EFFECT OF CHELATING AGENTS ON PHYTOXICITY AND BIOACCUMULATION OF HEAVY METALS IN VASCULAR PLANTS

MIROSLAV HORNÍK[1,2], JANA GULDANOVÁ[1], MARTIN PIPÍŠKA[1,2], JANA MAREŠOVÁ[1], JOZEF AUGUSTÍN[1,2]

[1]Department of Biotechnology, University of SS. Cyril and Methodius, J. Herdu 2, Trnava, SK-917 01, Slovak Republic (hornikm@ucm.sk)
[2] Consortium for Environmental Biotechnology and Environmental Chemistry, 418 Špačince, 919 51, Slovak Republic

Abstract: In this work the effect of chelating agents ethylenediaminetetraacetate (EDTA) and nitrilotriacetate (NTA) on phytotoxicity and bioaccumulation of Cd and Co in tobacco plants (*Nicotiana tabacum* L.) hydroponically grown in diluted Hoagland media (HM) spiked with ^{109}Cd and ^{60}Co was studied. Speciation analysis using a program Visual MINTEQ showed, that the portion of bioavailable ionic Me^{2+} forms significantly decreased in the presence of EDTA or NTA in 25% HM for account of $[Me\text{-}EDTA]^{2-}$ or $[Me\text{-}NTA]^{-}$ complexes. We found that the equimolar addition of EDTA or NTA to 50 μmol/dm^3 $CdCl_2$ or $CoCl_2$ in HM positively diminished phytotoxicity of Cd or Co on tobacco plants. Bioaccumulation of Cd by tobacco roots during 8 d cultivation was minimally affected in the presence of equimolar concentrations of EDTA or NTA to 10 μmol/dm^3 $CdCl_2$ in media. On the contrary, equimolar concentration of EDTA or NTA added into HM caused considerable decrease of Co uptake by tobacco roots. Cadmium showed higher mobility in conductive tissues of tobacco plants than cobalt and the transport ratio in the presence of EDTA or NTA increased 2-times or 3-times in comparison with control experiments (without addition of chelates), respectively. In the case of cobalt this effect was observed in a less extent. Obtained data suggest the possibilities and constraints in the use of chelating agents in phytoextraction technologies in term of phytotoxicity, uptake and translocation of metals in plant tissues.

Key words: cadmium, cobalt, bioaccumulation, phytotoxicity, *Nicotiana tabacum*, speciation

1. Introduction

Heavy metals soil pollution is a serious worldwide problem and can be potentially harmful to human health *via* the food chain. In most cases, metals soil contamination results from anthropogenic activities such as mining, smelting, fertilizer application, and in the case of radionuclides from typical operations of nuclear fuel cycle, nuclear weapon testing and occasional nuclear disasters.

The stricter implementation of environmental laws urges the development of cost-effective soil remediation methods. Traditional techniques of remediation are expensive and may cause secondary pollution. Phytoremediation, the use of green plants to remove pollutants from soil, is one cost-effective method to remediate metal and radionuclide contaminated soils. The technical aspects of phytoremediation are described in numbers of review papers (see latest works in this area e.g. PILON-SMITS and LEDUC, 2009; VANGRONSVELD *et al.*, 2009) and monographs (see e.g. MACKOVA *et al.*, 2006; WILLEY, 2007).

Phytoextraction as one of the most perspective phytoremediation method depends mainly on the bioavailability of toxic metals and radionuclides in the soil and the plant

capacity to accumulate these contaminants. Therefore, some researchers have found that complexing ligands (e.g. EDTA, NTA, EDDS and others) can solubilize the heavy metals with the purpose of making them available for uptake (SALT *et al.*, 1998), but they are not necessarily available for plant uptake (TANDY *et al.*, 2006). They redistribute surface contamination down the soil profile, causing a reduction in the concentration near the soil surface (ROBINSON *et al.*, 2003) and distribute the heavy metals within the entire root zone for uptake. In addition, some authors reported that complexes of Me-chelate were less phytotoxic than free Me^{2+} forms or protonated chelates (HERNÁNDEZ-ALLICA *et al.*, 2007).

Our previous works studied the bioaccumulation of Zn, Co, Cd or Cs from nutrient solutions by roots of hydroponically cultivated sunflower (*Helianthus annuus* L.), tobacco (*Nicotiana tabacum* L.) and celery (*Apium graveolens* L.) plants (HORNÍK *et al.*, 2005; HORNÍK *et al.*, 2007; HORNÍK *et al.*, 2008) and radiocesium accumulation from contaminated soil by autochthonous vegetation of plants (PIPÍŠKA *et al.*, 2005). The aim of the current work was to investigate the influence of chelating agents ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) on Cd and Co phytotoxicity and bioaccumulation in tissues of hydroponically cultivated tobacco plants (*Nicotiana tabacum* L.). Bioaccumulation of Cd and Co ions in plants was analyzed by gamma-spectrometry using ^{109}Cd and ^{60}Co as radiotracers.

2. Material and methods

2.1 Plant material

Seeds of tobacco (*N. tabacum* L.) were germinated and grown in pots filled with granulated perlite as an inert carrier and watered with 25% strength Hoagland nutrient medium (HOAGLAND, 1920) at light/dark photoperiod 12/12 h (2 000 lx) and 22°C. The composition of the full strength (100%) nutrient solution was (in mg/dm^3) $MgSO_4.7H_2O$ – 370; KNO_3 – 404; $CaCl_2$ – 444; $NaH_2PO_4.2H_2O$ – 292; $Na_2HPO_4.12H_2O$ – 46.5; $FeSO_4.7H_2O$ – 17.9; $NaNO_3$ – 340; NH_4Cl – 214; NH_4NO_3 – 160; H_3BO_3 – 8.5; $Na_2MoO_4.2H_2O$ – 0.06; $MnSO_4.5H_2O$ – 5.0; $ZnSO_4.7H_2O$ – 0.66; $CuSO_4.5H_2O$ – 0.8 (pH 6). After 8 weeks pre-cultivation seedlings were gently removed from perlite and roots were washed free of any adhering perlite granules by deionized water and used in bioaccumulation experiments.

2.2 Bioaccumulation experiments and cultivation conditions

Plants from pre-cultivation phase were transferred into vessels with a cover to protect plant roots against lights and cultivated for 8 days in 25% strength Hoagland medium containing $CdCl_2$ or $CoCl_2$ spiked with ^{109}Cd or ^{60}Co. There were two treatments without or with chelating agents EDTA or NTA. The pH of nutrient solutions was adjusted to 6.0 using 1 M NaOH. Bioaccumulation experiments were carried out in triplicate series at photoperiod 12/12 h (2000 lx) and 22°C. In time intervals samples of nutrient solution were taken and ^{109}Cd or ^{60}Co radioactivity was measured by gamma-spectrometry. At the end of the experiments plants were

harvested, roots were carefully rinsed in deionized water and incorporated radioactivity in roots, stems and leaves was measured. Plant parts were then oven dried (at 60°C for 24 hours) and dry weights were determined.

Growth value (GV) was calculated during the experiments as a ratio between m(i) – m(t) and m(i), where m(i) or m(t) are fresh weight of plants at the start or end of experiments, respectively.

2.3 Speciation modelling

For *in silico* estimations of Cd and Co speciation in nutrient solutions as a function of total salt concentration, the presence or absence of chelating agents (EDTA or NTA), solution pH and temperature were carried out with Visual MINTEQ (version 2.53). This speciation modelling program allows the calculation of complexes [Me-EDTA] or [Me-NTA] portion in cultivation media for specified conditions.

2.4 Radiometric analysis

Gamma spectrometric scintillation detectors 54BP54/2-X and 76BP76/3 with well type crystal NaI(Tl) (Scionix, Netherlands) and data processing software Scintivision32 (Ortec, USA) were used for ^{109}Cd and ^{60}Co determination in plant parts and cultivation media. A library of radionuclides was built by selecting characteristic γ-ray peaks (88.04 keV for ^{109}Cd and 1173.24 keV or 1332.50 keV for ^{60}Co) for energy and efficiency calibration. Standardized $^{109}CdCl_2$ and $^{60}CoCl_2$ solutions were obtained from Czech Metrological Institute (Czech Republic).

3. Results and discussion

3.1 Effect of chelating agents on Cd and Co phytotoxicity

In our work for calculation of Cd and Co ionic forms in nutrient media the program Visual MINTEQ ver. 2.53 we used. However, this evaluation has to be considered with caution, because the modelling program does not include the participation of the plant. Moreover, the high concentrations of EDTA and NTA used here likely also mask the effects of the exudates from the roots. Plant exudates can contribute to changing the pH, the Eh, and the predominant chemical species of the toxins of concern (NIU *et al*., 2007). Even so, we used the simulation results as a first approximation to the chemical mechanisms in the near-root zone.

We found that in used Hoagland media (HM) at pH 6.0 and 22°C more than 95% of Co was found as bioavailable Me^{2+} ions. Only 78% of Cd was found in the form of Cd^{2+} ions, whereby also significant proportion represented ionic $CdCl^+$ form (12.7%) and $CdHPO_4$ form (6.4%). Complexing ligands significantly decreased the concentration of cationic Co^{2+} or Cd^{2+} forms and the concentration of $[Me\text{-}EDTA]^{2-}$ or $[Me\text{-}NTA]^-$ complex forms increased.

For evaluation of Cd and Co phytotoxic effect on tobacco plants the calculation of growth value (the ratio of fresh weight of plants at the start or end of experiments

difference to fresh weight of plants at the start of experiments) and macro- and microscopy observation of phytotoxic symptoms on leaves were used.

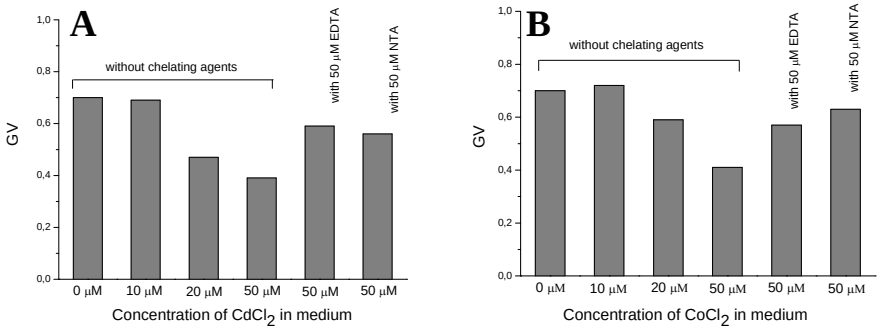

Fig. 1. The evaluation of phytotoxicity effect of Cd (A) and Co (B) on the basis of growth value (GV) of tobacco plants (*N. tabacum* L.) after 8 d cultivation in 25% HM without or with addition of EDTA or NTA at illumination 12/12 day/night period (2 000 lx), pH 6.0 and 22°C.

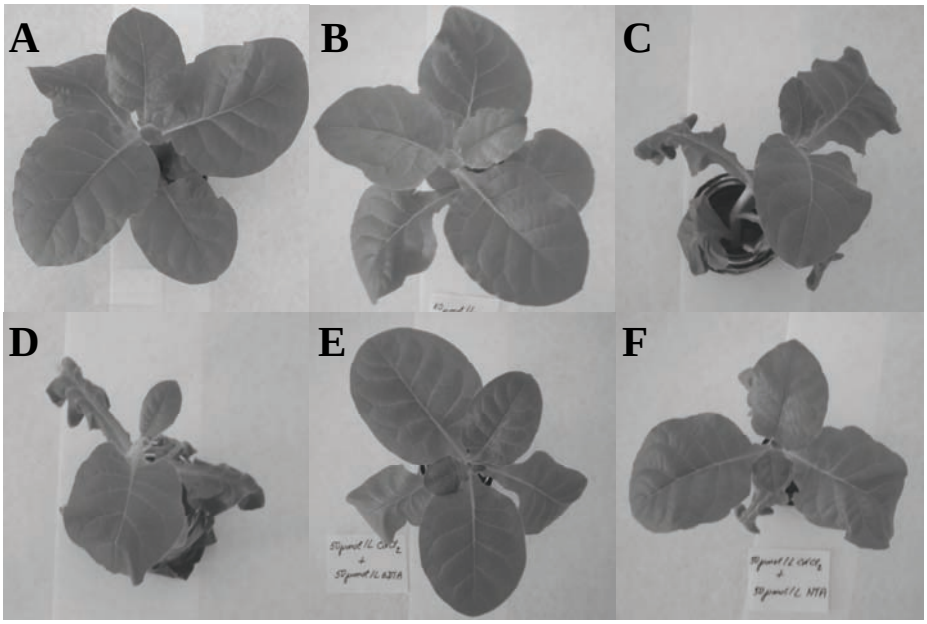

Fig. 2. Phytotoxicity effect of cadmium (die-back of leaves) in tobacco plants (*N. tabacum* L.) after 8 d cultivation at illumination 12/12 day/night period (2 000 lx) in 25% HM, pH 6 containing: A. 0 μmol/dm^3 $CdCl_2$; B. 10 μmol/dm^3 $CdCl_2$; C. 20 μmol/dm^3 $CdCl_2$; D. 50 μmol/dm^3 $CdCl_2$; E. 50 μmol/dm^3 $CdCl_2$ + 50 μmol/dm^3 EDTA; F. 50 μmol/dm^3 $CdCl_2$ + 50 μmol/dm^3 NTA. GV (growth value): A. 0.70; B. 0.69; C. 0.47; D. 0.39; E. 0.59; F. 0.56.

Fig. 1 shows, that the equimolar addition of EDTA or NTA to 50 μmol/dm^3 $CdCl_2$ or $CoCl_2$ in Hoagland media positively diminished phytotoxicity effect of Cd or Co on tobacco plants growth evaluated on the basis of growth values (GV) calculation.

Fig. 2 and 3 depict the die-back of plant leaves in the presence of Cd or chlorosis of leaves in the presence of Co in media as well as the effect of chelating agents on these processes. Phytotoxicity symptoms (die-back of leaves) and lower growth value were observed at 20 or 50 μmol/dm^3 $CdCl_2$ concentrations in media (Fig. 1A, 2C, 2D). After 50 μmol/dm^3 Co-treatment, plants yellowed with typical chlorotic ringspot symptoms and lower growth value than the control were found (Fig. 1B, 3D). Moreover, we found that this Co-treatment used in our experiment had impact on trichomes size and density (Fig. 4A). LEFÈVRE *et al.* (2009) found that more than 30% of accumulated Cd was found at the leaf surface and accumulated in trichomes. On the other side BAKKAUS *et al.* (2005) reported that cobalt was mainly located around the leaf veins. At the end of the 50 μmol/dm^3 Cd-treatment or Co-treatment with addition of EDTA or NTA in media, no sign of phytotoxicity was observed (Fig. 2E, 2F, 3E, 3F and 4B).

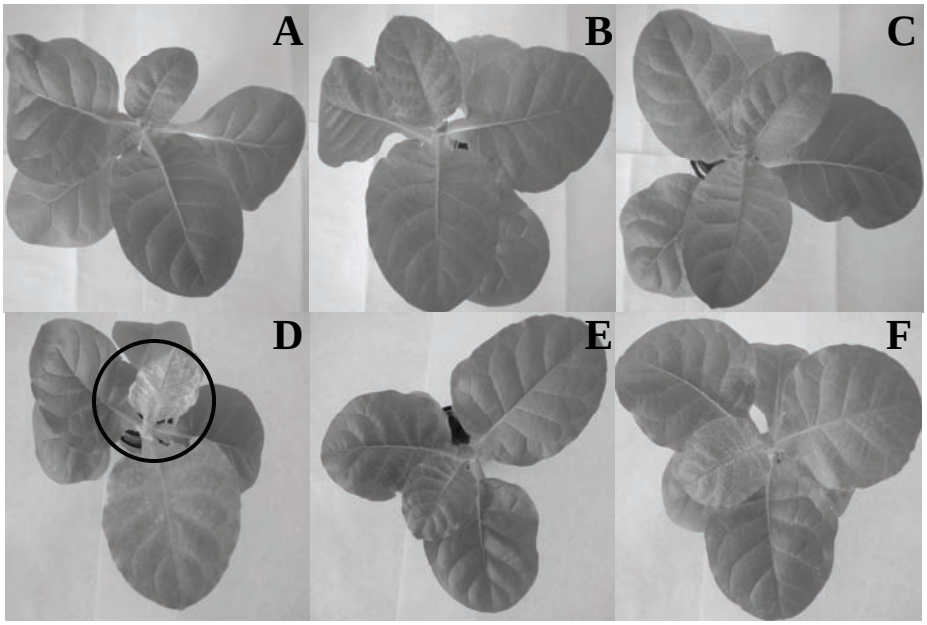

Fig. 3. Phytotoxicity effect of cobalt (chlorosis of leaves) in tobacco (*N. tabacum* L.) plants after 8 d cultivation at illumination 12/12 day/night period (2 000 lx) in 25% HM, pH 6 containing: A. 0 μmol/dm^3 $CoCl_2$; B. 10 μmol/dm^3 $CoCl_2$; C. 20 μmol/dm^3 $CoCl_2$; D. 50 μmol/dm^3 $CoCl_2$; E. 50 μmol/dm^3 $CoCl_2$ + 50 μmol/dm^3 EDTA; F. 50 μmol/dm^3 $CoCl_2$ + 50 μmol/dm^3 NTA. GV (growth value): A. 0.70; B. 0.72; C. 0.59; D. 0.41; E. 0.57; F. 0.63.

We suggest that the decrease of metal concentration in Me^{2+} form caused by chelating agents in nutrient media and the formation of [Me-ligand] complexes in media could diminish phytotoxic effect. Similar effect was also found by others authors (see e.g. HERNÁNDEZ-ALLICA *et al.*, 2007). This fact could be helpful in phytoremediation of contaminated soils with high concentration of toxic metals, where most plants can not grow under normal conditions.

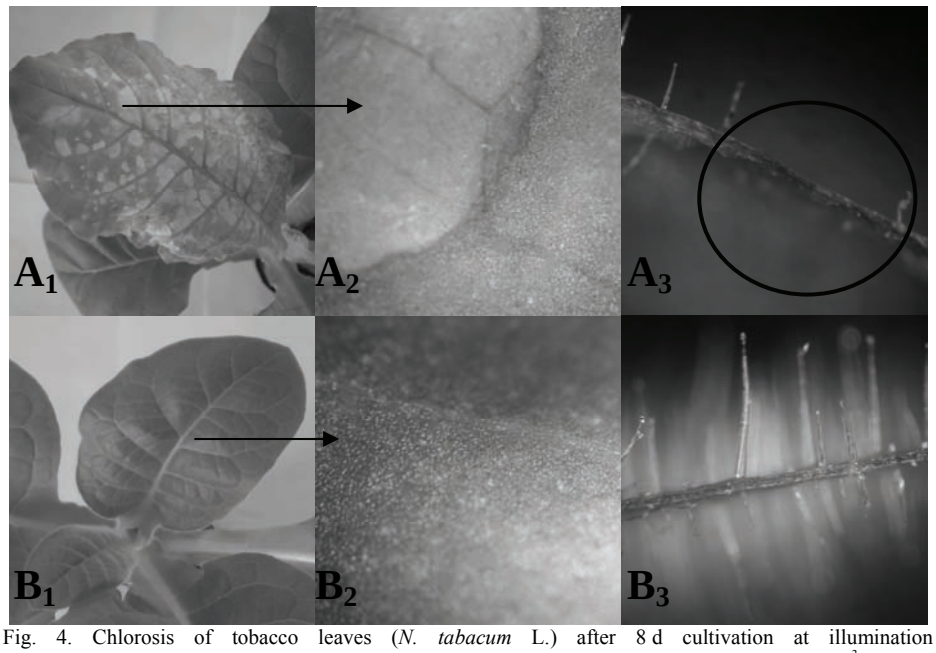

Fig. 4. Chlorosis of tobacco leaves (*N. tabacum* L.) after 8 d cultivation at illumination 12/12 day/night period (2 000 lx) in 25% HM, pH 6 containing: A. 50 μmol/dm^3 $CoCl_2$; B. 50 μmol/dm^3 $CoCl_2$ + 50 μmol/dm^3 EDTA. Macro- and microscopic photos: 1. second the youngest tobacco leaf; 2. surface of adaxial leaf section (magnification 50x); 3. adaxial and abaxial leaf trichomes (magnification 100x).

3.2 Effect of chelating agents on Cd and Co bioaccumulation

Bioaccumulation of Cd by tobacco roots during 8 d cultivation was minimally affected in the presence of EDTA or NTA equimolar concentrations to 10 μmol/dm^3 $CdCl_2$ in media. On the contrary, equimolar concentration of EDTA or NTA added into Hoagland media caused considerable decrease of Co uptake by tobacco roots (Fig. 5A). Calculated speciation of Cd and Co in used Hoagland medium (HM) is depicted in Fig. 5B.

For evaluation of metals mobility in conductive tissues of plants in the term of metal translocation efficiency we established non-dimensional transport ratio (*TR*), which represent the ratio of metal concentration in aboveground part of plants $[Me]_{shoot}$ to metal concentration in root system of plants $[Me]_{root}$. Cadmium showed higher mobility in conductive tissues of tobacco plants than cobalt and the transport ratio in the presence of EDTA or NTA increased 2-times or 3-times in comparison with control experiments (without addition of chelates), respectively (Fig. 6). In the case of cobalt this effect was observed in a less extent. Some authors reported that metal uptake is related to the availability of metals in soils and the addition of natural and/or synthetic chelators into soil could increase uptake and translocation of metals (see e.g. QUARTACCI *et al.* (2007). However, the amendment with non-biodegradable chelants of high metal-binding capacity, the high persistence of chelates and their

potential leaching to groundwater pose severe environmental concerns connected with the use of synthetic chelating agents such as EDTA and its derivatives. QUARTACCI *et al.* (2007) found that NTA and mainly EDDS were high effective in enhancing the concentrations of Cd, Cu, Pb and Zn in *Brassica carinata* shoots.

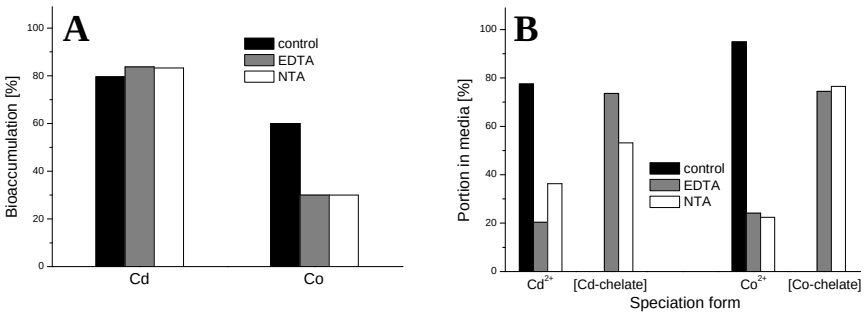

Fig. 5. A. Effect of EDTA and NTA on Cd and Co uptake by tobacco roots (*N. tabacum* L.). Data after 8 d cultivation at 22°C and illumination 12/12 day/night period (2 000 lx) in 25% HM pH 6.0 containing 10 μmol/dm^3 $CoCl_2$ or 10 μmol/dm^3 $CdCl_2$ and equimolar EDTA or NTA. B. Portion of Me^{2+} (Cd^{2+} or Co^{2+}) and [Me-chelate] ($[Me\text{-}EDTA]^{2-}$ or $[Me\text{-}NTA]^{-}$) forms in cultivation media at 22°C and pH 6.0. Speciation of Cd and Co was calculated by program Visual MINTEQ ver. 2.53.

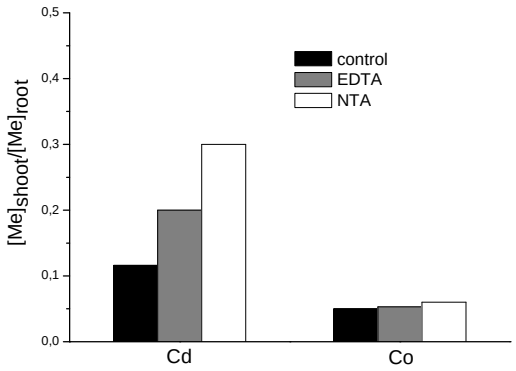

Fig. 6. Effect of EDTA and NTA on translocation of Cd and Co from roots to shoots (*TR*) of tobacco (*N. tabacum* L.) after 8 d plant cultivation in 25% HM containing 10 μmol/dm^3 $MeCl_2$ (Me = Cd or Co) without or with equimolar addition of EDTA or NTA, pH 6.0 at illumination 12/12 day/night period (2 000 lx) and 22°C.

4. Conclusions

The results from hydroponic cultivation of tobacco plants (*N. tabacum*) indicate that chelating agents (EDTA and NTA) are able to decrease of Cd and Co phytotoxicity, and on the other side these agents can increase of metal translocation from roots to shoots of tobacco plants. This fact can positively affect all processes involved in phytoremediation of contaminated environment with toxic metals or radionuclides.

References

BAKKAUS, E., GOUGET, B., GALLIEN, J.-P., KHODJA, H., CARROT, F., MOREL, J.L., COLLINS, R.: Concentration and distribution of cobalt in higher plants: The use of micro-PIXE spectroscopy. Nucl. Instrum. Methods Phys. Res. B, 231, 2005, 350-356.

HERNÁNDEZ-ALLICA, J., GARBISU, C., BARRUTIA, O., BECERRIL, J.M.: EDTA-induced heavy metal accumulation and phytotoxicity in cardoon plants. Environ. Exp. Bot., 60, 2007, s. 26-32.

HOAGLAND, D.R.: Optimum nutrient solution for plants. Science, 52, 1920, 562-564.

HORNÍK, M., PIPÍŠKA, M., SEKÁČOVÁ, J., AUGUSTÍN, J.: Determination of long distance transport of Cs^{+}, Co^{2+} and Zn^{2+} ions in vascular plants by autoradiography and gamma-spectrometry. Nova Biotechnol., VII-1, 2007, 33-40.

HORNÍK, M., PIPÍŠKA, M., VRTOCH, Ľ., SEKÁČOVÁ, J., AUGUSTÍN, J., LESNÝ, J.: Influence of complexing ligands on Zn uptake and translocation in tobacco and celery plants. Acta Agronomica Ovariensis, 2008, 50, 65-71.

HORNÍK, M., PIPÍŠKA, M., VRTOCH, Ľ., AUGUSTÍN, J., LESNÝ, J.: Bioaccumulation of ^{137}Cs and ^{60}Co by *Helianthus annuus*. Nukleonika, 50, 2005, S49-S52.

LEFÈVRE, I., MARCHAL, G., MEERTS, P., CORRÉAL, E., LUTTS, S.: Chloride salinity reduces cadmium accumulation by the Mediterranean halophyte species *Atriplex halimus* L. Environ. Exp. Bot., 65, 2009, 142-152.

MACKOVA, M., DOWLING, D., MACEK, T.: Phytoremediation Rhizoremediation. Springer, Dordrecht, The Netherlands, 2006, 300 pp.

NIU, Z.X., SUN, L.N., SUN, T.H., LI, Y.S., WANG, H.: Evaluation of phytoextracting cadmium and lead by sunflower, ricinus, alfalfa and mustard in hydroponic culture. J. Environ. Sci., 19, 2007, 961-967.

PILON-SMITS, E.A.H., LEDUC, D.L.: Phytoremediation of selenium using transgenic plants. Curr. Opin. Biotechnol., 20, 2009, 207-212.

PIPÍŠKA, M., HORNÍK, M, LESNÝ, J., AUGUSTÍN, J.: Transfer rádiocézia ^{137}Cs z kontaminovanej neobrábanej pôdy do voľne rastúcej vegetácie. Agriculture (Poľnohospodárstvo), 51, 2005, 470-478.

QUARTACCI, M.F., IRTELLI, B., BAKER, A.J.M., NAVARI-IZZO, F.: The use of NTA and EDDS for enhanced phytoextraction of metals from a multiply contaminated soil by *Brassica carinata*. Chemosphere, 68, 2007, 1920-1928.

SALT, D.E., SMITH, R.D., RASKIN, I.: Phytoremediation. Annu. Rev. Plant Physiol. Plant Mol. Biol., 49, 1998, 643-668.

VANGRONSVELD, J., HERZIG, R., WEYENS, N., BOULET, J., ADRIAENSEN, K., RUTTENS, A., THEWYS, T., VASSILEV, A., MEERS, E., NEHNEVAJOVA, E., VAN DER LELIE, D., MENCH, M.: Phytoremediation of contaminated soils and groundwater: lessons from the field. Environ. Sci. Poll. Res., (in press).

WILLEY, N.: Phytoremediation. Methods and Reviews. Humana Press, New Jersey, USA, 2007, 478 pp.