Deciphering Drought Resilience and Unravelling Mechanisms of Tolerance in Cotton: Selection by Physiological and Biochemical Markers

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Abstract

Cotton (Gossypium spp.) is vital raw product for the global textile industry. Its production is affected by drought stress, exacerbated by climate change. This study was designed to investigate the physiological and biochemical responses of cotton genotypes to drought, aiming to identify mechanisms of drought tolerance and susceptibility. Within a glasshouse, diverse genotypes were grown in polythene bags with a soil-sand mix, subjected to three drought stress levels i.e., control (100% field capacity), moderate stress (75% field capacity), and severe stress (50% field capacity), in a two-factor factorial design under a Completely Randomized Design (CRD). Physiological measurements and biochemical assays under varying drought conditions revealed significant decreases in growth related traits (shoot and root lengths, fresh and dry weights) with increased drought severity. Alongside antioxidant defence 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 71.83% of the variance, summarizing the major contribution by genotypes' drought responses. Cluster analysis further delineated the genotypes into three clusters, representing distinct drought response strategies. Notably, Cluster 1 (e.g., MNH-554, FH-113, FH-682, VH-295, AA-802) exhibited robust drought tolerance, whereas Cluster 3 (e.g., FH-114, FH-901, IR-3, MNH-552) included more drought-susceptible genotypes. This study revealed the complex dynamics between cotton's genetic makeup, physiological responses, and biochemical processes in facing drought conditions and to ascertain the drought tolerant and susceptible genotypes.

Keywords: Cotton, drought stress, drought seedling, morpho-physiological, proline, catalase

1 INTRODUCTION

Gossypium species display significant morphological diversity, ranging from small, trailing herbaceous perennials to towering 15m trees, each with its unique set of reproductive and vegetative traits. Predominantly, the commercially grown cotton varieties originate from two species: *G. hirsutum* and *G. barbadense*, with *G. hirsutum* accounting for 90% of global cotton output [31]. Recent statistics indicate a 14% reduction in cotton production, dropping from 13.98 million bales to drop 11.96 million bales over five years [3]. Drought significantly impairs cotton productivity, influencing root systems, exacerbating plant diseases, and increasing insect attacks. Specifically, Pakistan witnessed a 34% decline in cotton yield compared to the previous year due to the combined stress of heat and water scarcity [7]. The impact of drought on cotton, as with other crops, is pervasive and varies across different environments, affecting production levels. Drought stress alters plant physiology through various cellular and molecular mechanisms [20]. As water resources dwindle, the demand for drought-resistant cotton genotypes is escalating. Although cotton is a glycophyte and shows a fairly higher resistance to abiotic stresses than other major crops, severe environmental conditions, including drought, detrimentally influence its growth, yield, and fiber quality [41].

Drought tolerance means in plants are broadly categorized into four strategies: drought avoidance, drought tolerance, drought recovery, and drought escape, each playing a crucial role in plant survival under water scarcity [11]. Among these, drought avoidance and tolerance are the primary defences against drought stress. However, variability in drought tolerance within the cotton crop is somewhat constrained, with notable differences observable primarily at the seedling stage. Understanding plant responses is essential for developing drought-tolerant genotypes. Morphological traits have been utilized to distinguish between drought sensitivity and tolerance in upland cotton, highlighting the importance of traits assessments [23][29]. The role of root morphology in determining drought response is particularly significant [4][27]. Moreover, leaf water content has been identified as a key indicator of drought tolerance, with higher water content often associated with drought-tolerant plants [8].

Principal Component Analysis (PCA) is a powerful statistical tool that has significantly advanced the selection of cotton genotypes with enhanced drought tolerance. By transforming complex, multidimensional datasets into simplified, interpretable components, PCA enables researchers to identify the key traits that contribute to drought resilience in cotton [39]. This method effectively reduces the dimensionality of the data, highlighting the most variance-capturing

factors without significant loss of information, thus facilitating a more efficient and accurate selection of genotypes for breeding programs. By analysing traits collectively, researchers can pinpoint genotypes that are likely to perform well under water-limited conditions [43]. Furthermore, PCA's role extends beyond trait selection, aiding in the genetic basis to drought tolerance by correlating physiological and morphological traits with genomic markers [44]. Incorporating PCA into the selection process not only streamlines the identification of drought-tolerant genotypes but also enhances the overall efficiency of breeding programs aimed at developing cotton varieties capable of sustaining productivity under drought stress [45].

Relative water content, or RWC, is an important marker for drought-tolerant genotypes in cotton seedlings. Drought stress damages cotton's cell membrane stability, lowers RWC, alters the accumulation of dry matter, and decreases chlorophyll a and b [14]. Research on cotton genotypes under varying levels of water stress has shown that leaf water content and the quantum yield of photosystem-II decline as drought stress intensifies [40]. Water scarcity disrupts cellular growth [24][42], constrains leaf stem elongation, and reduces the number formation of buds [15][30]. Leaf water content directly affects cell expansion [37], leading to slower growth rates in stems and roots, ultimately impacting cotton yield [18][32]. Drought conditions also upset the ROS production and antioxidant production, potentially leading to ROS accumulation within the plant system [13][34], highlighting the complexity of drought responses in cotton.

Considering the significant impact of drought on cotton, this research aimed to pinpoint morpho-physiological markers of drought resistance in cotton at the seedling is a pivotal phase for the plant's growth and system development. Using Principal Component Analysis (PCA) and cluster analysis, we identified genotypes with enhanced drought resilience. This identification process will contribute to the formulation of breeding strategies aimed at enhancing drought resilience in cotton, an essential step towards developing climate-resilient crops capable of withstanding future environmental challenges.

2 MATERIALS AND METHODS

This study was carried out at the University of Agriculture, Faisalabad's Plant Breeding and Genetics Department Research area. Ayub Agricultural Research Institute (AARI), Cotton Research Institute (CRI), Central Cotton Research Institute (CCRI) in Multan, Nuclear Institute for Agriculture and Biology (NIAB), and the University of Agriculture Faisalabad (UAF) were among the prestigious institutions from which healthy seeds of 30 cotton genotypes were obtained for this study. The details of these genotypes are systematically catalogued in Table 2.1.

Table 2.1. list of genotypes that were employed in the study.

Sr. No.	Genotypes	Sr. No.	Genotype
G1	IUB-65	G16	FH-682
G2	FH-182	G17	VH-367
G3	FH-901	G18	NIAB-820
G4	VH-339	G19	MNH-552
G5	FH-634	G20	VH-295
G6	FH-114	G21	CRS-2007
G7	FH-170	G22	AA-802
G8	FH-115	G23	FH-118
G9	CIM-600	G24	RH-647
G10	MNH-605	G25	FH-142
G11	MNH-554	G26	BH-89
G12	IR-3	G27	FH-4243
G13	UH-148	G28	FH-444
G14	FH-113	G29	SLH-8
G15	VH-327	G30	IR-3701

Twenty-seven polythene bags for each cotton cultivar were categorized into three groups: a control group at 100% Field Capacity (FC), Stress1 at 75% FC, and Stress3 at 50% FC, with each treatment comprising three bags per replicate. These groups were initially irrigated daily at 100% FC till reaching the second true leaf stage to ensure uniform growth. At this pivotal stage, drought stress was introduced to test the cultivars' resilience. The control group continued to receive water to maintain 100% FC, applied whenever the soil's water content approached the Maximum Alloweable

Deficit (MAD), defined as 50% of the FC. In contrast, seedlings under drought stress conditions were regulated to remain at 75% and 50% FC for a duration of 10 days. To ensure precision, polythene bags were weighted up daily, and watering was adjusted accordingly to maintain the designated moisture levels. The duration of the experiment extended until the emergence of the fourth rising of main stem leaf, at which point the young plants were carefully extracted for the analysis of various morpho-physiological traits, as outlined in the methodology by [19].

Table 2.2: Examination of the Soil Employed in the Trial

Soil Characteristics	Values
Saturation Percentage	40.9%
pH	7.8
Electrical Conductivity (EC) (dSm−1)	1.5
Organic Matter (%)	0.74%
Sodium (mg Kg−1)	67
Chlorite (meq L−1)	1.5
Field Capacity (ml)	408
Carbonate	Nil
Bicarbonate (meq L−1)	2.5
Soil Texture	Silt Loam
Calcium & Magnesium (meq L−1)	25
Available Potassium (K) (mg Kg−1)	16
Calcium (meq L−1)	20

2.1 Morphological Parameters

As the fourth true leaf emerged, we precisely gathered data on key morphological parameters, for comprehensive analysis. To prepare for measurement, the length of shoot for each genotype were first thoroughly rinsed with distilled water. Subsequently, the shoot parts were separated, and their lengths were accurately measured in centimetres. The average SL for each genotype under every treatment condition was then calculated. In a similar manner, roots of every genotype were gathered, cleansed using distilled water, and measured for length. In order to compare morphological responses under various water availability conditions, the average RL was calculated. While DSW and DRW samples were measured after being in the oven for 42 hours and then weighted, FSW and FRW samples were weighted immediately after being cleaned and completely dried with tissue paper.

2.2 Physiological Parameters:

In order to determine the drought resilience of the cotton genotypes under investigation, the physiological responses to drought stress were carefully assessed in this work, with an emphasis on excised leaf water loss (ELWL) and relative water content (RWC). In order to determine their fresh weight, leaves removed at the fourth true leaf stage for ELWL were weighed right away. After that, the leaves were allowed to air dry in a controlled environment, and they were weighed on a regular basis to monitor water loss. This procedure was carried out repeatedly until the weight stabilised, indicating full evaporation of the water. ELWL was quantified using the formula:

$$ELWL = \frac{(Initial\ Fresh\ Weight - Final\ dry\ weight)}{initial\ fresh\ weight\ \times 100}$$

offering a precise measure of water loss efficiency under drought conditions.

To calculate RWC, a crucial measure of cellular hydration state, the fresh weight of leaves was compared to their fully turgid weight and their dry weight following desiccation. After completely rehydrating in distilled water, the leaves

were weighed to determine their turgid weight. To achieve the dry weight, leaves were then dried. The following formula was used to determine RWC:

$$RWC = \frac{(Fresh\ weight - Dry\ weight)}{Turgid\ weight - Dry\ weight} \times 100$$

2.3 Biochemical Analysis:

The genotypes at the fourth true leaf from each genotype's duplicates were gathered for the estimation of biochemical analysis to determine the response of biochemical chemicals after morpho-physiological examination. In order to extract antioxidant enzymes, 0.1 g of fresh leaf samples from each genotype of cotton were ground and then centrifuged for 10 minutes at 14,000 rpm in 1 ml of 50 mM cold phosphate buffer (pH 7.8). The amount of enzyme activity was measured using the supernatant that resulted. 95 ml of 0.1 N sodium chloride and 1 ml of dye reagent were combined with 5 ml of the extracted supernatant to estimate the amount of soluble protein. 0.02 g of Coomassie Brilliant Blue G-250 dye were dissolved in 10 ml of 95% ethanol and 20 ml of water to create the dye reagent 200 millilitres of diluted phosphoric acid. The protein-dye complex took five minutes to develop, and then the absorbance at 595 nm was measured.

Cotton leaves were emulsified in a solution containing 0.1 mM EDTA, 50 mM potassium phosphate buffer, and 1 mM dithiothreitol in order to estimate the activity of superoxide dismutase (SOD) [9]. Next, the ability of SOD to prevent the photochemical reduction of nitroblue tetrazolium was measured [16].

Cotton leaves were homogenised in a solution containing 50 mM potassium phosphate buffer, 0.1 mM EDTA, and 1 mM DTT in order to estimate the levels of peroxidase (POD) and catalase (CAT) activity [6]. The POD activity was measured according to Chance and Maehly's methodology. In a similar manner, Erel's approach was used to measure the activity of ascorbate peroxidase (APX) [10].

Cotton leaves were homogenised in a medium containing 3% sulfosalicylic acid (SSA) solution, centrifuged, and then processed again for proline measurement. After that, the supernatant was heated to 100°C for an hour while being combined with acid ninhydrin and glacial acetic acid. Toluene was added after cooling, and the organic phase's absorbance was measured at 520 nm [11]. Cotton leaves were homogenised in 0.1 M sodium phosphate buffer (pH 7.8) and centrifuged to estimate total soluble protein (TSP). Following a mixture of the supernatant and Coomassie Brilliant Blue G-250 dye reagent, the mixture was incubated at room temperature. With a spectrophotometer set at 595 nm, the absorbance was measured [14].

2.4 Statistical Analysis:

Using a completely randomised design (CRD) and a two-factor factorial analysis, the screening procedure was carried out in triplicate. The significant level ($p < 0.01$) was found using variance analysis. Additionally, Microsoft Excel was used in conjunction with XLSTAT version 2012.1.02, which is copyrighted by Addinsoft 1995–2021, to conduct principal component analysis (PCA), agglomerative hierarchical clustering, and simple correlation coefficients. acted as sources for the corresponding analyses [2] [12].

3 RESULTS

3.1 Morpho-physiological Traits:

ANOVA was used to determine the significance of the experimental parameters, and the results showed that there were statistically significant differences ($p < 0.01$) between the treatments. This indicates that the variation observed in the responses can be attributed to the different levels of the factors under investigation. These findings underscore the effectiveness of the experimental design in elucidating the effects of the treatments on the measured parameters. Additionally, the significance of the results reinforces the reliability of the conclusions drawn from the study, providing a robust basis for further interpretation and inference Table 3.1.

Root Length (RL) and Shoot Length (SL) also revealed highly significant differences among genotypes and treatments ($P < 0.0001$), including a significant genotype×treatment interaction, suggesting that drought stress substantially influences growth in terms of root and shoot lengths, with variable growth responses among genotypes to water scarcity.

Dry Root Weight (DRW) and Dry Shoot Weight (DSW) both exhibited highly significant differences among genotypes and treatment levels ($P < 0.0001$), highlighting the impact of drought stress on cotton's biomass accumulation. The interaction between genotype and treatment was significant, indicating varied responses among genotypes to different drought stress levels. Similarly, Fresh Root Weight (FRW) and Fresh Shoot Weight (FSW) showed significant

variation among genotypes and treatments (P < 0.0001), with a notable genotype treatment×interaction. This variation emphasizes the effect of drought stress on the fresh weight of cotton, reflecting differences in water content retention among genotypes under stress.

Peroxidase (POD) activity varied significantly across genotypes and treatment levels (P < 0.0001), with significant genotype*treatment interaction, indicating an enhanced antioxidant response to mitigate oxidative damage from drought stress. Total Soluble Proteins (TSP), Catalase (CAT) Activity, and Superoxide Dismutase (SOD) Activity showed significant differences by genotypes and treatment levels (P < 0.0001), with observed interactions. The increase in these biochemical markers under drought stress indicates a robust protective mechanism against oxidative stress induced by drought.

Electrolyte Leakage (ELWL) and Proline Content were significantly different among genotypes and treatments (P < 0.0001), with a marked interaction. Elevated proline levels and increased ELWL under drought stress suggest enhanced Osmo protective responses and changes in membrane permeability, respectively. Relative Water Content (RWC) demonstrated significant variations across genotypes and treatments (P < 0.0001), with a significant genotype*treatment interaction, underscoring differences in water use efficiency and hydration status among genotypes under drought conditions.

Table 3.1 | mean squares results of variance analysis for various characteristics.

Source of Variation	DF	SL	RL	FSW	DSW	FRW	DRW	SOD
Genotypes (G)	29	55.87	130.45	3.3987	1.4007	1.69562	0.8694	46.8
Treatment	2	1081.43	1877.43	8.07472	12.4864	5.87652	5.71784	1083.56
G * W	58	8.03	11.87	1.02842	1.0197	1.06379	1.08901	20.16
Error	180	0.59	0.59	0.00149	0.0015	0.00149	0.00023	0.59
Total	269	CV4.04%	CV4.93%	CV1.09%	CV1.98%	CV1.87%	CV1.31%	CV3.00%

Source of Variation	DF	POD	CAT	TSP	Proline	ELWL	RWC
Genotypes (G)	29	7.7153	85.904	7.946	5.1044	7.4667	925
Treatment	2	52.7508	946.29	188.667	41.5815	72.2815	18132.9
G * W	58	2.6773	20.027	2.249	1.3374	1.2421	240.3
Error	180	0.0015	0.591	0.001	0.0015	0.0015	0.6
Total	269	CV0.51%	3.57%	0.59%	0.90%	1.01%	1.37%

The degrees of freedom (DF) and mean square values for stem length (SL), Root length (RL), excised leave water loss (ELWL), proline, total soluble protein (TSP), peroxidase (POD), catalase (CAT), fresh shoot weight (FSW), dry shoot weight (DSW), fresh root weight (FRW), and relative water content (RWC) across genotypes (G), treatment, genotype by water interaction (G * W), and residual error.

3.2 Bar Graphs

Subjecting cotton drought stress it was observed how the application of varying stress levels influences key physiological traits compared to a control group. Our experimental layout was planned as control group, alongside with two treatment groups subjected to different stress intensities i.e., Stress 1 and Stress 2 respectively. Through meticulous analysis, it was observed discernible changes in several crucial traits as stress levels escalate from the control to Stress 1 and Stress 2 conditions as mentioned in Fig. 3.1.

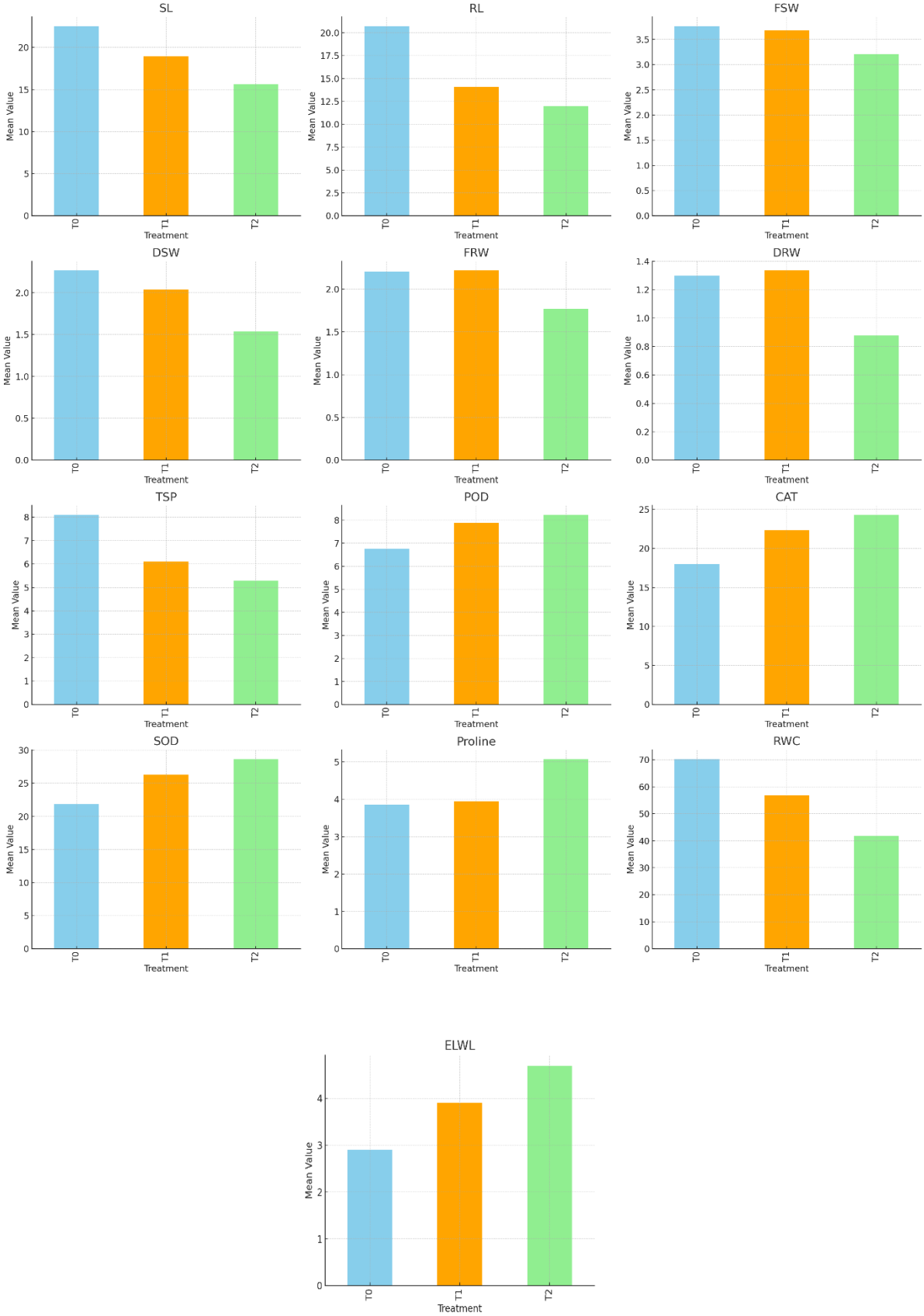

Fig. 3.1 | The bar graphs visually represent the mean values of each trait across the three treatments (T0, T1, T2)

Noting down the results from above figure 1. it was observed in Decrease in Growth Parameters: Shoot Length (SL), Root Length (RL), Fresh and Dry Weights (FSW, DSW, FRW, DRW) generally decrease from control (T0) to severe stress (T2), indicating that drought stress significantly inhibits plant growth. While for measuring the bbiochemical Response The values were increased in Total Soluble Protein (TSP), POD, CAT, and SOD activities under stress treatments (T1 and T2) compared to control (T0) suggests an enhanced antioxidant defence mechanism in response to oxidative stress caused by drought. while the values for proline and RWC it was observed that Proline levels were the potential indicator of stress tolerance, which were increased underdrought stress, which corroborates with the decrease in Relative Water Content (RWC), highlighting the physiological adjustments plants undergo to cope with drought. Meanwhile excised leaves water loss (ELWL) was noted as the increase in ELWL under stress treatments suggests higher water loss, indicating the drought stress impact on plant water regulation. These results suggested variability in genotype responses to drought stress, with some showing potential tolerance mechanisms through biochemical and physiological adaptations.

3.3 Box Plots

Box plots, in addition to bar graphs, provide a more detailed representation of the variability and distribution of trait responses under different levels of stress caused by the cotton drought. Additionally, box plots were utilised in our research to clarify the extent and variation of trait changes between the control group and Stress 1 and Stress 2 treatments. This allowed us to gain a thorough grasp of how drought stress affects cotton physiology Fig. 3.2.

Box plots were used to examine the physiological and biochemical responses of various cotton genotypes to differing degrees of drought stress. This analysis yielded important insights into the mechanisms of plant adaptation. Variations in genotype growth capacity were indicated by the large range of shoot length (SL) and root length (RL) recorded under normal conditions (T0). Under stress circumstances (T1 and T2), this variability decreased, and the median values decreased significantly, suggesting that dryness had a detrimental effect on plant growth. As stress levels grew, fresh and dry weights (FSW, DSW, FRW, and DRW) showed a similar trend, with median values falling, demonstrating the effect of water scarcity on biomass accumulation.

Biochemical markers showed an increase in median values from T0 to T2, including Total Soluble Protein (TSP), Peroxidase (POD), Catalase (CAT), and Superoxide Dismutase (SOD). This rise may indicate an enhanced antioxidant defence mechanism in response to oxidative stress brought on by drought circumstances. With the greatest median values and outliers in T2, proline—a crucial osmolyte recognised for its function in stress tolerance—exhibited a noteworthy rise during drought stress, demonstrating its key involvement in osmotic adjustment.

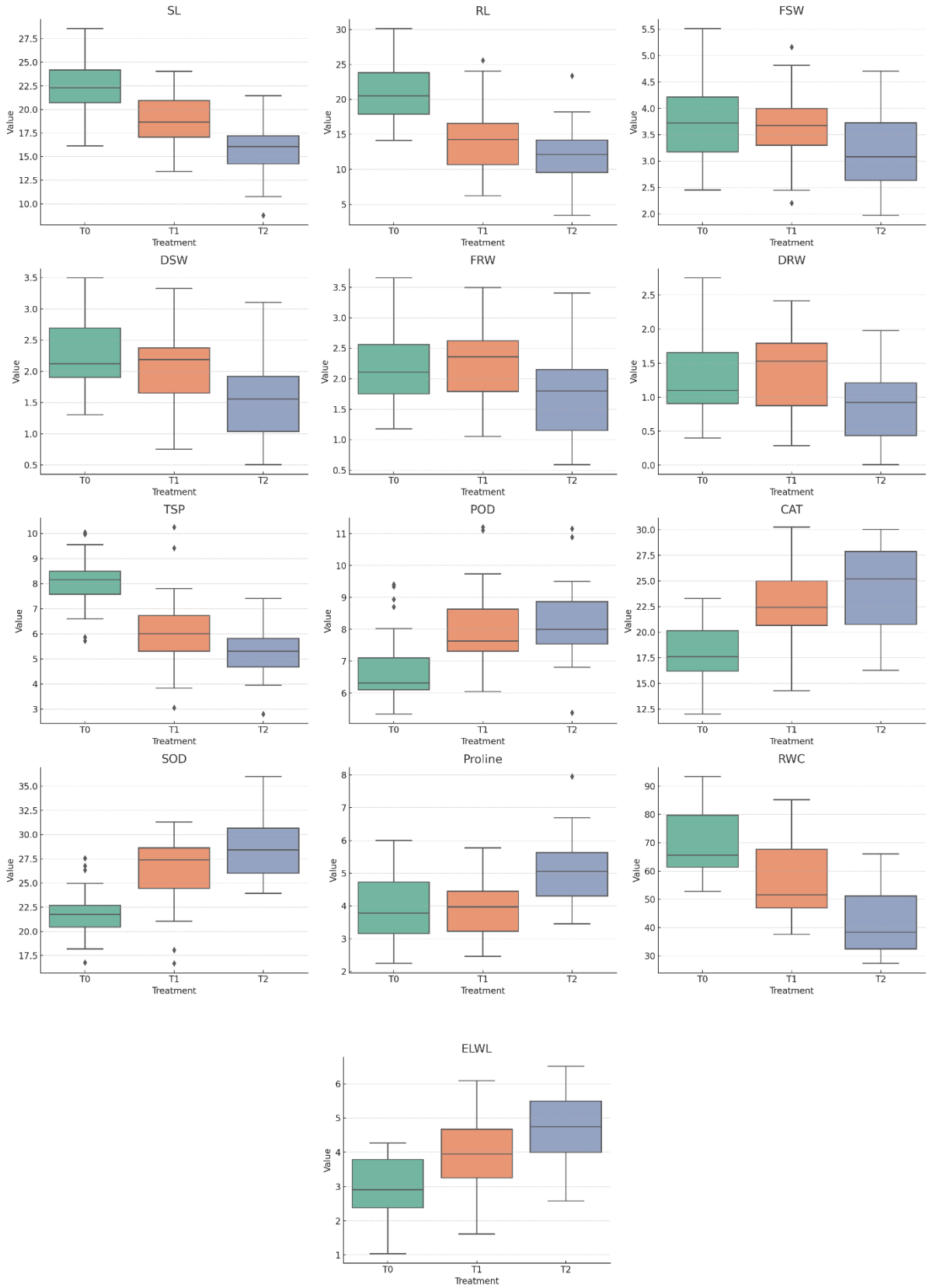

Fig. 3.2 | The box plots for each trait across treatments T0 (control), T1, and T2.

The plant's water status was affected by the drought, as seen by the declining trend in Relative Water Content (RWC) median values from T0 to T2. On the other hand, when stress levels rose, so did Evaporative Loss of Water (ELWL), highlighting the difficulty in retaining water during a drought. The existence of outliers in these parameters across treatments demonstrated the genetic variability among genotypes in their responses to drought, implying that breeding programmes may be able to take use of this variability to create cotton varieties that are resistant to drought.

3.4 Scree Plots

Principal component analysis (PCA) uses the Scree plot to display eigenvalue trends, which aids in determining the ideal number of principal components to keep. It aids in balancing information retention and overfitting, streamlining analysis for genotype selection in breeding programs, including those focused on cotton drought stress Fig. 3.3.

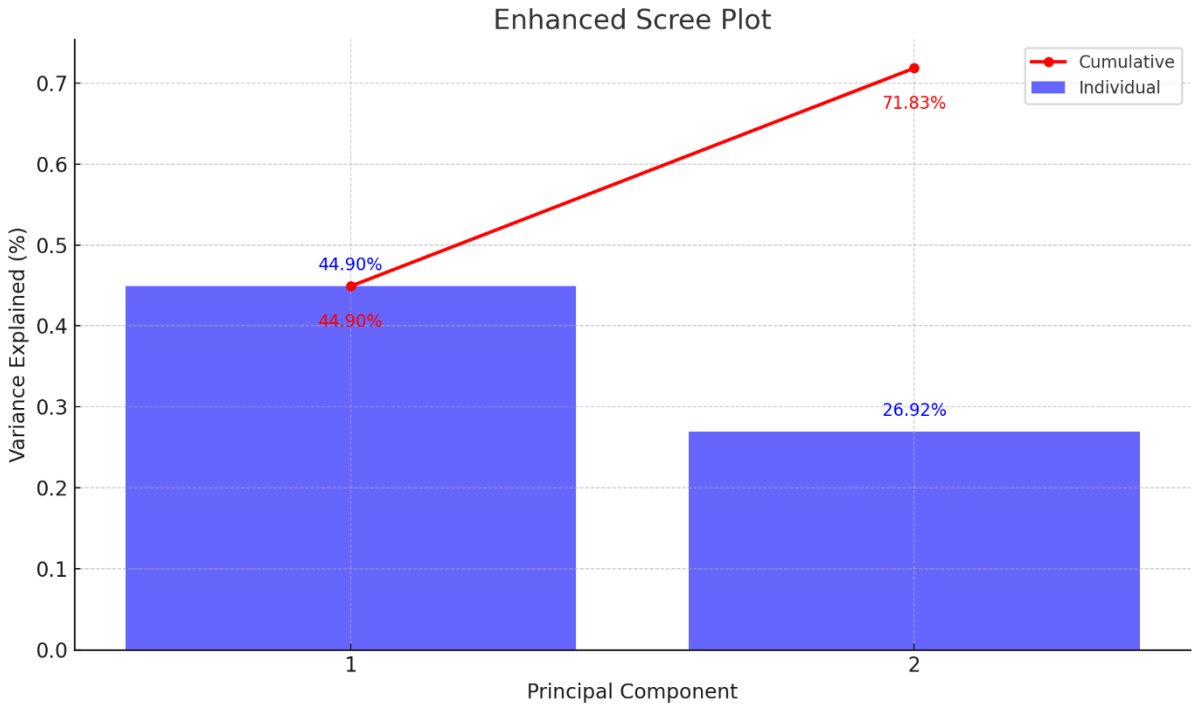

Fig. 3.3| The Scree Plot visually represents the variations each main component explains in the dataset.

The Scree Plot analysis within Principal Component Analysis (PCA) revealed that the The initial two main components were responsible for a noteworthy portion of the variance present in the dataset, approximately 71.83%, indicating these components' critical role in capturing the essence of the data. This significant variance coverage suggests a strong underlying pattern in the dataset, where these principal components succinctly encapsulate the primary variations in the genotypes' responses to drought stress. A notable decrease in variance explanation beyond the second component highlighted the diminishing returns from additional components, reinforcing the focus on the most significant components for a streamlined yet comprehensive data analysis.

The Scree Plot's detailed illustration of each principal component's contribution to the total variation exist in first component (PC1) alone representing about 44.90% of the variance emphasizes that PC1 predominantly captures the physiological and biochemical responses to drought stress in cotton genotypes. The second principal component (PC2) further enriches the explained variance by an additional 26.92%, collectively embodying a considerable portion of the dataset's variability with the first component. This analysis underscores PCA's utility in reducing the complexity of datasets while retaining essential information, facilitating a nuanced understanding of the genetic variability and drought response mechanisms.

3.5 Principle component analysis (PCA)

PCA and multivariate techniques play a crucial role in crop breeding programs for selecting genotypes with different levels of drought tolerance. PCA simplifies complex datasets by identifying key variables that contribute to trait variability, thereby aiding in the identification of genotypes with desirable traits. Multivariate techniques further

enhance genotype selection by grouping similar genotypes based on trait profiles, which helps in identifying drought-tolerant genotypes. These approaches work together to streamline genotype selection, speeding up the development of drought-tolerant crop varieties to address global food security challenges in the face of climate change-induced water scarcity Fig. 3.4.

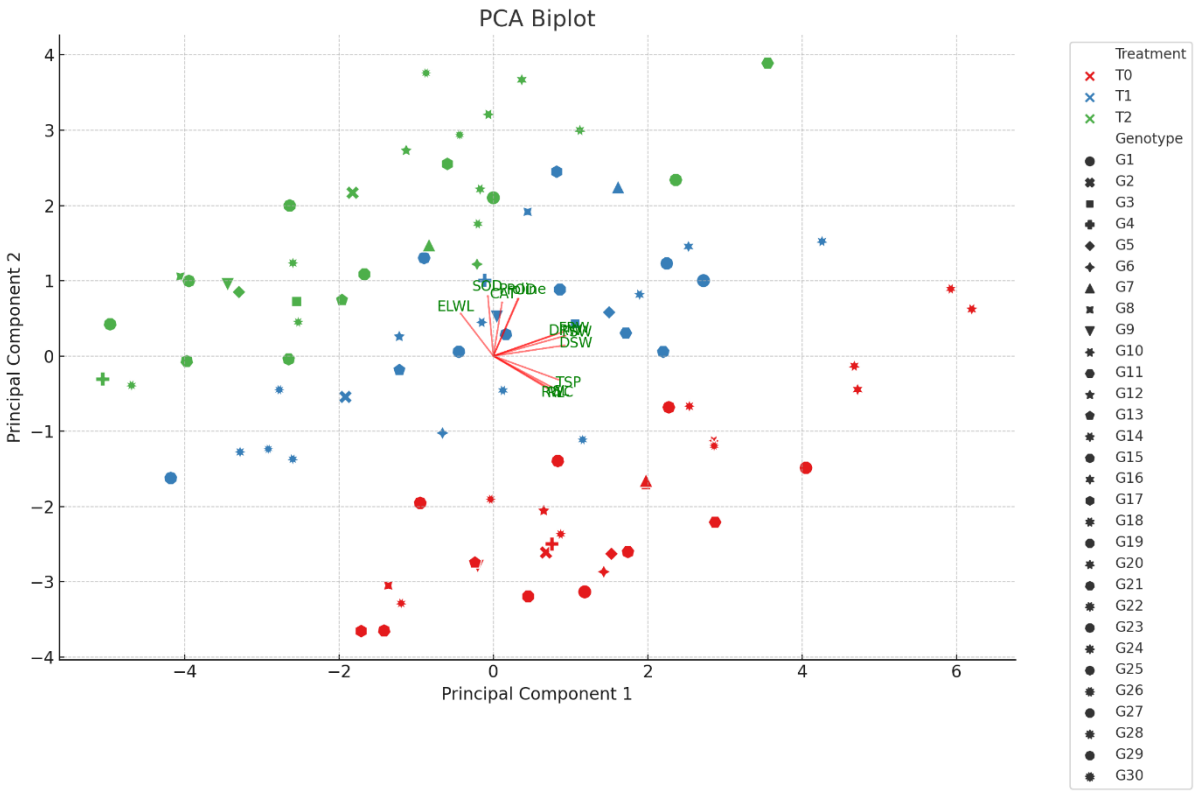

Fig.3.4 | The PCA biplot visually illustrating contribution of various morpho-physiological traits and genotypes distribution across plots

The PCA biplot analysis offers a detailed view of cotton genotypes under various drought treatments, represented by distinct colours for each treatment group. Trait vectors within the biplot indicate the relationship between specific traits and the principal components through their direction and magnitude, revealing the traits' influence on the dataset's variance. Positively correlated traits align in the same direction, while orthogonal vectors indicate no correlation. This pattern of vectors provides insights into how traits like proline, CAT, SOD, and POD, which serve as markers of stress response, contribute to the genotypes' overall drought resilience.

The biplot further highlights the variation in physiological and biochemical responses to drought stress by showing how genotypes are clustered based on treatment groups. This clustering indicates that different genotypes react differently to different drought levels, with comparable response patterns being displayed by genotypes that share treatment circumstances. Additionally, the distribution of genotypes within each cluster suggests that drought responses are naturally variable, providing possibilities to find genotypes with distinctive adaptation features appropriate for drought-tolerant breeding programmes.

The research clearly shows patterns of drought vulnerability and tolerance among the genotypes of cotton using PCA. Potentially more drought-tolerant genotypes include MNH-554, FH-113, FH-682, VH-295, and AA-802, which have the largest distance from the origin in the PCA space. These genotypes most likely have a combination of characteristics, including as effective water utilisation and strong antioxidant defences, that increase their resistance to drought. On the other hand, closer to the origin genotypes, such as FH-114, FH-901, IR-3, and MNH-552, seem more vulnerable to drought stress and may not have the defences in place to lessen its effects. The intricate interactions between genotypic features and drought stress responses are explained by this PCA-based evaluation, which also emphasises the possibility of using genetic diversity to increase cotton's resistance to drought.

3.6 Cluster Analysis

Cluster analysis is a powerful statistical approach that may be used to identify natural groups or clusters by analysing the similarity of observations within a dataset. In studies on cotton drought stress, it divides genotypes into groups according to trait profiles, which helps find potential genotypes for developing resilient cotton varieties Fig 3.5.

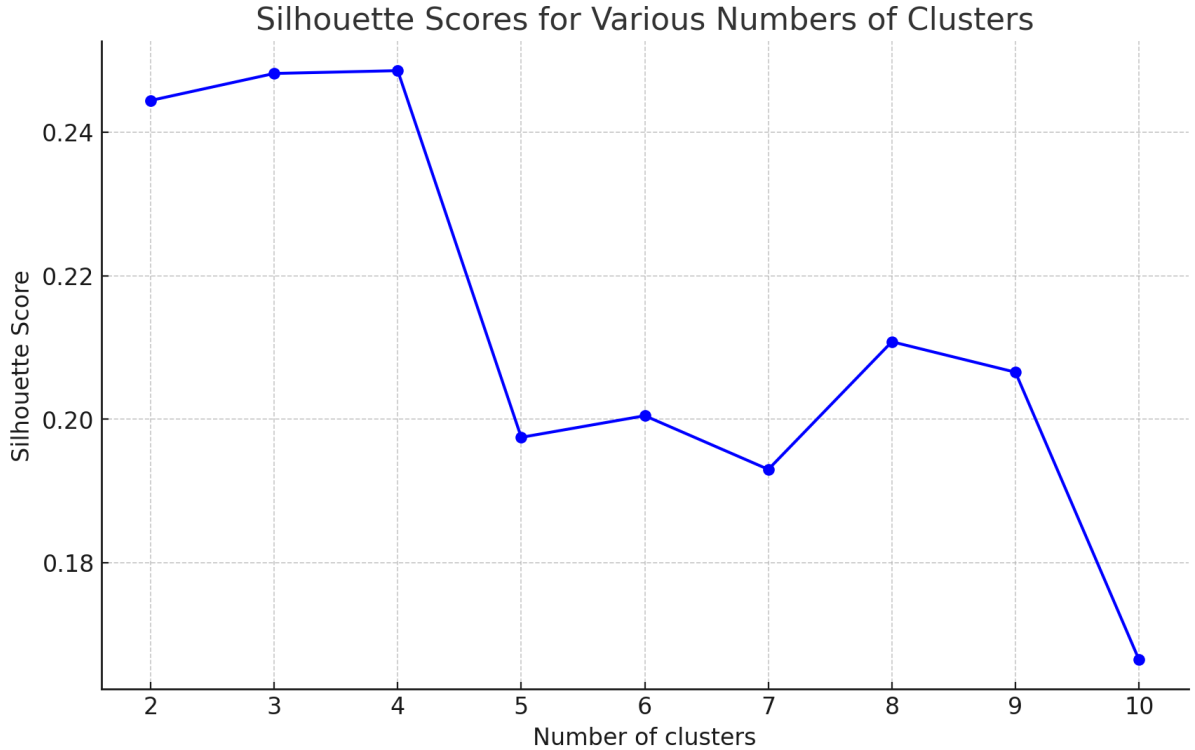

Fig. 3.5 | The cluster analysis, conducted through K-Means clustering and evaluated using silhouette scores

Cluster analysis of the dataset identified an optimal division into four groups, achieving a robust balance of similarity within clusters and distinctness between them, as indicated by silhouette scores. This division suggests a structured variation in the dataset, likely mirroring diverse physiological and biochemical responses to drought stress among cotton genotypes. Each cluster represents a unique combination of characteristics and drought responses, and the genotypes are uniformly distributed between these clusters, indicating a wide range of drought responses.

Different genotype groups with similar drought response features are revealed by the investigation. There are genotypes in some clusters that show strong resistance to drought, possibly even more tolerant, whereas there are genotypes in other clusters that appear more vulnerable to drought conditions. The processes supporting drought tolerance may become clearer with a thorough analysis of the characteristics in each cluster. In particular, genotypes in Cluster 1 had lower averages in growth metrics but higher averages in stress response indicators, suggesting that they are more vulnerable to drought. However, when drought circumstances are met, Cluster 2 genotypes show robust growth and elevated stress marker levels, indicating a potential for drought tolerance. Cluster 3 is distinguished by genotypes that efficiently sustain growth and water content during stressful conditions, exhibiting reduced activation of physiological stress responses, suggesting potential innate resistance to drought.

The clustering highlights the many methods that different cotton genotypes use to fend against drought stress. While the genotypes of Cluster 3 show sustained growth and water content, indicating innate resistance or stress avoidance, the genotypes of Cluster 2 show biochemical responses to stress, indicative of tolerance mechanisms. The genotypes in Cluster 1, which exhibit decreased growth and increased stress indicators, might be more susceptible to drought.

3.7 K-Means Three Clustering

The three-cluster solution, identified by Principal Component Analysis (PCA), offered a detailed illustration of the distribution of cotton genotypes under drought stress, plotted along the initial two principal components. This

configuration provided a clear picture of the genotypes' spatial clustering according to their physiological and biochemical responses to drought circumstances, with each cluster represented by a different colour (red for Cluster 1, green for Cluster 2, and blue for Cluster 3) Fig. 3.6.

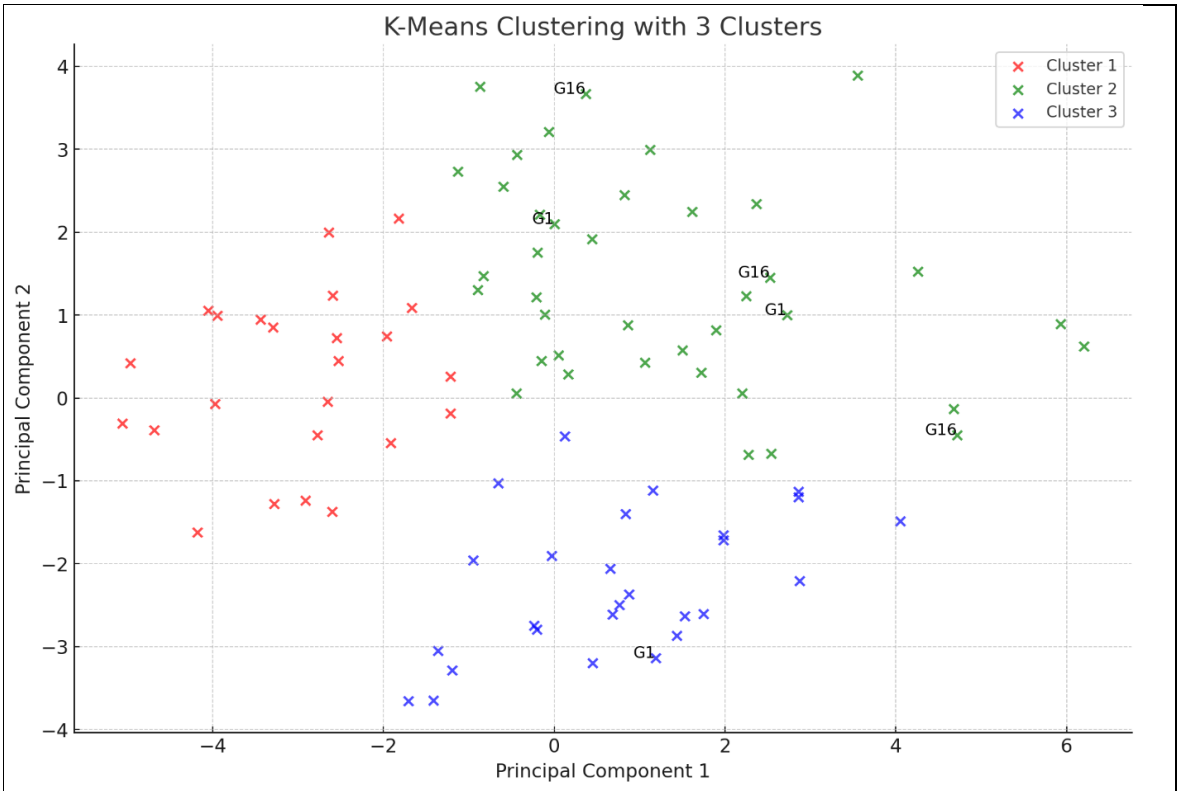

Fig. 3.6 | The plot visualizes the distribution of cotton genotypes within the 3-cluster solution, mapped along the first two PCs derived from the PCA analysis. Each cluster is represented by a distinct colour (red for Cluster 1, green for Cluster 2, and blue for Cluster 3),

Cotton genotypes were divided into three groups according to how they responded to drought stress by the K-means clustering analysis, which provided information about their adaptation mechanisms and potential for drought resilience.

Genotypes that exhibit a strong response to drought stress, combining rapid growth with an enhanced metabolic defence, set apart Cluster 1 (Red). The attributes that make up this category indicate that these genotypes have qualities that help them withstand drought, emphasising their resilience to biochemical changes and physiological robustness. The genotypes in Cluster 2 (Green) are particularly good at sustaining growth and water content during drought stress while requiring the least amount of activation of stress response systems. The characteristics of this cluster suggest that its members have an innate capacity to withstand drought and manage stress less dependently on physiological stress responses. Despite experiencing slower growth as a result of drought stress, genotypes in Cluster 3 (Blue) exhibit higher levels of stress-related biochemical markers. These genotypes are more vulnerable to drought stress, as seen by their reduced growth and elevated stress marker activity. This suggests a more pronounced response to stress conditions or a greater vulnerability to drought.

4 DISCUSSION

Our work explored how cotton genotypes respond to drought stress using a sophisticated analytical method that combined complex statistical analysis with in-depth physiological and biochemical observations. The extensive examination that included genotype selection, cluster analysis, ANOVA, box plots, bar graphs, scree plots, PCA, and genotype selection provided important new information about the physiological and biochemical processes underlying drought tolerance in cotton genotypes. Our results highlight how plants respond to drought stress in a variety of ways and are consistent with and build upon prior research.

The results showed a decrease in plant growth parameters under drought conditions as the level of drought stress increased from control to stress 2. This finding is consistent with previous research that has shown similar reductions in shoot length (SL) and root length (RL) among different genotypes [28][35]. Additionally, our study's increased antioxidant enzyme activity is consistent with research by Achard et al. (2006) [1] and Simonneau et al. (1993) [38], which emphasise the critical role these enzymes play in reducing oxidative damage brought on by drought stress.

In our study, we used Principal Component Analysis (PCA) to extract important variance from the dataset, demonstrating the usefulness of PCA in summarising genotype responses of cotton to drought stress. This method showed how PCA may identify important differences in plant stress responses, which lays a strong basis for focused breeding strategies. It is consistent with the findings of [17] Govindaraj et al. (2010) and Iqbal et al. (2011) [22]. We used PCA to identify genotypes that, based on their physiological and biochemical profiles, were more sensitive to drought circumstances (e.g., FH-114, FH-901, IR-3, and MNH-552) and those that showed notable drought tolerance (e.g., FH-113, FH-682, VH-295, and AA-802). Breeding programmes that attempt to improve cotton's resistance to drought by introducing features from robust genotype would benefit greatly from this distinctios.

Our research's cluster analysis successfully divided the cotton genotypes into three different groups according to their various drought response techniques, demonstrating the genotypes' variation in drought tolerance [21][33]. This analysis highlights the possibility for precision breeding techniques targeted at improving drought resilience and illustrates the significant genetic diversity seen in cotton [25][36]. Particularly, genotypes like MNH-554 and FH-113 belong to Cluster 1, which is characterised by great drought tolerance because of their robust growth and improved metabolic defences under stress (Govindaraj *et al.,* 2010). [22]. Genotypes like CIM-554 and FH-498, which exhibit innate resistance to drought through effective water management and low activation of stress markers, are characteristic of Cluster 2, suggesting the possibility of natural drought resilience [22]. On the other hand, genotypes such as FH-114 and FH-901 in Cluster 3 exhibit lower growth characteristics and higher stress markers, indicating a higher vulnerability to drought stress. The various differences between these clusters give strategic insights for breeding programmes as well as a greater understanding of cotton's drought adaptation processes. Our study opens the door for the creation of cotton cultivars more resistant to drought by identifying genotypes with advantageous features for drought tolerance, boosting sustainable cotton production in the face of climate change difficulties [5] [26].

5 CONCLUSION

Since drought is currently the primary factor restricting agricultural productivity and posing a threat to the future of agriculture, it is necessary to produce high-yielding and drought-tolerant cultivars. This work has yielded extensive insights into the drought stress responses of cotton genotypes by the application of morphological, physiological, and biochemical analysis methodologies. Through the application of ANOVA, PCA, and cluster analysis, we have investigated the mechanisms of drought tolerance in cotton. Our research yielded important results, such as differences in growth indices and antioxidant enzyme activity under heightened drought 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 drought, such FH-114, FH-901, IR-3, and MNH-552, and those that are more tolerant, like FH-113, FH-682, VH-295, and AA-802. This information can help breeding programmes improve cotton's resistance to drought. Furthermore, the incorporation of molecular markers into our findings has expanded our comprehension of the genetic foundation of these characteristics, providing specific avenues for genetic enhancement. With the help of cluster analysis, genotypes have been successfully divided into three different response groups, offering a clear plan for creating cotton cultivars with increased resistance to drought. In an era of climate change and water constraint, this work lays the door for the development of cotton cultivars that are better suited to tolerate drought, assuring sustainable cotton production.

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