Evaluation of proline content and enzymatic defense mechanism in response to drought stress in rice

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Abstract

To study proline content and enzyme defense system in response to drought stress in rice, a randomized complete block design with three replications was conducted in two separate environments (drought stress and non-stress). The rice cultivars used included two commercial cultivars, i.e., Ahlemi Tarom (low-yield and drought tolerant) and Sepidrood (high-yield and sensitive to drought) and three promising lines of fourth generation mutants (high-yield and drought tolerant), namely, No. 4, 94 and 95 tested on a research farm at Gonbad Kavous University in the 2018 crop year. Plant roots were separately sampled in each block at the reproductive stage. The proline content and activity of the enzymes involved in the defense system, including superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase were measured by extracting from each root sample. Results indicated that the drought-tolerant mutant lines had lower proline contents. The mutant lines and Ahlemi Tarom and Sepidrood cultivars had the highest to lowest levels of defense enzymes in the oxidative pathway, respectively. Correlation was found between the decrease in proline contents and increase in levels of defense enzymes of the oxidative stress pathway. The mutants possessed reliable genetic storages for tolerance to drought stress and had a significant superiority over the commercial cultivars at P≤0.05.

Keywords: ascorbate peroxidase; catalase; glutathione peroxidase; proline; rice; superoxide dismutase

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Introduction

Transferring electrons to molecular oxygen and forming active oxygen species in plant cells are among the consequences of membrane electron transport in chloroplasts, mitochondria, and plasmid membranes. Approximately, one percent of the oxygen consumed by plants is used to produce active oxygen species during normal

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cellular processes. On the other hand, biotic and abiotic stresses increase the formation of various active oxygen species in plant cells. The active oxygen species can lead to the destruction of oxidizers, including superoxide radicals, perhydroxyl radicals, hydrogen peroxide, hydroxyl radicals, alkoxyl radicals, peroxyl radicals, organic hydroperoxides, singlet oxygen, and excited carbonyl (Bhattacharjee, 2005). These radicals, especially at high concentrations, have the potential to oxidize vital cell molecules such as proteins, nucleic acids, and fats. By damaging a cell membrane and interfering with absorption exchanges, these radicals can change the osmotic potential of the cell (Golden et al., 2002). However, the balanced concentration of oxygen radicals is important in inducing the relative adaptability of the plant to provide tolerance to biotic and abiotic stresses. Among the known mechanisms are the activity of the inductive systematic tolerance and the function of the mentioned radicals as messenger transmission molecules in the process of gene expression (Gautam and Stein, 2011; Larkindale et al., 2005). Other disadvantages of the oxidative stress include the decomposition and reduction of protein concentrations in plant tissues which result in an increase in free amino acids such as proline (Ashraf et al., 1994). Generally, in many plants, a free proline accumulation occurs in response to the imposition of a wide range of biotic and abiotic stresses. By osmotic adjustment, proline can stabilize subcellular, cellular, and free radical structures (Ozturk et al., 2012). This is why plants use enzymatic and non-enzymatic defense systems against these stresses. Superoxide dismutase (SOD), catalase (CAT), Ascorbate peroxidase (APX) and glutathione peroxidase (GPX) are some of the most important enzymatic antioxidants. The SOD enzyme plays a unique role in purifying superoxide ions and converting them into water and hydrogen peroxide (Mackerness et al., 1999). Consequently, CAT, APX and GPX convert the formed hydrogen peroxide into water (Asada, 1990; Foyer and Harbinson, 1994; Mittler, 2002). The current study aimed to investigate proline contents and enzyme defense systems under normal and drought stress conditions in rice.

Materials and Methods

Seeds of two rice cultivars, i.e., Ahlemi Tarom (low-yield and drought tolerant) and Sepidrood (high-yield and sensitive to drought), and three promising lines of the fourth generation mutants (high-yield and drought tolerant) numbered 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, farm irrigation was completely cut off from the day 40 after the transplantation (a stage with maximum tillering) until the end of the growth period (Kazerani et al., 2018; Kazerani et al., 2019). During the experimental period, considering the soil moisture curve proportional to the research farm, samples were taken on days 50, 60, 70, 80 and 90 after the cultivation and soil moistures were estimated to be 32%, 24%, 18%, 8% and 4% of the soil water content proportional to -0.05, -0.12, -0.27, 0.72 and -1.1 MPa, respectively (the soil moisture curve is not presented). Since rice is sensitive to drought stress in its reproductive stage (Yoshida, 1981), stress was applied after its vegetative stage (the end of the tillering stage) that is equivalent to the day 40 after transplantation. To prevent any water penetrations from the margin of the farm, distances between the plots were considered two meters and a plastic cover prevented any water penetrations. To assay the biochemical index and antioxidant enzymes (SOD, CAT, APX and GPX) in the reproductive stage (seeding), each plant's root was separately sampled in each block under the stress and non-stress conditions.

To assay the proline content of the roots, Bates (1973) method was used. Initially, roots (500 mg) were ground down with 10 ml of 3% sulfosalicylic acid solution in a mortar. Then, the mixture was filtered with filter paper and 2 ml of the obtained extract was poured into an experiment tube and 2 ml of ninhydrin reagent (resulting from adding 1.25 g of ninhydrin to 30 ml of glacial acetic acid) and 2 ml of glacial acetic acid were added to it. In the next step, the tubes were placed in a bain-marie at 100 °C for one hour, after which the samples were kept in an ice bath for 30

minutes. Afterward, 4 ml of toluene was added to the contents of each tube and mixed by a vortex mixer for 30 seconds. The tubes were held at the room temperature for a while. At this stage, two separate layers were created and, finally, using toluene, the optical absorption of the upper layer at the 520 nm wavelength considered as the control was read by a spectrophotometer and the proline content was determined using a standard curve.

To determine the total amount of SOD, Giannopolitis and Ries (1977) method was used. In this regard, initially, 0.1 gr of the root sample was mixed and uniformed with 3 ml of the reaction solution, including 50 mM potassium phosphate buffer (pH=7.8), 13 mM methionine, 75 µM of tetrazolium, 2 µM riboflavin, 0.1 mM EDTA, and 100 µl of the extraction enzyme. The mixture was placed under bright light conditions of 5,000 lux for 15 minutes. The tetrazolium in the reaction mixture was regenerated under the white light radiation and converted into colored formazan. The maximum absorption of formazan is at the wavelength of 560 nm. On the other hand, the SOD enzyme decreases the amount of tetrazolium regeneration by using H^+ in the cell. Accordingly, increasing the concentration of enzyme extract in the regenerative environment of tetrazolium leads to a reduction in the formation of formazan. As a result, the absorption rate decreases at 560 nm (Beauchamp and Fridovich, 1971). Hence, the calibration was increased by adding 1 unit of superoxide enzyme for regenerating 50% of tetrazolium and the activity was recorded the wavelength of 560 nm spectrophotometrically. To evaluate the CAT activity, Aebi (1984) method was employed. Briefly, 0.1 gr of the root sample was mixed and uniformed with 3 ml of the reaction buffer, including 50 mM potassium phosphate buffer (pH=7.8), standard hydrogen peroxide, and 200 µl of the extraction enzyme. The maximum peroxide adsorption is at the wavelength of 240 nm. Therefore, when the CAT enzyme is present and active in the environment, hydrogen peroxide is degraded and its absorption is decreased at this wavelength by removing the oxygenated water (Aebi, 1984). This is why the absorbance of the solution was read by the spectrophotometer at 240 nm. Nakano and Asada's (1981) method was used to calculate the amount of APX enzyme activity. By applying this method, 0.1 gr of the root sample was mixed and uniformed in 1 ml of the reaction solution, including 50 mM of potassium phosphate buffer (pH=7.8), 0.5 mM of standard APX, 0.1 mM of hydrogen peroxide, and 200 µl of the extraction enzyme. APX breaks down the hydrogen peroxide to two water molecules using ascorbate as a substrate. The maximum absorption of ascorbate is at the 290 nm. Accordingly, with the presence and activity of the APX enzyme in the cell, the ascorbate is gradually oxidized and with a decrease in the ascorbate, the absorbance decreases at 290 nm (Nakano and Asada, 1987). Thus, the absorbance of the light was read at 290 nm and the correction of the values was performed by H_2O_2 without the enzyme.

Moreover, a modified version of the Nickel and Cunningham's (1969) method was used to estimate GPX activity. In this method, 0.1 gr of the root sample was mixed and uniformed with 3 ml of the reaction buffer, including 50 mM of potassium phosphate buffer (pH=7), 25 mM of guaiacol, 10 mM of hydrogen peroxide, and 100 µl of extraction enzyme. Peroxidase enzyme decomposes hydrogen peroxide into a water molecule using guaiacol as a substrate. For this purpose, the peroxidase enzyme oxidizes guaiacol to tetraguaiacol. The maximum tetraguaiacol absorption is at 470 nm. Therefore, with the activity of this enzyme at the mentioned wavelength, an increase in the presence of guaiacol peroxidase can be observed (Chance and Maehly, 1955). In this regard, the absorbance of the solution was read at 470 nm spectrophotometrically for 70 seconds.

After measuring the levels of proline and defense enzymes, the effects of drought stress on these traits were examined under the two environments using an analysis of combined variance. Moreover, the diversity of genotypes was studied. Then, by applying the LSD test, mean levels of these traits were calculated under the both drought stress conditions. All statistical analyses were performed by SAS and charts were drawn using EXCEL.

Table 1
Analyzing the combined variance of the proline content and enzymatic antioxidant systems

Source of Variation	df	Mean Square				
		Proline	SOD	CAT	APX	GPX
E (Environment)	1	0.98**	450.86**	1297.2**	0.96**	32.45**
E_1= Block(Environment)	4	0.001	0.91	1.3	0.001	0.08
G (Genotype)	4	0.53**	93.3**	153.9**	0.53**	12.4**
G×E	4	0.03**	5.12**	8.3**	0.03**	0.7**
E_2= Residual	16	0.002	0.97	1.6	0.002	0.1
cv		4.88	4.16	2.45	3.26	3.37

**: Significant at 1% probability levels.

Table 2
Comparing the means of the proline content and enzymatic antioxidant systems

	AhlemiTarom	Sepidrood	MutantNo. 94	MutantNo. 4	MutantNo. 95	LSD test
Proline (mg/gr FW)	0.4^{B}	0.44^{A}	0.3^{E}	0.34^{D}	0.37^{C}	0.022
SOD (standard unit/mg protein)	11.9^{C}	9.3^{D}	13.95^{A}	12.78^{B}	12.68^{B}	0.618
CAT (µmol/min/mg)	28.7^{D}	23.42^{E}	35.68^{A}	31.97^{B}	30.93^{C}	0.903
APX (µmol/gr FW)	0.29^{C}	0.25^{D}	0.49^{A}	0.46^{B}	0.45^{B}	0.015
GPX (standard unit/gr FW)	10.73^{B}	9.33^{C}	11.35^{A}	11.3^{A}	11.25^{A}	0.445

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

Results

Results obtained from the analysis of combined variance of proline content and enzymatic antioxidant systems demonstrated that the differences between the flooded and drought stress conditions were significant at P≤0.01 (Table 1). There was a significant difference among genotypes in terms of enzymatic antioxidant systems at P≤0.01 (Table 1). This indicated the existence of a genetic variation among genotypes in terms of the studied traits. The genotype interaction in the environment was significantly different at P≤0.01 in terms of the studied proline content and enzymatic antioxidant systems (Table 1). This showed the difference in the trend of variations in the mutants and cultivars in the two environments for proline content and enzymatic antioxidant systems (Table 2).

Decomposing proteins into free amino acids, including proline, is one of the drought stress-related effects. Proline consists of glutamate and ornithine. An increase in proline production is an osmotic adjustment strategy that can reduce plant growth (Mirza Masoumzadeh et al., 2012). Although osmotic adjustment is a kind of stress avoidance mechanism that increases water absorption, it does not play any roles in a plant's tolerance to stress and cannot be considered as a suitable criterion for drought tolerance. It can even be noted that this mechanism is specific to sensitive cultivars (Ahmadi and Sio-Se Mardeh, 2004). In other words, avoidance is not a real mechanism of tolerance to stress (Levitt, 1980). Therefore, increasing proline concentrations in sensitive cultivars under drought stress is associated with more protein decompositions (Ahmadi and Sio-Se Mardeh, 2004); hence, cultivars tolerant to drought stress resist to decomposing proteins into proline and, therefore, they maintain levels of protein concentration and cell interactions under a drought stress condition. Babaee et al., (2010) and Farhoudi and Makezadeh Tafti (2012) reported that, under the influence of drought stress, proline amino acid increased in thyme and chamomile, respectively. Increasing the proline production makes glutamate, a precursor of

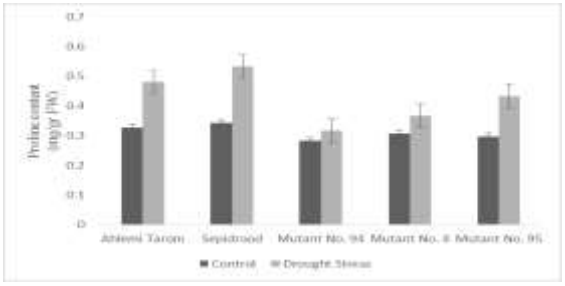

Fig. I. The amount of proline content in rice roots at seeding stage under the flooded and drought stress conditions; the value of the standard error is shown by a bar.

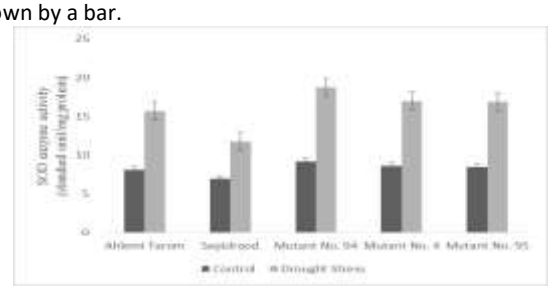

Fig. II. SOD enzyme content of rice roots during at seeding stage under the flooded and drought stress conditions. The value of the standard error is shown by a bar.

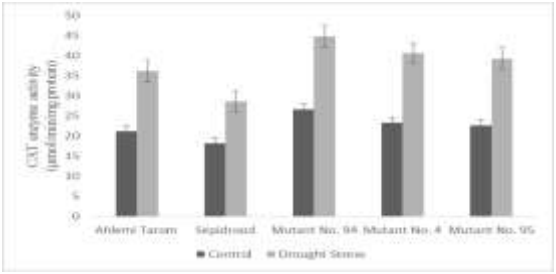

Fig. III. The amount of CAT enzyme in rice roots at seeding stage under the flooded and drought stress conditions; the value of the standard error is shown by a bar.

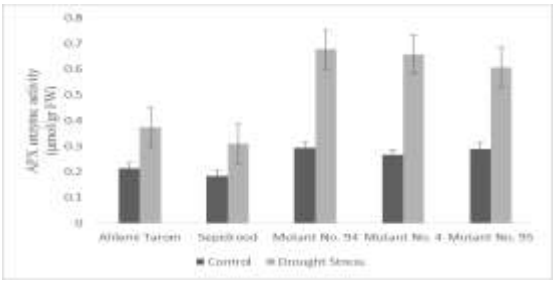

Fig. IV. The amount of APX enzyme in rice roots at seeding stage under the flooded and drought stress conditions. The value of the standard error is shown by a bar.

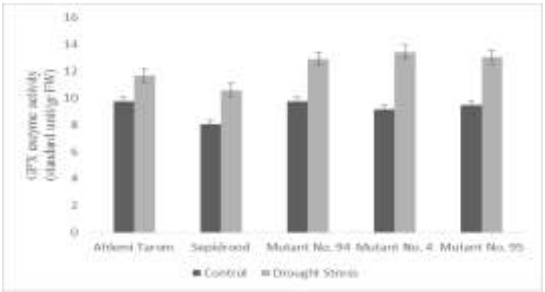

Fig. V. The amount of GPX enzyme in rice roots at seeding stage under the flooded and drought stress conditions; the value of the standard error is shown by a bar.

chlorophyll and proline less involved in chlorophyll biosynthesis pathway (Ghogdi et al., 2013).

The tolerant mutant No. 94 and the sensitive cultivar of Sepidrood with means of 0.3

and 0.44 mg/gr FW had the lowest and highest proline contents, respectively (Fig. I). The studied plant materials were significantly different in terms of proline content (Table 2). In the same line, Hosseini et al., (2017) stated that with increasing drought stress on wheat, a rising trend in proline content could be observed.

A SOD gene encodes a group of metalloenzyme proteins. The enzymatic product of this gene is capable of purifying superoxide ions. Superoxide ions are highly capable of conversion into hydrogen peroxide (Bowler et al., 1992). Converting superoxide to hydrogen peroxide is the first communicative bridge in the enzymatic purification of active oxygen radicals. SOD is regarded as a primary protective mechanism against active oxygen radicals (Asada, 1999). In cellular aging conditions, the increase in superoxide ions occurs as the first loop of the free-radical chain (Choi et al., 2007). Therefore, the role of SOD gene in reducing the frequency of superoxide ions and adjusting their concentration would be important in regulating the concentration of other active radicals, including hydrogen peroxide and hydroxyl radical, effectively (Neill et al., 2001). The double intensity of hydroxyl ion activity and its direct damage to DNA makes this issue really crucial (Bowler et al., 1992). The tolerant mutant No. 94 and the sensitive cultivar of Sepidrood had the highest and lowest SOD activities with means of 95.9 and 9.3 standard unit/mg protein, respectively (Fig. II). There was no significant difference between mutants No. 4 and 95 in terms of the activity of SOD (Table 2). In this regard, Navabpour et al., (2013) and Navabpour et al., (2016) stated that after applying oxidative stress, the exclusive SOD enzyme increased to sweep superoxide which is in line with the results of the present study.

CAT enzyme is found in almost all living organisms and is considered as a category of fermented proteins, and acts in plant and animal cells when the amount of peroxide is high in the cellular environment. This enzyme has a fourfold porphyrin protein structure with iron, which is responsible for the breakdown of free oxygen radicals, especially peroxide oxidation (Yang and Poovaiah, 2002; Du et al., 2007). CAT is present in the peroxisome and eliminates the hydrogen peroxide resulting from the use of photorespiration and β-oxidation of fatty acids (Mittler, 2002). Accordingly, it decomposes oxygenated water into water and oxygen (Du *et al.,* 2008). Considering the plants studied in the current study, the mutant No. 94 and the sensitive cultivar of Sepidrood had the highest and lowest CAT enzyme activities with means of 35.68 and 23.42 µmol/min/mg protein, respectively (Fig. III) and no similarities were observed in terms of the level of activity of this enzyme in the population (Table 2). In various studies carried out under drought stress conditions, it was found that by increasing oxidative stress, the CAT enzyme increased (Mazandarani et al., 2014). These are consistent with the results of this study.

Peroxidases are groups of enzymes that obtain hydrogen for regenerating and removing hydrogen peroxide from other metabolites, such as ascorbate and glutathione and are named according to their cofactors. Among these enzymes is APX (Esfandiari and Javadi, 2014) which is present in most of cellular organelles and plays an important role in the Mahler (Asada, 2000) and glutathione-ascorbate (Halliwell, 2006) cycles. One of the most significant locations for the activity of these cycles is chloroplast. In the cultivars sensitive to stress, such as Sepidrood, a decrease in the activity of APX occurs due to the lack of adjustment of the ratio of H^+, $NADP^+$/NADPH and the potential of chloroplast increases the production of active oxygen and causes severe oxidative stress (Esfandiari and Javadi, 2014). Reducing the activity of CAT and APX can result in the accumulation of hydrogen peroxide and, consequently, can decrease the activity of some of the enzymes of the Calvin cycle, such as ribulose monophosphate, kinase, and bisphosphatase (Amini et al., 2009). The mutant No. 94 and the sensitive cultivar of Sepidrood, with means of 0.49 and 0.25 µmol/gr FW, respectively had the highest and lowest APX activities (Fig. IV). There was no significant difference between the mutants No. 4 and 95, in terms of the activity of APX (Table 2). This is in accordance with the findings of Navabpour (2012).

The GPX enzyme contains the protein part of thiol and plays an important role in reacting to active oxygen radicals (Foyer and Noctor, 2005; Larkindale et al., 2005). This enzyme plays a prominent role in counteracting oxidative stress

and creating a relative equilibrium among ROSs. Such a role may be indirectly induced by a reaction without mediation or through an enzymatic mechanism by reducing the H_2O_2 concentration (Anne and Bruno, 1997; Pitzschke et al., 2006). This enzyme also has a role in inducing the activity of the glyoxylic acid cycle in the process of converting fatty acids to sugar and producing cellular energy (Ettinger et al., 1990). It also plays a very critical role in protecting cell membranes, especially under stress conditions (Maiorino et al., 1990; Herbette et al., 2002). Regarding the role of the cell membrane in the selective exchange of materials and ions, the protective mechanism and the role of GPX are very vital in protecting cell membranes and preventing peroxidation of membrane fats (Jung et al., 2002). This enzyme plays an exclusive role in the direct reduction of phospholipid hydrogenase and complex hydroxypropoxy to protect the membrane against oxidative stress (Herbette et al., 2002; Jung et al., 2002). The tolerant mutant No. 94 and the sensitive cultivar of Sepidrood, with means of 11.35 and 9.33 standard unit/gr FW, had the highest and lowest GPX activities, respectively (Fig. V). There was no significant difference in the activity of this enzyme among the mutants No. 94, 4 and 95 (Table 2). It was reported that the enzyme defense system degraded ROSs and provided an acceptable product under the drought stress condition (Navabpour, 2013). To a large extent, the above-mentioned results are in line with the results of the present study.

Discussion

Results of the current study indicated the active role of the enzymatic defense system of plants in response to drought stress. This is while many studies demonstrated the active role of this defense system in response to biotic and abiotic stresses. Overall, in this experiment, the mutant lines No. 94, 4 and 95 showed higher tolerance compared to Ahlemi Tarom and Sepidrood cultivars by indicating more favorable characteristics in terms of the activity of antioxidant enzymes under drought stress condition. Since tolerance to drought stress in plants is relative, genotypes that are superior to others with respect to tolerance to drought stress can be identified and introduced by evaluating the activity of the enzymatic and non-enzymatic systems and also examining other defense mechanisms, including the expression of drought tolerance genes. It seems that using the mutant cultivars examined in the present study, after being completely homozygous in hybridization projects, is justifiable and this can help us to apply the capacity of these genotypes to induce tolerance to drought stress.

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