THE EVALUATION OF HEAVY METAL TOXICITY IN PLANTS USING THE BIOCHEMICAL TESTS

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Abstract: The evaluation of the toxicity and stress caused by heavy metals on plants is very important part of the phytoremediation research. Several physiological parameters can be used to assess the heavy metal-induced stress such as germination, plant growth and biomass production, photosynthetic pigments, antioxidant enzymes or antioxidants. Published results of measured physiological parameters in plants exposed to metals were characterized from of the negative effects of metals point of view and compared with the experimental study of the metal (Cu, Cd, Zn) toxicity in flax (*Linum usitatissimum*) and China aster (*Callistephus chinensis*) using the biochemical tests under the laboratory conditions. The germination and biomass production of *C. chinensis* significantly decreased with the increase of metal concentration which is considered a very typical response, however in *L. usitatissimum* slight stimulation of germination and biomass production at low metal content was observed. From the two studied plants *C. chinensis* expressed typical symptoms of the heavy metal toxicity including the decrease of total chlorophyll, chlorophylls *a, b*. On the contrary, heavy metal ions affected positively the physiological parameters of *L. usitatissimum* when low metal concentrations were added for example slight increase of the chlorophyll concentration in leaves was recorded. The decrease of the chlorophyll content was observed only at the high metal content. On the other hand, the typical response of plants on the heavy metal stress – the increase in peroxidase activity – was observed only for *L. usitatissimum* but not for *C. chinensis* that in all other tests showed significant toxicity symptoms. So if only one physiological parameter would be considered incorrect interpretation could be concluded. With the increase of phytoremediation practical applications the more systematic tests of heavy metal stress are necessary to help scientists working in that field correctly interpret their results and understand the plant behaviour.

Key words*:* Phytoremediation, heavy metals, stress assessment, toxicity, *Linum usitatissimum*, *Callistephus chinensis*

1. Introduction

At present, phytoremediation is a method very often used to remove heavy metals from contaminated soils. High heavy metal concentrations in soils could be very toxic for physiology of plants and induce stress in plants (MACFARLANE and BURCHETT, 2001; ZHOU and QIU, 2005). In general, two responses to metal stress can be distinguished – metal sensitivity and metal tolerance. Sensitivity to metals results in injury or death of plants. Resistance means that in spite of metal toxicity plant responses in a way enabling it to survive high concentrations of metals, and to produce the next generation of plants (ORCUTT and NILSEN, 2000; KADUKOVA and KAVULICOVA, 2010).

Technologists dealing with phytoremediation often do not consider the biochemical aspects of the heavy metal-induced stress in plants. Therefore it is necessary to know not only the ability of plants to accumulate metals but also their

DOI 10.2478/v10296-012-0011-2

ability to alleviate stress (TAULAVUORI *et al*., 2005). There are a lot of results concerning the effect of elevated metal concentrations on physiological state of plants published in scientific literature, however, this information are often contradictory.

The aim of our study was to summarize and compare results of measured parameters in plants exposed to metals published in scientific literature – the toxic effects induced by heavy metals on plant physiology using biochemical tests. Combination of selected physiological parameters, such as germination, plant growth and biomass production, content of photosynthetic pigments, antioxidant enzymes or antioxidants from the negative effects of metals point of view expressing the influence on photosynthesis and cell oxidative status can be desirable for stress evaluation. These published results are compared with our results of physiological parameters measurement in *Linum usitatissimum* and *Callistephus chinensis* grown at the presence of Cu, Zn, and Cd.

2. Materials and methods

2.1. Experimental set-up

L. usitatissimum and *C. chinensis* seeds were germinated and grown under laboratory conditions in plastic pots (four seeds per each pot) filled with organic substrate (weight of substrate in each pot was 1 130g). The organic substrate used for plants was produced in Slovakia with the trade name Florcom. The main constituents were white sphagnum peat and black sphagnum peat blended with the nutrients. Substrate pH (H_2O) was 5.5 – 7.0, electrical conductivity 0.8 mS/cm, humidity max. 65%.

Plants were watered two times per week with approximately 200 mL tap water. At the beginning of the experiment plants were divided into three groups. Five pots each with four seeds were used as controls with uncontaminated soil substrate and 10 pots (two groups) were used for the treatment with Cu^{2+}, Cd^{2+} and Zn^{2+} ions in contaminated soils. Soil substrate in the second group (named Low metal content) was contaminated with amount of the metal ions 144.3, 220.0 and 0.9 $mg.kg^{-1}$ of dry weight of substrate per pot for Cu^{2+}, Zn^{2+} and Cd^{2+} ions, respectively. The third group was contaminated with five times higher concentrations of Cu^{2+}, Cd^{2+} and Zn^{2+} ions than in the second group and named High metal content. Cu^{2+}, Cd^{2+} and Zn^{2+} ions were separately added into each pot as an aqueous solutions of $CuSO_4$, $Cd(NO_3)_2$ and $Zn(NO_3)_2$ in one dose on the first day of the experiment.

2.2. Measurement of physiological parameters

Germination – Seeds of *L. usitatissimum* and *C. chinensis* were sown in the soil substrates mentioned above. Seeds were placed under the surface of the soil substrate in pots under the laboratory conditions, five replicates for each pot, and five pots for each treatment. The pots were watered with tap water daily till seed germination. Only daylight was used for illuminating. Temperature was measured once a day and was in the range of 20 – 23°C.

Plant length – The length of plants was measured every week regularly. At the end of the experiment all plants were excavated from all the sets of treatments, partitioned into shoots and roots, carefully washed with distilled water and oven dried at 80°C for 24 h, to determine the biomass (g $plant^{-1}$) of each plant part.

The chlorophyll content – The chlorophyll content was measured at the tenth week of experiment according to HARBORNE (1984). 0.05 g of fresh leaves was taken from each plant randomly, washed with distilled water and small cuttings were homogenized in 80% acetone. After double centrifugation (14 000 g, 1 min), direct determination of the absorbance of the supernatant was carried out at wavelengths of 663 and 646 nm.

Guaiacol peroxidase (GPX) activity – For measuring the GPX activity according to the modified method of ERDELSKÝ and FRIČ (1979) 0.5 g of fresh plant material was homogenised in 4 ml of cold 0.05M phosphate buffer (pH 5.8). The homogenate was centrifuged at 14 000 g for 20 min. The oxidation of guaiacol by peroxidase from the plant extract was observed by the monitoring of absorbance increase during 3 minutes of reaction. The change of absorbance per minute was used to quantify the amount of enzyme in the mixture using the extinction coefficient for tetraguaiacol ε = 26.6 $\mu M^{-1}.cm^{-1}$. Enzyme activity unit was expressed as the change in absorbance per minute (ΔA_{470}/min).

3. Results and discussion

3.1. Germination

Germination test is a basic method to assess heavy metals toxic effects on plants (DI SALVATORE *et al*., 2008). Seed germination and the early seedling growth are more sensitive to metal stress because some of the defence mechanisms have not developed and it is also strongly related to the seed coat permeability to metal ions (LIU *et al*., 2005). Germination test gives the picture about the metal toxicity but it is also important for the practical application of plants in phytoremediation under the field conditions. Negative effects (delay in germination, lower germination rate, inhibition of growth of roots and shoots, no germination, detrimental effect on germination) of metals exposure on seed germination have been often reported by several authors (DI SALVATORE *et al*., 2008; WIERZBICKA and OBIDZIŃSKA, 1998; ROUT *et al*., 2000; ABEDIN and MEHARG, 2002; RAHOUI *et al*., 2010). Some authors observed decreased germination only at higher metal content but at low metal content stimulation of germination was determined (ESPEN *et al*., 1997; LI *et al*., 2007). Among other factors which affected seed germination can be involved – replacing filter paper with agar resulting in improved sensitivity of germination, combination of several metals invokes detrimental effect on germination, adaptation of plants at germination, support of germination by less toxic metal (e.g. Zn) and differences in plant species. In our study it also was verified slight stimulation of germination at low metal content in *L. usitatissimum*, whereas China aster was more sensitive to the metal toxicity than flax (Fig. 1). The negative effect of metals on germination also depends on the kind of the metal and the metal form. Kind of metal salt has strong effect on germination and can influence the toxicity test results significantly. In general, nitrates alleviate negative effect of metal on germination (KADUKOVA and KAVULICOVA, 2010).

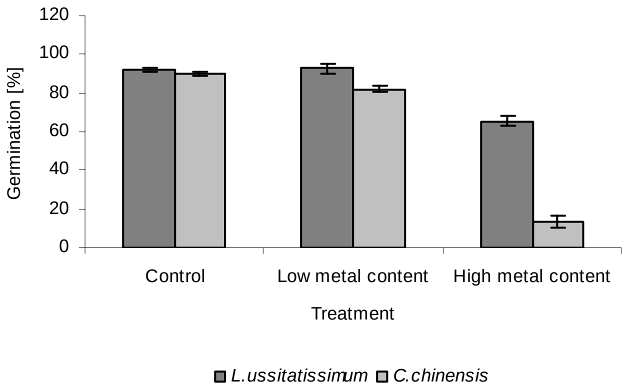

Fig. 1. Germination ability of *L. usitatissimum* and *C. chinensis* at Cu, Zn, and Cd treatment. Data are given as means (± S.E.) of five replicates.

3.2. Reduction in growth

The reduction in plant growth caused by heavy metal toxic effects on plants can be determined as reduced growth rate or decreased biomass production (BEGONIA *et al*., 1998). Negative effects of metals exposure on reduction in growth have been often observed by several authors (ARDUINI *et al*., 2004; GUO *et al*., 2007; VERNAY *et al*., 2008; CAO *et al*., 2009; OZTURK *et al*., 2010). Some of them (ARDUINI *et al*., 2004; CAO *et al*., 2009; OZTURK *et al*., 2010) determined that low metal content has stimulative effect on biomass production. For example in the case of *L. usitatissimum,* presence of low Cu, Cd and Zn concentrations stimulated the biomass production (Fig. 2A) but the same metal concentrations decreased the biomass production in *C. chinensis* (Fig. 2B). It shows that plants of *C. chinensis* are more sensitive to the metal toxicity than plants of *L. usitatissimum*.

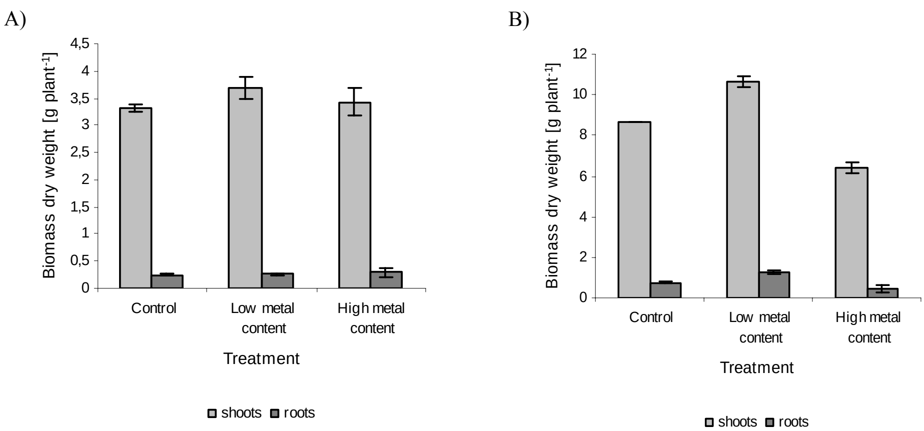

Fig. 2. The biomass dry weight of *L. usitatissimum* (A) and *C. chinensis* (B). Data are given as means (± S.E.) of five replicates.

The influence of metal on plant growth and biomass production significantly depends on the plant species and metal concentration. According to the comparison of published results (MILONE *et al*., 2003; ARDUINI *et al*., 2004; FAYIGA *et al*., 2004; AN 2006; ROONEY *et al*., 2007) it is visible that metal salt form does not influence plant growth and biomass production as significantly as it is in the case of germination. Although some positive effects of metals in the form of nitrates were observed in comparison with the negative effects of the same metal in the form of sulphates or chlorides. However, the negative effect of metal cation usually predominates over the effect of anion (KADUKOVA and KAVULICOVA, 2010). Considering this fact, we can conclude that this parameter – reduction in growth is not definitive physiological parameter for metal induced stress evaluation.

3.3. Photosynthetic pigments

A common response of plants to metal stress is a decrease of photosynthetic pigments (chlorophylls and carotenoids) in leaves of plants (MONTEIRO *et al*., 2009). The reduction at the levels of total chlorophyll, chlorophylls a, b and carotenoids on exposure to heavy metals has been observed in many species treated with different metals (MACFARLANE and BURCHETT, 2001; EKMEKÇI *et al*., 2008; GHNAYA *et al*., 2009; EKMEKÇI *et al*., 2008; MONFERRÁN *et al*., 2009). However, some authors observed no changes or slight increase of photosynthetic pigments in leaves at low metal content (ZHOU and QIU, 2005; GUPTA and SINHA, 2009). In our study, total chlorophyll, chlorophyll *a* and chlorophyll *b* contents were not different from the controls for all plants of *L. usitatissimum* under investigation (Fig. 3A). On the other hand, the chlorophyll content in the leaves of *C. chinensis* was slightly decreased by the metal mixture in comparison with controls (Fig. 3B).

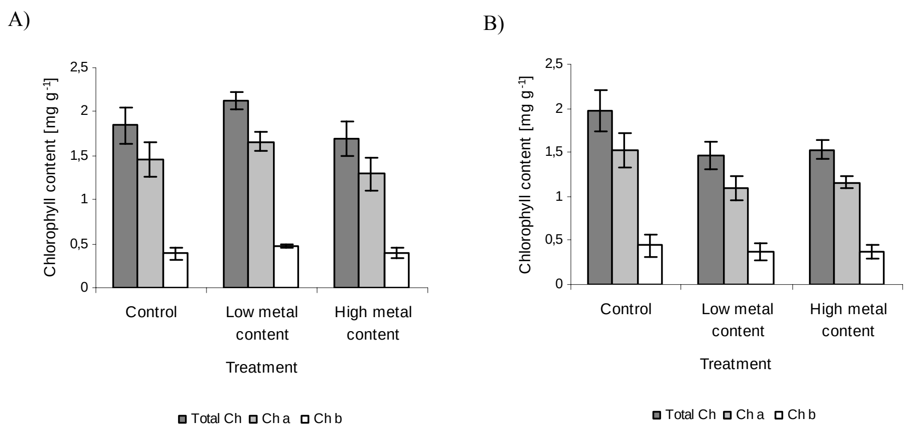

Fig. 3. The chlorophyll content in the fresh leaves of *L. usitatissimum* (A) and *C. chinensis* (B) (per g of fresh weight of the leaves). Data are given as means (± S.E.) of three replicates.

It is obvious that chlorophylls seem to be more sensitive to metals than carotenoids. Due to the ability of several metals to substitute the Mg in the chlorophyll molecule colour changes are not necessary to be observed after metal addition although the chlorophyll is non-functional. To evaluate the overall state of photosynthetic system the measurement of chlorophyll *a* fluorescence can give more precise view about the chlorophyll (KÜPPER *et al.*, 2000). According to published results it is possible to conclude that the influence of the metal on the chlorophyll content is not uniform and depends on several factors, such as plant species, kind of metal salt and its concentrations, time of exposure and presence of other chemicals or the specific mixture of the metals (KADUKOVA and KAVULICOVA, 2010).

3.4. Antioxidant enzymes and antioxidants

The formation of reactive oxygen species (ROS) causing oxidative damage to plants is one of the typical heavy metals toxicity symptoms in plants. To combat the oxidative damage plants have the antioxidant defense systems comprising of enzymes catalases (CAT), peroxidases (POD), superoxide dismutases (SOD) and antioxidants such as ascorbate (ASC), glutathione (GSH), α-tocopherol, flavonoids which remove, neutralize and scavenge ROS (SCHÜTZENDÜBEL and POLLE, 2002).

Measurement of the activity of antioxidant enzymes – catalases (CAT), peroxidases (POD), superoxide dismutases (SOD) can provide useful information about the stress level in plants. Amongst various enzymes involved in scavenging of the ROS activity, guaiacol peroxidase (GPX) plays a decisive role to evaluate the intensity of stress in plants (VERMA and DUBEY, 2003). Significant increase in peroxidase activity on exposure to heavy metals has been observed in many plant species treated with different metals (BACCOUCH *et al.*, 1998; MACFARLANE and BURCHETT, 2001; LEÓN *et al.*, 2002; BOOMINATHAN and DORAN, 2003; VERMA and DUBEY, 2003). In our study the content of guaiacol peroxidase of *L. usitatissimum* from the low and high metal treatment increased 1.5 and 3.5 times, respectively in comparison with the controls (Fig. 4A).

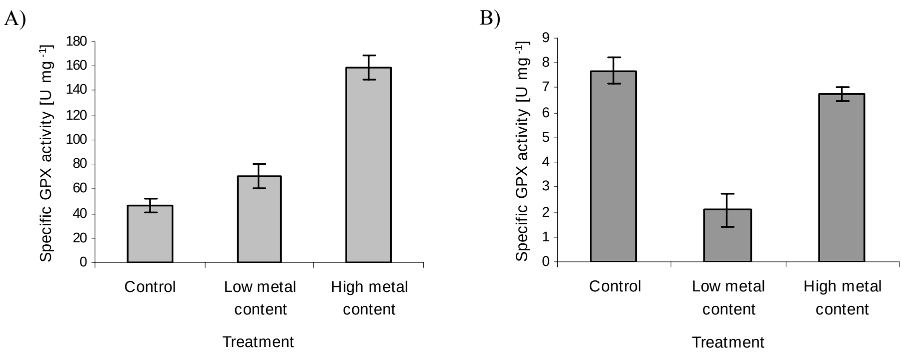

Fig. 4. The specific GPX activity in *L. usitatissimum* (A) and *C. chinensis* (B) under different treatment. Data are given as means (± S.E.) of three replicates.

In some cases the enzyme activity levels do not change or even can be decreased in the presence of metals (LEÓN *et al.*, 2002; ZHANG *et al.*, 2005). For example, the specific activity of GPX of *C. chinensis* was found to be from 1.1 to 4.3 times lower in the plants from all treatments (Fig. 4B). Low activity of enzyme may not denote the low stress level. It can be connected with the fact that under extreme conditions of metal stress, plants may be too weak to produce enough antioxidant enzymes to protect it (FAYIGA *et al.,* 2004). Some authors determined different changes in enzyme activity in different plant organs in the same plant species (MAZHOUDI *et al*., 1997; SHAH *et al*., 2001).

Amongst several antioxidants GSH along with ASC is involved in plant ascorbate-glutathione cycle and under the metal stress their levels usually increase, however, their contents depend on ion species, metal concentration and plant species. The increase at the levels of GSH and ASC on exposure to heavy metals has been observed in many plant species (ROMERO-PUERTAS *et al*., 2007; SUN *et al*., 2007; PONGRAC *et al*., 2009). Glutathione redox state is regarded as a good biomarker of the cellular redox state in metal stress. The decrease GSH/GSSG (GSSG - Glutathione disulfide) ratio indicates elevated sensitivity to metal stress in plant cells. α-Tocopherol and flavonoids are antioxidants and their increased contents can result in the protection of plants from oxidative stress. Due to complex metal stress evaluation in plants it would be necessary to assess several parameters – antioxidant enzymes and antioxidants together and separately for tolerant and sensitive plant species.

4. Conclusions

Phytoremediation is a potential remediation technology for cleaning-up polluted soils and water. Research related to this relatively new and promise technology needs to be widened by better understanding heavy metal-induced stress in plants. Symptoms of stress in plants depend on the particular metal (yet metal combination can act differently), plant species, but also on preliminary adaptation and other factors. The evaluation of heavy metal toxicity in plants using the biochemical tests is rather complex process. The influence of metals on physiological parameters is not uniform and changes of these parameters are difficult to be generalized. The combination of measurements of various physiological parameters is necessary to evaluate the metal stress in plants. There is still the demand to assess several selected key parameters (including their meaning) together which express the metal influence on photosynthesis and the degree of oxidative stress. It is desirable to prepare a standard protocol for clear stress evaluation for the researchers engaged in the field of phytoremediation without background in plant physiology.

Acknowledgement: This work was supported by Slovak Agency – project VEGA 1/0235/12.

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Presented at the 2nd International Conference "Biotechnology and Metals - 2011", September 22–23, 2011, Košice, Slovak Republic.