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ORIGINAL ARTICLE

Salicylic Acid Ameliorates the Effects of Oxidative Stress Induced by Water Deficit in Hydroponic Culture of *Nigella sativa*

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Osmotic stress associated with drought, and salinity is a serious problem that inhibits the growth of plants, mainly due to disturbance of the balance between production of ROS and antioxidant defense and causing oxidative stress. The results obtained in the last few years strongly prove that salicylic acid could be a very promising and protective compound for the reduction of biotic and abiotic stresses in sensitive of crops, because under certain conditions, it has been found to mitigate the damaging effects of various stress factors in plants. In this research, salicylic acid was used in control, and drought stressed plants, and the role of this compound in reduction of oxidative damages in *Nigella* plant was investigated. Data presented in this study indicated that SA application through the root medium brought on the increased levels of drought tolerance in black cumin seedlings. Plants pre-treated with SA exhibited slight injury symptoms whereas those that were not pre-treated with SA had moderate damage and lost considerable portions of their foliage. SA very profoundly inducing the activity of CAT, APX and GPX in plants, which led to reduction in H_2O_2 content, lipid peroxidation (MDA) and LOX activity so it seems that the application of SA greatly improves the dehydration tolerance through elevated activities of antioxidant systems or may be the expression of genes encoding some ROS-scavenging enzymes under drought stress, which would maintain the redox homeostasis and integrity of cellular components.

Key words: Antioxidant enzymes, Drought, Lipid peroxidation, Nigella, Reactive oxygen species

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Abbreviations: APX—ascorbate peroxidase; CAT—catalase; GPX—guaiacol peroxidase; MDA—malondealdehyde; ROS— reactive oxygen species, SA— Salicylic acid.

Water deficit is one of the most important environmental factors that regulate plant growth and development, and limit plant production. Plant can respond and adapt to water stress by alerting their cellular metabolism and invoking different defense mechanisms (Bohner and Jensen, 1996).

Survival under this stressful condition depends on the plant's ability to perceive the stimulus, generate and transmit the signal and initiate varying physiological and biochemical changes (Shinozaki and Yamaguchi, 1997). Increasing evidence suggest that drought stress induces oxidative stress through the production of reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals during stress conditions (Smirnoff, 1993). Electron leakage in the electron transport system in chloroplast and mitochondria is the main source of ROS. They are highly toxic and can damage many important cellular components such as lipid, proteins, DNA and RNA (Smirnoff, 1993). To control the level of reactive oxygen species, plants have evolved an antioxidant defense system comprising of enzymes such as a superoxide dismutase (SOD), Catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR) as well as non-enzymatic constituents such as ascorbate (ASA) and glutathione (GSH), which are responsible for scavenging excessively accumulated ROS in plants under stress conditions (Shi et al., 2007). The regulation of these antioxidant constituents by an exogenous substance might mediate the plant tolerance to drought stress.

Salicylic acid is (SA) is an endogenous growth regulator of phenolic nature, which participates in the regulation of physiological processes in plants. SA could be included in the category of phytohormones (Raskin, 1992). Exogenous application of SA may influence a range of diverse processes in plants, including seed germination, stomatal closure, ion uptake and transport, membrane permeability, photosynthetic and growth rate (Raskin, 1992; Senaratna et al., 2000). There are experimental data indicating participation of SA in signal regulation of gene expression during leaf senescence in Arabidopsis (Morris et al., 2000). Moreover, SA might serve as a regulator of gravitropism (Medvedev and Markova, 1991), inhibition of fruit ripening (Srivastava, and Dwivedi, 2000) and other processes. SA is also known as an important signal molecule for modulating plant responses to environmental stresses. It is now clear that SA provides protection against a number of biotic and abiotic stresses (Gue et al., 2007; Horvath et al., 2007). The alleviation of oxidative damage and increase resistance to environmental stresses are often correlated with an efficient anti oxidative system.

Nigella sativa L. is an annual herbaceous plant belonging to the Ranuculacea family. Seeds of black cumin (*Nigella sativa L*.) are used as a spice in cooking and in wide traditional medicinal uses, the seed volatile oil and its main active constituent, thymoquinone, are extensively reported to exhibit the protective effects against many diseases depending on its high antioxidant activity (El-Dakhakhny et al., 2000).

The objective of the present experiment was to investigate whether salicylic acid is involved in regulation of ROS metabolism in *Nigella* plant leaves and to elucidate the physiological mechanisms involving in increased *Nigella* plant tolerance to drought stress using different concentrations of exogenous SA.

MATERIALS AND METHODS

Plant material

Black cumins (*Nigella sativa*) were grown from seeds in pots filled with sand and were transferred to green house with day/night temperature of 22°C/18°C and a 16h photoperiod with a relative

humidity of 50%. The seedlings were irrigated with water once a day and half-strength Hoagland's solution once a week. After four weeks, the seedlings were transferred to bottles containing Hoagland's solution aerated with air pomp and were pretreated with 5 or 10µM salicylic acid (SA was added to nutrient solution). After 24 h, plants were subjected to in vitro drought stress. For this purpose, three seedlings were placed in aerated bottle containing distilled water served as a control and polyethylene glycol's solutions (PEG-6000) to achieve a drought (osmotic) stress level of -0.2, -0.4 and -0.6 MPa. After 48 h of root osmotic stress the shoots of plants were harvested and immediately frozen in liquid nitrogen and stored at -80°C for future analysis.

Hydrogen peroxide content

H_2O_2 content was measured colorimetrically after reaction with potassium iodide (KI) according to method of Alexieva et al., (2001).

Thiobarbituric acid reactive substance (TBARS)

One hundred mg of the leaf tissue of plants were homogenized in 10 ml of 0.1% TCA, and after that centrifuged at 10000×g for 15min. One ml of the supernatant was next swirled with 4 ml of 20% TCA containing 0.5% 2-thiobarbituric acid (TBA), and the solution was heated for 30 min at 90°C. Samples were cooled on ice for 5 mints and then re-centrifuged for 10min at 10000×g. For MDA measurement; the non-specific absorbance of the supernatant at 600nm was subtracted from the maximum absorbance at 532 nm, and an extinction coefficient (ε) of 155 $mM^{-1}Cm^{-1}$ was used for determination of MDA concentration (Heath and Packer, 1968).

Enzyme extraction and activity determination

500 mg leaves were homogenized in 50mM potassium phosphate buffer (pH 7.0) containing 1% soluble PVP, 1mM EDTA and 1mM PMSF with the addition of 10mM ascorbic acid in the case of the APX assay. The homogenate was centrifuged at 20000×g for 20 mints, and the supernatant used for assay of the activity of enzymes and protein content.

Catalase (CAT) (EC 1.11.1.6)

Catalase activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240nm using the extinction coefficient 40 mM^{-1} cm^{-1} for H_2O_2 (Velikova et al., 2000).

Guaiacol peroxidase (GPX) (EC1.11.1.7)

The GPX activity was determined using the method of Plewa et al. (1991) following the formation of tetra guaiacol by measuring the absorbance at 470nm and using an extinction coefficient 25.5 mM^{-1} cm^{-1}.

Ascorbate peroxidase (APX) (EC 1.11.1.11)

Ascorbate peroxidase was determined spectrophotometrically according to the oxidation of ASA. The reaction solution contained 50mM potassium phosphate buffer (pH 7.0), 0.5mM ascorbate, 0.1mM H_2O_2 and 150 µl enzyme extract. H_2O_2-dependent oxidation of AsA was followed by measuring the decrease in absorbance within 1min at 290 (extinction coefficient of 2.8 mM^{-1} cm^{-1}) (Nakano and Asada, 1981).

Lipoxygenase activity (LOX) (EC 1.13.11.12)

LOX activity was measured according to Minguez-mosquera et al. (1993) using an extinction coefficient 25000 M^{-1} cm^{-1}.

Total soluble proteins

Protein content was determined according to the method of Bradford (1976) using Bovine serum albumin as standard.

Proline determination

Determination of free proline content performed according to Bates et al (1973).

Statistical analysis

The experiment was conducted in a factorial arrangement based on completely randomized design with 12 treatments and three replications. Statistic assays were carried out by one-way ANOVA using LSD test to evaluate whether the means were significantly different, taking $p< 0.05$ as significant.

RESULTS

H_2O_2 accumulation: Water deficit at the levels of -0.4 and -0.6 MPa caused an increase in H_2O_2 content compared to control plants (Fig1). SA pretreatment had no significant effect in decreasing of H_2O_2 in control, and water stressed plant at -0.2 MPa. However, in those plants which were under drought stress at -0.4 and -0.6 MPa SA pretreatment decreased the amount of hydrogen peroxide.

Lipid peroxidation and lipoxygenase activity: MDA was measured as an indicator of lipid peroxidation. The data showed that drought induced increase in the amount of MDA (Fig 2-A). SA pretreatment decreased lipid peroxidation in drought stressed plants and had no effect on control plants. Lipoxygenase is an oxidative enzyme that contributes to oxidation of polyunsaturated fatty acids. The activity of this enzyme increased under-water deficit when compared with control (Fig 2-B). Activity of this enzyme decreased in SA pretreated plants under drought stress.

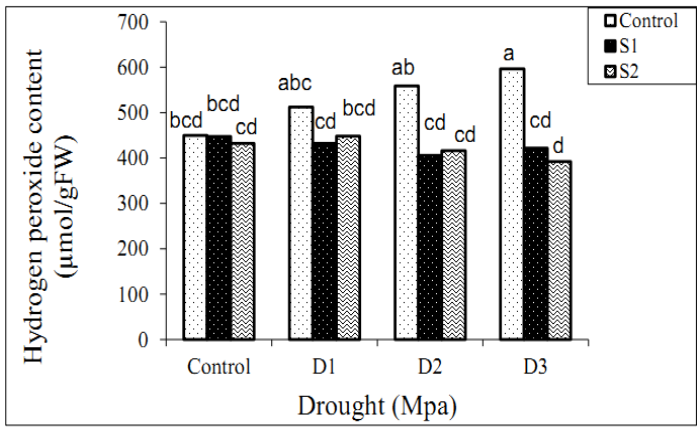

Figure 1: Effect of salicylic acid pretreatment on hydrogen peroxide content in *Nigella* plant leaves under control and drought stress conditions. The mean comparisons of treatments were done using LSD method at $p<0.05$ significant level. (S1: 5 µM SA, S2: 10 µM SA; D1: -0.2 Mpa, D2: -0.4 Mpa, D3: -0.6 Mpa levels of drought stress).

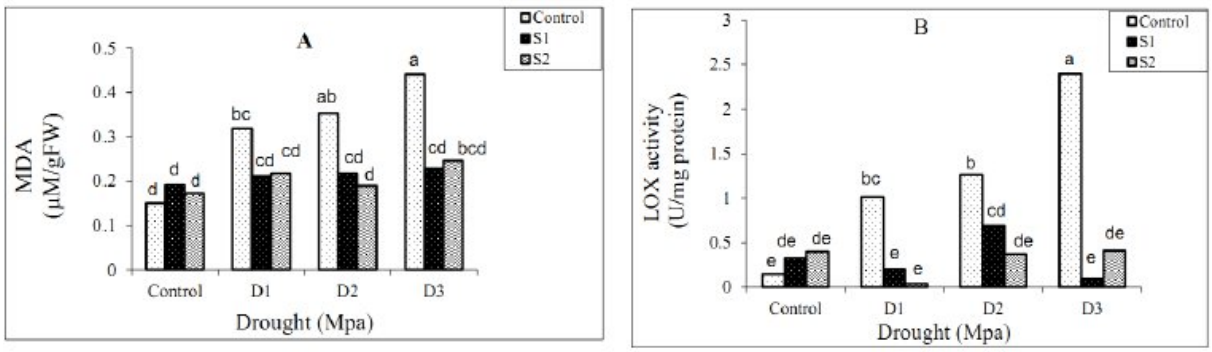

Figure 2: Effect of salicylic acid pretreatment on MDA content (A) and LOX activity (B) in *Nigella* plant leaves under control and drought stress conditions. The mean comparisons of treatments were done using LSD method at $p<0.05$ significant level. (S1: 5 µM SA, S2: 10 µM SA; D1: -0.2 Mpa, D2: -0.4 Mpa, D3: -0.6 Mpa levels of drought stress).

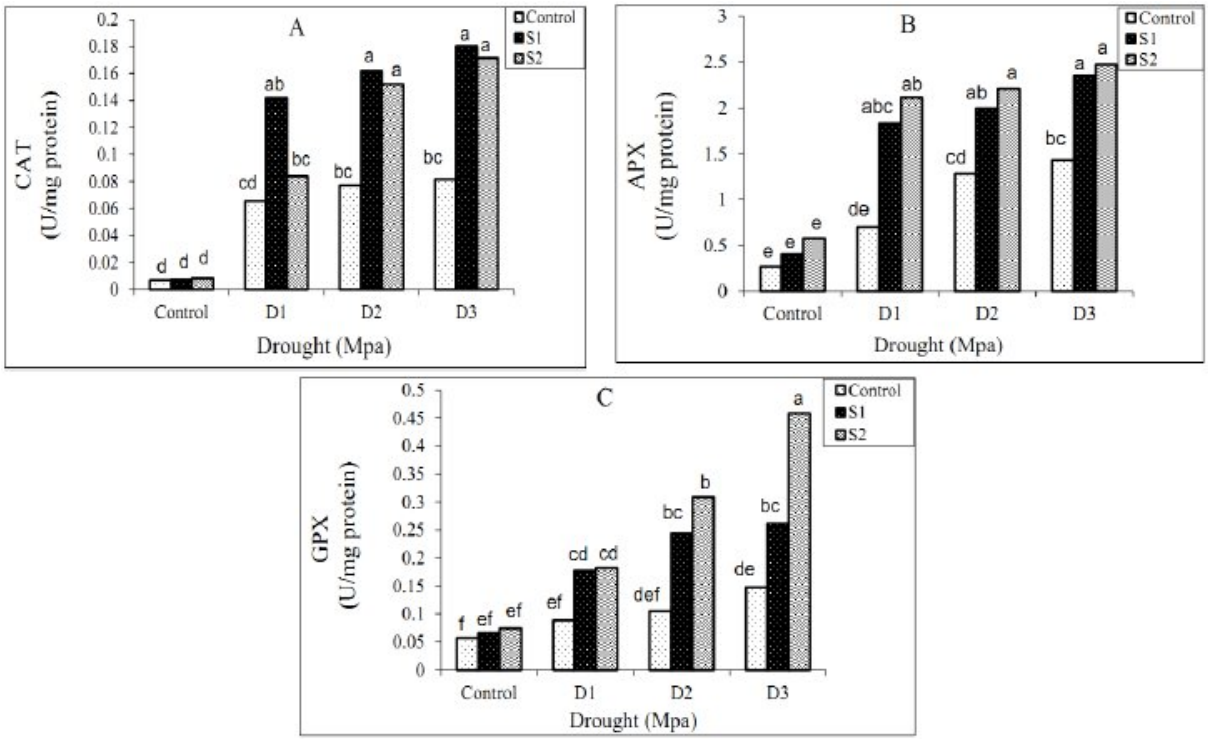

Figure 3: Effect of salicylic acid pretreatment on CAT (A), APX (B) and GPOX (C) activity in *Nigella* plant leaves under control and drought stress conditions. The mean comparisons of treatments were done using LSD method at $p<0.05$ significant level. (S1: 5 µM SA, S2: 10 µM SA; D1: -0.2 Mpa, D2: -0.4 Mpa, D3: -0.6Mpa levels of drought stress).

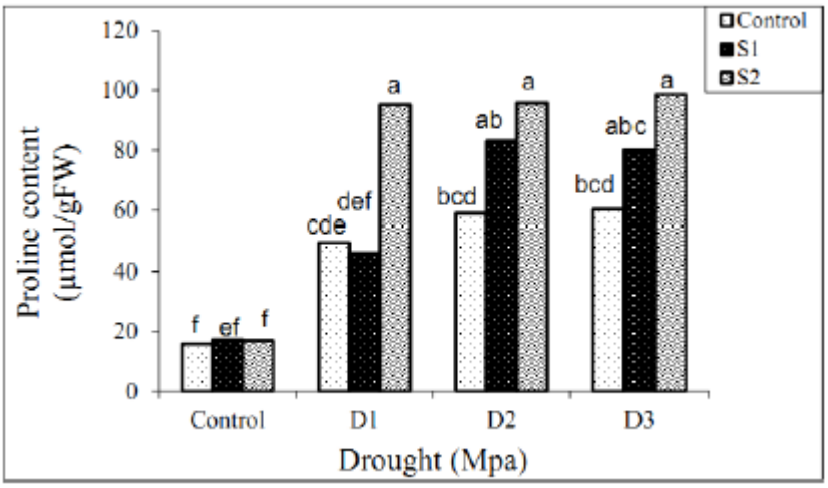

Figure 4: Effect of salicylic acid pretreatment on proline content in *Nigella* plant leaves under control and drought stress conditions. The mean comparisons of treatments were done using LSD method at $p<0.05$ significant level. (S1: 5 µM SA, S2: 10 µM SA; D1: -0.2 Mpa, D2: -0.4 Mpa, D3: -0.6 Mpa levels of drought stress).

Antioxidant enzyme activities: Change in specific activity of antioxidant enzymes is the consequence of oxidative stress. The effect of drought stress on CAT, GPX and APX in *Nigella* plant leaves, either with or without SA, pretreatment was assayed. As it is shown in (Fig 3-A, B and C) the activity of CAT, GPX and APX were higher in stressed plants (especially in -0.4 and -0.6 MPa) than those of the control groups, which may be a reflection of the oxidative burst under drought stress. Pretreatment of plants with 5 and 10µM SA increased the activity of CAT, APX and GPX, in those plants which were under drought stress and this is may be related to the key role of these enzymes in ROS detoxification under these conditions (only in the case of CAT the activity of this enzyme decreased when the plants were pretreated with 10µM SA). However, no significant different were observed between two concentrations of SA pretreatments. Two levels of SA pretreatment had no significant effects on enzyme's activity in control condition.

Proline content: The amounts of proline increased significantly under drought stress. Treatment of plants with 10µM SA (S2) significantly increased the proline content under drought stress.

DISCUSSION

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 and participates in the regulation of physiological processes, to study the role of this hormone in some physiological parameter under this stress. One of the described damages provoked by water deficit stress is the membrane injury and liberation of ions from the cell to extra cellular space (Halliwel and Gutteridge, 1984). This is a consequence of an oxidative burst leading to lipid peroxidation, membrane permeability and cell injury (Scandadalius, 1993). As shown in Fig2-A, MDA content increased in plants, which were subjected to drought. When plants were pretreated with SA, MDA decreased in drought condition, and

this effect is very important for drought stress tolerance, however, the effect of SA pretreatment in control plants was not statistically significant. Maintaining the integrity of cellular membranes under stress conditions is considered an integral part of salinity and drought tolerance mechanisms (Shakirova et al., 2003; Shinozaki and Yamaguchi, 1997).

Another effect of SA on lipid peroxidation related to lipoxygenase activity. Lipoxygenase is an oxidative enzyme which can contribute to lipid peroxidation. It is a non-heme enzyme that contains a single iron atom which is thought to oscillate between ferrous (inactive) and ferric (active) forms during each cycle of catalysis. The results showed that the activity of this enzyme increased in drought stress conditions while it decreased with SA pretreatment (Fig 2-B).

Under normal conditions, the total amount of ROS formed in the plants is determined by the balance between the multiple ROS producing pathways and the ability of the enzymatic and non-enzymatic mechanism to deal with them. Under stress conditions, ROS formation is higher than ability of plants to remove it, and this could result in oxidative damages (Laspina et al., 2005). In *Nigella* plants under -0.4 and -0.6 MPa of PEG solutions, APX and CAT activities were elevated over the controls, But GPX activity increased only at -0.6 MPa when compared with control plants(Fig 3-A, B and C). Therefore, we can assume that the plant antioxidant machinery was effectively struggling against stressful condition. Relatively higher activities of ROS-scavenging enzymes have been reported in tolerant genotypes when compared to susceptible ones, suggesting that the antioxidant system plays an important role in plant tolerance against environmental stresses (Shi et al., 2007). In addition, the results showed that under drought stress (-0.4 and -0.6 MPa) H2O2, content increased (Fig1). When SA was applied before drought stress, the activity of APX, CAT and GPX increased. In SA pretreatment, plant H_2O_2 content also declined, which may relate to antioxidant enzyme activity. In many studies, it was found that the function of SA alleviation of Oxidative stress was attributed to induction of various ROS-scavenging enzyme activities (Agarwal et al., 2005; Clark et al., 2002). The SA greatly improves the dehydration tolerance through the increment of proline content. However, this effect observed in high concentration of SA (10µM) and 5µM of SA had no significant effects on proline content.

Data presented in this study indicated that PEG induced drought stress could cause oxidative damage in *Nigella* leaves through excessive generation of ROS and exogenous SA application through the root medium increased levels of drought tolerance in black cumin seedlings. Plants pre-treated with SA exhibited slight injury symptoms whereas those that were not pre-treated with SA had moderate damage and lost considerable portions of their foliage. SA very profoundly decreased the severity of drought stress on all parameters measured by inducing the activity of CAT, APX and GPX in plants, which led to reduction in H_2O_2 content, lipid peroxidation (MDA) and LOX activity.

Based on the obtained results, it may be concluded that, application of exogenous SA can be a method to decrease water stress damages to plants. However, the application dose of SA needs further investigation according to different plant species and different growth stages.

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