

## Nitric Oxide and Salicylic Acid Mitigate Cadmium Stress in Wheat Seedlings

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Nitric oxide (NO) and salicylic acid (SA) are well known as signaling and ubiquitous bioactive molecules that play a key role in tolerance of plant to abiotic stress by regulating the various plant cellular mechanisms. Therefore, the present experiment was conducted to study the interactive effect of NO and/or SA in tolerance of wheat (*Triticum aestivum* L. cv. Samma) under cadmium (Cd) stress. The results indicated that plants grown in Cd-containing medium exhibited reduced growth characteristics (plant height, fresh weight, dry weight and leaf area), carbonic anhydrase activity and content of essential nutrients [nitrogen (N), phosphorus (P) and potassium (K)] nutrients and chlorophyll (Chl) *a* and *b* by enhancing MDA and Cd accumulation. However, combined application of NO and SA was more effective in suppression of deleterious effect of Cd stress by inducing the activities of enzymes [carbonic anhydrase (CA), superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT)] and accumulation of osmoprotectant (Pro) and by maintaining nutrients homeostasis, leading to the reduction of MDA accumulation and improvement of photosynthetic pigments (Chl *a* and Chl *b*) resulted in a better plant growth performance under Cd stress. The present study revealed that application of both NO and SA together improved the plant growth and development by reducing formation of reactive oxygen species by improving antioxidant enzymes and CA activity and balance supply of nutrients.

**Key words:** Cadmium stress, Nitric Oxide, Salicylic acid, Photosynthetic pigments, Essential nutrients, Carbonic anhydrase, Antioxidant, *Triticum aestivum*.

A few studies deal with role of nitric oxide (NO) and salicylic acid (SA) in plants under heavy metal stress. Cadmium (Cd) is one of the most toxic heavy metals for animal, human and plants by entering the environment may be through phosphate fertilizers and waste disposal (Yilmaz *et al.*, 2006; Mahmood *et al.*, 2009). It is clear that cadmium (Cd) is a strong environmental pollutant with high toxicity to animal and plant (di Toppi and Gabbrielli, 1999).

Although, the replacement with the mechanisms of cytoplasmic toxicity are identical in all organisms, different plant species and varieties show a wide range of plasticity in cadmium tolerance (Methwally *et al.*, 2003). Salin, (1988) stated that Cd cannot generate reactive oxygen species (ROS) via Haber-Weiss or Fenton type reactions. Cd toxicity results in the alteration of oxidant level in plant, including the generation of toxic reactive oxygen species (ROS) like hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical (OH<sup>•</sup>), superoxide radical (O<sub>2</sub><sup>-•</sup>) etc. (Choudhury and Sanjib, 2004). Plants have various defense mechanisms by which they can scavenge these ROS. Cd enhances the activity of some antioxidant enzymes like catalase, peroxidase, superoxide dismutase and glutathione

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reeducates and non-enzymes like acrobate, glutathione,  $\pm$ -tocopherol (Panda, 2002).

Salicylic acid (SA) is considered as a hormone-like endogenous regulator, which influences a range of diverse processes in plants including seeds germination, ion uptake and transport, membrane permeability and photosynthesis. Salicylic acid may acts as important signal molecule for modifying plant responses to environmental stressors (Simaei *et al.*, 2011). The mode of SA action is related to the inhibition of catalase (CAT) and ascorbate peroxidase (APX), two major  $H_2O_2$  scavenging enzymes, the inhibition might cause the cellular concentration of  $H_2O_2$  to rise. Subsequently,  $H_2O_2$  may act a second messenger and activating defense – related gene (Chen *et al.*, 1993). Probably, this mode of action not acceptable (Methwally *et al.*, 2003).

Nitric Oxide (NO) plays an important role in diverse physiological processes. It has been reported that the NO can regulates many mechanisms in plants and stress responses. NO promotes normal growth and development of plant at low concentration (Beligni and Lamattina, 2001). Also, NO alleviates the content of SA that plays a significant role in signaling pathways during biotic stresses (Klessig *et al.*, 2000). There are several reports on the involvement of exogenous NO in protecting plants against oxidative stress induced by heavy metals (Hsu and Kao, 2004; Wang and Zang, 2005; Kopyra *et al.*, 2006; Mahmood *et al.*, 2009).

Therefore, aim of the present study to investigate the role of SA and NO in tolerance of plant to Cd stress.

## MATERIALS AND METHODS

### Plant materials and cultivation conditions

This experiment was carried out under growth chamber condition using wheat (*Triticum aestivum* L. cv. Samma) obtained from a local market in Riyadh, Saudi Arabia. Healthy seeds were surface sterilized with 1% sodium hypochlorite for 10 min, and then vigorously rinsed with sterilized double distilled water (DDW) before sowing. The seeds were sown in plastic pots (25 cm diameter, 25 cm height) filled with perlite and supplied with Raukura's nutrient solution (Smith *et al.*, 1983).

The pots were arranged in a simple randomized design in the growth chamber with a single factor and five replicates. One week after sowing, seedlings were thinned so that each pot contained healthy plants of uniform size. Pots were irrigated every two days with DDW (100 ml) to keep the perlite moist.

When the plants were at the stage of two to three true leaves, start treatments with salicylic acid (SA), Nitric oxide (NO) and cadmium (Cd) solution was added to the pots with experimental wheat plants to attain the final concentration from 0 to 1 mM. The treatments were given as follows:

- (1)  $Cd_0$  mM +  $No_0$  mM +  $SA_0$  mM {control},
- (2)  $Cd_1$  mM +  $No_0$  mM +  $SA_0$  mM,
- (3)  $Cd_0$  mM +  $NO_{0.5}$  mM +  $SA_0$  mM,
- (4)  $Cd_0$  mM +  $NO_0$  mM +  $SA_{0.5}$  mM,
- (5)  $Cd_1$  mM +  $NO_{0.5}$  mM +  $SA_0$  mM,
- (6)  $Cd_1$  mM +  $NO_0$  mM +  $SA_{0.5}$  mM,
- (7)  $Cd_0$  mM +  $NO_{0.5}$  mM +  $SA_{0.5}$  mM,
- (8)  $Cd_1$  mM +  $NO_{0.5}$  mM +  $SA_{0.5}$  mM.

The source of Cd was cadmium chloride ( $CdCl_2$ ). The plants were sampled at 30 days after sowing to assess their growth characteristics [plant height, shoot fresh plant<sup>-1</sup> (shoot FW), dry weight plant<sup>-1</sup> (shoot DW)] and area leaf<sup>-1</sup> and physio-biochemical attributes [chlorophyll (Chl) a and b, proline content (Pro), malondialdehyde (MDA) content, carbonic anhydrase activity (CA) and activity of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD)].

### Plant growth characteristics

Plant height was measured using a meter scale after removal from the pots. After recording fresh weight (FW) with balance, plants were placed in a 60°C oven for 48 h and then were weighed for dry weight (DW). Leaf area (LA) was measured using a LI-3000 Portable Leaf Area Meter (LI-COR, Lincoln, NE, USA).

### Physiological and biochemical parameters

Chl was extracted from fresh leaves using the dimethylsulphoxide (DMSO) method of Barnes *et al.*, (1992). Chl a and Chl b concentrations were calculated based on the absorbance of the extract at 663.8 and 646.8 nm. On the other hand, Proline concentration was determined spectrophotometrically by adopting the ninhydrin method of Bates *et al.*, (1973). We first homogenized 300 mg of fresh leaf samples in sulphosalicylic acid, then

added 2 mL each of acid ninhydrin and glacial acetic acid. The samples were heated at 100 °C. The mixture was extracted with toluene and the free toluene was quantified at 528 nm using L-proline as a standard. The activity of CA (EC 4.2.1.1) was determined by the method of Dwivedi and Randhawa (1974). Leaf samples were cut into small pieces and suspended in cysteine hydrochloride solution. The samples were incubated at 40° C for 20 min. The pieces were blotted and transferred to test tubes containing phosphate buffer (pH 6.8), then alkaline bicarbonate solution and bromothymol blue indicator were added. The test tubes were incubated at 50° C for 20 min. After the addition of 0.2 mL of methyl red indicator,

The reaction mixture was titrated against 0.05 N HCl. The results were expressed as  $\mu\text{mol CO}_2 \text{ kg}^{-1} \text{ FW s}^{-1}$ . MDA content was determined according to the method of Heath and Packer (1968). Leaves were weighed and homogenates containing 10% trichloroacetic acid (TCA) and 0.65% 2-thiobarbituric acid were heated at 95°C for 60 min, cooled to room temperature and then centrifuged at  $10,000 \times g$  for 10 min. The absorbance of the supernatant was read at 532 and 600 nm against a reagent blank.

To determine the enzymatic activities of the antioxidant proteins, a crude enzyme extract was prepared by homogenizing 500 mg of leaf tissue in extraction buffer containing 0.5% Triton X-100 and 1% polyvinyl pyrrolidone in 100 mM potassium phosphate buffer (pH 7.0) using a chilled mortar and pestle. The homogenate was centrifuged at  $15,000 \times g$  for 20 min at 4°C. The supernatant was used for the enzymatic assays described below. We used the method of Chance and Maehly (1955) to determine POD (E.C. 1.11.1.7) activity using 5 ml of an assay mixture containing phosphate buffer (pH 6.8), 50 M of pyrogallol, 50 mM of  $\text{H}_2\text{O}_2$ , and 1 ml of the enzyme extract diluted 20X. This was incubated for 5 min at 25°C, after which the reaction was stopped by adding 0.5 ml of 5% (v/v)  $\text{H}_2\text{SO}_4$ . The amount of purpurogallin formed was determined by measuring absorbance at 420 nm. A unit of peroxidase activity was the amount of purpurogallin formed per mg protein per minute.

Furthermore, Aebi (1984) method was used to measure CAT (EC 1.11.1.6) activity. The decomposition of  $\text{H}_2\text{O}_2$  was monitored by the

decrease in absorbance at 240 nm. For the assay, a 50 mM phosphate buffer (pH 7.8) and 10 mM  $\text{H}_2\text{O}_2$  were used. The activity of SOD (EC 1.15.1.1) was determined by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT) according to the methods of Giannopolitis and Ries (1977). The reaction solution (3 ml) contained 50  $\mu\text{mol}$  NBT, 1.3  $\mu\text{mol}$  riboflavin, 13 mmol methionine, 75 nmol ethylene diaminetetraacetic acid (EDTA), 50 mmol phosphate buffer (pH 7.8), and 20 to 50  $\mu\text{L}$  enzyme extract. The reaction solution was irradiated under a bank of fluorescent lights at 75  $\mu\text{mol m}^{-2} \text{ s}^{-1}$  for 15 min. The absorbance at 560 nm was read against a blank (non-irradiated reaction solution). One unit of SOD activity was defined as the amount of enzyme that inhibited 50% of NBT photoreduction.

To determine N, P, K and Cd concentrations, we followed the digestion approach of Zheljazkov and Nielson (1996) as modified by Hseu (2004). A leaf sample (0.5 g) was placed in a 250 mL digestion tube, and 10 mL of 2:1 concentrated nitric acid: perchloric acid was added. Samples were heated for 45 min at 90 °C for 2–3 h until a clear solution was obtained. At intervals, 5 mL of concentrated nitric acid: perchloric acid with hydrogen peroxide were added to the sample (at least three times), and the digestion continued until the volume was reduced to about 1 mL. The interior walls of the tube were washed down with a little DDW and the tubes were swirled throughout the digestion to keep the walls clean and prevent loss of the samples. After cooling, 5 mL of 1%  $\text{HNO}_3$  was added to each sample.

Thereafter, the solution was filtered through Whatman No. 42 filter paper and  $<0.45 \mu\text{m}$  Millipore filter paper. The filtrate was diluted to a total of 25 mL with distilled water. After dilution, the content of N and P determined by using spectrophotometrically, content of K was determined by using with an atomic absorption spectrometer (Model 300, Perkin-Elmer, Waltham, MA, USA) and Cd was determined with the help of Inductively Coupled Plasma Optical Emission Spectroscopy (Model iCAP6000, Thermo-Scientific, Thermo-Fisher Scientific, Waltham, MA, USA).

#### Statistical analysis

Each pot was treated as one replicate and all the treatments were repeated five times. The

data were analyzed statistically with SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Means were statistically compared by Duncan's multiple-range test at  $p < 0.05\%$  level.

## RESULTS

Application of Cadmium reduced seedlings height, fresh and dry weight and leaf area. Salicylic acid significantly increased seedling height, fresh and dry weight and leaf area (Table 1). Also, similar results were found with NO. Application of NO significantly increased seedlings height, fresh and dry weight and leaf area under non-cadmium conditions. Also, combined application of SA and NO in the absent of cadmium

application significantly enhanced the seedlings height. Under cadmium stress, alone application of SA and NO showed similar results for seedlings height, fresh and dry weight (Table 1). However, combined application of SA and NO enhanced seedlings height, fresh and dry weight and leaf area.

Table 2 shows that the treatment with Cd decreased significantly the level of chlorophyll a and b, while, the NO and SA treatment slightly increased in the content of chlorophyll a and b. Plants treated with SA and NO together significantly induced the synthesis of photosynthetic pigments (Chl *a* and *b*) under non-stress condition. However, alone application of NO showed higher value for Chl *b* in comparison

**Table 1.** Effect of Nitric oxide, Salicylic acid and the interaction of nitric oxide and salicylic acid on plant height, shoot fresh and dry weight and leaf area of wheat seedlings under cadmium stress.

Treatment	Plant Height (cm) Plant <sup>-1</sup>	shoot fresh weight (g) Plant <sup>-1</sup>	shoot dry weight (g) Plant <sup>-1</sup>	Leaf area (cm <sup>2</sup> )
Control	11.33±0.33bc	0.38±0.10d	0.10±0.00c	2.63±0.01a
No	11.33±0.33bc	0.96±0.02ab	0.19±0.00b	1.95±0.02d
SA	12.00±0.00b	1.06±0.02a	0.22±0.00a	2.38±0.04c
Cd	8.66±0.33e	0.54±0.02c	0.06±0.00e	1.55±0.00f
No + SA	15.66±0.32a	0.30±0.00de	0.22±0.00a	2.48±0.02b
No + Cd	10.00±0.57d	0.21±0.01e	0.09±0.00cd	1.58±0.02f
SA + Cd	10.66±0.35cd	0.23±0.00e	0.08±0.00d	1.89±0.02d
No+SA+Cd	12.33±0.33b	0.89±0.03b	0.06±0.00e	1.72±0.00e

Same letters show no statistical difference at  $p < 0.05$  (Duncan multiple-range test)

**Table 2.** Effect of Nitric oxide, Salicylic acid and the interaction of nitric oxide and salicylic acid on chlorophyll (a) and (b) content and proline content of wheat seedlings under cadmium stress

Treatment	Chl a (mg g <sup>-1</sup> FW)	Chl b (mg g <sup>-1</sup> FW)	Proline content (µg <sup>-1</sup> FW)
Control	1.16±0.33d	0.36±0.03cd	6.33±.33f
No	1.57±0.34b	0.73±0.03a	22.33±0.33b
SA	1.36±0.36c	0.56±0.03b	19.66±0.33c
Cd	0.83±0.36e	0.36±0.03cd	25.66±0.88a
No + SA	1.83±0.33a	0.66±0.03a	20.33±0.33bc
No + Cd	1.16±0.33d	0.43±0.03c	16.33±0.33d
SA + Cd	1.26±0.35cd	0.30±0.01d	15.00±0.57d
No+SA+Cd	1.36±0.33c	0.53±0.03b	12.00±1.52e

Same letters show no statistical difference at  $p < 0.05$  (Duncan multiple-range test)

to alone application of SA but both treatment showed statistically similar results for Chl a under Cd stress. Moreover, under Cd stress, combined application proved superior by inducing the synthesis of Chl a and b (Table 2).

The data presented in Table 2 reveal that the Cd application increased significantly proline accumulation in the wheat seedlings leaf. Treatment by NO and SA alone have also increased the accumulation of free proline. Under non-stress condition, combined application of SA and NO enhanced the accumulation of proline as compared to control. Under stress, alone application of SA and NO showed similar value. However, application SA and NO together increased proline accumulation as compared to control (Table 2).

Application of Cd increased malondialdehyde (MDA) content as compared with other treatments (Table 3). However, application of SA and NO alone as well as in combination significantly reduced MDA content in leaf (Table 3). The lowest content was recorded in wheat seedlings leaf treated with both NO and SA combined together.

It is also clear from Table (3) that the application of cadmium significantly decreased the carbonic anhydrase activity (CA) content in wheat seedlings leaf. However, application of SA and NO alone as well as in combination significantly increased CA content in leaf. The highest content was recorded in seedlings treated with NO and SA. Under non-stress condition, plants treated

**Table 3.** Effect of Nitric oxide, Salicylic acid and the interaction of nitric oxide and salicylic acid on Malondialdehyde activity, Carbonic anhydrase activity, Peroxidase activity, Catalase activity and Superoxide dismutase activity of wheat seedlings under cadmium stress

Treatment	Malondialdehyde concentration (nmol g <sup>-1</sup> FW)	Carbonic Anhydrase activity [(μmol (CO <sub>2</sub> )kg <sup>-1</sup> (FW)s <sup>-1</sup> ]	Peroxidase (U mg <sup>-1</sup> Protein)	Catalase activity (U mg <sup>-1</sup> Protein)	Superoxide dismutase (U mg <sup>-1</sup> Protein)
Control	15.33±0.33e	225±2.88g	157.66±5.36e	184.66±2.60f	3.66±0.33d
NO	13.33±0.33f	410±5.77c	187.33±4.33d	235.00±2.88d	8.66±0.35c
SA	10.66±0.33g	454.6±2.6b	133.66±1.85f	239.00±5.50d	8.33±0.38c
Cd	40.33±0.33a	157±4.65h	177.00±3.60d	250.66±1.76c	8.00±0.57c
NO + SA	9.33±0.33g	554.3±2.33a	144.66±2.40ef	223.33±1.66e	11.66±0.33b
NO + Cd	31.33±0.88c	356.6±3.52e	238.00±5.68b	255.00±2.88c	8.66±0.33c
SA + Cd	35.66±0.66b	289.6±5.48f	219.33±5.81c	276.00±3.78b	11.66±0.39b
NO+SA+Cd	25.66±0.66d	378±5.68d	271.00±7.00a	320.00±5.77a	14.77±0.88a

Same letters show no statistical difference at p<0.05 (Duncan multiple-range test)

**Table 4.** Effect of Nitric oxide, Salicylic acid and the interaction of nitric oxide and salicylic acid on Cd content, N content, P content and K content of wheat seedlings under cadmium stress

Treatment	Cd content μmol g <sup>-1</sup> DW	N content μmol g <sup>-1</sup> DW	P content μmol g <sup>-1</sup> DW	K content μmol g <sup>-1</sup> DW
Control	0.01±0.00e	284.33±2.33d	272.00±1.15c	2.05±0.02c
NO	0.01±0.00e	292.66±2.66c	281.66±1.66b	1.83±0.02d
SA	0.01±0.00e	305.00±2.88b	291.33±1.85a	2.26±0.03b
Cd	1.16±0.01a	259.33±1.76f	257.33±1.33e	1.43±0.00f
NO + SA	0.01±0.00e	317.33±4.09a	257.66±1.33e	2.36±0.02a
NO + Cd	0.93±0.00b	246.66±1.66g	259.66±2.60de	1.48±0.03f
SA + Cd	0.53±0.01d	268.00±2.08e	293.66±1.85a	1.77±0.02d
NO+SA+Cd	0.66±0.01c	282.66±3.71d	263.66±2.72d	1.60±0.00e

Same letters show no statistical difference at p<0.05 (Duncan multiple-range test)



with SA and NO together gave the higher value for CA activity. Under stress condition, combined application of SA and NO was found more effective by giving enhanced value for CA activity as compared to alone application and control (Table 3). In addition, application of cadmium and nitric oxide significantly increased the Peroxidase content in wheat seedlings leaf, whereas, the application of SA significantly decreased the peroxidase content in the leaf. Under Cd stress, application of SA and NO alone as well as in combination significantly increased peroxidase activity (Table 3).

In the present study Table (3) shows that the application of Cd increased catalase content in wheat seedlings leaf more than the application of nitric oxide and salicylic acid alone as well as in combination. Application of SA and NO alone and together significantly induced CAT activity as compared to control. However, combined application of SA and NO proved superior by giving higher value for CAT as compared to alone application under Cd stress (Table 3).

Application of Cd significantly increased superoxide dismutase (SOD) content in wheat leaves; moreover, the nitric oxide and salicylic acid application has the same effect as compared with control. The combined application of NO and SA significantly increased SOD content more than alone application of SA and NO. Under stress condition, plants treated with SA and NO together exhibited maximum SOD activity as compared to the plants treated with alone SA and NO (Table 3).

Table 4 reveals that plants treated with Cd showed maximum value for Cd content as compared to control plants. However, application SA and NO alone as well as in combination reduced the content of Cd under Cd stress. Under Cd stress, nutrients content (N, P and K) was found decreased. Application of SA and NO alone as well as in combination enhanced these nutrients content under stress and non-stress condition as compared to controls. However, combined application of SA and NA was found more effective for N and P content as compared to alone application. While, alone application of SA proved superior for K content as compared to alone application of NO and combined application of SA and NO under Cd stress.

## DISCUSSION

Cadmium is known to inhibit plant growth (Aidid and Okamoto, 1993; Veselov *et al.*, 2003), and it is known that the accumulation of cadmium in plant tissues may cause a changes in various physiological processes resulting in weak growth and development of plants and reduction of the productivity (di Toppi and Gabbriellini, 1999; Rodríguez-Serrano *et al.*, 2009; Perfus-Barbeoch *et al.*, 2002).

The individual Cd application significantly decreased growth parameters of wheat seedlings (Table 1) as compared to control and other treatments, this result is in agreement with Gaafar *et al.*, (2012). Many studies reported that Ca stress is extremely phytotoxic and causes inhibition of plant growth (Vassilev *et al.*, 1995; Sandalio *et al.*, 2001; Dong *et al.*, 2005; Kurtyka *et al.*, 2008). The growth reduction produced by cadmium stress may be due to degradation of chlorophyll molecules (Sandalio *et al.*, 2001). However, application of SA and NO alone as well as in combination significantly increased growth traits of wheat plants. Cd growth inhibition could be due to the inhibition of cell division and elongation rate of cells that mainly occurs by an irreversible inhibition of proton pump responsible for the process (Aidid and Okamoto, 1993; Liu *et al.*, 2003). The chlorophyll a and b are important macromolecules produced by plant, and play an important role in photosynthesis, which is responsible for plant growth. In the present study (Table 2) the significant reduction of chlorophyll a and b content enhanced by cadmium stress might be due to several metabolically parameter such as, destruction of chloroplast and photosynthetic apparatus (Vassilev *et al.*, 1995) and inhibition of some chlorophyll biosynthesis enzymes (Shalygo *et al.*, 1999). Therefore, the application of Cd decreased the content of chlorophyll a and b as compared to controls (Table 2). Application of SA and NO alone as well as together significantly ameliorated the photosynthetic pigments over Cd stress (Table 2). This finding is agreement with Gaafar *et al.*, (2012).

These results probably suggested that the exogenous application of SA and NO led to the promotion of protective mechanisms by enhanced photosynthetic pigments that could induce the pre-

adaptive response to Cd stress. This result has been confirmed by El-Tayed (2005), Singh *et al.*, (2008) and Simaei *et al.*, (2011).

Under Cd stress, the enhanced proline accumulation was observed. However, application of SA and NO proved beneficial in alleviating the adverse effect of Cd by improving proline accumulation (Table 2). This result is in agreement with Simaei *et al.*, (2011) who reported that proline is able to scavenge hydroxyl radical and stabilize the structure and function of macromolecules such as DNA, protein and membranes via interactions with these macromolecules. The protective role of nitric oxide and salicylic acid may be related to its regulation effects on proline contents (Table 2).

Cadmium content in wheat seedlings leaf was very high at alone Cd treatment. Cd content was the same in nitric oxide, salicylic acid and control leaf plants (Table 4). These results are in good agreement with other workers such as (Metwally *et al.*, 2003; Erdem *et al.*, 2012). Cadmium treatment of the leaf individually increased the cadmium contents but when nitric oxide or salicylic acid were applied individually or together the cadmium content was significantly decreased. Therefore, differences in the cadmium content are observed between the treatment, under nitric oxide and salicylic acid treatment, the cadmium content is higher than in control. However, it could be said that nitric oxide and salicylic acid individually or combined can reduce cadmium contents in the treated wheat seedlings leaf with cadmium; nitric oxide or salicylic acid can then tolerate the accumulation of cadmium in the leaf. Decreased potassium ( $K^+$ ) content with cadmium treatment (Table 4), may be due to effects on the ATPase, responsible for the proton gradient needed to take up  $K^+$  actively. Also,  $K^+$  plays a key role in reduction of ROS production by reducing activity of antioxidant enzymes under stress (Khan *et al.*, 2010; McAinsh *et al.*, 1996), and maintaining photosynthetic electron transport (Cakmak, 2005). Treatment of cadmium decreased significantly the dry matter weight, but the changes in total nitrogen and phosphorous contents with nitric oxide or salicylic acid alone as well as in combination showed no significant changes in response to increased cadmium applications (Table 4). Probably, cadmium stress dose not interfere with the uptake of stated plant nutrition. Therefore, it could say

that no changes in nitrogen and phosphorous content of wheat seedling leaves with cadmium stress, which may indicate that cadmium stress does not have any mutual effect with nutrients in anion form.

The application of cadmium significantly increased MDA content as compared to control and other treatment, however, application of nitric oxide, salicylic acid and the combined application of nitric oxide and salicylic acid significantly decreased MDA content in wheat leaf (Table 3), decreased content of MDA content was recorded in wheat leaf seedlings treated with combined nitric oxide and salicylic acid. In the present study (Table 3), it is clear that under non-stress as well as cadmium-stress conditions application of nitric oxide and salicylic acid significantly enhanced the activity of antioxidant enzymes such as CA, peroxidase, CAT and SOD.

Cadmium stress showed increased value for MDA concentration in wheat seedling leaf (Table 3), because cadmium produces excessive reactive oxygen species including superoxide radicals ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical (OH) causing cell death due to oxidative stress (Shak *et al.*, 2001). However, application of nitric oxide and salicylic acid were very effective in reduction of MDA accumulation under cadmium stress (Table 3). It may be due to the improvement of antioxidant enzymes activity such as CA, peroxidase, CAT and SOD. Cadmium is known to promote the generation of superoxide anion ( $O_2^-$ ), hydroxyl radical (OH) and hydrogen peroxide ( $H_2O_2$ ) excessive levels of which cause oxidative damage of biomolecules like lipid, proteins and nucleic acid (Yamamoto *et al.*, 2001; di Toppi and Gabbriellini, 1999). A crucial plant response to cope with cadmium – induced oxidative stress is the up regulation of antioxidant enzymes like catalase, superoxide dismutase and glutathione reductase (Pereira *et al.*, 2002). There have been recent reports on the involvement of exogenous nitric oxide in protecting plants against oxidative stress induced by heavy metals, (Mohmood *et al.*, 2009).

However, in the present study the application of SA and NO alone as well as in combination was very effective in reduction of the MDA accumulation under cadmium stress. Probably due to the improvement of antioxidant

enzymes activity such as CA, peroxidase, CAT and SOD (Table 3). This increase in the activity of CA, peroxidase, CAT and SOD was observed at alone as well as in combination of SA and NO that the maximum CA, peroxidase, CAT and SOD activity in wheat seedlings leaf under cadmium stress could increase the ability to scavenge membrane damage. This finding is in a good agreement with other works such as (Agarwal and Pandey, 2004; Gaafar *et al.*, 2012).

Application of SA and NO may be explained on the basis of its role in the formation of macromolecules, have been proven to provide tolerance of plant to cadmium stress in plants by increasing enzymes activity. The antioxidant system is one of the important defense mechanisms of plants, through which plants perform normally under different environmental conditions by scavenging ROS. Therefore, it could say that Nitric Oxide and Salicylic acid treatment mitigated the damage effect of cadmium stress and adjusted the plants to perform normally.

### CONCLUSION

On the basis of the present results, it can be concluded that the application of nitric oxide, salicylic acid and combined (NO + SA) decreases the MDA activity and enhanced the activity of antioxidant enzymes, chlorophyll a and b the total content of proline as compared with control plants, which suggest that they could enhance the tolerance capacity of plants against the oxidative damage induced by Cd stress. The increased photosynthetic pigment, accumulation of proline and activity of CAT and SOD in wheat seedlings leaf treated with nitric oxide, salicylic acid could be responsible for improved plant height and fresh and dry weight production under cadmium stress. The results indicated that nitric oxide, salicylic acid and the synergistic (NO + SA) application mitigated the damage effect of cadmium stress and adjust the plants to perform normally.

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