HOKKAIDO UNIVERSITY

Title	Respiratory burst oxidase-D Expression and Biochemical Responses in Festuca arundinacea under Drought Stress
Author(s)	Rohollahi, Iman; Khoshkholghsima, Nayer; Nagano, Hironori et al.
Relation	The title of this article is change into the publisher's version .The original title of the Author's version is "Differential expression of respiratory burst oxidase-1 D gene and biochemical responses between two contrasting accessions of Festuca arundinacea under drought stress"
Citation	Crop science, 58(1), 435-442 https://doi.org/10.2135/cropsci2017.07.0416
Issue Date	2018-01-15
Doc URL	https://hdl.handle.net/2115/68622
Type	journal article
File Information	Iman et al_final.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Differential expression of *respiratory burst oxidase-D* gene and biochemical responses between two contrasting accessions of *Festuca arundinacea* under drought stress

Running title: Gene expression and biochemical responses in *Festuca arundinacea* under drought stress

Abstract

NADPH oxidases (NOX) catalyze the production of superoxide, a type of reactive oxygen species (ROS). In plants, the NOX homologs have been identified as respiratory burst oxidase homologs (Rboh). They are involved in ROS production in response to drought stress. Three entries of *Festuca arundinacea* Schreb. (tall fescue), tolerant ('Isfahan') and sensitive ('Quchan') accessions to drought during the germination stage which were selected from 14 wild populations in Iran as well as cv. 'Barvado' as control were used for analyses in the present sudy. Partial sequence of the *Festuca respiratory burst oxidase-D* (*FrbohD*) gene was isolated from 'Barvado'. We compared expression levels of *FrbohD* gene as well as H_2O_2, catalase activity and some biochemical responses between the three entries. Gene expression was evaluated for leaf and shoot samples subjected to 3, 6, and 9 days without water. The transcript level of *FrbohD*, H_2O_2 content, and catalase activity increased in 'Quchan' under drought stress. It appears that lower levels of *FrbohD* gene transcription and H_2O_2 concentration in *F. arundinacea* leaves contributed to drought-stress tolerance in 'Isfahan'. Total protein and total soluble carbohydrate content also increased significantly in 'Isfahan' when subjected to drought stress. 'Isfahan' exhibited drought resistance through various strategies, which could serve as selection criteria for improving drought resistance in turf grass breeding program.

Keywords: Drought tolerance; Emergence; *Festuca arundinacea*; Transcript level.

Abbreviations

Festuca respiratory burst oxidase-D, FrbohD; field soil moisture capacity, FC; Final emergence, FE; Germination rate, GR; NADPH oxidases, NOX; Nicotinamide adenine dinucleotide phosphate, NADPH; Polymerase chain reaction, PCR; reactive oxygen species, ROS; seedling vigor index, SVI

INTRODUCTION

Festuca L. is one of the largest genera in Poaceae in temperate regions of the world (Saha et al. 2005; Yamada 2011). Several taxa within the family are used as forage and turf grasses in a wide range of soil and climatic conditions. When exposed to low-water conditions, *Festuca arundinacea* Schreb. was considered to be more drought tolerant than seven other grass species due to it having a lower rate of leaf expansion, higher root number, and greater root weight (Wilman et al. 1998). Although *F. arundinacea* is considered to be relatively drought tolerant (Pessarakli 2008), there is still considerable genetic variation for this trait among *F. arundinacea* genotypes and populations (Severmutlu et al. 2011). A major advance in turfgrass improvement has been the identification of drought-tolerant germplasm. Considerable progress in the genetic breeding improvement of a number of turfgrass species over the past five decades has resulted in the development of higher turf quality, increased abiotic and biotic stress tolerances and reduced maintenance requirements (Meyer and Funk 1989).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX), also known as respiratory burst oxidases (RBO), is a protein complex that catalyzes the production of superoxide, a type of reactive oxygen species (ROS) (Takahashi, 2012). Reactive oxygen species have been shown to play many important roles in signaling and development in plants, such as plant defense response, cell death, stomatal closure, and

abiotic stress (Wong et al. 2007). In *Arabidopsis*, 10 *Rboh* genes have been identified, and among these, RbohD and RbohF function in ROS-dependent ABA signaling for stomatal closure (Kwak et al. 2003). The rice (*Oryza sativa* L.) *RbohA* gene was the first *Rboh* gene isolated from a plant (Groom et al. 1996). Consequently, *Rboh* genes have been isolated from several plant species including *Arabidopsis*, tobacco (*Nicotiana tabacum* L.) and potato (*Solanum tuberosum* L.) (Yoshioka, 2003).

Plants have evolved mechanisms of ROS generation as signaling for rapid cell-to-cell communication in biotic and abiotic stresses, which are dependent on the *respiratory burst oxidase-D* (*RbohD*) gene (Miller et al. 2009). The equilibrium between activities of antioxidative enzymes and/or ROS production defines whether oxidative signaling or damage will occur (Moller et al. 2007). In rice, drought-tolerant varieties generated lower H_2O_2 levels compared to drought-sensitive varieties (Guo et al. 2006; Rabello et al. 2008). Plants have evolved enzymatic and non-enzymatic systems to scavenge ROS. In enzymatic systems, for instance, superoxide dismutase, catalase, and ascorbate peroxidase can break down H_2O_2 (Jiang and Huang 2001). In non-enzymatic systems, higher levels of proline and carbohydrate in drought-tolerant rice varieties, relative to drought-sensitive ones, reveal that these compounds could contribute to drought-stress tolerance (Choudhary et al. 2005).

Final germination percentage, mean germination time, and time to 25- 75% germination are important for the successful establishment of grass species (Larsen and Bibby 2004). Investigation of seedling emergence and performance by a modified method of maintaining low-soil-moisture conditions, which replicates represent post-seeding rangeland conditions, is an efficient and simple technique for screening cool-season grass genotypes for drought-stress tolerance (Gazanchian et al. 2006). Rohollahi et al. (2015) reported significant variation in drought resistance at the germination and seedling stages of tall fescue accessions derived from different ecosystems in Iran.

Although some accessions had more rapid and greater final germination and establishment, their drought-tolerance mechanisms after establishment have not been fully characterized.

Khoshkholghsima and Rohollahi (2015) showed that *F. arundinacea* maintained higher relative water content levels under drought stress more than *Agropyron cristatum* (L.) Gaertn., *Festuca ovina* L., *Cynodon dactylon* L., and *Bromus inermis* Leyss. They speculated that drought-stress tolerance is associated with higher accumulation of compatible solutes and H_2O_2 signaling. In this study, we isolated and identified *Festuca respiratory burst oxidase-D* (*FrbohD*) gene from *F. arundinacea* cv. 'Barvado' and compared its expression levels under drought stress in two populations, 'Isfahan' and 'Quchan' with cv. 'Barvado' as control, when subjected to low soil-moisture conditions., 'Isfahan' and 'Quchan' which are drought tolerant and drought sensitive at the germination stage, respectively, were selected from 14 populations in Iran. We also quantified H_2O_2, total protein content, carbohydrate content, and catalase activity of plants under drought stress after establishment. To our understanding**,** this is the first report on the cloning of *RbohD* gene from *F. arundinacea* and its gene expression analysis under drought stress. Such results will allow for the provisioning of new genetic resources for *F. arundinacea* breeding research.

MATERIAL AND METHODS

Evaluation of Populations: Sixteen entries were evaluated in germination study. Seeds of 14 wild *F. arundinacea* populations were collected from cold, arid and semiarid regions throughout Iran. In addition, two commercial cultivars ('Barvado' and 'Barleroy') from Barenbrug Holding B.V. in Netherlands were used as controls. Descriptions of collection sites and seed characteristics can be found in Rohollahi et al. (2015). In the germination experiment, effects of varying soil moisture content (40, 60,

80 and 100% field soil moisture capacity [FC]) on final emergence (FE), germination rate (GR) root and leaf length, and seedling vigor index (SVI) were determined. Each pot (9-cm diameter by 10-cm depth) was filled with 300 g of sifted dry sandy loam soil based on the methodology of Gazanchian et al. (2006). A single factorial experiment was carried out based on a completely randomized design with four replicates.

Germination and seedling establishment were monitored for 20 days. Emergence was recorded at the detection of the leaf above the soil surface of the seedlings in each pot. The leaf length and the maximum root length for each emerged seedling were measured for each pot at the end of the experiment. In this study, GR was calculated as described by Maguire (1962). Seedling vigor index was calculated (Abdul-Baki and Anderson 1973) by multiplying the percentage of emergence for each accession by the mean length (cm) of the seedling (root plus leaf).

A population collected from a dry region in Iran, which was identified as 'Isfahan', exhibited high germination, growth, and SVI under drought stress. Another population labeled as 'Quchan' showed the low final germination and SVI. Consequently, these two contrasting accessions and a commercial cultivar, 'Bravado', were selected for post-establishment evaluation under drought stress (Table 1) and also gene expression. Selected genotypes were planted in polyethylene pots (top diameter = 20 cm, height = 30 cm) filled with field soil. Plants were established for 2 months with regular irrigation in a uniform greenhouse environment condition (18/22 °C under natural day light and 60-70% relative humidity). After establishment, some pots were deprived of water 3, 6 and 9 days upon initiation of drought stress treatments. Soil volumetric water content was determined by weighing the pots during the experimental periods for each treatment (Turner and Begg 1978). The second experiment was also factorial design based on complete randomization with three replications.

Biochemical Analysis: H_2O_2 and carbohydrate content concentrations were measured based on the methods of Warm and Laties (1982) and Cizkova et al. (1996). Carbohydrate content was extracted using ethanol. Dried leaf samples (0.3 g) were extracted with 80% (v/v) ethanol at 85-90°C for 2 hrs and the extracts were evaporated to dryness. Samples were then taken up in warm water and de-proteinized by adding 5 ml of 7.2% zinc sulfate heptahydrate. The solution was neutralized by adding 5 ml of 0.1N NaOH. The samples were filtered and total soluble carbohydrate content was measured at 485 nm using a spectrophotometer. Catalase activity was measured using the method of Chance and Maehly (1954). For catalase activity, the decomposition of H_2O_2 was measured by the decline in absorbance at 240 nm for 1 min. Protein content was determined by the method of Bradford (1976). Briefly, 100 ml of leaf sample was mixed with 5 ml of protein reagent (Sigma, St. Louis, MO, USA), and the absorbance was measured at 595 nm after 2 min using a spectrophotometer. Bovine serum albumin was used as a standard (Sigma, St. Louis, MO).

Statistical Analysis: The comparisons of means were done by a least-squares means test. Treatment effects were determined by analysis of variance according to the general linear model procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA).

Sequence Analysis of *RbohD* from *Festuca*: We used the small-scale CTAB (cetyltrimethylammonium bromide) method for DNA extraction of *F. arundinacea* cv. 'Barvado' (Murray et al. 1980). The primers, FrbohDF1-F5 and FrbohDR1-R6 (Table 2), were used for the cloning of the RbohD core DNA fragment. The forward and reverse primers were designed according to the *RbohD* sequence in rice (accession number: AK072353, Wong et al. 2007) for the cloning of *FrbohD*. The polymerase chain reaction (PCR) (Applied Biosystem, Foster City, CA, USA) was carried out with the primer set (Table 2) and LA Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan).

PCR condition for the screening were as follows: 5 min of denaturation at 94°C, 35 cycles of 95°C for 30 sec, 65°C for 30 sec and 72°C for 9 min. PCR products were purified with Nucleo Spin Extract Kit (TaKaRa Bio Inc., Shiga, Japan) prior to sequence analysis. The PCR amplification products were cloned into the pGEM-T Easy vector (Promega Corp., Tokyo, Japan). Vectors containing DNA fragments were amplified using *Escherchia coli* strain JM109 (Promega Corp. , Tokyo, Japan). After overnight culture, plasmids were isolated using High Pure Plasmid Isolation Kit (Roche Applied Science, Mannheim, Germany). The DNA sequencing of plasmids and PCR products were determined by primer walking with an automatic sequencer (ABI Prisma 3130 Genetic Analyzer, Applied Biosystem, Foster City, CA, USA).

RNA Extraction: Total RNA was extracted from ground leaf tissues from each sampling time by PureLink® Plant RNA Reagent (Invitrogen, Waltham, USA). Total RNA was quantified and checked for quality.

Real Time RT-PCR: Real time (RT)-PCR was carried out using StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Several pairs of primers were designed based on the sequenced of *FrbohD*. After testing, primers FrbohDF6 and FrbohDR7 (Table 2) were used to analyze *FrbohD* gene expression. The *F. arundinacea*-specific GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Table 2), used as a reference gene, was amplified in parallel with the target gene, allowing gene expression normalization and providing quantification. Detection of RT-PCR products was done using the SYBR Green universal master mix kit (Applied Biosystem, Foster City, CA, USA) following the manufacturer recommendations. To ensure the specificity of PCR products, a dissociation curve analysis was performed for each sample (Ririe et al. 1997). In addition, each sample was run in 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Data Analysis: We used StepOnePlus (Applied Biosystems, Foster City, CA, USA) to collect the fluorescence data. The cycle threshold, C_T, which is the cycle at which the fluorescent signal is statistically different from the background, was determined for each reaction. All replicates were pooled to estimate average C_T and the standard deviation of C_T for each sample. In addition, any replicate showing nonspecific products in the dissociation curve analysis was removed. At least two of three technical replicates and six of the total replicates were included in the average C_T calculations. Raw expression values were calculated in Microsoft Excel using the average C_T values.

RESULTS

Germination at Different Soil Moisture Content

Although all of the populations emerged at 60% and 40% FC, the highest FE (100%) was exhibited by accession 'Isfahan' at 40% FC (Table 1). At 40% FC the FE values for 'Quchan', 'Yasuj', 'Borujen' and 'Kamyaran' were 6.7, 13.3, 28.3 and 35.0 %, respectively (Table 1). The FE was 50% in 'Barvado' at 40% FC (Table 1). A comparison of the two soil water content treatments (100 % FC and 40% FC), revealed a significant decrease of GR (60%) averaged across all populations at 40% FC (Table 1). The average SVI decreased by 42% with decreasing soil water content (Table 1). The highest SVI measurements occurred for 'Isfahan' and 'Gonabad' and the lowest for 'Quchan' at 40 % FC (Table 1). 'Sanadaj', 'Gonabad', and 'Isfahan' accessions respectively had the least reduction in leaf and root length (Table 1). For all populations, the leaf and root length of seedling decreased significantly when FC decreased to 40 % (Table 1).

Biochemical Analysis

Folloing the above evaluation, three entries were used for further analyses. Relative to the control, leaf H_2O_2 content increased 36, 41, and 40% in leaves of treated 'Quchan' plants after 3, 6 , and 9 days of water deprivation, respectively (Fig. 1 A, B, and C). Also, leaf H_2O_2 content in 'Isfahan' increased by 20% under drought stress after 6 days of withholding water relative to the control (Fig. 1 B). In addition, after 3 days without water, 'Quchan' showed higher H_2O_2 levels under drought stress compared to the 'Isfahan' genotype. Also, leaf H_2O_2 content decreased in 'Barvado' under drought stress compared to the control. Catalase activity significantly increased in 'Quchan' under the non-irrigated treatment compared to the control (Fig. 1 D, E and F). 'Quchan' also showed the highest amount of catalase activity at all drought levels compared to 'Isfahan' and 'Barvado' (Fig. 1D, E and F). Catalase activity substantively decreased in 'Isfahan' under drought stress compared to the control (Fig. 1D, E and F).

Total leaf protein content in 'Isfahan' increased 9 days after withholding water relative to the control, but it decreased in 'Quchan' and 'Barvado' under drought stress compared to the control (Fig. 2 C). Total leaf carbohydrate increased in 'Isfahan' 37, 34 and 47%, respectively 3, 6, and 9 days after no irrigation relative to the control (Fig. 2 D, E and F). In addition, 'Isfahan' showed significantly the highest amount of carbohydrate 3 and 9 days after water deprivation compared to 'Quchan' and 'Barvado' under drought stress (Fig. 2 D, E and F).

Sequencing of *FrbohD*

Partial sequence analysis of *FrbohD* indicated it was 3,882 bp nucleotide long. *FrbohD* gene partial sequence (NCBI, accession number: KF811502) analysis showed 76.8% similarity to *RbohD* in *Oryza sativa* (GenBank/EMBL accession number AK072353 or Phytozome database, LOC_Os05g38980) (Wong et al. 2007). One single

heterozygous polymorphism was identified at position 3426, resulting in two similar *FrbohD* alleles

***FrbohD* Gene Expression under Drought Stress**

FrbohD expression in both leaves and shoots was observed at 3, 6 and 9 days after withholding water (Fig. 3 A-F). In leaves of 'Quchan', *FrbohD* expression levels increased 2.2-fold after 3 days without water (Fig.3 A), followed by a 1.4-fold-increase 9 days without water relative to the control (Fig. 3 C). Moreover, expression levels increased at 6 and 9 days after water deprivation in stem samples of 'Quchan' compared to the control (Fig. 3E and F). On the other hand, 'Isfahan' showed low expression levels of *FrbohD* in leaves and stems under drought stress (Fig 3 D-F). No differences in the gene expression levels were detected in the leaves of 'Barvado' with the exception of *FrbohD* expression in stems after 6 days without water (Fig. 3 E). Altogether, 'Quchan' showed the highest level of *FrbohD* expression in leaves and stems after water deprivation compared to 'Isfahan' and 'Barvado' under drought stress. Also, *FrbohD* expression always decreased in 'Isfahan' and increased in 'Quchan' under drought stress, relative to the control.

DISCUSSION

In the initial germination study, we found that SVI and FE of the drought-tolerant genotype "Isfhan" were the highest under lower soil moisture levels (Table 1). Studies on identification indices of drought resistance indicated that FE, leaf length, root length and SVI were the primary indicators of establishment in *F. arundinacea* (Rohollahi et al. 2015). 'Quchan' was selected as drought-susceptible genotype and cv. 'Barvado' as a mid-point check genotype.

Analyzing the functions of the drought-inducible genes among the genotypes is important not only for further understanding the underlying molecular mechanism of

stress tolerance, but also for identifying potentially useful gentic resource for a turfgrass breeding program. Additionally, consistent with germination results, *FrbohD* was up-regulated in 'Quchan', the drought-sensitive genotype, and down-regulated in 'Isfahan', the drought-resistant genotype, relative to the control plant under drought stress. Similar to our results, Zhang et al. (2012) also showed that *RbohH* was down-regulated in drought-tolerant varieties, but up-regulated in sensitive varieties when exposed to drought conditions. This up-regulated induction might be due to higher ROS accumulation and catalase activity in 'Quchan' genotype under drought stress compared to the control (Fig 1. A, B, C). The products of *Rboh* genes are thought to function not only in stress tolerance, but also in the regulation of gene expression and signal transduction (Wong et al. 2007). Wong et al. (2007) found that *RbohD* did not express in rice leaves, but our results showed that *FRbohD* up-regulated in 'Quchan' leaves and down-regulated in 'Isfahan' leaves when exposed to drought, relative to control (Fig 3. A, B, C). Similar to our results, Moller et al. (2007) indicated that the ROS level during drought stress may indicate the potential of oxidative stress or signaling cascades in plants.

Our findings suggest H_2O_2 could trigger the activation of defense mechanisms, including increasing antioxidant levels, which could help to alleviate damage and improve plant growth performance and seedling development under drought. Among the various ROS, H_2O_2 acts as a central player in stress signal transduction cascade due to its highest half-life (Gechev et al. 2006; Hossain et al. 2015). Polidoros and Scandalios (1999) showed high concentrations of H_2O_2-induced catalase and GST1 (Glutathione S-transferase -1) expression, directly. On the other hand Fu and Huang (2001) reported that superoxide dismutase, catalase, and peroxidase activities decreased with increasing duration of drought stress.

Based on our results, carbohydrate and protein content increased significantly in 'Isfahan' relative to control after 9 days without water (Fig. 2 C, D). Increases in H_2O_2 content, total carbohydrates, and sucrose content in *F. arundinacea* under drought stress were also reported by Khoshkholghsima and Rohollahi (2015). Increase in the sucrose concentration and hexoses during drought were also observed in *F. arundinacea* leaves (Karsten and MacAdam 2001). Consistent with our results, protein synthesis increased in drought-stressed plants of *F. arundinacea* after 10 days of drought stress (Jiang and Huang 2002). Alteration of protein synthesis or degradation is one of the fundamental metabolic processes that may influence drought tolerance (Jiang and Huang 2002).

In summary, our work revealed significant variation among *F. arundinacea* genotypes from different ecological regions of Iran in response to low soil water content. The 'Isfahan' genotype exhibited the best emergence, growth, and SVI under drought conditions, while 'Quchan' showed the lowest FE and SVI. After establishment, the 'Isfahan' genotype had the most total protein and carbohydrate content under drought conditions, while 'Quchan' showed the most *FrbohH* expression, H_2O_2 content, and catalase activity under drought stress compared to the control. Both strategies of higher total protein and carbohydrate content and less H_2O_2 content under drought stress in the 'Isfahan' genotype could be some key factors influencing its drought resistance. This genotype ('Isfahan') could be a useful genetic resource for development of superior cultivars for establishment in arid and semiarid regions.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

306 We gratefully acknowledge Dr. J. Ryan Stewart, Brigham Young University, Provo,
307 Utah, USA for critical reading of the manuscript.
308

References

Abdul-Baki, A.A., and J.D. Anderson. 1973: Relationship between decarboxylation of glutamic acid and vigor in soybean seed. Crop Sci. 13: 227–232. doi: 10.2135/cropsci1973.0011183X001300020023x

Bradford, M.M. 1976: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72: 248–254. doi:org/10.1016/003-2697(76)90527-3

Chance, B., and A.C. Maehly. 1955: Assay of catalases and peroxidases. Method Enzymol. 31: 764–775. doi: org/10.1016/S0076-6879(55)02300-8

Choudhary, N.L., R.K. Sairam., and A. Tyagi. 2005: Expression of D-pyrroline-5-carboxylate synthetase gene during drought in rice (*Oryza sativa* L.). Indian J. Biochem. Biophys. 42: 366–370. http://nopr.niscair.res.in/bitstream. (accessed 3 July 2017).

Cizkova, H., J. Lukavska., K. Pribian., J. Kopecky., and H. Brabcova. 1996: carbohydrate levels in rhizomes of *phragmites australis* at an oligotrophic and eutrophic site: A preliminary study. Folia Geobot. 31: 111–118. https://www.jstor.org/stable/4181422. (accessed 3 July).

Gazanchian, A., N.A. KhoshkholghSima., M.A. Malboobi., and E. Majidi Heravan.2006: Relationships between emergence and soil water content for perennial cool-season grasses native to Iran. Crop Sci. 46: 544–553. doi: 10.2135/cropsci2005.04-0357.

Gechev, T. S., F. Van Breusegem., J.M. Stone., I. Denev., and C. Laloi. 2006: Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. BioEssays 28:1091–1101. doi: 10.1002/bies.20493.

Groom, Q.J., M.A. Torres., A.P. Fordham-Skelton., K.E. Hammond-Kosack., N.J. Rabinson., and J.D. Jones. 1996: rbohA, a rice homologue of the mammalian

335 gp91phox respiratory burst oxidase gene. Plant J. 10: 515-522. doi:
336 10.1093/jb/mvs044.
337 Guo, Z., W. Ou., S. Lu., and Q. Zhong. 2006: Differential responses of antioxidative
338 system to chilling and drought in four rice cultivars differing in sensitivity. Plant
339 Physiol. Bioch. 44: 828–836. doi:10.1016/j.plaphy.2006.10.024
340 Hossain, M.A., S. Bhattacharjee., S.M. Armin., P. Qian., H.Y. Li., D.J. Burritt, and
341 L.S.P. Tran. 2015: Hydrogen peroxide priming modulates abiotic oxidative stress
342 tolerance: insights from ROS detoxification and scavenging. Front. Plant Sci. 6: 420.
343 doi:10.3389/fpls.2015.00420.
344 Jiang, Y., and B. Huang. 2002: Protein alterations in tall fescue in response to drought
345 stress and abscisic acid. Crop Sci. 42: 202–207.doi:10.2135/cropsci2002.2020.
346 Jiang, Y., and B. Huang. 2001. Drought and heat stress injury to two cool-season
347 turfgrasses in relation to antioxidant metabolism and lipid peroxidation. Crop Sci. 41:
348 436–442. doi:10.2135/cropsci2001.41246x.
349 Karsten, H.D., and J.W. MacAdam. 2001: Effect of drought on growth, carbohydrates,
350 and soil water use by perennial ryegrass, tall fescue, and white clover. Crop Sci. 41:
351 156–166. doi:10.2135/cropsci2001.411156x.
352 Khoshkholghsima, N.A., and I. Rohollahi. 2015: Evaluating biochemical response of
353 some selected perennial grasses under drought stress in Iran. Hortic. Environ. Biote.
354 56: 383–390. doi:10.1007/s13580-015-0010-8.
355 Kwak, J.M., I.C. Mori., Z.M. Pei., N. Leonhardt., M.A. Torres., J.L. Dangl., R.E.
356 Bloom., S. Bodde., J.D.G. Jones., and J.I. Schroeder. 2003: NADPH oxidase
357 *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in AtrbohD
358 and AtrbohF genes in ROS-dependent ABA signaling in *Arabidopsis*. EMBO J. 22:
359 2623-2633. doi: 10.1093/emboj/cdg277.

Larsen, S.U., and B.M. Bibby. 2004: Use of germination curves to describe variation in germination characteristics in three turfgrass species. Crop Sci. 44: 891–899. doi:10.2135/cropsci2004.8910.

Maguire, J.D. 1962. Speed of germination aid in selection and evaluation for seedling emergence and vigor. Crop Sci. 2: 176–177. doi:org/10.2135/cropsci1962.0011183x000200020033x.

Meyer, W.A., and C.R. Funk. 1989. Progress and benefits to humidity from breeding cool-season grasses for turf. In contributions from breeding forage and turf grasses. Madison, WI: Crop Sci. Soc. Am. Special Publication. no. 15. doi: 10.2135/cssaspecpub15.c4

Miller, G., K. Schlauch., R. Tam., D. Cortes., M.A. Torres., V. Shulaev., J.L. Dangl., and R. Mittler. 2009: The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. Sci. Signal. 2(84), ra45. doi:10.1126/scisignal.2000448

Moller, I.M., P.E. Jensen., and A. Hansson. 2007: Oxidative modifications to cellular components in plants. Annu. Rev. Plant Biol. 58: 459–481. doi:10.1146/annurev.arplant.58.032806.103946.

Murray, M.G., and W.F. Thompson. 1980: Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321–4326. doi:org/10.1093/nar/8.19.4321.

Pessarakli, M. 2007: Handbook of turfgrass management and physiology. CRC press. P 690, Tucson, Arizona, USA.

Polidoros, A.N., and J.G. Scandalios. 1999: Role of hydrogen peroxide and different classes of antioxidants in the regulation of catalase and glutathione S-transferase gene expression in maize (*Zea mays* L.). Physiol. Plant. 106: 112–120. doi:10.1034/j.1399-3054.1999.106116.x.

385 Rabello, A.R., C.M. Guimarães., P.H. Rangel., F.R. da Silva., D. Seixas., E. de Souza.,
386 A.C. Brasileiro., C.R. Spehar., M.E. Ferreira., and Â. Mehta. 2008: Identification of
387 drought-responsive genes in roots of upland rice (*Oryza sativa* L). BMC Genomics.
388 9: 485. doi:10.1186/1471-2164-9-485.
389 Rohollahi, I., N.A. Khoshkholghsima., T. Yamada., M. Kafi., Y.Hoshino., A. Liaghat.,
390 and A.A. Jafari. 2015: Evaluation of seedling emergence and relative DNA content
391 under dry soil conditions of wild *Festuca arundinacea* populations collected in Iran.
392 Grassl. Sci. 61: 6–14. doi:10.1111/grs.12074.
393 Ririe, K.M., R.P. Rasmussen., and C.T. Wittwer. 1997: Product differentiation by analysis
394 of DNA melting curves during the polymerase chain reaction. Anal Biochem. 245:154–
395 160. doi:10.1006/abio.1996.9916
396 Saha, M.C., R. Mian., J.C. Zwonitzer., K. Chekhovskiy., and A.A. Hopkins. 2005: An
397 SSR-and AFLP-based genetic linkage map of tall fescue (*Festuca arundinacea*). Theor.
398 Appl. Genet. 110: 323–336. doi:10.1007/s00122-004-1843-1
399 Severmutlu, S., N. Mutlu., E. Gurbuz, O. Gulsen., M. Hocagil., O. Karaguzel., T. Heng-
400 Moss., R.C. Shearman., and R.E. Gaussoin. 2011: Drought resistance of warm-
401 season turfgrasses grown in Mediterranean region of Turkey. HortTechnology. 21:
402 726–736. http://horttech.ashspublications.org/content/21/6/726.full.pdf+html.
403 (accessed 3 July 2017).
404 Si, Y., F. Dane., A. Rashotte., K. Kang., and N.K. Singh. 2010: Cloning and expression
405 analysis of the Ccrboh gene encoding respiratory burst oxidase in *Citrullus*
406 *colocynthis* and grafting onto *Citrullus lanatus* (watermelon). J. Exp. Bot. 61: 1635–
407 1642. doi:10.1093/jxb/erq031.
408 Takahashi, S., S. Kimura., H. Kaya., A. Lizuka., H.L. Wong., and K. Shimamoto., K.
409 Kuchitsu. 2012: Reactive oxygen species production and activation mechanism of

410 the rice NADPH oxidase OsRbohB. J. Biochem. 152:1635–1642.
411 doi:10.1093/jb/mvs044.
412 Turner, N.C., and E. Begg. 1978: Responses of pasture plants to water deficits. Plant
413 relations in patures. Melbourne. http://agris.fao.org/agris-search (accessed 3 July
414 2017).
415 Wilman, D., Y. Gao., and M.H. Leitch. 1998: Some differences between eight grasses
416 within the *Lolium-Festuca* complex when grown in conditions of severe water
417 shortage. Grass Forage Sci. 53: 57–65. doi:10.1046/j.1365-2494.1998.00104.x.
418 Wong, H.L., R. Pinontoan., K. Hayashi., R. Tabata., T. Yaeno., K. Hasegawa., C.
419 Kojima., H. Yoshioka., K. Iba., T. Kawasaki., and K. Shimamoto. 2007: Regulation
420 of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant
421 Cell. 19: 4022–4034. doi:10.1105/tpc.107.055624.
422 Yamada T. *Festuca*, In: Kole, C. 2011: Wild Crop Relatives: Genomic and Breeding
423 Resources: Millets and Grasses. Springer. New York, USA.
424 Yoshioka, H., N. Numata., K. Nakajima., S. Katou., K. Kawakita., O. Rowland., and N.
425 Doke. 2003: *Nicotiana benthamiana* gp91^{phox} homologs *NbrbohA* and *NbrbohB*
426 participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. Plant
427 Cell. 15: 706–718. doi:10.1105/tpc.008680
428 Zhang, H.W., X.W. Pan., Y.C. Li., L.Y. Wan., X.X. Li., and R.F. Huang. 2012:
429 Comparison of differentially expressed genes involved in drought response between
430 two elite rice varieties. Mol. Plant. 5: 1403–1405. doi:10.1093/mp/sss053.
431 Warm, E., and G.G. Laties. 1982: Quantification of hydrogen peroxide in plant extracts
432 by the chemiluminescence reaction with luminol. Phytochemistry. 21: 827–831. doi:
433 org/10.1016/0031-9422(82)80073-3.
434
435

436

437

438

439

440

441

442

443

444

445

Figure captions

Fig. 1. Drought stress and genotype interaction; (A, B, C) Drought stress and genotype interaction on leaf H_2O_2 at 3rd, 6th , and 9th day after water deprivation respectively; (D, E, F) Drought stress and genotype interaction on catalase in leaf at 3rd, 6th , and 9th day after water deprivation respectively. Bars with different letters within each preservative and within each group are significantly different in a least-squares means test.

Fig. 2. Drought stress and genotype interaction; (A, B, C) Drought stress and genotype interaction on total leaf protein at 3rd, 6th ,and 9th day after water deprivation respectively; (D, E, F) Drought stress and genotype interaction on total leaf soluble carbohydrate at 3rd, 6th , and 9th day after water deprivation respectively. Bars with different letters within each preservative and within each group are significantly different in a least-squares means test.

Fig 3. Relative expression levels of *Festuca respiratory burst oxidase-D* (*FrbohD*) in leaves and stems of *Festuca arundinacea*; (A) Expression analysis of *FrbohD* in leaf at 3rd day after water deprivation; (B) Expression analysis of *FrbohD* in leaf at 6th day after water deprivation; (C) Expression analysis of *FrbohD* in leaf at 9th day after water deprivation; (D) Expression analysis of *FrbohD* in stem at 3rd day after water deprivation (E) Expression analysis of *FrbohD* in stem at 6th day after water deprivation; (F) Expression analysis of *FrbohD* in stem at 9th day after water deprivation. Gene expression was normalized by comparing $\Delta\Delta C_T$ to control for each genotype. The error bars indicate SE (standard error).

Table 1. The effect of different treatment for soil moisture content in emergence and seedling stage on final emergence（FE）, germination rate（GR）, leaf and root length, seedling vigor index (SVI) in seedling stage.

Entries	FE		GR		Leaf length		Root length		SVI	
	100%FC§	40%	100%	40%	100%	40%	100%	40%	100%	40%
Barvado	96.6 bc	50.0 de	15.5 a	2.5 jk	14.5 b	7.0 efg	8.4 a-d	6.0 abc	22.2 ab	6.5 f
Isfahan	100.0 a	100.0 a	15.5 a	12.2 a	13.4 bcd	8.8 a	8.9 ab	6.4 a	22.3ab	15.2a
Quchan	56.7 e	6.7 h	3.9 g	0.1 k	8.0 i	2.3 i	7.6 de	2.3 f	8.8 h	0.03i
Sanandaj	96.6 abc	85.0 ab	14.3 abc	8.4 bc	10.6 gh	8.0 abcd	7.8 cde	5.9 a-d	17.8 def	11.8 bc
Gonabad	93.3 abc	88.3 a	12.2 b-e	7.2 cd	14.4 bc	8.6 ab	8.9 ab	6.1 abc	21.7 ab	13.0 ab
Semirom	98.3 ab	55.0 cd	13.7 a- d	3.9 fgh	14.2 bc	8.2 abc	8.8 abc	6.4 a	22.6 a	8.0 def
Borujen	58.3 e	28.3 fg	6.0 fg	1.6 ijk	11.8 efg	3.9 h	7.2 e	5.6 bcd	11.1 h	2.7 gh
Kamyaran	75.0 d	35.0 ef	8.0 f	1.2 ijk	10.0 h	4.6 h	9.0 ab	5.2 de	14.3 g	3.7 g
Mashhad	96.6 abc	70.0 bc	13.0 a-d	5.0 d -g	13.0 cde	7.4 cde	8.2 a-d	5.4 cd	20.9 abc	9.0 de
Ardabil	78.3 d	60.0 cd	12.4 b-e	4.9 efg	16.0 a	7.2 de	9.3 a	6.0 abc	19.7 bcd	8.0 def
Karaj	90.0 bc	65.0 cd	10.7 e-h	4.3 e -h	13.6 bcd	6.2 g	8.7 abc	5.2 de	20.1 abc	7.4 ef
Yasuj	93.3 abc	13.3 gh	11.2 de	0.6 gh	11.8 efg	1.6 i	8.5 a-d	1.9 f	19.0 cde	0.5 hi
Barleroy	96.6 abc	90.0 a	12.8 b-e	6.4 hij	12.5 def	6.3 fg	8.3 a-d	4.6 e	20.1 a-d	9.8 cde
Tiran	88.3 c	63.3 cd	11.9 cde	2.9 ghi	10.8 gh	7.1 ef	7.8 cde	5.3 cde	16.4 efg	7.9 def
Daran	98.3 ab	95.0 a	14.5 ab	9.7 b	12.5 def	7.2 de	7.9 b-e	5.8 a-d	20.0 a-d	12.3 b
Yazdabad	78.3 d	65.0 d	11.2 de	5.2 ghi	11.4 fgh	7.8 bcde	8.2 b-e	6.1 ab	16.4 efg	7.9 def

§ 100% and 40% Field soil moisture capacity

The different letters indicate that the values were significantly different within each treatment.

Table 2. Oligonucleotide primer sequence for *FrbohD* DNA cloning and Real-time PCR.

Primer name	**Sequence (5′- 3′)**	AT (°C)
FrbohDF1	CAAGTTTGTGCAGTACAGTA	52.2
FrbohDR1	AGTGCTTGCAACCAGCGAT	56.1
FrbohDF2	CGCATCTTCATCGTACCCACTGAA	60.5
FrbohDR2	CCCATTGCTCTTAAAGGTGCAGC	60.5
FrbohDF3	CAGGAGAGCTTCGCCAAATGCATC	62.2
FrbohDF4	GTATTTTTCCTACATCCATTTGGCAAGAG	59.1
FrbohDR3	CATTAGAAGGGTTCAGTGGGTACGATG	62.0
FrbohDF4	GTGCAACTCCTTCATCAGCATACTGAA	60.5
FrbohDR4	TTTTCTACTGACCTTCCCAAATACGTTC	59.1
FrbohDF5	TGTGACAAGAACGGTGACGGAAAGCT	62.1
FrbohDR5	TTTGAAACGAAACTCCACGGATATGC	58.9
FrbohDR6	CT TGTGGA AATGGA ACCGAGTCGTT	60.5
FrbohDF6	CCTTGCGAAGACAATGGTTCCTTCC	62.1
FrbohDR7	TATAAGGGCCATGTTCAGCTTGGTTG	60.5
GAPDHF1	TGGGTTATGTTGAGGAGGATTTGGTC	60.5
GAPDHR1	AAGCTTGACGAAGTTGTCGTTCAGAG	60.5

Fig.1

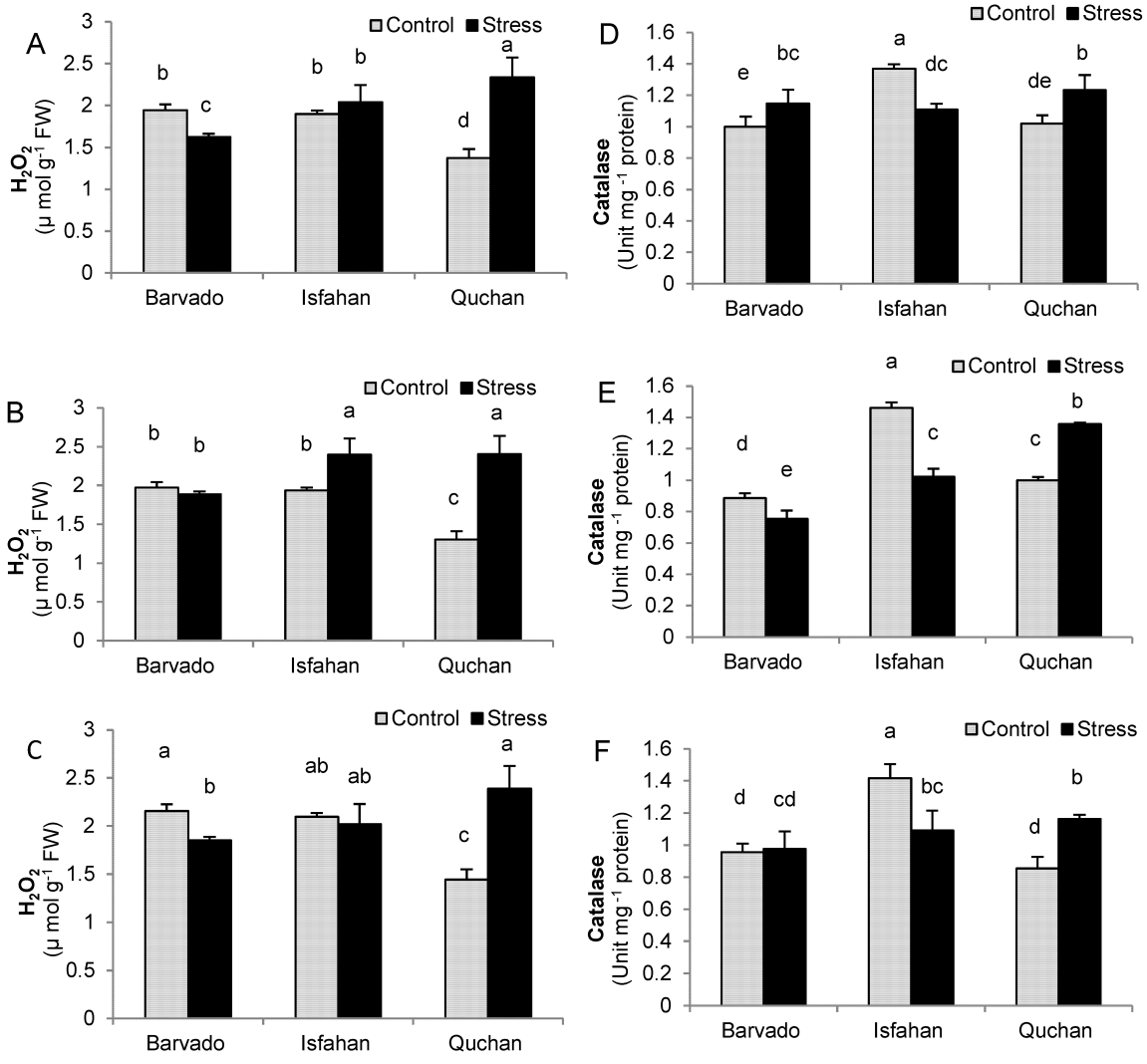

A
B
C
D
E
F
Control
Stress
H_2O_2 (μ mol g^{-1} FW)
Catalase (Unit mg^{-1} protein)
Barvado
Isfahan
Quchan

Fig.2

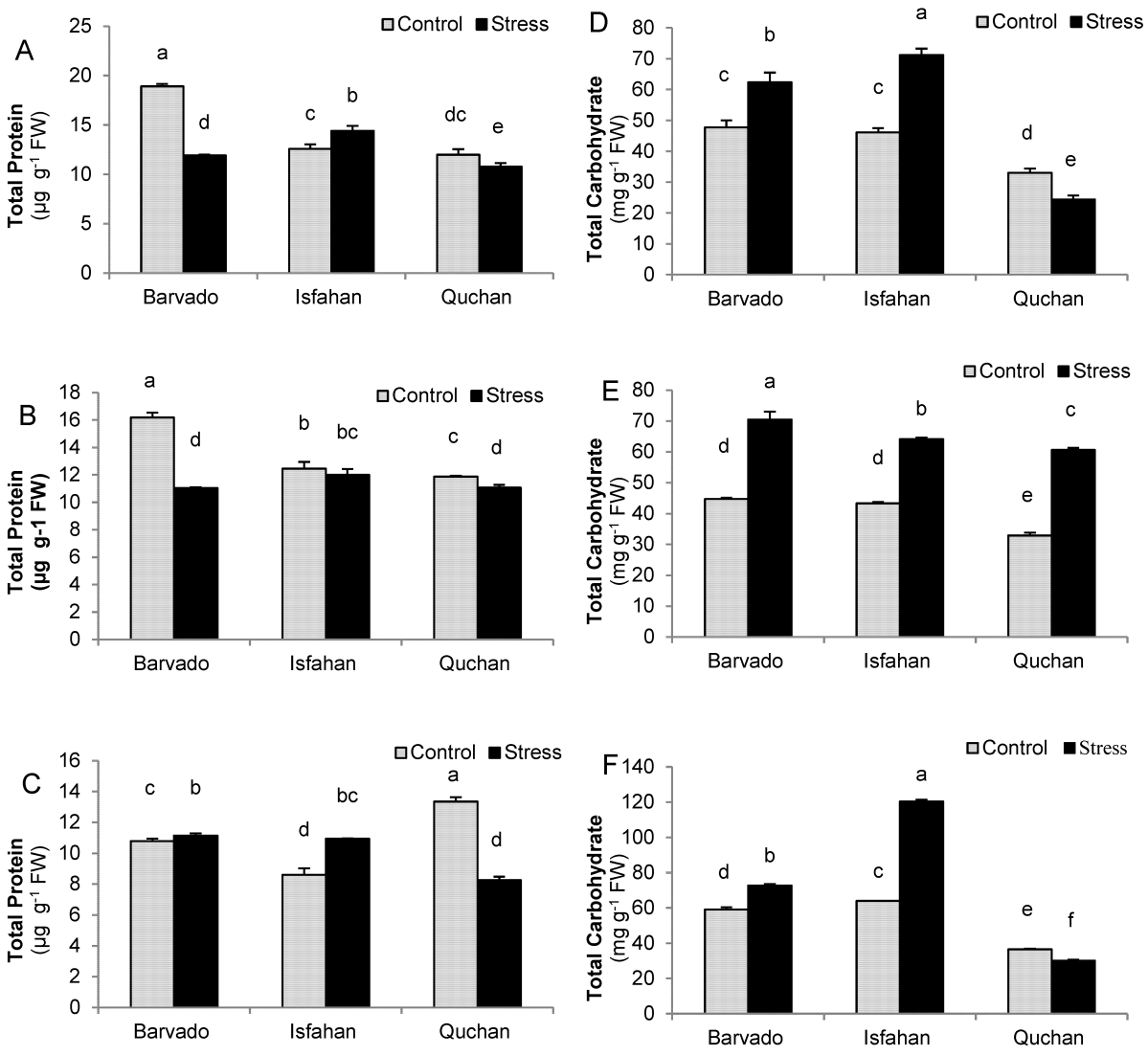
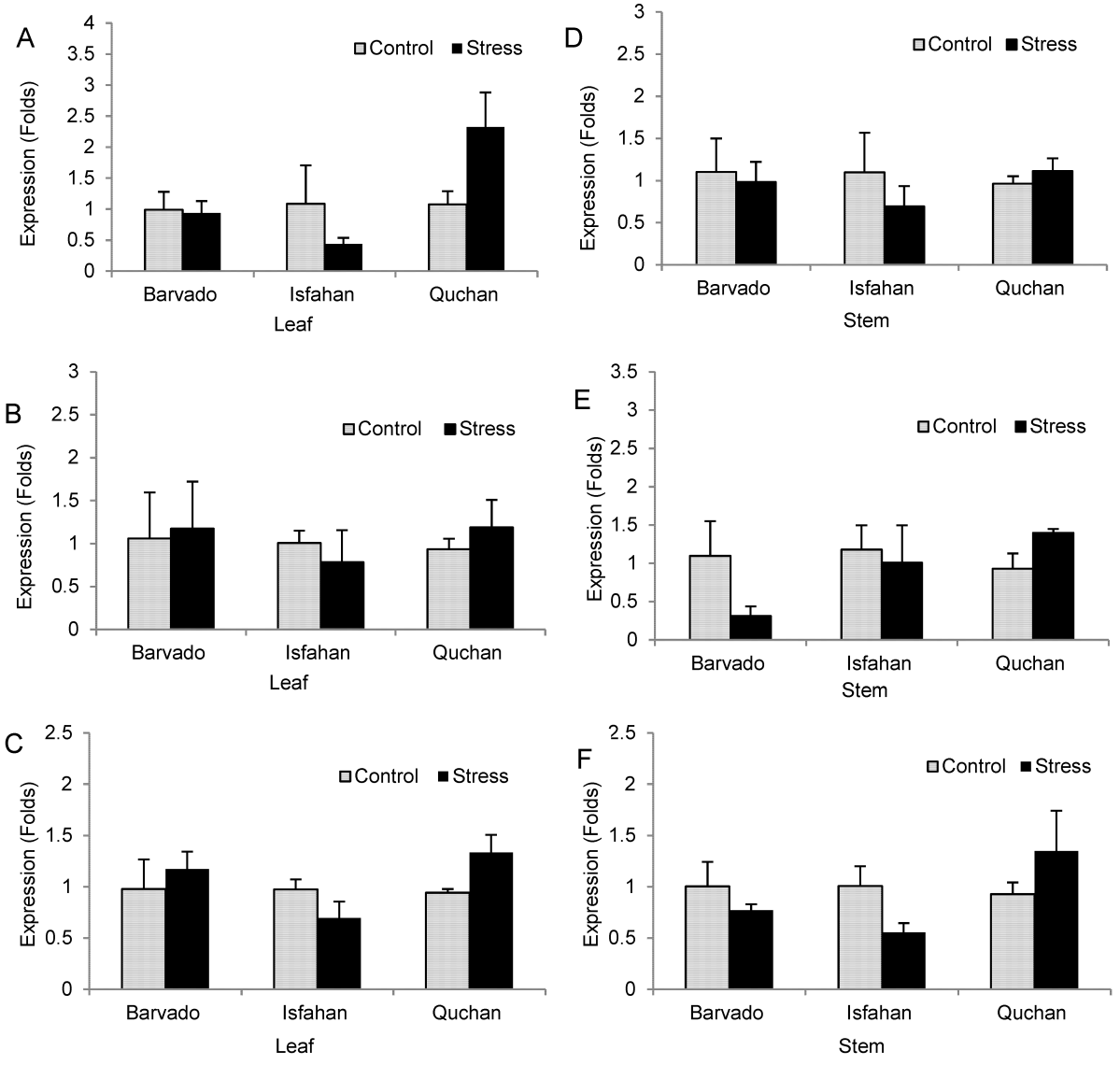

A
Total Protein (µg g-1 FW)
Control
Stress
Barvado
Isfahan
Quchan
B
Total Protein (µg g-1 FW)
C
Total Protein (µg g-1 FW)
D
Total Carbohydrate (mg g-1 FW)
E
Total Carbohydrate (mg g-1 FW)
F
Total Carbohydrate (mg g-1 FW)

Fig.3


A
Control
Stress
Expression (Folds)
4
3.5
3
2.5
2
1.5
1
0.5
0
Barvado
Isfahan
Quchan
Leaf
D
Control
Stress
Expression (Folds)
3
2.5
2
1.5
1
0.5
0
Barvado
Isfahan
Quchan
Stem
B
Control
Stress
Expression (Folds)
3
2.5
2
1.5
1
0.5
0
Barvado
Isfahan
Quchan
Leaf
E
Control
Stress
Expression (Folds)
3.5
3
2.5
2
1.5
1
0.5
0
Barvado
Isfahan
Quchan
Stem
C
Control
Stress
Expression (Folds)
2.5
2
1.5
1
0.5
0
Barvado
Isfahan
Quchan
Leaf
F
Control
Stress
Expression (Folds)
2.5
2
1.5
1
0.5
0
Barvado
Isfahan
Quchan
Stem