Responses of *Linum usitatissimum* and *Callistephus Chinensis* on copper contaminated substrate

KAVULIČOVÁ JANA[1, a)], SEDLÁKOVÁ-KADUKOVÁ JANA[2, b)], IVÁNOVÁ DANA[1,c)], REPICKÝ VLADIMÍR[1, d)]
[1]Institute of Metallurgy,
Faculty of Materials, Metallurgy and Recycling
Technical University of Košice
Letná 9, 042 00 Košice
SLOVAKIA

[2]Department of Microbiology
Institute of Biology and Ecology
Faculty of Science
Pavol Jozef Šafárik University
Šrobárova 2, 041 54 Košice
SLOVAKIA
[a)]jana.kavulicova@tuke.sk, [b)]jana.sedlakova@upjs.sk, [c)]dana.ivanova@tuke.sk,
[d)]vladimirrepicky@orangemail.sk

Abstract: The phytoremediation potential of two plants *Linum usitatissimum* (flax) and *Callistephus Chinensis* (China aster) was evaluated for their ability to accumulate copper in contaminated soil substrate under laboratory conditions. The results showed that the total amount of copper was accumulated significantly more in *L. usitatissimum* than *C. Chinensis*. The maximum highest accumulation of copper in *L. usitatissimum* was found of 280 mg kg^{-1} at copper concentration 2520 mg kg^{-1} in soil substrate. In both plants, the roots accumulated primarily higher amounts of copper compared to the amount translocated in the shoots. Phytoremediation research also includes the assessment of copper toxicity and stress caused in plants. The physiological parameters can be used to assess copper-induced stress such as germination and biomass production. Low toxicity symptoms in *L. usitatissimum* are indicated as an increase in biomass production in relation to increasing copper concentration along with low sensitivity in seed germination. The toxic effects of copper in *C. Chinensis* include the inhibition of germination as well as the reduction of biomass production at increasing copper concentrations in soil substrate. Our results indicate that the mixed culture of *L. usitatissimum* and *C. Chinensis* can be considered effective for the phytostabilization of copper from contaminated soil with low copper concentration.

Key-Words: phytoremediation, *Linum usitatissimum, Callistephus Chinensis,* copper, germination, biomass production

1 Introduction

Nowadays, contamination of soil with heavy and trace metals from the industrial activities is one of the most serious environmental problems [1, 2, 3]. Because of the potential toxicity and high persistence of the metals, the cleaning of contaminated soils is one of the most difficult tasks for environmental engineers [2, 4]. Among heavy metals, copper is an important engineering material, but it is considered to be one of the first known potentially toxic elements [5]. Higher copper concentration in the environment is not only the result of its increased use in industrial activities such as mining and smelting, but also of its use as a pesticide and its presence in sewage sludge treatment [2, 6]. Copper contamination is regarded a harmful to human health and the environment due to its nonbiodegradability, biomagnification potential and persistence in the environment [5,7]. Various physico-chemical methods have been used to reduce the concentration of available copper in soil, such as chemical precipitation, electrokinetic remediation, oxidation/reduction and immobilization [8]. One of perspective methods in copper contaminated soil treatment includes in situ remediation technology phytoremediation. Phytoremediation could be a great alternative to chemical or mechanical methods for cleaning of copper contamination. Phytoremediation has been defined as the use of green plants and their associated rhizospheric microorganisms, soil amendments, and agronomic techniques to remove, degrade, or detoxify harmful environmental pollutants [2, 8, 10]. Selection of appropriate plant species is an important in phytoremediation of metal contaminated soils. Phytoextraction technologies require plants that combine high metal uptake and high roots to shoot transport of these metals with high biomass [11]. Some plants have a natural ability to hyperaccumulate specific heavy metals. These plants are known as natural hyperaccumulators. van der Ent et al. [12] proposed that the criteria for hyperaccumulation of these metals were reduced. They recommend the following concentration criterion for copper in a dried leaf with plants growing in their natural environment: 300 mg kg^{-1} for copper. In general, the hyperaccumulators achieve a 100-fold higher concentration of the shoot metal (without reducing the yield) compared to a conventional non-accumulator plants [13]. Copper is essential mineral nutrient for plant growth and development at low concentrations, which plays key roles in photosynthetic and respiratory electron transport chains, even in ethylene sensing, cell wall metabolism and oxidative stress protection. However, excessive quantities of copper can lead to phytotoxicity such as leaf chlorosis and growth inhibition [14, 15]. Two plants, *Linum usitatissimum* (flax) and *Callistephus chinensis* (China aster) were chosen for experimental work. The possibility of using *Linum usitatissimum* [16, 17] and garden ornamental and flowering plants, like *Callistephus chinensis* [18] in phytoremediation has been reported by several authors.

The purpose of our study was to apply phytoremediation under the laboratory conditions for the removal of copper from contaminated substrate by using selected plants; to examine the effect of different concentrations of copper on the sensitivity of the seed germination and biomass production and to evaluate copper phytoremediation potential of *L. usitatissimum* and *C. chinensis* in copper contaminated soils.

2 Materials and Methods

2.1 Experimental set-up

The pot experiment in the laboratory was carried out from half of May until the beginning of September and lasted for 120 days. *Linum usitatissimum* (variety Merkur*)* and *Callistephus chinensis* seeds were germinated and grown under the laboratory conditions in plastic pots (four seeds of *L. usitatissimum* and four seeds of *C. chinensis* per each pot) filled with organic soil substrate. Organic soil substrate used for plants was produced in Slovakia with the trade name Florcom, garden soil substrate. 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^{-1}, humidity max. 65%. The content of background elements in substrate (e.g. Cd, Pb, Hg, As, Cr, Cu, Ni, Zn) was bellow limits for agricultural soil. Each pot was filled with the same amount of soil substrate. After seed germination only two plants of *L. usitatissimum* and two plants of *C. chinensis* were kept in each pot for the experiments (other plants were removed). Plants were watered two times per week with tap water (or more if it was necessary). Approximately 200 ml of water was added into each pot carefully not to overflow from the pots.

At the beginning of the experimental phase plants were divided into six groups; 2 pots per each group. The first group was a control group with uncontaminated soil substrate, the soil substrate in the second group was contaminated with the same amount of copper as was determined by the study of the Regional Office of the Public Health in agricultural soil in village Geča, near Košice in Eastern Slovakia. The monitoring and biomonitoring research was oriented on the study of the influence of the Incineration plant in Košice on the environment and the health of people living in neighbour villages [19]. In the third group soil substrate was contaminated with 2.5 times higher concentration than content of copper in the second group. Treatment of the second and third group is named Lab-low. The fourth, fifth and sixth groups were contaminated with 5, 10 and 21 times higher concentrations of copper, respectively, than was found in agricultural soil in village Geča and named Lab-high. Aqueous solutions of $CuSO_4.5H_2O$ were separately added into each pot in concentrations of Cu^{2+} listed in Table 1 calculated per dry weight of the soil in one dose on the first day of the experiment. At the end of the experiment (after 120 days) plants were harvested, washed with distilled water, oven dried, weighted and analysed for metal content.

Table 1. The concentration of Cu^{2+} added into the experimental soil substrates

Concentration of Cu^{2+} (mg kg^{-1})		Sample	
		Linum usitatissimum	*Callistephus chinensis*
Lab-low	0	L1	C1
	120	L2	C2
	300	L3	C3
Lab-high	600	L4	C4
	1200	L5	C5
	2520	L6	C6

Limit: The law NR SR n. 220/2004 Statutes at Large: About preservation and using of agricultural land [20]

2.2 Metal content analyses in plant tissues

Plant material for the metal content measurements by AAS spectroscopy was prepared according tothe modified method of Soon [21]. 0.5 g of powdered plant tissue was ashed in the muffle furnace for 4.5 hours at 600°C. Ash was dissolved in 10 ml of 2M HNO_3 solution, consequently was filtered and diluted with distilled water to 50 ml. Samples were stored in the refrigerator until measurements. The copper concentrations were carried out by AAS flame technique using the Perkin- Elmer Model 3100.

2.3 Measurements of physiological parameters

2.3.1 Determination of seed germination

Standard seed germination test according to the protocol of Slovak standard method [22] was carried out. The seeds were placed on two layers of the filter paper in glass Petri dishes. 100 seeds of *L. usitatissimum* and 30 seeds of *C. chinensis* were placed in Petri dishes containing $CuSO_4.5H_2O$ solution. The concentration of copper was the same as used at the Lab-low and Lab-high treatments. The control set of Petri dishes filled only with distilled water was used. Petri dishes with the seeds of *L. usitatissimum* and *C. chinensis* were maintained for 7 days at temperature 23-25°C and humidity 60-70%. Only daylight was used for illuminating. Seeds were considered as germinated when the shoots were longer than 2 mm.

2.3.2 Biomass production measurement

Plants were grown at room temperature (23-25 °C), with a14/10day/night photoperiod. 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 fresh weight was determined. The plant material was oven dried at 80°C until no weight change was observed any more (for 24 h), in order to determine biomass dry weight (g $plant^{-1}$) of each plant part.

3 Results and discussion

3.1 Copper accumulation in plant tissues

The copper concentration accumulated in plants was determined after 120 days of copper treatments. The accumulation of copper in plants of *L. usitatissimum* and *C. chinensis* was increased by increasing the copper concentration in the soil substrate (Fig. 1). Metal absorption in plants is correlated with increasing content of copper in soil substrate [23].

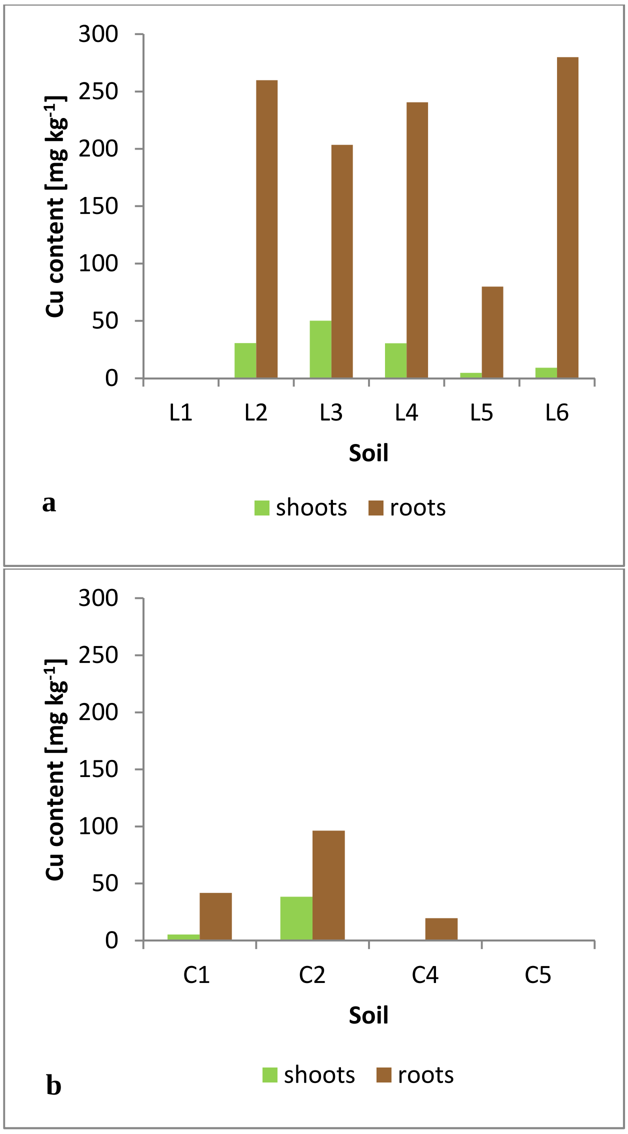

Fig. 1 Cu concentration in plants (shoots and roots) of a) *L. usitatissimum* and b) *C. chinensis* at copper treatment and accumulated from soil at different copper concentration

The copper concentration in *L. usitatissimum* was higher in all plants under treatments in comparison with controls. Cu accumulation reached values markedly higher in the roots than in the shoots (Fig. 1a). The maximum uptake in the roots exposed to the highest Cu concentration in the soil substrate (2520 mg kg^{-1}) was recorded with the value of 280 mg kg^{-1} dry weight. If copper is primarily accumulated mainly in the roots of *L. usitatissimum* and also the translocation to shoots is limited, *L. usitatissimum* can be regarded for phytostabilization of copper contaminated soil with a low concentration in soil. Results of our study were corresponded with other authors [24], where *L. usitatissimum* over-accumulated cadmium in its roots and can therefore be considered as Cd excluder by phytostabilization mechanism. On the contrary, the amount of copper in the shoots and the roots in *C. chinensis* varied considerably depending on the applied concentration as outlined in Fig. 1b. The significant decreases of the copper amount were observed in the shoots of C. *chinensis* growing at Cu concentration named Lab-high (C4-C6), which may be related to the fact that high copper concentrations in the soil had strong toxic effects on plant physiology. This supports the assumption that copper was within detoxifying mechanisms transported into the lower leaves and after leaf drying was removed from plants by the leaf-fall [25]. Remarkably, in both Cu treated plants; the difference in copper accumulation was significantly different when the same copper concentrations in soil substrate were applied.

3.2 Determination of seed germination by standard germination test

The germination test is an essential procedure to determine the toxic effects of heavy metals on plants [26]. Seed germination is more sensitive to metal stress because some of the defence mechanisms have not developed and it is also strongly related to the permeability of seed coatings to metal ions [27]. Germination test provides a picture of the toxicity to metals but it is also important for the practical use of plants in phytoremediation in the field conditions.
The seeds of *L. usitatissimum* and *C. chinensis* were exposed to different concentrations of copper to determine their effects on germination. After 7 d, total germination capacity of *L. usitatissimum* and *C. chinensis* at Cu treatment was evaluated. The seeds of *L. usitatissimum* and *C. chinensis* started to germinate on the first day of the experiment on filter paper under laboratory conditions. Compared to control, the germination capacity of *L. usitatissimum* was decreased by 42% and 61% at 600 and 1200 mg kg^{-1} of Cu^{2+} (L4 and L5) on filter paper, respectively. On the contrary, the germination capacity of *C. chinensis* seeds ranged from 33% to 17% at 120 to 1200 mg kg^{-1} of Cu^{2+} (C2-C5) compared to control. The highest concentration of Cu^{2+} was the main contributor to the decrease in germination by 83% and 96% in the case of *L. usitatissimum* and *C. chinensis*, respectively. According to results, toxic concentration of Cu^{2+} was from 120 and 1200 mg kg^{-1} of Cu^{2+} for *C. chinensis* and *L. usitatissimum*, respectively (Fig. 2). In our experiments, the greater sensitivity of seed germination test of *C. chinensis* than *L. usitatissimum* was evaluated.

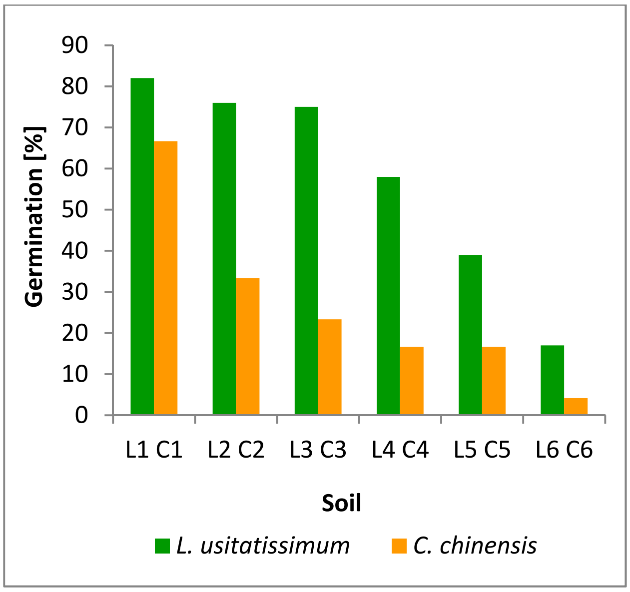

Fig. 2 Effects of copper on germination capacity and the subsequent growth of *L. usitatissimum* and *C. chinensis* seedlings subjected to 7 d of copper treatment

Figure 2 shows that copper is highly toxic to germination of *C. chinensis*. The negative impact of copper on germination capacity of *C. chinensis* is likely to be strongly related to the significantly higher permeability of the seed coating to copper compared to *L. usitatissimum*.

3.3 Dry biomass production

Negative effects of metals are usually readily observable by the changes in plant growth, which can be expressed, for example, by a reduction in biomass production [28]. There are several reasons can be for this behaviour – the specific toxicity of metal to plant, antagonism with other nutrients in plant, or inhibition of the root penetration in soil

[29]. In our study using two plants, a slight stimulation of biomass production for one and a decrease of biomass production for the other, were observed. Figure 3 shows the pot experiment in laboratory where plants of *L. usitatissimum* and *C. chinensis* are treated with Cu after ten weeks of the sowing.

Fig. 3 The pot experiment in laboratory. Plants of *L. usitatissimum* and *C. chinensis* at Cu treatment after ten weeks

Plants of *L. usitatissimum* produced more biomass in the presence of copper than without it. Very little differences in the amount of biomass were observed between copper treatments (Fig. 4a). The slight increase in biomass production ranged from 22% to 55% relative to control. The exception was the copper concentration in soil (L5), where a significant increase in biomass (146%) was determined. Copper is essential micronutrient required for normal growth, so that the low copper concentrations in soil can be stimulating for plants of *L. usitatissimum*. Similarly, an increase of biomass in the presence of metals has also been observed in other plants; however this effect also depends on concentration of metal. Chakravarty and Srivastava [30] revealed that low concentrations (0.1mM) of metals e.g. Cu, Ni stimulated the growth of *Helianthus annuus*. On the other hand, the growth of *C. chinensis* plants was negatively affected by the presence of copper (Fig. 4b). The amount of biomass with increasing copper concentration decreased markedly. The significant reduction in biomass production for copper concentration of 600 mg kg^{-1} (C4) and 1200 mg kg^{-1} (C5) was by 75% and 92% compared to control, respectively. It shows that plants of *C. chinensis* are much more sensitive to the metal toxicity than plants of *L. usitatissimum*. For both plants, increasing copper concentrations had a more negative effect on the biomass of their roots than on the biomass their shoots (Fig. 4).

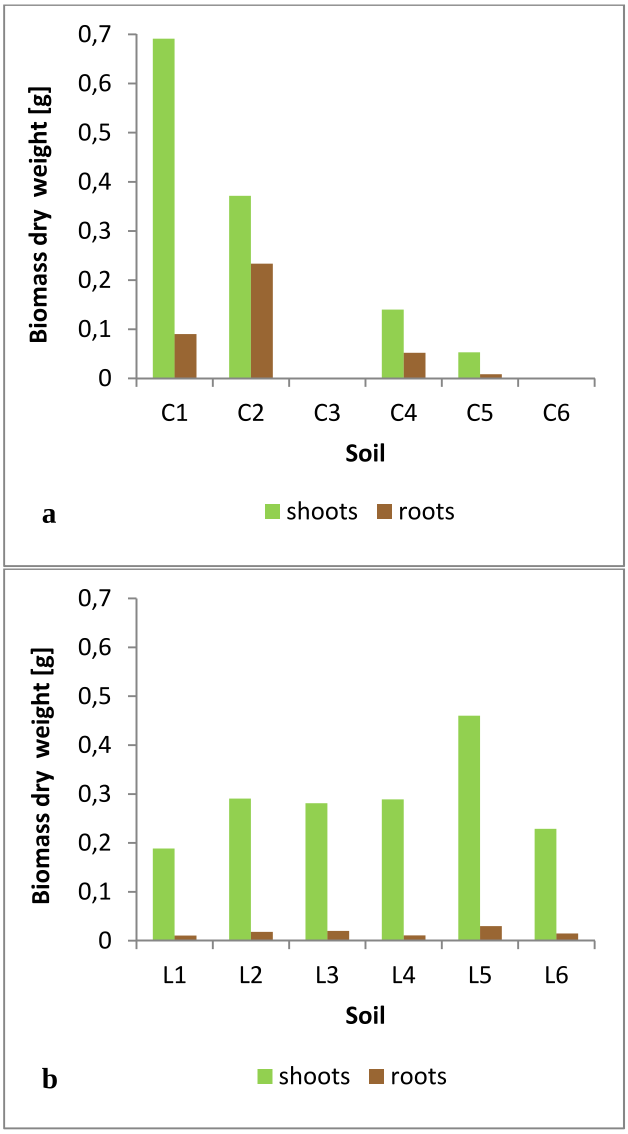

Fig. 4 Biomass dry weight (shoots and roots) at the end of the experiment for a) *L. usitatissimum* and b) *C. chinensis*

4 Conclusions

The assessing plant stress in the presence of heavy metals is a very important part of the research with the direct implication to phytoremediation. The studied parameters, such as seed germination and dry biomass production have been used to evaluate copper toxicity in *L. usitatissimum* and *C. chinensis*. The tolerance of *L. usitatissimum* to copper stress is expressed as the increase of biomass production along with low sensitivity in seed germination. *L.usitatissimum* used for phytoremediation study showed very little presence of toxicity symptoms. This might be caused by the higher metal tolerance of plants of *L.usitatissimum*. The symptoms of toxicity of *C. chinensis* to copper stress are reflected in the inhibition of germination and the reduction of biomass production. Due to the high accumulation of copper in the roots of *L.usitatissimum*, this plant is usable for the phytostabilization of contaminated soils with low copper concentration in soil. Based on the results, it may be concluded that mixed culture of *L. usitatissimum* and *C. chinensis* is suitable for cleaning up the soils with low copper contamination.

Acknowledgments:
This work was supported by the Slovak Research and Development Agency under the contract No. APVV-0351-12.

References:

[1] F. Gambale, M. Bregante, A. Paganetto, P. Magistrelli, L. Martella, G.A. Sacchi, A. Rivetta, M. Cocucci, A pilot phytoremediation system for the decontamination of lead-polluted soils, *Phytoremediation, Wetlands and Sediments*, Vol 6 (5), A. Leeson, E. Foote, K. Bank, V. Magar (eds), Battelle Press, Columbus, Ohio, USA, 2001, pp. 157-163.

[2] J. Kadukova, J. Kavulicova, Phytoremediation and stress: Evaluation of Heavy Metal-Induced Stress in Plants. New York: Nova Science Publishers, 2010, p. 134.

[3] K.Ghazaryan, H. Movsesyan, N.Ghazaryan, B.A. Watts, Copper phytoremediation potential of wild plant species growing in the mine polluted areas of Armenia, *Environmental Pollution*, 249, 2019, pp. 491-501.

[4] L.H. Wu, Y.M. Luo, X.R. Xing, P. Christie, EDTA-enhanced phytoremediation of heavy metal contaminated soil with Indian mustard and associated potential leaching risk. *Agriculture, Ecosystems and Environment*, 102, 2004, pp. 307-318.

[5] J.Chua, J.M.Banua, I.Arcilla, A.Orbecido, M.E. de Castro, N.Ledesma, C.Deocaris, C.Madrazo, L.Belo,Phytoremediation potential and copper uptake kinetics of Philippine bamboo species in copper contaminated substrate, *Heliyon,* 5 (9), 2019, e02440.

[6] S.-S. Ke, Effects of Copper on the Photosynthesis and Oxidative Metabolism of *Amaranthus tricolor* Seedlings, *Agricultural Sciences in China*, 6 (10), 2007, pp. 1182-1192.

[7] A.K. Shrivastava, A review on copper pollution and its removal from water bodies by pollution control Technologies, *Indian J. Environ. Prot*., 29 (6), 2009, pp. 552-560.

[8] W.-h. Zhang, R.-b. Sun, L. Xu, J.-n. Liang, T.-y. Wu, J. Zhou, Effects of micro-/nano-hydroxyapatite and phytoremediation on fungal community structure in copper contaminated soil, *Ecotoxicology and Environmental Safety,*174 (15), 2019, pp. 100-109.

[9] Y. Ouyang, Phytoremediation: modelling plant uptake and contaminant transport in the soil-plant-atmosphere continuum. *Journal of Hydrology*, 266 (1), 2002, pp. 66-82.

[10] C. Chigbo, L.Batty, R.Bartlett, Interactions of copper and pyrene on phytoremediation potential of *Brassica juncea* in copper–pyrene co-contaminated soil, *Chemosphere*, 90 (10), 2013, pp. 2542-2548.

[11] C. Poschenrieder, A. Lombini, J. Bech, M. Llugany, E. Dinelli, J. Barceló, *Smilax aspera L*. an evergreen Mediterranean climber for phytoremediation, *Journal of Geochemical Exploration* 123, 2012, pp. 41-44.

[12] A. van der Ent, A.J.M. Baker, R.D. Reeves, A.J. Pollard, H. Schat, Hyperaccumulators of metal and metalloid trace elements: facts and fiction. *Plant Soil,* 362, 2013, pp. 319-334.

[13] H. Ali, E.Khan, M.A Sajad, Phytoremediation of heavy metals-Concepts and applications, *Chemosphere* 91(7), 2013, pp. 869-881.

[14] X. Li, H. Ma, P. Jia, J. Wang, L. Jia, T. Zhang, Y. Yang, H. Chen, X. Wei, Responses of seedling growth and antioxidant activity to excess iron and copper in *Triticum aestivum* L., *Ecotoxicology and Environmental Safety,* 86, 2012, pp. 47-53.

[15] G. Feigl, D. Kumar, N. Lehotai, N. Tugyi, Á. Molnár, A. ördög, Á. Szepesi, K. Gémes, G. Laskay, L. Erdei, Z.Kolbert,: Physiological and morphological responses of the root system of Indian mustard (*Brassica juncea* L. Czern.) and rapeseed (*Brassica napus* L.) to copper stress, *Ecotoxicology and Environmental Safety*, 94, 2013, pp. 179-189.

[16] O. Douchiche, W. Chaïbi, C. Morvan, Cadmium tolerance and accumulation characteristics of mature flax, cv. Hermes: Contribution of the basal stem compared to the root, *Journal of Hazardous Materials*, 235-236, 2012, pp. 101-107.

[17] V. Angelova, R. Ivanova, V. Delibaltova, K.I Vanov, Bio-accumulation and distribution of heavy metals infibre crops (flax, cotton and hemp), *Idustrial crops and products*, 19 (3), 2004, pp.197-205.

[18] A. Cristaldi, G.O. Conti, E. H. Jho, P. Zuccarello, A. Grasso, C. Copat, M. Ferrante, Phytoremediation of contaminated soils by heavy metals and PAHs. A brief review, *Environmental Technology & Innovation*, 8, 2017, pp. 309-326.

[19] Z. Dietzová, J. Labancová, Vplyv emisií spaľovne TKO (KOSIT a.s. Košice) na kvalitu ovzdušia, pôdy, vody a zdravia obyvateľov okolitých obcí (Effect of emissions from incinerator (KOSIT a.s. Košice) on air, soil, water and health of inhabitants of surrounding villages). Regionálny úrad verejného zdravotníctva so sídlom v Košiciach (the Regional Office of the Public Health), 2008.

[20] Act No. 220/2004 Coll. of NC Slovak Republic on *Protection and Exploitation of Agricultural Soil* (Appendix 2). Part 96, p. 2290.

[21] Y.K. Soon, Determination of cadmium, chromium, cobalt, lead and nickel in plant tissue. *Kalra, P.Yash.* (Ed.), Handbook of Reference Methods for Plant Analysis, Boca Raton: CRC Press, 1998, pp. 193-198.

[22] STN 838303 (Slovak Technical Standard). Testing of dangerous properties of wastes. Ecotoxicity. Acute toxicity tests on aquatic organisms and growth inhibition tests of algae and higher cultivated plants.

[23] N.Ahsan, D.-G.Lee, S.-H-Lee, K.Y.Kang, J.J.Lee, P.J.Kim, H.-S. Yoon, J.-S.Kim, B.-H. Lee, Excess copper induced physiological and proteomic changes in germinating rice seeds, *Chemosphere*, 67 (6), 2007, pp. 1182-1193.

[24] M. E. Hosman, S. S. El-Feky, M. I. Elshahawy, E. M. Shaker, Mechanism of Phytoremediation Potential of Flax (*Linum usitatissimum L.*) to Pb, Cd and Zn, *Asian Journal of Plant Science and Research*, 7 (4), 2017, pp. 30-40.

[25] J. Kavuličová, J. Kaduková, J. Podracký, D. Ivánová, H. Horváthová, K. Flórián, Z. Dietzová, Čistenie pôdy kontaminovanej ťažkými kovmi s využitím rastlín, *Waste forum*, 4, 2010, pp. 422-427.

[26] M. Di Salvatore, A.M. Carafa, G. Carratů, Assessment of heavy metals phytotoxicity using seed germination and root elongation tests: A comparison of two growth substrates, *Chemosphere,* 73 (9), 2008, pp. 1461-1464.

[27] X. Liu, S. Zhang, X. Shan, Y.-G. Zhu, Toxicity of arsenate and arsenite on germination seedling growth and amylolytic activity of wheat, *Chemosphere*, 61 (2), 2005, pp. 293-301.

[28] A. Chaoui, S. Mazhoudi, M.H. Ghorbal, E. El-Ferjani, Cadmium and zinc induction of lipid peroxidation and effects of antioxidant enzyme activities on beans (*Phaseolus vulgaris* L.), *Plant Science*, 127, 1997, pp. 139-147.

[29] G.B. Begonia, C.D. Davis, M.F.T. Begonia, C.N. Gray, Growth Responses of Indian Mustard [*Brassica juncea* (L.) Czern.] and its Phytoextraction of Lead from a Contaminated Soil, *Bulletin of Environmental Contamination Toxicology*, 61 (1), 1998, pp. 38-43.

[30] B. Chakravarty, S. Srivastava, Toxicity of some heavy metals in vivo and in vitro in *Helianthus annuus*, *Mutation Research Letters,* 283 (4), 1992, pp.287-294.