Int. J. Electrochem. Sci., 6 **(2011)** 6011 - 6031

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Cadmium(II) and Zinc(II) Ions Effects on Maize Plants revealed by Spectroscopy and Electrochemistry

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Received: 2 June 2011 / *Accepted:* 11 October 2011 / *Published:* 1 December 2011

Toxicity of cadmium has been described in numerous papers and includes especially ability to generate reactive oxygen species (ROS), which are responsible for damaging of biomolecules and loss of their biological functions, however, mutual effect of zinc(II) and cadmium(II) ions is still unclear. Due to this fact, we focused on studying of joint effect of cadmium(II) and zinc(II) ions (0 μM Zn^{2+} + 0 μM Cd^{2+}; 100 μM Zn^{2+} + 0 μM Cd^{2+}; 0 μM Zn^{2+} + 100 μM Cd^{2+}; 10 μM Zn^{2+} + 100 μM Cd^{2+}; 50 μM Zn^{2+} + 100 μM Cd^{2+}; 75 μM Zn^{2+} + 100 μM Cd^{2+} and 100 μM Zn^{2+} + 100 μM Cd^{2+}) on experimental plants represented by *Zea mays* cv. CE220. Growth parameters (root/shoot length, fresh and dry weight), and stress markers and enzymes (oxidized/reduced glutathione, phytochelatins, total content of thiols, activities of glutathione-S-transferase (GST), alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP)) were monitored during the treatment. In addition, total content of zinc(II) and cadmium(II) ions was determined in plant tissues electrochemically using differential pulse anodic stripping voltammetry. Moreover, content of reduced (GSH) and oxidized (GSSG) glutathione, and phytochelatin2 and phytochelatin5 were quantified using high performance liquid chromatography with electrochemical detection (HPLC-ED). Firstly, we studied the influence of metal ions on the growth parameters, i.e. fresh/dry weight and root/shoot length. Application of both metals led to the reduction of root as well as shoot biomass compared to control untreated plants. These adverse effects were found also in length of shoot and roots as well as dry weight. Content of both cadmium and zinc was determined in roots and shoots of experimental plants using Differential Pulse Anodic Stripping Voltammetry (DPASV). The enhanced content of both zinc(II) and cadmium(II) ions in roots and shoots was identified. In comparison of cadmium and zinc, plants taken up higher amounts of zinc(II) ions. It was found that the plants treated with 100 μM Zn^{2+} + 0 μM Cd^{2+} took less Zn in the second collection day on average 0.8 - 6 times and in the tenth day of collection 0.1 - 1.1 times in aboveground parts and in the second day of sampling averaged 0.3 - 4.8 times and in the tenth day of collection 0.7 - 2.2 times in roots. Antioxidant activity was determined in roots and shoots of control and experimental maize plants exposed to cadmium(II) and/or zinc(II) ions. To determine

the antioxidant activity, we chose three methods as FRAP, DPPH and ABTS, and correlated the obtained results. The monitored enzyme activities (AST, ALT and ALP) were also correlated and we found that the activity of these enzymes enhanced with the increasing dose of both ions. In addition, we monitored GST activity correlation with total content of thiol compounds (-SH) measured spectrometrically using Ellman´s method. Amount of thiol compounds as well as GST activity was two times higher in roots compared to shoots. Aiming our attention on content of particular thiols, significant increasing of total content of GSH was observed at both 2nd and 10th day of the treatment. The most significant changes were evident in the case of the highest joint concentrations of zinc(II) and cadmium(II) ions - 100 μM Zn^{2+} + 100 μM Cd^{2+}. Treatment with both cadmium(II) and/or zinc(II) ions led to the increase of PC2 and PC5 in experimental plants. Higher amount of phytochelatins in shoots compared to roots indicates their role in chelatation and transport of both heavy metals to aerial parts.

Keywords: maize; cadmium; zinc; antioxidant activity; electrochemistry

1. INTRODUCTION

Cadmium is an important contaminant of the environment occurring as a minority component of zinc ores [1,2]. Due to its many industrial applications, especially in electrotechnologies, cadmium represents one of the most hazardous elements. It has many chemical similarities to zinc; however, cadmium is less reactive with acids compared to zinc. Toxicity of cadmium has been described in numerous papers and includes especially ability to generate reactive oxygen species (ROS), which are responsible for damaging of biomolecules and loss of their biological functions [3-5]. In plants, cadmium-induced stress may be characterized by stress markers (including oxidized/reduced glutathione, phytochelatins, metallothionein like proteins etc.), or by enzymes involved in the scavenging of ROS [6,7]. Zinc is in contrast to cadmium an essential biogenic element for microorganisms, plants, fungi and animals with many functions. Enzymes with zinc atom/atoms in reactive centre are widely distributed. In addition, zinc(II) ions serve as structural ions in transcriptional factors called zinc fingers [8]. Zinc fingers and their role in the stabilization of DNA, RNA and proteins have been widely discussed [9-11]. From the point of view of plants only, zinc is one of the essential micronutrients essential for optimum plant growth (biosynthesis of carbohydrates, chlorophyll formation, auxin metabolism, root development) and plant protective mechanisms against oxidative stress, thus, zinc represents common component of fertilizers [12-16]. On the other hand, fertilizers usually contain not only mixture of elements including zinc, but also trace concentrations of toxic heavy metals including cadmium [17]. It has been demonstrated that cadmium ions may be taken up and transported by the same mechanisms, which are involved in zinc uptake and transport. These facts are well evident in the case of members of the *Brassicaceae*, where common zinc/cadmium transporters, such as HMA2 (AtHMA2), HMA3 (AtHMA3, TcHMA3), HMA4 (AtHMA4, TcHMA4), NRAMP4 (AtNRAMP4, TcNRAMP4), YSL (BjYSL) have been identified [18-27]. It was assumed that cadmium had no biological role in an organism, however, marine diatoms (*Thalassiosira weissflogii*) contain cadmium-dependent enzyme carbonic anhydrase CDCA1, i.e. enzyme that catalyses the reversible hydration of carbon dioxide [28-31]. Cadmium performs the same function as

zinc in this enzyme. It seems that cadmium is involved in function of this enzyme instead zinc ions, which may be replaced by cadmium(II) ions [32,33]. Replacement of zinc(II) ions by cadmium(II) ones in enzymes, which is closely associated with the loss of the enzyme function, must be carefully considered as one of the possible mechanisms of cadmium toxicity. However, data on interactions between zinc(II) and cadmium(II) ions during their uptake and transport are still missing. Due to this fact, we focused on studying of joint effect of cadmium(II) and zinc(II) ions (0 μM Zn^{2+} + 0 μM Cd^{2+}; 100 μM Zn^{2+} + 0 μM Cd^{2+}; 0 μM Zn^{2+} + 100 μM Cd^{2+}; 10 μM Zn^{2+} + 100 μM Cd^{2+}; 50 μM Zn^{2+} + 100 μM Cd^{2+}; 75 μM Zn^{2+} + 100 μM Cd^{2+} and 100 μM Zn^{2+} + 100 μM Cd^{2+}) on experimental plants represented by *Zea mays* cv. CE220. Growth parameters (root/shoot length, fresh and dry weight), and stress markers and enzymes (oxidized/reduced glutathione, phytochelatins, total content of thiols, GST, ALT, AST, and ALP) were monitored during the treatment. In addition, total content of zinc(II) and cadmium(II) ions was determined in plant tissues electrochemically using differential pulse anodic stripping voltammetry. Moreover, content of reduced (GSH) and oxidized (GSSG) glutathione, and phytochelatin2 and phytochelatin5 were quantified using high performance liquid chromatography with electrochemical detection (HPLC-ED).

2. EXPERIMENTAL PART

2.1 Chemicals

Reduced (GSH) and oxidized (GSSG) glutathione were purchased from Sigma-Aldrich (USA). Phytochelatin2 (PC2) (γ-Glu-Cys)2-Gly was synthesized in Clonestar Biotech (Czech Republic) with a purity above 90%. HPLC-grade methanol (>99.9%; v/v) was from Merck (Germany). All other chemicals used were purchased from Sigma-Aldrich (USA) unless noted otherwise. Stock standard solutions of the thiols (1 mg/ml) were prepared with ACS water (Sigma-Aldrich, USA) and stored in dark at -20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through 0.45 μm Nylon filter discs (Millipore, USA) prior to HPLC analysis. The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Germany).

2.2 Plant cultivation and treatment

Maize plants - (*Zea mays* L.) cv. CE 220 of the same stage of ontological development, morphological appearance and weight were used in our experiment. Maize grains were germinated and grown in vermiculite in cultivation box under strictly defined conditions: temperature 25 ± 1 °C (light)/18 °C ± 1 °C (dark), photoperiod 16 hours (6 – 22 h), light flux 40 $\mu mol.m^{-2}.s^{-1}$ and relative humidity 80%. The plants were cultivated in hydroponic culture vessels (20 l) (PT 300, Nutriculture, UK). These vessels were filled with 15 l of medium and 36 plants were cultivated in one vessel. Amount of 0.42 l of culture medium was per one plant. Five-day seedlings were placed in these

vessels, which were filled with Richter's nutrient solution (zinc and cadmium free) and further cultivated under the same conditions. After next seven days, cultivation medium was replaced by the new cultivation medium, which was supplemented by cadmium(II)/zinc(II) ions as follows: 0 μM Zn^{2+} + 0 μM Cd^{2+}; 100 μM Zn^{2+} + 0 μM Cd^{2+}; 0 μM Zn^{2+} + 100 μM Cd^{2+}; 10 μM Zn^{2+} + 100 μM Cd^{2+}; 50 μM Zn^{2+} + 100 μM Cd^{2+}; 75 μM Zn^{2+} + 100 μM Cd^{2+} and 100 μM Zn^{2+} + 100 μM Cd^{2+}. Fresh stock solutions of corresponding salts - $Zn(NO_3)_2$ and $Cd(NO_3)_2$, concentration (10 mM $Zn(NO_3)_2$ and 10 mM $Cd(NO_3)_2$) were used (Sigma-Aldrich, USA). Plants were cultivated under above-described cultivation conditions for 10 days. Six plants from each experimental variant were harvested every second day. Samples of harvested plants were three-times rinsed by distilled water and divided into root and aerial parts. The fresh weight of the samples was measured on a Sartorius R160P scales (Sartorius GmbH, Germany) immediately after rinsing. The length of roots and shoots was determined by placing of each part of the plant to roller. Roots and shoots were dried at 45 °C for 24 hours in thermostat (UNB 300, Memmert, Germany) and then dry weight was determined on a Sartorius R160P scales (Sartorius GmbH, Germany).

2.3 Changes in plant anatomy – fluorescence microscopy

Maize plants were immediately after sampling carefully rinsed by distilled water and fixed in FAA fixation (10:1:2:7, v/v ethanol: glacial acetic acid: formaldehyde: deionised water, all from Sigma-Aldrich, USA). Just before staining, fixed plant material was carefully rinsed by distilled water. Hand-made radial sections (roots as well as stems at the half of organ length) were rinsed in distilled water, stained by acridine orange (0.1%, *w*/*w*, Sigma-Aldrich, USA) observed under a fluorescence microscope (Axioskop 40, Carl Zeiss, Gernany) equipped by a set of filters (Carl Zeiss, Germany). Photographs were taken on digital camera (Olympus Camedia 750, Olympus, Japan).

2.4 Preparation of biological samples

2.4.1 Electrochemical measurement

Leaves and root tissues of maize were dried at 45 °C for 24 hours in thermostat (UNB 300, Memmert, Germany). Samples of homogenized roots (0.1 g) or leaves (0.5 g) were used for microwave digestion in concentrated nitric acid (Sigma-Aldrich, USA). To prepare the samples microwave digestion were used according to recently published papers [34-37]. Briefly, the mineralization of samples took place in a microwave system Multiwave3000 (Anton-Paar GmbH, Austria). A sample (100 mg) was placed into glass vials MG5 and 900 μl of nitric acid (65%, w/w) were added. Prepared samples were sealed and placed into the rotor 64MG5 (Anton-Paar GmbH, Austria). Rotor with the samples was inserted into the microwave system and the microwave digestion was carried out under the following conditions: power 50 W – 10 min., power 100 W – 30 min., cooling (power 0 W) – 10 min., maximum temperature 80 °C. Sample preparation for subsequent electrochemical measurements was as follows: 15 μl mineralized sample was pipetted into Eppendorf

tubes with 985 µl acetate buffer (pH = 5.00). A blank digestion was simultaneously carried out in the same way.

2.4.2 Spectrophotometric measurement

Weighed plant tissues (roots/shoots, approximately 0.1 g of fresh weight) were transferred to test-tube (2 ml) (Eppendorf, Germany), and liquid nitrogen was added. The samples were frozen to disrupt the cells. The frozen sample was transferred to mortar and grinded for 1 min with pestle. Then, 2 ml of 0.2 M phosphate buffer (pH 7.0) was added and the sample was homogenized for 5 min. Further, homogenate was transferred to a new test-tube.

The mixture was further homogenised by shaking on a Vortex–2 Genie (Scientific Industries, USA) at 4 °C for 15 min. The homogenate was centrifuged (15 000 prm for 15 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, , Germany). Supernatant was filtered through a membrane filter (0.45 µm Nylon filter disk, Millipore, USA) prior to analysis [38,39].

2.4.3 Chromatographic measurement

Approximately 0.5 g of shoot/root tissue was frozen using liquid nitrogen and subsequently homogenized with liquid nitrogen and with 0.5 ml of phosphate buffer (pH 7.0). In addition, 0.5 ml of phosphate buffer was added.

The homogenate was centrifuged (15 000 prm) for 15 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Germany). Supernatant was collected and used for chromatographic analysis.

2.5 Electrochemical determination of zinc and cadmium in plants samples

Electrochemical measurements were performed on the device Autolab (EcoChemie, Netherland) with VA-Stand 663 (Metrohm, Switzerland). Three-electrode system, which consisted of hanging mercury drop electrode (HMDE) with working electrode surface 0.4 mm^2, silver-chloride electrode (Ag/AgCl/3 $mol.l^{-1}$ KCl) as reference electrode and platinum wire auxiliary electrode was used. GPES software (EcoChemie, Netherland) was used for data processing. Acetate buffer (pH 3.6, 0.2 $mol \cdot l^{-1}$ CH_3COOH + CH_3COONa) was used as the supporting electrolyte.

Electrolyte with samples was deoxygenated by argon (99.999%) for 120 s. The concentration of cadmium and zinc was measured using differential pulse anodic stripping voltammetry (DPASV). Anodic scan started at -0.7 V and finished at -0.4 V. Cadmium was accumulated on HMDE at 0.7 V with a 120 s accumulation at room temperature. The solution was mixed at 1450 $rev.min^{-1}$. Other parameters were as follows: modulations time 0.02 s, step potential 1.05 mV, and modulation amplitude 49.5 mV.

2.6 Spectrophotometric measurements

Spectrometric measurements were carried using an automated chemical analyser BS-200 (Mindray, China). Reagents and samples were placed at cooled sample holder (4 °C) and automatically pipetted directly into plastic cuvettes. Incubation proceeded at 37 °C. Mixture was consequently stirred. The washing steps with distilled water (18 mΩ) were performed in the midst of the pipetting. Contamination is reduced due to rinsing system, which is based on rinsing of the dosing needle as well as the stirrer by MilliQ water. The instrument was operated using software BS-200 (Mindray, China).

2.6.1 Protein determination – Bradford reaction

Determination of total proteins in the accordance with Bradford method is described in details in the following paper [40]. Briefly, reagent Coomassie Brilliant blue G-250 (0.01% Coomassie Brilliant Blue G-250, 4.7% CH_3CH_2OH, 8.5% H_3PO_4) in volume of 190 µl was pipetted. Further, sample in volume of 10 µl was added. Mixture was incubated for 10 min at 37 °C. Absorbance was measured at 595 nm, reagent itself was used as a blank. Obtained values of absorbance (blank, mixture after 10-min incubation) were used for the determination of total proteins.

2.6.2 Protein determination – Pyrogallol Red reaction

Reagent R1 (100 mM succinic acid, 6.94 mM sodium benzoate, 0.12 mM sodium molybdate, 2.09 mM sodium oxalate) in volume of 200 µl was pipetted into plastic cuvette for protein determination. Furthermore, 20 µl of sample was added. Pyrogallol red with sodium molybdate is bound in the complex with proteins in a succinic buffer at pH 2.5. This complex results in a shift of the absorption peak from 460 nm (agent) to 600 nm (complex). The absorption at wavelength $\lambda = 605$ nm was measured at 10^{th} minute of incubation at 37 °C. The absorbance values were used to calculate the absorption of reagent itself and absorbance values after 10 minutes of incubation with the sample.

2.6.3 Determination of antioxidant capacity – DPPH, FRAP, ABTS

Determination of antioxidant activity by the DPPH test

$DPPH^{\bullet}$ test is based on ability of stable free radical of 2,2-**di**-**p**icryl**h**ydrazyl to react with donors of hydrogen. $DPPH^{\bullet}$ radical demonstrates intense absorption in UV-VIS spectrum. In this test, solution of radical was decolourized after reduction with antioxidant (AH) or radical ($R^{\bullet}$) in accordance with the following scheme: $DPPH^{\bullet} + AH \rightarrow DPPH^{\bullet}\text{-}H + A^{\bullet}$, $DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH^{\bullet}\text{-}R$ [41]. This method is very simple and time-undemanding for manual analysis. A volume of $DPPH^{\bullet}$ reagent (200 µl) was incubated with sample (20 µl). Absorbance was measured after 15 min of incubation at $\lambda = 510$ nm. For calculating the antioxidant activity value of absorbance of reagent itself (A_0) and the value of absorbance after 15 min of incubation (A_{15}) were used. Resulting value was calculated according to the formula: $A = A_{15} - A_0$ [42].

Determination of antioxidant activity by the FRAP method (**F**erric **R**educing **A**ntioxidant **P**ower) is based on the reduction of ferric complexes of TPTZ (2,4,6-tripyridyl-S-triazine) with ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), which are almost colourless, eventually slightly brownish. This chemical form blue ferrous complexes after its reduction. Method has its limitations, especially measurement under non-physiological values of pH. In addition, this method cannot be used for the determination of only slowly reactive polyphenolics and thiols [43,44].

A volume of $FRAP^{\bullet}$ reagent (245 μl) was pipetted into a plastic cuvette with subsequent addition of sample (5 μl). Absorbance was measured at λ = 578 nm after 15 min. For calculating the antioxidant activity, we used the value of absorbance of reagent itself (A_0) and the value of absorbance after 15 min of incubation (A_{15}). Resulting value was calculated according to the following formula: $A = A_{15} - A_0$ [42].

Determination of antioxidant activity by the ABTS test

The ABTS radical method is one of the most used assays for the determination of the concentration of free radicals. It is based on the neutralization of a radical-cation arising from the one-electron oxidation of the synthetic chromophore 2,2'-**a**zino-**b**is(3-e**t**hylbenzothiazoline-6-**s**ulfonic acid) (ABTS): $ABTS^{\bullet} - e^- \rightarrow ABTS^{\bullet+}$. This reaction is monitored spectrophotometrically by the change of the absorption value.

A volume of $ABTS^{\bullet}$ reagent (245 μl) was pipetted into a plastic cuvette with subsequent addition of sample (5 μl). Absorbance was measured at λ = 670 nm after 15 min. For calculating the antioxidant activity, we used the value of absorbance of reagent itself (A_0) and the value of absorbance after 15 min of incubation (A_{15}). Resulting value was calculated according to the following formula: $A = A_{15} - A_0$ [42].

2.6.4 Determination of ALT activity

Determination of Alanine aminotransferase (ALT) consists of twostep reaction. In the first reaction catalysed by ALT alanine is converted to pyruvate. Formed pyruvate is by the action of Lactate dehydrogenase (LDH) reduced to lactate what is accompanied with oxidation of NADH to NAD^+. ALT activity is determined kinetically and is based on the rate of NADH concentration decrease during reaction, which can be measured by decrease of absorbance at 340 nm. Catalytic concentration of ALT is proportional to absorbance decrease.

150 μl volume of solution R_1 (100 mM Tris buffer pH 7.5, 500 mM L-alanine, 1200 U/l LDH) is pipetted into a plastic cuvette with subsequent addition of a 15 μl sample. This solution is incubated for 270 seconds. Subsequently, 30 μl of solution R_2 (15 mM 2-oxoglutarate, 0.18 mM NADH) is added and the solution is incubated for 90 seconds and then measurement of absorbance starts and lasts 180 seconds. The mean decrease of absorbance per minute is calculated.

2.6.5 Determination of AST activity

Determination of Aspartate aminotransferase (AST) consists of twostep reaction. In the first reaction catalysed by AST aspartate is converted to oxalacetate. Formed oxalacetate is by the action of Malate dehydrogenase (MDH) reduced to malate what is accompanied with oxidation of NADH to NAD^+. AST activity is determined kinetically and is based on the rate of NADH concentration decrease during reaction, which can be measured by decrease of absorbance at 340 nm. Catalytic concentration of ASAT is proportional to absorbance decrease.

150 µl volume of solution R1 (80mM Tris buffer pH 7.8, 240 mM L-aspartate, 1200 U/l MDH) is pipetted into a plastic cuvette with subsequent addition of a 15 µl sample. This solution is incubated for 270 seconds. Subsequently, 30 µl of solution R2 (15 mM 2-oxoglutarate, 0.18 mM NADH) is added and the solution is incubated for 90 seconds and then measurement of absorbance starts and lasts 180 seconds. The mean decrease of absorbance per minute is calculated.

2.6.6 Determination of ALP activity

To determine the alkaline phosphatase activity, 100 µl of reagent R1 (0.9 M 2-amino-2-methyl-1-propanol, pH 10.4; 1.6 mM magnesium sulphate, zinc sulphate 0.4 mM, 2 mM HEDTA) was pipetted and mixed with 2 µl of sample. After incubation (1 min), absorbance was measured at $\lambda = 405$ nm. Subsequently, volume of 20 µl of reagent R_2 (16 mM *p*-nitrophenylphosphate) was added and the mixture was incubated for 10 minutes. The value of ALP activity was calculated from absorbance values of the first (R_1 + sample) and the final absorbance value ($R_1 + R_2$ + sample).

2.6.7 Determination of glutathione-S-transferase activity

The method is based on the GST-catalysed reaction between GSH and GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB), which has the broadest range of isoenzyme detectability (e.g. alpha-, mu-, pi- and other GST isoforms). Under certain conditions, the interaction between glutathione and CDNB is very dependent on the presence of active GST. The GST-catalysed formation of GS-DNB produces a dinitrophenylthioether, which can be detected spectrometrically at 340 nm. Volume of 180 µl reactants consisted of 2 mM CDNB and PBS (1:19, *v*/*v*, 37 °C) was added to sample to a plastic microtube. After it, 30 µl of 12.5 mM GSH in 0.1 M phosphate buffer (pH 7.4) was added. The microtube was carefully stirred and loaded into an automatic biochemical analyzer BS-200 (Mindray, China) and measured at $\lambda = 340$ nm.

2.6.8 Determination of total thiol compounds content – Ellman´s reaction

Ellman´s spectrophotometric method was used for the determination of sulfhydryl (-SH) groups [45]. Ellman´s reagent (277 µl, reagent 1, R_1 – 2 mM 5.5'- dithiobis(2-nitrobenzoic) acid (DTNB) in 50 mM $Na_2(CH_3COO)_2$) was mixed with sample (45 µl). After it, 33 µl of reagent R2 (1 M Tris

base®: CH_3COOH) (Sigma-Aldrich, USA) was added. Mixture was incubated for 10 min at 37 °C, absorbance was measured at λ = 405 nm. Values of absorbance of reagent R_1 itself – blank – and mixture after 10-min incubation were used for the calculation of total -SH content.

2.7 High performance liquid chromatography with electrochemical detection (HPLC-ED)

HPLC-ED system consisted of two solvent delivery pumps operating in the range of 0.001-9.999 $ml.min^{-1}$ (Model 582 ESA Inc., Chelmsford, MA), Zorbax eclipse AAA C18 (150 × 4.6; 3.5 μm particles, Agilent Technologies, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector includes one flow cell (Model 6210, ESA, USA). Each cell consists of four working carbon porous electrodes, each one with auxiliary and dry Pd/H_2 reference electrodes. Both the detector and the reaction coil/column were thermostated. The sample (20 μl) was injected using autosampler (Model 542 HPLC, ESA, USA). Samples were kept in the carousel at 8 °C during the analysis. The column was thermostated at 32 °C. Mobile phase consisted of 80 mM TFA (A) and methanol (B). The compounds of interest were separated by the following linear gradient: 0 → 1 min. (3% B), 1 → 2 min. (10% B), 2 → 5 min. (30% B), 5 → 6 min (98% B). Flow rate of the mobile phase was 1 $ml.min^{-1}$ and working electrode potential was set to 900 mV [46-50]. Time of analysis was 20 min.

3. RESULTS AND DISCUSSION

3.1 Plant growth

Our study was focused on studying of joint effect of cadmium(II) and zinc(II) ions on experimental plants of maize (*Zea mays* L. cv. CE 220) treated with the following concentrations: 0 μM Zn^{2+} + 0 μM Cd^{2+}; 100 μM Zn^{2+} + 0 μM Cd^{2+}; 0 μM Zn^{2+} + 100 μM Cd^{2+}; 10 μM Zn^{2+} + 100 μM Cd^{2+}; 50 μM Zn^{2+} + 100 μM Cd^{2+}; 75 μM Zn^{2+} + 100 μM Cd^{2+} and 100 μM Zn^{2+} + 100 μM Cd^{2+}. Firstly, we studied the influence of metal ions on the growth parameters, i.e. fresh/dry weight and root/shoot length. Application of both metals led to the reduction of root as well as shoot biomass compared to control untreated plants (Figs. 1A and B). The most significant growth inhibition was observed in plants treated with combination of the highest concentrations of Zn^{2+} and Cd^{2+} ions (100 μM). Regression lines and their slopes (Table 1-3) confirmed the same trend. The highest slope was observed in control group in both shoot and root. The values reached 0.413 (shoot) and 0.068 (root) respectively. Other trends had decreasing tendency. Experimental plants treated with 100 μM cadmium(II) along with different zinc concentrations demonstrated distinct growth inhibition. These results were also confirmed by negative slopes. The lowest slope was observed in the concentration combination 100 μM zinc(II) and 100 μM cadmium(II) in both part of plant (-0.100 (shoot) and -0.033(root)).

Similar tendencies were observed in the case of dry weight, which is important for determination of water content (Figs. 1C and D). The highest value was detected in control group in both roots and shoots (slope 0.053 (shoots) and 0.007 (roots)). Furthermore, the tendency was

decreasing in the following order: 0 μM Zn^{2+} + 100 μM Cd^{2+}, 100 μM Zn^{2+} + 0 μM Cd^{2+}, 10 μM Zn^{2+} + 100 μM Cd^{2+}; 50 μM Zn^{2+} + 100 μM Cd^{2+}; 75 μM Zn^{2+} + 100 μM Cd^{2+} and 100 μM Zn^{2+} + 100 μM Cd^{2+} (0.042, 0.010, 0.010, 0.008, -0.009 and -0.018 shoots) and 0.005, 0.001, -0.001, -0.001, -0.003 and -0.004 (roots) respectively).

Moreover, we monitored the influence of all used concentrations on length of shoots and roots of maize. Only minimal growth inhibition after addition of 100 μM zinc(II) and 0 μM cadmium(II) (slope 14.013 against control 32.838 in shoots and 10.238 and 29.363 in roots respectively) was observed. This fact supports biogenic role of zinc and its low toxicity in chosen concentration. On the other hand, significant growth inhibition compared to control was observed in group 0 μM Zn^{2+} + 100 μM Cd^{2+} (8.900 in shoot and 6.275 in root). Mutual influence of both metals resulted in growth inhibition in all concentration combinations (Figs. 1E and F).

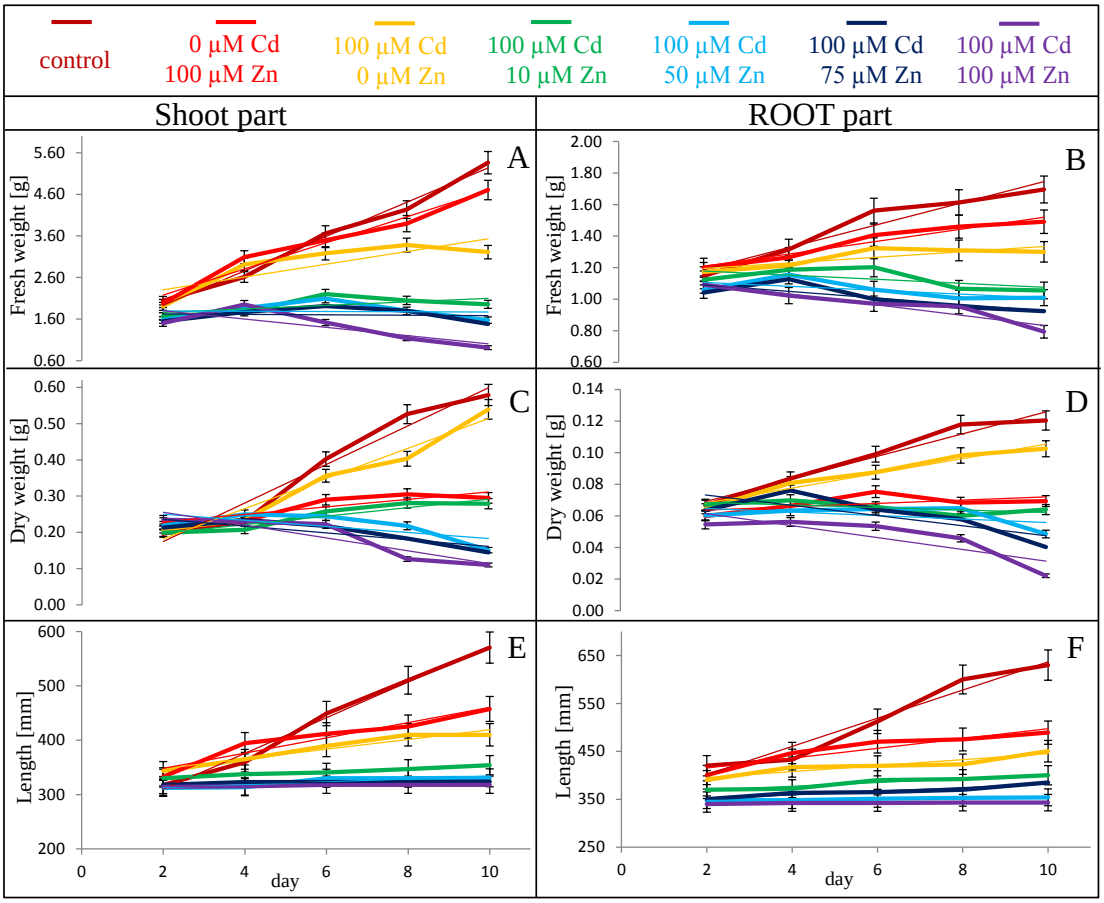

Figure 1. Growth curves of maize plant. Dependence of fresh weight (A and B), dry weight (C and D) and length (E and F) of shoots and roots, respectively, on the time of the treatment with the following concentrations of cadmium(II) and zinc(II) ions: 0 μM Zn^{2+} + 100 μM Cd^{2+}, 100 μM Zn^{2+} + 0 μM Cd^{2+}, 10 μM Zn^{2+} + 100 μM Cd^{2+}; 50 μM Zn^{2+} + 100 μM Cd^{2+}; 75 μM Zn^{2+} + 100 μM Cd^{2+} and 100 μM Zn^{2+} + 100 μM Cd^{2+}.

These results correspond to morphology of plants, which are shown in Fig. 2A, in which the maize plants are shown in second and tenth day of experiment. Application of cadmium and zinc led to

the significant changes in root morphology, especially formation of adventitious roots. The most distinct changes were observed under 100 μM cadmium concentration and at all joint treatments with zinc(II) ions. Combination 100 μM Zn^{2+} + 100 μM Cd^{2+} led to the decline of roots, chlorosis of leaves and general desiccation of shoots.

Due to above-mentioned facts, we investigated also anatomical changes of roots and stems because of cadmium and/or zinc treatment. Staining by acridine orange was used for visualisation of both primary cell walls (cellulose) and lignification (lignin) (Fig. 2B). Roots of control, hydroponically cultivated plants demonstrated anatomy typical for monocots – rhizodermis as the primary dermal tissue, ground tissue represented by cortex and stele with radial vascular bundles. In control plants, sclerification of exodermis (outermost part of cortex) and formation of intercellular spaces bordered with cells with lignified cell walls in mesodermis was well observable. No changes in formation of vascular tissue were observed. Increased lignification of parenchyma of stele was evident in cadmium and zinc treatment as well as their combinations.

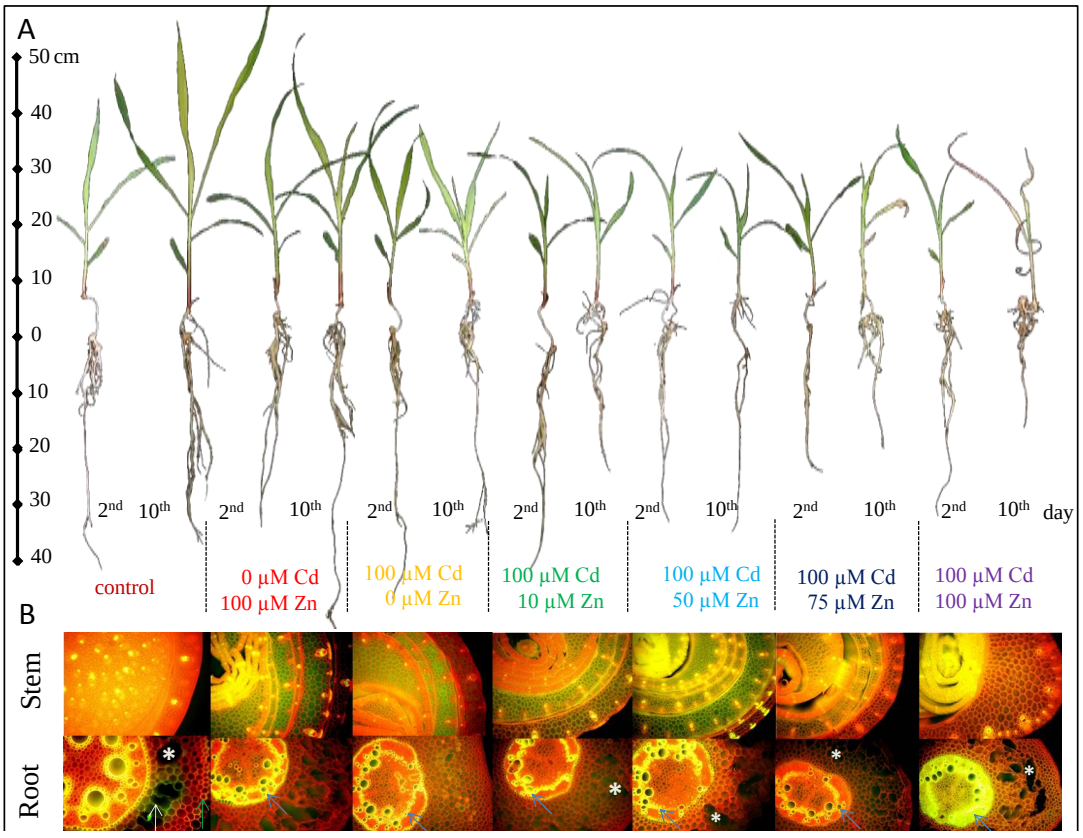

Figure 2. The influence of cadmium and/or zinc in different concentrations on maize at 2nd and 10th day of experiment. Morphology of experimental plants (A). Anatomical changes of stems and roots (B). Changes in formation of vessels are indicated by yellow arrow, star indicates intercellular spaces, which are in control plants bordered by cells with lignified cell walls – white arrows. Green arrow indicates sclerification of exodermis in control plants.

However, the cadmium treatment in combination with zinc led to the changes in formation of metaxylem vessels and lignification of parenchyma. The most significant changes were observable in the combination 100 μM Zn^{2+} + 100 μM Cd^{2+}. In this case, lignification of pericycle as a latent meristem as well as almost all parenchyma cells of stele was clearly noticeable. In addition, this combination caused decline of rhizodermis and formation of large radial intercellular spaces leading from root surface almost to endodermis. On the other hand, treatment of maize plants with cadmium itself and by combination 10 μM Zn^{2+} + 100 μM Cd^{2+} inhibited formation of intercellular spaces.

Table 1. Regression lines of fresh weight and their slopes.

Fresh weight [g]	Above-ground part	Root part
control	0.413x + 1.1	0.068x + 10.49
0 Cd + 100Zn	0.313x + 1.5	0.039x + 11.31
100 Cd + 0 Zn	0.154x + 1.9	0.017x + 11.60
100 Cd + 10 Zn	-0.003x + 1.8	-0.013 + 12.04
100 Cd + 50 Zn	-0.005x + 1.7	-0.012x + 11.32
100 Cd + 75 Zn	-0.043x + 1.6	-0.021x + 11.32
100 Cd + 100 Zn	-0.100x + 2	-0.033x + 1.17

Table 2. Regression lines of dry weight and their slopes.

Dry weight [g]	Above-ground part	Root part
control	0.053x + 0.06	0.007x + 0.05
0 Cd + 100Zn	0.010x + 0.2	0.001x + 0.06
100 Cd + 0 Zn	0.042x + 0.09	0.005x + 0.06
100 Cd + 10 Zn	0.010x + 0.17	-0.001x + 0.07
100 Cd + 50 Zn	0.008x + 0.26	-0.001x + 0.07
100 Cd + 75 Zn	-0.009x + 0.25	-0.003x + 0.08
100 Cd + 100 Zn	-0.018x + 0.29	-0.004x + 0.07

Table 3. Regression lines of plant length and their slopes.

Length [mm]	Above-ground part	Root part
control	32.838x + 244	29.363x + 342
0 Cd + 100Zn	14.013x + 320	10.238x + 394
100 Cd + 0 Zn	8.900x + 330	6.275x + 382
100 Cd + 10 Zn	2.875x + 324	4.000x + 360
100 Cd + 50 Zn	2.687x + 307	0.850x + 345
100 Cd + 75 Zn	0.600x + 318	3.825x + 343
100 Cd + 100 Zn	0.500x + 313	0.350x + 339

This effect was the most evident in the treatment by cadmium itself, where lignification of mesodermal cells was noticeable. In the case of aerial parts, shoots, changes in lignification of mechanical tissue represented by cells opposite to vascular bundles were observable. Enlargement of

cells of leaf sheath and formation of atypical small vascular bundles was evident in the combination of 100 μM Zn^{2+} + 100 μM Cd^{2+}. In conclusion, cadmium and zinc have a synergistic effect on root and stem anatomy.

3.2 Zinc and cadmium determination

Content of both cadmium and zinc was determined in roots and shoots of experimental plants using DPASV. The enhanced content of both zinc(II) and cadmium(II) ions in roots and shoots was identified. In comparison of cadmium and zinc, plants taken up higher amounts of zinc(II) ions. This fact is connected with physiological role of zinc. In addition, competition in cadmium/zinc uptake and transport must be carefully considered. Highest amount of both cadmium and zinc was found in roots. This fact is closely connected with direct contact of this organ with cultivation media containing these elements. It was found that significant amounts of heavy metals might be deposited in cell walls in roots as a result of their interactions with constituents of cell wall and in intercellular spaces due to formation of precipitates with inorganic anions [51-53]. It was found that the plants treated with 100 μM Zn^{2+} + 0 μM Cd^{2+} took less Zn in the second collection day on average 0.8 - 6 times and in the tenth day of collection 0.1 - 1.1 times in aboveground parts and in the second day of sampling averaged 0.3 - 4.8 times and in the tenth day of collection 0.7 - 2.2 times in roots (Figs. 3A and B).

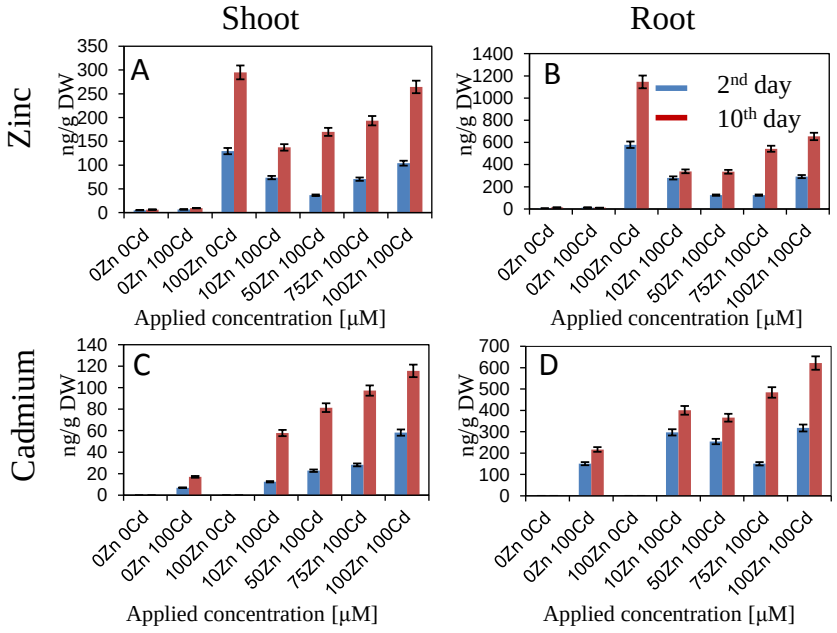

Figure 3. Zinc content in the aboveground parts (A) and roots (B) of maize plants in the 2nd and 10th day of cultivation. Cadmium content in the aboveground parts (C) and roots (D) of maize plants in the 2nd and 10th day of cultivation.

Plants treated with cadmium(II) ions in combination with zinc(II) ions demonstrated enhanced cadmium concentration with the increasing zinc concentrations in shoots (Fig. 3C) at the second and tenth day of treatment and in roots at the tenth day of treatment (Fig. 3D). The interesting finding was

that the presence of Zn in the culture solution increases the intake of Cd maize plants. Based on the comparison of results obtained from the second and tenth day of the experiment it can be concluded that despite the growth inhibition caused by metals, accumulation of Zn and Cd occurs in both plant parts.

3.2 Antioxidant activity

Antioxidant activity was determined in roots and shoots of control and experimental maize plants exposed to cadmium(II) and/or zinc(II) ions. To determine the antioxidant activity, we chose three methods as FRAP, DPPH and ABTS, which differ in the sensitivity on the spectrum of molecules with possible antioxidant activity. The obtained results were recalculated to equivalent of gallic acid (GAE) and protein content, which was determined by two independent methods - Bradford and Pyrogallol Red reaction.

All results were correlated on the base of the publication from Sulc et al. [54] dealing with the selection of appropriate methods for determining the antioxidant activity of different potato varieties, including their correlations. Authors compared the antioxidant activity of red-, purple- and yellow-fleshed potato varieties by the ABTS, DPPH and FRAP methods. Purple- and red-fleshed potatoes showed from 1.5 to 2.6 times higher antioxidant activity compared with the yellow-fleshed ones. However, the choice of matrix, in which antioxidant activity was determined, affected the obtained results. Significant differences between varieties and the localities, on which the potatoes were cultivated, were determined. The highest linear correlation between ABTS and FRAP arrays was found (R^2=0.94); these arrays appear to be useful for the determination of antioxidant activity of potatoes. Statistically significant differences between the antioxidant activity of lyophilizate and juice of fresh potato tubers were found in some varieties [54]. Based on these results we processed our data accordingly. In the first step, activities measured using FRAP and ABTS were compared. The increased concentrations of zinc(II) together with cadmium(II) ions led to the increased antioxidant activity detected using both FRAP and ABTS methods. This tendency was noticeable in roots as well as shoots. The highest value of antioxidant activity (FRAP/ABTS) was detected in experimental plants treated with 100 μM Zn^{2+} + 100 μM Cd^{2+} (32/12 mg of GAE/l/g of protein at the 2nd experimental day and 36/28 mg of GAE/l/g of protein at the 10th experimental day in shoots and 26/42 mg of GAE/l/g of protein (2nd) and 54/45 mg of GAE/l/g of protein (10th) in roots (Bradford). The highest value was detected by Pyrogallol Red method in 100 μM Zn^{2+} + 100 μM Cd^{2+} (246/190 mg of GAE/l/g of protein in shoots and 273/348 mg of GAE/l/g of protein (10th) in roots. Higher value detected in roots compared to shoots is probably connected with direct contact of roots with both heavy metals, which could generate reactive oxygen species, i.e. oxidative stress. This fact was confirmed in recalculation to total protein content using both Brandford and Pyrogallol Red methods, see Figs. 4A and B. Regression equation and well as Pearson coefficient are in agreement with above-mentioned fact - r_P = 0.7967 (Bradford) and 0.7957 (Pyrogallol Red) (Fig. 4C).

Similar tendency was observable using DPPH and ABTS methods, where enhancement of antioxidant activity at both 2nd and 10th experimental day was well evident. The highest values

(DPPH/ABTS) were detected in 100 µM Zn^{2+} + 100 µM Cd^{2+} (8.68/28.20 mg of GAE/l/g of protein in shoots and 17/45 mg of GAE/l/g of protein (10^{th}) in roots by Bradford method (Fig. 4D) and 59/190 mg of GAE/l/g of protein in shoots and 86/348 in roots by Pyrogallol Red method (Fig. 4E). Pearson´s coefficients predicate about mutual correlation of both DPPH and ABTS methods - r_P = 0.8292 (Bradford) and 0.8737 (Pyrogallol Red) (Fig. 4F).

DPPH and FRAP were the last methods used for the determination of antioxidant activity. No differences were observable at the 2^{nd} and 10^{th} experimental days. Only slight enhancement of antioxidant activity (DPPH/FRAP) was observable in 100 µM Zn^{2+} + 100 µM Cd^{2+} in comparison with control plants (9/36 mg of GAE/l/g of protein in shoots and 17/54 mg of GAE/l/g of protein in roots (Fig. 4G) by Bradford method and 58.67/246.43 mg of GAE/l/g of protein in shoots and 273/86 mg of GAE/l/g of protein in roots by Pyrogallol Red method (Fig. 4H). Comparison of DPPH and FRAP methods were found highest correlation of Pearson´s coefficients (0.9407 – Bradford and 0.9211 Pyrogallol Red), suggesting a very high dependence of the two methods. This is in well agreement with Sulc et al. [54].

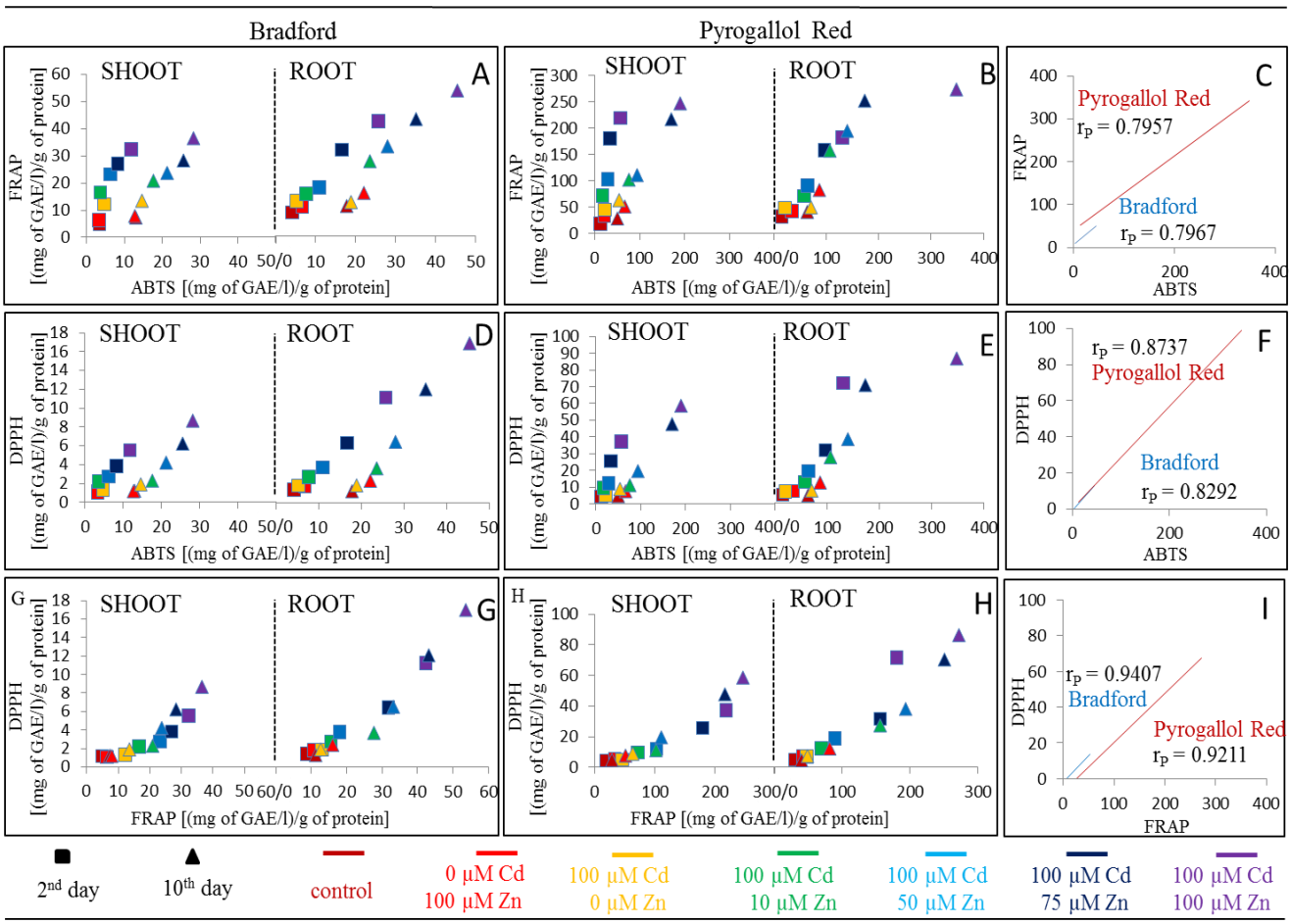

Figure 4. A-C comparison of FRAP and ABTS method, D-F comparison of DPPH and ABTS method, G-I comparison of DPPH and FRAP method.

3.3 ALT, AST and ALP activities

Data about the significance of some commonly analysed enzymes as markers of stress reactions in plants are still missing. In several papers, we have demonstrated that some enzymes (such as aminotransferases) can participate in plant stress reactions [55-60]. Thus, our attention was focused on the determination of activities of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP). Transaminases catalyse the transfer of the amino groups of amino acids to 2-oxoacids. In plants, transaminases very effectively participate in transformations of nitrogen compounds. They are important for the biosynthesis of amino acids from oxo-acids in the citrate cycle and for other crucial biochemical pathways, such as synthesis of chlorophyll. Alkaline phosphatase is a ubiquitous enzyme in all organisms and catalyses hydrolysis of orthophosphate monoesters; their physiological functions remain unclear, but they are considered to play a role in phosphate uptake [61].

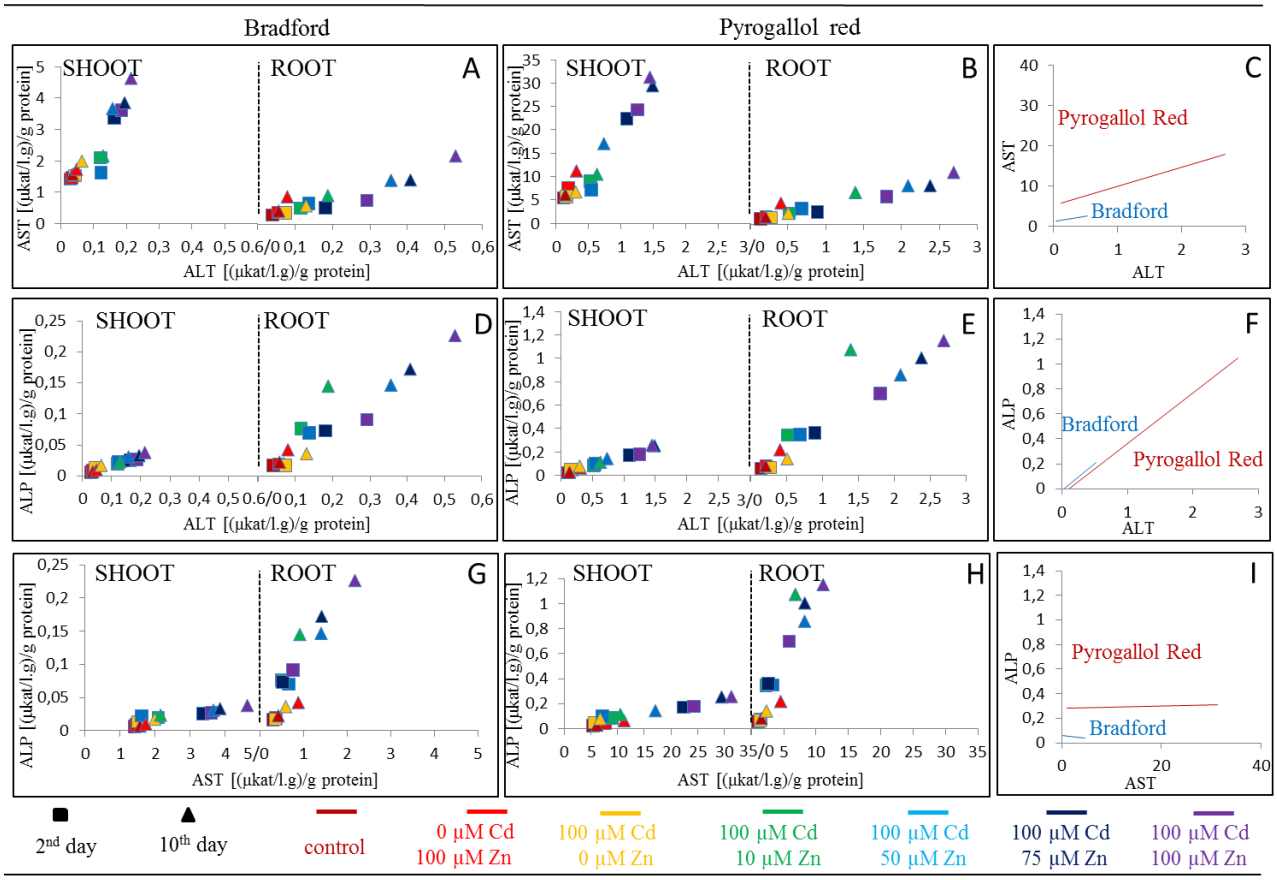

Figure 5. A-C Comparison of AST/ALT activity, D-F comparison of ALP/ALT activity, G-I comparison of ALP/AST activity.

In the cross-comparison of activities of AST and ALT, we determined the contents of individual enzymes in maize treated with different concentrations of zinc(II) and cadmium(II) ions in root and shoot in second and tenth day of the experiment (Figs. 5A and B). The highest values of the ALT/AST in comparison with control plants (1.57/0.04 (µkat/l.g)/g protein (shoot) and 0.42/0.06

(µkat/l.g)/g protein (root) - recalculated by Bradford (Fig. 5A) and 6.13/0.14 (µkat/l.g)/g protein (shoot) and 1.54/0.21 (µkat/l.g)/g protein (root) - recalculated by Pyrogallol Red (Fig. 5B)) were found in plants treated with 100 µM Zn^{2+} + 100 µM Cd^{2+} (4.63/0.21 (µkat/l.g)/g protein (shoot) and 2.17/0.53 (µkat/l.g)/g protein (root) - recalculated by Bradford (Fig. 5A) and 31.30/1.44 (µkat/l.g)/g protein (shoot) and 11.08/2.68 (µkat/l.g)/g protein (root) - recalculated by Pyrogallol Red (Fig. 5B)). This increase corresponds well with the higher metabolic activity (Fig. 5C).

ALP and ALT values showed the noticeable increase in comparison with control. Group treated with the highest concentration of zinc and cadmium also reached the highest values (100 µM Zn^{2+} + 100 µM Cd^{2+}), i.e. 0.03/0.21 (µkat/l.g)/g protein (ALP/ALT) in shoot and 0.23/0.53 (µkat/l.g)/g protein in root by Bradford (Fig. 5D) and 0.25/1.48 (µkat/l.g)/g protein in shoot and 1.15/2.68 (µkat/l.g)/g protein in root by Pyrogallol Red, respectively (Fig. 5E). Correlations of the results are shown in Fig. 5F.The methods ALP/AST showed the similar trend as increasing in their concentration with the increasing concentration of metal compared with control (Figs 5. G and H). It clearly follows from the results obtained that the higher level of AST in shoots and higher level of ALT and ALP in roots. These results were similar to recalculation to both Bradford and Pyrogallol Red (Fig. 5I).

3.4 Determination of thiol compounds

We were also focused on the monitoring of the GST activity, enzyme, which plays an important role in detoxification of xenobiotics and heavy metals by catalysing the their conjugation with a tripeptide glutathione [62]. In addition, we monitored its correlation with total content of thiol compounds (-SH) measured spectrometrically using Ellman´s method. Amount of thiol compounds as well as GST activity was two times higher in roots compared to shoots. Moderate increase was observed at 10th day of experiment in comparison to second day. The highest values of GST/SH were observed in plants treated with 100 µM Zn^{2+} + 100 µM Cd^{2+} (i.e. 0.03 µmol GST/min/mg protein, 0.37 SH mmol/g protein in shoot and 0.09 µmol GST/min/mg protein , 1.02 SH mmol/g protein in roots by Bradford (Fig. 6A) and 0.21 µmol GST/min/mg protein ; 2.50 SH mmol/g protein in shoots and 0.44 µmol GST/min/mg protein, 5.18 SH mmol/g protein in roots by Pyrogallol Red) (Fig. 6B). Correlations of the results are shown in Fig. 6C.

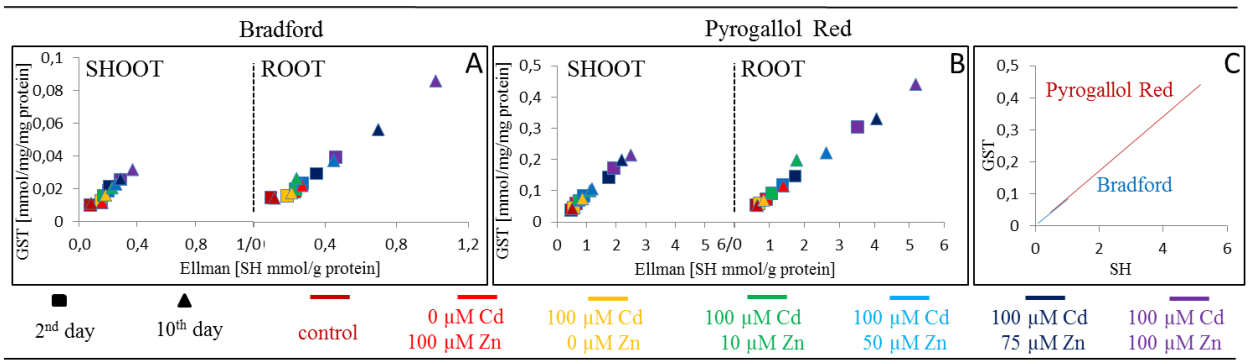

Figure 6. A-C Comparison of enzyme GST activity with total content of thiols according to Ellman.

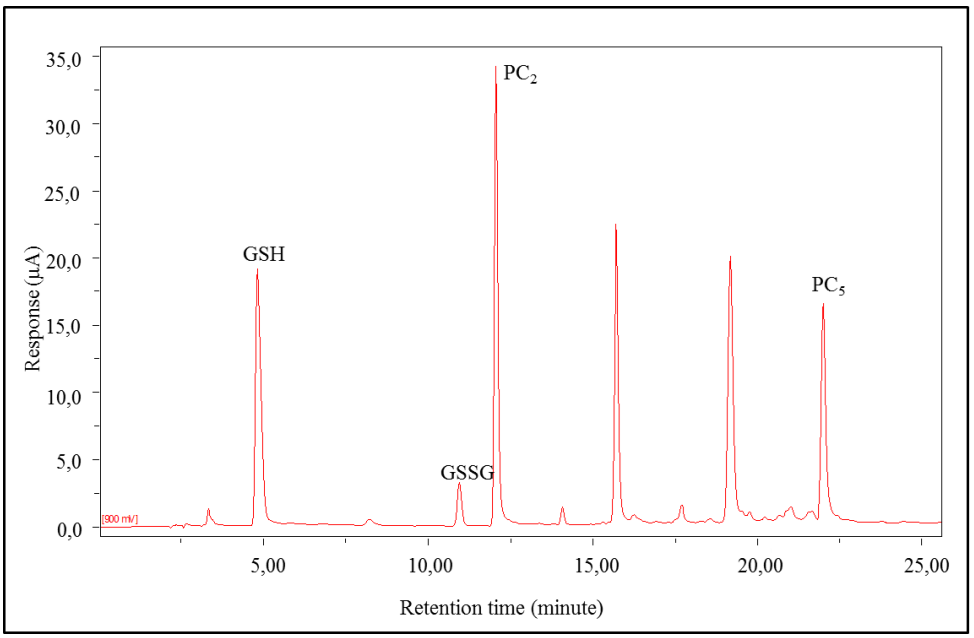

Figure 7. Chromatogram of GSH, GSSG, PC2, PC3, PC4, PC5. Their retention times [minute] were as follows: GSH [4.8], GSSG [10.95], PC2 [12.01], PC3 [15.7], PC4 [20.7] and PC5 [22.01].

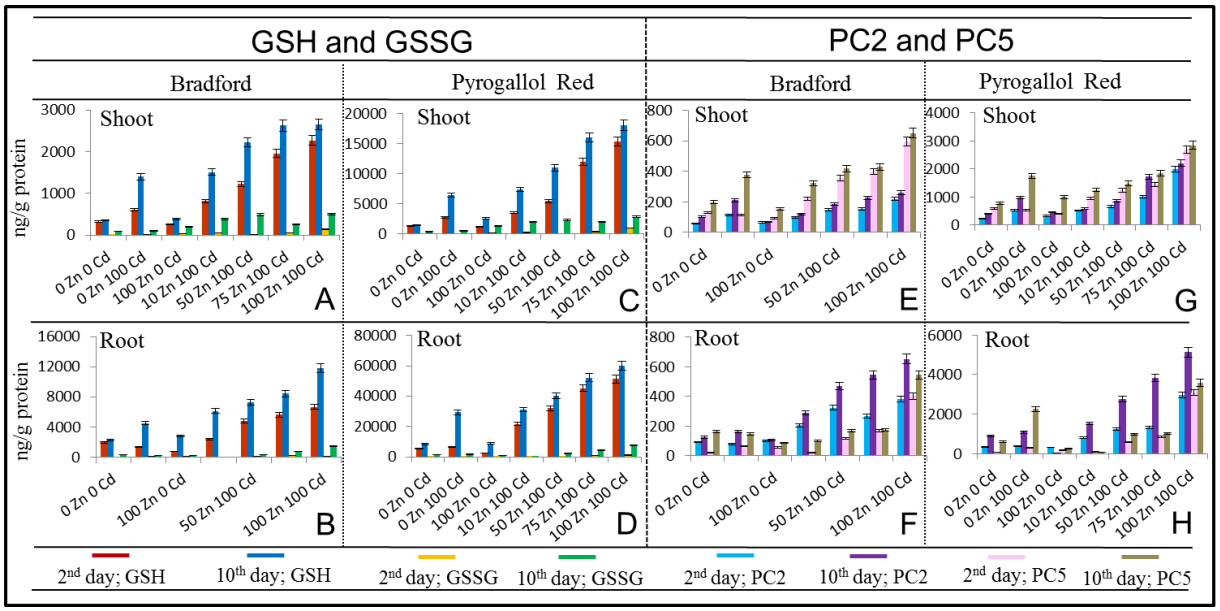

Figure 8. A-D Determination of GSH and GSSG content, E-H determination of PC2 and PC5 content. With increasing concentration of zinc(II) and cadmium(II) the content of GSH, GSSG and PC2, PC5 increases in the ^{2nd} and 10^{th} day of experiment.

Content of thiol compounds, such as reduced (GSH) and oxidized (GSSG) glutathione, and phytochelatins (PC2, PC5) was determined by HPLC–ED. Typical chromatogram of GSH, GSSG and PCs including their retention times is shown in Fig. 7. Significant increasing of total content of GSH was observed at both 2^{nd} and 10^{th} day of the treatment. The most significant changes were evident in

the case of the highest joint concentrations of zinc(II) and cadmium(II) ions - 100 μM Zn^{2+} + 100 μM Cd^{2+}. Mutual rate of GSH and GSSG was 5:1 for roots and this ratio increased with the increasing concentrations of metals.

In comparison with control group recalculated to total proteins determined by Bradford method at the 10th day of experiment, values varied from 350 ng/g protein of GSH and 90 ng/g protein of GSSG (control) to 2,650 ng/g protein of GSH and 500 ng/g protein of GSSG (100 μM Zn^{2+} + 100 μM Cd^{2+}) in shoots (Fig. 8A) and from 2,300 ng/g protein of GSH and 360 ng/g protein of GSSG (by control) to 11,800 ng/g protein of GSH and 1,500 ng/g protein of GSSG (100 μM Zn^{2+} + 100 μM Cd^{2+}) in roots (Fig. 8B). In a case of recalculation to total proteins determined by Pyrogallol Red method, these values varied from 1,400 ng/g protein of GSH and 350 ng/g protein of GSSG in shoots and 8,400 ng/g protein of GSH and 1,300 ng/g protein of GSSG in roots in control group (Fig. 8C).

The highest values (by Pyrogallol Red method) were detected in variant 100 μM Zn^{2+} + 100 μM Cd^{2+} i.e. 18,000 ng/g protein of GSH and 2,800 ng/g protein of GSSG in shoots and 60,000 ng/g protein of GSH and 7,600 ng/g protein GSSG in roots (Fig. 8D). PC2 and PC5 are involved especially in detoxification and transport of heavy metals [63,64]. However, their biosynthesis indicates accumulation of heavy metals in the cytoplasm. Treatment with both cadmium(II) and/or zinc(II) ions led to the increase of PC2 and PC5 in experimental plants, which was the most evident 10th day of treatment (Figs. 8E-F). Higher amount of phytochelatins in shoots compared to roots indicates their role in chelatation and transport of both heavy metals to aerial parts (Figs. 8E-H).

4. CONCLUSIONS

Increasing amount of pollutants of various nature and origin in the environment leads to the activation of detoxification mechanisms in organism. Total growth inhibition of maize plants exposed to zinc(II) and cadmium(II) ions was probably due to activation of defence reactions, mainly due to the synthesis of plant protection substances instead of the biosynthesis of substances necessary for plant growth. Moreover we can conclude that inhibition of the root is probably associated with the transport of cadmium in the root system. The content of metal ions in the plant increased with the increasing time of cultivation. An interesting finding in the experiment was that in the presence of zinc(II) ions, there was an increased intake of cadmium in maize plants.

ACKNOWLEDGEMENTS
The financial support from the following project MOBIMAERO IGA AF MENDELU TP 7/2011 and CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged. The author (PS) is „Holder of Brno PhD Talent Financial Aid“. The results were presented at 11th Workshop of Physical Chemists end Electrochemists held in Brno, Czech Republic.

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