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Chelate-assisted phytoextraction: effect of EDTA and EDDS on copper uptake by *Brassica napus* L.

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Abstract: Chelate-assisted phytoextraction is proposed as an effective approach for the removal of heavy metals from contaminated soil through the use of high biomass plants. The aim of the present study was to compare the efficiency of the two chelators: EDTA and biodegradable EDDS in enhancing Cu uptake and translocation by *Brassica napus* L. grown on moderately contaminated soil and treated with increasing concentrations of EDTA or EDDS. Increasing amounts of EDDS caused serious growth suppression of *B. napus* and an increase in shoot metal concentrations. Growth suppression limited the actual amount of phytoextracted Cu at high concentrations of EDDS. The maximum amount of extracted Cu was achieved by the application of 8.0 and 4.0+4.0 mmol kg^{-1} EDDS. The shoot Cu concentrations after EDTA application were much lower than with EDDS at the same doses. According to these experiments, EDTA does not appear to be an efficient amendment if Cu phytoextraction with *B. napus* is considered but EDDS is.

Keywords: phytoextraction; copper; EDTA; EDDS; *Brassica napus* L.

INTRODUCTION

Soil pollution by heavy metals is a widespread problem posing considerable threats to the environment. Copper (Cu) enters the soil by deposition from local foundries and smelters, through manuring with contaminated sludges and from application of fungicides. With its known antifungal and algaecidal properties, elevated levels of Cu in soil adversely affect microbially mediated soil processes.[1]

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Although clean-up of contaminated sites is necessary, often the application of environmental remediation strategies is very expensive and intrusive.[2] Thus, the development of a low-cost and environmentally friendly strategy is needed.

Phytoremediation is a method for *in situ* clean-up of contaminated soils. This technique uses the ability of certain plants to accumulate heavy metals in high concentrations in their above-ground parts.[3] The development of large-scale phytoextraction techniques could consider crop species as bioaccumulators of heavy metals; in fact, some of them can accumulate heavy metals while producing high biomass in response to established agricultural management.[4,5]

The major problem hindering plant remediation efficiency is that some of the metals are immobile in soils and their availability and phytoextraction rates are limited by solubility and diffusion to the root surface.[6,7] Synthetic chelators, *e.g.*, ethylenediamine tetraacetic acid (EDTA), have been used to artificially enhance heavy metals solubility in soil solution from the soil solid phase and thus to increase phytoavailability of heavy metals. The addition of chelators into the soil induces phytoextraction and translocation of heavy metals from the roots to harvestable, above-ground parts of plants.[8] The use of chelators is especially important for induced phytoextraction of Cu, since in general, the Cu concentration of plants tends to be internally rather than externally regulated. Plants use an exclusion strategy, comprising the avoidance of metal uptake and restriction of metal translocation from roots to the shoots, to adapt to toxic Cu concentrations in soil. Only high concentrations of phytoavailable Cu, *e.g.*, achieved by chelator addition, result in a breakdown of the exclusion mechanism and enhanced Cu uptake.[6]

One of the main drawbacks of chelator-induced phytoextraction is that most synthetic chelators, such as EDTA, form chemically and microbiologically stable complexes with heavy metals that pose a threat of groundwater contamination.[9,10] Ethylenediamine disuccinic acid (EDDS) is a structural isomer of EDTA and has two chiral carbon atoms and three stereoisomenrs.[11] Among them, only the (*S*,*S*) isomer is readily biodegradable. It is a low-toxic chelator with strong chemical affinity for heavy metals that produces benign degradation products,[12] which makes it a potentially suitable replacement of EDTA in chelate-assisted phytoextraction. Meers *et al*.[13] describe a high degree of biodegradability for EDDS with observed half lives ranging from 3.8 to 7.5 days, depending on the application rates.

Phytoextraction with *Brassica napus* L. has the potential to become a profitable enterprise when combined with biofuel production, especially in view of the expected increasing oil prices over the coming years. The aim of the present study was to compare the efficiency of the two chelators: EDTA and biodegradable EDDS in enhancing Cu-uptake and translocation by *B. napus* L. grown on moderately contaminated soil.

EXPERIMENTAL

The contaminated soil used in these experiments was collected from a former vineyard in a vine growing area near Novi Sad, Serbia with an over five-decade history of soil contamination with Cu-containing pesticides. The soil was air-dried, homogenized and sieved through a 2-mm stainless sieve before analysis.

The water and potential soil pH, organic matter content, free $CaCO_3$ content, specific electrical conductivity (*EC*), cation exchange capacity (*CEC*) and the content of exchangeable cations (Ca, Mg, K and Na) in the soil were determined in accordance with ISO methods for soil quality.[14-18] The particle size distribution was determined in the < 2 mm fraction by the internationally recognized pipette method.

Total soil Cu concentration was determined by microwave assisted digestion using the Usepa method 3051A[19] employing a Milestone Ethos 1 microwave sample preparation system. Analysis was subsequently performed using inductively coupled plasma-optical emission spectrometer, ICP-OES, (Varian Vista Pro-axial).

To assess the distribution of Cu among various components of the soil, a fractionation analysis was performed according to the sequential procedure of Tessier *et al.*[20] The exchangeable fraction was released with 1 M $MgCl_2$ at pH 7, the carbonate fraction with 1 mol dm^{-3} CH_3COONa (pH 5), the reducible fraction with 40 mol m^{-3} $NH_2OH{\cdot}HCl$ in 25 % CH_3COOH (95 °C) and the oxidizable fraction with 30 % H_2O_2 in 20 mol m^{-3} HNO_3 (pH ~2 and 85 °C).

Pot experiments were performed during April–June in an outdoor vegetation hall. The pots were filled with 5 kg of air dried soil and brought to 2/3 of field capacity with deionized water. Subsequently, ten seeds of a spring variety of *B. napus* were sown in the pots and after germination, thinned to two plants per pot. Considering the duration of the pot experiments (11 weeks), all pots were fertilized with a mineral fertilizer solution to avoid limiting nutritional conditions. The nutrient solution contained 1.00 g of N (2.86 g of NH_4NO_3) per pot. The soil moisture content was maintained constant at 2/3 of field capacity. After 7 weeks of growth, the pots were treated with the soil amendments outlined in Table I.

TABLE I. Chelator concentrations used for the treatments in the plant experiments (applied 4 weeks before harvest). Ctrl presents the untreated control, treatments A4 and A5 received a second application 7 days after the initial treatment

Treatment	Concentration, mmol kg^{-1}	Chelator
Control	0.0	–
A1	2.0	EDDS
A2	4.0	EDDS
A3	8.0	EDDS
A4	2.0 + 2.0	EDDS
A5	4.0 + 4.0	EDDS
B1	2.0	EDTA
B2	4.0	EDTA
B3	8.0	EDTA

The second application of EDDS was performed 7 days after the first based on EDDS data on half lives ranging from 3.8 to 7.5 days.[13] EDTA and EDDS, in the form of Na-salts, were dissolved in deionized water and applied to the top of the pot. Chelate treatment closer to the harvest was preferred as opposed to pre-sow or post-germination treatment to avoid possible growth suppressions. As observed by Meers *et al.*[21] and Lesage *et al.*,[22] phytotoxic effects by metal mobilization in a pre-sow or post-germination treatment considerably limited the success of metal extraction due to severely reduced biomass production. The harvest time was selected to be 4 weeks after chelate addition based on results given in literature,[13,23] in which a surge in metal accumulation by plants was observed 3 weeks after chelate addition.

The plants were harvested 11 weeks after sowing, oven dried at 60 °C to constant mass and weighed to determine the dry weight biomass production. The plant roots were separated from the soil, washed three times with deionized water, oven dried at 60 °C to constant mass and weighed. The total concentrations of Cu in the plant tissues were determined by ICP-OES (Varian, Vista-Pro) after digestion in a mixture of 10 ml of HNO_3 (65 %) and 2 ml of H_2O_2 (30 %) using the microwave technique.

To study the effects of the various amendments on the translocation of Cu, the translocation efficiency (τ), defined as the fraction that after root absorption was successfully translocated to the above-ground plant parts was used, *i.e.*,:

$$\tau\,(\%) = 100\times Cu\mathrm{shoot}\times DEW\mathrm{shoot}/(Cu\mathrm{shoot}\times Da_{\mathrm{shpot}} + Cu\mathrm{root}\times DEW\mathrm{root})$$

where *Cu*shoot and *Cu*root are the heavy metal concentration in the shoot and root (μg g^{-1}), respectively, and *DW*shoot and *DW*root are the dry weight production in the shoot and root (g), respectively.[21]

Statistical analysis was performed using Statistica 7 (StatSoft, Inc. Corporation, Tulsa, OK, USA) and Excel (Microsoft Inc., Seattle, NY, USA) software packages. Means of replicates and evaluation of significance of differences between the various treatments were determined by descriptive statistics and one-way Anova analysis, followed by the Tukey *post hoc* test (α = 0.05). Correlations between amendment concentration, dry weight production and shoot heavy metal concentrations were evaluated using Pearson's correlation coefficient.

TABLE II. Basic physical and chemical properties of the soil under study

pH-H_2O	–	8.22
pH-KCl	–	7.15
EC	μS cm^{-1}	107.3
Clay (< 2 μm)	%	17.2
Silt (< 20 μm)	%	32.1
Fine sand (20–200 μm)	%	47.2
Sand (200–2000 μm)	%	2.90
$CaCO_3$	%	3.82
OM	%	2.44
CEC	$cmol_{(+)}$ kg^{-1}	23.7
Exchangeable Ca	$cmol_{(+)}$ kg^{-1}	14.8
Exchangeable Mg	$cmol_{(+)}$ kg^{-1}	2.28
Exchangeable Na	$cmol_{(+)}$ kg^{-1}	0.10
Exchangeable K	$cmol_{(+)}$ kg^{-1}	1.45
Total Cu	mg kg^{-1}	256.4
Cu in soil fractions		
Exchangeable	mg kg^{-1}	1.2
Precipitated with carbonates	mg kg^{-1}	23.3
Bound to Fe + Mn oxides	mg kg^{-1}	124.0
Bound to organic matter	mg kg^{-1}	39.3

RESULTS AND DISCUSSION

According to the basic physical and chemical characteristics summarized in Table II, the soil used in the pot experiments can be classified as alkaline with a medium content of organic matter. The soil texture was clay loam. The total Cu content was 2.5 times higher than the maximum allowable concentration (*MAC*) of 100 mg kg^{-1} for agricultural soils, as prescribed by the laws of the Republic of Serbia.[24] The chemical fractionation of Cu in the soil enabled the determination of Cu concentrations in the exchangeable, carbonate, Fe + Mn oxides and organic matter fractions of the soil. As shown in Table II, Cu was predominantly bound to Fe and Mn oxides. The very low concentrations of Cu in bioavailable forms (exchangeable and precipitated with carbonates) limit its phytoavailability.

The dry matter yields of *B. napus* are shown in Fig. 1. When no chelates were added to the soil, all of the plants showed normal development without visual symptoms of metal toxicity. The treatments with 2.0 mmol kg^{-1} soil EDTA, 2.0 and 2.0 + 2.0 mmol kg^{-1} soil EDDS had no significant effect on the shoot biomass. However, the treatments with 4.0, 8.0 and 4.0 + 4.0 mmol kg^{-1} soil EDDS significantly affected plant growth and the shoot dry matter yields decreased to 63, 35 and 41 % of the control plants, respectively. Serious growth suppression upon EDDS addition at higher doses indicates that the plants were

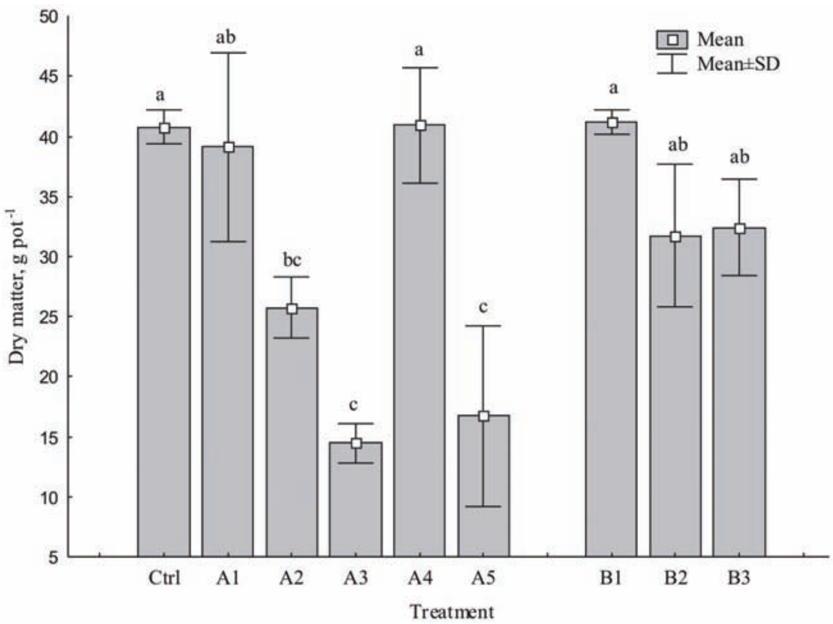

Fig 1. Effects of the application of chelates on the dry matter yields of shoots in *B. napus*. The values are means ±*SD* (n = 3); the superscript letters (a, b, ab, c) denote statistically different treatments according to the Tukey test (P = 0.05). (For detailed description of the treatments, *cf*. Table I).

subjected to heavy metal stress. This is supported by the significant negative correlation between dry-weight production of *B. napus* and the shoot Cu concentration (Table III).

The treatments with 4.0 and 8.0 mmol kg^{-1} soil EDTA appeared to be less toxic to *B. napus* compared to EDDS, decreasing the shoot dry matter yields to 78 and 79 % of the values for the control plants, respectively, which is similar to the results reported by Luo *et al*.[25] for the effect of 5.0 mmol kg^{-1} EDTA on the shoot dry matter of *Zea mays* L. and *Phaseolus vulgaris* L. The smaller effect of EDTA on plant growth is also visible through the lower coefficient of correlation between the EDTA dose and the dry weight production of *B. napus* compared with the same coefficient for EDDS (Table III).

TABLE III. Pearson's correlation coefficients between amendment concentrations, dry weight production of *B. napus* (DW), Cu concentrations in shoot and root and the phytoextracted amount of Cu

	EDDS	DW	Cu_{shoot}	Cu_{root}	$Cu_{phytoextracted}$
EDDS	–	–0.812[a]	0.811[a]	–0.626[a]	0.754[a]
DW		–	–0.790[a]	0.681[a]	–0.635[a]
Cu_{shoot}			–	–0.657[a]	0.898[a]
Cu_{root}				–	–0.662[a]
$Cu_{phytoextracted}$					–
	EDTA	DW	Cu_{shoot}	Cu_{root}	$Cu_{phytoextracted}$
EDTA	–	–0.672[b]	0.735[b]	0.558	0.678[b]
DW		–	–0.648[b]	–0.274	–0.342
Cu_{shoot}			–	0.254	0.925[a]
Cu_{root}				–	0.272
$Cu_{phytoextracted}$					–

[a]Correlation is significant at the 0.01 level; [b]correlation is significant at the 0.05 level

At harvest, the concentration of Cu in the control plants was 16.5 mg kg^{-1} dry weight in the shoots and 220.6 mg kg^{-1} dry weight in the roots, which is in good agreement with the results of experiments on copper uptake by *B. napus* when no amendments were applied.[26,27] These results indicate that Cu uptake and translocation from roots to shoots was limited in the absence of amendments.

In the present study, the most significant increase in Cu concentration in the plant shoots occurred at the doses of 4.0+4.0 and 8.0 mmol kg^{-1} EDDS, when the Cu shoot concentration was approximately 18 times higher than in the control plants and the application of 4.0 mmol kg^{-1} EDDS increased Cu uptake by approximately 8 times (Table IV). In the present experiments, the treatment with 4.0 mmol kg^{-1} EDDS resulted in a much greater Cu uptake than was the case in experiments by other authors who studied Cu uptake by other species of the family *Brassicaceae* at 3.0 and 5.0 mmol kg^{-1} EDDS and found that Cu shoot uptake increased three to four times.[13,28,29] It is possible that the increased uptake of Cu shown here was a result of the fact that the present experiments were set up in a way that simulated field conditions to a great extent (growing season, outdoors, natural light, a small number of plants per pot).

TABLE IV. Shoot and root Cu concentration (mg kg^{-1} dry weight) and translocation efficiency (%) with application of EDTA and EDDS. Values are means ±*SD* (*n* = 3); the superscript letters (a, b, ab, c) denote statistically different treatments according to the Tukey test (*P* = 0.05)

Treatment	Cu_{shoot}	Cu_{root}	Translocation efficiency
Untreated control	16.6 ± 2.3^{a}	220.6 ± 18.1^{ab}	33.6 ± 8.9^{a}
EDDS, mmol kg^{-1} soil:			
2.0	38.6 ± 16.8^{a}	244.4 ± 30.3^{ab}	52.2 ± 6.3^{ab}
4.0	131.5 ± 6.3^{ab}	91.2 ± 13.0^{a}	92.7 ± 4.4^{c}
8.0	316.4 ± 208.2^{b}	78.1 ± 32.1^{a}	93.1 ± 8.3^{c}
2.0 + 2.0	40.0 ± 10.7^{a}	236.6 ± 78.4^{ab}	55.7 ± 12.8^{b}
4 + 4	295.6 ± 43.9^{b}	143.7 ± 6.9^{ab}	89.1 ± 0.8^{c}
EDTA, mmol kg^{-1} soil:			
2.0	34.2 ± 2.8^{a}	202.1 ± 111.5^{ab}	53.4 ± 15.7^{a}
4.0	51.5 ± 19.3^{a}	287.8 ± 117.9^{ab}	52.5 ± 20.1^{a}
8.0	52.0 ± 11.6^{a}	390.6 ± 186.2^{b}	41.6 ± 10.6^{a}

There was no statistically significant increase in shoot Cu concentration compared to the control at the doses of 2.0 and 2.0+2.0 mmol kg^{-1} EDDS and it may be speculated that these treatments were insufficient to break down the uptake barriers of the plant under the conditions of the present experiments. The significant difference in metal uptake when 4.0 mmol kg^{-1} EDDS was applied in a single and split dose can be explained in light of ligand half lives;[13] the half lives in soil were estimated to be 4.7 days for 2.4 mmol kg^{-1} and 7.5 days for 4.0 mmol kg^{-1} EDDS. According to these findings, when EDDS was applied at a dose of 2.0 mmol kg^{-1}, the concentration of metal-chelate complex would be significantly decreased before the second application, performed 7 days after the first, keeping the metal–chelate concentration too low to break down the plant uptake barriers. On the other hand, there was no statistically significant difference in metal uptake between single and split applications of 8.0 mmol kg^{-1} EDTA, which was probably due to the prolonged ligand half life at the higher concentration.

The addition of EDTA to the soil at doses of 4.0 and 8.0 mmol kg^{-1} increased the Cu uptake by approximately 3 times, which is in good agreement with results of other studies in which application of EDTA at 3.0 to 5.0 mmol kg^{-1} increased the Cu uptake by *Brassicaceae* by 2 to 3.5 times.[10,28,30] However, the shoot Cu concentration was 2.5 and 6 times lower than with EDDS at the same doses. This observation was consistent with the observation that EDTA was less toxic to *B. napus* than EDDS (Fig. 1), which is also supported by the less significant correlations between EDTA dose and shoot Cu concentrations.

The results of the present experiments suggest that EDDS can be regarded as a better candidate chelate for the phytoextraction of Cu in soils. The effectiveness of chelate-enhanced metal accumulation by *B. napus* was consistent with the greater ability of EDDS than EDTA to solubilize soil metals.[13,25] The higher observed mobilization of Cu by EDDS could not be explained by its respective stability constants with the two chelators: log K = 18.7 for Cu–EDTA and log K = 18.4 for Cu–EDDS. These stability constants would suggest equal or better mobilezation of Cu by EDTA. The higher mobilization of Cu by EDDS in the current experiments can be explained by lower affinity (based on stability constants) of EDDS for competitor ions, such as: Ca^{2+} (log $K_{Ca–EDDS}$ = 4.2; log $K_{Ca–EDTA}$ = = 10.6), Mg^{2+} (log $K_{Mg–EDDS}$ = 5.8; log $K_{Mg–EDTA}$ = 8.8), Fe^{3+} (log $K_{Fe–EDDS}$ = = 22.0; log $K_{Fe–EDTA}$ = 25.0) and Mn^{2+} (log $K_{Mn–EDDS}$ = 9.0; log $K_{Mn–EDTA}$ = = 13.8).[31]

The limited translocation of heavy metals following absorption by the roots is one of the bottlenecks limiting the overall efficiency of phytoextraction. In their patent on the induced hyper accumulation of metals in plant shoots, Ensley *et al*.[32] described chemically enhanced phytoextraction as a two-step process. The plants first accumulate metals in their roots. Induction is then applied, which enhances the transfer of the metals to the shoots. This transfer is attributed to a disruption of the plant's metabolism, which regulates the transport of metal to shoots. The respective translocation efficiency values are presented in Table IV. The translocation efficiency for Cu in the untreated control was 33 %, which was lower than that reported by Marschiol *et al*.[26] when *B. napus* grown on soil polluted with 280 mg kg^{-1} Cu with no amendments achieved an efficiency of 57 %. The results of the present experiments indicate that the application of EDDS can dramatically increase the translocation of Cu from the roots to the shoots of *B. napus*. No statistically significant improvement was observed only at the dose of 2.0 mmol kg^{-1}, medium translocation efficiency was observed after the treatment with 2.0+2.0 mmol kg^{-1} EDDS, and when 4.0, 8.0, and 4.0+4.0 mmol kg^{-1} EDDS was applied, the translocation efficiency increased from 33 to 93 %. Similar efficiency in Cu translocation after the application of 5.0 mmol kg^{-1} EDDS was reported in the literature for corn (from 8.5 to 83 %) and beans (from 10.2 to 93 %).[25] It appears that only at very high phytoavailable Cu concentrations can the breakdown of the exclusion mechanisms result in a greatly enhanced Cu uptake. The increases of the translocation efficiency after the application of 2.0, 4.0 and 8.0 mmol kg^{-1} EDTA were very small and statistically insignificant compared to the control. Moreover, this is in good agreement with efficiencies that Luo *et al*.[25] obtained for corn (39 %) and bean (50 %) after the application of 5.0 mmol kg^{-1} EDTA.

The phytoextracted amount of Cu is the product of the metal concentration in the shoots and the dry-weight yield of the plant (Fig. 2). Although increasing doses of EDDS resulted in increased shoot Cu concentrations, up to 18 times, compared to the control, the phytoextracted amount of Cu did not follow the same order of magnitude due to growth suppression at high EDDS concentrations. The only statistically significant increase in phytoextracted Cu compared to the control was achieved after the application of 8.0 and 4.0+4.0 mmol kg^{-1} EDDS, when totals of 4.6 and 4.5 mg Cu per pot were phytoextracted, respectively.

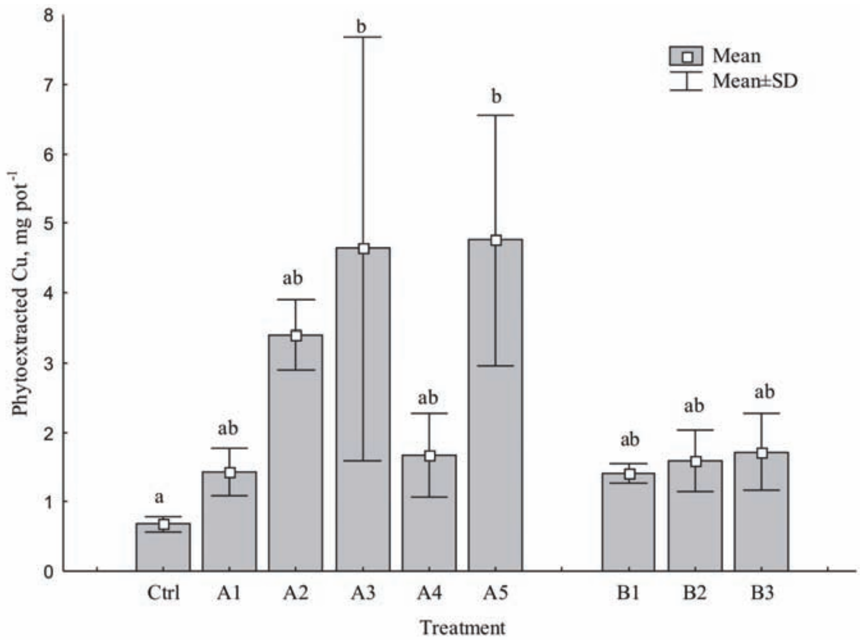

Fig. 2. Phytoextracted amount of copper (mg pot^{-1}) at different amendment concentrations The values are means ±*SD* ($n = 3$); the superscript letters (a, b, ab) denote statistically different treatments according to the Tukey test ($P = 0.05$). (For detailed descriptionof the treatments, *cf*. Table I.).

The amount of phytoextracted Cu after EDTA application did not differ statistically from the control even at the highest dose, although growth suppression was smaller than in the treatment with EDDS, as the Cu concentrations in the above-ground plant parts were only 2 to 3.5 higher than in the control.

Considerably smaller metal extraction rates were also found in other studies and they may be related to toxicity problems leading to yield reduction.[10,13,26] It could be, therefore, realistically hypothesized that they could perform better in the case of light soil pollution.

CONCLUSIONS

The two main important bottlenecks in the phytoextraction process are the limited bioavailability of heavy metals in soils and the limited translocation to the shoots. The presented experiments tried to overcome these bottlenecks by adding EDDS or EDTA to Cu-polluted soil. The soil originated from a former vineyard and contained low concentrations of Cu in an exchangeable form. Increasing amounts of EDDS caused serious growth suppression of *B. napus* and an increase in shoot metal concentrations, leading to the assumption that plants suffered heavy metal stress. Growth suppression limited the actual amount of phytoextracted Cu at high concentrations of EDDS. The maximum amount of extracted Cu was achieved by the application of 8.0 and 4.0+4.0 mmol kg^{-1} EDDS. The shoot Cu concentrations after EDTA application were much lower than with EDDS at the same doses and there was no statistical difference in phytoextracted amount of Cu between the control and EDTA treatments.

According to the performed experiments, EDTA does not appear to be an efficient amendment if Cu phytoextraction with *B. napus* is considered but EDDS does.

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ИЗВОД

ФИТОЕКСТРАКЦИЈА ПОТПОМОГНУТА ХЕЛАТОРИМА: ЕФЕКАТ EDTA И EDDS НА ВЕЗИВАЊЕ БАКРА КОД *Brassica napus* L.

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Сматра се да употреба биљака са високом биомасом за фитоекстракцију потпомогнуту хелаторима може представљати ефикасан начин за уклањање тешких метала из контаминираног земљишта. Циљ овог истраживања је био да се упореди ефикасност два хелатора: EDTA и биодеградабилног EDDS у повећању везивања и транслокације бакра код врсте *Brassica napus* L. гајене на умерено загађеном земљишту. Растуће концентрације EDDS су изазвале и повећано везивања бакра и изражен застој у порасту надземног дела биљке *B. napus* L. Количина фитоекстрахованог бакра при високим концентрацијама EDDS је била ограничена застојем у порасту надземног дела биљке. Највећа количина фитоекстрахованог бакра је постигнута са применом 8,0 и 4,0+4,0 mmol kg^{-1} EDDS. Концентрација бакра у надземном делу након примене EDTA је била много нижа него приликом примене EDDS у истим концетрацијама. На основу резултата добијених у овом експерименту, утврђено је да, за разлику од EDDS, EDTA није довољно ефикасан хелатор за фитоекстракцију бакра помоћу *B. napus* L.

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