

## TISSUE ORGANIZATION AND CELL ULTRASTRUCTURE IN THE ROOTS OF THREE *ARABIDOPSIS* SPECIES GROWN AT DIFFERENT ZINC CONCENTRATIONS

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**Abstract.** The model plant *Arabidopsis thaliana* is known to be heavy metal-sensitive in contrast to its relative species *A. arenosa* and *A. halleri* classified as pseudometallophytes. Quantitative differences in primary root anatomy previously found between *A. thaliana* and the non-metallicolous (NM) and metallicolous (M) populations of the non-model *Arabidopsis* species necessitated further research at cellular and ultrastructural levels. Seedlings of *A. thaliana*, ecotype Columbia and a natural population Ratkovo, the NM and M populations of *A. arenosa* and *A. halleri* were grown on agar medium containing 10  $\mu\text{M}$  (control) and 1000  $\mu\text{M}$   $\text{Zn}^{2+}$  for 5 days. Light microscopy confirmed the higher number of cells in the endodermal, cortical and epidermal layers and a higher incidence of additional cell tiers, the so-called middle cortex (MC) in the tolerant genotypes. Such differences were present in untreated plants and even more pronounced in plants exposed to excess of zinc (Zn). Electron microscopy of the root tissues at comparable distances from the root tip showed Casparian bands only in the radial cell walls of endodermis of *A. halleri* M population originating from severely (Cu, Cd and Pb) contaminated site. Casparian bands were not differentiated yet in the roots of the other species and populations, and they were not formed in the cell walls between endodermis and MC cells. In the apical cytoplasm of trichoblast bulges, autophagic vacuoles were found only in the sensitive *A. thaliana* and small vacuoles in the other genotypes. The enhanced concentration of Zn confirmed the higher metal sensitivity of the model species and did not substantially disturb the root cell ultrastructure of the tolerant *Arabidopsis* species.

**Keywords:** *Arabidopsis thaliana*, *Arabidopsis arenosa*, *Arabidopsis halleri*, non-metallicolous and metallicolous populations, root anatomy, cell ultrastructure, zinc

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### Introduction

The species *Arabidopsis thaliana* (L.) Heynh. due to its metal sensitivity, was found only in non-metalliferous sites in natural conditions (STAŇOVÁ *et al.* 2010, 2012). In contrast, the relative species, *A. halleri* (L.) O’Kane et Al-Shehbaz and *A. arenosa* (L.) Lawalrée are metal-tolerant and can occupy natural localities with the soils contaminated with heavy metals. Both are classified as pseudometallophytes having non-metallicolous and metallicolous populations growing on non-contaminated and contaminated soils respectively, found also in the territory of Slovakia (BANÁSOVÁ *et al.* 2006; TURISOVÁ *et al.* 2013). Natural variation of Zn tolerance was reported in *A. thaliana* accessions (RICHARD *et al.* 2011). Comparing the responses of relative *Arabidopsis* species and their populations can provide us with important data on mechanisms of metal

tolerance (ROOSENS *et al.* 2008). In addition, the existence of non-metallicolous (NM) and metallicolous (M) populations or ecotypes of the pseudometallophytes can serve as relevant model to study microevolutionary adaptive processes occurring within a species (DECHAMPS *et al.* 2010).

Root tissue organization has been described for the known model species *A. thaliana* (DOLAN *et al.* 1993) and the root developmental zones were characterized into detail (VERBELEN *et al.* 2006). The radial pattern of the root tissues (DOLAN *et al.* 1993; SCHERES *et al.* 1994; BAUM *et al.* 2002) as well as ultrastructure of root cells particularly within the apical meristematic region (DOLAN *et al.* 1993; ZHU *et al.* 1998) are also well known for the model plant *A. thaliana*. Formation of Casparian bands in the radial endodermal cell walls occurs at a distance of 1500 to 1600  $\mu\text{m}$  from the root apex in *A. thaliana* ecotypes Columbia

(ALASSIMONE *et al.* 2010) and Landsberg *erecta* (MARTINKA *et al.* 2012). Similar characteristics of the non-model *Arabidopsis* species are scarce.

The NM and M populations of *A. arenosa* and *A. halleri* from the localities in the territory of Slovakia (STAŇOVÁ *et al.* 2010) were compared with the sensitive *A. thaliana* ecotype Columbia (Col) and a natural population Ratkovo to characterize the responses of root system morphology and anatomy to varying contents of heavy metals in the root media (STAŇOVÁ *et al.* 2012). Growth and physiological responses also confirmed the differences in metal sensitivity of these *Arabidopsis* species (KENDEREŠOVÁ *et al.* 2012). Cytological, genetical and ecological characteristics of the non-model *Arabidopsis* species were reviewed and provide basis for further investigations of the genus *Arabidopsis* (CLAUS & KOCH 2006). The aim of this study was to extend the data on root anatomical traits in the three *Arabidopsis* species with an exploration of the responses to enhanced zinc concentration at tissue and cell ultrastructure levels.

### Material and methods

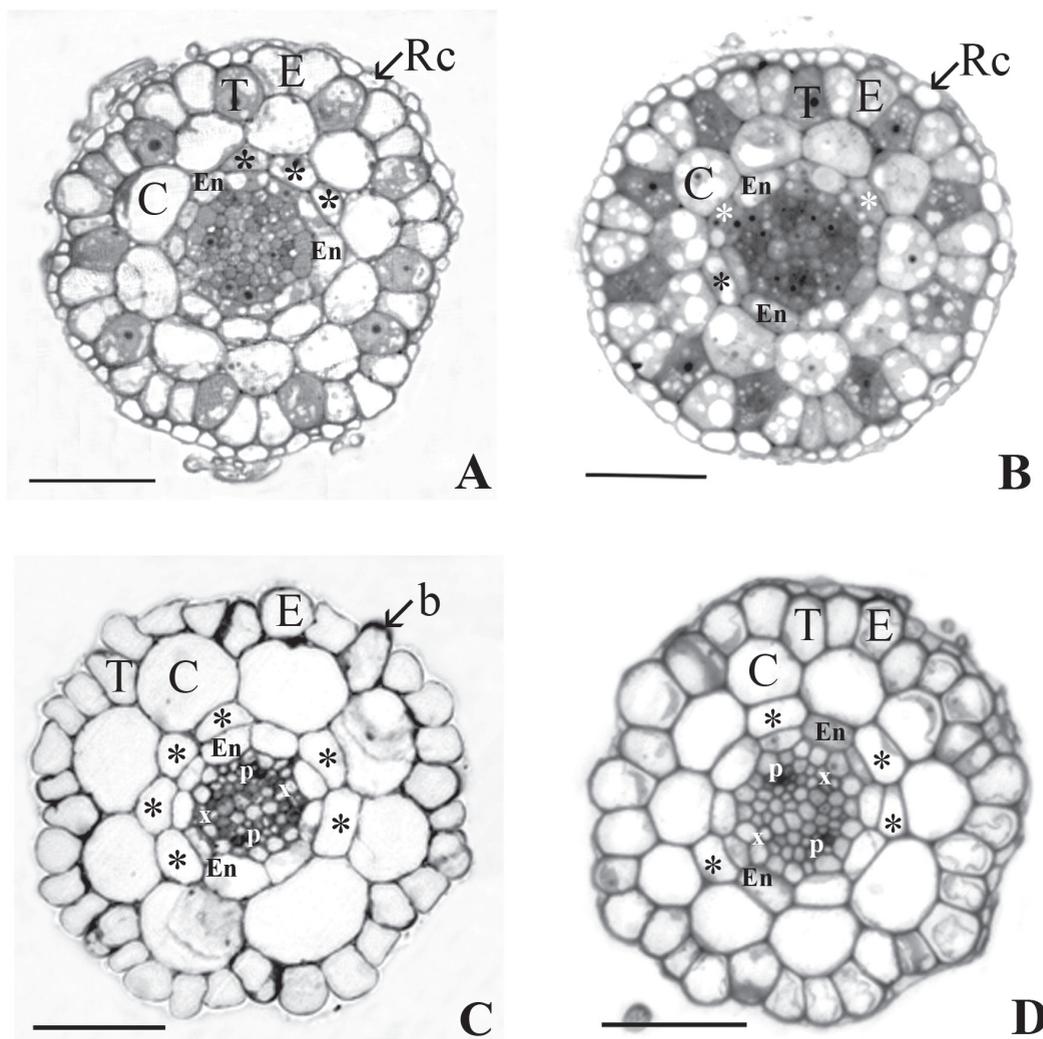
The plant material and populations of *A. thaliana*, *A. arenosa*, and *A. halleri* originated from the seeds collected in natural non-metalliferous and metalliferous localities in Slovakia described in our previous work (STAŇOVÁ *et al.* 2010). We used *A. thaliana* Col and a population from natural non-contaminated site Ratkovo (abandoned field), *A. arenosa* NM population Richtárová lúka (meadow) and M population Terézia (mine heap) from Central Slovakia and *A. halleri* NM population Úhorná (meadow) and M population Kropachy (meadow close to copper smelter) from East Slovakia. The metal contents of non-contaminated localities did not exceed the permitted values of heavy metal concentration in soils. Total metal concentrations (in mg·kg<sup>-1</sup>) in soils of the metalliferous sites were 2368 Zn, 3925 Pb, 22 Cd, 269 Cu with the M population *A. arenosa* Terézia, and 186 Zn, 283 Pb, 1.32 Cd, 501 Cu with the M population *A. halleri* Kropachy. The available fractions after EDTA

extraction are given by STAŇOVÁ *et al.* (2012).

Treatments and microscopical techniques were described by STAŇOVÁ *et al.* (2012). Briefly, the seedlings were grown on agar medium containing 10 µM (control) and 1000 µM Zn<sup>2+</sup> for 5 days. Apical part of the primary roots were fixed with Karnovsky procedure, dehydrated in ethanol series and embedded in Spurr resin. Serial semithin sections were taken from about 250 µm distance from the root tip (DFT) up to the site of the first trichoblast bulge appearance between 600 and 1300 µm DFT, depending on species and Zn concentration. The semithin sections were stained with Toluidine blue and the tissue organization was viewed with Olympus BX51 microscope. Ultrastructure of epidermal, cortical and endodermal cells was investigated in ultrathin sections stained with uranyl acetate and Pb citrate using Tesla BS500 TEM.

### Results and discussion

The relatively simple root tissue pattern of the well known species *A. thaliana* ecotype Col (DOLAN *et al.* 1993) was found also in the population originating from the natural site Ratkovo. Root tissue organization of the other two species *A. arenosa* and *A. halleri*, and their non-metalliferous and metalliferous populations was less regular and differed in a higher number of cells in the individual tissue layers, the epidermis, cortex, and endodermis (STAŇOVÁ *et al.* 2012). In addition, the cortex acquired additional cellular tiers, the so called middle cortex located at the xylem pole (Fig. 1), which resulted from periclinal divisions of endodermal cells (BAUM *et al.* 2002). In *A. thaliana* populations, only one middle cortex (MC) cell in a root cross section occurred rarely, within limited distances from the root tip (about 700 µm DFT). On the contrary, in heavy metal tolerant *A. arenosa* and *A. halleri* the MC tiers were more frequent in the sections of young, undifferentiated tissues (for example the NM and M population of *A. halleri*, Fig. 1 A, B) as well as in the regions of more differentiated cells and first trichoblast bulges appearance (indicating root hair emergence) at 700 to 1300 µm DFT



**Fig. 1.** Cross sections of *Arabidopsis* primary roots grown on agar medium with 10  $\mu\text{M}$  Zn (A, B) and 1000  $\mu\text{M}$  Zn (C, D). Region of undifferentiated cells in the root of *A. halleri*, NM population at 500  $\mu\text{m}$  DFT (A), and *A. halleri*, M population at 300  $\mu\text{m}$  DFT (B). Site of root hair emergence in the roots of *A. arenosa* NM population at 720  $\mu\text{m}$  DFT (C) and *A. halleri*, M population at 600  $\mu\text{m}$  DFT (D). Note the middle cortex (MC) cells (asterisks). **b** – bulge; **C** – cortex; **E** – epidermis; **En** – endodermis; **p** – phloem pole; **Rc** – root cap cells; **T** – trichoblast; **x** – xylem pole. Bars represent 0.05 mm.

depending on population and Zn concentration (Fig. 1 C, D). Except of *A. thaliana* populations, the occurrence of the first bulges were recorded slightly but not significantly closer to the root tip in higher Zn concentration than in control.

The increased 1000  $\mu\text{M}$  Zn concentration did not affect the root tissue pattern of the studied species or populations. Our previous

quantitative evaluation of the root anatomy showed higher number and size of the root cells and area of the root tissues of the tolerant species comparing to the non-tolerant *A. thaliana* (STAŇOVÁ *et al.* 2012). Differences in adventitious root tissue area were recorded between *Salix caprea* L. isolates originating from non-metalliferous and metalliferous localities.

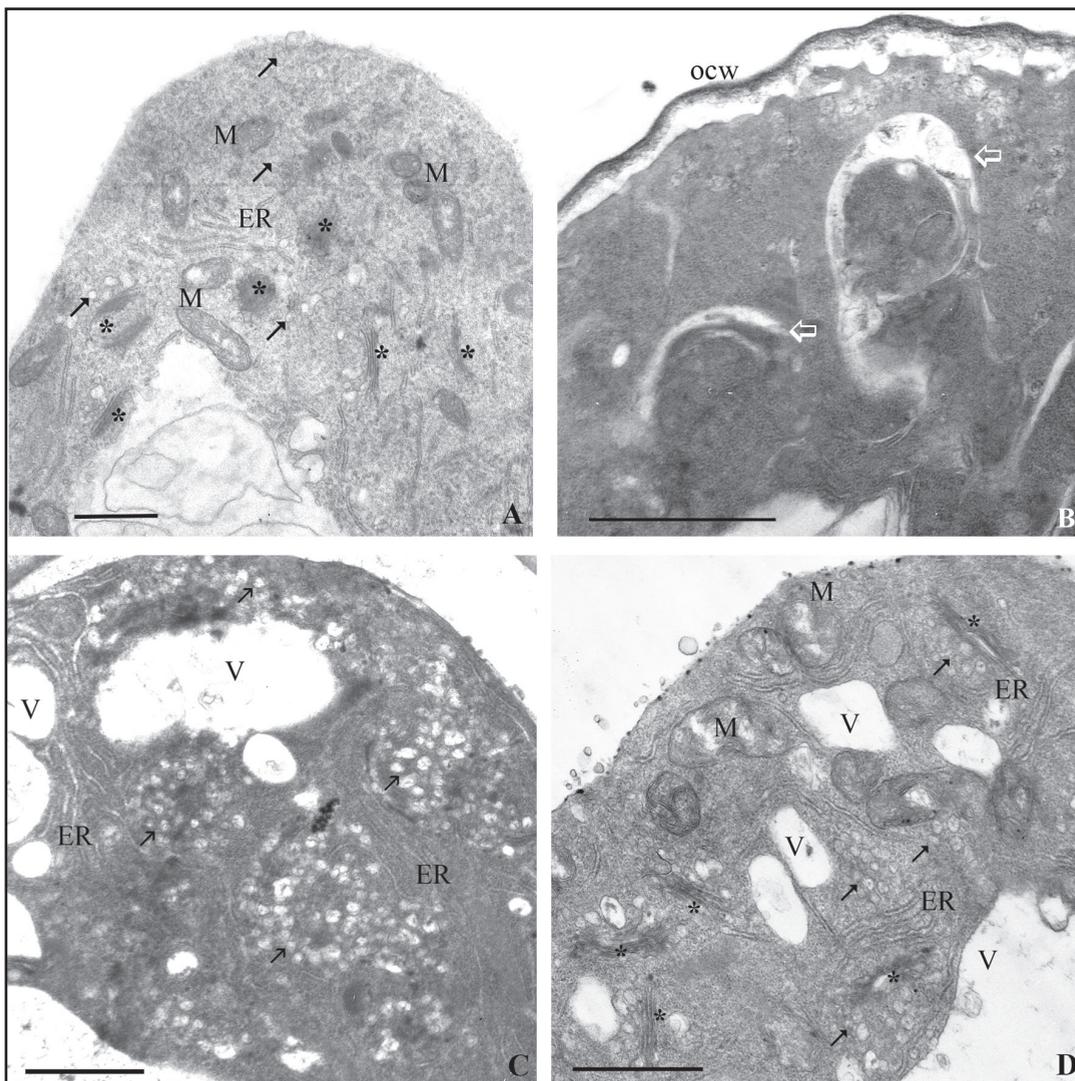
In contrast to our *Arabidopsis* seedlings, in both isolates, the treatment with heavy metals (Zn, Cd) resulted in a decrease of root tissue area (VACULÍK *et al.* 2012). There is not much known about root tissue proportions responding to metal toxicity and the results available show great variability depending on plant species and metal concentration (LUX *et al.* 2011; VACULÍK *et al.* 2012, and literature cited therein). Concerning root anatomy, more attention has been paid to differentiation of structural apoplasmic barriers in diverse genotypes and their responses to heavy metal toxicity (BROADLEY *et al.* 2007; ZELKO *et al.* 2008; MARTINKA *et al.* 2014). In case of our *Arabidopsis* species and populations, the concentration 1000  $\mu\text{M}$  Zn had minor effect on root tissue organization. Therefore, the possible effects at ultrastructural level were investigated.

The cells of rhizodermis and cortex, including endodermis, were investigated at the site of the first trichoblast bulge/root hair appearance that occurred within 600 to 1300  $\mu\text{m}$  behind the root tip. Although extremely sensitive to changes of their immediate environment, the root hairs were formed by the roots of sensitive (*A. thaliana*) and tolerant (*A. arenosa*, *A. halleri*) populations. The cytoplasm of the apical part of emerging root hairs was similar to that described by GALWAY *et al.* (1997). Comparing to control (Fig. 2 A) the exposure to 1000  $\mu\text{M}$  Zn induced formation of autophagic vacuoles in the apical part of root hair bulges in the sensitive *A. thaliana* ecotype Col (Fig. 2 B). Similar morphology of vacuole formed from dilated part of ER was shown in *A. thaliana* root meristem treated with coumarin. In these cells also autophagic vacuoles containing acid phosphatase were documented (KUPIDŁOWSKA 2001). This may indicate a process of cytoplasm disintegration as the autophagic vacuoles play a role in sequestration of cytoplasmic fragments and their subsequent digestion in the lytic vacuole as a stress response (BASSHAM *et al.* 2006). At this concentration, also the root growth was reduced only in *A. thaliana* and was unaffected in *A. arenosa* and *A. halleri* (KENDEREŠOVÁ *et al.* 2012). In the apical root hair cytoplasm, small vacuoles often appeared in the tolerant

populations (Fig. 2 C, D), which also could possess an autophagic function. However, the Zn concentration used did not induce damage to cellular organelles present in the cytoplasm of this root hair region in either sensitive or the tolerant species and populations.

At the distances where the first bulges appeared, the cortical cells under control 10  $\mu\text{M}$  Zn concentration contained a large central vacuole and well developed organelles in the cytoplasmic layer along the cell walls. Comparing to control the ultrastructure of these cells was not changed under the increased 1000  $\mu\text{M}$  Zn concentration in the agar medium (Fig. 3). The only remarkable difference was the occurrence of structures filled with homogeneous material, in the cytoplasmic layer along the walls of cortical cells (Fig. 3C). These structures might represent lipid bodies or electron-dense material in the form of globules induced by elevated concentration of Zn, found in the roots of *Paulownia tomentosa* (Thunb.) Steud. (AZARELLO *et al.* 2012). Their morphology resembled also the so called ER bodies that play a role in plant responses to environmental stresses (HAYASHI *et al.* 2001).

Ultrastructure of endodermal cells (Fig. 3) agrees with that characterized by MARTINKA *et al.* (2012) in *A. thaliana* ecotype Ler. Regardless of Zn concentration in the root media, the Casparian bands were not differentiated yet in the radial cell walls of endodermis of the populations of *A. thaliana* (Fig. 3 B), *A. arenosa* and the NM population Úhorná of *A. halleri*. The reasons might be that (1) the sections were taken closer (500 to 900  $\mu\text{m}$ ) than the distance 1600  $\mu\text{m}$  from the root cap junction where the Casparian bands appeared in *A. thaliana* (ALASSIMONE *et al.* 2010; MARTINKA *et al.* 2012) and, (2) the 1000  $\mu\text{M}$  Zn concentration was not high enough to induce the Casparian band differentiation closer to the root tips as reported with other species (BROADLEY *et al.* 2007; VACULÍK *et al.* 2012). The only exception was the M population of *A. halleri*, in which the Casparian bands were formed in the radial walls of endodermis cells already at about 840  $\mu\text{m}$  and 720  $\mu\text{m}$  from the root cap junction under 10  $\mu\text{M}$  (Fig. 3 A) and 1000  $\mu\text{M}$  Zn (Fig. 3 C, D)

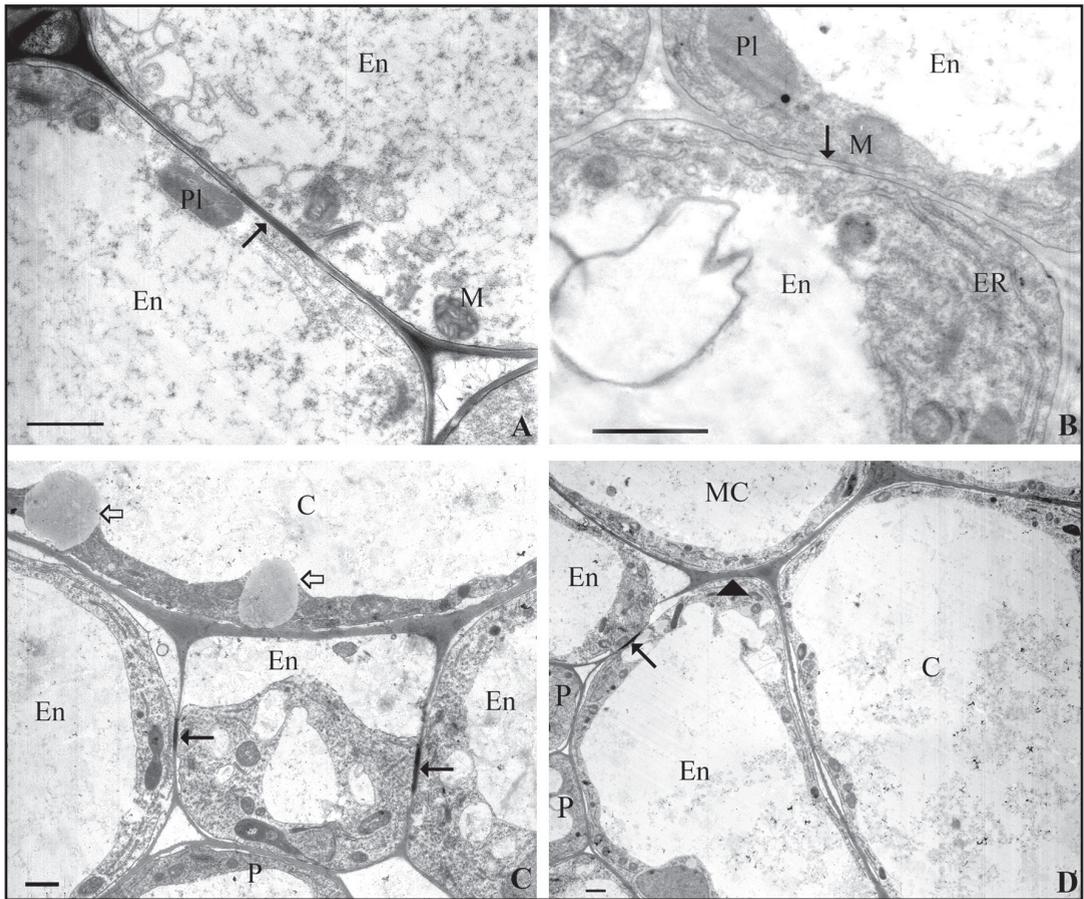


**Fig. 2.** Cytoplasm in the apical part of trichoblast bulges (emerging root hairs) in *Arabidopsis* primary roots grown on agar medium with 10  $\mu\text{M}$  Zn (A) and 1000  $\mu\text{M}$  Zn (B, C, D). A – cytoplasm rich in organelles. B – autophagic vacuoles (white arrows) in Zn-treated *A. thaliana* Col root. C, D – cytoplasm with organelles, prominent Golgi bodies (asterisks), numerous Golgi-derived vesicles (arrows) and small vacuoles in the apical region of trichoblast bulge in the roots of *A. arenosa* M population (C) and *A. halleri* NM population (D). ER – endoplasmic reticulum; M – mitochondria; ocw – outer cell wall; V – vacuole. Bars represent 1  $\mu\text{m}$ .

respectively. The formation of Casparian bands in this metallicolous population might relate to its adaptation to soil conditions severely contaminated with heavy metals, particularly copper in the locality Krompachy (STAŇOVÁ *et al.* 2012).

Development of peri-endodermal cells with thickened and lignified walls was documented in

the roots of tolerant hyperaccumulator *Thlaspi caerulescens* J. Presl et C. Presl but not in the non-tolerant *T. arvense* L. (ZELKO *et al.* 2008). The authors discuss the role of this cell layer as a possible structural barrier in radial ion transport. As the additional cortical tiers occurred more frequently in the tolerant *Arabidopsis* species, we inspected the cell walls between the endodermis



**Fig. 3.** Ultrastructure of cortical and endodermal cells of the roots grown under 10  $\mu\text{M}$  Zn (**A**) and 1000  $\mu\text{M}$  Zn (**B, C, D**). **A** – radial cell wall with Casparian band (**arrow**) in the endodermis of *A. halleri* M population. **B** – radial cell wall (**arrow**) lacking Casparian band in the endodermis of *A. thaliana* population from natural locality Ratkovo. **C** – Casparian bands (**arrows**) in the endodermis and globules with electron-dense material (**thick arrows**) in cortical cells of *A. halleri* M population. **D** – Casparian band present in the cell wall between endodermal cells (**arrow**), but absent in the cell wall between the endodermal and MC cell (**arrowhead**). **C** – cortex; **En** – endodermis; **ER** – endoplasmic reticulum; **M** – mitochondria; **P** – pericycle; **Pl** – plastid. Bars represent 1  $\mu\text{m}$ .

cells and their derivatives, the MC cells. Our results show that while the Casparian bands were clearly defined in the radial walls between the endodermal cells in *A. halleri* population Krompachy (Fig. 3 C, D), the cell walls between the endodermis and the cells of the additive middle cortex lacked this barrier (Fig. 3 D).

In conclusion, our results confirmed the previously described details of root anatomy of the *A. thaliana* relatives, the non-model species *A. arenosa* and *A. halleri*, and their populations originating from the natural localities with either non-metalliferous or metalliferous soils.

We extended the knowledge with data on root cell ultrastructure of the *Arabidopsis* pseudo-metallophytes and their NM and M populations, which have not been previously reported in the plant literature. Formation of Casparian bands at the comparable DFT was found only in the root endodermis of the *A. halleri* M population originating from the locality severely contaminated with heavy metals. There were no remarkable effects of the 1000  $\mu\text{M}$  Zn concentration except of the formation of autophagic vacuoles in the cytoplasm of trichoblast bulges of the sensitive *A. thaliana*.

Higher diversity among the populations could be expected in their responses to higher concentration of a variety of heavy metals.

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