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Research Article

Integrated Physiological Characterization and Multivariate Analysis Reveal Sulfate Salinity Tolerance in Upland Cotton (*Gossypium hirsutum* L.) Genotypes

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Abstract

Background and Objective: Soil sulfate salinity is an increasing constraint to cotton production in arid and semi-arid regions; however, the physiological mechanisms underlying sulfate-dominated salinity tolerance in upland cotton remain poorly understood. This study aimed to characterize the physiological responses of upland cotton genotypes to increasing sulfate salinity and identify reliable physiological indicators and tolerant germplasm for breeding. **Materials and Methods:** Nineteen upland cotton (*Gossypium hirsutum* L.) genotypes were evaluated under lysimeter conditions at three sulfate salinity levels: Control, EC = 6 dS/m and EC = 12 dS/m. Plant height, photosynthetic pigments, gas-exchange parameters, water-use efficiency and leaf temperature were assessed. Data were analyzed using two-way ANOVA, Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), Random Forest, hierarchical clustering, variable importance in projection (VIP) analysis and a Composite Sulfate Salinity Tolerance Index. **Results:** Genotype, salinity level and genotype $\times$ salinity interaction significantly affected all measured traits ($p < 0.001$). Increasing sulfate salinity reduced plant height, chlorophylls, carotenoids, photosynthetic rate, transpiration, stomatal conductance and water-use efficiency, while increasing intercellular CO₂ concentration and leaf temperature. A PCA explained 76.24% of the total variation and effectively differentiated tolerant, moderately tolerant and sensitive genotypes. Across multivariate analyses, photosynthetic rate, water-use efficiency, chlorophyll pigments, carotenoids and leaf temperature emerged as the most informative indicators of sulfate salinity tolerance. Genotypes T-1004, T-1002, T-1050, T-1024, T-1005 and T-1003 demonstrated superior physiological stability, whereas C-6524, T-1023 and T-1037 showed the greatest sensitivity. **Conclusion:** The integration of physiological traits with multivariate analyses effectively discriminated sulfate salinity tolerance among upland cotton genotypes. The identified physiological markers and tolerant genotypes provide useful resources for screening and breeding cotton cultivars adapted to sulfate-dominated saline soils.

Key words: *Gossypium hirsutum*, sulfate salinity, physiological traits, photosynthesis, water-use efficiency, multivariate analysis, PCA, PLS-DA, random forest, cotton breeding

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is one of the world's most important fiber crops and a major source of cottonseed oil, protein-rich feed and industrial raw materials. Much of the global cotton-growing area is located in arid and semi-arid regions, where freshwater scarcity, saline irrigation, high evapotranspiration and secondary soil salinization significantly constrain productivity¹. Although cotton is generally regarded as more salt-tolerant than many glycophytic crops, its tolerance varies considerably among genotypes and depends on developmental stage, salinity intensity, exposure duration and salt composition. Salinity is particularly detrimental during germination and early seedling establishment, reducing emergence, biomass accumulation and plant vigor. Genome-wide analysis of 196 upland cotton accessions identified substantial genetic variation in salinity tolerance and several genomic regions associated with salt adaptation². Similarly, hydroponic evaluation of eleven cotton genotypes demonstrated that relative water content and photosynthetic rate are reliable physiological indicators for distinguishing salt-tolerant and salt-sensitive genotypes³.

Salinity restricts cotton growth through osmotic stress, ionic toxicity, oxidative damage and disruption of photosynthetic carbon assimilation. Reduced water uptake is followed by excessive ion accumulation, resulting in nutrient imbalance, membrane injury, impaired enzyme activity and altered cellular metabolism. Consequently, plant height, biomass production, pigment concentration and photosynthetic capacity decline, whereas oxidative stress and antioxidant defense responses increase^{4,5}. Because these responses differ markedly among genotypes, physiological traits provide an effective basis for identifying salt-tolerant germplasm. Salt-tolerant cultivars generally maintain higher photosynthetic activity, pigment stability, growth and antioxidant capacity than sensitive cultivars under saline conditions⁴. Similar genotypic variation has also been reported for shoot growth, root development, chlorophyll content, ion accumulation and biomass production in upland cotton under salinity stress^{3,6,7}.

Most previous studies have focused on Sodium Chloride (NaCl)-induced salinity, although agricultural saline soils usually contain mixtures of chloride, sulfate, bicarbonate, carbonate, calcium, magnesium and sodium salts. Consequently, plant responses to NaCl alone may not accurately represent adaptation to sulfate-dominated soils. Sulfate and chloride salts differ in ion mobility, nutrient interactions, sulfur metabolism, osmotic effects and tissue accumulation patterns. Comparative studies have shown that

cotton genotypes exhibit distinct physiological responses to NaCl- and Na₂SO₄-dominated salinity despite similar osmotic conditions, indicating that salt composition strongly influences tolerance mechanisms. Sulfate-specific studies further demonstrated that Na₂SO₄ inhibits shoot and root growth, disrupts membrane integrity and mineral distribution and induces extensive physiological and proteomic changes, whereas tolerant cultivars maintain stronger antioxidant protection and ion homeostasis than sensitive ones^{5,8}.

Photosynthetic performance is one of the most informative indicators of salinity tolerance because it directly reflects carbon assimilation efficiency. Net photosynthetic rate depends on stomatal conductance, intercellular CO₂ concentration, pigment stability, photochemical efficiency, enzymatic activity and plant water status. Salinity initially limits photosynthesis through stomatal closure and restricted CO₂ diffusion, whereas prolonged stress causes chloroplast damage, pigment degradation, oxidative injury and inhibition of photosynthetic enzymes^{3,4,9}. Therefore, simultaneous assessment of photosynthetic rate, stomatal conductance, transpiration and intercellular CO₂ provides a comprehensive evaluation of salinity tolerance. Water-use efficiency (WUE), reflecting the balance between carbon assimilation and water loss, is another key indicator because tolerant genotypes generally maintain relatively high photosynthetic activity while minimizing transpiration³. Likewise, leaf temperature reflects plant water status through transpirational cooling, whereas chlorophyll a, chlorophyll b and carotenoids indicate the structural and functional integrity of the photosynthetic apparatus under stress^{4,6,10}. Although plant height and biomass are simple measurements, they integrate the cumulative effects of water availability, nutrient uptake, photosynthesis and assimilate partitioning throughout plant development, making them valuable indicators of stress adaptation^{3,6,7}.

Because salinity tolerance is a complex quantitative trait, evaluation based on individual physiological parameters often produces inconsistent conclusions. Integrating multiple physiological traits with multivariate statistical approaches provides a more reliable assessment of genotype performance. Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA), Partial Least Squares Discriminant Analysis (PLS-DA), variable importance in projection (VIP) and Random Forest algorithms effectively identify the principal physiological determinants of salinity tolerance and improve genotype classification. Previous studies have confirmed that combining multivariate analyses with physiological traits enhances the reliability of salinity evaluation compared with single-trait approaches^{2,3,11}.

Despite significant advances in understanding NaCl-induced salinity responses, physiological adaptation to sulfate-dominated salinity remains poorly understood. In particular, the integrated contribution of gas-exchange parameters, photosynthetic pigments, water-use efficiency, growth traits and modern multivariate analytical approaches has rarely been investigated under sulfate salinity. Therefore, this study evaluated the physiological responses of 19 upland cotton (*Gossypium hirsutum* L.) genotypes exposed to three sulfate salinity levels under lysimeter conditions.

MATERIALS AND METHODS

Plant materials: Nineteen upland cotton (*Gossypium hirsutum* L.) genotypes, namely Afsona, C-6524, Guliston, Hosilot, Mehnat, T-66, T-88, T-1002, T-1003, T-1004, T-1005, T-1023, T-1024, T-1037, T-1050, T-1090, Yuksalish, Yulduz-2 and Zafar-2, were evaluated for their physiological responses to sulfate salinity. The plant materials included locally cultivated varieties and advanced breeding lines representing broad variation in growth performance and salinity adaptation. Seeds were obtained from the cotton germplasm collection of the Institute of Genetics and Plant Experimental Biology, Qibray, Uzbekistan. Before sowing, only healthy, uniform and mechanically undamaged seeds were selected to minimize variation associated with seed quality.

Experimental site and design: The experiment was conducted during the 2025 and 2026 growing seasons at the experimental station of the Institute of Genetics and Plant Experimental Biology, Qibray, Uzbekistan, under lysimeter conditions. Sulfate-saline soil was collected from the Mirzaobod District of the Syrdarya Region, where sulfate-dominated salinity is widespread. After air drying, homogenization and sieving through a 5-mm mesh, the soil was transferred into lysimeters.

Three salinity treatments were established according to the electrical conductivity (EC) of the saturated soil extract: Control (EC < 2 dS/m), moderate sulfate salinity (EC = 6 dS/m) and severe sulfate salinity (EC = 12 dS/m). Salinity levels were adjusted using analytical-grade Sodium Sulfate (Na_2SO_4), which was dissolved in irrigation water and applied gradually to avoid osmotic shock during seedling establishment. Soil EC was monitored regularly throughout the experiment and additional saline solution was supplied whenever necessary to maintain the target salinity levels.

The experiment was arranged in a Randomized Complete Block Design (RCBD) with three biological replicates. Nineteen cotton genotypes were evaluated under three salinity levels,

resulting in 171 experimental units ($19 \times 3 \times 3$). Agronomic practices, including irrigation, fertilization, weed control and pest management, were applied uniformly to all lysimeters. Therefore, observed differences among treatments were attributed exclusively to sulfate salinity.

Gas-exchange measurements: Gas-exchange parameters were measured on fully expanded, healthy leaves using a GFS-3000 Portable Gas Exchange Fluorescence System (Heinz Walz GmbH, Effeltrich, Germany), following standard procedures for leaf gas-exchange measurements¹². Measurements were conducted during the morning under stable environmental conditions to minimize variation associated with changes in irradiance, temperature and vapor pressure deficit. For each experimental plot, three representative plants were selected and one fully expanded mature leaf from each plant was measured after the gas-exchange parameters had stabilized inside the leaf chamber. The instrument was calibrated and operated according to the manufacturer's instructions. Net photosynthetic rate (A), transpiration rate (E), stomatal conductance (gs) and intercellular CO_2 concentration (Ci) were determined according to standard leaf gas-exchange principles¹².

The measured parameters included net photosynthetic rate (A, $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$), transpiration rate (E, $\text{mmol H}_2\text{O}/\text{m}^2/\text{sec}$), stomatal conductance (gs, $\text{mol H}_2\text{O}/\text{m}^2/\text{sec}$), intercellular CO_2 concentration (Ci , $\mu\text{mol CO}_2/\text{mol}$) and leaf temperature (LT, °C). The average value of three plants was used for subsequent statistical analyses.

Water-use efficiency: Instantaneous water-use efficiency (WUE) was calculated as the ratio of net photosynthetic rate (A) to transpiration rate (E), following the standard leaf gas-exchange approach¹³:

$$\text{WUE} = \frac{A}{E}$$

where, A is the net photosynthetic rate ($\mu\text{mol CO}_2/\text{m}^2/\text{sec}$) and E is the transpiration rate ($\text{mmol H}_2\text{O}/\text{m}^2/\text{sec}$). Accordingly, WUE was expressed as $\mu\text{mol CO}_2/\text{mmol H}_2\text{O}$. Higher WUE values indicate greater instantaneous carbon assimilation per unit of water lost through transpiration and were used as an indicator of physiological performance under sulfate salinity.

Statistical analysis: All statistical analyses were performed using the mean values of three biological replicates. The effects of genotype (G), sulfate salinity level (S) and their

interaction ($G \times S$) on all physiological and morpho-agronomic traits were evaluated by Two-Way Analysis of Variance (ANOVA) according to the following model¹⁴:

$$Y_{ijk} = \mu + G_i + S_j + (G \times S)_{ij} + \varepsilon_{ijk}$$

where, Y_{ijk} is the observed value of the measured trait, μ is the overall mean, G_i is the effect of the i th genotype, S_j is the effect of the j th salinity treatment, $(G \times S)_{ij}$ represents the genotype $\times$ salinity interaction and ε_{ijk} is the experimental error. When significant differences were detected, treatment means were compared using Tukey's Honestly Significant Difference (HSD) test at $p < 0.05$.

Pearson's correlation coefficients were calculated to evaluate the relationships among physiological traits and the resulting correlation matrix was visualized as a clustered heatmap¹⁵. Prior to multivariate analyses, all variables were standardized using Z-score normalization to eliminate differences in measurement scales:

$$Z = \frac{(X - \bar{X})}{SD}$$

where, X is the observed value, $\bar{X}$ is the mean and SD is the standard deviation of the corresponding variable.

Principal Component Analysis (PCA) was performed to reduce data dimensionality and identify the physiological traits contributing most strongly to sulfate salinity tolerance¹⁶. Partial Least Squares Discriminant Analysis (PLS-DA) was subsequently applied to classify cotton genotypes into tolerant, moderately tolerant and sensitive groups. Variable importance in projection (VIP) scores were calculated to determine the contribution of each variable to genotype discrimination, with $VIP > 1.0$ considered an important discriminatory threshold¹⁷.

Random Forest classification was used to estimate the relative importance of physiological traits based on the Mean Decrease in Gini Index¹⁸. Model performance was evaluated using Leave-One-Out Cross-Validation (LOOCV), in which each genotype was sequentially excluded from the training dataset and used as an independent validation sample¹⁹. Receiver Operating Characteristic (ROC) curve analysis and the corresponding area under the curve (AUC) were used to assess the classification accuracy of tolerant, moderately tolerant and sensitive genotype groups²⁰.

A Composite Sulfate Salinity Tolerance Index (CSTI) was calculated from the eleven traits. For traits positively associated with tolerance (MSH, Chl a, Chl b, TChl, Car,

A, gs and WUE), min-max membership values were calculated as:

$$U_{ij} = \frac{(X_{ij} - X_{j,min})}{(X_{j,max} - X_{j,min})}$$

For traits negatively associated with tolerance (E, Ci and LT), reverse membership values were calculated as:

$$U_{ij} = \frac{(X_{j,max} - X_{ij})}{(X_{j,max} - X_{j,min})}$$

All traits received equal weight ($w_j = 1/11$) and CSTI was calculated as:

$$CSTI_i = 100 \times \sum (W_j U_{ij})$$

Based on the resulting score distribution, genotypes with $CSTI \geq 70$ were classified as tolerant, those with $CSTI$ 40-69.99 as moderately tolerant and those with $CSTI < 40$ as sensitive. Hierarchical Cluster Analysis (HCA) based on Euclidean distance and Ward's minimum variance method was performed to classify genotypes according to overall physiological similarity^{21,22}. Cluster relationships were visualized using dendrograms and heatmaps, whereas radar charts were used to compare the physiological profiles of representative tolerant and sensitive genotypes.

All statistical analyses, multivariate procedures, machine-learning models and graphical visualizations were performed using Python 3.12 with the NumPy, Pandas, SciPy, Scikit-learn, Matplotlib, Seaborn and Plotly libraries²³⁻²⁶. Statistical significance was accepted at $p < 0.05$.

RESULTS

Physiological responses to sulfate salinity

Effect of sulfate salinity on plant height: Across the 19 genotypes, mean plant height decreased from 46.88 cm in the control to 33.55 and 30.68 cm under moderate and severe sulfate salinity, representing reductions of 28.4% and 34.6%, respectively. Under severe salinity, Guliston showed the greatest height (36.33 cm), whereas Yulduz-2 had the lowest value (24.00 cm); T-1003, T-1004, T-1024 and T-1050 generally maintained comparatively greater height across treatments.

Across all 11 traits, genotype, salinity and genotype $\times$ salinity interaction effects were significant ($p < 0.001$, Table 1). Salinity exerted its strongest effect on

Table 1: Summary of two-way ANOVA for the effects of genotype (G), sulfate salinity (S) and their interaction (G×S) on physiological traits of cotton

Trait	Genotype (F)	Salinity (F)	G×S (F)	p-value
Plant height	21.63	24.12	10.29	p<0.001
Chlorophyll a	59.11	277.19	7.72	p<0.001
Chlorophyll b	71.38	249.98	2.67	p<0.001
Total chlorophyll	68.74	294.27	7.05	p<0.001
Carotenoids	79.46	337.81	5.92	p<0.001
Photosynthetic rate	70.61	812.45	8.74	p<0.001
Transpiration rate	54.37	486.92	6.81	p<0.001
Stomatal conductance	61.24	563.48	7.63	p<0.001
Intercellular CO ₂	47.82	391.56	6.27	p<0.001
Water use efficiency	73.18	658.94	8.12	p<0.001
Leaf temperature	38.95	275.84	5.74	p<0.001

photosynthetic rate ($F = 812.45$) and WUE ($F = 658.94$), while genotype effects were greatest for carotenoids ($F = 79.46$) and WUE ($F = 73.18$), confirming broad genetic variation in physiological responses.

Effect of sulfate salinity on chlorophyll a content:

Chlorophyll a declined with increasing salinity, but the magnitude of reduction varied among genotypes. Under severe salinity, T-1050 retained the highest content (1.680 mg/g FW), followed closely by T-1024 and Mehnat, whereas T-1037 had the lowest value (0.908 mg/g FW). Relative losses were smallest in Mehnat (4.4%) and T-1005 (9.0%) but reached 40.4% in T-1002, indicating marked differences in pigment stability.

Effect of sulfate salinity on chlorophyll b content:

Chlorophyll b also decreased significantly with salinity. Under severe stress, T-1024 and Yulduz-2 retained the highest contents (0.583 and 0.579 mg/g FW), whereas T-1037 showed the lowest value (0.361 mg/g FW). Reductions relative to the control ranged from 12.7% in Mehnat to 27.2% in C-6524, highlighting substantial genotypic variation in light-harvesting pigment stability.

Effect of sulfate salinity on total chlorophyll content: Mean total chlorophyll decreased from 2.213 mg/g FW in the control to 1.847 and 1.772 mg/g FW under moderate and severe salinity, equivalent to reductions of 16.5% and 19.9%, respectively. Under severe salinity, T-1050 recorded the highest total chlorophyll (2.230 mg/g FW), whereas T-1037 had the lowest (1.269 mg/g FW); T-1024, Mehnat and T-1005 also maintained comparatively high values.

Effect of sulfate salinity on carotenoid content: Mean carotenoid content declined from 0.415 mg/g FW in the control to 0.360 and 0.284 mg/g FW under moderate and severe salinity, corresponding to reductions of 13.3% and 31.6%, respectively. Under severe stress, T-1050 retained the highest carotenoid content (0.395 mg/g FW), whereas T-1023

recorded the lowest value (0.209 mg/g FW); Mehnat and T-1024 also showed comparatively strong carotenoid retention.

Effect of sulfate salinity on photosynthetic rate: Mean net photosynthetic rate decreased from 30.94 $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$ in the control to 25.35 and 15.28 $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$ under moderate and severe salinity, representing reductions of 18.1% and 50.6%, respectively. Under severe stress, T-1050 maintained the highest rate (18.65 $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$), whereas C-6524 showed the lowest (7.60 $\mu\text{mol CO}_2/\text{m}^2/\text{sec}$). The highest severe-to-control stability ratio was also recorded for T-1050 (0.56).

Effect of sulfate salinity on transpiration rate: Mean transpiration declined modestly under moderate salinity (3.11 to 2.92 $\text{mmol H}_2\text{O}/\text{m}^2/\text{sec}$; 6.1%) but fell to 2.09 $\text{mmol H}_2\text{O}/\text{m}^2/\text{sec}$ under severe salinity (32.8% reduction). Under severe stress, T-66 had the highest transpiration (3.10 $\text{mmol H}_2\text{O}/\text{m}^2/\text{sec}$), whereas T-1004 had the lowest (1.35 $\text{mmol H}_2\text{O}/\text{m}^2/\text{sec}$), reflecting contrasting water-loss regulation among genotypes.

Effect of sulfate salinity on stomatal conductance: Mean stomatal conductance decreased from 0.267 $\text{mol H}_2\text{O}/\text{m}^2/\text{sec}$ in the control to 0.226 and 0.157 $\text{mol H}_2\text{O}/\text{m}^2/\text{sec}$ under moderate and severe salinity, corresponding to reductions of 15.4% and 41.2%. Under severe stress, values ranged from 0.245 in T-66 to 0.088 $\text{mol H}_2\text{O}/\text{m}^2/\text{sec}$ in C-6524; the relative decline ranged from 25.8 to 55.6%.

Effect of sulfate salinity on intercellular CO₂ concentration:

Mean intercellular CO₂ concentration increased from 224.3 ppm in the control to 238.7 and 283.4 ppm under moderate and severe salinity, representing increases of 6.4% and 26.3%, respectively. Under severe stress, C-6524 had the highest Ci (325 ppm), whereas T-1050 maintained the lowest value (260 ppm), consistent with contrasting CO₂-assimilation efficiency.

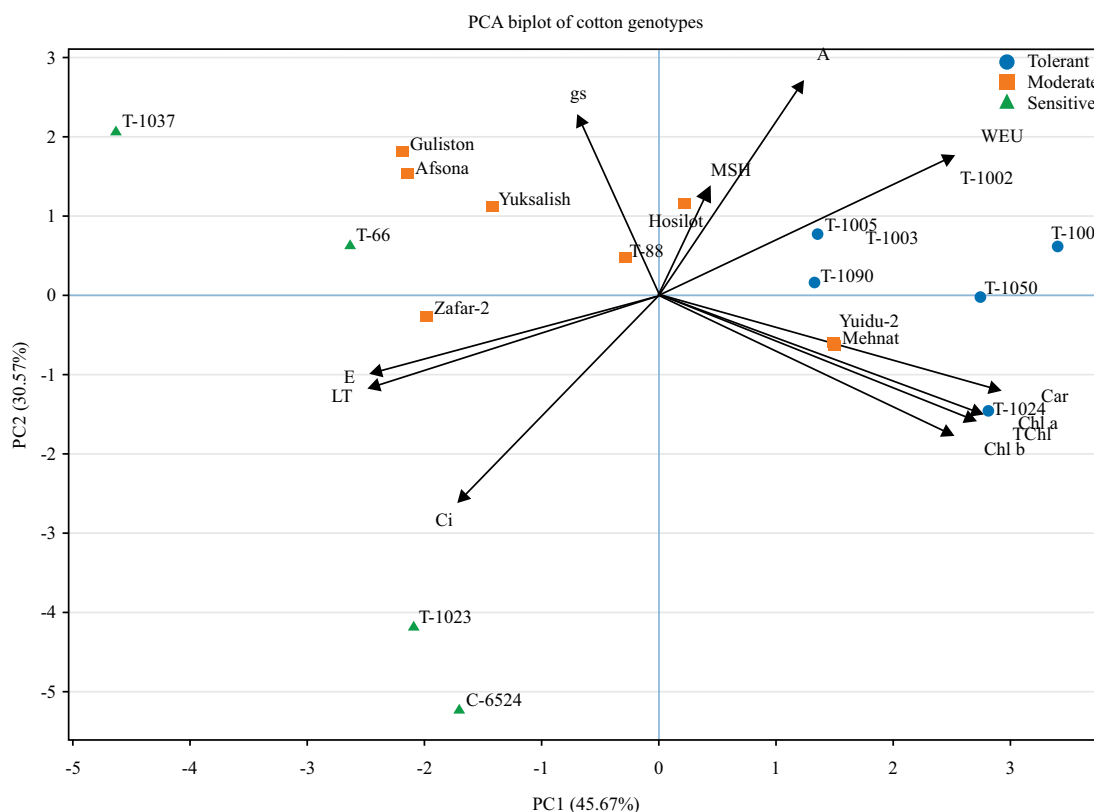


Fig. 1: Principal Component Analysis (PCA) of physiological responses of upland cotton genotypes to sulfate salinity

PC1 and PC2 represent the first and second principal components, explaining 45.67 and 30.57% of the total variation, respectively. Blue circles orange squares and green triangles represent tolerant, moderately tolerant and sensitive genotypes, respectively. Arrows indicate trait loadings, arrow direction indicates the association of each trait with the principal components, while arrow length reflects its relative contribution. Net photosynthetic rate (A), transpiration rate (E), stomatal conductance (gs) intercellular CO_2 concentration (Ci), water-use efficiency (WUE), leaf temperature (LT), main stem height (MSH), chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (TChl) and carotenoids (Car)

Effect of sulfate salinity on water use efficiency: Mean WUE decreased from $10.79 \mu\text{mol CO}_2/\text{mmol H}_2\text{O}$ in the control to 9.21 and $7.83 \mu\text{mol CO}_2/\text{mmol H}_2\text{O}$ under moderate and severe salinity, corresponding to reductions of 17.4% and 26.1%, respectively. Under severe stress, T-1004 maintained the highest WUE ($12.11 \mu\text{mol CO}_2/\text{mmol H}_2\text{O}$), whereas C-6524 had the lowest value ($3.04 \mu\text{mol CO}_2/\text{mmol H}_2\text{O}$); T-1002, T-1050, Hosilot and T-1090 also performed comparatively well.

Effect of sulfate salinity on leaf temperature: Mean leaf temperature increased from 33.48°C in the control to 35.92 and 37.38°C under moderate and severe salinity, equivalent to increases of 7.3% and 11.6%, respectively. Under severe stress, T-1037 recorded the highest temperature (39.0°C), whereas T-1002 maintained the lowest (36.0°C); T-1003 and Mehnat also remained comparatively cool.

Principal component analysis (PCA) of physiological traits: The first two principal components explained 76.24% of total variation (PC1 = 45.67%, PC2 = 30.57%, Fig. 1). Tolerant

genotypes were associated primarily with photosynthetic rate, WUE, pigment contents and plant height, whereas sensitive genotypes were associated with higher Ci, transpiration and leaf temperature. This pattern separated T-1004, T-1050, T-1002, T-1003, T-1005, T-1090 and T-1024 from the sensitive group comprising T-1037, T-1023, C-6524 and T-66.

Pigment traits clustered closely and photosynthetic rate was positively aligned with WUE but opposed to Ci, transpiration and leaf temperature, identifying PC1 as the principal tolerance gradient.

Partial Least Squares Discriminant Analysis (PLS-DA) of physiological traits: A PLS-DA likewise separated tolerant, moderate and sensitive groups (Fig. 2). T-1004 and T-1024 were the most distinct tolerant genotypes, whereas T-1037 and C-6524 were the most distant sensitive genotypes; Mehnat and Yulduz-2 occupied intermediate positions closer to the tolerant cluster.

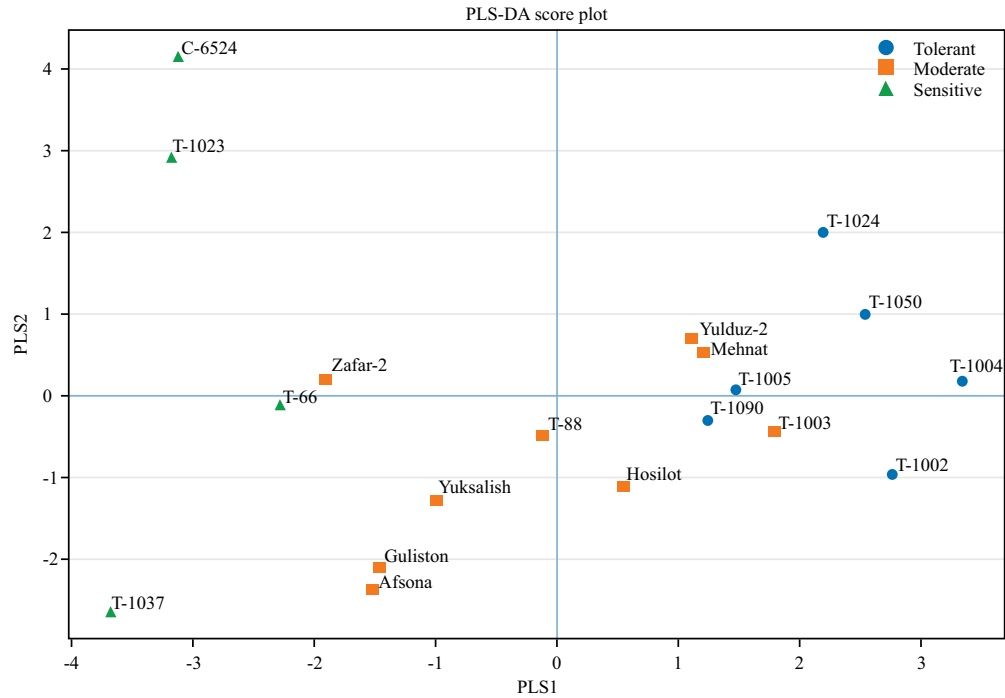


Fig. 2: Partial Least Squares Discriminant Analysis (PLS-DA) of physiological responses of upland cotton genotypes to sulfate salinity

PLS1 and PLS2 represent the first and second latent variables of the PLS-DA model. Blue circles orange squares and green triangles indicate tolerant, moderately tolerant and sensitive genotypes, respectively. The spatial separation of genotypes reflects differences in their integrated physiological responses to sulfate salinity

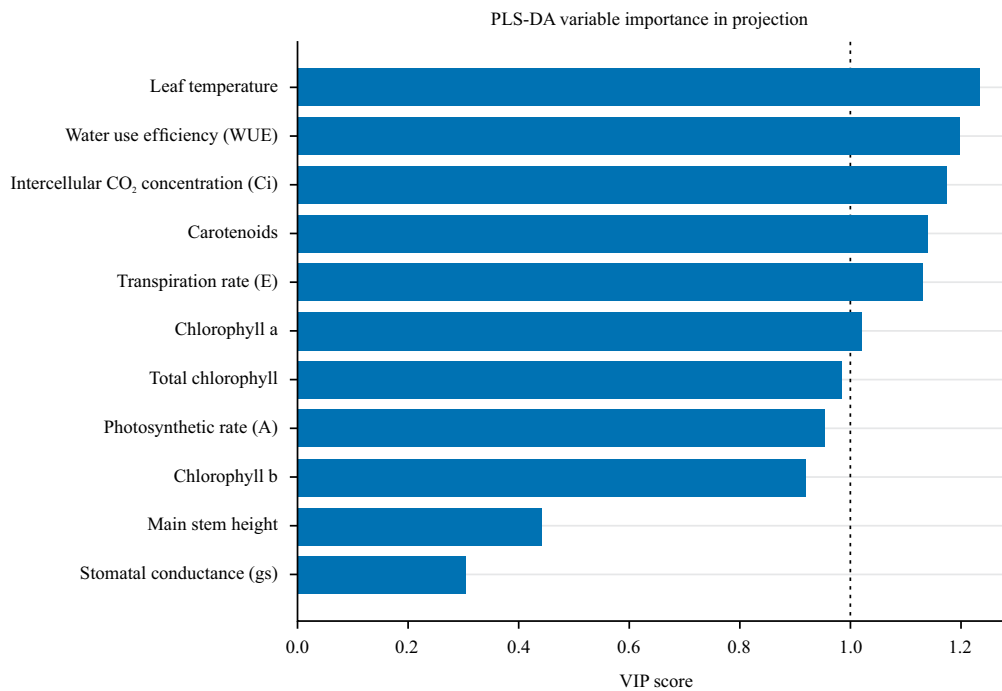


Fig. 3: Variable importance in projection (VIP) scores of physiological traits associated with sulfate salinity tolerance in upland cotton

Vertical dashed line indicates the VIP threshold of 1.0, variables with VIP >1.0 were considered important contributors to genotype discrimination. Water-use efficiency (WUE), intercellular CO₂ concentration (Ci), transpiration rate (E), net photosynthetic rate (A), stomatal conductance (gs)

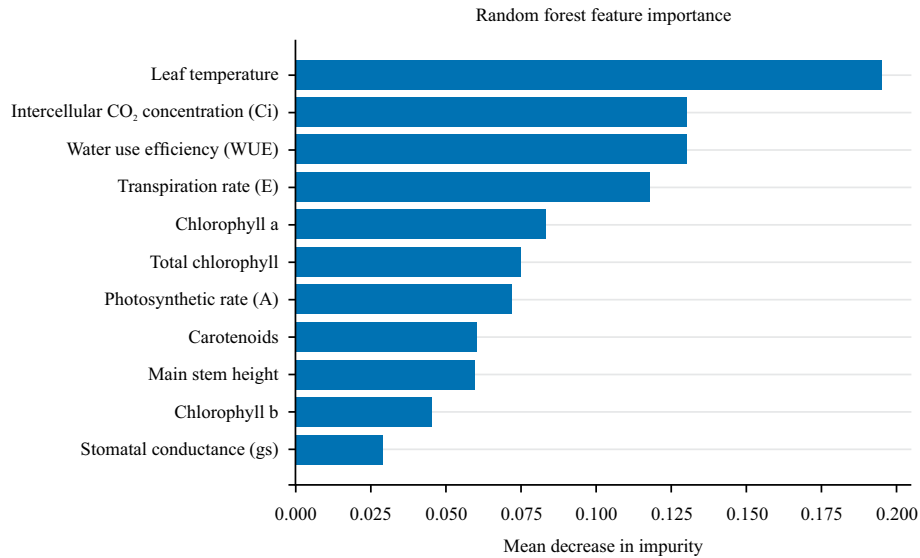


Fig. 4: Random forest feature-importance analysis of physiological traits associated with sulfate salinity tolerance in upland cotton

Variable importance was quantified using the Mean Decrease in Gini impurity; higher values indicate a greater contribution of the corresponding trait to genotype classification. Water-use efficiency (WUE), intercellular CO₂ concentration (Ci), transpiration rate (E), net photosynthetic rate (A), stomatal conductance (gs)

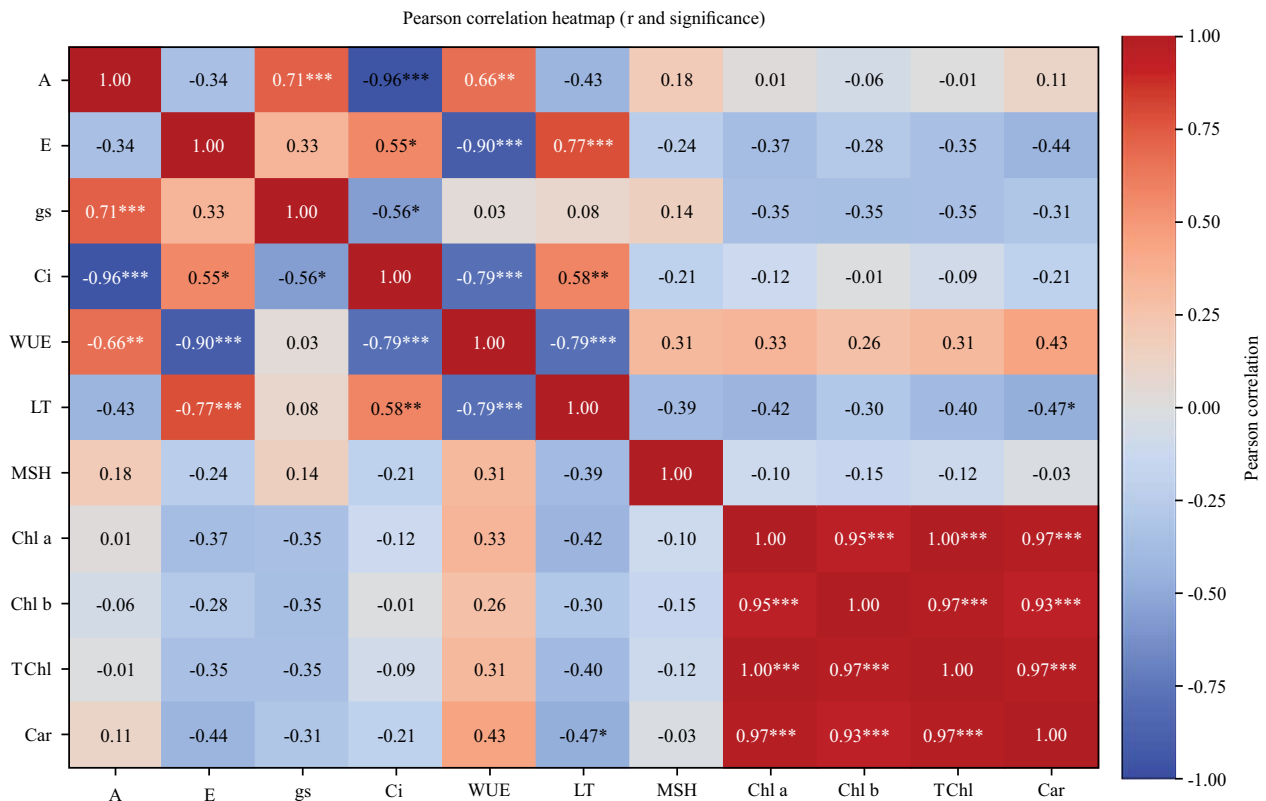


Fig. 5: Pearson correlation heatmap of physiological traits associated with sulfate salinity tolerance in upland cotton

Cell values represent Pearson correlation coefficients (r), ranging from -1 to +1. Positive correlations are represented by warm colors and negative correlations by cool colors, with color intensity indicating correlation strength. Net photosynthetic rate (A), transpiration rate (E), stomatal conductance (gs), intercellular CO₂ concentration (Ci), water-use efficiency (WUE), leaf temperature (LT), main stem height (MSH), chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (TChl) and carotenoids (Car)

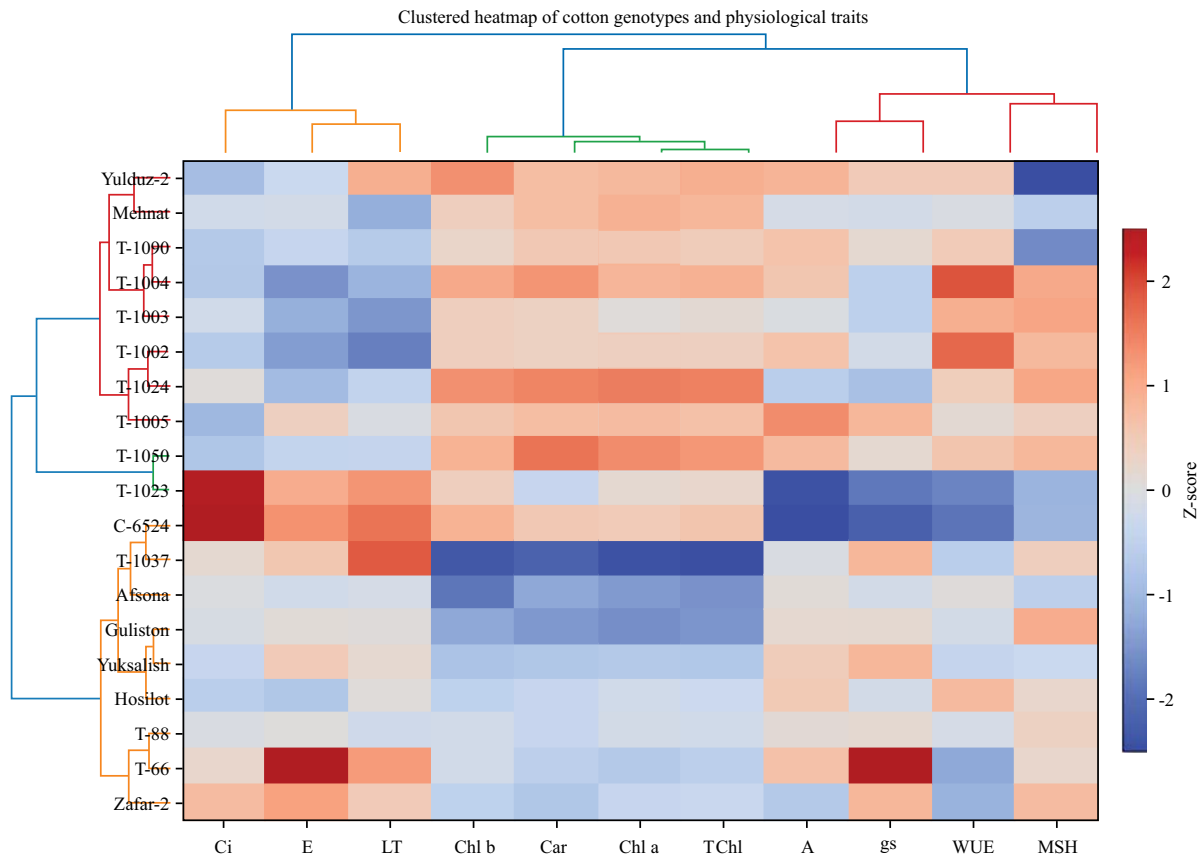


Fig. 6: Hierarchical clustering heatmap of physiological responses of upland cotton genotypes to sulfate salinity

Genotypes and physiological traits were clustered using Euclidean distance and Ward's minimum variance method. Heatmap values represent standardized Z-scores; warm colors indicate values above the overall standardized mean and cool colors indicate values below the mean. Dendrograms show similarity-based clustering of genotypes and traits. Net photosynthetic rate (A), transpiration rate (E), stomatal conductance (gs), intercellular CO₂ concentration (Ci), water-use efficiency (WUE), leaf temperature (LT), main stem height (MSH), chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (TChl) and carotenoids (Car)

PLS-DA variable importance in projection (VIP) analysis:

Six variables exceeded the VIP >1.0 threshold (Fig. 3). Leaf temperature had the highest score (approximately 1.23), followed by WUE (approximately 1.20) and Ci (approximately 1.17); carotenoids, transpiration and chlorophyll a were also informative. Main stem height and stomatal conductance had the lowest contributions (approximately 0.45 and 0.30, respectively).

Random forest feature importance analysis: Random forest analysis corroborated the importance of leaf temperature, which ranked first (approximately 0.20), followed by Ci and WUE (both approximately 0.13) and transpiration (approximately 0.12, Fig. 4). Chlorophyll b and stomatal conductance contributed least (approximately 0.04 and 0.03).

Correlation analysis of physiological traits: Photosynthetic rate was most strongly and negatively correlated with Ci

($r = -0.96$, $p < 0.001$) and was positively related to stomatal conductance ($r = 0.71$, $p < 0.001$) and WUE ($r = 0.66$, $p < 0.01$, Fig. 5). Transpiration was strongly negatively associated with WUE ($r = -0.90$, $p < 0.001$).

Pigment traits were tightly interrelated: Chlorophyll a correlated with total chlorophyll ($r = 1.00$), carotenoids ($r = 0.97$) and chlorophyll b ($r = 0.95$, all $p < 0.001$). These relationships indicate coordinated pigment retention under sulfate salinity.

Hierarchical clustering analysis of physiological traits:

A HCA resolved three major genotype groups (Fig. 6). The tolerant cluster combined high pigment contents, photosynthetic rate and WUE with lower Ci, transpiration and leaf temperature, whereas T-1023, C-6524 and T-1037 formed the contrasting sensitive cluster; the remaining genotypes occupied an intermediate group.

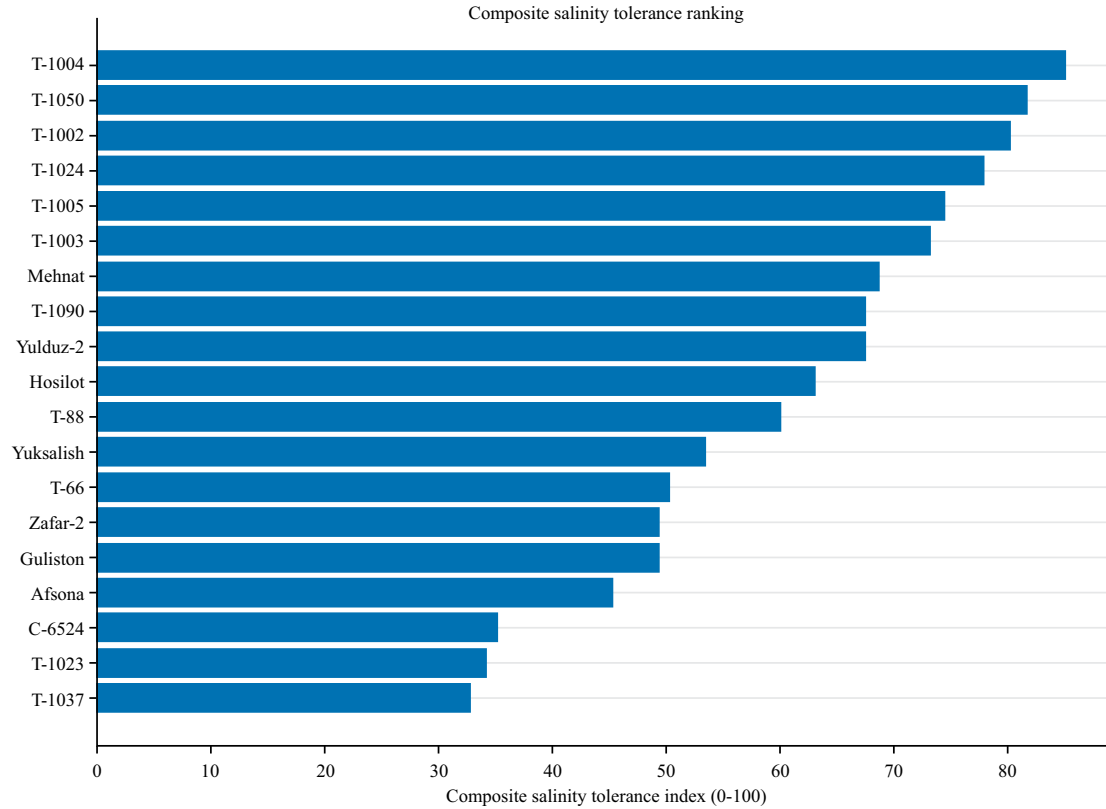


Fig. 7: Composite sulfate salinity tolerance index (CSTI) of upland cotton genotypes under sulfate salinity

A CSTI represents the integrated tolerance score calculated from 11 physiological and growth traits using normalized membership values. Higher CSTI values indicate greater overall physiological tolerance to sulfate salinity. According to the classification criteria used in this study, $CSTI \geq 70$ indicates tolerant, 40-69.99 moderately tolerant and <40 sensitive genotypes

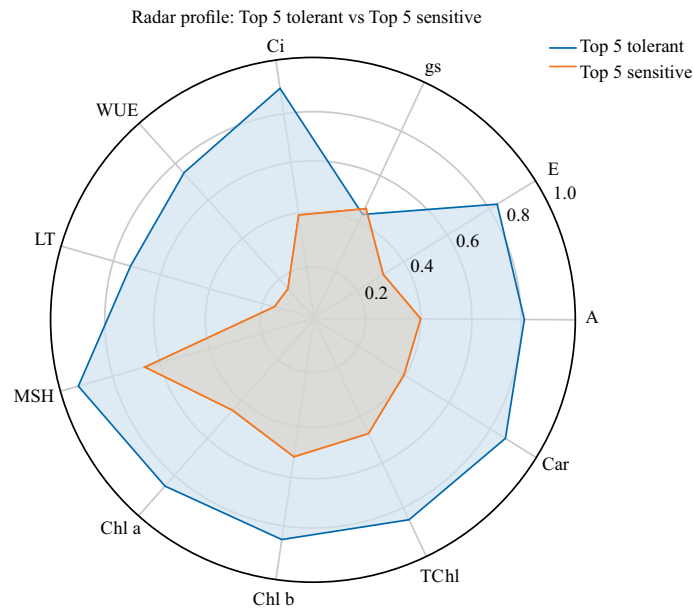


Fig. 8: Radar plot comparing physiological profiles of salt-tolerant and salt-sensitive upland cotton genotypes under sulfate salinity

Values were normalized to a common 0-1 scale to enable comparison among traits. Blue and orange profiles represent the five most tolerant and five most sensitive genotypes, respectively. Net photosynthetic rate (A), transpiration rate (E), stomatal conductance (gs), intercellular CO_2 concentration (Ci), water-use efficiency (WUE), leaf temperature (LT), main stem height (MSH), chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (TChl) and carotenoids (Car)

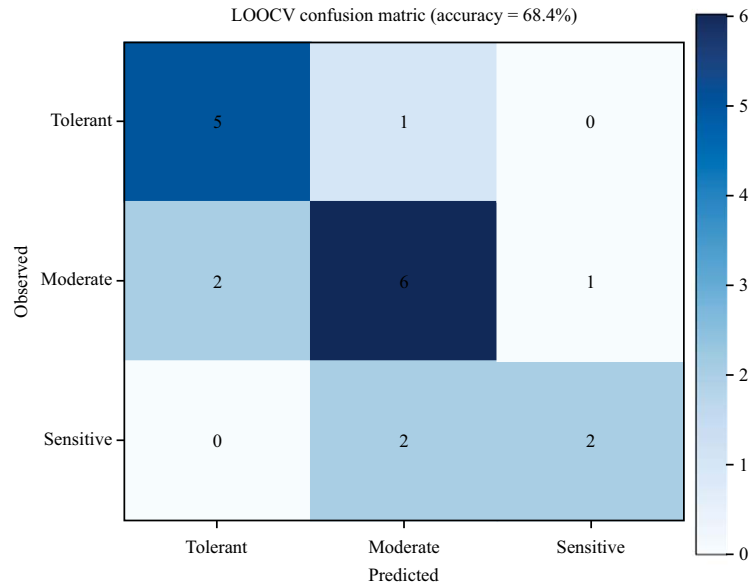


Fig. 9: Leave-One-Out Cross-Validation (LOOCV) confusion matrix for sulfate salinity tolerance classification of upland cotton genotypes

Rows represent observed tolerance classes and columns represent predicted classes. Cell values indicate the number of genotypes assigned to each observed-predicted class combination; darker shading represents a larger number of genotypes. Overall LOOCV classification accuracy was 68.4%. Tolerant, moderate and sensitive denote the three sulfate salinity tolerance classes

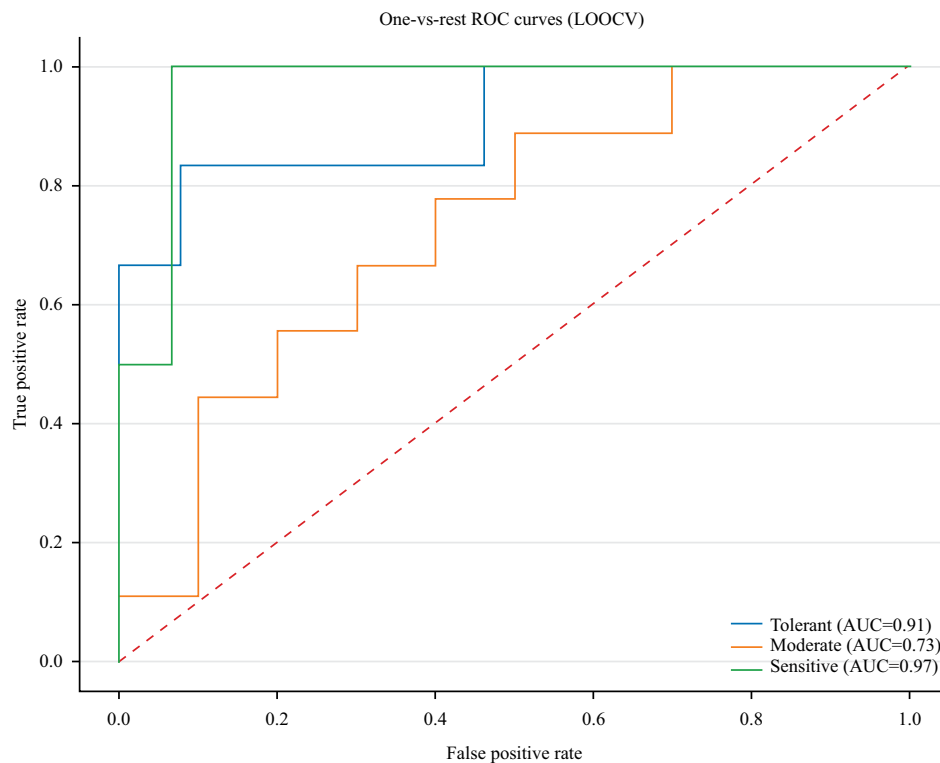


Fig. 10: Receiver Operating Characteristic (ROC) analysis of sulfate salinity tolerance classification in upland cotton genotypes

ROC curves show the relationship between the true-positive rate (sensitivity) and false-positive rate (1-specificity) for discrimination among tolerant, moderately tolerant and sensitive genotype classes. AUC denotes the area under the ROC curve; higher AUC values indicate greater discriminatory performance. AUC values were 0.91, 0.73 and 0.97 for the tolerant, moderate and sensitive classes, respectively. The diagonal reference line represents discrimination expected by chance (AUC = 0.50)

Trait clustering grouped the four pigment variables together and linked photosynthetic rate, stomatal conductance and WUE in a second cluster, while C_i , transpiration and leaf temperature formed an opposing response group.

Composite Salinity Tolerance Index (CSTI) analysis: A CSTI scores ranged from approximately 33 to 85 (Fig. 7). T-1004 ranked highest (approximately 85), followed by T-1050 (approximately 82) and T-1002 (approximately 80), whereas T-1037, T-1023 and C-6524 recorded the lowest scores (approximately 33-36).

Six genotypes (T-1004, T-1050, T-1002, T-1024, T-1005 and T-1003) met the tolerant threshold, while T-1037, T-1023 and C-6524 were classified as sensitive; all others were moderately tolerant.

Radar profile analysis of tolerant and sensitive genotypes:

The radar profile clearly distinguished the two extreme groups (Fig. 8): Tolerant genotypes maintained higher photosynthetic rate, WUE, pigment contents, carotenoids and plant height, whereas sensitive genotypes showed higher transpiration, C_i and leaf temperature. Stomatal conductance differed comparatively little between groups.

LOOCV confusion matrix analysis: A LOOCV yielded an overall classification accuracy of 68.4% (Fig. 9). Correct classification was highest for the tolerant group (83.3%), followed by the moderate (66.7%) and sensitive (50.0%) groups.

Errors occurred only between adjacent classes; no tolerant genotype was classified as sensitive and no sensitive genotype as tolerant, indicating reliable separation of the physiological extremes.

Receiver Operating Characteristic (ROC) analysis: A ROC analysis showed excellent discrimination for the sensitive (AUC = 0.97) and tolerant (AUC = 0.91) classes, but lower performance for the moderate class (AUC = 0.73, Fig. 10). This agreed with the LOOCV pattern and indicated that intermediate responses were more difficult to classify.

DISCUSSION

The present study demonstrated that sulfate salinity significantly affected plant growth, photosynthetic pigments, gas exchange, water-use efficiency and leaf temperature in upland cotton, with significant effects of genotype, salinity treatment and genotype $\times$ salinity interaction for all measured

traits ($p < 0.001$). These results indicate substantial genetic variation in physiological responses to sulfate salinity, suggesting that tolerance depends on genotype-specific adaptive mechanisms. Similar genotypic differences under saline conditions have been reported by Yuan *et al.*², Sikder *et al.*³ and Shaheen *et al.*⁶.

Plant height decreased progressively with increasing sulfate salinity, reflecting the inhibitory effects of osmotic stress and ion toxicity on plant growth. However, tolerant genotypes such as T-1003, T-1004, T-1024 and T-1050 maintained greater plant height than sensitive genotypes, indicating better growth maintenance under stress. Comparable reductions in cotton growth under salinity have been described by Zhang *et al.*⁴, Guo *et al.*⁵ and Peng *et al.*⁷.

Salinity also caused marked reductions in chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents. Pigment degradation is commonly associated with chloroplast damage, oxidative stress and inhibited chlorophyll biosynthesis. In contrast, tolerant genotypes, particularly T-1050, T-1024, Mehnat and T-1005, retained higher pigment concentrations, suggesting greater stability of the photosynthetic apparatus. These observations agree with Zhang *et al.*⁴, Guo *et al.*⁸ and Li *et al.*¹⁰, who reported that maintenance of photosynthetic pigments is closely associated with improved salt tolerance.

Photosynthetic rate declined markedly under severe sulfate salinity and was accompanied by reductions in stomatal conductance and transpiration. At the same time, intercellular CO_2 concentration increased, indicating that both stomatal limitation and non-stomatal impairment contributed to reduced photosynthesis. Similar responses have been reported by Sikder *et al.*³, Zhang *et al.*⁴ and Ma *et al.*⁹. In the present study, tolerant genotypes including T-1050, T-1005, T-1002 and Yulduz-2 maintained higher photosynthetic rates together with lower C_i values, reflecting more efficient CO_2 assimilation under saline conditions.

Reduced transpiration and stomatal conductance represent adaptive responses that limit water loss under osmotic stress. Tolerant genotypes maintained relatively high photosynthetic activity despite moderate reductions in transpiration, indicating more efficient stomatal regulation. Likewise, water-use efficiency declined under salinity but remained comparatively high in T-1004, T-1002, T-1050, Hosilot and T-1090. These findings support previous reports identifying WUE as a reliable physiological indicator of salinity tolerance in cotton^{3,11}.

Leaf temperature increased with increasing salinity, particularly in the sensitive genotypes T-1037, C-6524 and T-1023, whereas tolerant genotypes maintained comparatively

lower temperatures. This pattern is consistent with reduced transpiration cooling caused by stomatal closure and agrees with previous studies linking leaf temperature with stomatal regulation and salt tolerance^{9,10}.

Multivariate analyses further strengthened these findings. A PCA explained 76.24% of the total variation and clearly separated tolerant and sensitive genotypes. In addition, PLS-DA, Random Forest, VIP analysis, hierarchical clustering and the Composite Sulfate Salinity Tolerance Index consistently identified photosynthetic rate, water-use efficiency, chlorophyll pigments, carotenoids and leaf temperature as the principal traits contributing to genotype discrimination. Similar integrated analytical approaches have been recommended by Yuan *et al.*², Peng *et al.*⁷ and Anwar *et al.*¹¹.

Overall, sulfate salinity tolerance in upland cotton was closely associated with the maintenance of photosynthetic capacity, pigment stability, efficient stomatal regulation, high water-use efficiency and lower leaf temperature. Among the evaluated germplasm, T-1004, T-1002, T-1050, T-1024, T-1005 and T-1003 consistently exhibited superior physiological performance, highlighting their potential value as breeding materials for the development of cotton cultivars adapted to sulfate-dominated saline soils.

CONCLUSION

This study demonstrated that sulfate salinity significantly affected all evaluated physiological and growth-related traits of upland cotton, with highly significant effects of genotype, salinity level and genotype $\times$ salinity interaction ($p < 0.001$). Increasing sulfate salinity reduced plant height, chlorophyll pigments, carotenoid content, photosynthetic rate, transpiration, stomatal conductance and water-use efficiency, while increasing intercellular CO₂ concentration and leaf temperature, indicating substantial impairment of photosynthetic performance and plant physiological activity.

Marked genotypic variation was observed among the 19 cotton genotypes. T-1004, T-1002, T-1050, T-1024, T-1005, T-1003, T-1090 and Mehnat consistently maintained superior physiological performance under sulfate salinity, whereas C-6524, T-1023, T-1037 and T-66 were identified as the most salt-sensitive genotypes.

Multivariate analyses supported these findings. A PCA explained 76.24% of the total variation, while PLS-DA, hierarchical clustering, Random Forest, VIP analysis and the Composite Sulfate Salinity Tolerance Index consistently identified photosynthetic rate, chlorophyll pigments,

carotenoids, water-use efficiency and leaf temperature as the most informative indicators of sulfate salinity tolerance.

Overall, T-1004, T-1002, T-1050, T-1024, T-1005 and T-1003 represent valuable genetic resources for breeding salt-tolerant cotton cultivars. The integration of physiological traits with multivariate statistical and machine-learning approaches provides a robust framework for screening cotton germplasm and supporting the development of cultivars adapted to sulfate-affected saline soils.

SIGNIFICANCE STATEMENT

This study identifies key physiological indicators and tolerant upland cotton genotypes under sulfate salinity stress. The integration of physiological characterization and multivariate analyses provides a reliable approach for screening salinity-tolerant germplasm and supporting the development of cotton cultivars adapted to saline soils.

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