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Accumulation of Cu in Root Apoplasm and Re-translocation to Young Leaves in Rice, Maize and Sunflower with Different Toxicity Tolerance

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Abstract: The influence of Cu toxicity on growth and accumulation of Cu in three crop species including rice (*Oryza sativa* L. cv. T. Hashemi), maize (*Zea mays* L. cv. SC 704) and sunflower (*Helianthus annuus* L. cv. Mehr) was investigated in hydroponic culture media under environmentally controlled conditions. As indicated by the growth parameters, the tolerance of studied species to Cu toxicity effect was in order of rice>maize>sunflower. However, the accumulation of Cu in roots and shoots was rather adversely related to the tolerance of plants. The copper accumulation in root apoplasm in rice and sunflower was much higher than that in maize. It was likely the cause of high copper translocation into shoots in the former plants. Root length was the most sensitive parameter to Cu toxicity and could be considered as an indicator of responses of studied species to excess Cu. Root respiration increased in rice and maize, but decreased in sunflower plants in response to Cu toxicity. In rice, leakage of K⁺ from roots, in contrast to other two species, was not affected significantly by excess Cu. Supplemental Ca ameliorated toxicity effect of Cu depends on species, however, this effect was not necessarily associated with the reduction of uptake and transport of Cu. Growth of plants under high light intensity compared to low light conditions results in an alleviation of toxicity because of dilution effect and likely higher antioxidant pool of tissues. Re-translocation of Cu in plants loaded with toxic levels of Cu indicated that, firstly a substantial portion of Cu was remobilized and translocated from mature to young leaves. Secondly, the extent of retranslocation was species dependent, the highest and lowest amounts were observed in maize and rice respectively. Results suggested that different redistribution of Cu among leaves of different growth status in association with different tolerance at tissue levels could substantially contribute in growth responses of species to excess Cu.

Key words: Cu toxicity, rice, maize, sunflower, retranslocation

INTRODUCTION

Copper, an abundant transition metal in soils, is an essential nutrient for plants. It is a constituent micronutrient of protein components of several enzymes, mainly of those particularly in electron flow, catalyzing the redox reaction in mitochondria and chloroplasts^[1]. When absorbed in excess amounts, however, Cu can cause damage leading to total inhibition of growth^[2-4]. The normal Cu concentration in plant tissues is 5-20 mg kg⁻¹ and above the upper limit toxicity effects are likely to occur^[5].

For various reasons there is increasing concern about Cu toxicity in agriculture^[6]. These include the high Cu contents in soils caused by the long-term use of Cu-containing fungicides, industrial and urban activities (air pollution, city waste and sewage sludge) and the application of pig and poultry slurries having high amounts of Cu. Mechanisms of Cu tolerance in plants

are therefore of interest for crop production on Cu-polluted soils^[1].

The physiological cause of the phytotoxic effects of heavy metals, including Cu, has been the subject of numerous studies^[3,4,7-9]. The response of plants to Cu may depend on how the ions are distributed between shoot and root and between tissues within organs^[10]. A large proportion of the Cu absorbed by the plants is retained in the roots^[11]. It was also found that roots reflected Cu concentrations in the soil, but Cu levels in the shoot did not show significant changes in response to change in soil Cu concentration^[12].

Before reaching the plasma membrane of root cells, ions have to pass through the apoplasmic space of root. Binding of heavy-metal cations in the root apoplasm can be highly specific^[1], because the cell wall contains nitrogen-containing groups of either glycoproteins or proteins of ectoenzymes^[13,14]. This Cu binding in the apoplasm can contribute significantly to the total copper

content of roots^[1] as shown also by studies of the uptake of other polyvalent cations such as Zn and Fe^[15].

The uptake of trace elements in roots depends on the ionic status of the rhizosphere. Absorption of Cd²⁺ by roots of *Tamarix aphylla* was inhibited by increasing concentrations of Ca²⁺ in the solution^[16]. Magnesium ions were less effective in reducing Cd²⁺ uptake. Monovalent ions (Na⁺, K⁺ and Li⁺) also reduced Cd²⁺ uptake but with a lesser extent than divalent cations^[17]. In some plants Ca²⁺ but not Mg²⁺ had an antagonistic effect on Pb²⁺ uptake^[18]. On the other hand, many toxic effects of trace elements result from their replacement of Ca at vital sites of cell membranes in root cells. An optimal supply of calcium can considerably alleviate the toxicity of some trace elements^[16,17].

Copper is a potent inhibitor for photosynthesis and chlorophyll synthesis^[11] in some plants such as barley and spinach. However, no significant effect of Cu toxicity on chlorophyll synthesis in maize was reported^[19]. Though activity of Cu makes it an ideal cofactor in cellular energy transfer reaction, it also confers the potential to cause oxidative damage when present in excess^[20-23]. A well-known harmful effect of oxidative damage is the alteration of the plasma membrane permeability of cells leading to leakage of ions like potassium and other solutes. Rapid K⁺ efflux has been widely interpreted as a symptom of toxicity resulting from Cu induced oxidative damage to the plasma membrane^[24].

Besides direct and indirect effects on photosynthesis, Cu is also reported to inhibit respiration^[11]. However, studies on the effects of Cu on respiration were conducted using algae or aquatic angiosperms such as *Vallisneria*^[25]. In terrestrial plants, the effect of some heavy metals on respiration of roots was studied, however, the effect of Cu was not reported. A significant portion of the carbohydrates produced in photosynthesis is respired through the roots. The magnitude varies with age, species and environmental conditions. The respiration of maize root tips decreases by up to 40% after exposure to 20 $\mu\text{mol Pb}^{[26]}$ and the degree of inhibition increased with the duration of exposure. Concomitant with the decrease in respiration of the root was a decrease in the energy charge of the treated tissue^[26]. Cadmium, Zn^[27] and Al^[28] also appear to inhibit seedling mitochondrial respiration in a manner similar to that of lead.

The toxic effects of heavy metals have been linked to the production of free radicals and the oxidative damage caused by redox metals such as Cu as they produce hydroxyl radicals (Fenton-type reaction)^[29]. Some enzymes and compounds are involved in the scavenging of reactive oxygen species in the leaves^[30,31]. Leaf antioxidant capacity is modified by long-term light acclimation^[32] and

might confer tolerance to oxidative stresses. High light acclimated plants with a more efficient antioxidant and photoprotective system are better protected against heavy metal toxicity as compared to low light grown plants^[33]. However, a contradictory result was obtained in other work, in that a higher susceptibility to heavy metal toxicity was observed in high-light grown plants though having a higher concentrations of antioxidants^[33]. Therefore, it is important to explore how that response is modified by light intensity among different species suffering Cu-toxicity stress.

Retranslocation and phloem mobility of metals with nutritional role in plants such as Zn was studied by some workers. Translocation of foliar applied ⁶⁵Zn in phloem was studied to characterize the role of Zn nutritional status of plants on the extent of phloem mobility of Zn and to determine the relationship between phloem mobility of Zn and Zn efficiency of wheat cultivars^[34]. In another study using root-fed plants, the extent of ⁶⁵Zn remobilization from mature to young leaves in relation to Zn efficiency trait and Zn nutritional status of rice cultivars was investigated^[35]. Retranslocation of root- or foliar-applied ¹⁰⁹Cd at non-toxic^[36] moderately-toxic^[37] levels from leaves to grains was also studied in short term experiments. However, the significance of retranslocation of heavy metals toxicity effects among species in a long term experiment was not studied. It is possible that, susceptibility to metal toxicity is partly due to the higher retranslocation of metals from old parts of plants to growing young leaves.

Thus the main aim of this study was to compare the tolerance and accumulation of Cu in three different plant species. On the other hand, the physiological responses of selected crop plants were studied in order to evaluate the importance of each mechanisms for tolerance depending on different species.

MATERIALS AND METHODS

Three crop species were used in this study including rice (*Oryza sativa* L. cv. Tarom-Hashemi), maize (*Zea mays* L. cv. SC 704) and sunflower (*Helianthus annuus* L. cv. Mehr). Seeds were provided by the Rice Research Institute (Guilan, Iran) and Seed and Plant Improvement Institute (SPII) (Karaj, Iran). Experiments were conducted during the year 2003-2004.

Plants culture and treatments: The experiments were conducted in a growth chamber with a temperature regime of 25°/18°C day/night, 14/10 h light/dark period, a relative humidity of 70/80% and at a light intensity of 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (PPFD). Surface-sterilized seeds were

germinated in the dark on sand, moistened with distilled water and CaSO_4 at 0.05 mmol. The 7-days-old seedlings with uniform size were transferred to hydroponic culture in plastic container with 2L of nutrient solution (25%) and pre-cultured for 3 days. Copper treatments were started for 10-days-old plants, consisted of five levels of CuSO_4 at 0 (control), 25, 50, 75 and 100 μmol . Plants were treated for 12 days and nutrient solutions were completely changed every 3 days. Composition of the nutrient solutions were used according to Yoshida^[38] for rice, Fortmeier and Schubert^[39] for maize and Dannel^[40] for sunflower plants. pH of nutrient solutions were 6.5 and were adjusted every day.

For study of the effect of different light intensities, plants were grown simultaneously under two light intensities: high light (HL = 500 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PPF) and low light (LL = 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PPF). The effect of supplemental Ca in the growth medium, was studied using two levels of Ca as control (Ca = 0 mmol) and treatment (Ca = 5 mmol).

Harvesting: After 12 days treatment, plants were harvested. For removing of the apoplasmic Cu from roots, plants were placed for 1h in 5 mmol CaCl_2 +25% nutrient solution without Cu. Thereafter, plants were divided into shoots and roots, roots were washed with distilled water, weighed and blotted dry on filter paper and dried at 70°C for 2 days to determine plant dry weight. For estimation of Cu amounts bound in the root apoplasm, in the second group of plants the roots were washed only shortly (1 min) with distilled water. The difference of Cu content values in roots between these two group of plants was regarded as root apoplasmic Cu and was reported as μg Cu per mg of root DW.

For determination of Cu content, oven-dried samples were ashed in a muffle furnace at 550°C for 8 h and then digested in 1:3 HNO_3 . The digested samples were dried on a heating plate and subsequently ashed at 550°C for another 3 h. Samples were resuspended in 2 mL 10% HCl and made up to volume (25 mL) so that the end HCl concentration was 1%. Copper concentration was determined by atomic absorption spectrophotometry (Shimadzu, AA 6500).

For determination of chlorophyll concentration, third leaves (pair of leaves in sunflower) immediately after harvesting were used to extract of chlorophyll by acetone. For determination of root length, fresh roots were used according to the method of Tennant^[41]. The Cation Exchange Capacity (CEC) of roots was determined using control plants at harvest according to Shone and Flood^[42].

Root respiration rate: Root respiration rate (O_2 consumption) of plants was measured using an oxygen

electrode (Consort, Z921) at harvest, 12 days after Cu treatments. The root system was excised and placed in a water-filled glass container, cut into 25-50 mm segments and after mixing, 100-300 mg FW was collected by sieving and placed in 70 mL of air- saturated 2 mmol $\text{Ca}(\text{NO}_3)_2$ solution. The respiration rate was measured for 10 min at 22°C with continuous stirring by following the linear decrease of O_2 concentration over the time between the third and eight minute of the measurement period. Four replicates taken from plants of four pots were used for each treatment. Afterthat, fresh and dry weight of the root segments were determined. Respiration rate was expressed as $\text{nmol O}_2 \text{ consumption min}^{-1} \text{ g}^{-1}$ root dry weight (RDW).

Potassium leakage from roots: In order to study of the *in vivo* effect of Cu on plasma membrane permeability of root cells, the experimental procedure described by De Vos^[43] was used. After washing with distilled water for 1 min, the whole root system of intact plants grown previously for 12 days at 5 Cu levels, were transferred to the loading solution containing 0.5 mmol CaSO_4 , 0.1 mmol KCl and 1.5 mmol MES at pH = 6.0. After pre-incubation, the plants were transferred to 100 mL fresh nutrient solution and the experiment was started (t = 0). The solution was aerated and kept at 22°C using water bath. The K^+ concentration of the 2 mL aliquots which were taken every 20 min for 2 h, was determined by flame photometry (JENWAY, PFP7). The values of K^+ concentrations of aliquots showed the same tendency in all Cu treatments and time courses, therefore K^+ data was only reported for samples taken after 60 min from root medium of plants grown for 12 days at 100 μmol Cu.

Retranslocation of Cu to young leaves: To study the possible differential Cu translocation from mature (source) leaves to the youngest (sink) leaf in three studied species, an experiment was conducted according to the method of Hajiboland and Römheld^[35]. After pre-culture for 7 days, plants were transferred to nutrient solution with the same composition as used for pre-culture with the exception of that 100 μmol Cu (as CuSO_4) were applied for 24 h. After this period, nutrient solutions were substituted by 0.5 mmol CaSO_4 as root washing solution for 1h and thereafter by control nutrient solution. The first harvest was performed after root washing and the following harvests were carried out each time after that a new leaf (or leaf pairs in sunflower) had emerged and fully expanded i.e. without weight increase at the following harvests. Samples were weighed and washed with distilled water and were analyzed for Cu as was described above. Data of leaf weights confirmed that, each harvest was carried out at correct time (data not shown).

Statistical analysis: All experiments were carried out using randomized complete block design, with four replications. Statistical analysis were performed by Sigma-Stat (version 2.03) computer program using one way ANOVA and the Duncan test, to compare the means of treatments at $p < 0.05$.

RESULTS

Copper tolerance and growth: Shoot and root biomass per plant revealed progressive decline with increasing Cu concentrations in the nutrient solution in all of studied plants. However, the most inhibition of growth was observed in sunflower and maize. The highly significant reduction was observed in shoot and root dry mass even at the lowest Cu treatment (25 μmol) (Fig. 1). However, in rice, a significant decrease in shoot and root dry mass was only observed at the Cu concentration of 75 μmol and above. At the highest Cu level (100 μmol), a great loss of shoot and root biomass (by 85 and 92%, respectively) was observed in sunflower. Growth reduction was 79 and 73% in maize and 55 and 69% in rice in shoot and root, respectively (Fig. 1). Therefore, the tolerance of these three crop species was in order of rice>maize>sunflower. In the experiment for study of the effect of supplemental Ca and various light intensities the same order of tolerance were obtained.

In the contrary of growth results, in rice, chlorosis appeared in the Cu treatment as low as 25 μmol , whereas in sunflower, chlorosis was not observed in any of the Cu treatments. In the latter plant chlorophyll content was rather increased in response to Cu toxicity. In maize, chlorophyll content was also decreased particularly at the Cu levels of 100 μmol (Fig. 1).

Concentration and accumulation of Cu: Table 1 shows that Cu was accumulated to various extents, depending on the Cu concentration of the medium and species as well as plant organs. The highest concentrations of Cu was found in the roots of all studied plants. In contrast to growth results, rice accumulated more Cu than other two species in both shoot and root. The accumulation of Cu in the shoots was in order of rice>sunflower>maize and in the roots was in order of rice>maize>sunflower (Table 1). Roots of rice accumulated Cu up to approximately 4 mg g^{-1} of dry mass in the presence of 100 μmol Cu.

The estimated apoplasmic Cu accumulation in roots indicated a large difference among studied plants (Table 2). The highest amount of Cu in root apoplasm was observed in rice. Maize accumulated the lowest amount of Cu in this compartment. These data were in accordance with the corresponding CEC data of roots which showed the highest value in rice and the lowest in maize (Table 2).

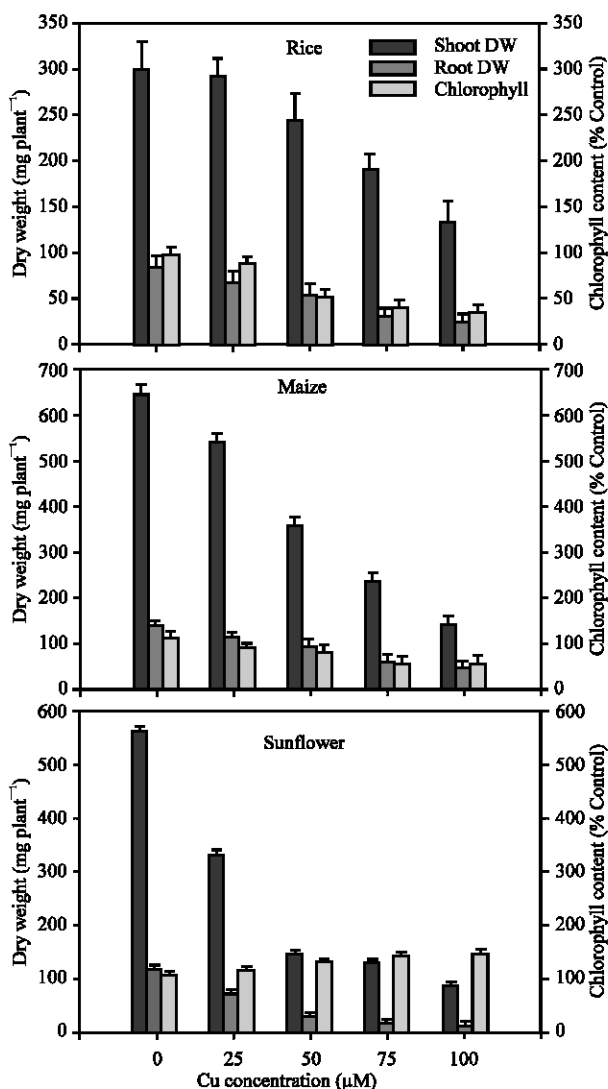


Fig. 1: Dry weight (mg plant^{-1}) of shoot and root and chlorophyll concentration (% control) in rice, maize and sunflower plants grown in various toxic levels of Cu in the nutrient solution

Root length, respiration and membrane integrity: Root length was the most sensitive parameter responding to the Cu toxicity than shoot and root dry mass in all tested species (Fig. 2). Significant depression in root length (62 and 54%) was observed at Cu treatment as low as 25 μmol in maize and sunflower, respectively. In rice, the root length was reduced only 24% at the 25 μmol Cu treatment. At the higher Cu level (100 μmol), a near complete inhibition of root elongation was observed in maize and sunflower giving a 90 and 93% reduction of root length as compared to the control, respectively (Fig. 2).

Root respiration rate showed significant stimulation in response to Cu in rice and maize, but decreased under

Table 1: Copper concentration ($\mu\text{g g}^{-1}\text{DW}$) in shoot and root of rice, maize and sunflower plants grown in various toxic levels of this metal in the nutrient solution

Cu treatment (μmol)	Rice		Maize		Sunflower	
	Shoot	Root	Shoot	Root	Shoot	Root
0	45±2e	206±5e	8±1e	32±2e	5±1e	32±1e
25	160±4d	697±11d	12±1d	81±1d	20±1d	173±5d
50	248±8c	1089±13c	28±1c	210±4c	77±3c	839±31c
75	359±12b	2136±12b	73±1b	418±7b	129±4b	1368±49b
100	695±18a	3993±181a	154±4a	850±10a	233±7a	1629±51a

Values in each column following by different letters are significantly different ($p < 0.05$)

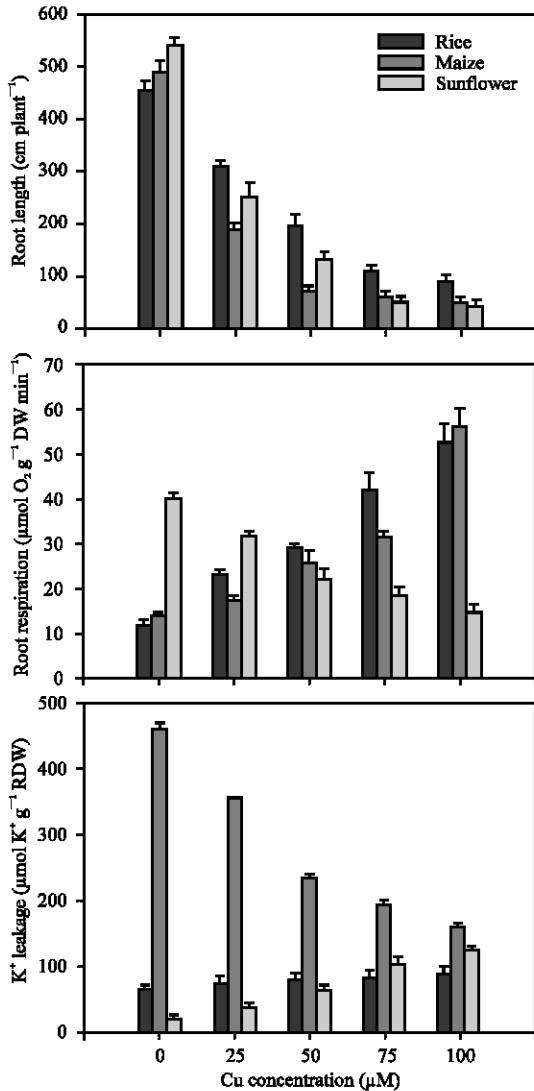


Fig. 2: Length (cm plant⁻¹), respiration ($\mu\text{mol O}_2 \text{ g}^{-1} \text{ DW min}^{-1}$) of and K⁺ leakage from roots in rice, maize and sunflower plants grown in various toxic levels of Cu in the nutrient solution

these conditions in the sunflower (Fig. 2). Release of K⁺ as an indicator of membrane integrity, which surprisingly decreased in maize, increased in sunflower and remained unchanged in rice (Fig. 2).

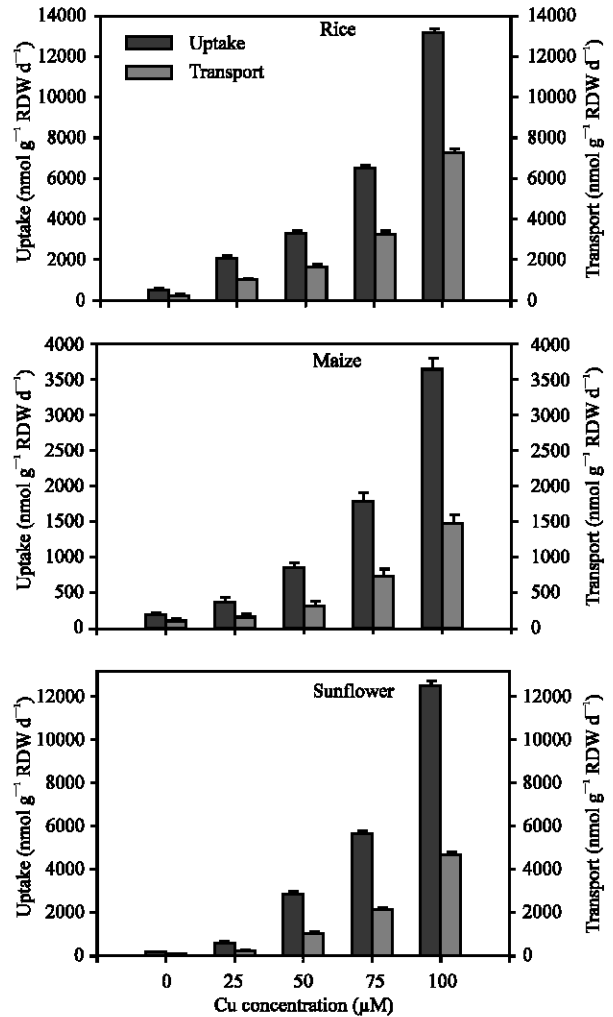


Fig. 3: Uptake and transport ($\text{nmol g}^{-1} \text{ RDW d}^{-1}$) of Cu in rice, maize and sunflower plants grown in various toxic levels of Cu in the nutrient solution

Ameliorating effect of supplemental Ca: The positive effect of Ca on growth parameters, was only shown in accompanying with the toxic levels of Cu in the medium. In control nutrient solutions, growth parameters were rather decreased in response to supplemental Ca, the most inhibitory effect of Ca in the absence of toxic levels of Cu was shown in rice (Table 3).

Table 2: Cation exchange capacity (meq 100 g⁻¹ RDW) of root and copper accumulation (µg mg⁻¹RDW) in root apoplasm of rice, maize and sunflower plants grown in various toxic levels of this metal in the nutrient solution

Cu treatment (µmol)	Rice		Maize		Sunflower	
	CEC	Cu in apoplasm	CEC	Cu in apoplasm	CEC	Cu in apoplasm
0	3.92±1.0	0.14±0.02e	0.85±0.09	nd	1.5±0.08	nd
25		0.25±0.01d		nd		nd
50		3.86±0.52c		0.51±0.04c		0.85±0.02c
75		19.20±2.61b		4.81±1.34b		14.15±2.31b
100		85.61±8.27a		8.78±1.69a		30.90±7.12a

Values in each column following different letters are significantly different (p<0.05), Nd: Not detectable

Table 3: The effect of supplemental Ca (mmol) on the growth parameters of rice, maize and sunflower plants in the presence of toxic levels of Cu in the nutrient solution

	Rice			Maize			Sunflower		
	Shoot DW (g plant ⁻¹)	Root DW (g plant ⁻¹)	Root length (cm plant ⁻¹)	Shoot DW (g plant ⁻¹)	Root DW (g plant ⁻¹)	Root length (cm plant ⁻¹)	Shoot DW (g plant ⁻¹)	Root DW (g plant ⁻¹)	Root length (cm plant ⁻¹)
Ca = 0 mmol									
0 µmol (Cu)	239±18a	64±4a	500±23a	719±28a	143±16a	393±17a	562±13a	212±21a	498±15a
50	121±13c	41±1b	150±19d	173±13c	55±11b	29±4d	119±8c	43±8b	77±5c
100	98±6d	24±1c	100±7e	88±14d	29±9c	29±9d	84±6d	25±6d	56±9d
Ca = 5 mmol									
0 µmol (Cu)	176±11b	35±3bc	452±12b	714±37a	110±19ab	226±9b	561±14a	213±17a	364±11b
50	139±12c	31±4c	196±14c	278±18b	86±11b	157±15c	194±19b	38±14b	42±3d
100	97±4d	19±5c	153±10d	160±14c	25±7c	34±6d	175±12d	28±10b	39±8d

Values in each column within each species following different letters are significantly different (p<0.05)

Table 4: The effect of two levels of light intensities (low light: 100 µmol m⁻²s⁻¹ and high light: 500 µmol m⁻²s⁻¹) on the growth parameters of rice, maize and sunflower plants in the presence of toxic levels of Cu in the nutrient solution

	Rice			Maize			Sunflower		
	Shoot DW (g plant ⁻¹)	Root DW (g plant ⁻¹)	Root length (cm plant ⁻¹)	Shoot DW (g plant ⁻¹)	Root DW (g plant ⁻¹)	Root length (cm plant ⁻¹)	Shoot DW (g plant ⁻¹)	Root DW (g plant ⁻¹)	Root length (cm plant ⁻¹)
Low light									
0 µmol (Cu)	148±13d	42±3bc	121±12c	438±26b	57±11b	342±14b	424±28b	125±14b	372±29d
50	114±8e	34±6c	57±6de	64±9d	11±3d	38±3c	158±21d	25±5c	45±12c
100	98±7f	21±5c	37±4e	27±8e	6±1e	19±2d	131±18d	23±6c	8±2e
High light									
0 µmol (Cu)	584±43a	154±21a	547±26a	1148±124a	178±29a	616±15a	649±26a	250±19a	839±23a
50	372±18b	110±12a	244±19b	59±46c	26±6c	39±10c	463±24b	142±14b	403±19b
100	195±21c	60±14b	73±11d	109±68c	20±8d	56±14c	217±16c	32±8c	18±4d

Values in each column within each species following different letters are significantly different (p<0.05)

Root length in the presence of toxic levels of Cu, was positively affected by supplemental Ca and most significant effect was shown in rice and no effect was observed in sunflower. In rice grown in the nutrient solution supplemented with 5 mmol Ca, reduction of shoot and root growth was only 45% compared with 62% in the absence of Ca and presence of 100 µmol Cu (Table 3). The effect of Ca on the alleviation of toxic effects of Cu in other two species was only prominent in the shoots.

Effect of various light intensity: Generally, under the low light conditions, shoot and root growth of all three species were inhibited (Table 4). Therefore, it seems likely that in our experiment the optimum light intensity is supplied only in HL conditions. In rice, the extent of toxic effects of Cu on growth parameters, was different under two different light conditions. The reduction of shoot and root DW of rice plants grown at Low Light intensity (LL plants), was lower (34 and 50%, respectively)

than that in plants grown under High Light intensities (HL plants) (67 and 61%, respectively). This tendency was not observed in other two species and the growth reduction was almost similar for plants under different light conditions. However, independent from corresponding control plants, growth of HL plants under all of Cu treatments was significantly higher than LL plants.

Uptake and transport of Cu: Figure 3 shows the rate of uptake and transport of Cu, expressed as nmol g⁻¹ DWd⁻¹ in three studied plants as a function of the concentration of Cu in the external medium. The highest uptake and transport rate was shown in the rice and the lowest values in maize (Fig. 3). Supplemental Ca decreased uptake rate of Cu, in the exception of uptake in maize at 100 µmol Cu. Transport rate did not generally respond to Ca, in the exception of maize grown at 100 µmol Cu, in which Cu transport similar to uptake rate showed an increasing effect rather reduction (Fig. 4). Growth at

Table 5: Cu content (ng leaf⁻¹) in the distinct leaves and roots of rice, maize and sunflower at first and last harvest time. Plants were grown in Cu-free nutrient solution after 2 days of Cu loading (100 μmol). Each harvest was performed as one fully expanded leaf (leaf pairs in sunflower) was appeared

	Rice	Maize	Sunflower
1st leaf			
First harvest	2.6±0.26	2.4±0.05	2.3±0.12
Last harvest	1.5±0.05	0.9±0.02	1.6±0.12
Reduction(%)	42.0	63.0	30.0
2nd leaf			
First harvest	3.2±0.35	3.2±0.16	4.7±0.17
Last harvest	2.2±0.17	1.6±0.17	2.7±0.18
Reduction(%)	31.0	50.0	43.0
3rd leaf			
First harvest	2.0±0.33	3.2±0.15	4.2±0.12
Last harvest	2.3±0.21	2.5±0.10	3.2±0.30
Reduction(%)	28.0	26.0	24.0
4th leaf			
First harvest	3.2±0.17	3.3±0.15	4.1±0.21
Last harvest	2.8±0.16	2.1±0.13	3.3±0.22
Reduction(%)	13.0	36.0	20.0
Root			
First harvest	27.4±0.86	28.0±0.34	32.1±1.09
Last harvest	6.2±0.14	8.5±0.33	6.7±0.17
Reduction(%)	77.0	70.0	79.0

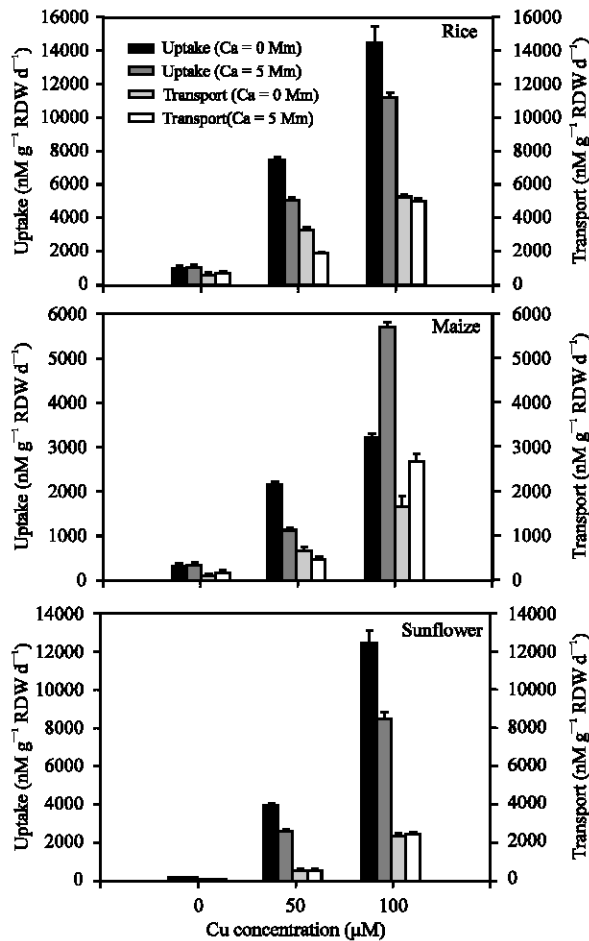


Fig. 4: Uptake and transport (nmol g⁻¹ RDW d⁻¹) of Cu in rice, maize and sunflower plants grown in various toxic levels of Cu in the nutrient solution supplemented with 5 mmol Ca

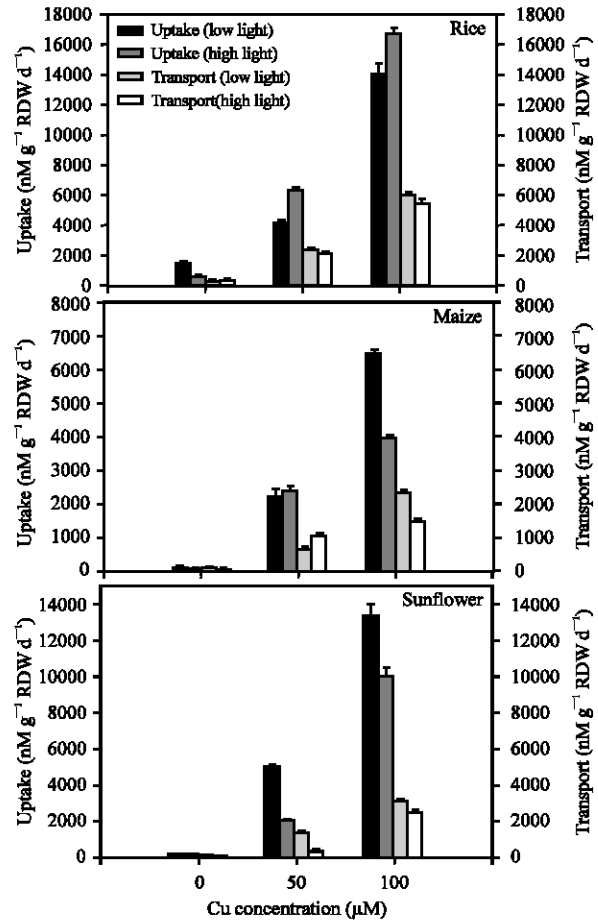


Fig. 5: Uptake and transport (nmol g⁻¹ RDW d⁻¹) of Cu in rice, maize and sunflower plants grown in various toxic levels of Cu in the nutrient solution under low (100 μmol m⁻²s⁻¹) and high (500 μmol m⁻²s⁻¹) light intensities

higher light intensity results in a general reduction of uptake and transport rate in maize and sunflower particularly at higher Cu concentrations, but increase uptake in rice (Fig. 5).

Different retranslocation of Cu: The reduction of Cu content of mature leaves as the result of retranslocation into younger leaves was more prominent in the maize than other two species (Table 5). In the contrary, the lowest reduction of Cu of roots was observed in this plant. Rice in particular, showed the lowest retranslocation of Cu to growing leaves (Table 5). First and second leaves compared to 3rd and 4th leaves in all studied species, had the highest potential to remobilize the Cu and transport it into the youngest leaves. Calculation of the Cu content of distinct leaves as % total shoot Cu content, showed that a significant

redistribution of Cu within shoot was occurred in all studied species, particularly in maize (Table 5).

DISCUSSION

The higher susceptibility of root growth compared to shoot growth to Cu toxicity is mentioned by some authors, either when the plant is grown on soil^[44] or in culture solution^[19]. This is probably a consequence of Cu accumulation in the roots, due to its reduced translocation to the shoot. In the present research, the highest Cu accumulation in all studied species was also shown in roots. The retention of Cu in roots is considered as one of the mechanisms for Cu tolerance in some crop species. So that, differences between plant species in Cu tolerance was assumed to be directly related to the Cu content of the shoots^[45]. In this study, shoot exclusion was observed in all studied species and Cu concentrations was 2-5 times higher in roots than in shoots. However, a correlation between shoot Cu concentration and tolerance of species was proposed by Marschner^[1] not shown in this study. Rice, accumulated a higher amount of Cu in both shoot and root, simultaneous with a relative high tolerance to toxicity. On the other hand, maize which did not accumulate Cu higher than the critical level for many other crops, showed a great inhibition of growth in the presence of Cu as low as 25 μmol . For most crop species, the critical level of Cu in the leaves is above 20-30 $\mu\text{g g}^{-1}$ DW^[46,47]. It could be concluded that, differences between rice and two other species are related to differences in Cu tolerance at tissue levels, e.g., compartmentation or detoxification mechanisms. The magnitude of Cu accumulation in rice was in the range of some hyperaccumulator species such as *Faraoa chalcophila*^[48].

On the other hand, a significant amount of Cu was accumulated in root apoplasmic space of all three studied plants, so that, the concentration values of non-washed roots for example in rice was as high as 11 mg g^{-1} RDW, i.e., up to 2.8 times higher than roots which were washed for 1h for removing excess copper from apoplasm. Additionally, a high accumulation of Cu in root apoplasm of rice and sunflower, compared to maize, was associated with high metal transport to shoots. It was reported that, the root apoplasm can serve as transient storage pool for heavy metals such as Fe and Zn which can be translocated subsequently into the shoots^[49]. The size of this storage pool for Fe plays a role for genotypical differences in sensitivity to Fe deficiency in soybean^[50].

In contrast to dry weight data, the chlorophyll content of plants responded more negatively in rice than other two species. In sunflower, not only the chlorosis was not observed, but also an increase was detected.

Leaves of this plant became dark green in the presence of Cu up to 100 μmol . Copper toxicity may induce chlorosis as the result of induction of an iron deficiency^[51,52]. Chlorosis can also be a direct result of the action of high Cu concentrations on lipid peroxidation and thus the destruction of the membranes^[53,54]. However, in this study, any reduction of chlorophyll content in sunflower, could be the result of a protecting mechanism, which cause rather an increase of chlorophyll as the result of growth inhibition. Further studies are necessary for confirmation of this hypothesis.

Root elongation appears to be more sensitive to Cu toxicity than root initiation^[55]. Inhibited elongation might be related to the sharp decrease in IAA oxidase activity in roots exposed to high Cu concentrations^[56]. In addition, damages of the plasma membrane of root cells, as reflected by enhanced K^+ efflux, are immediate responses to a high Cu supply^[16,43,57]. The increased permeability of the cell plasma membrane is considered to constitute the primary toxic effect of Cu^[16]. Tolerance to Cu in *silene vulgaris* involves differences in the susceptibility of cell membrane to damage. In this research, in agreement with the growth results, the K^+ efflux from roots of rice was not significantly increased in response to Cu treatments. This parameter was increased in sunflower, as the result of loss of membranes integrity. According to Murphy^[24], short-term (up to 4 h) Cu-induced efflux is mediated by channels, while peroxide-induced K^+ efflux represents leakage through non-specific lesions in the lipid bilayer during a long-term Cu treatment. Consequently, because of a long-term Cu treatment in our experiment, the cause of K^+ leakage from roots is peroxidation of membranes by free radicals produced in the presence of excess Cu. Surprisingly, in maize plants the constitutive K^+ leakage was higher than other species and decreased in response to Cu toxicity. These experiments were carried out in 5 time intervals and similar tendency was observed. Therefore, any experimental error could be ruled out. It is possible that, because of an extreme damage occurred in the presence of Cu, any K^+ could be adsorbed to exchange sites on roots during the loading period. Therefore, a tendency to a lower K^+ pool in the root apoplasmic space toward the higher Cu treatments, in turn, could cause a reduction of leakage to medium. This hypothesis should be further studied.

The presence of supplemental Ca restored partially the shoot growth only in maize and sunflower. However, it was not accompanied by lower transport of Cu, in contrast, it rather increased in maize. It could be assumed that Ca increased tissue tolerance to Cu. Ameliorating effect of Ca on root length was significant only in rice. Reduction of calculated Cu uptake rate in response to Ca

in rice and sunflower (Fig. 3), was not due to reduction of RDW (Table 3), but was the result of decreased Cu content of roots. It means that, Ca prevented the Cu uptake to roots in rice and sunflower. The effect of Ca on the inhibition of Cu uptake and transport, could be the result of competition between Cu and Ca for the exchange sites of root cells, which could cause a reduction in Cu influx into roots and consequently transport to shoot. An antagonistic effect of Ca^{2+} and with lesser extent of Mg^{2+} and monovalent cations on the uptake of toxic metals such as Cd^{2+} and Pb^{2+} was reported^[16-18]. However, in maize, Ca increases both uptake and transport rate values, also not due to reduction in RDW but only due to increase of shoot and root content of Cu. The cause of different responses of these species to Ca is not known. It seems that, in maize, because of severe toxicity effect of Cu at 100 μmol , Ca at 5 mmol acts also as another stress factor disturbing the uptake processes. This assumption is in agreement with the fact that, at 50 μmol Cu, Ca decreased uptake and transport of Cu in maize similar with that in two other species at 100 μmol Cu.

Changes in the calculated uptake rates under higher light intensity in all studied plants, was also the result of change in Cu content. It means that, Cu influx into roots was increased in high light grown rice plants and in contrast, decreased in two other species. However, these changes were not correlated with growth responses of plants to interaction of various light intensity with Cu toxicity effect. The cause of change in Cu uptake of plants grown at different light intensities is not known. It could be the result of some complex interactions between plant hormones, other macronutrients and change in root or shoot demand.

The higher values for growth parameters in HL plants in all studied species under Cu toxicity effect, was accompanied by a significant dilution of Cu in shoot and root. On the other hand, growth under high light conditions could improve acclimation of plants to production of free radicals, which is of high significance in Cu toxicity response. It was also reported that, because of a higher antioxidants capacity, toxicity effect of heavy metals could be alleviated in preacclimated plants to high light conditions^[58].

The effect of various Al concentrations on respiration of whole root system in both sensitive and tolerant cultivars of wheat^[20] and sorghum was studied^[59,60]. At low concentrations, a stimulation and at high concentrations an inhibition of respiration of whole root system was observed^[20]. This was in accordance with the effects of Al on growth, which was not affected by the low Al concentrations. In this work, stimulation of respiration of whole root system in rice and maize, is the

result of modified root metabolism in the presence of toxic levels of Cu. It was suggested that, the presence of Al imposes a demand for additional respiratory energy for roots^[61]. However, the increased respiratory costs have little to do with mechanism of tolerance^[61].

A significant reduction of root respiration rate in sunflower, could be the result of a greater modification of metabolism of root. This reduction which was observed in Cu levels as low as 25 μmol , could be a reflection of higher sensitivity of sunflower roots to Cu toxicity which is in accordance with growth rate of root.

This is the first report on the retranslocation of a heavy metal at toxic levels in a long term experiment using root-fed plants. A higher retranslocation potential for Cu in maize and sunflower in association with a high sensitivity at tissue level (lower critical concentration) could be one of the important causes of stronger inhibition of growth in these plants. The distribution of Cu among leaves of various age i.e., different effect on growth response, could be important than total shoot Cu content.

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