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ARTICLE

Exogenous salicylic acid alleviates oxidative damage of barley plants under drought stress

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ABSTRACT This paper reports the effects of 500 µM salicylic acid (SA) application on drought stress acclimation of barley (*Hordeum vulgare* L. *cv* Nosrat) plants grown in soil culture. In these experiments the following treatments were used: CK (control), DR (drought), SA (500 µM) and DSA (SA+drought). The results showed that drought stress decreased the dry mass and net CO_2 assimilation rate (*A*) of plants, which were all increased by the addition of SA. Under drought conditions, the improvement of photosynthesis of barley plants treated with SA was associated with an increase in g_s, whereas the maximal quantum yield of PSII (F_v/F_m) did not change with SA treatment. Malondialdehyde (MDA) content remained unchanged in DSA plants because of an efficient scavenging of reactive oxygen species (ROS) following a significant enhancement of some antioxidative enzyme activities. The present work suggests that the improvement of SA on drought tolerance of barley plants was associated with the increase of antioxidant defense abilities and maintenance of photosynthesis under drought, which may elucidate the physiological mechanism of SA in improvement of drought tolerance of barley plants.
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KEY WORDS

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Stress factors such as drought trigger common reactions in plants and lead to cellular damages mediated by reactive oxygen species (ROS) (Mano 2002). Oxidative stress results in a serious imbalance between the production and removal of ROS. Accumulation of ROS induces oxidative damage to proteins, membrane lipids and other cellular components (Creissen and Mullineaux 2002). It also inhibits the photochemical activities and decreases the activities of enzymes in the Calvin cycle (Monakhova and Chernyad'ev 2002).

Under environmental stress conditions such as drought, high activities of antioxidant enzymes and high levels of nonenzymatic antioxidant compounds are important for plants to tolerate stresses (Gong et al. 2005). However, respons of antioxidative enzymes to water deficiency is variable and depends on the intensity of the imposed water stress (Habibi and Hajiboland 2011). Antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) play an important role against oxidative stress (Apel and Hirt 2004). Plants containing high activities of antioxidant enzymes have shown considerable resistance to oxidative damage caused by ROS (Khan et al. 2007; Gapinska et al. 2008; Frary et al. 2010).

Salicylic acid (SA) is a naturally occurring plant hormone, influences various physiological and biochemical functions in plants. It can act as an important signaling molecule and has diverse effects on tolerance to biotic and abiotic stresses (Arfan et al. 2007; Wang et al. 2010). Its role in plant tolerance to abiotic stresses such as ozone, heat, heavy metal and osmotic stress (El-Tayeb 2005; Szepesi et al. 2005; Wang et al. 2010; Liu et al. 2011; Kadioglu et al. 2011) has been reported by several authors.

A survey of literature indicates that salicylic acid plays a key role in providing tolerance to the plants, exposed to water stress (Hayat et al. 2008; Kadioglu et al. 2011). Salicylic acid was found to enhance the activities of antioxidant enzymes such as peroxidase (POD), SOD and CAT, when sprayed exogenously to the drought stressed plants of tomato (Hayat et al. 2008) or to the salinity stressed plants (Szepesi et al. 2008; Yusuf et al. 2008). The exogenous SA application also enhanced the growth and photosynthetic rate in wheat (Hussein et al. 2007) under water stress. However, numerous studies have demonstrated that the effect of exogenous SA depends on various factors, including the species and developmental stage, the mode of application and the concentration of SA (Vanacker et al. 2001; Horváth et al. 2007).

In recent years the role of SA in the induction of tolerance against paraquat-induced oxidative damage in barley has been described (Ananieva et al. 2004). With respect to drought stress, relevant work is limited especially in case of barley, which is an important crop plant with a number of agro-industrial uses. This is of great interest since increasing the amount of irrigated land is difficult; water is scarce and only the most efficient agricultural systems are likely to receive

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inputs of irrigation water (Fereres et al. 2003). There is no information about the physiological responses of the barley (*cv* Nosrat) to SA under water stress, which is supposed to influence many physiological processes in drought stressed plants. The aim of this study was to investigate the effect of salicylic acid on barley growth, photosynthesis and defense system under drought stress. In addition of monitoring the growth, relative water content, chlorophyll fluorescence parameters and gas exchange pattern, the present work examined the effect of SA on the antioxidant defense system during drought stress in barley plants.

Materials and Methods

Plant material and treatments

Barley (*Hordeum vulgare* L. *cv* Nosrat) seeds were planted in pots (14 cm in diameter and 105 cm in depth), each pot containing 15 kg of sandy loam soil. The field capacity (FC) of soil was measured by gravimetric method. Before sowing, 15 kg of soil was fertilized, and 200 mg nitrogen kg^{-1} soil as NH_4NO_3, and 50 and 62.5 mg phosphorus and potassium kg^{-1} soil as KH_2PO_4 were applied to the soil cultures. The following treatments were used in the experiments: CK (control), DR (drought), SA (500 µM) and DSA (SA+drought). Water stress was initiated by withholding irrigation. Control plants were grown at 90% and drought stressed plants at 40% of FC. Plants grown in pots were kept in a greenhouse under natural day/night conditions with photosynthetically active radiation (PAR) of 800 ± 100 µmol m^{-2} s^{-1} and average day/night temperature of 30 ± 2/18 ± 2°C. After emergence, the seedlings were thinned to forty plants per pot and watered every 4-5 days to maintain 90% FC of the soil until fifteen days after sowing. From then on, the seedlings were watered every 1-2 days to maintain 90% FC in the CK soil and 40% FC in the drought treatments (DR and DSA). Salicylic acid (SA) was dissolved in absolute ethanol then added drop wise to water (ethanol/water: 1/1000 v/v) (Williams et al. 2003). Fifteen days after sowing, SA was applied on the foliage at a concentration of 500 µM with a hand sprayer. The appropriate controls without SA treatment were sprayed with ethanol/water (1/1000 v/v). The volume of the spray was 25 ml per pot. At 30 days after sowing (15 d after SA application and drought treatment), measurements were done and the young fully expanded leaves were collected and frozen in liquid N_2 immediately until analysis.

Analysis of water relations

Leaves were washed with distilled water, blotted dry on filter paper and after determination of fresh weight (FW) were dried for 48 h at 70°C for determination of dry weight (DW). Relative water content (RWC) was measured and calculated according to Lara et al. (2003) all in the second youngest leaf harvested at 1 h after light on in the greenhouse.

Measurements of photosynthetic gas exchange and chlorophyll fluorescence

Before harvest gas exchange parameters were measured. Net CO_2 fixation (A, µmol m^{-2} s^{-1}), transpiration rate (E, mmol m^{-2} s^{-1}) and stomatal conductance to water vapor (g_s, mol m^{-2} s^{-1}) were measured with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) after 5 h of the light period and sealed in the leaf chamber under a photon flux density of 800 ± 100 µmol m^{-2} s^{-1} in greenhouse conditions. Chlorophyll fluorescence parameters were recorded using a portable fluorometer (OSF1, ADC Bioscientific Ltd., UK) for both dark adapted and light adapted leaves. Leaves were acclimated to dark for 30 min using leaf clips before measurements were taken. Initial (F_0), maximum (F_m), variable ($F_v = F_m - F_0$) fluorescence as well as maximum quantum yield of PSII (F_v/F_m) were recorded. Light adapted leaves (400 µmol m^{-2} s^{-1}) were used for measurement of "steady-state" (F_s) and maximum (F'_m) fluorescence. Calculations were made for F'_0 ($F'_0 = F_0/[(F_v/F_m)+(F_0/F'_m)]$), effective quantum yield of PSII, Φ_{PSII} $[(F'_m - F_s)/F'_m]$, photochemical quenching, qP $[(F'_m - F_s)/(F'_m - F'_0)]$ and non-photochemical quenching, qN $(1-[(F'_m - F'_0)/(F_m - F_0)])$ (Krall and Edwards 1992).

Assay of enzymes activity and related metabolites

Determination of the activity of antioxidant enzymes and concentration of related metabolits were undertaken according to the methods described by Habibi and Hajiboland (2010). Fresh samples were ground in the presence of liquid nitrogen and measurements were undertaken using spectrophotometer (Specord 200, Analytical Jena, Germany). Superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated according to the method of Giannopolitis and Ries (1977). Enzyme was extracted in 25 mM HEPES pH 7.8 with 0.1 mM EDTA and the supernatant was added to the reaction mixture containing 0.1 mM EDTA, 50 mM Na_2CO_3 (pH 10.2), 13 mM methionine, 63 µM nitroblue tetrazolium chloride (NBT), 13 µM riboflavin. One unit of SOD was defined as the amount of enzyme which produced a 50% inhibition of NBT reduction under assay conditions. For the determination of catalase (CAT, EC 1.11.1.6) activity, samples were homogenized with 50 mM phosphate buffer pH 7.0 and assayed spectrophotometrically by following the degradation of H_2O_2 at 240 nm according to the method of Simon et al. (1974). Reaction medium contained 50 mM phosphate buffer (pH 7) and 10 mM H_2O_2. Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test at 470 nm (Chance and Maehly 1995). The enzyme was extracted by 10 mM phosphate buffer pH 7.0 and assayed in a solution containing 10 mM phosphate buffer, 5 mM H_2O_2 and 4 mM guaiacol. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed by following reduction in absorbance at 290 nm as ascorbate was oxidized according to the method of Boominathan and Doran (2002). The reaction

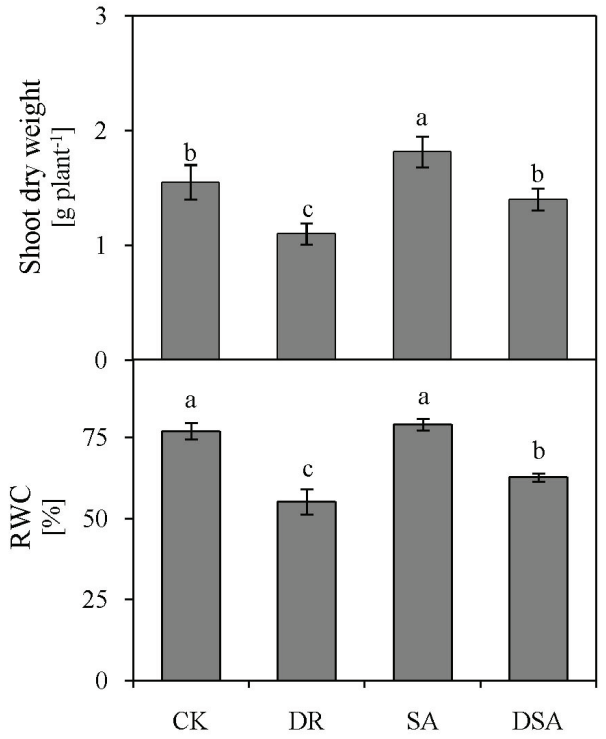

Figure 1. Dry weight (mg plant^{-1}) of leaves and relative leaf water content (RWC, %) under different treatments: CK (control), DR (drought), SA (500 µM) and DSA (SA+drought). Each value is the mean ± SD of 4 replicates. Data of each column indicated by the same letters are not significantly different (P < 0.05).

mixture contained 50 mM phosphate buffer (pH 7), 0.2 mM EDTA, 0.5 mM ascorbic acid and 50 µg bovine serum albumin (BSA). Lipid peroxidation was estimated at 532 nm from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid (Sigma). MDA levels were calculated from a 1,1,3,3-tetraethoxypropane (Sigma) standard curve. The concentration of H_2O_2 was determined using potassium titanium-oxalate at 508 nm (Habibi et al. 2010). Soluble protein was estimated spectrophotometrically by the Bradford method (1976).

Statistical analysis

Experiments were undertaken in complete randomized block design with 4 replications. Statistical analysis was carried out using Sigma Stat (3.5) with Tukey test (P < 0.05). Correlation analysis using Spearman Rank Order Correlation in Sigma Stat (3.5) were conducted to determine the relationship between maximum quantum yield of PSII (F_v/F_m) and stomatal conductance (g_s).

Results

Both relative water content (RWC) and dry weight decreased dramatically in water-stressed plants (Fig. 1). In contrast to drought, SA spraying increased dry matter accumulation in well-watered plants, as compared with control plants. Moreover, the plants after SA treatment showed higher dry weight and water content compared to plants without application of SA under drought conditions. Salicylic acid treatment did not increase relative water content in well-watered plants, as compared with controls.

The study of PSII photochemistry in the dark-adapted leaves showed that there was no significant difference in the maximal quantum yield of PSII (F_v/F_m) between control and SA-treated plants under well-watered conditions (Table 1). However, reduction of maximal efficiency of PSII in dark-adapted leaves (F_v/F_m) and effective quantum yield of PSII (Φ_{PSII}) were detectable in leaves of water-stressed plants. In addition, there was a good linear correlation (r = 0.84, P < 0.01) between stomatal conductance to water vapor (g_s) and F_v/F_m in water-stressed plants (Fig. 4). Photochemical quenching (*qP*) and non-photochemical quenching (*qN*) were not influenced under SA spraying and drought conditions. However, a decrease in *qN* was observed in SA applied plants compared with those without application of SA under drought conditions.

Table 1. Leaf physiological traits of barley plants under different treatments: CK (control), DR (drought), SA (500 µM) and DSA (SA+drought). *A* net photosynthetic rate, *E* transpiration rate, g_s stomatal conductance, *WUE* (*A*/*E*) water use efficiency, F_v/F_m maximum quantum yield of PSII, *qP* photochemical quenching, *qN* non-photochemical quenching, Φ_{PSII} effective quantum yield of PSII. Each value is the mean ± SD of 4 replicates. Data of each row indicated by the same letters are not significantly different (P < 0.05).

Photochemistry	CK	DR	SA	DSA
F_v/F_m	0.83 ± 0.01 ^{a}	0.77 ± 0.04 ^{b}	0.84 ± 0.01 ^{a}	0.80 ± 0.02 ^{ab}
qP	0.96 ± 0.01 ^{a}	0.94 ± 0.06 ^{a}	0.97 ± 0.02 ^{a}	0.96 ± 0.03 ^{a}
qN	0.21 ± 0.03 ^{ab}	0.31 ± 0.08 ^{a}	0.19 ± 0.08 ^{b}	0.22 ± 0.05 ^{a}
Φ_{PSII}	0.78 ± 0.02 ^{ab}	0.74 ± 0.03 ^{b}	0.81 ± 0.02 ^{a}	0.74 ± 0.05 ^{b}
Gas exchange	CK	DR	SA	DSA
A (µmol m^{-2} s^{-1})	12.6 ± 2.13 ^{ab}	4.83 ± 1.54 ^{c}	15.8 ± 1.49 ^{a}	9.53 ± 1.92 ^{b}
E (mmol m^{-2} s^{-1})	4.81 ± 0.38 ^{ab}	3.54 ± 1.10 ^{b}	5.53 ± 0.45 ^{a}	4.99 ± 0.61 ^{ab}
g_s (mol m^{-2} s^{-1})	0.41 ± 0.02 ^{b}	0.26 ± 0.01 ^{c}	0.49 ± 0.12 ^{a}	0.41 ± 0.03 ^{b}
WUE (µmol mmol^{-1})	2.61 ± 0.34 ^{ab}	1.37 ± 0.16 ^{c}	2.86 ± 0.20 ^{a}	1.95 ± 0.59 ^{bc}

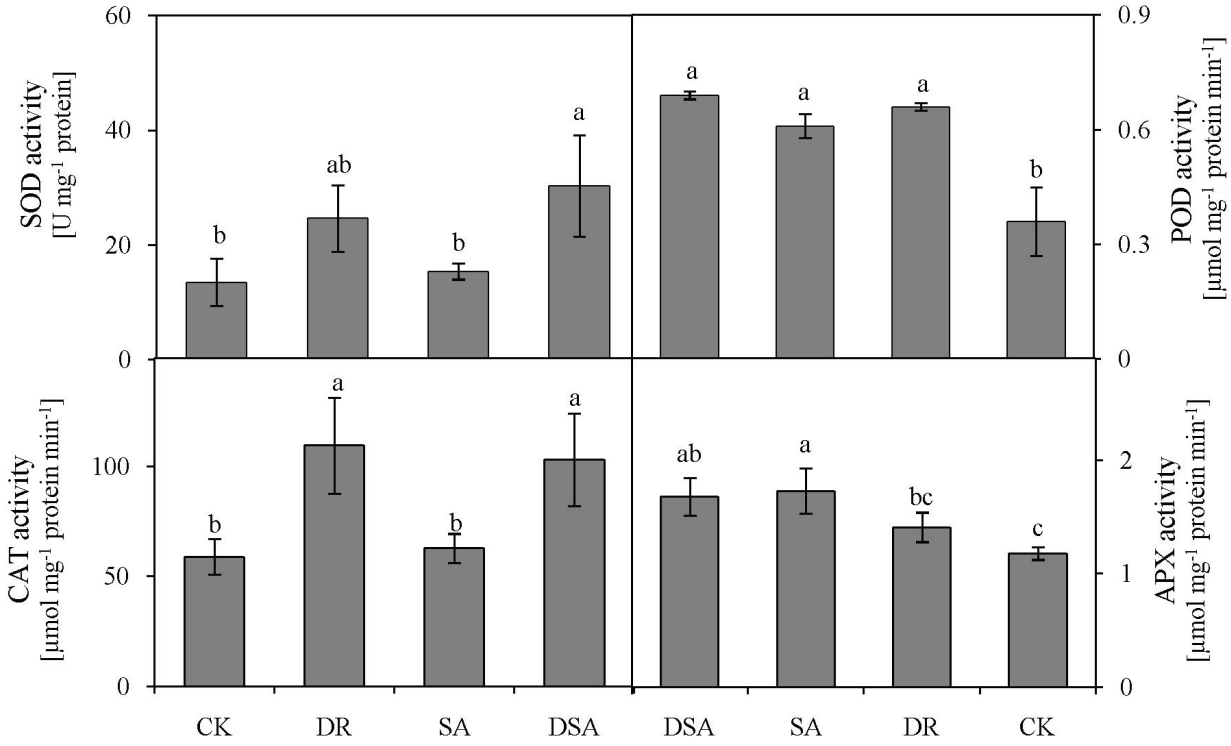

Figure 2. Antioxidant index of barley plants under different treatments: CK (control), DR (drought), SA (500 µM) and DSA (SA+drought). SOD superoxide dismutase, CAT catalase, POD peroxidase, APX ascorbate peroxidase. Each value is the mean ± SD of 4 replicates. Bars indicated with the same letter are not significantly different (P<0.05).

Net CO_2 assimilation rate (*A*) was not influenced by SA spraying, but was reduced by drought (Table 1). In this study, a remarkable reduction in leaf dry weight in drought stressed plants was associated with significant reduction of *A*. In addition, a significantly rise in the net assimilation rate was observed in the SA-supplemented water-stressed samples relative to water-deficit treatment. Transpiration rate (*E*) was only slightly affected by water stress, and this slight reduction was alleviated by SA under water stress. Stomatal conductance (g_s) was reduced strongly under drought conditions but increased by SA. SA-treated plants still maintain higher g_s value compared to those without application of SA under drought, which indicated that application of SA had positive effects on stomatal conductance of barley plants. Water use efficiency was significantly lower in drought-stressed plants. Thus, compared with the transpiration rate, the water use efficiency showed a greater decrease during water deficit.

Drought stress caused a significant increase of POD and CAT activities relative to control plants, whereas SOD and APX activities did not change (Fig. 2). Under well-watered conditions, SA stimulated APX and POD activities in comparison with the control, whereas CAT and SOD activities were not affected. Activity of APX and POD in water-stressed plants did not differ from that in SA-supplemented water-deficit plants. However, a significantly rise in the activity of SOD and CAT was observed in the SA-supplemented water-deficit samples relative to water-deficit treatment. Drought stress without SA spraying caused significant accumulation of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) (Fig. 3). Drought stress enhanced H_2O_2 content in both SA treated and non-SA treated plants under water stress conditions. In contrast to DR plants, in DSA plants, MDA level did not change at the end of the experiment.

Discussion

In this work, similarly with that observed for wheat (Hayat et al. 2005; Shakirova 2007) and maize (Khodary 2004), SA increased shoot dry weight in barley plants. Drought stress reduced growth activity of barley (Fig. 1), as it was observed in other plants species (Degu et al. 2008; Gao et al. 2011). It is a well-known fact that SA potentially generates a wide array of metabolic responses in plants and also affects plant water relations (Hayat et al. 2010). In this study, similarly with that observed for wheat (Singh and Usha 2003) and *Ctenanthe setosa* plants grown under drought conditions (Kadioglu et al. 2011), the results showed that barley plants sprayed with 500 µmol SA solution could maintain higher RWC compared with those plants which had not been treated with SA before

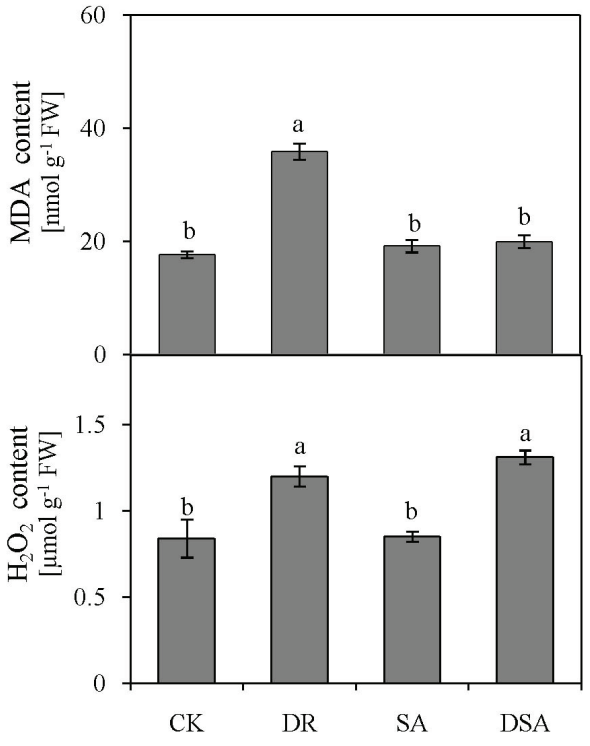

Figure 3. Leaf content of MDA (nmol g^{-1} FW) and H_2O_2 (µmol g^{-1} FW) in barley leaves at different treatments: CK (control), DR (drought), SA (500 µM) and DSA (SA+drought). Bars indicated with the same letter are not significantly different (P<0.05).

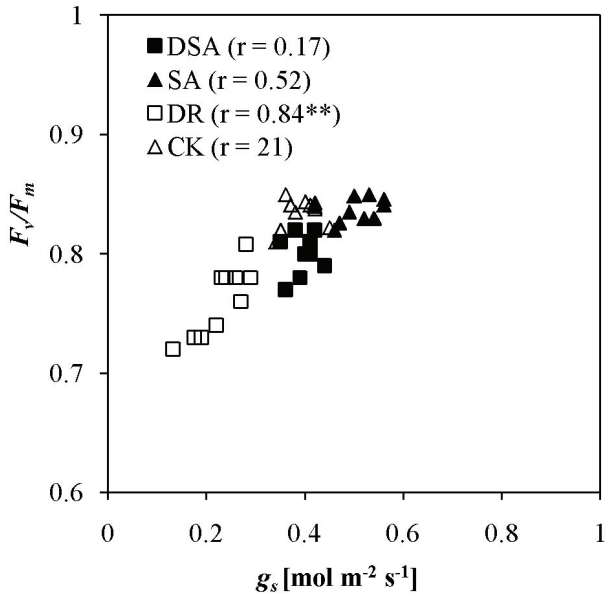

Figure 4. Correlation between maximum quantum yield of PSII (F_v/F_m) and stomatal conductance (g_s) in barley plants at different treatments. ns, *, and **: non-significant and significant, at the 5% and 1% levels of probability, respectively.

drought stress. These results show that application of SA is useful for drought tolerance improvement of barley plants.

Many authors suggested that application of chlorophyll a fluorescence analysis as a reliable method to assess the changes in the function of PSII under stress conditions (Price and Hendry 1991; Broetto et al. 2007). Lower photosynthetic activity could be a consequence of low photochemical efficiency of PSII, as shown by its lower quantum yield (Pieters and Souki 2005). We found that the significant decrease in F_v/F_m was observed under water stress conditions, which was possibly due to the reduction of g_s and restriction of CO_2 for photosynthesis and indicated photoinhibition. These results are consistent with the findings of Ranjbarfordoei et al. (2006), Gunes et al. (2007) and Boughalleb and Hajlaoui (2011) that declining F_v/F_m values implies that photochemical conversion efficiency could indicate the possibility of photoinhibition.

Inhibition of photosynthesis of plants caused by drought stress includes stomatal and non-stomatal factors. However, which of them is the main factor is associated with plant species and stress conditions (Gong et al. 2005). In the present study, the stomatal density decreased significantly with water stress. Reduction of stomatal conductance (g_s) inhibits supply of CO_2 and consequently reduces CO_2 assimilation (A) is a well known phenomenon in drought stressed plants (Lawlor and Cornic 2002). Reductions in photosynthetic performance under water stress have also been observed by Tognetti et al. (2005), Bacelar et al. (2006) and Ben Ahmed et al. (2009). The significant correlation between F_v/F_m and g_s confirmed the idea that stomatal closure that limits CO_2 availability for dark reactions may be one of mechanisms for photoinhibition in drought-stressed leaves. On the other hand, reduction of photosynthetic rate in drought-stressed plants in this work could not only be attributed to the stomatal limitation but may also be explained by inhibited leaf photochemistry as well as metabolic impairment *i.e.* reduction of Rubisco activity and ATP synthesis (Reddy et al. 2004).

Following the drought stress, SA-supplemented plants showed a higher g_s value in response to drought stress when compared with water-deficit plants. This is in accordance with the effect of SA on photosynthetic activity and stomatal conductance of tomato under salt stress (Poór et. al. 2011). The present study shows that stomatal limitation is involved in SA-induced alleviation of the negative effects drought stress on photosynthesis in barley leaves. This conclusion was based on the observations that (1) SA treatment led to a significant increase in A in water-stressed plants, which was highly associated with the increase in g_s, and (2) F_v/F_m and effective quantum yield of PSII (Φ_{PSII}) did not change with SA treatment (Table 1). Therefore, under drought stress, the improvement of photosynthesis of barley plants exposed to SA was associated with stomatal factors.

The water status of the barley leaves was expressed by water use efficiency (WUE) and relative water content (RWC) parameters. WUE decreased significantly because of relatively high transpiration rate (*E*) under water stress in both treatments tested. The net photosynthetic rate and stomatal conductance of higher plants leaves are known to decrease as RWC decrease (Lawlor and Cornic 2002).

Plants are able to protect their tissues from the harmful effects of drought-accumulated reactive oxygen species (ROS) using enzymes such as SOD, APX and CAT (Verhagen et al. 2004). The results showed that SA has induced all antioxidant enzyme activities in DSA plants under drought condition, which may be related to the induction of antioxidant responses that protect the plant from oxidative damage. There is data supporting that SA increases the activity of antioxidant enzymes such as CAT, POD and SOD (Hayat et al. 2008 and 2010), which in turn protect plants against ROS generation and lipid peroxidation. On the other hand, it was reported an increase in activities of SOD and APX but an inhibition of CAT activity following SA treatment (Janda et al. 2003; Shakirova 2007). In this work, CAT activity did not decrease in SA-treated plants under well-watered condition. This finding was in agreement with those of Farooq et al. (2009), who reported that CAT inhibition by SA cannot be validated in all plants. In this work, a significant rise in the activity of CAT and SOD in the DSA samples relative to DR treatment revealed that SA exerts beneficial effects on stress tolerance of barely by enhancing their antioxidative capacity. The results showed that drought stress independently and in combination with SA led to an increase in the activity CAT and H_2O_2 content. The observed higher activity of the photorespiratory enzymes such as CAT in treated plants was most probably due to enhanced energy dissipation through photorespiration in the stressed plants (Yordanova and Popova 2007). These results are consistent with the findings of Agarwal et al. (2005) and Mora-Herrera et al. (2005) who revealed that H_2O_2 accumulation induced by SA was not related to inhibition of CAT activity. Amounts of MDA remained unchanged in SA-supplemented plants exposed to water-deficit because of an efficient scavenging of ROS following significant enhancement of SOD, CAT, POD and APX activities. It indicates that antioxidant defense system induced by SA may protect plants under water-stress conditions, while under drought without SA spraying, an imbalance between production and scavenging of ROS may cause oxidative stress as could be judged by the accumulation of MDA.

In summary, the results showed that drought stress decreased the plant dry mass and net CO_2 assimilation rate, which were all increased by addition of SA. This was associated with the increase of water content and enhancement of antioxidant enzymes activities of plants by addition of SA. The results of the present work also suggested that the improvement of SA on drought tolerance of barely plants was associated with the increase of antioxidant defense capacity of tissues, alleviation of oxidative damage of functional molecules and maintenance of many physiological processes such as photosynthesis under drought. From this conclusion we can summarize that SA application can improve antioxidant defense system under drought stress conditions, and it may be recommended in arid and semiarid regions.

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