Influence of exogenously applied nitric oxide on strawberry (Fragaria × ananassa) plants grown under iron deficiency and/or saline stress

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Received 24 May 2018; revised 1 August 2018
doi:10.1111/ppl.12818

A study was carried out to assess the protective effects of exogenously applied nitric oxide (NO) in the form of its donor sodium nitroprusside (SNP) to strawberry seedlings (Fragaria × ananassa cv. Camarosa) grown under iron deficiency (ID), salinity stress or combination of both. The experimental design contained control, 0.1 mM FeSO₄ (ID, Fe deficiency); 50 mM NaCl (S, Salinity) and ID+S. Plants were sprayed with 0.1 mM SNP or 0.1 mM sodium ferrocyanide, an analogue of SNP containing no NO. The deleterious effects of ID+S treatments on plant fresh and dry matters, total chlorophyll and chlorophyll fluorescence were more striking than those caused by the ID or S treatment alone. Furthermore, combination of salinity and iron stress exacerbated electrolyte leakage (EL) and the levels of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) in plant leaves compared to those in plants grown with either of the single stresses. NO treatment effectively reduced EL, MDA and H₂O₂ in plants grown under stress conditions applied singly or in combination. Salt stress alone and with ID reduced the superoxide dismutase (EC1.15.1.1) and catalase (EC 1.11.1.6) activities but increased that of POD (EC 1.17.1.7). Exogenously applied NO led to significant changes in antioxidant enzyme activities in either ID or S than those by ID+S. Overall, exogenously applied NO was more effective in mitigating the stress-induced adverse effects on the strawberry plants exposed to a single stress than those due to the combination of both stresses.

Introduction

Mineral nutrient shortages and salinity are important limitations that restrict plant growth in the world’s agricultural soils (Maathuis et al. 2003, Tester and Davenport 2003). In the past and even more recently, plant biologists have studied abiotic stresses, such as salinity, drought or mineral nutrient deficiency individually to gain crucial information relating their effects on plant growth and nutrient uptake, concentration and distribution. However, it is clear that these results cannot be exclusively used to understand the effects of two or more combined stresses on plants (Mittler 2006, Suzuki et al. 2014), because in field conditions, plants are exposed to more than one abiotic stress factor at the same time (Zandalinas et al. 2018). Considerable progress has been

Abbreviations – CAT, catalase; EC, electrical conductivity; Fₒ, initial fluorescence of photosystem II in darkness; Fₘ, maximum fluorescence of photosystem II; Fᵥ, maximum variable fluorescence of photosystem II; Fᵥ/Fₘ, maximum quantum efficiency of photosystem II; FW, fresh weight; H₂O₂, hydrogen peroxide; ID, iron deficiency; MDA, malondialdehyde; NO, nitric oxide; POD, peroxidase; ΦPSII, quantum efficiency of photosystem II; SF, sodium ferrocyanide; SNP, sodium nitroprusside; SOD, superoxide dismutase.

Iron deficiency (ID) is one of the important microelement deficiencies that adversely affect growth of several crops, mostly due to poor uptake and utilization of this nutrient element by these crops rather than due to the presence of low levels of iron in soils (Kochian 2000). The presence of high amounts of calcium carbonate in calcareous soils results in high levels of bicarbonate ions, which cause ID (Pestana et al. 2005, Pestana et al. 2012). In general, the demand of iron by the plant and solubility of iron in soil is imbalanced, and it is considered as one of the principal reasons of ID-induced chlorosis. Moreover, Fe is a limiting factor in well-aerated soils containing high amount of iron, because at pH 7.0, it is primarily converted into highly insoluble ferric compounds (Rout and Sahoo 2015). Dicots plants reduce Fe(III) using a ferric reductase to Fe(II) which is taken up by the plants through a Fe(II) transporter (Curie and Briat 2003, Ding et al. 2009, 2010).

Iron is one of the essential nutrients for plant growth, which plays a crucial role in various cellular and physiological processes in plants (Grotz and Guerinot 2002, Rabhi et al. 2007). It is also known to be part of some important enzymes and proteins involved in basic biochemical processes such as photosynthesis, respiration, chlorophyll biosynthesis and oxygen transport (Buchanan et al. 2000, Graziano and Lamattina 2007, Li and Lan 2017). ID causes reduction in photosynthetic pigments such as chlorophyll but increases the levels of carotenoids thereby causing yellowing of leaves and chlorosis (Abadía et al. 2011).

Furthermore, in saline soils, deficiencies of micronutrients are also a severe problem due to low solubility of those nutrients (Rabhi et al. 2007) because a high level of sodium in the soil restricts potassium, iron and other mineral nutrients by inducing hyperosmotic stress in the plant as well as oxidative stress (Zhu 2001). Therefore, salinity stress is also another major soil problem deleteriously affecting plant growth and development (Wang et al. 2004, Pandolfi et al. 2012). It has been reported that salinity affects about 7% of the world’s agricultural land and the affected areas are expected to be enlarged in the future (Panta et al. 2014). Salinity imposes two direct effects on plants: osmotic stress and ionic stress. Osmotic stress reduces the uptake of water and minerals such as K⁺ and Ca²⁺ (Glenn et al. 1997, Munns et al. 2006, Khan et al. 2012). Ionic stress, caused by ions like Na or Cl accumulating in high amounts in the cytosol during saline conditions, damages the cell membrane and causes electrolytic leaching, which adversely affect metabolic activities in the cytosol such as photosynthesis, protein synthesis, lipid metabolism and nitrogen assimilation (Zhu 2001, Chen et al. 2007, Ahmad 2010, Pandolfi et al. 2012, Ahmad et al. 2014, Hashem et al. 2014). Salinity stress also generates reactive oxygen species (ROS) that can readily damage cellular membranes through oxidation of lipids, proteins and nucleic acids (Foyer and Noctor 2000). As a result, the leaves undergo early ageing and the photosynthetic activity decreases, which reduces carbon assimilation and plant yield (Khan et al. 2012). For this reason, there must be a balance between ROS production and antioxidants, controlled by enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), as well as by a variety of non-enzymatic antioxidants which detoxify ROS enabling the plants to survive under stress conditions. Plants also produce compounds such as proline (Pro) and glycine betaine (GB) to overcome the adverse effects of osmotic stress caused by salinity stress. In addition, ionic regulation is one of the mechanisms involved to maintain normal cellular functions under salt stress by reducing toxic ions into the cytosol or allowing ions to be kept in the apoplast or in the vacuole (Mittler 2002, Tester and Davenport 2003, Kopyra and Gwóźdź 2004, Khan et al. 2012, Baxter et al. 2014).

For healthy and sustainable plant growth, mineral nutrients required by the plants must be taken up from the soil in sufficient quantity by the roots, which are the main parts involved in mineral nutrient uptake, that interact with the area surrounding plant roots called rhizosphere (Xu and Shi 2006, Akram and Ashraf 2011). The rhizosphere helps the plant to perceive and react to the environment around the root zone. Plants have developed signaling systems to adapt to such stress conditions (Vert et al. 2003, Ashraf and Harris 2004, Zhu 2016). Over the past decade, scientific research in the field of salt tolerance, mineral nutrient acquisition and utilization has led to the identification of structural genes involved in ion transport and activation of enzymes playing a role in ion assimilation (Wang et al. 2002).

Nitric oxide (NO), as a signaling molecule, is believed to play an important role in the responses of plants to biotic and abiotic stresses (Neill et al. 2003, Wendehenne et al. 2004, Delledonne 2005, Kader and Lindberg 2005, Correa-Aragunde et al. 2007, Sheokand et al. 2010, Siddiqui et al. 2011). The role of NO in protecting the biomolecules such as proteins, DNA, lipids and chlorophyll in plants has been well studied (Lamattina et al. 2003). NO has also been reported to be linked with Fe homeostasis in plants by mediating iron-dependent ferritin expression (Murgia et al. 2002, Arnaud et al. 2006, Khan et al. 2007, Pestana et al. 2008, Cvikrova et al. 2013, Ahmad et al. 2014, Perdomo et al. 2014).
2006), by preventing Fe deficiency-induced chlorosis (Graziano et al. 2002, Graziano and Lamattina 2005), and by inducing Fe deficiency responses (Graziano and Lamattina 2007, García et al. 2011). The growth regulatory effect of NO has also been examined in plants grown under saline stress (Yang et al. 2011, Mostofa et al. 2015, Ahmad et al. 2016). Although NO has been contemplated as a key signaling molecule in alleviating ID or salinity stress individually, there seems to be no report in the literature relating to the effect of NO on both stresses when applied in combination. Therefore, the present investigation was aimed to assess the possible role of NO as anti-stress signaling molecule in strawberry plants grown in Fe deficiency and saline stress regimes individually or in combination and examine the functioning of antioxidant machinery to counteract both stress-induced oxidative stresses in strawberry plants.

Materials and methods

Plant growth conditions and treatments

Plants of strawberry (Fragaria × ananassa Duch) cultivar Camarosa were grown in plastic pots under glasshouse conditions under natural sunlight. During summer, strawberry plants with approximately four leaves and 10 cm height were planted in pots containing 5 kg of mixture of well washed sand and perlite at equal rate. Before transplanting the plants to the pots, all the dead leaves, runners, flowers and fruits of plants are pruned. Each treatment comprised of three replicates and each replicate or pot contained one strawberry plant. Two weeks after planting, different treatments were initiated. For minimizing both evaporation and algal growth, a black plastic sheet was laid on each pot.

Simulated Fe deficient condition was achieved by adding 0.1 mM FeSO₄ instead of 0.1 mM EDTA-Fe to Hoagland’s nutrient solution (Hoagland and Amon 1938). The experimental design comprised the control, 0.1 mM FeSO₄ (ID), 50 mM NaCl (S, salinity) and ID + S in Hoagland’s nutrient solution. Plants were sprayed with 0.1 mM sodium nitroprusside (SNP) or 0.1 mM sodium ferrocyanide (SF), an analogue of SNP containing no NO (20 ml pot⁻¹) prepared in 0.01% Tween-20 after 10-day growth. Each treatment was replicated three times and each replicate comprised six plants, i.e. 18 plants per treatment. The excess of the nutrient solution was allowed to flow from the containers so that the NaCl stress could be provided at the desired concentration in the root zone and at the same time the excess water was prevented from causing anoxia by waterlogging. The initial pH value of the nutrient solution was maintained at 6.3 by providing a small volume of 1.0 mM NaOH. Depending on the plant size, between 100 and 200 ml of nutrient solution per plant per day was given to the root zone.

During the experiment, the strawberry plants were grown in the glasshouse with the light period of 11 h each day. Plants were exposed to atmospheric temperature at 30±1°C/25±1°C (day/night) and relative humidity at 65–70%.

After 28 days of treatment, two plants were randomly selected from each replicate, shoots and roots were separated and they were dried in an oven at 75°C for 2 days to record the dry mass of the plants. The remaining four plants in each replicate were used to determine the characteristics described below.

Chlorophyll contents

Chlorophyll determination was made according to the method of Strain and Svec (1966). At harvest time, 1 g leaf sample was extracted in a mixture of 5 ml of acetone: water (90% v/v). The extraction was then filtered and stored in light-tight tubes. Chlorophyll extracts were read at 663 nm and 645 nm for chlorophyll a and b, respectively, and expressed as mg g⁻¹ fresh weight (FW).

The following formulas were used to calculate different types of chlorophyll:

\[
\text{Chl } a \ (\text{mg g}^{-1} \text{FW}) = \frac{[11.64 \times (A663) - 2.16 \times (A645)] \times TV}{FW}
\]

\[
\text{Chl } b \ (\text{mg g}^{-1} \text{FW}) = \frac{[20.97 \times (A645) - 3.94 \times (A663)] \times TV}{FW}
\]

where A663 is the absorbance value at 663 nm; A645, the absorbance value at 645 nm; TV is the total volume of the extract (ml) and FW is the weight of the fresh leaf tissue (g).

Chlorophyll fluorescence

For measuring this parameter, the strawberry leaves were subjected to a dark and light cycle following the standard instructions. Then fluorescence readings were taken using a chlorophyll fluorometer (Mini-PAM, Walz, Germany). Before taking readings, the leaves were kept in dark for 30 min. Data for minimum fluorescence (F₀), maximal fluorescence (Fm) and variable fluorescence (Fv) were recorded and then maximum quantum efficiency of photosystem II (PSII) as Fv/Fm was worked out.

Measurement of total and active Fe in leaves

Total Fe was analyzed according to the method of Lei et al. (2014). For this purpose, leaves of plants
were cleaned up with 1.5 mM CaCl₂ solution and then samples were digested in a solution containing HNO₃/HClO₄ (4:1, v/v). Total Fe was determined by using inductively coupled plasma (ICP) atomic emission spectroscopy.

The protocol described by Takker and Kaur (1984) was employed to measure active iron content in the leaves. Briefly, fresh leaf tissues (200 mg) were cut into small pieces and then extracted using 1 N HCl (20 ml) in 1/10 tissue/extractant and filtered after 5 h shaking. Fe content in the extracts was determined by using an ICP.

**Ferric reductase activity in the roots**

Ferric reductase activity in the roots of strawberry plants was measured according to the method described by Li et al. (2000). Briefly, before transferring the roots of strawberry plants to Hoagland’s nutrient solution containing 0.1 mM Fe-EDTA and 0.4 mM 2, 2-bipyridyl, pH 5.0, they were submerged into solution of saturated CaSO₄ for 5 min and then cleaned with deionized water. Ferric reductase activity in the roots was determined by measuring the concentration of Fe(II)-dipyridyl complex formed using a spectrophotometer at A523.

**Electrolyte leakage**

The protocol originally outlined by Dionisio-Sese and Tobita (1998) was employed. For this purpose, a fresh leaf (200 mg) was cut into uniform small pieces and placed into a test tube containing 10 ml of distilled water. After 2 h of incubation in a water bath, the initial electrical conductivity (EC₁) was determined using an EC meter. Thereafter, the samples were autoclaved at 121°C for 20 min to get all electrolytes released. When the temperature dropped down to 25°C, EC₂ was determined.

**Antioxidant enzymes**

Fresh leaf material (each 500 mg) was ground well in Na-P buffer (50 mM) containing soluble polyvinyl pyrrolidine (1%). The mixture was centrifuged at 20,000 g at 4°C for 15 min. The activity of CAT in the supernatant was determined following Kraus and Fletcher (1994), POD following Chance and Maehly (1955) and that of SOD following Beauchamp and Fridovich (1971). The Bradford (1976) protocol was employed to quantify total soluble proteins.

**Leaf malondialdehyde**

Malondialdehyde (MDA), a lipid peroxidation product, was determined as described by Heath and Packer (1968). Fresh leaf tissues (each 100 mg) were homogenized by adding 0.5 ml 0.1% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 15,000 g at 4°C for 10 min. The supernatant liquid was diluted with 20% TCA, mixed with 0.5 ml of 1.5% thiobarbituric acid and incubated in a water bath at 95°C for 25 min. The final sample was incubated on ice. The absorbance was read at 532 nm.

**Hydrogen peroxide**

Hydrogen peroxide (H₂O₂) in the leaf samples was assessed as described by Loreto and Velikova (2001). A proportion (0.5 g) for fresh leaf material was pulverized in a pestle and mortar containing 3 ml of TCA (1% w/v). Thereafter, the extract was centrifuged, and 0.75 ml of the filtrate reacted with 0.75 ml of 1 M KI. The OD of the all treated samples was recorded at 390 nm.

**NO determination**

Leaf NO was assayed according to the method described by Hu et al. (2003) and modified by Zhou et al. (2005). In brief, fresh leaf tissue (each 600 mg) was extracted in a pestle and mortar using a solution containing cool acetic acid buffer (3 ml of 50 mM, pH 3.6) and zinc diacetate (4%). A supernatant was taken from homogenate after centrifugation at 10,000 g for 15 min at 4°C. The supernatant 0.1 g of charcoal was added. Following filtration and vortexing, 1 ml of each filtrate was incubated at room temperature for 30 min with the Griess reagent. NO was determined by reading the absorbance at 540 nm.

**Chemical analysis**

For the quantification of different inorganic elements such as Na, K and Ca, finely ground leaf samples were ashed in a muffle furnace at 550°C. A total volume of 5 ml of 2 M hot HCl was added to each sample ash, and the final volume of the sample was brought to 50 ml by adding distilled deionized water (Chapman and Pratt 1982). An ICP was used to analyze all mineral elements in digested samples. The chloride in the plant extracted with hot water was quantified by coloring with potassium chromate indicator and titrating with AgNO₃ (Johnson and Ulrich 1959).

**Statistical analysis**

Two-way ANOVA of data was carried out using the program CoStat version 6.303 (CoHort Software, Sun Microsystems, Inc. Monterey, CA). Mean square values
were presented in Table 1. Mean data along with SE were presented in Figs 1–7 and compared at the 5% probability level. Treatment means were compared using the Duncan's Multiple Range Test (DMR) test to work out significant differences among them.

**Results**

Saline stress (50 mM NaCl) and ID (0.1 mM FeSO₄) applied individually or in combination, significantly \((P \leq 0.001)\) reduced the shoot and root dry mass as well as total dry biomass of strawberry plants. A maximal reduction was observed when both (salt + ID) stresses were applied together. However, SNP improved all these growth attributes except root DM under stress conditions (Table 1; Fig. 1). The effect of SF on all growth attributes remained non-significant, confirming that the improvement in these attributes were due to NO released from SNP.

Root/shoot dry mass ratio increased significantly \((P \leq 0.001)\) under both ID and saline stress conditions (Table 1; Fig. 1). Foliar-applied NO considerably suppressed the root/shoot dry mass ratio of strawberry plants subjected to stress conditions.

Chlorophyll \(a\) and \(b\) contents of strawberry plants declined significantly under both stresses. Lowest values of chlorophyll \(a\) and \(b\) contents were observed when both stresses were applied in combination (Table 1; Fig. 2). Exogenously applied NO as SNP was effective in enhancing the chlorophyll \(a\) contents under different stresses, while no change was observed in chlorophyll \(b\) contents under stress and non-stress conditions.

A significant decrease of total chlorophyll \((a + b)\) contents was monitored both in single and combined stresses. Exogenously applied NO as SNP was effective in enhancing the total chlorophyll contents. No change in chlorophyll \(a/b\) ratio was observed upon both stresses (saline and ID) or foliar-applied NO (Table 1; Fig. 2).

Chlorophyll fluorescence attributes such as \(F_o\), \(F_v/F_m\) and PSII \((P \leq 0.001, 0.01, 0.001,\) respectively) decreased significantly, particularly when both stresses were applied together (Table 1; Fig. 3). Exogenously applied SNP was effective in enhancing the efficiency all these photosystem parameters, while no change was observed after exogenous application of SF, confirming that the regulation in these attributes was due to NO only.

Active as well as total iron (Fe) contents of strawberry plants decreased significantly under all stressful regimes \((P \leq 0.001)\). Although exogenously applied both SF and SNP considerably improved the above-mentioned attributes under stress as well as non-stress conditions.

### Table 1. ANOVA (mean squares) of the data for different growth and biochemical attributes of strawberry plants treated with NO as a foliar spray. *ns*, non-significant; *, ** and *** = significant at 0.05, 0.01 and 0.001 levels, respectively.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Total dry mass</th>
<th>Shoot dry mass</th>
<th>Root dry mass</th>
<th>Root/shoot ratio</th>
<th>Chlorophyll a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress treatments (STs)</td>
<td>3</td>
<td>490.3***</td>
<td>315.2***</td>
<td>19.53***</td>
<td>0.007***</td>
<td>0.305***</td>
</tr>
<tr>
<td>NO treatments</td>
<td>2</td>
<td>43.4***</td>
<td>46.7***</td>
<td>0.363 ns</td>
<td>0.03***</td>
<td>0.0315***</td>
</tr>
<tr>
<td>ST × NO</td>
<td>6</td>
<td>2.59 ns</td>
<td>3.87*</td>
<td>0.41 ns</td>
<td>0.005***</td>
<td>0.006***</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>2.054</td>
<td>1.221</td>
<td>0.199</td>
<td>0.0004</td>
<td>0.001</td>
</tr>
<tr>
<td>STs</td>
<td>3</td>
<td>0.039***</td>
<td>0.555***</td>
<td>0.142***</td>
<td>726.2***</td>
<td>0.047***</td>
</tr>
<tr>
<td>NO treatments</td>
<td>2</td>
<td>0.002 ns</td>
<td>0.051***</td>
<td>0.013 ns</td>
<td>98.008***</td>
<td>0.005*</td>
</tr>
<tr>
<td>ST × NO</td>
<td>6</td>
<td>0.0009 ns</td>
<td>0.011**</td>
<td>0.008 ns</td>
<td>9.123*</td>
<td>0.0002 ns</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>0.0009</td>
<td>0.002</td>
<td>0.006</td>
<td>3.275</td>
<td>0.001</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td></td>
<td>Chl (a + b)</td>
<td>Chl (a/b)</td>
<td>(F_o)</td>
<td>(F_v/F_m)</td>
<td></td>
</tr>
<tr>
<td>STs</td>
<td>3</td>
<td>0.056***</td>
<td>6697.3***</td>
<td>31.69*</td>
<td>1192.6***</td>
<td>0.132***</td>
</tr>
<tr>
<td>NO treatments</td>
<td>2</td>
<td>0.010***</td>
<td>2056.3***</td>
<td>32.07***</td>
<td>503.02***</td>
<td>0.04***</td>
</tr>
<tr>
<td>ST × NO</td>
<td>6</td>
<td>0.0007 ns</td>
<td>249.0***</td>
<td>31.69*</td>
<td>3200.9***</td>
<td>0.235 ns</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>0.001</td>
<td>27.11</td>
<td>8.777</td>
<td>3200.9***</td>
<td>0.121</td>
</tr>
<tr>
<td>SOD</td>
<td></td>
<td>CAT</td>
<td>POD</td>
<td>(H_2O_2)</td>
<td>MDA</td>
<td></td>
</tr>
<tr>
<td>STs</td>
<td>3</td>
<td>2034.4***</td>
<td>75.36***</td>
<td>0.527***</td>
<td>1407.0***</td>
<td>8912.8***</td>
</tr>
<tr>
<td>NO treatments</td>
<td>2</td>
<td>38.36 ns</td>
<td>12.28***</td>
<td>0.087***</td>
<td>208.6***</td>
<td>146.1**</td>
</tr>
<tr>
<td>ST × NO</td>
<td>6</td>
<td>376.4***</td>
<td>2.911**</td>
<td>0.067</td>
<td>208.6***</td>
<td>146.1**</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>19.52</td>
<td>0.765</td>
<td>0.070</td>
<td>19.52</td>
<td>33.38</td>
</tr>
<tr>
<td>EL</td>
<td></td>
<td>Na(^+)</td>
<td>Cl(^-)</td>
<td>(Ca^{2+})</td>
<td>K(^+)</td>
<td></td>
</tr>
<tr>
<td>STs</td>
<td>3</td>
<td>2721.5***</td>
<td>4.577***</td>
<td>278.8***</td>
<td>101.7***</td>
<td>728.7***</td>
</tr>
<tr>
<td>NO treatments</td>
<td>2</td>
<td>304.3***</td>
<td>0.294***</td>
<td>6.888*</td>
<td>7.017***</td>
<td>62.27***</td>
</tr>
<tr>
<td>ST × NO</td>
<td>6</td>
<td>41.58*</td>
<td>0.131***</td>
<td>3.103 ns</td>
<td>3.147**</td>
<td>9.583***</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>12.69</td>
<td>0.002</td>
<td>1.246</td>
<td>0.663</td>
<td>1.551</td>
</tr>
</tbody>
</table>
SNP was more effective than SF in improving the active, as well as total Fe contents (Table 1; Fig. 4), again confirming the role of NO.

Ferric reductase activity decreased considerably \((P \leq 0.001)\) in strawberry plants subjected to stress conditions (Table 1; Fig. 4). Application of NO was effective in enhancing the ferric reductase activity under stress and non-stress conditions. A maximal activity of ferric reductase was observed when SNP was applied as a foliar spray.

A significant \((P \leq 0.001)\) decline in NO contents in the leaves of strawberry plants was observed under different stress conditions (Table 1; Fig. 5). No change was observed in NO contents due to foliar applied SF, while in contrast, a significant increase was observed due to 0.1 mM SNP particularly under stress conditions.

A significant \((P \leq 0.001)\) increase was observed in \(\text{H}_2\text{O}_2\) and MDA contents in strawberry plants under different stress conditions (Table 1; Fig. 5). No change was observed in MDA or \(\text{H}_2\text{O}_2\) contents due to foliar-applied SF, while a significant decrease was observed due to exogenously applied 0.1 mM SNP, particularly under stress conditions.

A considerable increase was observed in electrolyte leakage (EL) in the strawberry plants upon exposure to saline, ID as well as saline+ID conditions (Table 1; Fig. 5). A maximal reduction was observed when both stresses were applied in combination. Exogenously applied NO as SNP significantly suppressed the EL of strawberry plants.

ANOVA of the data for the activities of SOD, POD and CAT enzymes of all strawberry plants demonstrated that, under iron deficit conditions, a significant increase was observed only for the SOD activity. While the activity of CAT was reduced under salt stress and combined application of both (ID+salt) stresses (Table 1; Fig. 6). However, the activity of POD increased significantly \((P \leq 0.001)\) under salt stress as well as in combination of salt and ID conditions. No change was observed in the activity of SOD due to foliar-applied SNP. However, SNP treatment improved the activity of CAT enzyme under salt stress and combined application of both (ID+S) stresses, while the activity of POD enzyme improved only under the ID treatment.

A marked increase \((P \leq 0.001)\) in leaf Na\(^+\) and Cl\(^-\) contents was observed in strawberry plants subjected to S

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**Fig. 1.** Shoot and root dry mass, total dry mass and root/shoot ratio of plants sprayed with SF or SNP under ID, salinity (S) or a combination of both stresses (ID + S) applied individually or in combination (mean ± si); different letters (a–f) on bars show significant difference among means within each parameter.
Fig. 2. Chlorophyll a, b, a+b and a/b ratio of plants sprayed with SF or SNP under ID, salinity (S) or a combination of both stresses (ID+S) applied individually or in combination (mean ± SE). Different letters (a–h) on bars show significant difference among means within each parameter.

Discussion

The premier objective of this investigation was whether or not the foliar application of NO could alleviate the injurious effects of S and ID stresses when applied individually or in combination on strawberry plants. In our study, S as well as ID stress imposed, either separately or in combination, decreased the dry biomass and enhanced the root/shoot dry mass ratio of strawberry plants. Since in most of earlier published studies, the effect of S or ID stress on different crops has been observed separately, so we can relate our results to either S-induced or ID-induced adverse effects observed in different reports on different crops. For example, salt-induced adverse effects have been observed in *Schizonepeta tenuifolia* (Zhou et al. 2018), tomato (Siddiqui et al. 2017), cumin (Rebey et al. 2017), soybean (Egbichi et al. 2014) and *Mentha pulegium* (Oueslati et al. 2010) where it has been shown that salinity markedly decreased plant biomass. ID was also reported to be a potential growth retardant as observed in flax (Salama et al. 2009) and maize (Kanai et al. 2009). Acosta-Motos et al. (2017) have reported that salinity can enhance the root to shoot ratio because the roots store high amount of toxic ions under saline conditions and the translocation of nutrients to shoot is hampered so as to protect it. For example, in different studies, salt-induced enhanced root to shoot ratio has been observed, e.g. in *Euonymus japonica* (Gómez-Bellot et al. 2013), *Callistemon citrinus* (Álvarez and Sánchez-Blanco 2014) and *Myrtus communis* (Acosta-Motos et al. 2015). Astolfi et al. (2006) and Zhang et al. (2012) have reported that ID stress also enhanced the root to shoot ratio in barley and peanut plants, respectively. However, foliar-applied NO enhanced the growth and decreased the root/shoot dry mass ratio of strawberry plants under saline and ID stress conditions when applied singly or in combination. Similarly, in tomato (Manai et al. 2014) and...
Fig. 3. Chlorophyll fluorescence parameters ($F_o$, $F'/F_m$ and $\Phi_{PSII}$) of plants sprayed with SF or SNP under ID, salinity (S) or a combination of both stresses (ID + S) applied individually or in combination (mean ± SE). Different letters (a–h) on bars show significant difference among means within each parameter.

Fig. 4. Active Fe, total Fe content in the leaves and ferric reductase activity in the roots of plants sprayed with SF or SNP under ID, salinity (S) or a combination of both stresses (ID + S) applied individually or in combination (mean ± SE). Different letters (a–g) on bars show significant difference among means within each parameter.

soybean (Egbichi et al. 2014), exogenously applied NO improved the growth of plants mainly by acting as a signaling molecule (Molassiotis et al. 2010). Particularly, under ID stress, exogenous application of NO improved the growth of Arabidopsis thaliana plants by improving the ID and reducing leaf chlorosis (Koen et al. 2012).

Photosynthetic activity of most plants decreases under saline conditions because PSII activity is disturbed and thylakoid membrane along with its pigments is damaged due to formation of ROS under salinity stress (Dombrowski 2003, Naeem et al. 2010, Shaheen et al. 2012, Wu et al. 2012, Deinlein et al. 2014, AbdElgawad et al. 2016, Askary et al. 2017, Gomes et al. 2017). Like many other stresses, the deficiency of iron can also generate ROS and disturb the electron transport chain in the chloroplast because iron plays an important role in it (Graziano and Lamattina 2005). In the present study, chlorophyll a, b and total chlorophyll contents as well as chlorophyll fluorescence of strawberry plants decreased under salinity and ID stresses. It is parallel to what has already been observed in radish (Jamil et al. 2007),
Fig. 5. NO, MDA, EL and H₂O₂ contents of plants sprayed with SF or SNP under ID, salinity (S) or a combination of both stresses (ID+S) applied individually or in combination (mean ± SE). Different letters (a–f) on bars show significant difference among means within each parameter.

NO (nmol g⁻¹ FW)

MDA (nmol g⁻¹ FW)

EL (%)

H₂O₂ (nmol g⁻¹ FW)

tomato (Ciobanu and Sumalan 2009), oilseed rape (Naeem et al. 2010), wheat (Kanwal et al. 2011), eggplant (Wu et al. 2012) and sunflower (Akram et al. 2009, Heidari et al. 2014) under saline stress. Our results are similar to those found in Phytolacca americana (Liang et al. 2010), Arabidopsis (Koen et al. 2012) and peanut (Zhang et al. 2012) under ID. On the other hand, in the present study, exogenous application of NO enhanced chlorophyll a, total chlorophyll content and chlorophyll fluorescence in the strawberry plants under both stresses. Exogenously applied NO enhanced the chlorophyll content and chlorophyll fluorescence in rice (Habib et al. 2013) and tomato (Wu et al. 2011) as it is believed to play an important role in stomatal closure by inducing the synthesis of abscisic acid. Zhang et al. (2012) observed that exogenous application of NO enhanced the chlorophyll content by improving chlorophyll synthesis and chlorophyll fluorescence by protecting PSII under iron deficit conditions, as observed in peanut.

EL from biological membranes increases in plants subjected to saline stress (Demidchik et al. 2014) as well as under iron deficit regimes (Kong et al. 2014), which was ascribed to the generation of excessive amount of ROS. In this investigation, both stresses increased the EL in strawberry plants. Likewise, enhanced EL has been reported in cotton (Dong et al. 2014), barley (Velarde-Buendía et al. 2012) and wheat (Cuin et al. 2008) under saline conditions, and cotton (Liu et al. 2013) under ID stress. However, exogenously applied NO reduced the EL of strawberry plants under all stressful regimes as earlier observed in wheat (Zheng et al. 2009), sorghum (Jasid et al. 2008) and chickpea (Ahmad et al. 2016). Similarly, Liu et al. (2013) reported that exogenous application of NO could reduce the EL in cotton plants under ID. NO is believed to protect the cellular membranes by improving their permeability (Fan et al. 2007) and indirectly activates the stress-related genes (Neill et al. 2008, Ahmad et al. 2016).

Zhang et al. (2012) reported that under ID stress, total iron content and activity of ferric reductase enzyme decreases due to the conversion of Fe-III into Fe-II in the roots which then moves through the plasma membrane of roots into soil. Ksouri et al. (2007) have reported that salinity increases the pH of soil which in turn reduces iron contents. In our investigation, all stress conditions decreased the total iron content and activity of ferric reductase in strawberry plants. Similar results have been earlier observed in Mentha piperita (Askary et al. 2017) under saline conditions and peanut (Zhang et al. 2012) and tomato (Ye et al. 2015) under ID stress. However,
Fig. 6. Activities of SOD, CAT and POD enzymes of plants sprayed with SF or SNP under ID, salinity (S) or a combination of both stresses (ID + S) applied individually or in combination (mean ± SE). Different letters (a–h) on bars show significant difference among means within each parameter.

exogenous application of NO enhanced the total iron content and activity of ferric reductase in strawberry plants under all stressful conditions as have been earlier observed in peanut (Zhang et al. 2012, Song et al. 2017) and tomato (Ye et al. 2015) under ID stress. Graziano et al. (2002) have reported that NO reacts with iron to form Fe(III)-NO complex in aqueous media which changes into Fe(II) cation and enhances iron content of plants.

Acosta-Motos et al. (2017) have reported that NO is involved in the plant responses to saline conditions. In the present study, stress conditions decreased the NO contents of strawberry plants. Likewise, in Salvinia auriculata, Gomes et al. (2017) showed that exposure to high salinity regimes increased extracellular NO level after 2 h, while it decreased when tested after 5 days of salinity stress. Contrarily, in tomato, Manai et al. (2014) reported that exogenous application of SNP enhanced salinity stress tolerance which was ascribed to NO-induced activation of antioxidant enzymes. In view of these contrasting reports, it could be inferred with caution that salt-induced regulation in tissue NO levels depends on the type of plant species, duration or the intensity of a stress applied.

Under S and ID conditions, oxidative stress (ROS like H₂O₂) is produced which can effectively cause lipid peroxidation (Ebrahimzadeh et al. 2008, Shu et al. 2013, Kong et al. 2014). In our study, contents of H₂O₂ and MDA of strawberry plants increased under S and ID stresses applied individually or in combination. A similar trend in both biomolecules has been observed in cucumber (Shu et al. 2013) and M. piperita (Askary et al. 2017) under saline conditions, and in sunflower (Ranieri et al. 2001), flax (Salama et al. 2009) and peanut (Zhang et al. 2012, Kong et al. 2014) under ID. However, exogenous application of NO significantly reduced the contents of H₂O₂ and MDA in strawberry plants under S and ID stresses. Similar findings have been reported in soybean (Egbichi et al. 2014) under saline conditions, and in peanut (Zhang et al. 2012, Kong et al. 2014) under ID. Liu et al. (2007) found that NO is involved in the activation of key antioxidative enzymes.

In the present investigation, salinity alone or in combination with ID stress reduced the activity of CAT and enhanced that of POD, while the SOD activity was enhanced under ID stress. In some earlier studies, a decline in CAT activity has been observed in different plants such as M. piperita (Askary et al. 2017), wheat (Hameed et al. 2008), sesame (Koca et al. 2007) under saline conditions, and in flax (Salama et al. 2009) and peanut (Zhang et al. 2012, Kong et al. 2014) under iron deficient conditions. As in our study, enhancement in
Fig. 7. Sodium (Na\(^+\)), chloride (Cl\(^-\)), potassium (K\(^+\)) and calcium (Ca\(^{2+}\)) contents in the leaves of plants sprayed with SF or SNP under ID, salinity (S) or a combination of both stresses (ID + S) applied individually or in combination (mean ± SE). Different letters (a–h) on bars show significant difference among means within each parameter.

POD activity was found in wheat (Hameed et al. 2008) and sesame (Koca et al. 2007) under saline conditions, but in contrast suppression in POD activity was noted in flax (Salama et al. 2009) and peanut (Zhang et al. 2012, Kong et al. 2014) under iron deficit conditions. The reason for decrease in POD and CAT activities under ID stress could be that they are iron-containing enzymes, so under ID, it is logical to expect a decline in their activity. While exogenously applied NO enhanced the CAT and POD activities under all stress conditions, as has been earlier reported in maize (Sun et al. 2007), barley (Li et al. 2008), wheat (Zheng et al. 2009), tomato (Manai et al. 2014) and cucumber (Fan et al. 2013) under saline conditions, and in peanut (Zhang et al. 2012, Kong et al. 2014, Song et al. 2017) under ID.

Both stresses enhanced the tissue Na\(^+\) and Cl\(^-\) contents in the leaves of strawberry plants, while exogenous application of NO reduced the Na\(^+\) and Cl\(^-\) contents under both stresses. Earlier, Dong et al. (2014) observed that exogenously applied NO caused a marked suppression in tissue Na\(^+\) in cotton plants grown under saline regimes. This could be due to the accelerated function of Na\(^+\) pumps at the biological membranes under NO treatment (Wang et al. 2009).

Potassium (K\(^+\)) and calcium (Ca\(^{2+}\)) ions are very important for optimum growth of plants but under salinity, due to accumulation of toxic ions, these ions are substantially reduced (Acosta-Motos et al. 2017). In this study, both stresses reduced the contents of leaf K\(^+\) and Ca\(^{2+}\) of strawberry plants. However, exogenously applied NO enhanced the contents of K\(^+\) and Ca\(^{2+}\) in strawberry plants under both stresses. Chen et al. (2013) have reported that exogenous application of NO on mangroves under salinity stress enhanced the K\(^+\) uptake and reduced the K\(^+\) loss from the cytosol. The increase in K\(^+\) may be due to an enhanced transcription of K\(^+\) channels by NO treatment (Xia et al. 2014).

Our results showed that the combined effects of salinity and ID are more harmful than those monitored upon single stress treatment. Rabhi et al. (2007) reported that combined salinity and ID stress had more harmful effects on photosynthesis and growth of Medicago ciliaris than when they were applied separately. They attributed the reason of these results to the observation that under combined application of ID and saline stress, shoots

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accumulate more amounts of Na\(^+\) ions and decrease root acidification impacting negatively the nutrient uptake. Similarly, Abbas et al. (2015) found that saline and iron deficit conditions in combination reduced the growth, rate of photosynthesis and transpiration in rice plants more than that caused individually by ID and S stress. They determined that under combined S and ID conditions, the amount of Na\(^+\) ions is enhanced prominently in the roots and shoots which thereby reduce the uptake of Fe and K from the soil.

**Conclusions**

Overall, ID conditions and S stress whether applied individually or in combination reduced the plant biomass, chlorophyll content and chlorophyll fluorescence, leaf calcium, potassium, and iron contents, ferric reductase activity and contents of NO in the leaves while enhancing the activities of antioxidants, EL, root to shoot ratio, and levels of \(\text{H}_2\text{O}_2\) and MDA in strawberry plants. However, exogenous application of NO enhanced the plant biomass, chlorophyll content, chlorophyll fluorescence, contents of leaf calcium and potassium ions, iron content, ferric reductase activity, activity of antioxidants and contents of NO while reduced the EL, root to shoot biomass ratio, and levels of \(\text{H}_2\text{O}_2\) and MDA in strawberry plants. In conclusion, exogenous application of NO as a foliar spray could be a good mitigating agent under ID and S conditions.

**Author contributions**

C.K. and N.A.A. conducted the experimentation and data analysis, respectively. Also, C.K. and N.A.A. jointly wrote up the manuscript. M.A. helped in designing the study and edited critically the whole manuscript.

**Acknowledgement** – The authors wish to thank the University of Harran (Turkey) and GC University, Faisalabad (Pakistan) for supporting the present study.

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