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Effects of foliar applications of nitric oxide and spermidine on chlorophyll fluorescence, photosynthesis and antioxidant enzyme activities of citrus seedlings under salinity stress

D. KHOSHBAKHT[*, +], M. R. ASGHARI[*], and M. HAGHIGHI[**]

Department of Horticultural Science, College of Agriculture, Urmia University, West Azarbaijan, Iran[*]
Department of Horticulture Science, College of Agriculture, Isfahan University of Technology, Isfahan, Iran[**]

Abstract

The effects of exogenous sodium nitroprusside (SNP), as nitric oxide donor, and spermidine (Spd) on growth and photosynthetic characteristics of Bakraii seedlings (*Citrus reticulata* × *Citrus limetta*) were studied under NaCl stress. In citrus plants, SNP- and Spd-induced growth improvement was found to be associated with reduced electrolyte leakage, malondialdehyde, hydrogen peroxide content, and leaf Na^+ and Cl^- concentration. However, we found increased leaf Ca^{2+}, Mg^{2+}, and K^+ concentrations, relative water content, chlorophyll fluorescence parameters, antioxidant enzyme activities, such as ascorbate peroxidase, catalase, superoxide dismutase and peroxidase, as well as higher photosynthetic rate, intercellular CO_2 concentration, stomatal conductance, and transpiration rate under saline regime. Foliar application of SNP and Spd alone mitigated the adverse effect of salinity, while the combined application proved to be even more effective.

Additional key words: abiotic stress; biomass; gas exchange; oxidative stress; photosystem II efficiency.

Introduction

Salinity is one of the major environmental stresses for plants that can severely limit crop production throughout the world (Sudhir and Murthy 2004, Ravindran *et al.* 2007, Munns and Tester 2008). Salinity stress has received increasing attention in recent years because it greatly reduces agricultural productivity (Parihar *et al.* 2015). Salinity causes various injuries in plants, such as tissue burning, yield reduction, and finally plant death (Romero-Aranda *et al.* 2001), causes leaf senescence, reduction PSII activities (Khayyat *et al.* 2016), enhances membrane permeability (Dhindsa *et al.* 1981), nutritional imbalances, and toxicity (Khoshbakht *et al.* 2014). NaCl inhibits photosynthetic rate (P_N) as a consequence of osmotic stress, which leads to a decrease in water potential and stomatal conductance (g_s), sugar accumulation, which causes feedback inhibition, and ion toxicity (*e.g.*, an excess of Na^+ and Cl^-) accompanied by a reduction of K^+ and Ca^{2+} (Khoshbakht and Asgharei 2015a). Moreover, salinity can cause oxidative stress through excessive production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) (Wu *et al.* 2011). The accelerated accumulation of ROS is able to induce oxidative damage to many cellular constituents, such as proteins, membrane lipids, and nucleic acids (Mittler 2002). One of the most destructive impacts of oxidative damage is peroxidation of membrane lipids, which leads to the accompanying generation of malondialdehyde (MDA) (Wu *et al.* 2011). Consequently, a high MDA content is a helpful biomarker of lipid peroxidation and is therefore often used to detect oxidative

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[+]Corresponding author; phone: +98 311 3913447, fax: +98 311 3913356, e-mail: davod.khoshbakht@gmail.com
Abbreviations: APX – ascorbate peroxidase; C_a – atmospheric CO_2 concentration; C_i – intercellular CO_2 concentration; CAT – catalase; Chl – chlorophyll; E – transpiration rate; EL – electrolyte leakage; F_0 – minimal fluorescence yield of the dark-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; F_v – variable fluorescence; F_v/F_m – maximum photochemical efficiency of PSII; g_s – stomatal conductance; LN – number of leaves per plant; MDA – malondialdehyde; NPQ – nonphotochemical quenching; PAs – polyamines; Put – putrescine; POD – peroxidase; P_N – net photosynthetic rate; q_P – photochemical quenching; ROS – reactive oxygen species; RWC – relative water content; SL – length of shoot; SNP – sodium nitroprusside; SOD – superoxide dismutase; S – salinity stress; Spd – spermidine; Spm – spermine; TPDM – total plant dry mass; TPFM – total plant fresh mass.
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stress situations induced by salinity (Almansa *et al.* 2002). Salt stress alters the critical balance between the production of ROS and the quenching activity of antioxidants, resulting in oxidative stress that causes damage to plants (Hernández *et al.* 1999). To control the content of ROS, plants possess a well-developed and intricate antioxidant defense system including enzymatic and nonenzymatic antioxidative processes (Blokhina *et al.* 2003). Citrus plants are known to be sensitive to salts because of the specific toxicity of Cl^- and/or Na^+ and to the osmotic effect caused by the high concentration of salts (Koshbakht *et al.* 2015b).

The polyamines (PAs) such as putrescine (Put), Spd and spermine (Spm) are low-molecular-mass polycations ubiquities in all living organisms (Kusano *et al.* 2008). Some reports have indicated the relationships between PAs and environmental stress (Galston *et al.* 1997, Bouchereau *et al.* 1999). Biosynthesis of PAs may be an integral part of plant's response to salinity stress (Alcázar *et al.* 2010). Increasing PAs biosynthesis might protect the plants from salinity by removing free radicals, maintaining membrane and cellular structures, keeping a cation–anion balance (Bouchereau *et al.* 1999), regulation of ion channels and induction of ATP synthesis (Lopatin *et al.* 1994).

Nitric oxide (NO) is a bioactive molecule that plays a critical role in many physiological processes including germination, growth, and flowering (Lamattina *et al.* 2003). It was reported that application of the SNP, as nitric oxide (NO) donor, significantly increased salt tolerance through stimulating the activities of H^+-ATPase and Na^+/H^+ antiporter in the tonoplast (Zhao *et al.* 2004). The protective function of NO includes the regulation of ROS and antioxidants, induction of gene expression, and absorption and distribution of elements (Arasimowicz and Floryszak-Wieczorek 2007). We investigated the effects of SNP, as NO donor, and Spd on alleviating the injury to citrus seedlings and its relevant mechanism under NaCl stress.

Materials and methods

Plant material, growth conditions, salinity, Spd and SNP treatments: The experiment was carried out under a greenhouse conditions. After germination, seedlings (six-month-old plants) of Iranian mandarin Bakraii (*Citrus reticulata* × *Citrus limetta*) were transplanted into 30 cm wide plastic pots containing fine sand, then placed in a greenhouse at 25–28/17°C (day/night) temperatures, 800 μmol(photon) m^{-2} s^{-1} maximum PAR, 60/70 ± 5% relative humidity, and a 12-h photoperiod. Plants were irrigated with 0.6 L of water and fertilized with a commercial water soluble fertilizer containing macro- and micronutrients (*Floral Mixed fertilizer*, *IFO*, Italy). After the acclimation period of at least six months, (twelve months after seed germination), seedlings were sprayed at two-week interval, (for the first time it was two weeks before the onset of salinity treatment), with water (control) or with 0.5 mM SNP or/and with 0.5 mM Spd until both sides of all their leaves were completely wet (run-off). The NaCl, SNP, and Spd treatments were as follows:

Treatment	NaCl [mM]	SNP [mM]	Spd [mM]
Control (C)	0	0	0
NaCl (S)	75	0	0
S + SNP	75	0.5	0
S + Spd	75	0	0.5
S + SNP + Spd	75	0.5	0.5

Two weeks after the foliar application of SNP or/and Spd, all seedlings were exposed to salt treatments. Salt treatments (0 and 75 mM of NaCl) were added to the pots using 0.5 L of irrigation water. To avoid osmotic shock, the NaCl concentration was increased gradually. 60 d after the salt treatment, various analyses were performed.

Growth characteristics: At the end of experiment, after measuring the shoot length (SL), plants were harvested and leaf number (LN) was counted. Then plants were washed with distilled water in order to remove adhering foreign particles. The total plant fresh mass (TPFM) were recorded, and the samples were dried separately at 80°C for 48 h; total plant dry mass (TPDM) were then recorded.

Leaf tissue mineral analysis: At the end of experiment, leaves of each plant were separated and washed with deionized water. Tissues were oven dried at 70°C for 3 d and dried parts were milled to a powder for mineral nutrient analysis. Ground samples were ashed in porcelain crucibles at 550°C for 6 h. The white ash was mixed with 2 ml of hot HCl, filtered into a 100-ml volumetric flask, and made up to 100 ml with distilled water. Concentrations of Na^+ and K^+ were determined by flame photometry (*Model PFP7*, *Jenway,* UK). Ca^{2+} and Mg^{2+} concentrations were measured by using an atomic absorption spectrophotometer (*Perkin Elmper Analyst Model 200,* USA). Cl^- was extracted from 500 mg DM of leaf tissue with 0.1 M HNO_3 in 10% (v/v) glacial acetic acid and samples were incubated overnight at room temperature and then filtered. Finally, Cl^- concentration was determined by silver ion titration (Moya *et al.* 1999).

Assays of the antioxidant enzyme activities: Frozen leaf segments (0.5 g) were homogenized in 10 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% soluble polyvinylpyrrolidone (PVP), with the addition of 1 mM ascorbate in the case of APX assay. The homogenate was centrifuged at 15,000 × *g* for 20 min at 4°C and the supernatant was immediately frozen under liquid N_2 and

stored at –70°C for the following assays.

Total protein content of leaf samples was determined using the method described by Bradford (1976). The tubes containing the extract and Bradford reagent were incubated at room temperature for 30 min, thoroughly mixed, and measured at 595 nm by spectrophotometer (*U-2000, Hitachi Instruments,* Tokyo, Japan) and compared to bovine serum albumin (BSA) as a standard.

Total superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich (1971). The 3 ml of reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA, and 100 µl of enzyme extract. The reaction mixtures were illuminated for 15 min at a light intensity of 15 W fluorescent lamps for the initiation of reaction. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT monitored at 560 nm using a spectrophotometer (*U-2000, Hitachi Instruments,* Tokyo, Japan). The amount of enzyme that inhibited 50% of NBT photo reduction was expressed as one unit of SOD activity. SOD activity was expressed as unit per mg of protein.

Catalase (CAT, EC 1.11.1.6) activity was determined spectrophotometrically (*U-2000, Hitachi Instruments,* Tokyo, Japan) by following the decrease of absorbance of H_2O_2 at 240 nm (extinction coefficient is 39.4 mM^{-1} cm^{-1}) as described by Aebi (1984) with slight modifications. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and 200 µl of enzyme extract. The amount of CAT required to decompose 1.0 µM of H_2O_2 per min was defined as one unit of CAT activity.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM AsA (vitamin C), 0.1 mM H_2O_2, and 200 µl of enzyme extract. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol(AsA) per mg protein and per min. The activity was expressed as unit per mg of protein.

Peroxidase (POD, EC 1.11.1.7) activity was measured using guaiacol as a substrate (Nickel and Cunningham 1969). The increase in absorbance at 470 nm (extinction coefficient 26.6 mM^{-1} cm^{-1}) due to the guaiacol oxidation was recorded for 3 min. The reaction mixture contained 25 mM phosphate buffer (pH 7.0), 0.05% guaiacol, 1.0 mM H_2O_2, and 0.1 ml of enzyme extract. One unit of POD activity indicates the amount of enzyme that catalyses the oxidation of 1.0 µM of guaiacol in 1 min.

Measurement of lipid peroxidation and H_2O_2 production: Lipid peroxidation was determined by measuring the amount of produced MDA by the thiobarbituric acid reaction as described by Jiang and Zhang (2001). The crude extract was mixed with the same volume of a 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) tricholoroacetic acid. The homogenate was heated at 95°C for 30 min and then immediately cooled. The mixture was centrifuged at 3,000 × *g* for 10 min and the absorbance of the supernatant was monitored at 532 and 600 nm (*U-2000, Hitachi Instruments,* Tokyo, Japan). After subtracting the nonspecific absorbance (600 nm), the MDA content was determined by its molar extinction coefficient (155 Mm^{-1} cm^{-1}) and expressed as nmol g^{-1}(FM).

H_2O_2 content was determined according to Velikova *et al.* (2000). Frozen leaf sample (1 g) was ground in an ice bath with 5 ml 0.1 % (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 × *g* for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7). The absorbance of the supernatant was measured at 390 nm using a spectrophotometer (*U-2000, Hitachi Instruments,* Tokyo, Japan). H_2O_2 content was determined using the extinction coefficient 0.28 μM^{-1} cm^{-1} and the amount expressed as µmol g^{-1}(FM).

Starch analysis: Fresh leaf sample (the youngest fully-expanded leaves) was homogenized in 80% ethanol. After extraction, the concentration of starch was measured by the method of Hedge and Hofreiter (1962). The absorbance was measured at 630 nm using a spectrophotometer (*U-2000, Hitachi Instruments,* Tokyo, Japan). Glucose was used as standard solution. Starch content was expressed per FM.

Electrolyte leakage (EL) was used to assess membrane permeability. EL was measured using an electrical conductivity meter (*CC-501; Elmetron*, Zabrze, Poland). Five leaf discs were taken from the youngest fully-expanded leaf on one randomly chosen plant per replicate sample (pot). After three washes with distilled water to remove surface contamination, five leaf discs were then placed in test tube containing 10 ml of distilled water. These samples were incubated for 24 h on a shaker at room temperature. The electrical conductivity (EC) of the solution (EC1) was read after incubation. The same samples were then placed in an autoclave at 120°C for 20 min and the second EC reading (EC2) was taken after cooling the solution to room temperature. EL was then calculated as EC1/EC2, and expressed as a percentage (Lutts *et al.* 1995).

Relative water content (RWC) of leaves was determined following the method suggested by Barrs and Weatherley (1962). Leaf discs weighed (FM) and washed three times with double distilled water were placed into a 10-ml conical flask. Leaf discs were immersed in 10 ml of distilled water for 4 h at 4°C in dark. Turgid mass (TM) of leaf discs was then measured and samples were dried in

hot air oven at 70°C until constant mass (DM) was achieved. RWC was estimated using the following equation: RWC (%) = [(FM − DM)/(TM − DM)] × 100.

Chlorophyll (Chl) was determined using fully expanded leaves. A fresh leaf sample of 0.5 g was ground and extracted with 5 ml of 80% (v/v) acetone in the dark. The slurry was filtered, centrifuged at 5,000 × *g* for 10 min and absorbance was determined at 645 and 663 nm, for Chl *a* and Chl *b* concentrations, respectively, using a spectrophotometer (*U-2000, Hitachi Instruments,* Tokyo, Japan). Concentrations of Chl *a*, Chl *b*, and Chl_{tot} were determined according to Lichtenthaler and Wellburn (1983).

Proline accumulation (Pro) was determined according to the method described by Bates *et al.* (1973). Seedlings (0.5 g of fresh shoot material) were homogenized in 10 ml of 3% (v/v) aqueous sulphosalicylic acid and filtered through a Whatman No. 2 filter paper. The filtrate (2 ml) was then mixed with 2 ml of acid-ninhydrin reagent and 2 ml of glacial acetic acid in a test tube and the mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 4 ml of toluene, and the chromophore-containing toluene fraction was aspirated, cooled to room temperature, and its absorbance measured at 520 nm using a spectrophotometer (*U-2000, Hitachi Instruments,* Tokyo, Japan). Appropriate Pro standards (*Sigma Chemical Co.,* St. Louis, MO, USA) were included in order to calculate the concentration of Pro in each shoot tissue sample.

Chl fluorescence was measured in the dark- and light-adapted leaves between 9:00–11:00 h, with a portable fluorometer (*PAM-2500*, *Walz*, Effeltrich, Germany). After 30 min of dark adaptation, F_v/F_m was calculated as $(F_m - F_0)/F_m$, where F_m [induced by a short pulse (0.6 s) of saturating light of 3,450 μmol(photon) m^{-2} s^{-1}] and F_0 were the maximal and minimal fluorescence, respectively (Genty *et al.* 1989). After 4 min of illumination with continuous red, nonsaturating actinic light [447 μmol(photon) m^{-2} s^{-1}] and saturating pulses every 25 s, the maximum (F_m') and the steady state (F_s) fluorescence signals were measured in the light-adapted leaves. Then, the actinic light was turned off and the far-red pulse was applied to obtain the minimal fluorescence after the PSI excitation (F_0'). Photochemical quenching coefficient (q_P) was calculated as $(F_m' - F_s)/(F_m' - F_0')$ (van Kooten and Snel 1990). Nonphotochemical quenching, NPQ, which is proportional to the rate constant of the thermal energy dissipation, was estimated as $(F_m - F_m')/F_m'$ (Bilger *et al.* 2001).

Gas-exchange parameters, such as net photosynthetic rate (P_N), intercellular CO_2 concentration (C_i), transpiration (E), and stomatal conductance (g_s), were measured between 9:00–11:00 h at the end of the experiment using a portable photosynthetic system (*LI-6200, LI-COR Inc.,* Lincoln, NE, USA). Top fully expanded leaf was clamped to the leaf chamber and the observations were recorded when RH and atmospheric CO_2 concentration (C_a) reached a stable value. PAR, air temperature, relative humidity, and CO_2 concentration inside the sensor head were set at 1,000 ± 100 μmol(photon) m^{-2} s^{-1}, 28 ± 2ºC, 60%, and 380–400 μmol m^{-1}, respectively, when measuring P_N.

Soluble sugar: The concentration of soluble sugars was measured by the anthrone method (Palma *et al.* 2009). Approximately 0.15 g of fresh assimilating leaf sample was sheared into fine pieces and incubated in 15 ml of distilled water in a water bath at 100°C for 1 h. This extraction solution (0.05 ml) was added to 0.15 ml of distilled water and mixed with 0.05 ml of anthrone ethyl acetate. To develop colour, 0.5 ml of concentrated sulphuric acid was added to the mixture, which was then immediately boiled for 1 min. The absorbance of the solution was measured at 630 nm (*U-2000, Hitachi Instruments,* Tokyo, Japan) and the concentration of soluble sugars determined using a standard curve prepared from a sucrose standard.

Statistical analysis: All data were presented as means ± SD of 4 replicate seedlings. Statistical analyses were performed by one-way analysis of variance (*ANOVA*) based on completely randomized design using the *SAS* program *version 9.1* (*SAS Inc.,* Cary, NC, USA). Differences between treatments were separated by the least significant differences (LSD) test at $P<0.05$.

Results and discussion

Growth parameters: The plants grown in the soil supplemented with 75 mM of NaCl showed a significant decrease in the values of all the growth biomarkers (LN, SL, TPFM, TPDM) in comparison with control plants. The spray of Spd or SNP alone or SNP + Spd to the foliage of stress-free plants significantly increased all the growth markers. Plant LN of with Spd or SNP alone or SNP + Spd treatments increased compared with NaCl alone (S) treatment. SL of S-treated Bakraii seedlings decreased with increasing salt concentration but different spray with Spd or SNP alone or Spd + SNP improved the growth of the plants. The maximum increase of SL was recorded for the plants treated by Spd + SNP, while the minimum SL was recorded for the seedlings at S (75 mM NaCl) treatments. There was a significant decrease of TPFM and TPDM in plants treated with 75 mM NaCl. When plants treated with Spd or SNP alone or Spd + SNP under S stress, the TPFM and TPDM increased compared with S treatment (Table 1).

Growth inhibition by salinity has been attributed to the disturbance in water and osmotic potential, toxicity of excessive Na^+ and Cl^-, disturbance in the accumulation of

nutrients, disruption in the structure and the activity of the enzymes, damage in cell organelles and plasma membrane, disturbances in photosynthesis, respiration, and protein synthesis (Feng *et al*. 2002, Munns 2005). It is interesting to observe that co-application of SNP and Spd proved to be more effective in the improvement of growth parameters under salt stress; perhaps, it could be due to their synergistic or additive effects. It has been suggested that the main role of PAs is to maintain a cation–anion balance during long-term salinity (Santa-Cruz *et al.* 1997). Hence, the use of exogenous PAs supported the physiological activity by increasing growth (Parvin *et al*. 2014). NO acted on the phospholipid bilayer, improved the fluidness of the membrane, cell enlargement, and plant growth (Leshem and Wills 1998). Foliar applied NO acts as a growth regulator and is able to modulate the plant metabolism and the production of metabolites involved in stress tolerance (Takahama and Oniki 1997). In this regard, Huaifu *et al.* (2007) suggested that SNP-mediated increase in plant growth could be related to SNP-induced enhancement in the P_N under salt stress. Exogenous Put, Spd and Spm pre-treatment to *Arabidopsis* seedlings incresed NO concentration (Wimalasekera *et al.* 2011), which reduces the harmfulness of the reactive oxygen species (ROS). These results are in agreement with present findings that combination of SNP (as a NO donor) and Spd have beneficial effects on growth.

Physiological and biochemical parameters: Leaf Na^+ and Cl^- concentrations were significantly elevated with S treatment. The foliar application of Spd or SNP alone or Spd + SNP resulted in the significant reduction of the Na^+ and Cl^- contents in the leaves. Salinity reduced K^+, Ca^{2+}, and Mg^{2+} concentrations in leaves. The lowest K^+, Ca^{2+}, and Mg^{2+} contents were related to non-Spd or SNP treate Bakraii seedlings under S treatment. Leaf Ca^{2+}, Mg^{2+}, and K^+ significantly increased by the exogenous application of Spd or SNP alone or Spd + SNP (Table 2). The ratio of Na^+/K^+ has been proposed as an indicator of salinity tolerance. This index increased considerably after the S treatment with respect to control. Spd or SNP alone or Spd + SNP applied together with 75 mM NaCl decreased this ratio above the value obtained in the plants treated exclusively with S treatment (Table 2).

The presence of NaCl in the water used for irrigation changes the nutritional balance of plants, leading to high ratios of Na^+/Ca^{+2}, Na^+/Mg^{+2}, and Na^+/K^+ that can cause an inhibition of the growth in citrus. The main saline ions Na^+ and Cl^- can influence the nutrient absorption through competitive interactions or by influencing the ion selectivity of membranes (Behboudian *et al.* 1986). This result was in agreement with previous studies that exogenous Spd could alleviate salt-induced ion toxicity and nutrition imbalance by reducing the Na^+ influx and K^+, Ca^{2+}, and Mg^{2+} efflux (Roy *et al.* 2005). Due to their polycationic nature, PAs, especially Spd, contribute to maintain cellular cation–anion balance, and may act as a signaling regulator responsible for inward rectification of rectifying K^+ channels and certain Ca^{2+}-permeable channels (Yamaguchi *et al*. 2007). Consequently, exogenous Spd pre-treatment might promote salinity tolerance by regulating the ion metabolism in Bakraii. Increased K^+ and Ca^{2+} contents due to application of SNP may be explained based on the property of K^+ and Ca^{2+} to replace Na^+, as a result of mutual competition between the two ions for a transport site on a carrier protein, and maintain a high capacity of restricting salt uptake and transport under salt stress, and inhibition of membrane-associated carrier protein thereby maintaining its integrity (Khan *et al.* 2010, López-Carrión *et al* 2008).

Table 1. Growth characteristics and biomass of Iranian mandarin Bakraii seedlings treated with Spd, SNP, and Spd + SNP under saline conditions. Each value was mean ± SD ($n = 4$). Mean values followed by *the same letters* within a column are not significantly different at $p<0.05$. C – control; LN – leaf number; S – 75 mM NaCl; SL – shoot length; SNP – sodium nitroprusside; Spd – spermidine; TPDM – total plant dry mass; TPFDM – total plant fresh mass; CV – coefficient of variation.

Treatment	LN	TPDM [g]	TPFM [g]	SL [cm]
C	38.0 ± 2.94^{c}	13.8 ± 0.29^{c}	46.8 ± 1.82^{c}	34.7 ± 0.85^{c}
Spd	44.0 ± 3.65^{ab}	15.8 ± 0.36^{b}	48.9 ± 0.72^{ab}	38.2 ± 0.82^{ab}
SNP	41.0 ± 1.84^{ab}	15.0 ± 0.52^{b}	47.2 ± 0.85^{bc}	37.9 ± 1.64^{b}
Spd + SNP	46.0 ± 3.65^{a}	16.6 ± 0.73^{a}	49.8 ± 0.83^{a}	40.4 ± 2.58^{a}
S	15.0 ± 2.94^{f}	05.4 ± 0.88^{g}	21.2 ± 1.82^{f}	19.0 ± 1.68^{f}
S + Spd	23.7 ± 1.72^{e}	09.8 ± 0.67^{e}	35.3 ± 0.85^{e}	25.0 ± 0.36^{e}
S + SNP	25.0 ± 2.58^{de}	08.9 ± 0.34^{f}	33.9 ± 0.92^{e}	25.8 ± 0.67^{e}
S + Spd + SNP	28.0 ± 1.82^{d}	11.9 ± 0.26^{d}	39.1 ± 1.73^{d}	28.3 ± 2.75^{d}
ANOVA				
Treatment	***	**	**	***
CV [%]	8.4	4.5	3.2	5.2

Table 2. Leaf Cl^-, Na^+, K^+, Ca^{2+}, Mg^{2+}, and Na^+/K^+ratio in Iranian mandarin Bakraii seedlings treated with Spd, SNP, and Spd + SNP under saline conditions. Each value was mean ± SD (n = 4). Mean values followed by *the same letters* within a column are not significantly different at p<0.05. C – control; S – 75 mM NaCl; SNP – sodium nitroprusside; Spd – spermidine; CV – coefficient of variation.

Treatment	Cl^- [%]	Na^+ [%]	K^+ [%]	Ca^{2+} [%]	Mg^{2+} [%]	Na^+/K^+ ratio
C	0.76 ± 1.24^{d}	0.18 ± 0.05^{d}	2.0 ± 0.16^{b}	2.20 ± 0.03^{bc}	1.7 ± 0.51^{b}	0.09 ± 0.01^{d}
Spd	0.54 ± 1.28^{d}	0.13 ± 0.04^{d}	2.5 ± 0.08^{ab}	2.60 ± 0.02^{ab}	1.8 ± 0.26^{ab}	0.05 ± 0.03^{d}
SNP	0.62 ± 1.22^{d}	0.15 ± 0.03^{d}	2.3 ± 0.06^{ab}	2.40 ± 0.04^{ab}	1.8 ± 0.36^{ab}	0.07 ± 0.02^{d}
Spd + SNP	0.43 ± 1.24^{d}	0.11 ± 0.05^{d}	2.6 ± 0.09^{a}	2.90 ± 0.03^{a}	2.2 ± 0.25^{a}	0.04 ± 0.04^{d}
S	4.30 ± 1.24^{a}	2.40 ± 0.47^{a}	1.2 ± 0.16^{c}	1.20 ± 0.36^{d}	0.7 ± 0.29^{c}	2.00 ± 0.36^{a}
S + Spd	2.80 ± 1.24^{b}	1.30 ± 0. 29^{b}	2.6 ± 0.16^{ab}	1.80 ± 0.47^{c}	1.4 ± 0.36^{b}	0.52 ± 0.21^{bc}
S + SNP	3.20 ± 1.28^{b}	1.35 ± 0.51^{b}	2.3 ± 0.08^{ab}	1.82 ± 0.51^{c}	1.5 ± 0.55^{b}	0.64 ± 0.12^{b}
S + Spd + SNP	2.20 ± 1.22^{c}	0.92 ± 0.43^{c}	2.8 ± 0.06^{a}	2.10 ± 0.44^{bc}	1.5 ± 0.18^{b}	0.34 ± 0.29^{c}
ANOVA						
Treatment	**	**	*	**	**	**
CV [%]	16.8	18.2	18.9	17.9	21.5	22.4

Salt stress significantly increased EL and Pro with maximum increase observed in the plants grown with 75 mM NaCl. The application of Spd or SNP alone or Spd + SNP significantly decreased EL and Pro in leaf discs, with the largest decrease in EL and Pro measured when 0.50 mM Spd + SNP were applied. RWC was reduced by S treatment, but foliar application of SNP alone or Spd + SNP improved this factor (Table 3).

In the present study, membrane permeability was determined by measuring EL. An increase in EL indicates elevated leakiness of ions due to a loss of membrane integrity. This is an inherent feature of plants which are exposed to stresses such as salinity (Sharma *et al.* 2011). This study showed that foliar Spd or SNP alone or Spd + SNP application reduced the ion leakage (measured as electrolytes) in S-stressed Bakraii plants indicating that Spd and SNP treatment facilitated the maintenance of membrane functions under stress conditions. According to the results, it was found that the combined use of Spd + SNP is more effective than their individual use for reducing the effects of salt stress. It is clear that both Spd and NO crosstalk to stimulate the stress-inducing factors in the plant under stress conditions. Maintaining integrity of cellular membranes under salt stress is considered an integral part of salinity-tolerance mechanism (Stevens *et al.* 2006). This is probably due to the fact that PAs causes increases in the activities of antioxidant enzymes which, in turn, protect plants against the generation of ROS and membrane injury, or may result in the synthesis of other substances which have a protective effect on plants growing under salt stress (Upchurch 2008). In addition to their properties as free radical scavengers, PAs were also reported to stabilize biological membrane by direct binding to membrane phospholipids in stress conditions (Todorova *et al.* 2007). In the present study, lower EL with SNP may originate from salinity induced constraints on water availability and ion uptake, thus resulting in the reduction of root pressure-driven xylem transport rates of water and mineral nutrients to the shoot. Reports suggested a reduction in electrolyte leakage may be explained on the basis of SNP-facilitated maintenance of membrane functions through induction of antioxidant mechanisms and elevated ion uptake, thereby protecting the plants against the oxidative damage (Liu *et al*. 2014). Huaifu *et al.* (2007) reported that exogenous NO reduced the membrane permeability and membrane lipid peroxidation, and prevented the electrolyte leakage.

Table 3. Leaf characteristics of Iranian mandarin Bakraii seedlings treated with Spd, SNP, and Spd + SNP under saline conditions. Each value was mean ± SD (n = 4). Mean values followed by *the same letters* within a column are not significantly different at p<0.05. C – control; EL – electrolyte leakage; Pro – proline content; RWC – relative water content; S – 75 mM NaCl; SNP – sodium nitroprusside; Spd – spermidine; CV – coefficient of variation.

Treatment	EL [%]	RWC [%]	Pro [μmol g^{-1}(FM)]
C	12.8 ± 1.84^{d}	86.2 ± 1.03^{a}	22.0 ± 2.54^{d}
Spd	10.3 ± 1.33^{de}	88.3 ± 0.82^{a}	19.8 ± 2.98^{d}
SNP	11.3 ± 1.63^{de}	87.4 ± 1.62^{a}	20.2 ± 4.12^{d}
Spd + SNP	09.4 ± 1.31^{e}	89.2 ± 0.88^{a}	18.8 ± 1.83^{d}
S	41.4 ± 1.23^{a}	42.3 ± 3.77^{d}	72.0 ± 3.69^{a}
S + Spd	27.4 ± 1.84^{bc}	59.2 ± 1.63^{c}	40.2 ± 4.94^{bc}
S + SNP	29.9 ± 1.33^{b}	57.2 ± 1.82^{c}	45.2 ± 2.58^{b}
S + Spd + SNP	25.2 ± 1.29^{c}	65.4 ± 1.62^{b}	34.8 ± 4.16^{c}
ANOVA			
Treatment	***	**	*
CV [%]	9.1	10.8	18.3

The RWC is a useful measure of the physiological water status of plants. RWC in leaves is known as an alternative measure of plant water status, reflecting the metabolic activity in tissues (González and González-Vilar 2001). RWC reduction indicated a loss of turgor that resulted in limited water availability for the cell extension

process (Katerji *et al.* 1997) that may be caused by lower water availability under stress conditions (Shalhevet 1993), and/or root systems which are not able to compensate for water lost by transpiration through a reduction of the absorbing surface (Gadallah 2000). The protective effect of PAs on RWC under NaCl treatments has been reported earlier (Sheokand *et al.* 2008). García-Mata and Lamattina (2001) reported that SNP-treated water-stressed wheat seedlings tended to retain more water than that of water-stressed seedlings.

Results showed that under salinity stress, the Pro concentration of Bakraii leaves increased. Pro has multiple functions, such as osmotic pressure regulation, protection of membrane integrity, stabilization of enzymes/proteins, maintaining appropriate $NADP^+$/NADPH ratios and scavenger of free radicals (Hare and Cress 1997). In this study, Spd or SNP alone or Spd + SNP application reduced salt-induced Pro accumulation which suggested that salt-imposed stress might be partially alleviated without requiring the accumulation of Pro to high concentrations. This appears to be supported by Jiménez-Bremont *et al.* (2006) who reported that exogenous PAs decreased Pro accumulation in salinity-exposed *Phaseolus vulgaris* L. The Pro degradation seems to be beneficial in the response to stress, given that the degradation of Pro to glutamate generates reducing equivalents that support mitochondrial oxidative phosphorylation (Hare and Cress 1997). Furthermore, the proline dehydrogenase activity is capable of consuming $O_2^{\bullet-}$ (Hare and Cress 1997), and perhaps could reduce the oxidizing power of the cell and in turn possibly generate ROS (Rosales *et al.* 2007, López-Carrión *et al.* 2008). On the other hand, SNP appears to be capable of mitigating damage associated with salinity stress by reducing oxidative stress and inducing Pro degradation, mechanisms that permit the plant to adapt under these conditions (López-Carrión *et al*. 2008).

The protein, soluble sugar and starch content were significantly influenced by the salt stress and Spd or SNP alone or Spd + SNP application improved these parameters (Table 4).

In general, under salt stress, starch content decreases (Chaves *et al.* 2009), which is in accordance with the present study. Photosynthesis is one of the phenomena inhibited under the stress resulting in a reduction of starch and soluble carbohydrates within the leaves (Demetriou *et al.* 2007, Ruiz *et al.* 1997). Another implication of the starch and sucrose depletion promoted by the different levels of salinity is that leaves could change from source to sink under these adverse conditions (Arbona *et al.* 2005). In plants, PAs seem to play an important role *via* interaction with many anionic molecules, such as DNA, RNA, proteins, and membrane lipids, due to the polycationic nature of PAs (Bouchereau *et al.* 1999). Liu *et al* (2014) reported that foliage application of SNP accelerated accumulation of proteins and soluble sugars under salt stress. Moreover, free radicals such as H_2O_2 produced under salt stress conjugate to proteins and lead to the destruction of their structure. Therefore, the impact of Spd + SNP might be due to either preventing the production of free radicals or free radicals scavenging mechanisms and subsequently protecting the proteins (Peltzer *et al.* 2002, Sheokand *et al.* 2008).

The activity of SOD, CAT, POD, and APX increased with salinity and application of Spd or SNP alone or Spd + SNP had an additive effect on the activities of these enzymes. In the present study, we observed higher activities of SOD, CAT, POD, and APX content in the plants exposed to NaCl stress with Spd + SNP treatments (Table 5). Lipid peroxidation is one of the first indications of oxidative damage, and production of MDA can be used as an indicator of oxidative stress. Exposure to NaCl stress increased MDA compared with control leaves. However, Spd or SNP alone or Spd + SNP significantly reduced the contents of MDA under NaCl stress. H_2O_2 content also illustrated the same tendency (Table 6).

Salt stress, like other abiotic stresses, can lead to oxidative stress through the increase in ROS, which could potentiate the accumulation of MDA, an indicator of salt-induced oxidative damage to membranes (Hernández and Almansa 2002). CAT is an important antioxidant enzyme that catabolizes hydrogen peroxide (Larsen *et al.* 1988). H_2O_2 is one of the major and most stable ROS and its high concentration leads to oxidative stress through an increase of lipid peroxidation and modification of membrane permeability (Upchurch 2008) and Spd or SNP alone or SNP + Spd alleviate oxidative injury in plants by acting as direct free radical scavengers (Beligni *et al.* 2002, Wu *et al.* 2011). Salinity has been reported to increase SOD activity in cucumber plants (Huaifu *et al.* 2007) and in citrus (Almansa *et al.* 2002). Salt stress accelerated the $O_2^{\bullet-}$ production and induced a great increase in the SOD activity. Spd or SNP alone or Spd + SNP under NaCl stress improved the SOD activity in leaves, which demonstrated that Spd or SNP alleviated the injury to citrus seedlings under NaCl stress. This finding confirms the report of Parida and Das (2005) in regard to the mutual relationship between higher antioxidant activity and salinity tolerance. Our results showed the additive effects of Spd + SNP for reduction of ROS and induction of synthesis of antioxidant enzymes under salinity stress. Wimalasekera *et al.* (2011) observed that the PAs, Spd and Spm, greatly increased NO release in *Arabidopsis thaliana* seedlings. It is well documented that PAs counteract oxidative damage in plants by acting as direct free radical scavengers or binding to antioxidant enzyme molecules to scavenge free radical (Bors *et al.* 1989). Previous finding proposed that transgenic tobacco may significantly induce the expression of antioxidant enzymes by elevating the PAs content (Wi *et al.* 2006), resulting in tolerance to different abiotic stresses. It is evident that PAs exhibit their antioxidant

Table 4. Chl *a*, Chl *b*, Chl_{tot}, Chl *a*/*b*, protein, starch, and soluble sugar (SSU) in leaves of Iranian mandarin Bakraii seedlings treated with Spd, SNP, and Spd + SNP under saline conditions. Each value was mean ± SD (n = 4). Mean values followed by *the same letters* within a column are not significantly different at p<0.05. C – control; Chl_{tot} – total chlorophyll concentration; S – 75 mM NaCl; SSU – soluble sugar; SNP – sodium nitroprusside; Spd – spermidine; CV – coefficient of variation.

Treatments	Chl *a* [mg g^{-1}(FM)]	Chl *b* [mg g^{-1}(FM)]	Chl_{tot} [mg g^{-1}(FM)]	Chl *a*/*b* [mg g^{-1}(FM)]	Protein [mg g^{-1} (FM)]	Starch [mg g^{-1} (FM)]	SSU [mg g^{-1} (FM)]
C	0.64 ± 0.07^{b}	0.30 ± 0.08^{a}	0.94 ± 0.04^{b}	2.14 ± 0.12^{ab}	2.34 ± 0.47^{ab}	75.6 ± 4.6^{b}	26.0 ± 3.65^{bc}
Spd	0.64 ± 0.01^{b}	0.31 ± 0.06^{a}	0.95 ± 0.02^{b}	2.06 ± 0.05^{b}	2.57 ± 0.42^{a}	78.2 ± 5.9^{ab}	27.8 ± 1.82^{b}
SNP	0.67 ± 0.02^{b}	0.31 ± 0.04^{a}	0.98 ± 0.03^{ab}	2.17 ± 0.09^{ab}	2.43 ± 0.33^{ab}	77.6 ± 6.2^{b}	26.7 ± 2.58^{bc}
Spd + SNP	0.76 ± 0.04^{a}	0.33 ± 0.05^{a}	1.09 ± 0.03^{a}	2.30 ± 0.08^{a}	2.66 ± 0.17^{a}	$86.2 \pm 5.\ 7^{a}$	34.7 ± 3.37^{a}
S	0.22 ± 0.01^{d}	0.18 ± 0.02^{c}	0.40 ± 0.05^{d}	1.22 ± 0.03^{d}	1.05 ± 0.39^{d}	22.7 ± 3.6^{e}	14.2 ± 3.08^{e}
S + Spd	0.37 ± 0.06^{c}	0.23 ± 0.04^{b}	0.60 ± 0.02^{c}	1.62 ± 0.04^{c}	1.61 ± 0.36^{c}	48.3 ± 1.8^{cd}	19.1 ± 2.58^{d}
S + SNP	0.37 ± 0.06^{c}	0.24 ± 0.03^{b}	0.61 ± 0.04^{c}	1.55 ± 0.05^{c}	1.62 ± 0.46^{c}	42.3 ± 9.6^{d}	19.9 ± 4.16^{d}
S + Spd + SNP	0.42 ± 0.02^{c}	0.24 ± 0.02^{b}	0.66 ± 0.03^{c}	1.76 ± 0.07^{c}	1.89 ± 0.18^{bc}	54.3 ± 3.4^{c}	22.3 ± 3.65^{cd}
ANOVA							
Treatment	**	**	**	**	***	***	*
CV [%]	9.9	12.2	10.4	7.9	18.3	9.4	13.4

Table 5. Antioxidant enzyme activities in leaves of Iranian mandarin Bakraii seedlings treated with Spd, SNP, and Spd + SNP under saline conditions. Each value was mean ± SD (n = 5). Mean values followed by *the same letters* within a column are not significantly different at p<0.05. APX – ascorbate peroxidase; C – control; CAT – catalase; POD – peroxidase; S – 75 mM NaCl; SNP – sodium nitroprusside; SOD – superoxide dismutase; Spd – spermidine; CV – coefficient of variation.

Treatments	CAT [unit mg^{-1} protein]	SOD [unit mg^{-1} protein]	APX [unit mg^{-1} protein]	POD [unit mg^{-1} protein]
C	0.08 ± 0.01^{e}	12 ± 2.64^{f}	1.10 ± 3.36^{f}	0.20 ± 0.08^{d}
Spd	0.16 ± 0.09^{de}	18 ± 5.94^{de}	1.32 ± 1.82^{ef}	0.30 ± 0.18^{d}
SNP	0.14 ± 0.04^{de}	15 ± 4.39^{ef}	1.36 ± 2.58^{def}	0.28 ± 0.02^{d}
Spd + SNP	0.20 ± 0.03^{d}	21 ± 3.36^{d}	1.53 ± 4.39^{cde}	0.40 ± 0.20^{cd}
S	0.44 ± 0.03^{c}	34 ± 3.65^{c}	1.74 ± 1.82^{bcd}	0.64 ± 0.16^{bc}
S + Spd	0.62 ± 0.08^{b}	44 ± 3.36^{ab}	1.96 ± 2.82^{ab}	0.83 ± 0.25^{ab}
S + SNP	0.57 ± 0.06^{b}	41 ± 2.58^{b}	1.88 ± 1.63^{abc}	0.82 ± 0.23^{ab}
S + Spd + SNP	0.82 ± 0.08^{a}	48 ± 1.82^{a}	2.15 ± 2.45^{a}	0.94 ± 0.18^{a}
ANOVA				
Treatment	**	**	**	*
CV [%]	16.5	12.6	17.3	15.6

effect by inducing the expression of genes encoding antioxidant enzymes. Therefore, PAs may function not only as scavengers of ROS, but also as activators of the expressions of genes encoding antioxidant enzymes (Parvin *et al.* 2014). NO disproportioned $O_2^{\bullet -}$ to H_2O_2, so the content of H_2O_2 increased with the improved SOD activity. Under NaCl stress, H_2O_2 was eliminated by some enzymes, such as POD, CAT, APX, and their activity increased. Exogenous SNP could markedly improve the SOD, POD, CAT, APX activities, thereby improving the ability of scavenging free radicals and alleviating the injury, and decreasing membrane permeability and MDA content (Beligni *et al*. 2002, Verma and Mishra 2005). Because of the existence of an unpaired electron within the molecule, NO can react directly with some ROS, such as H_2O_2, superoxide anion ($O_2^{\bullet -}$), and the hydroxyl radical ($^{\bullet}OH$). Reaction of NO with $O_2^{\bullet -}$ produces peroxynitrite ($ONOO^{\bullet}$), which is a toxic product. However, $ONOO^{\bullet}$ can be protonated and decomposed to a nitrate anion and a proton or it can react with hydrogen peroxide to yield a nitrite anion and oxygen (Laspina 2005).

Photosynthetic parameters: P_N, E, g_s, and C_i were significantly reduced by the treatment of NaCl in comparison with the control plants. The application of Spd or SNP alone or SNP + Spd to the plants significantly increased the value of P_N, E, g_s, and C_i compared with the NaCl-treated plants (Table 7). Chl_{tot} was significantly affected by salinity and Spd or SNP alone or SNP + Spd pretreatment. There was a significant decrease in Chl *a* and Chl *b* content after the treatment with 75 mM NaCl. When treated with Spd or SNP alone or SNP + Spd in the presence of NaCl, the Chl_{tot} content increased compared with the sole NaCl treatment (Table 4). The fluorescence

Table 6. MDA and H_2O_2 in Iranian mandarin Bakraii seedlings treated with Spd, SNP, and Spd + SNP under saline conditions. Each value was mean ± SD (n = 4). Mean values followed by *the same letters* within a column are not significantly different at p<0.05. C – control; H_2O_2 – hydrogen peroxide; MDA – malondialdehyde; S – 75 mM NaCl; SNP – sodium nitroprusside; Spd – spermidine; CV – coefficient of variation.

Treatment	MDA [nmol g^{-1}(FM)]	H_2O_2 [μmol g^{-1}(FM)]
C	15.5 ± 4.14^{d}	20.6 ± 2.05^{d}
Spd	11.2 ± 2.62^{de}	21.0 ± 1.82^{d}
SNP	12.3 ± 1.26^{de}	20.0 ± 3.65^{d}
Spd + SNP	11.0 ± 3.26^{e}	20.0 ± 4.08^{d}
S	42.8 ± 2.45^{a}	38.5 ± 3.03^{a}
S + Spd	28.4 ± 3.44^{bc}	27.0 ± 2.58^{bc}
S + SNP	31.3 ± 1.62^{b}	30.7 ± 2.98^{b}
S + Spd + SNP	25.5 ± 1.26^{c}	26.0 ± 3.65^{c}
ANOVA		
Treatment	**	**
CV [%]	13.4	12.1

parameters were used as a nondestructive method to determine the functional state of the photosynthetic machinery. The fluorescence parameters were significantly affected by salt stress and Spd or SNP alone or Spd + SNP concentration (Table 8). Salinity stress decreased F_v/F_m and F_v in the leaves of Bakraii seedlings. These results indicate harmful effects of NaCl on metabolic processes ultimately resulting in reduced efficiency of PSII. The F_v/F_m in the dark-adapted state significantly decreased due to the salt-induced increase of F_0 and the marked decline of F_m. Salt stress significantly decreased q_P in the light-adapted state but markedly elevated NPQ. The pretreatment by Spd or SNP alone or Spd + SNP to the NaCl-treated plants completely recovered the loss of fluorescence parameters (Table 8).

A decrease in leaf Chl content has been described in citrus rootstocks irrigated with high NaCl concentration (García-Sánchez *et al.* 2002, Koshbakht and Asgharei 2015a). This loss of Chl content could be associated with accumulation of Cl^- and Na^+ in the leaves (Anjum 2007). The decrease in leaf Chl concentration could occur due to changes in the lipid protein ratio of pigment–protein complexes (Rao and Rao 1981), increased chlorophyllase activity and degradation (Singh and Dubey 1995), and an inhibition in the synthesis of photosynthetic pigments (García-Sánchez *et al.* 2002). Photosynthetic pigments present in the photosystems are believed to be damaged by stress factors resulting in a reduced light-absorbing efficiency of both photosystems (PSI and PSII) and hence a reduced photosynthetic capacity (Zhang *et al*. 2011). This is attributed to the rate of CO_2 assimilation which is generally reduced in response to salinity and this reduction is partially due to reduced g_s and due to the direct effects of NaCl on the photosynthetic apparatus independently of stomatal closure, which has also been reported for several plant species, both halophytes and nonhalophytes (Torrecillas *et al*. 1995). In the present study, salinity-induced reductions in Chl, P_N, g_s, and C_i were alleviated by exogenous PAs and SNP, in accordance with a similar experiment, implying that exogenous PAs (Anjum 2009) and SNP (Huaifa *et al.* 2007) alleviates the damage of photosynthetic apparatus under salinity conditions. The combination of Spd + SNP was better than Spd or SNP alone. The F_v/F_m is correlated with the efficiency of leaf photosynthesis. A decline in this ratio provides an indicator of photoinhibitory damage caused by the incident photon flux density when plants are subjected to a wide range of environmental stresses (Björkman and Demming 1987). The salt-induced increase of F_0 and the marked decline of F_m imply the inhibitory effects on PSII reaction center although the elevated NPQ may dissipate excess excitation energy (Wang *et al.* 2007). The reduction of F_v along with an increase in F_0 is considered to be characteristic for inhibition of the acceptor side of PSII (Setlík *et al.* 1990). The reduction in F_v/F_m and q_p were correlated with an increase in NPQ suggesting that salt treatment induced dissipation of damaging excessive energy. This energy could be dissipated at antennae through heat emission although other mechanisms are also involved in energy dissipation, *e.g.*, related to a transmembrane H^+ gradient generated by ATPase activity and uncoupling of electron transport, which may lead to oxidative stress (Maxwell and Johnson 2000). Earlier studies have indicated that salt stress reduced P_N and g_s in the leaves of many crops (Syeed *et al.* 2011). A decline in photosynthetic capacity under salt stress is often associated with the generation of ROS (Noreen *et al.* 2010). Salt stress hinders P_N at multiple levels, such as pigments, stomatal functioning, gaseous exchange, structure and function of thylakoid membrane, electron transport, and enzyme activities, by preventing the oxidative stress reduction mechanisms and cellular metabolism of plants (Sudhir and Murthy 2004). Excessive salt concentrations might cause the closure of stomata, thereby decreasing the partial CO_2 pressure and C_i and consequently resulting in a decreased P_N (Bethke and Drew 1992). It is well documented that PAs are involved in protection against different environmental stresses in plants (Bouchereau *et al*. 1999). There have been several reports establishing that PAs, especially the thylakoid-bound PAs, participate in the regulation of structure and function of photosynthetic apparatus under environmental conditions, such as UV-B radiation (Lütz *et al.* 2005), low temperature (Sfakianaki *et al.* 2006), and salinity (Demetriou *et al.* 2007). PAs exert positive effects on photosynthetic efficiency under stress conditions due to their acid-neutralizing and antioxidant properties, as well as their membrane- and cell wall-stabilizing activity (Mapelli *et al.* 2008). PAs with a high net positive charge can stabilize PSII proteins such as D1 and D2 under photoinhibition conditions. PAs binding to membrane

Table 7. Gas-exchange characteristics in leaves of Iranian mandarin Bakraii seedlings treated with Spd, SNP, and Spd + SNP under saline conditions. Each value was mean ± SD (n = 4). Mean values followed by *the same letters* within a column are not significantly different at p<0.05. C – control; C_i – intercellular CO_2 concentration; E – transpiration rate; g_s – stomatal conductance; P_N – net photosynthetic rate; S – 75 mM NaCl; SNP – sodium nitroprusside; Spd – spermidine; CV – coefficient of variation.

Treatment	P_N [μmol(CO_2) m^{-2} s^{-1}]	g_s [mmol(H_2O) m^{-2} s^{-1}]	E [mmol(H_2O) m^{-2} s^{-1}]	C_i [μmol mol^{-1}]
C	7.9 ± 1.82^{b}	0.36 ± 0.08^{b}	2.4 ± 0.36^{cb}	350 ± 18.2^{c}
Spd	8.8 ± 1.80^{ab}	0.42 ± 0.03^{ab}	2.9 ± 0.18^{ab}	450 ± 09.1^{a}
SNP	8.4 ± 1.61^{ab}	0.39 ± 0.02^{b}	2.8 ± 0.29^{b}	410 ± 09.6^{b}
Spd + SNP	9.8 ± 0.82^{a}	0.47 ± 0.07^{a}	3.4 ± 0.46^{a}	470 ± 29.4^{a}
S	2.1 ± 0.96^{e}	0.12 ± 0.01^{e}	0.9 ± 0.18^{e}	146 ± 06.4^{f}
S + Spd	4.0 ± 0.56^{d}	0.23 ± 0.04^{cd}	1.7 ± 0.33^{d}	265 ± 09. 4^{ed}
S + SNP	3.9 ± 0.86^{d}	0.20 ± 0.02^{d}	1.5 ± 0.56^{d}	250 ± 14.8^{e}
S + Spd + SNP	5.8 ± 0.46^{c}	0.28 ± 0.03^{c}	1.9 ± 0.19^{cd}	274 ± 05.9^{d}
ANOVA				
Treatment	***	**	**	*
CV [%]	19.7	15.1	15.8	8.5

Table 8. Fluorescence parameters in leaves of Iranian mandarin Bakraii seedlings treated with Spd, SNP, and Spd + SNP under saline conditions. Each value was mean ± SD (n = 4). Mean values followed by *the same letters* within a column are not significantly different at p<0.05. C – control; F_m – maximal fluorescence yield of the dark-adapted state; F_0 – minimal fluorescence yield of the dark-adapted state; F_v – variable fluorescence; F_v/F_m – maximum photochemical efficiency of PSII; NPQ – nonphotochemical quenching; q_P – photochemical quenching; S – 75 mM NaCl; Spd – spermidine; CV – coefficient of variation.

Treatment	F_v/F_m	F_O	F_m	F_v	q_P	NPQ
C	0.79 ± 0.04^{a}	402 ± 15.23^{c}	1982 ±14.08^{b}	1580 ± 25.16^{b}	0.75 ±0.04^{a}	0.52 ± 0.08^{cd}
Spd	0.79 ± 0.03^{a}	410 ± 17.14^{c}	1988 ± 17.64^{b}	1578 ± 16.08^{b}	0.77 ± 0.06^{a}	0.44 ± 0.09^{d}
SNP	0.78 ±0 .02^{a}	408 ± 10.26^{c}	1977 ± 18.03^{b}	1569 ±18.13^{b}	0.76 ± 0.01^{a}	0.42 ± 0.04^{d}
Spd + SNP	0.80 ± 0.05^{a}	410 ± 18.63^{c}	2078 ± 27.34^{a}	1668 ± 21.63^{a}	0.77 ± 0.05^{a}	0.50 ± 0.07^{d}
S	0.51 ± 0.02^{e}	515 ± 14.08^{a}	1064 ± 24.28^{f}	549 ± 32.98^{f}	0.40 ± 0.06^{c}	1.30 ± 0.20^{a}
S + Spd	0.67 ± 0.04^{c}	474 ± 16.63^{b}	1428 ±22.18^{d}	954 ± 28.26^{d}	0.57 ±0.04^{b}	0.75 ± 0.10^{b}
S + SNP	0.62 ± 0.01^{d}	480 ± 17.44^{b}	1266 ± 15.42^{e}	786 ± 16.32^{e}	0.59 ± 0.02^{b}	0.78 ± 0.08^{bc}
S + Spd + SNP	0.69 ±0 .02^{b}	470 ± 18.26^{b}	1531 ± 25.08^{c}	1061 ±29.32^{c}	0.58 ± 0.01^{b}	0.70 ± 0.18^{bc}
ANOVA						
Treatment	**	***	***	**	***	**
CV [%]	4.8	4.6	4.4	5.8	9.5	4.3

proteins may stabilize the protein structure during stress and consequently preserve photosynthetic activity (Hamdani *et al.* 2011). Exogenous SNP reduced the decrease of photosynthesis in cells of leaves because of salt stress, and improved Chl, P_N, E, g_s, C_i, F_v/F_m, and q_P but decreased NPQ. Higher g_s values showed higher transduction ability of the substrate in relation to net P_N, for assimilating more photosynthetic production. Increase of E enhanced the power of absorption and transportation for water, which was beneficial to the increase of photosynthesis and salt tolerance (Liu *et al*. 2014). Huaifa *et al.* (2007) reported that exogenous NO might enhance the salt resistance of cucumber seedlings by improving the photosynthesis in leaves. The results of Wimalasekera *et al.* (2011), showed that NO may be a link between PA-mediated stress responses filling a gap between many known physiological effects of PAs and amelioration of stresses. Therefore, SNP (as NO donor) along with Spd showed synergistic effect under in salinity stress in citrus, which could alleviate the salinity stress and help in improving the photosynthesis in citrus.

Conclusion: In summary, this investigation study revealed that salt stress had inhibitory effects on growth, gas exchange, and Chl fluorescence of Bakraii seedlings. Spd and SNP played important roles in alleviation NaCl toxicity in the citrus seedlings. Intractive effects of Spd + SNP were more effective compared to the seprate applications of Spd + SNP. Spd, SNP, and Spd + SNP significantly ameliorated the adverse effects of NaCl stress by enhanced activities of antioxidative enzymes, and promoted photosynthesis.

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