DOES CHELATED COPPER AMELIORATE THE GREENING OF IRON-DEFICIENT CUCUMBER PLANTS THROUGH NITRIC OXIDE SIGNALING? COMPARISON WITH CHEMICAL FORMS OF ZINC

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Summary. Recently the role of nitrogen monoxide (NO) in iron homeostasis has been proved by means of exogenously-applied different NO donors that can provoke leaf re-greening under conditions of iron deficiency. It was also supposed, that Fe-deficiency itself could stimulate the endogenous production of NO in stressed plants (Graziano and Lamattina, 2007). In this study, the comparison between effects of NO-donor sodium nitroprusside (SNP and related structural compounds) and chelated forms of copper and zinc [Cu(II)HEDTA and Zn(II)HEDTA, all applied at equimicromolar concentrations in the nutrient solution] towards leaf chlorosis of Fe-deficient cucumber plants was investigated. Plant variants (–Fe+2 μM Cu^{2+}) and (–Fe+ 20 μM Zn^{2+}) with very strong chlorosis were chosen for experiments to follow the recovery of leaf greening after treatment with substances or inhibitors of NO production and action. Treatment with SNP, Fe(II)CN and Cu(II)HEDTA produced leaf greening in both variants, especially strong for the newly developed leaf, as it was shown with chlorophyll measurements. KCN, Fe(III)CN and Zn(II)HEDTA were without greening effect. Using specific NO-inhibitor cPTIO it was shown that Cu(II)HEDTA treatment of iron-deficient cucumber plants could provoke leaf greening by means of endogenous

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NO production. The suggestion was made for NO formation from apoplastic nitrates through non-enzymatic way or by the action of nitrate-reductase activity after treatment with ferrocyanide and cupric-chelate.

Key words: iron deficiency, *Cucumis sativus* L., nitrogen monoxide, ionic and chelated forms of copper and zinc.

INTRODUCTION

Iron deficiency in plant nutrition is a widely spread problem for crops and human beings. In spite of the huge availability of ferric compounds in different soils, their solubility is restricted and the lack of suitable for uptake iron provokes various disorders in plant metabolism (Curie and Briat, 2005; Hell and Stevens, 2003). Several steps in photosynthetic pigment metabolism and chloroplast ultrastructure, are dependent on iron (Briat et al., 2007). The main characteristic symptom of plant iron deficiency is chlorosis, a leaf yellowing that is caused by a decrease in chlorophyll content. Due to human activities, often iron limitation can be combined with increasing copper level in soils and then in plant tissues after application of fungicides or other compounds for plant protection (He et al., 2005). Thus, the study of plant metabolic disorders under conditions of iron deficiency and Cu excess as important agricultural problems deserves attention. Copper toxicity in (–Fe) stressed plants after copper chemicals supply can provoke additional strong chlorosis and growth reduction depending on copper concentration (Boycheva and Babalakova, 2006). In our earlier studies we have demonstrated that chelated copper supply in the nutrient solution of Fe-deficient cucumbers altered stress responses, improved chlorophyll synthesis and enhanced plant growth in contrast to high inhibitory action of the same concentrations of ionic copper (Boycheva and Babalakova, 2006; Boycheva et al., 2008**).** Also, our preliminary data have shown, that chelated zinc supply in the nutrient solution of Fe-deficient cucumbers also provoked the similar ameliorating effect on leaf chlorosis, in contrast to highly inhibitory action of free Zn-ions (Babalakova and Boycheva,

unpublished data). The mechanism underlying the ameliorating effect of continuous treatment with micromolar concentrations of cupric- or zinc-chelates on leaf chlorosis of Fe-deficient cucumbers is not clear.

Recently it has been shown that exogenous application of nitric oxide (NO) donor like sodium nitroprusside (SNP) improves leaf chlorosis of iron-deficient maize plants. NO promotes a significant increase in chlorophyll content and chloroplast membrane density in maize plants growing with very low iron concentration (Graziano et al., 2002). Nitric oxide as a lipophylic diatomic gas allows rapid membrane diffusion and interaction with different cellular substances and metabolites (Stamler et al., 1992; Durnal and Klessig, 1999). It has been proposed that nitric oxide as a diffusible molecular messenger can participate in plant signalling processes involved in seed germination (Beligni and Lamattina, 2000; Bethke et al., 2006), regulation of growth and organogenesis (Pagnussat et al., 2002), hormone action (Guo et al., 2003), plant defence against oxidative stress (Lamattina et al., 2003), defence responses against pathogens and damage leading to cell death (Durner and Klessig, 1999; Murgia et al., 2004). The relationship between NO and iron homeostasis has been suggested in different experiments and the role of NO as a new player in plant iron metabolism has been proved (Graziano and Lamattina, 2005). It was supposed that iron-deficit itself could stimulate the endogenous production of NO in stressed plants (Graziano and Lamattina, 2007). On the base of the finding that exogenously-applied nitric oxide stimulates leaf greening in iron-deficient plants, we decided to compare the effect of NO donor and chelated copper and zinc on chlorophyll synthesis in iron-limited plants. For experiments, we have chosen Fe-deficient cucumber plants with very strong chlorosis (received after plant pre-treatment with ionic forms of Cu and Zn) to follow the leaf greening of those plants after application of nitric oxide donor or chelated forms of both metals. Using different inhibitors of NO production and action we have compared the effects of both metal chelates.

MATERIALS AND METHODS

Plant material and growth conditions of (+Fe) and (–Fe) cucumber variants

Seeds of cucumber (*Cucumis sativus* L. cv. Gergana) were germinated in Petri dishes on moistened with 0.1 mM $CaCl_2$ filter paper, in the dark at 28˚C for 3 days. Four seedlings were placed in 400 ml plastic pots filled with Hoagland-Arnon I nutrient solution (pH 6.0) in an environmental chamber. The complete nutrient solution (CNS) contained 5 mM $Ca(NO_3)_2.4H_2O$, 5 mM KNO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4.7H_2O$. The concentrations of micronutrients in μM were: H_3BO_4 – 10, $MnCl_2$ – 0.5, $ZnSO_4$ – 0.5, $CuSO_4$ – 0.2, Na_2MoO_4 – 0.1 and Fe(III)HEDTA – 20. The seedlings grew in environmental room under 12-h light at PPFD of 120 μmol m^{-2} sec^{-1} provided by fluorescent tubes, 60 % RH, at 26 ºC day / 22 ºC night temperature. The complete nutrient solution for control (+Fe) plants was supplemented with 20 μM Fe(III)-HEDTA [$FeCl_3$ complex of N-(2-hydroxyethyl) ethylenediamine triacetic acid - HEDTA] and 0.2 μM of $CuSO_4$. Fe-deficient plants (–Fe) used nutrient solution without iron (Babalakova et al., 2005; Boycheva and Babalakova, 2006). The seedlings of (+Fe) and (–Fe) variants were treated during the next 7 days with ionic copper or zinc (2 μM $CuSO_4$ or 20 μM ZnSO4, to produce strong leaf chlorosis) or with intact Cu(II)- or Zn(II)-HEDTA complexes (applied at equimolar concentrations of 2 and 20 μM).

Exogenous application of mononitric oxide donor sodium nitroprusside and related compounds, as well as copper- and zing chelates in solutions of Fe-deficient cucumbers

Fe-deficient cucumber variants (–Fe+Cu) and (–Fe+Zn) were chosen to follow leaf greening after treatment with exogenous NO donor SNP [or nitroferric-cyanide –$Fe(CN)_5(NO)$]. The effect of related ferrous- and ferric-cyanides as well as potassium cyanide [Fe(II)CN, Fe(III)CN, KCN)] on leaf greening was also tested. The inhibitor of nitric oxide synthase - methylene blue (MB – tetramethyl-thionine chloride, Swiss blue, Mayer et al., 1993) or NO-specific scavenger - 2-(4-carboxy-phenyl)-4,4,5,5-tetramethyl- imida- zoline-1-oxyl-3-oxide (cPTIO, Goldstein et al., 2003) were also applied. Fe(II)CN and Fe(III)CN have both been used as negative controls in experiments where SNP has been used as an NO donor (Oh and McCaslin, 1995; Graziano et al., 2002). Eleven days-old cucumbers were

treated with 50 μM SNP, Fe(II)CN, Fe(III)CN and KCN as well as with 50 μM Cu(II)HEDTA and Zn(II)HEDTA, mixed with nutrient solution (pH 6). The complexes Cu(II)HEDTA and Zn(II)HEDTA were prepared as stock solutions, at a molar metal to ligand ratio 1:1.25, pH 6.0 with Tris-KOH (Babalakova et al., 2005). The inhibitor MB was applied at concentration of 20 or 50 μM and c-PTIO - of 100 μM. After 3 days treatment with compounds, plant roots were washed several times with distilled water and cucumber seedlings were transferred to new pots with fresh CNS without iron for the next 2 days, and then the plants were analysed.

Pigment content assay

First and second leaves of treated with sodium nitroprusside and other substances cucumber plant variants were used for pigment extraction in acidified methanol (HCl:methanol, 1:99, v/v) according to the method of Nogues and Baker (2000). Absorption spectra of the extracts were determined after centrifugation using spectrophotometers Shimadzu UV/VIS 1600 or Multiscan Spectrum REW Cuvette (Termo electron, Finland). Content of chlorophyll *a* was estimated from the absorbance at 654 nm and was expressed as Abs g^{-1} leaf FW.

Statistical analysis

The experiments were repeated at least 4-5 times and the results in the figures presented the mean values ± SE (standard error), analysed by ANOVA. Differences between variants were considered significant when $P < 0.05$ (Tukey -test).

RESULTS AND DISCUSSION

Cucumber plants, grown for seven days without iron in the nutrient solution developed moderate chlorosis on the first and next leaves, with chlorophyll reduction about 50 % in comparison with (+Fe) plants but had green, well expanded cotyledons (data not shown). Additional presence of 2 μM cupric ions in solutions of Fe-deficient plants brought to appearance

of very strong chlorosis with only about 15 to 20 % chlorophyll content of the control (+Fe) level (Fig.1). The same or even higher decline in chlorophyll concentration of Fe-deficient cucumbers was achieved with 20 μM Zn-ions supply (Fig.2). Chelated forms of Cu and Zn, applied continuously on Fe-deficient cucumbers kept the pigment content to a higher level in comparison with the control (–Fe) plants (Babalakova and Boycheva, unpublished data). Searching for some explanation of chelated copper and zinc beneficial effect on the pigment content in Fe-deficient cucumbers, we have compared their action with that of nitric oxide donor sodium nitroprusside and related substances, all applied at equimolar concentrations in the nutrient solution. Fe(II)CN and Fe(III)CN share structural features with SNP, but lack a nitroso group and thus the ability to generate NO. SNP has several advantages when compared on other NO donors. At first SNP delivers NO for many hours and it produces complexes

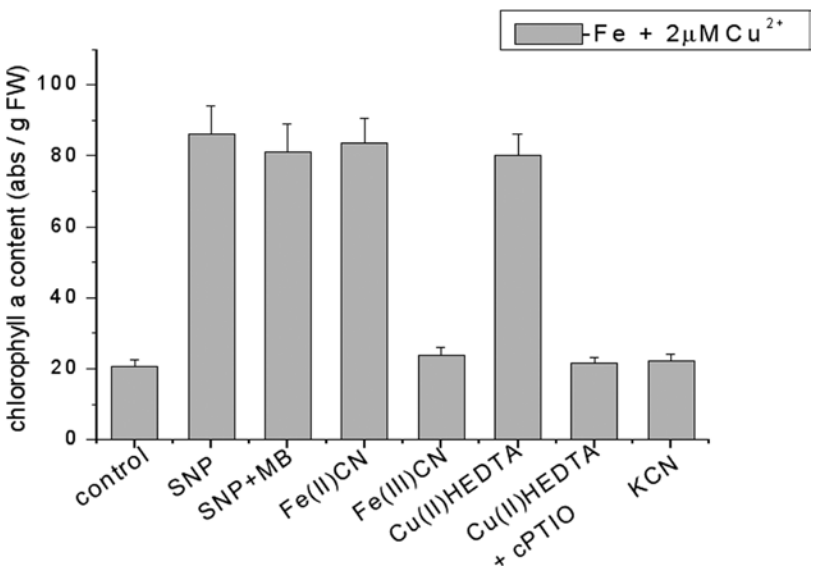

Fig. 1. Chlorophyll ***a*** content in leaves of Fe-deficient cucumber plants, pre-treated with copper (–Fe+Cu, control), after 3 days supply with NO donor sodium nitroprusside (SNP), or SNP plus MB, other cyanide-containing compounds [Fe(II)CN, Fe(III)CN and KCN], or with chelated copper Cu(II)HEDTA and Cu(II)HEDTA plus NO inhibitor cPTIO.

of cyanide with iron (Oh and McCaslin, 1995). After 3 to 5 days, treatment with SNP, Fe(II)CN and Cu(II)HEDTA produced leaf greening in both variants, especially for the newly developed leaf, as it was shown with chlorophyll *a* content measurements (Fig.1 and Fig.2). KCN and Fe(III)CN were without effect in both variants (–Fe+Cu and –Fe+Zn). Methylene Blue (MB), known as cyanide killer and inhibitor of NO-synthase and soluble guanylate-cyclase (sGC-ase), applied together with SNP, did not prevent greening (Fig.1 and Fig.2). Specific NO-scavenger cPTIO, however, stopped leaf greening of cucumber variants treated with SNP or Cu(II) HEDTA demonstrating that NO production could participate in the process of greening by remobilization of apoplastic and cellular iron pools (Fig.1). In spite of very strong chlorosis in variant (–Fe+Cu) before treatment with the substances, leaf greening started soon after application of NO-donor SNP, but also after treatment with ferrous cyanide. Cu(II)HEDTA treatment of cucumbers brought to some delay of leaf greening, but the extent was

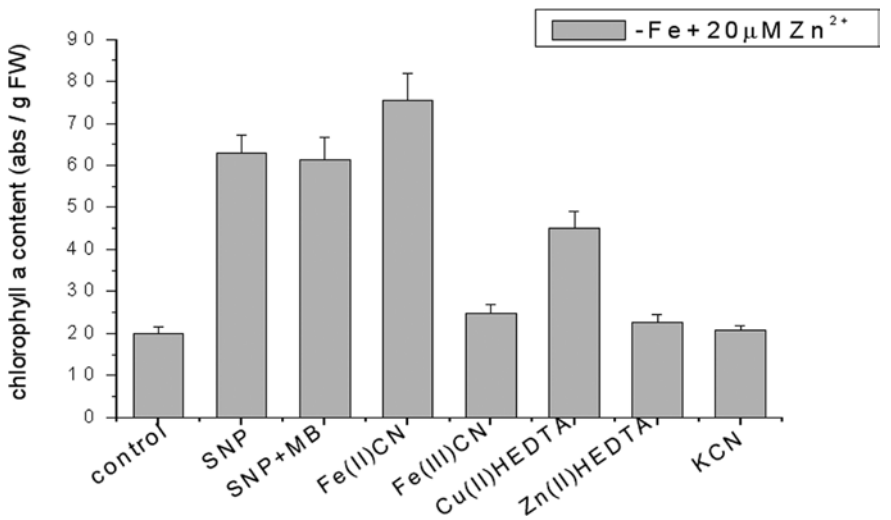

Fig. 2. Chlorophyll ***a*** concentration (abs/g FW) in the second leaf of Fe-deficient cucumber plants, pre-treated with zinc (–Fe+Zn) after 3 days supply with mononitric oxide donor sodium nitroprusside (SNP), or SNP plus MB, ferrocyanide [Fe(II)CN], ferricyanide [Fe(III)CN] or with chelated copper [Cu(II)HEDTA] and chelated zinc [Zn(II)HEDTA], and potassium cyanide (KCN).

similar (Fig.1). Using the same experimental scheme for the other variant (–Fe+Zn) with pale-yellow first leaf, some differences after application of cupric- and zinc-chelates were received (Fig.2). The highest positive effect on leaf greening was achieved with application of Fe(II)CN, followed by SNP and SNP+MB treatment. Cu(II)HEDTA supply produced smaller, but still positive effect, and Zn(II)HEDTA supply did not ameliorate leaf chlorosis (Fig.2). The duration of our experiment might be not sufficient to remedy leaf chlorosis of cucumbers, treated with zinc-chelate. Other authors received that plant roots treated with zinc did not show fast response in NO production, like copper (Bartha et al., 2005). Fe(III)CN, KCN and Zn(II) HEDTA supply were without greening effect on cucumber leaves. In both experimental variants the remedying effect of ferrous cyanide on leaf chlorosis was unexpectedly high (Fig.1 and Fig.2). In spite of the suggestion that ferrous- and ferric-cyanide can be used as negative controls in animal SNP experiments (Oh and McCaslin, 1995) or being without effect on maize leaf chlorosis (Graziano et al., 2002), only ferric-cyanide was a negative control in our experiments (without effect on leaf chlorosis). Recently Bethke et al. (2006) have received that cyanide compounds, nitrite and nitrate can stimulate seed germination in a NO-dependent manner together with SNP. The authors have accepted diverse mechanisms of action of CN^- and SNP. They have also used low pH treatment, facilitating decomposition and release of cyanides from SNP and other compounds (Bethke et al., 2006; Sarath et al., 2006). We have used pH 6.0 for SNP, KCN and Fe(III)CN and under those conditions the cyanide release is negligible (Al-Sadoni and Ferro, 2004). Sodium nitroprusside as an iron-nitrosyl complex has been used clinically as a vasodilatator. However, SNP does not liberate NO spontaneously in vitro. It requires partial reduction by different reducing agents like thiols (Grossi and D'Angelo, 2005, Fig. 3-1). SNP can also be decomposed to NO by light and alkaline conditions. To explain some of our results, we have proposed a scheme with possible ways of endogenous NO production during our experiments with hydroponic cucumbers or after using exogenous NO donor SNP (Fig.3). Sodium nitroprusside is actually a source of nitrosonium cation (NO^+) and thus behaves as a nitrosating electrophylic species (Fig. 3-1). Released NO^+ can react with transition metals in root apoplast [Fe(II) and Cu(I)] to produce

NO• radical, transforming in plant cells into monometal nytrosyl- or dimetal-nitrosyl complexes (FeNOC, DNOFeC) or interacting with sulfhydryl groups of proteins, yielding S-nitrosothiols, which lead to a change in protein function or activity. S-nitrosylation of proteins is rapidly reversible, making it an attractive candidate for involvement in signal transduction. It is uncertain, which of these two processes with NO is the more dominant modification in plants or what are their targets (Lindermayr et al., 2005). In our experiments the presence of copper ions in the medium facilitates formation of metal-nitrosyl complexes that can start the signaling cascade to the labile iron pools in cell apoplast or plastids, leading to Fe

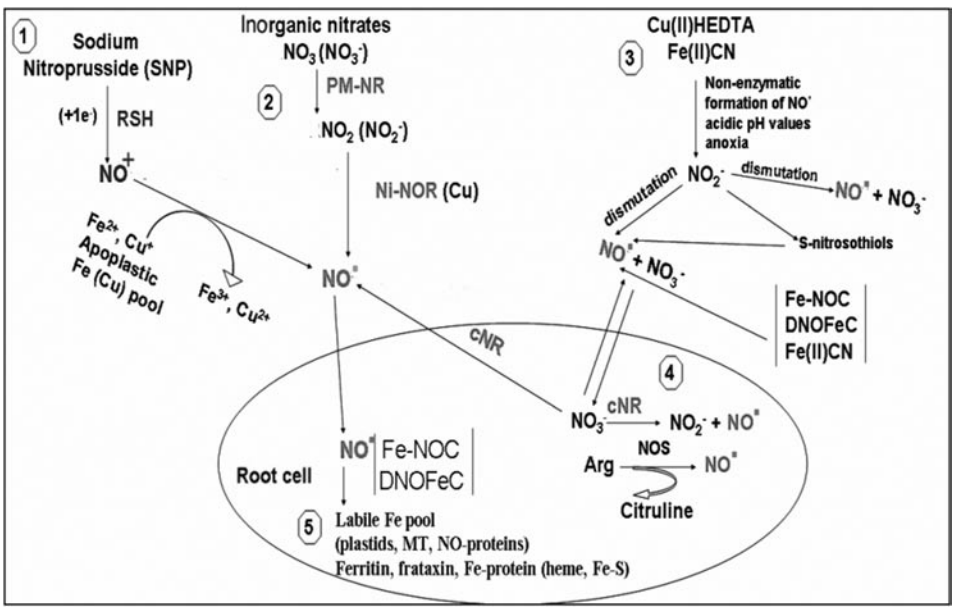

Fig. 3. (1-5). Scheme of the nitric oxide generation and transformation, proposed for the hydroponically-grown plants. **1** – Exogenously applied donor SNP releases nitrosonium cation NO^+ ; **2** – Nitric oxide production in the root apoplast from nitrate by plasma membrane-bound nitrate reductase (PM-NR) and nitrite : NO reductase (NI-NOR); **3** – Non-enzymatic formation of NO by dismutation of NO_2- to NO and NO_3- (acidic pH, anoxia, or Cu(II)HEDTA and Fe(II)CN action by unknown mechanisms; **4** – NO generation in the root cells by cytosolic nitrate reductase (cNR) and NO-synthase (NOS); **5** – Mobilisation of Fe-pool in plant cells.

remobilization for improved chlorophyll biosynthesis in treated Fe-deficient cucumbers (Fig. 3-1 and 5). Recently it has been reported that copper ions in nutrient solution stimulate appearance of NO in roots of Brassica and pea plants, whereas zinc ions do not show the fast response (Bartha et al., 2005). Another way for NO generation in nutrient solutions containing inorganic nitrates is thought to be fulfilled by the action of plasma membrane-bound nitrate reductase (PM-NR, Stoehr et al., 2001) and nitrite:NO reductase (NI-NOR, Fig.3-2). After nitrates or nitrites uptake in the cells, cytoplasmic NR (cNR) converts them to nitrite and then to NO (Stoehr and Ulrich, 2002, Fig.3-4). Nitric oxide can also be synthesized in plant root apoplast by nonenzymatic mechanism by dismutation of nitrites (NO_2^-) to NO and nitrates under acidic pH values (Stoehr et al., 2001; Bethke et al., 2004), or anoxia (Morard et al., 2004, Fig.3-3). In this scheme we can include cupric-chelate Cu(II)HEDTA as an effector of NO synthesis and also Fe(II)CN (Fig.3-3). In our experiments, treatment of iron-deficient plants with chelated copper brings to proton release by intact roots, thus sustaining low pH that facilitated non-enzymatic production of NO in solutions. On the other hand, our experiment for 3 days treatment with substances may provoke some hypoxia in the solutions that may also mediate NO production. The similar suggestion is possible for ferrocyanide treatment – after its supply, pH of the solution becomes more acidic. Nitric oxide can also be generated in root cells by the action of cytosolic nitrate reductase (cNR) or nitric oxide synthase (NOS, Fig.3-4, del Rio et al., 2004). Plant NO-synthase-type enzymes have been supposed to be involved in root NO production or connected to hormonal signaling (Guo et al., 2003). Our results indicate that NO generation in response to iron deficiency might primarily involve NR activity or non-enzymatic production in roots of cucumber plants, because MB inhibitor of soluble NOS was without effect. The use of specific NO inhibitor cPTIO demonstrates that the action of both SNP and Cu(II)HEDTA on leaf greening is connected with NO production. Released NO can react with iron-containing proteins, or through dinitrosyl-iron complexes (formed with Fe^{2+}, NO and low-molecular-weight thiols) can participate in re-mobilization of labile iron pool in root or leaf apoplast and ferritin (the main iron storage protein in plants) or mitochondrial fratexin and so on (Fig.3-5). In conclusion we suppose that

the more probable way for NO production after treatment with compounds under conditions of our experiment with Fe-deficient cucumbers, includes non-enzymatic generation of nitric oxide in root apoplast by NO_3 and NO_2 in solutions or by the action of NRs. The precise mechanism for NO synthesis in iron-deficient cucumber plants after cupric-chelate or ferrocyanide application is not possible to be described at the moment and needs further experiments.

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