Effect of Exogenous Salicylic Acid on Some Physiological Parameters and Alleviation of Drought Stress in *Nigella sativa* Plant under Hydroponic Culture

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Abstract

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To study the effect of salicylic acid on photosynthetic pigments (chlorophyll *a*, *b*, total chlorophyll, and carotenoids), polyphenol compounds, anthocyanin, flavonoids, phenylalanine ammonia-lyase activity, malondialdehyde, lipoxygenase activity, electrolyte leakage, relative water content, soluble sugar contents, and protein content of black cumin (*Nigella sativa*) under drought stress in hydroponic culture, an experiment was conducted as a completely randomised design in a factorial arrangement with three replicates. Experimental treatments included salicylic acid at three levels (0, 5, and 10μM) and drought stress (induced by polyethylene glycol 6000) at four levels (0, –0.2, –0.4, and –0.6 MPa). Results showed that salicylic acid application through the root medium increased drought tolerance of black cumin seedlings. Plants pre-treated with salicylic acid exhibited slight injury symptoms whereas those not pre-treated with salicylic acid had moderate damage and lost considerable portions of their foliage. In conclusion, salicylic acid could protect the *Nigella* plant against drought stress through increasing of all the mentioned traits, and 10μM salicylic acid was the most effective level under both conditions.

Keywords: black cumin; lipid peroxidation; phenolic compounds; osmotic stress; oxidative stress

Abbreviations: Chl – chlorophyll; LOX – lipoxygenase; MDA – malondialdehyde; PAL – phenylalanine ammonia-lyase; ROS – reactive oxygen species; RWC – leaf relative water content; SA – salicylic acid

Drought stress is one of the most important environmental factors that regulate plant growth and development, and limit plant production. Plant can respond and adapt to drought stress by alerting their cellular metabolism and invoking various defense mechanisms (Bohnert & Jensen 1996). Better understanding of the mechanisms that able plant to adapt to drought stress is necessary to make the best management of drought conditions. Plants have different mechanisms to avoid the water deficit. Drought stress also reduces photosynthesis, for a number of reasons: (*i*) hydroactive stomatal closure reduces the CO_2 supply to the leaves; (*ii*) water deficiency damages the cytoplasm ultrastructure and enzyme activity; (*iii*) dehydrated cuticles, cell walls, and plasma membranes are less permeable for CO_2 (Neocleous & Nasilakakis 2007). The reason of that environmental stress such as drought inhibits the growth and photosynthetic abilities of plant is the disturbance of the balance between the production of reactive oxygen species (ROS) and the antioxidant defense, causing accumulation of ROS, which induces oxidative stress and damages protein, membrane lipids, and other cellular components (Monakhova & Chernyad'v 2002). In order to survive, aerobic organisms have evolved enzymatic and non-enzymatic defense mechanisms against oxidative stress. To control the level of ROS and to protect cells under stress conditions, plant tissues contain several en-

zymes scavenging ROS and a network of low molecular weight antioxidants (ascorbate, glutathione, phenolic compounds, tocopherols, and carotenoids).

Salicylic acid (SA) is a signaling molecule with ubiquitous distribution in plants, and participates in plant physiology processes. Recently effects of SA on plants have been studied. A large body of evidence indicated that SA plays critical roles in plants including respiration, stomatal movement, photomorphogenesis, seed germination, and senescence. It is more important that SA has been demonstrated to be messenger involved in signal transduction in response to biotic and abiotic stresses (Clarke *et al*. 2000).

Nigella sativa (black cumin) is an annual flowering plant, native to southwest Asia and used widely in traditional and industrial pharmacology. It is reported that intact black cumin seeds or their extracts contain anti-diabetic, antihistaminic, anti-hypertensive, anti-inflammatory, anti-microbial, antitumor, galactagogue and insect repellent effects (D'Antuno *et al*. 2002). Detailed investigations on medicinal plants have been less frequent than on agricultural crops especially under drought stress; therefore, their tolerance assessment for cultivation in arid and semi-arid areas is very important.

The objective of the present experiment was to investigate the effects of salicylic acid pretreatment on alleviation of oxidative damages induced by drought stress. Comparing these responses can be useful in understanding the physiological and biochemical mechanisms of this compound in plants which have to cope with drought stress.

MATERIAL AND METHODS

Plant material. The seeds of black cumin were cultivated in pots which filled with sand and irrigated daily. After the emergence of cotyledons, they were irrigated with half-strength Hoagland's solution once a week. After four weeks, the seedlings were transferred to hydroponic culture which is aerated with air pomp in order to application of treatments. After the optimising of salicylic acid (SA) and polyethylene glycol (PEG-6000; Scharlau, Barcelona, Spain) concentrations, 5 and 10μM SA concentrations and –0.2, –0.4 and –0.6 MPa for osmotic potential were considered. The seedlings were pretreated with 5 and 10μM SA that were added to nutrient solution under hydroponic culture. After 24 h, in order to application of *in vitro* drought stress, the seedlings were subjected to PEG solutions to obtain osmotic stress at the levels of –0.2, –0.4, and –0.6 MPa. Distilled water was used as a control. After 48 h, the shoots of plants were gathered and immediately frozen in liquid nitrogen and stored at –80°C for subsequent analysis.

Leaf relative water content *(RWC)* was calculated as follows: RWC = [(fresh weight – dry weight)/(saturated weight – dry weight)] × 100 (Wheutherley 1950).

Electrolyte leakage. The electrolyte leakage was determined as described by Ben Hamed *et al.* (2007). Shoot samples (0.2 g) were placed in test tubes containing 10 ml of double distilled water. The tubes were incubated in a water bath at 32°C for 2 h and the initial electrical conductivity of the medium (EC_1) was measured by an EC meter (Metrohm, Filderstadt, Germany). The samples were autoclaved at 121°C for 20 min to release all the electrolytes, cooled at 25°C, and then the final electrical conductivity (EC_2) of each was measured. The electrolyte leakage (EL) was calculated by using the following formula: $EL = (EC_1/EC_2) \times 100$.

Thiobarbituric acid reactive substance *(TBARS).* The level of lipid peroxidation in plant tissues was measured by determination of malondialdehyde (MDA) and other aldehydes which are known to be breakdown products of lipid peroxidation. For MDA measurement, the non-specific absorbance of supernatant at 600 nm was subtracted from the maximum absorbance at 532 nm and an extinction coefficient (ε) of 1.55×10^5 $M^{-1}Cm^{-1}$ was used for determination of MDA concentration (Heath & Packer 1968). The extinction coefficient of 0.457×10^5 $M^{-1}Cm^{-1}$ (Meirs *et al*. 1992) was used for calculation of other aldehydes concentration.

Enzyme extraction and activity determination and total soluble proteins. Frozen shoot samples (0.5 g) were homogenised in 2.5 ml of 50mM phosphate buffer (pH = 7) containing 1M ethylenediamine tetraacetic acid (EDTA), 1mM phenylmethylsulfonyl fluoride (PMSF), and 1% polyvinyl pyrrolidone (PVP). The homogenate solution was centrifuged at 20 000 *g* at 4°C for 20 min and the clear supernatant was used directly for the assay of enzyme activity and estimation of protein. The supernatant was used for measurement of total soluble protein according to Bradford (1976) using bovine serum albumin as standard.

Phenylalanine ammonia-lyase (PAL) activity assay *(EC 4.3.1.5)*. PAL activity was assayed according to the method of D'Cünha (1996). The reaction mixture contained 100mM Tris-HCl buffer (pH 8.5), 1mM 2-mercaptoethanol, 50mM L-phenylalanine, and 100 μl of enzyme extract. The mixture was incubated at 30°C for 15 minutes. The reaction was terminated

by the addition of 0.5 ml 6M HCl and absorbance of the supernatant was measured at 290 nm. One unit of enzyme represents the conversion of 1 µmol substrate to cinammic acid per minute.

Estimation of flavonoids content. To determine the content of flavonoids, 0.1 g of leaf tissue was extracted in 15-ml glass centrifuge tubes containing 10 ml of acidified ethanol (ethanol:acetic acid, 99:1 (v/v)). The samples were gently boiled in a water bath at 80°C for 10 min and brought up to volume. Absorbance was measured at three wavelengths: 270, 300, and 330 nm with UV-VIS spectrophotometer (Krizek *et al.* 1998).

Determination of anthocyanins content. For determination of anthocyanins content, frozen tissue samples (100 mg) were soaked immediately in 10 ml of acidified methanol (methanol:HCl 99:1 (v/v)). Tissues were crushed using a glass pestle and kept at 25°C for 24 h in the dark. The extract was then centrifuged at 4000 *g* for 5 min at room temperature and absorption at 550 nm of the supernatant was read by an UV-VIS spectrophotometer (Varian Cary 50; Varian GmbH, Darmstadt, Germany). For the calculation of the amount of anthocyanins, the extinction coefficient of 33 000 $M^{-1}Cm^{-1}$ was used (Wagner 1979).

Determination of total phenol content. The total phenol content in leaves was determined by the method of Folin-Ciocalteu reduction, using gallic acid as standard. The phenol content was expressed as gallic acid equivalents in 1 mg dry weight (DW) (Gao *et al.* 2000).

Estimation of chlorophyll content. Chlorophyll (Chl) content was determined using the methods of Lichtenthaler (1987). In this method, Chl was extracted in the 80% acetone. Extracts were centrifuged at 3000 *g*, and the absorbance of the supernatant was measured at 663.2, 646.8, and 470 nm with a spectrophotometer (Cary 50; Varian Instruments, Walnut Creek, USA).

Determination of soluble sugar content. Frozen samples (0.1 g) were grinded and extracted with 2.5 ml of 80% (v/v) ethanol at 90°C for 60 min, followed by centrifugation at 10 000 *g* at 4°C for 10 minutes. The process was repeated for complete extraction. Total soluble sugar content was determined using anthrone reagent and glucose as standard (Roe 1955). Results were expressed as mg soluble sugar/g DW.

Statistical analysis. The experiments were performed in a randomised order. Data were expressed as means of three replicates with standard error. Statistical assays were carried out by one-way ANOVA using Duncan test to evaluate whether the means were significantly different, taking $P < 0.05$ as significant.

RESULTS

Leaf relative water content *(RWC).* Polyethylene glycol caused a significant reduction in RWC. SA pretreatment markedly alleviated the effect of drought stress and also increased this trait (Figure 1). There was no significant difference between SA concentrations and control under normal condition, but SA applied through the root medium was more effective in both concentrations at all levels of drought stress (Figure 1).

Lipid peroxidation, membrane stability index, and LOX activity. Increment of MDA and other aldehydes interpreted as reason for an increased lipid peroxidation under drought stress condition. The data indicated that drought induced an increase in the amount of MDA and other aldehydes (Figures 2A and 2B). Pretreatment with SA reduced lipid peroxidation in plants which were subjected to drought stress and had no effect on control plants. In order to investigation, the effect of drought stress on membrane permeability, electrolyte leakage was measured. Base on obtained results, drought stress caused an increasing on electrolyte leakage to intercellular space and SA pretreatment reduced this leakage at all the levels of drought stress (Figure 2C).

Estimation of polyphenols compounds, anthocyanin, flavonoids, and PAL activity. Dught treatments at the potentials of –0.4 and –0.6 MPa caused a reduction (23.2%) in polyphenol compounds compared with control (Figure 3A). Pretreatment of plants with SA had no significant effect on these compounds under control and –0.2 MPa conditions, while SA pretreatment increased these compounds at the drought levels of –0.4 and –0.6 MPa. Results showed that drought

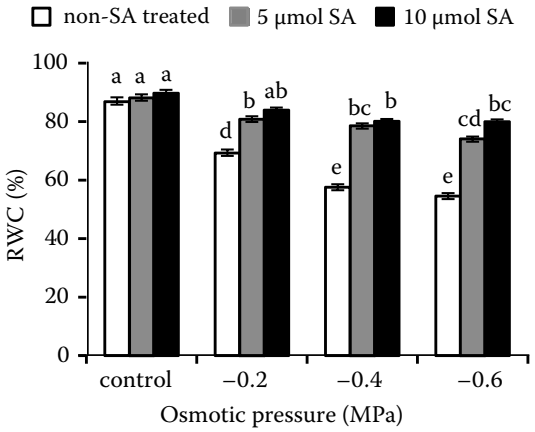

Figure1. Effect of salicylic acid pretreatment on relative water content in *Nigella* plant leaves under control and drought stress conditions (the mean comparisons of treatments were done using *LSD* method at $P < 0.05$ significance level)

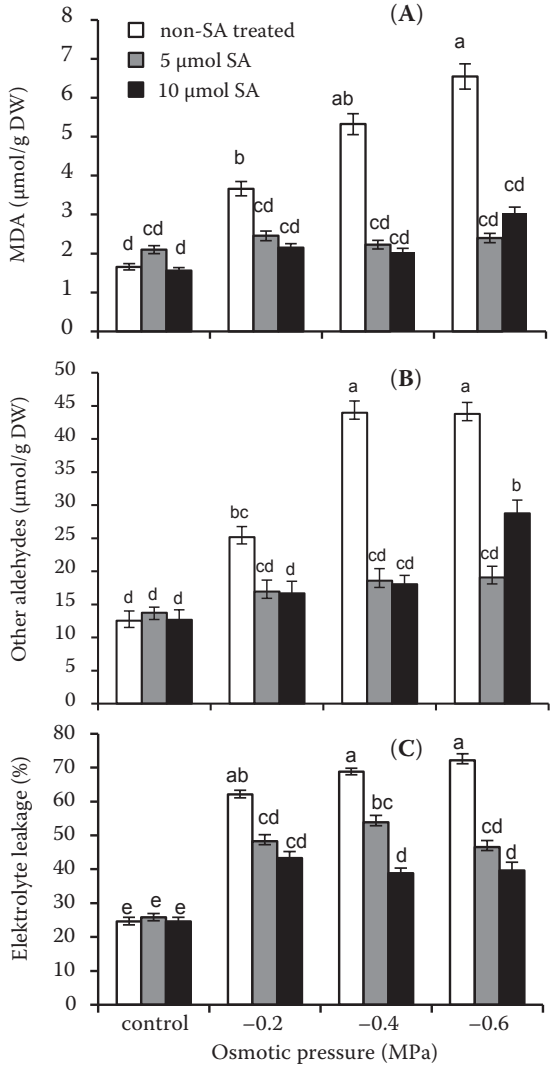

Figure 2. Effect of salicylic acid pretreatment on MDA (**A**), other aldehydes content (**B**), and electrolyte leakage (**C**) in *Nigella* plant leaves under control and drought stress conditions (the mean comparisons of treatments were done using *LSD* method at $P < 0.05$ significance level)

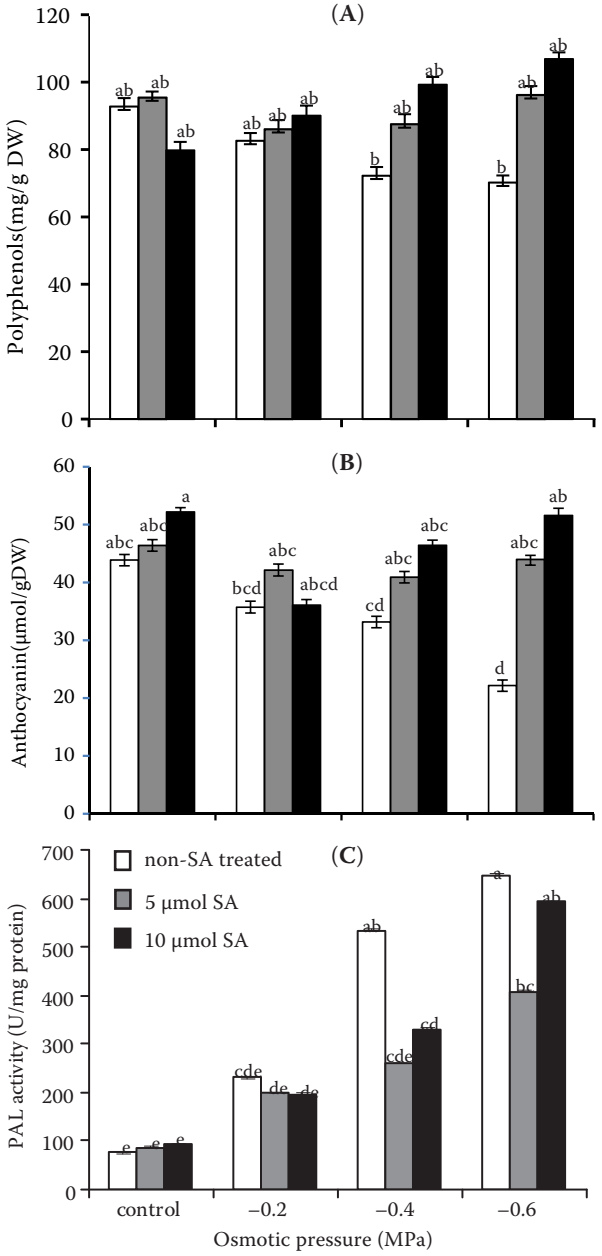

Figure 3. Effect of salicylic acid pretreatment on polyphenol compounds (**A**), anthocyanin (**B**), and PAL activity (**C**) in *Nigella* plant leaves under control and drought stress conditions (the mean comparisons of treatments were done using *LSD* method at $P < 0.05$ significance level)

stress induced by PEG caused the reduction of anthocyanin, but pretreatment with SA increased this pigment under stress conditions (Figure 3B). The effect of osmotic potential on phenylalanine ammonia-lyase (PAL) activity was shown in Figure 2C. Based on results; the higher levels of drought stress led to a remarkable increase in PAL activity. At the –0.4 MPa level both SA concentrations decreased the PAL activity, while under –0.6 MPa only the lower SA concentration caused decrease in PAL activity compared to the non SA-treated plants. Flavonoids contents were reduced by increasing drought stress, especially at the levels of –0.4 and –0.6 MPa (Figure 4). As expected, spectrophotometer measurements at all three wavelengths – 270 nm (Figure 4A), 300 nm (Figure 4B), and 330 nm (Figure 4C) revealed that SA application in plants under the mentioned levels of drought stress increased the flavonoids contents. The results indicated that different concentration of SA had the same effect on different absorbance percentages (Figure 4).

Protein content. The protein contents were reduced under drought stress. Treatment of plants with 10µM SA significantly increased the protein content under drought stress (Figure 5).

Chlorophyll and carotenoids content. Drought stress only at –0.6 MPa level reduced Chl *a*, *b*, total Chl, and carotenoids by approximately 42, 37, 40.2, and 63.1%, respectively, compared to control. Pretreatment of plants with SA was more effective under severe drought stress (–0.4 and –0.6 MPa) than in moderate and control conditions. Chl and carotenoids contents increased in plants, which were pretreated with SA compared with non-treated plants (Figure 6).

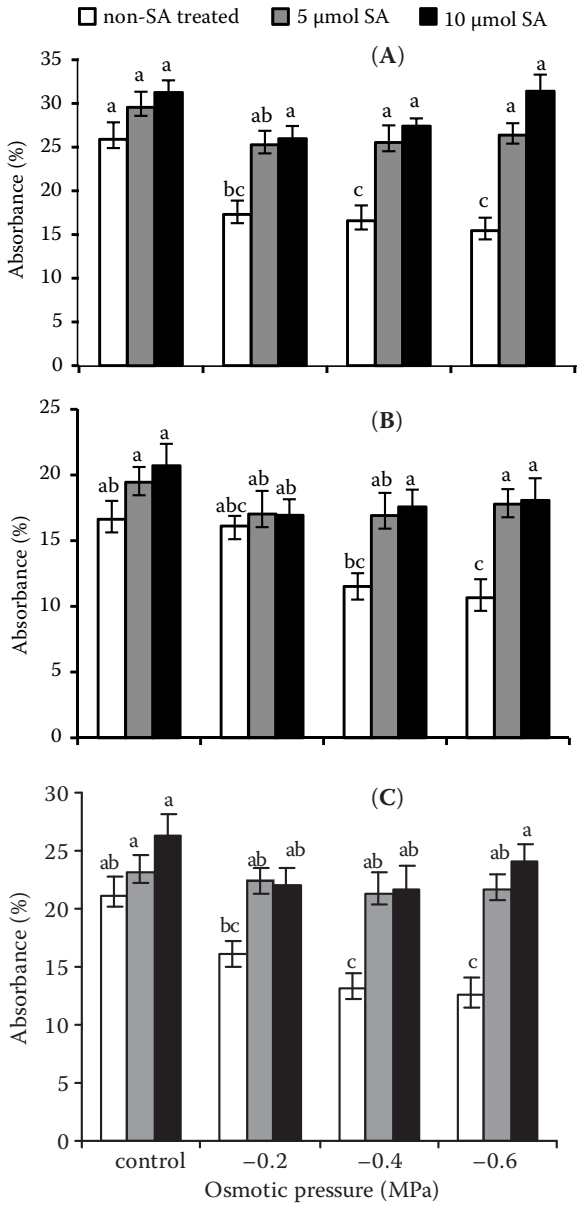

Figure 4. Effect of salicylic acid pretreatment on flavonoids measured spectrophotometrically at wavelengths of 270 nm (**A**), 300 nm (**B**), and 330 nm (**C**) in *Nigella* plant leaves under control and drought stress conditions (the mean comparisons of treatments were done using *LSD* method at $P < 0.05$ significance level)

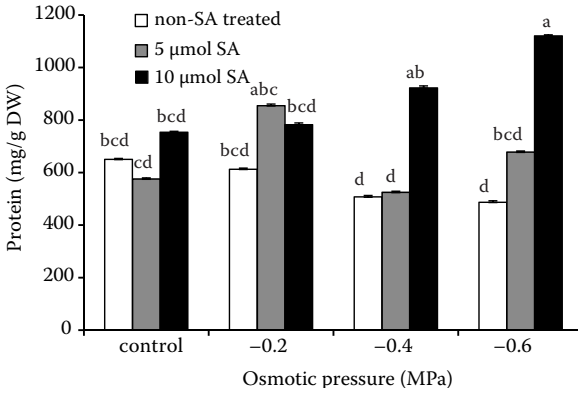

Figure 5. Effect of salicylic acid pretreatment on protein content in *Nigella* plant leaves under control and drought stress conditions (the mean comparisons of treatments were done using *LSD* method at $P < 0.05$ significance level)

Soluble sugar content. The results showed that soluble sugar content was increased under drought stress. SA pretreatment with the concentration of 5 and 10µM caused the reduction of this trait at –0.4 and –0.6 MPa treatments, but in the –0.2 MPa treatment SA pretreatment had no significant effects on carbohydrates content when compared with non-pretreated plants (Figure 7).

DISCUSSION

Drought is an important factor influencing the growth and physiological characteristics of plants (Xiangwen *et al.* 2009). The responses of plants to drought stress depend on the species and genotype, the length and severity of water deficit, and the age and stage of development (Bray 1997). Stress factors are well-known to cause a shift in the antioxidant balance in plant cells. This shift is due to an increase in the rate of generation of ROS, which induce lipid peroxidation in the membrane structures of the cells. Compounds that are able to reduce the damaging effects of various stresses are prominent in both theoretical and practical points of view. In this research, SA was used as an important signal molecule for modulating plant responses to drought stress which participates in the regulation of physiological processes, to study the effect of this phytohormone on some physiological parameters of plants under stress.

Drought stress induced by PEG decreased RWC (Figure 1). Similar results were obtained in barley (Kocheva *et al.* 2005), wheat (Lei *et al.* 2007), and rice (Hsu & Kao 2003) under drought stress. Lower water uptake by roots resulted in decrease of RWC. The decrease in water potential gradient between roots and their surrounding media due to the effects

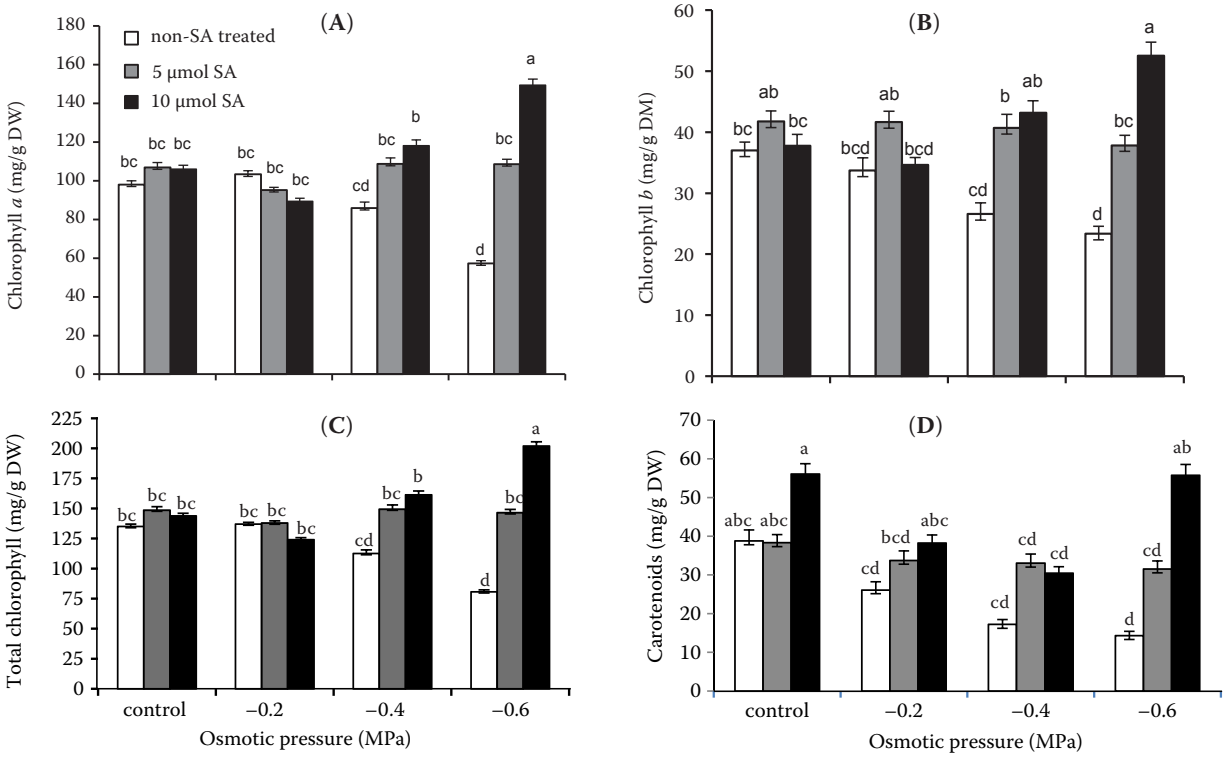

Figure 6. Effect of salicylic acid pretreatment on chlorophyll (Chl) *a* (**A**), Chl *b* (**B**), total Chl (**C**), and carotenoids (**D**) contents in *Nigella* plant leaves under control and drought stress conditions (the mean comparisons of treatments were done using *LSD* method at $P < 0.05$ significance level)

of PEG_{6000} adversely affected RWC. SA pretreatment caused the RWC increase under drought stress (Figure 1). These results were in agreement with the findings of Singh and Usha (2003). Increasing of RWC may be related to the role of SA in accumulation of compatible osmolytes in plants subjected to drought stress.

MDA and other aldehydes are often used as a measure of free radical damage to cell membranes under stress conditions (Halliwel & Gutteridge 1984). MDA and other aldehydes products of lipid peroxidation and an indicator of membrane damage were significantly increased on plants which were not treated with SA under drought stress. LOX is responsible for membrane degradation because catalyse the deoxygenation of polyunsaturated fatty acids that are toxic for cell. The comparative rates of lipid peroxidation were assayed in the leaves of control and PEG-treated black cumin by determining the levels of MDA and other aldehydes content. In case of control samples, the level of MDA and other aldehydes was nearly the same in all concentrations of SA. Upon PEG imposition, the other aldehydes level was increased on non-SA treated plants (Figure 2A). In this investigation SA pretreatment decreased the electrolyte leakage percentage. Several studies showed that MDA content and electrolyte leakage in susceptible plants were higher than in resistant plants (Juan *et al.* 2005). In previous studies, effect of pretreatment with SA was evidenced by a reduction in the level of lipid peroxidation and leakage of electrolytes from plant tissues as well as by more intensive growth processes as compared to control plants (Hayat & Ahmad 2007).

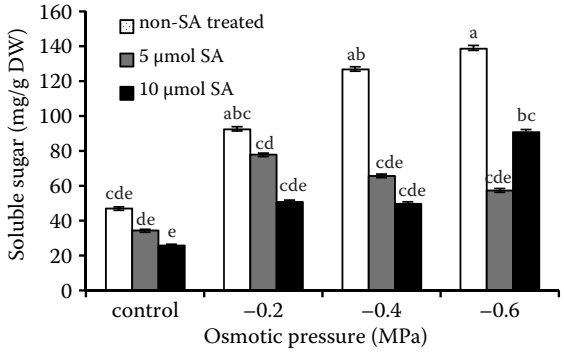

Figure 7. Effect of salicylic acid pretreatment on soluble sugar content in *Nigella* plant leaves under control and drought stress conditions (the mean comparisons of treatments were done using *LSD* method at $P < 0.05$ significant level)

The phenylpropanoid pathway is one of the important pathways of plant secondary metabolism,

which yields a variety of phenolics with structural and defense-related functions. These phenolic compounds include phenolic acid, anthocyanin, and flavonoids, which act as scavengers of free radicals and other oxidative species through their hydrogen donating (antioxidant) potential (Syvacy & Sokmen 2004). In *Nigella* plant, drought resulted in a decline in phenol compounds (Figure 3A). One of the possible reasons to explain the reduction of these compounds under drought stress is related to the antioxidant characteristics of these compounds to scavenging of ROS under drought stress (Sakihamai *et al.* 2002). Phenylalanine ammonia-lyase (PAL) is a crucial enzyme of phenylpropanoid metabolism, catalysing the formation of *trans*-cinnamic acid by L-deamination of phenylalanine. This enzyme was induced by various, biotic as well as abiotic, stresses which resulted in the accumulation of such phenolic compounds as phenolic acids and flavonoids (Solecka 1997). However, application of PAL inhibitor, 2-amino-2-indanophonic acid (AIP) could decrease PAL activity. As a result, the contents of the phenolics were markedly decreased and the resistance to the stresses was weakened. Results of this study showed that the activity of PAL enzyme increased in plants, which were under drought stress while the amount of phenolic compounds decreased under this situation, which exhibited the antioxidant role of these compounds against ROS production. It seems that the application of SA in a drought-stressed plant could alleviate the degree of drought and, therefore, the amounts of phenolic compounds increased significantly.

A possible reason for the reduction of photosynthetic pigments might be related to drought stress and this reduction is dependent upon several factors such as intensity, duration, and phonological phase of growth and genetic resistance capacity of plants. Reduction of photosynthetic pigments in drought stress could be related to degradation of chloroplast structure and photosynthetic apparatus, chlorophyll photo oxidation, destruction of chlorophyll substrate, inhibition of chlorophyll biosynthesis, and the increase of chlorophyllase activity. In this research, drought stress caused the reduction of photosynthetic pigments but SA pretreatment increased Chl (as the main part of photosynthetic structure) and carotenoids contents in both conditions (Figure 6). Costa *et al.* (2005) also reported that the activation of LOX caused the degradation of Chl. The reduction of carotenoids content under drought stress was related to the degradation of β-carotene (Sultana *et al*. 1999). Under drought stress, reduction of carotenoids could be related to its protection role in the photosynthetic apparatus, because carotenoids were responsible for scavenging of ROS, preventing lipid peroxidation, and ultimately mitigation of oxidative stress (Koyro 2006). SA applied in satisfactory concentrations may temporarily lower the level of oxidative stress in plants, which acts as a hardening process, improving the antioxidative capacity of the plants and helping to induce the synthesis of protective compounds (such as carotenoids) (Hayat & Ahmad 2007). Application of SA in *Zea mays* (Khodary 2004), barely (El-Tayeb 2005), wheat (Agrawal *et al.* 2005), and *Brassica napus* (Ghai *et al.* 2002) increased Chl *a*. Enhancement of the level of chlorophyll and carotenoid pigments, photosynthetic rate, carboxylase activity of Rubisco, and modification of the activity of some of the important enzymes – these are the roles assigned to SA (Hayat & Ahmad 2007).

Data presented in Figure 5 demonstrated that protein content decreased under drought stress. The reduction of protein content was the prevalent phenomenon in drought stress, because water deficiency had a major effect on the nitrogen metabolism. There is a considerable decline in protein synthesis in drought-stressed plants, due to the reduced number of polysomal complexes in tissues with lower water content. In this study, the generation of ROS caused the oxidation of amino acids and could burst the protein structure; therefore, oxidative stress was the most important reason for the reduction of proteins in *Nigella* plants under drought stress. However, SA pretreatment increased protein contents under both conditions (Figure 5). SA caused the increment of protein content on soybean (Kumar *et al.* 1999) and wheat (Singh & Usha 2003). Increasing the activation of nitrate reductase and nitrate contents caused the increment of protein content on SA treated plants (Fariduddin *et al.* 2003). It seems that the character of SA effect on the state of the hormonal system may well contribute to protective reactions of plants, acceleration of reparative processes, and the effect on protein contents (Hayat & Ahmad 2007).

Soluble sugars accumulated in plants for osmotic adjustment in response to drought and salinity stress and caused the protection of macromolecules and DNA structures (Juan *et al.* 2005). Nevertheless, results concerning the effect of drought and salinity stress on carbohydrate accumulation differ. Some researchers reported that carbohydrate contents increased under stress conditions (Jones & Turner 1980), some believed that this trait was reduced under stress conditions (Hanson & Hitz 1982), and some others reported that soluble sugar contents remained stable (Morgan 1992). Data presented

throughout this study indicated that drought stress caused the increment of soluble sugar contents, and pretreatment with SA had no effect on sugars under control condition (Figure 7). In the severe drought stress (–0.6 MPa), pretreatment of plant with 5µM SA decreased the sugar content but 10µM SA had no significant effect. In plant under –0.2 and –0.4 MPa drought, 10µM SA reduced the sugar content.

In conclusion, investigation of physiological parameters in *Nigella* showed that this plant is more sensitive to drought, and the application of SA could reduce the drought damages, especially at high levels of stress.

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