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Research Article

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Role of sulfur in pyrroline-5-carboxylate synthase (*P5CS)* gene expression, proline accumulation, and antioxidant enzyme activity of wheat under water deficit conditions

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ABSTRACT- Sulfur (S) plays a crucial role in plant growth and development and serves numerous biological functions in plants. To investigate the role of sulfur in drought stress protection, two experiments were conducted under greenhouse and growth chamber conditions in 2021 Also, to evaluate the influence of foliar application of sulfur on the expression of *Pyrroline-5-carboxylate synthase,* (*P5CS*), proline concentration, and antioxidant enzyme activities, the plants of wheat Sardari and Ivan cultivars were exposed to two levels of water regimes. Plants under well-watered irrigation and drought stress conditions showed different *P5CS* expression levels and accumulated proline. The *P5CS* expression level in the Ivan cultivar was significantly increased by foliar application of S, while the same application had no significant effect on *P5CS* expression in the Sardari cultivar. Plants exposed to drought stress and foliar-sprayed with S showed a higher proline accumulation The activities of antioxidant enzymes, including peroxidase (POD), catalase, glutathione peroxidase (GP), and glutathione reductase , were increased by 134 %, 40.4 %, 45.4 %, and 77.4 %, respectively, in plants exposed to drought stress. POD and GR were significantly increased by 19.6 % and 51.8 %, respectively, due to S application. Drought-induced plants that were foliar-sprayed with S exhibited a significantly lower rate of ion leakage and higher leaf-relative water content than those of control plants. Based on the results of the present research, it was revealed that S substantially enhances the expression rate of the *P5CS* gene, leading to proline accumulation, and activates defense reactions in water-stressed wheat.

INTRODUCTION

Drought affects various aspects of plant growth, development, and yield (Mirdoraghi et al., 2022). In recent decades, the importance of sulfur application for promoting normal growth, enhancing yield, and diversifying crops has been widely recognized. Sulfur has been proven effective in increasing the yield and yield components of different crops including wheat (Scherer, 2001; Aula et al., 2019). Approximately, 2 to 3 kg of sulfur per 1000 kg of grain produced is required to produce 1000 kg of wheat seeds (Zhao et al., 1999). Sulfur application has also been shown to improve drought tolerance in various crop plants (Samanta et al., 2020). It has also reported that the application of sulfur enhances yields in plants like sunflower under drought conditions (Shafiq al., 2021).

Plants primarily absorb sulfur in the form of sulfate (SO_4), which is the main source of sulfur for plants when present in low levels in soils (Narayan et al., 2023). Studies have suggested that S acts as a signaling molecule in both biotic and abiotic stress responses (Narayan et al., 2023). Plants have a higher demand for sulfur during vegetative growth and seed development (Narayan et al., 2023).

Sulfur plays numerous roles in plants, and is involved in important biological functions such as photoprotection, photosynthesis, metabolic reactions, and energy generation (Lee et al., 2016; Borpatragohain et al., 2019). Furthermore, sulfur influences the content of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme and photosynthetic pigments (Muneer et al., 2014; Lee et al., 2016). Meanwhile, sulfur deficiency leads to a loss of chlorophyll in young leaves. This deficiency has been reported to inhibit protein synthesis and photosynthesis rate (Muneer et al., 2014; Lee et al., 2016).

Sulfur is a precursor of protein synthesis and contributes to the synthesis of various organic products, including methionine, cysteine, and glutathione (Li et. al., 2020). Several studies have elucidated that sulfur can modulate the ratio of reduced glutathione to oxidized glutathione (Lee et al., 2016). Glutathione plays a role in eliminating reactive oxygen-containing compounds (Astolfi and Zuchi, 2013).

Sulfur is present in chloroplasts, important enzymes, and proteins. Previous research has demonstrated that photosynthesis is reduced under sulfur-deficient conditions (Muneer et al., 2014). Sulfur deficiency leads to a decrease in Ribulose bisphosphate Carboxylase-Oxygenase (RuBisCO) protein (an enzyme present in plant chloroplasts, involved in fixing atmospheric carbon dioxide during photosynthesis) content and net photosynthesis rate (Lee et al., 2016) results in the development of chlorosis (Gilbert et al., 1997).

It has been shown that under S deficiency, plants exhibited a higher accumulation of the reactive oxygen species (ROS) messenger (Lunde et al., 2008). It has also been found that sulfur application enhanced the activity of

enzymatic antioxidant enzymes (Shafiq et al., 2021), and improved photosynthetic capacity (Fatma et al., 2014) and photosynthetic activity in plants (Lee et al., 2016).

Sulfur is a structural component of amino acids, protein disulfide bonds, and vitamins and is necessary for chlorophyll formation (Narayan et al., 2023). Proteins and amino acids that contain S are significantly affected by S deficiency (Usmani et al., 2020; Li et al., 2021; Shafiq et al., 2021). It has been reported that the application of sulfur alleviates the adverse effects of drought stress on maize (Usmani et al., 2020), wheat (Li et al., 2021), and sunflower (Shafiq et al., 2021). Alleviation of drought stress by sulfur has been attributed to reduced oxidative stress (Li et al., 2021; Shafiq et al., 2021).

Compared to unstressed conditions, water-stressed plants accumulate higher concentrations of proline, the preferred compatible osmolytes in many plants (Hare and Cress 1997). Among known compatible osmotic adjustment solutes, proline is an indicator parameter for selecting drought-resistant varieties (Bates et al., 1973; Mwadzingeni et al., 2016; Arteaga et al., 2020). Proline has an important role in osmotic adjustments as well as the protection of enzymes and membranes, thereby counteracting the adverse effects of osmotic stress. In this regard, it has been reported that proline accumulation caused an improvement in osmotic stress tolerance in wheat (Ullah et al., 2022).

The biosynthesis of proline is controlled by two key enzymes including Δ1-pyrroline-5-carboxylate (*P5C*) synthetase (*P5CS*) and *P5C* reductase (*P5CR*) (Furlan et al. 2020). Most plants, such as wheat, have two genes (*P5CS* and *P5CR*) responsible for encoding *P5CS* and *P5CR* enzymes. Up-regulation of proline biosynthesis in water-stressed wheat has been reported (Jamshidi Goharrizi et al., 2023; Jamshidi Goharrizi et al., 2020; Maghsoudi et al., 2018). It has been proposed that some nutrients can induce gene expression (Curtis et al., 2019; Raffan et al., 2020). Nutrients can affect gene expression by taking part in the gene regulatory mechanisms (Raffan et al., 2020; Zarea and Karimi, 2023). Curtis et al. (2019) reported that gene-encoding enzymes of nitrogen assimilation were up-regulated in grains when wheat plants were supplied with sulfur. Raffan et al. (2020) investigated the efficacy of sulfur application on the expression of glutamate-cysteine ligase in wheat. That study claimed that supplied sulfur significantly affected the expression of glutamate-cysteine ligase.

However, sulfur's physiological and molecular regulatory effects are still poorly understood, especially under abiotic stresses such as salinity and drought. The current study aimed to elucidate the wheat response to S application at the molecular and physiological levels under non-limited and limited irrigation conditions.

MATERIALS and METHODS

Description of the experiments' conduction

Two experiments were conducted to elucidate the regulating effect of sulfur (S) on the molecular, biochemical, and physiological responses of two winter wheat cultivars. Experiment 1 clarified the effect of S via foliar application on *P5CS* gene expression, proline accumulation, and antioxidant enzyme activity. Experiment 2 detected the electrolyte leakage (EL), leaf relative water content (RWC), and chlorophyll (chl) pigments, including chl a and chl b and total chl, as well as carotenoid pigments' responses to S under well-watered (WW) conditions (80% of water holding capacity) and water deficit conditions (25% of water holding capacity) at anthesis stage. Two dry-land winter wheat cultivars, Sardari and Ivan, were chosen for this study. These cultivars are high-yielding wheat cultivars under moderate semi-arid conditions and are widely cultivated by Iranian wheat farmers, including in the semi-arid areas of western Iran. A few characteristics of the studied cultivars are shown in Table 1. Soil samples for potting experiments were collected from the Ilam University Agricultural Research farm's 0- to 30-cm soil layers. Some chemical and physical properties of the soil used for the experiments are outlined in Table 2.

Experiment 1

Experiment number 1 was conducted from December 2021 to June 2022 in the growth chamber, Department of Agronomy and Crop Breeding, Faculty of Agriculture, Ilam University, Ilam, Iran. Each Pot was filled with 0.7 kg soil. Fifteen seeds were sown in each pot, which was thinned to eight seedlings after sprouting and producing seedlings.

Combination treatments of S foliar application and drought were carried out in a completely randomized design (CRD). Treatments were replicated three times. All pots were well watered (80% of field capacity) until drought treatmentwas imposed. Irrigation treatment was imposed at two different levels, i.e., 80% of soil water holding capacity (WHC), which served as a control treatment, and irrigation at 25% of WHC as drought treatment. Plants were exposed to 80% WHC or 25% WHC 21 days after sowing. Sulfur at 1% (w/v) solution was sprayed on wheat seedling foliage 15 and 23 days after seed sowing. The second foliar spray was done four days after drought treatment was imposed. One group was foliar sprayed with S at 1% (w/v) concentration at the rate of 40 ml pot^{-1}. Control plants (the second group) were foliar sprayed with distilled water at the rate volume of 40 ml pot^{-1}.

At 14 days after drought imposition, leaf samples were taken to measure wheat seedlings' molecular, physiological, and biochemical responses to S-foliar application under adequate water supply (well-watered) and drought conditions. The fully-expanded leaves from each pot were randomly collected 14 days after drought-stress imposition. Two days after drought imposition, fresh, fully expanded leaf samples were also sampled from each pot to measure the S and N contents of the leaf, proline concentration, and antioxidant enzyme activities and isolate total RNA.

RNA Isolation and Gene Quantification

The total RNA was isolated from fully expanded leaves using an RNA isolation kit (RiboEx Total RNA-301-001; GeneAll Biotech) according to the manufacturer's instructions. The total RNA of the leaf samples (0.1 g) was extracted by grinding in liquid nitrogen. The quantification of the extracted RNA (the ratio A 260/ A 280 and A 260/ A 230) was checked using the NanoDrop 1000 Spectrophotometer instrument (Thermo scientific, USA). RNA samples that yielded an A260/A280 ratio between 1.8 and 2.1 were selected for cDNA synthesis. Genomic DNA was digested by DNaseI using a DNaseI Kit (Thermo Fisher Scientific). For this, isolated RNA samples were incubated at 37 ℃ for 30 min with DNaseI enzyme. To terminate reaction 1 µL ethylenediaminetetraacetic acid (EDTA) was added to samples and incubated at 65 ℃ for 10 min.

Total isolated RNA was copied into the first DNA strand using the cDNA Excel RT™ Reverse Transcription

Kit (SMOBIO) according to the manufacturer's instructions. The same amount of RNA concentration (250 ng) of all samples was used to reverse-transcribed in cDNA.

Transcription levels were measured using SYBR green in a master mix in a qRT-PCR machine. Quantitative real-time PCR (qRT-PCR) was performed using an ABI Step One Plus apparatus (Applied Bioscience, USA). Real qRT-PCR was performed in a total volume of 12.5 µL containing 6.5 µL volume of Plus 2x Master Mix Green (Cat. No. A325402, Pishgam, Iran), 50 ng cDNA, and 0.25 µL of each primer (10 µM). The amplification reactions were carried out in an ABI Step One plus apparatus with initial denaturing of 95℃ for 10 min, followed by 40 cycles of 95 ℃ for 20 s, annealing temperature of 60 ℃ for 40 s, and extension for 30 s at 72 ℃. After the PCR reaction, the amplifications' specificity was checked based on melting curve analysis by heating the amplicons from 65 ℃ to 95 ℃. The primer sequences and annealing temperature of primers used to amplify gene encoding proline synthesis-related enzyme (*P5CS*) with a product length of 120 bp and the Actin gene as an internal control (housekeeping gene) are shown in Table 3.

Proline Measurement

Leaf proline concentration was measured spectrophotometrically in the leaves of plant seedlings following the ninhydrin-based method described by Bates et al. (1973). Proline from fresh leaf samples was extracted with 3% sulfosalicylic acid. The absorbance of the proline extract in toluene was recorded at 520 nm. Proline was expressed as µ mol g^{-1} fresh weight.

Photosynthetic Pigments Measurement

The chlorophyll pigments (chlorophyll a, chlorophyll b, and total chlorophyll) values were determined on the fully expanded leaves, according to the method described by Arnon (1949). Chlorophyll pigments were extracted with acetone (80%). The chlorophyll pigment extracts' absorbance was measured at 645, 663, and 470 nm wavelengths (Lichtenthaler, 1987). To measure the chlorophyll concentrations in the samples the following calculation was made:

Chlorophyll a (mg mL^{-1}) = 12.7 A663 - 2.69 A645

Chlorophyll b (mg mL^{-1}) = 22.9 A645 - 4.68 A663

where A663 is the absorbance at a wavelength of 663 nm and A645 is the absorbance at a wavelength of 645 nm. The chlorophyll pigments content from the leaf tissue (original tissue sample) was calculated according to the following equation:

Total Chlorophyll a (mg) = Chlorophyll a (mg/mL) × final volume (mL)

Total Chlorophyll b (mg) = Chlorophyll b (mg/mL) × final volume (mL)

Carotenoids concentration was calculated in samples according to the following equation:

Carotenoids (mg mL^{-1}) = [(1000 A470 - 1.8 Chlorophyll a - 85.02 Chlorophyll b)/198]V/1000 W.

where V is the final volume of chlorophyll extract in 80 % acetone (20 mL), and W is the fresh weight of tissue extracted (0.5 g).

Antioxidant Enzyme Activities Determination

Catalase (CAT) activity was determined in samples of fresh leaves two weeks after drought stress treatment. The CAT activity was estimated according to the method described by Luck (1974). CAT activity was calculated based on the rate of H_2O_2 consumption and expressed as unit g^{-1} of fresh weight (f.w.). The reaction mixture consisted of 50 mM phosphate buffer with a pH of 7, 15 mM peroxide hydrogen, and 0.1 ml of enzyme extraction. The decline in absorbance was taken at 240 nm for 1 min using a spectrophotometer (Analytik Jena Specord 50 plus).

The method of Chance and Maehly (1955) was used to measure the activity rate of the peroxidase (POD) enzyme. The reaction mixture consisted of 0.1 ml of enzyme extraction, 50 mM potassium phosphate buffer (pH 7.0), and 1 mL of 20 mM guaiacol plus 40 mM H_2O_2. The POD activity (U g^{-1} leaf f.w.) was determined based on the rate of guaiacol oxidation at 470 nm for 1 min.

Glutathione reductase (GR) was measured based on the rate of nicotinamide adenine dinucleotide phosphate (NADPH) consumption as previously described in the method of Foyer and Halliwell (1976). The activity rates of the GR enzyme were expressed as unit mg^{-1} g^{-1} f. w. One unit of GR activity was set as the amount of enzyme required for oxidation of 1 µmol of NADPH min^{-1}.

The method of Rotruck et al. (1973) was followed to estimate the glutathione peroxidase (GP) activity. The initial mixture consisted of 500 µL potassium phosphate buffer (0.05 M, pH 7.4), 50 µL of sample homogenate, 200 µL of 4.0 mM reduced glutathione (GSH), and 0.1 ml H_2O_2 (2.5 mM). The mixture was incubated for 10 min at 37 ℃. The reaction was stopped by adding 0.5 ml (10%) trichloroacetic acid. The mixture was centrifuged at a centrifugal force of ۳۵۸ × *g* for 15 min. Three ml of 0.8 mM disodium hydrogen and 0.1 ml dithionitrobenzoic acid (DTNB) of a 0.4% solution were added to the collected supernatant, and the absorbance of the developed color was taken at a wavelength of 420 nm.

Experiment 2

The treatments for experiment No. 2 were the same as those in experiment 1, except S which was applied thrice. The factorial experiment was based on a randomized complete block design (RCBD) with four replications. Each pot was sprayed with sulfur solution (1%, w/v) three times: at tillering, stem elongation, and flowering stage at the rate of 40 ml per stage. Control seedlings were sprayed with distilled water alone. Proline accumulation in the leaf, chlorophyll pigments, electrolyte leakage (EL), and RWC were measured in flag leaves at the flowering stage.

Table 1. Some characteristics of the two bread wheat cultivars used in this study

Cultivar	Year of release	Growth habit	Origin	Plant height	1000-grain weight (g)	grain yield potential (kg/ha)*	Drought tolerance
Sardari	1931	Winter	Iran	Semi-dwarf	44	1932	Resistant
Ivan	2017	Winter	Iran	Semi-dwarf	34	2426	Resistant

*Under rain-fed condition

Table 2. Physical and chemical properties of the soil used in this study

Soil texture	EC (dS/m)	pH (1:2 H_2O)	Organic C (%)	N (%)	K	P	S
					mg kg^{-1}		

Table 3. Characteristics of *Pyrroline-5-carboxylate synthase,(*P5CS) gene and *Actin* gene primers used in qRT-PCR test

Gene name	NCBI accession no.	Primer sequence (5' to 3')	Amplicon (base pair)	annealing (temperature ^{0}C)
P5CS	AY888045.1	P5CS-F:5 CGGTGCTGAGGTTGGCATAAG3 P5CS-R:5TTGTCACCATTCACCACTTGCCC 3	22 23	56
Actin	AB181991.1	Actin-F:5 GTGTACCCTCAGAGGAATAAGG 3 Actin-R:5 GTACCACACAATGTCGCTTAGG 3	22 22	55

F: forward strand; R: reverse strand; qRT-PCR: quantitative real-time polymerase chain reaction

Clay loam	0.3	7	1.2	0.12	420	8.5	60

The method of Ritchie et al. (1990) was adopted to determine RWC (%). Flag leaves were randomly collected from each pot. The leaf samples were immediately weighed. The samples were then hydrated in the dark for 24 hours at room temperature and then weighed. The dry weight of these leaf samples was determined after these samples were oven-dried at 75°C for 24 h. The equation (Eq.) 1 was used to determine the percentage of flag leaf RWC: as follows

RWC (%) = (leaf fresh weight – leaf dry weight)/(leaf saturated weight – leaf dry mass) × 100 Eq. 1

Electrolyte leakage percentage (EL,%) was determined in flag leaves using the techniques of Lutts et al. (1996). Briefly, three leaves from three different seedlings per pot were fully collected and saturated by immersing them underwater at room temperature in the dark for 4 h. After immersing, the initial conductivity was determined using a conductivity meter. The leaf samples were then incubated in a water bath at 100°C for 60 min, whereby the absolute conductivity was determined. The Eq. 2 was used to calculate the EL,% as follows:

EL (%) = (initial conductivity / absolute conductivity) × 100 Eq. 2

Data analysis

The variance (ANOVA) analysis of all data recorded from the experiments was performed using SAS software, version 9.3. The least significant difference (LSD) test at a 0.05 significance level was used to compare treatment means.

RESULTS AND DISCUSSION

Analysis of variance (ANOVA) of drought imposition, cultivar, sulfur foliar application effects, and their interactions (Experiment number 1) on leaf sulfur (S) concentration, nitrogen (N) content, proline (Pr) accumulation, chlorophyll (Chl) content, carotenoids pigment (Cart) and antioxidant activity of catalase (CAT), peroxidase (POX), glutathione reductase (GR) and glutathione peroxidase (GP) on two wheat cultivars at seedling growth stage are presented in Table 4.

Sulfur and nitrogen content

Foliar-applied S significantly affected S concentration in leaves, so that S concentration in the leaves elevated by 30.6%. The Ivan cultivar had higher S concentrations in the leaf (35%) than that of the Sardari cultivar. A significant interaction was detected between two main factors, cultivar and S application, on leaf S concentrations (Table 4). The highest S concentration in leaves was recorded with foliar-applied S in Ivan cultivar plants (Fig. 1). It postulates that increased S content in plants following S foliar application indicated that the leaf absorbed the applied S through the stomata and/or cuticular area. For instance, previous study showed that foliar-applied zinc can be absorbed through the leaf cuticle and stomata (Li et al., 2019)

Drought reduced the N concentration in leaves by about 37%. Foliar-applied S plants under well-watered (WW) conditions had higher N content (14.5%) in leaves than those of control plants. The Ivan cultivar plants had higher concentrations of N than the Sardari cultivar plants under WD conditions. The lowest N content in leaves was detected in the water-stressed Sardari cultivar plants of the control treatment. However, the plants of this cultivar sprayed with S had a higher N content than that of the control plants under drought stress conditions (Fig. 2). Foliar-applied S had no significant influence on the N content in Ivan under drought conditions (Fig. 2). Salvagiotti et al. (2009) noted a synergistic interaction between S and N in wheat. These authors reported that soil-applied S elevated the N uptake by the plant. Ahmad et al. (2016) reported that, under drought conditions, higher levels of S are more allocated from leaves to the roots to enable roots to produce greater biomass.

Table 4. Mean square (MS) of the source of variation for the studied traits including leaf sulfur (S) concentration, nitrogen (N) content, proline (Pr) accumulation, chlorophyll (chl) content, carotenoids pigment (Cart) and antioxidant activity of catalase (CAT), peroxidase (POX), glutathione reductase (GR) and glutathione peroxidase (GP)

Treatment	S concentration	N content	Pr accumulation	Chl a content	Ch b content	total Chl content	Cart	CAT	POX	GR	GP
Sulfur(S)	0.02**	2.6**	1328*	0.58^{ns}	0.1^{ns}	0.18^{ns}	0.009^{ns}	4.2**	4071**	1.1**	0.3**
Cultivar(C)	0.03**	0.88*	36.9**	2.4*	0.1^{ns}	1.4^{ns}	1.07**	6.7**	35.1^{ns}	0.7**	0.3**
Drought(D)	0.0007^{ns}	19.2**	46.5**	150**	7.7**	227**	14.1**	92.6**	15882**	1.9**	0.6**
S×C	0.002**	0.01^{ns}	35.2**	0.008^{ns}	0.33ns	0.08^{ns}	0.15^{ns}	0.01^{ns}	930**	0.0001^{ns}	0.09**
S×D	0.0005 ^{ns}	0.23^{ns}	43.1**	0.34^{ns}	0.001^{ns}	0.38^{ns}	0.32^{ns}	0.3^{ns}	3.1^{ns}	0.28**	0.04**
C×D	0.000004^{ns}	0.000004^{ns}	2.1*	0.97^{ns}	0.42*	2.6*	0.03^{ns}	0.0003^{ns}	298*	0.08**	0.2**
S×C×D	0.0005^{ns}	0.99*	1.9*	0.43^{ns}	0.007^{ns}	0.14^{ns}	0.67*	7.19**	462*	0.008**	0.02*
SE	0.017	0.13	0.4	0.38	0.076	0.37	0.11	0.7	70.1	0.001	0.004
C.V. (%)	3.1	7.4	5.8	8.6	17.1	6.9	14.3	7.1	5.1	3.1	7.9

Ns; non-significant, *; significant difference at $p < 0.05$ level, and **; significant difference at $p < 0.01$ level

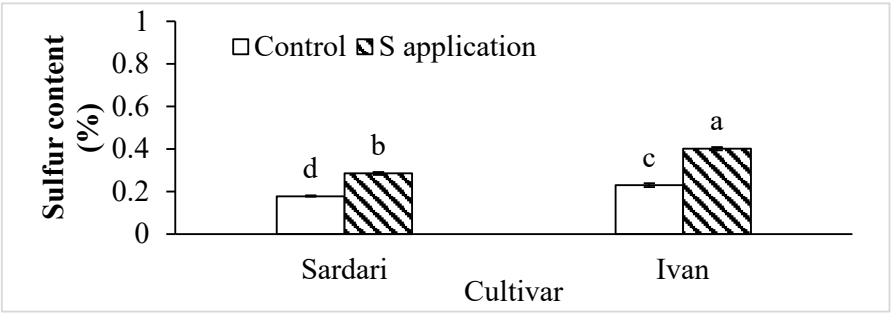

Fig. 1. Concentration of S in leaves of two wheat cultivars seedlings two days after foliar application of sulfur .Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

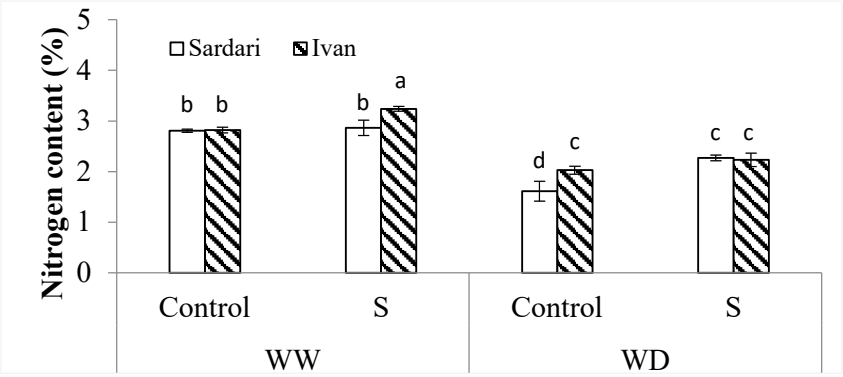

Fig. 2.Nitogent content in leaves of two wheat cultivars in response to sprayed S under well-watered (WW) and water-deficit (WD) conditions. Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

Proline-related-(*P5CS*) gene biosynthesis expression and proline accumulation

Fig. 3 exhibits the melting curve analysis chart of quantitative RT-PCR and agarose gel electrophoresis image of *P5CS* expression pattern in wheat seedling leaves in response to imposed drought and S-spraying. Expression analysis results of the *P5CS* gene under WW and WD conditions along with non-S-spray (control) and S-spraying (S) in Sardari and Ivan wheat cultivars are presented in Fig. 4. ANOVA revealed no significant difference between the two cultivars in terms of *P5CS* expression under WW conditions. However, drought significantly increased *P5CS* expression in Ivan cultivar plants in both S-spraying and control plants. Ivan cultivar plants exhibited higher *P5CS* expression under WD conditions than the same cultivar plants under WW conditions, especially when plants were foliar-applied with S (Fig. 4). However, applied S did not significantly affect the *P5CS* expression level of Sardari cultivar plants under both WW and WD conditions (Fig. 4).

Fig. (5) indicates the effect of foliar-applied S and water conditions on proline concentration in two tested wheat cultivars. Proline was determined two days after water deficit imposition (25% of WHC). Proline was statistically affected by S spray treatments under the drought conditions. As seen in Fig. 5, the imposed water deficit significantly augmented proline concentration in the leaves of two wheat cultivars. However, under WW conditions, plants that had received and not received S exhibited similar proline concentrations in leaves.

The trend of proline concentration to foliar-applied S under sufficient--watered and drought conditions was similar in the two tested cultivars. Accumulation of proline seems to be an effective way to sustain crop plant production in areas suffering from drought.

Proline acts as an osmo-protector and helps wheat plants to maintain osmotic balance (Hasan et al., 2020; Nadeem,2020; Nardino et al. 2022). Proline is also involved in the scavenging reactive oxygen species (Qayyum et al. 2021). Mwadzingeniet al. (2016) and Nardino et al. (2022) claimed that proline accumulation increased osmotic stress tolerance in wheat plants. Plants accumulate proline and boost the rate of antioxidant activity to cope with stressful conditions. Proline accumulation is a preliminary common response of many plants to stressful conditions, such as drought and salinity stress.

In the present study, foliar-applied S resulted in the up-regulation of *P5CS* expression in Ivan cultivar plants. At the same time, it had no significant effect on enhancing the expression level of this gene in the Sardari cultivar plants, indicating that the *P5CS* expression response to S spraying was variety-specific. Ornithine and glutamate are the two pathways for proline biosynthesis. Glutamate is converted to proline by two consecutive reactions. The two enzymes including Δ1-pyrroline-5-carboxylate synthetase (*P5CS*) and Δ1-pyrroline-5-carboxylate reductase catalyze the conversion of glutamate to proline (Meena et al., 2019). Over-expression of the *P5CS* in response to osmotic stress has been shown in wheat (Maghsoudi et al., 2018). Previous studies on wheat indicated a correlation between proline accumulation and *P5CS* gene expression (Jamshidi Goharrizi et al., 2023; Jamshidi Goharrizi et al., 2020; Maghsoudi et al., 2018). The over-expression of the *P5CS* gene encoding the *P5CS* enzyme has also been reported in transgenic wheat in response to water deficit imposition (Vendruscolo et al., 2007). The variation in genotypes in response to S application can be attributed to each cultivar's different tolerance mechanism and defense strategy. Differences in proline accumulation may also exist between genotypes of the same wheat species (Vuković et al., 2022). In the current study, the cultivars examined differed in proline accumulation. Previous investigations have also found that different plant species concentrate different osmolytes (Szabados et al., 2001; Vuković et al., 2022).

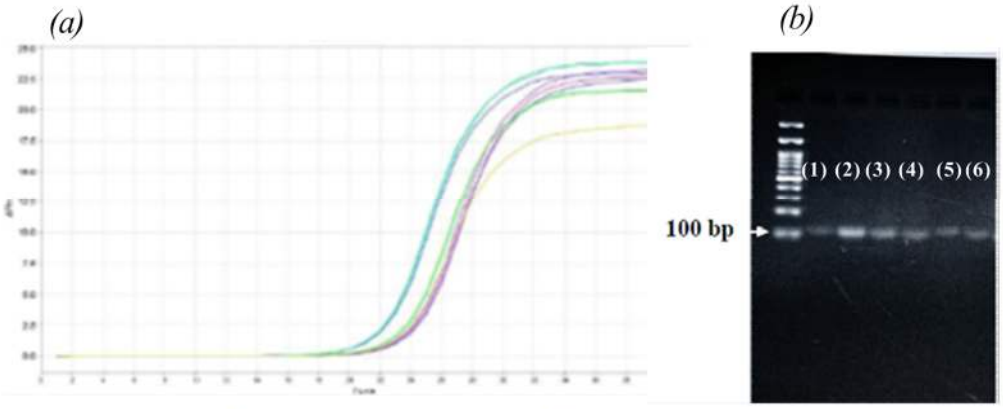

Fig. 3. (a), the melting curve analysis chart of quantitative RT-PCR and (b) agarose gel electrophoresis image of *P5CS* expression pattern of (1), Ivan cultivar under water-deficit-conditions; (2), Sulfur-sprayed plants of Ivan cultivar exposed to water-deficit-conditions; (3), S-applied Sardari cultivar under water deficit-conditions; (4) Unsprayed (control) Sardari cultivar exposed to water deficit-conditions; (5) S-sprayed Sardari cultivar under WW conditions; (6): Unsprayed Sardari cultivar under WW conditions as shown by differenr intemsity of an approximately 100 bp DNA band amplificd in quantitative RT-PCR

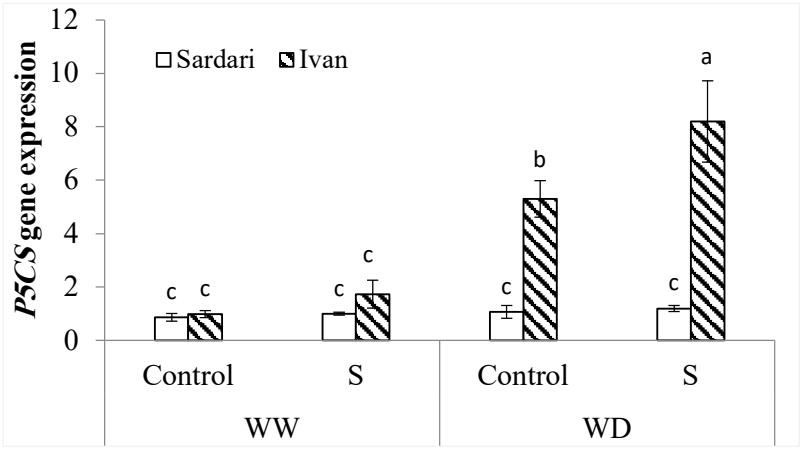

Fig. 4. *P5CS* expression level in seedling leaves of wheat Sardari and Ivan cultivars(in response to imposed water-deficit (WD) and well-watered (WW) conditions along with S spraying (S) or withour S spraying (control) . Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

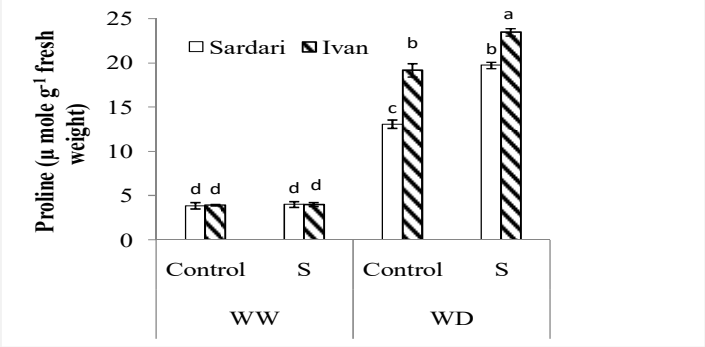

Fig. 5. Proline accumulation in seedling leaves of Sardari and Ivan wheat cultivars in response to imposed water-deficit (WD) and well-watered (WW) conditions and S spraying (S). Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

Chlorophyll (Chl) a and b, total Chl, and carotenoid contents were not influenced by the foliar-applied S (Table 4). However, drought significantly reduced Chl a, Chl b, total Chl, and carotenoids' pigments by 52%, 109%, 107%, and 98%, respectively, compared to WW conditions. Chl b, and total Chl were significantly affected by cultivar × drought interaction (Table 4). Ivan cultivar plants had significantly higher Chl b content than that of Sardari cultivar plants under WW conditions (Fig. 6). Total Chl content was higher in Sardari cultivar plants than that in the Ivan cultivar plants under drought conditions (Fig. 7). Sardri cultivar plants showed better performance under drought stress conditions in terms of carotenoids compared to Ivan cultivar plants (Fig. 8). Under drought conditions, higher concentrations of carotenoids were detected in foliar-S applied Sardari cultivar plants compared to control plants. However, there was not a significant difference in concentrations of carotenoids between the plants of the two treatments above. (Fig. 8).

The present results indicated that the applied S did not significantly decline the decrease in photosynthetic pigments (Chl a and b, and total Chl) in wheat plants under the WD conditions. This finding is in contrast to the results of Shah et al. (2022) and Resurreccion et al. (2001) who reported that foliar application of sulfur enhanced the chlorophyll pigment contents in mungbean and rice, respectively. It has been reported that ROS over-production causes damage to photosynthetic pigments under drought-stress conditions (Nxele et al. 2017). Although foliar-applied S plants exhibited a higher rate of antioxidant activities, proline content, and leaf relative water content than those of control plants, these increased defense reactions did not decrease the decline of photosynthetic pigments. The non-significant effect of applied S on protecting Chl pigments may be related to the severe prolonged drought imposed in the present study.

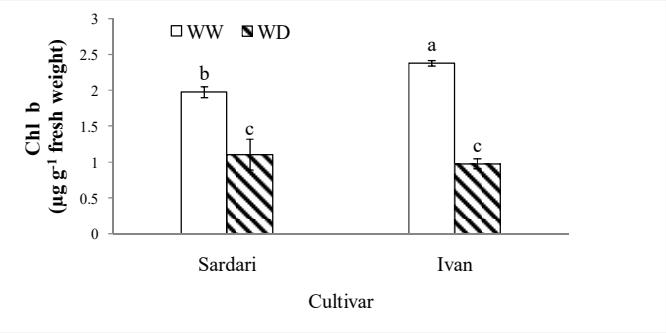

Fig. 6. Chlorophyll a content in leaves of Sardari and Ivan wheat cultivars under two water regimes (80% of water holding capacity and 25 %of water holding capacity) Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

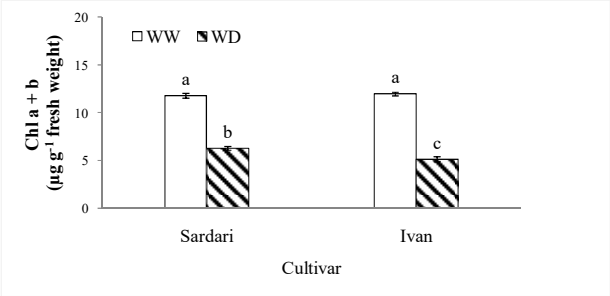

Fig. 7. Chlorophyll a+b (total Chl) content in leaves of Sardari and Ivan wheat cultivars under two water regimes (80% of water holding capacity and 25 %of water holding capacity) .Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

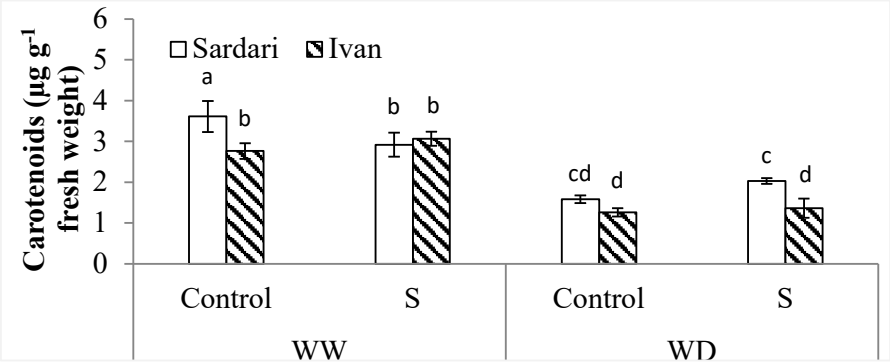

Fig. 8. Cartonoides content in leaves of Sardari and Ivan wheat cultivars under two water regimes (80% of water holding capacityand 25 %of water holding capacity) .Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

Antioxidant activity

The impacts of drought stress, S application, and their interaction on CAT activity in the Sardari and Ivan wheat cultivars are shown in Fig. 9. According to the results, the Sardari and Ivan wheat cultivars responded differently under control (WW) and drought stress conditions to foliar-applied S. Drought enhanced CAT activity regardless of cultivar type and S application. Water stress boosted the CAT activity by 39.2%. Applied S increased CAT activity up to 7.2%. Foliar-applied S significantly enhanced CAT activity in Sardari under WW conditions. Foliar-applied S had no significant effect on CAT activity in Sardari under drought conditions. However, compared with the control plants, the S application significantly boosted CAT activity in Ivan cultivar plants under WD. The highest rate of CAT activity was recorded with foliar-applied S in the Ivan cultivar under drought stress.The lowest CAT activity was recorded in the sufficient-watered control Sardari cultivar.

S foliar-sprayed plants of the Ivan cultivar revealed a higher rate of POX in leaves as compared to S-sprayed plants of Sardari cultivar under WW conditions (Fig. 10). Drought imposition significantly enhanced the activities of POX. The highest POX activities were recorded in foliar-S applied Sardari plants under drought conditions. The minimum activity of POX was recorded in unsprayed Sardari plants under WW conditions. Foliar-applied S did not significantly affect the POX activity rate in Ivan cultivar plants compared to the respective control plants under drought stress conditions.

Fig. 11 displays the effect of foliar-applied S on glutathione peroxidase (GP) activity in Sardari and Ivan wheat cultivars under well-watered and water deficiency conditions. Drought and applied S significantly increased GP activity in seedlings of both tested cultivars. Applied S as foliar spray significantly enhanced the activity of GP under imposed drought stress conditions The highest GP activity was observed in Ivan cultivar plants that were foliar-applied with sulfur and exposed to drought stress (Fig. 11).

Fig. 12 indicates the effect of S application and drought stress on glutathione reductase (GR) in the two studied wheat cultivars. S and drought significantly enhanced the GR activity in leaves. Under drought conditions, foliar-applied S resulted in a greater increase in GR activity in Sardari cultivar seedlings than in Ivan cultivar seedlings.

In the present study, foliar-applied S stimulated the enzymatic antioxidant system, indicating the capability of sulfur in the up-regulation of catalase, peroxidases, GP, and GR.

It has been shown that the antioxidant defense system sustains the equilibrium between reactive oxygen species (ROS) production and detoxification of ROS in plant cells (Vuković et al., 2022). Sulfur is a pivotal nutrient for glutathione and a main reserve for non-protein-reduced S. Sulfur is involved in the chemical structure of several amino acids. For instance, S is a constituent of methionine and cysteine. These two amino acids contain reduced sulfur. Methionine and cysteine are precursors of glutathione. Glutathione acts as a substrate in detoxifying enzyme mechanisms. Thus, in the current study, it was postulated that applying S to plants may be involved in methionine and cysteine biosynthesis and, consequently, biosynthesized glutathione.

Glutathione peroxidase is the most important intracellular agent of the antioxidant enzyme defense system, which catalyzes the reduction of H_2O_2 to H_2O and O_2 (Zhai et al., 2013). It has been shown that exogenous glutathione supplementation lowered the ROS production in wheat (Hasanuzzaman et al., 2018). Exogenous application of sulfur (S) has also been shown to reduce the H_2O_2 content in shoots of *Erysimum allionii* L. and *Isatiscappadocica* Desv (Arianmehr 2022). Das et al. (2021) reported that the supplementation of sulfur in Cd-stressed alfalfa triggered glutathione accumulation.

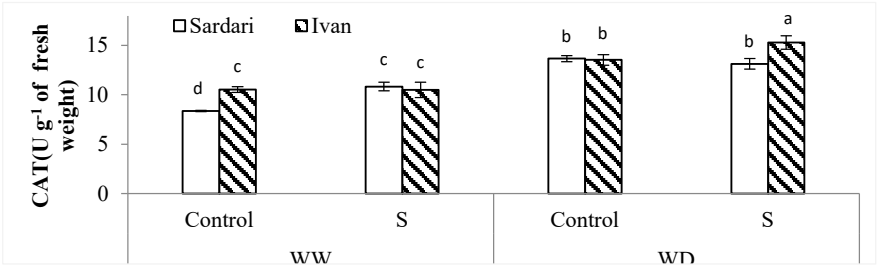

Fig. 9. Effect of applied sulfur (S) on catalase (CAT) enzyme activity under well-watered and water deficiency in Sardari and Ivan wheat cultivars. Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

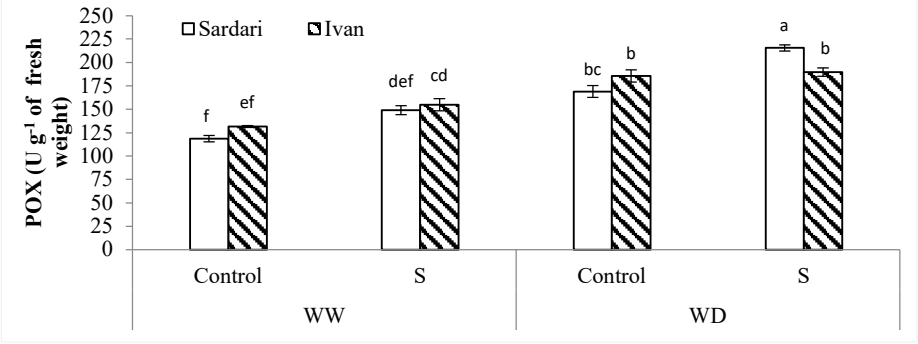

Fig. 10. Effect of applied sulfur (S) on peroxidase (POX enzyme activity under well-watered and water deficiency in Sardari and Ivan wheat cultivars. Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

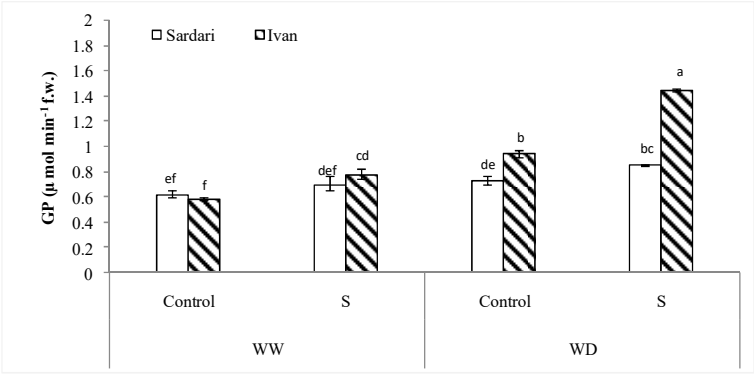

Fig. 11. Effect of applied sulfur (S) on glutathione peroxidase (GP) enzyme activity under well-watered and water deficiency in Sardari and Ivan wheat cultivars. Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

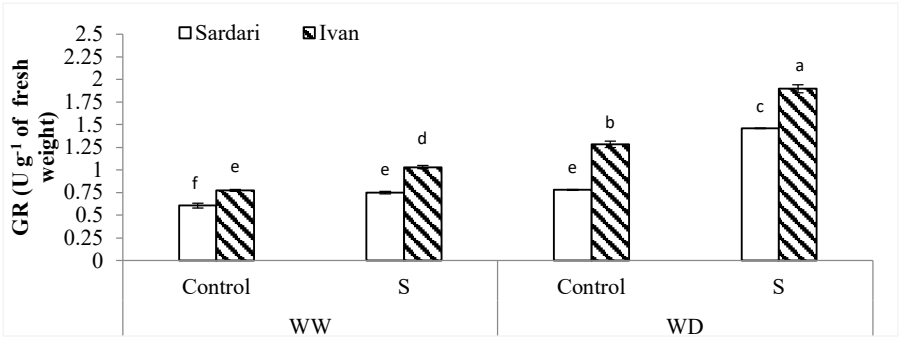

Fig. 12. Effect of applied sulfur (S) on glutathione reductase (GR) enzyme activity under well-watered and water deficiency in Sardari and Ivan wheat cultivars. Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

Leaf relative water content, electrolyte leakage, and proline accumulation in response to foliar-applied S at the anthesis stage

Analysis of variance (ANOVA) of drought imposition, cultivar, sulfur foliar application effects, and their interactions (experiment No. 2) on fag leaf relative water content (LRWC), electrolyte leakage (EL), and proline accumulation at the flowering stage on two wheat cultivars are presented in Table 5.

Electrolyte leakage (EL) and leaf relative water content (RWC) were significantly influenced by water deficiency as well as wheat cultivar and their interaction (Table 5). As displayed in Fig. 13, RWC was significantly decreased due to water-deficit conditions. Applied S did not affect RWC in Ivan cultivar plants compared to untreated plants under well-watered condition. However, RWC in plants of both cultivars positively responded to foliar-applied S and exhibited significantly higher RWC than that of control plants under the WD conditions. In this case, S application partially mitigated the adverse effect of drought by reducing the EL of leaves.

Plants foliar–sprayed with S exhibited significantly lower EL in both cultivar plants under WD conditions (Fig. 14). Ivan cultivar plants displayed lower EL than that of Sardari plants under drought conditions, so that under drought conditions, the lowest value of EL was detected in Ivan cultivar plants sprayed with S (Fig. 14).

Proline accumulation change in response to foliar-applied S, cultivar, and drought stress was significant (Table 5). Proline, measured at the anthesis stage, significantly increased by drought imposition (350%). The proline concentration was 1.2-fold higher in S-treated plants than in control plants. Ivan had higher proline accumulation in leaves (0.19 µ mol/ g f.w.) than Sardari (0.14 µ mol/ g f.w.). Under well-watered conditions, the trend of proline accumulation in response to foliar-applied sulfur was similar for both cultivars (Fig. 15). Under water deficit conditions, the highest proline content was recorded by Ivan plants sprayed with S (Fig. 15).

Leaf relative water content is an index of water deficit tolerance in leaves and water status within plants (Schonfeld et al., 1988; Vuković et al. 2022). S foliar-applied seedlings exhibited a far greater relative water content and accumulated a higher proline concentration in the leaf under soil water deficit conditions.

Table 5. Mean square (MS) of the source of variation for leaf relative water content (LRWC), electrolyte leakage (EL), and proline (Pr) accumulation in response to foliar-applied S at the anthesis stage

Treatment	RWC	LK	Pr
Sulfur (S)	4.02^{ns}	350**	0.010**
Cultivra (C)	117.6**	14^{ns}	0.016**
Drought (D)	3834.9**	7245**	0.323**
S×C	6.1^{ns}	31^{ns}	0.0001^{ns}
S×D	3.7^{ns}	238**	0.004**
C×D	379.8**	318**	0.008**
S×C×D	18.3*	73*	0.0016*
SE	4.2	17	0.00036
C.V. (%)	2.9	11	11.3

Ns; non-significant, *; significant difference at $p < 0.05$ level, and **; significant difference at $p < 0.01$ level

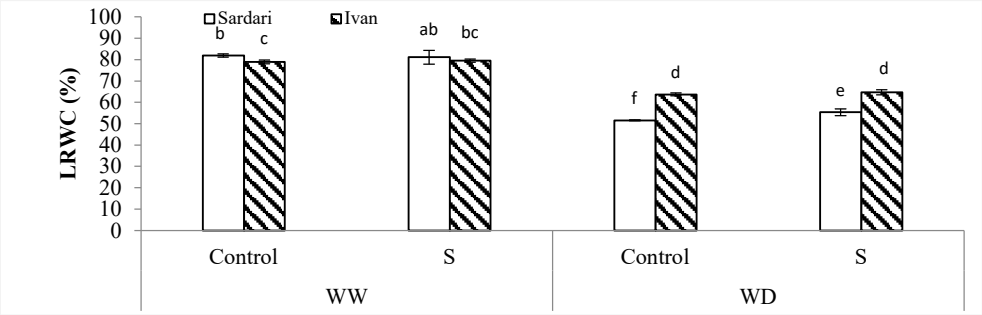

Fig. 13. Effect of foliar S application and drought stress on leaf relative water content in leaves of Sardari and Ivan wheat cultivar at anthesis stage .Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

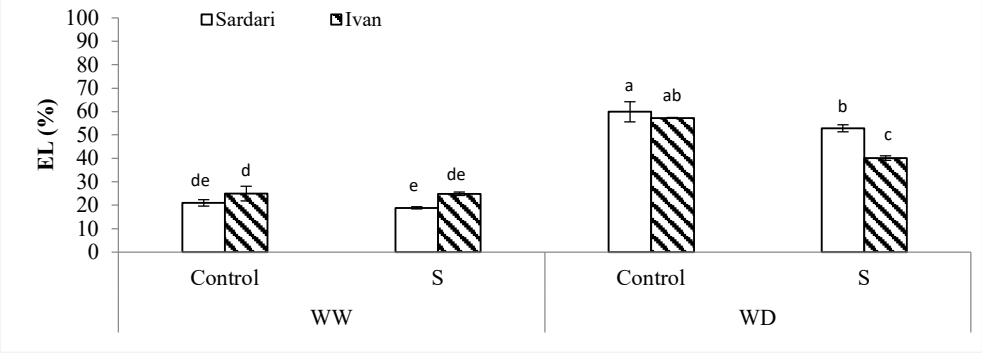

Fig. 14. Effect of foliar S application and drought stress on electrolyte leakage in leaves of Sardari and Ivan wheat cultivar at anthesis stage.Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

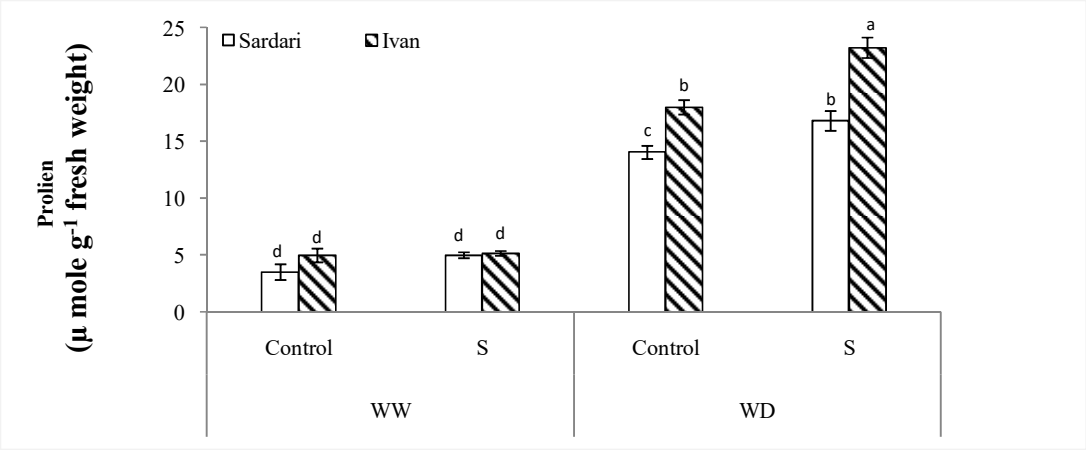

Fig. 15. Effect of foliar S application and drought stress on proline concentration in leaves of Sardari and Ivan wheat cultivar at anthesis stage .Treatments showing different letters on the related columns are significantly different (LSD test at a probability level of $p \leq 0.05$).

CONCLUSIONS

Drought stress is the most important leading environmental constraint to wheat production in semi-arid and arid areas of the world. In the present study, the effect of foliar application of sulfur (1%) on the water-stress responses of two wheat cultivars was elucidated. The molecular and biochemical analysis from the present study revealed that applied S could substantially enhance the evaluated gene (pyrroline-5-carboxylate synthase) along with proline accumulation, which can effectively diminish the adverse effect of water-defect stress. S application helped reactive oxygen species scavenging by activating antioxidant defense reactions that can indemnify the adverse effect of water stress. Water stress increased electrolyte leakage, but the foliar application of S alleviated the increase. The present study supports the important role of sulfur in conferring resistance to water stress in wheat which would probably be helpful for wheat growers to get better wheat performance in water-deficit soil conditions.

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Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable

Availability of data and materials

All data analyzed and generated during this study are included in this published article and its supplementary information files

Code availability

Not applicable

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication (include appropriate statements)

Not applicable

Author contributions

Performing the experiments and data collection by Zohreh Karimi; Supervision by Mohammad Javad Zarea; Advising by Arash Fazeli; Advising by Batool Zarei; All authors contributed to the article and approved the submitted version.

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نقش سولفور در بیان ژن پرولین-۵-کربوکسیلیت سنتاز (*P5CS*)، تجمع پرولین و فعالیت آنزیم‌های آنتی‌اکسیدانتی گندم در شرایط تنش کمبود آب

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چکیده - سولفور نقش کلیدی در رشد و نمو گیاه دارد و در بسیاری از کارکردهای زیستی گیاه مورد استفاده قرار می‌گیرد. برای بررسی نقش سولفور در حفاظت گیاه در مقابل تنش خشکی، دو آزمایش در شرایط گلخانه و اتاقک رشد در سال ۱۳۹۹ انجام شد. همچنین برای ارزیابی تاثیر محلول پاشی با عنصر سولفور بر بیان ژنهای مرتبط با ساخت پرولین(پرولین-۵-کربوکسیلات سنتاز، (*P5CS*)، تجمع پرولین، و فعالیت های آنتی_اکسیدانتی، گیاهان دو رقم سرداری و ایوان گندم در معرض دو سطح رژیم آبی قرار گرفتند. گیاهان در معرض تنش آبی در مقایسه با کیاهانی که تحت آبیاری مطلوب قرار گرفته بودند سطوح مختلف بیان ژن *P5CS* و تجمع پرولین را نشان دادند. هرچند محلول پاشی با گوگرد منجر به تغییر معنی دار در بیان ژن *P5CS* در رقم سرداری نگردید اما موجب افزایش معنی دار سطح بیان این ژن در رقم ایوان گردید. اعمال تنش آبی همراه با محلول‌پاشی سولفور موجب تجمع بیشتر پرولین گردید. اعمال تنش خشکی موجب افزایش فعالیت آنزیمهای آنتی اکسدانتی شامل پراکسیداز، کاتالاز، گلوتاتیون پراکسیداز و گلاتاتیون ریدوکتاز به ترتیب به میزان ۱۳۴، ۴۰/۴، ۵۴/۴ و ۷۷ /۴ درصد گردید. پراکسیداز و گلاتاتیون ریدوکتاز در اثر کاربرد سولفور به ترتیب به میزان ۱۹/۶ و ۵۱/۸ درصد افزایش معنی دار داشتند.گیاهان محلول پاشی شده با سولفور از نشت یونی کمتر و میزان بیشتر محتوای آب نسبی برگ نسبت به گیاهان شاهد برخوردار بودند. بر اساس نتایج این تحقیق مشخص گردید که سولفور موجب افزایش معنی دار بیان ژن *P5CS* و تجمع بیشتر پرولین گردید. همچنین گوگرد موجب فعال سازی واکنش‌های دفاعی مرتبط با اثرات سوء تنش کم آبی در گندم گردید.