

CONSIDERATIONS ON THE INFLUENCE OF COMPLEXATION IN THE COPPER UPTAKE AND TRANSLOCATION

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Abstract: The actual knowledge about food and the environment underlines the fact that agricultural and environmental sciences must solve various problems regarding copper uptake from soil to plants and its bioaccumulation, being important issues for copper concentration in crops and also for phytoremediation of polluted soils. We studied the relation between the form in which copper is applied to soil and the consequential copper bioavailability, uptake and translocation, using as examples simple and complex copper compounds. The copper concentration in basil plants harvested from soils treated with copper compounds and the calculated values of transfer coefficient, translocation factor, bioaccumulation factor, and uptake coefficient demonstrated that the ionic copper (from simple salts) is not necessarily easier to uptake than complex ions, but is easier translocated in plants, while the copper given as complex ions is most likely to be retained by roots. The results indicated that the involvement of copper complexes in agricultural treatments is a solution for soils phytoremediation, concerning the phytostabilization technology.

Keywords: *bioavailability, copper complexes, transfer coefficient, translocation factor, uptake coefficient*

INTRODUCTION

Copper is an essential metal for normal plant growth, even if it is also potentially toxic. Thus, copper plays an important role in the electron transport in the photosynthesis processes; it is a constituent of the chloroplast protein, and it is also known to be an important constituent of many enzymes of the oxidation reduction reactions, *i.e.*, copper is a cofactor in enzymes like plastocyanin and superoxide dismutase. The copper's ability to transfer electrons also renders it toxic when is present in excess, by inducing the formation of toxic reactive oxygen species. Copper has got a stabilizing influence on chloroplast, protecting it from being destructed, but the photosynthetic function is highly sensitive to copper toxicity. Disturbances in copper supply can cause a significant modification of the biochemical processes in plants leading to lower yields and lower quality of agricultural crops. An excessive supply of copper causes symptoms of chlorosis which are similar to the symptoms of iron deficiency [1-3].

It is obviously that the metals requirement of plants is provided by soil. Thus, the metals bioavailability is an essential factor for their provisions of plants. The capacity of plants to accumulate (bioaccumulate) metals is also important because it represents the basis of a relatively new method for soil remediation, named phytoremediation. Bioaccumulation is the process by which a chemical is taken up by an organism, either from direct exposure to a contaminated medium or by consumption of contaminated food [4]. The bioaccumulation of metals in plants is strongly dependent of the bioavailability of metals in soils.

Therefore, the bioavailability of metals in soil is a very important issue in agricultural and environmental studies, being interesting both for understanding the processes involved in the metals uptake by plants and for finding the most reliable methods for the prediction of availability of a given element to plants, and in particular to crop plants. The metals uptake is also important in the phytoremediation process [5].

Copper occurs in the Earth's crust at concentrations between 25–75 mg·kg⁻¹ and copper content of soils ranges from 2 to 100 mg·kg⁻¹, with an average value of about 30 mg·kg⁻¹, and most of them is an unavailable mineral form [6, 7].

In soil, the behavior, phytobioavailability and toxicity of copper (a hardly available and slowly translocating element), are influenced by its species, and are not a function of its total concentration. In addition, several soil variables control the copper solubility and thus bioavailability, including *pH*, oxidation and reduction potential, organic matter, soil texture, mineral composition, temperature, and water regime, dissolved organic matter having a great affinity to fix copper and thus to inhibit its sorption in soils. These phenomena are attributed to the formation of soluble Cu-organic complexes. Organic matter binds copper more tightly than any other micronutrients, and this reduces copper fixation by soil minerals and also availability to crops. The copper deficiency in crop plants due to low contents of copper in soils has been reported for many countries since the 19th century [6, 8].

Soils phytoremediation, defined as the use of green plants to remove pollutants from the environment by concentrating them in the harvestable parts, or to render them harmless, has emerged as a cost-effective, environment-friendly clean up alternative. Typically, there are two basic strategies of phytoextraction: chelate assisted phytoextraction (induced phytoextraction) and long-term continuous phytoextraction. Chelating agents have been shown to desorb metals from the soil matrix into the soil solution, and to

facilitate metal translocation from roots to shoots; the effect may vary from plant and metal species. They are frequently used to increase the bioavailability of heavy metals, thus enhancing their uptake by plants, though this may also decrease their biomass because of the toxicity. Moreover, chelators can mobilize soil nutrients, since the fertilizing action of chelators has been reported [9, 10].

For phytoremediation to be successfully, a strategy should be considered that combines rapid screening of plant species possessing the ability to tolerate and accumulate toxic metals with agronomic practices that enhance shoot biomass production and/or increase metal bioavailability in the rhizosphere [9, 11, 12]. Plants ideal for phytoextraction should possess multiple traits like ability to grow outside their area of collection, fast growth, high biomass, easy harvesting and accumulation of a range of metals in their harvestable parts, but no plant is presently known that fulfills all these criteria. Four processes are generally believed to be crucial for accumulation of metals in plants: (i) uptake of metals by roots, (ii) transport of metals from roots to shoot, (iii) complexation with chelating molecules, and (iv) compartmentalization into the vacuole [13].

In our study we correlated the uptake of copper, its bioavailability and translocation, with the form in which it was applied to the soil. This correlation is important both for finding the conditions for a proper copper concentration in plants and for increasing the efficiency of soil phytoremediation. We studied four copper compounds as sources for copper in application to agricultural soils, modeling the phytoremediation of soils polluted by agricultural treatments. We used the basil (*Ocimum basilicum*), which was previously studied as accumulator for other phytoremediation tests (e.g. phytoremediation of water to remove nutrients [14]).

MATERIALS AND METHODS

Materials

All the reagents were purchased from commercial sources and used as received. The basil seeds were also purchased from commercial sources.

Soil treatment and analysis

The soil studied in our experiments was chernozem, representative for Romanian agricultural land, taken from Constanta County, Romania. We used treated soil, a mixture of chernozem and compost based on dried horse manure, prepared for flowers and vegetables cultivation in green houses, in the year before experiments.

The soil was dried indoors until it could be crumbled to pass through a 4 mm sieve for pots experiment and a 2 mm sieve for analyses of properties. Soil was transferred to 25 cm diameter plant pots (2.5 kg of soil in each).

The soil water content was determined by gravimetric method. The soil pH was determined using standard method for greenhouse soils [11, 15]. The original soils characteristics (electrical conductivity, carbonates, chlorides, calcium, magnesium, total organic matters) were determined for aqueous extracts using standard methods [4]. We used a Consort 535 pH-meter for pH determination and a Consort K600 conductometer for electrical conductivity determination.

The soil total copper was extracted by a mixture of HNO_3 , H_2O_2 , and HCl . The bioavailable copper fraction from original soil was extracted with a 0.01 M CaCl_2 solution [16].

All determination were made triplicate and the mean values are reported.

Plant growth experiments

The seeds of *Ocimum basilicum* were sterilized with HgCl_2 0.1 % solution, by vigorous shaking for 1 min and washed in abundance with deionized water. The sterilized seeds were germinated on Petri dishes (10 seeds/dish) lined with filter paper and impregnated with 6 mL of distilled water. After the plants had grown for 14 days, three plants were selected, each with similar lengths (between 5 – 10 cm) and a pot experiment was carried out under laboratory conditions (natural light, 25 °C temperature) [17]. All experiments were made triplicate.

The plants were treated with four copper compounds ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4 \cdot \text{H}_2\text{O}$, $\text{K}_2[\text{Cu}(\text{C}_2\text{O}_4)_2] \cdot 2\text{H}_2\text{O}$, $[\text{Cu}(\text{en})_2]\text{SO}_4 \cdot \text{H}_2\text{O}$ where en = ethylenediamine). The treatment started after two weeks and consisted in watering the plants with copper salts solutions as pollutants (100 mL per week) of different concentrations ($50 \text{ mg} \cdot \text{L}^{-1}$, $100 \text{ mg} \cdot \text{L}^{-1}$, $150 \text{ mg} \cdot \text{L}^{-1}$). The treatment was done for five weeks (500 mL per plant pot), meaning 10, 20, respective 30 mg per kg soil.

After 45 days since starting treatment the plants were harvested. Plant samples (roots and shoots) were separated and thoroughly rinsed in abundant deionized water after mechanical cleaning, then dried at 70 °C until the stabilization of weight (48 hours) and weighed. The obtained tissue samples were crushed and digested using a H_2O_2 / HNO_3 mixture (10/8, v/v) in a Digesdahl Digestion Apparatus, 230 Vac (Hach). The resulting solutions were prepared for copper determination.

Determination of Cu in plant and soil samples

The copper content in plants and soil samples was determined using molecular absorption spectroscopy.

In order to increase the sensitivity of the analytical determination, we used the method of the standard addition which is applied for low concentrations of compounds, in chemical matrices that are not constant in the samples to be analyzed. Copper was determined as $[\text{Cu}(\text{NH}_3)_4]^{2+}$, obtained in the reaction with ammonia at $\text{pH} = 10$, and the absorbance was measured at 600 nm in a 1 cm long quartz cuvette using a Jasco V 550 spectrophotometer [18].

RESULTS AND DISCUSSION

Copper compounds

Copper compounds are used in agriculture as fertilizers (for soils which contain less than $5 \text{ mg} \cdot \text{kg}^{-1}$ available copper) and pesticides (because of their fungicidal and bactericidal properties). Copper fertilizers are available as oxides (Cu_2O , CuO), simple salts ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and complex compounds (Na_2CuEDTA) [7]. Copper pesticides

include copper oxides, copper hydroxide, simple salts (as sulphate, carbonate) and complex compounds (like copper ethanolamine complex, copper ethylenediamine complex, copper triethanolamine complex etc.) [19].

In our study we used four copper compounds, namely a copper salt ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, from Sigma Aldrich) which generates ionic copper, and three complex compounds ($[\text{Cu}(\text{NH}_3)_4]\text{SO}_4 \cdot \text{H}_2\text{O}$ from Sigma Aldrich, $\text{K}_2[\text{Cu}(\text{C}_2\text{O}_4)_2] \cdot 2\text{H}_2\text{O}$ and $[\text{Cu}(\text{en})_2]\text{SO}_4 \cdot \text{H}_2\text{O}$ prepared in our laboratory by usual methods [20, 21]) which generate complex copper ions. The stereochemistry of complex ions is distorted tetrahedral, with oxygen as donor in oxalate and nitrogen in NH_3 and ethylenediamine; the oxalate ion and ethylenediamine act as chelating ligands and form chelate complexes with the copper ion. The stability of chelate complexes is higher than corresponding complexes with monodentate ligands having identical donor atoms (*i.e.*, the stability of $[\text{Cu}(\text{NH}_3)_4]^{2+}$ is higher than $[\text{Cu}(\text{en})_2]^{2+}$) [22].

Most of $\text{Cu}(\text{II})$ salts dissolve readily in water and give the aqua ion, with molecular formula $[\text{Cu}(\text{H}_2\text{O})_6]^{2+}$. Addition of ligands to such aqueous solutions leads to the formation of complexes by successive displacement of water molecules [23]. This situation may be found in the soil solution, specifying that complex ions remain unchanged in aqueous solution. The fate of ion complexes in soil is dependent on their stability. The synthesis of chelated copper is the basis of chelate assisted phytoextraction, chelating agents having the role to increase the metal bioavailability. The complexes studied in this report, or other complexes with the same coordination environment of metal, may be synthesised in soil solution, especially as the effect of adding a chelator in the phytoremediation process.

Soil preliminary characterization

The characteristics of the soil, before the copper treatment, are presented in Table 1. We also used such soil samples in other experiments [24]. The original soil applied in this study contains $22.66 \text{ mg} \cdot \text{kg}^{-1} \text{ d.w.}$ (Table 1). It is recognized that the average copper concentration in soils is $30 \text{ mg} \cdot \text{kg}^{-1} \text{ d.w.}$ and the common range for soils is $2 - 100 \text{ mg} \cdot \text{kg}^{-1} \text{ d.w.}$ [6] thus the studied soils may be considered as uncontaminated soils prior to spiking with copper as pollutant.

Table 1. Characterization of original soil

Characteristics	Values	Characteristics	Values
pH_{water}	7.67	Calcium ($\text{mg} \cdot \text{kg}^{-1} \text{ d.w.}$)	700.00
Humidity %	2.00	Magnesium ($\text{mg} \cdot \text{kg}^{-1} \text{ d.w.}$)	0.46
Electrical conductivity _{water} ($\text{dS} \cdot \text{m}^{-1}$)	0.57	Total organic matters ($\text{g O}_2 \text{ kg}^{-1} \text{ d.w.}$)	5.81
Carbonates ($\text{g} \cdot \text{kg}^{-1} \text{ d.w.}$)	5.20	Copper ($\text{mg} \cdot \text{kg}^{-1} \text{ d.w.}$)	22.66
Chlorides ($\text{g} \cdot \text{kg}^{-1} \text{ d.w.}$)	2.96	Bioavailable copper ($\text{mg} \cdot \text{kg}^{-1} \text{ d.w.}$)	1.26

The bioavailable (mobile) copper fraction in soil was considered to be the fraction that is not tightly bound to soil [12].

Copper concentrations in plants

Copper has limited transport in plants; therefore, the highest concentrations are often in root tissues. Copper most likely enters roots in dissociated forms but is present in root tissues as a complex. Within roots, copper is associated principally with cell walls due to its affinity for carbonylic, carboxylic, phenolic, and sulfhydryl groups as well as by coordination bonds with N, O, and S atoms. At high copper supply, significant percentages of copper can be bound to the cell wall fractions. Within green tissues, copper is bound in plastocyanin and protein fractions. As much as 50 % or more of plant copper localized in chloroplasts is bound to plastocyanin. The highest concentrations of shoot copper usually occur during phases of intense growth and high copper supply [25].

The determined copper concentration in dry plants is presented in Table 2.

Table 2. The concentration of copper ($\text{mg} \cdot \text{kg}^{-1}$ dry weight) in roots, shoots, stems and leaves

Added compound	Concentration of added Cu(II) $\text{mg} \cdot \text{L}^{-1}$	Copper concentration ($\text{mg} \cdot \text{kg}^{-1}$ d.w.)			
		Roots	Shoots, as follows:	stems	leaves
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1)	50	89.26	65.84	61.38	68.96
	100	146.73	121.66	106.89	133.48
	150	168.73	158.06	146.72	167.06
$[\text{Cu}(\text{NH}_3)_4]\text{SO}_4 \cdot \text{H}_2\text{O}$ (2)	50	155.7	68.36	66.30	70.30
	100	185.15	89.18	51.52	115.10
	150	328.94	104.14	58.62	138.20
$\text{K}_2[\text{Cu}(\text{C}_2\text{O}_4)_2] \cdot 2\text{H}_2\text{O}$ (3)	50	149.36	90.41	62.47	119.04
	100	176.85	103.69	87.46	120.78
	150	236.56	118.12	102.71	132.04
$[\text{Cu}(\text{en})_2]\text{SO}_4 \cdot \text{H}_2\text{O}$ (4)	50	207.45	43.24	33.27	51.56
	100	213.82	46.70	37.45	55.74
	150	311.01	101.44	99.85	103.20
Distilled water	0.00	25.76	17.37	9.57	23.02

The data in the table show some characteristics of copper bioavailability and bioaccumulation in experimental conditions:

- the concentration of copper in plants increased with the increasing of copper concentration in soil, meaning that the rising of total copper concentration in soil also involve the rising of bioavailable fraction;
- a great difference between the copper determined in roots compared to shoots was observed for plants grown in soils treated with complex compounds, meaning a concentration and immobilization of copper in roots. The difference was small for ionic copper treatment, meaning that the copper is translocated in the aerial part of plants, being not immobilized in roots;
- the copper concentration in plants strongly depends to the copper source. Thus, the highest concentration in roots was achieved for the plants harvested from soil treated with complex compounds, especially with complexes $[\text{Cu}(\text{NH}_3)_4]\text{SO}_4$ and $[\text{Cu}(\text{en})_2]\text{SO}_4$, while the highest concentration in shoot was achieved for ionic copper, namely CuSO_4 . The copper concentration for plants treated with $[\text{Cu}(\text{C}_2\text{O}_4)_2]^{2-}$ was lowest in roots by

highest in shoots, compared with the two other complexes, and the explanation may be related to the solubility, the steric requirements or the charge of complex.

Copper uptake and translocation

Calculated values of some factors as translocation factor, transfer coefficient, bioaccumulation factor, and uptake coefficient can be used as measures for plants ability to uptake, accumulate and translocate copper, in experimental conditions.

The transfer of metal ions from the roots to the shoots is discussed in terms of *translocation factor* (TF). The translocation factor (TF) is defined as the ratio of total metal concentration in the plants shoot to that in the roots [26].

Another factor that allows comparative characterization of metals accumulation in the aerial parts of plant is the *transfer coefficient* (TC), calculated as the ratio of metal concentration in the plants shoot to total amount of metal (in the roots and shoot).

Like TF, the TC also expresses the transfer of metal (taken by the roots from soil) in aerial parts. The amount of metals transferred from roots to the aerial parts may be influenced by the species of metal from the soil, solubility in soil solution and the ability of roots species to takeover metals, and also the plant will determine whether the physiological characteristics of the metal can be transferred and accumulated in a particular part (as leaves, flowers, fruits) or if storage remains in roots [27].

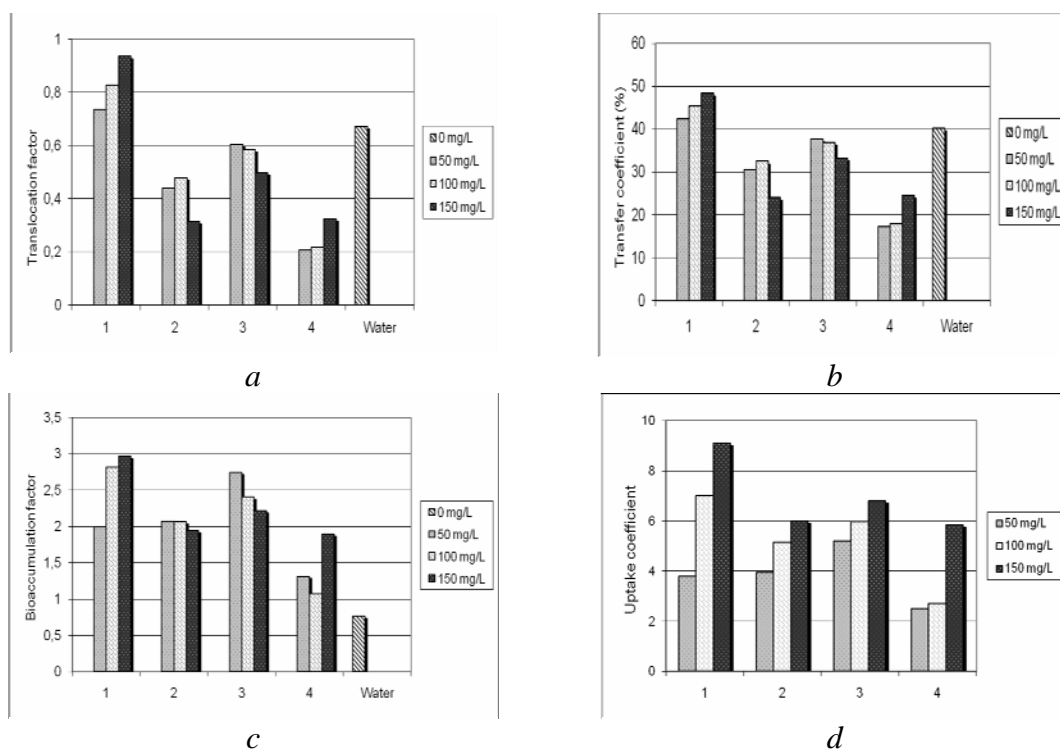


Figure 1. The TF (a), TC (b), BAF (c), and UC (d) values for *Sinapis alba* grown on soils treated with different quantities of CuSO₄·5H₂O (1), [Cu(NH₃)₄]SO₄·H₂O (2), K₂[Cu(C₂O₄)₂]·2H₂O (3), [Cu(en)₂]SO₄·H₂O (4)

The *bioaccumulation factor* (BAF), also known as the bioconcentration factor, was defined as the ratio of total metal concentration in shoots to that in soil, which is a measure of the ability of a plant to uptake and transport metals towards the shoots [26, 28]. The BAF, as defined above, may be interpreted as a transfer factor from soil to shoots or as the bioconcentration factor (ratio) in shoots. It shall be deemed that plant species exhibiting TF and BAF greater than one are suitable for phytoextraction [26].

The United States Environmental Protection Agency introduces a metals takeover coefficient by plants, noted as *uptake coefficient* (UC), which express the amount of a metal taken up by a plant compared with the amount applied to the soil. This factor is obtained from the ratio between the amount of metals accumulated in some tissues of plants grown on land, fields under varying degrees of impact with polluting material against the same plant species grown on blank soil unpolluted [27].

The values of TF, TC, BAF, and UC for all experiments are plotted in Figure 1.

From the data presented in Figure 1 we can make some remarks:

- the highest values for TF, TC, and BAF were determined for plants harvested on soil treated with (1), meaning the best copper translocation and bioaccumulation in aerial parts for ionic copper (from simple salt). For this soil, the values uniformly increased with concentration;
- the increasing of TF, TC and BAF with copper concentration in soil is not observed in all experiments. In some cases the highest copper concentration means the lower translocation, high quantities of copper being retained in roots;
- the lowest concentrations of copper in roots of plants (Table 2) treated with (3) may be correlated with highest values for TF, TC, and BAF, meaning an easier translocation of copper, compared with the two others complexes;
- the value of BAF calculated for unpolluted soil is smaller compared with values for all other experiments. An explanation may be the form of copper in soil, probably in insoluble inorganic compounds, which led to a low copper bioavailability. This supposition is sustained by the small value of bioavailable copper ($1.26 \text{ mg}\cdot\text{kg}^{-1} \text{ d.w.}$, Table 1) in original soil, which means that only 5.56 % of total copper is in a bioavailable form;
- for polluted soils, the values of BAF are greater than one, indicating that *Ocimum basilicum* is suitable for phytoextraction of copper. In the same time, the TF values are lower than one, probably because of copper immobilisation in roots. This result confirms that, for copper polluted soils, the preferred phytoremediation technology is the phytoremediation;
- the UC values increased with copper concentration in soil, irrespective of copper source. The highest values for UC were determined for copper sulphate, because the UC refers to the concentration in shoot, which is highest for ionic copper (Table 2).

The results are important both for controlling the copper concentration in crops and for phytoremediation of soils.

CONCLUSIONS

The experiments revealed the importance of copper source in the uptake, bioaccumulation, and translocation of copper. Thus, the application of copper as copper sulphate had as effect a high concentration in aerial part of basil plants and a low

concentration in roots. The copper taken as complexes was retained in roots, meaning that it is easier to be uptake by plants, but it is hardly available and slowly translocated, as a result it being concentrated in roots and not translocated to shoots.

This means that ionic copper (from simple salts, like sulphate) is more available to be translocated from roots to shoots than copper complexes. On the contrary, copper complexes make the metal more bioavailable and easier to be uptaken. Hence, the complexation increases the bioavailability but decreases the translocation of copper in plants; an explanation may be the complexes stability and hence a low probability for the reaction of copper complexes with ligands which have the role to transport it in plants.

Between tested complexes, the copper as $[\text{Cu}(\text{C}_2\text{O}_4)_2]^{2-}$ ion is the most easily to be uptaken by basil and translocated in aerial plants. The explanations may regard the charge of complex ion (is the only anion between the studied complexes), solubility, stability, stereochemistry, which can have a favorable effect in the transportation of copper from soil to roots and from roots to shoots.

This behavior of copper complexes supports the utilization of chelating agents (like oxalate anion) in the phytoremediation of copper polluted soils, involving the phytostabilization technology, meaning that the plants stabilize, rather than remove contaminants by plant roots metal retention.

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