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Evaluation of Toxic Effects and Bioaccumulation of Cadmium and Copper in Spring Barley (*Hordeum vulgare* L.)

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This paper deals with the analysis of toxic effects of cadmium and copper on the growth of spring barley (*Hordeum vulgare* L.) cultivated in hydroponics. The seedlings of barley were treated with four different concentrations of cadmium and copper, ranging from 0.1 to 10 mg L^{-1}. The aim of the study was to assess toxic effects of cadmium (Cd) and copper (Cu) on the growth of spring barley, and to determine metal accumulation in above-ground and underground parts of the plant. The impact of Cu and Cd on photosynthetic pigments (chlorophyll *a*, *b*), the content of malondialdehyde (MDA), and the essential micronutrients (Mn, Fe) were examined. Metal treatment reduced the growth of roots (by 60%), shoots (Cd – 48 %, Cu – 57%) and dry weight (Cd – 47 %, Cu – 52%) of barley. Exposure to metals altered the content of photosynthetic pigments and caused lipid peroxidation. Regression analysis revealed that there was significant negative relationship between MDA content and biomass of barley treated with Cu (r=-0.99, p=0.01). The examined heavy metals were accumulated mainly in the roots and bioconcentration of Cu there was higher than that of Cd, indicating that roots tended to accumulate higher amounts of Cu than Cd. Though translocation of Cd from roots to above-ground tissues was higher, higher levels of Cd were observed in leaves.

Key words: *bioaccumulation, growth, heavy metal, Hordeum vulgare, toxic effect.*

1. Introduction

Among numerous inorganic chemical pollutants, heavy metals are prevalent. One of the most serious global problems causing environmental concern is heavy metal pollution. Contamination by heavy metals mostly comes from industrial and energy production, waste disposal, vehicle exhaust, fuel combustion, fertilizers application. Soil and water contamination with copper and cadmium is widespread due to their intensive industrial and agricultural use. Heavy metals are non-biodegradable, possess the potential to accumulate in living organisms and can be incorporated into the food chain. Although some heavy metals like copper are essential trace elements in plant nutrition, many heavy metals do not play any significant role in plants physiology. Many researchers have shown that plants are capable of accumulating relatively high levels of heavy metals from the soil (Ali et al. 2004**;** Bidar et al. 2007**;** Mattina et al. 2003)**.** An adverse impact of heavy metals on commercial agricultural crops highlights the increased risk of human exposure to heavy metals through the food chain (Mueller 1994).

Cadmium (Cd) is a non-essential, strongly phytotoxic element causing various severe biochemical, physiological and morphological effects even at very low levels. Cd reduces the plant growth, inhibits biomass production and yield, disturbs the photosynthesis and transpiration (Larbi et al. 2002**;** López-Millán et al. 2009; Januškaitienė 2012**).**

Unlike cadmium, copper (Cu) is an essential micronutrient for the normal plant growth. It constitutes plant enzymes triggering a variety of physiological processes in plants (respiration, photosynthesis, cell wall metabolism, seed production, etc.). A slightly greater than optimal concentration for the plant growth leads Cu to become phytotoxic. A critical level for Cu toxicity

in crops is above 20-30 µg g^{-1} dry weight of leaves (Alaoui-Sossé et al. 2006**).** Cu excess affects plant biochemical and physiological processes, such as photosynthesis, nutrients metabolism, and reduces the plant growth (Xiong et al. 2006**;** Martínez-Peñalver et al. 2012**).**

Heavy metals have received considerable attention in toxicity studies. The symptoms of cadmium and copper poisoning in plants are linked with oxidative stress. Cd and Cu were shown to produce reactive oxygen species (ROS) and induce oxidative stress in plants (Foyer et al. 1994**;** Gaetke and Chow 2003; Romero-Puertas et al. 2004). In the presence of heavy metals, activities of various antioxidant enzymes, such as superoxide dismutase, catalase, peroxidase, glutathione reductase can be changed. Additionally, heavy metals negatively affect biosynthesis of secondary metabolites, which belong to non-enzymatic cell defense system compounds.

The main aim of this study has been to assess toxic effects of cadmium and copper on the growth of spring barley (*Hordeum vulgare* L.) and to determine metal accumulation in above-ground and underground parts of the plant.

2. Materials and Methods

Spring barley (*Hordeum vulgare* L. cv. Aura DS) after seed sterilization was germinated on moisture filter paper in the dark at 20±1 ºC for 3 days. After germination seedlings were grown in hydroponics filled with the half strength aerated Hoagland's nutrient solution (Terry 1980**)**. Plants were exposed for 5 days to cadmium and copper separately. The media were supplemented with different Cu (as $CuCl_2{\times}2H_2O$) and Cd (as $CdCl_2$) concentrations, namely 0, 0.1, 1, 5 and 10 mg L^{-1}. Three replicates for each heavy metal treatment (4 concentrations) and a control were used. Experiments were performed in climate controlled chambers: temperature of 22±1 °C in the daytime and 16±1 ° C at night, photoperiod – 14 hours, relative humidity – 65%, light intensity of 14000 Lx.

The following endpoints were evaluated: plant growth characteristics including shoot height and root length, total dry weight, amount of photosynthetic pigments (chlorophyll *a*, *b*), content of malondialdehyde (MDA), amount of accumulated Cu, Cd, Mn and Fe in plant leaves and roots.

The total content of chlorophylls (*a*, *b*) was determined spectrophotometrically after extraction of leaves in 100 % acetone (von Wettstein 1957). Concentration of MDA, the end-product of lipid peroxidation, was assessed by reaction with thiobarbituric acid (Buege and Aust 1978). The sample of leaves tissue was homogenized with Tris-HCl (pH 7.4) buffer solution containing 1.5% of PVPP and centrifuged at 10 000 g for 30 min at 4 ºC. Tissue extract was mixed with 0.5% thiobarbituric acid in 20% trichloroacetic acid (w/v) and heated at 95 ºC for 30 min. After centrifugation of the reaction mixture, absorbance of the supernatant was measured at 532 nm and corrected for unspecific turbidity by subtracting the value of absorbance at 600 nm. The concentration of MDA was expressed in nmol g-1 fresh weight using an extinction coefficient of 155 mM-1cm-1.

Plant samples were dried for 24 h at 70°C temperature and digested using the Milestone Ethos One closed vessel microwave system. About 200 mg of the sample was placed in a Teflon vessel and digested with 8 mL of HNO_3 (65%) and 2 mL of H_2O_2 (30%) in a microwave digestion system for 25 min. For the quantitative determination of metals in plant material a Shimadzu AA-6800 atomic absorption spectrometer equipped with deuterium background correction, and single-element hollow-cathode lamps as radiation sources were used. All instrumental settings were those recommended in the manufacturer's manual. An atomizer with an air/acetylene burner was used for determining all the elements investigated. The wavelengths (nm) used for determination of the analytes were as follows: Cu 324.8, Cd 228.8, Mn 279.5 and Fe 248.3. All reagents were of analytical reagent grade, unless otherwise stated. Deionized water (18.2 M Ωcm^{-1} resistivity) was used for all dilutions. All the plastic and glassware were cleaned by soaking in 5% HNO_3 for 24 hours and rinsed with distilled water prior to use. The element standard solutions were prepared by diluting a stock solution of 1000 mg L^{-1} (Cu, Cd, Mn, Fe) supplied by Sharlau (Spain).

Bioconcentration factors (BCF) of Cu and Cd of above-ground and underground plant tissues were calculated using the following equations (Mattina et al. 2003):

$$\mathrm{BCF} = \frac{C_{\mathrm{shoots\,(mg\,kg^{-1})}}}{C_{\mathrm{solution\,(mg\,L^{-1})}}} \quad (1)$$

$$\mathrm{BCF}' = \frac{C_{\mathrm{roots\,(mg\,kg^{-1})}}}{C_{\mathrm{solution\,(mg\,L^{-1})}}} \quad (2)$$

where c_{shoots} and c_{roots} are heavy metal concentrations in above-ground and underground plant parts, respectively, c_{solution} is a concentration of heavy metal in the solution.

Translocation factor (TF) was calculated using the following formula:

$$\mathrm{TF} = \frac{C_{\mathrm{shoots\,(mg\,kg^{-1})}}}{C_{\mathrm{roots\,(mg\,kg^{-1})}}} \quad (3)$$

Data were subjected to the analysis of variance (one-way ANOVA) for each endpoint. Significant differences between control and contaminated specimens were determined by Student's t-test and values $p<0.05$ considered significant.

3. Results and Discussion

An increase in Cu and Cd concentrations in the solution had a significant impact on the shoot growth of *H. vulgare* (Cu: $F = 23.19$, $p < 0.001$; Cd: $F = 16.22$, $p < 0.001$) (Fig. 1a). The growth of shoots of *H. vulgare* treated with the highest concentration of Cu and Cd was inhibited by 57.39% and 48.30%, respectively.

The roots possessed higher susceptibility than the shoot growth, and the length of roots decreased along with the metals concentration in the solution (Cu: $F = 44.80$, $p < 0.001$; Cd: $F = 19.56$, $p < 0.001$) (Fig. 1b). 0.1 mg Cu L^{-1} has evoked a stimulatory effect on the root growth. An increase in Cu concentration from 0.1 to 1 mg L^{-1} resulted in a very sharp decrease in the root length. The length of *H. vulgare* roots, exposed to the highest concentration of Cu and Cd, was 62.23% and 60.34%, respectively, lower than that of control plants.

The treatment with Cu and Cd produced a significant impact on the dry weight of *H. vulgare* (Cu $F = 23.30$, $p < 0.001$; Cd $F = 10.53$, $p < 0.001$) (Fig. 1c). In the 0.1 mg L^{-1} treatment the dry weight of barley was not adversely affected by the metals in the solution and an inhibitory effect on the dry weight was recorded in the treatments of 1-10 mg L^{-1}. Response of spring barley to Cu and Cd was very similar. The dry weight of *H. vulgare* treated with the highest Cu and Cd concentration was inhibited by 51.87% and 47.13%, respectively

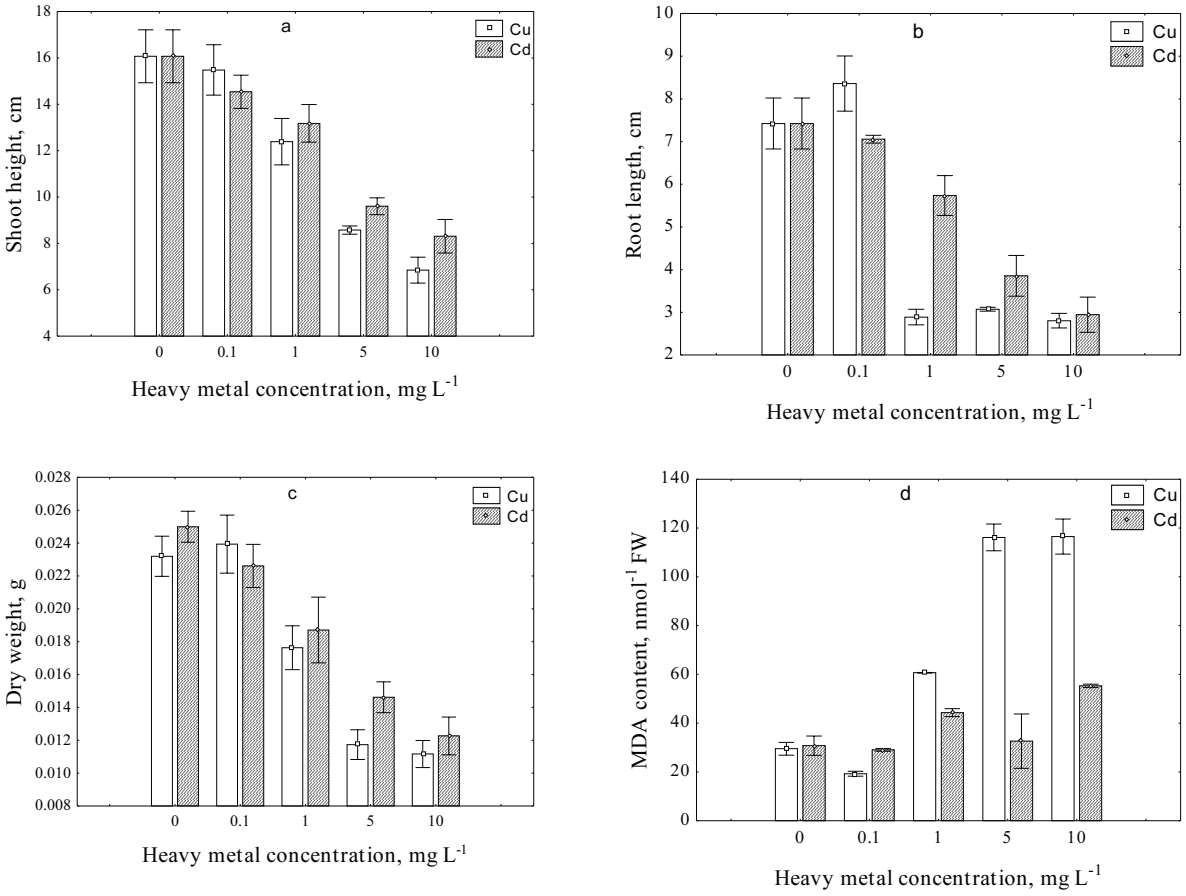

Fig. 1. Shoot height (a), root length (b), dry weight (c) of H. vulgare and MDA content (d) in the leaves of H. vulgare after exposure to Cu and Cd. Data are mean ± SE

Negative impact of Cu and Cd on the growth of crops and wild plants was shown in numerous studies (Monni et al. 2000**;** Xiong et al. 2006**;** Juknys et al. 2009). The impaired growth of barley seedlings may lead to lower biomass production and reduced yield. Moreover, our study has shown that an essential micronutrient Cu is phytotoxic at very low levels. The results of our study indicate that roots have been more adversely affected than shoots, indicating that roots are a primary target of exposure. It is proved by the results of analysis of bioaccumulation of Cu and Cd in roots and shoots (Fig. 2).

The content of heavy metals in plant tissues was significantly affected by heavy metal concentrations in the solutions and increased along with the external metal concentration (Cu: shoots $F = 7.34$, $p = 0.009$, roots $F = 98.09$, $p < 0.001$; Cd: shoots $F = 9.73$, $p = 0.004$, roots $F = 22.73$, $p < 0.001$) Concentrations of bioaccumulated Cu and Cd in roots were always significantly higher than those in shoots ($p < 0.05$). Additionally, the difference between the Cu and Cd amount in roots and shoots increased with the metal concentration in the solution. Significant high positive correlations were found between heavy metals content in the solution

and metals content in the roots (Cu: r = 0.99, p < 0.001, Cd: r = 0.95, p < 0.001) and in the shoots (Cu: r = 0.69, p = 0.026, Cd: r = 0.76, p = 0.01).

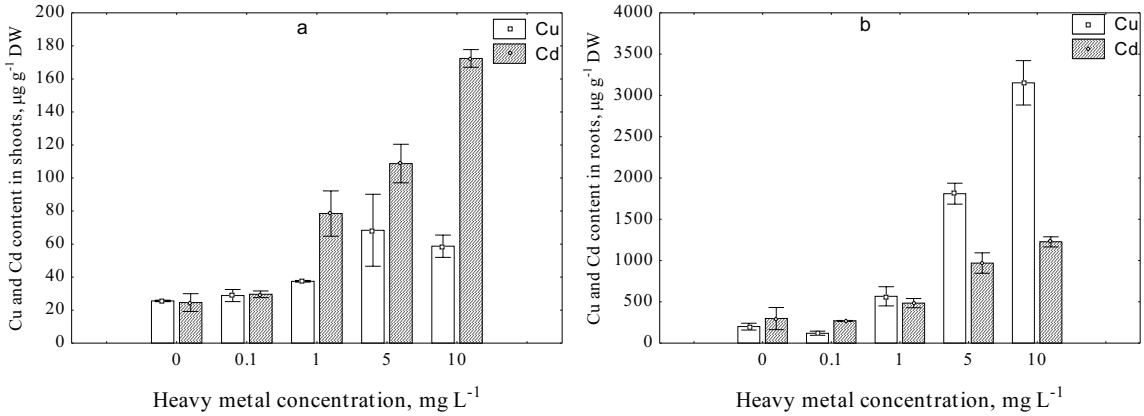

Fig. 2. Heavy metal accumulation in shoots (a) and roots (b) of H. vulgare after 5 days of exposure to Cu and Cd. Data are mean ± SE.

For the evaluation of spring barley's capability to extract from the solution and accumulate Cd and Cu in the plant, a bioconcentration factor (BCF) was calculated (Table 1). In underground tissues BCF varied from 122.7±8.7 to 2710.5±73.6 for Cd and from 315.3±38.0 to 1208.0±352.3 for Cu. It indicates that the roots tend to accumulate higher amounts of Cu than Cd. Obtained bioconcentration factors decreased along with metals concentration in the solution. The BCF in above-ground tissues were lower than in roots and ranged from 17.2±0.8 to 296.2±29.0 for cadmium and from 5.9±1.0 to 288.4±51.8 for copper. It indicates that accumulation of Cd in the leaves of barley is higher than that of Cu.

Table 1. Translocation and bioconcentration factors of above-ground (BCF) and underground (BCF') plant tissues after treatment with Cd and Cu

Concentration of exposed heavy metal, mg L^{-1}	Treatment with Cd			Treatment with Cu		
	BCF	BCF'	TF	BCF	BCF'	TF
0.1	296.2±29.0	2710.5±73.6	0.11	288.4±51.8	1208.0±352.3	0.26
1.0	78.5±19.4	485.4±78.8	0.17	37.5±0.7	567.8±164.5	0.07
5.0	21.7±3.3	194.0±34.8	0.11	13.7±6.2	387.4±35.8	0.04
10.0	17.2±0.8	122.7±8.7	0.14	5.9±1.0	315.3±38.0	0.02

Ability of spring barley to translocate Cd and Cu from roots to shoots was further confirmed by calculating a translocation factor (TF). TF values less than 1 imply higher accumulation of heavy metals in roots while a value greater than 1 indicates rapid heavy metal transportation from roots to shoots. In the current study, all evaluated TF values were less than 1, implying that the roots are a primary target of metals accumulation and transportation to the shoots is limited (Table 1). The TF value of Cu decreased from 0.26 to 0.02 with increasing Cu in the solution, indicating that in the case of low external concentrations of copper (r = -0.65, p = 0.042) it is essential for plant metabolism and Cu is highly absorbed and translocated (Kim et al. 2003). Whereas, in the case of higher external concentrations the uptake of Cu is limited, and translocation is very negligible. The tendency of Cu to accumulate in the roots rather than translocate was observed in *Arabidopsis thaliana* (Martínez-Peñalver et al. 2012). While the TF values of Cd ranged from 0.11 to 0.14, indicating a more intense translocation in comparison with Cu, no clear relationship with external Cd concentration was detected. It can be suggested that above-ground plant parts reached saturation, and a translocation rate was more or less static during higher exposure doses of Cd. Earlier studies proved that spring barley accumulated higher amounts of Cu and Cd in their roots than in their shoots, and their accumulation increased with the increased external concentration (Smýkalová and Zámečníková 2003; Ali et al. 2004**)**. Anyhow, it has been shown that plants may accumulate Cd in roots in a non-active form, and this may reduce its toxicity for roots (Sandalio et al. 2001). This phenomenon may partially explain the same level of roots length reduction due to exposure to equal concentrations of Cd and Cu (Fig. 1b).

Lipid peroxidation is a sensitive measure of

oxidative membrane damage and is useful as a biomarker for membrane damage induced by ROS. The treatment with Cu had a significant impact on MDA content in above-ground part of *H. vulgare* ($F = 134.51$, $p < 0.001$), whereas the treatment with Cd had no significant impact on the MDA level in above-ground part of the plant ($F = 4.36$, $p=0.07$) (Fig. 1d). The examined heavy metals differ in their oxidative capacity: Cu is a redox-active metal enhancing reactive oxygen species (ROS) formation via Fenton-Haber-Weiss reactions, whereas Cd has no redox activity, but exhibits the ability to produce ROS in plants (Hegedüs et al. 2001; Sandalio et al. 2001). The level of MDA has increased 4 times upon increasing the Cu concentration from 0 to 5 mg L^{-1}, and a further increase in Cu concentration from 5 to 10 mg L^{-1} had no significant impact on the changes of MDA content. Significant negative relationship between MDA content in leaves of spring barley and shoots dry weight was detected in the case of the treatment with Cu ($r = -0.99$, $p = 0.01$). It shows that the oxidative membrane damage results in the lower biomass production and this may lead to the lower yield of crops. MDA content in leaves of *H. vulgare* treated with 10 mg Cd L^{-1} was significantly higher than that in the control (1.8-fold increase, $p = 0.026$), however the changes of MDA level with the increasing Cd concentration was insignificant ($p = 0.15$) and the dose-effect relationship could not be detected. As Cd is a non-redox active metal, we may presume that only higher levels of Cd or longer duration of exposure, in comparison with other redox – active metals, may stimulate significant lipid peroxidation. Dong et al. (2006) have estimated that the lowest observed effect level of Cd for MDA content on leaves of *Lycopersicum esculentum* was 0.1 µM Cd, and MDA level increased with time.

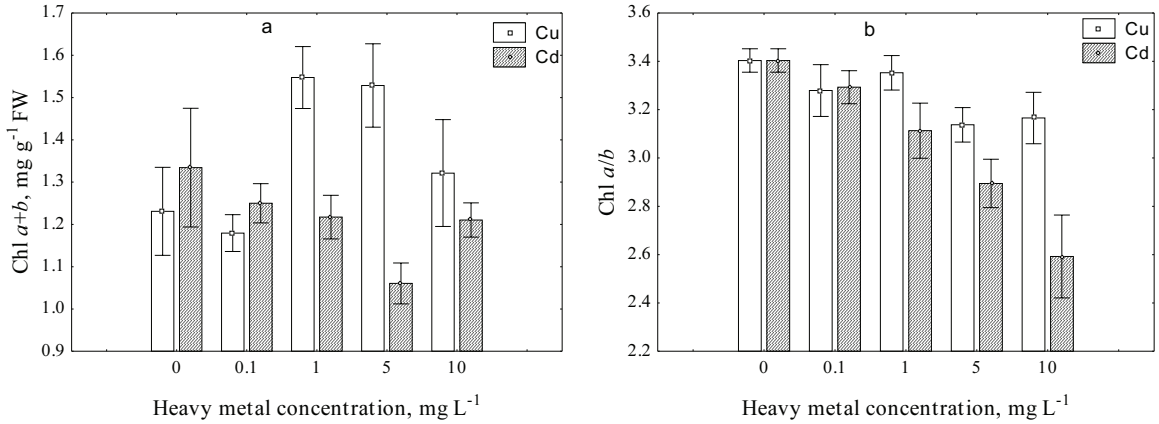

Fig. 3. The content of chlorophyll a+b (a) and chlorophyll a/b ratio (b) in the leaves of H. vulgare after exposure to Cu and Cd. Data are mean ± SE

Total chlorophyll content in leaves of *H. vulgare* was very scattered and no clear relationship between the concentration of metal in the solution and the content of chlorophyll *a+b* was detected (Fig. 3). Cu possessed a slight stimulatory effect on chlorophyll *a+b* content. It may be explained by the fact that Cu is an essential microelement. However, exposure to Cd led to significant reduction ($p<0.05$) of the chlorophyll *a*/*b* ratio in comparison with the control, demonstrating that chlorophyll *a* was more influenced than chlorophyll *b*. Our results indicate that lipid peroxidation is a more sensitive endpoint than the content of chlorophyll *a+b,* and moreover, the total content of chlorophyll *a+b* may underestimate the heavy metals impact on the photosynthesis rate. Exposure to 0-50 µM of $CdCl_2$ reduces the rate of photosynthesis in pea (*Pisum sativum* L.) by 6 times, but the content of chlorophyll was reduced only approximately by 40-50 % (Sandalio et al. 2001). As the content of photosynthetic pigments was not severely affected, we presume that growth reduction of barley was not related to the Cu and Cd effect on the photosystem. Lipid peroxidation may be one of the main reasons of growth impairment.

The content of micronutrients Fe and Mn in above-ground and underground plant tissues after treatment with Cd and Cu are presented in Fig. 4.

The exposure to Cu or Cd had no significant impact on Fe content in roots of *H. vulgare* and the content of Fe in leaves had significantly increased ($r=0.83$, $p<0.05$) after exposure to Cu. No effect of the Cd impact on Fe content in roots was reported for *Pisum sativum*, while in leaves a decrease in Fe content was observed (Sandalio et al. 2001). Decrease in Fe content in leaves and roots of *Lycopersicum ecsulentum* was recorded after exposure to 0-10 µM of Cd (Dong et al. 2006). Addition of Cd to the solution had a significant impact on increased Mn content in shoots of spring barley ($p=0.017$). Dong et al. (2006) observed that Mn content in plants was increased at low concentrations of Cd and sharply decreased at higher levels of exposed metal. The exposure to Cu resulted in a significant decrease in Mn in roots ($p<0.05$). A very similar pattern of Fe and Mn partition was observed in organs of *Elsholtzia splendens* exposed to Cu (Yang et al. 2002).

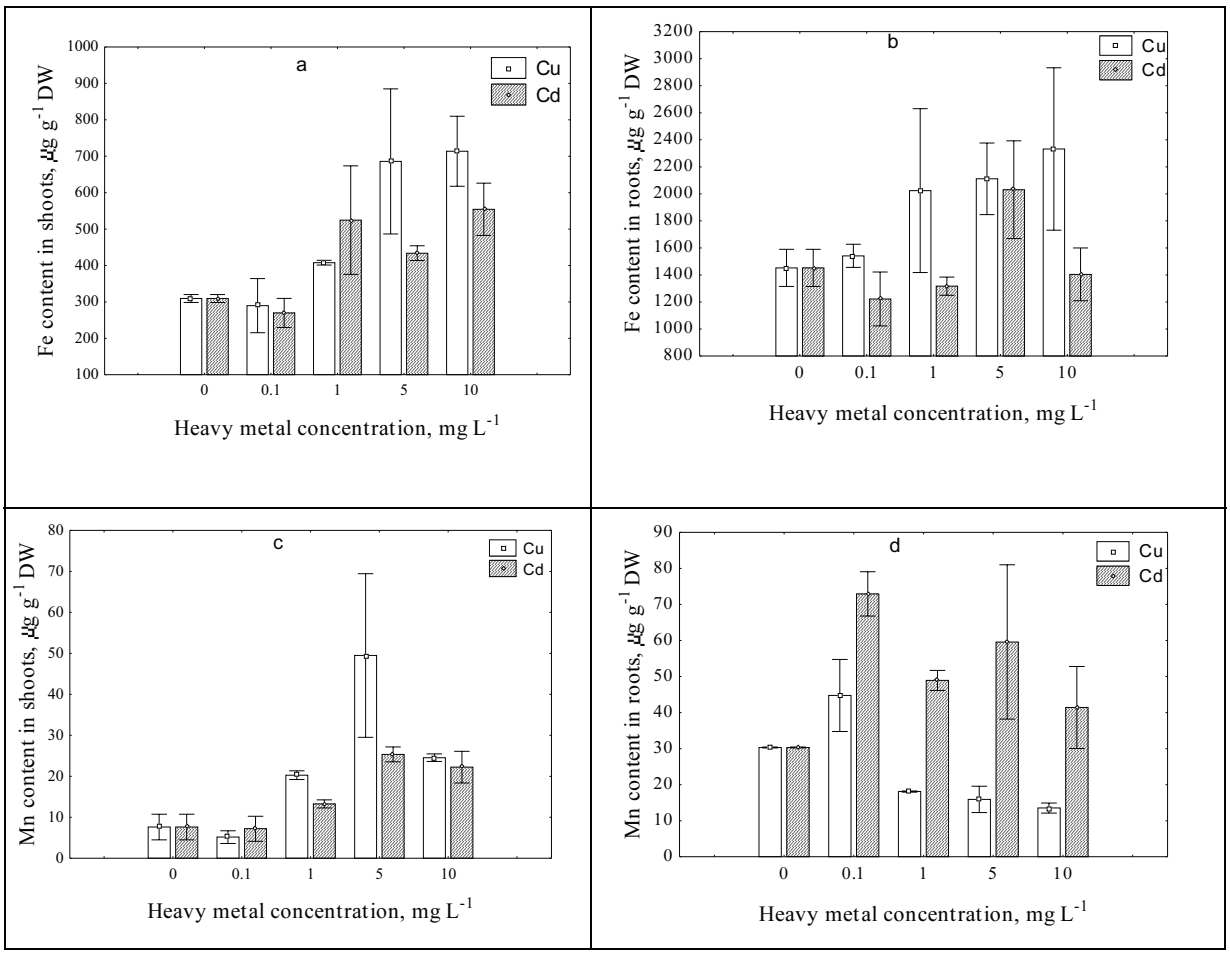

Fig. 4. The content of micronutrients in shoots (a, c) and roots (b, d) (DW basis) of H. vulgare after 5 days of exposure to Cu and Cd. Data are mean ± SE

4. Conclusions

Our results indicated that the growth of *H. vulgare* was adversely affected by either essential micronutrient copper or nonessential cadmium. The obtained results showed that Cu and Cd treatment (1-10 mg L^{-1}) significantly reduced the growth of roots and shoots as well as biomass production. It was observed that the growth of roots was the most sensitive endpoint, as roots are a primary target of heavy metals in the plants, and the exposure to Cu and Cd reduced the root length approximately by 60 %.

Cu and Cd were mainly accumulated in roots of barley and the internal Cu and Cd concentration increased along with the external one. Roots tend to accumulate higher amounts of Cu rather than Cd and, on the contrary, in leaves the bioconcentration of Cd is higher than that of Cu, implying the more intense translocation of cadmium. The excessive Cd and Cu accumulation led to the changes in micronutrients (Fe, Mn) content and exhibited a decrease in chlorophyll content.

Both heavy metals induced oxidative damage to cell membranes, the treatment with Cu and Cd resulted in 4-fold and 1.8-fold increase in the level of MDA, respectively. The significant negative relationship between MDA content in leaves of spring barley and dry weight of shoots indicate that oxidative damage is one of main factors determining the reduced plant growth.

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Vario ir kadmio toksinių poveikių ir bioakumuliacijos įvertinimas paprastuosiuose miežiuose (*Hordeum vulgare* L.)

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Straipsnyje analizuojami kadmio ir vario toksiški poveikiai paprastųjų miežių (*Hordeum vulgare* L.), išaugintų hidroponikose, vystymuisi. Miežių daigai buvo paveikti keturiomis skirtingomis kadmio ir vario koncentracijomis, svyruojančiomis nuo 0,1 iki 10 mg l^{-1}. Darbo tikslas buvo įvertinti toksiškus kadmio (Cd) ir vario (Cu) poveikius paprastojo miežio augimui ir nustatyti metalų susikaupimą antžeminėje ir požeminėje augalo dalyse. Ištirtas Cu ir Cd poveikis fotosintezės pigmentų (chlorofilo *a*, *b*), malondialdehido (MDA) ir būtinųjų mikroelementų (Mn, Fe) koncentracijoms. Metalų veikimas sulėtino miežių šaknų vystymąsi (60 proc.), daigų augimą (Cd – 48 proc., Cu – 57 proc.), sumažėjo biomasė (Cd – 47 proc., Cu – 52 proc.). Dėl metalų poveikio pakito fotosintezės pigmentų koncentracija ir buvo sukelta lipidų peroksidacija. Regresinė analizė parodė, kad buvo reikšmingas neigiamas ryšys tarp MDA koncentracijos ir miežių biomasės, kurie paveikti variu Cu (r=–0,99, p=0,01). Analizuoti sunkieji metalai daugiausia buvo sukaupti šaknyse. Vario biokoncentracija, kuri buvo didesnė nei kadmio, rodo, kad šaknys linkusios sukaupti didesnius kiekius vario negu kadmio. Tačiau kadmio pernaša iš šaknų į antžeminės dalies audinius buvo didesnė, taip pat buvo pastebėta didesnė Cd koncentracija lapuose.