Exogenous nitric oxide alleviates iron-deficiency chlorosis in peanut growing on calcareous soil

X.W. Zhang[1], Y.J. Dong[1,2], X.K. Qiu[1], G.Q. Hu[1], Y.H. Wang[1], Q.H. Wang[1]

[1]*College of Resources and Environment, Shandong Agricultural University, Tai'an, P.R. China*

[2]*Chinese National Engineering Research Center for Slow/Controlled Release Fertilizers, Tai'an, P.R. China*

ABSTRACT

Sodium nitroprusside (SNP), a nitric oxide (NO) donor, was added into controlled release fertilizer (CRF) or sprayed on leaves to supply NO on iron deficiency stress in peanut (*Arachis hypogaea* Linn) plants growing on calcareous soils. Iron deficiency reduced plant growth and chlorophyll content. NO improved plant growth and alleviated leaf interveinal chlorosis, and increased the activity of root Fe^{III} reductase and the concentration of available iron in cultured soil, suggesting that NO action could be related to iron availability to the plant. The actual photochemical efficiency (ΦPSII) and photochemical maximum efficiency of PSII (Fv/Fm) were increased, and minimum fluorescence yield (Fo) was decreased under NO-treated condition, which supported the protective effect of NO on photosystem II (PSII) in peanut leaves. NO increased the activities of antioxidant enzymes, and reduced malondialdehyde (MDA) accumulation. These results suggest that exogenous NO could alleviate iron deficiency induced chlorosis of peanut plants growing on calcareous soil.

Keywords: *Arachis hypogaea* Linn; active iron; Fe^{III} reductase; chlorophyll; antioxidant enzymes

Iron (Fe) is an essential nutrient element with a crucial function in plants. It takes part in photosynthesis, respiration, DNA synthesis, and hormone structure and action (Graziano and Lamattina 2007). Therefore, Fe availability maintains a direct correlation with plant productivity. Chlorosis, because of the unavailability of Fe in calcareous soils, is a major agricultural problem that results in diminished crop yields in an estimated 30% of calcareous soils worldwide (Mori 1999).

Fe deficiency impairs chlorophyll biosynthesis and chloroplast development in both dicotyledonous and monocotyledonous species (Graziano et al. 2002). Fe deficiency is reported to affect the expression and the activity of certain peroxidase isoenzymes and induced secondary oxidative stress in dicotyledonous species (Ranieri et al. 2001). Studies on physiological responses to Fe deficiency already made significant progress in tomato, maize and some other crops (Graziano et al. 2002, Graziano and Lamattina 2007). Plant species differ in their efficiency of Fe absorption or utilization and also in Fe requirements at different growth stages. Non-graminaceous plants (strategy I) enhance acidification of the extracellular medium and increase both root ferric-reducing capacity and uptake of ferrous Fe. In contrast, graminaceous plants possess the ability to secrete phytosiderophores to enhance Fe uptake from soils (strategy II). Peanut belongs to Strategy I plant, and Fe^{III} is initially solubilized by reduction and Fe^{II} is then transported across the plasma membrane. Therefore, the physiological responses of peanut to Fe-deficiency stress include the release of hydrogen ions and reductants from roots and the increased reduction of Fe^{III} at the plasmalemma of roots (Römheld and Marschner 1983).

Supported by the National Natural Science Foundation of China, Project No. 40701094; by the National Science and Technology Pillar Program during the Twelfth Five-Year Plan Period, Projects No. 2011BAD11B01 and 2011BAD11B02; and by the Key Projects in the National '948' Program during the Twelfth Five-year Plan Period, Grant No. 2011-G30.

Nitric oxide (NO) is a bioactive free radical which plays important roles in many physiological processes in plants, such as growth, development, senescence and adaptive responses to multiple stresses (Beligni and Lamattina 2001, Graziano and Lamattina 2007, Misra et al. 2010). NO is reported to ameliorate stress responses in plants (Misra et al. 2011). Not only do plants produce significant amounts of NO, but they also respond to atmospheric NO either directly, by reaction with effector molecules or indirectly, by modifying the redox state of the cell. NO can readily form complexes with transition metal ions in aqueous solutions or those present in diverse nucleophylic compounds such as metalloproteins. The $Fe^{III}NO$ complex appears to undergo a charge transfer reaction to form $Fe^{II}NO^{+}$ and can increase the availability of Fe in plants (Graziano et al. 2002). Our previous studies also indicated that NO improves peanuts tolerance to Fe deficiency stress (Zhang et al. 2011).

In northern China, Fe-deficiency chlorosis is still a severe and common problem in peanut producing areas on calcareous soil which have not been resolved. Fe which was added into the soil or sprayed on leaves was apt to be oxidized. Additionally, the studies of utilizing NO to resolve Fe deficiency chlorosis in peanut are limited. Based on these known chemical properties of NO on other plants, we wished to investigate the mechanism of NO in alleviating Fe deficiency-induced chlorosis in peanut growing on calcareous soil. In this study, we applied new approach to supply NO for peanut growing on calcareous soil. Sodium nitroprusside (SNP, NO donor) was added into controlled release fertilizer (CRF) as base fertilizer or directly sprayed on peanut leaves. Our overall objective was to investigate the physiological mechanism of exogenous NO increasing peanut tolerance to Fe deficiency stress on calcareous soil. The specific objectives of this study were to examine (i) whether NO increased the Fe availability within the plant; (ii) how NO protected the PS^{II} in peanut leaves; and (iii) the effects of NO on modulating the antioxidant enzymes in the leaves of peanut plants.

MATERIAL AND METHODS

Plant material and growth conditions. Pot experiment was conducted on calcareous soil at a farm near Dezhou City in northern China, where the Fe-deficiency chlorosis in peanut already appeared in previous years. The soil parent material was the sediments from the Yellow River, and the soil texture was sandy with the following properties: total nitrogen (N) 0.6 g/kg, Olsen phosphorus (P) 53.01 mg/kg, available potassium (K) 76.84 mg/kg, DTPA-Fe 3.00 mg/kg, calcium carbonate ($CaCO_3$) 13.6%, and pH (H_2O) 8.2.

Seeds of peanut (*Arachis hypogaea* Linn. hsuji) were seeded on May 10, 2010. Plants were grown in plastic pots (four plants per container) with a capacity of 2.5 kg air dried soil. Pots were arranged in randomized block designs with three replicates. During the growing season, plants were managed under commonly used agronomic and irrigation practices.

The experimental design is given in Table 1. The $FeSO_4$ and SNP were supplied by being added into CRF in the process of CRF production or sprayed on leaves directly. The controlled release fertilizers were provided by the Chinese National Engineering Research Center for Slow/Controlled Release Fertilizers and the compound fertilizer used in this experiment was provided by the Shandong Kingenta Ecological Engineering Co., Ltd. The CRF consists of 25% (by weight) sulfur-coated material. The nutrient content of the CF which used to produce CRF was 15% N, 15% P_2O_5, and 15% K_2O. The content of $FeSO_4$ and SNP added into CRF was 18.90 and 5.63 mg, respectively, for every 1.0 g CRF. The consumption of the CF used to producing CRF as base fertilizer was 4.76 g per container.

The treatment of spraying $FeSO_4$, SNP and $FeSO_4$ + SNP solution on leaves was performed when the seedlings were 10-day-old. The performance was conducted in the evening every three days, and proceeded for three successive times at each critical

Table 1. The experimental design

No.	Treatment
CK	an CRF contains neither $FeSO_4$ nor SNP, and describe it as CRF1
T1	an CRF contains $FeSO_4$, and describe it as CRF2
T2	an CRF contains SNP, and describe it as CRF3
T3	an CRF both contains $FeSO_4$ and SNP, and describe it as CRF4
T4	CRF1 and sprayed leaves with $FeSO_4$ solution 0.1% (w/v)
T5	CRF1 and sprayed leaves with SNP 1.0 mmol
T6	CRF1 and sprayed leaves with $FeSO_4$ solution 0.1% (w/v) and SNP 1.0 mmol

All the treatments of CK and T1 to T6 in other Tables and Figures were in accordance with the description of Table 1

growth stage (seedling, flowering, podding, and maturity). The consumption was 10 mL every pot for each time. The experiment was designed with the same contents of CF, and the total quantity of $FeSO_4$ and SNP sprayed on leaves was consistent with that added into CRF.

At each growth stage after emergence (seedling, flowering, podding, and maturity), all chemical and biochemical analysis were carried out on young leaves of control and treated plants. The crop was harvested at maturity stage, and the pod yield and fat percentage content of peanut were determined.

Determination of available Fe content in the soil. Soil samples of root zone were taken at seedling and maturity stage, and available Fe was extracted by DTPA (pH 7.3) and quantified by atomic absorption spectrophotometry (Lindsay and Norvell 1978).

Assay of Fe^{III} reductase activity. Root samples were taken at seedling stage for assay of Fe^{III} reductase activity. The roots rinsed several times in distilled water were transferred to a beaker with saturated $CaSO_4$ under vigorous aeration. After 5 min, the plant was rinsed several times in distilled water, then placed with its root system in a beaker with nutrient solution which contained 0.1 mmol Fe^{III}-EDTA, and 0.4 mmol 2,2'-bipyridyl. After 2 h, Fe^{III} reductase activity was determined by measuring the concentration of Fe^{II}-dipyridyl complex formed at A_{523} in a spectrophotometer (Shimadzu UV-2210, Tokyo, Japan) (Xu et al. 1998).

Determination of active Fe content. Active Fe content was determined according to the procedure of Takker and Kaur (1984). Fresh young leaves taken at each growth stage (seedling, flowering, podding, and maturity) were cut into pieces and extracted with 1 mol/L HCl (in 1:10 tissue: extractant), shaken for 5 h and filtered, and the Fe concentration in the filtrate was measured with atomic absorption spectrophotometry (PE-2100B, Perkin Elmer Co. Ltd., Wellesley, USA).

Fat contents assay. Samples of peanut were taken at harvest stage. Fat percentage content was measured according to Ali and Khan (1988).

Determination of chlorophyll content, net photosynthetic rate (P_n), and transpiration rate (T_r). Young leaves (0.5 g of fresh weight) taken at flowering, podding, and maturity stage were powdered with liquid nitrogen, and pigments were extracted with 4 volumes of 80% (v/v) acetone until complete bleaching. The concentrations of chlorophyll were determined according to Arnon (1949).

The young leaves were selected to measure net photosynthetic rate (P_n) and transpiration rate (T_r) by using CIRAS-2 (Amesbury, MA, USA), between 9:00–10:00 AM at the flowering and podding stage. Three plants per pot were selected each time.

Determination of chlorophyll fluorescence. Young leaves were selected to measure chlorophyll fluorescence by using the pulse amplitude modulated system (model FMS2, Hansatech Instruments, Norfolk, UK). Fluorescence measurements were done between 9:00–10:00 AM at the flowering stage of the plants.

Antioxidant enzyme, MDA, soluble protein extraction and assay. The second fully expanded leaves of the plants were sampled for enzymatic analysis at seedling and maturity stage. Samples were homogenized in 0.05 mol/L phosphate buffer (pH 7.8) by grinding with a mortar and pestle under chilled condition with liquid nitrogen. The homogenate was filtered through four layers of muslin cloth and centrifuged at 12 000 × for 10 min at 4°C, and the supernatants were used for measurements of SOD, CAT, POD activities and MDA concentration according to Zhang (1992). Soluble protein content was measured by the Coomassie Brilliant Blue G-250 method (Bradford 1976).

Data analysis. The Tukey's test was used to test the effect of different treatment, and the least significant difference (*LSD*) was calculated to compare the differences between means in each treatment. Correlative analysis was done using the SPSS software (SPSS 11.5).

RESULTS

Plant growth. As shown in Figure 1, Fe deficiency inhibited the growth of plants which were produced on calcareous soil. NO supplementation significantly alleviated the inhibition, producing an increase of 8.6% (T2) and 8.2% (T5) in FW compared with the control plants (Figure 1A). Even under Fe treated conditions, the increase of NO on FW was significant. The root: top ratio (R/T) was increased when plants were subjected to Fe deficiency stress, which was beneficial to nutrient absorption. Supplementation with NO further increased the R/T (Figure 1B).

NO regulates pH, available Fe content of calcareous soil and improves Fe reduction capacity. Table 2 shows that Fe deficiency resulted in the decrease of pH, and NO supplementation further reduced pH significantly by 0.21 (T2) and 0.18 (T5) compared with control at seedling stage. While under Fe treated conditions, the decrease

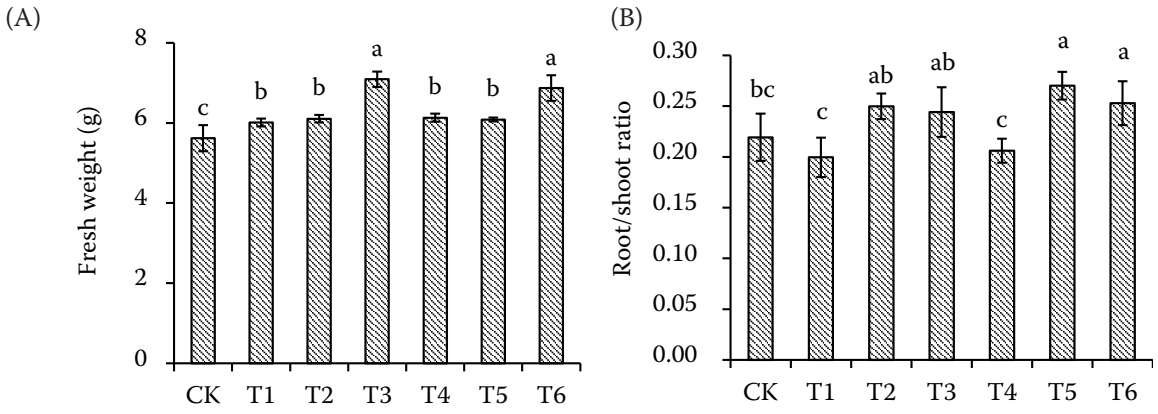

Figure 1. Effects of exogenous NO on fresh weight (FW) (A) and root/shoot ratio (B) of peanut at seedling stage

was slight. The pH of culture soil was elevated at maturity stage, and at this stage, spraying SNP on leaves was more efficient than fertilizing CRF with SNP. That is because the SNP decomposed in the soil solution.

Compared with control, NO supplementation significantly increased available Fe content in the culture soil at seedling stage. Spraying SNP on leaves was more efficient than fertilizing CRF with SNP at maturity stage. That is because at later growth stage, SNP decomposed in the soil solution. The effect was not significant when adding $FeSO_4$ alone to CRF, while it was dramatic when applied with SNP together (Table 2).

Reduction of Fe^{III} to Fe^{II} by Fe^{III} chelate reductase is thought to be an obligatory step in Fe uptake as well as the primary factor in making Fe available for absorption by all plants except grasses (Yi and Guerinot 1996). As shown in Table 2, Fe deficiency resulted in an increase of Fe^{III}-reductase activity. And the increase was further promoted by NO supply, producing a 54.2% (T2) and a 166.9% (T5) increase in the Fe^{III} reductase activity compared with control.

NO increases active Fe content, pod yield and fat content of peanut. As shown in Table 3, peanut was the most sensitive to Fe-deficiency stress at flowering stage, and the content of active Fe was the lowest at this stage. While NO alleviated the Fe-deficiency stress significantly, producing a 95.7% (T2) and a 47.8% (T5) increase in active Fe content compared with control at this stage. Even under $FeSO_4$ treated conditions, the active Fe was greatly increased. NO supplementation increased the active Fe content by 111.6% (T3) and 127.2% (T6) compared with control at flowering stage. At different growth stages, the efficiency of these two approaches of supplementation NO for peanut was distinct. At later stage, treatment of spraying SNP on leaves was more effective than adding SNP to CRF.

The present study shows that in NO treated plants, the pod yield of each plant was larger than control. The fat percentage content was increased by 8.0% (T2) and 13.5% (T5) compared with control (Table 3). A significant (r^2 = 0.850) correlation between pod yield and the content of active Fe was

Table 2. Effects of NO on pH, the content of available iron in the soil, and the activity of Fe^{III} reductase in peanut root at seedling stage. Soil samples of root zone were taken at seedling and maturity stage

Treatment	pH		Available iron/(mg/kg)		Fe^{III} reductase activity (μmol/g 2 h)
	seedling stage	maturity stage	seedling stage	maturity stage	
CK	7.88 ± 0.03^{bc}	8.79 ± 0.08^{b}	1.24 ± 0.11^{c}	1.81 ± 0.05^{d}	0.68 ± 0.26^{d}
T1	8.05 ± 0.10^{a}	8.92 ± 0.16^{ab}	1.28 ± 0.02^{bc}	2.13 ± 0.20^{cd}	0.61 ± 0.13^{d}
T2	7.67 ± 0.04^{e}	8.89 ± 0.03^{ab}	1.34 ± 0.02^{b}	2.01 ± 0.25^{cd}	1.04 ± 0.18^{c}
T3	7.97 ± 0.01^{ab}	8.84 ± 0.05^{ab}	1.35 ± 0.05^{b}	2.50 ± 0.29^{b}	1.09 ± 0.15^{c}
T4	8.00 ± 0.14^{ab}	8.95 ± 0.04^{a}	1.30 ± 0.05^{bc}	2.29 ± 0.13^{bc}	0.50 ± 0.04^{d}
T5	7.70 ± 0.03^{de}	8.35 ± 0.04^{d}	1.37 ± 0.04^{b}	2.19 ± 0.07^{bc}	1.81 ± 0.03^{a}
T6	7.82 ± 0.03^{cd}	8.59 ± 0.06^{c}	1.65 ± 0.04^{a}	2.91 ± 0.32^{a}	1.53 ± 0.04^{b}

Different letters within the same column means significant at 5% level. The same below

Table 3. Effects of NO on the content of active Fe, pod yield of each plant and fat of peanut

Treatment	Active Fe content (mg/kg FW)				Pod yield of each plant (g/plant)	Fat (%)
	seedling stage	flowering stage	podding stage	maturity stage		
CK	23.24 ± 1.88^{c}	9.91 ± 0.73^{e}	12.58 ± 1.29^{d}	13.47 ± 2.59^{c}	6.68 ± 0.16^{d}	40.89 ± 2.50^{e}
T1	24.84 ± 1.62^{c}	11.91 ± 2.14^{de}	12.90 ± 0.91^{d}	13.88 ± 2.63^{c}	6.85 ± 0.13^{cd}	42.50 ± 2.40^{de}
T2	26.18 ± 4.39^{c}	19.39± 2.42^{ab}	12.69 ± 2.30^{d}	13.74 ± 0.46^{c}	7.25 ± 0.17^{cd}	44.16 ± 2.45^{cd}
T3	28.18 ± 3.68^{c}	20.97 ± 3.37^{ab}	20.37 ± 3.12^{c}	16.94 ± 2.22^{c}	8.54 ± 0.69^{a}	47.55 ± 0.53^{ab}
T4	28.17 ± 2.31^{c}	17.87 ± 1.31^{bc}	32.17 ± 0.75^{b}	63.67 ± 6.62^{a}	7.45 ± 0.35^{bc}	43.78 ± 0.68^{cde}
T5	49.28 ± 3.53^{b}	14.64 ± 0.62^{cd}	28.79 ± 3.91^{b}	29.60 ± 1.98^{b}	7.39 ± 0.36^{bc}	46.39 ± 1.29^{bc}
T6	65.50 ± 5.88^{a}	22.51 ± 3.86^{a}	50.21 ± 9.65^{a}	71.30 ± 13.38^{a}	7.99 ± 0.36^{ab}	49.45 ± 0.32^{a}

The contents of active iron in leaves were investigated at seedling, flowering, podding and maturity stage. The plants were harvested at maturity stage, and the pod yield of each plant and fat percentage content were determined

obtained at flowering stage. Similarly, significant correlation between fat percentage and active Fe at seedling (r^2 = 0.791) and flowering (r^2 = 0.828) stage was also observed (Table 4).

Chlorophyll content. As shown in Figure 2, NO supplementation avoided Fe-deficiency induced chlorosis of plants growing on calcareous soil without $FeSO_4$ treatment. It increased the content of chlorophyll by 26.2% (T2, chlorophyll a), 10.8% (T2, chlorophyll b), 32.0% (T5, chlorophyll a) and 14.5% (T5, chlorophyll b) relative to control at flowering stage (Figure 2A–B). At different growth stages, the efficiency of these two approaches of supplementation $FeSO_4$ and SNP for peanut were discrepant. Under $FeSO_4$ treated conditions, chlorophyll content was also increased. Fe-deficiency slightly reduced the ratio chlorophyll a/b, and NO supply increased the ratio chlorophyll a/b significantly (Figure 2D). After flowering stage, the content of chlorophyll was decreased. This was because at this stage, there was great rainfall in northern China, which induced the nutrient loss in the soil. Thus, various nutrient deficiency stresses resulted in the reduction of chlorophyll content. Whereas, NO greatly increased the content of chlorophyll and the level of ratio chlorophyll a/b (Figures 2A–D).

NO protects photosynthetical system. Nutrient uptake came to a climax at podding stage, the pegs interposed into the soil to have pod, and the photosynthesis was most vigorous. Fe deficiency resulted in a decrease of P_n and T_r, and NO supply alleviated the decrease, producing an increase of 13.8% (T2) and 53.7% (T5) compared to control. Similarly, T_r was also promoted by NO, despite the increase was slight (Table 5).

As shown in Table 5, the ratio Fv/Fm was 0.69 in leaves of control plants, and NO supplementation increased the ratio to 0.76–0.77, which was similar to the Fe treated ones. Fe deficiency induced an increase of Fo, and a decrease of ΦPSII. However, NO application alleviated the effects.

Antioxidant enzymes activity. Figure 3 shows that Fe deficiency inhibited the activities of CAT and POD. However, the effect was different on SOD. The activity of SOD was slightly increased under Fe deficiency stress at seedling stage, whereas it was decreased at maturity stage. NO supplementation further promoted the activity of SOD significantly. It increased the activity of SOD by 54.8% (T2) and 36.9% (T5) compared with control at seedling stage (Figure 3A). The activity of CAT was decreased at maturity stage compared with that of at seedling stage. However, the result of POD was contrary. NO supplementation increased the activities of CAT and POD at seedling stage. However, spraying leaves with $FeSO_4$ + SNP decreased the activity of POD by 34.4% compared to that of spraying leaves with $FeSO_4$ alone at maturity stage (Figures 3B–C).

Table 4. Correlations between pod yield of each plant, fat and active Fe content in leaves

Items	Active Fe content			
	seedling stage	flowering stage	podding stage	maturity stage
Pod yield of each plant	0.406	0.850*	0.513	0.349
Fat	0.791*	0.828*	0.752	0.514

*P < 0.05; **P < 0.01

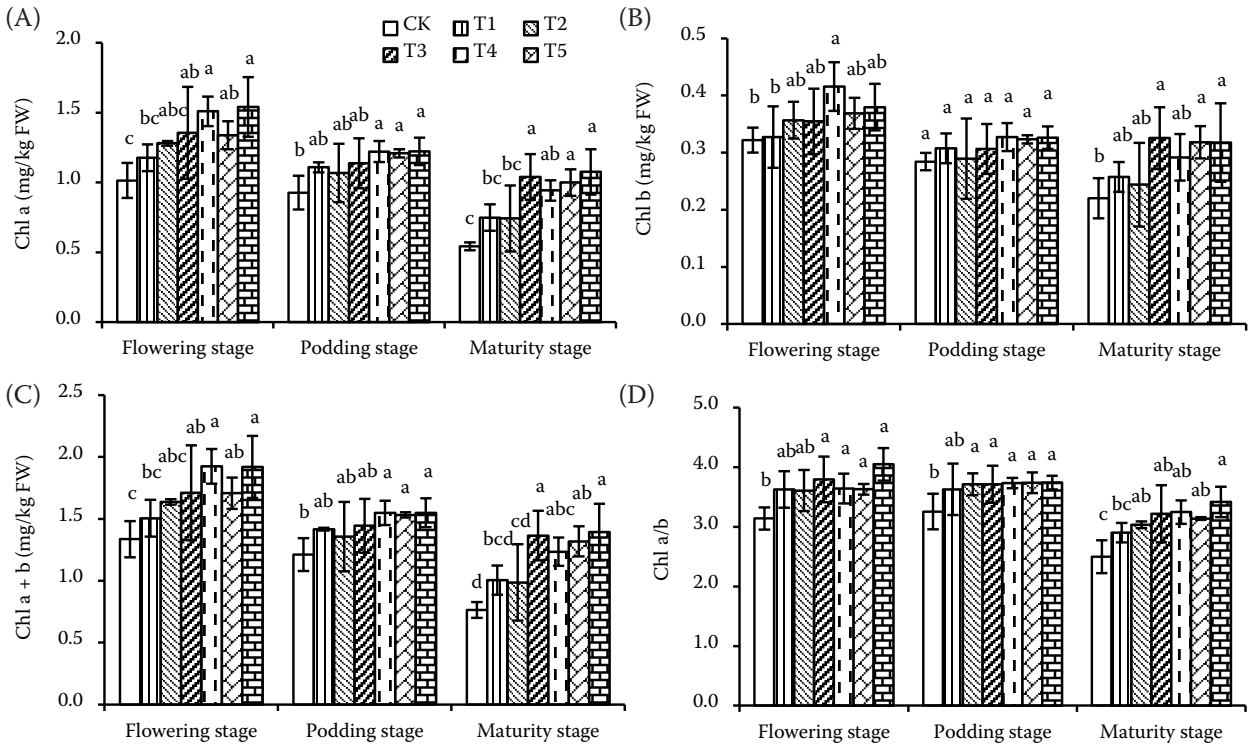

Figure 2. Effects of exogenous NO on the content of chlorophyll a (A), chlorophyll b (B), chlorophyll a + b (C) and chlorophyl a/b (D). The contents of chlorophyll in peanut leaves were investigated at flowering, podding and maturity stage

MDA and soluble protein contents. Fe deficiency increased the accumulation of MDA, and decreased the content of soluble protein in peanut leaves. NO supplementation alleviated the effect (Figures 4A–B). At later growth stage, the content of MDA greatly increased, whereas spraying leaves with SNP dramatically alleviated the increase (Figure 4A). In NO-treated plants, the contents of soluble protein were higher than that of the untreated ones. At seedling stage, NO increased the content of soluble protein by 69.7% (T2) and 68.4% (T5) compared with the control (Figure 4B).

DISCUSSION

SNP was widely used as NO donor. Previous report confirmed that Fe deficiency reduced the NO content in maize leaves, whereas the content of NO in leaves treated with SNP was higher than in those without SNP treatment (Sun et al. 2007).

Table 5. Effects of NO on the net photosynthetic rate (P_n), transpiration rate (T_r) and chlorophyll fluorescence parameters in leaves of peanut. P_n and T_r were determined at flowering and podding stage. Fo, ΦPS Ⅱ and Fv/Fm were investigated at flowering stage

Treatment	P_n/(μmol/m^2/s)		T_r/(mmol/m^2/s)		Fo	ΦPS Ⅱ	Fv/Fm
	flowering	podding	flowering	podding			
CK	2.42 ± 0.29^{d}	13.42 ± 0.31^{e}	0.22 ± 0.02^{c}	1.11 ± 0.21^{d}	82.3 ± 6.1^{a}	0.55 ± 0.02^{d}	0.69 ± 0.05^{c}
T1	4.06 ± 1.15^{bc}	16.05 ± 2.27^{cde}	0.20 ± 0.03^{c}	1.12 ± 0.11^{d}	76.5 ± 8.5^{ab}	0.61 ± 0.01^{c}	0.72 ± 0.01^{bc}
T2	3.59 ± 0.34^{cd}	15.27 ± 0.55^{de}	0.22 ± 0.06^{c}	1.16 ± 0.04^{cd}	73.0 ± 5.0^{abc}	0.61 ± 0.01^{c}	0.76 ± 0.05^{ab}
T3	4.88 ± 1.00^{abc}	18.57 ± 0.10^{bc}	0.44 ± 0.03^{b}	1.45 ± 0.06^{b}	65.5 ± 2.5^{c}	0.62 ± 0.02^{bc}	0.78 ± 0.01^{a}
T4	5.32 ± 0.43^{ab}	17.85 ± 1.87^{bcd}	0.70 ± 0.01^{a}	1.26 ± 0.08^{cd}	75.5 ± 6.5^{ab}	0.64 ± 0.00^{b}	0.77 ± 0.01^{ab}
T5	5.26 ± 1.27^{ab}	20.62 ± 1.43^{ab}	0.77 ± 0.03^{a}	1.33 ± 0.04^{bc}	77.5 ± 0.5^{a}	0.67 ± 0.02^{a}	0.77 ± 0.01^{a}
T6	5.48 ± 0.32^{a}	22.67 ± 2.72^{a}	0.70 ± 0.10^{a}	1.69 ± 0.01^{a}	68.0 ± 4.0^{bc}	0.70 ± 0.00^{a}	0.78 ± 0.01^{a}

Different letters within the same column means significant at 5% level. The same below

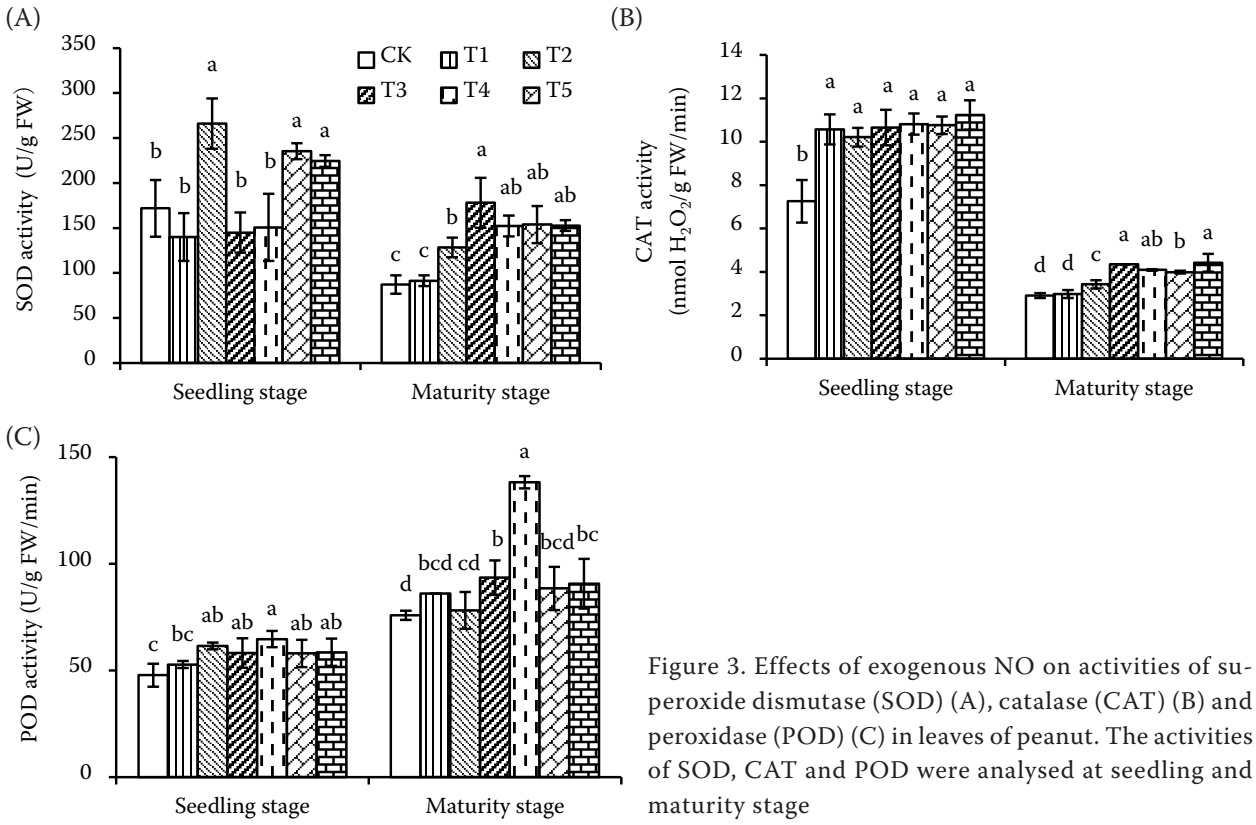

Figure 3. Effects of exogenous NO on activities of superoxide dismutase (SOD) (A), catalase (CAT) (B) and peroxidase (POD) (C) in leaves of peanut. The activities of SOD, CAT and POD were analysed at seedling and maturity stage

This work presents a strong evidence supporting a role for exogenous NO in plant Fe nutrition. Results obtained with Fe-deficient peanut plants suggested that NO action should be related to Fe acquisition and availability inside the plant. It also indicates that NO plays a protective effect against Fe deficiency stress. The occurrence of Fe deficiency symptom is known to be related to the content of calcium carbonate and available Fe form in soil. The reactivity of soil carbonate affects the solubility of Fe and, especially, the concentration of the bicarbonate ion, which is a strong buffer and may neutralize the H^+ released by proton pumps of the root plasmalemma (Šrámek and Dubský 2011). The $FeSO_4$ which was added into the CRF and sprayed on leaves was apt to be oxidized. The present study showed that NO supplementation significantly reduced pH of cultured soil, and available Fe concentration was higher in NO-treated soil than that of the non NO-treated ones (Table 2). In addition, Fe transport across the plasmamembrane is initiated by a plasmalemma located Fe^{III} reductase. Its activity is pH dependent and at alkaline pH supposed to be much depressed (Mengel 1994). NO modulates the activities of H^+-ATPase and H^+-PPase in wheat seedling roots and promotes the salt tolerance against salt stress (Ruan et al. 2004). Further experiment showed

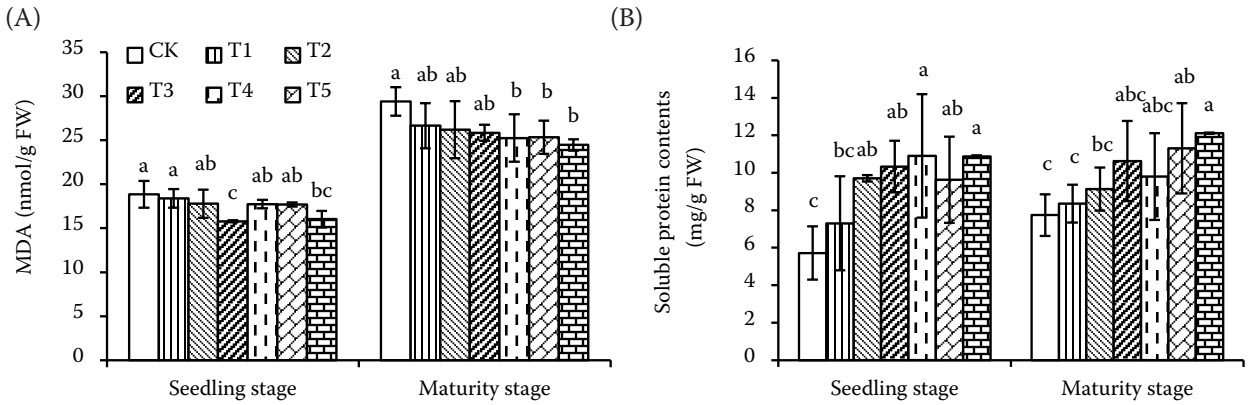

Figure 4. Effects of exogenous NO on the contents of malondialdehyde (MDA) and soluble protein in leaves of peanut. The contents of MDA and soluble protein were determined at seedling and maturity stage of peanut

that plasma membrane H^+-ATPase was involved in regulating rhizosphere pH (Yang et al. 2011). In the present work, NO supplementation dramatically promoted the activity of Fe^{III} reductase in peanut roots both under Fe or non-Fe treated conditions (Table 2). Therefore, the function of NO alleviation of Fe deficiency was related to decrease of pH. It increased the concentration of available Fe in cultured soil. On the other hand, the induction of Fe^{III} reductase activity was ascribed to pH reduction. Both were beneficial to Fe acquisition from the soil.

Oserkowsky (1933) showed that the active Fe is involved in the chlorophyll biosynthesis and provides a reliable indication of plant Fe status. In this study, the chlorosis symptoms were observed in young leaves of peanut growing on calcareous soil treated with CRF1. However, the chlorosis was alleviated in the presence of NO. The content of active Fe in young leaves was increased compared with that of the chlorotic ones (Table 3 and Figure 2). Previous report showed that the active Fe participates in various physiological actions inside the plants, such as the electron transport. Various Fe-containing proteins are involved in electron transfer processes in energy-transducing membranes. In the photosynthetic electron transport chain, heme-containing proteins (cytochromes) and non-heme Fe-containing proteins (Fd-like proteins) can be found (Sandman and Malkin 1983). In this sense, Fe deficiency is associated with inhibition of the photosynthesis, and consequently, leads to a decrease of pod yield. A significant correlation was observed between the concentration of active Fe with the pod yield and fat percentage at flowering stage (Tables 3 and 4). All the results suggest a physiological function for NO in Fe availability within plant.

Fe deficiency impairs chlorophyll biosynthesis and chloroplast development (Graziano et al. 2002). Results obtained with Fe-deficient peanut suggested that NO action should be related to Fe availability within the plant. Under Fe-deficient conditions, NO supplementation increased the chlorophyll content of peanut leaves, achieving a similar level to those growing under Fe treated conditions (Figures 2A–C). Previous report showed that chlorophyll was in a significant relation to the conversion of light energy. At room temperature, the chlorophyll fluorescence emanated from Chlorophyll *a* primarily. In this study, NO supply increased the chlorophyll a/b and P_n in peanut subjected to Fe deficiency (Figure 2D and Table 5). It was in accordance with the previous report that the increase of chlorophyll a/b was beneficial to absorbance and conversion of light energy (Liang et al. 2010). Transpiration pull is the prime impetus of Fe transport in xylem. The present experiment showed that NO supplementation promoted T_r in leaves (Table 5). Therefore, the increase of T_r both promoted Fe transport in xylem and Fe absorption from the soil. Despite the NO donor SNP contains Fe in its molecule (1 mol ferrocyanide/mol compound), previous report proved that the Fe in SNP did not augment chlorophyll content in plant (Graziano et al. 2002). Thus we can rule out possible physiological causes of Fe homeostasis in SNP treatment.

The ratio Fv/Fm is proportional within a similar range, 0.76 to 0.85, to normal conditions. Nevertheless, the ratio decreased under stress (Liang et al. 2010). Thus, this parameter was often used to evaluate the photoinhibition and the damage of PSII complex. Results showed that Fe deficiency induced a decrease of Fv/Fm and an increase of Fo, which indicate the destruction or inactivation of PSII reaction center. However, NO supplementation alleviated the effect (Table 5). ΦPSII measures the proportion of the light absorbed by chlorophyll associated with PSII which is used in photochemistry. As such, it can give a measure of the rate of linear electron transport and an indication of overall photosynthesis. Under laboratory conditions, there is a strong linear relationship between this parameter and the efficiency of carbon fixation. However, due to changes in the rate of photorespiration or pseudocyclic electron transport, a discrepancy between these two parameters may occur under certain stress conditions (Fryer et al. 1998). Fe deficiency reduced the ΦPSII in peanut leaves, whereas NO promoted this parameter dramatically (Table 5). Thus, it can be inferred that NO alleviated the damage in the photosynthetic apparatus under Fe-deficient stress. NO is shown to enhance electron transport in chloroplast thylakoid (Vladkova et al. 2011).

SOD, CAT, and POD are all important enzymes involved in antioxidation processes and presents in different organelles in plants, protecting plants from oxidative stress. Fe deficiency can induce an increase of SOD activity, but a decrease of CAT and POD activities at seedling stage (Figure 3A), suggesting that CAT and POD may not play essential roles in detoxifying ROS under Fe deficiency. That is because CAT and POD are all heme-containing enzymes, their activities are likely to be affected by Fe deficiency (Ranieri et al. 2001). NO was found to interfere with plant photo-oxidative stress by detoxifying reactive oxygen species

(Beligni and Lamattina 2002). Our previous work confirmed that NO could promote the activities of SOD, POD and CAT (Zhang et al. 2011). The present study showed that NO supplementation promoted the activities of CAT and POD at seedling stage of peanut plants under Fe deficiency stress (Figures 3B–C). It indicates that these two enzymes play important roles in scavenging ROS under Fe deficiency stress.

Fe was found in heme compounds, such as various cytochromes, peroxidase, and catalase, and in phytoferritin and ferredoxin; these compounds may have reduced activities under Fe deficiency conditions. Other enzymes may require active Fe for activation (Ranieri et al. 2001). In the study, NO supplementation increased the content of active Fe in peanut leaves (Table 3). Therefore, we can infer that the increase of active Fe was an important factor which promoted the activity of antioxidant enzymes. It was reported that POD-H_2O_2 breakdown system participates in chlorophyll decomposition. In the present experiment, we observed that spraying leaves with SNP inhibited the activity of POD under $FeSO_4$ treated conditions at maturity stage (Figure 3C). The result indicates that NO can inhibit the activity of POD at later growth stage of peanut, which consequently postponed the breakdown of chlorophyll in the leaves. MDA is the end product of membrane lipid peroxidation when plants are under stresses, and its content reflects injury extent of plant cell membrane. In the present experiment, Fe deficiency increased the accumulation of MDA, and decreased the content of soluble protein. However, the effects were alleviated in the presence of SNP (Figure 4). These results indicated that exogenous NO reduced the production of MDA and enhanced the soluble protein accumulation, thereby improving the Fe deficiency tolerance of peanut plants.

The results of this study showed that exogenous NO could improve fresh weight of peanut plants subjected to Fe deficiency stress. The higher level of active Fe and chlorophyll content of SNP treatment showed that NO increased the Fe availability within the plant. By determining the activities of SOD, CAT and POD, we learned that NO protected plants by increasing the activities of some antioxidative enzymes. All of them were strong evidence to support the physiological role for exogenous NO in plant Fe nutrition. Future study will analyse the level of hydroxyl radical (OH^-) and superoxide anion ($O_2^{\bullet -}$), and further elucidate the effects of different superoxides on the growth of Fe deficiency-stressed plants.

Acknowledgement

The authors thank the lecturer Xiujuan Wang, College of Foreign Languages, Shandong Agricultural University, China, for her critical reading and revision of the manuscript. Special acknowledgements are given to the editors and reviewers.

REFERENCES

Ali R., Khan M.N. (1988): Modified butyrometric method for rapid determination of fat in seeds. Journal of the American Oil Chemists' Society, *65*: 1951–1952.

Arnon D.I. (1949): Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiology, *24*: 1–15.

Beligni M.V., Lamattina L. (2001): Nitric oxide: a non-traditional regulator of plant growth. Trends in Plant Science, *6*: 508–509.

Beligni M.V., Lamattina L. (2002): Nitric oxide interferes with plant photo-oxidative stress by detoxifying reactive oxygen species. Plant, Cell and Environment, *25*: 737–748.

Bradford M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, *72*: 248–254.

Fryer M.J., Andrews J.R., Oxborough K., Blowers D.A., Baker N.R. (1998): Relationship between CO_2 assimilation, photosynthetic electron transport, and active O_2 metabolism in leaves of maize in the field during periods of low temperature. Plant Physiology, *116*: 571–580.

Graziano M., Lamattina L. (2007): Nitric oxide accumulation is required for molecular and physiological responses to iron deficiency in tomato roots. Plant Journal, *52*: 949–960.

Graziano M., Beligni M.V., Lamattina L. (2002): Nitric oxide improves internal iron availability in plants. Plant Physiology, *130*: 1852–1859.

Liang W.B., Xue S.G., Shen J.H., Wang P. (2010): Effects of manganese stress on photosythesis and chlorophyll fluorescence parameters of *Phytolacca americana*. Acta Ecologica Sinica, *30*: 619–625.

Lindsay W.L., Norvell W.A. (1978): Development of a DTPA soil test for zinc, iron, manganese and copper. Soil Science Society of America Journal, *42*: 421–428.

Mengel K. (1994): Iron availability in plant tissues – iron chlorosis on calcareous soils. Plant and Soil, *165*: 275–283.

Misra A.N., Misra M., Singh R. (2010): Nitric oxide biochemistry, mode of action and signaling in plants. Journal of Medicinal Plants Research, *4*: 2729–2739.

Misra A.N., Misra M., Singh R. (2011): Nitric oxide ameliorates stress responses in plants. Plant, Soil and Environment, *57*: 95–100.

Mori S. (1999): Iron acquisition by plants. Current Opinion in Plant Biology, *2*: 250–253.

Oserkowsky J. (1933): Quantitative relation between chlorophyll and iron in green and chlorotic pear leaves. Plant Physiology, *8*: 449–468.

Ranieri A., Castagna A., Baldan B., Soldatini G.F. (2001): Iron deficiency differently affects peroxidase isoforms in sunflower. Journal of Experimental Botany, *52*: 25–35.

Römheld V., Marschner H. (1983): Mechanism of iron uptake by peanut plants. Plant Physiology, *71*: 949–954.

Ruan H.H., Shen W.B., Xu L.L. (2004): Nitric oxide modulates the activities of plasma membrane H^+-ATPase and PPase in wheat seedling roots and promotes the salt tolerance against salt stress. Acta Botanica Sinica, *46*: 415–422.

Sandman G., Malkin R. (1983): Iron-sulfur centers and activities of the photosynthetic electron transport chain in iron-deficient cultures of the blue-green alga *Aphanocapsa*. Plant Physiology, *73*: 724–728.

Šrámek F., Dubský M. (2011): Occurrence and correction of lime-induced chlorosis in petunia plants. Plant, Soil and Environment, *57*: 180–185.

Sun B.T., Jing Y., Chen K.M., Song L.L., Chen F.J., Zhang L.X. (2007): Protective effect of nitric oxide on iron deficiency-induced oxidative stress in maize (*Zea mays*). Journal of Plant Physiology, *164*: 536–543.

Takker P.N., Kaur N.P. (1984): HCl method for Fe^{2+} estimation to resolve iron chlorosis in plants. Journal of Plant Nutrition, *7*: 81–90.

Vladkova R., Dobrikova A.G., Singh R., Misra A.N., Apostolova E. (2011): Photoelectron transport ability of chloroplast thylakoid membranes treated with NO donor SNP: changes in flash oxygen evolution and chlorophyll fluorescence. Nitric Oxide, *24*: 84–90.

Xu L.Z., Zhang F.S., Li C.J. (1998): 2,2'-bipyridyl-colorimetric method for measurement of Fe(III) reductase activity in roots of dicotyls. Plant Nutrition and Fertilizer Science, *4*: 63–66.

Yang Y., Wang Q.L., Geng M.J., Guo Z.H., Zhao Z. (2011): Rhizosphere pH difference regulated by plasma membrane H^+-ATPase is related to differential Al-tolerance of two wheat cultivars. Plant, Soil and Environment, *57*: 201–206.

Yi Y., Guerinot M.L. (1996): Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency. Plant Journal, *10*: 835–844.

Zhang X.W., Zhang M., Wang Q.H., Qiu X.K., Hu G.Q., Dong Y.J. (2011): Effect of exogenous nitric oxide on physiological characteristic of peanut under iron-deficient stress. Plant Nutrition and Fertilizer Science, *17*: 665–673.

Zhang X.Z. (1992): Research Methodology of Crop Physiology. Agriculture Press, Beijing, 208–212.

Received on June 3, 2011

Corresponding author:

Yuanjie Dong, Shandong Agricultural University, College of Resources and Environment, Tai'an, 271018, P.R. China
e-mail: yjdong@sdau.edu.cn