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SUBCELLULAR LOCALISATION OF CADMIUM CORRELATES WITH TOLERANCE RATE OF DIFFERENT WHEAT VARIETIES

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ABSTRACT

Distribution and allocation of metals within different cellular compartments significantly impact plant responses to toxic elements like cadmium. In this work, the subcellular accumulation of cadmium was analysed using gammaspectrometry in wheat tissue samples derived from 7 plants of early developmental stage. We assessed distribution of cadmium in 9 wheat varieties. Similarly to roots, the highest ^{109}Cd content in shoot cells accumulated in the cytosol/vacuolar fraction, ranging between 40-60% of the total ^{109}Cd accumulated. The lowest values of cytosol/vacuolar accumulation were detected in the case of Durgalova variety, the highest in Ilona and Sunanka varieties. The lowest portion (less than 20%) of cadmium was accumulated in the organelle fraction of both shoots and roots. In the shoot cell walls 20-40% of cadmium was deposited, the highest values were observed in the case of Zirnitra and Peruna varieties, and the lowest values in the Sunanka and Ilona. Our data revealed that tolerance based on shoot growth negatively correlates with Cd deposition in shoot cell walls (-0.48) and in shoot organelles (-0.31). On the other hand, positive correlation (0.48) was detected with metal deposition in shoot cytosol. Our approach opens new avenues for investigating the metal accumulation dynamics in plants, facilitating the development of resilient and environmentally sustainable agricultural practices.

Keywords: cell compartments, cell fractionation, cellular localisation, metal toxicity, *Triticum*

INTRODUCTION

Phosphate fertilisers are the primary source of cadmium in agricultural soils and its accumulation in cultivated plants represents the main input of this toxic element into the animal organism, including human (**Clemens, 2006**). Despite this risk, the European Community recently increased the maximum permitted Cd level in wheat grains and today the Cd levels in spring bread wheat fluctuate around 100 µg.kg^{-1} (**Stolt *et al.*, 2016**). For health safety reasons, Cd levels need to be decreased in cereals and other crops. Different external factors (including climate conditions, soil properties but also nutrition supply) affect wheat tolerance to cadmium and its ability to withstand and accumulate in tissues (**Smith *et al.*, 2023**). In addition, genetic heredity determines Cd accumulation rate and sequestration strategies (**Smith *et al.*, 2023**). There are large differences in Cd accumulation by different plant species (**Yi *et al.*, 2023**). Furthermore, an inter-varietal difference has been reported in both bread wheat (*Triticum aestivum L.*) and durum wheat (*Triticum turgidum* var. *durum*) (**Smith *et al.*, 2023**). Numerous publications have addressed the conditions and mechanisms of Cd accumulation in plants, and reported on the genes (proteins) responsible for Cd uptake, transport, storage, and detoxification. Gained knowledge focused on identifying (generation of) cultivars with limited Cd uptake and accumulation in edible parts (**Sterckeman and Thomine, 2020**).

Cadmium accumulation in plants entails Cd uptake by root epidermis or exodermis from soil, root-to-shoot translocation via the xylem, intracellular Cd sequestration, and (re)distribution of Cd in above-ground tissues (**Yi *et al.*, 2023**). The first step is dependent on the Cd (bio)availability and soil properties and is controlled by plant genetic factors. Numerous metal transporters (including those for calcium ions) have been recognized in these processes (**Yi *et al.*, 2023**). Subsequent loading of metals to the root xylem allows for root-to-shoot translocation via symplastic and/or apoplastic transport, mainly in the form of Cd-complexes with various chelates (**Lux *et al.*, 2011**). This highly costly process is mediated by heavy metal ATPases (HMAs) and some other proteins (**Yi *et al.*, 2023**).
Plants possess different sequestration strategies to prevent Cd translocation and reduce Cd accumulation in sensitive aboveground parts, including cereal grains. The cell wall is a key sequestration site in plants. As the first structure that comes into contact with heavy metals enables heavy metal binding and retention due to its components, such as cellulose, hemicellulose, lignin, and pectin (**Shi *et al.*, 2019**). **Guo *et al.* (2022)** suggested that cell walls can prevent 36–70% of Cd from being transported into protoplasts. Metal binding capacity of cell walls is limited and genetically determined (**Xiao *et al.*, 2020**). For example, intraspecific variations in cell wall composition of the hyperaccumulating species *Arabidopsis halleri* affected Cd tolerance and accumulation (**Meyer *et al.*, 2015**). In addition, Cd resistance of oat varieties has been shown to coincide with greatest Cd storage in the cell walls of aerial parts (**Uraguchi, Kiyono, *et al.*, 2009**). Within the cells, Cd^{2+} forms complexes with low-molecular chelating compounds (e.g., various amino acids, glutathione or nicotianamine) to promote its transfer into the vacuoles (**DalCorso *et al.*, 2008**). In many plant species, phytochelatins are synthetized in the presence of cadmium and the formed Cd-phytochelatin complexes are sequestered in the vacuole (**Ernst *et al.*, 2008**). Cd sequestration in cell walls and in vacuoles is not only a factor controlling Cd distribution and storage among tissues and organs but also a way to reduce Cd toxicity in plants (**Stolt *et al.*, 2003; Yi *et al.*, 2023**). Finally, the main route for transporting Cd to grain, fruits or even roots is phloem (**Kato *et al.*, 2010**). **Tian *et al.* (2016)** provided evidence that phloem reallocates Cd from old leaves (storage tissues) to newly grown tissues, again in a form of complexes with glutathione, phytochelatins and nicotinamide (**Mendoza-Cózatl *et al.*, 2008**). Several low-affinity cation transporters are involved in this transport and have been described especially in rice (**Uraguchi *et al.*, 2011; Yamaji *et al.*, 2013**).
Most literature reports on cadmium content and distribution in different tissue types or organs; there are general conclusion e.g., on dominant metal allocation in roots and limited transfer to shoots in non-hyperaccumulating species. **Vásquez *et al.* (1992)** demonstrated that the main sites of Cd accumulation in the roots of pennycress (*Noccaea caerulescens*) were the cell walls and the middle lamella, suggesting that Cd is bound to ion exchange sites. The presence of Cd in the cell walls and middle lamella was also confirmed by **Khan *et al.* (1984)** in maize (*Zea mays*) plants. Other locations for Cd^{2+} ions accumulation included vacuoles and membrane organelles. Recently, high Cd-tolerance of rapeseed genotypes has been suggested to result from retainment of Cd in leaf cell walls and vacuoles,

mainly in the form of pectate/protein-integrated Cd (**Liao *et al*., 2024**). These authors suggested specific metal deposition in certain compartments is a key detoxication mechanism at cellular level. Therefore, we studied here cadmium allocation at cellular level in different tissues of 9 wheat varieties. The observed Cd deposition pattern we correlated with metal tolerance rate. Instead of atomic absorption spectrometry we used highly selective and sensitive radioanalytical approach using ^{109}Cd as a radioisotope tracer. The use of radioisotope tracers for highly selective and sensitive detection allows the study of the concentration range of metals in samples as well as the study of the allocation of metals at the level of its individual parts, but also at the level of cells or even cellular fractions (organelles) (**Rodríguez-Castrillón *et al*., 2008; Liao *et al*., 2024**). In addition, it requires much less plant biomass for analyses. Gaining knowledge on coincidence of metal allocation in cell compartments with plant tolerance or sensitivity adds to better efficiency of screening wheat varieties capable to tolerate and accumulate metals, hence those representing risk for safety of food (in the case of metals without biological function), but also in biotechnological programmes where metal uptake is desirable such as biofortification by zinc or selene.

MATERIAL AND METHODS

Plant material

A set of 9 varieties of wheat, which included 4 varieties of winter summer wheat (*Triticum aestivum*) (varieties Slovenka, Sunanka, Ilona and Escoria), 3 varieties of double grain wheat (*Triticum dicoccum*) (varieties Durgalova, Zirnitra, Badurka) and 2 varieties of spelt (*Triticum spelta*) (varieties Mislina, Peruna). Seeds were provided by the Gene Bank SK (Research Institute of Plant Production in Piešťany, NPPC Lužianky). Wheat seeds were sterilized before germination using a 5% (v/v) sodium hypochlorite solution for 10 min. Subsequently, the seeds were rinsed three times with sterile deionized water. In sterile containers (10x10x16 cm) with two layers of sterile filter paper moistened with sterile deionized water, we cultured the plants in a KBWF 720 growth chamber (Binder, DE) for 3 d at 24/18 °C. Subsequently, the seedlings were transferred into sterile plastic containers with 500 ml of 25% strength Hoagland's solution (Hoagland 1920). Plants were further cultivated for 10 d in a growth chamber under standard conditions (relative humidity 60%, maximum temperature 24/18 °C; photoperiod 16/8 h; maximum light intensity 11 450 lx).
Uniform plants were replaced to 25% Hoagland solution containing 50 mg.g^{-1} fw Cd^{2+} in the form of $CdCl_2$. After 10 d cultivation, the tissues of the whole plants were dried to constant weight and analysed for Cd content. The experiments were prepared in three replicates.
Tolerance indexes to cadmium were determined as the ratio between shoot fresh weights under Cd stress and the corresponding controls and expressed in %.

Determination of photosynthetic pigments

Determination of photosynthetic pigment concentrations was carried out according to **Lichtenthaler and Wellburn (1987)**, converting pigment concentrations to mg.g^{-1} fw of acetone.

Atomic absorption spectrometry

Roots were washed with a solution of 5 mM EDTA and subsequently in distilled water twice. Root and shoot tissues of 3 plants per variant were pooled (respectively) to give a sample. Samples were oven dried for 72 h at 70°C to generate completely dried samples. Dried plant tissue (200 mg) was mineralized in 8 ml of concentrated HNO_3 (Sigma) using Anton Paar Multiwave 3000 microwave system. Atomic absorption spectrometry (AAS) was performed using iCETM 3000 (Thermo ScientificTM) device using a certified plant reference material ERMCD281 (Merck).

Radioanalytical analyses

For radioanalytical studies, the metal solution for plant cultivation was spiked with exact amount of the radioisotope ^{109}Cd (Czech Metrological Institute, Czech Republic) (expressed as Bq/g DW) in 5 mL vial tubes. Metal exposure was for 10 days. Gradual fractional centrifugation method by **Zhao *et al*. (2017)** was adapted for radianalytical approach. Root tissues were separated with a scalpel and immersed in 20 mM Na_2-EDTA for 15 min and rinsed with deionized water to remove Cd^{2+} from the surface. The shoots and roots of seedlings were frozen with liquid nitrogen. For the gammaspectrometry analysis, tissue from 7 plants was pooled to form a replicate. Frozen tissues were homogenized in a cold buffer composed of 50 mM Tris-HCl, 250 mM sucrose and 1 mM dithioerythritol. The homogenate was centrifuged at 5,000 rpm for 15 min to obtain the cell wall fraction (F1). The supernatant was then pipetted into a new tube and centrifuged again at 15,000 rpm for 45 minutes, at which time organelles, such as nucleus, plastids, mitochondria, and others were obtained. The pellet obtained contained organelle fraction (F2). The final supernatant was collected as a fraction containing cytosol and vacuoles (F3). The fractions obtained were analyzed using a scintillation gammaspectrometer type 76BP76/3 (Scionix, The Netherland) with well type NaI(Tl) crystal. For energy and efficiency calibration, a library of radionuclides was created by selecting the characteristic γ-ray peaks for ^{109}Cd (Eγ = 88.04 keV), ^{137}Cs (Eγ = 661.66 keV), and ^{60}Co (Eγ = 1,173.24 keV), . The calibration was performed using standard solutions of $^{109}CdCl_2$ with known radioactivity and half-life of the radionuclide ($T_{1/2}$ = 462.6 d). The peak areas of the samples and references were determined at the same geometry. The accumulation of ^{109}Cd was expressed as % of the isotope depleted from the growth media by the plants, or re-calculated to the total amount of ^{109}Cd accumulated in tissue (Bq.g^{-1} DW).

Data processing

Data processing, visualisation and correlation analysis were done using Excell program.

RESULTS AND DISCUSSION

Environmental contamination by heavy metals poses risks for the production of contaminated plants and subsequently for food. The intake of cadmium by cereals depends on multiple factors, and its extent cannot be predicted even with available molecular markers. Analyzing 9 wheat varieties regarding their abilities to tolerate and accumulate Cd^{2+} ions allowed the assessing tolerance and accumulation strategies of each variety. The fresh weight of 9 wheat varieties was variably affected in presence of cadmium in growth media, whereby the most affected were the varieties Peruna and Durgalova. In contrast, the most tolerant appeared the varieties Escoria, Slovenka and Mislina (Figure 1). The observed variability among the wheat varieties aligns with previous studies (**Yue *et al*., 2018**). Several morphological characteristics emphasize the negative impact of cadmium toxicity on plants (**You *et al*., 2018**). Content of photosynthetic pigments also represents a good physiological indicator of the metal stress acting on plants (**Kalaji *et al*., 2016**) indicating (in)proper function or damage of the photosynthetic apparatus (**Yotsova *et al*., 2020**). Suppressed total chlorophyll content pointed on sensitivity of Sunanka, Badurka and Ilona varieties. Cd^{2+} ions cause a decrease in chlorophyll content by preventing the formation of the photoactive complex of protochlorophyll reductase enzyme and the synthesis of aminolevulinic acid (**Öncel *et al*., 2000**).
The negative impact of heavy metals on pigment content in plants was described by **Yotsova *et al*. (2020)**. In contrast, the varieties Slovenka and Peruna seemed tolerant to metal toxicity (Figure 1). The increase in total chlorophyll content has also been observed by others (**Chandra *et al*., 2009**), while temporary increase was attributed to the presence of nutrients and other substances in the environment (wastewater supply), whose beneficial effects outweighed the toxicity of the contaminants (**Chandra *et al*., 2009**). Compensation growth has been suggested to be a plant strategy to gain further resources (**Dobroviczká *et al*., 2013, Mészáros *et al*., 2013**). In one variety, Peruna, there was no change in any photosynthetic parameter. These results indicate a relatively mild toxicity of the applied dose and form of Cd. Longer exposure or a higher dose of cadmium would likely cause more pronounced plant responses and thus allow for clearer differentiation of the varieties according to their sensitivity to cadmium (**Öncel *et al*., 2000**).

Determination of the total Cd uptake and accumulation in wheat tissues

The cadmium content decreased significantly in the growth medium after 10 days in all wheat varieties tested (Figure 2). The most pronounced decrease was observed in the case of the Escoria and Peruna varieties, while the lowest decrease was observed in the Sunanka variety. However, Cd depletion is not necessarily a reflection of root uptake and/or accumulation rates in different tissues. In fact, despite the high Cd depletion from the medium, the total Cd content in the shoots of Escoria and Peruna was relatively low compared to the other cultivars, even the lowest in the case of Escoria (Figure 2). On the contrary, the shoots of the cultivars Zirnitra and Sunanka accumulated the most Cd (Figure 2). Variability in Cd uptake and accumulation has often been described in different plant species, including wheat (**Yotsova *et al*., 2020; Yi *et al*., 2023**).

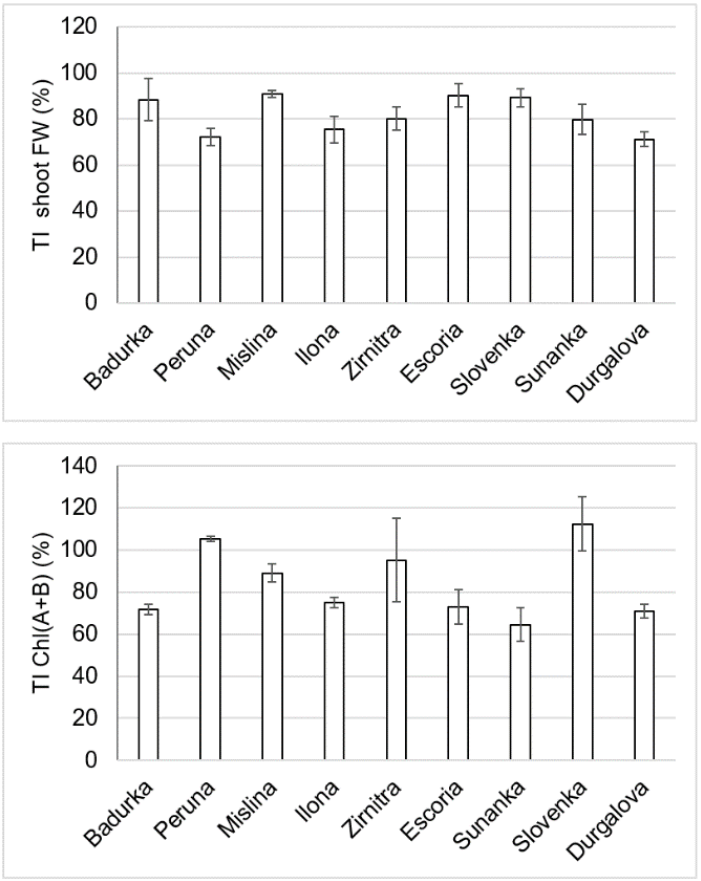

Figure 1 Wheat sensitivity to presence of cadmium in growth media. Tolerance indexes, expressed as the ratio of values in Cd-treated and corresponding control plants, were expressed for fresh weight of shoots (upper panel) and total chlorophyll *a* and *b* content (lower panel). The data represent average values ± SE (n=3).

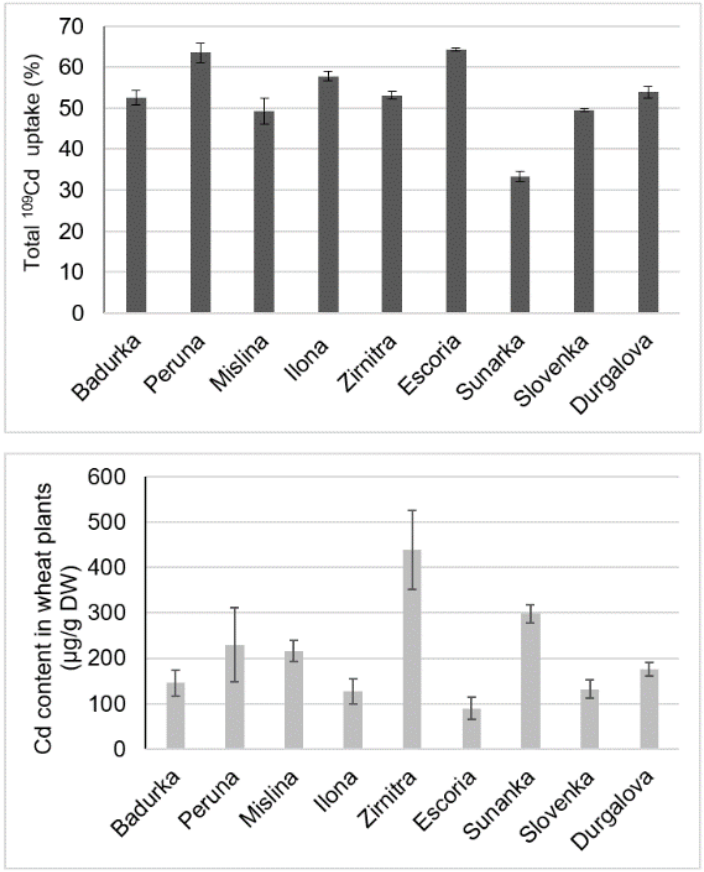

Figure 2 Uptake of cadmium by wheat plants from the growth media. Rate of total ^{109}Cd uptake from media (upper panel) and corresponding Cd content in wheat shoots. The data represent average values ± SE (n=3).

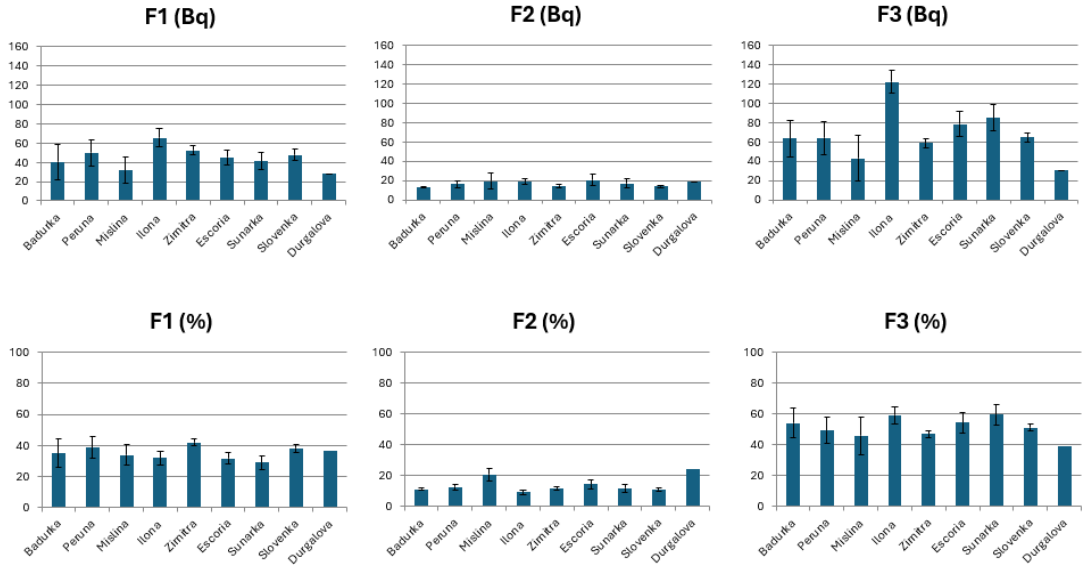

Figure 3 Cadmium content in different shoot cell fractions (in Bq) and relative content comparing with the other cell fractions (in %). Data indicate amounts of ^{109}Cd in cell wall (F1), organelle (F2) and vacuole (F3) fractions and represent the mean value ± SE with n = 3. For the variety Durgalova, single replicate was available only due to pathogen contamination.

Determination of Cd allocation in wheat tissues at cellular level

There are relatively limited number of studies which underpin the subcellular localisation of toxic metal as a tolerance strategy. For example, **Meyer *et al.* (2015)** correlated toxicity-induced modification in the cell wall with intraspecific variations in Cd tolerance and accumulation of the hyperaccumulating species *Arabidopsis helleri*. We studied the content of absorbed ^{109}Cd in different cellular fractions, in roots, and also in the shoots. After homogenization in liquid nitrogen and extraction solution, we obtained cell wall fractions (F1), organelles (F2), and cytosol and vacuoles (F3) by sequential centrifugation. Gammaspectrometry analyses showed that the distribution of Cd in the cellular compartments is not uniform among varieties, while differs also when individual organs are compared. In roots, the highest ^{109}Cd content, corresponding to 50-63 % of partial distribution, was quantified in the cytosol/vacuolar fraction across all varieties (Figure 3). In contrast, the lowest content (less than 20%) accumulated in the organelle fraction. Data indicate that the varieties Durgalova, Zirnitra and Slovenka accumulate the least Cd in cellular organelles (Figure 3), while Sunanka accumulates the most in this fraction (Figure 3). Up to 20-40% of ^{109}Cd accumulated in roots was deposited in cell walls. In the case tea seedling roots, ~12 to 30% of metal was deposited in the cell walls (**Cao, Yang, *et al.*, 2018**). Enhanced content of pectin and hemicelluloses in the root cell wall increased Cd accumulation in the root cell walls

in rice (**Xiong *et al*., 2009**). **Xu *et al*. (2015)** showed that Cd itself induces the changes in polysaccharide contents leading to increased binding capacity of root cell walls in *Arabidopsis thaliana*.
In shoots, relatively higher variability was observed in partial distribution of ^{109}Cd in cell compartments. Similarly to roots, the highest ^{109}Cd content in shoot cells accumulated in the cytosol/vacuolar fraction, ranging between 40-60% of total ^{109}Cd (Figure 4). The lowest values were detected in Durgalova variety, highest in Ilona and Sunanka varieties (Figure 4). The lowest portion (less than 20%) of cadmium accumulated in the organelle fraction, as was observed in roots. In the shoot cell walls 20-40% of cadmium was deposited (Figure 4), most in the Zirnitra and Peruna varieties, least in the Sunanka and Ilona. Previously, in oilseed rape (*Brassica napus*) leaves, only 11% of the metal content has been reported in this compartment (Carrier *et al*., 2003). In contrast, cell wall represented up to 83.4% of the very low Cd content in some tea seedlings leaves (**Cao, Yang, *et al*., 2018**), and 69.4% in leaves in two rice genotypes (**Guo *et al*., 2022**). The latter authors assumed different cell wall composition (by means of functional groups capable to chelate metals) was responsible for low-Cd accumulating nature of corresponding rice leaves.

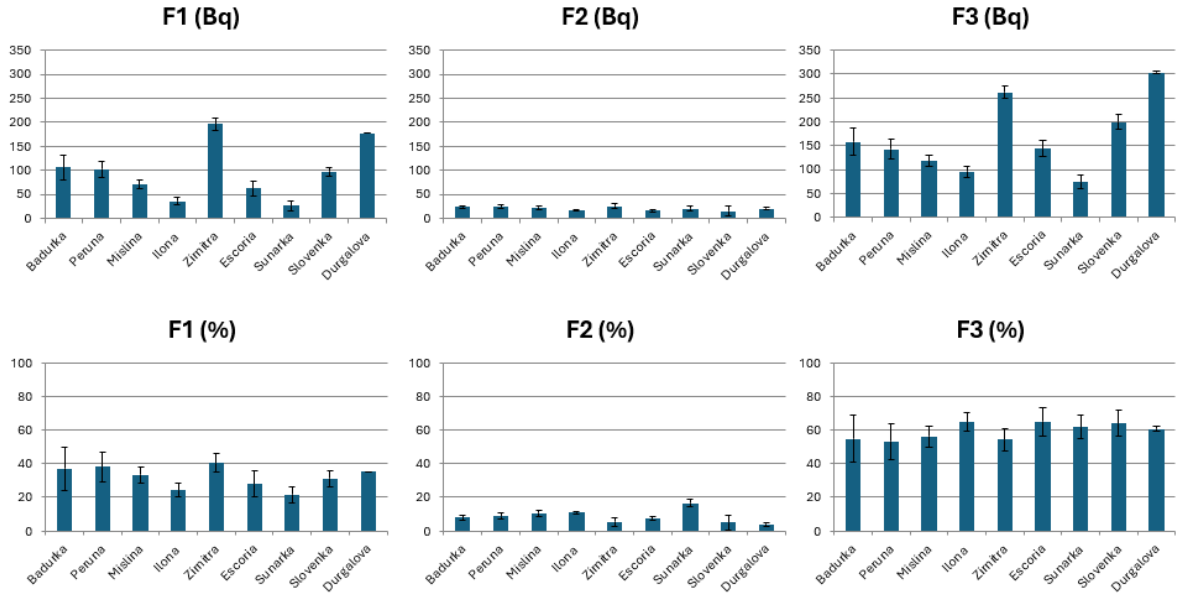

Figure 3 Cadmium content in different root cell fractions (in Bq) and relative content comparing with the other cell fractions (in %). Data indicate amounts of ^{109}Cd in cell wall (F1), organelle (F2) and vacuole (F3) fractions and represent the mean value ± SE with n = 3. For the variety Durgalova, single replicate was available only due to pathogen contamination

Correlation between Cd content, allocation and wheat tolerance

Tolerance indexes (TI) were correlated with metal content data. As expected, wheat tolerance negatively correlated with total Cd uptake (-0.378), and also with Cd content in both shoot and root tissue (for both <-0.2). More interestingly, TI values showed no correlation with Cd allocation in any of the root cell fraction (data not shown). Our data contradict previous reports, which metal tolerance had linked with dominant deposition of cadmium in cell wall fraction of roots (**Cao, Yang, *et al*., 2018; Guo *et al*., 2022**). Pectin-like compounds, cellulose, and hemicellulose have been shown to fix more Cd in Cd-tolerant *Arabidopsis thaliana* ecotype (**Xiao *et al*., 2020**). The process of Cd adsorption onto the isolated cell wall in rice leaves is likely primarily based on chemisorption (**Guo *et al*., 2022**). **Parrotta *et al*. (2015)** noted, however, that evidence for the cell wall acting as a barrier against Cd (and other heavy metals) is not immediately clear from every study and the corresponding resistance mechanisms in which cell walls are involved might include not only forming a barrier organ (for example the root), but also acting as modulator of transport capacity of the contaminant, sorption material of Cd or the impediment of Cd to penetrate through the plasma membrane.
Wheat tolerance to cadmium appeared to negatively correlate with Cd deposition in shoot cell walls (-0.48) and in shoot organelles (-0.31) and with chlorophyll content (-0.32). On the other hand, positive correlation of 0.48 was detected with metal deposition in shoot cytosol. The increased accumulation of Cd in vacuoles and cellular organelles is likely a mechanism to inhibit the transport of Cd to the xylem and, consequently, to the aerial parts of the plants (**Gao *et al*., 2015**).
Metal allocation strategies vary not only among species but also within wheat species (**Xiao *et al*., 2020; Halim *et al*., 2021; this work**), and even within the same plant (**Liao *et al*., 2024, this work**). In the screening of 55 rapeseed genotypes, Cd concentrations in the shoot cell wall and vacuole were significantly higher in high Cd accumulating genotypes than in low Cd accumulating genotypes, whereas the opposite trend was observed in the roots (**Liao *et al*., 2024**). The tolerant wheat cultivar (Mustang) chelated Cd in the nonedible parts of plants in variable fractions (**Halim *et al*., 2021**).

Comparing the methods used for measurements of Cd content

Gao *et al*. (2015) examined the cellular and subcellular localization of Cd^{2+} ions in wheat using immunohistochemistry by chelating free Cd in the tissue and subsequently converting it to a Cd^{2+}-EDTA complex labeled with colloidal gold. The authors observed distinct gold particles in the middle lamella and epidermal cell walls, as well as in the cortex and endodermal cells of the roots. Here gammaspectrometry and radioisotope ^{109}Cd were used to measure the cadmium content in plant samples to reduce the amount of needed plant material. Analyses of cell fractions by AAS require relatively high amount of plant material, therefore the data obtained are impossible to link to individual plants. Gammaspetrometry allowed to measure ^{109}Cd signal in cell fractions originating from a pool of 7 plantlets of very young age. Cadmium content was measured in roots of 3 varieties with both AAS and gammaspectrometry. Data show good coherence between the methods, though the latter method provided more variable data (Figure 4). The reason can be better sensitivity and also variance from smaller set of plants pooled. Though the radioanalytical method was not validated, reproducibility of data allows to identify the occurring trends and suggest a good potential for routine application.

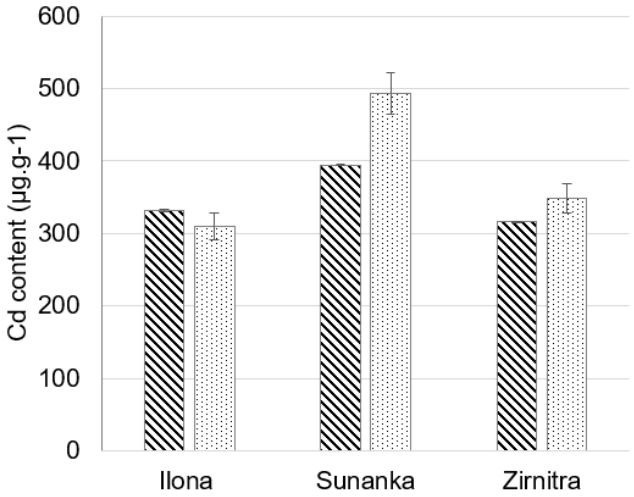

Figure 4 Measurement of cadmium content in wheat roots using AAS (hatched columns) and gammaspectrometry (dotted values). Data represent the mean value ± SE with n = 3.

CONCLUSION

Analyses using gammaspectrometry indicated that the allocation of metals in cell compartments plays a role in the ability of cells to protect sensitive structures from metal toxicity. Cadmium accumulation in cell walls likely contributes to the protection of the more sensitive components like the photosynthetic apparatus, which can generate resources even in the presence of toxic elements. Resources are essential for mounting a costly induced defense against prolonged exposure to heavy metal stress. Wheat varieties accumulating significant amounts of cadmium (e.g. Peruna, Zirnitra and Escoria) possess efficient mechanism to accumulate metal in roots while limit metal translocation to shoots. These varieties thus constitute a group of cultivars that are relatively safe in terms of producing contaminated grain and consequently food. Screening for metal allocation strategies at subcellular level might support breeding programs to develop cultivars that are either more resilient to contaminated soils or more efficient in biofortification programs (in case of essential metals). This practical application of this research enhances its significance and utility in real-world agricultural and environmental contexts.

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