

The effect of melatonin on physiological, biochemical, and enzymatic properties and the expression of antioxidant genes under different irrigation regimes in wheat

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Abstract

A split-plot experiment was conducted based on a randomized complete block design with four replications in two years to study the effect of melatonin on the biochemical properties and the gene expression related to antioxidant enzyme activity in bread wheat. Irrigation levels (normal (FC = 80%), mild stress (FC = 60%), and severe stress (FC = 40%)) were assigned to the main plots, and melatonin foliar applications (zero, 50, 100, 150, and 200 μ M) were assigned to Subplots. Results showed that, with the intensification of water stress, the 1000 kernel weight decreased, and the activity of ascorbate peroxidase enzyme and flavonoid content increased. Also, the level of 100 μ M melatonin had the highest 1000 kernel weight, flavonoid content, and ascorbate peroxidase enzyme activity. In this study, the highest number of grains per spike, biological yield, grain yield, chlorophyll a, chlorophyll b, carotenoids, and the lowest amount of malondialdehyde recorded for foliar treatment with 50 μ M of melatonin under normal irrigation conditions and the highest proline content, total phenol, superoxide dismutase, and catalase were allocated to the 100 μ M melatonin foliar treatment under severe water stress conditions. The synergistic effect of water deficit stress and melatonin foliar application increased the activity and expression level of genes related to antioxidant enzymes. So, the content of superoxide dismutase (21.30% and 65.16% respectively) and catalase (50.60% and 54.44% respectively) enzyme activity increased significantly under 100- and 150-mM melatonin foliar application in water severe water stress compared to the corresponding control treatment. Furthermore, mentioned melatonin levels increased the gene expression levels of superoxide dismutase (16.67% and 38.19% respectively), ascorbate peroxidase (73.76% and 47.57% respectively), polyphenol oxidase (39.32 and 51.15%) and catalase (39.95% and 50.0% respectively) under an extreme water shortage compared with corresponding control treatment. In general, the application of 100- and 150-mM melatonin induced resistance to water deficit stress in wheat by increasing the expression of antioxidant genes.

Keywords: antioxidant; gene expression; melatonin; water deficit; wheat

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Introduction

Wheat is a widely used staple food crop worldwide. However, it fails to yield the required amount due to abiotic stresses such as drought, salinity, and heat stress (dos Santos *et al.*, 2022). All abiotic stresses in the environment have adverse effects on the growth and production of wheat. However, of all the stresses, drought stress is the most detrimental to crops. (Chowdhury *et al.*, 2021). The effects of climate change have led to a rise in global temperatures, making drought stress more threatening. In areas with little rainfall, higher temperatures lead to increased water evaporation rates, thereby increasing the risk of drought or prolonging periods of drought. Water scarcity impacts all stages of growth, but the reproductive stage and grain filling are especially vulnerable. This leads to a reduction in the number of grains and a decrease in the size of wheat grains (Elkelish *et al.*, 2021). During grain filling, the production of essential carbohydrates such as sucrose and starch can be inhibited by specific necessary enzymes (Lu *et al.*, 2019). Drought reduces plant growth and productivity by impacting water potential, nutrient uptake, and photosynthesis. It also causes oxidative damage through increased reactive oxygen species (ROS) (Bi *et al.*, 2017).

Melatonin (N-acetyl-5-methoxytryptamine) is a plant growth regulator primarily found in various organisms, including plants, bacteria, fungi, and algae (Debnath *et al.*, 2020). Numerous studies have shown that melatonin plays a crucial role in various biological processes in plants, resulting in significant outcomes such as seed germination (Chen *et al.*, 2021), root growth (Boyko *et al.*, 2020), flowering (Kolář *et al.*, 2003), leaf senescence (Ahmad *et al.*, 2020), enhanced photosynthesis capacity (Ahmad *et al.*, 2019), and reduction of oxidative damage (Siddiqui *et al.*, 2020). Moreover, several studies have shown that melatonin's antioxidant properties play a significant role in enhancing plants' resistance to abiotic stressors such as cold stress (Wang *et al.*, 2020), heat stress (Wei *et al.*, 2015), salt stress, UV stress (Zhang *et al.*, 2020), and drought stress (Sharma *et al.*, 2020). Recent research on cotton has found that melatonin can balance carbohydrates in anthers under drought stress, leading to increased pollen fertility (Hu *et al.*, 2020). In addition, regulating various processes, such as carbon and nitrogen metabolism by melatonin, enhances the ability of plants to withstand drought stress. Melatonin plays a critical role in reducing the effects of drought stress by boosting the performance of antioxidants, enhancing cellular redox homeostasis, and supporting photosynthesis (Ye *et al.*, 2016). Studies have shown that melatonin has improved economic yield in various plants under water stress conditions (Zahedi *et al.*, 2021). According to a study by Miao *et al.* (2020), drought-resistant and drought-sensitive cultivars experienced a decrease in grain yield due to drought stress. However, the study found that low melatonin levels (less than 100 μmol) significantly improved yield under normal and drought conditions. It has been reported that drought stress can reduce wheat crop yield and water content. Still, melatonin can increase leaf pigments, proline, sugars, and protein. Furthermore, melatonin improves antioxidant enzyme activity (Sattar *et al.*, 2023). Researchers use various techniques, including physiological, molecular, and genetic, to better understand how plants grow and respond to stress (Sheoran *et al.*, 2015). Understanding the relationship between antioxidant enzyme activity, gene expression, and genetic variation in drought tolerance can help uncover the factors that govern antioxidant defence. Water stress and wheat studies have only examined how antioxidant genes respond during seedling.

Various studies have investigated the effects of growth stimulants on the physiological characteristics and yield of wheat under different stress conditions. In these experiments, only the physiological effects of growth stimulants have been studied, and the impact of growth stimulants on the expression of genes involved in stress resistance in wheat has rarely been evaluated. Melatonin is a newly identified growth stimulant, but its effect on the physiological characteristics of wheat has not yet been studied. In this study, researchers investigated the impact of melatonin on antioxidant gene expression for the first time. The study clarifies how melatonin treatment induces resistance to water deficit stress on genes and enzymes. Therefore, the current research was designed and implemented to study the effect of melatonin on physiological, biochemical, and enzymatic properties and the expression of antioxidant genes under different irrigation regimes in wheat.

Materials and Methods

The study was conducted at Mahabad University's Faculty of Agriculture in North West Iran during the 2020- 2021 growing seasons at 1320 meters above sea level and coordinates of 36°10'N and 45°43'. The climate in the research area has an average annual rainfall of 286 mm and an average annual temperature of 11 °C, with the highest temperature of 16.5 °C and the lowest of 4.3 °C (Figure 1).

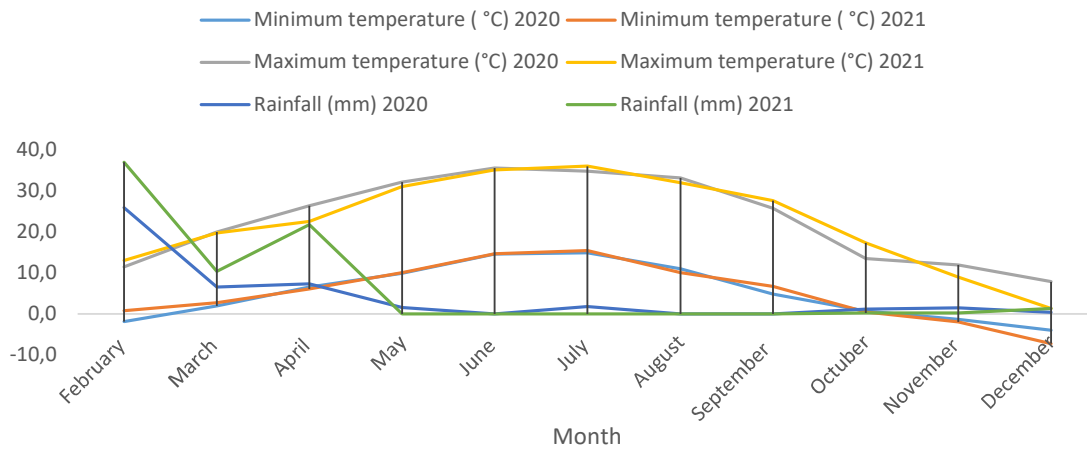


Figure 1. Rainfall (mm), Minimum and maximum temperature (°C) records of experimental site during 2020-2021

The land was prepared by plowing and levelling it to reach the ideal moisture level, also known as field capacity. The fertilizers were applied based on the soil analysis conducted on the research farm and the plant fertilization program (Table 1). The experiment was a Split plot design based on a randomized complete block with four replications in both years of the experiment.

Table 1. Physical and chemical analysis of the experimental soil

Parameters	Value
Sp%	44
EC (ds/m)	1.38
F.C 1/3 A	27.4
W.P	12.5
B.D	1.3
pH	8.08
T. N. V%	4.73
O.C%	1.3
N%	0.13
P (ppm)	14.62
K (ppm)	444
Sand %	16
Silt %	54
Clay %	28
Soil texture	Silt clay loam

The treatment included different levels of irrigation regime and melatonin. The irrigation regime at three levels (Normal (FC = 80%), mild stress (FC = 60%), and severe stress (FC = 40%)) were assigned to the main plot. The foliar application of melatonin in five levels (zero level, 50, 100, 150, and 200 μM) was assigned to the Subplot. The variety used in this experiment was 'Pishgam' (*Triticum aestivum*, cv 'Pishgam', a bread-making variety), planted in the fall (late October) in both years. This variety was obtained from the Agricultural Research Center of West Azarbaijan Province - Iran.

Application of treatments

The water deficit treatments were applied coinciding with the plant's stem growth stage in both years. Before this stage, three irrigations were done, including one irrigation at the planting time and two adequate irrigations (To compensate for the lack of moisture to the extent of Field capacity). The amount of water used in these three rounds of irrigation was equal to 550 m^3 .

To determine the moisture weight percentage of soil in the experimental units, multiple random samples were collected before each irrigation and sent to the laboratory for analysis. The amount of water required in subsequent irrigations was measured based on bringing the soil moisture to the field capacity (FC= 80, 60, and 40 percent) by using the following formula (1):

$$I_n = (fc - ai) \times D \times b$$

where I_n = irrigation water depth (mm), F_c = moisture of soil in the field capacity (percentage by weight), a_i = soil moisture before irrigation (weight percent), D = depth of root (mm) (1000 mm was considered for wheat), b : apparent specific mass (g cm^{-3}). Field soil moisture was considered constant during the experiment. After determining the irrigation water depth, the amount required for irrigation was estimated based on the following formula (2); this amount was controlled by the meter at the beginning of the irrigation pipes.

$$V = \left(\frac{I_n}{1000} \right) \times A$$

where I_n = depth of irrigation water (mm), V = amount of irrigation water (m^3), and A = plot area (m^2).

The total water consumed under normal irrigation and water deficit stress was measured by a meter. The estimated water usage for the irrigation treatments at 80% FC, 60% FC, and 80% FC were 4290, 3880, and 3435 m^3 , respectively.

After seven and fourteen days of inducing drought stress, in two stages melatonin was applied to the leaves.

Traits measurement

In the mature physiological stage, ten plants were randomly selected from each experimental unit to measure the traits by removing the effect of margins. Samples were taken from fresh green flag leaf to measure chlorophyll a, b, and carotenoid; the samples were digested using 80% acetone and then centrifuged before measuring their absorbance using a spectrophotometer (Lichtenthaler and Wellburn, 1993). A fresh green flag leaf weighing 0.5 g was used to estimate proline. To grind the sample, a mortar and pestle that was pre-chilled and had a buffer with a pH of 7.02 was utilized. The method of Mishra and Abidi (2010) was used to determine proline. Total phenolic and flavonoid content in the extracts using the method (Shin *et al.*, 2007).

Antioxidants activity

2 mL of extraction buffer containing 0.1 M potassium phosphate (pH 7), 1 mM EDTA, and 1% PVP (w/v) was used to homogenize 0.1 g frozen wheat samples. After centrifuging the samples at 10,000 g for 15 minutes at 4 °C, the supernatant was utilized to measure SOD, POX, and CAT activities. (Araz *et al.*, 2022). The spectrophotometric measurement of CAT activity was taken for three minutes at 240 nm after adding 20 mM H_2O_2 . The unit of CAT is defined as the activity that decomposes one micromole of H_2O_2 per minute at 25 °C and pH 7.0. (Araz *et al.*, 2022). Scanning the prevention of depletion of P-Nitro-blue tetrazolium

chloride (NBT) was used to assay the activity of SOD (Shams *et al.*, 2022). The APX activity was measured by monitoring the absorbance decrease at 290 nm for 1 minute in the reaction mixture containing ascorbate. (Shams *et al.*, 2023)

To measure wheat's grain yield and biological yield, a 1 m² plot was harvested at the end of spring (mid-July 2021) while avoiding the borders on each side. The seeds on ten plants and ten spikes were counted during harvesting to determine the kernel number per Spike. From each plot, ten random spikes were collected and hand-shelled to determine the thousand-grain weight.

Gene expression analysis

In the second year of the experiment, leaf tissue sampling was done after applying water stress treatments and melatonin foliar application, and the samples were immediately stored at -82 degrees Celsius until RNA extraction. First, all the tools needed for RNA extraction were sterilized to prevent the effect of RNase enzyme and RNA degradation.

Total RNA was extracted using RNX- Plus™ kit (Sina Gene Company - Iran). To determine the quality and quantity of RNA, 1% of gel electrophoresis and NanoDrop spectrophotometry (Thermoscientific 2000c, USA) were used, respectively. All RNAs will be treated using Ferments' DNaseI kit according to the manufacturer's instructions to remove possible genomic DNA contamination from the extracted RNAs. According to the manufacturer's instructions, the GeneAll Hyper Script kit will synthesize the cDNA synthesis reaction.

3 µg of RNA was treated by DNaseI (Thermofisher, USA), and 1 µg of treated RNA was subjected to complementary DNA (cDNA) Synthesis (Fermentas, Revert Aid™ First Strand cDNA, USA) in total volume 20 µl containing 1 mM dNTPs, 0.5 µM dT primer and 1 µl reverse transcriptase enzyme (Nasirzadeh *et al.*, 2021).

The online PrimerQuest software (Thornton and Basu 2011) was used to design primers based on the cDNA sequence. Primer designing was carried out by online PrimerQuest software (Thornton and Basu 2011) based on cDNA sequence (Table 2).

qRT-PCR was done in Step One Plus Real-Time PCR in a total volume of 20 µl consisting of 0.2 U/µl Taq DNA polymerase, 0.2 mM dNTPs, 0.25 µM of each primer and 200 ng cDNA under the thermal program of initial denaturation 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 20 s. Melt curve analysis was conducted to evaluate the PCR specificity of all reactions The experiment was conducted with three independent replications of each sample.

Table 2. Name and sequence of oligonucleotide primers used for gene amplification in this research

Gene	Primer	(5' - 3') sequence	Sequence reference	Reference
<i>TaSOD</i>	<i>Sod-F</i>	5'-AAGCACCACGCCACCTAC-3'	<i>TAU72212</i>	Hyun Baek and Skinner (2003)
	<i>Sod-R</i>	5'-TGGGCTTGAGGTTCTTCC-3'		
<i>TaAPX</i>	<i>Apx-F</i>	5'-CTGACAGCGTTCAAGGTAT-3'	<i>AJ006358</i>	Nasirzadeh <i>et al.</i> (2021)
	<i>Apx-R</i>	5'-GTTGGACGGATGGTACTGA-3'		
<i>TaCAT</i>	<i>Cat-F</i>	5'-GTTGGACGGATGGTACTGA-3'	<i>X94352</i>	Nasirzadeh <i>et al.</i> (2021)
	<i>Cat-R</i>	5'-AAGACGGTGCCTTTGGGT-3'		
<i>TAPPO</i>	<i>PPO-F</i>	5'-CGATCTACGCCAACAGGTCGTC-3'	<i>AY515506</i>	Hyun Baek and Skinner (2003)
	<i>PPO-R</i>	5'-CACTGGAGTCAAGGTCGGTCAGCA-3'		

Statistical analysis

The SAS 9.4 software was used to analyze variance. Tukey's test was used with a significance level of $P \leq 0.05$ to identify any significant difference among mean values. The gene expression data were analyzed using the Relative Expression Software Tool (REST).

Results

The combined analysis of variance revealed that the irrigation levels and foliar application of melatonin significantly affected all traits with a probability level of 1%. The difference between irrigation with melatonin foliar application interaction treatments in terms of the effect on chlorophyll a, chlorophyll b, carotenoid, proline, phenol content, the activity of superoxide dismutase, catalase, malondialdehyde, and grain yield at the level of 1% probability and in terms of the effect on the number of grains per spike and biological yield was significant at the 5% probability level (Table 3).

Table 3. Combined analysis of variance for the studied traits in the wheat

SOV	MS							
	DF	Chlorophyll a	Chlorophyll b	Carotenoid	Proline	Total phenol	Flanoid	Superoxide dismutase
Year (Y)	1	5.28 ^{ns}	3.15 ^{ns}	2.90 ^{**}	0.012 ^{**}	29.17 ^{ns}	52.14 ^{ns}	401.18 ^{ns}
Repetition (Year)	3	1.46	0.15	0.12	0.004	37.25	30.25	7684.5
Irrigation (I)	2	133.21 ^{**}	9.01 ^{**}	13.21 ^{**}	0.317 ^{**}	3694.35 ^{**}	3921.12 ^{**}	15610.2 [*]
Y×I	2	5.21 ^{ns}	0.01 ^{ns}	0.02 ^{ns}	0.0009 ^{ns}	112.51 ^{ns}	52.14 ^{ns}	854.81 ^{ns}
Ea	12	1.43	0.05	0.08	0.0005	84.34	81.30	4460.2
Melatonin (M)	5	5.25 ^{**}	0.41 ^{**}	0.52 ^{**}	0.077 ^{**}	184.67 ^{**}	346.13 ^{**}	14963.3 ^{**}
Y×M	5	1.25 ^{ns}	0.01 ^{ns}	0.02 ^{ns}	0.011 ^{ns}	12.21 ^{ns}	55.17 ^{ns}	1402.98 ^{ns}
I×M	10	4.59 ^{**}	0.28 [*]	0.46 ^{**}	0.23 ^{**}	136.69 ^{**}	5.96 ^{ns}	7576.9 ^{**}
Y×I×M	10	1.02 ^{ns}	0.11 ^{ns}	0.10 ^{ns}	0.0008 ^{ns}	21.18 ^{ns}	14.55 ^{ns}	673.18 ^{ns}
Eb	90	1.25	0.10	0.07	0.006	34.43	33.83	1529.7
CV%	-	12.21	13.98	9.24	17.31	20.84	14.14	18.65
*, **and *** show insignificance and significance at the $p < 0.05$ and $p < 0.01$ levels, respectively								
SOV	MS							
	DF	Catalase	Ascorbate peroxidase	Malondialdehyde	Number of grains per spike	Thousand kernel weight	Biological yield	Grain yield
Year (Y)	1	561.16 ^{ns}	1420.15 ^{ns}	18.41 ^{ns}	17.91 ^{ns}	18.21 ^{ns}	15.81 ^{ns}	1.29 ^{ns}
Repetition (Year)	3	271.56	710.3	5.50	9.59	5.19	8.14	0.77
Irrigation (I)	2	9008.6 ^{**}	30793.1 ^{**}	823.82 ^{**}	408.92 ^{**}	321.52 ^{**}	175.85 ^{**}	21.48 ^{**}
Y×I	2	351.60 ^{ns}	5150.60 ^{ns}	115.51 ^{ns}	54.93 ^{ns}	121.18 ^{ns}	54.16 ^{ns}	1.17 ^{ns}
Ea	12	214.80	461.2	10.01	10.44	8.55	3.43	0.36
Melatonin (M)	5	2568.2 ^{**}	5469.8 ^{**}	80.08 ^{**}	42.61 ^{**}	51.16 ^{**}	68.74 ^{**}	7.60 ^{**}
Y×M	5	89.11 ^{ns}	156.14 ^{ns}	11.21 ^{ns}	102.17 ^{ns}	15.23 ^{ns}	5.21 ^{ns}	0.87 ^{ns}
I×M	10	661.78 ^{**}	725.0 ^{ns}	38.53 ^{**}	22.59 [*]	39.19 ^{ns}	18.56 [*]	2.67 ^{**}
Y×I×M	10	51.44 ^{ns}	318.01 ^{ns}	13.52 ^{ns}	84.27 ^{ns}	7.35 ^{ns}	9.34 ^{ns}	1.09 ^{ns}
Eb	90	176.45	446.4	8.91	97.72	10.32	7.40	0.77
CV%	-	13.31	22.93	18.87	11.39	15.41	17.58	16.97
*, **and *** show insignificance and significance at the $p < 0.05$ and $p < 0.01$ levels, respectively								

Chlorophyll a

Our data showed that the plant that received 100 μM melatonin under 80% FC treatment had the highest (13.25 mg g^{-1} FW) chlorophyll content between the mentioned treatment and the treatments of 50 and 150 μM melatonin under 80% FC; no significant difference was detected. The lowest chlorophyll content

was attributed to the 40% FC conditions with the control and foliar application of 150 μM melatonin (5.25 and 5.50 mg g^{-1} FW) (Table 4).

Table 4. Mean comparison of irrigation with melatonin foliar application interaction treatments in terms of the effect on the traits in wheat

Irrigation	Melatonin	Number of grains per spike	Biological yield (Kg ha^{-1})	Grain yield (Kg ha^{-1})	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)	Proline (mg g^{-1} FW)	Phenol content (mg Galic acid g^{-1} DW)	Superoxide dismutase activity ($\mu\text{mol g}^{-1}$ FW)	Catalase activity ($\mu\text{mol g}^{-1}$ FW)	Malondialdehyde content ($\mu\text{mol g}^{-1}$ FW)
Normal (FC80%)	Control	28.00cde	13910def	5516.3bc	9.75cd	2.43bcd	3.04d	0.25g	25.78g	135.69g	43.12h	15.01de
	50 μM	35.25a	23864a	7954.5a	12.25ab	3.06a	3.82ab	0.35fg	35.85ef	156.78efg	64.12fg	9.56f
	100 μM	33.75ab	21281ab	7593.7a	13.25a	3.31a	4.14a	0.42ef	30.93fg	193.30defg	62.71fg	16.24de
	150 μM	29.25bcd	15796cd	5265.2bc	12.00ab	3.12a	3.78ab	0.50b-c	25.35g	163.23efg	54.28gh	13.73ef
	200 μM	31.50abc	16581cd	5526.9bc	11.00bc	2.87ab	3.46bc	0.43def	35.15ef	234.59bcd	77.32def	15.49de
Mild stress (FC60%)	Control	22.50fg	11790ef	3929.9de	8.12ef	2.38cd	2.34c	0.45c-f	42.45de	144.03fg	65.29efg	22.30b
	50 μM	28.00cde	16503cd	5501.0bc	10.00cd	2.52bc	3.13cd	0.364fg	50.71cd	209.52cde	93.84cd	21.77b
	100 μM	30.00bcd	16967cd	5655.7bc	9.25de	2.56bc	2.95d	0.45c-f	43.68de	199.44def	83.81de	17.15cde
	150 μM	32.25abc	14658de	6276.5b	9.75cd	2.43bcd	3.04d	0.57ab	45.70d	209.52cde	79.78def	16.84cde
	200 μM	26.37def	18830bc	4886.1cd	9.75cd	2.38cd	3.03d	0.62ab	36.44ef	264.59abc	83.81de	19.07bcd
Severe stress (FC 40%)	Control	20.50g	11579ef	3516.9e	5.50h	1.37fg	1.64h	0.37f	45.70d	196.92defg	57.61gh	27.27a
	50 μM	24.00efg	13409def	3859.5de	7.75efg	1.81ef	2.39ef	0.56abc	57.42bc	291.55ab	105.84bc	28.64a
	100 μM	23.50efg	14359de	4786.3cd	6.50gh	1.75efg	2.06fg	0.67a	67.03a	268.11abc	116.62ab	21.01bc
	150 μM	20.75g	10551f	4469.7cde	5.25h	1.31g	1.71gh	0.54bcd	56.71bc	316.16a	126.46a	22.22b
	200 μM	22.12fg	11980ef	3993.3de	7.50fg	1.87c	2.34ef	0.56abc	62.10ab	162.33efg	107.24abc	22.83b

Means in each column, followed by similar letter(s), are not significantly different at the 5% probability level

Chlorophyll b

The highest chlorophyll b content was obtained from 50, 100, and 150 μM (3.06, 3.31, and 3.12 mg g^{-1} FW respectively) treatments under 80% FC conditions. In this study, the lowest chlorophyll content (1.37 and 1.31 mg g^{-1} FW respectively) was assigned a 40% FC level with the control treatment and using 150 μM melatonin. The results also showed that under 80% FC and 60% FC irrigation, treatment with 50, 100, and 150 μM melatonin enhanced chlorophyll b content over the corresponding control (Table 4).

Carotenoid

According to mean comparison results, plants treated with 80% FC irrigation and 50, 100, and 200 μM melatonin (3.82, 4.14, and 3.78 mg g^{-1} FW respectively) produced the highest, and untreated plants with melatonin under 40% FC irrigation (1.64 mg g^{-1} FW respectively) produced the lowest content carotenoid. In our study, melatonin foliar application significantly improved carotenoid content in all three irrigation treatments, especially the 50, 100, and 200 μM levels (Table 4).

Proline

The highest proline content was recorded in foliar-applied plants with 100 μM melatonin under 40% FC irrigation (0.67 mg g^{-1} FW). No treatment of plants with melatonin under 80% FC irrigation (0.25 mg g^{-1} FW) resulted in the minimum proline content (Table 4).

Phenol content

Total phenol content was significantly increased with the deficit irrigation; in addition, Foliar application of 100 and 200 μM melatonin under 40% FC conditions had the highest phenol content (67.1 $\text{mg Galic acid g}^{-1}$ DW) (Figure 1). The least phenol content was related to the control and foliar application of 150 μM melatonin under 80% FC irrigation treatment (25.78 $\text{mg Galic acid g}^{-1}$ DW) (Table 4).

Flavonoid content

It is evident Figure 7 that 60% FC and 40% FC irrigation treatment increased leaf flavonoid content by 49.19% and 101.30%, respectively, compared to 80% irrigation conditions (Figure 2).

In this research, the levels of 100 and 150 μM of melatonin (45.76 and 45.49 mg Galic acid g^{-1} DW) and the control treatment (34.18 mg Galic acid g^{-1} DW) produced the largest and least flavonoid content (Figure 3).

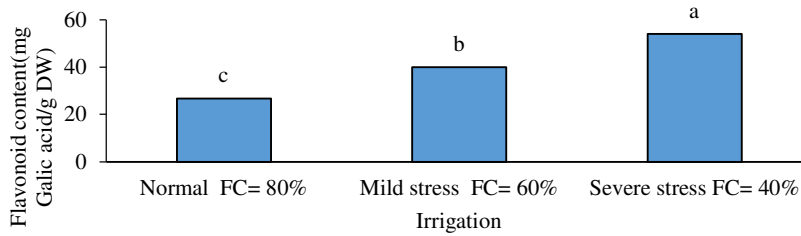


Figure 2. Means comparison for irrigation treatment on flavonoid content ($p < 0.05$)

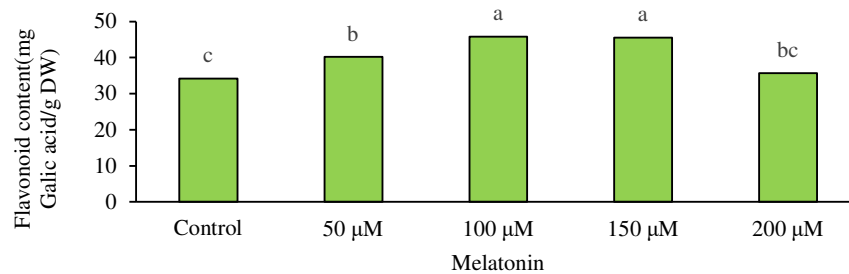


Figure 3. Means comparison effect on flavonoid content ($p < 0.05$)

Superoxide Dismutase Enzyme

Based on the findings, it was observed that the highest level of superoxide dismutase enzyme activity was achieved through foliar application of 50, 100, and 150 μM while subjected to 40% FC irrigation treatment (291.55, 268.16, 1316.16 μmol respectively). Also, the minimum activity was related to control treatment under 40% FC condition (135.69 μmol) (Table 4).

Catalase

Based on the study data, foliar application with 100 and 150 μM melatonin under 40% FC irrigation treatment (116.62 and 126.46 μmol respectively) achieved the highest, and the control treatment under 40% FC had the lowest (43.12 μmol) amount of catalase activity. The results showed that applying all four melatonin levels significantly increased catalase enzyme activity under 40% FC and 60% FC irrigation treatment. Still, under 80% FC irrigation conditions, level 150 could not increase catalase activity compared to the control (Table 4).

Ascorbate Peroxidase

The results showed that the activity of the ascorbate peroxidase enzyme was induced by irrigation deficit so that the severe water deficit treatment (40% FC) and normal conditions (80% Fc) gained the maximum (133.23 μmol) and minimum (55.15 μmol) enzyme activity, respectively (Figure 4). The mean comparison of melatonin levels in terms of the effect on ascorbate peroxidase activity showed that the levels of 50, 100, 150, and 200 μM increased the activity of this enzyme by 24.31, 89.02, 49.20, and 31.99 percent, respectively, compared to untreated plants (Figure 5).

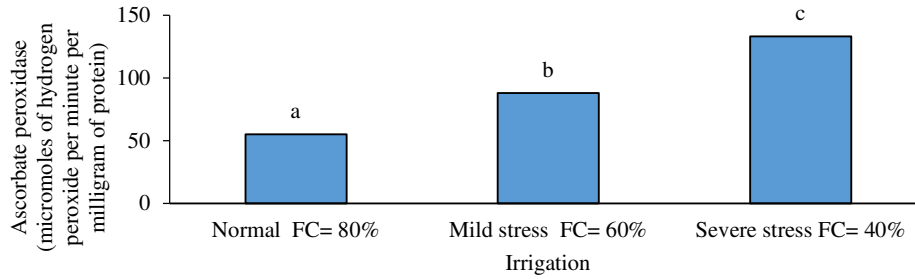


Figure 4. Means comparison for the effect of irrigation on ascorbate peroxidase activity ($p < 0.05$)

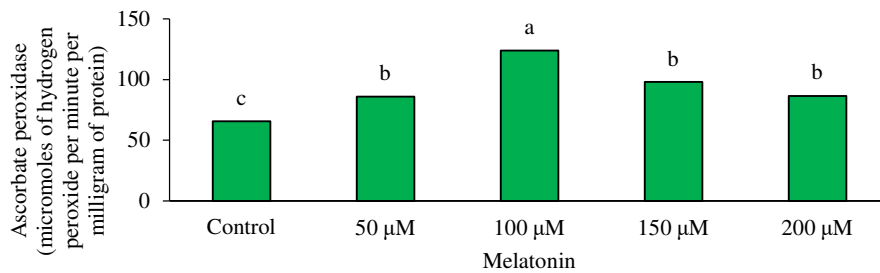


Figure 5. Means comparison for the interactive effect of melatonin on ascorbate peroxidase activity ($p < 0.05$)

Peroxidation of cell membrane lipids (Malondialdehyde)

In this study, the lowest Malondialdehyde (MAD) was related to the foliar application of 50 µM melatonin under 80% FC irrigation treatment; in contrast, the highest lipid peroxidation indicator was recorded in control and of 50 µM melatonin under 40% FC irrigation condition (Table 4).

Grain numbers per spike

In our study, 50 and 100 µM foliar applications of melatonin under 80% FC conditions (35.25 and 33.75 grains, respectively) produced the highest, and 150 µM foliar applications under 40% FC conditions (20.75 grains) produced the minimum number of grains. The results showed that although the number of grains decreased significantly with the intensification of the water deficit, the foliar application of 50, 100, and 200 µM melatonin under 60% FC could increase the number of grains over than control treatment. Under severe water deficit stress, melatonin foliar application levels did not have a significant advantage compared to the control treatment (Table 4).

Thousand kernel weight

In our work, severe irrigation deficit (40% FC) reduced the thousand kernel weight versus 80% and 60% FC irrigation treatment by 12.33 and 11.80%, respectively (Figure 6). We found that the thousand kernel weight positively reacted to all levels of melatonin foliar application (Figure 7). Hence, 50, 100, 150, and 200 µM levels enhanced this attribute by 19.44, 28.57, 20.44, and 16.54 versus control plants.

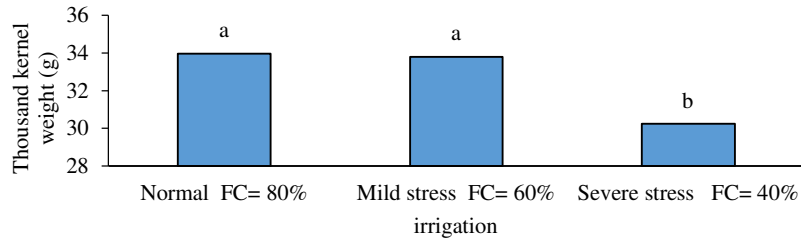


Figure 6. Means comparison for irrigation on thousand kernel weight ($p < 0.05$)

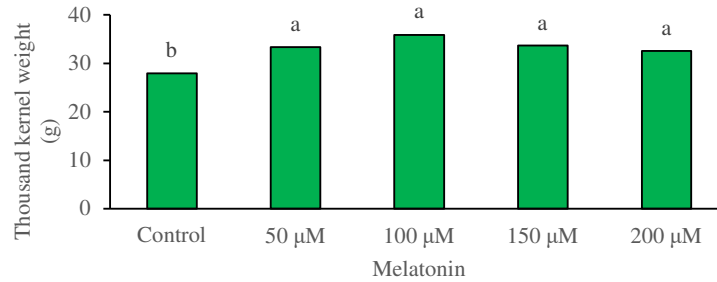


Figure 7. Means comparison for melatonin on thousand kernel weight ($p < 0.05$)

Biological yield

The highest biological yield related to the 50 and 100 μM foliar application under 80% FC conditions (23864.22 and 21281.50 kg ha^{-1} respectively) and the lowest value related to the control treatment and the application of 150 μM melatonin under 40% FC conditions (10551.17 and 11579.23 kg ha^{-1} respectively). The results also showed that the levels of 50 and 100 μM melatonin under all three irrigation conditions increased the biological yield remarkably compared to the corresponding control treatment (Table 4).

Grain yield

The mean comparison results revealed that foliar application of 50 and 100 μM melatonin under 80% FC conditions (7954.5 and 7593.7 kg ha^{-1} respectively) had the highest grain yield. While the lowest grain yield (3516.9 kg ha^{-1}) was assigned to the control treatment (no foliar application) under 40% FC conditions. In this experiment, foliar application of 100 μM melatonin in all three irrigation treatments significantly increased grain yield versus the corresponding control treatment. It moderated the adverse effect of drought stress on grain yield (Table 4).

Expression of antioxidant genes

In this study, irrigation treatments affected the expression of ascorbate peroxidase, catalase, and polyphenol oxidase genes at the probability level of 1% and the expression of the superoxide dismutase gene at the probability level of 5%. The effect of melatonin levels on the gene expression of all four enzymes was significant at the probability level of 1%. The interaction effect of irrigation with melatonin foliar application treatments on ascorbate peroxidase, catalase and polyphenol oxidase gene expression was significant at a 1% probability level and on superoxide dismutase gene expression at a 5% probability level (Table 5).

Table 5. Variance analysis of characteristics related to the expression of antioxidant genes under irrigation and melatonin treatments

SOV	Means of Squares				
	DF	Superoxide dismutase gene expression	Ascorbate peroxidase gene expression	Catalase gene expression	Polyphenol oxidase gene expression
Repetition	3	591.66	256.7	2.71	931.40
Irrigation (I)	2	3222.30*	14454.4**	182.72**	3694.35**
Ea	6	337.41	135.8	2.14	84.34
Melatonin (M)	5	1436.76**	1162.4**	31.17**	184.67**
I×M	8	390.76*	1334.4**	8.78**	136.69
Eb	36	149.88	183.6	1.76	32.43
CV%	-	19.26	24.16	12.61	14.21

*, **and *** show insignificance and significance at the $p < 0.05$ and $p < 0.01$ levels, respectively

Superoxide dismutase (SOD) gene expression

According to the results, the SOD enzyme had the highest relative gene expression when 150 μM melatonin was applied to the leaves and the plant was irrigated at 40% FC. The difference in SOD gene expression between this treatment and other melatonin levels (50, 100, and 200 μM) under similar irrigation conditions was insignificant. Under 40% FC conditions, the control treatment showed the least SOD enzyme gene expression. Melatonin foliar application at 150 μM showed the best impact on SOD enzyme expression in all irrigation conditions (Figure 8).

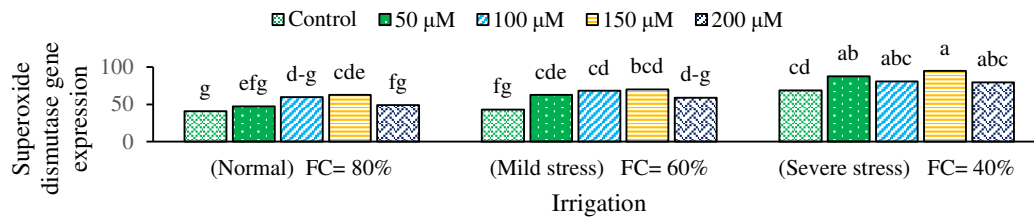


Figure 8. Means comparison for the interactive effect of irrigation \times melatonin on Superoxide dismutase gene expression value ($p < 0.05$)

Ascorbate peroxidase (APX) gene expression

Our data showed that plants treated with 50, 100, and 150 μM melatonin under 40% FC conditions had the highest, and plants untreated with melatonin under 80% FC conditions had the lowest ascorbate peroxidase gene expression. Melatonin at 100-200 μM levels increased APX gene activity significantly, especially at 60% and 40% FC irrigation (Figure 9).

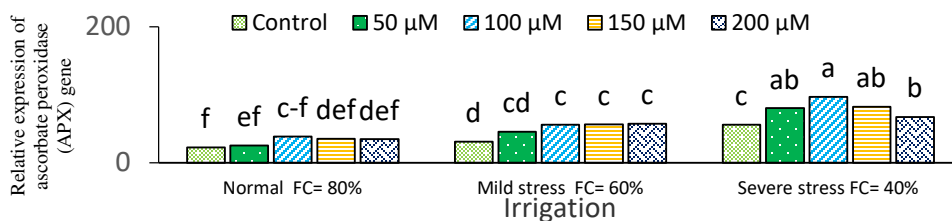


Figure 9. Means comparison for the interactive effect of irrigation \times melatonin on Ascorbate peroxidase gene expression value ($p < 0.05$)

Polyphenol Oxidase (PPO) gene expression

The mean comparisons showed that the plants treated with 100- and 150-mM melatonin exhibited the highest PPO gene expression under 40% FC conditions. The control treatment and foliar application of 200 μM under 80% FC irrigation showed the lowest expression of this gene (Figure 10).

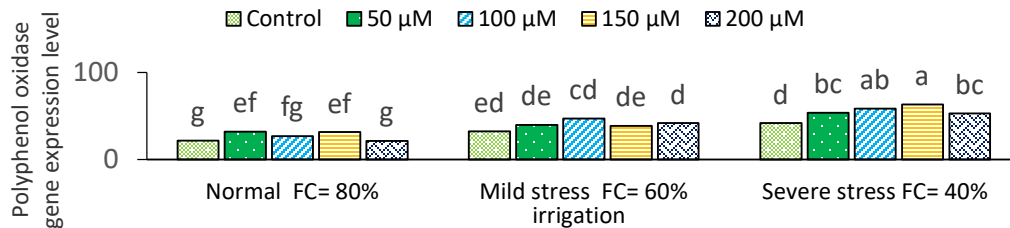


Figure 10. Means comparison for the interactive effect of irrigation × melatonin on polyphenol oxidase gene expression value ($p < 0.05$)

Catalase (CAT) gene expression

In the current experiment, foliar application of 100, 150, and 200 μM melatonin under 40% FC conditions had the maximum, and non-use of melatonin under 80% FC conditions had the minimum expression of the catalase enzyme gene. The results also revealed that irrigation deficit and melatonin, especially the level of 50 μM, synergistically affected the expression of the catalase gene (Figure 11).

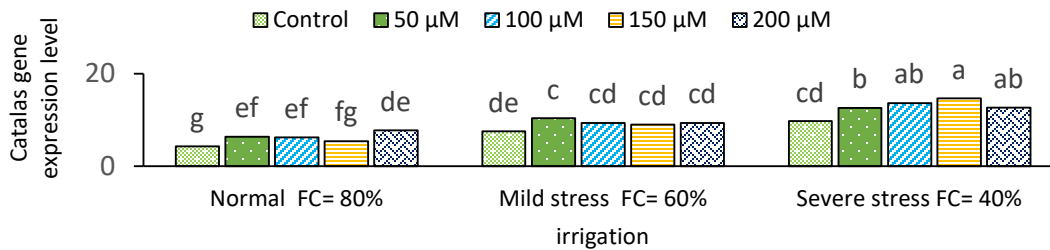


Figure 11. Means comparison for the interactive effect of irrigation × melatonin on Catalase gene expression value ($p < 0.05$)

Discussion

Photosynthetic pigments and proline

In the current study, the content of photosynthetic pigments decreased with the intensification of irrigation deficit from 80% FC to 40% FC, while proline content increased at the same time; also, under irrigation conditions, 60% FC and 40% FC, foliar application of 50 μM melatonin compared to other levels had a beneficial effect on the increase of photosynthetic pigments, while under the mentioned conditions, the maximum proline was recorded in 150 μM melatonin foliar application treatment.

The study found that irrigation deficit caused a significant reduction in photosynthetic pigments. This was due to an increase in the activity of the chlorophyllase enzyme, which led to the destruction of chlorophyll contents under drought-stress conditions (Manzoor *et al.*, 2022). The efficiency of photosynthesis is directly correlated with the content of chlorophyll (Berry *et al.*, 2018), which can impact the overall production of assimilates. When plants experience drought stress, their leaf surface decreases, and their photosynthetic

pigments are destroyed. This disruption in photosynthesis directly leads to decreased plant growth (Liang *et al.*, 2019). Treatment of plants with melatonin preserves photosynthetic pigments and thus enhances photosynthesis in plants grown under unfavourable conditions (Cui *et al.*, 2017). It has been reported that melatonin treatment prevents the activity of the enzyme (Pao) Pheophorbide-a oxygenase (a key enzyme in chlorophyll decomposition) (Turk *et al.*, 2014). The results of previous research on barley (Li *et al.*, 2012) and corn (Ahmad *et al.*, 2019) reported that melatonin increases the durability of leaf chlorophyll under water shortage conditions. Zahra *et al.* (2022) reported that using melatonin externally can regulate chlorophyll catabolism, but it also inhibits the activities of chlorophyll catalyzing. In another study by Sattar *et al.* (2023), the application of melatonin significantly improved chlorophyll contents.

In our study, irrigation deficit increased proline content, and plants increased their proline content during irrigation deficit; this process aids in managing the water content of cells during drought stress and promotes the development of resistance to drought (Ghouri *et al.*, 2022). Wang *et al.* (2019) reported that wheat plants with more osmolytes in their cells maintain water better, improving photosynthesis, less oxidative stress, and higher grain yield even in drought. Proline accumulates to defend against oxidative stress, preventing dehydration, adjusting osmotic pressure, and scavenging ROS (Yanlei *et al.*, 2017). Nasirzadeh *et al.* (2021) study has also confirmed the rise of leaf proline content in wheat cultivars.

Our results revealed that Melatonin at 150 μM increased proline content in leaves; Melatonin can cause an increase in proline levels by stimulating the production of pyrroline-5-carboxylate synthetase 1 (P5CS1), an enzyme that plays a role in proline biosynthesis (Alyammahi and Gururani, 2020). The improvement of proline concentration in response to simultaneous treatment with melatonin and water deficit has been documented in another research (Ahmad *et al.*, 2019; Jafari *et al.*, 2022).

Antioxidant traits

The content of total phenol, flavonoid, superoxide dismutase, catalase, and ascorbate peroxidase increased considerably with a deficit in irrigation. Among melatonin levels, 100 μM foliar application treatment exhibited the most positive effect on flavonoid content and ascorbate peroxidase; also, the level of 100 μM improved the content of total phenol and catalase in both 60% FC and 40% FC conditions versus the corresponding control. In addition, it was observed that applying 50 μM through the leaves under 60% FC and 40% FC irrigation resulted in a significant rise in superoxide dismutase levels compared to the control. Plants can produce reactive oxygen species (ROS) in response to external stress, which helps to combat free radicals.

Other studies have confirmed that various plants experience an increase in the activity of their antioxidant enzymes when subjected to water stress conditions (Du *et al.*, 2020; Gujjar *et al.*, 2021). Previous studies reported that applying melatonin in various crops increases stress tolerance by protecting the photosynthetic mechanism, increasing antioxidant capacity, and improving water retention capacity (Cui *et al.*, 2017). On the other hand, melatonin is considered a broad-spectrum antioxidant and a scavenger of free radicals (Dawood and El-Awadi, 2015).

In this study, the content of malondialdehyde increased with the intensification of water scarcity stress; however, melatonin foliar application, especially 100 and 150 μM under 60% FC and 40% FC, irrigation significantly reduced the content of malondialdehyde. Under severe stress, ROS quantity increases, destroying plant cells (Khan *et al.*, 2021).

Our research found that irrigation deficit increased ROS and MDA production; stress caused more ROS and MDA production, which damages plant membranes and increases electrolyte leakage (Ozturk *et al.*, 2021). Improved production of antioxidant enzymes reduces ROS production when melatonin is applied (Mushtaq *et al.*, 2020) and the optimization of membranes. Researchers have found that melatonin plays a vital role in preventing lipid peroxidation through its ability to react with lipid peroxy (LOO.) and lipid alkyl (LO.) radicals so that the cycle of peroxidation is interrupted and stopped (Waszczak *et al.*, 2018). Water

deficit stews cause higher levels of H₂O₂ and MDA in wheat leaves (Marcek *et al.*, 2019; Dudziak *et al.*, 2019; Nasirzadeh *et al.*, 2021). Studies have shown that melatonin can preserve membrane integrity while reducing the production of lipid peroxidation products and electrolyte leakage in cucumber seedlings experiencing drought stress (Zhang *et al.*, 2020).

Reducing the amount of lipid peroxidation under water stress conditions as a result of melatonin foliar application has been proven in other studies (Jafari *et al.*, 2022). Silicon and melatonin produced the highest levels of antioxidants under optimal and water-stress conditions (Sattar *et al.*, 2023).

Agronomic traits

This research showed that the highest grain yield and components were obtained under optimal irrigation conditions (80% FC). Using 100 µM melatonin improved the number of grains under 60% FC conditions; grain and biological yield were increased under 60% FC and 40% FC conditions by foliar application of 100 µM melatonin versus the corresponding control treatment. Thousand kernel weight also positively reacted to treatment with 100 µM melatonin. Drought stress can be harmful, particularly during the final grain formation stage. At this point, the grain's ability to gather nutrients decreases, resulting in smaller grain size and lower yield. Drought stress can decrease chlorophyll and photosynthesis, lowering grain weight and yield (Ozturk *et al.*, 2021; Wang *et al.*, 2022).

It has been shown that indole compounds, including melatonin, affect plant growth and biomass production by affecting processes such as photosynthesis, respiration, ion transport, improving membrane permeability, improving the activity of enzymes and hormones (Zhang *et al.*, 2015; Li *et al.*, 2014). It has been reported that, under water shortage conditions, the treated plants with melatonin had a higher nitrogen and phosphorus content in leaves than untreated plants (Al-Huqail *et al.*, 2020).

It has also been reported that foliar application of melatonin due to induced antioxidant properties increases plant tolerance to stressful conditions; by stimulating the production of antioxidant enzymes, melatonin directly counteracts the adverse effects of reactive oxygen species and thus improves the antioxidant capacity of the plant (Ahmad *et al.*, 2020; Zahedi *et al.*, 2021). In research on peas (Szafraska *et al.*, 2016) and tomatoes (Martinez *et al.*, 2018; Liang *et al.*, 2019), the application of melatonin reduced the destructive effect of drought stress on crop yield, which is consistent with our results. Melatonin boosts antioxidant activity, chlorophyll content, and photosynthesis efficiency, leading to higher biomass yield.

Characteristics related to gene expression

When plants experience stress, they may produce too much ROS, harming their structure and interfering with their natural metabolic processes (Tavanti *et al.*, 2021; Nadarajah *et al.*, 2020). Plants prevent these disorders by regulating the expression of genes related to stress, physiological and biochemical responses, and antioxidant defence systems (Gholamin *et al.*, 2020; Hesami *et al.*, 2019). According to a recent study, the genes *SOD*, *APX*, *PPO*, and *CAT* showed the highest expression levels when plants were exposed to drought stress at 40% FC and foliar application of melatonin at 100 and 150 µM levels. This experiment recorded the highest activity level of antioxidant enzymes (especially superoxide dismutase and catalase enzymes) under extreme water deficit conditions and foliar application of 100 and 150 µM melatonin. Based on the findings, it appears that exposure to water deficit and melatonin treatments has boosted the production of antioxidant enzymes within cells. As a result, these enzymes have become more effective in protecting against the negative effects of water deficiency. According to Luna's (2004) research, there is a connection between *CAT* gene expression and *CAT* enzyme activity in wheat cultivars experiencing water deficit. In addition, in the study of Nasirzadeh *et al.* (2023) drought stress increased the expression of antioxidant genes in wheat.

Conclusions

Foliar application of melatonin at 100 and 150 μM levels increased the expression levels of antioxidant enzyme genes, including superoxide dismutase, ascorbate peroxidase, polyphenol oxidase, and catalase, as well as the synthesis and activity of these enzymes. They increased the synthesis and activity of these enzymes. The increase in these enzymes' activity causes ROS detoxification and the reduction of the peroxidation of cell membrane lipids and the content of malondialdehyde. Inducing resistance to water shortage stress by melatonin treatments caused the synthesis and persistence of photosynthetic pigments, which are the place for the photosynthetic products. Therefore, improving yield components and grain yield in melatonin foliar treatment under water stress conditions is related to increased expression of antioxidant genes that induce resistance to drought stress.

Authors' Contributions

Hamze H, and Khalili M, designed the experiment. Hasso Mohammad M, and Hamze H, wrote the manuscript with support from Khalili M. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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