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Foliar Application of Nano Tetraammine Copper (II) Sulfate Complex Influences Cu and Fe Homeostasis, Phenolics and Lignin Biosynthesis in Tobacco (Nicotiana rustica) Plants

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Abstract

Copper (Cu) is an essential micronutrient for higher plants and is required for cell redox homeostasis, free radical scavenging, function of electron transport chains and cell wall lignification. Copper deficiency is a widespread nutritional disorder in plants and its adequate supply is necessary for an optimum crop production. In order to evaluate the efficacy of nano tetraammine copper (II) sulfate complex ($[Cu(NH_3)_4]SO_4$) (NCu) in the meeting of plants Cu requirement, Cu-sufficient (+Cu) and Cu-deficient (–Cu) tobacco (*Ncotiana rustica* L.) plants were treated with 0.5 µM NCu complex through leaves. The shoot and root biomass and photosynthesis of –Cu plants were restored by foliar application of NCu complex, while in the +Cu plants the shoot biomass was repressed likely due to a supra optimal Cu level. Foliar application of NCu complex restored almost completely the activity of Cu-containing enzymes, superoxide dismutase, polyphenol oxidase and diamine oxidase. Iron (Fe) homeostasis was also significantly influenced by both Cu starvation and foliar application as could be confirmed by Fe concentration data and activity of Fe-enzymes, peroxidase and polyamine oxidase. The activity of phenylalanine ammonia lyase and the levels of phenolics and lignin were markedly decreased in the –Cu plants. These parameters, however, were completely restored or even exceeded that of the +Cu plants upon foliar application. Our results suggest that, foliar application of NCu is a feasible method for a rapid and efficient compensation of Cu deficiency symptoms due to a high penetration ability and a sufficient retranslocation of applied Cu in the phloem.

Keywords: Cu-containing enzymes; Phenolics; Lignin; Foliar Application; Nano Complex.

Introduction

Copper (Cu) is an essential nutrient for higher plants and as a redox active cofactor is found in a wide variety of plant proteins including plastocyanin, cytochrome *c* oxidase, Cu/Zn-superoxide dismutase (Cu/Zn-SOD),

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laccases, ascorbate and amine oxidases, and polyphenol oxidases (PPOs) [1]. Copper deficiency in plants causes reduced growth rate, chlorosis, curling of leaf margins, and defects in pollen development and viability [1]. One of the most obvious symptoms of Cu deficiency is impaired lignification of cell walls. The inhibition of lignification in Cu-deficient tissues is mainly related to the direct role of the PPOs as Cu-containing enzymes in catalyzing the oxidation of phenolics as precursors of lignin [2]. Lignification decreases even under mild Cu deficiency, and is resumed rapidly by Cu resupply, thus, is a suitable indicator of the Cu nutritional status of a plant [1].

Copper in soil exists in different forms, including water soluble, exchangeable, organically-bound and those associated with carbonates and hydrous oxides of iron (Fe), manganese and aluminum [3]. Copper deficiency is widespread in agricultural soils high in organic matter content [4]. Sandy and highly leached soils with low total Cu contents, calcareous, and other high pH and saline soils are also often associated with Cu deficiency. If diagnosed early, Cu deficiency is easily corrected with addition of Cu sulfate or chelate [3].

Foliar nutrition is recognized as an important method of fertilizer application, because it facilitates the rapid absorption of mineral elements. It has been demonstrated that foliar application of Cu fertilizers provides both agronomic and economic solutions to Cu deficiency [3] and increases the uptake of Cu and grain yield and quality [5]. In wheat (*Triricum aestivum* L.), foliar spray of Cu (in the sulfate form), applied under field conditions in a calcareous soil, increased grain yield [6]. A significant increase of 20% on grain yield and harvest index was also recorded with a foliar spray of Cu (in the sulfate form) in barley (*Hordeum vulgare* L.) [7]. In grape vine (*Vitis vinifera* L.) foliar spray of a mixture of Cu and chelated Fe, increased leaf photosynthesis, improved uniformity of leaves, and the levels of soluble solids, soluble sugars, and vitamin C in the grapes [8].

Penetration of foliar fertilizers is dependent on the nature of the applied materials, i.e. size, *hydrophilicity, lipophilicity* and net charge. Reduction of particle size that increases specific surface area of a fertilizer, consequently increases its contact with plant surfaces and leads to higher nutrient uptake [9]. Nanoparticles (NPs) are atomic or molecular aggregates with at least one dimension between 1 and 100 nm [10]. This small size gives rise to properties different from those exhibited by the bulk of the material of the same composition [10]. Application of NPs in agriculture both as fertilizer and pesticide is becoming more common worldwide [11]. The impact of NPs on plants depends on many factors, such as the composition, concentration, size, the physical and chemical properties, and even the plant species under study [12]. Application of CuO NPs increased the flavonoid content and a significant induction of genes related to the responses to oxidative stress in *Arabidopsis thaliana* [13] and increased fruit quality in tomato [12]. The toxic effect of Cu and CuO NPs has also been investigated in rice [14], mung bean and wheat [15]. The effect of nano Cu complexes, however, has not been investigated so far and their effectiveness for correction of Cu deficiency symptoms and utilization within plants has not been evaluated.

Tobacco (*Nicotiana* spp.) is the most important non-food crop species in the world and is produced in more than 100 countries under various climatic conditions with different soil types [16]. In Iran, tobacco is cultivated within an area of about 9-12 thousand hectares [16] using Ncotiana rustica L. as the most common species in the country. A high efficacy of foliar application of bulk and nano Fe complexes in the alleviation of Fe-deficiency symptoms has been reported in this species [17]. Application of K through leaves had also a significant ameliorative effect in tobacco under drought stress [18].These data suggest a high potential of foliar fertilization for the correction of nutrients deficiency, improvement of growth and stress tolerance in this species.

In order to explore the effect of leaf application of nano Cu complex ($[Cu(NH_3)_4]SO_4$) and its efficacy in the meeting Cu requirement in tobacco, this experiment was undertaken in the plants with different Cu nutritional status. In addition to growth, Cu content and activity of Cu-containing enzymes, the effect of Cu resupply was studied on the Fe homeostasis, lignification and activity of related enzymes.

Materials and Methods

Plants culture and treatments

Seeds of tobacco (Ncotiana rustica L.) plants were surface-sterilized using 1% active hypochlorite and germinated on perlite in dark and moistened by distilled water. After emergence of primary leaves, seedlings were transferred to light and irrigated with 50% Hoagland nutrient solution. Thirty-day-old young seedlings were transferred to hydroponic medium in 10 L plastic pots (five plants per pot) and pre-cultured for 30 days.

One group of plants was supplied with adequate Cu (0.5 µM as $CuSO_4 \cdot 5H_2O$) throughout the experiment until harvest (+Cu plants). The second group was supplied with low Cu (0.125 µM) (Cu-deficient plants,

–Cu plants) for 10 days, and in order to induce deficiency symptoms, Cu was completely eliminated from the nutrient solution during a 20-day pre-culture period.

Thirty days after different Cu nutrition, plants were treated with Cu as nano$[Cu(NH_3)_4]SO_4$ solution (pH 5.8), at 0.5 µM through leaf (foliar application, FA). This treatment was performed gradually on the middle-aged, fully-expanded leaf (treated leaf) within three days to avoid leaf damage. Control plants were sprayed with distilled water.

Plants were grown under controlled environmental conditions with a temperature regime of 25 °C/18 °C day/night, 14/10 h light/dark period, a relative humidity of 50%–60% and at a photon flux density of about 400 µmol m^{-2} s^{-1}.

One week after finishing leaf spray (10 days after starting application treatments, 70 days after sowing), plants were harvested. After determination of the total fresh weight (FW), plants were divided into four different fractions, including treated leaf (Treated L), upper leaves (Upper L, leaves above the treated leaf including new leaves emerged after starting the treatment), lower leaves (Lower L, leaves below the treated leaf) and roots.

Measurement of Photosynthesis rate

The photosynthesis rate (A, µmol CO_2 $m^{-2}s^{-1}$) of the treated leaves was measured before harvest with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) between 10:00 and 13:00. The measurements were conducted with photosynthetically active radiation intensity at the leaf surface of 300–400 µmol $m^{-2}s^{-1}$.

Synthesis of nano Cu complex

One gram of ground $CuSO_4 \cdot 5H_2O$ was added to 25 mL of NH_4OH: water (3:2 v/v) under ultrasonic radiation. In order to decrease the solubility, 15 mL of ethanol was poured to the solution slowly while stirring. After cooling for three hours, the formed dark blue nano crystals ($[Cu(NH_3)_4]SO_4$) was filtered through filter paper using a Büchner funnel and washed with 25 mL of NH_4OH: ethanol (1:1 v/v) solution, rinsed with ethanol and air-dried.

Structural characterization of nano Cu complex

Structural characteristics of the Cu nano-complex were determined using scanning electron microscopy (SEM, TESCAN FEG-SEM MIRA3, Czech Republic) and Fourier-transform infrared spectroscopy (FT-IR, Bruker IR-spectrometer, Tensor 27, Bruker, Germany). According to the SEM image, ($[Cu(NH_3)_4]SO_4$) nanoparticles were spherical in shape (Fig. 1). The FT-IR spectra of ($[Cu(NH_3)_4]SO_4$) nanoparticles, confirmed the existence of the asymmetric and symmetric stretching, degenerate distortion, symmetric distortion, and rocking vibrations of the coordinated NH_3 in the regions of 3,488, 3,383, 1,626, 1,373 and 931 cm^{-1}, which are lower than those of free NH_3 molecules (Fig. 1). In addition, after coordination, the N–H bond of NH_3 is weakened and the stretching frequencies of coordinated NH_3 are shifted to lower frequencies due to the formation of Cu–N bonds which can be detected by a peak at 478 cm^{-1}. Moreover, the two characteristic peaks at 1,201 and 641 cm^{-1} can be attributed to stretching and deformation of S–O bonds in SO_4^{2-} ions [19-20].

Determination of Cu and Fe concentration

Leaf and root samples were dried at 70 °C for two days and, after determination of dry weight (DW), were transferred to porcelain crucibles and dry-ashed at 550 °C for 5 h, resolved in 0.5 M HCl and made up to volume with double-distilled water. Copper and Fe concentrations were determined by atomic absorption spectroscopy (AA6300, Shimadzu, Japan).

Biochemical analyses

Activity of Cu-containing enzymes: superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed using mono formazan formation, and one unit was defined as the amount of enzyme required to induce a 50% inhibition of reaction compared with control samples without enzyme aliquot [21]. Activity of polyphenol oxidase (PPO, EC 1.10.3.1) was determined by following the change in the absorbance at 495 nm due to oxidation of caffeic acid and the extinction coefficient of 2062 M^{-1} cm^{-1} [22]. Diamine oxidase(DAO, EC 1.4.3.6) activity was assayed through spectrophotometric determination of Δ-pyrroline at 430 nm using putrescine as substrate and the extinction coefficient of 1860 M^{-1} cm^{-1} [23].

Activity of Fe-containing enzymes: polyamine oxidase (PAO, EC 1.4.3.4) was estimated based on the colorimetric assay of Δ-pyrrolin at 430 nm using spermidine as a substrate [24]. Peroxidase (POD, EC 1.11.1.7) activity was determined using guaiacol as substrate [25].

Concentration of phenolic compounds and metabolism

Phenylalanine ammonia lyase (PAL, EC 4.3.1.24) activity was assayed as the rate of conversion of L-phenylalanine to trans-cinnamic acid using its extinction coefficient of 9630 M^{-1} cm^{-1} [26]. Folin–Ciocalteau reagent was used for determination of phenolics using

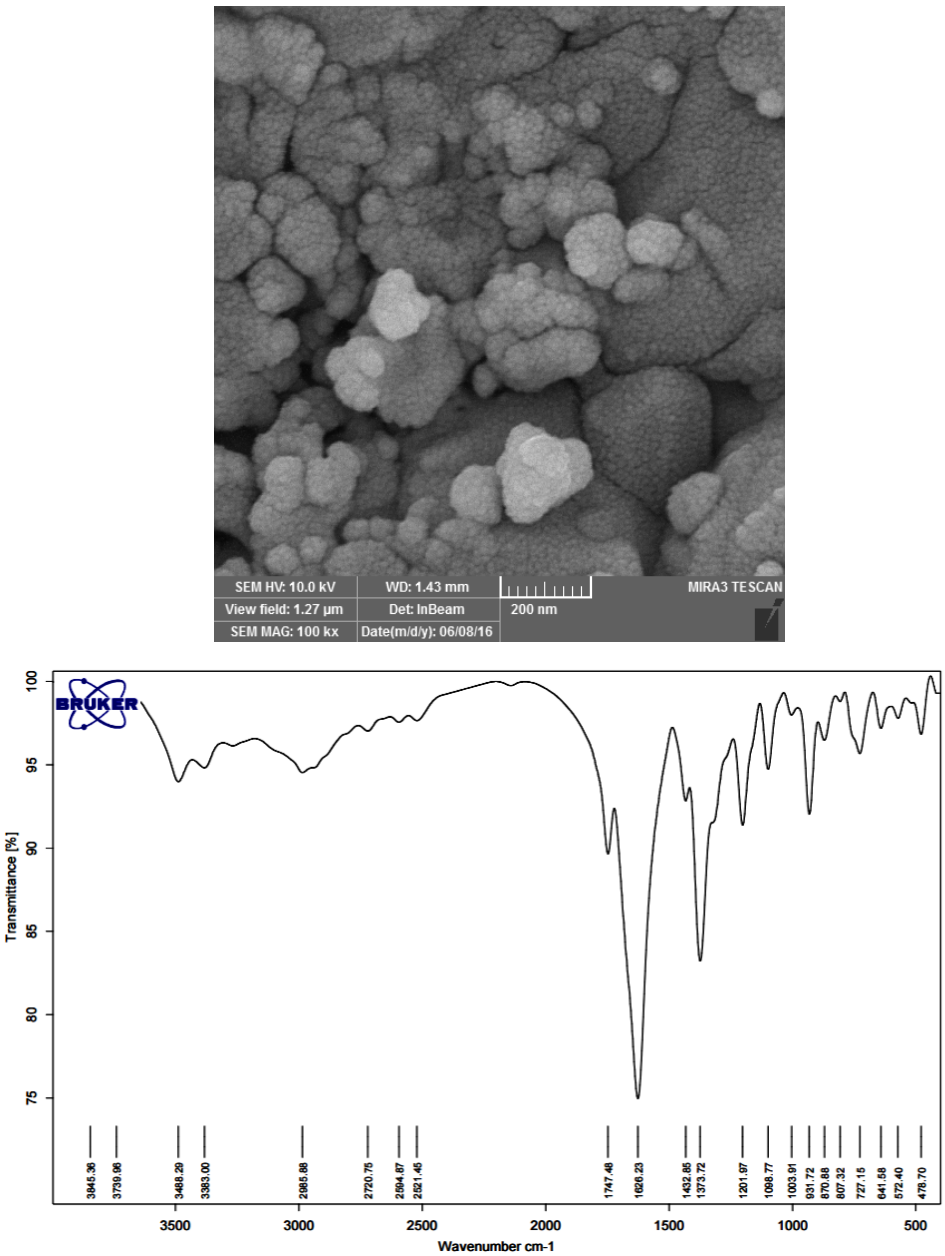

Figure 1. Scanning electron microscopy (SEM) image (upper panel) and Fourier-transform infra-red (FT-IR) spectrophotometry (lower panel) of the synthesized compound.

gallic acid as the standard [27]. Lignin was extracted according to Brinkmann et al. [28]. Lignin content in the extract was determined using acetyl bromide method by measuring of absorbance at 280 nm using the specific absorption coefficient value 8.4 L g^{-1} cm^{-1} [29].

Statistical analyses

The experiment was undertaken in complete randomized block design with four independent replications. Statistical analyses were carried out using Sigma Stat (3.02) with Tukey test ($P < 0.05$).

Results

Copper deficiency decreased shoot and root biomass of tobacco plants. Application of NCu to the –Cu plants improved both shoot and root biomass while the shoot

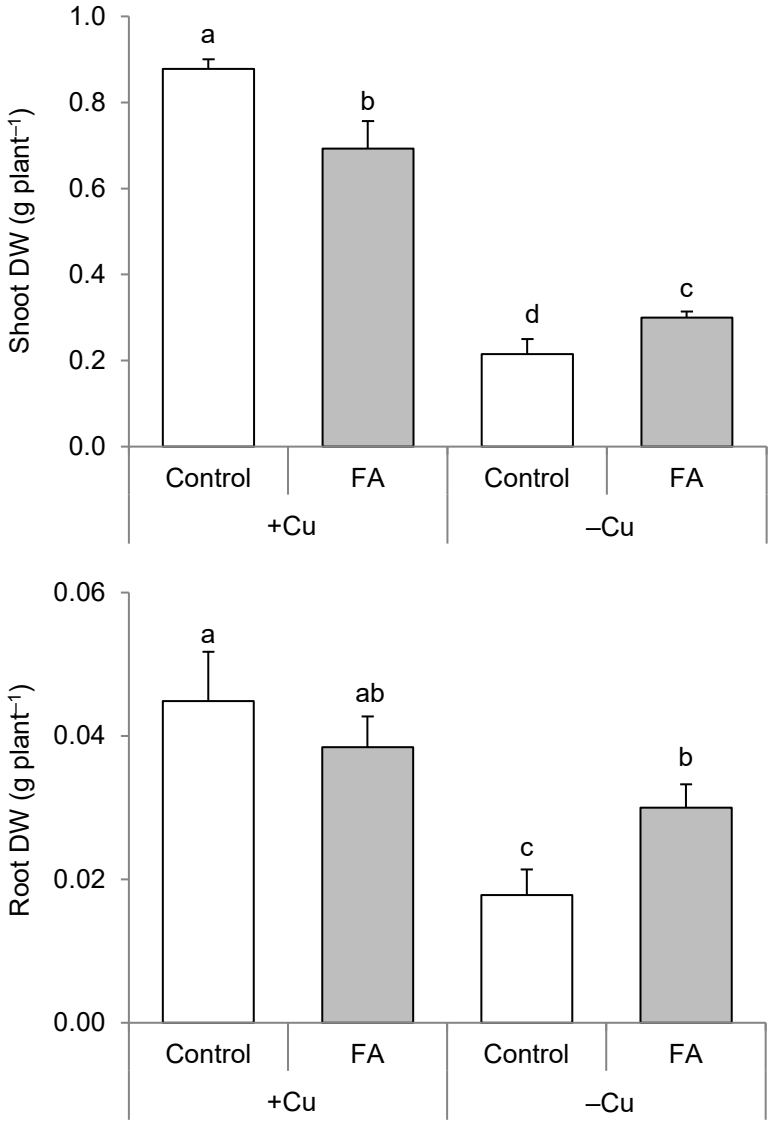

Figure 2. Biomass of tobacco (*Nicotiana rustica*) plants grown under sufficient Cu supply (+Cu) or deficient supply (–Cu), and were sprayed with distilled water (Control) or with nano Cu complex ($[Cu(NH_3)_4]SO_4$) through leaves (FA). Differences among data indicated by different letters are statistically signifcant ($P<0.05$).

biomass was significantly repressed by NCu application in +Cu plants (Fig. 2).

The net photosynthesis rate decreased under Cu deficiency conditions. A significant effect, however, was observed in the middle-aged leaf (Treated L). Application of NCu to the leaves of –Cu plants improved significantly the leaf photosynthesis rate (Fig. 3). In contrast to the effect on biomass, NCu application did not reduce the photosynthesis of Upper L and Treated L in the +Cu plants and rather increased it in the Lower L (Fig. 3).

Copper deficiency reduced the levels of SOD activity; this effect was significant in the Upper L and Lower L. Application of NCu increased the SOD activity not only in the –Cu plants but also in the +Cu ones that was significant in the Upper L (Table 1).

In the Upper L and Treated L, –Cu plants showed lower PPO activity than +Cu ones. This parameter, however, was rather higher in the –Cu plants compared with +Cu ones in the Lower L and remained unchanged in the roots. Foliar application of Cu resulted in an increase in the PPO activity of –Cu plants that was

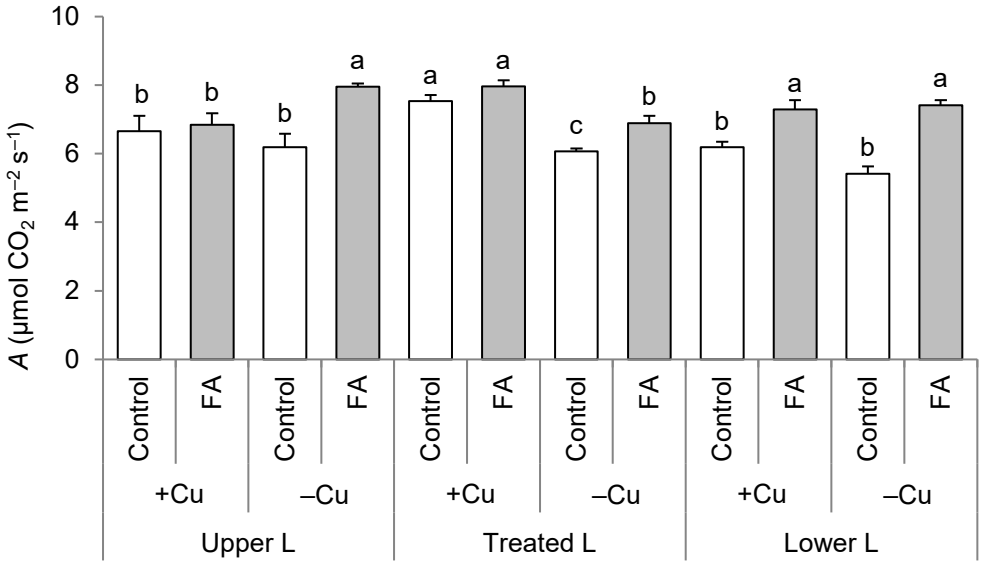

Figure 3. Net photosynthesis rate (*A*) in tobacco (*Nicotiana rustica*) plants grown under sufficient Cu supply (+Cu) or deficient supply (–Cu), and were sprayed with distilled water (Control) or with nano Cu complex ($[Cu(NH_3)_4]SO_4$) through leaves (FA). Differences among data of each organ indicated by different letters are statistically signifcant (P<0.05).

Table 1. Activity of superoxide dismutase (SOD), polyphenol oxidase (PPO) and diamine oxidase (DAO) in the middle-aged (treated) leaf (Treated L), the leaves located above (Upper L) and below (Lower L) it and in the roots of tobacco (*Nicotiana rustica*) plants grown under sufficient Cu supply (+Cu) or deficient supply (–Cu), and were sprayed with distilled water (Control) or with nano Cu complex ($[Cu(NH_3)_4]SO_4$) through leaves (FA). Differences among data of each organ indicated by different letters are statistically signifcant (P<0.05). n.d. not determined.

Nutritional status	Application treatment	SOD (Unit mg^{-1} protein)			
		Upper L	Treated L	Lower L	Roots
+Cu	**Control**	146±6.8 ^{b}	127±12.3 ^{ab}	104±4.3 ^{ab}	103±4.3 ^{b}
	FA	168±4.2 ^{a}	135±14.4 ^{a}	122±14.1 ^{a}	116±10.3 ^{ab}
–Cu	**Control**	107±8.7 ^{c}	102±15.2 ^{b}	23.2±4.8 ^{c}	100±8.9 ^{b}
	FA	155±8.3 ^{ab}	148±11.8 ^{a}	96±10.4 ^{b}	129±2.7 ^{a}
		PPO (nmol mg^{-1} pr min^{-1})			
		Upper L	Treated L	Lower L	Roots
+Cu	**Control**	631±83 ^{b}	560±27 ^{a}	502±75 ^{c}	1302±183 ^{a}
	FA	320±28 ^{c}	433±33 ^{b}	612±32 ^{c}	679±43 ^{b}
–Cu	**Control**	338±37 ^{c}	423±86 ^{b}	1164±109 ^{b}	1131±238 ^{a}
	FA	894±113 ^{a}	510±54 ^{ab}	1391±104 ^{a}	1457±109 ^{a}
		DAO (pmol mg^{-1} pr min^{-1})			
		Upper L	Treated L	Lower L	Roots
+Cu	**Control**	34.9±2.5 ^{a}	32.2±5.4 ^{a}	27.7±3.8 ^{a}	27.6±8.2 ^{a}
	FA	n.d.	n.d.	n.d.	n.d.
–Cu	**Control**	19.1±2.1 ^{b}	13.9±4.8 ^{b}	12.8±7.7 ^{b}	18.1±2.4 ^{a}
	FA	29.6±5.4 ^{a}	31.1±6.9 ^{a}	19.8±6.5 ^{ab}	20.6±5.9 ^{a}

significant in the Upper L and Lower L. In contrast to SOD, the PPO activity decreased or remained unchanged by NCu application in the +Cu plants (Table 1).

The same effect of Cu deficiency was observed on the activity of DAO, and NCu was effective in the increasing DAO activity that was significant in the Upper L and Treated L (Table 1).

The activity of PAO was lower in the –Cu plants compared with +Cu ones; a significant effect was observed in the Treated L. Application of NCu increased the PAO activity in the –Cu plants while decreased it in the +Cu ones both in the leaves and roots (Table 2). The activity of POD did not differ between

Table 2. Activity of polyamine oxidase (PAO) and peroxidase (POD) in the middle-aged (treated) leaf (Treated L), the leaves located above (Upper L) and below (Lower L) it and in the roots of tobacco (*Nicotianarustica* L.) plants grown under sufficient Cu supply (+Cu) or deficient supply (–Cu), and were sprayed with distilled water (Control) or with nano Cu complex ($[Cu(NH_3)_4]SO_4$) through leaves (FA). Bars indicated by different letters are significantly different ($P<0.05$). n.d. not determined.

Nutritional status	Application treatment	PAO (pmol mg^{-1} protein min^{-1})			
		Upper L	Treated L	Lower L	Roots
+Cu	**Control**	n.d	0.45±0.09 [a]	n.d	0.13±0.10 [ab]
	FA	n.d	0.13±0.05 [b]	n.d	0.03±0.01 [b]
–Cu	**Control**	n.d	0.25±0.03 [b]	n.d	0.05±0.00 [b]
	FA	n.d	0.51±0.07 [a]	n.d	0.19±0.02 [a]
		POD (mmol mg^{-1} protein min^{-1})			
		Upper L	Treated L	Lower L	Roots
+Cu	**Control**	5.34±1.42 [b]	4.68±1.15 [b]	5.69±1.90 [a]	2108±402 [a]
	FA	n.d	n.d	n.d	n.d
–Cu	**Control**	4.98±0.49 [b]	5.48±0.92 [ab]	5.65±0.46 [a]	1280±213 [b]
	FA	7.92±1.28 [a]	6.87±0.82 [a]	4.79±0.92 [a]	2063±338 [a]

+Cu and –Cu plants in the leaves, but similar to PAO, increased by application of NCu in the Upper L and roots (Table 2).

The activity of PAL was hardly influenced by Cu starvation. Application of NCu complex did not also influence the PAL activity in the –Cu plants. In the +Cu plants, in contrast, application of NCu decreased PAL activity that was significant in the Treated L and Lower L (Fig. 4). The phenolics and lignin concentrations decreased upon –Cu conditions. This effect was significant in the leaves of different ages and in the roots except for root phenolics concentration. Application of NCu in the –Cu plants significantly increased the phenolics and lignin concentrations in all analyzed plant fractions. In the +Cu plants, however, phenolics concentration decreased upon NCu application that was significant in the Treated L and Lower L (Fig. 4).

Copper concentration was lower in the –Cu plants compared with +Cu ones, this effect was significant in all analyzed shoot fractions (Fig. 5). Application of NCu to the –Cu plants resulted in a significant increase in the Cu concentration of all analyzed plant fractions (Fig. 5). In the +Cu plants, however, application of NCu caused a significant increase in the Cu concentration only in the Treated L compared to the respective control plants (Fig. 5).

The Fe concentration was decreased under Cu starvation in the Lower L while increased in the roots. Application of NCu increased Fe concentration in the leaves of different age while decreased it in the roots (Fig. 5).

Discussion

Effect of NCu application on the biomass and photosynthesis

Plants dry matter production in this work was expectedly repressed under Cu-deficient conditions that could be the result of disturbances in several cellular and metabolic processes, including electron transport, proton pumping and photosynthesis [1, 4]. Application of NCu to the –Cu plants increased shoot and root biomass significantly, however, the biomass was not completely recovered that could be related to a relatively short exposure (3 days) and recovery (7 days) times before harvest.

Leaf photosynthesis was also restored by NCu application in –Cu plants; this parameter even exceeded that of +Cu control ones in the Upper L and Lower L. Significantly higher photosynthesis rate in the leaves of FA plants confirms a high efficacy of leaf application of NCu in the restoration of photosynthesis likely because of a rapid uptake and incorporation into the Cu-containing macromolecules such as plastocyanin. Penetration of leaf-applied nutrients into the leaf is a complex process and acts as the most limiting step for the efficacy of foliar fertigation [30]. Physico-chemical properties of the spray formulation such as molecular size, solubility, electric charge, pH, surface tension, retention and spreading all play major roles in the efficacy of nutrients uptake by the leaves [30]. Complete restoration of leaf photosynthesis by NCu in our tobacco plants suggests a high leaf penetration ability of this complex. It is noteworthy that the lack of a negative effect of NCu application on the leaf photosynthesis in +Cu plants, contrary to the biomass

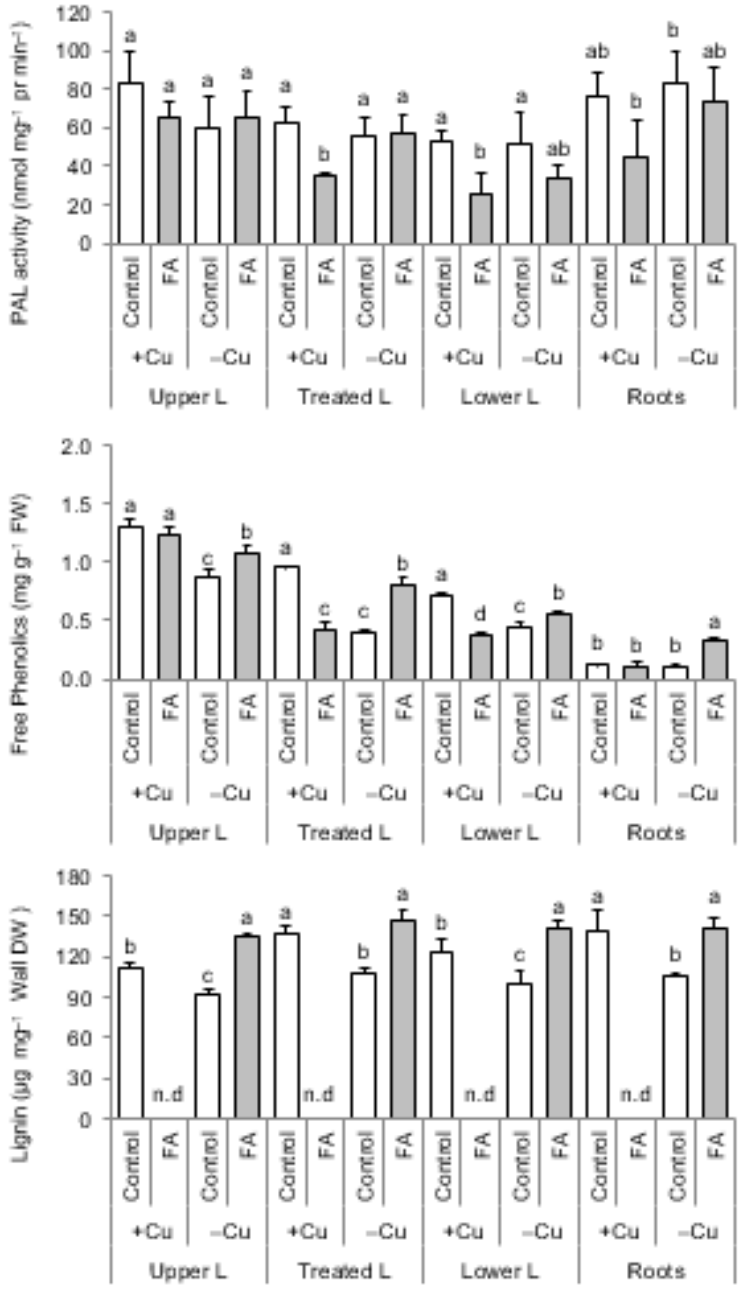

Figure 4. Activity of phenylalanine ammonia lyase (PAL) and concentrations of phenolics and lignin in the middle-aged (treated) leaf (Treated L), the leaves located above (Upper L) and below (Lower L) it and in the roots of tobacco (*Nicotianarustica* L.) plants grown under sufficient Cu supply (+Cu) or deficient supply (–Cu), and were sprayed with distilled water (Control) or with nano Cu complex ($[Cu(NH_3)_4]SO_4$) through leaves (FA). Differences among data of each organ indicated by different letters are statistically signifcant ($P<0.05$). n.d. not determined.

response, could be likely related to higher Cu requirement of photosynthesis *per se* compared with biomass production and/or to a relatively short exposure and recovery time.

Effect of NCu application on the Cu and Fe concentrations

Foliar-applied Cu was readily retranslocated into the

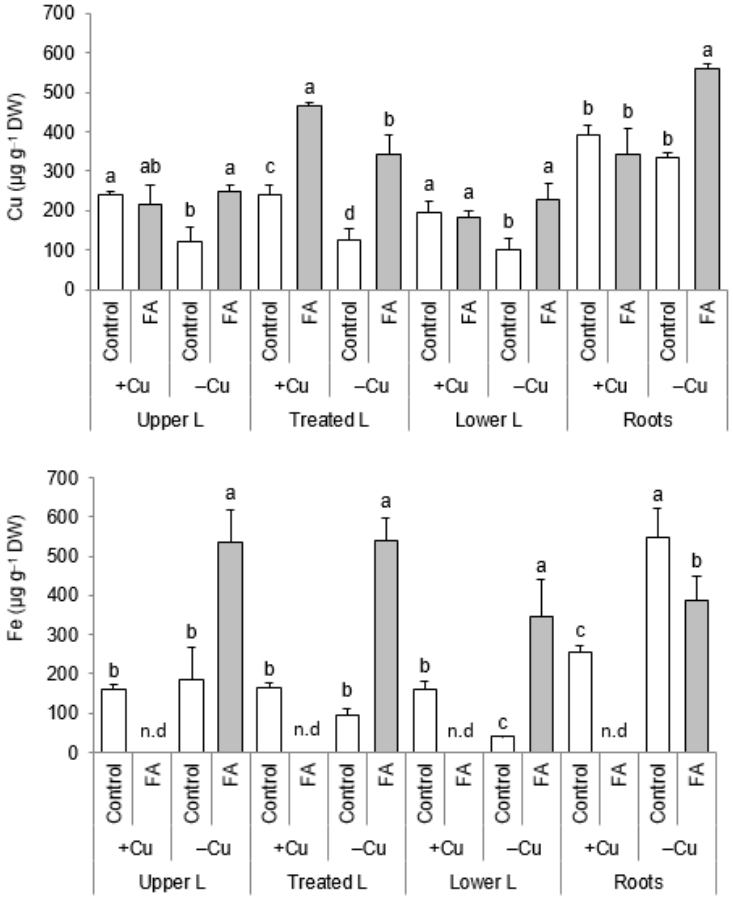

Figure 5. Concentration of Cu and Fe in the middle-aged (treated) leaf (Treated L), the leaves located above (Upper L) and below (Lower L) it and in the roots of tobacco (*Nicotianarustica* L.) plants grown under sufficient Cu supply (+Cu) or deficient supply (–Cu), and were sprayed with distilled water (Control) or with nano Cu complex ($[Cu(NH_3)_4]SO_4$) through leaves (FA). Differences among data of each organ indicated by different letters are statistically signifcant ($P<0.05$). n.d. not determined.

Upper L, Lower L and roots confirmed by significantly higher Cu content in these fractions compared with the respected control plants. In addition, a significant improvement of the activity of Cu-containing enzymes in the leaves not directly treated with NCu, suggests also a rapid re-translocation of applied Cu through phloem to other leaves. Contrary to earlier reports suggesting a poor retranslocation ability for Cu in plants [31], recent investigations showed that Cu is efficiently remobilized at both vegetative [32, 33] and reproductive [34] stages. In this work, a rapid and almost even distribution of Cu among leaves of different ages and roots in the FA treatments confirmed a high phloem mobility of this nutrient in plants.

The Fe concentration was influenced by both Cu nutritional status and application treatment. Reduction of Fe in the –Cu shoots was associated with its accumulation in the roots suggesting an impaired root-shoot translocation of Fe under Cu starvation in our tobacco plants. The occurrence of secondary Fe deficiency in Cu-deficient plants similar with that for other organisms has been documented in *Arabidopsis* [35]. Recently, the involvement of a ferroxidase activity (Fe(II) to Fe(III) conversion) in the delivery step of Fe to the xylem, prior to the formation of the mobile FeIII-citarte complex in the xylem sap, has been postulated [36]. Such ferroxidase activity has been shown to be reduced in Cu-deficient plants because it belongs to one Cu-containing laccase-like enzymatic group [37]. Thus, foliar application of NCu was effective in the increasing

Fe concentration of leaves likely because of restoration of the root ferroxidase activity.

Effect of NCu application on the activity of Cu-containing and Fe-containing enzymes

Foliar application of NCu was successful in the increasing of the activity of Cu-containing enzymes, SOD, PPO and DAO in the –Cu plants. Copper availability is a major factor in the expression of Cu/ZnSOD genes (*CSD1* and *CSD2*) [38]. When Cu supply is insufficient, *CSD1* and *CSD2* and the Cu chaperone, *CCS*, are all downregulated, allowing plastocyanin and other essential Cu proteins to remain active over a wide range of Cu concentrations [38]. Although the molecular pathway of response to Cu deficiency has not been explored for PPO and DAO, there are reports on the reduction of the activity of these enzymes in Cu-starved plants and its restoration by Cu resupply [39].

Despite that PAO and POD are not Cu enzymes, their activity decreased under Cu starvation and increased by FA treatment. Since POD and PAO are Fe-containing enzymes, modifications in their activity by Cu deficiency and resupply is most likely related to the corresponding modifications in the Fe homeostasis by Cu starvation and resupply discussed above.

The mechanism for the inhibitory effect of NCu application in +Cu plants observed on the PPO but not SOD activity, is not known. It may primarily suggest a different susceptibility of these two enzymes to the supra-optimal Cu supply. As an alternative explanation, PPO activity may be indirectly influenced by some factors that are in turn modified by Cu nutritional status. Considering the same negative effect of NCu on the PAL activity and phenolics concentration in +Cu plants, modifications in the formation and/or inter-conversion of phenolics were probably responsible for the observed inhibitory effect of NCu in +Cu plants.

Effect of NCu application on the phenolics and lignin concentration

The most conspicuous effect of NCu in this work was its effect on the phenolics metabolism and lignin. The lignin biosynthesis pathway can be divided into two parts. The first is the general phenylpropanoid pathway that is started by PAL and generates precursors for synthesis of lignin monomers (monolignolalcohols). The second step is the oxidation of the monolignol molecule that is catalyzed by one or more PODs, i.e. Fe-containing enzymes that require H_2O_2 for their reactions and/or laccases, i.e. a PPO type Cu-containing enzyme [40]. Diamine oxidases and PAO that oxidize amines (putrescine, spermidine) reduce O_2 to H_2O_2 and provide H_2O_2 for lignin biosynthesis [24]. Thus, lower lignin concentration in the –Cu tobacco plants in our work, was the result of reduction in the activity of related enzymes and limitations in the phenolics substrate and H_2O_2 under these conditions. Our data showed that leaf application of NCu complex is able to restore all these parameters and the FA plants had similar or even higher lignin concentration only after 3 days of leaf application. Lignification is needed for plant defense, structural rigidity and for differentiation of xylem thus water and solutes transport into the shoot [41].

Conclusion

Our data showed that leaf application of nano tetraammine copper (II) sulfate complex ($[Cu(NH_3)_4]SO_4$) is capable to be absorbed by the leaves and retranslocated through phloem. Other nano Cu compounds, such as nano Cu-EDTA complex, need to be tested for their xylem and phloem mobility. This work is important from a practical point of view. Foliar application of nutrients has the advantage over the soil-incorporated fertilizers, because leaf fertigation is not affected by soil components, precipitation, adsorption onto soil surfaces or risk of loss by erosion. To avoid any risk to human health and the environment, however, the fate of nano fertilizers applied to the leaves or soil, needs to be thoroughly investigated.

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