Current Trends in Natural Sciences Vol. 4, Issue 8, pp. 37-44, 2015

Current Trends in Natural Sciences (on-line)
ISSN: 2284-953X
ISSN-L: 2284-9521

Current Trends in Natural Sciences (CD-Rom)
ISSN: 2284-9521
ISSN-L: 2284-9521

INFLUENCE OF HEAVY METALS ON GAMETOPHYTE DIFFERENTIATION IN TWO *DRYOPTERIS* SPECIES IN ROMANIA'S FLORA

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Abstract
In this work we aimed to study the influence of Zn, Cu and Pb compounds on spore germination and gametophyte development in the species Dryopteris affinis (Lowe) Fraser-Jenkins and Dryopteris filix-mas (L.) Schott. The following initial variants were prepared: V_1Cu 140 mg·L^{-1} Knop solution, V_1Zn 300 mg·L^{-1} Knop solution, V_1Pb 300 mg·L^{-1} Knop solution. From these variants we considered V_2 and $V_3$2 as the concentrations, respectively, 5 and 10 times higher than the initial ones. The percentage of spores germinated was noted to decrease with the increase in the metal concentration in the solution so that there are significant differences between the control and metal variants; in some variants there was no germination reported: in V_3Pb in both species and in V_2Pb in the Dryopteris filix-mas. With regard to the gametophyte differentiation in very few variants, the stage of chordate prothallus was reached (C, V_1Pb, V_1Zn: in the two species). In V_2 and V_3, regardless of the species and metal, the spores and filaments turn to necrosis.

Keywords: pollution, heavy metals, ferns.

1. INTRODUCTION

Heavy metals are natural substances that are found in varying degrees in the earth crust. Some of them are essential in the development of organisms (e.g. Fe, Cu, Zn, Ni, Mo, Co, Cr), and are called trace elements or micronutrients, except for Cd, Pb, Hg, which have no biological role. Through their activities, people have altered the natural cycles of these elements, resulting in increased exposure rate. Extraction and processing of metals date back to ancient times: Cu since the year 5000 BC. Hr., Pb since 4000 BC, Fe in 2000 BC. Other metals were used in alloys and after a long period of time they were found in their metallic forms: bracelets made of an Ag-Zn alloy that date from the year 200 BC were discovered in Rhodes, while the metal element Zn became available on a commercial scale after the 14th century (International Zinc Association).

Comparing the data from International Lead and Zinc Study Group in terms of zinc mining production, an increase of exploitation was found; from 13,054,000 tons in 2013 to 13,512,000 tones in 2014. Unlike Zn, Pb mining production decreased from 5,264,000 tons (2013) to 4,944,000 tones (2014). The decrease in primary production is due to the increase in the weight of the secondary production that provides a share of 80% of the annual US needs and 60% in Europe (International Lead Association). Zinc is used in a 50% proportion in galvanizing processes, and the main use of lead is the manufacture of batteries (80%) International Lead and Zinc Study Group .

Globally, the main reserves are in Chile: 29% of the 700 million metric tons (United States Geological Survey). Although it is considered "the most highly recycled resource" (Cooper

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Development Association Inc.), the mining production of Cu presented a growing trend: from 18.254 million metric tons metric tons in 2013 to 18514 million tons in 2014 (International Copper Study Group). Copper and its alloys are used mainly in electrical engineering (60%).

Zinc is an essential element for human health, going into the composition of many enzymes such as: carbonic anhydrase, superoxide dismutase, alcohol dehydrogenase (ADH), alkaline phostase, carboxypeptidase, etc. It also influences the activity of vitamin A, pancreas (being part of the composition of insulin), immune system (it quickens wound healing), cell division, DNA synthesis, RNA and protein synthesis.

Copper is a trace mineral that ensures the optimal functioning of the thyroid, is involved in the production of melanin, has a role in the formation of connective tissue, allows the absorption and utilization of iron, is necessary to fix calcium in the bones, maintains, through its balance with Zn, the immune system and the nervous system within functional parameters.

Cu and Zn have an important role in plants because they are cofactors or activators for many enzymes. If these micronutrients are in excess, they destroy chloroplasts, reduce chlorophyll synthesis affecting the rate of photosynthesis, and produce chlorosis (Maksymiec, 1997; Yruela, 2005; Broadley et al., 2007).

Pb can change the cell`s membrane permeability, inhibit respiration and ATP-production, reduce the rate of photosynthesis, affect population genetics and damage DNA (Greene, 1993; Pourrut et al., 2011).

Fern spores can be successfully used in the tests devised to determine heavy metal toxicity and to identify opportunities for phyto-rehabilitation. The aim of this work is to study the influence of Zn, Cu and Pb compounds on spore germination and gametophyte differentiation in the following species: *Dryopteris affinis* (Lowe) Fraser-Jenkins and *Dryopteris filix-mas* (L.) Schott.

2. MATERIALS AND METHODS

Biological material. Mature leaves of the two *Dryopteris* species (*D. affinis and D. filix-mas*) were collected from individuals from different sites in the Vâlsan River Valley. In the laboratory, the leaves were kept at room temperature, with the bottom down on paper, so that they release the spores from the sporangia.

Table 1. Experimental variants

Variant	Concentration
Control (C)	Knop solution
V_1Cu	140 mg $CuSO_4 \cdot L^{-1}$ Knop solution
V_2Cu	700 mg $CuSO_4 \cdot L^{-1}$ Knop solution
V_3Cu	1400 mg $CuSO_4 \cdot L^{-1}$ Knop solution
V_1Pb	300 mg $Pb(CH_3CO_2)_2 \cdot L^{-1}$ Knop solution
V_2Pb	1500 mg $Pb(CH_3CO_2)_2 \cdot L^{-1}$ Knop solution
V_3Pb	3000 mg $Pb(CH_3CO_2)_2 \cdot L^{-1}$ Knop solution
V_1Zn	300 mg $ZnSO_4 \cdot L^{-1}$ Knop solution
V_2Zn	1500 mg $ZnSO_4 \cdot L^{-1}$ Knop solution
V_3Zn	3000 mg $ZnSO_4 \cdot L^{-1}$ Knop solution

The metals used. In this experiment compounds with heavy metals were used, i.e. Zn sulfate, Cu sulfate and Pb acetate. The spores from each species were grown in Knop (1865) solution [$Ca(NO_3)_2$: 1.00 $g \cdot L^{-1}$, $MgSO_4$: 0.25 $g \cdot L^{-1}$, KH_2PO_4: 0.25$g \cdot L^{-1}$, KNO_3: 0.25$g \cdot L^{-1}$] with various concentrations of the substances mentioned above to give the variants in Table 1.

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The culture vessels, covered and sealed with Parafilm, were kept in the growth chamber under controlled conditions of temperature (25°C in daytime and 15°C at night), in constant humidity and light (photoperiod: 16 hours light, dark 8:00).

Spore germination and gametophyte differentiation. For each variant three duplicates were made, out of which spores were selected at random for the microscopic preparations that allow determining the germination percentage. For the statistical interpreting the mean value and the standard deviation were calculated, and comparisons were made between mean values using SPSS (Version 16 for Windows). Gametophyte differentiation was monitored periodically, and the stages of development were written down. The micrographs were made by means of an OPTIKA B275 microscope with an A630 Canon Power Shoot camera.

3. RESULTS AND DISCUSSIONS

Spore germination was influenced by heavy metals that were present in the culture medium (Table 2); this influence is expressed, on the one hand, by a lowering of the number of spores germinated with an increase in the metal concentration, and, on the other hand, by the time delay in the germination in the variants V_3 compared to the control. The time delay in germination of spores and the reducing of the percentage of spores germinated under the influence of heavy metals have been reported in the literature: Cu in concentrations of 5 and 50 ppm delayed the germination of spores as compared to control in the species *Pteris vittata*, and at concentration of 50 ppm they remained in a single-cell (spore) stage (Pal and Chunari, 2015).

It can be noted that in the species *D. affinis* the percentage of germinated spores is much higher than in *D. filix-mas* for all variants except V_2Cu, where there are percentages of 6 and 8, respectively. By comparing the means by means of the Duncan test, we note that there are no significant differences between variants V_2Cu and V_3Cu in the species *D. affinis*.

In V_3Pb no germination was noted in either species; instead, in the species *D. filix-mas* we could not apply the Duncan test for the Pb variants because of insufficient data: germination was reported only in variant V_1 (17.3). Soare et al. (2014) found that Pb acetate affects spore germination and gametophyte development in *D. affinis.*

For both species the highest percentages of germinated spores were determined in the Zn variants: in *D. affinis* values were recorded ranging between 60.6 (V_1) and 30.6 (V_3). In *D. filix-mas* the maximum was 56 (V_1Zn), and there are no significant differences between variants V_2Zn and V_3Zn (Duncan test).

The fact was confirmed that $CuSO_4$ (Fig.13-25) is more toxic than $ZnSO_4$ (Fig.10-22), a result previously obtained by Francis and Petersen, (1983), Biesinger and Christensen (1972), Baskaran and Jeyachandran (2012).

Table 2. Influence of heavy metals on the germination of spores

	Experimental variants									
Species	Control	V_1Cu	V_2Cu	V_3Cu	V_1Pb	V_2Pb	V_3Pb	V_1Zn	V_2Zn	V_3Zn
	Percentage of germinated spores (mean ± standard deviation)									
D.a.	82±3,6[a]	54±4[b]	6±3[c]	7,3±4[c]	49,3±5,5[b]	24,3±3[c]	-	60,6±8[b]	49,3±3,7[c]	30,6±5[d]
D.f.m.	69±1,7[a]	29±4,3[b]	8±2[c]	3,6±1,1[d]	17,3±9	-	-	56±1[b]	13,6±3,5[c]	15,6±3,5[c]

Legend: The values are the means of 3 replicates ± standard deviation; a, b, c, d - the results obtained from the Duncan test: the comparisons were made between control and V_{1-3} for each metal. *D.a.-Dryopteris affinis, D.f.m.-Dryopteris filix-mas.*

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ISSN-L: 2284-9521

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Gametophyte differentiation. To monitor the development of the gametophyte observations were made on the stages of development at intervals of 1 month, 2 months, 4 months (Tables 3 and 4). After the first month the most common development stage is the prothallus blade stage that occurs in the control (Fig.1-14) and in V_1 (Fig.11-17-20-23) for all metals, in both species. In *D. affinis* V_1Pb there also appear three-dimensional cellular masses (Fig.5), and in V_1Zn young chordate prothalli (Fig.8). Also in this species prothallus filaments were observed in V_2Pb and V_2Zn. For the remaining variants, spores are either germinated or not germinated.

Table 3. **Gametophyte differentiatin to *Dryopteris affinis***

Variants	Period		
	1 month	**2 months**	**4 months**
Control	prothallic blades	prothallic blades, chordate prothallia, antheridia	prothallic blades, chordate prothallia, antheridia
V_1Cu	prothallic filaments to blades, germinated spores	necrotic filaments and protallic blades	necrotic prothallic blades
V_2Cu	necrotic germinated spores	necrotic germinated spores	necrotic germinated spores
V_3Cu	few germinated spores	necrotic spores	necrotic spores
V_1Pb	prothallic blades, rare three-dimensional cell masses	chordate prothallia	chordate prothallia, antheridia and prothallic filaments
V_2Pb	prothallic filaments (3-4 cells)	necrotic filament	spores and discollored filaments
V_3Pb	-	-	-
V_1Zn	prothallic blades (some with short rhizoids and necrotic cells), young chordate prothallia	branched chordate	chordate prothallia: necrotic (partial/total), viable antherozoids
V_2Zn	short filaments (2-3 cells), some necrotic	necrotic short filaments	spores and discollored filaments
V_3Zn	germinated spores	necrotic spores	necrotic spores

The evolution of prothallus blades after 2 months can be summed up in two situations, namely the shift to the chordate prothallus in V_1Pb (Fig.6-18) and V_1Zn (Fig.9-21) in both species, and the emergence of antheridia in the controls (Fig.2-3-15), or their necrosis for V_1Cu in both species (Fig.12-24). Also during that period the spores germinated in the two species in the variants V_2Cu, V_3Cu, V_3Zn, are noted to develop necrosis.

In the control, for both species after 4 months, the chordate prothalli branched significantly and antheridia continued to appear (Fig.4, Fig.16); chordate prothalli also appeared in V_1Pb in the two species (Fig.7, Fig.19) and in V_1Zn *D. filix-mas* (Fig.22). Other important changes for that date were observed in V_1Zn *D. affinis*, where the chordate prothalli undergoes partial/total necrosis and antherozoides appear (Fig.10).

Cheruiyot (2008) tested the toxicity of Zn, Cu and Pb in concentrations of 0, 5, 10, 15, 25, 50, 100, 200, 500, 1000 ppm of the species *Ceratopteris richardii*. She noted that, in the variants in which Cu was used, the spores germinated only in the 5 ppm concentration, and gametophyte development was affected, as it was smaller and yellow-brown in colour. The spores germinated and developed normally at concentrations of 5 ppm Zn and at 5, 10, 15, 25, 50, 100 ppm Pb. For the variants ranging from 10 to 25 ppm Zn and at 200 ppm Pb concentration the spores germinated, but gametophyte growth and development was affected: it had a small size and was brown.

Table 4. **Gametophyte differetiation to *Dryopteris filix-mas***

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ISSN: 2284-953X
ISSN-L: 2284-9521

Current Trends in Natural Sciences (CD-Rom)
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Variants	Period		
	1 month	2 months	4 months
Control	differentiation of prothallic blades and prothallic blades	prothallic blades, chordate prothallia, antheridia	prothallic blade, chordate prothallia, antheridia
V_1Cu	filament and prothallic blades	necrotic filaments and prothallic blades	necrotic filament and prothallic blade
V_2Cu	necrotic germinated spores	necrotic germinated spores	necrotic germinated spores
V_3Cu	few germinated spores	necrotic spores	necrotic spores
V_1Pb	prothallic blades	chordate prothallia	chordate prothallia
V_2Pb	-	-	-
V_3Pb	-	-	-
V_1Zn	prothallic blades	chordate prothallia	chordate prothallia
V_2Zn	germinated spores	necrotic germinated spores	necrotic germinated spores
V_3Zn	germinated spores	necrotic germinated spores	necrotic germinated spores

Fig.1 D.a. C-one month (x100, orig.)

Fig.2 D.a. C-2months (x100, orig.)

Fig.3 D.a. C-2months (x400, orig.)

Fig.4 D.a. C-4months (x40, orig.)

Fig.5 D.a. V_1Pb-one month (x40, orig.)

Fig.6 D.a. V_1Pb-2months (x40, orig.)

Fig.7 D.a. V_1Pb-4months (x40, orig.)

Fig.8 D.a.V_1Zn-one month (x40, orig.)

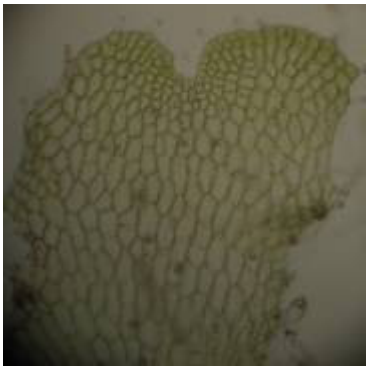

Fig.9 D.a. V_1Zn-2months (x40, orig.)

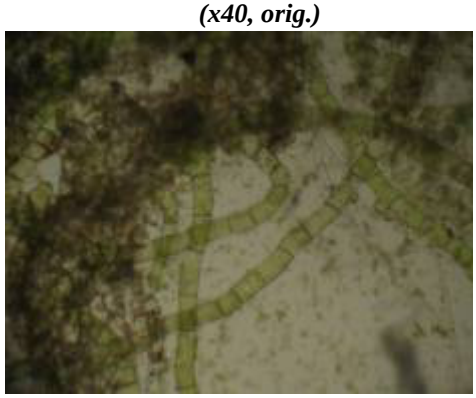

Fig.10 D.a. V_1Zn-4months (x40, orig.)

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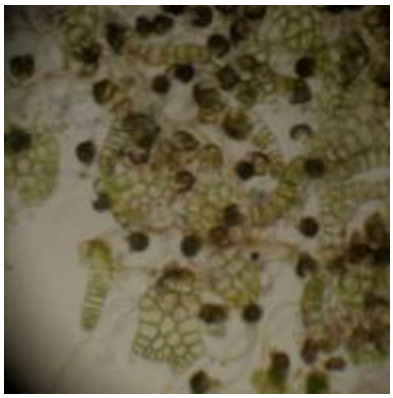

Fig.11 D.a. V_1Cu-one month (x40, orig.)

Fig.12 D.a. V_1Cu-2months (x100, orig.)

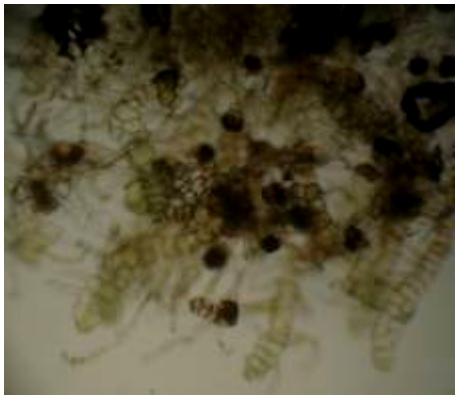

Fig.13 D.a. V_1Cu-4months (x40, orig.)

Fig.14 D.f.m C-one month (x40, orig.)

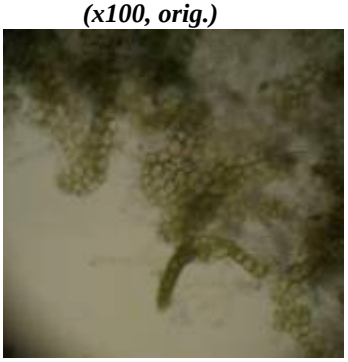

Fig.15 D.f.m. C-2months (x40, orig.)

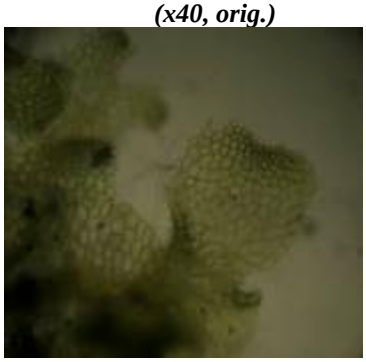

Fig.16 D.f.m. C-4months (x40, orig.)

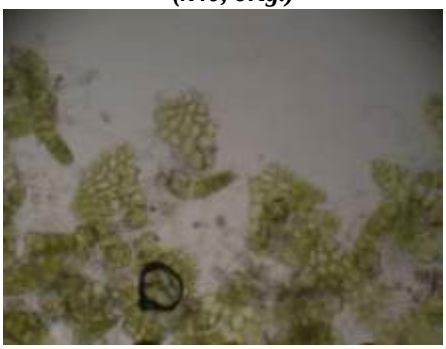

Fig.17 D.f.m. V_1Pb-one month (x40, orig.)

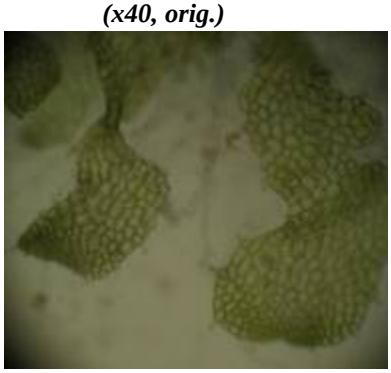

Fig.18 D.f.m. V_1Pb-2months (x40, orig.)

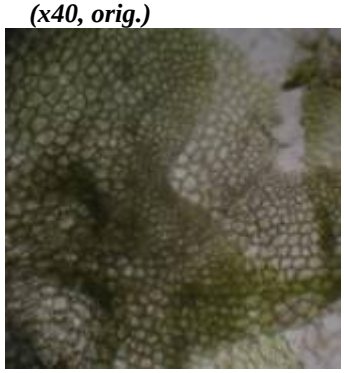

Fig.19 D.f.m. V_1Pb-4months (x40, orig.)

Fig.20 D.f.m. V_1Zn-one month (x100 orig.)

Fig.21D.f.m. V_1Zn-2months (x40, orig.)

Fig.22 D.f.m.V_1Zn-4months (x40, orig.)

Current Trends in Natural Sciences (on-line)
ISSN: 2284-953X
ISSN-L: 2284-9521

Current Trends in Natural Sciences (CD-Rom)
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ISSN-L: 2284-9521

Fig.23 D.f.m. V_1Cu-one month (x40, orig.)

Fig.24 D.f.m. V_1Cu-2months (x100, orig.)

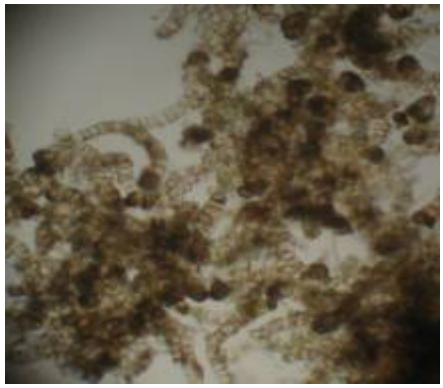

Fig.25 D.f.m.V_1Cu-4months (x40, orig.)

As a result of the experiment conducted by Muccifora (2008) it was established that Cu has an inhibitory effect with respect to spore germination (germination within 40 days of cultivation, germination percentage of only 25%) and determines changes in the gametophyte differentiation (re-orientation of growth), which accumulates a large amount of Cu.

4. CONCLUSIONS

It was noted that the percentage of germinated spores decreases with the increase in the metal concentration in the solution, so that there are significant differences between the control and the metal variants; in some variants there was no germination: in V_3Pb in both species, and in V_2Pb for *Dryopteris filix-mas*. As far as gametophyte differentiation is concerned, it was in very few variants that the chordate prothallus stage was reached (M, V_1Pb, V_1Zn - in both species). For V_2 and V_3, regardless of species

5. ACKNOWLEDGEMENTS

The biological material was provided by Romanian Pteridological Association.

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ISSN-L: 2284-9521

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