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BRIEF COMMUNICATION

Nitric oxide alleviates Fe deficiency-induced stress in *Solanum nigrum*

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Abstract

The possible involvement of nitric oxide in response of *Solanum nigrum* seedlings to Fe deficiency was investigated. Iron deficiency resulted in decreased shoot height and chlorophyll content and increased proliferation of root hairs and H_2O_2, K^+ and Ca^{2+} content. NO donor S-nitrosoglutathione (GSNO) was effective in preventing Fe deficiency-induced increase in content of H_2O_2 and the ion uptake. The protective effects of GSNO were reversed by cPTIO, an NO scavenger, and tungstate, a nitrate reductase (NR) inhibitor.

Additional key words: ion uptake, reactive oxygen species, S-nitrosoglutathione.

Nitric oxide is a widespread intracellular and intercellular signaling molecule that is involved in developmental, hormonal and environmental responses in plants (Zhang *et al*. 2006, Lopez-Carrion *et al.* 2008). NO promotes plant growth and development in a variety of ways (Beligni and Lamattina 2001), *e.g.*, by stimulating seed germination, root growth and lateral root development, and also by retarding senescence (Leshem *et al.* 1998, Libourel *et al.* 2006, Kolbert *et al.* 2008). However, exposure to relatively high doses of NO influences normal metabolism by reducing respiration (Zottini *et al.* 2002) and net photosynthesis (Leshem *et al.* 1998). There are several NO sources in plants, including nitrate reductase (NR) (Desikan *et al.* 2002), the nitric oxide synthase-like (NOS-like) enzyme (Guo *et al*. 2003) and non-enzymatic pathways (Bethke *et al.* 2004).

Iron (Fe) is an essential nutrient element with a crucial function in plants (Graziano and Lamattina 2007). It acts as a co-factor for many enzymes and proteins. Although iron is abundant in the earth crust, its availability is always limited, especially in alkaline soil. Iron starvation in plants decreases the chlorophyll content and thus leads to severe reductions in crop yield (Graziano *et al*. 2002). In response to iron deficiency, plants can increase iron solubility in soil by proton extrusion and enhance iron absorption through root hair proliferation (Curie and Briat 2003, Graziano and Lamattina 2007). Ranieri *et al*. (2001) reported that Fe deficiency enhanced H_2O_2 accumulation in sunflower leaves. Iron deficiency reduces the activity of ascorbate peroxidase (APX; EC 1.11.1.11) and increases the contents of glutathione (GSH) and ascorbate (ASC), indicating that iron deficiency induces secondary oxidative stress in plants (Ranieri *et al.* 2001, Zaharieva *et al*. 2004, Sun *et al*. 2007). Navarre *et al.* (2000) reported that NO inhibits the activity of tobacco aconitase, an Fe-containing enzyme that regulates iron homeostasis, suggesting that NO plays a role in modulating iron accumulation in plants. Graziano and Lamattina (2007) reported that nitric oxide (NO) was produced rapidly in the root epidermis of tomato under iron deprivation. Additionally, supplementation with NO improved plant growth without increasing total iron content by modulating the expression of iron uptake-related genes. Sun *et al.* (2007) reported that NO could protect maize plants from iron deficiency-induced oxidative stress by reacting with reactive oxygen species (ROS) directly or by changing

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Abbreviations: cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAB - 3-diaminobenzidine; DAF-2 DA - 4,5-diaminofluorescein diacetate; GSNO - S-nitrosoglutathione; L-NAME - N^G-nitro-*L*-Arg-methyl ester; NR - nitrate reductase; NOS - nitric oxide synthase; ROS - reactive oxygen species; TCA - trichloroacetic acid.
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the activities of ROS-scavenging enzymes. In the present study, we investigated the possible involvement of NO in response of *Solanum nigrum* to iron deficiency-induced stress.

Seeds of *S. nigrum* were first treated with 70 % ethanol for 30 s and then immersed in 1 % (m/v) sodium hypochlorite for 8 min, rinsed five times with sterile distilled water, and then left to germinate on one-half strength hormone-free Murashige and Skoog (1962; MS) medium supplemented with 30 g dm^{-3} sucrose and 7 g dm^{-3} agar (*Sigma*). Five-day old seedlings were transferred to Hoagland solution (Graziano and Lamattina 2007). The seedlings were grown for 5 d and transferred to fresh Hoagland solution that contain either 0.1 μM (Fe deficiency) or 50 μM (Fe sufficiency) FeNaEDTA for 7 d. Cultures were maintained at 22 ± 3 °C under a 16-h photoperiod with a photosynthetic photon flux (PPF) density of 45 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. S-nitrosoglutathione (GSNO), an NO donor, and an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 100 μM), as well as an NO synthetase (NOS) inhibitor, N^G-nitro-*L*-Arg-methyl ester (L-NAME, 100 μM), and a nitrate reductase (NR) inhibitor, tungstate (100 μM), were used to determine whether NO was able to mediate the physiological responses of the *S. nigrum* seedlings to Fe deficiency-induced stress. NO was monitored by incubating roots from 12-d-old seedlings with 15 μM of the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA) in 20 mM Hepes-NaOH (pH 7.5). Thereafter, the roots were washed twice for 5 min with fresh buffer and observed under a laser scanning confocal microscope (excitation 490 nm; emission 515 nm). For localizing H_2O_2 produced by *S. nigrum* roots, treated roots were immersed into 1 mg cm^{-3} of 3-diaminobenzidine (DAB)-HCl (pH 3.8) for 5 h and cleared by boiling in alcohol (95 %, v/v) for 5 min. The H_2O_2 contents were determined as described by Velikova *et al*. (2000). Roots (0.1 g) were homogenized in 5 cm^3 of trichloroacetic acid (TCA, 0.1 %, m/v) at 4 °C and centrifuged at 12 000 *g* for 10 min. The 0.5 cm^3 of supernatant was added to 0.5 cm^3 of phosphate buffer (pH 7.0) and 1 cm^3 of 1 M KI. The reaction mixture was measured at 390 nm. Chlorophyll was determined by the method of Arnon (1949). Root hair length was evaluated in a 1-mm section (1 mm above root tip) of *S. nigrum* roots. Seedlings were oven-dried at 65 - 70°C for one week. A total of 50 mg of dried plant tissues was ground up and digested in 1 cm^3 of concentrated nitric acid for 2 - 3 d at room temperature. Samples were then boiled for 1 - 2 h until they were completely digested. After adding 4 cm^3 of *Millipore*-filtered deionized water and brief centrifugation, the contents of K, Ca and Fe in each sample were determined by atomic absorption spectrometry (*Shimadzu AA-6300*, Tokyo, Japan). Each experiment was repeated at least three times with six plantlets. Statistical analysis was done by Duncan's test ($P < 0.05$).

Fe deficiency induced root hair proliferation and reduced the growth of *S. nigrum* seedlings significantly compared with those sufficiently supplied with Fe (Fig. 1, Table 1). The height and chlorophyll content of 17-d-old *S. nigrum* plants grown in 0.1 μM FeNaEDTA were reduced to 74.58 % and 56.58 %, respectively, of those of plants grown in 50 μM FeNaEDTA. Fe deficiency induced the over-accumulation of NO and a strong increase of H_2O_2 content in seedling roots (Fig. 1, Table 1). These results indicate that roots responded to iron starvation by accumulating both NO and H_2O_2.

As Fe deficiency induced endogenous NO accumulation in the roots, we further examined the effect of NO on the tolerance to Fe deficiency of *S. nigrum* plantlets. For this purpose, the NO donor GSNO was applied to determine whether NO was able to mediate the physiological responses of *S. nigrum* seedlings to iron deficiency-induced stress. Supplement with the nutrient solution with 100 μM GSNO improved chlorophyll content and further promoted root hair elongation in iron-deficient *S. nigrum* seedlings. Addition of 100 μM GSNO also reduced the H_2O_2 accumulation in *S. nigrum* seedlings (Table 1).

Table 1. Effects of 100 μM GSNO, cPTIO, L-NAME or tungstate on endogenous NO content, H_2O_2 accumulation, root hair length, plantlets height and chlorophyll content in *S. nigrum* seedlings under Fe deficiency. Means ± SE, $n = 6$. Different letters within each column indicate significant differences at $P < 0.05$.

Treatment	NO relative fluorescence [%]	H_2O_2 content [nmol g^{-1}(f.m.)]	Root hair length [μm]	Plantlet height [cm]	Chl content [mg g^{-1}(f.m.)]
Control	21 ± 3.0 f	148 ± 51 e	60 ± 5.4 e	6.57 ± 0.37 a	1.52 ± 0.12 a
-Fe	100 ± 12.0 b	464 ± 33 c	170 ± 13 ab	4.90 ± 0.50 b	0.86 ± 0.13 c
-Fe + GSNO	133 ± 12.4 a	419 ± 13 d	192 ± 9 a	6.30 ± 0.46 a	0.99 ± 0.03 bc
-Fe + cPTIO	42 ± 6.0 e	732 ± 56 a	47 ± 6.7 f	3.40 ± 0.60 c	0.73 ± 0.02 d
-Fe + cPTIO + GSNO	75 ± 9.5 c	621 ± 52 ab	92 ± 9.9 d	4.23 ± 0.60 bc	1.13 ± 0.12 b
-Fe + tungstate	95 ± 10.5 b	432 ± 39 cd	129 ± 5.4 c	4.67 ± 0.26 b	0.81 ± 0.05 cd
-Fe + tungstate + GSNO	120 ± 14.0 ab	393 ± 26 d	145 ± 6 b	6.00 ± 0.48 a	0.90 ± 0.05 c
-Fe + L-NAME	36 ± 5.3 e	661 ± 41 ab	46 ± 2.9 f	2.39 ± 0.26	0.46 ± 0.09 e
-Fe + L-NAME + GSNO	62 ± 7.8 d	593 ± 32 b	83 ± 3.1 d	4.50 ± 0.46 b	0.81 ± 0.05 cd

To investigate whether or not the protective effect induced by GSNO treatment was the result of the production of NO and whether or not endogenous NO is central to this process, 100 μM cPTIO (an NO scavenger) was applied alone or with GSNO. The endogenous NO content was reduced to 42 % by the NO scavenger cPTIO (Table 1), suggesting that the DAF-2DA-dependent fluorescence is related to endogenous NO. Supplementation with cPTIO inhibited root hair proliferation during iron starvation significantly. Chlorophyll content and plantlet growth were further reduced, and H_2O_2 accumulation was significantly elevated in the presence of cPTIO under iron deficiency. The inhibitory effects of cPTIO were reversed by supplementation with the NO donor GSNO (Table 1), suggesting that NO is involved in alleviating iron deficiency-induced stress in *S. nigrum* by reducing H_2O_2 accumulation and hence it limiting oxidative damage.

NO synthesis in plants occurs by different pathways containing the main enzymes: nitrate reductase (NR) (Desikan *et al*. 2002) or nitric oxide synthase-like (NOS-like) enzyme (Guo *et al*. 2003). To differentiate these pathways in *S. nigrum* roots, the NR inhibitor, tungstate, and the NOS inhibitor, L-NAME were used. Production of NO was detected using DAF-2 DA fluorescence probe. The 100 μM tungstate suppressed the accumulation of NO induced by Fe-deficiency significantly, however, L-NAME treatment only slightly reduced the NO content in Fe-deficiency roots. In agreement with these results, tungstate showed higher inhibitory effects on root hair extension and plantlet growth and higher accumulation of H_2O_2 than L-NAME (Table 1).

Fe deficiency induced endogenous NO accumulation. NO has a high affinity for iron and the Fe-nitrosyl complex is central in NO biochemistry. Studies have indicated that NO is an important for plant iron homeostasis (Graziano and Lamattina 2005). In order to address the question of whether or not NO improves ion absorption during iron-deficiency in *S. nigrum* plantlets, exogenous NO (GSNO) and NO scavengers (cPTIO, tungstate and L-NAME) were applied to assess the relationship between NO and ion accumulation. Fe starvation increased the contents of K and Ca, and reduced Fe accumulation. Application of the GSNO further increased the K and Ca contents, however, the Fe content was only slightly elevated. Inhibition of NO accumulation by cPTIO and tungstate reversed the effects of NO on the ion absorption in *S. nigrum*.

NO has been shown to be a critical for inducing adaptive responses to iron deficiency and root hair proliferation in tomato roots. Graziano and Lamattina (2007) speculated that there are likely other molecules that act in the pathway upstream or downstream from the site of NO action, or in concert with NO. Our results indicate that intracellular accumulation of H_2O_2 and NO were the responses of roots to Fe starvation, and that these molecules are involved in iron deficiency-induced root hair proliferation in *S. nigrum* seedlings. We also found that supplementation with the NO scavengers cPTIO and tungstate increased H_2O_2 accumulation significantly while reducing root hair proliferation. This implies that appropriate H_2O_2 level are required for root hair development, and that over-accumulation of H_2O_2 inhibits root hair initiation and elongation (Table 1).

NO treatment improves the plantlet growth under the condition of Fe starvation; however, Fe absorption shows only slight increase by NO supplementation. Similar result was also reported by Graziano and Lamattina (2007) in tomato plants, where exogenous NO did not increase total iron content. They hypothesized that NO might facilitate the delivery of iron between and within plant cells through the formation of iron-nitrosyl complexes and thereby improve the tolerance of seedlings to iron starvation. Future research is needed to elucidate the molecular mechanisms of NO-mediated iron assimilation in plants under iron starvation. Fe starvation resulted in the generation of NO (Fig. 1), which increased the absorption of Ca and K in *S. nigrum*. NO supplementation produced similar effects (Table 2). However, inhibition of NO accumulation by cPTIO and tungstate reversed the effects of Fe deficiency on K and Ca accumulation (Table 2). Inhibition of NO production by specific inhibitors also indicated that NO was mainly produced due to increased NR activity in low iron-supplying *S. nigrum* seedlings,

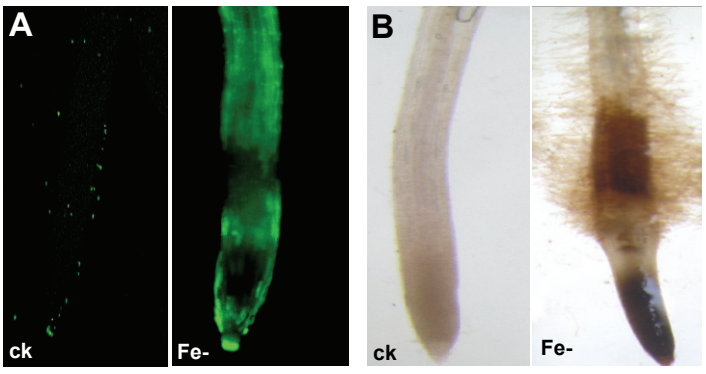

Fig. 1. Nitric oxide (NO) and H_2O_2 accumulation are early responses of roots to iron starvation in *S. nigrum* seedlings: *A* - NO production in *S. nigrum* roots during iron deficiency shown as green fluorescence from DAF-2 DA in representative roots; *B* - H_2O_2 accumulation revealed by DAB staining in *S. nigrum* roots during iron deficiency (ck: control, Fe-: iron deficiency).

Table 2. Effects of Fe deficiency on the contents of Fe, K and Ca in *S. nigrum* seedlings in the presence or absence of NO donor (GSNO) and NO scavengers (cPTIO, L-NAME and tungstate). The concentrations of GSNO, cPITO, L-NAME and tungstate were 100 μM. Means ± SE, $n = 3$. Different letters within each column indicate significant at $P < 0.05$.

Treatment	Fe [μg g^{-1}(d.m.)]	K [μg g^{-1}(d.m.)]	Ca [μg g^{-1}(d.m.)]
Control	576.20 ± 23.4 a	413.33 ± 22.6 c	17.22 ± 3.40 c
-Fe	150.79 ± 12.2 bc	442.55 ± 26.7 ab	37.66 ± 6.75 b
-Fe + GSNO	195.74 ± 46.2 b	463.42 ± 13.5 a	52.40 ± 5.30 a
-Fe + cPTIO	143.25 ± 17.5 c	385.00 ± 21.5 d	8.79 ± 1.20 e
-Fe + cPTIO + GSNO	172.60 ± 14.2 bc	436.00 ± 17.0 b	12.00 ± 4.10 d
-Fe + tungstate	139.97 ± 8.74 c	419.54 ± 12.9 c	12.44 ± 2.36 d
-Fe + tungstate + GSNO	169.12 ± 16 bc	440.00 ± 12.3 ab	17.90 ± 4.40 c
-Fe + L-NAME	148.20 ± 21.3 bc	438.77 ± 15.9 b	35.98 ± 4.90 b
-Fe + L-NAME + GSNO	143.68 ± 17.9 c	441.43 ± 12.6 ab	37.00 ± 5.80 b

which was consistent with previous reports (Graziano and Lamattina 2007). Ca is a vital signaling molecule in plants responding to stress (Xu *et al.* 2009). NO was shown to interact with Ca^{2+} channels in plants (Neill *et al*. 2002; Wendehenne *et al*. 2004). Therefore, the increased Ca content in *S. nigrum* is important for Fe starvation tolerance. K^+ is an essential element that plays vital roles in various aspects of plant cell growth and metabolism and is needed in large quantities (Zhu *et al*. 1998). Appropriate concentration of K is advantageous for the accumulation of divalent cations (Khan and Hanson 1957). From these results, we concluded that NO could bring tolerance to Fe deficiency-induced stress in *S. nigrum* by limiting the oxidative damage and maintaining intracellular ion equilibrium.

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