

Research Article

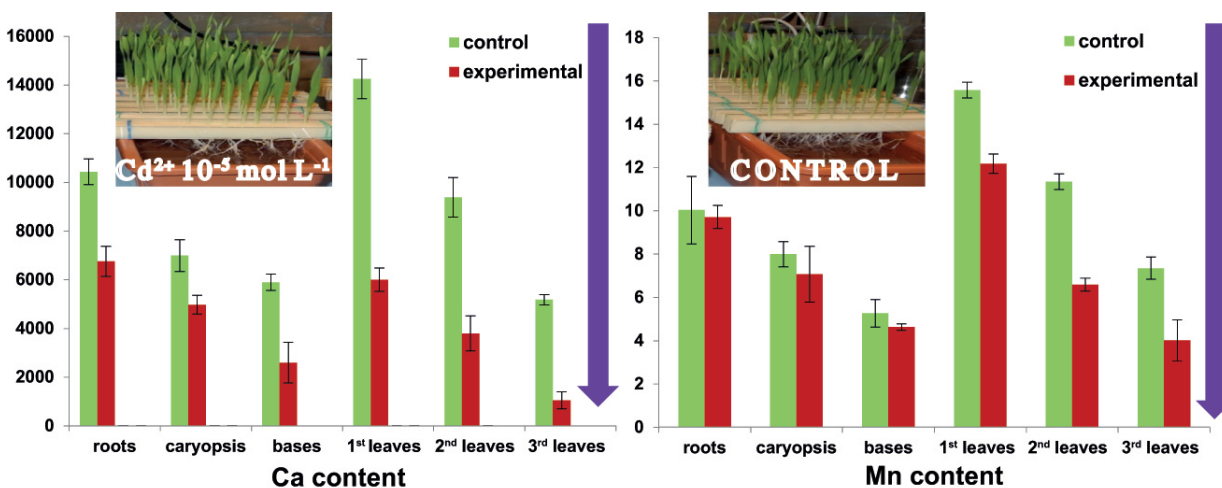
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Effect of cadmium stress on barley tissue damage and essential metal transport into plant

Abstract: Conductometric assessment of electrolyte leakage from spring barley plant organs subjected to higher cadmium concentrations 10^{-2} – 10^{-4} mol l⁻¹ revealed serious tissue damage. Therefore plants were grown under controlled environment conditions in a phytotron in Knop's nutrient solution in two variants – the control variant without cadmium and experimental variant treated during the growth of plants with CdCl₂ solution of 10^{-5} mol l⁻¹. Barley plants were then separated into roots, residual caryopses, leaf bases, and leaves. Lyophilized samples were analyzed for the uptake and redistribution of cadmium, calcium, copper, iron, zinc, and manganese in plant organs by spectrometric

methods. Cadmium caused significant decrease of calcium and manganese levels in all analyzed plant parts indicating that cadmium was a distinctive antagonist to intake of these metals. Cadmium treatment also lowered amounts of zinc and iron in roots. An opposite trend was characteristic for Cu, where Cd stress caused significant Cu increase and retention in roots and significant decrease in leaves. The combined usage of the electroanalytical technique - conductometry and spectrometric methods (ICP-OES, AAS) proved to be a useful tool for monitoring of tissue damage and intake and transfer of cadmium and macro- and microelements into barley organs.



Keywords: Barley; Cadmium; Calcium; Zinc; Iron; Copper; Metal transfer; Conductometry; Spectrometry

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1 Introduction

Plants are closely tied to the surrounding environment. In plants toxic metals induce abiotic oxidative stresses.

Stress factors generally applied at higher levels may cause irreversible changes in physiological processes [1]; deficit of water causes stomatal closure or low temperature slows down the biochemical processes. Low levels of toxic metals such as cadmium also slow growth and affect biochemical processes. Strength and duration of stress exposure can also cause permanent changes [1,2]. In addition to toxic metals, changes in the membranes of plant cells are mostly affected by water stress, changes in temperature and by frost. Toxicity of the metals (such as cadmium), can cause an accumulation in tissues, which consequently affects the metabolism of plants, particularly the photosynthetic apparatus [3]. Higher levels of stress also damage the membranes. Damage of membranes at the monitored concentrations of cadmium can then be detected by simple discharge of cell contents [4]. Measurement of discharge of ions is the basis for the conductometric method that can be used to detect damage caused by toxic ions, but also by drought and frost [5-7].

Toxic heavy metal contamination in soils and crop plants is of major concern due to their negative health effects on humans and other animals [8]. Interactions among the elements occurring at root surface and within the plant also affect the uptake and translocation. This association of cadmium, which is unessential to plants, and essential elements such as calcium (Ca), copper (Cu), iron (Fe), zinc (Zn) and manganese (Mn) in the environment together with their chemical similarity may lead to their interactions and different transfer and accumulation in plant organs. Cd is a nonessential element that is one of the most toxic heavy metals, causing serious physiological disorders associated with oxidative stress - damage to root tips, reduction of photosynthesis, growth inhibition and antioxidant response in all plant organs [9,10]. Cd may interfere with a number of metabolic processes, reduces the intake of water and nutrients, causes chlorosis and necrosis of leaves, reduces the efficiency of electron transport, chlorophyll biosynthesis, and induces overproduction of reactive oxygen species and lipid peroxidation in plant leaves and roots [9,11].

The aim of this study was to determine the levels of Cd that causes membrane injury in barley plant organs by a combination of electrochemical and spectroscopic methods (flame atomization atomic absorption spectrometry, electrothermal atomization atomic absorption spectrometry and inductively coupled plasma optical emission spectrometry) for determination uptake and transfer of some essential elements (Ca, Cu, Fe, Zn and Mn) into different organs of barley. Some of them (bivalent) may have a protective role against oxidative stress caused by Cd and may reduce its uptake and accumulation into the root system.

2 Experimental Procedure

2.1 Plant material and its preparation

Plants were grown hydroponically in culture vessels of 5500 ml volume. Spring barley cultivar Sebastian has been used for the experiment. Germination proceeded on filter paper in germinators and all germinators were moved to a thermostatic chamber at 20°C (three layers of filter paper were placed in the bottom and either 18 ml (if pre-soaked seeds were germinated) or 23 ml water (when dry seeds were used) was added. There were 80 seeds per germinator). Sprouted seeds - plants (coleoptiles should not be longer than 10 mm, 4th day after beginning of germination) were replanted into culture containers, sealed with foam. Plants were grown in air-conditioned rooms with artificial lighting. Plant roots were immersed in the Knop's nutrient solution. As the light sources pressure sodium lamps, light bulbs and energy saving lamps have been used. Exposure time periods of light and darkness were set as 14/10 hours and temperature values 22/16°C. Irradiance during cultivation was in average 400 mmol m² s⁻¹. Humidity of the air was controlled by mist maker and a ventilator supplying the 70% relative humidity. Seventh-day of plant growth, final concentration of CdCl₂ · 2.5 H₂O solution was 10⁻⁵ mol l⁻¹. All plants were supplemented with full basic nutrient and vessels were refilled and completed with distilled water and thereafter they were aerated and lighted. On the eighteenth-day of growing, the plants were separated into roots, residual caryopsis, leaf bases and the first, second and third leaf. These materials were consequently used for spectroscopic measurements.

2.2 Sample preparation and conductivity measurement of electrolyte leakage

Plants of spring barley were germinated on germinators for 5 days at 19°C and then transplanted and grown in hydroponic culture using Knop's nutrient solution. The third day after transplantation doses of CdCl₂ · 2.5 H₂O at 10⁻⁵ mol l⁻¹ were administered. On the 9th day after transplantation doses of CdCl₂ · 2.5 H₂O at a concentration of 10⁻³ and 10⁻² mol l⁻¹ were administered. Twelfth-day electrolyte conductivity of the ions leakage of tissue was measured with conductivity electrode connected to conductometer Elteca 3100A (TMS, Malá Skála, Czech Republic). The plants at the third stage were harvested. From these plants 100 mm of roots were taken from the middle of the root system; from the oldest leaf (the 1st leaf) and the youngest leaf (the 3rd leaf) two

segments of 100 mm in three replicates were collected. These segments were inserted into the test tubes with 3 ml of distilled water, shaken horizontally 150 cycles per minute at room temperature 24°C for up to 90 minutes and thereafter, after boiling, the conductivity of solutions was measured again. Since on the cut surfaces of leaves and roots effluent electrolyte also occurred in the control variant, it was necessary for the final calculation use an index of damage (I_t) adjusted by Flint [12] and Prášil and Zámečník [7] expressed as the relative conductivity and relative damage.

$$I_t = (R_t - R_0) / (R_m - R_0) \quad (1)$$

where I_t = index of damage after exposure to cadmium;
 R_0 = relative electrolyte leakage of the control variant without cadmium exposure levels relative to value after boiling;

R_t = relative leakage of electrolyte after exposure to cadmium relative to value after boiling;

R_m = relative electrolyte leakage after exposure to the maximum concentration of cadmium relative to value after boiling.

In the case that the values of the effluent electrolyte after exposure to higher Cd concentrations were similar to values after boiling, it can be assumed that the first $R_m = 1$.

$$I_t (\%) = (R_t - R_0) / (1 - R_0) \quad (2)$$

2.3 Sample preparation and decomposition for spectral analyses

After about three days of freeze-drying, the samples were milled in the analytical mill IKA A11 Basic. Freeze-dried samples were ground finely and microwave digested in an acid solution using MWS-3+ (Berghof Products + Instruments, Germany). Samples of 150 mg were weighted into the Teflon digestion vessel DAP-60S and 2 ml of nitric acid 67%, p.a. ISO (Merck) and 3 ml H_2O_2 30%, TraceSelect (Fluka) was added. The mixture was shaken carefully and vessel was after half hour waiting closed and heated in the microwave oven. The decomposition proceeded for 1 h in the temperature range 100-190°C. The digest obtained was transferred into the 50 ml silica beaker and evaporated to wet residue. Obtained residue was dissolved in 1.5% HNO_3 ; the dissolving was accelerated by sonication. Then digests were transferred to probes and adjusted with 1.5% HNO_3 to 10 ml.

2.4 Determination of cadmium concentration in the digest by electrothermal atomisation - atomic absorption spectrometry (ETA-AAS)

Cadmium concentrations in the digests prepared from the plants cultivated without presence of cadmium were

measured with electrothermal atomisation (ETA-AAS) using a spectrometer Varian SpectrAA 280Z (Varian, Ltd., Mulgrave, Australia) with graphite atomiser and programmable sample dispenser Varian 120. The determination of Cd concentration was carried out in argon atmosphere in a pyrolytic graphite tube with platform.

2.5 Determination of calcium concentration in the digest by flame atomisation atomic absorption spectrometry (F-AAS)

Calcium concentrations in the digests were measured using AAS with air-acetylene flame technique using a spectrometer Varian SpectrAA 110 at wavelength 422.7 nm with deuterium background correction. Standard solution ASTASOL (Analytika, CR) of calcium was used in the preparation of a calibration curve for the measurement. For the determination of calcium 2 ml of original sample were pipetted to probes. To avoid negative effects of phosphates, 1.5 ml of lanthanum nitrate solution [0.07 mol l^{-1} $La(NO_3)_3 \cdot 6 H_2O$] was added to each sample and adjusted with 1.5% HNO_3 to 10 ml.

2.6 Determination of copper, zinc, iron, manganese and higher concentrations of cadmium in the digest by inductively coupled plasma optical emission spectrometry (ICP-OES)

Inductively coupled plasma-atomic emission spectrometry (ICP-OES, Varian, VistaPro, Australia) equipped with a two channel peristaltic pump, a Struman-Masters spray chamber and a V-groove pneumatic nebulizer made of inert material (experimental conditions: power of 1.2 kW, plasma flow of 15.0 $l \text{ min}^{-1}$, auxiliary flow of 0.75 $l \text{ min}^{-1}$, nebulizer flow of 0.9 $l \text{ min}^{-1}$) was applied. The elements were determined at the spectral lines $\lambda = 214.4 \text{ nm}$ for Cd, $\lambda = 327.4 \text{ nm}$ for Cu, $\lambda = 238.3 \text{ nm}$ for Fe, $\lambda = 257.6 \text{ nm}$ for Mn, and $\lambda = 206.2 \text{ nm}$ for Zn. Calibration solutions were prepared in 1.5% HNO_3 as follows: 5–50 $\mu\text{g l}^{-1}$ for Cd and 50–500 $\mu\text{g l}^{-1}$ for Cu, 1–10 mg l^{-1} for Mn, Fe, and Zn.

The plant material samples were analysed in three replicates. The quality of analytical data was assessed by simultaneous analysis of certified reference material 1567a (Wheat Flour) (8% of all the samples). All data obtained were in the confidence intervals given by certified reference material producer. The background of the trace element laboratory was monitored by analysis of 8% blanks prepared under the same conditions, but without

samples, and experimental data were corrected by mean concentration of analytes in blanks, and compared with detection limits (mean \pm 3SD of blanks) which were 0.07, 3.24, 7.25, 10.37, 2.12, 155.7 ng ml⁻¹ for Cd, Cu, Zn, Fe, Mn and Ca, respectively.

2.7 Statistical analysis

Statistical analyses were performed using Statistica 7.0 (StatSoft). Parametrical test P values <0.05 were considered statistically significant. One-way analysis of variance ANOVA ($\alpha = 0.05$; Tukey's test Post Hoc HSD T-test) was used for statistical evaluation.

3 Results and Discussion

3.1 Plant membrane damage determined by conductometric measurement of electrolyte leakage

The extent of plant damage (Fig. 1) and the determination of conductivity of leakage of electrolyte were used for the final evaluation (Fig. 2). It is apparent that at low Cd concentration 10^{-5} mol l⁻¹ damage of the roots was low. However higher concentrations of Cd caused damage on the roots significantly earlier than other plant parts (leaves). The damage of the root at higher concentrations of Cd was higher than the damage of the first leaf. The damage of the first leaf was higher in comparison to damage of second and the third leaf. In the original work [12] measurement method based on leakage of ions from plant material is described. In this study the ion leakage method was tested on several plant species, where damage to the membrane system was detected. This method often was used for the evaluation of frost injury on cereals [7];

the authors tried to optimize the method for wheat. The interpretation of results related to intensity of damage based on the calculation of the relative conductivity enabled comparisons of damage of different plant organs. Damage of membranes in conditions of water stress and more detailed investigation of leakage of ions from symplast into the apoplast and then to the outer solution for barley tissues has been further studied; the results demonstrate the suitability of this method [5]. In another work [3] the effect of low water content to membrane damage has been described. The values for damage control variant in our experiment showed that ions can pass from injured cells by cutting of the leaf segments and the most obviously in the roots. The process of membrane damage, particularly damaged and destroyed the components of the protein enzymes [13], but the damage may also lead to the alteration of lipid structures [14]. High linear correlations between Cd content and injury damage in the roots ($R^2 = 0.959$) and the 1st leaves ($R^2 = 0.998$) and logarithmic correlation in the 2nd and the 3rd leaves ($R^2 = 0.817$) were determined.

The application of experimental Cd²⁺ concentration of 10^{-5} mol l⁻¹ has been chosen because only a tendency towards root damage occurred, while at higher concentrations significant conclusive damage of plants was observed. Analyses of determined metals were performed in three experimental replicates and obtained results are shown in Figs. 3, 4 and 5. The Cd bioaccumulation decreased in the order roots > residual caryopsis > bases of leaves > 1st leaves and 2nd leaves > 3rd leaves (Fig. 3). The cadmium levels transported to higher plant organs generally declined.

Statistical evaluation with ANOVA one-way analysis of variance by Tukey's test at the level of significance $\alpha=0.05$ revealed significant differences of relative conductivity between control or experimental leaf samples ($P < 0.05$) using low Cd concentrations (10^{-5} and 10^{-3} mol l⁻¹) and

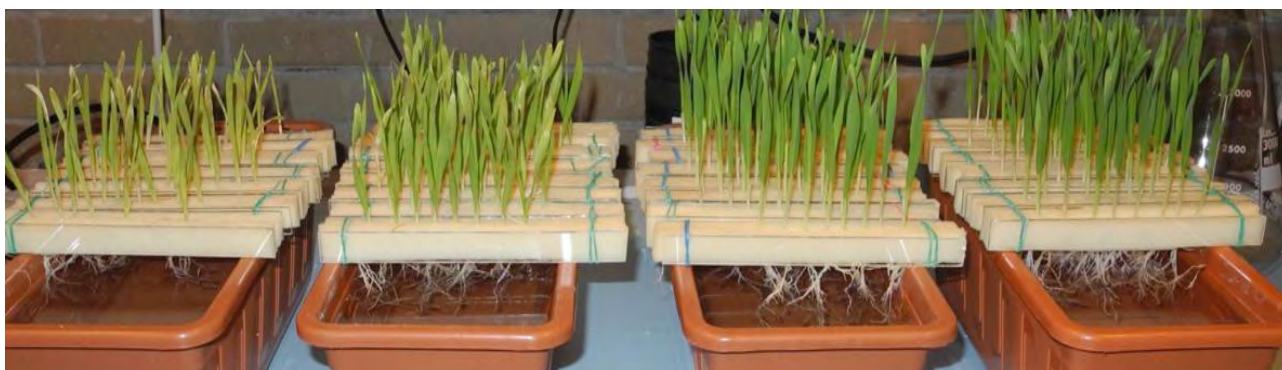


Figure 1: Barley stress responses in plant development on the 3rd day of stress (from the left Cd²⁺ concentrations 10^{-2} mol l⁻¹, 10^{-3} mol l⁻¹, 10^{-5} mol l⁻¹ and control without cadmium treatment).

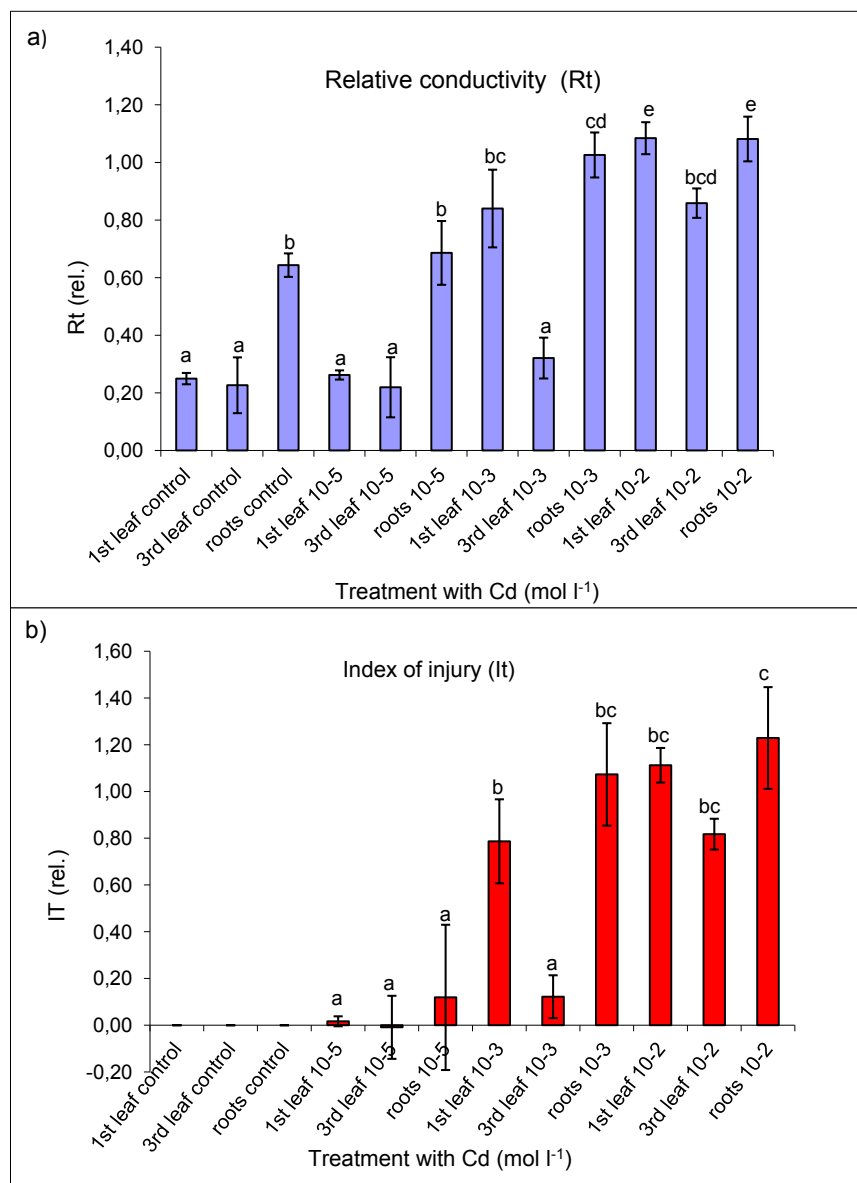


Figure 2: Relative conductivity (R_t) (2a) and index of injury (I_t) (2b) of the selected barley organs after cultivation in $\text{CdCl}_2 \cdot 2.5 \text{H}_2\text{O}$ solutions of 10^{-5} , 10^{-3} and $10^{-2} \text{ mol l}^{-1}$ concentrations.

high Cd concentration ($10^{-2} \text{ mol l}^{-1}$). Likewise significant differences between root samples were found. Index of injury was significantly different in the Cd treatments at 10^{-5} and $10^{-2} \text{ mol l}^{-1}$ concentrations ($P < 0.05$).

3.2 Effect of Cd on the content of Ca

A pronounced decrease of calcium was observed in all organs in experimental variants influenced by cadmium (Fig. 3). When compared with the control variant, a statistically significant decrease ($P < 0.001$) was characteristic for the 1st leaves and for the 3rd leaves, and distinct decreases for the 2nd leaves; generally the decrease of calcium was also found

in other organs – roots and residual caryopsis. Ca was the most accumulated in the oldest leaves of plants. Ca shares many physical similarities with Cd, has a similar charge and ionic radius. Mutual antagonism between these elements has also been published by other authors [10]. It has been reported that Cd application resulted in a decrease of Ca content in various plant species [15]. These results suggest that Ca competes with Cd for uptake through Ca transporters [16]. Calcium plays a major role in many aspects of plant growth and development, including stress responses. In addition to its function as an essential plant nutrient and as a structural element in the plant cell wall, Ca^{2+} is one of the intracellular messengers used to relay information

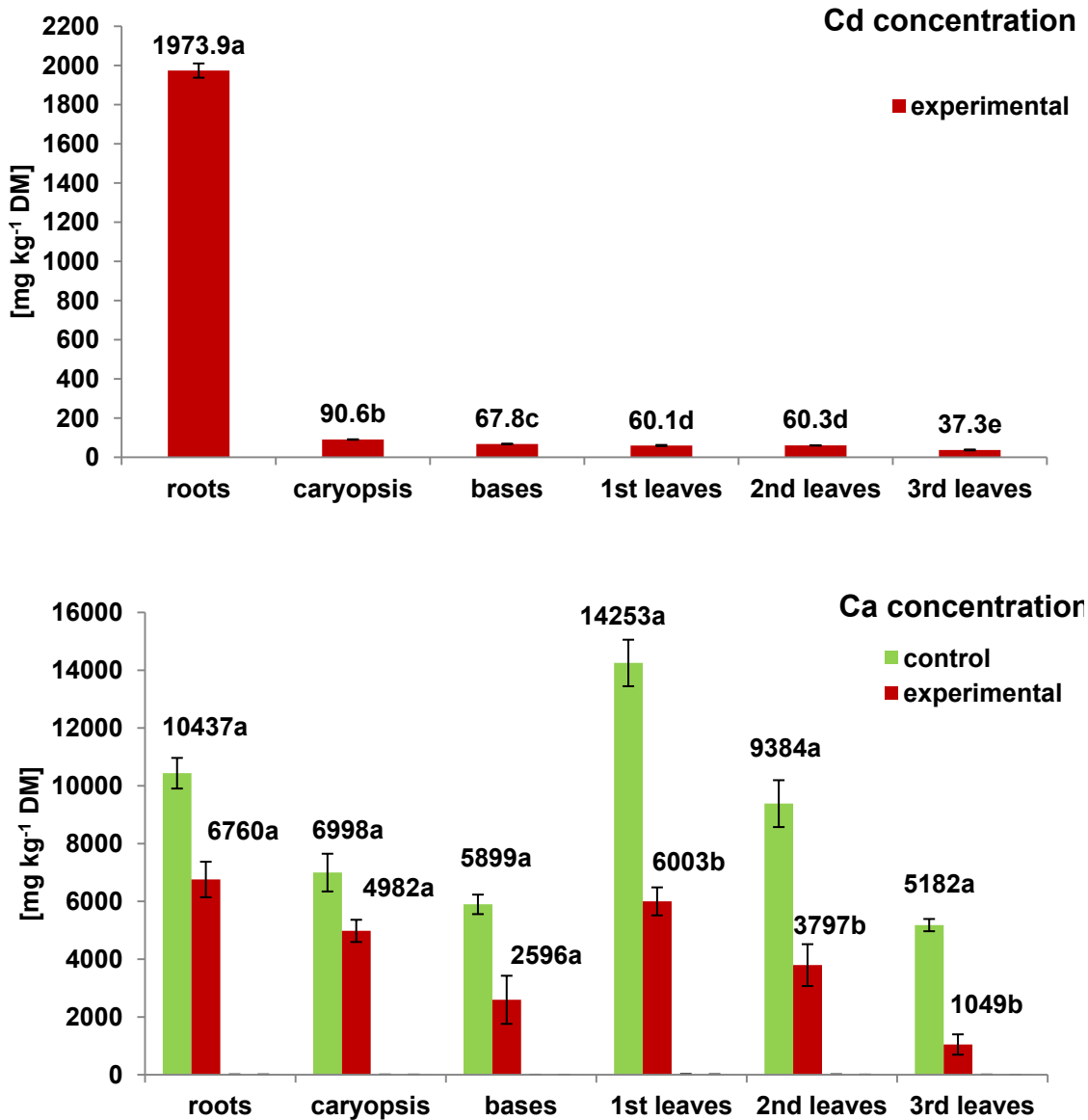


Figure 3: Average values of cadmium and calcium in mg kg⁻¹ DM in control and experimental barley organs obtained from three replicates. Columns denoted with different letters are significantly different at P<0.001 (control versus experimental for Ca content, experimental for Cd content).

from diverse developmental and environmental stimuli to the cellular machinery responsible for mounting biological responses.

3.3 Effect of Cd on the content of Cu

Application of Cd in the experimental variant caused a significant increase of the Cu content in the roots, and non-significant increases in bases of leaves were detected (Fig. 4). Conversely, in the leaves Cu content decreased, and in the first and second leaves Cu content decreased significantly at P<0.001. Recently it was suggested and

indicated from experiments with plasma membrane preparations obtained from roots of barley (*Hordeum vulgare* L. cv. Minorimugi) that Cu association to plasma vesicles occurs quicker than Cd and this resulted in hindering of the access of Cd to plasma membrane vesicles [17]. Copper is a microelement that has roles in photosynthesis, respiration, antioxidant activity, cell wall metabolism and hormone perception. In plants, Cu is a cofactor for plastocyanin, copper/zinc superoxide dismutase (Cu/ZnSOD), cytochrome-c oxidase, and the ethylene receptors for the apoplastic oxidases: ascorbate oxidase, diamine oxidase and polyphenol oxidase [18].

3.4 Effect of Cd on the content of Fe

The highest iron content was measured in the plant roots (Fig. 4). Cd-induced stress resulted in a significant reduction of iron in barley roots (a decline from 1598 ± 18.6 mg kg⁻¹ DM to 1076 ± 9.2 mg kg⁻¹ DM) at $P < 0.001$ has been detected). Lesser increases of iron levels were determined in the leaves, bases and residual caryopses in experimental treatments. Iron content accumulated in roots represented 85.8% of total iron contained in plants. Liu *et al.* [19] on the basis of experiments with rice cultivars consider that the presence of iron plaque on plant roots inhibits uptake and accumulation of heavy metals by plants, although they admit that the functions of plaque

are limited and only effective at relatively low Cd levels. The lesser Fe levels in leaves and stems may be caused by the Fe³⁺ inhibition of the reductase, which leads to a lack of an acceptable form of Fe²⁺ and thus reduces efficiency of photosynthesis.

3.5 Effect of Cd on the content of Zn

The effect of Cd on Zn content resulted in the important decrease of Zn content in the roots (48%), bases (47%) and residual caryopsis (22%) and increase in the leaves from older to younger ones (an increase of 36%, 52% and 37% in the 1st, 2nd and 3rd leaves (Fig. 5). The found differences are important although they are

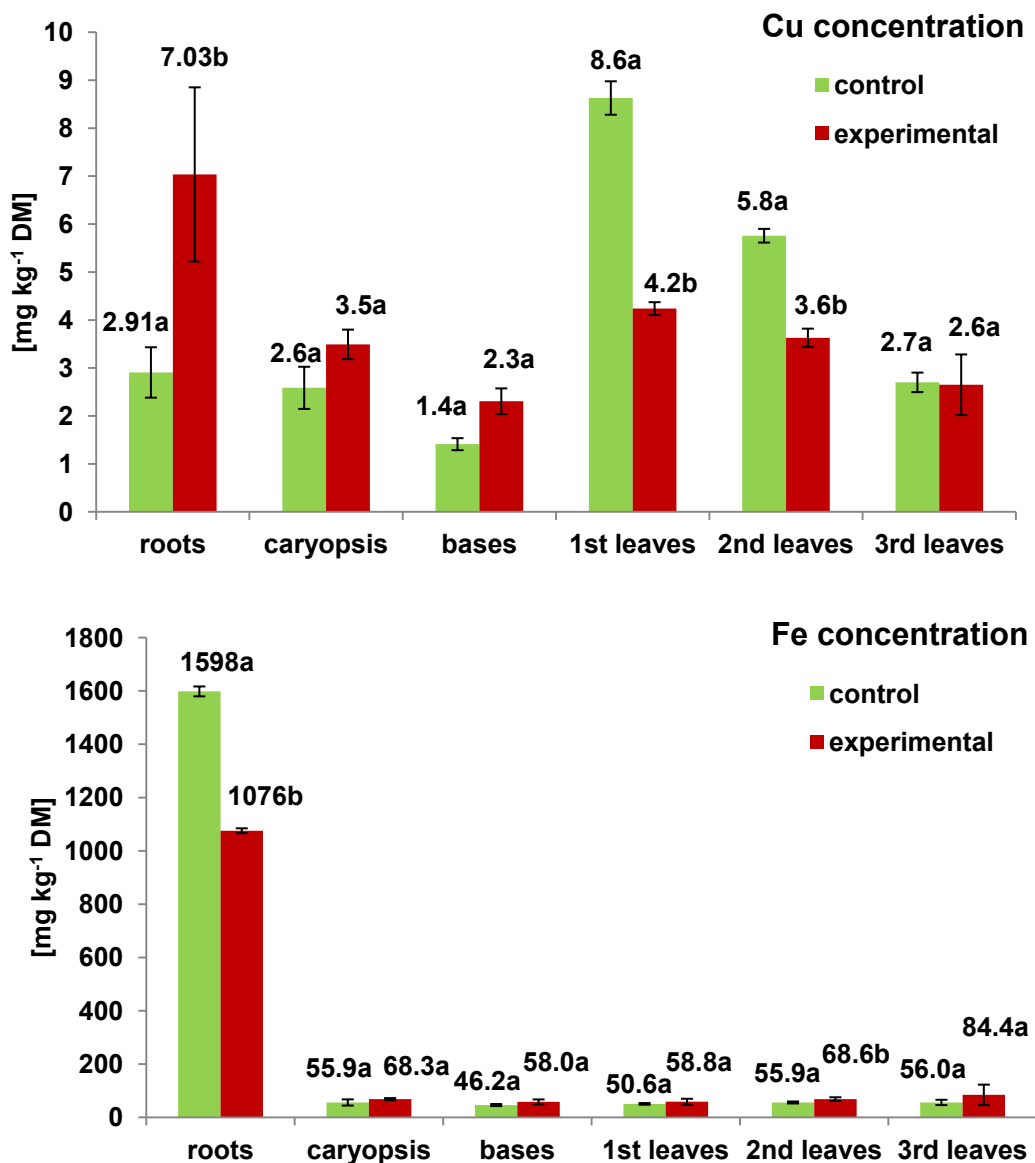


Figure 4: Average values of copper and iron in mg kg⁻¹ DM in control and experimental barley organs obtained from three replicates. Columns denoted with different letters are significantly different at $P < 0.001$ (control versus experimental content).

not significant. Strong interaction between Cd and Zn has been observed by Cherif et al. [11] and they found that Zn was preferentially accepted by plants and, to a certain extent, can protect plants from the toxic effects of cadmium. This suggests that synergism and additivity of Cd-Zn interactions might be related to inadequate compartmentation of cadmium and zinc burdens and the metal levels are concentrated in the sensitive sites. Synergistic effects of Cd and Zn were also found under field conditions in cultivated spring wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.), in which increasing Cd and Zn contents in soils could increase the accumulations of both metals [20]. The effect of Cd and Zn interactions monitored in tolerant plantlets

from in vitro cultures of linseed (*Linum usitatissimum*) and estimation of metal uptake showed greater Cd uptake in the roots than shoots, whereas Zn was found to be translocated from roots to shoots [21]. In the roots Cd was accumulated in the cytoplasm, while Zn was localised in the cell walls, in contrast to shoots where an equal Zn amount was found in the cell wall and the cytoplasm. Zinc is an essential element for plants, where it plays an essential role as an integral co-factor of over 300 enzymes involved in biosynthesis and turnover of proteins, nucleic acids, carbohydrates, and lipids. Furthermore, Zn has a critical structural role in many proteins and this resulted in isolation and identification of three new Zn²⁺ transporters from barley [22].

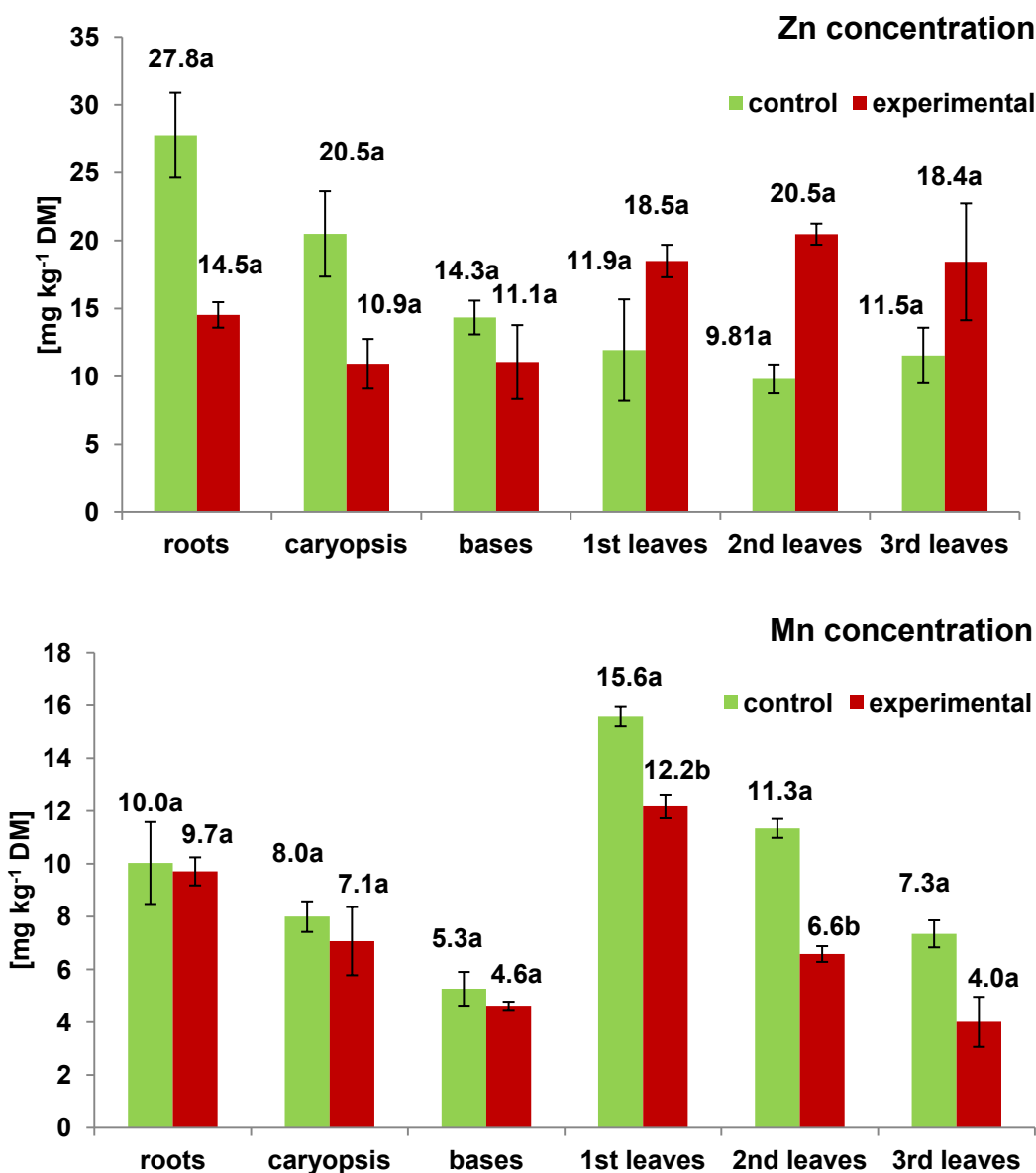


Figure 5: Average values of zinc and manganese in mg kg⁻¹ DM in control and experimental barley organs obtained from three replicates. Columns denoted with different letters are significantly different at P<0.001 (control versus experimental content).

3.6 Effect of Cd on the content of Mn

For the accumulation of Mn under Cd supply, a similar trend to that of Ca was observed. However, the accumulation of Mn under Cd supply does not follow the same trend as the accumulation of Zn in leaves. The Zn content was higher in the experimental while the Mn content in the control. High levels were found in the oldest leaves (1st and 2nd) and roots of plants. Cd stress caused decreases of Mn contents; statistically significant decline was found in the 1st leaves by 21.8% and the 2nd leaves by 41.7%, and was also evident in the 3rd leaves – by 45.4%. In our experiment Mn levels decreased both in roots as well as leaves and other aboveground parts (Fig. 5). Manganese is an essential element necessary for enzymatic activities in all subcellular compartments, and a reduction of Mn uptake and transport in the presence of Cd has been reported [23]. Martin *et al.* [24] showed that manganese dramatically reduced cadmium intake, and that this is associated with the inhibition of activation of extracellular signal-regulated kinase, characteristic of cadmium intoxication; thus manganese transporter system(s) may be responsible for cadmium entry into cells. In the manganese accumulator *Lupinus albus*, when the Mn supply was adequate, the plants showed few symptoms of Cd toxicity and a Mn:Cd ratio of up to 20 was enough to minimise Cd stress in the leaves, and in summary, high leaf Mn concentrations seem to render white lupinus more tolerant to Cd stress and this implies a protective role of Mn in photosynthetic tissues [25].

4 Conclusions

We have verified the suitability of the method based on the outflow of electrolyte and conductometric measurements of cells for the evaluation of index of membrane damage to the roots and leaves of young plants of spring barley cultivated in conditions of increasing concentrations of cadmium. Most roots, the oldest leaves and to a lesser extent the youngest leaves were damaged. This work shows that the method of discharge ions is suitable for monitoring the damage to the membranes. Cadmium stress induced by addition of cadmium chloride in nutrient solution at a concentration of 10^{-5} mol L⁻¹ showed in different organs of cultivated barley cv. Sebastian (roots, residual caryopsis, bases of the leaves, and the 1st, 2nd and 3rd leaves) different changes in the content and distribution of Cd and selected micro- and macroelements – Cu, Zn, Fe, Mn, and Ca – in barley plants. Cd significantly decreased Ca and Mn levels in all barley organs tested, while its impact on Zn and Fe

in individual organs was different. After Cd treatment in experimental variants Fe and Zn amounts in roots decreased, while conversely, Cd treatment moderately enhanced leaf Fe and to a greater extent Zn accumulation in both older and younger leaves. Cadmium stress caused significant Cu increase in roots, remaining caryopses and bases of leaves, however, conversely, it lead to a significant Cu decrease in the leaves. In summary, Cd acts as a Ca and Mn antagonist, decreasing their levels in all plant organs, as well as decreasing Cu in above-ground biomass, which implies that environments with higher Cd concentrations may be treated with Ca and Mn supplementations. The decrease of Zn and Fe in roots and gradual increase from older to younger leaves suggest their transfer in shoots when competing with the cadmium sorption to root plasma membrane. The opposite trend was found for Cd treatment – the increase of Cu levels after Cd addition suggests quicker Cu sorption into root plasma membrane in comparison to Cd. The obtained results indicate many factors affecting intake and transport mechanisms of investigated metals, such as formation of Fe/Mn plaque on roots, synergistic or antagonistic pattern in their uptake and translocation mechanisms into different plant parts. Non-metallothionein mechanisms as well as metallothionein induction, the forms, in which they are mainly stored in plant organs (soluble and insoluble form), vacuolar compartmentation, quickness of sorption on plasma membrane and many other factors should be considered in other comprehensive studies of these complex metal uptake and transfer processes.

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