

Alleviation of photosynthetic inhibition in copper-stressed tomatoes through rebalance of ion content by exogenous nitric oxide

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Abstract: Copper (Cu) is an essential element that plays a very important role in plant growth and development. Its accumulation in soil is now considered a serious environmental problem endangering agricultural production and human health. In this study, the influence of exogenous nitric oxide (NO) on photosynthesis, Cu, magnesium (Mg), and iron (Fe) contents was studied in tomato seedlings under Cu stress grown in hydroponic culture. The results showed that the addition of sodium nitroprusside (SNP), a donor of exogenous NO, significantly alleviated photosynthetic inhibition in Cu-stressed tomatoes, including the recovery of photosynthetic pigment content and chlorophyll a fluorescence and the improvement of net photosynthetic rate, stomatal conductance, and transpiration rate. Application of exogenous NO by SNP reduced the excessive accumulation of Cu and recovered Mg and Fe contents in Cu-stressed tomatoes. This positive involvement of exogenous NO in tomato tolerance to Cu stress was further confirmed by addition of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a specific NO scavenger, which resulted in further reduction of photosynthesis and more severe disturbance of ion homeostasis in Cu-stressed seedlings. These results indicate that rebalance of ion content was involved in alleviation of photosynthesis inhibition in Cu-stressed tomatoes by exogenous NO.

Key words: cPTIO, essential elements, photosynthesis, sodium nitroprusside

1. Introduction

Copper (Cu) is not only a heavy metal element but also an essential nutrient for plant growth and development (Thounaojam et al., 2012). Insufficient supply of Cu can result in abnormal plant growth and development, both of which have been widely reported in previous studies (Yu and Rengel, 1999; Andrés-Colás et al., 2013). However, an excess of Cu, a serious environmental problem arising and spreading together with industrialization and the improper management of fertilizers and irrigation in agriculture in the past decades, can also adversely affect physiological processes and biochemical reactions in organisms and endanger the environment and, more particularly, human health (Hirayama et al., 2012; Wen et al., 2013). Therefore, increasing knowledge about how plants respond to Cu stress can greatly contribute to applied technologies, such as metal phytoextraction, to improve crop yield and quality in contaminated soil and to protect environmental safety (Seth et al., 2011).

Photosynthesis, a fundamental and critical metabolic process for plant growth, development, and yield, is very sensitive to unfavorable conditions. For example, severe inhibition of photosynthesis has been reported in cadmium (Cd)-stressed and nickel (Ni)-stressed sunflowers due to the degradation of chlorophyll, the destruction of chloroplast ultrastructure, and the inactivation of CO₂-fixation enzymes (Laspina et al., 2005; Gill et al., 2013a). Excessive accumulation of reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, hydroxyl radicals, and superoxide radicals is considered the main cause of a series of damages to the photosynthetic apparatus and subsequent inhibition of plant growth under heavy metal stress (Xiong et al., 2010; Gill et al., 2013a; Bharwana et al., 2014).

Nitric oxide (NO) is a small and highly diffusible gas that can freely penetrate lipid bilayer membranes and be easily transported inside cells. These chemical properties make NO a versatile signal molecule that functions

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through interactions with cellular targets via either redox or additive chemistry (Mur et al., 2013). Recently, growing evidence suggests that NO is involved in not only plant growth and development but also in responses to abiotic and biotic stress (Hong et al., 2008; Kausar et al., 2013; Tran et al., 2013; Ali and Ismail, 2014). For example, the application of exogenous NO can enhance photosynthetic pigment content in leaves of Cd-treated *Brassica napus* plants (Jhanji et al., 2012), alleviate the inhibitory effect of heavy metals on root growth in *Lupinus luteus* (Kopyra and Gwóźdź, 2003), and reduce Cu-induced ammonium (NH_4^+) accumulation in rice leaves (Yu et al., 2005) through improving antioxidant capacity (Xiong et al., 2010). Besides improvement of the redox system, regulation of NO of ion homeostasis is also an important mechanism for plant tolerance to heavy metal stress (Xiong et al., 2010). Our previous studies have demonstrated that exogenous NO induces tomato tolerance to Cu toxicity through improvement of antioxidant enzyme activity and accumulation of metallothionein, an organic chemical that can bind heavy metals and thus reduce their toxicity (Wang et al., 2010a, 2010b). However, it is still ambiguous whether this application of exogenous NO could regulate ion homeostasis in Cu-stressed tomato plants, especially at the subcellular level.

Ion homeostasis in plants can be influenced by ion interaction such as exchange, cotransportation, and competition in the process of nutrient absorption and subsequent distribution under heavy metal stress (Xiong et al., 2010; Mihailovic and Drazic, 2011; Siddiqui et al., 2011). For example, the content of calcium (Ca) and zinc (Zn) has been shown to be significantly decreased under Ni stress due to the competitive absorption of excessive Ni with Ca and Zn ions (Mihailovic and Drazic, 2011). NO addition can regulate ion homeostasis by sequestration of Ni in the roots and stimulation of Ca and Zn absorption, thus alleviating Ni toxicity in plants (Mihailovic and Drazic, 2011; Kazemi, 2012). Competitive absorption of Cu with magnesium (Mg) and Fe, 2 essential metal elements that are closely associated with light reaction in plant photosynthesis, has also been well demonstrated, especially under stress conditions (Pätsikkä et al., 2002; Alaoui-Sossé et al., 2004; Chen et al., 2004). We thus proposed a hypothesis that rebalance of ion content might also contribute to the alleviation of photosynthetic inhibition and growth reduction of Cu-stressed tomato seedlings. The objective of this research was to elucidate whether modulation of ion homeostasis was involved in alleviation of photosynthetic inhibition in Cu-stressed tomatoes.

2. Materials and methods

2.1. Plants, growth conditions, and experimental design

The germinated seeds of tomato (*Solanum lycopersicum* L. 'Zhongshu No. 4') were raised in well-washed quartz sand in the greenhouse of Shandong Agricultural University and irrigated with tap water from April to June and August to October in 2011.

Tomato seedlings with 1 fully expanded leaf were then removed to hydroponic boxes (40 cm × 30 cm × 12 cm, 8 plants per box) containing a complete tomato nutrient solution: 3.85 mM $\text{Ca}(\text{NO}_3)_2$, 2.35 mM KNO_3 , 1.36 mM KH_2PO_4 , 2 mM MgSO_4 , 46.3 μM H_3BO_3 , 9.55 μM MnSO_4 , 50 μM Fe-EDTA, 0.76 μM ZnSO_4 , 0.02 μM H_2MoO_4 , and 0.32 μM CuSO_4 , pH 6.0 ± 0.5 . The solution was continually aerated with an electric pump and completely renewed every 4 days. Air temperature was 25–30 °C during the day and 18–25 °C during the night; relative humidity was 65%–75%.

The design for this study consisted of a control (normal growth conditions) and 3 treatments [10 μM CuSO_4 (Cu); 10 μM CuSO_4 + 100 μM sodium nitroprusside (SNP) (Cu+SNP); 10 μM CuSO_4 + 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) (Cu+cPTIO)]. Each treatment was arranged in a randomized block design with 6 replicates, with 6 plants per replication. SNP and cPTIO (Sigma, USA) were used as a donor of exogenous NO and a specific NO scavenger, respectively. When the tomato seedlings had 6 or 7 true leaves, extra CuSO_4 with or without SNP was added to the nutrient solution. For inhibitory experiments, cPTIO was added to the nutrient solution 2 days earlier than the extra CuSO_4 addition. Over the course of the treatment, the nutrient solution was renewed daily to maintain sufficient stress strength.

At 7 days after treatment, the third or fourth fully expanded leaves (counted from the top of seedlings) of tomato plants grown under different treatments were used for the measurement of photosynthesis, photosynthetic pigment content, chlorophyll *a* fluorescence, and heavy metal element content. Main stems and lateral roots of tomato plants grown under different treatments were also sampled for the measurement of heavy metal element content.

2.2. Determination of photosynthetic parameters

Photosynthetic rate (P_n), stomatal conductance (G_s), internal CO_2 concentration (C_i), and transpiration rate (T_r) of individual leaves were measured at 1000 hours by an open photosynthesis system (Ciras-II, PPsystems, UK), which was set to 2.5 cm^2 of leaf area, 25 °C leaf temperature, 90% relative humidity, 200 mbar of leaf to air vapor pressure, 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, and 400 $\mu\text{mol mol}^{-1}$ of CO_2 .

2.3. Determination of photosynthetic pigment content

About 0.5 g of fresh leaves was used for photosynthetic pigment extraction by 80% acetone. The absorbance of the centrifuged extract was measured at 663 nm, 645 nm, and 440 nm. The content of chlorophyll *a*, chlorophyll *b*, and carotenoids was calculated according to the methods introduced by Strain and Svec (1966) and Ikan (1969).

2.4. Determination of chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence was determined by a portable fluorometer (FMS-2, Hansatech, UK) at room temperature according to the protocol described by Yang et al. (2009). Briefly, dark-adapted leaves were first subjected to a modulated light and then to a saturating pulse of 8000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for determination of dark-adapted minimal fluorescence (F_0) and maximal fluorescence (F_m). Thereafter, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of white actinic and another saturating pulse were applied to determine fluorescence in steady state (F_s) and light-adapted maximal fluorescence (F_m'). Short-term far red light was used for determination of minimal light-adapted fluorescence level (F_0'). The individual parameters of chlorophyll *a* fluorescence were calculated according to Chen et al. (2013) as follows:

Actual PSII efficiency (ΦPSII), $\Phi\text{PSII} = (F_m' - F_s) / F_m'$

Photochemical quenching (qP), $qP = (F_m' - F_s) / (F_m' - F_0')$

Relative electron transport rate (ETR), $\text{ETR} = (F_m' - F_s) / F_m' \times \text{PAR} \times 0.84 \times 0.5$

Nonphotochemical quenching (qN), $qN = 1 - (F_m' - F_0') / (F_m - F_0)$

2.5. Organ and subcellular distribution of heavy metal elements

Leaf, stem, and root samples were oven-dried at 70 °C until the weight reached a constant for the measurement of Cu, Fe, and Mg contents. At least 6 plants were analyzed for each treatment. The oven-dried samples were digested with 4 mL of HNO_3 plus 2 mL of HClO_4 and then analyzed using an atomic absorption spectrophotometer (AA-6800, Shimadzu Corporation, Japan).

For the measurement of subcellular distributions of Cu, Mg, and Fe, 2 g of fresh samples was ground well with precooled homogenate [250 mM sucrose, 50 mM Tris-HCL (pH 7.4), 1 mM dithioerythritol ($\text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$)] based on a sample-to-solution ratio of 1:10 at 4 °C. The homogenate was subsequently centrifuged at 600 rpm for 10 min, at 1000 rpm for 15 min, and at 10,000 rpm for 20 min, respectively, in order to obtain the cell wall component (F1), the chloroplast and plastid component for leaves or the proplastid and plastid component for roots (F2), the other organelles and membrane component (F3), and the final supernatant (F4), which was the soluble

substance containing macromolecules in vacuoles, macromolecular organic matter, and inorganic ions. The 4 components were subjected to determination of Cu, Fe, and Mg contents based on the method described above.

2.6. Statistical analysis

The results were subjected to one-way analysis of variance (ANOVA) and least significant difference test with SAS software (Statistica version 6.1, StatSoft, USA) and are presented as means \pm standard error of 6 replicates. Different letters indicate a significant difference from the control at the 0.05 probability level.

3. Results

3.1. Photosynthesis

Photosynthesis of tomato seedlings grown under normal conditions, Cu stress, Cu stress with SNP, and Cu stress with cPTIO was determined at 7 days after treatment (Figure 1). Compared with the control, the Pn, Gs, and Tr of Cu-stressed tomato leaves were decreased by 31%, 31%, and 36%, respectively, whereas Ci was increased by 8%. Significant increases of Pn, Gs, and Tr and decreases of Ci were observed in the leaves of Cu+SNP tomatoes compared to the Cu-stressed plants, though Pn, Gs, and Tr were still lower than those of the control. Replacement of SNP with cPTIO, a specific scavenger for NO, in a nutrient solution with excessive Cu decreased Pn by 24%, Gs by 48%, and Tr by 28%, respectively, compared to the Cu-stressed tomatoes. At the same time, a significant increase of Ci was observed in the leaves of Cu+cPTIO tomatoes compared with the Cu-treated ones.

3.2. Photosynthetic pigment content

Changes of chlorophyll *a*, chlorophyll *b*, and carotenoid contents in the leaves of tomato seedlings grown under normal conditions, Cu stress, Cu+SNP, and Cu+cPTIO were investigated at 7 days after stress exposure (Figure 2). Chlorophyll *a*, chlorophyll *b*, and carotenoid contents in the leaves of Cu-stressed tomato seedlings were decreased by 42%, 52%, and 66%, respectively, with respect to control. When 100 μM SNP was added along with excessive Cu to nutrient solution, Cu toxicity in tomato seedlings was alleviated and chlorophyll *a*, chlorophyll *b*, and carotenoid contents were thus increased significantly compared to the Cu-stress plants, though all 3 contents were still lower than in the control. Chlorophyll *a*, chlorophyll *b*, and carotenoid contents were further decreased in the leaves of Cu+cPTIO plants by 31%, 24%, and 36%, respectively, compared with the Cu-stressed ones.

3.3. Chlorophyll *a* fluorescence

Figure 3 shows changes of chlorophyll *a* fluorescence in the leaves of tomato seedlings exposed to normal conditions, Cu stress, Cu+SNP, and Cu+cPTIO, respectively. The significant decrease of ΦPSII , qP , and ETR was observed

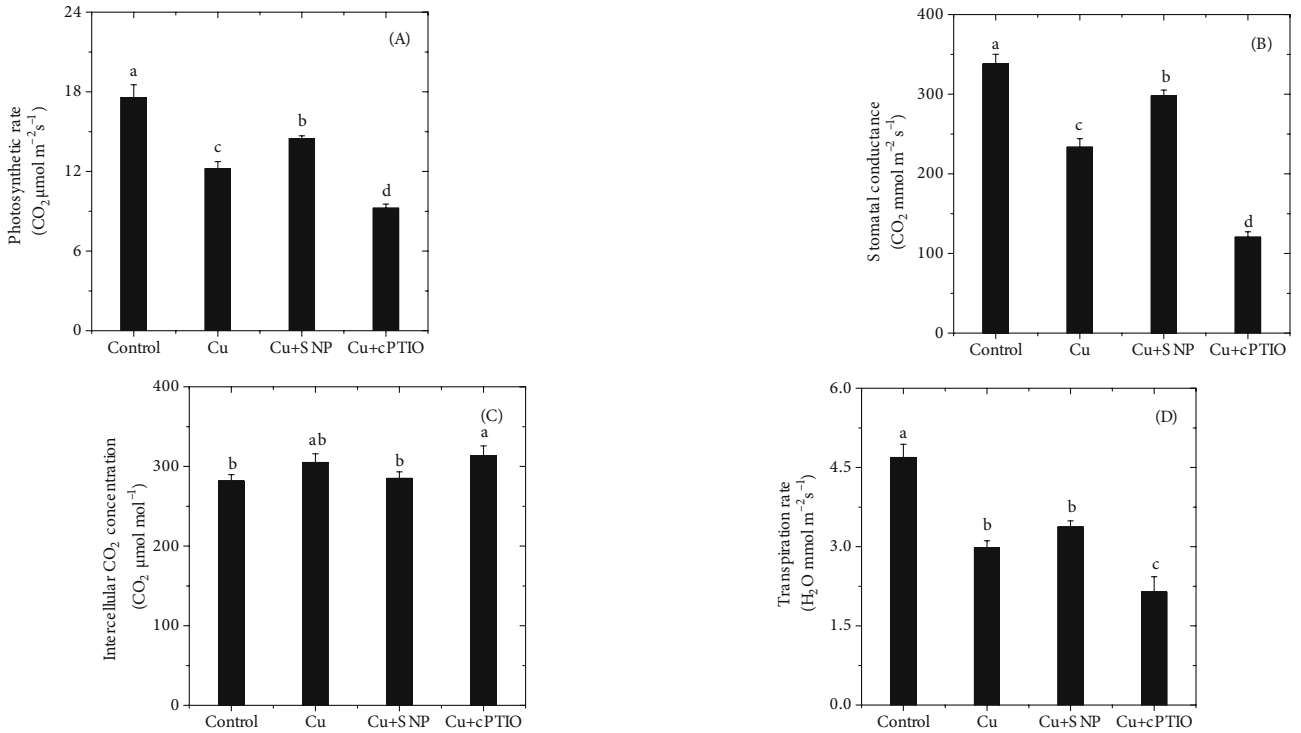


Figure 1. Effects of exogenous NO on net photosynthetic rate (A), stomatal conductance (B), intercellular CO_2 concentration (C), and transpiration rate (D) in tomato leaves under Cu stress. Tomato plants with 6 or 7 true leaves were grown under normal conditions (Control), Cu stress (Cu), Cu stress with $100 \mu\text{M}$ SNP (Cu+SNP), and Cu stress with $100 \mu\text{M}$ cPTIO (Cu+cPTIO). At 7 days after treatment, photosynthetic parameters in the tomato leaves were measured. Vertical bars represent the standard errors ($n = 6$). Different letters indicate significant differences between the treatments at 0.05 level.

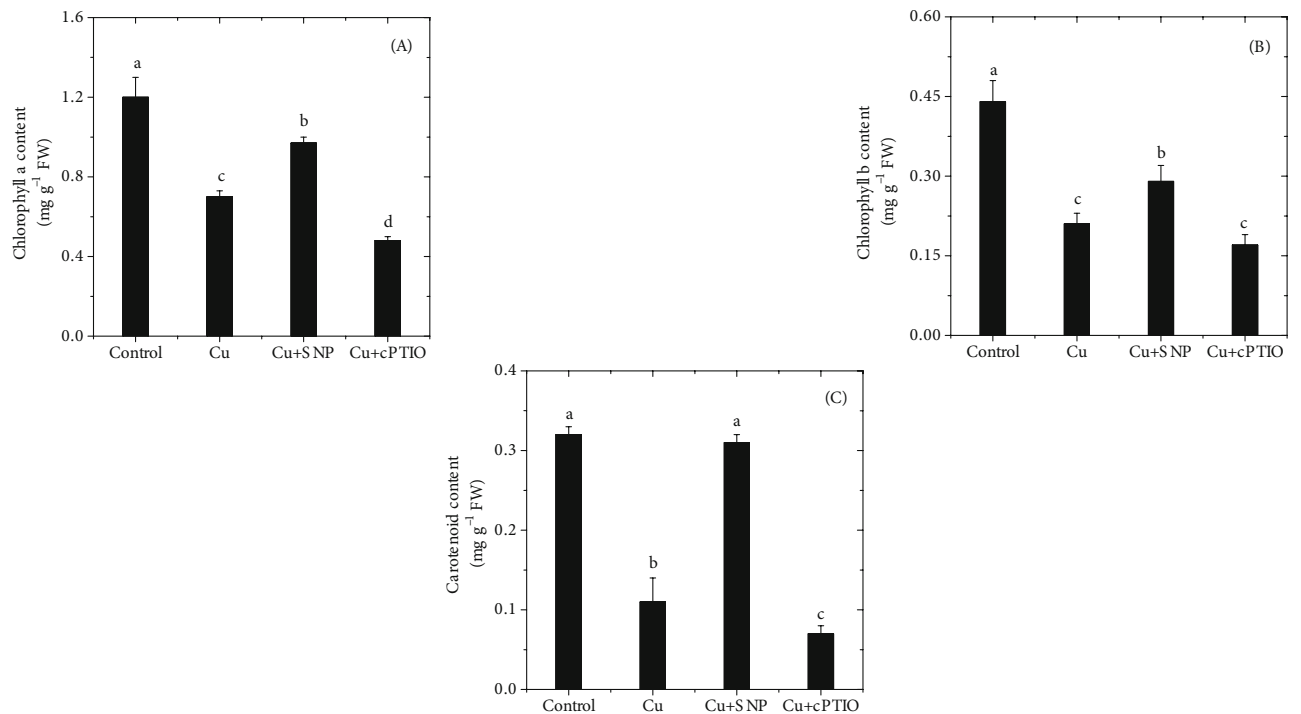


Figure 2. Effects of exogenous NO on the content of chlorophyll a (A), chlorophyll b (B), and carotenoids (C) in tomato leaves under Cu stress. Tomato plants with 6 or 7 true leaves were grown under normal conditions (Control), Cu stress (Cu), Cu stress with $100 \mu\text{M}$ SNP (Cu+SNP), and Cu stress with $100 \mu\text{M}$ cPTIO (Cu+cPTIO). At 7 days after treatment, photosynthetic pigment content in the tomato leaves was measured. Vertical bars represent the standard errors ($n = 6$). Different letters indicate significant differences between the treatments at 0.05 level.

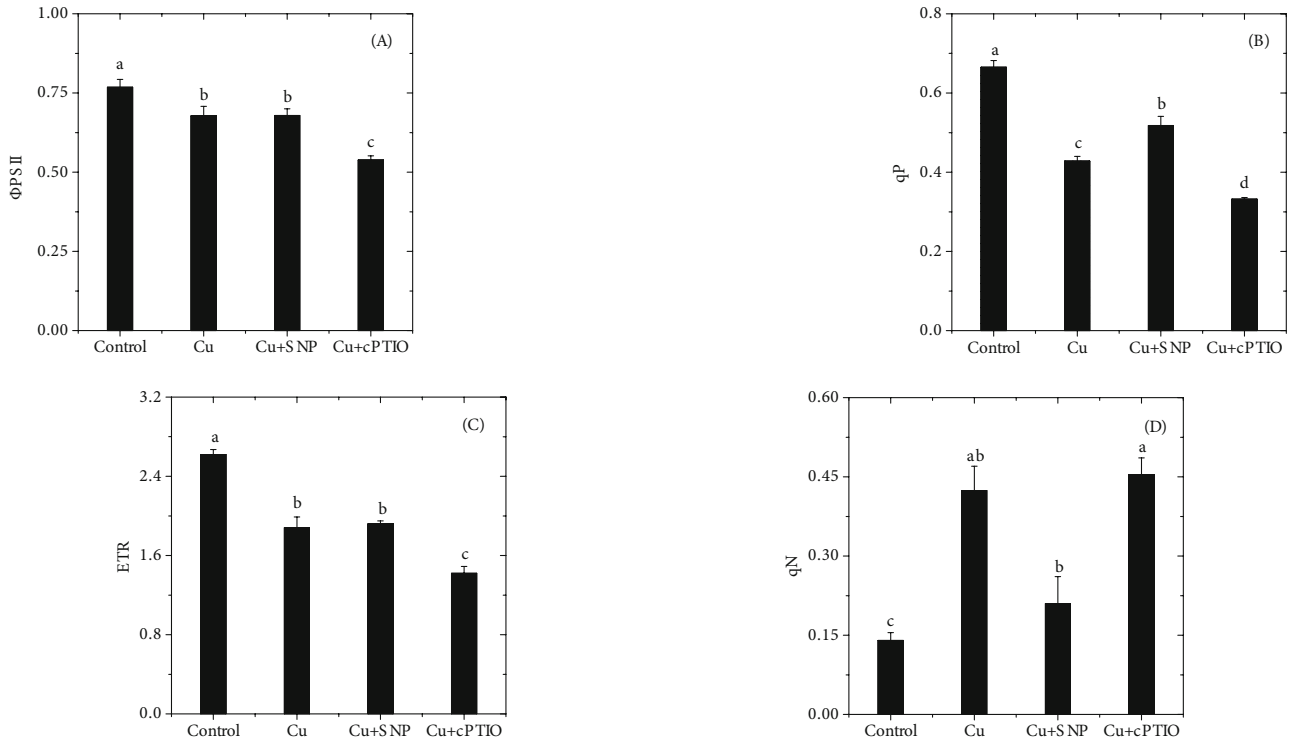


Figure 3. Effects of exogenous NO on PSII efficiency (Φ_{PSII} , **A**), photochemical quenching (qP, **B**), relative electron transport rate (ETR, **C**), and nonphotochemical quenching (qN, **D**) in tomato leaves under Cu stress. Tomato plants with 6 or 7 true leaves were grown under normal conditions (Control), Cu stress (Cu), Cu stress with 100 μ M SNP (Cu+SNP), and Cu stress with 100 μ M cPTIO (Cu+cPTIO). At 7 days after treatment, chlorophyll *a* fluorescence in the tomato leaves was measured. Vertical bars represent the standard errors (n = 6). Different letters indicate significant differences between the treatments at 0.05 level.

by 12%, 36%, and 28%, respectively, whereas qN was increased by about 2 times in the leaves of Cu-stressed plants with respect to the control. Compared with Cu-stressed seedlings, the addition of SNP alleviated Cu toxicity in the photosynthetic apparatus to different extents, though these values were still lower or higher than those of control. Further reductions in Φ_{PSII} , qP, and ETR and enhancement in qN were observed in the Cu+cPTIO seedlings.

3.4. Copper content

The Cu content in the leaves, stems, and roots of Cu-stressed tomatoes were 2.70, 1.19, and 14.64 times higher than those of the control, respectively, and the highest Cu content was observed in the roots, followed by leaves and stems (Figure 4). The Cu content in the leaves, stems, and roots of seedlings subjected to the Cu+SNP treatment was decreased by 43%, 1%, and 12%, respectively, compared to the Cu treatment. On the contrary, the Cu content in the leaves, stems, and roots of plants subjected to the Cu+cPTIO treatment was increased by 25%, 99%, and 63%, respectively, compared to the Cu treatment.

The Cu subcellular distribution in different cell components in the leaves and roots of Cu-stressed seedlings was significantly increased compared to control

(Figure 5). Cu content was the highest in the cell wall component (F1), followed by the soluble component (F4). Compared with the Cu-stressed plants, Cu content in F1, the chloroplast and plastid component (F2), the other organelles and membrane component (F3), and F4 in the leaves of tomatoes treated with Cu+SNP was decreased by 32%, 10%, 17%, and 32%, respectively. Cu content in F1, F2, F3, and F4 in the roots treated with Cu+SNP was decreased by 50%, 22%, 52%, and 25%, respectively. Substitution of SNP with cPTIO resulted in the increase of Cu content in F1, F2, F3, and F4 by 26%, 34%, 27%, and 28% for the leaves and by 0.5%, 47%, 41%, and 16% for the roots compared to the Cu-stressed plants, respectively.

3.5. Magnesium content

Mg content in the leaves, stems, and roots of tomato seedlings treated with Cu was decreased by 31%, 20%, and 10%, respectively, compared to control seedlings (Figure 6). Mg content in the leaves, stems, and roots of tomato seedlings treated with Cu+SNP was increased by 12%, 7%, and 2%, respectively, compared with the Cu-stressed ones. Substitution of SNP with cPTIO resulted in further reduction of Mg content by 15% for leaves, 17% for stems, and 2% for roots with respect to the Cu-stressed plants.

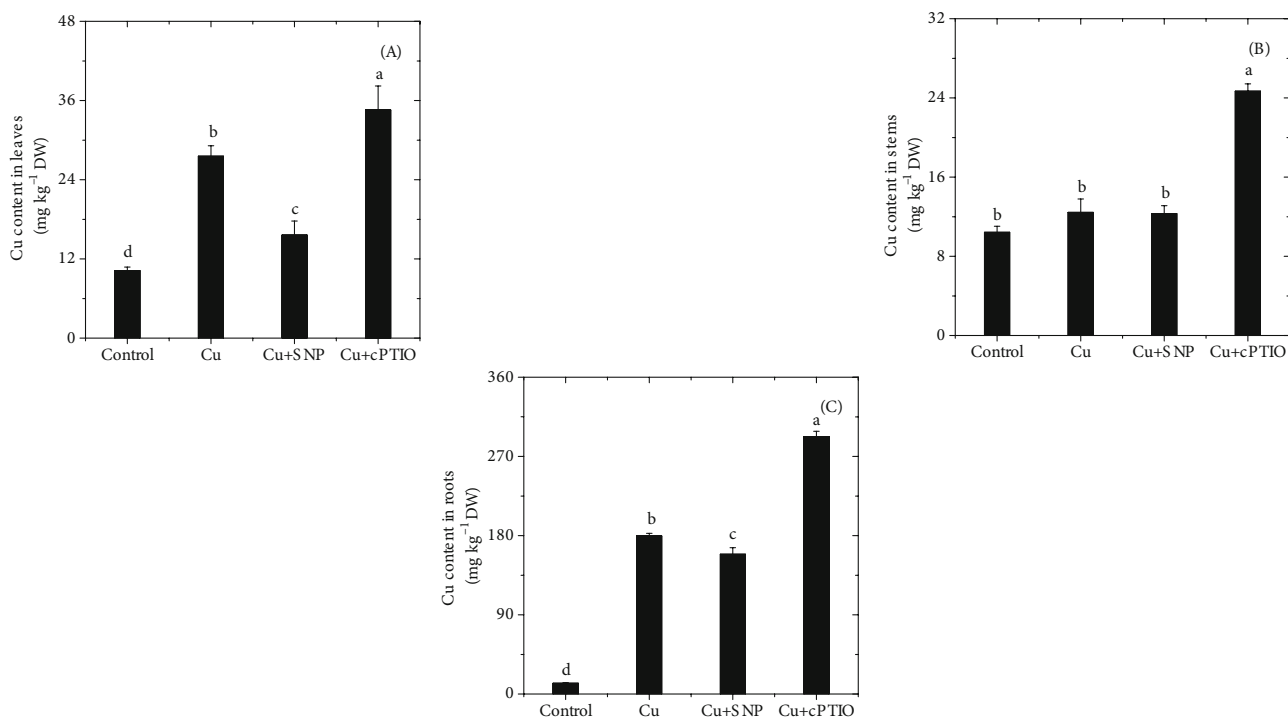


Figure 4. Effects of exogenous NO on Cu content in leaves (A), stems (B), and roots (C) of tomato seedlings under Cu stress. Tomato plants with 6 or 7 true leaves were grown under normal conditions (Control), Cu stress (Cu), Cu stress with 100 μ M SNP (Cu+SNP), and Cu stress with 100 μ M cPTIO (Cu+cPTIO). At 7 days after treatment, Cu content in the leaves, stems, and roots of tomato plants was measured. Vertical bars represent the standard errors ($n = 6$). Different letters indicate significant differences between the treatments at 0.05 level.

The Mg subcellular distribution in different cell components in the leaves and roots of Cu-stressed seedlings was decreased compared to the control to different extents (Figure 7). The greatest reduction of Mg content was observed in F2 followed by F1 in the leaves and in F3 followed by F2 in the roots. Compared with the Cu-stressed ones, Mg content in F1, F2, F3, and F4 in the leaves of tomatoes treated with Cu+SNP was increased by 28%, 83%, 14%, and 25%, respectively. A significant increase of Mg content was observed in F4 in the roots treated with Cu+SNP together with slight fluctuation of Mg content in F1, F2, and F3 compared with the Cu-stressed seedlings. Cu+cPTIO treatment resulted in further decrease of Mg content in 4 cell components in the leaves with respect to the Cu-stressed plants. No significant influences of Cu-cPTIO treatment were observed on Mg content in the 4 cell components in the roots compared with the Cu treatment group.

3.6. Iron content

The Fe content in the leaves, stems, and roots of Cu-stressed tomatoes was decreased by 47%, 28%, and 19% with respect to those of control, respectively (Figure 8). The addition of SNP to the nutrient solution with excessive Cu caused the increase of Fe content by 33% for the leaves, 28% for the stems, and 18% for the roots compared with the Cu-stressed seedlings. On the contrary, the Fe content

in the leaves, stems, and roots of the plants subjected to the Cu+cPTIO treatment decreased by 25%, 25%, and 3%, respectively, with respect to the Cu treatment. Fe content was significantly decreased by Cu stress in all 4 cell components in the leaves and in F1, F2, and F4 in the roots compared to the control (Figure 9). The greatest reduction of Fe content was observed in F2 followed by F1 in the leaves and F4 followed by F2 in the roots. Cu+SNP treatment caused significant increases of Fe content by 41% in F1, 144% in F2, and 25% in F4 in the leaves compared with the Cu-stressed ones. An increase of Fe content in Cu+SNP treatments was also observed in the 4 cell components in the roots, to different extents than in the Cu-stressed seedlings. The addition of cPTIO to the nutrient solution containing excessive Cu resulted in further decreases of the Fe content in F1, F2, F3, and F4 by 56%, 19%, 11%, and 17% for the leaves and by 3%, 12%, 29%, and 13% for the roots compared to the Cu-stressed plants, respectively.

4. Discussion

Being different from salt stress, ion toxicity is considered the main cause for a series of abnormal responses in plants under trace element-mediated stress such as Cd, Mn, and Cu (Cuyper et al., 2012; Chen et al., 2013; Gill et al., 2013a). Nonstomatal factors might thus be the key

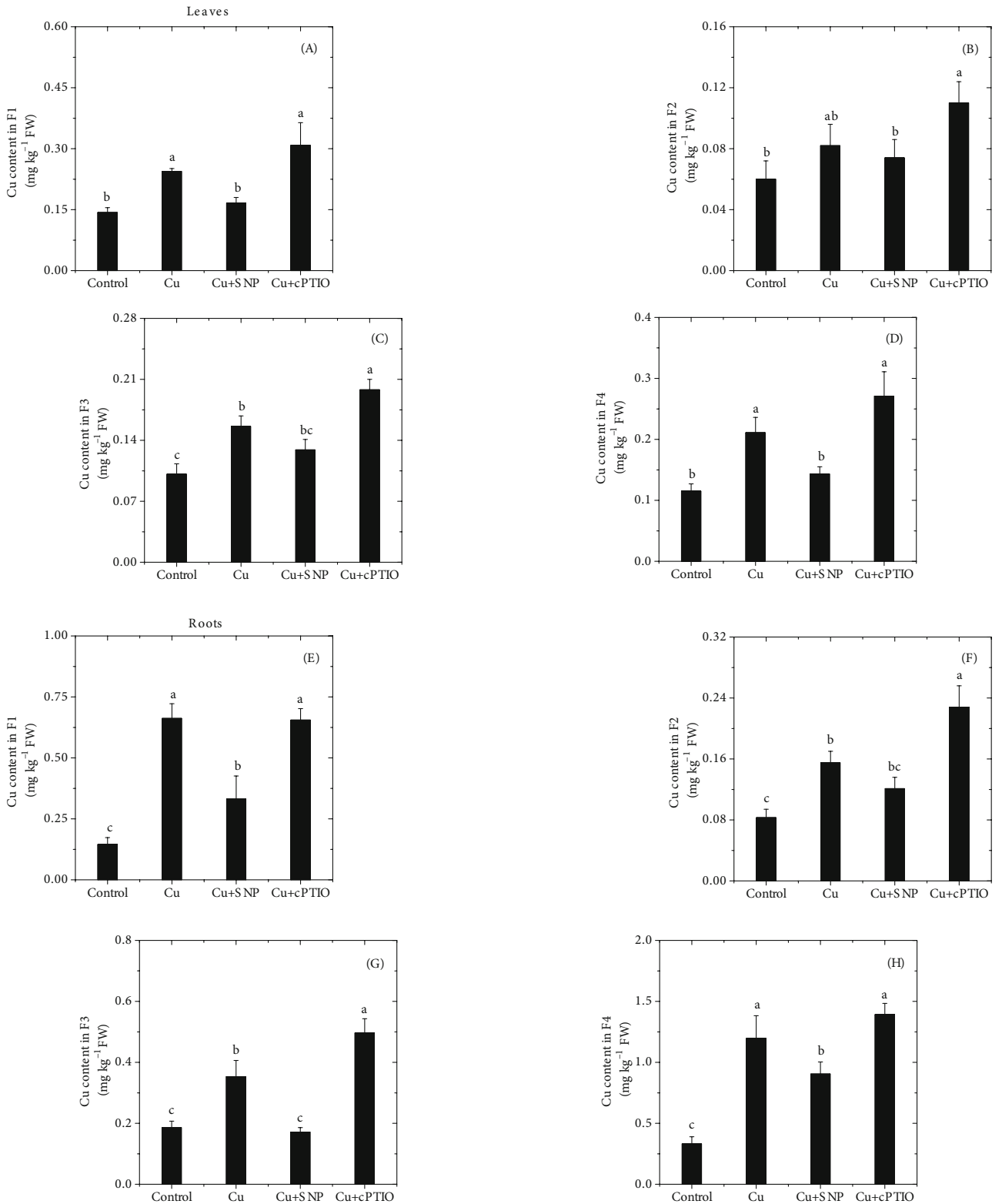


Figure 5. Effects of exogenous NO on Cu subcellular distribution in leaves (A–D) and roots (E–H) of tomato seedlings under Cu stress. Tomato plants with 6 or 7 true leaves were grown under normal conditions (Control), Cu stress (Cu), Cu stress with 100 μ M SNP (Cu+SNP), and Cu stress with 100 μ M cPTIO (Cu+cPTIO). At 7 days after treatment, Cu contents in F1 (cell wall component, A and E), F2 (chloroplast and plastid component for leaves or proplastid and plastid component for roots, B and F), F3 (other organelles and membrane component, C and G), and F4 (soluble component, D and H) were measured. Vertical bars represent the standard errors ($n = 6$). Different letters indicate significant differences between the treatments at 0.05 level.

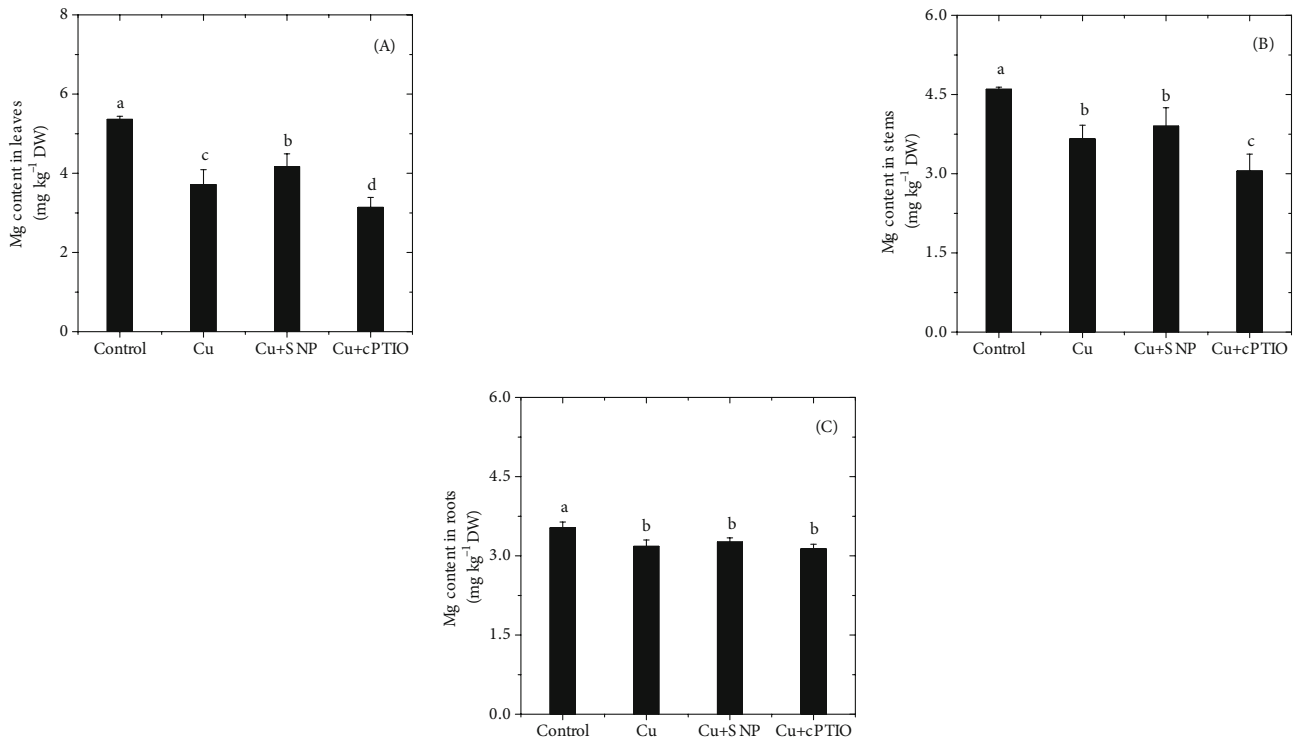


Figure 6. Effects of exogenous NO on Mg content in leaves (A), stems (B), and roots (C) of tomato seedlings under Cu stress. Tomato plants with 6 or 7 true leaves were grown under normal conditions (Control), Cu stress (Cu), Cu stress with 100 μ M SNP (Cu+SNP), and Cu stress with 100 μ M cPTIO (Cu+cPTIO). At 7 days after treatment, Mg content in the leaves, stems, and roots of tomato plants was measured. Vertical bars represent the standard errors ($n = 6$). Different letters indicate significant differences between the treatments at 0.05 level.

factors for substantial decrease of Pn, Gs, and Tr in the leaves of tomatoes grown under Cu stress (Figure 1). In order to confirm this presumption, photosynthetic pigment content and chlorophyll *a* fluorescence were investigated. Significant decrease of chlorophyll *a*, chlorophyll *b*, and carotenoid contents was observed in the leaves of Cu-stressed tomatoes (Figure 2), possibly due to an acceleration in chlorophyll degradation (Parasad and Hagemeyer, 1999) and/or the blocking of chlorophyll synthesis (Connan and Stengel, 2011). At the same time, Φ PSII, qP, and ETR were significantly decreased in the leaves of Cu-stressed tomatoes (Figures 3A–C), suggesting that electron transport was severely inhibited. qN, a photoprotective mechanism in plants, was thus significantly stimulated in tomato leaves under Cu stress (Figure 3D) in order to quench the excessive accumulation of solar energy through heat dissipation, because this accumulation can potentially stimulate the production of ROS and result in severe damages to the photosynthetic apparatus (Gill et al., 2013b). Based on these results, we concluded that Cu stress might inhibit photosynthesis through its direct effect on the photosynthetic apparatus in tomato seedlings.

The maintenance of ion homeostasis is essential for plant growth and development. Here we analyzed ion distribution in the leaves, stems, and roots of Cu-stressed tomatoes. The results showed that Cu accumulated mostly in the roots, followed by leaves and stems (Figure 4). Mg and Fe contents were significantly decreased in the leaves, stems, and roots of Cu-stressed tomatoes at the same time (Figures 6 and 8). Compared with ion accumulation at the organ level, subcellular accumulation may better reflect ion toxicity in plants grown under unfavorable ion conditions because biochemical and physiological processes mostly happen in the cytoplasm. Leaves and roots are the most active sites for bioreactions in plants. We thus analyzed Cu, Mg, and Fe distribution in different cell components of tomato leaves and roots as well (Figures 5, 7, and 9). The highest Cu content was observed in F1, followed by F4. Significant decreases of Mg and Fe contents were found in F1, F2, F3, and F4. The likely explanation for these observations is that excessive Cu may accumulate near the root surface, compete with Mg and Fe for membrane uptake sites (Pätsikkä et al., 2002; Alaoui-Sossé et al., 2004; Chen et al., 2004), and thus cause disturbance of ion homeostasis in Cu-stressed tissues. Consequently,

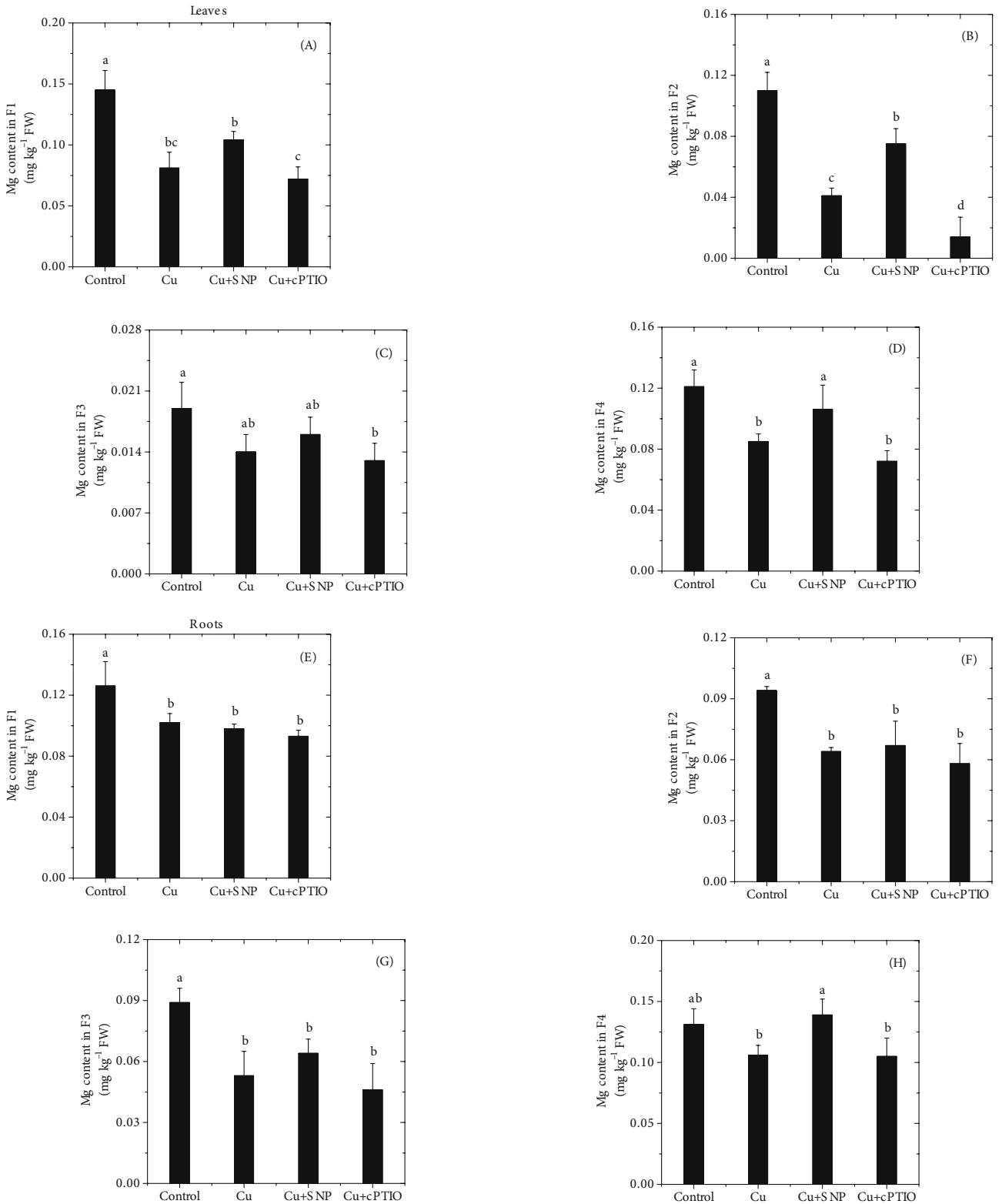


Figure 7. Effects of exogenous NO on Mg subcellular distribution in leaves (A–D) and roots (E–H) of tomato seedlings under Cu stress. Tomato plants with 6 or 7 true leaves were grown under normal conditions (Control), Cu stress (Cu), Cu stress with 100 μ M SNP (Cu+SNP), and Cu stress with 100 μ M cPTIO (Cu+cPTIO). At 7 days after treatment, Mg contents in F1 (cell wall component, A and E), F2 (chloroplast and plastid component for leaves or proplastid and plastid component for roots, B and F), F3 (other organelles and membrane component, C and G), and F4 (soluble component, D and H) were measured. Vertical bars represent the standard errors ($n = 6$). Different letters indicate significant differences between the treatments at 0.05 level.

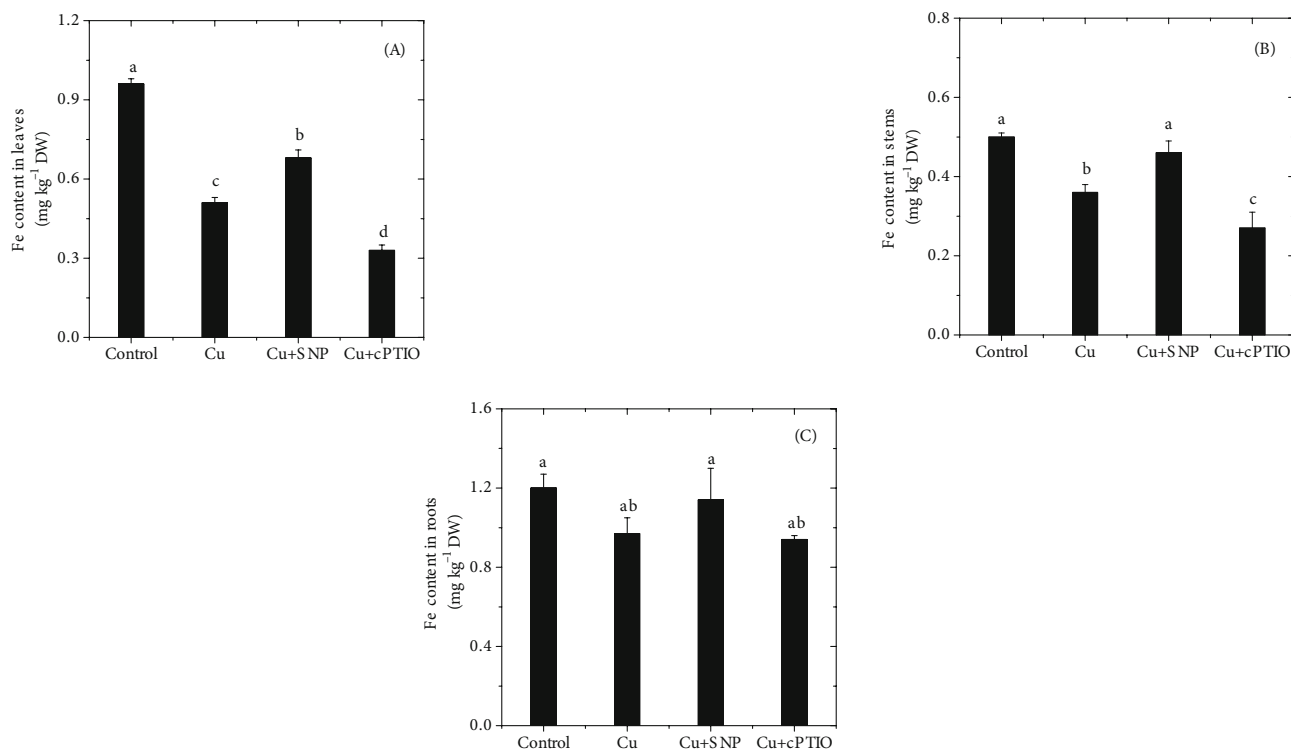


Figure 8. Effects of exogenous NO on Fe content in leaves (A), stems (B), and roots (C) of tomato seedlings under Cu stress. Tomato plants with 6 or 7 true leaves were grown under normal conditions (Control), Cu stress (Cu), Cu stress with 100 μ M SNP (Cu+SNP), and Cu stress with 100 μ M cPTIO (Cu+cPTIO). At 7 days after treatment, Fe content in the leaves, stems, and roots of tomato plants was measured. Vertical bars represent the standard errors ($n = 6$). Different letters indicate significant differences between the treatments at 0.05 level.

carbohydrate synthesis and growth of tomatoes were severely inhibited by Cu stress (Figure 1).

The involvement of NO in the alleviation of photosynthesis and growth inhibition by abiotic stress has been widely reported in previous studies (Beligni and Lamattina, 2002; Procházková et al., 2013). In the present study, the alleviation of photosynthesis reduction by exogenous NO was observed in the leaves of Cu+SNP tomatoes but not in Cu+cPTIO plants (Figure 1). The reported mechanisms for the alleviation of this kind might include promotion of antioxidant enzyme activities and detoxification through NO-mediated activation of metallothionein transcription and expression, which play a key role in the chelating of heavy metals in Cu-stressed tomatoes (Wang et al., 2010a, 2010b). Here we found that rebalance of ion content by exogenous NO might contribute to growth recovery of Cu-stressed tomatoes as well. A potential explanation for this ion rebalance is that there is an enhancement of Cu exclusion and Mg and Fe absorption in Cu-stressed tomatoes after the application of exogenous NO. Several pieces of evidence can support this hypothesis. First, Cu content was decreased in 4 cell parts

of leaves and roots in Cu+SNP seedlings compared with those of Cu-stressed tomatoes (Figure 5). These findings are similar with aluminum (Al) extrusion in rice through NO-mediated ratio changes of cell wall components (Yang et al., 2008). Cellulose is a central component in plant cell walls (Xiong et al., 2010), and previous studies showed that the cellulose content in tomato roots can be influenced by NO in a dose-dependent manner (Correa-Aragunde et al., 2008; Taylor, 2008), which might contribute to the stimulation of Cu extrusion by exogenous NO in Cu-stressed tomatoes. Another supporting fact is that the application of exogenous NO had no significant effect on the transpiration of Cu-stressed tomatoes (Figure 1), which could inhibit the entry of excessive Cu into stressed plants to some extent because heavy metal translocation from plant root to shoot has been suggested to be driven by transpiration (Salt et al., 1995). Finally, the application of exogenous NO can enhance the accumulation of mRNAs responsible for Mg-transporter and Fe-transporter production (Ikari et al., 2002; Graziano and Lamattina, 2007) and induce the activity of H⁺-ATPase and H⁺-PPase in plasma membrane or tonoplast in tomato plants (Siddiqui et al., 2011), which might be positively involved

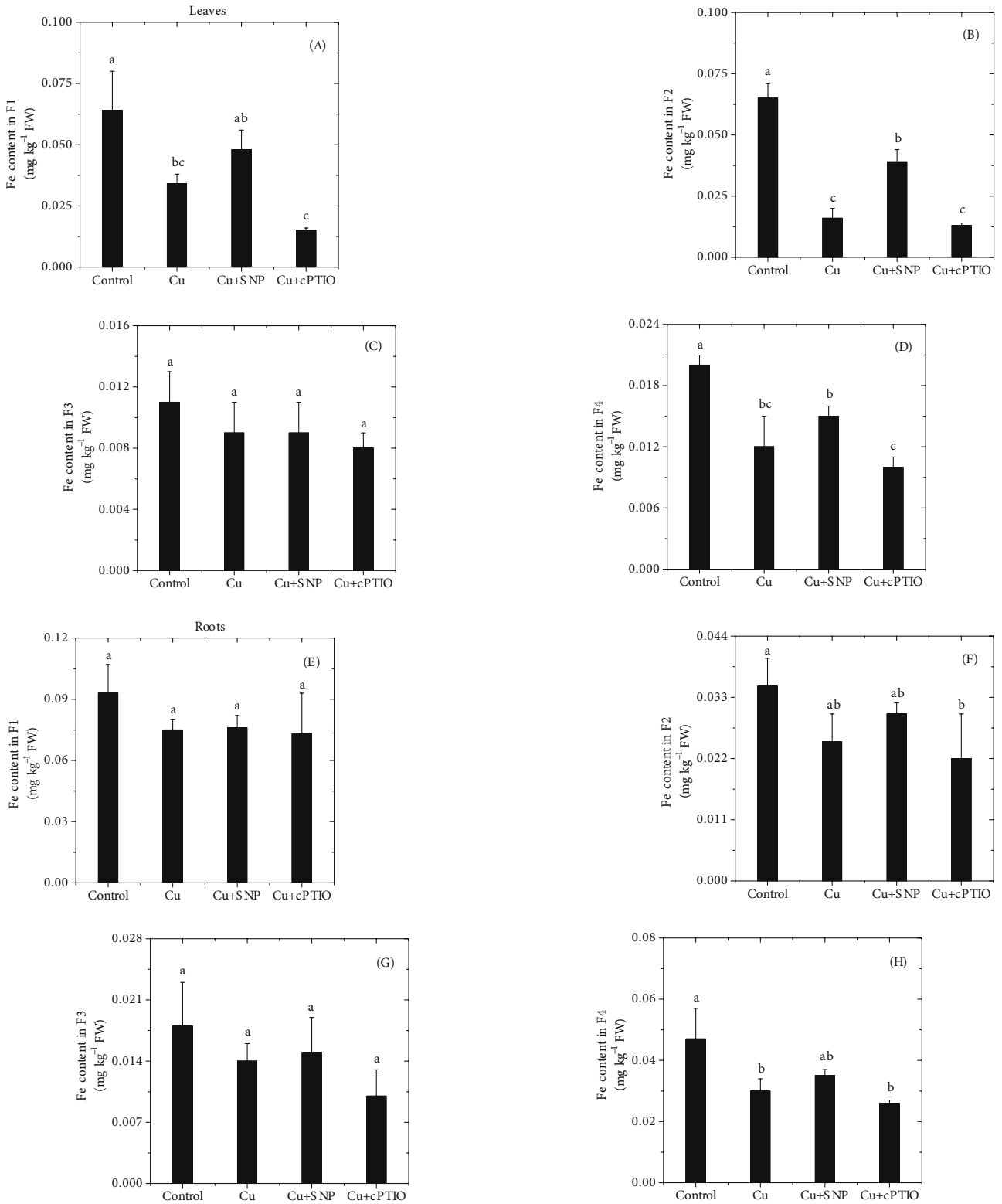


Figure 9. Effects of exogenous NO on Fe subcellular distribution in leaves (A–D) and roots (E–H) of tomato seedlings under Cu stress. Tomato plants with 6 or 7 true leaves were grown under normal conditions (Control), Cu stress (Cu), Cu stress with 100 μ M SNP (Cu+SNP), and Cu stress with 100 μ M cPTIO (Cu+cPTIO). At 7 days after treatment, Fe contents in F1 (cell wall component, A and E), F2 (chloroplast and plastid component for leaves or proplastid and plastid component for roots, B and F), F3 (other organelles and membrane component, C and G), and F4 (soluble component, D and H) were measured. Vertical bars represent the standard errors (n = 6). Different letters indicate significant differences between the treatments at 0.05 level.

in the recovery of Mg and Fe contents in Cu+SNP treatment (Figures 6–9). Based on these results, we conclude that NO-mediated rebalance of ion content might be involved in alleviation of photosynthetic inhibition in Cu-stressed tomatoes.

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