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Differential Responses of Antioxidative System to Soil Water Shortage in Barley (*Hordeum vulgare* L.) Genotypes

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Abstract

Drought is one of the major factors limiting the yield and quality of crops in the world. The activity of antioxidative system to tolerate the drought stress is significant in plants. In the present study, the activities and isoform profiles of catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and superoxide dismutase (SOD) were analyzed in four barley genotypes grown under soil water restriction. Drought stress caused increase in the activities of CAT and SOD in all studied genotypes, while APX activity decreased. The total GR activity increased substantially in genotypes K 2778 and St.Garabag 7 and decreased in No. 77 local and St.Pallidum 596 genotypes under conditions of severe water stress. No detectable differences were observed in the isoenzyme pattern (the appearance of a new isoenzymes and disappearance of another one) between control plants and those subjected to soil drought. However, intensification of corresponding isoforms in electrophoretic spectra was observed in stressed barley leaves relative to watered ones. The obtained results possibly suggest that antioxidant protection in barley plants under drought conditions could be attributed mainly to SOD and CAT.

Keywords

***Hordeum vulgare* L., Drought, Reactive Oxygen Species, Antioxidant Enzymes**

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1. Introduction

Barley, in comparison with other cereal crops, has a better fodder value including both grain and straw. The major environmental factor that constrains the productivity and stability of crop plants is water stress [1] [2]. According to the different scenarios predicted by the Intergovernmental Panel on Climate Change [3] it is expected that there will be a reduction in precipitation and rising evapotranspiration rates.

When plants are exposed to environmental stresses such as drought, reactive oxygen species (ROS) such as superoxide (O_2•), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•) and singlet oxygen ($^1O_2$) are produced [4]. The balance between the production of ROS and the quenching activity of the antioxidants is disturbed and this often results in oxidative damage. ROS are highly reactive to membrane lipids, protein and DNA [5]. Plants have developed efficient antioxidant system that can protect plants from this disaster [6]. The toxic effects of ROS are counteracted by enzymatic as well as non-enzymatic antioxidative system such as: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbic acid (AsA), tocopherol, glutathione and phenolic compounds, etc. Normally, each cellular compartment contains more than one enzymatic activity that detoxifies a particular ROS. Presence of these enzymes in almost all cellular compartments clears their crucial role of ROS detoxification for the survival of the plant [7] [8]. During the past few years, considerable progress has been made in understanding how plants protect themselves against oxidative stresses. In plants, over 150 genes encode for different ROS-detoxifying or ROS-producing enzymes forming well organized ROS gene web [9]. Several genes encoding for plant antioxidant enzymes have been cloned, characterized, and used in the construction of transgenic lines [10]. Genetic analysis of the paraquat-tolerant *Conyza bonariensis* indicated that all the three enzymes of HalliwelI-Asada pathway, *i.e.* SOD, APX and CAT, co-segregate [11]. Manipulation of genes that protect and maintain cellular functions or those maintain structure of cellular components has been the major target of attempts to produce plants that have enhanced stress tolerance.

Analysis of numerous literature data on this topic suggests that the common response to stress does not exist [4] [12]-[14]. The response of the antioxidant system to the stress effect of the same type depends on the plant species since the reaction of antioxidant system in leaves of various plants differs at the same level of drought. Activities of cytosolic ascorbate peroxidase and MDA-reductase decreased, whereas SOD and CAT activities remained unchanged in sorghum. In contrast, activities of chloroplastic peroxidase and catalase increased in sunflower [15]. Besides, the response of the antioxidant system is characterized by the degree and duration of stress. For example, in the two grass species activities of SOD, CAT and POD increased under soil drought, but when stress intensified, under severe stress the enzyme activities decreased, which correlated with the chlorophyll loss and destruction of membranes [16]. The reaction of the antioxidant system to stress probably depends on the initial level of antioxidant activity, which in turn is determined by the physiological state of the plant [17]. The activity of the antioxidant system could also be stipulated by the plant age and cultivation conditions [18].

Considering the above-mentioned, the aim of this work was to study the antioxidant enzymes, including catalase, ascorbate peroxidase, glutathione reductase and superoxide dismutase in barley genotypes grown under soil water deficit.

2. Materials and Methods

2.1. Plant Materials

St.Garabag 7 and No. 77 local species of Nutans specific diversity and St.Pallidum 596 and K-2778 genotypes of Palladium specific diversity were chosen as the investigation objects. The plants were cultivated at the Jalilabad Regional Experimental Station of the Azerbaijan Research Institute of Crop Husbandry under normal and drought conditions.

2.2. Enzyme Extraction and Activity Determination

Enzyme extract was prepared by homogenizing leaf material (1 g fr wt) with a pestle in an ice-cold mortar with 0.05 M Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0). The homogenates were filtered through four layers of cheesecloth and then centrifuged at 4˚C. The supernatant were collected and used for the assays of enzymatic activities.

2.3. CAT

The activity of catalase was determined as a decrease in absorbance at 240 nm for 1 min following the decom-

position of H_2O_2 as described by Kumar and Knowles [19]. The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2. The reaction was initiated by adding the enzyme extract. CAT activity (μmol·min^{-1}·mg^{-1}·protein) was defined using molecular extinction coefficient ε = 39.4 mM^{-1}·sm^{-1}.

2.4. APX

The activity of ascorbate peroxidase was assayed according to Nakano and Asada [20]. The assay mixture consisted of 0.05 mM ASA, 0.1 mM H_2O_2, 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.6), and 0.3 mL enzyme extract. The activity was measured as a decrease in absorbance at 290 nm for 30 sec. APX activity (μmol·min^{-1}·mg^{-1}·protein) was defined using molecular extinction coefficient ε = 2.8 mM^{-1}·sm^{-1}.

2.5. GR

Glutathione reductase activity was determined at 340 nm for 10 min in reaction mixture containing 100 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 0.2 mM NADPH and 0.5 mM GSSG [21]. GR activity (μmol·min^{-1}·mg^{-1}·protein) was defined using molecular extinction coefficient ε = 6.2 mM^{-1}·sm^{-1}.

2.6. SOD

Superoxide dismutase activity was estimated by using SOD Assay Kit-WST (Sigma-Aldrich, USA). The absorbance was recorded at 450 nm and one enzyme unit of SOD activity was defined as the amount of the enzyme required to cause 50% inhibition of the rate of NBT reduction.

Protein content was determined according to Sedmak [22] by using bovine serum albumin as a standard.

2.7. Determination of the Isoenzyme Composition of Enzymes

Qualitative changes of the enzyme activity were investigated with native polyacrylamide gel (PAGE) using the Laemmli method [23] of gel electrophoresis with some modifications. The protein amount was determined by the method of Sedmak [22]. Bovine serum albumin was used for the construction of standard curve. An equal amount of the enzyme extract was mixed with bromophenol blue and glycerol to a final concentration of 12.5% (v/v). To determine the isoform composition of enzymes the method of electrophoresis in 10% (for APX) and 7% (for CAT) polyacrylamide gel under non-denaturating conditions in Tris-HCl buffer (pH 8.3), 4˚C, 3 h supply of constant current of 30 mA was used. 2 mM sodium ascorbate was added to the electrode buffer to detect APX activity. Visualization of APX isoforms was conducted according to Mittler and Zilinskas [24]. For visualization of ascorbate peroxidase, the gel was incubated in a solution containing 50 mM potassium phosphate buffer (pH 7.0) and 2 mM ascorbate-Na, for 30 min. Then the gel was incubated in a solution containing 50 mM potassium phosphate buffer (pH 7.0), 4 mM sodium ascorbate and 2 mM H_2O_2, for 20 min and stained in a solution containing 50 mM potassium phosphate buffer (pH 7.8), 28 mM TEMED and 2.45 mM nitro blue tetrazolium, for 15 min by mixing on a shaker. The gel was washed with distilled water to remove excess stain and was photographed. Catalase was stained on the gel by incubation in the dark for 20 min in the substrate (3.27 mM H_2O_2) and developing (1% potassium ferrocyanide and 1% ferric chloride) solutions [25].

3. Results and Discussion

Significant variations for antioxidant activity were found out among genotypes grown under normal water supply and drought stress. One of the antioxidant enzymes playing the main role in the plant protection system against oxidative stress is catalase. This enzyme provides a rapid utilisation of hydrogen peroxide [26]. Thus, catalase activity was assayed under normal and stress conditions in St.Garabag-7 and No. 77 local species of Nutans specific diversity and St.Pallidum-596 and K-2778 genotypes of Palladium specific diversity (**Figure 1**). Significant differences were not observed for catalase activity in normally watered plants. Catalase activity was higher in St.Garabag-7 (140 ± 12 unit/mg protein) and lower in St.Pallidum 596 (90 ± 10 unit/mg protein) relative to other genotypes. In No. 77 local and K-2778 genotypes catalase activity was almost the same under normal conditions.

CAT activity increased in all genotypes subjected to drought stress. CAT activity in St.Garabag-7 genotype was found to be 260 ± 24 unit/mg protein, indicating 2-fold increase in the enzyme activity under drought compared with the normal conditions. Similar to normal conditions increases in catalase activity under stress

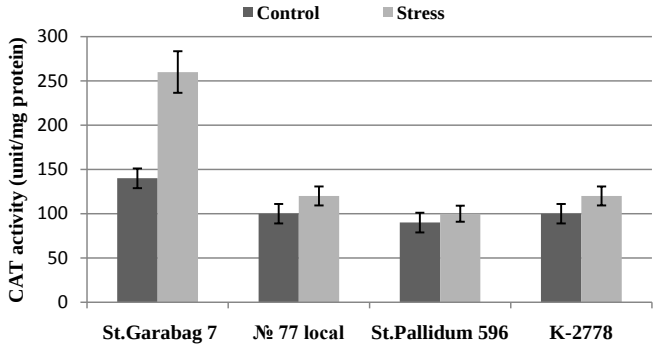

Figure 1. CAT activity of barley leaves under soil water shortage.

were almost the same (1.2 times) for No. 77 local and K-2778 genotypes. Water deficiency caused relatively small increase in CAT activity in St.Pallidum 596 (approx. 1.1 times), which reached 100 ± 12 unit/mg protein. The high activity of catalase under drought shows its protective role against stress.

Catalase is a chromoproteid thet contains oxidized heme as a prosthetic group (non-protein). Certain concentrations of hydrogen peroxide formed during exchange reactions have toxic effects on cells. Catalase neutralizes hydrogen peroxide, converting it into water and inactive molecular oxygen [7].

Another enzyme neutralizing hydrogen peroxide in living cells is peroxidase. However, it has been proven that catalase fulfills its catalytic function independently of peroxidase. Unlike peroxidases functioning at low peroxide levels, catalase can also act effectively at high concentrations of peroxides.

Ascorbate peroxidase can also plays an important role in plant protection against oxidative stress [27] [28]. APX is a key enzyme in the utilization of hydrogen peroxide in chloroplasts and cytosol of plant cells. The activity of hydrogen peroxide in barley genotypes has been analysed under normal and drought conditions (**Figure 2**). Among studied genotypes St.Garabag-7 was characterized by the maximum catalase and minimum ascorbate peroxidase activity (280 ± 22 unit/mg protein) under normal conditions. Despite belonging to different specific diversities ascorbate peroxidase activity was almost the same in No. 77 (640 ± 52 unit/mg protein) local and K-2778 (640 ± 66 unit/mg protein) genotypes of barley. It is interesting that catalase activity was also the same in these genotypes. Maximum activity of ascorbate peroxidase (660 ± 56 mMol/mg min) and minimum activity of CAT were detected in St.Pallidum 596 genotype of barley under normal conditions.

Figure 2 shows the influence of drought on ascorbate peroxidase activity. As seen in the figure contrary to catalase, ascorbate peroxidase activity decreased in all the genotypes under water deficiency. The minimum activity of the enzyme was observed in St.Garabag 7 (240 ± 21 unit/mg protein), and maximum activity in K-2778 genotype (360 ± 33 unit/mg protein). In genotype St.Garabag 7 water deficiency led to slight decrease in ascorbate peroxidase activity compared with the control plants. Whereas in genotype K-2778, characterized by the maximum activity of ascorbate peroxidase under water deficiency, it decreased approximately twice compared with the control plants. Ascorbate peroxidase activity was the same in barley genotypes No. 77 and St.Pallidum 596 under stress showing 2.2- and 2.3-fold decreases in activity, respectively.

APX catalyses oxidation of ascorbate leading to the formation of monodehydroascorbate (MDA) radical. According to the intracellular compartmentation APX is divided into four types: APX dissolved in the chloroplast stroma (sAPX), APX connected to thylakoid (tAPX), cytosolic form (cAPX) and glyoxisome membrane form (gmAPX). Different authors indicated changes in APX activity under drought and heat stress [29]. Hydrogen peroxide is also known to induce the gene of APX cytosolic function and participate in signaling under oxidative stress [30].

Glutathione reductase is considered as an important enzyme in the protection of the plant antioxidant system. It catalyses the reduction of glutathione, oxidised in the presence of $NADPH^+$ in the glutathione ascorbate cycle [31]. Glutathione reductase activity was also determined (**Figure 3**). Among normal watered plants maximum and minimum activities of GR were observed in No. 77 local (99 ± 10 unit/mg protein) and K-2778 (42 ± 6 unit/mg protein) genotypes, respectively. Genotypes St.Garabag 7 and St.Pallidum 596 occupy intermediate positions for this parameter.

Figure 3 shows changes in glutathione reductase activities in various barley genotypes under water deficiency. Maximum and minimum activities of GR were observed for St.Garabag 7 (156 ± 13 unit/mg protein) and

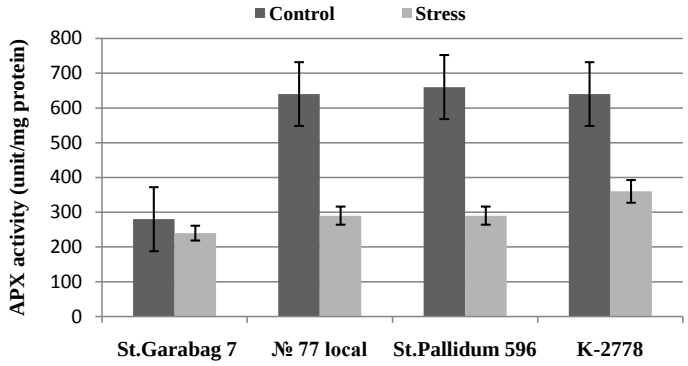

Figure 2. APX activity of barley leaves under soil water shortage.

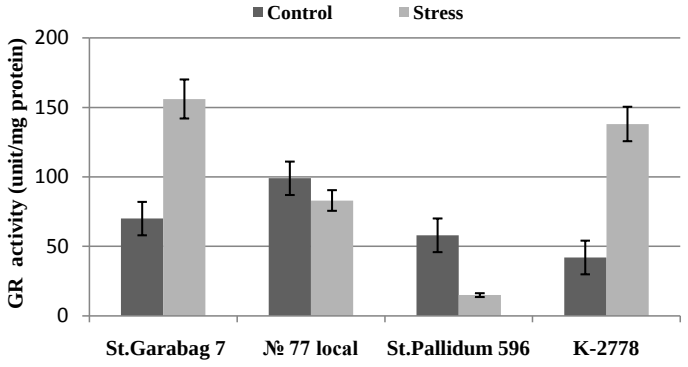

Figure 3. GR activity of barley leaves under soil water shortage.

St.Pallidum 596 (15 ± 2 unit/mg protein), respectively. Genotypes K-2778 and No. 77 occupy intermediate positions for this parameter. It is noteworthy that the same tendency exists for CAT activity in this genotypes. Comparison of the stress and control variants showed that the highest increase (more than 3 times) in GR activity occured in genotype K-2778 under drought. Enzyme activity in stressed genotype St.Garabag 7 increased almost 2 times, decreased 0.2 times in genotype No. 77 4 times in genotype St.Pallidum 596.

Four isoforms of GR associated with different cell compartments exist in plants. The most amount of this enzyme is associated with chloroplasts. However, its isoenzymes were also detected in cytosol and mitichondria [31] [32]. Hydrogen peroxide formed by the reduction of the superoxide radical is released in cytoplasma, chloroplasts and membranes with participation of ascorbate peroxidase and oxidation of ascorbate occurs: $2H_2O_2$ + ascorbate → dehydroascorbate + H_2O + O_2. Dehydroascorbate forms ascorbate in the presence of reduced glutathione (GSH): 2GSH + dehydroascorbate → GSSG + ascorbate. Oxidised glutathione (GSSG) in turn is regenerated forming the reduced glutathione in the presence of NADPH(H^+): GSSG + NADPH+H+ → 2GSH + NADP.

Superoxide dismutase (SOD) catalyzes dismutase reaction of the superoxide radical $O_2 \cdot^-$ with the formation of molecular oxygen and hydrogen peroxide. SOD is one of the most important enzymes in the protection system of plants against stress and occurs in every cell of all plants [6] [33].

Figure 4 shows the effect of water deficiency on superoxide dismutase activity in different barley genotypes. The same maximum activity of superoxide dismutase was observed in genotypes No. 77 local and K-2778 under normal conditions. While minimum activity of the enzyme was observed for genotype St.Pallidum 596 (82 ± 9 unit/mg protein).

As seen in **Figure 4** superoxide dismutase activity increased in all studied barley genotypes under water deficiency. Maximum and minimum values for SOD activity were observed in genotypes St.Garabag 7 (100 ± 13 unit/mg protein) and St.Pallidum 596 (93 ± 8 unit/mg protein), respectively. It is interesting that the same tendency was observed for glutathione reductase activity under stress. Genotypes No. 77 local and K-2778 occupy intermediate positions. It should be noted that the comparison of stressed and control variants showed different patterns of activity changes. Thus, the highest increase in superoxide dismutase activity (~1.2 times)

was observed in St.Garabag 7 genotype under stress.

Some authors emphasized an important role of SOD in the antioxidant protection system under the influence of different stressors [33] [34]. But there are various isoforms of SOD reacting differently to water deficiency. Activities of MnSOD and FeSOD increased rapidly in response to water deficiency, while Cu/ZnSOD activity remained unchanged in cowpea plants [35]. SOD is known to be a multimer metalloprotein which has different isoforms characterized by the metal type, localized in the emzyme active center. According to the literature data the most common isoforms of SOD are those contained copper-zinc (Cu/ZnSOD), manganese (MnSOD), iron (FeSOD) and nickel (NiSOD) in the active center [4]. Induction of SOD in plant cells against different stress factors indicates its important role in the plant protection system. In general, SOD activity increases under water deficiency for the utilization of superoxide radicals. Under the influence of salinity and other adverse environmental factors different mechanisms are known to function against oxidative stress. In addition to the formation of a substrate for SOD, oxygen oxidation triggers different mechanisms.

Thus, the analysis of the main enzymes of the antioxidant protection system in four barley genotypes under normal and drought conditions showed that drought caused increases in catalase and SOD activities and a decrease in ascorbate peroxidase activity in all the studied genotypes. The activity of glutahtione reductase increased in genotypes St.Garabag 7 və K-2778 and decreased in genotypes No. 77 local and St.Pallidum in response to drought.

Electrophoretic spectra of antioxidant enzymes from barley plants exposed to soil drought were also studied (**Figure 5**). Marked qualitative changes (appearance or disappearance of additional lines on electrophoregram were not revealed in the enzyme electrophoretic spectra. However, the intensification of the colors of the respective isoforms from barley leaves exposed to drought was detected in the electrophoretic spectra (**Figure 5** and **Figure 6**).

Two isoforms of APX differing in their mobility (Rf 0.89 and Rf 0.96) and one isoform of catalase from barley seedlings were observed in electrophoretic spectra. Anological results were also obtained by other authors [36] [37].

Thus, the obtained results suggest that drought tolerance of barley is closely related to the antioxidant protection system. Biochemical study of some oxidative enzymes and their multiple isoenzymes allows us to assess their enzymatic activities in barley plants and to coordinate this parameter with physiological and

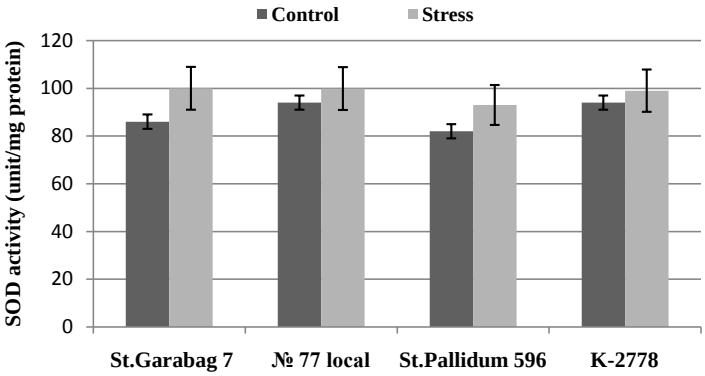

Figure 4. SOD activity of barley leaves under soil water shortage.

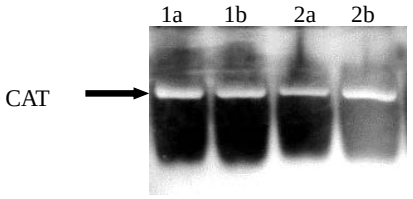

Figure 5. Isoenzyme pattern of CAT in the leaves of K-2778 (1) and St.Pallidum 596 (2) barley genotypes: a—normal irrigation , b—drought.

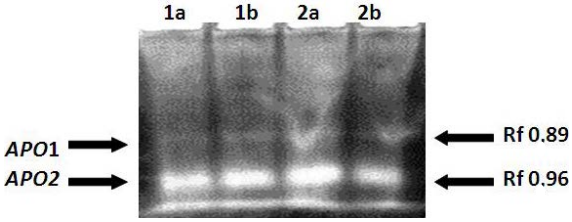

Figure 6. Isoenzyme patterns of APX in the leaves of K-2778 (1) and 2-St.Pallidum 596 (2) barley genotypes: a—normal irrigation, b—drought.

morphological processes. Barley plants maintain their life functions and homeostasis under extremal conditions due to qualitative and quantitative changes in activities and isoenzyme contents of antioxidant enzymes. The obtained data can play a role of theoretical basis for creation of new test systems to assess drought tolerance.

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