



## Original Article

# Effects of Exogenous Nitric Oxide on Germination and Physiological Properties of Basil under Salinity Stress

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## Abstract

Nitric oxide (NO) is a bioactive molecule, which was found to have several physiological roles, including antioxidant. To have a better understanding of the effects of NO concentrations (0, 0.1 and 0.2 mM) on germination, growth, photosynthetic pigments, lipid peroxidation and antioxidant activity of basil (*Ocimum basilicum* L.) under different salinity concentrations (0, 100 and 200 mM of NaCl), a factorial experiment based on completely randomized design was carried out. Results revealed that salinity caused a significant decrease in germination characteristics and growth of basil. Increasing salinity concentration led to significant increase in the activity of superoxide dismutase (SOD), catalase (CAT), Ascorbate peroxidase (APX), proline content, malondialdehyde (MDA) and electrolyte leakage while content of photosynthetic pigments and relative water content were reduced. Application of NO (0.1 and 0.2 mM) under salinity stress improved germination traits, increased dry weight, chlorophyll content, antioxidant activity and proline content, while MDA content and electrolyte leakage were decreased. These results suggest that NO might induce salt tolerance in basil by preventing oxidative damage.

**Key words:** Antioxidant activity, Basil, Nitric oxide, MDA, Salinity stress

**Abbreviations:** NO: Nitric Oxide, SOD: Superoxide Dismutase, CAT: Catalase, APX:Ascorbate Peroxidase, MDA:malondialdehyde, RWC: Relative Water Content

## Introduction

Salinity is one of the most important limiting factors for agriculture development around the world [1]. About 77 million hectares of agricultural lands (5%) are suffering from saline conditions [2]. Several physiological processes have been affected by salinity stress which results to a decreased growth and productivity [3]. The mechanism of salinity tolerance varies at cellular, molecular and the whole-plant levels [4].

Plants exposed to stressful environmental conditions can increase the reactive oxygen species (ROS) production. Plant cells evolve an

antioxidative defense system to protect cellular structures from oxidative damage. The importance of cellular antioxidant machinery for protection against various environmental stresses has been well documented [5,6]. Enzymatic antioxidants (such as SOD, CAT, APX and GR) and non-enzymatic antioxidants (included GSH and carotenoids) are the main components of antioxidants defense system [7].

Nitric oxide (NO) is known as an important messenger in plant defense signaling and it is shown that it has a crucial role in plant physiological processes regulation, including germination, flowering, fruit ripening and organ

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senescence [8] stomatal closure, growth and development [9-11].

Although the investigations about the effects of NO on physiological properties of plants under salinity stress is limited and also contradictory, there are some reports implied that it may alleviate hazardous effects of salinity to some extent. For instance, in reed (*Phragmites communis* Trin.) plants under 200 mM NaCl treatment addition of NO donor led to the stimulated expression of the plasma membrane H<sup>+</sup>-ATPase. This result revealed salt resistance-inducing role of NO by increasing the K<sup>+</sup>/Na<sup>+</sup> ratio [12].

In another study, seed pre-incubation with the NO donor improved germination properties of *Lupinus luteus* L. which were reduced due to saline conditions [13]. There is also some other reports with similar results in wheat [14] and rice [15] plants under salinity stress and treated by exogenous NO donor.

Basil (*Ocimum basilicum* L.) is an important medicinal plant belongs to Lamiaceae family, which is a widely grown aromatic crop cultivated either for the production of essential oil, dry leaves for the market, or as an ornamental [16,17]. Basil is a native plant to the tropical and subtropical regions of Asia, Africa and South America as an annual and perennial herb [18]. Basil leaves and seeds have been widely used in indigenous system of medicine for several problems such as headaches, coughs, stomach-ache and kidney malfunction [17]. In addition, both the fresh and dry leaves of the plant are very commonly used in food and spice industries. Basil is also known as an important source of aroma compounds and used for its important biochemical properties including antibacterial, antifungal and antioxidant properties [19-21].

According to the roles of NO on physiological properties of plants and in order to find a method for improving the salt resistance of seeds and seedlings of basil as a valuable medicinal plant, seed germination and some physiological characteristics of *Ocimum basilicum* were studied.

## Materials and methods

### Plant Materials

Two separate experiments were conducted to investigate the effects of NO on basil plants under saline conditions at germination stage and seedling growth. Germination experiment was performed with three levels of NO (0, 0.1 and 0.2 mM) and

three levels of salinity (0, 100 and 200 mM) as a factorial plan based on a completely randomized design. Glasshouse experiment was conducted with the same treatments. Seeds in both experiments were surface sterilized with 1% sodium hypochlorite (NaClO) for 5 minutes and washed 3 times with distilled water extensively. Then they were primed with different levels of NO (0, 0.1 and 0.2 mM) and placed in Petri dishes and pots for each experiment. Distilled water was used as control treatments. In germination experiment, salinity was applied in Petri dishes and germination properties were recorded daily. After twelve days, seedlings were used to determine seedling fresh and dry weight. In glasshouse experiment, salinity was applied after 15 days when plants were established completely. Plants were harvested after 45 days. Samples were used for dry weight determination and leaf samples were used for chemical analysis.

### Relative Water Content Determination

Relative water content (RWC) was determined using fresh leaf discs with 2 cm<sup>2</sup> area. After weighting, the leaf discs were immersed in deionized water for 24 hours. Saturated leaf weight was recorded and the dry mass was noted after drying at 70 °C for 48 h. The following formula was used to calculate RWC [22]:

$$\text{RWC} = \frac{\text{Fresh weight} - \text{dry weight}}{\text{Turgor weight} - \text{dry weight}} \times 100$$

### Biochemical Analysis

#### Chlorophyll Measurement

Samples (100 mg leaves) were homogenized in chilled 80% (v/v) acetone and centrifuged at 10,000 g for 10 min at 4 °C. Absorbance of the acetone extracts was measured at 663 and 645. The contents of chlorophyll a and chlorophyll b were calculated as described by Lichtenthaler [23].

#### Proline Measurement

Proline content was determined based on the method of Bates et al. [24]. 100 mg of Leaf tissue was homogenized with 10 ml of 3% aqueous sulfosalicylic acid and centrifuged at 10,000×g for 10 min, 2 ml of supernatant were mixed with 2 ml of glacial acetic acid and 2 ml of acid ninhydrin for 1 h at 100 °C. The developed colour was extracted in 4 ml toluene and measured colourimetrically at 520 nm. A standard curve with l-proline was used for the final calculations. Content of proline was expressed as mol g<sup>-1</sup> FW (fresh weight).

#### Determination of Lipids Peroxidation

Membrane Lipid peroxidation was measured in terms of malondialdehyde (MDA) content [19]. Approximately 0.2 g of leaf and root tissues from control and treated plants were cut into small pieces and homogenized by the addition of 1ml of 5% trichloroacetic acid (TCA) solution. The homogenized leaf and root tissues were ground using cold mortar and pestle in ice bath. The homogenates were transferred to fresh tubes and centrifuged at 12,000 rpm for 15 minutes at room temperature. One ml of supernatant was mixed with 5 ml of 20% (v/v) trichloroacetic acid containing 0.5% thiobarbituric acid (TBA). The mixture was heated at 100°C for 30 minutes, quickly cooled and centrifuged at  $10,000 \times g$  for 10 min. The absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated by means of an extinction coefficient of  $155 \text{ mM}^{-1}\text{cm}^{-1}$  [25]. The results were expressed as  $\mu\text{mol MDA g}^{-1} \text{FW}$ .

#### Enzyme Assays

Leaves tissue (100 mg FW) were placed into liquid nitrogen and then homogenized with a prechilled mortar and pestle under ice cold-conditions in 4 ml 50 mM potassium phosphate buffer, pH 7.0, with the addition of 1 mM EDTA. The homogenate was centrifuged at 15,000 rpm, at 4 °C for 20 min. The supernatant was stored at -20 °C and used for the assay of enzyme activity.

#### *Superoxide Dismutase Activity*

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by measuring the inhibition of photochemical reduction of NBT [26]. The color was developed by adding the following reagents: 2.4 ml of 50 mM potassium phosphate buffer solution (pH 7.8), 0.2mL of 195mM methionine, 0.1mL of 0.3 mM EDTA, 50 $\mu$ L enzyme extract, 0.2 ml of 1.125 mM NBT and 0.2 ml of 60 $\mu$ M riboflavin. Reaction mixtures were illuminated for 15 min at light intensity of 5000 lux. The absorbance of solution was measured at 560 nm. One unit of SOD was defined as the amount of enzyme causing half-maximal inhibition of the NBT reduction under the assay condition.

#### *Ascorbate Peroxidase Activity*

For Ascorbate peroxidase (APX, EC 1.11.1.11) activity measurement the reactive solution contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM  $\text{H}_2\text{O}_2$  and 10  $\mu$ l of enzyme extracts. The decrease in absorbance at 290 nm was read. Activity was calculated using the

extinction coefficient of  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ . One unit of APX was defined as the amount of degrading 1  $\mu\text{mol}$  of ascorbate  $\text{min}^{-1} \text{ mg protein}^{-1}$  under the assay conditions [27].

#### *Catalase Activity*

Catalase (CAT, EC 1.11.1.6) activity was determined by following the consumption of  $\text{H}_2\text{O}_2$  at 240 nm for 1 min [28]. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM  $\text{H}_2\text{O}_2$  and 50  $\mu$ l of enzyme extract in a 3 ml volume. The enzyme activity was calculated using the extinction coefficient ( $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ ) and expressed as units (1  $\mu\text{mol}$  of  $\text{H}_2\text{O}_2$  decomposed per minute) per mg protein.

#### Statistical Analysis

All data were analyzed using Analysis of Variance (ANOVA) and the means were compared by LSD at  $P = 0.05$ .

## Results

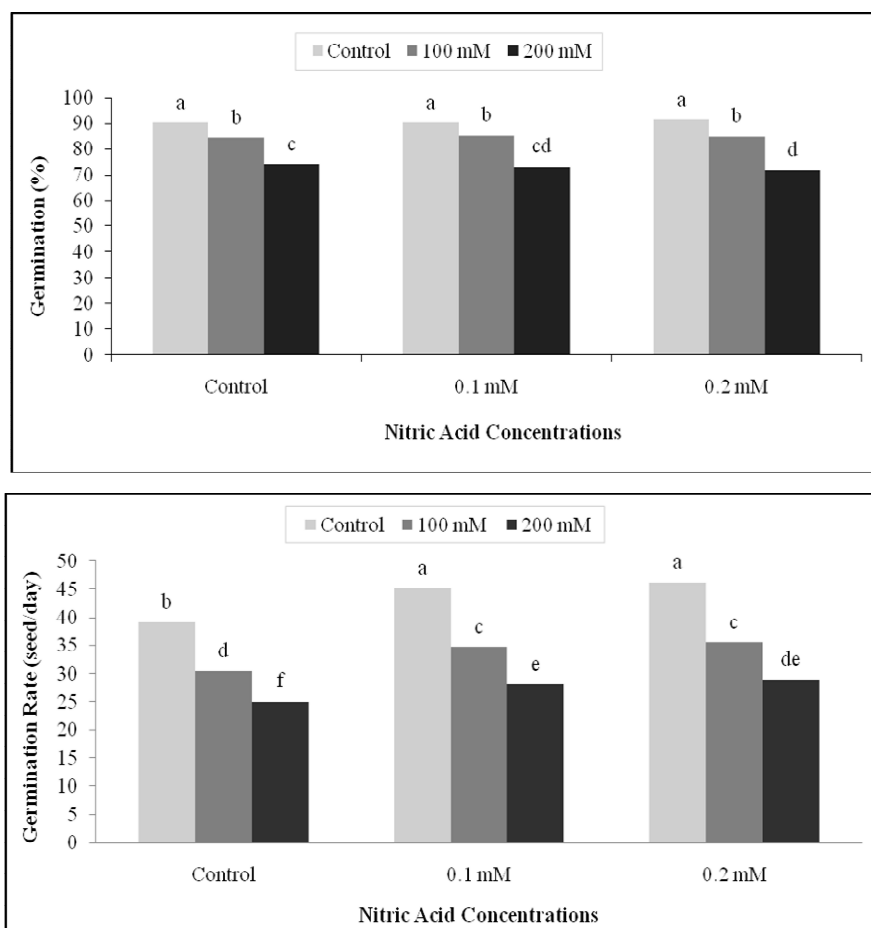
### Germination Properties

Germination percentage was significantly reduced due to the applied salinity levels, but exogenous application of NO could not improve germination ability of basil seeds (Fig. 1). Although the germination percentage was increased with increasing NO levels, the differences were not significant. NO increased germination rate of seeds in different levels of salinity (Fig. 1), although the difference between NO concentration levels was not significant. Applied Salinity treatments highly reduced Seed Stamina Index (SSI), but it was improved by exogenous application of NO (Fig. 2). Similarly, two concentrations of NO did not show significant differences. Seedling growth was also affected by the different levels of salinity and NO. In fact, reduced seedling fresh weight as a result of salinity was significantly alleviated by applied NO levels (Fig. 2). Dry weight of seedling which was decreased due to the salinity treatments was also improved by exogenous application of NO (Fig. 2), even though the difference between NO levels was not significant in both seedling fresh and dry weight.

### Glasshouse Experiment

#### *Shoot Dry Weight*

Shoot dry weight as an important index of plant growth was affected by the interactions of salinity and NO.



**Fig. 1** Interaction of salinity and nitric oxide on germination percentage and germination rate of basil

Salinity-reduced shoot dry weight was improved by applied NO treatments. Enhancing effect of NO on shoot dry weight was not elevated with increasing NO level and it was even slightly decreased.

#### *Chlorophyll and Carotenoid Content*

Deleterious effect of salinity treatments on chlorophyll content was obvious (Fig. 3). But exogenous application of NO improved both chlorophyll content of a and b under both salinity levels. In contrary with chlorophyll a, increasing NO concentration improved chlorophyll b content of plants, significantly.

Carotenoid content was also improved by NO treatments in both normal and salinity stress conditions. The trend of reducing carotenoid content of plants under salinity stress was slower when plants exposed to different levels of NO.

#### *Proline Content*

Accumulation of proline in response to salinity and NO application was considerable (Fig. 4). Proline accumulation was effectively elevated as a result of saline conditions in both NO levels and control treatment. Regards to the NO concentrations, 0.1 mM NO level enhanced proline content in both

salinity levels, but it was declined with increasing NO level to 0.2 mM, significantly.

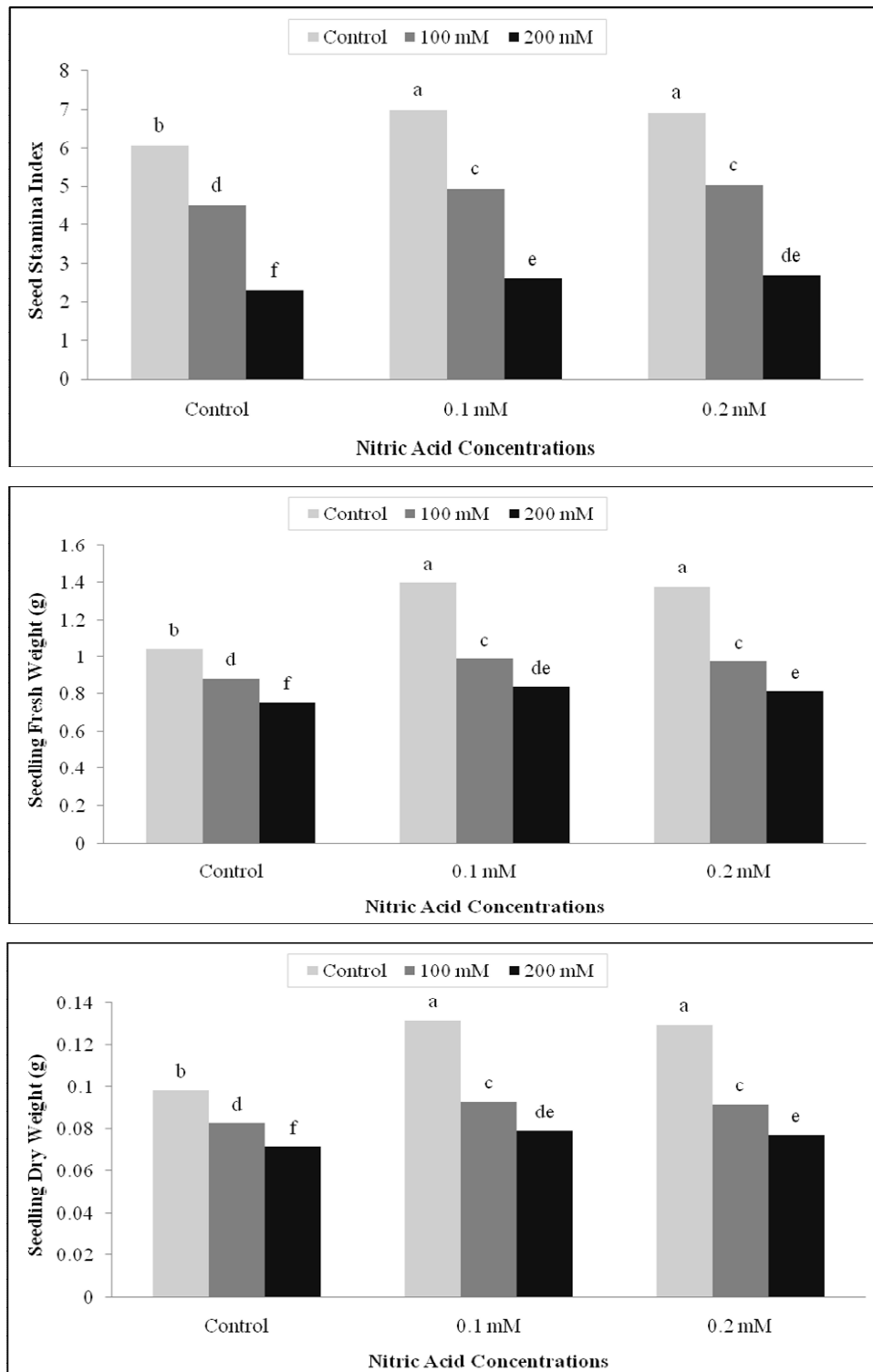
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#### *MDA Content*

MDA content in basil leaves increased with increasing salinity concentration. As shown in Fig. 4, in no salinity condition there was no significant difference between NO levels for this trait. Applying the first level of NO (0.1 mM) under salinity condition resulted in a significant decrease in MDA content compared to control. The reduction (%) in MDA content at the level of 0.2 mM NO was more than that of 0.1 mM. Results of membrane damage based on MDA content showed that lipid peroxidation in leaves of basil increased along with increasing stress severity.





**Fig. 2** Interaction of salinity and nitric oxide on seedling fresh weight, seedling dry weight and seed stamina index of basil

#### *Electrolyte Leakage and RWC*

The electrolyte leakage of basil was assessed to study the extent of membrane damage due to NaCl. Electrolyte leakage also increased with increasing concentration of NaCl (Fig. 4). However, the percentage of electrolyte leakage was much higher in non NO treated plants as compared to NO treated plant.

The interaction between NO and salinity for RWC was significant (Fig. 4). Results showed that RWC decreased with increasing in salinity levels so that the highest and lowest of RWC recorded for 0 mM and 200 mM salinity respectively (Fig. 4). Seed priming with concentrations of 0.1 and 0.2 mM NO resulted in increase in RWC.

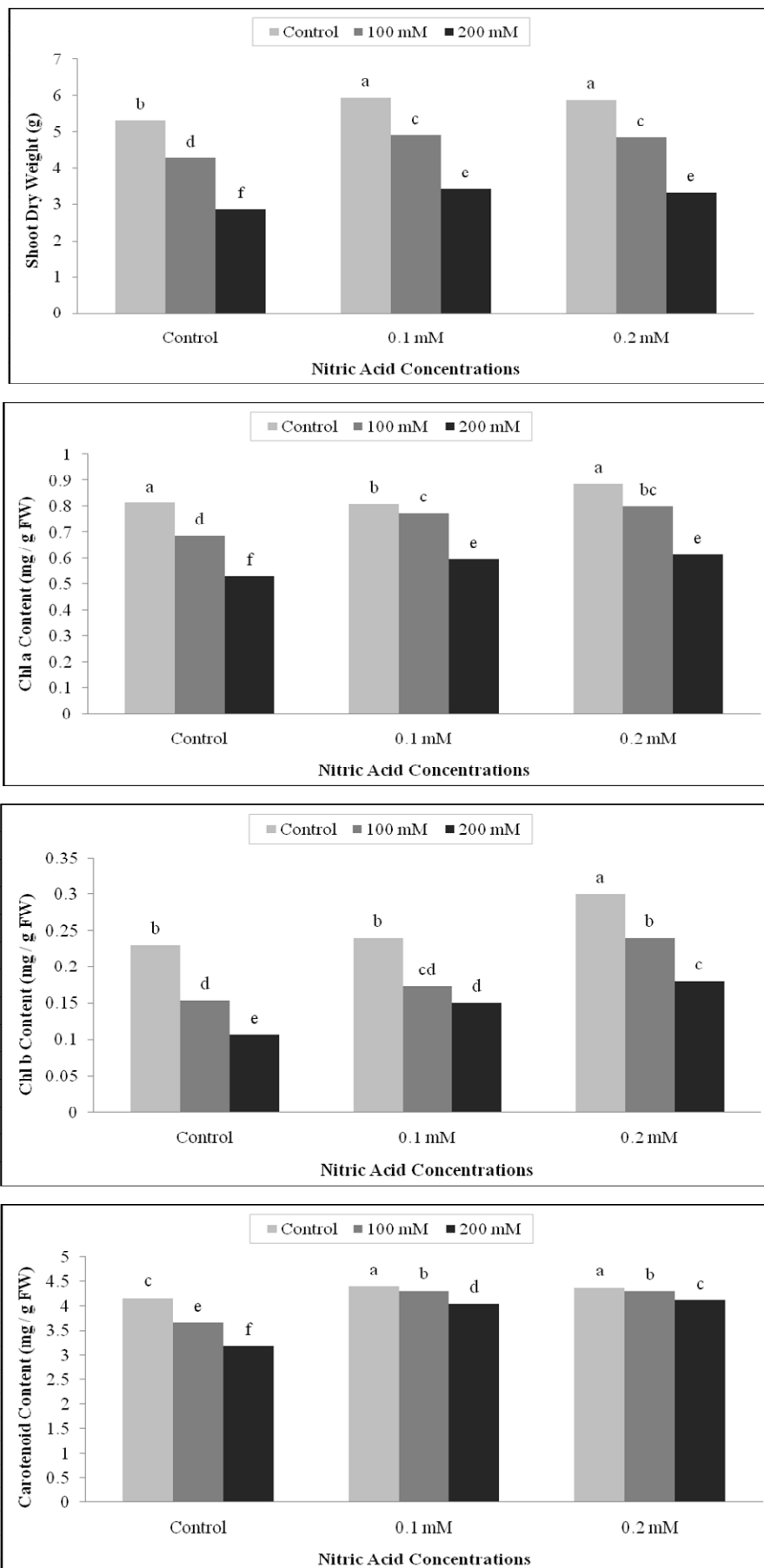
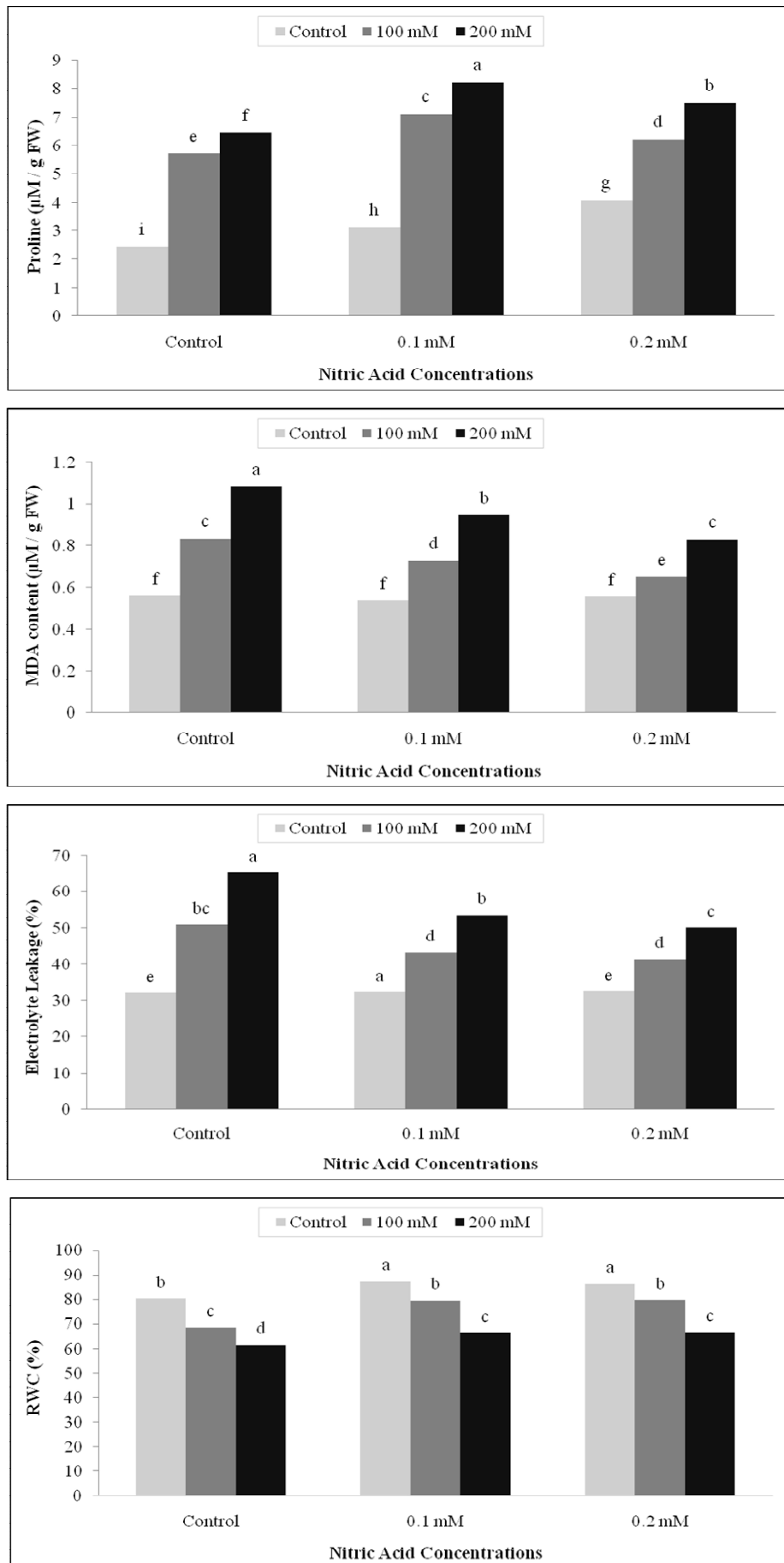
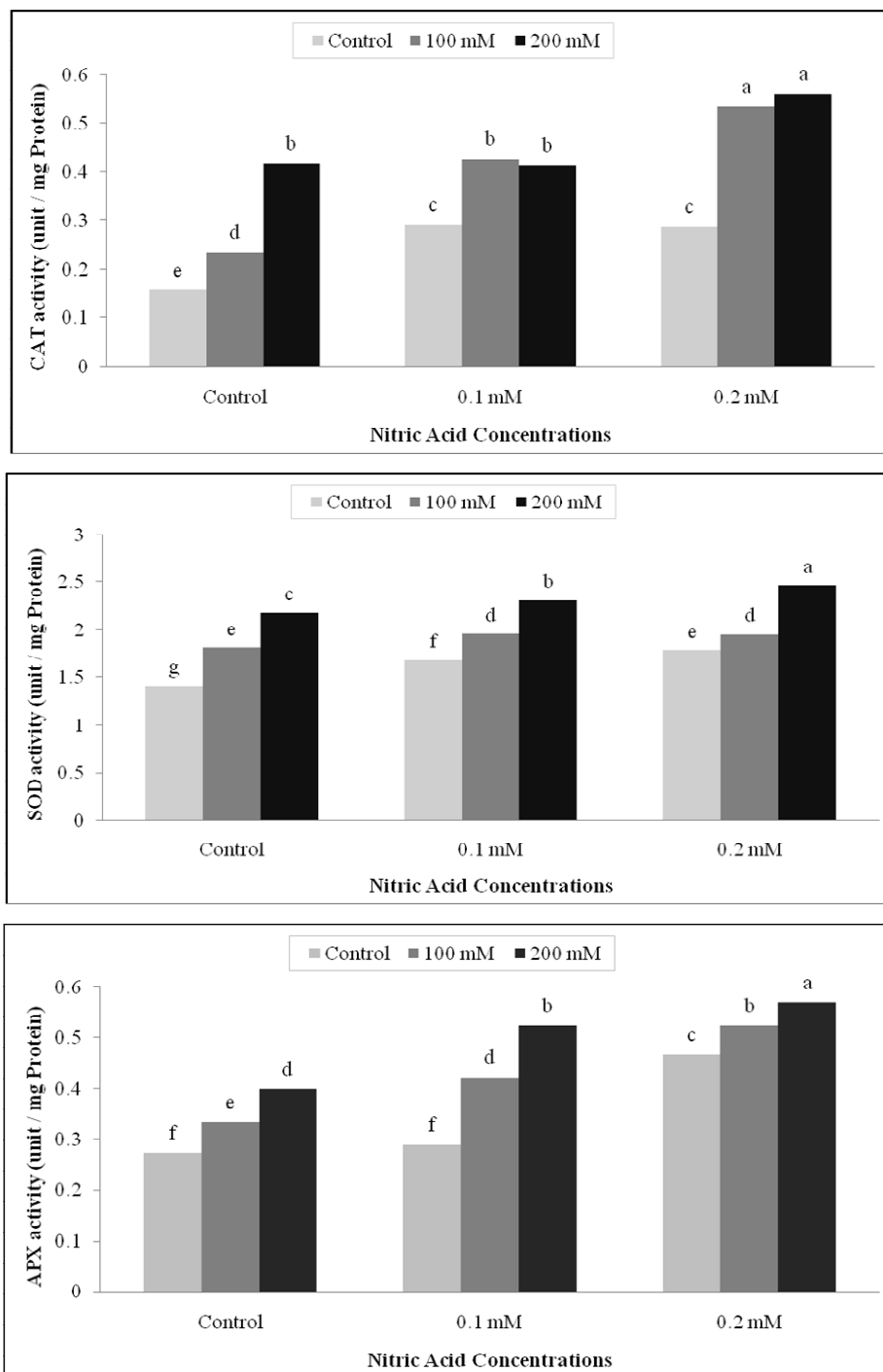


Fig. 3 Interaction of salinity and nitric oxide on chl a, chl b, carotenoid and shoot dry weight of basil



**Fig. 4** Interaction of salinity and nitric oxide on MDA, proline, electrolyte leakage and RWC of basil



**Fig. 5** Interaction of salinity and nitric oxide on CAT, SOD and APX activity of basil

#### *Antioxidant Activity*

As it was shown in Fig. 5, the CAT activity increased with increasing in salinity concentration. Priming with the first level of NO (0.1 mM) in no salinity condition resulted in a significant increase in CAT activity compared to control plants. The highest and lowest CAT activity in all NO concentrations belonged to 200 mM and 0 mM salinity respectively.

The interaction of NO and salinity for SOD activity was statistically significant. Means comparison for

this trait (Fig. 5) shows that activity of SOD increased under salinity condition compared to control plants. Also in the same salinity levels, plants treated with NO had the higher SOD activity compared to no treated plants.

Increasing salinity stress significantly increased APX activity of basil leaves compared to the control. The highest activity of APX belonged to level of 0.2 mM NO in all salinity levels.

Plant tolerance to salt stress is highly depending on the antioxidant defense systems enhancement

including enzymatic and non-enzymatic antioxidants. Antioxidative enzymes could protect cell structures from ROS damages produced under stress condition. According to these results, a significant increase in activities of CAT, SOD and APX in leaves of *basil* seedlings was obvious as a result of saline condition compared with control treatments.

## Discussion

Nitric oxide is known as a highly unstable gaseous free radical, which could be considered as a cytotoxin and as a cytoprotectant in plants tissues [29,30]. It was demonstrated that germination process has been affected by exogenous application of NO under stress conditions. For instance, Zheng *et al.* [31] found that a germination property of wheat which was substantially reduced due to high salinity stress was improved as a result of NO treatment. This is mainly because of rapid increase in  $\beta$ -amylase activity [32,33].

Exogenous application of NO especially in low concentrations has been reported to improve the plants performance under a wide range of environmental stress [34]. It is also believed that a large group of plants are able to produce substantial amounts of NO in their natural environments [14]. There are some reports about the alleviating effects of NO on reduced growth and dry matter accumulation of plants under saline conditions [35]. Protective role of exogenous application of NO has been revealed by some studies [36], even though the studies related to the salinity and NO interactions on chlorophyll and photosynthesis apparatus is very limited.

Osmolytes and especially proline accumulation is one of the most important responses of plants exposed to environmental stress and known as an adaptive mechanism under stress condition [37]. An enhanced accumulation of proline by exogenous application of NO under salt stress was previously documented [38]. Similar results were also observed in wheat plants under exogenous NO and osmotic stress [39].

It was observed that activities of CAT, SOD and APX in leaf were significantly increased by exogenous NO treatment. This might be attributed to the alleviative role of NO on oxidative stress and therefore improved germinating rate under salt stress [40-43].

These results demonstrated that exogenous NO treatment reduced the oxidative stress damage in basil imposed by salt stress. It is due to the fact that the activities of CAT, SOD and APX in the presence of NO under salt stress were higher than those under salt stress alone. This finding was somehow similar with the results of Akio Uchida [15] who found that NO induced AOS (active oxygen scavenging) activities in rice under salt stress. The observed differences implies salinity stress effects on antioxidative system is complex and depends on different parameters including plant treatment time, plant tissues, plant species and genotypes.

Mittova *et al.* [44] reported that MDA content of tomato leaf was increased due to saline conditions, which is mainly because of overproduction of ROS. In the present study, it was observed that exogenous NO treatment significantly declined MDA contents in the basil leaves under salt stress. A similar role of exogenous application of NO was also observed in salt-stressed barley plants [45].

Cell membrane stability has been widely used as criteria to differentiate stress tolerant and susceptible cultivars and in some cases higher membrane stability could be correlated with abiotic stress tolerance [46,47]. Sreenivasulu *et al.* [48] observed electrolyte leakage as a function of NaCl in salt stressed seedlings of foxtail millet. However, in basil sensitivity may be due to increase in membrane permeability or loss of membrane integrity leading to an increase in solute leakage.

Increased H<sub>2</sub>O<sub>2</sub> accumulation and lipid peroxidation due to salinity stress resulted in significant increase in electrolyte leakage. Membrane stability and extent of lipid peroxidation have been used as indices of salt injury and salt tolerance in *Amaranthus* [49]. It has been suggested that decrease in membrane stability reflects the extent of lipid peroxidation caused by reactive oxygen species [50].

RWC in leaves is considered as an alternative measure of plant water status, reflecting the metabolic activity in plant tissues [51]. Results show that salt stress significantly declined RWC compared to the control treatment. The decrease in RWC under salinity stress has already been reported [52]. This decrease could be attributed to root systems which are not able to compensate for water lost by transpiration through a reduction of the absorbing surface [5,48]. NO treatments elevated RWC to a level higher than the non-treated

salt stressed plants. Increased RWC by NO application not reported under salinity stress.

The decrease in RWC under salinity stress in basil under salinity stress is in confirmation of already reported results [53].

In general, it can be concluded that exogenous application of NO is an effective approach to alleviate hazardous effects of salinity on basil plants and it is mainly through the changes in physiological properties and antioxidative system.

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