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The Effect of Water Stress on the Antioxidant Content, Protective Enzyme Activities, Proline Content and Lipid Peroxidation in Wheat Seedling

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Abstract: An investigation was conducted to study the effect of water stress on the antioxidant content, protective enzyme activities, proline content and lipid peroxidation in wheat seedlings. Drought stress increases the amount of Reactive Oxygen Species (ROS), leading to metabolic disorders. It is now known that higher levels of activity- protective mechanisms render the cells more enduring against environmental stress including drought. Two widely cultivated cultivars of wheat in Iran, Sab. and N. Sar. were grown up according to the hydroponic method. Having reached the stage of 4-5 leaves growth; the plants were kept under 4, 8 and 12 bars potential resulting from using Polyethylene Glycol 8000 (PEG 8000). Hogland solution was used as the control. Then the amount of ascorbate, glutathione, superoxide dismutase and catalase activity, proline and lipid Peroxidation was measured in cut samples of the leaves. The result indicated an increase in the amount of Ascorbate and Glutathione as the stress was intensified in the case of Sab. Moreover, the reduced form of Ascorbate (ASC) and Glutathione (GSH) were higher in Sab. at 8 and 12 bars. The amount of Proline accumulation was considerably higher in Sab. than N. Sar. SOD activity, on the other hand, diminished at 8 and 12 bar levels. CAT activity is also regarded as a limiting factor. Lipid peroxidation was also geared up as the stress was intensified. These limiting factors rendered N. Sar. cultivar more sensitive to water stress resulting from PEG8000 compared to Sab.

Key words: Antioxidant, antioxidant enzyme, lipid peroxidation, proline, wheat seedling

INTRODUCTION

As one of the most important crops in Iran, wheat has a significant role in food security in the country. Most of the wheat-cultivated area in Iran is placed on dry lands where, unfortunately, there is not a regular pattern for precipitation. For this reason, drought stress is regarded as the most important limiting factor in these areas, being revealed in two periods of wheat life, i.e. in the seedling stage in fall and in the end of growth period (bolting to maturity) (Esfandiari *et al*., 2007a).

Reactive oxygen species (ROS) are regarded as the main source of damage to cells under drought stress which are produced in vital processes such as photosynthesis, photorespiration and respiration (Jimenez *et al*., 1998; Kornyeyev *et al*., 2003; Mittler, 2002; Taylor *et al*, 2003; Vaidyanathan *et al*., 2003; Uchida *et al*., 2002). ROS are partially-reduced forms of atmospheric oxygen (O_2). They typically result from the excitation of O_2 to form singlet oxygen ($^1O_2$) or from the transfer of one, two and three electrons to O_2 to form, respectively, Superoxide Radical (O_2^-), Hydrogen Peroxide (H_2O_2) or a Hydroxyl Radical (HO) (Mittler, 2002). In contrast to atmospheric oxygen, ROS are highly cytotoxic and can seriously react with vital biomolecules such as lipids, proteins, nucleic acids, etc., causing lipid peroxidation, protein denaturing and DNA mutation amount to, respectively (Breusegem *et al*., 2001: Quiles and López, 2004; Scandalios, 1993). Evidence suggests that membranes are the primary sites of water deficit injury to cells and organelles (Candan and Tarhan, 2003), because ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasmalemma or intracellular organelles (Karabal *et al*., 2003; Stewart and Bewley, 1980). Peroxidation of plasmalemma leads to the leakage of cellular contents, rapid desiccation and cell death. Intracellular membrane damage can affect the respiratory activity in mitochondria, causing pigment to break down and leading to the loss of carbon-fixing ability in Chloroplasts (Scandalios, 1993).

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Fortunately, plants have evolved both enzymatic and non-enzymatic protective mechanisms to eliminate or reduce ROS, which are effective at different levels of stress-induced deterioration (Beak and Skinner, 2003). Enzymatic antioxidant system is one of the protective mechanisms including Superoxide Dismutase (SOD: EC 1.15.1.1), which can be found in various cell compartments. It catalyses the disproportionation of two $O_2^{\cdot-}$ radicals to H_2O_2 and O_2 (Scandalios, 1993). H_2O_2 is eliminated by various antioxidant enzymes such as Catalases (CAT: EC 1.11.1.6) (Kono and Fridivich, 1983; Scandalios, 1993) and Peroxidases (POX: EC 1.11.1.7) (Gara *et al*., 2003; Jablonski and Anderson, 1982) that, in turn, convert H_2O_2 to water. Other enzymes that are very important in ROS scavenging system and function in Ascorbate-Glutathione cycle are Glutathione Reductase (GR:EC1.6.4.2), Monodehydro Ascorbate Reductase (MDHAR: EC1.6.5.4) and Dehydroascorbate Reductase (DHAR: EC 1.8.5.1) (Candan and Tarhan, 2003; Yoshimura *et al*., 2000). Low molecular mass antioxidants such as Ascorbate and Glutathione make up the other part of ROS scavenging system (Reddy *et al*., 2005). These antioxidants can directly interact with and detoxify oxygen-free radicals like Superoxid and Hydroxyl radical. Moreover, Ascorbate and Glutathione are cofactors of Ascorbate Peroxidase (APX) and Glutathione Peroxidase (GPX) respectively, which play a critical role in Ascorbate-Glutathione and water-water cycles (Mittler *et al*., 2004). These cycles are considered very important in plant cells, which contribute to detoxifying ROS.

Furthermore, ROS are inevitable by-products of normal cell metabolism (Martinez *et al*., 2001), but, under normal conditions, production and destruction of ROS is well regulated in cell metabolism (Mittler, 2002). When a plant is encountered with harsh conditions, ROS production will overcome scavenging systems and oxidative stress will burst.

The accumulation of free proline in plant cells is regarded as a conspicuous reaction of the plant when encountered with environmental stress such as drought and salt so that the amount of proline in cells is a few times more than the rest of amino acids. In addition to reducing the water potential in the cell, proline accumulation appears to render membranes more enduring (Mansour, 1998; Sairam *et al*., 2002). On the other hand, the consumption of $NADPH,H^+$ through biosynthesis leads to a more balanced ratio of $NADP^+/NADPH,H^+$, resulting in an increased redox potential as well as reduced acidity in the cell (Sánchez *et al*., 2002). Therefore, proline accumulation makes photoinhibition in chloroplast and ROS production in other organelles less likely to happen (Sánchez *et al*., 2002).

As mentioned before, drought stress is one of the most important environmental limiting factors in Iran. Furthermore, wheat is a strategic crop, which plays a crucial role in food security and sustainability. On the other hand, drought stress causes oxidative stress in plant cells to degrade vital biomolecules. These facts explain the necessity of research about water-stress-tolerance mechanisms, especially ROS scavenging systems in wheat. In this research two wheat cultivars (Sabalan (Sab.) and normal Sardari (N. Sar.)) were selected and cultivated in an extensive area in the North and North-West of Iran where they were grown hydroponically. Wheat seedlings were treated with Hoagland's solution. PEG treatments of 4, 8 and 12 bars were applied, together with a control for 10 days. With the respect of Anti-oxidant enzymes importance and their role in abiotic stress, especially drought, the activity levels of SOD, CAT and total amount of Ascorbate (ASC+DHA) and Glutathione (GSH+GSSG), with reduced form of Ascorbate (ASC) and glutathione (GSH) and proline content and Lipid Peroxidation were measured as this period was elapsed.

MATERIALS AND METHODS

The stock of wheat seeds (Sab. and N. Sar. cultivars) were obtained from International Center for Agricultural Research in the Dry Areas (ICARDA), Maragheh, Iran, during the year 2005. They were surface-sterilized with 0.1% Sodium Dodecyl Sulphate (SDS) solution on a magnetic stirrer for 20 min and thoroughly washed with de-ionized water. Then, the wheat seeds were immersed in distilled water for 6 h. After that, the seeds were kept in a dark environment with the temperature of 20±0.5°C until the seeds started to germinate. At this stage, the germinated seeds were homogenized and transferred to special pots for hydroponic growing. Wheat seedlings were grown in Hoagland's solution until they got 4-5 leaves. At this stage, 4, 8 and 12 osmotic bars adapted from Michel's (1983) model were created using PEG8000. Hoagland's solution, on other hand, was used as control. The seedlings received the treatment for 10 days. With the elapse of the period, the samples of the absolutely young leaves were taken and immersed in liquid nitrogen. The samples were preserved at -70°C until the parameters had to be measured. It needs mentioning that the greenhouse temperature was adjusted at 20±2°C, the lighting period was 12 h with the light intensity of 200 μmol m^{-2} sec^{-1} during the growing period of seedlings.

Measuring biochemical and physiological parameters: The amount of Ascorbate, Glotathione and Proline were

measured according to the methods presented by Law *et al.* (1983), Fadzilla *et al.* (1997) and Bates (1973), respectively. CAT and SOD were extracted according to Esfandiari (2007), while their activities and the amount of lipid peroxidation were measured through the methods offered by Aebi (1984), Sen Gupta *et al.* (1993) and Stewart and Bewley (1980).

RESULTS AND DISCUSSION

Unlike Sab., the amount of ASC+DHA in N. Sar. was reduced in drought stress conditions (Table 1). Meanwhile, the amount of the ASC declined as the water stress level was increased in both Sab. and N. Sar., though N. Sar. appeared more sensitive toward water stress, causing the control to show a significant difference with all other water stress ($p<5\%$). Besides, the minimum amount of ASC was achieved at 12 bars with a relatively slower reduction of ASC in the case of Sab., i.e., a significant difference occurred only at 8 and 12 bars with the control and 4 bars ($p<5\%$). Moreover, there appeared to be more ASC in Sab. compared to N. Sar. at all levels except the control (Table 1).

The amount of GSH+GSSG in N. Sar. was constant at 8 bars while the amount declined significantly at 12 bars in comparison to other levels ($p<5\%$). In Sab., on the other hand, the amount grew bigger with increasing water stress in a way that the highest amount was observed at the level of 12 bars (Table 2). The amount of GSH in N. Sar. declined as the intensity of water stress was increased so that there was a significant difference ($p<5\%$) between 8 and 12-bar levels on the one hand and control and 4-bar levels on the other. However, the variations in the amount of GSH in Sab. did not follow any particular pattern and there appeared to be a significant difference between the amount of GSH and the control at 12 bars level ($p<5\%$).

The highest amount of proline was observed at 12 bars in the case of both cultivars studied. This can be indicative of the fact that proline is accumulated as the water stress is magnified. However, the amount of accumulated proline at 12-bar level was significantly ($p<5\%$) bigger in the case of Sab. than that of N. Sar. Furthermore, the difference in terms of proline accumulation in the case of N. Sar. turned out to be significantly between 12 bars and control, only while Sab. cultivar showed a significant difference in terms of proline accumulation between 12-bar level and all other levels ($p<5\%$) (Fig. 1).

SOD variations at all levels of water stress did not appear to be significant in Sab. while the SOD activity at 8 and 12-bar levels was considerably lower at 4-bar level and the control in the case of N. Sar. SOD activity, on the other hand, in N. Sar. and Sab. exhibited significant difference only at 8 and 12-bar levels (Fig. 2).

CAT activity measurement in N. Sar. indicated identical variational trend for all levels of water stress, but in the case of Sab., CAT activity at 12-bar level was significantly ($p<5\%$) higher compared to that of other levels (Fig. 3).

In the same way, lipid peroxidation in both N. Sar. and Sab. increased as water stress was intensified. A significant difference was also observed between the two cultivars at all levels except the control. Furthermore, lipid peroxidation turned out to be higher in N. Sar. at all levels except the control (Fig. 4).

Ascorbate and glutathione are believed to be present in two forms-- reduced and oxide-- in all cell organelles (Noctor *et al.*, 1998). These antioxidants, in their reduced forms, have the tendency to react directly with ROS (Reddy *et al.*, 2005; Potters *et al.*, 2002). It should be reminded that ascorbate and glutathione are the key elements in Mehler (Asada, 2006), xanthophyll (Potters *et al.*, 2005) and ascorbate-glutathione cycles (Jiang *et al.*, 2006). The higher the amount of GSH/GSH+GSSG and ASC/ASC+DHA is, the higher redox potential the cell enjoys and the better position it will be in (Granczarska, 2005; Noctor *et al.*, 1998). A decrease in the ASC+DHA and GSH+GSSG pool size with their reduced form of water stress conditions is considered a limiting factor in N. Sar. Therefore, ROS is encumbered as

Table 1: The effect of water stress on total ascorbate (ASC+DHA) and reduced form of ascorbate (ASC) values in wheat seedling

	ASC+DHA (μmol g DW)				ASC (μmol g DW)			
Cultivars	Control	4 bar	8 bar	12 bar	Control	4 bar	8 bar	12 bar
Sab.	76.98	97.06	92.17	83.44	69.24	72.91	55.63	43.67
N. Sar.	102.04	94.04	66.68	57.87	89.27	67.13	32.98	20.85
$LSD_{5\%}$	18.15				13.29			

Table 2: The effect of water stress on total glutathione (GSH+GSSG) and reduced form of glutathione (GSH) values in wheat seedling.

	GSH+GSSG (μmol g DW)				GSH (μmol g DW)			
Cultivars	Control	4 bar	8 bar	12 bar	Control	4 bar	8 bar	12 bar
Sab.	19.68	27.12	27.69	31.90	18.50	23.55	13.26	18.28
N. Sar.	26.45	25.39	23.65	19.70	23.81	19.95	6.41	2.89
$LSD_{5\%}$	3.77				4.33			

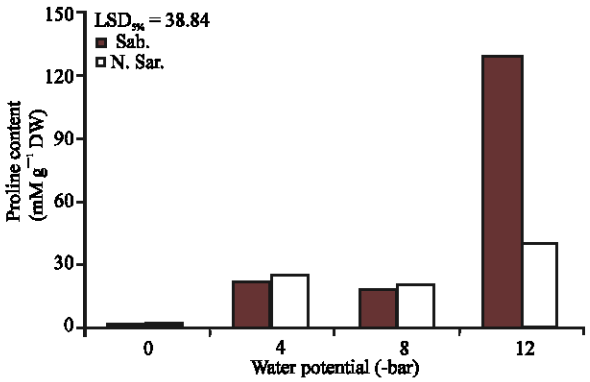

Fig. 1: Proline content of two wheat cultivars at different water potential levels

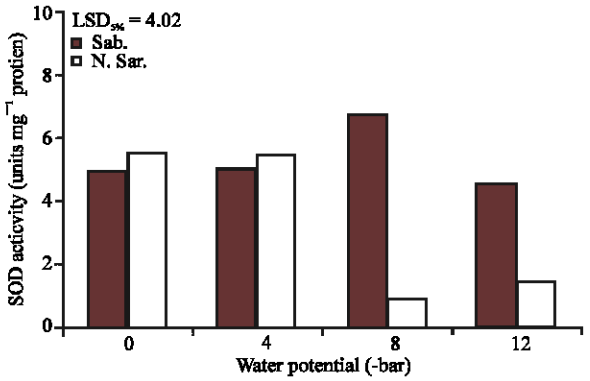

Fig. 2: Superoxide dismutase activity of two wheat cultivars at different water potential levels

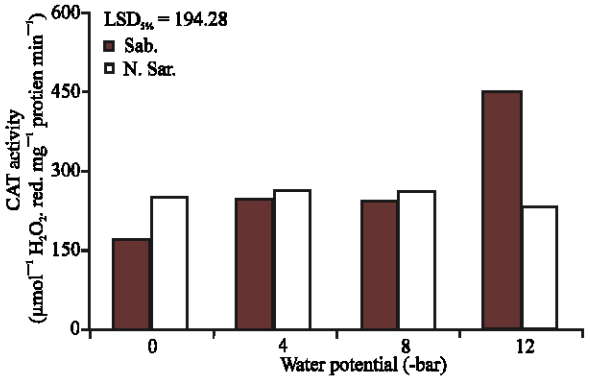

Fig. 3: Catalase activity of two wheat cultivars at different water potential levels

a result of defensive mechanisms, increasing the risk of harm to biomolecules (Esfandiari, 2007).

SOD activity is significantly ($p<5\%$) dropped at higher levels of water stress in the case of N. Sar. (Fig. 2).

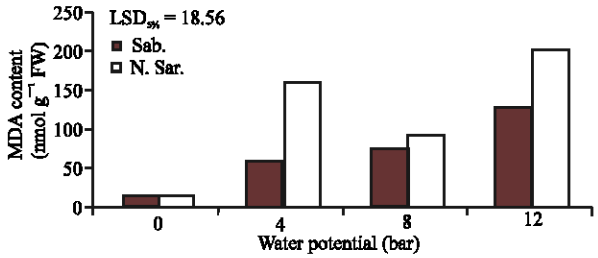

Fig. 4: Malondialdehyde content of two wheat cultivars at different water potential levels

The drop in SOD activity level leads to the accumulation of $O_2^{\cdot-}$ radical and the occurrence of Haber-Weiz reaction, which in turn results in a highly toxic hydroxyl radical to appear (Mittler, 2002). These active oxygen radicals are extremely toxic for cells, invading critical and vital points in the cell (Mittler *et al*., 2004). The increase in the amount of radicals causes photosynthesis to diminish as a result of destroying protein D_1 in PS II (Lu and Zhang, 1999). Moreover, the decrease in the amount of antioxidant enzymes as well as disturbed metabolism can result from the diminished activity level of SOD. It can be concluded that the low SOD activity is the cause of intensified oxidative tension in N. Sar. compared to that of Sab.

CAT is another important antioxidant enzyme that converts H_2O_2 to water in the Peroxysomes (McCord and Fridivich, 1969; Fridivich, 1989; Mittler *et al*., 2004). In this organelle, H_2O_2 is produced from β-oxidation of fatty acids and photorespiration (Morita *et al*., 1994). The activity level of this enzyme in N. Sar. was identical at all levels of water stress while the activity level turned out to be the highest at 12-bar level in the case of Sab. (Fig. 3). The lower activity level of CAT at 12 bars may have caused H_2O_2 to be accumulated in N. Sar. much more than that of Sab. while the APX in N. Sar. was lower than that of Sab (the related data is not presented). The accumulation of H_2O_2 is diminished as a result of reduced APX and CAT activity as well as the drop in the activity level of Calvin cycle enzymes such as Ribulose Monophosphate Kinase and Biphosphatase, which are believed to be very sensitive to higher amounts of H_2O_2 (Yamazaki *et al*., 2003). The activity drop in these vital enzymes results in the lower CO_2 fixation, $NADPH,H^+$ non-use and diminished $NADP^+/NADPH,H^+$. As a result, ROS over production in chloroplast causes damage to thylakoid leading to photoinhibition. Since the Fe-SOD and Cu/Zn-SOD isozymes are sensitive to higher levels of H_2O_2, there will be a decrease in their activity levels (Milone *et al*., 2003). The lower CAT activity level in N. Sar. seems to have intensified oxidative stress level by

affecting some other points compared to that of Sab., leading ultimately to intensified oxidative stress.

The result of the study indicates that the amount of damage to cell membrane in both cultivars increased as the water stress was magnified, though the damage was larger in the case of N. Sar. (Fig. 4). The explanation can be that, in N. Sar., the mechanisms responsible for scavenging ROS and those responsible for preventing ROS-generation are limited by the decrease in the pool size of ASC+DHA and GSH+GSSG (Table 1, 2), as well as the drop in the activity levels of such vital enzymes as SOD and CAT (Fig. 2, 3). As a result, the protective mechanisms act less efficiently in N. Sar. cells than in Sab. cells so that the scavenging mechanisms of ROS are overcome by the amount of generated ROS, leading to the destruction of critical points by ROS and increased damage to the membranes in N. Sar. (Fig. 4). Sab, on the other hand, exhibits a higher amount of antioxidants and better antioxidant enzyme activity in conditions of high water stress in comparison to N. Sar. (Table 1, 2, Fig. 2, 3). The findings of the study correspond to the other findings indicating a negative correlation between the activity level of protective mechanisms in cells and the amount of damage to membranes as reported by Bor *et al*., (2003), Shao *et al.* (2005) and Esfandiari *et al.* (2007a, b).

The maximum amount of proline in both Sab and N. Sar. cultivars appeared at 12 bars amounting to 109.91 and 26.8 times as much as that of the control, respectively (Fig. 1). Sànchez *et al.* (2002) reported the desirable amount of proline accumulation in stressed environment variable between 3 to 300 in different plants and cultivars. Lacerda *et al.* (2005) maintain that the amount of proline is higher in stress-resisting cultivars than the stress-sensitive ones, which is in accordance with the findings of the present study. The increased amount of proline in Sab. is an indicative of the increased activity level of proline biosynthesizing enzymes at higher water levels compared to that of N. Sar. proline is commonly used as a physiological factor in selecting those plant cultivars sensitive to environmental stress. So, proline accumulation increases the $NADP^+/NADPH,H^+$ ratio, increasing the redox potential of cells (Ashraf and Foolad, 2007). Furthermore proline accumulation leads to the stability of membranes and enzyme structures inside the cell (Sairam *et al*., 2002). The results from proline accumulation in Sab. and higher stability in its membranes are in accordance with each other (Sairam *et al*., 2002), indicating the key role of proline in cutting ROS generation and decreasing the damage to biomolecules accordingly (Sànchez *et al*., 2002).

By and large, it can be concluded that ROS target the critical points on plant cells. Damage to these cells disturbs metabolism, resulting, in turn, in further production of ROS. So, the amount of produced ROS overcomes the protective mechanisms leading to a more intensified oxidative stress. That is, the explanation why N. Sar. cultivar in this study grew more sensitive to the water stress resulting from PEG8000, in comparison to Sab.

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