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Impact of reduced irrigation on physiological, photosynthetic, and enzymatic activities in wheat (*Triticum aestivum* L.) exposed to water stress at varying plant densities

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Abstract. Drought stress is one of the most critical factors reducing the performance of crop plants in arid and semi-arid regions globally, and understanding underlying mechanisms is important to be able to implement measures for alleviating the negative impacts of drought on yield and yield quality. The present experiment aimed to investigate the effect of drought stress and planting density on the physiological characteristics, photosynthetic pigments, and enzymatic activity of wheat plants exposed to water stress. The experiment was conducted in a factorial arrangement based on a completely randomized block design with three replications. The treatments included irrigation cessation at three levels (control, i.e., full irrigation, irrigation until flowering (IUF), and irrigation until the dough stage (IUD)), and plant density (300, 400, 500, and 600 plants m^{-2}). The results indicate that leaf relative water content, soluble sugars, cell membrane stability index, chlorophyll a, b, and carotenoids significantly decreased under IUF, while these parameters increased at higher plant densities. Additionally, the interaction of drought stress and plant density significantly affected leaf proline and flavonoid content, total chlorophyll, and catalase activity. The highest leaf proline content (3.88 mg g^{-1} FW) was observed in IUF and a density of 600 plants m^{-2}, representing a 192% increase compared to the control. Additionally, the highest total chlorophyll content (3.66 mg g^{-1} FW) was recorded at no-stress conditions at a density of 500 plants m^{-2}. The activity of antioxidant enzymes increased under water stress. Overall, our results indicate that a density of 500 plants m^{-2} is optimal for maintaining stable growth conditions in wheat in the semi-arid to arid climate of Iran. These findings provide valuable insights to develop agronomic strategies for coping with drought in wheat cultivation, particularly in arid and semi-arid regions.

Keywords: drought stress, proline, soluble sugar, wheat, flavonoid.

HIGHLIGHTS

- Drought stress negatively affects the productivity of crop plants
- Plants are more sensitive to water stress during the flowering stage compared to the dough phase

– Optimal plant densities, such as 500 plants m^{-2}, can help mitigate drought stress.

1. INTRODUCTION

Wheat is cultivated on a large scale worldwide and ranks first among other cereals in terms of production and acreage, due to its high genetic flexibility and diversity (van Frank et al., 2020). Notably, wheat plays a vital role in Iran's food security as it is a staple food consumed daily, making it essential to the national diet (Dadrasi et al. 2023). Furthermore, Iran's policy of self-sufficiency in wheat production aims at ensuring a stable food supply and reducing dependency on imports (Ghaziani et al. 2023). The country cultivates 6 million hectares of wheat, with 2 million hectares being irrigated and 4 million hectares rain-fed. The total wheat production stands at 13.5 tons ha^{-1}, with an average yield of 4.44 tons ha^{-1}in rain-fed wheat cultivation (Dadrasi et al. 2023). In Iran, winter wheat is cultivated using three different cultivation methods: conventional, semi-mechanized, and mechanized Rain-fed cultivation, which enables economically viable production at lower costs, is found in areas with high rainfall, such as the Zagros and Alborz mountain ranges. Local irrigated wheat varieties, which are grown mainly in the temperate climate zones of the country, are Baharan, Pishgam, Sirvan, Mihan, and Heydari, while rainfed wheat varieties sare Azar, Karaj and Zagros. In addition, some international varieties such as Sultan and Kalyansona (which originate from Turkey and India, respectively) are also grown in Iran. Typical sowing dates are between November 1 and 20, and what is usually harvested in early July (Ghahremaninejad et al. 2021). The usual plant density is 450 to 500 seeds per m^2, which is about 10% below the optimal density (Ghahremaninejad et al. 2021).

The performance and physiological characteristics of wheat are influenced by factors such as water availability and plant density (Safar-Noori et al., 2018). Climate change has caused an increase in drought events, salinity problems, heat waves, and the occurrence of excessive radiation, all of which have worsened the conditions for wheat cultivation in many regions of the world (Ghadirnezhad Shiade et al., 2020; Taghavi Ghasemkheili et al., 2023) and has been responsible for a significant decline in agricultural productivity and quality (Brito et al., 2019). Given the fact that Iran is characterized by arid and semi-arid climatic conditions, droughts occur in decadal cycles (Hamarash et al. 2022). Prolonged and successive droughts significantly influence wheat yield and production, such that the country's production in dry years declines to 9 million tons, a much lower level than the 15 million tons typically harvested in wetter years (Ghaziani et al. 2023). Hence, irrigated wheat cultivation has gained importance to alleviate droughts and improve yield components (Zhao et al., 2020).

Drought stress negatively affects the growth, yield, biochemical, and physiological characteristics of crop plants (Safar-Noori et al., 2018). it impacts yield through various physiological processes, with the effects differing depending on the plant species (and cultivars), as well as the timing and severity of the drought (Seleiman et al., 2021). Water deficiency leads to reduced plant growth and yield due to increasing osmotic pressure, increased plant respiration, reduced photosynthesis, and consequently reduced cell division (Seleiman et al., 2021). In addition, various types of reactive oxygen species (ROS) are produced under stress conditions, acting as secondary messengers and playing an important role in transmitting stress signals (Ghadirnezhad Shiade et al., 2020; Ghadirnezhad Shiade et al., 2022). Plants possess antioxidant systems to mitigate the damages caused by increased production of ROS. These antioxidant systems in plants include non-enzymatic antioxidants such as proline, ascorbic acid, alpha-tocopherol, anthocyanins, phenols, flavonoids and some antioxidant enzymes such as catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and glutathione reductase (GR), which are responsible for scavenging ROS under various conditions (Møller et al., 2007; Ghadirnezhad Shiade et al., 2020). Plants with high levels of enzymatic antioxidants exhibit further resistance to oxidative damage (Kibria et al., 2017). Nasirzadeh et al., (2021) exposed wheat plants to three three levels of water stress, i.e., no stress (control), medium stress (volumetric soil water content at 40% of Field capacity (FC)), and severe stress (volumetric soil water content at 25% of FC). They noted that drought stress led to reduced relative water content (RWC), enhanced proline content, and antioxidant enzyme activities (catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX)). Similarly, increased soluble sugar and proline content, while decreased RWC, photosynthetic pigments content, and the membrane stability index (MSI) were observed in wheat plants subjected to various irrigation deficit patterns (Amoah & Seo, 2021).

One of the influential factors for increasing plant performance in agricultural management is the alteration of plant density, as this can lead to changes in crop yield and yield components (Li et al., 2020). In fact, plant density is one of the most critical factors in the ability of crops to utilize environmental resources because it affects the biochemical and physiological characteristics of the plant (D. Li et al., 2020). Optimal planting density varies depending on local or regional settings, planting date, climatic conditions (especially rainfall distribution), soil type, and wheat variety (Elhani et al., 2007). Previous studies have shown that adjusting plant density can help mitigate the adverse effects of drought. For instance, Li et al., (2020) conducted field experiments with three plant densities (480–570, 360–390, and 240–270 10^4 plants hm^{-2}) and three irrigation levels (no irrigation, 80.0 mm only at the jointing stage, and 60.0 mm each at the jointing and flowering stages). They found that increasing plant density can compensate for the adverse effects of water deficit under limited irrigation. The authors explained this result by suggesting that higher plant density enhances competition for resources, prompting the plants to optimize their use of water and nutrients.

To effectively identify the mechanisms of drought-tolerant plants, a comprehensive understanding of the physiological and antioxidant processes occurring in the crop under drought conditions is essential. These traits can used as valuable selection markers. Previous studies have largely overlooked the effects of drought stress on physiological parameters, photosynthetic pigments, and enzymatic activity of wheat at different planting densities. To fill this gap, this study aimed to: 1) Examine antioxidant levels in wheat exposed to different water stress levels and investigate the physiological, photosynthetic, and antioxidant mechanisms underlying drought tolerance in wheat; and, 2) Determine the optimal planting density to maximize yield under drought conditions.

Figure 1. Map of Iran indicating the geographical location of the Borujerd County (Lorestan Province).

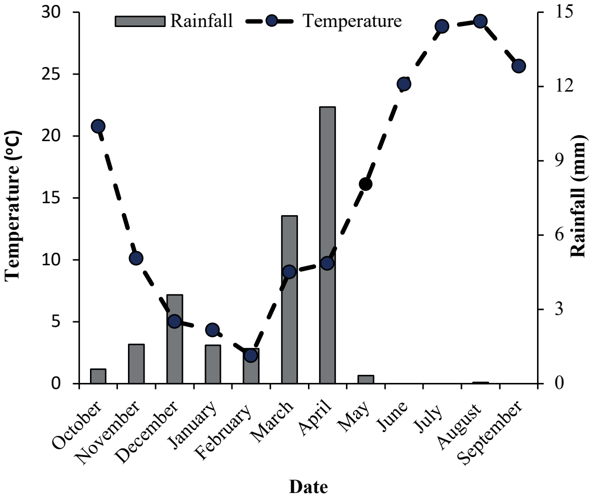

Figure. 2 Average rainfall (bars; scale on the right-hand side) and temperature (dashed line with dots; scale on the left-hand side) in the study area of Borujerd county.

2. MATERIALS AND METHODS

2.1. Experimental area and design

The experiment was conducted at the Agricultural Research Station of Borujerd County (48°8'E, 33°9'N, 1550 m above sea level), located in the Agricultural and Natural Resources Research Center of Lorestan Province, Iran (Figure 1). The seasonal evolution of average rainfall and temperature in the study area is shown in Figure. 2. The experiment ran from October 2020 to September 2021. The planting date was November 6, and the harvest date was June 26. The approximate flowering date was May 4, and the dough stage occurred on June 4. The experiment was carried out in a factorial design based on a completely randomized block design with three replications. The experimental treatments contained three levels of irrigation (control, i.e., full irrigation, irrigation until flowering (IUF), and irrigation until the dough stage (IUD)), and four plant densities (300, 400, 500, and 600 plants per m^2). Plants were irrigated by flood irrigation, which in IUF and IUD was discontinued based on when the corresponding developmental stages were reached. Irrigations were applied on a weekly basis when soil water fell below field capacity (FC) (i.e., between 20% and 30% of the soil's volume). The physical and chemical analysis of the soil in study area are (0–30 cm depth) presented in Table 1.

Table 1. Physicochemical properties of the soil in areas of Borujerd, Iran.

Sand	Silt	Clay	Fe	NH_4	K	P	OC	EC	pH
	%			ppm			%	ds m^{-1}	
16.11	34.75	49.14	7.11	5.14	278	10.4	1.17	0.51	7.91

Fe: Iron;.NH4: Ammonium, K: potassium; P: phosphorus; OC: organic carbon; EC: electrical conductivity.

2.2. Measured physiological parameters

2.2.1. Cell membrane stability index (CMSI)

Three plants were randomly selected from each plot and the upper third part of their leaves were harvested. These leaves were individually wrapped in plastic bags and transferred to the laboratory. Leaf discs were prepared from the samples. Next, each sample (0.1 g) was mixed with distilled water (10 mL) and autoclaved at 100 °C for 15 minutes. Similarly, another set of samples was placed in Falcon tubes and kept at 40 °C for 30 min. Then, the samples were maintained in the laboratory environment until their temperature reached 25°C. Subsequently, the EC (electrical conductivity) of each Falcon tube was measured using an EC meter, and finally, the membrane stability index was calculated using Eq. (1) (Amoah & Seo, 2021):

$$CMSI = [(1- c_1/c_2) \times 100] \quad (1)$$

where, c_1 and c_2 represent the EC values at 40 and 100 °C, respectively.

2.2.2. Relative Water Content (RWC) of the leaves

Three plants were randomly selected from each plot and the upper third of their leaves were harvested at the same age. These leaves were individually packed in plastic bags and transferred to the laboratory. Each leaf was measured (fresh weight (FW)) and then placed in distilled water at 4 °C in the refrigerator for 24 hours. The leaves were then removed from the distilled water, dried with filter paper, and weighed again (saturation weight (TW)). They were then placed in an oven at 75 °C for 48 hours and weighed again (dry weight (DW)). The relative water content was calculated using Equation 2 (Ghadirnezhad Shiade et al., 2023)

$$RWC = [(FW - DW)/(TW - DW)] \times 100 \quad (2)$$

2.2.3. Measurement of soluble sugars

Dried plant samples were powdered and mixed with 80% ethanol (8 mL) and incubated for 30 minutes at 80 °C in a water bath. Afterward, the tubes were allowed to cool, followed by centrifugation at 2000 rpm for 20 minutes. The extraction process was repeated three times. To the collected liquid phase, 5.3 mL of 5% zinc sulfate ($ZnSO_4$) and 5.3 mL of 0.3 N barium hydroxide ($Ba(OH)_2$) were sequentially added to remove pigments. The liquid phase was then centrifuged again at 2000 rpm for 20 minutes. The liquid phase was transferred to a volumetric flask of 100 mL, and 2 mL of the resulting solution was used to determine the concentration of soluble sugars using the phenol-sulfuric acid method. The absorption of samples was measured using a spectrophotometer (model 6305 Jenway), at 485 nm. According to (Amoah & Seo, 2021).

2.2.4. Measurement of proline

Proline content was measured using the method described by Bates et al. (1973). The leaf sample (1 g) was homogenized with 10 mL of 3% sulfosalicylic acid in a mortar and centrifuged at 4000 rpm for 10 minutes. Then, 2 mL glacial acetic acid and 2 mL ninhydric acid were added and incubated in a boiling water bath at 100 °C for one hour. Then 4 mL of toluene was added to the sample and the optical absorbance was measured at 520 nm using a spectrophotometer.

2.2.5. Measurement of anthocyanin and flavonoids

The measurement of anthocyanins and flavonoids in the leaves was performed spectrophotometrically using the method described by (Wagner, 1979) and (Krizek et al., 1998).

2.3. Leaf chlorophyll and carotenoids

The leaf samples were randomly taken from three plants in each plot. The measurement of chlorophyll (chlorophyll a, chlorophyll b, and total chlorophyll) and carotenoids was carried out using a non-destructive method. Leaf samples (0.5 g) were placed in 5 mL of dimethyl sulfoxide at 65 °C for 4 hours. Then, chlorophyll was measured using a spectrophotometer. The absorption levels at wavelengths of 665 (A665), 645 (A645), and 470 (A470) nm were recorded, respectively. Subsequently, the amounts of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Total Chl), and carotenoids were calculated using Eqs. 3-6 (Ghadirnezhad Shiade et al., 2023):

$$\text{Chl a} = (12.19 \times \text{A665}) - (3.45 \times \text{A645}) \tag{3}$$

$$\text{Chl b} = (21.99 \times \text{A645} - 5.32 \times \text{A665}) \tag{4}$$

$$\text{Total Chl} = \text{Chl a} + \text{Chl b} \tag{5}$$

$$\text{Carotenoeid} = (1000 \times \text{A470} - 2.14 \times \text{Chla} - 70.16 \times \text{Chlb}) / 220 \tag{6}$$

2.4. Measurement of enzymatic activity

To measure the activity of the catalase (CAT) enzyme, a leaf sample (1 g) was mixed with 3 mL of buffer, extracted, and then centrifuged. Absorbance at 240 nm wavelength was measured for two minutes, and the initial minute data, with an extinction coefficient of 0.0394/0 mM cm^{-1}, was utilized to calculate catalase activity (Chance & Maehly, 1955). For peroxidase (POD) enzyme activity, a leaf sample (2 g) was mixed with 4 mL of buffer, extracted, and after centrifugation, absorbance at 470 nm wavelength was measured for 4 minutes. The initial minute data, with an extinction coefficient of 47.2 mMol cm^{-1}, was used to calculate POD (Chance & Maehly, 1955). The assay for superoxide dismutase (SOD) activity included 0.055 M of nitroblue tetrazolium, 1.42% Triton X-100, 0.1 mM of Ethylenediaminetetraacetic Acid (EDTA), and 16 mMol pyrogallol. After adding tissue sample extract, the change in absorbance at 560 nm wavelength was evaluated using a spectrophotometer (Masayasu & Hiroshi, 1979). Moreover, the assay for Glutathione Reductase (GR) activity on the sample containing 100 mMol potassium phosphate, 1 mMol EDTA, 2.0 polyvinyl phosphate, 2 mMol EDTA, 1.5 mMol magnesium chloride, 5.0 mMol oxidized glutathione, and 50 mMol of Nicotinamide adenine dinucleotide, reduced form (NADH) was read at 340 nm wavelength (Sgherri et al. 1994).

2.5. Data analysis

Data analysis was performed using SAS software, and the graphs were plotted using Origin Pro 2021 software. The Least Significant Difference (LSD) method at a 5% significance level was employed for mean data comparison.

3. RESULTS

The analysis of variance revealed that the main effects of drought stress and planting density significantly impacted all studied traits. Furthermore, the interaction effects of these factors influenced proline content, flavonoid levels, total chlorophyll, and catalase activity (Table 2).

3.1. Physiological parameters

The study findings indicated that the CMSI was affected by drought stress treatment (Table 3). Specifically, the highest value (85.8%) was observed in the control treatment, decreasing by 23% when terminating irrigation at the IUF stage, and by 16% when irrigation was terminated at the IUD stage. Additionally, the highest value of this parameter (82.21%) was recorded at a plant density of 500 plants m^{-2}, whereas the lowest value (69.35%) was reported at a density of 300 plants m^{-2} (Table 3). Similarly, the highest RWC value (81.21%) was observed in the control, decreasing by 19% and 11% under IUF and IUD, respectively (Table 3). Moreover, a plant density of 500 plants m^{-2} resulted in the highest RWC (76.89%), while the lowest value was observed at a density of 300 plants m^{-2}, at 59.25% (Table 3).

In addition, the study revealed an increase in the soluble sugar content of wheat under water stress at different growth stages (Table 3). Initially, the highest concentration of 1.95 μg g^{-1} FW was observed under the IUF treatment, which subsequently decreased by 45% to 1.52 μg g^{-1} FW under the IUD treatment. Additionally, a plant density of 600 plants m^{-2} triggered the highest parameter value (1.72 μg g^{-1} FW), which then reduced by 44% to the lowest value (0.95 μg g^{-1} FW) at a density of 500 plants m^{-2} (Table 3). The lab data showed that the highest level of leaf proline (3.88 mg g^{-1} FW) was achieved in the IUF and 600 plants m^2 density, which was statistically significantly different from the other experimental treatments. The lowest amount was recorded at the interaction effect of control and 500 plants m^{-2} density (1.41 mg g^{-1} FW) (Figure 3).

Besides, it was observed that the anthocyanin content in wheat was at its lowest level under non-water deficit conditions (34.1 mg g^{-1}). However, with increasing drought stress severity, the production of anthocyanins increased, with the highest level of anthocyanin production (2.34 mg g^{-1}) under the IUF treatment, decreasing

Table 2. Analysis of variance for physiological parameters, photosynthetic pigments, and enzymatic activity of wheat plants under drought stress and different plant densities.

SOV	df	CMSI	RWC	Soluble sugars	Proline content	Anthocyanin	Flavonoid	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid	Catalase	Peroxidase	Superoxide dismutase	Glutathione reductase
Block	2	8.59	49.11	0.04	0.15	0.007	0.02	0.021	0.0009	0.001	0.006	58028	5.7	0.001	0.005
Irrigation	2	959*	659**	1.33**	4.11*	2.98**	10.5**	3.31*	0.81**	9.33**	4.24*	17144743**	336*	3.54**	3.63**
Density	3	99**	139**	0.10**	0.85*	0.1**	0.57**	1.11*	0.091*	1.2**	0.46*	169692**	198	0.04**	0.05*
Irrigation × Density	6	11.33	1.11	0.005	0.19*	0.005	0.03*	0.031	0.006	0.44**	0.021	51199*	40	0.005	0.01
Error	18	39.26	8.11	0.011	0.03	0.003	0.009	0.028	0.004	0.021	0.008	13165	58	0.003	0.008
CV		9.21	8.66	12.2	7.5	3.07	1.85	10.11	9.11	10.11	8.12	5.57	19	5.43	9.77

ns: no significant, * significant at 5% level, ** significant at 1% level.

Table 3. Physiological traits for the examined irrigation treatments and plant densities.

Treatments	CMSI (%)	RWC (%)	Soluble sugars ($\mu g\ g^{-1}$ FW)	Anthocyanin ($\mu g\ g^{-1}$ FW)
Irrigation				
Control	85.8 a	81.2 a	1.12 c	13.4 c
IUF	65.1 c	65.32 c	1.95 a	2.34 a
IUD	71.2 b	71.68 b	1.52 b	1.77 b
Plant density				
300 plant m^{-2}	69.35 c	59.25 d	1.40 b	1.71 b
400 plant m^{-2}	71.16 bc	64.41 c	1.11 bc	1.83 ab
500 plant m^{-2}	82.21 a	76.89 a	0.95 c	1.97 a
600 plant m^{-2}	75.15 ab	69.15 b	1.72 a	1.76 b

CMSI: cell membrane stability index, RWC: relative water content.

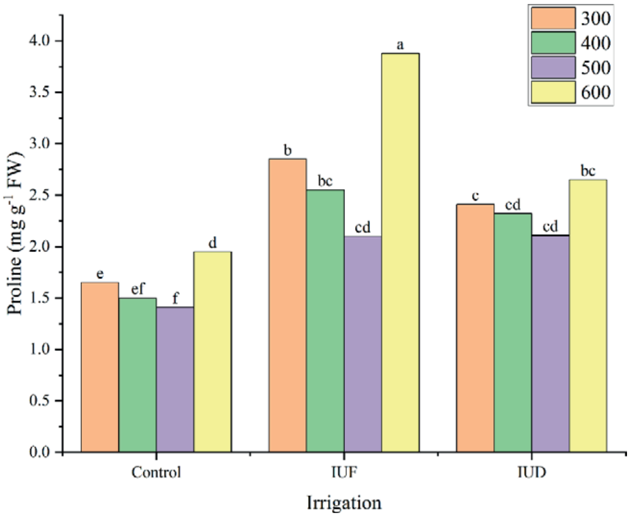

Figure 3. Interaction effects of irrigation practices (control, IUF and IUD) and plant densities (300-600 plant m^{-2}; colors) on proline content. IUF (Irrigation until the flowering stage), IUD (Irrigation until the dough stage).

(1.77 mg g^{-1}) under the IUD treatment (Table 3). Furthermore, the highest value of this parameter (1.97 mg g^{-1}) was observed at 500 plant m^{-2}, while no significant difference was observed in other treatments (all showing approximately 1.80 mg g^{-1}). On the other hand, flavonoid content in wheat leaves was influenced by the interactive effects of drought stress and plant density per unit area (Figure 4). Under non-drought stress conditions, the flavonoid content was higher at all plant densities than in the water deficit stress treatments. Additionally, the results showed that increasing plant density up to 500 plants m^{-2} led to an increase in flavonoid content in all drought stress treatments, while at a density of 600 plants

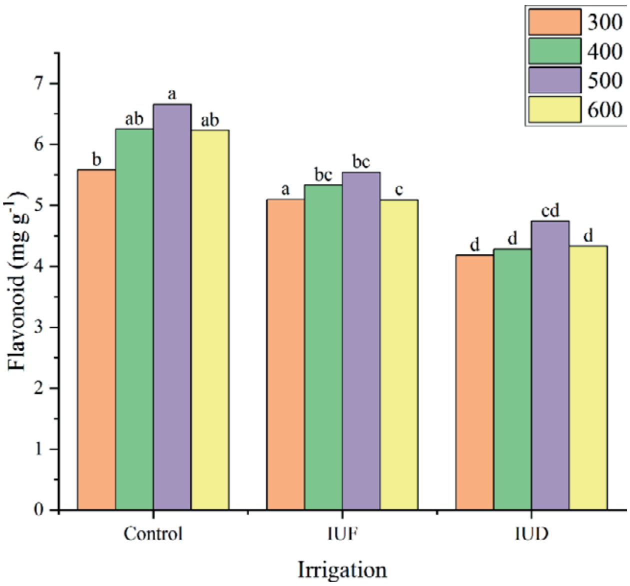

Figure 4. Interaction effects of irrigation practices (control, IUF and IUD) and plant densities (300-600 plant m^{-2}; colors) on flavonoid content. IUF (Irrigation until the flowering stage), IUD (Irrigation until the dough stage).

m^{-2}, there was a noticeable decrease in flavonoid content across all drought stress levels. The highest flavonoid content (6.6 mg g^{-1}) was observed under non-drought stress conditions and a plant density of 500 plants m^{-2}. This treatment differed significantly from other experimental treatments except for plant densities of 400 and 600 plants m^{-2} under the same level of drought stress. When irrigation continued until IUD, the flavonoid content in leaf tissues reached its lowest level across various plant densities (4.38 mg g^{-1}), (Figure 4).

3.2. Photosynthetic pigments

Our results revealed that chlorophyll a content was highest under the control treatment (1.92 mg g^{-1} FW), decreasing by 50% under IUF (0.95 mg g^{-1} FW)Under IUD, a less severe reduction of 23% was observed (1.46 mg g^{-1} FW) (Table 4). Furthermore, the chlorophyll a content increased with increasing plant density up to 500 plants m^{-2} (1.72 mg g^{-1} FW), but it decreased again at a density of 600 plants m^{-2} by 52% (1.34 mg g^{-1} FW). The lowest chlorophyll a content (1.11 mg g^{-1} FW) was observed at a density of 300 plants m^{-2} (Table 4). The same trend was recorded for Chl b under both drought and plant density treatments. Accordingly, the highest value (0.88 mg g^{-1} FW) was observed in the control treatment, decreasing by 60% under the IUF and 40% under IUD treatments. In relation to plant density, the highest value (0.73 mg g^{-1} FW) was recorded at 500 plants m^{-2}, while the lowest value (0.51 mg g^{-1} FW) was recorded at 600 plants m^{-2}.

Table 4. Photosynthetic pigments for the examined irrigation treatments and plant densities.

Treatments	Chlorophyll a(mg g^{-1} FW)	Chlorophyll b (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)
Irrigation			
Control	1.92 a	0.88 a	2.11 a
IUF	0.95 c	0.35 c	0.92 c
IUD	1.46 b	0.52 b	1.52 b
Plant density			
300 plant m^{-2}	1.11 c	0.42 d	1.25 d
400 plant m^{-2}	1.59 ab	0.62 b	1.39 c
500 plant m^{-2}	1.72 a	0.73 a	1.48 b
600 plant m^{-2}	1.34 b	0.51 c	1.71 a

As for catenoid content, the highest level (2.11 mg g^{-1} FW) was obtained under conditions of no drought stress (Table 4). Additionally, the results indicated that with irrigation cut-off, the level of leaf carotenoids decreased 0.92 mg g^{-1} FW in IUF and 1.52 mg g^{-1} FW in IUD, respectively (Table 4). Furthermore, it was shown that the leaf carotenoid content was higher (1.71 mg g^{-1} FW) at 600 plants m^{-2} than at other densities. Decreasing the plant density from 600 to 300 plants m^{-2} resulted in a reduction in leaf carotenoid content by 18% (Table 4).

Our data indicate that the total chlorophyll content was influenced by the interaction of drought stress and plant density (Figure 5). Although the IUF treatment resulted in a greater reduction in this parameter compared to the IUD and control treatments, the highest value (3.66 mg g^{-1} FW) was measured at a plant density of 500 plants m^{-2}. Under IUF, the total chlorophyll content decreased for all studied plant densities. Accordingly, the lowest total chlorophyll content (1.52 mg g^{-1} FW) was observed in the treatment with a plant density of 300 plants m^{-2} at the IUD stage (Figure 5).

3.3. Enzymatic activity

The results indicated that in all planting densities, the CAT enzyme activity increased with the severity of drought stress (Figure 6). The increase in enzyme activity was significantly greater under IUF compared to both IUD and the control treatment. Additionally, further increasing the plant density contributed to this enhanced enzyme activity. The highest CAT activity (3956 nmol/g/min) was observed under IUF and a plant density of 600 plants m^{-2}, significantly differing from

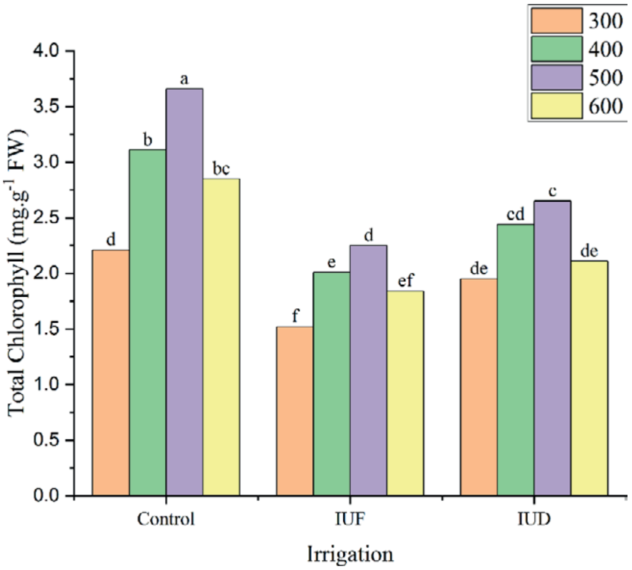

Figure 5. Interaction effects of irrigation practices (control, IUF and IUD) and plant densities (300-600 plant m^{-2}; colors) on total chrophyll content. IUF (Irrigation until the flowering stage), IUD (Irrigation until the dough stage).

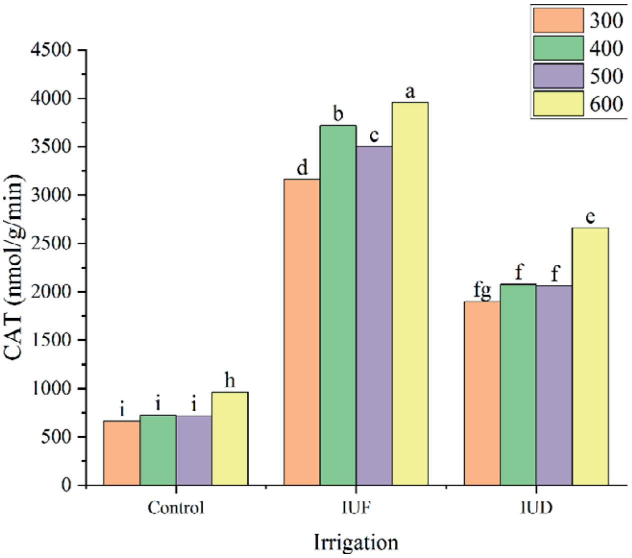

Figure 6. Interaction effects of irrigation practices (control, IUF and IUD) and plant densities (300-600 plant m^{-2}; colors) on Catalase (CAT) activity. IUF (Irrigation until the flowering stage), IUD (Irrigation until the dough stage).

other treatments, whereas the lowest value (660 nmol/g/min) was found in the control treatment and at a plant density of 300 plants m^{-2} (Figure 6).

Table 5. Enzymatic activity for the examined irrigation treatment and plant densities.

Treatments	POD (nmol/g/min)	SOD (µmol/g/min)	GR (nmol/g/min)
Irrigation			
Control	39 a	0.61 b	0.35 c
IUF	43 a	0.73 b	0.84 b
IUD	31 b	1.75 a	1.61 a
Plant density			
300 plant m^{-2}	33.8 b	0.96 b	0.87 b
400 plant m^{-2}	43 a	1.1 a	1.02 a
500 plant m^{-2}	45 a	1.4 a	1.06 a
600 plant m^{-2}	37.5 ab	1.02 b	0.92 b

The results reported in Table 5 indicate that the drought conditions led to an increase in the POD activity with the highest value (43 nmol/g/min) obtained under the IUF treatment. On the other hand, the lowest POD activity was observed under IUD (31 nmol/g/min). Additionally, the results showed that this enzyme activity was lower at a plant density of 300 m^{-2} (33.8 nmol/g/min) than under the other experimental treatments. Although no statistically significant difference was observed among the three densities of 400 - 600 plants m^{-2}, the results show that the highest activity of the POD enzyme was achieved at a density of 500 m^{-2} (45 nmol/g/min) (Table 5). As for SOD enzyme activity, the data show that it was lower under control conditions (61 µmol/g/min) than in conditions of water stress. Furthermore, under both IUF and IUD, the activity of this enzyme increased, with the highest increase observed in IUD (1.75 µmol/g/min) (Table 5). Similar changes were recorded for GR, with the highest activity (1.61 µmol/g/min) in IUD, and the lowest activity (0.35 µmol/g/min) in the control experiment (Table 5).

DISCUSSION

This study aimed to evaluate the effects of irrigation and plant densities on the physiological characteristics of wheat. The results revealed that both the individual treatments as well as their interactions significantly impacted the studied parameters. Our results indicated a considerable reduction of the CMSI under water stress conditions. Accordingly, water deficit stress increased electrolyte leakage from the cell walls and decreased cell membrane stability. In addition, our study showed a positive relation between increased cell membrane stability and other traits such as proline and soluble sugars. Specifically, we found that wheat leaves grown at a density of 500 plants m^{-2} had the highest cell membrane stability. Interestingly, these plants had the lowest levels of soluble sugars and proline. This suggests that higher plant densities might create conditions that better maintain cell membrane stability, possibly by optimizing resource use and reducing stress at the cellular level. Our results are consistent with those of Amoah & Seo, (2021) who documented reduced CMSI under drought stress in wheat plants.

According to our results, the RWC of the leaves was reduced under water deficit. Water deprivation starting at the flowering stage (IUF) compared to water deprivation starting at the dough stage (IUD) resulted in longer-lasting water deficit exposure of the wheat plants, leading to more severe drought stress and a greater reduction in RWC in IUF than IUD. The imbalance between water supply and demand in plants is the most probable reason for the reduction in RWC under drought conditions. Similar results were reported by (Amoah & Seo, 2021) on wheat and Ghadirnezhad Shiade et al., (2023) on rice under salinity stress. Furthermore, Nasiri et al., (2017) observed similar results regarding RWC in higher plant densities (60 and 80 plants m^{-2}) on RWC. RWC is a critical parameter for assessing plant water status, quantifying the ratio of current water content to the plant's maximum water-holding capacity. Under drought conditions, a further decrease in RWC often signifies a substantial disruption in plant water relations. This disruption occurs because the plant's capacity to absorb and retain water from the soil is compromised, leading to reduced turgor pressure and impaired physiological processes. Consequently, the plant's water content diminishes, which points to the plant's difficulty in maintaining homeostasis. This decline in RWC can significantly impact various aspects of plant health, including nutrient uptake, photosynthetic efficiency, and overall growth. Additionally, under water deficit conditions, the roots cannot compensate for the water lost through transpiration, leading to a reduction in leaf water potential. Leaf relative water content directly correlates with leaf turgidity and plant water potential.

The increased anthocyanin content in wheat leaves under drought stress conditions can be attributed to the role of anthocyanins as UV-absorbing pigments in the leaf epidermis, protecting plant tissues from damage caused by these radiations (Hatier & Gould, 2008). Furthermore, it has been suggested that anthocyanin compounds accumulate in vacuoles or bind to the cuticle wall, serving as a protective response of plants against UV radiation (Carvalho et al., 2010). Additionally, the results of this study showed that the anthocyanin content in wheat leaves increased with density plant density increasing from 300 to 500 plants m^{-2}, decreasing again when plant density was further increased to 600 plants m^{-2}. The increase in anthocyanin content for plant densities up to 500 plants m^{-2} can be attributed to enhanced light absorption efficiency and improved protection of the photosynthetic system, thereby positively affecting dry matter production. Further increasing the plant density up to 600 plants m^{-2} appears to enhance shading within the wheat canopy. This reduced UV penetration into the canopy lowers anthocyanin production in the plants. Similar results were documented by Onjai-uea et al., (2022) who studied plant spacing, variety, and harvesting age effects on purple Napier grass.

Moreover, we observed that drought stress and plant density interactions also influenced the flavonoid content of wheat leaves. Under control conditions, the flavonoid content of leaves was higher in denser plant habitats compared to those under drought stress treatments. Moreover, flavonoid content increased with plant density up to 500 plants m^{-2} under drought stress but decreased significantly when plant density was further increased to 600 plants m^{-2}. Flavonoids play a crucial role in plant stress responses, including antioxidant activity and protection against oxidative stress caused by droughts (Taheri Asghari& Hossein, 2014; Onjai-uea et al., 2022). Under no drought stress, higher plant densities promote flavonoid accumulation due to efficient resource use. Under drought conditions, moderate densities (up to 500 plants m^{-2}) trigger stress responses that enhance flavonoid production, whereas excessive densities (600 plants m^{-2}) lead to reduced flavonoid content due to heightened competition and resource scarcity. Similar results were reported by Taheri Asghari& Hossein, (2014) who noted enhanced Kaempferol (a kind of flavonoid) content in Chicory (*Cichorium intybus* L.) which was exposed to water deficit conditions and cultivated at various planting densities. The antioxidative property of flavonoids, conferred by the hydroxyl group attached to a ring structure, involves the transfer of an electron to free radicals, reducing their potential. Flavonoids serve as effective scavengers for peroxide radicals, reducing the potential of alkyl peroxide radicals and ultimately acting as potent inhibitors of lipid peroxidation (Molor et al., 2016).

This study not only found that drought stress leads to an increase in proline content in the leaves, but also that an increase in plant density up to 500 plants m^{-2} leads to a decrease in proline content in the leaves at all drought stress levels. However, with an increase in plant density to 600 plants m^{-2}, the proline content of the leaves increased significantly. Regardless of drought stress effects, optimal plant growth conditions were achieved with an increase in plant density of up to 500 plants m^{-2}, leading to a decrease in leaf proline content.

However, at the higher density, stressful conditions were created due to increased competition between plants for water and nutrients as well as shading from overlapping plants, resulting in higher proline content. In this respect, our results are consistent with those of Nasiri et al., (2017).

In this study, discontinuing irrigation at different growth stages of wheat enhanced soluble sugar content. This phenomenon can be attributed to the establishment of osmotic regulation, with enhanced soluble sugar content resulting in reduced water loss from the plant and aiding in maintaining the stability of plant cells under drought-stress conditions (Shiade et al., 2024). Compatible solutes such as soluble sugars do not interfere with normal biochemical reactions of the cell and act as osmotic protectants during osmotic stress (Shiade et al., 2024). Similar results were reported by Li et al., (2014). Furthermore, it was found that increasing plant density from 300 to 500 plants m^{-2} decreased soluble sugar content, while further increasing plant density to 600 plants m^{-2} resulted in a greater increase in this compound. This can be attributed to the plant's resilient condition at 500 plants m^{-2} density.

Both the IUF and IUD treatments adversely affected the synthesis and stability of photosynthetic pigments, resulting in decreased content. Similar reductions in chlorophyll content under drought stress conditions have been reported in other studies (Nasiri et al., 2017). Taheri Asghari & Hossein (2014) also reported a reduction in chlorophyll content under drought stress conditions, noting that plants not suffering from drought stress present fewer damages from free radicals and higher leaf chlorophyll content. The negative effect of drought stress on chlorophyll content in wheat plants can be explained by the destruction of thylakoid membranes in chloroplasts and the photo-destruction of chlorophyll due to increased activity of oxygen species and chlorophyllase enzymes (Naroui Rad et al., 2012). Additionally, the activity of chlorophyllase is stimulated by the increase in some growth regulators such as ethylene and abscisic acid under drought stress (Amoah & Seo, 2021). An increase in plant density of up to 500 plants m^{-2} led to elevated photosynthetic pigment content, but the level of photosynthetic pigments was reduced under higher plant densities (600 plants m^{-2}). This phenomenon can be attributed to internal factors within the plant resulting from competition between plants for nutrient uptake in the soil. Another reason could be the reduction in leaf area due to excessive plant density, which leads to a decrease in photosynthetic pigment content. Similar results were reported by Nasiri et al., (2017) and Amoah & Seo, (2021).

The IUF treatment exposed plants to a prolonged water deficit compared to the IUD treatment. This phenomenon resulted in lipid peroxidation in the plants, caused by increased production of ROS and subsequent destruction of the cell membrane. Therefore, lant's reduced access to water availability, especially at higher densities, led to an increased antioxidant enzyme activity to scavenge ROS. Kibria et al., (2017) documented that under severe stress conditions, the CAT activity increased due to the higher lipid peroxidation and the increased production of hydrogen peroxide, as this enzyme is mainly contributing to hydrogen peroxide decomposition. Also, in other studies, an increase in CAT enzyme activity has been reported under stressful conditions (Ghadirnezhad Shiade, et al., 2023). POD enzymes play a very important role in deactivating ROS in plant cells during various stress situations, such as drought, and their activity level changes depending on the plant species and the intensity of stress in plants (Sharma et al., 2019). The increase in plant density (beyond optimal levels) was associated with increased intra-species competition, which led to the creation of stress conditions in the plant, and as a result, increased activity of antioxidant enzymes (Gao et al., 2018).

CONCLUSION

In the present study, it was found that the physiological characteristics and photosynthetic pigments of wheat are influenced by both drought stress and plant density. The results show that drought stress led to a decrease in chlorophyll content, carotenoids, relative water content, and cell membrane stability index, while it increased the levels of proline and soluble sugars. Furthermore, enzymatic activities were enhanced at higher drought stress and plant densities. Overall, our results indicated that a density of 500 plants m^{-2} can be optimal to maintain stable growth conditions in wheat under the drought conditions simulated in our field experiment. This optimal density establishes a balance between plant competition and resource utilization and allows for improved underlying responses and resilience to water scarcity.

In general, our findings reveal that the flowering stage was more sensitive to water deficit conditions than the dough stage. Accordingly, it is recommended to prioritize irrigation during the flowering stage, as wheat can better tolerate water stress afterward. Considering the limitations of water availability in Iran, continuous irrigation is not feasible in this country. Therefore, to balance water use with crop performance, irrigation should ideally be applied until the end of the flowering stage, after which moderate drought stress can be tolerated without severe yield penalties.

It is notable that inadequate irrigation and suboptimal plant density significantly impact wheat quality and yield, with water stress leading to reduced grain size and quality, and increased susceptibility to pests and diseases, which further diminishes marketability and crop productivity. On the other hand, high plant density can cause excessive competition for resources, resulting in stunted growth and poor grain quality, while low density can lead to inefficient use of resources.

The broader significance of this study lies in its implications for managing water scarcity in arid and semi-arid regions. Our findings emphasize the fact that optimizing irrigation practices along with plant density can enhance resource efficiency and crop resilience. Current climate change scenarios for Iran indicate for the future more frequent and intense droughts, along with shifts in precipitation patterns toward less frequent but heavier rainfall events. Our findings stress the importance of targeted irrigation during sensitive phases for adapting wheat production to these changes and helping farmers cope with increased drought incidence. It is recommended that future studies should explore additional strategies for managing drought, including breeding to enhance drought resistance and resilience and selecting varieties with shorter growing stages, which could help avoid water stress conditions particularly around and after flowering. Further research into the molecular responses of plants under drought stress is also needed to better prepare wheat cultivation for the expected consequences of climate change in Iran and elsewhere.

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