



Differential effects of copper sulfate (CuSO_4) and copper tetraamine sulfate complex ($[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$) on Cu-enzyme activities, Cu and Fe levels, and lignin deposition in Cu-deficient tobacco (*Nicotiana rustica*) plants

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Abstract

Knowledge of the effect of different copper (Cu) chemical forms and their application mode on meeting the plant's Cu requirement is much more limited than that of other micronutrients. In this work, hydroponically-grown tobacco (*Nicotiana rustica* L.) plants were pre-cultured for thirty days under Cu-sufficient and Cu-deficient conditions, then the Cu-deficient plants were resupplied with 0.5 μM CuSO_4 (CuSu) or Cu tetraamine sulfate complex ($[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$) (CuAm) through roots or leaves. The biomass of plants was resumed almost equally by both chemical forms of Cu. Cu's leaf and root concentrations exhibited a more pronounced response to CuSu application. In contrast, the restoration of the activities of Cu-containing enzymes (superoxide dismutase, polyphenol oxidase, diamine oxidase) was either similar to or, in some cases, even higher when CuAm was applied. The leaf iron concentration was also diminished under Cu starvation and increased by Cu resupply more effectively by CuAm. The activity of phenylalanine ammonia-lyase and peroxidase, phenolics accumulation, and lignin deposition was significantly influenced by Cu deficiency and resupply. Foliar-applied CuAm and root-applied CuSu were the most effective treatments for the resumption of lignin concentration. Results showed efficient re-translocation of foliar-applied Cu. However, the activity of defense enzymes and lignin content suggests that foliar spraying likely induced mechanical stress in the leaves. Furthermore, the effect of the accompanying ion (NH_4^+) was likely the mechanism for the superior effect of CuAm in the induction of structural strength through lignin deposition and improvement of Fe uptake in plants compared with CuSu.

Keywords Cell wall-bound phenolics · Copper-containing enzymes · Copper–iron interaction · Diamine oxidase · Lignin · Polyphenol oxidase

Introduction

Copper (Cu), a redox-active transition metal, is an essential nutrient for higher plants and is involved in many physiological processes, including photosynthesis, respiration, ethylene perception, antioxidant defense, cell expansion, and cell wall lignification (Cakmak et al. 2023). Copper ions act as cofactors in several proteins and enzymes,

such as plastocyanin, Cu/Zn superoxide dismutase (SOD), cytochrome c oxidase, diamine oxidase, and polyphenol oxidase (Cakmak et al. 2023). Polyphenol oxidase (PPO) refers to three different enzymes, including catecholase (EC 1.10.3.2), laccase (EC 1.10.3.1), and cresolase (EC 1.14.18.1). These enzymes contain a dinuclear Cu center, act in the metabolic processes of phenolic compounds derived from the shikimate pathway, including anthocyanins and lignin biosynthesis, and are involved in cell wall modeling (Aniszewski et al. 2008).

Inadequate supply of Cu to plants leads to stunted growth, leaf chlorosis and impaired photosynthesis, wilting (Kopsell and Kopsell 2007) and inhibition of lignification (Cakmak et al. 2023). Molecular genetic studies showed that the status of Cu in plant tissues is sensed by SQUAMOSA promoter

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binding protein-like 7 (SPL7) that contains Cu-response elements and is active under low Cu availability (Yamasaki et al. 2009). SPL7 is required for the expression of multiple Cu-microRNAs, *miR397*, *miR398*, *miR408*, and *miR857*, that are involved in the regulation of genes encoding Cu transporters, Cu chaperone, and Cu proteins, indicating their central role in Cu homeostasis (Shahbaz and Pilon 2019; Mir et al. 2021).

Copper deficiency often occurs in soils rich in organic matter, sandy-textured, excessively leached soils, and in alkaline and saline soils with high amounts of carbonates (Kopsell and Kopsell 2007). Like other nutrient deficiencies, applying fertilizers to meet plant Cu demand is a common agricultural practice in crop production. However, in contrast to micronutrients such as Fe, limited information is available on the different behaviors of various Cu compounds in soil.

The Cu fertilizers used in agricultural soils differ in physical state, chemical reactivity, plant availability, and cost. Commonly used fertilizers for meeting plants demand are Cu sulfate (CuSO_4), Cu-EDTA (Cu-ethylenediaminetetraacetate), Cu acetate ($\text{Cu}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$), Cu oxychloride ($\text{Cu}_2(\text{OH})_3\text{Cl}$), cupric oxide (CuO), and cuprous oxide (Cu_2O) (Malhi et al. 2005; Malhi and Karamanos 2006). Although the highest quantities of Cu in labile forms in the soil and total Cu uptake in plants are achieved upon application of Cu chelates such as Cu-EDTA (Obrador et al. 2013), Cu sulfate is the preferred source of Cu fertilizer because of its low cost compared to chelated fertilizers (Malhi and Karamanos 2006). In hydroponic studies, CuSO_4 is also the primary form of Cu used in almost all physiological studies. Copper tetraamine sulfate ($[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$) is a water-soluble Cu complex that is dissociated in aqueous solution at $\text{pH} < 6.0$ and releases Cu^{2+} and ammonia. This compound is closely related to Schweizer's reagent (tetraamine Cu dihydroxide, $[\text{Cu}(\text{NH}_3)_4(\text{H}_2\text{O})_2](\text{OH})_2$) that is used in purifying cellulose and the production of cellulose fibers (Burchard et al. 1994). There is no information on the uptake and utilization of $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ in plants except a work undertaken with foliar applied nano form of $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ (Bahrami-Rad et al. 2022). However, the effect of the bulk form of this compound and its difference with the most widely used Cu form (CuSO_4) has not been investigated so far.

Copper and Fe share similarities in their cellular functions, such as redox activity, scavenging reactive oxygen species (ROS) through related SOD isoforms (CuSOD or FeSOD), and dependence on a reduction step before uptake. It may be suggested that there could be some links in plant responses to Cu and Fe deficiencies. However, reports on the effect of Cu deficiency on Fe uptake and shoot–root transport are contradictory. Available data show that Cu-deficiency either increases (Waters and McInturf 2014), decreases (Carrió-Seguí et al. 2019a), or does not change the Fe uptake

(Waters and Armbrust 2013), or even differently influences Fe content in the leaves and roots (Perea-García et al. 2020).

Foliar nutrition is an essential method of fertilization with many advantages over root fertilization such as rapid absorption of mineral nutrients without probability of losing through erosion, adsorption onto soil minerals or precipitation (Fernández and Brown 2013). Foliar nutrients usually penetrate through leaf stomata and hydrophilic pores within the leaf cuticle and then enter the cells. Depending on plant species and environmental conditions, differences in stomata size and variable extent of stomatal opening significantly affect the penetration of fluids applied to leaf surfaces (Eichert and Fernández 2023). Foliar application is recommended for micronutrients because they are needed in lower quantities and are more prone to sequestration by soil organic and inorganic molecules, leading to decreased plant-available fractions (Niu et al. 2021). Because Cu deficiency in crops often occurs in irregular patches within fields, foliar application has been suggested as the most practical and economical way to correct Cu deficiency during the growing season (Malhi et al. 2005).

In contrast to Fe, reports on the effect of foliar application of Cu are minimal, and information is scarce on the difference among various Cu compounds in their efficacy for meeting plants' Cu demand. Most studies on foliar Cu fertilization have been undertaken under field conditions on plants with unknown Cu nutritional status, leading to either higher yield (Barbosa et al. 2013) or no effect on plant yield and Cu content (Korzeniowska and Stanisławska-Głubiak 2011). In recent years, nano Cu compounds such as metal CuO or $\text{Cu}(\text{OH})_2$ have also been included in foliar application studies and provided evidence on the activation of plant defense and enhancement of secondary compounds (Lafmejani et al. 2018; Elmer et al. 2021; Nekoukhou et al. 2023; López-Luna et al. 2023; Kusiak et al. 2023). However, because of the insoluble and colloidal nature of nano compounds and their unknown and different behavior than bulk compounds in the solutions and plants (Hong et al. 2021), and due to the application of high amounts of nano Cu compounds ($400\text{--}1500 \text{ mg L}^{-1}$), data of such studies must be explained cautiously from the perspective of plant nutrition. In addition, these studies have not defined the used plants as Cu starved. Hence, the observed responses are unrelated to meeting plants' Cu demand, and these studies could not be relevant to plants' Cu nutritional physiology. To the best of knowledge, no report compares different Cu compounds or between foliar application with root fertilization in Cu-deficient plants.

Tobacco (*Nicotiana* spp.) is the world's most crucial non-food crop species and is produced in more than 100 countries under various climatic conditions with different soil types (FAO 2023). To investigate the efficacy of CuSO_4 and $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ for meeting plants' Cu requirement

and to compare the foliar application with root fertilization, Cu-starved tobacco plants were resupplied with either of these compounds through root or leaf. Biomass production, Cu and Fe uptake, activity of Cu enzymes and lignification were studied in the Cu-resupplied plants. We hypothesized that these two Cu forms differ in uptake and distribution within plants and in meeting plants' nutritional requirements due to distinct chemical behaviors in solution and within plants and differing accompanying ions.

Materials and methods

Plant culture and treatments

Tobacco seeds (*Nicotiana rustica* L.) were surface sterilized using 1% active hypochlorite, germinated on perlite in the dark, and moistened with distilled water. After the emergence of primary leaves, seedlings were transferred to light and irrigated with 50% Hoagland nutrient solution.

Thirty-day-old young seedlings of similar size were transferred to 10 L plastic containers (five plants per container) filled with 100% nutrient solution and pre-cultured with two levels of Cu, including adequate (+Cu) and low (–Cu) Cu treatments. +Cu plants were supplied with 0.5 μM Cu (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) throughout the experiment until harvest, while –Cu plants were supplied with low Cu (0.125 μM) for 10 days, and to induce deficiency symptoms, Cu was eliminated from the nutrient solution during the further 20-day pre-culture period.

Thirty days after different Cu nutrition in the pre-culture medium, Cu-deficient plants were randomly assigned to five application treatments. The first group was left untreated and served as the control group (–Cu), while four other groups were treated with Cu either as CuSO_4 (CuSu) or Cu tetraamine sulfate complex ($[\text{Cu}(\text{NH}_3)_4]\text{SO}_4 \cdot \text{H}_2\text{O}$) (CuAm) solutions (pH 5.8), both at 0.5 μM through the leaf (leaf application, LA) or root (root application, RA). LA was performed gradually on the young, fully expanded (middle-aged) leaf within three days to avoid damage. Six different treatments were applied to plants, and each treatment consisted of four independent containers as four replicates.

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

Plants harvest

Plants were harvested ten days after starting leaf or root application treatments (70 days after sowing). Whole shoots and roots were excised separately, rinsed with

deionized water, and blotted dry on filter paper. The shoots were divided into three different fractions, including middle-aged leaves (ML), upper leaves (UL, leaves above the ML including new leaves that emerged after starting the treatment), and lower leaves (LL, leaves below the ML).

Measurement of photosynthesis rate

The photosynthesis rate (A , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) of the middle-aged leaf was measured 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 $\mu\text{mol m}^{-2} \text{ s}^{-1}$.

Biochemical analyses

The activity of Cu-containing enzymes: superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed using monoformazan 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 (Sarker and Oba 2018). The polyphenol oxidase activity (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 $\text{M}^{-1} \text{cm}^{-1}$ (Kampatsikas et al. 2019). Diamine oxidase (DAO or CuAO, EC 1.4.3.6) activity was assayed through the spectrophotometric determination of Δ -pyrroline at 430 nm using putrescine as substrate and the extinction coefficient of 1860 $\text{M}^{-1} \text{cm}^{-1}$ (Asthir et al. 2002).

The activity of phenolics metabolizing enzymes: 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 $\text{M}^{-1} \text{cm}^{-1}$ (Hajiboland et al. 2017). An assay of peroxidase (POD, EC 1.11.1.7) was performed using guaiacol as substrate (Sarker and Oba 2018). The soluble protein concentration was determined using Bradford reagent and bovine albumin serum (BSA) as standard.

The concentration of phenolic compounds and lignin: Water-soluble and cell wall-bound phenolics were extracted according to the previously described method (Hajiboland et al. 2017). Folin–Ciocalteu reagent was used to determine phenolics using gallic acid as the standard (Blainski et al. 2013). Lignin was extracted and determined using the acetyl bromide method by measuring absorbance at 280 nm using the specific absorption coefficient value 8.4 $\text{L g}^{-1} \text{cm}^{-1}$ (Brinkmann et al. 2002).

Estimation of Cu concentration in the root symplast and apoplast

A washing procedure described by Zhou et al. (2007) was employed to determine Cu concentration in the root symplast and apoplast. For this purpose, the $-Cu$ plants were supplied either with 1.0 μM CuSu or CuAm through roots for 48 h. Concentration data of unwashed and EDTA-washed roots were considered total Cu and symplastic Cu, respectively. The difference between total and the symplastic Cu estimated the apoplastic Cu. Four independent plants as four replicates were used for this analysis.

Determination of Cu and Fe content

Leaf and root samples were dried at 70 °C for two days and, after determination of dry weight, 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).

Microscopic observation of the leaves

Samples of 5 mm around leaf midribs were excised and immediately fixed in formalin-acetic acid-alcohol (FAA) and stored at 4 °C until sectioning. After preparation of free hand sections, samples were washed with distilled water and placed in 5% sodium hypochlorite solution for 20 min for clearing and rinsed with distilled water. After washing in 10% acetic acid for 2 min, sections were stained with methyl green for lignin identification. Sections were embedded and mounted in glycerol. Thin cut sections were observed under a microscope fitted with a digital camera attached to a computer.

Statistical analyses

The experiment was undertaken in a complete randomized block design with six different treatments and four independent replications for each treatment. Statistical analyses were carried out using Sigma Stat (3.02) with the Tukey test ($P < 0.05$).

Results

Stunted growth in the Cu-deficient plants was observed 2–3 days after the complete elimination of Cu from the nutrient solution, while yellowing of the young leaves was observed later, about 5–7 days after the appearance of visual differences in shoot growth between Cu-sufficient

and Cu-deficient plants. Cu-deficient plants showed 63 and 59% reduction in the shoot and root dry biomass at harvest compared with Cu-sufficient plants, respectively. The application of Cu significantly improved the shoot biomass without the difference between the two Cu forms. Leaf treatment, however, was significantly more effective than root treatment in the resumption of shoot growth (Fig. 1A). Root growth, in contrast, was significantly improved only by leaf application of Cu without difference between two Cu compounds (Fig. 1B).

The photosynthesis rate was decreased by 56% under Cu-deficient conditions, and applying both Cu compounds restored this parameter significantly. CuSu was more effective than CuAm in restoring leaf photosynthesis. However, a significant difference between the two Cu forms was observed only in the RA plants (Fig. 1C).

The activity of SOD decreased by Cu deficiency; this effect was significant in the UL, LL, and in the roots. Resupply of Cu through leaves significantly increased the leaf SOD activity while did not affect this parameter in the roots. CuAm was more effective in improving the SOD activity of ML and LL compared with CuSu. In contrast to leaf application, resupply of Cu through the roots did not significantly influence the SOD activity or only led to a slight increase (Fig. 2A).

The activity of PPO was significantly decreased by Cu deficiency and resumed by its resupply in the UL and ML. Like SOD, LA was more effective than RA in resuming PPO activity but without a difference between CuSu and CuAm (Fig. 2B).

The effect of Cu deficiency on the reduction of DAO activity was significant in the leaves and roots. Similar to SOD and PPO, the activity of DAO was resumed by Cu resupply. In contrast to SOD and PPO, however, DAO exhibited a more pronounced response to RA than LA, as observed in the UL and ML. Two Cu forms did not differ in the resumption of leaf DAO activity in the LA or RA plants. In the roots, however, CuSu was more effective in the LA plants but less effective than CuAm in the RA ones (Fig. 2C). The constitutive activity of DAO in the LL was lower compared with other shoot fractions, and Cu resupply was not successful in the resumption of its DAO activity either as LA or RA treatment (Fig. 2C).

Activity of PAL was slightly or significantly lower in the $-Cu$ plants compared with $+Cu$ ones. Two Cu compounds differently influenced the PAL activity in the LA and RA treatments. CuSu was the most effective compound in the leaves in the LA, while CuAm was mainly effective in the RA treatment. However, the difference between the Cu compounds was only significant in the UL. In the roots, two Cu compounds did not significantly differ in the resumption of PAL activity (Fig. 3A).

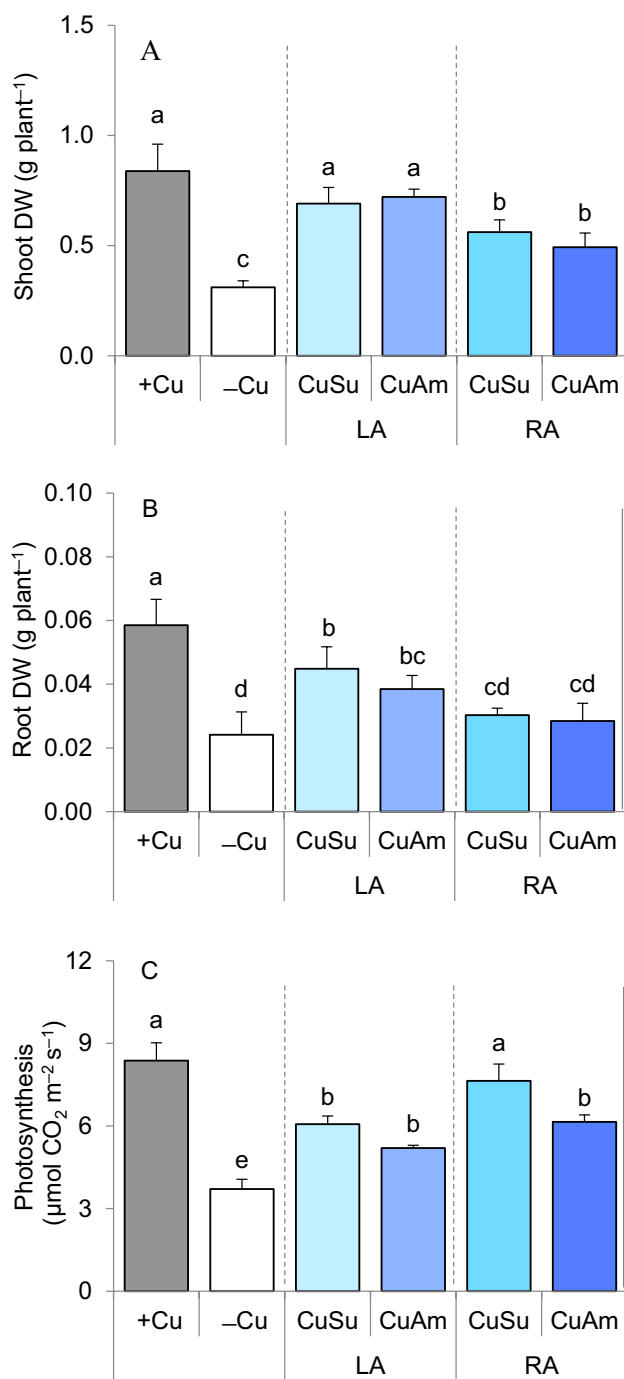


Fig. 1 Shoot and root biomass (A, B) and photosynthesis rate (C) in tobacco (*Nicotiana rustica*) plants grown under Cu deficiency conditions and treated with Cu sulfate (CuSu, CuSO₄) or Cu ammonium sulfate complex ([Cu(NH₃)₄]SO₄, CuAm) through leaves (LA) or roots (RA). Cu-sufficient (+Cu) and Cu-deficient (-Cu) plants were left untreated as control plants. Differences among data indicated by different letters are statistically significant ($P < 0.05$)

The activity of POD was decreased by 22–50% in the -Cu plants, which was significant in the UL, ML, and roots. Copper resupply effectively restored POD activity in the

leaves and roots, except CuAm, which did not influence the leaf POD activity when applied to the roots. In contrast to PAL, the resupply of Cu as CuSu was more effective in restoring leaf POD activity when applied through roots, while CuAm demonstrated superiority in the LA treatment observed in the UL.

In the roots, POD activity was more responsive to the RA with no discernible difference between the two Cu compounds (Fig. 3B).

The concentrations of free phenolics decreased upon Cu deficiency conditions up to 54% in the ML and LL but not in the UL and roots. Copper resupply influenced the free phenolics levels differently depending on the Cu form and application mode. CuSu in the LA plants and CuAm in the RA ones significantly increased the free phenolics concentration in the ML and LL, while root-applied CuSu and foliar spray of CuAm did not influence or decrease the leaf phenolics concentration. In the roots, the concentration of free phenolics was not influenced by Cu deficiency but increased by root application of Cu, particularly by CuAm (Fig. 4A).

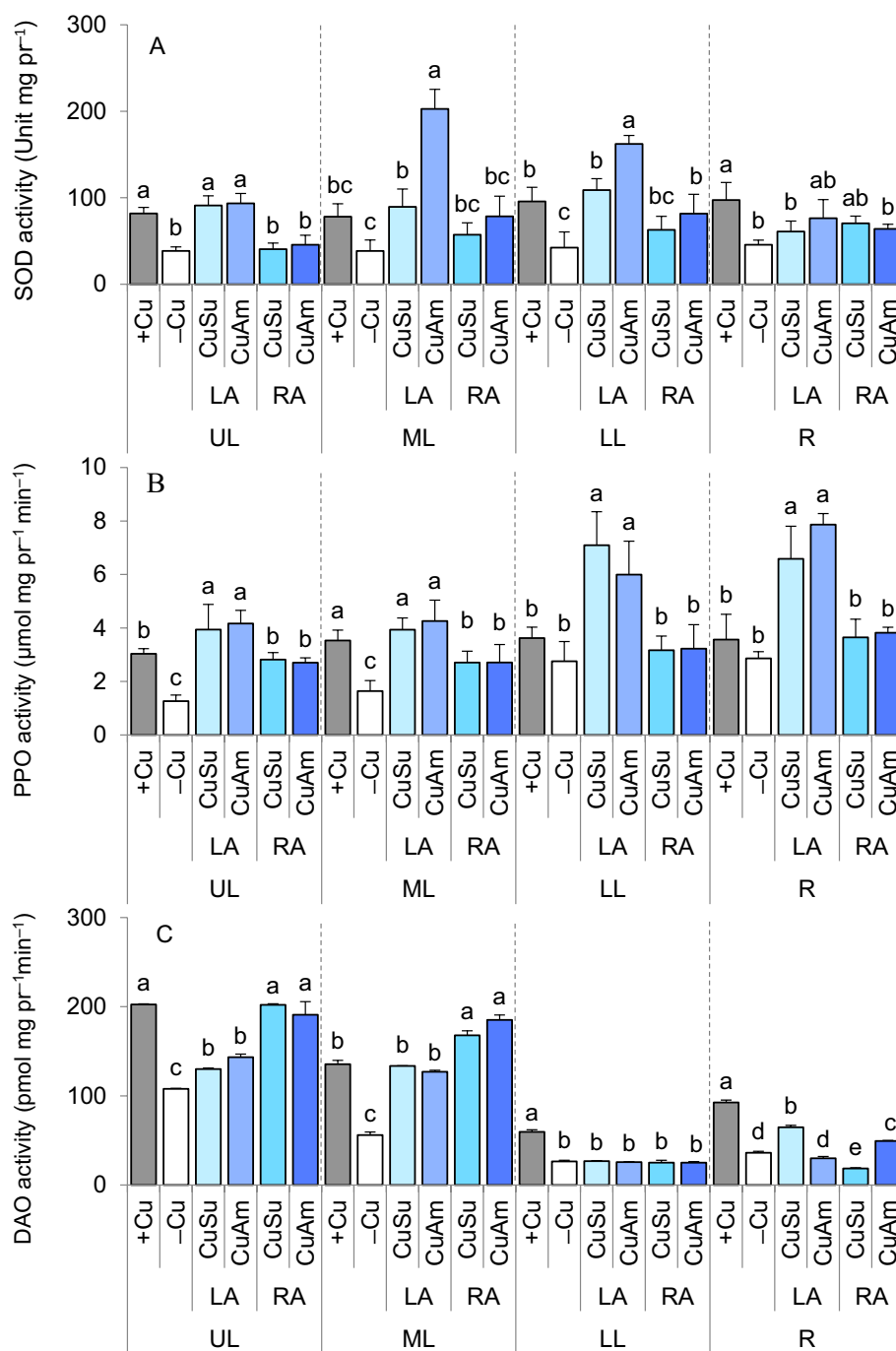
The effect of Cu deficiency on reduced CW-bound phenolics was observed in the leaves of different ages, but a significant effect was found only in the UL. Application of Cu through roots did not significantly affect the CW-bound phenolics in the leaves and roots. The leaf-applied CuAm did not influence the concentration of CW-bound phenolics, while CuSu decreased this parameter, that was significant in the ML, LL, and roots (Fig. 4B).

The leaf and root lignin content significantly decreased under Cu-starvation. Similar to that observed for the free and CW-bound phenolics, the effect of Cu depended on the Cu form and application mode. In the LA plants, CuAm resumed lignin concentration in the leaves and roots, while CuSu did not influence it in the leaves. In the RA plants, in contrast, CuSu was the effective Cu form in restoring the leaf and root lignin level, while CuAm could not resume the lignin concentration in the ML, LL, and roots (Fig. 4C).

Lower lignification of the leaf tracheary elements was evident in the Cu-deficient plants compared with the Cu-sufficient ones (Fig. 5). Lignification in the leaves of RA plants treated with CuSu and that of LA plants resupplied with CuAm complex was almost similar to the +Cu plants. Nevertheless, leaf tracheary elements in plants treated with the CuAm complex as RA and CuSu as LA exhibited significantly reduced lignification, with no substantial difference compared to those in Cu-deficient plants (Fig. 5).

The comparison of six different treatments for the leaf Cu concentration did not show a significant difference between +Cu and -Cu plants. However, a significant reduction of Cu concentration in the UL, ML, and LL was observed by comparing +Cu and -Cu plants (t -test, $P < 0.05$). Application treatments increased the Cu

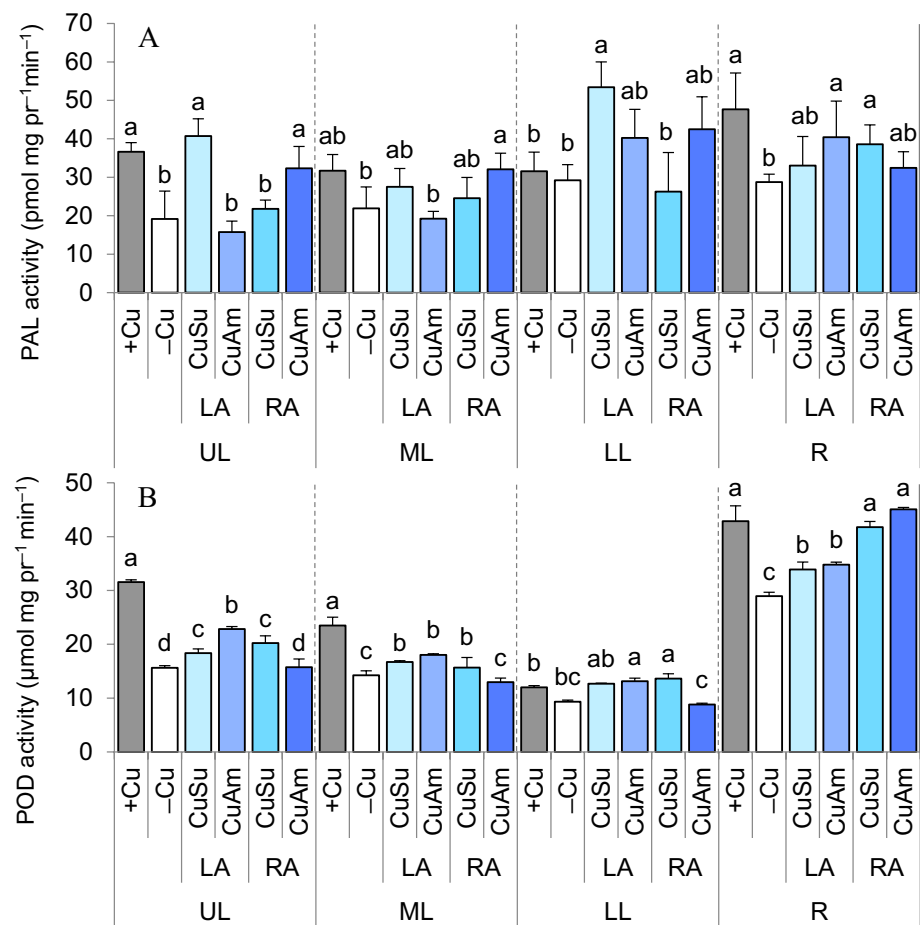
Fig. 2 Activity of superoxide dismutase (SOD) (A), polyphenol oxidase (PPO) (B) and diamine oxidase (DAO) (C) in tobacco (*Nicotiana rustica*) plants grown under Cu deficiency conditions and treated with Cu sulfate (CuSu, CuSO₄) or Cu ammonium sulfate complex ([Cu(NH₃)₄]SO₄, CuAm) through leaves (LA) or roots (RA). Cu-sufficient (+Cu) and Cu-deficient (-Cu) plants were left untreated as control plants. Differences among data of each organ indicated by different letters are statistically significant ($P < 0.05$)



concentration of all analyzed plant fractions. However, a statistically significant effect in the RA plants was found through their comparison with -Cu treatment for both Cu forms in the ML and CuSu in the LL. Leaf application of CuSu was more effective than CuAm in the increasing Cu concentration in both leaves and roots (Fig. 6). When applied to the roots, the impact of CuSu on increasing Cu concentration did not exhibit a significant difference from CuAm or was only slightly higher than CuAm (Fig. 6A).

Leaf Fe concentration in the -Cu plants was similar to or lower than the +Cu plants but increased with Cu resupply. In general, Fe concentration in the leaves of CuAm-fed plants was higher than that in the CuSu-fed ones; however, the difference between the two Cu compounds was significant only for the LA plants. Root Fe concentration, in contrast, increased by Cu starvation and remained higher after Cu resupply except for the effect of CuAm applied to the roots that decreased root Fe concentration (Fig. 6B).

Fig. 3 Activity of phenylalanine ammonia lyase (PAL) (A) and peroxidase (POD) (B) in tobacco (*Nicotiana rustica*) plants grown under Cu deficiency conditions and treated with Cu sulfate (CuSu, CuSO₄) or Cu ammonium sulfate complex ([Cu(NH₃)₄]SO₄, CuAm) through leaves (LA) or roots (RA). Cu-sufficient (+Cu) and Cu-deficient (-Cu) plants were left untreated as control plants. Differences among data of each organ indicated by different letters are statistically significant ($P < 0.05$)



To test the probable differential sequestration of two Cu forms in the root apoplast and their effect on the Cu concentration of aerial parts, Cu in the roots was analyzed without (total) and after washing with EDTA (symplastic Cu) (Fig. 7). The total root Cu concentration was significantly lower when plants were loaded with CuAm solution. In comparison, Cu concentration in the root symplast did not differ between the two Cu forms. The concentration of Cu in the root apoplast was significantly lower in CuAm-supplied plants compared to CuSu-loaded ones (Fig. 7).

Discussion

Cu deficiency symptoms in tobacco plants

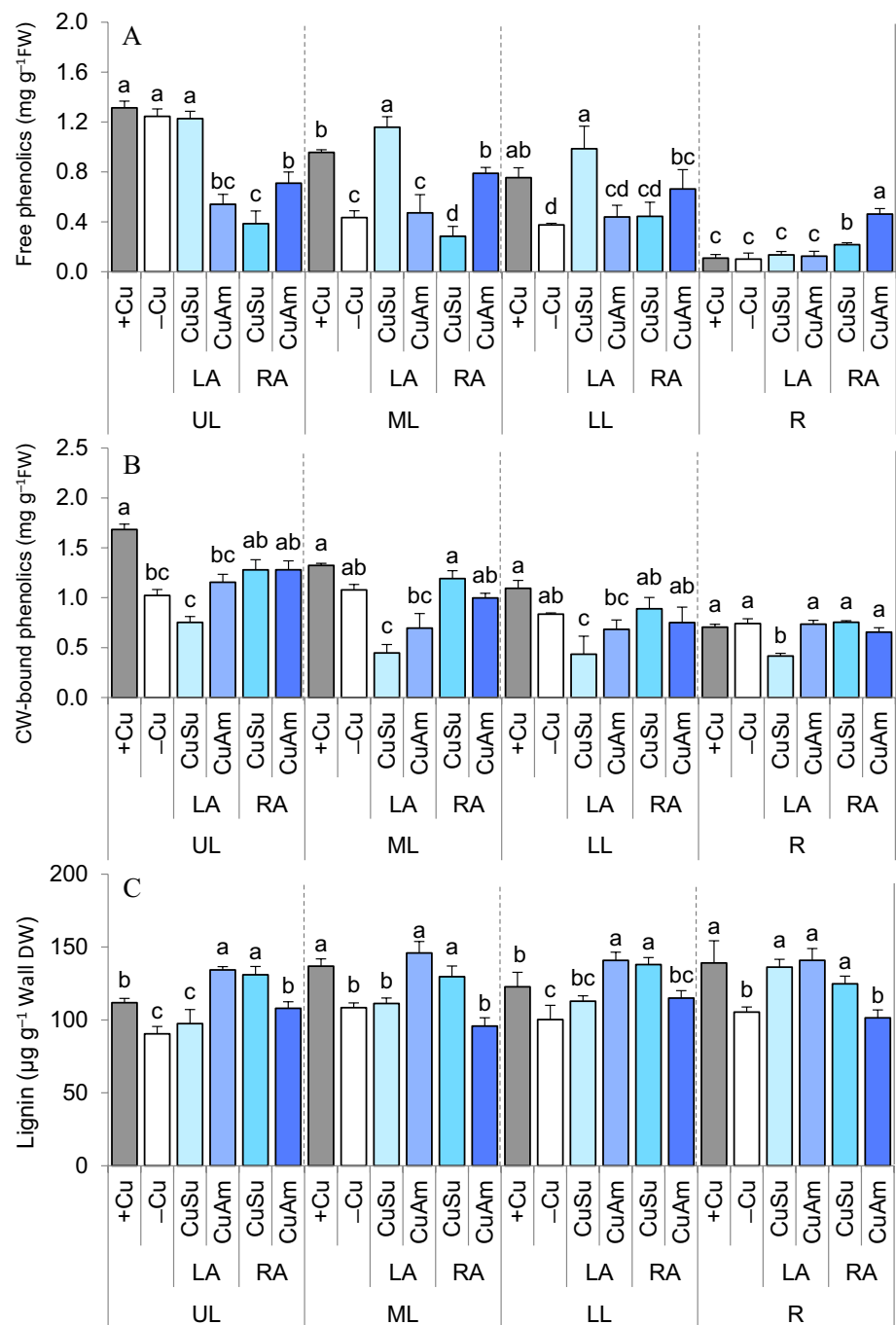
Plants' growth was expectedly depressed by Cu deficiency. Reduction of dry matter production in Cu-starved plants is the consequence of disturbances in many cellular functions, including cell expansion, mitochondrial and plastid electron transport, proton pumping and nitrogen assimilation (Da Silva and Williams 2001; Burkhead et al. 2009; Cakmak et al. 2023).

Leaf photosynthesis is often the most conspicuous effect of Cu starvation, as observed in the tobacco plants in this work. Plastocyanin is one of the most abundant Cu protein in higher plants (Shahbaz and Pilon 2019). The involvement of this protein in the electron transfer between the cytochrome b₆f complex and photosystem I (Burkhead et al. 2009) was likely the mechanism for Cu deficiency-mediated reduction of photosynthesis. Copper may also play a role in thylakoid grana stacking (Burkhead et al. 2009). In addition, Cu is likely involved in synthesizing Fe-S clusters through the SUF (sulfur assimilation proteins) machinery, which is destabilized under Cu deficiency. Leaf proteomics studies revealed an upregulation of ferredoxin and Rieske protein, which contain Fe-S clusters to compensate for a loss of activity under Cu deficiency conditions (Billard et al. 2014).

Difference between two Cu forms and application modes on the biomass and photosynthesis rate

Higher biomass recovery when Cu was applied to the leaves than the plants treated through roots was likely related to the higher efficiency of this treatment in the increasing Cu concentration of different plant parts, compared with the

Fig. 4 The concentration of free (A) and cell wall-bound phenolics (B) and lignin (C) in tobacco (*Nicotiana rustica*) plants grown under Cu deficiency conditions and treated with Cu sulfate (CuSu, CuSO₄) or Cu ammonium sulfate complex ([Cu(NH₃)₄]SO₄, CuAm) through leaves (LA) or roots (RA). Cu-sufficient (+Cu) and Cu-deficient (-Cu) plants were left untreated as control plants. Differences among data of each organ indicated by different letters are statistically significant ($P < 0.05$)

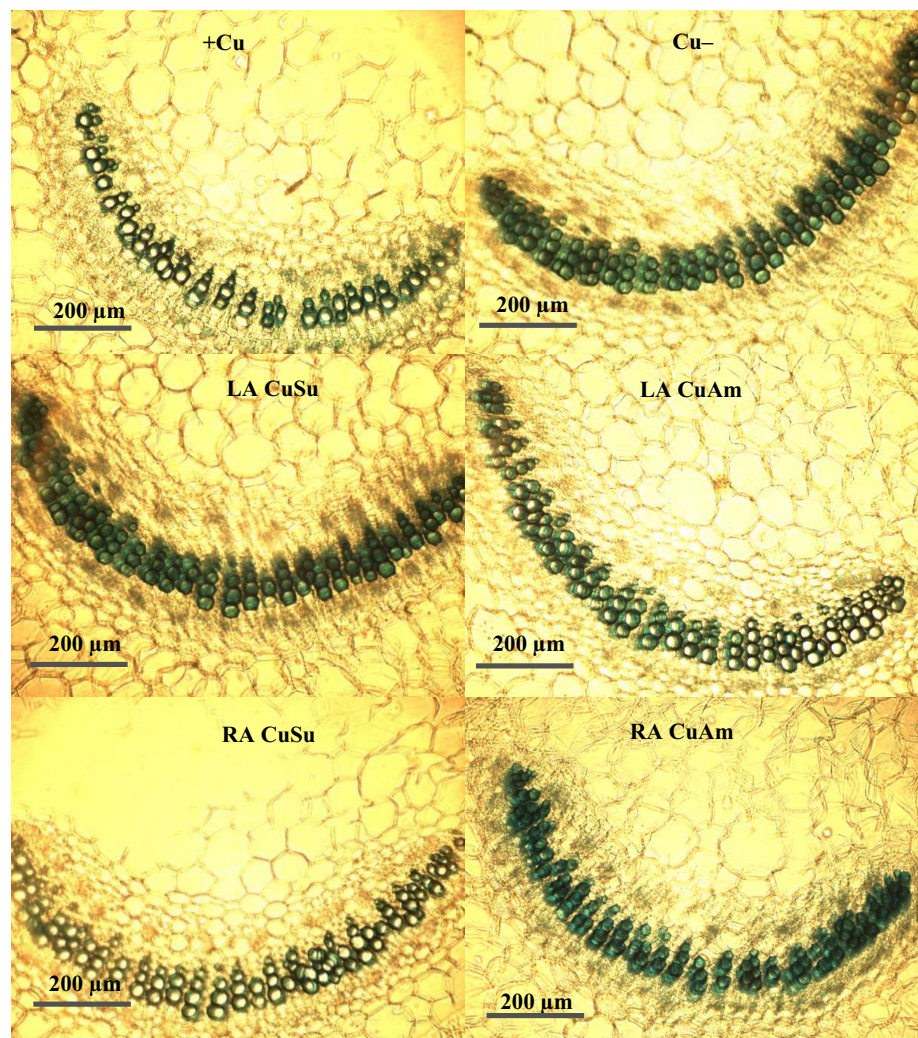


root application treatment (Fig. 6A) that also implied a high remobilization rate of applied Cu to the leaves (see also below).

The superior effect of CuSu over CuAm on the recovery of photosynthesis was well correlated with higher leaf Cu concentration in the CuSu-fed plants compared with the CuAm-fed ones. However, slightly or significantly ($P < 0.05$) higher recovery of photosynthesis in the RA compared with their LA counterparts (Fig. 1) that was observed despite much lower tissue Cu level in the former plants (Fig. 6),

may be likely due to the fulfillment of Cu requirement of photosynthesis with this level of tissue Cu due to an efficient intracellular Cu distribution under these conditions. It has been observed that in Cu-starved plants, the Cu transport across chloroplast membrane is stimulated through the upregulation of P-type ATPase, HMA1 (Billard et al. 2014). Interestingly, *miR408* that coordinates responses to Cu status through the SPL7 module in Arabidopsis (Zhang et al. 2014), is a positive regulator of photosynthesis and its overexpression increases the content of Cu and plastocyanin

Fig. 5 Free-hand cross-sections of leaf petioles in tobacco (*Nicotiana rustica*) plants grown under Cu deficiency conditions and treated with CuSO_4 (CuSu) or $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ complex (CuAm) through leaves (LA) or roots (RA). Cu-sufficient (+Cu) and Cu-deficient (-Cu) plants were left untreated as control plants. Sections were stained with methyl green for identification of lignin



in chloroplasts and induces the expression of photosynthetic genes in various species, including tobacco (Pan et al. 2018).

Considering the irreplaceable role of Fe in photosynthesis, a concomitant increase in the leaf Fe concentration upon Cu resupply (Fig. 6) may also significantly contribute to the photosynthesis recovery of Cu-resupplied plants. Nevertheless, the superior effect of CuSu over CuAm in restoring photosynthesis could only be explained by an efficient increase in the leaf Cu concentration since Fe concentration was more increased by CuAm than CuSu.

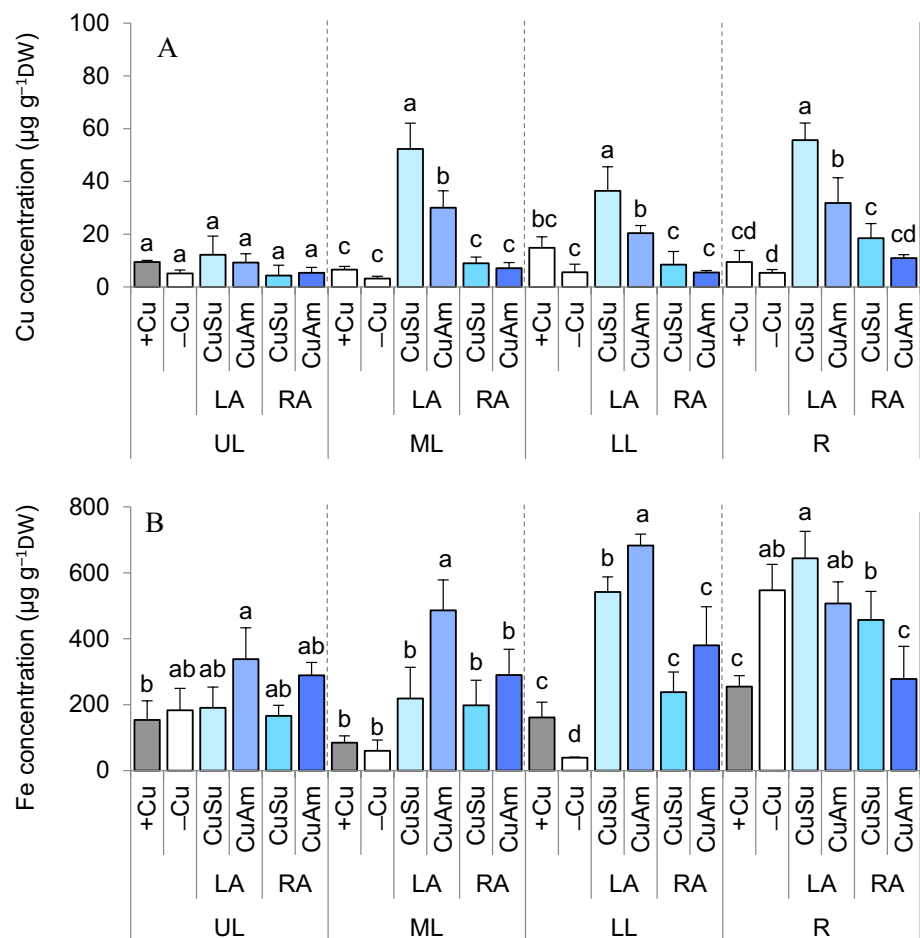
Effect of Cu deficiency and resupply on the Cu concentration

A comparison of two Cu compounds showed the superior effect of CuSu for increasing the Cu concentration of plant fractions compared with CuAm in both LA and RA plants. In the LA plants, this could be explained by lower penetration ability of CuAm through leaf cuticles and/or stomata than CuSu. Such circumstances may lead to the retention of a

significant portion of the applied CuAm, with subsequent removal occurring through surface washing. In the case of dissociation of $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ before penetration into the leaf, a slow release rate of Cu^{2+} from the $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ complex during the short time exposure employed in this work could also be involved in the lower Cu concentration of CuAm-fed plants compared with CuSu ones. Since the exact penetration mode of $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$, i.e., as a free Cu^{2+} ion or as an undissociated complex, is not known, the relative contribution of the mechanisms mentioned above to the lower Cu concentration in the CuAm-sprayed plants is unclear.

In the RA plants, lower root uptake of Cu in the CuAm-supplied plants may be related to the slow release of Cu^{2+} ion from the $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ complex in the nutrient solution because of its tight binding to the nitrogen of NH_3 as its ligand. Thus, the release of Cu^{2+} from the complex acted as a limiting step and influenced both root and shoot concentration of Cu provided as $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ complex. The root apoplast likely mediates an additional mechanism.

Fig. 6 Concentration of Cu (A) and Fe (B) in tobacco (*Nicotiana rustica*) plants grown under Cu deficiency conditions and treated with Cu sulfate (CuSu, CuSO₄) or Cu ammonium sulfate complex ([Cu(NH₃)₄]SO₄, CuAm) through leaves (LA) or roots (RA). Cu-sufficient (+Cu) and Cu-deficient (-Cu) plants were left untreated as control plants. Differences among data of each organ indicated by different letters are statistically significant ($P < 0.05$)



Lower Cu concentration in the root apoplast of plants supplied by CuAm (Fig. 7) is likely a reflection of lower adsorption of [Cu(NH₃)₄]²⁺ onto the free pectin groups and

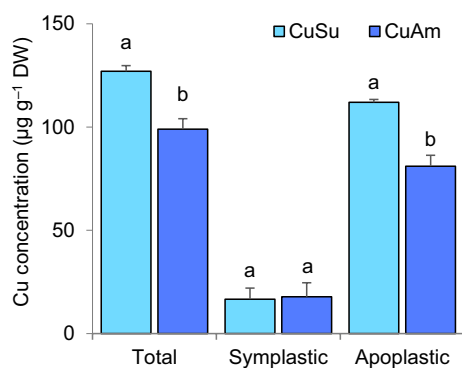


Fig. 7 Concentration of Cu in the roots without washing (Total Cu) and in the EDTA-washed roots (Symplastic Cu) and the difference between two values (Apoplastic Cu) in tobacco (*Nicotiana rustica*) plants grown under Cu deficiency conditions and loaded with Cu sulfate (CuSu, CuSO₄) or Cu ammonium sulfate complex ([Cu(NH₃)₄]SO₄, CuAm) through roots for 48 h. Differences between two Cu forms within each fraction indicated by different letters are statistically significant ($P < 0.05$)

less retention and sequestration of this complex in the free spaces of the roots. Apoplastic Cu is a temporary pool for Cu²⁺ ions and is essential for the extent of Cu transported into the shoot (Printz et al. 2016).

The higher Cu concentration observed in the LA compared to the RA plants cannot be attributed to surface contamination due to the washing procedure conducted before plant analysis (see M&M). A high Cu concentration was also detected in the LL and roots, which were not directly exposed to Cu treatment. Such rapid and almost even distribution of Cu among leaves of different ages and roots in the LA treatments implied a high phloem mobility of Cu applied to the leaves in contrast to the generally stated low Cu retranslocation except for the senescing leaves (Cakmak et al. 2023). The high-affinity Cu transporter COPT6 is expressed in the vasculature of stems and leaves and is responsible for Cu redistribution in shoots (Garcia-Molina et al. 2013). In addition to COPT6, recent evidence showed that OPT3 (OLIGO PEPTIDE TRANSPORTER 3), a Fe transporter, is also involved in the phloem loading of Cu in Arabidopsis (Chia et al. 2023).

The up to six-fold higher Cu concentration observed in the LA compared to RA plants could be explained by different

activity and regulatory patterns of the transporters involved in the internalization of Cu derived from leaves or roots. In contrast to root-localized COPT1 and COPT2, which are upregulated in response to Cu deficiency (Yamasaki et al. 2009), COPT6 is also present under Cu-sufficient conditions (Garcia-Molina et al. 2013). We postulate that due to a relatively mild Cu deficiency in the tobacco plants, the Cu-starvation pathway was presumably not adequately upregulated in Cu-deficient plants, and as a result, root absorption of Cu was not considerably fast and effective for increasing the Cu concentration level of RA plants.

In contrast, a constitutive expression of the transporter(s) responsible for Cu redistribution in shoots, i.e., COPT6 (Garcia-Molina et al. 2013), facilitated the transit of Cu from the treated leaves (ML) to other organs in the LA plants. In Arabidopsis, a Cu-starvation-induced molecular pathway could be activated only under severe Cu deficiency, achieved by growing Arabidopsis *spl7-2* mutants under complete Cu elimination (Bernal et al. 2012). It is also likely that the root transporters, in contrast to the leaf transporters, are rapidly downregulated upon Cu resupply, as was observed for COPT1 and COPT2, unlike COPT3–5 (Sancenón et al. 2003); thus, Cu accumulation occurred only in the LA plants. Detailed studies are needed to identify the transporters involved in the internalization of Cu derived from leaves or roots and their regulatory patterns.

Although the increase in Cu concentration in –Cu plants was more significant when foliar fertigation was used, the Cu concentration range in the leaves of LA plants, ranging from 20 to 50 $\mu\text{g g}^{-1}$ DW, did not induce any toxicity symptoms. This was further supported by the relatively higher biomass response (Fig. 1A) and the absence of increased activity in antioxidant enzymes such as POD (Fig. 3B). In addition, considering a high re-translocation of Cu mentioned above, the leaf-loaded Cu is expected to be diluted quickly following plant growth.

Effect of Cu deficiency and resupply on the activity of Cu-containing enzymes

The total SOD activity determined in this work comprises the activity of three independent SOD isoforms classified by the related metal cofactors, including Mn-SOD, Fe-SOD, and isoforms of CuZn-SOD that are distributed in different cellular compartments (Alscher et al. 2002; Zelko et al. 2002). Under Cu limitation and in a *miR398*-dependent pathway, plastid-localized Fe-SOD (FSD1) is upregulated. At the same time, cytosolic and plastidial isoforms of CuZn-SOD (CSD1 and CSD2) are downregulated, allowing plastocyanin and other essential Cu proteins to remain active over a wide range of Cu concentrations (Yamasaki et al. 2007; Abdel-Ghany and Pilon 2008). The reduction in total SOD levels observed

under Cu deficiency conditions in this study may imply the absence of or inadequate compensatory higher Fe-SOD levels in the tobacco plants (Fig. 6B).

PPOs are intracellular multicopper oxidases with a coupled binuclear type-3 copper center that catalyze the oxidation of monophenols and/or o-diphenols to o-quinones (Solomon et al. 1996). Reduction of PPO activity under Cu starvation and its restoration by Cu resupply (as CuSO_4) has been reported in clover (Delhaize et al. 1985). However, the molecular pathways regulating PPO's transcript levels and/or activity under Cu deficiency have not been investigated. The involvement of the SPL7-mediated pathway in the post-transcriptional downregulation of LAC mRNAs encoding for different but closely related enzymatic group laccase has been reported in Arabidopsis (Abdel-Ghany and Pilon 2008). It is noteworthy that, similar to other related research, we determined here the activity of PPO of the tyrosinase type that is not clearly distinguishable from the laccase activity, particularly in a background of peroxidase activity, as noted by other authors (Mayer and Staples 2002).

The higher efficacy of Cu resupply through leaves in restoring SOD and PPO activity showed that Cu reaches the catalytic leaf sites in the LA treatments faster than RA ones. This assumption is supported by the higher Cu concentration of various shoot fractions and roots of LA plants compared to the RA plants. This observation again emphasizes Cu's impressive phloem mobility when administered to the leaves, as previously detailed.

Diamine oxidases (CuAOs) are homodimers harboring a tightly-bound Cu ion and a quinone moiety in each subunit (Aniszewski et al. 2008). In Arabidopsis, eight genes (AtCuAOs) encode putative functional CuAOs, and the related proteins are localized in the apoplast, peroxisome, or vacuole (Planas-Portell et al. 2013). In Cu-resupplied clover plants, active holoenzymes of DAO can only be synthesized in leaf blades during the very early stages of their development (Delhaize et al. 1985). These findings align with the data, which demonstrates lower DAO activity in LL (senescing leaves) compared to UL and ML and indicates the absence of its restoration upon Cu resupply (Fig. 2C). However, despite contrasting with the Cu concentration data, the reason for the greater efficacy of RA treatment compared to LA in restoring DAO activity remains unclear.

Higher leaf Fe concentration in CuAm-fed plants was likely the mechanism for the higher effectiveness of CuAm treatment in the restoration of leaf SOD activity than CuSu. Considering the compensatory pathway for increased transcription of the FeSOD gene (Abdel-Ghany and Pilon 2008) under Cu starvation, as previously described, it is probable that the observed total SOD activity results from both heightened CuZn-SOD and Fe-SOD activities. Another plausible mechanism explaining the higher SOD activity in response to CuAm is the influence of the accompanying

ammonium ion (NH_4) in the CuAm complex (as elaborated below).

Effect of Cu deficiency and resupply on the activity of phenolics metabolizing enzymes and lignin

Although PAL and POD are not Cu enzymes, their response to Cu deficiency is most likely an indication of a disturbance in the regulatory pathway of phenolic metabolism and ROS homeostasis in Cu-deficient plants. Analysis of PAL genes and phenotyping loss-of-function mutations in Arabidopsis demonstrated the conspicuous role of this enzyme not only in the biosynthesis of anthocyanins, flavonoids, and lignin but also in a broad spectrum of plant processes, including stress adaptation and salicylic acid synthesis (Huang et al. 2010). The response of PAL activity to Cu deficiency and resupply, thus, may imply its contribution to the Cu-related plant stress response. Some authors have suggested a functional role for Cu homeostasis in plant signaling and defense regulation (Rodriguez et al. 1999; Fraudentali et al. 2021). More evidence supporting a link between phenolics synthesis and Cu signaling has been provided by anthocyanin accumulating phenotype of apple plant overexpressing a Cu-miRNA (*miR408*) that is associated with increased Cu content and positive regulation of Cu-binding genes in the overexpressing plants (Hu et al. 2023).

Lignin reduction in Cu-starved tobacco plants in this work could be well explained by the lower activity of enzymes involved in its biosynthesis, including Cu-containing enzymes (PPO and DAO), PAL and POD. The lignin biosynthesis pathway starts with PAL and is followed by the oxidation of the monolignol molecule through laccases, i.e., a PPO-type Cu-binding enzyme and/or one or more PODs, i.e., Fe-containing enzymes require H_2O_2 (Schuetz et al. 2014) that could be provided by diamine oxidases (Asthir et al. 2002). Recently, the role of two other Arabidopsis multicopper oxidase-like proteins, skewed 5 (SKU5) and its homolog SKU5-similar 1 (SKS1), has been shown to contribute to root cell wall formation through modulating ROS homeostasis (Chen et al. 2023).

Foliar-applied CuAm and root-applied CuSu were the most effective treatments for the resumption of lignin concentration, accompanied by similar differential responses of SOD, PPO, and POD. Such different effects of Cu compounds and application modes are likely the result of a complex interaction among multiple players responsible for plant defense and lignin metabolism.

Since the enhanced activity of phenolics metabolizing enzymes and lignin deposition is from plants' characteristic responses to stress (Moura et al. 2010), the changes observed in these parameters may also be attributed, at least partly, to the induction of mild mechanical stress in plants supplied through leaf spray. Tobacco is very sensitive to

leaf mechanical disturbance, and mild mechanical stress activates enzymes of phenolics metabolism (PAL, PPO, and POD) and causes lignification of the leaves in this species (Hajiboland et al. 2017).

The effect of the accompanying ion (NH_4^+) of the CuAm compound could not be overlooked. The release of 2 mM NH_4^+ by application of 0.5 mM $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ may considerably influence plants defense responses. There is evidence of the activation of plant defense machinery by NH_4^+ (versus NO_3^-) applied to plants as a nitrogen source (Fernandez-Crespo et al. 2014; Hajiboland unpublished results).

Collectively, it can be inferred that the enhanced effectiveness of CuAm in comparison to CuSu concerning the activities of SOD, PPO, and POD, as well as the deposition of lignin in the leaves of LA plants, could be attributed to both the stress induced by foliar spraying and the influence of NH_4^+ . Consequently, the most significant levels of these parameters were attained through the synergistic interaction of both these factors. In the case of CuAm applied to the roots, in contrast, NH_4^+ ions may be almost wholly assimilated in the roots, and thus, the leaf biochemical factors were primarily influenced by Cu supply to the plants. Consequently, a higher response of biochemical parameters in the RA plants is observed for CuSu because it provides Cu more efficiently than CuAm.

Interactions between Cu and Fe supply

Copper deficiency leads to a so-called 'secondary Fe deficiency' and leaf chlorosis in Arabidopsis (Bernal et al. 2012). In this work, however, Cu-deficiency-mediated reduction of leaf Fe concentration was not observed in the UL and ML, likely due to mild Cu deficiency in the tobacco plants. An increased expression of Fe deficiency markers *IRT1* and *FRO2* and reduced catalase activity in Cu-starved Arabidopsis could only be detected in *spl7* mutants that experience severe Cu deficiency because of a defect in sensing Cu deficiency (Bernal et al. 2012).

The most conspicuous effect on leaf Fe concentration was observed by Cu resupply. The contribution of Cu proteins in root-shoot Fe translocation has been reported in Arabidopsis (Perea-García et al. 2020). Considering that the primary form of Fe in the xylem is a Fe(III)-citrate complex, it appears that Fe(II) must be oxidized to Fe(III) prior to Fe(III) chelation by citrate (Roschztardt et al. 2011). A ferroxidase (Fe(II) to Fe(III) conversion) activity has been detected in a PPO-like (laccase) enzyme (LAC12) that is probably located in the procambium and may be the protein carrying out this function prior to Fe(III) chelation by citrate, thus participating in root-to-shoot Fe translocation (Bernal and Kramer 2021). Arabidopsis *lac12* mutant lines showed less Fe concentration in the shoot but higher Fe in the roots than

wild-type plants (Bernal and Kramer 2021). Considering significantly reduced root ferroxidase activity in the *spl7* mutant under Cu deficiency conditions (Bernal et al. 2012) and the role of *miR408* in direct and negative regulation of ferroxidase expression (Carrió-Seguí et al. 2019b), it is speculated that enhanced Cu availability through its resupply to -Cu plants may restore or even increase its activity and contribute to higher root-shoot Fe transport in tobacco plants. These data confirm that an adequate Cu nutritional status is required to maintain Fe partitioning (Wairich et al. 2022).

CuAm was more effective in increasing Fe concentration in our work.

The accompanying NH_4^+ ion likely involved in the higher leaf Fe concentration in CuAm-supplied plants. One probable mechanism for the effect of NH_4^+ is the reduction of rhizosphere pH because of a direct proton release following root NH_4^+ absorption (Zhou et al. 2015). This explanation, however, could not be applied to the LA plants. Recently, NH_4^+ has been a new player in plant response to Fe deficiency (Zhu et al. 2019). In cucumber, a conspicuous effect of 2 mM NH_4^+ on the activation of Fe-acquisition machinery, including ferric chelate reductase (FRO) activity, rhizosphere acidification, and upregulation of AHA1, FRO2, and FIT, was observed, even when applied to the leaves (Hajiboland, unpublished results).

The critical toxicity concentration of Fe in the leaves is above $500 \mu\text{g g}^{-1}$ DW (Cakmak et al. 2023). In the tobacco plants, the leaf Fe concentration was elevated up to five-fold upon leaf Cu application, particularly in the CuAm-supplied plants that reached $680 \mu\text{g g}^{-1}$ DW at harvest. Nevertheless, Fe toxicity does not seem to occur in the tobacco plants, at least under the experimental conditions. In tobacco grown under field conditions in sandy soil and red soil, the leaf Fe concentration was $400\text{--}500 \mu\text{g g}^{-1}$ DW, and the first toxicity symptom, such as leaf browning, was observed when it exceeded $1000 \mu\text{g g}^{-1}$ DW (Li et al. 2019).

Conclusion

The leaf application of Cu is an efficient alternative to root fertilization due to its ability to enhance plant biomass rapidly. This efficiency can be attributed, at least partially, to the high re-translocation of Cu from treated leaves and its efficient partitioning to various plant parts. Compared to CuSO_4 , the $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ complex exhibits a lower release of Cu^{2+} and reduced retention in the root apoplast, resulting in lower Cu concentrations in the CuAm-treated plants. Nonetheless, the equal effectiveness of the $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ complex with CuSO_4 in promoting growth and photosynthesis in -Cu plants indicates that the former chemical form is a suitable option, mainly when used as a

foliar fertilizer. Additionally, $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ demonstrates superiority over CuSO_4 in enhancing the mechanical strength of plant tissues and improving Fe nutrition.

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Data availability The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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