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The Effect of Some Heavy Metals (Cd, Cu, Pb, Zn) and Substrates on Chelidonium majus L. Seed Germination and Seedling Growth

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Abstract. The increasing heavy metal presence in the environment affects plant growth and physiology, seed production and crop quality. Moreover, it could indirectly influence people's health and the quality of the environment. *Chelidonium majus* L. is an object of study in order to investigate its sensitivity to the toxic effects of heavy metals because of its growth in populated areas and common occurrence. The results of this study revealed that the heavy metal influence on seed germination and seedling growth depended on the type and concentration of the heavy metals and the type of the substrate they supplemented. The studied heavy metals (Cd, Cu, Pb, Zn) did not inhibit the germination of the seeds on filter paper but retarded root and hypocotyl growth on this substrate, except Pb^{2+}, which did not. On agar substrate the metals inhibited both the germination (excl. Pb^{2+}) and seedling growth regardless of concentrations applied.

Key words: Greater celandine, heavy metal toxicity, tolerance index, filter paper, water agar.

Introduction

Heavy metals are among the main pollutants in the environment. They affect plant development, seed germination, slow down photosynthesis and decrease yield, seed production and crop quality. Apart from their direct influences, soil quality could indirectly influence people's health and the quality of the environment (Oliver, 1997; Stasinos et al., 2014). Anthropogenic activities such as mining, smelting, burning of fossil fuels and agricultural practices involving the excessive use of pesticides, fungicides, fertilizers and sewage sludge have led to an increase in heavy metal content in soil in different areas (Ahmad et al., 2012; Chen et al., 2005). The increasing environmental pollution requires an environmentally-friendly technology such as phytoremediation. It is a low-cost sustainable approach which is based on the capacity of some plant species to purify contaminated soils and water (Laghlimi et al., 2015). Screening for plant species with promising characteristics for phytoremediation is the first but basic and important step in broadening the knowledge of phytoremediation and its future perspectives. *Chelidonium majus* L. is a perennial medicinal species which is widely distributed across Europe (Euro+Med, 2006-2020). In Bulgaria the species is distributed across rocky and damp shadowed habitats, hedgerows and as a ruderal species beside woodlands. It is widely distributed across the country,

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except along the Black sea coast, Struma valley, Tundzha hilly plain up to 1,500 m above sea level (Jordanov, 1970).

The species' fast growth, relatively high biomass production and common occurrence in populated areas make the species an appropriate object of study in order to investigate its sensitivity to the toxic effects of heavy metals as a potential species for phytoremediation. A few reports suggested the species' ability to accumulate organic and inorganic soil pollutants such as phenanthrene, arsenic, lead, mercury and cadmium (Badea, 2015; Zhang et al., 2011, 2014). The germination and seedling growth are frequently used to assess the tolerance of a plant species to heavy metals because these charactaristics often are very sensitive to the environmental conditions (Peralta et al., 2001; Seregin & Ivanov, 2001).

The aim of the study was to evaluate seed germination ability and seedling development on two types of substrates supplemented with different concentrations of Cd, Cu, Pb, Zn in order to define the species tolerance towards them. The heavy metal sensitivity is species-specific, therefore, the measures of germination, plant growth and tolerance index were used as indicators to assess the metal toxicity to *Ch. majus*. It is known that different substrates and conditions could impact the germination and root growth. Filter paper which is most commonly used in germination experiments is considered to interfere with metal ions (Di Salvatore et al., 2008). Therefore, it was necessary to compare it to another substrate (which is water agar) to analyze the influence of the substrate type and this type of *in vitro* conditions on seed germination in this species and to assess the substrate influence on metal toxicity. The studied Cd and Pb are among the commonly occurring heavy metals. They do not participate in physiological processes in plants and are very toxic metals which are harmful even in minor quantities. On the other hand, Cu and Zn are essential microelements necessary for plant growth and development, but they are toxic in excessive concentrations.

The studies on *Ch. majus* were mainly connected with its medicinal properties and effects in use based on the alkaloids which the species biosynthesize (Maji & Banerji, 2015). Up to now studies on heavy metal toxicity to *Ch. majus* germination and seedling growth have not been reported.

Material and Methods

Seed origin. Seeds were gathered from native plants of *Ch. majus* grown in the village of Mramor, near Sofia.

Seed sterilization. Seeds were pre-soaked in distilled water for 24 hours. They were first surface sterilized with 70% ethanol for 2 minutes, then were soaked in 0.1% $HgCl_2$ for 2 minutes and rinsed three times in sterile distilled water. As a last step in surface sterilization seeds were sterilized in commercial bleach (chlorine < 2.5%) half diluted with sterile water for 10 minutes and afterwards the seeds were rinsed again three times with sterile distilled water.

Seed germination and seedling growth conditions. Compounds which were used to create metallic stress were $Pb(NO_3)_2$, $ZnSO_4 \cdot 7H_2O$, $CdCl_2 \cdot 2½H_2O$ and $CuSO_4 \cdot 5H_2O$. They were applied in three concentrations of Pb^{2+}, Zn^{2+} and Cu^{2+} ions: 100; 150; 250 mg/l, and Cd^{2+} ions were added in concentrations of 1; 5 and 10 mg/l. The chosen concentrations were determined taking into account the Bulgarian standards for permissible content of harmful elements in soils (Ordinance № 3, 2008). The sterilized seeds were put onto two types of substrate – filter paper and water agar. Sterilized glass petri dishes (90 mm in diameter), were covered with two layers of sterilized filter paper (Whatman № 1), moistened with 4 ml of water solutions of heavy metals in the different concentrations or only with distilled water for the control. For the agar medium, water solutions of the heavy metals were solidified with 7 g/l agar (Duchefa, NL) and autoclaved at 121ºC for 20 minutes. In the water agar experiment $CuSO_4 \cdot 5H_2O$ was not added because of the severe decrease in pH and the incapability of agar to harden. The petri dishes used were 90 mm in diameter. The control variants contained distilled water agar only.

All petri dishes were wrapped with Parafilm. They were kept in dark at 8±2°C for 7 days. After that, they were placed at 23±2°C under dark conditions for an additional 2 weeks and then were cultivated at 23±2°C with photoperiod of 16h light/8h dark.

Each treatment had 3 or 2 replicates (for filter paper and water agar dishes, respectively) with 20 seeds in each. The seed coat protrusion was considered a sign of germination.

Statistical analyses. The statistical analyses were made using SigmaPlot v. 14.0 and statistical significance was evaluated with Student's t-test (p=0.05).

Studied heavy metal toxicity parameters. Several parameters were measured in order to determine the species' ability to germinate and develop in heavy metal presence: germination percentage, relative germination rate, mean root length, mean hypocotyl length, root/shoot ratio, tolerance index, phytotoxicity percentage (Rasafi et al., 2016).

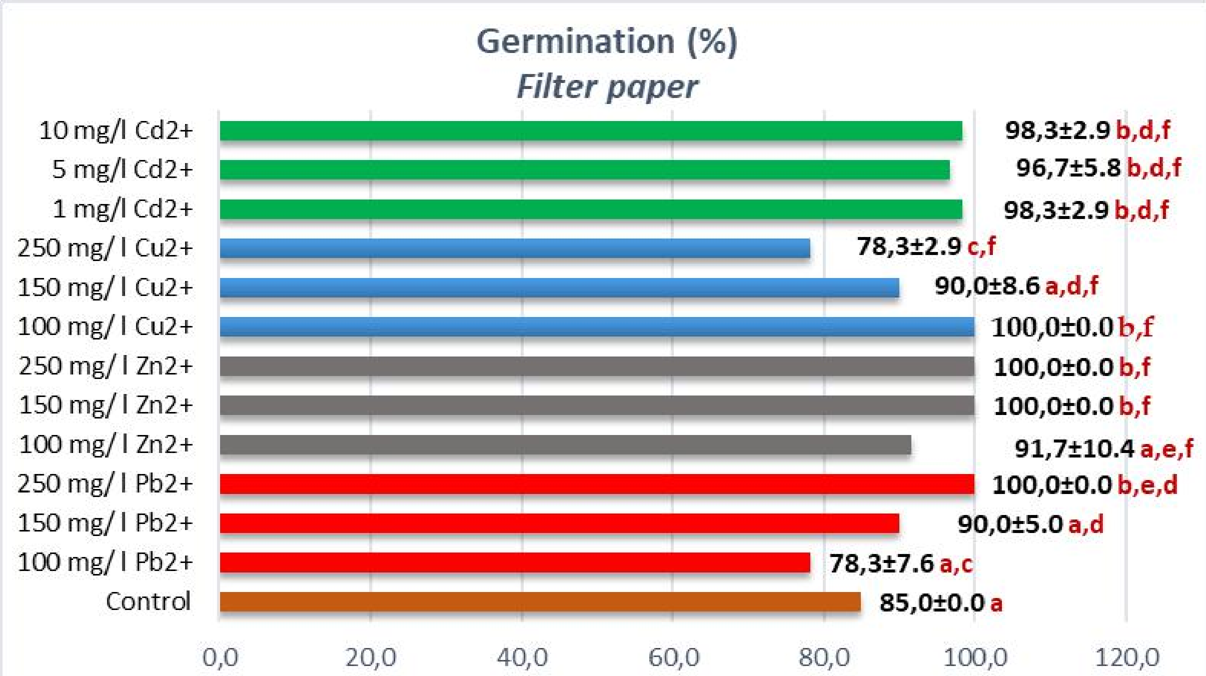
Results

Germination percentage. The germination percentage of the seeds sown on filter paper was high (over 75%) in all concentrations applied and for all heavy metals used (Fig. 1-A). On agar substrate the germination was low – less than 50%. In contrast, only for Pb^{2+} in all its applied concentrations the germination was 75% and more, which was even higher than the control (Fig. 1-B).

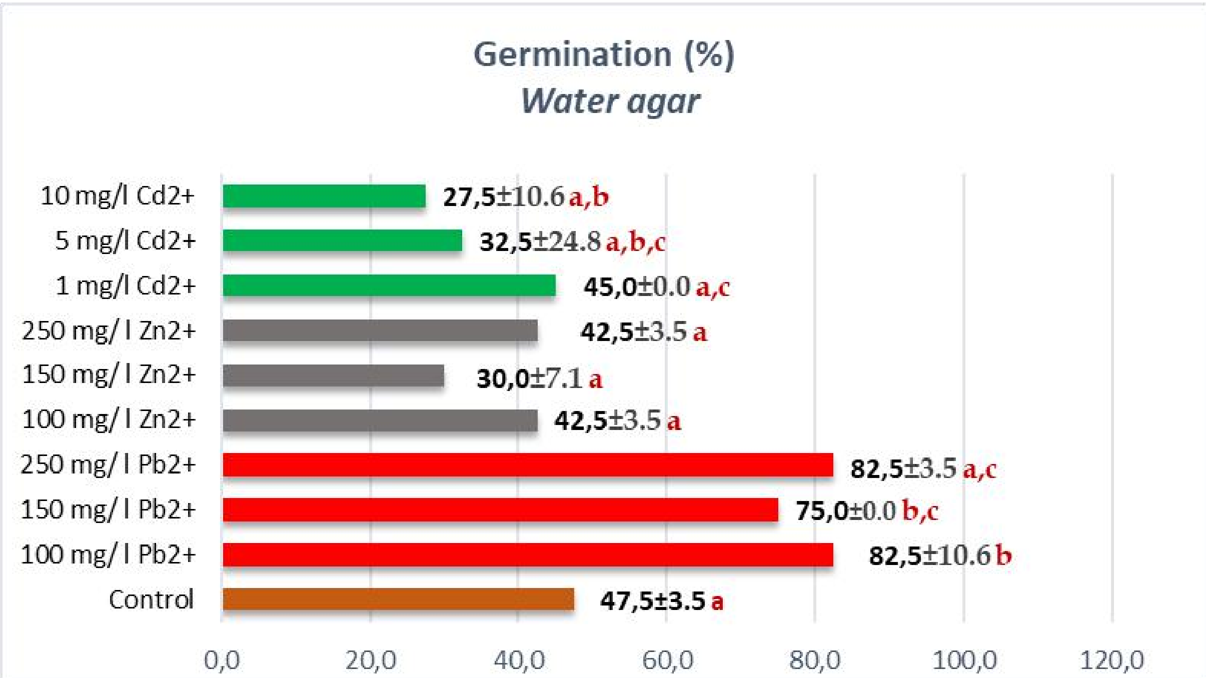
Effect of the applied heavy metals on relative germination rate. There was a strong inhibiting influence of the heavy metals in water agar. Germination did not decrease with the increase in heavy metal concentrations on filter paper. However, there was a rise in the germination percentages for all heavy metals used and compared with the control (Table. 1). On filter paper only for Cu^{2+} was observed progressive inhibition of the germination with the increase in Cu^{2+} concentration. In water agar this tendency was observed for the Cd^{2+} variants.

Mean root length under heavy metal stress. The roots of seedlings which were growing on filter paper moistened with Pb^{2+} solution were significantly longer than all variants on this type of substrate (Fig. 2-A). There was a little decrease in length at the highest Pb^{2+} concentration even though it remained higher than that of the control. Seedlings grown on filter paper dampened with the lowest Cd^{2+} concentration had roots with comparatively high length, but it sharply decreased at the higher concentrations used. In terms of the variant with Zn^{2+}, there was a significant reduction in root length, which was proportional to the increase in Zn concentration. Root growth was completely halted in the variant where Cu^{2+} was used. The inhibiting influence of heavy metals was considerably more expressed in the substrate of water agar (Fig. 2-B). When it was supplemented with Pb^{2+} and Zn^{2+}, the roots were black and underdeveloped, except for Cd^{2+}, where the inhibiting effect was weaker.

Mean hypocotyl length under heavy metal stress. For all heavy metals, the higher applied concentrations were, the shorter the hypocotyl length was. It was higher at lower concentrations of lead and cadmium than it was in the control seedlings. Zn^{2+} and Cu^{2+} inhibited hypocotyl growth, where the most significant inhibition occurred for Cu^{2+} treated seedlings and was observed even at the lowest concentrations of the element applied (Fig. 3-A). The growth preventing effect of heavy metals on hypocotyls was again significantly stronger in water agar. The highest hypocotyl length of seedlings developed in water agar supplemented with heavy metals was observed in 1 mg/l Cd^{2+} treatment. In concentrations of 5 and 10 mg/l Cd^{2+} the inhibiting effect was more prominent, close to that observed in the variant treated with lead and Zn^{2+}. Hypocotyls did not develop at the highest Zn^{2+} concentration (Fig. 3-B).

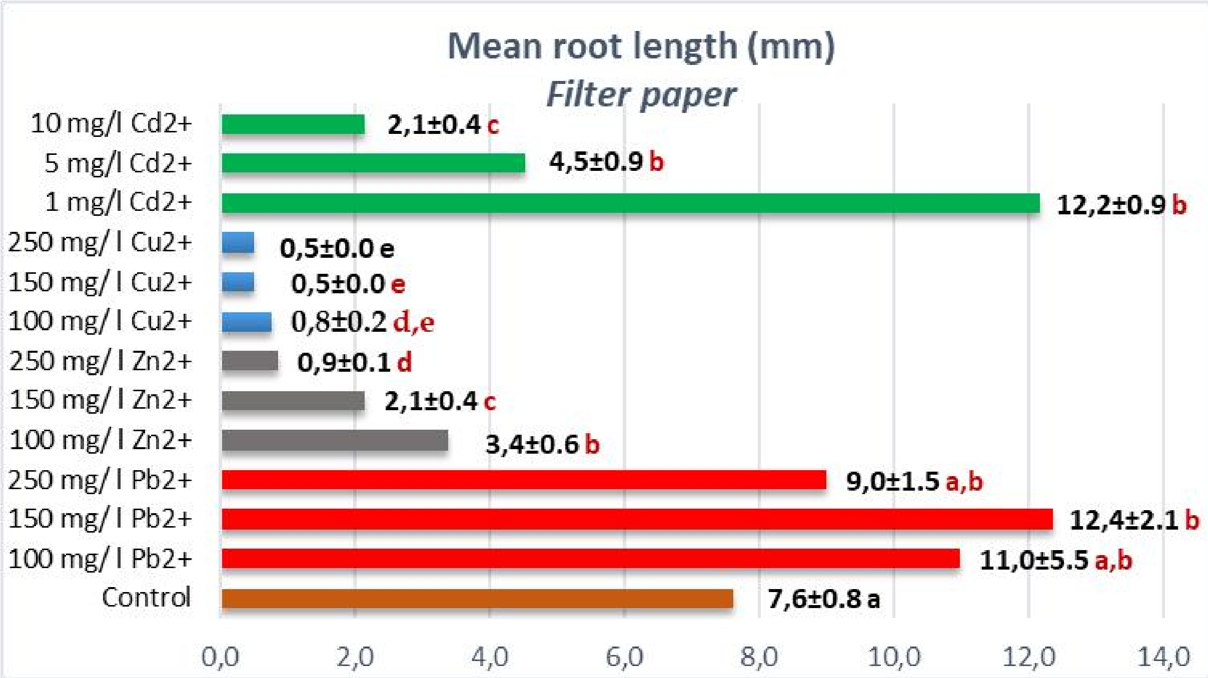


A)

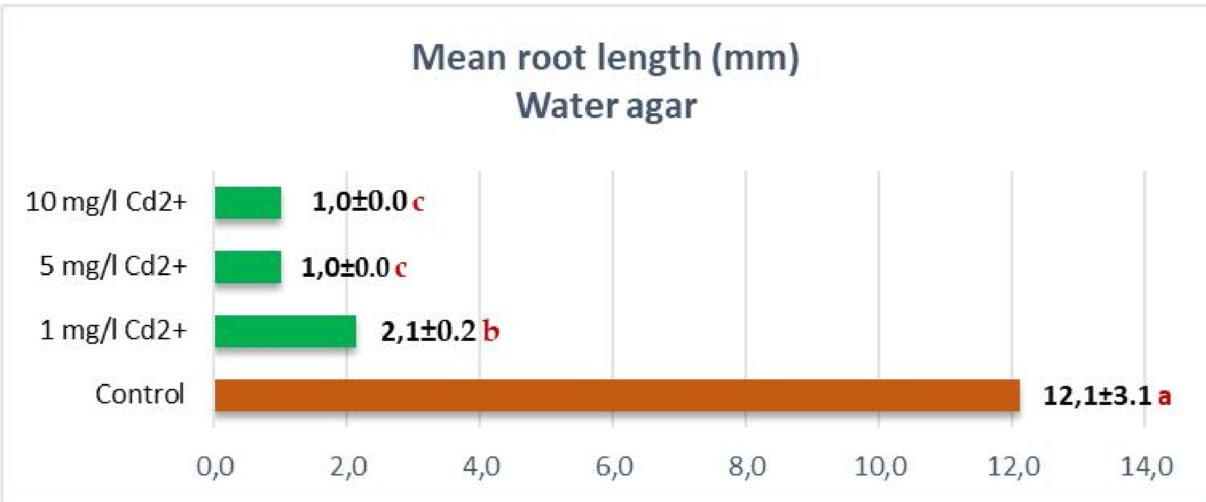


B)

Fig. 1. Germination percentage (%) ± Standart deviation on filter paper **(A)** and water agar **(B)**. Values with different letters are significantly different.



A)



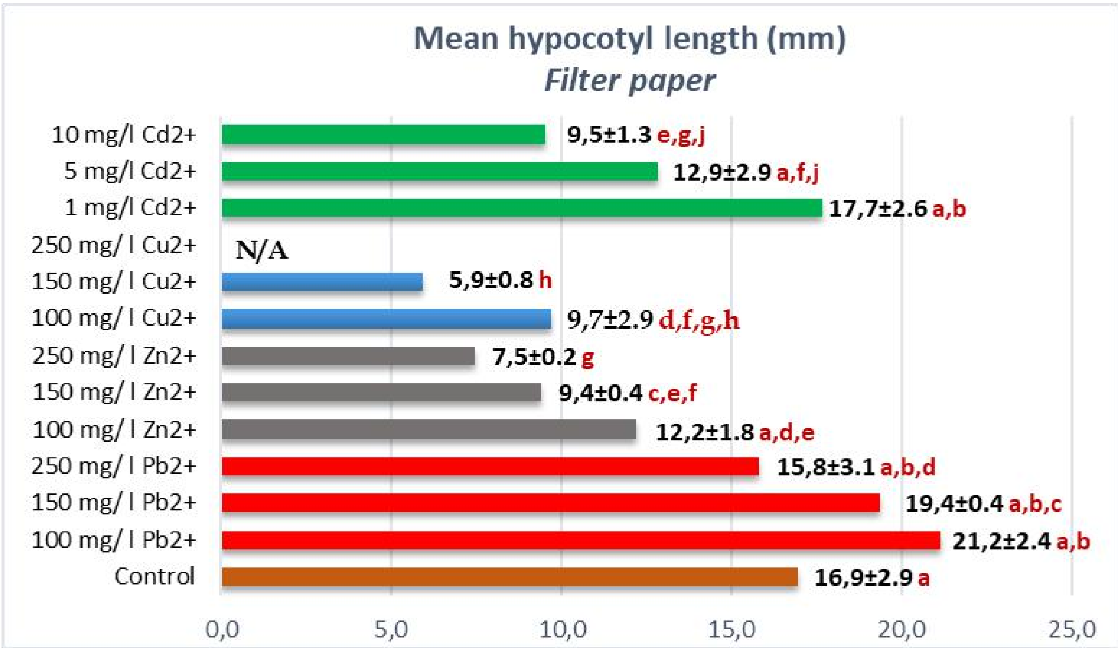
B)

Fig. 2. Mean root length (mm) of seedlings grown on filter paper (A) and water agar (B) supplemented with different concentrations of heavy metals. Values with different letters are significantly different.

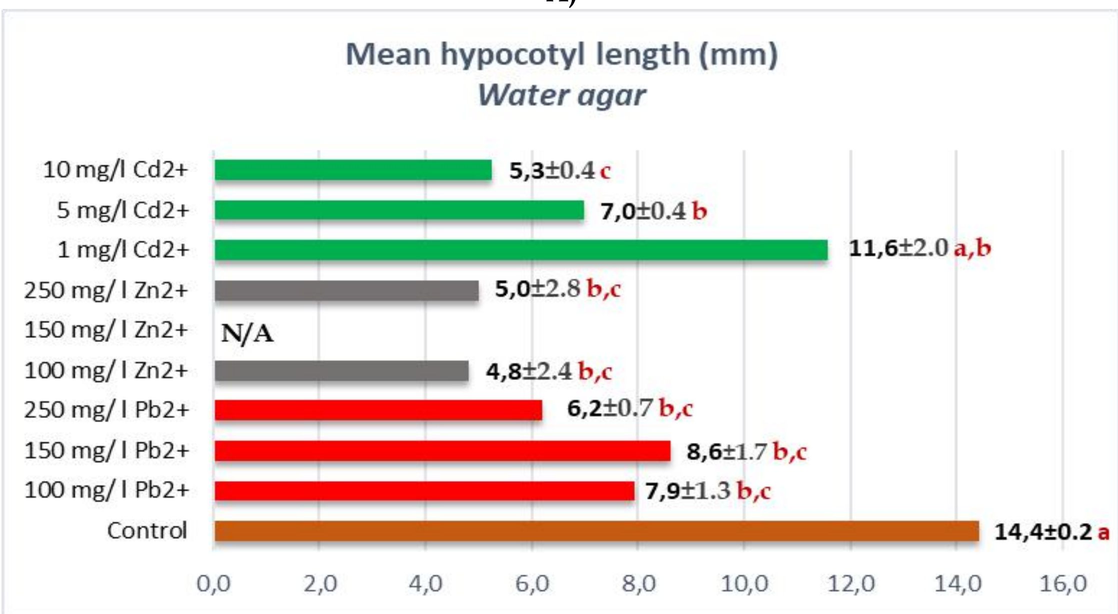
Root/shoot ratio. This indicator showed that the Pb^{2+} effect on seedling development did not vary widely at the different concentrations on filter paper, but it was absolutely detrimental for the root development on agar substrate (Table. 1). Of all applied metals on filter paper, Cu^{2+} most strongly inhibited seedling growth. The ratio tended to decrease for Zn^{2+} and Cd^{2+} with the increase in their concentrations on filter paper. The toxic influence was huge on water agar regardless of the concentration applied.

Tolerance index. The tolerance index decreased with the concentration increase of all heavy metals although their influence on germination was small. On filter paper the lowest tolerance of the plants was for Cu^{2+} and for Cd^{2+} there was a sharp drop at the highest concentration of 10 mg/l Cd^{2+} (Table. 1). Zn^{2+} and Pb^{2+} ions completely halted seedling development on the water agar substrate and there was low tolerance for Cd^{2+} on the same substrate.

Phytotoxicity. Cu^{2+} had the strongest phytotoxic effect on *Ch. majus*, followed by Zn^{2+}, Cd^{2+} and Pb^{2+} in filter paper. Phytotoxicity increased when the heavy metals were added to the agar medium. Cd^{2+} was less phytotoxic in filter paper, but as it can be seen, Cd^{2+} was less phytotoxic than Pb^{2+} and Zn^{2+} in water agar instead of in filter paper (Table. 1).



A)



B)

Fig. 3. Mean hypocotyl length (mm) of seedlings grown on filter paper (A) and water agar (B) supplemented with heavy metals. Values with different letters are significantly different.

Table 1. Values of some of the studied parameters on filter paper and water agar supplemented with heavy metals. *Legend:* Values with different letters are significantly different.

Concentration (mg/l)	Relative germination rate		Tolerance index		Phytotoxicity percentage (%)		Root/shoot ratio	
	Filter paper	Water agar	Filter paper	Water agar	Filter paper	Water agar	Filter paper	Water agar
Control								
-	7.6±0.8	12.1±3.1	16.9±2.9	14.4	0.0	0.0	0.5	0.8
Pb^{2+}								
100	10.9±5.5	N/A	21.2±2.4	7.9	-0.4	N/A	0.5	N/A
150	12.4±2.3	N/A	19.4±0.4	8.6	-0.6	N/A	0.6	N/A
250	9.0±1.5	N/A	15.8±3.1	6.2	-0.2	N/A	0.5	N/A
Zn^{2+}								
100	3.4±0.6	N/A	12.2±1.8	4.8	0.6	N/A	0.3	N/A
150	2.2±0.4	N/A	9.4±0.4	5.0	0.7	N/A	0.2	N/A
250	0.9±0.1	N/A	7.5±0.2	N/A	0.9	N/A	0.1	N/A
Cu^{2+}								
100	0.8±0.2	N/A	9.7±2.9	N/A	0.9	N/A	0.1	N/A
150	0.5±0.0	N/A	5.9±0.8	N/A	0.9	N/A	0.1	N/A
250	0.5±0.0	N/A	N/A	N/A	0.9	N/A	N/A	N/A
Cd^{2+}								
1	12.2±0.9	2.1±0.2	17.7±2.6	11.6	-0.6	0.8	0.7	0.2
5	4.5±0.9	1.0±0.0	12.9±2.9	6.9	0.4	0.9	0.4	0.1
10	2.2±0.4	1.0±0.0	9.5±1.3	5.3	0.7	0.9	0.2	0.2

Discussion

The results obtained showed that the heavy metal influence on seed germination and seedling growth depended on the type and concentration of the heavy metals and the type of the substrate they supplemented.

Overall, heavy metals did not inhibit the germination of the seeds which were germinated on filter paper, but retarded root and hypocotyl growth on this substrate. Such a tendency has been observed in *Arabidopsis thaliana*, *Glycine max*, *Triticum aestivum* (Araujo & Monteiro, 2005; Li et al., 2005; Yang et al., 2010). It was considered to be related to the seed coat. Many chemicals could not be absorbed by the seeds due to the seed coat. Moreover, their reserves make the germination possible even in unfavourable conditions (Kapustka, 1997). Besides this, the seed coat protrusion does not necessarily mean growth through cell division, but it could be due to cell elongation (Chon et al., 2004; Haber & Luippold, 1960).

It is known that root growth is more sensitive to heavy metal influence than germination (Araujo & Monteiro, 2005). The inhibiting effect of heavy metals on seedling root growth after germination is observed in a wide variety of studied species. The diminished root and hypocotyl growth, which affected the values of the other calculated parameters, revealed the extent of the toxic effect of a given element on the species. The retarted growth of hypocotyls and roots could be due to the impact of heavy metals on cell division and metabolite activity (Hargemeyer & Breckle, 1996; Naseer et al., 2001). The strong reduction in root growth might be because of their greater susceptibility to heavy metals being the first contact point which is exposed to their toxic impact (Shah et al., 2010; Yang et al., 2010).

Roots absorb and accumulate metals more often and that is why metals impact roots more strongly than the aboveground parts of the plant (Öncel et al., 2000). The different presence of heavy metals in plant organs resulted in the different degree of root and hypocotyl growth inhibition, because the heavy metals remain in the cell walls and close to the place of intake. Metals are found to be more concentrated in roots than in stems (Greger, 2004).

In this study it was shown that the substrate type is a factor which influences the toxicity of the heavy metals and that is why it is important to be taken into account. All studied parameters of seeds germinated and developed on agar substrate were worse than those on filter paper, which revealed the higher toxic impact of the heavy metals on agar substrate. The lower heavy metal toxicity on filter paper substrate might be due to the interaction of metal ions with the filter paper, which led to the decrease in the heavy metal concentration, lower bioavailability and subsequently weaker impact on the seeds and seedling growth on filter paper than on water agar (Di Salvatore et al., 2008). Perhaps Pb^{2+} binded to the filter paper more easily than the other metals, which could be the reason for better seedling development.

The *in vitro* conditions themselves on agar substrate might influence germination negatively because the percentage of the control, that was just water agar, was low, too. We could explain this observation with the conditions (such as temperature and humidity in cultivation vessels), which don't seem to be favourable for *Ch majus*' seed germination.

These adverse conditions in combination with the negative impact of the heavy metals resulted in poor seedling development. This was observed even in the agar supplemented with Pb^{2+}, which initially had stimulating impact on germination, but subsequently the Pb^{2+} presence in the substrate inhibited seedling growth.

Research of seed germination and seedling growth of *Ch. majus* is the first step of a study on its heavy metal tolerance. In this respect, it is necessary to take into account the higher degree of severity of the metal impact in laboratory conditions because of their direct influence on the seeds and seedling development. Generally, heavy metals bind in different degrees in soil conditions which decreases their bioavailability. As a continuation of this study seed germination and seedling growth are going to be explored in soils supplemented with heavy metals. Moreover, the impact of metals on *in vitro* cultivated plants is going to be investigated.

Conclusion

The results showed that the heavy metal type, concentration and substrate interact to form the conditions which the species seeds have to overcome in order to germinate and develop. Taking into account all studied parameters, the heavy metals applied in filter paper could be put in the following toxicity scale according to degree of toxicity and impact on seedling development: Cu>Zn>Cd>Pb. The heavy metals supplemented in agar could be sequenced in the scale as follows: Zn>Pb>Cd.

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