THE INFLUENCE OF NICKEL AND CADMIUM COMPOUNDS ON GAMETOPHYTE DIFFERENTIATION IN *Dryopteris affinis* (Lowe) Fraser-Jenkins AND *Dryopteris filix-mas* (L.) Schott

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Abstract. The aim of this research was to study the influence of compounds containing Ni and Cd on gametophyte differentiation in two native ferns, *Dryopteris affinis* (Lowe) Fraser-Jenkins and *D. filix-mas* (L.) Schott. The variants tested were V_1Cd: 3 mg·L^{-1} Cd acetate, V_2Cd: 15 mg·L^{-1} Cd acetate, V_3Cd: 30 mg·L^{-1} Cd acetate, V_1Ni: 75 mg·L^{-1} Ni sulphate, V_2Ni: 375 mg·L^{-1} Ni sulphate, V_3Ni: 750 mg·L^{-1} Ni sulphate and Control. The Pearson correlation index showed a significant negative correlation between the percentage of germinated spores and the heavy metal concentration in the culture medium, in both species. The main changes observed in the gametophyte were: a longer period needed to reach the characteristic stages, damage to rhizoid elongation, the formation of three-dimensional cell masses, chlorosis and necrosis of the gametophyte cells, and accumulation of crystal structures on the surface of the gametophyte.

Keywords: spore germination, chlorosis, necrosis, metal accumulation.

Rezumat. Influența compușilor cu nichel și cadmiu asupra diferențierii gametofitului la *Dryopteris affinis* (Lowe) Fraser-Jenkins și *Dryopteris filix-mas* (L.) Schott. Scopul acestei lucrări a fost acela de a studia influența compușilor ce conțin nichel și cadmiu asupra diferențierii gametofitului la două specii native de ferigi, *Dryopteris affinis* (Lowe) Fraser-Jenkins și *D. filix-mas* (L.) Schott. Variantele experimentale testate au fost V_1Cd: 3 mg·L^{-1} acetate de Cd, V_2Cd: 15 mg·L^{-1} acetate de Cd, V_3Cd: 30 mg·L^{-1} acetate de Cd, V_1Ni: 75 mg·L^{-1} sulfat de Ni, V_2Ni: 375mg·L^{-1} sulfat de Ni, V_3Ni: 750 mg·L^{-1} sulfat de Ni și varianta Martor. Indicele de corelație Pearson arată o corelație negativă între procentul de spori germinați și concentrația metalului greu din mediul de cultură, în cazul ambelor specii. Principalele modificări observate la nivelul gametofitului au fost: o perioadă mai lungă de timp necesară pentru atingerea stadiilor caracteristice, alterarea alungirii rizoizilor, formarea de mase celulare tridimensionale, cloroze și necroze ale celulelor gametofitului, acumularea de structuri cristaline pe suprafața gametofitului.

Cuvinte cheie: germinația sporilor, cloroze, necroze, acumulare de metal.

INTRODUCTION

Nickel (Ni) and cadmium (Cd) are heavy metals found in the Earth's crust, occupying, in point of their relative abundance, in 24th and, 64th place, respectively. The natural sources of pollution are volcanic emissions, vegetation fires, dust or powder resulting from weather conditions affecting rocks and soil. The anthropogenic sources are varied: Cd results from mining and ore processing, from chemical plants, fertilizers, paints, metal plating, dyes, and oil refining, and Ni - from the pulp and paper industry, dyeing and textile printing, chemical fertilizer industry, and waste incineration (AGARWAL, 2009), as well as from other common sources: metallurgy, coal burning, and Ni-Cd batteries. Sewage sludge and the effluents used to irrigate crops lead to increased concentrations of heavy metals in the soil: thus, CHAUDRI et al. (2001) and AHMAD et al. (2015) concluded that the relationship between the concentration of soluble Cd in the soil and grain crops became more linear after the application of sludge, and SMOLEN et al. (2010) and AHMAD et al. (2015) found that there are moderate to strong positive correlations between the concentrations of Fe, Cd, Co and Pb in the crops (spinach and lettuce) and the soil.

According to the United States Geological Survey (USGS), the countries that produce the largest amount of Cd are in Asia: China, Korea, and Japan. It is again Asia that dominates with respect to the primary production of Ni in 2013, with 921 tonnes, followed by Europe with 495.4 tonnes, and America with 268.6 tonnes (according to the International Nickel Study Group - INSG).

Heavy metals can be transmitted along the food chain, affecting both producers and consumers. Cd inhibits seed germination (HEIDARI & SARANI, 2011; ZIAR-UR-REHMAN et al., 2015) and also affects root growth (HAOUARI et al., 2012; ZIAR-UR-REHMAN et al., 2015) and absorption of nutrients; it induces oxidative stress by releasing free radicals (KHAN et al., 2007; ZIAR-UR-REHMAN et al., 2015). Cd is "the only metal that can affect human or animal health even if concentrations in plant tissues are not phytotoxic" (PEIJNENBURG et al., 2000; PERALTA-VIDEA et al., 2009).

Nickel is a heavy metal essential for higher plants, indeed less important than Zn or Cu, yet having a special role as a constituent of urease (SEREGIN & KOZHEVNIKOVA, 2006). Excess Ni inhibits seed germination, photosynthesis and transpiration, affecting plant metabolism, nutrient absorption, and causing ultrastructural changes (AHMAD & ASHRAF, 2011).

In order to study the influence of heavy metals on superior plants one can perform phytotoxicity tests that use fern spores (CATALÁ et al., 2011). Ferns are excellent models for study in several ways: 1. biodiversity - there are over 250 genera and 10,000 species of ferns (RATHINASABAPATHI, 2006); 2. adjustment to extreme environments - resistance to aridity, such as certain species of the families Actiniopteridaceae, Sinopteridaceae, Pteridaceae and

Selaginellaceae (POREMBSKI & BARTHLOTT, 2000; RATHINASABAPATHI, 2006), tolerance to salinity - *Acrostichum aureum* is a halophilous species (MEDINA et al., 1990; RATHINASABAPATHI, 2006); 3. invasive and competitive capacity: *Lygodium microphyllum* in North and South America, and the species of the genus *Salvinia* threaten all water bodies in the world (ABBASI & NIPANEY, 1986; RATHINASABAPATHI, 2006); 4. capacity of forming numerous spores, which are easy to collect and preserve; 5. the gametophyte is small and grows rapidly; 6. acute and chronic toxicity tests are not expensive and require common laboratory equipment (CATALÁ et al., 2011).

The aim of this research was to study the influence of a number of heavy metals on gametophyte differentiation in two native species of the genus *Dryopteris*: *D. affinis* (Lowe) Fraser-Jenkins (*Da*) and *D. filix-mas* (L.) Schott (*Dfm*).

MATERIALS AND METHODS

The biological material was collected from individuals located in the Vâlsan Valley, a protected area of national interest (Argeş County, Romania). Mature leaves of both species were collected from individuals in different sites, then wrapped in paper and carried in plastic bags. In the laboratory, the leaves were maintained at room temperature with the lower part down on paper, in order to release the spores from the sporangia.

In the experiment we used compounds containing heavy metals, namely cadmium acetate and nickel sulphate, compounds that are carcinogenic (as indicated by the International Agency for Research on Cancer - Group I: cadmium and cadmium compounds, and nickel compounds). According to Directive 76/464/EEC and daughter directives, these metals are on the list of priority substances/hazardous, i.e.: Cd and its compounds are comprised in List I (Directive 83/513 of 26 September 1983), and Ni and its compounds in List II. Under Directive 86/278/EEC, which regulates the use of sewage sludge in agriculture, the limit values for heavy metal concentrations in the soil are established (Cd: 1-3 $mg \cdot Kg^{-1}$, Ni 30-75 $mg \cdot Kg^{-1}$ of dry matter in a representative soil sample). For each species we prepared the following variants (Table 1).

Table 1. Experimental variants.

Variants	Concentration
Control (C)	Knop solution
DaV_1Cd/*Dfm* V_1Cd	3 mg cadmium acetate$\cdot L^{-1}$ Knop solution
DaV_2Cd/*Dfm*V_2Cd	15 mg cadmium acetate$\cdot L^{-1}$ Knop solution
DaV_3Cd/*Dfm*V_3Cd	30 mg cadmium acetate$\cdot L^{-1}$ Knop solution
DaV_1Ni/*Dfm*V_1Ni	75 mg nickel sulfate$\cdot L^{-1}$ Knop solution
DaV_2Ni/*Dfm*V_2Ni	375 mg nickel sulfate$\cdot L^{-1}$ Knop solution
DaV_3Ni/*Dfm*V_3Ni	750 mg nickel sulfate$\cdot L^{-1}$ Knop solution

The concentrations were determined by taking account of Order 756 of 3 November 1997 approving the Rules on the assessment of environmental pollution, where alert soil thresholds for Cd and Ni are established at 3 $mg \cdot Kg^{-1}$, and 75 $mg \cdot Kg^{-1}$ dry matter.

The spores were grown on the surface of the liquid culture medium. The culture dishes, covered and sealed with Parafilm, were kept in a POL EKO 350 growth chamber. The temperature values were: 25°C during the day, and 15°C at night, and the humidity and lighting conditions were controlled (photoperiod: 16 hours of light, and 8 hours of dark). After one week from the initiation of the study, the percentage of germinating spores was determined. It is safe to consider that the spores have germinated if the rhizoid cell can be identified microscopically. Three repetitions were performed for each variant, from which spores were randomly selected, for the microscopic spore preparations to determine the percentage of germination. The statistical interpretation was performed using SPSS (version 16 for Windows). We calculated: the mean value, the standard deviation, and the P (Pearson) correlation factor. We also performed the comparisons between the mean values using the Duncan test. Gametophyte differentiation was monitored periodically. The biological material was microphotographed under a B275 OPTIKA microscope with an A630 Canon Power Shoot camera.

RESULTS AND DISCUSSION

The data obtained one week after the initiation of the experiment showed that spore germination was influenced by the presence of heavy metal compounds in the culture medium. There were significant differences in the germination percentages in the Control and the other experimental variants (Table 2). For *Da*, the differences between germination percentages ranged from -11 (C-V_1Cd) to -76 (C-V_3Cd), and for the species *Dfm* the differences ranged from -13.40 (C-V_1Cd) to -54 (C-V_2Ni). By comparing the results obtained using the Duncan test, we found that for *Da* the only variants between which there are no significant differences are the Control and V_1Cd, and for *Dfm* the differences are between the variants V_1Cd and V_2Cd, and V_2Ni and V_3Ni. The Pearson correlation index showed a significant negative correlation between the percentage of spores germinated and the heavy metal concentration in the culture medium, in both species (*Da*: R= -0.958 for the Cd acetate variants, and R= -0.976 for the nickel sulphate variants, and for *Dfm*: R= -0.958 for Cd acetate variants, and R= -0.790 for the Ni sulfate ones; $p<0.01$).

The literature mentions the fact that the gametophyte is the most sensitive stage in the life cycle of the pteridophytes (GUPTA et al., 1992), as numerous exogenous factors affect spore germination. Among these there are

pollutants such as pesticides (KEARY et al., 2000; SHEFFIELD, 2002; LUO & IKEDA, 2007; CASSANEGO et al. 2010; DROSTE et al., 2010), metals (NISHIZONO et al., 1987; SELA et al., 1989; GUPTA & DEVI, 1992; GUPTA et al., 1992; MA et al., 2001; KAMACHI et al., 2005; MUCCIFORA, 2008; KIELING-RUBIO et al., 2011; SOARE et al., 2013c), etc. Lead acetate (10 mg/100ml, respectively 50 mg/100ml Knop solution) affected spore germination and gametophyte differentiation in the species *Athyrium filix-femina*, *Dryopteris affinis* and *D. carthusiana* (SOARE et al., 2014). Nickel in a concentration of 0.05 to 100 mg L^{-1} affected spore germination in *Regnellidium diphyllum* (KIELING-RUBIO et al., 2012). The spore germination of *Ceratopteris thalictroides*, *Drynaria quercifolia*, *Cristella parasitica*, *Pteris ensiformis*, *Amelopters prolifera* and *Adiantum lunulatum* was affected by different Cadmium concentrations (GUPTA & DEVI, 1992).

Table 2. The influence of heavy metals on the germination of spores.

Experimental variants							
Species	C	V_1Cd	V_2Cd	V_3Cd	V_1Ni	V_2Ni	V_3Ni
Germination percent (mean±SD)							
Da	82±3.6^a	71±3^a	59±10.5^b	6±2.6^c	66.6±2.5^b	45±5.1^c	19±6.5^d
Dfm	69±1.7^a	55.6±4.7^b	48.3±5.5^b	15.3±3.5^c	42.3±9.2^b	15±2^c	18±10.1^c

Legend: The values are the means of 3 repetitions ± standard deviation; a, b, c, d - Duncan test results: the comparisons were made between Control and V_{1-3}Cd, and Control and V_{1-3} Ni for each species.

Throughout the monitoring period in terms of gametophyte differentiation, differences were observed both between the two species, and between the experimental variants set for the same species. Thus, one month after the initiation of the experiment, in both species, the Control was at the stage of prothallium blade, and the other variants were at less advanced stages (Table 3). We found that there was a tendency of prothallium filaments to branch (*Da*: V_1Cd and V_1Ni). In some variants, we also observed the tendency of the gametophyte to form three-dimensional cell masses (*Da*: V_1Cd, V_2Cd, V_1Ni, *Dfm*: V_2Cd and V_1Ni). The V_3Cd variants of *Da* and the V_2Ni and V_3Ni variants of both species were at the stage of germinated spores. Another change observed in the *Da* gametophyte, in the V_1Ni variant, was that the rhizoids were fewer and short in comparison with the other variants.

Table 3. Stages of gametophyte differentiation one month after the initiation of the experiment.

Variants	**Species**	
	Dryopteris affinis	***Dryopteris filix-mas***
C	prothallium blade	prothallium blade
V_1Cd	prothallium filament, blade differentiation, branching prothallium filaments, three-dimensional cell masses	blades differentiation
V_2Cd	short filament, three-dimensional cell masses	prothallium filament, three-dimensional cell masses
V_3Cd	germinated spores	filaments differentiation
V_1Ni	branched filaments (short rhizoid), three-dimensional cell masses	filaments differentiation, three-dimensional cell masses
V_2Ni	germinated spores	germinated spores
V_3Ni	germinated spores	germinated spores

Two months after the start of the experiment, the differences in the stages of gametophyte differentiation maintained in both species (Table 4). As far as *Da* was concerned, C, V_1Cd and V_2Cd were at the stage of chordate prothallia, while V_1Ni was at the stage of prothallium filaments and blades, whereas the other variants were at the stage of germinated spores. In *Dfm*, the stages of differentiation were the prothallium blades for C, V_1Cd, prothallium filaments for V_2Cd, V_3Cd, V_1Ni, and germinated spores for V_2Ni and V_3Ni. As far as the V_2Cd and V_1Ni variants are concerned, we observed a tendency to form three-dimensional cell masses, a phenomenon reported by SOARE et al. (2013a) in *Athyrium filix-femina* and *Polypodium vulgare* gametophytes treated with bifenthrin.

Also, three-dimensional cellular masses were found in abnormal gametophytes that developed from aged spores (BALLESTEROS et al., 2011, 2012; SMITH & ROBINSON, 1975). Similarly, the chlorophyll pigments in *Da*V_1Ni were also affected, the filaments and prothallium blades were discoloured. The variants that had a higher concentration of the metal compound (V_2Ni, V_3Ni) resulted in the necrosis of the germinated spores. WAHID et al. (2008) showed that Cd stress produces chlorosis, necrosis and loss of pigments in *Vigna radiata* leaves. Chlorosis of leaves was reported by PERVEEN et al. (2011) as Cd toxicity symptoms in *Zea mays*. Also, the decrease of photosynthetic pigments in *Pisum sativum* leaves was noticed by SINGH (2014) in response to Cd toxicity.

When the last monitoring of the gametophyte differentiation was conducted after four months, the differences in the development stages were maintained both between the species and the experimental variants (Table 5). In both species, variants containing Ni had a stronger negative influence on the gametophyte, while the control variants were at the stage of chordate prothallium. For V_3Cd, V_2Ni and V_3Ni, the germinated spores became necrotic, thus ceasing the development cycle.

Table 4. Stages of gametophyte differentiation after two months from the start of the experiment.

Variants	Species	
	Dryopteris affinis	***Dryopteris filix-mas***
C	prothallium blade, chordate prothallia, antheridia	prothallium blade, chordate prothallia
V_1Cd	chordate prothallia	prothallium blade, young chordate prothallia
V_2Cd	prothallium blade, chordate prothallia, three-dimensional cell masses	prothallium blade, young chordate prothallia
V_3Cd	germinated spores (necrotic)	prothallium blade, chordate prothallia, antheridia
V_1Ni	prothallium filament and discoloured prothallium blade (necrotic)	prothallium filament and prothallium blade (necrotic)
V_2Ni	germinated spores (necrotic)	germinated spores (necrotic)
V_3Ni	germinated spores (necrotic)	germinated spores (necrotic)

Table 5. Stages of gametophyte differentiation after four months from the start of the experiment.

Variants	Species	
	Dryopteris affinis	***Dryopteris filix-mas***
C	prothallium blade, branched chordate prothallia, antheridia	chordate prothallia
V_1Cd	branched chordate prothallia	chordate prothallia (necrotic)
V_2Cd	chordate prothallia	chordate prothallia
V_3Cd	germinated spores (necrotic)	prothallium blade, chordate prothallia
V_1Ni	prothallium blade (discoloured)	prothallium filament and prothallium blade (discoloured)
V_2Ni	germinated spores (necrotic)	germinated spores (necrotic)
V_3Ni	germinated spores (necrotic)	germinated spores (necrotic)

After two months from the start of the experiment we observed an accumulation of metal on the surface of the gametophyte in some Cd variants (*Da*V_1Cd, *Dfm*V_3Cd) (Fig. 1) and the precipitation of metals. The latter was due to biosorption to cell walls, or precipitation of metal compounds (GADD, 2010).

We believe that, in the particular case of the gametophyte of the leptosporangiate pteridophytes the deposit could be mostly favoured by their small size and the single-layered structure, as almost all cells are exposed directly to the environment. Precipitation of crystalline structures around the rhizoids of the prothallia of *Athyrium filix-femina* was noticed in gametophytes treated with a copper fungicide (SOARE et al., 2013a), as well as at the tip of the papillae in the gametophytes of *Asplenium scolopendrium*, the latter with a fungicide containing 20% metallic copper (SOARE et al., 2013b). This property may have a particular importance in the bioremediation of environments contaminated with metals.

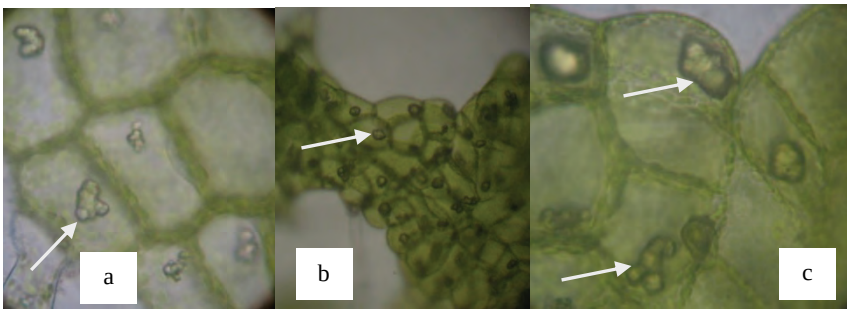

Figure 1. Gametophyte of *Dryopteris affinis* (Lowe) Fraser-Jenkins, V_1Cd experimental variant: two months (a) and four months after sowing the spores (b, c). Accumulation of metal (arrow) on the gametophyte cells (a, c x400; b x100; original).

CONCLUSIONS

In conclusion, cadmium acetate and nickel sulphate affect both the germination of spores and the differentiation of the gametophyte in the ferns *Dryopteris affinis* and *D. filix-mas.* The main changes noticed in the process of gametophyte differentiation were as follows: a longer period of time necessary to reach the characteristic stages, damage to rhizoid elongation, the formation of three-dimensional cell masses, chlorosis and necrosis of the gametophyte cells and an accumulation of crystal structures on the surface of the gametophyte. This accumulation may have a particular importance in the bioremediation of environments contaminated with metals.

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Received: March 31, 2016
Accepted: May 29, 2016