

## Article

# Analysis of Physio-Biochemical Responses and Expressional Profiling Antioxidant-Related Genes in Some Neglected *Aegilops* Species under Salinity Stress

Bitá Jamshidi <sup>1,\*</sup>, Alireza Pour-Aboughadareh <sup>2,\*</sup>, Jan Bocianowski <sup>3</sup>, Lia Shooshtari <sup>4</sup>, Henryk Bujak <sup>5,6</sup>, Aras Türkoğlu <sup>7</sup> and Kamila Nowosad <sup>5,\*</sup>

- <sup>1</sup> Department of Food Security and Public Health, Khabat Technical Institute, Erbil Polytechnic University, Erbil 44001, Iraq
- <sup>2</sup> Seed and Plant Improvement Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj 31588-54119, Iran
- <sup>3</sup> Department of Mathematical and Statistical Methods, Poznań University of Life Sciences, Wojska Polskiego 28, 60-637 Poznań, Poland; jan.bocianowski@up.poznan.pl
- <sup>4</sup> Department of Plant Breeding and Biotechnology, Kermanshah Branch, Islamic Azad University, Kermanshah 67187-73654, Iran; lia.shooshtari@iau.ac.ir
- <sup>5</sup> Department of Genetics, Plant Breeding and Seed Production, Wrocław University of Environmental and Life Sciences, Plac Grunwaldzki 24A, 53-535 Wrocław, Poland; henryk.bujak@upwr.edu.pl or h.bujak@coboru.gov.pl
- <sup>6</sup> Research Centre for Cultivar Testing, Słupia Wielka 34, 63-022 Słupia Wielka, Poland
- <sup>7</sup> Department of Field Crops, Faculty of Agriculture, Necmettin Erbakan University, Konya 42310, Türkiye; aras.turkoglu@erbakan.edu.tr
- \* Correspondence: bita.alimer@epu.edu.iq (B.J.); a.poraboghadareh@edu.ikiu.ac.ir (A.P.-A.); kamila.nowosad@upwr.edu.pl (K.N.)



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**Abstract:** Wild common wheat species represent a significant pool of resistance genes to various environmental stresses. In this study, we examined several physiological traits and the activity of three antioxidant enzymes—namely, catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX)—as well as the expression patterns of their encoding genes in three neglected *Aegilops* species with alien genomes (including *Ae. triuncialis* (UUCG-genome), *Ae. neglecta* (UUMM-genome) and *Ae. umbellulata* (UU-genome)) under two control (0 mM NaCl) and salinity (250 mM NaCl) conditions. The results of the analysis of variance (ANOVA) showed highly significant effects of salinity stress, accessions, and their interaction on most physio-biochemical traits, root and shoot dry biomasses, and antioxidant-related gene expression level. As a result of comparison between *Aegilops* species and a bread wheat cultivar (cv. Narin as a salt-tolerant reference variety), *Ae. triuncialis* responded well to salinity stress, maintaining both ionic homeostasis capability and biochemical ability. Moreover, transcriptional data revealed the prominence of *Ae. triuncialis* over other *Aegilops* species and salt-tolerant bread wheat [cv. Narin] in terms of the level of expression of antioxidant genes (APX, SOD, and CAT). This result was further supported by a biplot rendered based on principal component analysis (PCA), where this wild relative showed a positive association with most measured traits under salinity stress. Moreover, we speculate that this accession can be subjected to physiological and molecular studies, and that it can provide new insights into the use of the alien genomes in future wheat breeding programs.

**Keywords:** ancestor species; wheat; salt tolerance; biochemical markers; gene expression

## 1. Introduction

Among edaphic stresses, soil salinity is considered one of the global threats that drastically limits agriculture production. Near 20% of arable land and 33% of irrigated land are affected by salinity, leading to a severe reduction in crop yield and quality [1]. As a

consequence of the green revolution, the use of fertilizer-responsive high-yielding cultivars of main staple crops, such as wheat, increased worldwide [2]. It has been reported that around 1128 Mha of area worldwide is affected by salinity and soil sodicity. The areas most affected by salt are in the Middle East (189 Mha), followed by Australia (169 Mha), North Africa (144 Mha), and South Asia (52 Mha) [3]. According to the literature, the area of saline-affected soil on the planet is increasing over time, and the current area affected by salinity stress will be nearly tripled by 2050 [3,4].

Salinity tolerance is a complex treatment, and it is controlled by several processes and pathways [5]. As has been proved, two important regulatory pathways are involved in inducing the salt tolerance mechanism: (i) the osmotic pathway, which consists of antioxidant metabolism, osmoprotectant biosynthesis, ion homeostasis adjustment, hormone signaling, and photosynthesis regulation, and (ii) water uptake [6]. Since sodium chloride (NaCl) is one of the main salts of the various soil layers, most tolerance mechanisms focus on the transportation and partitioning of sodium ions ( $\text{Na}^+$ ). Increasing the concentration of  $\text{Na}^+$  around the roots induces both ionic and osmotic toxicity [7]. Therefore, maintaining a low  $\text{Na}^+$  content is one of the important mechanisms for plant survival under salinity stress [8]. Munns et al. [9] classified the plant response to salinity stress into two main phases. The first phase is called the osmotic or ion-independent one, which begins as soon as the salt concentration around plant roots increases, which, in turn, leads to restrictions in the transfer of water from the soil to the roots, a reduction in the flow of ions from the roots to the shoots, closure of the stomatal apparatuses, and, ultimately, a reduction in the rate of shoot growth [10]. Ion toxicity or ion dependency is the second phase associated with the accumulation of cytotoxic ions and occurs over several days, and it causes an imbalance in metabolic processes, thereby increasing leaf senescence and ultimately plant death [11].

One of the important changes that occurs at the cellular level during salinity stress is the increase in the level of reactive oxygen species (ROS). A partial decrease in atmospheric oxygen results in the creation of ROSs, and the accumulation of this type of oxygen finally leads to oxidative stress [12]. It has been shown that ROSs can be divided into four types: single oxygen ( $^1\text{O}_2$ ), superoxide radical ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydrogen radical ( $\text{HO}^\bullet$ ) [13]. Excessive accumulation of any type of these ROS can cause various damage at molecular and cellular levels. When plants are subjected to stressful conditions, several defense mechanisms enable them to overcome excessive ROS accumulation. One of the most important defense mechanisms is the scavenging system, which is divided into two main types: enzymatic and non-enzymatic antioxidant systems. The enzymatic system consists of the activity of several enzymes, such as catalase (CAT), ascorbate peroxidase (APX), superoxidase dismutase (SOD), peroxidase (POD), glutathione reductase (GR), and malondialdehyde (MDA). Tocopherols, carotenoids, ascorbate acid (AsA), and glutathions (GSH) are grouped in the non-enzymatic system. Both antioxidant systems have critical roles in improving the tolerance of plants coping with various environmental stresses, and they can also be used as useful biomarkers for the selection of tolerant plant materials [14–19]. Hence, the regulation of antioxidant-related genes could increase plant tolerance to salinity stress [20].

In wheat, numerous studies have demonstrated alternations in the activity of the antioxidant system to decrease the effects of oxidative stress that are induced by abiotic stresses. For instance, Barakat [21] reported an increasing trend for APX and CAT activities in wheat plants treated with high levels of salinity stress. Suneja et al. [18] reported that among antioxidant enzymes, SOD is one of the most important biochemical indicators that can serve as an indirect selection criteria for screening and selecting salt-tolerant wheat genotypes at the seedling stage. Moreover, Sharma et al. [22] highlighted the role of APX activity in increasing the tolerance of wheat and other cereals to various environmental stresses. In another study, SOD, CAT, and APX enzymes in some wild relatives of bread wheat revealed greater activities in response to drought stress conditions [23]. It has been reported that an increase in the SOD transcript in response to heat treatment resulted in the enhancement of tolerance to the high levels of heat stress in wheat [24]. Moreover, as

mentioned by Matsumura et al. [25], the wheat's *CAT* gene expressed in transgenic rice increases tolerance against cold stress. Zhai et al. [26] showed that *GPX* genes play key roles in increasing salt tolerance and ABA-signaling cascades in wheat plants when they were faced with high levels of salt concentrations.

Numerous studies have revealed that the wild relatives of wheat can be used as a significant gene pool for the improvement of the tolerance of common wheats against various environmental stresses [27–35]. Indeed, these germplasm resources, due to their adaptation to various environmental conditions over a long period of time, are interesting genes for the improvement of wheat [36]. Wheat germ consists of two main genera: *Aegilops* and *Triticum*. Of these, the genus *Aegilops* includes several species with diverse genomic structures, and can be donor genes that confer valuable agronomic traits [36]. Despite many physiological and biochemical studies on salinity stress in wild wheat species (see [36]), antioxidants and the expression patterns of their coding genes have not been thoroughly investigated in neglected species, such as *Ae. neglecta*, *Ae. triuncialis*, and *Ae. umbellulata*. Therefore, this study aimed to compare the three mentioned *Aegilops* species along with a cultivated bread wheat cultivar (cv. Narin as salt tolerant) in terms of several physiological and biochemical markers under two conditions of control and salinity stress.

## 2. Materials and Methods

### 2.1. Genetic Materials and Experimental Setup

In this investigation, three neglected *Aegilops* species, including *Ae. triuncialis* (UUC-genome; accession code: IUGB-00318), *Ae. neglecta* (UUMM-genome; accession code: IUGB-00387), and *Ae. umbellulata* (UU-genome; accession code: IUGB-00966), along with a salt-tolerant bread wheat cultivar (cv. Narin as a salt-tolerant reference), were evaluated under optimal glasshouse conditions. Five seeds of each genetic material were planted in plastic pots with a 20 cm diameter and a 50 cm height that were filled with sand. All pots were kept under optimal photoperiod and temperature conditions. All greened seeds were irrigated with Hoagland medium at half strength [37]. After the seedlings germinated and the second leaf appeared, full-power medium was applied three times a week. Two weeks after germination, all pots were arranged in a factorial experiment based on a randomized complete block design with three replicates. Stress included control consisted (0 mM NaCl) and severe salinity stress (250 mM NaCl). The non-stressed and stressed seedlings were irrigated with medium without NaCl and with NaCl, respectively. Salinity was applied gradually, and it eventually reached 250 mM after five steps. After two weeks of cultivation under salinity stress, the seedlings were sampled, and their leaves were harvested and stored at  $-80^{\circ}\text{C}$ .

### 2.2. Determination of Root and Shoot Dry Weight

First, root and shoot tissues of healthy seedlings were cut in order to determine the dry biomasses of roots and shoots. The collected root and shoot tissues were dried for 72 h at  $70^{\circ}\text{C}$ , and then their dry weight was recorded as root and shoot dry biomasses, respectively.

### 2.3. Determine of Root and Shoot $\text{Na}^+$ and $\text{K}^+$ Contents

To determine the  $\text{Na}^+$  and  $\text{K}^+$  content of roots and shoots, 10 mg of dried samples were digested with 10 mL 0.5 N nitric acid. All samples were then incubated in a water bath for 2 h at  $85^{\circ}\text{C}$ . The digested solutions were then filtered, and their supernatant phases were subjected to determination of  $\text{Na}^+$  and  $\text{K}^+$  content ( $\text{mmol g}^{-1}$  dry weight) using a flame photometry device (Sherwood Scientific Flame Photometer 420, Cambridge, UK) [38].

### 2.4. Determination of Antioxidant Enzyme Activities

To determine the activity of some antioxidant enzymes (superoxide dismutase—SOD; ascorbate peroxidase—APX; and catalase—CAT), crude enzymes were extracted from young leaves [39]. A total of 50 mg of fresh leaves were digested in 1 mL extraction buffer containing 3 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1.5% *w/v* polyvinylpyrrolidone (PVP), and

0.05 M Tris-HCl buffer (pH 7.5). For the APX assay, 0.2 mM ascorbate was added to the extraction buffer. The extracted solution was filtered and centrifuged for 20 min at 12,000 RPM, and the separated supernatant was used to determine the activity of the mentioned enzymes. In the next step, the activity of APX, CAT, and SOD were determined based on the manuals developed by Nakano and Asada [40], Chance and Maehly [41], and Dhindsa et al. [42], respectively.

### 2.5. Estimation of Gene Expression Patterns

Based on previous studies, we identified some antioxidant-related genes in wheat. To estimate the relative expression of APX (Forward: 5'-GCAGCTGCTGAAGGAGAAGT-3'/Reverse: 5'-CACTGGGGCCACTCACTAAT-3' [43]), CAT (Forward: 5'-CCATGAGATCA-AGGCCATCT-3'/Reverse: 5'-ATCTTACATGCTCGGCTTGG-3' [43]), and SOD (Forward: 5'-CAGAGGGTGCTGCTTTACAA-3'/Reverse: 5'-GGTCACAAGAGGGTCCTGAT-3' [43]) genes and compare their patterns under two control and salinity stress conditions, total RNAs were extracted from 100 mg of leaf using a RNX-Plus™ kit (DENAZIST ASIA, Tehran, Iran) according to the manufacturer's guidelines. A Nano-Drop Spectrophotometers (Thermo Scientific-2000C, Waltham, MA, USA) was used to test the concentration of extracted RNA. In the next step, the cDNAs were synthesized using Easy™ cDNA Synthesis Kit (Parstos, Tehran, Iran). Real-time PCR was performed in a 20 µL volume containing 10 µL of 2× RealQ Plus 2× Master Mix Green (Ampliqon, Odense, Denmark), 4 µL of RNase-free water, 4 µL of cDNA (50 ng µL<sup>-1</sup>), and 1 µL of forward and reverse primers. All amplifications were run in a MiniOpticon™ Real-Time PCR device (Bio-Rad, Hercules, CA, USA). In addition, the *α-tubulin* gene (forward: 5'-AGTGTCCTGTCCACCCACTC-3'/reverse: 5'-ATTCAGAGCACCGTCAAACC-3' [44]) was used to normalize the expression of the tested genes under both growth conditions. The obtained CTs were used to calculate the relative expression of each gene, as described by Pfaffl [45].

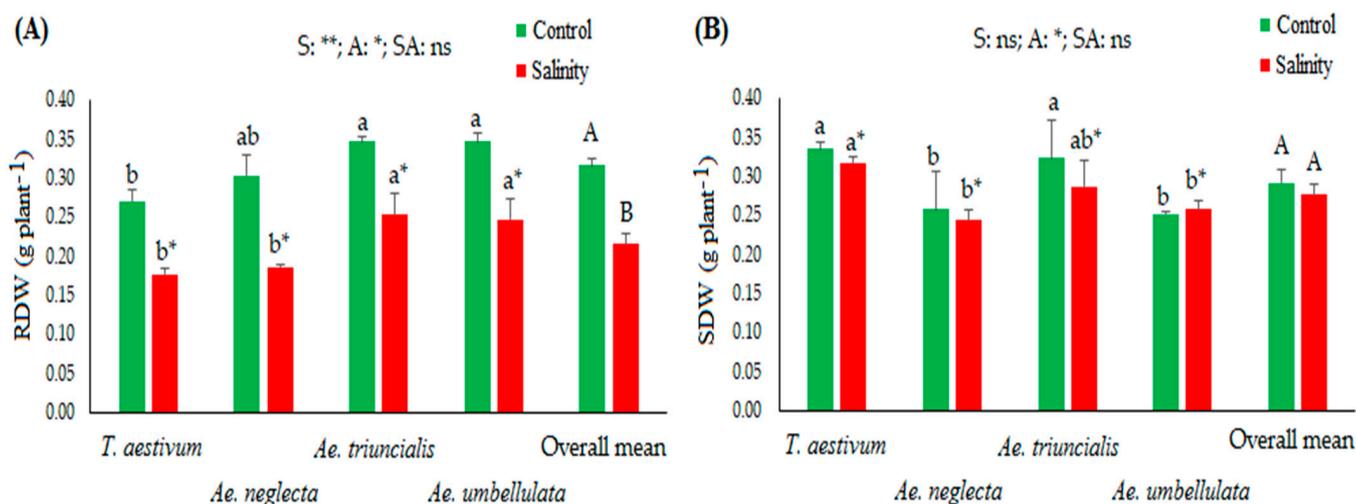
### 2.6. Data Analysis

An analysis of variance (ANOVA) was performed to test the effects of salinity stress, accessions, and their interaction based on the measured traits and the gene expression data using SAS software [46]. The means comparisons were conducted using Duncan's test at the level of probability of significance ( $p < 0.05$  and  $p < 0.01$ ) for each effect. Interrelationships among the experimental data were determined through a biplot rendered from a principal component analysis (PCA).

## 3. Results

### 3.1. Root and Shoot Biomass and Their Ion Concentrations

ANOVA showed that the main effects of salinity treatment (S) and accessions (A) were significant for root dry weight (Figure 1A). The main effect of stress and accession interaction (SA) was not statistically significant (Figure 1A). Overall, for all species, RDW was higher for the controls than for salinity. The highest RDW values were observed for *Ae. triuncialis*, and *Ae. umbellulata*, both for the control (0.347 g plant<sup>-1</sup>) and salinity (0.253 and 0.246 g plant<sup>-1</sup>, respectively) conditions. The lowest RDW values were observed for *T. aestivum*, and they were 0.270 and 0.176 g plant<sup>-1</sup> for control and salinity, respectively. ANOVA indicated that the main effects of stress treatment, stress, and accession interaction were not significant for shoot dry weight (Figure 1B). However, the main effect of accession was statistically significant (Figure 1B). Overall, for all species (except *Ae. umbellulata*), SDW was higher for the controls than for salinity. The highest SDW values were observed for *T. aestivum*, both for control (0.377 g plant<sup>-1</sup>) and salinity (0.356 g plant<sup>-1</sup>). The lowest SDW value for control was observed for *Ae. umbellulata* (0.283 g plant<sup>-1</sup>), while for salinity the lowest value was for *Ae. neglecta* (0.273 g plant<sup>-1</sup>).

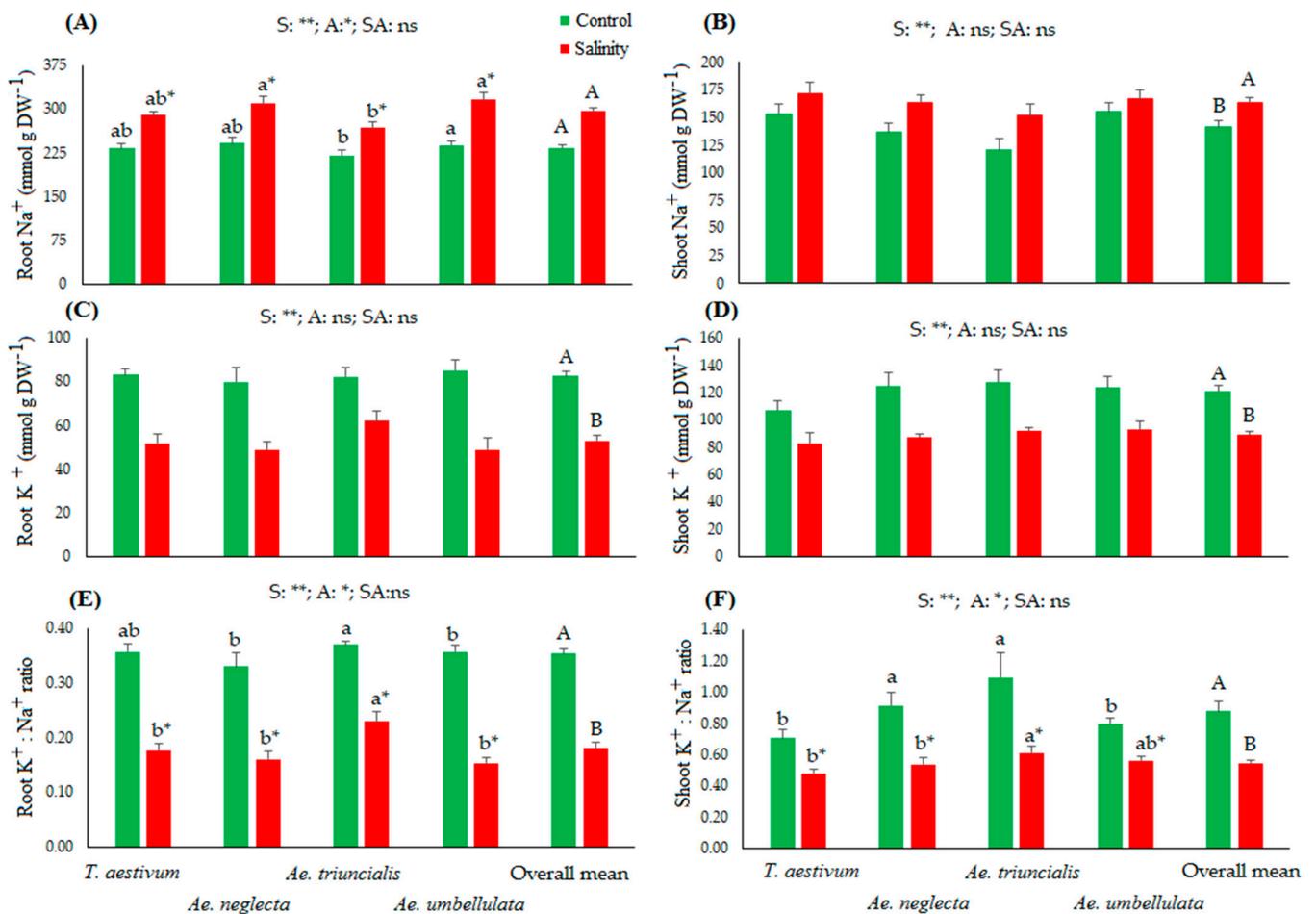


**Figure 1.** Means comparison between the control (0 mM NaCl) and salinity (250 mM NaCl) conditions as well as among investigated accessions in terms of (A) root dry weight (RDW) and (B) shoot dry weight (SDW). S, A, and SA indicate accessions main, salinity stress treatments, and interaction between accessions and salinity stress treatment effects, respectively. ns, \* and \*\* indicate not significant, significant differences at  $p < 0.05$  and  $p < 0.01$ , respectively. The different letters show significant differences among species under the control conditions. The different letters with a star (\*) show significant differences among species under salinity conditions. The different capital letters show significant differences among the control and salinity stress conditions.

ANOVA indicated that the main effect of salinity stress (S) was significant for the root  $\text{Na}^+$ , root  $\text{K}^+$ , and root  $\text{K}^+:\text{Na}^+$  ratio, as well as the shoot  $\text{Na}^+$ , shoot  $\text{K}^+$ , and shoot  $\text{K}^+:\text{Na}^+$  ratio (Figure 2). The main effect of accession (A) was statistically significant only for the root  $\text{Na}^+$  (Figure 2A), root, and shoot  $\text{K}^+:\text{Na}^+$  ratio (Figure 2E,F). No significant SA interaction was observed for any of the ion concentrations (Figure 2A–E). Root  $\text{Na}^+$  were always higher in salinity than in the control (Figure 2A). Under the control conditions, root  $\text{Na}^+$  ranged from 221  $\text{mmol g DW}^{-1}$  (for *Ae. triuncialis*) to 241  $\text{mmol g DW}^{-1}$  (for *Ae. umbellulata*), but for salinity, ranged from 268.33  $\text{mmol g DW}^{-1}$  (for *Ae. triuncialis*) to 316.33  $\text{mmol g DW}^{-1}$  (for *Ae. umbellulata*). In terms of shoot  $\text{Na}^+$ , significant differences were observed only for overall averages (Figure 2B). A significantly higher mean value was observed for salinity (296.42  $\text{mmol g DW}^{-1}$ ) than for the control (233.42  $\text{mmol g DW}^{-1}$ ). In terms of root  $\text{K}^+$  (Figure 2C) and shoot  $\text{K}^+$  (Figure 2D), significant differences were observed only for overall averages. In both cases, higher values were observed with control conditions than for salinity. Larger values of root  $\text{K}^+:\text{Na}^+$  ratio and shoot  $\text{K}^+:\text{Na}^+$  ratio were observed for the control than for salinity (Figure 2E,F). Root  $\text{K}^+:\text{Na}^+$  ratio for the control ranged from 0.33 (for *Ae. neglecta*) to 0.37 (for *Ae. triuncialis*). However, for salinity, the root  $\text{K}^+:\text{Na}^+$  ratio ranged from 0.15 (for *Ae. umbellulata*) to 0.23 (for *Ae. triuncialis*). Shoot  $\text{K}^+:\text{Na}^+$  ratio under control conditions ranged from 0.71 (for *T. aestivum*) to 1.09 (for *Ae. triuncialis*). However, for salinity, shoot  $\text{K}^+:\text{Na}^+$  ratio ranged from 0.48 (for *T. aestivum*) to 0.61 (for *Ae. triuncialis*).

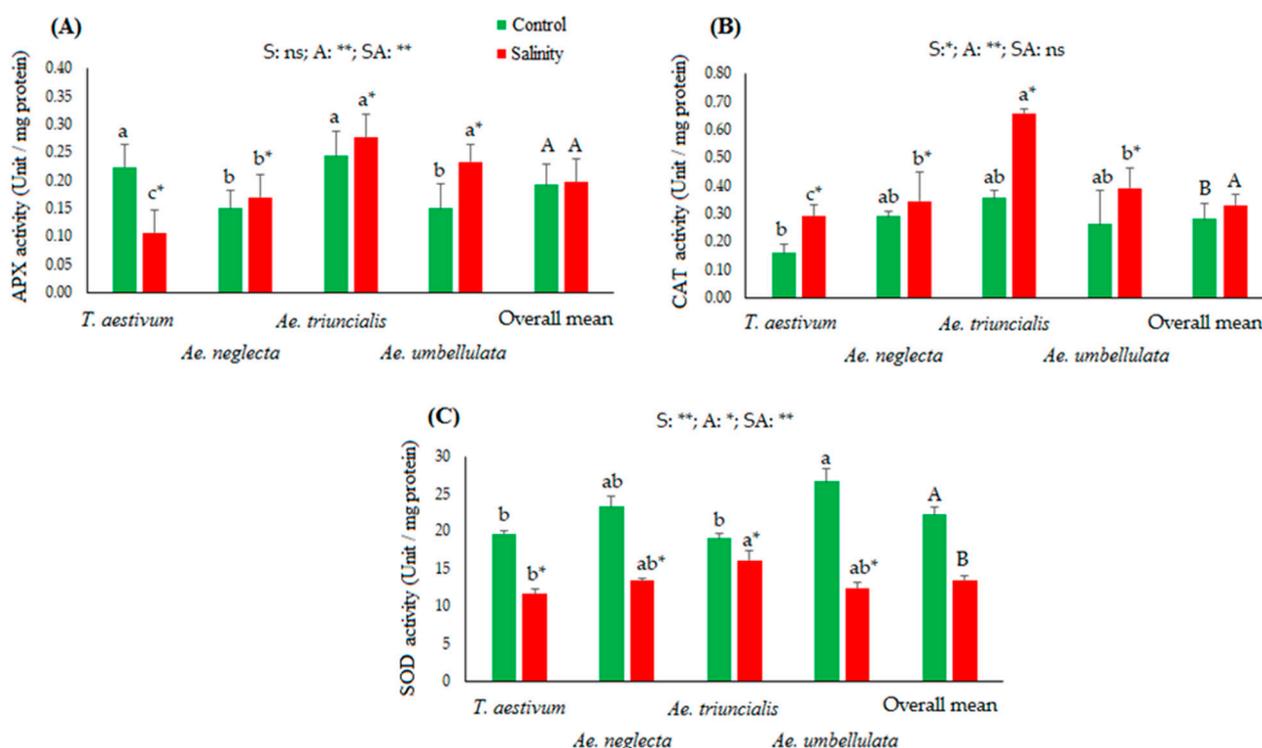
### 3.2. Biochemical Activities in Studied Species

ANOVA showed that the main effect of accession (A) was significant for APX activity, CAT activity, and SOD activity (Figure 3). The main effect of stress treatment (S) was significant for CAT activity (Figure 3B) and for SOD activity (Figure 3C). The SA interaction was significant for APX activity (Figure 3A) and for SOD activity (Figure 3C).



**Figure 2.** Means comparison between the control (0 mM NaCl) and salinity (250 mM NaCl) conditions as well as among investigated accessions in terms of (A) root Na<sup>+</sup> content, (B) shoot Na<sup>+</sup> content, (C) Root K<sup>+</sup> content, (D) shoot K<sup>+</sup> content, (E) root K<sup>+</sup>:Na<sup>+</sup> ratio, and (F) shoot K<sup>+</sup>:Na<sup>+</sup> ratio traits. S, A, and SA indicate accessions main, salinity stress treatments, and interaction between accessions and salinity stress treatment effects, respectively. ns, \* and \*\* indicate not significant, significant differences at  $p < 0.05$  and  $p < 0.01$ , respectively. The different letters show significant differences among species under control conditions. The different letters with a star (\*) show significant differences among species under salinity conditions. The different capital letters show significant differences among control and salinity stress conditions.

APX activity for the control ranged from 0.15 Unit mg protein<sup>-1</sup> (for *Ae. umbellulata* and *Ae. neglecta*) to 0.24 unit/mg protein<sup>-1</sup> (for *Ae. triuncialis*). However, for salinity, APX activity ranged from 0.11 unit/mg protein<sup>-1</sup> (for *T. aestivum*) to 0.28 unit/mg protein<sup>-1</sup> (for *Ae. triuncialis*) (Figure 3A). CAT activity under control conditions ranged from 0.16 Unit mg protein<sup>-1</sup> (for *T. aestivum*) to 0.36 unit/mg protein<sup>-1</sup> (for *Ae. triuncialis*). However, for salinity CAT activity ranged from 0.29 unit/mg protein<sup>-1</sup> (for *T. aestivum*) to 0.66 unit/mg protein<sup>-1</sup> (for *Ae. triuncialis*) (Figure 3B). SOD activity under control conditions ranged from 18.94 unit/mg protein<sup>-1</sup> (for *Ae. triuncialis*) to 26.65 unit/mg protein<sup>-1</sup> (for *Ae. umbellulata*). However, for salinity SOD activity ranged from 11.61 (for *T. aestivum*) to 16.1 (for *Ae. triuncialis*) (Figure 3C).



**Figure 3.** Means comparison between control (0 mM NaCl) and salinity (250 mM NaCl) conditions as well as among investigated accessions in terms of APX (A), CAT (B), and SOD (C) activity. S, A, and SA indicate accessions main, salinity stress treatments, and interaction between accessions and salinity stress treatment effects, respectively. ns, \* and \*\* indicate not significant, significant differences at  $p < 0.05$  and  $p < 0.01$ , respectively. The different letters show significant differences among species under control conditions. The different letters with a star (\*) show significant differences among species under salinity conditions. The different capital letters with show significant differences among control and salinity stress conditions.

### 3.3. Gene Expression Evaluation in Studied Accessions

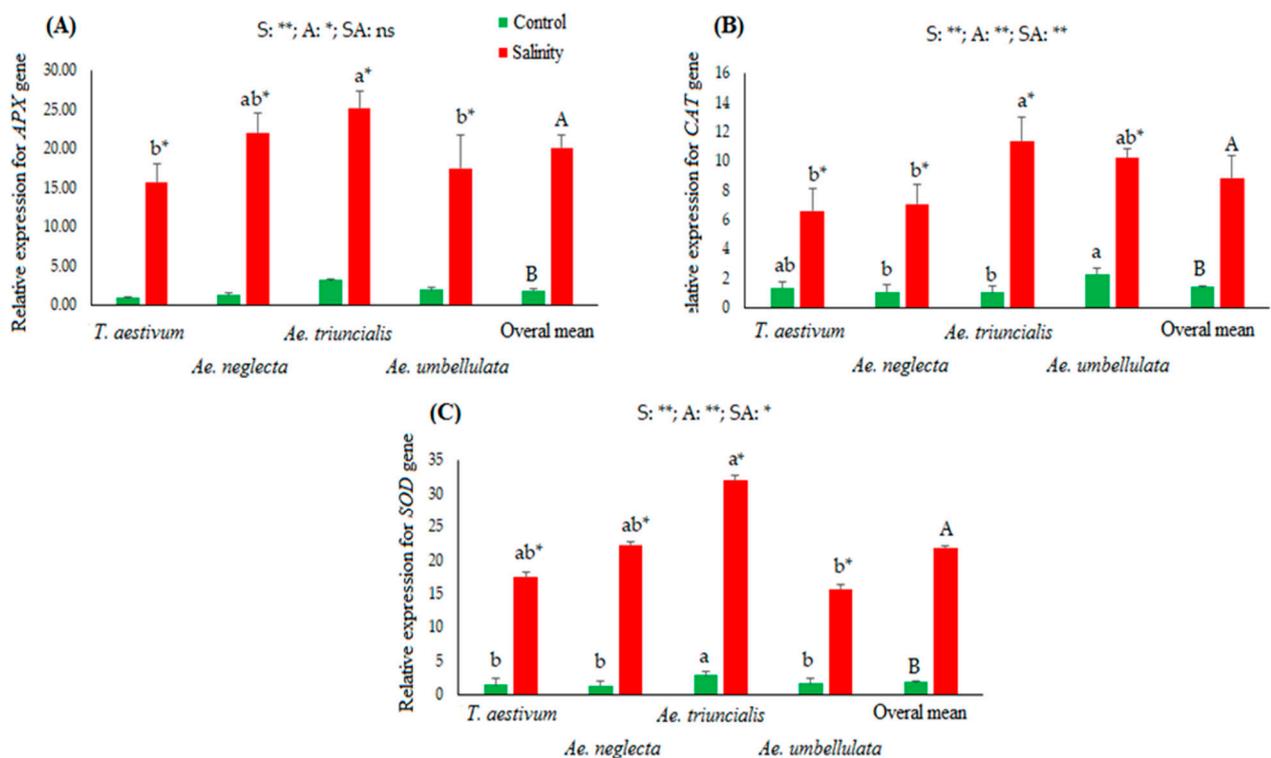
The result of ANOVA for the relative expression of studied genes indicated that the main effects of stress treatment (S) and accession (A) were significant for relative expression for the APX gene, the CAT gene, and the SOD gene (Figure 4). SA interaction was significant for relative expression for the CAT (Figure 4B) and the SOD (Figure 4C) genes.

The relative expression for the APX gene under control conditions varied between 0.95 (for *T. aestivum*) and 3.13 (for *Ae. triuncialis*) (Figure 4A), while under salinity conditions, it ranged from 15.67 (for *T. aestivum*) to 15.10 (for *Ae. triuncialis*). The relative expression for the CAT gene under control conditions ranged from 1.04 (*Ae. triuncialis*) to 2.29 (for *Ae. umbellulata*). However, for salinity conditions, the relative expression for the CAT gene ranged from 6.62 (for *T. aestivum*) to 11.38 (for *Ae. triuncialis*) (Figure 4B). The relative expression for the SOD gene under control conditions ranged from 1.29 (for *Ae. triuncialis*) to 2.85 (for *Ae. triuncialis*). However, for salinity relative expression for SOD gene ranged from 15.66 (for *Ae. umbellulata*) to 31.93 (for *Ae. triuncialis*) (Figure 4C).

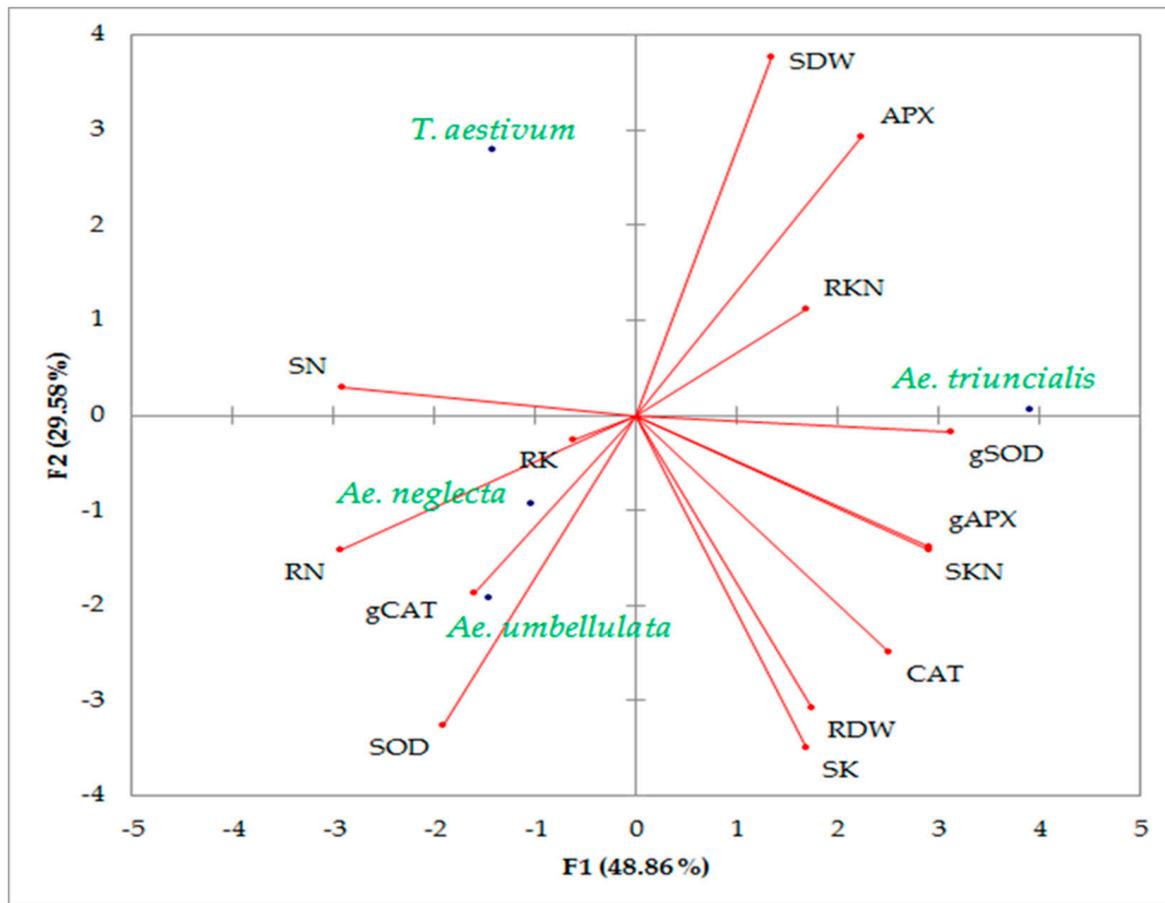
### 3.4. Association among Measured Trait under Control and Salinity Conditions

The PCA was used to assess the relationships among various measured traits under both the control and salinity stress conditions (Figure 5). The results showed that the first two PCs explained 78.44% (PC1: 48.86% and PC: 29.58%) of the total variation. In the PCA loading plot, PC1 was strongly and positively associated with SK, SKN, RKN, RDW, SDW, APX, and CAT activities, as well as the relative expression of the APX and SOD genes. On the other hand, PC2 was strongly affected by RDW, SDW, and APX activity. The

angle between the trait vectors showed the relationship patterns among them. The angle between the vectors for SDW, APX activity, and RKN was less than  $90^\circ$ , so they correlated with each other positively. Moreover, association among SK, SKN, RDW, and CAT activity and the relative expression of the *SOD* and *APX* genes was positive. The association among SN, RK, RN, and SOD activity and the relative expression of the *CAT* gene was positive. A  $180^\circ$  angle shows a negative association between two traits. For example, SN and RN negatively correlated with SKN and RKN, respectively. Other relationships among measured traits are shown in Figure 5. Another result of this biplot was that there was exploration of the association between the studied accessions and the measured traits. *T. aestivum* positively associated with SDW and SN. *Ae. umbellulata* and *Ae. neglecta* showed the positive correlation with RK, RN, SN, and SOD activity and the relative expression of the *CAT* gene. As a superior accession, *Ae. triuncialis* showed a positive association with RDW, SDW, RKN, SKN, and SK activity of the APX and CAT enzymes, and relative expression of the *APX* and *SOD* genes.



**Figure 4.** Mean comparison between control (0 mM NaCl) and salinity (250 mM NaCl) conditions as well as among investigated accessions in terms of the relative expression of *APX* (A), *CAT* (B), and *SOD* (C) genes. S, A, and SA indicate accessions main, salinity stress treatments, and interaction between accessions and salinity stress treatment effects, respectively. ns, \* and \*\* indicate not significant, significant differences at  $p < 0.05$  and  $p < 0.01$ , respectively. The different letters show significant differences among species under control conditions. The different letters with a star (\*) show significant differences among species under salinity conditions. The different capital letters show significant differences among the control and salinity stress conditions.



**Figure 5.** The PCA-based biplot shows the interrelationships among the measured traits and transcriptional data in some neglected *Aegilops* species under salinity stress. RDW, SDW, RN, RK, RKN, SN, SK, SKN, APX, CAT, SOD, gAPX, gCAT, and gSOD indicated root dry weight, shoot dry weight, root Na<sup>+</sup> content, root K<sup>+</sup> content, root K<sup>+</sup>: Na<sup>+</sup> ratio, shoot Na<sup>+</sup> content, shoot K<sup>+</sup> content, shoot K<sup>+</sup>:Na<sup>+</sup> ration, APX activity, CAT activity, SOD activity, relative expression of APX gene, relative expression of CAT gene, and relative expression of the SOD gene, respectively.

#### 4. Discussion

Soil salinity represents a significant challenge to global agriculture, impacting both crop productivity and quality. Recently, there has been an increased interest in studying physio-biochemical responses and gene expression profiling associated with antioxidants in neglected *Aegilops* species under salt stress conditions [16,27,28,47,48]. Studying these species can provide valuable insights into the mechanisms by which plants adapt to extreme environments, and can serve as a basis for developing breeding strategies to increase salt tolerance in crops [15,17,33,34,36]. This study comparatively revealed the response of three neglected *Aegilops* species (*Ae. neglecta*, *Ae. triuncialis*, and *Ae. umbellulata*) along with a bread wheat cultivar (*T. aestivum* cv. Narin) to salt stress at physiological and molecular levels. Our results showed that the physiological and the transcriptional pattern of changes is significantly associated with growth conditions. On the other hand, the tested cultivars showed different responses to salinity stress compared to the control conditions for most of the measured traits and transcriptional tests (Figures 1–4). The analysis of the physio-biochemical responses reveals distinct variations among neglected *Aegilops* species in their capacity to withstand salt stress [27,29,49–51].

In response to this stress, plants deploy various defense mechanisms aimed at mitigating the detrimental effects of excessive salt concentration in the soil. An essential indicator of salt stress is the intracellular accumulation of salt ions. The studied *Aegilops* species have

exhibited the ability to accumulate salt ions, suggesting the presence of specific mechanisms for salt ion transport [29]. Elucidating these mechanisms can provide valuable insights into ion homeostasis regulation and its implications for salt tolerance [52]. In the present study, the lowest Na<sup>+</sup> content and the highest K<sup>+</sup> content in root and shoot tissues, as well as the highest K<sup>+</sup>:Na<sup>+</sup> ratio values in roots and shoots, were recorded for *Ae. triuncialis* (Figure 2). In accordance with our results, Ahmadi et al. [16] also reported the good capability of this species in response to a high level of salinity stress (350 mM NaCl). Another critical aspect of the physio-biochemical response is the maintenance of water homeostasis within plant cells [18]. Salt stress disrupts the water balance, leading to water loss and desiccation. The investigated *Aegilops* species have demonstrated regulatory mechanisms to uphold water economy under salt stress conditions, which contributes to their resilience and survival. These mechanisms encompass reduced transpiration rates through stomatal closure [53,54] as well as the accumulation of osmolytes [48,54], such as proline and salt components, which aid in maintaining water balance [32,55].

Plants activate various defense mechanisms in response to salt stress, including the synthesis of antioxidants and increased activity of antioxidant enzymes [56]. Salt stress induces an increase in the production of reactive oxygen species in plant cells, leading to structural and metabolic disorders. Plants employ antioxidant enzymes such as superoxide peroxidase (POD), dismutase (SOD), and catalase (CAT) [57], as well as antioxidants such as polyphenols and flavonoids, to neutralize and eliminate these reactive oxygen species. In this study, salinity stress significantly increased the activity of CAT, APX, and SOD activities (Figure 3). As a result of a comparison of studied species, the mentioned antioxidants were more active in *Ae. triuncialis* compared to other species and to salt-tolerant species. Indeed, analysis of the antioxidant enzyme activity and accumulation of antioxidants in the examined *Aegilops* species has revealed their efficacy at combating oxidative stress induced by salinity, which, in turn, accords with the previous studies [27,58,59]. Precise regulation of gene expression associated with antioxidant production and antioxidant enzyme activity plays a pivotal role in determining salt tolerance in cultivated plants [60,61].

Gene expression profiling constitutes a powerful tool in deciphering salt tolerance mechanisms [62,63]. Analysis of gene expression provides insights into differential gene regulation among distinct *Aegilops* species and different accessions within the same species in response to salt stress. Differential gene expression highlights the pivotal role of certain genes in modulating salt tolerance [30,64]. Identification of these genes can yield valuable information regarding the molecular mechanisms governing plant adaptation to salt stress. Gene expression analysis can also unravel key metabolic and regulatory pathways that are implicated in salt tolerance [16]. It also enables the identification of genes involved in antioxidant synthesis, the regulation of antioxidant enzyme activity, and salt ion transport [6]. A comprehensive understanding of these metabolic and regulatory pathways enhances our comprehension of plant adaptation strategies to salt stress, and it facilitates the development of breeding strategies aimed at improving salt tolerance in cultivated plants [65,66]. In the present study, the results of transcriptional data supported the biochemical analysis, where *Ae. triuncialis* showed a significant potential to cope with salinity stress compared to bread wheat and other *Aegilops* species.

The findings derived from the analysis of physio-biochemical responses and gene expression profiling offer substantial implications for plant breeding programs and sustainable agriculture. The identified genes associated with antioxidant production [52], antioxidant enzyme activity [17,58], and salt ion transport [46] can serve as valuable genetic resources for breeding programs aimed at developing novel crop varieties with enhanced salt tolerance. Enhancing salt tolerance is of utmost importance for sustainable agriculture, particularly in regions that are characterized by limited access to fresh water. The utilization of neglected *Aegilops* species in plant breeding endeavors can contribute significantly to mitigating the adverse effects of salt stress on agricultural productivity and enhancing resilience in the face of changing environmental conditions [67,68]. The activity of SOD, GPX, APX, catalase, and GR is very often studied in wheat [69–72] as well as in other species,

such as lettuce [73], tobacco [74], rice [75], barley [76], common bean [77], *Arabidopsis* [78], tomato [79], *Salvinia* [80], maize [81], and *Brassica juncea* [82].

## 5. Conclusions

The investigation of the physio-biochemical responses and the gene expression profiling that are related to antioxidants in neglected *Aegilops* species under salt stress conditions provides invaluable insights into the adaptive mechanisms of these plants to extreme environmental conditions. The physio-biochemical responses highlight the capacity of these species, especially *Ae. triuncialis*, to regulate ion balance and water homeostasis, and to mount effective defenses against oxidative stress. The acquired knowledge holds significant potential for the development of innovative breeding strategies targeting enhanced salt tolerance in cultivated crops. The outcomes of these studies have crucial implications for sustainable agriculture, particularly in the context of evolving climatic conditions and the escalating threat of soil salinity.

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