The Indian Journal of Agricultural Sciences **96** (8): 998–1006, August 2026/Article
https://doi.org/10.56093/ijas.v96i8.169528

Integrative insights into salinity stress mitigation and physio-molecular mechanisms in sugarcane (*Saccharum officinarum*)

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Received: 25 July 2025; Accepted: 28 July 2026

ABSTRACT

Sugarcane (*Saccharum officinarum* L.) is a major tropical and subtropical crop which is highly sensitive to salinity stress. Hence, the study was aimed to identify salt-tolerant genotypes and associated physio-molecular mechanisms of sugarcane by evaluating the five genotypes (Co 0118, Co 13035, Co 15023, Co 15025, and Co 0238), each maintained under control (non-saline) and saline irrigation conditions (EC_{iw} ~4, 8, and 10 dS/m). The continuous saline irrigation at EC_{iw} levels of ~4, 8, and 10 dS/m progressively increased soil ECe from an initial value of 0.81 dS/m (control) to 2.89, 6.91, and 8.79 dS/m, respectively, resulted in substantial salt buildup in the soil. Salinity reduced physiological performance; however, Co 15023 maintained higher chlorophyll content (18–25% higher SPAD values). Antioxidant enzymes were significantly enhanced, with Co 13035 showing 3.2-fold higher superoxide dismutase (SOD) and 2.8-fold higher catalase (CAT) activity under stress. At the molecular level, genes of the Salt Overly Sensitive (SOS pathway) (*SOS1, SOS2, SOS3*), along with *NHX1* (Na^+/H^+ exchanger 1) and *HKT* (High-affinity K^+ transporter), were significantly upregulated (fold change ≥5; $p \leq 0.05$) in Co 13035. Notably, *SOS1* expression showed up to 12-fold upregulation, indicating efficient ion homeostasis. Overall, Co 13035 was identified as the most salt-tolerant genotype due to its superior antioxidant defence and ion regulation, making it a promising candidate for cultivation and breeding in salt-affected regions.

Keywords: Ion transporter, Lipid peroxidation, Salt stress, Sugarcane, Salt overly sensitive

Sugarcane (*Saccharum officinarum* L.) is extensively grown worldwide for sugar and bioethanol production, covering approximately 26–27 mha globally with a total production exceeding 2.0 billion tonnes (FAO 2023–24); however, the adverse climatic and poor soil health conditions pose serious implications for its growth and development (Dhansu *et al.* 2022). Sugarcane is a glycophyte or salt sensitive crop that shows higher vulnerability to salinity stress at various growth phases i.e. germination, formative/ tillering, growth, and maturity (Pooja *et al.* 2020) causing morphological, physiological, metabolic, and molecular changes. Salt water of >8 dS/m and soil EC of >1.7 dS/m caused significant reduction in physiological traits at critical growth stages and reduced cane yield upto 50% (Dhansu *et al.* 2022) and every unit increase beyond ECe - 1.7 dS/m limits yield to the tune of 5.9% (Rao *et al.* 2021).

To adapt and survive in salt stress conditions, plants developed various physiological, biochemical and molecular mechanisms to cope with salt stress that mainly varies depending on the exposure, intensity, and developmental stage (Dhansu *et al.* 2022). Multiple signaling pathways modulate transcription of genes, which play key roles in regulating various physiological and biochemical processes consequently affecting plant growth under salt stress (Mann *et al.* 2023). The NHX (Na^+/H^+ Antiporters) and HKT (Histidine Kinase Transporter) genes, which encode K^+ transporters and channels, are involved in various biological responses in plants (Gupta *et al.* 2021). NHX1, located in the tonoplast, is vital for active K^+ uptake, stomatal function, and turgor regulation (Barragán *et al.* 2012). The HKT family improves salt tolerance by regulating ion transportation (Contiliani *et al.* 2022). Furthermore, the Salt Overly Sensitive (SOS) signaling pathway is a major regulatory mechanism for ion homeostasis under high salinity condition (Brindha *et al.* 2021). In addition to the role of ion transporters, salinity stress induces reactive

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oxygen species (ROS) (O_2^-, OH^-, and H_2O_2) formation in plant cells that causes oxidative damage. The antioxidant system plays a vital role in the plant's defence against salt stress, participating in this defence response by mitigating the damaging effects of ROS (Kaushik *et al.* 2024). This study investigated the role of these antioxidants both at the transcript and metabolite/activity levels. Non-enzymatic antioxidants, such as flavonoids, alkaloids, phenolic compounds, tocopherol, and carotenoids, also contribute by donating electrons to enzymatic antioxidants, ensuring a coordinated defence response (Pooja *et al.* 2020, Kumar *et al.* 2021). The present study investigated antioxidant responses at transcriptomic, enzymatic activity, and metabolite levels (ascorbate, glutathione, and carotenoids), alongside physiological traits. Five high-yielding sugarcane genotypes (Co 0118, Co 13035, Co 15023, Co 15025, and Co 0238) were evaluated under progressive salinity (EC_{iw} 4–10 dS/m) to elucidate genotype-specific tolerance mechanisms through integrated physio-biochemical-molecular analyses.

MATERIALS AND METHODS

Plant material and treatments: The experiment was conducted during 2022 and 2023 at ICAR-Sugarcane Breeding Institute, Regional Centre, Karnal, Haryana using five sugarcane genotypes (Co 0118, Co 13035, Co 15023, Co 15025, and Co 0238) under a randomised block design (RBD) with three biological and three technical replicates. Cemented pots (50 kg capacity) filled with soil (*p*H 7.9, ECe 0.55 dS/m, field capacity 28% v/v, bulk density 1.45 g/cc, porosity ~40%) were planted with single-budded sets. Chloride dominated salinity (7:3) stress was imposed using a defined salt mixture comprising NaCl, $CaCl_2 \cdot 2H_2O$, $MgCl_2.6H_2O$, and $MgSO_4.7H_2O$ in proportions of Na:(Ca+Mg) – 1:1, Ca:Mg – 1:3 and Cl:SO_4 – 7:3 (Datta *et al.* 2001). The salts were dissolved in irrigation water to achieve target irrigation water salinity (EC_{iw}) levels of 4 dS/m (moderate), 8 dS/m (severe), and 10 dS/m (extreme), along with control were maintained. Initially the pots were fully saturated with irrigation of 35 L water and thereafter a fixed volume of 5 L/pot/irrigation was applied at fortnight interval. Soil salinity (ECe) was monitored at 15-day intervals using the saturated paste extract method with a conductivity meter. Pots were closed at the bottom to avoid leaching and to maintain consistent salinity levels. Data on physiological, biochemical, and molecular traits were recorded at the tillering phase from three biological replicates.

Physio-biochemical observations: Fresh top visible dewlap (TVD) leaves from each replicate were collected early in the morning for estimation of total chlorophyll, carotenoid, and protein content. Total chlorophyll was determined following Gu *et al.* (2015) and carotenoids by Lichtenthaler and Buschmann (2001). Total soluble protein (TSP) was estimated using the method of Bradford (1976). Malondialdehyde (MDA) content was estimated as per Heath and Packer (1968) and H_2O_2 content was estimated by the method of Bindschedler *et al.* (2006). Superoxide dismutase (SOD, EC1.15.1.1) was assayed following Beauchamp and Fridovich (1971) by monitoring inhibition of photochemical reduction of nitroblue tetrazolium (NBT). Catalase (CAT, EC1.11.1.6) activity was determined according to Aebi (1984). Ascorbate peroxidase (APX, EC1.11.1.11) was estimated as per Nakano and Asada (1981) and Peroxidase (POX, EC1.11.1.7) activity was estimated following Rao *et al.* (1998). Ascorbic acid (AsA) content was estimated following Chinoy *et al.* (1978). AsA content was expressed as mg/g FW. Reduced glutathione (GSH) was determined following Law *et al.* (1983) and absorbance was read at 412 nm.

Differential gene expression: Total RNA was extracted from leaves using TRIzol reagent (Hi-Media). DNase I-treated RNA (1 µg) was reverse transcribed into cDNA using the iScript™ cDNA synthesis kit (Bio-Rad). The synthesized cDNA was diluted (10×) and used as a template for RT-qPCR analysis. Gene-specific primers were designed using PrimerQuest (IDT, https://eu.idtdna.com) and all primer sequences, including those of the housekeeping gene, are provided in the (Supplementary Table 1). Quantitative PCR was performed on a CFX96 Real-Time PCR System (Bio-Rad) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) in a 20 µl reaction volume. The thermal cycling conditions included an initial denaturation at 95°C for 20 s, followed by 40 cycles of 95°C for 3s and 62°C for 30s. Gene expression levels were normalised using GAPDH as the internal control (housekeeping) gene, selected based on its stable expression across treatments. Relative expression was calculated using the $2^{-}\Delta\Delta Ct$ method as described by Livak and Schmittgen (2001).

Statistical analysis: Data were tested for normality using the Shapiro-Wilk test, and non-normal variables were appropriately transformed. Two-way ANOVA was performed using SAS software (v9.3; SAS Institute Inc., USA) on triplicate data. Mean comparisons were made using Duncan's multiple range test (DMRT) at a 5% significance level.

RESULTS AND DISCUSSION

Build-up of salinity in soil due to prolonged saline irrigation: Continued irrigation with saline water of EC_{iw} ~ 4, 8, and 10 dS/m led to a progressive buildup of soil electrical conductivity (ECe) over time compared to the initial ECe value (0.63 dS/m before sowing). At the formative phase, a critical stage for sugarcane growth, soil ECe significantly increased with rising irrigation salinity from 0.76 dS/m under control to 2.21, 5.54, and 7.87 dS/m under EC_{iw} levels of 4, 8, and 10 dS/m, respectively. By harvest, the ECe further escalated, reaching 0.81 dS/m in the control, and 2.89, 6.91, and 8.79 dS/m under EC_{iw} ~4, 8, and 10 dS/m treatments, respectively (Supplementary Fig. 1). The ECe rise observed during the formative stage, a key phase for tiller formation and root development that could interfere with water and nutrient uptake, potentially reducing plant vigour and final yield.

Physio-biochemical traits: The results showed that chlorophyll (photosynthetic pigments) significantly decreased under salinity stress, with reductions of 5.92%,

19.85%, and 25.72% under irrigation water salinity of 4, 8 and 10 dS/m, respectively (Fig. 1A). Among the genotypes, Co 15023 exhibited the highest total chlorophyll content, whereas Co 0238 recorded the lowest (Fig. 1A). In contrast, SPAD values (greenness index) were highest in Co 13035 (47.10), followed by Co 0238 (45.62) under control conditions (Fig. 1B). The variation between chlorophyll content and SPAD values may be attributed to differences in leaf thickness, internal structure, and optical properties, as SPAD provides a relative estimate rather than absolute quantification of chlorophyll. Salinity stress significantly reduced SPAD values by 8.78%, 18.16%, and 27.31% at EC_{iw} ~ 4, 8, and 10 dS/m, respectively. Based on individual parameter responses, Co 13035 showed better maintenance of greenness under stress, whereas Co 0238 exhibited comparatively lower chlorophyll content despite relatively higher SPAD values, indicating genotypic variation in physiological response (Rao *et al.* 2021).

Moreover, similar declining trend was observed in carotenoid content with increasing salinity stress. Specifically, genotype Co 0238 was found to have the highest content of carotenoids, with genotype Co 13035 following closely (Fig. 1C). Carotenoids, known to scavenge excess energy and protect the photosystems from oxidative damage (Mitra 2012), were relatively stable in these genotypes, suggesting an effective ROS-quenching mechanism under salinity. The downward trend in photosynthetic pigment content across genotypes under salinity stress highlights the importance of considering physiological traits such as chlorophyll and carotenoid levels when selecting for salinity tolerance in plant breeding programmemes (Hammami *et al.* 2024).

Soluble protein content: Under control condition, the mean genotypic concentration of total soluble proteins (TSPs) was approximately 9.35 mg/g FW while intensified stress conditions demonstrated a reduction of 9.30% at EC_{iw} ~ 4 dS/m, 22.82% at EC_{iw} ~ 8 dS/m and a substantial 40.10% at EC_{iw} ~ 10 dS/m (Fig. 1D). The reduction in protein content is attributed to ionic toxicity and osmotic stress, which impair protein structure and metabolism, in line with previous studies (Passamani *et al.* 2017). Among the genotypes, Co-cane 13035 exhibited the least decline in TSP at EC_{iw}~4 dS/m with a marginal reduction of 3.82%, whereas Co-cane 15023 experienced the most significant decrease of 40.62%. At EC_{iw} ~8 dS/m, the mean genotypic reduction was observed to be 22.45%, and an even more pronounced reduction of 39.87% was recorded at EC_{iw} ~10 dS/m (Fig. 1) (Supplementary Table 2). This suggests that these genotypes may possess efficient protein-protection mechanisms or increased synthesis of stress-related proteins (Arefian *et al.* 2019). The maintenance of higher protein levels in Co 13035, in particular, reflects an enhanced osmotic adjustment capacity and possibly greater expression of protective proteins under stress (Sarker *et al.* 2019).

MDA and H_2O_2 content: The mean MDA concentration increased as salinity increased, i.e. 0.360, 0.430, and 0.546 µg/g FW at EC_{iw} of approximately 4 dS/m, ~8 dS/m, and ~10 dS/m, respectively. Among sugarcane genotype, Co-cane 15025 had the highest MDA (0.439 µg/g FW), followed by Co-cane Co 15023 (0.420 µg/g FW), with the least noted in Co 0118 (0.317 µg/g FW) (Table 1).

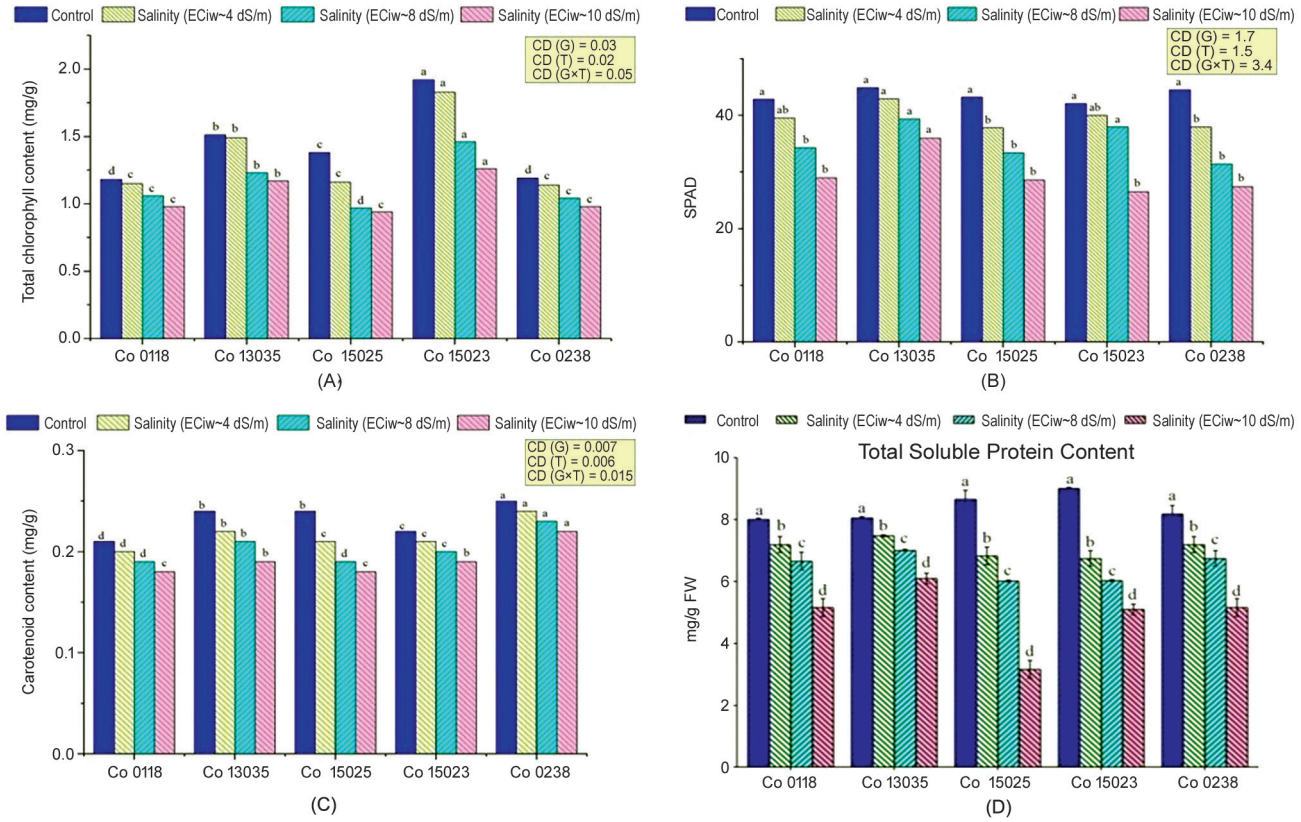

Fig. 1 Effect of varying irrigation water salinity on photosynthetic pigments and total soluble protein content of sugarcane genotypes.

Table 1 Effect of different saline water irrigation on MDA and H_2O_2 content of sugarcane genotypes

Treatments/Traits	MDA	H_2O_2
Irrigation water salinity (T)		
Control	0.235[d]	528.51[d]
EC_{iw} ~4 dS/m	0.360[c]	1019.94[c]
EC_{iw} ~8 dS/m	0.430[b]	1321.85[b]
EC_{iw} ~10 dS/m	0.546[a]	1673.04[a]
LSD (p=0.05) (T)	0.02	31.74
Genotypes (G)		
Co 0118	0.317[d]	1056.46[c]
Co 13035	0.362[c]	1018.94[d]
Co 15025	0.439[a]	1406.48[a]
Co 15023	0.420[b]	1139.89[b]
Co 0238	0.413[b]	1057.46[c]
LSD (p=0.05) (G)	0.017	35.49
LSD (p=0.05) (T × G)	0.03	70.97

Means having at least one common letter are non-significant statistically (p<0.05) by Dunkan`s Test.

MDA, Malondialdehyde content.

MDA serves as an important marker of membrane lipid peroxidation and is indicative of cellular damage caused by oxidative stress (Saleem *et al.* 2022). However, H_2O_2 content escalated with increased salinity, registering 92.98%, 150.11%, and 216.56% increases at EC_{iw} ~4 dS/m, ~8 dS/m, and ~10 dS/m, with respect to the control. The maximum H_2O_2 content was observed in Co-cane 15025 (1406.48 µg/g FW), and the minimum in Co-cane 13035 (1018.94 µg/g FW) (Table 1). H_2O_2 acts both as a damaging agent and a signaling molecule. Its controlled accumulation triggers antioxidant defence response, while excessive levels result in oxidative injury (Saleem *et al.* 2022).

Enzymatic and non-enzymatic antioxidant: SOD activity was enhanced under salt stress conditions with respective increases of 96.70% at EC_{iw} ~4 dS/m, 135.44% at EC_{iw} ~8 dS/m, and 199.32% at EC_{iw} ~10 dS/m. Co-cane 13035 exhibited maximum SOD (34.45 U/min/mg protein) activity, whereas Co-cane 15025 had the minimum SOD (12.02 U/min/mg protein) activity (Table 2).

This upregulation of SOD activity under salinity indicates an active first-line defence mechanism against superoxide radicals, converting them into H_2O_2 in chloroplasts (Duarte *et al.* 2013). Both CAT and POX activity exhibited an increase in response to salinity; with a 27.18% and 54.66% increase at EC_{iw} ~ 4 dS/m, 79.49% and 148.26% increases at EC_{iw} ~8 dS/m, and 170.26% and 311.75% increase at EC_{iw} ~10 dS/m. The highest CAT activity was observed in Co 13035 (5.04 U/min/mg protein), along with the highest POX activity (227.36 U/min/mg/protein). In contrast, the lowest CAT activity was found in Co 15025 (1.99 U/min/mg/protein), and the lowest POX activity was also observed in Co 15025 (56.15 U/min/mg/protein) (Table 2). The observed increase in CAT and POX activity supports the role of these enzymes in mitigating oxidative damage by decomposing H_2O_2 into water, with CAT functioning mainly in the cytoplasm and POX operating across various cellular compartments (Gill and Tuteja 2010).

The activity of APX increased significantly under salt stress in five Co-cane genotypes (Table 2). Compared to control conditions, APX activity showed a 65.11% increase at EC_{iw} ~4 dS/m, a 144.19% increase at EC_{iw} ~8 dS/m and a 279.07% increase at an EC_{iw} of ~10 dS/m. Co-cane 13035 had the highest APX activity (1.06 U/min/mg/

Table 2 Effect of different saline water irrigation on enzymatic and non-enzymatic antioxidant content of sugarcane genotypes

Treatments/Traits	SOD	CAT	APX	POX	AsA	GSH
Irrigation water salinity (T)						
Control	11.82[d]	1.95[a]	0.43[d]	56.77[d]	0.35[d]	2.55[b]
EC_{iw} ~4 dS/m	23.25[c]	2.48[b]	0.71[c]	87.80[c]	0.63[c]	4.43[c]
EC_{iw} ~8 dS/m	27.83[b]	3.50[c]	1.05[b]	140.94[b]	0.86[b]	5.45[b]
EC_{iw} ~10 dS/m	35.38[a]	5.27[a]	1.63[a]	233.75[a]	1.30[a]	6.56[a]
LSD (p=0.05) (T)	1.240	0.07	0.04	3.83	0.03	0.11
Genotypes (G)						
Co 0118	28.62[b]	3.20[c]	0.99[b]	129.47[c]	0.43[e]	5.14[b]
Co 13035	34.45[a]	5.04[a]	1.06[a]	227.36[a]	0.29[d]	5.79[a]
Co 15025	12.02[d]	1.99[e]	0.87[d]	56.15[e]	1.14[a]	3.98[e]
Co 15023	23.57[c]	2.64[d]	0.92[cd]	102.25[d]	0.95[b]	4.16[d]
Co 0238	24.21[c]	3.63[b]	0.93[c]	133.84[b]	0.80[c]	4.79[c]
LSD (p=0.05) (G)	1.39	0.08	0.05	4.28	0.03	0.13
LSD (p=0.05) (T × G)	2.77	0.16	0.10	8.57	0.06	0.25

Means having at least one common letter are non-significant statistically (p<0.05) by Dunkan`s Test. SOD, Superoxidase dismutase; CAT, Catalase activity; APX, Ascorbate peroxidase; POX, Peroxidase activity; AsA, Ascorbic acid; GSH, Glutathione.

protein), followed by Co 0118 (0.99 U/min/mg/protein) and Co 0238 (0.93 U/min/mg/protein) (Table 2). APX, primarily functioning in the chloroplast, plays a crucial role in reducing H_2O_2 using ascorbate as an electron donor in the ascorbate-glutathione cycle (Noctor and Foyer 1998). The pronounced APX activity under stress further highlights the activation of this cycle, which is a critical anti-oxidative defence mechanism in plants under salinity stress. GSH content showed an incremental increase in relation to the control, with a 73.72% increase at EC_{iw} ~4 dS/m, 113.72% at EC_{iw} ~8 dS/m, and 157.25% at EC_{iw} ~10 dS/m. The highest GSH content was found in Co-cane 13035 (5.79 mg/g FW), while the lowest was recorded in Co-cane 15025 (3.98 mg/g FW) (Table 2). Glutathione, a key non-enzymatic antioxidant, plays an important role in redox homeostasis and acts directly in quenching ROS. Its increase under stress conditions is a significant indicator of enhanced anti-oxidative potential (Bistgani *et al.* 2019). AsA content also exhibited an upward trend under salt stress, showing a 80% increase at EC_{iw} ~4 dS/m, 145.71% increase at EC_{iw} ~8 dS/m, and 271.43% increase at EC_{iw} ~10 dS/m in comparison to the control. The highest ascorbic acid content was noted in Co-cane 15025 (1.14 mg/g FW), while the lowest was in Co-cane 13035 (0.29 mg/g FW) (Table 2). The elevated ascorbate levels under salinity stress reaffirm its role in cellular protection. Increased levels of ascorbic acid under salinity have also been observed in crops like *Brassica rapa* (Mittal *et al.* 2018). The cumulative enhancement in both enzymatic (SOD, CAT, APX, POX) and non-enzymatic (GSH, ASA) antioxidants observed in this study confirms the activation of a robust antioxidative defence mechanism in sugarcane genotypes under saline conditions. Co-cane 13035 maintained higher enzymatic and GSH levels, suggesting superior oxidative stress management, whereas Co 15025, despite exhibiting the highest AsA accumulation (1.14 mg/g FW), showed comparatively lower enzymatic activities (SOD: 12.02; CAT: 1.99 U/min/mg protein), indicating an uncoupled antioxidant defence system. This suggests that although non-enzymatic antioxidants were elevated, insufficient activity of key antioxidant enzymes may have limited the efficient regeneration of AsA via the AsA–GSH cycle. Consequently, the lack of coordination between enzymatic and non-enzymatic components likely impaired ROS detoxification, thereby explaining the relatively poor stress tolerance of this genotype despite high ascorbate levels (Hasanuzzaman *et al.* 2019).

Gene expression analysis: The three *SOS* (salt overly sensitive) genes were differentially expressed in leaves under salinity stress in studied Co-canes. The interaction between salinity and genotype was highly significant. Genotypes Co 13035 and Co 0118 exhibited the highest expression of *SOS1*, with increases of 5 and 4.5-fold at EC_{iw} ~ 4 dS/m, and 12 and 10-fold at EC_{iw} ~ 8 dS/m, respectively, compared to the control (Fig. 2A–C). This pattern indicates their enhanced capability for Na^+ efflux, a key component of salt stress tolerance. These findings support the established role of SOS1 as a plasma membrane Na^+/H^+ antiporter that actively extrudes Na^+ from the cytoplasm, thereby maintaining electrochemical homeostasis and enhancing salinity tolerance (Zhou *et al.* 2016). Similarly, the *SOS2* showed similar trend under varied salinity stress with maximum expression was observed in Co 15023, followed by Co 13035, with 6-fold and 5-fold upregulation at EC_{iw} ~ 4 dS/m, respectively as compared to control. At EC_{iw} ~ 8 dS/m, the highest *SOS2* expression was observed in Co 13035, followed by Co 0118, showing 14-fold and 12.5-fold upregulation, respectively as compared to control. This aligns with previous evidence that SOS2, a serine/threonine protein kinase activated by salt-induced Ca^{2+} signals, is essential for downstream SOS pathway activation in salt-tolerant genotypes (Zhao *et al.* 2021). At EC_{iw} ~ 4 dS/m, the highest *SOS3* expression was observed in Co 13035 and Co 15023, with 5-fold and 4.5-fold upregulation, respectively as compared to control. At EC_{iw} ~ 8 dS/m, the highest expression levels were observed in Co 0238 and Co 13035, with 11-fold and 10.5-fold upregulation, respectively as compared to control. SOS3, a Ca^{2+}-binding protein, acts as a sensor in the SOS pathway, activating SOS2 in response to ionic stress. Its higher expression, especially in Co 0238, reflects genotype-specific adaptability. These findings corroborate with Aslam *et al.* (2019), who reported increased *SOS3* expression in pineapple under salt treatment.

In addition to the *SOS* gene, the *NHX1* and *HKT* genes play crucial roles in plant salt stress tolerance by regulating sodium (Na^+) and potassium (K^+) transport, thereby maintaining ion homeostasis under saline conditions. At EC_{iw} ~8 dS/m, the expression of *NHX1* was significantly upregulated in Co 0238, Co 13035, and Co 0118, with upregulation of 11-fold, 9-fold, and 8.5-fold, respectively as compared to control (Fig. 2D). This suggests vacuolar sequestration of Na^+ as a mechanism of salt tolerance. *NHX1* encodes a vacuolar Na^+/H^+ exchanger that reduces cytosolic Na^+ concentration by sequestering excess ions into vacuoles (Sharma *et al.* 2022). Similarly, the *HKT* expression pattern was almost similar across all genotypes at EC_{iw} ~ 4 dS/m. whereas, at EC_{iw}~8 dS/m, *HKT* expression was highly upregulated in Co13035 and Co 0238, showing 11-fold and 10-fold upregulations, respectively as compared to the control (Fig. 2E). HKT transporters play a pivotal role in Na^+ exclusion and K^+ retention, crucial for maintaining a high K^+/Na^+ ratio under saline conditions (Jabeen *et al.* 2022). Collectively, these gene expression patterns demonstrate both common and genotype-specific responses to salinity. Salt stress induces both ionic and osmotic imbalances, and the activation of ion transporters and antiporters like *SOS1, NHX1,* and *HKT* are essential for restoring ion homeostasis (Brindha *et al.* 2021). The most salt-tolerant genotypes, particularly Co 13035 and Co 0118, consistently exhibited higher expression levels of all key genes, emphasising their robust ionic regulation systems. These results further reinforce the view that genotypic variability in expression of ion transport-related genes directly contributes to the observed differences in salt tolerance across Co-canes.

The expression level of antioxidant enzymatic genes

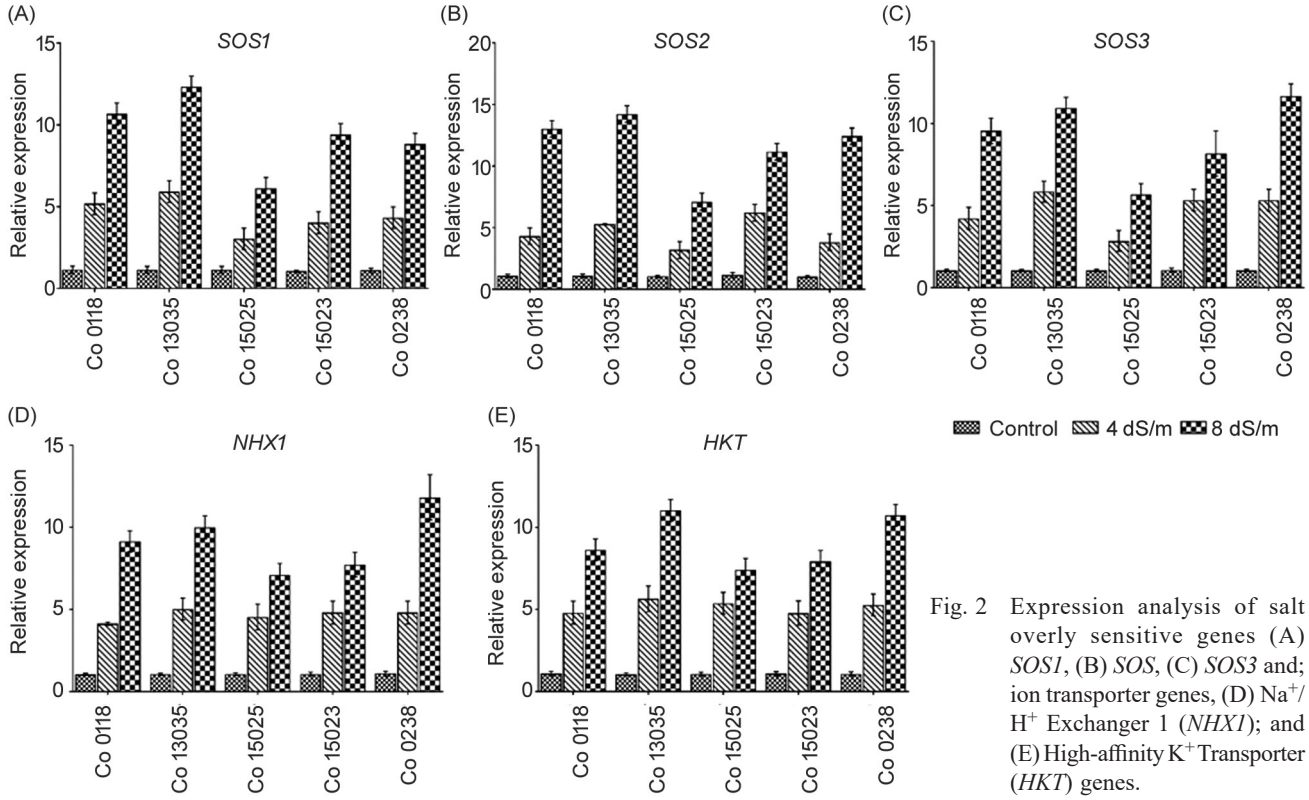

Fig. 2 Expression analysis of salt overly sensitive genes (A) *SOS1*, (B) *SOS*, (C) *SOS3* and; ion transporter genes, (D) Na^+/H^+ Exchanger 1 (*NHX1*); and (E) High-affinity K^+ Transporter (*HKT*) genes.

such as *SOD, CAT, POX, APX*, and *GR* were analysed (Fig. 3A–E). The expression of *SOD* gene showed maximum upregulated expression, increasing by 2.50-fold in Co 13035 and 2.10-fold in Co 0118, as compared to control at ECiw~8 dS/m. A similar pattern was observed for *CAT* and *POX* expression. Co 0118 and Co 13035 exhibited the highest *CAT* expression levels, with increases of 2.10-fold and 2.00-fold and 1.90-fold and 1.80-fold at EC_{iw}~4 dS/m and EC_{iw}~8 dS/m, respectively, compared to their respective controls. POX gene expression was upregulated under both salinity treatments (EC_{iw} ~4 dS/m and EC_{iw} ~8 dS/m), but the maximum upregulation was noted in Co 13035 and

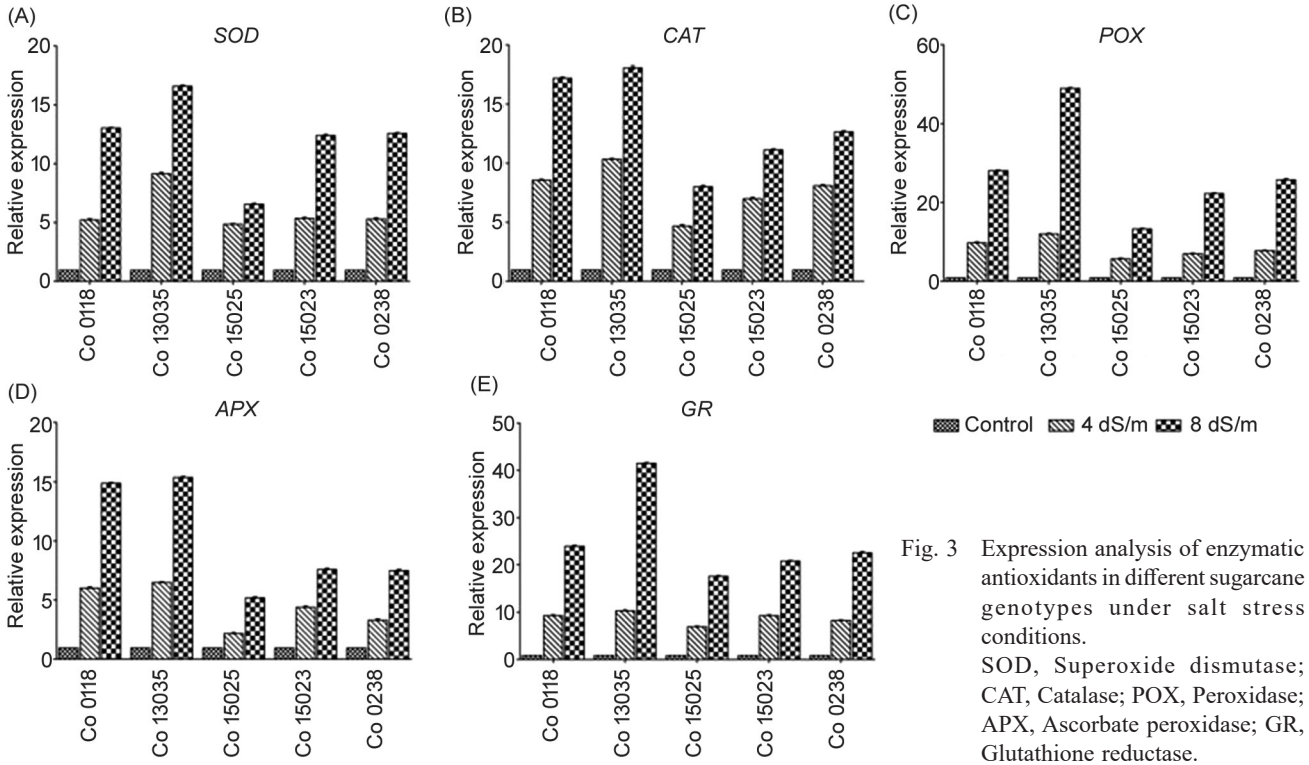

Fig. 3 Expression analysis of enzymatic antioxidants in different sugarcane genotypes under salt stress conditions.
SOD, Superoxide dismutase; CAT, Catalase; POX, Peroxidase; APX, Ascorbate peroxidase; GR, Glutathione reductase.

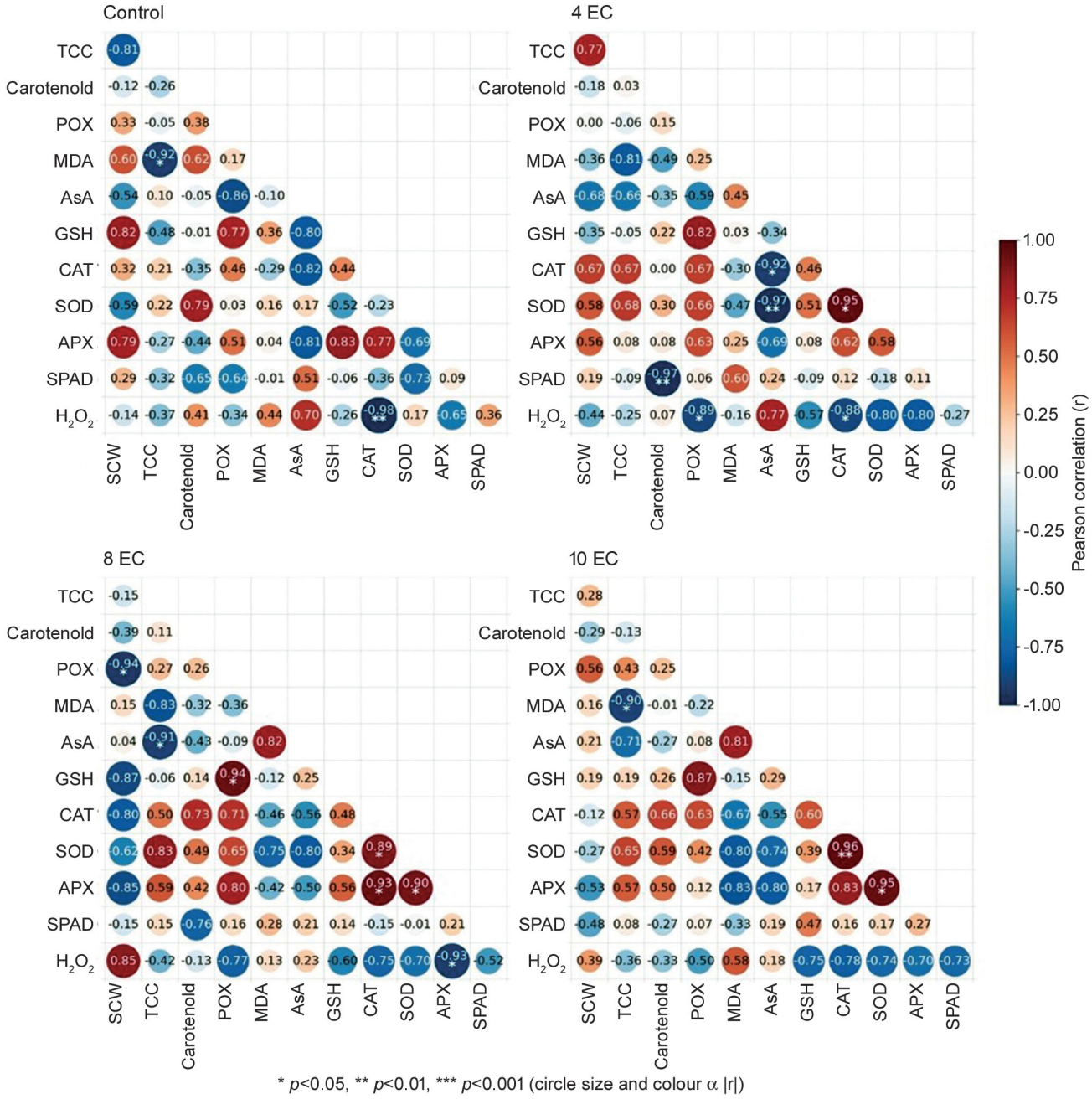

Fig. 4 Scatter plots, correlation matrices, and box-and-whisker plots generated for traits associated with single cane weight (SCW) under four different environmental conditions.
TCC, Total chlorophyll content; SCW, Single cane weight; POX, Peroxidase; MDA, Malondialdehyde content; AsA, Ascorbic acid; GSH, Glutathione; CAT, Catalase; SOD, Superoxide dismutase; APX, Ascorbate peroxidase; SPAD, Soil plant analysis development.

Co 0118 at EC_{iw} ~ 8 dS/m, showing increases of 2.60-fold and 2.00-fold, respectively, compared to the control. The *APX* gene also showed maximum expression at EC_{iw} ~8 dS/m, increasing by 2.00-fold in Co 13035 and by 1.75-fold in Co 0118, while the minimum upregulation was observed in Co 15025 among all genotypes. *GR* gene expression was most significantly upregulated in Co 13035 and Co 0118, showing increases of 1.70-fold and 1.45-fold at EC_{iw}~4 dS/m, compared to the control. At EC_{iw} ~8 dS/m, *GR* expression was maximum in Co 13035, followed by Co 0238, with increases of 2.65-fold and 2.20-fold, respectively, compared to the control. These results align with earlier findings suggesting that antioxidant gene induction is a vital defence mechanism to neutralise ROS under saline conditions (Brindha *et al.* 2021, Zhao *et al.* 2021).

Moreover, the increased expression of antioxidative genes such as *SoSOD*, *SoCAT*, *SoPOD*, *SoAPX*, and *SoGR* indicates an adaptive strategy to mitigate the damaging effects of salt-induced oxidative stress. The increasing salinity levels led to a significant rise in relative gene

expression of enzymatic antioxidants compared to plants grown in non-saline soils (Lamizadeh *et al.* 2019).

Pearson's correlation analysis: In the visual representation, box-and-whisker plots for the control and all three salinity stress conditions (4, 8, and 10 dS/m) were placed on the right side of the canvas, while area plots were aligned along the diagonal to depict the distribution trends of each trait. Under salinity stress, the boxplots revealed a consistent increase in H_2O_2, MDA, SOD, CAT, APX, POD, AsA, and GSH, along with a decline in TCC, SPAD values, carotenoid content, and single cane weight (Fig. 4). Correlation analysis under 4 dS/m salinity and control conditions indicated that single cane weight (SCW) exhibited strong positive correlations with SPAD, TCC, CAT, and APX. Additionally, a significant positive correlation was observed between GSH and SCW under control conditions. Under higher salinity levels (8 and 10 dS/m), H_2O_2 showed a positive correlation with SCW. Given its role as a reactive oxygen species (ROS) and a signaling molecule, H_2O_2 likely contributes to stress tolerance by inducing the expression of antioxidant enzymes (CAT, APX, SOD, POD) and modulating abscisic acid (ABA)-mediated stomatal closure, thereby conserving water and supporting stress resilience. In the current population, this response appears to play a crucial role in sustaining cane yield under stress. AsA showed a negative correlation with SCW under control and mild stress (4 dS/m), suggesting that excessive ascorbic acid under non-stress conditions might interfere with ROS signaling necessary for optimal growth.

The diagonal elements represent area plots showing the distribution of each trait. The upper triangle of the matrix displays Pearson correlation coefficients across all four conditions, while the lower triangle contains the corresponding scatter plots. The statistical significance of correlation coefficients is indicated by asterisks positioned above the values, with *** for $p \leq 0.001$; ** for $p \leq 0.01$; and * for $p \leq 0.05$.

Despite this, a positive correlation between AsA and H_2O_2 across all treatment's highlights AsA's consistent role in ROS scavenging (Fig. 4). TCC was positively correlated with antioxidant enzymes SOD and CAT across all conditions and with APX specifically under salinity stress. Functionally, SOD catalyzes the conversion of superoxide radicals (O_2^-) into H_2O_2, which is subsequently broken down by CAT into water and oxygen, thus mitigating oxidative stress.

In conclusion, the findings highlight the importance of genetic diversity in stress adaptation and suggest that Co 13035 possesses inherent mechanisms that confer resilience to salinity stress. Continued research on genetic manipulation and breeding strategies could lead to developing salt-tolerant sugarcane varieties for future agricultural sustainability.

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