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Differences in cadmium accumulation and induced changes in root anatomical structures in plants used for food

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Abstract. A rapid urbanization passes all over the world thus the effect of chemicals, including heavy metals, increases on plants. Heavy metal pollution poses a serious hazard to humans' health, and it uptake into plants is the primary way through which it can enter the food chain. The goal of this study was to investigate the impact of cadmium (Cd) contamination on plant growth responces, Cd uptake, and changes in the root anatomical structures as species-specific reaction to Cd stress. The vegetation experiment was carried out with monocotyledon *Hordeum vulgare* L. and dicotyledonous *Lactuca sativa* L. The plants were grown in quartz sand under controlled optimal growth conditions. Changes in the root structure and Cd accumulation were studied at five levels of Cd added as $Cd(NO_3)_2$ 4 H_2O solution in substrate. The level of Cd in the air-dry plant material was estimated by an atomic absorption spectrometer. To identify structural changes in the plant roots which were caused by Cd accumulation cross sections were cut using microtome and stained with Astra Blue/Safranin for observations using a light microscope. Barley and lettuce growth and development were significantly influenced by increasing the amount of Cd in substrate. There were differences in the ability to accumulate Cd in above-ground plant parts depending on a model object. Substrate contamination with Cd caused significant changes in the root anatomical structures. The obtained results confirmed significance of anatomical and physiological studies to reveal species-specific plant response to Cd stress to avoid heavy metal entrance in the food.

Key words: barley, lettuce, cadmium uptake, root anatomy, growth responses.

INTRODUCTION

Soil contamination with heavy metals has occurred over thousands of years. Soil can be contaminated as the result of natural processes or human activity (Meuser & Van der Graaff, 2011; Swartjes, 2011). European Environmental Agency has found out that there are 2.5 million potentially contaminated sites in Europe and about 14% of them are expected as contaminated. The main types of local sources of contamination in Europe are waste disposal (municipal and industrial waste disposal), industrial and commercial activities (mining, oil extraction and production, power plants), military activities (military sites, war affected zones), storages (oil storage, obsolete chemicals storage, other storages), transport spills on land (sites with spills of oil and other hazardous substances), nuclear (nuclear operations) and other sources (van Liedekerke et al., 2014).

In Europe the main contaminant groups are heavy metals (34.8%) and mineral oils (23.8%) (van Liedekerke et al., 2014).

Among phytotoxic heavy metals, Cd is one of considerable importance due to high water solubility, mobility, and toxicity even in low levels. Severe toxicity of Cd is based on mutagenic and carcinogenic features and high accumulation capacity in plants tissues (Siedlecka, 1995; di Toppi & Gabbrielli, 1999; Lux et al., 2011b). Thus Cd has become one of the most harmful pollutants in agricultural soils with high potential to enter the food chain (Peralta-Videa et al., 2009; McLaughlin et al., 2011) and cause a negative effect on humans' health (Swartjes & Cornelis, 2011). In general, plants have different ability to accumulate heavy metals from soil – vegetable species can be listed in increasing order of Cd accumulation: French bean, beetroot, radish, pea, carrot, and broccoli < potato, tomato, zucchini, and sweetcorn < onion, leek, parsnip < turnip < cabbage, kale < lettuce, spinach (McLaughlin et al., 2011).

The interaction of heavy metals with various biochemical and physiological processes in plants is widely studied (Hall, 2002; Clemens, 2006), however the general overview of processes, taking place in heavy metal-affected cells, are only partially understood. Martin et al. (2006) have revealed that heavy metals reduce shoot: root biomass ratio in vegetables. It is well documented that heavy metals compete with nutrients, causing mineral disturbance in plants, and as a result their growth becomes slower (Siedlecka, 1995; Vollenweider et al., 2006; Ghnaya et al., 2007; Titov et al., 2007). Visual symptoms of Cd phytotoxicity as leaf necrosis, browning of roots, reducting of root diameter and branching, increasing fragility have been found for *Pisum sativum, Lactuca sativa, Spinacia oleracea* (Fusconi et al., 2006; Osvalde & Paegle, 2007).

There are two kinds of heavy metals which are referred to micronutrients essential for plant metabolism (Fe, Cu, Zn, Mn and Mo) and not essential, such as Cd, Hg and Pb (Marschner, 1995; Siedlecka, 1995). Both can cause toxicities when they are present in excess. In spite of the different mobility of metals in soil and plants, the root system, in general, accumulates significantly higher concentrations of heavy metals (Abe et al., 2008) and it is one of the main targets of the toxic effects. Consequently, the disturbed root metabolism directly or indirectly affects other physiological processes in plants (Titov et al., 2007).

During the last years particular scientific attention was paid on phytoremediation techniques of contaminated soils as well as on plants with exceptional metal-accumulating capacity – hyperaccumulators (Raskin & Ensley, 2000). In general, metal hyperaccumulating species have been identified at least in 45 plant families and new species or populations continue to be discovered (Kraemer, 2003). Economically important crop species including barley (*Hordeum vulgare*) recently have been identified as plants for efficient uptake and accumulation of heavy metals with a phytoremediation potential (Vassilev et al., 1998; Sękara et al., 2005; Nadgórska-Socha et al., 2013).

The physiological mechanisms and main strategies of different species related to the metal accumulation, compartmentalization, and detoxification have still not been investigated in detail. Many studies have revealed the changes in the morphological traits of plants and internal structures of roots as epidermis, cortex parenchyma, exodermis, vascular cynlinder, sclerenchyma, Casparian strips, and the xylem pipes under heavy metal stress in the range of plants (Hose et al., 2001; Enstone et al., 2003; Probst et al.,

2009). These changes, occurring in plants, can be species and tissue specific (Hermle et al., 2006; Lux et al., 2011a).

Although, response of plants to Cd treatment is intensively studied there is still limited knowledge concerning the particular role of root anatomical structure of monocotyledons and dicotyledons in fixing and transport of metal ions. Cd can cause changes in the root morphology and anatomy of plants. There are mechanisms – physiological, anatomical and physical – how plants prevent Cd uptake in the root and transport to xylem (Lux et al., 2011a; Lux et al., 2011b). Some of them are: production of Cd-chelates, accelerated development of endodermis and exodermis (Hose et al., 2001; Enstone et al., 2003; Lux et al., 2011a), formation of hypodermal periderm (Lux et al., 2011b) and peri-endodermal layer of cells with lignified cell walls (Zelko et al., 2008). The increased thickness of the abaxial and adaxial sclerenchyma and pericycle tissues in *Brachiaria decumbens*, caused by heavy metals, could be related to adsorption of metals in the cell walls, constituting an alternative pathway for allocation of these ions and preventing their translocation to photosynthetic tissues (Gomes et al., 2011).

Another changes in the root anatomical structures caused by presence of heavy metals were observed by Vollenweider et al. (2006). Roots of *Salix viminalis* cultivated in the presence of Cd formed thickened cell walls of the collenchymas and pericycle, with higher concentrations of metal than the other tissues.

It is known from previous studies that monocotyledons and dicotyledons can accumulate different Cd and lead (Pb) amount from soil (Martin et. al., 2006; Osvalde & Paegle, 2007; Abe et al., 2008; Chang et al., 2014). As morphologically differentiated inner cell layer of cortex – root endodermis has an important effect on the transport of soil solution between the root cortex and vascular cylinder, endodermis typical for monocotyledons with its Casparian stripes acts as an important barrier that holds up heavy metals (Enstone et al., 2003; Gomes et al., 2011).

A morphologically differentiated inner cell layer of cortex – endodermis typically surrounds the pericycle (Evert, 2006). In the roots, in which the secondary growth is not present, endodermis has several steps of differentiation. The first is formation of thickened and lignified Casparian strips in anticlinal walls of endodermis (Evert, 2006). During the second step the cell wall covers with a thin suberin film to an inner side of cell thus separating the Casparian strips from cytoplasm. The third step characterizes by an irregular thickening of endodermis cell wall. If the thickening occurs in all the cell wall except the outer one, the U – like endodermis forms. Most of monocots and just some dicots have the third step of endodermis differentiation (Evert, 2006).

Since a lot of field crops are monocots with endodermis consisting of U – like form cells, endodermis may have a relevant role in preventing a free penetration of pollutants through the apoplast.

Therefore, knowledge on modifications in the structure of root tissues could provide insights on differences in the root system functioning and capability of heavy metal accumulation among the plant species. The goal of this study was to investigate the impact of Cd contamination on plant growth responses, Cd uptake, and changes in the root anatomical structures as species specific reaction to Cd stress.

MATERIALS AND METHODS

The vegetation experiment was carried out with a monocotyledon barley (*Hordeum vulgare* L., cv. 'Ansis') and a dicotyledonous lettuce (*Lactuca sativa* L., cv. 'Grand Rapid') as model plants. These plants were grown up in 1 L polyethylene containers from seeds (18 barley plants per pot and three lettuce plants per pot) under controlled growth conditions – day/night temperature + 20/18 °C, photoperiod light/dark 16/8 h, humidity of substrate 60–65%, photon flux density 160 μmol m^{-2} s^{-1} supplied by fluorescent tubes). Quartz sand was used as substrate. Humidity of the substrate was maintained throughout the experiment gravimetrically using deionized water.

Five levels of Cd (added as $Cd(NO_3)_2 \cdot 4\ H_2O$ solution): 0, 3, 6, 9, 12 mg L^{-1} for barley and 0, 1, 2, 4, 6 mg L^{-1} for lettuce were added in substrate. Nutrient solution, containing optimal concentrations of macroelements and microelements (in mg L^{-1}: N 120, P 60, K 150, Ca 800, Mg 50, S 60, Mn 1.5, Zn 1, Cu 0.5, Mo 0.02, B 0.2, Fe 30) (Osvalde, 2011), was added in substrate for all treatments. All the nutrients were provided with complete nutrient solution, Ca as $CaCO_3$ in each pot at the beginning of the experiment.

The vegetation experiment ran for 43 days. Plants were collected on the day of 16^{th}, 23^{rd}, 29^{th}, 36^{th} and 43^{rd} of the experiment. Biomass of plans was estimated during the experiment. Roots were separated from shoots and washed in distilled water. Fresh weight of leaves and roots was determined for one plant.

Plant material was dried at 64 °C to a constant weight. Plant material was dry mineralized with HNO_3 vapours and dissolved in HCl (Rinkis et al., 1987). The level of Cd in air-dry plant material was estimated by an atomic absorption spectrometer Perkin Elmer AAnalyst 700A, acetylene-air flame. For control treatment Cd analyses were conducted on a graphite furnace equipped atomic absorption spectrometer Perkin Elmer AAnalyst 700 (Anonymous, 2000).

To identify structural changes in the plant roots, caused by Cd accumulation, 15 μm cross sections were cut using Leica SM 2010R microtome and stained with Astra Blue/Safranin. Cross sections were made in two regions – at the distance of 10 mm from a root apex and 5 mm from a root base. Investigation and photographs of micro slides were made using digital microscope Leica DM5500 equipped with a digital camera Leica DFC490. Images were analysed using graphic workstation Dell Precision T7400 and software Image-Pro Plus 6.2.

The statistical analysis of results was done using MS Excel 2013. Standard errors (SE) were calculated in order to reflect the mean of the results. The Student's *t*-test 'Two-Sample Assuming Equal Variances' ($p < 0.05$) was used for testing the differences between the treatments.

RESULTS AND DISCUSSION

Heavy metal pollution in soil had different impact on the process of biomass production of barley and lettuce. At control conditions Cd0 barley biomass gradually raised up to the fifth leaf stage, at Cd3 – to the sixth leaf stage, at Cd6, Cd9 and C12 – to the fourth leaf stage, while lettuce biomass of control treatment Cd0 gradually raised up to the ninth leaf stage, at Cd1 and Cd2 – to the eighth leaf stage and Cd4 and Cd6 – to the sixth leaf stage.

In general, plant exposure to Cd treatments resulted in decreasing fresh biomass of plant leaves and roots significantly. At the highest pollution level (Cd12) biomass of barley leaves was 55.45% and of roots 36.79% from the control (Cd0) at the end of the experiment (Fig. 1, A, B). There was no significant impact on biomass of barley leaves and roots at the low pollution level of Cd 3 mg L^{-1} in the substrate on the 29th day (*t*-test, $p < 0.05$).

In plant fresh weight a considerable decrease was also found for lettuce (Fig. 2, A, B). At the end of the experiment, Cd6 treatment resulted in massive inhibition of leaf and root biomass (29.50% and 21.76%, respectively, of the control level).

It is notable that the highest root biomass was found for low Cd concentrations in substrate (for barley Cd3, for lettuce Cd1 and Cd2). For lettuce this phenomenon was typical at the beginning of ontogenesis.

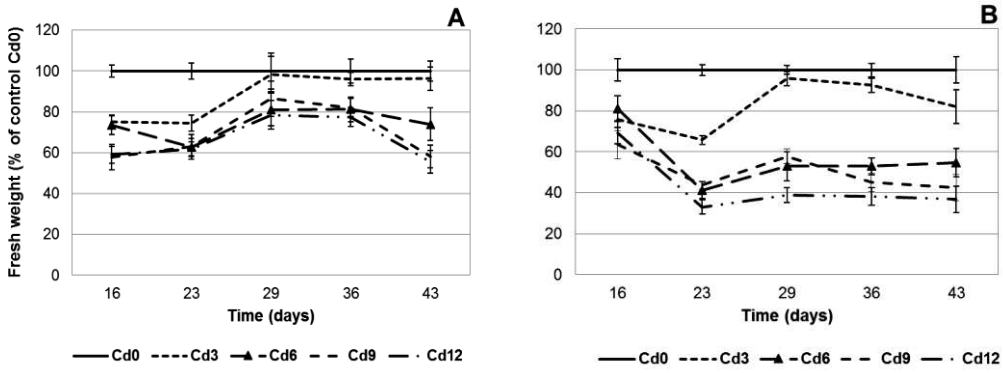

Figure 1. Fresh weight (% of control) of the leaves (A) and roots (B) of *H. vulgare* L. at five levels of Cd added in substrate, ± SE.

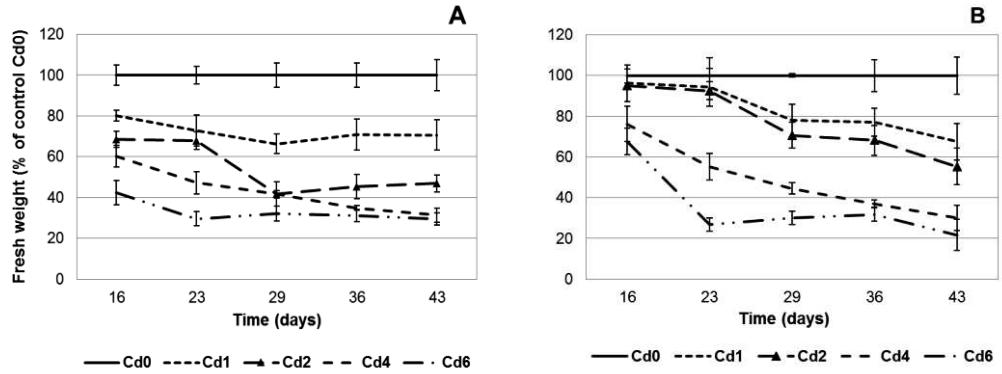

Figure 2. Fresh weight (% of control) of the leaves (A) and roots (B) of *L. sativa* L. at five levels of Cd added in substrate, ± SE.

Similar tendencies of changes in plant biomass of leaves and roots under Cd pollution were confirmed by other studies which found that low concentrations of Cd could contribute to the activation of physiological processes in the plant, while at high concentrations there was high possibility of irreversible, even structural changes (Hart et al., 1998; Wójcik & Tukiendorf, 1999, Titov et al., 2007). In our study the results revealed that significant changes were typical for barley at Cd pollution level from 6 to 12 mg L^{-1} in substrate, for lettuce from 2 to 6 mg L^{-1} in substrate.

During the recent years increasing experimental evidence has been associated to Cd toxicity with antagonistic reactions of Cd and essential nutrients (N, P, K, Ca, Mg, Fe, Cu, B, Mn and Zn), alteration of membrane permeability, generation of oxidative stress. Thus, Cd toxicity may result from: a) disturbances in Zn uptake and transport, due to similar pathways; b) replacing of K and inactivation of stomata; c) inactivation of photosynthesis (Cd inactivates water photolysis, reaction-centers of photosystems, electron transport, carboxylation enzyme *Rubisco* and phosphoglycerol synthetase activity) etc. (Siedlecka & Krupa, 1996; Grant et al., 1998; Arazi et al., 1999; di Toppi & Gabbrielli, 1999; Siedlecka & Krupa, 1999; Clemens, 2001; Šeršeň & Královά, 2001). The plant vitality and biomass reduced in the result of inactivation of these physiological processes. Barceló et al. (1990) and Sandalio et al. (2001) have mentioned that reduction of plant biomass has been the result of nutrition and water unbalance in plants. During the experiment, it was observed that both barley and lettuce roots changed their colour (they became darker yellow) and became thinner and more fragile. As well as there were visual changes in leaves as chlorotic spots observed and at the end of the experiment leaves became necrotic.

Plant exposure in increasing levels of Cd resulted in a progressive inhibition of growth and simultaneous accumulation of Cd both in leaves and roots of plants. The highest concentrations of Cd were established for both barley and lettuce roots and leaves (Figs 3, 4) at the beginning of ontogenesis (from the 16th to 23rd day of the experiment).

Although, Cd concentrations in the roots were much higher than in leaves of studied plants, lettuce and barley exhibited specific differences in the ability to accumulate Cd in leaves. In general, Cd accumulation occurred more intensively in lettuce leaves, compared to barley. Thus, on the 29th day of the experiment under a 6 mg L^{-1} level of Cd in the substrate (Cd6), the concentration of Cd in lettuce leaves reached 56.0 mg kg^{-1} while in barley leaves 25.4 mg kg^{-1} (Figs 3, A; 4, A). More seriously affected leaf biomass of lettuce was also found in these conditions: fresh weight of lettuce aboveground parts was 2.5 times lower than for barley and reached only 30% of control level. Our data support previous findings that leafy vegetables can accumulate the highest value of heavy metals (Pandey & Pandey, 2009; Ngole, 2011; Liu et al., 2012; Chang et al., 2014).

The obtained results confirmed that metal accumulation mainly depends on plant species. Although, Cd is considered to be one of the most mobile heavy metals with high translocation possibilities to plant above-ground tissues (Lux et al., 2011a; (Vassilev et al., 2004; Sękara et al., 2005;), it mainly accumulates in the plant roots. At the end of the experiment Cd concentration in barley roots under treatments, respectively, Cd3, Cd6, Cd9, Cd12 was 12.7, 16.6, 11.8, and 13.9 times higher than in the leaves (Fig. 3, B). These ratios for lettuce were significantly lower - at the end of the experiment Cd level of lettuce roots under treatment Cd1 was 3.2 times higher than in the leaves, Cd2 – 3.8, Cd4 – 5.2 and Cd6 – 6.0 (Fig. 4, B). As reduced translocation of heavy metals to the

plant shoots a possible mechanism of metal tolerance appears (Lux et al., 2011a), the obtained results confirmed that barley roots act as a physiologically active protection barrier restricting root-shoot translocation of Cd.

Uptake and transport processes have been recognized as the central mechanism of metal detoxification and tolerance in plants (di Toppi & Gabbrielli, 1999; Hall, 2002; Enstone et al., 2003; Sękara et al., 2005; Clemens, 2006; Gomes et al., 2011; Lux et al., 2011a; Lux et al., 2011b; Vaculík et al., 2012). Therefore, the investigations were done to reveal the particular role of the root anatomical structure in fixing and transport of Cd to provide more insights in differences of monocotyledons and dicotyledons.

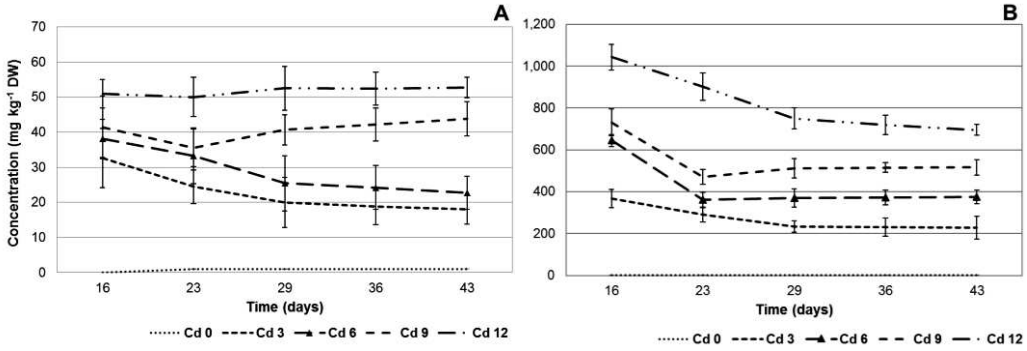

Figure 3. Cadmium concentrations (mg kg^{-1} DW) in the leaves (A) and roots (B) of *H. vulgare* L. at five levels of Cd added in substrate, ± SE.

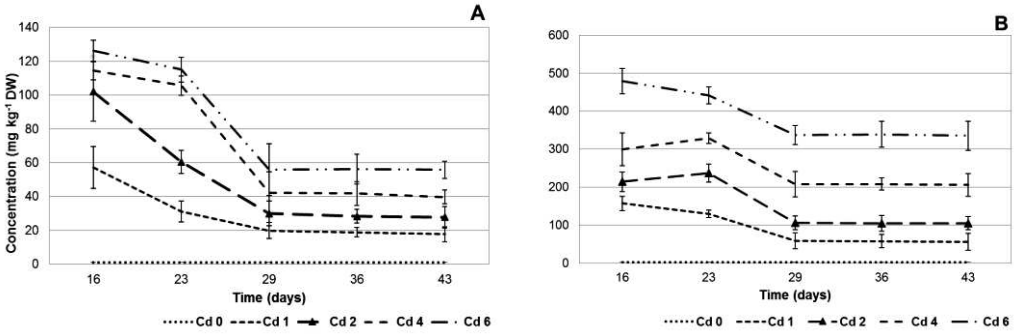

Figure 4. Cadmium concentrations (mg kg^{-1} DW) in the leaves (A) and roots (B) of *L. sativa* L. at five levels of Cd added in substrate, ± SE.

Current study suggests that at the beginning of ontogenesis Cd transport from the roots to the leaves in the barley (monocotyledons) can be delayed by Casparian stripes in endoderm, later by the cell wall thickening of endoderm and pericycle cells (Fig. 5). At the beginning of ontogenesis the cell wall thickening was less expressed in lettuce roots (Fig. 5, B). The differences in development of endodermis under equal level of Cd contamination (Cd6) were found – while endodermis of barley formed lignified anticlinal and inner periclinal U-shape cell walls as well as peri-endodermis (Fig. 5, A), only Casparian bands in the root endodermis cells of lettuce plants were present and formation of peri-endodermis absent (Fig. 5, B).

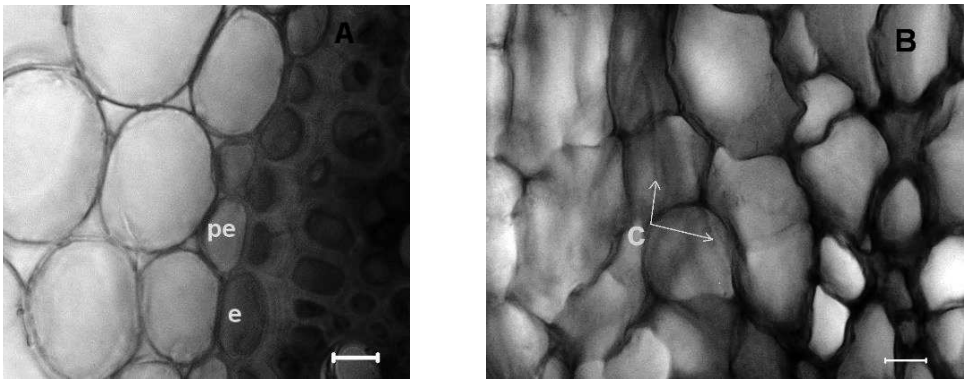

Figure 5. Cross section of *H. vulgare* L. root base (A) and cross section of *L. sativa* L. main root base (B) exposed to Cd6 and on the 29th day of the experiment. Abbreviations: e – endodermis; pe – peri-endodermis; c – Casparian bands. Scale bar: 5 µm (A), 20 µm (B).

Our study has shown that a peri-endodermal layer of cells with lignified cell walls was present in the base of barley root starting from the 23rd day of the experiment (Fig. 6). Although, some authors report that these apoplasmic barriers develop closer to the root apex when roots are exposed to high concentrations of potentially toxic elements (Zelko et al., 2008; Lux et al., 2011a). The impact of Cd contamination level Cd3 on the formation of peri-endodermis can be observed starting from the 43rd day of the experiment when 3 cells were found. The number of peri-endoderm cells increases depending on Cd treatment level. Most relevant differences in number of peri-endoderm cells were stated from the 43rd day of the experiment – while they were absent in the control plants, in Cd3, Cd6, Cd9 and Cd12 treatments 3, 6, 11 and 15 cells were found respectively (Fig. 6). Formation of peri-endodermis in lettuce roots was not observed.

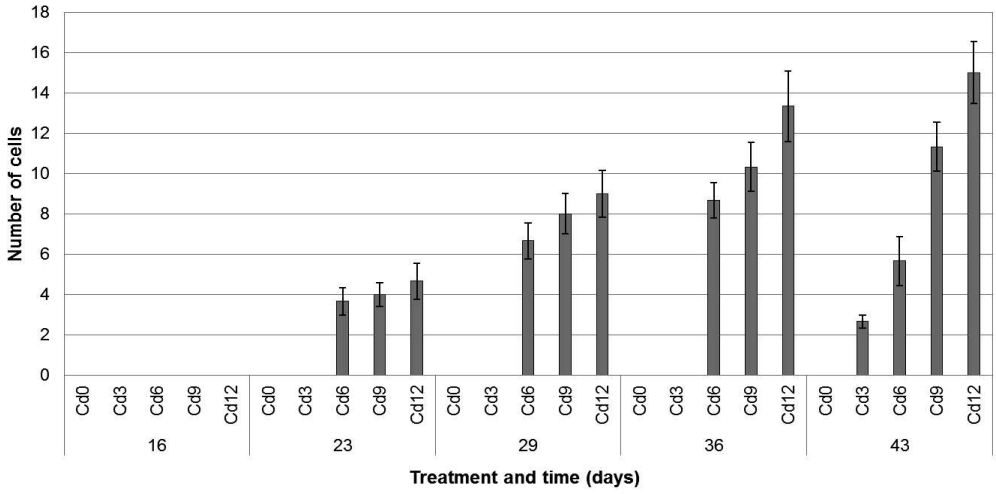

Figure 6. Number of peri-endodermis cells in *H. vulgare* L. root base exposed to five levels of Cd added in substrate, ± SE.

The studies on changes of the root anatomical structure in conditions of Cd toxicity reveal the presence of lignified pericycle cell walls and the lack of passage cells in endodermis of barley under Cd6 (Fig. 7, A), while under Cd0 passage cells are present and proximal pericycle cell walls remain unlignified and maintained parenchymatic character (Fig. 7, B). This available evidence suggests that passage cells of endodermis allow the passing solutions towards vascular cylinder by symplast to play an active role in ion uptake and could be important for Cd uptake and transfer (Peterson & Enstone, 1996). Penetrating the soil solution is ensured by the transpiration in tracheary elements of the radial vascular bundle of root, the passage cells in their turn are located opposite to xylem elements and ensure the penetration in vascular cylinder only via the symplast (Gomes et al., 2011).

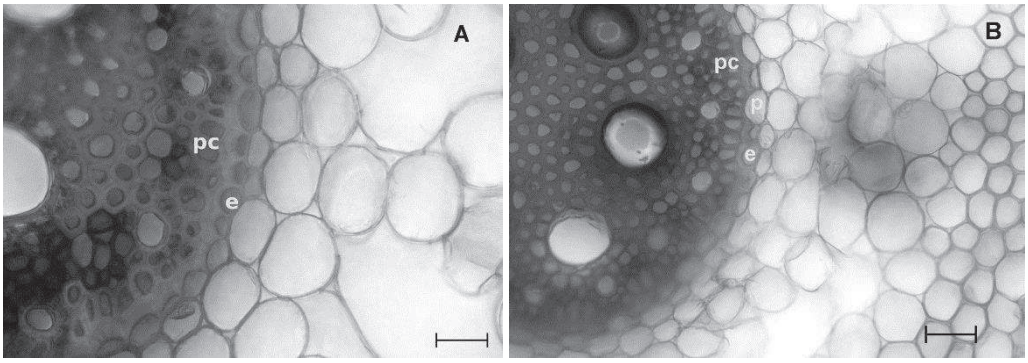

Figure 7. Cross section of *H. vulgare* L. root base exposed to Cd6 (A) and Cd0 (B) on the 29th day of the experiment. Abbreviations: e – endodermis; pc – pericycle; p – passage cells. Scale bar: 30 µm (A), 50 µm (B).

According to Nagahashi et al. (1974) the effectiveness of the Casparian strips as a barrier to apoplastic movement has been demonstrated at the electron microscope level by the use of maize roots. Thus, heavy metals cannot penetrate the endodermis to enter the stele through apoplast – it is allowed only by symplastic structures of passage cells (Enstone et al., 2003; Gomes et al., 2011).

CONCLUSIONS

This study suggests that Cd accumulation in monocotyledon and dicotyledon plant leaves and roots differs depending on changes in the root anatomical structures caused by Cd pollution. Barley and lettuce exposure to Cd treatments resulted in decreasing fresh biomass of leaves and roots significantly. Significant changes were characteristic for barley at Cd pollution level from 6 to 12 mg L^{-1} in substrate, for lettuce from 2 to 6 mg L^{-1} in substrate. The obtained results confirm that metal accumulation highly depends on plant species: lettuce and barley exhibit specific differences in the ability to accumulate Cd in their leaves. Changes in anatomical structures of barley roots – endoderm cell walls thickening and lignification, lack of passage cells in endodermis, pericycle cell walls thickening as well as formation of peri-endodermis – act as an active

protection barrier restricting root-shoot translocation of cadmium. At the beginning of ontogenesis the corresponding responses were less expressed in lettuce roots.

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