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Research Article

Multivariate analysis of agronomic and physiological traits in a wheat Recombinant Inbred Lines (RILs) under drought stress

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ABSTRACT- Drought stress is a primary constraint on global wheat production, necessitating the development of resilient cultivars. This study investigated the genetic basis of drought tolerance by evaluating a population of 169 wheat recombinant inbred lines (RILs) across two growing seasons under well-watered and drought-stressed conditions. We assessed seven key agronomic and physiological traits: relative water content (RWC), chlorophyll content (SPAD), plant height (PH), thousand grain weight (TGW), biological yield (BY), grain yield (GY), and harvest index (HI). Combined analysis of variance (ANOVA) revealed highly significant effects of genotype, year, and irrigation treatment on all traits. Drought stress consistently and significantly reduced all measured traits, with mean GY declining by approximately 25–30%. Correlation and principal component analyses (PCA) demonstrated structured relationships among traits under drought. Although BY remained a key determinant of yield, RWC was identified as a reliable positive indicator of sustained GY and BY under drought stress. In contrast, SPAD emerged as a prominent factor in the selection of drought-tolerant genotypes. Cluster analysis identified distinct subpopulations, highlighting RILs such as RIL_101 and RIL_41 that exhibited constitutive traits for high yield potential and robust water maintenance across environments (defined by the combination of irrigation treatment and year). Our findings demonstrated that drought resilience is governed by the maintenance of water status coupled with yield stability, rather than by vegetative greenness alone. The identified elite RILs provide valuable germplasm for breeding programs and for mapping quantitative trait loci (QTLs) associated with drought tolerance, thereby offering a pathway to the development of high-yielding, drought-resilient wheat varieties.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is a cornerstone of global food security, serving as a primary source of nourishment for over 4.5 billion people by supplying more than twenty percent of the world's dietary calories and protein (Khalid et al., 2023). Despite its paramount importance, the sustainable production of this vital cereal is continually threatened by a wide range of abiotic stresses. Among these, drought represents the most pervasive and devastating constraint, severely limiting yield potential across major wheat-growing regions (Khan et al., 2025). This challenge is expected to intensify under projected climate change scenarios, which forecast rising temperatures, increasingly erratic rainfall patterns, and a higher frequency of extreme weather events. Together, these factors will exacerbate water scarcity and place unprecedented pressure on agricultural systems worldwide (Seleiman et al., 2021). Consequently, there is an urgent need to develop high-yielding wheat cultivars with enhanced resilience to drought stress in order to safeguard future global food supplies. The nature of drought tolerance in wheat is inherently complex, as it does not correspond to a single trait but rather to a multifaceted physiological phenomenon. It encompasses a suite of morphological, biochemical, and phenological adaptations, including the development of deep root systems, efficient stomatal regulation, effective osmotic adjustment, and the optimal partitioning of photoassimilates to developing grains (Ahmad et al., 2018; Mohi-Ud-Din et al., 2024). This complexity is further compounded by strong environmental influences, i.e., the timing, intensity, and duration of drought stress, along with pronounced genotype-by-environment (G × E) interactions, which have

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historically masked genetic gains and rendered conventional breeding a slow and challenging process. To address this complexity, breeders rely on secondary physiological traits that serve as crucial indicators of plant performance under stress (Pereyra et al., 2021; Vieira et al., 2025). RWC, which provides a direct measure of plant water status and turgor maintenance; chlorophyll content, which reflects photosynthetic capacity and senescence dynamics; and HI, which quantifies partitioning efficiency, are among the key traits linked to drought resilience (González-Espíndola et al., 2024). These physiological processes ultimately integrate into yield components such as GY, BY, and TGW. Importantly, the genetic correlations among these traits can shift markedly under drought, revealing fundamental biological trade-offs. For instance, a strategy that promotes high vegetative biological yield may be advantageous under well-watered conditions but can become detrimental under terminal drought if it occurs at the expense of reproductive sink strength and grain filling (Senapati et al., 2019).

To dissect this intricate web of traits and interactions, advanced statistical techniques are indispensable. Multivariate analyses, such as PCA, are powerful tools for reducing the dimensionality of complex phenotypic datasets (Kim et al., 2018). PCA achieves this by revealing the primary, independent axes of variation, known as principal components, that explain the majority of variability among genotypes. This enables the visualization of the genetic architecture of a population, the identification of traits that contribute most to differentiation, and the uncovering of hidden patterns and relationships among traits (Elhaik et al., 2022). Cluster analysis further complements PCA by grouping genotypes based on trait similarity, thereby enabling the identification of distinct physiological profiles—from highly susceptible to deeply resilient—and facilitating the selection of elite breeding candidates from each group. The utility of these methods is greatly enhanced by the use of robust genetic populations such as RILs. RILs are particularly valuable due to their nearly homozygous and genetically fixed nature, which allows for precise replication of genotypes across multiple environments and seasons. This stability is critical for disentangling genetic variance from environmental noise, enabling the accurate quantification of genetic variation, the mapping of QTLs, and a clearer understanding of the genetic control of complex traits such as drought tolerance (Sharma et al., 2024).

This study employs a comprehensive multivariate approach to elucidate the genetic and physiological basis of drought tolerance in wheat. A population of 169 RILs was meticulously evaluated over two growing seasons under both well-watered and drought-stressed conditions. The specific objectives of this research were to: (1) quantify the phenotypic variation and assess the impact of drought stress on critical agronomic and physiological traits and (2) elucidate the dynamic correlation structure among these traits under both water regimes in order to identify key physiological indicators of drought tolerance.

MATERIALS AND METHODS

The research assessed 169 wheat RILs developed from a cross between SeriM82 and Babax cultivars. These lines were studied at the Research Farm of Plant Production and Genetics in Bajgah research station, Shiraz, Iran (29°435N, 52°3528E) over two consecutive growing seasons (2020-2021 and 2021-2022). SeriM82 is a semi-dwarf spring wheat cultivar originating from the “Veery” cross (KVZ/BUHO//KAL/BB). Babax was developed through the “Babax” crossing program (BOW/NAC//VEE/3/BJY/COC) (Olivares-Villegas et al., 2007; Pinto et al., 2010). SeriM82 possesses the 1BL/1RS (rye) translocation and is characterized by a high yield potential, while Babax is distinguished for its drought tolerance (McIntyre et al., 2010; Pinto et al., 2010). The RIL population was cultivated under both normal irrigation and drought stress treatments, with each environment arranged in an α-lattice design and three replicates. Sowings took place on November 4th and 6th for the first and second years, respectively, and harvesting was conducted on June 30th and July 6th in the first and second seasons. Individual plots comprised two 1.5-meter-long rows spaced 30 cm apart. Fertilizer was applied during the season to optimize yield: 150 kg/ha of diammonium phosphate was used at planting, followed by 200 kg/ha of urea at both the start of tillering and during the stem elongation phase. For irrigation, a strip system with drippers were placed 10 cm apart. These drippers emitted 1.8 to 2 liters of water per hour at a pressure of 1 bar. Drought treatment was imposed by withholding irrigation at the booting stage, corresponding to the Zadoks decimal code Z45 (Zadoks et al., 1974). The Zadoks scale is a two-digit system (00–99) in which the first digit denotes the principal growth stage (0–9), and the second digit specifies a secondary stage within that phase. This scale provides a precise framework for describing developmental milestones in cereals, encompassing germination (00–09), seedling growth (10–19), tillering (20–29), stem elongation (30–39), booting (40–49), heading (50–59), flowering (60–69), grain filling (70–79), dough development (80–89), and ripening (90–99).

Measurements

RWC

RWC was determined by randomly collecting mature flag leaves from wheat plants in each plot and treatment. The fresh weight of each sample was recorded immediately after collection. The leaves were then submerged in distilled water overnight to allow full saturation, after which they were gently blotted to remove surface moisture and weighed again to obtain the turgid weight. Finally, the samples were oven-dried at 70 °C until a constant weight was reached, and the dry weight was recorded. RWC was then calculated using the following equation (Farooq et al., 2009):

$$RWC= ((FW-DW))/((TW-DW)) \times 100 \qquad \text{Eq. (1)}$$

where FW (g) is the fresh weight of the leaf immediately after collection, TW (g) is the turgid weight of the leaf after full saturation, and DW (g) is the dry weight of the leaf after oven-drying at 70 °C.

Estimation of chlorophyll using SPAD

A chlorophyll meter (SPAD-502, Minolta, Japan) measured leaf chlorophyll concentration, expressed as the SPAD value. One month after the onset of drought stress, five plants were randomly selected from each plot, and SPAD readings were taken from the central portion of the upper surface of fully expanded flag leaves.

Agronomic and yield components

At harvest, plant height (PH; cm) was measured from the soil surface to the tip of the main spike once the plants reached physiological maturity. For thousand grain weight (TGW; g), ten plants were randomly selected from the central rows of each plot to minimize edge effects. Plants from the two middle rows were harvested to determine biological yield (BY; kg m^{-2}) and grain yield (GY; kg m^{-2}). The harvest index (HI; %) was then calculated using the following equation:

Harvest index (%) = (Grain yield (g $plant^{-1}$)) / (Biological yield (g $plant^{-1}$)) × 100 Eq. (2)

Statistical analysis

Descriptive statistics, such as means and standard deviations, were calculated using the Statistical Analysis System (SAS, Version 9.3, SAS Institute Inc., Cary, NC, USA). The PROC GLM procedure assisted in combined analysis of variances (c-ANOVA) in SAS software (Dodig et al., 2008). An analysis of variance was performed on the alpha lattice design using the R software package agridat. All statistical analyses were performed using the R programming language (version 4.2.3). To assess the linear relationships between the measured traits, a correlation analysis was conducted. Pairwise Pearson correlation coefficients (*r*) were calculated for all variable combinations using the “rcorr()” function from the “Hmisc” package. The resulting correlation matrix was visualized using a heatmap, created with the “ggplot2” package (version 3.4.0). To investigate the genetic relationships and group the genotypes based on their multivariate phenotypic profiles, an unsupervised hierarchical cluster analysis (HCA) was performed. A pairwise Euclidean distance matrix was first computed to quantify the dissimilarity between each genotype across all measured traits. This distance matrix was then used as an input for clustering via Ward’s minimum variance method (method = “ward.D2”). This agglomerative algorithm was selected for its tendency to create compact, spherical clusters of relatively equal size. A PCA was performed using the “FactoMineR” package in R. The number of significant principal components (PCs) to retain was determined using the Kaiser-Guttman criterion, which retains components with eigenvalues greater than 1.

RESULTS AND DISCUSSION

Phenotypic variation

The results of the c-ANOVA indicated that the main effects of year, irrigation, and RILs were highly significant ($P < 0.01$) for all measured traits (Supplementary Table 1). Descriptive statistics showed a clear and consistent negative impact of drought stress across traits (Table 1). Both SPAD and RWC were significantly reduced under drought stress. SPAD values decreased from a mean of 43.6 to 29.0, with greater variability evident in the second season of drought stress, while RWC declined from about 67% to 49%. Plant height was reduced by an average of 10 cm, though data from the first well-watered season were skewed by an extreme maximum value of 450.00 cm. Drought stress further reduced TGW by an average of 5.8 g, BY by roughly 183 g/m^2, and GY by approximately 25–30%, with mean GY decreasing from 343.77 g/m^2 to 252.29 g/m^2. The effect on HI was less consistent: a minimal reduction occurred in the first year, but a more substantial decrease of 6.56% was observed in the second season. The second growing season was also generally more favorable, leading to higher mean values for TGW, BY, and GY compared to the first season. Substantial genetic variation within the population was reflected in high standard deviations, particularly for BY (97.79–236.00) and GY (38.67–67.42) (Table 1).

Correlation analysis

Correlation analysis revealed distinct relationships among the seven agronomic traits under well-watered and drought-stressed conditions (Fig. 1). Under well-watered conditions, the strongest positive correlation was observed between GY and BY ($r = 0.87$, $P < 0.001$). RWC also showed strong correlations with both BY ($r = 0.66$, $P < 0.001$) and GY ($r = 0.68$, $P < 0.001$), highlighting the central role of plant water status in sustaining productivity. A moderate positive correlation was detected between HI and GY ($r = 0.43$, $P < 0.001$), suggesting that partitioning efficiency contributed to yield. In contrast, SPAD exhibited weak but significant negative correlations with both GY and HI ($r = –0.19$ and –0.20, respectively; $P < 0.001$), which may have indicated a link between delayed senescence and reduced nutrient remobilization to the grain. TGW and PH showed generally weak and non-significant associations with other variables (Fig. 1A). Under drought stress, the strong positive correlation between BY and GY persisted, though with slightly reduced strength ($r = 0.86$, $P < 0.001$). RWC again showed strong, significant correlations with GY ($r = 0.68$, $P < 0.001$) and BY ($r = 0.67$, $P < 0.001$), indicating that genotypes maintaining better hydration were able to sustain growth and partition resources more effectively to the grain. In contrast, SPAD showed no significant relationship with either RWC or GY, suggesting that it may not serve as a reliable selection criterion for drought tolerance in this RIL population (Fig. 1B).

PCA

The PCA identified the primary sources of trait variation among 169 RILs evaluated under well-watered conditions based on seven agronomic and physiological traits (Fig. 2A–F). The analysis extracted five principal components that together explained 94.70% of the total variance, with the first two components being the most meaningful for interpretation (Fig. 2A). The PC1 (eigenvalue: 2.70; 38.60% variance) was defined as a yield potential axis, as it was strongly associated with positive correlations of GY (32.79% contribution), BY (28.46%), and RWC (26.24%). This indicated that high-yielding RILs under optimal water conditions also exhibited robust biomass production and maintained tissue water content (Fig. 2B). The PC2 (eigenvalue: 1.20; 17.50% variance) represented a physiological and architectural axis. It was dominated by a strong negative correlation with HI (52.70% contribution) alongside positive contributions from SPAD and PH, revealing a key trade-off in which RILs with high positive scores tended to be taller and have higher chlorophyll content but a lower harvest index (Fig. 2C and Fig. 2D). Together, PC1 and PC2 explained 56.10% of the total variance, providing a reliable framework for visualizing genetic relationships. The distribution of RILs allowed for the identification of elite candidates, such as RIL_12, RIL_101, RIL_41, RIL_7, RIL_114, and RIL_67 (Fig. 2E and Fig. 2F). Under drought stress, seven principal components were extracted, with the first five collectively explaining 94.75% of the total variance (Fig. 3A). PC1 was the most significant (eigenvalue: 2.60; 37.60% variance), followed by PC2 (eigenvalue: 1.10; 16.10% variance). Together, these two components explained 53.70% of the variation, providing a sufficient basis for constructing a biplot to visualize genetic relationships under stress. Analysis of trait contributions and correlations revealed a distinct structure shaped by the drought environment. PC1 functioned as a yield and water status component, defined by traits associated with sustained productivity and hydration. It was primarily characterized by strong positive correlations with grain yield (34.78% contribution, r = 0.956), biological yield (29.83%, *r* = 0.886), and RWC (26.78%, *r* = 0.839) (Fig. 3B). In contrast to the well-watered analysis, PC2 emerged as a partitioning efficiency axis, driven largely by a strong negative correlation with HI (54.58% contribution, *r* = –0.784) (Fig. 3C and Fig. 3D). This highlighted that the second major source of variation under drought was the efficiency with which RILs converted biological yield into grain, with plant architecture (PH: 28.33% contribution, *r* = 0.565) playing a secondary role.

The distribution of the 169 RILs on the PC1–PC2 plane enabled the identification of breeding candidates with superior stress tolerance. RILs with high positive scores on PC1, such as RIL_41 (4.63), RIL_101 (4.56), and RIL_30 (3.10), were prime candidates for stress resilience due to their ability to maintain high grain yield, biological yield, and relative water content under drought. RILs with strong negative coefficients on PC2, including RIL_68 (–2.58), RIL_78 (–2.52), and RIL_36 (–2.21), were distinguished by a high harvest index. The most desirable ideotype for stress conditions would occupy the top-right quadrant of the biplot, combining high yield stability (high PC1) with efficient partitioning (low PC2). RIL_101 stood out as an exceptional example, exhibiting a very high PC1 score coupled with a moderately positive PC2 score. In addition, RILs with extreme values on other components, such as RIL_17 on PC3 (–3.28) or RIL_57 on PC2 (–2.32) and PC1 (–3.16), represented unique physiological responses to stress (Fig. 3E and Fig. 3F).

HCA

Under well-watered conditions (Fig. 4A and Table 2), Cluster 3 emerged as the high-performing group, characterized by the most desirable and tightly constrained ranges for yield components, with GY between 363 and 474 g/m^2, BY from 1071 to 1553.83 g/m^2, and HI spanning 29.88 to 36.88%. Importantly, RILs in this cluster also showed the highest lower limit for RWC (0.684–0.803%), reflecting a consistent capacity to maintain tissue water status and suggesting strong resilience to drought stress. By contrast, Cluster 2 displayed the greatest variability and the least favorable extremes, with the broadest BY range (758.67–1461.67 g/m^2) and the lowest minimum values for both GY (234.67 g/m^2) and RWC (0.422%), underscoring its composition of more vulnerable and unstable RILs. Cluster 1 occupied an intermediate position, showing moderate but stable performance. Its yield range (GY: 221.83–407.00 g/m^2) surpassed the lower bounds of Cluster 2 but did not reach the elite levels of Cluster 3, while its RWC range (0.515–0.825%) reflected a mid-tier status. The ranges for PH and SPAD were broadly similar across all clusters, indicating that these traits were less discriminatory for grouping than yield and RWC (Table 2).

Under drought stress conditions, the clustering analysis revealed a clear re-ranking of RILs compared to the well-watered environment, with Cluster 3 (21 RILs) emerging as the stress-resilient group (Fig. 4B and Table 3). This cluster was distinguished by its superior and stable yield potential, exhibiting the highest mean values for GY (263.83 g/m^2) and BY (931.67 g/m^2), along with a strong HI (25.75–32.55%). However, the RWC range within this cluster was relatively broad (0.413–0.672%), suggesting that the best-performing RILs in the group were those capable of effectively maintaining hydration under stress. Cluster 1 (80 RILs), the largest group, represented moderate responders, with a wide range across all traits but generally lower yield metrics than Cluster 3. Cluster 2 (68 RILs) was identified as the drought-susceptible group, containing RILs with the lowest values for key traits, including GY (minimum 174.67 g/m^2), BY (minimum 663.33 g/m^2), and RWC (minimum 0.292%) (Table 3).

Table 1. Descriptive statistics for various agronomic traits of 169 wheat recombinant inbred lines (RILs) evaluated in the growing seasons of 2020-2021 (first years) and 2021-2022 (second years) under well-watered (WW) and drought-stressed (DS) condition.

Traits	Year	Condition	Mean	Maximum	Minimum	Std Dev	Skewness	Kurtosis
SPAD	First year	WW	43.75	55.80	36.13	4.09	0.46	-0.33
		DS	29.64	37.87	21.33	3.55	0.15	-0.52
	Second year	WW	43.39	56.60	32.80	4.97	0.04	-0.42
		DS	28.27	46.03	10.97	7.13	0.12	-0.28
RWC (%)	First year	WW	0.61	0.92	0.37	0.09	-0.49	0.33
		DS	0.44	0.64	0.26	0.08	-0.08	-0.42
	Second year	WW	0.72	1.03	0.46	0.09	-0.49	0.36
		DS	0.53	0.74	0.30	0.09	-0.34	-0.28
PH (cm)	First year	WW	96.53	450.00	62.60	30.50	1.32	1.26
		DS	86.01	109.00	56.73	11.53	-0.14	-0.60
	Second year	WW	98.24	123.47	67.60	13.92	-0.23	-0.77
		DS	89.10	114.93	61.20	12.69	-0.10	-0.58
TGW (g)	First year	WW	32.82	42.64	26.76	2.81	0.30	0.30
		DS	26.92	33.29	20.69	2.29	0.16	-0.23
	Second year	WW	37.45	45.08	26.31	2.89	-0.30	0.73
		DS	29.09	35.65	23.82	2.26	0.05	0.05
HI (%)	First year	WW	28.17	35.71	21.28	3.20	0.02	-0.60
		DS	26.55	33.03	33.03	2.58	0.06	-0.34
	Second year	WW	35.72	40.50	27.93	2.64	-0.51	-0.12
		DS	29.16	40.56	20.59	2.80	0.12	1.56
BY (g/m^2)	First year	WW	1021.37	1630.33	455.67	236.00	0.14	-0.28
		DS	802.47	1286.67	1286.67	196.02	0.12	-0.15
	Second year	WW	1128.98	1495.33	797.33	129.15	0.34	0.03
		DS	1015.65	1350.00	802.00	97.79	0.38	0.25
GY (g/m^2)	First year	WW	285.86	523.00	136.00	67.42	0.28	-0.003
		DS	212.25	370.00	370.00	53.67	0.25	-0.17
	Second year	WW	401.68	593.33	259.33	58.46	0.41	0.45
		DS	292.33	428.00	180.67	38.67	0.47	1.23

SPAD: Determination of chlorophyll, RWC: Relative water content, PH: Plant height, TGW: Thousand grain weight, HI: Harvest index, BY: Biological yield, and GY: Grain yield.

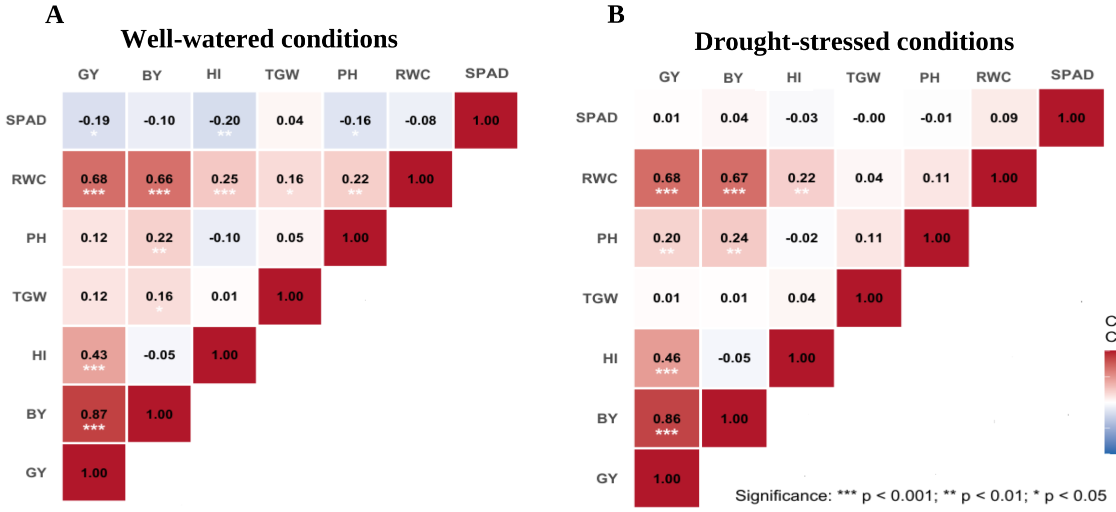

Fig. 1. Pearson's correlation coefficients of agronomic traits of 169 wheat Recombinant Inbred Lines (RILs) evaluated under well-watered (A) and drought-stressed (B) condition over two years. SPAD: Determination of chlorophyll, RWC: Relative water content, PH: Plant height, TGW: Thousand grain weight, HI: Harvest index, BY: Biological yield, and GY: Grain yield. * Significant at $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

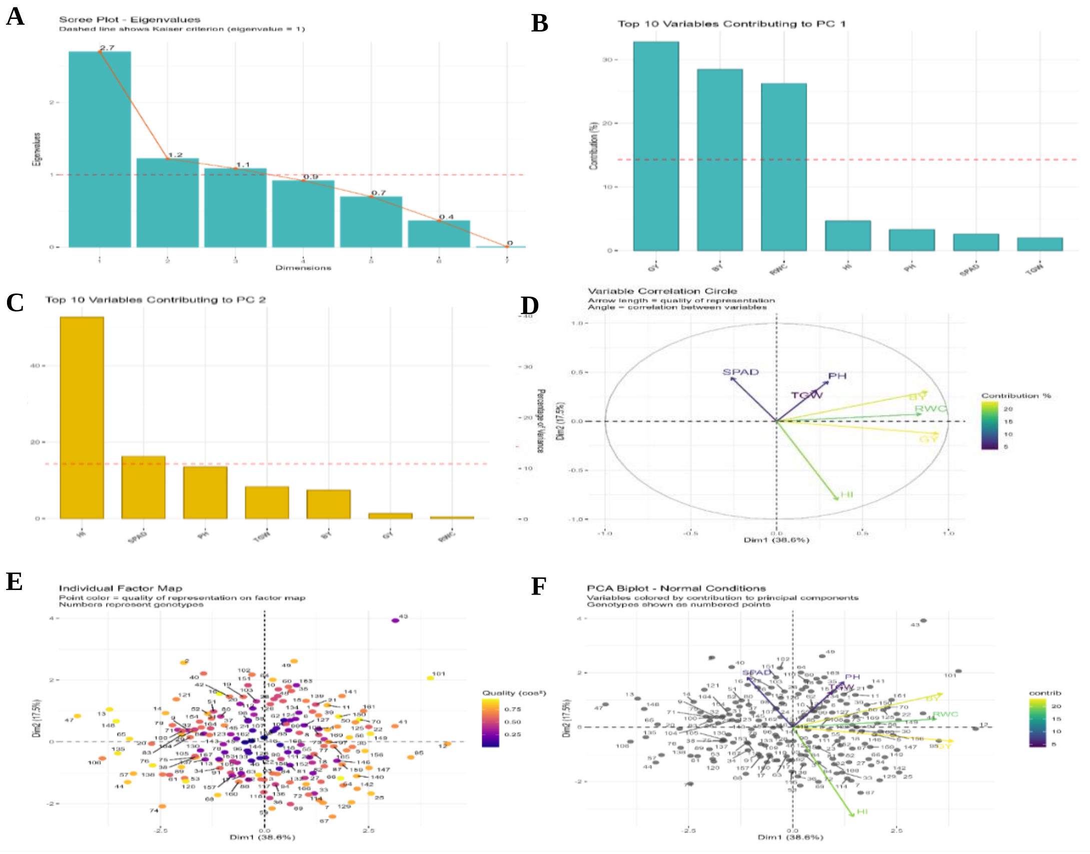

Fig. 2. Comprehensive Principal Component Analysis (PCA) of agronomic traits in 169 recombinant inbred lines (RILs) under well-watered conditions. Panel A shows the scree plot displaying the eigenvalues of the first principal components, with the dashed line indicating the Kaiser criterion (eigenvalue > 1) used to identify the most significant components. Panels B and C present bar plots of the percentage contributions of the top 10 variables to the first and second principal components (PC1 and PC2), respectively. Panel D illustrates the variable correlation circle, which shows the correlations between the original variables and the first two PCs. The direction of each vector indicates the correlation with the axes, while its length represents the quality of representation on the factor map. The cosine of the angle between vectors approximates their correlation, such that acute angles indicate positive correlation and obtuse angles indicate negative correlation. Panels E and F display the PCA biplot, which overlays the variable vectors (shown in red) with the RILs, each represented as a numbered point. The color of the points reflects the quality of representation of each RIL on the factor plane. This biplot enables the direct identification of high-performing RILs as well as the interrelationships among traits.

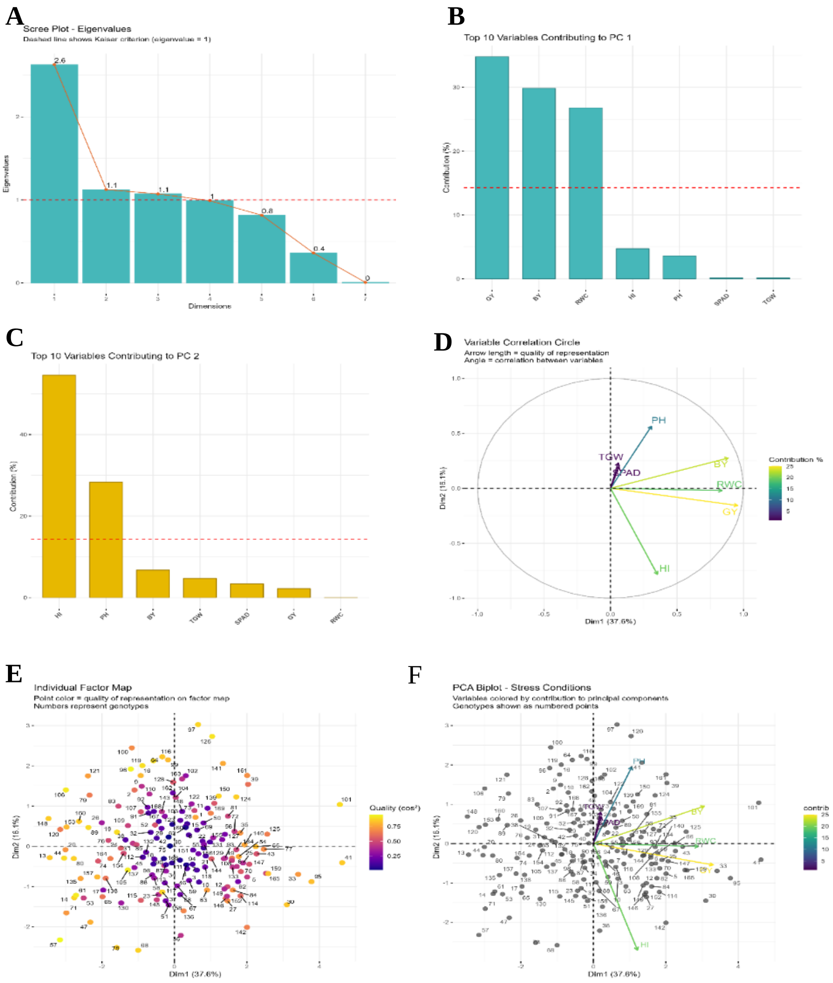

Fig. 3. Comprehensive Principal Component Analysis (PCA) of agronomic traits in 169 Recombinant Inbred Lines (RILs) under drought-stressed conditions. (A) Scree Plot: Displays the eigenvalues of the first principal components. The dashed line indicates the Kaiser criterion (eigenvalue > 1), which was used to identify the most significant components. (B) Variable Contributions to PC1: A bar plot presenting the percentage contribution of the top 10 variables to the first principal component (PC1). (C) Variable Contributions to PC2: A bar plot showing the percentage contribution of the top 10 variables to the second principal component (PC2). (D) Variable Correlation Circle: Depicts the correlations between the original variables and the first two PCs. The direction of each variable vector indicates its correlation with each axis, while the length represents the quality of its representation on the factor map. The cosine of the angle between any two vectors approximates their correlation, with acute angles indicating positive correlations and obtuse angles indicating negative correlations. (E and F) PCA Biplot: The core visualization, combining the results from panels (E) and (F). Variable vectors (in red) display the direction and strength of trait contributions, while each RIL is represented as a numbered point. The color of the points reflects the quality of representation of each RIL on the factor plane. This biplot enables the direct identification of high-performing RILs and provides insight into the interrelationships among traits.

Phenotypic variation in wheat RILs under drought stress conditions

The phenotypic variation observed in this study of 169 wheat RILs across two growing seasons under contrasting water regimes provides valuable insights into the complex responses of wheat to drought stress. The significant reductions recorded in key agronomic traits clearly demonstrate the detrimental effects of water deficit conditions on wheat productivity while simultaneously revealing substantial genetic variability within this breeding RIL population. Our findings are consistent with earlier studies highlighting drought as one of the most critical abiotic stresses affecting wheat production worldwide, particularly in the context of climate change and the intensifying challenge of water scarcity (Zhang et al., 2018; Seleiman et al., 2021). The use of RILs in this study proved particularly advantageous for dissecting the genetic architecture of drought tolerance traits, as the near-homozygous nature of such populations enables precise characterization of QTLs underlying complex responses to environmental stresses (Li et al., 2020; Liu et al., 2025).

Impact of drought stress on physiological and yield-related traits

The significant decline in SPAD values under drought stress observed in our study (from a mean of 43.6 to 29.0) reflected the impairment of photosynthesis typically associated with water deficiency. A reduction in chlorophyll content directly affects photosynthetic capacity, limiting carbohydrate assimilation and ultimately influencing growth and yield formation (Seleiman et al., 2021; Xu et al., 2023). The greater variability in SPAD values under drought stress during the second season (Std Dev: 7.13) suggests that genetic differences in chlorophyll maintenance are more pronounced under certain environments, creating an opportunity to select genotypes with superior chlorophyll retention under stress (Xu et al., 2023). The observed reduction in SPAD can be attributed to physiological mechanisms such as chlorophyll degradation and inhibited synthesis driven by oxidative stress (Seleiman et al., 2021). Since maintaining chlorophyll content under drought has been linked to improved photosynthetic , inhibited growth, and ultimately yield losses (Zhang et al., 2018). performance, it has been proposed as a selection criterion for drought tolerance in wheat (Ahmed et al., 2022). However, despite the significant genetic variation in SPAD maintenance identified in this study, our correlation analysis produced results inconsistent with SPAD as a reliable selection marker for drought resilience. Similarly, the observed reduction in RWC from approximately 67% to 49% reflects a decline in turgor pressure and cellular water status, both of which are essential for sustaining physiological processes under water-limited conditions. RWC is widely considered a reliable indicator of plant water status and has been positively correlated with drought tolerance in wheat and other cereals (Ahmed et al., 2022; Kettani et al., 2023). The significant reduction in RWC observed here aligns with previous reports demonstrating that water deficit disrupts cellular water relations, leading to impaired cell expansion

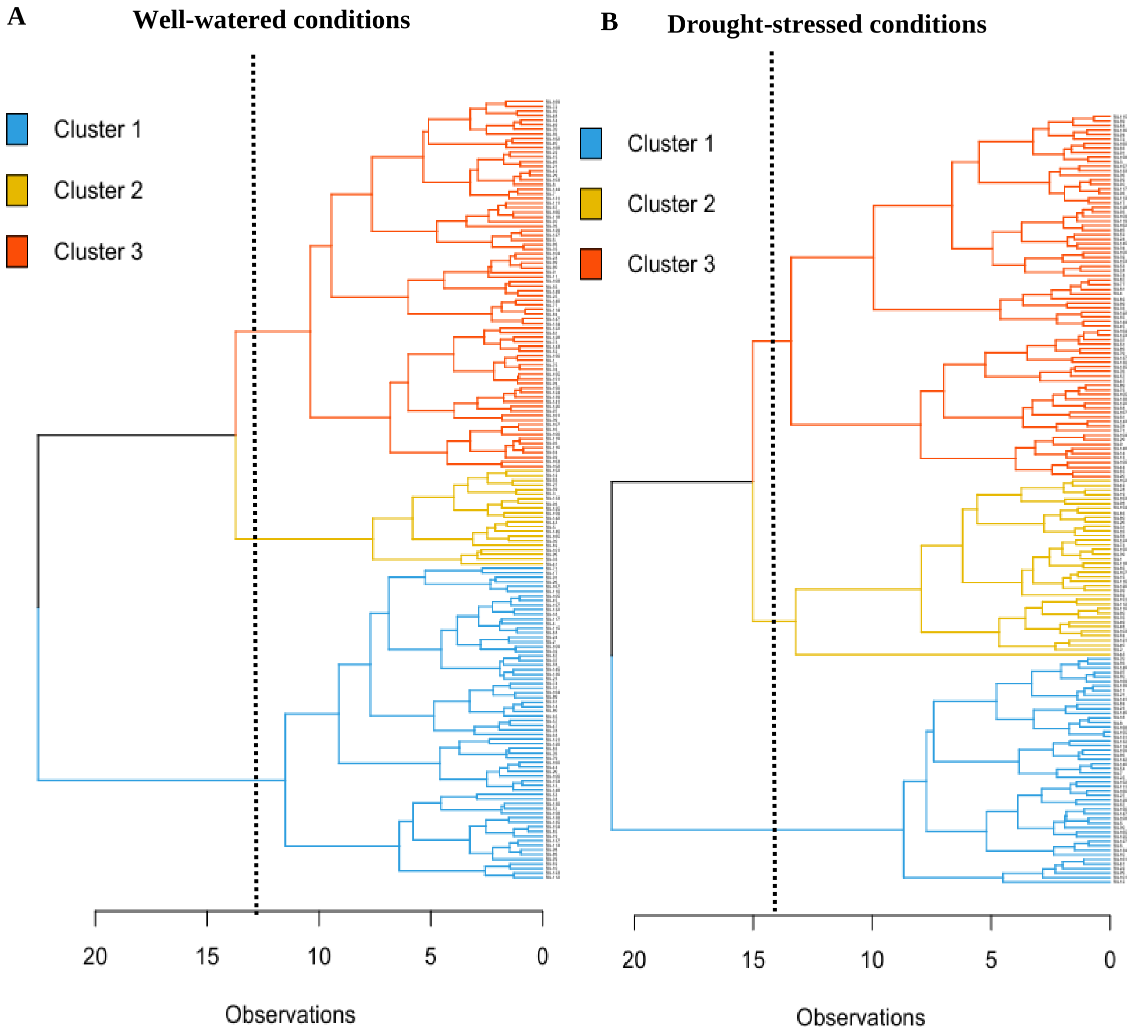

Fig. 4. Hierarchical cluster analysis based on agronomic traits of 169 wheat Recombinant Inbred Lines (RILs) evaluated under (A) well-watered and (B) drought-stressed condition over two years. SPAD: Determination of chlorophyll, RWC: Relative water content, PH: Plant height, TGW: Thousand grain weight, HI: Harvest index, BY: Biological yield, and GY: Grain yield.

Table 2. Cluster mean and range for different agronomic traits of the 169-wheat recombinant inbred lines (RILs) under well-watered conditions during two years

Trait	**Range (Min - Max)**		
	Cluster 1 (39 RILs)	**Cluster 2 (80 RILs)**	**Cluster 3 (50 RILs)**
SPAD	44.59 (36.40 - 52.77)	44 (36.40 - 51.60)	43.81 (37.22 - 50.40)
RWC (%)	0.67 (0.51 - 0.82)	0.62 (0.42 - 0.81)	0.74 (0.684 - 0.803)
PH (cm)	93.39 (65.10 - 121.67)	94.49 (67.90 - 121.07)	92.89 (65.10 - 120.67)
TGW (g)	36.91 (30.59 - 43.23)	35.12 (28.79 - 41.44)	36.72 (31.99 - 41.44)
HI (%)	31.54 (27.58 - 35.49)	32.53 (28.91 - 36.14)	33.38 (29.88 - 36.88)
BY (g/m^2)	1037.50 (797.50 - 1277.50)	1110.17 (758.67 - 1461.67)	1312.42 (1071.00 - 1553.83)
GY (g/m^2)	314.42 (221.83 - 407.00)	331.84 (234.67 - 429.00)	418.50 (363.00 - 474.00)

SPAD: Determination of chlorophyll, RWC: Relative water content, PH: Plant height, TGW: Thousand grain weight, HI: Harvest index, BY: Biological yield, and GY: Grain yield. The traits highlighted in bold were severely reduced by drought stress.

Table 3. Cluster mean and range for different agronomic traits of the 169-wheat recombinant inbred lines (RILs) under drought stress conditions during two years

Trait	**Range (Min - Max)**		
	Cluster 1 (80 RILs)	**Cluster 2 (68 RILs)**	**Cluster 3 (21 RILs)**
SPAD	28.36 (18.10 - 38.62)	29.77 (20.07 - 39.47)	26.94 (18.10 - 35.78)
RWC (%)	0.53 (0.37 - 0.69)	0.46 (0.29 - 0.63)	0.54 (0.41 - 0.67)
PH (cm)	85.64 (60.37 - 110.90)	85.34 (60.70 - 109.97)	85.64 (60.37 - 110.90)
TGW (g)	28.84 (23.47 - 34.21)	28.05 (23.36 - 32.73)	28.13 (23.47 - 32.79)
HI (%)	27.98 (23.50 - 32.46)	27.41 (23.49 - 31.33)	29.15 (25.75 - 32.55)
BY (g/m^2)	982.09 (724.00 - 1240.17)	875 (663.33 - 1086.67)	1065.17 (931.67 - 1198.67)
GY (g/m^2)	274.25 (180.67 - 367.83)	242.92 (174.67 - 311.17)	311.25 (263.83 - 358.67)

SPAD: Determination of chlorophyll, RWC: Relative water content, PH: Plant height, TGW: Thousand grain weight, HI: Harvest index, BY: Biological yield, and GY: Grain yield. The traits highlighted in bold were severely reduced by drought stress.

The profound effect of drought stress on yield components was evident in the substantial reductions in TGW, BY, and GY. The average reduction of 5.8 g in TGW reflects a significant limitation in assimilate supply during the grain-filling stage, a developmental phase particularly sensitive to water deficit (Li et al., 2020). The greater decline in BY (approximately 183 g/m^2) compared to the reduction in GY highlights how drought primarily constrains total biomass accumulation through impaired photosynthesis and reduced vegetative growth (Pantha et al., 2024). The 25–30% decrease in GY observed in this study is consistent with the findings from previous meta-analyses that reported yield losses of a similar magnitude under drought conditions in wheat (Zhang et al., 2018). This pronounced yield reduction underscores both the economic consequences of drought stress for wheat production and the urgent need for breeding drought-resilient cultivars. Interestingly, the maintenance of HI in the first year despite drought stress suggests that some genotypes were able to prioritize assimilate allocation to grains rather than vegetative structures, reflecting a potentially adaptive strategy under water deficit (Sareen et al., 2023). However, the marked drop in HI (6.56%) observed in the second year indicates that this capacity is not universal but likely environment-dependent, influenced by factors such as stress severity, timing, and interaction with other environmental variables.

Trait associations under divergent irrigation regimes

The correlation analysis revealed a fundamental restructuring of relationships among key agronomic traits under drought stress, reflecting shifts in the physiological strategies governing yield formation (Tardieu et al., 2018). Under well-watered conditions, the strong positive associations among grain yield, biological yield, and relative water content indicate that productivity is largely driven by overall biomass accumulation supported by optimal plant water status (Shao et al., 2008). The positive correlation between harvest index and grain yield further suggests that efficient assimilate partitioning to reproductive sinks contributes to yield formation even under non-stress conditions (Richards et al., 2010). By contrast, the weak negative correlations observed between chlorophyll content and yield components may reflect a subtle trade-off, where delayed senescence is accompanied by reduced nutrient remobilization efficiency during grain filling, a process more apparent under favorable growth conditions (Khalid, 2020).

Under drought-stressed conditions, the correlation structure shifted significantly; however, the strong positive correlation between BY and GY was maintained, underscoring that biomass accumulation remained a central determinant of yield even under stress. The pivotal role of RWC in drought tolerance was clearly evident, as it showed strong positive correlations with both GY and BY, indicating that genotypes capable of maintaining higher tissue hydration were able to sustain growth and partition resources more effectively to the grain. In contrast, SPAD demonstrated no significant relationship with either RWC or GY, confirming that chlorophyll content was not a reliable selection criterion for drought tolerance within this RIL population. These findings support a breeding paradigm focused on selecting genotypes with superior water conservation mechanisms that underpin both sustained growth and efficient assimilate partitioning, rather than relying on vegetative greenness alone (Blum, 2009; Richards et al., 2010). The PCA of 169 RILs across both well-watered and drought-stressed conditions provided a powerful multidimensional overview of the genetic architecture and the key physiological trade-offs shaping trait expression in this population. A particularly striking feature of our results was the consistency of PC1 across both water regimes and growing seasons. In all cases, PC1 represented a “Yield and Water Status” axis, defined primarily by GY, BY, and RWC. This consistent positive correlation structure under both optimal and stress conditions suggests that the physiological mechanisms underlying high yield are intrinsically linked to the plant’s capacity to maintain tissue hydration. This finding aligns with the well-established principle that sustained water status is critical for photosynthetic activity, nutrient assimilation, biomass accumulation, and ultimately grain filling (Blum, 2005). The strong correlation between GY and RWC under drought, in particular, highlights the value of RWC as both a key indicator of drought avoidance mechanisms and a practical marker for yield potential in breeding programs targeting water-limited environments (Richards et al., 2010). The identification of elite lines such as RIL_101 and RIL_41, which consistently scored high on PC1 under both watering regimes, is especially significant. These RILs appear to possess constitutive traits for both high yield potential and superior water maintenance, making them promising candidates for the development of cultivars with broad adaptation and inherent drought resilience. The most striking contrast between environments was observed in the interpretation of the second principal component (PC2). Under well-watered conditions, PC2 represented a “Physiological and Architectural” trade-off, wherein taller plants with higher chlorophyll content were associated with lower harvest index values. This relationship was particularly evident in well-fertilized, non-stressed environments, where plants could afford to allocate resources to vegetative growth without directly compromising grain yield (Lopes et al., 2012).

Discrimination of elite stress-resilient RILs for breeding

The clustering of RILs based on physiological traits under both well-watered and drought-stressed conditions successfully delineated distinct subpopulations with contrasting adaptive strategies and breeding values. The results confirm that drought resilience is not determined by a single trait but rather by a complex physiological syndrome, in which the maintenance of water status is intrinsically linked to yield stability. Under well-watered conditions, Cluster 3 stood out as a cohort of elite, high-performing lines, characterized by superior and relatively constrained ranges for GY, BY, and HI. Notably, this group also exhibited the highest lower limit for RWC, suggesting that its high yield potential is coupled with an inherent capacity for maintaining tissue hydration. This observation aligns with the well-established principle that RWC is a primary indicator of plant water status and a key correlating factor of both drought tolerance and yield stability in cereals (Georgii et al., 2017). The strong association of high yield and high RWC within this cluster makes it a valuable resource for breeding programs aimed at developing high-performing, resilient varieties adapted to favorable environments with occasional terminal drought. In contrast, Cluster 2 displayed the greatest variability and included the least favorable extremes, particularly for RWC and yield. This group represents a heterogeneous pool of stress-susceptible genotypes. The presence of lines with very low RWC indicates failures in osmoregulation or root water uptake, leading to severe tissue dehydration and, consequently, yield loss. Such susceptibility illustrates the genetic vulnerability present within the population and highlights the risks of including such material in a breeding program without stringent selection (Tardieu, 2012). At the same time, the high variability within this cluster could still be exploited for rare, beneficial alleles through detailed genetic analysis. The stability of Cluster 1, which performed intermediately but consistently across key traits, represents another important finding. These “stable-but-moderate” performers are highly valuable for breeding programs targeting marginal environments characterized by consistently low inputs or high stress, where reliability and resilience are often prioritized over maximum yield potential (Elsayed, 2025). Based on phenotypic performance under drought stress, a targeted inbreeding program should prioritize RIL_101 and RIL_41 as primary candidates for line development and direct evaluation, given their exceptional maintenance of GY, BY, and RWC under water-limited conditions. To integrate superior partitioning efficiency, RIL_68 and RIL_78 also emerge as prime candidates due to their consistently high harvest index under drought stress. Furthermore, RILs such as RIL_17 and RIL_57, which exhibited extreme and unique physiological responses, should be incorporated into breeding programs to generate stable genetic stocks and provide valuable material for investigating novel mechanisms of stress tolerance.

CONCLUSION

This study evaluated the complex phenotype of drought tolerance in wheat, demonstrating that resilience is governed by an integrated suite of traits rather than by any single characteristic. The identification of elite lines such as RIL_101 and RIL_41, which constitutively maintain high yield, biological yield, and RWC, provides validated candidates for both cultivar development and pre-breeding efforts. The strong correlation between sustained tissue hydration and yield stability under stress establishes RWC as a powerful phenotypic criterion for selection in water-limited environments. In contrast, the inconsistency of chlorophyll content as a marker highlights the risks of relying on simplistic visual indicators in breeding programs. The pronounced genetic variation observed, together with the distinct physiological profiles revealed through clustering, represents a valuable resource for subsequent genetic mapping. This work thus provides a solid phenotypic foundation for identifying the QTLs that control key mechanisms such as water conservation and partitioning efficiency. In the short term, these findings support the advancement of elite RILs into breeding pipelines, while the long-term impact lies in leveraging this population to elucidate the genetic architecture of drought tolerance, thereby accelerating the development of climate-resilient wheat varieties.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICAL STATEMENT

Not applicable.

DATA AVILABILITY

All data analyzed and generated during this study are included in this published article.

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