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Role of Nitric Oxide on Iron Homeostasis, Chlorophyll Biosynthesis and Antioxidants System in Two Wheat Cultivars

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Abstract: The present study was aimed to investigate the role of NO in iron homeostasis and to evaluate the ability of NO to cope with iron deficiency or toxicity symptoms in two wheat (*Triticum vulgaris* L. Sids-1 and Gimmieza-7) cultivars. Seeds were germinated and grown in vermiculite watered with nutrient solution. Iron was supplied in different concentrations (zero, 20, 40, 80 and 160 μM) as Fe-EDTA. Sodium Nitro-Prusside (SNP) was used as NO donors in a 100 μM concentration and 100 μM Methylene Blue (MB) was used as NO scavengers. The solutions were supplied to plants by irrigation once a week included in the nutrient solution. The results revealed that NO plays a significant role in enhancing the availability of iron in iron-deficient conditions. It also ameliorates the adverse effect of excess iron in the two cultivars under investigation by increasing the activities of catalase and ascorbic acid peroxidase as well as NO concentrations and decreasing lipid peroxidation and protein degradation. Moreover, NO declined iron stress-induced chlorosis symptoms by increasing Chl. a+b concentration. NO was found to affect Chl. a+b content by enhancement of the conversion of Mg-protoporphyrin to pchlide and subsequently to chlorophyll. The role of NO was approved by using NO scavenger (MB) which reverses all NO effects when applied.

Key words: NO, iron stress, antioxidants, porphyrins, chlorophyll biosynthesis

INTRODUCTION

When grown on highly calcareous soils, most plant species of the so called Strategy I type (Marschner and Römheld, 1994) respond to lack of iron (Fe) by developing characteristic symptoms of Fe deficiency chlorosis, primarily in young leaves. The concentration of Fe expressed on a dry weight leaf basis and the amount of Fe per leaf frequently decreases in chlorotic leaves, although the Fe concentration can sometimes be the same or even higher in chlorotic leaves as compared with green ones (Morales *et al*., 1998; Römheld, 2000; Nikolic and Römheld, 2002). Various soil factors (e.g., CO_2, ethylene, low temperature, high water content and drought) resulting in severe inhibition of root growth might be responsible for triggering a restriction in leaf expansion growth, which in turn elevates the Fe concentration in these chlorotic leaves as a consequence of the diminished dilution of Fe concentration (Römheld, 2000).

Whereas Fe deficiency results in chlorosis, Fe excess is believed to generate oxidative stress (Halliwell and Gutteridge, 1984). Toxic reduced O_2 species are inevitable by-products of biological oxidations. The toxicity of the relatively unreactive superoxide radicals and H_2O_2 arises by the Fe-dependent conversion into the extremely reactive hydroxyl radicals (Haber-Weiss reaction) that cause severe damage on membranes, proteins and DNA (Halliwell and Gutteridge, 1984). Because hydroxyl radicals are by far too reactive to be controlled directly, aerobic organisms eliminate the less reactive O_2 species as efficiently as possible. H_2O_2 is removed by catalases, ascorbic acid peroxidases (ASPX) and GSH peroxidases. The dismutation of superoxide radicals to O_2 and H_2O_2 is catalyzed by metalcontaining SODs (Bowler *et al*., 1992).

As the intrinsic constituent or metal cofactor, iron is actively involved in cellular detoxification reactions catalysed by catalase, phenolic-dependent peroxidases (non-specific peroxidases), ascorbate peroxidase and Fe superoxide dismutase (Fe-SOD), which scavenges hydrogen peroxide and superoxide, thus protecting the cell from oxidative injury. Under stress conditions, the enhanced peroxidase activity in the intercellular spaces, stimulating cell wall stiffening (Gaspar *et al*., 1985), probably reduces cell growth which might represent a mechanical adaptation to adverse conditions (Ranieri *et al*., 1995).

Iron deficiency impairs chlorophyll biosynthesis and chloroplast development in both dicotyledonous and monocotyledonous species. Its availability maintains a direct correlation with plant productivity. Chlorosis because of unavailability of iron is a major agricultural problem that results in diminished crop yields in an estimated 30% of calcareous soils worldwide (Mori, 1999).

Nitric Oxide (NO) is a bioactive free radical implicated in a number of physiological functions, including intra- and intercellular mediation of some animal responses (Anbar, 1995). In plants, NO is involved in the signaling of growth, development and adaptive responses to multiple stresses (Durner and Klessig, 1999; Beligni and Lamattina, 2001b) and in a number of cytotoxic and cytoprotective effects (Beligni and Lamattina, 1999a, b, 2001a). NO can readily form complexes with transition metal ions in aqueous solutions or those present in diverse nucleophylic compounds such as metalloproteins. Metal-nitrosyl complexes are formed under neutral physiological conditions and were proposed to act as a link between the different redox states of NO (Stamler *et al*., 1992).

The biological chemistry of NO is dominated by free-radical reactions and interaction of NO with other free-radical species leads to either inhibition or potentiation of oxidative damage (Beckman *et al*., 1990). One mechanism by which NO can inhibit lipid peroxidation has been postulated to involve reaction between NO and lipid-derived radicals (e.g., peroxyl, alkoxyl radicals) (Hogg *et al*., 1993; Rubbo *et al*., 1994). NO has been shown to inhibit the activity of tobacco aconitase, an iron-sulphur containing enzyme that regulates iron homeostasis, suggesting a role for NO in modulating iron levels in plants (Navarre *et al*., 2000).

The present study was aimed to investigate the role of NO in iron homeostasis and to evaluate the ability of NO to cope with iron deficiency or toxicity symptoms in two wheat (*Triticum vulgaris* L.) cultivars. The present study was also aimed to explore further the relation between NO and chlorophyll biosynthesis under iron stress conditions.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wheat seeds (*Triticum aestivum* L.) cultivars (Sids-1 and Gimmieza-7) were supplied by National Research Center (NRC), Dokki-Giza, Egypt. The study was conducted at (2006) in botanical garden of botany department, Faculty of Science, Suez Canal University, Ismailia, Egypt. Seeds were surface sterilized in 1.8% (v/v) sodium hypochlorite and rinsed several times in distilled water. Seeds were germinated and grown in vermiculite watered with nutrient solution. The nutrient solution had the following composition: 5.25 mM KNO_3, 7.75 mM $Ca(NO_3)_2$, 4.06 mM $MgSO_4$ and 1.0 mM KH_2PO_4; micronutrients: 46 μM H_3BO_4, 9.18 μM $MnSO_4$, 5.4 μM $ZnSO_4$, 9.0 μM $CuSO_4$ and 2.0 μM Na_2MoO_4. Iron was supplied in different concentrations (zero, 20, 40, 80 and 160 μM) as Fe-EDTA. The nutrient solution was adjusted to pH 5.5 and renewed once a week. Plants were grown in an open air at 14/10 h (25°C/22°C) day/night regime. 28-day-old plants were harvested, weighed, frozen and stored in a deep-freezer for further analysis.

NO Treatments and Chemicals

Sodium nitroprusside (SNP) was used as NO donors in a 100 μM concentration (Graziano *et al*., 2002); 100 μM Methylene Blue (MB) was used as NO scavengers which inhibit NO production

and/or action (Cragan, 1999). The solutions were supplied to plants by irrigation once a week included in the nutrient solution.

Iron Content Determination

Leaves and roots samples of the two cultivars were oven-dried at 65°C for 48 h and mineralized by wet open digestion in HNO_3:H_2SO_4:$HClO_4$ (5:1:1, 2-5 mL for 0.5-1.5 g fresh weight). Total iron concentration in the digest was estimated by atomic absorption spectroscopy.

Antioxidant enzymes and lipid peroxidation enzymes extracts were prepared in 20 mL chilled extraction buffer (pH 7.5). Enzyme assays were conducted immediately following extraction. Peroxidase activity was determined by following the dehydrogenation of guaiacol at 436 nm (Malik and Singh, 1980). Catalase activity was assayed in a method following Aebi (1983). Activity was determined by following the decomposition of H_2O_2 at 240 nm. ASPX activity was determined using the method of Nakano and Asada (1987). Activity was determined by following the H_2O_2- dependent decomposition of ascorbate at 290 nm. Total protein content was extracted with trichloroacetic acid and NaOH and estimated spectrophotometrically according to Lowery *et al.* (1951).

Estimation of lipid peroxidation was assayed spectrophotometrically using TBA-MDA assay. (Hodges *et al*., 1999).

Hydrogen peroxide (H_2O_2) and NO estimation concentration in the treated leaf extract was measured by the FOX^2 method (Jiang *et al*., 1990; Wolff, 1994) based on the peroxide-mediated oxidation of Fe^{2+} with xylenol orange.

Nitric oxide concentration was directly measured using the method of Ridnour *et al.* (2000). Assay mixture consisted of 100 µL tissue extract mixed with equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthalethylenediamine dihydrochloride). The solution was immediately mixed by inversion incubated at room temperature for 3 min and then $A_{496\ nm}$ was measured. NO concentration of the solution was calculated using an extinction coefficient of 6600 M^{-1} cm^{-1}.

Porphyrins Extraction and Quantitation

Plant leaves (0.5 g) were harvested and porphyrins were extracted based on the method of Rebeiz *et al.* (1970). Tissue was homogenized in 2.5 mL of cold acetone:0.1 M NH_4OH (90/10, v/v) and centrifuged at 30,000*g* for 10 min. The pellet was then re-extracted in 1.5 mL of solvent and centrifuged again. The supernatants were combined and washed successively with an equal volume and a one-third volume of hexane prior to spectrophotometric analysis. From Hexane fraction, Pchlide concentrations were determined using a molar absorption coefficient of 31,100 M cm^{-1} at 626 nm (Kahn, 1983) and Proto IX was determined using a molar absorption coefficient of 16,200 M cm^1 at 503 nm (Gough, 1972). The acetone:NH_4OH fraction (containing metal porphyrins) was re-extracted with 30 mL of petroleum ether and the ether fraction was used to determine Coproporphyrine and Mg-porphyrine using a molar absorption coefficient of 4.7×10^5 mM cm^{-1} at 401 for coproporphyrine. Mg-porphyrine was calculated using the equation: nmol/mL Mg-porphyrin = 56.61 A_{583}-13.24 A_{624}-7.79 A_{648}.

Chlorophyll Quantification

The pigments were extracted in 80% chilled acetone. The amount of total Chl a and b was estimated spectrophotometrically according to Lichtenthaler (1987).

Statistical Analysis

Analysis of variance (ANOVA) and student t-test was performed on all data using SPSS program ver. 11.0.

RESULTS

Iron Concentration

When applied to iron deficiency conditions, SNP increased iron concentration in shoots and roots of both wheat cultivars. Moreover, at Fe toxicity levels, the same application reduced Fe content when compared to control except at gimmieza cultivar treated with 80 and 160 μM Fe. MB application, however, induced a significant decrease in Fe concentration at sids cultivar's roots while it caused a non significant change in iron concentration at gimmieza cultivar's roots Table 1).

Catalase Activity

SNP treated cultivars showed an enhancement catalase activity in all iron concentrations treatments. MB treatment, however, induced a significant decrease in catalase activity as iron concentrations increased (Fig. 1a and b).

Peroxidase Activity

The deacrease in peroxidase activity was more pronounced at the toxic levels of iron (80 and 160 μM) in Sids cultivar. In Gimmieza cultivar, both iron deficiency and toxicity induced significant decreases in peroxidase activity when compared with normal iron concentration (40 μM) (Fig. 2a and b).

SNP treatment increased peroxidase activity in toxic levels of iron in both cultivars however, MB treatment induced a highly significant reduction in peroxidase activity as iron concentration increased when compared with non-treated and SNP treated plants.

Ascorbic Acid Peroxidase (ASPX) Activity

SNP treatment induced a highly significant increase in ASPX activity in zero, 20 and 40 μM Fe. High iron concentration 80 and 160 μM+SNP treatment exhibited a decline in ASPX activity when compared with non treated or MB treated plants (Fig. 3a and b).

Lipid peroxidation, expressed as MDA levels, significantly increased at high iron concentrations (80 and 160 μM Fe) and in the presense of MB treatment. SNP application, however, reduced MDA levels in all applied iron concentrations. The decrease was much more pronounced at 20 uM Fe in sides cultivar and at 80 μM Fe in gimmeieza cultivar (Fig. 4a and b).

Table 1: Iron concentration expressed as μg g^{-1} dry wt. in shoot and root of two wheat cultivars treated with different iron concentrations alone or with either 100 μM SNP (NO doner) or 100 μM MB (NO scavenger). Iron was supplied in different concentrations (zero, 20, 40, 80 and 160 μM) as Fe-EDTA. SNP and MB solutions were supplied to plants by irrigation once a week included in the nutrient solution. Values are the means of three replicates ±SE

		Iron concentration (μg g^{-1})					
		Shoot			Root		
Wheat cultivar	Treatments	Control	+SNP	+MB	Control	+SNP	+MB
Sids	Zero	58.51±1.16	224.6±3.17	76.7±2.02	1053.8±2.6	1386.0±3.76	1076.0±2.6
	20	49.61±1.41	250.7±2.89	114.4±3.18	1220.0±2.02	1497.0±1.73	1221.8±2.03
	40	218.2±1.46	1133.2±1.46	296.9±1.46	1316.3±1.76	1689.5±3.76	1327.5±1.73
	80	303.3±2.35	568.2±2.03	322.7±1.44	1544.0±3.18	1300.1±7.81	1495.1±3.18
	160	461.3±1.73	491.1±3.46	478.2±1.73	1628.5±2.31	1244.3±5.78	1646.2±2.6
	F	10639.8	18305.4	6292.3	9378.9	1234.7	8149.1
Gimmieza	Zero	292.6±4.6	379.3±1.73	235.7±2.6	1251.0±4.6	1326.3±2.3	1255.0±2.89
	20	458.7±1.74	529.3±1.69	321.3±3.18	1273.5±4.04	1346.5±2.6	1354.1±1.15
	40	535.5±2.60	639.9±1.19	338.4±2.06	1336.5±2.3	1439.5±2.03	1232.0±1.73
	80	649.8±2.03	868.1±2.40	343.8±1.78	1415.0±2.6	1327.5±3.18	1417.5±3.19
	160	696.3±2.60	1432.1±1.70	510.9±1.15	1448.8±2.58	1120.5±2.03	1437.3±2.30
	F	3065.93	52128.3	1940.1	649.9	1983.12	1524.77

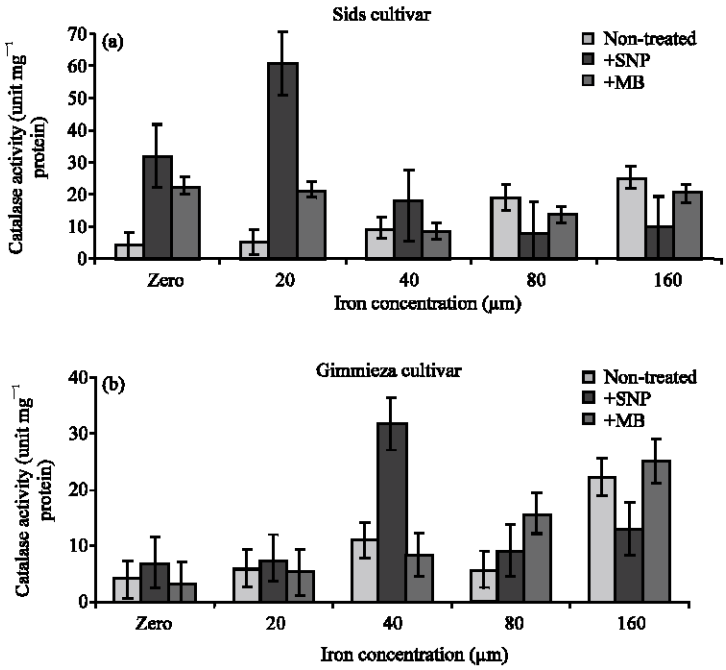

Fig. 1a and b: Catalase activity, expressed as unit mg^{-1} proteins, in the leaf extract of two wheat cultivars treated with different iron concentrations alone or with either 100 µM SNP (NO doner) or 100 µM MB (NO scavenger). Iron was supplied in different concentrations (zero, 20, 40, 80 and 160 µM) as Fe-EDTA. SNP and MB solutions were supplied to plants by irrigation once a week included in the nutrient solution. Values are the means of three replicates ±SE

Protein Content

Increasing iron concentration from zero to 40 µM Fe induced a significant increase in protein content in both wheat cultivars. Iron toxicity, however, signficantly decreased protein content. SNP application enhanced protein contents over that of the non-treated or MB treated plants (Fig. 5a and b).

H_2O_2 Content

It was noticed that iron concentration (160 µM) induced a highly significant increase in H_2O_2 content comparing with normal iron concentration (40 µM) at sids cultivar while all iron treatments increased H_2O_2 concentration in gimmieza cultivar when compared to control iron concentration (40 µM) Applications of SNP and MB induced highly significant increases in H_2O_2 content at all iron concentrations (zero-160 µM) in both cultivars (Table 2).

NO Levels

The data showed that application of SNP induced a significant increase in NO level at all iron concentrations. The highest NO level was observed at 80 µM Fe. On the other hand, MB treatment significantly reduced NO level at all iron concentrations in both cultivars.

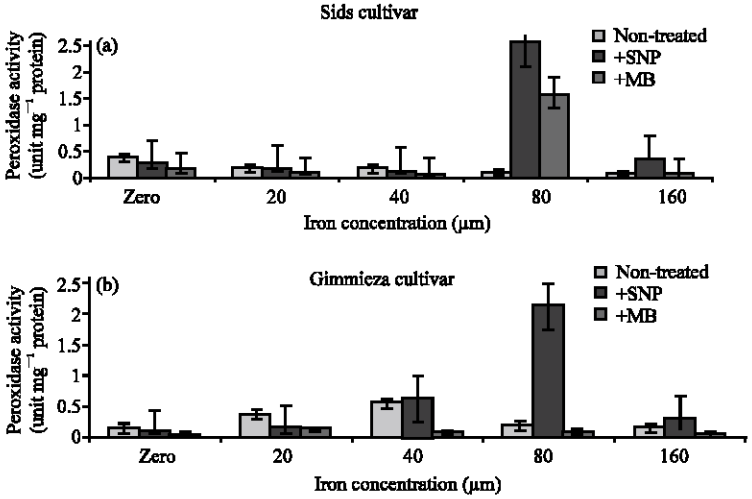

Fig. 2a and b: Guaiacol-dependent peroxidase activity, expressed as unit mg^{-1} proteins, in the leaf extract of two wheat cultivars treated with different iron concentrations alone or with either 100 µM SNP (NO doner) or 100 µM MB (NO scavenger). Iron was supplied in different concentrations (zero, 20, 40, 80 and 160 µM) as Fe-EDTA. SNP and MB solutions were supplied to plants by irrigation once a week included in the nutrient solution. Values are the means of three replicates±SE

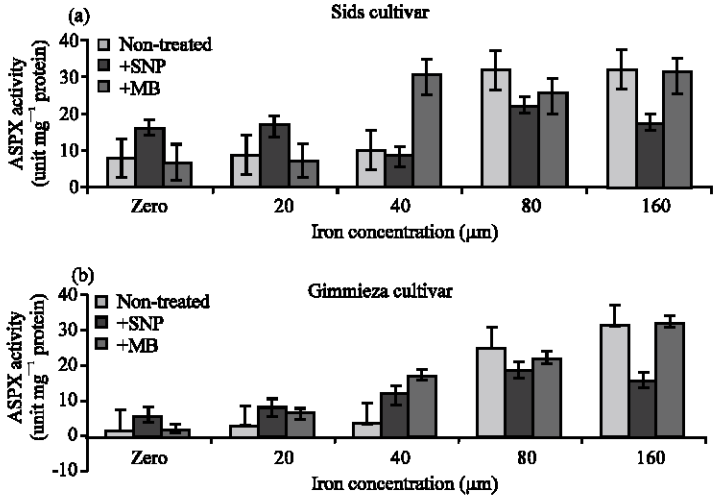

Fig. 3a and b: Changes in the activity of ASPX in two wheat cultivars treated with different iron concentrations alone or with either 100 µM SNP (NO donor) or 100 µM MB (NO scavenger). Iron was supplied in different concentrations (zero, 20, 40, 80 and 160 µM) as Fe-EDTA. SNP and MB solutions were supplied to plants by irrigation once a week included in the nutrient solution. Values are the means of three replicates±SE

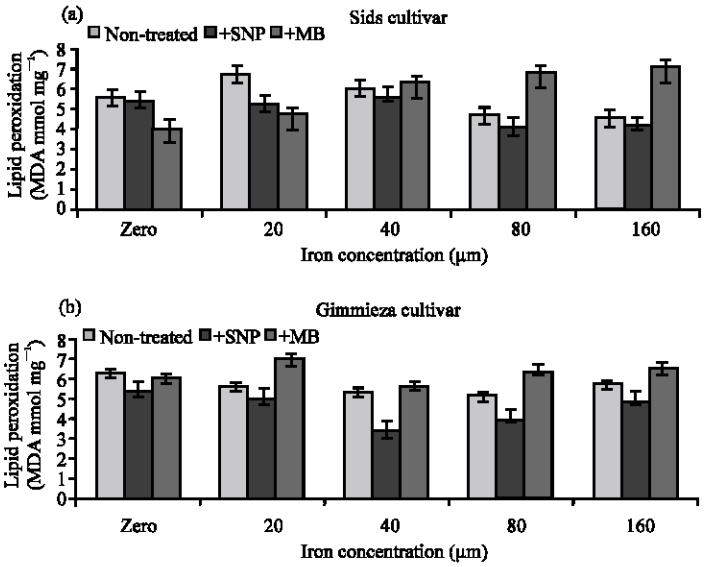

Fig. 4a and b: Lipid peroxidation, expressed as MDA levels (µmol/g) in two wheat cultivars treated with different iron concentration alone or with either 100 µM SNP (NO doner) or 100 µM MB (NO scavenger). Iron was supplied in different concentrations (zero, 20, 40, 80 and 160 µM) as Fe-EDTA. SNP and MB solutions were supplied to plants by irrigation once a week included in the nutrient solution. Values are the means of three replicates ±SE

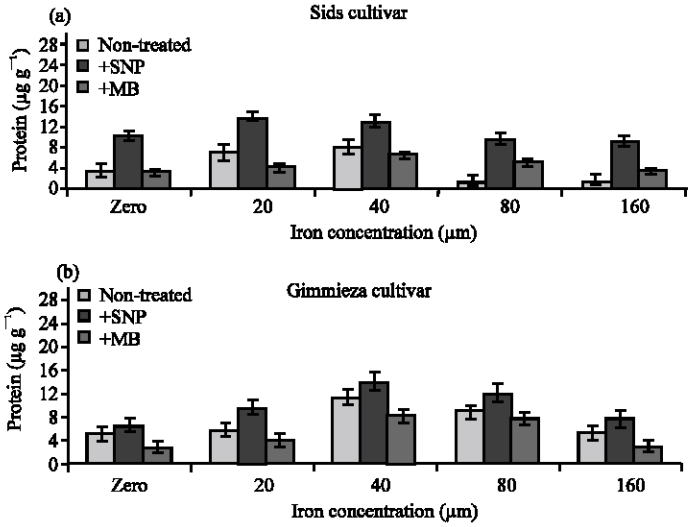

Fig. 5a and b: Protein content, expressed as (µg g^{-1}) in two wheat cultivars treated with different iron concentration alone or with either 100 µM SNP (NO doner) or 100 µM MB (NO scavenger). Iron was supplied in different concentrations (zero, 20, 40, 80 and 160 µM) as Fe-EDTA. SNP and MB solutions were supplied to plants by irrigation once a week included in the nutrient solution. Values are the means of three replicates ±SE

Table 2: H_2O_2 concentration expressed as μmol g^{-1} fresh wt. and NO concentration recorded as (mmol mg^{-1}) in leaves of two wheat cultivars treated with different iron concentrations alone or with either 100 μM SNP (NO donor) or 100 μM MB (NO scavenger). Iron was supplied in different concentrations (zero, 20, 40, 80 and 160 μM) as Fe-EDTA. SNP and MB solutions were supplied to plants by irrigation once a week included in the nutrient solution. Values are the means of three replicates ±SE

Wheat cultivars	Treatments	H_2O_2 (μmol g^{-1})			NO concentration (mmol mg^{-1})		
		Control	+SNP	+MB	Control	+SNP	+MB
Sids	Zero	9.41±0.318	28.00±1.01	92.91±1.26	13.0±0.26	21.8±0.31	2.0±0.21
	20	15.46±0.319	31.54±0.319	55.54±0.728	13.3±0.35	24.6±0.41	3.0±0.08
	40	13.10±0.289	29.18±0.580	39.18±0.587	14.5±0.27	29.9±1.02	3.5±0.116
	80	10.02±0.280	13.58±0.289	33.18±1.16	16.5±0.35	33.0±0.87	4.1±0.173
	160	25.36±0.319	29.18±1.16	37.82±0.746	14.6±0.36	15.6±0.23	3.8±0.24
	F	445.9	90.35	677.4	20.23	104.6	30.14
Gimmieza	Zero	84.01±1.15	56.91±1.19	83.51±1.16	15.5±1.57	17.7±0.12	6.3±0.173
	20	38.71±1.45	53.61±0.46	80.61±0.94	16.5±0.26	16.5±0.346	5.7±0.26
	40	29.18±1.16	43.70±0.86	76.62±1.17	17.5±0.56	19.4±0.35	4.0±0.116
	80	41.18±1.46	75.64±2.06	83.72±1.74	13.9±0.72	28.4±1.01	3.6±0.231
	160	54.72±1.18	73.64±1.49	72.87±1.19	7.0±0.145	25.9±0.26	3.0±0.08
	F	275.69	100.40	13.72	27.041	66.82	57.82

Chlorophyll Biosynthesis

In order to investigate the relation between NO and iron availability and some biosynthetic precursors of chlorophyll, Coproporphyrin, proto-IX, Mg-protoporphyrin, Pchlide and subsequently Chl. a+b were analyzed. The data in Fig. (6 I-j) showed that SNP treatment enhanced the formation of coproporphyrin more than that of control or MB treatments Fig. 6a and b). The same treatment (SNP) caused decrease in proto-IX concentration in all Fe treatments (Fig. 6c and d).

Pchlide formation from Mg-protoporphyrin was enhanced in the presence of SNP treatments in the two wheat cultivars (Fig. 6g and h). MB application, however, caused an inhibition of the conversion of proto-IX to Mg-protoporphyrin and lead to accumulation of proto-IX in all Fe treatments at both wheat cultivars (Fig. 6c-f). SNP, an NO doner, produced a significant increase in the chl. a+b content compared with control plants (Fig. 6i and j). While, MB (NO-scavenger) was able to block the effect of SNP. The effect of SNP was much more pronounced in Fe deficient treatments.

DISCUSSION

Iron deficiency responses involve several physiological plant adaptations (Guerinot and Yi, 1994; Mori, 1999). Iron is transported in the xylem as Fe(III) (Guerinot and Yi, 1994) and reduction of Fe(III) to Fe(II) is an essential requisite to cross the plasma membrane. The enzyme involved in this reaction is the plasma membrane-bound iron (III)-chelate reductase, whose activity seems to depend on the apoplastic pH and light (González-Vallejo *et al*., 2000). In addition, Fe(III) reduction *in vivo* may be aided by intermediate superoxide radical formation (Brüggemann *et al*., 1993), indicating that changes in the redox state of the apoplast might be involved in Fe(III) reduction. Moreover, a high leaf apoplastic pH depresses the activity of Fe^{3+} reductase, less Fe^{2+} can be transported across the plasma membrane into the leaf symplast, resulting in Fe deficiency chlorosis (Kosegarten *et al*., 1999).

Application of SNP in the present study increased iron concentrations during iron deficiency conditions. This effect may be due to nitric oxide-induced decrease apoplastic pH and this enhances acidification of the extracellular medium and increase both root ferric-reducing capacity and uptake of ferrous iron. Proton pumping into the cell wall establishes a favorable pH surrounding for the reductase and prevents repulsion of negatively charged Fe ions at the plasma membrane, which represent the major iron species in the xylem (Abadia *et al*., 2002). In addition, acidification of the apoplast might contribute to the regulation of iron transport across the plasma membrane. Moreover, NO-mediated ferritin regulation has been shown in *Arabidopsis* (Murgia *et al*., 2002). In addition to a role in the

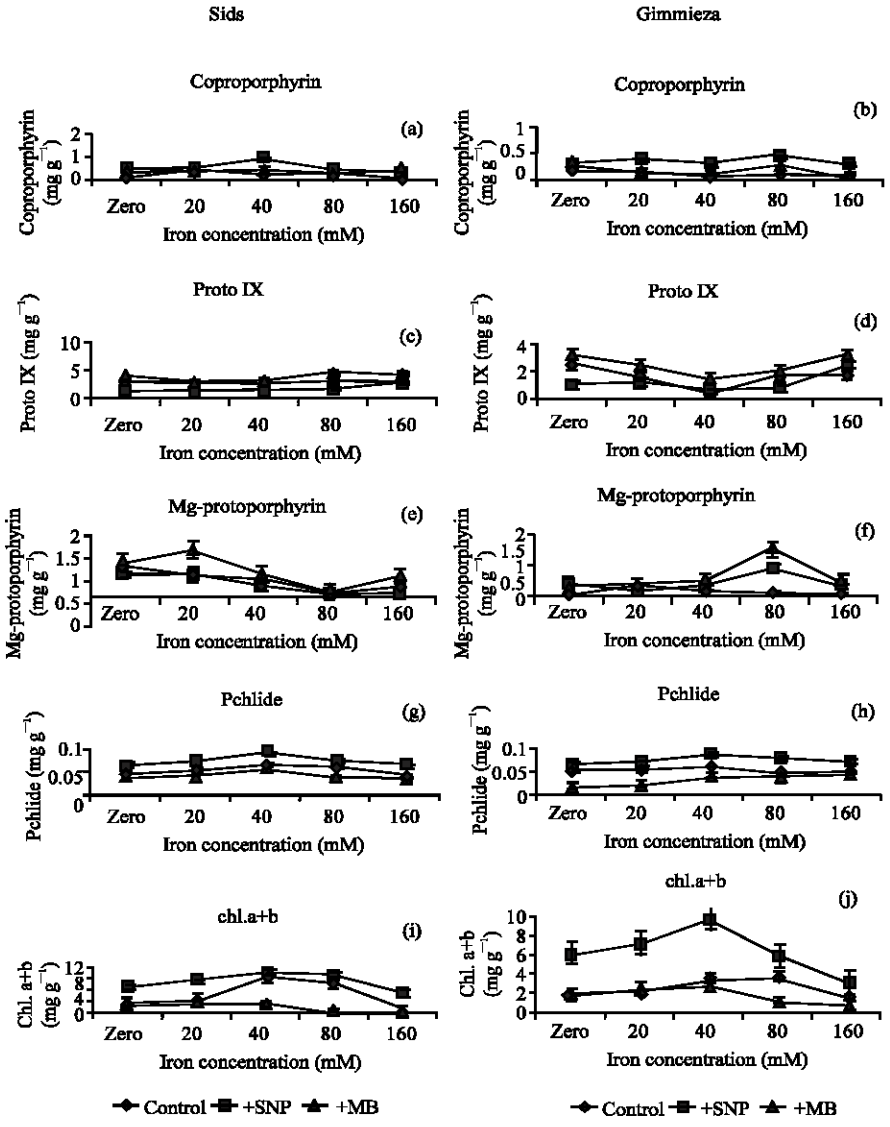

Fig. 6a-j: Coproporphyrines, Proto IX, Mg-porphyrins, pchlide and Chl. a+b concentrations expressed as (μg g^{-1}) in two wheat cultivars treated with different iron concentration alone or with either 100 μM SNP (NO doner) or 100 μM MB (NO scavenger). Iron was supplied in different concentrations (zero, 20, 40, 80 and 160 μM) as Fe-EDTA. SNP and MB solutions were supplied to plants by irrigation once a week included in the nutrient solution. Each point corresponds to the mean of two to four independent experiments; bars indicate the SDs

regulation of ferritin expression, NO improves the availability of iron within the plant, possibly by the formation of iron nitrosyl complexes (Graziano *et al*., 2002). NO action is achieved either directly, by reaction with effector molecules or indirectly, modifying the redox state of the cell. It was also found

that SNP application maintains a moderate iron levels in plants subjected to toxic iron concentrations. NO could modulate oxidative reactions, particularly the generation of cytotoxic oxygen species or tetravalent iron. Kanner *et al*. (1991) also proposed that these reactions included (I) modulation of the reactivity of iron-heme and non-heme compounds; (ii) chelation of free iron in a form that prevents or decreases its potential to generate cytotoxic oxygen species and (iii) scavenging of free radicals.

Iron toxicity levels (80 and 160 μM Fe) were found to induce oxidative stress by increasing MDA levels and decreasing protein content in the two wheat cultivars (Fig. 4 and 5). Application of SNP (as NO doner) reduced the ameliorative effect of excess iron by maintaining protein content near control conditions (40 μM Fe) and decreasing MDA levels (lipid peroxidation byproducts). NO can abate lipid peroxidation, perhaps by intercepting various lipid radicals forming ROONO adducts, which results in the termination of the chain propagation reactions, as was suggested by (Wink *et al*., 1995). Thus, it might be possible that the interaction of NO-iron is the main reactions responsible for NO inhibition of lipid peroxidation after exposure to SNP (Fig. 4a and b). Moreover, it was suggested that NO can protect cells from the deleterious effects of the reactive oxygen species generated through the reaction of ferryl intermediates with NO to avoid the formation of ferric species (Ames *et al*., 1993). An alternative to this explanation is that lipid peroxidation in biological membranes is decreased after NO exposure as a result of a different mechanism that involves cytochrome P_{450} inactivation. Puntarulo and Cederbaum (1997) showed that NO-related decrease in P_{450} content in microsomal membranes affected O_2^- production and iron release from ferritin. Moreover, the interaction between an iron compound and NO has complementary effects that change the reactivity of both the iron ion and NO toward other molecules. Since the rate constant of NO with iron-heme and non-heme compounds seems to reach a diffusion controlled limit (Kanner *et al*., 1991), once the NO-iron adducts are generated they are very stable (Moore and Gibson, 1976).

The H_2O_2 content underwent a significant increase following the iron starvation or toxicity treatment (Table 3). Although H_2O_2 is not particularly detrimental to cell metabolism, it can be reduced to extremely reactive OH· radicals through the Fenton reaction in the presence of iron in the free form or bound to small molecules, such as amino acids, nucleotides and organic acids (Halliwell and Gutteridge, 1989). The high levels of H_2O_2 may be due to decreased capacity to peroxide detoxification and/or an active production of peroxide with a consequent rise of oxidative cell status. NO also inhibits catalase and ascorbate peroxidase activity (Clark *et al*., 2000) and this may lead to the increase in H_2O_2 concentration. Moreover, NO generation has been detected under conditions in which H_2O_2 generation is stimulated (Delledonne *et al*., 1998) and it may well turn out that these two molecules are commonly present during various stresses. Thus, stress responses may reflect responses to both H_2O_2 and NO. Clark *et al*. (2000) propose that NO, like SA, plays a role in regulating H_2O_2 levels during the resistance response by reversibly inhibiting catalase and APX. NO production may even precede the accumulation of H_2O_2, as recently observed by *in vivo* imaging of an elicitor-induced NO burst in tobacco (Foissner *et al*., 2000).

SNP application induced an increase in catalase activity in iron deficiency treatment (zero and 20 μM Fe) while excess iron decreased catalase activity at both cultivars under investigation (Fig. 1a and b). The increase in catalase activity under Fe excess treatments may be due to a stimulation of photorespiration. The present results are supported by that of Kampfenkel *et al*. (1995), who reported a doubled increase in catalase activity and an inhibition in photosynthesis in iron excess treated *Nicotiana plumbaginifolia* plant.

The diminished ASPX activity due to iron deprivation (Fig. 3a and b) may result as a consequence of the high request for iron from the ASPX molecule, as it contains, in addition to the haem group, also a non-haem iron atom. Similarly, Iturbe-Ormaetxe *et al*. (1995) found a marked fall in this enzymatic activity when the total extract of iron-deficient pea leaves was assayed.

The regulation of Chl biochemical pathway is generally considered to be exerted most effectively at the enzymatic step which produces the first compound unique to that pathway. Chl and heme are thought to share a common biosynthetic pathway from ALA to protoporphyrin IX (Duggan and Gassman 1974). The decrease in chlorophyll a+b during iron deficiency observed in the present study (Fig. 6a-j) may be resulted from the increase in Mg-proto which inhibits ALA biosynthesis. The stimulation of Proto and Mg-proto synthesis from Glu by a,a'-DP (an iron chelator) occurs because the step between Glu and ALA is inhibited by heme and heme synthesis in the plastids is prevented by Fe sequestering agents such as a,a'-DP. Mg-protoporphyrin IX and, possibly, Mg-Proto Me are also effective feedback inhibitors of ALA synthesis (Chereskin and Castelfranco, 1982). The concentration range in which these metabolites are effective *in vitro* leads to the postulatation that heme and Mg-protoporphyrins are likewise feedback regulators of tetrapyrrole synthesis *in vivo*.

Vleck and Gassman (1979) have shown that a,a'-DP (iron chelator) prevents the conversion of Mg-Proto Me to Pchlide in detached bean leaves. So SNP application, which increased iron availability in iron deficient conditions and reduced iron toxicity-induced oxidative stress, alleviates the destructive effect on chlorophyll biosynthesis by stimulating the conversion of Mg-Proto Me to Pchlide and consequently Chl. a and b concentrations as shown in Fig. (6 a-j). The occurrence of chlorophyll binding sites in PSI-K suggests a mechanism for Fe responsive signal transduction to the antenna reaction center complex. Specifically, it could be proposed that the chlorophyll binding sites of PSI-K are sensitive to flux through the chlorophyll biosynthetic pathway, which in turn is affected by the activity of the Fe-requiring aerobic oxidative cyclase. This would lead to functional uncoupling between antenna and PSI in cells with reduced cyclase activity. Recently, one Fe-containing subunit of this enzyme was identified in *Rubrivivax gelatinosus* (Pinta *et al*., 2002).

It could be concluded that NO plays a significant role in enhancing the availability of iron in iron deficient conditions. It also ameliorates the adverse effect of excess iron in the two cultivars under investigation by increasing NO concentrations and the activities of catalase and ascorbic acid peroxidase and decreasing lipid peroxidation and protein degradation. Moreover, NO declines iron stress-induced chlorosis symptoms by increasing Chl.a+b concentration. NO was found to affect Chl. a+b contents by enhancement of the conversion of Mg-protoporphyrin to pchlide and subsequently to chlorophyll. The role of NO was approved by using NO scavenger (MB) which reverses all NO effects when applied. The results support the idea that NO is closely related to iron metabolism, transport and/or availability and consequently, to chlorophyll biosynthesis.

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