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Effects of Cadmium(II) Ions on Early Somatic Embryos of Norway spruce Studied by Using Electrochemical Techniques and Nuclear Magnetic Resonance

Dalibor Huska [1], *Ondrej Zitka* [1], *Olga Krystofova* [1], *Vojtech Adam* [1], *Petr Babula* [2], *Josef Zehnalek* [1], *Karel Bartusek* [3], *Miroslava Beklova* [4], *Ladislav Havel* [5] *and Rene Kizek* [1, *]

[1] Department of Chemistry and Biochemistry, and [5] Department of Plant Biology, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic
[2] Department of Natural Drugs, Faculty of Pharmacy, and [4] Department of Veterinary Ecology and Environmental Protection, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences, Palackeho 1-3, CZ-612 42 Brno, Czech Republic
[3] Institute of Scientific Instruments, Academy of Sciences of the Czech Republic, Kralovopolska 147, CZ-612 00 Brno, Czech Republic
[*]E-mail: kizek@sci.muni.cz

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Studying of metal ions effects on plant cells and whole plants are still topical. Early somatic embryos (ESEs) of spruce represent the unique plant model system, which can be used to study of various types of environmental stresses including metal ions under well controlled experimental conditions. In the paper nuclear magnetic resonance (NMR) and electrochemical techniques were employed to investigate the effect of cadmium(II) ions (0, 50, 150, 250, 500 and 1,000 μM) on early somatic embryos (ESEs) of Norway spruce, clone 2/32. The ESEs were treated with cadmium(II) ions for fourteen days. The increase in cluster area and viability were determined by image analysis and fluorescein diacetate/propidium iodide double staining, respectively. Growth characteristics revealed the ESEs treated with cadmium-ethylene diamine tetra-acetic acid (Cd-EDTA) showed an increase in growth-inhibiting effect due to cadmium(II). Furthermore, total thiol content and certain low molecular mass plant thiols (cysteine, reduced glutathione, oxidized glutathione and phytochelatin2) were determined using Brdicka reaction and liquid chromatography with electrochemical detection, respectively. The highest level of phytochelatin2 synthesis was determined in ESEs treated with 50 μM Cd-EDTA. The average content of phytochelatin2 varied from 850 to 1800 nM/g FW. We also attempted to determine changes in water content in the ESEs treated with cadmium(II) ions using NMR. The highest increase in water content was observed at clusters treated with 250 μM Cd-EDTA. The clusters treated with higher cadmium concentrations (more than 250 μM) demonstrate only slow increase in the water content. Water metabolism at woody plants and their embryos exposed to cadmium(II) ions has not yet been described. Based on the results obtained it can conclude that the cells of embryonic clusters attempted to excrete cadmium(II) ions or, more precisely, decrease its

concentration within the cluster. This phenomenon can be considered as one of the detoxification mechanisms of plants use to deal with the presence of heavy metal contamination. The escalation of water uptake continuously progress as long as the thiols are synthesized.

Keywords: Plant; Norway Spruce, Somatic Embryos; Water; Thiol; Nuclear Magnetic Resonance; Voltammetry

Abbreviations: CCD charge-coupled device; Cys cysteine; DPV differential pulse voltammetry; EDTA ethylene diamine tetra-acetic acid; ESE early somatic embryo; FDA fluorescein diacetate; GSH reduced glutathione; GSSG oxidised glutathione; HPLC high performance liquid chromatography; NMR nuclear magnetic resonance; PCs phytochelatins; PI propidium iodide

1. INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is a non-invasive imaging tool used by many to study molecules [1,2]. Proton NMR, ^{1}H, is a common application of nuclear magnetic resonance in NMR spectroscopy used today [3]. NMR spectroscopy measures the interaction of an oscillating radio-frequency electromagnetic field with a collection of nuclei immersed in a strong external magnetic field to study molecules [4]. While NMR is frequently used in medicine, it is also used in biological, biochemical and chemical research. In plant biology, NMR is utilized to study water and mineral compounds transported within a plant [5-12], the determination of plant metabolites [13-17], the investigation of cellular processes [18-20] and for examining the growth and development of plants [21-26]. NMR use also monitors water changes in early somatic embryos of spruce [27]. Early somatic embryos of spruce represent unique plant model systems used in investigating the influence of environmental stress on woody plant species [28-31]. Plants evolved protective mechanisms which enable them to control the levels of metal ions, both essential and toxic pollutants of the environment [32], in their system. Thiols such as glutathione and phytochelatins partake in plant stress response to heavy metals toxicity [33-36]. Moreover, thiols act as scavengers of reactive oxygen species, produced by heavy metals [37].

To investigate the effect of cadmium(II) ions on ESEs, six concentrations of cadmium(II) ions (50, 100, 150, 250, 500 and 1,000 μM) were chosen and compared with untreated, controlled ESEs. Natural cadmium(II) concentrations in water is below the indicated levels, however its ion content in soil can be greater then 1 mg/kg [38]. Based on previously published results [27,29] ESEs are highly resistant to the presence of heavy metals. Therefore a wide range of cadmium(II) concentrations including 1,000 μM were utilized to study morphological changes within a short period of time. Nuclear magnetic resonance was employed to investigate the effects of cadmium(II) ions on ESEs. Additionally, the level of protective thiols in treated embryos was determined using electrochemical techniques.

2. MATERIAL AND METHODS

2.1. Chemicals and pH measurements

Phytochelatin2 (PC2, $(\gamma\text{-Glu-Cys})_2$-Gly was synthesized in Clonestar Biotech (Brno, Czech Republic) with a purity above 90%. HPLC-grade methanol (>99.9%; v/v) from Merck (Dortmund, Germany) was used. Fluorescein diacetate (FDA), propidium iodide (PI) and all other used reagents of ACS purity were purchased from Sigma Aldrich (Sigma Aldrich Chemical Corp.,USA) unless noted otherwise. All solutions were prepared using deionised water (18.2 MΩ, Millipore, France). Culture media were prepared using plant cell culture chemicals purchased from Duchefa Biochemie BV (Haarlem, The Netherlands). Pipettes, tips and micro test tubes were purchased from Eppendorf (Hamburg, Germany). The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by the personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0 – 14/3 mol/dm3 KCl) was calibrated by set of WTW buffers (Weilheim, Germany).

2.2. Plant material and cultivation conditions

Early somatic embryos (ESEs) of the Norway spruce (*Picea abies* /L./ Karst.), clone 2/32 from the collection of the Department of Plant Biology of Mendel University in Brno, was used. The embryonic culture was maintained on a semisolid (half-strength LP medium Gelrite Gellan Gum, Merck, Germany) with modifications in accordance with Havel and Durzan [39]. The concentration of 2,4-dichlorofenoxyacetic acid and N^6-benzyladenine was 4.4 and 9 μM, respectively [40]. The pH value was adjusted to 5.7-5.8 before autoclaving (121°C, 100 kPa, 20 min). The organic part of the medium, excluding saccharose, was sterilized by ultrafiltration through a 0.2 μm polyethylensulfone membrane (Whatman, Puradisc 25 AS). Sub-cultivations of stock cultures were carried out at two week intervals. The stock and experimental cultures were maintained in a cultivation box in the dark at 23±2°C. The clusters of embryos were cultivated on the semisolid medium without cadmium(II) addition for 14 days and then transferred onto cultivation medium with an addition of cadmium(II) chelate (Cd-ethylene diamine tetra-acetic acid) at concentrations of 0 (only EDTA without Cd(II) ions), 50, 150, 250, 500 and 1,000 μM. A stock solution of Cd-EDTA was prepared by mixing $Cd(NO_3)_2$ with ethylene diamine tetra-acetic acid (EDTA) in a 1:1 molar ratio and stirred at 50°C for 1 h. The filter-sterilized Cd-EDTA complex was added to the autoclaved culture medium [41]. The samples were collected at the very beginning of the experiment and at 3^{rd}, 6^{th}, 8^{th}, 10^{th} and 14^{th} day of the treatment. All experiments were carried in triplicates.

2.3. Computer image analysis

We used a charge-coupled device (CCD) camera for the observation of growth of spruce ESEs. The images of ESEs clusters were recorded at the beginning of the treatment and in certain intervals according to the end of the treatment. The data were converted to a digital image in the Grab–IT (version 1.3) program. The area size of ESEs clusters in digital images was calculated by program

Image–Pro Plus, (Sony, ver. 1.3). The data were processed in Excel (Microsoft). For other details reported on IA see in Ref. [29].

2.4. Photography of ESEs in bright field

ESEs (about 0.1 mg) were collected by a scalpel and transferred onto microscopic slides. The ESEs was spread and superimposed by cover glass. The sample was then placed and examined under a microscope (Olympus AX 70, Japan). The images were forty times magnified by digital camera Olympus 4040 and converted to digital image in the Grab–IT (version 1.3) program.

2.5. Double staining

Modified double staining with FDA and PI for the determination of the viability of ESEs was used [29]. FDA causes green fluorescence in viable cells because the non-fluorescent FDA easily penetrates into viable cells where it is hydrolyzed to a brightly fluorescent fluorescein ($\lambda_{excitation}$= 490 nm and $\lambda_{emission}$ = 514 nm) that does not diffuse out readily through the cytoplasmatic membrane. The red fluorescence of PI ($\lambda_{excitation}$ = 536 nm and $\lambda_{emission}$ = 620 nm) in cells shows these cells are dead since this compound is unable to pass through the functional cytoplasmatic membrane. ESEs (~1 mg) were harvested and diluted with water with a final volume of 50 µl. The stock solutions of PI and FDA were added to a final concentration of 20 µg/mL and 1 µg/mL respectively. After 5 min of incubation at room temperature, the percentage of dead (red-stained cells) and viable cells (green-stained cells) were evaluated using an Olympus AX 70 fluorescence microscope with an Olympus cube U-MWU coupled with the digital camera. The percentage quantification of red (dead cells) and green areas (viable cells) in compact embryonic group of single embryos were determined using method IA (Image–Pro Plus was used, ver. 1.3, Sony).

2.6. Electrochemical determination of total thiols content

Electrochemical measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). 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 glassy carbon electrode was auxiliary electrode. The supporting electrolyte (1 mM $[Co(NH_3)_6]Cl_3$ and 1 M ammonium buffer; NH_3(aq) and NH_4Cl, pH 9.6) was changed after five measurements. The DPV parameters were as follows: sample volume 1 µl, supporting electrolyte volume 1.9 ml, time of accumulation 120 s, initial potential of –0.7 V, end potential of –1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude -250 mV, E_{ads} = 0 V. All experiments were carried out at temperature 4 °C (Julabo F12, Germany). For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed. The optimization of the experimental conditions was published by [42].

ESEs samples were prepared according to procedure published in [27]. Briefly, ESEs (~100 mg) were harvested and diluted with 0.066 M phosphate buffer (pH 7.0) with a final volume of

1,000 µl. The mixture was prepared by hand-operated homogenizer ULTRA-TURRAX T8 (IKA, Germany) at 25,000 rpm for 3 minutes. The homogenate was transferred to a new test-tube. The mixture was further homogenised on a Vortex–2 Genie (Scientific Industries, New York, USA) at 4 °C for 30 min. The homogenate was centrifuged (14 000 *g*) for 30 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). Prior to analysis the supernatant was filtered through a membrane filter (0.45 µm Nylon filter disk, Millipore, Billerica, Mass., USA).

2.7. Nuclear magnetic resonance

Tomography working on magnetic resonance-base was used to study of the changes of ^{1}H nucleus spin density in treated ESEs. Experiments were performed on a home-built MR imaging system equipped with a 4.7 T horizontal bore magnet with a bore diameter of 20 cm, operating at 200 MHz for ^{1}H built at Institute of Scientific Instruments, Academy of Sciences of the Czech Republic, Brno, Czech Republic. Current active shielding gradient coils providing up to 180 mT/m gradient strength. Measuring samples were cultivated in plastic Petri dish (50 mm in diameter). The MR images were acquired using a classic Spin Echo pulse sequence with the following parameters: echo time (TE) = 12 ms, repetition time (TR) = 3.8 s, matrix size = 256 × 256 pixels (30 × 30 mm with resolution 0.117 mm per pixel), slice thickness = 2 mm and number of averages of the MR signal was (NA) = 5. The data were processed in Marevisi program.

2.8. High performance liquid chromatography with electrochemical detection

High performance liquid chromatography with electrochemical detection (HPLC-ED) system consisted of two chromatographic pumps Model 582 ESA (working range 0.001-9.999 ml.min^{-1}, ESA Inc., Chelmsford, MA), reverse-phase chromatographic column Polaris C18A (150 × 4,6; 3 µm particles size, Varian Inc., CA, USA) and twelve-channel CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector includes three flow cells (Model 6210, ESA, USA). Each cell consists of four analytical cells containing working carbon porous electrode, two auxiliary and two reference electrodes. The samples were injected by autosampler (Model 542, ESA, USA) at 4 °C. The injected sample volume was 50 µl. HPLC-ED experimental conditions were as follows: mobile phase: 80 mmol.l^{-1} trifluoroacetic acid (solvent A) and methanol (solvent B); gradient: 3 % methanol constant for 8 minutes, then increase of methanol to 15 % during 1 min, after 8 minutes of constant methanol content (15 %) decrease to 3 % during 1 min; mobile phase flow rate 0.8 ml.min^{-1}, column and detector were thermostated at 25 °C, the potential set on working electrodes was 900 mV. The optimization of the experimental conditions for the separation and detection of the thiols was published by [43-46]. The samples were processed same as in Subchapter 2.6.

2.9. Statistical analysis

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). Statistical significances of the differences in single and total thiols content and water

content were determined using STATISTICA.CZ. Differences with $p < 0.05$ were considered significant and were determined by using of one way ANOVA test (particularly Scheffe test), which was applied for means comparison.

3. RESULTS AND DISCUSSION

3.1. Growth and viability of ESEs

Early somatic embryos of Norway spruce, clone 2/32, were cultivated on semisolid LP cultivation medium, where the embryonic cells formed clusters (inset in Fig. 1). The growth parameters of the clusters can be determined using computer image analysis as a non-destructive tool. Each cluster is scanned using a CCD camera. The scanned image is further transferred to the computer, where the acquired image is subsequently analysed and the area of cluster is determined [29]. The control clusters of ESEs, clone 2/32, grew well during the fourteen days of cultivation ($y = 18.151x - 8.1976$; $R^2 = 0.9929$, Fig. 1). The viability of the control ESEs determined using double staining method varied from 94.5 % to 99.5 % (97±2.5%). These viability parameters are the same as previously published [27,29,30,47]. The growth and viability characteristics of clone 2/32 determined in the present paper show the stability of the ESEs culture, which make it useful for the investigation of the influence of various types of environmental stress factors.

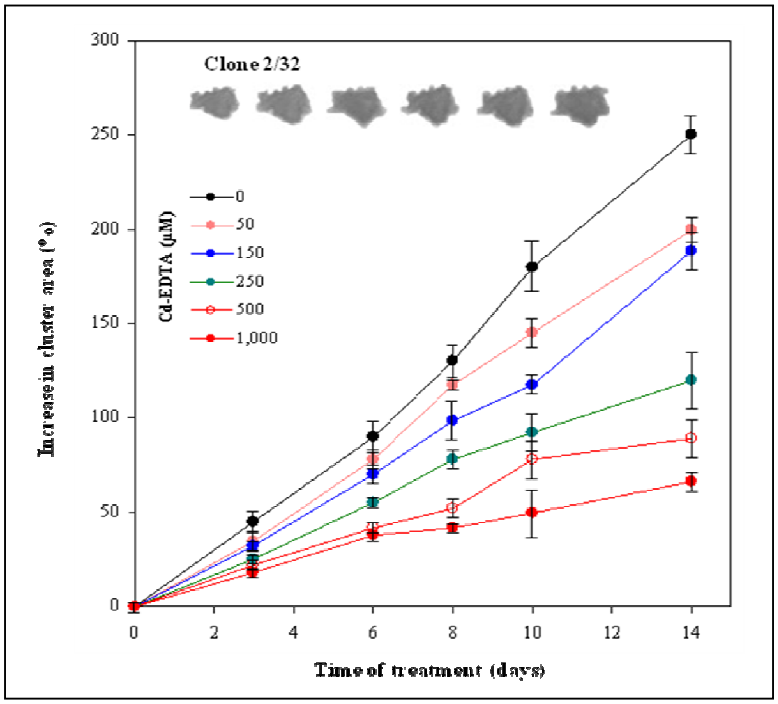

Figure 1. The dependence of increase in the ESEs clusters area on time of the treatment of ESEs by Cd-EDTA (0, 50, 150, 250, 500 and 1,000 μM Cd-EDTA). In inset: the typical images of the clusters at various times of the treatment (0^{th}, 3^{rd}, 6^{th}, 8^{th}, 10^{th} and 14^{th} day).

Cadmium(II) ions were applied in the chelated form since cadmium(II) inorganic salts precipitate out with other components of the cultivation medium which cannot be taken up by a plant cell [41]. The obtained growth characteristics of the ESEs treated with Cd-EDTA showed growth-inhibiting effect of cadmium(II) well determined by decrease in the cluster area (Fig. 1). At the end of the treatment, the inhibition could be quantified as the decrease in ESEs cluster area (DCA) about 0.5 % per 1 μM of Cd-EDTA compared with control ESEs (Fig. 1). The figure was calculated according to following equation:

$$DCA = \frac{\sum \frac{increase\,in\,cluster\,area_{control\,ESEs} - increase\,in\,cluster\,area_{ESEs\,treated\,with\,cadmium(II)\,ions}}{cadmium(II)\,dose}}{n}$$

where: n – number experimental groups excluding control, both increases in cluster area are measured at 14th day of the treatment.

Moreover, higher concentrations of Cd-EDTA resulted in significant morphological changes and decrease in viability of the embryonic cells (Fig. 2). The viability of the embryonic cells treated with the highest cadmium concentration (1,000 μM Cd-EDTA) decreased 47 ± 5 % at the end of the experiment. Karcz and Kurtyka investigated the effect of cadmium(II) ions on growth, proton extrusion and membrane potential in maize coleoptile segments [48]. They used similar concentrations of the heavy metal ions (0, 0.1, 1, 10, 100 and 1,000 μM). They also observed growth depression at concentrations higher than 100 μM, which is in good agreement with the present results. The cadmium(II) ions induced growth inhibition at almond seedlings [49], green microalga *Scenedesmus armatus* (Chod.) [50] and others.

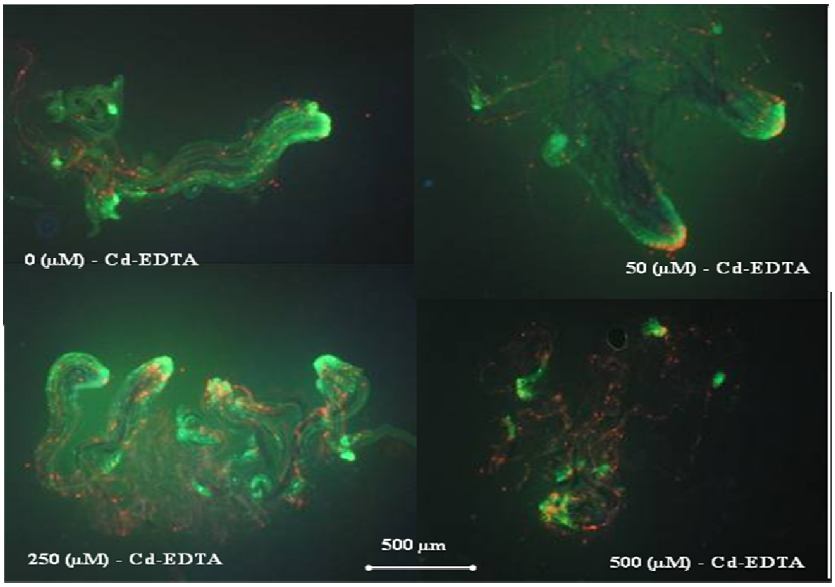

Figure 2. Changes in the viability and morphology of the somatic embryos cultivated in the presence of 0, 50, 250 and 500 μM Cd-EDTA at 3rd day of the treatment studied using FDA/PI double staining. FDA causes green fluorescence in viable cells. The red fluorescence of PI in cells shows that these cells are dead.

3.2. Total thiols content in ESEs

Based on the results obtained here and published previously [27] ESEs of Norway spruce, clone 2/32, demonstrate great resistance to complex Cd-EDTA. It is likely that synthesis of protective thiols involved in heavy metal induced stress reaction of a plant cell could closely be associated with this phenomenon. The electrochemical techniques such as differential pulse voltammetry (DPV) Brdicka reaction is a suitable tool for the analysis of thiols [33,41]. Peptides and proteins rich in free —SH group give specific catalytic signals in the presence of Co(III) complex and ammonium buffer called Brdicka reaction. One of these catalytic signals called Cat2 is proportional to thiols concentration and, thus, can be utilized for quantification of these molecules [28,42,51-54]. The typical DP voltammograms of the clusters of ESEs treated with 500 μM Cd-EDTA collected at 0^{th}, 10^{th} and 14^{th} day of the treatment are shown in inset in Fig. 3. The enhancing height of the Cat2 peaks with increasing time of the treatment is well evident. The differences between total content of thiols expressed as the enhancement of Cat2 peak in control and treated ESEs are shown in Fig. 3. It clearly follows from the results obtained that the content of thiols in the treated ESEs were lower compared to control ESEs at 3^{rd} day of the treatment. This phenomenon probably indicates the depletion of free thiols due to the binding of cadmium(II) ions entering plant cells as well as insufficient biosynthesis of the new thiols in the treated ESEs. At the sixth day of the treatment, the biosynthesis of thiols enhanced. Their increasing content was proportional to cadmium(II) concentrations and time of the treatment till the end of the experiment (Fig. 3). The rapid increase in thiol levels in the treated ESEs demonstrates their ability to withstand heavy metal toxicity over a long period of time (Fig. 1).

3.3. Content of low molecular plant thiols in ESEs

In spite of the fact that stationary electrochemical methods are commonly used for detection of numerous biologically active compounds [55-73], DPV Brdicka reaction cannot facilitate the exact identification of individual thiols [74]. Thiols (cysteine Cys, reduced glutathione GSH, oxidized glutathione GSSG and phytochelatin 2 PC2) involved in plant heavy metals detoxification processes were quantitatively determined using high performance liquid chromatography with electrochemical detection (HPLC-ED). HPLC-ED chromatogram of the thiols standards (Cys, GSH, GSSG and PC2, 1 μM) is shown in Fig. 4A. The HPLC-ED chromatograms of the samples prepared from the ESEs treated with 500 μM Cd-EDTA collected at 0^{th}, 10^{th} and 14^{th} day of the treatment are shown in Fig. 4B. The chromatograms obtained were repeatable. The peaks of the thiols were well separated and developed. The content of cysteine, one of the simplest thiols, increased up to the eighth day of the treatment, after that its content slowly decreased. This can be associated with the use of cysteine in biosynthetic pathways for phytochelatin and glutathione synthesis (Fig. 5A). In the case of GSH its content in the ESEs gradually enhanced with higher cadmium concentration and time of the treatment up to the sixth day of treatment (Fig. 5B). Similar trends in GSH content were determined in the cadmium hyperaccumulator *Sedum alfredii* Hance [75]. From this day on, the levels of GSH in ESEs exposed to Cd-EDTA were approximately well-balanced (within the range from 190 to 400 μM/g FW). GSSG level was significantly lower in all analysed samples (within the range from 10 to 35

µM/g FW). Moreover, the decreasing trend in GSSG content in ESEs exposed to Cd-EDTA was observed. This indicates the capability of the Norway spruce somatic embryos to bind heavy metals ions and thereby scavenge free oxygen radicals (Fig. 5C). Moreover Mendoza-Cozati and Moreno-Sanchez suggested a kinetic model of GSH and phytochelatin synthesis, which discussed the depletion of glutathione via synthesis of phytochelatins [76].

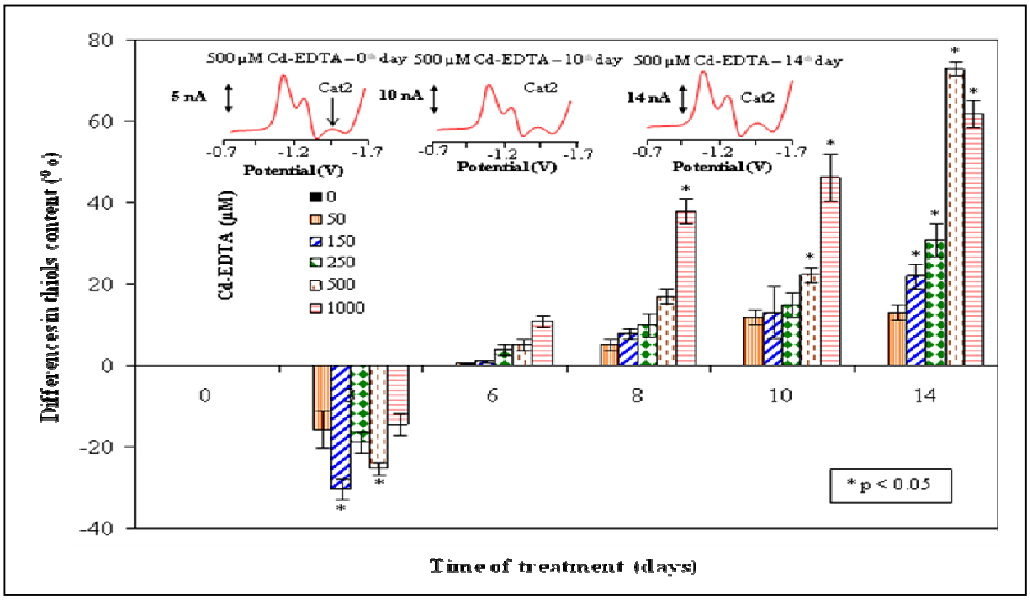

Figure 3. The changes in the total thiols content in ESEs treated with 0, 50, 150, 250, 500 and 1,000 µM Cd-EDTA. The total content of the thiols determined in the control samples was subtracted. In inset: typical DP voltammograms of the ESEs treated with 500 µM of Cd-EDTA at 0th, 10th and 14th day of the treatment. Measurements were carried out using DPV Brdicka reaction. For the measurements three clusters from three replicates were used. Changes in thiol content are expressed as percent differences from controls cultivated on Cd-free medium. The data measured were subtracted from this value.

In the case of the most important plant peptide involved in heavy metal detoxification processes called phytochelatin (PC2), its lowest concentration was determined at the third as well as at the sixth day of treatment. Based on the results obtained, it can be concluded the adaptation mechanisms of plant cells and PC2 synthesis were not sufficient to cover the total requirements of early somatic embryos to survive stress due to the presence of heavy metal ions. Applying cadmium ions after the sixth day, the biosynthesis of PC2 rapidly and significantly increased depending on time of the treatment as well as CD-EDTA concentration (Fig. 5D). The most intensive PC2 synthesis was determined in ESEs treated with 50 µM Cd-EDTA. Under effects of higher concentrations of Cd-EDTA, the content of PC2 was lower which can be associated with reducing the number of living cells. The average content of PC2 varied from 850 to 1800 nM/g FW in the ESEs. The content of PC2 is in good agreement with the levels determined at *Bacopa monnieri* L. treated with cadmium(II) ions

[77] The content of PC2 determined the ESEs is higher than the content detected in *in vitro* culture of the red spruce [78].

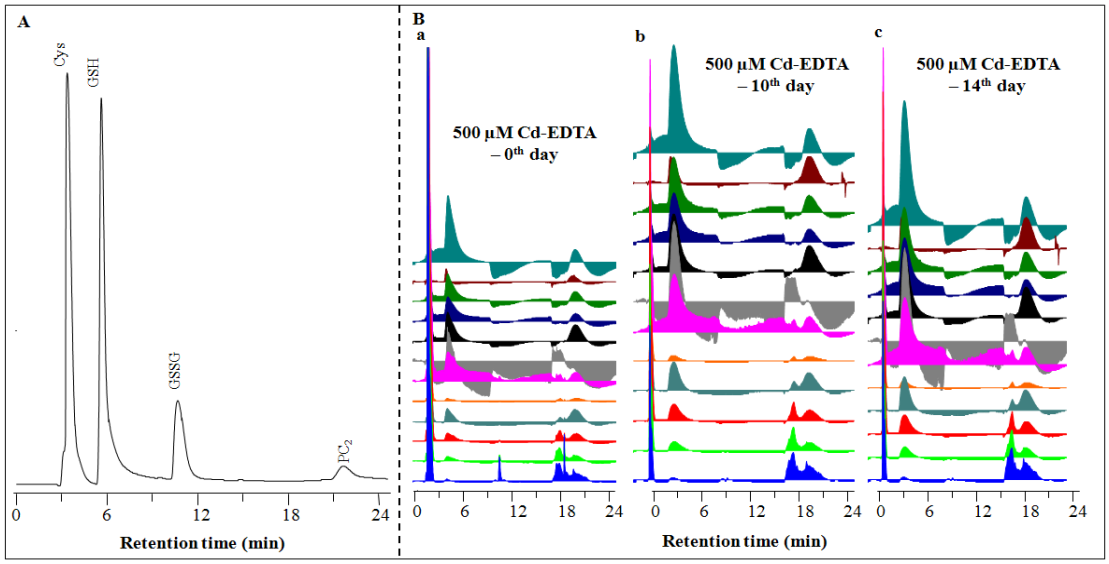

Figure 4. Typical HPLC-ED chromatograms of (**A**) Cys, GSH, GSSG and PC2 standards (1 µM) and (**B**) of real samples at 0th, 10th and 14th day of the clusters treatment with 500 µM Cd-EDTA. There can be recognized signals of all four thiols of interest according to their retention times found in standard chromatogram.

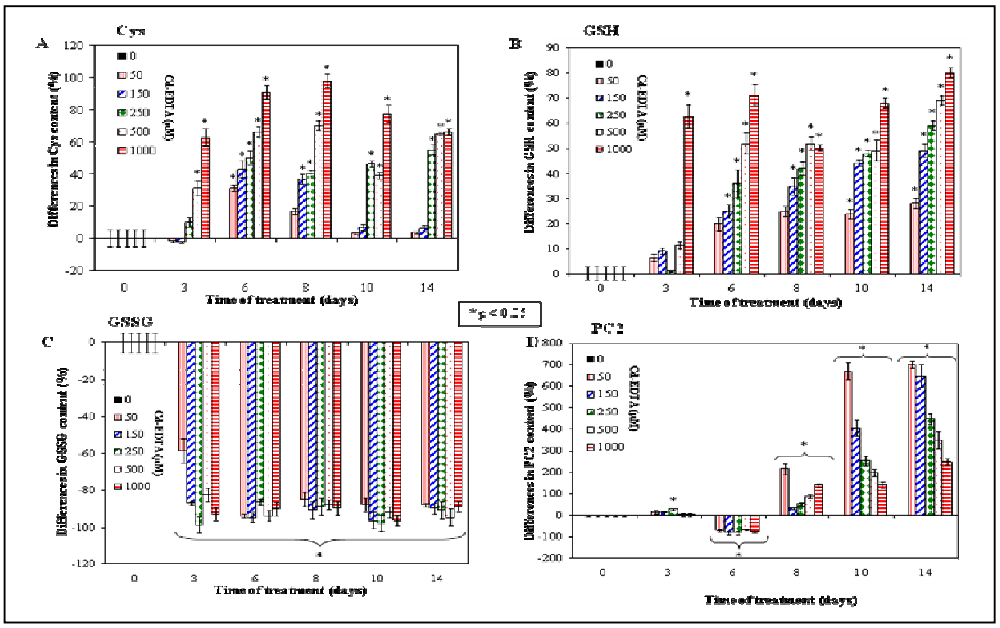

Figure 5. *The changes of the low molecular plant thiols content.* The content of (**A**) Cys, (**B**) GSH, (**C**) GSSG and (**D**) PC2 in the ESEs clusters treated with 0, 50, 150, 250, 500 and 1,000 µM Cd-EDTA. The thiols content detected in the control ESEs was subtracted. The resulting content was re-calculated on 1 gram of fresh weight of the cluster. Changes in thiol content are expressed as percent differences from controls cultivated on Cd-free medium. The data measured were subtracted from this value.

3.4. Nuclear magnetic resonance

Water content in the ESEs was studied using NMR. Brightness of the pixel image corresponded to the number of nuclei at the designated place. Therefore changes in number of proton nuclei (water content) during the cultivation of ESEs could be determined. As a control of the measuring system's sensitivity, the cuvette filled with water was placed near the measured cultures. The obtained images were standardized on the constant magnitude of the cuvette visual slice. The colour scale showing both water and dry weight content in the ESEs was estimated. Clusters of ESEs were placed on a Petri dish containing cultivation medium with various concentrations of Cd-EDTA. The whole Petri dish was put in the working space of a NMR scanner; five clusters were monitored in one scan. Typical NMR image of the individual clusters is shown in Fig. 6. Blue colour indicates the presence of water in the clusters, yellow and red tones show the decrease in the water content.

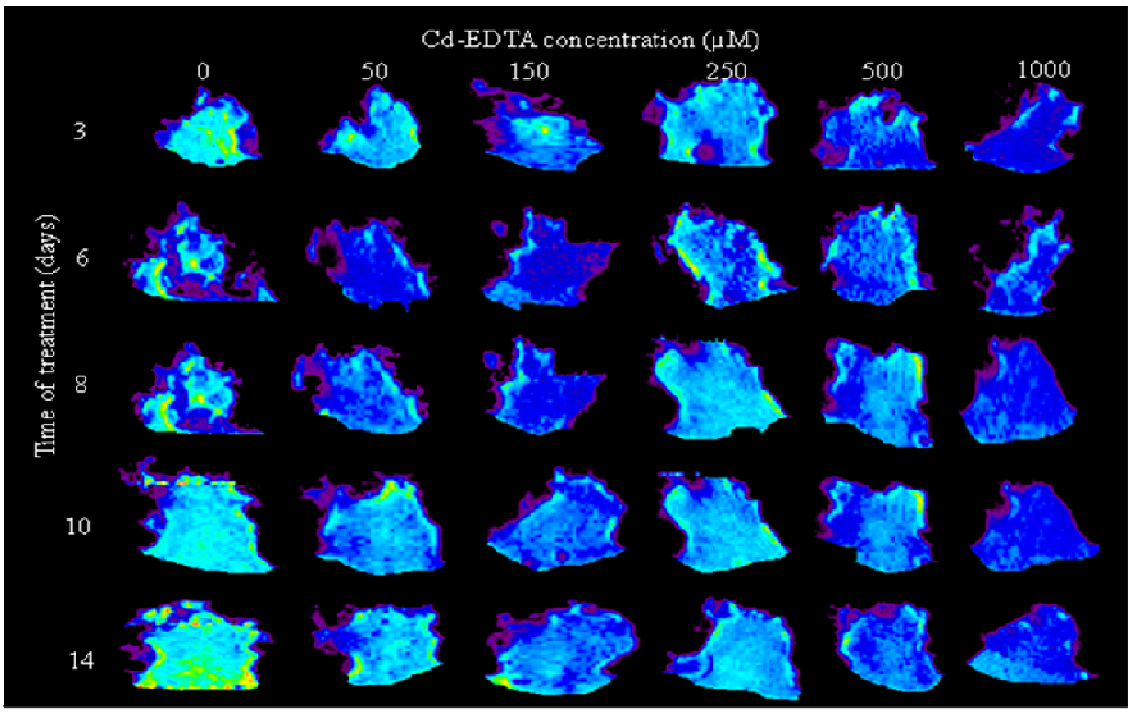

Figure 6. NMR scans of the ESEs clusters treated with 0, 50, 150, 250, 500 and 1,000 µM Cd-EDTA in time scale of the treatment.

The data given in Fig. 6 provided very interesting findings on water accumulation in the clusters of ESEs treated with higher Cd-EDTA concentrations. Marevisi computer program were employed to determine these changes. The changes indicated water content in the ESEs markedly oscillates during the first days of treatment. These oscillations did not depend on the cadmium concentration as well as the time of the treatment (Fig. 7). Over time the water content in the clusters significantly enhanced according to Cd-EDTA concentrations and the time of the treatment. The highest water content was observed at 10^{th} day of the treatment (Fig. 7). The average content of water

in the ESEs clusters treated by various cadmium concentrations compared to control clusters is shown in Fig.7. It clearly follows from obtained results the highest increase in water content was observed at the clusters treated with 250 μM Cd-EDTA. The clusters treated with higher cadmium(II) ions concentrations demonstrate lower increase in water content. The affect of water metabolism by cadmium(II) ions has not been intensively investigated. Milone et al. showed cadmium(II) ions did not cause changes in relative water content in wheat treated with cadmium(II) ions [79]. Devi et al. reported 50 μM of cadmium(II) ions induced water loss content in shoots and roots at the treated pea [80]. Similar results were described by past studies [81,82].

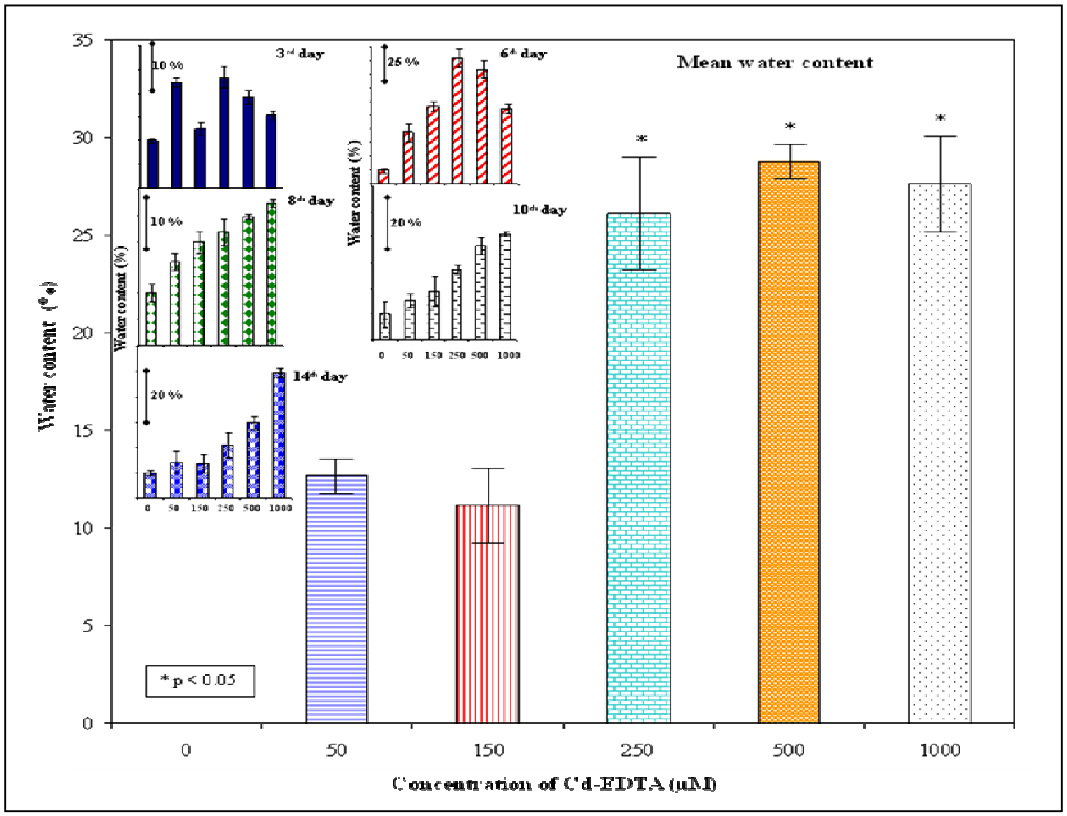

Figure 7. The average water contents in the ESEs clusters treated with Cd-EDTA. In inset: the dependence of the water content on the Cd-EDTA concentration and the time of the treatment. The water content was evaluated using computer program Marevisi.

4. CONCLUSIONS

Water metabolism at woody plants and their somatic embryos exposed to cadmium(II) ions has not yet been described. Based on the results obtained it can conclude that the cells of ESE clusters attempted to excrete cadmium(II) ions or, more precisely, decrease its concentration within the cluster. Previous observations indicate cadmium(II) treated plants transported toxic ions to older leaves [33]. However in order to lower cadmium concentration inside a cluster water uptake was seen to increase.

This phenomenon can be considered as one of the detoxification mechanisms of plants use to deal with the presence of heavy metal contamination. The escalation of water uptake continuously progress as long as the thiols are synthesized. These mechanisms provide the Norway spruce embryonic culture to survive against unfavourable conditions in the presence of heavy metal concentrations.

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References

1. D. J. Cosgrove, *Plant Physiol. Biochem.*, 38 (2000) 109.
2. H. K. Kim, Y. H. Choi and R. Verpoorte, *Nat. Protoc.*, 5 (2010) 536.
3. K. Ilvonen, L. Palva, M. Peramaki, R. Joensuu and R. Sepponen, *J. Magn. Reson.*, 149 (2001) 36.
4. R. S. Macomber, A Complete Introduction to Modern NMR Spectroscopy, John Wiley & Sons, Inc., New York, 1998.
5. M. J. Clearwater and C. J. Clark, *Plant Cell Environ.*, 26 (2003) 1205.
6. A. G. Perez-Donoso, L. C. Greve, J. H. Walton, K. A. Shackel and J. M. Labavitch, *Plant Physiol.*, 143 (2007) 1024.
7. T. W. J. Scheenen, F. J. Vergeldt, A. M. Heemskerk and H. Van As, *Plant Physiol.*, 144 (2007) 1157.
8. H. Van As, *J. Exp. Bot.*, 58 (2007) 743.
9. A. Van der Toorn, H. Zemah, H. Van As, P. Bendel and R. Kamenetsky, *J. Exp. Bot.*, 51 (2000) 1277.
10. L. Van der Weerd, M. Claessens, C. Efde and H. Van As, *Plant Cell Environ.*, 25 (2002) 1539.
11. C. W. Windt, F. J. Vergeldt, P. A. De Jager and H. Van As, *Plant Cell Environ.*, 29 (2006) 1715.
12. I. F. Ionenko, A. V. Anisimov and N. R. Dautova, *Biol. Plant.*, 54 (2010) 488.
13. J. A. Heyes and C. J. Clark, *Funct. Plant Biol.*, 30 (2003) 1089.
14. B. L. Tan, N. Reddy, V. Sarafis, G. A. C. Beattie and R. Spooner-Hart, *Hortscience*, 40 (2005) 720.
15. T. Y. Tse, R. M. Spanswick and L. W. Jelinski, *Protoplasma*, 194 (1996) 54.
16. Y. Q. Pu, F. Chen, A. Ziebell, B. H. Davison and A. J. Ragauskas, *BioEnergy Res.*, 2 (2009) 198.
17. K. G. Zulak, A. M. Weljie, H. J. Vogel and P. J. Facchini, *BMC Plant Biol.*, 8 (2008) 19.
18. P. V. Gersbach and N. Reddy, *Ann. Bot.*, 90 (2002) 253.
19. J. Lambert, P. Lampen, A. von Bohlen and R. Hergenroder, *Anal. Bioanal. Chem.*, 384 (2006) 231.
20. M. Stark, B. Manz, A. Ehlers, M. Kueppers, I. Riemann, F. Volke, U. Siebert, W. Weschke and K. Konig, *Microsc. Res. Tech.*, 70 (2007) 426.
21. S. M. Glidewell, M. Moller, G. Duncan, R. R. Mill, D. Masson and B. Williamson, *New Phytol.*, 154 (2002) 197.
22. N. R. Jagannathan, R. Jayasundar, V. Govindaraju and P. Raghunathan, *Proc. Indian Acad. Sci.-Chem. Sci.*, 106 (1994) 1595.
23. J. L. Maas and M. J. Line, *Hortscience*, 30 (1995) 1090.
24. M. I. Menzel, A. M. Oros-Peusquens, A. Pohlmeler, N. J. Shah, U. Schurr and H. U. Schneider, *J. Plant Nutr. Soil Sci.-Z. Pflanzenernahr. Bodenkd.*, 170 (2007) 24.
25. M. M. Millard, O. B. Veisz, D. T. Krizek and M. Line, *Plant Cell Environ.*, 18 (1995) 535.

26. J. A. Vozzo, J. M. Halloin, T. G. Cooper and E. J. Potchen, *Seed Sci. Technol.*, 24 (1996) 457.
27. V. Supalkova, J. Petrek, J. Baloun, V. Adam, K. Bartusek, L. Trnkova, M. Beklova, V. Diopan, L. Havel and R. Kizek, *Sensors*, 7 (2007) 743.
28. R. Mikelova, J. Baloun, J. Petrlova, V. Adam, L. Havel, H. Petrek, A. Horna and R. Kizek, *Bioelectrochemistry*, 70 (2007) 508.
29. J. Petrek, J. Vitecek, H. Vlasinova, R. Kizek, K. J. Kramer, V. Adam, B. Klejdus and L. Havel, *Anal. Bioanal. Chem.*, 383 (2005) 576.
30. J. Vitecek, V. Adam, J. Petrek, J. Vacek, R. Kizek and L. Havel, *Plant Cell Tissue Organ Cult.*, 79 (2004) 195.
31. M. F. Belmonte, G. Donald, D. M. Reid, E. C. Yeung and C. Stasolla, *J. Exp. Bot.*, 56 (2005) 2355.
32. E. Grill, E. L. Winnacker and M. H. Zenk, *Science*, 230 (1985) 674.
33. V. Supalkova, D. Huska, V. Diopan, P. Hanustiak, O. Zitka, K. Stejskal, J. Baloun, J. Pikula, L. Havel, J. Zehnalek, V. Adam, L. Trnkova, M. Beklova and R. Kizek, *Sensors*, 7 (2007) 932.
34. C. Cobbett and P. Goldsbrough, *Annu. Rev. Plant Biol.*, 53 (2002) 159.
35. A. Meister and M. E. Anderson, *Annu. Rev. Biochem.*, 52 (1983) 711.
36. P. Babula, V. Adam, R. Opatrilova, J. Zehnalek, L. Havel and R. Kizek, *Environ. Chem. Lett.*, 6 (2008) 189.
37. C. K. Sen and L. Packer, *Faseb J.*, 10 (1996) 709.
38. M. B. Kirkham, *Geoderma*, 137 (2006) 19.
39. L. Havel and D. J. Durzan, *Int. J. Plant Sci.*, 157 (1996) 8.
40. H. Vlasinova, M. Mikulecky and L. Havel, *Biol. Plant.*, 47 (2003) 475.
41. J. Vacek, J. Petrek, R. Kizek, L. Havel, B. Klejdus, L. Trnkova and F. Jelen, *Bioelectrochemistry*, 63 (2004) 347.
42. J. Petrlova, D. Potesil, R. Mikelova, O. Blastik, V. Adam, L. Trnkova, F. Jelen, R. Prusa, J. Kukacka and R. Kizek, *Electrochim. Acta*, 51 (2006) 5112.
43. J. Petrlova, R. Mikelova, K. Stejskal, A. Kleckerova, O. Zitka, J. Petrek, L. Havel, J. Zehnalek, V. Adam, L. Trnkova and R. Kizek, *J. Sep. Sci.*, 29 (2006) 1166.
44. D. Potesil, J. Petrlova, V. Adam, J. Vacek, B. Klejdus, J. Zehnalek, L. Trnkova, L. Havel and R. Kizek, *J. Chromatogr. A*, 1084 (2005) 134.
45. V. Diopan, K. Stejskal, M. Galiova, V. Adam, J. Kaiser, A. Horna, K. Novotny, M. Liska, L. Havel, J. Zehnalek and R. Kizek, *Electroanalysis*, 22 (2010) 1248.
46. O. Zitka, D. Huska, V. Adam, A. Horna, M. Beklova, Z. Svobodova and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 1082.
47. J. Vitecek, V. Adam, J. Petrek, P. Babula, P. Novotna, R. Kizek and L. Havel, *Chem. Listy*, 99 (2005) 496.
48. W. Karcz and R. Kurtyka, *Biol. Plant.*, 51 (2007) 713.
49. E. Nada, B. A. Ferjani, R. Ali, B. R. Bechir, M. Imed and B. Makki, *Acta Physiol. Plant.*, 29 (2007) 57.
50. Z. Tukaj, A. Bascik-Remisiewicz, T. Skowronski and C. Tukaj, *Environ. Exp. Bot.*, 60 (2007) 291.
51. V. Adam, O. Blastik, S. Krizkova, P. Lubal, J. Kukacka, R. Prusa and R. Kizek, *Chem. Listy*, 102 (2008) 51.
52. R. Kizek, J. Vacek, L. Trnkova, B. Klejdus and L. Havel, *Chem. Listy*, 98 (2004) 166.
53. J. Kukacka, J. Petrlova, R. Prusa, V. Adam, B. Sures, M. Beklova, L. Havel and R. Kizek, *Faseb J.*, 20 (2006) A75.
54. L. Trnkova, R. Kizek and J. Vacek, *Bioelectrochemistry*, 56 (2002) 57.
55. P. Daneshgar, P. Norouzi, M. R. Ganjali and F. Dousty, *Int. J. Electrochem. Sci.*, 4 (2009) 444.
56. A. A. Ensafi, M. Taei and T. Khayamian, *Int. J. Electrochem. Sci.*, 5 (2010) 116.
57. J. R. Esch, J. R. Friend and J. K. Kariuki, *Int. J. Electrochem. Sci.*, 5 (2010) 1464.

58. O. A. Farghaly, O. A. Hazzazi, E. M. Rabie and M. Khodari, *Int. J. Electrochem. Sci.*, 3 (2008) 1055.
59. M. R. Ganjali, F. Faridbod, E. Nasli-Esfahani, B. Larijani, H. Rashedi and P. Norouzi, *Int. J. Electrochem. Sci.*, 5 (2010) 1422.
60. J. G. Manjunatha, B. E. K. Swamy, G. P. Mamatha, S. S. Shankar, O. Gilbert, B. N. Chandrashekar and B. S. Sherigara, *Int. J. Electrochem. Sci.*, 4 (2009) 1469.
61. P. Norouzi, F. Faridbod, B. Larijani and M. R. Ganjali, *Int. J. Electrochem. Sci.*, 5 (2010) 1213.
62. P. Norouzi, M. Pirali-Hamedani, M. R. Ganjali and F. Faridbod, *Int. J. Electrochem. Sci.*, 5 (2010) 1434.
63. P. Norouzi, M. Qomi, A. Nemati and M. R. Ganjali, *Int. J. Electrochem. Sci.*, 4 (2009) 1248.
64. M. Ogunlesi, W. Okiei, A. S. Akanmu, T. Popoola, K. Okafor and O. Akore, *Int. J. Electrochem. Sci.*, 4 (2009) 1593.
65. I. Fabrik, J. Kukacka, J. Baloun, I. Sotornik, V. Adam, R. Prusa, D. Vajtr, P. Babula and R. Kizek, *Electroanalysis*, 21 (2009) 650.
66. D. Huska, V. Adam, O. Zitka, J. Kukacka, R. Prusa and R. Kizek, *Electroanalysis*, 21 (2009) 536.
67. D. Huska, J. Hubalek, V. Adam, D. Vajtr, A. Horna, L. Trnkova, L. Havel and R. Kizek, *Talanta*, 79 (2009) 402.
68. S. Krizkova, P. Blahova, J. Nakielna, I. Fabrik, V. Adam, T. Eckschlager, M. Beklova, Z. Svobodova, V. Horak and R. Kizek, *Electroanalysis*, 21 (2009) 2575.
69. S. Krizkova, I. Fabrik, V. Adam, J. Kukacka, R. Prusa, L. Trnkova, J. Strnadel, V. Horak and R. Kizek, *Electroanalysis*, 21 (2009) 640.
70. V. Adam, D. Huska, J. Hubalek and R. Kizek, *Microfluid. Nanofluid.*, 8 (2010) 329.
71. V. Adam, J. Petrlova, J. Wang, T. Eckschlager, L. Trnkova and R. Kizek, *PLoS ONE*, 5 (2010) e11441.
72. O. Krystofova, L. Trnkova, V. Adam, J. Zehnalek, J. Hubalek, P. Babula and R. Kizek, *Sensors*, 10 (2010) 5308.
73. J. Sochor, O. Zitka, H. Skutkova, D. Pavlik, P. Babula, B. Krska, A. Horna, V. Adam, I. Provaznik and R. Kizek, *Molecules*, 15 (2010) 6285.
74. V. Adam, I. Fabrik, V. Kohoutkova, P. Babula, J. Hubalek, R. Vrba, L. Trnkova and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 429.
75. Q. Sun, Z. H. Ye, X. R. Wang and M. H. Wong, *J. Plant Physiol.*, 164 (2007) 1489.
76. D. G. Mendoza-Cozatl and R. Moreno-Sanchez, *J. Theor. Biol.*, 238 (2006) 919.
77. S. Mishra, S. Srivastava, R. D. Tripathi, R. Govindarajan, S. V. Kuriakose and M. N. V. Prasad, *Plant Physiol. Biochem.*, 44 (2006) 25.
78. P. Thangavel, S. Long and R. Minocha, *Plant Cell Tissue Organ Cult.*, 88 (2007) 201.
79. M. T. Milone, C. Sgherri, H. Clijsters and F. Navari-Izzo, *Environ. Exp. Bot.*, 50 (2003) 265.
80. R. Devi, N. Munjral, A. K. Gupta and N. Kaur, *Environ. Exp. Bot.*, 61 (2007) 167.
81. J. Kovacik, J. Tomko, M. Backor and M. Repcak, *Plant Growth Regul.*, 50 (2006) 239.
82. E. LozanoRodriguez, L. E. Hernandez, P. Bonay and R. O. CarpenaRuiz, *J. Exp. Bot.*, 48 (1997) 123.