

STUDIES ON THE ACCUMULATION OF HEAVY METALS IN *Trifolium pratense* L. PLANTS ON AN ARTIFICIALLY POLLUTED SOIL IN THE GREENHOUSE

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Abstract

This study aims to investigate the accumulation of heavy metals (Cd, Pb, Zn and Cu) from soil in red clover (Trifolium pratense L.) plants in experiments with single-element induced pollution, carried out in the greenhouse. The soil used comes from an unpolluted permanent meadow in the Copsa Mica area and was analyzed from a physico-chemical point of view. The total contents of heavy metals fall within the normal limits according to Order 756/1997 on the assessment of environmental pollution. To perform the experiments with induced pollution, the soil material was treated with variable amounts of metal (Cd, Cu, Pb and Zn) from their soluble salts, and for each element 6 experimental variants were established depending on the metal content in the soil. The results show that for the total cadmium and lead contents in the soil and the metal content in the plant, extremely significant linear correlation indices were obtained and for zinc and copper very significant. The mobile metal contents in the soil and the metal content in Trifolium pratense plants have a very significant linear correlation index.

Key words: heavy metals, pollution, soil, *Trifolium pratense* L.

INTRODUCTION

Soil is a complex system composed of mineral and organic fractions, water and air, inhabited by diverse biota that vary depending on environmental and climatic conditions (Giunchino et al., 2026).

The rapid growth of industrialization in the last century has led to the intense and often uncontrolled exploitation of natural resources, having a significant impact on the environment. Industrial activities have led to the emergence of numerous pollutants, including soil contamination with heavy metals (Briffa et al., 2020, cited by Cakaj, 2023).

Heavy metal pollution is a major problem due to its persistence, high toxicity and bioaccumulative potential present in the environment (Abhinav et al., 2024). They accumulate very easily in soils and transform into more toxic methyl compounds, and their elimination is a long-term process, which can take tens or even hundreds of years (Abd Elnabi et al., 2023, cited by Cakaj, 2024; Li et al., 2018, cited by Zhao et al., 2022; Denchylia-Sakal et al., 2019).

Soils contaminated with heavy metals have a negative impact on living organisms and the food chain (Tomaskin et al., 2013; Zhao et al., 2022).

Forage crops grown on heavy metal contaminated soils tend to accumulate excessive amounts of heavy metals, posing a severe risk to human and animal health (Rattan et al., 2005; Kulhari et al., 2013, cited by Bhatti et al., 2016). Heavy metals, both essential and non-essential, have similar harmful effects on plants. Plants have developed detoxification mechanisms to reduce the negative effects of heavy metal exposure (Collin et al., 2022)

Although heavy metals such as zinc and copper are essential for the growth and development of plants and animals, in concentrations above maximum permissible limits, they disrupt the normal functioning of organisms (Ali et al., 2012; Jolly et al., 2013; Abuzaid et al., 2021).

Cadmium and lead are non-essential heavy metals, known to be highly toxic and carcinogenic to animals and humans (Rajaganpathy et al., 2011). Since the heavy metal content in soil is strongly affected by soil characteristics, and the bioaccumulation of

heavy metals in forage crops is a major pathway for heavy metals to enter livestock systems (Bhatti et al., 2016).

Cadmium is easily absorbed and accumulated by plants and affects physiological and biochemical processes such as seed germination, growth, photosynthesis, and antioxidant systems. It also blocks the absorption and transport of vital elements, leading to critical nutritional deficiencies (Haider et al., 2021; Wu et al., 2022).

The non-biodegradable nature of lead, like cadmium, is the main reason for its prolonged persistence in the environment (Flora et al., 2012; Alisha and Gupta, 2018).

The most polluted areas in Romania are Baia Mare, Zlatna and Copsa Mica where the highest levels of contamination are with cadmium, lead, copper and zinc, which mainly come from anthropogenic activities (Damian et al., 2008; Mihali et al., 2013).

When these heavy metals are present in the soil in low concentrations, plants continue to grow evenly despite the accumulation of these metals. The ability of plants to accumulate heavy metals in their organs can therefore be used to monitor soil pollution with heavy metals (Malizia et al., 2012).

Trifolium pratense L. is a perennial herbaceous species of the legume family (fabaceae) widely cultivated in temperate regions. It has a high protein content, large and tender leaves, and is a soil nitrogen fixer. It is widely used as pasture or for hay production, and is also used as a green manure and for soil and water conservation, contributing to the improvement of soil fertility (Meng et al., 2022; Mo et al., 2022; Mu et al., 2025; Valujeva et al., 2025).

Studies have shown that *Trifolium pratense* L. has a strong photosynthetic capacity, superior antioxidant activity and low root mobility towards heavy metals, indicating that it may have good potential to resist heavy metal toxicity (Meng et al., 2022; Mu et al., 2025).

Cakaj et al. (2024) note that *Trifolium pratense* L. has a high bioaccumulation potential for zinc, cadmium and nickel, both in the roots and leaves. Thus, due to its ability to accumulate heavy metals in the soil, it has been proposed as a potential bioindicator plant of heavy metals. Thus, it is used as an indicator plant for

biological monitoring of metals in polluted soils (Shahbaz et al., 2018).

The results presented by Kujawska et al. (2018) indicate that red clover plants accumulated high levels of copper, zinc, and nickel from soil-like substrates, but can be used as animal feed.

In studies conducted by Ali et al. (2012), it is shown that *Trifolium alexandrinum* has the ability to accumulate heavy metals (Zn, Pb, Cu and Cd) from artificially contaminated soils.

The values of the bioaccumulation coefficient in the roots were significantly higher, with the highest values for zinc. The translocation of heavy metals from the roots to the stems was limited, however, *Trifolium alexandrinum* plants are recommended in the phytoremediation of contaminated soils.

Bidar et al. (2009) indicate that the bioaccumulation of heavy metals from soil in *Trifolium repens* L. plants is mainly in the roots (Cd>Zn>Pb) compared to the aerial parts of the plants.

The results of the experiments with induced pollution (Cd and Zn) conducted by Stravinskiene & Racaitė (2014) showed that heavy metals have an inhibitory effect on the development of white clover plants (*Trifolium repens* L.). The inhibitory effect of cadmium was statistically significantly greater than that of zinc on the growth and development of white clover roots and shoots. In the studies conducted by Sotiriou et al. (2023) mention that *Trifolium pratense* L. has a moderate capacity to accumulate Cd and Zn compared to a weak capacity to accumulate Pb. Similar results are also shown by Lanier et al. (2016), emphasizing that cadmium accumulates more in roots than in leaves, and the presence of lead in the soil slightly reduces the bioaccumulation of cadmium in both the underground and aerial parts of the plant.

Memic et al. (2023) show that white clover behaves differently towards metals depending on the type of metal, its location in the soil and the season.

Trifolium pratense L. was selected for the experiment because it was one of the most common plant species in the legume family in the Copsa Mica area and known in the literature for its capacity to accumulate heavy metals. The aim of this study is to evaluate the effects of increasing concentrations of heavy metals (Cd,

Pb, Zn and Cu) in the soil on *Trifolium pratense* L. plants and to evaluate its capacity to accumulate these elements in plant tissues.

MATERIALS AND METHODS

This paper presents a study conducted in 2025, regarding the accumulation of heavy metals (Cd, Pb, Zn and Cu) in *Trifolium pratense* L. plants, in experiments with induced pollution. The experiments were carried out in the greenhouse of INCDPAPM-ICPA Bucharest, in pots with 10 kg of soil.

For the experiments, soil material was collected from an unpolluted permanent meadow in the Copsa Mica area. The soil material was harvested from the upper 0-20 cm layer, homogenized and dried at air temperature.

The experiments were carried out on artificially contaminated soil. Thus, the soil material was treated with amounts of metals (Cd, Pb, Zn and Cu) derived from their soluble salts (acetate). For each element, 5 experimental variants with treated soil and the control were established, as follows:

- Cd: control; 3 mg·kg⁻¹; 6 mg·kg⁻¹; 9 mg·kg⁻¹; 12 mg·kg⁻¹; 15 mg·kg⁻¹;
- Pb: control; 50 mg·kg⁻¹; 100 mg·kg⁻¹; 150 mg·kg⁻¹; 200 mg·kg⁻¹; 250 mg·kg⁻¹;
- Zn: control; 300 mg·kg⁻¹; 600 mg·kg⁻¹; 900 mg·kg⁻¹; 1200 mg·kg⁻¹; 1500 mg·kg⁻¹;
- Cu: control; 100 mg·kg⁻¹; 200 mg·kg⁻¹; 300 mg·kg⁻¹; 400 mg·kg⁻¹; 500 mg·kg⁻¹;

When establishing these concentrations, previous studies were taken into account, as well as the limit values from Order 756/1997. The soil was carefully prepared to ensure a uniform distribution of the applied treatments.

Trifolium pratense L. (red clover) of the David Liv variety from SCDA Livada was selected as the test plant. The plants were harvested 18 weeks after sowing.

Before preparing the experiments, the soil material used in the experiments was analyzed from a physicochemical point of view. The collected soil samples were dried at room temperature, then ground and sieved through a sieve with 2 mm and 0.2 mm mesh. The samples were carried out in four repetitions.

The soil texture was determined by the sieving and sedimentation method.

Soil pH was determined using the potentiometric method (soil: water ratio 1:2.5 g/v), organic matter content was determined using the Walkley-Black method, modified after Gogoasa. Total nitrogen was determined using the Kjeldahl method. Soil available phosphorus and potassium were determined using the Egner-Riehm-Domingo method.

For the determination of heavy metals content was used atomic absorption spectrometry, after the extraction by the aqua regia–microwave digestion method (Ethos Easy Microwave Dygestion System from Milestone).

Microwave digestion was performed using 10 mL of aqua regia (7.5 mL HCl and 2.5 mL HNO₃) at 140°C for 30 min, method developed according to SR ISO 11466:1999.

DTPA extractable heavy metals were extracted from soil (10 g) with 20 ml of extraction solution (0.05 M DTPA (diethylenetriaminopentacetic acid), 0.01 M CaCl₂, and 0.1 M TEA (tetraethylammonium), according to SR ISO 14870:2002. ICE3000 (1M) atomic absorption spectrophotometry was used.

Trifolium pratense L plant samples were harvested, oven-dried at 60°C, chopped, ground and treated with nitric acid in a microwave digestion system (Ethos Easy Microwave Dygestion System from Milestone).

Microwave digestion was performed using 10 mL mixture of HNO₃ + H₂O₂ (9 mL HNO₃ and 1 mL H₂O₂) at 210°C for 20 min, method developed in-house.

Microsoft Excel was used for statistical data processing.

RESULTS AND DISCUSSIONS

Table 1 presents the main characteristics of the soil material used in the induced pollution experiments. The results of laboratory determinations indicated a fine textured, medium clayey (LL) soil. As granulometric fractions (in mm, % of the mineral part of the soil), they contain 12.0% coarse sand (2.0-0.2 mm), 44.0% fine sand (0.2-0.02 mm), 19.3% silt (0.02-0.002 mm) and 24.2 clay (<0.002 mm).

The analysed soil has a weak alkaline reaction (pH = 7.94), low humus content (2.71%), medium nitrogen (0.183 %) and potassium (186

mg·kg⁻¹) input, and a high phosphorus content (53 mg·kg⁻¹).

Table 1. Physico-chemical characteristics of the soil material (n = 4)

Characteristics	Mean value
Coarse sand (%) 2.0-0.2 mm	12.0
Fine sand (%) 2.0-0.02 mm	44.0
Silt (%) 0.02-0.002 mm	19.3
Clay (%) < 0.002 mm	24.7
Texture	LL
pH _{water}	7.94
Humus (%)	2.71
Total N (%)	0.183
Available P (mg·kg ⁻¹)	53
Available K (mg·kg ⁻¹)	186
Cd (mg·kg ⁻¹)	0.771
Pb (mg·kg ⁻¹)	41.1
Zn (mg·kg ⁻¹)	124
Cu (mg·kg ⁻¹)	27.8
Cr (mg·kg ⁻¹)	43.9
Mn (mg·kg ⁻¹)	579
Ni (mg·kg ⁻¹)	37.2

In terms of heavy metal content, it is observed that the values obtained are within the normal limits according to Order 756/1997 on the assessment of environmental pollution. The heavy metal contents indicated the following average values: 0.771 mg·kg⁻¹ Cd, 41.1 mg·kg⁻¹ Pb, 124 mg·kg⁻¹ Zn, 27.8 mg·kg⁻¹ Cu, 43.9 mg·kg⁻¹ Cr, 579 mg·kg⁻¹ Mn and 37.2 mg·kg⁻¹ Ni.

The data obtained in the experiments with single-element induced pollution were used to parameterize and validate stochastic models, which allowed the estimation of the bioaccumulation of heavy metals (Cd, Pb, Zn and Cu) in *Trifolium pratense* L. plants depending on the total content of the metal in the soil and its mobile fraction (DTPA extractable form).

Figure 1 presents regression equations estimating the stochastic dependence between total soil cadmium content, mobile soil cadmium content and cadmium content in the aerial part of *Trifolium pratense* L. plants. The value of the linear correlation coefficient (Cd_{total} r=0.9891^{***}) is highly significant, showing a very strong correlation between total soil cadmium content and cadmium content accumulated in *Trifolium pratense* L. plants (Figure 1a). Figure 1b presents the logarithmic diagram between mobile soil cadmium content and cadmium content in the aerial part of *Trifolium pratense* L. plants, indicating a distinctly significant linear correlation coefficient (Cd_{DTPA} r=0.9618^{**}). Increasing the bioavailability of cadmium in the soil favors a more intense absorption and a higher accumulation of the metal in the tissues of *Trifolium pratense* L. plants.

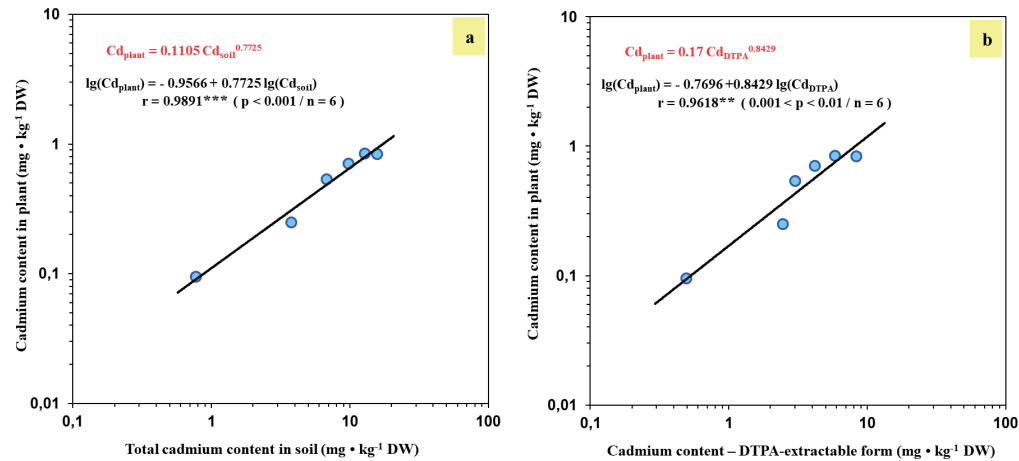


Figure 1. Regression equations estimating the stochastic dependence between total soil cadmium content (a), soil mobile cadmium content - DTPA extractable form (b) and cadmium content in the aerial part of *Trifolium pratense* L. plants harvested from an experiment with induced pollution

The regression equations estimating the stochastic dependence between the total lead content in the soil (Figure 2a), the mobile lead

content in the soil (Figure 2b) and the lead content in the aerial part of *Trifolium pratense* L. plants are shown in Figure 2. Extremely

significant linear correlation indices (Pb_{total} $r=0.9846^{***}$) and respectively distinctly significant (Pb_{mobile} $r=0.9735^{**}$) were obtained. The transfer of zinc content from soil to the aerial part of *Trifolium pratense* L. plants is represented by regression equations that estimate the stochastic dependence between the

zinc content in soil and that accumulated in plants (Figure 3). The results show a distinctly significant linear correlation between the total zinc content in soil (Figure 3a), the mobile zinc content in soil (Figure 3b) and that in the aerial part of plants, with high values in both cases (Zn_{total} $r=0.9535^{**}$ and Zn_{mobile} $r=0.9673^{**}$).

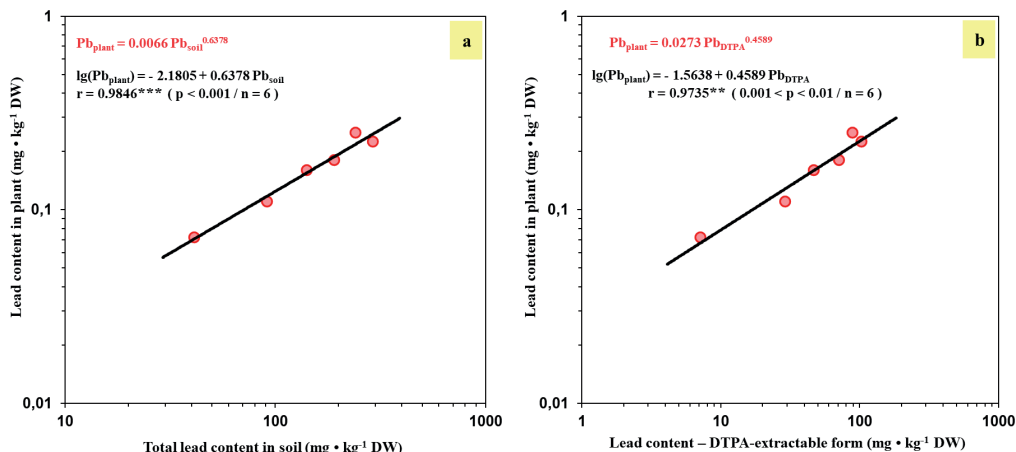


Figure 2. Regression equations estimating the stochastic dependence between total soil lead content (a), soil mobile lead content - DTPA extractable form (b) and lead content in the aerial part of *Trifolium pratense* L. plants harvested from an experiment with induced pollution

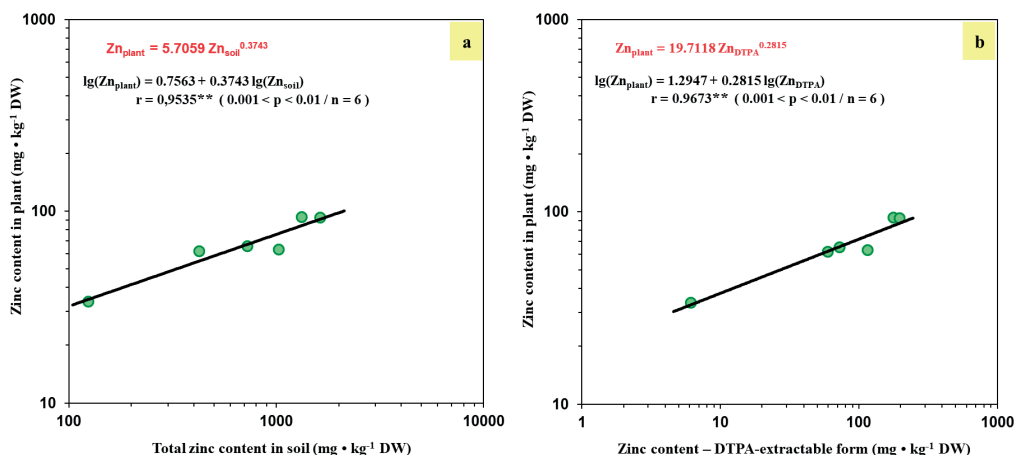


Figure 3. Regression equations estimating the stochastic dependence between total soil zinc content (a), soil mobile zinc content - DTPA extractable form (b) and zinc content in the aerial part of *Trifolium pratense* L. plants harvested from an experiment with induced pollution

Studies by Denchylia-Sakal et al. (2019) on soil artificially polluted with zinc and copper show that as the concentration of the metal in the soil increases, the leaf area decreases. *Trifolium pratense* L. plants can accumulate significant amounts of zinc, with higher values in the roots and leaves compared to the shoots.

Figure 4 shows regression equations that estimate the stochastic dependence between the total copper content in the soil (Figure 4a), the mobile copper content in the soil (Figure 4b) and the copper content in the aerial part of *Trifolium pratense* L. plants. The linear correlation index

is distinctly significant in both cases: C_{total} $r=0.965^{**}$ and C_{DTPA} $r=0.9518^{**}$.

Cakaj et al. (2023) showed that *Trifolium pratense* L. accumulated the highest concentration of copper in the roots – 20.38 $\text{mg} \cdot \text{kg}^{-1}$, while in the leaves 9.20 $\text{mg} \cdot \text{kg}^{-1}$.

Metal concentrations in the Zlatna and Copsa Mica areas exceed the maximum permissible limits according to Order 756/1997. In Copsa

Mica the maximum permissible limits for lead are exceeded by 5.61-27.68 times, zinc: 2.16-25 times and cadmium: 5.99-133.3 times. For Zn and Cd, the total concentrations exceeded both the maximum permissible limit and the intervention threshold in the clay-rich horizons, at variable depths, in both areas (Damian et al., 2008).

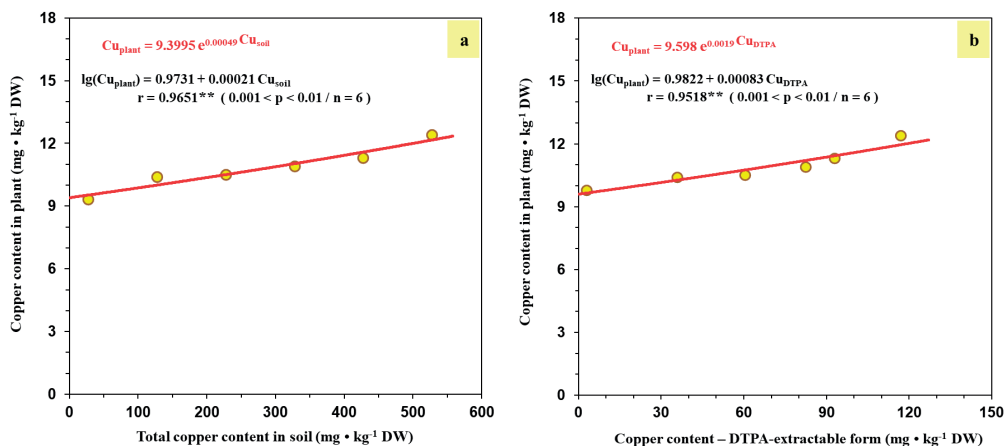


Figure 4. Regression equations estimating the stochastic dependence between total soil copper content (a), soil mobile copper content – DTPA extractable form (b) and copper content in the aerial part of *Trifolium pratense* L. plants harvested from an experiment with induced pollution

CONCLUSIONS

This study shows that *Trifolium pratense* L. has the capacity to accumulate heavy metals (Cd, Pb, Zn and Cu) from artificially contaminated soils. The soil material used for the experiments in the greenhouse is a soil with a fine texture, weak alkaline reaction and medium nutrient supply. The contents of heavy metals fall within the normal limits according to the order 756/1997 on the assessment of environmental pollution.

The results of the experiments carried out in the greenhouse show a strong correlation between the total and mobile content of heavy metals in the soil and that accumulated in the aerial part of *Trifolium pratense* L. plants. Also, a direct relationship was observed between the increase in the solubility of metals in the soil material and the applied doses. It is noted that for the total cadmium and lead contents in the soil and the metal content in the plant, very significant linear correlation indices were obtained, and for zinc and copper distinctly significant. The mobile

metal contents in the soil and the metal content in *Trifolium pratense* L. plants have distinctly significant linear correlation indices.

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