acclimate the adaptive mechanisms coupled with the mitigating capacity of pernicious ions (Na^+ and Cl^-) in Juncao under long-term salinity stress.

Herein, we established a sustainable saline agricultural management strategy (SSAMS) to assess the individual or integrative effects of the NaCl-SA regime on the morpho-physio-biochemical responses, ionic homeostasis maintenance, osmoregulation, antioxidative capacity, and secondary metabolism in Juncao. The purpose of this study was also to clarify whether Na^+ or Cl^- is primarily more detrimental to Juncao for effective management of NaCl-induced biotoxicity. Furthermore, we investigated for the first time another distinctive aspect of combining environmental (NaCl) and hormonal (SA) elicitors in association with a non-conventional crop for the stimulation and/or production of bioactive compounds/secondary metabolites. Plant biologists, environmentalists, and pharmacologists will be able to use the information gained from this study for uplifting the socio-economic status of salt-affected areas via plant growth stimulation, maximizing phytoremediation capacity, producing therapeutically bioactive compounds for animal husbandry, and restoration of salinized soil or marginal lands for arable purposes.

Materials and Methods

Test Plant, Growth Conditions, and Experimental Design

The current experiment was conducted from July to September 2019 in a multiplane glasshouse at Shanghai Jiao Tong University, Shanghai, China ($31^{\circ}11' \text{ N}$, $121^{\circ}36' \text{ E}$) under a 16 h light/8 h dark photoperiod, 25°C average temperature, and 70% relative humidity. *Pennisetum giganteum* (Giant Juncao) was used as a test plant due to its field morpho-physio-biochemical performance and NaCl tolerance as indicated earlier (Hayat et al. 2020). The soilless substrate/growth medium (meteorite: perlite: peat and organic fertilizer mixture, 2:2:2:1) used in the current study was obtained from the Shanghai Experimental, Teaching, and Demonstration Center Shanghai Jiao Tong University, Shanghai, China. The basic physicochemical properties of the substrate, the eco-physiological attributes of the plant, and the experimental conditions required for plant growth are available in our previously published article (Hayat et al. 2020). Plant propagules (4 cuttings pot^{-1}) were sown equidistantly in polyethylene plastic pots ($55 \times 35 \times 25 \text{ cm}$) containing 2.5 kg of substrate growth medium.

Following one month of plant germination, 6 treatments ($n=5$ biological replicates each) comprised three NaCl concentrations (0, 250 and 500 mM) and one salicylic acid (SA) concentration (1.00 mM) were applied using a completely randomized design (CRD) as follows: (1) 0 mM NaCl + 1.00 mM SA, (2) 250 mM NaCl application only, (3) 250 mM NaCl with exogenous 1.00 mM SA foliar application, (4) 500 mM NaCl application only, and (5) 500 mM NaCl with exogenous 1.00 mM SA foliar application, while (6) without NaCl and exogenous SA application (sprayed with ultrapure water containing 0.01% Tween-20) served as control treatment. The concentrations of NaCl (250 and 500 mM) used in the current experiment were based on our previous research work (Hayat et al. 2020). Similarly, the choice of a single SA concentration (1.00 mM) was based on the preliminary experiment (Hayat et al. unpublished) as well as referring to the previous literature (Aftab et al. 2010; Batista et al. 2019; Pirasteh-Anosheh et al. 2021) for modulating plant growth and salinity tolerance response.

Salinity stress was imposed by adding sodium chloride (NaCl, molecular weight [MW]: 58.44, Shanghai Lingfeng Chemical Reagent Company Limited, Shanghai, China) in 5 doses each of 50 mM and 100 mM per day to achieve the final concentrations of 250 mM and 500 mM, as well as to avoid osmotic shock (Miranda et al. 2017). While 13.812 g of salicylic acid (SA, $\text{C}_7\text{H}_6\text{O}_3$, MW:138.12; Sangon

Biotech Co. Ltd, Shanghai, P.R. China) was initially dissolved in 100 mL of 70% ethanol to make 1 M stock solution. Afterward, 1.00 mM SA (pH 6.0–6.5) working solution (v/v) was prepared using ultrapure water containing 0.01% Tween-20 (C₂₆H₅₀O₁₀, Sinopharm Chemical Reagent Co. Ltd, Shanghai, P.R. China), as per previously described method (Bukhat et al. 2020). 1.00 mM SA was exogenously sprayed (20 mL plant⁻¹) with a manual pump on both the adaxial and abaxial surface of Juncao leaves in the first and second days; however, it was re-sprayed in the seventh and eighth days after the beginning of NaCl treatment. Plants were kept for 60 days under the NaCl-SA regime; samples of each tissue were analyzed immediately, refrigerated (– 80 °C) for future use, or dried in an oven for elemental analysis.

Evaluation of Morpho-Physio-Biochemical Attributes

Plants were harvested along with an intact root system after 60 days of NaCl-SA treatments. Morpho-physio-biochemical indices of *P. giganteum* were evaluated following our previous study (Hayat et al. 2020). Briefly, root, shoot length (cm), and fresh weight (g) were manually measured by using a measuring tape or an electronic weighing balance (Sartorius BSA623S, Max. = 620 g, d = 1 mg). The plant tissues (root, stem and leaves) were then oven-dried at 70 °C for 3 days until they reached a consistent dry weight (g).

The relative water content of leaves was tested for evaluating plant water status using the Barrs and Weatherley (1962) method:

$$\text{LRWC (\%)} = \frac{(\text{Fresh weight} - \text{Dry weight})}{(\text{Fully turgid weight} - \text{Dry weight})} \times 100.$$

Fully expanded leaves (0.5 g each) were used for the extraction of photosynthetic pigments. Leaf chlorophyll (Chl *a* and *b*) and carotenoid extraction were performed in dimethyl sulfoxide (DMSO), following a slightly modified method (Hiscox and Israelstam 1979). Leaf photosynthetic pigments were quantified at 665, 649, and 480 nm wavelengths using TECAN-i-control (infinite M200 Pro) spectrophotometer.

Growth tolerance index (GTI) was used to assess the impact of SA on plant growth and NaCl tolerance capabilities in *P. giganteum* as Ali et al. (2017) described:

$$\text{GTI} = \frac{\text{Root or shoot length under stress conditions}}{\text{Root or shoot length under control conditions}}.$$

Estimation of Stress Indicators Followed by Antioxidative Responses in Plants Under NaCl-SA Regime

To evaluate stress indicators coupled with antioxidant activities, 0.5 g fresh leaf tissues were homogenized in an ice bath with 2 mL pre-chilled phosphate buffer solution (PBS, 0.1 M, pH 7.4). The supernatants were collected for further analysis after centrifugation at 4000 rpm (1753 g) for 15 minutes at 4 °C (Thermo Scientific Multifuge X1R centrifuge; Microliter 30 × 2 sealed rotor). According to the manufacturer's protocols, the levels of hydrogen peroxide (H₂O₂), malondialdehyde (MDA), electrolyte leakage (EL), free proline (PRO), and total protein (TP) were determined using commercial assay kits (A064-1-1, A003-3-1, A107-1-1, and A045-2-2; Nanjing Jiancheng Bioengineering Institute, China). Total protein (TP) determination was based on the Coomassie Brilliant Blue method and expressed as g prot L⁻¹. EL was calculated and expressed as a percentage by following the method of González and González-Vilar (2001):

$$\text{EL (\%)} = \frac{\text{Initial electrical conductivity (EC}_i\text{)}}{\text{Final electrical conductivity (EC}_f\text{)}} \times 100.$$

The EC_i and EC_f were measured by using a conductivity meter (ORION Star A212).

The activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) was assessed using the manufacturer's protocol of commercial reagent kits (A001-1, A084-3-1, A007-1-1, A123-1-1 and A062-1, Nanjing Jiancheng Bioengineering Institute, China) respectively, and expressed as U g⁻¹ FW and U mg⁻¹ prot. Total phenolic compounds (TPC) were quantified using Folin–Ciocalteu reagent (Singleton et al. 1999), and absorbance was measured at 725 nm using a TECAN-i-control (infinite M200 Pro) spectrophotometer. TPC was calculated as gallic acid equivalent (GAE) in mg g⁻¹ of fresh weight (FW). Total flavonoid contents (TFC) were estimated according to the Lamaison and Carnet (1990) described method. Absorbance was measured at 430 nm and expressed as quercetin equivalent (QE) in mg g⁻¹ of fresh weight (FW). Total phenolic acid (TPA) was estimated using Arnov's method with slight modifications (Vergun et al. 2019), and absorbance was measured at 490 nm by using TECAN-i-control (infinite M200 Pro) spectrophotometer. The TPA was expressed as caffeic acid equivalent (CAE) in mg g⁻¹ of fresh weight (FW).

UHPLC Analysis of Phenolic Contents/Secondary Metabolites

A total of nine polyphenolics/bioactive compounds, including syringic acid, ferulic acid, luteolin, chlorogenic acid, quercetin, rutin trihydrate, kaempferol, hydroxycinnamic

acids, and gallic acid, were quantified in both treated and untreated samples by using ultra-high-performance liquid chromatography (UHPLC) as per Rahman et al. (2020) method. Syringic acid, ferulic acid, luteolin, chlorogenic acid, quercetin, rutin trihydrate, kaempferol, hydroxycinnamic acids, and gallic acid were used as standards.

Determination of Ions Homeostasis Maintenance

For the assessment of ions accumulation as well as ions flux ratio in plant tissues under the NaCl-SA regime, 0.1 g of freeze-dried samples of root, stem, and leaves were subjected to either inductively coupled plasma emission spectrometry (ICP-OES, Optima 800, PerkinElmer, USA) or ion chromatography. ICP-OES was used to determine the ions Na^+ , K^+ , Ca^{2+} , and Mg^{2+} , whereas ion chromatography was used to quantify the contents of Cl^- ion.

Statistical Analysis

The acquired data from various indicators were statistically analyzed using Statistical Package for Social Science software, version 23.0 (SPSS, Chicago, IL, USA). In brief, the analysis of variance (ANOVA) test followed by the Student-Newman-Keuls (SNK) post hoc test was used for comparing the significance of the difference between means ($n = 5$; $p < 0.05$). For correlation analysis, heat maps were used using GraphPad Prism v9.0 (Origin Lab, Berkeley, CA, USA) software to fully understand the interrelationships between the studied plant attributes, different adaptive mechanisms, and ion uptake/accumulation flux ratio.

Results

NaCl Concentration-Dependent Influence of SA on Plant Growth and Developmental Attributes of Juncao Under Increasing NaCl Concentrations

Compared to control plants (without NaCl and SA), the morpho-physiological features such as root, shoot lengths (RL, SL), root fresh and dry weights (RFW and RDW), shoot fresh and dry weights (SFW and SDW), as well as root and shoot growth tolerance indices (RLTI and SLTI) of *Pennisetum giganteum* (*P. giganteum*; Juncao) were adversely affected by increasing level of NaCl stress (Fig. 1A–H). Supp. Fig. S1 provides a visual inspection/phenotype of each treatment. The reduction in growth and developmental attributes were more pronounced with 500 mM NaCl than 250 mM (Fig. 1A–I). In comparison to control plants (without NaCl and SA), 500 mM NaCl stress significantly ($p < 0.05$) decreased SL, RFW, and SFW by 32.75%, 180.39%, and 107.67%, respectively (Fig. 1B–D).

Also, 500 mM of NaCl stress declined RL, RDW, and SDW by 34.97%, 117.69%, and 59.69% than the control group, despite not statistically significant (Fig. 1A, E–F). Additionally, compared to the control plants, RLTI (%; not significant) and SLTI (%; $p < 0.05$) were radically reduced under both salinity levels (250 mM and 500 mM NaCl) (Fig. 1G–H). Likewise, both levels of salt stress decreased the root/shoot dry mass (RDM/SDM) ratio of Juncao, as compared to unstressed plants (not significant) (Fig. 1I).

According to Fig. 1A–I, integrative application of SA + 250 mM NaCl increased RDM/SDM ratio compared to only 250 mM NaCl-stressed plants (though non-significant). Interestingly, foliar application of SA mitigated the adverse effects of 500 mM NaCl by augmenting RL (by up to 7.25%), RFW (by up to 23.7%), SFW (by up to 37.02%), RDW (by up to 7.73%), and SDW (by up to 29.51%) compared to only 500 mM NaCl counterpart, though not statistically significant (Fig. 1A, C–F). Both RLTI (%) and SLTI (%) showed a declining trend under 250 mM NaCl + SA combined. However, exogenous SA slightly improved RLTI (%) of 500 mM NaCl-exposed plants than corresponding 500 mM salt-stressed plants (not significant) but did not alter SLTI (%). In a nutshell, exogenous SA had slight stimulatory effects on the growth and developmental attributes of the 500 mM NaCl-stressed group. At the same time, it showed inhibitory effects on unstressed and 250 mM NaCl-stressed Juncao plants compared to the corresponding control groups.

Salicylic Acid Positively Influenced Homeostasis of Ions in a NaCl Concentration-Dependent Manner

Salinity and SA treatments exhibited differential responses regarding ions accumulation in Juncao tissues (root, stem, and leaves). The Na^+ and Cl^- ion contents were substantially elevated in Juncao under both NaCl concentrations compared to control plants ($p < 0.05$; Fig. 2A–F). Interestingly, exogenous SA significantly ($p < 0.05$) reduced the Na^+ concentration in the root (by up to 17.2%) under 250 mM salt stress than its relative NaCl counterparts but considerably increased the Na^+ content of stem and leaves ($p < 0.05$; Fig. 2A–C). Nonetheless, compared to individual 500 mM NaCl treatment, foliar spray of SA significantly limited Na^+ content of stem (by up to 12%; $p < 0.05$) followed by a substantial increase in leaves Na^+ content under 500 mM of NaCl stress ($p < 0.05$) while showing no significant stimulatory and/or inhibitory effect on root Na^+ content (Fig. 2A–C). On the contrary, SA increased root Na^+ content under non-saline conditions but did not alter the Na^+ content of stem and leaves. In addition, integrative application of SA + 250 mM NaCl and SA + 500 mM NaCl remarkably ($p < 0.05$) declined Cl^- content in the root (by up to 48.33% and 12.54%), stem (by up to 42.14% and 28.57%), and leaves (by up to 59.79% and 35.19%), respectively, in comparison

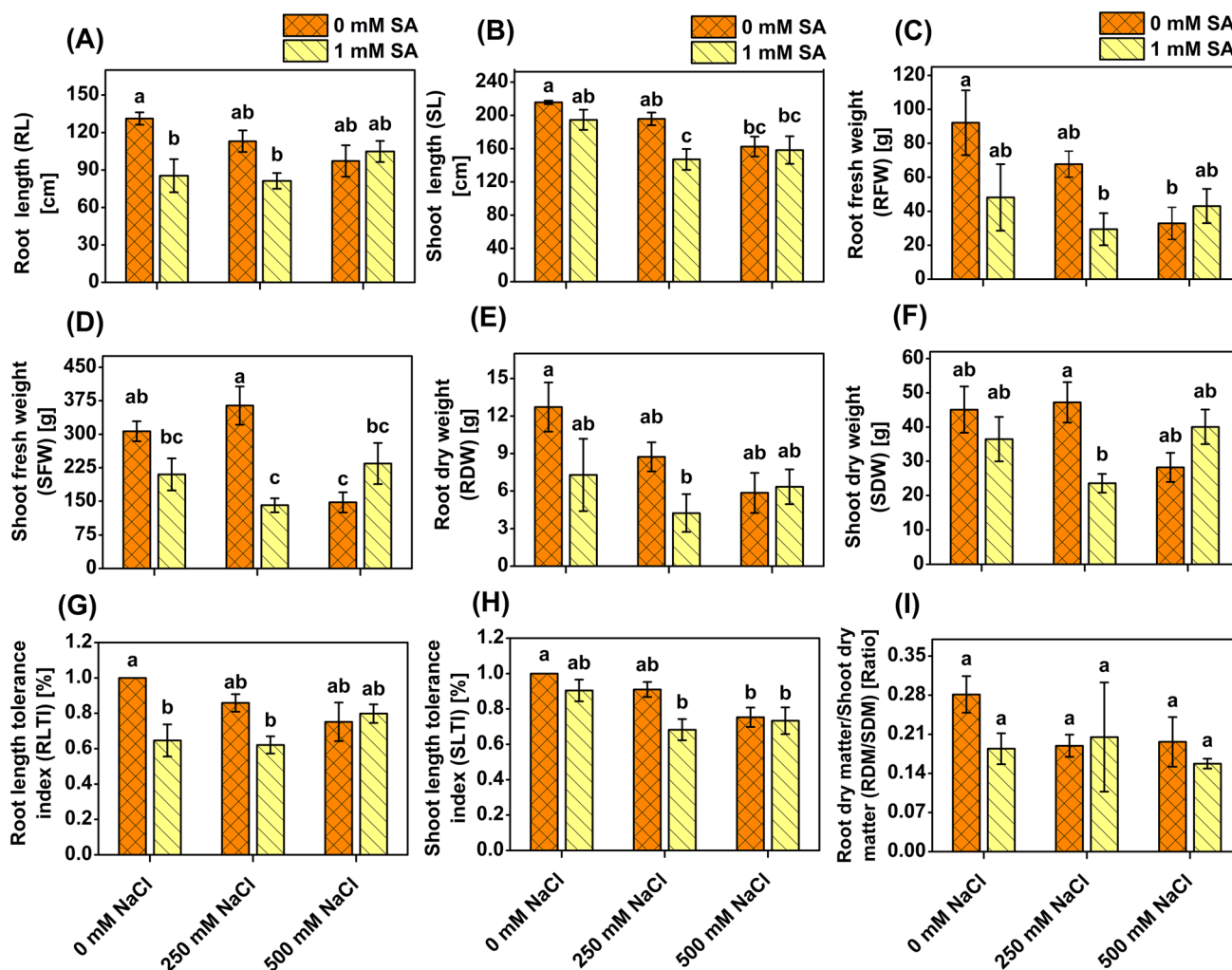


Fig. 1 Effect of salicylic acid on plant agronomic attributes (A and B; root and shoot length), biomass yield (C–F; root, shoot fresh and dry weight), tissue tolerance indices (G–H; root and shoot growth tolerance indices), and root/shoot dry matter ratio (I; RDM/SDM ratio) of *Pennisetum giganteum* under different NaCl concentrations. mM, mil-

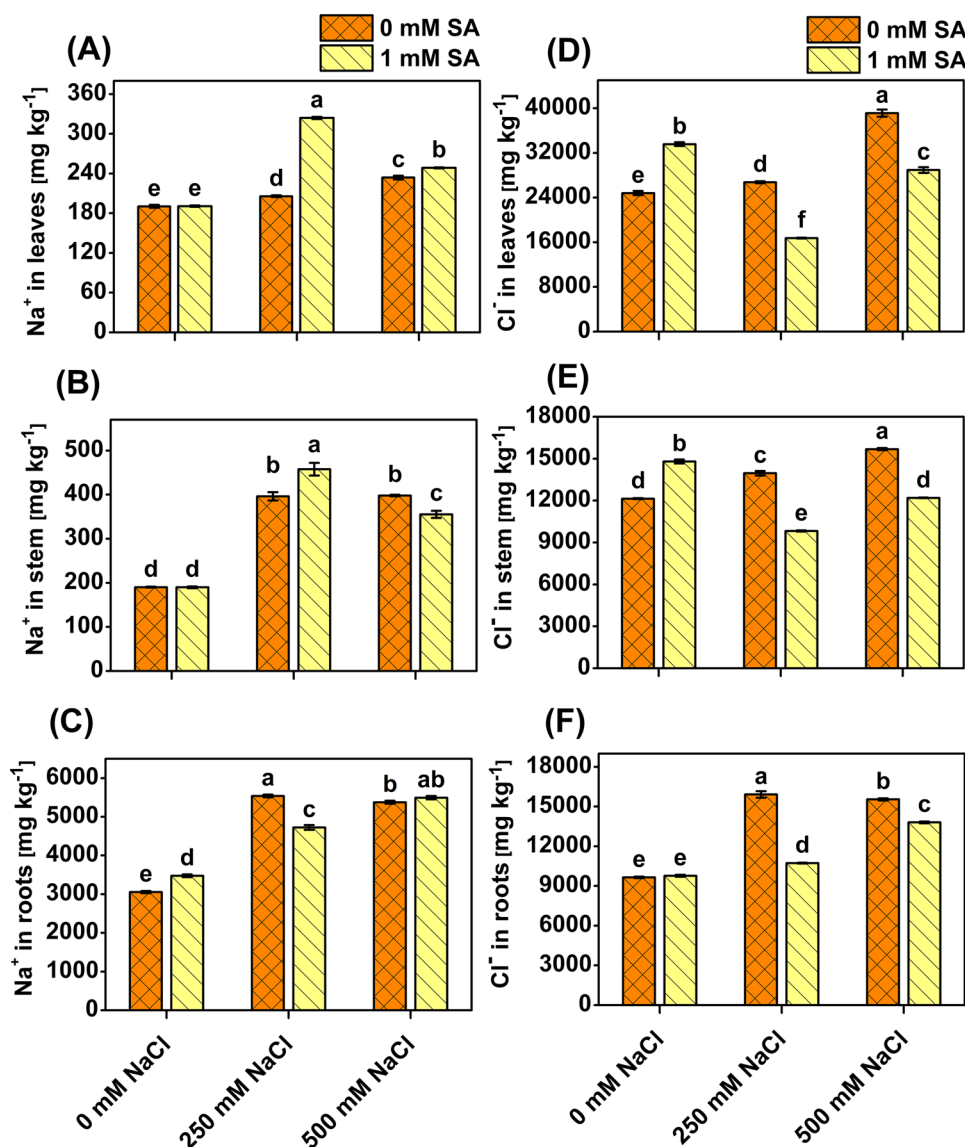
limole; SA, Salicylic acid. All the means sharing different letter(s) are significantly different at the $p < 0.05$ level, while all the means sharing the same letter(s) are insignificantly different at the $p < 0.05$ level. Vertical bars represent the standard error of means ($n = 5$)

to the corresponding NaCl-stressed groups (Fig. 2D–F). Moreover, SA considerably increased Cl^- concentration in the stem and leaves of the unstressed plant ($p < 0.05$), however, its unaffected root Cl^- content.

Furthermore, 250 mM salt treatment significantly increased root K^+ contents, as well as root, stem Ca^{2+} and Mg^{2+} concentrations than unstressed plants ($p < 0.05$), though non-significantly improved K^+ concentration in stem (Fig. 3A–I). Compared to control plants, Ca^{2+} and Mg^{2+} contents were remarkably reduced in Juncao leaves at 500 mM NaCl stress, whereas both salt levels significantly ($p < 0.05$) inhibited the K^+ content of leaves. On the other hand, SA significantly improved the K^+ content of root and stem under 250 mM saline condition. In contrast, it showed a significant decrement in tissue (roots, stem, and leaves) K^+

content under 500 mM of salinity stress compared to salt-stressed groups (Fig. 3A–C). Compared to corresponding NaCl-stressed counterparts, exogenous SA slightly improved Ca^{2+} concentration in Juncao root and stem by 8.5% and 5.05% under 250 mM salt stress (not significant). Notwithstanding, the combinatorial application of SA + 500 mM NaCl significantly ($p < 0.05$) enhanced the Ca^{2+} content in stem (by 27.22%) and leaves (by 17.01%), respectively, whereas non-significantly increased root Ca^{2+} concentration (by 14.97%) in comparison to 500 mM NaCl counterparts. Treating plants with SA under 250 mM saline condition significantly ($p < 0.05$) augmented Mg^{2+} concentration in Juncao stem (by 14.13%), however, slightly increased Mg^{2+} contents of root (by 1.91%) and leaves (by 5.16%), in comparison to 250 mM NaCl-stressed plants (non-significant).

Fig. 2 Effect of salicylic acid on toxic ions accumulation in leaves (A and D), stem (B and E), and roots (C and F) of *Penisetum giganteum* under NaCl stress conditions. mM, milli-mole; SA, Salicylic acid. All the means sharing different letter(s) are significantly different at the $p < 0.05$ level, while all the means sharing the same letter(s) are insignificantly different at the $p < 0.05$ level. Vertical bars represent the standard error of means ($n = 5$)



Likewise, relative to only 500 mM NaCl stress, stem Mg^{2+} contents were significantly ($p < 0.05$) increased by 22.70% under 500 mM + SA combined treatment. On the contrary, SA showed a significant inhibitory effect on root Mg^{2+} content under 500 mM saline conditions. Exogenous SA application significantly ($p < 0.05$) increased K^+ , Ca^{2+} , and Mg^{2+} concentrations in the roots of the non-stressed plants. However, leaves K^+ contents, as well as stem and leaves Ca^{2+} contents, were remarkably declined in the presence of SA under non-saline conditions (Fig. 3A–I).

Increasing salt stress exhibited a radical decline in nutrient ions ratios in Juncao tissues (root, stem, and leaves) compared to control plants (Figs. 4 and 5). Interestingly, compared to only 250 mM NaCl counterparts, integrative application of SA significantly ($p < 0.05$) modulated K^+/Na^+ , Ca^{2+}/Na^+ ratios in roots of 250 mM NaCl-stressed plants but significantly decreased these

ratios in leaves ($p < 0.05$). Compared to only 500 mM NaCl-stressed plants, exogenous SA considerably declined K^+/Na^+ ratio in root and leaves ($p < 0.05$) under 500 mM NaCl stress. It is worth noting that integrative application of SA + 500 mM NaCl significantly ($p < 0.05$) upregulated Ca^{2+}/Na^+ ratio in stem and leaves, but non-significantly increased Ca^{2+}/Na^+ ratio in the root, as compared to only 500 mM NaCl treatment. Moreover, SA significantly ($p < 0.05$) reduced the Mg^{2+}/Na^+ ratio in leaves in 250 mM NaCl exposure; however, it was remarkably enhanced in stem with the integrative application of SA + 500 mM salt (Fig. 4H). Nonetheless, SA application substantially increased K^+/Na^+ ratios in stem ($p < 0.05$), while it showed a decreasing trend in roots and leaves K^+/Na^+ ratios under non-saline conditions. In addition, SA significantly ($p < 0.05$) amended the Ca^{2+}/Na^+ ratio in the roots of unstressed plants, whereas it substantially

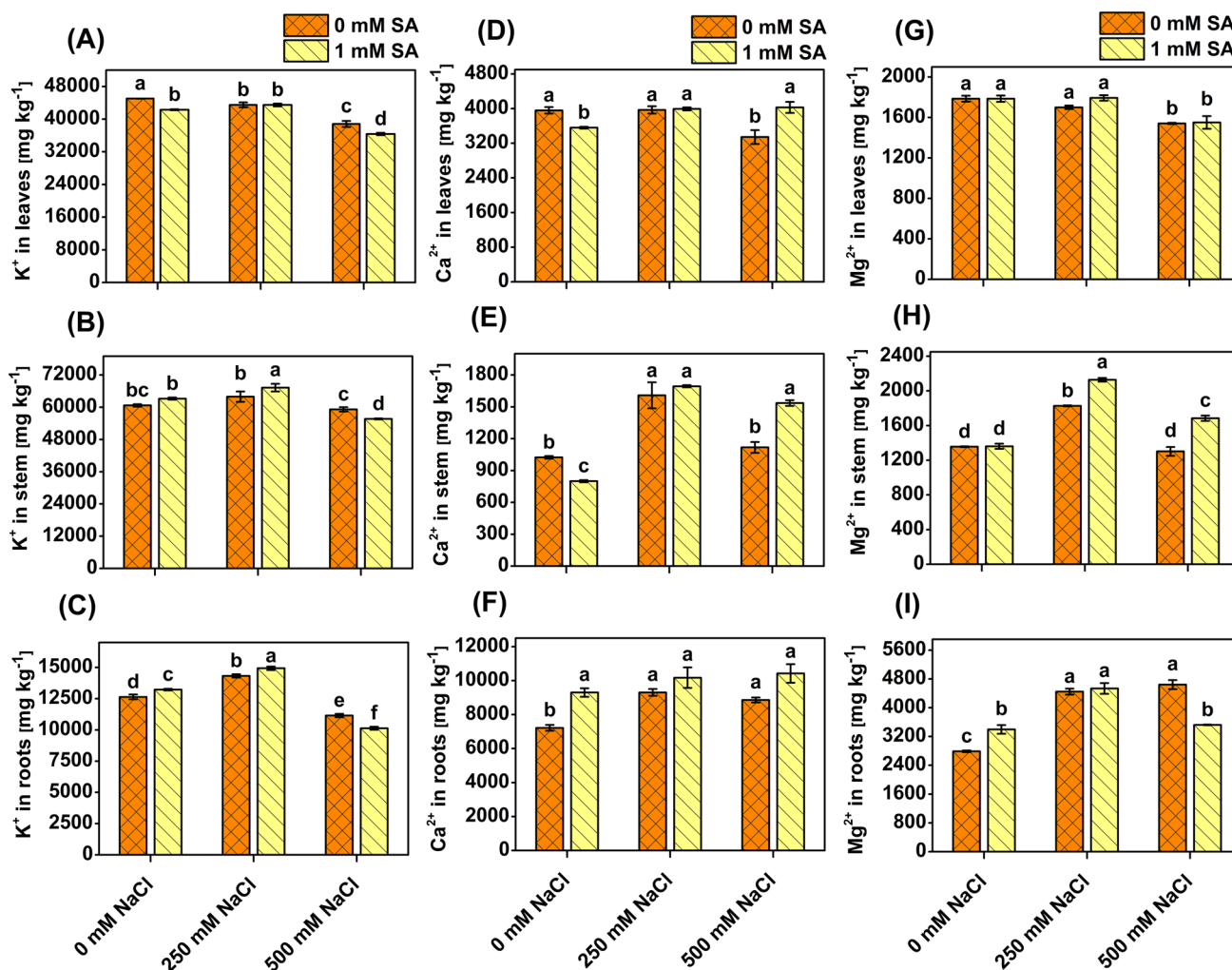


Fig. 3 Influence of salicylic acid on nutrient ions accumulation in leaves (A, D, and G), stem (B, E, and H), and roots (C, F, and I) of *Pennisetum giganteum* under two salt stress conditions. mM, millimole; SA, Salicylic acid. All the means sharing different letter(s) are

significantly different at the $p < 0.05$ level, while all the means sharing the same letter(s) are insignificantly different at the $p < 0.05$ level. Vertical bars represent the standard error of means ($n = 5$)

inhibited the $\text{Ca}^{2+}/\text{Na}^{+}$ ratio in stem and leaves under non-saline conditions.

Compared to salt-stressed groups, SA significantly ($p < 0.05$) improved the $\text{K}^{+}/\text{Cl}^{-}$ ratio in all tissues of *Juncao* under both saline conditions (250 and 500 mM NaCl) besides unaltered root $\text{K}^{+}/\text{Cl}^{-}$ ratio under SA + 500 mM NaCl combined treatment. Furthermore, SA noticeably reduced $\text{Cl}^{-}/\text{Ca}^{2+}$ and $\text{Cl}^{-}/\text{Mg}^{2+}$ ratios in all tissues of *Juncao* under both saline conditions compared to individual salt-stressed plants ($p < 0.05$), while SA significantly ($p < 0.05$) upregulated $\text{Cl}^{-}/\text{Mg}^{2+}$ ratio in the root of 500 mM NaCl-stressed plant. Under the non-saline condition, SA had no ameliorative impact on $\text{K}^{+}/\text{Cl}^{-}$, $\text{Cl}^{-}/\text{Ca}^{2+}$, and $\text{Cl}^{-}/\text{Mg}^{2+}$ ratios of stem and leaves while displaying a positive effect on these ratios in roots of *Juncao* (Figs. 4 and 5).

Integrative Effect of SA on Oxidative Stress Markers

To validate the oxidative stress response of *Juncao* plants when subjected to increasing saline stress conditions (with or without exogenous SA application), levels of hydrogen peroxide (H_2O_2), malonaldehyde (MDA), and passive electrolyte leakage (EL) were estimated (Fig. 6). Peak levels of H_2O_2 (35.38%; $p < 0.05$), MDA (28.94%), and EL (31.81%) were noticed under 250 mM NaCl stress (Fig. 6A–C), as compared to control plants (without NaCl). Surprisingly, SA treatment mitigated the adverse impact of salinity accrued repercussions by significantly reducing MDA level (59.84%; $p < 0.05$) combined with a non-significant reduction in the level of H_2O_2 (37.51%) and EL (5.8%) under 250 mM saline stress conditions. On the other hand, under 500 mM NaCl, SA increased H_2O_2 (non-significant) and MDA level

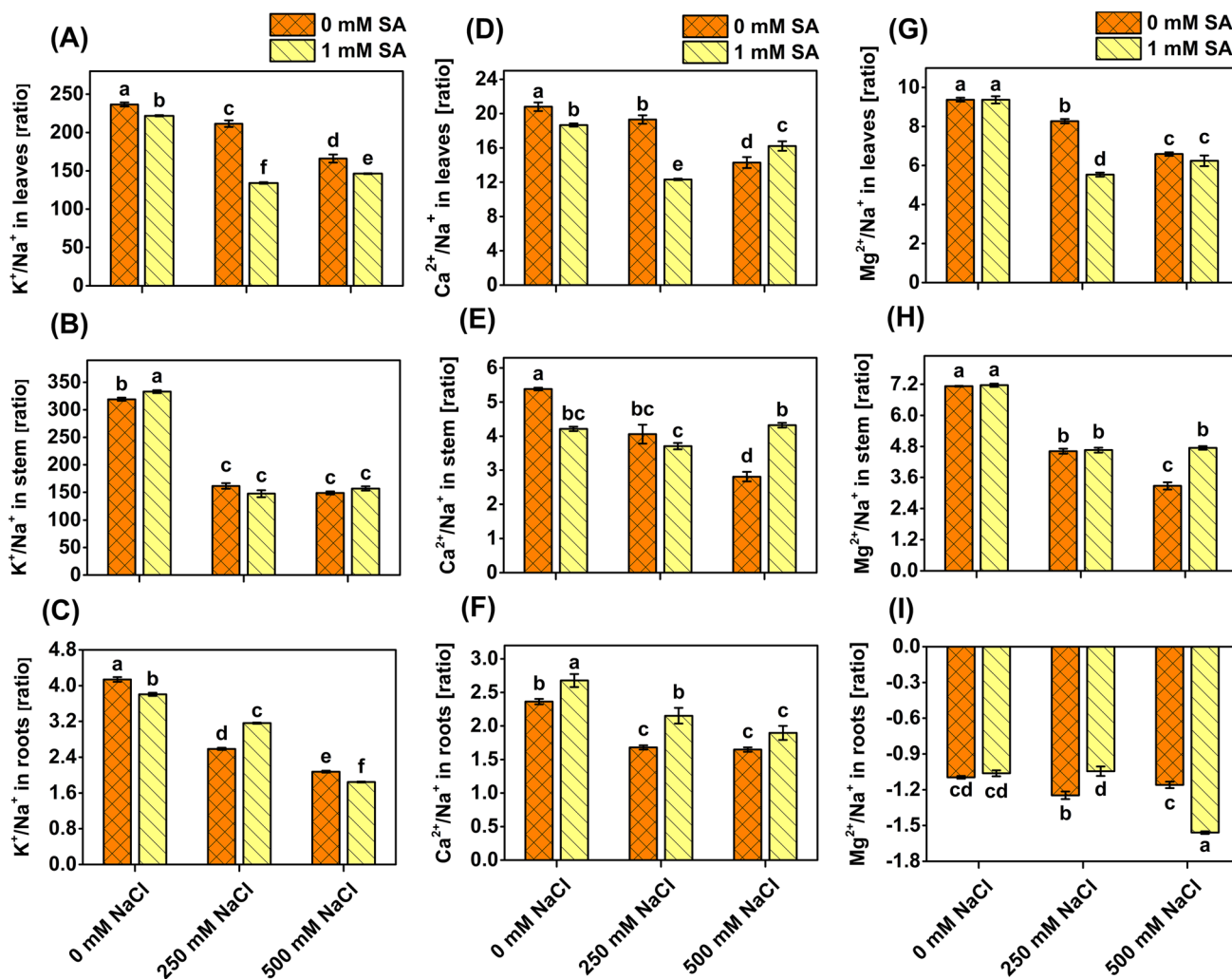


Fig. 4 Mitigating role of salicylic acid on nutrient ion ratios with respect to Na⁺ ion in leaves (A, D, and G), stem (B, E, and H) and roots (C, F, and I) of *Pennisetum giganteum*. mM, millimole; SA, Salicylic acid. All the means sharing different letter(s) are significantly different at the $p < 0.05$ level, while all the means sharing the same letter(s) are insignificantly different at the $p < 0.05$ level. Vertical bars represent the standard error of means ($n = 5$)

(significant), but not EL. Likewise, SA increased H₂O₂, MDA, and EL in non-stressed plants, though non-significant.

Effect of SA on Osmotic Adjustment, Osmoprotection and Chlorophyll Contents Under Increasing NaCl Stress Condition

Salinity substantially reduced leaf relative water content (LRWC; %) by 26.4% and 28.8% under 250 and 500 mM NaCl stress compared to non-stressed control plants ($p < 0.05$; Fig. 7A). Notably, SA remarkably improved LRWC by 30.2% and 37.2% in 250 and 500 mM NaCl-treated plants in comparison to relative salinity-stressed groups ($p < 0.05$). Proline content significantly improved in the 250 mM salt treatment than unstressed control plants (Fig. 7B). Compared to corresponding NaCl-stressed groups,

combinantly different at the $p < 0.05$ level, while all the means sharing the same letter(s) are insignificantly different at the $p < 0.05$ level. Vertical bars represent the standard error of means ($n = 5$)

combined SA + 250 mM NaCl and SA + 500 mM NaCl treatments significantly ($p < 0.05$) reduced proline accumulation by up to 54% and 110%, respectively. At the same time, total soluble protein (TSP) was increased in 250 mM salinity-exposed plants compared to unstressed plants (non-significant; Fig. 7C). In addition, the integrative application of SA + 500 mM NaCl slightly increased protein contents by 10.25% compared to only 500 mM NaCl treatment (non-significant). Under non-stress conditions, SA subsequently decreased LRWC (%), followed by a remarkable increase in proline content.

Moreover, chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (total Chl), and carotenoids (Car) were increased under both salt stress conditions, as compared to the control plants, despite not statistically significant. Nonetheless, chlorophyll *a/b* (Chl *a/b*) ratios were non-significantly decreased

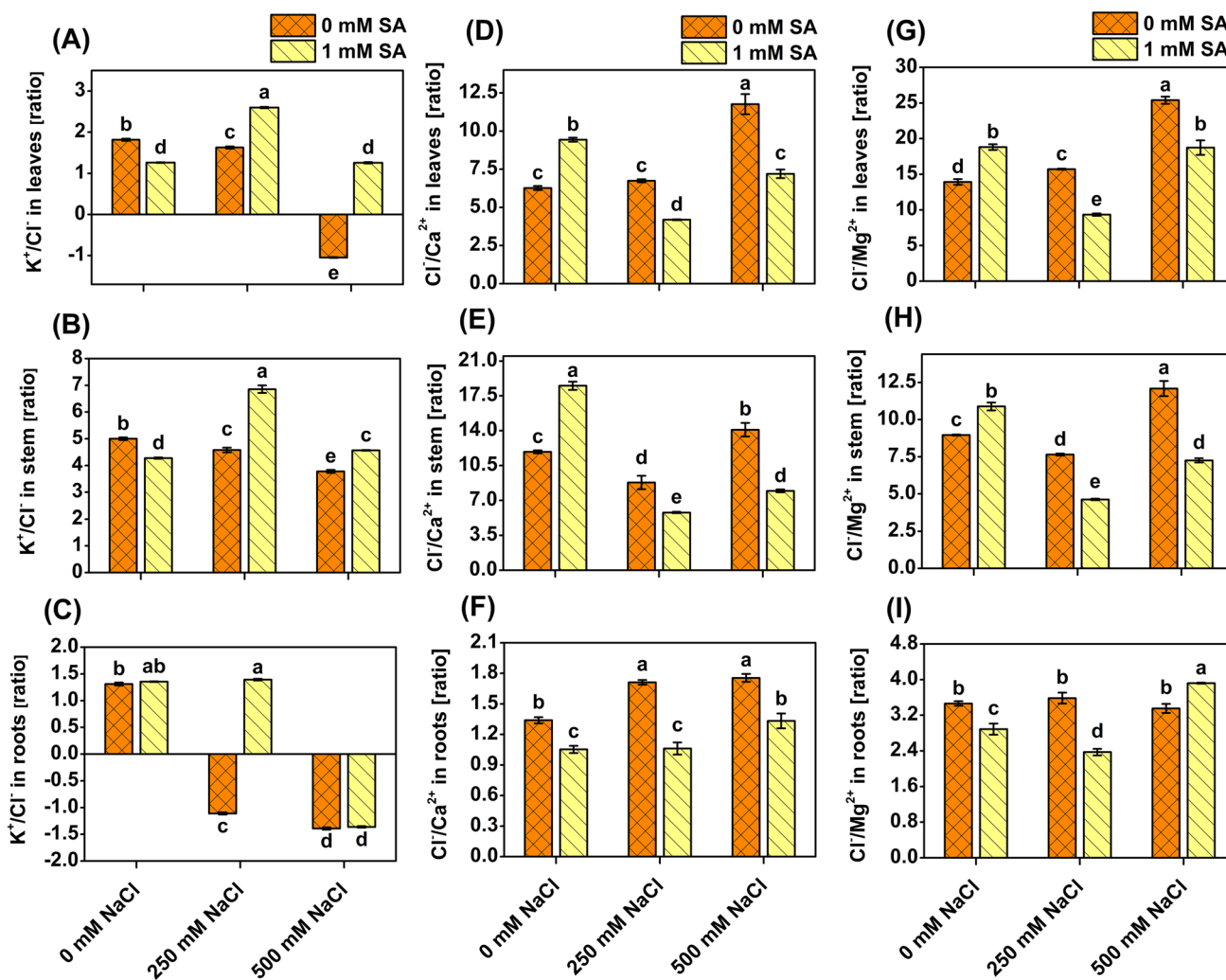


Fig. 5 Ameliorative role of salicylic acid on nutrient ion ratios with respect to Cl⁻ ion in leaves (A, D, and G), stem (B, E, and H) and roots (C, F, and I) of *Pennisetum giganteum*. mM, millimole; SA, Salicylic acid. All the means sharing different letter(s) are signifi-

cantly different at the $p < 0.05$ level, while all the means sharing the same letter(s) are insignificantly different at the $p < 0.05$ level. Vertical bars represent the standard error of means ($n = 5$)

under both saline conditions in comparison to unstressed plants (Table 1). Compared to corresponding NaCl-stressed plants, the *Chl a/b* ratio and carotenoid contents of *Juncao* were insignificantly increased with foliar spray of SA under both salt concentrations (250 and 500 mM NaCl). Contrarily, SA exhibited no apparent influence on *Chl a*, *b*, and total *Chl* under both salt levels compared to NaCl-stressed groups. Under the non-saline condition, SA significantly ($p < 0.05$) reduced photosynthetic pigments in *Juncao*, but the *Chl a/b* ratio was significantly increased ($p < 0.05$) compared to the control plant.

Antioxidative Response of *Juncao* Under NaCl-SA Association

The activities of antioxidant enzymes (such as SOD, POD, APX, CAT, GR) and non-enzymatic antioxidants (including flavonoid, total phenolic compounds, TPC; and total phenolic acid, TPA) unveiled stimulatory and/or inhibitory responses under NaCl-SA association (Fig. 8). Compared to non-stressed control plants, SOD activity was significantly ($p < 0.05$) upregulated, but APX activity was non-significantly increased under both NaCl levels (Fig. 8A).

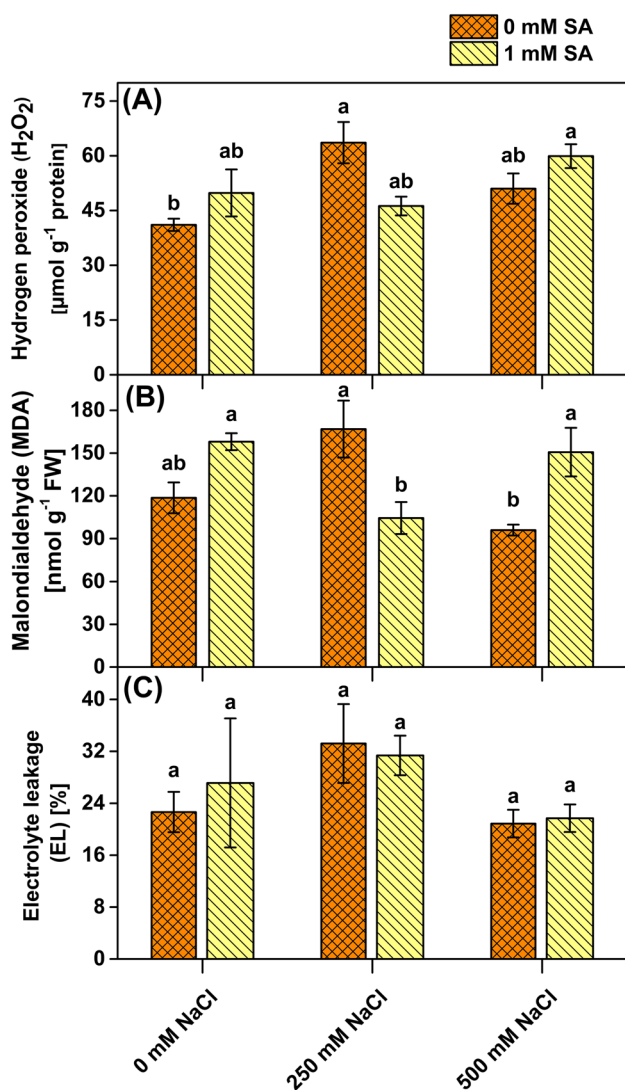


Fig. 6 Evaluating stress markers via **A** H₂O₂ generation, **B** lipid peroxidation (MDA), and **C** passive electrolyte leakage in *Pennisetum giganteum* under NaCl-SA regime. mM, millimole; SA, Salicylic acid. All the means sharing different letter(s) are significantly different at the $p < 0.05$ level, while all the means sharing the same letter(s) are insignificantly different at the $p < 0.05$ level. Vertical bars represent the standard error of means ($n = 5$)

Likewise, 250 mM salt treatment significantly enhanced flavonoid contents ($p < 0.05$; Fig. 8F) followed by increased CAT activity and TPA, in comparison to unstressed control plants (though not statistically significant; Fig. 8C, H), while POD was slightly declined (Fig. 8B), followed by reduced GR activities (non-significantly; Fig. 8E) under both NaCl concentrations (250 and 500 mM). TPC was significantly reduced under both NaCl concentrations ($p < 0.05$; Fig. 8G), whereas flavonoids showed a remarkable decline under the 500 mM saline stress condition. Also, in comparison to control, TPA was decreased in 500 mM NaCl-exposed plant (non-significant).

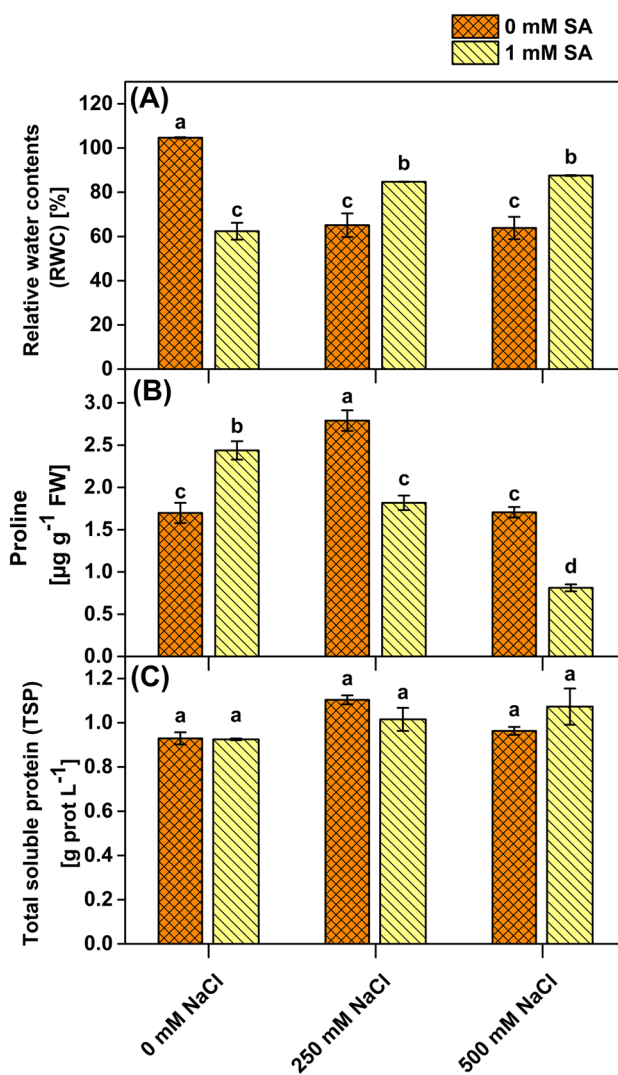


Fig. 7 Interactive effect of NaCl-salicylic acid association on osmoprotection and osmotic adjustment capability of *Pennisetum giganteum* via **A** leaf relative water contents (RWC), **B** proline, and **C** total soluble protein (TSP). mM, millimole; SA, Salicylic acid. All the means sharing different letter(s) are significantly different at the $p < 0.05$ level, while all the means sharing the same letter(s) are insignificantly different at the $p < 0.05$ level. Vertical bars represent the standard error of means ($n = 5$)

On the other hand, compared to only NaCl-stressed counterparts, SA application substantially reduced SOD activity by up to 178.8% and 80.52% of 250 mM and 500 mM NaCl-stressed plants, respectively. The integrative application of SA + 250 mM NaCl upregulated the activities of POD (by up to 8.66%) and CAT (by up to 9.76%), in comparison to only NaCl-treated plants (non-significant). Also, compared to the corresponding NaCl-stressed group, flavonoids were significantly reduced with SA application besides a minuscule abatement in APX and TPA under 250 mM salt stress.

Compared to the corresponding control group, the combinatorial application of SA + 500 mM NaCl increased

Table 1 Interactive effect of NaCl and salicylic acid on photosynthetic pigmentation of *Pennisetum giganteum*

| Treatments | 0 mM SA | 1 mM SA | 0 mM SA | 1 mM SA | 0 mM SA | 1 mM SA | 0 mM SA | 1 mM SA | | |
|-------------|---|--------------------------|---|-------------------------|--|--------------------------|--|-------------------------|--|-------------------------|
| 0 mM NaCl | Chl <i>a</i> (mg g ⁻¹) 26.9 ± 0.1 ^a | 19.9 ± 2.7 ^b | Chl <i>b</i> (mg g ⁻¹) 18.6 ± 0.6 ^a | 11.9 ± 2.1 ^b | Chl <i>a/b</i> ratio 1.46 ± 0.05 ^b | 1.84 ± 0.1 ^a | Total Chl (mg g ⁻¹) 45.6 ± 0.7 ^a | 32 ± 4.9 ^b | Carotenoid (mg g ⁻¹) 4.1 ± 0.1 ^a | 2.8 ± 0.3 ^b |
| 250 mM NaCl | 27.2 ± 0.05 ^a | 27.1 ± 0.06 ^a | 22.3 ± 0.3 ^a | 20.5 ± 0.7 ^a | 1.22 ± 0.02 ^b | 1.33 ± 0.05 ^b | 49.6 ± 0.3 ^a | 47.7 ± 0.7 ^a | 4.2 ± 0.03 ^a | 4.3 ± 0.03 ^a |
| 500 mM NaCl | 27 ± 0.04 ^a | 27 ± 0.06 ^a | 20 ± 1.2 ^a | 19 ± 0.7 ^a | 1.40 ± 0.08 ^b | 1.41 ± 0.05 ^b | 47.1 ± 1.3 ^a | 46.4 ± 0.8 ^a | 4.3 ± 0.1 ^a | 4.4 ± 0.04 ^a |

SA, Salicylic acid; Chl *a*, *b*, *a/b* ratio and total chlorophyll contents. All the means sharing different letter(s) are significantly different at the $p < 0.05$ level, while all the means sharing the same letter(s) are insignificantly different at the $p < 0.05$ level. Means ± standard errors (SE) of means ($n = 5$)

the activity of APX (by up to 8.38%) and CAT (by up to 27.22%), followed by the content of flavonoids (by 19.33%) and TPA (by 59.47%), despite not statistically significant. Compared to NaCl-stressed plants, POD activity significantly ($p < 0.05$) reduced under the combined SA + 500 mM NaCl application. Notably, SA application considerably increased TPC by up to 28.66% and 38.07% under 250 and 500 mM NaCl compared to their respective control plants ($p < 0.05$). Nonetheless, SA treatment has no influence on GR activity under both NaCl concentrations. In addition, SA application significantly upregulated both SOD and flavonoids ($p < 0.05$), besides the stimulatory impact on POD, GR, APX, CAT, and TPA antioxidants in unstressed plants (not significant).

Effect of Isolated or Integrative NaCl and SA Elicitation on Metabolic Responses of Juncao Leaves

Various polyphenolics/bioactive compounds, including syringic acid, ferulic acid, luteolin, chlorogenic acid, quercetin, rutin trihydrate, kaempferol, hydroxycinnamic acid, and gallic acid, were extensively studied in stressed and non-stressed plants (with either isolated or combined NaCl and SA elicitation) via UHPLC analysis. Compared to control plants (without NaCl and SA), salinity stress significantly ($p < 0.05$) impaired the production of luteolin (Supplementary Fig. S2C), followed by a gradual reduction in syringic acid and gallic acid production (Supplementary Fig. S2A, D), despite not statistically significant. Likewise, 250 mM salinity downregulated ferulic acid, whereas upregulated the production of rutin trihydrate in comparison to control plants (Supplementary Fig. S2B and S2F; not significant). Similarly, compared to non-stressed plants, 500 mM NaCl insignificantly reduced chlorogenic acid and rutin trihydrate (Supplementary Fig. S2D, F). However, salinity stress did not influence the remaining bioactive compounds.

Moreover, exogenous SA declined syringic acid biosynthesis compared to the 250 mM NaCl-stressed group (Supplementary Fig. S2A). Nonetheless, compared to 250 mM salt stress, SA upregulated ferulic acid and chlorogenic acid by 10.57% and 14.75% in 250 mM salt-stressed plants (Supplementary Fig. S2B and S2D; despite not statistically significant). Correspondingly, SA application augmented syringic acid by 12.76% at 500 mM NaCl than NaCl-stressed Juncao plants (Supplementary Fig. S2A; not statistically significant), whereas, in comparison to only 500 mM NaCl counterpart, integrative application of SA + 500 mM significantly upregulated chlorogenic acid by up to 17% ($p < 0.05$; Supplementary Fig. S2D). Similarly, SA inhibited the production of rutin trihydrate under both saline conditions, despite non-significant (Supplementary Fig. S2F). Under non-stress conditions, SA showed a cumulative effect on syringic acid and rutin trihydrate production than unstressed

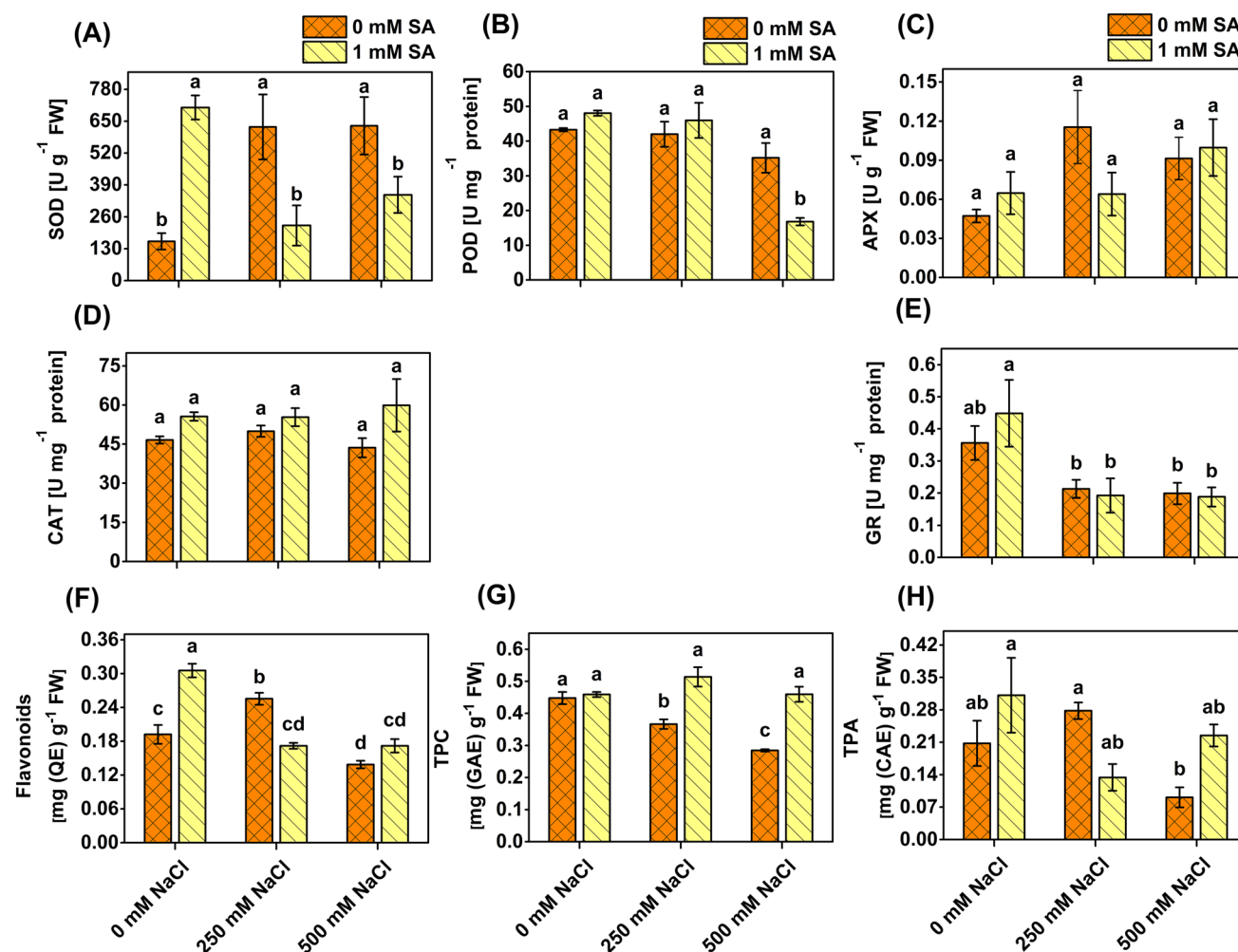


Fig. 8 Salicylic acid-mediated response on the battery of enzymatic (A–E) and non-enzymatic (F–H) antioxidants of *Pennisetum giganteum* under increasing NaCl concentrations. mM, millimole; SA, Salicylic acid; SOD, superoxide dismutase; POD, peroxidase; APX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase. mM,

millimole; SA, Salicylic acid. All the means sharing different letter(s) are significantly different at the $p < 0.05$ level, while all the means sharing the same letter(s) are insignificantly different at the $p < 0.05$ level. Vertical bars represent the standard error of means ($n = 5$)

plants, despite not significant (Supplementary Fig. S2A and S2F).

Correlation Analysis Among Different Plant Indices Under NaCl-SA Interaction

Correlation analyses (represented as heat maps) were performed to establish a positive or negative relationship among the studied *Pennisetum giganteum* attributes along with the accumulation and ion ratios in the tissues of plants under NaCl-SA interactions (Supplementary Figs. S3, and S4). A positive correlation was found between ascorbate peroxidase (APX) enzymatic antioxidant and Na⁺ accumulation in the root (Na⁺ R). In contrast, a negative correlation between the antioxidative activities of the glutathione reductase (GR) enzyme and Na⁺ accumulation in the root (Na⁺ R)

was observed. A strong negative correlation between luteolin (a secondary metabolite) and Na⁺ accumulation in the root (Na⁺ R) was also noticed. Likewise, GR antioxidant and syringic acid (secondary metabolite) activities showed a strong negative correlation with the contents of Na⁺ ion in the stem (Na⁺ S). Further, plant growth in terms of shoot length (SL) coupled with shoot tolerance index (SL TI) was negatively correlated with the contents of Na⁺ ion in Juncao leaves (Na⁺ L). On the contrary, passive electrolyte leakage (EL), as well as activity of peroxidase (POD) antioxidant, was positively correlated with the contents of K⁺ ion in root and stem (K⁺ R and S), while a strong positive correlation between rutin trihydrate (secondary metabolite) and K⁺ ion in the root (K⁺ R) was noted. Similarly, rutin trihydrate was also positively correlated to the contents of K⁺ ion in stem (K⁺ S). A positive correlation between POD activity and

K^+ ions in leaves (K^+ L) was observed, while gallic acid showed a strong positive correlation with the accumulation of K^+ ions in leaves (K^+ L). Root dry biomass (root DW) was negatively correlated to Ca^{2+} contents in the root (Ca^{2+} R). Moreover, total soluble protein (TP) and chlorogenic acid (secondary metabolite) were positively correlated with the contents of Ca^{2+} ions in stem (Ca^{2+} S). A negative correlation was noticed between GR activity and contents of Ca^{2+} ion in stem (Ca^{2+} S). Syringic acid was negatively correlated to the contents of Mg^{2+} ion in the root (Mg^{2+} R), whereas POD activity was positively correlated to the contents of Mg^{2+} ion in leaves (Mg^{2+} L). Interestingly, the contents of Cl^- ion in roots (Cl^- R) exhibited a strong positive correlation with the activities of APX enzymatic antioxidant was noticed. In comparison, activities of SOD enzymatic antioxidants were positively correlated with the accumulation of Cl^- ion in stem (Cl^- S) of Juncao plants. A highly positive correlation was noticed between net K^+/Na^+ ions flux ratio in roots (K^+/Na^+ R) and luteolin accumulation, followed by a positive correlation with gallic acid and a negative correlation with APX antioxidative activity. Similarly, the net K^+/Na^+ ions flux ratio in stem (S) was strongly correlated with GR activity and syringic acid accumulation, while showing a positive correlation with the luteolin accumulation. Likewise, the net Ca^{2+}/Na^+ ions flux ratio in roots (Ca^{2+}/Na^+ R) was positively correlated with GR activity while negatively correlated with chlorophyll b (Chl b) contents and hydroxycinnamic acid (secondary metabolite) accumulation. Nonetheless, root fresh biomass (root FW) was positively correlated with the net Ca^{2+}/Na^+ ions flux ratio in stem (Ca^{2+}/Na^+ S). Remarkably, POD activity exhibited a strong positive correlation with net Mg^{2+}/Na^+ flux ratio in roots (Mg^{2+}/Na^+ R). Likewise, a significant positive correlation was noticed between syringic acid accumulation and net Mg^{2+}/Na^+ ions flux ratio in stem (Mg^{2+}/Na^+ S). A positive correlation was observed between GR activity, luteolin accumulation, and net Mg^{2+}/Na^+ ions flux ratio in stem (Mg^{2+}/Na^+ S). Noticeably, plant growth in terms of SL and SL TI were found in strong positive correlation with K^+/Na^+ , Ca^{2+}/Na^+ , and Mg^{2+}/Na^+ ions flux ratios in leaves (K^+/Na^+ , Ca^{2+}/Na^+ , and Mg^{2+}/Na^+ L). Similarly, root fresh and dry biomass (root FW and DW) were positively correlated to the K^+/Na^+ , and Ca^{2+}/Na^+ ions flux ratio in leaves (K^+/Na^+ and Ca^{2+}/Na^+ L) while a positive correlation between shoot fresh, dry biomass (shoot FW and DW), and net Ca^{2+}/Na^+ ions flux ratio in leaves (Ca^{2+}/Na^+ L) was noticed. Furthermore, GR activity and syringic acid accumulation were positively correlated with the net Mg^{2+}/Na^+ ions flux ratio in leaves (Mg^{2+}/Na^+ L). Nevertheless, APX activity negatively correlated with the net K^+/Cl^- ions flux ratio in roots (K^+/Cl^- R), while TPC was positively correlated with the net K^+/Cl^- ions flux ratio in leaves (K^+/Cl^- L) of Juncao. It is worth noting that a significant positive correlation between

hydroxycinnamic acid and net Cl^-/Ca^{2+} ions flux ratio in roots (Cl^-/Ca^{2+} R) was observed, followed by a negative correlation between total phenolic contents (TPC) and net Cl^-/Ca^{2+} ions flux ratio in roots (Cl^-/Ca^{2+} R) was found in Juncao plants. Also, chlorogenic acid accumulation showed a strong negative correlation with the net Cl^-/Ca^{2+} ions flux ratio in stem (Cl^-/Ca^{2+} S). In contrast, a negative correlation between the net Cl^-/Mg^{2+} flux ratio in stem (Cl^-/Mg^{2+} S) and chlorogenic acid accumulation was noticed.

Discussion

Exogenous applications of SA can improve plant (mostly conventional crops) tolerance for salinity-induced stresses. In non-conventional crops, however, it is less studied so far. Therefore, we tried to evaluate the role of exogenous SA application in alleviating salt stress responses in *Pennisetum giganteum* (Juncao). Plant growth evaluation can successfully determine the negative impacts of salinity stress. A radical decline in the physiology of Juncao was observed in the current study, which corroborates the reported findings in a variety of Poaceae family members (Mir et al. 2018; Torun et al. 2020; Yadav et al. 2020). Interestingly, plant growth was also reduced with SA application per se when grown under control conditions (0 mM NaCl). The negative influence of SA application on plants' growth and developmental responses is a well-observed characteristic that reflects its biostimulatory function. In the current study, salinity induced detrimental effects on root development and perhaps the involved mitotic activity (cellular growth and expansion), which may have limited/reduced the nutrient uptake and eventually abrupt plant–water relationship resulting in osmotic and ionic stress. However, integrative application of SA alleviated the detrimental impacts of high saline stress by enhancing growth and developmental biomarkers of Juncao, which can be accredited to the mitigation of ion toxicity in the roots of the treated plants (mainly in 250 mM NaCl-treated plants). The improved performance of plants (treated with SA) under salinity stress conditions can be reflected as the induced resistance achieved from the exogenous application of SA.

Salinity tolerance in plants is primarily dependent on the acquisition or assimilation of essential ions (particularly K^+ , Ca^{2+} , and Mg^{2+}); as evident from the current study, and which may be constituted as a vital mechanism of homeostasis maintenance (de Freitas et al. 2019; Isayenkov and Maathuis 2019). Juncao plants exposed to 250 mM NaCl had slightly increased ion accumulations (both pernicious and essential ions); however, at 500 mM, the toxic ion accumulations increased, whereas the essential concentration ions accumulation significantly reduced. Notably, SA promoted nutrient (K^+ , Ca^{2+} and Mg^{2+}) acquisition and limited

toxic ion (Na^+ and Cl^-) accumulation in salt-stressed Juncao plants, indicating that ionic homeostasis is necessary to modulate the ionic flux and subsequent sequestration in plant tissues (Zhu et al. 2017). Under increasing salt concentrations, the SA-mediated higher K^+/Na^+ , $\text{Ca}^{2+}/\text{Na}^+$, $\text{Mg}^{2+}/\text{Na}^+$, K^+/Cl^- , and lower $\text{Cl}^-/\text{Ca}^{2+}$ and $\text{Cl}^-/\text{Mg}^{2+}$ ratios are deemed as a critical trait for ionic stress tolerance in plants (Gupta and Huang 2014; Nahar et al. 2016). In contrast to our findings, Nieves-Cordones et al. (2019) proposed that K^+/Na^+ , $\text{Ca}^{2+}/\text{Na}^+$, $\text{Mg}^{2+}/\text{Na}^+$, and K^+/Cl^- ratio below 1.0 advocate a high level of phytotoxicity, which may have a negative impact on critical metabolic and cellular processes in plants.

Meanwhile, a remarkable increase in K^+ , Ca^{2+} , and Mg^{2+} ions as well as higher K^+/Na^+ , and $\text{Ca}^{2+}/\text{Na}^+$ ratios with SA supplementation might as well be accredited to the stimulation of diverse mechanisms such as Na^+ exclusion via SOS or NHX pathway, H^+ -ATPase activity, low K^+ leakage through gated outwardly rectifying K (GORK) channel, and activation of high-affinity transporters (K^+ and Ca^{2+} -selective ion channels) in the plasma membrane and tonoplast (Ahmad et al. 2018; Ghassemi-Golezani and Farhangi-Abriz 2018; Isayenkov and Maathuis 2019; Rubio et al. 2020), supported by lower Na^+ levels in the root and stem of SA-applied Juncao plants. Despite high Na^+ concentrations in the leaves, Juncao plants exhibited tolerance with no apparent phytotoxic symptoms, corroborating our previous work (Hayat et al. 2020). However, the mechanisms of toxic ion transport in plant tissues at high NaCl concentrations are less well understood than those of nutrient ion transport (Teakle and Tyerman 2010). Nonetheless, SA mediated the K^+/Cl^- ratios in aboveground tissues (stem and leaves) and K^+/Na^+ ratio in the stem of 500 mM NaCl-stressed Juncao plants, highlighting the critical role of K^+ in regulating pernicious Na^+ and Cl^- ions in plant compartments. Comparable results have been reported in *Pennisetum glaucum*, *Triticum aestivum*, and *Zea mays* (El-Katony et al. 2019; Yadav et al. 2020).

Furthermore, the current study's findings indicated changed redox machinery in the plants in response to the applied NaCl and SA. A correlation to the inhibited growth of the observed plants established the role of redox potential. When the plants were exposed to increasing NaCl concentrations, an abundance in the H_2O_2 content, MDA content, and electrolyte leakage was observed. However, the integrative application of SA tended to reduce the accumulation. Under increasing salinity, a substantial reduction in H_2O_2 concentration, MDA accumulation, and EL with SA application has previously been documented (Kamran et al. 2020; Torun et al. 2020; Yadav et al. 2020), which corroborate our findings. In addition, the activity of enzymatic and non-enzymatic antioxidants was significantly altered in response to SA application under variable NaCl stresses.

The hyperactivity of SOD might be due to higher O_2^- formation, whereas the relatively high activities of APX could be due to the presence of a high level of ascorbate, which maintains the ascorbate–glutathione (ASC-GSH) cycle and eventually leads to an avoidance mechanism for the O_2^- -mediated damage in PSII, as well as helps scavenge H_2O_2 and retain highly reduced states of the plastoquinone pool (Wicwarz et al. 2018). Based on our findings, probably the amounts of proline and total soluble protein (TSP) accumulated in Juncao plants could be inadequate to relieve salinity-induced oxidative burst. Consequently, both enzymatic and non-enzymatic antioxidants contributed to counteract the adverse effects of salinity-induced oxidative stress biomarkers. Upregulation of antioxidant enzyme activity in saline conditions is a typical response of tolerant plants to pernicious ion accumulation in leaf cells or tissues (Foyer et al. 2017; Farhangi-Abriz and Ghassemi-Golezani 2018). Whether inherent or induced, plants acquire significant salt tolerance to defend themselves from oxidative damage via a powerful antioxidant system (Isayenkov and Maathuis 2019; Sharma et al. 2019).

Contrarily, GR activity as well as total phenolic contents (TPC) were decreased, followed by a slight decline in POD activity at both NaCl concentrations (250 and 500 mM), which are in agreement with the previous studies (Jiang et al. 2017; Torun et al. 2020). Nonetheless, the decline in GR might be ascribed to a reduction in GSH synthesis along with an increase in the rate of GSH breakdown in cell organelles. Similar to our study, upregulation of antioxidant enzymes improved salt tolerance in rice (Jini and Joseph 2017), wheat (Ahanger and Agarwal 2017), and sweet sorghum (Forghani et al. 2020). Furthermore, Valifard et al. (2014) and Zhou et al. (2018) validated our findings by stating that phenolic and flavonoid concentration decreased as salinity increased. However, it has previously been reported that antioxidants (except ascorbic acid) do not undergo a regeneration cycle. Keeping this in view, the antioxidant capacity of Juncao was drastically reduced in the presence of 500 mM NaCl stress, indicating that the rate of ROS production may have exceeded the detoxifying capacity of enzyme-controlled processes such the Asada cycle. To recapitulate, we postulate a strategic management of the plant's defense system in detoxifying ROS at their source, which may imply a substantial difference between the salt tolerance mechanism of C3 plants (such as glycophytic crops) and our test species (*P. giganteum*), which is a C4 plant. These findings tend to support Juncao's status as a salt-accumulator/tolerant non-traditional crop plant.

Apart from osmotic adjustments (Fig. 6), the SA supplementation significantly upregulated antioxidant activities in our test plant, which were inversely proportionate to H_2O_2 and MDA levels, intimating another prospect of enhanced morpho-physio-biochemical features in Juncao

plants under extreme saline conditions. It has been demonstrated that reduced oxidative damage in SA-treated plants is correlated with substantial activation of the antioxidant defense system to counteract salinity-induced ROS generation and improve salinity tolerance (Ahanger and Agarwal 2017). Consistent with our findings, SA is known to have two fundamental roles: it may act as an antioxidant (Simić et al. 2007) as well as a secondary messenger for signaling ROS-accrued stress events (Kim et al. 2018; Pirasteh-Anosheh et al. 2021). Concurrent to our findings, several studies have revealed the ameliorative role of SA on the stimulation of the NaCl-induced antioxidative system in plants (Ahanger et al. 2020; Ahmad et al. 2018; El-Esawi et al. 2017), which has been attributed to the modulation of the phenylpropanoid pathway or phenylalanine ammonia-lyase activity (PAL; an enzyme that initiates the synthesis of free phenolics and is responsible for phenylpropanoid metabolism), PSII activity, an increment in polyphenols, and improvement of Ca^{2+} uptake. TPC was higher in SA-treated plants than in control and NaCl-stressed plants in our study. The enhanced SA-mediated phenolic compound accumulation in Juncao could also be attributed to the hyperactivities of the *PAL* gene (unpublished data), which resulted in the accumulation of phenolic compounds in our test plant. The present work reaffirms the conclusions of numerous authors (Misra et al. 2014; Yücel and Heybet 2016), who reported that the application of SA enhanced antioxidant activity via phenol and flavonoid accumulation. Although Juncao is a salt-tolerant non-conventional crop, given that salinity stress modulated plant growth, ionic homeostasis, unaltered photosynthetic process, osmotic adjustment, and antioxidative responses; SA is an effective treatment that intriguingly mediated induced tolerance responses by regulating the key metabolic processes at the cellular and physiological level under high salt stress condition.

We postulate that the downregulation of H_2O_2 , MDA, and EL in NaCl-SA-treated plants may have also played a key role in metabolism maintenance and/or stress-induced tolerance (Romero-Romero et al. 2020), which subsequently contributed to enhanced plant growth, photosynthesis process, and directly arbitrated membrane damage by maintaining K^+ , Ca^{2+} , and Mg^{2+} flux ratio, as well as antioxidant battery in Juncao plants. Given that, K^+ and Ca^{2+} are well-known osmolytes/osmoticum that accumulate in guard cells during stomatal opening and sustain cell turgor (Inoue and Kinoshita 2017). Also, K^+ , Ca^{2+} , and Mg^{2+} play critical roles in signaling and osmoprotection by modulating hydraulic conductivity, photosynthetic rates, and membrane integrity (Wu et al. 2018; Sharma et al. 2019). Besides higher K^+ , Ca^{2+} , and Mg^{2+} contents in our test plant, the accumulation of proline and TSP had a significant role in osmotic adjustment and cellular homeostasis (Ahmed et al. 2019; Sharma et al.

2019), thereby, mitigating the adverse effects of salinity-induced oxidative stress.

Under salt stress conditions, salt-resistant/tolerant plants accumulate more osmolytes or organic solutes and have the lowest lipid peroxidation level, as highlighted in the current study. Upregulated osmolytes or compatible solutes have been considered a typical response of salinity-tolerant plants at the physiological level (Borrelli et al. 2018; Skliros et al. 2018). Compatible solutes, including proline, TSP, and total soluble sugar, play a pivotal role in plant tolerance by mediating water status, nitrogen supply, ROS-scavenging mechanisms, and membrane integrity protection (Cai and Gao 2020; Muchate et al. 2016). Cell membrane stability/integrity is a well-established metric for assessing plant resistance to abiotic stress (Kumar et al. 2016). In the current study, proline and TSP levels increased in the 250 mM NaCl-exposed plant but remained unchanged under 500 mM salt stress, while water contents reduced with increasing salinity level. An increasing trend in proline and TSP accumulation in salt-stressed Juncao plants allowed us to correlate these osmoprotectants, or compatible (organic) solutes to the NaCl-induced tolerance response, as previously reported in lettuce, maize, quinoa, and wheat (Iqbal et al. 2014; Ahmed et al. 2019; Jain and Vaishnav 2019; Cai and Gao 2020). In addition, salinity stress also mediates the activities of pyrroline-5 carboxylate synthase (P5CS) and proline dehydrogenase (PDH), a proline oxidation enzyme (Sharma et al. 2019, 2020), thereby might have upregulated proline biosynthesis in Juncao (Fig. 6B). Furthermore, salinity stress enhances the expression of transcription factors such as OsNAC5 and ZFP179, which are responsible for proline synthesis and accumulation, and therefore induce stress tolerance (Song et al. 2011). The current study has not reported any significant increase in protein production of salt-stressed plants. Meanwhile, NaCl stress had either a stimulatory or inhibitory impact on RWC, proline, and TSP of Juncao in NaCl-stressed plants, confirming the plant's inherent potential to exacerbate the devastating consequences of increasing salinity levels.

In addition, results of the current study showed that exogenous SA maintained lower osmolytes (proline) levels under increasing NaCl concentrations, which could be attributed to partial relief from salinity stress (Yadav et al. 2020) by adjusting water balance, chlorophyll biosynthesis (Fig. 6; Table 1), and restricting pernicious ions influx into a plant cell (Fig. 2). Similar results were found in barley (Torun et al. 2020), Ethiopian mustard (Husen et al. 2018), and soybean (Farhangi-Abriz and Ghassemi-Golezani 2018). The integrative application of SA + 500 mM NaCl enhanced protein content to a certain extent. This might aid in the maintenance of osmolarity in salinity-exposed cells (Mimouni et al. 2016). SA-modulated water content could be attributed to mediated ion transfer rate in plant tissues.

Variations in SA-mediated osmolyte accumulation, on the other hand, could be explained by Bose et al. (2017), who argued that SA activates multiple pathways, including a battery of antioxidant enzymes, to scavenge ROS in chloroplasts and protect photosynthetic machinery. The improvement in SA-mediated RWC of Juncao leaves (Fig. 6) under increasing salt stress could be directly linked to ions homeostasis maintenance (Fig. 2), which is consistent with previous research on barley cultivars (Torun et al. 2020). One of the most noticeable impacts of salt stress on plant growth is usually associated with the alteration in water balance together with the level of photosynthetic pigment (Maxwell and Johnson 2000).

Results of the current finding, however, revealed unaltered photosynthetic pigments (including Chl *a*, *b* and carotenoids) in Juncao, which was also attributable to the crosstalk between K^+ , Ca^{2+} , and Mg^{2+} ions coupled with mitigation effect on Na^+ or Cl^- induced phytotoxicity under escalated NaCl levels (Fig. 2 and 3). Na^+ and Cl^- are metabolically toxic to plants if accumulated in high concentrations in the cytoplasm (Teakle and Tyerman 2010). However, SA regulated photosynthetic pigments at a level comparable to non-stressed control plants, indicating SA-induced biostimulatory role in photoprotection. Moreover, chlorophyll content has a positive relationship with the electron transport chain during photosynthesis (Hassannejad et al. 2020). In comparison, carotenoids have a vital role in photoprotection and membrane protection from salinity-induced oxidative damages. Notably, the total Chl and Chl *a/b* ratios of our test plant were comparable to those of control plants under the SA-500 mM salinity regime, implying that SA application has no significant effect on photosynthetic pigments, except for a slight reduction in plant photosynthetic pigments in the absence of NaCl stress.

Interestingly, SA increased Chl *a/b* ratios in salt-stressed plants relative to corresponding NaCl counterparts, suggesting a preferential role of energy transferring among pigments, possibly due to salt stress-induced energy transfer from accessory pigment to principal pigment. This could also be attributed to the decrement of toxic ions in Juncao roots and shoots (Fig. 2), as previously observed in SA-treated soybean plants (Farhangi-Abriz and Ghassemi-Golezani 2018). The altered photosynthetic pigments in only SA-supplemented plants (without NaCl; Table 1), on the other hand, might be an adaptive strategy to avoid excessive light absorption, limiting ROS generation at the expense of photoinhibition. Hence, the increased Chl *a/b* ratio might refer to slight photoinhibition in the stressed plants. Comparable to our findings, foliar treatment of glycine betaine (GB) did not affect chlorophyll *a* and *b* content in canola under salinity (Athar et al. 2015), owing to variations in GB concentration, amount of exogenously applied GB on leaves,

and application technique (i.e., root or foliar). Arfan et al. (2007) prophesized that SA-induced photosynthetic enhancement in wheat would be associated with higher chlorophyll and carotenoid contents, which are dependent on a variety of non-stomatal and metabolic factors, such as Rubisco, PEP, and PSII activities, higher stomatal conductance, and CO_2 uptake. In general, maintaining water status and higher stomatal conductance improve CO_2 diffusion into the leaves, resulting in improved photosynthetic performance, and are the most intrinsic determinants of growth and plant responses to salinity stress (Li et al. 2014). On the contrary, reduction in chlorophyll content is a usual response of susceptible plants (including all glycophytic crops) to salinity-induced stress, which has been linked to the inhibition of the carbon assimilation process (Nieves-Cordones et al. 2019).

Based on their potential roles in the antioxidant and a myriad of therapeutic activities against plants and human diseases (Batista et al. 2019; Kim et al. 2006; Riedel et al. 2012), we investigated the abundance/performance of secondary metabolites including syringic acid, ferulic acid, luteolin, chlorogenic acid, quercetin, rutin trihydrate, kaempferol, hydroxycinnamic acid, and gallic acid using UHPLC analysis. However, our findings did not reveal any significant differences among the control and salt/SA-treated Juncao plants. Although it was observed that the overall abundance would show an increasing or decreasing trend as that of luteolin, chlorogenic acid, gallic acid and rutin trihydrate, however, the effects were of no statistical difference. Nevertheless, a high correlation was observed between these compounds and the Ca^{2+} contents in the stem, net ions flux ratios in plant tissues, and GR activity under the NaCl-SA regime. The increased production of bioactive compounds through environmental or genetic elicitors has been shown to protect plants under saline conditions through ROS scavenging and mitigating the ROS-induced damage due to their structural homology (Sharma et al. 2019).

Meanwhile, many secondary metabolites are stress induced, including several types of phenolics; however, it also depends on the species or variety of the plant (Sharma et al. 2019), as evidenced by the lower concentrations of syringic acid, luteolin, and gallic acid in Juncao following NaCl elicitation (Supplementary Fig. S2). These findings confirmed that not all bioactive compounds responded relatively similarly to varying levels of NaCl stress. The functions of these particular bioactive compounds, however, remained unclear in the current study as we did not observe any significant alterations except chlorogenic acid. This may be due to the genetic makeup of Juncao plants themselves which probably have different counteractive functions for regulating the NaCl-induced stress damage. Therefore, we intend to identify the main reason behind these findings in our future research work.

Conclusions

In conclusion, the current study evaluated the prevalence of NaCl concentrations that negatively impacted plant growth and developmental attributes of the Juncao plant. Meanwhile, SA treatment mitigated the detrimental effects of increasing NaCl concentrations on Juncao's morpho-physio-biochemical characteristics by modulating pleiotropic metabolic and cellular processes and enhancing nutrient ions (K^+ , Ca^{2+} , and Mg^{2+}). Furthermore, the cumulative accumulation of cationic contents exhibited a hormesis phenomenon in Juncao tissues under the NaCl-SA paradigm. A significantly higher amount of Ca^{2+} content was found in all tissues with NaCl-SA integration. Under increasing salinity, the integrative application of SA limited pernicious Cl^- ion accumulation in the root, stem, and leaves, as well as Na^+ content in the root and stem. In addition, the integrative application of SA enhanced the nutrient ion ratios (particularly K^+/Na^+ and K^+/Cl^-), followed by a substantial decrease in the net Cl^-/Ca^{2+} and Cl^-/Mg^{2+} ratios under increasing salinity stress. Also, SA supplementation mitigated the adverse effects of NaCl-induced ROS generation by maintaining MDA levels, redox homeostasis, and optimizing the battery of enzymatic and non-enzymatic antioxidants. Bioactive compounds showed variable responses towards the isolated or integrative application of NaCl and SA. Surprisingly, the integrative application of SA-NaCl enhanced chlorogenic acid concentrations in the Juncao plant, suggesting a way forward for local farmers, animal husbandry professionals, and pharmacologists. Consequently, our research will provide valuable insights on how to maximize putative phytochemical content in many other non-conventional crops, in addition to salt tolerance.

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Declarations

Conflict of interest The authors declare that they have no competing interests.

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