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Antioxidants Response to Salinity and Ameliorating its Effect by *Nigella Sativa*

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ABSTRACT

Salinity, a major stress in semiarid areas, caused significant decreases in growth (30%), photosynthetic pigments (45%) relative water content (RWC) (20%) and protein content (10%) of *Vicia faba* L. plants, while protein content, membrane permeability and activities of Catalase (CAT), superoxide dismutase (SOD) were increased significantly (P≥0.05). Treating seedlings with aqueous solution of *Nigella sativa* alleviated inhibitory effects of NaCl as it caused significant increases in growth and photosynthetic pigments as well as RWC. Antioxidant enzyme activities decreased with *Nigella sativa*. The results showed that *Nigella sativa* had antagonistic effect and could mitigate toxic effects of salinity on growth and physiology of plants.

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INTRODUCTION

A wide range of environmental stresses (such as high and low temperature, drought, salinity, UV stress and pathogen infection) are potentially harmful to the plants [1,2]. Salt stress in soil or water is one of the major stresses especially in arid and semi-arid regions and can severely limit plant growth and productivity [3-5]. Soil salinity is one among the major abiotic stresses that adversely affects plant productivity and metabolism [6]. Saline conditions reduce the water absorption ability of plants, cause rapid reductions in growth, and induce many metabolic changes similar to those caused by water stress [7, 8]. Cellular ionic imbalance in plant cells is the first consequence of salt stress.

Salinity tolerance has been studied in relation to regulatory mechanisms of osmotic and ionic homeostasis [11]. Salt stress, like other abiotic stresses, can also lead to oxidative stress through the increase in reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$), which are highly reactive and highly cytotoxic and can seriously react with vital biomolecules such as lipids, proteins, nucleic acid, etc, causing lipid peroxidation, protein denaturing and DNA mutation, respectively [12,13].

Reactive oxygen species (ROS) are regarded as the main source of damage to cell under biotic and abiotic stresses [2,13–15]. To minimize the effects of oxidative stress, plant cells have evolved a complex antioxidant system, which is composed of low-molecular mass antioxidants (glutathione, ascorbate and carotenoids) as well as ROS-scavenging enzymes, such as: superoxide dismutase (SOD), calatase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and glutathione reductase (GR) (Alscher *et al*., 1997; Apel and Hirt, 2004; Tunc-Ozdemir *et al.*, 2009). ROS-scavenging enzymes are present in different cellular compartments as isoenzymes [12,16–18].

Evidence suggests that membranes are the primary sites of salinity injury to cells and organelles [14] because ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasmalemma or intracellular organelles [19]. Peroxidation of plasmalemma leads to the leakage of cellular contents, rapid desiccation and cell death. Intracellular membrane damage can affect respiratory activity in mitochondria, causing pigment to break down and leading to the loss of the carbon fixing ability in chloroplasts [20].

Fortunately, plants have developed various protective mechanisms to eliminate or reduce ROS, which are effective at different levels of stress-induced deterioration [13,21].The enzymatic antioxidant system is one of the protective mechanisms including superoxide dismutase (SOD: EC 1.15.1.1), which can be found in various cell compartments and it catalyses the disproportion of two O2·- radicals to H_2O_2 and O_2 [20]. H_2O_2 is eliminated by various antioxidant enzymes such as catalases (CAT: EC 1.11.1.6) and peroxidases (POX: EC 1.11.1.7) which convert H_2O_2 to water [20,22].

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Moreover, ROS are inevitable byproducts of normal cell metabolism [23]. But under normal conditions production and destruction of ROS is well regulated in cell metabolism [15]. When a plant faces harsh conditions, ROS production will overcome scavenging systems and oxidative stress will burst. In these conditions, ROS attack vital biomolecules and disturb the cell metabolism and ultimately the cell causes its own death [24].

Peroxidation of lipids, commonly taken as an indicator of oxidative stress, disrupts the membrane integrity of the plant cell [25,26]. This means that essential solutes leak out from the organelles and from the cell and cause the damage of membrane function and metabolic imbalances [27]. In plants, there are many potential places for generation of ROS, such as chloroplasts [28], mitochondria and peroxisomes [29, 30].

The seeds of *Nigella sativa* L., commonly known as black seed, are extensively studied, both phytochemically and pharmacologically and they proved to have several biological activities [31]. The aqueous and oil extracts of these seeds have been shown to possess beneficial effects; such as antioxidant, anti-inflammatory, anticancer, analgesic and antimicrobial activities.

The aim of the present study was to investigate the effect of salinity (NaCl) on growth parameters, the activity of some key antioxidant enzymes and protein content during germination of *Faba bean* plant and the influence of Nigella sativa –treatment which may improve the salt tolerance of faba bean grown under different levels of NaCl.

MATERIALS AND METHODS

Plant Material and Salt Treatments:

Seeds of faba bean (*Vicia faba)* obtained from Department of Agronomy, Faculty of Agriculture, Alexandria University, were sterilized with 5% hypochlorite for 5 min washed extensively with distilled water and were placed on a filter paper in 9 cm Petri dishes, moistured with distilled water, germinated for two days, then seeds were transferred to plastic pots (15 cm3) filled with vermiculite soil. Pots were irrigated two days intervals with 1/2 strength Hoagland's nutrient containing different regimes of NaCl (0, 50, 75,100 and 200 mmol/L NaCl) to achieve soil water field capacity level, and germinated in controlled conditions 25±2 °C.

Relative water content was calculated by drying the fresh weight of plant organs in an oven at 65 0C for 72 hours [32].

Recovery:

After 12 days uniformly germinated Seedlings were selected and transferred to aqueous solution of *Nigella sativa* (powder of *N. sativa* were soaked for 3 days, then filtered to get a clear solution) for 3 days at the same germination condition.

Photosynthetic Pigments:

Total chlorophyll (Chl) and carotenoids (Car) contents of plants were extracted in 80% water acetone and determined spectrophotometrically [33]

Extraction of Protein:

The root, cotyledon, hypocotyls of seedlings were homogenized with ice in 0.1 mM sodium phosphate buffer (pH 6.8). The homogenates were then centrifuged at 13,000 rpm for 30 min at 4 ºC and supernatants were used for determination of total soluble protein content and total peroxidase enzyme assays. Protein content of the extracts were determined using bovin serum albumin as standard [34].

Determination of CAT Activity:

Catalase activity was determined according to the method of Chance et al. (1995). 0.5 g tissue was ground with liquid nitrogen by using cold mortar and pestle and then suspended with suspension solution containing 50 mM Tris-HCl (pH 7.8). After filtering through 2 layers of cheesecloth, the suspensions were centrifuged at 12000 g for 20 minutes at 4ºC. Supernatant was taken for the enzyme assay.

Enzyme extract containing 100 mg protein, was added into assay medium containing 50 mM potassium phosphate buffer (pH 7.0), 25 mM H_2O_2. The reaction was started by the addition of enzyme extract. The decrease in absorbance was recorded at 240 nm with Schimadzu double-beam spectrophotometer for 2 minutes. The enzyme activity was calculated from the initial rate of the enzyme. (Extinction coefficient of H_2O_2 = 40 $mM^{-1}cm^{-1}$ at 240 nm) [34].

Determination of SOD:

Activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride [22]. The assay mixture consisted of 50 µL of the enzyme extract, 50 mM phosphate buffer (pH 7.8), 0.1 µM EDTA, 13 mM methionine, 75 µM nitroblue tetrazolium and 2 µM riboflavin in a total volume of 1.5 mL. Riboflavin was added and tubes were shaken and placed under fluorescent lighting from two 20 W

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tubes. The reaction was allowed to proceed for 15 min, after which the lights were switched off and the tubes covered with a black cloth. Absorbance of the reaction mixture was read at 560 nm, and one unit of SOD activity (U) was defined as the amount of enzyme required to cause 50 % inhibition of the nitroblue tetrazolium photoreduction rate. The results were expressed as $U.mg^{-1}$ protein

Membrane Permeability:

Bean leaves were sliced into small discs 0.05 cm thick and washed three times with deionised water to remove surface-adhered electrolytes. After drying with filter paper, 10 discs were placed in closed vials containing 30 ml deionised water in closed vials containing 30 ml of deionised water and shaken at 25 C on a rotary shaker for 30 min; subsequently electrical conductivity of the solution was determined, using a conductivity meter (model DDSJ-308A, Shanghai Precision & Scientific Instrument Co., Ltd.,China). The vials with solution were then boiled for 10 min, quickly cooled and the total electrical conductivity was obtained. Relative leakage rate was expressed as percent of total electrolytes.

Statistical Analyses:

ANOVA was applied to test the significance of treatments using STATGRAPICS (Statisica 4). Data were log transformed prior to analysis to ensure that they were normally distributed.

RESULTS AND DISCUSSION

Figure 1 showed that shoot and root growth were much higher in recovered plants (25 and 40%, respectively, averaged between different NaCl levels).

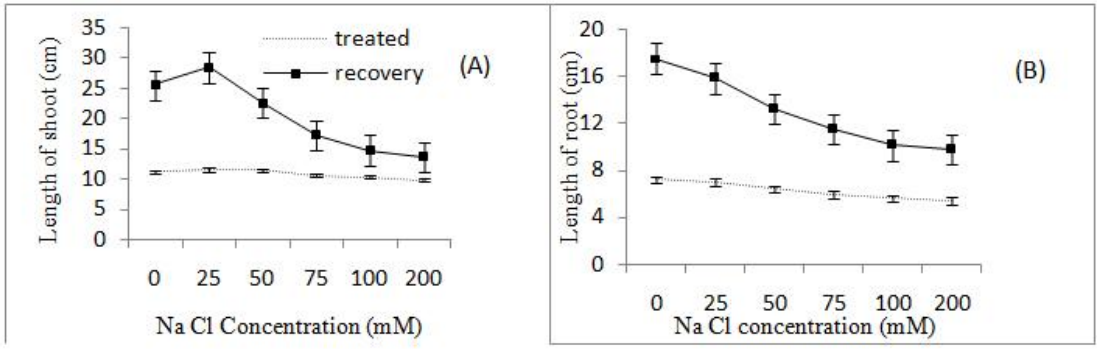

Fig. 1: Lengths of shoot (A) and roots (B) of treated and recovered bean seedlings (n = 20 + 1 SE).

The increase in length of shoot was reflected in a higher photosynthetic pigments, while Chl a and carotenoids were increased in plants treated with *Nigell sativa* by 40, 32, 20, and 18% and by 1-fold, 20, 9 and 20% in plants treated with 25, 50, 75 and 200 mmol NaCl, respectively, while untreated plants had lower concentrations of these pigments (P≥ 0.05) (Fig. 2).

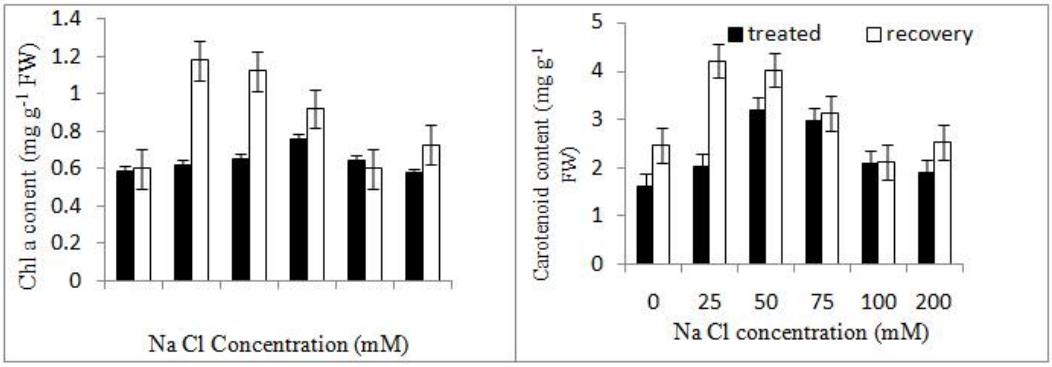

Fig. 2: Effects of Na Cl on photosynthetic pigments Carotenoid and Chlorophyll a contents (n = 10 + 1 SE).

It was found that treated plants had higher (P≤0.05) RWC than recovered plants (averaged 10% in roots and 30% in shoots) (fig. 3).

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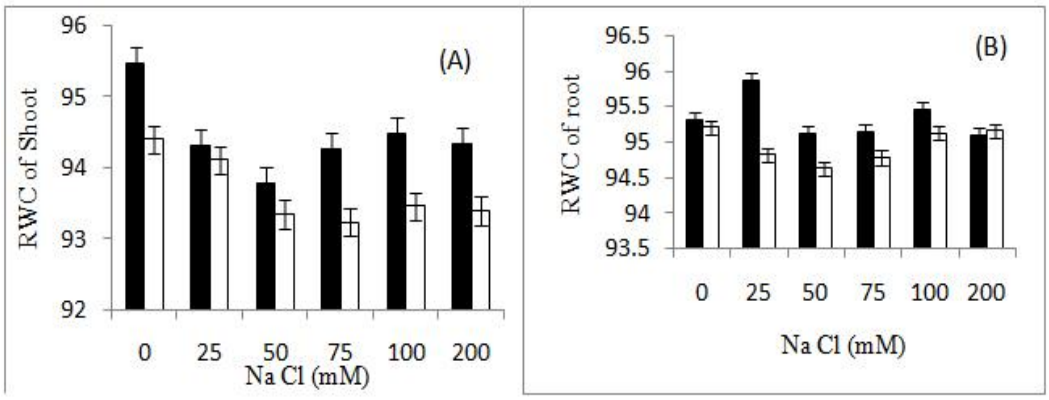

Fig. 3: Relative water content (RWC) of treated shoot (A) and roots (B). (Legends as Fig.2).

Figure 4 shows membrane permeability expressed as electrical conductance of shoot and root. It was found that exposure to NaCl caused an increase in EC by about 35% (averaged between different NaCl treatments in both shoot and root) compared to recovered plants.

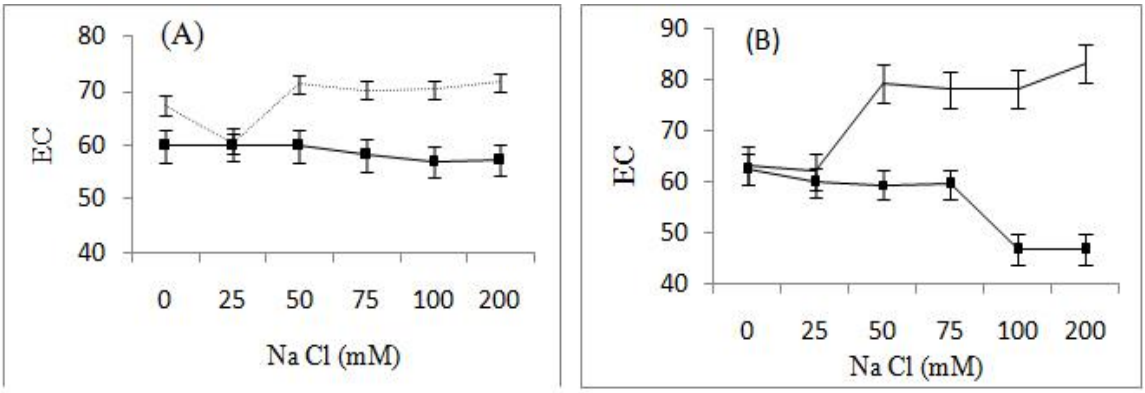

Fig. 4: Electrical conductivity (EC) of shoot (A) and root (B). Legends as Figure 1

Protein content of treated plants showed higher ($P \leq 0.05$) concentrations than recovered plants and averaged by about 12% in both organs (Fig 5).

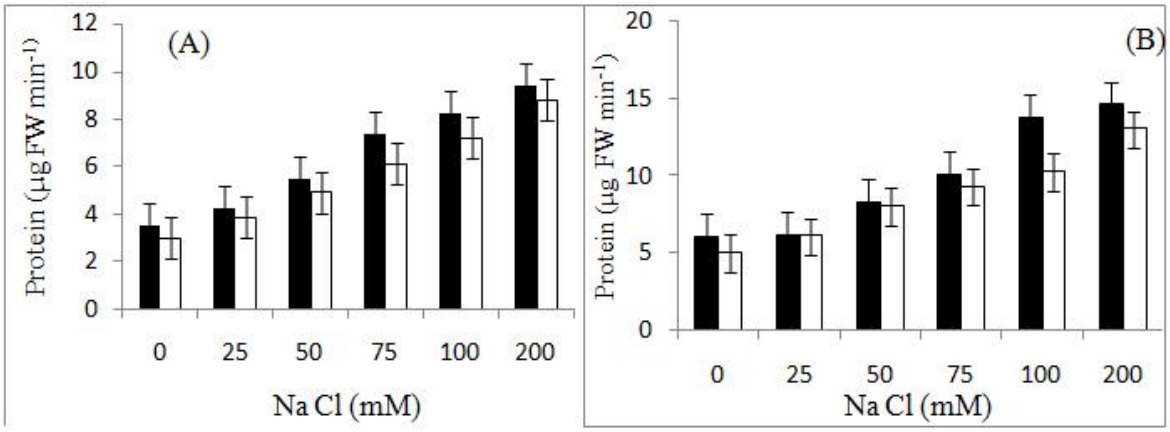

Fig. 5: Protein content of Shoot (A) and Roots (B) of bean seedlings. Legends as Fig.2.

SOD activity showed similar trend, where recovered plants had lower activity than treated ones and there was no significant effect ($P \geq 0.05$) between shots and roots (fig 6).

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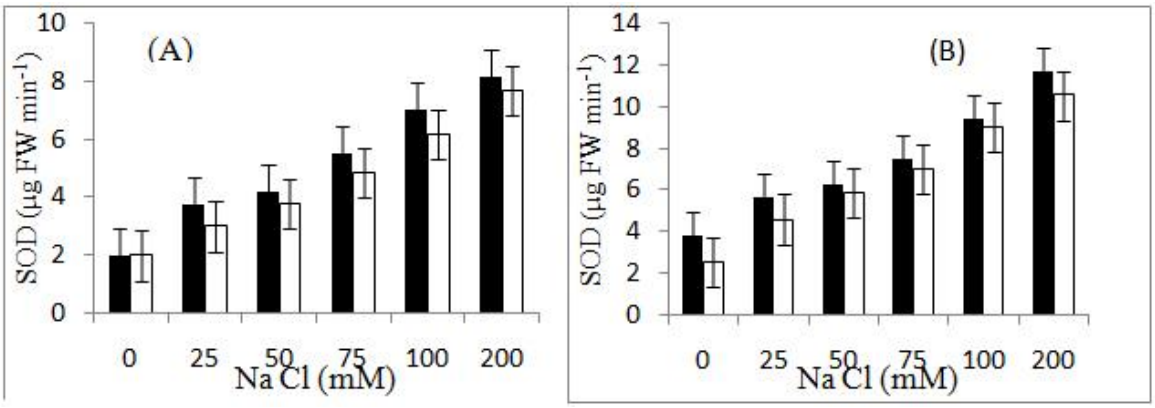

Fig. 6: SOD activity in shoots (A) and roots (B) in bean seedlings.

On the other hand, CAT activity was lower in shoots and roots of recovered plants compared to that in treated ones (Fig. 7). Moreover, Percentage of CAT activity in shoots of recovered plants were much lower (averaged between NaCl concentrations about 50%) than that in roots (averaged about 10%) (Fig 7).

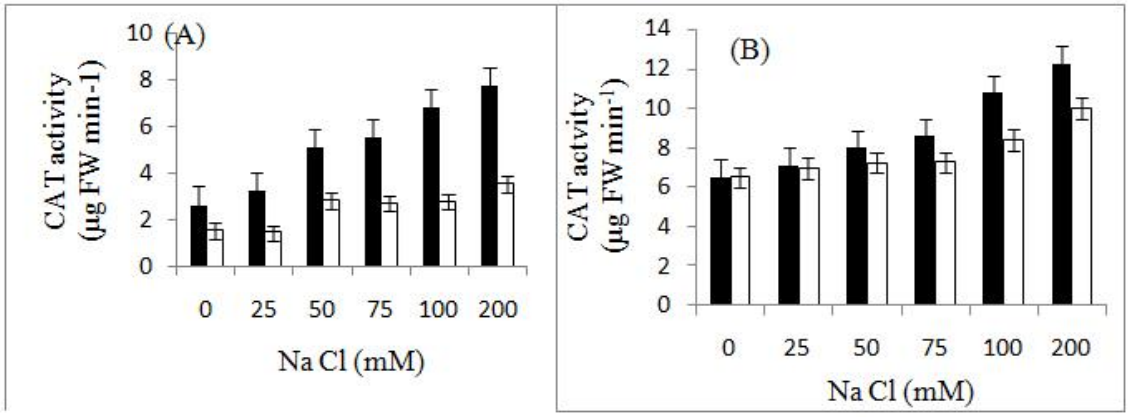

Fig. 7: CAT activity in shoots (A) and roots (B) of bean seedlings. Legends as Fig.2.

There is a large body in the literature about the effects of salinity on plants, all indicate that growth and other physiological parameters of plants were adversely affected by salt stress. [*e.g*. 23, 35 – 36]. Significant diminution in the chlorophyll content of plants grown under NaCl salinity was found in the present study; however, There was no changes in the chlorophyll content in 20-day salt stressed spinach (*Spinacia oleracea* L.) plants [37]. Addition of Ca2+ to the growing solution decreased the inhibitory effect of NaCl on chlorophyll content of the leaves. Consequently, a relatively higher photosynthetic rate was observed in NaCl-treated plants when a higher concentration of Ca^{2+} was present in the test solution [36, 38].

The morphological development of plants was affected by salinity (Fig.1). Plant height, root length and area of individual leaves were reduced with increasing stress level (data not shown). Altered morphology and reduced growth rate are known to occur under salinity in many crops causing economic reductions in the yield. Growth and development in a range of aromatic plants, including peppermint and spearmint [39], sage [40] and *Nigella sativa* [41] were demonstrated as well to reduce under salinity conditions. In the present study, the restricted development under salinity was accompanied by a progressive reduction of leaves, stems and root biomass production with increased NaCl concentration (data discussed elsewhere) .Recently, similar results were found on sweet basil [42]. It was found that growth of bean plants in extract of *N. sativa* significantly decreased the inhibitory effect of NaCl on the activities of SOD, POD, and CAT.

However, plants grown alone treatment alone did not change the activities of the above enzymes. These suggested that extract of *N. sativa* effectively controlled the activities of antioxidant enzymes only in stressed plants [36]. There are several potential sources, such as chloroplasts, mitochondria, peroxisomes, and cytosols, of ROS production in plants during normal cellular metabolism (13,15]. Many biotic and abiotic stresses including salt stress disrupt the cellular homeostasis of cells and further enhance the production of ROS in the plants cells [13, 43]. The production of ROS during stresses results from pathways, such as photorespiration, from the photosynthetic apparatus and from mitochondrial respiration [15]. The increased rate of ROS

production in chloroplasts of plants under salt stress is well-known [44, 45]. Salt stress is known to result in an extensive lipid peroxidation that has often been used as an indicator of salt-induced oxidative damage in membranes [46 - 48]. It has been shown that both osmotic and ionic effects are involved in NaCl salinity effect and limit the photosynthesis and respiration, leading to an increase in ROS generation. The increased rate of ROS generation and decreased scavenging of ROS contribute to overall oxidative stresses and damages like peroxidation of membrane lipids and loss of membrane Permeability. However, contrary to the present study, there are some reports [49, 50] showing an increased activity of antioxidant enzymes under stress conditions. For example, salinity-induced increase in SOD activity is often observed in higher plants [*e.g.*51, 52]. Variable responses of CAT, from a significant increase in CAT activity [52, 53] to no change [53, 54], have been found in plants under salt stress. In addition, treatments with 50 mmol L^{-1} NaCl did not alter the activities of antioxidant enzymes of loquat [46, 54]. The inconsistency among various reports on the responses of antioxidant enzymes to salt stress might be because of a large variability in genotype and adaptability of tested plant species to salt stress and the different experimental conditions of different studies.

Conclusion:

The enrichment of the growth medium with extract of *N. sativa* prevented chlorophyll loss, maintained optimum photosynthesis and growth, and preserved membrane permeability in bean plants grown under NaCl stress. The above protective effects of enrichment with Extract of *N. sativa* could be related to the improved activities of key antioxidant enzymes, and thereby their free radical scavenging in the stressed plants. The present study did not discriminate between osmotic and ionic effects of NaCl stress but the obtained results showed that calcium had a regulatory control over activities of antioxidant enzymes, and, thereby, salt stress tolerance of plants. Although a distinct protective effect of *N. sativa* extract on antioxidant enzymes and, thereby, overall better growth performance of the NaCl-treated plants were found, mechanism(s) involved in the process are largely unknown and need to be elucidated.

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