Effects of Soil Water Shortages on the Activity of Antioxidant Enzymes and the Contents of Chlorophylls and Proteins in Barley

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Abstract: Water is a key factor influencing the yield and quality of crops. Plants mainly adapt to water deficits by biochemical changes and osmotic regulation. In the present study, the activities of catalase (CAT), guaiacol peroxidase (POX), ascorbate peroxidase (APX), superoxide dismutase (SOD) and as well as the levels of protein and chlorophyll were studied at maturity stage of barley (Hordeum vulgare L.) subjected to different soil water levels (without stress, moderate stress and severe stress). The results indicated that drought stress depended on drought intensity, with more severe drought stress creating more serious effects on barley. Water stress induced a decrease in total chlorophyll and total protein contents which was much more marked in severe stress than in moderate stress. The activities of antioxidant enzymes which include CAT, POX and SOD were significantly high in the water-stressed treatments. Furthermore, the activities of POX and SOD enzymes were significantly high in severe stress than in moderate stress. However, the activity of APX did not significantly change in both of water-stressed levels compared with well-watered conditions. The present study possibly suggested that antioxidant protection in barley plants under drought conditions could be attributed mainly to SOD and POX.

Key words: Hordeum vulgare • Drought stress • Antioxidant enzymes • Chlorophyll • Protein

INTRODUCTION

Drought stress usually decreased crop production. It inhibits the photosynthesis of plants, caused changes of chlorophyll contents and components and damage of photosynthetic apparatus. It also inhibits the photochemical activities and decreases the activities of enzymes in the Calvin cycle [1].

When plants are exposed to environmental stresses such as drought, reactive oxygen species (ROS) such as superoxide (O2•−), hydrogen peroxide (H2O2), hydroxyl radicals (OH•) and singlet oxygen (1O2) are produced. The balance between the production of ROS and the quenching activity of the antioxidants is upset and this often results in oxidative damage [2]. ROS are highly reactive to membrane lipids, protein and DNA. They are believed to be the major contributing factors to stress injuries and to cause rapid cellular damage, particularly when plants are exposed to stress conditions [3].

The ability of tissues to cope with drought stress might be related to their ability to scavenge ROS by enhancing the activities of the antioxidant enzymes during water loss. In environmental stresses conditions such as drought, high activities of antioxidant enzymes are important for plants to tolerate stresses [1]. The protective mechanisms against the ROS involve the several functionally interrelated antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) and enzymes of the ascorbate-glutathione cycle such as ascorbate peroxidase (APX) [4].

There are many reports indicated that the significant relationship between enhanced antioxidant enzyme activities and increased resistance to environmental stress stresses in several plant species, such as rice [5], wheat [6] and sugar beet [7]. Nevertheless, most of the investigations in pot trials were limited to spatial growth of the root conditions, making it difficult to apply the conclusions to an agroecosystem under field conditions. In addition, most experiments applied the stress to early stages of plant development. Hence, additional information on understanding possible responses and the physiological mechanisms of barley at maturity stage to changes of soil water levels under increasing drought stress conditions is needed.
The objective of this work was to examine the effects of different soil water levels on contents of chlorophyll and protein and change of antioxidant enzyme activities in the leaves of barley at maturity stage under field natural conditions.

MATERIALS AND METHODS

Plants and Water-stress Treatments: The study was performed at the research farm of Ahar Branch, Islamic Azad University (Ahar, Iran). The farming experiments started on March 6 and ended on June 9, 2010. *Hordeum vulgare* L. accession M 3, provided by the Institute of Agriculture, Karaj, Iran, was used in this study. The experimental scheme was carried out using five-month-old plant planted in October 2009, spaced at 25 cm in the row with 25 m between rows and fertilized soil with 100 kg/ha biotic fertilizer.

Plants Were Divided in Three Treatments: Without stress (WS), moderate stress (MS) and severe stress (SS). WS plants were maintained in an optimal soil water condition (80% of relative water content (RWC)) during the whole experimental period, whereas MS and SS treatments subjected to a water shortage period starting from March 6 to May 15 (i.e. 60% RWC for MS and 35% RWC for SS). Containers of MS and SS were covered with plastic shelter in order to avoid rainfall infiltrations and evaporation from the soil surface.

Six different plants for each treatment were used and samples of leaves were taken from the same three plants. Fresh leaf samples were used for determinations of chlorophyll contents, whereas for assay of protein and enzymes, leaves were covered with an aluminium foil and put in a plastic envelope and then were frozen in liquid nitrogen immediately and stored at -80°C until enzyme assays.

Relative Water Content (RWC): Leaf samples which were collected of treatments were used for RWC assay. Relative water content was determined by drying the leaves at 80°C for 48 h and calculated using the following formula: [(fresh weight - dry weight) / (saturated weight - dry weight)] × 100.

Extraction and Assay of Total Chlorophyll: Fresh leaf (1 g) was immediately ground and mixed with 80% acetone and kept at -20°C for 1 h and then absorbance were recorded at 645 and 663 nm, respectively, with a spectrophotometer (LABOMED UVD-3200).

Preparation of Extracts for Enzyme Assays: Frozen leaf samples (1 g of fresh mass) were ground to a fine powder in liquid nitrogen and extracted with extraction buffer containing 50 mM potassium phosphate (pH 7.5) and 1 mM Sodium Metabisulfite. The extracts were centrifuged at 4°C for 20 min at 15,000 rpm and the resulting supernatants used as crude extracts.

Protein Determination: The protein concentration in leaf crude extracts was determined using bovine serum albumin as standard.

CAT Activity Assays: The enzyme extract (20 µl) was added to reaction mixture containing 750 µl of 70 mM hydrogen peroxide (H₂O₂) and 750 µl of 100 mM phosphate buffer (pH 7.0) adjusted to 3 ml with sterile distilled water. The absorbance was read at 240 nm [8]. CAT isoenzymes were analyzed on 6% non-denaturing PAGE at 4°C, 20 µg protein per lane was loaded.

POX Activity Assays: Peroxidase (POX) activity was determined specifically with guaiacol at 470 nm following the method of Choi *et al.*[9]. The enzyme extract (20 µl) was added to the reaction mixture containing 750 µl of 10 mM guaiacol solution, 750 µl of 70 mM hydrogen peroxide (H₂O₂) solution and 1500 µl of 100 mM potassium phosphate buffer solution (pH 7.0). POX isoenzymes were visualized in 12% (w/v) polyacrylamide gels by the chemical guaiacol staining method according to [10].

APX Activity Assays: Ascorbate peroxidase (APX) was spectrophotometrically assayed following a decrease in the absorbance at 290 nm [11]. The assay mixture contained: 750 µl of 5mM ascorbate, 750 µl of 2 mM H₂O₂ and 1500 µl of 100 mM phosphate buffer (pH 7.0) with 30 µl of the enzyme extract. Isoenzymes of APX were analyzed on 10% Native- PAGE at 4°C. The carrier buffer was supplemented with 2M ascorbate. For gel assay, equal amounts of 30 µg protein per lane were loaded.

SOD Activity Assays: Superoxide dismutase (SOD) activity was determined by following the photoreduction of Nitrotetrazolium Blue Chloride (NBT). The reaction mixture contained: 100mM phosphate buffer (pH 7.0), 0.1mM EDTA, 13mM methionine, 75 µM Nitrotetrazolium Blue Chloride, 2mM riboflavin and appropriate amounts of the supernatant. Riboflavin was added as the last component and the reaction was initiated by placing the
tubes under 15W fluorescent lamp. The reaction was terminated after 5 min by removing the reaction tubes from the light source. Non-illuminated and illuminated reactions without supernatant served as calibration standards. Reaction products were measured at 560 nm. Isoenzymes of SOD were separated on 10% non-denaturing PAGE at 4°C. Equal amounts of 40 µg protein per lane was loaded. After electrophoresis, SOD activities in gels were visualized according to Demirevska-Kepova et al. [12].

Statistical Analysis: All data obtained was analyzed by General Linear Model (GLM) and the mean differences were compared by Duncan’s multiple range tests by using SPSS program. Each data point was the mean of 18 replicates (six plans and three measurements per plant). Comparisons with P < 0.05 were considered significantly different. In all the figures the spread of values is shown as error bars representing standard errors of the means.

RESULTS

Relative Water Content: To understand how water status of barley plants was affected by water stressed treatment we monitored relative water content (RWC) of the leaves in well-water (WS) and water stressed (MS, SS) treatments (Table 1). Relative water content of barley leaves were decreased under drought stress. As compared with the control (WS), RWC of leaves in MS and SS declined by 25% and 57%, respectively.

Chlorophyll and Protein Contents: Oxidative stress due to the existence of the water deficits can be demonstrated by decreasing in chlorophyll and protein contents. The changes in chlorophyll contents during water shortages are shown in Table 1. Chlorophyll content of control non-water-stressed leaves was 3.20 mg g⁻¹ FW. It significantly unchanged in response to moderate stress but the plants exposed to sever stress showed a statistically significant decrease in chlorophyll content relative to controls. As shown in Table 1, protein concentration of plants exposed to moderate stress was not statistically different from controls. However, in sever stress treatment, protein showed a highly significant decrease compared with controls.

CAT Activity: The activity of CAT increased with water deficit and the highest amount of CAT activity by 167.08 µmol min⁻¹ mg⁻¹ proteins was obtained in severe stress treatment (Fig. 1); however there was no significantly statistic difference between moderate and severe stress treatments. The water shortage-mediated changes in CAT activity were smaller than that of SOD and POX.

POX Activity: As shown in Fig. 1, significant increases in POX activity in both moderate and severe stress treatments was observed compared with the controls. Furthermore, there was a significant difference between MS and SS treatments in the enhancement of POX activity. Total POX activities increased by two-fold in MS whereas it increased by three and half-fold in SS in comparison with control well water treatment.

APX Activity: There were no significant changes in APX activity of both treatment of the water deficit compared with control treatment (Fig. 1).

SOD Activity: Increases in total SOD activities were observed in response to drought stress, with a higher level of SOD activities in severe stress than in moderate stress (Fig. 1). In comparison with controls the mean increases in SOD activities ranged from 18% for MS to 27% for SS.

Isoenzyme Patterns of Enzymes: Isoenzyme profiles of antioxidative enzymes are presented in Figure 2. In order to analyze the changes of CAT isozymes under drought stress, tissue extracts were subjected to native page. CAT is revealed as one major isoform. Under water deficit stress, the intensity of CAT isoform was increased. In the profile of POX activity in severely stressed plants, three bands (POX1, POX2 and POX3) were detected. POX3

Table 1: Drought stress-induced changes on the contents of RWC (%), chlorophyll and protein (mg g⁻¹ fw)

<table>
<thead>
<tr>
<th></th>
<th>WS</th>
<th>MS</th>
<th>SS</th>
</tr>
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<tbody>
<tr>
<td>RWC</td>
<td>80.40±2.25'a</td>
<td>60.40±3.03'b</td>
<td>34.80±1.88c</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>3.20±0.17</td>
<td>2.93±0.18ab</td>
<td>2.53±0.18b</td>
</tr>
<tr>
<td>Protein</td>
<td>6.91±0.08'a</td>
<td>6.83±1a</td>
<td>6.56±0.07b</td>
</tr>
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Treatment notations indicate WS: without stress control plants; MS: moderate stressed plants; and SS: severe stressed plants. The data represent the means ± SE. of 18 replicates. Within row, means with the same letter are not significantly different at P < 0.05 by the Duncan’s multiple range tests.
Fig. 1: Activities of catalase (CAT), guaiacol peroxidase (POX), ascorbate peroxidase (APX) and superoxide dismutase (SOD) of barley leaves under drought conditions. The values (mean ± S.E.) are calculated from 18 replicates.

Fig. 2: Effects of drought stress on isoenzyme patterns of catalase (CAT), guaiacol peroxidase (POX), ascorbate peroxidase (APX) and superoxide dismutase (SOD). The lanes for the activity staining were loaded with the following treatments: WS (without stress); MS (moderate stress) and SS (sever stress).

Increased in both water deficit-stressed treatments (MS, SS), especially a strong induction in severely stressed plants. Two new bands (POX1, POX2) for POX were revealed at the highest drought stress treatment. The six APX bands were present in three treatments of barley plants. No major changes were observed for all APX isoenzymes at elevated drought stress levels. One major band (SOD4) for SOD activity observed in barley leaves. SOD4 increased in response to drought stress, with a slightly greater increase in moderately stressed plants.
SOD3 identified when plants exposed to drought stress. Moreover, the intensity of SOD3 was higher in severe stress treatment than in moderate stress treatment. Two additional SOD activity bands (SOD1, SOD2) were only observed in severely stressed leaves.

**Discussion**

Drought, due to its osmotic effect in natural and agricultural habitats can induce a wide number of responses ranging from increase the osmotic potential of the cell and growth inhibition to leads to the production of ROS such as O$_2^\cdot$ and H$_2$O$_2$ in plant tissues [13]. ROS are highly active molecules that can easily damage membrane and oxidize photosynthetic pigments, proteins and nucleic acids [1]. ROS-scavenging mechanisms of the cell include the enzymatic and non-enzymatic antioxidants. Scavenging mechanisms for ROS involve these enzymes: SOD, CAT, APX, POX and GR.

Water shortage stress enhances proteolytic degradation of Rubisco (ribulose-1, 5-bisphosphate carboxylase/oxygenase) protein [14]. Hence, decreases in soluble protein contents under water deficit could be largely due to a decline in Rubisco protein.

Chlorophyll concentrations were decreased in drought-stressed leaves of Barley (Table 1). The reduction in chlorophylls under drought stress are mainly because of the membrane disintegration and damage to chloroplasts by ROS [2]. In other studies, chlorophyll was declined during drought stress [2, 15].

Antioxidant enzymes such as SOD, POX, APX and CAT are reported to increase under various environmental stresses [6, 16, 17]. As a confirmation, in the present work, we also observed enhanced activities of SOD, POX and CAT under water-stressed conditions in compared with well watered conditions (Fig. 1).

Catalase eliminates H$_2$O$_2$ by breaking it down directly to form water and oxygen [1]. The drought-induced increases in CAT activities were observed in the present study (Fig 1). However, there were no significant differences between MS and SS treatments. The increased CAT activities during drought have been reported Noctor et al. [18] and Ramachandra Reddy et al. [19].

POX is among the major enzymes that scavenges H$_2$O$_2$ in chloroplasts which is produced through dismutation of O$_2^\cdot$ catalyzed by SOD [13]. Drought-stressed leaves strongly enhanced the activities of POX, which could be considered as a response to drought-induced oxidative damage, suggesting enzymatic removal of H$_2$O$_2$ by POX (Fig. 1). Increase in POX activity in response to drought stress has been reported in *Arabidopsis thaliana*, *Myrtus communis*, *Phillyrea angustifolia* [2, 20].

In this study, water deficit had no effect on APX activity (Fig. 1). [1] also reported that APX activity was not influenced by drought stress. It seems that the product of the SOD reaction, H$_2$O$_2$, was eliminated by higher induced levels of both CAT and POX activity in moderately stressed leaves and just induced levels of POX activity in severely stressed leaves.

SOD catalyzes the conversion of the superoxide anion to H$_2$O$_2$. In previous studies, changes in SOD activity have been shown depending on drought severity, duration and species. SOD induction has been observed in water stressed leaves of *Lotus corniculatus* [21] and wheat [22]. Confirming these studies, in the present study, SOD activity was increased under water shortage stresses (Fig. 1). Moreover, as compared with MS treatment, SS treatment significantly represented higher activities of SOD.

The important components of protective systems are enzymatic defenses such as SOD and CAT as well as POX which scavenge superoxide radical (O$_2^\cdot$) and hydrogen peroxide (H$_2$O$_2$), respectively [23]. POX plays an essential role in scavenging from the H$_2$O$_2$ toxicity. The combined action of CAT and SOD converts the toxic O$_2^\cdot$ and H$_2$O$_2$ to water and molecular oxygen (O$_2$), thus averting the cellular damage under unfavorable conditions like water stress [18, 19]. Yong et al. [3] found that the rules of SOD, CAT and POX activity change are similar, which indicated that these three enzymes cooperated with each other during water deficits. However, Reports on the effects of stresses on CAT activities vary. Increased, decreased, or unchanged CAT activities under drought stress have been observed by Jiang and Huang [24] and Sobkowiak et al. [25]. As a confirmation, in our study, we also observed higher induced activities POX, CAT and SOD under water shortage stress and enhanced activities of POX and SOD in severely stress treatment than in moderately stress treatment (Fig. 1).

It could be noticed that drought-induced changes of the isoenzyme patterns of POX, CAT, APX and SOD are accompanied with the recently described change in the activity of these enzymes (Fig. 2). POX isozyme pattern were strongly affected by water deficit stress, at which new isoenzymes, designated as POX1 and POX2 appeared. The activities of other isozyme (POX3) were enhanced compared to control. Jung [2] reported that the drought-stressed mature leaves of *Arabidopsis thaliana* were capable of greatly increasing the POX isozyme 3.
The activity of CAT isoenzyme increased under drought stress. Sobkowiak et al. [25] observed that the activity of CAT isoform increased gradually under oxidative stress. No detectable differences were observed in APX isoenzyme pattern between control plants and those treated with water shortage stress. A similar observation was noted in soybean and gourd plants under stress condition [25, 26]. The SOD isoenzyme pattern was significantly different at the water stress treatments compared with the control without stress plants. Jung [2] observed the high activity of the SOD isoforms particularly the Mn-SOD isoform during drought stress.

CONCLUSION

Results presented in Figures 1-2 suggested that the rules of SOD and POX activity change are similar, which indicated that these two enzymes cooperated with each other during water deficits. Also, among the studied enzymes, SOD and POX activities increase with enhancement of drought intensity and activities of them in severely stressed plants are significantly higher than in moderately stressed plants. These suggest that antioxidant protection in barley plants could be attributed mainly to SOD and POX. The combined action of POX and SOD converts the toxic superoxide radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) to water and molecular oxygen (O$_2$), thus averting the cellular damage under water stress conditions.

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REFERENCES


