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Differential Responses in Germination, Growth and Genes Expression of Cu/Zn- and Fe-superoxide Dismutase of Barley Under Salinity Stress

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Abstract

Soil salinity limits crop productivity by affecting the growth, physiology, and expression of stress-responsive genes. To evaluate which varieties of cultivated barley from Jordan are salt tolerant, five cultivars of barley (*Hordeum vulgare* L.) of different varieties and morphotypes (i.e., two-and six-rowed barley) were evaluated in terms of their germination, growth traits, and gene expression of Cu/Zn- and Fe-SODs to three levels of salinity (100, 200 or 300 NaCl mM). Germination and root length were significantly affected by moderate and high levels of salinity (200 and 300 mM NaCl) mainly in the varieties Athroh, Mutah, Acsad176, Rum, and to lesser extent in Yarmouk variety, possibly as a consequence of osmotic stress and/or ionic toxicity. Analysis of quantitative real-time PCR (qRT-PCR) showed differential expressions of both Cu/Zn- and Fe-SOD genes between varieties and genotypes (i.e., the six-rowed barleys-Athroh, Acsad176, and Rum-and two-rowed barley-Yarmouk and Mutah). Moreover, both genes were up-regulated by salinity of 300 mM NaCl in the Athroh, Yarmouk, and Acsad176 varieties. Altogether, the result revealed that responses to salinity in all traits of germination, root growth, and gene expression were dependent on the variety and genotype of studied barley. Accordingly, these results have helped us to distinguish between salt-tolerant and salt-susceptible genotypes of cultivated barley of Jordan that shall be useful to local farmers and breeders of barley.

Keywords: Salt tolerance; Barley; Gene expression; Cu/Zn-SOD; Fe-SOD; Jordan

Introduction

Improving crop performance in saline soil is an overarching goal of any breeding program where knowledge of differential responses of crop varieties to salinity is important to screen for stress-tolerant genotypes [1-3]. Soil salinity is a devastating stress factor that limits crop productivity by affecting the growth, and biochemical regulation, i.e., up-and down-regulation, of stress-responsive genes [4,5]. When a plant is exposed to high salinity, osmotic stress can occur, lowering the soil water potential and reducing the amount of water available to a plant's root system, ultimately inhibiting germination and reducing root growth [6]. Moreover, high salinity causes plant toxicity through the accumulation of toxic ions (Na^+ and Cl^-) that disrupt important metabolic and physiological changes [7].

In addition to its effects on germination and growth a plant, salinity induces overproduction of reactive oxygen species (ROS), including singlet oxygen ($1O_2$), hydroxyl radicals (.OH), and hydrogen peroxide (H_2O_2), that can cause oxidative damage to macromolecules and cellular membrane [8]. Stress-tolerant plants have efficient scavenging systems including several anti-oxidative enzymes that prevent the accumulation of ROS [9]. An important example of these enzymes is the superoxide dismutase family (SOD; EC1.15.1.1) which protects plant from abiotic stresses by catalyzing the dismutation of superoxide into oxygen (O_2) and hydrogen peroxide (H_2O_2), which is further degraded by other enzymes [10]. Different isomers of SODs, named based on their metal group cofactor, have been found in different cellular organelles of plants; copper zinc (Cu/Zn-SOD) is located in the cytoplasm and chloroplasts, and iron SOD (Fe-SOD) is located in the chloroplast [11,12]. Physiologically, many studies have indicated that changes in the SOD-enzymatic activities and some metabolites are reflected in an increase in SOD mRNA transcripts in response to stress factors [13]. Nonetheless, to the best of our knowledge, there is few if any, studies have examined the effect of NaCl on the levels SOD-mRNA transcripts in crop plants, in contrast to many studies that examined the effect of salinity on enzyme activities and metabolites [10,14-16].

The agroecosystem in Jordan is characterized by prevalence of both arid and semiarid areas with a fluctuating annual rainfall and high evapotranspiration that contributes to increased soil salinity [17]. Five different barley (*Hordeum vulgare* L.) varieties are currently cultivated in Jordan: Athroh, Rum, Acsad176 (six-rowed barley), and Yarmouk, and Mutah (two-rowed barley). Some of these cultivars are known for their response variation to drought [18], yet there is a limited knowledge of their performances under salinity stress. Accordingly, the main objective of this study was to elucidate the variation in salinity responses of the five varieties of barley as assessed by germination, growth, and the expression levels of the Cu/Zn-SOD and Fe-SOD.

Materials and Methods

Plant materials, stress treatment, growth and stomatal parameters

Uniformly sized seeds of five varieties of cultivated barley-Yarmouk, Acsad176, Athroh, Rum and Mutah-that have been developed and released in Jordan were used in this study. Initially, the seeds were obtained from the National Center for Agricultural Research and

Extension (NCARE) of Jordan. The seeds were surface-sterilized with 70% (v/v) ethanol for 5 min followed by a 2% commercial bleach (sodium hypochlorite) treatment for 3-5 min, and rinsed thoroughly in sterile distilled water. After stratification for a few days at approximately 4°C, ten seeds from each variety were germinated on 12 × 12 cm petri dishes onto a wet filter paper (Whatman No 1, Whatman International Ltd., Kent, UK) at 25°C in the dark with or without salt treatment at four NaCl levels (0 mM NaCl as a control, 100 NaCl mM, 200 NaCl mM, 300 NaCl mM). These four levels of salinity were choosen as they have been shown to cause several growth and physiological responses to discriminate differences in crop cultivars [19-22]. After 4 days of treatment, the percentage of germination was determined after radicle emergence, and the root lengths of germinated seedlings were measured after 9 days of treatments. For gene expression analysis, leaves from the barley varieties were collected on the third day after treatment with a 300 mM NaCl solution, and were quickly frozen in liquid nitrogen at -20°C until RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was extracted from frozen barley tissues using an IQeasy™ Plus plant RNA extraction mini kit (iNtRON Biotechnology, Korea). The concentrations of RNA in the samples were measured spectrophotometrically (260 nm/280 nm; Biochrom, Cambridge, UK). The first-strand cDNA was synthesized by mixing 2 µg of RNA with 4 µl of Prime Script™ RT reagent (Takara, Japan), and the final volume of the mixture was adjusted to 20 µl with RNase-free water (0.1% (v/v); diethylpyrocarbonate-treated water). The samples were then placed in a thermocycler (Biometra, Germany) for 45 minutes at 37°C, followed by 15 seconds at 85°C, and finally at 4°C for approximately 5 minutes. Amplified samples were then diluted to 50 ng/µl with sterile RNase-free water and stored at -20°C for gene expression analysis by quantitative real-time PCR (qRT-PCR).

Quantitative real-time PCR (qRT- PCR)

Differential expression analysis of SOD genes (Cu/Zn-SOD and Fe-SOD) was studied using qRT-PCR. PCR reactions were performed in a total volume of 25 µl consisting of 10 µl of Kappa Syber Fast qPCR reagent (KAPA Biosystems, MA, USA), 50 ng/µl synthesized template cDNAs, and 10 µM aliquots of each primer of the studied genes (Table 1) at each. The cycling conditions were as follows: 2 min/95°C, 10 s/95°C, 25 s/57°C, 25 s/60°C, and a final extension step of 2 min/60°C using a CFX96 touch real-time PCR system (Bio-Rad) instrument. This experimental procedure was conducted at least three times starting from cDNA synthesis. The threshold cycle (Ct) values of the triplicate PCRs were averaged, and relative quantification of the transcript levels was analyzed using the comparative Ct method [23].

Name	Accession number	Primer sequence 5′-3′	Amplicon Length(bp)	Tm (°C)
Actin	AY145451.1	CTCCATCATGAAGTGTGACGTG GACGACCTTGATCTTCATGCTG	151	61.77 61.83
Cu/Zn-SOD	HM537232.1	GGTGACACGACTAATGGATGC GGAATCTGGCTATCGACAATGG	164	61.50 61.40
Fe-SOD	AK375983.1	CTATCAACCCACTTGCTTTCGG CTGCTTTACAAGGGTCTGGATG	144	62.04 62.28

Table 1: Names of studied genes, their accession numbers, primer sequences, amplicon length, and temperature degree used in their amplification.

Statistical analysis

Data analysis was performed with SPSS for Mac (v20, SPSS Inc., Chicago, IL, USA). The differential responses to salinity at all parameters estimated in this study were executed by one- and two-way analyses of variance (ANOVAs) and Duncan's Multiple-Range test (DMRT). The values shown in the results section are represented as the arithmetic mean ± standard error (S.E.; n=3), with p value ≤ 0.05 regarded as a significant difference. Before the performing of data analysis, the normality was checked (Shapiro-Wilk test), and when normality and homogeneity of variance were violated, the logarithmic transform was taken for the raw data. To measure the level of gene expression of SODs, the actin gene transcript was used as an internal control to quantify the relative transcript level of each target gene in each sample [24].

Results and Discussion

Although the differential responses of crop species and the varieties of a species to salinity is well documented, yet elucidating variations in crop responses of different genotypes of a crop plant to salinity is important as it helps in distinguishing plant varieties into tolerant and susceptible genotypes that are useful in breeding for stress tolerance [1]. Five varieties of barley from Jordan were evaluated for their germination, growth, and molecular responses to salinity. Under control conditions (0 mM NaCl), the germination percentage was similar in all barley varieties, and treating them with 100 mM NaCl did not significantly alter the germination. Contrarily, at a relatively moderate level of salinity (200 mM NaCl), the germination percentage was decreased significantly in the Athroh genotype ($F_{1,4}=49$; $p<0.01$), whereas the higher salt concentration (300 mM NaCl) was found to inhibit germination in the Acsad176 ($F_{1,4}=12.50$; $p<0.05$), Athroh ($F_{1,4}=75$; $p<0.01$), and Rum ($F_{1,4}=19$; $p<0.05$) (Figure 1A). Two-way ANOVA was used to examine the effect of salinity on germination in relation to the variation of cultivars, and the results showed that the salinity effect on germination is variety dependent ($F_{12,40}=1.9$; $p<0.05$, Table 2). Moreover, the inhibitory effect of 200 and 300 mM NaCl salinity on germination was found to be significantly stronger in six-rowed barley varieties (Athroh, Acsad176, and Rum) than in two-rowed barley varieties (Yarmouk and Mutah) (Figure 1B). This inhibition of germination of all barley cultivars, except Yarmouk and Mutah, by salinity can be attributed to the water deficit caused by lower plant water uptake (known as osmotic stress) and by the cellular accumulation of toxic sodium and chloride ions that may interfere with the metabolic processes of germination [25,26]. However, it is also

possible that both osmotic stress and ionic toxicity synergistically affected the germination process, and to distinguish them from each other, an investigation is required to compare the relative effects of NaCl and an iso-osmotic solution of an inert osmoticum such as polyethylene-glycol (PEG) [27].

Source	Germination	Total root length
Salt treatment (T)	**	*
Genotype (G)	**	**
T × G interaction	*	NS

Table 2: Significance of the variation source (salt treatment, genotype, and treatment genotype interaction) in germination, total root length, and stomatal resistance of five varieties of barley under control or saline conditions.

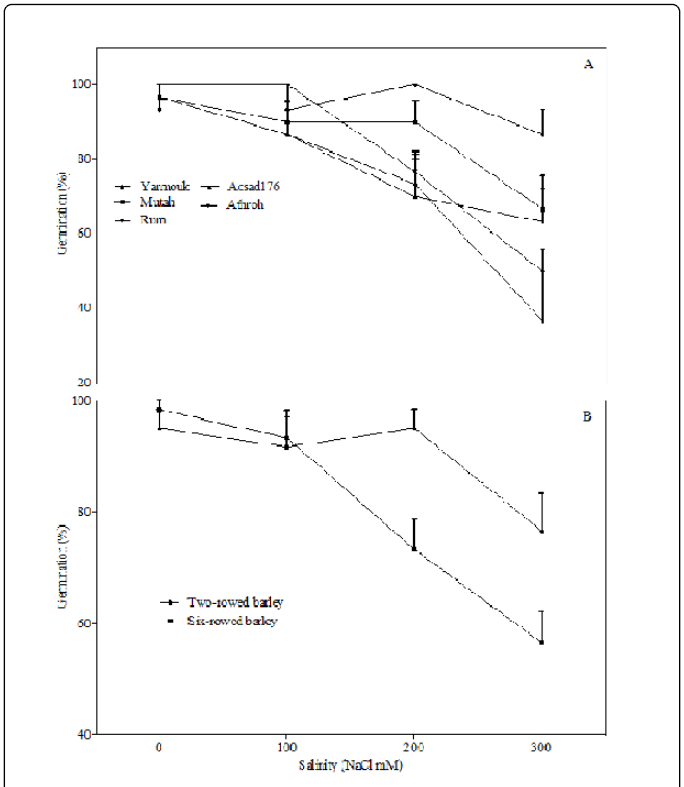

Figure 1: Germination percentage of five barley varieties (four days after sowing) supplemented with either 0, 100, 200, or 300 mM NaCl (A), and the difference between two-rowed and six-rowed cultivars in germination percentage (B). Values are the means of three experiments (±S.E.).

As the germination of two-row barley varieties was found to be more tolerant to salinity than that of six-row varieties, it seems that genetic variation exists within the studied varieties that enabled some genotypes (two-rowed varieties) to germinate more efficiently than others under salinity stress. In fact, a recent study, based on a quantitative trait locus (QTL) study, suggested that two different regions in two different chromosomes of barley govern the germination capacity under varied levels of salinity [28]. This leads us to hypothesize that the studied genotypes of barley have different genetic potentials that, together with environmental conditions (i.e., salinity stress), can determine germination capacity.

Root length was estimated in this study as an important determinant trait of successful seedling growth and development [29,30]. Root length in this study did not vary under normal conditions (i.e., 0 mM NaCl) and under salinity of 100 mM NaCl. Nonetheless, at a salinity of 200 mM NaCl root length was reduced significantly in accessions Mutah ($F_{1},4=25.47$; $p<0.01$) and Acsad176 ($F_{1},4=17.44$; $p<0.05$), while at 300 mM NaCl salinity, Yarmouk ($F_{1},4=15.13$, $p<0.05$), Mutah ($F_{1},4=57.77$; $p<0.01$), and Acsad176 ($F_{1},4=38.24$; $p<0.01$) were found to show reduced root lengths (Figure 2A). Additionally, two-way ANOVA indicated that the salinity effect on root length is variety dependent and that a salinity of 200 mM NaCl decreased the root length of six-rowed genotypes more than it decreased the root length of two-rowed genotypes ($F_{11,13}=4.10$; $p=0.067$ Figure 2B). The reduced root length caused by salinity may be due to the damage of seeds observed in 'low-vigor seeds', which is caused by the inhibition of cell division [4]. In addition, salinity can also reduce the water potential around the root system of a plant and thus decrease water and food availability to root cells [31].

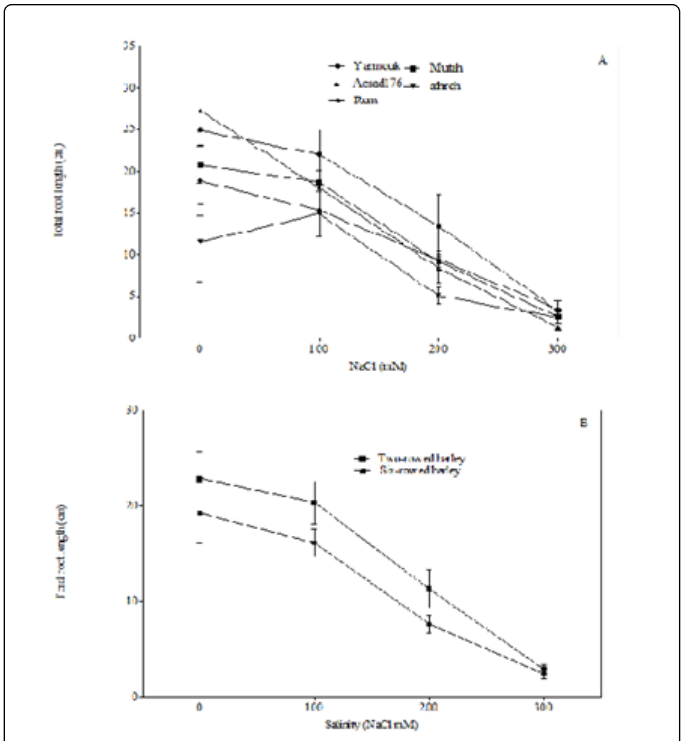

Figure 2: Root length of barley varieties (nine days after sowing) supplemented with either 0, 100, 200, or 300 mM NaCl (A), and differences between two-rowed and six-rowed cultivars in root length with increasing salinity (B). Values are the means of three experiments (±S.E.).

High salinity can also lead to the cellular accumulation of ROS that can damage certain macromolecules within a plant [8,32]. Such ROS are typically scavenged by a variety of anti-oxidative enzymatic families including superoxide dismutases (SOD) that are increased in activity in response to stress factors such as such salinity, drought, and

pathogens [8,11,33,34]. The increased activity of these enzymes is typically indicative of the induction and enhancement of stress-responsive genes [12,35,36]. In this study, the expression levels (i.e., transcription) of the genes encoding anti-oxidative enzymatic isomers of SODs, namely, Cu/Zn-SOD and Fe-SOD, were studied in the barley cultivars using quantitative real-time PCR (qRT-PCR). The transcription levels of the genes were quantified in leaf tissues of genotypes exposed to 300 mM NaCl and compared to their corresponding untreated controls (0 mM NaCl). The results showed a differential expression of both Cu/Zn SOD ($F_{4,10}$=5.76, $p<0.05$), and Fe-SOD genes ($F_{4,10}$=22.72, $p<0.001$) in the varieties studied. Although, the Fe-SOD expression level was higher than the expression level of Cu/Zn-SOD, both genes showed a significant up-regulation in the Athroh, Yarmouk, and Acsad176 accessions upon exposure to salinity (Figure 3A and 3B). This up-regulation of both genes may be attributed to an increased generation of ROS by salinity that causes high activities of Cu/Zn-SOD and Fe-SOD enzymatic scavengers [36,37]. Moreover, the mean gene expression of Fe-SOD was higher in six-rowed barley than in two-rowed barley (mean of 10.37 and 1.19; $F_{1,13}$=4.72, $p<0.05$), suggesting that expression response of Fe/SOD is genotype dependent. The increased activity of SODs upon exposure to salinity has often been correlated to the degree of stress tolerance between plant species and between genotypes within species [14]. Accordingly, the gene expression analysis in this paper can lead us to the conclusion that Athroh, Yarmouk, and Acsad176 are salt-tolerant genotypes and that possibly possess an efficient ROS-scavenging system. The differential responses of these genes may suggest that they are good candidates for modulation of their enzymatic activities or for over-expressing them in different plant varieties in order to develop salt resistant genotypes [38]. Similar results of an increased induction of stress-related genes were found in stressed barley and wheat [5], maize [39], and cotton [40].

The dependence of the differential responses of germination, root length, and genes expression on variety and genotype of studied cultivars make them useful traits to distinguish the cultivated varieties into salt-tolerant and salt-susceptible genotypes [4,41,42]. Accordingly, the studied genotypes can be ranked as NaCl tolerant (number of traits indicated tolerance)>susceptible (number of traits indicated tolerance); Yarmouk (tolerant at 3 traits out of 3 total traits)>Acsad176 (2 traits)>Mutah ≈ Athroh ≈ Rum (1 trait). Therefore, Yarmouk seems to be the most salt-tolerant, possibly because they are able to adjust osmotically to high salinity levels, and Mutah, Rum, and Athroh are the most sensitive genotype.

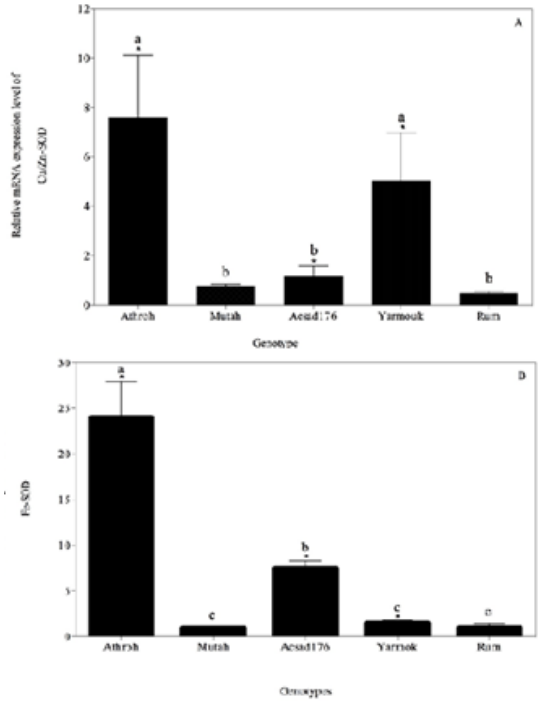

Figure 3: Expression levels of Cu/Zn-SOD (A) and Fe-SOD (B) in 7-day-old seedlings of five barley genotypes grown with either 0 or 300 mM NaCl. Values are the means (± S.E.) of three biological replicates of relative expression levels (log2 fold change) of 300 mM NaCl-treated samples relative to controls and normalized to the expression level of actin. Asterisks indicate significant differences ($p<0.05$) relative to the control within each variety; different letters indicate differences among NaCl-treated varieties.

Conclusion

This study provides knowledge on the performance of cultivated barleys from Jordan under differing salinity levels with respect to germination, growth, and gene expression. As the differential responses of most traits studied were found to depend on barley varieties and genotypes, it is, therefore, possible to distinguish the cultivated barley of Jordan into salt tolerant and salt susceptible genotypes that shall be useful to the local farmers and breeders of barley.

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Conflict of Interest

The author declares no conflict of interest.

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