# Effect of salicylic acid on photochemistry and antioxidant capacity in *Salvia nemorosa* plants subjected to water stress

**Ghader Habibi***

*Ghader Habibi, Biology Department, Payame Noor University, Tehran, Iran*

## Abstract

Oxidative stress is commonly induced when plants are grown under drought stress conditions. To analyze how salicylic acid (SA) can partly alleviate drought-induced oxidative stress and negative impacts of drought on physiology and growth of *Salvia nemorosa* plants, we investigated the physiological responses of *S. nemorosa* to SA application under drought stress. The treatments were composed of Co (control, 100% field capacity), Dr (drought, 50% field capacity), SA (500 µM) and DSA (SA + drought). Plant growth and relative water content (RWC) were negatively affected by drought stress; however, SA treatment significantly improved the growth rate and enhanced the drought tolerance of seedlings. This increased tolerance in SA-supplied plants was obtained by reduced damaging effect on performance index ($PI_{abs}$) and maximal quantum yield of photosystem II (PSII) ($F_v/F_m$) through improvement of reaction centers (RC/CS) with associated changes in excitation energy trapping (TRo/CS) and electron transport ($ET_0$/CS) per excited cross-section of leaf. Additionally, under drought condition, plants cultivated with SA exhibited better protection against oxidative damage because of higher catalase (CAT) and ascorbate peroxidase (APX) activities and lower levels of malondoialdehyde (MDA) and hydrogen peroxide ($H_2O_2$). The present study suggests that salicylic acid can play a protective role during drought stress by enhancing the photosynthetic capacity and the antioxidant defense system.

*Key words*: antioxidative enzymes; *Salvia nemorosa*; performance index; salicylic acid; water stress

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## Introduction

Plant productivity is influenced adversely by environmental stresses such as salinity and drought (Mittler and Blumwald, 2010). During drought stress, the rate of reactive oxygen species (ROS) generation is dramatically elevated. Accumulation of ROS induces oxidative stress to proteins, membrane lipids, and other cellular components (Creissen and Mullineaux, 2002). The plant cells respond to elevation in ROS levels by increasing the expression and activity of ROS-scavenging enzymes (Mano, 2002; Miller et al., 2010). However, response of antioxidative enzymes to water stress is variable and depends on the intensity of the imposed water stress (Habibi and Hajiboland, 2011). Exposure of plants to water stress leads to serious physiological and biochemical dysfunctions including reduction in turgor, growth, photosynthetic rate, stomatal

*Corresponding author
*E-mail address*: gader.habibi@gmail.com
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conductance, and damages of cellular components (Janda et al., 2007).

Salicylic acid (SA) is a naturally occurring plant hormone which influences various physiological and biochemical functions in plants, acts as an important signaling molecule, and has diverse effects on tolerance to biotic and abiotic stresses (Arfan et al., 2007; Wang et al., 2010). It was shown that salicylic acid plays a key role in providing tolerance for the plants exposed to water stress, heat, heavy metals, and osmotic stress (El-Tayeb, 2005; Wang et al., 2010; Liu et al., 2011; Hayat et al., 2008; Kadioglu et al., 2011). It has been reported that the wheat seedlings subjected to drought stress when treated with salicylic acid, exhibited higher water content and dry matter production compared to the control (Singh and Usha, 2003). In our previous experiment, the results suggested that improvement of SA on drought tolerance of barely plants was associated with the increase in antioxidant defense abilities, alleviation in oxidative damage of functional molecules, and maintenance of many physiological processes such as photosynthesis under drought (Habibi, 2012).

The purposes of this work were (*a*) to investigate the effect of salicylic acid on *S. nemorosa* growth and photosynthesis, and (*b*) to evaluate the correlation between the photochemistry and drought tolerance in *S. nemorosa*. This is, the first study focused on the influence of SA treatment on the antioxidant properties and photosynthesis of *S. nemorosa* under drought conditions.

## Materials and Methods

### Plant growth and treatments

*S. nemorosa* seeds were planted in pots 14 cm in diameter and 95 cm in depth containing sandy loam soil. The field capacity (FC) of soil, measured by weighing, was 14%. Before sowing, for the basal fertilization, 200 mg nitrogen $kg^{-1}$ soil as $NH_4NO_3$ and 50 mg and 62.5 mg phosphorus and potassium $kg^{-1}$ soil as $KH_2PO_4$ were applied, respectively. The treatments were composed of Co (control) Dr (drought), SA (500 µM) and DSA (SA +drought). The treatments of water stress consisted of control, 100% FC and drought, 50% 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 28 ± 2/18 ± 2° C. After emergence, the seedlings were watered every 4-5 days to maintain 100% FC of the soil until fifteen days after sowing. From then on, the seedlings were watered every 4 days to maintain 100% FC in the Co soil and 50% 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.

Non-SA applied plants were sprayed with ethanol/water (1/1000 v/v). The volume of the spray was 25 ml per pot. Thirty days after sowing (15 d after SA application and drought treatment), measurements were done and the recent fully expanded leaves were collected and frozen in liquid $N_2$ immediately until analysis.

### Measurements of water relations

Leaves were washed with distilled water, blotted dry on filter paper and after determination of fresh weight (FW) they 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).

### Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence transients (OJIP transients) were recorded with a Plant Efficiency Analyser (PEA, Hansatech Instruments Ltd., King's Lynn, Norfolk, PE 32 1JL, England) in dark-adapted (for at least 20 min) leaves of *S. nemorosa*. The OJIP transients were induced by a red light (peak at 627 nm) of 3500 µmol $m^{-2}$ $s^{-1}$ (sufficient excitation intensity to ensure closure of all PSII reaction centers to obtain a true fluorescence intensity of Fm) provided by the PEA through an array of six light-emitting diodes.

## The JIP-test

JIP-test (Strasser and Strasser, 1995; Strasser et al., 2004) was used to analyze each OJIP transient. The data used from the original fluorescence measurements involved maximal fluorescence intensity (Fm), fluorescence intensity at 50 µs (considered as $F_0$), the specific energy fluxes (per reaction center) for absorption (ABS/RC), trapping ($TR_0$/RC), dissipation at the level of the antenna chlorophylls ($DI_0$/RC), and electron transport ($ET_0$/RC), the flux ratios or yields, i.e. the maximum quantum yield of primary photochemistry ($\varphi_{Po}=TR_0/ABS=F_V/F_M$), the efficiency ($\psi_0=ET0/TR_0$) with which a trapped exciton can move an electron into the electron transport chain further than *Q*A, the quantum yield of electron transport ($\varphi_{Eo}=ET_0/ABS$), and the phenomenological energy fluxes (per excited cross-section of leaf, CS) for absorption (ABS/CS), trapping ($TR_0$/CS), dissipation ($DI_0$/CS) and electron transport ($ET_0$/CS).

The fraction of active PSII reaction centers per excited cross-section (RC/CS) was also calculated. In addition to above parameters, a multi-parametric expression, the performance index ($PI_{abs}$), was also calculated (Strasser et al., 2000). The $PI_{abs}$ considers the three main steps that regulate photosynthetic activity by a PSII reaction center (RC) complex, namely absorption of light energy (ABS), trapping of excitation energy (TR), and conversion of excitation energy to electron transport (ET).

## Assay of enzymes activity and related metabolites

Determination of the activity of antioxidant enzymes and concentration of related metabolites 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), and 13 µM riboflavin. One unit of SOD was defined as the amount of enzyme which had 50% inhibition of NBT reduction under assay conditions.

For 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, 10 mM $H_2O_2$ and one unit represented 1 µM $H_2O_2$ decomposed $min^{-1}$.

Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test at 470 nm (Chance and Maehly, 1955). The enzyme was extracted by 10 mM phosphate buffer pH 7.0 and assayed in a solution contained 10 mM phosphate buffer, 5 mM $H_2O_2$, and 4 mM guaiacol. The enzyme activity was calculated as enzyme protein required for the formation of 1 µM tetraguaiacol $min^{-1}$.

Ascorbate peroxidase (APX, EC 1.11.1.11) was measured by following the decrease in absorbance at 290 nm as ascorbate was oxidized according to the method of Boominathan and Doran (2002). The reaction mixture contained 50 mM phosphate buffer pH 7, 0.2 mM EDTA, 0.5 mM ascorbic acid and 50 µg BSA. One unit represented 1 µM ascorbate oxidized $min^{-1}$. Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid (Sigma) at 532 nm. MDA levels were calculated from a 1, 1,

Table 1
Physiological traits of plants under different treatments

| Growth | Control | Drought | SA | Drought+SA |
|---|---|---|---|---|
| FW (g $plant^{-1}$) | 2.83 ± 0.21 $^a$ | 1.79 ± 0.24 $^b$ | 2.94 ± 0.31 $^a$ | 2.20 ± 0.23 $^a$ |
| DW (g $plant^{-1}$) | 0.72 ± 0.11 $^a$ | 0.51 ± 0.14 $^b$ | 0.78 ± 0.12 $^a$ | 0.64 ± 0.13 $^a$ |
| RWC (%) | 89 ± 3 $^a$ | 59 ± 5 $^b$ | 91 ± 2 $^a$ | 72 ± 3 $^{ab}$ |

FW: fresh weight; DW: dry weight and RWC: relative leaf water content; 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).

3, 3-tetraethoxypropane (Sigma) standard curve. The concentration of $H_2O_2$ was determined using potassium titanium-oxalate at 508 nm (Habibi and Hajiboland, 2011). Soluble protein was estimated spectrophotometrically by method described by Bradford (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 ($P \leq 0.05$). Correlation analysis using Spearman Rank Order Correlation in sigma stat (3.5) were conducted to determine the relationship between $PI_{abs}$ and leaf RWC.

## Results

The data in Table 1 show that plant responses to the combined drought and SA spraying treatment were distinctly different from the responses when the drought stress was applied alone. Water stress applied alone remarkably reduced the shoot fresh matter and shoot dry matter. Drought stress also significantly decreased RWC. SA-supplemented plants showed higher dry weight and water content compared to those without application of SA under drought conditions.

To understand the precise effects of SA on the kinetics of recorded OJIP transients, data collected at water stress periods were analyzed in Fig. I. The effects of drought stress on the maximum quantum yield of primary

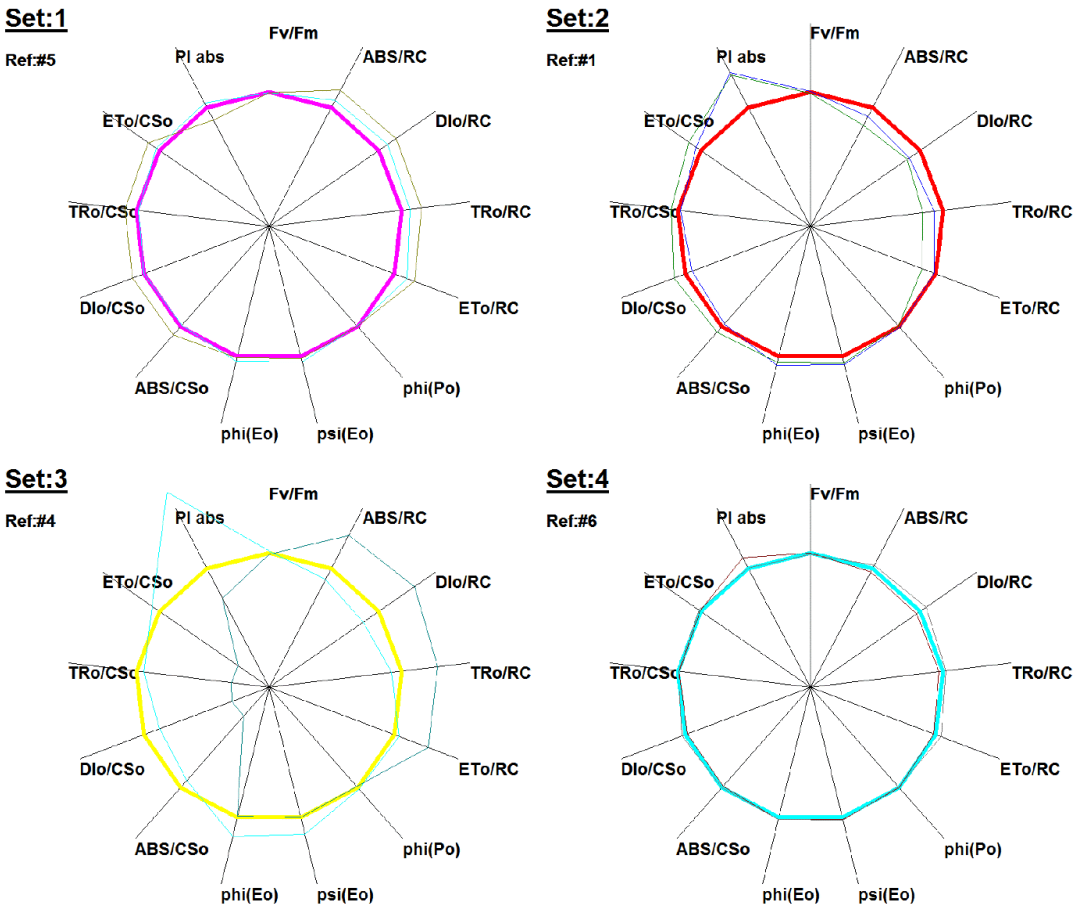


Fig. I. Radar plots depicting changes in the phenomenological (per CS) and specific (per RC) energy fluxes of absorption (ABS) excitation energy trapping (TR) and electron transport (ET); changes in quantum efficiency ($F_v/F_m$) and the performance indexes ($PI_{abs}$) are also shown. Set 1: control, Set 2: SA, Set 3: drought, and Set 4: drought + SA

photochemistry ($F_v/F_m$) and the specific and phenomenological energy fluxes for light absorption, excitation energy trapping, and electron transport are also shown in the form of a radar plot (Fig. I). Drought stress resulted in the deactivation of reaction centers (RC/CS) and decreased excitation energy trapping ($TR_0$/CS) and electron transport ($ET_0$/CS). Notably, the effects of drought stress on the increase in ABS/RC were alleviated by SA applications. Well-watered plants (control and SA treatments) had performance indexes ($PI_{abs}$) of 8-10; however, under drought conditions, the $PI_{abs}$ values in *S. nemorosa* were 400% lower than those recorded in control plants (Fig. I). SA-supplemented plants showed higher $PI_{abs}$ compared to those without application of SA under drought conditions. Correlation of the decline in plant water status with decreased performance index was observed in fully expanded leaves of *S. nemorosa* (Fig. II). CAT, SOD, and POD activities were stimulated under drought stress in comparison with the control. A significant rise in the activity of APX 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 malondoialdehyde (MDA) and hydrogen peroxide ($H_2O_2$) (Fig. III). However, hydrogen peroxide and malondoialdehyde levels decreased by SA application under drought stress.

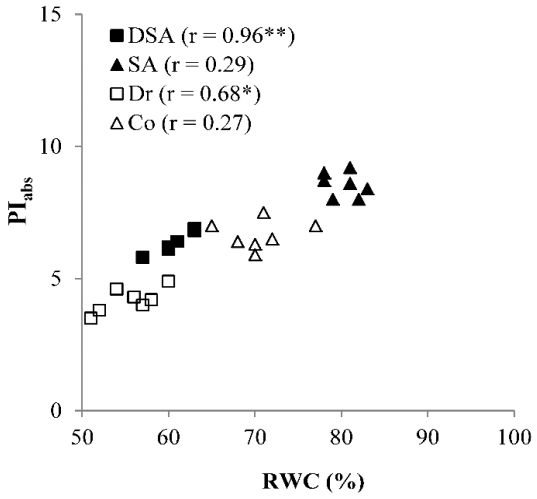


Fig. II. Correlations between the performance indexes ($PI_{abs}$) and the relative water content (RWC) in *Salvia nemorosa* plants grown with or without SA supplementation after 15 d drought treatment: ns, *, and **: non-significant, significant at the 5 % and 1 % levels of probability, respectively

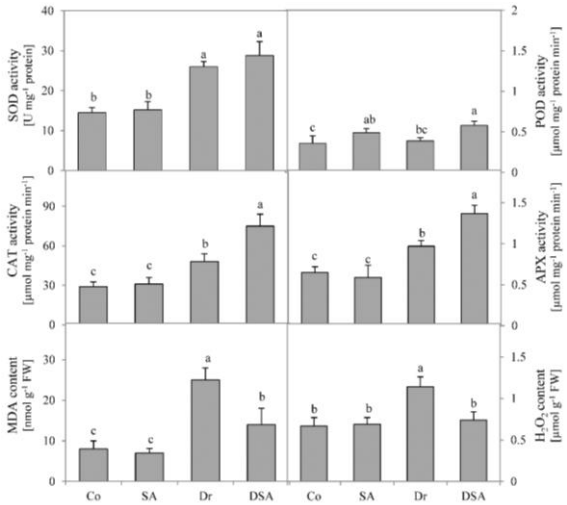


Fig. III. Antioxidant index of *S. nemorosa* plants under different treatments; SOD: superoxide dismutase; CAT: catalase; POD: peroxidase; APX: ascorbate peroxidase; MDA: malondoialdehyde; $H_2O_2$ hydrogen peroxide; each value is the mean ± SD of 4 replicates. Bars indicated with the same letter are not significantly different (P<0.05).

## Discussion

It was evident that drought significantly reduced the RWC and dry matter, as is was observed in other plants species (Degu et al., 2008; Gao et al., 2011). When SA spraying and drought stress were applied together, adverse effects of drought were less than those observed when the drought stress was applied singly (Singh and Usha, 2003; Hayat et al., 2010; Kadioglu et al., 2011). In addition, application of SA maintained a high dry matter and RWC under drought. These results show that application of SA is useful for drought tolerance improvement of S. nemorosa plants. In this study, reductions in photosynthetic performance under water stress were close with the findings of Lawlor and Cornic (2002), Tognetti et al. (2005), Bacelar et al. (2006), and Ben Ahmed et al. (2009).

Results suggested that the decrease in the $PI_{abs}$ and down-regulation of photochemical activity during water stress conditions may be interpreted as the evidence for PSII RC deactivation (Ivanov et al., 2006) and the $PI_{abs}$ was much more sensitive than the $F_v/F_m$ ratio. These results indicate that PSII RC's are functionally

altered by SA application through increase in density of active reaction centers, RC/CS.

Decline in photosynthetic performance in the unsprayed control might be partially due to lipid peroxidation of chloroplast membranes, as revealed by the higher hydrogen peroxide and malondoialdehyde levels. Chloroplasts are the major source of $H_2O_2$ production. $H_2O_2$ can inhibit $CO_2$ fixation through oxidative inactivation of the photosynthetic carbon reduction cycle enzymes (Djanaguiraman et al., 2010). Plants possess an enzymatic antioxidant defense system to protect themselves against ROS. There is data supporting the idea that SA increases the activity of antioxidant enzymes such as CAT, POD, and SOD (Hayat et al., 2008, 2010). In the present study, APX and CAT activities were increased in the SA supplemented water-deficit samples relative to water-deficit treatment (Fig. III). CAT can simultaneously have access two molecules of $H_2O_2$, thereby reducing the increased cytosolic concentration of $H_2O_2$ and oxidative damage (Djanaguiraman et al., 2010). Lipid peroxidation and membrane damage decreased with an increase in CAT and APX activity. Plants cultivated with SA have a strengthened antioxidative capacity due to the increased antioxidant enzyme activity and decreased MDA and $H_2O_2$ content (Xue et al., 2001; Djanaguiraman et al., 2010).

**Conclusion**

In summary, our results indicate that drought stress caused higher production of $H_2O_2$, MDA content, greater membrane damage and declines in photosynthetic rate, and finally resulting in lower dry matter production. From this conclusion we can say that foliar application of SA under water stress alleviates oxidative stress by enhancing antioxidant defense mechanism in *S. nemorosa* plants. This investigation showed that the down-regulation of photochemical activity during periods of water stress occurred through PSII RC deactivation, and rapid up-regulation of the same processes was observed following SA applications.

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