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DISSECTING SEEDLING-STAGE SALINITY TOLERANCE IN COTTON FOR MORPHOPHYSIOLOGICAL AND BIOCHEMICAL TRAITS USING MULTIVARIATE ANALYSIS

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SUMMARY

Salinity stress is a major constraint in the upland cotton (*Gossypium hirsutum* L.) productivity, particularly at the seedling stage, where ion toxicity, osmotic imbalance, and oxidative damage severely impair early growth. This study dissected salt tolerance mechanisms across 20 diverse cotton genotypes subjected to three salinity stress levels: control (1.6 dS/m), moderate (12 dS/m), and severe (17 dS/m). Plants, evaluated at the fourth true leaf stage, had their key morphophysiological and biochemical parameters checked. Elevated salinity significantly reduced shoot and root biomass, while sodium accumulation and Na^+/K^+ ratios sharply increased, indicating disrupted ionic homeostasis. For plants to cope with this stress, antioxidant enzyme activities—superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT)—markedly rose, reflecting activation of ROS detoxification pathways. The principal component analysis (PCA) differentiated tolerant and sensitive genotypes by integrating multiple traits. CIM-595, Mubarak, CIM-612, and FH-152 were distinct as tolerant genotypes with balanced ionic profiles and elevated antioxidant responses, whereas sensitive lines, such as SB-149, KZ-181, and AGC-999, performed poorly under severe salinity. These findings provide mechanistic insight into cotton's adaptive strategies and offer robust targets for breeding programs in saline-prone agroecosystems.

Keywords: Cotton (*G. hirsutum* L.), salinity, seedling stage, salt tolerance, PCA, morphophysiological and biochemical traits

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Key findings: This study identified salt-tolerant cotton (*G. hirsutum* L.) genotypes through principal component analysis, which identified CIM-595, Mubarak, CIM-612, and FH-152 as salt-tolerant genotypes. These lines maintained balanced Na^+/K^+ ratios and elevated antioxidant enzyme activities, indicating effective ionic regulation and oxidative stress mitigation. Their performance under high salinity supports their use in breeding salt-resilient cotton.

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is a globally cultivated fiber and oilseed crop, yet its productivity faces increasing threats from salinity stress, particularly in arid and semi-arid regions. In Pakistan, over 6.3 million hectares of irrigated land incur adverse effects from salinization, causing annual agricultural losses exceeding USD 71.264 million (20 billion PKR) (Mohanavelu *et al*., 2021; Basak *et al*., 2022). Salinity impairs crop performance primarily by disrupting ion balance and water uptake, triggering a cascade of physiological, morphological, and metabolic disturbances. At the seedling stage, excess Na^+ and Cl^- ions inhibit germination, reduce osmotic potential, and impair membrane function (Chen *et al*., 2020). As the stress intensifies during vegetative growth, plants exhibit reduced stomatal conductance, suppressed photosynthetic activity, and altered respiration rates, collectively reducing biomass accumulation (Koudahe *et al*., 2021). Morphologically, salinity leads to diminished root and shoot elongation, lower fresh and dry weight, and reduced leaf expansion. Prolonged exposure also causes premature abscission of reproductive organs, ultimately degrading fiber yield and quality (Ul-Allah *et al*., 2021). Beyond these morphological impairments, salinity further induces biochemical changes through the overproduction of reactive oxygen species (ROS), which plants attempt to mitigate via antioxidant enzymes. These effects become aggravated more by oxidative stress as ROS accumulate beyond detoxification capacity, damaging cellular structures unless counteracted by antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) (Syed *et al*., 2021).

Biochemical markers play a pivotal role in evaluating genotypic responses to salinity, particularly those linked to oxidative stress mitigation. High external concentrations of Na^+ disrupt cellular ion homeostasis and trigger excessive production of ROS, including superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH) (Qamer *et al.,* 2021). These ROS damage vital biomolecules, such as lipids, nucleic acids, and proteins, impairing cellular integrity. Counteractive actions cause plants to activate an enzymatic antioxidant defense system comprising superoxide dismutase (SOD), peroxidases (POD), and catalase (CAT), which scavenge and neutralize ROS. Specifically, SOD isoforms Cu/Zn-SOD (cytosolic), Mn-SOD (mitochondrial), and Fe-SOD (chloroplastic) catalyze the dismutation of O_2^- into H_2O_2. This entails subsequent detoxification by CAT and POD into harmless water and oxygen, maintaining redox balance. The efficiency of this detoxification cascade is a critical determinant of salinity tolerance, as also reported by Ul-Allah *et al*. (2021), who highlighted the role of enhanced antioxidant activity in mitigating salt-induced oxidative stress and sustaining cotton growth under saline environments (Peng *et al.,* 2016).

Addressing salinity stress requires integrative tools capable of analyzing complex trait interactions (Ahmed *et al*., 2024). Unlike univariate methods, multivariate approaches, such as principal component analysis (PCA), capture correlations among traits and genetic variation across genotypes, enabling effective identification of key contributors to salt tolerance (Yeater *et al*., 2015). Importantly, PCA can differentiate tolerant and sensitive genotypes by integrating multiple morphophysiological and biochemical traits, providing a robust basis for trait-based

Table 1. Genotypes analyzed in the study.

No.	Genotypes	S. No.	Genotypes
1	VH-282	11	FH-169
2	FH-172	12	AGC-999
3	CIM-612	13	MNH-1076
4	IUB-212	14	FH-942
5	FH-114	15	SB-149
6	CRS-456	16	FH-215
7	Mubarak	17	FH-154
8	S-3	18	MNH-129
9	KZ-181	19	NIAB-545
10	FH-171	20	CIM-595

screening (Zhang *et al*., 2018). PCA has been instrumental in evaluating morphophysiological and biochemical responses to abiotic stress, aiding the selection of superior genotypes under saline conditions (Zafar *et al*., 2022). However, the combined use of antioxidant activity, ion balance, and biomass traits remains underexploited in cotton breeding. This study applies PCA to integrate these traits, offering a refined strategy for selecting salt-resilient genotypes suited for saline-prone and marginal lands (Zhang *et al*., 2018).

MATERIALS AND METHODS

The experiment commenced at the Department of Plant Breeding and Genetics, University of Agriculture Faisalabad (UAF), under controlled conditions. Twenty diverse upland cotton (*G. hirsutum* L.) genotypes, selected based on salinity adaptability, came from UAF, Central Cotton Research Institute (CCRI), Cotton Research Institute (CRI), Ayub Agricultural Research Institute (AARI), and Nuclear Institute for Agriculture and Biology (NIAB) (Table 1).

Seeds reached delinting and soaking overnight in 15 mM $CaSO_4$ to improve germination uniformity and reduce ionic shock (Ali *et al*., 2008). The next day, sowing seeds continued in 20-cm perforated polyethylene cups filled with washed river sand, a non-saline substrate ensuring uniform ion distribution. Each cup received five seeds, later thinned to one seedling. The experiment followed a completely randomized design with three replicates per genotype per treatment. This resulted in a total of 540 experimental units (20 genotypes × 3 treatments × 3 replications × 3 plants). Salinity stress imposition ensued by irrigating with NaCl-enriched water at electrical conductivity levels of 12 dS/m (moderate) and 17 dS/m (severe), while maintaining the control at 1.6 dS/m. Gradual raising of salinity from 7.5 dS/m to the target levels avoided osmotic shock (Munawar *et al*., 2021). Plants received irrigation with half-strength Hoagland nutrient solution every other day (Arif *et al*., 2023). Seedlings grown for 25 days had a natural photoperiod at ~30±2 °C. When the fourth true leaf reached full development, recording morphological, physiological, and biochemical measurements occurred.

Measurement of morphophysiological parameters

At 25 days after sowing, seedlings sustained gentle removal and rinsing with deionized water. Separating shoots and roots proceeded at the cotyledonary node. Shoot length (SL) and root length (RL) measurements used a graduated ruler. Fresh weights of shoot and root tissues entailed immediate recording using a precision electronic balance. For dry biomass determination, samples underwent oven-drying at 70 °C ± 2 °C for 72 hours until constant weight, following Arif *et al*. (2023). For ionic analysis, dried leaf samples' fine grinding and digesting utilized a mixture of nitric and sulfuric acids (HNO_3:H_2SO_4) under controlled heating. The resulting extracts' filtration took place before use to determine sodium (Na^+) and potassium (K^+) concentrations via flame photometry, according to the protocol of Ali *et al*. (2008).

Measurement of biochemical parameters

Biochemical assays continued on fresh leaf tissues harvested at the fourth true leaf stage. For each genotype, 0.1 g of leaf material underwent homogenization in 1 mL of 50 mM potassium phosphate buffer (pH 7.4) and centrifugation at 14,000 × g for 10 min at 4 °C. The resulting supernatant served for duplicate enzyme activity assays (Munawar *et al*., 2021). Peroxidase (POD) activity measurement used the guaiacol-H_2O_2 method by recording absorbance at 470 nm every 20 seconds (Chance and Maehly, 1955). Catalase (CAT) activity determination proceeded by monitoring the decomposition of H_2O_2 at 240 nm, defining one unit as a 0.01 decrease in absorbance per minute (Aebi, 1984). Superoxide dismutase (SOD) activity assessment was via inhibition of nitroblue tetrazolium (NBT) photoreduction; one unit inhibited 50% of NBT reduction (Beauchamp and Fridovich, 1971). Total soluble protein (TSP) quantification followed by the Bradford method, with absorbance measured at 595 nm (Bradford, 1976). The measurement units were TSP (mg/g FW), POD (units/g FW), CAT (units/g FW), SOD (units/g FW), proline (μmol/g FW), and Na^+ and K^+ concentrations (ppm).

Statistical analysis

Descriptive statistics calculations emerged in Microsoft Excel. The conduct of two-way ANOVA proceeded in RStudio (v4.1.3) to assess the effects of genotype, salinity treatment, and their interaction in all traits, with significance set at $p < 0.05$ and $p < 0.01$. Data visualization, including histograms and violin plots, progressed using the ggplot2 package (Wickham *et al*., 2016). Principal component analysis (PCA) took place with 'factoextra' (Kassambara and Mundt, 2017) to identify key traits influencing salinity response. Pearson correlation coefficients and trait network generation used the 'qgraph' package (Epskamp *et al*., 2012).

RESULTS

Analysis of variance

The two-way ANOVA revealed highly significant ($p < 0.01$) effects of cotton (*G. hirsutum* L.) genotypes (G), salinity treatments (T), and their interactions (G × T) across all measured traits. These included shoot length (SL), root length (RL), fresh shoot weight (FSW), dry shoot weight (DSW), fresh root weight (FRW), dry root weight (DRW), total soluble proteins (TSP), antioxidant enzyme activities (POD, CAT, SOD), proline, and ionic content (Na^+, K^+, and Na^+/K^+ ratio) (Table 2). These findings indicate considerable genetic variability and differential physiological responses under salt stress, supporting the potential for genotype selection.

Descriptive analysis of cotton genotypes and their traits under salt stress

Descriptive statistics and violin plots (Figure 1) demonstrated clear shifts in morphophysiological and biochemical traits across salinity treatments. The SL and RL declined progressively from control (T0) to severe stress (T2), indicating inhibition of elongation growth. Similar reductions resulted in shoot and root biomass, as reflected in the FSW, DSW, FRW, and DRW, highlighting impaired water uptake and carbon assimilation in stress.

Total soluble protein (TSP) decreased with increasing salinity, reflecting reduced protein stability and synthesis. In contrast, antioxidant enzymes responded positively: POD, CAT, and SOD activities increased with stress intensity, peaking under T2, which signifies activation of enzymatic defenses against ROS.

Proline content rose steadily from T0 to T2, supporting its role in osmotic adjustment and protection of cellular structures in stress. Ionic traits showed opposite shifts: sodium

Table 2. Descriptive statistics and analysis of variance for three treatments and 14 morphological, physiological, and biochemical traits.

Traits	T0		T1		T2		ANOVA MSS		
	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Genotype (G)	Treatment (T)	Genotype × Treatment (G × T)
SL	19.44 - 31.46	24.01±3.47	15.25 - 27.07	19.77±3.24	11.62 - 21.69	17.17±3.19	74.152^{**}	569.153^{**}	3.665^{**}
RL	13.07 - 28.97	21.24±4.32	6.07 - 20.18	14.29±4.89	6.09 - 20.13	13.15±4.63	166.57^{**}	1334.98^{**}	12.57^{**}
FSW	2.42 - 6.34	4.48±0.66	2.32 - 5.43	4.06±0.80	2.32 - 5.93	4.22±0.89	4.98946^{**}	2.49842^{**}	0.81614^{**}
DSW	1.36 - 4.13	2.92±0.68	1.07 - 3.90	2.46±0.66	1.20 - 3.95	2.51±0.75	2.96701^{**}	5.81735^{**}	0.95729^{**}
FRW	1.23 - 4.60	3.02±0.56	0.94 - 3.72	3.18±0.61	1.20 - 4.21	2.72±0.84	3.35038^{**}	1.59603^{**}	0.71543^{**}
DRW	0.08 - 2.98	1.83±0.65	0.19 - 3.01	1.70±0.63	0.29 - 2.79	1.50±0.67	3.07464^{**}	0.71856^{**}	0.70548^{**}
TSP	5.62 - 10.53	8.64±1.37	3.05 - 7.55	5.82±1.10	3.89 - 8.07	5.99±1.39	11.921^{**}	121.558^{**}	1.504^{**}
POD	4.36 - 10.64	7.81±1.58	5.66 - 10.61	8.07±1.30	7.66 - 12.27	9.68±1.57	12.809^{**}	76.9091^{**}	2.9857^{**}
CAT	11.97 - 24.19	19.06±3.55	14.14 - 27.35	23.41±3.83	13.17 - 34.04	26.66±3.93	71.43^{**}	1023.34^{**}	32.47^{**}
SOD	17.04 - 31.73	23.08±3.84	19.13 - 33.35	27.56±3.56	27.36 - 35.73	30.77±2.63	41.115^{**}	962.667^{**}	25.233^{**}
Proline	3.03 - 6.30	4.49±0.63	2.71 - 7.13	4.68±0.91	4.40 - 7.87	5.91±1.32	5.0925^{**}	41.4084^{**}	1.4403^{**}
Na^+	9.65 - 32.26	22.79±7.55	19.98 - 66.80	44.69±18.86	29.97 - 100.21	65.84±24.32	2220.8^{**}	33684.6^{**}	193.7^{*}
K^+	119.64 - 165.99	141.20±17.28	83.65 - 129.89	101.97±12.49	73.68 - 127.80	94.01±16.58	1509.3^{*}	37937.8^{**}	260.9^{**}
Na^+/K^+	0.06 - 0.27	0.16±0.06	0.15 - 0.75	0.49±0.20	0.26 - 1.30	0.80±0.33	0.33352^{**}	6.15020^{**}	0.05580^{**}

Notes: ANOVA and descriptive statistics for 14 seedling traits of cotton under T0, T1, and T2 salinity. Includes MSS, SD, and p-values for genotype (G), treatment (T), and G × T interaction. *p < 0.05; **p < 0.01; and NS = nonsignificant.

(Na^+) accumulation increased sharply with rising salinity; potassium (K^+) levels decreased; and the Na^+/K^+ ratio rose substantially, confirming progressive ionic imbalance (Table 2, Figure 1).

Correlation

The Pearson correlation analysis under control (T0 = 1.6 dS/m), moderate (T1 = 12 dS/m), and severe (T2 = 17 dS/m) salinity stress revealed dynamic relationships among morphophysiological and biochemical traits, reflecting the progressive impact of salinity on cotton seedlings (Figure 2).

Under control conditions (T0), strong positive correlations appeared among growth traits (SL, RL, FSW, DSW, FRW, and DRW), indicating coordinated vegetative development. Antioxidant enzyme activities (CAT and SOD) also displayed interrelations, suggesting a tightly regulated oxidative defense even under non-stressed conditions. In contrast, Na^+ content showed negative associations with growth traits and K^+, implying early signs of ionic antagonism.

At moderate salinity (T1), these correlations weakened. Growth remained positively related but less robust. Proline showed stronger positive correlations with biomass traits, underscoring their role in osmotic adjustment under moderate stress. Na^+ became increasingly antagonistic to growth traits and to K^+, reinforcing the onset of ionic imbalance.

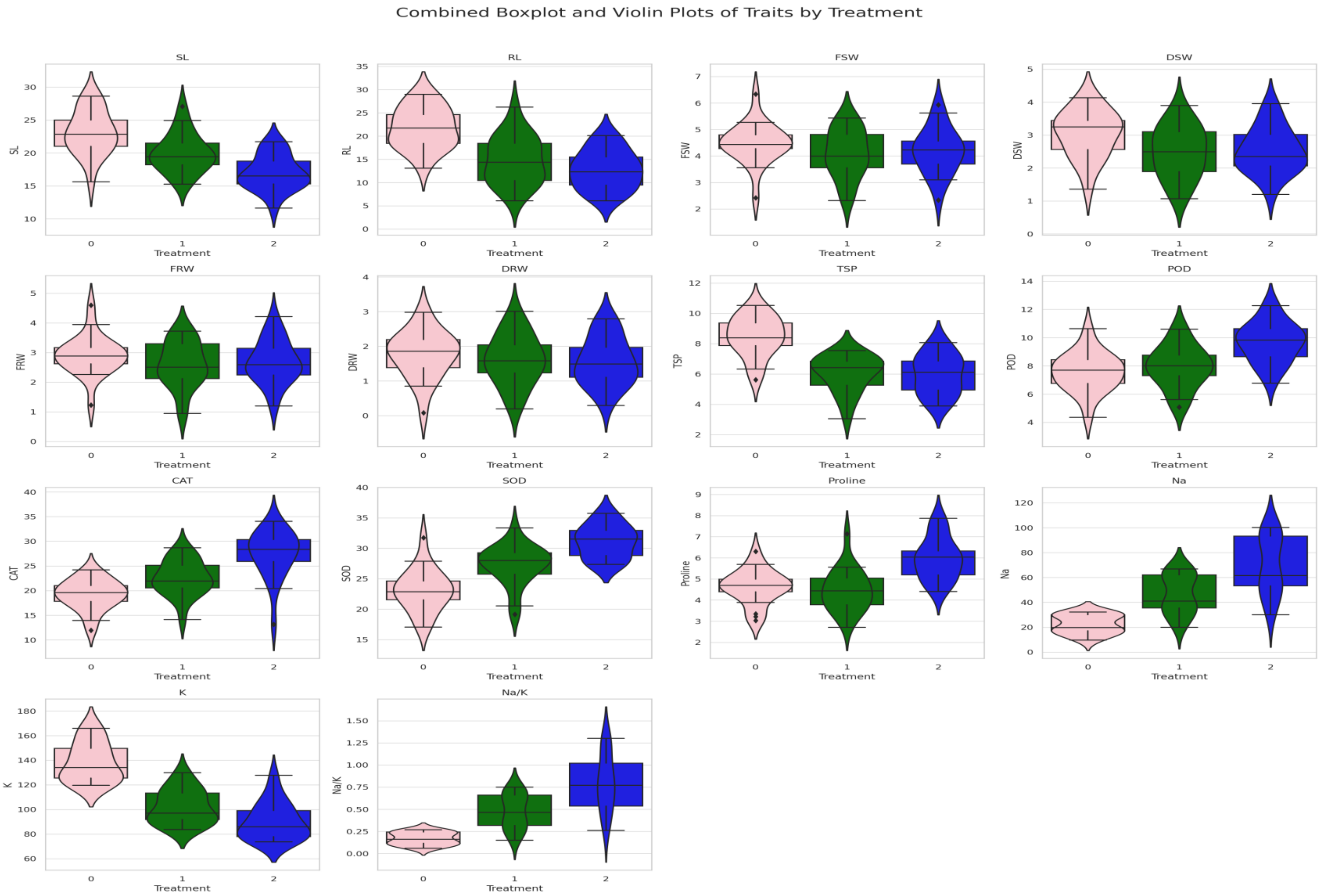

Figure 1. Box and violin plots showing the distribution of 14 morphological, physiological, and biochemical traits in 20 cotton genotypes under control, mild, and severe salinity stress.

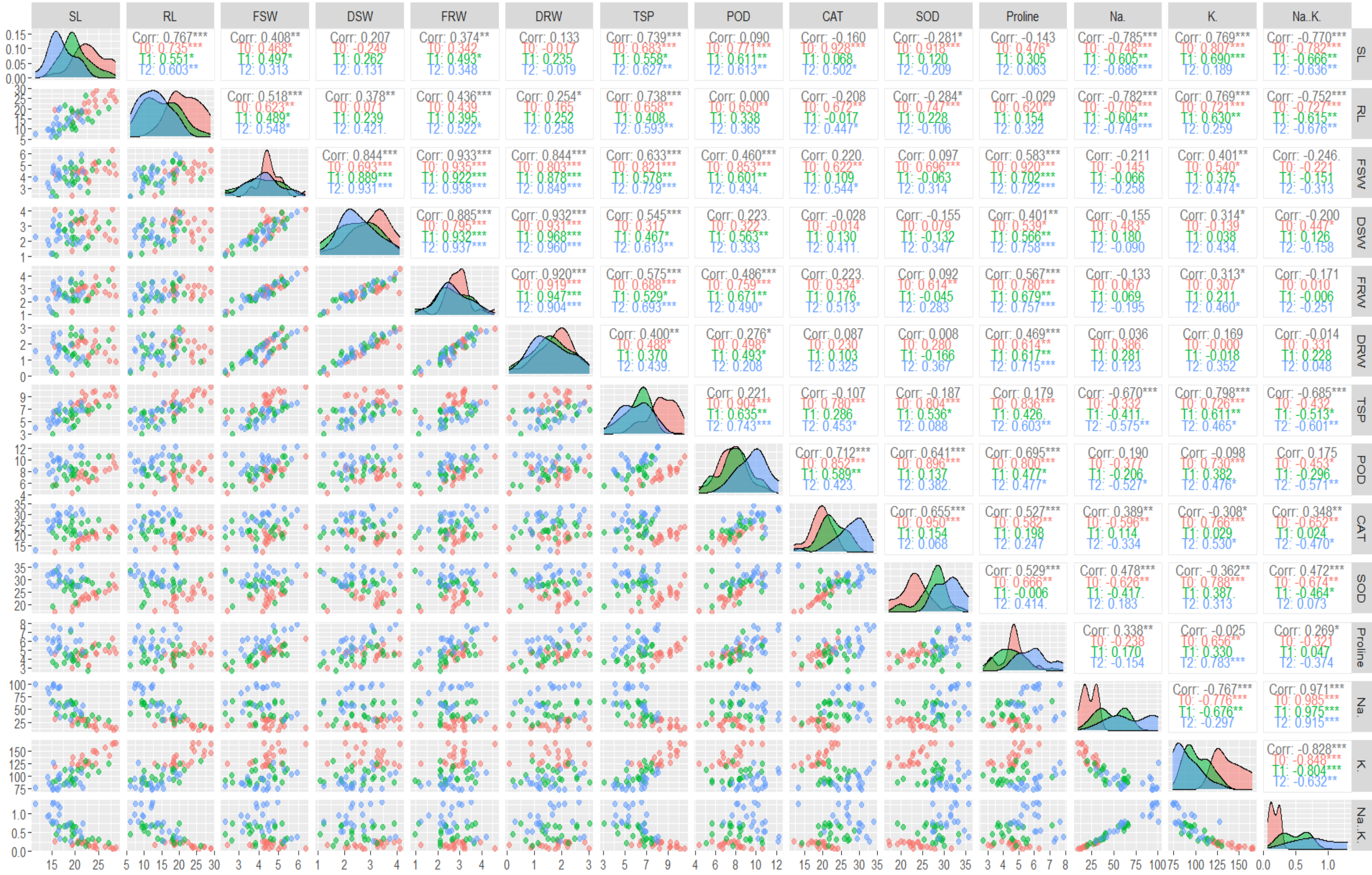

Figure 2. Scatter plot matrix showing correlations among 14 morphophysiological and biochemical traits of cotton genotypes under control (T0, red), moderate (T1, green), and severe (T2, blue) salinity stress; significant correlations are indicated by *, **, and ***.

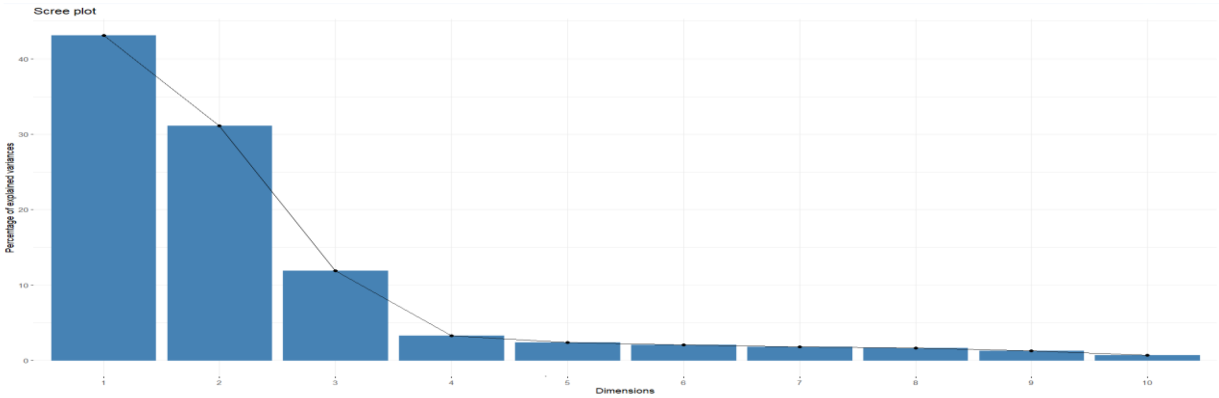

Figure 3A. Scree plot explaining the contribution percentage of principal components in salt stress.

Under severe salinity stress (T2), stress-induced shifts intensified. Growth traits, such as SL and RL, remained positively related, but Na^+ showed markedly negative correlations with both growth and K^+, confirming severe ionic disruption. Notably, proline's association with stress tolerance gained reinforcement, exhibiting strong positive correlations with biomass traits, suggesting its pivotal role in biomass preservation under high salinity. CAT and SOD remained positively correlated, highlighting continued antioxidant synergy under severe oxidative stress.

Multivariate analysis

Principal component analysis (PCA) employment helped explore trait interrelationships and classify cotton genotypes based on their responses to increasing salinity stress. The scree plot (Figure 3A) indicated the first two principal components, PC1 and PC2, captured 74.24% of the total variance, with PC1 explaining 43.14% and PC2 accounting for 31.10%. PC1 mainly represented growth and ionic traits, while PC2 captured antioxidant and osmotic adjustment responses, framing the biological meaning of these components. Thus, these two components became useful for biplot construction and genotype discrimination.

The PCA biplot (Figure 3B) visualizes genotypic distribution and trait associations under three treatments: T0 (control, blue), T1 (moderate stress, yellow), and T2 (severe stress, orange). Trait vectors aligned with PC1 and PC2 illustrate their contributions and interrelationships, where smaller angles (< 90°) denote positive correlations, orthogonal vectors (~90°) imply independence, and opposing directions indicate negative associations.

Under control conditions (T0), genotypes clustered in the positive quadrant of PC1 and revealed associations with growth-favorable traits, such as shoot length (SL), root length (RL), potassium (K^+), and total soluble proteins (TSP). This reflects vigorous growth and nutrient balance in non-saline environments. Conversely, traits such as sodium (Na^+), Na^+/K^+ ratio, superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and proline loaded strongly in stress conditions (T1, T2), indicating their roles in oxidative stress defense and ionic regulation under salinity.

Genotypic distribution and salt tolerance

Salt-tolerant genotypes, such as G20 (CIM-595) and G7 (Mubarak), clustered close to both stress-associated traits and their control counterparts, suggesting minimal performance decline under salinity. CIM-595 showed high associations with proline and SOD, indicating strong oxidative stress management. Mubarak maintained stable trait expression across treatments, reflecting robust adaptability. These genotypes represent promising candidates for salt-resilient cotton breeding programs. G3 (CIM-612) and G16 (FH-152)

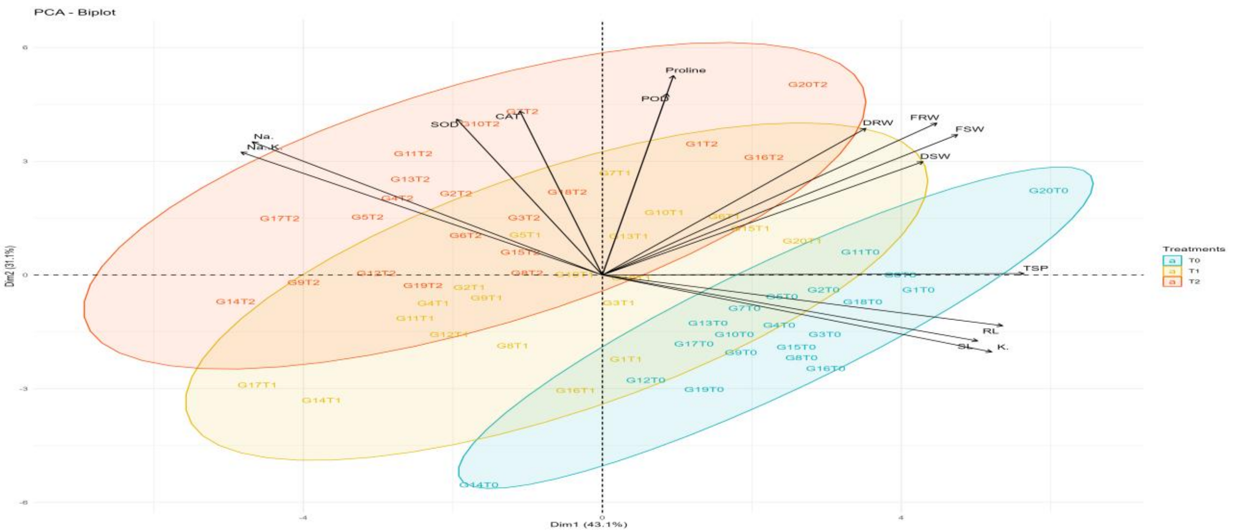

Figure 3B. PCA biplot (PC1 vs PC2) showing contributions of morphological and biochemical traits to variability in upland cotton under control (T0, blue), moderate (T1, yellow), and severe (T2, red) salinity stress.

displayed moderate to high tolerance, maintaining consistent performance and clustering between T0 and T2 zones. In contrast, G14 (SB-149), G9 (KZ-181), G10 (AGC-999), and G17 (FH-154) shifted distinctly toward stress-vulnerable zones in the biplot under T2. These genotypes expressed associations with higher Na^+ and Na^+/K^+ ratios and had lower scores for growth and defense traits, indicating limited tolerance.

DISCUSSION

Salinity is a complex environmental constraint that disrupts water uptake, nutrient acquisition, and cellular homeostasis in cotton. Although *Gossypium hirsutum* shows moderate salt tolerance (Wu *et al*., 2020), the early vegetative stage is particularly sensitive, with excess Na^+ surpassing the osmotic threshold and causing germination failure, reduced shoot-root growth, and ionic toxicity (Koudahe *et al*., 2021; Balasubramaniam *et al*., 2023).

Growth traits declined markedly under 12 and 17 dS/m NaCl treatments, reflecting osmotic inhibition and ionic injury. These effects align with previous reports linking salinity to stomatal closure, reduced CO_2 assimilation, and inhibited root elongation (Flowers and Colmer, 2008; Liu *et al*., 2010; Guo *et al*., 2020). Sensitive genotypes showed sharper reductions, confirming the dual-phase injury of osmotic stress followed by ionic toxicity (Malakar and Chattopadhyay, 2021).

Maintaining a favorable K^+/Na^+ ratio emerged as a central determinant of tolerance. CIM-595, Mubarak, CIM-612, and FH-152 exhibited lower Na^+ accumulation and better K^+ retention in stress, suggesting reliance on exclusion and vacuolar sequestration mechanisms (Gupta and Huang, 2014). This supports the established role of Na^+/K^+ balance as a predictive marker of tolerance in cotton and other crops (Flowers and Colmer, 2008; Sikder *et al*., 2020).

Salinity-induced ROS overproduction triggered strong antioxidant responses. Elevated activities of SOD, POD, and CAT, particularly in CIM-595 and FH-152, highlight activation of detoxification pathways that convert superoxide radicals to H_2O_2 and then to water and oxygen (Hasanuzzaman *et al*., 2021; Challabathula *et al*., 2022). Positive associations between antioxidant activity and biomass confirm their protective role in sustaining photosynthesis, consistent with earlier reports in cotton (Patil *et al*., 2024).

Proline accumulation further supported stress adaptation, functioning as an osmolyte and molecular chaperone. Its strong correlation with biomass indicates physiological significance, in agreement with earlier findings in cotton (Meloni *et al*., 2001; Qamer *et al*., 2021). The integration of proline with antioxidant activity and ion regulation underscores its utility as a biochemical marker of tolerance.

PCA provided an effective framework for differentiating genotypes. Together, PC1 and PC2 explained 74.24% of the total variation. PC1 mainly represented growth and ionic traits, while PC2 captured antioxidant and osmotic adjustment responses. Tolerant genotypes (CIM-595, Mubarak, CIM-612, and FH-152) clustered with traits such as SOD, CAT, and proline, highlighting adaptive synergy, while sensitive lines (SB-149, KZ-181, and AGC-999) grouped with high Na^+ and Na^+/K^+ ratios, confirming poor regulatory capacity. These results are consistent with earlier PCA-based evaluations of cotton in stress (Yeater *et al*., 2015; Zafar *et al*., 2022).

This study establishes a trait-based framework for identifying salt-tolerant cotton. However, since salinity tolerance is complex and polygenic, future work should integrate quantitative trait loci (QTL) mapping, genome-wide association studies (GWAS), and transcriptomics to uncover stress-responsive genes (e.g., *GhSOS1*, *GhNHX1*). Genomic selection and high-throughput phenotyping, combined with validation at reproductive and maturity stages under field conditions, will strengthen breeding applications.

CONCLUSIONS

This study successfully identified salt-tolerant cotton (*G. hirsutum* L.) genotypes, particularly CIM-595, Mubarak, CIM-612, and FH-152, through a comprehensive evaluation of morphophysiological and biochemical traits under progressive salinity stress. Key tolerance indicators included stable biomass retention, efficient Na^+/K^+ regulation, elevated antioxidant enzyme activities (SOD, POD, and CAT), and proline accumulation, all of which mitigated ionic and oxidative stress. Correlation and PCA analyses revealed strong trait interdependence and enabled genotype differentiation based on adaptive responses. These findings not only validate trait-based screening as a valuable selection tool but also provide foundational insight for future integration with genomic approaches. The identified genotypes represent promising candidates for breeding programs aimed at improving cotton resilience in saline-prone agroecosystems. However, this study focused only on the seedling stage, which is particularly sensitive to salinity. Tolerance expression may differ at reproductive and maturity stages; therefore, future research should extend to later developmental phases to confirm the stability and practical relevance of these findings. Future studies should expand to larger and more diverse germplasm panels and integrate molecular tools such as QTL mapping, GWAS, and transcriptomics. This will help identify key genes and pathways involved in salt tolerance and strengthen the practical use of trait-based screening in cotton breeding.

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