Assessment of Cotton Genotypes at the Seedling Stage: A Comparative Study of Morphophysiological and Biochemical Responses to Various Salinity Stress Levels

Nimra Gul1*, Asif Saeed1, Azeem Iqbal Khan1, Faisal Saeed Awan2
1Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan
2Center of Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Pakistan
*Corresponding author's email: nimratahir122@gmail.com

Abstract- Cotton (Gossypium spp.) is a vital raw product for the global textile industry. Its production is affected by salt stress. This study was designed to investigate the morph-physiological and biochemical responses of cotton genotypes to salt, aiming to identify mechanisms of salt tolerance and susceptibility. Within a glasshouse, diverse genotypes were grown in polythene bags filled with sand, subjected to three salt stress levels i.e., control, moderate stress (12dS/m), and severe stress (17dSm), in a two-factor factorial design under a Completely Randomized Design (CRD). Physiological measurements and biochemical assays under varying salt conditions revealed significant decreases in growth related traits (shoot and root lengths, fresh and dry weights) with increased salt severity. Alongside antioxidant defense mechanisms were upregulated, as evidenced by increased peroxidase (POD), catalase (CAT), superoxide Dismutase (SOD) activities, and proline levels, indicating adaptive responses to oxidative stress. Principal Component Analysis (PCA) showed the results for that the first two principal components accounted for approximately 76% of the variance, summarizing the major contribution by genotypes' salt responses. Cluster analysis further delineated the genotypes into three clusters, representing distinct salt response strategies. This study revealed the complex dynamics between cotton's genetic makeup, physiological responses, and biochemical processes in facing salt conditions and to ascertain the salt tolerant and susceptible genotypes.

Keywords: Cotton, salt stress, seedling, morpho-physiological, biochemical, proline, catalase

1 INTRODUCTION

Cotton, a delicate and soft staple fiber, originates within the protective boll close to the plant seeds and is classified under the genus Gossypium, belonging to the family Malvaceae, within the Malvales order, and part of the Gossypieae tribe [1]. However, its production faces several challenges, including both biotic factors like pest infestations and abiotic stresses. Among these, abiotic stress, induced by environmental conditions, detrimentally influences plant growth, development, productivity, and the quality of seeds [2].

Salinity stress, a result of elevated soil and water salt levels, significantly restricts agricultural output globally, affecting at least 20% of irrigated land [3]. This has led to an increasing focus on developing salt-resistant agricultural varieties, especially for upland cotton, a major global cash crop. Despite its extensive cultivation, upland cotton's yield is significantly reduced under salt stress, which disrupts plant growth, photosynthesis, causes ion toxicity, and leads to oxidative damage [4]. Addressing salinity and sodicity issues in major irrigation projects is crucial for enhancing productivity on salt-impacted lands, turning environmental challenges into economic assets [5]. Yet, the scarcity of clear salinity tolerance indicators hampers the efforts in breeding salt-resistant upland cotton varieties [6].

Saline soils are characterized by salt concentrations inhibiting plant growth, whereas soils with salt concentrations not disrupting soil structure are non-saline [7]. Salinity stress is a significant issue in arid and semi-arid areas, especially impacting soils in low-lying regions. Particularly, areas across Asia, the Pacific, and Australia are extensively affected by salt, with approximately 6% of the total land area experiencing salinity issues[8]. Saline soils are characterized by salt concentrations inhibiting plant growth, whereas soils with salt concentrations not disrupting soil structure are non-saline. Salt stress triggers a range of plant responses—morphological, biochemical, and physiological—to cope with the adverse effects [9] [10]. It has been documented that, within Pakistan's 22 million hectares of agricultural land, 6.28 million hectares are impacted by salt. Of this salt-affected land, approximately 60.5% is categorized as saline-sodic, while the remaining 39.5% suffers from salinity issues. [11][12]. The primary causes of salinity include inappropriate agricultural practices, insufficient rainfall, excessive evaporation, and poor drainage, alongside the misuse of brackish water for irrigation [13] [14]. With projections indicating that half of the cultivated lands may suffer from salt stress by 2050, it is crucial to address salinity at early plant stages, particularly during germination and seedling phases, where damage is most acute [15] [16].

Cotton's tolerance to abiotic stresses, such as salinity and drought, is comparatively higher than other major crops, though these conditions still significantly affect its growth, yield, and fiber quality[17] [18]. Early stages of plant growth

are critical for assessing salinity effects, with salt stress causing a range of damages, including water stress, nutritional imbalance, and cytotoxicity from excess sodium and chloride ions, leading to oxidative stress and affecting metabolic activities [19] [20]. Antioxidant enzymes are vital in mitigating oxidative stress caused by salinity. Elevated activities of enzymes like peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) are observed in salt-tolerant cotton varieties. This highlights their essential role in offering resistance against salinity[21] [22].

Principal Component Analysis (PCA) emerges as a valuable statistical tool in this context, enabling the identification of cotton genotypes with superior salt tolerance by simplifying complex data sets into interpretable components, thereby aiding in the efficient selection for breeding programs. This research focuses on identifying morpho-physiological markers for salt resistance in cotton at the seedling stage using PCA and cluster analysis, aiming to inform breeding strategies for improving salt resilience. This effort is critical for developing climate-resilient cotton capable of enduring future environmental challenges.

2 MATERIALS AND METHODS

The present research took place within the Department of Plant Breeding and Genetics at the University of Agriculture, Faisalabad. A total of twenty cotton genotypes were sourced from the department's seed bank and several other institutions, including the Central Cotton Research Institute Multan, the Nuclear Institute of Agriculture and Biology, the Cotton Research Station Multan, and the Ayub Agricultural Research Institute. Detailed information regarding these genotypes is meticulously organized in Table 2.1.

TABLE 2.1: Genotypes used in experiment.

Sr. No.	Genotypes	Sr. No.	Genotypes	Sr. No.	Genotypes
1.	VH-339	11.	VH-295	21.	MS-DIC
2.	FH-634	12.	CRS-2007	22.	NIAB-878
3.	FH-114	13.	BH-89	23.	VH-329
4.	FH-170	14.	FH-118	24.	IUB-75
5.	FH-115	15.	RH-647	25.	C-26
6.	IR-3	16.	FH-444	26.	CIM-599
7.	UH-148	17.	SLH-8	27.	CRS-1
8.	NIAB-820	18.	IR-3701	28.	CIM-602
9.	VH-327	19.	AGC-501	29.	VH-371
10.	MNH-552	20.	FH-35	30.	MNH-465

Seeds were sowed in polyethylene containers filled with sand. Before planting, each container was irrigated to field capacity. The seeds, with their lint removed, were immersed in an aerated 15 mM solution of CaSO4.2H2O for the entire night. The following morning, 5 to 6 seeds were planted about 2 cm deep in each sand container. Each plant variety was grown with three separate replications, under saline conditions of 0, 12, and 17 dS/m NaCl. The seedlings were irrigated every other day starting from 3 to 4 days post-planting. Fifteen days after planting, coinciding with the emergence of the first true leaves, an initial salinity treatment of 7.5 dS/m was administered via the irrigation water. A subsequent increase in salinity to 12 dS/m and then to 17 dS/m was achieved by applying additional salt with the irrigation water upon the beginning growth of the third true leaf, 28 days after sowing. Salt stress was applied through half strength Hoagland solution. The study was executed as a two-factor factorial experiment utilizing a completely randomized design (CRD), with each treatment replicated three times. The control group did not receive any NaCl addition. Following the treatments, various morphological, physiological, and biochemical parameters were measured.

2.1 Morphological Parameters

During the stage when plants developed three true leaves, morphological measurements were collected for analysis. Each plant genotype was thoroughly rinsed with deionized water before measuring the lengths of roots and shoots in centimeters. Average values for each genotype across different treatments were computed for detailed analysis. The weight of roots and shoots were measured with the help of weighing balance. After recording the fresh weight, plants were kept in drying oven at 70C for 48 h. Then, dry weights of roots and shoots were estimated on an electrical weighing balance.

2.2 Physiological Parameters

To evaluate the salt tolerance of the cotton varieties being studied, their physiological reactions to salt stress were meticulously analyzed, focusing on the concentrations of sodium (Na+) and potassium (K+) ions. The measurements of Na+ and K+ concentrations were carried out using a flame photometer (model: Jenway PFP 7). Initially, for the digestion of the dried plant material, 0.05g of dried leaf sample was placed into digestion tubes. Following this, 1 ml of concentrated H2SO4 was added to each dried sample to facilitate digestion. These tubes were then stored in a dark place for overnight incubation at ambient temperature. The following day, 0.5 ml of 35% H2O2 was introduced, and the tubes were transferred to a digestion block where they were heated at 350°C until fumes began to emerge. After heating for 30 minutes, the tubes were removed from the block to cool. Additional 0.5 ml of H2O2 was gradually added, and the tubes were returned to the digestion block. This process was repeated until the digested material became clear. The digest was then diluted to a final volume of 25 ml in volumetric flasks. Once filtered, this solution was used for the analysis of K+ and Na+ ion concentrations.

2.3 Biochemical Parameter

Following the morpho-physiological assessments, biochemical analyses were conducted on samples from the fourth true leaf stage of each cotton genotype to examine their biochemical response. For the extraction of antioxidant enzymes, 0.1 g of fresh leaf tissue from each cotton variant was pulverized and centrifuged at 14,000 rpm for 10 minutes in 1 ml of 50 mM phosphate buffer (pH 7.8) at cold temperature. Enzyme activity was quantified using the resultant supernatant. To measure soluble protein levels, 5 ml of this supernatant was mixed with 95 ml of 0.1 N sodium chloride and 1 ml of dye reagent, which was prepared by dissolving 0.02 g of Coomassie Brilliant Blue G-250 dye in a mixture of 10 ml of 95% ethanol, 20 ml of water, and 200 ml of diluted phosphoric acid. The protein-dye complex was allowed to form over five minutes, followed by measuring absorbance at 595 nm.

For the assessment of superoxide dismutase (SOD) activity, cotton leaf samples were emulsified in a solution comprising 0.1 mM EDTA, 50 mM potassium phosphate buffer, and 1 mM dithiothreitol. The SOD activity was evaluated based on its capacity to inhibit the photochemical reduction of nitroblue tetrazolium[23][24]. To determine peroxidase (POD) and catalase (CAT) levels, cotton leaves were homogenized in a mixture containing 50 mM potassium phosphate buffer, 0.1 mM EDTA, and 1 mM DTT. POD activity was measured using the method by Chance and Maehly, while Erel's protocol was employed to assess the activity of ascorbate peroxidase (APX)[25][26].

For proline content estimation, cotton leaves were processed in a 3% sulfosalicylic acid solution, centrifuged, and the supernatant was subsequently heated at 100°C for an hour with acid ninhydrin and glacial acetic acid. Post-cooling, toluene was added, and the absorbance of the organic phase was measured at 520 nm.[27]. Finally, to quantify total soluble protein (TSP), cotton leaves were homogenized in 0.1 M sodium phosphate buffer (pH 7.8) and centrifuged. The supernatant was then mixed with Coomassie Brilliant Blue G-250 dye reagent and incubated at room temperature. Absorbance was measured using a spectrophotometer at 595 nm [28].

2.4 Statistical Analysis

The screening process was conducted using a completely randomized design (CRD) alongside a two-factor factorial analysis, with each experiment replicated three times. A significant level of $p < 0.01$ was determined through analysis of variance. Furthermore, to perform principal component analysis (PCA), agglomerative hierarchical clustering, and the calculation of simple correlation coefficients, Microsoft Excel, integrated with XLSTAT version 2012.1.02 (copyrighted by Addinsoft from 1995 to 2021), was utilized as the analytical tool for these respective analyses.[29] [30].

3 RESULTS

The significance of the experimental parameters was assessed through 2-factor factorial ANOVA, which revealed statistically significant differences ($p < 0.01$) among the treatments. This indicated that the observed variations in responses were significantly influenced by the different levels of the factors under investigation. The significant interaction between genotypes and treatment levels further highlights the complex nature of genotype response to environmental stressors, emphasizing the need for targeted genetic improvement strategies to optimize cotton production under varying salt stress conditions.

The ANOVA results for root length (RL) and shoot length(SL) indicated significant effects on both genotypes and treatments, as well as their interaction in cotton under salt stress. Suggesting that different genotypes respond distinctly to salt stress, and the efficacy of any given treatment varies significantly across genotypes. The data underscores the critical role of selecting appropriate genotypes and treatments for optimal growth in salt-stressed

environments. The ANOVA results for other morphological parameters highlight statistically significant effects of genotypes, treatments, and their interactions across all measured parameters in cotton under salt stress.

For TSP (Total Soluble Protein), and POD (Peroxidase Activity), the ANOVA results indicated statistically significant effects of genotypes, treatments, and their interactions on these variables, across all factors in each analysis. For CAT (Catalase Activity), SOD (Superoxide Dismutase Activity), Proline, Na (Sodium), K (Potassium), and the Na/K ratio in cotton under salt stress revealed highly significant effects of genotypes, treatments, and their interactions across all parameters. These findings indicate a robust response of these physiological and biochemical traits to genetic variation and salt stress treatments, highlighting their critical roles in plant stress tolerance mechanisms.

TABLE 3.1 | Mean squares results of variance analysis for various characteristics.

Source of Variation	DF	SL	RL	FSW	DSW	FRW	DRW	TSP
Genotypes (G)	29	41.181	159.59	4.252	2.889	2.994	2.57	10.635
Treatment	2	928.3	2257.11	0.467	2.867	0.59	0.85	189.87
G * T	58	6.11	15.82	1.171	1.207	1.25	1.157	1.34
Error	180	0.58	0.59	0.001	0.0014	1.25	0.001	0.58
Total	269	CV3.86%	CV4.91%	CV0.87%	CV1.38%	CV1.39%	CV1.85%	CV10.03%

Source of Variation	DF	POD	CAT	SOD	PROLINE	Na+	K^+	$Na+/k^+$
Genotypes (G)	29	10.143	82.13	15.91	4.136	1828.5	1166.9	0.259
Treatment	2	113.249	1711.94	1359.77	61.9907	62662	61278.3	7.594
G * T	58	4.033	21.75	13.45	2.215	158.4	136.1	0.045
Error	180	0.586	0.59	0.59	0.0015	0.5	0.6	0.002
Total	269	CV8.78%	CV3.22%	CV2.83	CV0.75%	CV1.80%	CV0.67	CV3.67%

The degrees of freedom (DF) and mean square values for stem length (SL), Root length (RL), fresh shoot weight (FSW), dry shoot weight (DSW), fresh root weight (FRW), dry root weight (DRW), total soluble protein (TSP), peroxidase (POD), catalase (CAT), SOD, proline, sodium ion concentration(Na+) potassium ion concentration(K^+), sodium to potassium ratio ($Na+/k^+$) across genotypes (G), treatment, genotype by treatment interaction (G * T), and residual error.

3.1 Box Plot

In our study, we examined the effects of salinity stress on cotton seedlings, highlighting the intricate responses across a spectrum of morpho-physiological and biochemical traits. The investigation was methodically structured around three distinct salinity stress levels: control (Normal), moderate salinity (12 dS/m), and high salinity (17 dS/m), with the data visualization employing boxplots to elucidate the distribution patterns of each trait under these conditions.

For shoot length and root length, the normal condition (green box) exhibited the highest median values, symbolizing optimal growth in the absence of stress. As salinity increased to 12 dS/m (orange box), a noticeable reduction in median length was evident, which further declined under the 17 dS/m stress level (red box), indicating a pronounced sensitivity of these growth parameters to salinity stress.

The analysis of fresh and dry shoot weight, as well as fresh and dry root weight, mirrored this trend, with the weight measurements diminishing progressively with increased salinity. The shift in boxplot colors from green through orange to red not only visually represents a gradient of increasing stress but also corresponds to a decline in the robustness of these biomass indicators, underscoring the detrimental effects of saline environments on plant vigor. Biochemical traits such as total soluble protein, peroxidase (POD), catalase (CAT), and superoxide Dismutase (SOD) activities displayed a complex response pattern. Under control conditions, the green boxes showed relatively high median values, indicative of baseline metabolic activities. With moderate stress (12 dS/m, orange boxes), we observed a variable response where, in some cases, enzyme activities appeared to increase, possibly reflecting an initial adaptive response to counteract oxidative stress. However, at high salinity levels (17 dS/m, red boxes), the general trend

suggested a strain on these biochemical defenses, as evidenced by lower median values or increased variability within the populations, indicating a potential overburdening of the plant's stress response mechanisms.

Fig. 1: Box plots representing response of different traits to different salt stress levels.

Proline accumulation, a well-known Osmo protectant, increased with salinity levels, as indicated by the upward shift from green to red boxes. This trend underscores proline role in osmotic adjustment and stress mitigation. Sodium Ion Concentration followed an expected upward trajectory with increased salinity, which was visually captured by the transition from green through orange to red, reflecting the plant's exposure to and accumulation of Na+ ions. Conversely, Potassium Ion Concentration exhibited a nuanced response, with the highest values in control conditions (green box) and a decline under stress (orange and red boxes), illustrating the challenge of maintaining K+ homeostasis under saline conditions. The Sodium to Potassium Ratio (Na+/K+) significantly increased with salinity, as evidenced by the color gradation in the boxplots, signaling an escalating ionic imbalance detrimental to plant health.

The left side of the boxplots, denoting the lower quartile to the median (25th to 50th percentile), illustrates the central tendency and dispersion of data under each condition. This region is critical for understanding the range within which the majority of observations fall, providing insight into the trait's stability or variability under varying stress levels. In sum, this detailed trait-wise analysis via color-coded boxplots offers a nuanced understanding of cotton seedlings' adaptive and maladaptive responses to salinity stress. The visual and statistical patterns observed underscore the profound impact of salinity on cotton physiology and biochemistry, laying a foundation for targeted breeding and genetic engineering efforts aimed at enhancing salinity tolerance in cotton crops.

3.2 Bar Graphs

Bar graphs, in addition to box plots, provide a more detailed representation of the variability and distribution of trait responses under different levels of salt stress. In our investigation into the response of cotton seedlings to salinity stress, traits under study 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 Protein (TSP), Peroxidase Activity (POD), catalase activity (CAT), superoxide dismutase activity (SOD), proline content, and the ionic concentrations of sodium (Na^+) and potassium (K^+), alongside their ratio (Na^+/K^+).

Our findings reveal that under normal conditions (T0), the cotton seedlings exhibited robust growth and physiological activity, with mean values for shoot length and root length at 23.12cm and 21.19cm, respectively. However, upon exposure to moderate (T1) and severe (T2) salinity stress, a marked decrease in these parameters was observed. Specifically, the mean Shoot Length reduced to 19.72cm and 16.70cm under T1 and T2, respectively, underscoring the significant impact of elevated salinity levels on plant development. In the control group (T0), cotton seedlings exhibited optimal growth, with fresh root weight (FRW) and fresh shoot weight (FSW) presenting substantial biomass indicative of vigorous development. Conversely, as salinity levels escalated to T1 and T2, a noticeable decrement in both FRW and FSW was observed, underscoring the detrimental impact of salinity on plant growth. This trend was similarly reflected in the dry weights (DRW and DSW), affirming the overarching stress effect across both fresh and dry biomass assessments.

Similarly, biochemical adaptations to salinity stress were reflected in the activities of antioxidant enzymes and proline accumulation. For instance, peroxidase activity (POD) exhibited mean values of 7.72, 8.50, and 9,93 under T0, T1, and T2 conditions, respectively. This trend was mirrored across other biochemical traits, indicating an orchestrated defensive response to mitigate oxidative stress induced by salinity. Notably, the Na^+/K^+ ratio, a critical indicator of ionic homeostasis and salinity stress tolerance, showed a significant increase from 0.15 in control conditions to 0.42 and 0.73 under moderate and severe stress levels, respectively. This shift highlights the challenge of maintaining cellular ion balance under salinity stress, which is pivotal for sustaining plant growth and metabolic functions.

These results underscore the complex interplay between genetic constitution and environmental stress, providing valuable insights for breeding strategies aimed at enhancing salinity tolerance in cotton. Future research will focus on the genetic underpinnings of these phenotypic traits, aiming to unravel the molecular mechanisms that confer resilience against salinity stress, thereby contributing to the sustainable cultivation of cotton in salt-affected regions.

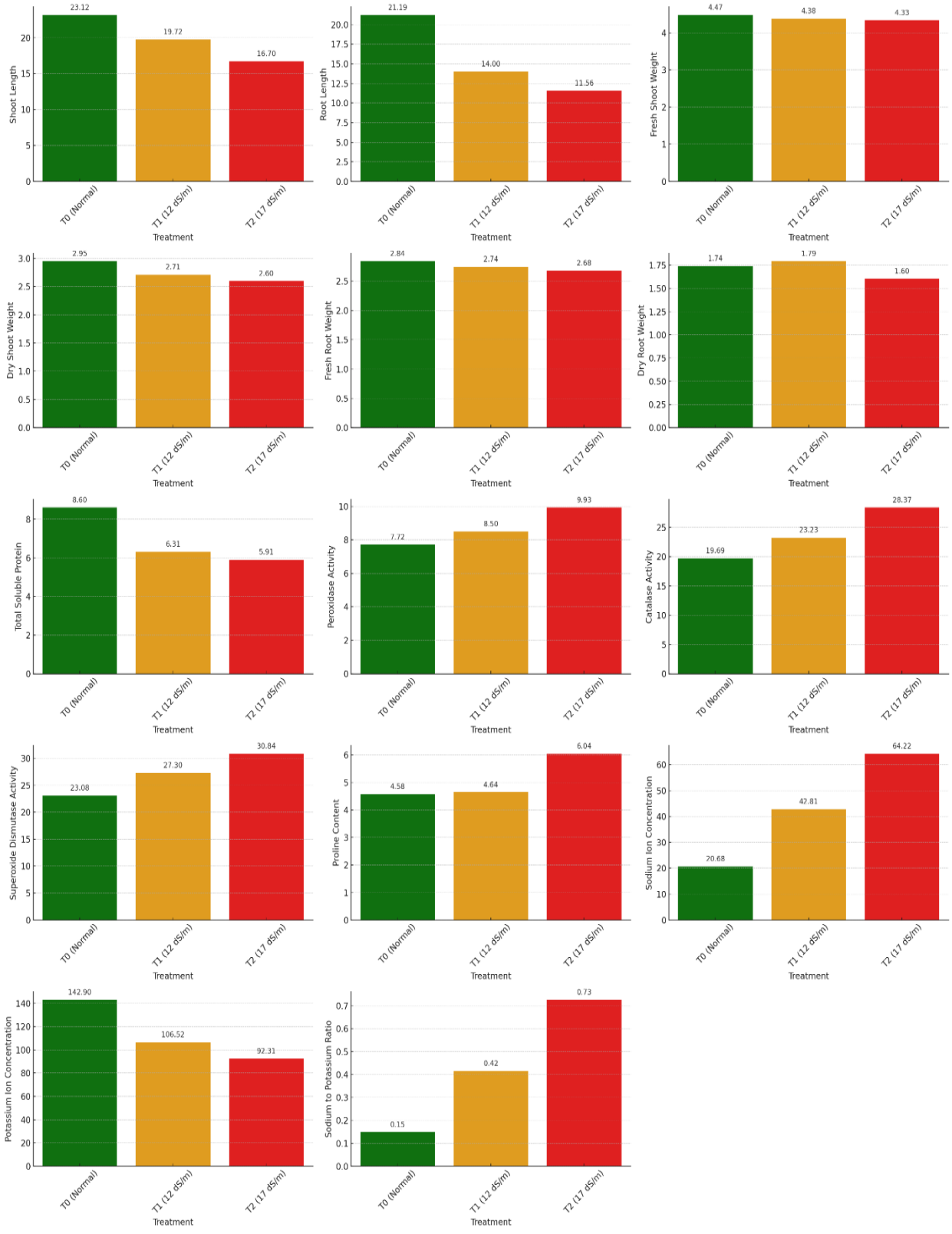

Fig. 2: Bar graphs representing response of different traits to different salt stress levels.

3.3 Scree Plot

The scree plot, a cornerstone of our Principal Component Analysis (PCA), vividly illustrates the variance captured by the initial 10 principal components derived from our dataset on cotton seedlings under varying levels of salinity stress. This visualization is instrumental in elucidating the dimensionality of our dataset, highlighting the significant proportion of total variance (approximately 76%) accounted for by the first two principal components alone. The first component captures 42.7% of the variance, primarily reflecting the broad-scale response of the cotton seedlings to salinity stress across the physiological and biochemical traits measured. The second component, accounting for an additional 33.3% of the variance, likely represents variations within these responses, possibly related to genotypic differences or specific physiological mechanisms of stress tolerance.

The subsequent components, though individually accounting for smaller percentages of the total variance (ranging from 10.8% for the third component to 0.8% for the tenth), collectively contribute to a more nuanced understanding of the dataset. These components may encapsulate specific traits or interactions not captured by the first two, underscoring the complexity of plant responses to environmental stresses. The diminishing variance explained by each successive component, as depicted in the scree plot with dark blue bars and annotated values, underscores the principle of diminishing returns in capturing new information with additional components.

This analysis not only reaffirms the multidimensional nature of salinity stress response in cotton seedlings but also highlights the utility of PCA in identifying key patterns and reducing data complexity. The scree plot, therefore, serves as a pivotal tool in our exploratory data analysis, guiding the selection of components for further investigation and modeling. It lays the groundwork for subsequent analyses aimed at deciphering the intricate web of physiological and biochemical responses to salinity stress, potentially unveiling genotypic traits conducive to enhanced tolerance and resilience in cotton crops.

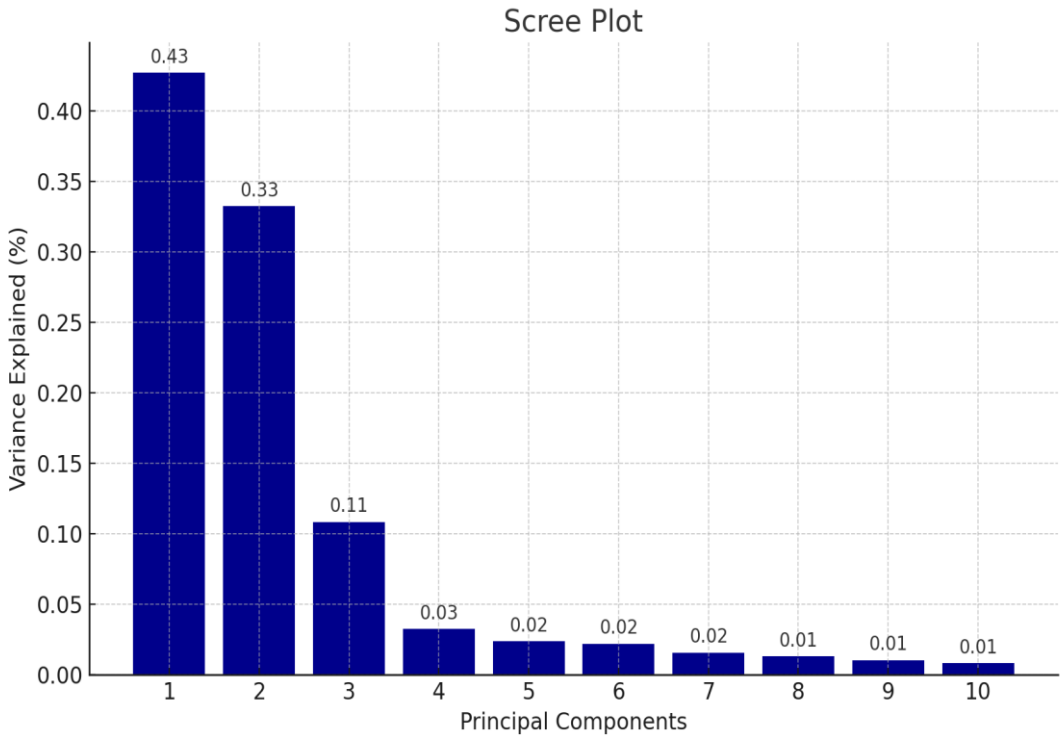

Fig. 3 Scree plot for different salt stress levels

3.4 PCA

In our research examining the impact of salinity stress on cotton seedlings, Principal Component Analysis (PCA) was pivotal in dissecting the dataset to uncover patterns across morphological, physiological, and biochemical traits. The PCA underscored that the initial two principal components significantly encapsulate the dataset's inherent variability, with Principal Component 1 (PC1) elucidating 42.74% and Principal Component 2 (PC2) revealing 33.26% of the total variance. This profound representation of data variance through the foremost components accentuates the intricate and multifaceted nature of the cotton seedlings' response to salinity stress, encompassing both morphological dimensions such as shoot and root lengths, and physiological and biochemical aspects including ion content and enzyme activity.

Distinct segregation was observed among the treatment groups within the PCA framework, delineated by color codes: green for the control group (T0), orange for mild salinity stress (T1, 12 dS/m), and red for severe stress conditions (T2, 17 dS/m). This demarcation highlighted the phenotypic variance induced by differing salinity levels, revealing a nuanced physiological and biochemical adjustment spectrum in response to increased salinity. Moreover, the differential clustering along the PC1 and PC2 axes not only provided a visual testament to the specific stress responses elicited by varying salinity concentrations but also underscored the potential of certain genotypes to better withstand salinity challenges.

This analytical approach facilitated a comprehensive exploration of the traits that are crucial for understanding salinity tolerance in cotton, offering a robust platform for subsequent genetic and molecular investigations aimed at fortifying the crop against salinity stress. Through illuminating the complex interplay between morphological, physiological, and biochemical traits under salinity stress, our PCA analysis has laid foundational insights for enhancing cotton's resilience to salinity.

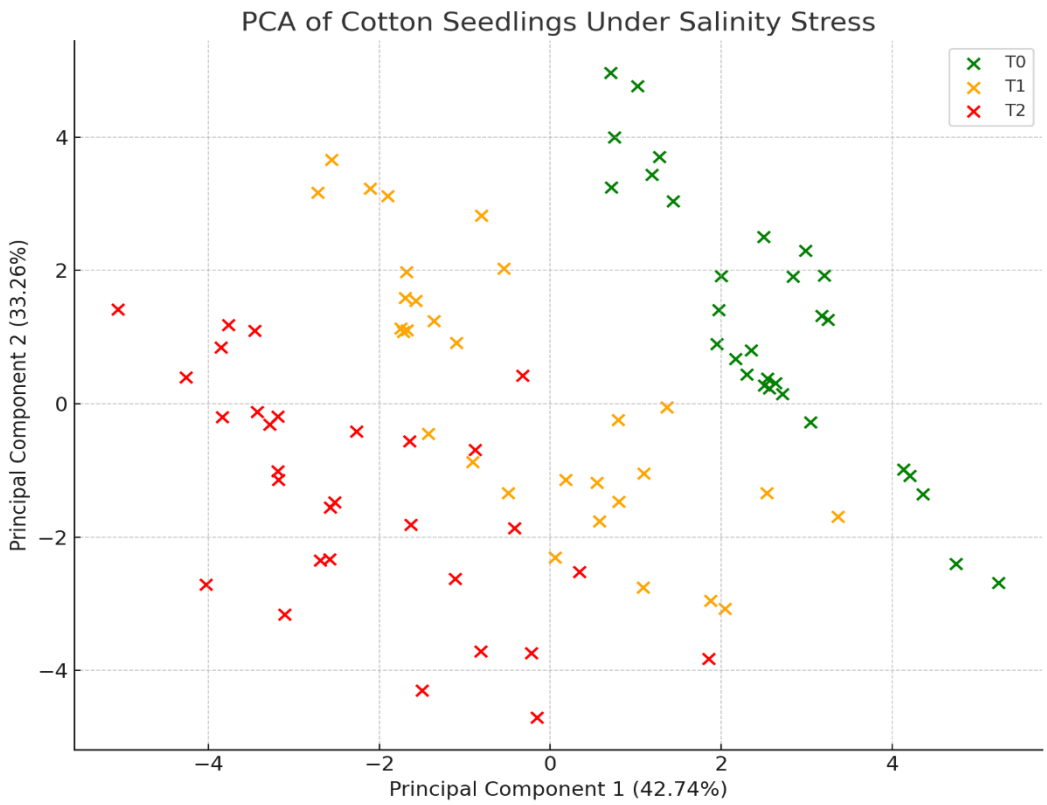

Fig. 4 Biplots representation of different salt stress levels

3.5 PCA Biplot

The PCA biplot analysis unveils the intricate interplay of morphological, physiological, and biochemical responses of cotton seedlings under salinity stress, highlighting how specific genotypes navigate the spectrum of stress tolerance. By capturing 76% of the dataset's variance through Principal Component 1 (42.74%) and Principal Component 2 (33.26%), this visual tool delineates the resilience or vulnerability of cotton genotypes to varying degrees of salinity, with the treatments—control (T0), mild stress (T1), and severe stress (T2)—color-coded for clarity.

Under mild salinity stress (T1), genotypes "FH-115, FH-118, IR-3, NIAB-878, VH-329" emerge as particularly resilient, maintaining key physiological and growth traits near optimal levels. Their positioning in the biplot suggests a robust ability to mitigate the effects of mild salinity through effective ionic regulation and osmotic balance. Conversely, in the face of severe salinity stress (T2), "FH-35, SLH-8, FH-170 and ACG-501" exhibit exceptional tolerance, potentially leveraging enhanced antioxidant defense mechanisms and efficient nutrient uptake to sustain growth and physiological functions.

The analysis also identifies genotypes "FH-634, VH-295 and CRS-2007" markedly sensitive to increased salinity levels. Their proximity to the severe stress indicators on the biplot underscores a significant disruption in physiological equilibrium under stress, highlighting the need for targeted breeding strategies to improve their salinity tolerance.

The directional vectors representing traits such as shoot and root length, proline content, and Na+/K+ ratio reveal the complex traits contributing to stress adaptation. Genotypes maintaining a central biplot position or showing minimal displacement toward stress-associated vectors likely possess a combination of adaptive traits, offering resilience against salinity stress. This nuanced understanding facilitates the identification of key traits for selection in breeding programs aimed at enhancing cotton's salinity tolerance.

This comprehensive PCA biplot analysis spotlights genotypes " FH-115, FH-118, IR-3, NIAB-878, VH-329" for their resilience under mild stress, and "BR-776," "KG-102" for their robustness under severe stress, positioning them as prime candidates for salinity tolerance breeding. The identified vulnerable genotypes " FH-35, SLH-8, FH-170 and ACG-501" mark the critical targets for genetic improvement efforts. Through strategic genotype selection and breeding, this research contributes significantly to developing cotton varieties with enhanced tolerance to salinity, paving the way for sustainable agriculture in salinity-affected regions.

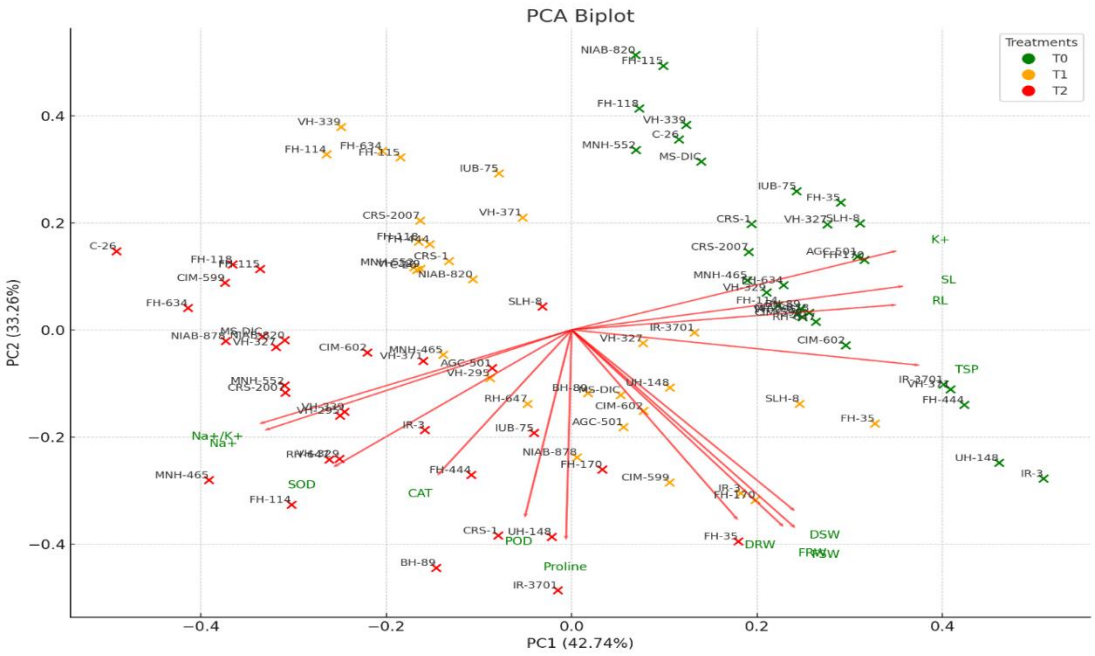

Fig. 5 PCA Biplots representation of genotypes to different salt stress levels.

3.6 Silhouette clustering

The silhouette analysis conducted on the PCA-transformed dataset of cotton seedlings under salinity stress provides a quantitative measure to infer the optimal number of clusters that represent distinct groupings within the data, based on the similarity of morphological, physiological, and biochemical traits. This method evaluates the cohesion within clusters and the separation between them, with silhouette scores ranging from -1 to 1, where a higher score indicates that samples are well matched to their own cluster and distinctly separated from others. Our analysis spanned a range of 2 to 10 clusters, revealing a nuanced landscape of silhouette scores that guide the identification of an optimal clustering structure. The silhouette scores exhibited a pattern, with certain numbers of clusters achieving higher scores, indicative of a more appropriate fit for the dataset's inherent structure. The plot of silhouette scores against the number of clusters presents a visual aid in determining the point at which the score is maximized, suggesting an optimal balance between cluster cohesion and separation.

The results from the silhouette analysis are pivotal for understanding the underlying structure of the dataset, potentially illuminating natural groupings of cotton genotypes based on their response to salinity stress. Identifying these clusters aids in pinpointing genotypes with similar stress response profiles, which can significantly impact the development of breeding strategies aimed at enhancing salinity tolerance. The optimal number of clusters, as indicated by the peak silhouette score, offers a strategic foundation for further genetic and physiological investigations, enabling focused studies on the mechanisms of stress tolerance and the identification of key traits for salinity resilience. This silhouette analysis not only augments our methodological arsenal for exploring complex biological data but also contributes significantly to our understanding of plant stress physiology, facilitating targeted research and breeding efforts to improve crop resilience to salinity stress.

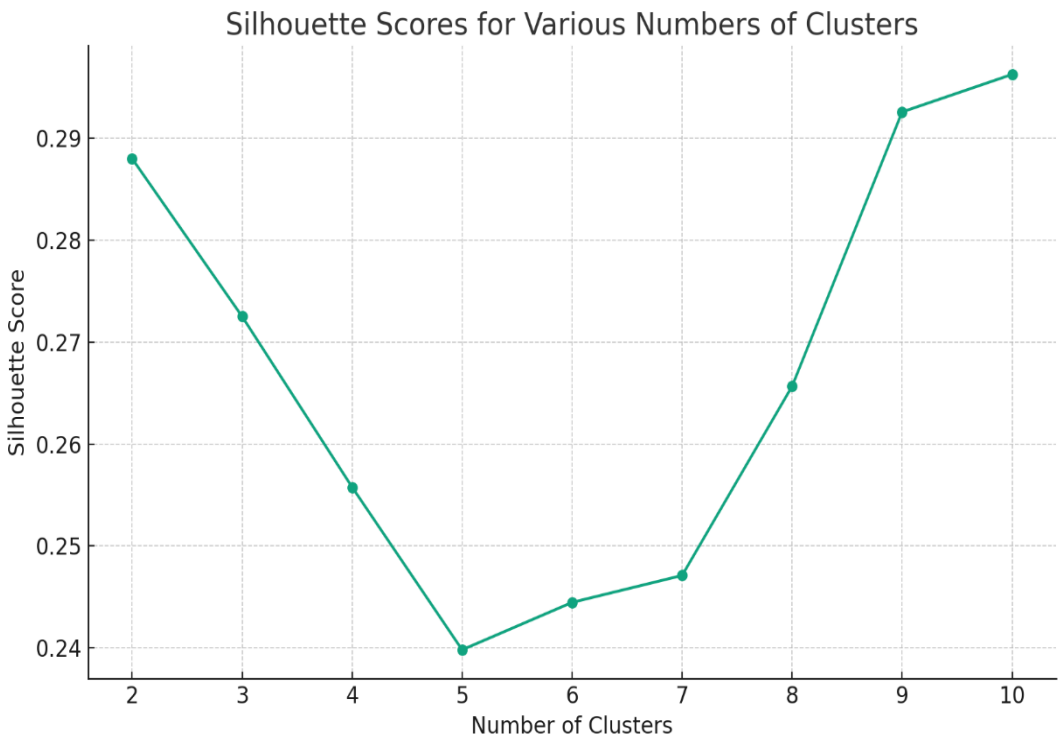

Fig. 6 Silhouette scores for various clusters

3.7 K-Means Cluster

In our research focusing on the salinity stress tolerance of cotton at the seedling stage, we employed K-Means clustering to analyze and group genotypes based on their physiological and biochemical responses. The analysis identified three distinct clusters, which were visualized using principal component analysis (PCA) to reduce the dimensionality of our multivariate dataset. This approach facilitated a clear separation of genotypes into three groups, represented in the visualization by different colors corresponding to their assigned clusters.

Cluster 1, predominantly characterized by a certain set of genotypes, displayed specific traits that suggest a unique response pattern to salinity stress. This cluster, along with the others, was differentiated based on key physiological and biochemical metrics, including shoot length, root length, fresh and dry weights of shoots and roots, and activities of antioxidant enzymes among others. Notably, genotypes in this cluster might represent a group with either higher tolerance or susceptibility to salinity stress, as inferred from their clustering pattern. Cluster 2 included genotypes that were distinctly separated from the others, indicating a potential difference in their salinity stress response mechanisms or overall resilience. This cluster's unique positioning in the PCA plot emphasizes the importance of genotype-specific responses to environmental stresses. Cluster 3 was marked by another set of genotypes, again highlighting the diverse genetic basis for salinity stress tolerance within cotton. The distribution and grouping of genotypes across these clusters underscore the complex interplay of genetic factors contributing to stress tolerance.

The clusters were color-coded based on the treatment levels (T0, T1, T2), which represented control, moderate, and high salinity stress conditions, respectively. This color coding, with T0 in green, T1 in orange, and T2 in red, not only facilitated an easy differentiation of the stress levels but also provided insights into how each genotype within the clusters responds to increasing salinity stress.

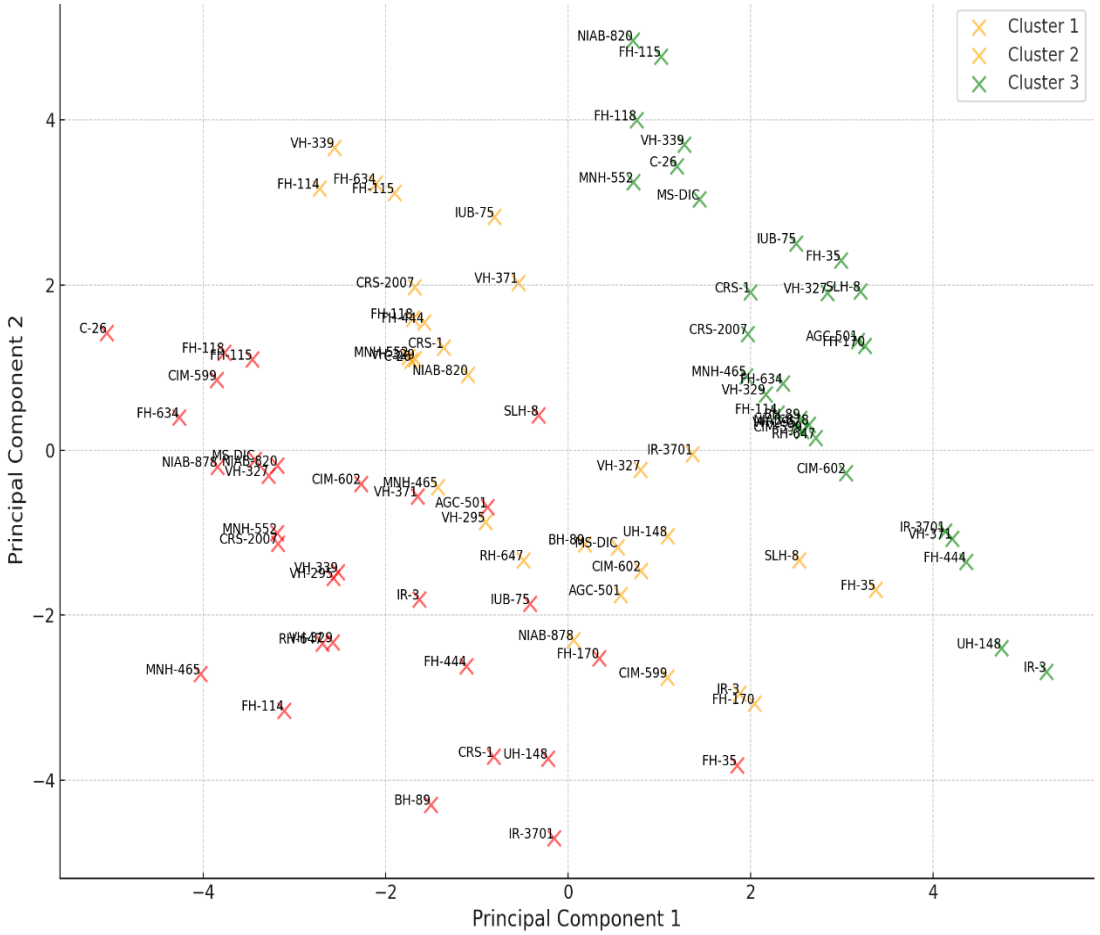

Fig. 7 K-Means clustering of genotypes.

4 DISCUSSION

In our comprehensive analysis we delved into the morphological, physiological and biochemical responses of 30 upland cotton varieties under varying salinity stress conditions, specifically control, T1 (12 dS/m), and T2 (17 dS/m). This study was conducted in the context of understanding salinity's multifaceted impact on plant growth at cellular, subcellular, and organ levels, drawing upon the foundational work of [31] and [32], which outlined the primary effects of salinity stress as osmotic stress through increased Na^+ and Cl^- concentrations. Our research aimed to build upon these insights by evaluating key parameters such as the activities of Superoxide Dismutase (SOD), Peroxidase (POD), Catalase (CAT), Proline concentration, Sodium (Na+), Potassium (K+) concentrations, and the Na+/K+ ratio, alongside physical growth measurements such as Stem Length (SL), Root Length (RL), and various weight metrics (FSW, DSW, FRW, DRW), and Total Soluble Protein (TSP).

Reflecting the challenges highlighted by [33] regarding ion toxicity's impact on biomass and potential programmed cell death, and the discussions by [34] and [35] on oxidative stress and lipid peroxidation, our study identified genotypes such as FH-115, FH-118, IR-3, NIAB-878, VH-329 as notably resilient. These genotypes demonstrated robust antioxidative enzyme activities, elevated Proline levels, and a balanced Na+/K+ ratio, indicative of an intrinsic capacity to withstand the oxidative stress and ion disequilibrium induced by high salinity. Conversely, genotypes FH-634, VH-295, CRS-2007, RH-647 and MNH-465 were categorized as less tolerant, underperforming in these critical markers and thus reflecting a higher susceptibility to salt-induced physiological and biochemical stress at the seedling stage.

This work not only contributes to the expanding body of literature on salt stress resilience, building upon the physiological and genetic mechanisms of tolerance explored by [36]and the phenotypic evaluation strategies discussed by [37]but also addresses the natural variation and the importance of accurate morpho-physiological indexing for salinity tolerance as described by [38] and [39]. By effectively identifying genotypes with pronounced tolerance and sensitivity to salinity stress, our research offers valuable insights for breeding strategies aimed at developing upland cotton varieties with enhanced resilience to salinity. This initiative not only promises sustainable cotton production in saline-affected areas but also sets a foundation for future genetic and molecular studies to decode the underlying mechanisms of salt tolerance in cotton, ultimately boosting agricultural productivity in the face of challenging environmental conditions.

In our research on the impact of salt stress on cotton at the seedling stage, Principal Component Analysis (PCA) was employed as a critical methodological approach to distill the complex, multidimensional data into a more interpretable form. By transforming the original variables into a set of linearly uncorrelated principal components, PCA enabled the identification of the most significant patterns and variations within the dataset [40]. Our K-Means clustering analysis effectively segregated the cotton genotypes into three clusters based on their response to salinity stress at the seedling stage. The distinct grouping and the specific genotypes contained within each cluster offer valuable insights into the genetic diversity and potential mechanisms of salinity tolerance in cotton. This information could be instrumental in guiding future breeding programs aimed at enhancing salinity tolerance in cotton cultivars. This reduction in dimensionality not only facilitated a clearer understanding of the underlying structure of the data but also highlighted the key morphological, physiological, and biochemical traits that contribute to cotton seedlings' resilience or susceptibility to salt stress.

6 CONCLUSION

Since salinity is currently a significant factor restricting agricultural productivity and posing a threat to the future of agriculture, it is necessary to produce high-yielding and salt-tolerant cultivars. This work has yielded extensive insights into the salinity stress responses of cotton genotypes through the application of morphological, physiological, and biochemical analysis methodologies. By utilizing ANOVA, PCA, and cluster analysis, we have investigated the mechanisms of salinity tolerance in cotton. Our research yielded important results, such as differences in growth indices and antioxidant enzyme activity under heightened salinity stress. These findings highlight the negative effects on plant development and the critical function of these enzymes in reducing oxidative damage.

By using PCA, we were able to differentiate between genotypes that are more resilient to salinity, such as FH-115, FH-118, IR-3, NIAB-878, VH-329, and those that are less tolerant, like FH-634, VH-295, CRS-2007, RH-647 and MNH-465. This information can assist breeding programs in improving cotton's resistance to salinity. Furthermore, the incorporation of molecular markers into our findings has expanded our understanding of the genetic foundation of these traits, providing specific avenues for genetic enhancement. With the help of cluster analysis, genotypes have been successfully divided into different response groups, offering a clear plan for creating cotton cultivars with increased resistance to salinity.

The identification of these genotypes provides a foundation for further research into the genetic mechanisms underlying salt stress tolerance, with potential applications in breeding programs aimed at enhancing cotton resilience to salinity. This could be particularly beneficial for improving crop yields in areas prone to high soil salinity, a common challenge in many cotton-growing regions. In an era of climate change and water constraint, this work lays the foundation for the development of cotton cultivars that are better suited to tolerate salinity, ensuring sustainable cotton production.

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***Nimra Gul**, Ph.D. Plant Breeding and Genetics, University of Agriculture, Faisalabad. Pakistan.