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Effect of sodium nitroprusside on responses of *Melissa officinalis* to bicarbonate exposure and direct Fe deficiency stress

R. AMOOAGHAIE+ and SH. ROOHOLLAHI

Biology Department, Science Faculty, Shahrekord University, Shahrekord, Iran

Abstract

In our study, one-month-old *Melissa officinalis* plants were subjected to Fe-deficiency treatments, such as 10 μM Fe (as direct iron deficiency, DD), and 30 μM Fe + 10 mM $NaHCO_3$ + 0.5 g l^{-1} $CaCO_3$ (as indirect iron deficiency, ID), and 30 μM Fe (as control) for 14 d. Both Fe-deficiency types reduced plant growth, photosynthetic pigment contents, an active Fe content in roots and leaves, root Fe(III)-reducing capacity, Fe-use efficiency, maximal quantum yield of PSII photochemistry, a ratio of variable to basic fluorescence, and activities of antioxidant enzymes, while they increased lipid peroxidation and a H_2O_2 content in leaves. These effects were more pronounced in plants exposed to ID with bicarbonate than those of DD plants. We showed that sodium nitroprusside (SNP), as NO donor, could ameliorate the adverse effects of bicarbonate on above traits. The methylene blue, as NO blocker, reversed the protective effects conferred by SNP in the ID-treated plants as well as DD plants. These findings suggests that NO protects photosynthesis and growth of ID-treated plants as well as DD plants by contribution in availability and/or delivery of metabolically active iron or by changing activities of reactive oxygen species-scavenging enzymes.

Additional key words: bicarbonate; iron; lemon balm; nitric oxide; chlorophyll fluorescence.

Introduction

Iron (Fe) is an important element for a large number of growth and developmental processes, such as chlorophyll (Chl) synthesis, electron transport reactions and oxido-reduction functions, nitrogen fixation, DNA and hormone synthesis (Guerinot 2010). Although Fe is abundant in soils, its availability is limited in alkaline soils and high bicarbonate content in theses soils is the basic factor inducing Fe chlorosis in plants. The typical response of plants using strategy I includes the coordinated induction of a core set of three activities (acidification, reduction, and transport) at the plasma membrane of root epidermal cells following the onset of iron limitation. Acidification of the rhizosphere serves to drive more Fe into solution. Fe transport across the plasmamembrane is initiated by a plasmalemma located Fe(III)-reductase. Its activity is pH dependent and supposed to be much depressed at alkaline pH. In contrast, bicarbonate plays a role of buffer neutralizing protons released by the root H^+-ATPase and inhibiting the root Fe(III)-reductase activity (M'sehli *et al.* 2008, Guerinot 2010, Jelali *et al.* 2010). Besides these harmful effects on the Fe uptake by roots, bicarbonate might lead to Fe inactivation *via* the alkalinization of the leaf apoplast pH (Mengel *et al.* 1994).

Iron deficiency considerably influences photosynthesis. Many components of photosynthesis, such as PSI, PSII, cytochromes b_{563}, *f*, and c_6, ferrodoxin NAD(P)H/PQ oxidoreductase, catalase, and ascorbate peroxidase, require iron (Guerinot *et al*. 2010). The bicarbonate as well as direct Fe deficiency impairs biosynthesis of Chls and carotenoids (Car) (Ding *et al.* 2008, Jelali *et al.* 2010). Leaves with a low Chl content not only have a weak photosynthetic capacity but also absorb more light per Chl. The excessively absorbed light energy could lead to photooxidative process and easily induces accumulation of reactive oxygen species (ROS). ROS production causes oxidative stress that can damage DNA, inactivate

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+Corresponding author; phone: 0098-381-4424419, fax: 0098-381-4424419, e-mail: Rayhanehamooaghaie@yahoo.com
Abbreviations: APX – ascorbate peroxidase; C – control; CAT – catalase; Car – carotenoids; Chl – chlorophyll; DD – direct Fe deficiency; DHAR – dehydroascorbate reductase; DM – dry mass; F_0 – minimal fluorescence yield of the dark-adapted state; F_v/F_m – maximal quantum yield of PSII photochemistry; FM – fresh mass; ID – indirect Fe deficiency; MDA – malondialdehyde; MB – methylene blue; NO – nitric oxide; POX – peroxidase; ROS – reactive oxygen species; SNAP – S-nitroso-N-acetylpenicillamine; SNP – sodium nitroprusside; SOD – superoxide dismutase.
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enzymes, and lead to lipid peroxidation (Morales *et al*. 2006). In order to control ROS concentration, plants possess the antioxidant defense system comprising of enzymes, such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POX), dehydroascorbate reductase (DHAR), and nonenzymatic constituents, such as glutathione, ascorbate, tocopherol, and glutathione, which are scavenging ROS in plants under stress conditions (Gill and Tuteja 2010). Therefore, the regulation of these antioxidant systems by an exogenous substances might contribute to tolerance of bicarbonate or direct iron deficiency in plants.

NO as a signal molecule participates in several growth and developmental processes, as well as in the plant response to biotic and abiotic stresses (Ramireza *et al*. 2011, Amooaghaie and Nikzad 2013, Amooaghaie *et al.* 2015).

There is an increasing amount of evidence about NO participation in iron homeostasis in plants. Graziano *et al.* (2002) showed for the first time that treatment with the artificial NO donors, such as sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) or with a gaseous NO-enriched air, prevented the typical interveinal chlorosis in maize plants grown under direct Fe deficiency. NO inhibitors, 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide potassium salt (cPTIO) and methylene blue (MB), reversed these responses. Moreover, plants of normal growth, grown on sufficient concentrations of iron, become chlorotic when endogenously produced NO was scavenged by cPTIO.

According to literature, several authors have monitored the effects of NO on the improvement of iron homeostasis in plants under direct Fe deficiency conditions (Sun *et al.* 2007, Ding *et al.* 2008, Kumar *et al.* 2010, Garcia *et al.* 2011, Jin *et al.* 2011). However, little studies investigated role of NO in tolerance of bicarbonate-induced Fe deficiency. Also, there is meagre information on NO-mediated modulation of antioxidant responses in bicarbonate-exposed plants. Therefore, in the present study, we compared the effects of NO on iron homeostasis and the changes of the antioxidant enzyme activities in bicarbonate-treated plants with ones in direct Fe-deficient plants. If the role of NO in Fe homeostasis of bicarbonate-treated plants is clearly understood, it might be feasible to engineer plants to be able to grow better in calcareous soils.

Iron deficiency is among the major problems affecting the yield of various species in Iran. Despite the abundance of this micronutrient in the most soils, it is not readily available for roots of many plants, since it is mainly found as stable Fe(III) compounds with low solubility at alkaline pH. Lemon balm (*Melissa officinalis)* is one of medicinal plants sensitive to Fe deficiency and severe Fe symptoms such as chlorosis is often observed in balm plants grown in the field. In this study, balm was selected as the test plant to examine whether nitric oxide could alleviate Fe deficiency stress and modify photosynthesis, iron status, and plant growth under direct or bicarbonate-induced iron efficiency.

Materials and methods

Plant growth conditions and treatments: Balm plants were grown in sand culture in a greenhouse. Sand in 2.5-L pots was washed with glass-distilled water. The seedlings of *M. officinalis* were grown in plastic pots at a temperature of 25 ± 1ºC, a relative humidity (RH) of 75 ± 5%, and a photon flux density at 500 µmol(photon) $m^{-2} s^{-1}$, during a 12-h photoperiod and irrigated with complete nutrient solution for 1 month. The composition of the nutrient solution was: 1.25 mM $Ca(NO_3)_2$, 1.25 mM KNO_3, 0.5 mM $MgSO_4$, 0.25 mM KH_2PO_4, and 10 µM H_3BO_3, 1 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.05 µM $(NH_4)_6Mo_7O_{24}$, and 0.4 µM $CuSO_4$. Iron was supplied in the form of Fe(III)-EDTA. The pH of the nutrient solution was adjusted to 6.5 ± 0.3 before supplying it to the plants.

After 1 month, three treatments were performed for two weeks. Plants in the group 1 continued to receive the complete nutrient solution at presence of 30 µM iron (Fe-sufficient control: C). Plants in the group 2 grew at presence of 10 µM iron (direct iron deficiency: DD), and plants in the group 3 received nutrient solution containing 30 µM Fe in the form of Fe (III)-EDTA and 0.5 g$(CaCO_3)$ L^{-1} + 10 mM $NaHCO_3$ (indirect iron deficiency: ID) added to the nutrient solution to mimic the effect of a calcareous soil. The pH was adjusted to 6.5 with NaOH for both C and DD treatments and at 8.5 for the ID treatment.

In each three groups, plants were divided further into four lots, each having three pots. Plants in lots 1, 2, 3 per each group, respectively, were supplied with 0, 50, or 100 µM sodium nitroprusside (SNP), as exogenous NO donor. Plants in lot 4 received 100 µM SNP plus 100 µM methylene blue (as NO blocker) in the nutrient solution. The nutrient solution was re-supplied every morning and pots were flushed each week with deionized water to remove root exudates. Measurements were made on fully expanded young leaves of plants 14 d after initiating various treatments.

Plant growth and active iron content determination: After 14 d of treatments, balm plants were harvested and root and shoot fresh masses (FM) were determined. Then, samples were dried at 60ºC for 72 h and root and shoot dry mass (DM) was measured.

For determining active Fe, extracts were prepared by homogenizing 1 g of fresh leaf or root material in 10 ml of 1 N HCl. The absorbance of the solution at 510 nm was determined by an atomic absorption spectrophotometer (*UV-2550, Shimadzu,* Japan).

The shoot Fe-use efficiency was defined as the ratio of shoot biomass [mg] to shoot Fe concentration [µg(Fe)].

Chl *a* fluorescence was measured on the second leaf of lemon balm plant by a portable pulse-amplitude modulated photosynthesis yield analyzer *(Mini-PAM, Heinz Walz,* Effeltrich, Germany*)* according to the protocol described by Kumari *et al.* (2005). All samples were dark-adapted for 20 min prior to fluorescence measurements. The intensity of saturation pulse was 6,000 µmol(photon) m^{-2} s^{-1} and pulse length was 0.8 s. Chl fluorescence was measured using actinic irradiation of 204 µmol(photon) m^{-2} s^{-1}. After the first saturation pulse, we obtained the minimal fluorescence (F_0) and maximal fluorescence (F_m), then calculated maximal photochemical efficiency of PSII reaction centers (F_v/F_m) based on variable fluorescence (F_v) = F_m – F_0. At least four individual plants were analyzed for Chl fluorescence for every time point or experimental treatment.

Car and Chl content: Chl *a*, Chl *b*, and Car contents were determined in fresh leaf samples (0.2 g) from randomly selected plants per replicate. The samples were homogenized with 5 ml of acetone (80%, v/v) using a mortar and pestle before being filtered through *Whatman No. 2* filter paper. The absorbance was measured using a UV-visible spectrophotometer (*UV-2550, Shimadzu,* Japan) at 663, 645, and 470 nm and the contents were calculated using following formula of Lichtenthaler (1987).

$$\text{Chl } a = 12.21 \times A_{665} - 2.81 \times A_{649}$$
$$\text{Chl } b = 20.13 \times A_{649} - 5.03 \times A_{665}$$
$$\text{Car} = [1{,}000 \times (A_{470} - 27) \times (\text{Chl } a - 104) \times \text{Chl } b]/229$$

Lipid peroxidation and H_2O_2: Method of Heath and Packer (1968) for measurement of malondialdehyde (MDA) was followed to determine lipid peroxidation levels. MDA is one of several low-molecular-mass end products formed *via* the decomposition of certain primary and secondary lipid peroxidation products. Leaf samples (0.5 g) were homogenized in 10 ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 15,000 × *g* for 10 min. Supernatant was mixed with 4.0 ml of 0.5% thiobarbituric acid, heated at 95ºC for 30 min, and then quickly cooled in an ice bath. After centrifugation at 10,000 × *g* for 10 min, the absorbance was recorded at 532 nm and nonspecific absorbance at 600 nm (*UV-2550, Shimadzu,* Japan) was subtracted. The MDA content was calculated using its absorbance coefficient of 155 $mmol^{-1}$ cm^{-1} and expressed as µmol(MDA) per gram of FM.

Method of Mukherjee and Choudhuri (1985) was followed for hydrogen peroxide measurements using spectrophotometr (*UV-2550, Shimadzu, Japan*) at 390 nm after reaction with potassium iodide. Supernatant of 0.5 ml of 0.1% TCA leaf extracts, 0.5 ml of 100 mM K-phosphate buffer, and 2 ml of reagent [1 M KI (w/v) in fresh double-distilled water] was used. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H_2O_2.

Ferric-chelate reductase activity in the roots was determined according to Chouliaras *et al*. (2004). Apical root segments rinsed several times in distilled water were transferred to a beaker containing 0.2 mM $CaSO_4$. After 5 min, roots were rinsed several times in distilled water and were incubated in dark at 23°C for 120 min in 5 ml of a solution with following composition: 0.2 mM $CaSO_4$, 0.1 mM Fe(III)-EDTA, and 0.3 mM Na-2-bathophenanthrolinedisulfonic acid (BPDS). The solution was buffered at pH 5.5 with 5 mM 2-(N-morpholino) ethanesulfonic acid (MES). Root-reducing capacity was expressed as the concentration of the resulting formation of Fe(II)-BPDS in the solution, and was estimated at 535 nm (*UV-2550, Shimadzu, Japan*) using a molecular extinction coefficient of 22.14 mM^{-1} cm^{-1}. The control consisted of a complete solution without roots (Chouliaras *et al*. 2004). Activity was expressed in µmole g^{-1}(DM) h^{-1}.

Antioxidant enzyme activities: Briefly, 0.1 g sample was homogenized in an ice bath and a pre-chilled mortar and pestle with 1.5 ml of extraction buffer [50 mM phosphate buffered saline (PBS, pH 7.8) and 4% polyvinyl polypyrroidone (PVP)]; after centrifugation (10,000 × *g* for 20 min at 4ºC) supernatant was collected and analyzed to determine activities of antioxidant enzymes. Catalase (CAT, EC 1.11.1.6) activity was determined spectrophotometrically at 240 nm (*UV-2550, Shimadzu,* Japan) using the method described by Aebi (1984). One unit of CAT gives a H_2O_2 decomposition rate of 1 µmol min^{-1} at 25ºC. The peroxidase (POX, EC 1.11.1.7) activity was determined using the method described by Doerge *et al*. (1997). The increase in the absorption at 470 nm (*UV-2550, Shimadzu,* Japan) was countinusely detected after adding H_2O_2. A unit of guaiacol peroxidase activity was defined as enzyme content which causes the formation of 1 µM tetraguaiacol in min. The activities of ascorbate peroxidase (APX, EC 1.11.1.11) and superoxide dismutase (SOD, EC 1.15.1.1) were measured using a procedure modified from that described previously by Nakano and Asada (1981). One unit APX activity was defined as oxidized ascorbic acid at a rate of 1 µmol min^{-1} at 25ºC. The SOD activity was defined as the amount of enzyme that inhibited 50% of NBT photoreduction. Activities of all enzymes were recorded as unit per gram of fresh mass [U g^{-1}(FM)].

Nitric oxide: NO production was determined by using Greiss reagent according to the method described by Zhou *et al*. (2005) with slight modifications. Samples were ground in a mortar and pestle in 3 ml of 50 mM cool acetic acid buffer (pH 3.6, containing 4% zinc diacetate). The homogenates were centrifuged at 10,000 × *g* for 15 min at 4ºC. The supernatant was collected. The pellet was washed by 1 ml of extraction buffer and centrifuged again. The two supernatants were combined and 0.1 g of charcoal was added. After vortexing and filtration, the filtrate was

leached and collected. The mixture of 1 ml of filtrate and 1 ml of the Greiss reagent was incubated at room temperature for 30 min. Meanwhile, another identical filtrate which was pretreated with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO), the specific scavenger of NO, for 15 min, was used as a blank control. Absorbance was assayed at 540 nm (*UV-2550, Shimadzu,* Japan). The NO content was estimated according to a standard curve of $NaNO_2$.

Statistical analysis: The whole experiment was set as factorial with completely randomized design with three replications. *SPSS version 13* was used to run analysis of variance (*ANOVA*). *Duncan*'s multiple range tests were used to compare the treatment means. Significance level was set at $P<0.05$.

Results

Plant growth: After 14 d, ID or DD reduced shoot FM by 40 and 65% and shoot DM by 37 and 58%, respectively. Exposure to DD and ID also declined root FM by 15 and 43.3%, and root DM by 25 and 40%, respectively (Fig. 1).

Exposure to 50 μM SNP improved root or shoot biomass significantly under DD and ID (Fig. 1). However, 100 μM SNP had a more noticeable effect on root and shoot growth. Application of 100 μM SNP increased shoot DM by 1.18, 1.89, and 2.5 folds in plants of C, DD, or ID groups when compared with the SNP-untreated plants in each group. Shoot FM and root FM and DM also illustrated the same tendency. The reverse trend for these attributes was evident in SNP + MB treatments under DD and ID exposure (Fig. 1).

Exposure to DD and ID increased the root/shoot ratio significantly. Indeed, application of 100 μM SNP increased the root/shoot ratio by 25 and 41% under DD and ID exposure, respectively. However, the application of 50 μM SNP did not affect the root/shoot ratio in C group (Fig. 2).

Photosynthetic pigments: The younger leaves were more drastically affected by iron deficiency than the older ones. Exposure to DD and ID declined contents of Chl *a* by 32 and 50% and of Chl *b* by 22 and 38%, respectively. Addition of 100 μM SNP, as NO donor, completely prevented leaf chlorosis, producing 1.7 and 2.34 fold increase in Chl *a*, and 1.2 and 1.5 fold increase in Chl *b*, respectively, in the plants subjected to DD and ID (Fig. 3*A*,*B*). Application of MB, as NO blocker, reversed the effect of the NO donor on photosynthetic pigment

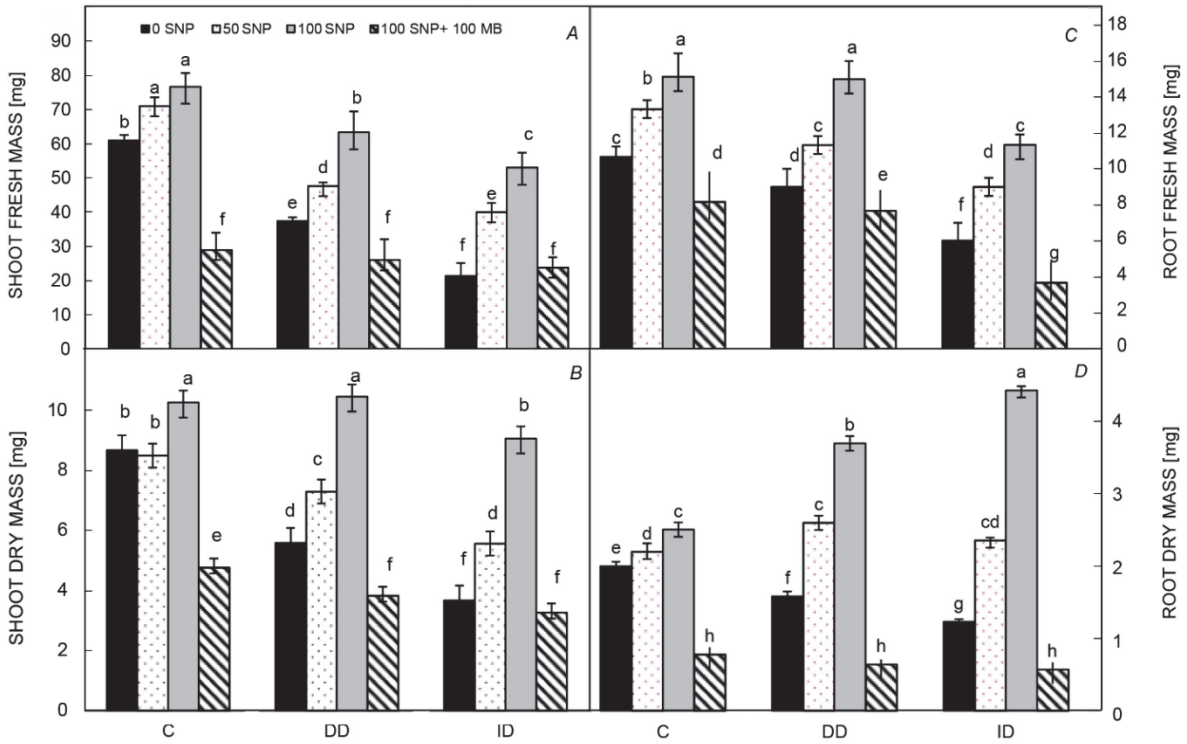

Fig. 1. Effect of various concentrations of sodium nitroprusside (SNP) on dry and fresh mass of roots and shoots in *Melissa officinalis* under bicarbonate exposure or direct iron deficiency. One-month-old plants subjected to three treatments: 30 μM Fe (as control: C), 10 μM Fe (as direct iron deficiency: DD), and 30 μM Fe + 10 mM $NaHCO_3$ + 0.5 g($CaCO_3$) L^{-1} (as indirect iron deficiency: ID) for 14 d. Some plants per each group were treated with 50 or 100 μM SNP, 100 μM SNP plus 100 μM methylene blue (MB). Bars with *different letters* are significantly different at $P<0.05$ according to *Duncan*'s multiple range tests.

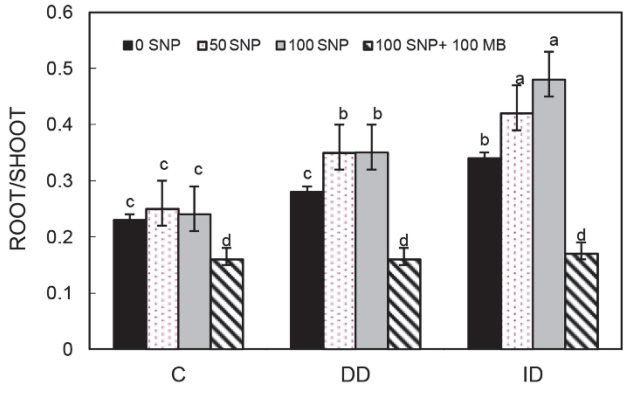

Fig. 2. Effect of various concentrations of sodium nitroprusside (SNP) on the root/shoot ratio in *Melissa officinalis* under bicarbonate exposure or direct iron deficiency. One-month-old plants subjected with three treatments: 30 μM Fe (as control: C), 10 μM Fe (as direct iron deficiency: DD), and 30 μM Fe + 10 mM $NaHCO_3$ +0.5 g($CaCO_3$) L^{-1} (as indirect iron deficiency: ID) for 14 d. Some plants per each group were treated with 50 or 100 μM SNP, 100 μM SNP plus 100 μM methylene blue (MB). Bars with *different letters* are significantly different at P<0.05 according to *Duncan*'s multiple range tests.

contents in both DD and ID (Fig. 3*A*,*B*). Both DD and ID exposure slightly reduced the ratio of Chl *a*/*b*, while NO supply increased this ratio significantly (Fig. 3*C*). Similar to Chl, Car contents were also greatly reduced by both treatments, while addition of SNP improved Car contents under C or DD and ID (Fig. 3*D*).

Active iron content and Fe-use efficiency: In plants subjected to DD and ID, active iron content in leaves decreased by 44 and 64.8%, respectively (Fig. 4*A*). Similar tendency was observed for active iron content in roots. ID exposure significantly declined ferric chelate reductase activity in roots (Fig. 4 *D*). SNP application increased Fe content in roots (Fig. 4*B*) and root Fe(III)-reducing capacity (Fig. 4 *D*) of Fe-deficient plants of balm, especially under ID exposure. The negative effects of DD and ID on active iron content of leaves (Fig. 4*A*) and Fe-use efficiency (Fig. 4*C*) were also improved by application of 100 μM SNP. For example, when compared to DD and ID treatments alone, the treatment with 100 μM SNP resulted in 26.6 and 84.2% increase in active iron content and in 45 and 52.9% increase in Fe-use efficiency, respectively. In order to assess whether endogenous NO is involved in responses induced by SNP, the NO blocker, MB, was applied. As shown in Fig. 4, a significant decrease of active iron content in roots and leaves as well as Fe-use efficiency and root Fe(III)-reducing capacity were recorded in the plants exposed to DD and ID, when MB was added (Fig. 4*A*–*D*). It correlared with the data from shoot biomass and Chl content.

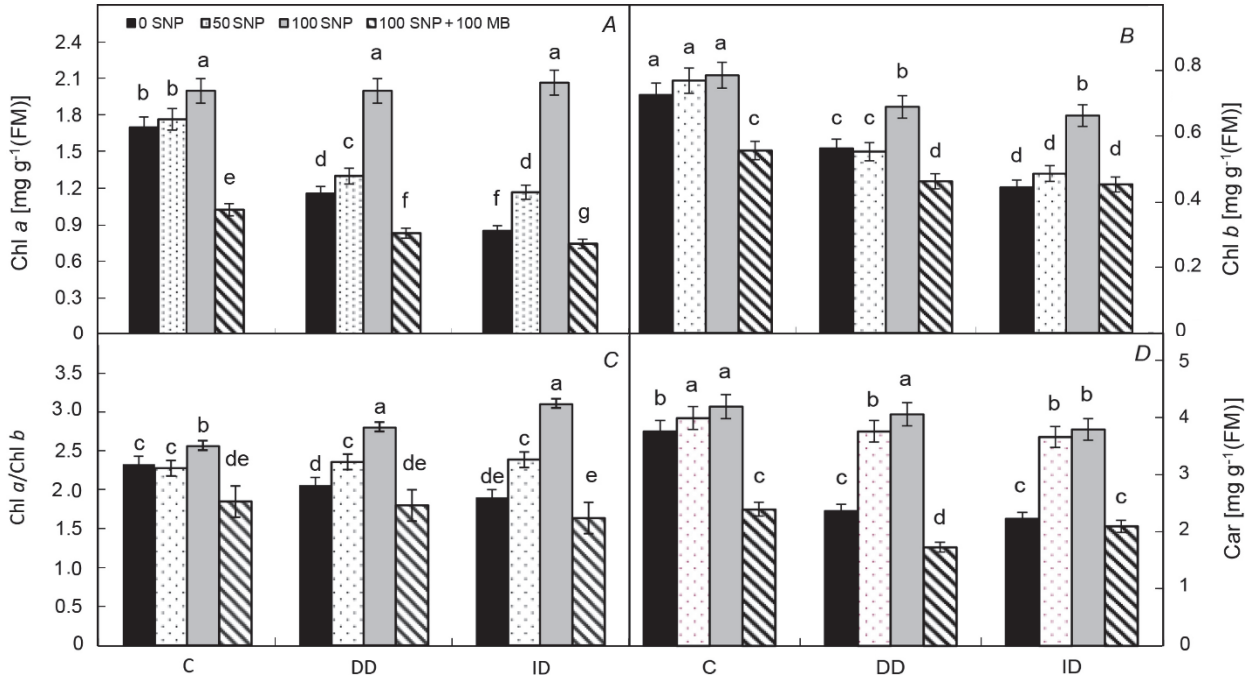

Fig. 3. Effect of various concentrations of sodium nitroprusside (SNP) on chlorophyll (Chl) *a*, Chl *b*, and carotenoids contents and the ratio of Chl *a*/*b* in leaves of *Melissa officinalis* under bicarbonate exposure or direct iron deficiency. One-month-old plants subjected to three treatments: 30 μM Fe (as control: C), 10 μM Fe (as direct iron deficiency: DD), and 30 μM Fe + 10 mM $NaHCO_3$ + 0.5 g L^{-1} $CaCO_3$ (as indirect iron deficiency: ID) for 14 d. Some plants per each group were treated with 50 or 100 μM SNP, 100 μM SNP plus 100 μM methylene blue (MB). Bars with *different letters* are significantly different at P<0.05 according to *Duncan*'s multiple range tests.

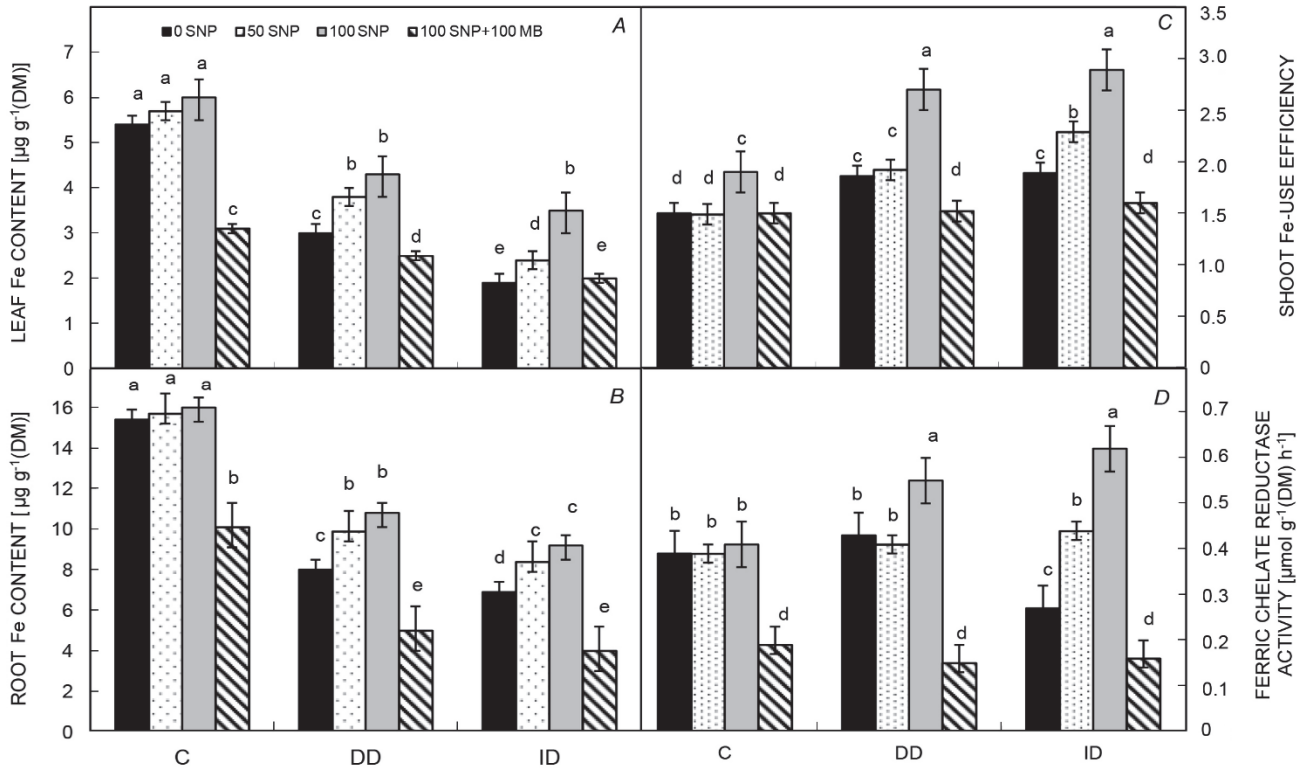

Fig. 4. Effect of various concentrations of sodium nitroprusside (SNP) on active Fe content in root and leaves and shoot Fe-use efficiency and ferric chelate reductase activity of roots in *Melissa officinalis* under bicarbonate exposure or direct iron deficiency. One-month-old plants subjected to three treatments: 30 µM Fe (as control: C), 10 µM Fe (as direct iron deficiency: DD), and 30 µM Fe + 10 mM $NaHCO_3$ + 0.5 g($CaCO_3$) L^{-1} (as indirect iron deficiency: ID) for 14 d. Some plants per each group were treated with 50 or 100 µM SNP, 100 µM SNP plus 100 µM methylene blue (MB). Bars with *different letters* are significantly different at $P<0.05$ according to *Duncan*'s multiple range tests.

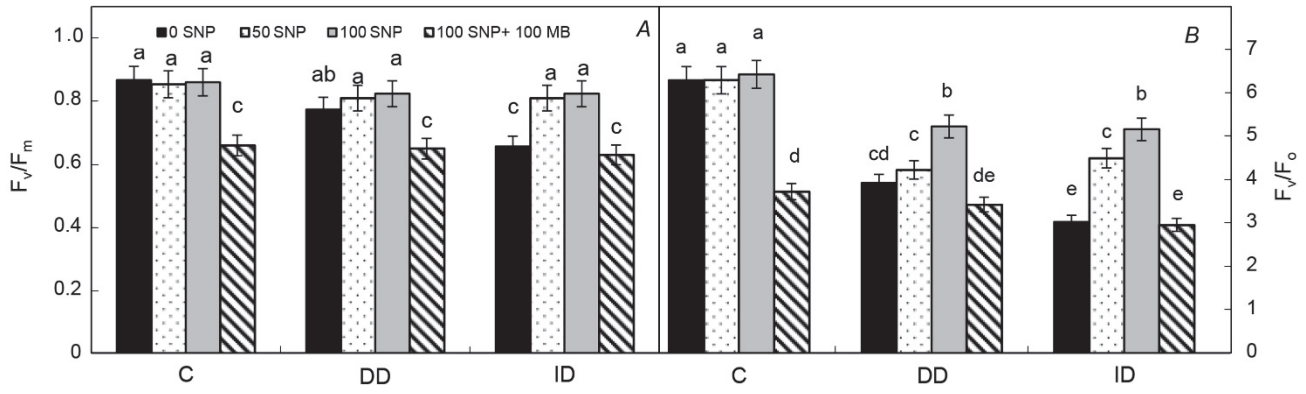

Fig. 5. Effect of various concentrations of sodium nitroprusside (SNP) on chlorophyll fluorescence parameters (F_v/F_m and F_v/F_0) in leaves of *Melissa officinalis* under bicarbonate exposure or direct iron deficiency. One-month-old plants subjected to three treatments: 30 µM Fe (as control: C), 10 µM Fe (as direct iron deficiency: DD), and 30 µM Fe + 10 mM $NaHCO_3$ + 0.5 g($CaCO_3$) L^{-1} (as indirect iron deficiency: ID) for 14 d. Some plants per each group were treated with 50 or 100 µM SNP, 100 µM SNP plus 100 µM methylene blue (MB). Bars with *different letters* are significantly different at $P<0.05$ according to *Duncan*'s multiple range tests.

Chl fluorescence parameters: As shown in Fig. 5, F_v/F_m and F_v/F_0 significantly declined by both Fe deficiency types. The higher decrease was observed in the ID plants. For example, DD and ID exposure lowered F_v/F_m values by 10.4 and 24.4%, respectively, and F_v/F_0 values by 38 and 52.4%, respectively, in comparison with to C. As expected, results showed that SNP was able to alleviate negative effects of DD and ID on F_v/F_m and F_v/F_0 in a dose-dependent manner (Fig. 5). For instance, the ratio of F_v/F_m was 0.67 in leaves under ID and 100 µM SNP

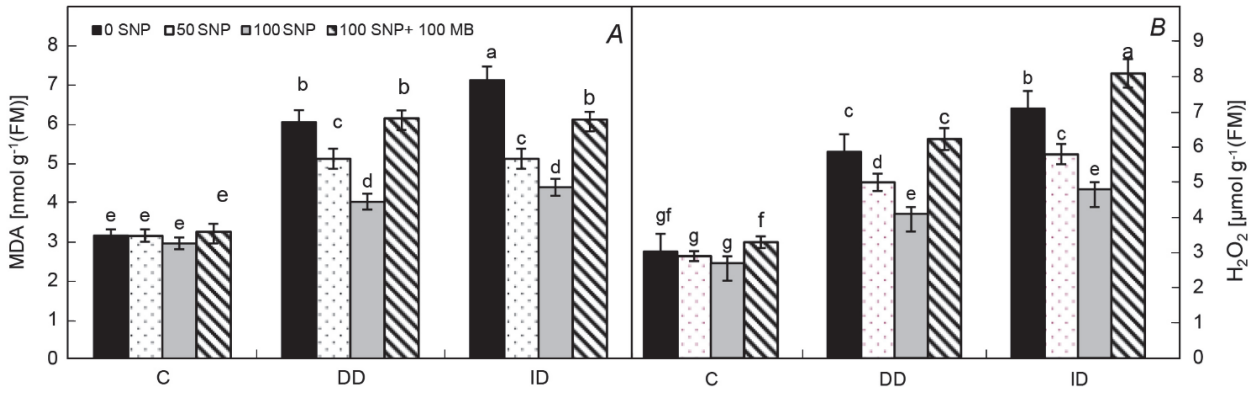

Fig. 6. Effect of various concentrations of sodium nitroprusside (SNP) on lipid peroxidation (MDA content) and H_2O_2 content in leaves of *Melissa officinalis* under bicarbonate exposure or direct iron deficiency. One-month-old plants subjected to three treatments: 30 μM Fe (as control: C), 10 μM Fe (as direct iron deficiency: DD), and 30 μM Fe + 10 mM $NaHCO_3$ + 0.5 g($CaCO_3$) L^{-1} (as indirect iron deficiency: ID) for 14 d. Some plants per each group were treated with 50 or 100 μM SNP, 100 μM SNP plus 100 μM methylene blue (MB). Bars with *different letters* are significantly different at $P<0.05$ according to *Duncan*'s multiple range tests.

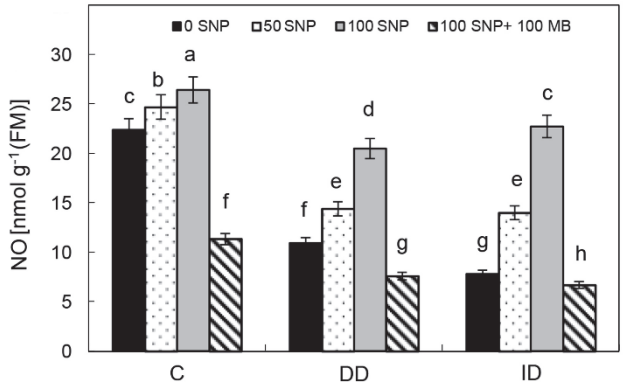

Fig. 7. Effect of various concentrations of sodium nitroprusside (SNP) on NO content in leaves of *Melissa officinalis* under bicarbonate exposure or direct iron deficiency. One-month-old plants subjected to three treatments: 30 μM Fe (as control: C), 10 μM Fe (as direct iron deficiency: DD), and 30 μM Fe + 10 mM $NaHCO_3$ + 0.5 g($CaCO_3$) L^{-1} (as indirect iron deficiency: ID) for 14 d. Some plants per each group were treated with 50 or 100 μM SNP, 100 μM SNP plus 100 μM methylene blue (MB). Bars with *different letters* are significantly different at $P<0.05$ according to *Duncan*'s multiple range tests.

supplementation increased the ratio to 0.82, which was similar to the C plants. However, application of 100 μM SNP alleviated the effects of ID and the ratio F_v/F_0 reached to 5. In contrast, application of MB in the presence of SNP decreased F_v/F_m and F_v/F_0 significantly under both DD and ID exposure (Fig. 5). These results suggested that changes in endogenous NO were likely involved in SNP-induced responses and that NO production might occur in plants and play an important role in this process.

Lipid peroxidation and H_2O_2 content: In plants subjected to DD or ID, the content of H_2O_2 and MDA were higher than in C (Fig. 6*A*,*B*). Exposure to DD and ID increased MDA by 1.9 and 2.22 fold compared with C leaves (Fig. 6*A*). However, 100 μM SNP significantly reduced the contents of H_2O_2 by about 29 and 38% under DD and ID, respectively. Lipid peroxidation also illustrated the same tendency (Fig. 6*A*). The application of MB associated to SNP reversed above mentioned effects and increased H_2O_2 and MAD contents under DD and ID exposure.

NO content: We measured the NO content in balm leaves in order to evaluate the role of NO in improving the growth of balm plants treated by DD and ID. Both treatments reduced the NO content in leaves. The content of NO in leaves treated with SNP was higher than that of C plants with SNP. In plants treated by DD and ID, the content of NO in leaves was 2 and 2.8 fold lower than that of C. As shown in Fig. 7, addition of 100 μM SNP, as NO donor, in plants subjected to DD and ID caused a 1.88 and 2.9 fold increase in NO content, respectively, and a significant decrease in the NO content was recorded in plants exposed to DD and ID, when MB was added.

The activities of antioxidant enzymes SOD, APX, POX, and CAT decreased approximately by 33.7, 29.2, 32.3, and 38.6%, respectively, under DD and by 31.9, 42.4, 42, and 51.2% under ID, respectively (Fig. 8*A–D*). Therefore negative effects of ID exposure on APX, POX, and CAT activities were significantly more pronounced than those of DD, but difference between effects of two treatments on SOD activity was not significant. The treatment with NO resulted in significantly increased activities of SOD, APX, POX, and CAT in leaves under both iron deficiency types. Application of 100 μM SNP increased the activities of SOD, APX, POX, and CAT approximately 1.4, 1.9, 1.7, and 2.3 fold, respectively, under DD and 1.4, 2.26, 1.85, and 2.6 fold under ID exposure, respectively (Fig. 8*A–D*). However, application of MB plus SNP significantly reduced the activities of SOD, APX, POX, and CAT under DD and ID exposure (Fig. 8).

Discussion

Bicarbonate is considered as one of the most important factors causing Fe chlorosis in „strategy I“ plants, mainly on calcareous soils. As anticipated by several authors (Alcántaraa *et al*. 2000, Pestana *et al*. 2005, Jelali *et al*. 2010), bicarbonate or decreasing Fe adversely affected the growth of balm plants (Fig. 1). As reported for *Pisum sativum* (Jelali *et al*. 2010), visual symptoms of chlorosis as well as Chl content were more pronounced in balm plants exposed to ID as compared to the DD plants (Fig. 3). The stronger leaf chlorosis of plants subjected to bicarbonate correlated with further decrease in active Fe concentration of their leaves (Fig. 4*A*), which suggested ID chlorosis resulting from restrictive effect of bicarbonate on iron availability. Besides, we added $CaCO_3$ for mimicking of condition in calcareous soils and the presence of Ca might lead to Fe precipitation which was increasing Fe unavailability and deficiency. The decrease of Chl contents might occur due to a role of Fe in the synthesis of γ-aminolevulinic acid and protochlorophyllide, the precursors of Chl biosynthesis (Guerinot 2010). Although, Xu *et al*. (2009) previously reported nitric oxide alleviated chlorosis in *Solanum nigrum* under direct Fe deficiency, our results showed for the first time that the ID-treated plants with 100 μM SNP exhibited the large increase in their Chl (Fig. 3*A*,*B*) and Car contents (Fig. 3*D*) and looked similar to the Fe-sufficient C plants.

As expected, the decline of active Fe content in leaves (Fig. 4*A*) and roots (Fig. 4*B*) of balm was more pronounced in presence of bicarbonate than direct Fe deficiency which might be relevant to more restrictive effect of bicarbonate on Fe(III)-reducing capacity of roots (Fig. 4*D*). Several authors (Alcántaraa *et al.* 2000, Chouliaras *et al.* 2004, Romera *et al*. 1997) also revealed that bicarbonate application decreased root Fe(III)-reducing capacity. Most of negative effects of bicarbonate have been attributed to its capacity to buffer high pH in soils, which can diminish both Fe solubility and root ferric reductase activity in Fe-deficient strategy I plants (M'sehli *et al*. 2008). Romero *et al*. (1997) illustrated that bicarbonate may inhibit the development of root Fe(III)-reducing capacity by diminishing the availability of certain metal ions required for this process. Lucena *et al*. (2007) demonstrated bicarbonate could induce Fe chlorosis by inhibiting the expression of ferric reductase, iron transporter, and H^+-ATPase genes. Our data also showed lower shoot Fe-use efficiency (Fig. 4*C*) in the ID-treated plants, indicating lower translocation rate of Fe from roots to shoots in these plants. Similar to our results, Nikolic *et al*.

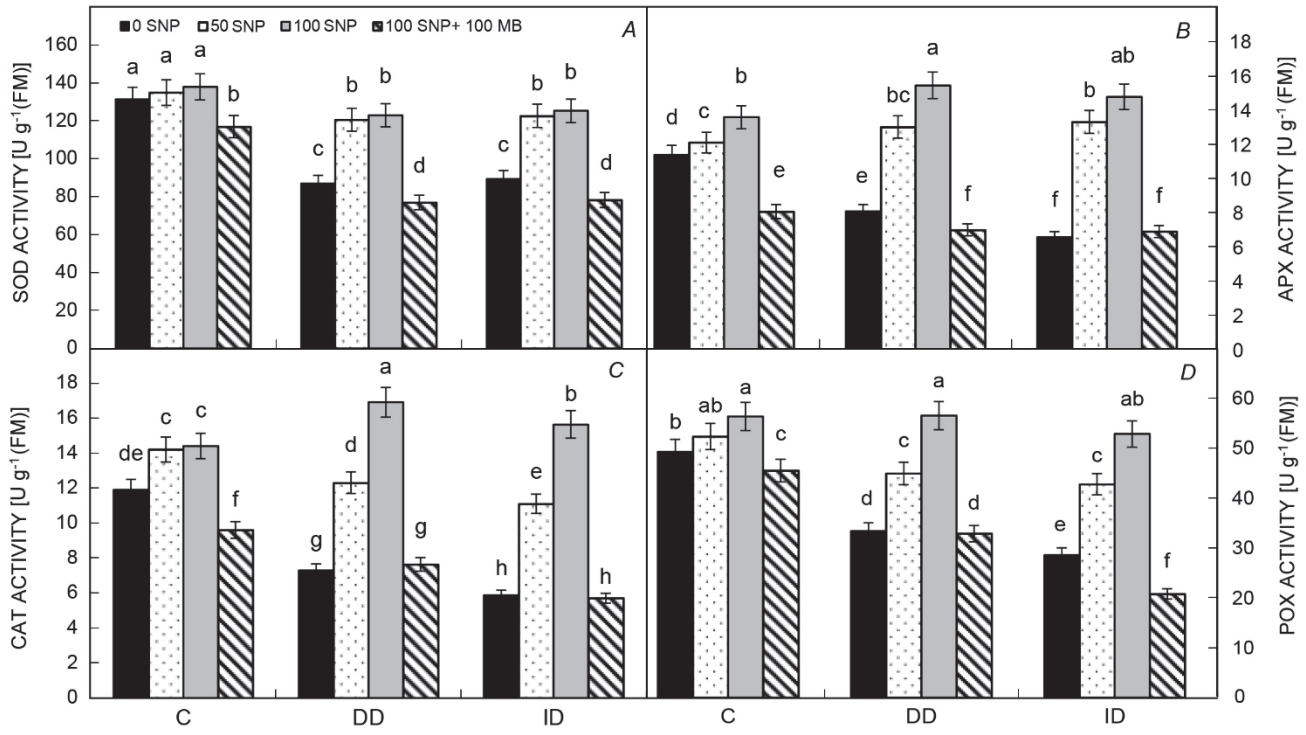

Fig. 8. Effect of various concentrations of sodium nitroprusside (SNP) on activities of antioxidant enzymes in leaves of *Melissa officinalis* under bicarbonate exposure or direct iron deficiency. One-month-old plants subjected to three treatments: 30 μM Fe (as control: C), 10 μM Fe (as direct iron deficiency: DD), and 30 μM Fe + 10 mM $NaHCO_3$ + 0.5 g($CaCO_3$) L^{-1} (as indirect iron deficiency: ID) for 14 d. Some plants per each group were treated with 50 or 100 μM SNP, 100 μM SNP plus 100 μM methylene blue (MB). Bars with *different letters* are significantly different at $P<0.05$ according to *Duncan*'s multiple range tests.

(2000) also found that bicarbonate reduced the Fe(III)-reducing capacity, ^{59}Fe uptake and translocation rate in *Vitis* genotypes and led to higher precipitation of Fe(III) as hydroxides or phosphates in the root.

In our study, SNP application improved active iron content of leaves and chlorosis in the ID-treated plants as well as DD plants (Fig. 4*A*). Graziano and Lamattina (2005) have suggested that NO might be involved in alleviation of iron chlorosis through at least two possible mechanisms: (*1*) contributing to the reduction of Fe^{3+} to Fe^{2+}, favoring the form of iron that is acquired, and (*2*) through the formation of dinitrosyl–iron complexes (DNICs) probably linked to glutathione, which could facilitate iron transport through cell membranes. Interestingly, our data for the first time showed that in presence of bicarbonate, available Fe concentration (Fig. 4*B*) as well as the root Fe(III)-reducing capacity (Fig. 4*D*) was higher in the SNP-treated plants. These observations were similar to results of Graziano *et al.* (2002) and Kumar *et al.* (2010) in plants exposed to direct Fe deficiency. Other researches revealed that NO upregulated the activities of H^+- ATPase and H^+-PPase in wheat seedling roots under salt stress (Ruan *et al*. 2004), ferric chelate reductase activity in response to direct iron deficiency in roots of peanut (Zhang *et al*. 2012) and *A. thaliana* (Chen *et al*. 2010). It also has been demonstrated that nitric oxide is involved in regulation of Fe-acquisition genes in strategy I plants (Garcia *et al.* 2011). Therefore, effect of SNP on alleviation of ID in balm may be related to the development of root Fe(III)-reducing capacity by NO and reducing apoplast and rhizosphere pH that both contribute to Fe uptake. Similar to our results, Graziano *et al*. (2002) proposed that NO improved Fe functionality in maize plants exposed to direct Fe deficiency by enhancing the chemical reduction of apoplastic Fe(III) to Fe(II). Our results showed that SNP enhanced shoot Fe-use efficiency in the ID-treated plants (Fig. 4*C*), indicating improvement of Fe mobilization from roots to shoots by NO. Based on our data and previous studies, we can suggest that SNP effect could be related to role of NO in the expression of the ferric reductase, the iron transporter, and the H^+-ATPase genes, which could improve both Fe uptake in roots and its translocation from roots to shoots and lead to enhanced leaf Fe content and the regreening process under ID. However, we did not explore gene expression and therefore further investigations should address this hypothesis.

On the other hand, ID as well as DD led to the significant decrease of the NO content in balm leaves (Fig. 7) as it was also found for maize (Sun *et al*. 2007) and prunus (Cellini *et al*. 2011) previously. Our results confirmed that under ID treatment or under DD conditions, iron concentration in leaves was positively correlated with the NO content. Our data proved that SNP increased the NO content (Fig. 7) and the root/shoot ratio (Fig. 2) in ID plants. Our results were similar as a report of Jin *et al*. (2011), which demonstrated nitric oxide acts downstream of auxin in regulating root branching induced by direct Fe-deficiency and thus enhances Fe-deficiency tolerance in tomato plants. These observations are suggesting that the increase in NO production contributes to the improvement of iron availability not only by regulating the physiological processes, but also by promoting morphological adaptive responses, such as increment of the root/shoot ratio that in turn leads to a more efficient exploration of the soil and contributes to the enhancement of Fe(III)-reductase activity.

Because the NO donor, SNP, contains iron in its molecule, it is important to exclude the effect of iron in SNP for the elucidation of NO function. Thus, we investigated effect of sodium ferrocyanide (an analog of SNP that did not release NO) on plants exposed to ID or under DD. The results showed that sodium ferrocyanide had no effect on Chl contents and chlorosis in the ID-treated plants or DD plants (data not shown) which confirmed NO was effective in reverting the ID-chlorosis in balm. The similar result has been observed in maize by Graziano *et al.* (2002) under direct iron deficiency. In order to assess whether endogenous NO is involved in responses induced by SNP, the NO blocker, MB, was applied. The MB prevents NO production and/or action (Cragan 1999). The significant decrease of endogenous nitric oxide, iron content, and Fe-use efficiency were recorded in the plants exposed to ID or DD stress when MB was added. In correlation with data from shoot biomass and Chl contents, we suggested that NO was effective in reverting the iron-deficiency chlorosis and NO improved iron availability for Chl synthesis. Failure of bicarbonate-treated plants and Fe-deficient plants in treatment of 100 mM SNP + 100 mM MB, to recover iron availability and Chl synthesis indicates that the SNP-induced recovery occurred due to NO supply and thus, was inhibited using MB, as the NO blocker.

In our experiment, Chl fluorescence kinetics indicated that F_v/F_m and F_v/F_0 in balm decreased by ID or DD (Fig. 5) which suggests the reduction of PSII efficiency. It is proposed that the distribution of PSII reaction center might be attributed to the alterations of Chl biosynthesis or an altered stoichiometry between PSI and PSII (Balakrishnan *et al*. 2000). Pestana *et al*. (2005) explained the decline observed in F_v/F_m under Fe-deficiency conditions by the changes in the electron transport chain, in addition to structural modifications of PSII. Additionally, the reduction of the F_v/F_0 ratio could be related to the structural damage of thylakoids that led to the reduction of photosynthesis (Vladkova *et al*. 2011). Application of SNP kept F_v/F_m and F_v/F_0 at similar level as in C, which indicated that NO alleviated the damage in the photosynthetic apparatus under ID exposure or DD stress. In this study, ID exposure or DD decreased the Chl *a*/*b* ratio and NO supply increased the Chl *a/b* ratio in balm subjected to ID (Fig. 3*C*). Liang *et al*. (2010) reported that the increase of Chl *a/b* ratio was beneficial to absorbance and conversion of light energy. Thus, it can be inferred that

NO alleviated the damage of the photosynthetic apparatus under ID. NO was shown to enhance electron transport in chloroplast thylakoids (Vladkova *et al*. 2011).

On the other hand, because iron is an important component of the electron transport chains in chloroplasts and mitochondria, ID or DD impair the electron transport and it probably leads to the production of ROS (Graziano and Lamattina, 2005) as was shown in balm leaves (Fig. 6). Our results demonstrated that NO reduced the accumulation of H_2O_2 and MDA under both iron-deficiency types (Fig. 6) confirming that NO protected membrane lipids against oxidative damage. 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). Indeed, NO can terminate the lipid peroxidation by reacting with lipid alcoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals, which are produced during lipid peroxidation. It has been reported that the reaction between NO and $LO^{\bullet}/LOO^{\bullet}$ is done in a direct and rapid route (Beligni and Lamattina, 1999). NO also increased the activities of antioxidant enzymes in balm plants in order to detoxify ROS induced by ID. Because, iron is an integral constituent or cofactor of many antioxidant enzymes, such as POX, CAT, APX, and Fe-SOD, iron deficiency inhibits their activities and enhancement of SOD, CAT, POX, and APX activities by SNP might be attributable to NO-mediated improved availability of iron in plants. Ding *et al.* (2008) reported that exogenous nitric oxide ameliorated the inhibition of photosynthesis and antioxidant enzyme activities in Chinese cabbage *(Brassica chinensis* L.) under direct iron deficiency. Kumar *et al.* (2010) and Sun *et al.* (2007) also observed a similar improvement in the activities of haem-Fe enzymes upon treating Fe-deficient maize plants with SNP, but they reported iron deficiency increased the activity in some isoforms of SOD and SNP application declined SOD activity. However, our result did not confirm this result. The difference in obtained results might depend on the degree of Fe deficiency, treatment period, and plant species. In summary, under ROS-related toxicity, NO may act as a chain breaker and thus limit the oxidative damage. NO-mediated cellular Fe availability may modulate oxidative status and antioxidant responses of plants under Fe-deficient conditions.

In conclusion, this work presented strong evidence supporting a role for NO in iron availability. NO improved chlorophyll biosynthesis, photosynthetic function, and PSII efficiency. It led to lower production of ROS and improvement of growth in *M. officinalis* not only under direct Fe deficiency but also under bicarbonate exposure. Also NO, as a bioactive antioxidant, protected the iron-deficient balm against oxidative stress probably by reacting with ROS directly or by changing activities of ROS-scavenging enzymes, especially in the bicarbonate-treated plants.

Our results opened the possibility of ameliorating the effects of calcareous soils by identifying or creating balm lines with enhanced NO production or by production of iron fertilizers containing NO donor. Further understanding of the mechanism for amelioration of bicarbonate symptoms by NO could enhance the prospects of achieving such goals.

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