



Atmospheric application of trace amounts of nitric oxide enhances tolerance to salt stress and improves nutritional quality in spinach (*Spinacia oleracea* L.)



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ABSTRACT

The increased salinity in greenhouses has become a problem of great concern. In this study, it was observed that the salt-induced oxidative damages (indicated by MDA, H₂O₂ and antioxidant enzymes, including POD, SOD and CAT) could be alleviated by application of NO gas. Consequently, although both photosynthesis and growth in plants were inhibited by NaCl stress, they were restored by NO gas application, and the fresh and dry biomasses of edible parts increased by 60% and 27% over NaCl stress treatment, respectively. Furthermore, gaseous NO application also significantly elevated the levels of several antioxidation-associated compounds such as proline, ascorbate, glutathione, total phenolics and flavonoids, as well as the total antioxidant capacity (indicated by DPPH scavenging activity) in NaCl-treated plants. Keeping in mind all of the above, we concluded that atmospheric application of trace amounts of nitric oxide gas could be an effective strategy for improving both biomass production and nutrition quality in spinach under salt stress.

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1. Introduction

Cultivation in greenhouses is a very good farming practice for achieving higher crop production, where climate and other extraneous variables affect crop production (Chang et al., 2011). Additionally, farmers are able to earn higher incomes relative to conventional cultivation, and therefore greenhouses have become an important type of covered agriculture, globally. However, greenhouse cultivation does not allow the rain to reach the surface of the soil and the evapotranspiration is high, which together with over-fertilisation could easily result in higher salinity compared to open-field conditions (Chen et al., 2004). Therefore, serious salinity issues often arise in greenhouses after several seasons of cultivation. For example, Darwish, Atallah, Moujabber & Khatibb (2005) observed that soil in greenhouses has a salinity sixfold higher than the soil outside. Soil salinity imposes both osmotic stress and ionic toxicity in plants and triggers the synthesis of reactive oxygen species (ROS) (Farooq, Siddique, & Schubert, 2013; Soussi, Ocaña, & Lluch, 1998), which may lead to oxidative destruction of cells (Meloni, Oliva, Martinez, & Cambraia, 2003) and may also disrupt normal metabolism in plants (Farooq et al., 2013). Consequently, salt tolerance often appears to be inversely related

to growth rate of plants and thereby the farmers' income. Therefore, the increased salinity in greenhouses has become a problem of great concern.

Various strategies for reclamation of salt-affected soils have been proposed and tried in response to the problem of salinity, e.g. irrigation practices (increasing the volume and frequency of irrigation, changing the method of water application and installing subsurface drainage), soil amendments (gypsum and calcium chloride dehydrate), transgenic crop breeding and crop rotations, etc. (Ondrasek, Rengel, & Veres, 2011). However, some of these measures have significant shortcomings. For example, increasing irrigation would lead to a higher atmospheric humidity inside greenhouses which could cause an increase in the incidence of insect pests and diseases (Rosenzweig, Lglesias, Yang, Epstein, & Chivian, 2001), and genetically modified plants might have potential risk for human beings (Dona & Arvanitoyannis, 2009). Ergo, an economical and practical method to alleviate salt stress on plant growth, especially in greenhouse agricultural production, is still a pressing need.

Nitric oxide (NO) is a freely diffusible gaseous compound, and has been widely agreed upon as an important signalling molecule in plants (Crawford & Guo 2005). Increasing evidences revealed that the endogenous NO in plants occupies a central place in the signalling network to induce tolerance against salinity. For instance, *Arabidopsis* mutant *Atnoa1* with a decreased endogenous

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NO accumulation exhibits a higher hypersensitivity to salt treatment than wild type plants (Zhao, Tian, & Zhang, 2007). Currently, an antioxidant-based mechanism of NO against salt stress has been proposed. Exogenous NO application elevates antioxidant enzyme activity, promotes the maintenance of cellular redox homeostasis, and consequently mitigates the oxidative damage produced by ROS in citrus plants under salinity (Tanou et al., 2009). These evidences provide an implication that developing NO-associated technology may be feasible in improving crop production in greenhouses that suffer from salinity issues. However, little information is available regarding the above assumption.

Because of the relatively sealed construction of greenhouses, application of trace amounts of NO in greenhouses could be operable. In our previous studies, we have successfully applied 200 nl l⁻¹ gaseous NO in a closed chamber, which exhibited a significant increase of biomass production on normal soil conditions (Jin, Du, Zhang, Tang, & Lin, 2009). Therefore, 200 nl l⁻¹ of NO was also used in the present study to investigate whether application of gaseous NO has a positive effect on alleviating salt stress in crops grown with saline growth medium. Our results show that application of trace amount of gaseous NO improves the growth of spinach (*Spinacia oleracea*) by elevating antioxidant capacity under salinity stress.

2. Materials and methods

2.1. Plant cultivation

The variety of spinach (*S. oleracea* L.) used in this study was the local circle-leaf variety “Chunye”. The seeds were germinated in trays filled with a sandy substrate. At the third true leaf stage, the seedlings of similar size were moved into 1 litre plastic containers (five plants per container) filled with an aerated, full-strength complete nutrient solution of either 0 or 200 mM NaCl. The other nutrients had the following composition: Ca(NO₃)₂ 1000 μM, (NH₄)₂SO₄ 1000 μM, MgSO₄ 500 μM, KH₂PO₄ 250 μM, K₂SO₄ 500 μM, H₃BO₃ 10 μM, ZnSO₄ 0.5 μM, CuSO₄ 0.1 μM, MnCl₂ 0.5 μM, (NH₄)₆(Mo₇)₂₄ 0.1 μM and Fe-EDTA 10 μM. The pH of the nutrient solution was monitored, and was adjusted to 5.7 ± 0.2 by NaOH or H₂SO₄ every day. Nutrient solution was renewed every three days. The plants were grown in two independent growth chambers: 1. Control (ambient atmosphere) and 2. Ambient atmosphere with 200 nl l⁻¹ added NO. The CO₂ concentration in the chamber was controlled at about 350 μl l⁻¹. The ambient atmosphere had concentrations of NO and NO₂ of < 4 nl l⁻¹ and < 18 nl l⁻¹ [the gases were determined using a chemiluminescence NO–NO₂–NO_x analyser (EC9841B, Ecotech, Inc., Knoxville, Victoria, Australia)], respectively. Such low levels of background NO and NO₂ in the ambient atmosphere ensured the reliability of the elevated NO treatment in this study. For the NO treatments, pure NO gas was first diluted by N₂ at the final concentration of 100 ml l⁻¹. The diluted NO gas was then injected into the growth chambers at the final concentration of 200 nl l⁻¹. The detected NO concentration in the growth chamber could maintained at 162–173 nl l⁻¹ after 3 h of NO injection. In addition, at the end of each day, the detected NO concentration in growth chamber was 117–135 nl l⁻¹. The NO in the growth chambers was refreshed daily at the beginning of the day. Other conditions in the chambers were the same and had a daily cycle of 25 °C, 14-h day and a 20 °C, 10-h night. The daytime light intensity was 300 μmol photons m⁻² s⁻¹. The experiment was independently performed five times.

2.2. Measurement of biomass, SPAD and photosynthetic rate

After 21 days of treatments, the chlorophyll concentrations were measured on three new fully expanded leaves per pot with a chloro-

phyll meter (SPAD-502, Minolta Camera Co., Osaka, Japan) and recorded as an SPAD reading. The SPAD makes rapid and non-destructive measurements to provide a relative indication of leaf chlorophyll concentration. The photosynthetic rate was also measured on three newly fully expanded leaves per pot after 3 h of NO injection by using an infra-red analyser (LI-6400 System, Li-COR Company, Lincoln, NE, USA). The photosynthetic measurements were conducted at a photon flux density of 1000 μmol m⁻² s⁻¹, and CO₂ concentration of 350 μl l⁻¹. The airflow rate was 500 ml min⁻¹. Leaf temperature was maintained at 25 °C and relative humidity at 70% in the leaf chamber. After chlorophyll concentration and photosynthetic rate measurements, the plants were harvested. The edible parts and roots were weighted and then dried in a 75 °C oven to a constant weight for measurement of dry weight.

2.3. Determination of POD, SOD and CAT activities

Peroxidase (POD, EC1.11.1.7) activity was assayed on the basis of the guaiacol oxidation method at 470 nm (Pinhero, Rao, Paliyath, Murr, & Fletcher, 1997). In brief, 0.5 g leaf samples were extracted by 5 ml 50 mM potassium phosphate buffer (pH 5.5). The extracts were then centrifuged at 10 000 g for 15 min at 4 °C. The reaction mixture contained 1 ml extraction buffer, 5 μl 30% H₂O₂, 5 μl guaiacol and 15 μl supernatant. Enzyme activity is expressed in units of mg⁻¹ protein min⁻¹. One unit of enzyme activity was defined as μM guaiacol oxidation per min. Protein content of the extracts was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a protein standard.

The catalase (CAT, EC 1.11.1.6) activity was assayed according to the method of Hossain, Hasanuzzaman, and Fujita (2010) by monitoring the decrease in absorbance at 240 nm for 1 min caused by the decomposition of H₂O₂ with a spectrophotometer. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 15 mM H₂O₂ and enzyme solution. The activity was defined as the amount of enzyme that oxidised H₂O₂ mg min⁻¹ mg⁻¹ protein.

The superoxide dismutase (SOD, EC 1.15.1.1) activity was measured at 560 nm by the method described in Rico et al. (2013) based on the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). 0.5 g leaf samples were homogenised with 3 ml of 50 mM phosphate buffer (pH 7.8, containing 0.1% polyvinylpyrrolidone), and homogenates were centrifuged at 10,000 rpm for 15 min at 4 °C. The reaction mixture contained 33 mM NBT, 10 mM methionine 0.66 mM EDTA and 3.3 mM riboflavin in 50 mM phosphate buffer, pH 7.8. Reactions were started by switching on the light and allowed to run for 30 min at 25 °C. One unit of enzyme activity was defined as the quantity of SOD required for 50% inhibition of NBT reduction. Control experiments (blank runs) were always carried out using the same procedure, but in the absence of superoxide dismutase.

2.4. Determination of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) concentrations

The level of lipid peroxidation was measured by estimating MDA, a decomposition product of the peroxidised polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the reactive material following the method of Hasanuzzaman, Hossain, and Fujita (2011). 0.5 g leaf samples were homogenised in 3 ml 5% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 11 500 g for 10 min. One ml supernatant was mixed with 4 ml of TBA reagent (0.5% [w/v] of TBA in 20% TCA). The reaction mixture was heated at 95 °C for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged at 11,500g for 15 min. The absorbance of the coloured supernatant

was monitored at 450, 532 and 600 nm. The concentration of MDA was calculated according to the following equation:

$$\text{Concentration}(\mu\text{mol l}^{-1}) = 6.45 \times (\text{OD}_{532} - \text{OD}_{600}) - 0.56 \times \text{OD}_{450}$$

where OD stands for optical density. Concentration of MDA in leaves was expressed as nmol of MDA g⁻¹ fresh weight.

H₂O₂ was assayed according to the method described by Hossain et al. (2010). H₂O₂ was extracted by homogenising 0.5 g of leaf samples with 3 ml of 50 mM K-phosphate buffer pH (6.5) at 4 °C. The homogenate was centrifuged at 11,500g for 15 min. 3 ml of supernatant was mixed with 1 ml of 0.1% TiCl₄ in 20% H₂SO₄ (v/v), and the mixture was then centrifuged at 11,500g for 12 min at room temperature. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm and expressed as $\mu\text{mol g}^{-1}$ fresh weight.

2.5. Determinations of proline, ascorbate, glutathione, total phenolics, flavonoids and DPPH

Proline was measured using the method of Zhao, Chen, Zhang, and Zhang (2009). Briefly, leaf samples were homogenised in 3% sulfosalicylic acid. 2 ml extraction was incubated with 2 ml of ninhydrin reagent (2.5% [w/v] ninhydrin, 60% [v/v] glacial acetic acid, and 40% 6 M phosphoric acid) and 2 ml of glacial acetic acid at 100 °C for 40 min, and the reaction was terminated in an ice bath. Following centrifugation, proline concentration from the supernatant was determined colorimetrically by measuring the absorbance at 515 nm. L-Proline was used as the standard.

Ascorbic acid was determined according to methods of Gupta and Prakash (2009). Briefly, 1.5 g of plant material was blended with 3% metaphosphoric acid, topped up to 10 ml and filtered. 5 ml of metaphosphoric acid extract was taken and titrated with 2,6-dichlorophenol indophenol dye to create a pink end-point that persisted for at least 15 s. The dye factor was calculated by titrating the standard ascorbic acid against the dye. Ascorbic acid was calculated using the titer value and the dye factor taking into account the suitable dilutions that were carried out.

Reduced glutathione (GSH) was assayed according to Comba, Benavides, and Tomaro (1998). Briefly, 0.5 g of leaves was homogenised in 5 ml of 0.1 M HCl (pH 2), and then centrifuged at 10,000 g for 10 min at 4 °C and 200 μl of supernatants were mixed with 800 μl of 0.5 M KH₂PO₄/K₂HPO₄ buffer (pH 8), 1 mM EDTA, 100 μl of 6 mM DTNB [5,5-dithio-bis-(2-nitrobenzoic acid), Sigma, USA] and incubated at 30 °C for 15 min. The absorbance was measured at 412 nm.

The total phenolics in the leaves was measured according to Jin, You, Tang, Wu, and Zheng (2007). Briefly, 1 g of plant material was ground in a mortar to a fine powder with liquid N₂ and extracted with 8 ml of 80% methanol in a sonication bath for 25 min. The solution was centrifuged at 5000g for 10 min. The total phenolic concentration in the supernatant was determined colorimetrically at 750 nm by Folin–Ciocalteu's method using gallic acid for calibration curve.

The concentration of total flavonoids was estimated colorimetrically (Dewanto, Wu, Adom, & Liu, 2002). Briefly, spinach leaves were homogenised with 80% acetone (1:2 w/v). Afterwards, 0.5 ml of plant extract or standard catechin solution was mixed with 1 ml of distilled water and 75 μl of a 5% NaNO₂ solution. After 6 min, 150 μl of a 10% AlCl₃·6H₂O solution was added. Five minutes later, 0.5 ml of 1 M NaOH was added, and the volume was brought up to 2.5 ml with distilled water. The solution was thoroughly mixed and the absorbance was immediately measured at 510 nm. Flavonoid contents were calculated using a standard calibration curve, prepared from catechin. The results are expressed as micrograms of catechin equivalents per gram of spinach.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity of the extract was evaluated using absolute ethanolic solution of DPPH following Kumar et al. (2010). The absorbance of samples (*A*_{sample}) was measured using a spectrophotometer (UV160A, Shimadzu, Japan) at 517 nm against an ethanolic blank. A negative control was run after adding DPPH solution to 0.1 ml of the extraction solvent (70% acetone). A decrease in absorbance at 517 nm showed a reduction of DPPH radicals. The percent inhibition of DPPH radicals by the extract was calculated using the formula:

DPPH free radical scavenging capacity(%)

$$= [1 - (A_{\text{sample}}/A_{\text{control}})] \times 100$$

where *A*_{control} is absorbance of the control and *A*_{sample} is the absorbance of the sample after incubation for 10 min at room temperature.

2.6. Statistic analysis

Values presented in this manuscript were the means of five replicated experiments. All data were processed by analysis of variance and mean separation was performed using *t*-tests at *p* < 0.05 levels.

3. Results

3.1. Effects of trace amounts of gaseous NO on biomass production

Under 200 mmol L⁻¹ NaCl stress, the fresh and dry weights of the edible parts of spinach decreased by 48% and 42%, respectively, compared with the control. However, application of 200 nl l⁻¹ NO reversed NaCl-inhibited growth. It increased the fresh and dry biomasses by 60% and 27% over the NaCl treatment, respectively (Fig. 1). The root growth was affected by either NaCl treatment or NaCl-NO co-treatment, as was growth of the edible parts of the plant (Fig. 1).

Chlorophyll synthesis has also been shown to be impaired under NaCl (Santos, 2004). This trait was also expressed in this study. The chlorophyll concentration, indicated by a SPAD reading, decreased by 34% in NaCl treatment compared with the control treatment (Fig. 2B). Application of NO gas in the NaCl treatment groups restored the chlorophyll synthesis. Alterations of either biomass production or chlorophyll concentration should be associated with an alteration in photosynthetic processes. This assumption was validated by measuring photosynthetic rate. In the NaCl treatment group, the photosynthetic rate decreased by 48% as compared with the control, while in NO-NaCl co-treatment, it was comparable to that in the control (Fig. 2C).

3.2. Effects of trace amounts of gaseous NO on salt-induced oxidative stress

Oxidative stress has been recognised as a mechanism underlying the inhibition of plant biomass production by salt stress. Therefore, the MDA and H₂O₂ levels in leaves were measured. As shown in Fig. 3, 200 mM NaCl treatment resulted in a much higher level of either MDA or H₂O₂ in leaves, compared with the levels in the control group. For MDA, it increased by more than 150%, while for H₂O₂, it increased by about 50%. Application of NO gas to the salt-treated plants lowered the levels of MDA and H₂O₂ in their leaves to 19%. These results indicated that trace amounts of NO gas has a positive effect on plants in terms of reducing oxidative stress.

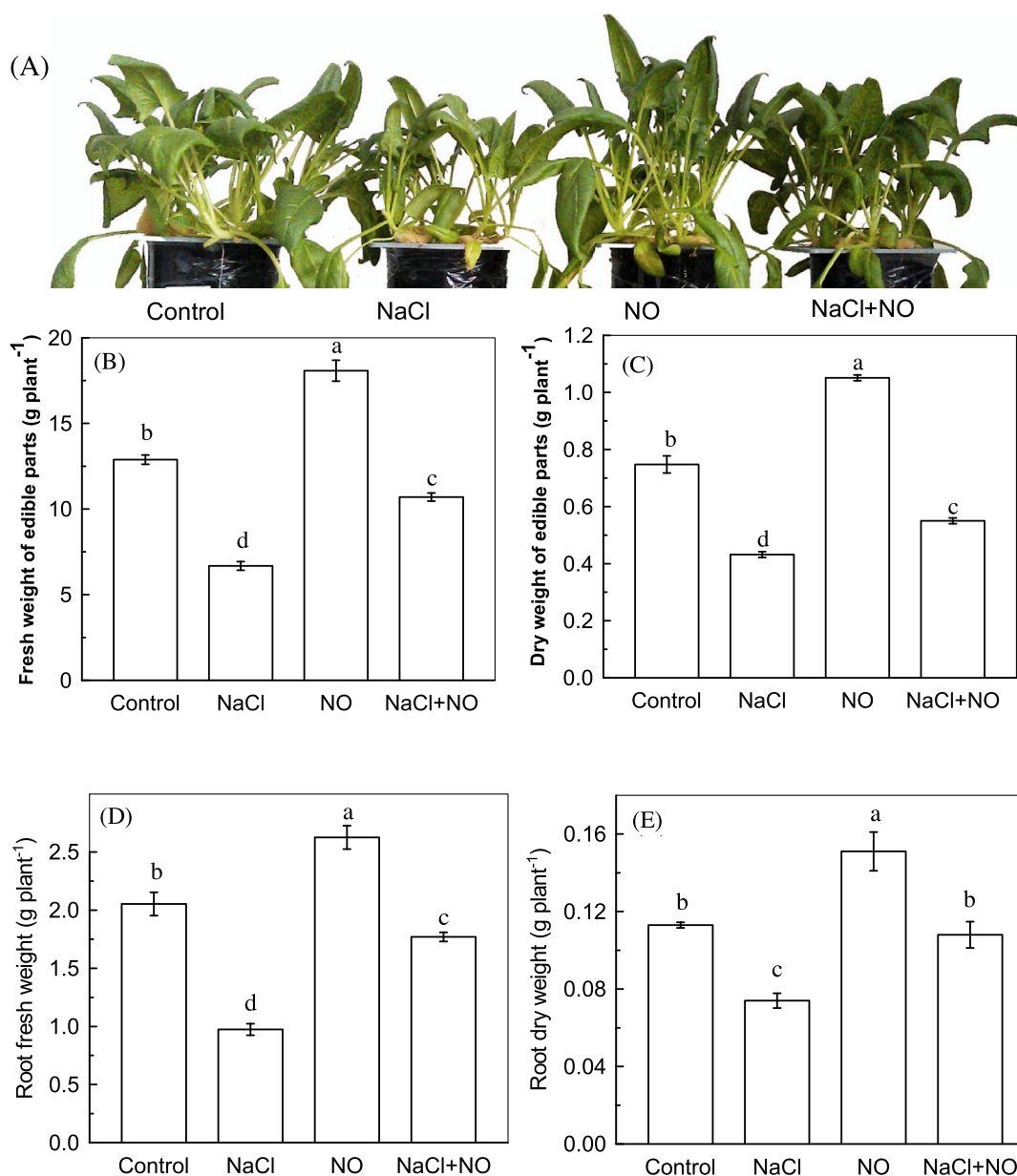


Fig. 1. Effects of NO gas treatments on the growth of spinach under salt stress. (A) The picture of the plants; (B) the fresh weight of the above-ground parts; (C) the dry weight of the edible parts; (D) the fresh weight of root and (E) the dry weight of root. Seedlings were subjected to the following treatments: control (NaCl 0 mmol l⁻¹, atmosphere), NaCl (NaCl 200 mmol l⁻¹, atmosphere), NO (NaCl 0 mmol l⁻¹, NO 200 nl l⁻¹) and NaCl + NO (NaCl 200 mmol l⁻¹, NO 200 nl l⁻¹). The other nutrients were added as presented in M&M. Plants were harvested 21 days after treatments. Values represent the means $\pm$ standard errors ($n = 5$). Different letters denote significant difference at $P < 0.05$.

3.3. Effect of trace amounts of gaseous NO on antioxidant systems under salinity stress

Studies have shown that the elevation of antioxidant capacity is a strategy used to increase plant tolerance to salt stress (Zhu, Wei, Li, Qian, & Yu, 2004). Therefore, the activities of peroxidases (POD), superoxide dismutase (SOD) and catalase (CAT) in leaves were measured (Fig. 4). Under NaCl stress, the activities of all of the three enzymes increased as compared with the control. Application of NO gas showed a further increase in the activities of these antioxidant enzymes in the leaves of NaCl-stressed plants.

Several compounds also play an important role in the antioxidant reactions in plants. Proline is not only an osmotic adjustment substance, but may also help eliminate ROS and improve the antioxidant ability (Ashraf & Foolad, 2007). Under NaCl stress, the proline level in leaves increased by more than 3 folds compared with

the control, while application of NO gas showed a further increase in proline levels by more than one fold. The level of total phenolic content in the leaves of salt-stressed plants was also slightly, but statistically significantly, higher than that of the control plants. Application of NO gas to salt-stressed plants further elevated the level of total phenolics in their leaves by 17%. In contrast to proline and total phenolics, NaCl treatment lowered the levels of ascorbate, glutathione and total flavonoids in leaves by 23%, 16% and 29% respectively, compared with control treatment (Table 1). However, application of NO gas almost completely reversed the above decreases.

DPPH free radical scavenging capacity was used to evaluate the total antioxidant capacity of the plant. As shown in Table 1, NaCl treatment decreased DPPH activity in leaves by 15% as compared with the control. However, application of NO gas elevated the DPPH scavenging activity by 12% in NaCl treatment.

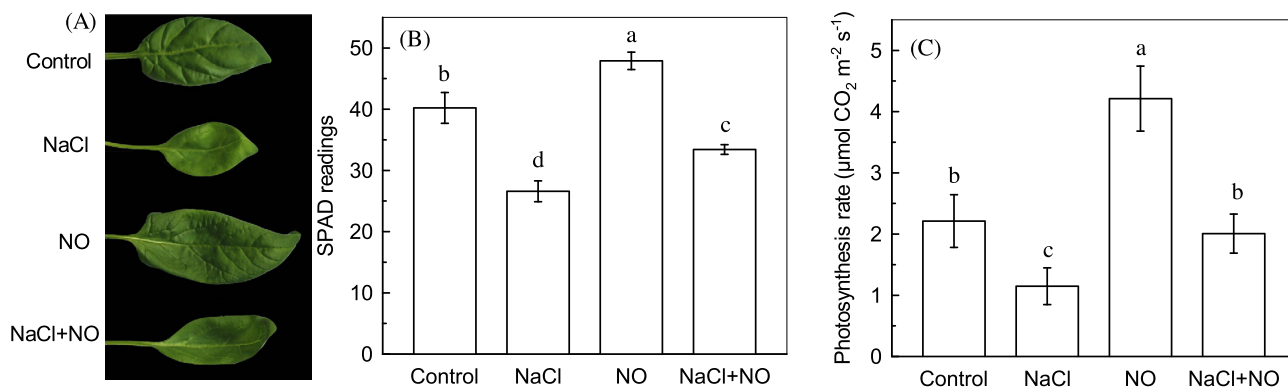


Fig. 2. Effects NO gas treatments on chlorophyll concentration and photosynthetic rate of spinach under salt stress. (A) The picture of the leaves; (B) chlorophyll concentration; (C) photosynthetic rate. Treatments are the same as in Fig. 1. Values represent the means $\pm$ standard errors ($n = 5$). Different letters denote significant difference at $P < 0.05$.

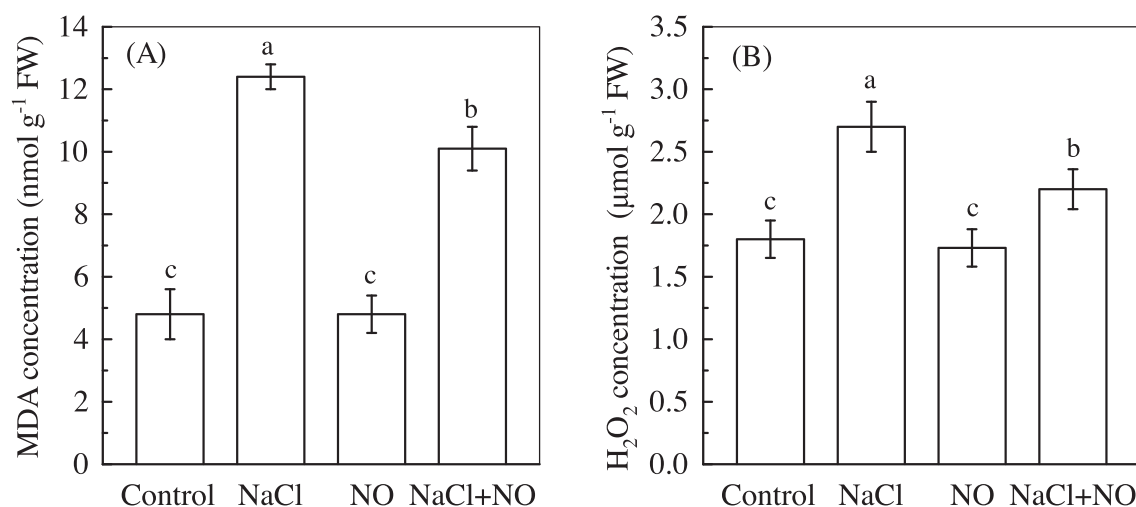


Fig. 3. Effects of NO gas treatments on salt-induced oxidative stress of spinach. Treatments are the same as in Fig. 1. (A) MDA concentration of the leaves; (B) H₂O₂ concentration of the leaves. Values represent the means $\pm$ standard errors ($n = 5$). Different letters denote significant difference at $P < 0.05$.

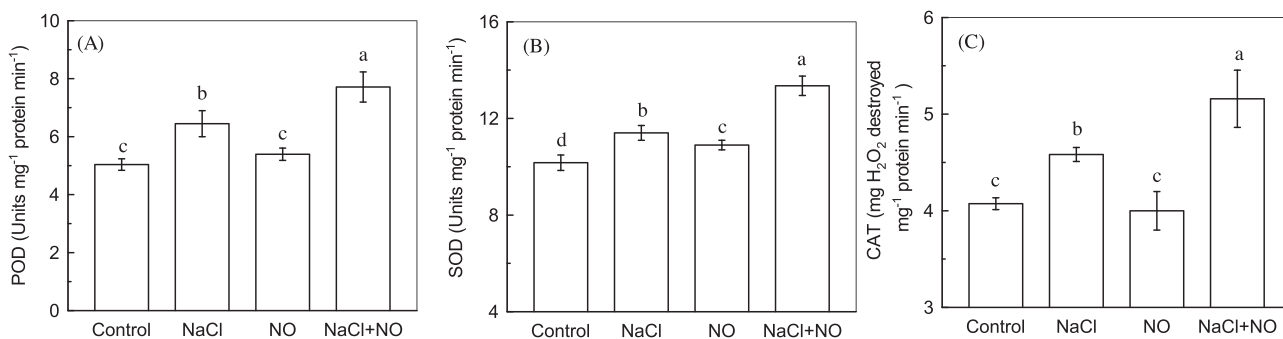


Fig. 4. Effects NO gas treatments on POD (A), SOD (B) and CAT (C) activities of spinach under salt stress. Treatments are the same as in Fig. 1. Values represent the means $\pm$ standard errors ($n = 5$). Different letters denote significant difference at $P < 0.05$.

4. Discussion

High salt conditions cause hyperosmotic as well as ionic stresses that in turn generate secondary effects such as oxidative damage (Zhu, 2000). In plants, the oxidative effect of salinity on important metabolic processes located in cells is well established (Hernández, Olmos, Corpas, Sevilla, & del Río, 1995). One of the most remarkable effects is that it can seriously affect leaf photosynthesis, which is the ultimate source of biomass production in

plants (Chaves, Flexas, & Pinheiro, 2009). Therefore, the extent of salt-induced oxidative stress is thought to be associated with crop yield. Interestingly, under NaCl stress, we observed that although the levels of both MDA and H₂O₂, two indicators of oxidative stress, were significantly elevated, they were restored by application of 200 nl l⁻¹ gaseous NO (Fig. 3). The activities of three antioxidative enzymes POD, SOD and CAT significantly increased under salt stress conditions, as shown in previous reports (Lee, Kim, & Lee, 2001), while application of NO caused a further increase (Fig. 4).

Table 1
Levels of proline, ascorbate, glutathione, total phenolics, flavonoids and DPPH scavenging in the leaves of spinach grown for 21 days under different treatments as described in Fig. 1. Values represent the means $\pm$ standard errors ($n = 5$). Different letters denote significant difference at $P < 0.05$.

Treatment	Proline ($\mu\text{g g}^{-1}$ FW)	Ascorbate ($\mu\text{mol g}^{-1}$ FW)	Glutathione ($\mu\text{mol g}^{-1}$ FW)	Total phenolics ($\mu\text{g gallic acid equivalent g}^{-1}$ FW)	Flavonoids ($\mu\text{mol (+)-catechin equivalent g}^{-1}$ FW)	DPPH scavenging activity (%)
Control	46.6 $\pm$ 0.4d	1.76 $\pm$ 0.21b	0.26 $\pm$ 0.01b	1.92 $\pm$ 0.11c	0.25 $\pm$ 0.02b	58.0 $\pm$ 0.5b
NaCl	202.3 $\pm$ 1.2b	1.35 $\pm$ 0.16c	0.22 $\pm$ 0.01c	2.15 $\pm$ 0.13b	0.18 $\pm$ 0.02c	49.5 $\pm$ 1.16d
NO	56.1 $\pm$ 0.1c	2.45 $\pm$ 0.19a	0.30 $\pm$ 0.02a	2.57 $\pm$ 0.08a	0.29 $\pm$ 0.01a	67.7 $\pm$ 0.4a
NaCl + NO	431.5 $\pm$ 0.3a	1.73 $\pm$ 0.09b	0.25 $\pm$ 0.01b	2.51 $\pm$ 0.17a	0.24 $\pm$ 0.02b	55.5 $\pm$ 0.6c

Moreover, proline level and DPPH free radical scavenging activity, common indicators in evaluating the resistance to oxidation in plants (Bae, Jayaprakasha, Jifon, & Patil, 2012), were all dramatically increased by NO exposure under salt stress conditions (Table 1). These alterations mean that the salt-induced oxidative stress of the plants treated with NO was improved.

The decrease in oxidative load in strengthening the resistance of photosynthesis to stress has been demonstrated in many studies using molecular genetic approaches to either disable antioxidant enzymes, boost their activities or alter the abundance of oxidants or reductants. For example, H_2O_2 has long been recognised as a potent inhibitor of photosynthesis due to its inhibition on CO_2 fixation (Foyer & Shigeoka, 2011); transgenic plants with simultaneous expression of three multiple antioxidant enzymes showed a lower photosynthetic efficiency loss than untransformed plants under stress conditions (Lee et al., 2007). Therefore increases of SPAD readings and photosynthetic rates in plants under salt stress were observed, as expected, in the NO co-treated group (Fig. 2). Accordingly, the plant growth could also be expected to be better due to the above positive alteration. In fact, the combined application of gaseous NO significantly reduced the inhibitory effect of salt stress on both fresh and dry weights of spinach (Fig. 1). Consequently, we considered that the increase of plant production under salt stress by trace amounts of NO gas treatment might be associated with the restoration of photosynthetic processes, which might benefit from the alleviation of oxidative stress.

Additionally, the quality characteristics of vegetables are very important for human health. Ascorbic acid and glutathione are universal reductants and antioxidants in plants (Ahmad, Sarwat, & Sharma, 2008). Phenolics are secondary plant metabolites, and also exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (Halliwell, 1996; Ksouri et al., 2007). Flavonoids also serve as good oxidant scavengers (Cook & Samman, 1996; Wang, Bunce, & Maas, 2003). The above antioxidants have been shown to have beneficial effects on human health by combating damage caused by oxidising agents (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005). Accordingly, the higher levels of ascorbate, glutathione, phenolics and flavonoids in salinity-stressed plants by gaseous NO co-treatment also meet these health requirements (Table 1).

The role of NO as a signal molecule mediating plant responses to abiotic stressors such as salinity (Zhao et al., 2004), drought (García-Mata & Lamattina, 2001), chilling and heat (Uchida, Jagendorf, Hibino, Takabe, & Takabe, 2002) have been well documented. Strong evidence that exogenous NO, applied via the roots in the form of NO donors (e.g. SNP, NONOate, GSNO), provide antioxidant protection against oxidative damage (Uchida et al., 2002). However, these NO donors are either expensive or environmentally toxic and meant for hydroponic cultivation only, which cannot be adopted for agricultural production. In this study, we provide evidence in support of the application of trace amounts of atmospheric NO gas to plants to achieve a positive effect on salt-induced oxidative stress and crop production as well as the NO donor application to roots (Figs. 1 and 3). NO gas could be easily applied in the relative hermetical construction of greenhouses.

Consequently, application of trace amounts of NO might be an attractive approach in agricultural management in salt-affected vegetable greenhouses. Besides the salinity in the growth environment, our previous study also revealed that NO gas application could also significantly stimulate the growth of spinach grown in normal soil (Jin et al., 2009). More than 90% of the greenhouses in China are used for vegetable production (Costa & Heuvelink, 2004). Therefore, our strategy to make use of gaseous NO application in greenhouse production is worth consideration and further study. One thing to be aware of is that our conclusion was supported by hydroponic growth chamber data and the extrapolation to greenhouse soil culture was still a leap.

In conclusion, the present study presents the comparative potential of exogenously applied gaseous NO, in high salinity greenhouses, in counteracting salinity stress, which not only increases vegetable productivity, but also elevates its nutritional qualities. The ability of NO to elevate the plants' antioxidant abilities might be a plausible mechanism for reversing salinity induced photosynthesis inhibition, which would ultimately improve plant growth.

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References

- Ahmad, P., Sarwat, M., & Sharma, S. (2008). Reactive oxygen species, antioxidants and signaling in plants. *Journal of Plant Biology*, 51, 167–173.
- Ashraf, M., & Foolad, M. R. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany*, 59, 206–216.
- Bae, H., Jayaprakasha, G. K., Jifon, J., & Patil, B. S. (2012). Variation of antioxidant activity and the levels of bioactive compounds in lipophilic and hydrophilic extracts from hot pepper (*Capsicum* spp.) cultivars. *Food Chemistry*, 134, 1912–1918.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram of proteins utilizing the principle of protein–dye–binding. *Analytical Biochemistry*, 72, 248–254.
- Chang, J., Wu, X., Liu, A. Q., Wang, Y., Xu, B., Yang, W., et al. (2011). Assessment of net ecosystem services of plastic greenhouse vegetable cultivation in China. *Ecological Economics*, 70, 740–748.
- Chaves, M. M., Flexas, J., & Pinheiro, C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany*, 103, 551–560.
- Chen, Q., Zhang, X. S., Zhang, H. Y., Christie, P., Li, X. L., Horlacher, D., et al. (2004). Evaluation of current fertilizer practice and soil fertility in vegetable production in the Beijing region. *Nutrient Cycling Agroecosystems*, 69, 51–58.
- Comba, M. E., Benavides, M. P., & Tomaro, M. L. (1998). Effect of salt stress on antioxidant defence system in soybean root nodules. *Australian Journal of Plant Physiology*, 25, 665–671.
- Cook, N. C., & Samman, S. (1996). Flavonoids—chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of Nutritional Biochemistry*, 7, 66–76.
- Costa, J. M., & Heuvelink, E. (2004). Protected cultivation rising in China. *Fruit and Vegetable Technology*, 4, 8–11.
- Crawford, N. M., & Guo, F. Q. (2005). New insights into nitric oxide metabolism and regulatory functions. *Trends in Plant Science*, 10, 195–200.

- Darwish, T., Atallah, T., El Moujabber, M., & Khatib, N. (2005). Salinity evolution and crop response to secondary soil salinity in two agro-climatic zones in Lebanon. *Agricultural Water Management*, 78, 152–164.
- Dewanto, V., Wu, X., Adom, K. K., & Liu, R. H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of Agricultural and Food Chemistry*, 50, 3010–3014.
- Dona, A., & Arvanitoyannis, I. (2009). Health risks of genetically modified foods. *Critical Review in Food Science and Nutrition*, 49, 164–175.
- Farooq, M., Siddique, K. H. M., & Schubert, S. (2013). Role of nitric oxide in improving plant resistance against salt stress. In *Ecophysiology and responses of plants under salt stress* (pp. 413–424). New York: Springer.
- Foyer, C. H., & Shigeoka, S. (2011). Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiology*, 155, 93–100.
- García-Mata, C., & Lamattina, L. (2001). Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. *Plant Physiology*, 126, 1196–1204.
- Gupta, S., & Prakash, J. (2009). Studies on Indian green leafy vegetables for their antioxidant activity. *Plant Foods for Human Nutrition*, 64, 39–45.
- Halliwel, B. (1996). Antioxidants in human health and disease. *Annual Review of Nutrition*, 16, 33–50.
- Hasanuzzaman, M., Hossain, M. A., & Fujita, M. (2011). Nitric oxide modulates antioxidant defense and the methylglyoxal detoxification system and reduces salinity-induced damage of wheat seedlings. *Plant Biotechnology Reports*, 5, 353–365.
- Hernández, J. A., Olmos, E., Corpas, F. J., Sevilla, F., & del Río, L. A. (1995). Salt-induced oxidative stress in chloroplasts of pea plants. *Plant Science*, 105, 151–167.
- Hossain, M. A., Hasanuzzaman, M., & Fujita, M. (2010). Up-regulation of antioxidant and glyoxalase systems by exogenous glycinebetaine and proline in mung bean confer tolerance to cadmium stress. *Physiology and Molecular Biology of Plants*, 26, 259–272.
- Jin, C. W., Du, S. T., Zhang, Y. S., Tang, C., & Lin, X. Y. (2009). Atmospheric nitric oxide stimulates plant growth and improves the quality of spinach (*Spinacia oleracea*). *Annals of Applied Biology*, 155, 113–120.
- Jin, C. W., You, G. Y., Tang, C. X., Wu, P., & Zheng, S. J. (2007). Iron-deficiency-induced secretion of phenolics facilitates the reutilization of root apoplastic iron in red clover (*Trifolium pratense* L.). *Plant Physiology*, 144, 278–285.
- Ksouri, R., Megdiche, W., Debez, A., Falleh, H., Grignon, C., & Abdelly, C. (2007). Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte *Cakile maritime*. *Plant Physiology and Biochemistry*, 45, 244–249.
- Kumar, B. S. A., Lakshman, K., Jayaveera, K. N., Shekar, D. S., Kumar, A. A., & Manoj, B. (2010). Antioxidant and antipyretic properties of methanolic extract of *Amaranthus spinosus* leaves. *Asian Pacific Journal of Tropical Medicine*, 3, 702–706.
- Lee, D. H., Kim, Y. S., & Lee, C. B. (2001). The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza Sativa* L.). *Journal of Plant Physiology*, 158, 737–745.
- Lee, Y. P., Kim, S. H., Bang, J. W., Lee, H. S., Kwak, S. S., & Kwon, S. Y. (2007). Enhanced tolerance to oxidative stress in transgenic tobacco plants expressing three antioxidant enzymes in chloroplasts. *Plant Cell Reports*, 26, 591–598.
- Meda, A., Lamien, C. E., Romito, M., Millogo, J., & Nacoulma, O. G. (2005). Determination of the total phenolic, and proline contents in Burkina Faso honey, as well as their radical scavenging activity. *Food Chemistry*, 91, 571–577.
- Meloni, D. A., Oliva, M. A., Martinez, C. A., & Cambraia, J. (2003). Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. *Environmental and Experimental Botany*, 49, 69–76.
- Ondrasek, G., Rengel, Z., & Veres, S. (2011). Soil salinization and salt stress in crop production. In A. Shanker & B. Venkateswarlu (Eds.), *Abiotic stress in plants – Mechanisms and adaptations* (Chapter 8). InTech: Publisher.
- Pinheiro, R. G., Rao, M. V., Paliyath, G., Murr, D. P., & Fletcher, R. A. (1997). Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance of maize seedlings. *Plant Physiology*, 114, 695–704.
- Rico, C. M., Morales, M. I., McCreary, R., Castillo-Michel, H., Barrios, A. C., Hong, J., et al. (2013). Cerium oxide nanoparticles modify the antioxidative stress enzyme activities and macromolecule composition in rice seedlings. *Environmental Science and Technology*, 47, 14110–14118.
- Rosenzweig, C., Iglesias, A., Yang, X. B., Epstein, P. R., & Chivian, E. (2001). Climate change and extreme weather events: implications for food production, plant diseases, and pests. *Global Change and Human Health*, 2, 90–104.
- Santos, C. V. (2004). Regulation of chlorophyll biosynthesis and degradation by salt stress in sunflower leaves. *Scientia Horticulturae*, 103, 93–99.
- Soussi, M., Ocaña, A., & Lluch, C. (1998). Effects of salt stress on growth, photosynthesis and nitrogen fixation in chick-pea (*Cicer arietinum* L.). *Journal of Experimental Botany*, 325, 1329–1337.
- Tanou, G., Job, C., Rajjou, L., Arc, E., Belghazi, M., & Diamantidis, G. (2009). Proteomics reveals the overlapping roles of hydrogen peroxide and nitric oxide in the acclimation of citrus plants to salinity. *Plant Journal*, 60, 795–804.
- Uchida, A., Jagendorf, A. T., Hibino, T., Takabe, T., & Takabe, T. (2002). Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice. *Plant Science*, 63, 515–523.
- Wang, S. Y., Bunce, J. A., & Maas, J. L. (2003). Elevated carbon dioxide increases contents of antioxidant compounds in field-grown strawberries. *Journal of Agricultural and Food Chemistry*, 51, 4315–4320.
- Zhao, L. Q., Zhang, F., Guo, J. K., Yang, Y. L., Li, B. B., & Zhang, L. (2004). Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed. *Plant Physiology*, 134, 849–857.
- Zhao, M. G., Chen, L., Zhang, L. L., & Zhang, W. H. (2009). Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing tolerance in *Arabidopsis*. *Plant Physiology*, 151, 755–767.
- Zhao, M. G., Tian, Q. Y., & Zhang, W. H. (2007). Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in *Arabidopsis*. *Plant Physiology*, 144, 206–217.
- Zhu, J. K. (2000). Genetic analysis of plant salt tolerance using *Arabidopsis*. *Plant Physiology*, 124, 941–948.
- Zhu, Z. J., Wei, G. Q., Li, J., Qian, Q. Q., & Yu, J. Q. (2004). Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (*Cucumis sativus* L.). *Plant Science*, 167, 527–533.