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Study of antioxidant defense genes expression in rice (*Oryza sativa* L.) cultivars in response to drought stress

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Abstract

Drought stress is one of the important factors that restrict crop production in the world. This study was conducted to investigate defense gene expression in response to drought stress, and also to evaluate the drought tolerance and its mechanism in rice cultivars based on randomized complete block design in two separate environments (drought stress and non-stress). The rice cultivars used included two commercial cultivars, i.e., Ahlemi-Tarom, Sepidrood, and three promising lines of fourth mutant generation called 4, 94 and 95 tested on the research farm at Gonbad Kavous University in 2018. For carrying out the drought stress-related experiment, irrigation was completely cut off 40 days after the transplantation (a stage with maximum tillering) until the end of the growth period. In seeding stage, each plant leaf was separately sampled in each block (under the stress and non-stress conditions). TBARM, superoxide and hydrogen peroxide contents and oxidative-stress related gene expression including *superoxide dismutase*, *catalase*, *ascorbate peroxidase* and *glutathione peroxidase* were measured. Results indicated that the drought-tolerant mutant lines had lower TBARM, superoxide and hydrogen peroxide contents compared to the studied cultivars. The mutant lines 94, 4 and 95 and Ahlemi-Tarom and Sepidrood had the highest to lowest levels of gene expression and yield in drought stress condition, sequentially. Accordingly, there was a complete correlation between the decreases in TBARM, superoxide and hydrogen peroxide and an increase in the level of gene expression. Mutant 94 had the highest yield and probably a suitable genetic potential in relation to drought tolerance. Therefore, the mentioned mutant is recommended for carrying out further studies.

Key words: *Ascorbate peroxidase, Catalase,* Abiotic stress, *Glutathione peroxidase,* Rice, *Superoxide dismutase*.

ABBREVIATIONS

ROS (Reactive Oxygen Species), SOD (Superoxide dismutase), CAT (Catalase), APX (Ascorbate peroxidase), GPX (Glutathione peroxidase), POD (Peroxidase), TBARM (Thiobarbituric Acid Reactive Material), TCA (Trichloroacetic Acid), O_2^- (Superoxide), H_2O_2 (Hydrogen peroxide), DEPC (Diethylpyrocarbonate), REST (Relative Expression Software Tool), MDA (Malondialdehyde).

INTRODUCTION

Drought stress is one of the most important limiting factors for crop production (Zhang *et al.,* 2018). Plants apply various strategies to deal with drought stress, among which the development of a large and deep root

system to increase the absorption of water from the depth of soil, the stomatal closure to reduce water loss, the accumulation of compatible soluble and protector proteins, and the increased level of antioxidants can be mentioned (Chaves *et al.,* 2003). Accordingly, relying on the genetic potential of cultivars and their improvement and modification genetically play major roles in inducing drought tolerance. Since the fundamental principles of each plant breeding program are determined by studying genetic parameters, being fully aware of the ways genes are effective in achieving success in the plant breeding programs is necessary (Navabpour, 2013). From a cellular perspective, in the time of drought stress, the stomata are closed to keep the existing moisture in the plant and this, while reducing the gas exchange in the leaves, leads to the formation of ROSs (Reactive Oxygen Species) in chloroplasts (Luna *et al.,* 2004). Indeed, the main essence of the stress process is creating an imbalance between producing ROSs and scavenging them in the cell, which occurs in both biotic and abiotic stresses. High and toxic concentrations of ROSs cause severe damage to the protein structure, inhibit the activity of several enzymes through metabolic pathways, and resulting in oxidized macromolecules, including lipids and DNA. Exacerbation and continuation of these undesirable events may result in programmed cellular death (Gill and Tuteja, 2010; Kar, 2011). Under growing conditions, cellular metabolism activities regularly lead to the production of ROSs; therefore, cells feel this uncontrolled increase in the level of ROSs and use it as a signaling mechanism to activate protective responses (Moller and Sweetlove, 2010). On the other hand, to neutralize the toxic and destructive effects of ROSs, enzymatic and non-enzymatic antioxidant defense systems are activated in plant cells, and the level of ROSs is adjusted by these systems (Gechev *et al.,* 2002). The enzymatic defense system includes SOD, CAT, and POD (*Ślesak et al.,* 2007; Foyer and Noctor, 2009; Sharma *et al.,* 2012; Szymańska *et al.,* 2017) and the non-enzymatic defense system consists of ascorbic, glutathione, α-tocopherol, and carotenoid (Foyer and Noctor, 2009; Sharma *et al.,* 2012; Szymańska *et al.,* 2017). From the molecular point of view, most environmental stresses have an increase in oxidative processes in common. In this sense, drought stress is no exception. Basically, the level of active oxygen radicals increases by drought stress, and if enzymatic and non-enzymatic reactions do not adjust the proper level of these radicals, oxidative stress occurs. As a consequence, a number of damages occur, such as cell membrane damage and excessive increase in fatty acid oxidation. By exacerbating this, vital molecules, such as proteins and DNA are also at risk and ultimately cellular aging, followed by necrosis and cell death occurs. Assessing the peroxidation of membrane fats under the stress condition is considered as an indicator of resistance (Hodges *et al.,* 1999; Kolupae *et al.,* 2008). Based on what was mentioned earlier, a simple and quick assessment of oxidative stress is crucial. In this regard, measuring TBARM, as an indicator of cellular oxidation and lipid peroxidation, has an acceptable efficacy (Hagege *et al.,* 1990). This index significantly increases in the final stages of growth under the influence of biotic and abiotic stresses (Navabpour *et al.,* 2003; Navabpour *et al.,* 2011; Navabpour, 2012a). ROSs, as secondary factors, are involved in important cellular processes. Superoxide ions play roles in the programmed cell death (PCD) and the hypersensitive defense response (Doke *et al.,* 1994). Hydrogen peroxide plays a significant role in the formation of lignin walls of plant cells (Lewis and Yamamoto, 1990). By increasing the activity of the metabolic pathway of the MAP-kinase, hydrogen peroxide regulates the aperture and closure patterns of stomata as a defense response to stress conditions (Neill *et al.,* 2001). Superoxide and hydrogen peroxide are of significant importance in controlling the production of ethylene, jasmonic and salicylic acid (Mackerness, 2000). Generally, the ROSs, especially superoxide and hydrogen peroxide, have two distinct roles: they are toxic in high concentrations and they can damage vital molecules, such as proteins, DNA, and lipids, by applying oxidative stress. On the other hand, they cause damage to the cell wall and disrupt the exchange process of the cell membrane (Golden *et al.,* 2002). This is while in balanced concentrations, they are vital to induce relative adaptation of the plant to create tolerance to biotic and abiotic stresses. Moreover, they act as signal molecules in the process of regulating the expression of genes and synthesizing proteins (Larkindale *et al.,* 2005). The *SOD* gene encodes a group of metalloenzyme proteins. The enzymatic product of this gene is capable of scavenging superoxide ions. Superoxide ions have high potentials of being converted to hydrogen peroxide (Leonowicz *et al.,* 2018). Converting superoxide to hydrogen peroxide is the first bridge in the enzymatic scavenging of the ROSs. The SOD enzyme is the primary defense mechanism against the ROSs (Alscher *et al.,* 2002; Sharma *et al.,* 2012). The SOD enzyme itself accelerates the conversion of superoxide to hydrogen peroxide in the cytoplasm, chloroplast and mitochondria (Beyer *et al.,* 1991). One of the main sources for producing hydrogen peroxide is the photorespiration process in

which the conversion of glycolate to glyoxylate occurs, and hydrogen peroxide is produced. The resulting hydrogen peroxide is catalyzed by the CAT enzyme in the peroxisome. Another important source for producing hydrogen peroxide is the β-oxidation process of fatty acids. The CAT in glyoxysome decomposes hydrogen peroxide (Kiani *et al.,* 2008). Therefore, the CAT is present in peroxisomes and glyoxysomes of plant and animal cells and plays a role in the decomposition of hydrogen peroxide to water and oxygen (Ahmed *et al.,* 2011; Kim *et al.,* 2014). It is noteworthy that if the concentration of hydrogen peroxide is high, the CAT will eliminate hydrogen peroxide (Ebadi Almas *et al.,* 2019). The APX enzyme is present in spermatophyte and algae and plays a role in eliminating hydrogen peroxide from the ascorbate-glutathione cycle, and its activity is located in the cytosol, chloroplast, mitochondria and peroxisome (Shigeoka *et al.,* 2002; Mittler *et al.,* 2004). Therefore, the APX plays an important role in scavenging hydrogen peroxide, which is carried out with the aid of ascorbic acid, as an electron donor for the reduction of hydrogen peroxide to water; during this reaction, ascorbic acid is converted to Monodehydroascorbate (Noctor and Foyer, 1998). The GPX enzyme can be found in all parts of plants and uses phenol, as an electron donor, and participates in the growth process, lignin synthesis, ethylene biosynthesis, and defending and wound healing process (Apel and Hirt, 2004; Michalak, 2006). The expression of the *GPX* gene is effective in inducing the expression of the *Malate Synthase* and *Isocitrate lyase* genes. These genes encode proteins that are important in the glyoxylic acid cycle. The activity of this cycle, along with β-oxidation and glycogenesis, can make it possible to convert fatty acids into glucose (Ettinger and Harada, 1990). The major difference between sensitive and tolerant plants to osmotic stresses is in the activity of these defense systems against stresses (Hsu and Kao, 2003). Hence, the concentration of these protective enzymes is considered as an indirect indicator for identifying tolerant plants and the expression of these defense genes significantly helps to determine the oxidative stress pathway. On the other hand, all important physiological processes and biochemical interactions at the cellular level are the results of gene activities, so it is important to investigate the differential pattern of gene expression. In this regard, one of the most important methods is the use of qRT-PCR. The qRT-PCR is an *in vitro* technique applied for measuring transcripts of known sequences. This technique has high sensitivity and flexibility. In practice, a cDNA sequence is made after extracting RNA from a target tissue and the amount of fluorescent produced is plotted in curves by a computer by linking ethidium bromide into each chain reaction cycle. Normally, the relative expression of the studied genes amplified with the help of specific primers is measured using the housekeeping gene that has acceptable stability of expression under different treatment conditions (Navabpour, 2012b). In this regard, this study was carried out in regard to the importance of the genetic pathway of enzymatic defense systems against drought stress in rice. On that account, the current study was conducted to examine the importance and evaluate the pattern of important defense genes in response to drought stress and to investigate the relationship between the expression of these genes with adjusting the concentration of ROSs.

MATERIALS AND METHODS

Plant material and growth condition

An initial cross between two rice cultivars of Ahlami-Tarom and Sepidrood was conducted at Gonbad Kavous University in 2008 (Sabouri *et al.,* 2008a; Sabouri *et al.,* 2008b). Ahlemi-Tarom is a low-yielding rice cultivar tolerant to drought stress and Sepidrood is a high-yielding rice cultivar sensitive to drought stress at the reproductive stage of growth (Sabouri *et al.,* 2011; Kazerani *et al.,* 2018). Initially, 300 F_9 lines were irradiated with Gammacell set. This set used a Cobalt-60 source and emitted radiation with gamma-ray (250 gray). Then, were cultivated under the normal and drought stress conditions on a research farm at Gonbad-e-Kavos University in 2015. In order to conduct the first screening, parental lines were cultivated as controls in the vicinity of the M_1 farm and based on the morphological characteristics and important agronomic traits, mutants with a significant superiority to their parents were selected. Accordingly, 134 superior M_2 mutants were cultivated in the vicinity of their parents in 2016 and the secondary screening was performed with regard to target traits. Finally, in 2017, 3 superior mutants of M_3 were cultivated in the vicinity of the primary parental cultivars (Ahlemi-Tarom and Sepidrood) to increase their genetic purity, consolidate the genes, and identify the most superior genotype in terms of drought stress tolerance. Seeds of two rice cultivars, i.e., Ahlemi-Tarom, Sepidrood and three promising lines of M_4 called 94, 4 and 95 were cultivated on a research farm at Gonbad Kavous University in 2018 using a randomized complete block design with three replications in two separate environments, i.e., flooded and drought stressed. When carrying out the drought stress-related experiment, irrigating the farm was completely cut off 40 days after the transplantation

(a stage with maximum tillering) until the end of the growth period (Kazerani *et al.,* 2018; Kazerani *et al.,* 2019b). During the experimental period, samples were taken on days 50, 60, 70, 80 and 90 after the cultivation in drought stress condition and weight moisture of soil were measured to be 32%, 24%, 18%, 8% and 4%, respectively. Based on this, the water potential of soil was estimated to be -0.05, -0.12, -0.27, -0.72 and -1.1 MPa, respectively. Since rice is sensitive to drought stress in its reproductive stage (Yoshida, 1981), stress was applied after its vegetative stage (at the end of the tillering stage) that is equivalent to the day 40 after the transplantation (Kazerani *et al.,* 2019a; Kazerani *et al.,* 2019c). To prevent any water penetrations from the margin of the farm, distances between the plots were considered two meters and a plastic barrier prevented any water penetrations. Leaves of each plant in each block (flooded and drought stressed conditions) were separately sampled in the reproductive stage (seeding) to measure the levels of ROSs (O_2^- and H_2O_2), TBARM and to quantify the expression of the antioxidant genes (*SOD*, *CAT*, *APX* and *GPX*). Fifteen treated leaves were randomly sampled in each replicate during the seeding stage. Fresh leaves were used to measure biochemical characteristics and samples frozen in liquid nitrogen (-80 °C) were used to estimate gene expression.

Measurement of Thiobarbituric Acid Reactive Material (TBARM)

To evaluate the TBARM index, which is a measure of estimating oxidative stress, the amount of thiobarbituric acid, i.e., the final and relatively stable product of oxidation reaction of large vital molecules, was assessed. To this end, a modified version of the Hagege *et al.* (1990) method was applied. Using this method, 0.5 g of leaf was homogenized and 1 ml of trichloroacetic acid (TCA) (15% w/v) was added. The obtained solution was then blended vigorously after adding 10 ml of acetone and was centrifuged at 4750 rpm for 15 min. The small sediment resulted from the centrifugation was washed by 5 ml of acetone. Then, after using a vortex mixer, the solution was again centrifuged at the same speed for 10 min. The last step was repeated four times. Afterwards, 3 ml of phosphoric acid (1% w/v) and 1 ml of thiobarbituric acid (0.6% w/v) were added and the solution was placed at 100 °C for 30 min. The reaction was stopped by rapid cooling of the tubes by placing them on ice and the absorbance of the obtained solution was measured at the following wavelengths, i.e., 532 nm and 590 nm, using a spectrophotometer.

Measurement of Superoxide ion (O_2^-)

A modified version of the Elstner and Heupel (1976) method was applied to assess the level of superoxide ion. Initially, 1 g of the leaf sample was uniformly ground and 3 ml of 65 mM potassium phosphate buffer (pH=7.8) was added. The resulting solution was centrifuged at 5000 rpm for 10 min. One ml of the above solution was removed and placed at 25 °C together with 0.9 ml of 65 mM potassium phosphate buffer, 0.1 ml of 10 mM hydroxylamine hydrochloride for 20 min. Subsequently, just as much ethyl ether as the solution was added and centrifuged at 1500 rpm for 5 min. The absorbance was read at 530 nm using a spectrophotometer. The standard curve was set in terms of nmol/min/g FW. The mentioned curve was prepared using the concentrations of superoxide produced from the chemical composition of NO_2^- and hydroxylamine.

Measurement of Hydrogen peroxide (H_2O_2)

H_2O_2 was measured using the Velikova *et al.* (2000) method. Leaf tissues (0.07 g) were homogenized in an ice bath with 5 mL of TCA (0.1% w/v). The homogenate was centrifuged at 12000 rpm for 15 min and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH=7) and 1 mL of 1 M KI. The absorbance of the supernatant was measured at 390 nm. The content of H_2O_2 was calculated by comparison with a standard calibration curve previously made by using different concentrations of H_2O_2 (Loreto and Velikova, 2001).

Evaluation of gene expression

To determine the pattern of the expression of oxidative stress pathway genes, the entire RNA was initially extracted from the leaf sample using the p-BIOZOL buffer (Bioflux, Japan) and according to the manufacturer's instructions. To eliminate the remaining DNA in the RNA samples, the DNase I enzyme (Fermentas) was used. The reverse transcription reaction was performed by the M-MuLV enzyme (Fermentas) according to the manufacturer's instructions. The quality and quantity of extracted RNA were assessed using agarose gel electrophoresis and spectrophotometer (Figure 1). qRT-PCR was applied to multiply the transcripts of defense genes in the oxidative stress pathway by using specific primers (Table 1) and the quantitative method conducted by the iQ5 device (Bio-Rad). The NCBI website (primer blast section) was used to determine the actual melting temperature of the primers, estimate the length of the fragment amplified by the primer, and to ensure specific primer amplification. A 20 µl of the reaction solution was prepared in special tubes. To prepare the cDNA, a method proposed by Fermantas Company was employed, such that, initially, 5 µl of each RNA sample (after DNase I treatment) were poured into each tube and 1 µl of oligo (dT) primer was added to each tube.

Table 1. The names and sequences of specific primers used for the qRT-PCR amplification.

Primer name	Sequence	Product length (cm)	Melting temperature (°C)	References
Actin For	5′-CTTCCTCATGCCATCCTGC-3′	140	58.28	Xu *et al.* (2015)
Actin Rev	5′-GCAAGCTTCTCCTTGATGTCC-3′		59.26	
SOD-Cu/Zn For	5′-GCTCTATTGCGTTGTATGCCA-3′	137	59.06	Rossatto *et al.* (2017)
SOD-Cu/Zn Rev	5′-GCTTGACTCCCAAATGGTGAC-3′		59.46	
CAT For	5′-AAGGCCAGACAATGTCAGAT-3′	203	56.81	Yoo *et al.* (2017)
CAT Rev	5′-GTGGCATTAATACGCCAGTA-3′		55.63	
APX For	5′-AGTGTGAACCAGCAGACTAC-3′	198	57.18	Yoo *et al.* (2017)
APX Rev	5′-TCAAGACAGTAGCAGGGGAA-3′		57.98	
GPX For	5′-AGCACCTACAAGGGGAAGGT-3′	199	60.48	Chen *et al.* (2015)
GPX Rev	5′-GAGTGCAAGCAAACTGGACA-3′		58.98	

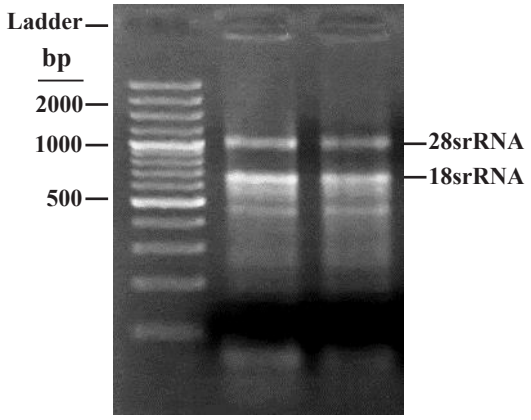

Figure 1. Sample gel electrophoresis image to quantify and qualify extracted RNA.

Afterwards, 5 µl of DEPC (Diethylpyrocarbonate) water were added to reach 11 µl and placed at 70 °C for 5 min. 4 µl of 5 × reaction buffer, 2 µl of dNTP mixture (10 mM), and 20 U/µl of Ribolock RNase inhibitor enzyme were added to reach 19 µL with DEPC water. Then, they were incubated at 37 °C for 5 min and 200 U/µl M-MuLV RT was added. The reaction mixture was heated at 42 °C for 1 h and placed at 70 °C for 10 min to stop the reaction. This cDNA sequence was used in the polymerase chain reaction. This solution contained 3 µl of cDNA, 0.5 µl of each primer (10 pmol), 9.9 µl of SYBR Green PCR Master Mix (2×), 0.5 µl of DMSO, and 5 U/µl of Taq DNA enzyme Polymerase. In this regard, the amount of the reaction solution was adjusted to 17 µl using DEPC water, RNase-free, and used for the reaction. The thermal reaction cycles (qRT-PCR) consisted of six stages. The first stage, called pre-denaturation, consisted of one cycle at 95 °C for 300 s. The second, third and fourth stages were 40 cycles including the denaturation stage at 95 °C for 10 s, annealing stage at primer annealing temperature for 20 s and the extension stage at 72 °C for 10 s. The final extension stage consisted of one cycle at 72 °C for 300 s. The specificity of the amplicons was checked by melting curve analysis (10 s at melting temperature) after 81 cycles (Kazerani and Navabpour, 2019). *Actin*, as a reference gene, and non-stressed samples, as control samples, were used to calculate the relative expression level of oxidative stress pathway genes. The relative level of expression of the studied genes compared to the reference control genes (*Actin*) was analyzed using REST (Relative Expression Software Tool: REST) (Pfaffl *et al.,* 2002) based on the following equation (Equation 1).

$$R=\frac{(E\ target)^{\Delta c\ P\ target\ (Mean\ control-Mean\ sample)}}{(E\ ref)^{\Delta c\ P\ ref\ (Mean\ control-Mean\ sample)}} \quad (1)$$

Data analysis

After extracting the measured data, their normalization was assured and, then, an analysis of variance and a comparison of means, using the least significant difference (LSD), were conducted by SAS. Charts were drawn by Excel.

RESULTS AND DISCUSSION

The results obtained from the combined variance analysis of the levels of yield, ROSs, TBARM and the expression of oxidative stress pathway genes indicated that the difference between the two flooded and drought stress conditions was significant in terms of all the traits at the 1% significance level (Table 2). The genotypes were significantly different in regard to all the traits at 1% significance level (Table 2). This showed the

Table 2. Analyzing the combined variance of the yield, TBARM, ROSs and the expression of antioxidative genes.

Source of variation	df	Mean of square							
		Yield	TBARM	O_2^-	H_2O_2	*SOD*	*CAT*	*APX*	*GPX*
E: Environment	1	8221567.5**	178.79**	0.161**	2.5**	124.56**	116.23**	63.48**	838.99**
E_1: Block (Environment)	4	308.8	0.43	0.0003	0.002	0.69	0.3	0.1	1.1
G: Genotype	4	1875825**	77.53**	0.074**	0.584**	73.1**	53.2**	27.3**	121.2**
G×E	4	1281630**	4.23**	0.003**	0.021**	4.32**	2.89**	1.54**	7.1**
E_2: Residual	16	7.8	0.58	0.0004	0.003	0.72	0.4	0.2	1.2
Coefficient of variation (%)		5.84	2.91	10.48	4.47	5.57	7.07	8.11	6.9

**: Significant at 1% probability levels.

Table 3. Comparing the means of the yield, TBARM, ROSs and the expression of antioxidative genes.

Traits	Ahlemi-Tarom	Sepidrood	Mutant No. 94	Mutant No. 4	Mutant No. 95	LSD test
Yield (Kg/ha)	4100^{e}	4250^{d}	5255^{a}	5187.5^{b}	5120^{c}	3.418
TBARM (µmol/g FW)	14.3^{b}	16.53^{a}	11.57^{e}	12.5^{d}	13.37^{c}	0.487
Superoxide (nmol/min/g FW)	0.21^{b}	0.25^{a}	0.15^{d}	0.18^{c}	0.18^{c}	0.025
Hydrogen Peroxide (µmol/g FW)	0.65^{b}	0.73^{a}	0.48^{e}	0.54^{d}	0.58^{c}	0.033
SOD	4.16^{c}	2.37^{d}	5.05^{a}	4.58^{b}	4.23^{c}	0.278
CAT	2.97^{c}	1.92^{d}	4.09^{a}	3.38^{b}	3.06^{c}	0.267
APX	3.14^{c}	2.18^{d}	4.22^{a}	3.56^{b}	2.83^{c}	0.316
GPX	4.44^{c}	3.3^{d}	10.4^{a}	8.36^{b}	7.92^{b}	0.581

Based on the LSD test, the means of each row with a letter in common did not significantly differ at the 5% significance level.

existence of a genetic variation among the genotypes in the studied traits. The genotype interaction in the environment was significantly different for all the traits at the 1% significance level (Table 2). This illustrated the difference in the trend of variations in the mutants and cultivars in the two environments for the studied traits (Table 3).

Yield

Sepidrood cultivar and mutants 94, 4, 95 and Ahlemi-Tarom cultivars yielded 5600, 5580, 5500, 5450 and 4400 Kg/ha, respectively, the highest to lowest yield under flooded condition (Figure 2). Mutants 94, 4 and 95 and Ahlemi-Tarom and Sepidrood cultivars yielded 4930, 4875, 4790, 3800 and 2900 Kg/ha, respectively, the highest to lowest yield under drought stress condition (Figure 2). Mutant 94 and Ahlemi-Tarom cultivars with the mean yield of 5255 and 4100 Kg/ha had the highest and lowest yield, respectively (Table 3).

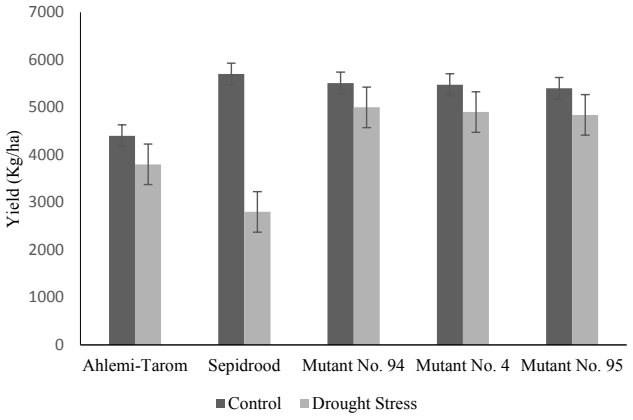

Figure 2. The amount of rice yield during the stage of seeding under the flooded and drought stress conditions. The value of the standard error is shown by a bar.

TBARM

With an increase in drought stress, TBARM was enhanced (Figure 3). The sensitive cultivar Sepidrood and the tolerant mutant 94 had the highest and lowest TBARM values with means of 16.73 and 11.59 µmol/g FW, respectively (Figure 3). The studied cultivars and mutants significantly differed in malondialdehyde (MDA) content (Table 3). MDA is highly stable in the cellular oxidation process. In different studies conducted to examine drought stress, it was found that TBARM increased by enhancing stress levels (Navabpour, 2012c; Navabpour, 2013; Navabpour *et*

al., 2015b; Navabpour *et al.,* 2016). The results of such studies are consistent with the results of the current study. In general, it can be stated that with increasing environmental stresses, an increase in the TBARM index was observed (Navabpour *et al.,* 2015b). The level of MDA increases by various oxidative stress factors, such as salinity stress (Kazemi *et al.,* 2012; Kamali *et al.,* 2015; Ghasemi Mesrami *et al.,* 2016a; Ghasemi Mesrami *et al.,* 2016b), abscisic acid treatment (Navabpour, 2012a), heavy metal poisoning (Navabpour and Mazandarani, 2017), and disease induction (Navabpour *et al.,* 2013; Navabpour *et al.,* 2015a). These results are consistent with the results of the present study.

ROSs

The sensitive cultivar of Sepidrood, the tolerant cultivar of Ahlemi-Tarom, and the following tolerant mutants, i.e., 95, 4 and 94 with means of 0.25, 0.21, 0.18, 0.18 and 0.15 nmol/min/g FW had the highest to lowest levels of superoxide ion, respectively (Figure 4). There was no significant difference between the tolerant mutants 4 and 95 in terms of superoxide ion (Table 3). The sensitive cultivar Sepidrood, the tolerant cultivar Ahlemi-Tarom, and the following tolerant mutants 95, 4 and 94, with means of 0.73, 0.65, 0.58, 0.54 and 0.48 µmol/g FW had the highest to lowest levels of hydrogen peroxide, respectively (Figure 5). The studied plant materials were significantly different in terms of hydrogen peroxide (Table 3). The results of Haddad and Kamangar (2015) can be mentioned in accordance with these results.

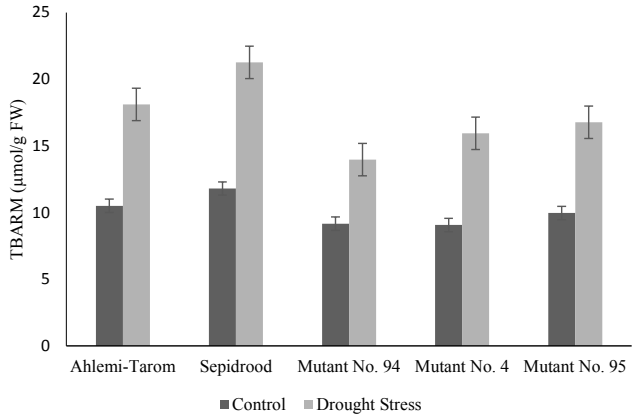

Figure 3. TBARM content in rice leaves during the stage of seeding under the flooded and drought stress conditions. The value of the standard error is shown by a bar.

***SOD* gene**

The differential pattern of the relative expression of the *SOD* gene, as an important defensive and exclusive gene in the pathway for superoxide ion, showed that the tolerant mutant 94 and the sensitive cultivar Sepidrood with means of 5.05 and 2.37 had the highest and lowest relative expression of this gene compared to the control in the studied population, respectively (Figure 6). The relative expression of this gene in the tolerant cultivar Ahlemi-Tarom was not different from that of the tolerant mutant 95 (Table 3). These findings are consistent with the results of a study conducted by Heidari and Haddad (2015).

***CAT* gene**

The differential pattern of the relative expression of the *CAT* gene, as an important induction gene in the pathway for the decomposition of hydrogen peroxide, demonstrated that the tolerant mutant 94 and the sensitive cultivar Sepidrood with means of 4.09 and

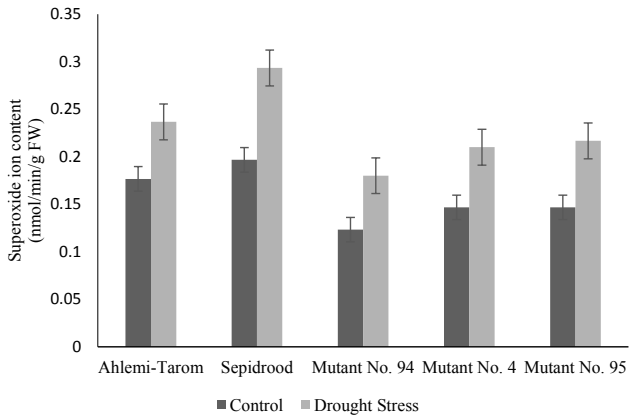

Figure 4. The superoxide ion content in rice leaves during the stage of seeding under the flooded and drought stress conditions. The value of the standard error is shown by a bar.

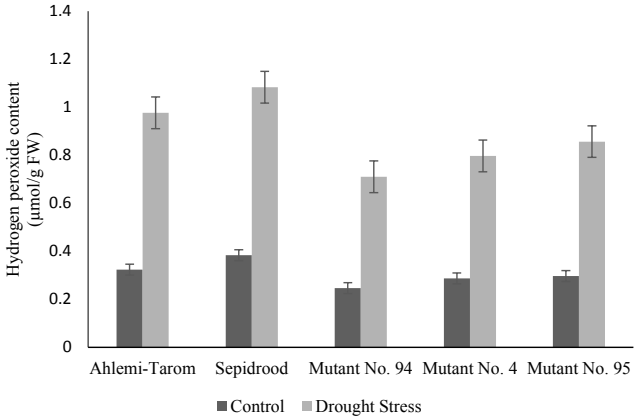

Figure 5. The hydrogen peroxide content in rice leaves during the stage of seeding under the flooded and drought stress conditions. The value of the standard error is shown by a bar.

1.92 indicated the relative expressions of the gene compared to the control, respectively (Figure 7). The relative expression of the *CAT* gene in the tolerant cultivar Ahlemi-Tarom was not significantly different from that of the tolerant mutant 95 (Table 3). In oxidative stresses, such as drought (Mazandarani *et al.,* 2014; Heidari and Haddad, 2015), salinity (Kazemi *et al.,* 2012), senescence (Haddad *et al.,* 2004) and disease (Navabpour *et al.,* 2015a), the *CAT* gene catalyzed hydrogen peroxide by increasing the expression and production of the enzyme. These findings are in line with the present results.

APX gene

The differential pattern of the relative expression of the *APX* gene showed that the tolerant mutant 94 and the sensitive cultivar Sepidrood with means of 4.22 and 2.18 had the highest and lowest relative expressions of the gene compared to the control, respectively (Figure 8). The relative expression of this gene in the tolerant cultivar Ahlemi-Tarom was not significantly different from that of the tolerant mutant 95 (Table 3). These findings are consistent with the results of a study conducted by Heidari and Haddad (2015).

GPX gene

The differential pattern of the relative expression of the *GPX* gene showed that the tolerant mutant 94 and the sensitive cultivar Sepidrood with means of 10.4 and 3.3 had the highest and lowest relative expression of this gene as compared to the control, respectively (Figure 9). The relative expression of this gene was not significantly different in the mutants 4 and 95 (Table 3). These findings are consistent with the results of a study conducted by Navabpour (2012c).

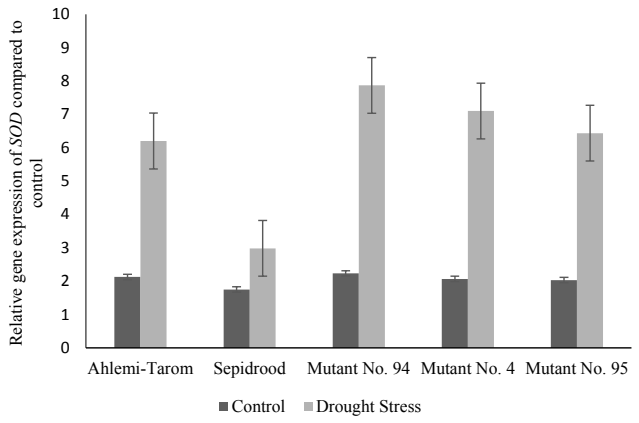

Figure 6. Relative gene expression pattern of *SOD* in rice leaves during the stage of seeding under the flooded and drought stress conditions. The value of the standard error is shown by a bar.

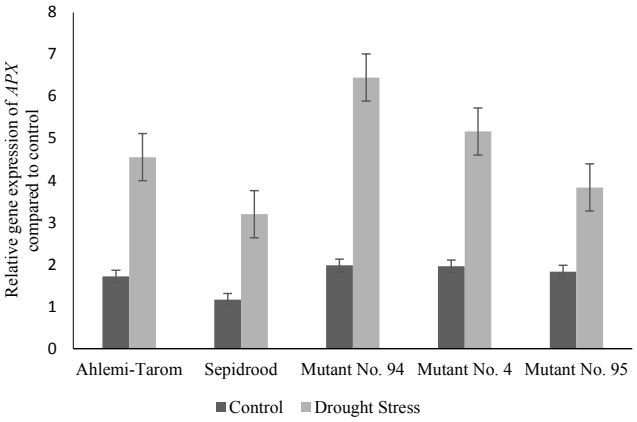

Figure 8. Relative *APX* gene expression in rice leaves during the stage of seeding under the flooded and drought stress conditions. The value of the standard error is shown by a bar.

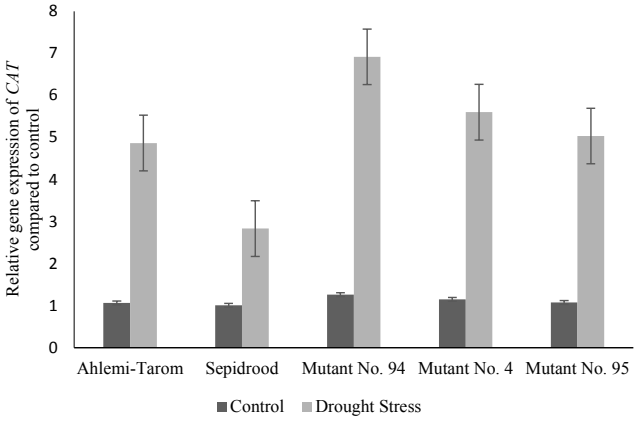

Figure 7. Relative *CAT* gene expression in rice leaves during the stage of seeding under the flooded and drought stress conditions. The value of the standard error is shown by a bar.

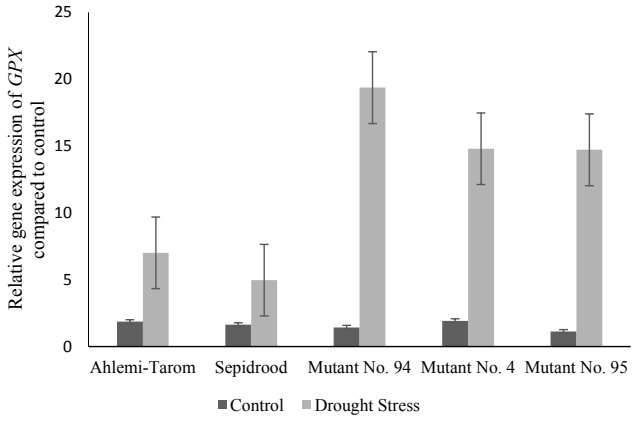

Figure 9. Relative *GPX* gene expression in rice leaves during the stage of seeding under the flooded and drought stress conditions. The value of the standard error is shown by a bar.

CONCLUSIONS

According to the results obtained from this study, the mutants 94, 4, 95 and the cultivars Ahlemi-Tarom and Sepidrood had the highest and lowest levels of defense gene expressions (*SOD*, *CAT*, *APX* and *GPX*) in response to drought stress. In terms of the TBARM index and ROSs (superoxide and hydrogen peroxide), the cultivars Sepidrood, Ahlemi-Tarom and the mutants 95, 4 and 94 had the highest and lowest levels under the drought stress condition. The mutants studied under the drought stress condition could reduce the damage caused by the ROSs by increasing the expression of the antioxidant genes and had a significant superiority (P<5%) compared to the commercial cultivars Sepidrood and Ahlemi-Tarom. In this study, the mutant 94 had the highest yield and probably and could be considered as a suitable genetic potential in terms of drought tolerance. The mentioned mutants in this research can be used as drought-tolerant cultivars after further/details tests, and be applied in rice breeding programs for drought stress conditions.

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