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Phenolic compounds, antioxidant enzymes, and oxidative stress in barley (*Hordeum vulgare* L.) genotypes under field drought-stress conditions

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Abstract

Climate change has exacerbated drought, making water scarcity a significant constraint on crop production. This study aimed to evaluate drought stress responses of 21 barley cultivars and breeding lines, using various traits of leaf oxidative stress [DPPH radical scavenging, malondialdehyde (MDA), and hydrogen peroxide (H_2O_2)], antioxidants (enzymes and polyphenols), photosynthetic pigments and chlorophyll fluorescence (F) [carotenoid (Car), chlorophyll (Chl), F_m, F_0, and F_v/F_m], relative water content (RWC), electrolyte leakage (EL), proline (Pro), protein content (PC), and grain yield. Field experiments were conducted under both normal and drought stress conditions. Significant effects of moisture conditions were observed for most of the traits, except for Chla/b, carotenoids, and EL. Syringic acid, gallic acid, chlorogenic acid, ferulic acid, ellagic acid, caffeic acid, vanillic acid, and *p*-coumaric acid were the prominent phenolic acids in barley genotypes. The predominant leaf flavonoids were luteolin, apigenin, and rutin. There was significant genetic variation among genotypes for all traits except Chla/b. Drought stress caused significant increases in DPPH, MDA, H_2O_2, total phenolic content, total flavonoid content, peroxidase, and Pro. While catalase, ascorbate peroxidase, Chla, Chlb, Tchl, F_v/F_m, F_0, F_m, RWC, PC, and grain yield were significantly decreased due to water stress. These findings offer key insights into barley genotypes' drought stress response, aiding breeders in identifying key physiological and biochemical traits as markers for developing drought-tolerant cultivars.

Keywords Antioxidants, Drought tolerance, Oxidative stress, Phenolics, Photosynthesis, Physiology

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Introduction

In recent years, climate change has amplified the severity of abiotic stress [1]. The scarcity of water resources and the consequences of climate change intensify the agricultural challenges of drought. These effects are compounded by factors such as the water-holding capacity of the rhizosphere, evapotranspiration rates, overexploitation of groundwater for irrigation, and inconsistent or irregular rainfall patterns [2]. Drought conditions have a detrimental effect on crop production and negatively influence food supply and farmer income [3, 4]. Barley, a member of the Triticeae tribe, is an important cereal crop, and serves as a model plant for studying the physiology, biochemistry, and molecular biology of abiotic stress tolerance of other tribe members such as wheat, maize, and rice. It is grown globally in a diverse range of environments, many of which are frequently subjected to severe drought conditions [5, 6].

Plants face a range of environmental stresses throughout their lives that jeopardize their survival [7]. Drought stress triggers significant changes in morphological, biochemical, physiological, and economic traits in plants [2, 3]. The most effective approach to handle the adverse effects of climate changes on plant production is cultivating tolerant genotypes to abiotic stresses. Plants employ various strategies to tolerate drought stress such as escaping, avoidance, tolerance, and recovery. These strategies involve a range of physiological and biochemical alterations in plant cells that enable the plants to survive under extreme dehydration, adjust their growth patterns to mitigate moisture stress and, maintain important physiological functions under drought stress [8, 9].

Drought stress leads to several critical negative effects on physiological functions in plants [2]. A key factor in cell death under severe drought stress is the loss of membrane integrity, which results in cell dehydration, and alters membrane structure and its permeability. This process initiates a cascade involving ion influx, elevated reactive oxygen species (ROS) levels, and oxidative damage which ultimately results in cell death [2, 3, 10]. Oxidative stress disrupts cells and their components by deactivating enzymes, damaging membranes, and harming organelles like mitochondria and chloroplasts in plants [7, 11].

Many global studies have shown that water shortage causes various changes in barley [1]. In barley, various evaluations of morphology, phenology, physiology, biochemical, and grain yield under water deficit [12–16] have been carried out. Phenolic and flavonoid compounds have been investigated in the grain of barley genotypes [17–20] and under stress [21–24]. These compounds have also been studied in the leaves of wheat, Aegilops, and their amphidiploid leaves under salt stress [25].

It was shown that physiological damage caused by drought stress leads to oxidative stress [7, 26], which affects grain yield [27]. Oxidative stress as a secondary consequence of environmental stress [14, 28] results from the accumulation of high amounts of ROS in cells [7, 28]. ROS can exert opposing influences based on their concentration within the cell. Maintaining low levels of ROS may integrate them into stress signaling pathways, activating responses for stress defense and acclimation [8, 29]. Generally, the basic levels of ROS produced under optimal environmental conditions typically do not result in cellular damage due to the activation of stress-responsive genes. Evidence suggests that this level of ROS generation may be linked to its constrained role in developmental processes [7, 10, 30] or in the regulation of morphogenetic activities associated with phytohormones [31]. Low to moderate levels of ROS can act as signaling molecules, assisting plants in developing defense mechanisms by mediating the perception of stress, integrating environmental signals, and activating stress response pathways. This process contributes positively to the regulation of tolerance towards both biotic and abiotic stresses [10, 30]. Conversely, an increase in ROS production in plant cells can result in oxidative damage, such as the peroxidation of lipids in cell membranes, contribute to membrane degradation [10, 14, 32] and ultimately cause cell death and yield reduction [14, 33].

Alterations in malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) can also be associated with drought stress. MDA resulting from lipid peroxidation is utilized as an indicator to evaluate the extent of lipid peroxidation in plant cells [8, 34, 35]. Through various mechanisms triggered by abiotic stresses, plants produce nonenzymatic and/or enzymatic antioxidants to protect their cells from free radicals in adverse environments [33, 36, 37]. Plant cells synthesize a variety of phenolic compounds (polyphenols such as flavonoids and tannins) that function as non-enzymatic antioxidants, assisting in the neutralization of ROS, and resulting in a decrease in peroxidation of the cell membrane [38, 39]. Phenolic compounds are essential for various metabolic, physiological, and developmental functions such as cell division, photosynthesis, synthesis of photosynthetic pigments, hormone regulation, reproduction, and nutrient mineralization. However, under stress situations, plants increase the production of polyphenols as antioxidants, which assist them in managing environmental challenges [40]. It was shown that drought stress regulates the biosynthetic pathways of flavonoids and phenolic acids, leading to a higher accumulation of these compounds. This enhancement contributes to their role as antioxidants, thereby mitigating the adverse effects associated with water deficit conditions in plants. The phenylpropanoid

biosynthetic pathway is triggered by abiotic stress, leading to the buildup of different phenolic compounds [40].

Phenolic compounds fulfill various functions in plants, including serving as signaling molecules, facilitating auxin transport, acting as antioxidants, bioremediation, scavenging free radicals, enhancing plant defense, allelochemical and antioxidants as food additives [30, 33, 40]. Plants that demonstrate enhanced synthesis of polyphenols in response to abiotic stresses, typically exhibit more adaptability to stressful environmental conditions [40].

To combat the ROS, plants may utilize specific mechanisms, including the activation of antioxidant enzymes like peroxidase (POX), catalase (CAT), and ascorbate peroxidase (APX) to minimize the oxidative damage to plants [28, 35, 41]. Additionally, the presence of additional non-enzymatic antioxidants like osmoprotectant compounds including mannitol, betaine, and proline contribute to scavenging free radicals in plant cells [8, 38]. Several studies suggest that reducing oxidative damage and enhancing drought tolerance are closely linked to a more effective antioxidant system [38, 42].

Water stress induces stomatal closure and restricts the entry of CO_2, which directly hampers the photosynthetic process. Furthermore, drought stress leads to structural rearrangements in the thylakoid membrane [43] which adversely affects both photosystem I (PSI) and photosystem II (PSII) at different growth stages of the plant. Notably, the operational quantum efficiency of PSII (ΦPSII) is highly sensitive to drought stress, making this trait a valuable early marker for detecting the water stress effect [44]. Chlorophyll fluorescence is one of the key traits currently considered for evaluating plant tolerance to abiotic stresses such as drought [45, 46]. Moreover, the ratio of F_v/F_m shows the maximum quantum efficiency of photosystem II and serves as an indicator of the overall performance of the optical system and generally, this ratio declines when the plant experiences environmental stress [45, 47].

In breeding programs, drought tolerance can be assessed through the traits that are linked to grain yield potential to find tolerant genotypes. Earlier research has explored drought tolerance in barley and investigated the ambiguity of physiological trait intolerance [48–53]. The present study aims to expand this understanding by examining the role of non-enzymatic and enzymatic antioxidants in mitigating oxidative stress using a diverse set of barley genotypes. Therefore, this research aimed to evaluate drought tolerance of different barley genotypes by analyzing physiological traits, oxidative stress responses, polyphenols, antioxidant activity, and grain yield. The results of this research can incorporate valuable findings into future breeding programs for drought tolerance in barley.

Materials and methods

Plant materials and experimental conditions

In this research, 21 cultivars and advanced breeding lines of barley (*Hordeum vulgare* ssp. *vulgare* L.), (hereafter called genotypes) were used. These genotypes were obtained from the Research and Education Center for Agriculture and Natural Resources of Isfahan Province, Iran. The names and pedigrees of these genotypes are listed in Table 1. The experiments were undertaken in the research farm of the Isfahan University of Technology, Isfahan, Iran. The location of the experiments was at a latitude of 32° 42′ North and a longitude of 51° 28′ East, with an altitude of 1624 m above sea level and an annual average precipitation of 122.6 mm. A randomized complete block design (RCBD) with two replications was implemented for each of the moisture regimes (normal and drought stress). All studied traits were measured in experiments that was conducted in 2021–2022, but the data for grain yield was collected from the conducted experiments over two years of 2019–2020 and 2021–2022 and their average were used for reporting and statistical analysis. Each experimental plot (replication) contained four rows (each row contains 22 plants), each 110 cm in length and 20 cm apart. The plant spacing within the rows was 5 cm. The seeds were manually planted at a depth of 5 cm.

Irrigation regimes

During the planting and establishment of plant growth phases, all plots were irrigated based on the crop water needs, ensuring that the soil water depletion did not exceed 50% to prevent plant mortalities. At Zadoks growth stage 13, two irrigation treatments were implemented based on the maximum allowable depletion (MAD) of available soil water (ASW), ranging between 0.03 and 1.5 MPa [54]. The control and water-deficit (drought stress) conditions were set at 55% and 85% depletion levels of the available soil water, respectively. By utilizing daily weather data from the Najaf-Abad Synoptic Station, and applying the FAO Penman–Monteith equation along with barley crop coefficients for various growth stages [55], soil moisture depletion was assessed by monitoring barley evapotranspiration over the growing season. Irrigation of the plots was conducted according to the MAD (maximum allowable depletion) threshold values, specifically for each irrigation treatment that was derived from the equation proposed by [56].

$$\theta_{irrig} = \theta_{fc} - (\theta_{fc} - \theta_{pwp}) \times MAD$$

Here, θ_{irrig} (m^3 m^{-3}) represents the soil water content threshold at the irrigation time for the designated MAD. Volume basis soil water content at field capacity is

Table 1 Some specifications and codes of the evaluated barley genotypes

Genotype name	Code number	Country of origin	Pedigree	Number of spike rows
Yusof	1	Iran	Lignee 527/Chn-01//Gustoe/4/Rhn-08/3/Deir Alla 106//Dl71/Strain	6
Goharan	2	Iran	Rhn-03//L.527/NK1272	6
34-p9697	3	Iran	Eneldo"S"/Yousef	6
14-p9698	4	Iran	KAROON/KAVIR//Rhodes'S'//Tb/Chzo/3/Gloria'S'/4/Karoon	6
Mehr	5	Iran	Roho/Mazurka//Rojo	6
15-Es9597	6	Iran	Trompilo/L.Moghan//Yousef	6
9-Es9598	7	Iran	Gorgan//Aths/BC/3/1-BC-80,631	6
Armaghan	8	Iran	Legia//Rhn/Lignee 527	6
khatam	9	Iran	LB.Iran/Una 8271//Gloria's'/Com's'/3/Kavir	6
Mahtab	10	Iran	Unumli Arpa/Azhar/Azhar/3/Beacher/Unumli Arpa/4/Tsiklon	6
Es-96-6	11	Iran	Kitchin/SLB60-35/3/Lignee 527/NK1272//JLB 70 – 63	6
Es-96-8	12	Iran	Warm	6
Es-96-13	13	Iran	KAROON/KAVIR//Rhodes'S'//Tb/Chzo/3/Gloria'S' /4/Lignee 527/NK1272//JLB 70 – 63/5/Fjr30	6
Es97-9	14	Iran	KAROON/KAVIR//Rhodes'S'//Tb/Chzo/3/Gloria'S'/4/Karoon	6
Es97-10	15	Iran	Bgs/Dajia//L 1242/3/(L.B.IRAN/Una8271//Gloria'S'/3/Alm/Una80//….)/4/ Nimrooz	2
P-97-3	16	Iran	Bereke-54's/3/Rhn-03//L.527/NK1272	6
p-97-19	17	Iran	82 S:510/3/Arinar/Aths//DS 29/4/Sahra	6
26-p98	18	Iran	Legia//Rhn/Lignee 527/3/Rhn03	6
29-p98	19	Iran	Rojo/3/LB.IRAN/Una8271//Gloria"S"/Com"S"/4/ Rojo/3/LB.IRAN/Una8271// Gloria"S"	6
43-p98	20	Iran	Anoidium/Arbayan-01/3/Lignee527/NK1272//JLB70-63/4/Bgs/Dujia//L.1242	6
44-p98	21	Iran	Anoidium/Arbayan-01/3/Lignee527/NK1272//JLB70-63/4/Ashar/Beecher	6

represented by θ_{fc} (m^3 m^{-3}), and at wilting point by θ_{pwp} (m^3 m^{-3}). MAD represents the proportion of the total available soil that can be utilized by plants from the root zone. The following equation was used to calculate irrigation depth based on the soil water content [56]:

$$D_{irrig} = (\theta_{fc} - \theta_{avg}) \times Z_e$$

Here, D_{irrig} (cm) represents the irrigation depth, θ_{avg} denotes the soil water content in the root zone before irrigation (measured in m^3 m^{-3}), and Ze stands for the root depth (cm). A drip irrigation system was used in which, each dripper released approximately 1.3 L of water per hour, as measured by an irrigation meter. The tapes were spaced 30 cm apart which can efficiently provide water to both planted rows in each plot.

To ensure for accurate implementation and monitoring of drought stress, the moisture levels in the soil were monitored daily using a portable moisture meter TDR (SM01, AKA, Azar-Khak-Ab Urmia Co.). Soil sampling was performed from a depth of 0 to 30 cm at the beginning of the growing season and from a depth of 0 to 60 cm at the end of the season. Field capacity (θ_{fc}) and permanent wilting point (θ_{PWP}) were determined to be 32.68% and 16.20%, respectively. Non stressed plots (control plots) were irrigated when 55 ± 1% of the available water (θ_{Fc}– θ_{PWP}) was depleted, while plots under drought stress were irrigated when 85 ± 1% of the available water was depleted. During the entire growing season, control plots were irrigated 8 times with a total of approximately 5500 m^3ha^{-1} of water, while plots under drought stress were irrigated 4 times with a total volume of approximately 3000 m^3ha^{-1}. The meteorological data starting from the application of stress at Zadoks stage 13 is shown in Table S1.

Measurement of traits

DPPH radical scavenging activity

The total antioxidant capacity of fresh leaf samples was determined using DPPH stable radicals, based on the method described by [57] with some modifications. In the beginning, frozen leaf samples of 200 mg were homogenized with 3 mL of methanol. The resulting homogenate was then centrifuged at 3770 × g for 10 min. Next, 500 µL of the supernatant from the methanolic extracts was combined with 500 µL of methanol and 500 µL of a 0.004% DPPH solution in plastic vials. The vials were incubated in a dark environment at room temperature for 30 min. A UV-visible spectrophotometer was used to measure the absorbance of each sample at 517 nm, with a blank serving as the control. The measurement of DPPH radical scavenging capacity was done by utilizing the formula below:

DPPH radical scavenging capacity= [(the absorbance of the blank– The sample's absorbance)/ the absorbance of the blank] ×100.

Lipid peroxidation (MDA)

The thiobarbituric acid (TCA) test was utilized to quantify MDA as the product of lipid peroxidation in leaf homogenates [58]. To perform the test, homogenization was performed on 0.30 g of fresh leaf sample in 5 mL of 0.1% TCA. Following this, the homogenate underwent centrifugation at 12,000×g for 10 min at 4 °C. Subsequently, 4 mL of 20% TCA comprised of 0.5% thiobarbituric acid (TBA) was combined with 1 mL of the supernatant. The mixture was heated for 30 min at 95 °C in a water bath and promptly chilled on ice. Following another centrifugation at 10,000×g for 10 min, the extract's absorbance was measured at 532 nm, with corrections made for non-specific turbidity by deducting the absorbance at 600 nm.

Hydrogen peroxide (H_2O_2)

H_2O_2 concentration was assessed, using the method described by [59]. Fresh leaf tissue of 0.30 g was thoroughly homogenized in 5 mL of trichloroacetic acid (TCA). The resulting mixture was then centrifuged at 12,000×g for 10 min at 4 °C, and the supernatant was neutralized using 5 M K_2CO_3 to achieve a pH of 5.6, with the addition of 100 µL of 0.3 M phosphate buffer (pH 5.6) and then allowed the reaction mixture to proceed for 1 h in the dark. Afterward, absorbance was recorded at 390 nm. Finally, the concentration of H_2O_2 was determined using a standard curve made from known H_2O_2 concentrations.

Total phenolic content (TPC)

TPC was determined using the Folin-Ciocalteu method as outlined by [60] with some modifications. Initially, 625 mg of dried and ground leaf samples were drenched in 12.5 mL of 80% methanol (Merck) beneath shaking conditions (150 rpm) for 24 h. Subsequently, each leaf sample extract was filtered and transferred into new containers. Next, a mixture of 100 µL of a five-fold diluted methanolic extract, 500 µL of 10% Folin-Ciocalteu reagent, and 400 µL of 7.5% sodium carbonate was prepared in plastic tubes. The mixture was subsequently warmed using a water bath at a constant temperature of 45 °C for 15 min. In the last step, the UV-visible spectrophotometer was used to measure the absorbance of each sample, and the TPC of the extracts was determined using a standard curve generated with tannic acid.

Total flavonoid content (TFC)

TFC was determined using the aluminum chloride reagent, based on the method described by [61], with some modifications. First, 100 µL of the methanolic extract was combined with 900 µL of distilled water and 60 µL of a 5% sodium nitrite solution. After 5 min, 120 µL of a 10% aluminum chloride solution was added, and the mixture was allowed to stand at room temperature for another 5 min. Subsequently, 400 µL of a 4% sodium hydroxide solution was added to the mixture followed by the addition of 400 µL of distilled water and then was left at room temperature for 15 min. The absorbance readings for each sample were then measured at 510 nm using a standard curve of quercetin.

Antioxidant enzyme activity

To assess enzymatic activity, 0.1 g of leaf tissue was sampled and immediately frozen in liquid nitrogen. The frozen leaves were immersed in 1 mL solution containing 50 mM phosphate buffer (pH 7.8), 1% Polyvinylpolypyrrolidone (w/v), 2 mM Ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100, 50 mM Tris hydrochloride (Tris–HCl), and 2 mM Dithiothreitol (DTT). Afterward, the homogenate was centrifuged at 14,000 rpm for 30 min to separate the components. The supernatant was carefully collected for enzyme activity assays. The activity of CAT was monitored by observing the reduction in absorbance at 240 nm for 2 min during the breakdown of H_2O_2 [62]. In addition, APX activity was determined by the decline in absorbance at 290 nm for 2 min [63]. POX activity was evaluated by monitoring the rise in absorbance at 470 nm over 2 min [64]. The activities of these enzymes were expressed per unit of protein content.

Carotenoid and chlorophyll content

To determine the content of chlorophyll (Chl) and carotenoids (Car), fresh leaf segments of 200 mg were used. These segments were placed in 10 mL of 80% (v/v) acetone and then centrifuged using a 5810R Eppendorf Refrigerated Centrifuge from Germany. The absorbencies of the acetone extracts were measured at 663 nm for Chla, 647 nm for Chlb, and 470 nm for Car using a U-1800 UV/VIS Spectrophotometer from Hitachi, Japan. Finally, the results were shown as mg of Chl or Car per gram of leaf fresh mass [65].

Chlorophyll fluorescence

Chlorophyll fluorescence was assessed by utilizing a portable fluorometer (Opti-Sciences OS30p Inc., Chlorophyll fluorometer, Hudson, NH, USA) on leaves (youngest fully expanded leaf) that had undergone 20 min of dark adaptation. Measurements were taken between 8 and 10 am under natural sunlight, with a PPFD exceeding 800 µmol m^{-2} s^{-1} in all experimental plots. The maximum (F_m) and minimum (F_0) fluorescence values, along with the maximum quantum efficiency of photosystem II (F_v/F_m), were determined after exposure to saturating light at an intensity of 3000 µmol (photon) m^{-2} s^{-1} for 2 s.

$$F_v / F_m = (F_m - F_0) / F_m$$

Relative water content (RWC)

Initially, the fresh weight (FW) of the leaf samples was measured. Subsequently, the leaves were hydrated in distilled water for 24 h at room temperature. Turgid weight (TW) was measured quickly after removing surface water with paper towels. After drying the leaves at 75 °C for 48 h, the dry weight (DW) was measured [66]. The following equation was used to calculate RWC:

$$RWC\ (\%) = \begin{bmatrix} (Fresh\ weight - Dry\ weight)\ / \\ (Turgid\ weight - Dry\ weight) \end{bmatrix} \times 100$$

Electrolyte leakage (EL)

Electrolyte leakage (EL) was employed to evaluate membrane permeability. This was done by using an electrical conductivity (EC) meter at the grain-filling stage. The percentage of EL (%EL) was calculated using the equation of EL (%) = [1– (EC_1/EC_2)] × 100, where EC_1 and EC_2 represent the electrical conductivities of the bathing solution of the fresh leaf before and after it was incubated in boiling water, respectively [67].

Total protein content

Protein content in leaf samples during the grain-filling stage was measured by using the Bradford method, with bovine serum albumin as the standard, and the enzymatic activities were expressed as units per milligram of protein [68]. In this experiment, fresh plant materials were used. Two milliliters of 0.1 M phosphate buffer was added to the fresh plant samples, followed by centrifugation at 15,000 rpm for 12 min at 4 °C. The absorbance was then measured at 595 nm using a spectrophotometer. The total protein content was reported as milligrams per milliliter of leaf fresh weight.

Proline content

To extract free proline from fresh leaves, leaf samples of each 0.5 g were quickly frozen and ground in liquid nitrogen. The ground samples were then homogenized in 10 mL of 3% sulfosalicylic acid (w/v) and centrifuged at 8,500 rpm for 10 min. Following centrifugation, 2 mL of the supernatant was combined with 2 mL of ninhydrin reagent and 2 mL of acetic acid. The mixture was then incubated in a water bath at 100 °C for 1 h. After incubation, 4.0 mL of toluene was added, and the absorbance was measured at 520 nm. The proline concentration was determined by applying a standard curve, following the method described by [69].

Quantification of components of phenolic compounds by HPLC

For measuring phenolic acid content of leaf samples, a high-performance liquid chromatography (HPLC) system was used. The analysis was conducted with Agilent Technologies HP 1090 series HPLC (USA) equipped with a Waters Symmetry C18 column (Waters Corp., Milford, MA, USA) (4.6 × 250 mm, 5 µm particle size), with sentry guard column (3.9 × 10 mm, 5 µm particle size) which was used at flow rate of 0.8 mL/min and a UV absorbance detector. The temperature of the column oven was adjusted to 25 °C. Initially, the methanolic extracts were filtered through a 0.22 µm CA sterile syringe filter and then placed into HPLC glass vials for analysis. Following this, the extract was injected into the analytical column in a 20 µL volume. The mobile phase consisted of eluent A (0.1% formic acid in water, v/v) and eluent B (0.1% formic acid in acetonitrile, v/v). The flow rate was maintained at 0.8 mL per minute, following this linear gradient program: transitioning from 10 to 26% solvent B over 20 min, holding at 65% solvent B for 40 min, and finally reaching 100% solvent B in 45 min. The DAD was calibrated to wavelengths of 350, 310, 270, and 520 nm for the live observation of peak intensity, while the complete spectra (190–650 nm) were recorded continuously for identifying plant components. Detection was carried out in the range of 200 to 400 nm using an absorbance detector. The identification and quantification of individual flavonoids, flavanols, and phenolic acids in the leaf samples were achieved by comparing their relative retention times with those of authentic standards [70]. The limit of detection (LOD) for the HPLC method was calculated using the standard deviation of the response and the slope of the calibration curve, which resulted in a value of 0.033 mg/L. Calibration curves were created for all polyphenolic standards, including syringic acid, gallic acid, chlorogenic acid, caffeic acid, ellagic acid, ferulic acid, vanillic acid, p-coumaric acid, luteolin, apigenin, and rutin, at concentrations with ranging from 20 to 200 mg/L (Fig. S1). The concentrations are reported in milligrams of phenolic acid per 100 g of dry weight (mg 100 g^{-1} DW).

Grain yield

At the physiological maturity stage of plants, the grains of the two middle rows of each plot were harvested, weighed and then grain yield was expressed as g m^{-2}.

Statistical analysis

The data was subjected to analysis of variance (ANOVA) through the PROC GLM procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC, United States). The Kolmogorov-Smirnov test ($p < 05$) was used to assess the normality of the data distribution. Bartlett's test was

utilized to examine the homogeneity of error variances. The Fisher's protected least significant difference (LSD) test was used for mean comparisons. A forward multivariate regression analysis for grain yield was conducted with PROC REG in SAS, where the maximal quantum efficiency of photosystem II (F_v/F_m), chlorophyll a content (Chla), and POX activities served as independent variables. PCA was used for principal component analysis, and then Stat Graphics (ver. 16.1.11) software was utilized to generate graphical representations. JMP software (version 13) was utilized to group genotypes and phenolic compounds through heat maps, following the Ward method [71].

Results

The results of the ANOVA (Tables S2 & S3) showed that the difference between the two moisture regimes was significant for the studied traits, except for the Chla/b ratio, carotenoid content, and EL. The genotypes showed significant differences for these traits, except for the ratio of Chla/b. A significant interaction between genotype and moisture regimes was observed for all traits except for the Chla/b ratio, F_0, F_m, RWC, and EL. The mean comparison revealed that most of the traits experienced a significant decrease due to drought stress. However, the traits of DPPH, MDA, H_2O_2, TPC, TFC, POX, and Pro exhibited a significant increase. No significant effect of drought was observed on Chla/b ratio, Car content, and EL traits (Tables 2 and 3). The most affected traits by drought stress were MDA and Pro, while Car content was the least affected one.

DPPH radical scavenging activity

Drought stress, genotype, and their interaction significantly affected the DPPH free radical scavenging activity (Table S2). The drought stress led to a significant enhancement in DPPH radical scavenging activity in most of the genotypes (except in genotypes 4, 14, 15, 16, 17, 19, and 20). Under stress conditions, genotypes 2 and 3 had the highest value of DPPH radical scavenging activity, while genotype 16 had the lowest one. Due to drought stress, genotypes 3 and 6 showed the greatest change (increase), while genotypes 10 and 18 had the least increase in DPPH radical scavenging activity (Table S4).

Lipid peroxidation (MDA)

The effect of drought stress, genotype, and their interaction were significant on MDA (Table S2). The leaf MDA content of genotypes in drought stress conditions was considerably higher than the control condition (about 216%) and it ranged from 2.02 nM g^{-1} FW under control conditions to 6.40 nM g^{-1} FW under drought stress (Table 2). Under stress conditions, genotypes 1 and 7 showed higher MDA levels, while genotypes 15, 18, and 20 exhibited lower ones. The greater increase in MDA content due to drought stress was observed in genotypes 6 and 7, while the lowest increase was found for genotype 9 (Table S4).

Hydrogen peroxide (H_2O_2)

Drought stress significantly increased leaf H_2O_2 content (about 29%) and it was 0.99 mM g^{-1} FW in control plants to 1.28 mM g^{-1} FW in stressed plants (Table 2). The H_2O_2 content was significantly different in genotypes and they showed different responses to drought stress for this trait (Table S2). Most genotypes exhibited an increase in H_2O_2 levels under drought stress conditions, with the higher H_2O_2 content in genotypes 3, 4, and 9, and the lower ones in genotypes 2, 15, 18, and 20. Genotypes 4 and 9 showed the most substantial increases in H_2O_2 due to drought stress, while genotypes 2, 15, 17, and 18 had no significant changes (Table S4).

Total phenolic and flavonoid content

The results showed that drought stress, genotype, and interaction significantly affected TFC and TPC (Table S2). Drought stress increased TPC (about 6%) and TFC (about 17%) in leaf tissue (Table 2). In stress conditions, genotypes 2 and 4 had a high value of TPC, while genotypes 3 and 12 showed elevated TFC. Genotype 16 had low TPC, and genotypes 5 and 6 had lower TFC. Genotype 15 exhibited the greatest increase in both TPC and TFC because of drought stress conditions (Table S4).

Table 2 Mean comparisons of DPPH radical scavenging assay, MDA, H_2O_2, non-enzymatic and enzymatic antioxidants averaged over 21 genotypes of barley for normal and drought stress conditions

Environmental condition	DPPH	MDA	H_2O_2	TPC	TFC	CAT	APX	POX
Control	48.82^b	2.02^b	0.99^b	60.76^b	17.76^b	0.87^a	1.11^a	3.11^b
Stress	58.89^a	6.40^a	1.28^a	64.49^a	20.92^a	0.43^b	0.87^b	4.04^a
LSD	0.52	0.44	0.05	2.22	0.29	0.12	0.14	0.39
Change (%)	20.63	216.83	29.29	6.14	17.79	-50.57	-21.62	29.90

DPPH: 2, 2-Dipheny- 1-picryl-hydrazyl- hydrate (%). MDA: Leaf malondialdehyde content (nMol g^{-1}FW). H_2O_2: Hydrogen peroxide (mMol g^{-1}FW)

TPC: Total phenolic content (mg g^{-1} DW). TFC: Total flavonoid content (mg g^{-1} DW). CAT: Catalase activities (unit mg^{-1} protein)

APX: Ascorbate peroxidase activities (unit mg^{-1} protein). POX: Peroxidase activities (unit mg^{-1} protein)

For each trait, means followed by the same letter are not significantly different according to LSD test (probability level of 5%)

Table 3 Mean comparisons of physiological properties and grain yield averaged over 21 genotypes of barley for normal and drought stress conditions

Environmental condition	Chla	Chlb	Tchl	Chla/b	Car	F_v/F_m	F_0	F_m	RWC	EL	PC	Pro	GY
Control	1.61[a]	0.64[a]	2.25[a]	2.56[a]	0.63[a]	0.71[a]	65.55[a]	225.57[a]	59.93[a]	30.21[a]	2.12[a]	0.81[b]	749.48[a]
Stress	1.36[b]	0.51[b]	1.87[b]	2.73[a]	0.61[a]	0.57[b]	51.67[b]	122.02[b]	45.39[b]	45.87[a]	1.64[b]	2.64[a]	396.93[b]
LSD	0.05	0.11	0.09	0.34	0.32	0.002	4.32	14.81	14.01	22.99	0.19	0.22	65.20
Change (%)	-15.53	-20.31	-16.89	6.64	-3.17	-19.72	-21.17	-46.90	-24.26	34.14	-22.64	225.92	-47.04

Chla: Chlorophyll a content (mg g^{-1}). Chlb: Chlorophyll b content (mg g^{-1}). Tchl: Total chlorophyll content (mg g^{-1}). Chla/b: Ratio of chla to chlb

Car: Carotenoid content (mg g^{-1}). F_v/F_m: Maximal quantum efficiency of photosystem II. F_0: Minimum Chl fluorescence. F_m: Maximum Chl fluorescence

RWC: Relative water content (%). EL: Electrolyte leakage (%). PC: Total protein content (mg mL^{-1}). Pro: Proline content (umol g^{-1} FW).GY: Grain yield (g m^{-2})

For each trait, means followed by the same letter are not significantly different according to LSD test (probability level of 5%)

Antioxidant enzyme activity

Drought stress, genotype, and their interaction significantly affected antioxidant enzyme activity (Table S2). Drought stress promoted significantly the POX activity (about 29%), while the activities of two other antioxidant enzymes (APX (about 21%) and CAT (about 50%)) were weakened in stress conditions (Table 2). The CAT enzyme in all genotypes showed a decrease in stress conditions, except in genotypes 7 and 8. The average content of CAT enzyme was 0.87 (unit mg^{-1} protein) in normal conditions and 0.43 (unit mg^{-1} protein) in stress conditions. The level of APX enzyme decreased in stress conditions for all genotypes, except in genotypes 1, 3, 7, 14, and 16. The average content of APX enzyme was 1.11 (unit mg^{-1} protein) under normal conditions and 0.87 (unit mg-1 protein) under stress conditions. POX enzyme levels increased under stress conditions in all genotypes, except in genotypes 4, 5, 7, 8, 11, and 21. The average POX enzyme content was 3.11 (unit mg^{-1} protein) under normal conditions and 4.04 (unit mg^{-1} protein) under stress conditions (Table S4).

Carotenoid and chlorophyll content

The genotype and its interaction with irrigation regimes significantly influenced carotenoid content. The interaction revealed that drought stress exerts varying effects on carotenoid content across the genotypes (Table S3). Under stress conditions, genotypes 16 and 20 had higher carotenoid content, while genotype 3 had the lowest value with the greatest reduction in carotenoid content due to stress (Table S5). The effects of irrigation regime, genotype, and their interaction were significant for Chla, Chlb, and Tchl, but not for the ratio of Chla/b (Table S3). Under drought stress, the content of Chla, Chlb, and Tchl significantly decreased, while Chla/b ratio remained unaffected (Table 3). Genotype 3 had the lowest levels of Chla, Chlb, and Tchl, while genotypes 21, 20, and 20 showed higher ones. Genotype 3 experienced the greatest reduction in Tchl, while genotype 15 had the lowest reduction due to drought stress (Table S5).

Chlorophyll fluorescence

Drought stress significantly reduced the F_v/F_m ratio (about 19%) (Table 3). Also, significant effects of genotype and their interaction were detected for this trait (Table S3). All genotypes exhibited lower F_v/F_m ratios under drought stress in comparison to normal conditions, indicating the damage to PSII and photoinhibition due to this stress. Genotype 19 showed the highest F_v/F_m ratio under drought stress, while genotypes 9 and 16 had significantly lower values. Among the genotypes, genotype 9 had the greatest reduction of this trait, whereas, genotype 4 showed the lowest value of reduction (Table S5).

Relative water content (RWC)

RWC was significantly decreased by 24.26% under drought stress when compared to normal conditions (Table 3). Genotypes showed significant differences for RWC (Table S3). Genotype 15 experienced the highest RWC reduction (28.85%), whereas genotype 14 had the lowest (0.77%) reduction of RWC because of drought stress. Under drought stress, genotype 14 had the highest RWC, while genotype 16 exhibited the least of this trait (Table S5).

Electrolyte leakage (EL)

The results showed that the irrigation regime had no significant effects on electrolyte leakage (EL) in genotypes. However, a significant difference was observed among genotypes for this trait (Table S3). Most genotypes, except genotype 3, exhibited higher EL under drought stress, but those changes were not statistically significant. Genotypes 5 and 10 had the highest EL value, while genotype 12 had the lowest. Under drought stress, genotype 20 showed the greatest increase in EL, whereas genotypes 5, 14, and 17 exhibited the least one (Table S5).

Protein content

The analysis of variance revealed a significant effect of irrigation regime, genotype, and their interaction on protein content in the leaf (Table S3). Under stress, most genotypes showed a decrease in protein content, while genotypes 4, 6, 7, 8, and 12 exhibited some increase. Genotype 21 had the lowest protein content in the leaf under drought stress, whereas genotypes 6 and 7 had a higher mean of this trait (Table S5). The lower reduction of protein content due to drought stress occurred in genotypes 10 and 14, while genotype 18 showed the largest decrease for this trait (Table S5).

Proline content

Drought stress significantly increased proline levels in genotypes (about 225%) (Table 3). Genotypes 4, 10, 16, and 17 exhibited the higher proline content under drought conditions, while genotype 12 had the lowest. Because of drought stress, genotype 15 showed the greatest increase in proline, whereas genotype 18 exhibited the lowest (Table S5).

Phenolic compounds

Phenolic acids (non-flavonoid polyphenols) and flavonoid compounds were influenced by drought stress, genotype, and their interaction (Table 5). This study identified eight key phenolic acids in leaves including chlorogenic acid, ferulic acid, ellagic acid, syringic acid, vanillic acid, *p*-coumaric acid, caffeic acid, and gallic acid, alongside three main flavonoids: Luteolin, apigenin (flavones), and rutin (flavanol).

Drought stress elicited both inhibitory and stimulatory effects on phenolic acid production, and generally drought stress caused an increase in most phenolic compounds, except for chlorogenic acid, caffeic acid, and apigenin. Notably, the concentrations of syringic acid, gallic acid, ellagic acid, and vanillic acid doubled due to drought stress, but chlorogenic acid remained unaffected by drought (Table 4). Under stress conditions, ellagic acid, rutin, and vanillic acid were the most abundant polyphenols, while chlorogenic acid and luteolin were the least prevalent. The genotypes demonstrated varying phenolic compound accumulation and the highest value of these compounds was observed for gallic acid in genotype 14, for ellagic acid in genotype 2, for luteolin in genotype 16, for vanillic acid in genotype 19, and for apigenin in genotype 1. Additionally, genotype 9 showed the highest value of syringic acid and caffeic acid levels, genotype 8 excelled in chlorogenic acid and p-coumaric acid, and genotype 5 exhibited the highest value of rutin and ferulic acid concentrations (Table S7).

Grain yield

Drought stress, genotype, and their interaction significantly affected grain yield (Table S3), leading to an overall yield loss of 47% due to stress (Table 3). All studied genotypes showed a significant loss in grain yield due to drought stress. Genotypes, 1, 17, and 18 had higher grain yields under drought stress, while genotypes 3, 10, and 14 showed the lower value of this trait. Genotypes 3 and 16 exhibited the highest and lowest reduction in grain yield due to drought stress, respectively (Table S5).

Table 4 Mean comparisons of phenolic acids and flavonoid contents of the leaves averaged over 21 genotypes of barley for normal and drought stress conditions

Content (mg 100 g $^{-1}$ DW)	Syringic acid	Gallic acid	Chlorogenic acid	Caffeic acid	Ellagic acid	Ferulic acid	Vanillic acid	*p*-Coumaric acid	Luteolin	Apigenin	Rutin
Control	2.43^{b}	2.52^{b}	3.61^{a}	13.47^{a}	235.65^{b}	15.17^{b}	94.96^{b}	19.85^{b}	0^{b}	7.68^{a}	157.49^{b}
Stress	4.19^{a}	4.20^{a}	3.55^{a}	9.29^{b}	563.06^{a}	17.68^{a}	160.09^{a}	24.13^{a}	0.16^{a}	6.33^{b}	175.52^{a}
LSD	0.39	0.78	1.08	0.94	30.85	1.07	0.34	1.61	0.03	0.33	2.30
Change (%)	72.43	66.67	-1.66	-31.03	138.94	16.54	68.59	21.56	100	-17.58	11.45

For each trait, means followed by the same letter are not significantly different according to LSD test (probability level of 5%)

Table 5 Results of multiple regression analysis for grain yield as a dependent variable in 21 genotypes of barley under drought stress conditions

Independent variable	Regression coefficient	Standard error	Partial R-Square	Model R-Square	C(p)	*P value*	Model adjusted R^2
F_v/F_m	142.43	158.87	0.920	0.920	16.02	0.0001	0.918
Chla	163.02	56.97	0.008	0.929	12.24	0.038	0.925
POX	23.00	8.05	0.012	0.941	5.56	0.007	0.936

F_v/F_m: Maximal quantum efficiency of photosystem II. Chla: Chlorophyll a content. POX: Peroxidase activities

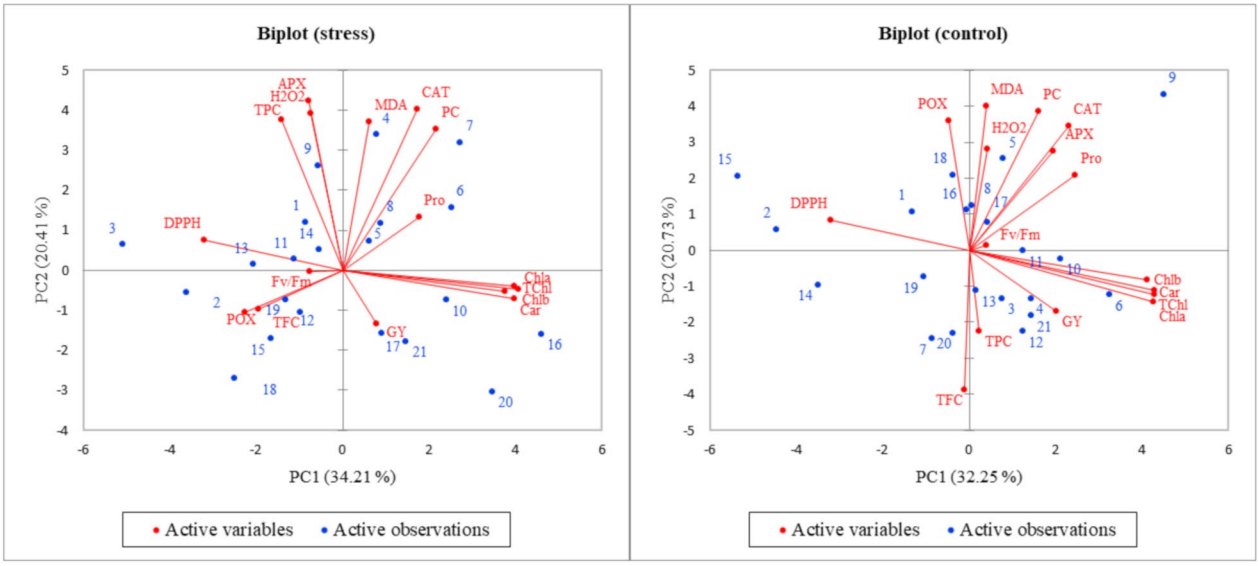

Fig. 1 Biplot display of plant DPPH: 2, 2-Dipheny-1-picryl-hydrazyl- hydrate. MDA: Leaf malondialdehyde content. H_2O_2: Hydrogen peroxide. TPC: Total phenolic content. TFC: Total flavonoid content. CAT: Catalase activities. APX: Ascorbate peroxidase activities. POX: Peroxidase activities. Chla: Chlorophyll a content. Chlb: Chlorophyll b content. Tchl: Total chlorophyll content. Car: Carotenoid content. F_v/F_m: Maximal quantum efficiency of photosystem II. PC: Total protein content. Pro: Proline content. GY: Grain yield, in 21 genotypes of barley for normal and drought stress conditions

Relationships of traits

A multivariate regression was employed to identify the most influential traits affecting grain yield under drought stress. Forward stepwise regression analysis revealed that F_v/F_m, Chla, and POX were the most effective traits on grain yield (Table 5).

Principal Component Analysis (PCA) was utilized to explore the relationships among traits and to identify favorable genotypes based on multiple characteristics. Under drought stress, the first principal component (PC_1) explained about 34% of the variation, while the second principal component (PC_2) accounted for 20%. In contrast, PC_1 and PC_2 under control conditions explained 32% and 20% of the variation, respectively, highlighting the significant role of these components in differentiating environmental responses (Fig. 1). Variable vectors indicated the direction and magnitude of their influence on the principal components. Under drought conditions, there was a positive correlation between PC_1 with Chla, Chlb, Tchl, and Car, but its correlation with F_v/F_m and DPPH was negative. The PC_2 showed a stronger correlation with APX, TPC, MDA, and H_2O_2. Conversely, under control conditions, PC_1 was positively associated with F_v/F_m, Chlb, Car, Tchl, and Chla, while the PC_2 was positively correlated to POX, MDA, and H_2O_2, but negatively with TPC and TFC (Fig. 1).

Under drought stress conditions, apigenin and rutin displayed long vectors along the first axis, highlighting their strong correlation with it, while chlorogenic acid, ellagic acid, and ferulic acid showed vectors aligned closely with the second axis (Fig. 2). Under control conditions, Caffeic acid had the greatest influence on the first component, while chlorogenic acid and ferulic acid played a more prominent role in the second component. Additionally, apigenin and rutin were positioned in the positive region of PC_1 and the negative region of PC_2, reflecting their distinct roles in genotypes 4 and 13 (Fig. 2).

Heatmapping analysis

Under normal conditions, the heat map (Fig. S2) categorized the 21 barley genotypes into three separate groups, based on their phenolic and flavonoid compound levels. The heat map revealed distinct variations in the accumulation of these compounds across the genotypes. It also illustrated that the quantified phenolic compounds could

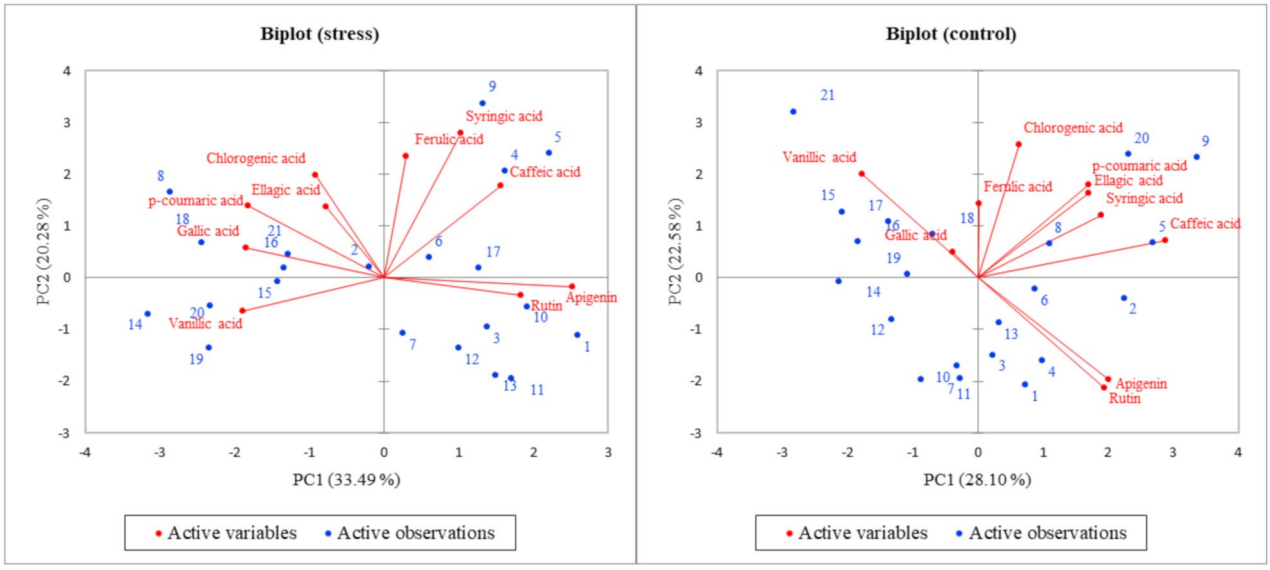

Fig. 2 Biplot display of phenolic acids and flavonoid contents in 21 genotypes of barley for normal and drought stress conditions

be separated into two main groups. The initial group of genotypes, which includes genotypes 1, 3, 7, 4, 10, and 11, exhibited significantly lower levels of most phenolic compounds (represented in blue), indicating reduced metabolic activity under non-stress conditions. The second groups of genotypes displayed moderate accumulation patterns and consists of genotypes 12, 13, 14, 19, 15, 16, 18, 17, and 21. The third group, which comprises genotypes 2, 6, 8, 20, 5, and 9, demonstrated higher quantities of several phenolic compounds, highlighted by a dark red color, signifying elevated levels of secondary metabolic activity. This clustering pattern emphasizes intrinsic genotypic differences in phenolic metabolism, even when there is no drought stress present.

Under drought stress conditions, a heatmap and hierarchical clustering analysis of phenolic and flavonoid compounds revealed significant differences among barley genotypes (Fig. S3). The genotypes were categorized into three main clusters based on their compound levels. The first group (genotypes 1, 3, 6, 7, 10, 11, 12, 13, and 17) exhibited higher concentrations of several phenolic acids and flavonoids, indicated by intense red areas in the heatmap. These genotypes appear to maintain or even increase the production of secondary metabolites in response to drought stress. In contrast, the third group (genotypes 2, 8, 14, 15, 16, 18, 19, 20, and 21) consistently showed low levels of most of the compounds, represented by blue areas, indicating a limited metabolic response to the stress. The second group (genotypes 4, 5, and 9) displayed moderate changes in various compounds. Overall, the clustering pattern suggests that drought stress has led to more differentiation among the genotypes regarding phenolic and flavonoid accumulation.

Discussion

Plants encounter various abiotic stresses during their life cycle, with drought stress standing out as one of the most critical abiotic challenges. This stress significantly reduces agricultural productivity, posing a serious threat to global food security [7, 72]. It has been shown that the response to water deficiency significantly triggers numerous physiological and biochemical changes in plants [2, 3]. The development of drought-tolerant cultivars is one of the most cost-effective and sustainable solutions for enhancing crop productivity under water deficiency conditions [8] and physiological and biochemical traits may be used as indicators of drought tolerance in breeding programs for genotype evaluation and screening.

DPPH radical scavenging activity

Different techniques exist for evaluating total antioxidant capacity. One such technique involves using the stable free radical 2-2-D-phenyl-1-picrylhydrazyl (DPPH) [73]. The findings of this study were in agreement with some other studies that have demonstrated environmental stresses such as drought can significantly increase antioxidant activities in plants to protect them against free radical-induced damage [41].

Under drought stress, our genotypes exhibited different responses in DPPH radical scavenging activity, highlighting the role of genetic variation in stress tolerance. Genotypes that show higher levels of DPPH radical scavenging activity under drought stress may have a higher capacity to cope with oxidative stress. In this study, genotypes 2 and 3 had a higher increase of DPPH than the others due to drought-stress conditions. However, it was observed that water deficit significantly increased DPPH in all

evaluated barley genotypes [74]. Other studies have also confirmed the increase of DPPH levels in plants under stress conditions [25].

Lipid peroxidation (MDA)

MDA and H_2O_2 levels are critical indicators of oxidative stress induced by drought [8, 35]. Some studies revealed a direct correlation between antioxidant activity, H_2O_2 production, and MDA concentrations with drought severity [75]. In cereals, enhanced stress tolerance is associated with reduced H_2O_2 and MDA levels [76]. The assessment of stress tolerance in genotypes can be made by measuring the MDA level. Elevated MDA levels under stress serve as a key indicator of membrane damage, with reduced membrane stability reflecting increased lipid peroxidation from ROS [77]. Our results revealed that some genotypes, showed greater tolerance to oxidative stress, since they produced lower MDA under drought stress. These genotypes with low MDA levels may possess superior mechanisms to cope with oxidative damage. In agreement with our findings, different genotype responses for MDA production under drought stress have been previously reported in barley [13, 75]. Overall, significant increases in MDA under stress conditions have been found in barley in different studies [12, 14, 27, 78].

Hydrogen peroxide (H_2O_2)

ROS, particularly H_2O_2, play a dual role in plants under drought stress. H_2O_2, elevated during water scarcity, especially in chloroplasts, peroxisomes, and mitochondria, activates an antioxidant defense system to regulate cellular redox balance. At low concentrations, ROS function as signaling molecules, but prolonged drought stress leads to excessive ROS accumulation, overwhelming antioxidant defenses, and causing damage [11, 14, 29, 30]. The findings of this study highlight the increased effect of drought stress on H_2O_2 accumulation in different barley genotypes, suggesting that oxidative stress happened as a response mechanism. However, a significant increase in H_2O_2 levels under drought stress emphasizes the role of drought in generating ROS and causing disruption in the cellular system. In this study, significant interaction of genotype by irrigation regimes implied the variability in oxidative stress responses among different genotypes. Genotypes that had higher levels of H_2O_2 under drought conditions may have a greater susceptibility to oxidative damage or may lack effective ROS-scavenging mechanisms. In contrast, genotypes that showed lower H_2O_2 accumulation due to drought stress may have a higher potential for tolerance through mechanisms such as increased antioxidant enzyme activity or structural flexibility.

Our findings were in agreement with those of other researchers reporting a significant increase in H_2O_2 levels in various barley genotypes under drought [13, 75, 79] and salinity stress [80].

Total phenolic and flavonoid content

Non-enzymatic antioxidants, particularly phenolic compounds, are vital for neutralizing ROS and maintaining redox balance, thereby improving stress tolerance [10, 81]. Drought stress can have a considerable impact on the phenylpropanoid pathway, especially by modifying the functions of essential enzymes like phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) and it was indicated that PAL activity increases in response to drought, resulting in a higher production of phenolic compounds which serve as antioxidants, safeguarding the plant from oxidative harm caused by reactive oxygen species (ROS) [82]. Likewise, CHS, which is involved in flavonoid biosynthesis, is elevated in response to drought to synthesize protective flavonoids. Variations in enzyme activity across different genotypes help clarify the specific patterns of phenolic accumulation noted under drought stress and emphasize the importance of these compounds in enhancing drought tolerance [82]. The variation in phenolic and flavonoid content among our genotypes under drought stress underscores the role of genetic factors in secondary metabolite production. Genotypes with high phenolic and flavonoid levels likely exhibit enhanced synthesis or accumulation mechanisms, potentially activating specific biosynthetic pathways that mitigate ROS and stabilize cellular structures, thereby improving oxidative stress resilience. Notably, genotype 15 displayed a significant increase in these compounds, indicating strong antioxidant activity and drought tolerance. Conversely, some genotypes showed minimal changes or slight decreases in these compounds which agreed with the results of other research on wild barley [83]. Similarly, another study found that salinity stress significantly raised TPC and TFC in *Aegilops* spp [25].

Antioxidant enzyme activity

Plants activate antioxidant enzymes like POX, CAT, and APX to further scavenge ROS levels and prevent oxidative damage [10, 28, 41]. The results of this study indicated that barley genotypes displayed varied responses to drought stress in terms of antioxidant enzyme activity, illustrating different thresholds and strategies for maintaining redox balance. These findings highlight that drought stress can have a complex and different effects on plants' antioxidant systems, which depends on genotypes, intensity of the stress and their interaction. Most of our genotypes showed increased POX activity under drought stress, while CAT and ascorbate APX activities

significantly decreased compared to the optimal irrigation conditions.

Outstandingly, genotypes 7 and 8 maintained relatively higher CAT activity under drought stress, unlike most genotypes that exhibited a decline. This suggests that these genotypes possess intrinsic mechanisms enabling them to better manage oxidative stress caused by drought. These genotype-specific variations highlight the critical role of genetic variation in drought tolerance, as some genotypes are better equipped to handle oxidative stress.

The decrease in CAT activity is believed to result from suppressed enzyme production or alterations in the enzyme subunits under stress conditions or likely enzyme degradation caused by active peroxisomal proteases or light-induced inactivation [84]. The reduction in CAT activity may suggest that it may be inactivated by the buildup of hydrogen peroxide caused by water stress which can be partially attributed to the enzyme's photoinactivation [85, 86].

CAT activity in genotypes exhibits diverse responses to abiotic stresses. In barley, drought stress led to elevated CAT activity only in some cultivars [13]. Conversely, a study reported that CAT activity was not significantly affected by barley's developmental stage or drought stress [87]. A study found a reduction in CAT activity in barley shoots under drought and temperature stresses [88]. It was reported that a decrease in CAT activity under drought stress led to the accumulation of H_2O_2 and increased lipid peroxidation, resulting in oxidative damage [89].

Certain genotypes of this study exhibited stable APX activity under drought stress, reflecting efficient antioxidant defenses and improved adaptation to oxidative stress. This may result from better regulation of antioxidant enzymes or enhanced ROS scavenging. In contrast, genotypes with lower APX activity struggle with ROS detoxification, leading to oxidative damage and impaired growth. These differing APX responses highlight the significance of genotype-specific adaptations for drought stress tolerance.

Studies have shown the diverse effects of drought on APX activity in plants. For wheat, mild drought increased APX activity, but prolonged drought reduced it due to higher MDA production [90]. In barley, drought stress affected APX activity variably among cultivars: most showed increased activity, one showed a decrease, and the rest remained unaffected [13]. Adaptive mechanisms may compensate for the imbalance in antioxidant enzymes, such as increased APX and GPX levels when CAT activity is reduced [91].

Plants under stress, particularly drought, prioritize essential processes like maintaining turgor pressure and repairing membranes over producing antioxidant enzymes. This shift indicates that drought alters antioxidant enzyme activity, leading to a reduction in key enzymes such as APX and CAT, which are crucial for reacting with ROS. The increase in POX activity in most genotypes of our study under drought stress, indicates its essential role in detoxifying ROS and protecting cellular integrity. The rise in POX is particularly important for breaking down H_2O_2 when CAT is inactive. In contrast, Genotype 13 showed no change in POX activity, which may indicate a stable antioxidant system or alternative stress response mechanisms. The increase of POX may reflect the plant's compensatory response to oxidative damage caused by drought stress, since the POX is involved in the breakdown of hydrogen peroxide and other ROS. The rise in POX activity could therefore be an adaptive mechanism to prevent oxidative damage to plant cells and help to maintain cell integrity under stress. However, this response appears to be affected by genotype, stress intensity and their interaction. Various studies have shown that the activity of the POX enzyme is significantly increased in barley genotypes due to drought stress [15, 78, 92]. However, a decrease of POX was happened in a few genotypes in our study. Different response for enzyme activity may suggest that these genotypes utilized different adapted alternative salvage pathways, including nonenzymatic antioxidants such as carotenoids, to scavenge H_2O_2 [93].

Our findings emphasize the complex interaction between genotype and drought stress in shaping antioxidant enzyme responses. It can be concluded that antioxidant enzyme activity plays a key role in barley genotypes' reaction to drought stress. The variation in enzyme activities across genotypes suggests the potential of selecting genotypes with stronger antioxidant systems, potentially enhancing drought tolerance in barley.

Carotenoid and chlorophyll content

Carotenoids, important pigments in plants, increase during drought and help protect against oxidative damage by neutralizing free radicals [28, 94], even under conditions induced by drought stress [37, 95]. In another study, it was observed that drought stress led to a reduction in carotenoid levels in barley, with a more pronounced reduction in the sensitive genotype compared to the tolerant one [15]. In contrast, another barley research [96] showed an increase in carotenoid levels under drought stress for most genotypes, while some showed a decrease. Other studies have also shown changes in carotenoid levels in plants as a result of stress [43, 97].

Chlorophyll, the key pigment for light absorption in leaves, is vital for photosynthesis and is essential for biochemical and physiological processes in plants. It also plays a critical role in plant responses to drought stress [35, 98, 99]. Chlorophyll content serves as a reliable

marker for identifying drought and salt-tolerant genotypes of barley [49]. This study revealed that drought stress reduced chlorophyll content in barley. The Chla/b ratio remained unchanged under drought conditions, indicating a coordinated stress effect on both chlorophyll types. Since the Chla/b ratio often reflects plant adaptation to environmental conditions, its stability suggests that these genotypes maintained a balanced allocation of chlorophyll types under drought stress.

In our study, genotype 3 exhibited the lowest levels of Chla, Chlb, and Tchl, indicating the reduced photosynthetic capacity and greater vulnerability to drought-induced damage in this genotype. Conversely, genotypes with higher chlorophyll levels under drought stress likely possess more photosynthetic capacity and drought tolerance. The effect of drought on chlorophyll levels was different in various barley genotypes and this was in agreement with those results reported in other studies [13, 15, 96].

Chlorophyll fluorescence

Measurement of chlorophyll fluorescence is used to assess the effects of environmental stress on the photosynthetic system [45, 46, 100]. The F_v/F_m ratio, a key chlorophyll fluorescence trait, has been recognized as a reliable indicator of light inhibition in PSII, especially under drought stress in barley [45, 101, 102].

The results of this research revealed that drought stress significantly reduced the F_v/F_m ratio in barley genotypes, highlighting the effect of genetic variation and genotype-environment interactions on this trait. The decline in F_v/F_m indicates photoinhibition and structural damage to PSII, impairing its function and reducing photosynthetic efficiency, plant growth, and yield [47]. Lower F_v/F_m ratios in some of our genotypes demonstrate their vulnerability to light damage and reduced photosynthetic capacity due to limited photoprotective or repair capabilities, leading to greater susceptibility to oxidative stress and long-term damage. These findings underscore the importance of selecting genotypes with robust photosynthetic performance under drought stress. It was reported that tolerant genotypes of wild barley [12] and barley [50] experienced a smaller decrease in F_v/F_m than susceptible genotypes, indicating more severe PSII photoinhibition and better energy transfer efficiency between chlorophyll a and b. These findings are consistent with other research on plant species under stress [45, 46, 103].

Relative water content (RWC)

Genotypes with significant reductions in RWC under drought stress are more sensitive to water scarcity [104]. In contrast, genotypes maintaining higher RWC in drought conditions show better drought tolerance through effective water retention and reduced electrolyte leakage. Genotype 14 demonstrated a strong capability to preserve cell turgor in its leaves during drought, consistent with the results regarding electrolyte leakage. Other studies consistently found that drought stress generally reduces RWC, reflecting a plant's stress tolerance [13, 105–107].

Electrolyte leakage (EL)

The observed genotypic differences for electrolyte leakage (EL) indicate consist of variation among genotypes for membrane stability. Some genotypes showed more EL and this may be associated with underlying oxidative stress processes, such as lipid peroxidation [76, 78]. Maintaining plasma membrane integrity is vital for drought tolerance, allowing plants to sustain physiological functions and protect grain yield under water-limited conditions [108, 109]. Cell membrane stability is widely recognized as a key indicator of plant tolerance to abiotic stress and is frequently used to evaluate drought tolerance in various genotypes [110]. Other studies on barley genotypes have shown that drought and salinity stress conditions significantly reduce cell membrane stability, indicating increased membrane damage and permeability [49, 80]. Some barley cultivars exhibit a marked increase in electrolyte leakage under stress, while others remain unaffected [13].

Protein content

Stress conditions typically reduce leaf protein content, as ROS primarily damage proteins [83, 111]. The results of this study showed that most genotypes showed reduced protein levels under stress, while some exhibited increased protein content, probably due to activation of stress-responsive mechanisms. Genotypes with higher protein content in stress conditions show better stress tolerance, while a significant reduction of protein in genotype 18 highlights its vulnerability to stress.

Some other research on barley and related species has shown varied effects of environmental stress on total protein content in leaves. Under combined heat and drought stress, barley cultivars experienced a significant decrease in leaf protein levels compared to the controls [88, 112]. Similarly, drought stress in wild barley led to a notable reduction in protein content [15].

Proline content

Proline accumulation in response to abiotic stress serves as a protective factor in plant and also can be used as an indicator of happening stress in plant which is depends on the type of stress and its severity. Proline accumulation enhances stress tolerance by aiding in osmotic regulation, stabilizing membranes and protein structures, reducing oxidative stress, and acting as a signaling molecule that triggers various adaptive responses [113].

Proline functions as an osmotic protector, an antioxidant, and a regulator of cytosolic pH, while also serving as a reservoir for carbon and nitrogen during recovery [114]. However, it was reported that in some genotypes, particularly in sensitive ones, elevated proline levels may simply reflect the extent of cellular damage rather than indicating the stress tolerance [113, 114]. This study revealed that leaf proline content was significantly affected by drought stress, and there was a genetic variation with genotype-specific responses for proline accumulation. Drought stress led to a significant increase in leaf proline content across all genotypes, aligning with findings of other studies in barley genotypes [13, 114]. In the other studies on barley, drought stress, caused a significant increase in proline content [12, 37, 53]. However, proline accumulation is not always indicative of greater drought stress tolerance, as higher levels may sometimes reflect sensitivity to stress [113]. Variability in findings regarding proline's role in stress tolerance can be attributed to factors such as genetic background [113], stress intensity, and plant developmental stage [115].

Polyphenol components

Abiotic stress profoundly affects the metabolic profiles of compounds synthesized via the phenylpropanoid pathway, especially phenolic compounds. These changes play a vital role as adaptive mechanisms, strengthening plant resilience and enhancing tolerance to diverse abiotic stress conditions [116–118].

Our results highlighted the significant impact of drought stress, genotype variability, and their interaction on the concentrations of phenolic acids and flavonoids in barley leaves (Table S6). Drought stress led to notable alterations in these compounds, demonstrating both stimulatory and inhibitory effects, reflecting the complex nature of the plant's metabolic response to environmental stressors. Importantly, most phenolic compounds increased under drought stress and these results were consistent with the results of [25, 34, 119]. These increases in phenolic compounds, including gallic acid, ellagic acid, syringic acid, and vanillic acid, may represent the plant's defense mechanisms against drought.

The response of phenolic compounds to drought stress was different. Chlorogenic acid showed no notable changes under drought conditions, while both caffeic acid and apigenin experienced marked reductions. This suggests metabolic shifts in the pathways responsible for these compounds under stress. Such reductions might reflect the plant's reallocation of resources, prioritizing specific metabolic pathways crucial for survival while downregulating the production of certain secondary metabolites. The stability of chlorogenic acid could imply that its initial concentration was sufficiently high, making any potential increase under stress negligible. This observation highlights that some phenolic acids, like chlorogenic acid, may exhibit lower sensitivity to environmental factors compared to others. It was reported that ferulic acid may play a crucial role as a metabolite in enhancing drought tolerance in plants [53]. The response of caffeic acid (CA) is contingent upon varying abiotic conditions. Different abiotic stressors can influence the synthesis, concentration, and functional efficacy of caffeic acid, highlighting the complex interactions between environmental factors and secondary metabolite production [118].

One key finding of our study was the genetic diversity among genotypes for the accumulation of phenolic compounds, which could be attributed to their capacity for response to drought stress. These findings show that distinct metabolic pathways are activated in different genotypes under stress and provide a potential approach for selecting a genotype for tolerance based on their polyphenolic profiles. High levels of vanillic acid and ellagic acid play a significant role in response to drought stress. In addition, high routine accumulation, a flavonoid, indicates that flavonoids, along with phenolic acids, play an important role in mitigating drought-induced oxidative damage. Flavonoids are famous for their antioxidant properties and their protective effects on vegetable tissues under environmental stress [81]. In another study, it was found that under salinity stress, ferulic acid and gallic acid were the most abundant polyphenols, whereas vanillic acid, ellagic acid, and chlorogenic acid exhibited the lowest levels among the analyzed compounds [25].

While HPLC-based phenolic profiling offers valuable insights into how plants respond to drought, its use in large-scale breeding programs may be limited due to its high cost, the requirement for specialized equipment, and the need for technical expertise. However, the HPLC analysis can be used for a few advanced breeding lines or developed cultivars.

Grain yield

Grain yield plays a crucial role in understanding the relations between biochemical responses in plants and stressors, as well as how this factor affects adaptation through genetic diversity [120]. Drought stress significantly impacted grain yield across all genotypes, with a 47% reduction observed, which aligns with previous studies attributing yield declines to physiological, biochemical, and metabolic changes under drought stress [27, 105, 121, 122]. The different responses of genotypes to drought stress revealed the interaction between genetics and environmental conditions. Some genotypes exhibited lower grain yield loss under drought stress, indicating greater physiological and biochemical plasticity and drought tolerance. However, from an economic perspective, genotypes that maintained higher grain yield

under drought stress demonstrated better adaptation to water deficit.

Genotype 3 exhibited the highest reduction in grain yield under drought stress, indicating its vulnerability due to a lack of physiological and biochemical plasticity required for drought resilience. In contrast, genotype 16 showed the least reduction in grain yield, demonstrating its genetic potential to cope with drought stress through adaptive strategies. This genotype has more drought tolerance and could be a good candidate with high potential for developing tolerant cultivars in breeding programs Overall, the genotypes have good plasticity under stress conditions enabling them to achieve economically viable grain yield despite environmental challenges. This adaptability makes them strong candidates for the development of drought-tolerant cultivars, which could significantly enhance sustainable grain production in stress-prone regions.

Relationships of traits

Stepwise regression analysis identified F_v/F_m, chlorophyll a (Chla), and POX as key predictors of grain yield, with F_v/F_m showing the strongest correlation ($r = 0.92**$). These variables collectively explained 94% of the grain yield variation, highlighting their importance in grain yield under stress conditions. Enhancing photochemical efficiency (F_v/F_m) and antioxidant defense mechanisms (e.g., POX) could be vital for improving crop yield under drought conditions. Chlorophyll fluorescence is widely recommended for evaluating plant responses to environmental stresses, particularly water stress, and assessing drought tolerance [46, 100]. Chlorophyll fluorescence is proposed as an effective tool for detecting drought stress susceptibility in barley [101].

Principal component analysis (PCA) simplifies complex data by consolidating variables into principal components (PCs) that capture the most variances. Under drought stress, photosynthetic variables (Chla, Chlb, and Car) positively influenced PC_1, highlighting the importance of preserving photosynthetic pigments to mitigate stress effects and sustain plant performance [37, 123]. Genotypes near these variables exhibited greater stress tolerance and adaptation. Antioxidant-related variables, such as DPPH and F_v/F_m, also strongly contributed to PC_1, underscoring their role in minimizing photochemical damage and maintaining photosystem efficiency [100].

In PC_2, antioxidant mechanisms (TPC, APX, H_2O_2) and oxidative stress markers (MDA) were most influential, emphasizing their critical role under stress conditions [35, 37]. The PCA distribution revealed genotypes (e.g., 17 and 21) near the GY transporter exhibited higher productivity, demonstrating the effect of physiological and biochemical stress responses in grain yield. Conversely, distant genotypes (e.g., genotype 3) were more drought-stress sensitive. This analysis highlights that antioxidant mechanisms and photosynthetic maintenance are critical determinants of stress tolerance, aiding in identifying tolerant genotypes to manage stress effects. Under control conditions, photosynthetic pigments (Chla, Chlb, Car, and TChl) significantly contributed to PC_1, reflecting their importance in maintaining normal physiological functions. Genotypes near these variables, such as 6 and 10, demonstrated higher photosynthetic efficiency and grain yield. Oxidative stress and antioxidant defense markers (MDA, H_2O_2, and POX) were positively correlated with PC_2, suggesting basal antioxidant activity under optimal conditions, which may enhance stress tolerance. Antioxidant capacity (DPPH) and photosystem efficiency (F_v/F_m) also aligned with PC_2, emphasizing their role in maintaining oxidative balance and photochemical efficiency. For instance, genotype 15, positioned near the DPPH vector, exhibited higher basal antioxidant capacity, while genotypes 17 and 11, closer to the center, displayed balanced traits and physiological stability.

Based on the PCA analysis in this study, genotypes 1 and 10, associated with flavonoids apigenin and rutin, demonstrated enhanced defense capacity in drought conditions. Genotypes 2, 8, 16, 18, and 21 showed close ties to chlorogenic acid, ellagic acid, and gallic acid, important for regulating defense signaling and cell protection. Genotypes 14, 15, 19, and 20, associated with vanillic acid, exhibited downregulation patterns. These findings underline the importance of phenolic and flavonoid profiles in improving plant performance under both control and stress conditions, offering to use the genetic potential of these genotypes for grain yield to enhance plant tolerance.

Hierarchical clustering and heat map analysis under drought conditions demonstrated a clear divergence in the response patterns of phenolic and flavonoid compounds among different barley genotypes. Some genotypes that in both normal and stressed conditions, showed an elevated level of bioactive compounds, suggesting their more capability to sustain or stimulate secondary metabolism during water deficit conditions. This may lead to improved their antioxidant defense and mechanisms for stress tolerance. The distinct clustering of genotypes supports the idea of using phenolic profiles as biochemical indicators for assessing barley genotype to recognize tolerant genotypes. It was mentioned that some significant genomic regions and potential genes in plan genome of some genotypes linked to drought tolerance, emphasizing different reaction of barley genotypes to water scarcity [122]. Evaluation of genotypes by physiological and biochemical traits can help to increase the accuracy and effectiveness for drought tolerance screening.

Conclusions

The findings of this study highlight the significant roles of genotype and environmental interactions in drought tolerance. Under drought stress, a significant increase in polyphenols has been closely linked to drought stress resilience of genotypes. This is attributed to their critical roles as signaling molecules, enhancing plant defense mechanisms, facilitating auxin transport, and scavenging free radicals. The enhanced antioxidant capacities of polyphenols combined with enzymatic antioxidants in tolerant barley genotypes are further evidenced by their reduced susceptibility to oxidative stress, as revealed by reduced levels of MDA and H_2O_2. Amid increasingly severe drought cycles driven by climate change, drought-tolerant barley lines hold strong potential for maintaining stable yields in arid regions, given barley's inherent resilience among cereal crops. These insights into barley's drought response can help breeders identify key traits for developing drought-tolerant cultivars, improving resilience and yield in water-limited environments. This research also lays a foundation for uncovering the molecular mechanisms of drought tolerance, supporting efforts to enhance food security.

Supplementary Information

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Supplementary Material 1

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Author contributions

G.S. and A.A. conceptualized the study; B.M. conducted the experiments; B.M. and G.S. administrated the project; B.M. conducted data analysis; B.M. implemented the software; B.M., G.S., and A.A. validated the impact of phenolic compounds on alleviating drought stress; B.M. wrote original manuscript draft; G.S. and A.A. reviewed and edited the manuscript; G.S. and A.A. supervised the research. All authors have reviewed and agreed to the published version of the manuscript.

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Data availability

All data relevant to the study are included in the article or uploaded as Supplementary Materials.

Declarations

Competing interests

The authors declare no competing interests.

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