Zinc Oxide Nanoparticles Enhance Drought Tolerance in Wheat via Physio-Biochemical Changes and Stress Genes Expression

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Background: Drought response in plants at molecular level, aiding them to overcome the adverse effects of drought, remarkably depends on the expression of a few regulator genes and signal transduction. For reducing the drought stress, nanoparticles show great promise compared to other commonly used methods, even though the underlying mechanisms are still unknown.

Objectives: This study was performed to investigate the expression analysis of genes involved in drought tolerance and the use of zinc oxide nanoparticles (ZnO NPs) to mitigate the undesirable effects of drought stress in wheat.

Materials and Methods: A factorial experiment based on completely randomized design (CRD) was performed with three replicates. The experiment was carried out in the greenhouse of Mohaghegh Ardabili University, Ardabil, Iran in 2017. The factorial combination of stress levels of water supply (including 85%, 60%, and 35% field capacity) and ZnO NPs (0, 0.5, and 1.0 g. L^{-1}) were used on three wheat cultivars (Mihan, Heidari, and Gascogne). Three days after spraying the ZnO NPs in the three-leaf stage, drought stress was applied for ten days and physio-biochemical traits and gene expression of wheat cultivars were investigated. The expression of *Wdhn13*, *DREB2*, *P5CS*, and *CAT1* genes in leaves were analyzed by real-time polymerase chain reaction (PCR).

Results: Generally, drought stress significantly enhanced total protein and lysine, soluble sugars, chlorophyll, carotenoid contents, antioxidant enzymes activities, and proline accumulation in plants treated with ZnO NPs. Moreover, the ZnO NPs increased the expression of the genes involved in proline biosynthesis (i.e., *P5CS*), catalase activity (i.e., *CAT1*), and dehydration-responsive genes *DREB2* and *Wdhn13*, which are known as drought-tolerance parameters.

Conclusions: According to our results, ZnO NP-treated wheat induced drought-tolerance genes and effectively facilitated deficiency tolerance. Therefore, under drought stress, we recommend spraying bread wheat with ZnO NPs (1 g. L^{-1}) in the growing season, which can improve wheat grain yield under dry conditions.

Keywords: Antioxidant enzymes, Drought stress, Osmoprotectants, Real-Time PCR, Wheat, ZnO NPs

1. Background

As the population significantly increases around the world, the demand for wheat, *Triticum aestivum*, as one of the most important crops, is increasing. However, due to severe global climate change, especially drought or water deficit, wheat productivity has been decreased in many arid and semi-arid regions of the world (1). One of the known effects of water deficit is the reduction of photosynthesis and photosynthetic matters needed to

grain filling due to the closure of the stomata. As stress increases, reactive oxygen species (ROS) accumulate in the cell, followed by lipid peroxidation, reduction, and degradation of photosynthetic pigments, proteins, and nucleic acids, disrupted cell mechanisms, and altered gene expression (2). Therefore, the protection of the structure and function of cell components under drought is of primary importance to enhance crop/ plant productivity in the climate change scenario

(3). In this regard, plants apply some physiological-biochemical responses to maintain cell ROS balance (3). In the biological system, the enzymes catalase (CAT), polyphenol oxidase (PPO), and peroxidase (POD) (2) are considered to be the first line of defense against ROS accumulation. On the other hand, the accumulation of some plant organic osmotics, such as proline, soluble carbohydrates, and amino acids in response to stress conditions, leads to cell readjustment and crop protection (4, 5). In addition to plant defense mechanisms, micronutrient fertilizers can enhance plant water tolerance. Zinc is known as an essential trace element after iron that plays a crucial physiological role in the biological system, such as activation of enzymes involved in many biochemical pathways as well as the synthesis and function of proteins, leaf photosynthesis, and stress tolerance, control of metabolism in plants, and plant growth and yield (6, 7). According to some recent studies, nanotechnology techniques, especially nanofertilizers, have a vital role in reducing stress-induced alterations in plants. Moreover, due to the important properties of nanomaterials in lower consumption and higher efficiency, spraying them can be a sustainable management approach to plant protection and nutrition (8). Today, application of the nanoparticles, including zinc oxide nanoparticles (ZnO NPs), has been widespread in the agricultural sector (9). ZnO NPs are largely involved in stress tolerance (10). ZnO NPs, by promoting crops tolerance against drought stress through several ways, including the expression of drought resistance genes, have proved to decrease the unfavorable impacts of drought stress more effectively compared to other approaches (11, 12). Transcription factors (TFs) are main regulators of gene expression in different genes involved in reducing and/or protecting cellular stress damage (13). In wheat, several genes encoding dehydrins have been determined, such as wheat dehydrin (*Wdhn13*) and dehydration responsive element binding factor (*DREB2*), most of which are induced by dehydration (14). *DREB* is one of the essential transcription factors involved in response to drought stress by controlling the expression of stress-related genes (15). Water deficit is effective in induction of the catalase gene (*CAT1*) and Δ1-pyrroline 5-carboxylate synthase (*P5CS*) genes (16, 17). Recently, Sun *et al.* (18) showed that ZnO NPs can induce tolerance to drought stress in maize by adjusting the up-regulation transcription of *CAT*, *APX*, and Cu/Zn *SOD* genes. Wheat is of great importance because it is one of the major food crops in the world today. Due to the great demand for this product and the arid and semi-arid climate of Iran, it seems that introducing cultivars with more tolerance to stress conditions and higher performance is necessary. However, the physico-biochemical mechanisms of the ZnO NPs-induced drought tolerance in wheat are still largely unknown. Most studies have focused individually on wheat nutritional response to foliar application of ZnO NPs or drought stress (19, 20) and few studies have been performed on the simultaneous evaluation of physiological, biochemical, and molecular mechanisms on plants subjected to spraying of zinc nanoxide under drought conditions.

2. Objectives

This study aimed to evaluate the influence of ZnO NPs on the expression of drought-related genes *Wdhn13*, *CAT1*, *P5CS*, and *DREB2*. It also attempted to investigate the effects of spraying ZnO NPs on biochemical responses, enzymatic activity, and quality in wheat cultivars under drought stress.

3. Materials and Methods

3.1. Experimental Design
The experiment was carried out in a greenhouse at the Mohaghegh Ardabili University, Faculty of Agriculture and Natural Resources, Ardabil, Iran, in 2017. A factorial experiment based on completely randomized design (CRD) was performed with three replicates. Water supplying levels including 85%, 60%, and 35% field capacity (FC) were used on three wheat cultivars. To be close to the actual field conditions, wheat (*Triticum aestivum* L.) cultivars, Mihan (drought-tolerant), Heidari (semi-drought tolerant), and Gascogne (drought-sensitive) were sown in 4 kg pots containing a mixture of soil, sand, and rotten leaf (2:2:1 v/v/v) with a density of 7 plants/pot (**Fig. 1**). Based on soil testing and plant requirements, the commercial fertilizer 20-20-20 containing N, P, and K at 1.5% (w/v), with the N from of urea (46% N), P from of calcium superphosphate (15% P_2O_5), and K from of potassium sulfate (K_2SO_4, 48% K_2O) was applied on plant leaves with uniform coverage in a solution volume of 0.096 L.m^{-2} using a hand sprayer at three-leaf stage (according to Page, Miller, and Keeney 1982). The texture of the
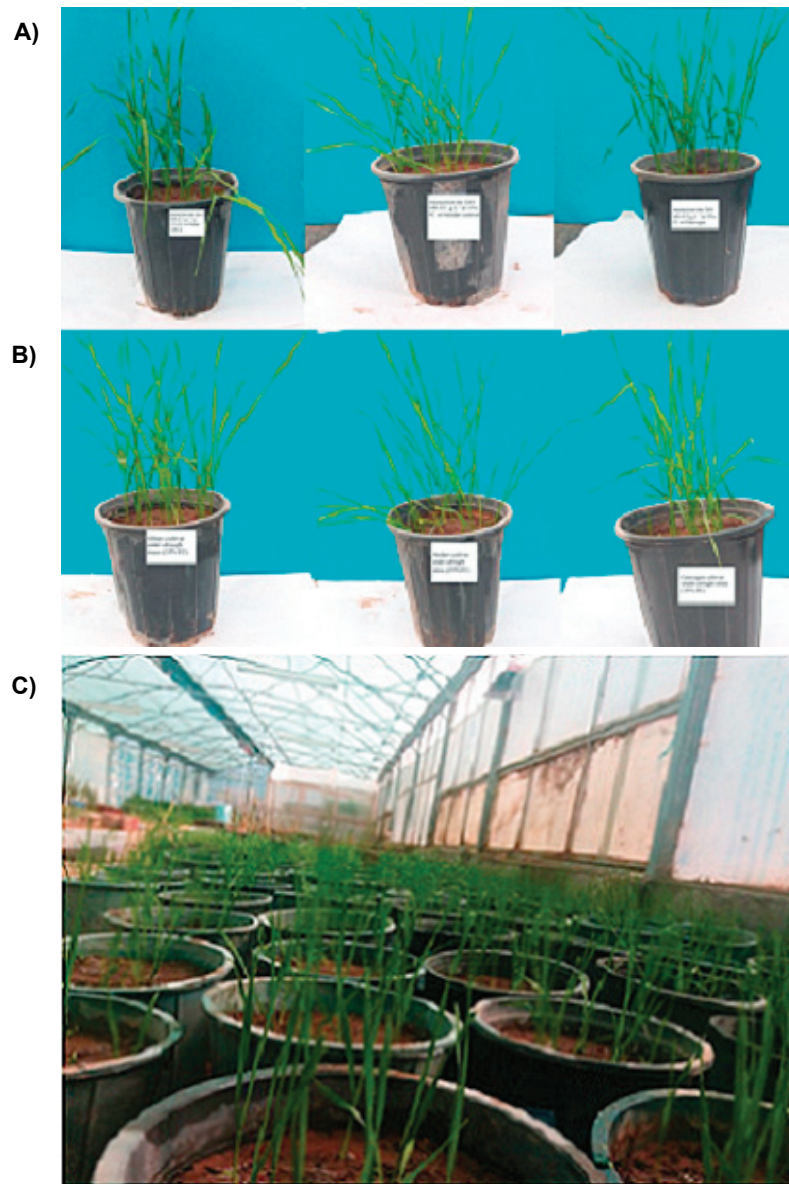
Figure 1. Plant samples sprayed with ZnO NPs 0.5 g. L^{-1} in drought stress (35% FC) **A)**, Wheat cultivars under drought stress (35% FC) **B)** and the three-leaf wheat stage in greenhouse **C)**

soil based on sandy-loam with (clay 10%, soil 80%, and sand 10%), pH 6.48, EC 2.4 dS.m^{-1}, total nitrogen 1.68%, available phosphorus, potassium, and zinc 19.8 mg.kg^{-1}, 212 mg.kg^{-1}, and 0.28, respectively.

Seeds of wheat cultivars were purchased from Seed and Plant Improvement Institute of Karaj, Iran. Surface sterilization of all seeds was done with NaOCl 5% solution for three min and then was rinsed two times with deionized water. The plants were kept under controlled conditions of greenhouse (55-60% relative humidity, 15-28 °C, and 16/8 h light/darkness photoperiod).

ZnO NPs were purchased from Nano-Material Pars-Gostaran company (NAMAGO, Iran) with purity of (by XRF): 97-98%, crystal structure: spherical, and CAS number: 1314-13-2. ZnO NPs of a mean size of 20-60 nm diameters were used in the study (**Fig. 2**). The unique surface of ZnO particles was more than 30 m^2. g^{-1}. After adding deionized water to ZnO NPs, Nano zinc oxide powder was (100 W and 40 kHz) on a shaker for better solution. Plants were sprayed with ZnO NPs (0, 0.5, and 1.0 g. L^{-1}) before flowering (three-leaf stage). Treatments were sprayed on the plant leaves with hand spray bottles in four directions of the pots, so that the plant was completely covered. Three days after
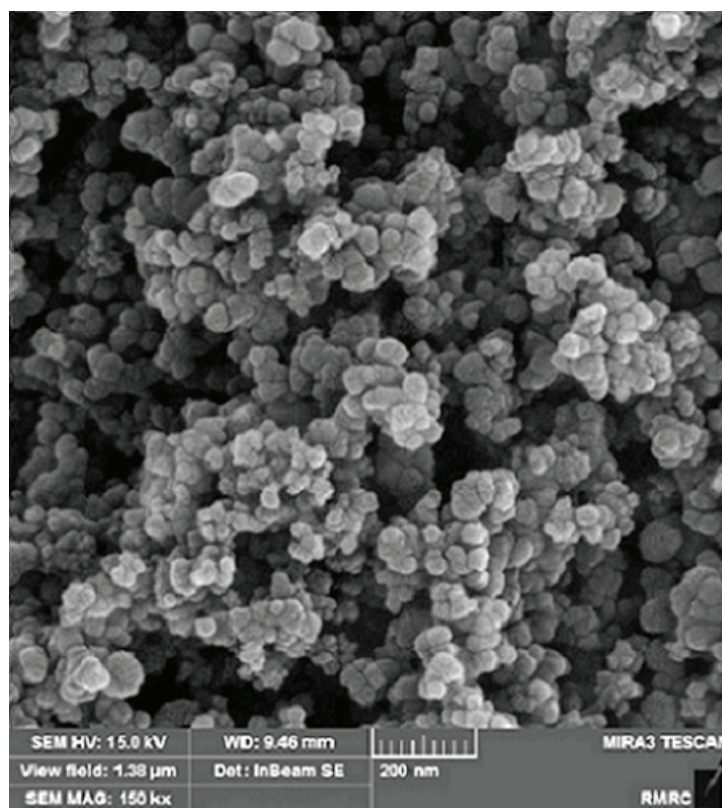
Figure 2. SEM image of zinc oxide nanoparticles

spraying, water stress was applied after 3-4 leaf stage to the pots for ten days. After ten days of stress, leaf samples were immediately transferred to aluminum foil in dry ice packs to measure physiological and biochemical traits, and some were transferred to refrigerator-80 for RNA extraction in liquid nitrogen flasks.

3.2. Measurement of Chlorophyll and Carotenoid Content

To measure the chlorophyll content, 0.2 g of fresh leaf tissue was ground by mortar and pestle in 80% acetone and the final volume of the resulted solution reached 20 mL. Thereafter, the solution was centrifuged for 10 min at 400 rpm followed by the absorption at 645, 663, and 470 nm by using the spectrophotometer (model UV- 160A- SHIMADZO, Japan). Chlorophyll and carotenoid contents were estimated based on the method described by Arnon (21).

3.3. Measurement of Antioxidant Enzymes and Total Protein

To measure the enzyme activity, 0.2 g of leaf fresh tissue was ground in liquid nitrogen and 1 mL of Tris–HCl (0.05 M, pH = 7.5) buffer was added. The obtained mixture was centrifuged at 4 °C and 13000 rpm for 20 min and the supernatant was employed for measuring enzyme activity. Polyphenol oxidase (PPO), peroxidase (POD), and catalase (CAT) activities were assayed based on the method of Kar and Mishra (23). Furthermore, protein evaluation was carried out by Bradford method (24), so that 0.2 g of tissue sample was squashed with 0.6 mL of extraction buffer and centrifuged at 4 °C and 11500 rpm for 20 min. The resulted supernatant was then transferred to the new tubes and centrifuged at 4000 rpm for 20 min. To assay protein amount, 10 μL of the extract was added to 5 μL of Bradford solution and 290 μL of extraction buffer. The absorption was performed at 595 nm, and bovine serum albumin (BSA) was used as standard.

3.4. Measurement of Malondialdehyde

Malondialdehyde (MDA) was measured according to Namaulema *et al.* (22). MDA content was determined with thiobarbituric acid (TBA) reaction. Then, 200 mg of tissue sample was homogenized in 25 mL 1-Butanol. The resulted homogenate was centrifuged for 10 min at 10000 rpm. Afterwards, 5 mL of the centrifuged solution was added to 5 mL of TBA reagent, and the

mixture was heated at 95 °C for 120 min and quickly cooled on ice bath. The absorbance was read at 530 nm by a spectrophotometer.

3.5. Measurement of Lysine and Methionine
The concentration of lysine and methionine was determined according to Lošák *et al.* (25). The absorbance was read at 570 nm wavelength by spectrophotometer.

3.6. Measurement of Soluble Sugars Content
Soluble sugars were determined according to Sudhakar *et al.* (26). Wavelength was performed by absorption at 625 nm. For this purpose, a mixture of 1.0 g of ground leaf tissue and 5 mL of ethanol 80% (v/v) was prepared and centrifuged at 1500 rpm for 15 min. Thereafter, 3 mL of 0.2% anthrone reagent (0.5 g of anthrone in 250 mL of 72% sulfuric acid) was added to 100 mL of ethanolic extract followed by incubation in boiling water (95 °C) for 10 min (27). After cooling on ice bath, absorbance of the samples was read at 620 nm.

3.7. Measurement of Proline Content
Proline was extracted from the leaves using the method described by Bates *et al.* (28). Briefly, 0.5 g of leaf sample was added into 10 mL of 3% sulfosalicylic acid and homogenized entirely at 3000 rpm for 10 min. afterwards, 2 mL of the filtered mixture was blended

with 2 mL of ninhydrin followed by incorporation of 2 mL of acetic acid into each tube. The samples were then maintained in a water bath for 1 h and immediately placed on ice bath for a few min. Finally, the supernatants were subjected to absorption at 520 nm.

3.8. RNA Extraction, cDNA Synthesis and Real-time PCR Gene Expression Analysis
The first leaf of three-leaf stage was excised to investigate the relative expression level of *Wdhn13*, *CAT1*, *P5CS*, and *DREB2* genes. Primers for target genes were designed based on NCBI mRNA sequences by using Primer3 software Ver. 0.4.0, and *18srRNA* reference gene primer was selected based on Sun *et al.* (18) (**Table 1**). Then, 50 mg of leaf tissue culture was powdered and prepared for RNA extraction by RNA isolation kit (Vivantis-Taiwan). Nano drop spectrophotometer and agarose gel electrophoresis (1.5%) were used to evaluate the quantity and quality of the extracted RNA, respectively. RNase-free DNase I (Fermentase, USA) was added to remove any DNA from RNA samples based on instruction protocol. For cDNA synthesis, 1 µg of RNA was mixed with 1 µL of Oligo dT primer, then the mixture was placed at 60 °C for 5 min. We added 10 µL reverse transcriptase mix (2 µL of dNTP, 1 µL of reverse transcriptase enzyme (SMOBIO, Taiwan), 1 µL of Rnase inhibitor, 4 µL of

Table 1. List of primers designed to examine gene expression patterns

Gene	Primers	Tm	Fragment size (bp)	Efficiency (%)
Wdhn13 (AB076807.1)	Forward: 5' GCTGCCTGGACAGCACTAAG 3' Revers: 5' GCDCTCCTCCATGAAAGCTT 3'	60	130	95
CAT1 (D86327.1)	Forward: 5' CATCTGGCTCTCCTACTGG 3' Revers: 5'AGAACTTGGACGGCCCTGA 3'	60	140	93
P5CS (KM044035.1)	Forward: 5' GGGCTACCCAAAAGCAGAT 3' Revers: 5' GAGTCCAAAACGAGCACCAT 3'	60	238	95
DREB2 (JQ004969.1)	Forward: 5' GGGGCCGACTTTTCTTTCTC 3' Revers: 5' TCGCAATCTTGTCGCCTGTT 3'	60	80	90
18srRNA (AH001810.2)	Forward: 5' GGAGTATGGTCGCAAGGCTGAAAC 3' Revers: 5'CTCAATCTGTCAATCCTCACTATGTCTGG 3'	60	133	98

buffer and 2 μL DEPC treated water) into the tubes and incubated at 45 °C for 1 h. The Real-time PCR was performed using ABI Step One (applied bioscience, USA) with the final volume of 10 μL containing 5 μL of High rox SYBR green mix (Ampliqon, Denmark), 0.5 μM of each primer, 2 mM of $MgcL_2$, 0.2 mM of dNTPs, and 100 ng of cDNA. The quantitative RT-PCR was run at 95 °C for 120 s, 40 cycles at 94 °C (30 s), 58 °C (30 s), and 72 °C (20 s). Data were analyzed by Step one software. Relative expression of each target gene was calculated based on $2^{-\Delta\Delta Ct}$ by normalization against control samples and reference gene (29). Also, the efficiencies were analyzed using LinReg (v.2013.0) software by Ruijter *et al.* (30).

3.9. Statistical Analysis
Differences between treatments were identified from the variance analysis by using the general linear model procedure in SAS (ver. 9.2, SAS). Separation of means was done by using the least significant difference (LSD) test at *P*<0.05 level. When F-test indicated statistical significance, the protected LSD was applied to separate drought stress effects. Significant interactions were separated by the slicing method (31).

4. Results

4.1. Chlorophyll and Carotenoids Contents
As shown in **Table 2**, interactive effects of drought stress × cultivars × zinc nanoxide are significant in relation to total protein content, activity of POD, CAT, and PPO antioxidant enzymes, lysine amino acids, proline, and some of pigments, including chlorophyll a, chlorophyll b, and carotenoid at 1% level and soluble sugar content at 5%.

Table 2. Variance analysis of (mean squares) the effects of ZnO NPs and drought stress on some qualitative, quantitative traits and gene expression in wheat cultivars

Source of variation	df	Chl. a	P-Value	Chl. b	P-Value	T Chl.	P-Value	Car	P-Value	PR	P-Value	SS	
Drought stress (D)	2	0.0455**	0.0017	0.00796**	≤0.0001	0.0910**	≤0.0001	2380.16**	0.0027	0.022ns	0.28	0.7459**	≤0.0001
Cultivar (C)	2	0.0086ns	0.2668	0.00132**	0.0003	0.0121ns	0.2001	1314.06*	0.0322	0.279**	≤0.0001	0.0339ns	0.5219
ZnO (Z)	2	0.0148ns	0.1065	0.00317**	≤0.0001	0.0272*	0.0302	987.97ns	0.0727	0.132**	0.001	0.0800ns	0.2208
D × C	4	0.0152ns	0.0606	0.00059**	0.0042	0.0199*	0.0380	856.14ns	0.0624	0.283**	≤0.0001	0.0373ns	0.5792
D × Z	4	0.0130ns	0.0991	0.00716**	≤0.0001	0.0391**	0.0010	2321.28**	0.0002	0.163**	≤0.0001	0.1321*	0.0485
Z × C	4	0.0532**	≤0.0001	0.00555**	≤0.0001	0.0923**	≤0.0001	2827.95**	≤0.0001	0.108**	0.0002	0.1903**	0.0099
D × C × Z	8	0.0207**	0.0041	0.00217**	≤0.0001	0.0309**	0.0005	1238.74**	0.0028	0.239**	≤0.0001	0.1423*	0.0123
Error	54	0.0063		0.00014		0.0073		358.80		0.017		0.0515	
CV (%)		**21.0**		**16.0**		**18.8**		**21.5**		**21.0**		**24.2**	

Source of variation	df	CAT	P-Value	POD	P-Value	PPO	P-Value	TP	P-Value	Lys	P-Value	Met	
Drought stress (D)	2	4.043**	≤0.0001	3.390**	≤0.0001	43.10**	≤0.0001	1440.45**	≤0.0001	0.0116**	≤0.0001	0.000010ns	0.4309
Cultivar (C)	2	4.210**	≤0.0001	1.241**	≤0.0001	119.90**	≤0.0001	393.76**	0.0093	0.0838**	≤0.0001	0.000027ns	0.1273
ZnO (Z)	2	1.051*	0.0362	0.662**	≤0.0001	4.66ns	0.0794	1299.62**	≤0.0001	0.0135**	≤0.0001	0.000033ns	0.0773
D × C	4	17.437**	≤0.0001	1.735**	≤0.0001	9.73**	0.0008	1435.22**	≤0.0001	0.0125**	≤0.0001	0.000015ns	0.3249
D × Z	4	2.798**	≤0.0001	2.396**	≤0.0001	28.55**	≤0.0001	1170.67**	≤0.0001	0.0079**	≤0.0001	0.000047**	0.0098
Z × C	4	10.321**	≤0.0001	1.704**	≤0.0001	56.11**	≤0.0001	803.16**	≤0.0001	0.030**	≤0.0001	0.000040*	0.0202
D × C × Z	8	5.294**	≤0.0001	0.632**	≤0.0001	23.08**	≤0.0001	735.35**	≤0.0001	0.0089**	≤0.0001	0.000025ns	0.0675
Error	54	0.298		0.055		1.75		77.07		0.0002		0.000013	
CV (%)		**16.9**		**19.3**		**16.6**		**14.1**		**19.1**		**8.8**	

Source of variation	df	MDA	P-Value	CAT1	P-Value	DREB2	P-Value	P5CS	P-Value	Wdhn13	P-Value
Drought stress (D)	2	0.00377**	≤0.0001	475.78**	≤0.0001	241.94**	≤0.0001	150.87**	≤0.0001	955.32**	≤0.0001
Cultivar (C)	2	0.00143**	0.0006	92.53**	≤0.0001	73.47**	≤0.0001	291.09**	≤0.0001	226.58**	≤0.0001
ZnO (Z)	2	0.00016ns	0.3910	190.38**	≤0.0001	19.92**	≤0.0001	59.23**	≤0.0001	12.47**	≤0.0001
D × C	4	0.00115**	0.0001	15.08**	≤0.0001	21.21**	≤0.0001	33.30**	≤0.0001	99.17**	≤0.0001
D × Z	4	0.00082**	0.0020	64.05**	≤0.0001	18.06**	≤0.0001	11.58**	≤0.0001	8.60**	≤0.0001
Z × C	4	0.00112**	0.0002	16.55**	≤0.0001	4.64**	0.0004	16.41**	≤0.0001	33.13**	≤0.0001
D × C × Z	8	0.00019ns	0.3422	10.78**	≤0.0001	16.94**	≤0.0001	5.66**	≤0.0001	19.92**	≤0.0001
Error	54	0.00016		0.50		0.76		0.69		0.52	
CV (%)		**15.2**		**11.2**		**15.0**		**20.7**		**9.2**	

, *, ** not significant or significant at the 0.05 or 0.01 probability level, respectively.
[a] TP= total protein; CAT= catalase; POD= peroxidase; PPO= polyphenol oxidase; PR= proline; Lys= lysine; Met= methionine; SS= soluble sugars; Chl.= chlorophyll Car= carotenoid; and MDA= Malondialdehyde.

Our findings showed that ZnO NPs increased the chlorophyll content in all cultivars. The nanoparticle treatments were able to significantly increase total chlorophyll, chlorophyll a, and chlorophyll b levels in all cultivars; however, this increment was more pronounced in stress (FC 35%). The highest total chlorophyll (0.733 mg. g^{-1} FW) was observed in tolerant cultivar (Mihan) with ZnO NPs 1 g. L^{-1}. In comparison, the lowest level (0.268 mg. g^{-1} FW) was observed in sensitive cultivar (Gascogne) under severe stress (FC 35%) (**Table 3**). The results indicated that with increasing ZnO NPs concentration, carotenoid increased in tolerant and semi-tolerant cultivars compared to the sensitive cultivar. The highest carotenoid content (145.1 mg. g^{-1} FW) belonged to Mihan under interaction stress FC 60% with 1 g. L^{-1} of ZnO NPs, while the lowest amount of carotenoid (48.3 mg. g^{-1}FW) belonged to Gascogne (sensitive cultivar) (**Table 3**).

4.2. Antioxidant Enzymes Activity

Our results showed that ZnO NPs increased CAT, POD, and PPO activities in Mihan and Heidari cultivars. At all stress levels, the nanoparticle treatments significantly increased the CAT, POD, and PPO antioxidant activities in cultivars; however, this increment was more pronounced in severe stress than the others. The highest amounts of the CAT (6.72 µM. g^{-1}min^{-1}) and POD (4.19 µM. g^{-1}min^{-1}) activities were observed in Heidari and Gascogne by ZnO NPs 1 g. L^{-1}, whereas the lowest level of CAT, POD, and PPO activities were observed by 0.5 g. L^{-1} of ZnO NPs under mild and severe stress conditions (**Table 4**).

4.3. Measurement of Malondialdehyde

As shown in **Table 2**, only interaction of drought stress × cultivar, drought stress × ZnO NPs and cultivar ×

Table 3. Interaction[a] of drought stress × cultivar × ZnO NPs on some qualitative responses of wheat cultivars

Treatments [b]			SS [c] (mg.g^{-1} FW)	Chl. a (mg.g^{-1} FW)	Chl. b (mg.g^{-1} FW)	T Chl. (mg.g^{-1} FW)	Car (mg.g^{-1} FW)
Drought stress	Cultivar	ZnO (g. lit^{-1})	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
	Mihan	0	0.95^{ab}±0.232	0.348^{ab}±0.002	0.060^{bc}±0.006	0.446^{ab}±0.0001	79.4^{ab}±4.8
		0.5	0.55^{d}±0.0004	0.375^{ab}±0.003	0.062^{bc}±0.002	0.447^{ab}±0.053	84.7^{ab}±1.3
		1	1.11^{a}±0.393	0.387^{ab}±0.046	0.071^{b}±0.003	0.570^{a}±0.063	89.4^{ab}±5.9
85% FC	Heidari	0	0.83^{bc}±0.050	0.367^{ab}±0.023	0.062^{bc}±0.004	0.410^{ab}±0.004	81.2^{ab}±5.8
		0.5	0.57^{cd}±0.055	0.244^{b}±0.129	0.065^{bc}±0.008	0.309^{b}±0.121	86.1^{ab}±2.2
		1	0.79^{bcd}±0.013	0.479^{a}±0.059	0.091^{a}±0.004	0.429^{ab}±0.027	109.3^{a}±12.1
	Gascogne	0	0.60^{cd}±0.084	0.316^{ab}±0.159	0.040^{d}±0.008	0.364^{b}±0.164	71.0^{b}±42.2
		0.5	0.64^{cd}±0.056	0.265^{b}±0.076	0.041^{d}±0.028	0.306^{b}±0.104	71.9^{b}±34.1
		1	0.67^{cd}±0.072	0.298^{b}±0.179	0.048^{cd}±0.005	0.338^{b}±0.180	56.8^{b}±20.7
LSD (0.05)			**0.27**	**0.167**	**0.018**	**0.173**	**34.4**
	Mihan	0	1.04^{bcd}±0.237	0.328^{cd}±0.144	0.057^{fe}±0.002	0.385^{ef}±0.146	74.0^{cd}±30.7
		0.5	1.17^{bc}±0.155	0.416^{bc}±0.057	0.084^{d}±0.002	0.694^{ab}±0.004	141.2^{a}±17.4
		1	1.50^{a}±0.001	0.540^{a}±0.003	0.194^{b}±0.008	0.733^{a}±0.005	145.1^{a}±18.2
60% FC	Heidari	0	0.92^{cde}±0.001	0.334^{cd}±0.025	0.048^{f}±0.007	0.383^{ef}±0.032	73.9^{cd}±3.7
		0.5	0.85^{de}±0.180	0.487^{a}±0.052	0.073^{de}±0.006	0.475^{de}±0.060	91.6^{bc}±16.7
		1	0.94^{cde}±0.134	0.537^{a}±0.008	0.157^{b}±0.004	0.488^{cde}±0.063	94.9^{bc}±17.8
	Gascogne	0	0.75^{e}±0.002	0.310^{d}±0.048	0.043^{f}±0.010	0.353^{f}±0.058	61.8^{d}±13.2
		0.5	0.85^{de}±0.197	0.401^{bcd}±0.066	0.074^{de}±0.006	0.544^{cd}±0.014	98.3^{bc}±1.0
		1	1.30^{ab}±0.249	0.460^{ab}±0.012	0.103^{c}±0.023	0.590^{bc}±0.070	108.7^{b}±8.3
LSD (0.05)			**0.27**	**0.105**	**0.016**	**0.112**	**28.2**
	Mihan	0	1.05^{a}±0.342	0.252^{c}±0.099	0.077^{a}±0.007	0.329^{bc}±0.092	60.7^{bc}±7.6
		0.5	1.05^{a}±0.003	0.413^{a}±0.067	0.078^{a}±0.008	0.479^{a}±0.077	96.4^{a}±18.7
		1	1.19^{a}±0.502	0.466^{a}±0.166	0.084^{a}±0.020	0.549^{a}±0.186	98.4^{a}±46.9
30% FC	Heidari	0	1.06^{a}±0.239	0.261^{bc}±0.020	0.033^{b}±0.0004	0.293^{c}±0.022	54.3^{c}±24.4
		0.5	0.83^{a}±0.120	0.390^{ab}±0.039	0.066^{a}±0.010	0.469^{ab}±0.047	91.6^{ab}±4.0
		1	1.18^{a}±0.498	0.405^{a}±0.040	0.069^{a}±0.001	0.474^{ab}±0.039	93.8^{ab}±12.9
	Gascogne	0	0.77^{a}±0.153	0.235^{c}±0.002	0.032^{b}±0.001	0.268^{c}±0.002	48.3^{c}±0.05
		0.5	1.01^{a}±0.0004	0.451^{a}±0.059	0.079^{a}±0.026	0.530^{a}±0.085	105.8^{a}±12.0
		1	1.15^{a}±0.467	0.487^{ab}±0.047	0.086^{a}±0.022	0.573^{a}±0.074	111.8^{a}±10.5
LSD (0.05)			**0.55**	**0.130**	**0.024**	**0.147**	**34.5**

[a] Interaction data were analyzed with Least Squares Means and means separated with LSD.
[b] Values in each column and each level of drought stress followed by same letter are insignificant at 5% level.
[c] SS=soluble sugars; Chl. =chlorophyll a, and Car=carotenoid.

ZnO NPs effects were significant ($P<0.01$) according to the LSD test. The highest amounts of MDA were observed in Gascogne under drought stress, and the lowest ones belonged to Heidari cultivar. MDA content was significantly decreased in ZnO NPs treated under mild stress (60% FC), implying that ZnO NPs have a negative effect on MDA content. The lowest levels of MDA were in 0.5 g. L⁻¹ of ZnO NPs in Gascogne and Mihan cultivars under the control conditions (FC 85%) (**Table S1,** supplementary file).

4.4. Soluble Sugars, Proline, and Total Protein Contents

ZnO NPs spraying (0.5 and 1 g. L⁻¹) significantly increased the soluble sugars content in Mihan and Gascogne cultivars (**Table 3**). In general, Mihan cultivar had the highest soluble sugars content (1.50

mg. g⁻¹ FW) by applying 1 g. L⁻¹ of ZnO NPs, and the lowest content (0.55 mg. g⁻¹.FW) in 85% FC.

Appling 1 g. L⁻¹ of ZnO NPs significantly increased proline in all wheat cultivars. The highest amount of proline (1.355 µM. g.⁻¹ FW) was observed in tolerance cultivar (Mihan) under severe stress (35% FC), whereas the lowest amount (0.248 µM. g.⁻¹ FW) was observed in Heidari (**Table 4**).

The total protein content decreased under drought stress, while the ZnO NPs–treated cultivars showed a significant increase under all drought levels. The highest protein content (114.2 µM. g.⁻¹ FW) was obtained from the tolerant cultivar under ZnO application. The lowest protein content (33.0 µM.g.⁻¹FW) was obtained by applying 1 g. L⁻¹ under drought stress (60% FC) in Heidari cultivar (**Table 4**).

Table 4. Interaction effects of drought stress × cultivar × ZnO NPs on some biochemical responses of wheat cultivars

Treatments [b]			TP [c] (µM.g⁻¹ FW)	CAT (µM.g⁻¹ pr.min⁻¹)	POD (µM.g⁻¹ pr.min⁻¹)	PPO (µM.g⁻¹ pr.min⁻¹)	PR (µM.g⁻¹ FW)	Lys (µM.g⁻¹ FW)
Drought stress	Cultivar	ZnO (g.lit⁻¹)	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
85% FC	Mihan	0	68.3ᵃ±3.8	1.88ᵉᶠ±0.092	1.16ᵈ±0.022	8.0ᶜ±0.034	0.514ᶜᵈ±0.033	0.040ᵈ±0.008
		0.5	47.1ᵇ±9.3	1.46ᶠ±0.015	1.27ᵇᶜᵈ±0.035	9.54ᵇᶜ±0.418	0.539ᶜᵈ±0.054	0.069ᶜ±0.002
		1	67.0ᵃ±5.8	2.14ᵉᶠ±0.143	1.64ᵇᶜ±0.218	11.96ᵃᵇ±0.430	0.588ᶜ±0.235	0.091ᶜ±0.012
	Heidari	0	52.5ᵇ±8.6	2.48ᵈᵉ±0.084	1.19ᵈ±0.414	8.34ᶜ±0.465	0.350ᵈ±0.044	0.004ᵉ±0.007
		0.5	49.7ᵇ±9.4	5.06ᵇᶜ±0.082	1.25ᶜᵈ±0.119	9.95ᵃᵇᶜ±0.569	0.400ᶜᵈ±0.001	0.014ᵉ±0.006
		1	68.5ᵃ±3.2	6.72ᵃ±0.717	1.62ᵇᶜ±0.239	11.39ᵃᵇ±1.823	0.802ᵇ±0.192	0.038ᵈ±0.014
	Gascogne	0	41.5ᵇ±4.3	4.45ᶜ±0.877	1.06ᵈ±0.304	12.13ᵃ±3.531	0.530ᶜᵈ±0.076	0.220ᵃ±0.002
		0.5	44.2ᵇ±10.7	3.19ᵈ±0.743	1.30ᵇᶜᵈ±0.053	3.47ᵈ±1.287	0.574ᶜ±0.077	0.196ᵇ±0.018
		1	46.6ᵇ±13.1	5.64ᵇ±0.471	4.19ᵃ±0.202	3.95ᵈ±0.920	1.035ᵃ±0.074	0.220ᵃ±0.026
LSD (0.05)			**14.1**	**0.83**	**0.37**	**2.50**	**0.194**	**0.022**
60% FC	Mihan	0	57.3ᵈ±0.7	2.01ᵈᵉ±0.144	1.17ᵇᶜ±0.142	4.87ᵉ±0.649	0.540ᶜᵈ±0.036	0.026ᵈ±0.002
		0.5	86.7ᵇ±15.0	1.80ᵉ±0.057	0.23ᵉ±0.060	7.20ᵈ±0.571	0.628ᶜ±0.194	0.002ᵉ±0.001
		1	114.2ᵃ±13.5	2.22ᶜᵈᵉ±0.278	0.97ᶜᵈ±0.036	17.65ᵃ±1.037	1.004ᵃ±0.007	0.004ᵉ±0.002
	Heidari	0	46.7ᵈ±6.8	2.52ᶜᵈᵉ±0.048	1.55ᵃᵇ±0.098	9.57ᶜ±0.357	0.373ᵉ±0.109	0.010ᵉ±0.0001
		0.5	33.0ᵉ±1.6	1.92ᵈᵉ±0.208	1.66ᵃ±0.017	10.20ᶜ±1.390	0.391ᵈᵉ±0.048	0.0001ᵉ±0.00004
		1	71.5ᶜ±11.4	2.92ᶜ±0.304	1.76ᵇᶜ±0.562	12.43ᵇ±1.161	0.476ᶜᵈᵉ±0.022	0.004ᵉ±0.004
	Gascogne	0	41.5ᵉ±4.1	2.67ᶜᵈ±0.036	0.70ᶜᵈᵉ±0.138	6.15ᵈᵉ±0.272	0.572ᶜ±0.018	0.107ᶜ±0.016
		0.5	77.0ᵇᶜ±4.1	4.70ᵇ±0.582	0.62ᵈᵉ±0.087	3.06ᶠ±0.501	0.631ᶜ±0.084	0.174ᵇ±0.008
		1	78.5ᵇᶜ±1.1	5.72ᵃ±1.278	0.72ᶜᵈ±0.572	6.51ᵈ±0.998	0.840ᵇ±0.115	0.281ᵃ±0.020
LSD (0.05)			**14.2**	**0.85**	**0.48**	**1.46**	**0.156**	**0.015**
30% FC	Mihan	0	52.2ᶜᵈ±4.0	2.08ᵇᶜ±0.170	1.30ᵃ±0.073	4.93ᶜᵈ±0.436	0.796ᵇᶜ±0.340	0.050c±0.002
		0.5	83.3ᵃᵇ±1.6	3.93ᵃ±0.325	0.51ᵉ±0.123	6.25ᵇᶜᵈ±0.368	0.977ᵇ±0.101	0.024ᶜᵈ±0.008
		1	87.1ᵃ±13.1	4.97ᵃ±0.367	0.53ᵈᵉ±0.033	6.87ᵇᶜ±0.579	1.355ᵃ±0.022	0.085ᵇ±0.002
	Heidari	0	45.4ᵈ±3.4	2.40ᵇ±0.735	1.21ᵃᵇ±0.126	10.10ᵃ±1.506	0.384ᵈᵉ±0.004	0.010ᵈ±0.006
		0.5	55.3ᶜᵈ±7.7	1.23ᶜ±0.731	0.87ᶜᵈ±0.176	6.54ᵇᶜᵈ±0.728	0.248ᵉ±0.046	0.028ᶜᵈ±0.008
		1	68.4ᵇᶜ±5.2	1.74ᵇᶜ±0.764	1.35ᵃ±0.370	8.61ᵃᵇ±1.592	0.476ᵈᵉ±0.006	0.030ᶜᵈ±0.014
	Gascogne	0	63.0ᶜ±23.5	4.03ᵃ±1.157	0.89ᵇᶜ±0.323	6.86ᵇᶜ±0.435	0.659ᶜᵈ±0.354	0.122ᵃ±0.053
		0.5	66.9ᵇᶜ±0.7	2.36ᵇ±0.015	1.07ᵃᵇᶜ±0.088	4.05ᵈ±0.573	0.399ᵈᵉ±0.014	0.091ᵃᵇ±0.008
		1	68.7ᵇᶜ±3.0	4.93ᵃ±0.681	1.20ᵃᵇᶜ±0.223	4.29ᶜᵈ±3.894	0.531ᶜᵈᵉ±0.086	0.099ᵃᵇ±0.012
LSD (0.05)			**16.7**	**1.10**	**0.34**	**2.66**	**0.292**	**0.033**

[a] Interaction data were analyzed with Least Squares Means and means separated with LSD.
[b] Values in each column and each level of drought stress followed by same letter are insignificant at 5% level.
[c] TP=total protein; CAT=catalase; POD=peroxidase; PPO=polyphenol oxidase; PR=proline; and Lys=lysine.

4.5. Lysine and Methionine Content
Our findings revealed that lysine content was significantly increased in ZnO NPs. The highest amounts of lysine (0.281 μM. g.$^{-1}$ FW) were observed in the interaction of ZnO NPs 1 g. L^{-1} at 60% FC in Gascogne cultivar and the lowest levels of lysine (0.0001 μM. g.$^{-1}$ FW) were demonstrated in mild stress (60% FC) with 0.5 g. L^{-1} of ZnO NPs (**Table 4**). As shown in **Table 2**, there was a significant interaction between drought stress × zinc nanoxide and zinc nanoxide × cultivars in methionine. Drought stress × ZnO indicates that methionine content was significantly increased in ZnO NPs 1 g. L^{-1} under drought stress. The highest amount of methionine was observed in Mihan by 1 g. L^{-1} of ZnO NPs and the lowest ones were seen in Heidari cultivar under 0.5 g. L^{-1} of ZnO NPs (**Table S2,** supplementary file).

4.6. Expression Analyses of Genes
As shown in **Table 2**, the three-way interaction effects of drought stress × cultivars × zinc nanoxide were significant (*P*<0.01) in relation to expression level of *Wdhn13*, *CAT1*, *P5CS*, and *DREB2* transcription factors. All these factors were significantly up-regulated after spraying with ZnO NPs (**Fig. 3**). In the tolerant genotype, the relative transcript abundances of *Wdhn13*, *CAT1*, *DREB2*, and *P5CS* transcripts in the wheat plants treated with ZnO NPs 1 g. L^{-1} were 2.2-fold, 1.5-fold, 1.4-fold, and 3-fold compared to those of control plants in 85% FC, respectively. Also, tolerant, semi-tolerant, and sensitive genotypes showed a similar pattern under different levels of drought stress (**Fig. 3**). Moreover, *DREB2* relative expression level was more than *P5CS*, indicating the importance of the *DREB2* transcription factor in response to drought stress. Although *DREB2* showed sharp increase in Heidari cultivar, it was down-regulated in Gascogne cultivar (**Fig. 3C, D**). Interestingly, we documented the positive effects of ZnO NPs on the expression level of *DREB2* and *Wdhn13* genes for the first time (**Fig. 3A, C**).

5. Discussion
Plants, as a sessile organism, have developed several mechanisms in perceiving signals and inducing different pathways to regulate molecular responses to better cope with abiotic stress conditions (17). Water shortage, for example, induces the ROS production and lipid peroxidation production in the cell membrane system through MDA production as oxidative damage marker (3, 17). The balance in producing and removing

oxidative agents is important to survive plant cells under stress (5). In this study, a considerable reduction was observed in chlorophyll and carotenoid contents under water deficit, which is generally due to ROS destructive effects on chloroplast (32), In this regard, Mejri *et al.* reported that drought stress causes perturbation in the ratio of chlorophyll a to b and degrades them preventing chloroplast activity (33). Hypothetically, plants may be inclined to decrease the chlorophyll content to prevent photooxidation. Also, they increase the MDA content indicating the peroxidation of membrane and cell damage under water stress (34, 35). It has been demonstrated that NP is involved in the production of ROS and activation of antioxidant system under abiotic stress. Interestingly, foliar spraying ZnO NPs inhibited chlorophyll degradation and significantly improved Chlorophyll a/b, and T-Chlorophyll contents in both drought-stressed and control plants. Similarly, Semida *et al.* (36) proved that spraying 50 and 100 ppm ZnO NPs improved the photosynthetic efficiency in water-stressed eggplant (*Solanum melongena* L) indicating its potency to stabilize membrane integrity, increasing RWC, and ceasing the chlorophyllase activity to preserve chlorophyll content, which then increases the photosynthesis efficiency. This mitigated the drought effects probably correlated with reducing MDA content and low lipid peroxidation, which was observed in Gascogne cultivar treated with 0.5 g. L^{-1} under drought. Likewise, it has been shown that ZnO NPs reduced MDA and lipid peroxidation in drought-treated maize, maintaining chloroplasts and mitochondria (18).

As a matter of fact, antioxidant system already was activated after perception of high ROS production by NADP oxidase under abiotic stress. It has been demonstrated that ZnO NPs effectively alleviate various abiotic stress, especially at low concentrations through activating antioxidant enzymes (37). Based on three-way interaction, the activity of CAT has been significantly increased by spraying 1 mg. L^{-1} ZnO in Mihan and Gascogne under 30% FC. Thus, it enhances ROS scavenging and decreases MDA level. However, PPO activity has been increased in Mihan cultivar sprayed with 1 mg. L^{-1} ZnO NPs under moderated stress indicating low toxicity and mitigating effects through incrementing ROS species to prevent oxidative stress in wheat (38). Comparatively, it was demonstrated that ZnO has low toxic effect than CeO$_2$ on soybean, and plants treated with ZnO were able to keep their growth
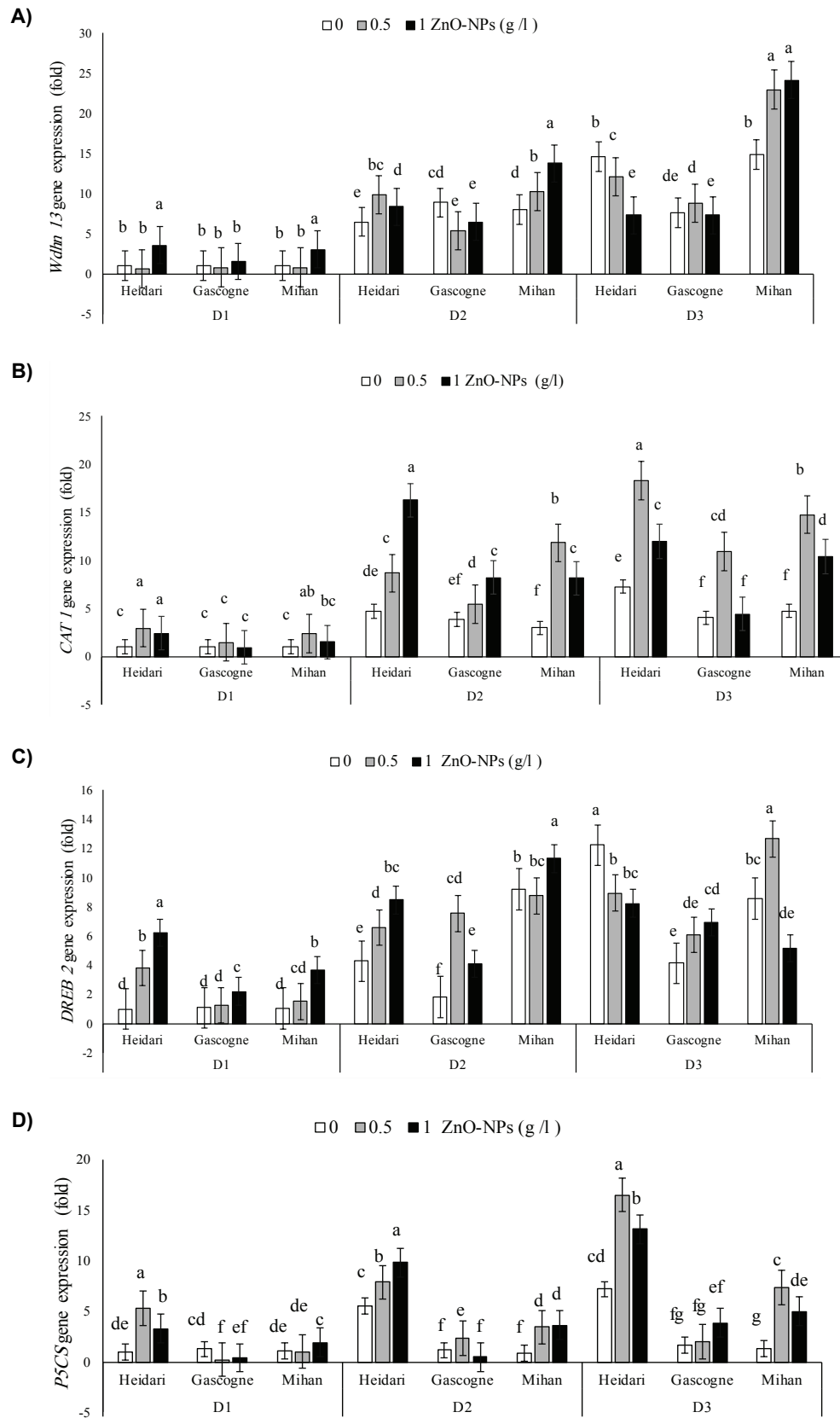
Figure 3. Three-way interactive effects of drought stress × cultivars × ZnO NPs on the expression of *Wdhn13* **A)**, *CAT1*, **B)**, *DREB2* **C)**, and *P5CS* **D)** genes in wheat cultivars. Bars with the same letter are not significantly different at $P<0.05$ based on the least significant difference (LSD) test. D1= 85% field capacity drought stress, D2= 60% field capacity drought stress, and D3= 30% field capacity drought stress.

and yield, along with trifle increase in ROS and lipid peroxidation (39). In this regard, Khan *et al.* reported that silicon NPs effectively improved the uptake mechanism and alleviated the detrimental effects of abiotic stress by promoting antioxidant activity of enzyme (40). Sedghi *et al.* (41) showed that simultaneously exposed soybean seeds to 1 g. L^{-1} of ZnO NPs and drought showed better germination rate and tolerance, as well as low residual fresh and dry weight. Improving the wheat tolerance by ZnO NP was carried out by increasing the antioxidant enzyme activity (SOD and CAT) under drought stress. Similarly, it has been reported that foliar application of fullerenol nanoparticles (FNPs) on maize alleviated the oxidative effects of drought by increasing the activity of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and ascorbate peroxidase (APX) in a concentration-dependent manner (42). The involvement of those genes in promoting plant responses to unfavorable conditions has been well established in many studies (16, 17). The positive effects of ZnO might be correlated with accumulation of proline and sugar content.

In the present study, soluble sugars and proline contents were significantly increased under drought stress. Proline is an osmoprotectant and essential substance for the pentose phosphate pathway, which regulates the cellular redox potency needed for preserving many antioxidants in the redox state. Therefore, this molecule plays an important role in ceasing oxidative damage, such as eliminating O_2 radicals in thylakoid membranes (5). At the biochemical level, key molecules such as carbohydrates and amino acids play important roles in stress tolerance and improve the plant adaptation by ROS scavenging, altering their membrane stabilization and osmoregulation (43). Our results indicated that all total soluble protein, soluble sugar, and proline content were higher in ZnO NPs-treated cultivars under drought, which proved that spraying ZnO NPs enhanced the osmotic regulation and helped to increase the structural stabilization of proteins. This study is in accordance with the findings of Sun *et al.* (18) in maize. Also, the increased content of soluble sugar in the ZnO NPs–treated wheat cultivars under drought may be due to several factors, including i) inhibiting the degradation of insoluble carbohydrates and its conversion to soluble sugars, ii) increment in sucrose transfer and decrement of the ratio of sucrose to starch, and iii) suppressing the decomposition of starch

outside the leaves for osmotic adjustment during short and long-term water deficit periods. In addition, zinc is involved in maintaining membrane potential and structure, the synthesis of tryptophan, and functions as a regulatory cofactor in protein synthesis (19). The increased lysine and methionine content under drought stress (as observed in this study) caused by degradation of proteins protects the structure of enzyme, produces coenzyme A and enters the Krebs cycle, and provides the energy needed for the cell to cope with stress (44).

In this study, increasing the CAT activity probably caused the up-regulation of *CAT1* in drought-tolerance cultivars during drought stress that is in agreement with the results obtained by Fleta-Soriano *et al.* (45). In addition, the expression level of *CAT1* was significantly elevated in ZnO NPs (0.5 g. L^{-1}) treated cultivar under drought stress. It seems that ZnO NPs are involved in ROS scavenging by stimulating antioxidant enzyme activities. Recently, Sun *et al.* (18) proved this hypothesis and demonstrated that the expression level of CAT was significantly enhanced in ZnO NP-treated plants under drought. Our study demonstrated a higher level of *P5CS* expression in drought-tolerant and semi-tolerant cultivars than that of drought-sensitive cultivar, which is consistent with Dudziak *et al.* (17). The exposure of Heidari cultivar to severe drought stress (35% FC) and its spraying with ZnO NP resulted in an immediate increase of *P5CS* expression levels. It proposed that metal-based NPs act as stress signaling molecules and induce the expression of drought-responsive genes, leading to defense system activation and tolerance to stress (12). Based on the results, the expression level of *Wdhn13* was the highest in ZnO-treated tolerant plants compared to other cultivars under two levels of water stress (60% and 35% FC). However, *CAT1* genes were increased more in semi-tolerant cultivar (Heidari) compared to others under the same conditions. Our results supported this hypothesis and indicated that both genes are involved in primary response to drought stress in wheat. Interestingly, this study is the first report on the positive effect of foliage spraying of ZnO NP on relative expression levels of *DREB2* and *Wdhn13* genes, triggering drought tolerance responses in Mihan and Heidari cultivars. Thus, it is supposed that ZnO NP-treated wheat plants represented an improved response to drought due to up-regulation of dehydration-responsive and antioxidant related genes, CAT, enhanced proline biosynthesis, and those genes

encoding the late embryogenesis abundant proteins. In this study, the combination of physiological responses and expression analysis of candidate genes may help understand the mechanism of ZnO NPs effects on wheat cultivars at a molecular level to mitigate drought stress.

6. Conclusion

Water deficit conditions largely affect the biochemical/ physiological processes, such as photosynthesis, nutrient and hormone metabolism, carbohydrates absorption, and a substantial reduction in the growth and productivity of crops. This research was conducted to investigate the impacts of ZnO NPs (0, 0.5 and 1 g.L^{-1}) and stress levels of water supply (including 85%, 60%, and 35% field capacity) on biochemical traits and gene expression of wheat cultivars (Mihan, Heidari, and Gascogne). Our findings revealed that ZnO NPs (0.5 and 1 g.L^{-1}) application significantly ameliorated some of the damaging impacts of drought stress and improved the activity of antioxidant enzymes and compatible metabolites, photosynthetic total pigments, total protein, soluble sugars, and lysine and methionine contents. Furthermore, the related genes were induced in both stress and ZnO NP-treated plants. Evaluated traits may indicate discrepancies in stress response mechanisms with potential contribution to drought stress. The differences in sensitivity of signaling steps causing defense gene expression requires further investigations.

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