Physiological and Ascorbate-Glutathione pathway-related genes responses under drought and heat stress in crested wheatgrass

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**ABSTRACT**

Drought and heat stress are two factors that limit the growth of cool-season plant species in many regions of the world. The objective of this experiment was to study the expression patterns of genes involved in ascorbate and glutathione pathways, while assessing the physiological responses of plants and their tolerance to drought stress and heat stress. The results of this study indicated that there were variations in the plants’ tolerance to drought and heat among the crested wheatgrass genotypes. Based on the real time-PCR results genes involved in the biosynthesis (GalLDH and γ-ECS) and recycling (APX, GR, DHAR, MDHAR) of ascorbate and glutathione, also DREBs were significantly affected by stress. The expression of the DREB2 gene increased substantially in all genotypes under drought and heat stress, which was also associated with high levels of expression genes involved in the Asc-Glu pathway. Based on our results, it seems that all of the genes involved in the Asc-Glu pathway probably had the DRE/CRT element in their promoter region for the DREB2 gene. Sequencing the DREB2 gene showed that leaky mutations occurred in two genotypes collected from cold and wet regions. As a result, the DREB2 probably cannot bind with the dehydration-responsive elements (DRE/CRT, as a cis-acting element) and, although the expression of the DREB2 gene was increased, there was no significant change in the level of expression genes involved in the Asc-Glu pathway. Based on physiological analyses, the ranking of the genotypes’ tolerance to drought would appear as ‘AC3’ > ‘AC5’ > ‘AC6’ > ‘AC1’ > ‘AC2’ > ‘AC4’ and the ranking of tolerance to heat stress would be ‘AC5’ > ‘AC1’ > ‘AC6’ > ‘AC4’ > ‘AC2’ > ‘AC3’. Finally, our results indicated that tolerance to drought and heat associated positively with the expression of genes involved in the Asc-Glu pathway and natural habitat of genotypes. It was also found that the DREB2 plays a key role in regulating the expression of genes involved in the Asc-Glu pathway.

1. Introduction

Heat stress and drought stress are two major environmental factors that can limit the management of turfgrass in semi-arid and arid regions during summer months (Du et al., 2013; Li et al., 2008). Models pertaining to the prediction of weather demonstrate that some regions in the world may be facing reduced amounts of rainfall due to the increase in average global temperatures, which could probably be associated with an increase in the incidence and persistence of drought periods in the coming years (Parrota et al., 2016; Xu and Zhou, 2006). Heat stress and drought stress cause oxidative damage to plant cells through increased production of reactive oxygen species (ROS) including hydrogen peroxide (H₂O₂), singlet oxygen (¹O₂) and hydroxyl radical (OH⁻) (Du et al., 2013). The balance between the production and removal of ROS may be disturbed by environmental stresses (Koca et al., 2007). To cope with reactive oxygen species during stress, the turfgrass has evolved an effective antioxidant defense system that includes both enzymatic and non-enzymatic antioxidants (Etemadi et al., 2015).
The ascorbate-glutathione cycle (Asc-Glu pathway) is involved extensively in the defense against oxidative stress (Neto et al., 2005). In the Asc-Glu pathway, ascorbate and glutathione are two crucial non-enzymatic compounds that can be recycled while H$_2$O$_2$ is scavenged. It is known that ascorbate and glutathione are constantly regenerated and biosynthesized in plants in order to scavenge reactive oxygen species (ROS) (Shan and Liang, 2010). L-galactono-1, 4-lactone dehydrogenase (GalLDH) and Gamma-glutamylcysteine synthetase (γ-ECS) are key enzymes for ascorbate and glutathione biosynthesis, respectively (Dringen, 2000; Wheeler et al., 1998). The enzymes of the Asc-Glu pathway comprise monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) as well as ascorbate peroxidase (APX) (Noctor and Foyer, 1998).

Transcription factors (TFs) are commonly known among regulators in all organisms. They play key roles in the regulation of gene expression, especially under stress conditions (Hassan et al., 2015; Nakashima et al., 2009). Dehydration-responsive element binding proteins (DREBs) constitute a large family of TFs that are involved in the transduction pathway of plant stress signaling. They can exclusively bind to dehydration-responsive elements (DRE/CRT, as a cis-acting element) and activate the expression of many stress-inducible genes (Rehman and Mahmood, 2015). Also, various studies have shown that the overexpression of the DREB gene in different plants caused the increase in tolerance to abiotic stress such as drought, heat, and salt (Liu et al., 2015).

Studies in recent years have shown that there are several methods of improving turfgrass’ tolerance to heat and drought. The use of species and genotypes that exhibit a stronger tolerance to heat and drought can be an important way to facilitate turfgrass management (Du et al., 2015). The control plants were situated in pots and the moisture of the soil was kept within the optimum temperatures for the growth of cool-season grasses. The control plants were kept at optimal temperature conditions (22 °C day / 18 °C night) and were watered daily. These temperatures are within the optimum temperatures for the growth of cool-season grasses. The control plants were subjected to 35 / 30 °C (day / night) and were watered daily. The plants were maintained at optimal temperature conditions (22 °C day / 18 °C night) and were not watered. Water stress was initiated by withholding water from the soil for 24 days. The third treatment was to apply heat stress. The plants were subjected to 35 / 30 °C (day / night) and were watered daily. Plants were situate in soils with a moisture of 60–80% of water holding capacity throughout the experiment (Abraham et al., 2008; Larkindale and Huang, 2004).

2.3. Measurements

Physiological and biochemical properties of the six Iranian crested wheatgrass genotypes were evaluated in response to drought stress and heat stress after 0, 8, 16, 24 days by measuring hydrogen peroxide (H$_2$O$_2$) content, malondialdehyde (MDA) content, electrolyte leakage (EL), relative water content (RWC), proline content, total nonstructural carbohydrates (TNC) content, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD) and turf quality.

2.3.1. Hydrogen peroxide contents (H$_2$O$_2$) and Malondialdehyde content (MDA)

Samples of Agropyron genotypes were ground finely by using liquid N$_2$ in ice-chilled mortar and pestle and were homogenized in 4% ice-cold trichloro acetic acid. The homogenate was then centrifuged at 15,000g for 15 min at 5 °C. The supernatant was used for the calculation of hydrogen peroxide content (H$_2$O$_2$; Guo et al., 2006) and malondialdehyde content (MDA; Heath and Parker, 1968).

2.3.2. Relative Water Content (RWC) and Electrolyte Leakage (EL)

The amount of water in leaves was determined by measuring the relative water content (RWC) calculated by the following formula. RWC (%) = (FW-DW)/(TW-DW) x 100, where FW is the leaf fresh weight, DW is leaf dry weight for tissues dried at 80 °C for 4 d, and TW is the turgid weight of leaves after being soaked in water for 4 h at 20 °C
maximal conductance of the dead tissue (Cmax) was calculated (Blum meter). The leaf samples were then killed at 140 °C for 20 min, and the calculated after the leaves were shaken for 24 h by using a conductivity (%) = 100 × Ci/Cmax. The conductivity of the solution (Ci) was calculated as EL (Barrs and Weatherley, 1962). Cell membrane stability was determined by Bates et al. (1973) with some modifications. Samples of 0.6 g were homogenized in 10 mL of 4% sulfosalicylic acid and then were centrifuged at 19,000g for 15 min. The supernatant was treated with 2.0 mL of acid ninhydrin dissolved in glacial acetic acid and boiled for 1 h. Absorbance was recorded at 520 nm with a calibration curve. The amount of Total Nonstructural Carbohydrates (TNC) was assayed according to the protocol of Fry et al. (1993) with slight modifications and was expressed as 0.2 g samples of Agropyron genotypes. Absorbance of the solution was read at 515 nm and was compared with a standard curve to determine the TNC content.

2.3.3. Proline content and total nonstructural carbohydrates (TNC) content
Proline content was determined according to the method described by Bates et al. (1973) with some modifications. Samples of 0.6 g were homogenized in 10 mL of 4% sulfosalicylic acid and then were centrifuged at 19,000g for 15 min. The supernatant was treated with 2.0 mL of acid ninhydrin dissolved in glacial acetic acid and boiled for 1 h. Absorbance was recorded at 520 nm with a calibration curve. The amount of Total Nonstructural Carbohydrates (TNC) was assayed according to the protocol of Fry et al. (1993) with slight modifications and was expressed as 0.2 g samples of Agropyron genotypes. Absorbance of the solution was read at 515 nm and was compared with a standard curve to determine the TNC content.

2.3.4. Antioxidant enzymes
Initially, 0.4 g sample of each Agropyron genotype was ground in 5 mL of 50 mM phosphate-buffer (pH 7.6) at 5 °C which was then centrifuged at 14,000g for 14 min. The supernatant was gathered for assays of enzyme activities. Extraction and assays of different antioxidant enzymes, ascorbate peroxidase activity (APX), superoxide dismutase activity (SOD), catalase activity (CAT) and peroxidase activity (POD) were performed by using the methods described by Chen et al. (2009) and Han et al. (2008).

2.3.5. Turf quality
Turf quality was rated on a 1-to-9 scale (NTEP) according to the canopy color, tissue, density and uniformity, where 0 represented a brown, absolutely dried necrotic turf covering, whereas the value 6 represented an acceptable quality for a home lawn, and the value 9 meant an optimum color, density and uniformity.

2.3.6. RNA preparation and cDNA synthesis
Total RNA was extracted from the leaves of wheatgrass by using the TRI reagent (Sigma) following the manufacturer’s recommendations. To remove genomic DNA contaminants and to purify the RNA, a digestion by RNase-free DNase (Qiagen) was performed on the RNA samples. The RNA concentration was determined by spectrophotometry at 260 nm using the NanoDrop machine. With SuperScript III reverse transcriptase (Invitrogen) and oligo (dT) 20 (Invitrogen), the first-strand complementary DNA (cDNA) were produced from 300 ng of total purified RNA following the manufacturer’s instructions. The quality and quantity of cDNA were evaluated by Nano Dot ND-3800.

2.3.7. Real-time PCR
Primers of DHAR, APX, and GR genes were synthesized based on previous reports (Shan and Liang, 2010) to be used in Real-time PCR (Table 2) and primers of DREB2, GaLDH, γ-ECS and MDHAR were designed with the assistance of the Primer3 software v 0.9. The specificity of each pair of primers was also validated by sequencing the PCR products randomly. In order to prevent the amplification of other DREB gene families, the DREB2 gene sequence was aligned with another DREB2 gene family in other plant species, and the primer was designed from non-conserved regions. Primers with amplification efficiencies of more than 95% were selected to be used in this study. In order to avoid errors, the real-time RT-PCR was typically normalized against the β-Actin housekeeping gene (Table 2).

The real-time PCR was carried out using a 7500 Real-Time PCR System (Applied Biosystems, CA, USA). Briefly, the multiplex PCR reaction master mix was prepared with 200 ng of cDNA, 300 nM each of forward and reverse primers, 10 µL SYBR green fast Ready Mix (Kapa Biosystems) and sterile distilled water were added so that the total volume reached 20 µL. The amplifications were conducted with an initial incubation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s (denaturation) and 53–58 °C for 30 s (annealing/extension) and eventually the process was terminated with an incubation at 72 °C for 10 min. Melting curve analysis was performed to check the presence of non-specific PCR products or the absence of primer dimers. The relative gene expression level was computed by using the Ct or 2−ΔΔCt method. Three biological replicates were carried out for each sample, and three technical replicates were produced for each biological replication. The data were statistically analyzed by the randomized block design (RBD) method via the SAS software program version 9.1 (SAS institute, Cary, NC, USA).

2.3.8. Cloning, sequencing and analysis of PCR products
The DREB2 gene of genotypes was amplified by PCR. The primer used for it is listed in Table 3. PCR products were purified by using the QIAEXII gel purification kits (Qiagen), according to the manufacturer’s instructions. They were cloned in ‘PCR II TOPO Vector’ included in the TA TOPO Cloning kit (Invitrogen), according to the manufacturer’s instructions. Plasmid DNA was sequenced by using the Big Dye® Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Samples were analyzed by an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The results obtained herein from the sequencing were compared with the DREB2 gene in the NCBI gene bank.

2.4. Data analysis
The experiment consisted of two factors (six genotypes and three treatments) with four replications for each genotype or treatment in a split plot design with treatments as main plots and genotypes as sub-plots. All pots were randomized within the growth chamber. Statistical significance was tested using the analysis of variance procedure in SAS 9.1 (SAS Institute Inc., Cary, NC). Differences between the mean values were determined by using the Fisher’s protected LSD test at the 5% probability level.
3. Results

3.1. Physiological responses of Iranian crested wheatgrass genotypes under drought stress and heat stress

Under drought and heat stresses, H2O2 production in all genotypes increased and the rate of increase differed between genotypes (Fig. 1). In this study, ‘AC3’ and ‘AC6’ under drought stress and ‘AC1’ and ‘AC5’ under heat stress had lower H2O2 contents compared to other genotypes (Fig. 1). During the experiment, the control plants did not show significant differences in MDA content (Fig. 1). By prolonging the durations of drought and heat, MDA production in all genotypes increased and the rate of increase differed between genotypes (Fig. 1). ‘AC3’ and ‘AC6’ under drought stress and ‘AC1’ and ‘AC5’ under heat stress had lower MDA contents than other genotypes during the experiment (Fig. 1).

After 8, 16 and 24 days of drought and heat treatments, the EL increased in all genotypes and the rate of increase differed among genotypes (Fig. 1). In this experiment, the ‘AC3’ and ‘AC6’ genotypes under drought stress and ‘AC1’ and ‘AC5’ under heat stress had lower EL compared to other genotypes (Fig. 1). RWC content of ‘AC3’ and ‘AC6’ under drought stress and ‘AC1’ and ‘AC5’ under heat stress were higher than other genotypes in this experiment (Fig. 2).

Results showed that the proline content in ‘AC3’ and ‘AC5’ genotypes increased by prolonging the drought period (8, 16 and 24 days of water-deficit treatments) (Fig. 2). During the experiment, no genotype under heat stress showed substantial differences in proline content (Fig. 2). The results showed that the TNC content increased in ‘AC3’ during drought stress, and likewise in ‘AC1’ and ‘AC5’ during heat stress at 8, 16 and 24 days of the stress treatments (Fig. 2). The results indicated that on the eighth and twenty-fourth day of the experiment, the TNC content increased in ‘AC6’ during drought stress (Fig. 2). After 16 and 24 days of stress treatments, the TNC content increased in ‘AC5’ under drought stress and in ‘AC6’ under heat stress (Fig. 2).

3.2. Antioxidant enzyme activities of Iranian crested wheatgrass genotypes under drought stress and heat stress

The results imply that SOD activity increased in the ‘AC3’ under drought stress, which also happened for ‘AC1’ under heat stress after 8, 16 and 24 days of treatments (Fig. 3). The SOD enzyme’s activity increased in ‘AC1’, ‘AC3’ and ‘AC6’ under drought stress, while it increased in ‘AC5’ under heat stress by prolonging the drought stress period (considering 8 and 16 days of water-deficit treatments). The present results showed that after 8, 16 and 24 days of water-deficit treatments, the activity of CAT enzyme increased in ‘AC3’ and ‘AC6’ during drought stress (Fig. 3). The CAT enzyme activity increased in ‘AC3’ and ‘AC5’ under heat stress when the value was measured on the sixteenth and twenty-fourth day of the experiment (Fig. 3).

Our results suggest that on the eighth, sixteenth and twenty-fourth day of drought treatments, the activity of APX increased in ‘AC3’ (Fig. 3). Heat stress increased enzymatic activity of APX in ‘AC1’ within 24 days of heat treatments (Fig. 3). After 8, 16 and 24 days of the experiment, the POD enzyme activity increased in ‘AC6’ and ‘AC1’ during drought and heat stress respectively (Fig. 3).

3.3. Turf quality of Iranian crested wheatgrass genotypes under drought stress and heat stress

Both drought and heat treatments caused a decline in turf quality compared to the control plants during the research and the rate of decline was different between genotypes (Fig. 4). The ‘AC3’ genotypes under drought stress and ‘AC1’ and ‘AC5’ genotypes under heat stress had significantly higher turf quality than other genotypes during the experiment and maintained acceptable turf quality (7.0 or higher) within 8 and 16 days of drought and heat treatments. However, the turf quality fell below the acceptable quality after 24 days of the treatment (Fig. 4).

3.4. Ascorbate Glutathione pathway and DREB genes expression profiles in Iranian crested wheatgrass genotypes under drought stress and heat stress

The study of GalLDH, APX, γ-ECS, GR, DHAR, MDHAR and DREB2 genes demonstrated that drought and heat stresses had significant effects on expression of these genes, whereas their expression had various patterns in different genotypes of the crested wheatgrass under those stresses (Figs. 5 and 6; Supplementary Figs. 1 and 2). Our results showed that the expression of genes for the Asc-Glu pathway in some of
the genotypes ('AC1', 'AC3', and 'AC5') were gradually up-regulated during drought stress (Fig. 5, Supplementary Fig. 1), whereas the expression of these genes in other genotypes ('AC2', 'AC4', and 'AC6') increased parallel to prolonging the period of drought stress (moderate drought stress). Subsequently, the expression of genes significantly decreased after 24 days of drought (Fig. 5, Supplementary Fig. 1). On the other hand, the 'AC3' genotype exhibited significantly higher expression levels of genes for the Asc-Glu pathway compared to other genotypes under drought stress (Fig. 5, Supplementary Fig. 1). Among the different genes mentioned above, by comparing the conditions before and after drought stress, the highest level (about 5.1 times) and the lowest level (about 0.57 times) of genes expression were observed in the γ-ECS gene (after 24 days of drought stress) and in the GalLDH gene (before drought stress), respectively (Fig. 5, Supplementary Fig. 1).

The results of sequencing the DREB2 gene showed that replacement mutations occurred in two genotypes collected from cold and wet regions. Based on the results of sequencing the DREB2 gene, leaky mutations occurred at nucleotides 486 (C instead of G) and 529 (C instead of T) in the 'AC2' and 'AC4', respectively (Fig. 8). The results obtained from nucleotides translated into the amino acid sequences in 'AC2' and 'AC4' genotypes (http://web.expasy.org/translate/) revealed that leaky mutations changed the type of amino acids compared to the basic sequence of DREB2 in NCBI (http://www.ncbi.nlm.nih.gov/) (Fig. 9).

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4. Discussion

4.1. Physiological responses of Iranian crested wheatgrass genotypes under drought stress and heat stress

Drought and excessive heat are considered as two of the most common and destructive environmental stresses for turfgrass (Jiang and Huang, 2001). Grasses develop different mechanisms in order to survive in arid and semi-arid regions with high-temperature environments. These mechanisms that are developed for survival include physiological, biochemical and molecular adaptations along with metabolic defense mechanisms (Du et al., 2013; Peng et al., 2012).

Under normal ambient conditions, potentially reactive oxygen species (ROS) are produced at a low level and there is a suitable balance between the production and removal of ROS (Apel and Hirt, 2004; Sheikh-Mohamadi et al., 2017a). Environmental stresses cause reactive oxygen species (ROS) such as singlet oxygen (\(^{1}\text{O}_2\)), hydrogen peroxide (\(\text{H}_2\text{O}_2\)), and hydroxyl radicals (\(\text{OH}^*\)) to be accumulated in plants, which can cause serious damage to vital cellular components (Lu et al., 2009; Xu et al., 2006). This study indicated that ‘AC3’ and ‘AC6’ under drought stress and ‘AC1’ and ‘AC5’ under heat stress show a lower \(\text{H}_2\text{O}_2\) content. In previous studies, genotypes that maintained low levels of \(\text{H}_2\text{O}_2\) content had stronger resistance to environmental stress (Jiang and Huang, 2001; Soliman et al., 2012).

The structure and function of the cell membranes are among the main targets of many environmental stresses, and it is generally known that the maintenance of integrity and stability under stress conditions is essential for plant survival (Du et al., 2009). Damage to the cell membrane may be associated with oxidative damage as a result of lipid peroxidation (Koyro et al., 2013). Malondialdehyde (MDA) is one of the
final secondary end products by the lipid peroxidation of the cell membrane, described as an indicator of the extent to which oxidative damages occur to the cell membrane and lipid peroxidation. The MDA could be used as a physiological indicator for the discrimination of drought and heat-tolerant or drought- and heat-susceptible genotypes (Han et al., 2014). In this experiment, the ‘AC3’ and ‘AC6’ under drought stress and ‘AC1’ and ‘AC5’ under heat stress showed a lower MDA content than other genotypes. When exposed to abiotic stress, genotypes that were susceptible to drought and heat tended to accumulate more MDA than genotypes that were tolerant to drought and heat. This was reported in the tall fescue [Festuca arundinacea L. (Xu et al., 2013)], wheat [Triticum sp. (Abid et al., 2016)], Kentucky bluegrass [Poa pratensis L. (Du et al., 2013)] and Bermudagrass [Cynodon dactylon L. (Hu et al., 2012)]. The results of this research indicate that drought- and heat-tolerant genotypes could exhibit better protection against oxidative damage to plant cells.

Osmotic adjustment is an important physiological mechanism for the maintenance of water potential in plant cells during periods of environmental stress (Koca et al., 2007; Sharbatkhari et al., 2016). Plants maintain the osmotic equilibrium by the accumulation of osmoprotectants including proline and total nonstructural carbohydrates, which reduce the osmotic potential and maintain the cell turgor needed for plant growth (Liu et al., 2011; Sheikh Mohammadi et al., 2017b). The results of this study showed that the ‘AC3’ and ‘AC5’ had higher leaf proline content compared to other genotypes under drought stress. Proline content remained unchanged in all genotypes under heat stress. High proline levels under drought stress were in line with balancing the osmotic strength of cytosol, preserving protein structures, protecting the integrity of cellular membranes and having antioxidant protective roles in plants (Zhang et al., 2009).

TNC include water soluble carbohydrates (such as glucose, fructose and sucrose) and storage carbohydrates (such as starch and fructan)
TNC accumulation has been widely used as a physiological index related to the resistance against abiotic stress because nonstructural carbohydrates provide energy and solutes for osmotic adjustment and energy reserves (Fu and Dernoeden, 2008). In our study, higher levels of TNC contents were observed in 'AC3' under drought stress, and likewise observed in 'AC1', 'AC5' and 'AC6' under heat stress, compared to other genotypes throughout the experiment. There are many reports about TNC accumulation under heat stress (Liu and Huang, 2000), salinity stress and drought and heat stress (Liu and Huang, 2000). The results of some studies have shown that more TNC accumulated in drought- and heat-tolerant cultivars than in drought- or heat-susceptible cultivars (DaCosta and Huang, 2006).

The electrolyte leakage is an indicator of cell membrane stability or cellular injury. It has been widely used as an evaluation and selection of species or genotypes that are tolerant to temperature stress and drought stress (Wang et al., 2011). In general, an increased electrolyte leakage is considered as a sign of cell membrane deterioration in response to abiotic stress (Yu et al., 2015). Throughout the experiment, 'AC3' and 'AC6' genotypes under drought stress and 'AC1' and 'AC5' genotypes under heat stress had lower EL compared to other genotypes. The superiority of stress tolerant genotypes has been attributed to their greater cell membrane integrity and stability associated with the accumulation of saturated fatty acids and a stronger antioxidant defense activity (Du et al., 2013).

Leaf RWC indicates an effective index of the status of water balance in a plant (Fu and Huang, 2001). RWC is a parameter extensively used for the measurement of the internal water status during environmental stresses (Yu et al., 2015). RWC correlates well with stress intensity and provides the earliest data about the physiological response to water deficiencies (Liu et al., 2008). This study indicated that 'AC3' and 'AC6' have higher RWC contents under drought stress, while the 'AC1' and 'AC5' have higher RWC contents under heat stress, compared to other genotypes. The maintenance of leaf water status is necessary for the continuation of physiological and biochemical mechanisms under abiotic conditions (Bhushan et al., 2007). Previous studies have reported that grass species and genotypes can have a higher resistance to drought or heat stress and can exhibit higher cellular hydration under stress conditions by maintaining high levels of RWC content (Chai et al., 2010).

4.2. Antioxidant enzyme activities of Iranian crested wheatgrass genotypes under drought stress and heat stress

Under optimal growth conditions, the balance between ROS production and scavenging is tightly controlled by an array of the antioxidant defense systems to reduce oxidative stress (Luna et al., 2005; Wahid et al., 2007). The production of ROS such as hydrogen peroxide ($H_2O_2$), induced by drought or heat stress, and suppressed antioxidant activities can lead to lipid peroxidation and oxidative damage (Du et al., 2013). To minimize and eliminate oxidative injury, plants are equipped with an antioxidant defense system comprising enzymatic antioxidant defense systems such as SOD, CAT, POD, APX and other non-enzymatic antioxidant defense systems such as GSH, whereby the antioxidants remove, neutralize and scavenge the ROS at different cellular locations.
POD enzyme activities were not observed in the
Drought and heat stress increased the POD enzyme activity in
antioxidant enzymes that can eliminate H2O2 toxicity, which converts
stress treatments. CAT is known as one of the most powerful plant
activity in
2007). In this experiment, drought stress increased the CAT enzyme
activity of heat treatments. POD is among the major enzymatic antioxidants
under heat stress after 8, 16 and 24 days of water-de.
activity in
2012). The damage caused by oxidative stress, induced
H2O2 to water (H2O) and molecular oxygen (O2) (Luna et al., 2005;
Sekmen et al., 2012). The damage caused by oxidative stress, induced
by H2O2, occurs when the hydroxyl radical is produced, which is very
dangerous for the cell, the DNA structure and function (Maricle et al.,
2007). In this experiment, drought stress increased the CAT enzyme
activity in ‘AC3’ under drought stress, and it also
increased in ‘AC1’ under heat stress after 8, 16 and 24 days following
stress treatments. The activities of CAT enzymes increased in ‘AC3’ and ‘AC5’
heat stress after 16 and 24 days of the experiment as compared to
other genotypes. APX is a very important antioxidant enzyme for con-
trolling ROS levels in the ascorbate-glutathione cycle. The enzyme APX
transforms H2O2 to H2O and O2 (Cruz and Carvalho, 2008; Sekmen et al.,
2012). After 8, 16 and 24 days of water-deficit treatments, the activity of the APX enzyme increased in ‘AC3’ under drought stress. Heat stress increased the APX enzyme activity in ‘AC1’ within 24 days of stress treatments. POD is among the major enzymatic antioxidants that scavenge H2O2 in chloroplasts, while the H2O2 is produced through the dismutation of O2+ – catalyzed by SOD (Sekmen et al., 2012). Drought and heat stress increased the POD enzyme activity in ‘AC6’ and ‘AC1’ within 8, 16 and 24 days of stress treatments respectively. Higher POD enzyme activities were not observed in the ‘AC1’, ‘AC2’ and ‘AC5’ under drought stress and likewise not observed in the ‘AC1’ under heat stress. Maintaining a high capacity of activities of antioxidant enzymes (SOD, APX, CAT, and POD) may contribute to the plants’ tolerance to heat and drought by improving the capacity of protection and a better cellular defense mechanism against oxidative injury (Du et al., 2013). However, changes in antioxidant enzyme activities under environmental stress depend on plant species, genotypes, the duration and intensity of the stress (Du et al., 2009; Xu et al., 2013). A more effective activity of antioxidative enzymes can contribute to the development of plants that are tolerant to abiotic stress. Such cases have also been re-
ported in several higher plants that are tolerant to drought and heat
stress (Maricle and Adler, 2011; Du et al., 2013; Rai et al., 2011; Walter et al., 2013). The results of antioxidant enzymes analysis showed that the content of antioxidant enzymes under direct stress conditions was closely related to the climatic conditions in the site from which the plant was collected (Table 1). Accordingly, the amount of antioxidant enzymes in plants collected from hot areas (AC1, AC5, AC6) under thermal stress conditions was significantly higher than those collected from cold regions (AC2, AC3, AC4). The amount of antioxidant enzymes in plants that were collected from arid regions (AC3, AC5, AC6) under conditions of drought stress was significantly higher than those collected from wet areas (AC2, AC4). Climatic changes often result in several effects because of the environmental stress on plants in their natural habitats. As a result of the response to stress, the accumulation of several phenolic compounds as “multifunctional” antioxidants can occur in different plant species (Sárosi et al., 2010). Our results are similar to those reported by Fang et al (2017) which showed that the tolerance to various forms of stress is related to habitat. Furthermore, our results are in line with previous research that showed how the differences in antioxidant levels can correlate with the habitat and conditions in which the genotypes naturally exist (Stanković et al.,

Fig. 6. Effect of heat stress on the expression of genes involved in the Asc-Glu pathway (GalDH and γ-ECS) in crested wheatgrass genotypes.

Fig. 7. Effects of drought stress and heat stress on the expression of the DREB gene in crested wheatgrass genotypes.
and wet regions (pathway under drought stress and heat stress is probably related to the found that the regulation of gene expression involved in the Asc-Glu pathway. Based on our results, we
other hand, the intensity of gene expression involved in the Asc-Glu pathway was higher in genotypes collected from
involved in the Asc-Glu pathway varied when either drought stress or heat stress a
involved in the Asc-Glu pathway probably had the DRE/CRT element (G/ACCGAC)
the Asc-Glu pathway and the content of antioxidant enzymes. Finally, we found that the habitats from which the genotypes were collected could affect the expression of genes that are involved in the Asc-Glu pathway and the content of antioxidant enzymes.
In the present study, we observed that the expression of DREB2 gene increased substantially in all of the genotypes under drought stress and heat stress. Also, we found that the level of gene expression involved in the Asc-Glu pathway was associated with the expression of DREB2. Based on our results, it seems that all of the measured genes involved in the Asc-Glu pathway probably had the DRE/CRT element (G/ACCGAC) as cis acting as a regulatory element in their promoter region for DREB2. Pandey et al (2015) identified the DRE/CRT in the regulatory regions of the MDHAR, DHAR, APX and GR genes pertaining to the Asc-Glu pathway by in silico analysis. Results from the present study supported those reported by Pandey et al (2015). Furthermore, we observed that the DREB2 expression increased in tolerant genotypes more than it did in other genotypes during drought stress and heat stress. Therefore, it seems that the tolerance to drought stress and heat stress is probably associated with the expression of DREB2 in the genotypes of crested wheatgrass in this research.

Fig. 8. Position of leaky mutations in ‘AC2’ and ‘AC4’ genotypes. A1, B1) Gene sequence of DREB2 in NCBI. A2, B2) sequenced part of DREB2 gene in ‘AC2’ and ‘AC4’ genotypes, respectively.

2017; Sárosi et al., 2010).

4.3. Turf quality of Iranian crested wheatgrass genotypes under drought stress and heat stress

When considering research on turfgrass and the environment, turf quality is investigated to evaluate the effects of stress on grass species and genotype. In our experiment, turf quality of all genotypes was maintained at a high level in the control group. In our study, the ‘AC3’ under drought stress and ‘AC1’ and ‘AC5’ under heat stress had significantly higher turf quality than other genotypes throughout the stress treatments. Plants that can maintain high levels of turf quality for a longer period of time under drought and heat stress are indicative of stronger resistance to drought and heat stress (Abraham et al., 2008; Du et al., 2009; Jiang and Huang, 2001).

4.4. Ascorbate Glutathione pathway and DREB genes expression profiles in Iranian crested wheatgrass genotypes under drought stress and heat stress

The results showed that the regulation of gene expression which was involved in the Asc-Glu pathway varied when either drought stress or heat stress affected the situation. Accordingly, our results indicated that the level of gene expression in drought stress was higher than that of the heat stress. On the one hand, we found that the level of gene expression involved in the Asc-Glu pathway was higher in genotypes collected from warm regions (‘AC5’ > ‘AC1’ > ‘AC6’) under heat stress. On the other hand, the intensity of gene expression involved in the Asc-Glu pathway under drought stress was higher in genotypes collected from arid regions (‘AC3’ < ‘AC5’ > ‘AC6’). Genotypes collected from cold and wet regions (‘AC2’ and ‘AC4’) had the lowest level of gene expression involved in the Asc-Glu pathway. Based on our results, we found that the regulation of gene expression involved in the Asc-Glu pathway under drought stress and heat stress is probably related to the natural habitat of genotypes. In this same way, our results showed that the genotypes which were collected from arid regions and warm regions showed stronger tolerance to drought and heat than the other genotypes which were collected from wet regions and cold regions under drought stress and heat stress. Generally, we found more tolerance to drought stress and heat stress in the genotypes of the crested wheatgrass which was positively associated with the gene expression involved in the Asc-Glu pathway and with the natural habitats of the genotypes. The results of gene expression in this study was similar to the results of our results on antioxidant enzymes. We found that genotypes which were collected from arid regions and warm regions showed a stronger tolerance to drought stress and heat stress (respectively) which can be linked to the increase in the expression of genes involved in the Asc-Glu pathway and the content of antioxidant enzymes. Finally, we found that the habitats from which the genotypes were collected could affect the expression of genes that are involved in the Asc-Glu pathway and the content of antioxidant enzymes.

In this study, we observed that the expression of DREB2 gene content of antioxidant enzymes.

Fig. 9. Position of amino acid sequence of DREB2 in crested wheatgrass. A1, B1) Part of amino acid sequence of DREB2 protein in crested wheatgrass. A2, B2) Part of amino acid sequence of DREB2 protein in ‘AC2’ and ‘AC4’ genotypes, respectively. Position of leaky mutations is shown by the yellow color in ‘AC2’ and ‘AC4’ genotypes (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
Based on the results of sequencing the DREB2 gene, we found that a
leaky mutation in the DREB2 gene can explain the reduced expression
of genes involved in the Asc-Glu pathway in ‘AC2’ and ‘AC4’ genotypes.
This leaky mutation may have resulted in the synthesis of a dysfunc-
tional protein lacking normal activity. The obtained results of nucleo-
tides translated into the amino acid sequences demonstrated that
changes in the type of amino acids in the DREB2 protein can alter the
function of the protein and can lead to the synthesis of a nonfunctional
DREB2 in ‘AC2’ and ‘AC4’ genotypes.

Several studies revealed that DREB2A proteins have the highest
binding affinity for ACCGAC, but there are reports on differences in the
binding specificity in some other plants (Sakuma et al., 2006a, b). Based
on the results of previous studies on grasses, the DREB2 transcripts have
key roles in controlling the drought and heat responses via alternate
splicing which may allow quick responses to stress. The expression of
stress-inducible DREB2 genes in some crops have been examined under
diverse abiotic stresses (Mizoi et al., 2012).

Parallel to our results, DREB2 genes were previously reported to be
significantly affected by drought, heat and salt treatments (Mizoi et al.,
2012). On the other hand, DREB2 genes are responsive to drought and
heat-induced stresses (Matsukura et al., 2010; Mizoi et al., 2012).
Collectively, our results indicate that DREB2 genes can play noteworthy
roles in developing new genotypes of the crested wheatgrass that can be
tolerant to drought or heat, and leaky mutations can lead to the survival
and dispersal of a species in different climatic conditions.

5. Conclusions

In summary, the majority of molecular, physiological and bio-
chemical parameters evaluated herein suggested that of the six crested
wheatgrass genotypes examined in this study, there was one genotype
with a better ability to survive drought stress and two genotypes with
better abilities to survive heat stress, which can be used in dry and
warm climates. Our results showed that drought and heat stress reduced
turfgrass quality, and the level of decline was different among geno-
types. The ‘AC3’ exhibited the best turf quality and a better tolerance to
drought stress, while the ‘AC5’ and ‘AC1’ yielded more promising re-
results than other genotypes under heat stress. A slower decrease in RWC
and also a slower increase in MDA and EL were observed in ‘AC3’
during the drought-stress period. The same was observed in the ‘AC5’
and ‘AC1’ during the heat-stress period, suggesting their greater toler-
ance to drought and heat and their greater potential for improving such
tolerance in breeding programs. Our results demonstrated that the su-
perior drought tolerance in ‘AC3’ and heat tolerance in ‘AC5’ and ‘AC1’
– as compared to other genotypes – are associated with a more efficient
osmotic adjustment (increased proline and TNC content). A greater
oxidative scavenging capacity resulted through the maintenance of
higher enzymatic antioxidant activity. Based on biochemical and phy-
siological analysis for drought and heat-tolerance, a substantial range of
drought and heat tolerance was observed among the Iranian crested
wheatgrass genotypes. The ‘AC3’ was drought-tolerant, ‘AC5’ and ‘AC6’
were moderately drought tolerant, while ‘AC1’, ‘AC2’ and ‘AC4’ were
susceptible to drought. Furthermore, ‘AC5’ and ‘AC1’ were heat-tol-
erant, ‘AC6’ was moderately heat-tolerant, while ‘AC4’, ‘AC2’ and ‘AC3’
were susceptible to heat. It was observed that the variations in the
expression of genes involved in the Asc-Glu pathway in various geno-
types under drought stress and heat stress may be due to the various
habitats in which the genotypes are naturally found. Similarly, it seems
that the leaky mutation in DREB2 was probably caused by different cli-
matic conditions which, in turn, cause changes to the protein function
in various genotypes. Generally, our results showed that genotypes
collected from arid and warm regions show greater tolerance to drought
and heat, compared to genotypes collected from cold and wet regions.
Results indicated that more tolerance to drought stress and heat stress
in some of the genotypes of crested wheatgrass are positively associated
with gene expression involved in the Asc-Glu pathway and with the
natural habitat of the genotypes. Also, the DREB2 protein regulates
gene expression involved in the Asc-Glu pathway. Based on the traits
measured in this study, it may be suggested that ‘AC3’ is the genotype
that is most tolerant to drought, while the ‘AC5’ and ‘AC1’ are most
tolerant to heat stress.

Conflict of interest

Authors declare there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

References

Improved tolerance to post-anthesis drought stress by pre-drought priming at vege-
tative stages in drought-tolerant and -sensitive wheat cultivars. Plant. Physiol.

hybrid bluegrass and Kentucky bluegrass to drought and heat stress. HortScience 43,
2191–2195.


Barry, H.D., Weatherley, P.E., 1962. A re-examination of the relative turbidity techniques


Bayat, H., Nemati, H., Tehranifar, A., Gazanchian, A., 2016. Screening different crested
wheatgrass (Agropyron cristatum (L.) Gaertner) Accessions for drought stress toler-

Bluhshan, D., Pandey, A., Choudhury, M.K., Datta, A., Chakraborty, S., Chakraborty, N.,
2007. Comparative proteomics analysis of differentially expressed proteins in
chickpea extracellular matrix during dehydration stress. Mol. Cell. Proteom. 6,
1868–1884.


Chai, Q., Jin, F., Merewitz, E., Huang, B., 2010. Growth and physiological traits asso-
ciated with drought survival and post-drought recovery in perennial turfgrass species.

mutant from seeded Bermuda grass and its physiological responses to drought stress.

Cruz, D., Carvalho, M.H., 2008. Drought stress and reactive oxygen species: production,

DaCosta, M., Huang, B., 2006. Changes in carbon partitioning and accumulation patterns
during drought and recovery for colonial bentgrass, creeping bentgrass, and velvet


turfgrass species to heat stress associated with antioxidant enzyme activity. J. Am.

associated with heat tolerance in a cool-season perennial grass species. Environ.
Exp. Bot. 87, 159–166.

Etemadi, N., Sheikh-Mohamadi, M.H., Nikbakht, A., Sabzalian, M.R., Pessarakli, M.,
2015. Influence of trioxapac-ethyl in improving drought resistance of wheatgrass

Seed germination of Caragana species from different regions is strongly driven by

Fry, J.D., Lang, S.N., Clifton, R., Maier, F.P., 1993. Freezing tolerance and carbohydrate
content of low temperature-acclimated and non-acclimated centipede grass. Crop.
Sci. 33, 1051–1055.

Fu, J., Dernoeden, P.H., 2008. Carbohydrate metabolism in creeping bentgrass as influ-

Fu, J., Huang, B., 2001. Involvement of antioxidants and lipid peroxidation in the
adaptation of two cool-season grasses to localized drought stress. Environ. Exp.

Guo, Z., Ou, W., Lu, S., Zhang, Q., 2006. Differential responses of antioxidative system to


