

Original Article

J Genet Appl Biotechnol, 2026: e2026017

DOI: <https://doi.org/10.66432/g17gf887>

Received: 28 Nov 2025; Revised: 15 Feb 2026; Accepted: 18 Feb 2025; Published online: 28 Feb 2026

**Moisture-level dependent trait profile of drought response in *Gossypium hirsutum***Tahreem Arif^{1*}, Maqbool Ahamd², Fahad Idrees³, Muhammad Tahir⁴, Rukhsar Shaheen², Abdul Manan⁵, Xinyan Zhao¹, Muhammad Junaid⁶, Can Zhang¹¹State Key Laboratory of Efficient Production of Forest Resources, Key Laboratory of Forest Silviculture and Conservation of the Ministry of Education, The College of Forestry, Beijing Forestry University, Beijing 100083, China²MARA Key Laboratory of Crop Ecophysiology and Farming System in the Middle Reaches of the Yangtze River, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, China³National Key Lab of Crop Genetic Improvement and College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China⁴Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants, Ministry of Education, College of Biological Sciences and Biotechnology, Beijing Forestry University, Beijing 100083, China⁵Institute of Crop Science, College of Agriculture and Biotechnology, Zhejiang Provincial Key Laboratory of Crop Germplasm, Zhejiang University, Hangzhou 310058, China⁶Department of Climate Change, College of Agriculture, University of Haripur, Haripur, Pakistan*Corresponding author: Tahreem Arif; tahreem939@gmail.com**Abstract**

Drought is one of the major abiotic stresses limiting cotton productivity, particularly in arid and semi-arid regions. The screening at the seedling stage has been extensively utilized to accelerate the process of selection, but the role of growth, water status, and oxidative traits can change with moisture conditions. Understanding this dependence on moisture-level is essential for trait-based phenotyping and drought-resilient breeding. A greenhouse study was conducted using forty upland cotton accessions grown under 100%, 75%, and 50% field capacity in a two-replications factorial completely randomized design. After two weeks of sowing, stress was imposed, and measurements were recorded at 45 days. Analysis of variance, regime-specific trait summaries, principal component analysis, and K-means clustering were used to interpret the dataset. Drought had decreased the shoot length and relative water content and changed the covariance pattern among traits. ANOVA revealed that genotypes, moisture treatment, and the interaction of genotype and treatment had significant effects on all traits. Multivariate analysis revealed a trait architecture shift as moisture decreased. ELWL and RL were observed to play a major role in principal component structure under normal water conditions, whereas POD and H₂O₂ had a dominant role under moderate and severe drought. Cluster centroids further distinguish between profiles related to higher antioxidant activity. The findings revealed that cotton seedlings are moisture-level specific instead of being determined by a hierarchical set of traits under drought stress. This study can be useful for selecting drought screening traits in cotton.

Keywords: Cotton; Drought; Excised Leaf Water Loss; Relative Water Content; Peroxidase; Hydrogen Peroxide

How to cite this article: Arif T, Ahamd M, Idrees F, Tahir M, Shaheen R, Manan A, Zhao X, Junaid M, Zhang C. Moisture-level dependent trait profile of drought response in *Gossypium hirsutum*. J. Genet. Appl. Biotechnol. 2026: e2026017. <https://doi.org/10.66432/g17gf887>

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Introduction

One of the most significant abiotic factors that curtails crop productivity in the world is drought, which is especially destructive in arid and semi-arid agricultural systems where rainfall is inconsistent and where the evaporation rate is high (1,2). Drought stress has been shown to decrease biomass accumulation, retard development, lower lint yield, and decrease fibre quality in cotton, and thus drought adaptation is a key objective of modern breeding programs (3,4). Since

the upland cotton is mostly grown in regions with limited water, the understanding of the physiological basis of drought response is essential in enhancing resilience and persisting productivity.

At the physiological and cellular levels, drought perturbs plant water relations, restricts stomatal conductance, suppresses photosynthesis, and disrupts metabolic homeostasis (1,5). Dehydration promotes the formation of reactive oxygen species (ROS), including superoxide

radicals, hydrogen peroxide (H_2O_2), and hydroxyl radicals, mainly through disruption of chloroplast, mitochondrial, and peroxisomal electron transport chains. (6,7). Despite the fact that over-accumulation of ROS may cause lipids, proteins, pigments, and nucleic acids damage, ROS also play a crucial role as important signaling molecules used to regulate the perception of stress, defense activation, and acclimation responses (7,8). Therefore, the effects of drought on plant performance are based on the relationship between the generation of ROS and detoxification, and not just on the accumulation of ROS.

Oxidative regulation is particularly found in cotton, where drought tolerance has been consistently linked to enhanced antioxidant defense and redox homeostasis. Peroxidases (POD), catalase, and superoxide dismutase are some of the enzymes that facilitate ROS detoxification and aid in the preservation of cellular processes under water deficit condition (9,10). Cotton research has demonstrated that drought-tolerant genotypes favor the view that oxidative balance is a significant determinant of stress adaptation (11–13). Although oxidative responses are not the sole aspect of a general drought phenotype that also encompasses growth maintenance and water-status regulation.

Drought stress is a multifaceted quantitative characteristic that is a result of the combination of morphological, physiological, and biochemical reactions (2,14). Root length (RL) and shoot length (SL) are growth-related traits that indicate the biomass allocation and growth persistence during the limited water supply. Water-status traits, like relative water content (RWC), and excised leaf water loss (ELWL), give insight into tissue hydration avoidance, and the ability to restrict water loss (15,16). The redox aspect of stress response is biochemical markers, such as the concentration of H_2O_2 and POD activity. Notably, these characteristics act as a system, and their biological importance not only depends on the values of the individual but also on the variability in these values based on stress environments.

The Seedling stage screening has practical benefits to cotton breeding as it is an efficient technique, reproducible, and appropriate in assessing large germplasm collections under controlled conditions (4,17). However, the intensity and duration of stress, stages of development, and the environmental background have a strong effect on the drought responses (2). A trait that is highly informative in the presence of moderate moisture constraint may become less important when severe drought occurs, and other traits may be more important as the physiological context of stress response changes to include constraints on growth and to include oxidative and water economizing changes.

Despite substantial progress in cotton drought research, comparatively less attention has been given to how contrasting moisture levels reorganize overall architecture and alter trait interdependencies. Most studies emphasize mean trait responses or genotype ranking under one stress condition, whereas fewer studies address how drought intensity reshapes the multivariate phenotype space itself. This remains an important gap because the interpretation of any given trait is context dependent; its contribution to

drought response can vary according to the moisture environment in which it is expressed.

Unlike previous cotton drought studies that mainly used PCA, correlation analysis, or trait-association approaches to rank genotypes under a single stress condition, the present study compares trait structure separately across well-watered, moderate-stress, and severe-stress regimes. Therefore, the novelty of this work is not the use of multivariate analysis alone, but the moisture-regime-specific interpretation of how growth, water status, and oxidative traits change their relative importance across drought intensities. This approach provides a more targeted framework for selecting drought-screening traits in cotton seedlings. (18). In this context, the present study aimed to refine the interpretation of a greenhouse seedling dataset by focusing on the moisture-level dependent trait profile of drought response in *G.hirsutum*. Specifically, the objectives were to quantify changes in major growth, water-status, and oxidative traits across moisture regimes, evaluate the effect of genotype, and genotype $\times$ treatment interaction, and characterize the reorganization of multivariate trait structure across moisture levels using PCA and clustering. We hypothesized that progressive drought stress would shift the dominant trait profile from growth and water-loss-related dimensions toward oxidative regulation and redox balance.

Materials and Methods

Plant material and experimental conditions

The study was based on a greenhouse experiment comprising 40 upland cotton (*Gossypium hirsutum* L.) genotypes evaluated under three soil-moisture regimes corresponding to 100%, 75%, and 50% field capacity. The names/codes of the 40 upland cotton accessions are available in (19). The experiment followed a factorial completely randomized design with two replications. The two-replication design was used to allow screening of a relatively large genotype set across three controlled moisture regimes while maintaining uniform greenhouse conditions. Seeds were sown in polythene cups filled with sand, and seedlings were thinned to one plant per cup after emergence. Water stress was imposed two weeks after sowing. Field capacity of the sand-filled polythene cups was determined before stress imposition by saturating the medium with water, allowing free drainage, and recording the water content/weight corresponding to 100% field capacity. The 75% and 50% field capacity treatments were calculated relative to this value. Moisture levels were monitored daily using a moisture meter, and water was added as required to restore each cup to its target moisture level. This procedure was continued until trait measurement at 45 days after sowing.

Trait measurement

The root length and shoot length were measured on uprooted seedlings and were articulated in centimeters. Relative water content (RWC) was determined using fresh, turgid, and dry mass based on the technique (15). ELWL was determined based on fresh, wilted, and dry weights (16). Peroxidase activity (POD) was determined spectrophotometrically using the guaiacol/ H_2O_2 assay described (20). The concentration of

Hydrogen peroxide (H_2O_2) was determined by a potassium iodide-based assay (21).

Statistical analysis

The analysis of variance (ANOVA) was computed based on standard biometrical procedures to the effects of genotype, moisture treatment, and the interaction of genotype and treatment on each trait (22). Along with the ANOVA results, means and standard deviations of traits were summed up for each moisture regime. Prior to PCA, trait data were standardized to z-scores within each moisture regime because the measured traits had different units and scales.

PCA was then performed separately for 100%, 75%, and 50% field capacity treatments. Principal components with eigenvalues greater than 1 were retained, and the percentage variance explained by PC1 and PC2 was reported for each moisture regime (23). K-means clustering was performed separately within each moisture regime using standardized trait values. Several k values were compared using the elbow method based on within-cluster sum of squares, and $k = 8$ was selected because it provided interpretable phenotype groups while allowing comparison across moisture regimes.

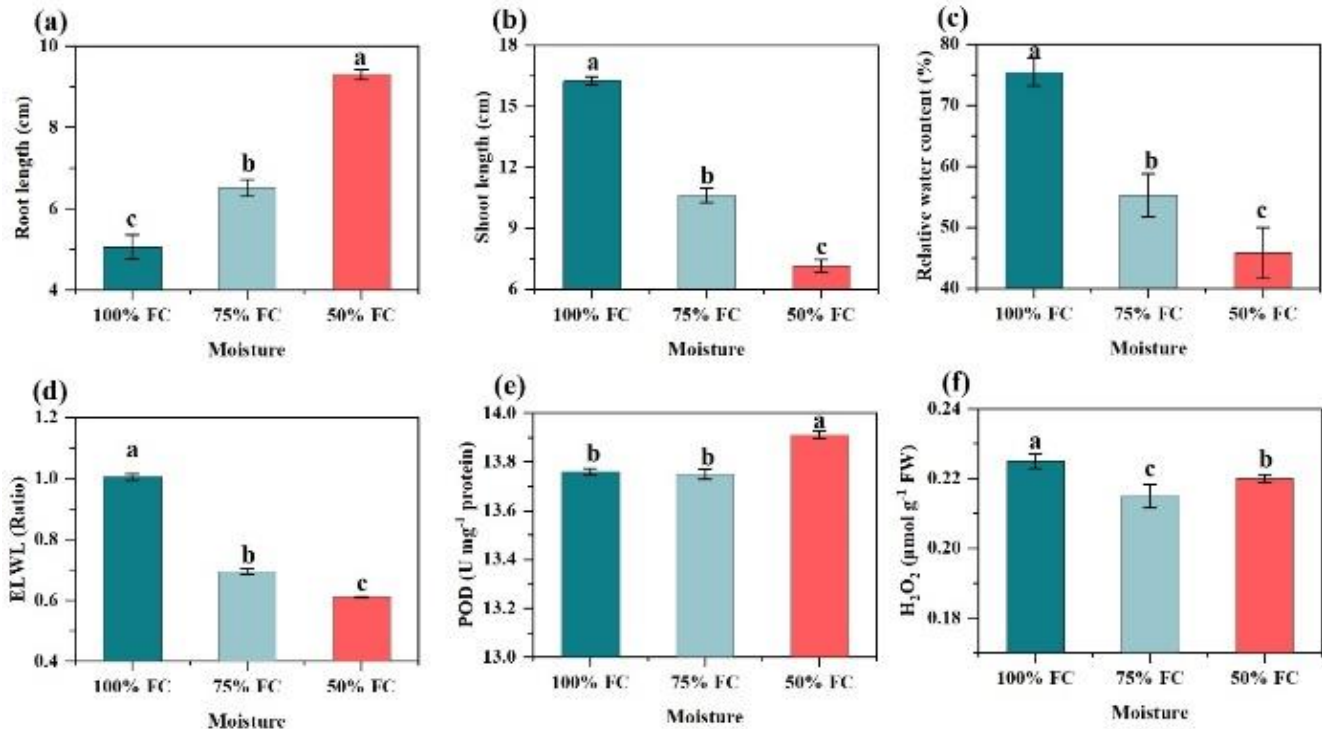


Figure 1: Effect of moisture levels (100%, 75%, and 50% field capacity) on various seedling traits in cotton. (a) Root length, (b) Shoot length, (c) Relative water content, (d) Excised leaf water loss (ELWL), (e) Peroxidase activity (POD), and (f) Hydrogen peroxide (H_2O_2) concentration.

Table 1. Mean squares from factorial analysis of variance for seedling traits of 40 upland cotton genotypes evaluated under three moisture regimes.

Sources of Variation	Degree of Freedom	Root length	Shoot length	RWC	ELWL	POD	H_2O_2
Genotypes	39	40.29**	41.92**	9.82**	19.06**	2.34**	21.81**
Treatments	2	2616.54**	6610.77**	2295.66**	170.77**	0.23**	59.31**
Genotype × Treatments	78	4.61**	12.06**	4.97**	4.15**	2.67**	422.78**
Error	120						
Total	239						

Values represent mean squares. *, ** indicate significance at $P < 0.05$ and $P < 0.01$, respectively. RL = root length; SL = shoot length; RWC = relative water content; ELWL = excised leaf water loss; POD = peroxidase activity; H_2O_2 = hydrogen peroxide concentration.

Results

Effects of moisture regime on trait means

The intensity of drought had a great impact on the average expression of growth, water status, and oxidative attributes in 40 genotypes. The root length increased from 5.06 cm at 100% field capacity (FC) to 9.30 cm at 50% FC, which showed a change in the biomass distribution to root growth under drought conditions (Figure 1a). Shoot length reduced with water stress from 16.25 cm at 100% FC to 7.15 cm at 50% FC, which indicated a high level of inhibition of the growth of shoots (Figure 1b). Relative water content (RWC) decreased significantly with an initial value of 75.44% at 100% FC and a final value of 45.76% at 50% FC (Figure 1c). Excised leaf water loss (ELWL) decreased from 1.005 at 100% FC to 0.611 at 50% FC (Figure 1d). Peroxidase activity (POD) remained constant across moisture treatments, although its importance increased at lower moisture levels (Figure 1e). The concentration of Hydrogen peroxide (H_2O_2) showed very less changes across moisture regimes, but its discriminatory value became more significant under moderate and severe drought conditions (Figure 1f).

Analysis of variance

The ANOVA (Table 1) of all traits showed highly significant effects of genotype, moisture treatment, and genotype $\times$ treatment interaction indicating significant genetic variability and genotype response to moisture level. The treatment effect was more pronounced on root length, shoot length, relative water content, and H_2O_2 , whereas genotype $\times$ treatment interaction was also significant on all the traits, which implies that the performance of the genotype was not stable across environments. These findings confirm that interpretation that drought response in cotton seedlings is highly context-dependent and that genotype ranking can vary with the intensity of moisture.

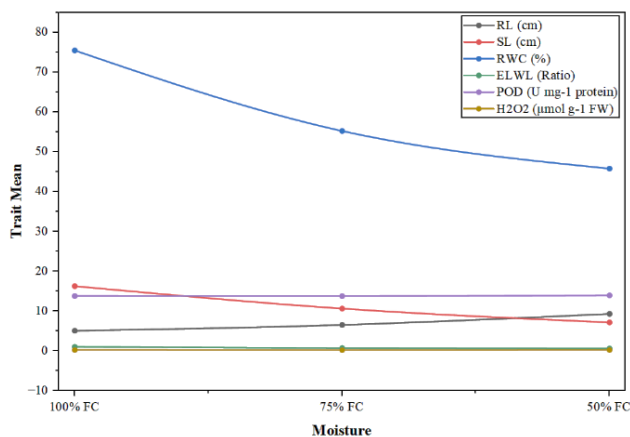


Figure 2. Principal component analysis of seedling traits under three moisture regimes: (a) 100% field capacity, (b) 75% field capacity, and (c) 50% field capacity. Trait vectors indicate the direction and relative contribution of RL, SL, RWC, ELWL, POD, and H_2O_2 to genotype separation. Percentages on each axis indicate the variance explained by PC1 and PC2. RL; root length, SL; shoot length, RWC; relative water content, ELWL; excised leaf water loss, POD; peroxidase activity, H_2O_2 ; hydrogen peroxide concentration.

Moisture-dependent changes in multivariate trait structure

Principal component analysis (Figure 2) revealed that the sources of phenotypic variation were mainly different in moisture levels. ELWL and RL were strong contributors under 100% field capacity compared to the principal component structure, which suggests that the differences between genotypes were linked to a characteristic of growth and water loss in the non-stress conditions. At 75% field capacity, POD and H_2O_2 made strong contributions to PC1 and PC2, indicating the growing importance of oxidative regulation with depleting soil moisture. Under 50% field capacity, H_2O_2 loaded strongly on PC2, and POD positively contributed to the same axis, indicating that redox balance is a significant aspect of genotype separation in severe drought. Altogether, these patterns suggest that the multivariate phenotype space was reorganized by drought intensity rather than simply compressed under stress.

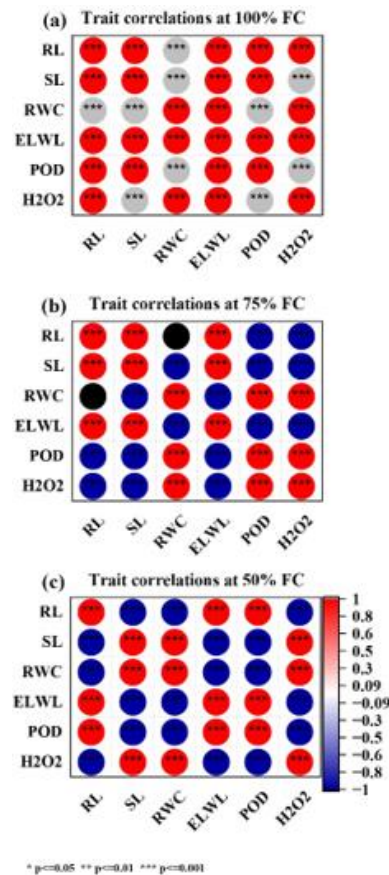


Figure 3. Pearson correlation heatmaps among seedling traits under three moisture regimes: (a) 100% field capacity, (b) 75% field capacity, and (c) 50% field capacity. Red and blue colors indicate positive and negative correlations, respectively, and circle size/color intensity represents the strength of association. Asterisks indicate statistical significance. Correlations represent trait associations and should not be interpreted as direct causal relationships. RL; root length, SL; shoot length, RWC; relative water content, ELWL; excised leaf water loss, POD; peroxidase activity, H_2O_2 ; hydrogen peroxide concentration.

Cluster-centroid trait profiles

K-means clustering justifies the presence of moisture-specific phenotype profiles. Centroid values distinguished clusters primarily by root length, shoot length, RWC, and ELWL at 100% field capacity (Table 2), and clusters at 75% field capacity (Table 3), separated more distinctly based on combinations of RWC, shoot growth, and oxidative traits. Below 50% field capacity, (Table 4) different centroid profiles of clusters with relatively high POD activity and clusters with relatively high H₂O₂ concentrations appeared, indicating different physiological responses to extreme stress. Some clusters that integrated a long root and a relatively good water status, whereas others were characterized by high oxidative markers. These contrasting profiles support the opinion that drought tolerance at the seedling stage is multidimensional and relies on the integration of growth, water status, and oxidative characteristics.

Trait correlations matrix

The heatmap (Figure 3) presents the relationships between different traits (Root Length (RL), Shoot Length (SL), Relative Water Content (RWC), Excised Leaf Water Loss (ELWL), Peroxidase Activity (POD), and Hydrogen Peroxide (H₂O₂) in three moisture levels like 100%, 75%, and 50% field capacity (FC).

Strong positive correlations (red) were found among most of the traits at 100% FC (Figure 3a), indicating that they are equally affected by the well-watered conditions. At 75% FC when we plot correlations (Figure 3b), the correlations are more variable, with few negative correlations (blue) appearing, suggesting a change in trait relationships with high drought stress. Strong negative correlations observed at 50% FC (Figure 3c) between growth-related traits (e.g., SL) and oxidative stress markers (e.g., H₂O₂), reflecting the plant's adaptive shift towards oxidative regulation and stress response as moisture availability decreases. The overall trend suggests that drought stress reorganizes trait relationships, with increased oxidative stress playing a central role under severe stress.

Discussion

This study shows that drought response in upland cotton seedlings is best understood as a moisture-level dependent reorganization of trait priorities rather than as a fixed, one-dimensional tolerance syndrome. As soil moisture declined, shoot growth and relative water content were substantially restricted, more strongly than root extension, and can promote a relative shift in assimilate allocation toward belowground structures to enhance water acquisition (2,5,14). The marked decline in RWC across the three moisture regimes confirms progressive impairment of plant water status under stress. Because RWC reflects the hydration state of leaf tissues, its reduction indicates that drought altered the balance between water uptake and transpirational loss. The concurrent decrease in ELWL suggests that genotypes under stronger stress tended to exhibit lower post-excision water loss, which may reflect adaptive adjustments related to tissue water retention and dehydration avoidance. Similar water-status responses have been widely used in crop drought screening because

they integrate plant hydraulic behavior with leaf-level stress expression (15–17).

Table 2. cluster-centroid trait mean at 100% field capacity

Cluster	RL	SL	RWC	ELWL	POD	H ₂ O ₂
Cluster 1	8.8	18.6	80.5	1.91	11.5	0.15
Cluster 2	4.79	16.0	82.5	1.01	16.25	0.3
Cluster 3	5.12	15.6	74.1	0.89	10.0	0.24
Cluster 4	5.62	16.2	77.9	1.08	12.9	0.23
Cluster 5	4.45	16.5	74.2	0.98	13.5	0.18
Cluster 6	4.84	15.9	75.1	1.06	13.76	0.22
Cluster 7	5.08	17.1	73.9	0.91	14.5	0.18
Cluster 8	5.3	14.4	66.3	0.81	14.25	0.25

RL; root length, SL; shoot length, RWC; relative water content, ELWL; excised leaf water loss, POD; peroxidase activity, H₂O₂; hydrogen peroxide concentration

Table 3. cluster-centroid trait means at 75% field capacity

Cluster	RL	SL	RWC	ELWL	POD	H ₂ O ₂
Cluster 1	5.2	8.2	33.7	0.44	15.0	0.15
Cluster 2	5.5	10.3	40.5	0.59	13.0	0.36
Cluster 3	8.03	13.8	58.2	0.83	14.5	0.19
Cluster 4	8.02	9.8	60.2	0.78	14.3	0.2
Cluster 5	6.3	11.1	58.1	0.65	12.5	0.18
Cluster 6	6.3	10.02	54.4	0.56	13.8	0.2
Cluster 7	5.9	13.8	53.2	0.89	10.5	0.29
Cluster 8	6.8	9.7	49.7	0.59	14.5	0.22

RL; root length, SL; shoot length, RWC; relative water content, ELWL; excised leaf water loss, POD; peroxidase activity, H₂O₂; hydrogen peroxide concentration

Table 4. cluster-centroid trait means at 50% field capacity

Cluster	RL	SL	RWC	ELWL	POD	H ₂ O ₂
Cluster 1	12.79	14.4	56.8	1.1	15.5	0.28
Cluster 2	9.19	7.8	51.9	0.7	11.08	0.22
Cluster 3	9.5	7.4	49.1	0.8	15.1	0.16
Cluster 4	7.9	7.2	46.3	0.7	21.0	0.2
Cluster 5	9.1	6.7	45.7	0.6	10.5	0.23
Cluster 6	9.0	6.1	42.7	0.5	13.7	0.21
Cluster 7	9.3	7.6	43.6	0.5	14.7	0.22
Cluster 8	8.4	5.9	34.8	0.4	12.0	0.32

RL; root length, SL; shoot length, RWC; relative water content, ELWL; excised leaf water loss, POD; peroxidase activity, H₂O₂; hydrogen peroxide concentration

Although mean POD activity and H₂O₂ changed less dramatically than growth and water-status traits, the multivariate analyses indicate that oxidative traits became more influential as drought intensified. This distinction is important. A trait may show only modest changes in population means yet still become highly informative for differentiating genotypes when stress reshapes the covariance structure of the phenotype. The increasing importance of POD and H₂O₂ in the PCA under 75% and 50% field capacity is consistent with current understanding that oxidative adjustment is central to drought acclimation and that antioxidant metabolism modulates both ROS signaling (7,10,24). Integrated omics analyses. Integrated omics analyses in cotton also indicates that drought

tolerance emerges from coordinated regulation of antioxidant metabolism, carbon status, and stress-responsive signaling networks rather than from isolated biochemical markers (12).

The significant genotype $\times$ treatment interactions observed for all traits further indicate that drought response cannot be generalized across moisturized environments. Instead, the physiological basis of genotype performance appears to shift along with the moisture gradient. Under well-watered conditions, genotype differences were associated more strongly with growth and water-loss dimensions. Under moderate and severe drought, redox-related traits gained interpretive value, implying that oxidative balance became a stronger determinant of phenotypic separation. This finding supports the idea that trait-based screening should be matched to the target drought scenario rather than relying on a universal set of indicators. Current multivariate analyses of cotton under water, heat, and salt stress also demonstrate that trait utility is environment-specific and that integrative selection models are better than single-trait models in screening large sets of germplasm (25,26)

The cluster-centroid profiles give an even more system-level frame of reference in identifying regime-specific phenotype clusters. In extreme drought, clusters characterized by higher POD activity may represent genotypes with increased antioxidant buffering, whereas clusters with increased H_2O_2 may reflect greater oxidative burden or alternative critical stress signals (6,8). From a breeding perspective, these profiles can be used for genotypes that maintain performance through oxidative control from those that experience greater stress injury.

This study also has some practical implications for phenotyping strategy. If trait contributions alter with drought intensity, then the effectiveness of seedlings-stage screening depends on how closely the screening environment reflects the target production scenario. Growth and water-status traits may be sufficient under mild to moderate stress, whereas oxidative traits become increasingly valuable for identifying biologically meaningful variation under severe moisture limitations. Therefore, multivariate and interaction-aware analyses should complement single-trait comparisons when selecting parents or designing screening protocols. From a breeding perspective, the results suggest that cotton drought screening should not rely on a single universal trait across all moisture environments. Under well-watered or mild-stress conditions, growth and water-status traits such as root length, shoot length, RWC, and ELWL may be useful for early differentiation of genotypes. Under stronger drought stress, oxidative traits such as POD activity and H_2O_2 concentration should be interpreted together to distinguish genotypes with stronger antioxidant buffering from those experiencing greater oxidative burden. Therefore, breeding programs may benefit from applying moisture-intensity-specific trait indices rather than using the same selection criteria across all drought scenarios. A limitation of this study is that the experiment was conducted with two replications under controlled

greenhouse conditions. Therefore, the genotype $\times$ treatment interaction should be interpreted as evidence of differential seedling response within this experimental system rather than as a final field-based ranking of drought tolerance. Further validation across seasons, soil types, and field environments will be necessary before specific genotypes are recommended for breeding deployment.

Conclusion

In conclusion, the drought response of upland cotton seedlings was strongly dependent on moisture levels, with progressive water limitation reshaping the relative contribution of growth, water status, and oxidative traits. Shoot growth and RWC declined with increasing stress, root length increased, and multivariate analyses showed that oxidative traits became more important discriminators under lower moisture regimes. Significant genotype, treatment, and genotype $\times$ treatment effects across all traits further indicate that drought response is not governed by a fixed trait hierarchy. These findings support the use of moisture-intensity-aware phenotyping frameworks for cotton drought screening and a stronger basis for identifying informative traits for stress-resilient breeding.

Acknowledgement: The first author expresses gratitude to the Government of Punjab, Pakistan, and the University of Agriculture, Faisalabad.

Disclaimer: None.

Conflicts of Interest: The authors declare no conflict of interest.

Data availability: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Source of Funding: The funding was provided by the University of Faisalabad under the master's degree research student project.

Ethics approval statement: Not Applicable

Authors' Contribution Statements: Tahreem Arif: supervision, writing original draft, conducting experiments, visualization, proof-reading, supervision, funding, Maqbool Ahamd: Formal analysis, proof-reading, Software, Fahad Idrees: Review and editing, Muhammad Tahir: Review and editing, language, Rukhsar Shaheen: Formal analysis, Abdul Manan: Proofreading, Formal analysis, Zhao Xinyan: Formal analysis, proof reading, Muhammad Junaid: Language, Visualization, Zhang Can: Review and editing.

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