

STRATEGIES OF HEAVY METAL UPTAKE BY *PHASEOLUS VULGARIS* SEEDS GROWING IN METALLIFEROUS AND NON-METALLIFEROUS AREAS

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ABSTRACT. This study focuses on the accumulation of metal ions (Pb^{2+} , Zn^{2+} , Cu^{2+} , Fe^{2+}) in *Phaseolus vulgaris* seeds (*Fabaceae* family) collected from metalliferous / non- metalliferous areas in Maramures County. By “metalliferous areas” we will understand following in this research paper only areas displaying such pollution, as opposite to the other areas, consequently labeled as “non-metalliferous”. Four different concentrations of metal ions were used and their imbibition's degree into the seeds was investigated. The accumulation of metal ions in the seeds of *P. vulgaris* increases as the initial concentration of metal ions is higher. Even more, the seeds growing in non-metalliferous areas display a higher absorption of all metal ions, except the Fe^{2+} , than the seeds growing in metalliferous areas. From an anatomical point of view, we have observed that regardless of concentration, the metal ions were able to penetrate the seed through the hilum, up to the coat.

Keywords: *Phaseolus vulgaris*, lead, copper, iron, zinc, heavy metal accumulation, atomic absorption spectrometry, optical microscopy

INTRODUCTION

Plants usually show the ability to accumulate large amounts of metals without visible changes in their appearance or yield. In many plants, the level of metal accumulation can exceed even several hundred times the maximum level permissible for human beings, without having any negative effect on their growth or yield [1]. Therefore, it seems that plants can endure a level of environmental pollution that might be even several times higher than the level observed nowadays.

The persistence of metallic pollutants in soil is the most difficult issue. The presence of metals accumulated in soil will span hundreds of years, impacting to a lesser or greater extent plants grown on such sites [2]. The plants

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growing in metalliferous habitats probably have the ability to inactivate the heavy metal by binding the excess metal ions and/or by changing the chemical composition and physical organization of their cell membranes [3]. There are numerous investigations [4, 5] on the influence of heavy metals on the plants metabolism, but data remains scarce about the accumulation of heavy metals in the reproductive organs of plants [6, 7]. Stefanov *et. al.* [3], which studied the accumulation of lead, zinc and cadmium in different plants seeds, showed that plants accumulate selectively heavy metal ions in their seeds. Peanut and corn seeds accumulate mainly lead, pea seeds accumulate mainly cadmium and wheat seeds accumulate mainly zinc. On the other hand, Lane and Martin [8] showed that the seed coats of *Raphanus sativus* were a strong barrier to lead and helped prevent contamination of embryos until the seed coat was torn apart by the germinating embryonic root. There are reports on the inhibitory effect of lead on the germination of seeds of the following species *Lupinus luteus* [9], *Oryza sativa* [10] and *Sinapis alba* [11]. The fact that a method of measuring the tolerance of plants to metals entails sowing the seeds of tolerant and sensitive species on metal-contaminated soil points to a significant effect of heavy metals on germination [12]. The above studies, unlike those by Lane and Martin [8], point to the significant influence of lead on seed germination. In this situation it seems probable that the effect of lead on germination depends on interspecies differences in seed structure-in particular, on differences in the structure of seed coats. As it is well known, the role of the seed coat is to protect the embryo from harmful external factors. But seed coats have a wide range of anatomic forms that exist in no other plant organ or tissue [13]. Wierzbicka *et. al* [1] showed that from all plants families tested, the *Fabaceae* family is very susceptible to the higher concentration of metal ions.

The aims of this study were to evidence the role playing the seeds coat of bean, but also the vulnerability of these seeds to the heavy metals presence. The role of the seed coats as heavy metal barriers is often bypassed by the high concentration of these chemical species, this fact generating a risk of these seeds consumption. We present a selection of research data on the accumulation of heavy metals (Pb^{2+} , Zn^{2+} , Cu^{2+} , Fe^{2+}) in *P. vulgaris* seeds growing in metalliferous and non-metalliferous areas in Maramures county. Investigating how the initial concentration of metal ions impacts the imbibition capacity of seeds and, on the other hand, establishing the degree to which those ions have penetrated the seeds were our key research goals. We have also researched how the imbibition capacity of seeds varies depending on their area of origin (metalliferous or not).

RESULTS AND DISCUSSION

Figure 1 displays the *P. vulgaris* seeds, as a whole and split, impregnated with different ions in different concentrations and originating from metallic and non – metallic areas. All images reveal the imbibition of metal ions into the

seeds as taking place starting with the hilum and going up to the cotyledon, as the colored zones on the seeds split demonstrate. Moreover, as substantiated in all images by the change in color of the tegument of seeds in whole, the penetration of metal ions takes place up to the tegument of the seed. On the other hand, the analysis of images of seeds impregnated with the same metal ion (Figures 1(a)-(b), 1(c)-(d), 1(e)-(f) and 1(g)-(h) respectively) reveals that the intensity of color inside the seed increases as the concentration of metal ions is higher (namely green for Fe^{2+} , red-brown for Zn^{2+} , red for Cu^{2+} and yellow for Pb^{2+}). The optical microscopic analyses confirm this observation (see below).

The macroscopic views of seeds as illustrated in Figures 1 (h) and (i) reveal that seeds impregnated with Pb^{2+} ions, at the same concentration (10.000 mg/L), display different colors depending on the source of each seed (coming either from a metalliferous area – Ferneziu, in our particular case, or from a non-metalliferous one – Oarta de Jos, in our case). The images demonstrate that in seeds originating in a metalliferous area (as in Figure 1 (i)) Pb^{2+} has prevalently accumulated in the tegument and in the superior part of the cotyledons, while the accumulation in seeds originating from a non-metalliferous area take place in the whole cotyledons and tegument.

Thus, the absorption capacity of seeds originating from non-metalliferous areas exceeds the absorption capacity of those from metalliferous ground. This happens as plants growing in metalliferous sites have the ability to inactivate the excess of metal ions and/or to alter the chemical composition and/or physical layout of membranes of their cells [3].

At the lowest concentration in Fe^{2+} we have tested (see Figure 2 (a)) no presence of the Fe^{2+} ions into seed structures was microscopically observed. In opposition to this, at 50 mg/L in concentration (see Figure 2 (b)) the penetration of Fe^{2+} into the cotyledon and going up to the tegument can be noticed, to stabilize in the area between tegument and cotyledon. A more or less homogeneous distribution of metal ions in the cotyledon is obvious in Figure 2(d). The green color of the cotyledon at the highest concentration of Fe^{2+} , as in Figure 2(d), is clearly more intense than the color displayed at lower concentration of Fe^{2+} , as in Figure 2(c). The imbibition's degree trends up, as the concentration in Fe^{2+} increases. The analysis of Fe^{2+} ions in terms of their imbibition capacity depending on the origin of the seeds (as in Figures 2(a) and (b)), has led us to conclude that the seeds originating from a non-metalliferous area have a higher capacity to absorb Fe^{2+} ions than those originating from a metalliferous area. In particular, Fe^{2+} ions penetrate the seeds originating from a non-metalliferous area up to the cotyledon, to a smaller extent the tegument and agglomerate in the area between the tegument and the cotyledon (the green layer). We have remarked no Fe^{2+} ions penetration at all, however, in seeds originating from a metalliferous area.

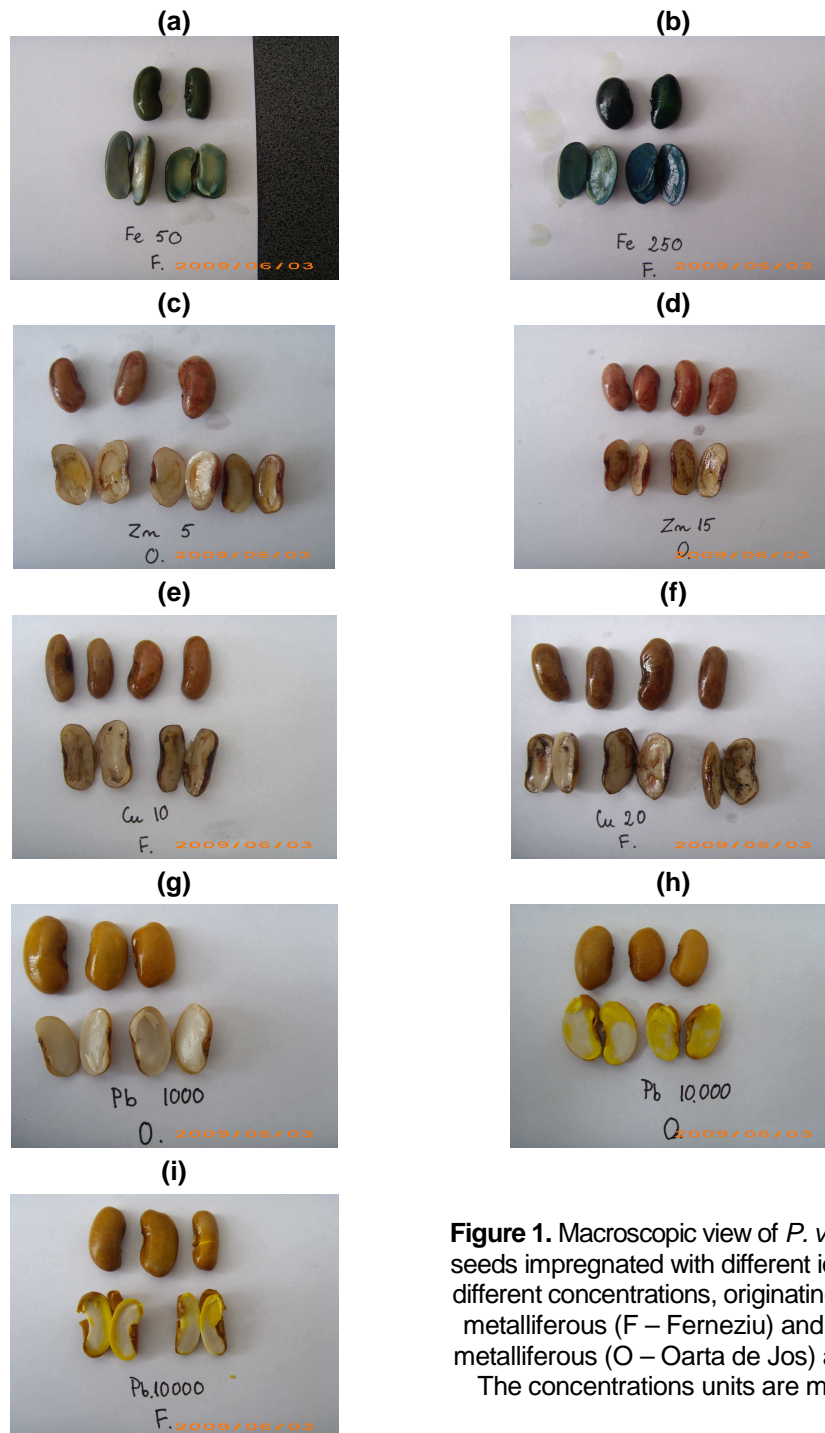


Figure 1. Macroscopic view of *P. vulgaris* seeds impregnated with different ions, at different concentrations, originating from metalliferous (F – Fernezuiu) and non-metalliferous (O – Oarta de Jos) areas. The concentrations units are mg/L.

This behavior is explained by the fact that the iron (known as essential trace element for plants [14]) diminish the toxic effect of the other metals by compete with these for binding sites on the cell membranes, followed by penetration and accumulation into the cells [15, 16, 17]. This ionic exchange process is part of the plant strategy to survive on soils strongly polluted with heavy metals.

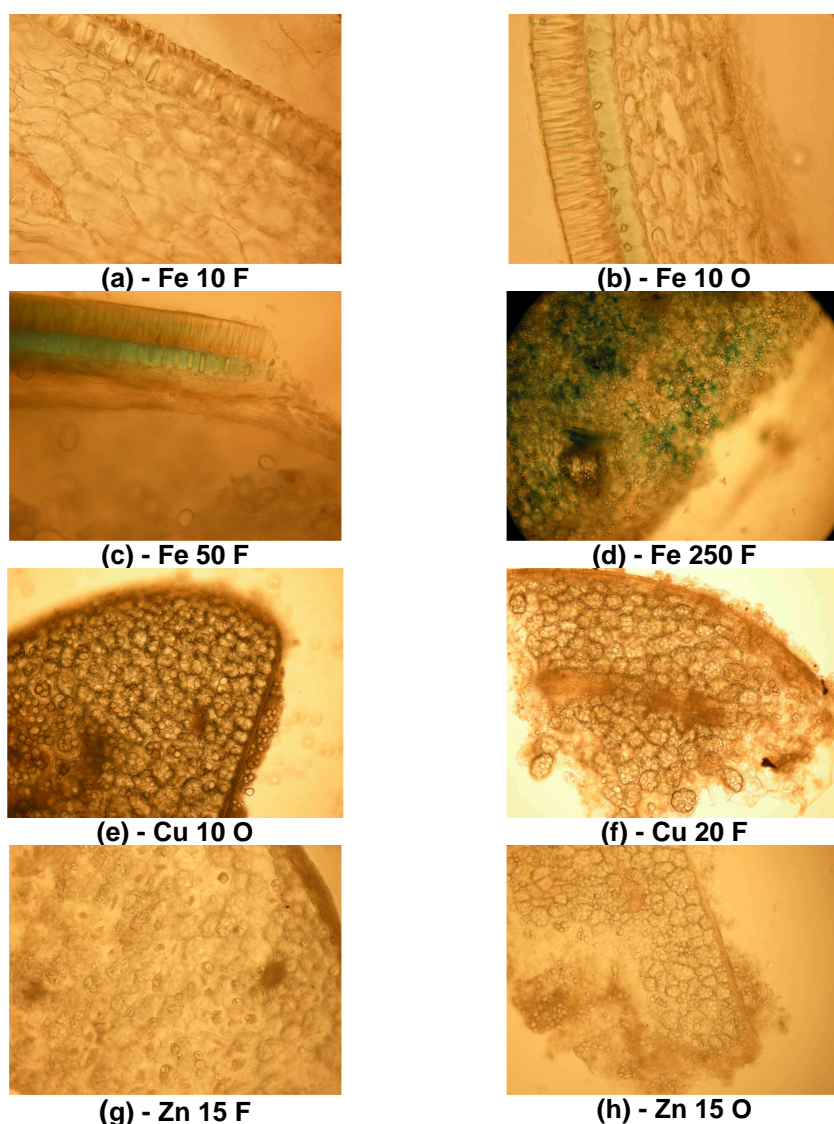


Figure 2. *P. vulgaris* seed – cross sections as captured in optical microscope images at 10 X magnification to detect how the concentration of metal ions impacts the imbibition degree.

As we have monitored the process of imbibition of seeds with Cu^{2+} ions (see Figures 2(e) and (f)) we have noticed the trend of these ions to agglomerate on the cellular wall [3, 5, 18] (red lines between cells) and in the tegument, regardless of the concentration and origination of seeds.

We have conducted the microscopic analysis of seeds impregnated with Zn^{2+} ions (see Figures 2(g) and (h)) and Pb^{2+} ions (not shown) exactly as we did to research the imbibition process with Cu^{2+} ions. We have in this case noticed that regardless of concentration and origination of seeds, the ions agglomerate on the cellular wall (brown lines between cells, for Zn^{2+}) and on the tegument.

Table 1 gives the range of concentrations we have used for the metal ions. We have concluded, as outcome valid for all the metal ions, that the higher their initial concentration was (as in Figure 3), the higher the concentration of metal in seeds is.

We have prepared our initial solutions with the pH ranging between 3,99 and 5. 41 (as revealed in Table 1) and with weak acidity. The process by which *Pistacia vera* L. removes Pb^{2+} ions from aqueous solutions takes place with maximum efficiency (ca. 93,8–95,1%) at a pH ranging from 3,5 to 5,5, as demonstrated by Yetilmezsoy *et. al.* [19]. The absorption of metal ions into seeds subsides at low pH (namely at high concentrations of hydrogen), as hydrogen ions compete with those of metals in the attempt to penetrate the plant [20]. As the pH grows above 7, a decline in mobility of the metal ions in aqueous solutions translates into those ions lower probability to reach and penetrate the seed. [21].

The influence of *P. vulgaris* seeds provenience on the Cu content in seeds is illustrated in Figure 4. Seeds originating in non-metalliferous area (Oarta de Jos) display a higher concentration in copper than seeds in metalliferous area (Fernezuiu). Zinc and lead displays a behavior comparable to copper, as confirmed during our optical microscopic analyses. The ability of plants to inactivate the heavy metal by binding the excess and/or by changing the chemical composition and physical organization of their cell membranes [3] stands as explanation for this outcome. Moreover, the plants growing on polluted areas develop, in time, some mechanisms to survive in drastically ecologic conditions. The selection of this kind of resistant plants constitutes during the time a particularly ecotype.

Iron makes the exception to the above – mentioned behavior. Figure 5 reveals how the origin of seeds has an influence on the Fe content. Seeds originating in non-metalliferous areas (Oarta de Jos) display a lower Fe concentration than the seeds from metalliferous sites (Fernezuiu).

Table 1. Particularities of the metal ions investigated in *P. vulgaris* seeds.

Sample ID	Seed Provenience	[Metal ion] ²⁺ _{initial} /pH (mg / L)	[Metal ion] ²⁺ _{soil} (mg / Kg)	[Metal ion] ²⁺ _{seed} (mg/Kg)	RSD (%) [*]
Fe-10 F	Ferneziu	10 / 4,46	15 287,5	40,4	1,9
Fe-50 F		50 / 3. 99		138,2	7,3
Fe-250 F		250 / 3,51		979,2	1,8
Reference		-		30,5	4,7
Cu-10 F		10 / 4,62	153,2	36,9	1,6
Cu-20 F		20 / 4,58		37,6	0,2
Cu-25 F		25 / 4,65		115,5	0,2
Reference		-		0	0
Zn-5 F		5 / 5,07	93,64	63,6	6,5
Zn-10 F		10 / 5,41		93,3	1,8
Zn-15 F		15 / 4,7		150,4	0,8
Reference		-		29,4	0,2
Pb-100 F		100 / 4,84	357,89	5. 067,4	7,3
Pb-1. 000 F		1. 000 / 4,68		13. 793,4	0,3
Pb-10. 000 F		10. 000 / 4,33		92. 247, 1	1,4
Reference		-		8,1	14,5
Fe-10 O	Oarta de Jos	10 / 4,46	11 952,52	56,1	3,4
Fe-50 O		50 / 3. 99		60,5	16,1
Fe-250 O		250 / 3,51		454,5	4,0
Reference		-		29,5	4,0
Cu-10 O		10 / 4,62	14,61	72,4	1,2
Cu-20 O		20 / 4,58		104,3	0,3
Cu-25 O		25 / 4,65		151,2	0,01
Reference		-		0	0
Zn-5 O		5 / 5,07	40,41	86,9	3,1
Zn-10 O		10 / 5,41		102,5	1,0
Zn-15 O		15 / 4,7		172,7	1,3
Reference		-		23,4	0,5
Pb-100 O		100 / 4,84	40,13	3. 989,4	2,4
Pb-1. 000 O		1. 000 / 4,68		18. 396,1	0,9
Pb-10. 000 O		10. 000 / 4,33		87. 442,1	0,9
Reference		-		8,2	21,3

^{*} RSD was determined from three parallel measurements.

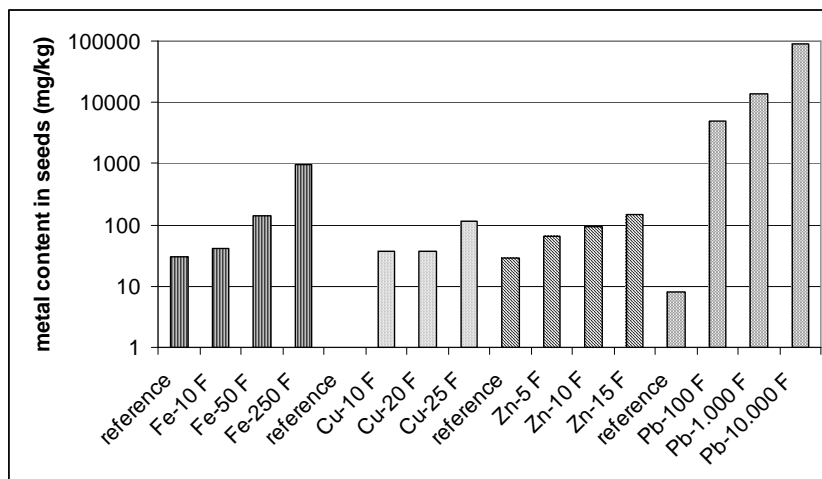


Figure 3. Influence of the initial concentration on the metal contents in *P. vulgaris* seeds originating in Ferneziu

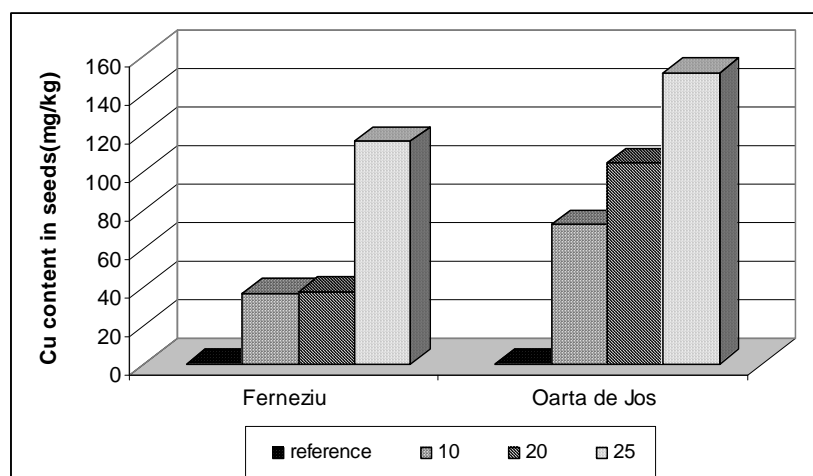


Figure 4. Influence of provenience of the *P. vulgaris* seeds on the Cu content in seeds

Various levels of concentration in Fe ions could generate on a plant substantially different outcomes. In small concentrations, Fe ions play a beneficial role, as they are essential to the well-being of the plant. In higher concentrations, however, Fe ions become a source of stress, as at sub-cellular level the concentration of metabolic compounds that react with oxygen goes up [22].

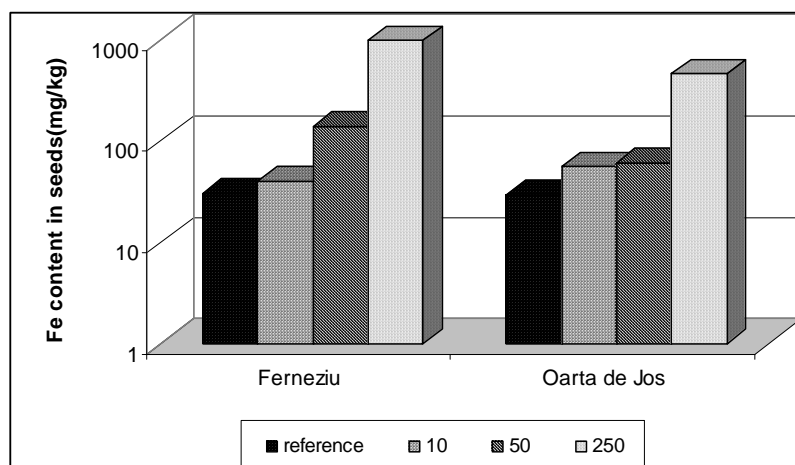


Figure 5. Dependence of the Fe content in seeds on the origin of *P. vulgaris* seeds

CONCLUSIONS

The accumulation of metal ions (Pb^{2+} , Zn^{2+} , Cu^{2+} , Fe^{2+}) in *Phaseolus vulgaris* seeds (Fabacea family) collected from metalliferous and non-metalliferous areas in Maramures County is the key focus of this study. In this respect, we have used four different concentrations of metal ions and we have investigated their respective degree of imbibition into the seeds.

- ◆ The intensity of color of the seed (green for Fe^{2+} , red-brown for Zn^{2+} , red for Cu^{2+} and yellow for Pb^{2+}) grows as the concentration of metal ions increases.
- ◆ Seeds originating in non-metalliferous areas display a higher adsorption capacity of metal ions (except iron) than seeds from metalliferous areas
- ◆ The plants growing on polluted areas develop during the time, mechanisms to survive in drastically ecologic conditions, following a selection process, thus resulting a particularly ecotype.

EXPERIMENTAL SECTION

Seed Material

Ferneziu is a neighborhood of Baia Mare and also a metalliferous area due to the mining and ore processing activity. The soils originating from this area is very polluted, containing heavy metals (Table 1) and toxic anions which influence the vegetation and fauna growing and propagation. Oarta de Jos is located at approximately 50 km away from Baia Mare, in the

south-east of the Maramures County (see the map in Figure 6) and is a non-metalliferous area due to the fact that there is no any mining and industrial activity. Moreover, the village is at approximately 10 km away from the main road, which is also considered a source of pollution. The concentrations of the reference metal ions in soil are presented in Table 1.

We have collected *P. vulgaris* seeds out of the crop harvested in Ferneziu and respectively in Oarta de Jos, in the fall of 2008.

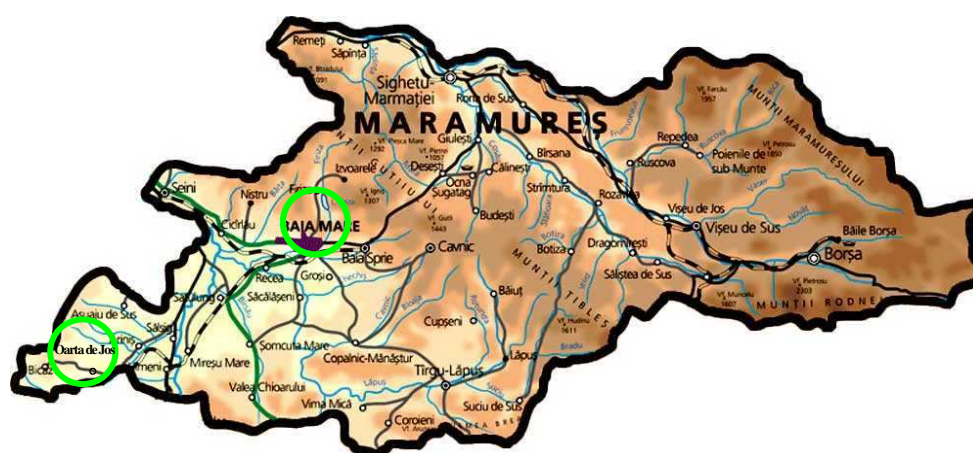


Figure 6. Map of Maramures County. Green circles identify the locations from where we have collected the seeds of *P. vulgaris* for the research interests of this paper.

Series of fifty seeds, collected in the above-mentioned areas, were washed three times with distilled water and immersed in solutions with different concentrations of heavy metal ions. The solutions containing the mentioned metal ions were prepared using FeSO_4 , $\text{Pb}(\text{NO}_3)_2$, CuSO_4 , ZnSO_4 salts. The initial concentrations of those solutions were established having in view the same metal ions concentration in soil and are presented in Table 1.

Analyses on heavy metals

After 7 days of immersion at 22°C in the solution with ions of heavy metals, we have washed three times the seeds with distilled water before initiating our analyses, conducted in two ways.

1. We have relied on a German – made Krüss Optronic binocular microscope in order to investigate the degree of imbibition by optical microscopy. This method has entailed the use of a special reagent to color each category of metal ions under scrutiny. Table 2 gives the color assigned to each metal ion and corresponding to each reagent.

Table 2. The chemical reagents used to identify the iron, copper, zinc and lead ions and the colors obtained

Heavy metal	Reagent	Color
Fe	K ₃ [Fe(CN) ₆] 5%	Green
Cu	Ditizone 0,12% in CHCl ₃	Red
Zn	Ditizone 0,12% in CHCl ₃	Brown
Pb	KI 5%	Yellow

We have maintained the seeds for 10 minutes immersed in reagent, and have dried them up for another 10 minutes in open air. A manual MIC – 500 – type microtome has allowed us to produce mono-cellular microscopic sections. In order to use the microtome efficiently, before sectioning we have immersed the seeds in a paraffin bath, to get them fastened.

2. A Perkin Elmer AAS 800 Spectrometer has allowed us to apply a spectrometric method in order to measure the concentration of metals in seeds. The drying process, in air at 1200°C for 2 hours, took place in a US-made Binder drying oven. A German – made Retsch RM 100 grinding machine has completed the next step in the process. The mineralization of the powder of seeds ($m_{\text{powder}} = 5 \text{ g}$), thus produced has entailed the chemical treatment with 28 ml of acid mixture (HNO₃ 65%, $d=1,4 \text{ kg/L}$ (Lach-Ner): HCl 37%, $d=1,19 \text{ kg/L}$ (Merck) = 1 : 3, volume proportion). We have ensured the adequate homogeneity of the mixture after a half an hour process and we have brought to 100 ml volumetric flask with distilled water the mix that we have finally filtered.

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