Hortic. Environ. Biotechnol. 58(6):537-547. 2017.
DOI 10.1007/s13580-017-0353-4

ISSN (print) : 2211-3452
ISSN (online) : 2211-3460

Research Report

Exogenous Application of Nitric Oxide and Spermidine Reduces the Negative Effects of Salt Stress on Tomato

Manzer H. Siddiqui[1*], Saud A. Alamri[1], Mutahhar Y. Al-Khaishany[1], Mohammed A. Al-Qutami[1], Hayssam M. Ali[1], Hala AL-Rabiah[1], and Hazem M. Kalaji[2]

[1]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, 2455, Saudi Arabia
[2]Department of Plant Physiology, Faculty of Agriculture and Biology, Warsaw University of Life Sciences (WULS-SGGW), Nowoursynowska 159, 02-776 Warsaw, Poland

*Corresponding author: manzerhs@yahoo.co.in

Received November 21, 2016 / Revised April 25, 2017 / Accepted May 1, 2017

Abstract. Due to increasing soil salinity, the world agricultural output is being threatened by the shrinking area of fertile land. In the present study, we explored the interactive roles of nitric oxide (NO; 100 μM) and spermidine (SP; 200 μM) in ameliorating the effects of salt stress (NaCl; 100 mM) in tomato (*Solanum lycopersicum* L. *var.* Five Star) seedlings. NaCl stress reduced shoot and root length, shoot and root fresh weight, shoot and root dry weight plant^{-1} and leaf area leaf^{-1}. NaCl stress also suppressed the biosynthesis of photosynthetic pigments (*Chlorophyll a* and *b*) and increased proline (Pro) content, membrane damage and lipid peroxidation by inducing reactive oxygen species (H_2O_2 and $O_2^{\bullet-}$) generation in roots and leaves, as well as electrolyte leakage (EL) and malondialdehyde (MDA) accumulation in leaves. However, applying NO and/or SP increased the activities of catalase, peroxidase, superoxide dismutase, glutathione reductase and ascorbate peroxidase and increased photosynthetic pigment (chlorophyll *a* and *b*) and Pro accumulation, as well as reducing H_2O_2, $O_2^{\bullet-}$ and MDA content and EL, under salt stress. When tomato plants were treated with NO and SP simultaneously, NO signaling was further enhanced, which was confirmed by the addition of cPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; NO scavenger].

Additional key words: antioxidant system, nitric oxide, salinity, salt resistance, spermidine, tomato

Introduction

The increasing salinity in agricultural soils on a global scale is one of the major threats to crop productivity and poses a serious challenge to feeding the ever-growing world population. Anthropogenic activities, including reduced rainfall resulting from climate change and the use of irrigation systems with high levels of naturally occurring salts in irrigation water, have led to the conversion of a large amount of fertile lands to saline lands. According to the FAO (2005), increased salinity affects agricultural production in over 397 million hectares of land. The continuous increase in salt levels in arable soils has destructive global consequences, which will result in a 30% land loss within 25 years, rising to 50% by the year 2050 (Wang et al., 2003). Nearly 20-27% of irrigated land is currently affected by salt, with crop yields well below their genetic potential (Siddiqui et al., 2009; 2010).

Increasing salinization has become a major environmental threat, marked by a severe slowdown in the sustainable improvement of crop production. The degree of impact of salt stress on plant growth and development depends on the concentration of salt, the sensitivity of crops and the genetic makeup of plants for resisting and overcoming the adverse effects of salt stress (Siddiqui et al., 2009; Munns 2005). Salinity leads to many changes in plants at the physiological and molecular levels. Salinity disturbs photosynthetic activity, respiration, osmotic balance, ion homeostasis, protein and nucleic acid synthesis, enzyme activity, organic solute accumulation and hormonal balance (Siddiqui et al., 2012; Gupta and Huang 2014). In addition, salinity disturbs the water absorption capacity of plants, resulting in a paucity of water in leaves due to osmotic (hyperosmotic) stress, which is brought on by the accumulation of high salts in soil and plants. Osmotic stress caused by salinity alters plant root architecture and inhibits root elongation by disturbing cell

size, cell division and the expansion of cells in the root elongation zone. Thus, it is crucial to investigate the physio-biochemical mechanisms of plants involved in salt stress tolerance. Compounds and polyamines (PAs) containing nitrogen play a key role in ion and osmotic homeostasis, in addition to promoting the fortification of cellular macromolecules, which leads to a greater capacity of plants to scavenge and detoxify reactive oxygen species (ROS), resulting in increased abiotic stress tolerance.

Polyamines (PAs) are nitrogenous compounds that are ubiquitous in all living cells and have diverse functions in many cellular processes (Thomas and Thomas 2001; Zhao and Yang 2008). Among compatible solutes, PAs enable plants to tolerate environmental stresses by protecting their cellular components, such as nucleic acids, proteins and phospholipids (Groppa and Benavides 2008; Kusano et al., 2008; Nahar et al., 2016) and by regulating gene expression under various stress conditions (Wen et al., 2011). Among PAs, spermidine (SP) (triamine), putrescine (diamine) and spermine (tetramine) are synthesized in response to different abiotic stresses in plant tissues and play a vital role in various physiological functions in plants. However, the specific role played by PAs in stress mitigation remains unknown and needs to be further explored.

PAs and nitric oxide (NO) might play a cumulative role in plants (Fan et al., 2013; Wang et al., 2016). NO acts as a signaling molecule and has antioxidant properties (Beligni et al., 2002). Evidence suggests that NO protects plants from oxidative damage by regulating cellular redox homeostasis and promoting the scavenging capacity of plants by inducing antioxidant enzymes (Siddiqui et al., 2011). Both PAs and NO have signaling properties, play multiple biological functions in plant growth and development and interact with hormones (Nahar et al., 2016; Wang et al., 2016). PA and NO biosynthesis might be interlinked, as PAs (spermine and SP) induce NO generation in *Arabidopsis thaliana* seedlings (Tun et al., 2006). Under stress conditions, NO might act as a downstream signal for PAs in plants (Arasimowicz-Jelonek et al., 2009). Also, both spermine and SP have an inhibitory effect on NO biosynthesis in *Araucaria angustifolia* (Silveira et al., 2006). However, the cumulative role and functional properties of both biogenic molecules (SP and NO) under salinity have, to the best of our knowledge, yet to be confirmed. Therefore, it is important to discover the possible interrelationship between SP and NO and to explore the cumulative effect of SP and/or NO on growth and development and on the antioxidant system in tomato plants under stress, which has yet to be studied. The aim of the present study is to determine whether the application of SP alone, as well as in combination with NO, enhances the physio-morphological attributes of tomato plants by improving their tolerance to NaCl stress.

Materials and Methods

Plant Culture

Tomato seeds (*Solanum lycopersicum* L. *var.* Five Star) were obtained from a local market. Before sowing, the seeds were surface sterilized with a solution containing 49% sterile double-distilled water (DDW), 50% ethanol and 1% sodium hypochlorite for 5 minutes, followed by vigorous rinsing with sterile DDW. The tomato seeds were germinated on two sheets of sterilized moistened filter paper in a Petri dish (12-cm diameter). The Petri dishes were kept in the dark for germination.

SP and NO Treatment Under NaCl Stress

After two weeks of germination, the seedlings were transferred to fresh Petri dishes and subjected to the following treatments: (i) 0 μM NO + 0 μM SP, (control), (ii) 100 μM NO, (iii) 200 μM SP, (iv) 100 μM NO + 200 μM SP, (v) 100 mM NaCl, (vi) 100 μM NO + 100 mM NaCl, (vii) 200 μM SP + 100 mM NaCl, (viii) 100 μM NO + 200 μM SP + 100 mM NaCl and (ix) 200 μM cPTIO + 100 μM NO + 200 μM SP + 100 mM NaCl. Analytical grade chemicals were used in this study. Sodium nitroprusside [SNP: (Na_2 [Fe $(CN)_5NO].2H_2O$] was used as the NO donor, while cPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] was used as the NO scavenger. NaCl treatment was applied to the Petri dish containing tomato seedlings in order to attain the proper final concentration. All Petri dishes were incubated in a growth chamber (temperature 25 ± 3°C, relative humidity 50 to 60% and light conditions of 450 μmol of photons $m^{-2} \cdot s^{-1}$ under a 16/8-h light/dark cycle).

Determination of Morphological Characteristics of Plants

Seedlings were harvested after 30 days of treatment and subjected to growth, physiological and biochemical analysis. The growth performance of tomato seedlings was evaluated in terms of shoot length (SL) and root length (RL) $plant^{-1}$, shoot (SFW) and root fresh weight (RFW) $plant^{-1}$, shoot (SDW) and root dry weight (RDW) $plant^{-1}$ and area $leaf^{-1}$. The leaf area was recorded directly using a Leaf Area Meter (LI-COR Inc., USA). The areas of three leaves (upper, middle and lower) from each plant in a sample (consisting of five plants) were measured.

Determination of Physiological and Biochemical Characteristics of Plants

***Chlorophyll*:** Chlorophyll (Chl) *a* and *b* were extracted from the fresh leaves of plants using the DMSO method of Barnes et al. (1992).

***Proline (Pro)*:** Proline (Pro) levels were estimated as described by Bates et al. (1973). Leaves were sampled from tomato plants, and 50 mg leaf tissue (per treatment) was homogenized in 3% sulfosalicylic acid and filtered. The filtrate (2 mL) was transferred to a test tube containing ninhydrin reagent (1.25 mg ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid), after which the samples were heated to 100°C. The reaction mixture was separated with toluene, and free toluene was quantified at 520 nm.

***Malondialdehyde (MDA)*:** Leaf samples were collected and crushed in liquid nitrogen. Fine leaf powder was suspended in a test tube containing 10% trichloroacetic acid and 0.65% 2-thiobarbituric acid and heated at 95°C for 60 minutes. The homogenate was centrifuged at 10,000 g for 10 minutes after cooling. The absorbance of the supernatant was read at 532 nm and 600 nm against a reagent blank (Heath and Packer, 1968).

Electrolyte Leakage

To evaluate membrane permeability, electrolyte leakage (EL) was measured according to the method of Lutts et al. (1995). EL was defined as $EC_1/EC_2 \times 100$ and expressed as a percentage.

Determination of Antioxidant Enzyme Activity

To determine the activities of antioxidant enzymes, each leaf sample was crushed into a fine powder in liquid N_2 using a mortar and pestle. The homogenized leaf tissue was suspended in extraction buffer (0.5% Triton X-100 and 1% polyvinylpyrrolidone) and in 100 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 15,000 g for 20 minutes at 4°C, and the supernatant was used for the enzymatic assays. The Bradford method (1976) was used for protein quantification.

Peroxidase (POD; EC 1.11.1.7) activity was assayed according to the method of Chance and Maehly (1955), with slight modifications. The enzyme reaction mixture (5 mL) contained 5 mM pyrogallol, 100 mM phosphate buffer (pH 6.8), 5 mM H_2O_2 and 1 mL of enzyme extract diluted 20-fold. After incubating the assay mixture for 5 minutes at 25°C, the reaction was stopped by adding 0.5 mL of 5% (v/v) H_2SO_4. The amount of purpurogallin formed was estimated spectrophotometrically at 420 nm. One unit of POD activity was determined based on the amount of purpurogallin formed per mg of protein per minute.

The method of Aebi (1984) was followed to determine catalase (CAT; EC 1.11.1.6) activity.The reaction solution included 50 mM phosphate buffer (pH 7.8) and 10 mM H_2O_2. The decomposition of H_2O_2 was determined based on the decrease in absorbance at 240 nm.

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured by observing the inhibition of nitro blue tetrazolium (NBT) photoreduction (Giannopolitis and Ries, 1977). In this assay, the reaction solution comprised 50 mM NBT, 1.3 mM riboflavin, 13 mM methionine, 75 μM ethylenediamine tetraacetic acid (EDTA), 50 mM phosphate buffer (pH 7.8) and 50-100 μL enzyme extract. The reaction solution was illuminated under fluorescent light at 75 μM $m^{-2} \cdot s^{-1}$ for 15 minutes. 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 inhibiting 50% of NBT photoreduction at 560 nm.

APX (EC 1.11.1.11) activity was measured using the method of Nakano and Asada (1981) as follow: the reaction was initiated by adding 200 μL enzyme extract to reaction buffer solution containing 50 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. The hydrogen peroxide-dependent oxidation of ascorbate was followed by measuring the decrease in the absorbance at 290 nm (absorbance coefficient at 2.8 $mM^{-1} \cdot cm^{-1}$) for min^{-1} in mL^{-1} of reaction mixture.

GR (EC 1.6.4.2) activity was determined as described by Foyer and Halliwell (1976), with minor modifications. The assay mixture contained 50 μL enzyme extract, 100 mM phosphate buffer (pH 7.8), 0.1 μM EDTA, 0.05 mM NADPH and 3.0 mM oxidized glutathione in a total volume of 1.0 mL. After incubating the reaction mixture for 10 min. at 25°C, the NADPH oxidation rate was monitored by reading the absorbance at 340 nm at the moment of H_2O_2 addition and then 1 minute later. The difference in absorbance (A340) was divided by the NADPH molar extinction coefficient (6.22 $mM^{-1} \cdot cm^{-1}$) and the enzyme activity was expressed as μM of NADPH $min^{-1} \cdot mg^{-1}$ protein.

Histochemical Detection of ROS and NO in Roots by Fluorescence Microscopy

NO was detected in the roots of each treated seedling using 4,5-diaminofluorescein diacetate (DAF-2DA) under a fluorescence microscope (Nikon Eclipse Ni-U, Nikon, Tokyo, Japan). Root segments (10 mm from the root tip) were incubated in a solution containing the fluorescent probe DAF-2DA (prepared in 10 mM Tris-HCl [pH 7.4]) for 1 hour in the dark at room temperature. The roots were then washed three times in fresh buffer without DAF-2 DA and examined at excitation and emission wavelengths of 490 nm and 525 nm, respectively, under a microscope (Rodríguez-Serrano, 2006).

Hydrogen peroxide (H_2O_2) was detected using the fluorescent probe 2′, 7′-dichlorofluorescein diacetate (DCF-DA) following the method of Schopfer et al. (2001). H_2O_2 was detected by incubating the roots of experimental seedlings in 25 μM DCF-DA (prepared in 10 mM Tris-HCl) for 30 minutes at

37°C. The roots were then washed three times with buffer and imaged under a fluorescence microscope (Eclipse Ni-U, Nikon, Tokyo, Japan) at excitation and emission wavelengths of 480 and 530 nm, respectively. Images were captured with a camera (DS-Ri1).

Histochemical Detection of ROS in Leaves

$O_2^{\bullet-}$ and H_2O_2 were detected in tomato leaves as described by Wang et al. (2011) and Mostofa and Fujita (2013), respectively. Briefly, the second leaves of treated plants were incubated in 0.1% nitro blue tetrazolium (NBT) and 1% 3,3-diaminobenzidine (DAB) solutions (prepared in a 50 mM Tris acetate buffer [pH 5.8]) for 12 hours at room temperature in the dark and in the light, respectively. Thereafter, incubated leaves were rinsed in DDW. To remove chlorophyll, the leaves were boiled in bleaching solution containing glycerol, acetic acid and ethanol (1:1:4) to detect blue insoluble formazan (for $O_2^{\bullet-}$) and a deep brown polymerization product (for H_2O_2). After cooling, images of leaves were taken by placing the leaves between two glass plates.

The experiment was performed using a randomized sample design with a single factor of six replicates. The data were subjected to one-way analysis of variance using SPSS Ver. 17 statistical software. The means were compared statistically using Duncan's multiple-range test at the $p < 0.05$ level. Each value shows the mean of six replicates (n=6) ± standard error (SE).

Results

In the present study, we evaluated the performance of plants under NaCl stress based on their growth and physiological and biochemical characteristics. We also validated the interacting role of NO and SP in restoring plant growth and development under salinity stress.

Effect of NO and/or SP on Plant Growth Characteristics

We measured seven growth parameters in tomato seeds (SL, RL, SFW, RFW, SDW, RDW and LA; Figs. 1 and 2). Separate and combined treatments with NO and SP enhanced all growth characteristics in plants under normal conditions.

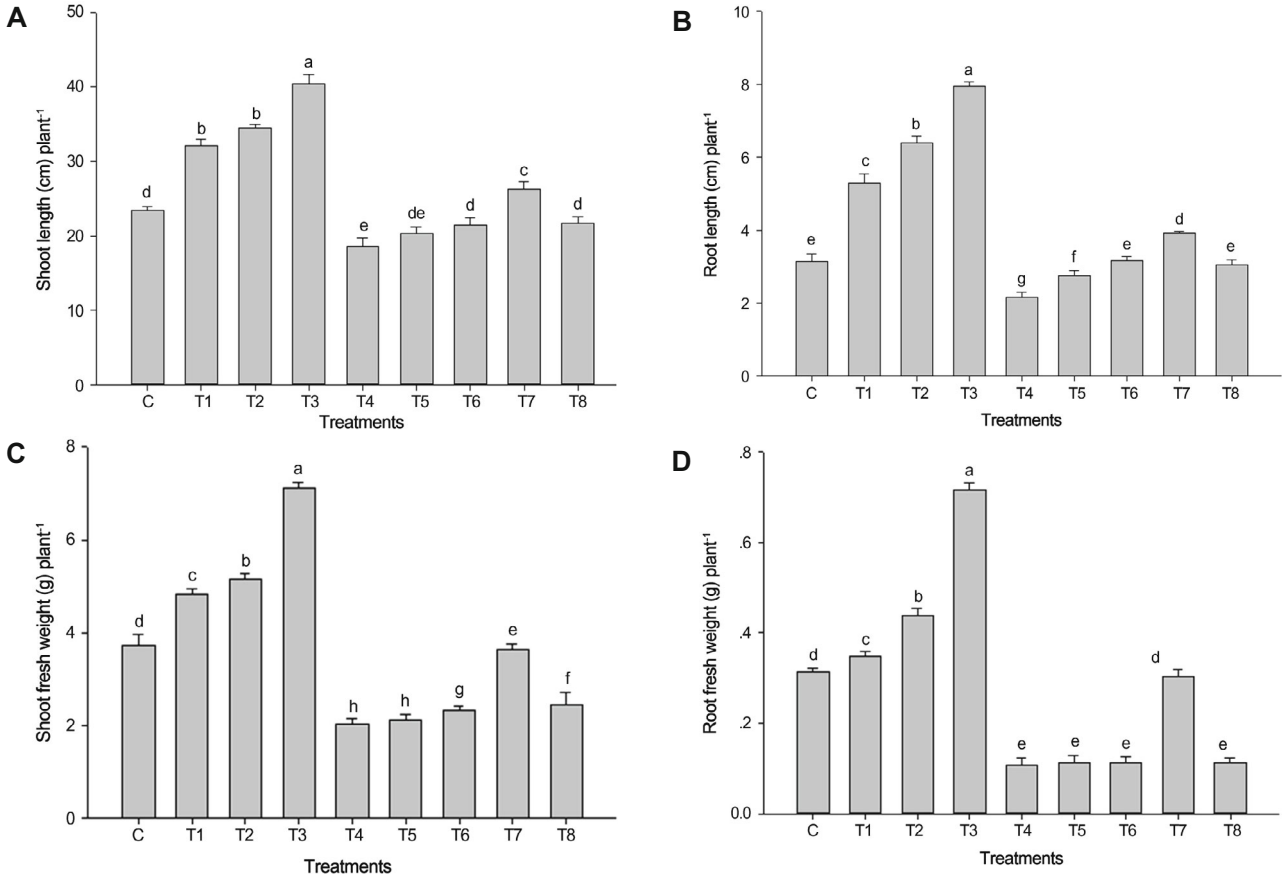

Fig. 1. Effect of nitric oxide and spermidine on (A) shoot length, (B) root length, (C) shoot fresh weight and (D) root fresh weight plant^{-1} in tomato plants under salinity stress. Bars followed by the same letter do not differ statistically at $p < 0.05$ (Duncan Multiple Range Test). Average of six determinations are presented with bars indicating S.E. Control (C), NO (T1), SP (T2), NO+SP (T3), S (T4), NO+S (T5), SP+S (T6), NO+SP+S (T7) and cPTIO+NO+SP+S (T8).

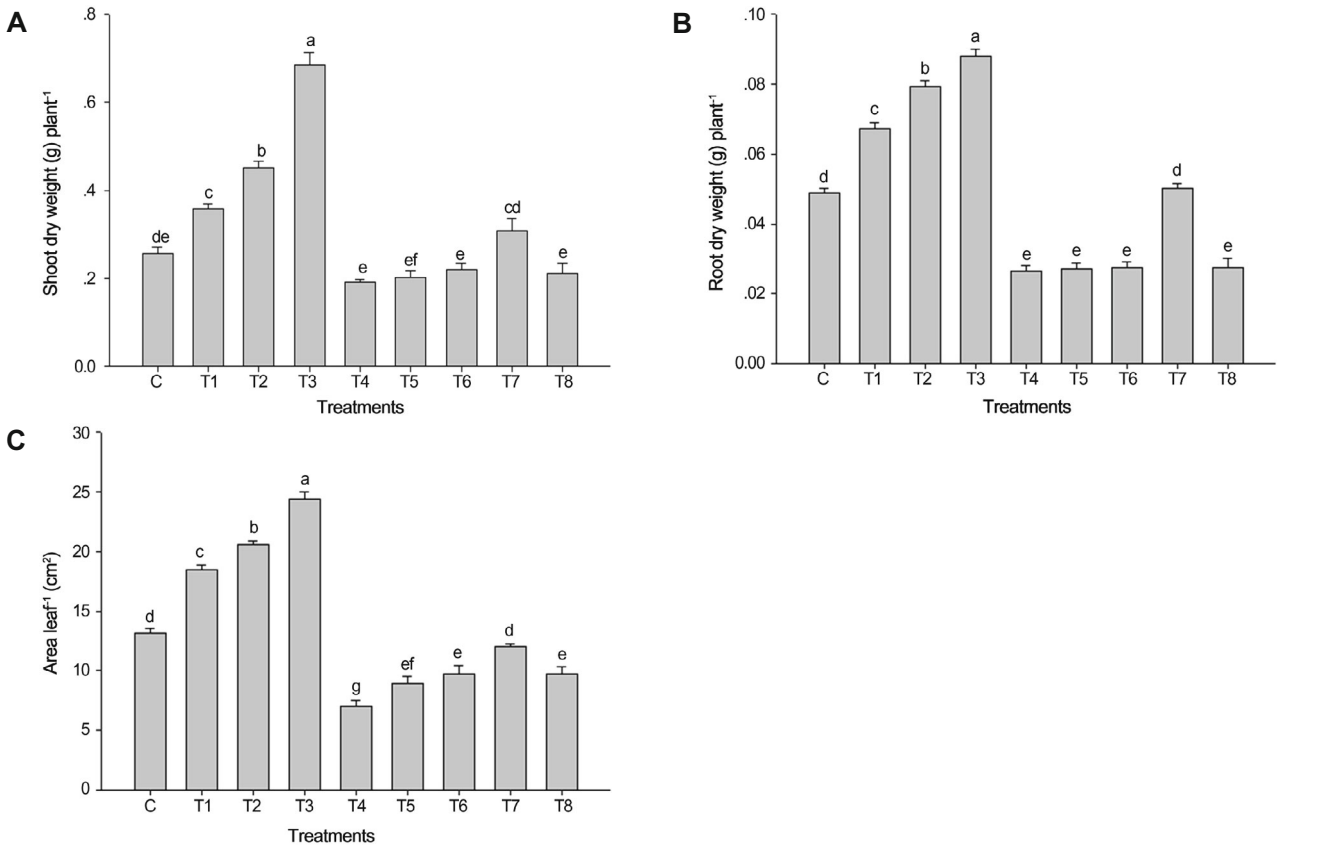

Fig. 2. Effect of nitric oxide and spermidine on (A) shoot dry weight, (B) root dry weight plant^{-1} and (C) leaf area in tomato plants under salinity stress. Bars followed by the same letter do not differ statistically at $p < 0.05$ (Duncan Multiple Range Test). Average of six determinations are presented with bars indicating S.E. Control (C), NO (T1), SP (T2), NO+SP (T3), S (T4), NO+S (T5), SP+S (T6), NO+SP+S (T7) and cPTIO+NO+SP+S (T8).

NO combined with SP was more effective and yielded greater values for all measured growth traits under both salinity and non-salinity conditions compared to separate treatments. However, the application of NaCl significantly inhibited all parameters compared with the control. The application of NO and SP together was more effective in improving plant growth traits under salt stress than separate treatments. To confirm the role of NO, we applied cPTIO to the seedlings, which reduced growth parameters, even in the presence of SP.

Effect of NO and/or SP on Physio-biochemical Parameters

The application of NO alone, as well as in combination with SP, significantly increased the accumulation of both photosynthetic pigments (*Chl a* and *Chl b*) under non-stress conditions (Fig. 3A and B). The combined application of NO and SP yielded higher values for both parameters than the application of these treatments separately. NaCl treatment reduced the biosynthesis of both pigments compared with the control. Moreover, the combined application of NO and SP significantly improved *Chl a* and *Chl b* biosynthesis under salinity stress. In the present study, we confirmed that the addition of cPTIO in a solution containing NO reverses the ameliorating effect of NO under salt stress, even in the presence of SP. Similarly, the application of NO and SP separately, as well as in combination, increased the accumulation of Pro over the control, especially the combined application of NO and SP (Fig. 3C). Under salt stress, NO + SP-treated plants had higher Pro contents than the control. To confirm the role of NO in this process, we applied the NO scavenger, cPTIO, to the seedlings, which led to reduced Pro accumulation.

Under non-stress conditions, EL and the contents of MDA and H_2O_2 decreased in seedlings treated with NO and SP separately, as well as in combination (Fig. 4A, B and C), over the controls. In general, NaCl-stressed tomato seedlings showed a significant increase in MDA and H_2O_2 contents, as well as EL, compared to the controls. The combined application of NO and SP was more efficient in limiting the accumulation of MDA and H_2O_2 than NO and SP alone under salinity stress. Moreover, when cPTIO, an inhibitor of NO, was added, the levels of MDA, H_2O_2 and EL increased, even in the presence of SP and NO.

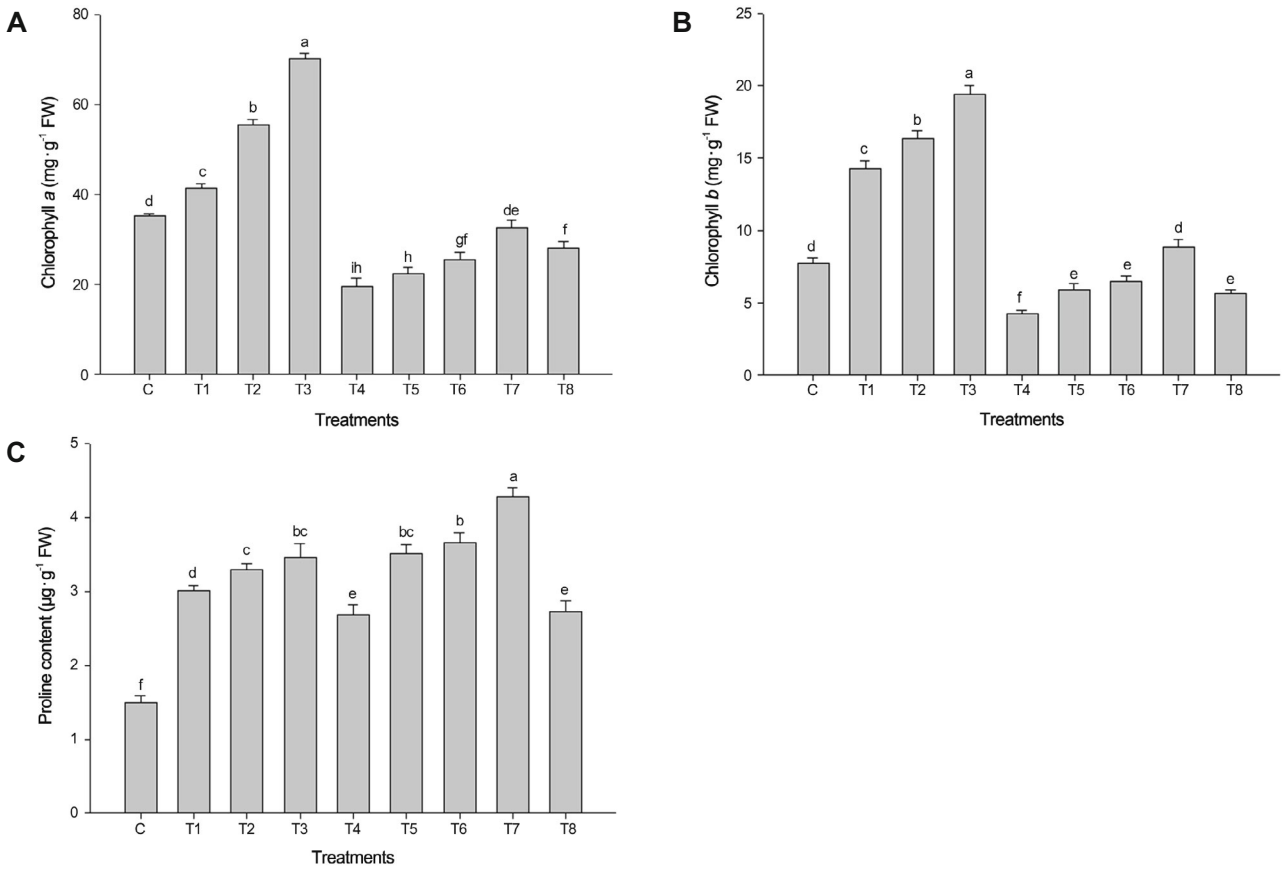

Fig. 3. Effect of nitric oxide and spermidine on the contents of (A) chlorophyll *a*, (B) chlorophyll *b* and (C) proline in tomato leaves under salinity stress. Bars followed by the same letter do not differ statistically at $p < 0.05$ (Duncan Multiple Range Test). Average of six determinations are presented with bars indicating S.E. Control (C), NO (T1), SP (T2), NO+SP (T3), S (T4), NO+S (T5), SP+S (T6), NO+SP+S (T7) and cPTIO+NO+SP+S (T8).

We detected the *in situ* generation of ROS in the leaves of tomato seedlings under salinity stress using DAB staining, as shown in Figure 5. We detected greater generation of DAB-H_2O_2 (brown color, due to DAB polymerization) in the intact leaves of NaCl-treated plants compared to the lower levels of DAB-H_2O_2 in the leaves of non-stress plants (Fig. 5A). However, less H_2O_2 generation was detected in the leaves of seedlings treated with NO + SP compared with the control and cPTIO-treated plants under stress and non-stress conditions. We detected the production of $O_2^{\bullet-}$ in leaves in the form of dark blue insoluble formazan using NBT staining (Fig. 5B). Parallel results were obtained in tomato seedlings treated with NO and/or SP under stress and non-stress conditions. We observed trace levels of $O_2^{\bullet-}$ production in the leaves of seedlings treated with NO and/or SP compared with the control and cPTIO-treated plants. Seedlings treated with the combined application of NO and SP showed lower $O_2^{\bullet-}$ levels in their leaves compared to NaCl-treated plants.

We detected ROS (H_2O_2) production in the roots of tomato seedlings under both conditions (Fig. 6A). A sharp increase in green DCF fluorescence in roots was detected in NaCl-stressed seedling roots compared to the roots of non-stressed seedlings. Lower levels of green DCF fluorescence were observed in the roots of tomato seedlings treated with NO + SP under salinity stress. The inclusion of cPTIO with NO + SP treatment reversed the increase in green fluorescence to levels similar to those of the control. These results suggest that the application of NO combined with SP is an effective way to mitigate ROS accumulation.

The pattern of NO accumulation in the roots of tomato seedlings (as detected using DAF-2DA) was opposite that of ROS production in roots. As shown in Figure 6B, NO production was greater in the roots of seedlings treated with NO and/or SP compared to the control and NaCl-treated seedlings. We detected a sharp increase in NO-dependent fluorescence in the roots of seedlings treated with NO combined with SP under both non-stress and NaCl-stress conditions. Under salinity stress, the NO-dependent increase in fluorescence was eliminated in the presence of cPTIO, a NO scavenger.

To further investigate the mechanism underlying plant tolerance to salt stress, we measured the activities of antioxidant enzymes (CAT, POD, SOD, GR and APX). The activities of

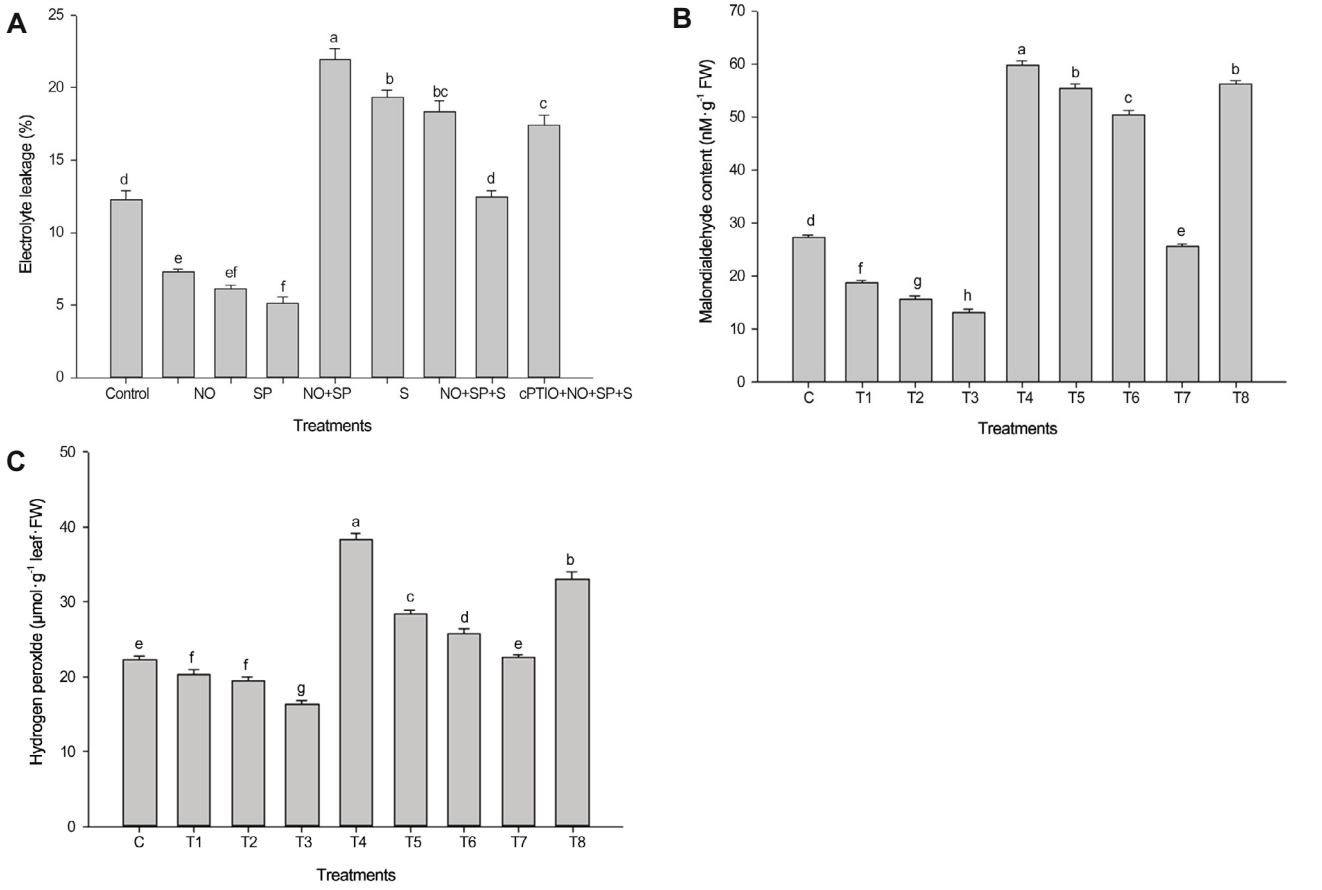

Fig. 4. Effect of nitric oxide and spermidine on (A) electrolyte leakage (B) malondialdehyde contents and (C) H_2O_2 contents in tomato plants under salinity stress. Bars followed by the same letter do not differ statistically at $p < 0.05$ (Duncan Multiple Range Test). Average of six determinations are presented with bars indicating S.E. Control (C), NO (T1), SP (T2), NO+SP (T3), S (T4), NO+S (T5), SP+S (T6), NO+SP+S (T7) and cPTIO+NO+SP+S (T8).

these enzymes increased in tomato seedlings treated with NaCl compared to the control. These antioxidant enzyme activities increased even further when NO and/or SP were added to the medium under both non-stress and stress conditions (Fig. 7A and B). The combined application of NO + SP increased the activities of these enzymes more strongly than their separate application under non-stress conditions. Finally, the activities of these enzymes were higher in NO+SP-treated tomato seedlings compared to NaCl-treated seedlings.

Discussion

In the present study, we found that the application of NO and/or SP enhanced plant growth traits under non-stress condition (Figs. 1 and 2). A maximum increase in SL, RL, SFW, RFW, SDW, RDW and LA was detected using the combined application of NO and SP. Salt stress limits plant growth and development by altering many physiological and biochemical processes. In the current study, NaCl stress disturbed tomato plant growth attributes such as SL, RL, SFW, RFW, SDW, RDW and LA (Figs. 1 and 2), whereas the application of NO with SP helped restore normal plant growth. Seedlings treated with the NO scavenger cPTIO exhibited a sharp decrease in growth characteristics, even in the presence of NO and SP. Therefore, we propose that the application of NO with SP increases the tolerance of tomato plants to salt stress. The efficacy of NO and SP in improving plant growth and development may be traced to their roles. Under stress, NO may protect plants by both directly and indirectly regulating the enzymatic and transcriptional modulation of Pro and polyamine biosynthetic pathways (Filippou et al., 2013). The NO-induced increase in plant growth parameters may be due to the role of NO in cell elongation, cell division and tissue differentiation (Fernández-Marcos et al., 2012; Siddiqui et al., 2011)]. The application of NO might upregulate SP biosynthesis (Wang et al., 2016), while conversely, SP might induce NO biosynthesis (Tun et al., 2006). Moreover, SP accelerates cell division and root formation in plants (Bouchereau et al., 1999; Imai et al., 2004). Plant tolerance to salt stress may be associated with the coordinating roles of both NO and SP in plant growth.

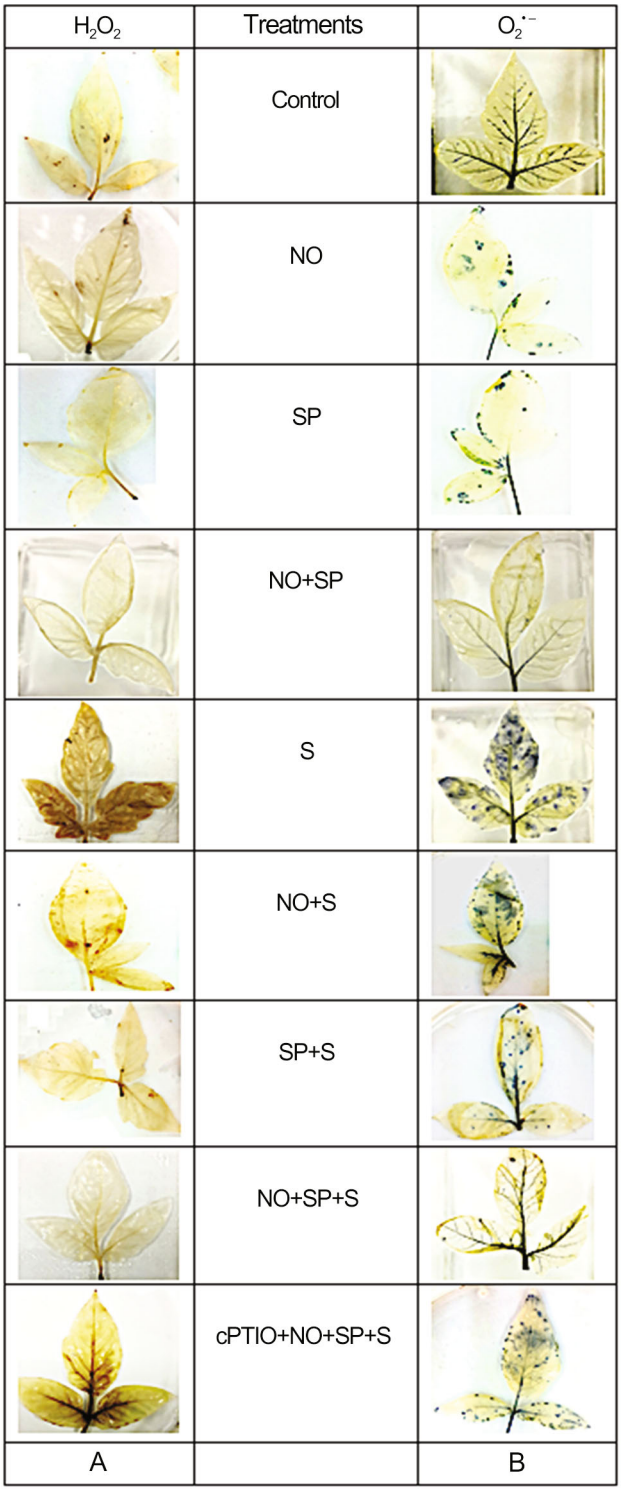

Fig. 5. *In situ* visualization of (A) H_2O_2 production in leaves using DAB staining and (B) $O_2^{\bullet-}$ formation in the leaves of tomato plants under salinity stress using NBT staining.

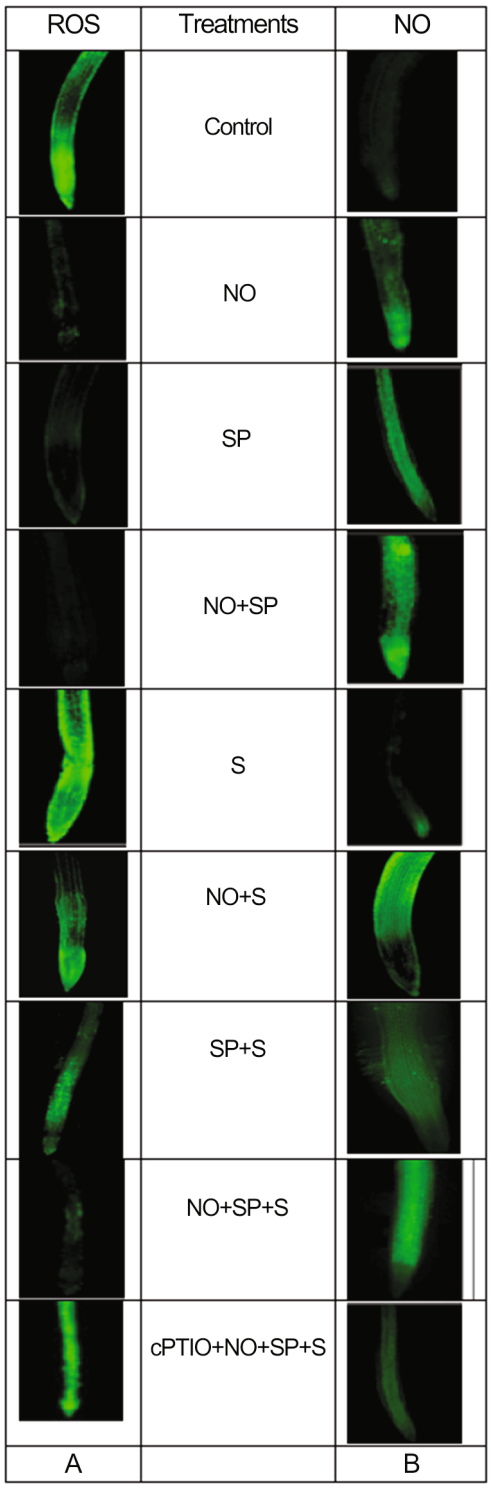

Fig. 6. *In situ* visualization of (A) ROS formation in primary roots using the fluorescent probe DCF-DA and (B) NO generation in primary roots of tomato plants using DAF-2DA under salinity stress.

Chl a and *Chl b* are key pigments for photosynthesis, an important process for plant growth and development. We explored the interacting roles of NO and SP in the accumulation of both photosynthetic pigments under stress and non-stress conditions (Fig. 3A and B). The application of NO and/or SP significantly increased the contents of both pigments in tomato seedlings under stress and non-stress conditions, while NaCl decreased the levels of both pigments. The combined application of NO and SP was more effective

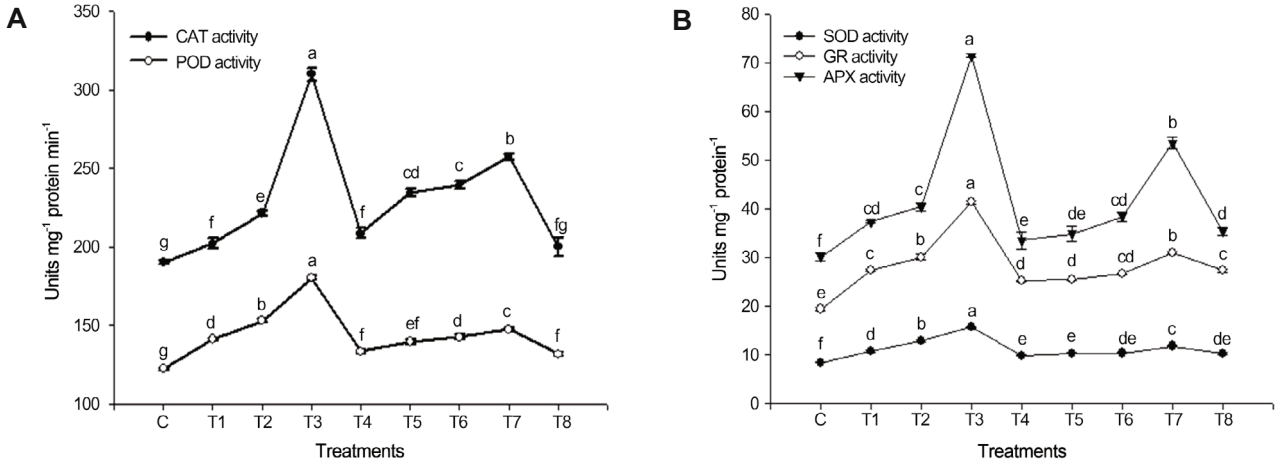

Fig. 7. Effect of nitric oxide and spermidine on the activity of (A) catalase and peroxidase and (B) superoxide dismutase, glutathione reductase and ascorbate peroxidase in tomato plants under salinity. Bars followed by the same letter do not differ significantly at $p < 0.05$ (Duncan Multiple Range Test). Average of six determinations are presented with bars indicating S.E. Control (C), NO (T1), SP (T2), NO+SP (T3), S (T4), NO+S (T5), SP+S (T6), NO+SP+S (T7) and cPTIO+NO+SP+S (T8).

in increasing the accumulation of both pigments in seedlings than individual treatments under non-stress conditions. Under salt stress, the application of NO and SP together was also more effective at enhancing the accumulation of both pigments compared to individual treatments, thereby enhancing plant tolerance to salt stress. The inclusion of cPTIO with NO+SP decreased the levels of both pigments, confirming the cumulative roles of NO and SP in salt stress tolerance. These results confirm the findings of Khan et al. (2012) and Siddiqui et al. (2013). NO regulates iron homeostasis and induces internal iron transport, leading to the synthesis of photosynthetic pigments and chloroplast development (Graziano and Lamattina 2005; Liu et al., 2007) and improving the photosynthetic characteristics of plants (Wu et al., 2013). SP plays a key role in chlorophyll accumulation and photosystem II activity, and it protects chloroplasts and protein D1 (an important component of photosystem II) under stress conditions. SP treatment is highly efficient in restoring photochemical efficiency (Fv/Fm), thereby increasing salt stress tolerance in tomato seedlings (Shu et al., 2012; Hu et al., 2015).

Pro is an important compatible solute that adjusts cellular osmolarity under stress. In the present study, NaCl treatment enhanced Pro accumulation in tomato seedlings, whereas the application of NO and/or SP increased it even further compared to the control (Fig. 3C). The application of NO with SP increased Pro biosynthesis, perhaps due to the role of NO in regulating the expression of *P5CS1*, a Pro biosynthetic gene. The NO and SP biosynthesis pathways have a common precursor, i.e., arginine, via nitric oxide synthase and arginase, respectively (Verslues and Sharma, 2010). Pro is involved in the scavenging of free radicals in addition to protecting enzymes (Siddiqui et al., 2012; Fu et al., 2014). Pro provides energy and N to plants for their consumption upon exposure to salinity stress, resulting in improved tolerance via increasing plant growth (Figs. 1 and 2).

MDA, H_2O_2, $O_2^{\bullet-}$, and EL are biomarkers for oxidative damage in plants under various environmental conditions. In the present study, analysis of ROS, MDA and EL in leaf extracts and ROS in leaf as well as root tissues *in vivo* by microscopy revealed high levels of ROS generation induced by salt stress (Figs. 4A, B and C and 5A and B). Siddiqui et al. (2009; 2012) also observed increased levels of MDA, H_2O_2, $O_2^{\bullet-}$ and EL in plants under NaCl stress. NaCl-induced overproduction of ROS, such as H_2O_2 and $O_2^{\bullet-}$, triggers the accumulation of MDA, which causes membrane lipid peroxidation in plants. However, the application of NO and SP together inhibited oxidative damage by suppressing the formation of ROS and MDA, as well as EL, which we also observed in the leaves and roots of tomato seedlings via microscopy (Figs. 5A and B, and 6A). PAs, including SP, are ubiquitous nitrogen compounds that play multiple roles both directly and indirectly in protection against membrane damage, as they interact with various macromolecules and membranes, and they also form a complex with Fe^{+} and phospholipids (Nahar et al., 2016; Imai et al., 2004). Both NO and SP have multifunctional properties and are involved in the tolerance of plants to stress (Siddiqui et al., 2017; Nahar et al., 2016; Imai et al., 2004).

As shown in Figure 6B, NO and SP treatment, both separately and in combination, enhanced the accumulation of NO in the roots of tomato seedlings under both stress and non-stress conditions. When we applied cPTIO, a NO scavenger, together with NO + SP, NO formation was reduced in the roots of tomato seedlings, while in the absence of cPTIO, both NO + SP triggered the production of NO, suggesting that the enhanced salt stress tolerance induced by

NO might be linked to the coordinated roles of both NO and SP in tomato plants. NO itself acts as an antioxidant and scavenges ROS (Figs. 4C, 5A and B, and 6A), resulting in reduced lipid peroxidation (Fig. 4B). This substantiates the findings of Nahar et al. (2016) using mung bean treated with putrescine and NO under cadmium stress, Li et al. (2014) using ginger treated with SP + NO under cold stress and Arasimowicz-Jelonek et al. (2009) using cucumber treated with PAs and NO under drought stress.

As shown in Figure 7A and B, a maximum increase in antioxidant enzyme (CAT, POD, SOD, GR and APX) activity was detected in tomato seedlings treated with NO + SP under both stress and non-stress conditions. These results are in agreement with the findings of Khan et al. (2012). The tolerance of plants to stress depends on ROS scavenging performed by antioxidant enzymes. We found that the changes in antioxidant enzyme activity under stress were significantly greater in the presence of NO and SP versus the control. To validate these results, we included cPTIO, a NO scavenger, which resulted in a noticeable decrease in enzyme activity. Thus, we postulate that both NO and SP have beneficial effects by enhancing antioxidant enzyme activity and functioning in defense against ROS by removing H_2O_2 and $O_2^{\bullet-}$ (Figs. 4C, 5A and B, and 6A).

Our results indicate that NO and SP increase salt stress tolerance in tomato plants, as revealed by improved growth and photosynthetic pigment contents, by regulating the levels of Pro, a compatible solute. The inclusion of cPTIO confirmed the functional commonality and coordination of the roles NO and SP. The combined application of NO and SP improve the resistance of tomato plants to salt stress by limiting ROS formation and mitigating lipid peroxidation. ROS scavenging and the inhibition of membrane damage might be due to the increased antioxidant enzyme activity and Pro levels in tomato seedlings treated with NO and SP under salt stress. However, since the potential relationship between PAs and NO has not been confirmed at both the molecular and physiological levels, it is important to further investigate the functional relationship between PAs and NO.

Acknowledgment: The authors are thankful to the Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia [project number RGP-VPP-153].

References

Aebi H (1984) Catalase in vitro. Meth Enzymol 105:121-126

Arasimowicz-Jelonek M, Floryszak-Wieczorek J, KubiŚ J (2009) Interaction between polyamine and nitric oxide signaling in adaptive responses to drought in cucumber. J Plant Growth Regul 28:177-186

Barnes JD, Balaguer L, Manrique E, Elvira S, Davison AW (1992) A reappraisal of the use of DMSO for the extraction and determination of chlorophylls *a* and *b* in lichens and higher plants. Environ Exp Bot 32:85-100

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free Proline for water-stress studies. Plant Soil 39:205-207

Beligni MV, Fath A, Bethke PC, Lamattina L, Jones RL (2002). Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone Layers[1]. Plant Physiol 129:1642-1650

Bouchereau A, Aziz A, Larher F, Martin-Tanguy J (1999). Polyamines and environmental challenges: recent development. Plant Sci 140: 103-125

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Chance B, Maehly AC (1955). Assay of catalase and peroxidases. Meth Enzymol 2:764-775

FAO (2005) Global Network on Integrated Soil Management for Sustainable Use of Salt-affected Soils. Rome: FAO Land and Plant Nutrition Management Service. Available online at: http://www.fao.org/ag/agl/agll/spush

Fan HF, Du CX, Guo SR (2013) Nitric oxide enhances salt tolerance in cucumber seedlings by regulating free polyamine content. Environ Exp Bot 86:52-59

Fernández-Marcos M, Sanz L, Lorenzo Ó (2012) Nitric oxide: An emerging regulator of cell elongation during primary root growth. Plant Signal Behav 7:196-200

Filippou P, Antoniou C, Fotopoulos V (2013). The nitric oxide donor sodium nitroprusside regulates polyamine and proline metabolism in leaves of *Medicago truncatula* plants. Free Radic Biol Med 56:172-183

Foyer C, Halliwell B (1976). The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. Planta 133:21-25

Fu JJ, Sun YF, Chu XT, Yang LY, Xu YF, Hu TM (2014). Exogenous nitric oxide alleviates shade-induced oxidative stress in tall fescue (*Festuca arundinacea* Schreb.). J Hortic Sci Biotechnol 89:193-200

Giannopolitis CN, Ries SK (1977) Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol 59:309-314

Graziano M, Lamattina L (2005) Nitric oxide and iron in plants: an emerging and converging story. Trends Plant Sci 10:4-8

Groppa MD, Benavides MP (2008) Polyamines and abiotic stress: recent advances. Amino Acids 34:35-45

Gupta B, Huang B (2014). Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. Int J Genomics 701596

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189-198

Hu L, Xiang L, Li S, Zou Z, Hu XH (2015) Beneficial role of spermidine in chlorophyll metabolism and D1 protein content in tomato seedlings under salinity-alkalinity stress. Physiol Plant 156: 468-477

Imai A, Matsuyama T, Hanzawa Y, Akiyama T, Tamaoki M, Saji H, Shirano Y, Kato T, Hayashi H, Shibata D, Tabata S, Komeda Y, Takahashi T (2004) Spermidine synthase genes are essential for survival of *Arabidopsis*. Plant Physiol 135:1565-1573

Khan MN, Siddiqui MH, Mohammad F, Naeem M (2012). Interactive role of nitric oxide and calcium chloride in enhancing tolerance to salt stress. Nitric Oxide 27:210-218

Kusano T, Berberich T, Tateda C, Takahashi Y (2008) Polyamines: essential factors for growth and survival. Planta 228:367-381

Li X, Gong B, Xu K (2014) Interaction of nitric oxide and polyamines involves antioxidants and physiological strategies against chilling-induced oxidative damage in *Zingiber officinale* Roscoe. Sci Hortic 170:237-248

Liu Y, Wu R, Wan Q, Xie G, Bi Y (2007) Glucose-6-phosphate dehydrogenase plays a pivotal role in nitric oxide-involved defense

against oxidative stress under salt stress in red kidney bean roots. Plant Cell Physiol 48:511-522

Lutts S, Kinet JM, Bouharmont J (1995) Changes in plant response to NaCl during development of rice (*Oryza sativa* L.) varieties differing in salinity resistance. J Exp Bot 46:1843-1852

Mostofa MG, Fujita M (2013) Salicylic acid alleviates copper toxicity in rice (*Oryza sativa* L.) seedlings by up-regulating antioxidative and glyoxalase systems. Ecotoxicology 22:959-973

Munns R (2005) Genes and salt tolerance: bringing them together. New Phytol 167:645-663

Nahar K, Hasanuzzaman M, Alam MM, Rahman A, Suzuki T, Fujita M (2016). Polyamine and nitricoxide crosstalk: Antagonistic effects on cadmium Toxicity in mung bean plants through upregulating the metal detoxification, antioxidant defense and methylglyoxal detoxification systems. Ecotoxicol Environ Saf 126:245-255

Nakano G, Asada K (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidise in spinach chloroplasts. Plant Cell Physiol 22:867-880

Rodríguez-Serrano MA RÍA, Romero-Puertas MC, Zabalza ANA, Corpas FJ, Gómez M, Del Rio LA, Sandalio LM (2006) Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation *in vivo*. Plant Cell Environ 29:1532-1544

Schopfer P, Plachy C, Frahry G (2001) Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. Plant Physiol 125:1591-1602

Shu S, Guo SR, Yuan LY (2012) A review: polyamines and photosynthesis. In: Najafpour MM (ed) Advances in Photosynthesis-Fundamental Aspects. InTech, Rijeka, chapter 21, pp 439-464

Siddiqui MH, Al-Whaibi MH, Ali HM, Sakran AM, Basalah MO, Al Khaishany MYY (2013) Mitigation of nickel stress by the exogenous application of salicylic acid and nitric oxide in wheat. Aust J Crop Sci 7:1780-1788

Siddiqui MH, Al-Whaibi MH, Basalah MO (2011). Role of nitric oxide in tolerance of plants to abiotic stress. Protoplasma 248: 447-455

Siddiqui MH, Mohammad F, Khan MMA, Al-Whaibi MH (2012) Cumulative effect of nitrogen and sulphur on *Brassica juncea* L. genotypes under NaCl stress. Protoplasma 249:139-153

Siddiqui MH, Mohammad F, Khan MN (2009). Morphologicalandphysio biochemical characterization of *Brassica juncea* L. Czern.&Coss. genotypes under salt stress. J Plant Interact 4:67-80

Siddiqui MH, Mohammad F, Khan MN, Al-Whaibi MH, Bahkali AHA (2010) Nitrogen in relation to photosynthetic capacity and accumulation of osmoprotectant and nutrients in brassica genotypes grown under salt stress. Agric Sci China 9:71-680

Siddiqui MH, Alamri SA, Al-Khaishany MYY, Al-Qutami MA, Ali HM, Khan MN (2017). Sodium nitroprusside and indole acetic acid improve the tolerance of tomato plants to heat stress by protecting against DNA damage. J Plant Interact 12:177-186

Silveira V, Santa-Catarina C, Tun NN, Scherer GFE, Handro W, Guerra MP, Floh EIS (2006) Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert.) O. Ktze. Plant Sci 171:91-98

Thomas T, Thomas TJ (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cell Mol Life Sci 58:244-258

Tun NN, Santa-Catarina C, Begum T, Silveira V, Handro W, Floh IS, Scherer FE (2006) Polyamines induce rapid biosynthesis of nitric oxide (NO) in Arabidopsis thaliana seedlings. Plant Cell Physiol 47:346-354

Verslues PE, Sharma S (2010). Proline metabolism and its implications for plant-environment interaction. Arabidopsis Book 8:e0140

Wang C, Zhang S, Wang P, Hou J, Qian J, Ao Y, Lu J, Li L (2011) Salicylic acid involved in the regulation of nutrient elements uptake and oxidative stress in *Vallisneria natans* (Lour.) *Hara* under Pb stress. Chemosphere 84:136-142

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218:1-14

Wang Y, Luo Z, Mao L, Ying T (2016) Contribution of polyamines metabolism and GABA shunt to chilling tolerance induced by nitric oxide in cold-stored banana fruit. Food Chem 197:333-339

Wen XP, Ban Y, Pang XM, Moriguchi T (2011) Identification of differentially-expressed genes potentially related to stress tolerance in a transgenic line of European pear over-expressing an apple spermidine synthase gene (*MdSPDS1*). J Hortic Sci Biotechnol 86: 146-152

Wu XX, Zhu XH, Chen JL, Yang SJ, Ding HD, Zha DS (2013). Nitric oxide alleviates adverse salt-induced effects by improving the photosynthetic performance and increasing the anti-oxidant capacity of eggplant (*Solanum melongena* L.). J Hortic Sci Biotechnol 88: 352-360

Zhao H, Yang H (2008) Exogenous polyamines alleviate the lipid peroxidation induced by cadmium chloride stress in *Malus hupehensis* Rehd. Sci Hortic 116:442-447