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RESEARCH ARTICLE

Assessing drought tolerance of cotton (*Gossypium* spp.) RIL populations using agrophysiological and stress indices

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Abstract

In this study, 80 samples (F_6) of recombinant inbred line (RIL) populations derived from the wild cotton species *Gossypium tomentosum* and *Gossypium mustelinum* were comprehensively evaluated under control and drought conditions. The main objective of the study was to identify lines that were tolerant, sensitive and stable to drought stress conditions. The stress tolerance of the lines was assessed using physiological and yield-based stress indices. The main evaluated physiological parameters were relative leaf water content (RWC), excised leaf water loss (ELWL) and yield (Yp, Ys). Stress indices, including STI, GMP, MP, SSI, YI, YSI and TOL, were also calculated. Based on the obtained data, the level of stress tolerance of RIL samples was determined using principal component analysis (PCA). According to the results of the analysis, RIL-36 (STI = 1.44, YSI = 1.44, SSI = –2.07, TOL = –12.15), RIL-45 (Ys = 58.17, YI = 1.62, GMP = 59.90) and RIL-27 (Ys = 56.93, STI = 1.20, YSI = 1.20) were found to be resistant lines. RIL-25 (YSI = 1.24, SSI = –1.12), RIL-63 (SSI = –0.47), RIL-76 (YSI = 1.10) and RIL-24 (YSI = 1.05) lines also showed positive results. In contrast, RIL-19 (Ys = 19.66, STI = 0.39, SSI = 2.90), RIL-07 (YSI = 0.47, TOL = 33.94), RIL-06, RIL-39 and RIL-72 were evaluated as sensitive lines with low Ys and high SSI. In conclusion, the integration of agrophysiological parameters with the stress tolerance index has been shown to be effective in differentiating drought-tolerant, tolerant, sensitive and stable RIL genotypes. The inclusion of wild *Gossypium* species in the breeding stock will expand the genetic base of cultivated cotton and increase the possibility of creating water-stress-tolerant varieties.

Keywords: drought conditions; *Gossypium mustelinum*; *Gossypium tomentosum*; PCA; population; recombinant inbred line; stress indices

Introduction

Cotton (*Gossypium* spp.) is a major economic crop in more than 30 countries, with China, India, the United States and Pakistan accounting for the largest share. Cotton is mainly grown in warm climates (1). As a glycophytic plant, cotton exhibits higher tolerance to abiotic stresses than other major crops (2). However, extreme environmental conditions such as drought negatively impact cotton growth, yield and fibre quality (2). Drought tolerance traits are genetically determined to affect various morphological and physiological traits of crops. This leads to a significant decrease in cotton yield and fibre quality (3, 4). Irrigation during the flowering and boll stages of cotton is the most important period that determines yield (5). Short-term water deficit stress at this stage significantly affects various physiological and biochemical properties of cotton plants, such as leaf expansion, photosynthesis, carbon and nitrogen metabolism and antioxidant metabolism (6–8). In addition, changes in stomatal closure and increased water accumulation in leaf tissues contribute to ensuring photosynthetic activity in cotton, as well as an increase in chlorophyll content and relative water content (RWC) (9, 10). There is a positive correlation between physiological and agronomic traits of the plant, namely relative leaf water content, leaf water loss capacity, cell membrane stability, number of bolls per plant, boll weight and cotton yield. Therefore, cotton germplasm samples with such traits can be used to identify genotypes resistant to water deficit stress (11). Research reported that disruption of physiological processes, including relative leaf water content and excised leaf water loss capacity, under soil drought conditions during the flowering period of cotton (12). The decline in crop yields due to abiotic and biotic stresses is a significant challenge for the global economy and food security. Therefore, molecular genetics and breeding programs are focusing on selecting genotypes with high yield indices under water stress. However, changes in yield characteristics may be due not only to drought tolerance but also to adaptation factors (13). It is challenging to include all samples when analysing drought tolerance in large populations. However, principal component analysis (PCA) can be used to determine the significance of each value and helps identify principal components that control all variable values (14). Drought indices provide a measure of drought based on yield loss under water-deficit conditions relative to normal conditions and are used to analyse drought-tolerant genotypes (15). The drought stress sensitivity index (SSI) has been proposed as a tool to assess the yield performance of cotton varieties under drought conditions (16). Low values of SSI indicated low yield variation under both stressed and control conditions, demonstrating high yield stability. In contrast, high values of SSI ≥ 1 indicated high sensitivity, identifying a new progressive index (STI : stress tolerance index) that can be used to identify high-yielding genotypes under both stressed and non-stressed conditions (17–20). He stated that selection based on STI leads to genotypes with high stress tolerance and high yield potential (20). GM indices are mathematical products of yield. Additionally, the best indicator for assessing drought tolerance is not relative efficiency, but rather genotypes with high yields under both conditions are considered drought-tolerant (21). Genotypes with a high yield stability index (YSI) have been reported to exhibit resistance to environmental stresses (22). Cotton breeding for drought tolerance and water use efficiency has been limited by the species' limited genetic diversity, a result of intensive selection for high lint yields. Studying the genomes of wild tetraploid cotton species, including *G. tomentosum*, *G. darwinii* and *G. mustelinum*, may identify additional alleles valuable for increasing drought tolerance (23).

This study utilised 80 samples of the wild cotton species *G. tomentosum* and *G. mustelinum*. RIL populations (F_6) were analysed under drought conditions utilising physiological and yield-based stress parameters through PCA, cluster and correlation analysis. According to the studies, RIL population samples were categorised into drought-tolerant, sensitive and stable categories. This category of population sample has not been examined in relation to abiotic and biotic stresses, nor in other analytical studies.

Materials and Methods

In this study, the RIL population was derived from crossing *G. tomentosum* and *G. mustelinum* by serial selfing up to the F_6 generation, which consisted of 80 lines. The experiment was conducted in 2024 at the field experimental site of the Genomics and Bioinformatics Centre of the Academy of Sciences of the Republic of Uzbekistan under two conditions (41.380978°N, 69.342885°E): A) control (control-opt) and B) drought (drought-dry). In both environments, lines were planted 4 m long, with a row spacing of 76 cm and a plant spacing of 25 cm based on a randomised block design (RBD). The control environment was irrigated 8 times using the 2-4-2 method. To create a drought environment, less water was given, irrigated 3 times using the 2-1-0 method. During the growing season, weather data were obtained from announcements on the Uzhydrometeorology Centre's website and average temperature, precipitation and soil moisture were monitored.

Traits studied

Three replicates of leaf samples were collected from each line and physiological traits such as relative leaf water content (RWC) and leaf water loss (ELWL) and agronomic traits such as average yield per plant were studied (24, 25). To further assess drought tolerance or sensitivity, the following stress indices were calculated. Stress sensitivity index (SSI), stress tolerance index (STI), tolerance (TOL), mean yield (MP), geometric mean yield (GMP), yield index and yield stress index (YSI) (16, 20, 26–28).

Statistical analyses

Experimental data were analysed using OriginPro-2022 (Version 9.9, SR1; OriginLab Corporation, Northampton, MA, USA). The following statistical criteria and thresholds were used to ensure the reliability of the results: PCA was performed based on the correlation matrix of agrophysiological traits and stress indices. Only principal components with eigenvalues >1.0 were considered significant for explaining the total variance. Cluster analysis: Hierarchical clustering was performed using the unweighted pair group method (UPGMA) with arithmetic mean. Euclidean distance was used as the dissimilarity metric to determine the genetic and phenotypic distances between the 80 RILs. Classification criteria: RILs were divided into three groups (tolerant, stable and sensitive) based on STI and SSI. Genotypes with STI values > mean + standard deviation (SD) were classified as tolerant, while those with STI < mean – SD were classified as susceptible. Significance testing: All data were subjected to two-way analysis of variance (ANOVA). Mean differences between genotypes and environments (control and drought) were confirmed at the significance level using Duncan's multiple range test (DMRT) ($P < 0.05$).

Results

In this study, 80 samples of RIL populations of wild cotton species *G. tomentosum* and *G. mustelinum* were analysed for physiological (RWC, ELWL), agronomic (average yield per plant) and yield-based stress indices under control and drought conditions. Based on drought tolerance indices (STI, SSI and YI), population samples were divided into three groups: tolerant, sensitive and stable. In the study, the analysis of the relative leaf water content (RWC) of the physiological traits showed that under control conditions, the highest RWC value was observed in RIL-56 (98.89 %) and the lowest in RIL-30 (33.53 %). Under drought conditions, the highest RWC was recorded in RIL-20 (78.37 %) and RIL-07 (73.35 %) and the lowest value was recorded in RIL-56 (32.26 %). The lines with the most stable RWC in both environments were RIL-03 and RIL-55, RIL-36 and their RWC values remained high under drought conditions.

Also, when leaf water loss (ELWL) was analysed, the lowest ELWL value was observed in RIL-08 and RIL-30 (0.30) and the highest ELWL value was observed in RIL-49 (1.56) under control conditions. Under drought conditions, the highest ELWL value was recorded in RIL-44 (1.00) and the lowest in RIL-17 (0.30). In addition, the rate of change of ELWL in RIL-29, RIL-55 and RIL-44 samples was very low, which indicates that they are balanced against stress. The results of yield-based analyses of population samples showed that under control conditions, the highest yield (Yp) was determined in RIL-01 (74.12) and the lowest yield was determined in RIL-62 (24.71). Under drought conditions, the highest yield value was determined in RIL-45 (58.17) and the lowest yield was determined in RIL-19 (19.66). RIL-12, RIL-36 and RIL-63 lines were stable in terms of yield value in both environments.

The following results were obtained from the yield-based stress index analyses. The STI analysis found that RIL-36 had the highest value (1.44) and RIL-19 had the lowest value (0.39). The lowest SSI was observed in RIL-36 (-2.07) and the highest SSI was observed in RIL-19 (2.90), which indicates their stress tolerance and sensitivity. As a result of YSI analysis, the highest value was observed in RIL-36 (1.44) and the lowest value was observed in

RIL-72 (0.39). The lowest TOL index values were recorded in RIL-36 (-12.15) and the highest in RIL-07 (33.94), which indicates the degree of yield loss of these RILs in drought. As a result of MP analysis, the highest value was observed in RIL-45 (59.93) and the lowest value was observed in RIL-13 (27.01). As a result of GMP analysis, the highest value was observed in RIL-45 (59.9) and the lowest value was observed in RIL-13 (26.53). The highest value of YI was observed in line RIL-45 (1.62) and the lowest value was in line RIL-19 (0.55).

PCA-biplot analysis of physiological and yield-based stress indices of population samples under control and drought conditions

As a result of PCA biplot analysis, the yield (Yp, Ys), yield-based stress indices (YI, YSI, MP, GMP, STI, TOL, SSI) and physiological traits (RWC, ELWL) obtained from 80 samples of the RIL population under control and drought conditions were divided into two principal components. The first component (PC1) accounted for 38.52 % of the total variance and the second component (PC2) accounted for 30.63 %, explaining 69.15 % of the total variability. The PC1 component was positively correlated with yield and stress tolerance indices such as STI, GMP, MP, Yp and Ys and was important in identifying high-performing lines. PC2 was associated with TOL and SSI scores and represented the level of stress sensitivity. According to the results of PCA-biplot analysis, RIL-27, RIL-65 and RIL-76 samples had high Yp, Ys, GMP and STI values, which were identified as stress-resistant and high-yielding lines. In addition, samples such as RIL-19, RIL-09 and RIL-53 were distinguished by high stress sensitivity and low yield (Fig. 1).

Pearson correlation analysis was performed on the studied traits. As a result, there is a positive but weak correlation between RWC_opt and RWC_dry (r = 0.24). This means that the relative water content of the leaf in the control condition is not very stable during drought. The correlation between ELWL_opt and ELWL_dry (r = 0.03) is almost zero; that is, there is no correlation. It was shown that the level of water loss is an independent characteristic in the two conditions. There is a low negative correlation between RWC_dry and ELWL_opt (r = –0.26) and leaves that retain water well (RWC_dry) lose less water (ELWL_opt). A moderate positive correlation was found between Yp and Ys (r = 0.28). RIL samples that gave high yields under control conditions usually give relatively high yields even under drought. It can be seen that there is a direct or strong relationship between Ys and YI (r = 1.00), between Ys and YSI (r = 0.55) and between YI and YSI. Also, among STI and Ys (r = 0.55), STI and YI (r = 0.55), STI and YSI (r = 1.00), STI is the best elasticity index and is a useful indicator in distinguishing resistant samples. The correlations between indices such as SSI and TOL (r = 0.96), SSI and STI (r = –1.00) and SSI and YSI (r = –1.00) are inversely correlated; that is, when SSI is high, elasticity is low (STI, YSI). There is an almost complete correlation between MP and GMP (r = 0.99) and these two indices give very close values. Lines with high yields between TOL and Yp (r = 0.73) usually also experience significant yield loss under stress conditions (TOL), indicating that they are susceptible rather than resistant.

Analysis results of 80 samples of the RIL population

Tolerant

The following were selected as drought-tolerant RILs. RIL-27, RIL-45, RIL-36, RIL-76, RIL-63, RIL-25 and RIL-24 were found to be drought-tolerant: RIL-27 line Ys = 56.93 – high yield under drought conditions, Yp = 47.31 – average yield under optimal conditions, STI = 1.20 – high yield retention under stress, YSI = 1.20 – very high stress tolerance, SSI – low – low sensitivity to stress. In the RIL-45 line, Ys = 58.17, Yp = 61.69 – very high yield in both conditions, YI = 1.62 – general stress resistance at the highest level, GMP = 59.90 –

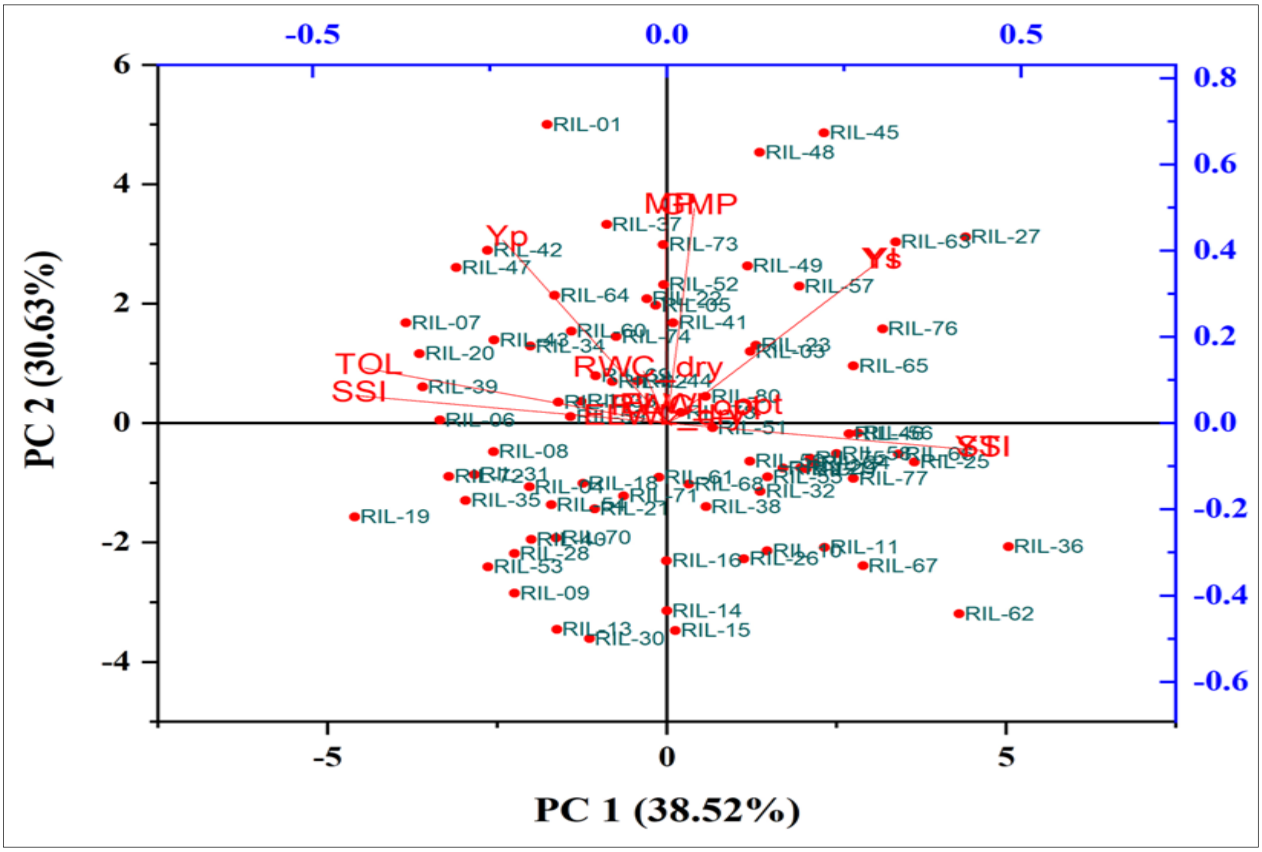

Fig. 1. PCA-biplot analysis of physiological and yield-based stress indices in RIL population samples under control and drought conditions ($p \leq 0.05$).

the average yield in both environments is high, YSI = 0.94 – good stability, RIL-36 line STI = 1.44 – the highest value, YSI = 1.44 – the yield was almost not lost under stress, SSI = –2.07, TOL = –12.15 – negative values, i.e. the yield even increased under stress. RIL-76 line Ys = 49.50, STI = 1.10, YSI = 1.10 – has good tolerance, GMP = 47.21 – has a balanced yield. RIL-63 line Ys = 54.35, GMP = 51.84 – high and stable yield, STI = 1.10, SSI = –0.47 – low yield loss under stress, RIL-25 line STI = 1.24, YSI = 1.24, SSI = –1.12 – very good stability, RIL-24 line STI = 1.05, YSI = 1.05, SSI = –0.24, Ys = 39.35 – stable and resistant properties.

Sensitive and stable

The following lines were selected as drought-tolerant RILs. RIL-19, RIL-07, RIL-06, RIL-39 and RIL-72 were distinguished by the following negative indicators. RIL-19 line Ys = 19.66 – the lowest yield, STI = 0.39, YSI = 0.39 – poor yield preservation, SSI = 2.90 – very sensitive to stress, RIL-07 line Yp = 64.14 – has a very high yield at the optimum, but a sharp decrease in drought was observed Ys = 30.20, TOL = 33.94 – the value of yield loss is large, SSI = 2.50, YSI = 0.47 – low stability. RIL-06 line Ys = 27.03, SSI = 2.37, TOL = 27.29 – sensitivity is clearly expressed, RIL-39 line Ys = 28.50, SSI = 2.47, TOL = 31.42 – yield is strongly reduced, RIL-72 line Yp = 50.57, Ys = 25.17, TOL = 25.40, YSI = 0.50 – yield is halved under stress. When comparing the values of RIL samples in both environments, the RIL -12 line has Yp = 37.28, Ys = 37.95, SSI = –0.09, YSI = 1.02 – almost 100 % stability. The RIL-24 line has Yp = 37.44, Ys = 39.35, SSI = –0.24, YSI = 1.05 – slightly higher. The RIL-25 line has SSI = –1.12, YSI = 1.24 – the yield does not decrease under stress, but increases STI = 1.24, the GMP indicator is stable. RIL-55 line Yp = 36.80, Ys = 36.73, SSI = 0.01, YSI = 1.00 – excellent stability, RIL-63 line Ys = 54.35, SSI = –0.47, YSI = 1.10, GMP = 51.84 – acceptable stability and high yield value. RIL-36 line STI = 1.44, SSI = –2.07, YSI = 1.44 – increased yield value under stress, RIL-76 line Ys = 49.50, SSI = –0.47 and YSI = 1.10 – showed stable and resistant properties.

Cluster (dendrogram) analysis

Hierarchical cluster analysis based on the complex indicators of 80 RIL lines under control and drought stress conditions confirmed the genetic-physiological heterogeneity of the population. In the study, cluster (dendrogram) analysis was performed using the hierarchical structure in the Origin-2022 program and was divided into 6 main clusters. Each cluster included RIL samples that were close to each other. Cluster 1 included lines such as RIL-25, RIL-36, RIL-59, RIL-58, RIL-33, RIL-44 and RIL-65, which indicates their mutual similarity. Cluster 2 included lines such as RIL-12, RIL-35, RIL-28, RIL-13, RIL-71 and RIL-38 and there were also short distances between them. Cluster 3 includes lines such as RIL-45, RIL-56, RIL-01, RIL-42, RIL-47, RIL-06 and RIL-20, which are relatively close to each other in terms of characteristics. Cluster 4 also includes lines such as RIL-05, RIL-37, RIL-57, RIL-48, RIL-12 and RIL-26. Cluster 5 includes lines such as RIL-07, RIL-23, RIL-61, RIL-68, RIL -77, RIL-74, RIL-33 and RIL-65. Cluster 6 includes lines such as RIL-19, RIL-35, RIL-31, RIL-04, RIL-09, RIL-53 and RIL-18. Furthermore, the RIL-45 line is located very close to the central point of the dendrogram, on a branch that is distinct from all other lines, indicating that it is distinct from the others in terms of phenotypic or genetic characteristics.

Clusters I and II (light green and green)

Lines belonging to this group (e.g. RIL-04, RIL-64, RIL-73) showed the highest STI and stable agrophysiological parameters. These genotypes maintained high RWC and chlorophyll content even under water deficit conditions. Clusters III and IV (yellow and brown): These groups have a moderate level of tolerance and have shown moderate yield losses under drought conditions. Clusters V and VI (purple and dark purple): These clusters, which contained the largest number of lines (RIL-01, RIL-42, RIL-67), were characterised by high sensitivity to stress. These lines had high SSI and severe yield loss. Experimental outliers: RIL-45 (blue) and RIL-56 (black) lines are located at a significant distance (d > 50) from all other clusters and were found to have a unique agrophysiological profile. This may indicate the presence of unique alleles in the genetic makeup of these lines. The dendrogram structure shows that lines belonging to similar clusters use similar physiological mechanisms under water deficit conditions. These results serve as a key criterion for selecting parental forms for drought tolerance gene mapping (QTL mapping) (Fig. 2).

Discussion

In this study, 80 recombinant inbred lines (RILs) were evaluated under control and drought conditions based on physiological and yield stress tolerance indices (STI, GMP, MP, YI, YSI, SSI and TOL). Previous studies confirm the obtained results, which clearly demonstrate genetic variability in drought response among populations. Classification of RIL populations based on drought indices revealed a high degree of variability in stress response (15). The genotypes RIL-45, RIL-36 and RIL-25 were characterised by high STI (>1.0), GMP, MP and YSI (>1.0) values. These indices indicate that these lines not only have high yield stability, but also have robust physiological mechanisms to maintain productivity under water deficit conditions. The effectiveness of these indices in identifying drought-tolerant genotypes is well documented. Research indicates that GMP, MP and STI are the most reliable parameters for assessing genotypic adaptability under different moisture levels (15). Our findings support this, as these high-performing RILs maintain a physiological balance between water conservation and biomass production. From a mechanistic perspective, high STI and YSI indicate that these genotypes have the genetic capacity to mitigate oxidative stress or maintain cell turgor, which is crucial for the continuity of photosynthesis under prolonged drought.

Similarly, research reported high MP and GMP as key selection criteria for drought-tolerant cotton genotypes (29). However, unlike some studies that focus solely on yield, our use of integrated physiological traits (RWC and ELWL) along with these indicators provides a more holistic understanding of drought tolerance. PCA and stress indices PCA biplot analysis provided a clear spatial distribution of genotypes based on adaptive strategies. The clustering of RIL-36, RIL-25 and RIL-45 along the yield index axis indicates a high genetic capacity to maintain yield under water stress conditions. Research indicates that yield stability and physiological resilience are key contributors to drought tolerance in cotton (30). In our study, the clustering of RWC and ELWL close to the centre of the PCA highlights their role as integrative physiological markers and supports the hypothesis that water status maintenance is important for stress adaptation.

Mechanistic explanations for tolerance, the superior performance of the resistant lines (RIL-36, RIL-45) compared to susceptible genotypes such as RIL-19 (YSI = 0.39, SSI = 2.90), may

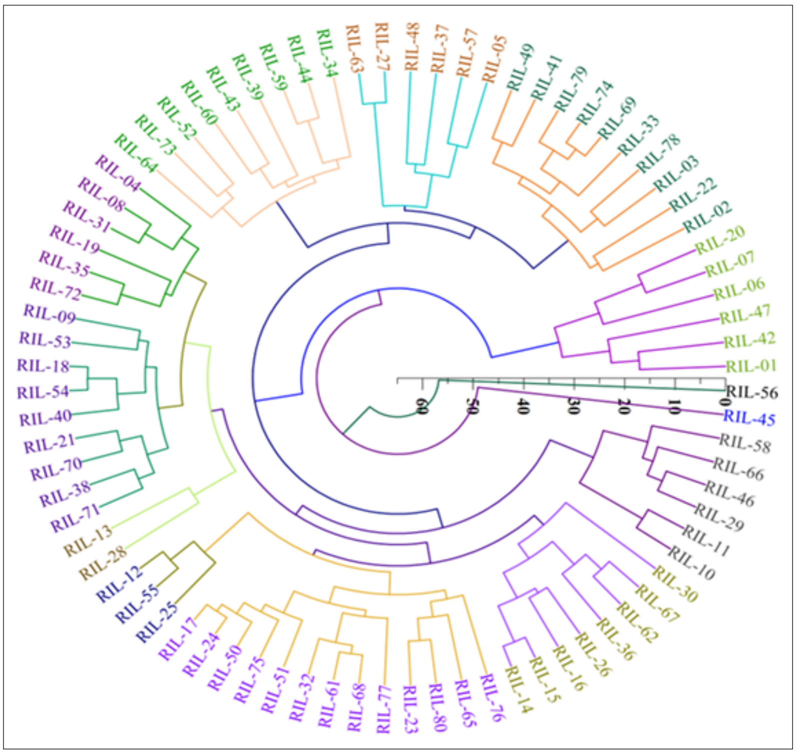

Fig. 2. Cluster (dendrogram) analysis of physiological and yield-based stress indices in RIL population samples under control and drought conditions.

be due to efficient physiological homeostasis. Mechanistically, the higher RWC and lower ELWL in these lines indicate increased osmotic adaptation and cuticle efficiency that prevent cell dehydration. These findings contradict some traditional views that emphasise only biomass; instead, they support a model in which survival is due to the activation of stress-sensitive alleles inherited from the wild ancestor (*G. tomentosum*). The sharp difference between resistant and susceptible lines (RIL-19) confirms that this RIL population is an ideal resource for QTL mapping. The identified resistant lines (RIL-36, RIL-25 and RIL-45) demonstrate the necessary genetic stability to serve as elite parental donors in breeding programs aimed at creating climate-resistant cotton varieties. Overall, the integration of yield-based indices and PCA provides a comprehensive framework for characterising drought tolerance in this RIL population. The identification of highly resistant lines such as RIL-36, RIL-25 and RIL-45 offers valuable genetic resources for future breeding programs. These lines are recommended as elite parental material for incorporation into high-yielding cotton varieties with the aim of developing climate-tolerant varieties with improved water use efficiency.

Conclusion

This study revealed the physiological and genetic basis of drought tolerance in 80 RIL populations of G. *tomentosum* and G. *mustelinum*. The results obtained provide important methodological and practical insights for early screening of drought-tolerant genotypes in cotton breeding. The study confirmed that physiological parameters such as RWC and ELWL are reliable markers for assessing drought stress. In particular, lines such as RIL-08, RIL-12 and RIL-24, which have effective water conservation mechanisms, were found to have high potential to maintain physiological homeostasis under water deficit conditions. The identified lines RIL-36, RIL-25 and RIL-45 are characterised not only by high stress indices (STI and YSI) but also by genetic immunity. The three samples play a strategic role as donor genotypes in the creation of new drought-tolerant varieties. The study showed that the use of stress indices (STI, SSI and PCA) in combination with physiological indicators reduces the probability of errors in genotype selection. This approach is recommended as a more accurate and reliable methodology in breeding programs. The most resistant and most sensitive lines identified in this study will serve as the main targets for future drought tolerance gene mapping (QTL mapping) and genome-wide association studies (GWAS). This, in turn, will accelerate the implementation of marker-based selection (MAS) technology in the development of stress-tolerant cotton varieties.

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Authors' contributions

RIM conducted the laboratory and field experiments, collected the data, performed statistical analysis and wrote the manuscript. AKM, ISN, UAB, JKN, NNK, SIM, ABM, YAM, IEB, BKR and RKA participated in the experiments and revision of the manuscript. ZTB revised and approved the final manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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