



Changes in antioxidant enzyme activities and gene expression profiles under drought stress in tolerant, intermediate, and susceptible wheat genotypes

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Abstract

Drought is a common abiotic stress that influences crop production to a large extent all over the world. Wheat crop experiences drought at main stages during its life cycle, which induces oxidative stress in the plants. The antioxidant mechanisms of the plant have a significant role in providing tolerance against the water stress. The objective of this work was to investigate the antioxidant activity and transcript profile of antioxidant enzyme related genes in three wheat genotypes to be TN4228 (drought tolerant), TN1399 (moderately tolerant) and TN3737 (susceptible) at control [80% Field capacity (FC)] medium (40% FC) and severe stress (25% FC). Water-limited conditions led to a decrease in relative water content (RWC). However, drought increased malondialdehyde (MDA), hydrogen peroxide (H₂O₂), proline content, and antioxidant enzyme activities [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX)]. In addition, we observed the enhancement of the delta-1-pyrroline-5-carboxylate synthase (*P5CS*), *CAT*, *APX* and *SOD* gene expressions at drought stress conditions. Both moderate and severe stresses caused genotype-specific responses, which were dependent on stress severity. According to drought stress and defense systems in TN4228, it was obtained that the engaged genes and enzymes play significant roles in defense responses and could be viable targets to determine the level of drought tolerance.

Keywords Drought · Wheat · Antioxidant enzymes · Gene expression · Proline

Abbreviations

CAT	Catalase
SOD	Superoxide dismutase
APX	Ascorbate peroxidase
MDA	Malondialdehyde
H ₂ O ₂	Hydrogen peroxide
P5CS	Delta-1-pyrroline-5-carboxylate synthase
RWC	Relative water content
FC	Field capacity
ROS	Reactive oxygen species

Introduction

Drought is a major abiotic stress that adversely influences crop production and leads to economic losses in the world (Dolferus et al. 2019). Wheat (*Triticum aestivum* L.) is an economically important food for about 1/3 of the world's population (Dudziak et al. 2019). Recently, the importance of drought-resistant cultivars is more highlighted due to increasing the adverse impact of climate change particularly in arid and semiarid areas such as most parts of Iran (Fang and Xiong 2015). Genotypic variations for resistance to drought stress are known to exist in different crops. The development of resistant cultivars, however, is hampered by low heritability for drought tolerance and lack of effective selection strategies (Sheoran et al. 2015). The response of resistant and susceptible wheat genotypes to water deficit-induced oxidative stress and antioxidant management at a particular growth stage and under controlled growth conditions has been reported in the literature (Lascano et al. 2001; Sheoran et al. 2015; Rahimi et al. 2019).

Drought stress induces morpho-physiological and molecular variability in plants (Shinozaki and

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Yamaguchi-Shinozaki 2007). Moderate to severe stress significantly influences physiological and biochemical status in wheat such as relative water content (RWC) and antioxidant enzyme activities. Upon exposure to drought, reactive oxygen species (ROS) levels significantly increase, which is because of their higher generations than detoxifications, leading to oxidative burst (Sheoran et al. 2015). Excessive ROS concentration at any form induces the damages in DNA, protein and membrane lipid or even cell death (Mattos and Moretti 2015). To minimize the ROS concentration and preserve plant cells from oxidative damage, there are enzymatic and non-enzymatic antioxidant defense systems that are able to scavenge the ROS efficiently. In addition to antioxidant system, non-antioxidant mechanisms such as proline synthesized from glutamate through delta-1-pyrroline-5-carboxylate synthase (P5CS) is required as an osmolyte for maintaining cell turgidity (Yanlei et al. 2017) and even has an essential role as a ROS scavenger. Malondialdehyde (MDA) can be used as an index of membrane damage because of being ultimate product of lipid peroxidation (Dudziak et al. 2019).

The strategies combining physiological, molecular and genetic techniques have been increasing to elucidate plant growth and tolerance to stresses (Sheoran et al. 2015). Understanding the association of antioxidant enzyme activity and gene expression with genetic variation in drought tolerance is important for further deciphering the factors controlling antioxidant defense. However, most studies evaluating the effect of water stress in wheat have been restricted to morphological, physiological and biochemical responses of antioxidant genes at the seedling stage only. Therefore, the present work was designed (a) to identify the sensitive and tolerate genotypes of wheat under drought, (b) to assess the effect of oxidative stress on physiological traits and antioxidant responses, integrating physiological, biochemical and molecular approaches at anthesis stage of the plants. Our hypothesis is that the genotypes have different responses to drought stress in terms of physiological and biochemical properties and also the expression of several stress response-related genes including delta-1-pyrroline-5-carboxylate synthase (*P5CS*), *CAT*, *APX*, and *SOD* genes is different under drought levels.

Materials and methods

Plant materials and growth conditions

Seeds of three wheat genotypes including highly tolerant (TN4228), moderately tolerant (TN1399) and susceptible (TN3737) were obtained from National Plant Gene Bank, Iran. The pot experiment was conducted in Karaj, Iran as a complete randomized block design (CRBD) with three replications. Five

seeds of each genotype were sown in plastic pots with 5 L size in a mixture of coco peat, peat moss, and perlite (2:2:1). The average temperature was between 18 and 28 °C, and the relative humidity varied from 55 to 60%. Until anthesis stage (BBCH-61), all plants were evenly irrigated. At anthesis stage (on July 17, 2018), the plants were subjected to drought by deficit irrigation, in a way that soil moisture content declined gradually and reached 40% field capacity (FC) as moderate and 25% FC as severe drought treatments, 20 days after the last normal irrigation. Control plants were irrigated normally. The sampling of leaves was done in dough developmental stage. To measure antioxidant enzyme activities, lipid peroxidation and gene transcription levels, harvested flag leaves were transferred in liquid nitrogen and kept at – 80 °C until they were used in the laboratory.

Relative water content (RWC) measurement

The RWC of leaves was calculated as a percentage according to the method of Heath and Packer (1968) as follows:

$$\text{RWC} = \frac{(\text{FW} - \text{DW})}{(\text{SW} - \text{DW})} \times 100$$

where FW is fresh weight, SW is leaf weight after soaking for 24 h at room temperature and DW is leaf dry weight after drying for 24 h at 75 °C.

Malondialdehyde (MDA) concentration

To determine the MDA content, the samples were extracted with phosphate buffer and centrifuged at 14,000 rpm for 30 min. After that, the thiobarbituric acid (0.5% w/v) containing 20% w/v trichloroacetic acid was added to the mixture. The samples were placed in a hot water bath for 30 min and then were immediately cooled with ice and finally centrifuged at 10,000 rpm for 10 min. Samples were read at 532 and 600 nm wavelengths (Alexieva et al. 2001).

Measurements of H₂O₂ production

H₂O₂ was determined by 3,3'-diaminobenzidine (DAB) staining as described by Guan and Scandalios (2000). Wheat leaf samples were incubated in DAB solution (1 mg/ml, pH 3.8; Sigma-Aldrich) for 8 h in dark at 28 °C, and then dipped into boiling 80% (v/v) ethanol for 10 min. The samples were extracted with 80% (v/v) ethanol after cooling, and photographed.

Proline concentration

To measure proline content, 0.5 g fresh leaf sample was mixed with 10 ml of sulfosalicylic acid (3% w/v). The

mixture containing the sample were centrifuged at $4000\times g$ for 20 min, and then 2 ml of ninhydrin acid and 2 ml of glacial acetic acid were added. Simultaneously, 2 ml of standard 0, 4, 8, 12, 16, 20 mg l^{-1} proline and 2 ml ninhydrin acid and 2 ml acetic acid were mixed. All samples were heated in a hot water bath for 60 min and then placed on ice to be cooled completely. Four milliliters of toluene was added to the solution and stirred for 20 s. Using 0, 4, 8, 12, 16, and 20 mg l^{-1} proline standards, the standard curve was determined spectrophotometrically at 520 nm. The toluene-soluble proline was sufficiently measured at 520 nm expressed as $\mu\text{mol proline g}^{-1}$ FW (Bates et al. 1973).

Enzyme assay

The extraction procedures were carried out at 0–4 °C. Fresh samples (0.5 g) were homogenized in a Heidolph, Diax 900 homogenizer in 5 ml 100 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA- Na_2 and 0.5 mM ascorbate. Because ascorbate peroxidase (APX) is labile in the absence of ascorbate, 0.5 mM ascorbate was included for the extraction of this enzyme with the above procedure. The homogenized shoot samples were centrifuged at 10,000 g for 5 min. The supernatant was used as a crude enzyme extract. All colorimetric measurements were made at room temperature in a Shimadzu UV/VIS 1201 spectrophotometer.

Catalase (CAT) activity was measured with initial rate for H_2O_2 disappearance (Dhindsa et al. 1981). The reaction mixture contained 3% H_2O_2 and 0.1 mM ethylenediaminetetraacetic acid (EDTA) in 0.05 M Na-phosphate buffer (pH 7). The reduction in H_2O_2 concentration was determined as a decline in optical density at 240 nm, and the activity was measured as $\mu\text{mol H}_2\text{O}_2$ decomposed per minute.

Superoxide dismutase (SOD) activity was determined as described by Beyer and Fridovich (1987). The reaction mixture consisted of 1.17×10^{-6} M riboflavin, 0.1 M methionine, 2×10^{-5} M potassium cyanide (KCN) and 5.6×10^{-5} M

nitroblue tetrazolium salt (NBT), which was dissolved in 3 ml of 0.05 M sodium phosphate buffer (pH 7.8). 1 ml of enzyme extract was enriched with 3 ml of the reaction. Illumination was started to initiate the reaction at 30 °C for 60 min. The blanks were identical solutions that were kept under dark condition. The absorbance was determined at 560 nm with the spectrophotometer against the blank.

Ascorbate peroxidase (APX) activity was calculated by following the decrease of ascorbate and measuring the change in absorbance at 290 nm for 1 min in 2 ml of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA- Na_2 , 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 50 μl of crude enzyme extract (Nakano and Asada 1981). The activity was calculated from the extinction coefficient ($2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) for the ascorbate.

Gene expression analysis

Total RNA was extracted using GeneAll® Ribospin™ kit (BioFrontier, Korea). RNA quality and quantity were measured by 1% agarose gel electrophoresis and NanoDrop spectrophotometry (Thermoscientific 2000c, USA), respectively. 3 μg of RNA was treated by DNaseI (ThermoFisher, USA) and 1 μg of treated RNA 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. Primer designing was carried out by online PrimerQuest software (Thornton and Basu 2011) based on cDNA sequence (Table 1). Wheat *actin* gene (AB181991.1) was used as an internal standard for gene expression. qRT-PCR was done in StepOne Plus Real Time PCR (ABI, USA) in total volume 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 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. Finally, melt curve analysis was

Table 1 Primers sequence, melting temperature (T_m), size, and accession number

Gene	Primer	Sequence (5'–3')	T_m (°C)	Product size (bp)	Accession number
Actin	F	CACGCTTCCTCATGCTATC	58	123	AB181991.1
	R	CTGACAATTTCCCGCTCAG			
Cu/Zn SOD	F	GGGCACCTGAAGATGAAATC	58	120	FJ890986.1
	R	TTGAATTTGGTCCAGTAAGGG			
APX	F	CCAGCACCAACAAGTGATAC	58	121	AK331910.1 EF184291.1 AY513263.1 AY513262.1 AF532974.1
	R	CCAGCACCAACAAGTGATAC			
CAT	F	GCGAGAAGATGGTGATCG	58	143	KM523672.1 D86327.1
	R	CTTGATCTCATGGGTGAGG			
P5CS	F	CCAGGAAAGATAGCAAGSS	58	101	AK454018.1 KM523670.1 KC175596.1 AB193551.1 AY888045.1
	R	AAACCATCAGCAACCTCTG			

implemented to assess the PCR specificity for all reactions. Every sample was replicated three times independently.

Statistical analysis

Analysis of variance was conducted using SAS software. Tukey's test at $P < 0.05$ was utilized for identifying the significant difference among mean values. Data of gene expressions were analyzed by Relative Expression Software Tool (REST).

Results

RWC, H₂O₂, MDA, and proline concentrations

Drought, especially severe stress, resulted in a significant decline of RWC. Under both severe and moderate stresses, the rate of RWC reduction was similar in drought-tolerant (TN4228) and intermediate (TN1399) genotypes, but it was lower when compared to susceptible (TN3737) genotype (Fig. 1a). Compared to normal irrigation, the severe

and moderate stress caused higher accumulation of H₂O₂ and MDA in flag leaves of all plants. However, TN3737 genotype contained higher H₂O₂ and MDA content than the other ones, and no significant difference was observed between TN4228 and TN1399 (Fig. 1b, c). Severe drought led to higher proline accumulation compared to moderate stress. Under moderate drought stress, both TN4228 and TN1399 genotypes showed a similar level of proline content (Fig. 1d).

Enzyme activity

Drought stress increased the antioxidant enzyme activities. In TN4228, moderate stress increased CAT activity by 73% compared to control (Fig. 2a). For SOD, we obtained a significant enhancement when plants were treated with both moderate and severe stresses. In TN1399, it was found two-fold enhancement of SOD activity under moderate stress in comparison with control (Fig. 2b). TN4228 and TN3737 represented the highest and lowest APX activity, respectively (Fig. 2c).

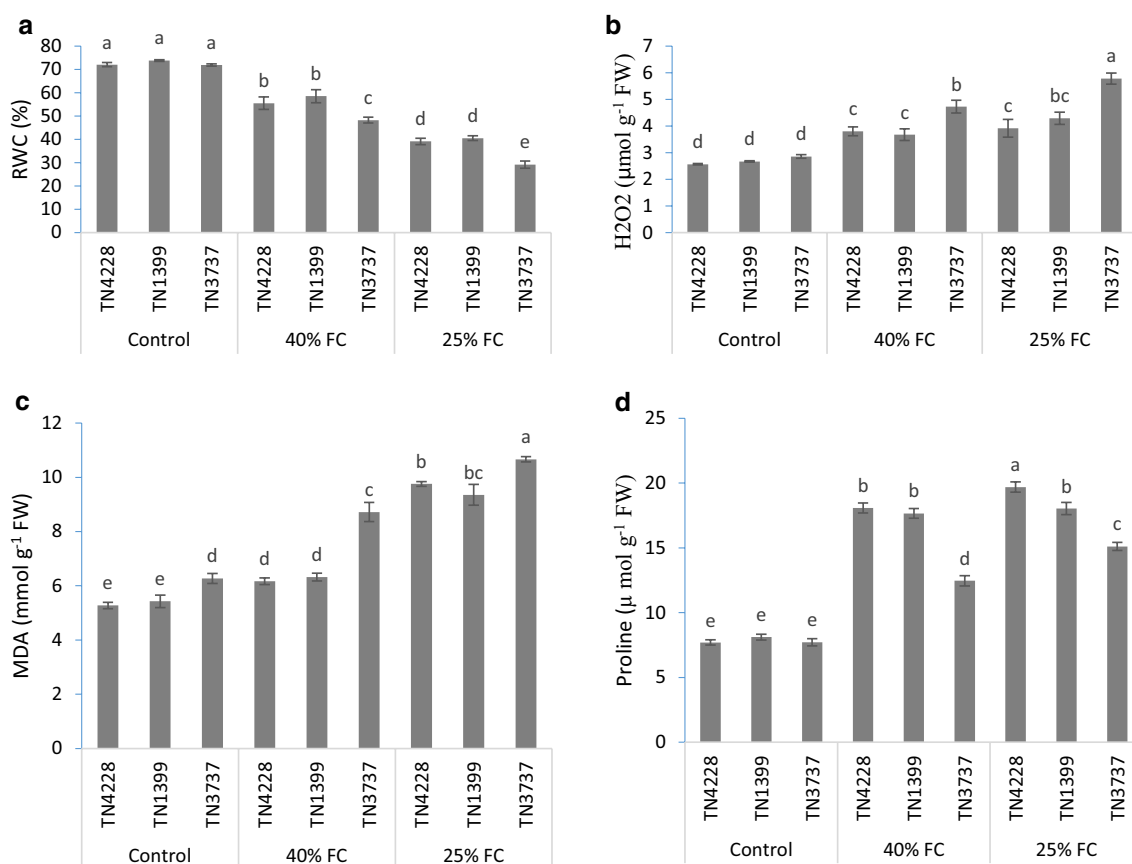


Fig. 1 Relative water content (RWC, **a**), hydrogen peroxide (H₂O₂, **b**), malondialdehyde (MDA, **c**), and proline content (**d**) in three wheat genotypes of drought-tolerant (TN4228), intermediate

(TN1399), and susceptible (TN3737) under drought stress at anthesis stage for a 20-day period. The values are represented with mean \pm SE, $n = 3$

Gene expression

Severe drought increased *P5CS* gene expression up to 16, 6, and fourfold in TN4228, TN1399, and TN3737, respectively, and moderate stress upregulated *P5CS* gene expression up to fivefold in TN4228 and TN1399 (Fig. 3a). The antioxidant enzymes exhibited activity variations under severe stress compared to moderate drought conditions, and TN4228 and TN1399 genotypes indicated the highest CAT, APX, and SOD activity under both drought conditions. In TN3737, APX activity remained unchanged at severe stress, while decreased at moderate drought (Fig. 2c). Different patterns of gene expression were detected for *SOD*, *CAT* and *APX* during exposure to drought treatments (Fig. 3b–d). In TN4228, *CAT* gene was highly overexpressed under drought treatments, while in TN1399, only upregulated under severe stress condition. In TN3737, *CAT* transcript level increased highly under moderate drought, but slightly under severe drought. TN4228 and TN1399 indicated a higher increase in *APX* transcript level under moderate drought as compared to severe stress. In TN3737, *APX* gene was upregulated to twofold at moderate stress conditions, whereas downregulated in

severe drought-stressed plants. The transcript level of *SOD* gene increased significantly in TN4228 and TN1399 as they were exposed to severe drought. In TN1399, *SOD* gene was upregulated up to threefold under moderate drought, but its expression was not affected by severe drought stress. In TN3737, *SOD* gene was slightly downregulated at moderate stress condition, whereas upregulated to threefold under severe stress.

Discussion

RWC as an important indicator for plant water condition is associated with metabolic activities and physiological processes (Huang et al. 2013). In this research, RWC was found dependent on drought intensity in all genotypes. Higher tolerance in TN4228 and TN1399 could be attributed to their higher RWC and their abilities in preserving water content under drought conditions, which is necessary for the maintenance of plants optimum physiological and metabolic functions (Eftekhari et al. 2017). In line with our findings, several studies have demonstrated the connections

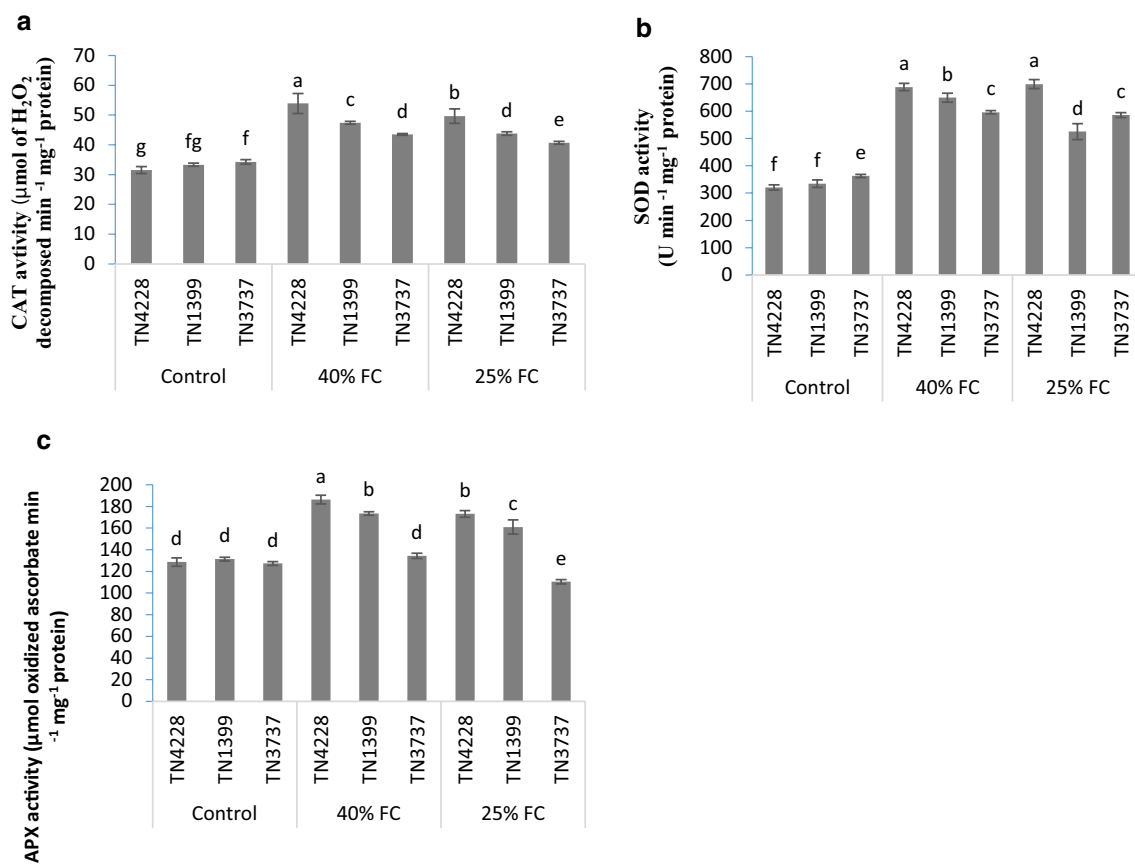


Fig. 2 The activity of catalase (CAT, **a**), superoxide dismutase (SOD, **b**), and ascorbate peroxidase (APX, **c**) in three wheat genotypes of drought-tolerant (TN4228), intermediate (TN1399) and susceptible

(TN3737) under drought stress at anthesis stage for a 20-day period. The values are represented with mean \pm SE, $n = 3$

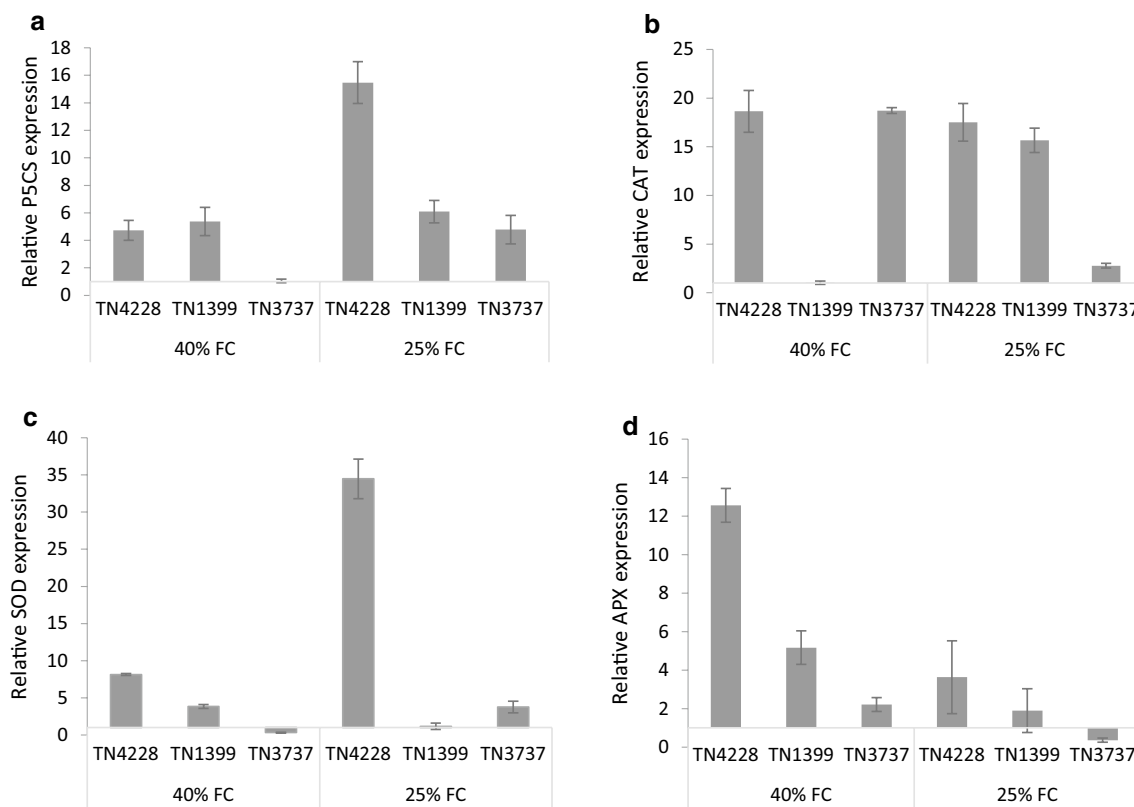


Fig. 3 Gene expression of delta-1-pyrroline-5-carboxylate synthase (*P5CS*, **a**), catalase (*CAT*, **b**), superoxide dismutase (*SOD*, **c**), and ascorbate peroxidase (*APX*, **d**) in three wheat genotypes of drought-

tolerant (TN4228), intermediate (TN1399) and susceptible (TN3737) under drought stress at anthesis stage for a 20-day period. The values are represented with mean \pm SE, $n=3$

between the maintenance of higher RWC under drought and the higher drought tolerance in wheat (Marcek et al. 2019; Eftekhari et al. 2017).

The lower H_2O_2 and MDA quantity, as stress indexes, in tolerant and intermediate genotypes could be contributed to their higher activities of antioxidant enzymes. It could be suggested that more tolerant genotypes possess high efficient antioxidant defense system at the cellular level to protect them against oxidative stress. Similar to our results, different studies on wheat reported that lower soil water content resulted in higher H_2O_2 and MDA concentrations in leaf cells (Marcek et al. 2019; Dudziak et al. 2019).

Proline accumulation has a defensive function in oxidative stress due to its important role in osmotic adjustment, withstanding cellular dehydration and ROS scavenging (Yanlei et al. 2017). Our results indicated higher proline content under drought treatments, which can be explained by the restriction of its oxidation or/and the stimulation of its biosynthesis. In fact, TN4228 and TN1399 tolerant genotypes had higher proline content and *P5CS* expression when compared to TN3737 susceptible genotype. Thus, it could be suggested that under drought, the upregulation of *P5CS* gene can enhance drought tolerance by causing higher proline

concentration. Similar investigations also approve this result (Wang et al. 2018; Bandurska et al. 2017; Chakraborty and Pradhan 2012). In addition, higher *P5CS* expression and more tolerance under drought conditions have been reported in wheat (Dudziak et al. 2019) and barley (Bandurska et al. 2017). The elevated level of *P5CS* transcripts under osmotic stress can be attributed to H_2O_2 -derived signals and ABA-dependent pathways (Bandurska et al. 2017).

Lower *CAT*, *APX*, and *SOD* activities under severe drought compared to moderate drought implies that severe drought weakened the capability of genotypes in H_2O_2 scavenging, which may be a result of synthesis inhibition and/or degradation of *CAT*, *APX* and *SOD* enzymes. Similar to our results, Sofo et al. (2015) observed that the *CAT* and *APX* activity is associated with the degree of drought. The elevated level of *CAT*, *APX*, and *SOD* activity under water-limited conditions has been attributed to a higher drought tolerance in previous researches on rice (Wang et al. 2019), barley (Marok et al. 2013), ramie (Huang et al. 2013), and wheat (Chakraborty and Pradhan 2012). Down regulation of *APX* gene under water shortage has been also reported in studies on *Arachis hypogaea* L. (Furlan et al. 2014) and *P. pratensis* (Bian and Jiang 2009). Harb et al. (2015)

reported that the drought-induced pattern of *SOD* gene expression was specific for each genotype in barley, which is in agreement with our findings. Furthermore, indicated a significant increase in transcript levels of *SOD* gene in more drought-tolerant orchard grass genotypes. In this study, it was observed that in drought-tolerant genotype, the activities of studied antioxidant enzymes had a better relative consistency with their corresponding genes in comparison with intermediate and susceptible genotypes under both drought treatments. Luna (2004) reported a correlation between *CAT* gene expression and *CAT* enzyme activity in wheat cultivars under water deficit.

In summary, both drought stress conditions caused genotype-specific responses, which were dependent on drought severity. Considering all studied drought-induced responses together, superior upregulation of defense systems in tolerant TN4228 indicated that the studied genes and enzymes play meaningful roles in drought defense responses and could be viable targets to identify drought tolerance level.

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