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Enhancement of metal(loid)s phytoextraction by *Cannabis sativa* L.

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Abstract

The key factor for the phytoextraction efficiency is bioavailability of extracted element. Mobility and bioavailability of metal(loid)s is affected by numerous soil factors. To improve the metal(loid)s accumulation capacities, the addition of chelating agents has been proposed. The objectives of this research was to investigate the ability of different chelating agents (EDTA, humic substances) and glutathione to enhance the metal(loid)s phytoextraction by four *Cannabis sativa* L. cultivars. Our results showed that metal(loid)s accumulation in plants increased with increasing concentration of metal(loid)s in growing solution; although, the metal distribution in plant parts was various. Generally, all metal(loid)s were accumulated mainly in roots except arsenic that was detected primarily in shoots. However, our results showed that metal(loid)s accumulation depended on chosen cultivar and there was no existing strategy for metal detoxification in *C.sativa*. Tested chelates enhanced the transfer from roots to shoots. Assuming that EDTA had a positive effect on the metal(loid)s mobility, a larger amount of metal(loid)s is taken up and translocated to the shoots, while an effect of humic substances wasn't statistically proved. Nevertheless, glutathione application increased metal(loid)s accumulation in roots. *C.sativa* plants demonstrated to possess the ability to transfer arsenic, cadmium, copper and zinc from root to shoot, one of the criteria that must be met to consider a plant well suited for phytoextraction.

Key words: Phytoextraction, chelate, glutathione, *Cannabis sativa* L., metal.

Introduction

Low contaminated sites near the industrial plants are not suitable for food crops cultivation [1]. Contaminants accumulated in food crop may cause danger to human health. Though, low contaminated sites seem to be applicable for other useful plants. Nowadays, there is a huge energy demand; therefore, the growth of energy crops on polluted sites can be feasible.

Industrial hemp is a crop with a broad range of applications. Its best known products are based on hemp fibers. Generally, the fiber content in plant is about 25% [2], the rest of the wood material is pressed into briquettes or it is used as thermal insulating materials [3]. Due to a high biomass production, hemp can be used as an energy crop and at the same time it can clean up the environment. Its growth on contaminated soil can reduce the pollution through a transfer of the contaminant from soil to harvestable plant parts [4]. Green technologies as phytoremediation have recently attracted a great deal of attention as an alternative means of soil and water pollution [5-8]. For example, phytoextraction technique uses an ability of plants to uptake of toxic metal(loid)s from soil [9-11]. A potential of different plant species for phytoextraction was broadly studied [12, 13]. Phytoextraction by hemp was studied for cadmium, lead, zinc, copper, chromium and nickel [14-16]. Fewer studies focused on a fibre quality. Linger *et al.* [6] have recently shown no effect of heavy metal contamination on fibre fineness and strength. According to other authors, hemp is suitable for growing in industrially polluted regions, and it can be used for reclamation of heavy metal contaminated soils with further utilization of biomass in the industry [4, 17]. Hemp is also feasible for anaerobic sewage sludge management. It can remove heavy metals from sludge and at the same time the addition of sludge increases hemp biomass production [18].

Plants accumulate from soil metal(loid)s that have either essential or nonessential function. Each plant species requires different element composition and concentration. Generally, plants take up macronutrients as nitrate and phosphate, essential elements such as chromium, copper, iron, manganese and zinc [19], nonessential elements such as cadmium, cobalt, mercury, lead and vanadium [9, 16] and radioactive elements such as cesium, strontium and uranium [20, 21]. Lack of nutrients can cause serious crop production problems [22]. Such conditions lead to a synthesis of specific substances that can make the lacking elements bioavailable. For instance, roots of higher plants cultivated in solution without nutrients could secrete proteases and increase the level of free amino acids in the soil solution as a source of nitrogen [23]. However, phytotoxic amounts of both essential and nonessential metal(loid)s in plants can lead to an inhibition of plant growth [24, 25].

Metal(loid)s affect enzyme mechanisms by binding to sulphydryl groups in proteins or by displacing of the essential metals in metalloproteins or metalprotein complexes [26]. Some metals affect chloroplast apparatus. For instance, cadmium affects chlorophyll synthesis, water splitting, Calvin cycle enzymes and regulation of energy distribution of PS 2 [27]. Indeed, plants response to the stress with increasing production of reactive oxygen species (ROS) [28, 29]. Low molecular weight thiols, as glutathione (GSH), play important role in defense mechanisms against ROS. GSH detoxifies xenobiotics by prior activation and conjugation with such compounds [30, 31]. Further, GSH acts as a precursor for the synthesis of phytochelatins [32].

The effectivity of phytoextraction is strongly dependent on the biomass production, metal(loid)s concentrations in plant tissues and bioavailability of extracted element [33]. The key factor is

bioavailability. Mobility and bioavailability of metal(loid)s is affected by numerous soil factors, such as cation exchange capacity, pH and organic matter content [34, 35]. To improve the metal(loid)s accumulation capacities, and the enhancement of metal(loid)s availability in soil, the addition of chelating agents has been proposed. Recently, the synthetic chelating agents such as ethylenediaminetetraacetic acid (EDTA) have been used [36]. Nowadays, the biodegradable chelating agents such as ethylenediamine disuccinate (EDDS) have been tested [14, 33]. As an alternative to above mentioned chelates natural sources such as humic substances (HS) can be used [37]. They are composed from three main fractions: humic acid, fulvic acid and humin [38]. Soluble complexes of HS and metal ions reduce metal absorption onto soil surface while increase the metal uptake into plants [39]. Humic acids (HA) were found more effective in enhancing metals uptake than EDTA in sunflower and corn [40]. Furthermore, it was stated that HAs are ideal soil amendments for phytoextraction enhancement but more studies are needed to prove the potential of HS to become effective phytoextraction enhancers [41].

The objectives of this research were to investigate the ability of different chelating agents (EDTA, humic substances) and glutathione to enhance the metal(loid)s phytoextraction by four *Cannabis sativa* L. cultivars.

Materials and Methods

Plant material: *Cannabis sativa* L. cv. Beniko, Bialobrzskie, Fibrol and Monoica, fibre hemp cultivars (Agritec, Ltd.), were cultivated in a cultivation room under controlled conditions (23°C, humidity about 60%, daily light phase of 16 hours) with supplementary light (irradiance of 115 µmol/m^2 s) in Hoagland's solution [42]. Four weeks old plants were replaced into solution with toxic metal(loid)s. The experiments were performed in triplicates.

Firstly, an ability of phytoextraction of hemp cultivars was tested. Plants were cultivated in the solution with ions of arsenic ($NaAsO_2$), cadmium ($Cd(NO_3)_2 \cdot 4H_2O$), copper ($Cu(NO_3)_2 \cdot 3H_2O$), lead ($Pb(NO_3)_2$) or zinc ($Zn(NO_3)_2 \cdot 6H_2O$) at metal(loid)s' concentrations 50, 100, 200, 500, 1000, 2000 and 5000 µmol/l.

Secondly, amendment effects on metal(loid)s accumulation were studied. Plants grew in a modified solution. The modification was realized as an amendment of 100 µmol/l of EDTA salt ($C_{10}H_{14}N_2Na_2O_8.2H_2O$), GSH (Sigma-Aldrich), or 100 mg/l of a mixture of humic and fulvic acids (Lignohumate AM - AMARGO s.r.o., Czech Republic) to Hoagland's solution. The solution also contained 100 µmol/l of metal(loid).

After one week, the plants were harvested. Roots were washed subsequently in double distilled water, in solution of EDTA (0.1 mol/l) and double distilled water. Plant leaves, stems and roots were separated, frozen at -70°C in liquid nitrogen and freeze-dried.

Metal determination: The dried plant tissues were ground to a powder and digested in 5 ml of acid mixture of 65% HNO_3 and 60% $HClO_4$ (v/v ratio 85/15) [43]. Contents of cadmium, copper, lead and zinc were measured by atomic absorption spectroscopy (SensAA, GBS, Australia). Determination of arsenic was realized by measurement at Optima 2000 DV ICP spectrometer (Perkin Elmer, USA). Metal(loid)s concentrations were calculated as a proportion of the metal(loid) amount to dry weight (DW) of the plant part.

Data analysis: The statistical treatment included calculation of mean concentrations of elements and analysis of variances to estimate statistically significant differences between groups of samples. The significance of differences was determined using Student's t-test for $\alpha \geq 0.05$.

The differences among amendment treatments of particular cultivars were tested by one-way ANOVA with Tukey's HSD multiple comparison test. Significance level P was 0.05 for both analyses. Each treatment was represented by three biological replicates. STATISTICA 8 (StatSoft, Tulsa, OK, USA) software was used for all the computations.

Results and Discussion

Phytoextraction of metal(loid)s: Our results showed that metal(loid)s accumulation in plants increased with increasing concentration in the growing solution, although the metal(loid)s distribution in plant parts was various. Tested metals (cadmium, copper, and zinc) were accumulated primarily in roots while arsenic occurred mostly in shoots. Moreover, accumulation trends of cadmium, copper, and zinc were very similar to each other. Higher concentrations of metal(loid)s in solution increased their transfer to the shoots. Metal absorption and the restriction of translocation to the shoots may be the avoidance of toxic effect of the metal on the roots [44].

Arsenic: When cultivated with the addition of arsenite, plants in 5000 µmol/l solution began to wilt next day in 2000 µmol/l, third day in 1000 µmol/l, and sixth day in other concentrations (Table 1). Arsenite ions reduced relative growth rate and developed severe browning which progressed to necrosis. At low concentrations (up to 200 µmol/l) arsenic was accumulated mostly in stems of tested cultivars, and at higher concentrations in leaves (Table 2). At 5000 µmol/l, the highest arsenic concentration was detected in the leaves of Beniko cultivar (18 mg/g DW) and the lowest in the stems of Fibrol and Monoica cultivars (both, 3 mg/g DW). In the cultivar Bialobrzskie the stems and the leaves arsenic contents were comparable, and moreover at the highest concentration the metalloid value in the stems exceeded its value in the leaves (15 and 13 mg/g DW, respectively). Conversely, it was reported that arsenic is stored primarily in roots [45, 46]. However, different arsenic translocation in plant parts depends on their sensitivity or resistance to arsenic.

The accumulation of arsenic in hyperaccumulator *P. vittata* was much lower in the roots than in the fronds, while the opposite was true in non-hyperaccumulator *P. ensiformis* [47]. It was demonstrated that tomato plants stored arsenic in roots, while arsenic in bean plants was readily transported into the shoots and accumulated in high levels in the leaves [48]. Limiting of arsenic transfer to shoots helped tomato plants against arsenic phytotoxicity, so they were more tolerant to arsenic than bean plants. Phytotoxicity of arsenite is in agreement with published data [25, 49]. It is known that arsenite is mobile and it is taken up into the plant by P-independent mechanism [50]. Once inside the plant, arsenite bound to thiol groups and interferes with enzymes mechanisms [45]. Its reaction with sulphydryl groups of proteins causes disruption of root functions, and even cellular death [51]. Transport to the shoots might be supported by a complex of As-thiol. It was demonstrated the formation of As (III)-S complexes in the roots of *Prosopis*, which were freely transported into the shoots [52]. Arsenic uptake and distribution is also strongly dependent on the type of arsenic.

According to Carbonell *et al.* [49], the ability to uptake of arsenic into the roots of *Spartina alterniflora* grew in order: dimethylarsenic acid (DMAA) < monomethylarsenic acid (MMAA) <As (V) <As (III). Inorganic arsenic and MMAA were accumulated mainly in the root system, while DMAA was readily translocated to the shoots.

Cadmium: The addition of cadmium led to gradual wilting of plants (Table 1). Third day plants started to wilt in 5000 and 2000 µmol/l cadmium solution, fourth day in 1000 µmol/l and sixth day in 500 and 200 µmol/l. Roots became brownish, less branching and reduced growth. Toxicity symptoms as stunting and chlorosis also appeared. Cadmium toxicity might cause translocation to the shoots as the avoidance of toxic effect of the metal on the roots. At the highest concentration (5000 µmol/l), the amount of the metal in the stems greatly raised up to the root levels (Table 3). Furthermore, cadmium content in the stem of Bialobrzskie cultivar was twice more higher than in the root. The plants from the solutions with other concentrations accumulated cadmium chiefly in roots. The amount in the roots varied between 22 mg/g DW (Monoica) and 30 mg/g DW (Beniko, Bialobrzskie, Fibrol). It corresponds to the literature. It was found that cannabis plants accumulated 4 g/g in root and 0.1 g/g in shoots at the cadmium concentration of 200 mg/kg in the cultivation medium [53]. Energy plants accumulated cadmium more in root, less in shoots. These results are consistent with the general assumption that the metals deposited especially in roots [46], which is probably part of the defense mechanism of plants against to toxic substances. It was found that roots of lettuce released much more of their absorbed cadmium for translocation to the roots than ryegrass or orchardgrass [54]. The effect of the increased cadmium accumulation in shoot was confirmed in *Ipomoea* stem [55]. Cadmium translocation to the shoots may be the avoidance of toxic effect of the metal on the roots [44]. Cadmium detrimental effect on photosynthetic activity, chlorophyll content, and plant growth was also proved [27, 56]. Metal induced oxidative stress that led to protein degradation through amino acid metabolism resulting in decrease of plant growth [16].

Copper: Copper is an essential element and thus plants were more tolerant to its presence in solution. Tested concentrations of copper did not cause evident toxic symptoms (Table 1). It is known that copper plays roles in photosynthesis, respiration, antioxidant activity, cell wall metabolism and hormone perception [57]. However, it was reported that copper concentration in non-contaminated soils is about 20-30 µg/g but in contaminated soils can reach levels one hundred times higher [58]. Our tested concentrations were higher than natural background and copper was concentrated mainly in the roots (Table 4). On the other hand, the amount of copper in the roots was independent on the amount of copper in solution. It was evident that all four cultivars reached similar copper level in the roots (about 15 mg/g DW) and even the higher concentration in solution did not increase it. Moreover, at the highest concentration (5000 µmol/l) the amount of the metal in the stems greatly raised up (from 15 mg/g DW for Bialobrzskie to 30 mg/g DW for Fibrol and Monoica) so copper content in the stems was bigger than in the roots. It was demonstrated that toxicity symptoms induced by copper (1500 µmol/l) were linked to a sharp rise of copper content in leaf of *Hordeum vulgare*, accompanied by oxidative stress [59]. However, any data regarding copper toxicity on *C.sativa* have not been published. Hemp plants seemed to be more resistant comparing to barley. Our results corresponded with the accumulation and distribution of copper in *Elsholtzia splendens* [60], which is known for its tolerance to high concentrations of this metal. The amount of copper in plant parts also decreased in the line: root > stem > leaf. At 500 µmol/l copper content in roots was about 8 mg/g, 1 mg/g in stems and 0.25 mg/g in leaves. The amounts of copper in plant parts of *E. splendens* agreed with our results (9-12 mg/g DW in roots, 1.7-3.8 mg/g DW in stems, and 0.2-2.4 mg/g DW in leaves).

Plant species have different tolerance strategies that protected themselves against copper toxicity. It was described by researchers that compared plants naturally growing on contaminated site [61]. In *Malva sylvestris*, exclusion of copper from the roots or its stabilization in the soil restricted its toxicity effects. *Chenopodium ambrosioides* accumulated copper in roots and then in leaves and in *Datura stramonium* most of copper was accumulated in leaves. Moreover, *D. stramonium* and *C. ambrosioides* elevated their antioxidative enzyme activities in response to copper toxicity. The protection strategy in *C. sativa* seems to be similar to *C. ambrosioides*. Due to easily binding of copper to the sulfhydryl groups of membrane proteins, a damage of the proteins can be provoked [62]. A model for effect of copper on *C. sativa* roots was also proposed [63]. Aldo/keto reductase is the first protein interacting with copper ions, it could reduce copper to Cu(I), so ions could be available for interaction with other partner proteins, like phytochelatins, which usually bind Cu(I), and from this location copper can be transported to the vacuole.

Lead: Toxic effect of lead appeared third day (Table 1). Plants wilted gradually in solution concentration of above 1000 µmol/l. Roots became brownish and reduced growth. Sixth day, wilting of plants was visible also at lower concentrations (200 and 500 µmol/l). Wilting of plants growing in solution with higher concentrations of lead was caused by an increase of lead amount in the stems (Table 5). Lead concentrations in the leaves varied from 7 to 24 mg/g DW, and at lower concentrations were comparable to the control plants (from 3 to 14 mg/g DW). Generally, lead was accumulated primarily in the roots, and then in the stems. At 5000 µmol/l, the highest lead concentration was detected in the roots of Fibrol and Monoica cultivars (both 60 mg/g DW) and the lowest one in Beniko cultivar (30 mg/g DW), Our results are in agreement with literature [4]. *Linum usitatissimum* and *C.sativa* plants growing at an industrially polluted region accumulated lead mainly in roots and less in stem and in leaves. Similarly, the accumulation of lead by *Elsholtzia* sp. also decreased in line root> stem> leaf [64]. For example, at 200 µmol/l concentration plants reached lead concentration of 20 mg/g in roots, 2 mg/g in stems and 0.15 mg/g in leaves. In our experiment we detected in roots 12–30, in stems 0.3- 0.6 and in leaves 0.002 – 0.010 mg/g DW of lead. It is evident that *C. sativa* plants transported less metal in the shoots, while plants of *Elsholtzia* sp. transported more lead in the shoots which corresponded with their known high tolerance to heavy metals. It was reported that once absorbed by the roots, lead is rather immobile, showing very limited translocation into shoots [65, 66]. According to literature, lead retention in roots is based on the binding of lead to ion exchange sites on the root cell walls and extracellular precipitation, mainly in the form of Pb carbonates [67]. Lead treatment of 300 µmol/l reduced root elongation of *Triticum*

Table 1. Wilting of plants during 7 days exposure to arsenic, cadmium, copper, lead, and zinc at 0, 50, 100, 200, 500, 1000, 2000, or 5000 [µmol/l] concentration (+ means no visible toxic symptoms, - means visible toxic symptoms).

Solution [µmol/l] / day	As							Cd							Cu							Pb							Zn						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
100	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
200	+	+	+	+	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+
500	+	+	+	+	-	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+
1000	+	+	-	-	-	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+
2000	+	+	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+
5000	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+

Table 2. Arsenic concentration [µg/g] in root, shoot, and leaf of four *Cannabis sativa* L. cultivars after 7 days of cultivation in solution with 50, 100, 200, 500, 1000, 2000, or 5000 [µmol/l] arsenic concentration; control mean Hoagland solution (arsenic concentration under limit of quantification); DW mean dry weight; standard deviation is represented as ± S.D. (n=3).

Solution [µmol/l]	Beniko			Bialobrzskie			Fibrol			Monoica		
	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]
control	5.22 ± 1.15	1.53 ± 0.37	1.22± 0.26	4.69 ± 1.46	1.64 ± 1.43	0.921 ± 0.077	0.601 ± 0.111	0.491 ± 0.046	0.425 ± 0.127	4.79 ± 0.369	1.49 ± 0.32	1.08 ± 0.405
50	64.9 ± 5.62	151 ± 7.18	29.3± 1.72	36.1 ± 4.19	181 ± 23.5	50.9 ± 3.27	34.6 ± 9.16	32.3 ± 2.75	16.6 ± 0.668	124 ± 7.47	123 ± 4.34	162 ± 8.76
100	112 ± 22.6	308 ± 45.1	55.4± 3.06	65.5 ± 5.07	259 ± 2.45	6.21 ± 0.405	65.6 ± 14.0	410 ± 12.6	58.9 ± 15.7	87.9 ± 1.43	336 ± 18.9	79.5 ± 8.03
200	161 ± 25.3	425 ± 98.1	118 ± 8.61	185 ± 9.59	365 ± 15.4	29.3 ± 1.95	128 ± 9.50	408 ± 19.4	534 ± 20.8	127 ± 6.74	286 ± 46.6	129 ± 3.02
500	292 ± 25.2	915 ± 25.1	1487 ± 442	216 ± 22.04	583 ±81.4	453 ± 28.1	244 ± 5.35	539 ± 20.3	1444 ± 196	296 ± 42.5	309 ± 14.4	1142 ± 97.8
1000	399 ± 14.2	2519 ± 85.8	3199 ± 339	518 ± 10.2	2411 ± 68.5	2107 ± 47.6	520 ± 20.1	920 ± 24.5	2247 ± 616	622 ± 27.6	582 ± 22.5	2148 ± 97.2
2000	826 ± 134	4396 ± 133	6635 ± 227	1296 ± 38.0	6430 ± 133	6180 ± 58.9	321 ± 18.3	1387 ± 145	4295 ± 896	940 ± 14.1	1937 ± 122	5085 ± 125
5000	2241 ± 279	5067 ± 314	17673 ± 2516	2913 ± 265	15347 ± 2484	12671 ± 40.1	1900 ± 80.2	3453 ± 122	8652 ± 235	1323 ± 105	3008 ± 126	12716 ± 1410

Table 3. Cadmium concentration [µg/g] in root, shoot, and leaf of four *Cannabis sativa* L. cultivars after 7 days of cultivation in solution with 50, 100, 200, 500, 1000, 2000, or 5000 [µmol/l] concentration; control mean Hoagland solution (cadmium concentration under limit of quantification); DW mean dry weight; standard deviation is represented as ± S.D. (n = 3).

Solution [µmol/l]	Beniko			Bialobrzskie			Fibrol			Monoica		
	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]
control	4.00 ± 1.04	0.441 ± 0.022	0.58 ± 0.10	1.99 ± 0.912	0.581 ± 0.282	1.06 ± 0.171	1.16 ± 0.195	0.321 ± 0.036	0.664 ± 0.422	4.91 ± 3.02	1.59 ± 0.296	0.873 ± 0.139
50	710 ± 50.6	263 ± 12.1	36.5 ± 3.20	826 ± 14.9	311 ± 54.7	31.2 ± 2.85	547 ± 31.4	211 ± 23.7	2.47 ± 0.451	657 ± 16.7	283 ± 12.5	46.4 ± 0.609
100	2561 ± 113	676 ± 43.4	188 ± 68.6	1848 ± 257	660 ± 80.4	154 ± 45.0	1437 ± 153	753 ± 19.9	124 ± 1.01	1490 ± 41.9	760 ± 35.3	67.3 ± 1.12
200	3048 ± 103	1034 ± 114	317 ± 17.9	2416 ± 218	993 ± 170	74.0 ± 2.29	3009 ± 847	636 ± 12.7	242 ± 34.3	3073 ± 175	1661 ± 223	319 ± 33.4
500	11730 ± 1204	2656 ± 628	423 ± 58.5	7091 ± 164	2335 ± 168	145 ± 10.1	9283 ± 479	6486 ± 459	529 ± 45.8	6091 ± 116	4345 ± 141	867 ± 54.1
1000	15918 ± 5.97	7960 ± 128	644 ± 43.0	19421 ± 102	7399 ± 225	665 ± 17.0	16447 ± 850	5860 ± 477	758 ± 67.6	11746 ± 231	9186 ± 194	1847 ± 42.6
2000	30385 ± 3999	14283 ± 3721	776 ± 51.4	24533 ± 1871	22556 ± 2692	732 ± 33.5	19902 ± 2357	12447 ± 1117	1025 ± 361	16676 ± 872	18497 ± 3686	3859 ± 432
5000	33457± 1432	30772 ± 8008	1813 ± 67.3	26455 ± 5276	55678 ± 7832	5180 ± 188	28972 ± 562	32293 ± 1601	3273 ± 285	22025 ± 850	22994 ± 277	14592 ± 3804

aestivum L. more than three times and significantly increased antioxidative enzymes activities [68]. It was shown that 500 µmol/l lead concentration is moderately toxic and 1000 µmol/l highly toxic to *Oryza sativa* L. plants [69]. On the other hand, *C.sativa* plants seemed to be more tolerant since the plant survived even at 5000 µmol/l concentration.

Zinc: Comparing to cadmium and lead, zinc did not led to a perishing of *C. sativa* plants (Table 1). Zinc content in plant parts increased with the increase of zinc exposure (Table 6). The accumulation trend in plant parts decreased in the line: root > stem > leaf, and the trend was equal to cadmium, copper and lead accumulation. Plants of Beniko and Bialobrzskie cultivated in 500 and 1000 µmol/l solution showed a sharp increase of zinc amount in the roots. At the highest zinc concentration (5000 µmol/l) the amount of zinc in the roots varied from 25 mg/g DW (Monoica) to 45 mg/g DW (Fibrol). Surprisingly, higher concentrations of zinc (500, 1000 µmol/l) did not led to a raise of zinc accumulation in the roots but led to a steep increase of zinc amount in the stems. Zinc content in the stems greatly rose up to the root levels in Beniko and Bialobrzskie cultivars and zinc content in the stem at 5000 µmol/l was 37and 27 mg/g DW, respectively, and in the roots 30 mg/g DW, each. It was proved that zinc translocation grew with its amount in the soil, and at higher concentration of zinc greater amount was accumulated in *C.sativa* plants [18]. Zinc content in plant parts of *C. sativa* declined in the following sequence: root > leaf > stem.

Hydroponically growing plants of *Brassica napus* and *Trifolium repens* translocated most of the zinc in the leaves and less into the stems, whereas *Agrostis stolonifera* distributed the metal equally [70]. All results confirmed that excess of zinc was transferred from the root into the shoot while zinc distribution in the shoot depended on plant species. Zinc as an essential element plays an important role in plant metabolism by an involvement in the activation of many enzymes and supply of a link between enzyme and substrate [71]. However, excess of zinc raises specific physiological and morphological changes such as an inhibition of photosynthesis, root system reduction, aerial part dwarfism, chlorosis formation or disruption of mitochondrial structures [72]. Resistant plants such as *Datura innoxia* didn't show any visible injury at 5000 µmol/l but high amount of zinc decreased the photosynthesis and stomatal conductance [73]. Other plants were more sensitive. Growth inhibition and decrease of chlorophyll content in leaves of *S. lycopersicum* was observed at concentration of 150 µmol/l [74]. Moreover, ascorbate oxidation occurred in the leaves of *Phaseolus vulgaris* L. treated by 50 µmol/l [75]. Plants of *C.sativa* seemed to belong to resistant plants since the highest tested concentration (5000 µmol/l) did not show any visible toxic symptoms.

Amendments effect: Amendments of EDTA, GSH or humic acids mixture in solutions had no significant effect on plants growth. In the presence of arsenic, cadmium and lead ions plants wilted as was mentioned previously. On the other hand, our results demonstrated that the solution enhancement had an influence on metal(loid)s accumulation and distribution in plants. EDTA increased the transfer from roots to shoots, while GSH increased metal(loid)s accumulation in roots.

Chelates: The presence of EDTA in the solution had no effect on arsenic uptake (Fig.1) but it had an effect on uptake of other metals (Figs 2-5). It significantly reduced the amount of accumulated cadmium, copper and zinc in the roots and the stems, and at the same time it increased metals transport into leaves (Figs 2, 3 and 5). Indeed, the metal contents in the roots were approximately twice (cadmium), three times (copper) or four times lower (zinc), whereas the metal contents in the leaves were approximately twice (zinc) or six times (copper) higher. The increase of cadmium content in the leaves was strongly dependent on the cultivar and varied from 0.2 to 0.7 mg/g DW, whereas its content in plants grown in solution without EDTA varied from 0.003 to 0.17 mg/g DW. Moreover, a significant increase of lead was observed in the leaves (Fig.4), the content was forty times higher comparing to the plants treated by solution without EDTA. On the other hand, humic substances (HS) had only a merely effect on metal(loid)s accumulation. The most pronounced effect was observed in roots and stems (Fig.1). For example, a significant increase (twice more) was observed in the accumulation of lead in the roots and stems of Bialobrzskie cultivar. Moreover, in the roots of the cultivar an increase of arsenic amount was detected. In contrast, a decrease in arsenic and zinc accumulation was measured in the stems of Bialobrzskie and Monoica cultivars.

It was stated that EDTA not only increased the solubility and hence biological availability of metals in the soil [76, 77], but it also participated in the transport of metals in plant parts [35, 76, 78]. The effect of EDTA on lead accumulation in the shoots of *Helianthus annuus* was demonstrated. EDTA treatment increased lead content twelve times, and significantly bigger amount of lead was allocated in the shoots [79]. The effect on other metals distribution was also reported. The addition of EDTA increased shoot concentration of cadmium, lead and zinc in *Helianthus annuus*, *Cannabis sativa* and *Brassica rapa* [12]. It was reported that lead in the roots of *Zea mays* with EDTA addition was mostly distributed in the apoplast, while zinc was mostly located in the symplast; therefore, the capacity of EDTA to enhance the nonselective apoplastic transport of metal may be most important for chelates enhanced phytoextraction [80]. According to literature, effectiveness of EDTA depends on its rate, contamination level of lead as well as complementary metals present in soils and method of its application [81]. Although EDTA has been shown to be effective, its toxic effect on soil microorganisms was proved [36].

Humic substances (HS) were used as an alternative to EDTA, HS had more advantages than disadvantages. It was reported that HS mitigated damaging effects of radiation, pesticides and excess of mineral fertilizers [82]. Several studies showed that HS applied into the soil increased metal transfer into the shoot. Direct HS addition significantly enhanced cadmium uptake by *Nicotiana tabacum* [37]. The addition of HS significantly increased the copper content in roots, and shoots of *Elodea nuttallii*, whereas the presence of HS in the soil had exactly opposite effect on cadmium content in the roots [83]. Moreover, it was proved that irrigation with water containing HS increased metals availability that led to increased lead and cadmium accumulation in *Triticum aestivum* [39]. Those studies indicated that the enhancement effect is strongly dependent not only on plant species, but also on the concentration of HS. However, gradual application of small doses of chelates can considerably reduce the toxicity and environmental problems associated with its utilization [84].

Table 4. Copper concentration[µg/g] in root, shoot, and leaf of four *Cannabis sativa* L. cultivars after 7 days of cultivation in solution with 50, 100, 200, 500, 1000, 2000, or 5000 [µmol/l] concentration; control mean Hoagland solution (copper concentration 3.3 µmol/l); DW mean dry weight; standard deviation is represented as ± S.D. (n = 3).

Solution	Beniko			Bialobrzskie			Fibrol			Monoica		
[µmol/l]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]
control	534 ± 16.2	16.7 ± 0.86	8.89 ± 1.30	275 ± 76.5	13.6 ± 4.20	6.93 ± 0.345	1191 ± 180	25.9 ± 0.860	4.46 ± 0.241	191 ± 76.1	26.3 ± 8.66	10.4 ± 1.01
50	1868 ± 482	171 ± 50.5	32.9 ± 1.20	1485 ± 158	96.1 ± 4.27	20.9 ± 7.14	2093 ± 111	256 ± 1.91	56.7 ± 1.08	1026 ± 20.8	229 ± 6.72	27.9 ± 3.07
100	4322 ± 88.6	453 ± 13.1	28.2 ± 1.81	3413 ± 367	349 ± 46.2	29.6 ± 4.00	4583 ± 632	467 ± 68.5	92.1 ± 9.48	2044 ± 137	423 ± 16.7	29.0 ± 1.91
200	5642 ± 114.9	1001 ± 290	40.5 ± 3.44	5682 ± 419	821 ± 19.5	84.1 ± 19.6	10058 ± 1047	1471 ± 41.5	128 ± 1.74	4594 ± 67.6	899 ± 24.3	84.4 ± 8.06
500	10964 ± 463	2510 ± 620	181 ± 1.40	9600 ± 557	1724 ± 419	235 ± 12.8	12452 ± 195	3486 ± 73.0	182 ± 48.4	8470 ± 312	3790 ± 111	238 ± 25.2
1000	12741 ± 170.2	3956 ± 687	206 ± 27.6	11749 ± 1210	3411 ± 117	480 ± 15.7	15543 ± 157	3718 ± 358	243 ± 37.8	11321 ± 1171	5345 ± 201	441 ± 38.9
2000	12412 ± 2072	7928 ± 986	443 ± 21.1	13374 ± 1636	7073 ± 85.4	692 ± 10.2	14927 ± 700	12612 ± 1270	781 ± 76.3	9578 ± 281	13068 ± 477	710 ± 75.0
5000	12790 ± 2193	19022 ± 1934	900 ± 58.3	13205 ± 2714	15320 ± 3627	1722 ± 447	16240 ± 2869	29914 ± 3105	1649 ± 69.4	12039 ± 1870	27243 ± 2955	2900 ± 66.1

Table 5. Lead concentration[µg/g] in root, shoot, and leaf of four *Cannabis sativa* L. cultivars after 7 days of cultivation in solution with 50, 100, 200, 500, 1000, 2000, or 5000 [µmol/l] concentration; control mean Hoagland solution (lead concentration under limit of quantification); DW mean dry weight; standard deviation is represented as ± S.D. (n = 3).

Solution	Beniko			Bialobrzskie			Fibrol			Monoica		
[µmol/l]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]
control	74.5 ± 25.3	14.4 ± 3.94	5.25 ± 0.79	1.94 ± 0.66	0.825 ± 0.191	3.05 ± 0.362	46.9 ± 5.45	6.50 ±0.825	5.66 ± 0.33	74.9 ± 3.40	17.3 ± 0.538	14.2 ± 4.01
50	3738 ± 598	125 ± 66.8	2.99 ± 0.713	7045 ± 612	146 ± 9.09	3.44 ± 0.171	5319 ± 818	288 ± 16.2	1.38 ± 0.135	5069 ± 102	104 ± 23.3	6.26 ± 0.760
100	8246 ± 206	185 ± 19.4	5.26 ± 0.109	5715 ± 882	760 ± 12.0	6.42 ± 0.259	16606 ± 2399	184 ± 9.23	1.23 ± 0.617	11164 ± 4139	451 ± 18.1	14.6 ± 2.83
200	23477 ± 4328	588 ± 15.1	5.43 ± 0.461	12921 ± 119	329 ± 25.3	4.53 ± 0.271	30225 ± 4338	572 ± 25.8	2.28 ± 0.823	12395 ± 725	315 ± 13.8	9.68 ± 0.459
500	27768 ± 3208	619 ± 12.5	6.01 ± 0.503	939 ± 47.8	471 ± 56.5	2.08 ± 0.168	22592 ± 2309	366 ± 69.9	1.32 ± 0.717	26844 ± 4832	772 ± 15.0	23.4 ± 1.40
1000	30079 ±4015	765 ± 27.5	11.5 ± 2.12	45822 ± 1092	649 ± 64.3	6.78 ± 0.517	37700 ± 1305	1258 ± 45.4	8.04 ± 0.894	25610 ± 7335	520 ± 25.9	16.0 ± 0.972
2000	27748 ± 968	2017 ± 80.9	10.8 ± 1.31	1355 ± 185	546 ± 14.1	5.06 ± 0.509	40766 ± 3251	5532 ± 39.6	10.13 ± 0.175	37771 ± 6790	1123 ± 84.8	21.6 ± 4.57
5000	32084 ± 5880	5132 ± 50.4	7.05 ± 0.207	40268 ± 904	9627 ± 116.9	20.8 ± 1.20	66280 ± 5393	7724 ± 91.2	13.10 ± 1.42	59837 ± 2351	6831 ± 158	24.0 ± 8.02

Table 6. Zinc concentration[µg/g] in root, shoot, and leaf of four *Cannabis sativa* L. cultivars after 7 days of cultivation in solution with 50, 100, 200, 500, 1000, 2000, or 5000 [µmol/l] concentration; control mean Hoagland solution (zinc concentration 2.3 µmol/l); DW mean dry weight; standard deviation is represented as ± S.D. (n = 3).

Solution	Beniko			Bialobrzskie			Fibrol			Monoica		
[µmol/l]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]	Root [µg/g DW]	Stem [µg/g DW]	Leaf [µg/g DW]
control	435 ± 67.0	50.8 ± 2.47	47.3 ± 2.72	512 ± 36.5	66.1 ± 10.6	51.7 ± 3.86	30.5 ± 0.743	33.0 ± 2.01	34.2 ± 2.59	774 ± 16.0	144 ± 4.56	91.8 ± 5.79
50	1630 ± 100	182 ± 17.6	70.1 ± 2.06	663 ± 4.21	220 ± 17.3	57.1 ± 8.19	377 ± 47.0	79.0 ± 1.97	39.2 ± 2.58	1823 ± 54.0	349 ± 11.5	137 ± 13.2
100	3718 ± 254	371 ± 88.6	122 ± 9.73	2959 ± 97.6	322 ± 64.8	83.5 ± 5.93	1146 ± 160	112 ± 24.1	46.0 ± 1.90	1944± 26.5	450 ± 18.8	198 ± 7.65
200	4972 ± 530	330 ± 62.0	463 ± 24.3	2985 ±14.7	521 ± 41.6	113 ± 6.18	5915 ± 176	615 ± 33.5	335 ± 15.1	3107 ± 104	1215 ± 149	276 ± 9.28
500	16212 ± 1493	2315 ± 83.3	823 ± 12.0	15754 ± 486	1506 ± 25.3	301 ± 13.7	9932 ± 641	2203 ± 107	670 ± 15.7	9086 ± 191	3038 ± 258	462 ± 10.7
1000	23535 ± 755	5925 ± 124	1459 ± 67.3	23786 ± 326	3860 ± 84.5	1454 ± 164	13548 ± 714	4750 ± 102	1420 ± 45.4	9804 ± 173	3589 ± 402	1081 ± 91.3
2000	25875 ± 545	9506 ± 145	4615 ± 16.4	29162 ± 997	6523 ± 280	3174 ± 548	19560 ± 1693	6497 ± 254	3186 ± 376	11960 ± 2058	9680 ± 965	1506 ± 31.3
5000	31208 ± 4173	37440 ± 499	7253 ± 74.7	31366 ± 1961	27190 ± 6905	5528 ± 170	45449 ± 2581	10153 ± 3031	5526 ± 217	22564 ± 4394	16183 ± 1861	3334 ± 396

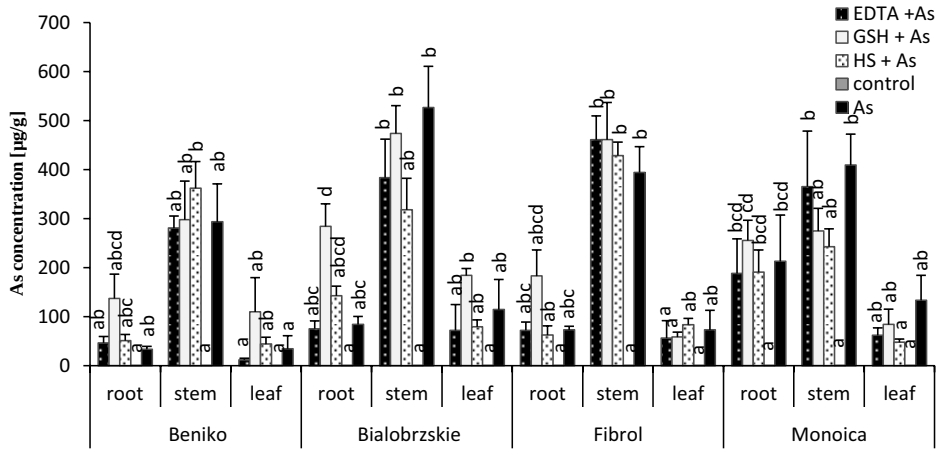

Figure 1. Arsenic concentration [µg/g] in stem and leaf of four *Cannabis sativa* L. cultivars after 7 days of cultivation in solution with 100 [µmol/l] of metal(loid) concentration with different additions (EDTA, GSH, or HS). Control means solution without metal(loid) and without amendments; standard deviation is represented as ± S.D. (n = 3). The differences among amendments treatments of the cultivars were tested by one-way ANOVA with Tukey's HSD multiple comparison test (significance level P=0.05).

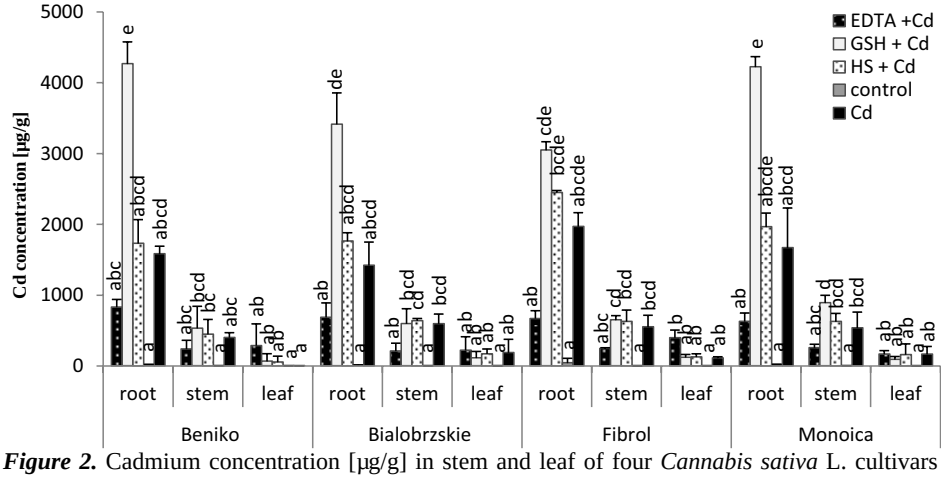

Figure 2. Cadmium concentration [µg/g] in stem and leaf of four *Cannabis sativa* L. cultivars after 7 days of cultivation in solution with 100 [µmol/l] of metal(loid) concentration with different additions (EDTA, GSH, or HS). Control means solution without metal(loid) and without amendments; standard deviation is represented as ± S.D. (n = 3). The differences among amendments treatments of the cultivars were tested by one-way ANOVA with Tukey's HSD multiple comparison test (significance level P = 0.05).

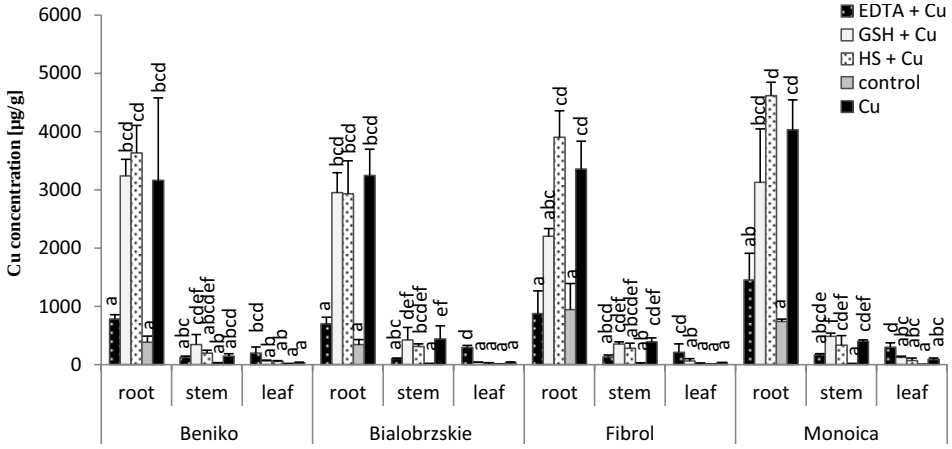

Figure 3. Copper concentration [µg/g] in stem and leaf of four *Cannabis sativa* L. cultivars after 7 days of cultivation in solution with 100 [µmol/l] of metal(loid) concentration with different additions (EDTA, GSH, or HS). Control means solution without metal(loid) and without amendments; standard deviation is represented as ± S.D. (n = 3). The differences among amendments treatments of the cultivars were tested by one-way ANOVA with Tukey's HSD multiple comparison test (significance level P = 0.05).

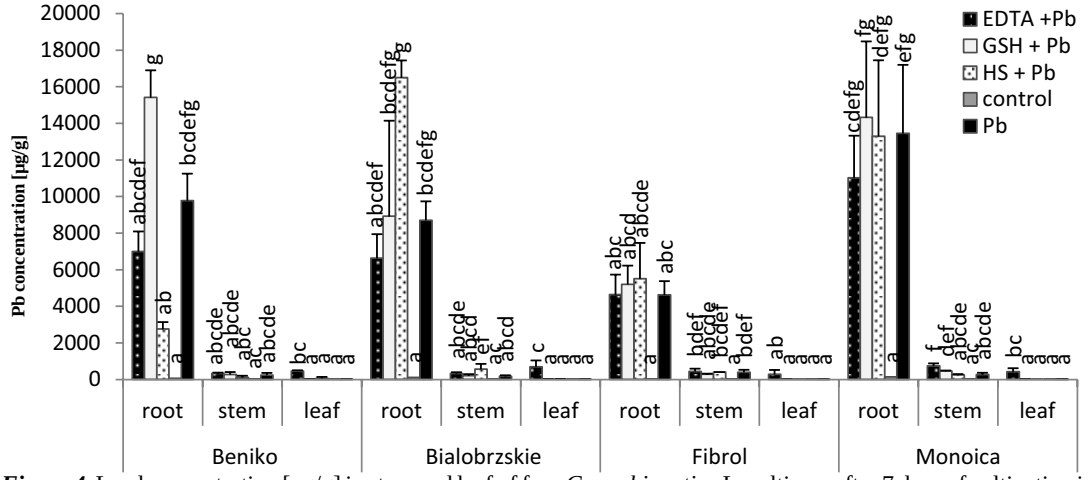

Figure 4. Lead concentration [µg/g] in stem and leaf of four *Cannabis sativa* L. cultivars after 7 days of cultivation in solution with 100 [µmol/l] of metal(loid) concentration with different additions (EDTA, GSH, or HS). Control means solution without metal(loid) and without amendments; standard deviation is represented as ± S.D. (n = 3). The differences among amendments treatments of the cultivars were tested by one-way ANOVA with Tukey's HSD multiple comparison test (significance level P = 0.05).

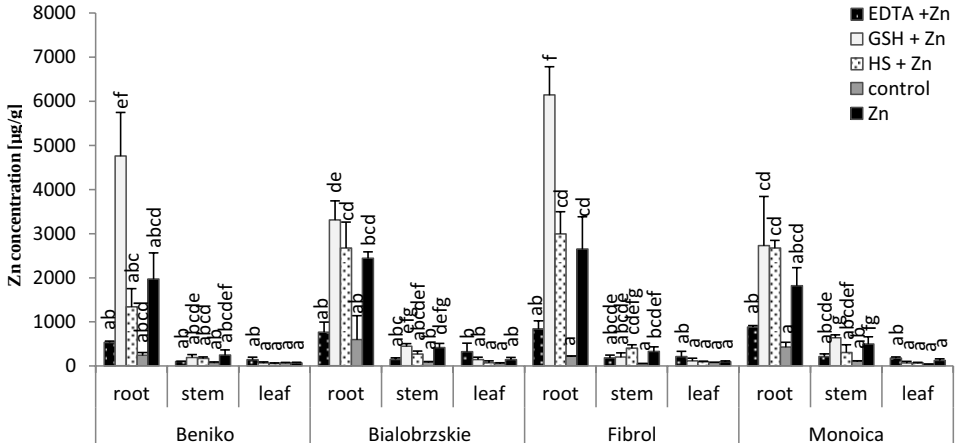

Figure 5. Zinc concentration [µg/g] in stem and leaf of four *Cannabis sativa* L. cultivars after 7 days of cultivation in solution with 100 [µmol/l] of metal(loid) concentration with different additions (EDTA, GSH, or HS). Control means solution without metal(loid) and without amendments; standard deviation is represented as ± S.D. (n = 3). The differences among amendments treatments of the cultivars were tested by one-way ANOVA with Tukey's HSD multiple comparison test (significance level P = 0.05).

Glutathione: The addition of GSH had an impact on uptake of all metal(loid)s (Figs 1 - 5). It increased cadmium and zinc amounts in the roots of all cultivars (Figs 2 and 5). Besides, an increase of lead accumulation in the roots of three cultivars (Beniko, Bialobrzskie and Monoica) was detected (Fig. 4). Moreover, enhanced arsenic uptake was observed in the roots of cultivars Beniko, Bialobrzskie and Fibrol (Fig. 1). The transfer of metal(loid)s to the shoots was not so obvious. An increase of arsenic in the leaves (Beniko, Bialobrzskie) and copper in the stems (Bialobrzskie) was identified (Figs 1 and 3). Increased arsenic transfer corresponded with literature. It was demonstrated that low level of GSH (0.4 mmol/l) increased arsenic uptake by *Pteris vittata* plants, while higher concentration (0.8 mmol/l) had no effect on the uptake [85]. Nevertheless, the addition of GSH helped arsenic transport from the root to the shoot of *P. vittata* (transfer factor rose twice). It has been presented that glutathione is involved in the reactions forming phytochelatins [32]. Phytochelatins induced on exposure to arsenic formed a complex with arsenite ions [45, 86]. High cadmium levels in *Brassica juncea* were also associated with a rapid accumulation of phytochelatins in roots [87]. The increase in glutathione synthetase activity was dependent on the initial cadmium concentration. It was found that the accumulation dynamic of cadmium or lead bound with GSH was changed in *Zea mays* and *Brassica napus* roots [88]. Observed inhibition of lead uptake in the presence of increasing GSH concentration and the apparent up-regulation of lead uptake following pre-exposure to GSH were consistent with a transport via a peptide transporter, that did not differentiated between GSH and the metal-GSH complex. Therefore, higher metal(loid)s contents in *C.sativa* might be caused by GSH involvement in production of phytochelatins.

Conclusions

Our results showed that metal(loid)s accumulation significantly varied with chosen cultivar. Generally, all metal(loid)s were accumulated mainly in roots except arsenic. Arsenic accumulation trend was similar to arsenic hyperaccumulator *P. vittata*. Other metals showed different tendency. Cadmium content in the shoots increased with increasing concentration of cadmium in solution. Copper was accumulated differently. It reached similar level in the roots of cultivars and even the higher concentration in solution

did not increase it. Moreover, at the highest concentration the amount of the metal in the stems greatly raised up to the roots levels. Zinc accumulation trend was comparable to copper. Higher concentrations of zinc did not led to a raise of zinc accumulation in the roots but led to a steep increase of zinc amount in the stems. Its translocation to the shoots might be the avoidance of toxic effect of the metal on the roots. In contrast, lead was rather immobile, showing very limited translocation into the shoots. From the distribution of metals we can suggest that probably there is no existing strategy for metal detoxification in *C.sativa*. Nevertheless, hemp demonstrated to possess the ability to transfer arsenic, cadmium, copper and zinc from root to shoot, one of the criteria that must be met to consider a plant well suited for phytoextraction. However, studies at real contaminated sites could give more information about the phytoextraction process.

The effect of the amendments on the accumulation of metal(loid)s varied depending on the cultivar and the element. The chelates enhanced the nonselective apoplastic transport of metal(loid)s. Our results showed that EDTA had a positive effect on the metals mobility, a larger amount of metals was taken up and translocated into the shoots, while an effect of humic substances wasn't statistically significant. On the other hand, the addition of GSH increased metal(loid)s amounts in the roots of plants. Higher metal(loid)s contents in *C.sativa* may be caused by GSH involvement in production of phytochelatins, but further studies are needed to explain the mechanisms.

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