The nitric oxide production and NADPH-diaphorase activity in root tips of *Vicia faba* L. under copper toxicity

Ting Zou[1], Li Ping Zheng[2], Hui Yan Yuan[2], Ya Fei Yuan[1], Jian Wen Wang[1*]

[1]**College of Pharmaceutical Sciences, Soochow University, Suzhou 215123, China**
[2]**College of Architecture and Urban Environment, Soochow University, Suzhou 215123, China**

*Corresponding author: jwwang@suda.edu.cn

Abstract

Copper (Cu) toxicity on plants has become a major problem with increasing agricultural and environmental pollution. The effect of Cu on NADPH-diaphorase (NADPH-d) activity, commonly employed as a marker for nitric oxide synthase (NOS) activity and nitric oxide (NO) production, was investigated in root tips of *Vicia faba* L. We found that Cu stress stunted root growth and development of root hair. Cu treatment also caused an increase in NADPH-d activity and NO release in roots. Application of the NO donor sodium nitroprusside (SNP) efficiently alleviated the copper toxicity effects. The Cu-induced NO in vascular bundles was associated primarily with the presence of the induced NADPH-d activity. Our results suggested that NOS-like enzyme, but not the nitrate reductase, was the source of inducible NO generation in roots of *V. faba* under Cu stress. This is the first report on NADPH-d activity and its distribution in plants under Cu toxicity.

Keywords: Copper; Faba bean; NADPH-diaphorase; Nitric oxide; Root growth.
Abbreviation: Cd, cadmium; Cu, Copper; DAF-2 DA, 4,5-diaminofluorescein diacetate; H_2O_2, hydrogen peroxide; NADPH-d, NADPH-diaphorase; L-NAME, N-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; ROS, reactive oxygen species; SA, salicylic acid; SNP, sodium nitroprusside, Zn; zinc.

Introduction

Copper (Cu) is an essential trace element for growth and development of plants (Maksymiec, 1997; Waraich et al., 2011), but it is also toxic even at very low concentration (above 0.01 mM) to most plants (Fernandes and Henriques, 1991). Recently, due to its involvement with the wide spread use of fungicides, pesticides and fertilizers and so on, Cu pollution has become increasingly a major environmental problem (Kaplan, 1999). An inhibition of plant growth, chlorosis, bronzing and necrosis are usual reported as the generally symptoms of a Cu excess. This growth inhibition is a result of numerous physiological and metabolic disturbances, including reduction of Fe uptake (Marschner, 1995), destruction of the lipid and protein constituents of membrane (Maksymiec, 1997), inhibition of photosynthetic rate (Pätsikkä et al., 2002), increase in the activity of root peroxidases (Chaoui et al., 2004), alteration of plant hormonal status and inhibition of cell division (Lequeux et al., 2010). As a redox-active metal, Cu renders its toxicity with increased levels of reactive oxygen species (ROS) including H_2O_2, $O_2^{\bullet-}$ and $\bullet HO$ in a Fenton-type reaction, reacting with proteins, nucleic acids and lipids in plant cells (Fernandes and Henriques, 1991). To counteract the oxidative injury by copper, plants have developed the strategy by activating activities or transcription levels of antioxidant enzymes such as superoxide dismutase, catalase and ascorbate peroxidase (Sudo et al., 2008; Karuppanapandian et al., 2011). At the same time ROS generation can trigger a variety of cellular responses through signaling events including the burst of nitric oxide (NO). NO, a reactive nitrogen species, is believed to act as a signal molecule mediating responses to both biotic and abiotic stresses in plants (Wendehenne et al., 2004; Delledonne, 2005). Recently, a few studies suggested that NO can play a role in protecting plants from oxidative stresses under the stress of heavy metals such as cadmium (Cd), zinc (Zn) or Cu (Bartha et al., 2005; Rodriguez-Serrano et al., 2006), and NO donor sodium nitroprusside (SNP) treatment can protect plants from the damage by regulating the activity of antioxidative enzymes (Kopyra and Gwóźdź, 2003). In a previous study, using 4,5-diaminofluorescein diacetate (DAF-2 DA), a specific NO dye, Bartha et al. (2005) demonstrated a biphasic NO induction, namely, a fast burst of NO release within 2 hr followed by a slow increase within 48 h of Cu treatment at 100 μM in the root tips of *Brassica juncea* L. and *Pisum sativum* L. The oxidative damage in rice leaves caused by excess CuSO$_4$ (10 mM) could be reduced by the exogenous application of NO donor SNP (100 μM) (Yu et al., 2005). Moreover, a compensatory increase in either ROS or NO accumulation was found to sustain in phenylalanine ammonium-lyase (PAL) activity for anti-oxidative protection in lignification and related processes in Cu-exposed roots of chamomile (*Matricaria chamomilla* L.) (Kováčik et al., 2010). In plants, the possible sources of NO enzymatic generation have been reported, including NO synthase (NOS)-like enzyme (Guo and Crawford, 2005), nitrate reductase (NR) (Desikan et al., 2002) and nitrit:NO reductase (Stőhr and Stremlau, 2006). Non-enzymatic reduction of apoplastic nitrite also play role in

NO genesis in plants (Bethke et al., 2004). With the treatment of NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) or the NR inhibitor tungstate on tomato seedlings under the Cu stress, Wang et al. (2010) demonstrated that Cu-induced NO accumulation may be mediated by proteins similar to mammalian NOS enzymes and NR. Bartha et al. (2005) reported an inverse relationship between NR activity and NO levels induced by 100 μM Cu in roots of *B. juncea* and *P. sativum*. Although the molecular identity of plant NOS is unknown, NADPH-diaphorase (NADPH-d) activity was identified as an indicator of NOS activity in plant cells (Cueto et al., 1996; Simontacchi et al., 2004). During indole-3-butyric acid-induced adventitious root formation of mung bean, endogenous NO and NADPH-d activity showed overall similarities in their tissue localization (Huang et al., 2007). Valentovičová et al. (2010) examined the histological distribution of NADPH-d activity in barley root tips during Cd treatment and found NADPH-d signals had a similar distribution as endogenous NO. However, little has been reported about NADPH-d activity in Cu toxicity to date. The distribution and source of NO in roots under Cu stress still needs to be examined. In continuation of our characterization of NO action induced by abiotic or biotic elicitors (Wang and Wu, 2004; Wang and Wu, 2005; Wang et al., 2006), the present work was carried out to investigate the NADPH-d activity in faba bean root tips during Cu treatment and compare its histochemical localization to the NO production in the roots. NR activity was also examined for the analysis on possible NO sources.

Results

Cu-induced root growth inhibition

The obvious inhibitory effect of CuSO$_4$ on root growth was observed in Fig. 1A. Treatment of CuSO$_4$ (1 mM) for 4 h resulted in approximately 63.6% inhibition of root elongation after 24 h recovery culture (Fig. 1B). In addition, root hair development was also significantly inhibited under Cu toxicity (Fig. 1C).

Cu-induced NO production and its effect on root growth

There was a significant increase in NO content in *V. faba* roots exposed to Cu treatment (Fig. 2). In general, NO content after Cu treatment (4 h) was more than control by 200 and 270% in the elongation zone (root segment 1-4 mm behind the root apex) and the beginning of the differentiation zone (root segment 4-7 mm behind the root apex), respectively. Supply of NO donor SNP alone also enhanced generation of NO in roots. However, addition of SNP to Cu-treatment solution resulted in a significant decrease in NO content compared to Cu treatment. Although application of NOS inhibitor L-NNA to Cu-treatment solution reduced NO generation, the NR inhibitor tungstate had no significant effect on the Cu-induced NO production (Cu+tungstate in Fig. 2B). Correspondingly, SNP supplementation recuperated the reduction in the root length to only 36.4% (Fig. 1B). To confirm the protective effect of NO on the root growth, the NOS inhibitor L-NNA was used. Our experiment showed that the alleviating effect of NO on Cu-induced inhibition of root elongation could be revised (Cu+L-NNA in Fig. 1). In addition, root hair development in faba bean seedlings was significantly inhibited by CuSO$_4$ and L-NNA (Fig. 1C). However, this inhibition was attenuated by SNP supplement (Cu+SNP in Fig. 1C).

Cu-induced NADPH-d activity

The analysis on NADPH-d activity of the crude extract from 11 mm-long roots revealed that the enzyme activity increased by 42.1% in the roots exposed to Cu (Fig. 3A). And NADPH-d activity was detected in both cytoplasmic and microsomal fractions of the roots (Fig. 3B, 3C). However, the cytoplasmic NADPH-d activity showed a different pattern in comparison to the microsomal one in both the control and Cu-treated roots. In the cytoplasmic fraction, the lower level of NADPH-d activity was detected in the beginning of the differentiation zone, where the higher level of NADPH-d activity was found to the microsomal fraction. After Cu treatment, the induced cytoplasmic NADPH-d activity was detected only in the differentiation zone. However, the induced NADPH-d activity in microsomal membrane fraction was observed in the root apex and the elongation zone. Upon supplementation with SNP (Cu+SNP in Fig. 3), there was a decrease in the enzyme activity in both cytoplasmic and microsomal membrane fraction of the elongation zone. The NOS inhibitor L-NNA significantly inhibited NADPH-d activity in the two fractions, suggesting that NADPH-diaphorase-mediated NO production is a main source for endogenous NO in the roots.

### Histochemical observation on NADPH-d activity and NO production

In situ analysis of NADPH-d activity in control roots revealed that the enzyme was localized mainly in the pericycle and primary phloem elements, and also in some cells of primary xylem at a distance of 4-7 mm behind the root apexs (Fig. 4A). After Cu treatment, SNP treatment alone or in combination, it was detected already in the elongation zone. The Cu-induced NO generation was observed in some cells of pericycle and phloem, and stronger DAF fluorescence emerged in the differentiation zone (Fig. 4B). Addition of SNP decreased the Cu-induced fluorescence density. The NOS inhibitor L-NNA suppressed both NADPH-d activity and NO production. NO-specific green fluorescence was not observed in control roots.

### Inhibition of NR activity by Cu toxicity

The activity of the enzyme was higher (90.6 nmol NO^{2-}/g fw/hr) in the differentiation zone of root tips in control group (Fig. 5). Addition of SNP had little effect on the NR activity. However, Cu-treatment for 4 hr reduced NR activity significantly about 100-fold in this zone. The inhibition of NR activity induced by Cu toxicity was alleviated by NO donor SNP and NOS inhibitor L-NNA, especially in the differentiation zone of roots.

Discussion

In mammals, NADPH-d is an oxidative enzyme localized to neurons that resist the toxic effects of excitatory amino acids and hypoxia and survive the degenerative processes of Huntington and Alzheimer diseases (Koh et al., 1986; Kowall and Beal, 1988). This enzyme was discovered to display some NOS activity and the identity of NOS with it was subsequently shown by co-localization at the mRNA and protein level (Dawson et al., 1991). NADPH-d activity can be demonstrated by the reduction of tetrazolium salts to a visible blue reaction product. It is believed that NADPH-d staining reflects NOS activity (Bredt et al., 1991; Hope et al., 1991). Recently, NADPH-d activity was also identified as an indicator of NOS activity in plant cells (Cueto et al., 1996; Simontacchi et al., 2004; Huang et al., 2007; Valentovičová et al., 2010).
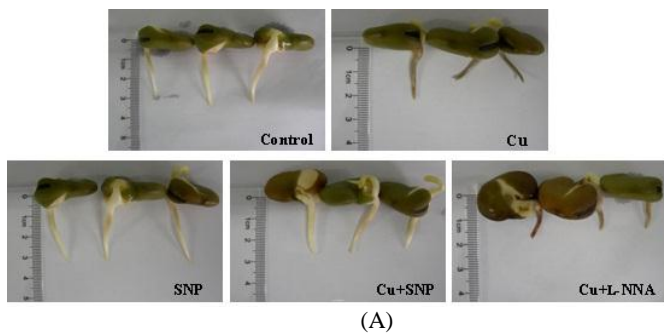
(A)
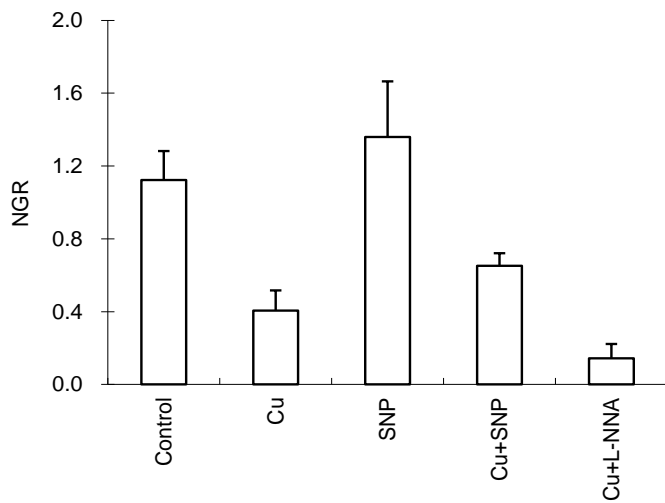
(B)
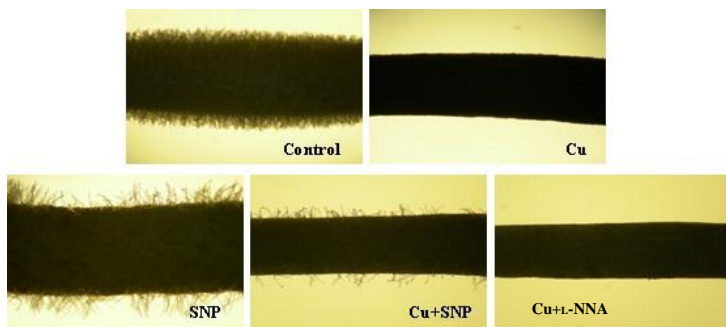
(C)

Fig 1. The effect of Cu and NO on root growth of *Vicia faba*. (A) Root length; (B) Net growth rates (NGR); (C) Root hair development. Roots were treated with 1.0 mM CuSO₄ (Cu); 1.0 mM SNP; 1.0 mM CuSO₄+1.0 mM SNP (Cu+SNP) and 1.0 mM CuSO₄+12.0 mM L-NNA (Cu+L-NNA) for 4 h, and then maintained in distilled water for 24 h recovery. All experimental groups were kept in an incubator at $25\pm1°C$, and the control group was incubated in distilled water. Mean values $\pm$ SD (n=20). *Values significantly different for $P \leq 0.05$ and **Values significantly different for $P \leq 0.01$.
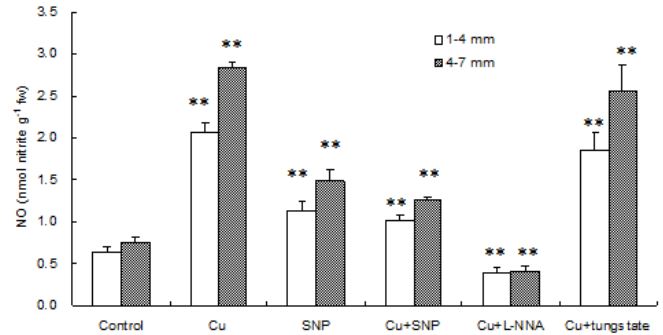

Fig 2. Nitric oxide content in the division zone (root segment 1-4 mm behind the root apex) and the beginning of the elongation zone (root segment 4-7 mm behind the root apex) of root of *Vicia faba*. Roots were treated with 1.0 mM CuSO₄ (Cu); 1.0 mM SNP; 1.0 mM CuSO₄+1.0 mM SNP (Cu+SNP) and 1.0 mM CuSO₄+12.0 mM L-NNA (Cu+L-NNA) and 1.0 mM CuSO₄+ 0.1 mM tungstate for 4 h. All experimental groups were kept in an incubator at $25\pm1°C$, and the control group was incubated in distilled water. Bar Values represent the mean $\pm$ S.D. of triplicate samples. * Values significantly different for $P \leq 0.05$ and ** Values significantly different for $P \leq 0.01$

With the pharmaceutical experiments using NOS inhibitor L-NAME or L-NNA, plant NOS was found the key enzyme involved in NO production induced by heavy metal stress such as Cd, Zn and Cu (Bartha et al., 2005; Rodriquez-Serrano et al., 2006). Valentovičová et al. (2010) demonstrated that NO was induced by Cd toxicity in barley roots and the dynamic distribution of endogenous NO was similar with the induced NADPH-d activity in roots. The present investigation showed that the CuSO₄ treatment at 1 mM for 4 hr can significantly stimulate NADPH-d activity by 42.1% in roots of faba bean. This is the first report on the NADPH-d activity under Cu toxicity. The analysis of crude extract of the 11-mm-long root tip revealed that the Cu-induced NADPH-d activity started in the microsomal membrane fraction in elongation zone (1-4 mm behind the root apex) and increased further in the microsomal and cytoplasmic fraction of the differentiation zone (4-7 mm behind the root apex). We showed that Cu-induced NADPH-d activity is associated with both the cytoplasmic and microsomal cell fractions where NO was detected by previous reports (Corpas et al., 2004; Valentovičová et al., 2010). In view of the co-localization of NO synthase and NADPH-d activity, we examined the histological distribution of NO production and NADPH-d activity during Cu-stress. In general, Cu-induced NADPH-d signals had a similar distribution as endogenous NO, being specifically located in the vascular bundles of roots (Fig. 4). Similarly, Cd-induced NO generation was observed in some cells of percycle, parenchymatic stellar cells and companion cells of protopholem among the metaxylem vessels in the elongation zone of barley roots (Valentovičová et al., 2010). In *V. faba* salicylic acid (SA), hydrogen peroxide (H₂O₂) and fungal elicitor chitooctaose could induce increases of DAF fluorescence observed most strongly in companion cells and sieve elements in the phloem (Gaupels et al., 2008). Our present results showed that NOS inhibitor L-NNA promoted the inhibition of Cu on root growth and reduced both NADPH-d staining and NO fluorescence in root vascular bundles. These results suggested that NO plays a vital role in Cu-induced root growth inhibition. Also, the production of NO in this process may be catalyzed by a NOS-like enzyme.
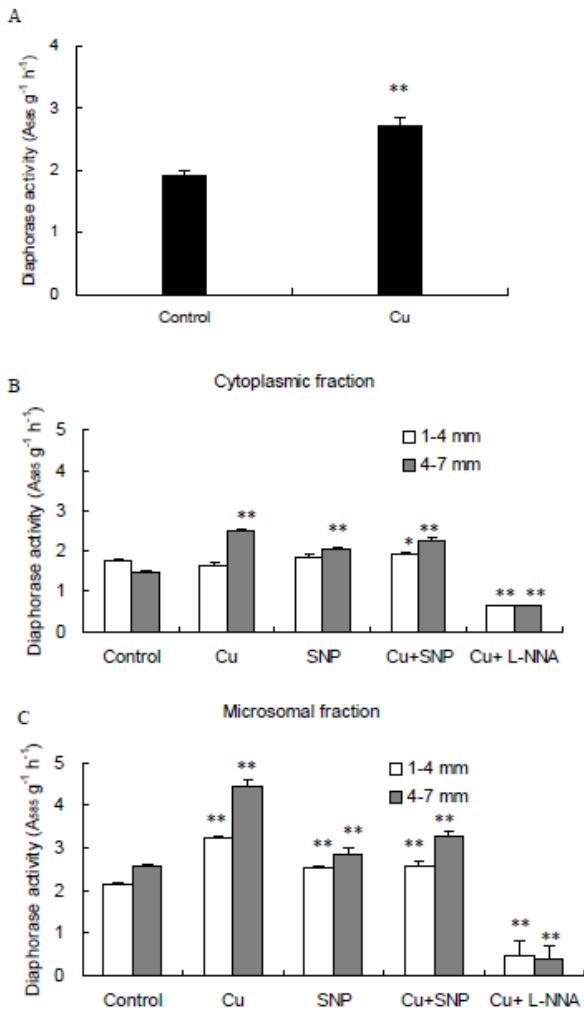
Fig 3. The NADPH-diaphorase activity in crude extract in the root tip (11 mm) (A), the cytoplasmic (B) and microsomal fractions (C) along the longitudinal root axis. Treatment procedure, dosage and symbols were the same as specified in Fig 2.

Meanwhile, we found the Cu-treatment reduced the NR activity significantly in the differentiation zone of roots (Fig. 5). To investigate whether NR as a potential source of NO or not, the root tissue was incubated with 0.1 mM tungstate. The NR inhibitor had no effect on the Cu-induced increase of NO production (Fig. 2). Although treatment with the NR inhibitor tungstate blocked the beneficial effect of SNP on copper-induced damage to tomato leaves, repressed the SNP-induced increase of chlorophyll content and root activities, there was no direct evidence of NR involved in Cu-induced NO production (Wang et al., 2010). Bartha et al. (2005) also reported an inverse relationship between NR activity and NO levels induced by 100 μM Cu in roots of *B. juncea* and *P. sativum.* Gaupels et al. (2008) found that tungstate had no effect on the H_2O_2-induced increase in DAF fluorescence and sodium nitrite, the substrate of NR, couldn't elicit the NO production in *V. faba* phloem cells. Use of another NR inhibitor sodium azide also had no effect on developmentally regulated NO production in vascular tissue (Gabaldon et al., 2005).

Although there were evidences to show that NR-mediated NO production is involved in pathogen attack, salt stress, and freezing tolerance (Molodo et al., 2005; Liu et al., 2007; Zhao et al., 2009), our study suggested that NOS-like enzyme was as a main source of inducible NO generation in roots of *V. faba* under Cu stress. The application of the NO donor to plants was proved to assuage the growth inhibition under different stresses (Lombardo et al., 2006). We also found that the NO donor compound, SNP efficiently alleviated the copper toxicity effect, as shown by increases of root growth and root hair formation (Fig. 1). The exogenous NO-mediated reduction of Cu-toxicity relies mainly on its ability to scavenge ROS and stimulate antioxidant systems in plants (Sudo et al., 2008). The further study is needed to investigate the mechanism of modulating the NADPH-d activity and NO protection under Cu stress. On the other hand, the different NO donors could be chemical synthesized and modified to be used as a priming elicitor to ameliorate the damages by heavy metals on crops.

Material and methods

Plant material and growth conditions

Faba bean (*Vicia faba* L.) seeds were soaked for 24 h in distilled water and allowed to germinate between two layers of moist gauze at 25°C in the dark. When the newly emerged roots were of 1.00-2.00 cm in length, they were used in the experiment. Growing roots were treated with 1.0 mM $CuSO_4$ for 4 h, and then maintained in distilled water for 24 h recovery. To study the NO effects on Cu exposure, growing roots were treated with 1.0 mM $CuSO_4$; 1.0 mM SNP (a NO donor); 1.0 mM $CuSO_4$+1.0 mM SNP; 1.0 mM $CuSO_4$+12.0 mM N_ω-nitro-L-arginine (L-NNA, a NOS inhibitor) and 1.0 mM $CuSO_4$+0.1 mM tungstate (a NR inhibitor) for 4 h, and then maintained in distilled water for 24 h recovery. All experimental groups were kept in an incubator at 25±1°C, and the control group was incubated in distilled water. All assays were replicated at least three times; each replicate was carried out on 50 seeds for germination and 20-30 seeds for growth measurements. All chemicals were purchased from Sigma Co. (St. Louis, MO, USA). Net growth rates (NGR) were calculated according to the method of Pan et al. (2001) as follows: (final length−initial length) / initial length. The seminal root length of seeds in each treatment was measured before and after 4 hr exposure to test solutions and 24 hr recovery culture. Root segments (0.5 cm) were excised from each seeds at 1.0 cm behind the root tip and placed onto microscope slides for root hair observation by a Nikon stereo microscope (Model C-DSS230).

Nitric oxide (NO) content

NO content was determined using the method described by Zhou et al. (2005). Briefly, root tissues (0.1 g) 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 r/min for 15 min at 4°C. The supernatant was collected. The pellet was washed by 1 mL of extraction buffer and centrifuged as before. The two supernatants were combined and 0.1 g of charcoal was added. After vortex and filtration, the filtrate was leached and collected. The mixture of 1 mL of filtrate and 1 mL of the Greiss reagent (1%
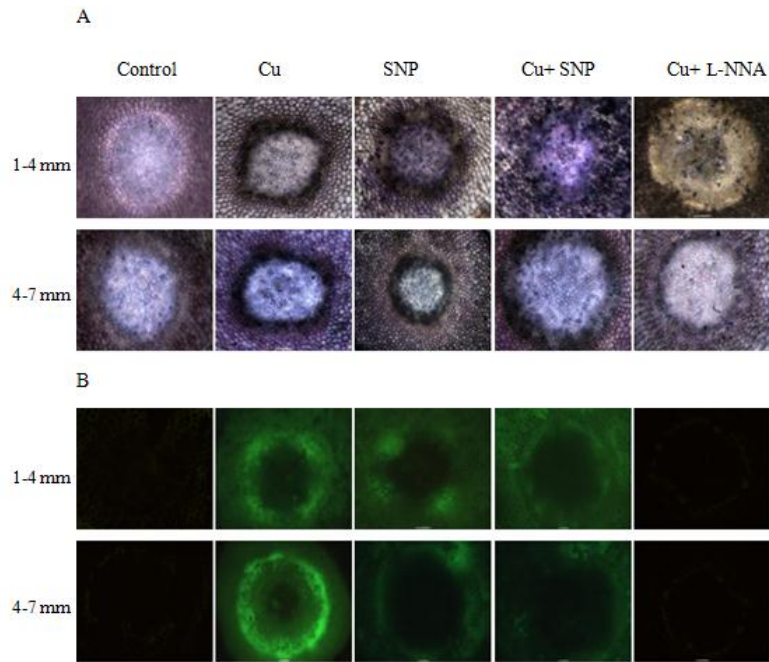
Fig 4. NADPH-diaphorase (A) and nitric oxide (B) stain of the elongation zone (root segment 1-4 mm behind the root apex) and the beginning of the differentiation zone (root segment 4-7 mm behind the root apex) of root of *Vicia faba*. Treatment procedure, dosage and symbols were the same as specified in Fig. 2.

Fig 5. Nitrate reductase activity in the elongation zone (root segment 1-4 mm behind the root apex) and the beginning of the differentiation zone (root segment 4-7 mm behind the root apex) of root of *Vicia faba*. Treatment procedure, dosage and symbols were the same as specified in Fig. 2.
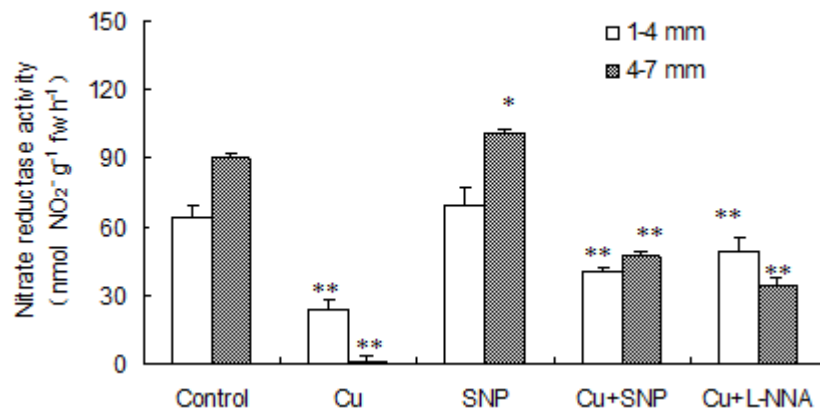
sulphanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)-ethylenedia-minedihydrochloride) was incubated at room temperature for 30 min. Absorbance was determined at 540 nm. NO content was calculated by comparison to a standard curve of $NaNO_2$.

NADPH-d activity assay

Root segments were homogenized gently in a pre-cooled mortar with homogenization buffer containing 1.0 mM EDTA, 1.0 mM dithiothreitol and 1.0 mM protease inhibitors phenylmenthylsulphonyl fluoride (from 50 mM stock solution in isopropanol) in 100 mM Tris-HCl, pH 8.0. All subsequent procedures were performed at 4°C. The homogenate was centrifuged at 5,000 r/min for 5 min followed by 12,000 r/min

for 10 min to obtain the crude extract. The cytoplasmic and microsomal membrane fractions were obtained by ultracentrifugation of crude extract at 100,000 r/min for 30 min. Membranes were washed once with 10 mL of the homogenization buffer and centrifuged at 150,000 r/min for 30 min. Pellet was resuspended in 0.5 mL of homogenization buffer for further analysis. The proteins were quantified with bovine serum albumin as the calibration standard according to the method of Bradford (1976). NADPH-d activity was measured by a colorimetric method according to Simontacchi et al. (2004).

Histochemical observation

In situ localization of NADPH-d in root tissue was monitored

119

by immersing 0.5-mm-thick root sections (after washing in 50 mM Tris-HCl pH 8.0 for 10 min) into the reaction solution (1 mM NADPH, 0.5 mM MgCl$_2$, 0.2 mM CaCl$_2$, 0.5 mM NBT in 50 mM Tris-HCl buffer pH 8.0) for 5 min. NO production was monitored by immersing 0.5-mm-thick root sections, after washing in 10 mM Tris-HCl pH 7.4, for 10 min into a 20 mM DAF-DA (from 5mM stock solution in DMSO) in 10 mM Tris-HCl pH 7.4 for 60 min by fluorescence microscopy on an Olympus BX51 microscope mounted with an exciter filter BP 460-490 and a barrier filter BA 515.

Nitrate reductase (NR) activity

NR activity was assayed as described by Poonnachit and Darnell (2004) with some modifications. Root tissues (100 mg fresh weight) were incubated in 2 mL of assay solution containing 2% 1-propanol, 100 mM KH$_2$PO$_4$ (pH 7.5) and 30 mM KNO$_3$ at 31°C for 1 h in the dark. After incubation, the assay solution with roots was filtered and a 1 mL aliquot from each sample was removed to the reaction solutions (1 mL sulfanilamide (1% w/v in 1.5 M HCl) and 1 mL N-(1-naphthyl)-ethylenedia-minedihydrochloride (0.02% w/v in 0.2 M HCl). The samples were incubated at room temperature for 30 min. The absorbance at 540 nm was determined with a spectrophotometer. The activity of NR was expressed as μmol of NO$_2^-$ produced per hour and per gram of fresh weight.

Conclusion

The results from the present study clearly showed that Cu stress stunted growth and development of *V. faba* roots. The Cu-induced NO in vascular bundles was associated primarily with the presence of the induced NADPH-d activity. Our results suggested that NOS-like enzyme, but not the nitrate reductase, was the source of inducible NO generation in roots of *V. faba* under Cu stress. This study also provided more effective strategies for the alleviation of Cu damage by NO donor SNP.

Acknowledgements

References

Bartha B, Kolbert Z, Erdei L (2005) Nitric oxide production induced by heavy metals in *Brassica juncea* L. Czern. and *Pisum sativum* L. Acta Biol Szeged 49: 9-12

Bethke PC, Badger MR, Jones RL (2004) Apoplastic synthesis of nitric oxide by plant tissues. Plant Cell 16: 332-341

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Chem 72: 248-254

Bredt DS, Glatt CE, Hwang PM, Fotuhi M, Dawson TM, Snyder SH (1991) Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. Neuron 7: 615-624

Chaoui A, Jarrar B, EL Ferjani E (2004) Effects of cadmium and copper on peroxidase, NADH oxidase and IAA oxidase activities in cell wall, soluble and microsomal membrane fractions of pea roots. J Plant Physiol 161: 1225-1234

Corpas FJ, Barroso JB, Carreras A, Quirós M, León AM, Romero-Puertas MC, Esteban FJ, Valderrama R, Palma JM, Sandalio LM, Gómez M, del Río LA (2004) Cellular and subcellular localization of endogenous nitric oxide in young and senescent pea leaves. Plant Physiol 136: 2722-2733

Cueto M, Hernández-Perera O, Martín R, Bentura ML, Rodrigo J, Lamas S, Golvano MP (1996) Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. FEBS Lett 398: 159-164

Dawson TM, Bredt DS, Fotuhi M, Hwang PH, Snyder SH (1991) Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci USA 88: 7797-7801

Delledonne M (2005) NO news is good news for plants. Curr Opin Plant Biology 8: 1-7

Desikan R, Griffihts R, Hancock J, Neill S (2002) A new role for an old enzyme: Nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 99: 16314-16318

Fernandes JC, Henriques FS (1991) Biochemical, physiological, and structural effects of excess copper in plants. Bot Rev 57: 246-273

Gabaldon C, Gomez Ros LV, Pedreno MA, Ros Barcelo A (2005) Nitric oxide production by the differentiating xylem of *Zinnia elegans*. New Phytol 165: 121-130

Gaupels F, Furch ACU, Will T, Mur LAJ, Kogel KH, van Bel AJE (2008) Nitric oxide generation in *Vicia faba* phloem cells reveals them to be sensitive detectors as well as possible systemic transducers of stress signals. New Phytol 178: 634-646

Guo FQ, Crawford NM (2005) *Arabidopsis* nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. Plant Cell 17: 3436-3450

Hope BT, Michael GJ, Knigge KM, Vincent SR (1991) Neuronal NADPH-diaphorase is a nitric oxide synthase. Proc Natl Acad Sci USA 88: 2811-2814

Huang AX, She XP, Huang C, Song TS (2007) The dynamic distribution of NO and NADPH-diaphorase activity during IBA-induced adventitious root formation. Physiol Plantarum 130: 240-249

Kaplan M (1999) Accumulation of Cu in soils and leaves of tomato plants in greenhouses in Turkey. J Plant Nutr 22: 237-244

Karuppanapandian T, Moon J-C, Kim C, Manoharan K, Kim W (2011) Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms. Aust J Crop Sci 5: 709-725

Koh JY, Peters S, Choi DW (1986) Neurones containing NADPH-diaphorase are selectively resistant to quinolinate toxicity. Science 234: 73-76

Kopyra M, Gwóźdź EA (2003) Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of *Lupinus luteus*. Plant Physiol Biochem 41: 1011-1017

Kováčik J, Grúz J, Klejdus B, Štorka F, Marchiosi R, Ferrarese-Filhod O, 2010. Lignification and related parameters in copper-exposed *Matricaria chamomilla* roots: role of H$_2$O$_2$ and NO in this process. Plant Sci 179: 383-389

Kowall NW, Beal MF (1988) Cortical somatostatin, neuropeptide Y, and NADPH diaphorase neurons: normal anatomy and alterations in Alzheimer's disease. Ann Neurol 23: 105-114

Lequeux H, Hermans C, Lutts S, Verbruggen N (2010) Response to copper excess in *Arabidopsis thaliana*: Impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile. Plant Physiol Biochem 48: 673-682.

Liu YG, Wu RR, Wan Q, Xie GQ, Bi YR (2007) Glucose-6-phosphate dehydrogenase plays a pivotal role in nitric oxide-involved defense against oxidative stress under salt stress in red kidney bean roots. Plant Cell Physiol 48: 511-522

Lombardo MC, Graziano M, Polacco JC, Lamattina L (2006) Nitric oxide functions as a positive regulator of root hair development. Plant Signal Behav 1: 28-33

Maksymiec W (1997) Effect of copper on cellular process in higher plants. Photosynthetica 34: 321-342

Marschner H (1995) In: Mineral Nutrition of Higher Plants (ed.). Academic Press, London, 461-478

Molodo LV, Augusto O, Almeida IMG, Magalhaes JR, Salgado I (2005) Nitrite as the major source of nitric oxide production by *Arabidopsis thaliana* in responses to *Pseudomonas syringae*. FEBS Lett 579: 3814-3820

Pan JW, Zhu MY, Chen H (2001) Aluminum-induced cell death in root-tip cells of barley. Environ Exp Bot 46: 71-79

Pätsikkä E, Kairavuo M, Šeršen F, Aro EM, Tyysstjärvi E (2002) Excess copper predisposes photosystem II to photoinhibition in vivo by outcompeting iron and causing decrease in leaf chlorophyll. Plant Physiol 129: 1359-1367

Poonnachit U, Darnell R (2004) Effect of ammonium and nitrate on ferric chelate reductase and nitrate reductase in *Vaccinium* species. Ann Bot 93: 399-405

Rodriquez-Serrano M, Romero-Puertas MC, Zabalza A, Corpas FJ, Gomez M, Del Rio LA, Sandalio LM (2006) Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo. Plant Cell Environ 29: 1532-1544

Simontacchi M, Jasid S, Puntarulo S (2004) Nitric oxide generation during early germination of sorghum seeds. Plant Sci 167: 839-847

Stőhr C, Stremlau S (2006) Formation and possible roles of nitric oxide in plant roots. J Exp Bot 57: 463-470

Sudo E, Itouga M, Yoshida-Hatanaka K, Ono Y, Sakakibara H (2008) Gene expression and sensitivity in response to copper stress in rice leaves. J Exp Bot 59: 3465-3474

Valentovičová K, Halušková L, Huttová J, Mistrík I, Tamás L (2010) Effect of cadmium on diaphorase activity and nitric oxide production in barley root tips. J Plant Physiol 167: 10-14

Wang JW, Wu JY (2004) Involvement of nitric oxide in elicitor-induced defense responses and secondary metabolism of *Taxus chinensis* cells. Nitric Oxide 11: 298-306

Wang JW, Wu JY (2005) Nitric oxide is involved in methyl jasmonate induced defense responses and secondary metabolism activities of *Taxus* cells. Plant Cell Physiol 46: 923-930

Wang JW, Zheng LP, Wu JY, Tan RX (2006) Involvement of nitric oxide in oxidative burst, phenylalanine ammonia-lyase activation and Taxol production induced by low-energy ultrasound in *Taxus yunnanensis* cell suspension cultures. Nitric Oxide 15: 351-358

Wang LN, Yang LM, Yang FJ, Li XG, Song YP, Wang XF, Hu XY (2010) Involvement of H_2O_2 and metallothionein in NO-mediated tomato tolerance to copper toxicity. J Plant Physiol 167: 1298-1306

Waraich EA, Ahmad R, Saifullah, Ashraf MY, Ehsanullah (2011) Role of mineral nutrition in alleviation of drought stress in plants. Aust J Crop Sci 5: 764-777

Wendehenne D, Durner J, Klessig DF (2004) Nitric oxide: a new player in plant signaling and defence responses. Curr Opin Plant Biol 7: 449-455

Yu CC, Hung KT, Kao CH (2005) Nitric oxide reduces Cu toxicity and Cu-induced NH_4^+ accumulation in rice leaves. J Plant Physiol 162: 1319-1330

Zhao M-G, Chen L, Zhang L-L, Zhang W-H (2009) Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing tolerance in *Arabidopsis*. Plant Physiol 151: 755-767

Zhou BY, Guo ZF, Xing JP, Huang BR (2005) Nitric oxide is involved in abscisic acid-induced antioxidant activities in *Stylosanthes guianensis*. J Exp Bot 56: 3223-3228