

Article

Wild Relatives of Wheat Respond Well to Water Deficit Stress: A Comparative Study of Antioxidant Enzyme Activities and Their Encoding Gene Expression

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Abstract: Previous studies have revealed that some wild wheat accessions respond well to water deficit treatments and have a good potential in terms of photosynthetic parameters, root system architecture, and several physiological properties. However, the biochemical responses and molecular mechanisms of antioxidant-encoding genes remain to be elucidated. Herein, we investigated the most tolerant accessions from *A. crassa*, *Ae. tauschii*, and *Ae. cylindrica* previously identified from a core collection in previous studies, along with a control variety of bread wheat (*T. aestivum* cv. Sirvan) through measuring the shoot fresh and dry biomasses; the activities of antioxidant enzymes (including ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (GPX), and peroxidase (POD)); and the relative expression of *CAT*, superoxide dismutase (*MnSOD*), and *GPX* and *APX* genes under control and water deficit conditions. Water deficit stress caused a significant decrease in the shoot biomasses but resulted in an increase in the activity of all antioxidant enzymes and relative expression of antioxidant enzyme-encoding genes. Principal component analysis showed a strong association between the shoot dry biomass and the activity of *CAT*, *POD*, and *APX*, as well as *MnSOD* gene expression. Thus, these traits can be used as biomarkers to screen the tolerant plant material in the early growth stage. Taken together, our findings exposed the fact that *Ae. tauschii* and *Ae. crassa* respond better to water deficit stress than *Ae. cylindrica* and a control variety. Furthermore, these accessions can be subjected to further molecular investigation.

Keywords: *Aegilops* spp.; oxidative stress; gene expression; physio-chemical properties; enzyme activity

1. Introduction

Limitation in water availability for plants is the main challenge in agriculture, and furthermore their ability to cope with drought stress effects is of special economic importance. Hence, water scarcity is one of the most detrimental abiotic stress factors which negatively affects plant growth and production. Water-stress tolerance mechanisms involve various subtle physio-chemical changes [1].

Indeed, the changes due to water deficiency are generally dependent on both the severity and duration of stress [2]. Thus, the comprehensive detection of the physio-chemical mechanisms and molecular responses to this stress is indispensable for a complete view of the tolerance mechanisms for water-limited conditions in plants [3].

When plants undergo water deficit conditions, oxidative stress is the second stress by appearance through the increasing generation of reactive oxygen species (ROS) [4]. The ROS family has different members, including singlet oxygen ($^1\text{O}_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH). These ROS immediately accumulate in different cell organelles, such as peroxisomes, chloroplasts, and mitochondria [5]. The excessive accumulation of ROSs has negative effects on enzymatic activities and metabolic pathways, and finally leads to cell death [6]. Plants have several protective mechanisms which enable them to detoxify additional ROS. One of these mechanisms is the enzymatic group, which includes monodehydroascorbate reductase (MDHAR), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), the peroxidation detoxification product malondialdehyde (MDA), and glutathione reductase (GR). The non-enzymatic group encompasses carotenoids, tocopherols, ascorbate acid (AsA), and glutathione (GSH) [7]. CAT, APX, SOD, and POD play important roles in inducing tolerance to abiotic stress, and in many studies are considered as biomarkers for the fast screening of tolerant genetic materials even at the seedling stage [8–14]. Therefore, the regulation of the antioxidant enzyme-encoding gene expression could enhance plant tolerance to water-deficient conditions. The regulation of gene expression controls numerous physio-chemical responses to water-deficit stress [15]. In recent years, advancements in biotechnology and molecular biology have led to the expression and regulation of key genes, and are considered a powerful tool to enhance plant tolerance to cope with various stresses [10,16]. Hence, the overexpression of genes encoding antioxidant enzymes may promote tolerance and oxidative stress induced by water deficit stress.

The wild relatives of crop plants may provide a significant gene pool for maintaining sustainable agro-ecosystems and improving agricultural production. Their natural selection in diverse conditions can enrich valuable traits, especially tolerance to both abiotic and biotic stresses that can be introduced into crop plants by crossing [17]. Indeed, this claim is supported by numerous reports, which have clearly revealed that crop wild relatives (CRWs) have a relatively higher level of tolerance to environmental stresses compared to bred plants. Food security for the upcoming centuries will likely depend on important genetic resources as the result of human-induced climate change, which has led to greater instability in the ecosystem [18].

Among cereal crops, bread wheat (*Triticum aestivum* L.) is grown almost all over the world, and its yield is influenced by both biotic and abiotic stresses [19]. The global production of wheat is 763 million tonnes [20], and 20% of the daily calories and protein requirements of 4.5 billion people is based on this production [21]. It has been proven that wheat has a huge germplasm compared to other crop plants. In the Tririceae tribe, the *Aegilops* genus is a very important source of variability and has crucial role in the evolution of both durum and bread wheats. This genus has 22 species with different genomic constitutions and levels of ploidy, including diploid, tetraploid, and hexaploid [22]. Some *Aegilops* species have been shown to be exploitable due to their rich gene pool, providing donor genes that confer useful traits [23]. As such, it seems that the introgression of the allelic repertoire existing in the germplasm of the wild relatives could improve tolerance to water-deficit stress in wheat cultivars.

Although much physiological and biochemical research has been conducted on water deficit tolerance in cultivated wheat genotypes and their wild relatives, the expression patterns of key antioxidant genes have not been studied in detail. Previous studies have reported that several wild wheats, such as *Ae. crassa*, *Ae. cylindrica*, and *Ae. tauschii*, physiologically responded better to severe drought stress and revealed an acceptable tolerance compared to drought-tolerant cultivars and other species [23–25]. Here, we selected three tolerant wheat accessions from the aforementioned species, along with a drought-tolerant genotype (*T. aestivum* cv. Sirvan), with an objective comparison of their

antioxidant enzyme activities and antioxidant enzyme-encoding gene expression patterns under two control and water-deficit stress conditions.

2. Materials and Methods

2.1. Plant Material and Experimental Procedures

The genetic material consisted of one accession form *Ae. tauschii* (DD-genome; Genbank code: TN-01-2010), *Ae. cylindrica* (DDCC-genome; Genbank code: IUGB-1592), and *Ae. crassa* (DDMM-genome; genebank code: IUGB-1589), along with a drought-tolerant genotype (*T. aestivum* cv. Sirvan) as a control. A core collection of this material and other wild species has previously been subjected to screening for genetic diversity for acquired drought tolerance with respect to different physio-chemical properties and root system architectures [23,25,26]. The experimental material were accessed from the Ilam University Genbank (IUGB; Latitude 33° 39' N, Longitude 46° 22' E, and altitude of 1445 m above sea level). To break dormancy, seeds were placed at 4 °C for 72 h before sowing. Five seeds per accession were placed in pots (20 cm × 10 cm) that were filled with a mixture of sand, soil, and humus added in equal proportions. The pots (2 × 3 × 4 = 24 pots) were arranged in an experimental layout following a factorial randomized complete block assortment in three replications with a 16h photoperiod and an air temperature set to 25 ± 5 °C during the day and 15 ± 5 °C during the night. Seedlings were exposed to the following water treatments at the three-leaf growth stage: (1) well-watered (full field capacity (FC) = 95 ± 5%) as the control, and (2) water-stressed (FC = 30 ± 5%) conditions [25]. The determination of FC was performed according to an approach proposed by Souza et al. [27]. After the appearance of stress signs (10 days after applying stress treatment), leaf segments were collected and stored immediately at −80 °C. Moreover, harvested shoot samples were immediately weighted to determine the shoot fresh biomass (SFB) and afterwards were exposed to 70 °C for 72 h to measure the shoot dry biomass (SDB) [23].

2.2. Determination of Enzyme Activities

To determinate the enzyme activities, crude enzymes were extracted from leaf tissues (three-leaf stage) according to a method described by Pagariya et al. [28]. Briefly, 0.1 g of fresh leaf was homogenized in 1 mL of extraction buffer containing phosphate buffer (pH 7.4), 0.1 mM of EDTA, 1% polyvinyl pyrrolidone (PVP), and 0.1% x-Triton. The extracts were filtered and centrifuged at 15,000 g for 20 min at 4 °C. The supernatants were used to determine antioxidant enzymes activities. The activities of peroxidase (POD) [29], catalase (CAT) [30], guaiacol peroxidase (GPX) [31], and ascorbate peroxidase (APX) [32] were measured. All the activities were expressed as unit per mL (U mL^{−1}).

2.3. RNA Extraction and RT-qPCR Analyses

The RNX-Plus™ Kit (Sinaclon, Iran, Tehran) was used to extract total RNA from 100 mg of leaf tissues according to the manufacturer's instructions [10]. Samples were treated with RNase-free DNase-I (ThermoFisher Scientific, USA, Waltham) according to the manufacturer's instructions to remove DNA impurity. cDNA was synthesized from 1 µg of the treated RNA using the Hyper Script™ Reverse Transcriptase Kit (GeneAll, Korea, Seoul). Quantitative real-time PCR (RT-qPCR) was performed in a 15 µL volume containing 5.5 µL of RNase-free water, 1 (0.5l M) of diluted cDNA (50 ng µL^{−1}), 0.5 µL of forward and reverse primers (0.5l M), and 7.5 µL of 2x RealQ Plus Master Mix Green (Ampliqon). RT-qPCR reactions were conducted in a MiniOpticon™ Real-Time PCR System (Bio-Rad, USA). The RT-qPCR was carried out with an amplification profile of 95 °C for 3 min followed by 45 cycles of 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 30 s. After 45 cycles, a melting curve analysis was performed (65–95 °C) to verify the specificity of the amplicons. Specific primers for antioxidant genes were designed according to Baek and Skinner [33] and were synthesized by the Bioneer Company (Daejeon, South Korea). The housekeeping gene "18S rRNA" was used as a reference to normalize the

expression data for antioxidant genes [15]. All the primer sequences are listed in Table 1. Pfaffl's [34] $2^{-\Delta\Delta CT}$ equation was used to estimate the relative expression of each gene.

Table 1. Sequence of specific primers used in this study [15,33].

Gene		Sequence (5'-3')
APX	Forward	GCAGCTGCTGAAGGAGAAGT
	Reverse	CACTGGGGCCACTACTAAT
CAT	Forward	CCATGAGATCAAGGCCATCT
	Reverse	ATCTTACATGCTCGGCTTGG
MnSOD	Forward	CAGAGGGTGCTGCTTTACAA
	Reverse	GGTCACAAGAGGGTCCTGAT
GPX	Forward	CCCCCTGTACAAGTTCCTGA
	Reverse	GTCAACAACGTGACCCTCCT
18S rRNA	Forward	GGCCGCTCCTAGCCCTAATTG
	Reverse	TGAGCACTCTAATTTCTTCAAAGTACG

2.4. Data Analysis

An analysis of variance (ANOVA) was carried out with MSTATC software [35]. Duncan's Multiple Range Test (DMRT) at $p < 0.05$ was conducted to compare the mean data. A principal component analysis (PCA) was performed with XLSTAT package (Addinsoft, France, Paris).

3. Results

3.1. Effects of Water Deficit Treatments on Shoot Biomasses and Antioxidant Activities

Significant differences were observed between the water treatments and accessions in terms of the shoot fresh and dry biomasses, with significant differences among accessions based on the analysis of variance (ANOVA). The interaction between the water treatment and accession was not significant (Table 2). The water deficit stress decreased the means of the SFBs and SDBs by 38.65% and 58.06%, respectively, relative to the controls (Table 2). Under control conditions, the SFB ranged from 0.84 to 1.62 g plant⁻¹, while it ranged from 0.61 to 0.88 g plant⁻¹ under water deficit conditions. Under both growth conditions, the control variety (*T. aestivum* cv. Sirvan) and *Ae. tauschii* recorded the highest SFB, while *Ae. cylindrica* and *Ae. crassa* recorded the lowest fresh biomasses (Figure 1a). In contrast, SDB showed the highest variability among the investigated accessions, varying between 0.53 and 0.72 g plant⁻¹ in the control conditions. Under the water-deficit stress conditions, the range of this trait was between 0.08 and 0.44 g plant⁻¹. As shown in Figure 1b, under both growth conditions *Ae. tauschii* and *Ae. crassa* better maintained their dry biomasses compared to a tolerant variety (cv. Sirvan) and *Ae. cylindrica*.

Table 2. Analysis of variance, mean values, and relative change in biomasses, antioxidant enzyme activities, and antioxidant enzyme-encoding gene expressions in the four investigated wheat accessions.

Source of Variation	Biomass		Antioxidant Activity				Relative Expression			
	SFB	SDB	APX	POD	GPX	CAT	APXg	MnSODg	GPXg	CATg
R	0.076 ^{ns}	0.021 ^{ns}	0.001 ^{ns}	0.0001 ^{ns}	9.32 ^{ns}	0.0001 ^{ns}	8.294 ^{ns}	1.144 ^{ns}	0.994 ^{ns}	0.214 ^{ns}
WT	1.302 ^{***}	0.784 ^{***}	0.006 ^{***}	0.022 ^{***}	830.18 ^{***}	0.019 ^{***}	2671.142 ^{***}	2259.096 ^{***}	756.119 ^{***}	18.727 ^{***}
A	0.304 [*]	0.067 [*]	0.011 ^{***}	0.003 ^{***}	254.21 ^{***}	0.003 ^{***}	187.535 ^{***}	136.500 ^{***}	20.201 ^{***}	1.665 ^{***}
Int.	0.090 ^{ns}	0.022 ^{ns}	0.008 ^{***}	0.001 ^{***}	72.55 [*]	0.003 ^{***}	203.474 ^{***}	113.804 ^{***}	20.034 ^{***}	1.548 ^{***}
MCC	1.19	0.62	0.11	0.06	12.32	0.07	2.34	2.36	2.28	1.54
MSC	0.73	0.26	0.14	0.12	24.08	0.13	20.62	19.16	12.01	3.07
†RC	38.65	58.06	−27.27	−100	−95.45	−85.71	8.81-fold	8.12-fold	5.27-fold	1.99-fold

ns: non-significant; * and *** significant at $p < 0.05$ and $p < 0.001$, respectively. R, replicates; WT, water-deficit stress treatment; A, accession; Int., interaction effect. SFB, shoot fresh biomass (g plant^{-1}); SDB, shoot dry biomass (g plant^{-1}); APX, ascorbate peroxidase (U mL^{-1}); POD, peroxidase (U mL^{-1}); CAT, catalase (U mL^{-1}); GPX, guaiacol peroxidase (U mL^{-1}); APXg: APX gene; MnSODg: MnSOD gene; GPXg: GPX gene; CATg: CAT gene. MCC, mean values for control conditions; MSC, mean values for water deficit stress conditions; RC, relative change due to stress relatively to the control conditions. † Negative values indicate an increasing trend in the water deficit stress compared to the control conditions.

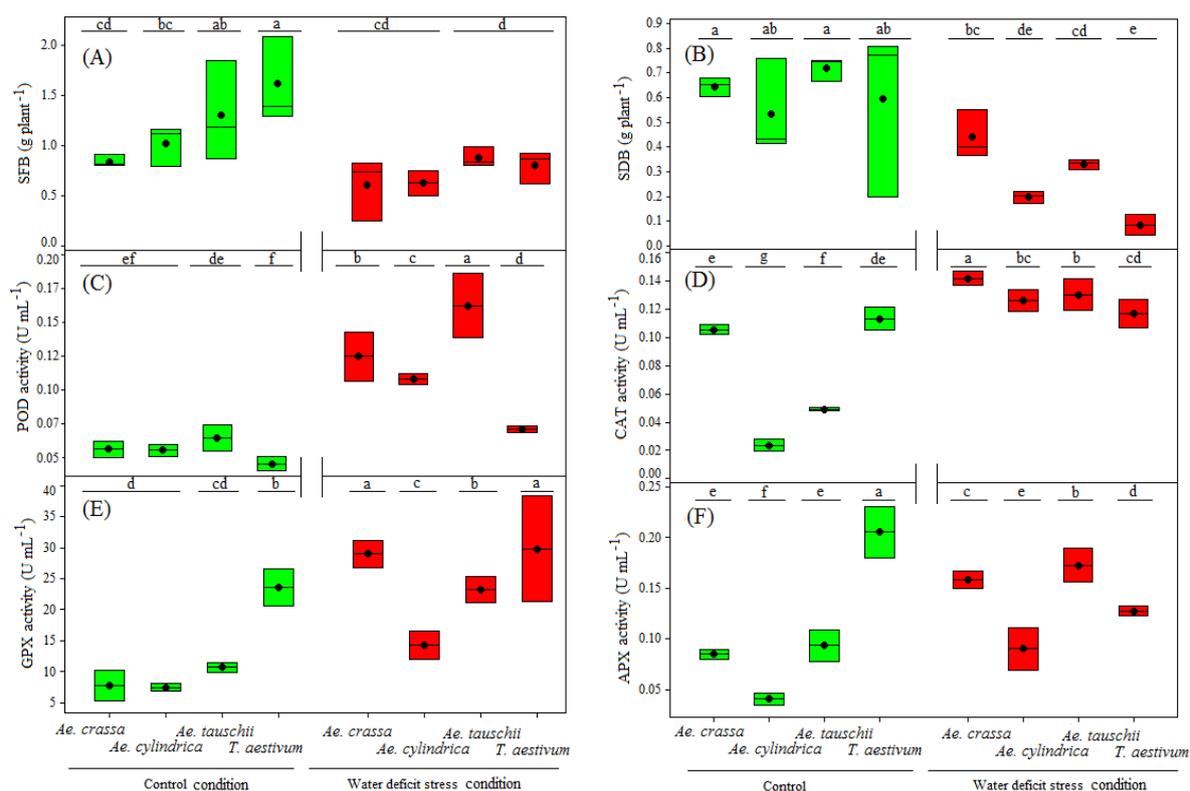


Figure 1. Box plots of investigated accessions for (A) SFB (shoot fresh biomass), (B) SDB (shoot dry biomass), (C) POD (peroxidase activity), (D) CAT (catalase activity), (E) GPX (guaiacol peroxidase activity), and (F) APX (ascorbate peroxidase activity) under the control and water deficit stress conditions. Different letters on each box indicate a significant difference at $p \leq 0.01$. In each dot box, the horizontal bar within the box and the internal boxes indicate the mean, median, and the first and third quartiles, respectively.

3.2. Effects of Water Deficit Treatments on Biochemical Traits

Significant differences were observed in the activity of the CAT, GPX, POD, and APX antioxidant enzymes between the water deficit stress conditions, accessions, and their interactions (Table 2). Water deficit stress increased the POD, GPX, CAT, and APX activities by 100%, 95.45%, 85.71%, and 27.27%, respectively, relative to the corresponding means under control conditions (Table 2). Under control conditions, the POD activity varied between 0.05 and 0.06 U mL^{-1} , whereas it ranged from 0.07 to 0.16 U mL^{-1} under water deficit conditions. The highest activity of POD was recorded in *Ae. tauschii* under control conditions, whereas the lowest activity occurred in *T. aestivum* (the tolerant control variety) (Figure 1c). The CAT activity ranged from 0.02 to 0.11 U mL^{-1} under control conditions, and from 0.12 to 0.14 U mL^{-1} under water-stress conditions. With respect to significant interaction effects, the *Ae. crassa* and *Ae. cylindrica* accessions showed the highest and lowest activity of this antioxidant under water deficit and control conditions, respectively (Figure 1d). The GPX activity showed a high level of variation in the control samples, ranging from 7.42 to 23.51 U mL^{-1} . Under water-deficit stress conditions, for this enzyme activity the range of variability was found to be between 14.29 and 29.82 U mL^{-1} . Under water-deficit conditions, the highest activity of GXP occurred in *Ae. crassa* and *T. aestivum*, while under control conditions *Ae. crassa* and *Ae. cylindrica* showed the lowest activity (Figure 1e). APX was strongly affected by interaction between the water deficit stress conditions and accessions. APX ranged from 0.04 to 0.21 U mL^{-1} in the control, and from 0.09 to 0.17 U mL^{-1} in the water deficit conditions. However, the highest and lowest activities were recorded for *T. aestivum* and *Ae. cylindrica* under the water deficit stress and control conditions, respectively (Figure 1f).

3.3. Effects of Water Deficit Treatments on Gene Expression Profiles

The ANOVA results indicated that the stress treatment affected the relative transcript abundance of the *CAT*, *APX*, *GPX*, and *MnSOD* genes, with significant differences among accessions. Furthermore, the two-way interaction effect between the water-deficit stress conditions and accessions for all genes was significant (Table 2). Water deficit stress significantly increased the mean expressions by 8.81 (*APX*), 8.12 (*MnSOD*), 5.27 (*GPX*), and 1.99 (*CAT*)-fold, relative to the corresponding values under the control conditions. Under the control conditions, all the assessed genes (except *CAT* gene) showed a similar pattern, whereas in the water deficit stress conditions the trend of expression was in the following order: *APX* (20.62) > *MnSOD* (19.16) > *GPX* (12.01) > *CAT* (3.07) (Table 2). Under the control conditions, the highest mRNA transcript of the *CAT* gene was recorded in the leaves of *T. aestivum* (Figure 2d).

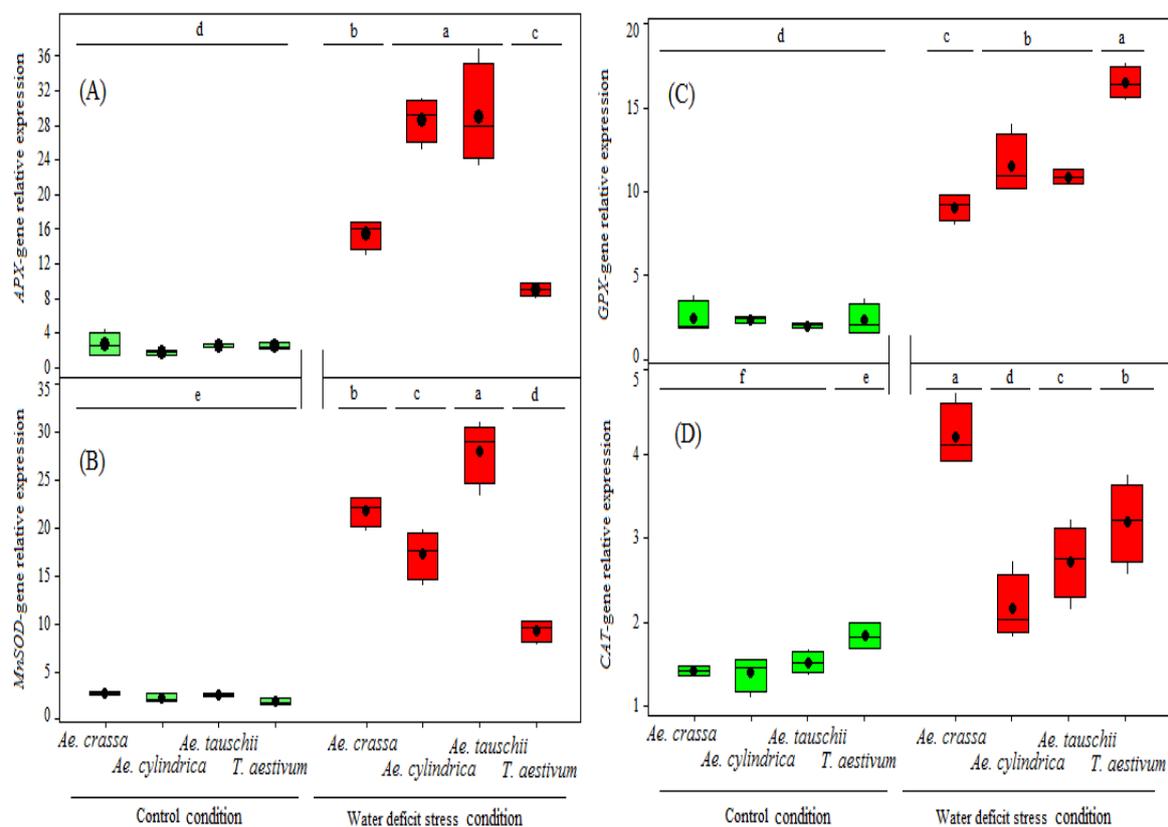


Figure 2. Box plots of the investigated accessions for the (A) *APX*, (B) *MnSOD*, (C) *GPX*, and (D) *CAT* gene relative expression in the control and water deficit stress conditions. Different letters on each box indicate significant difference at $p \leq 0.01$. Each dot box, horizontal bar within box, and internal boxes and whisker indicate the mean, median, the first and third quartiles, and minimal/maximal values, respectively.

Under the water deficit conditions, a significant increase in the relative expression of the *APX* gene was found in the leaves of *Ae. cylindrica* (17.01-fold) and *Ae. tauschii* (11.21-fold) relative to corresponding expressions in the control conditions (Figure 2a). Furthermore, the level of mRNA transcription of *MnSOD* gene was increased due to the water-deficit stress, and the highest relative expression of this gene was found in *Ae. tauschii* (10.74-fold) and *Ae. crassa* (8.09-fold) as compared to the control conditions (Figure 2b). *T. aestivum* (7.08-fold) and *Ae. tauschii* (5.42-fold) showed a higher expression of the *GPX* gene than the other accessions (Figure 2c). Moreover, *Ae. crassa* (2.97-fold) and *T. aestivum* (1.79-fold) showed the maximum numbers of *CAT* gene transcripts compared to the other accessions, when compared with the control conditions (Figure 2d).

3.4. Association between Shoot Biomasses with Biochemical Activities and Relative Gene Expressions

To depict the relationships among the shoot biomasses, biochemical activities, and gene expression patterns, a principal component analysis (PCA) was computed. The results showed three components with eigenvalues of 5.05, 3.06, and 1.89, accounting for all the total variation in the biochemical and relative expression patterns under water deficit stress conditions. The first component (PC1), accounting for 50.49% of the total variation, was mainly affected by the SDB, POD, and CAT enzyme activities, and the relative expression of the *MnSOD*, *APX*, and *GPX* genes. The second component (PC2) justified 30.56% of the total variation and was strongly influenced by the *GPX* and *APX* enzyme activities, as well as the expression of the *APX* and *CAT* genes. The third component (PC3) accounted for 18.95% of the total of variation and was significantly associated with the SFB and *APX* activity (factor loading values are not shown). Furthermore, a PCA-based biplot was rendered based on the first two PCs, with the aim of discovering association among characters (Figure 3a). In this way, a small angle shows a strong positive association, whereas a large angle explains a weak association. On the other hand, no and strong negative associations are displayed at 90° and 180°, respectively. Accordingly, positive strong associations were found between the following characters: POD activity and *MnSOD* gene expression; SDB and CAT activity; and SFB and *GPX* gene expression. Moreover, the associations among the *MnSOD* gene expression, activity of POD, CAT and *APX* enzymes, and SDB were positive. The activities of *APX* and *GPX* enzymes along with the *GPX* gene expression showed association with each other. The activity of *GPX* enzyme showed a positive association with the *GXP* gene expression and SFB. Since selection based on high values of extracted PCs leads to the identification of the best accessions, a considerable result for *Ae. tauschii* was identified as the most tolerant accession compared with the others (Figure b).

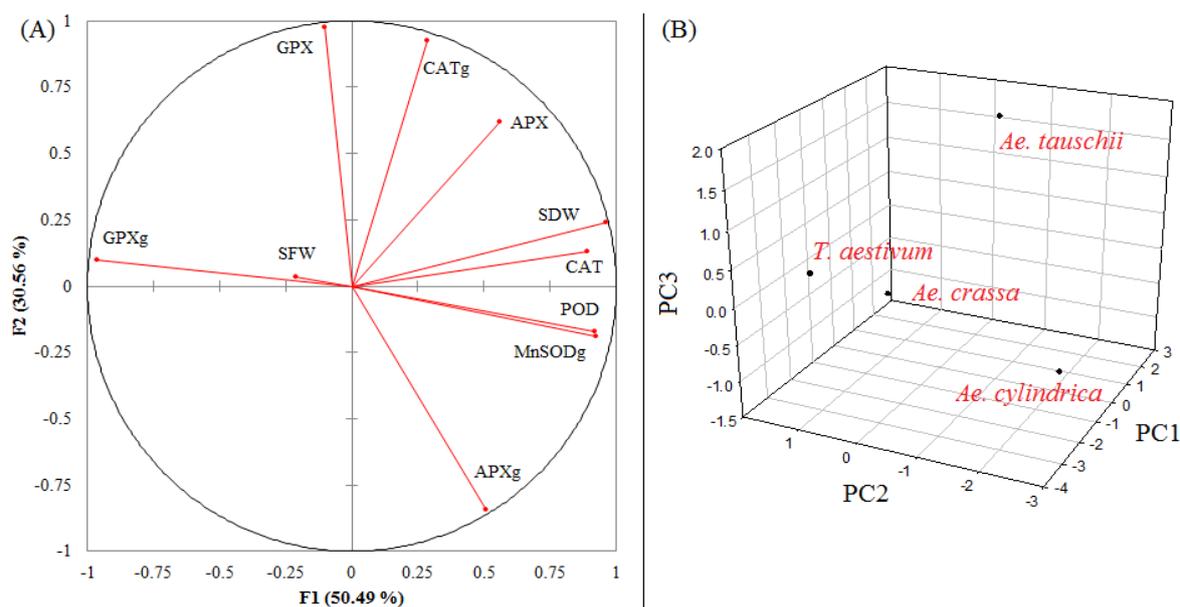


Figure 3. (A) Biplot rendered by the first two components to show associations among the measured characteristics. (B) A PCA-based 3D plot indicates the separation of the investigated accessions based on their factor loadings. SFB, shoot fresh biomass (g plant⁻¹); SDB, shoot dry biomass (g plant⁻¹); APX, ascorbate peroxidase (U mL⁻¹); POD, peroxidase (U mL⁻¹); CAT, catalase (U mL⁻¹); GPX, guaiacol peroxidase (U mL⁻¹); *APXg*, *APX* gene; *MnSODg*, *MnSOD* gene; *GPXg*, *GPX* gene; *CATg*, *CAT* gene.

4. Discussion

Extensive climate changes will continue to pose serious problems in the global food supply in coming decades. In these circumstances, the development of new varieties of food crops with a high performance that are also adapted to diverse environments is required for continued agricultural

sustainability [36]. Over many years, the replacement of landraces with a few modern varieties that have uniformity in their genetic base genetic variability has finally caused genetic vulnerability. In addition to this challenge, the narrowing of the genetic base of cultivated varieties is another major bottleneck for breeding programs and crop improvement tasks. Therefore, the use of CWRs can be offered as a hopeful approach to re-enrich the genetic base of cultivated crops. Indeed, these natural resources offer an interesting array of desirable traits which could be transferred into susceptible cultivated varieties through conventional breeding programs and transgenesis, and/or even other emerging technologies.

Among crop plants, wheat has a large gene pool and possesses many interesting wild relatives. There are numerous reports about the potential of wild wheat species in response to different abiotic stresses, pests, and diseases [10,13,23,24,26,37,38]. In the present study, we examined the physico-chemical potential of three ancestral species of bread wheat, along with a commercial cultivar (*T. aestivum* cv. Sirvan), in response to a severe level of water deficit stress (FC = 30%) at an early growth stage. Our results showed that there are significant differences between the water deficit treatments, and that the investigated accessions responded differently to stress (Table 2).

As a result, water stress diminishes the shoot fresh and dry biomasses by 38.65% and 58.06% compared to the control conditions. The reduction in fresh biomass in this study was in accordance with the results of other studies. For example, Ahmadi et al. [24] indicated that water deficit stress resulted in a reduction in the shoot fresh weight in different landraces and wild relatives of wheat seedlings when they were subjected to medium drought stress (50% field capacity) and severe drought stress (25% field capacity). Indeed, a significant reduction in fresh biomass under water deficiency suggests in this way that some species were very sensitive to water stress. In the present study, the percentage reduction in shoot biomass in all the investigated accessions may be the result of differential growth in the root zone, as reported by Ahmadi et al. [26]. On the other hand, no differences in fresh biomass in the studied accessions may be ascribed to their drought tolerance potential. Moreover, the reduction in the shoot dry biomass due to water deficit stress was in accordance with the results obtained by other researchers in wheat and its wild relatives [13,23–26]. Similarly, Senuja et al. [13] reported a reduction trend in the shoot dry mass (~27%) in a set of accessions of *Ae. tauschii*, *T. dicoccoides*, and *T. aestivum* subjected to different levels of water stress. Similarly, Pour-Aboughadareh et al. [25] showed that *Aegilops* ssp. seedlings grown under water deficiency conditions undergo reductions in their shoot dry masses.

Drought stress or water deficiency conditions adversely affect plant growth and development. To cope with these negative effects, plants have developed many defense lines by the activation of numerous stress-responsive genes and transcription factors (TFs) [39]. As soon as drought stress hits plants, the production of intensive reactive oxygen species (ROS) is an inevitable event, defined as an “oxidative burst”. Hence, removing excess ROS to sustain a fine balance between ROS scavenging and ROS production pathways, as well as to protect plant cells, is a key strategy for tolerant genotypes under adverse conditions [7]. Several antioxidant enzymes, such as APX, CAT, GPX, POD, and SOD, are known as ROS-scavenging enzymes and significantly reduce the levels of ROS in plants [7,40,41]. Our results showed that the antioxidant activities, including APX, POD, GPX, and CAT, increased significantly under water deficit stress conditions when compared with the control (Table 2). In a recent study conducted by Pour-Aboughadareh et al. [11], the Polyethylene glycol (PEG)-induced water deficit stress significantly increased the antioxidant activities in a set of Iranian durum wheat landraces and breeding genotypes. SOD is the first frontline antioxidant system against oxidative injury, catalyzing the dismutation of superoxide and generating hydrogen peroxide, which is converted to H₂O and O₂ by CAT [42]. Our results showed a strong correlation between the *MnSOD* gene transcripts and CAT and POD enzyme activities under water deficit stress conditions (Figure 3a). In this experiment, when the relative expression of *MnSOD* gene was compared, the highest numbers of transcript were observed in *Ae. tauschii* and *Ae. crassa* compared to other accessions (Figure 2b). The *MnSOD* gene has three isoforms, which are activated in the different organelles, such as chloroplast, mitochondria,

cytosol, and peroxisome [43]. Based on our results, the increased expression of the *MnSOD* gene in *Ae. crassa* and *Ae. tauschii* suggests a key defense mechanism targeted to organelles that provide a condition for its activation. Furthermore, our findings indicated that the CAT activity was increased by water deficit stress in all four wheat accessions, and the rate of increment was higher in *Ae. crassa*. This result was supported by relative expression data, where the maximum numbers of CAT gene transcripts were found in *Ae. crassa* (Figure 2d).

POD has been described as a biomarker in plant physiology research, and its activity rate is used as the criteria for estimating oxidative stress. This antioxidant oxidizes numerous substrates using H₂O₂ and prevents the extra accumulation of H₂O₂ produced by stress conditions [44]. Hence, POD can enhance plant tolerance due to catalyzing the oxido-reduction between H₂O₂ and various reductants [45]. Our results reveal a significant increase in POD activity in all the investigated accessions under water-deficit stress (Table 2). Several previous reports agree with our findings, having described increased POD activity under water-stress conditions in various plants, like poplar [46], oilseed rape [8], bread wheat [47], cassava [48] and durum wheat [11]. Similar to our results, stressed *Ae. crassa* and *Ae. tauschii* showed the highest POD activity when compared with those under control conditions (Figure 1c). GPX is another critical antioxidant enzyme which is located in the chloroplast and it has an important role in the defence of cells against stressor factors through eliminating H₂O₂ [49]. Based on our results, *T. aestivum* and *Ae. crassa* accessions showed the highest activities of GPX under water deficit conditions compared to the other accessions (Figure 1e). However, the RT-qPCR data indicated that stress conditions more dramatically increased the numbers of GPX transcripts in *Ae. cylindrica* and *T. aestivum* compared to other accessions (Figure 2C). This selective difference was further supported by the PCA-based biplot, where we observed a weak correlation between GPX activity and its relative expression (Figure 3a). It has been reported that there is a strong correlation between the scavenging of H₂O₂ and the activity of APX enzyme [44]. Two molecules of ascorbate play a significant role as an electron donor for scavenging H₂O₂ [50]. Our results indicate that the changing trend of APX activity and its gene expression under water deficit stress conditions was mainly dependent on tested accessions. As shown in Figure 1f, there is a significant interaction between water stress treatment and accession for APX activity. The highest activity of APX was observed in *T. aestivum* under the control conditions and in *Ae. tauschii* under water deficit stress. Nonetheless, *Ae. tauschii* and *Ae. cylindrica* showed an increase in their APX expression trend due to water stress compared to other accessions (Figure 2a).

Changes in the antioxidant enzyme activities and their encoding genes in response to water deficit stress as a means to retain the fine balance between the generation and detoxification of ROSs at the intracellular level, have been reported in several wheat plants grown under glasshouse and field conditions [12–14,24]. Although tolerant genotypes commonly have high antioxidant capacity, the genetic potential is a main factor to alleviate oxidative damage and balance the structural integrity of cell components [51]. For instance, several experiments have indicated that drought and salt-tolerant wild relatives of wheats with alien genome exhibited higher antioxidant capacity than cultivated genotypes [10,13,24,52]. Likewise, our results revealed that, among the tested accessions, *Ae. crassa* responded well to severe water deficit stress compared to other *Aegilops* accessions and the tolerant bread wheat genotype (cv. Sirvan). This result was confirmed by a 3D plot, which was rendered based on the first three components (Figure 3b). As can be seen, the accession *Ae. tauschii* followed by *Ae. crassa* with high values for extracted PCs separated far from the origin of biplot and other accessions. Hence, these accessions can be desirable candidates for further complimentary evaluation with the aim of exploiting it in crossing programs.

5. Conclusions

The present study revealed that shoot biomasses, antioxidant enzyme activities, and antioxidant-encoding genes can be used as selective traits for discriminating between tolerant and susceptible wheat accessions at the early seedling stage. As a significant result, the current study indicated that *Ae. tauschii* and *Ae. crassa*

responded well to water deficit stress and could be candidates for further complementary experiments. Hence, the identified accessions represent a valuable genetic resource for wheat which can be used for chromosomal localization and the isolation of new drought tolerance-associated genes that might aid in developing new wheat varieties that physiologically responded well to adverse conditions and produce high yields in drought-prone zones. We anticipate that our results can contribute toward the better exploitation of wild relatives in wheat breeding programs.

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