Research on the *in vitro* Bioaccumulation Capacity of Lead in Some Pteridophyte Species of the Romanian Flora

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The aim of this article is to study the in vitro bioaccumulation capacity of lead in four autochthnous pteridophyte species: Asplenium scolopendrium (As), Asplenium trichomanes-ramosum (At), Cystopteris fragilis (Cf), Polypodium vulgare (Pv), with a view to use them in phytoremediation processes. The polyphenol and flavonoid amounts determined in the material obtained after four months of in vitro culture, in a medium that contained 57.36 mg lead·kg^{-1} Knop medium (V1), 286.82 lead·kg^{-1} Knop medium (V2), 573.64 lead · kg^{-1} Knop medium (V3) were lower than the amounts determined in the control variants (C). The higher polyphenol concentrations of the V3 variants as compared with the V2 variants can be explained by the fact that phenolics provide protection against metal ions. In the V1 experimental variant, the highest lead concentration was determined for the species Polypodium vulgare, in the V2 variant the highest lead concentration was determined for the species Cystopteris fragilis and in the V3 variant the highest lead concentration was determined for the species Asplenium scolopendrium. The bioaccumulation coefficient varied from 0.78 in the Asplenium trichomanes-ramosum V2 variant to 1.97 in the Asplenium scolopendrium V3 variant. The results obtained in vitro, the amount of lead accumulated in the gametophyte and/or sporophyte tissues and the bioaccumulation coefficient obtained for the tested species, respectively should be completed by ex vitro tests performed on mature plants.

Keywords: in vitro bioaccumulation, lead, Asplenium scolopendrium, Asplenium trichomanes-ramosum, Cystopteris fragilis, Polypodium vulgare

Heavy metals such as iron, molibdenum, zinc, nickel, copper, vanadium and cobalt are essential trace elements, necessary for plant metabolism. Nevertheless, they can become toxic when they exceed a certain concentration level. Other elements, such as the arsenic, mercury, silver, antimony, cadmium, lead and uranium are heavy metals with an increased toxicity level for plants [1]. Increased amounts of heavy metals resulting from human industrial, agricultural and household activities have entered the environment and accumulated in both land and aquatic ecosystems [2]. The presence of diverse pollutants in the environment that could affect primary productivity and consequently animal and human populations [3]. Currently, many studies are oriented toward the capacity of plants to accumulate metals from the environment and to transport them to different organs [4].

Many plant species have developed genetic and physiological tolerance to survive in metal polluted environment [5]. Phytoremediation is the use of a plant's capacity to accumulate, degrade, or remove toxic chemicals and pollutants from the environment [6]. Some plant species can accumulate high amounts of metals. Ma et al. [7]. showed that *P. vittata* accumulates in its leaves an amount of arsenic of up to 2% of dry weight. Hyperaccumulation of heavy metals has been reported by now in over 400 terrestrial plant species [8]. Many pteridophytes species have been identified to accumulate toxic metals [7, 9]. Both sporophytes and gametophytes of pteridophyte species can tolerate and accumulate different metals. Gametophytes of *Pteris vittata* and *Athyrium yokoscense* were shown to tolerate and accumulate lead [10]. Gametophytes of *P. vittata* were also tolerant of high levels of arsenic and showed arsenic hyperaccumulation [10, 11]. *In vitro* experiments showed that *Azolla filiculoides* can accumulate up to 10,000 ppm cadmium, 1,990 ppm chromium, 9,000 ppm copper, 9,000 ppm nickel and 6,500 ppm zinc from their environment [12].

Among the responses that plants have to different stress factors in the environment we should also mention the changes induced in the metabolism of phenylpropanoids [13]. The specialized literature has recorded an increase in the amount of polyphenols when plants are subjected to metal pollution [14, 15]. For example, in *Vaccinium myrtillus*, increased accumulation of foliar phenolics was detected in response to leaf Zn, Pb and Cd accumulation [14]. The aim of this paper is to study the *in vitro* bioaccumulation capacity of lead in four autochthonous pteridophyte species with a view to the possibility of using them in phytoremediation processes.

Experimental part

Materials and methods

Biological material: gametophyte/ gametophyte + sporophyte obtained *in vitro* from the species *Asplenium scolopendrium, Asplenium trichomanes-ramosum, Cystopteris fragilis, Polypodium vulgare* (table 1). The

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Table 1
BIOLOGICAL MATERIAL

Species	Explant
Asplenium scolopendrium (As)	gametophyte
Asplenium trichomanes-ramosum (At)	gametophyte
Cystopteris fragilis (Cf)	gametophyte
Polypodium vulgare (Pv)	gametophyte + sporophyte

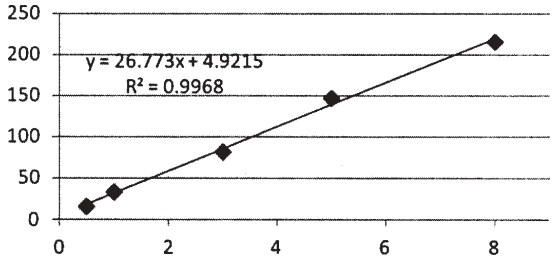

Fig. Calibration curve for determination of lead

Table 2
EXPERIMENTAL VARIANTS

Experimental variant	Composition mg Pb·kg^{-1} Knop medium
AsV1	57.36
AsV2	286.82
AsV3	573.64
AsC (Control)	Knop medium
AtV1	57.36
AtV2	286.82
AtV3	573.64
AtC (Control)	Knop medium
CfV1	57.36
CfV2	286.82
CfV3	573.64
CfC (Control)	Knop medium
PvV1	57.36
PvV2	286.82
PvV3	573.64
PvC (Control)	Knop medium

Table 3
CONCENTRATIONS AND SIGNAL INTENSITY OF THE STANDARD SOLUTIONS

Standard solutions	0.5 ppm	1 ppm	3 ppm	5 ppm	8 ppm
Intensity of the signal	16.05	33.41	81.78	146.2	215.7

biological material was obtained *in vitro* through the culture of green sporangia (sori) in Murashige-Skoog medium [16].

Substance used: lead acetate $(CH_3COO)_2Pb\cdot 2H_2O$. Taking into account the level of lead allowed in the soil in Romania, 20 mg Pb·kg^{-1} (according to Order no. 756/1997), we established the following experimental variants (table 2).

From the material obtained *in vitro* after three months of culture, we prepared the explants used in the experiment (fragmented colonies of gametophyte/ gametophyte + sporophyte); these were cultured in Knop medium (1865) [17] using agar, according to the experimental variants presented in table 2. The culture vesels were placed in the growth chamber under controlled temperature, humidity and illumination conditions. The polyphenols and flavonoids content of the plant material, the lead content of the material regenerated *in vitro* and the bioaccumulation coefficient were determined 4 months after the initiation of *in vitro* cultures.

Polyphenols content. The total polyphenols content was determined according to the method of Singleton and Rosi (1965) with Folin-Ciocalteu reagent. Gallic acid was employed as calibration standard; the results were expressed as gr gallic acid equivalents (GAE)/100 gr DW [18].

Flavonoids content was determined according to the method of Zhishen (1999) [19].; the results were expressed as gr catechin equivalents (CE)/100 gr DW.

Lead content. The lead content was determined using inductively coupled plasma *atomic emission spectrometry [20].* To determine the accumulated lead, the plant material obtained after four months of *in vitro* culture was dried at 100°C in the thermoscale. For ICP-AES (inductively coupled plasma atomic emission spectrometry) analyses we used a Varian, Liberty 110 spectrometer for quantitative determinations of lead. The instrument has a 40.68 MHz RF Generator and a 0.75 m Czerny-Turner Monochromator with 1800 grooves/mm Holographic grating. The operating parameters of the instrument are: plasma gas flow 12 L/min, V-Groove nebulizer, 15 rpm pump rate, 10 s integration time, and automatic background. The wavelength used for the measurement of lead was 220.353 nm. For the mineralization of the materials we used a microwave digestion system Speed wave from Berghof. The reagents used were nitric acid (67%-7 mL) and hydrogen peroxide (3 mL) p.a. from Merck for the digestion of the samples and bi-distilled water to bring the volume to volumetric flasks. For the sample measurement, first a Rapid Quant (semi-quantitative analysis) is performed, in order to approximate the concentration in which the elements are present. For the calibration of the spectrometer we used five standards solutions with different concentrations, obtained by dilution of a multi-element standard solution (ICP multi-element standard solution IV) with a concentration of 1000 mg/L. The standard solutions used for the calibration curve (fig. 1) have presented the signals displayed in table 3.

Bioaccumulation coefficient. To estimate the metal bioaccumulation from medium to plant tissues (gametophyte/gametophyte+sporophyte), we calculated the bioaccumulation coefficient (BC). The bioaccumulation coefficient (BC) is the ratio of plant heavy metal concentration to its quantity in soil (medium) [21, 22].

Results and discussions

Polyphenols and flavonoids content

The amount of polyphenols registered in the gametophytes of the species *Asplenium scolopendrium*, *A. trichomanes-ramosum*, *Cystopteris fragilis*'i *Polypodium vulgare* cultivated in a medium with lead was lower than the amount determined in the control variants (table 4). The same tendency was also noticed for the flavonoids content, except for the species *Polypodium vulgare,* a case in which the explant included both the gametophyte and the sporophyte. For the CfV2 and PvV3 experimental variants, the amount of polyphenols was near the level determined in the control variant. The haploid gametophyte can have a lower capacity of synthesizing polyphenols and flavonoids as compared with the diploid sporophyte of the same species. Moreover, within the plant sporophyte the

No.	Species	Experimental variants	Total polyphenols (g GAE/100 g DW)	Flavonoids (g CE/100 g DW)
1.	*Asplenium scolopendrium*	Control	1.89±0.04	2.00±0.02
2.		V1	1.49±0.02	1.44±0.04
3.		V2	1.09±0.03	1.17±0.02
4.		V3	1.44±0.03	1.56±0.06
5.	*Asplenium trichomanes-*	Control	3.34±0.05	3.70±0.03
6.	*ramosum*	V1	2.84±0.07	3.24±0.05
7.		V2	2.04±0.05	1.90±0.02
8.		V3	2.46±0.03	2.58±0.03
9.	*Cystopteris fragilis*	Control	3.65±0.05	5.92±0.02
10.		V1	3.40±0.02	5.42±0.03
11.		V2	3.49±0.01	5.34±0.03
12.		V3	-*	-*
13.	*Polypodium vulgare*	Control	5.05±0.05	6.04±0.05
14.		V1	2.95±0.04	3.03±0.05
15.		V2	3.14±0.04	4.85±0.05
16.		V3	4.95±0.05	8.04±0.06

* - material affected by necrosis

Table 4
POLYPHENOLS AND FLAVONOIDS CONTENTS

Table 5
LEAD CONTENT

No.	Sample	Lead concentration (mg·kg^{-1})
1	CfC	Under DL
2	AtrC	Under DL
3	AsC	Under DL
4	PvC	Under DL
5	CfV1	91.16±6.27
6	AsV1	48.33±3.32
7	AtV1	58.33±4.01
8	PvV1	110.50±7.60
9	CfV2	395.33±27.19
10	AsV2	318.16±21.88
11	AtrV2	225.16±15.49
12	PvV2	310.50±21.36
13	AsV3	1130.66±77.78
14	AtV3	850.66±58.52
15	PvV3	820.66± 56.46

Note: The results are presented as average of three determinations + expanded uncertainty. The detection limit is 0.4 mg/L for lead proved by previous validations

Experimental variants	Lead concentration in culture medium (mg·kg^{-1})	Lead concentration in plant tissues (mg·kg^{-1})	Bioaccumulation coefficient
CfV1	57.36	91.16	1.58
CfV2	286.82	395.33	1.37
CfV3*	573.64	-	-
AsV1	57.36	48.33	0.84
AsV2	286.82	318.16	1.10
AsV3	573.64	1130.66	1.97
AtV1	57.36	58.33	1.01
AtV2	286.82	225.16	0.78
AtV3	573.64	850.66	1.48
PvV1	57.36	110.50	1.92
PvV2	286.82	310.50	1.08
PvV3	573.64	820.66	1.43

* - *material affected by necrosis*

Table 6
BIOACCUMULATION COEFFICIENT

accumulation of polyphenols varies in its different parts (root, stem and leaves). For example, bilberry leaves generally contain more of phenolics than stems [23, 24]. Generally, heavy metals lead to an increase in soluble polyphenol levels [25-27]. The higher polyphenols concentrations of the V3 (AsV3, AtV3, CfV3, PvV3) variants as compared with the V2 (AsV2, AtV2, CfV2, PvV2) variants may be explained by the fact that phenolics provide protection from metal ions. They can act as metal chelators and can directly scavenge free radicals produced by metal ions [14, 28], thus protecting the deterioration of biomolecules [29]. The involvement of condensed tannins in Pb accumulation in *Athyrium yokoscense* gametophytes has also been mentioned [10].

The lead content accumulated in the plant material

The samples were measured on the calibration curve and the results are shown in table 5. The obtained results indicate that in the control samples (which did not have lead in their medium), the amount of lead was below the detection limit (DL). In the V1 experimental variant, the highest lead concentration was recorded in the species *Polypodium vulgare* (PvV1); the concentration of lead increases in the following order: AsV1>AtV1> CfV1> PvV1. For the V2 experimental variant, the recorded concentration of lead increased in the following order: AtV2> PvV2>AsV2>CfV2. The experimental variants that had the highest concentrations of lead in their media (V3) also had the highest concentration of lead in their plant material. There is a positive correlation between the concentration of lead acetate in the medium and the concentration of lead accumulated in the plant material. Thus, the calculated correlation index was +0.9764 for the variants containing *Asplenium scolopendrium*, +0.9668 for those containing *Asplenium trichomanes-ramosum*, 1 for *Cystopteris fragilis* and +0.9833 for those with *Polypodium vulgare*.

The bioaccumulation coefficient (BC) varied from 0.78 for the AtV2 variant to 1.97 for the AsV3 variant (table 6).

PvV1 had a bioaccumulation coefficient close to that of the AsV3 variant. While in the species *Asplenium scolopendrium* the bioaccumulation coefficient increases proportionately to the increase in lead in the medium, from 0.84 for AsV1 to 1.97 for AsV3, the other species had a different variation of BC.

Many fern species have been identified to absorb and accumulate toxic metals [7, 9] Metals accumulate in different amounts in fern organs. Thus, Zhu et al. [30] demonstrated that in the medicinal species *Blechnum orientale* the accumulation of Cu, Zn, Pb, Cd, As, and Hg in the root was significantly higher than in the leaf. The lead concentration in the leaves of some fern species (from Phu Soi Dao National Park, Phitsanulok Province, Thailand) ranged from 0.96 to 138.39 mg·kg^{-1} [31]. The aquatic fern *Salvinia minima* accumulated a high concentration of lead in its tissues [32]; twofold higher concentrations of lead were attained in the submerged leaf than in the floating leaves [33]. The results obtained *in vitro,* namely the amount of lead accumulated in the regenerated gametophytre and sporophyte tissues and the accumulation coefficient obtained for the tested species, respectively should be completed with *ex vitro* tests for mature plants.

Conclusions

The species included in the experiment, *Asplenium scolopendrium, Asplenium trichomanes-ramosum, Cystopteris fragilis, Polypodium vulgare* tolerated the lead concentrations used for the three experimental variants. An exception was the species *Cystopteris fragilis*; in its V3 variant (573.64 mg Pb·kg^{-1} Knop medium), gametophyte explants were affected by necrosis. The amount of lead determined in the plant material which was grown *in vitro* for four months in a medium containing lead acetate increased proportionately with the lead concentration of the medium. The highest bioaccumulation coefficient, 1.97, was calculated for the AsV3 variant. The presence of stress factors in the environment, in our case the presence of lead in the culture medium has produced changes in the metabolism of phenylpropanoids. The higher polyphenols concentrations of the V3 variants as compared with the V2 variants may be explained by the fact that phenolics provide protection from metal ions. The results obtained *in vitro* should be completed with *ex vitro* tests for mature plants.

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