THE EFFECTS OF HEAVY METALS ON SEED GERMINATION AND SEEDLING GROWTH OF LACTUCA SATIVA L. AND SPINACIA OLERACEA L.

A. MOȚ[1,2], Roxana MADJAR[1], Liliana BĂDULESCU[3], M. MIHALACHE[1]
[1] University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Agriculture
[2] University of Agronomic Sciences and Veterinary Medicine of Bucharest, Research Center for Studies of Food and Agricultural Products Quality
[3] University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Horticulture
mot_andrei@yahoo.com

Abstract. *Seed germination is very sensitive to unfavourable environmental conditions. An important factor of the environment, which inhibits most metabolic processes and, consequently, limits the growth and productivity of plants, is the presence of heavy metals. This study was conducted to determine the effects of the following heavy metals Cd, Cu, Ni, Pb, Co and Zn on germination and later, the growth of lettuce (Lactuca sativa L.) and spinach (Spinacia oleracea L.) seedlings. The experiment was performed using soluble salts of the metals mentioned above, that dissociate in water ($Cd(NO_3)_2•4H_2O$, $Cu(NO_3)_2•3H_2O$, $Ni(NO_3)_2•6H_2O$, $Pb(CH_3COO)_2•3H_2O$, $Co(NO_3)_2•6H_2O$, $Zn(CH_3COO)_2•2H_2O$). Five solutions with different concentrations were tested. Distilled water was used as control variant. The experimental variants consist on mixtures of the six metals in quantities ranging from reference values to values that exceeds intervention threshold for sensitive soils. Concentrations ranges as follows: Cd (3 to 20 ppm), Cu (20 to 200 ppm), Ni (25 to 200 ppm), Pb (30 to 150ppm), Co (20 to 100 ppm) and Zn (100 to 700 ppm). The abundance of this elements was chosen according to the values for trace chemical elements in soil (Order 756/1997). For conducting the study, 20 cm outer diameter Petri dishes were used, with 2 filter paper disks soaked in heavy metal solutions. Each repetition had a total of 25 seeds, that was replicated 4 times, in the pot resulting in a total of 100 seeds per plant species. A total of 1200 seeds were used. Seed germination tests were performed in accordance to ISTA - International Seed Testing Association (1999). A series of physiological indicators associated with germination and seedling vigour were calculated. The dynamics of the germination was followed for 6 days for Lactuca sativa L. and 10 days for Spinacia oleracea L., starting from the first day of germination. It has been observed that the influence of heavy metals on the germination and early development of the seedlings is very complex and determines a multitude of effects on many parameters, which in most situations do not have a standard or predictable distribution.*

Keywords*: germination, Lactuca sativa L., Spinacia oleracea L., toxicity, heavy metals*

INTRODUCTION

Although heavy metals are naturally found in small quantities in all ecosystems, they have become one of the major contaminants of plant and animal products. This is due to the anthropic activities, which have increased soil pollution worldwide. Even agricultural practices play a major role in the contamination of the environment, through the pesticides and fertilizers used (LAMASTRA et al., 2018; IEEP, 2019). The reason why heavy metals are considered a real risk is because they have a very high presence in the environment, over 1000 years, they have teratogenic and carcinogenic effects and they are also bioaccumulated and bioamplified in vegetal and animal organisms. (MANZETTI et al., 2014).

Heavy metals act differently in plant organs, generating multiple physiological, morphological or biochemical responses. Depending on the specificity of the plant, these responses may be more pronounced or less visible. These changes in plant behaviour have been studied for different plants, finding that the absorption and accumulation of heavy metals in the soil varies depending on the species, metal, concentration and organ of the plant (CU et al., 2015; AMER et al., 2017; GARÓFALO CHAVES et al., 2011). Lettuce (*Lactuca sativa L.*) and spinach (*Spinacia oleracea* L.) are two versatile species that have been used in many laboratory studies. The short vegetation period, the easy cultivation mode and the physiological particularities of these species have facilitated the study of many mechanisms of toxicity actions (BAGUR-GONZÁLEZ et al., 2010; ALIA et al., 2015). Even they are not considered hyperaccumulator plants, spinach and salad have been used in phytoremediation or bioremediation experiments, due to the short vegetation period and the fact that they accumulate a significant amount of heavy metals (MUSA et al., 2017; ABHILASH et al., 2016). Highlighting the effects of heavy metals in laboratory tests is a fast and efficient method for phytotoxicity testing on germination and growth stages of seedlings. Also, it can be used as a preliminary method for "in situ" use of plants for treating contaminated soils.

Germination, as an initial stage of the plant's life cycle, plays a major role in the plant's life. The germination potential of the seeds is influenced by different external or internal factors. Understanding these factors correlated with the biology of the species is necessary to ensure conditions within the tolerance limits of the plant. From a physiological point of view, a seed is considered to have germinated when the radicle has pierced the tegument, this stage being followed by the growth process (DELIAN et al., 2010).

The metals existing in the ecological systems are available for absorption process by plants in a certain proportion of the amount of metal existing in the soil, sediment, wateror atmosphere. Plants can easily uptake metals that are dissolved in the soil solution, either in ionic form, chelated or complexed (FAIRBROTHER et al., 2007). The main ways of uptaking the metals by plants are root uptaking and, less, leaf uptaking. Root absorption is performed both passively and actively. By the passive take-up path are absorbed Pb, Ni, and by the active take-up path are absorbed Cu, Mo, Zn. For Cd, there are assumptions that the transfer is made both active and passive (TUAN AND POPOVA, 2013). There are many types of metals found in soils, as micronutrients essential for normal growth (Fe, Mn, Zn, Cu, Mg, Mo and Ni, Co) and non-essential elements with unknown biological functions (Cd, Sb, Cr, Pb, Ace, Ag, Se and Hg). This study aimed to observe the behavior of seeds and seedlings in the presence of increasing concentrations of Zn, Cu, Ni, Co, Pb and Cd. The concentrations start from values close to the reference values of Order 756, 1997, and gradually increase until they exceed Intervention threshold for sensitive use of soils, established in the same order.

MATERIAL AND METHODS

The biological material was represented by the lettuce seeds *(Lactuca sativa L.)*, variety *Attraction* and spinach seeds (*Spinacia oleracea L.*), variety *Matador*. To establish the effect of heavy metals on seed germination and seedling growth, six soluble salts were used: $Cd(NO_3)_2{\bullet}4H_2O$, $Cu(NO_3)_2{\bullet}3H_2O$, $Ni(NO_3)_2{\bullet}6H_2O$, $Pb(CH_3COO)_2{\bullet}3H_2O$, $Co(NO_3)_2{\bullet}6H_2O$, $Zn(CH_3COO)_2{\bullet}2H_2O$ as soluble form of Cd, Cu, Ni, Pb, Co and Zn. Five mixtures were prepared, with different concentration of each metal, resulting five variants of treatments, denoted as C1, C2, C3, C4, C5 (Table 1). The concentrations were chosen to cover as much as possible the whole interval, from reference value to values that exceeds intervention threshold for sensitive use of soils, as described by Romanian regulation (Order 756/1997). Distilled water was used as control variant (M). To carry out the study, glass Petri dishes with an external diameter of 20 cm were used, containing 2 filter paper disks soaked in the heavy metal solutions. The vessels were previously disinfected with a hydroalcoholic solution (ethanol/distilled water 80/20). Each variant was replicated 4 times and each replicate counted 25 seeds. A total of 1200 seeds were used.

Table 1

Variants of concentrations used in experiment, relative to regulation values (Order 756/1997) (ppm)

Metal	Variants						Reference value in soils	Alert threshold sensitive soils	Intervention threshold sensitive soils
	M	C1	C2	C3	C4	C5			
Ni	0	25	75	100	150	200	20	75	150
Cu	0	20	60	100	150	200	20	100	200
Cd	0	3	5	10	15	20	1	3	5
Zn	0	100	300	450	600	700	100	300	600
Pb	0	30	50	75	100	150	20	50	100
Co	0	20	30	50	75	100	15	30	50

The experimental scheme is presented in the table below (Table 2).

Table 2

Experimental scheme

Seed Number	Lettuce				Spinach			
Replicate / Variant	R1	R2	R3	R4	R1	R2	R3	R4
M	25	25	25	25	25	25	25	25
C1	25	25	25	25	25	25	25	25
C2	25	25	25	25	25	25	25	25
C3	25	25	25	25	25	25	25	25
C4	25	25	25	25	25	25	25	25
C5	25	25	25	25	25	25	25	25
	Total number of lettuce seeds: 600				Total number of spinach seeds: 600			
	Total number of seeds: 1200							

In the first 24 hours after seed placement, the vessels were kept in dark conditions, afterwards being left in natural light at an average temperature of 21-22 °C on daytime and 18-19 °C on night, until the end of the experiment.

A series of physiological indicators associated with germination and seedling growth were calculated. Those are presented in the table 3.

Table 3

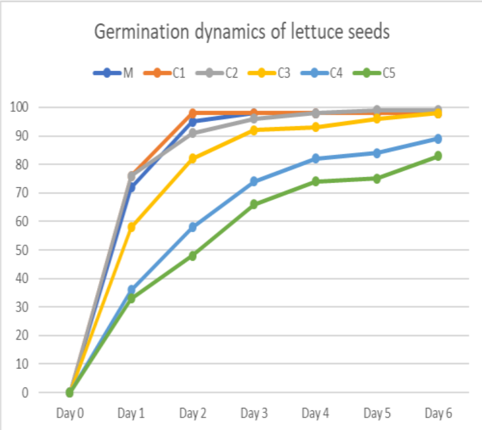
List of physiological indicators associated with germination and seedling vigor

Indicator	Unit	Formula	Source
Germination			
Final germination percentage	FGP (%)	(total number of germinated seeds / total number of planted seeds) x100	Ranal and Santana, 2006, according to ISTA, 1999
Germination speed	GS	Sum (number of germinated seeds on day ti / ti) (not cumulated from one day to another)	Maguire, 1962
Germination speed modified	GSmod	GS/FGP*100	Evetts and Burnside, 1927
Maximum germination value („peak value”)	PV	FGP / the number of days required to reach the maximum germination value	Czabator, 1962
Physiological indicators regarding the effects of toxicity on early seedling development			
Seedling growth inhibition	SGI (%)	(Seedling length of control variant - Seedling length of tested variant) / length of control variant x100	Ali et al., 2015
Final percentage of viable seedlings	FPP (%)	(Number of viable seedlings at the end of the experiment / total number of seeds) x100	Mangena et al., 2019

RESULTS AND DISCUSSIONS

Germination dynamics of seeds

Regarding the germination dynamics, the results are shown in Figure 1. The seed germination started on the second day after the seeds were placed in the Petri dishes, excepting spinach seeds from C2 variant.



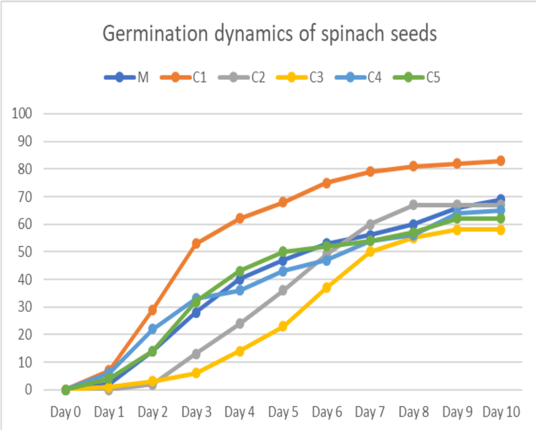


Figure 1. Germination dynamics of lettuce seeds (A) and spinach seeds (B)

Also, as can be seen in Figure 1, the number of germinated seeds is influenced by the concentration levels. An interesting fact has been shown by C1variant for spinach (Figure 1A), which has a better evolution than all variants, including the control. A reason for this evolution can attributed to the low level of contamination that did not affect negatively the seeds, but on the other hand, the low levels of beneficial elements helped to stimulate germination. The difference between this variant and the others will be analyzed on the following indicators.

Final germination percentage

There were counted the seeds whose radicle reached a length of at least 2 mm. At this time the seeds were considered germinated. Final germination percentage (FGP) is shown in Figure 2.A and 2.B.

The maximum final germination percentage (FGP) for lettuce seeds was reported for C2 treatment (99%), better than the control variant which reported a value of 98%. Yet, the ANOVA unifactorial test ($\alpha=0.05$) showed that are no significant difference between control variant (M) and C1, C2, C3 variants (p-value>0.05). C4 variant showed a significant difference beside M variant (p-value=0,0311) and C5 showed a highly significant difference (p-value< 0,01) beside control variant (M) (Figure 2.A).

The maximum final germination percentage (FGP) for spinach seeds was reported for C1 treatment (83%). The control variant reported a value of 69%. Using the ANOVA unifactorial test ($\alpha=0.05$) showed that are no significant difference between control variant (M) and C2, C3, C4 and C5 variants (p-value>0.05), but C1 variant showed a significant difference beside M variant (p-value=0.7331) (Figure 2.B).

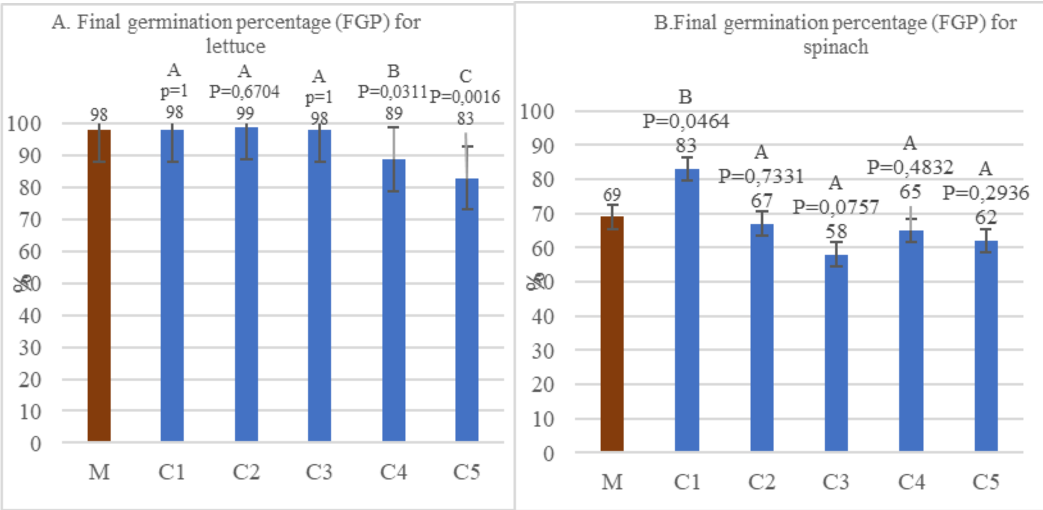


Figure 2. Final germination percentage (FGP) of lettuce seeds (A) and spinach(B) (means±SE)

Germination speed and Germination speed modified

Germination speed can be used as an indicator to evaluate the seedlings vigour. High values obtained using this expression mean higher seedling vigour of one sample. It can pe observed in Figure 3.A that the speed of germination of lettuce has a descendent way, according with the levels of contamination. Germination speed modified introduce a correction to this factor, evaluating the values in concordance with final germination percentage. In this way, the errors caused by non-germinated seeds are eliminated. In Figure 3.B it can be observed that introducing that correction, the values of germination speed modified form are improved.

Also, it can be observed that the C2 and C3 variant had lower values than the extremity variants (M, C1, C4, C5).

For lettuce, from statistical view point (ANOVA test unifactorial test (α=0.05)), there were not registered significant differences for speed germination between control and lower concentrations (C1, C2, C3), but there was a highly significant difference regarding high concentrations (C4, C5).

For spinach, there was observed a significant difference for speed germination between control and C1 variant, and no significant differences for control and the rest of the variants (C2, C3, C4, C5).

Table 4

ANOVA P-value comparing control variant with each variant for germination speed modified formula

Variant	M	C1	C2	C3	C4	C5
Lettuce P-value	A	A 0,1464	A 0,7999	A 0,0260	C 0,0004	C 0,0001
Spinach P-value	A	B 0,0048	A 0,0221	A 0,0373	A 0,1393	A 0,1812

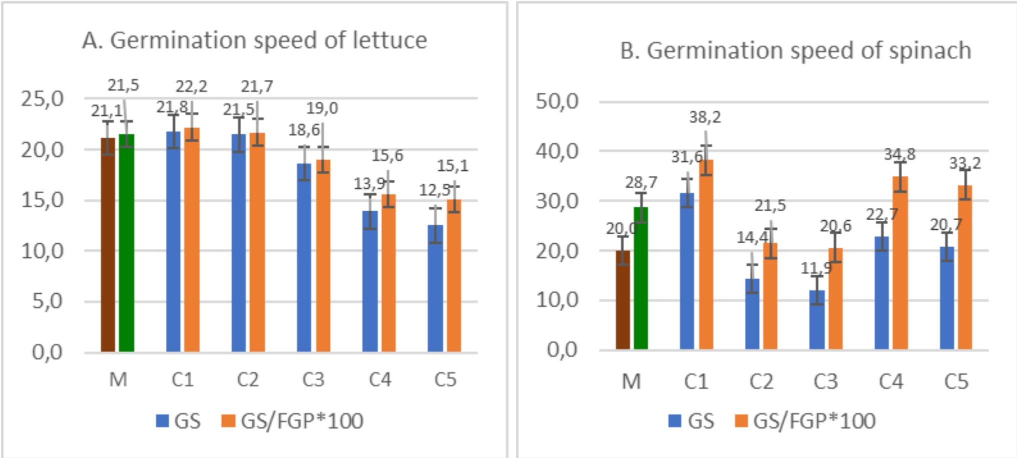


Figure 3. Germination speed of lettuce (A) and spinach (B) (means ±SE)

Maximum germination value

For all variants excepting spinach seeds from C2, germination started on the second day after the seeds were placed in the Petri dishes. On lettuce experiment, the maximum germination value was reported on C1 variant (49), followed by the control variant (36.83). Running ANOVA test unifactorial test (α=0.05), it is shown that there are significant difference between control variant and C1. The same response was obtained between control and C2 variant. The maximum germination value for C4 and C5 variants are lower, here is a highly significant difference beside control. The lowest germination value was observed on C5 variant (13.83).

On spinach experiment, the maximum germination value was reported on C1 variant as well (9.61), and the minimum value was observed on C3 variant (6,63). Yet, there are no significant difference between control and any of other variants. (Figure 4).

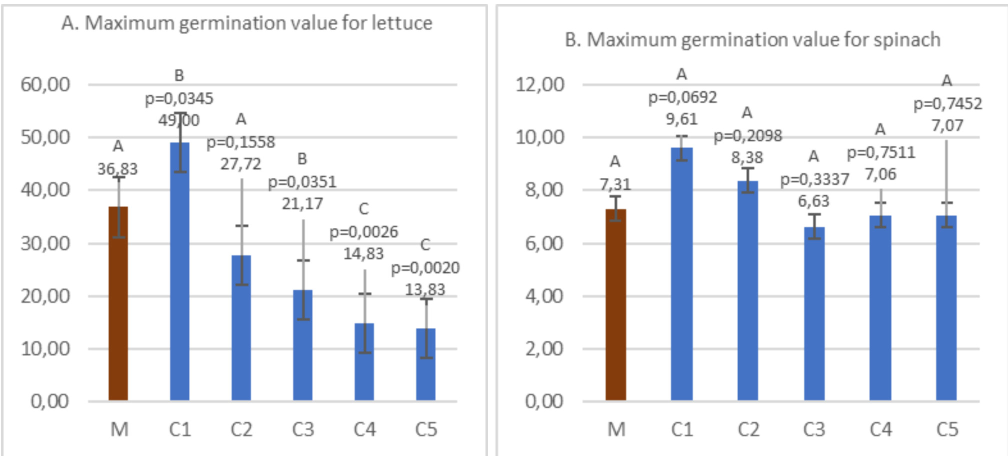


Figure 4. Maximum germination value of lettuce (A) and spinach (B) (means ±SE)

Seedling growth inhibition

At the end of the seed germination test, depending on the state of viability, measurements for each variant and repetition were performed for 4 seedlings on seedling length and radicle length. A comparison of evolution of seedlings under the six treatments, at the final moment of the study is pointed out in the figure 5.A for lettuce seedlings and figure 5.B for spinach.

Figure 5. Seedling growth under heavy metal treatments. Lettuce (A) Spinach (B)

Considering that the control variant which was treated only with distilled water is not inhibited at all (SGI index is 0 for control) is can be easily observed both from the figure 5 and from the mean values of SGI index (table 5) that the inhibition process is emphasizing with the level of contamination with heavy metals. For C5 variant, in both cases lettuce and spinach, the growth inhibition exceeds 97%. Running ANOVA unifactorial test (α=0.05) test over the SGI index of all variants, the control is significantly different form all other variants, both for lettuce and spinach (Figure 6).

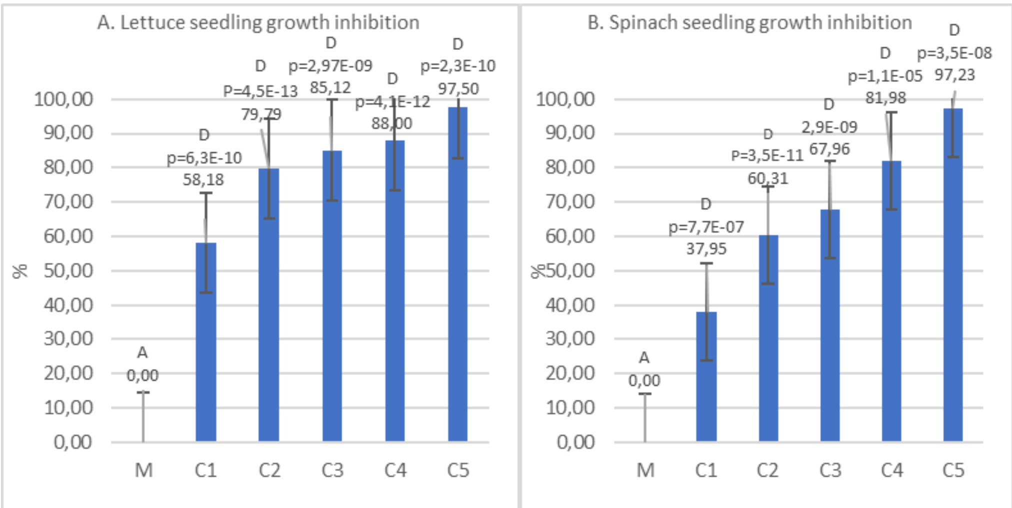


Figure 6. Seedling growth inhibition for lettuce (A) and spinach (B) (means ±SE)

Final percentage of viable seedlings

The number of viable seedlings was evaluated at the end of germination and was considered to be complete when the seedlings no longer exhibited new symptoms of toxicity, and their growth continued. It was noted that in the case of C5 variants, at the end of the specific period, the viability of the seedlings was just 1%, for both lettuce and spinach seedlings. ANOVA test reported that are no significant difference between control variant and C1 variant ($p>0.05$), for both studied species. For lettuce, there are highly significant differences between control and C2, C3, C4 and C5 variants. For spinach, there is a significant difference between C2 and control, but also, highly significant differences between control and C3, C4 and C5 variants (Figure 7).

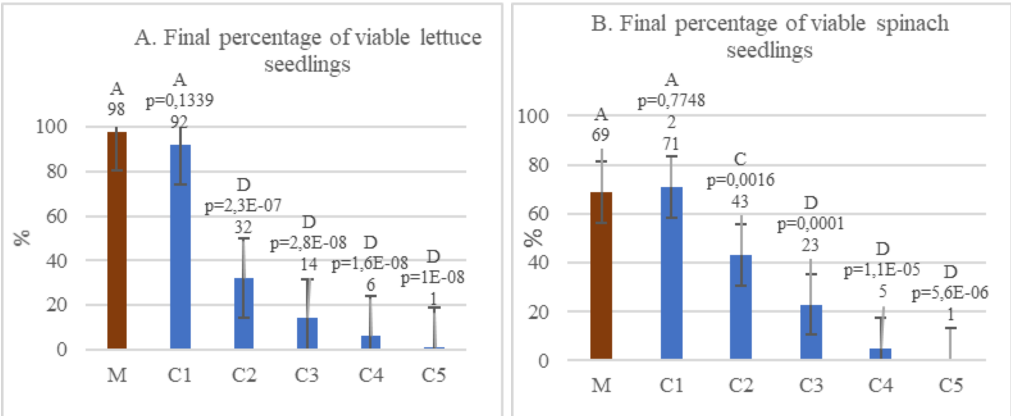


Figure 7. Final percentage of viable seedlings of lettuce (A) and spinach (B) (means ±SE)

CONCLUSIONS

On germination:

Regarding the Final Germination Percent in uncontaminated environment, the lettuce had a better result (98) than spinach (69%).

The results show that the final germination percent is not influenced in a significant way at lower levels of contamination with heavy metals, but at high levels, the germination decreases gradually. Also, at lower levels, the germination seems to be accelerated for both lettuce and spinach seeds. At high concentration, the speed of germination is slower than the control for lettuce seeds, but for spinach seed in seems to keep the same value as control variant.

On early seedling development:

Regarding the growth inhibition, even a small quantity of heavy metals can induce the inhibition. The growth of seedlings was significantly reduced from the first level of contamination and this trend has been maintained to the maximum levels of contamination for both species.

The final percentage of viable seedlings for lettuce decreased with the increase of concentration of heavy metals. At lower concentrations the viability maintained the same direction as the control variant, but at maximum levels the viability aims to 0%.

The spinach seedlings viability was higher at the first level of contamination than the control variant, but not significantly. For the rest of the concentration, the viability followed the same direction as the lettuce seedlings.

BIBLIOGRAPHY

ABHILASH, M.R., SRIKANTASWAMY, S., SHIVA KUMAR, D., JAGADISH, K., SHRUTHI, L., 2016 - Phytoremediation of Heavy Metal Industrial contaminated soil by Spinacia oleracea L and Zeamays L. IRA-International Journal of Applied Sciences, 4 (1):192-199

ALI, A., PHULLB, A.R., ZIAC, M., SHAHB, A.M.A., MALIKA, N.M., HAQ, I., 2015 - Phytotoxicity of River Chenab sediments: In vitro morphological and biochemical response of Brassica napus L. Environmental Nanotechnology, Monitoring & Management, 4: 74-84

ALIA, N., SARDAR, K., SAID, M., SALMA, K., SADIA, A., SADAF, S., TOQEER, A., MIKLAS, S., 2015 - Toxicity and Bioaccumulation of Heavy Metals in Spinach (Spinacia oleracea) Grown in a Controlled Environment. Int J Environ Res Public Health, 12(7): 7400–7416

AMER, M., TYLER, A., FOUDA, T., HUNTER, P., ELMETWALLI, A., WILSON, C., VALLEJO-MARIN, M., 2017 - Spectral characteristics for estimation heavy metals accumulation in wheat plants and grain. Scientific Papers Series Management, Economic Engineering in Agriculture and Rural Development, 17 (3): 47-56

BAGUR-GONZÁLEZ, M.G., ESTEPA-MOLINA, C., MARTÍN-PEINADO, F., MORALES-RUANO, S., 2011 - Toxicity assessment using Lactuca sativa L. bioassay of the metal(loid)s As, Cu, Mn, Pb and Zn in soluble-in-water saturated soil extracts from an abandoned mining site. Journal of soils and sediments 11 (2): 281-289

CU, N.X., 2015 - Effect of Heavy Metals on Plant Growth and Ability to Use Fertilizing Substances to Reduce Heavy Metal Accumulation by Brassica Juncea L. Czern. Global Journal of Science Frontier Research: Agriculture and Veterinary, 15 (3): 35-40

CZABATOR, F.J., 1962 - Germination Value: An Index Combining Speed and Completeness of Pine Seed Germination. Forest Science, 8:386-396

DELIAN E., BĂDULESCU, L., DOBRESCU, A., 2012 - Fiziologia plantelor. Lucrări practice. Ediţia a II-a. Editura Universitară. 145 pag. Bucuresti

EVETTS, L.L., BURNSIDE, O.C, 1972 - Germination and Seedling Development of Common Milkweed and Other Species. Weed Science 20(4):371-378

GARÓFALO CHAVES, L.H., ESTRELA, M.A., DE SOUZA, R.S., 2011 - Effect on plant growth and heavy metal accumulation by sunflower. Journal of Phytology, 3(12): 04-09

IEEP, Report on the Implementation of the Sewage Sludge Directive 86/278/EEC, May 2009

MAGUIRE, J.D., 1962 - Speed of germination - Aid in selection and evaluation for seedling emergence and vigor. Crop Science, 2(2):176—177

MANZETTI, S, van der SPOEL, R., van der SPOEL, D., 2014 - Chemical Properties, Environmental Fate, and Degradation of Seven Classes of Pollutants. Chemical Research in Toxicology, 27(5): 713-737

MANGENA, P., MOKWALA, P.W., 2019 - The Influence of Seed Viability on the Germination and In Vitro Multiple Shoot Regeneration of Soybean (Glycine max L.). Agriculture 9 (3): 1-12

MUSA, D.M., GARBA, Y., YUSUF M.S., DANJUMA, M.S., 2017 - Potential of lettuce(Latuca sativa) for phytoremediation of Cd, Cu, Cr and Pb in contaminated soil along river Salanta. Dutse Journal of Pure and Applied Sciences (DUJOPAS), 3 (2): 258-269

ORDER no. 756, November 3, 1997 for the approval of the Regulation regarding the assessment of environmental pollution, Romania

RANAL, M., De SANTANA, D.G., 2006 - How and why to measure the germination process?. Revista Brasil. Bot., 29 (1): 1-11

TUAN, T.A., POPOVA, L.P., 2013 - Functions and toxicity of cadmium in plants: Recent advances and future prospects. Turkish Journal of Botany 37(1):1-13

FAIRBROTHER, A., WENSTEL, R., SAPPINGTON, K., WOOD, W., 2007 - Framework for Metals Risk Assessment. Ecotoxicology and Environmental Safety 68:145–227