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The effect of equal Cd and Cu exposure in peat substrate on growth and bioaccumulation of *Hordeum vulgare*

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Environment pollution with heavy metals is becoming more important these days. In this work we investigated the impact of soil contaminated with various concentrations of two heavy metals Cd and Cu (100; 200; 400; 800 and 1 600 mg/kg soil) on barley (*Hordeum vulgare*) growth and bioaccumulation. Investigated plants were sown in vegetative pots with the prepared peat and sand substrate (ratio 2:1), and 4 days after germination the plants were watered with appropriate metal concentration solutions. The experiment lasted for 14 days until the second true leaf unfolded. At the end of the experiment, the content of pigments was measured using a spectrophotometer, MDA was determined using the thiobarbituric acid method, and plants height and dry mass were also detected. Increasing of metal concentrations decreased plant height and biomass, and the Cd effect on morphological parameters was higher than that of Cu when a correlation between barley height and Cd concentration was –0.74 ($p < 0.05$) and between barley height and Cu concentration –0.39 ($p < 0.05$). Increasing metal concentrations effect on photosynthesis pigments was different, i. e. under the Cu effect the content of pigments (a+b) increased and a correlation was 0.47 ($p > 0.05$), while under the Cd effect it decreased and a correlation was –0.13 ($p > 0.05$), but statistically insignificant. Between MDA concentration in barley leaves and heavy metals concentration in the substrate a strong correlation ($p < 0.05$) was estimated, which was slightly stronger under the copper impact. At lower concentrations lower MDA levels were detected there, compared to those of the control plants, when the concentration increased up to 1 600 mg Cu and Cd/kg substrate, the MDA concentrations increased statistically significantly by 68 and 32%, respectively. Up to 800 mg/kg concentration in the substrate accumulation of copper was more willing than accumulation of cadmium in barley leaves ($p < 0.05$), but when the concentration rises up to 1 600 mg/kg, the plant starts to accumulate more Cd than Cu.

Key words: cadmium, copper, malondialdehyde, pigments, dry biomass, bioaccumulation factor

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INTRODUCTION

Heavy metals, which naturally occur at low levels in the environment, tend to accumulate to toxic concentrations as a consequence of mining, smelting, as well as excessive use of phosphate fertilizers and sewage sludge in agriculture. It is well known that Cd^{2+} is one of the most toxic heavy metals without any metabolic significance. It can be transferred to the food chain by plant uptake. Studies carried out in different plant species have revealed that Cd^{2+} can interfere with a number of metabolic processes. It diminishes water and nutrient uptake (Li et al., 2008; Cherif et al., 2012), results in visible symptoms of injury in plants such as chlorosis and necrosis of leaves, reduced length and browning of roots (Cherif et al., 2012).

Copper (Cu) is considered as a micronutrient for plants (Thomas et al., 1998) and plays an important role in CO_2 assimilation and ATP synthesis. Cu is also an essential component of various proteins like plastocyanin of the photosynthetic system and cytochrome oxidase of the respiratory electron transport chain (Demirevska-Kepova et al., 2004). But enhanced industrial and mining activities have contributed to the increasing occurrence of Cu in ecosystems. Cu is also added to soils from different human activities, including mining and smelting of Cu-containing ores. Mining activities generate a large amount of waste rocks and tailings, which get deposited at the surface. Excess of Cu in soil plays a cytotoxic role, induces stress and causes injury to plants. This leads to plant growth retardation and leaf chlorosis (Lewis et al., 2001). Exposure of plants to excess Cu generates oxidative stress and ROS (Stadtman, Oliver, 1991). Oxidative stress causes disturbance of metabolic pathways and damage to macromolecules (Hegedus et al., 2001; Yadav, 2009).

Plant's ability to accumulate heavy metals and tolerate excess depends on the plant species, growth conditions and stage of development (Lorraine et al., 2000; Januškaitienė, 2012; Januškaitienė, Dikšaitytė, 2014). Copper deficiency is a rare phenomenon, and the excess copper is common and due to poor soils, and a result of human activities. For copper overload, like iron deficiency in plants, leaves change color, weakening enzyme activity, reactive oxygen species are being produced by this damaging the membrane, K^+ transfer is being disrupted, inhibition of cell elongation and sharing of forming copper sulphide can be noticed, copper replaces enzyme calcium or iron. Due to copper excess abundant short, hairy, brownish lateral roots are formed (Kramer et al., 2007).

The responses to Cd can be different and sometimes opposite according to the species. Net photosynthesis is also sensitive to Cd because it directly affects chlorophyll biosynthesis and the proper development of the chloroplast ultrastructure (Rascio et al., 2011). However, the main targets of the influence of Cd appear to be ribulose 1.5-bisphosphate carboxylase (RuBPC) and phosphoenolpyruvate carboxylase (PEPC). As describes Singh et al. (1996), metals generally affect chlorophylls more than carotenoids. It has been demonstrated that Cd^{2+} also induces changes in the antioxidant status in plants (Balestrasse et al., 2006; Smiri et al., 2010).

Some plants, such as *H. vulgare*, has no good detox system, so they inactivate heavy metals, combining them into chelates in the cytoplasm or storing them into the vacuole (Clemens, 2001; Lindon et al., 1993). These plants quickly and effectively transfer metals from the roots to the shoots through the xylem, increasing quantities of heavy metals not only above the ground but also in the grain (Rascio, 2011). So the aim of this research is to compare the effect of equal concentrations of two heavy metals Cd and Cu on spring barley morphological and biochemical parameters.

MATERIALS AND METHODS

Spring barley (*Hordeum vulgare* L.) was chosen for investigation. Experiments were carried out in a vegetation room with the controlled environment: photoperiod 14 h, average temperature of night and day 20 and 25 °C, relative

humidity 60%. Philips Master Green Power CG T 600 W lamps, light intensity at the level of plants 14 000 Lx, provided light.

Barley was sown in a 2 liters pot with a prepared substrate of peat and sand (2 : 1) with 40 seeds each. In each treatment there were three pots of replication. Seeds were germinated and grown for 4 days.

Four days after germination plants were spaced out, leaving only 30 (1.5 cm) of plants, and the first 400 ml of the appropriate concentration were watered. The studied metal concentrations are prepared using $CuSO_4 \cdot 5H_2O$ and $CdSO_4 \cdot 8 / 3H_2O$ salts. It was calculated that the metal content of the substrate would meet 100, 200, 400, 800, and 1 600 mg/kg of metal pollution to the substrate. In the middle of the experiment, i. e. after 7 days, once again barley was watered with the same solutions. The duration of experiments was 14 days.

On the last day of the experiment, the height of plants, masses, determined contents of pigments and malondialdehyde (MDA) were measured in leaves. The photosynthetic pigments were analyzed using a spectrophotometer (Genesys 6, ThermoSpectronic, USA) and 100% acetone extracts were prepared according to the Wettstein's method (Wettstein, 1957). Photosynthetic pigments were expressed in mg/g of fresh weight. Malondialdehide (MDA), the end-product of lipid peroxidation, was used as a biomarker of membrane oxidative damage. MDA content was determined by reaction with thiobarbituric acid (TBA) giving a pink-colour compound after heating. The sample of leaf tissue was homogenized with a Tris-HCl buffer solution, pH 7.4, containing 1.5% of polyvinylpyrrolidone (PVPP), and centrifuged at 10 000 g for 30 min at 4 °C. Equal amounts of tissue extract and 0.5% TBA in 20% trichloroacetic acid (TCA) (w/v) were mixed and heated at 95 °C for 30 min. The reaction was stopped by transferring tubes on ice. After centrifugation of the reaction mixture at 10 000 g for 15 min, absorbance of the coloured 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^{-1} cm^{-1} (Buege, Aust, 1978; Blokhina et al., 2003).

At the end of the experiment the plants were harvested. Shoots were dried in an oven at 70 °C until a constant biomass of dry shoots was obtained. The biomass was expressed in mg $plant^{-1}$.

Dried plant samples were digested using the Milestone Ethos in the 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 the 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. 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 1 000 mg L^{-1} (Cu, Cd) supplied by Sharlau (Spain) (Žaltauskaitė, Šliumpaitė, 2013).

Bioaccumulation factors (BAF) of Cu and Cd of barley plants were calculated using the following equation (Mattina et al., 2003):

$$BAF = C_{DryMass}/C_{Substrate}, \quad (1)$$

where $C_{DryMass}$ is the concentration of metal (mg/kg) found in the plant after the experiment and $C_{Substrate}$ is added heavy metal concentrations (mg/kg) in the growth substrate (Žaltauskaitė, Šliumpaitė, 2013).

The Pearson correlation coefficient, *r*, *p*-values, and linear regression were used for the assessment of the metal contamination effect on

the investigated plant parameters. And for a comparison of independent variables the Student's *t* test was used. All analyses were performed by STATISTICA and EXEL and the results were expressed as mean values and their standart error (SE).

RESULTS

It was found that increasing of both copper ($r = -0.39$) ($p < 0.05$) and cadmium ($r = -0.74$) ($p < 0.05$) concentrations in the substrate negatively affected the plant height (Fig. 1). At the highest Cd and Cu concentrations impact on the height of barley plants decreased by 17.3% ($p < 0.05$) and 30% ($p < 0.05$), respectively, compared to that of control plants.

The increase of both heavy metals in the growth substrate also increased the negative effect on biomass accumulation (Fig. 2). Cu concentration increase affected a little bit higher reduction of biomass, when detected *r* was –0.84,

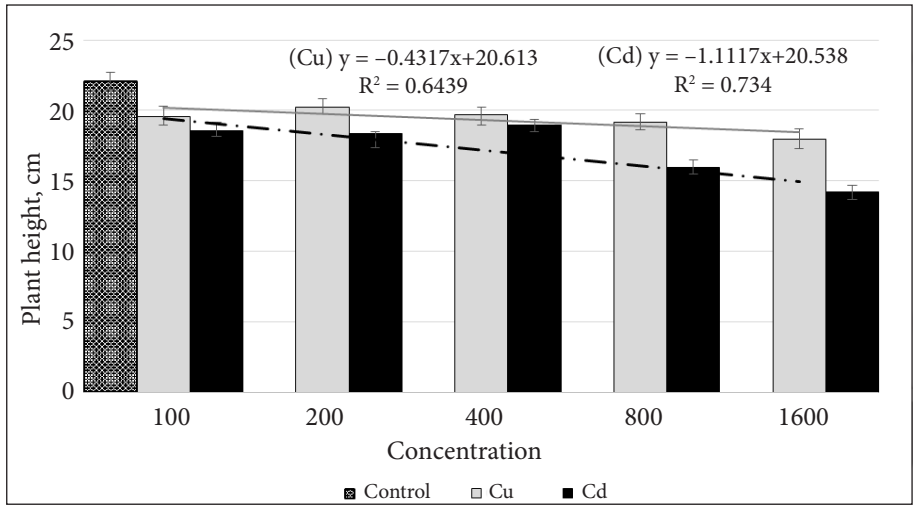

Fig. 1. The changes of barley height under the impact of different Cu and Cd concentrations. The values are means ± SE

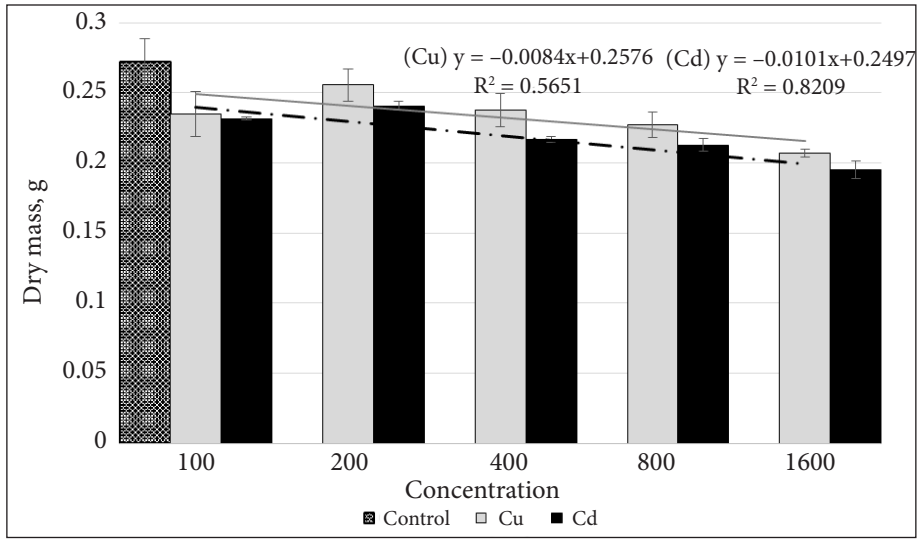

Fig. 2. The changes of barley dry biomass under the impact of different Cu and Cd concentrations. The values are means ± SE

and for Cd $r = -0.81$ ($p < 0.05$). If we compare only the highest 1 600 mg of Cd and Cu/kg substrate effect, the tendency was the same, i. e. dry biomass decreased by 23% ($p < 0.05$) and 28% ($p < 0.05$), respectively, compared to that of control plants.

Figure 3 presents the changes of the content of chlorophyll ($a + b$) in the leaves of barley. It was found that increasing concentration of copper led to the increase of pigment content ($r = 0.47$) ($p > 0.05$), while the increase of cadmium had a contrary effect, i. e. Chl ($a + b$) content decreased ($r = -0.13$) ($p > 0.05$), but not statistically significantly.

The increasing concentration of copper tends to decrease the chlorophyll a and b ratio constantly ($r = -0.71$) ($p > 0.05$) (Fig. 4). Only at the 200 mg/kg concentration effect the a/b ratio decreased by 1.2% more than at 400 mg/kg. The chlorophyll a/b ratio at cadmium exposure is decreasing ($r = -0.5$) ($p > 0.05$), but statistically insignificantly too.

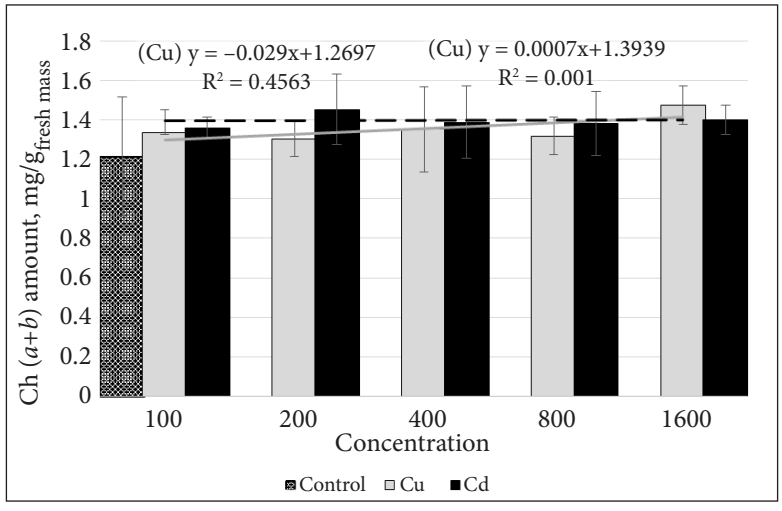

Fig. 3. The changes of the content of chlorophyll ($a + b$) mg/g fresh weight in barley leaves under the impact of different Cu and Cd concentrations. The values are means ± SE

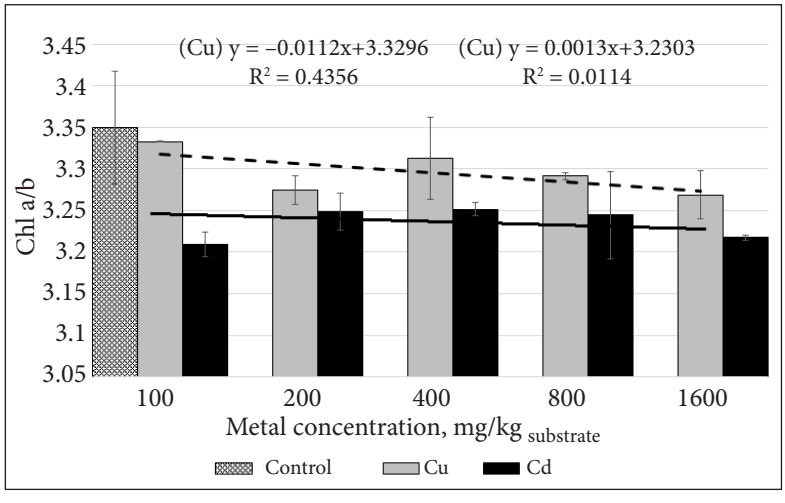

Fig. 4. The changes of the chlorophyll a/b ratio in barley leaves under the impact of different Cu and Cd concentrations. The values are means ± SE

At both copper and cadmium exposures the content of carotenoids tended to grow (Fig. 5): Cu ($r = -0.67$) ($p > 0.05$), Cd ($r = -0.42$) ($p > 0.05$). The exposures of the highest heavy metal concentrations increased the content of carotenoids by 22% (Cu) and 20% (Cd), compared to that of the control plants.

The MDA content in barley leaves continued to rise when the concentrations of heavy metals increased (Fig. 6). At lower concentrations effects of Cu and Cd MDA in barley leaves were even lower than in the control plants, and under the effect of 1 600 mg Cu and Cd in the kg of the substrate MDA concentration increased by 68% ($p < 0.05$) and 32% ($p < 0.05$), respectively. The multiple regression analysis showed a statistically significant positive correlation between the MDA content and heavy metal concentration in the growth substrate when detected r for Cu was 0.89 ($p < 0.05$) and for Cd 0.86 ($p < 0.05$).

The sample dry biomass analysis for determination of the accumulation of heavy metals in plants has shown that with increasing

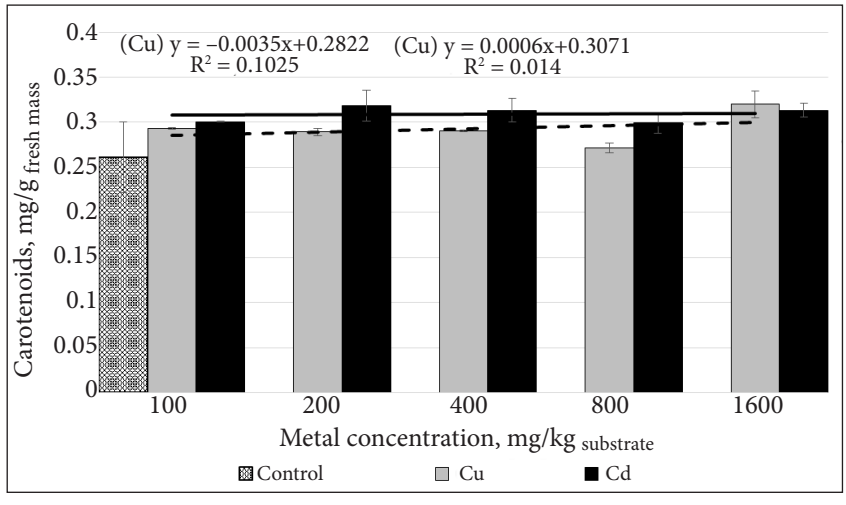

Fig. 5. The changes of the content of carotenoid mg/g fresh weight in barley leaves under the impact of different Cu and Cd concentrations. The values are means ± SE

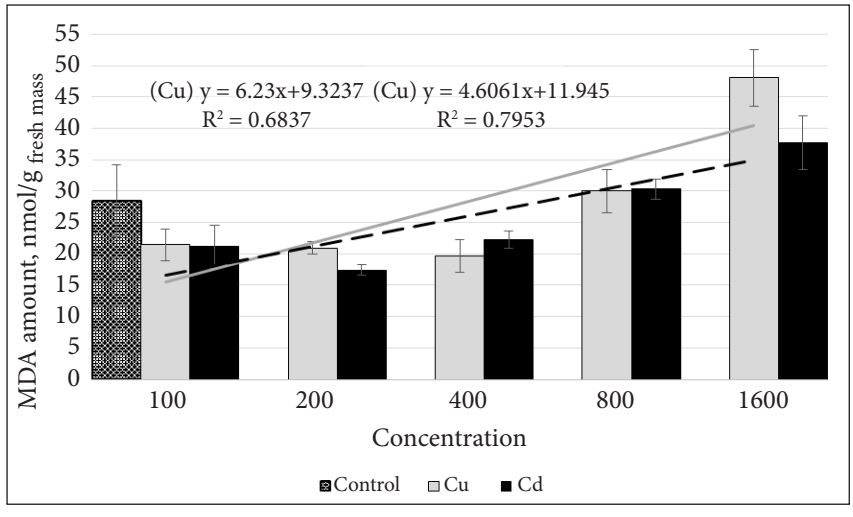

Fig. 6. The changes of the content of MDA (nmol/g fresh mass) in barley leaves under the impact of different Cu and Cd concentrations. The values are means ± SE

concentrations of the two metals the accumulated metal content in the dry biomass of barley also increased, but exponentially (Cu $r = 0.99$), (Cd $r = 0.99$) ($p < 0, 05$) (Fig. 7). Cadmium was not detected at 100 mg/kg of substrate contamination. But at the same and 200 mg/kg Cu concentration up to 0.29 μg/g dry biomass was detected, which can explain the necessity of copper as a microelement. The maximum accumulation was determined at 1 600 mg/kg of both metals: Cu 5 300 μg/g and Cd 5 442 μg/g dry mass.

From the detected concentrations of accumulated heavy metal in the dry biomass a bioaccumulation factor was calculated (Table). Up to 800 mg/kg concentration in the substrate accumulation of copper was greater than accumulation of cadmium in barley leaves ($p < 0.05$), but when concentration rises up to 1 600 mg/kg, the plant starts to accumulate more Cd than Cu.

Table. Cu and Cd bioaccumulation factor in barley

Used metal concentration	BAF	
	Cu	Cd
100	0.00103	0.00000
200	0.00147	0.00068
400	0.00264	0.00089
800	0.00271	0.00252
1 600	0.00331	0.00335

DISCUSSION

Natural systems have traditionally received metal contaminations from anthropogenic sources including treated sewage discharges, mining operations and runoffs from metal-refining industries. Plants may require some of essentials (Fe, Zn, Cu or Mo) in trace quantities, but at higher concentrations they may become toxic (Gallego et al., 2012). Cadmium is toxic to plants. It has been proven that it does not participate in metabolism, but some plants can accumulate up to 100 mg/kg dry weight (Benavides et al., 2005; White, Brown, 2010). This metal enters into plants through roots and leaves, and water dishes circulated around the plant, is distributed to all organs (Seregin, Ivanov, 2001). Unlike cadmium, copper is necessary for photosynthesis in plant growth and root development. However, the excess can disrupt the development of roots, cause tissue damage and reduce the efficiency of photosynthesis (Xuedong et al., 2012; Schutzendubel, Polle, 2002; Saxena, Shekhawat, 2013). For example, in this study, Cd made bigger damage than Cu, i. e. at Cd exposure the height and dry biomass inhibition was stronger (Figs. 1, 2). Metals entering a plant reduce its growth rate, water and mineral uptake, cell reproduction (Tamas et al., 2008), membrane function,

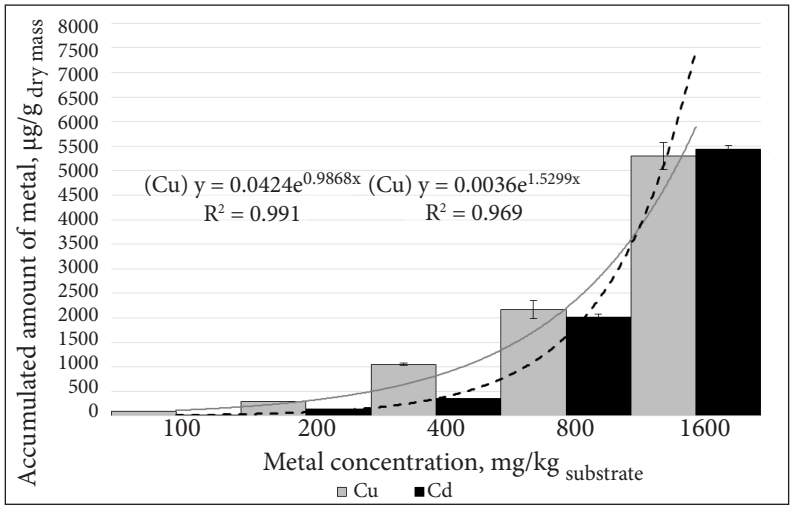

Fig. 7. The content of Cu and Cd accumulated in barley. The values are means ± SE

inhibit the enzyme activity, protein binding, even causing DNA damage and cell death (Benavides et al., 2005; Galleo et al., 2012). In this study there was a linear dependence on dry weight loss with increasing amounts of metal substrate (Fig. 2). The dry weight of both metals decreased similarly, but for Cd the effect was by 5% stronger. It is known that toxic heavy metals enter plant cells through transport systems involved in micronutrient uptake (Clemens, 2006). Additionally, the following metals disturb the nutrient passage through the root membrane affecting ATPase other proteins or carriers (Greger et al., 1991). On the other hand, these nutrient deficiencies can be caused by ion leakage from damaged roots, thereby causing the deficit in shoots (Siedleska, 1995; Roth et al., 2006).

The photosynthetic pigments are one of the major targets of toxic effects of Cd and Cu. Reduced chlorophyll content in Cd treated plants is due to the inhibition of its synthesis. Cu treated plants are not as strongly affected, because copper is less likely reacting to Fe and Mn (Vassilev et al., 2002). The photosynthetic potential of plants is directly dependent on the chlorophyll content and its production in leaves (Datt, 1998). In this study it was found that the changes of the pigment content in leaves of barley under heavy metals exposure were statistically insignificant (Figs. 3–5). Thus, plants, which synthesize higher content of chlorophylls or keep it unchanged under different stress effect, are more tolerant to them (Smith et al., 2000). As was described above, metals generally affect chlorophylls more than carotenoids (Singh et al., 1996) and this also agrees with the results of this research.

Metal toxicity is ascribed to direct interaction with proteins due to their affinities for thioyl-, histidyl- and carboxyl-groups, causing metals to target structural, catalytic and transport sites of the cell, and stimulate generation of reactive oxygen species (ROS) that modify the antioxidant defence and elicit oxidative stress (Sharma, Dietz, 2008; Flores-Caceresa et al., 2015). Plasma membrane damage caused by oxidative stress is very well reflected in the end product of lipid peroxidation, malondialdehyde (MDA) concentration in plants (Ortega-Villasante et al., 2005). In this research malondialdehyde (MDA) concentrations in barley leaves also had a statistically significant strong correlation with the heavy metal content in the substrate, but statistically significant differences between the control and treated plants were only at the maximum content of heavy metal in the substrate (Fig. 6). It can be assumed that the plants are trying to cope with toxic effects of the metals by inactivating them, i. e. connecting them to the chelates in the cytoplasm or storage on vacuoles (Clemens, 2001), and by activating both enzymatic and non-enzymatic antioxidant systems (Yadav, 2009).

Increasing concentrations of investigated metals in the growth substrate also increased the content of accumulated metal in the dry biomass of barley (Fig. 7). Ali et al. (2004) and Singh, Agrawal (2007) found that Cu and other metals were mainly accumulated in roots and then transferred to leaves. But other studies showed that in *Hordeum vulgare*, *Zea mays*, and *Triticum aestivum* leaves the concentrations of Cd, Cu and Zn increased significantly by increasing the concentration of heavy metal in sand substrate when 40% Cu was transported from roots to shoots. Although Chakroun et al. (2010) noted that more Cu and Zn are transferred into leaves than Cd or Pb. Similar results were also found by Žaltauskaitė and Šliumpaitė (2013). But others, Ali et al. (2004), Chakroun et al. (2010) and Rezvani et al. (2011), in their researches reported that accumulation of Cu in *Hordeum vulgare* and *Aeluropus littoralis* leaves was higher than that of Cd. So this research shows that Cd only at very high concentrations was intensively transported from the roots to the leaves compared to that of Cu.

CONCLUSIONS

With increasing the concentration of metal in the growth substrate the negative effects on barley plants also increased, and the effect of Cd on morphological parameters was higher than that of Cu when the correlation between

barley height and Cd concentration was –0.74 ($p < 0.05$), and between plant height and Cu concentration –0.39 ($p < 0.05$). The highest 1 600 mg/kg concentration effect on the height of copper plants height decreased by 17.3% ($p < 0.05$), and the same concentration of cadmium by 30% ($p < 0.05$), compared to that of the control plants.

Increasing metal concentrations effect on photosynthesis pigments was different, i. e. under the Cu effect the content of pigments ($a + b$) increased and the correlation was 0.47 ($p > 0.05$), while under the Cd effect it decreased and correlation was –0.13 ($p > 0.05$), but statistically insignificant.

There was a strong and statistically significant correlation between the MDA concentration in barley leaves and the heavy metals concentration in the substrate, which was slightly stronger under the copper impact (Cu: $r = 0.89$; Cd: $r = 0.86$) ($p < 0.05$). At lower concentrations effect lower MDA levels were detected, compared to those of the control plants, and when the concentration increased up to 1 600 mg Cu and Cd/kg substrate, the MDA concentrations increased statistically significantly by 68% and 32%, respectively.

In the case of up to 800 mg/kg concentration in the substrate the accumulation of copper was greater than the accumulation of cadmium in barley leaves ($p < 0.05$), but when the concentration rises up to 1 600 mg/kg, a plant starts to accumulate more Cd than Cu.

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VIENODOS Cd IR Cu TARŠOS DURPIŲ SUBSTRATE POVEIKIS *Hordeum vulgare* AUGIMUI IR BIOAKUMULIACIJAI

Santrauka

Intensyvi pramonės veikla, žemdirbystė kelia grėsmę užteršti aplinką sunkiaisiais metalais. Šiame darbe buvo tiriama dviejų sunkiųjų metalų (Cd ir Cu) įvairiomis koncentracijomis (100; 200; 400; 800 ir 1 600 mg/kg substrato) užteršto durpių substrato daroma įtaka vasarinių miežių (*Hordeum vulgare*) augimui ir metalų bioakumuliacijai. Tiriamieji miežiai buvo sėjami į vegetacinius indus su paruoštu durpių ir smėlio (santykiu 2:1) substratu ir 4 dieną po sudygimo palaistyti atitinkama metalo tirpalo koncentracija. Eksperimentas tęsėsi 14 dienų, t. y. kol augalai išleido du tikruosius lapus. Eksperimento pabaigoje išmatuoti augalų aukščiai, pigmentų kiekiai apskaičiuoti spektrofotometriškai, MDA nustatytas naudojant tiobarbitūrinės rūgšties metodą, įvertinta vidutinė augalo sausa biomasė. Cd koncentracijų poveikis morfologinius parametrus mažino stipriau nei Cu, kai koreliacijos koeficientas tarp miežių aukščio ir Cd koncentracijų buvo –0,74 ($p < 0,05$), o tarp aukščio ir Cu – –0,39 ($p < 0,05$). Didėjančios metalų koncentracijos pigmentų kiekius miežių lapuose veikė skirtingai, t. y. dėl Cu poveikio pigmentų ($a+b$) kiekis didėjo, gautas net 0,47 ($p > 0,05$) koreliacijos koeficientas, o dėl Cd – mažėjo –0,13 ($p > 0,05$), tačiau koreliacijos koeficientai statistiškai nereikšmingi. Tarp MDA koncentracijos miežių lapuose ir sunkiųjų metalų kiekio substrate nustatytas stiprus koreliacinis ryšys ($p < 0,05$), kuris buvo šiek tiek stipresnis dėl vario poveikio, nors esant mažesnei koncentracijai nustatyti ir mažesni MDA kiekiai, palyginti su kontroliniais augalais. Koncentracijai padidėjus iki 1 600 mg, Cu ir Cd/kg substrato gauti atitinkamai 68 ir 32 % statistiškai reikšmingai didesni ir MDA kiekiai. Esant mažesnėms (iki 800 mg metalų/kg) substrato koncentracijoms, vario akumuliacija lapuose buvo intensyvesnė nei kadmio ($p < 0,05$), bet kai koncentracija substrate išauga iki 1 600 mg/kg, augalai intensyviau akumuliuoja Cd nei Cu.

Raktažodžiai: kadmis, varis, malondialdehidas, pigmentai, sausa biomasė, bioakumiliacija