

EFFECTS OF CHRONIC TOXICITY INDUCED BY CADMIUM ON THE GAMETOPHYTE OF TWO FERN SPECIES

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Abstract

The aim of the present study was to determine the effect of chronic cadmium (Cd) action on the germination of spores and gametophyte differentiation in species *Athyrium filix-femina* (L.) Roth and *Dryopteris filix-mas* (L.) Schott, on different culture media (Knop solution, soil) for a period of 3 months. Cadmium was used in the following concentrations: C=0 mg Cd · L⁻¹ Knop solution/kg⁻¹ soil, V₁=25 mg Cd · L⁻¹ Knop solution/kg⁻¹ soil, V₂=50 mg Cd · L⁻¹ Knop solution/kg⁻¹ soil, V₃=100 mg Cd · L⁻¹ Knop solution/kg⁻¹ soil, V₄=150 mg Cd · L⁻¹ Knop solution/kg⁻¹ soil. The percentage of germinated spores was found to decrease with the increasing Cd concentration in the environment, while germination is delayed in time. Unlike the Knop solution variants, in the soil variants gametophyte development was not significantly affected; for V₁-2 soil concentrations the sporophyte appears in *Athyrium filix-femina*, a stage that was also noted for the Knop solution control, for the same species. In the case of the variants grown on Knop solution, although the spores did germinate and the gametophyte began to differentiate, Cd-induced chronic stress cannot be compensated by the gametophyte, so that the cells lose their membrane integrity, and their survival is compromised.

Key words: heavy metals, *Athyrium filix-femina*, *Dryopteris filix-mas*.

INTRODUCTION

Cadmium is one of the most important heavy metals, and it is usually encountered: on the International Agency for Research on Cancer. list due to its carcinogenic properties, on toxic substance list of Agency for Toxic Substances and Disease Registry (CAS ID #:7440-43-9), among the top 126 priority pollutants, according to the United States Environmental Protection Agency (Flora, 2014).

It is a transition metal (block d), which presents chemical similarities to zinc (Zn) – in fact, they both belong to the same group (12). These similarities can cause the toxicity of Cd: replacement of Zn, a trace element, by Cd affects metabolic processes (Wuana and Okieimen, 2011).

According United States Geological Survey, to estimate Cd reserves, Zn reserves are checked, while taking into account this aspect: Cd is approximately 0.003% of Zn ores.

Cadmium pollution is due to natural sources, the contribution of which varies between 10-50% of total emissions, and also to anthropogenic sources. For example, the mining of zinc is estimated to release approximately 6 million tones, as a byproduct

of Cd (Raza et al., 2015). In order to present the main anthropogenic sources International Cadmium Association proposes a classification that takes into account the presence of Cd as impurity – non-Cd products: iron and steel, fossil fuels, cement, phosphate-based fertilizers, and, as a necessity: NiCd batteries, pigments, Cd alloys, electronic Cd compounds, etc.

Determining Cd toxicity on living beings is performed using acute and chronic toxicity tests. Acute toxicity refers to short-period exposure of an organism to the action of one or more toxic agents. Within this framework, lethal concentration (LC₅₀) is determined, i.e. the concentration that leads to the death of 50% of the test organisms. In nature, most pollutants manifests their action after a long time, and pollution is usually in non-lethal concentrations. Chronic toxicity is the "capacity of a substance or a solution to induce adverse effects for a long time, after repeated or continuous exposure, sometimes over the whole lifetime of an organism" (United States Environmental Protection Agency).

The best-known and most severe form of chronic exposure to the action of Cd is that occurring in Japan: consumption of rice contaminated with Cd leads to the disease

called "Itai-Itai", which is characterized by kidney damage and disorders of the bone system (osteomalacia and osteoporosis) (Nordberg et al., 2015).

According to Pavlik (1997) 90% of the Cd taken up by plants comes from the ground, and only 10% from the atmosphere, as the main paths of penetration are the roots and leaves.

Catalá et al. (2011) recommended using ferns in toxicity tests, both acute and chronic, because the results can be extrapolated to wild plants or cultivated plants, they are found in different habitats (ecological or organic relevance), and growing spores and development gametophyte can be made on different media (solution, soil, etc.).

In order to know pteridospore sensitivity in a chronic toxicity testing of different substances and environmental samples should be used (Catalá and Rodríguez-Gil, 2011).

The aim of this paper was to determine the chronic effect of the action of Cd on the germination of spores and gametophyte differentiation in species *Athyrium filix-femina* (L.) Roth (*Aff*) and *Dryopteris filix-mas* (L.) Schott (*Dfm*) on different culture media.

MATERIALS AND METHODS

In order to obtain the spores of the two species, the author took several study trips along the Vâlsan Valley over the period August 2015. Mature leaves were collected from several individuals in different sites in order to ensure genetic diversity. After releasing the spores in the sporangia, there followed their collecting and preserving in a refrigerator at 4°C.

Testing media:

Two test media were used: Knop solution [$\text{Ca}(\text{NO}_3)_2$: 1.00 g·L⁻¹; MgSO_4 : 0.25 g·L⁻¹; KH_2PO_4 : 0.25g·L⁻¹; KNO_3 : 0.25g·L⁻¹] and flower earth Florisol obtained by processing from the deposit in Dersca-Dorohoi, with a pH between 6.5-7, humidity 60-70% N: 410 ppm, P: 192 ppm, K: 1350 ppm; organic substance min 70% dry product. The soil was sterilized at 60°C.

Tested substance: The substance tested was Cd acetate in various concentrations; reporting was done per L for the samples in Knop solution, and per kg for the soil variants: Control (C)=0 mg Cd·L⁻¹ kg⁻¹, V₁=25 mg Cd·L⁻¹ kg⁻¹, V₂=50

mg Cd·L⁻¹ kg⁻¹, V₃=100 mg Cd L⁻¹ kg⁻¹, V₄=150 mg Cd·L⁻¹ kg⁻¹.

To ensure optimal conditions for development, the culture vessels were kept in growth chamber at 25°C in the daytime, and 15°C at night, with constant humidity and illumination (photoperiod: 16 hours of light, and 8 hours of dark). The soil variants were placed in Petri dishes and periodically watered with distilled water.

The experiment had 3 repeats. For the Knop solution variants quantitative determinations were made: the percentage of germinated spores was determined, and to do the statistical interpretation the SPSS program, version 16 was used, with which the average and the standard deviation were calculated. Comparisons were made using Duncan's test. To monitor the differentiation of the gametophyte in all variants, observations were made at regular intervals, and photomicrographs were made under an OPTIKA B275 microscope with an A630 Canon Power Shoot camera and under a OPTIKA stereo-microscop.

RESULTS AND DISCUSSIONS

Germination of spores is influenced by a number of factors such as light, phytohormones, ions of metal, temperature (Suo et al., 2015).

As far as the cultures of spores are concerned, which used the Knop solution, Cd significantly affected germination, primarily by reducing the percentage of germinated spores. All experimental variants were affected, except V₂Cd *Dfm*, where there were 7 percent more spores than in the controls, and in *Aff* – between V₃Cd and the control there were no significant differences (see Table 1). Also, spore germination was delayed in time in the V₄Cd variant, in both species: germination was reported after a month compared to the control. Also, in this variant the lowest percentage of spores germinated was obtained: 4 for *Dfm* and 15 for *Aff*.

Time delay and a lower percentage of spores germinated due to the presence of various concentrations of Cd were also reported by Gupta and Devi (1992), and Biswas et al. (2015) in several species of ferns.

Gupta et al. (1992) found that, in concentrations of 2.5 and 5 mg Cd L⁻¹ spore germination was inhibited, and the development of the gametophyte was discontinued at the stage of prothallium blade in the species *Ceratopteris thalictroides*.

Table.1. Influence of heavy metals on the germination of spores

Species	C	V ₁ Cd	V ₂ Cd	V ₃ Cd	V ₄ Cd
Percentage of germinated spores (mean ± standard deviation)					
<i>Athyrium filix-femina</i>	90.3±2 ^a	26.6±5 ^c	45±1 ^b	92.3±1 ^a	15±1 ^c
<i>Dryopteris filix-mas</i>	81.6±2 ^b	75.3±4 ^c	89.3±5 ^a	54±3 ^d	4±1 ^c

Legend: The values are the means of 3 replicates ± standard deviation; a, b, c, d, e – the results obtained from the Duncan test: the comparisons were made between control and V₁₋₄ for each metal.

In Table 2 and Table 3 the gametophyte differentiation after one month is shown, both on the soil and on the Knop solution, in both species.

Table 2. Gametophyte differentiation of *A. filix-femina* (one month after experiment initiation)

Variants	Knop solution	Soil
Control	blades differentiation, antheridia	young chordate prothallia, antheridia
V ₁ Cd	filaments differentiation, germinated spores	prothallium blade, antheridia
V ₂ Cd	prothallium blade, three-dimensional cell masses, antheridia	chordate prothallia
V ₃ Cd	prothallium filament, three-dimensional cell masses	chordate prothallia, rare prothallium blade, antheridia
V ₄ Cd	filaments differentiation	prothallium blade, antheridia

Table 3. Gametophyte differentiation of *D. filix-mas* (one month after experiment initiation)

Variants	Knop solution	Soil
Control	prothallium blade	young chordate prothallia
V ₁ Cd	damaged filaments and blades differentiation, short rhizoid, three-dimensional cell masses	chordate prothallia
V ₂ Cd	blades differentiation, three-dimensional cell masses	chordate prothallia
V ₃ Cd	filaments, blades differentiation	prothallium blade and filament
V ₄ Cd	germinated spores	prothallium blade and filament

It was found that gametophyte development was much faster in the soil-grown variants, where the following stages were noted:

chordate prothallia in the controls of both species (Figure 14, 17) V₂Cd (Figure 23) and V₃Cd in *Athyrium*, V₁Cd and V₂Cd in *Dryopteris*, blade in V₁Cd (Figure 20) and V₄Cd in *A. filix-femina*, and for the second species, filaments in V₃₋₄Cd. In *Athyrium*, antheridia with viable antherosoids were observed, in all cases, except V₂Cd. In the Knop solution variants the most advanced stage of development was the prothallian blade one, which occurred in the controls of the two species (Figure 1, 4, 5) and in V₂Cd *Athyrium* (Figure 7). The filament stage usually occurred at high concentrations V₃₋₄ (Figure 10).

Due to the influence of Cd, the gametophyte development was affected: the filaments (V₁Cd in both species) and blades were partially damaged (V₂Cd in *Dryopteris* - Figure 12) and three-dimensional cell masses were formed (Figure 8). The abnormal growth of the prothallium blade was reported by Gupta and Devi (1994), as well, in the species *Pteris vittata*, where the gametophyte is much more sensitive to Cd action than the sporophyte.

Table 4. Gametophyte differentiation of *A. filix-femina* (three months after experiment initiation)

Variants	Knop solution	Soil
Control	chordate prothallia with sporophyte	chordate prothallia with archegonia
V ₁ Cd	damaged filaments, germinated spores	chordate prothallia and sporophyte with embryonic leaf
V ₂ Cd	damaged prothallia	chordate prothallia and sporophyte
V ₃ Cd	damaged filaments	mature chordate prothallia with archegonia, young chordate prothallia with antheridia, fecundation
V ₄ Cd	damaged filaments, germinated spores	prothallia, antheridia

Table 5. Gametophyte differentiation of *D. filix-mas* (three months after experiment initiation)

Variants	Knop solution	Soil
Control	young elongated prothallia	chordate prothallia, antheridia
V ₁ Cd	damaged filaments	chordate prothallia,
V ₂ Cd	damaged filaments and blades	chordate prothallia, archegonia
V ₃ Cd	germinated spores, damaged filament	young chordate prothallia
V ₄ Cd	few germinated spores	young chordate prothallia (small)



Figure 1. *Dfm*, C, one month (x100).



Figure 2. *Dfm*, M, 3 months (x100).



Figure 3. *Dfm*, C, 3 months (x400).

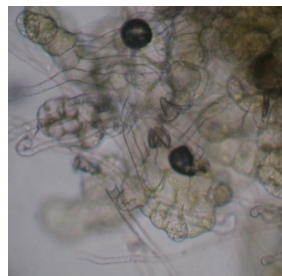


Figure 4. *Aff* C, one month (x100).

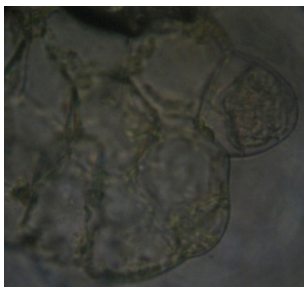


Figure 5. *Aff* C, one month (x400).

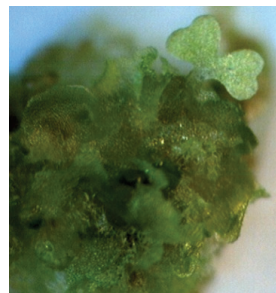


Figure 6. *Aff* C, 3 months (x10).



Figure 7. *Aff* V₂, one month (x100).



Figure 8. *Aff* V₂, one month (x100).



Figure 9. *Aff* V₂, 3 months (x100).



Figure10. *Dfm* V₃, one month (x100).

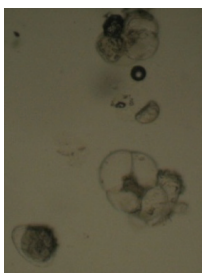


Figure11. *Dfm* V₃, 3 months (x100).

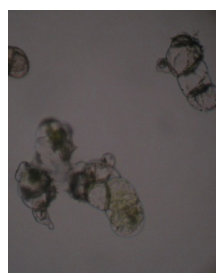


Figure12. *Dfm* V₂, one month (x100).

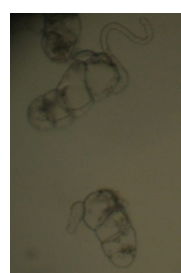


Figure13. *Dfm* V₂, 3 months (x100).

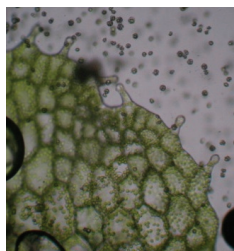


Figure14. *Dfm C*, soil, one month (x100).

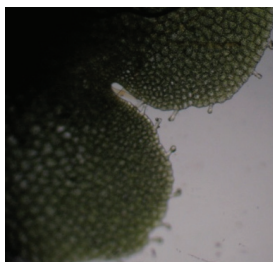


Figure15. *Dfm C*, soil, 3 months (x40).

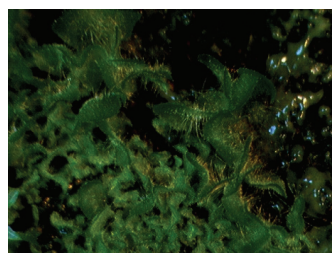


Figure16. *Dfm C*, soil, 3 months (x10).



Figure17. *Aff C*, soil, one month 10x10 (x100).

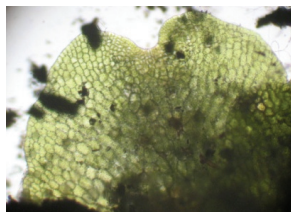


Figure18. *Aff C*, soil, 3 months (x40)

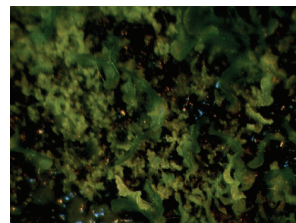


Figure19. *Aff C*, soil, 3 months (x10).



Figure 20. *Aff V₁*, soil, one month (x100).

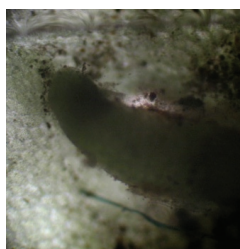


Figure 21. *Aff V₁*, soil, 3 months (x100).



Figure 22. *Aff V₁*, soil, 3 months (x10).

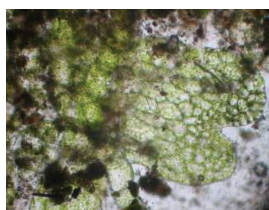


Figure 23. *Aff V₂*, soil, one month (x100).



Figure 24. *Aff V₂*, soil, 3 month (x10).

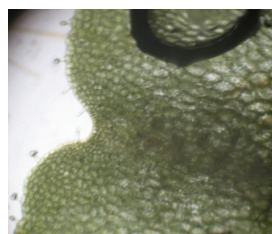


Figure 25. *Dfm V₂*, 3 months (x40).

After 3 months from the initiation of the experiment, in *A. filix-femina* as control, the Knop solution variant, the sporophyte was formed (Figure 6), while *D. filix-mas* was still in the gametophyte stage: prothallium blade (Figure 2) with antheridia (Figure 3). According to Table 4 and Table 5, regardless of species and experimental variant, after 3 months the gametophyte development was

stopped in the stage of chordate prothallium (Figure 9), with damaged blades and filaments (Figure 11, 13). For low concentrations (V_{1-2}), in the soil-grown variants, Cd stimulated the development so that within 3 months the sporophyte was formed on the gametophyte (Figure 22, 24), in which the juvenile leaves can be noted (Figure 21). In the remaining Cd variants, and also in the control, the

gametophyte was still in the stage of heart-shaped prothallium of different sizes (Figure 15, 16, 19) with/without antheridia and/or archegonia (Figure 18, 25).

CONCLUSIONS

Cd affected the percentage of spores germinated and gametophyte development in the experimental variants grown on Knop solution.

The influence of Cd on gametophyte development was far more significant in the solution variants (damaged blades and chordate prothallia) as compared to those grown on soil, and between species, in *Dryopteris filix-mas* as compared to *Athyrium filix-femina*.

In V_{1-2} concentrations for soil-grown *Athyrium* the occurrence of the sporophyte is noted. In the case of the variants grown on Knop solution, although the spores did germinate and the gametophyte began to differentiate, Cd-induced chronic stress cannot be compensated by the gametophyte, so that the cells lose their membrane integrity, and their survival is compromised.

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