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Physiological, biochemical and molecular responses of durum wheat under salt stress

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Abstract

The yield of durum wheat extremely reduces in response to salinity stress because of several variations in biochemical, physiological and molecular traits in this unfavorable condition. According to the agro-physiological traits under salinity stress, the most resistant and sensitive cultivars were selected from 10 genotypes of durum wheat over a period of 2 years. Afterwards, the molecular, biochemical, and physiological traits in these two genotypes were evaluated. The seedlings (3–4 leaves) were exposed to salinity through irrigating with 0.5 × Hoagland solution containing 200 mM NaCl until physiological maturity. Principal components analysis for the agronomic characteristics and stress resistance index led to identifying Behrang and Arya as the most tolerant and sensitive genotypes, respectively. In these two genotypes in response to salinity stress, osmolyte contents (proline, total soluble carbohydrates and total soluble proteins) and enzymatic antioxidant defence system activities (ascorbate peroxidase, catalase and guaiacol peroxidase) were much higher in the most tolerant genotype than those of the sensitive cultivar. Moreover, the most tolerant genotype showed less amount of oxidative stress parameters (hydrogen peroxide, electrolyte leakage, malondialdehyde and other aldehydes) than the sensitive one. *Pyrroline-5-carboxylate reductase* (*P5CR*) and *delta-1-pyrroline-5-carboxylate synthase* (*P5CS*) genes expression increased under salinity stress (considering much higher increase in the most tolerant cultivar). Also, proline content was shown to have a significant positive correlation with *P5CS* and *P5CR* genes expression levels. Our result not only identified Behrang cultivar as a superior genotype for durum wheat breeding programs, but also represented several efficient mechanisms involved in salt tolerance.

Keywords: agronomic traits, durum wheat, molecular responses, *P5CR*, *P5CS*, physio-biochemical mechanisms

Introduction

Durum wheat is one of the most important crops in the world that has 734 million tons of production, annually (Fao, 2018); however, the production of this valuable crop has considerably reduced because of salinity stress. Salt stress (SS) is one of the main abiotic stresses which can reduce plant yield (Kandil *et al*., 2012; Sharma, 2015). Salinity extremely affects the physio-biochemical traits (Jamshidi Goharrizi *et al*., 2019; Jamshidi Goharrizi *et al*., 2020a, c) and also can change the gene expression pattern (Jamshidi Goharrizi *et al*., 2018) and proteomic profile of plants (Jamshidi Goharrizi *et al*., 2020b). All of these changes under salinity stress will eventually reduce the plant yield. There are some approaches to enhance wheat yield under the saline regions and in salt-tolerant cultivars that are known as the most efficient procedures for overcoming the grain yield (GY) losses under SS conditions (Munns and Filmer, 2007). Plant yield decreases when the pH or electrical conductivity (EC) of soil goes up to 8.5 or 4 dS/m, respectively (Sairam and Srivastava, 2002). Salinity

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induces water deficit stress even in the soils that have been well-irrigated because of the reduced osmotic potential of the soil. Therefore, it makes it hard for roots to take out enough water from their surroundings (Sairam *et al.*, 2002; Goudarzi and Pakniyat, 2008). The outcome of salinity on plants could be observed such as reducing economic yield and performance (Parida and Das, 2005). Under salinity stress, some mechanisms are activated in plants which lead to an acceptable yield and performance such as stability of cell membrane, enzymatic, and non-enzymatic defence systems (Flowers, 2004; Roy *et al.*, 2014). Thus, SS affects many physiological and biochemical processes (Munns and Tester, 2008) and results in a lot of changes in cellular and organelle level responses. Several physiological, biochemical and molecular characteristics have been known and utilized as indicators to assess the salt tolerance in plants, including salt-responsive genes, antioxidant systems, K^+ and Na^+ content, relative water content, chlorophyll and carotenoids quantification, photosynthesis rate, dry and fresh weight of roots and shoots, spike number, leaf number and length, and germination rate (El-Hendawy *et al.*, 2005; Tajbakhsh *et al.*, 2006).

Proline has been known as a balancing factor for reducing the harmful effect of salinity and water deficit stresses. Also, it serves as an organic nitrogen source that could be used in the recovery period after salinity (Sairam and Tyagi, 2004). Proline accumulates in a higher amount than other amino acids in plants which suffered from stress (Munns and Tester, 2008), and its accumulation mainly is resulted from increasing glutamate synthesis through two sequential reactions catalysed via *pyrroline-5-carboxylate reductase* (*P5CR*) and *pyrroline-5-carboxylate synthesis* (*P5CS*).

According to the agro-physiological traits, from 10 genotypes, for 2 years and under salinity stress, this study aimed to select the most resistant and sensitive durum wheat genotypes. Afterwards, we assessed physiological, biochemical and molecular responses of these two genotypes (the most resistant and susceptible durum wheat genotypes) under salinity.

Material and methods

Plant materials and growth conditions

A total of 10 durum wheat genotypes including Shabrang (cultivar, No. 1), Dehdasht (cultivar, No. 2), DW11 (promising line, No. 3), Behrang (cultivar, No. 4), Yava (cultivar, No. 5), DW18 (promising line, No. 6), Dena (cultivar, No. 7), DW7 (promising line, No. 8), Karkhe (cultivar, No. 9) and Arya (cultivar No. 10) were used in this study. These genotypes have been procured by the Plant Improvement Institute (SPII). Seeds of these genotypes were carefully surface-sterilized (Singh *et al.*, 2015) and then they were cultured in the pots (15 cm height) and filled up with farmyard manure, sand, and soil in equal proportions. In each pot, five seeds were cultured and all traits were calculated based on the average of these five plants achieved from those seeds. The pots were placed in the control condition (16 h photoperiod, 60 ± 5% relative humidity and 25°C) in a greenhouse at SPII until the 4-leave-seedlings were achieved. These 4-leave-seedlings in pots were transferred to the free environment (outside of the greenhouse) and kept until physiological maturity. This experiment was repeated in 2 years. Different concentrations of salinity including 200 mM NaCl (for treated plants) and 0 mM NaCl (for untreated plants) were selected as salinity stress levels (Tammam *et al.*, 2008; Kumar *et al.*, 2017). The salinity treatment started from the 4-leave-seedlings and continued until the physiological maturity (Nazari *et al.*, 2019). The seedlings were irrigated using 0.5 X Hoagland solution containing 200 mM NaCl every 7 d. After 3 times irrigation (Hoagland solution + NaCl), all pots were completely irrigated with distilled water to remove the accumulated NaCl. At the appointed time (physiological maturity), flag leaf samples of untreated and treated plants were collected from the three replicates, rapidly frozen in liquid nitrogen and then stored at −80°C for the next measurements. All physio-biochemical traits and gene expression levels were measured on the most tolerant and sensitive genotypes, which were selected based on agronomic traits and stress resistance indexes. In order to measure physio-biochemical and molecular traits in the most resistant and sensitive genotypes, flag leaves were used.

Agronomic and physio-biochemical measurements

The data on GY, seeds per spike (SS), thousand kernel weight (TKW), number of fertile tillers per plant (NT) and plant height (PH) were measured by field observations.

Photosynthetic pigments

For evaluation of carotenoid and chlorophyll contents, 0.1 g of flag leaf was ground in a mortar containing 3 ml of 80% acetone. Afterwards, the volume of the extract reached 15 ml. The extract was centrifuged at 5000 rpm for 10 min, then the absorbance was recorded at 480, 645 and 663 nm, using a UV-visible Spectrophotometer to quantify carotenoids (Car), chlorophyll a (Chl *a*) and chlorophyll b (Chl *b*) content, respectively. Total chlorophyll was achieved by this equilibrium; Chl *a* + Chl *b* (Arnon, 1949). The photosynthetic pigments were reported as mg pigment per fresh weight sample (g).

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Osmolyte contents

To extract the proline content, 0.2 g flag leaf was placed in 10 ml sulfosalicylic acid (3%) and the resulting mixture was completely homogenized in a mortar (Bates *et al.*, 1973). In the next step, 2 ml of the filtered mixture was mixed with 2 ml of ninhydrin and then 2 ml of acetic acid was added to each tube. The samples were placed in the hot bath 100°C for 1 h and immediately placed in the ice bath for a few minutes. Then, 4 ml of toluene was added to each tube and the samples were mixed for 15 s to become completely uniform. Finally, the supernatant was used to determine the proline concentration according to the proline standard curve at 520 nm.

To evaluate the content of soluble carbohydrates, 500 mg leaf sample was ground with 5 ml of 95% ethanol (Wardlaw and Willenbrink, 1994). Then, 100 µl of the alcoholic extract was added to 3 ml anthrone solution (100 ml of sulphuric acid (72%) and 150 mg anthrone). Next, the samples were heated for 15 min in a boiling water bath. Total soluble carbohydrates (TSC) were calculated based on the absorption at 625 nm and the glucose standard curve. The final results were reported as mg/g of fresh weight samples.

To assess the protein content (PC) of flag leaf, 500 mg of the fresh leaf was homogenized in 5 ml of 10 mM potassium phosphate buffer (pH 7.0) with 4% (w/v) polyvinylpyrrolidone (PVP), and then the resulted solution was centrifuged at 16,000 g at 4°C for 25 min, and then the supernatant was used for estimating the leaf PC (Bradford, 1976). The supernatant (20 µl) was mixed with Bradford reagent (980 µl). In the next step, the 595-nm wavelength of absorbance was recorded. Finally, the results of PC were reported as µg/g fresh weight samples (µg/g FW).

Oxidative stress parameters

To estimate the level of malondialdehyde (MDA), as an output derived from lipid peroxidation and the other aldehydes (OLD), the extracts were made through the Cakmak and Horst (1991) method. Fresh tissue (200 mg) was homogenized using 3 ml of 50 mM phosphate buffer with pH 7.0 and was centrifuged at 16,500 ***g*** for 22 min. Afterwards, the aliquot of the supernatant (1 ml) was mixed with 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) (2 ml). The mixture was heated in a water bath (94°C for 36 min), after heating, the samples were immediately cooled in an ice bath and the absorbance of the supernatant was recorded at 532 and 600 nm. Average readings in triplicate was utilized for estimating the MDA level by extinction coefficient of 155/mM/cm and the formula as follows:

$$\text{MDA (nM)} = \frac{\Delta A_{(532-600)}}{1.56 \times 10^5}.$$

The extinction coefficient for OLD content was 457/mM/cm (Meir *et al.*, 1992). The MDA and OLD contents were reported as nmol/g of fresh weight samples.

The electrolyte leakage (EL) was measured based on the Lutts *et al.*, (1996) method. Leaf samples (300 mg) were washed with deionized water, placed in tubes containing 15 ml deionized water and incubated for 2 h at 25°C, and the EC of the solution (EC_1) was determined. Next, samples were heated at 100°C for 30 min in a water bath, and the final conductivity (EC_2) was recorded after the samples were cooled to 25°C. The EL was calculated as the following formula:

$$\text{EL} = \left(\frac{EC_1}{EC_2}\right) \times 100.$$

To evaluate the quantity of H_2O_2, 250 mg of the fresh leaf was ground in 3 ml of 5% TCA with 0.1 g of activated charcoal, and then the resulted homogenate was centrifuged for 22 min at 15,000 g (Velikova *et al.*, 2000). Next, 0.75 ml of 1 M KI along with 0.5 ml of 10 mM potassium phosphate buffer was mixed with 0.5 ml of the obtained supernatant. Finally, Hydrogen peroxide content was measured by reading spectrophotometrically at 390 nm and using 0.28/µM/cm extinction coefficient. The hydrogen peroxide amount was represented as µmol/g fresh weight samples.

Antioxidant enzyme specific activities

Enzymes extraction

To prepare the enzyme extract, 0.5 g of fresh leaves were homogenized in 8 ml of the extraction buffer (50 mM phosphate buffer with pH 7.7, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 1% PVP) using a mortar and pestle. The homogenate centrifugation was performed at 10,000 g at 4°C for 25 min. Then, the supernatant was utilized as an extract for estimating enzyme specific activities.

Ascorbate peroxidase enzyme activity assay

To measure ascorbate peroxidase activity (APX), the oxidation of ascorbate (extinction coefficient = 2.8/mM/cm) was recorded at 290 nm for 3 min (Nakano and Asada, 1981). The absorbance of the assay medium (consisting of 200 µl enzymes extract, 1200 µl potassium phosphate buffer (50 mM), 200 µl ascorbate, 200 µl EDTA, 200 µl of 0.1 mM H_2O_2) was measured by the Cary 60 UV-Visible Spectrophotometer (Agilent Technologies, USA). One unit of APX activity was specified as the necessary amount of enzyme for the oxidation of 1 µmol of ascorbate per minute.

Catalase enzyme activity assay

The catalase activity (CAT) was evaluated by the Chandlee and Scandalios (1984) method. First, 6.05% (v/v) of potassium phosphate (50 mM, pH 7.0), 0.93% (v/v) of H_2O_2 (15 mM) and 93% (v/v) of enzyme extract were mixed. Next,

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the conversion rate of H_2O_2 (extinction coefficient = 39.4/mM/cm) to water and oxygen molecules was monitored by reducing the absorbance at 240 nm for 1 min. The necessary amount of CAT to decompose 1.0 µM of H_2O_2/min was reported as one unit of CAT activity.

Guaiacol peroxidase enzyme activity assay

The assay medium was made by mixing enzyme extract with guaiacol (9 mM), H_2O_2 (19 mM), and phosphate buffer (pH 7.0, 50 mM) (Lin and Kao, 1999). The guaiacol peroxidase (GPX activity) was determined by measuring the peroxidation of H_2O_2 (extinction coefficient = 26.61/mM/cm) with Guaiacol as an electron donor. The increase in absorbance at 470 nm, due to the degradation of H_2O_2, was monitored for 1 min spectrophotometrically. One unit of GPX activity exhibits the amount of enzyme that catalyses the oxidation of 1.0 µM of Guaiacol in 1 min.

RNA extraction, cDNA synthesis and real-time polymerase chain reactions (RT-PCR)

The flag leaf tissues were used for RNA extraction. Total RNA was extracted from 50 mg of leaf tissue via Biozol reagent (BIOER, China, Cat#: BSC51S1) (Abbasi *et al.*, 2015). The quantity and quality of the extracted RNA were examined through a Nanodrop spectrophotometer and 1% agarose gel electrophoresis, respectively. For cDNA synthesis, 1 µg of RNA was mixed with 1 µg of Oligo dT primer, and the mixture volume was diluted to 12 µl and placed at 65°C for 5 min. Then, 2 µl of dNTP and 1 µl reverse transcriptase enzyme (Fermentase), 1 µl of Rnase inhibitor and 4 µl of buffer were incorporated into the tubes and were kept for 1 h at 45°C. The expression of *P5CR* and *P5CS* genes was estimated in the flag leaf tissues. The *P5CR* and *P5CS* primers were designed through Primer3 software Ver. 0.4.0 (Rozen and Skaletsky, 2000). As *Actin* (Act) reference gene is a stable internal control gene under stress (Lekshmy and Jha, 2017), (Paolacci *et al.*, 2009), (Tenea *et al.*, 2011) and (Borges *et al.*, 2012), this housekeeping was selected to calculate the expression of *P5CR* and *P5CS* genes. The primers' sequences of the desired genes were as follows:

P5CS: Forward: GAGACAAGTCCCGTGTTGGT,

Reverse: CCCCACGGAGAACTTTAACA (product size: 135 bp);

P5CR: Forward: GAAGCAGGTTCTGGTTGAGC,

Reverse: GCCAGTAACCGCATCAAAAT (product size: 280 bp);

Actin: Forward: CGTGTTGGATTCTGGTGATG,

Reverse: AGCCACATATGCGAGCTTCT (product size: 208 bp).

RT-PCR was used to evaluate the expression of *P5CR* and *P5CS* using the Icycler device (Bio-Rad, USA). Each reaction was composed of 5 µl of cDNA, 1 µl of reverse and forward primers (10 pmol), SYBR Biopars, 10 ml of buffer 2X, as well as 3 µl of distilled water. The device program included the first step: 180 s at 95°C, the second step: 10 s at 95°C, 10 s at 60°C as well as 30 s at 72°C for 35 cycles (Ma *et al.*, 2008). Data were analysed by using the mathematical model as elucidated by Pfaffl (2001): $\text{Ratio} = (E_{\text{target}})^{\Delta cp}_{\text{target(Control}-\text{Sample)}}/(E_{\text{ref}})^{\Delta cp}_{\text{ref(Control}-\text{Sample)}}$, in which Ratio (R) represents the difference in the genes expression levels between the two studied samples (Pfaffl, 2001). Finally, the amplification accuracy of the target sequences was verified via gel electrophoresis.

Stress tolerant indices

Stress resistance index (SI) index was calculated for each genotype based on their GY under stress and control irrigation conditions according to the following formula (Lan, 1998):

$$\text{SI} = \frac{Y_s \times \left(\frac{Y_s}{Y_p}\right)}{\overline{Y_s}}$$

In these equations, Y_p and Y_s were the yield obtained under the control and stress conditions, respectively. Also, the $\overline{Y_s}$ was the average of yield overall genotypes in stress conditions, respectively.

Statistical analysis

All the data were subjected to the combined analysis of variance using the GLM procedure of the SAS statistical program (ver. 9.1; SAS Institute Inc., Cary, NC, USA) according to the RCBD model for the analysis of durum wheat genotypes. Significant differences among the means (pairwise comparisons) were determined using the least significant difference (LSD) test ($P < 0.05$). Principal component analysis (PCA) was performed by Stat Graphics (ver. 16.1.11) software to identify the interrelationships among the genotypes/traits and to group the different genotypes. The biplot analysis for the morphological traits was carried out to identify the most tolerant and sensitive genotypes. The primers used in this study were designed using the primer3 program (Rozen and Skaletsky, 2000) and RT-PCR data were calculated based on the REST software (Pfaffl *et al.*, 2002) that works based on the mathematical model of Pfaffl (Pfaffl, 2001).

Results

Agronomic traits

The results of the combined analysis of variance indicated that SS and genotypes (G) had a significant influence on all agronomic traits (online Supplementary Table S1). The effects of $G \times \text{SS} \times Y$(year) interactions were also significant

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($P \leqslant 0.01$) for all the traits except PH. The interaction of $Y \times$ SS had a significant effect on NT, SNS and PH (online Supplementary Table S1). The significant effect of G × SS for all the traits showed that the response of genotypes to SS was different. The average of 2 years showed that salinity stress significantly reduced GY (36%) and its components such as NT (20%), PH (13%), SS (27%) and TKW (33%). Based on the average of 2 years, the highest GY was achieved for Behrang (5.35 g) under SS condition, while Karkhe had the highest GY (7.11 g) under the control condition. Arya and Dehdasht had the lowest GY at stress and non-stress conditions, respectively (online Supplementary Table S2). Regarding SI, a higher value was observed in DW-18 (1.37) and Behrang (1.28) genotypes compared to the other ones. In contrast, under SS condition, Behrang genotype had a higher value of SS, TKW and pH (online Supplementary Table S2).

Selection of the most tolerant and sensitive genotype

In order to decrease the data dimensionality and achieve a superior comprehension of the efficacy of agronomic-plant characteristics, PCA was carried out (Fig. 1). The first two components, explaining 86.85% of the total variance, were considered as the favourable representation of fundamental information. The first component (PC1) accounted for the main part of the whole variation with 61.26% and the second component (PC2) was 25.59%.

Physio-biochemical mechanism

In this study, a considerable variation was recorded between control and stress conditions, as well as between the tolerance and sensitive genotypes (online Supplementary Table S3). Variance analysis showed diversity among conditions, genotypes and interaction of them for all studied physio-biochemical traits (online Supplementary Table S3). The results of ANOVA indicated that there was a significant influence of salinity stress on all the physio-biochemical traits, and also the effect of genotypes for these characteristics was significantly based on the variance analysis except Chl *a*, Chl *b*, TChl, Car, CAT and OLD (online Supplementary Table S3). The significant effect of salinity × genotype for these traits (except OLD, CAT, Car, Chl *a*, Chl *b* and TChl) showed that the response of genotypes to stress and non-stress conditions was different. For more information, comparison of means in genotypes (online Supplementary Table S4–S7) indicated that SS caused a significant reduction in physiological traits (Chl *a*, Chl *b* and TChl) and an increase in biochemical characteristics (El, MDA, OLD, H_2O_2, TSC, Pro, GPX, APX and CAT) in all genotypes.

Photosynthesis pigments

The effect of SS on photosynthesis pigments content was found significant (online Supplementary Table S3). However, there was not a significant difference between Behrang and Arya genotypes. The mean comparison of the effect of treatment on carotenoid and chlorophyll content revealed that salinity significantly reduced the content of photosynthetic pigment. The reduction level under SS condition in comparison with the control condition for Chl *a*, Chl *b*, TChl and Car was 63, 65, 64 and 46%, respectively (online Supplementary Table S4).

Osmolyte contents

Based on the results of variance analysis, the protein (PC), TSC, proline (Pro) content indicated a significant difference at 1% level between Behrang tolerant and Arya sensitive genotypes (online Supplementary Table S3). TSC was largely influenced by salt concentration so that the SS significantly increased TSC. Behrang tolerant genotype indicated more amount of TSC than that of Arya sensitive genotype. The soluble carbohydrates content increased up to 2.6-fold in Behrang genotype under stress conditions than that of control, whereas this trait increased up to 1.9-fold in Arya genotype under salinity than that of control (online Supplementary Table S5). The content of proline was significantly increased in Behrang genotype, up to 3.2-fold compared to the non-stress condition. The total PC of leaf significantly decreased in the sensitive genotype (29%), whereas the tolerant genotype (Behrang) kept the level of PC constant under salinity stress. Overall, Behrang genotype was found to be tolerant in response to SS due to the mechanisms involved in the accumulation of TSC as well as Pro, which are not degraded by stress.

Oxidative stress parameters

According to the outputs of variance analysis, the (EL, MDA and H_2O_2 content indicated a significant difference at 1% level between Behrang tolerant and Arya sensitive genotypes (online Supplementary Table S3). All stress oxidative parameters were largely affected by salinity, so that the SS significantly increased these parameters (EL, MDA, OLD and H_2O_2). Behrang tolerant genotype showed lower oxidative damage than that of Arya sensitive genotype. The EL, MDA, OLD and H_2O_2 content increased 350, 100, 63 and 111% in Behrang genotype under stress conditions than those of control, respectively (online Supplementary Table S6), whereas these parameters increased 704, 440, 138 and 405% in Arya genotype under salinity than those of control, respectively. Therefore, Behrang genotype can be identified as the more tolerant genotype under salinity

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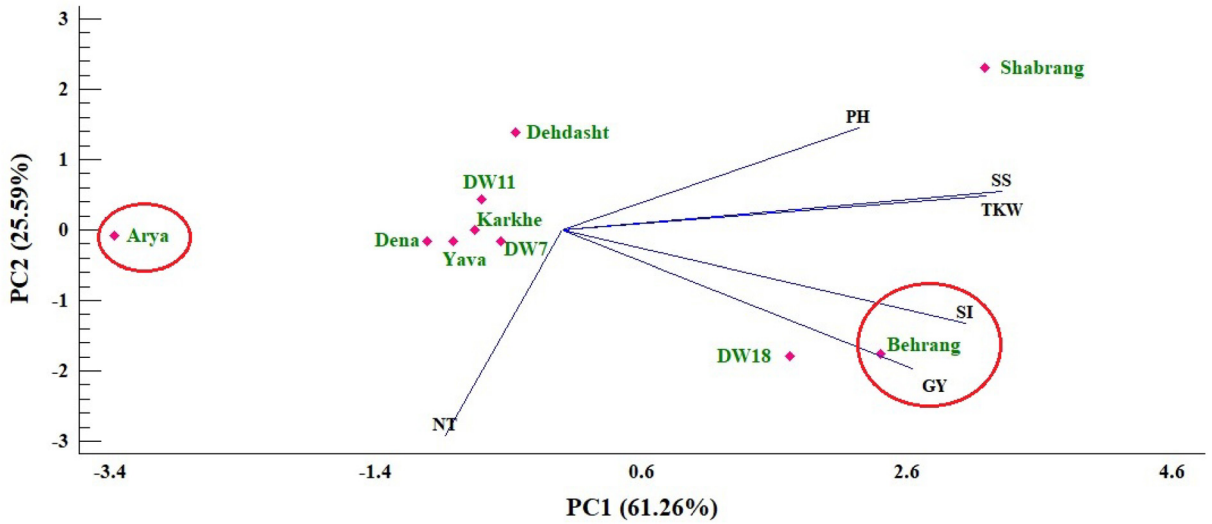

Fig. 1. The biplot display of agronomic traits under salt stress condition (grain yield (GY), seed per spike (SS), thousand kernel weight (TKW), number of fertile tillers per plant (NT), plant height (PH) and stress tolerance index (SI)).

stress condition than Arya sensitive genotype due to the mechanisms involved in response to oxidative stress.

Antioxidant enzyme specific activities

The analysis of variance revealed a significant difference at 1% level between Behrang tolerant and Arya sensitive genotypes for the activity of APX and GPX (online Supplementary Table S3). The activity of GPX, CAT, and APX enzymes increased up to 4.1, 4.3 and 3.4-fold in Behrang tolerant genotype under salinity than those of control, respectively, which correspond to 1.3, 1.4 and 1.5-fold in Arya sensitive genotype (online Supplementary Table S7). As a result, Behrang genotype showed the high activity of antioxidant enzymes in salinity condition, which reflects the powerful antioxidant mechanisms that can protect the plants under SS against free radicals derived from oxidative stress.

Genes expression

The ANOVA findings exhibited that in the tolerant and sensitive genotypes, the expression levels of *P5CR and P5CS* genes significantly increased under SS although the induced levels of both genes expression in the tolerant genotype were much higher than the sensitive one (online Supplementary Table S3). The expression of *P5CS and P5CR* genes increased by 662 and 471% in Behrang tolerant genotype under salinity stress than those of control, respectively. These genes expression levels increased up to 91 and 84% in Arya sensitive genotype under salinity compared to the control condition, respectively (online Supplementary Table S8). Based on the findings, Behrang genotype could be a promising and appropriate gene resource to improve the wheat salt-tolerant genotypes in future breeding programs. Furthermore, the results related to *P5CR and P5CS* expression can increase our knowledge of the mechanisms involved in the salt-tolerant wheat genotypes.

PCA under SS condition

To explore the interrelationships among the traits and the collinearity between the tolerant and sensitive genotypes and the measured traits, PCA was performed on the averaged data over 2 years under SS condition. The PCA was performed to study the likely relationships among the measured traits and tolerant/sensitive genotypes (Fig. 2). Most of the variation (89.34%) was explained by the first two principal components (PC1 and PC2). The first PC (PC1) showed 74.70% of the total variation and exhibited positive correlations with SS, TKW, PH, and GY as well as GPX, APX, CAT, TSC, Pro, PC, *P5CS* and *P5CR* genes besides negative correlations with EL, MDA, OLD and H_2O_2 (Fig. 2). PC2 results explained 14.65% of the total variation and were negatively correlated with Chl *a*, Chl *b* and TChl. Based on this analysis, the tolerant (Behrang) and sensitive (Arya) genotypes were classified into two completely distinct groups according to the agronomic, physiological, biochemical characteristics and gene expression. The tolerant genotype was characterized by higher quantities of agronomic traits (especially GY, SS, TKW and PH), enzyme activities, osmolyte contents, and genes expression besides lower values of oxidative stress parameters. The sensitive genotype formed a distinct group identified for their higher

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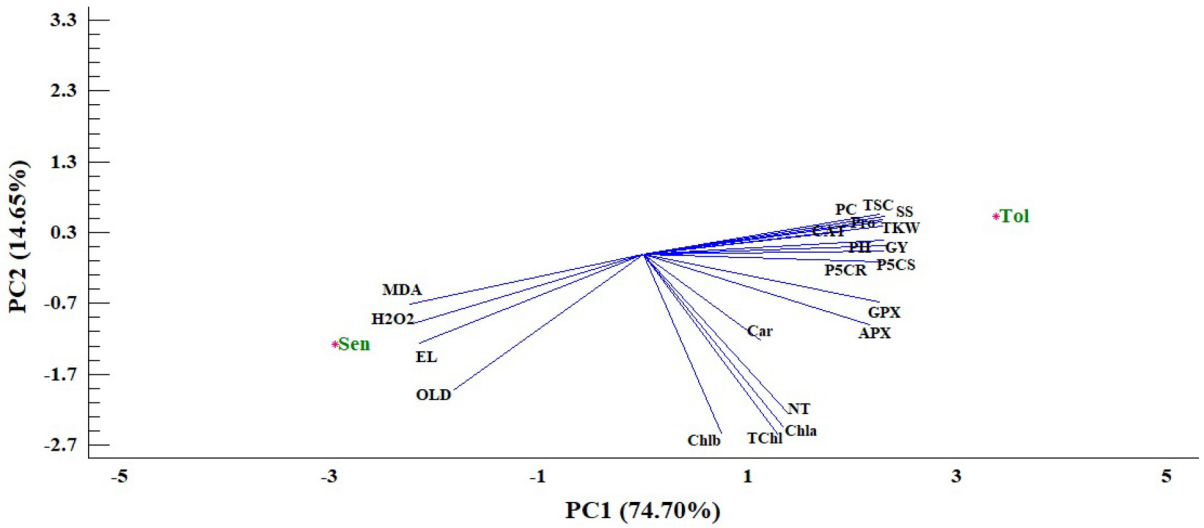

Fig. 2. The biplot display of all studied traits under salt stress condition for tolerant (Tol) and sensitive (Sen) genotypes (grain yield (GY), seed per spike (SS), thousand kernel weight (TKW), number of fertile tillers per plant (NT), plant height (PH), chlorophyll a (Chl a),chlorophyll b (Chl b), chlorophyll *a* + *b* (TChl), carotenoids (Car), ascorbate peroxidase activity (APX), catalase activity (CAT), guaiacol peroxidase activity (GPX), hydrogen peroxide (H_2O_2), electrolyte leakage (EL), malondialdehyde (MDA), other aldehydes (OLD), total soluble carbohydrate (TSC), proline content (Pro), protein content (PC), the expression of P5CS and P5CR genes).

levels of oxidative stress parameters as well as lower values of agronomic traits, enzyme activities, osmolyte contents, and gene expression (Fig. 2). Based on the test results and the above observations, the tolerant and sensitive durum wheat genotypes were successfully distinguished from each other in terms of their antioxidant activity, GY and its components, genes expression, photosynthetic pigments, and oxidative stress parameters. The degree between traits vectors in Fig. 2 showed clearly the positive correlation between GY and its components as well as osmolyte content, photosynthesis pigment and enzyme activities. On the other hand, there was a negative correlation between oxidative stress parameters and other traits (Fig. 2).

Discussion

Soil salinity is considered as one of the most important abiotic stresses which restricts the productivity and growth of crops in semi-arid and arid regions, worldwide. Durum wheat is subjected to loss of yield due to this scenario. As salinity is a significant environmental factor restricting plant growth via suppressing biological processes like nutrient uptake and assimilation, it is urgent to enhance our knowledge of physiological and molecular mechanisms to select crops that remain highly productive under NaCl accumulation in the soil (Guellim *et al.*, 2019). In this study, PCA and SI led to Behrang and Arya being identified as the most tolerant and sensitive genotypes, respectively. Then, we aimed to investigate some of the molecular, biochemical, and physiological responses of these two genotypes and to recognize the most important mechanisms in SS tolerance. Our findings revealed that some traits including the GY, SS, NT, PH, TKW, Chl *a*, Chl *b*, TChl, Car and PC decreased under SS, while EL, MDA, OLD, H_2O_2, proline, TSC, GPX, CAT and APX increased.

A major cause of the decrease in yield under water deficit can be reduced photosynthesis activities due to the stomatal closure and limited carbon dioxide uptake (Grieve *et al.*, 2012). Since chlorophylls preservation is considerably significant for photosynthesis in environments suffering from a variety of stresses (Dugasa *et al.*, 2019), the selection of genotypes based on the chlorophylls level is an important duty in the stress studies. In agreement with our results, Tounsi *et al.*, (2017) showed that a salt-tolerant genotype has higher chlorophyll content than a sensitive one, although it was not significant statistically. They also observed that photosynthetic pigments do not undergo significant changes under salinity stress (Tounsi *et al.*, 2017). The carotenoids are responsible for singlet oxygen scavenging under salinity stress. Thus, in plants, a higher level of carotenoids may grant more tolerance under this condition. However, no significant difference was observed between tolerant and sensitive genotypes on carotenoid contents in this study. A study showed that the tolerant genotype had more carotenoids levels than the sensitive one (Chandrasekar *et al.*, 2000). The highest amount of the photosynthesis pigments was recorded in

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the control condition and the lowest in the stress condition. In line with our results, it was proven that SS has resulted in a significant decrease in these pigments content (Al-Khaishany *et al.*, 2018; Dugasa *et al.*, 2019). The reason for photosynthesis pigment decrease is a reduction in the degradation when plant suffered from stresses and then transferred the nutrients elements such as magnesium to younger leaves from older ones (Dugasa *et al.*, 2019).

The accumulation of protein is very important for cell survival against stress conditions because it causes the membranes to be stabilized under such situations (Jamshidi Goharrizi *et al.*, 2020d). Moreover, it was reported that protein accumulation under stress results in nitrogen storage for recovery after removing stress symptoms (Qasim *et al.*, 2003). Besides, another study demonstrated that many proteins are synthesized by plants in response to SS (Qasim *et al.*, 2003). The total PC of leaf significantly declined in the sensitive genotype, whereas the tolerant genotype kept its proteins under salt conditions in comparison with the sensitive one. Similarly, up-regulation of proteins in okra (*Abelmoschus esculentus* L.) seedlings under SS was reported recently (Zhan *et al.*, 2019).

We observed that salinity significantly increased proline content only in Behrang salt-tolerant genotype for balancing the harmful effect of salinity and serving as an organic nitrogen resource which could be used within the recovery period after salinity (Sairam and Tyagi, 2004). In line with our findings, previous studies also revealed that high accumulation of intracellular proteins, proline, and soluble sugar prevent excessive water loss, maintain normal cell turgor, improve the cell and enhance the salt resistance in plants (Al-Khaishany *et al.*, 2018). Moreover, enhancement of the accumulation of proline as an osmolyte was reported in tomato plants under salinity stress (Chanratana *et al.*, 2019).

SS led to a significant increase in EL, MDA, OLD and H_2O_2, which can be attributed to lipid peroxidation and thereby membrane damage. Thus, it was demonstrated that plant cell membranes are damaged and lose their integrity in salinity condition (Blokhina *et al.*, 2003), and the increasing content of EL and aldehydes happen, eventually. EL, MDA, OLD and H_2O_2 increased much more in the sensitive genotype (Arya) under salinity condition than those of the tolerant genotype (Behrang), respectively.

Based on our results, Behrang genotype revealed the high activity of antioxidant enzymes in salinity condition, which reflects the potent oxidant mechanisms protecting the stressed plants against free radicals derived from oxidative stress. These findings were also proved in the previous studies (Badridze *et al.*, 2009; Singh *et al.*, 2015), which also showed a significant increase in the GPX, APX and CAT activities in the salt-resistant genotypes. In a study, it was also observed that salt-tolerant durum wheat cultivars efficiently induce antioxidant enzymes such as SOD as well as CAT enzyme activities when compared to sensitive cultivars (Tounsi *et al.*, 2017).

The findings exhibited that in the tolerant and sensitive genotypes, the expression level of *P5CR and P5CS* significantly increased during SS although the induced level of both gene expressions in the tolerant genotype was much higher than the sensitive one. Based on the findings, durum wheat could be a promising and appropriate gene resource to improve the salt-tolerant wheat genotypes. Furthermore, the results related to *P5CR and P5CS* expression can increase our knowledge of the mechanisms involved in salt tolerance in wheat. *P5CR* and *P5CS* gene expression levels and proline content in our study were induced by SS. Proline accumulation in Behrang genotype may be largely resulted from increasing glutamate synthesis through two sequential reactions catalysed via *pyrroline-5-carboxylate reductase (P5CR)* and *pyrroline-5-carboxylate synthesis (P5CS)*. It has been reported that the *P5CS* gene and proline content are induced under ABA treatment, salt, and drought stresses (Yoshiba *et al.*, 1995). In a study, *P5CS* and *P5CR* gene expression levels and proline content were induced by salinity stress (Jamshidi Goharrizi *et al.*, 2020d). Based on our findings, *P5CR* and *P5CS* genes were most likely induced in response to osmotic stress for increasing the free content of proline as an osmotic adjustment under conditions suffered from the high concentrations of salt.

The GY is an important purpose in wheat breeding programs (Munns *et al.*, 2000). Several crop features and environmental factors may affect GY. It is controlled by several genes and also affected highly by environmental situations (Kaya *et al.*, 2012). Thus, evaluation of physio-biochemical traits specifying GY is a key step in these breeding programs (Munns and James, 2003). The GY components (TKW and SS), TSC, PC, proline, enzyme activities characteristics as well as *P5CS* and *P5CR* gene expression displayed a significant positive correlation with GY under salinity stress. A significant negative correlation between GY and total oxidative parameters was found. Consistent with the recent work, another researcher suggested that resistance of salt-tolerant wheat genotypes is associated with the less amount of EL and MDA, which improved the capacity of GPX and SOD, and increased activities of APX and CAT for scavenging the reactive oxygen species (Dugasa *et al.*, 2019). These results showed a significant negative correlation between oxidative stress parameters and the antioxidant enzymes activities. Besides the significant positive correlations among agronomic traits, osmolyte contents and enzymes activities, these parameters also had a positive correlation with *P5CS* and *P5CR* genes expression. The positive correlation between proline and genes expression may reflect the fact that proline accumulation is largely derived from the higher activity of *pyrroline-5-carboxylate reductase (P5CR)* and *pyrroline-5-carboxylate synthesis (P5CS)* enzymes (Ma *et al.*, 2008).

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Conclusion

In this study, Behrang and Arya genotypes were selected as the most tolerant and sensitive genotypes based on the agronomic traits and SI in two growing seasons. SS greatly influenced the agronomic, physiological and biochemical functions of durum wheat genotypes; however, this influence depends on plant genotypes. The results showed that the photosynthetic pigments, osmolyte contents, specific enzyme activities, and *P5CR* and *P5CS* genes in wheat had a strong relationship with salt tolerance. The proline level was shown to have a significant positive correlation with *P5CS* and *P5CR* genes expression levels. In this study, both the sensitive and tolerant genotypes have survived in response to SS. In our opinion, the higher levels of photosynthesis pigments, non-enzymatic and enzymatic antioxidant defence systems and compatible solutes as well as the lower quantities of oxidative stress parameters in Behrang genotype (the most tolerant genotype) are the main reasons for salt tolerance of this cultivar. Also, based on our results, these traits under salinity stress could be considered as the most valuable indexes for the screening of tolerant cultivars. Furthermore, Behrang genotype can be used by plant breeders to improve the tolerance of salinity sensitive cultivars in breeding programs.

Conflict of interest

The authors declare that they have no conflict of interests

Supplementary material
To view supplementary material for this article, please visit https://doi.org/10.1017/S1479262120000416

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References

Abbasi A, Mohammadi B, Sarvestani R and Mirataei F (2015) Expression analysis of candidate genes in Common Vetch (*Vicia sativa* L.) under drought stress. *Journal of Agricultural Science and Technology* 17: 1291–1302.

Al-Khaishany MY, Al-Qurainy FH, Alaraidh IA, Barakat MN, Ahmed A, Elshafei MH, Alamri SA, Ali HM, Alsahli AA and Alzahrani SM (2018) Genetic variation of wheat for salt tolerance based on physiological and agronomic traits. *International Journal of Agriculture and Biology* 20: 2853–2861.

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. *Plant Physiology* 24: 1.

Badridze G, Weidner A, Asch F and Börner A (2009) Variation in salt tolerance within a Georgian wheat germplasm collection. *Genetic Resources and Crop Evolution* 56: 1125–1130.

Bates LS, Waldren RP and Teare I (1973) Rapid determination of free proline for water-stress studies. *Plant and Soil* 39: 205–207.

Blokhina O, Virolainen E and Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Annals of Botany* 91: 179–194.

Borges A, Tsai SM and Caldas DGG (2012) Validation of reference genes for RT-qPCR normalization in common bean during biotic and abiotic stresses. *Plant Cell Reports* 31: 827–838.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.

Cakmak I and Horst WJ (1991) Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). *Physiologia Plantarum* 83: 463–468.

Chandlee J and Scandalios J (1984) Analysis of variants affecting the catalase developmental program in maize scutellum. *Theoretical and Applied Genetics* 69: 71–77.

Chandrasekar V, K. Sairam R and Srivastava G (2000) Physiological and biochemical responses of hexaploid and tetraploid wheat to drought stress. *Journal of Agronomy and Crop Science* 185: 219–227.

Chanratana M, Joe MM, Choudhury AR, Anandham R, Krishnamoorthy R, Kim K, Jeon S, Choi J, Choi J and Sa T (2019) Physiological response of tomato plant to chitosan-immobilized aggregated *Methylobacterium oryzae* CBMB20 inoculation under salinity stress. 3 *Biotech* 9: 397.

Dugasa MT, Cao F, Ibrahim W and Wu F (2019) Differences in physiological and biochemical characteristics in response to single and combined drought and salinity stresses between wheat genotypes differing in salt tolerance. *Physiologia Plantarum* 165: 134–143.

El-Hendawy SE, Hu Y, Yakout GM, Awad AM, Hafiz SE and Schmidhalter U (2005) Evaluating salt tolerance of wheat genotypes using multiple parameters. *European Journal of Agronomy* 22: 243–253.

Fao I (2018) WFP. 2018. *The State of Food Insecurity in the World* 1–62.

Flowers T (2004) Improving crop salt tolerance. *Journal of Experimental Botany* 55: 307–319.

Goudarzi M and Pakniyat H (2008) Evaluation of wheat cultivars under salinity stress based on some agronomic and physiological traits. *Journal of Agriculture and Social Science* 4: 35–38.

Grieve CM, Grattan SR and Maas EV (2012) Plant salt tolerance. In: *Agricultural Salinity Assessment and Management*. 2nd edn. Reston, VA: ASCE, Manual and Reports on Engineering Practice vol. 71. pp. 405–459.

Guellim A, Catterou M, Chabrerie O, Tetu T, Hirel B, Dubois F, Ben Ahmed H and Kichey T (2019) Identification of phenotypic and physiological markers of salt stress tolerance in Durum wheat (*Triticum durum* Desf.) through Integrated Analyses. *Agronomy* 9: 844.

Jamshidi Goharrizi K, Wilde HD, Amirmahani F, Moemeni MM, Zaboli M, Nazari M, Moosavi SS and Jamalvandi M (2018) Selection and validation of reference genes for normalization of qRT-PCR gene expression in wheat (*Triticum durum* L.) under drought and salt stresses. *Journal of Genetics* 97: 1433–1444.

Jamshidi Goharrizi K, Riahi-Madvar A, Rezaee F, Pakzad R, Jadid Bonyad F and Ghazizadeh Ahsaei M (2019) Effect of salinity stress on enzymes' activity, ions concentration, oxidative

stress parameters, biochemical traits, content of sulforaphane, and CYP79F1 gene expression level in *Lepidium draba* plant. *Journal of Plant Growth Regulation* 6: 1–20.

Jamshidi Goharrizi K, Amirmahani F and Salehi F (2020a) Assessment of changes in physiological and biochemical traits in four pistachio rootstocks under drought, salinity and drought + salinity stresses. *Physiologia Plantarum* 168: 973–989.

Jamshidi Goharrizi K, Baghizadeh A, Kalantar M and Fatehi F (2020b) Assessment of changes in some biochemical traits and proteomic profile of UCB-1 pistachio rootstock leaf under salinity stress. *Journal of Plant Growth Regulation* 39: 608–630.

Jamshidi Goharrizi K, Baghizadeh A, Kalantar M and Fatehi F (2020c) Combined effects of salinity and drought on physiological and biochemical characteristics of pistachio rootstocks. *Scientia Horticulturae* 261: 108970.

Jamshidi Goharrizi K, Moosavi SS, Amirmahani F, Salehi F and Nazari M (2020d) Assessment of changes in growth traits, oxidative stress parameters, and enzymatic and non-enzymatic antioxidant defense mechanisms in *Lepidium draba* plant under osmotic stress induced by polyethylene glycol. *Protoplasma* 257: 459–473.

Kandil A, Sharief A and Elokda M (2012) Germination and seedling characters of different wheat cultivars under salinity stress. *Journal of Basic & Applied Sciences* 8: 585–596.

Kaya Y, Jocic S and Miladinovic D (2012) Sunflower *Technological Innovations in Major World Oil Crops*, vol. 1. New York, NY: Springer, pp. 85–129.

Kumar S, Beena A, Awana M and Singh A (2017) Physiological, biochemical, epigenetic and molecular analyses of wheat (*Triticum aestivum*) genotypes with contrasting salt tolerance. *Frontiers in Plant Science* 8: 1151.

Lan J (1998) Comparison of evaluating methods for agronomic drought resistance in crops. *Acta Agriculturae Boreali-Occidentalis Sinica* 7: 85–87.

Lekshmy S and Jha S (2017) Selection of reference genes suitable for qRT-PCR expression profiling of biotic stress, nutrient deficiency and plant hormone responsive genes in bread wheat. *Indian Journal of Plant Physiology* 22: 101–106.

Lin CC and Kao CH (1999) Nacl induced changes in ionically bound peroxidase activity in roots of rice seedlings. *Plant and Soil* 216: 147.

Lutts S, Kinet J and Bouharmont J (1996) NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Annals of Botany* 78: 389–398.

Ma L, Zhou E, Gao L, Mao X, Zhou R and Jia J (2008) Isolation, expression analysis and chromosomal location of P5CR gene in common wheat (*Triticum aestivum* L.). *South African Journal of Botany* 74: 705–712.

Meir S, Philosoph-Hadas S and Aharoni N (1992) Ethylene-increased accumulation of fluorescent lipid-peroxidation products detected during senescence of parsley by a newly developed method. *Journal of the American Society for Horticultural science* 117: 128–132.

Munns R and Filmer M (2007) Paving the way for salt-tolerant wheat. *Farming Ahead* 186: 50–53.

Munns R and James RA (2003) Screening methods for salinity tolerance: a case study with tetraploid wheat. *Plant and Soil* 253: 201–218.

Munns R and Tester M (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Biology* 59: 651–681.

Munns R, Hare R, James R and Rebetzke G (2000) Genetic variation for improving the salt tolerance of durum wheat. *Australian Journal of Agricultural Research* 51: 69–74.

Nakano Y and Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology* 22: 867–880.

Nazari M, Goharrizi KJ, Moosavi SS and Maleki M (2019) Expression changes in the TaNAC2 and TaNAC69-1 transcription factors in drought stress tolerant and susceptible accessions of *Triticum boeoticum*. *Plant Genetic Resources: Characterization and Utilization* 17: 471–479.

Paolacci AR, Tanzarella OA, Porceddu E and Ciaffi M (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. *BMC Molecular Biology* 10: 11.

Parida AK and Das AB (2005) Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety* 60: 324–349.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* 29: e45–e45.

Pfaffl MW, Horgan GW and Dempfle L (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* 30: e36–e36.

Qasim M, Ashraf M, Ashraf M, Rehman S-U and Rha E (2003) Salt-induced changes in two canola cultivars differing in salt tolerance. *Biologia Plantarum* 46: 629–632.

Roy SJ, Negrão S and Tester M (2014) Salt resistant crop plants. *Current opinion in Biotechnology* 26: 115–124.

Rozen S and Skaletsky H (2000) *Primer3 on the WWW for General Users and for Biologist Programmers Bioinformatics Methods and Protocols*. Totowa, NJ: Humana Press, pp. 365–386.

Sairam R and Srivastava G (2002) Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. *Plant Science* 162: 897–904.

Sairam R and Tyagi A (2004) Physiology and molecular biology of salinity stress tolerance in plants. *Current Science* 10: 407–421.

Sairam RK, Rao KV and Srivastava G (2002) Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. *Plant Science* 163: 1037–1046.

Sharma R (2015) Genotypic response to salt stress: I–relative tolerance of certain wheat cultivars to salinity. *Advances in Crop Science and Technology* 3: 192.

Singh A, Bhushan B, Gaikwad K, Yadav O, Kumar S and Rai R (2015) Induced defence responses of contrasting bread wheat genotypes under differential salt stress imposition.

Tajbakhsh M, Zhou M, Chen Z and Mendham N (2006) Physiological and cytological response of salt-tolerant and non-tolerant barley to salinity during germination and early growth. *Australian Journal of Experimental Agriculture* 46: 555–562.

Tammam AA, Alhamd MA and Hemeda MM (2008) Study of salt tolerance in wheat (*Triticum aestium* L.) cultivar Banysoif 1. *Australian Journal of Crop Science* 1: 115–125.

Tenea GN, Bota AP, Raposo FC and Maquet A (2011) Reference genes for gene expression studies in wheat flag leaves grown under different farming conditions. *BMC Research Notes* 4: 373.

Tounsi S, Feki K, Hmidi D, Masmoudi K and Brini F (2017) Salt stress reveals differential physiological, biochemical and molecular responses in *T. monococcum* and *T. durum* wheat

genotypes. *Physiology and Molecular Biology of Plants* 23: 517–528.

Velikova V, Yordanov I and Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science* 151: 59–66.

Wardlaw IF and Willenbrink J (1994) Carbohydrate storage and mobilisation by the culm of wheat between heading and grain maturity: the relation to sucrose synthase and sucrose-phosphate synthase. *Functional Plant Biology* 21: 255–271.

Yoshiba Y, Kiyosue T, Katagiri T, Ueda H, Mizoguchi T, Yamaguchi-Shinozaki K, Wada K, Harada Y and Shinozaki K (1995) Correlation between the induction of a gene for Δ1-pyrroline-5-carboxylate synthetase and the accumulation of proline in Arabidopsis thaliana under osmotic stress. *The Plant Journal* 7: 751–760.

Zhan Y, Wu Q, Chen Y, Tang M, Sun C, Sun J and Yu C (2019) Comparative proteomic analysis of okra (*Abelmoschus esculentus* L.) seedlings under salt stress. *BMC genomics* 20: 381.