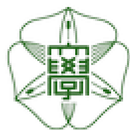
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Title	Respiratory burst oxidase-D Expression and Biochemical Responses in Festuca arundinacea under Drought Stress
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Citation	Crop science, 58(1), 435-442 https://doi.org/10.2135/cropsci2017.07.0416 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"
Issue Date	2018-01-15
Doc URL	http://hdl.handle.net/2115/68622
Type	article (author version)
File Information	Iman et al_final.pdf

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

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

6 **Abstract**

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

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

26 **Abbreviations**

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

31

32 **INTRODUCTION**

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

46 Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX), also
47 known as respiratory burst oxidases (RBO), is a protein complex that catalyzes the
48 production of superoxide, a type of reactive oxygen species (ROS) (Takahashi, 2012).
49 Reactive oxygen species have been shown to play many important roles in signaling and
50 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.

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

80 Khoshkholghsima and Rohollahi (2015) showed that *F. arundinacea* maintained
81 higher relative water content levels under drought stress more than *Agropyron cristatum*
82 (L.) Gaertn., *Festuca ovina* L., *Cynodon dactylon* L., and *Bromus inermis* Leyss. They
83 speculated that drought-stress tolerance is associated with higher accumulation of
84 compatible solutes and H_2O_2 signaling. In this study, we isolated and identified
85 *Festuca respiratory burst oxidase-D* (*FrbohD*) gene from *F. arundinacea* cv. 'Barvado'
86 and compared its expression levels under drought stress in two populations, 'Isfahan'
87 and 'Quchan' with cv. 'Barvado' as control, when subjected to low soil-moisture
88 conditions., 'Isfahan' and 'Quchan' which are drought tolerant and drought sensitive at
89 the germination stage, respectively, were selected from 14 populations in Iran. We also
90 quantified H_2O_2, total protein content, carbohydrate content, and catalase activity of
91 plants under drought stress after establishment. To our understanding**,** this is the first
92 report on the cloning of *RbohD* gene from *F. arundinacea* and its gene expression
93 analysis under drought stress. Such results will allow for the provisioning of new
94 genetic resources for *F. arundinacea* breeding research.
95

96 **MATERIAL AND METHODS**

97 **Evaluation of Populations:** Sixteen entries were evaluated in germination study.
98 Seeds of 14 wild *F. arundinacea* populations were collected from cold, arid and
99 semiarid regions throughout Iran. In addition, two commercial cultivars ('Barvado' and
100 'Barleroy') from Barenbrug Holding B.V. in Netherlands were used as controls.
101 Descriptions of collection sites and seed characteristics can be found in Rohollahi et al.
102 (2015). In the germination experiment, effects of varying soil moisture content (40, 60,

103 80 and 100% field soil moisture capacity [FC]) on final emergence (FE), germination
104 rate (GR) root and leaf length, and seedling vigor index (SVI) were determined. Each
105 pot (9-cm diameter by 10-cm depth) was filled with 300 g of sifted dry sandy loam soil
106 based on the methodology of Gazanchian et al. (2006). A single factorial experiment
107 was carried out based on a completely randomized design with four replicates.

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

115 A population collected from a dry region in Iran, which was identified as ‘Isfahan’,
116 exhibited high germination, growth, and SVI under drought stress. Another population
117 labeled as ‘Quchan’ showed the low final germination and SVI. Consequently, these
118 two contrasting accessions and a commercial cultivar, ‘Bravado’, were selected for
119 post-establishment evaluation under drought stress (Table 1) and also gene expression.
120 Selected genotypes were planted in polyethylene pots (top diameter = 20 cm, height =
121 30 cm) filled with field soil. Plants were established for 2 months with regular irrigation
122 in a uniform greenhouse environment condition (18/22 °C under natural day light and
123 60-70% relative humidity). After establishment, some pots were deprived of water 3, 6
124 and 9 days upon initiation of drought stress treatments. Soil volumetric water content
125 was determined by weighing the pots during the experimental periods for each treatment
126 (Turner and Begg 1978). The second experiment was also factorial design based on
127 complete randomization with three replications.

128 **Biochemical Analysis**: H_2O_2 and carbohydrate content concentrations were
129 measured based on the methods of Warm and Laties (1982) and Cizkova et al. (1996).
130 Carbohydrate content was extracted using ethanol. Dried leaf samples (0.3 g) were
131 extracted with 80% (v/v) ethanol at 85-90°C for 2 hrs and the extracts were evaporated
132 to dryness. Samples were then taken up in warm water and de-proteinized by adding 5
133 ml of 7.2% zinc sulfate heptahydrate. The solution was neutralized by adding 5 ml of
134 0.1N NaOH. The samples were filtered and total soluble carbohydrate content was
135 measured at 485 nm using a spectrophotometer. Catalase activity was measured using
136 the method of Chance and Maehly (1954). For catalase activity, the decomposition of
137 H_2O_2 was measured by the decline in absorbance at 240 nm for 1 min. Protein content
138 was determined by the method of Bradford (1976). Briefly, 100 ml of leaf sample was
139 mixed with 5 ml of protein reagent (Sigma, St. Louis, MO, USA), and the absorbance
140 was measured at 595 nm after 2 min using a spectrophotometer. Bovine serum albumin
141 was used as a standard (Sigma, St. Louis, MO).
142 **Statistical Analysis:** The comparisons of means were done by a least-squares
143 means test. Treatment effects were determined by analysis of variance according to the
144 general linear model procedure of the Statistical Analysis System (SAS Institute Inc.,
145 Cary, NC, USA).
146 **Sequence Analysis of *RbohD* from *Festuca***: We used the small-scale CTAB
147 (cetyltrimethylammonium bromide) method for DNA extraction of *F. arundinacea* cv.
148 'Barvado' (Murray et al. 1980). The primers, FrbohDF1-F5 and FrbohDR1-R6 (Table
149 2), were used for the cloning of the RbohD core DNA fragment. The forward and
150 reverse primers were designed according to the *RbohD* sequence in rice (accession
151 number: AK072353, Wong et al. 2007) for the cloning of *FrbohD*. The polymerase
152 chain reaction (PCR) (Applied Biosystem, Foster City, CA, USA) was carried out with
153 the primer set (Table 2) and LA Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan).

154 PCR condition for the screening were as follows: 5 min of denaturation at 94°C, 35
155 cycles of 95°C for 30 sec, 65°C for 30 sec and 72°C for 9 min. PCR products were
156 purified with Nucleo Spin Extract Kit (TaKaRa Bio Inc., Shiga, Japan) prior to
157 sequence analysis. The PCR amplification products were cloned into the pGEM-T Easy
158 vector (Promega Corp., Tokyo, Japan). Vectors containing DNA fragments were
159 amplified using *Escherchia coli* strain JM109 (Promega Corp. , Tokyo, Japan). After
160 overnight culture, plasmids were isolated using High Pure Plasmid Isolation Kit (Roche
161 Applied Science, Mannheim, Germany). The DNA sequencing of plasmids and PCR
162 products were determined by primer walking with an automatic sequencer (ABI Prisma
163 3130 Genetic Analyzer, Applied Biosystem, Foster City, CA, USA).
164 **RNA Extraction:** Total RNA was extracted from ground leaf tissues from each
165 sampling time by PureLink® Plant RNA Reagent (Invitrogen, Waltham, USA). Total
166 RNA was quantified and checked for quality.
167 **Real Time RT-PCR:** Real time (RT)-PCR was carried out using StepOnePlus Real-
168 Time PCR System (Applied Biosystems, Foster City, CA, USA). Several pairs of
169 primers were designed based on the sequenced of *FrbohD*. After testing, primers
170 FrbohDF6 and FrbohDR7 (Table 2) were used to analyze *FrbohD* gene expression. The
171 *F. arundinacea*-specific GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Table
172 2), used as a reference gene, was amplified in parallel with the target gene, allowing
173 gene expression normalization and providing quantification. Detection of RT-PCR
174 products was done using the SYBR Green universal master mix kit (Applied Biosystem,
175 Foster City, CA, USA) following the manufacturer recommendations. To ensure the
176 specificity of PCR products, a dissociation curve analysis was performed for each
177 sample (Ririe et al. 1997). In addition, each sample was run in 2% agarose gel
178 electrophoresis and visualized by ethidium bromide staining.

179 **Data Analysis:** We used StepOnePlus (Applied Biosystems, Foster City, CA,
180 USA) to collect the fluorescence data. The cycle threshold, C_T, which is the cycle at
181 which the fluorescent signal is statistically different from the background, was
182 determined for each reaction. All replicates were pooled to estimate average C_T and the
183 standard deviation of C_T for each sample. In addition, any replicate showing
184 nonspecific products in the dissociation curve analysis was removed. At least two of
185 three technical replicates and six of the total replicates were included in the average C_T
186 calculations. Raw expression values were calculated in Microsoft Excel using the
187 average C_T values.
188
189 **RESULTS**
190 **Germination at Different Soil Moisture Content**
191 Although all of the populations emerged at 60% and 40% FC, the highest FE
192 (100%) was exhibited by accession 'Isfahan' at 40% FC (Table 1). At 40% FC the FE
193 values for 'Quchan', 'Yasuj', 'Borujen' and 'Kamyaran' were 6.7, 13.3, 28.3 and
194 35.0 %, respectively (Table 1). The FE was 50% in 'Barvado' at 40% FC (Table 1). A
195 comparison of the two soil water content treatments (100 % FC and 40% FC), revealed
196 a significant decrease of GR (60%) averaged across all populations at 40% FC (Table 1).
197 The average SVI decreased by 42% with decreasing soil water content (Table 1). The
198 highest SVI measurements occurred for 'Isfahan' and 'Gonabad' and the lowest for
199 'Quchan' at 40 % FC (Table 1). 'Sanadaj', 'Gonabad', and 'Isfahan' accessions
200 respectively had the least reduction in leaf and root length (Table 1). For all populations,
201 the leaf and root length of seedling decreased significantly when FC decreased to 40 %
202 (Table 1).
203
204 **Biochemical Analysis**

205 Folloing the above evaluation, three entries were used for further analyses. Relative
206 to the control, leaf H_2O_2 content increased 36, 41, and 40% in leaves of treated 'Quchan'
207 plants after 3, 6 , and 9 days of water deprivation, respectively (Fig. 1 A, B, and C).
208 Also, leaf H_2O_2 content in 'Isfahan' increased by 20% under drought stress after 6 days
209 of withholding water relative to the control (Fig. 1 B). In addition, after 3 days without
210 water, 'Quchan' showed higher H_2O_2 levels under drought stress compared to the
211 'Isfahan' genotype. Also, leaf H_2O_2 content decreased in 'Barvado' under drought
212 stress compared to the control. Catalase activity significantly increased in 'Quchan'
213 under the non-irrigated treatment compared to the control (Fig. 1 D, E and F). 'Quchan'
214 also showed the highest amount of catalase activity at all drought levels compared to
215 'Isfahan' and 'Barvado' (Fig. 1D, E and F). Catalase activity substantively decreased in
216 'Isfahan' under drought stress compared to the control (Fig. 1D, E and F).
217 Total leaf protein content in 'Isfahan' increased 9 days after withholding water
218 relative to the control, but it decreased in 'Quchan' and 'Barvado' under drought stress
219 compared to the control (Fig. 2 C). Total leaf carbohydrate increased in 'Isfahan' 37, 34
220 and 47%, respectively 3, 6, and 9 days after no irrigation relative to the control (Fig. 2 D,
221 E and F). In addition, 'Isfahan' showed significantly the highest amount of carbohydrate
222 3 and 9 days after water deprivation compared to 'Quchan' and 'Barvado' under
223 drought stress (Fig. 2 D, E and F).
224

225 **Sequencing of *FrbohD***

226 Partial sequence analysis of *FrbohD* indicated it was 3,882 bp nucleotide long.
227 *FrbohD* gene partial sequence (NCBI, accession number: KF811502) analysis showed
228 76.8% similarity to *RbohD* in *Oryza sativa* (GenBank/EMBL accession number
229 AK072353 or Phytozome database, LOC_Os05g38980) (Wong et al. 2007). One single

230 heterozygous polymorphism was identified at position 3426, resulting in two similar
231 *FrbohD* alleles
232

233 *FrbohD* Gene Expression under Drought Stress

234 *FrbohD* expression in both leaves and shoots was observed at 3, 6 and 9 days after
235 withholding water (Fig. 3 A-F). In leaves of ‘Quchan’, *FrbohD* expression levels
236 increased 2.2-fold after 3 days without water (Fig.3 A), followed by a 1.4-fold-increase
237 9 days without water relative to the control (Fig. 3 C). Moreover, expression levels
238 increased at 6 and 9 days after water deprivation in stem samples of ‘Quchan’ compared
239 to the control (Fig. 3E and F). On the other hand, ‘Isfahan’ showed low expression
240 levels of *FrbohD* in leaves and stems under drought stress (Fig 3 D-F). No differences
241 in the gene expression levels were detected in the leaves of ‘Barvado’ with the
242 exception of *FrbohD* expression in stems after 6 days without water (Fig. 3 E).
243 Altogether, ‘Quchan’ showed the highest level of *FrbohD* expression in leaves and
244 stems after water deprivation compared to ‘Isfahan’ and ‘Barvado’ under drought stress.
245 Also, *FrbohD* expression always decreased in ‘Isfahan’ and increased in ‘Quchan’
246 under drought stress, relative to the control.

247 DISCUSSION

248 In the initial germination study, we found that SVI and FE of the drought-tolerant
249 genotype “Isfhan” were the highest under lower soil moisture levels (Table 1). Studies
250 on identification indices of drought resistance indicated that FE, leaf length, root length
251 and SVI were the primary indicators of establishment in *F. arundinacea* (Rohollahi et al.
252 2015). ‘Quchan’ was selected as drought-susceptible genotype and cv. ‘Barvado’ as a
253 mid-point check genotype.
254 Analyzing the functions of the drought-inducible genes among the genotypes is
255 important not only for further understanding the underlying molecular mechanism of

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

272 Our findings suggest H_2O_2 could trigger the activation of defense mechanisms,
273 including increasing antioxidant levels, which could help to alleviate damage and
274 improve plant growth performance and seedling development under drought. Among
275 the various ROS, H_2O_2 acts as a central player in stress signal transduction cascade due
276 to its highest half-life (Gechev et al. 2006; Hossain et al. 2015). Polidoros and
277 Scandalios (1999) showed high concentrations of H_2O_2-induced catalase and GST1
278 (Glutathione S-transferase -1) expression, directly. On the other hand Fu and Huang
279 (2001) reported that superoxide dismutase, catalase, and peroxidase activities decreased
280 with increasing duration of drought stress.

281 Based on our results, carbohydrate and protein content increased significantly in
282 'Isfahan' relative to control after 9 days without water (Fig. 2 C, D). Increases in H_2O_2
283 content, total carbohydrates, and sucrose content in *F. arundinacea* under drought stress
284 were also reported by Khoshkholghsima and Rohollahi (2015). Increase in the sucrose
285 concentration and hexoses during drought were also observed in *F. arundinacea* leaves
286 (Karsten and MacAdam 2001). Consistent with our results, protein synthesis increased in
287 drought-stressed plants of *F. arundinacea* after 10 days of drought stress (Jiang and Huang
288 2002). Alteration of protein synthesis or degradation is one of the fundamental metabolic
289 processes that may influence drought tolerance (Jiang and Huang 2002).
290 In summary, our work revealed significant variation among *F. arundinacea*
291 genotypes from different ecological regions of Iran in response to low soil water content.
292 The 'Isfahan' genotype exhibited the best emergence, growth, and SVI under drought
293 conditions, while 'Quchan' showed the lowest FE and SVI. After establishment, the
294 'Isfahan' genotype had the most total protein and carbohydrate content under drought
295 conditions, while 'Quchan' showed the most *FrbohH* expression, H_2O_2 content, and
296 catalase activity under drought stress compared to the control. Both strategies of higher
297 total protein and carbohydrate content and less H_2O_2 content under drought stress in
298 the 'Isfahan' genotype could be some key factors influencing its drought resistance.
299 This genotype ('Isfahan') could be a useful genetic resource for development of
300 superior cultivars for establishment in arid and semiarid regions.
301
302 **Conflict of interest**
303 The authors declare that they have no conflict of interest.
304
305 **Acknowledgments**

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

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446 Figure captions

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

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

461

462 Fig 3. Relative expression levels of *Festuca respiratory burst oxidase-D* (*FrbohD*) in
463 leaves and stems of *Festuca arundinacea*; (A) Expression analysis of *FrbohD* in leaf at
464 3rd day after water deprivation; (B) Expression analysis of *FrbohD* in leaf at 6th day
465 after water deprivation; (C) Expression analysis of *FrbohD* in leaf at 9th day after water
466 deprivation; (D) Expression analysis of *FrbohD* in stem at 3rd day after water
467 deprivation (E) Expression analysis of *FrbohD* in stem at 6th day after water
468 deprivation; (F) Expression analysis of *FrbohD* in stem at 9th day after water
469 deprivation. Gene expression was normalized by comparing $\Delta\Delta C_T$ to control for each
470 genotype. The error bars indicate SE (standard error).

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475 Table 1. The effect of different treatment for soil moisture content in emergence and seedling stage on final
476 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]	22.3[ab]	15.2[a]
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]	0.03[i]
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]

477 § 100% and 40% Field soil moisture capacity
478 The different letters indicate that the values were significantly different within each treatment.
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487 Table 2. Oligonucleotide primer sequence for *FrbohD* DNA cloning and Real-time PCR.

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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

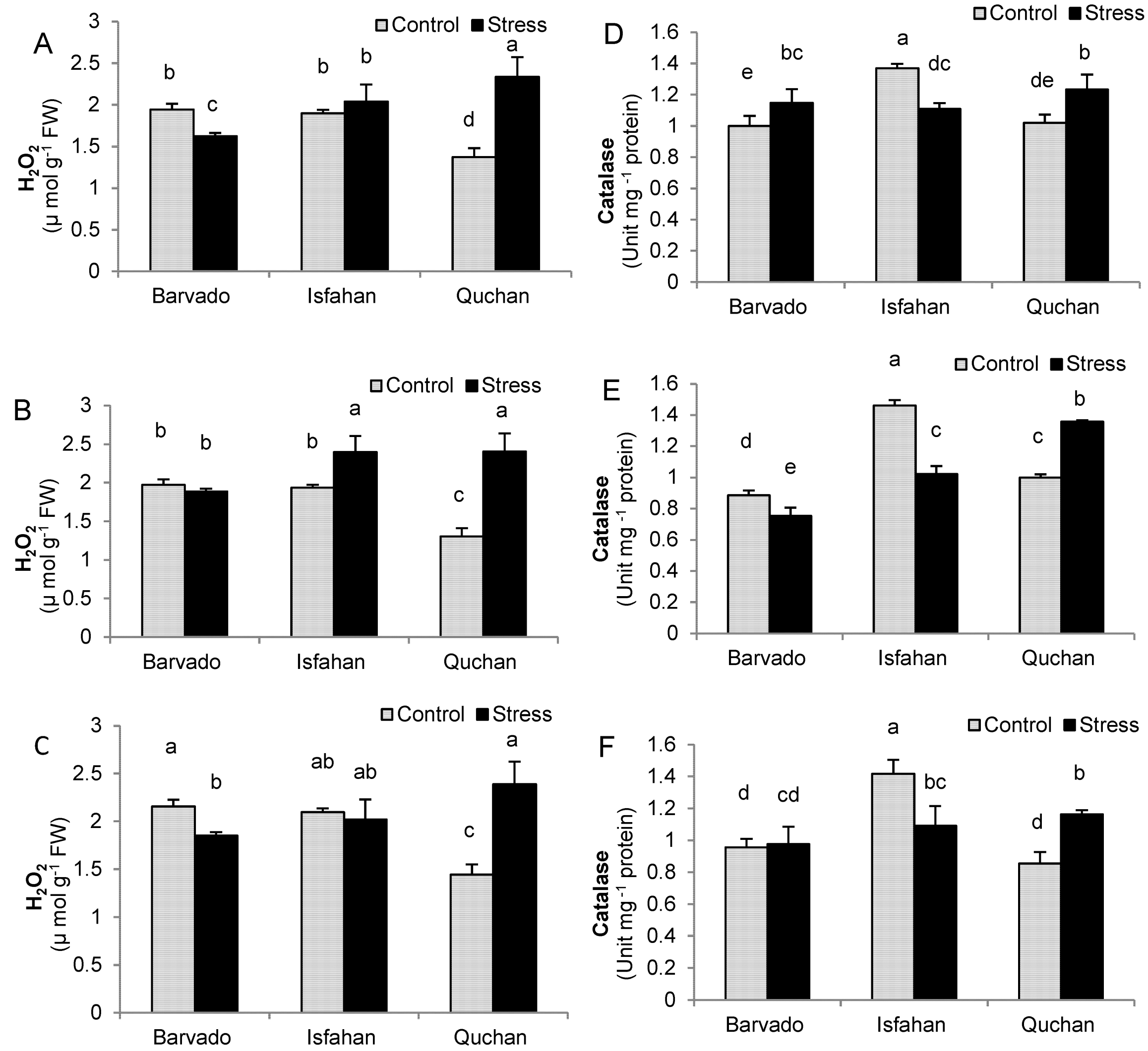

Fig.2

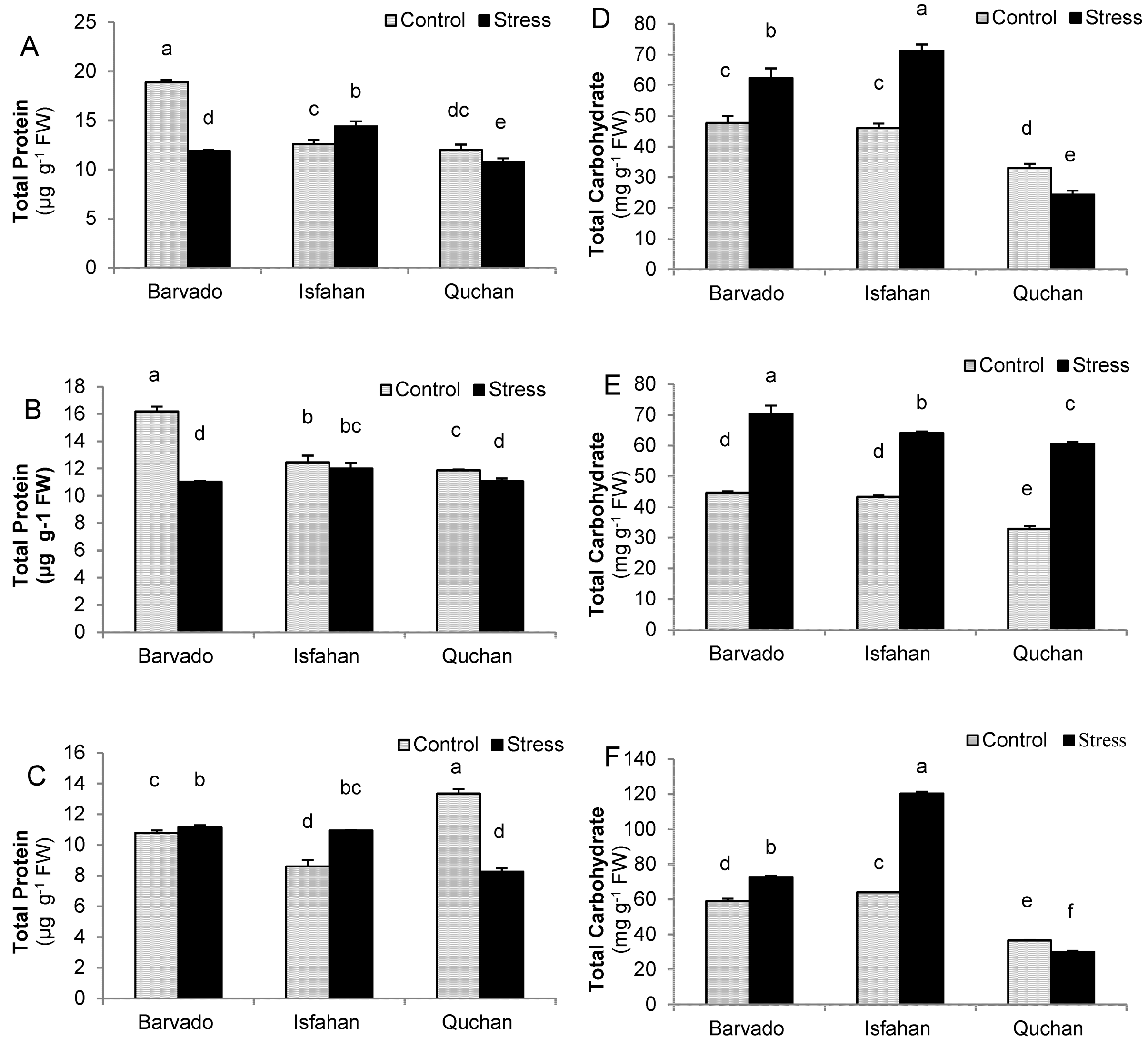

Fig.3