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Employment of Electrochemical Methods for Assessment of the Maize (*Zea mays* L.) and Pea (*Pisum sativum* L.) Response to Treatment with Platinum(IV)

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Platinum group elements (PGEs) belong to the group of heavy metals, which represents potential high risk for the environment. They get into the environment mainly from the still increasing transport, however, they are used also in chemical, electrical and glass industry. In this study, we aimed our attention at using electrochemical methods as such differential pulse voltammetry and high performance liquid chromatography with electrochemical detection for determination of Pt(IV) content, amino acids, reduced (GSH) and oxidized (GSSG) glutathione levels, and phytochelatin synthase (PCS) activity in maize and pea plants treated with various doses of Pt(IV). The results revealed that amino acid levels, thiol contents and PCS activity increased markedly with the increasing concentration of Pt(IV). The amino acid content was the highest in maize and pea plants treated with 25 μM Pt(IV) in 8th and 12th days of treatment. GSH showed an 80% increase (compared to control) in plants treated with 100 μM after 12th days long exposure. The most significant increase of GSH was determined in roots pea treated with 25, 50 and 100 μM. GSSG showed an increase of 100% (compared to control) in roots of pea treated with 100 μM after 8th days exposure. Different tendency was determined in the maize plants. The GSH/GSSG ratio was increased in shoots of maize treated with 25 μM at both sampling times. In both cultivars, PCS activity was increased, mainly due to 25, 50 and 100 μM of Pt(IV) exposure in roots and shoots. The comparison of the trends of slopes of dependences of total thiols and activity of PCS showed that maize roots and shoots were the obviously the same sensitive as shoots and roots of pea in thiol content but different situation was observed at PCS activity. Our data indicated that both species have different strategies to withstand the stress caused by Pt(IV).

Keywords: Differential Pulse Voltammetry; High Performance Liquid Chromatography with Electrochemical Detection; Ion-Exchange Chromatography; Platinum; *Zea mays* L.; *Pisum sativum* L.; Phytotoxicity; Biochemical Markers; Amino Acid; Phytochelatin Synthase

1. INTRODUCTION

Due to its specific properties, platinum has found application in many industrial branches (chemical, electrochemical, petrochemical) and in jewellery. In an effort to reduce the amount of pollutants resulting from the combustion of fuels, automobile catalytic converters, where a mixture of platinum group elements (PGEs, especially platinum (Pt), palladium (Pd) and rhodium (Rh)) is used as a catalyst, were introduced. Large temperature fluctuations in automobile catalytic converters together with a presence of various (rare) metals lead to redox reactions and subsequent PGEs release into the environment [1]. Contamination of soils in the vicinity of roads is primarily connected with their runoff from road surface. Rainwater runoff from the road surface represents one of the most important ways of PGEs entries to the soil, surface water and sediments [2-6]. It is not surprise that the increasing levels of platinum have being found in the environment [2,7]. This fact instigates concern about possible health risks, mainly in the connection with food chains and their possible contamination [8]. In this light, plants represent the first trophic level in the food chain endangered by platinum. Despite the fact that platinum is considered nontoxic and chemically stable, it has been established that almost 10 % of Pt emitted to the environment undergoes chemical transformation under the formation of soluble and thus mobile forms [9-11]. The bioavailability and generally behaviour of Pt in the environment depend on many parameters. pH value is determinative for the Pt bioavailability in the aquatic and soil environments, where acidic environment significantly increases Pt mobility. In addition, PGEs are able to form chemically stable complexes with organic compounds present in both aquatic and soil environments (fulvoacids, humic acids). However, the presence of soil particles in soils and water sediments are responsible for PGMs adsorption and possible surface complexation [12,13]. In the case of Pt, the most unavailable is metallic Pt, but bioavailability markedly increases for the $PtCl_4$ and the highest is for $Pt(NH_3)_4(NO_3)_2$ complex [2,3,14,15].

Heavy metals (including PGEs) are taken up from soil by roots together with other compounds/metal ions, or via surface of aerial parts from atmosphere [16], and may interfere with numerous biochemical and physiological processes including photosynthesis, respiration, nitrogen and protein metabolism, and nutrient uptake and cause cellular damage to vital macromolecules and membranes by inducing oxidative stress as production of lipid peroxides and reactive oxygen species (ROS, O_2^- , H_2O_2, OH-) [17]. The knowledge of the plants ability to withstand metal ions uptake includes several cellular defence mechanisms, such as plasma membrane exclusion, vacuolar compartmentalization and cell wall immobilization [18]. Upon exposure to metals, plants often synthesize a set of diverse metabolites, particularly specific amino acids, such as cysteine, proline, and histidine [19]. Nowadays, modern methods for separation and quantitation of free amino acids include ion exchange chromatography, high performance liquid chromatography, gas chromatography, and capillary electrophoresis [20]. Thiol-peptide compounds, mostly reduced glutathione (GSH), which on average contain an elevated percentage of cysteine sulfhydryl residues, play a key role in heavy metal detoxification. GSH is also the direct precursor of phytochelatins (PC), the principal heavy metal-

complexing peptides of plants [21]. The PC are synthesized by the enzyme phytochelatin synthase (PCS) and have general structure of (γ-Glu-Cys)n-X, where n equals to 2–11 and X is usually Gly. They are the most important molecules for metal detoxification in plants [22]. These are enzymatically synthesized from reduced glutathione (GSH) and related thiols in a γ -glutamyl-cysteinyl transpeptidation reaction catalysed by PCS. This activity rises markedly with the increasing concentration of heavy metal. Therefore, PCS activity is used as marker of pollution metal. There are currently various methods for determining PCS activity, but electrochemical detection (ED) is an attractive alternative method for electroactive species detection, because of its inherent advantages of simplicity, ease of miniaturization, high sensitivity and relatively low cost. If electrochemical detector is coupled with effective separation method such as high performance liquid chromatography (HPLC), therefore the HLPC-ED is ultrasensitive and faster method for PCS detection [23,24].

The aim of this study was to determine a change in the content of amino acids by ion exchange liquid chromatography (IEC), in the activity of PCS determined by our previously published method [23,24] and other biochemical parameters, when two important cultivars plants, pea (*Pisum sativum* L.) as the dicot plant and maize (*Zea mays* L.) as the monocot plant, were treated with different concentrations of Pt(IV) in the form of $PtCl_4$ and demonstrate that these detection methods are suitable for revealing of the effects of platinum(IV) ions on a plant.

2. EXPERIMENTAL PART

2.1 Chemicals and pH measurement

Reduced (GSH) and oxidized (GSSG) glutathione, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, USA). Phytochelatin2 (PC2) (γ-Glu-Cys)2-Gly was synthesized in Clonestar Biotech (Brno, Czech Republic) with a purity above 90 %. HPLC-grade methanol (>99.9%; v/v) was from Merck (Dortmund, Germany) were used. Other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless noted otherwise. Stock standard solutions of the thiols (1 $mg \cdot ml^{-1}$) 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, Billerica, Mass., USA) prior to HPLC analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany).

2.2 Biological material

Growth test was performed for the evaluation of the toxic effect of platinum on experimental plants. This test is based on the exposition of seeds/corns to tested compound with evaluation of germination and further development of seedlings. Seeds of pea (*Pisum sativum L.*) and maize (*Zea mays L.*) were exposed to $PtCl_4$ of the following doses 0; 5; 10; 25; 50; and 100 μM. One hundred seeds/corns were used for each concentration. In addition, experiment was carried out in triplicates. Germination plates placed in the boxes (volume 500 ml) were used in experiments. Final volume of applied solution was 300 ml. Germination proceeded for 8 and 12 days at dark in the cultivation box

under strictly defined conditions: temperature 25±1 °C and air moisture 60±5 %. Samples were taken in the strictly defined time intervals at 8 and 12 days.

For the preparation of samples, 500 µg of fresh plant material was homogenized with 100 µl of phosphate buffer (0.2 M, pH 7.2) according to [25,26]. Briefly, plant tissues were disrupted by liquid nitrogen. Homogenized sample was quantitatively transferred into Eppendorf tube (volume 2 ml) and vortexed at 4 °C for 20 min (Vortex-2 Genie, USA). Subsequently, homogenate was ultrasonicated for 10 min at 50% power (Bandelin Sonorex Digital 10P Ultrasonic bath) and centrifuged (15 min at 4 °C, 16.400 rpm; Eppendorf 5402, USA). Supernatant was collected into Eppendorf tubes (volume 1.5 ml) and immediately used for the measurements.

2.3 Total platinum content determination

Shoots and roots parts of pea and maize were dried at 45 °C for 24 hours in thermostat (UNB 300, Memmert, Germany). Samples of plants (100 mg) were placed into glass vials MG5 with 700 µl of nitric acid (60%, *w*/*w*) and 300 µl peroxide (30%, *v*/*v*). Prepared samples were sealed and placed into the rotor 64MG5 (Anton-Paar GmbH, Austria) and the microwave digestion was carried out under the following conditions: power 150 W – 10 min., power 2 200 W – 20 min., cooling power 0 W – 10 min. For subsequent electrochemical measurements, 985 µl of acetate buffer and 15 µl of mineralized sample was pipetted into Eppendorf tubes [27,28].

Determination of platinum were performed with a 797 VA Computrace instrument connected to 813 Compact Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and a platinum electrode was auxiliary. 797 VA Computrace software (Metrohm) was employed for data processing. Software GPES 4.9 supplied by Metrohm was employed for smoothing and baseline correction. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%), saturated with water for 120 s. Platinum was determined by adsorptive stripping differential pulse voltammetry in the presence of 2 ml of 0.36 M sulphuric acid, containing 0.24 ml of hydrazine (10 mM) and 0.01 ml of formaldehyde (37% aqueous solution). After 2 min purging of the sample, the Pt(II)-formazone-complex, which is formed in the electrolyte solution, is accumulated for 15 s at the hanging mercury drop electrode (HMDE) (0 V vs. Ag/AgCl, with stirring). Then the potential was scanned from -0.5 to -1.2 V at a sweep rate of 10 mV/s, and the catalytic hydrogen wave at -0.9 V is measured. Volume of injected sample: 20 µl, volume of measurement cell 2 ml (20 µl of sample + 1980 µl electrolyte). Other parameters of method were: modulation time 0.057 s, interval time 0.1 s, step potential 1.95 mV, scan rate 10 mV/s, modulation amplitude 49.5 mV.

2.4 Ion exchange chromatography

A dried sample 0.5 or 25mg was dissolved in the presence of 500 µl 6 M HCl. Acid hydrolysis was carried out in a microwave reactor (Anton Paar, Germany). The experimental parameters were as follows: power 80 W, Ramp 15 min., Hold 90 min., Max 120 °C, Max pressure 25 bar, Rotor-XF-100-

6 (Anton Paar, Germany). Then sample was analysed by ion exchange chromatography (AAA-400, Ingos, Czech Republic).

For aminoacid determination we used an ion-exchange liquid chromatography (Model AAA-400, Ingos) with post column derivatization with ninhydrin and VIS detector was used. A glass column with inner diameter of 3.7 mm and 350 mm in length was filled manually with a strong cation exchanger in sodium cycle LG ANB (Ingos) with approximately 12 μm particles and 8% porosity. The column was tempered within the range from 35 to 95 °C. The elution of the amino acids of interest was carried out with the column temperature set to 74°C. A double channel VIS detector with inner cell of volume 5 μl was set to two wavelengths, 440 and 570 nm. A solution of ninhydrin (Ingos) was prepared in 75% v/v methylcelosolve (Ingos) and in 2% v/v 4 M acetic buffer (pH 5.5). Tin chloride (SnCl2) was used as a reducing agent. The prepared solution of ninhydrin was stored under an inert atmosphere (N_2) in the dark at 4 °C. The flow rate was 0.25 ml/min. and the reactor temperature was 120 °C.

2.5 Determination of total thiol compounds content

Spectrophotometric measurements of thiol compounds content were carried out using an automated chemical analyser BS-400 (Mindray, China). It is composed of cuvette space tempered to 37±1 °C, reagent space with a carousel for reagents (tempered to 4±1 °C), sample space with a carousel for preparation of samples and an optical detector. Transfer of samples and reagents is provided by robotic arm equipped with a dosing needle (error of dosage up to 5% of volume). Cuvette contents are mixed by an automatic mixer including a stirrer immediately after addition of reagents or samples. Contamination is reduced due to its rinsing system, including rinsing of the dosing needle as well. Ellman's spectrophotometric method was used for the determination of sulfhydryl (-SH) groups. Ellman's reagent (277 μl, reagent 1, R1 – 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 R1 itself – blank – and mixture after 10-min incubation were used for the calculation of total -SH content [29,30].

2.6 High performance liquid chromatography with electrochemical detection

Chromatographic analysis was determined using 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 nm particles, Agilent Technologies, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector includes one flow cell (Model 6210, ESA, USA). The 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 [31-33].

2.6.1 Preparation of samples for the determination of GSH and GSSG using HPLC-ED

Approximately 0.5 g sample of shoots or roots parts of pea and maize was frozen by liquid nitrogen and subsequently homogenized with 1000 µl of phosphate buffer (pH 7.0). The homogenized samples were transferred into Eppendorf test tube (2 ml, Germany) and vortexed (Vortex-2 Genie, Scientific Industries, USA) for 10 min. Finally, samples were centrifuged (16 400 rpm, 15 min, 4 °C; Universal 32 R centrifuge, Hettich-Zentrifugen GmbH, Germany). Supernatant was filtered through a membrane filter (0.45 µm Nylon filter disk, Millipore, USA) and used for chromatographic analysis.

2.6.2 Preparation of samples for the determination of PCS using HPLC-ED

Estimation of the PCS activity was carried out according [23]. The plant material in fresh weight 0.050 g of pea or maize was ground in mortar under liquid nitrogen for 2 min. Then 250 µl of 20 mM phosphate buffer (pH 7.5) and 1 mM tris(2-carboxyethyl)phosphine (TCEP) was added. Subsequently the homogenate was centrifuged (16400 g, 20 min, 4 °C). Supernatant was divided into two aliquots (control and reaction mixture). Control 50 µl was mixed with 50 µl of 20 mM phosphate buffer (pH 7.5) and 1 mM TCEP. Reaction mixture was mixed 50 µl of supernatant with 50 µl of mixture of 5 mM GSH in 20 mM phosphate buffer (pH 7.5) and 1 mM TCEP. Then all variants were incubated at 35 °C at 300 rpm using termomixer (Eppendorf, Germany). After that 2 µl of 5 mM 5-sulfosalycilic acid was added to stop the reaction. After the stopping the reaction, HPLC-ED analysis was carried out. The amount of synthesized PC_2 correlated with PCS activity [23,34].

3. RESULTS AND DISCUSSION

Our experiments were focused on studying of change in the content of amino acids determined by IEC and PCS determined by HPLC-ED in both cultivars exposed to different doses of Pt(IV) with respect to reveal the effect of this noble metal on the used plants and to demonstrate that the selected detection methods are suitable for determining the effects on plants.

3.1 Platinum accumulation

Fresh weight of pea and maize plants at the 8^{th} and 12^{th} day of exposition to Pt(IV) was significantly reduced in comparison with control unexposed plants (data not shown). Content of Pt(IV) accumulated in experimental plants was evaluated electrochemically. This method is low-cost and especially undemanding compared to others. In addition, it is highly applicable for the determination of metal content in biological matrixes of both plant/animal origins. The high sensitivity belongs to the next advantages of electrochemical methods [8,35-40]. Signals corresponding to Pt(IV) detected at potential of – 0.95 V were well developed. We constructed dependence of Pt(IV) peak height on its concentration within the range from 0.1 to 50 ng/ml (Fig. 1A). The height of the peak was linearly

proportional to concentration of Pt(IV). The dependence obtained was strictly linear (y = 3.6282x, R^2 = 0.9935, Fig. 1A). Fig. 1B shows peak height corresponding to the Pt(IV) content in fresh root samples of maize treated with 10 µM of Pt(IV), demonstrating be a good method for determination of Pt(IV) in these biological samples. Fig. 2 shows accumulation of Pt(IV) in experimental plants. It is well evident that Pt(IV) is accumulated in the concentration- and time-dependent manners.

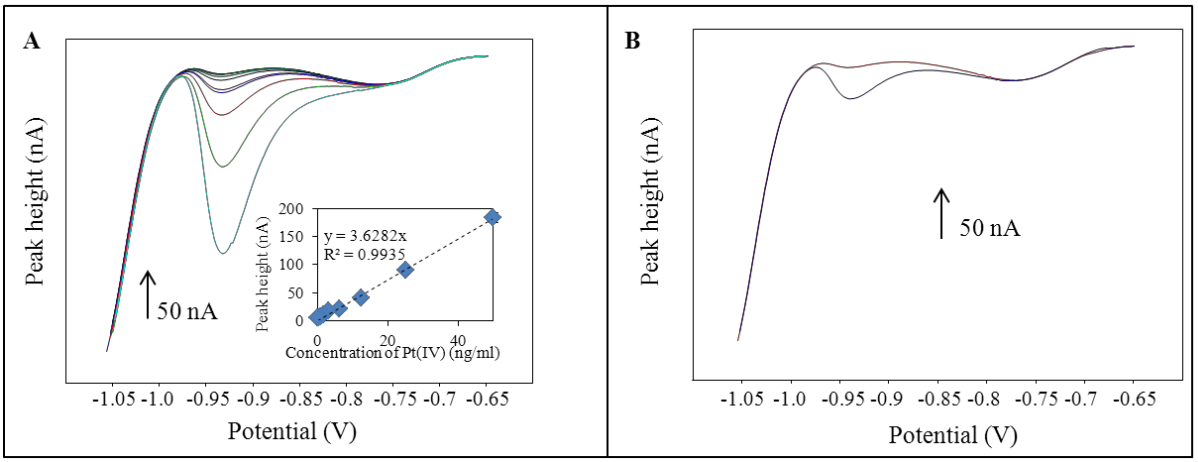

Figure 1. (**A**) DP voltammograms of Pt(IV) within concentration range from 0.1 to 50 ng/ml; in inset: calibration curve measured within concentration range from 0.1 to 50 ng/ml. (**B**) DP voltammograms of roots of maize treated with 10 µM (red line) or 100 µM (blue line) Pt(IV), both diluted 10000 ×. For other experimental details see in Section 2.3.

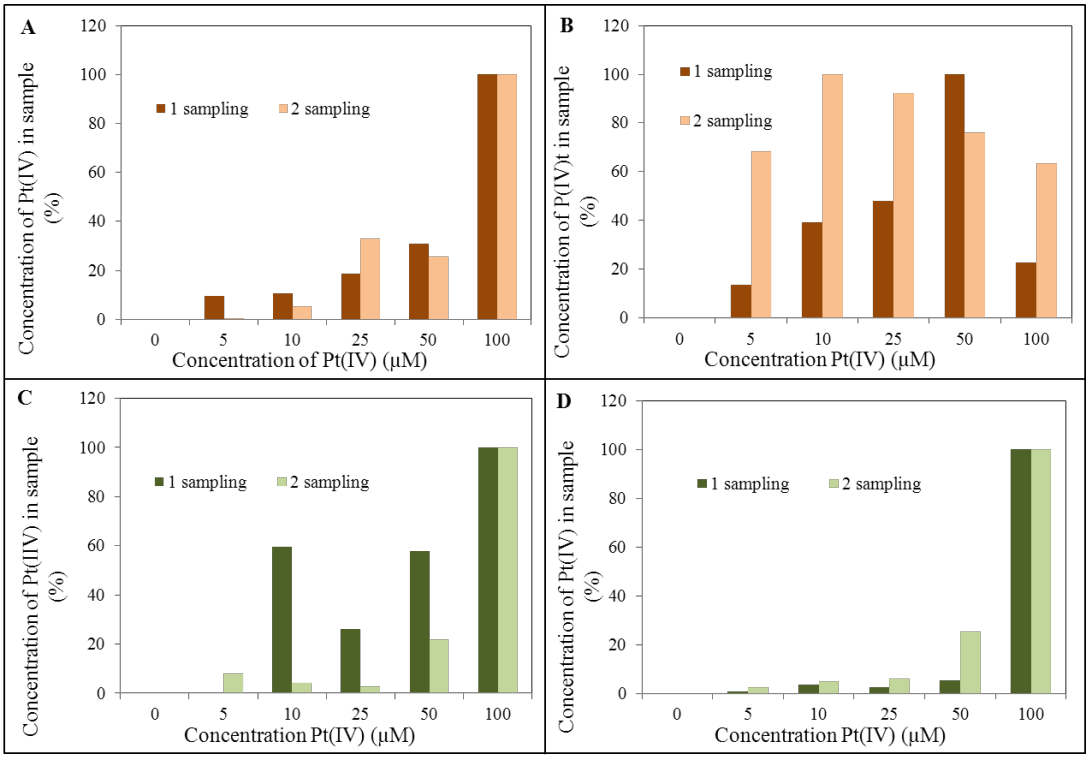

Figure 2. Concentration of total platinum in (**A**) maize roots, (**B**) pea roots, (**C**), maize shoot and (**D**) pea shoot. The plants were treated with $PtCl_4$ within the concentration range 0; 5; 10; 25; 50; and 100 µM. The plants were harvested after 8 (1st sampling) or 12 (2nd sampling) days of the treatment. The platinum was determined using differential pulse voltammetry. For other experimental details see in Sections 2.2 and 2.3.

Platinum may be transported from roots to the shoot, again in the time- and concentration-dependent manner. Significant accumulation of Pt(IV) in maize roots was well evident especially in the highest applied concentrations 50 and 100 μM (Fig. 2A) in both times. Increase of Pt(IV) content in the roots of experimental pea plants was well evident especially in the low and higher Pt(IV) concentrations (10, 25, 50 and 100 μM) at the 8th day of the exposure (Fig. 2B), while the Pt(IV) contents increased in plants treated with 5 μM Pt(IV) and showed no significant change during treatment. In shoots, maize and pea plants showed a higher content of Pt(IV) after treatment with 50 and 100 μM at 12th day of the exposure (Figs. 2C and D). However, our study showed different contents of Pt(IV) depending on the species. The shoots of maize plants showed higher Pt content that pea plants and the roots of pea plants showed less Pt(IV) content in plants treated with higher dose of Pt(IV) than maize plants.

3.2 Determination of amino acid content

In this study, ion-exchange chromatography followed by post-column derivatization with nynhidrin was used for determination of amino acid content in roots and shoots of both plants. This method is robust and low cost [41]. In Figs. 3 and 4 there is total amino acid contents (Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, His, Lys and Arg) determined in maize and pea plants roots and shoots treated with different concentration of Pt(IV) in both times of the sampling. In maize and pea plants, the total amino acid content was the highest in plants treated with 25 μM Pt(IV) at 8th and 12th days of treatment. However, total amino acid contents decreased in plants treated with 50 and 100 μM. In roots of maize plants (Figs. 3A and B), Asp, Glu, Pro, Ile, Leu, Lys and Arg contents increased by the effect of Pt(IV), the remaining amino acids showed no significant change. In spite of the fact that Asp, Ser, Glu, Pro and Lys showed an increase in shoots of maize plants at 8th days (Fig. 3C), this increase was lower after 12th days long treatment (Fig. 3D). Between all amino acids studied, Pro was the most abundant in 8th and 12th days, 21% and 23%, respectively in plants treated with 25 μM Pt(IV). Pro is an important osmoprotectant when plants are exposed to abiotic stresses, as such heavy metal. Different levels of protein and amino acids and especially a higher level of Pro can be therefore directly correlated with the degree of metal tolerance [42,43]. The possibility of Pro involvement in the chelation of metal ions was studied by Sharma et al., with a Cd specific electrode, and revealed that the proline-dependent enzyme protection was based on a reduction of free metal ion activity in the assay buffer due to formation of a metal-Pro complex [19]. As we found in this study, His also increased with Pt(IV) concentrations. Kramer et al. showed that the free His is a metal chelator in plants [44].

Concerning pea plants, Asp, Cys and His was more abundant in 8th day of treatment in plants treated with 25 μM Pt in roots (22, 13 and 20%, respectively, Figs. 4A and B); and Asp, Pro and Cys in shoots of pea plants (Figs. 4C and D). Pt(IV) toxicity also perturbed amino acid metabolism in plants and depends on which plants are monocotyledonous or dicotyledonous, and shoot or root. Considering content of Cys, it is likely that PCs are involved in Pt(IV) detoxification in this specie [45,46].

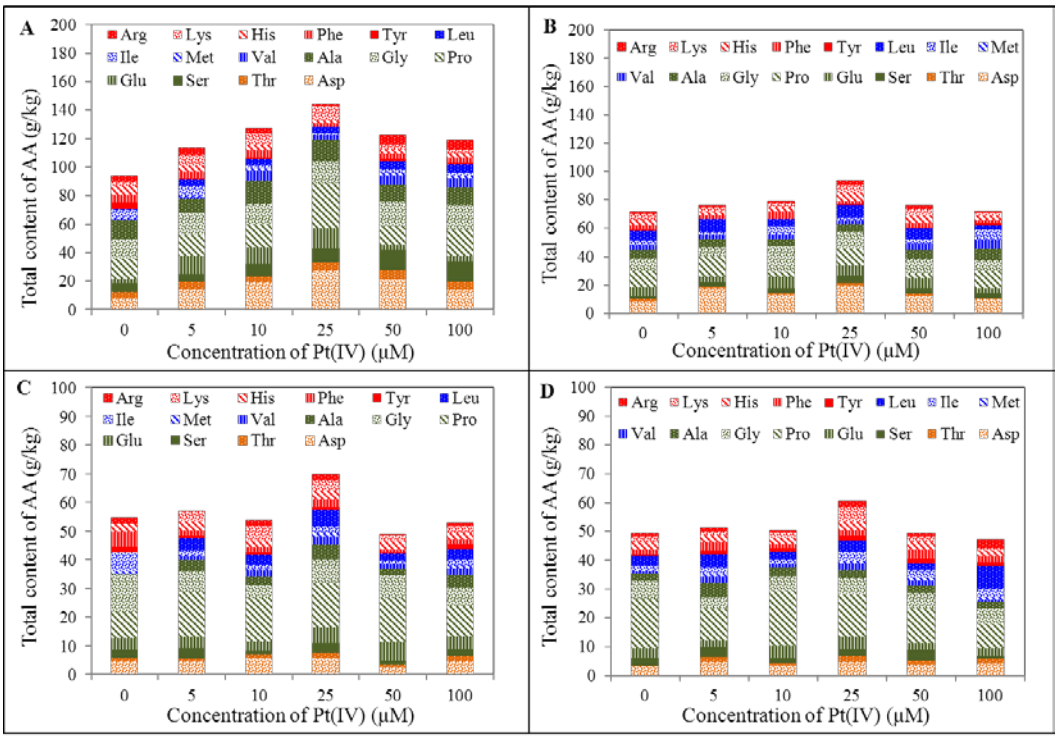

Figure 3. Aminoacids content in treated maize roots harvested (**A**) after 8 days (1st sampling), or (**B**) after 12 days (2nd sampling). Aminoacids content in treated maize shoots harvested (**C**) after 8 days (1st sampling), or (**D**) after 12 days (2nd sampling). For other experimental details see in Section 2.5.

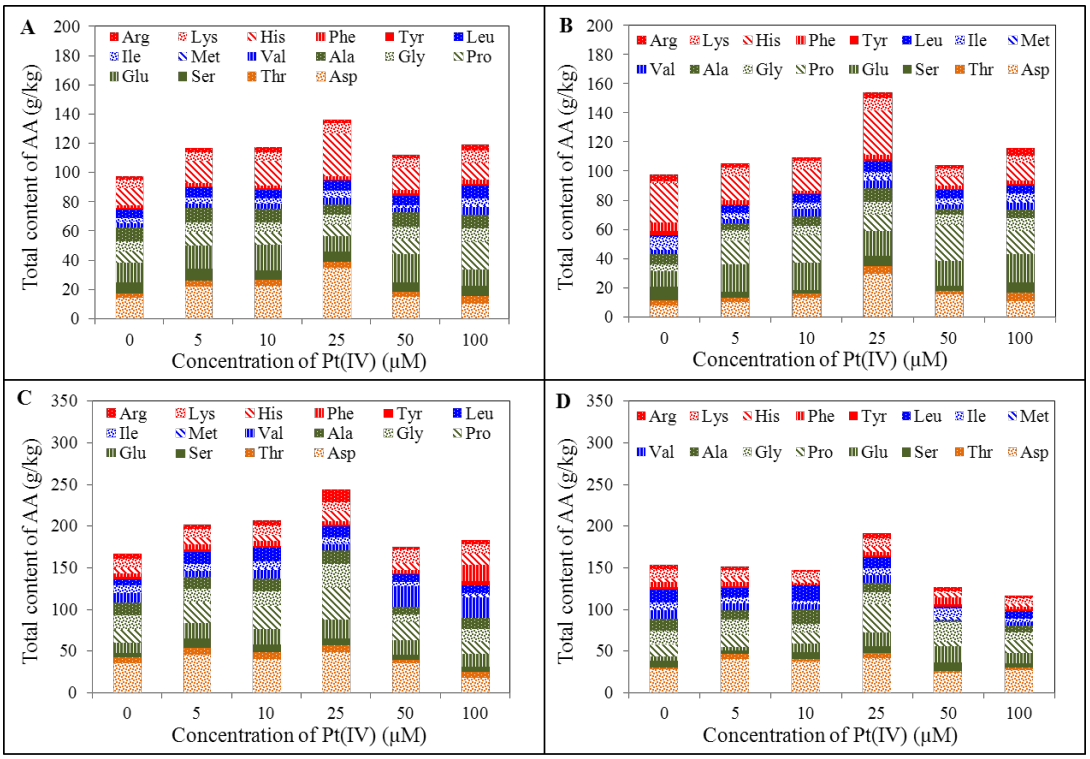

Figure 3. Aminoacids content in treated maize roots harvested (**A**) after 8 days (1st sampling), or (**B**) after 12 days (2nd sampling). Aminoacids content in treated maize shoots harvested (**C**) after 8 days (1st sampling), or (**D**) after 12 days (2nd sampling). For other experimental details see in Section 2.5.

3.3 Determination of GSH and GSSG

High performance liquid chromatography with electrochemical detection (HPLC-ED) was used for the determination of reduced (GSH) and oxidized (GSSH) glutathione. In addition, GSH/GSSG ratio was determined as an important biochemical marker [47]. Generally, thiols and especially glutathione are involved in the detoxification of heavy metals including PGEs. Klueppel et al. cultivated English ryegrass (*Lolium perenne* L.) in the soil enriched by $[Pt(NH_3)_4](NO_3)_2$. The highest Pt(IV) levels were determined in roots; in addition, the majority of Pt was found in the bound form with the sulfhydryl groups-containing compounds, such as phytochelatins and low-molecular peptides [3,48].

Primarily, we observed effect of Pt(IV) on the glutathione levels in shoots and roots of pea and maize plants. It is well evident that the application of the highest Pt(IV) concentration led to the almost two fold increase in GSH level compared to control by untreated pea plants after 8^{th} days of exposure (not shown). After 12^{th} days of exposure, the highest increase of GSH was determined in the case of two highest Pt(IV) concentrations (50 and 100 μM, for 80% in the shoots compared to control). The most significant increase in GSH in roots was determined in plants treated with 25, 50 and 100 μM.

Different tendency was determined in the maize plants. GSH levels were significantly reduced with the increasing Pt(IV) concentration (in concentration-dependent manner) after 8 days of exposure. Longer exposure (12^{th} days) caused significant increase in GSH only in the two low Pt(IV) concentrations as 10 and 25 μM in shoots. In the case of roots, GSH increase was determined in plants treated with the two highest Pt(IV) concentrations (50 and 100 μM) after 8 days of exposure, later especially under the exposition by 10 μM Pt(IV) concentration.

In pea plants, the most significant GSSG increase was determined after 8 days of exposure to the highest Pt(IV) concentration tested (100 μM). This increase was 100% compared to control. Later, the two fold increase compared to control was determined in plants treated with 50 μM concentration after 12 days of exposure. In maize plants, shoot showed moderate increase in GSSG compared to control. In the lowest concentration (5 μM), the increase was 47% at 8^{th} day compared to control, 79% at 12^{th} day.

The ratio between GSH and GSSG serves as an important stress parameter. The GSH/GSSG rate increased in the maize shoots treated with 25 μM at both days. In the case of roots, the increase in GSH/GSSG rate was determined at 8th day of the Pt(IV) exposure in the highest Pt(IV) concentration, later (12th day) the moderate decrease (for 30%) was determined. Decrease in the GSH/GSSG rate at 12th day of the exposure may be explained by the depletion of GSH as a protective mechanism due to exposure to platinum.

3.4 Determination of PCS

To understand the metabolism of thiol compounds and formation of peptides called phytochelatins, PCS activity determination is crucial as we published elsewhere [23,34]. Presented dependencies (Fig. 5) shows the PCS activity (taken as $fkat_{PCS}$ = $fmol_{PC2}$/second) in the percents with respect to maximum content (25 μM of Pt(IV)) in shoots and roots treated with different Pt(IV) concentration in both times. The PCS activity increased markedly with the increasing concentration of

Pt(IV) in both roots and shoots of maize and pea plants. The highest activity of PCS was interestingly observed both plants while treated with 25 µM of Pt(IV) (100%). The plants treated with concentrations exceeding 25 µM showed overall slightly decreasing PCS activity at 8th and 12th days of treatment but still was higher than control (0 µM Pt(IV)) (Fig. 5). The roots of maize plants showed higher PCS activity (Figs. 5A and C) in 0, 5 and 10 µM of Pt(IV) (50-60%) than shoots maize (10-30%) at 8th and 12th day's of treatment. However, PCS activity was similar in 25, 50 and 100 µM of Pt(IV) in both plants. When comparing both species, the activity of PCS in root was similar in both times, while roots and shoots of pea showed a reduction of PCS activity at 50 and 100 µM of Pt(IV) at both sampling times (Figs. 5C and D). It was more pronounced on 12th day (Fig. 5D) by pea shoot and opposite by pea roots (Fig. 5B). This is the main finding that the difference between trends of the increasing PCS activity is very similar by different sampling time by maize root and shoot. There is quite different situation in pea plant. The literature contains many contradictions on the relationship between heavy-metal tolerance and PC synthesis and PCS activity [49-55]. Ours results may be related to differences in the strategies to uptake Pt(IV) between both cultivars. The different distribution of Pt(IV) among the tissues could explain the differences in sensitivity to this toxic metal in higher concentrations between maize and pea, and the increases in PCS activity. These data confirmed that HPLC with electrochemical detection is suitable for determination of PCS activity in both species of plants when treated with Pt(IV).

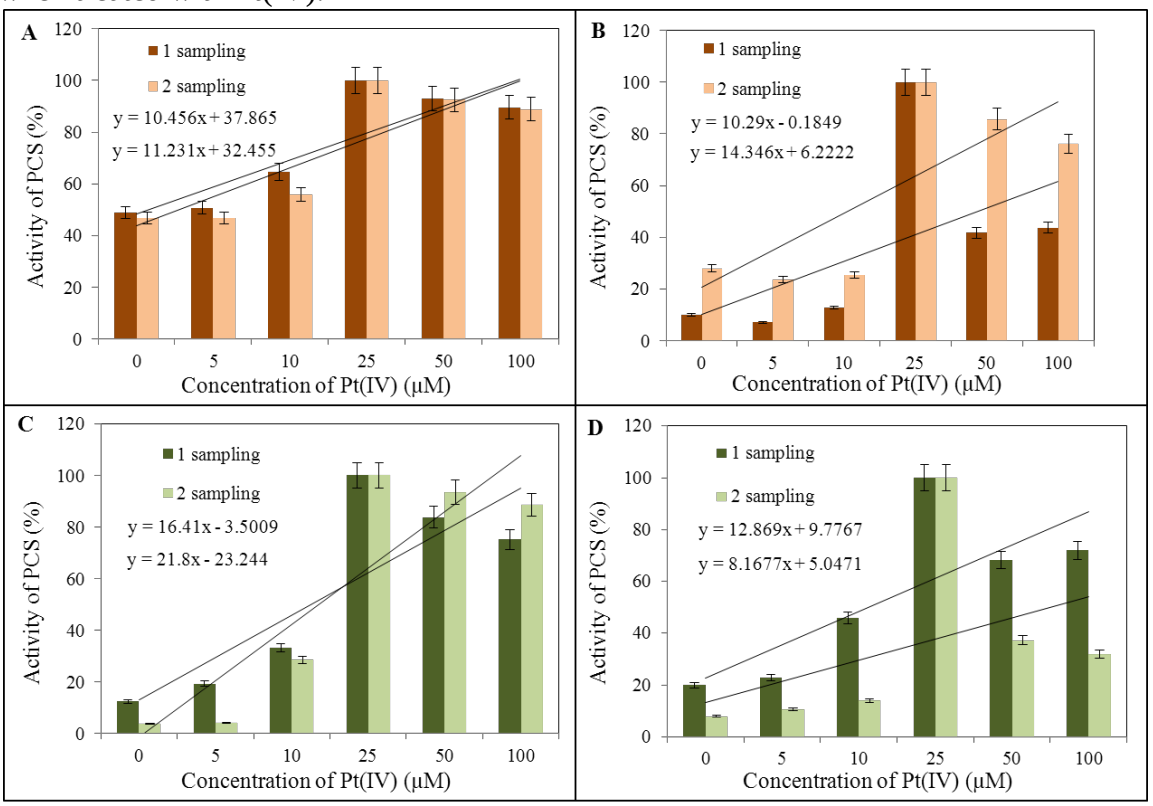

Figure 5. Phytochelatin synthase (PCS) activity dependence on applied Pt(IV) concentration (for sampling 1 – 8th day of exposure and sampling 2 – 12th day of exposure) measured in roots of (**A**) maize and (**B**) pea plants, and shoots of (**C**) maize and (**D**) pea plants. The overall trends are expressed as slopes from regression equations. For other experimental details see in Section 2.7.

3.5 Determination of total thiol compounds against PCS activity

Ellman's method provides rapid and simple colorimetric determination of total thiol compounds [34]. Thiol compounds in the plant tissue relates to activity of PCS. Thus we decided to compare total thiol compounds, which were detected using Ellman´s reagent, to PCS activity data presented in the Chapter 3.4. For overall consideration of the data we decided to provide the slopes from dependences of each determined parameters (Ellman´s reagent or PCS activity) on the applied concentration of Pt(IV). Owing to the difference of the both organisms we compared only basic trends between 8th day and 12th day of exposure. All values of first point represented by 8th day of exposure were normalized to 0 and second point represented by 12th day of exposure was moved by the same constant as the first point. The overall slope values were than normalized on the percentage values because of difference of data format from both used methods (Ellman´s reagent – mM of equivalent cysteine, or PCS activity – $fkat_{PCS}$). The slopes are demonstrating the change of the observed parameter and thus the sensitivity of the part of plant to determined parameter. Finally we obtained line segments for pea and maize by roots and shoots for different samplings 1(8th day) and 2 (12th day). It is shown in Fig. 6A that maize roots and shoots were the obviously the same sensitive as shoots and roots of pea in thiol content but different situation was observed at PCS activity (Fig. 6B) where the difference between sampling two was both positive but shoots were more sensitive. The presented data proves that maize has complex mechanisms for detoxification of platinum and the roots and shoots mechanisms are almost similar.

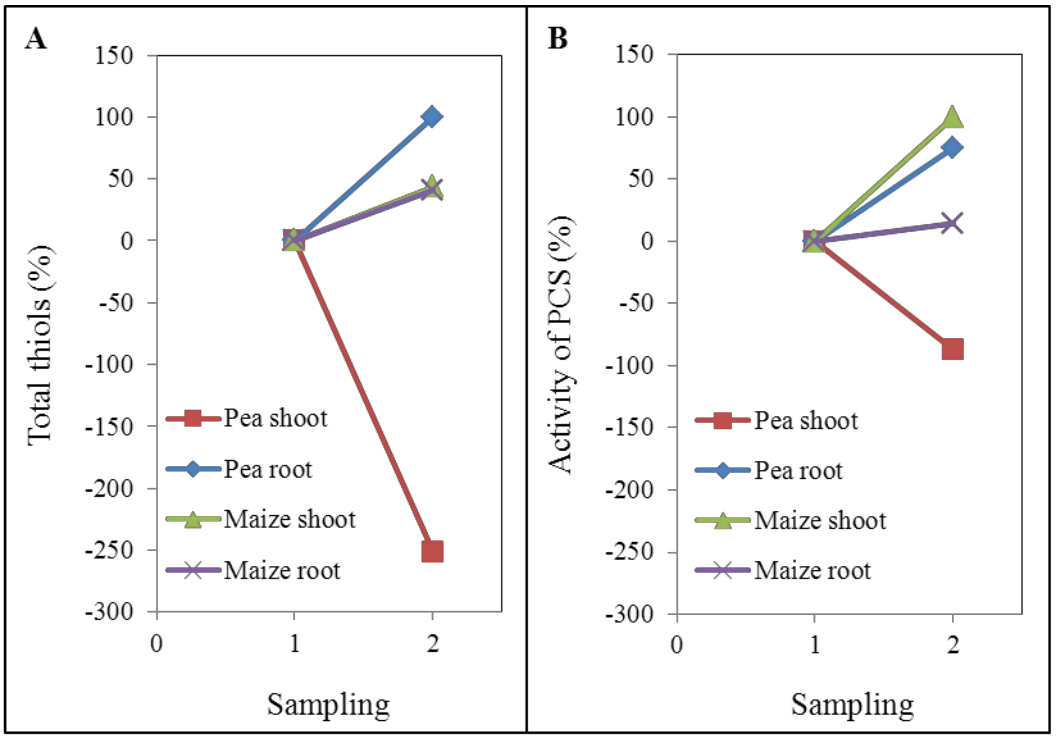

Figure 6. The comparison of the trends of slopes of dependences of (**A**) total thiols content and (B) activity of phytochelatin synthase measured in roots and shoots of maize and pea plants treated with 0; 5; 10; 25; 50; and 100 µM of $PtCl_4$ on sampling time (1 – 8th day of exposure and 2 – 12th day of exposure).

Different manners was observed in pea where roots demonstrated the highest sensitivity to increasing concentration of Pt(IV) thanks to positive slope but this was rapidly decreased in pea shoot, which indicates that plants were not willing to rapid synthetize thiols (Fig. 6A). The same situation is confirmed by PCS activity values (Fig. 6B). From the presented comparison it is well evident that maize protects itslef against the increasing concentration of Pt(IV) similarly in the level of both parts of plant but the pea plant is mostly using the roots compared to shoots.

4. CONCLUSIONS

Our study shows that electrochemical methods such as differential pulse voltammetry (DPV) and HPLC-ED are fast and suitable methods for the determination of the toxic effects inducing by Pt(IV) in different higher plants. Pt(IV) are currently being intensively studied in plants due to the increase of this element in environment. The high concentration of Pt(IV) induce stress in both studied plants, activating protective mechanisms. This study represents the first investigation from two different species, monocot and dicot, in the context of Pt(IV) detoxification. Further studies in this direction would reveal other components involved in metal detoxification mechanism of maize and pea. Thus, theses electrochemical method represents suitable tool that significantly contributes to the possibilities of analytical purposes of such applications.

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